

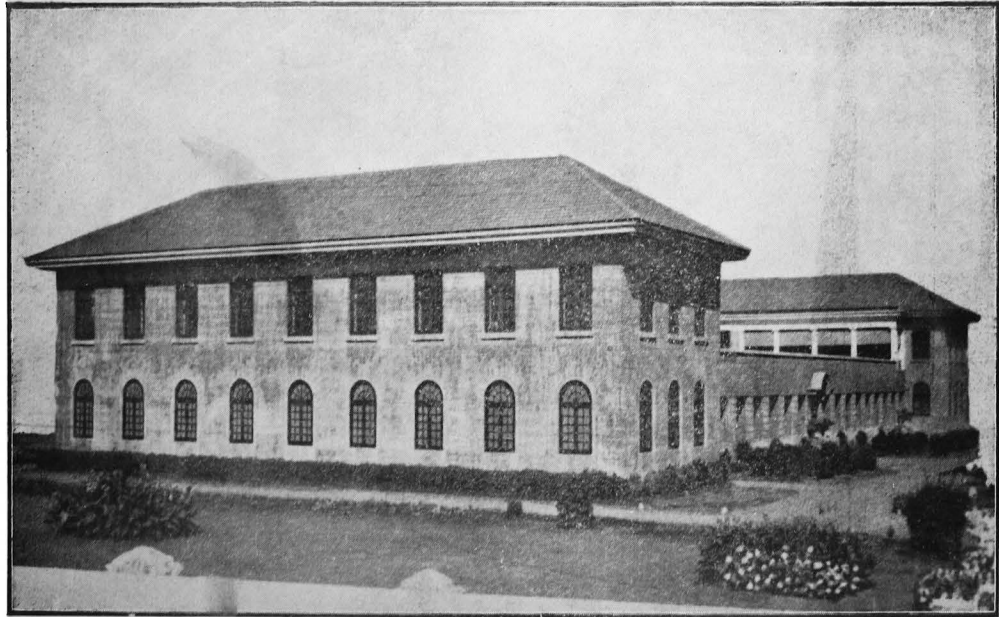
**Nowrosjee Wadia College, Poona**  
**Department of Bio-Chemistry**



**RESEARCH PAPERS**  
**ON**  
**FOOD PROBLEMS**

**SEPTEMBER**  
**1949**

With greetings  
The Newsjee W. ...  
+  
Compliments of the Editor  
to  
S. G. Vakey



The Vicaji D. B. Taraporevala Institute of Science.

**Nowrosjee Wadia College, Poona**  
**Department of Bio-Chemistry**



**RESEARCH PAPERS**  
**ON**  
**FOOD PROBLEMS**  
**1938 - 1948**

*Under the guidance of*

**Rao Bahadur Dr. D. L. Sahasrabuddhe, D.Sc., M.Sc., M.Ag., F.A.Sc.,**

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*and*

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at the Nowrosjee Wadia College, Poona.

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**The late Mr. Vicaji D. B. Taraporevala.**

To

The Memory of

The late Mr. VICAJI D. B. TARAPOREVALA

whose generosity enabled the College

to provide an excellent home for its Science Departments

in

The Vicaji D. B. Taraporevala Institute of Science.

This volume of research work done in the Institute

is

Dedicated

*As a token of gratitude.*



**His Excellency Raja Sir Maharaj Singh,**  
*Governor of Bombay and*  
*Chancellor of the University of Poona.*



## FOREWORD

When I was asked by Principal V.K. Joag to write a foreword to this useful and interesting publication, I readily agreed for what seemed to me to be two good reasons: First, because the publication is appearing at a very opportune moment, and second, because it is, I understand, the first book of its kind which has been compiled in Maharashtra since the establishment of the University of Poona.

In my recent Convocation address to that University I emphasised the great value of research work not only for the interest which it rouses in the students but also for the national interests which it promotes. We have been repeatedly told that one of the gravest problems facing India at present is the shortage of food, and our leaders have by word and example been doing all that lies within their power to step up the production of food so as to make our country self-sufficient in the near future. At such a moment, the publication of the result of years of research in problems concerning food is not only opportune, but shows very commendable public-spirit. The vital importance of nutritional analysis and bio-chemical research cannot be overestimated.

I congratulate those students of the Nowrosjee Wadia College, Poona, who have, under the able guidance of Dr. D.L. Sahasrabudhe and Dr. N.V. Bhide, contributed their thought and their talent to this valuable work. A meed of praise is also due to the authorities of the College on their encouragement and assistance, without which this contribution would have remained unknown to the public.

GOVERNMENT HOUSE  
Ganeshkhind,  
September 12, 1949.

MAHARAJ SINGH  
Governor of Bombay.

encouraged his students to undertake research on problems relating to food.

During these 12 years, Dr. Sahasrabuddhe and his students have worked on many such problems and have produced valuable results. The students have received recognition from the University of Bombay for their work. They have been awarded the M.Sc. and Ph.D. degrees.

The theses submitted by these students for their degrees are now being published by the College at its own cost. Some of them were originally published in the Bombay University Journal and similar other periodicals. They have been revised, condensed, and made suitable for inclusion in this publication.

We are happy that within a few months of the establishment of the University of Poona we have been able to bring out this volume of research papers. We believe, that this is a happy augury for the new University. We have no doubt that the University will encourage its constituent Colleges to continue such work and thus supplement its own efforts for the promotion of research and advancement of learning.

We thought that it would be very appropriate that we should have for the volume a foreword from the Chancellor of the new University, H. E. Raja Sir Maharaj Singh, the Governor of Bombay and so we approached him and we are happy to state that he readily agreed and sent his foreword. We take this opportunity to convey to H. E. our grateful thanks.

Our thanks are also due to the authorities of the Bombay University and the Editors of the Journals in which some of the papers were first published for permission to publish these papers in this volume.

The provision of an excellent building for housing our Science Laboratories was made possible by the generous donation of Rs. 80,000/- given by the late Mr. Vicaji D. B. Taraporevala towards its cost. Without a suitable building it would not have been easy for the College to under-

take such research work. We have, therefore, thought it appropriate that this first publication of its kind undertaken by our College should be dedicated to the memory of Mr. Taraporevala. It is to us a matter of deep regret that Mr. Taraporevala is not alive to see this publication.

We cannot close this preface without expressing our thanks to Mr. M. M. Thatte, M.Sc. and Mr. V. S. Kulkarni, M.Sc. for the valuable help they gave in preparing the papers for the Press and reading the proofs. Thanks are also due to the Proprietor and the Managers of the Israelite Press for the readiness with which they undertook and the thoroughness with which they carried out the printing work of this volume. But for their ready help it would have been difficult for the College to produce the book in such an attractive form.

NOWROSJEE WADIA COLLEGE }  
15th September 1949. }

V. K. JOAG.

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**Rao Bahadur Dr. D. L. Sahasrabudhe, D.Sc.**



# INTRODUCTION

By

RAO BHADUR DR. D. L. SAHASRABUDDHE, D. Sc.

From the analysis of the animal bodies it has been ascertained that the organs consist chiefly of carbon, hydrogen, oxygen, nitrogen and mineral matter. The bones and teeth contain, in addition, calcium and phosphorus in large quantities while the blood also contains iron, sodium and chlorine. The object of food eaten is to supply all these elements to the body in a suitable form.

There are four chief groups of chemical compounds which can supply the requirements of the animal body and therefore constitute the bulk of the body. Three of these groups are (1) carbohydrates, (2) fats, and (3) proteids from which the supply of carbon, hydrogen and nitrogen is chiefly derived while the fourth group consisting of mineral matter provides calcium, phosphorus, sodium, chlorine, iron and a few other elements. It is the proportion of each of these constituents in the food-stuff that is more important than the actual presence of these and this is what an analyst attempts to ascertain, since every food-stuff contains at least a small quantity of each of these. A better judgement of the value of a food-stuff can be obtained if the proportion of the digestibility of the constituents is known.

The object of food is not only to supply material for growth and to replace the waste of the body but the object is also to supply body heat and energy for internal and external work. The energy supplied by the food can be calculated by adding the energy supplied by all the constituents in the food. By actually burning purified constituents in calorimeter the heat-giving or the energy-giving value of each constituent has been determined in calories per gram.

An example may explain this as follows :—

		Small calories	Large calories.
One gram of carbohydrate	...	4,100	4.1
One gram of protein	...	4,100	4.1
One gram of fat	... ..	9,300	9.3

If we take wheat-bran we can find out the heat energy supplied by one hundred grams of the material.

TABLE No. 1

	Wheat-bran. per cent.		Large calories in 1 gram of constituent.		Total large calories.
Moisture	... 7.90		—		—
Ether extract (fat)	... 4.00	×	9.3	=	37.20
Albuminoids	... 14.43	×	4.1	=	59.16
Digestible Carbohydrates.	57.02	×	4.1	=	233.78
Fibre	... .. 11.85	×	4.1	=	48.58
Total energy in 100 grams of wheat bran					= 378.72

The whole of the total energy shown is not useful to the body as part of the constituent is voided in dung undigested. The energy which is utilised in the body is determined from the digestible material and is called "available energy". In most of the papers included in this publication, this availability percentage of the various food-stuffs studied has been indicated. In Europe and U. S. A. they go even still further and make allowance for that part of the available energy which is utilised in digesting the food itself. We have, however, not gone to that extent and have only determined the availability.

The International League of Food Experts has given that in temperate climate 24,00 large calories of heat and 60 to 70 grams of proteids should be made available to every average man per day. But if the individual is doing work he should get an extra allowance of 150 calories. In India which is a hot country 2200 to 2300 calories of heat are sufficient for an ordinary individual. The usual food-stuffs in common use are

- (1) *Jowar (Andropogon sorghum)*
- (2) *Bajri (Pennisetum typhoideum)*
- (3) *Wheat (Triticum sativum)*
- (4) *Rice (Oryza sativa).*

Some pulses are also used along with these food-stuffs.

If we take one pound of any of these grains we can obtain 1575 calories. This means that in order to obtain 2200 to 2300 calories of heat  $1\frac{1}{2}$  pounds of grains is a sufficient quantity of food-stuffs. The quantity of food-stuffs mentioned can supply 67 grams of proteid material. If the grains supplied contain less of proteid material, this deficiency can be made up by the addition of a small quantity of rice-polish or a small quantity of ground-nut cake.

#### Functions of important constituents:—

(1) **Proteids or albuminoids:**—Proteids or albuminoids occurring in grains, roots and other forms of vegetable food are similar in general composition to those in milk; blood and flesh. From the albuminoids of the blood are formed not only the albuminoids of the animal frame but also the gelatinoids, hair, wool, horn etc., and under some circumstances the fat. By the combustion of albuminoids in the body heat and mechanical force will also be developed. One gram of albuminoids yields 4.1 large calories. Albuminoids thus supply in themselves most of the requirements of the animals—a statement which can be made of no other constituent. The albuminoids of food are frequently described as “Flesh formers”.

An animal even when not increasing in weight, will always require a certain constant supply of albuminoids in its food to replace the waste of nitrogenous tissues which is always going on even during rest. When the nitrogenous tissues of the animal or the albuminoids consumed as food are oxidised in the body, the nitrogen they contain is not burnt but excreted in the form of a nitrogenous substance—urea.

(2) **Fat:**—Fats contained in the food are similar to those found in the animal body. An animal is apparently capable of selecting certain fats in the food for storing up and even of transforming one kind of fat into another. The fat in the food

is either burnt in the animal system or it is stored up as reserved matter. Fat has a greater value as a heat producer than any other ingredient. Its calorific value is 9.3.

(3) **Carbohydrates**:—The carbohydrates of food are chiefly starches, sugars, celluloses etc. Carbohydrates form the largest part of every vegetable food. Carbohydrates are not permanently stored in the animal body but serve for the production of heat and mechanical work. When consumed in excess of normal requirements, they may be converted into fat. 1 gram of carbohydrate gives 4.1 large calories.

(4) **Mineral matter**:—The ash constituents present in the food are the same as those found in the animal body. All that is accomplished by the animal is to select from the digested constituents those of which it is in want. The fact that the ash of feeding stuffs is of great importance to animals is shown by feeding them on mineral-free foods in which case they die of mineral starvation. During such starvation nervous system suffers first. Mineral matter is present in all vital parts. Nuclei of the cells are rich in phosphorus. Bones contain 70 per cent. of calcium phosphate. Blood serum contains sodium chloride and other sodium salts while blood corpuscles contain potassium and iron salts.

(5) **Vitamins**:—In a general way if a human being or any animal is provided with the necessary proteins, mineral matter and calories of heat in the form of carbohydrates and fats he should be able to get along but in the feeding experiments carried on by feeding pure constituents of food to guinea-pigs or rats it was found that the animals suffered in health and they recovered their condition when fed on the quantities of food constituents in the form of ordinary food-stuffs (not purified). For a long time this difference could not be explained but it has now been discovered that the deficiency of vitamins in the purified constituents is at the bottom of the trouble. The vitamins are known as "A", "B", "C", "D" etc. for want of better names. Now a large number of vitamins have come to light and have joined the family and we cannot predict to what extent the number will increase. The vitamins have compelled us to reform our ideas on nutri-

\*

tion of adults and babies and we must now pick out our foods with a new angle of vision.

In 1937, a plan was made to start some investigation work on food-stuffs in the chemical laboratory of the Nowrosjee Wadia College. The idea in starting investigation work was to help students going up for the M.Sc. and Ph. D. examinations of the Bombay University by thesis and secondly also to carry on some investigation in which members of the chemistry staff can take part and spend their spare time usefully. It was not possible for each worker to take over full problem and carry it out himself. It was therefore decided to take one problem and carry it out as a team work. The old idea of one man one problem is going in the background and team-work is coming into vogue in most of the laboratories doing research work. In the Wadia College Laboratory the investigation of the proportion of the constituents in the usual food-stuffs consumed in the Bombay Deccan and a relative capacity of these constituents to break down into simpler compounds were considered as important and interesting. It is these simpler compounds that can be absorbed into the human body and the animal body in general, through the valves of the alimentary canal and this is exactly what happens during digestion.

Usually breaking down of the constituents is brought about by hydrolysis. Digestibility of food-stuffs is found out by actually feeding the animals with the food-stuffs to be studied. In Wadia College the investigation work was not conducted in-vivo but in-vitro. There are advantages and disadvantages of the invitro system. The advantages of the in-vivo experiments are that the digestibility is studied by actually feeding animals and determining the proportion of food digested. Since it is done on the living animals there is no reason to doubt in any way the results obtained. On the other hand the experiments done invitro can be carried on under any suitable condition. The temperature, the proportion of reagents, and other conditions can be controlled and the results obtained are always comparable. It was therefore decided to carry on experiments under controlled conditions

in vitro. It would have been better if the results would have been confirmed by experiments in vivo. It is therefore that the results as obtained in the laboratory are placed before the public in the form in which they were obtained.

The following materials were studied. They can be divided into separate groups as:—(i) Cereals (ii) Pulses (iii) Oil seeds (iv) Materials known as dry fruits (v) Ghees—natural and artificial and (vi) Edible oils. Besides these some enzymes also were studied.

The work can be divided according to the reacting agents such as acids, enzymes and so on. The work on enzymes has been dealt with in separate papers. In the animal body it is the enzymes that act as reagents to break down highly complex compounds into simpler ones by their special power of hydrolysis.

In the human body all the enzymes bring about breaking of complex compounds at the body temperature which is about 37°C. and hence all the reactions of the various agents have been worked at 37 degrees centigrade temperature.

The following were the materials (with their botanical names) which were studied in this laboratory:—

- (1) Cereals—(i) *Bajri*—(*Pennisetum typhoideum*).  
 (ii) *Vari*—(*Panicum miliaceum*).  
 (iii) *Wheat*—(*Triticum sativum*).  
 (iv) *Jowar*—(*Andropogon sorghum*).  
 (v) *Rajgira*—(*Amaranthus paniculatus*).  
 (vi) *Nachani*—(*Eleusine coracana*).  
 (vii) *Rala*—(*Sataria italica*).  
 (viii) *Harik*—(*Paspalum scrobiculatum*).
- (2) Pulses—(i) *Gram*—(*Cicer arietinum*).  
 (ii) *Black-gram*—(*Phaseolus radiatus*).  
 (*Udid*)
- (3) Oil seeds:—  
 (i) *Linseed*—(*Linum usitatissimum*).  
 (ii) *Ground-nut*—(*Arachis hypogaea*).  
 (iii) *Safflower*—(*Carthamus tinctorius*).  
 (iv) *Cocoanut*—(*Cocos nucifera*).

## (4) Materials known as dry fruits:—

- (i) Cashew nut—(*Anacardium occidentale*)
- (ii) Almond—(*Prunus amygdalus*)
- (iii) Pistachio-nut—(*Pistacia vera*)

## (5) Roots and Tubers:—

- (i) Carrot—(*Daucus carota*).
- (ii) Sweet Potato—(*Ipōmea batata*)
- (iii) Yam—(*Dioscorea alata*).
- (iv) Suran—(*Amorphophallus campanulatus*).
- (v) Potato—(*Solanum tuberosum*)

Taking each group and comparing materials in that group with one another we are able to find out their gradation of breaking down. We can also then compare different groups with one another. In order to explain this treatment more thoroughly we might say that taking the group of cereals we can find out whether rice starch breaks down more easily than *bajri* starch or *jowar* starch and arrange them in order of their power to breaking down. This will be a comparison in the materials in the same group. But now if we take two groups such as cereals and pulses in groups and compare the power of breaking down of the cereals with the power of breaking down of the pulses we can obtain the comparative breaking down of the starches in these two groups.

Along with the food grains a study was made of the hydrolysis of the starches of the materials known as dry fruits. It may be mentioned that edible roots and tubers were also studied as they form an important section of human food materials.

Amongst the various reagents used for the study of starch-hydrolysis hydrochloric acid is the one that is able to break the whole of the starch material and all sorts of carbohydrates into a simple monosaccharose viz. glucose. This gives us the proportion of the total carbohydrates in each material while the action of enzyme gives us the actual amount of carbohydrates available for absorption in the body. The following table gives us the general analysis of the materials studied.

TABLE NO. 2

Material	Moisture %	Ether extract %	Proteins % @	Carbo- hydrate %	Crude fibre %	Ash* %
<i>Bajri</i> ...	7.44	5.66	9.60	74.74	0.92	1.70
<i>Vari</i> ...	10.10	4.36	7.53	70.69	4.62	2.70
Wheat ...	9.97	2.05	11.25	73.66	1.36	1.70
<i>Jowar</i> ...	7.98	3.25	7.83	76.64	1.25	3.05
<i>Rajgira</i> ...	9.35	5.03	15.01	65.72	2.83	2.05
<i>Nachani</i> ...	8.55	1.75	5.71	78.27	2.88	2.84
Gram ...	8.24	4.52	20.03	61.22	3.89	2.10
Black-gram	7.23	2.20	23.21	59.16	3.93	4.27
Cashew-nut	6.00	44.70	21.30	22.40	2.10	3.50
Almond ...	5.90	53.50	20.70	13.40	2.70	3.80
Pistachio ...	5.70	50.70	19.60	18.10	2.80	3.10
Carrot ...	7.70	1.72	4.30	73.20	6.56	6.52
Sweet-potato	7.50	3.90	4.50	78.85	1.75	3.50
Yam ...	8.12	0.27	3.88	82.21	1.48	4.04
<i>Suran</i> ...	8.02	0.31	3.98	77.55	4.10	6.04
Potato ...	7.62	0.11	4.68	79.51	2.88	5.20
<i>Rala</i> ...	9.51	3.64	9.58	74.48	1.36	1.43
<i>Harik</i> ...	9.92	1.87	6.22	80.14	0.75	1.10

@ Proteins are determined by finding out nitrogen and multiplying the figure by 6.25.

\* Ash includes sand also.



TABLE No. 3

Disintegration of starches as in flours by 1 per cent. diastase in 3 hours (expressed on the basis of total hydrolysable starch) :

	Material	Starch hydrolysed per cent.	Total hydrolys- able starch %
<b>Cereals :-</b>			
	<i>Bajri</i>	... 45.56	59.82
	<i>Vari</i>	... 49.18	56.23
	Wheat	... 29.81	74.29
	<i>Jowar</i>	... 30.57	77.13
	<i>Rajgrā</i>	... 33.52	59.67
	<i>Nachani</i>	... 34.57	71.67
	<i>Rala</i>	... 39.23	66.83
	<i>Harik</i>	... 35.48	70.52
<b>Pulses :-</b>			
	Gram	... 88.64	42.32
	Black-gram	... 36.60	50.37
<b>Materials known as dry fruits :-</b>			
	Cashew nut	... 30.59	17.34
	Almond	... 23.17	7.78
	Pistachio-nut	... 19.74	6.53

TABLE No. 4

Disintegration of starches as in flours by one per cent. pancreatin in three hours (expressed on the basis of total hydrolysable starch)-

	Material	Starch hydrolysed per cent.	Total hydrolys- able starch %
<b>Cereals :-</b>			
	<i>Bajri</i>	... 55.93	59.82
	<i>Vari</i>	... 50.16	56.23
	Wheat	... 14.58	74.29
	<i>Jowar</i>	... 25.66	77.13

x

<i>Rajgira</i>	...	33.60	59.67
<i>Nachani</i>	...	30.16	71.67
<i>Rala</i>	...	53.54	66.83
<i>Hark</i>	...	43.28	70.52

Pulses :-

Gram	...	50.16	42.32
Black-gram	...	46.44	50.37

Materials known as dry fruits :-

Cashew-nut	...	33.98	17.34
Almond	...	27.12	7.78
Pistachio-nut	...	21.72	6.53

In using food materials human beings take certain salts into their body. It was therefore considered interesting to see whether the salts had any effect on the hydrolysis of food materials.

TABLE No. 5

**Effect of common salt on the disintegration of starches by one per cent. diastase in three hours.**

Material		Starch hydrolysed (without salt) %	Starch hydrolysed (with salt 0.15 M) %	Total hydrolyzable starch %
<i>Jowar</i>	...	30.57	32.49	77.13
<i>Bajri</i>	...	33.51	33.51	59.67
<i>Nachani</i>	...	34.56	33.92	71.67
Wheat	...	29.86	31.09	74.29

It is seen from these figures that the disintegration of starches is favourably affected in the case of wheat and *jowar* and adversely affected in the case of *nachani* and no effect on *bajri*.

TABLE No. 6

**Effect of common salt on the disintegration of starches by pancreatin one per cent. in three hours.**

Material	Starch hydrolysed (without salt) %	Starch hydrolysed (with salt 0.15 M) %	Total hydrolysable starch %
Wheat ...	12.15	9.87	74.29
<i>Jowar</i> ...	21.38	26.68	77.13

It is seen from these figures that the disintegration of starches is favourably affected in the case of *Jowar* adversely affected in the case of Wheat.

Milk is intended for the nourishment of newly born animals. If milk is to serve exclusively for nourishing the young, it must of course contain all the nutrients required for the animal organism for its maintenance and growth and there are proteins, fats, carbohydrates and inorganic salts. In addition to these, milk also contains small quantities of various substances known as vitamins which although they possess no direct feeding value have nevertheless an important physiological effect. The milk of all species of animals contains all the constituents mentioned above but the quantities in which they are present vary in different cases.

Ass's milk was studied because it is used for feeding children and is considered to be medicinally good. Its importance to children is due to the fact that it contains a high percentage of sugar and a very low percentage of fat. Children therefore, can easily digest it.

TABLE No. 7.

## Composition of milk of different animals.

	Origin	Total solids	Fat	Milk-sugar	Proteids	Ash	Density
Godbole & Sadgopal- (Benares)	Buffalo	18.0 -22.5	3.75 -6.50	5.0 -5.4	5.3 -6.0	0.70 -0.93	1.0320 -1.0420
	Cow	12.9 -14.5	3.00 -3.85	4.5 -5.2	3.4 -4.0	0.45 -0.65	1.0260 -1.0350
Chem. Lab. N. Wadia College	Sheep	14.08 -19.28	4.7 -8.6	3.40 -3.83	4.55 -5.38	0.67 -1.10	— —
	Goat	12.94 -18.28	2.94 -8.15	3.313 -4.606	3.82 -5.11	— —	— —
„	Aus	8.88 -10.26	0.10 -1.48	5.61 -6.58	1.83 -2.15	0.38 -0.52	1.0316 -1.0844

Work on hydrolysis of fats was also carried out. The fats are broken into simple fatty acids.

TABLE No. 8

**Hydrolysis of ghees, edible oils, and vegetable ghees with enzyme lipase.**

Sample.	Melting Pt. (degrees C.)	Iodine value	Hydrolysis after 48 hours %
Buffalo ghee ...	37.2	28.08	53.20
Sheep ghee ...	29.9	34.75	20.00
Goat ghee ...	23.8	25.87	44.53
Cow ghee ...	39.1	36.64	41.66
Linseed oil ...	solidifies at - 20	165.54	70.00
Safflower oil ...	solidifies at - 18	141.50	23.98
Cocoanut oil ...	25.3	9.96	26.48
Vegetable ghee (A) ...	39.50	65.49	17.99
do. (B) ...	40.2	57.94	17.66
do. (C) ...	39.2	61.10	29.96

If the conditions under which the hydrolysis of fat is carried out are changed then the percentage of hydrolysis is considerably changed.

**Proteids:—**Proteids are hydrolysed chiefly by two enzymes, namely, pepsin and trypsin. Pepsin acts in acid medium while trypsin acts in alkaline medium. These enzymes do not act on different substances equally well but act more quickly on some than on others. When the seeds of cashew nut, almond, pistachio nut were acted upon by pepsin in acid medium it was found that pistachio nut was acted upon the most while the cashew nut was acted upon the least. While

almond stood between the two. This order of disintegration remained the same when trypsin was used to superimpose pepsin.

**Enzymes;**—Study of the enzymes was done by different workers in connection with those enzymes with which they had to deal. They studied first of all the proper temperature, pH value, strength and time required for the reaction. The enzymes have a specific action on definite constituents and although two enzymes may act on one and the same constituent yet the conditions under which they work are different such as pepsin and trypsin, although pepsin and trypsin both hydrolyse proteid substance yet pepsin works in an acid medium while trypsin works in an alkaline medium.

Starch is hydrolysed and broken down by amylase. We however find that there are two kinds of enzymes—alpha and beta. The beta amylase works well up to a temperature of 70 degrees centigrade while the alpha amylase works well above this temperature. But it is also found that at a temperature a little below 70 degrees Centigrade and a little above 70 degrees Centigrade, both beta and alpha show some activity and therefore overlap the work of each other.

A general study of the *Ricinus* lipase from castor seed was carried out with a special reference to the nature of the different buffers on the activity and the optimum pH of the *Ricinus* lipase. It was found that optimum pH does not change much but the activity is the highest in acetate buffer. Since the activity of the lipase changes with time, an investigation was carried out in order to find out a modified form of lipase which will keep its activity constant for a fairly long time. It was found that acetone dried lipase keeps the activity constant for a long time and hence it was used for other experiments.

The synthesis and hydrolysis of aliphatic esters of homologous series starting from methyl to amyl butyrate, were carried out. It was found that synthesis increases from methyl to amyl whereas it is reversed in case of hydrolysis. A

distinction was also found between normal and iso forms. It was found that the percentage synthesis or the hydrolysis only depends upon the number of carbon atoms in alcoholic group but not in the acidic group.

The action of different salts was studied on the synthesis and hydrolysis of esters and the salts were divided into three groups as accelerators, retarders and poisons. No definite result had been found as regards the effect of nature of the ions on the synthesis and hydrolysis of esters.

It is a well known fact that the phosphates in the soils form an important plant food material. But the phosphoric acid in the soil is not always available to the plants or it may be available in different degrees. It is therefore important to know what proportion of phosphoric acid in the soil is available to plants. There are different methods by which this can be judged. But there is no method which can be used to give a correct idea of their availability. An attempt was made in the N. Wadia college laboratory to ascertain this. Some typical soils of Bombay Province were obtained and comparative methods of phosphoric acid availability were tried to find out which method gives consistent results and which is yet easily worked out in a laboratory.

Nitrogen is obtained generally from organic materials and only in a few cases from sulphate of ammonia and sodium nitrate. The organic materials which have been tried in the Bombay Province are farm-yard manures, oil cakes, fish and bone-manures. There are substances other than these which are all derived from animals' remnants such as horns, bone-grit and other waste which can be used with advantage as fertilisers but about which nothing definitely is known as to how long they take in disintegrating and making the nitrogen available for plants. An attempt was therefore made by submitting these substances of animal origin mixed with different soils to ascertain as to how long it takes to make the nitrogen available to plants when mixed with different soils of the Bombay Province.

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## PAPER I

# COMPARATIVE DISINTEGRATION OF FLOURS AND STARCHES WITH HYDROCHLORIC ACID, DIASTASE AND PANCREATIN.

BY

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and also of the Agri. College, Poona, Magazine).

In all civilised countries where scientific work is carried out there are laboratories which are specially devoted to the study of digestibilities of important foodstuffs. In India a large amount of work on nutrition is being carried out at Coonoor, Bangalore, Madras, Calcutta, etc. The nutritive values of foodstuffs depend upon two factors, firstly the quantity of the essential constituents such as carbohydrates, proteins, fats, etc., which the foodstuffs contain and secondly on the digestibility of the constituents.

The earliest investigation about the disintegration of the foodstuffs made with a view to find the digestibility, dates back to the year 1895. Among the various workers in this field who studied the disintegration of the starches, the names of Stone (1) Barnetzky (2) and Langsworthy and Deul (3) must be mentioned.

Stone (*loc. cit.*) carried out work with a view to study the degree of susceptibility of different starches to the action of different enzymes. Langsworthy and Deul (*loc. cit.*) carried out an interesting work on the digestibilities of certain raw starches like those of wheat and potato on human subjects. Rockwood (4) studied the action of pancreatin, ptylin and diastase on the starch from bleached and unbleached wheat flours.

Similarly the study of proteins from the various grains has been carried out by using enzymes *in vitro* or *in vivo* by feeding the animals with the foodstuffs. In India some of the



workers in the study of proteins are Bhagwat and Shrinivasan (5), Neogi, Narayana and Desai (6), Damodharan and Krishnaswami (7), etc., while among the workers in the foreign countries the names of Cohn and White (8) and Watermann and Johns (9) may be mentioned.

It will be seen from the above review that some of the foodgrains have been studied by parts i. e. the starches and the proteins isolated from them. An investigation of this type although of great importance as it indicates the nutritive value of the different parts of the grains is not adequate enough in as much as it does not give any comparative idea of the utility of the flours as a whole.

The digestibility of the starch which is found in the absence of the other constituents of the flours may differ from one obtained in the presence of the other constituents such as proteins, fats etc.; Sahasrabuddhe and Kibe (10) have shown that the various constituents of flour have a mutual positive or negative effect on the course of the disintegration. So the values obtained for the pure starches or isolated proteins will not be the same as those which may be obtained when these substances are taken together with the other constituents of the flours. And it is the flours that are actually eaten and not the isolated constituents of the flours and hence a study of the flours as such must be done in order to get an exact idea of their comparative values.

It is necessary, if the general idea of the comparative utility of the food-grains is to be obtained, to subject them to the action of as many disintegrating reagents as possible with time variation to include a large number of stages of hydrolysis. From the results of the disintegration of the constituents the comparative digestibility of the different grains and hence their comparative value can easily be judged. As it has already been mentioned that the different constituents of the grains have some effect on the disintegration of each other, it is necessary to study them separately and also together so that some definite idea of such mutual effects may be obtained. In the present paper the disintegration of flours and starches has

been studied by using hydrochloric acid, diastase, and pancreatin on *Bajri* (*Pennisetum typhoideum*), *Vari* (*Panicum miliacium*), Gram (*Cicer arietinum*) and Black Gram (*Phaseolus radiatus*).

The disintegration of the proteins from the flours of the same grains with proteolytic enzymes has been already published by the authors (11).

### Preparation of the flours and starches

These were prepared from the grains by first removing the impurities. The grains were washed with water, cleaned and dried. These were then crushed to flours in a hand-mill, steeped in a solution of 0.4 per cent. NaOH, filtered through muslin cloth and kept overnight. The supernatant liquid was then removed next day and the bulk was acidified with HCl and repeatedly washed with distilled water till it was free from chlorides. It was then filtered through filter paper and dried. It was powdered and passed through 200 mesh sieve and preserved with some quantity of toluene. The flours prepared from cleaned grains were similarly made in a hand-mill and passed through 200 mesh sieve. The grains were obtained from the local market.

The general analysis of the flours which were used for the disintegration is given in Table 1.

Reducing sugars were determined by Bertrand's method and the other constituents such as starch, proteins, crude fibre, etc. were determined according to the A. O. A. C. methods (12).

TABLE 1  
General analysis of the flours.

	<i>Bajri</i> per cent	<i>Vari</i> per cent	Gram per cent	Black gram per cent
Moisture	7.44	10.10	8.24	7.23
Ether extracts	5.60	4.36	4.52	2.20
Proteins	9.60	7.53	20.03	23.21
Carbohydrates	74.74	70.69	61.22	59.16
Crude fibre	0.92	4.62	3.89	3.93
Ash	1.70	2.70	2.10	4.27

## Disintegration of starches with hydrochloric acid

Although it is of great importance to study the disintegration of flours, as it is the usual form in which the grains are used, still the study of the disintegration of the pure starches is of no less importance. In the study of the disintegration of the flours it is quite likely that the constituents of the grains other than the starches may exhibit a certain amount of influence on the values of disintegration. Effront (13) in his studies has made a similar observation. So the results obtained may be too high or too low but if the disintegration of the pure starches is known the amount of the influence exerted by the other constituents of the grains may be judged. Moreover the problem is interesting from another point of view. A large amount of work has been done by many workers on similar lines as mentioned before, and they have found that the starches from different origins differ considerably in their susceptibility to the different ferments. Although it is a fact that the susceptibilities of the different starches were found with respect to different enzymes it is not found with hydrochloric acid. As hydrochloric acid can hydrolyse the starches to give rise to reducing sugars it was thought interesting to study the action of hydrochloric acid on different starches.

Half a gram of each of the starches was exactly weighed out in a conical flask and made into a thin paste with 10 c. c. of water and 10 c. c. of HCl were then added to it. The flasks were kept for definite periods at room temperature which varied from 27° to 30°C. After the fixed period the excess of hydrochloric acid was neutralised with sodium carbonate, clarified with a small amount of zinc oxide and the volume made to 100 c. c. It was then filtered on a dry filter paper and 10 c. c. of the filtrate used for the determination of reducing sugars by the Bertrand method. The reducing sugars thus obtained were multiplied by 0.93, the conversion factor for the starch and the percentage of hydrolysis calculated. The strengths of the acid were 25 per cent., 37.5 per cent. and 50 per cent.; and the periods were 24 hours, 48 hours and 72 hours. The results obtained in each case are given in Table 2.

TABLE 2

Disintegration of different starches with hydrochloric acid.  
Expressed on the basis of the total hydrolysable starch.

	25 per cent HCl			37.5 per cent HCl				50 per cent HCl			Total starch per cent
	24 hours per cent	48 hours per cent	72 hours per cent	24 hours per cent	48 hours per cent	72 hours per cent	24 hours per cent	48 hours per cent	72 hours per cent		
<i>Bajri</i> starch	3.04	3.47	4.62	4.95	13.64	24.75	25.80	49.90	62.81	90.31	
<i>Vari</i> starch	1.65	3.31	4.41	4.62	13.09	23.21	20.13	35.31	50.05	92.04	
Gram starch	2.95	5.31	3.04	5.83	16.72	23.98	26.73	44.33	65.77	88.60	
Black gram starch	2.34	2.95	1.97	4.69	14.74	22.11	27.80	44.11	60.06	89.13	

From the figures from 25 per cent. HCl it is seen that there is little difference in the values for *bajri* and *vari* after 72 hours. Between gram starch and black gram starch, gram starch is more easily attacked than black gram starch. Of all the four starches gram starch gives the maximum hydrolysis value while black gram starch gives the minimum value. With an increase in the strength of the acid to 37.5 per cent., the values for the hydrolysis in all cases are very much increased. The values after 24 hours in this case are nearly equal to the values obtained after 72 hours in the case of 25 per cent. HCl. The differences in the various values of hydrolysis with 37.5 per cent. HCl for all the starches are very nearly equal which was not so when the hydrolysis was carried out with 25 per cent. HCl. It is interesting to note that values obtained after 24 hours of hydrolysis with 50 per cent. HCl are nearly equal to the values which are obtained with 37.5 per cent. HCl after 72 hours. It is observed in general that between starches from the two cereals, *bajri* starch is more easily attacked, while between the starches from the pulses, gram starch is more easily hydrolysed. In general it is observed that of the four starches gram starch is more easily attacked with 50 per cent.

HCl. and 25 per cent. HCl. while black gram starch is least attacked by 37.5 per cent. HCl.

### Disintegration of the starchy material of the flours with hydrochloric acid

After having studied the disintegration of the starches extracted from the different grains, the flours themselves were taken and experiments were carried out with a view to find out if there was any influence of the non-starchy material of the grains, especially the proteins, on the disintegration of starch. The experimental details were exactly the same as described above. Except that one gram of the flour was used in each case. The results were calculated on the basis of the total hydrolysable starch. The results are given in Table 3.

TABLE 3  
Hydrolysis of the flours with HCl.

*Expressed on the basis of the total hydrolysable starch*

	25 per cent HCl			
	24 hours per cent	48 hours per cent	72 hours per cent	Total
<i>Bajri</i> flour	5.64	7.58	12.95	59.82
<i>Vari</i> flour	2.20	4.22	6.20	56.23
Gram flour	13.82	14.81	18.17	42.32
Black gram flour	16.72	18.96	19.52	50.37
	37.5 per cent HCl			
	per cent	per cent	per cent	per cent
<i>Bajri</i> flour	19.21	31.96	38.08	59.82
<i>Vari</i> flour	18.62	31.16	37.81	56.23
Gram flour	20.93	33.35	34.50	42.32
Black gram flour	21.20	34.60	38.40	50.37
	50 per cent HCl			
	per cent	per cent	per cent	per cent
<i>Bajri</i> flour	40.12	69.19	78.23	59.82
<i>Vari</i> flour	27.93	67.03	78.23	59.82
Gram flour	40.71	70.15	86.74	42.32
Black gram flour	38.08	69.77	80.41	50.37

For all the strengths of the acid *bajri* flour is more easily hydrolysed than *vari* flour. The differences between the values go on decreasing with increasing strengths of the acid. Between gram flour and black gram flour, black gram flour gives higher

values for the 25 per cent and 37.5 per cent HCl, while gram flour gives higher values only with 50 per cent HCl. Considering the values for all the flours in general it is seen that black gram flour is most easily attacked whereas *vari* flour is attacked with difficulty. In all cases the values for the cereal grains are lower than the values for the pulses. When these figures are compared with those obtained for the hydrolysis of the different starches it is noted that for every corresponding period and every corresponding strength, each value of the flour is considerably more than that for the starch from the same grain. For instance the values for the *bajri* starch with 37.5 per cent HCl are 4.95, 13.69 and 24.75 whereas the corresponding values for the flour are 19.21, 31.96 and 38.08. The observation in the case of other grains is quite similar to this. This observation is supported by the observation of Effront (*loc. cit.*) who has found that the proteins of the flours have a decided beneficial effect upon the course of the hydrolysis of the starchy material. The observation was made with special reference to diastase, but here we find that it is repeated even in the case of disintegration of the starches with HCl. The greater difference in the values for the pulse flours and pulse-starches is obviously due to the greater quantity of the proteins in the pulse grains than the cereals. This shows that the non-starchy bodies in the grains have a good deal of influence on the rate of hydrolysis of the starchy material. A similar observation in the case of rice was made by Sahasrabudde and Kibe (*loc. cit.*).

### Disintegration of starches with diastase.

In order to find out the amount of influence exerted by the non-carbohydrate material, it is necessary to get the values of hydrolysis of the pure starches with the same ferment and under similar conditions of experiment, so that the difference between the values of disintegration for the flours expressed as the percentages on the basis of the total hydrolysable starch contained by the grains and those for the pure starch of the grain may be regarded as the total amount of the influence of the non-carbohydrate bodies, and hence the study of the diastase hydrolysis of the starch was followed.

One-fourth of a gram of the oven dry starch of each kind was exactly weighed into a conical flask and made into a thin paste by adding 10 c. c. of water. To this paste 50 c. c. of boiling water were added and the flask was kept in the boiling water bath for three minutes. It was then cooled and 30 c. c. of the acetate buffer of pH 4.8 together with 10 c. c. of diastase solution of different strengths were added. It was then incubated at 37° for the fixed period, at the end of which the enzyme was killed by a few drops of 10 per cent NaOH and the solution was clarified with zinc oxide (14). The volume was made to 100 c. c. and filtered on a dry filter paper and 10 c. c. of the filtrate were used for the estimation of the reducing sugar by Bertrand's method. The digestibilities were calculated on the basis of the total hydrolysable starch. The results are given in Table 4.

TABLE 4  
Disintegration of starches with diastase  
*Expressed on the basis of the total hydrolysable starch.*  
(Starches)

	0.2 per cent			0.5 per cent			1.0 per cent			Total per cent
	1 hour per cent	2 hours per cent	3 hours per cent	1 hour per cent	2 hours per cent	3 hours per cent	1 hour per cent	2 hours per cent	3 hours per cent	
<i>Bajri</i> starch	21.12	22.66	23.28	23.98	31.02	31.90	31.02	35.20	35.64	90.31
<i>Vari</i> starch	22.66	23.65	23.98	29.43	32.01	32.98	36.98	35.64	37.62	92.04
Gram starch	26.84	27.72	28.16	31.02	32.12	33.00	31.02	31.02	35.42	88.60
Black gram starch	26.62	27.50	27.94	29.92	30.03	31.79	30.25	31.25	33.22	89.13
	(Flours)									
<i>Bajri</i> flour	28.56	31.96	35.02	30.60	35.19	37.70	35.02	39.44	45.56	59.82
<i>Vari</i> flour	29.64	34.20	36.48	31.92	34.48	39.14	38.38	43.58	49.18	56.23
Gram flour	34.12	34.27	35.19	34.27	34.73	36.57	34.50	35.85	38.64	42.32
Black gram flour	30.60	31.80	32.40	31.20	31.60	33.20	36.00	36.40	36.60	50.37

Between *bajri* and *vari* starches there is little difference in the values 0.2 per cent. diastase. The values of hydrolysis for the same strength in the case of the pulse starches are higher than those for the cereal starches. As the strength of the enzyme is increased the difference in the various values for the starches goes on decreasing. For 1 per cent. diastase *bajri* starch and gram starch are equally attacked. Between *bajri* and *vari* starch, *vari* starch is more easily attacked than the *bajri* starch and between gram starch and black gram starch, gram starch is more easily disintegrated than the black gram starch.

#### Disintegration of flours with diastase.

The disintegration of the flours was studied with diastase with the same object in view as in the last case i. e. to find out if there was any influence of the non-starchy bodies on the amount of the hydrolysis of the starchy material. The experimental details were the same as in the case of the starches. The results are given in Table 4.

The values of hydrolysis for the various flours in general are higher than the corresponding values for the starches. Between *bajri* flour and *vari* flour, *vari* flour is more easily attacked by the enzyme than *bajri* flour, and between gram flour and black gram, gram flour is more easily attacked by the lower strength of the enzyme, i. e. 0.2 and 0.5 per cent, diastase, while for 1 per cent diastase black gram flour gives very nearly the same value. Among the various flours *vari* is most easily attacked while gram flour is least easily attacked.

Here again the observation similar to the previous one was made regarding the influence of the non-starchy material on the hydrolysis of the starchy material. The difference in the values for the starches and flours is higher in the case of the cereal grains than in the case of the pulses.

#### Disintegration of starches and flours with pancreatin.

Pancreatin is a ferment which resembles diastase in giving similar end products of the starch hydrolysis. In the



hydrolysis of the starches the end product is the reducing sugar which may have been formed by varying stages of hydrolysis the number and nature of which may differ from ferment to ferment. It is, however, essential to find out their comparative diastatic powers so that we may be enabled to compare the values for the two ferments, after bringing them to the common basis. The diastatic power for pancreatin was therefore compared with that of the standard diastase which was used in the foregoing work.

For comparing the diastatic powers of the different enzymes, various methods based on the Fehling solution were used. Shermann (15) had developed a method to compare the diastatic values of different enzymes which could be expressed on the Linthar scale. The following procedure was followed in the present case.

A 2 per cent. solution of the soluble potato starch was prepared in the usual way by pouring boiling water over the paste and boiling it for 10 minutes in a boiling water-bath. 50 c. c. of the solution thus prepared were taken in different flasks and to one was added 10 c. c. of 0.5 per cent. solution of diastase and to another 10 c. c. of pancreatin together with 40 c. c. of the acetate buffer solution of pH 4.8. The flasks were incubated at 37°C for one hour, the enzymes were killed by a few drops of 10 per cent. NaOH and the volume was made to 250 c. c. It was clarified with zinc oxide and filtered on a dry filter paper. 10 c. c. of the filtrate were used for the determination of the reducing sugars by the Bertrand method. The ratio of the reducing sugars produced was taken as the ratio of the diastatic powers of the enzymes. This ratio was found to be 6:5 which was therefore taken as the ratio of the enzyme powers. The diastatic power of pancreatin was therefore  $\frac{5}{6}$ th of the diastase.

So in order to make the results obtained with pancreatin comparable with those of the diastase, all the values were multiplied by  $\frac{6}{5}$ . The experimental procedure was exactly similar to the procedure as in the case of the diastase. The results are calculated on the basis of the total hydrolysable starch (oven dry).

In all, three strengths of pancreatin were chosen, viz 0.2, 0.5 and 1 per cent., as in the case of the diastase. The periods of hydrolysis were one hour, two hours and three hours, the temperature being 37°C. The results are given in Table 5.

TABLE 5

Disintegration of starches and flours with pancreatin  
*Expressed on the basis of the total hydrolysable starch*  
(starches)

	0.2 per cent			0.5 per cent			1.0 per cent			Total per cent
	1 hour per cent	2 hours per cent	3 hours per cent	1 hour per cent	2 hours per cent	3 hours per cent	1 hour per cent	2 hours per cent	3 hours per cent	
Bajri starch	19.58	20.46	21.12	20.13	22.36	23.98	21.12	26.84	30.58	90.38
Vari starch	21.13	21.89	22.66	20.27	22.56	22.66	18.48	27.28	31.91	88.04
Gram starch	19.91	22.44	24.42	21.12	29.48	29.59	22.56	28.28	32.33	88.60
Black gram starch	19.69	22.66	24.46	21.19	26.84	28.38	22.67	28.16	32.23	89.13
	(Flours)									
Bajri flour	22.40	27.71	32.64	34.47	36.72	39.90	42.84	48.11	55.93	59.82
Vari flour	24.32	28.69	33.06	33.16	37.54	37.88	31.92	34.77	50.16	56.23
Gram flour	27.33	29.90	37.49	36.34	39.56	40.22	38.64	41.53	50.16	42.32
Black gram flour	24.40	25.60	32.70	33.84	36.96	37.40	33.80	39.70	46.44	50.37

From the figures it is seen that all the flours are better digested by pancreatin than the diastase, while all the starches in general are better digested by the diastase than the pancreatin. The catalytic effect of the non-carbohydrate bodies was better seen in the case of the pancreatin than in the case of diastase. In the case of the diastase the difference in the values of the cereals and the pulses was greater than in the

case of the pancreatin. The pancreatin therefore attacks the cereals more easily than the diastase, but it attacks the pulses far better than the diastase.

### Conclusions

1. From the results of all the experiments described so far it has been found that with the increasing strengths of the hydrolysing agents the values of disintegration gradually increase.

2. The values of the disintegration both for the starches and flours are found to increase when the period of reaction is increased, although the increase is not exactly proportional to the time.

3. It is noticed in many cases the *vari* flour requires a little more time to start the reaction. But once the reaction starts it proceeds with the increasing periods of reaction.

4. In the case of the diastase hydrolysis of the flours, *vari* flour is more easily disintegrated than *bajri* flour while in the case of pancreatin and hydrochloric acid *bajri* flour is more easily disintegrated than the *vari* flour.

5. With diastase and the pancreatin as hydrolysing agents gram flour is more easily disintegrated than black gram flour.

6. The values of disintegration of the flours are in general higher in the case of pancreatin than in the case of diastase.

7. The values of the disintegration of the cereal flours for hydrochloric acid are nearly equal to those of the pulse flours while for diastase and pancreatin the values for cereal flours are greater than those for the pulse flours.

8. In the hydrolysis of the starches it has been observed that there is very little difference in the values for hydrolysis with different reagents, for any particular strength of the hydrolysing agents or the period of the reaction. The values of disintegration, however, go on increasing with the period of the reaction or the strength of the hydrolysing agent.

9. The values of disintegration for the flours are in all cases higher than those for the starches from the same grains under similar conditions of experiment. This shows that there is a good deal of effect of non-carbohydrate bodies on the course of the hydrolysis. The maximum difference between the values for the flours and starches and hence the maximum effect is found in the case of the hydrochloric acid disintegration, while minimum difference is noticed in the case of diastase hydrolysis. In the case of pancreatin as the hydrolysing agent the difference in the values for the flours and starches is intermediate of the two mentioned above.

10. As regards the disintegrating capacity of the various reagents hydrochloric acid is found to be the best while pancreatin is of the third order, as far as the flours are concerned.

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**PAPER II****A STUDY IN THE HYDROLYSIS OF THE  
STARCHES OF SOME IMPORTANT CEREALS**

By D. N. GODBOLE, M. Sc.

*(With the kind permission of the authorities of Bombay University)***SECTION I****Introduction**

The work described in this paper forms a part of the series of investigations—undertaken at the Biochemistry laboratory of the Nowrosjee Wadia College, Poona—Viz. Disintegration of the different food-stuffs. The present work, however, is restricted to the study of the hydrolysis of the starches in different grains, especially the cereals.

It has been definitely established that the nutritive value of the food-stuffs depends upon the quantity of the essential constituents of the food-stuffs, such as carbohydrates, proteins, fats etc. which the food-stuffs contain, and also on how much of each of the constituents is broken down.

The cereals are rich in carbohydrates, particularly in starch, while they contain less albuminoids than the pulses. Wheat, however, contains more albuminoids than the other cereals. (Bull. No. 124, 1925, Dept. of Agri. Bombay. The chemical composition of food grains, vegetables and fruits of Western India by Dr. D. L. Sahasrabuddhe.)

The work described in the following pages is mainly restricted to the study of the carbohydrates or starchy portion of the food grains, and to what extent it is available when acted upon by the enzymes which are present in the alimentary canal, and which hydrolyse the carbohydrates into simpler material.

While studying the disintegration of the food grains we must know, not only the percentages of the different ingredients in them but we must also know how much of each is available and in what way it is available. We must also know the limits

of disintegrations of the grains during their passage through the alimentary canal. W.E. Stone (U.S.A. Dept. Agri. 1896, Bull 34 . Bernetzky (quoted by Stone L.C.), C. F. Langsworthy (J. Biol. Chem. LII-1920) and H. J. Deual (J. Biol. Chem. 1920) studied the disintegration of starches with the same view to find their digestibilities while the chemical transformation, as such, was studied by C. T. Lathier (J.C.S. 1895 LXVII)—Brown (J.C.S. 1890, LVII, p. 458), Morris (J.C.S. 1890 LVII, p. 458) and others. W. E. Stone (L.C.) established an order of decreasing susceptibility of various starches to the action of the ferments he had used. Bernetzsky and Lenberg (quoted by Stone L.C.) and Hammerstein (Ber. 1883 quoted by Stone) however, working on the same line had, each of them got different order of saccharification. No explanation is given. The only thing we can say that the nature of susceptibility of the starches depends upon the types of ferments and the types of starches.

In 1920 the biological part of starch digestion was studied by C.F. Langsworthy (L.C.) and H. J. Dueul (L.C.) and they found that raw starches, like those of wheat, corn and potato were completely assimilated. Potato starch caused some physiological disturbance.

Rockwood (J. Biol. Chem. 1910 P. 32) found by starch iodine reaction that Pancreatin, Ptylin, and diastase hydrolyse the starches from bleached and unbleached wheat flours at equal speeds. The chemical study of the products of hydrolysis of starch has been carried out by a number of workers such as Brown and Morris (L.C.) Baker and Lind (J.C.S. 1895 LXVII p. 702 to 708) while the conditions of the action the ferments of starch have been studied by Detmar (Z. Physiol. Chem. VII 1882) Effont (Comptes Rendus 1892 CXV) Kjeldhal (Z. Gas. Brauw 1880) and others.

Patwardhan and Norris (J. Indian Inst. Sci. 1923. 11 A 127) found activation in starch hydrolysis with melted cholam diastase and inactivation with unmelted cholam diastase at high salt concentration. Eadie (Bio. Chem. J. 1926, 20, 1016) observed slight activation at salt concentrations of about

i. 62 N, Sherman Caldwell and Adams (J. Am. Chem. Soc., 50,2538, 1928) found the effect of neutral salts on Pancreatic amylase in starch hydrolysis. Neutral salts are necessary to the activity of pancreatic amylase. Anions are more influential than cations, so the values obtained for the pure starches or isolated proteins will not be the same as those which may be obtained when these substances are taken together with the other constituents as found in the grains. Sabasrabuddhe and Bhide (Agri. College Mag. Dec. 1943. Vol. XXXV No. 1 and 2) studied, with the same idea, the hydrolysis of the starches in grains without separating starches from them and compared with the hydrolysis of their starches separated from the other constituents and found a definite effect of the other constituent on starch hydrolysis. Even the origin and nature of starch have definite effect on their hydrolysis. It is generally recognised that the starch consists of two components amylose and amylopectin. The investigation of Semec and his collaborators have shown that the ratio of amylose to amylopectin varies with the origin of the starch. So the starches from various origin have different nature of enzymic hydrolysis.

Since it is customary to eat flours or grains as such and not the starches or proteins in isolated form the study, of the disintegration of flours of grains as such, must be done in order to get an exact idea of their usefulness. This utility can be studied in two ways. One with the aid of animals by feeding them and studying the changes. The other is a laboratory method. The laboratory method consists of imitating as far as possible the process, which takes place in the alimentary canal. Even if we are unable to copy out the exact process, we can study the nature of reactions that take place and thereby find out the relative values. Thus in the in vitro method we get better comparable results. Another great advantage in choosing this method is that we can study the reactions by stages. However it is always desirable to confirm the results thus obtained in the in vitro experiments, by verifying them on the animals.

From the above discussion it will be seen that the in vitro experiments would give us a true comparative picture of the

disintegration of the constituents of the various grains! On this line a number of workers have attempted at different times to judge the food values. All these attempts were made in a scattered manner and the results they have obtained were of conflicting nature. But with the help of the recent findings many of the discrepancies can be explained. The two-enzyme theory, first proposed by Wijman (Rec. Tran. Chem. 1890) shows that the diastase consists of two enzymes, one hydrolyses starch to maltose and erythrogranulose and the other converts this erythrogranulose to leuco-dextrin and starch to malto-dextron. But Chrzasztes (Biol. Chem. Z. 1923, 142, 417; Ibid 1924, 150, 60) considers three distinct stages of starch hydrolysis (1) Liquefaction (2) Dextrin formation and (3) sugar formation. In the light of the above findings we can say that some workers have gone up to a certain stage of hydrolysis and drawn their conclusions while the others have gone to some different stage of hydrolysis and formed their conclusions. So it is necessary to try as many hydrolysing agents as possible in order to get a general idea about the utility of the food-stuff from the digestion point of view. We must also study the hydrolysis at different stages.

As we have restricted ourselves to the study of the hydrolysis of the starchy portion of the food grain we have chosen the cereals for our investigations. The flours we have studied are:- wheat, *jowar*, *rajgira* and *nachani*. All these flours were subjected to the action of hydrochloric acid, diastase and pancreatin.

In the case of hydrochloric acid hydrolysis we have selected three strengths—25%, 37.5% and 50%—and the periods were 24 hours, 48 hours and 72 hours. All the reactions were studied at the room temperature that is at 26 to 28°C. In the case of diastatic and pancreatic reactions, we have chosen three strengths 0.5%, 1.0% and 1.5% and the periods were 1 hour, two hours and three hours. The temperature of the reaction mixture was kept at 37°C in all experiments which is the normal temperature of the human body.

#### **Material:—**

Four varieties of food grain were chosen for the study of the



starch hydrolysis. They are wheat, *jowar*, *rajgira* and *nachani*. All of them are cereals. All of these grains are grown and consumed by the people of Bombay Presidency.

Wheat:—Botanical name—*Triticum sativum*. 2. *Jowar*:—Botanical name—*Andropogon sorghum*. 3. *Rajgira*:—Botanical name—*Amaranthus paniculatus*.

### Preparation of the Material :

The grains were first washed with water, dried in the sun and turned into flour by a hand mill. The flour was passed through 200 mesh screen and stored in the stoppered bottles with a few drops of toluene. The following is the general analysis of investigation.

TABLE No. 1

	Wheat	<i>Jowar</i>	<i>Rajgira</i>	<i>Nachani</i>
Moisture	9.97	7.98	9.35	8.55
Ether extract	2.05	3.25	5.03	1.75
Proteins	11.25	7.83	15.01	5.71
Carbohydrates	73.66	76.64	65.72	78.27
Crude fibre	1.36	1.25	2.83	2.88
Ash	1.70	3.05	2.05	2.84

Reducing sugars were determined by Bertrand's method, the other constituents, such as total hydrolysable starch, proteins, crude fibre etc., have been determined according to the methods of analysis of the Association of the Official Agricultural Chemists of U. S. A. 1926.

The buffer solutions were prepared according to the Snell's colorimetric estimation. For pH. 4.8 acetate buffer was used. When the reactions were carried out at various pH disodium and citric acid buffers were used as these solutions give a wide range of pH. Diastase used was E. Merk's 1.60. Pancreatin used was E. Merck 1.40.

## SECTION II.

## THE DISINTEGRATION OF STARCHES IN DIFFERENT FLOURS BY HYDROCHLORIC ACID.

In order to get the general idea of disintegration of the starches, hydrochloric acid the common reagent for the hydrolysis of starches, was used in these experiments. This gave the general nature of disintegration of the starches in different flours.

### Procedure :

1 gram of the flour under study (oven dry) was taken in a conical flask and 100 c. cs. of distilled water were added and heated on water bath for 3 minutes in order to gelatinise the flour. Then 10 c.cs. of each of 25%, 37.5% and 50% of hydrochloric acid were added to the flask and kept at room temperature for 24 hours, 48 hours and 72 hours. At the end of the fixed periods the reaction mixture was neutralised with sodium carbonate and was clarified with zinc oxide. The volume was made up to 100 c.cs. and filtered on dry filter paper. 10 c.cs. of the filtrate were taken for the estimation of the reducing sugars by Bertrand method. The figures thus obtained were multiplied by 0.83 the conversion factor for starch and the percentage hydrolysis was calculated on the basis of total hydrolysable starch (oven dry).

TABLE No. 2

Per cent. disintegration of the flours with 10 c.cs. of 25% hydrochloric acid, expressed on the basis of the total hydrolysable starch (oven dry).

	24 hours	48 hours	72 hours	Total starch hydrolysable
Wheat ...	22.05	23.95	25.72	74.29
Jowar ...	6.53	10.19	13.63	77.13
Rajgira ...	17.64	23.11	26.89	59.67
Nachani...	6.45	9.18	11.37	71.67

TABLE No. 3 -

Per cent. disintegration of the flours with 10 c.cs. of 37.5% hydrochloric acid, expressed on the basis of hydrolysable starch (oven dry).

	24 hours.	48 hours.	72 hours.	Total starch hydrolysable
Wheat ...	24.75	27.68	29.61	74.29
Jowar ...	8.44	11.63	14.68	77.13
Rajgira ...	22.23	25.74	28.07	59.67
Nachani ...	7.54	11.37	13.03	71.67

TABLE No. 4

Per cent. disintegration of the flours with 10 c.cs. of 50% hydrochloric acid expressed on the basis of hydrolysable starch (oven dry).

	24 hours.	48 hours.	72 hours.	Total starch hydrolysable
Wheat ...	28.66	31.60	33.83	74.29
Jowar ...	11.62	14.75	17.10	77.13
Rajgira ...	23.11	31.22	31.71	59.67
Nachani ...	9.56	13.56	17.99	71.67

From the above tables we see that Wheat flour is the most susceptible to the action of hydrochloric acid while Jowar and Nachani are the least susceptible to the action. Practically there is very slight difference between the figures of Jowar and Nachani flours. Both are hydrolysed the least during the three periods and with three strengths.

Rajgira flour shows a peculiar mode of reaction. With 25% acid within 24 hours it is hydrolysed to the extent of 17% but on increasing the strength to 37.5% or period up to 48 hours the figure rises to 23% which is nearly equal to that of wheat flour at that period and at that strength and on further increasing the strength and period the rate of hydrolysis is found to be nearly the same as that of Wheat flour. So Rajgira flour is a bit more difficult to disintegrate than wheat flour in the beginning and then it is hydrolysed with equal ease as the wheat flour.

It is noticed that flours containing more proteins are more susceptible to the action of hydrochloric acid than the flours which contain less proteins. Here Wheat and Rajgira contain 11.2 per cent. and 15% proteins respectively and in their flours we see more disintegration than Jowar and Nachani flours which contain 7.8% and 5.7% proteins respectively. Bhide and Sahasrabudde ( Ph. D. thesis 1942, University of Bombay ) made a similar observation. Pulses which contain more proteins are more easily attacked by hydrochloric acid than cereals which contain less proteins. This observation is in accordance with the observation made by Effront (comptes Rendus 1892 6 XV ) who has found that the proteins of the flours have decided beneficial effect upon the course of hydrolysis. This observation was made with special reference to the action of diastase but here we find a similar type of behaviour in the case of disintegration by hydrochloric acid also.

### SECTION III A

## DETERMINATION OF OPTIMUM pH FOR DIASTASE.

### Introduction

Modern workers have found the importance of the pH in the enzyme reactions. Different enzymes work at different pH values and temperatures for their maximum effect. The optimum pH for diastase is found to be 4.8 by the workers such as Sherman (J. A. C. S. 1919, 41, 231), Maslow (1926 J. Bio. Chem. 68, 83, 0), Core (1925 J. A. Chem. Soc. 47, 28). This value of the optimum pH was determined by various workers by using pure starch as the substrate. As we are going to use the flours as such as the substrate it was thought desirable to find whether there is any effect on the optimum pH value due to the constituents other than the starch in the grains.

#### *Determination of optimum pH.*

Experiment:- 1 gram of the flour was taken in conical flask of 150 c. cs. capacity. 10 c. cs. of distilled water were added and heated on a water bath for 3 minutes to gelatinise

the flour. Then 10 c. cs. of buffer solutions (phosphate) of different pH were added together with 10 c. cs. of 1% diastase - solution and kept in an incubator at 37°C. At the end of an hour the flasks were taken out and 10 drops of 10% caustic soda solution were added to check the further reaction. The reaction - mixture was clarified with zinc oxide. The volume was made up to 100 c. cs. and filtered on dry filter paper. 10 c. cs. of the filtrate were taken for the estimation of reducing sugars by Bertrand's method.

TABLE No. 5.

Following are the quantities in c.cs. of  $\text{KMnO}_4$  (0.1 N) required for 10 c.cs. of the filtrate.

pH	Wheat	Jowar	Nachani	Rajgira
2.2	2.2	1.50	-	-
4.3	6.85	6.50	3.90	4.2
4.8	7.60	8.50	7.40	5.1
5.4	7.0	6.80	5.40	4.8
5.7	2.6	5.30	-	3.0
6.5	6.2	4.60	4.40	4.1
6.9	6.09	5.20	4.50	4.9
7.2	6.00	5.9	5.00	4.5
8.0	5.85	4.60	3.4	-
8.3	5.70	4.4	-	-

From table No. 5 and graph No. 4 it is seen that there are two optimum values for all the flours used. One is at 4.8 in all flours, which is greater than in the other.

#### COMPARATIVE DISINTEGRATION OF THE FLOURS MADE WITH THE HELP OF DIASTASE

Now in the present section a study of comparative disintegration of the flours is made with the help of diastase.

1 gram of the flour (oven dry) of each type was accurately weighed on a conical flask and 20 c.cs. of distilled water were added and heated in water bath for 3 minutes to gelatinise. It was then cooled and 10 c.cs. of acetate buffer solution of

4.8 pH were added (the optimum pH for diastase found in the last section) 10 c.cs of the diastase solution (0.5%, 1.0%, 1.5%) were added. A few drops of toluene were added to prevent any bacterial action. At the end of the fixed period, 5 drops of 10% NaOH were added to check the further reaction.

The reaction mixture was clarified with zinc oxide. The volume made up to 100 c.cs. and filtered on a dry filter paper. 10 c. cs. of filtrate were taken for the estimation of the reducing sugars by Bertrand's method. The figures thus obtained were multiplied by 0.93, the conversion factor for the starch and the percentage hydrolysis was calculated on the basis of the total hydrolysable starch (oven dry).

In all three strengths (0.5%, 1% and 1.5%) of the diastase were used—10 c.cs. of each strength were taken at a time—and the periods allowed were one hour, two hours and three hours. The diastase used was E. Merk 60/100. For every strength and period a blank experiment was made and the reading of the blank was subtracted from the readings obtained.

The results obtained in various trials are given below.

TABLE No. 6

Per cent. hydrolysis of the flours with 10 c.cs. of 0.5% diastase expressed on the basis of total hydrolysable starch (oven dry).

Name of the flour.	1 hour.	2 hours.	3 hours.	Total starch hydrolysable.
Wheat ...	20.85	23.61	27.62	74.29
Jowar ...	24.94	26.85	28.74	77.13
Rajgira ...	21.74	29.49	31.92	59.67
Nachani ...	27.91	31.34	32.00	71.67

The action of 0.5% diastase appears to be almost equal on all the flours. Nearly 30% of the total hydrolysable starch of the flours is hydrolysed at the end of 3rd hour. The Wheat and Jowar flours were hydrolysed a bit less than 30% of their total hydrolysable starches while Rajgira and Nachani flours

were hydrolysed more than 30% of their total hydrolysable starches. During the 1st hour Wheat and Rajgira flours are disintegrated up to 21% while Jowar and Nachani flours are hydrolysed to the extent of 25% and 28% respectively.

If the difference between the figures of first and second hours, be taken as the rate of hydrolysis, during the second hour, the rate of hydrolysis of Jowar flour is only 1.6% while that of Rajgira flours is 7.75% and in the case of Wheat and Nachani it is 2.76% and 3.43% respectively. The Rajgira flour is more easily attacked by the enzyme during 2nd period.

At the 3rd hour the rate of hydrolysis (the difference between the figures of second and third hour) is more i. e. 4.01% in the case of wheat flour and in the case of Nachani flour it is the least i. e. 0.66%. The rates of Jowar and Rajgira flours are 2.19% and 2.13% respectively which are intermediate between Wheat and Nachani flours.

Considering the figures for Wheat flour, it is seen that during the first hour 20.85% of the total hydrolysable starch is hydrolysed and during second and third hour the rate drops down considerably. The rise in the second hour is only 2.8 and the difference between second and third hour is 4.0.

The percentage distribution of hydrolysis over the various periods was 20.85% during the first hour 2.08% during the second hour, and 4% during the last hour. If these figures are looked upon as the rate of hydrolysis during the various periods then it may be said that the reaction proceeds with a great velocity during the first period, it then falls to about 1/9 of the original rate which again increases to about 1/6 of the original rate, in the last period.

Similar observation is made in the case of Jowar flour. The percentage distribution, during the first period was 24.9%, 1.6 %, and 2.2% during the second and the third periods. The rate of hydrolysis shows that it is 25% during the first period and drops down to 1/15 and again increases to 1/10 of the original rate.

In the case of Rajgira the percentage distribution is 22% in the first hour, then the rate drops down to 7.5% and 2.5% in the second and third periods. Comparing the figures of hydrolysis of the other flours we find that Rajgira flour which is hydrolysed more easily than wheat flour but less than Jowar and Nachani flours in the first hour, is disintegrated more easily than Wheat and Jowar flours in the second hour and in the third hour.

As regards Nachani flour it is disintegrated more easily in all the periods. The percentage distribution is 28% in the first hour and then the rate drops down to 2.5% and 7% in the second and third hours.

So in the first hour the order of increasing susceptibility is Wheat, Rajgira, Jowar and Nachani. In the second hour the order is Wheat, Jowar, Rajgira and Nachani and the same is maintained in the third hour. This shows that the periods of the reaction mixture affect the extent of hydrolysis of the different flours.

Now, it would be interesting to see whether changes in the strength of the enzyme has any effect on the alteration of the orders of susceptibility. So experiments were carried out with similar experimental details with a change of the strength of the diastase.

TABLE No. 7

Percentage hydrolysis of the flours with 10 c.cs. of diastase 1%, expressed on the basis of total hydrolysable starch (oven dry).

Name of the flour	1 hour.	2 hours.	3 hours.	Total starch hydrolysable
Wheat flour	26.92	28.17	22.81	74.29
Jowar ..	27.78	29.54	30.57	77.13
Rajgira ..	26.76	31.93	33.52	59.67
Nachani ..	29.01	33.11	34.57	71.67



On increasing the strength of the diastase there is little difference in hydrolysis of flours of Jowar and Nachani. The rise is of about 2% in the case of Wheat flour while in the case of Rajgira flour the rise is more i.e. 5% during the first hour as compared with the figures of 0.5% diastase in the same hour. In the case of Wheat the rise in the figure is nearly constant i.e. 1.3 between 1st and 2nd hour and 1.7% between 2nd and 3rd hour. Similar is the case of Jowar.

In the case of Rajgira and Nachani the difference between 1st and 2nd hour is 5.67 in the case of Rajgira and 4.1 in the case of Nachani. But the rate during the third hour drops to only 1.4%.

Comparing all these figures, Wheat and Jowar are hydrolysed more easily during the first hour and there is very slow reaction after that i.e. 1/20 and 1/15th of that in the first hour respectively. In the case of Nachani and Rajgira the same effect is observed but the reaction between first and second hour is also appreciable i.e. 1/5th and 1/7th of that of first hour respectively. Here also in the case of Rajgira which stood second in the order of increasing susceptibility (Wheat, Rajgira, Jowar and Nachani) in the first hour is changed in order as Wheat, Jowar, Rajgira and Nachani as in the case of 0.5% diastase.

TABLE No. 8

Percentage hydrolysis of the flours with 10 c.cs. of 1.5% diastase expressed on the basis of total hydrolysable starch (oven dry).

Name of the flour	1 hour.	2 hours.	3 hours.	Total starch hydrolysable
Wheat flour	28.17	31.09	33.01	74.29
Jowar ,,	28.94	31.52	33.15	77.13
Rajgira ,,	29.66	32.98	34.57	59.57
Nachani ,,	33.11	34.99	38.09	71.67

On further increasing the strength of the diastase (1.5%) there is not an appreciable change as compared to 1% diastase,

Only the figures are slightly higher. Wheat and Jowar show the same way of reaction, while in the case of Rajgira and Nachani, the difference which was appreciable during first and second hour is lowered down. In the case of 1.5% diastase the order of increasing susceptibility does not change during the three periods as in the case of 0.5 and 1.0% diastase. The order is Wheat, Jowar and Nachani during the three periods. If we compare all the tables of 0.5%, 1% and 1.5% diastase, we find that major portion of the hydrolysable starch, is disintegrated during the first hour and the reaction drops down during the subsequent periods. This may be due to the accumulation of the reaction product, which perhaps checks the further reaction and also to the removal of the large amount of substrate during the first hour and also due to the weakening of the enzyme.

Now comparing the flour among themselves, we can see their order of decreasing susceptibility as follows :—

Nachani, Rajgira, Jowar and Wheat.

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#### SECTION IV

### COMPARATIVE DISINTEGRATION OF FLOURS AS JUDGED BY THE HYDROLYSIS WITH THE HELP OF PANCREATIN.

In the last section we considered the effect of the hydrolysing action of Diastase using different strengths for different periods. Now in this section it is proposed to study the hydrolysis of the flours by using Pancreatin amylase.

#### **Standardisation of the pancreatin for its diastatic power:—**

Pancreatin is a ferment which resembles the diastase in giving end products of hydrolysis of starches. In the hydrolysis of starch the end product is the reducing sugar, which may have been formed by various stages of hydrolysis, the quantity and nature of which may differ from ferment to ferment. The object of the present investigation is to study

the comparative digestibility of the flours. It is necessary therefore to study the values of hydrolysis obtained with different hydrolysing agents in a comparative way. Since we are using other ferment it is but natural and essential to find out their comparative diastatic powers, so that we may be able to compare the values for the two ferments after bringing them to common basis. The diastatic power of the pancreatin was therefore, compared with that of the standard diastase, which was used by us in the foregoing work.

### Comparison of the diastase and the Pancreatin

For comparing the diastatic powers of the different reagents various methods based on the use of Fehling solution are in use. Sherman (1910, J. A. Chem. Sc. 32, 1082) had developed a method to compare the diastatic values of different agents which could be expressed on the Linthard scale. The following procedure was followed in the present case.

**Experimental:**—A 2 per cent. solution of the soluble potato starch was prepared in the usual way by pouring boiling water over a paste and boiling it for 10 minutes on a water bath. 50 c.cs. of the solution thus prepared were taken in different flasks and to one was added 10 c.cs. of the 0.5% solution of diastase and to the other 10 c.cs. of the 0.5% solution of Pancreatin together with 40 c.cs. of the acetate buffer solution of 4.8 pH. The flasks were incubated at 37°C for an hour. The enzyme was killed by a few drops of 10% NaOH and the volume made to 250 c.cs. The reducing sugars were estimated after clarification etc. in the usual way by Bertrand's method. The ratio of the amounts of the reducing sugars produced was taken as the diastatic ratio. The diastatic power of the Pancreatin was found to be  $\frac{5}{6}$ th of the diastase. In order to compare the results obtained with Pancreatin to that of diastase we have to multiply the figures by  $\frac{6}{5}$  or 1.2. All values obtained for pancreatin were multiplied by the factor 1.2.

The experimental procedure is exactly similar to that of the last section. The results are also calculated on the basis of the total hydrolysable starch (oven dry).

TABLE No. 9

Percentage hydrolysis of the flours with 10 c.cs. of 0.5% pancreatin calculated on the basis of the total hydrolysable starch (oven dry).

Name of the flour.	1 hour	2 hours	3 hours	Total starch hydrolysable
Wheat ...	6.816	7.716	9.996	74.29
Jowar ...	11.292	12.996	22.32	77.13
Rajgira ...	14.504	14.476	25.704	59.67
Nachani ...	13.368	14.712	25.572	71.67

TABLE No. 10

Percentage hydrolysis of the flours with 10 c.cs. of 1% Pancreatin calculated on the basis of total hydrolysable starch (oven dry)

Name of the flour.	1 hour	2 hours	3 hours	Total starch hydrolysable
Wheat ...	7.88	12.38	14.58	74.29
Jowar ...	13.34	22.94	25.66	77.13
Rajgira ...	16.49	29.57	33.60	59.67
Nachani ...	15.68	27.26	30.16	71.67

TABLE No. 11

Percentage hydrolysis of the flours with 10 c.cs of 1.5% pancreatin calculated on the basis of total hydrolysable starch (oven dry).

Name of the flour.	1 hour	2 hours	3 hours	Total starch hydrolysable
Wheat ...	10.67	16.87	19.26	74.29
Jowar ...	18.52	27.49	30.72	77.13
Rajgira ...	20.49	30.84	38.44	59.67
Nachani ...	20.40	33.76	35.70	71.67

0.5% Pancreatin (10 c.cs of 0.5% pancreatin) attacked Rajgira and Nachani flours more easily than Wheat and Jowar

flours. During the first hour the order of decreasing susceptibility is Rajgira, Nachani, Jowar and Wheat and the same order is maintained in the second and third hour.

In the case of Wheat flour the velocity of the reaction is very slow. It is 6.8 per cent. during the first hour and 0.9% and 2.28% during the second and the third hour. But in the case of Jowar, Rajgira and Nachani flours the pancreatin attacks more than 10% in the first hour, then the rate drops down to 1% during the second hour, and during third hour the rate again increases to more than 10%. Looking to all these values we can say the value of the Wheat flour is considerably lower than other flours. Wheat is least attacked by 0.5% Pancreatin.

Now with increased strength of the Pancreatin (i.e. 1.0%) we observed the same mode of reaction. Only the values are a bit more in all the periods and with all the flours. The same order of susceptibility is maintained.

On further increasing the strength of the ferment to 1.5% we find the same order of susceptibility and the values are increased by about 5% in all periods and for all flours except the Wheat flour which shows the rise of 2.8% in the value during the first hour and Rajgira flour which shows the difference of 1.3% in the values during the second hour. During second hour the order of decreasing susceptibility is Nachani, Rajgira, Jowar and Wheat. However the order is changed to Rajgira, Nachani, Jowar and Wheat during the third hour.

Comparing these values with that of Diastase it is found that more than 20% of the starch of the flours is hydrolysed by Diastase during the first hour, while Pancreatin attacks about 12% of the starch during the first hour, with 0.5% strength. With higher strengths more hydrolysis is observed in the case of diastase, but during the third hour the values both in the case of Diastase and Pancreatin, approach each other very nearly, except the Wheat flour which is little susceptible to the action of Pancreatin.

In the case of Diastase the order of decreasing susceptibility is Nachani, Jowar, Rajgira and Wheat, but here in

the case of Pancreatin we find the order is Nachani, Rajgira, Jowar and Wheat. In the case of hydrolysis with hydrochloric acid the order is Wheat, Rajgira, Jowar and Nachani. This shows that the course of reaction considerably depends upon the nature of the ferment.

The observation which we have just now made regarding the differences in the order of susceptibility of different flours with various enzymes, is of the nature of the observation made by Stone (Loc. Cit.) with special reference to the digestion of the pure starches. His observation appears to have been made on studying the action of the different ferments on the various starches for any one fixed period. We have seen that by using the same ferment but with different strengths the order of susceptibility can be changed. We have also seen that the order can be changed by the use of the same ferment and the same strength but different period. This nature of the reaction was observed by Sahasrabuddhe and Bhide (Loc. Cit.) with reference to starches and flours also.

It is seen, therefore, that it is not only the change of the ferment that is responsible for the change of order of susceptibility but the strength of the ferment as well as the period of reaction are equally responsible for the change in the order of susceptibility of the different grains.

### Conclusions

1. From the results of all the experiments described so far, it has been found that with the increasing strength of the hydrolysing agent, the values of the disintegration of the carbohydrates of the flours of Wheat, Jowar, Rajgira and Nachani gradually increase.

2. The values of the disintegration of starches in the flours are found to increase when the period of the reaction is increased, although the increase is not exactly proportional to the time.

3. When hydrochloric acid is used as a hydrolysing agent the following order of decreasing susceptibility is observed—Wheat, Rajgira, Jowar and Nachani.

4. In the case of the diastatic hydrolysis of the starches in the flours the order of decreasing susceptibility is Nachani, Rajgira, Jowar and Wheat.

5. Pancreatin when used as a hydrolysing agent the following order of Nachani, Rajgira, Jowar and Wheat is found as the order of decreasing susceptibility.

6. It is noticed in many cases that the starch in the Rajgira flour requires a little more time than starches in the other flours, to start the reaction but once the reaction starts it proceeds with increasing rates with the increasing period of reaction.

7. The values of the disintegration of starches in the flours are, in general, higher in the case of diastase than in the case of pancreatin and hydrochloric acid.

8. As mentioned in the introduction W. E. Stones, Bernetzsky Lenberg and Hammerstein had worked on the disintegration of the starches from different grains. They had found different order of susceptibility to the different enzymes. In the present investigation similar results are obtained. It is further demonstrated by the experiments that as soon as hydrolysing agent is changed the order of susceptibility also changes. This is in agreement with the observation made by above-mentioned authors. Moreover it is also found that the order of susceptibility changes with the duration of the hydrolysis.

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## PAPER III

A STUDY OF COMPARATIVE DISINTEGRATION  
OF THE CONSTITUENTS OF THE GRAINS OF  
RALA AND HARIK

BY

M. V. SATHE, M.Sc.

*(With the kind permission of the authorities of the Bombay  
University)*

**Introduction**

The work described in this paper forms a part of a series of investigations undertaken in the Biochemistry Laboratory of the Nowrosjee Wadia College, Poona, viz. the disintegration of different food-grains. The present work, however, is restricted to the study of comparative disintegration of the flours of some food-grains.

For studying the disintegration, two millets *Rala* (*Setaria Italica*) and *Harik* (*Paspalum Scrobiculatum*) were chosen. To get the idea of comparative disintegration both of them were subjected to the action of different enzymes of different strengths and for different periods of reactions.

**Description of the grains:—**

(i) *Rala* or Italian millet:—This is recognised in the Bombay Province by the name Kangli, Rale, Rala, Kang, Chenna and Vavari. This millet is largely cultivated in India in plains and hills. It is both wild and cultivated in India and other warm countries. The word Kang indicates its antiquity as cultivated in India. It is always sown as a Kharip. But the same land is sown twice. Grains are yellowish in colour. In Bombay Province the crop is becoming more popular and the area of cultivation has increased considerably. Ahmednagar, Satara and Karnatak collectorate are chief centres of cultivation.

The grains are much esteemed as articles of food in many parts of India. It is eaten in the form of cakes, porridges in



North West Provinces. In Madras Province it is considered to be excellent material for making pastry. Boiled with milk it is used as a pleasant meal for the invalids.

(ii) *Harik—Kodra or Kodo millet*:—This is a native of India and extensively cultivated in many parts of India during the rainy seasons. In North West Provinces it is grown far extensively than other millets. It is chiefly cultivated in Districts south of Jumna, Allahabad and Lalitpur. It is sown at the beginning of rains. Careful weeding is needed to secure good outturn which is estimated from 10 to 12 maunds per acre. In Bombay Province it takes the seventh place among the cereals. Gujrat and Konkan claim as largest cultivating areas.

In Bombay Province several varieties are recognized by the natives, the difference being due to the nature of the soil and method of cultivation. The grains contain in some seasons a poisonous narcotic principle which is due to an alkaloid development under certain conditions of climate and seasons. A damp cloudy weather towards the harvest time, a damp season and a damped undrained soil are apt to produce poisonous Kodra.

*General Analysis*:—The general analysis of the flours which are used for the present investigation is as follows:—

		Rala	Harik
Moisture	...	9.51%	9.92%
Ether extract	...	3.64%	1.87%
Proteins	...	9.58%	6.22%
Carbohydrates	...	74.48%	80.14%
Crude fibre	...	1.36%	0.75%
Ash	...	1.43%	1.1%

The other constituents such as total hydrolysable starch etc. have been determined according to the methods of analysis of the Association for Official Agricultural Chemists of U.S.A. (1926 and 1945.)

### Total Hydrolysable starch

The Rala Flour as such	...	66.83%	oven dry basis
The Harik flour as such	...	70.52%	..
Starch (from Rala flour)	...	94.12%	..
Starch (from Harik flour)	...	98.23%	..

### SECTION I

#### A. The comparative disintegration of the flours as such by hydrochloric acid.

The percentage hydrolysis of the flours by 10 c. cs. of 25%, 37.5% and 50% hydrochloric acid, expressed on total hydrolysable starch basis. (oven-dry).

TABLE No. 1

Strength of HCl.	Name of the flour.	Hours 24	Hours 48	Hours 72	Total starch hydrolysable.
25%	Rala	1.623%	2.258%	2.978%	66.83%
25%	Harik	1.051%	2.21%	2.46%	70.52%
37.5%	Rala	2.562%	3.462%	4.43%	66.83%
37.5%	Harik	2.138%	3.223%	3.778%	70.52%
50%	Rala	3.294%	4.48%	6.218%	66.83%
50%	Harik	2.452%	3.66%	6.02%	70.52%

So from the above results it is observed that the Rala flour is, in general, more easily acted upon than the Harik flour by hydrochloric acid.

#### B. Comparative disintegration of the isolated starches of the flours by Hydrochloric acid.

The percentage hydrolysis of the isolated starches of the flours by 10 c. c. of 25%, 37.5% and 50%, hydrochloric acid expressed on total hydrolysable starch basis (oven dry).

TABLE No. 2

Strength of HCl.	Name of the Starch.	Hours 24	Hours 48	Hours 72	Total Hydrolysable starch.
25%	Rala	0.939%	1.331%	1.823%	94.12%
25%	Harik	0.712%	1.121%	1.642%	98.23%
37.5%	Rala	1.742%	2.322%	2.992%	94.12%
37.5%	Harik	1.452%	2.198%	2.623%	98.23%
50%	Rala	2.175%	3.037%	4.246%	94.12%
50%	Harik	1.648%	2.682%	3.75%	98.23%

From the above results it is observed that the isolated starch from the Rala flour, in general is more easily acted upon than the isolated starch from the Harik flour by hydrochloric acid.

In case of both the flours the presence of ingredients other than the carbohydrates exhibit some influence on the process of hydrolysis by hydrochloric acid.

## SECTION II

### A STUDY OF COMPARATIVE DISINTEGRATION OF THE FLOURS AS SUCH AND THEIR ISOLATED STARCHES WITH DIASTASE.

#### A. Comparative disintegration of the flours as such judged by hydrolysis with diastase:—

The percentage hydrolysis of the flours by 10 c. cs. of (0.2%, 0.5% and 1.0%) diastase at pH 4.8 expressed on the total hydrolysable starch basis (oven dry).

TABLE No. 3.

Strength of the enzyme	Name of the flour	Hour 1	Hours 2	Hours 3	Total Hydrolysable starch
0.2%	Rala	24.36%	28.21%	31.43%	66.83%
0.2%	Harik	27.92%	31.33%	33.52%	70.52%
0.5%	Rala	30.43%	33.72%	36.13%	66.83%
0.5%	Harik	29.35%	32.47%	34.53%	70.52%
1.0%	Rala	32.33%	36.85%	39.23%	66.83%
1.0%	Harik	30.82%	33.84%	35.48%	70.52%

From the above results it is observed that the major portion of the total hydrolysable starch is hydrolysed during the first interval and there is no considerable difference in the values of the percentage hydrolysis of the first and the second interval. This shows that the enzyme must be losing its strength after the first interval or the concentration of the mass falls down. The values of percentage hydrolysis for different strengths of diastase are different from those for hydrochloric acid of different strengths. It seems therefore that the manner in which a particular hydrolysing agent acts is different from that in which another hydrolysing agent acts. In order to explain such differences in the hydrolysing actions of different hydrolysing agents some definite knowledge of the structure of the starch of the grain is essential. It is also observed that the susceptibility of the flours is changed by a change in the concentration of the hydrolysing agent. In addition to this it is observed that according to the nature of the starch the rate of hydrolysis may be different at different intervals i. e. in some cases it may be greater in the first interval or in some cases it may take time to start and the rate may be greater in the second interval. Finally it is noted that the carbohydrate constituents of the Rala flour in general, are more easily acted upon than those of the Harik flour by diastase.

## SECTION II

### B. Comparative disintegration of the isolated starch from each of the flours as judged by the Hydrolysis with diastase.

TABLE No. 4

The percentage hydrolysis of the isolated starch of the flours by 10 c.c. of (0.2%, 0.5% and 1.0%) diastase at pH 4.8 expressed on total hydrolysable starch basis (oven dry).

Strength of the enzyme.	Name of the starch.	Hour 1	Hours 2	Hours 3	Total hydrolysable-Starch
0.2%	Rala	19.6%	20.23%	27.77%	94.12%
0.2%	Harik	16.82%	18.43%	19.76%	98.23%
0.5%	Rala	19.12%	24.22%	28.1%	94.12%
0.5%	Harik	20.8%	22.12%	24.87%	98.23%
1.0%	Rala	28.72%	30.76%	31.62%	94.12%
1.0%	Harik	22.28%	25.45%	27.43%	98.23%

From the above results it is ascertained that the values of the percentage hydrolysis of the flours as such are higher than those for the isolated starch of each of the flours when acted upon by diastase. This means that the removal of the non-carbohydrate bodies have some effect on the hydrolysis of the isolated starch of each of the flours. The isolated starch from the Rala flour is more easily acted upon than the isolated starch from the Harik flour by diastase.

### SECTION III

## A STUDY OF COMPARATIVE DISINTEGRATION OF THE FLOURS AS SUCH AND THEIR ISOLATED STARCHES WITH PANCREATIN.

### A. The comparative disintegration of the flours as such as judged by the use of pancreatin:—

Percentage hydrolysis of the flours as such at pH 6.8 by 10 c.c. of (0.2 percent, 0.5 percent and 1.0 percent) pancreatin expressed on total hydrolysable starch basis (oven dry).

TABLE No. 3

(The values in the following table are obtained by multiplying the values of percentage hydrolysis with pancreatin by the factor  $6/5$  to make them comparable with diastase).

Strength of the enzyme	Name of the flour	Hour 1	Hours 2	Hours 3	Total Hydrolysable starch.
0.2%	Rala	10.8%	19.48%	24.71%	66.83%
0.2%	Harik	9.7%	14.52%	17.82%	70.52%
0.5%	Rala	30.56%	39.61%	47.52%	66.83%
0.5%	Harik	24.86%	30.24%	33.12%	70.52%
1.0%	Rala	47.51%	51.26%	53.54%	66.83%
1.0%	Harik	39.58%	41.84%	43.28%	70.52%

From the above Table and results—it is observed that the values of percentage hydrolysis obtained in case of pancreatin are more than those for diastase. This means that the manner in which a particular hydrolysing agent acts is different from that in which the other hydrolysing agent acts. It is observed from the hydrolysis by diastase and pancreatin that not only the susceptibility of the flours to the ferment changes according to the strength of the hydrolysing agent but also due to the change of the disintegrating agent. The Rala flour is more easily acted upon than the Harik flour. In case of the Rala flour the percentage hydrolysis has reached to 53%. The Harik flour is acted upon nearly equally well by diastase and pancreatin. The extent of percentage hydrolysis of Harik flour has reached to 43 per cent by hydrolysis with pancreatin.

### B. Comparative disintegration of the isolated starch of each of the flours by pancreatin:—

In the previous sections the comparative disintegration of the flours as such is studied and it is found that the non-carbohydrate constituents of the flours have a considerable effect on the course of hydrolysis. In order to see whether such observation can be made with pancreatin as hydrolysing agent it was thought interesting to carry the experiments with pancreatin.

The percentage hydrolysis of the isolated starch of each of the flours at pH 6.8 by 10 c. cs. of (0.2%, 0.5% and 1.0%) pancreatin expressed on total hydrolysable starch basis (oven dry).

TABLE No. 6.

(The values in the table are obtained by multiplying the values obtained with pancreatin by the factor 6/5 to make them comparable with diastase).

Strength of the enzyme	Name of the starch	Hour 1	Hours 2	Hours 3	Total Hydrolysable Starch.
0.2%	Rala	8.13%	14.17%	16.19%	94.12%
0.2%	Harik	5.72%	8.76%	11.4%	98.23%
0.5%	Rala	20.11%	28.12%	33.52%	94.12%
0.5%	Harik	18.24%	22.18%	25.88%	98.23%
1.0%	Rala	37.63%	40.56%	41.54%	94.12%
1.0%	Harik	34.85%	36.76%	38.46%	98.23%

From the above results it is observed that both the flours and the isolated starches are more easily acted upon by pancreatin than by diastase under similar experimental conditions. The effect of the removal of the non-carbohydrate bodies is seen better in the case of the hydrolysis by Pancreatin than by Diastase. The isolated starch from the Bala flour is more easily acted upon than the isolated starch from the Harik flour by different strengths of pancreatin.

#### SECTION IV

### A STUDY OF COMPARATIVE DISINTEGRATION OF THE PROTEIN PART OF THE FLOURS AS SUCH WITH PEPSIN ALONE AND PEPSIN SUPERIMPOSED WITH TRYPSIN.

#### A. Disintegration of the protein part of the flours as such by Pepsin:—

**Experimental:—**2 grams of each sample (oven dry) were suspended into 25 c. c. of decinormal hydrochloric acid and 25 c. c. of 0.2% pepsin prepared in decinormal hydrochloric acid were added to the suspension. The reaction was carried out at (temperature) 37°C and pH 3.8 —4. for required time i.e. 1, 2 and 3 hours.

After incubating for the fixed period 5 c. c. of normal sodium hydroxide were added to neutralise the acid and 2 c. c. of colloidal iron were added to coagulate the proteins. 5 c. c. of magnesium sulphate solution (5 per cent.) were added to help coagulation. Clarification was hastened by adding drop by drop basic lead acetate solution. The volume was then made to 100 c. c. and filtered. 5 c. c. of the filtrate were taken for the estimation of the amino-nitrogen by the Van Slyke's method (1912, Jr. Biol. Chem. 12, 399). The amount of nitrogen obtained was changed to normal temperature and pressure and then the disintegration was expressed as milligrams of nitrogen for 100 grams of the sample. (A blank experiment was run side by side for every period and strength of pepsin.

The results of the blank experiments were subtracted from the final values obtained).

**Disintegration of the Protein part of the flours as such, as judged by Pepsin :—**

Hydrolysis of the protein part of the flours as such by 10 c. c. of 0.2 per cent., 0.5 per cent. and 1.0 per cent. pepsin expressed as milligrams of nitrogen per 100 grams of the flours (oven dry).

TABLE No. 7

Strength of the enzyme.	Name of the flour.	Hour 1	Hours 2	Hours 3	Total Proteins.
0.2%	Rala	62.51 mg.	173.33 mg.	193.74mg.	9.58%
0.2%	Harik	43.24 ,,	95.56 ,,	121.23 ,,	6.22%
0.5%	Rala	132.35 ,,	182.39 ,,	210.23 ,,	9.58%
0.5%	Harik	73.78 ,,	116.27 ,,	154.12 ,,	6.22%
1.0%	Rala	151.17 ,,	191.27 ,,	224.67 ,,	9.58%
1.0%	Harik	90.81 ,,	146.26 ,,	170.93 ,,	6.22%

In case of the hydrolysis by 0.2%, 0.5% and 1% pepsin the Rala flour is more easily acted upon than the Harik flour as the protein part of the flour is concerned. In both the cases the reaction is slow in the first interval.

**B. The effect of superimposed trypsin on the hydrolysis of the protein part of the flours:—**

*Experimental:—*The hydrolysis of the flours was carried out as before. 50 c. c. of decinormal hydrochloric acid were neutralised by 5 c. c. of normal sodium hydroxide and 5 c. c. of 6 per cent. trypsin prepared in decinormal solution of sodium hydroxide were added. The resulting 60 c.c. contained 0.5 per cent. trypsin and hydroxyl concentration was 1/20. The activity of the enzyme was killed by heating it to 80 degrees centigrade for 5 minutes. Then on cooling, 5 c.c. of colloidal iron and 2 c.c. of magnesium sulphate solution (5 per cent.) were added to hasten clarification. The



volume was made to 100 c.c. and 5 c.c. of the filtrate were taken for the estimation of amino-nitrogen by the Van Slyke's method. The results are expressed as milligrams of nitrogen per 100 grams of the sample. Digestion by 0.5 per cent. pepsin was allowed for one hour and the same was used for trypsin hydrolysis.

TABLE No. 8

Hydrolysis of the protein part of the flours as such with 0.5 per cent. pepsin for one hour - and then with superimposed trypsin (6 per cent.) for one hour expressed as milligrams of nitrogen per 100 grams of the flours (oven dry).

Name of the flour.	Nitrogen for pepsin.	Nitrogen for pepsin superimposed by - Trypsin.	Total proteins.
Rala	132.35 mg.	187.41 mg.	9.58%
Harik	73.78 ,,	101.28 ,,	6.22%

From the above table it is observed that there is a considerable difference in the values of peptic and tryptic digestion. The difference in the case of the Rala flour is more than that in the case of the Harik flour. So from all the above discussion it is observed that the Rala flour is more easily acted upon than the Harik flour by proteolytic enzymes.

## SECTION V

## CONCLUSIONS AND SUMMARY

1. From the results of all the experiments so far described it has been observed that when there is an increase in the strengths of the hydrolysing agent the values of disintegration gradually increase.
2. The values of disintegration of both the flours as such and of the isolated starch of each of the flours are found to increase when the period of reaction is increased. However the increase is not proportional to the time.

3. It is found that in the case of the hydrolysis of the flours by pancreatin and the isolated starch of each of the flours by pancreatin, the hydrolysis is rather slow in the first interval i. e. the reaction takes some time for starting.

4. In the case of the hydrolysis of the flours as such by diastase the Rala flour is more easily acted upon than the Harik flour. Similarly the Rala flour is more easily acted upon than the Harik flour by hydrochloric acid and pancreatin.

5. The values of percentage hydrolysis of the Rala flour by pancreatin are in general higher than those in the case of the hydrolysis by diastase.

6. In the case of the hydrolysis of the isolated starch of each of the flours, the Rala starch is more easily acted upon than the Harik starch. The values of percentage hydrolysis go on increasing with increase in the periods of reaction and concentrations of the different hydrolysing agents. However the increase in the values is neither proportional to the time nor to the concentrations of the hydrolysing agents.

7. The values of percentage hydrolysis for the flours such in all the cases are higher than those for the isolated starch from the same grain, under similar experimental conditions. This means that there is some effect of non-carbohydrate bodies of the flours on the course of hydrolysis. There is in general maximum difference in the values of percentage hydrolysis of the flours as such and the isolated starch of the same flour in case of the hydrolysis by pancreatin. The minimum effect is observed in the case of their hydrolysis by hydrochloric acid. In the case of diastase as the hydrolysing agent the difference in the values of percentage hydrolysis of the flours as such and the isolated starch of the same grain is more than that existing in the case of the hydrolysis by hydrochloric acid.

8. The pure isolated starch of each of the flours is more easily acted upon by pancreatin than by diastase and hydrochloric acid.

9. As regards the disintegration capacity of each of the disintegrating agents pancreatin is found to be the best while diastase of the second order and hydrochloric acid of the third order.

10. In the case of the hydrolysis of the protein part of the flours as such pepsin acts more easily upon the Rala flour than the Harik flour. In the case of these flours there is distinct difference between the figures obtained by pepsin and pepsin superimposed by trypsin.

### **Comparison of the grains among themselves:—**

The utility of the grains can be judged from the amount of its material which is available to the body system. This can be found out from the quantities of disintegration which have been given in the various tables. All these values have been calculated on the basis of the total hydrolysable starch. These figures are more useful as they will give some idea of the comparative disintegration by the various disintegrating agents on a similar basis. As the utility of the grain from the percentage hydrolysis of the flour useful for the body system all the figures are not to be converted to the basis of total Hydrolysable starch but to the basis of flours as it is the flour which is used and consumed and not the starches. It has been also found out that the order of the values of the disintegration changes from enzyme to enzyme. Hence comparison should be considered under the same experimental conditions. For instance in the case of the Rala flour the value of percentage hydrolysis for 1 per cent. diastase and three hours as the period of reaction comes to be 39.23 expressed on total hydrolysable starch basis which is 66.83. This means that out of 100 grams of the total hydrolysable starch only 39.23 grams are capable of being absorbed. Now in 100 grams of Rala flour the total hydrolysable starch is 66.83. So if you want to calculate the quantity of the material which may be useful to the body system from 100 grams of the Rala flour we have to calculate the quantity of the available substance in 66.83. So that in 100 grams of the Rala flour the quantity available is 39.23. The corresponding quantity for

39.23 comes to be as 26.18. This means that if 100 grams of Rala flour are taken only 26.18 grams of the carbohydrates are useful for the body system strictly under the conditions mentioned above. Similarly the figure for the Harik flour is 24.927. The order of utility among the grains themselves comes to be as:-

Rala flour	... 26.18 per cent.
Harik flour	... 24.927 per cent.

(as far as carbohydrates are concerned).

Similar order can be found out for other disintegrating agents of different concentrations and for different period of reactions. If a comparison is to be made among themselves by 1 per cent. pancreatin and for three hours as time of reaction the order of utility comes to be as:—

Rala flour	... 35.47 per cent.
Harik flour	... 30.456 per cent.

(as far as carbohydrates are concerned).

This brings about more clearly the difference in the flours as regards the order of susceptibility to the hydrolysing agent.

If we want to compare the flours from the point of breaking down the protein part of the flours as such we get the following order if the strength of pepsin is 1 per cent. and period of reaction is three hours:—

Rala flour	... 132.35
Harik flour	... 73.78

(the figures are for amino-nitrogen in milligrams per 100 grams of the flour).

Taking the condition of the experiment as 0.5 per cent. pepsin and superimposed by 0.5 per cent. trypsin (one hour for pepsin digestion and one hour for trypsin digestion) the order comes to be as follows:—

Rala flour	... 187.41
Harik flour	... 101.28

(the figures are of amino-nitrogen in milligrams per 100 grams of the flour).

From the above discussion it is observed that the Rala flour is more easily acted upon than the Harik flour by diastase and pancreatin as far as disintegration of the protein part of the flours as such is concerned. Rala flour is more easily acted upon than the Harik flour by Pepsin alone and pepsin superimposed by Trypsin.

## PAPER IV

### A STUDY OF COMPARATIVE DISINTEGRATION OF THE CARBOHYDRATES FROM DRY FRUITS

By

DR. R. S. KALE, Ph.D.

#### Introduction

With the recent advances in the science of Biochemistry the subject of nutrition has been the favourite field for many research workers all over the world. All the food stuffs depend upon the quantity and quality of the various constituents such as carbohydrates, proteins, fats, vitamins and mineral matters which the food stuffs contain and secondly how much of these constituents become available to and assimilated by the human beings for their upkeep.

A large number of Indians are purely vegetarians; but even with the so called non-vegetarian population of the world, vegetables and articles of vegetable origin, form the major portion of their every-day dish. Fruits, in particular, are cherished by all.

For studying the comparative disintegration, all three varieties of dried fruits—mostly containing a large percentage of oil—were selected. Viz.

#### Materials.

Cashew nut	— (Anacardium occidentale)
Almond	— (Prunus amygdalus)
Pistachio nut	— (Pistacia vera)

To call them fruits is a misnomer. According to botanical nomenclature these are seeds and not fruits, but in a layman's terminology they are universally called as "dried fruits" and this has been maintained throughout the present work for the sake of convenience.

The general analysis of the food stuffs used for the present investigation is as follows :—

	<i>CHO</i>	<i>Proteins</i>	<i>Fats</i>	<i>Moisture</i>	<i>Ash</i>	<i>Fibre</i>	
Cashew	22.4	21.3	44.7	6.0	3.5	2.1	100/
Almond	13.4	20.7	53.5	5.9	3.8	2.7	100/
Pistachio	18.1	19.6	50.7	5.7	3.1	2.8	100/

The other constituents such as the total hydrolysable starch etc. have been determined according to the methods of analysis of the Association of the Official Agricultural Chemists of U.S.A. 1926.

#### Total Hydrolysable starch :

Cashew nut	...	17.34%	...	oven dry flour basis
Almond	...	7.777%	...	" "
Pistachio nut	...	6.531%	...	" "

#### SECTION I

A study of the comparative disintegration of carbohydrates from the three dry fruits with hydrochloric acid:

The strengths of the acid used were 25%, 37.5% and 50% and the periods allowed for hydrolysis were 24, 48 and 72 hours. Temperature of the reaction was 37.5°C.

#### Percentage hydrolysis of the carbohydrate constituents with 25% HCL

TABLE No. 1

Name of the sample	24 hrs.	48 hrs.	72 hrs.	Total hydroly-sable starch
Cashew nut	7.27 41.95	7.36 42.47	7.84 45.25	17.34
Almond	4.04 51.99	4.84 62.35	5.5 70.78	7.777
Pistachio nut	3.42 52.32	4.04 61.81	4.27 65.4	6.531

Figures in the nominator denote the percentage hydrolysis of the carbohydrate material on the oven dry flour basis while those in the denominator denote the percentage hydrolysis on the total hydrolysable starch basis.

### Percentage hydrolysis of the Carbohydrate

constituents with 37.5 % HCl

TABLE No. 2.

Name of the sample.	24 hours.	48 hours.	72 hours.	Total hydrolysable starch
Cashew nut ...	<u>7.36</u> 42.47	<u>8.17</u> 47.14	<u>8.74</u> 50.43	17.34
Almond ...	<u>4.56</u> 58.78	<u>5.22</u> 67.18	<u>5.5</u> 70.78	7.777
Pistachio nut ...	<u>3.7</u> 56.68	<u>4.27</u> 65.40	<u>4.27</u> 65.4	6.531

### Percentage Hydrolysis of the Carbohydrate constituents with 50 % HCl.

TABLE No. 3.

Name of the sample	24 hours.	48 hours.	72 hours.	Total hydrolysable starch
Cashew nut ...	<u>8.44</u> 48.7	<u>10.64</u> 61.38	<u>11.4</u> 65.78	17.34
Almond ...	<u>5.22</u> 67.18	<u>5.5</u> 70.78	<u>5.5</u> 70.78	7.777
Pistachio nut ...	<u>4.04</u> 61.81	<u>4.27</u> 65.40	<u>4.27</u> 65.40	6.531

Comparing the three fruits among themselves we find that:-

1. Cashew nut, Almond and Pistachio nut stand in the descending order for hydrolysis on the flour basis. While

the order is changed to Almond, Cashew and Pistachio on the total hydrolysable starch basis, the order of Cashew and Pistachio nut being almost equal.

2. The rate of hydrolysis falls as the hydrolysis proceeds in all cases. The maximum limit of hydrolysis is reached in the second interval in the case of Almonds and Pistachio nuts.

3. Comparing the velocities we find that in all intervals the descending order is Cashew nut, Almonds and Pistachio nut on flour basis, while on the total hydrolysable starch basis the order in the 1st interval is Almond, Pistachio and Cashew. In the 2nd and 3rd intervals it is Cashew, Almond and Pistachio, the amount of Pistachio and Almond hydrolysed being the same in the 2nd and 3rd intervals.

4. With the increase in the strengths of the hydrolysing agent there is also an increase in the total amounts of the substrates hydrolysed.

5. Major portion of the total hydrolysable starch is hydrolysed in the 1st interval alone, the rate then suddenly falls in the following intervals.

## SECTION II.

### **Comparative disintegration of the carbohydrates from the dry fruits with diastase :**

In the present section, a further study of the comparative disintegration of the carbohydrates from the dry fruits is made by using the amylolytic enzyme 'Diastase'. A study of this nature is more important because it resembles the natural process of splitting up of food stuffs occurring in the body to a certain extent. Many workers such as Stone (loc. cit.) and Langworthy (loc. cit.) have studied the action of different ferments on pure starches and not on the whole meal. Naturally, the results obtained with flours would be more significant—since the foodstuffs are consumed as a whole and not as isolated starch or protein.



**Percentage hydrolysis of the carbohydrates  
with 0.5 per cent. Diastase.**

TABLE No. 4.

Name of the sample	1 hour.	2 hours.	3 hours.
Cashew nut ...	<u>2.85</u>	<u>3.42</u>	<u>4.27</u>
	16.45	19.73	24.64
Almond ...	<u>0.59</u>	<u>0.72</u>	<u>1.04</u>
	7.59	9.27	13.38
Pistachio nut ...	<u>0.29</u>	<u>0.43</u>	<u>0.72</u>
	4.43	6.58	11.02

**Percentage hydrolysis of the carbohydrates with  
1 per cent. Diastase.**

TABLE No. 5

Name of the sample.	1 hour.	2 hours.	3 hours.
Cashew nut ...	<u>3.23</u>	<u>4.08</u>	<u>5.32</u>
	18.65	23.54	30.59
Almond ...	<u>0.88</u>	<u>1.29</u>	<u>1.8</u>
	11.33	16.60	23.17
Pistachio nut ...	<u>0.59</u>	<u>0.88</u>	<u>1.29</u>
	9.03	13.46	19.74

**Percentage hydrolysis of the carbohydrates with  
1.5 per cent. Diastase.**

TABLE No. 6

Name of the sample.	1 hour.	2 hours.	3 hours.
Cashew nut ...	<u>4.09</u>	<u>5.22</u>	<u>6.84</u>
	23.70	30.12	39.46
Almond ...	<u>1.29</u>	<u>1.8</u>	<u>2.38</u>
	16.60	23.17	30.63
Pistachio nut ...	<u>1.04</u>	<u>1.42</u>	<u>1.9</u>
	15.91	21.72	29.07

Figures in the nominator denote the percentage hydrolysis of the carbohydrate material on the oven dry flour basis while figures in the denominator denote the percentage hydrolysis on the total hydrolysable starch basis.

Comparing the fruits among themselves we find that:—

1. The order of susceptibility is still maintained as Cashew nut, Almond and Pistachio nut on the flour basis and total hydrolysable starch basis.
2. The velocity is maximum in the 1st, minimum in the 2nd and intermediate in the 3rd interval.
3. Comparing the velocities we notice that in 1st and 3rd interval the order is Cashew nut, Almond and Pistachio nut on both flour basis as well as total hydrolysable starch basis. But in the 2nd interval the order is—Cashew nut, Almond and Pistachio nut on the flour basis and Almond, Pistachio and Cashew on the total hydrolysable starch basis.
4. With the increase in the strength of the hydrolysing agent the amount of substrates hydrolysed are also increased.
5. Major portion of the starch is hydrolysed in the 1st interval. Percentages of starch hydrolysed in the case of Almond and Pistachio nut from time to time are almost equal.

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### SECTION III

## COMPARATIVE DISINTEGRATION OF THE CARBOHYDRATES FROM DRY FRUITS WITH PANCREATIN.

**Percentage hydrolysis of the carbohydrates from the dry  
fruits with 0.5% pancreatin**

TABLE No. 7

Name of the sample	1 hour	2 hours	3 hours
Cashew nut ...	<u>3.16</u>	<u>3.92</u>	<u>4.98</u>
	18.23	22.62	28.73
Almond ...	<u>0.72</u>	<u>0.88</u>	<u>1.21</u>
	9.27	11.33	15.57
Pistachio nut ...	<u>0.43</u>	<u>0.59</u>	<u>0.88</u>
	6.58	9.03	13.46

**Percentage Hydrolysis of the carbohydrates from the dry fruits with 1 %Pancreatin.**

TABLE No. 8

Name of the sample	1 hour	2 hours	3 hours
Cashew nut ...	<u>3.8</u>	<u>4.83</u>	<u>5.89</u>
	21.93	27.87	33.98
Almond ...	<u>1.04</u>	<u>1.51</u>	<u>2.11</u>
	13.38	19.43	27.12
Pistachio nut ...	<u>0.72</u>	<u>1.04</u>	<u>1.42</u>
	11.02	15.91	21.72

**Percentage Hydrolysis of the Carbohydrates in the dry fruits with 1.5 %Pancreatin.**

TABLE No. 9.

Name of the sample	1 hour	2 hours	3 hours
Cashew nut ...	<u>4.66</u>	<u>5.89</u>	<u>7.55</u>
	26.89	33.98	43.56
Almond ...	<u>1.66</u>	<u>2.27</u>	<u>3.02</u>
	21.36	28.77	38.87
Pistachio nut ...	<u>1.29</u>	<u>1.81</u>	<u>2.41</u>
	19.74	27.69	38.87

Figures in the nominator denote the percentage hydrolysis of the carbohydrate material on the oven dry flour basis, while the figures in the denominator denote the percentage hydrolysis on the total hydrolysable starch basis.

Comparing the dry fruits among themselves we find that:—

1. Cashew nut, Almond and Pistachio nut is the descending order of susceptibility on flour basis. That on total hydrolysable starch basis Cashew stands first and Almond and Pistachio are hydrolysed to the same extent.
2. The velocity is maximum in the 1st, falls to the minimum in the 2nd and rises again in the third interval. Major portion of the starch is hydrolysed in the 1st interval.
3. Comparing the velocities—Cashew nut, Almond and Pistachio nut is the descending order in all intervals on the flour basis. It is the same in the 1st interval on total hydrolysable starch basis but in the 2nd and 3rd interval the order changes to Pistachio nut, Almond and Cashew nut.
4. With the increase in the strength of the hydrolysing agent there is also an increase in the amounts of the starch hydrolysed.
5. Comparing the figures with that of 1.5% diastase we find that the figures for Pancreatin are higher than those for diastase. The descending order for divergency is Cashew nut, Almond and Pistachio nut—the difference is highest in the third interval—thus increasing as the hydrolysis proceeds.

#### SECTION IV

#### Effect of removing fat from the dry fruits on the enzymic hydrolysis of carbohydrates.

The dry fruits under consideration contain a large percentage of oil. It was therefore thought to study how these oils affect the disintegration of the non-fatty material in the dry fruits by the enzymes. Food material is often mixed with a foreign fat of animal or vegetable origin i.e. ghee, butter or many vegetable oils as sweet oil, coconut oil etc. They make

the food delicious, at the same time supply the fatty contents necessary for the growth and also wear and tear of the body. The dry fruits under consideration are often fried in Ghee and ingested with addition of salt, pepper etc. Of course the added fat is of foreign origin. Many workers have studied the action of such foreign fats on the hydrolysis of different food stuffs.

In the present chapter we intend to study how the fatty material of the same origin affect the digestion of the non-fatty material in the same food stuff by different enzymes.

#### Preparation of the sample :

Dried fruits were pounded in a mortar with pestle to a sufficiently uniform consistency. The powders were then put in the soxhlet and were run over by ether for a day or two till all the fatty material was extracted by ether. The fat free powders were then subjected to the action of diastase and pancreatin as before.

#### Comparative disintegration of the carbohydrates from the fat-free material with 1% Diastase.

TABLE No. 10.

Name of the sample	1 hour	2 hours	3 hours
Cashew nut	...	<u>2.87</u>	<u>3.92</u>
		16.56	22.62
Almond	...	<u>0.59</u>	<u>1.21</u>
		7.59	15.57
Pistachio nut	...	<u>0.43</u>	<u>0.88</u>
		6.58	11.02
			13.46

#### Comparative disintegration of the carbohydrates from the fat-free material with 1% Pancreatin.

TABLE No. 11

Name of the sample	1 hour	2 hours	3 hours
Cashew nut	...	<u>3.16</u>	<u>4.09</u>
		18.23	23.70
Almond	...	<u>0.72</u>	<u>1.29</u>
		9.27	16.60
Pistachio nut		<u>0.59</u>	<u>1.04</u>
		9.03	13.46
			15.91

Figures in the denominator denote the percentage hydrolysis on the total hydrolysable starch basis and those in the nominator denote the percentage hydrolysis on the flour basis.

4. Comparing the figures with those corresponding figures—in the table of fat containing sample we notice that the figures in the 'fat-free' table are lower than those of 'fat containing' sample showing thereby that the fatty material aids the hydrolysis of non-fatty material with the enzyme.

Thus in conclusion we see that the presence of fats helps the hydrolysis of carbohydrates with the enzymes—Diastase and Pancreatin.

## PAPER V

# STUDY OF THE HYDROLYSIS OF STARCHES CONTAINED IN SOME EDIBLE ROOTS AND TUBERS

BY

DR. U. A. SANT, M.Sc., Ph. D.

*(With the kind permission of the authorities of the Bombay University).*

### Introduction

Much work on the disintegration of starches by means of enzyme in case of grains has been carried out, but little information is available regarding roots and tubers, as compared with that of grain. An attempt is therefore made to study the hydrolysis of the constituents in some roots and tubers, and the material selected for the purpose is Carrot (*Daucus Carota*), Sweet Potato (*Ipomea Batata*), Yam (*Dioscorea alata*), Elephant Foot or Suran (*Amorphophallus Campanulatus*), and Potato (*Solanum Tuberosum*), etc.—Sahasrabudhe and Kibe (Govt. Memo. Imp. Coun. New Delhi, 1935), have shown that the various constituents of the grain have a mutual positive or

negative effect on the course of the reaction. So the values obtained for the pure starches which will be in absence of other constituents of the grain, will not be the same as those which may be obtained when these substances are taken together with the other constituents of the grain. Disintegration was therefore carried out in the presence of disintegrating reagents such as hydrochloric acid, Diastase, Pancreatin in the case of roots and tubers by using dried flours as such of these materials. In the presence of Pancreatin, salts such as sodium chloride, calcium phosphate (mono), and an organic salt like potassium tartrate was also used in order to get a comparative idea of their disintegration in the presence of these salts.

## SECTION 1

### Materials and methods of Analysis

For studying the comparative disintegration in roots and tubers, the following were selected:—

- |                               |  |
|-------------------------------|--|
| (1) Carrot                    | ( <i>Daucus Carota</i> )               |
| (2) Sweet Potato              | ( <i>Ipomea Batata</i> )               |
| (3) Yam                       | ( <i>Dioscoreaalata</i> )              |
| (4) Elephant foot<br>or Suran | ( <i>Amorphophallus Campanulatus</i> ) |
| (5) Potato                    | ( <i>Solanum Tuberosum</i> )           |

Description of material:—

#### Carrot:—

It is cultivated throughout India. The carrot appears to have been used in India from fairly ancient times. The proper time for sowing carrot seeds is from the month of September to October. They take three months to mature. The so-called root constitutes an important vegetable in the market. Carrot-tops afford a useful fodder. It is also used for human consumption as a vegetable.

#### Sweet Potato:—

Presumably a native of America, but is extensively cultivated in India. Two forms are met with, one with red, the

other with white tubers. The area under the sweet Potato in India cannot be definitely ascertained. It is grown all over the country. Taking India as a whole, it is planted from August to November and reaped from December to May. The crop does not thrive unless the land be naturally dry. The sweet potato contains more dry, starchy and sugary matter than ordinary potato, but less nitrogenous substance.

#### **Yam:—**

Yam produces tubers of good quality, but so deeply situated in the soil as to render harvesting exceedingly difficult. It is a common article of food as vegetable. There appears to be a difference of opinion as to whether the tuberous organ is a true tuber or tuberous root. Commonly it is referred to as a tuber, but certain characteristics are more important in keeping with a tuberous root. Yams are starchy like potatoes.

#### **Elephant foot or Suran:—**

It is cultivated more abundantly in Bombay Province. In medicine the corms are used as external irritants to relieve rheumatic swellings. The cultivated corms are cooked and form a common article of food. They are boiled like potatoes. The tubers are usually planted out in May to June and in twelve months they are fit to be dug up.

#### **Potato:—**

Since its introduction in Europe from the highlands of Sicily and Peru towards the close of the sixteenth century, potato has had a profound influence on the civilization of temperate regions. A great number of varieties have been produced by breeding and cultivation. The chief points of difference are—size, form, depth of eyes, colour and starch contents. So a particular type of potato called "Mahabalesh-wari Potato" was selected.

#### **Preparation of the material:—**

Disintegration of the carbohydrates present in the crop was studied on the starches contained in the flours. The disintegration was judged from the amount of reducing sugar formed by using various hydrolysing agents.



**The Flours :—**

The vegetables were first of all cleaned by washing with water. The skin was removed by rasping except in the case of carrots from all sides and then cut into thin slices. In the case of carrot the middle portion was removed instead of the outer layer and then cut into thin slices. After the slices were dried under shade, all these dried materials were then ground to flours in a hand-mill and passed through two-hundred mesh sieve and preserved with a sufficient quantity of toluene in glass stoppered bottles.

The general analysis of the flours which are used for the present investigation is given in the following Table.

Figures given in Table No. 1 represent percentages of Proteins, fats, etc.

TABLE No. -1

**Analysis of the Flours.**

Name of the flour	Carrot	Sweet-potato	Yam	Suran	Potato
Moisture	... 7.70	7.50	8.12	8.02	7.62
Ether extract	... 1.72	3.90	0.27	0.31	0.11
Proteins	... 4.30	4.50	3.88	3.98	4.68
Digestible Carbohydrates	... 73.20	78.85	82.21	77.55	79.51
Crude fibre	... 6.56	1.75	1.48	4.10	2.88
Ash	... 6.52	3.50	4.04	6.04	5.20

Analytical procedure was followed according to A.O.A.C. methods.

**Methods of Sugar Estimation :—**

Out of the various methods suggested in the literature for the purpose, Bertrand's Method was followed for the estimation of sugar formed during hydrolysis of starch,

### Determination of Reducing and Non-reducing sugars initially present in the flours.

Among carbohydrates, starch is the chief material, but along with it, in certain materials, sugars of various types are also present. They may be classified in general as reducing and non-reducing sugars. Before studying the hydrolysis of starches from the flours by means of hydrolysing agents, it was thought necessary to determine the amount of reducing and non-reducing sugars initially present in the flours.

#### Experimental :-

To obtain sugars in solution, material was extracted with water. To the known amount of the flour was added a known volume of distilled water for extracting sugar from the flour. The experimental procedure was carried out according to A. O. A. C. methods. Total hydrolysable starch contained in the flours was also determined according to the A. O. A. C. methods.

TABLE No. 2

#### Reducing and Non-reducing Sugars and Starch contained in flours.

Name of the flour	Reducing sugar %	Non-reducing sugar %	Total starch contained in flours. %
Carrot	25.200	16.537	8.223
Sweet Potato	4.880	6.120	59.875
Yam	4.724	4.410	63.316
Suran	4.576	3.150	58.424
Potato	0.620	1.260	72.170

Taking into consideration the percentage of different sugars present in the flours, it is found that carrot contains highest percentage of sugar. Next to carrot is sweet potato, followed by yam, suran and potato.

Comparing the amount of starches present in the different flours, potato contains highest percentage of starch.

From the results obtained it is observed that all these flours contain both reducing and non-reducing sugar, and these flours also contain a large proportion of starch. Carrot flour contains too small a proportion of starch. Sweet potato contains approximately 60% starch, while yam flour contains approximately 63%; and suran flour 58% approximately.

## SECTION II

### DISINTEGRATION OF DIFFERENT FLOURS BY MEANS OF HYDROCHLORIC ACID.

The object of using Hydrochloric acid was to find out the total hydrolysable starch in the flours. In the preliminary trials the acid strengths used were different; and it was found that some of them attacked the flours immediately, while others were found to be too weak. So the strengths which were selected, were 25%; 37½%; and 50%. The periods allowed for hydrolysis were 24, 48, and 72 hours. The temperature varied from 28°C to 30°C.

#### Procedure :—

One gramme of the air-dry flour was exactly weighed in a conical flask and 10 c. c. of water were added to make a paste of the flour. Exactly 10 c. c. of Hydrochloric acid of different strengths were added to each flask and shaken for some time. After allowing the hydrolysis to proceed for the fixed period, the excess of hydrochloric acid, was neutralised with alkali, and clarified with zinc oxide, and the volume was made up to 100 c. c. The solution was then filtered on a dry filter paper and 20 c. c. of the filtrate were then used for the determination of the reducing sugar by Bertrand's method. After making the necessary corrections the amount of starch hydrolysed was calculated.

TABLE No. 3

**Hydrolysis of the flours by means of 25% Hydrochloric acid expressed on the basis of the hydrolysable starch (oven dry).**

Name of the flour	time in hours	starch hydrolysed %	Total starch contained in flours. %
Carrot	24	2.363	8.223
"	48	2.778	"
"	72	3.143	"
Sweet Potato	24	0.020	59.875
" "	48	0.064	"
" "	72	0.103	"
Yam	24	0.087	63.316
"	48	0.316	"
"	72	0.552	"
Suran	24	0.306	58.424
"	48	0.385	"
"	72	0.465	"
Potato	24	2.427	72.170
"	48	2.978	"
"	72	3.075	"

From the figures in the above table it is observed that between carrot and sweet potato, carrot is more easily disintegrated than sweet potato.

Now considering the figures for yam and suran flours, it is seen that the yam flour is slightly more disintegrated than suran flour.

In general, among these flours, potato starch for this strength of hydrochloric acid suffers more disintegration than other flours. Starch present in carrot and potato flour is more disintegrated, while starch present in sweet potato flour is much less disintegrated. The order of susceptibility is found as mentioned below:—

Carrot, potato, yam, suran and sweet potato.

In order to see whether the observations made above are repeated with increase in strength of Hydrochloric acid, hydrolysis was carried out with 37½% Hydrochloric acid. The results obtained with 37½% Hydrochloric acid are given in Table No. 4.

TABLE No. 4.

Hydrolysis of the flours by means of 37½% Hydrochloric acid expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	time in hours	starch hydrolysed %	Total starch contained in flours. %
Carrot	24	3.623	8.223
"	48	3.780	"
"	72	3.888	"
Sweet Potato	24	0.103	59.875
"  "	48	0.182	"
"  "	72	0.221	"
Yam	24	0.316	63.316
"	48	1.103	"
"	72	1.497	"
Suran	24	0.621	58.424
"	48	0.700	"
"	72	0.780	"
Potato	24	2.506	72.170
"	48	3.215	"
"	72	3.372	"

From the values of hydrolysis it is seen that with 37½% hydrochloric acid strength, the following is the order of susceptibility:—

Carrot, potato, yam, suran and sweet potato. The order of disintegration remains the same as before.

Hydrolysis was then carried out with 50% strength of Hydrochloric acid and the results obtained are given in Table No. 5.

TABLE No. 5

Hydrolysis of the flours by means of 50% Hydrochloric acid expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	time in hours	Starch hydrolysed %	Total starch contained in flours. %
Carrot	24	4.568	8.223
"	48	4.961	"
"	72	5.906	"
Sweet potato	24	0.182	59.875
" "	48	0.261	"
" "	72	0.339	"
Yam	24	0.709	63.316
"	48	1.891	"
"	72	2.678	"
Suran	24	0.936	58.424
"	48	1.094	"
"	72	1.172	"
Potato	24	2.585	72.170
"	48	3.603	"
"	72	3.923	"

From the results obtained in Table No. 5, it is seen that for further increase in strength of Hydrochloric acid, the rate of hydrolysis is also increased. The increase in the rate of hydrolysis in some cases appears to be more. From the values of hydrolysis obtained, it will be seen that out of the whole lot, the most easily disintegrated is starch present in carrot flour, followed by potato, yam, suran and sweet potato. With increased strength of Hydrochloric acid, the order of susceptibility remains the same as before.

In general it is found that even with 50% strength of Hydrochloric acid, the starch present in different flours is not very much hydrolysed except in the case of carrot flour.

## SECTION III

## HYDROLYSIS OF THE FLOURS WITH DIASTASE

It has been generally believed that raw starches are only digested a little if at all by the human body. Thorpe (Dict. of Appl. Chem. Lond.) quoting from Roberts says, "Starch in raw state is to man an almost indigestible substance". He further states, "Diastase has only comparatively feeble action on the unbroken starch granule, even at the temperature of the body." Fofanow (Z. Klin. Med. 1911, LXXii257) conducted a series of experiments on human subject with small quantities of raw wheat, oat, rice and potato starches. He found that three starches were practically completely assimilated, while raw potato starch was from two and one half to four times less well digested.

Among roots and tubers much work seems to have been carried out in the case of potato. Diastase is an enzyme which acts on starch breaking it down to reducing sugars. It is therefore a good reagent to measure the saccharogenic values of disintegration. The strength of the Diastase used was 0.25%, 0.5%, and 1%. The action was tried for one, two and three hours at a constant temperature of 37°C. The object in selecting 37°C as the temperature was to study the disintegration of starches by the enzyme at the body temperature.

In the present work study of the hydrolysis of the flours of carrot, sweet potato, yam, suran and potato has been made with Diastase (a starch splitting enzyme). Many workers have studied the action of Diastase on the starches, but the action of Diastase does not seem to have been carried out on the flours.

**Experimental:—**

0.25 gm. of the dry flour of each type was accurately weighed into a conical flask and made into a thin paste by adding 10 c.c. of water. To this paste 50 c.c. of boiling water were added and the flask was kept in a boiling water-bath for three minutes. It was then cooled to the room temperature and

30 c.c. of Acetate Buffer solution of pH 4.8—which is the optimum pH of Diastase, were added. Flasks were then kept in an incubator and when the required temperature was reached 10 c.c. of the Diastase solution of different strengths were added, which had also attained the desired temperature, and the flasks were kept at 37°C for the fixed periods. A few drops of toluene were added to prevent any bacterial action. At the end of the fixed period, the enzyme was killed by a few drops of 10% alkali. It was then clarified with zinc oxide. The volume was made up to 100 c.c. and filtered. 20 c.c. of the filtrate were used for the estimation of reducing sugar, and the percentage of starch hydrolysed was calculated. Blank experiment was carried out with Diastase to ascertain if any quantity of reducing sugar was produced by the enzyme, and to make necessary corrections in the results obtained.

The results obtained with Diastase in the case of different flours in the various trials are given in Table No. 6.

TABLE No. 6

Hydrolysis of the flours with 0.25% Diastase expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	Time in hours	Starch hydrolysed %	Total starch contained in flours %
Carrot	1	1.561	8.222
"	2	2.209	"
"	3	2.720	"
Sweet potato	1	9.882	59.875
" "	2	11.080	"
" "	3	12.246	"
Yam	1	9.680	63.316
"	2	11.042	"
"	3	12.160	"
Suran	1	5.821	58.424
"	2	6.239	"
"	3	8.161	"
Potato	1	10.171	72.170
"	2	11.460	"
"	3	12.540	"



From the values of hydrolysis obtained it is clear that the order of susceptibility is as follows:—

Potato, sweet potato, yam, suran and carrot.

Hydrolysis was then carried out with 0.5% Diastase, and the results obtained are given in Table No. 7.

TABLE No. 7

Hydrolysis of the flours with 0.5% Diastase expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	Time in hours	Starch hydrolysed %	Total starch contained in flours. %
Carrot	1	2.205	8.223
„	2	2.834	„
„	3	3.070	„
Sweet potato	1	18.320	59.875
„ „	2	21.786	„
„ „	3	25.460	„
Yam	1	16.230	63.316
„	2	18.261	„
„	3	20.804	„
Suran	1	11.790	58.424
„	2	12.637	„
„	3	14.115	„
Potato	1	19.410	72.170
„	2	22.210	„
„	3	26.320	„

Although by increasing twice the strength the values of hydrolysis are doubled in the case of potato and sweet potato, yet the order of susceptibility so far as the flours are concerned remains the same as before. Taking into consideration the amount of starch hydrolysed at the end of the third

period and the amount of starch initially present, the order of the susceptibility is as follows :—

Sweet potato, carrot, potato, yam and suran. With 0.25% strength the order was as follows:—

Carrot, sweet potato, yam, potato and suran.

TABLE No. 8

Hydrolysis of the flours with 1% Diastase expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	Time in hours	Starch hydrolysed %	Total starch contained in flours %
Carrot	1	3.470	8.223
"	2	4.095	"
"	3	5.040	"
Sweet potato	1	26.460	59.875
" "	2	29.204	"
" "	3	32.035	"
Yam	1	26.160	63.316
"	2	28.213	"
"	3	30.133	"
Suran	1	18.114	58.424
"	2	20.660	"
"	3	23.418	"
Potato	1	23.101	72.170
"	2	30.050	"
"	3	33.010	"

From the results obtained although in general it appears that carrot starch undergoes lowest hydrolysis, yet taking into consideration the amount of starch initially present in these flours, and the starch hydrolysed at the end of the third period, the order is as follows :—

Carrot, sweet potato, yam, potato and suran.

## SECTION IV

HYDROLYSIS OF THE FLOURS WITH  
PANCREATIN.

Pancreatin is another enzyme which acts on starch, and hence the action of this also was tried. Rockwood (J. Biol. Chem. 1910,32) studied the action of Pancreatin on the starch from bleached and unbleached wheat flours. Many workers have studied the action of Pancreatin on starches, but similar action does not seem to have been studied in the case of the flours. It was thought desirable to study the action of Pancreatin on the flours to see if any similar effect was noticed. As in the last case three strengths of Pancreatin were chosen. These were 0.25%, 0.5%, and 1%, periods and temperature were the same as in the case of Diastase. Sugar formed from time to time was estimated and percentage hydrolysis was calculated, and the results obtained were compared—A blank experiment was also run side by side. Experimental procedure followed was the same as before. Buffer used was 6.8 (pH 6.8).

TABLE No. 9

Hydrolysis of the flours with 0.25% Pancreatin expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	Time in hours	Starch hydrolysed %	Total starch contained in flours. %
Carrot	1	1.260	8.223
"	2	1.575	"
"	3	1.890	"
Sweet potato	1	8.560	59.875
" "	2	10.502	"
" "	3	10.989	"
Yam	1	8.440	63.316
"	2	9.840	"
"	3	10.432	"
Suran	1	5.190	58.424
"	2	6.183	"
"	3	7.210	"
Potato	1	8.130	72.170
"	2	9.820	"
"	3	11.160	"

In order to study further the effect of increasing the strength of Pancreatin, the reaction was carried out further by employing 0.5% as the strength of Pancreatin, and the results obtained are given in the following Table.

TABLE No. 10

Hydrolysis of the flours with 0.5% Pancreatin expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	Time in hours	Starch hydrolysed %	Total starch contained in flours. %
Carrot	1	1.890	8.223
"	2	2.205	"
"	3	2.834	"
Sweet potato	1	15.124	59.875
" "	2	17.007	"
" "	3	20.824	"
Yam	1	13.227	63.316
"	2	14.220	"
"	3	17.001	"
Suran	1	10.452	58.424
"	2	11.566	"
"	3	13.234	"
Potato	1	16.108	72.170
"	2	18.518	"
"	3	21.925	"

TABLE No. 11

Hydrolysis of the flours with 1% Pancreatin expressed on the basis of the total hydrolysable starch (oven dry).

Name of the flour	Time in hours	Starch hydrolysed %	Total starch contained in flours. %
Carrot	1	3.150	8.223
"	2	3.470	"
"	3	4.095	"
Sweet potato	1	21.735	59.875
" "	2	24.629	"
" "	3	26.827	"
Yam	1	19.900	63.316
"	2	23.620	"
"	3	25.120	"
Suran	1	16.520	58.424
"	2	18.060	"
"	3	21.115	"
Potato	1	23.435	72.170
"	2	25.380	"
"	3	27.121	"

From the results of the experiments with Diastase and Pancreatin, it has been found that with increasing strengths of the hydrolysing agents, the values of disintegration also increase.

The values of hydrolysis are found to increase when the period of the reaction is also increased, although the increase is not exactly proportional to the time.

Values of hydrolysis obtained with Diastase are higher than those obtained with Pancreatin.

If the values of hydrolysis of the flours as such are considered, carrot undergoes much less hydrolysis than other flours.

Taking into consideration the total amount of starch initially present and the starch hydrolysed at the end of the

third hour, carrot undergoes more hydrolysis than the other flours.

Order of susceptibility so far as the flours are concerned, does not alter.

By an increase in strength order of susceptibility in some of the flours also changes, when hydrolysis is considered in relation to starch originally contained in the flour.

## SECTION V

### HYDROLYSIS OF THE FLOURS WITH PANCREATIN IN THE PRESENCE OF SALTS

Human food consists of a variety of substances derived from animal and vegetable kingdom. They are classified according to their chemical nature consisting of proteins, fats, carbohydrates, inorganic salts, etc. Along with the cooked or uncooked material, salts are also consumed. Enzymes act on cooked as well as on uncooked starch.

It is well known that many enzyme reactions are accelerated or retarded by salts. A lot of controversy exists regarding the action of neutral salts. In some cases the influence of the salts has been studied on soluble starches in presence of Diastase or Pancreatin with a view to determine the effect which influences enzyme activity. The present investigation was undertaken to study the effect produced by Pancreatin in presence of salts on starch contained in the flours and not on merely separated starches in some edible roots and tubers such as, sweet potato, yam and suran. The object is to judge whether salts are helpful to the disintegration of the starch contained in the flour as such. As much work on potato has been carried out in presence of salts in case of potato, and as carrot contains a small proportion of starch, experiments were carried out in the case of sweet potato, yam and suran flours.

#### Experimental :-

0.25 gm. of the flour was made into a thin paste by adding 10 c.c. of distilled water in a conical flask. 40 c.c. of boiling

water were then added to the flask and the flask was held in a boiling water bath for exactly three minutes to gelatinise starch contained in the flour. It was then cooled and was kept in an incubator. 20 c.c. of the buffer solution were then added, which were also kept in an incubator to attain the desired temperature (37° C). Phosphate buffer having pH 6.8 (being the optimum pH of Pancreatin) was used as this was found to be suitable for the purpose. Phosphate buffer produced no measurable difference in the hydrolysis of starch. Then 20 c.c. of the salt solution containing known amounts of sodium chloride were added. When the temperature of the mixture was 37° C, 10 c.c. of the solution of Pancreatin which had also attained the required temperature, were added. The strength of the solution of Pancreatin was 0.5%. The total volume of the mixture was 100 c.c. The reaction mixtures prepared accordingly were then kept for 1 and 2 hours respectively. Few drops of toluene were also added to the mixture. The flasks were removed at an interval of one hour. The reaction was then stopped by adding a few drops of alkali and the sugar formed was then estimated as usual. A blank experiment was run side by side containing no salt, so that activation due to salt could be very well determined. As no appreciable influence is produced by the Phosphate buffer during the first two hours, the reaction is studied for two hours only.

TABLE No. 12.

Hydrolysis of sweet potato flour with Pancreatin in presence of sodium chloride.

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysis without salt. %	Starch hydrolysed in presence of salt. %	Total starch contained in flours %
Sweet potato	0.5	1	15.124	16.482	59.875
„	„	2	17.007	18.845	„

From these results it is clearly observed that salt has an influence in accelerating the starch hydrolysis.

The results obtained show that the activity of Pancreatin is slightly affected with this concentration of the salt employed. As 0.5% concentration of sodium chloride, facilitated the hydrolysis of Pancreatin slightly more, it was therefore thought that by employing a higher concentration of the salt, whether the hydrolysis produced is diminished or increased as before, the reaction was therefore carried out further. With a view to see if the concentration of the salt is increased five to six times more, whether the salt has a remarkable accelerating or retarding influence upon the hydrolysis of the starch contained in the flours, the reaction was therefore carried out further at a salt concentration of 3%, other conditions of the experiment remaining unaltered. Experimental procedure adopted was the same as before.

The results obtained are given in the following Table :—

TABLE No. 13

**Hydrolysis of sweet potato flour with Pancreatin in presence of sodium chloride.**

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysed without salt. %	Starch hydrolysed in presence of salt. %	Total starch contained in flours %
Sweet potato	3	1	15.124	18.882	59.875
	"	2	17.007	21.562	"

From the results thus obtained it is seen that with increased concentration of salt, the values of hydrolysis are also increased. It is observed that in the case of sodium chloride at a salt concentration of 3%, the hydrolysis is more than in the case of 0.5%, and the activity of Pancreatin is greater in the presence of 3% sodium chloride than in the case of 0.5% sodium chloride.

It is well known that H ion concentration has also an influence upon enzymic hydrolysis of starch in presence of salt. Acceleration or retardation by natural salts depends upon the



H ion concentration of the medium. In the case of sodium chloride, Sherman (Loc. Cit.) has determined an Optimum  $pH$  7.1—7.2 for particular concentration of sodium chloride, who carried out investigations on Pancreatin amylase. The results obtained with the concentration of the salt employed by him were found out by him in the case of starches. But the Optimum  $pH$  determined for starch, may or may not differ from the Optimum  $pH$  in the case of the flour. That is it may or may not accelerate or retard the action. It was therefore thought to find out first the Optimum  $pH$  for the flour in the presence of salt and then to determine the values of disintegration of the starches contained in the flours.

In the case of sodium chloride the activity of Pancreatin was studied in the presence of 0.5% concentration of sodium chloride at closely graded H ion activities viz.  $pH$  6.8, 7.1, 7.4 and 8.0. The results obtained are given in Table No. 14. The reaction was allowed to proceed for one hour.

TABLE No. 14.

**Hydrolysis of sweet potato flour with Pancreatin in presence of sodium chloride at varying  $pH$ .**

Name of the flour	Concentration of salt employed (%)	Buffer used ( $pH$ )	Starch hydrolysed %	Total starch contained in flours %
Sweet potato	0.5	6.8	16.482	59.875
„ „	„	7.1	16.691	„
„ „	„	7.4	16.610	„
„ „	„	8.0	16.101	„

From the results obtained it is seen that the Optimum  $pH$  for the flour is 7.1. When the salt concentration is 0.5%, the Optimum  $pH$  is 7.1. By suitably varying the salt concentration to 3%, it was found that the Optimum  $pH$  was also found to be 7.1. The reactions were therefore, carried out further at  $pH$  7.1, other experimental conditions, remaining unaltered, in the case of sweet potato, jam and suran flours,

TABLE No. 15

**Hydrolysis of different flours with Pancreatin in  
presence of sodium chloride.**

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysed without salt (pH6.8) %	Starch hydrolysed in presence of salt %	Total starch contained in flours %
Sweet potato	0.5	1	15.124	16.691	59.875
" "	"	2	17.007	19.102	" "
Yam	"	1	13.227	13.826	63.316
"	"	2	14.220	14.329	" "
Suran	"	1	10.452	10.657	58.424
"	"	2	11.566	11.765	" "

From the figures obtained in the above table, it is evident that if the values of hydrolysis obtained due to Pancreatin alone are deducted from these, the difference will account for the increase in the values of hydrolysis, and from the increase in the values, it will be possible to judge more definitely, which of these starches contained in different flours undergo more or less disintegration.

The values of hydrolysis are slightly greater in presence of salt than those obtained without the addition of sodium chloride in case of Pancreatin indicating thereby that 0.5% sodium chloride slightly accelerates the disintegration of starches contained in the different flours. Among these flours with this strength of sodium chloride, starch contained in the flour of sweet potato is more hydrolysed than the starch contained in the flour of yam and suran. In this case it was also noticed that if the values of hydrolysis obtained due to Pancreatin after two hours are deducted from the values obtained at the end of the second hour with Pancreatin in the presence of salt, from the increase in the values of hydrolysis it is also found that yam flour is more attacked than suran flour, and sweet potato flour is more hydrolysed than these two flours.

In order to see whether with slightly more increase in the concentration of sodium chloride similar types of results are obtained, or whether there is any variation in the values of hydrolysis, experiments were carried out by employing 1% concentration of sodium chloride. The results obtained are given in Table No. 16.

TABLE No. 16  
Hydrolysis of different flours with Pancreatin in presence of sodium chloride.

Name of the flour	Concentration of salt employed %	Time in hours	Starch hydrolysed without salt (pH 6.8) %	Starch hydrolysed in presence of salt %	Total starch contained in flours %
Sweet potato	1	1	15.124	17.542	59.875
" "	"	2	17.007	20.301	"
Yam	"	1	13.227	14.242	63.816
" "	"	2	14.220	14.939	"
Suran	"	1	10.452	10.946	58.424
" "	"	2	11.566	12.027	"

From the increase in the values it is found that sweet potato flour does not undergo much hydrolysis as is observed in the case of yam and suran flour. Of course the total value of hydrolysis in the case of sweet potato flour is more with 0.5% and 1% salt concentration than with other flours. From the total increase in the values of hydrolysis the order of susceptibility is the same as was found in the case of 0.5% sodium chloride. But taking into consideration two times increase in the salt concentration of sodium chloride, the increase in the case of sweet potato flour is not much. The presence of sodium chloride has an accelerating effect when starch is hydrolysed with Pancreatin. In this case also if the values of hydrolysis obtained with Pancreatin alone at the end of the second hour are deducted from the results obtained at the end of the second period from the increase in the values of hydrolysis also it will be observed that sweet potato flour is more attacked than yam and suran flour respectively.

The values of hydrolysis in two cases that is either by employing a concentration of 0.5% or by employing a concentration of 1%, the values of hydrolysis obtained are not proportional to the concentration of the salt employed. Similarly the hydrolysis produced is also not proportional to time. This was observed in the case of sweet potato, yam and suran flours. In order to see whether by employing three times more i. e. 3% concentration of sodium chloride, the latter has a marked accelerating influence, the reaction was carried out in the presence of 3% sodium chloride. The results obtained with Pancreatin by employing 3% concentration of sodium chloride are given in Table No. 17.

TABLE No. 17.

**Hydrolysis of different flours with Pancreatin in presence of sodium chloride.**

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysed without salt (pH 6.8) %	Starch hydrolysed in presence of salt %	Total starch contained in flours %
Sweet potato	3	1	15.124	19.098	59.875
" "	"	2	17.007	22.039	"
Yam	"	1	13.227	15.256	63.316
"	"	2	14.220	16.238	"
Suran	"	1	10.452	11.307	58.424
"	"	2	11.566	12.662	"

From the values of hydrolysis it is clear that the values of hydrolysis also increase with an increase in the concentration of sodium chloride.

From the results obtained it is observed that sodium chloride accelerates the action of Pancreatin on starch. The accelerating effect of sodium chloride on enzymic hydrolysis of starch is also influenced by the concentration of the salt employed. From the concentration of sodium chloride employ-

ed, it was found that with increase in the concentration of the salt, hydrolysis also increases, but the increase in the values of hydrolysis was not found to be proportional to the concentration of the salt employed.

The order of susceptibility was as follows :—

Sweet potato, yam and suran.

It was also observed that H ion concentration containing varying concentration of sodium chloride, the reaction of the medium was not altered by the presence of salt in the concentration employed.

Sodium chloride is capable of accelerating the velocity of hydrolysis of starch depending upon the pH of the medium. At pH 7.1 the reaction was accelerated, and further retarded at pH 8.0.

It is likely that the salts accelerate the hydrolysis of starch by protecting the enzyme against inactivation or from inhibiting substances produced during the reaction.

#### **Hydrolysis of different flours with Pancreatin in presence of Calcium Phosphate (mono).**

Having studied the effect of sodium chloride on the acceleration of enzymic hydrolysis of starch contained in different flours, the next salt used for studying the reaction was calcium phosphate (mono). As no measurable effect was found with 1% calcium phosphate, results obtained with 3% concentration have been given.

#### **Experimental Procedure.—**

0.25 gm. of the flour was made into a thin paste by adding 10 c. c. of water. 40 c. c. of boiling water were then added to the flask and the flask was held in a boiling waterbath for three minutes to gelatinise starch contained in the flour. It was then cooled and was kept in an incubator. 20 c. c. of the buffer solution were then added, which had also attained the required temperature. Phosphate buffer having pH 7.1

was used. Then weighed amount of salt was added and also 20 c. c. of distilled water were added. When the required temperature ( $37^{\circ}$ ) was reached, 10 c. c. of the solution of pancreatin (having 0.5% strength) were added. It had already attained the desired temperature. The total volume of the mixture was 100 c. c. The reaction mixtures prepared accordingly were then kept for 1 and 2 hours respectively. A few drops of toluene were added, sugar formed was estimated as usual. As in the case of sodium chloride, a controlled experiment was also run side by side. The results obtained are given in the following Table.

TABLE No. 18.

**Hydrolysis of different flours with Pancreatin in presence of Calcium Phosphate.**

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysed without salt pH 6.8 %	Starch hydrolysed in presence of salt %	Total starch contained in flours %
Sweet potato	3	1	15.124	15.160	59.875
" "	"	2	17.007	17.044	"
Yam	"	1	13.227	13.240	63.316
"	"	2	14.220	14.235	"
Suran	"	1	10.452	10.460	58.424
"	"	2	11.566	11.572	"

From the results obtained it will be observed that calcium phosphate has a slight effect in accelerating the enzymic hydrolysis of starch contained in flours.

If these results are compared with those obtained in the presence of 3% sodium chloride, it is found that calcium phosphate has a slight effect in accelerating the enzymic hydrolysis of starch.

**Hydrolysis of different flours with Pancreatin in presence of Potassium Tartrate.**

The next salt used was potassium tartrate. The experimental procedure adopted was the same as was followed in the case of calcium phosphate.

As no measurable effect was found with 0.5% potassium tartrate, in the case of yam and suran flours, the result obtained with 0.5% salt concentration in the case of sweet potato has been given in Table No. 19.

TABLE No. 19

**Hydrolysis of the flours with Pancreatin in presence of Potassium Tartrate.**

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysed without salt pH 6.8 %	Starch hydrolysed in presence of salt %	Total starch contained in flours %
Sweet potato	0.5	1	15.124	15.930	59.875
" "	"	2	17.007	17.816	"

From the results it is observed that the results obtained with 0.5% sodium chloride are much higher than those obtained with 0.5% potassium tartrate.

Experiments were then carried out by employing 3% concentration of potassium tartrate, and the results obtained are given below.

TABLE No. 20

**Hydrolysis of the flours with Pancreatin in presence  
of Potassium Tartrate.**

Name of the flour	Concentration of salt employed (%)	Time in hours	Starch hydrolysed without salt pH6.8 %	Starch hydrolysed in presence of salt %	Total starch contained in flours %
Sweet potato	3	1	15.124	16.540	59.875
" "	"	2	17.007	19.434	"
Yam	"	1	13.227	13.340	63.316
"	"	2	14.220	14.335	"
Suran	"	1	10.452	10.539	58.424
"	"	2	11.566	11.651	"

From the results obtained in Table No. 18 and 20, it is seen that potassium tartrate has a greater accelerating effect than calcium phosphate. However, among the three salts used, it is found that sodium chloride has an appreciable effect in accelerating the hydrolysis of starch contained in different flours.

### SUMMARY AND CONCLUSION

The object of this paper was to study the hydrolysis of starches from typical roots and tubers, and the reagents used were HCl, Diastase, Pancreatin. In order to determine the total hydrolysable starch contained in the flour, reactions with hydrochloric acid were studied. It was found that with increase in strength, the values of disintegration increase very slowly, and the increase was not proportional to the time allotted.

As these substances were rich in starch, starch-splitting enzymes were used namely Diastase and Pancreatin under varying conditions of time and with different strengths. From the results of the experiments with Diastase and Pancreatin, the following observations were made:—



with increase of strength of Diastase or Pancreatin, the values of disintegration also increased. The values of hydrolysis were found to increase when the period of the reaction was also increased, although the increase was not proportional to the time.

Values of hydrolysis obtained with Diastase were higher than those obtained with Pancreatin.

If the values of hydrolysis of the flours as such were considered, carrot flour had undergone much less hydrolysis than other flours. However, taking into consideration the total amount of starch initially present and the starch hydrolysed at the end of the third period, carrot had undergone much more hydrolysis than the flours.

Order of susceptibility so far as the flours were concerned, did not alter. By an increase in strength there was a change in order of susceptibility in some of the flours, when hydrolysis was considered in relation to starch originally contained in the flour.

In order to study whether addition of salts had any influence on enzyme activity, experiments were carried out in the case of three salts, namely sodium chloride, calcium phosphate (mono), and potassium tartrate upon the flours of sweet potato, yam and suran with Pancreatin.

The values of hydrolysis were slightly higher than those obtained in the case of Pancreatin alone indicating thereby that salts facilitate hydrolysis of flours with Pancreatin.

Enzyme showed greater activity in presence of sodium chloride than with other salts.

It was found that with increase in the concentration of sodium chloride, hydrolysis also increased, but the increase in the values of hydrolysis was not found to be proportional to the concentration of the salt employed.

The order of the susceptibility was as follows:- Sweet potato, yam and suran.

It is likely that the salt accelerates the hydrolysis of starch by protecting the enzyme against inactivation.

## PAPER VI

## THE INFLUENCE OF SOME ADDED SUBSTANCES ON THE HYDROLYSIS OF THE STARCHES IN DIFFERENT FLOURS

By

D. N. GODBOLE, M. Sc.

*(With the kind permission of the authorities of the Bombay University.)*

**Introduction :**

When we take some starchy food such as cooked rice, we find that the addition of the common salt increases the palatability. The object of this work is to find out whether the addition of common salt increases the palatability of the stuff and thereby increases the secretions in the body, or it actually helps in breaking down the material in the alimentary canal, and if it helps, to what extent it helps, and this can be judged in-vitro by studying the hydrolysis of the flours when acted upon by the enzymes in the presence of sodium chloride.

Nasse (Pflugers Arch 1875, 11, 138) first observed that the animal amylase becomes more active in the presence of the neutral salts, but Sherman, Caldwell and Cleaveland (J. A. Chem. Soc. 52, 2436, 1930) have shown that for the activity of the malt amylase neutral salts are not essential. Sherman, Caldwell and Admans (J. Am. Chem. Soc. 1928, 2538, 50) found that the effect of neutral salt on the activity of the pancreatic amylase does not depend on the purity of the enzyme. Neutral salts are necessary to the activity of the pancreatic amylase. Sherman and co-workers (J. A. Chem. Soc. 1927, 49, 2596, 1928, 50, 2539, 2535, 2538) and Willstatter, Waldschmidt, Leitz Hesse, (Z. Physical. Chem. 1923, 136, 143) investigated the activating effect of the neutral salts on the activation of salivary amylase. Michaelis and Pechastein (Bio. Chem. Z. 1914, 59, 77) observed that the diastase is inactive in the absence

of the salts. Myrback (Z. Physiol. Chem. 1926, 159, 1) however observed that salivary amylase is active even in absence of salts.

As regards effect of salts on vegetable amylase, Eadie (Bio. Chem. J. 1926, 20, 1016) observed activation with barley malt amylase. So also Patwardhan and Norris (J. Ind. Inst. Sci. 1928, 11A, 63) with *colum* malt diastase, Narayanmurty (Ibid, 1930, 13A, 63) with *colum* malt amylase and Sherman, Caldwell and Cleaveland (J. Am. Chem. Soc. 1930, 52, 2406) with barley malt amylase, and Lisbonne (Soc. Biol. 1912, 72, 936) with electrodyalysed malt amylase found the activation by the presence of salts.

Fricke and Kaja (Ber. 1925, 57, 310, 313), Narayanmurty and Norris (J. Ind. Inst. Sci. 1928, 11A, 104) observed inactivation with electrolysed barley malt amylase and *colum* malt diastase respectively. Karmarkar and Patwardhan (Ibid. 1930, 13A, 159) found no effect with wheat amylase. James and Cattle (Bio. Chem. J. 1933, 27, 1805) found that in potato amylase the part responsible for breaking down the starchy molecule to dextrins was capable of activation. Heen and Schweigart (Bio-Chem. Z. 1923, 143, 516) separated free and active amylase from potato amylase.

A quantitative study of the influence of sodium acetate, sodium borate, sodium citrate, sodium phosphate was made by Sherman, Caldwell and Dale (J. Am. Chem. Soc. 1927, 49, 2596). The influence of neutral salt on the enzyme hydrolysis of starch under controlled conditions of buffer strengths and salt strengths was studied by Giri and Shrikhande (J. Ind. Chem. Soc. Vol. XII, 273, 1935) and it was found that the accelerating effect of neutral salt increased with the increase in concentration of the salt to a certain extent, but on further increase of salt concentration the effect was less. The buffer salt concentration has also influence on the hydrolysis of starch in enzyme reaction (Giri and Shrikhande).

With all these findings we see that there is a lot of controversy as regards the effect of neutral salts. In this section

we are going to study to what extent sodium chloride influences the hydrolysis of the starches in the flours both at optimum pH and at different pH values.

## SECTION I

### THE EFFECT OF SODIUM CHLORIDE ON THE HYDROLYSIS OF THE FLOURS BY DIASTASE AT THE OPTIMUM pH 4.8.

In the first paper we have studied the hydrolysis of starches in flours with the aid of diastase. But the salts have definite effect on the hydrolysis of starches by diastase. Here we want to find out to what extent the common salt activates the hydrolysis of starches in the flours by diastase. For this we have chosen three periods namely one hour, two hours, and three hours and the salt concentrations are 0.05 M, 0.15 M and 0.3 M. 1.0% diastase solution is used in these experiments.

#### Experimental:

1 gram of flour was gelatinised with 10 c. cs. of distilled water by heating on water bath for three minutes. Then 10 c. cs. of the acetate buffer of 4.8 pH were added. Then after putting in the required c. cs. of sodium chloride solution and 10 c. cs. of 1.0% diastase solution, the final volume was made up to 60 c. cs. Final molar concentration of the salt was 0.05, 0.1, 0.15 and 0.3. Final concentration of buffer solution in the reaction mixture was 0.004 mol. acetate. Few drops of toluene were added as an antiseptic. The flasks were kept at 37°C for one hour. After keeping the flasks for the required period few c. cs. of 10% NaOH were added to check the reaction. The clarification was done by zinc oxide and estimation of reducing sugars was carried out according to Bertrand's method as given in the first part.

TABLE No. 1

Hydrolysis of the wheat flour with 10 c. cs. of 1.0% diastase and with the different salt concentrations (NaCl)

expressed on the basis of total hydrolysable starch (oven dry) pH 4.8.

	1 Hr.	2 Hrs.	3 Hrs.	Total starch hydrolysable
Without salt	26.92	28.17	29.86	
with NaCl 0.05 M	27.62	31.09	31.09	
"  "  0.1 M	26.92	29.91	31.09	74.29
"  "  0.15 M	25.66	27.89	31.09	

We have seen that in the case of hydrolysis of wheat by 1.0% diastase, the rate of hydrolysis is 27% during the first hour and then the rate falls down to 1.28% during the second hour and again rises slightly to 1.69% during the third hour. On addition of 0.05 mol. sodium chloride the rate during the first hour now becomes 27.62% as against 26.92% which is without the use of common salt. Similarly the rate corresponding to the rate of hydrolysis during the second period is 3.5% while in the last period there is practically no difference. This indicates that almost the whole of the disintegration is completed by the end of the second hour in the presence of 0.05 M salt concentration, whereas in its absence the reaction proceeds further and attains its maximum value at the end of the third hour. Comparing these figures we observe the activation effect, and this can be expressed by the difference between the figures obtained with and without salt at that particular period. Thus the activation by 0.05 M is 0.7 during the first hour 2.9 during the second hour and 1.2 during the third hour. The effect is maximum during the second hour.

On increasing the salt concentration to 0.1 M the rate of hydrolysis is 26.92% during the first hour, 2.89% during the second hour and 1.28% during the third hour as against 26.92%, 1.25% and 1.69% during the first, second and third hour without salt. Now if we consider the activating effect, it is zero during the first hour, 1.64 during the second hour and 1.23 during the third hour. With higher salt concentration of 0.15 Mol, there is a drop in the value of hydrolysis during the first hour (25.66% against 28.17%). But during the third hour we observe the activation.

If we consider the values obtained during the first hour, the addition of 0.05 Mol. sodium chloride increases the rate of hydrolysis, then on further addition of the salt (0.1 M) we see no activation but a retardation in the rate of hydrolysis. The same effect of retardation is also observed with more concentration of salt (0.15 M). During the second hour, as in the case of the first hour, 0.5 M salt concentration shows the maximum activating effect and further addition of salt shows a drop in the value of hydrolysis. The only difference is that with 0.1 M salt concentration the value is a bit higher than that without addition of salt. During the third hour the addition of salt favours the disintegration. During this period we observe only 31.09% hydrolysis with all the concentrations of the salt (0.05 M, 0.1 M and 0.15 M). This shows that the reaction is practically complete, when only 31.09% of the total hydrolysable starch is disintegrated. This may perhaps be due to the accumulation of reaction products which check further action. Similarly weakening of the enzyme and lessening of the substrate may arrest the further reaction.

From these observations we find that the optimum salt concentration for wheat flour is 0.05 M and the reaction is practically stopped at the end of second hour. Further increase of salt concentration retards the velocity of reaction.

TABLE No. 2

**Hydrolysis of Jowar flour by 10 c.c.s. of 1.0% diastase and with different salt concentrations expressed on the basis of the total hydrolysable starch oven dry.**

	1 Hr.	2 Hrs.	3 Hrs.	Total starch hydrolysable
without salt	27.78	29.54	30.57	
NaCl. 0.05 M.	29.54	30.40	31.98	77.13
„ 0.10 M	30.16	30.52	32.42	
„ 0.15 M	30.57	31.06	32.49	
„ 0.30 M	27.55	28.17	32.49	

With the salt concentration of 0.05 M, the activation of the diastase hydrolysis is observed during all periods. If we compare the values obtained with 0.05 M salt concentration to those obtained without addition of the salt it is observed that during the first hour the velocity of the hydrolysis is increased to 29.54% from 27.78%. The activation is 1.76 during the first hour, 0.86 during the second hour and 1.41 during the third hour. With 0.10 M salt concentration the activation is 2.36 during the first hour, 0.98 during the second hour and 1.85 during the third hour. With 0.15 M concentration the activation is 2.79 during the first hour, 1.52 during the second hour and 1.92 during the third hour.

But with higher salt concentration of 0.3 M we observe the retardation during the first and second hour, while during the third hour there is an activation of 1.92.

Comparing all these values we find that the optimum salt concentration for Jowar flour to be hydrolysed by diastase is 0.15 M and the period three hours. The maximum value of hydrolysis is 32.49% which is attained at the end of the third hour.

TABLE No. 3

**Hydrolysis of the Rajgira flour with 10 c.cs. of 1% diastase and with different salt concentrations.**

	1 Hr.	2 Hrs.	3 Hrs.	Total starch hydrolysable
without salt	26.76	31.92	33.51	
NaCl 0.05 M	26.76	29.88	33.51	59.67
„ 0.10 M	25.54	29.61	33.51	
„ 0.15 M	24.51	29.51	33.51	

TABLE No. 4

**Hydrolysis of the Nachani Flour with 10 c. cs. of 1% Diastase and with Different Salt Concentrations.**

		1 Hr.	2 Hrs.	3 Hrs.	Total starch hydrolysable.
without salt	...	29.0	33.11	34.56	
with NaCl	0.05 M	31.15	33.92	35.18	71.67
„ „	0.10 M	30.67	32.47	33.92	
„ „	0.15 M	30.14	31.15	33.92	

In case of Rajgira no increase is observed in the velocity of the reaction at the salt concentration of 0.05 M, during all the three periods. Even at higher salt concentrations we observe the retardation in the velocity of the hydrolysis. The salt has got a negative effect on the hydrolysis of the Rajgira flour by diastase. At the end of third hour, there is no difference in the values of hydrolysis with or without salt. This shows that the salt has no effect in the third hour on the hydrolysis of starch in the Rajgira flour by diastase.

In the case of Nachani flour, we get higher values with salt concentrations of 0.05 M, 0.10 M and 0.15 M, than those obtained without salt; but maximum value is observed at the salt concentration of 0.05 M. During all the three hours the values at salt concentration of 0.1 M and 0.15 M are lower than those without salt. But the values at 0.05 M are higher. Thus the activation in the hydrolysis of the Nachani flour is observed at the salt concentration 0.05 M and the period of three hours.

Comparing all these tables we find that for wheat flour hydrolysis, the optimum salt concentration is 0.05 M sodium chloride, for Jowar flour 0.15 M, for Nachani flour it is 0.05 M. For Rajgira flour, however, addition of salt retards the hydrolysis by diastase. Among all the flours Rajgira has the least quantity



of the total hydrolysable starch and addition of salt has retarding effect on its hydrolysis.

In the case of Jowar flour which contains maximum amount of total hydrolysable starch, the activation of salt in the enzymic hydrolysis is observed up to the salt concentration of 0.15 Mol. A larger quantity of salt is required to give maximum activation. This may perhaps be due to the larger quantity of the hydrolysable starch in the Jowar flour.

## SECTION II

### EFFECT OF ADDITION OF SODIUM CHLORIDE ON THE HYDROLYSIS OF THE FLOURS BY PANCREATIN AT 4.8 pH.

In Section I of this paper we have studied the activating effect of the salt on the hydrolysis of the flours by diastase and found the optimum salt concentration and optimum period. Now it is to be seen whether the change of the hydrolysing agent will affect the effects of salt concentration observed in the flours hydrolysed by diastase. For this two flours, namely of Wheat and Jowar were selected. Experimental procedure was the same as in 1st section. The strength of the Pancreatin used was 1% and the quantity used for each experiment was 10 c. cs.

TABLE No. 5.

Hydrolysis of the Wheat flour with 10 c. cs. of 1% Pancreatin with and without addition of Sodium Chloride, calculated on the basis of total hydrolysable starch (oven dry).

		1 Hr.	2 Hrs.	3 Hrs.	Total starch hydrolys- able.
without salt	...	6.57	10.32	12.15	
with NaCl.	0.05 M	7.05	10.32	12.15	74.29
„	„ 0.10 M	5.64	9.40	11.90	
„	„ 0.15 M	4.70	8.46	9.87	

TABLE No. 6

**Hydrolysis of the Jowar flour with 10 c.c.s of 1% Pancreatin with and without addition of Sodium Chloride, calculated on the basis of total hydrolysable starch (oven dry).**

	1 Hr.	2 Hrs.	3 Hrs.	Total starch hydrolysable
without salt	11.12	19.12	21.38	
with NaCl .05 M	17.04	19.27	26.24	77.13
„ „ .10 M	20.75	20.75	26.68	
„ „ .15 M	20.75	20.75	26.68	
„ „ .30 M	19.27	20.01	24.45	

With 1% pancreatin (10 c. cs. of 1% pancreatin solution) the rate of hydrolysis of the wheat flour is 6.57% during the first hour then it falls to 3.75% and 1.83% during the second and third hours without addition of salt. In presence of sodium chloride (0.05 M) the rate of hydrolysis is increased to 7.05% and during second and third hour there is no change in the figures of disintegration obtained with or without salt. Thus the activating effect of salt is observed during the first hour only.

Now with higher concentration of 0.1 M and 0.15 M we find no increase in the velocity of reaction but on the contrary there is a retardation in the disintegration of the flour. The rate is changed to 5.64% and 4.7% with the salt concentration of 0.1 M and 0.15 M respectively, as against 6.57% during the first hour and 10.32 during the second hour. During the third hour also, a drop in the rate of reaction is observed. Thus with increase in the salt concentration the wheat flour disintegration decreases. Thus the optimum salt concentration is 0.05 M and the activation is observed during the first hour only.

If we compare these figures of disintegration with the help of pancreatin, with those of diastase, we find the optimum

salt concentration is 0.05 M in the case of both the hydrolysing agents. But with the diastatic hydrolysis the activating effect of salt is found during the second and third hour, while in the case of pancreatin hydrolysis no activation is observed during second and third hours. With higher salt concentration of 0.1 M and 0.15 M there is retardation in the hydrolysis with the help of diastase and pancreatin.

**Jowar:-** Without addition of salt the rate of disintegration of the jowar flour by pancreatin is 11.12% during the first hour, 8% during the second and 2.26% during the third hour.

With the salt concentration of 0.05 M the rate becomes 17.4% during the first hour, 2.23% and 6.97% during the second and the third hour. Thus the activating effect of salt is 5.92 during the first hour, 0.15 and 4.86 during the second and the third hour. With salt concentration of 0.1 M and 0.15 M the activation by the salt in the hydrolysis of the flour is maximum. It is 9.63 during the first hour, 1.63 and 3.30 during the second and the third hour. The activating effect with both the salt concentrations of 0.1 M and 0.15 is the same for all the three periods. It is maximum during the first hour and minimum during the second hour.

If we increase the salt concentration to 0.3 M we observe the activating effect, but the values are lower than those with 0.1 M and 0.15 M salt concentrations. This shows that if we increase the salt concentration the activating effect is lowered down. Thus the optimum salt concentration is 0.1 M to 0.15 M.

Comparing these figures with those of diastase we find that in the diastatic hydrolysis the optimum salt concentration is 0.15 M and the period is the third hour, but in the case of pancreatin hydrolysis the range of optimum concentration is increased. It is 0.10 M to 0.15 M and maximum hydrolysis takes place during the third hour. Thus the effect of salt concentration on the hydrolysis of starches in flours changes with change in hydrolysing agent.

## SECTION III

**EFFECT OF ADDED OIL AND PEPTONE ON THE  
DISINTEGRATION OF THE FLOURS WHEN  
ACTED UPON BY DIASTASE AND  
PANCREATIN**

Sahasrabuddhe and Kibe (M. Sc. thesis) have shown that the various constituents of the grains have a mutual positive or negative effect on the course of hydrolysis. Basu and Sarkar (Jour. Ind. Med. Res. 1935, 22, 759) have investigated the digestibility of rice starch and suggested that the uniformly higher contents of fat and protein characterising the 'aman' type varieties of rice are responsible for their difficult digestibility. Similarly Bhide and Sahasrabuddhe (Agri. College Mag. 1943, Vol. XXXV No. 1 and 2) studied the hydrolysis of the starches isolated from the flours and the flours as such. They have shown that the ingredients in the flours other than starches have a good deal of effect on the enzyme hydrolysis of the starch. It will be, therefore, of some use if we study the effect of added oils or fats, and proteins on the course of hydrolysis of the flours. So two flours, namely Wheat flour and Jowar flour were selected for the study. The hydrolysing agents were diastase and pancreatin. The oil selected was pure ground-nut oil and pure peptone for proteinous material.

Experimental procedure was the same as in the last section. The pH of the mixture was 4.8 and the hydrolysis was carried for one hour at the temperature of 37°C. 10 c. cs of 1% diastase and pancreatin were used. The buffer concentration was 0.04 M phosphate.

TABLE No. 7

**Percentage hydrolysis of the flours with 10 c. cs. of  
1% diastase solution and with different oil concentrations,  
expressed on the basis of total hydrolysable starch.**

Flour	without oil	added oil.			
		.0295 gm.	.059 gm	.0885 gm.	.118 gm
Wheat	26.92	28.17	29.81	31.09	30.43
Jowar	27.78	28.36	28.36	28.36	—

In the case of wheat flour hydrolysis the effect of added oil is appreciable. The hydrolysis - value goes on increasing up to the oil concentration of 0.0885 grams. Further addition of the oil has retarding effect.

As regards Jowar flour the addition of oil favours the disintegration but no change in the value is observed with increase in oil concentration. Thus the addition of oil has got some activating effect on the hydrolysis of the flours by diastase.

TABLE No. 8.

Percentage hydrolysis of the flours with 10 c. cs. of pancreatin solution and with different oil concentrations expressed on the basis of the total hydrolysable starch.

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Flour	without oil	with oil added.		
		.0295 gm.	.059 gm.	.088 gm.
Wheat	6.57	5.68	5.68	4.42
Jowar	11.12	11.31	11.12	10.87

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In the case of the hydrolysis of the wheat flour by pancreatin the oil has no activating effect, but it retards the hydrolysis. The more oil we add the more hydrolysis is checked. But in the case of the hydrolysis of the Jowar flour 0.0295 gram of the oil increases the rate of hydrolysis, but on further increasing the oil concentration the velocity of reaction decreases.

Comparing tables 23 and 24, it is observed that the effect of oil is different in different enzymic hydrolysis and on different flours.

TABLE No. 9.

**Percentage hydrolysis of the flours with 10 c. ca. of 1% diastase solution and with different peptone concentrations expressed on the basis of total hydrolysable starch.**

Flour	without peptone	with peptone added.		
		0.05 gm.	0.1 gm.	0.15 gm.
Wheat	26.92	26.65	25.95	19.73
Jowar	27.78	27.20	26.04	24.38

TABLE No. 10.

**Percentage hydrolysis of the flours with 10 c. ca. of 1% pancreatin solution and different salt concentrations expressed on the basis of the total hydrolysable starch.**

Flour	without peptone	with peptone added		
		0.05 gm.	0.1 gm.	0.15 gm.
Wheat	6.57	6.43	5.68	5.10
Jowar	11.12	11.27	11.27	11.27

Comparing tables 25 and 26, it is observed that peptone has no effect on the wheat flour hydrolysis both in the case of diastase and pancreatin. The rate of hydrolysis drops down as the concentration of the peptone increases.

In the case of the hydrolysis of the flour by diastase the addition of peptone is found to have a retarding effect on the course of hydrolysis. But in the case of pancreatin as a hydrolysing agent the value of hydrolysis is increased with the addition of peptone but the rate of hydrolysis is found to be independent of the quantity of the peptone added.

### Conclusions

1. Sodium chloride shows a strong influence on the hydrolysis of the starches in the flours studied with the help of diastase and pancreatin.

2. In the enzymic hydrolysis of the starches in the different flours at the optimum pH of 4.8 it is observed that the

optimum salt concentration varies from flour to flour. 0.05 Mol. concentration is required for the maximum hydrolysis of the starches in the flours of Wheat and Nachani i.e. 0.05 Mol. is the optimum salt concentration for wheat and Nachani flour. Similarly for Jowar flour the optimum salt concentration is 0.15 M. But the salt has got no activating effect on the hydrolysis of the starch in the Rajgira flour.

3. Change of hydrolysing agent changes the range of optimum salt concentration.

4. It is observed that different durations of reaction have different optimum salt concentrations.

5. In the case of starch in the Wheat flour the maximum value of hydrolysis by diastase is obtained at the end of two hours with 0.05 M salt concentration. Further increase in the salt concentration retards the velocity of reaction. So the optimum salt concentration for the Wheat flour is 0.05 M.

6. The optimum salt concentration for the Jowar flour to be hydrolysed by diastase is 0.15 M and the period three hours.

7. The salt has no effect or rather has a retarding effect on the hydrolysis of Rajgira flour by diastase.

8. When pancreatin is used as an hydrolysing agent it is observed in the case of the Wheat flour that optimum salt concentration is 0.05 M which is the same as that for diastase. But in the case of hydrolysis by diastase the activating effect of the salt is also observed in the second and third hour, while in the case of Pancreatin hydrolysis no activation is marked in second and third hour.

9. In the case of the hydrolysis of the Jowar flour by pancreatin the optimum salt concentration is from 0.1 M to 0.15 M. Thus the range of optimum salt concentration is widened in the case of pancreatic hydrolysis.

10. The salt concentration has different effects at different hydrogen ion concentrations on the hydrolysis of starches in the flours by diastase.

10. In the case of hydrolysis of the wheat flour by diastase the optimum salt concentration is 0.1 M near the optimum pH. At other pH values the optimum salt concentration is 0.05 M. Similarly in the case of the jowar flour the optimum salt concentration is 0.15 M near the optimum pH values and 0.1 M at other pH values. So also in the case of nachani flour it is 0.05 M near the optimum pH and 0.1 M at other H ion concentrations.

11. Out of the two optima of pH observed in the case of diastase, the optimum pH of 4.8 is common for all flours with different salt concentration; but the second optimum pH changes from flour to flour and from concentration to concentration.

12. With regard to the effect of the presence of vegetable oil on the hydrolysis of the starches only ground-nut oil was tried and in the case of two flours namely, wheat and jowar. Diastase and pancreatin were the hydrolysing agents used. In the case of wheat flour hydrolysis with diastase the values of hydrolysis goes on increasing up to the concentration of 0.0883 gm. of oil. A further increase in the concentration of the oil shows a tendency of retarding effect. In the case of pancreatin reagent the oil has a retarding effect.

In the case of jowar flour the addition of the oil favours the hydrolysis by diastase. But the effect does not increase with the increase in the concentration. In the case of hydrolysis of Jowar flour with pancreatin the presence of oil shows but very little effect.

The presence of peptone in the disintegration of the wheat flour with diastase has no effect. The same is observed even with pancreatin used as an hydrolysing agent. In hydrolysing jowar flour peptone has retarding effect with diastase and has but very little effect with pancreatin as the hydrolysing agent.

A number of workers such as Sherman, Caldwell and Adman, Eadie; Patwardhan and Norris; Narayanmurty, Giri and Shrikhande have studied the effect of salt concentration



on the hydrolysis of starches. Here the effect of sodium chloride on the hydrolysis of starchy material in the different flours by diastase and pancreatin is studied and the observations made above are similar to their observations. Giri and Shrikhande (J. Ind. Chem. Soc. 1935, Vol. 12, p. 273) studied the activating effect of sodium chloride on the hydrolysis of starch by sweet potato amylase and they have shown that sodium chloride does not accelerate the activity of the enzyme at the optimum pH of 6.0. In the present work we have studied the effect of salt on the hydrolysis of the flours as such at different pH values. It is however observed that there is a definite activating effect at different pH values including the optimum pH. This may be due to the fact that Giri and Shrikhande have used pure soluble starch as substrate and sweet potato amylase as hydrolysing agent, while in the present investigation the flours as such were used as substrate and the enzyme chosen were diastase and pancreatin.

It is seen that non-carbohydrate bodies in the grain have a good deal of effect on the course of hydrolysis. This observation is in agreement with the one recorded by Sahasrabuddhe and Kibe, Sahasrabuddhe and Bhide, and Basu and Sarkar.

## PAPER VII

### THE EFFECT OF ADDITION OF POTASSIUM CHLORIDE AND SODIUM CHLORIDE ON THE HYDROLYSIS OF THE FLOURS OF RALA AND HARIK WITH DIASTASE AT pH 4.8 AND PANCREATIN AT pH 6.8

BY

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*(With the kind permission of the authorities of the Bombay University)*

#### Introduction

Common salt is one of the most essential requirements of the living tissues. It is mostly digested with every kind of food-stuff to which it is added as adjunct in order to make it

tasty and palatable. Common salt as it is known causes an increased secretion of saliva and the gastric juices, and thereby brings about into action an additional quantity of enzymes to react with the food-stuffs. Naturally there is bound to be an increased hydrolysis of the food constituents. The object of the present work is to find out to what extent the addition of sodium chloride and potassium chloride helps the digestion of the material in the alimentary canal. In vitro experiments would give some idea about the effect of the addition of sodium chloride and potassium chloride on the hydrolysis of the flour by the enzymes.

In this part it is intended to study to what extent sodium chloride and potassium chloride influence the hydrolysis of the flours as such by diastase and pancreatin at optimum pH values.

## SECTION I.

### THE EFFECT OF ADDITION OF SODIUM CHLORIDE AND POTASSIUM CHLORIDE ON THE HYDROLYSIS OF THE RALA AND HARIK FLOURS BY DIASTASE AT pH 4.8.

#### A. Effect of addition of sodium chloride on the hydrolysis of Rala flour by diastase at pH 4.8.

In the previous part the hydrolysis of the flours as such by diastase is studied. Now we want to find out to what extent the addition of sodium chloride and potassium chloride affects the hydrolysis. For this purpose three periods 1, 2 and 3 hours were chosen. The salt concentrations were 0.05 molar, 0.1 molar, 0.15 molar, 0.2 molar and 0.3 molar. In these experiments 0.5 per cent. diastase solution was used.

*Experimental*:—1 gram of the Rala flour (oven dry) was gelatinised with 20 c. c. of water by heating it on a hot water bath for 3 minutes. On cooling it, 10 c. c. of the buffer solution of pH 4.8 were added. After adding the required c. cs. of sodium chloride 10 c. c. of 0.5 per cent. diastase were added.

The final volume was 50 c. c. and the final molar concentration of the salts were 0.05 M, 0.1 M, 0.15 M, 0.2 M, and 0.3 M. Then a few drops of toluene were added to prevent bacterial reactions. It was then incubated at 37°C for fixed periods at the end of which the enzyme was killed by a few drops of 10 per cent. sodium hydroxide. It was then clarified by zinc oxide and the volume was made to 100 c. c. and filtered through a dry filtered paper. 10 c. c. of the filtrate were used for the estimation of the reducing sugar by Bertrand's method. The figures obtained were multiplied by 0.93 (conversion factor for starch) and then the percentage hydrolysis was calculated on the basis of total hydrolysable starch (oven dry).

TABLE No. 1

Percentage hydrolysis of the Rala flour at pH 4.8 by 10 c. c. of 0.5 per cent. diastase and with different salt (sodium chloride) concentrations expressed on total hydrolysable starch basis (oven dry).

	Hour 1	Hours 2	Hours * 3
without salt	28.43%	32.82%	34.21%
with 0.05 M NaCl	31.1%	36.41%	36.58%
with 0.1 M NaCl	34.21%	38.21%	49.83%
with 0.2 M NaCl	30.57%	34.23%	36.62%
with 0.3 M NaCl	29.45%	33.84%	34.88%

\* (Total hydrolysable starch ---66.83%).

It is noticed that the beneficial effect of this salt (NaCl) is there up to the extent of 0.05 M and 0.1 M (NaCl). On increasing the salt concentration the beneficial effects are not increased correspondingly but there is retardations. However this is not harmful as the values at these concentrations are not less than those in the case of the hydrolysis without using the salt.

**B. Effect of the addition of potassium chloride on the hydrolysis of the Rala flour by diastase at pH 4.8.**

It was thought interesting to study what change takes place during the hydrolysis of the rala flour if the salt is changed. Hence experiments were carried out with different concentrations of potassium chloride and the results obtained are as follows :-

**TABLE No. 2**

Percentage hydrolysis of the rala flour at pH 4.8 by 10 c.c. of 0.5 per cent. diastase and with salt concentrations (KCl) expressed on total hydrolysable starch basis (oven dry).

	Hour 1	Hours 2	Hours. 3 *
Without salt	28.43%	32.82%	34.21%
Without 0.05 M KCl	34.92%	37.23%	39.82%
With 0.1 M KCl	37.41%	42.17%	42.41%
With 0.2 M KCl	32.65%	35.58%	37.38%
With 0.3 M KCl	30.21%	33.96%	36.61%

\* (Total hydrolysable starch 66.83%).

It is noticed that the beneficial effects of the salt (KCl) are there up to the extent of 0.05 M and 0.1 M (KCl). On increasing the salt concentration to 0.2 or 0.3 M (KCl) the beneficial effects do not increase correspondingly. However the values obtained for these concentrations are not less than those in the case of the hydrolysis without using the salt. This means that there will not be at least any harm if the concentrations are increased.

The optimum salt concentration (KCl) for rala flour is 0.1 M (KCl) when hydrolysed by 10 c.c. of 0.5% diastase at pH 4.8.

### C. Effect of addition of sodium chloride on hydrolysis of the harik flour by diastase at pH 4.8.

It was thought interesting to study the effects of addition of sodium chloride if the substrate is changed. For this purpose harik flour was chosen as a substrate and the experimental procedure followed was similar to that of the rala flour.

TABLE No. 3.

Percentage hydrolysis of the harik flour at pH 4.8 by 10 c.c. of 0.5 per cent. diastase and with different salt concentrations (NaCl) expressed on total hydrolysable starch basis (oven dry).

	Hour 1	Hours 2	Hours. * 3
Without salt	27.35%	31.45%	33.51%
With 0.05 M (NaCl)	34.51%	36.78%	37.98%
With. 15 M (NaCl)	37.23%	39.81%	40.24%
With 0.2 M (NaCl)	30.12%	35.43%	37.21%
With 0.3 M (NaCl)	29.97%	32.61%	35.64%

\* (Total hydrolysable starch 70.52%).

Beneficial effects of NaCl are there up to the extent of 0.05 M and 0.15 M (NaCl). If the concentrations are increased more the beneficial effects do not increase correspondingly. However the values for these higher concentrations of the salt are not less than those in the case of the hydrolysis without using the salt. This means if the concentrations are more, the beneficial effects are reduced but they are not harmful. The optimum salt concentration (NaCl) for the hydrolysis of the harik flour by 10 c.c. of 0.5 per cent. diastase at pH 4.8 is 0.15 M (NaCl).

**D. The effect of addition of potassium chloride on the hydrolysis of the harik flour by diastase at pH 4.8.**

TABLE No. 4.

The percentage hydrolysis of the harik flour at pH 4.8 by 10 c.c. of 0.5 per cent. diastase and with different salt concentrations (KCl) expressed on total hydrolysable starch basis (oven dry).

	Hour 1	Hours 2	Hours. 3
Without salt.	27.35%	31.45%	33.51%
With 0.05 M (KCl)	29.22%	32.93%	35.12%
With 0.15 M (KCl)	30.58%	35.12%	36.81%
With 0.2 M (KCl)	28.42%	33.63%	34.48%
With 0.3 M (KCl)	27.78%	31.58%	33.81%

\* (Total hydrolysable starch...70.52%).

On comparing the results for NaCl and KCl, it is observed that the effect of addition of NaCl is more than that of KCl in case of the hydrolysis of the harik flour by 0.5 per cent. diastase at pH 4.8. In case of the harik flour the beneficial effects of KCl are there to the extent of 0.05 M, and 0.15 M KCl. When the concentrations are increased more the beneficial effects do not increase correspondingly however the values at these concentrations are more than those in the case of the hydrolysis without using the salt. This means that the beneficial effects are reduced at these concentrations but there is no harm with these increased concentrations. The optimum salt concentrations of KCl for the hydrolysis of the harik flour by 10 c. c. of 0.5 per cent. diastase at pH 4.8 is 0.15 M KCl. This shows that when a substrate is changed there is a change in the optimum molar concentration of the salts.

## SECTION II

THE EFFECT OF ADDITION OF SODIUM CHLORIDE AND POTASSIUM CHLORIDE ON THE HYDROLYSIS OF THE FLOURS OF RALA AND HARIK WITH PANCREATIN AT pH 6.8

A. Effect of addition of sodium chloride on the hydrolysis of the rala flour by pancreatin at pH 6.8.

From the previous section it was thought interesting to see what effect would be observed if the hydrolysing agent is changed. In this case pancreatin was used as the hydrolysing agent and the procedure followed was similar to that in the previous section.

TABLE No. 5

Percentage hydrolysis of the rala flour at pH 6.8 by 10 c. c. of 0.5 per cent. pancreatin and with addition of salts of different strengths (NaCl) expressed on total hydrolysable starch basis (oven dry).

	Hour 1	Hours 2	Hours * 3
Without salt	30.56%	39.61%	47.52%
With 0.05 M (NaCl)	33.67%	44.23%	50.73%
With 0.15 M (NaCl)	36.83%	46.92%	51.82%
With 0.2 M (NaCl)	32.63%	41.38%	49.22%
With 0.3 M (NaCl)	31.15%	40.92%	49.11%

\* (Total hydrolysable starch 66.83%).

It is noticed that the beneficial effects of NaCl are there up to 0.15 M NaCl. When the concentration is increased further there is a retardation with respect to 0.05 M and 0.15 M NaCl. However there is activation with respect to the values obtained for the hydrolysis without using the salt. Even though the salt concentration is six times of 0.05 M NaCl the beneficial

effects are not increased correspondingly, but there is retardation. However these concentrations are not harmful as the values obtained are more than those of the hydrolysis without using the salt. The optimum salt concentration (NaCl) for the hydrolysis of rala flour by 0.5 per cent. pancreatin at pH 6.8 is 0.15 M NaCl. If the effect of sodium chloride on hydrolysis of rala flour by diastase and pancreatin is compared it is nearly the same. However the optimum molar salt concentrations are different which might be due to the change of hydrolysing agent.

**B. Effect of addition of potassium chloride on the hydrolysis of Rala flour by Pancreatin at pH 6.8.**

TABLE No. 6

Percentage hydrolysis of the rala flour at pH 6.8 by 10 c.c. of 0.5 per cent. pancreatin and with addition of salt concentrations (KCl) of different strengths expressed on total hydrolysable starch-basis (oven dry).

	Hour 1	Hours 2	Hours • 3
Without Salt	... 30.56%	39.61%	47.52%
With 0.05 M KCl	... 33.78%	43.17%	49.81%
With 0.15 M KCl	... 34.62%	45.93%	51.43%
With 0.2 M KCl	... 31.87%	42.27%	50.12%
With 0.3 M KCl	... 31.17%	40.27%	48.0 %

• (Total hydrolysable starch 66.83%)

The beneficial effects of adding KCl are there up to the extent of 0.15 M KCl. When the concentration is increased further even up to six times of 0.05 M KCl, there is retardation. These higher concentrations are not harmful even though the beneficial effects are reduced as the values at these concentrations are more than those for the hydrolysis without using the salt. If we compare the effect of addition of potassium chloride on the hydrolysis of the rala flour by diastase and



pancreatin the effect is comparatively more during the hydrolysis by diastase. This effect might be due to change of hydrolysing agent. The optimum salt concentration (KCl) for maximum hydrolysis of the rala flour by 0.5 per cent. pancreatin at pH 6.8, is 0.15 M KCl.

**C. Effect of addition of sodium chloride on the hydrolysis of the Harik flour by Pancreatin at pH 6.8.**

TABLE No. 7

Percentage hydrolysis of the harik flour at pH 6.8 by 10 c.c. of 0.5 per cent. pancreatin and with salt (NaCl) of different concentrations expressed on total hydrolysable starch basis (oven dry)

	Hour 1	Hours 2	Hours. * 3
Without salt	24.86%	30.24%	33.12%
With 0.05 M NaCl	27.43%	35.12%	38.23%
With 0.1 M NaCl	30.13%	35.31%	38.43%
With 0.2 M NaCl	26.97%	33.52%	35.43%
With 0.3 M NaCl	25.23%	31.52%	33.19%

\* (Total hydrolysable starch...70.52%).

From the above results it is observed that the beneficial effects of NaCl are there up to the extent of 0.1 M NaCl. On increasing the salt concentration to 0.3 M NaCl i.e. six times that of 0.05 M NaCl the effects are not increased correspondingly, but they are reduced. The values of percentage hydrolysis at these higher concentrations are more than those obtained for the hydrolysis without using the salt. This shows that the beneficial effect of addition of sodium chloride are reduced at these concentrations yet these concentrations are not quite harmful. The optimum salt concentration (NaCl) for the hydrolysis of the harik flour by 10 c.c. of 0.5% pancreatin at pH 6.8 is 0.1 M NaCl.

**D. Effect of addition of potassium chloride on the hydrolysis of the harik flour by pancreatin at pH 6.8.**

TABLE No. 8

Percentage hydrolysis of the harik flour at pH 6.8 by 10 c.c. of 0.5 per cent. pancreatin and with different salt concentrations (KCl) expressed on total hydrolysable starch basis (oven dry)

	Hour 1	Hours 2	Hours. * 3
Without salt	24.86%	30.24%	33.12%
With 0.05 M KCl	26.21%	33.12%	34.48%
With 0.1 M KCl	28.82%	33.87%	36.23%
With 0.2 M KCl	25.93%	32.13%	35.31%
With 0.3 M KCl	25.14%	30.98%	33.91%

\* (Total hydrolysable starch 70.52%).

It is observed that the beneficial effects of addition of potassium chloride are there up to the extent of 0.1 M KCl. However if the concentrations are increased more, the beneficial effects do not increase correspondingly. These salt concentrations are not harmful as the values of percentage hydrolysis at these concentrations are more than those of the hydrolysis without using the salt. The optimum salt concentration (KCl) for the hydrolysis of the harik flour at pH 6.8 by pancreatin is 0.1 M KCl.

#### CONCLUSIONS AND SUMMARY.

1. The addition of sodium chloride and potassium chloride shows considerable effect on the hydrolysis of the flours by diastase and pancreatin.

2. In the case of the hydrolysis of the flours by diastase at pH 4.8, the optimum salt concentrations change from flour to flour. 0.1 M KCl and 0.1 M NaCl are optimum salt con-

centrations for the maximum hydrolysis of the rala flour whereas for the maximum hydrolysis of the harik flour the optimum salt concentrations are 0.15 M NaCl and 0.15 M KCl.

3. It seems that change of hydrolysing agent brings about a change in the optimum salt concentration. In case of the hydrolysis of the flours by pancreatin at pH 6.8 the optimum salt concentrations for the maximum hydrolysis of the rala flour are 0.15 M NaCl and 0.15 M KCl, whereas for the harik flour they are 0.1 M KCl. and 0.1 M NaCl.

4. It is observed that at optimum salt concentration the time of reaction is different for maximum activation.

5. During the hydrolysis of the rala flour by diastase at pH 4.8 the activation effect at optimum sodium chloride concentration is less than that at optimum potassium chloride concentration. In the case of the hydrolysis of the harik flour by diastase at pH 4.8 the activation at optimum sodium chloride concentration is more than that at optimum potassium chloride concentration.

6. During the hydrolysis of the rala flour by pancreatin at pH 6.8, the optimum sodium chloride concentration is more than that at optimum potassium chloride concentration. In case of the hydrolysis of the harik flour by pancreatin at pH 6.8 the activation at potassium chloride concentration is less than that at optimum sodium chloride concentration.

7. Even though the salt concentrations are increased twice or thrice of the optimum salt concentration the beneficial effects are comparatively reduced, but there is no harm due to this increased concentration.

8. The addition of the salts of different molar concentrations helps the hydrolysis of the flours and the effects are beneficial at the optimum salt concentration which varies from flour to flour and also from enzyme to enzyme.

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## PAPER VIII

A STUDY OF COMPARATIVE DISINTEGRATION  
OF PROTEINS OF THE DRY FRUITS

BY

DR. R. S. KALE, Ph. D.

(With the kind permission of the authorities of the Bombay University)

**Introduction :—**

In the present section we intend to study the comparative disintegration of the proteins from the dry fruits with different enzymes mainly occurring in the animal intestinal canal.

The nutritional value of any protein depends upon the following facts:—(i) its disintegration into amino acids which will determine the proportion which will be absorbed in the blood (ii) its assimilability i.e. the extent to which it is able to produce—for growth and repair—this factor, known as the biological value of protein—will depend upon the nature of its amino acid contents. The procedure that has been followed is similar with some alterations—to that used by Waterman and John (l. c.). The disintegration of the proteins is expressed as milligrams of amino-nitrogen by Van Slyke's method for 100 grams of the sample used.

## SECTION I

**Disintegration of proteins of dry fruits with pepsin.**

*Experimental* :—2 grams of each sample were suspended in 25 c.cs. of 0.1 N HCl and 25 c.cs. of 0.5 % pepsin prepared in 0.1 N HCl were added to the suspension. It was then incubated at 37°C for the required period i.e. 1, 2, 3 hours. After the incubation for fixed periods 5 c.cs. of 1 N NaOH were added to neutralise acid and 5 c.cs. of the colloidal iron were added to coagulate the proteins.

1 c.c. of magnesium sulphate solution (5%) was added when complete coagulation took place. Clarification was hastened by adding few drops of basic lead acetate solution. The volume was made to 100 c. cs. and filtered. 5 c. cs. of the

filtrate were used for the estimation of amino nitrogen by Van Slyke method (1912, J. Biol. Chem. 12, 399). The disintegration was expressed as milligrams of nitrogen for 100 grams of the sample. A blank experiment was run side by side for every period and for every strength of the pepsin used and the results of the blank experiment together with error for the apparatus were subtracted from the value obtained.

**Hydrolysis of the proteins from the dry fruits with 0.5% pepsin expressed as mgms. of nitrogen per 100 gms. of the flour.**

TABLE No. 1

Name of the sample	...	1 hour	2 hours	3 hours.
Cashew nut	...	112.2	157.08	224.4
Almond	...	145.86	213.18	269.28
Pistachio nut	...	168.30	266.88	334.20

**Hydrolysis of the proteins from the dry fruits with 1% pepsin expressed as mgms. of nitrogen per 100 grams of the flour.**

TABLE No. 2

Name of the sample	...	1 hour	2 hours	3 hours.
Cashew nut	...	190.74	246.84	314.16
Almond	...	258.06	347.82	426.36
Pistachio nut	...	336.6	493.68	583.44

**Hydrolysis of proteins from the dry fruits with 1.5% pepsin expressed as mgms. of nitrogen per 100 grams of the flour.**

TABLE No. 3

Name of the sample	...	1 hour	2 hours	3 hours.
Cashew nut	...	359.04	561.0	684.42
Almond.	...	504.90	661.98	807.84
Pistachio nut	...	729.3	841.5	908.82

Here in this case we find that (i) the descending order of susceptibility is pistachio nut, almond, and cashew nut (ii) the velocity of reaction in all the cases falls gradually as the hydrolysis proceeds. (iii) major portion of the hydrolysis takes place in the 1st interval (iv) with the increase in the strength of the hydrolysing agent and the time of reaction there is also an increase in the total amounts of proteins split up. (v) The sudden drop in the rates of hydrolysis may be due to the fact that the hydrolysis takes place in two stages; during the first there is a rapid hydrolysis of the proteins to proteases and peptones while during the second stage the primary degradation products undergo a slow hydrolysis to similar products. An observation of this type has been made by McFartare (1927, *J. Gen. Physio.* 10, 437) Similarly Miller and Calvery (*J. Biol. Chem.* 1936, 116, 393) have shown that the hydrolysis of lactalbumin by pepsin proceeds rapidly for first few hours.

## SECTION II

### The Effect of superimposed trypsin:

The end products of the peptic digestion of the proteins are mainly peptides of varying degrees of complexity (Calvery and Schock 1936, *J. Biol. Chem.* 113, 5) although free amino acids may be liberated. Complete digestion of the proteins does not take place in one stage but in a series. The end products of the peptic digestion mainly occurring in the start are further carried out into simpler units by the enzymes of the alimentary canal which are secreted by the pancreas and the intestines.

Trypsin is able to effect hydrolysis of high molecular weight protamines. Calvery (1933, *J. Biol. Chem.* 102, 73) has found that the enzyme is unable to bring about hydrolysis in albumin solutions in which pepsin digestion has been previously allowed to proceed to its maximum. Miller and Calvery (1936, *J. Biol. Chem.* 116, 393) have found that trypsin is able to effect a slight further hydrolysis although the final degree of hydrolysis was approximately the same as when pepsin was

allowed to react to its limit. So the pure pancreatic proteinase brings about the same amount of hydrolysis as pepsin in some cases.

In the light of the observations made by various workers it was thought interesting to see this.

TABLE No. 4

Name of the sample.	Pepsin.	Superimposed Trypsin.
Cashew nut ...	112.2	572.22
Almond ...	145.86	695.64
Pistachio nut ...	168.30	785.40

Comparing the figures in the above table we find that the pistachio nut sample is most easily attacked by superimposed trypsin, while cashew nut is the least attacked of all the three. Difference in the case of pistachio nut is 617.10, in the case of almond 549.78 and in the case of cashew nut it is 460.02. Comparing the figures of the peptic digestion and those of superimposed trypsin—we see that in all the cases—tryptic digestion is quick and predominant.

### SECTION III

#### **Effect of removing fats on the hydrolysis of proteins of the dry fruits.**

We have studied the effect of removing fat from the dry fruits on the hydrolysis of carbohydrates with different amyolytic enzymes. In the present section we intend to study similarly the effect of removing fat on the disintegration of proteins by the enzymes.

The fat-free sample (as prepared before) was similarly subjected to the action of pepsin and pepsin superimposed by trypsin. The results are as given below in table No. 5.

**Hydrolysis of the proteins from the fat-free material of the dry fruits with 0.5% pepsin expressed as mgms. of nitrogen per 100 gms of the flour.**

TABLE No. 5

Name of the sample.	1 hour.	2 hours.	3 hours.
Cashew nut ...	78.54	134.64	179.52
Almond ...	123.42	168.30	224.4
Pistachio nut ...	134.64	201.96	246.84

**Effect of superimposed Trypsin on the disintegration of the proteins from the fat-free material :—**

TABLE No. 6

Name of the sample.	Pepsin.	Superimposed Trypsin
Cashew nut ...	78.54	516.12
Almond ...	123.42	639.54
Pistachio nut ...	134.64	729.3

**3 :: 1.5 :: 1. The ratio of the total proteins split up from time to time is 1 :: 1.5 :: 1.8.**

Comparing the figures with the corresponding figures in the table—where the whole 'fat containing' material was disintegrated by 0.5 % pepsin we see that—

The total amount of proteins split up from time to time are much less in the case of 'fat-free material' than in the case of 'fat containing' sample—showing thereby that the fats in the dry fruits have definitely an accelerating effect on the disintegration of the proteins. Table No. 6 shows the hydrolysis of the proteins from the fat-free material of the dry fruits with 0.5% pepsin for one hour and then with superimposed trypsin 6% for one hour—expressed as mgms of nitrogen per 100 grams of the flour.

The total amount of proteins split up in fat-free material is much less than that in the whole fat containing material.



## PAPER IX

## INFLUENCE OF COMMON SALT (SODIUM CHLORIDE AND VITAMINS ON THE ENZYMIC HYDROLYSIS OF FATS AND PROTEINS)

BY

DR. R. S. KALE, Ph. D.

*(With the kind permission of the authorities of the Bombay University)*

## SECTION 1

Common salt is one of the most essential requirements of living tissues. It is mostly ingested with every kind of food-stuff to which it is added as an adjuvant in order to make the stuff tasty and palatable. Dry fruits are often roasted and salted before use. Common salt as we know, causes an increased secretion of saliva and gastric juice thereby bringing into action an additional quantity of enzyme to react with the food-stuffs. Naturally there is bound to be an increased hydrolysis of the food constituents. Leaving aside this property, it is intended to study in this section, whether the addition of salt itself, has any influence on the digestibility of the dry fruits.

**Procedure:-**

2 c.cs. of olive oil were taken in a conical flask and to it were added - 5 c.cs. of phosphate buffer solutions of  $pH$  3 i.e. acidic side of optimum  $pH$ -, 4.8 - i.e. the optimum  $pH$  of castor bean lipase and 6 - i.e. alkaline side of optimum  $pH$ . 0.5. gram of the castor bean lipase was then put in each reaction mixture. Lastly 5 c.cs. of sodium chloride stock solutions of strengths 0.1 M and 0.5 M were added. The reaction was allowed to proceed for 24 hours. A blank experiment was run side by side in each case. The extent of hydrolysis was then determined at the end of reaction period by adding neutral alcohol and titrating against 0.1 N  $NaOH$ . The extent of hydrolysis is expressed in terms of c.cs. of 0.1 N  $NaOH$ ,

**Reaction Mixture :-**

2 c.cs. of olive oil and 0.5 gram lipase powder with 5 c.cs. buffer solution and 5 c.cs. salt solution.

TABLE No. 1.

pH.	3	4.8	6
Without salt	3.5	4.9	4.4
With 0.1 M salt	4.2	4.9	4.4
With 0.5 M salt	3.8	4.8	4.1

From the above table we find that:-

1. At the optimum pH, the salt has practically no effect on the hydrolysis of the oil.
2. On the acidic side of the optimum pH, the addition of salt causes increased hydrolysis of the oil. An increase with concentration of the salt, however, causes a diminution in the accelerating effect of the salt.
3. On the alkaline side of the optimum pH, addition of salt solution of 0.1 M strength has no effect but a more concentrated solution of 0.5 M strength inhibits the enzyme action.

## SECTION II.

**Influence of common salt (sodium chloride) on the enzyme hydrolysis of the proteins.**

In the last section, the influence of adding common salt on the enzyme hydrolysis of fats was studied. In the present section it is intended to study the effect of adding common salt on the hydrolysis of proteins.

**Procedure.**

20 c.cs. of 5% solution of peptone were taken in a conical flask. To it were added 10 c.cs. of acetate buffer of pH 1.2, 1.8, & 4.7 together with 0.5 grams of pepsin. Lastly 10 c.cs.

of NaCl stock solution of strength 0.1 M and 0.5 M were added. The reaction mixture was kept in an incubator at 37°C and the reaction was allowed to proceed for 24 hours. The extent of hydrolysis was finally determined with the Sorensen's formaldehyde titration method and the results are expressed in terms of N/10 NaOH. A blank experiment was run side by side in each case.

TABLE No. 2

<i>p</i> H.	1.2	1.8	4.7
Without salt	5.3	6.5	4.9
With 0.1 M salt	5.9	6.5	4.9
With 0.5 M salt	5.5	6.4	4.7

Here again we find that :-

1. At the optimum *p*H (1.8), the salt has practically no effect on the hydrolysis of the proteins by pepsin.
2. On the acidic side of the optimum *p*H, the addition of salt causes increased hydrolysis of the proteins; however an increase in the concentration of the salt causes a diminution in the accelerating effect of the salt.
3. On the alkaline side of the optimum *p*H, addition of salt solution of strength 0.1 M has no effect, but a more concentrated solution of 0.5 M strength inhibits the enzyme action.

### SECTION III

#### **Influence of Vitamins on the Enzymic Hydrolysis of Fats and Proteins.**

Until the early years of the twentieth century it was customary to consider a perfect diet as made up of a mixture, in fairly definite proportions, of proteins, fats, carbohydrates and water. Although it was realised that sodium chloride appeared to be absolutely necessary it could be included under none of

these groups. Experiments to maintain normal life on a such a mixture, however, utterly failed. It was, therefore, concluded, that there must be still an unknown substance or substances of vital importance to normal life. Several groups of investigators tried to make advances towards a complete and definite knowledge of these food factors called 'Vitamins.'

The vitamins can be broadly separated into two groups: one of which is soluble in water and the other is soluble in fats and fat-solvents.

Fat soluble	...	Vitamin A, D, E, K.
Water soluble	...	Vitamins B (complex), C.

None of these vitamins is affected by digestion. They are absorbed as such from the intestinal canal and utilised in the systems for growth and repair of the tissues. However, their presence affects the digestibility of the other food constituents.

Thus the effect of the following Vitamins on the enzymic hydrolysis of proteins and fats is studied.

1. Thiamin chloride ... Vitamin B, Antineuritic or Antiberi-beri Vitamin.
2. Nicotinic acid ... Vitamin B, (complex, P. P. factor), Antipellagra vitamin factor.
3. Ascorbic acid ... Vitamin C, Antiscorbutic Vitamin.
4. Vitamin D. ... Antirachitic vitamin.
5. Vitamin E. ... Antisterility or Fertility Vitamin.
6. Vitamin K ... Antihemorrhagic or Coagulation Vitamin.

#### A. Effect of Vitamins on the hydrolysis of fats :—

*Procedure* :—The procedure adopted was the same as in the case of "Common salt" (vide last chapter)—except that

instead of common salt the necessary amount of Vitamin was added. Initial acidity of the vitamin products was determined by dissolving them either in water or neutral alcohol. Strengths of vitamins used were 20 mgms and 50 mgms.

*Reaction Mixture :—*

2 c.cs. of olive oil, 0.5 grams lipase, powder, 5 c.cs. phosphate buffer of pH 4.8 and 20 mgms or 50 mgms of vitamin kept in an incubator at 37°C for 24 hours. The extent of hydrolysis is expressed in terms of c.cs. of N/10 NaOH.

TABLE No. 3

Blank expt. (without vitamin).....4.9 c.cs. N/10 NaOH.

<i>With Vitamins</i> (after deducting the initial acidity)	20 mgms.	50 mgms
1. Thiamin chloride	... 5.3	5.5
2. Nicotinic acid	... 5.4	5.7
3. Ascorbic acid	... 5.6	5.8
4. Vitamin D.	... 5.1	5.2
5. Vitamin E.	... 4.9	4.9
6. Vitamin K.	... 4.9	4.9

From the above table we find that :—

Thiamin chloride, Nicotinic acid, Ascorbic acid and Vitamin D have an accelerating effect on the hydrolysis of the oil by castor bean lipase and that Vitamin D, thiamin chloride, Nicotinic acid and Ascorbic acid stand in the ascending serial order as far as the accelerating effect is concerned.

Vitamin E and K have no effect on the hydrolysis.

**B. Effect of Vitamins on the enzymic Hydrolysis of Proteins.**

*Procedure :—*

The procedure adopted was the same as that in the case of common salt.

**Reaction Mixture :—**

20 c.cs. of 5% peptone solution and 10 c.cs. of acetate buffer of pH 1.8, 0.5 gram of pepsin, 20 mgms. or 50 mgms. of vitamin product.

The reaction mixture was kept in an incubator at 37°C for 24 hours. Extent of hydrolysis is expressed in terms of c.cs. of N/10 NaOH as determined by Sorensen formaldehyde titration method.

TABLE No. 4

Blank Expt (without Vitamin).....6.5 N/10 NaOH.		
With Vitamins (after deducting the initial acidity)	20 mgms.	50 mgms.
1. Thiamin chloride	... 7.0	7.3
2. Nicotinic acid	... 7.1	7.4
3. Ascorbic acid	... 7.3	7.5
4. Vitamin D.	... 6.5	6.5
5. Vitamin E.	... 6.5	6.5
6. Vitamin K.	... 6.5	6.5

From the above table we find that—

1. Thiamin chloride, nicotinic acid and ascorbic acid have an accelerating effect on the hydrolysis of peptone by pepsin and stand in the ascending serial order as far as the accelerating effect is concerned.

2. Vitamins D, E and K have no effect on the hydrolysis.

## PAPER X

## "A STUDY OF GOAT-MILK"

BY

M. M. THATTE, M. SC.

*(With the kind permission of the authorities of the Bombay University)*

## SECTION I

**A study of a few samples of milk from goats at random.**

In every civilized country of the world an adequate national food policy presupposes an abundant supply of milk to foster the healthy growth of every individual in the nation. Taking into consideration this importance of milk as a necessary factor of food material, much of the scientific study on milk has been done from the physiological and biological points of view. The importance of milk is, moreover, due to the easily assimilable fat-contents in it.

Milk is naturally intended for the nourishment of newly-born animals. If milk is to serve exclusively for nourishing the young, it must, of course, contain all the nutrients required by the animal organism for its maintenance and growth and these are proteins, fats, carbohydrates and inorganic salts. In addition to these, milk also contains small quantities of various substances which, although they possess no direct feeding-value have nevertheless an important physiological effect. Milk of all species of animals contains the whole of the constituents mentioned above, but the quantities in which they are present vary in different cases.

As cows and buffaloes are the common milking animals importance is given to the analytical study of milk and its products obtained from these two species, and consequently that branch is developed in details.

The composition of milk secreted by an animal does not remain constant over the whole lactation period. Moreover there are many factors which affect the amount and the composition of milk secreted by an animal. The three factors namely the feeding stuff, the seasonal change and the stage of the lactation period are comparatively important. The feeding - stuff and the seasonal change have an important bearing on the yield of milk. The stage of lactation period is the most important factor affecting the composition of milk. The percentage of fat and the percentage of nitrogenous compounds such as casein, albumin etc., are very high in the milk samples during the first few days of lactation period as these components are essential for the growth of newly - born young ones. As the stage of lactation period advances, the proportion of carbohydrates increases while fat and nitrogen compounds lower down and these then become almost constant. These changes in the components of milk during the lactation period of an animal are very interesting to study. In the case of cow and buffalo the study has been very much advanced, but a few people had done work on goat - milk and it is for this purpose goat - milk was taken for study.

Goat - milk is used for human consumption through both choice and necessity in many parts of the world. It is used either without consideration of any special characteristic it may possess or because of its specific properties. Some persons who manifest allergic reactions to the ingestion of cow milk have no such difficulty with goat - milk. There is a prevailing opinion that goat - milk is more easily digested than milk of some other species, particularly cow - milk. The foreign literature contains clinical evidence to support this opinion. Its value is high in the treatment of intestinal troubles because of its ease of digestion. The use of it is made by a sanatorium in the treatment of digestive troubles. It is reported that curds formed in the stomachs of infants fed on goat - milk are smaller and more flocculent than in those fed on cow - milk. The curd of goat - milk differs from that of cow - milk in that it is granular rather than a rubbery type and undoubtedly forms smaller particles in the infant's stomach.



It is for this reason considered that goat - milk is desirable for infant feeding.

General analysis of a few samples of goat-milk was carried out with a view to find out the proportion of different constituents and the possibility of any simple relationship between the different substances that are commonly present in goat-milk. Every possible care was taken to get a sample of genuine goat-milk. The analysis of the samples was carried out by the methods mostly taken from "The association of official agricultural chemists" and "The methods used in the examination of milk and dairy products" by C. Barthel.

In taking a sample of milk for the purpose of analysis certain precautions must be observed owing to the fact that the larger fat globules immediately begin to rise to the surface even when the milk has stood only for a short time.

#### **Preparation of sample :—**

The animal is completely milked and from the total amount of milk, 100-110 c.c. are withdrawn after pouring the whole amount of milk from one clean dry vessel into another clean dry vessel 4 or 5 times. Before the time of actual experiment the sample is again well mixed by pouring from one clean dry bottle into another clean dry bottle 4 or 5 times. If cream is formed on the milk, mixing should be continued until all cream is detached from the sides of the vessel and evenly emulsified throughout the liquid. The necessary amount of milk is pipetted for analysis. If the same sample is to be kept for analysis for next day a suitable preservative is to be added. Potassium dichromate is used for the preservation of milk samples. About 0.5 gm. to one litre of milk is sufficient to preserve the sample.

For general analysis of goat-milk samples, nine goats were selected locally at random. None of these goats was specially fed and all of them used to be out for the whole day. Samples of milk were collected when every goat had come to the normal lactation stage. These samples were collected from

morning milk during the same season. Table No. 1 gives the analytical results of different samples of goat-milk.

The above samples are arranged according to the ascending order of their fat content. Among the samples studied the percentage of fat as low as 2.5 and as high as 8.15 was found out. But the figure—total solids—not fat—is practically the same. It does not depend upon the percentage of fat in normal milk. The average value of total solids—not fat—seems to be about 9.93. Tocher ("variations in the composition of milk", p.p. 58) states that average values of percentage of total solids—not fat in cow-milk associated with the particular values of percentage of butter fat are linear in character. There is a positive correlation between butter fat and solids—not fat. In our present work no such relationship was found out in goat-milk.

The greater the percentage of butter fat the greater the percentage of total nitrogen. This conclusion is borne out by a few samples. In some cases it is seen that if the percentage of lactose is higher then the percentage of total nitrogen is lower. There is practically no relationship between the percentage of casein and the percentage of lactose. The above conclusions are rather rough and qualitative. The definite relationship without any variation is not found out.

E. B. Bailey found lactose content from 35 samples of milk. It ranged from 2.74 to 4.46 (chemical abstract. 32; p.p. 7147; 1938). In our experiments lactose content was found ranging from 3.31 to 4.60. The ratio of casein to albumin, as shown by Table No. 3, is not constant. The minimum and maximum ratios are 2.84 : 1 and 4.40 : 1 and the average ratio is 3.50 : 1.

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TABLE No. 1  
Analysis of a few samples of goat-milk.

Sample No.	Fat %	Total solids %	Water %	Total solids not-fat %	Lactose %	Total-Proteids %	Casein %	Albumin %	Casein/Albumin
1	2.95	18.12	86.88	10.17	4.606	3.819	2.958	0.861	3.44
2	3.20	18.58	86.42	10.3	3.729	4.249	3.207	1.042	3.08
3	4.2	12.94	87.06	8.74	3.957	3.984	2.564	0.820	3.13
4	5.0	15.12	84.88	10.12	4.458	3.941	3.214	0.727	4.40
5	5.15	15.12	84.88	9.97	3.685	4.278	3.311	0.967	3.43
6	5.6	15.81	84.19	10.21	3.988	4.608	3.407	1.201	2.84
7	5.9	16.19	83.81	10.29	3.918	5.110	4.038	1.072	3.77
8	7.5	16.78	83.22	9.28	3.739	3.815	3.107	0.708	4.39
9	8.15	18.28	81.72	10.13	3.567	4.882	3.664	1.218	3.00
<b>Average</b>	<b>5.29</b>	<b>15.22</b>	<b>84.78</b>	<b>9.93</b>	<b>3.882</b>	<b>4.232</b>	<b>3.275</b>	<b>0.957</b>	<b>3.50</b>

## SECTION II

**A study of milk of two goats during the lactation period.**

The composition of milk secreted by an animal does not remain constant over the whole lactation period. Moreover there are many factors which affect the amount and the composition of milk secreted by an animal. Dwight Espe ("Secretion of Milk") states the following important factors affecting the amount and the composition of milk.

- (1) Intra and interbreed difference.
- (2) Change in food-stuff.
- (3) Condition and dry period.
- (4) The stage of lactation.
- (5) Effect of pregnancy.
- (6) Season of the year.
- (7) Estrus.
- (8) Interval between milking.
- (9) Age, size and exercise.
- (10) Diseases etc.

Even within the same individual considerable variation may occur in the production of milk from season to season as well as in the percentage of fat from milking to milking and even between quarters or halves of the same udder. These variations occur in spite of every effort to keep physiological and environmental factors constant.

Following parturition the daily production of milk tends to increase to become maximum. The time required to reach maximum production with high producing animals is usually longer than that required for low producing animals. Towards the end of the lactation period the production of milk begins to decline gradually. Factors besides individuality and breed which affect the decline in milk production are frequency of milking, age, seasonal changes, state of nutrition, pregnancy, general management, etc.

The three factors, namely the feeding stuff, the seasonal change and the stage of lactation are comparatively more important than the rest. The feeding stuff and the seasonal change affect the yield of milk rather than its composition. The percentage of fat in milk is due to inherited characteristics, which cannot be greatly changed by any method of feeding. The stage of lactation is the most important factor affecting the composition of milk.

A study of the lactation period of buffaloes and cows has been done by a number of chemists. This branch has developed in details in India and outside. Comparatively a little work has been done on the complete lactation period of goats. In our work the study of lactation period of two goats was carried out. An attempt was made to secure goats from the Government Live-stock Department, Poona. It was found that the farm does not maintain goats. Hence only two goats were selected from local men. It became a troublesome job to convince these men to give the first milk for analysis. Two or three goats which were selected for this work had been given up as no first milk was obtained. Finally a successful attempt was made in securing the first milk in one case and that goat was selected for the study of lactation period. In another case the study was started from ninth day after the delivery of the kids.

The two goats selected were named as 'A' and 'B' goat. Both of them were from the breed generally found on Deccan land. The lactation period was studied during the fourth lactation of 'A' goat and during the third lactation of 'B' goat. These goats used to be out for grazing for the whole day and they were fed on a daily ration of half a pound mixture of Jawar and Bajri.

As the changes in the composition of milk during the early days of lactation period, particularly in the first week, are very rapid, the milk samples of 'A' goat were analysed every day. The first milk, colostrum, and the morning milk in the next four days were analysed. After that the milk sample was analysed at intervals of about 15 days during the normal period of

lactation. In the case of 'B' goat the analysis of milk samples was started after a week from the delivery of kids.

The first milk after kidding, known as colostrum, was very different from normal milk. Its colour was yellowish, the consistency was sticky and the taste was saltish. Table No. 2 gives the composition of the first few milk-samples from 'A' goat.

TABLE No. 2

## Composition of the first few milk samples.

Percentage	First day	Second day	Third day	Fourth day	Fifth day
Fat	7.3	5.7	5.3	5.1	5.9
Water	72.30	81.75	81.10	82.48	81.63
Total solids	27.70	18.25	18.90	17.52	18.37
Total solids not-fat	20.40	12.55	13.60	12.42	12.47
Ash	0.9088	0.904	0.9122	0.7627	0.7244
Lactose	2.846	3.578	3.719	4.064	3.904
Proteids	14.65	7.12	6.819	5.946	6.401

Table No. 2 shows that:-

**Fat:-** The fat percentage is very high i.e. 7.3 in the first milk. It then rapidly falls to 5.7 on the next day and in the next two days it slowly comes down after which there is a slight rise.

**Total solids—not fat:-** In the first milk it is abnormal i.e. 20.40% and on the next day there is a rapid fall to 12.55%. Then in the next three days there is little variation.

**Proteids:-** The total proteids contents in the first milk are found to be 14.65%. On the next day there is a rapid fall to 7.12% i.e. in the first milk the proteid contents are more than

twice that present in the next day's sample. In the next two days they slowly fall down after which there is a little rise.

*Lactose*:- The lactose percentage in the first milk is 2.846. On the next day it comes to 3.578 and then there is a little rise in the next two samples after which it slightly falls down.

From the figures we can say that colostrum or the first milk is abnormal in all its constituents. The second milk sample shows wide variations from the first milk, especially in the proteid contents. The second milk and the subsequent milk samples show little variations and the general trend of these samples is to become normal in all its constituents.

But on the fifth day there is a rise in fat percentage and proteid content and a slight fall in lactose.

Dr. D. L. Sahasrabuddhe in his note on "Variation in the composition of milk of individual buffaloes" (Poona Agricultural College reprint No. 17) gives the results of his experiments on the milk of buffaloes. The figures given by him show that all the constituents in the first milk are very abnormal when compared with normal milk; the greatest difference being in albumin which is ten times higher than that present in normal milk.

The lactation period of 'A' goat continued for 109 days and that of 'B' goat for 174 days. Table No. 3 gives the composition of milk samples from 'A' goat collected at intervals of about 15 days during the normal period of lactation and the period towards the end of lactation. Table No. 4 gives the composition of milk samples from 'B' goat collected as in the case of 'A' goat.

In the first 3 or 4 days of lactation period and towards the end of lactation, percentage values of different constituents are different from those found in milk samples in normal period. Unlike the first milk the final milk is not obtained at the end of any definite period as the animal usually does not go dry all

TABLE No. 8

Composition of milk-samples during the lactation period of 'A' goat.

Obs. No.	Days	1	2	3	4	5	16	12	32	51	65	79	98	109
1	Fat%	7.8	5.7	5.3	5.1	5.9	8.0	8.4	4.25	3.8	4.25	3.4	3.2	5.2
2	Water%	72.80	81.75	81.10	82.84	81.63	85.99	86.25	85.50	85.85	85.70	86.80	86.85	82.76
3	Total solids%	27.70	18.25	18.90	17.52	18.37	14.01	13.75	14.50	41.15	14.80	13.20	13.65	17.24
4	Total solids not fat%													
5	Ash%	20.40	12.55	13.60	12.42	12.47	11.01	10.35	10.25	10.35	10.05	9.80	10.45	12.04
6	Lactose%	0.9088	0.9040	0.9122	0.7627	0.7244	0.7821	0.7276	0.7907	0.8141	0.6753	0.7096	0.7793	0.5478
7	Proteids%	2.316	3.578	3.719	4.064	3.904	4.122	3.977	4.820	4.189	4.130	3.842	3.969	2.052
		14.65	7.120	6.819	5.946	6.401	4.042	3.788	3.644	3.613	3.721	3.519	4.454	7.658



TABLE No. 4

Composition of milk-samples during the lactation period of 'B' goat.

Obs. No.	Days	9	23	39	53	67	81	95	112	123	130	146	154	167	174
1	Fat%	3.4	1.70	1.75	1.95	2.25	1.9	2.2	2.75	4.85	3.85	4.2	5.95	4.4	5.95
2	Water%	85.16	89.22	88.81	88.29	88.20	88.67	88.31	87.84	85.97	87.47	86.48	84.30	85.02	82.92
3	Total solids%	14.84	10.78	11.19	11.71	11.80	11.33	11.69	12.16	14.03	12.58	13.52	15.70	14.98	17.08
4	Total solids-not-fat%	11.44	9.03	9.44	9.76	9.55	9.43	9.49	9.41	9.68	9.18	9.32	9.75	10.58	11.13
5	Ash%	0.7524	0.7802	0.7950	0.8814	0.8780	0.8345	0.7178	0.9296	0.8561	0.8174	0.6985	0.6165	...	0.7567
6	Lactose%	4.143	4.143	3.961	3.782	4.009	3.619	3.473	3.612	4.935	3.961	3.812	3.775	5.642	3.340
7	Proteids%	3.289	3.289	3.212	3.588	3.454	3.654	3.599	3.609	4.015	4.116	4.028	4.316	5.633	6.469

at once. The result of the analysis of milk sample in the last week shows marked variations in the constituents of milk and hence the last sample of milk may show abnormal variations in its constituents like the first milk.

Dr. D. L. Sahasrabuddhe in his note on "Variation in the composition of milk of individual buffaloes" (Poona Agricultural College reprint No. 17) states that fat regularly, though slowly, increases up to the end of lactation period and the solids—not-fat are fairly constant. The total proteids and casein increase slightly with the advance of lactation period.

The variations in the composition of cow-milk during the lactation period have been studied by different workers in foreign countries. Similar changes in the constituents of milk during the lactation period were obtained in the case of cows.

The daily yield of milk from 'A' goat during the normal period of lactation was about three pounds and that from 'B' goat was about one and a half pounds. The yield of milk went on increasing during the early stages and then in second or third week it became nearly constant. The decrease in the yield of milk was gradual in the last month of lactation period and in the last week the decrease in the yield of milk was considerable. W. Tayler and A.D. Husband (quoted from 'variations in the composition of milk' p.p. 63 Tocher) found that for 13 days in the beginning of the lactation period of a goat there was an upward trend for the percentage of lactose accompanied by an upward trend in the daily volume of milk. They also found that towards the end of lactation period there was a distinct fall in the daily volume of milk accompanied by a slight fall in the percentage of lactose. In our experiments also the percentage of lactose increased accompanied by the volume of milk in the early stages of lactation period. There was also a slight fall in lactose along with the fall in daily volume of milk towards the end of lactation period.

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## PAPER XI

## "A STUDY OF SHEEP-MILK"

By

P. G. TULPULE, M. Sc.

*(With the kind permission of the authorities of the  
Bombay University)*

**Introduction**

Milk is an indispensable article of human diet and for the healthy growth of every individual abundant supply of milk is necessary. Taking into consideration this importance of milk as a necessary factor of the food material, much of the scientific study of milk has been done from the physiological and biochemical points of view. As cow and buffalo are the common milking animals, the analytical study of the milk and milk products obtained from these two species is given much importance and consequently that branch is developed in details.

There are milking animals, other than cows and buffaloes, which secrete milk from milk glands from the time of the birth of the young ones. These are sheep, goat, mare, camel, elephant, human being, etc. The milk obtained from any of these species is made up of the same constituents such as:—

- (1) Water.
- (2) Fat.
- (3) Milk-sugar or Lactose.
- (4) Albuminoids consisting of casein, lactalbumin and globulin.
- (5) Salts consisting of phosphates, citrates and chlorides of metals like calcium, potassium and sodium and traces of some other metals.

In addition to these, there are also some enzymes, like oxidase and catalase, and vitamins.

But these constituents are contained in different proportions in different milks. The variation in constituents depends upon the nature of the animal and its requirements for the growth of its young ones. Again in the case of the same animal, it has been found that the constituents vary in their percentage contents, according to the requirements of its young ones at different periods of its growth.

The composition of milk secreted by an animal does not remain constant over the whole lactation period. Moreover there are number of factors which affect the amount and the composition of milk secreted by an animal.

These factors are as follows:—

(From "Secretion of Milk" by Dwight Espe)

- (1) Intra and interbreed difference.
- (2) Due to the changes in the feeding stuff which is supplied to the animal.
- (3) Condition and dry period.
- (4) The stage of lactation.
- (5) Effect of pregnancy.
- (6) Season of the year.
- (7) Interval between milking.
- (8) Estrus.
- (9) Age, size, exercise, disease, etc.

The composition of milk of cow and buffalo has been studied in detail and much light is thrown on the nature of variation in the composition of milk due to the factors mentioned above.

The three factors, namely the feeding-stuff, the seasonal change and the stage of lactation are comparatively more important than the rest. The feeding-stuff and the seasonal change have an important bearing on the yield of milk or milk production and therefore, these two factors affect the yield of the milk more than its composition.

The stage of lactation is the most important factor affecting the composition of milk. The percentages of fat and nitrogenous compounds, such as casein, albumin etc., are very high during the earlier days of lactation as these components are essential for the growth of the new born young ones. As the period of lactation advances the proportion of carbohydrate contents is increased while the proportion of fat and nitrogen compounds is lowered down and then remain almost constant during the normal period of lactation. These changes in the components of milk, during the lactation period of an animal, are very interesting to study. - In the case of common milking animals like cows and buffaloes this study is much advanced, but very few people in India have done any work on sheep milk and it was for this purpose the study of Sheep-Milk was taken up.

The study of sheep milk was undertaken with the following two main objects:-

The first object was to study the effect of the stage of lactation on the composition of sheep milk with a special reference to the colostrum and the milk during the first week of the lactation period.

The second object was to see whether the differences in breed of the animal have any effect on the mode of variation of different constituents of milk during the lactation period.

With these two objects the composition of milk during the whole lactation periods of three sheep of different breeds was studied.

In the work so far done in different countries the attention is mainly concentrated on the yield of the milk and its relation to the growth of lambs. Especially the study of colostrum and the milk during the first few days of lactation period is not thoroughly done. This study is interesting as the changes in the composition of milk during this period of lactation are very rapid. Also it struck to our mind that we have contributed very little information about the study of

milk and milk products of Indian sheep though the sheep milk and sheep-ghee are consumed in some Indian villages in considerable quantities.

These facts will clearly indicate that the study of the composition of milk during the complete lactation period of sheep of different breeds will contribute a great deal to this branch. From this point of view three sheep of different breeds grazing on Deccan lands, were selected for milk study. During the study of the lactation period particular importance was given to colostrum and the milk during the first few days of lactation period.

The three sheep selected for the study of milk were:-

- (1) Deccani Sheep
- (2) Back cross sheep
- (3) First Cross of Deccani & Merino

## SECTION I

### **A Study of Six Samples of Sheep-milk with reference to the ratio of Casein to Albumin & Globulin.**

Before studying the lactation period, complete analysis of six samples of sheep milk was carried out with a view to establish some relationship between different constituents. The milk samples were studied during the normal period of lactation of the animals. The six sheep were under the same conditions of feeding. The nitrogen of casein and total proteids was determined for each sample and from the results the percentages of casein and proteids were calculated. The percentages of albumin plus globulin was derived by the method of difference. The analytical results of the composition of milk samples are given in Table No. 1. The percentages of casein and albumin and also the ratio of casein to albumin are mentioned in Table No. 2.

TABLE No. 1

Composition of six milk samples obtained from six different sheep.

Constituents.	Sheep.					
	1	2	3	4	5	6
Total Solids ...	19.28	16.31	14.08	16.90	18.17	15.38
Water ...	80.72	83.69	85.92	83.10	81.83	84.62
Fat ...	8.6	5.8	4.7	5.7	7.5	5.1
Proteids ...	5.03	4.55	4.97	5.38	5.37	5.24
Lactose ...	3.54	3.82	3.44	3.83	3.50	3.40
Ash ...	0.68	0.67	0.93	0.98	1.1	0.99
Solids-not-fat ...	9.25	9.04	9.21	10.20	9.97	9.63

TABLE No. 2

Ratio of Casein to Albumin in Sheep-milk.

Sheep No.	Proteids %	Casein %	Albumin + Globulin %	Ratio of Casein : Albumin + Glo.
1	5.033	3.801	1.232	3 : 1
2	4.55	3.548	1.002	3.5 : 1
3	4.977	3.913	1.064	3.9 : 1
4	5.383	4.104	1.284	3.4 : 1
5	5.370	4.032	1.338	3 : 1
6	5.243	4.127	1.116	3.7 : 1

Among the samples studied the percentage of fat, as low as 4.7 and as high as 8.6 was found. But solids-not-fat were practically constant and the mean of the six samples was 9.71. Thus the figure solids-not-fat was independent of the fat contents of the milk samples. A study of the ash obtained by incinerating the solids and thus removing the organic matter is very interesting. The percentage of ash in milk indicates the presence of the salts of metals like calcium, magnesium, potassium, sodium, iron and also chlorine, phosphorus and sulphur in combined form. The average percentage of ash in sheep milk is 0.88.

The nitrogen distribution in terms of casein, albumin and globulin was studied for the six samples of normal milk. The results are given in Table No. 2. The ratio of casein to albumin plus globulin showed a slight variation from sample to sample.

The first and the fifth sheep showed the ratio as 3:1 while the third sheep secreted milk containing casein in higher proportion. The ratio of casein to albumin in that case was 3.9:1. The average ratio for the six samples was 3.5:1. Dr. N. N. Godbole and Sadgopal quoted 2.8:1 as the average ratio for sheep milk.

Out of the three main constituents of milk viz. fat, proteids and sugar the former two were found in different proportions in samples of milk from different animals while the milk sugar was the least variable factor. The maximum and the minimum percentages of milk sugar were found to be 3.837 and 3.404. This proved that the carbohydrate contents in sheep milk were comparatively low and in no case the lactose contents were greater than the percentage of proteids or fat.

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## SECTION II

## A STUDY OF MILK OF TWO SHEEP DURING THE LACTATION PERIOD.

Three sheep of different breeds were selected for the study of milk by the kind permission of the Superintendent of Sheep Breeding Research Station, Poona, working under the supervision of the Imperial Control of Agricultural Research.

The breeds of the three sheep were as follows:

- (1) Deccani Sheep—Pure line selection.
- (2) Back Cross Sheep—Cross breed sheep holding  $3/4$  Deccani blood and  $1/4$  Merino.
- (3) First Cross Sheep—Deccani  $\times$  Merino.

A special precaution was taken to keep constant the factors which change the composition of milk of an animal. These factors are particularly the food stuff and the seasonal change. The intervals between the two successive milkings were also kept constant. The three sheep were fed on similar diet and also the material was kept constant quantitatively for the whole lactation period. The animals under experimental conditions were studied in the same season, that is winter. Therefore, these factors did not interfere with the changes in the composition of milk of the individual sheep, and the variation in the constituents of milk was only due to the stage of lactation and hence the results of the three breeds were comparable.

**General Conditions on the Farm.**

There are three lambing seasons in Deccan. First is August or Shrawani, as called by the Shepherds, second is Divali or winter lambing and the third is summer lambing. Winter lambing is favoured by all shepherds. Summer lambing is a calamity as there is no green food and majority of lambs die for want of milk. The August lambing season is only useful to the shepherds in dry areas. The milk analysis of winter lambing was undertaken as it represented the major season of the Deccan.

Food-stuff:—The flocks of sheep on the research station subsisted mainly on grazing available on hill-sides, which was termed as Forest Kuran. This area was representative of the typical grass-land of the transition tract of the Deccan. This grazing was available for six months of the year i.e. from July to December and for the remaining period the flocks moved on the cultivated areas, grazing on the after-maths of the kharif crops. The grazing mostly consisted of weeds and particularly Haraly (Sanidon Dactilon). Thus during the first part of the observations of milk samples the sheep were grazing on the hill-sides and from January to March their main food was weeds in the field.

These sheep received supplementary food in the farm. It consisted of three ozs. of ground-nut cake, from the time of their lambing. All the animals under trial received the same treatment. The supply of the ground-nut cake varied slightly from month to month as given in the table No. 3. This increase or decrease in the quantity of ground-nut cake was dependent on the change of grazing.

TABLE No. 3

Ground-nut cake	Nov.	Dec.	Jan.	Feb.	March.
Ozs. per sheep per day.	2	3.25	4	4	4

Daily supplementary ration to the sheep during the lactation period.

Occasionally the flock was given a salt mixture consisting of common salt, iron sulphate, sulphur, and traces of copper-sulphate. Besides the common-salt the rest of the ingredients were added as anthelmintic and also they helped towards the growth of wool.

The lactation periods of three different sheep were studied.

The information about the three sheep is tabulated in table No. 4.

TABLE No. 4

Breed of Sheep.	Age	Lambing turn	No. of Lambs.	Lactation period in days.
Deccani	3½	4	1	92
Back Cross	3	3	2	79
First Cross	2½	2	1	70

The quantity of milk obtained from an individual sheep was very scanty and just sufficient for the lambs. For this reason the study of the changes in the yield of milk during the lactation period of an individual sheep was not possible. The lambs would have starved if all the quantity of milk available at a time was taken as a sample. To overcome this difficulty, small quantity of milk, just sufficient for analysis, was hand-milked and used as a sample.

From the observations we could conclude that

(1) The quantity of milk yielded by the Deccani sheep is more than that of Merino or the cross of Merino and Deccani under the circumstances and the climate of Deccan Land.

(2) The lactation period of the Deccani sheep is longer than that of the Merino or the cross of Deccani and Merino.

(b) Methods adopted for sampling and analysis of sheep-milk.

The methods recommended by the Association of Official Agricultural Chemists of U. S. A. 1946 were mostly adopted for all determinations.

In this section we propose to give the account of the study of sheep's milk from three different points of view.

(1) Variations in different constituents of milk during the lactation periods of three sheep belonging to different breeds.

(2) Comparison of the mode of variation in the constituents of milk during the lactation period of three sheep.

(3) Comparison of the lactation period of sheep to the lactation period of buffalo.

As mentioned in the previous section three sheep of different breeds were selected for the study of milk during their lactation periods.

These sheep were selected in such a way that their breeds held the Deccani sheep blood in different proportions and the Deccani breed characteristics are reduced from Sheep No. (1) to sheep No. (3).

Sheep No. (1) Deccani Sheep.

Sheep No. (2) Back Cross Sheep.

Sheep No. (3) First Cross sheep of Deccani & Merino.

By studying the composition of milk during the lactation period of three sheep we have to see whether definite relation lies between the characteristics of the breed and the milk obtained from the animal in the breed.

The results of the composition of milk during the lactation period of three sheep are given in Table Nos. 5, 6 and 7. As the changes in the composition of milk during the earlier days of lactation, particularly in the first week, were very rapid, the milk samples of the individual sheep were analysed every day till the figures showed that the composition had become normal and no more rapid variations could be marked. When once the milk was normal the changes were considerably slow and for this reason the milk was analysed at the interval of one week during the normal period of lactation. This procedure was followed for the three sheep under experimental conditions.

TABLE No. 5

## Composition of Milk during the Lactation Period of Deccani Sheep

Composition.	DAYS.												
	Colostrum	2	3	4	5	11	19	30	39	47	63	82	92
Total Solids %	... 28.38	20.16	16.87	15.91	15.15	14.42	14.59	16.51	16.70	18.01	21.72	20.93	18.58
Water %	... 71.62	79.84	83.13	84.09	84.85	85.58	85.51	83.49	83.30	81.99	78.28	79.04	81.42
Fat %	... 9.2	8.3	5.8	5.4	5.1	4.2	4.3	5.35	5.65	7.4	9.7	9.1	...
Lactose %	... 2.332	2.61	3.147	3.859	3.93	4.12	3.643	3.772	3.757	3.77	3.864	4.322	4.261
Proteids %	... 16.07	8.11	6.728	5.44	4.98	5.07	...	4.706	4.78	4.83	4.72	4.401	5.265
Ash %	... 0.71	0.77	0.772	0.989	0.91	0.87	...	0.85	0.83	0.84	0.83	0.90	0.81
Total Solid -not fat %	... 19.11	11.49	10.64	10.28	9.82	10.1	...	9.32	9.367	9.14	9.412	9.623	10.336

TABLE No. 6

Composition of Milk during the Lactation Period of Back-Cross Sheep.

Composition.	DAYS.											
	Colos- trum	2	3	4	12	20	28	36	50	57	65	79
total Solids %	25.98	19.77	16.85	16.04	14.87	13.76	12.98	15.17	18.53	18.94	16.41	18.89
Water %	74.07	80.23	83.15	83.96	85.13	86.24	87.02	84.83	81.47	81.06	83.59	81.61
Fat %	8.9	8.2	6.4	5.9	4.8	3.4	2.7	4.8	8.3	8.4	6.7	7.7
Lactose %	2.613	3.045	3.170	3.222	3.477	3.997	3.940	4.082	4.227	3.887	4.308	3.698
Proteids %	12.82	6.883	5.644	5.808	5.123	4.774	4.931	4.910	4.214	...	5.285	4.921
Ash %	1.1	0.93	0.91	0.78	1.05	0.9	0.69	0.77	1.08	0.79	0.93	1.1
Total Solid - not fat - %	16.53	10.86	9.724	9.810	9.65	9.671	9.561	9.712	9.471	...	10.523	9.719

TABLE No. 7

## Composition of Milk during the Lactation Period of First-Cross Sheep.

Composition	DAYS.												
	Colostrum	2	3	4	11	18	27	35	42	49	63	70	
total solids %	...	26.72	21.03	18.94	18.82	17.81	17.28	18.72	19.23	21.44	21.07	20.66	20.05
water %	...	73.28	79.97	81.06	81.18	82.19	92.72	81.28	80.77	78.50	78.93	79.34	79.95
fat %	...	9.4	8.9	8.4	8.4	6.8	6.6	7.1	8.05	9.4	9.1	8.4	8.1
lactose %	...	2.292	2.611	2.988	2.999	3.390	3.404	3.440	3.556	3.203	3.872	4.104	4.244
Proteids. %	...	14.17	8.31	5.47	5.213	5.022	5.161	5.181	5.188	5.302	5.716	4.610	4.330
Ash %	...	0.98	0.90	0.91	0.81	0.70	0.77	0.70	0.62	0.63	0.71	0.68	0.71
Total Solid-not fat %	...	17.392	11.821	9.313	9.022	9.112	9.335	9.321	9.364	9.185	9.298	9.394	9.284

The colostrum was very much different from the normal milk. It was thick and tasted saltish. All the constituents except Lactose were very high. If we study the composition of colostrum and that of the first normal milk sample, side by side, we find that the composition of colostrum does not show any relation to the breed of the animal. It is moreover an individual factor. The percentages of total solids and fat in colostrum of deccani sheep are identical with the percentages of these two constituents of colostrum of first-cross-sheep. On the other hand the results of the first normal milk samples show a definite relationship in the breeds.

The figures in these tables show the rapid change in the composition of milk in first few days.

During the normal period of lactation no rapid variations could be marked in the milk samples. A few samples at the end of the lactation period were somewhat abnormal. Fat and sugar varied irregularly during the last 15 days of lactation. In the deccani and first cross sheep the fat contents were decreased at the end of the lactation period and in back-cross sheep both fat and lactose showed the same tendency.

These results make it clear that the mode of variation of different constituents of milk during the lactation periods of sheep, belonging to different breeds, is identical. The results also prove that the first merino cross gave the richest milk, then in decreasing order are ranked the back-cross sheep and the deccani sheep. The average fat contents in the three cases were respectively 8.25, 6.43 and 6.4%. In the deccani sheep the percentage of fat in colostrum was 9.2, while in back-cross sheep and first cross sheep the percentages were found to be 8.9 and 9.4 respectively. The fat contents in the first normal milk samples of three sheep were 5.4%, 6.4% and 8.4% respectively. Thus, in the normal period of lactation the tendency of secreting milk rich in fat contents was increased from deccani to merino breed though the daily yield of milk of merino cross sheep was less than that of deccani sheep.



The percentage of fat in milk varied from sheep to sheep and this variation was fairly regular throughout the lactation cycle. It was highest in concentration in colostrum, then the concentration had fallen to a minimum in about three weeks, rising later to a figure intermediate between this minimum and the colostrum value. It showed some signs of increase towards the end of the lactation period.

Similarly the mode of variation of other two constituents of milk, namely sugar and proteids, in the three varieties of sheep, was identical. Percentage of lactose increased gradually throughout the lactation period and in the three cases the minimum and the maximum percentages are respectively

- (i) 2.332 and 4.3 in deccani sheep.
- (ii) 2.613 and 4.308 in back cross sheep.
- (iii) 2.292 and 4.244 in first cross sheep.

The figures indicated that the breed differences of sheep had no effect on the lactose contents of milk.

We can summarise the study of variation in the composition of milk during the lactation period of the three sheep as :-

(1) The mode of variation of different constituents of milk during the lactation period is independent of the breed of the sheep.

(2) In general, sheep milk is rich in fat contents but it is especially so in merino cross breeds.

(3) Colostrum or the first milk after lambing is very different from the normal milk. All the constituents except milk sugar or lactose are very high in colostrum, as compared to the normal milk.

(4) In all the three cases milk becomes normal in 3 or 4 days.

(5) Solids-not-fat are practically constant over the normal period of lactation and the average values in the three breeds of sheep are almost identical.

(6) Percentage of lactose increases throughout the lactation period. The increase during the first few days is comparatively rapid. The breed differences of sheep have no effect on the lactose contents of milk.

(7) During the normal period of lactation, an inverse relationship, exists between proteids and lactose.

(8) The total proteids when once become normal are found to be the least variable constituents.

(9) The percentage of ash is greater during the early days of lactation than the later days.

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## PAPER XII

## "A STUDY OF MILK OF ASS"

By

- RAJADHYAKSHYA, M. Sc.

*(With the kind permission of the authorities of the Bombay University)*

**(A) Introduction.**

It is common knowledge that ass's milk is generally given to children who are suffering from rickets and whooping cough and other similar diseases and where cow's milk is not tolerated. Some fantastic and queer notions exist regarding the ass's milk. Ass is generally looked upon as an animal devoid of brain and the milk from that animal is taken rather with suspicion. There is also a strong belief even to day among the illiterate as well as some literate people that the milk if it is to be taken should be taken as soon as the animal is milked as otherwise on keeping the milk vessel even for a short time on the ground it gets full of insects. This is a wrong belief as we ourselves have observed no insects present of any kind whatsoever even though the milk is kept for days under normal atmospheric condition. The milk merely gets fermented as expected but no insects are observed. In fact the preservative qualities of the milk appear to be superior as it was observed in the laboratory that when fresh samples of buffalo's and ass's milk were kept in the refrigerator (temperature about 9°C.) the former curdled earlier than the latter.

Taking into consideration the food value of the ass's milk and some strange ideas associated with it we decided to investigate the ass's milk in detail.

Before proceeding to give out the detailed procedure and the working methods by which we tackled the problem it will be worth while to say something about the workers and their study in this branch. A reference to the Analyst 1896,

Vol. 21, page 88, reveals the mean composition of ass's milk given by H. D. Richmond as total solids—10.23%, fat 1.18%, sugar 6.86%, proteins 1.74% and ash 0.45%, while in 1898, A. Schlossmann examined 16 samples of asses' milk and found the specific gravity to vary from 1.031 to 1.036. The mean percentage results of the total solids were 11.5, of ash 0.40, and of sugar 4.94. The fat varied from 0.15% to 0.60% and the proteins from 1.4% to 1.72%. In 1907 Wagner gave the average results of five years of the amount of fat found in the milk of a herd of asses. The analyses were made in the summer months, the animals only giving milk from May to September and of the 30 animals constituting the herd nine or ten were giving milk at the same time. In all 392 determinations were made the average percentage quantities of fat found being 1902, 0.23; 1903, 0.14; 1904, 0.10; 1905, 0.11; 1906, 0.10. The average for 5 years 0.125%. The actual quantities found varied from nothing to 0.70%. The majority of the results, however, approached the above given average. Other workers like G. D. Elsdon, G. H. Walker, Teichert, Konig have also made valuable contribution in this field. Table No. 1, will show the mean composition of as's milk given by different above mentioned analysts.

TABLE No. 1.  
Composition of As's Milk.

Authority	Water %	Fat %	Sugar %	Total Solids %	Proteins %	Ash %
Elsdon and Walker.	...	1.00	6.80	9.95	1.70	0.45
Teichert ...	89.70	1.50	6.00	10.30	2.40	0.40
Teichert ...	90.12	1.37	6.19	9.88	1.85	0.47
Konig ...	89.64	1.64	5.99	...	2.22	0.51

In India Godbole & Sadgopal and C. P. Antharakrishnan of Department of Biochemistry, Indian Institute of Science, Bangalore have done work on ass's milk. Table No. 2, will show the analysis carried out by Godbole and Sadgopal on a few samples of ass's milk.

TABLE No. 2

Specific Gravity.	Total Solids. %	Fat. %	Proteins. %	Lactose %	Ash. %
1.023	9.16	1.3	1.6	6.28	0.40
1.035.	9.53	1.5	2.0	6.80	0.48

The only somewhat systematic study of ass's milk appears to have been undertaken recently by C. P. Anthrakrishnan of Department of Biochemistry, Indian Institute of Science, Bangalore in his paper published in the Journal of Dairy Research 1941, Volume 12, Page 119, in which he gives the percentage composition of ass's milk as follows:-

TABLE No. 3

Sample No.	Total Solids. %	Fat. %	Lactose. %	Ash. %
1.	8.47	0.68	6.23	0.40
2.	8.91	0.63	6.06	0.37
3.	8.42	0.59	6.20	0.38
4.	8.51	0.71	6.50	0.47
5.	8.46	0.64	6.23	0.38
6.	9.10	0.60	5.98	0.40
7.	7.80	0.66	5.85	0.42
8.	8.46	0.54	6.00	0.37
9.	8.75	0.63	6.24	0.40

From above it can be seen that though there are not a few workers who have studied the composition of ass's milk, yet it appears that the study of variation of the composition of each of the constituents of the milk during the whole lactation period has not been undertaken by any of the above workers. It need hardly be said that the stage of lactation has some influence upon the composition of milk, particularly upon the fat content. The Solids-not-fat content is higher for the first week or two of the lactation and then falls rapidly for about a month to a level which is maintained almost constant until 2 or 3 months from the end of the lactation (Vide 'Food' September 1946).

It was therefore thought proper that it would be an interesting problem to study the variations of each of the constituents of ass's milk during the lactation period and accordingly the work was taken in hand.

TABLE No. 4

## Composition of milk-samples during the lactation period of Sample No. 1

Obs. No.	Date	6-8-45	13-8-45	20-8-45	27-8-45	3-9-45	14-9-45	17-9-45	24-9-45	1-10-45	8-10-45	16-10-45
1	Specific Gravity	1.0270	1.0300	1.0380	1.0685	1.0337	1.0337	1.0301	1.0338	1.0332	1.0305	1.0321
2	Total Solids %	10.22	10.39	8.76	12.07	10.06	9.81	11.46	9.67	9.83	11.17	9.50
3	Fat %	1.40	1.70	0.20	3.30	1.40	1.00	2.40	1.03	0.70	2.40	0.80
4	Solids-not-fat %	8.82	8.69	8.56	8.77	8.66	8.81	9.06	8.67	9.13	8.77	8.70
5	Proteins %	1.97	1.85	1.70	1.69	1.69	1.91	1.79	1.79	2.03	1.93	1.79
6	Lactose %	6.60	6.48	6.61	6.90	6.77	6.57	6.61	6.46	6.96	6.43	6.20
7	Ash %	0.38	0.41	0.37	0.39	0.32	0.39	0.39	0.40	0.40	0.40	0.36
8	Casein %	0.52	0.60	0.51	0.55	0.70	0.83	0.80	0.79	0.89	0.79	0.55
9	Albumin & Globulin %	1.62	1.59	1.56	1.00	1.21	1.14	1.14	1.16	1.42	1.00	1.34

TABLE No. 6

## Composition of milk-samples during the lactation period of Sample No. 3

Obs. No.	Date	26-8-46	7-9-46	11-10-46	16-10-46	21-10-46	29-10-46	4-11-46	11-11-46	18-11-46	25-11-46	2-12-46	9-12-46	28-12-46
1	Specific Gravity	1.0480	1.0400	1.0346	1.0390	1.0361	1.0260	1.0256	1.0320	1.0280	1.0305	1.0280	1.0360	1.0380
2	Total Solids %	9.89	9.87	9.40	9.05	9.53	9.14	7.13	7.61	9.00	8.47	7.56	9.19	9.49
3	Fat %	nil	nil	0.10	nil	0.10	0.10	0.20	0.10	0.10	0.15	nil	nil	0.40
4	Solids-not-fat %	9.89	9.87	9.30	9.05	9.43	9.04	6.93	7.61	6.90	8.52	7.56	9.19	9.09
5	Proteins %	2.94	3.30	2.70	2.91	2.62	2.86	1.70	1.61	1.61	1.61	1.52	1.70	1.52
6	Lactose %	6.98	6.73	5.97	5.74	6.08	5.71	4.11	4.48	6.30	5.85	4.59	6.10	6.25
7	Ash %	0.56	0.62	0.63	0.53	0.55	0.50	0.49	0.52	0.45	0.54	0.59	0.40	0.44



TABLE No. 5

## Composition of milk-samples during the lactation period of Sample No. 2

Obs. No.	Date	19-11-45	28-11-45	8-12-45	10-12-45	17-12-45	27-12-45	31-12-45	7-1-46	14-1-46	21-1-46	28-1-46	4-2-46	11-2-46	18-2-46	14-3-46	19-3-46	26-3-46	1-4-46
1	Specific Gravity	1.0290	1.0300	1.0314	1.0318	1.0318	1.0318	1.0319	1.0325	1.0367	1.0331	1.0344	1.0332	1.0351	1.0342	1.0334	1.0345	1.0340	1.0334
2	Total Solids %	7.33	9.05	9.95	11.29	11.00	11.54	10.23	9.94	11.81	9.84	9.11	9.15	9.08	8.96	8.67	8.97	8.79	8.70
3	Fat %	0.80	0.40	0.90	2.50	2.30	3.10	1.50	0.90	2.10	1.20	0.30	0.20	nil	0.20	0.30	0.20	0.20	0.20
4	Solids-not-fat %	7.69	8.65	9.05	8.79	8.70	8.44	8.73	9.04	9.71	8.64	8.81	8.95	9.08	8.76	8.37	8.77	8.59	8.50
5	Proteins %	2.14	1.92	1.92	1.90	1.85	1.77	1.76	1.87	1.96	1.96	1.96	1.98	1.98	2.06	1.90	1.94	1.90	2.08
6	Lactose %	6.38	6.44	6.56	6.53	6.86	6.24	6.59	6.31	6.71	6.44	6.40	6.14	6.18	6.06	6.36	6.47	6.05	6.31
6	Ash %	0.38	0.37	0.38	0.44	0.38	0.37	0.34	0.39	0.39	0.43	0.38	0.36	0.42	0.38	0.34	0.35	0.45	0.45
8	Casein %	0.54	...	...	...	0.67	...	...	...	0.81	...	...	...	0.59	...	...	...	...	1.05
9	Albumin & Globulin %	0.90	...	...	...	1.03	...	...	...	1.34	...	...	...	1.33	..	...	...	...	1.27

TABLE No. 7

Composition of milk-samples during the lactation period of Sample No. 4

Date	Specific Gravity %	Total Solids %	Fat %	Solids-not-fat %	Proteins %	Lactose %	Ash %	Cassin %	Albumin & Globulin %
9-10-45	1.0346	12.22	3.20	9.02	2.71	5.78	0.58	0.98	2.04
15-10-45	1.0372	9.85	0.10	9.75	2.60	6.23	0.54	1.24	1.51
22-10-45	1.0345	9.86	0.70	9.16	...	6.22	0.58	1.18	1.84
29-10-45	1.0351	11.16	1.10	10.06	...	6.87	0.60	...	...
7-11-45	1.0350	9.34	0.10	9.24	2.19	6.89	0.55	...	...

TABLE No. 8

Composition of milk-samples during the lactation period of Sample No. 5

12-11-45	1.0398	9.96	1.40	8.58	1.82	6.20	0.44	0.93	1.83
28-11-45	1.0392	10.54	1.70	8.84	2.21	6.23	0.45	...	...

**(B) A Study of the variation of each constituent of ass's milk with the lactation period.**

Each sample was analysed week after week with a view to study the variation of each constituent of the milk during the animal's lactation period. A general view of the Table No. 4 will show that there is too much variation in the case of total solids and fat. The specific gravity has a tendency of increasing gradually with the lactation period for about eight weeks the rise being from 1.0270 to 1.0337. The specific gravity afterwards falls gradually, the maximum and minimum figures for the specific gravity are 1.0270 and 1.0337 respectively, the average being 1.0318.

Too much variation in the fat has been observed, in sample No. 1, the minimum and maximum limits in this sample being 0.2% and 3.3% respectively. The average fat for the whole lactation period is 1.48%. In all milking animals there is a tendency for the fat percentage to vary according to the kind of food supplied and the mood of the animal. If the oil cake and cotton seed are regularly supplied in food the fat percentage naturally increases but in the absence of these the fat goes down and if the animal does not get any concentrate, the fat percentage may decrease severely. As the ass is not fed with any care for its milk the variation is great and excepting the extreme limits nothing can be said about the weekly changes found in the table. The average fat recorded in no way represents the correct average of the fat due to its great variability. The fact that on some occasions either the fore milk or the strippings had to be analysed should also be taken into consideration. The figures for the Solids-not fat-remain practically constant with a slight variation from week to week. The minimum and maximum figures obtained during the lactation period are 8.56 and 9.13, the average being 8.79. Although the variation in the fat is great still the figures for solids-not fat-remain practically constant. The protein figure in general remains more or less constant throughout the lactation period, decreasing gradually for a few weeks then remaining constant for sometime and then slowly rising till the end

of the lactation period with a slight variation from week to week. 1.8% is an average figure for the proteins in ass's milk. The other constituent besides solids-not fat-which remains practically constituent throughout the whole lactation period is the lactose of ass's milk. The variations in the lactose are very small, the minimum and maximum figures obtained are 6.20 and 6.96 respectively. In general the ass's milk should safely be said to contain 6.6% of the lactose. The high percentage of lactose and its consistency in proportion throughout the animal's lactation period are perhaps the chief characteristics of the ass's milk. The ass's milk can readily be distinguished from the milks of other common milking animals due to its large percentage of lactose. Some consistency is also exhibited by the ash of the ass's milk there being very little variation as the lactation period advances. The average percentage of ash of ass's milk may be taken as 0.38, the minimum and maximum figures being 0.32 to 0.41 respectively.

#### **Casein and Albumin and Globulin:—**

Unlike the casein of cow's or buffalo's milk the casein of ass's milk is not readily curdled on which account it is sometimes used as a substitute for human milk. Table No. 4, will show that the albumin and globulin percentages are higher than casein percentage. Godbole and Sadgopal give the limits of percentages of casein and albumin and globulin in ass's milk as 1.2 - 1.3, and 0.4 - 0.7 respectively while Leach quotes 0.67% and 1.55% as the contents of casein and albumin. Bung also gives 0.7 and 1.6 as the percentages for casein and albumin. We have recorded 0.69% and 1.29% as the average figures for casein and albumin respectively. Taking separately casein on one hand and albumin and globulin on the other the latter are higher than the former, the variation in albumin being from 1.00% to 1.56% while the variation in casein is from 0.51% to 0.89%. In the figures obtained by Godbole and Sadgopal for casein and albumin the percentage of casein is higher than that of globulin. The samples analysed by us are therefore more in agreement with the foreign samples and differ in this respect from the samples analysed by Godbole and Sadgopal.

## Sample No. 2.

As in sample No. 1, the specific gravity increases gradually for about 10 weeks the actual figures recorded being 1.0290 and 1.0381. It then slowly falls down till the end of the lactation period, the minimum and maximum figures obtained throughout the whole lactation period are 1.0290 and 1.0381, the average being 1.0326.

In this sample also variations in the total solids and fat are observed. The total solids increase gradually as the lactation period advances and at the end of the lactation period they have the tendency to decrease slowly. The maximum and minimum figures obtained are 11.81% and 7.83%, the average being 9.66%. The figures for solids-not fat, - as in the previous sample, remain fairly constant throughout the animal's lactation period. The maximum and minimum figures recorded are 9.71 and 7.63. Though in this sample the range between the extreme limits is high the average figure for the solids-not fat-e. g. 8.73 approaches closely to that of the first sample.

Fat, like total solids showing a great variation, remains practically constant until five<sup>a</sup> weeks from the end of the lactation period. On 11-2-1946 no fat was detected in the milk of the animal. This confirms the results obtained by Wagner as quoted by Richmond in his book where Wagner has recorded 0.00% and 0.70% as the extreme figures for the fat of ass's milk. Proteins, lactose and ash as in the previous sample remain more or less constant throughout the entire lactation period. The protein in this case also has the tendency of decreasing gradually with the lactation period remaining practically constant for about 2 weeks and then slowly rising till the end of the lactation period, the minimum and maximum figures obtained in this case are 1.77 and 2.14. The average figure for protein is 1.93. The lactose also shows a remarkable consistency which is a common characteristic for the ass's milk. The average figure for lactose in this sample is 6.38, the minimum and maximum figures obtained are 6.05 and 6.71

respectively. The ash in this sample remains fairly constant throughout the lactation period. The maximum and minimum figures recorded are 0.45 and 0.34, the average being 0.39.

### Sample No. 3

Sample No. 3, differs widely from other two samples in that no fat being detected for most of the week and the figures for protein, lactose and ash differing considerably from the other two samples. We have, however, given the figures obtained during the lactation period. These figures do not indicate anything in particular as there is no regularity in any way though it shows that the figures obtained have a general agreement with samples of other two animals analysed by us. The figures show no regularity probably on account of very careless manner in which the animal was kept. This shows that if the animal is not properly looked after then the milk may show variations beyond conceptions. Taking for example the lactose which ought to remain constant we find that in this sample the variation is from 4.11 to 6.3. Specific gravity in this sample decreases slowly as the lactation period advances becoming practically constant for about 2 to 3 weeks and then increasing slowly as the lactation period advances. The minimum and maximum figures obtained for specific gravity are 1.0258 and 1.0430. The average being 1.0344. Total solids in this sample show a tendency to decrease as the lactation period advances for about 5 weeks and then to increase till the end of the lactation period. Very low figures of total solids e.g. 7.13, 7.61 and 7.56 on 4-11-1946. 11-11-1946 and 2-12-1946 have been recorded, the corresponding figures for lactose also being proportionately low. This fact may be explained as a peculiarity of the individual animal. As already said the animal yielded fat which was negligible in quantity, its variation being from nil to 0.2. Particular care was taken in this case to obtain the entire bulk of milk which the animal gave at one time so that in this case the question of fore-milk or the strippings did not arise at all. The solids-not fat-content is higher for the first five weeks then falling rapidly to a level which is almost maintained constant till 2 weeks

before the end of lactation period. As low figures as 6.93, 7.51, 7.56 for solids-not fat-have been recorded in this sample. Similar low figures are also obtained by Anthrakrishnan (loc. cit.) where he gets the maximum and minimum figures for solids-not fat - as 8.50 and 7.14. This shows that there is a great variation in the constitution of the milk from animal to animal. The average figure for solids-not-fat in this sample, however, approaches very closely to that obtained for the other two samples. Protein content of the animal also shows a peculiarity. For about first six weeks it is very high compared to that recorded during the latter half of the lactation period, the minimum and maximum figures obtained throughout the lactation period being 1.52 and 3.30. The peculiarity of the ass's milk that its lactose content shows a remarkable consistency throughout the lactation period is not exhibited by this sample. Very low figures such as 4.11, 4.48 have been obtained which can only be attributed to the peculiarity of the animal. The figures of ash in this sample also are higher as compared with the other two samples.. Whereas the figures for the ash in the first two samples varied from 0.32 to 0.45 in this case the variation is from 0.40 to 0.63, the average being 0.52.

In addition to the above three samples two more animals were put to test. The analysis in one case could be made only for five weeks and in the other only for two weeks because of the unavoidable circumstances. In one case the young one of the animal met with a fatal accident as a result of which the animal stopped giving milk. The analytical results of the milk of these two animals are given in Table Nos. 7 and 8. In these two samples also the behaviour of each of the constituents of milk is analogous to the first two samples and even though the entire lactation period is not covered in the case of these two samples, the same characteristics e.g. the variation in the fat, the consistency of lactose and ash and to some extent of the proteins are also exhibited by these two samples. Unlike the sample No. 1, the casein and albumin and globulin figures of sample No. 4 are close to each other though the latter is definitely higher than the former.

**(C) A Comparison of each of the constituents of ass's milk with human milk.**

For comparison the average figures of each of the constituents of ass's milk from the samples analysed are taken.

*A Comparison with human milk:*—A number of workers has analysed the human milk. Table No. 9, shows the mean composition of each of the constituents of human milk as given by Richmond in his book on page 64.

TABLE No. 9.

**Mean Composition of Human Milk.**

Water %	Fat %	Sugar %	Protein %	Ash %
86.69 - 88.53	2.41 - 4.16	4.67 - 7.51	1.10 - 2.35	0.19 - 0.34

According to Godbole and Sadgopal the following composition for human milk has been noted.

TABLE No. 10.

Specific Gravity	Total Solids %	Fat %	Sugar %	Proteins %	Ash %
1.030 - 1.034	11.5 - 13.5	2.00 - 3.65	5.8 - 6.5	1.0 - 1.65	0.15 - 0.25

The same authors give the average composition of human milk from foreign source.



TABLE No. 11.

Specific Gravity	Water %	Fat %	Protein %	Sugar %	Ash %	Total Solids %
1.0298	87.58	3.74	2.01	6.37	0.30	12.42

Table No. 12, will show the figures for each of the constituents of ass's milk obtained by taking the average figures in the case of the three animals whose entire lactation period was studied by us.

TABLE No. 12.

### Composition of ass's milk.

Specific Gravity	Total Solids	Fat %	Proteins %	Sugar %	Ash %
1.0316	8.88	0.10	1.83	5.61	0.38
1.0344	10.26	1.48	2.15	6.58	0.52

Human milk is similar in character to ass's milk in that it coagulates into small granular and flocculent masses whilst with that of the ruminants the coagulation takes place into a hard mass. As stated by Richmond a number of series of analysis has been published in which the variation in the composition of human milk with the stage of lactation has been noted. It is observed that the percentage of fat increases as the lactation advances whilst there is a diminution in the amount of protein and ash. There is some evidence to show that the amount of sugar tends to increase. As already said the variation of fat in the case of ass's milk with the stage of lactation is great whilst the protein and ash figures, with a slight variation from week to week, almost remain constant. There is also no evidence to show that the amount of lactose increases with the

lactation period. On the other hand the amount of lactose in ass's milk has no effect on the stage of lactation. The specific gravity in both human and ass's milk is more or less the same. As regards the fat contents in the case of ass's and human milk nothing can be said in particular due to the variability of the fat contents in the case of the former. Whereas Godbole and Sadgopal have given 2.0 and 3.65 as the minimum and maximum figures for human milk, we have recorded 0.10 and 1.35 as the corresponding figures for the ass's milk. As will be shown in the chapter of analysis of fat there is a similarity between fat of human and ass's milk in that both contain low percentage of the volatile fatty acids. The sugar percentages in both ass's and human milk do not differ much. We have recorded 5.61 - 6.58 as the minimum and maximum figures for the lactose of ass's milk while the corresponding figures for the human milk as recorded by Godbole and Sadgopal are 5.8 and 6.5.

In general it is known that the proteins of the animal-origin are more easily digestible than the same from the vegetable sources. As has already been said that in the case of human milk casein is less easily precipitated than the other caseins as in the case of elephant's and buffalo's milks. The same is the case with casein of the ass's milk in that it is less easily precipitated and is much lighter than the casein of buffalo's milk showing thereby that ass's milk contains casein which is more finely divided and hence also more digestible. Again casein is less easily digestible than the other proteins present in milk e.g. lacta-albumin and lacta-globulin. The percentage of the latter, as has been shown, is more than the casein in the case of ass's milk. Considering these facts the ass's milk should be easily digestible than even the human milk as in the latter the percentage of casein is found to be more than the percentage of albumin and globulin. It may be probably on this account that the ass's milk is given to the rickety children and those suffering from some other troubles.

The percentage of ash in the case of ass's milk has been obtained as varying from 0.38 to 0.52.

Ass's milk does not resemble in any respect to buffalo's milk as all the constituents of the buffalo's milk show higher percentage as against those observed in the case of ass's milk. Buffalo's milk is more rich in proteins compared with all the other animals. The proportion of casein to lacta-albumin and lacta-globulin in the case of buffalo's milk is roughly 5:1 while in the case of ass's milk the corresponding proportion is roughly 1:2 where we have found that percentage of lacta-albumin and lacta-globulin is more than the percentage of casein.

Like buffalo's milk, goat's milk also shows no resemblance to ass's milk. All the constituents of goat's milk show a higher percentage than that found in the latter excepting the percentage of lactose. Ass's milk contains a greater percentage of lactose than any of the common milking animals. Again the percentage of fat in ass's and mare's milk is very low compared to the milks of other animals. So it can be inferred that in the case of hardy animals which have to do a bit of rough work as in the case of mare and the ass the percentage of the fat is always low.

From the above comparison of ass's milk with human, cow's, buffalo's and goat's milk we can say that ass's milk approaches more or less human milk rather than cow's milk. It has no resemblance whatsoever with either buffalo's or goat's milk.

**(D) A Comparison of the ratio of lactose, proteins and ash in ass's milk to the corresponding ratio in human, buffalo's and cow's milk.**

Dr. Vieth (reference:- Dairy Chemistry by Richmond, 4th edition, page 129) enunciated the proposition that the ratio of lactose, proteins and mineral matter (ash) in cow's milk was 13:9:2 and that this ratio was very exact, varying little from milk to milk. Again Pappel and Richmond for Egyptian buffaloes and Lather for Indian animals have each pointed out that the ratio of milk sugar, proteins and ash is on the average

12:10:2 as against 13:9:2 in cow's milk (reference:- Richmond, page 70). Dr. D. L. Sahasrabuddhe has also found the same ratio for Indian breed cows as 12.2:10.4:2. The corresponding ratio for human milk as obtained by taking the mean values given by Godbole and Sadgopal for Indian samples comes roughly to 60:13:2. We have obtained the ratio for the three samples of ass's milk which we have analysed as (1) 34:10:2 (2) 32:10:2 (3) 20:8:2. As can be seen from these figures the ratio for the first two samples of ass's milk shows a close similarity while the third sample does not show any resemblance to the first two samples. As previously stated this fact may be due to the peculiarity of the individual animal. The Vieth ratio obtained for ass's milk shows no similarity with any of the corresponding ratio obtained for cow's, buffalo's or human milk. This is natural due to the fact that whereas in ass's milk the percentage of lactose is more than in either of the above animals, the ash percentage in the case of human milk is very low compared to cow's, buffalo's and ass's milk. For that reason the last two figures of the ratio in the case of the latter three animals shows a close resemblance whereas in all the animals the first figure of the ratio is quite different from one another.

**(E) Superiority of ass's milk as a nourishing food over the milk of other animals.**

Milk is regarded as an excellent food due to the vitamins present in it. Practically all the vitamins are present in milks of various animals. Vitamin potency of fresh milk is the highest in comparison with the heated milk as the heat destroys many a vitamin. Ass's milk is generally given to children soon after the animal is milked. It should also be noted that ass's milk when heated gets curdled before it reaches the boiling.

It can therefore be seen that milk is an excellent food containing all the ingredients in proper proportions required for healthy and proper growth of man. For a proportionate and healthy growth of a human body normal food should consist of (a) carbohydrates (b) fat and (c) proteins in certain definite

proportions. The calories of food required for the daily work have got to be distributed between carbohydrates, fats and albuminoids in the ratio of about roughly 5:1:1, all of which together supply the necessary amount of energy required by an adult. Table No. 13 will show that in ass's milk the ratio of carbohydrates, fat and albuminoid is 5:1:1 and hence it is superior even to human as well as to cow's milk. (reference:- Butter-fat by Godbole and Sadgopal, page 14).

TABLE No. 13

Animal.	Goat	Buffalo	Cow	Mother	Ass
Carbohydrates:	1.5:1:1	1:1.5:1	1.5:1:1	5:2:1	5:1:1
Fat: Albuminoids.					

In the samples which we have analysed the ratio also roughly comes to 5:1:1 so it can safely be said that from the point of view of carbohydrates fats, albuminoids, salts and vitamins ass's milk forms an ideal food both for adults and children. It may probably be on this account that the ass's milk is given to children suffering from rickets and whooping coughs. In our opinion it should also be given to persons suffering from jaundice, where the patient is given as little fat as possible, as more carbohydrates as possible and where he is not allowed to take buffalo's or even cow's milk but instead buttermilk which contains hardly 0.5% fat. Ass's milk which also is very deficient in fat and which contains by far the greatest amount of carbohydrates and which also contains albuminoids in certain definite proportion should serve a useful food for such persons. Ass's milk is much used in France and Germany. In Dresden there are regular establishments for its sale; but as the yield of milk is poor it is very expensive. In India it has its value only as a medicine and especially given to children suffering from rickets and whooping coughs. It is not popular as food among the adults due to its peculiar taste and smell.

**Conclusions:-**

1. Ass's milk has a white colour and has its own peculiar taste. On heating it gets curdled before it reaches the boiling point due to, perhaps, greater proportion of lacta-albumin and lacta-globulin present in the milk than the casein.

2. Fat in ass's milk shows a great variation throughout the lactation period probably on account of the fact that the animal is not fed properly and with care. The other constituents of the milk specially the solids-not-fat and the lactose remain fairly constant throughout the animal's lactation period. Total-solids of samples of ass's milk range from 8.88 -- 10.26 and fat from 0.10 -- 1.48.

3. Vieth ratio found for the ass's milk is 34:10:2.

4. The proportion of lacta-albumin and lacta-globulin being more than the casein and also the ratio of carbohydrates, fat and albumin being 5:1:1 ass's milk should serve an ideal food both for adults and children.

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## PAPER XIII

COMPARISON OF THE COMPOSITION OF  
SHEEP GHEE AND BUFFALO GHEE

By

P. G. TULPULE, M. SC.

*(With the kind permission of the authorities of the Bombay University)*

## Introduction

Before we examine the analytical and the physical constants of the two butter-fats, it is necessary to understand that both oils and fats, whether of animal origin or vegetable origin, are mixtures of glycerides of fatty acids of low or high molecular weights. In these again, the glycerides of higher molecular weights are of either saturated acids or of unsaturated acids. Whenever the oils or fats are introduced into the human system, their assimilability and digestibility are dependent upon the nature of the fatty acids. The glycerides of the saturated acids of high molecular weights are difficultly digestible and therefore load the human system. They are likely to do, therefore, harm to the system and do no good whatsoever. This is mainly due to the fact that the glycerides of saturated acids with high molecular weights have melting points higher than that of the body temperature. According to Lewkowitsch the melting points of the saturated glycerides of high molecular weights vary between 56.5°C to 75°C.

The liquid glycerides of unsaturated acids of high molecular weights, particularly those of Oleic and Linoleic acids, (occurring in butter-fat) on the contrary are not harmful as those of the corresponding Stearic glycerides of about the same molecular weight.

The glycerides of saturated acids with low molecular weights generally occur in the butter-fats of cow, buffalo and other milking animals. They range from those of butyric to myristic acids and are known to be easily assimilated by the human system because of their low melting points. These fatty acids of low molecular weights are further differentiated as volatile soluble and volatile insoluble acids.

The proportionate percentage of the glycerides of saturated acids of high molecular weights on one hand and that of the glycerides of either unsaturated acids or acids of low molecular weights on the other hand, decides the comparative assimilability and digestibility of any given oil or fat.

Taking into consideration the above information the butter fat of sheep and buffalo are examined and the results obtained are compared to each other from the point of view of assimilability and digestibility.

## SECTION I

### **Comparative merits of sheep butter fat and buffalo butter fat as a human food material.**

The buffalo butter fat or ghee is commonly used as a food material in India and therefore the comparative merits of butter fats obtained from cow and buffalo are well studied, with a view to see which of the two animals deserves greater attention and care. But while studying the sheep milk and its products, it is brought to notice that during the lambing season of sheep, the milk supply is abundant and at many places the butter fat obtained from sheep milk is brought to the market either mixed with cow or buffalo ghee. Therefore, to know the value of the sheep butter fat as a human food material, it is compared with the butter fat of buffalo.

Table No. (1) gives the analytical results of the two butter fats under consideration.



TABLE No. 1

**Analytical Values of Sheep and Buffalo Ghee.**

Properties.	Sheep ghee.	Buffalo ghee.
Sp. Gravity	0.9132	0.8977
Melting point	29.9	37.2
Saponification Value	254.1	231.3
Acid Value	1.036	0.63
Iodine Value	34.75	28.06
R. M. Value	23.26	30.02
Polenske Value	6.54	2.216
Percentage of Liquid		
Acids	34.92	31.7
Percentage of Solid acids	50.02	43.33

**Comparison of buffalo-ghee and sheep-ghee:—**

The analytical results show that the saponification value, Polenske value and iodine value of the sheep ghee sample are slightly higher than those of buffalo ghee; on the other hand R. M. value of buffalo butter fat is much higher than that of sheep butter fat. The difference in R. M. value is 6.76 and as the R. M. value represents the presence of most easily digestible volatile soluble fatty acids in fat, it would naturally be a most important factor to be considered while comparing the merits of the two butter fats. As the R. M. value of buffalo ghee is much higher than that of sheep ghee it is obvious that it contains higher percentage of easily assimilable fatty acids. The iodine value and Polenske value differ by 6.69 and 4.324 respectively. The presence of liquid acids in sheep ghee is only 3.22% more than in buffalo ghee, while the difficultly digestible saturated fatty acids with high molecular weights are about 7% more in sheep ghee than in buffalo ghee. The Polenske value proves that the volatile insoluble acids like lauric and myristic acids are present in higher proportion in sheep ghee.

From the above information it can be concluded that the percentage of volatile soluble acids being greater in buffalo ghee it is naturally more easily digested. The proportions of liquid acids in two butter fats under consideration differ very slightly and hence do not count much in comparison, but the sheep ghee is found inferior due to the presence of the glycerides of difficultly digestible solid fatty acids in comparatively higher proportion.

The results of hydrolysis of the two butter fats obtained from sheep and buffalo by the enzyme lipase are given later and they indicate that the buffalo butter fat is hydrolysed easily and to a greater extent than the butter fat of sheep.

## SECTION II

### Study of the hydrolytic rancidity of sheep - ghee and buffalo - ghee.

The subject rancidity i. e. the development of an acrid taste and unpleasant odour in oils and fats, rendering the same unfit for edible purposes, has attracted the attention of chemists for a very long time. It has been studied particularly with an attention to the decomposition of oils and fats on keeping. But even though numerous explanations have been put forward from time to time the subject is still kept somewhat vague.

In the work so far done in this field, it is observed that the preliminary values and physico - chemical properties of fat, under experimental conditions are noted after a long interval of keeping and then the conclusions are drawn with regard to rancidity. Very little is known about the changes in various preliminary values of fat, such as acid value, iodine value etc. occurring during different intervals of keeping. Taking into consideration this fact the work on rancidity of sheep ghee and buffalo ghee was undertaken. Acid values and iodine values of various ghee samples, kept under different physical and chemical conditions were determined at an interval of one

month. Variations in acid values indicated the changes in free acidity of the ghee samples at different intervals of keeping, while the variations in iodine value indicated the changes which occurred in unsaturated fatty acids of ghee. Both these factors, free acidity and proportions of unsaturated acids in glycerides are important from the point of view of rancidity.

#### (A) The Effect of Aeration on Rancidity.

The oxidation of the unsaturated fatty acids or their glycerides is the most common form of rancidity. To study the effect of oxygen on buffalo ghee a fresh sample of ghee, about 300 grams was kept in a conical flask and air was bubbled through it for 30 minutes every day. The process was continued for six months and the acid value and iodine value of the ghee sample were determined at an interval of one month.

Before passing air, the container was warmed in a water-bath as the process of air-passing was easier in melted ghee. The appliance of vacuum suction pump was used for air passing. A blank experiment was conducted side by side. 300 grams of the same ghee were preserved in another bottle and the acid value and iodine value of that sample were determined at an interval of one month. This sample received the same treatment, except air passing.

The acid values and iodine values are given in Tables Nos. (2) and (3) in comparison with the blank experiment conducted side by side.

TABLE No. 2.

#### The change in acid value of buffalo ghee due to Aeration.

Interval in months.	Blank	Air-Passed Sample
0	0.517	0.517
1	0.64	0.616
2	0.651	0.653
3	0.683	0.770
4	0.706	0.848
5	0.924	0.866
6	0.95	0.929

TABLE No. 3

**The Change in Iodine Value of Buffalo-ghee due to Aeration.**

Interval in months.	Blank	Air-passed Sample
0	26.46	26.46
1	26.77	27.02
2	26.58	26.56
3	26.14	25.89
4	25.82	25.23
5	25.11	24.58

We find that in the blank experiment the increase in acid value during the period of six months was 0.433, while in the case of the ghee sample through which air was passed regularly, the increase was 0.412. When the butter fat is aerated it does not show as much acidity as when it is not aerated. The change in acid value was maximum during the third month. For the first few months the increase was practically identical in the blank and the experimental samples, but during the third month the increase in acid value of the experimental sample was greater. In the blank sample the acid value changed from 0.651 to 0.683 during the third month of keeping and in the sample, through which air was passed, the change was from 0.653 to 0.770. The appearance of ghee changed during this period. It turned viscous and lost the flavour completely. The colour changed from white to yellow and the crystalline condition was lost. It had an irritating smell of rancid fat.

The changes observed in iodine value of the aerated sample were very interesting. It slightly increased during the first month and then there was a regular fall for the rest of the period of keeping. After five months the iodine value of the aerated sample of ghee showed a fall of 1.88 while in the blank experiment it was 1.22.

Similar experiments of aeration of oils, viz. linseed, safflower and coconut-oil, were conducted in the Wadia College Bio-chemistry Department by Miss S. P. Naegamvalla. After five months the change in acid value was recorded as given in table No. 4.

TABLE No. 4

**The Change in Acid Values of the Oil Samples after Five Months' Aeration.**

Acid Value	Linseed oil	Safflower oil	Cocoanut oil
Initial Acid Value	6.30	1.48	1.39
Acid Value after 5 months	13.84	6.62	2.07

**(2) A comparative study of the composition of buffalo ghee and sheep ghee due to hydrolytic rancidity and the effect of the substances like common-salt, casein, sugar and lipase on their decomposition.**

About 100 grams of each of the ghee samples, of buffalo and sheep were taken in a number of bottles and two grams of common salt, casein (obtained from cow milk) sugar and lipase were added to each of the two ghee samples and then kept in an incubator at 37°C for four months. The acid values and the iodine values of ghee samples under experimental conditions were determined at an interval of one month. The acid value is expressed in number of milligrams of KOH required to neutralise the acidity in one gram of ghee. The iodine values were determined with a view to see whether the substances added have any effect on oxidation of unsaturated fatty acids in ghee.

The effects of the addition of common salt, casein, sugar and lipase on rancidity of buffalo ghee and sheep ghee:—

(1) Effect on Acid Values :-

In Table No. 5 and 6 the acid values of buffalo and sheep ghee samples under experimental conditions are noted.

TABLE No. 5

**Changes in Acid Values of buffalo ghee on keeping with NaCl, Casein, Sugar & Lipase**

Ghee Sample.	Months.				
	0	1	2	3	4
Blank	0.75	0.981	1.097	1.123	1.446
NaCl added	0.75	1.077	1.240	1.260	1.390
Casein added	0.75	1.239	1.250	1.386	1.694
Sugar added	0.75	1.539	1.694	1.880	1.921
Lipase added	0.75	18.672	26.180	33.120	37.710

TABLE No. 6

**Changes in Acid Values of sheep ghee on keeping with NaCl, Casein, Sugar & Lipase.**

Ghee Sample	Months				
	0	1	2	3	4
Blank	1.036	1.241	1.300	1.563	1.860
NaCl added	1.036	1.253	1.611	1.730	1.950
Casein added	1.036	1.272	1.540	1.632	1.800
Sugar added	1.036	1.320	1.631	1.722	1.976
Lipase added	1.036	10.600	16.812	21.414	25.500

(2) Effect on Iodine Value:—

In Tables No. (7) and (8) the iodine values of buffalo and sheep ghee samples under experimental conditions are noted.

TABLE No. 7

**Changes in Iodine Value of buffalo ghee on keeping with NaCl, Casein, Sugar & Lipase.**

Ghee Sample	Months.				
	0	1	2	3	4
Blank	25.61	26.77	25.58	25.14	24.82
NaCl	25.61	26.16	25.72	25.17	24.77
Casein	25.61	28.35	25.90	25.25	...
Sugar	25.61	25.84	26.32	25.83	25.01
Lipase	25.61	26.24	25.73	25.44	24.81

TABLE No. 8

**Changes in Iodine Value of Sheep ghee on keeping with  
NaCl, Casein, Sugar & Lipase.**

Ghee Sample	Months.				
	0	1	2	3	4
Blank	34.75	34.41	34.60	34.13	33.82
NaCl	34.75	34.83	32.22	32.71	32.50
Casein	34.75	36.07	34.32	33.93	33.75
Sugar	34.75	34.82	34.74	33.10	33.40
Lipase	34.75	35.68	35.11	33.52	33.48

(1) In the aerated sample of ghee, iodine value was affected more than the acid value. An appreciable decrease in iodine value proved that due to aeration the unsaturated fatty acids in ghee might be undergoing the process of oxidation. When the process of aeration was continued for five months, the general appearance of ghee was completely changed.

(2) In both, buffalo ghee and sheep ghee, common salt distinctly showed the inhibiting effect.

(3) Casein and sugar in the presence of air, moisture and light showed a slight increase in acid value but the taste and aroma were effected to a very great extent.

(4) With all the substances, NaCl, casein, sugar and lipase, in both sheep and buffalo ghee, the increase in acid value was maximum during the first month of keeping.

(5) In the case of common salt, casein and sugar, the rate of decomposition was almost constant during the last three months of keeping while with lipase, the rate decreased from month to month.

(6) Lipase was very important from the point of view of hydrolytic rancidity.

(7) Sheep ghee was turned rancid to a lesser extent than buffalo ghee with casein, sugar and lipase.

(8) The four substances can be arranged in the following order according to their effects on iodine value. (i) common salt, (ii) lipase, (iii) sugar and (iv) casein.

### SECTION III

#### Hydrolysis of Sheep Ghee and Buffalo Ghee with Enzyme 'Lipase.'

In this section it is intended to describe the work done on disintegration of different types of ghees by the action of castor seed lipase. The castor seed lipase was prepared by Tanaka's method.

#### Experimental Part.

The work on hydrolysis of buffalo and sheep ghee is divided into the following groups:—

- (1) Disintegration of buffalo ghee and sheep ghee in comparison with an edible oil, viz. linseed oil.
- (2) Comparative disintegration of
  - (i) Fresh ghee sample.
  - (ii) A ghee sample kept for 8 months.
  - (iii) A ghee sample heated to 200°C.

Before starting the work on hydrolysis, the acid value, saponification value, sp. gravity and refractive index of different ghee samples were determined. To obtain comparable results similar conditions for hydrolysing ghee with lipase were strictly followed in all cases. The procedure of the method adopted for hydrolysis of ghee with lipase:—

0.1 Gram of lipase, exactly weighed was taken in five conical flasks. One of these flasks was used for the blank



experiment and to the other four flasks 2 c. cs. of ghee sample and 10 c. cs. of phosphate buffer (4.8 pH) were added in order. The contents of the flasks were shaken and then incubated at 37°C. At the intervals of 2, 4, 24 and 48 hours, one of the flasks was taken out and the fatty acids separated due to hydrolysis of ghee were determined in terms of N/10 sodium hydroxide Solution. Before titration 20 c. cs. of redistilled rectified spirit were added to the flask and it was heated to 90°C in a water-bath.

The difference between the blank and the experimental reading gave the amount of hydrolysis that had taken place in the interval and finally the results were expressed in the percentage of fat hydrolysed during the time of reaction, taking the c. cs. of N/10 alkali required for the complete saponification of the same quantity of fat as hundred per cent. hydrolysis.

(1) Comparison of the disintegration of

- (i) buffalo ghee, (ii) sheep ghee and (iii) edible oil (linseed oil), with lipase.

TABLE No. 9.

**Hydrolysis of Buffalo Ghee with Lipase.**

Time in hours.	Titration reading.	Amount of hydrolysis in c. cs. of 0.1 N NaOH	Velocity of reaction in the interval.	% of fat hydrolysed.
0	19.1	...	...	...
2	30.4	11.3	11.3	13.2
4	37.4	18.3	7.0	21.7
24	53.6	34.5	16.2	41.2
48	63.6	44.5	10.0	53.2

**Hydrolysis of Sheep Ghee with Lipase.**

Time in hours.	Titration reading.	Amount of hydrolysis in c. cs. of 0.1 N NaOH	Velocity of reaction in the interval.	% of fat hydrolysed.
0	19.1	...	...	...
2	27.7	8.6	8.6	9.2
4	30.8	11.7	3.1	12.7
24	36.5	17.4	5.7	19.0
48	37.4	18.8	1.4	20.0

**Hydrolysis of Linseed Oil with Lipase.**

Time in hours.	Titration reading.	Amount of hydrolysis in c. cs. of 0.1 N NaOH	Velocity of reaction in the interval.	% of fat hydrolysed.
0	19.1	...	...	...
2	36.0	16.9	16.9	21.7
4	43.2	24.1	7.2	32.5
24	65.5	46.4	22.3	66.1
48	72.3	53.2	6.8	70.0

- (1) Linseed oil out of the three is most easily attacked and to the greatest extent also. Then comes in order buffalo ghee and shee ghee.
- (2) The percentage hydrolysis in 48 hours is respectively 70%, 53.2% and 20% in the above three cases.

**(2) Comparative hydrolysis of**

- (i) Fresh ghee sample.
- (ii) Ghee sample preserved for 8 months.
- (iii) Ghee sample heated to 200°C for half an hour with the enzyme 'lipase'.

TABLE No. 10.

**(i) Hydrolysis of a Fresh Ghee Sample.**

Time in hours.	Titration reading.	Amount of hydrolysis in c.cs, of 0.1 N NaOH.	Velocity of reaction in the interval.	% of fat hydrolysed.
0	19.1	...	...	...
2	30.4	11.3	11.3	13.25
4	37.4	18.3	7.0	27.7
24	53.6	34.5	16.2	41.2
48	63.6	44.5	10.0	53.3

**(ii) Hydrolysis of Ghee Sample Preserved for 8 Months.**

0	19.1	...	...	...
2	30.5	11.4	11.4	13.2
4	35.4	16.3	4.9	19.3
24	55.2	36.1	19.8	43.1
48	63.0	43.9	7.78	52.5

**(iii) Hydrolysis of Ghee Sample Heated to 200°C.**

0	19.1	...	...	...
2	27.0	7.9	7.9	9.6
4	29.8	10.7	2.8	13.1
24	39.1	20.0	9.3	23.5
48	40.2	21.1	1.1	26.1

From the figures in Table No. (10) we see that:-

The first two samples viz. fresh ghee and a ghee sample preserved for eight months were hydrolysed to the same extent in first forty-eight hours. The percentages of hydrolysis at the end of the last interval i. e. after 48 hours were respectively 53.3 and 52.5 for the first two samples and only 26.1% of the ghee sample heated to 200°C. was hydrolysed during that interval.

It clearly showed that heating had definitely some effect on the glycerides of volatile and insoluble fatty acids.

## PAPER XIV

**“A COMPARATIVE STUDY OF GOAT - GHEE  
AND COW - GHEE”**

BY

M. M. THATTE, M.Sc.

*(With the kind permission of the authorities of the Bombay  
University)*

## SECTION I

**A comparative study of goat-ghee and cow-ghee with  
respect to preliminary values.**

It is well-known that ghee forms an important and necessary nourishing food article. On account of its high nourishing value, it becomes an important constituent of daily food and hence it is consumed by many people who can afford to have it.

In our experiments the process followed to obtain ghee was the one that is followed in local areas. Milk was slowly heated to get almost all fat floating on the surface of the milk in the form of thick layer. This thick layer together with some quantity of milk was poured in one clean vessel and was subjected to the process of curdling by adding small quantity of butter milk. After two days it was churned after adding about equal quantity of water. When cream was removed for butter preparation, the quantity of milk left behind was also subjected to the same process and these two lots of butter were mixed together and washed with water for a number of times.

Butter was transformed into liquid ghee by melting. The liquid ghee became viscous after 3 to 4 hours. Every sample was cent per cent. genuine and every process was done under our personal supervision and with direct help.

The methods employed for the determination of several physical and chemical values of the samples under study are mostly taken from the standard books on dairy chemistry among which 'Vegetable fats and oils' by Jamieson and 'Chemical technology and analysis of oils, fats and waxes' by Lewkowitsch may be mentioned.

Goat-ghee was prepared from milk of 'A' goat. A second sample of goat-ghee was obtained from Sanand. The live-stock expert was requested to supply a genuine sample of goat-ghee from Gujrath and the sample sent by him was studied at Poona in Nowrosjee Wadia College, to see if the goat-ghee from Gujrath side differs in any way from the goat-ghee from Maharashtra. The following table gives the preliminary values of Maharashtra goat ghee and Gujrath goat-ghee.

TABLE No. 1

## Preliminary values of two samples of goat-ghee.

Observation No.	Analytical value	Maharashtra goat-ghee	Gujrath goat-ghee
1	Melting point	23.8°C	30°C
2	B. R. Reading at 40°C	40.5	42
3	ND at 40°C	1.4527	1.4538
4	Acid value	1.086	1.059
5	Sap. value	243.3	236.5
6	Iodine value	25.87	31.47
7	R. M. value	24.51	28.65
8	Polenske - value	9.538	9.549
9	M.P. of insoluble acids	40.5°C	44 2°C
10	Neutralisation value	237.9	212.5
11	Mean molecular weight	235.9	263.9

The above table shows that the melting point, B. M. value, iodine value and mean molecular weight of Maharashtra goat-ghee are lower than those of Gujrath goat-ghee. The sap. value is higher in Maharashtra goat-ghee but the Polenske value and acid value are practically the same in both these samples. The Polenske value is considerably high in both the samples.

When freshly prepared ghee is kept in a vessel, especially in summer, it is separated in three layers. The topmost layer is yellowish oily liquid. At the bottom of the vessel white thick and semi solid particles of ghee are settled. The intermediate portion consists of semi-liquid layer of ghee. It was thought that these three layers may show some difference in their preliminary values. These three layers in goat-ghee were studied separately with a view to find out the range of variations in them.

Goat-ghee from 'A' goat was kept in a 250 c.c. cylinder with stopper. After about 5 to 6 days, the liquid layer was pipetted carefully. The remaining portion was semi-solid. The usual sample was supposed to be the mixed layer or intermediate layer. The following table gives the range of variations in these three layers.

TABLE No. 2  
The variations in goat-ghee layers.

Obs. No.	Analytical value	Liquid layer	Mixed layer	Thick layer
1	Melting point	13 6°C	23.8°C	34.1°C
2	B. R. reading at 40°C	40.1	40.5	40.6
3	ND at 40°C	1.4525	1.4527	1.4528
4	Acid value	1.077	1.086	0.9988
5	Sap. value	247.5	243.3	242.4
6	Iodine value	27.56	25.87	25.17
7	R. M. value	28.62	24 51	24.13
8	Polenske value	8.948	9.538	9.616
9	Melting - point of insoluble acids	40.8°C	40.5°C	40.0°C
10	Neutralisation value	233.4	237.9	241.8
11	Mean molecular weight	240.4	235.9	231.9

The figures from the above table show that the melting point of liquid layer is lowest and that of the thick layer is highest while that of the mixed layer lies between these two values.

In the case of B. R. reading there is practically very little difference. There is no considerable difference in the free acid value of these three layers. The sap. value of the liquid is the highest one while that of the thick layer is the lowest one and that of the mixed layer is the intermediate one. The sap. value of the liquid layer differs from that of the mixed layer by 4.2 and that of the mixed layer differs from the value of thick

layer by only 0.9. This shows that in this case the mixed layer is nearer to the thick layer, than to the liquid layer.

The liquid layer has the highest iodine value. The difference in this value of the remaining two layers is not considerable. The iodine value of the liquid layer is greater by 1.69 than that of the mixed layer. In this respect also the mixed layer is nearer to the thick layer, than to the liquid layer.

The R.M. value is distinctly higher in the liquid layer, than that in the mixed layer. The difference of this value between the liquid layer and the mixed layer is 4.11. The R. M. value of the mixed layer is slightly greater than that of the thick layer i. e. by 0.38 only. In the case of polenske value the order is changed. The polenske value of the liquid layer is lowest and that of the thick layer is slightly higher than that of the mixed layer. The neutralisation value of the insoluble acids goes on increasing from the liquid layer through the mixed layer towards the thick layer.

In our later work it is shown that the liquid layer is more hydrolysed than the mixed layer and the mixed layer is more hydrolysed than the thick layer. The values of maximum hydrolysis after 48 hours are 36.42; 34.53 and 32.95 respectively. If hydrolysis is taken as a measure of digestibility then it can be said that the liquid layer is more easily digestible than the mixed layer while the mixed layer is more digestible than the thick layer. Hence it seems that the liquid layer is superior to the remaining two layers in this respect.

From the above discussion it is clear that the mixed layer has all these values which are intermediate between the values of the other two layers.

## (2) Comparison of goat-ghee with cow-ghee.

Goat-milk and cow-milk do not differ from each other very much in their proportions of the constituents. It is, therefore, worth comparing ghee samples, milk products, obtained from goat-milk and cow-milk. Goat ghee for comparative study was obtained from the milk of 'A' goat and cow ghee was obtained from a cow specially kept for this purpose. A general comparison only in preliminary values was made. The following



table gives the ranges of variations in the preliminary values of these two samples. (See table No. 3 below).

The figures from table No. 3 show that:—In goat-ghee the melting point, B. R. reading, free acidity and iodine value are distinctly lower than the corresponding values in cow-ghee. In melting point the difference is of 15.6°C and in iodine value the difference is of 6.7. The difference in acid value is about 0.54. The Sap. value, the R. M. value, the Polenske value and the neutralisation value of the insoluble acids all of them are higher in goat-ghee. In Sap. value this difference is of 33.3 and in R. M. value it is of 1.6. But in Polenske value the difference is very high. This is about 7.8. In our later work it is shown that cow-ghee is more easily hydrolysed than goat-ghee. All these results go to show that goat-ghee and cow-ghee differ from each other in their preliminary values.

TABLE No. 3.  
Preliminary values of goat-ghee and cow-ghee.

Obs. No.	Analytical value	Goat-ghee	Cow-ghee
1	Melting point ...	23.8°C	39.4°C
2	B. R. reading at 40° ...	40.5	41.7
3	ND at 40°C ...	1.4527	1.4536
4	Acid value ...	1.086	1.62
5	Sap. value ...	243.3	210.0
6	Iodine value ...	25.87	32.58
7	R. M. value ...	24.51	22.90
8	Polenske value ...	9.538	1.747
9	Melting point of insoluble acids	40.5°C	42.3°C
10	Neutralisation value ...	237.9	213.8
11	Mean molecular Wt....	235.9	259.4

## SECTION II

**Rancidity in Goat-Ghee and Cow-Ghee**

Fats which have developed an objectionable odour and taste on keeping are said to be rancid. Edible oils and fats are liable to become rancid in course of time if special precautions are not adopted.

Lewkowitsch defines, as rancid, those oils and fats the free fatty acids of which have been oxidised by the oxygen of the air in the presence of light. On the other hand Wanger found that fats kept in an atmosphere of nitrogen for two years and exposed to light become rancid and concluded that light is the determining factor in the development of rancidity. D. C. Dyer (*Jour. Agri. Res.* 1916; 6; p.p. 927) analysed the air contained in butter before and after cold storage and found that oxygen content decreased and the carbon dioxide content increased indicating oxidation changes in the fat. The quantity of carbon dioxide found was directly proportional to the amount of non-fatty material present in butter and the conclusion was drawn that the unpleasant flavours of rancid butter are produced by oxidation not of the fat itself but of the non-fatty ingredients.

The rancidity of pure fats is now generally understood to be due to a product of hydrolysis and oxidation and it is usually characterised by the loss of natural sweetness and flavour of the fat, the formation of compounds of unpleasant odours and taste and a development of acidity in the fat. However, the acidity of a fat is not a perfect measure of its rancidity or non-rancidity for it is possible to have examples of fats showing extremely low acidity and yet being very rancid; whereas other cases may occur in which fats show a much higher free fatty-acid content and yet are perfectly sweet and non-rancid.

Rancidity is of various types :—(1) Simple hydrolysis of glycerides into free fatty acids. (2) Oxidation of the saturated fatty acids with the production of ketones. (3) Oxidation at the double bonds of the unsaturated neutral glycerides or unsaturated fatty acids. The first two types are

almost exclusively the result of enzyme action while the last is usually due to exposure to air and may also be influenced by the presence of enzymes.

The first type of rancidity which may be called "hydrolytic rancidity" is caused by exposure of fats to the moisture in presence of lipolytic enzymes. In seeds lipase is always present and consequently seed - fats are very prone to develop free acidity if they are kept under damp conditions. Adipose tissue fats of animals, on the other hand, are not so liable to become hydrolysed. Hydrolytic rancidity of such fats when it occurs, is usually due to the growth of moulds (*penicillium Aspergillus*) which contains lipolytic enzymes in abundance.

Dr. N. N. Godbole ("Butter fat"—1939) from the results of a number of experiments has stated that diffused sun-light as such alone is not sufficient to produce rancidity. It plays a part of a positive catalyst. He states that micro-organisms, enzymes or bacteria cannot be held responsible as initial starters of the process of rancidity. Of course, once the game has been started by moisture, then air, enzymes, micro-organisms and bacteria may act as positive catalysts. The process of rancidification is accelerated very much by the presence of free fatty acids and also by the addition of previously rancid fat. Another important point noted by Bavis (J. S. C. I. 417 T; 1923) is that the presence of free oleic acid increases the rate of the compounds causing kreis reaction.

For stabilising the fats or for inhibiting rancidity of the fats, common salt has been employed by Dr. N. N. Godbole. This salt has the preservative action. Aromatic amines, non-hydroxy aromatic nitro derivatives are frequently employed for inhibiting rancidity.

In our work, it was intended to study the hydrolytic rancidity, oxidation at the double bond and their inhibition in goat - ghee and cow - ghee. Five wide mouth bottles were filled with goat - ghee. They were labelled as 1, 2, 3, 4 and 5. Ghee in bottle No. 1 was mixed with castor - seed lipase (2%) Ghee sample in bottle No. 2 was mixed with cane sugar (2%).

That in bottle No. 3 was mixed with common - salt (2%) and casein (2%) was added to that in bottle No. 4 while ghee sample in bottle No. 5 was kept for blank experiment. These bottles were kept open in an incubator at 37°C.

The acids produced by hydrolysis on keeping and by the addition of above substances were determined by dissolving about 5 grams of the sample separately from each bottle, in neutral alcohol and titrating the whole bulk against N/10 alkali. Iodine values of all these samples were also found out with a view to see whether the addition of the above substances has any effect on oxidation at the double bond. The hydrolytic rancidity and the iodine values were determined after every month in the case of goat-ghee. The following table gives the results of the hydrolytic rancidity. Figures represent c.c. of N/10 NaOH required to neutralise the free fatty acids present in one gram of the sample.

TABLE No. 4

## Hydrolytic rancidity in the case of goat-ghee.

Days	Blank	Lipase	Sugar	Common Salt	Casein
0	0.1888	0.1888	0.1888	0.1888	0.1888
31	0.2255	3.701	0.2211	0.2639	0.2608
61	0.2444	4.658	0.2442	0.2618	0.2608
90	0.4840	6.658	0.4204	0.4986	0.4753
122	0.8720	8.258	0.6441	0.8081	0.7903

The figures from the above table show that:—

In the beginning i.e. for first 61 days the acidity that is produced is not appreciable; but later on it goes on increasing and after 122 days it is considerable. For the first 61 days

addition of cane sugar shows no sign of inhibition of hydrolytic rancidity. But later on it shows retarding effect. Common salt and casein have no effect up to first 90 days but afterwards they show a little inhibition of hydrolytic rancidity. Addition of lipase decomposes goat-ghee without any regularity.

If these substances are arranged in descending order of hydrolysing action the arrangement will be as follows:- Lipase, common salt, casein and sugar.

The following table shows the effect of these substances at the double bond. The figures represent the iodine values of the different samples to which different substances were added.

TABLE No. 5

Days	Blank	Lipase	Sugar	Common salt	Casein
0	31.47	31.47	31.47	31.47	31.47
31	31.89	31.71	31.30	31.23	31.19
61	30.07	29.93	29.94	29.98	29.52
90	27.68	28.44	28.54	27.87	27.73

The figures in the table show that:- After 90 days there are appreciable differences in iodine values. After 31 days in the case of blank experiment and in the lipase added sample there is a slight increase in iodine value. Common salt and casein do not affect the double bond. Lipase and sugar have a slight inhibiting action. The difference in the iodine value in the blank experiment after 3 months is 4.79.

In the case of cow-ghee similar experiments were carried out. But there were six bottles. The sixth one contained cow-ghee mixed with rancid-ghee (2%).

TABLE No. 6

**Hydrolytic rancidity in the case of cow-ghee.**

Days	Blank	Lipase	Sugar	Common salt	Casein	Rancid ghee
0	0.2887	0.2887	0.2887	0.2887	0.2887	0.2887
59	0.3718	2.561	0.3621	0.3713	0.3591	0.4531
93	0.4051	3.558	0.3963	0.4141	0.3649	0.5168
124	0.4364	4.205	0.4336	0.4325	0.3824	0.5279
153	0.7246	4.917	0.6573	0.6219	0.5717	0.7688

From the above table we find that in the blank experiment there is no appreciable change in the free acidity for the first four months, but in the fifth month there is a considerable change in the free acidity. Rancid ghee helps hydrolysis in the case of cow-ghee. Sugar and common salt have no effect in the early stages of experiment under the experimental conditions, but later on they show inhibiting effect. Casein under these conditions retards decomposition from the very beginning. If these substances are arranged in descending order of hydrolysing action, the order is as follows :- Lipase, rancid ghee, sugar, common salt and casein. In the sample where lipase was added rate of decomposition of the fat in the beginning is higher and then it slowly falls down.

If we look to the figures in the blank experiments both in goat-ghee and cow-ghee we see that goat-ghee is more prone to become rancid only if hydrolytic rancidity is taken into consideration. In the second part of this paper it is shown that cow-ghee is more hydrolysed by lipase than goat-ghee. But the acidity developed in the sample to which lipase was added was more in goat-ghee than in the cow-ghee. This may be due to deterioration of the lipase powder kept for a long time in contact with ghee.

As in goat-ghee samples, the iodine values of cow-ghee samples were found out and are given in the following table :-

TABLE No. 7

**Changes in the iodine values of cow-ghee samples.**

Days	Blank	Lipase	Sugar	Common salt	Casein	Rancid ghee
0	32.58	32.58	32.58	32.58	32.58	32.58
59	32.88	32.99	32.78	32.63	32.76	33.06
93	32.28	32.12	31.98	31.85	32.15	31.63
124	30.56	29.97	31.10	30.84	31.77	29.86

The figures from the above table show that after 124 days there are appreciable differences in the iodine value. After 59 days in all cases there is a slight increase in iodine value. After 124 days the sample in which rancid ghee was added has iodine value lower than that of any other sample. Here again it shows that casein has inhibiting action. The difference in the iodine value in the blank experiment after four months is 2.02.

If these substances are arranged according to their effect at the double bond the arrangement may be as follows :- Rancid ghee, lipase, common salt, sugar and casein. Addition of common salt, sugar and casein inhibits oxidation at the double bond. There is no great difference in iodine value of the lipase added sample and that of the blank experiment. This indicates that lipase does not affect the double bond in its action of hydrolysis of the fat under our experimental conditions. Up till now only two properties of lipase are known (1) hydrolysis (2) synthesis. The oxidation at double bond or the breaking up of the double bond by lipase is not remarkably indicated. If lipase had been acted upon the

double bond, then there would have been a considerable difference in iodine value.

If we look to the figures of iodine values in the blank experiment both in goat-ghee and cow-ghee we find that goat-ghee is more prone to the effect of oxidation of unsaturated glycerides or unsaturated fatty acids.

Air is one of the factors in producing rancidity. Samples exposed to open air become rancid after a few months. Effect of aeration must, therefore, be marked in such samples. In our experiment we studied the effect of aeration on cow-ghee. Every day melted cow-ghee was subjected to aeration for 15 to 20 minutes. Aeration was carried out mechanically with the help of suction pump. The flask containing cow-ghee was fitted with a rubber cork having two glass tubes open at both ends. One end of one tube was dipped in cow-ghee and the end of second tube was kept above the surface of ghee. The second tube was joined to the suction pump. Atmospheric air was thus allowed to bubble into the melted ghee sample. Shaking was also continued from time to time at the time of aeration. Acidity and iodine value were found out at different intervals of days. Sample of the same ghee was kept aside to carry out blank experiment. The following table gives the iodine values and the number of c.c. of N/10 NaOH required to neutralise the free fatty acids present in one gram of the sample of ghee. (See table No. 8 on the next page).

The figures from the table (No 8) show that the free acidity in the blank experiment increases very slowly. In the aerated sample for the first 133 days it increases slowly but after that period it increases rapidly. In the case of iodine value in the blank experiment there is a decrease in the first 30 days and then up to 165 days it remains practically constant. But from 165 days to 225 days there is a noticeable decrease in iodine value. In the aerated sample the fall in iodine value is started from the beginning and total fall in 225 days is 5.21 while that in the blank experiment is 2.43. The temperature at which aeration was carried out was not high. The temperature was at about the melting point of ghee. Hence it can be



said that aeration affects the sample and thereby differences are found in acid value and iodine value showing the decomposition of the glyceride and the oxidation at the double bond.

TABLE No. 8.

## Results showing the effects of aeration.

Days	Blank		Aeration	
	C.C. of NaOH	Iodine value	C.C. of NaOH	Iodine value
0	0.0956	33.32	...	...
30	0.1213	32.43	0.1237	32.27
70	0.1375	32.47	0.1445	31.83
103	0.1555	32.23	0.1629	30.83
133	0.1592	32.19	0.1858	29.97
165	0.1658	32.15	0.2818	29.50
196	0.1677	31.41	0.4200	28.01
225	0.1832	30.89	0.5076	27.11

In similar way aeration was carried out in cocoanut oil, safflower oil and linseed oil by Miss Naegamvala in our laboratory. She found that the effect of aeration in these oils is not marked in the beginning but later on the effect is remarkable. The effect is the greatest in linseed oil and a slight effect is seen in cocoanut oil. Linseed oil-drying oil-becomes viscous earlier than safflower oil-semi drying oil.-cocoanut oil-non drying oil-does not become viscous.

Hence it can be concluded that aeration affects the oil or fat and the effect is more pronounced when the oil is a drying one.

## PAPER XV

## "HYDROLYSIS OF A FEW FAT-SAMPLES"

BY

M. M. THATTE, M. SC.

*(With the kind permission of the authorities of the Bombay University)*

## SECTION I

**A study of hydrolysis of goat-ghee and cow-ghee in comparison with a few edible oils and vegetable ghee by castor-seed lipase.**

It has been observed and proved that oil-seeds contain an enzyme 'lipase' which by its virtue of lipolytic activity brings about hydrolysis of the oils on keeping.

The freshly extracted oils from the crushed seeds contain very negligible amount of acid, but on keeping for some days appreciable quantities of free acid can be detected and the amount tends to increase gradually as the period increases. The decomposition of the oil into acid and glycerol has been attributed to the presence of a ferment which has been termed "lipase".

It is well known that in the alimentary canal of the higher animals, glycerides undergo hydrolysis by the catalytic agency of a fat-splitting enzyme 'lipase' secreted by pancreas. The breaking down of fats by this agent is greatly accelerated by the physical action of the bile salts—sodium glycocholate, sodium taurocholate and bile pigments—Bilirubin and Biliverdin. These by their marked power of lowering surface tension tend to increase the dispersion of the oily droplets and to give rise to emulsions. The hydrolysis of the glycerides starts as soon as the fatty acid molecule enters the living cells of the adsorbing surface. The adsorption of the majority of the edible oils and fats is almost quantitative i.e. values ranging

from 95% to 100% are obtained for the digestibility coefficients. Lower values have been recorded for some fats, more particularly in cases where the melting point approaches or is higher than the body temperature so that the emulsification of the fatty globules in the intestinal contents may possibly be less satisfactory than in the case of oils (oils, fats and fatty foods J. C. Brumond, Professor of Biochemistry, University of London).

Numerous methods have been devised for obtaining a product with a hydrolysing power greater than that of the crushed seeds. All these methods consist in removing from the seeds the materials such as oil and albuminoid substances devoid of hydrolysing properties.

1. Method of Nieloux (C. R. 1904, 138; 1112, 1175, 1288, 1352)
2. Hoyer's extraction method (Ber. 1904, 37, 1436)
3. Taylor's extraction method (Jour. Biol. Chem. 1906, 2, 87)
4. Armstrong and Ormerod's extraction method  
(Proc. R. S. 1906, B.78; 376)
5. Falk's method (Jour. Am. Chem. Soc. 1912, 34, 735; 1913, 35, 624, 1897)
6. Tanaka's method (Eighth Int. Congress of Applied Chem. 1912, Sec. Vol. 11, 37; J.S.C.I. 1912, 31; 884)

For the present work we adopted Tanaka's method. *Tanaka's method* :—A necessary amount of fresh seeds was purchased locally and after decortication these were ground finely in a mortar for a considerable period. The whole mass was packed in different packets of ordinary filter paper. The above packets were dipped in ether for three days to remove oil from finely ground mass. The last traces of oil were removed by Soxhlet method. The packets were opened and the contents were exposed to open atmosphere for some time to remove ether. The contents were then passed through a fine sieve. 100 grams of this product were triturated with 600-700 c.c. of 0.1 N acetic acid at 30°-35°C for 30 minutes. The milky liquid was filtered and the residue thoroughly

washed with water till free from acidity and dried at a temperature not exceeding 40°C. The dried product was again ground in mortar and passed through a fine sieve. The lipase powder thus obtained was white, odourless and quite free from soluble matter. The lipase powder thus obtained was preserved in glass stoppered bottle with the addition of a few drops of toluene. Care was taken not to allow the lipase powder to be dried in the sun because such an exposure spoils the activity of the lipase powder.

The researches of Folk (Jour. Am. Chem. Soc. 1913, 35, 1904) and of Folk and Sognira (ibid. 1915, 37, 217) show that castor-beans contain two distinct lipolytic enzymes. One of these is soluble in water and has a comparatively greater hydrolysing effect on esters like ethyl butyrate than on glycerides like triacetin. The other is insoluble and is more active towards triacetin than towards ethyl butyrate. The soluble enzyme is termed as 'esterase' and the insoluble as 'lipase.'

The optimum pH of the castor-bean lipase has already been found out and is between 4.7 to 5. In our work we had used phosphate buffer with pH 4.8

To set up the necessary conditions certain preliminary experiments were carried out. The first experiment was carried out to fix the amount of lipase to be used every time. An increase in the amount of lipase accelerates the hydrolysis but the velocity of decomposition is by no means proportional to the amount of lipase used, as small quantity acts relatively more energetically than larger (Cf. Fokin J. S. C. I. 1904, 23, 1152).

In our experiments we had taken from 0.1 gram to 0.8 gram of lipase powder in eight different flasks and found that the amount of hydrolysis goes on increasing as the quantity of lipase goes on increasing. But the velocity of decomposition is not proportional to the quantity of lipase used. 0.1 gram of lipase acted more energetically than the larger quantities. Hence it was thought best to use every time 0.1 gram of lipase powder.

Neutral alcohol is a solvent for fatty acids originally present and those which are obtained after hydrolysis. We had carried two sets of experiments. In one set 10 c.c. of absolute alcohol were added in the beginning when the flasks were incubated and in another set 10 c. c. of absolute alcohol were added at the end of the reaction and in their place 10 c.c. of distilled water were added at the time of setting the experiment. The results of these two sets of experiments undoubtedly showed that alcohol acts as a deterrent to the working of castor-seed lipase. Hence alcohol was added at the end of the reaction every time.

The yield of hydrolysis depends upon the formation of a good emulsion. The results of the experiments of Constein, Hoyer and Wartenberg pointed out that the amount of water compared with the quantity of lipase and oil plays an important part in the rate of hydrolysis. They stated that at least three times the theoretical amount of water should be used. Hence instead of adding water, only necessary amount of buffer solution was used. We tried different quantities of buffer solution and arrived at a conclusion that if 6 c. c. of buffer solution are used practically constant results are obtained.

These preliminary experiments established the necessary conditions to give maximum hydrolysis. In each case 0.1 gram of lipase and 2 c. cs. of ghee were well mixed and then to that mixture 6 c.cs. of phosphate buffer ( $pH$  4.8) were added and the whole mixture was again shaken for one minute. The flasks (100 c. cs. capacity) were then incubated at  $37^{\circ}C$  for the required period and were well shaken after every half an hour during first four hours.

At the end of the reaction, after a certain period, 10 c. c. of neutral alcohol were added into the flask and the contents of the flask were heated to  $70-75^{\circ}C$  for one minute. The addition of alcohol and heating the flasks served two purposes. Alcohol dissolved the separated fatty acids and the activity of lipase was stopped after heating. The contents of the flasks

were then titrated against standard alkali. Free acidity and Sap. value of ghee were found out on the same day of the experiment.

On account of shortage of absolute alcohol or even rectified spirit we used distilled methylated spirit in our actual experimental work to dissolve the fatty acids. The small 100 c. c. flasks were not suitable for titration where 35 or 40 c. c. N/10 NaOH were required. Hence a new procedure was adopted in which the contents of the small flasks were mixed with 10 c. c. of neutral distilled spirit and the flask was kept in boiling water for one minute. The contents of the flask were poured into a 250 c. c. conical flask and the small flask was washed twice using every time 10 c. c. of neutral distilled spirit. The washings were added to the contents of the conical flask and the whole mixture was titrated against N/10 NaOH using phenolphthalein as an indicator.

In our work all the experiments were carried on a small scale and no shaking machine nor mechanical stirrers used and hence the results were low.

In India a very important question has always been raised and discussed regarding the comparative merits of butter fats, (obtained from cows, buffaloes and goats) edible oils and vegetable ghees with a view to find out their comparative values as food material. The use of any oil or fat depends upon the degree of its absorbability, assimilability and digestibility. The oil or fat which loads the digestive system instead of being digested is definitely not useful to the body. Not only that but much depends on the rate of digestibility. The higher the rate of digestibility the greater the use of the fat as food material.

The glycerides are decomposed by ferment called 'lipase' into fatty acids and glycerol. These fatty acids are then absorbed in the body as they are produced. This decomposition or oil-splitting or hydrolysis depends upon the physical nature, chemical nature and also on the other ingredients present in the oil or fat. During the last war, to supply the demand of ghee, a number of factories manufacturing vegetable ghees

were erected and the sums of capital locked up in their working were so large that it appears that people may be misguided by the information based on propagandist nature.

Luehring ('Butter fat' p. p. 21-N. N. Godbole and Sadgopal) tried to ascertain the relation between the relative velocities of saponification of oils and fats and their relative digestibility. This argument of drawing an inference regarding the digestibility from the saponification velocity is open to one serious objection and that is that in estimating saponification value strong alcoholic solutions of caustic potash are used which cannot be compared with the small, though active, quantities of ferments present in the human body.

The higher fatty acids like palmitic and stearic, it is found, pass off through human system unused and unabsorbed. But if oleic acid is present, it is taken up and absorbed by the system. Not only that but its mere presence also helps partly to absorb the otherwise indigestible acids like palmitic and stearic. From this it seems that the more the oil or fat unsaturated the more it is digestible. Of course, it is only one of the factors influencing the digestibility of the oils or fats.

It is also observed that oils and fats whose melting points lie below the normal temperature of the body (about 36°C) are absorbed and digested in the intestines almost up to 97 to 98 per cent., whereas tri-stearin, having a melting point 71°C, is only 9 to 14 per cent. used up. The remaining portion only helps in loading the system. The digestibility coefficients of oils hydrogenated to different degrees of hardness are determined by A. D. Holmes and H. J. Duel (Analyst, 1921, 46 p.p. 503) with the following results. Cotton-seed oil hydrogenated to melt at 35°, 38.6°, 46°C showed digestibility coefficients of 96.8, 95.5, and 94.9% respectively. In the case of arachis oil, melting at 37, 39, 50, and 52.4 degrees centigrade, the respective digestibility coefficients were 98.1, 95.1, 92.0, 79.0 per cent. These results show clearly that digestibility depends more or less on the melting point of the fat or the oil.

The glycerides of saturated acids of low molecular weights ranging from those of butyric to those of myristic acids are known to be easily assimilated by the system because of their low melting points. To conclude we say that the digestibility of any oil or fat depends upon its melting point and unsaturated nature.

The absorption of fat from the intestine has long been the subject of controversy. The results of investigation of Munk go to show that fats are not directly absorbed but are first hydrolysed and then resynthesised into glycerides (cf. Moore. Proc. Roy. Soc. A. 1903, 72, p.p. 134), Franzer (Analyst 1938, 63, p.p. 308) states that there are essentially two ways by which fat can be conveyed from the intestine into the body, the first is via the lacteals, the intestinal lymphatics and the thoracic duct into the systemic circulation. This route avoids the liver, and the fatty material goes direct to the general body tissues. The second route is by the capillaries; thence into the portal vein and to the liver. He concludes that fat is absorbed both as fat and fatty acid. Absorption of fat favours passage by the lacteal-lymphatic route to the fat depots. Absorption as fatty acid is by the capillary pathway and material passes direct to the liver where it may be further metabolised. Lyman (Jour. Biol. Chem. 1917, 32, 7) fed dogs with glyceryl stearate and palmitate and observed great difference in absorption. He doubts whether absorption is dependent on the melting point of the fat.

Hydrolysis of goat-ghee, cow-ghee, edible oils and vegetable ghees was carried out.

*Hydrolysis of goat-ghee*:—Goat-ghee selected for this work was obtained from the milk of 'A' goat. Detailed information about its layers has been given in the previous work. The three layers were subjected to hydrolysis using 2 c.c. of ghee, 0.1 gram lipase and 6 c.c. of buffer. The flasks were incubated at 37°C for different intervals of time.

The results obtained are given in table No. 1



TABLE No. 1.

**Hydrolysis of goat-ghee layers.**

Name of layers	Percentage hydrolysis after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
Liquid ...	5.671	9.298	26.11	36.42
Mixed ...	5.442	9.156	27.34	34.53
Thick ...	5.025	9.059	26.85	32.25

All these results show that the hydrolysis is rapid during the early stages of reaction and then it slowly falls down. According to the degree of hydrolysis, the liquid layer is better than the other two layers. Then stands the mixed layer and finally the thick layer.

The iodine values of the liquid layer, the mixed layer and the thick layer are 27.56, 25.87 and 25.17 respectively. The M.Ps. are 13.6°C, 23.8°C and 34.1°C respectively. These values show that the liquid layer has the highest iodine value and the lowest melting point.

The thick layer has the lowest values while the mixed layer has the intermediate values. On examining these values we can say that taking one and the same sample of ghee and dividing it into three layers according to its liquidity the percentage hydrolysis of these layers depends on the iodine value and the melting point. The layer which has more iodine value and lower melting point is hydrolysed more than the layer which has lower iodine value and higher melting point.

**Hydrolysis of Cow-Ghee :—**

As in the case of goat-ghee the three layers of cow-ghee were subjected to hydrolysis using 2 c. c. of ghee, 0.1 gram

lipase and 6 c. c. buffer. The results obtained are given in table No. 2.

TABLE No. 2

## Hydrolysis of Cow-Ghee Layers.

Name of layer.	Percentage hydrolysis after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
Liquid ...	6.27	10.37	31.32	44.83
Mixed ...	5.34	8.45	32.71	41.66
Thick ...	2.78	6.01	18.53	28.49

The figures from Table No. 2 show that —

On the whole it seems that the hydrolysis is rapid during the early stages of reaction and afterwards it slowly falls down. According to the degree of hydrolysis the liquid layer stands first, then the mixed layer and finally the thick layer. In the liquid layer and in the mixed layer the percentage hydrolysis even at the end of 24 hours is greater than the percentage hydrolysis after 48 hours in the thick layer. The melting points of these layers are 16.2°, 39.1°C and 43.0°C respectively. The iodine values are 40.17, 36.64 and 33.39 respectively. The layer having lower melting point and higher iodine value is hydrolysed to a greater extent than the other two layers.

For comparison two edible oils and two vegetable ghees were selected. The oils were safflower and coconut. The vegetable ghees are named as 'A' and 'B' and these are in common use. Results are shown in Table No. 3.

TABLE No. 3.

**Hydrolysis of Goat-Ghee, Cow-Ghee, Edible Oils and Vegetable Ghees.**

Name of sample	Percentage hydrolysis after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
Goat-ghee ...	5.442	9.156	27.34	34.53
Cow-ghee ...	5.34	8.45	32.71	41.66
Safflower oil ...	5.37	9.001	19.73	23.99
Cocoanut oil ...	5.590	8.414	21.12	26.48
Vegetable ghee 'A'	3.256	6.357	12.01	17.99
Vegetable ghee 'B'	1.996	3.610	12.13	17.66

Here again it is seen that the hydrolysis is rapid during the early stages of reaction and then slowly falls down. After 48 hours goat-ghee is 34.53 per cent. hydrolysed, cow-ghee is 41.66 per cent. hydrolysed, safflower oil is 23.99 per cent. hydrolysed, cocoanut oil 26.48 per cent., vegetable ghee 'A' 17.99 per cent. and vegetable ghee 'B' 17.66 per cent. According to the descending order of percentage hydrolysis the order in which they can be put will be as follows:— Cow-ghee, goat-ghee, cocoanut-oil, safflower oil, vegetable ghee 'A' and vegetable ghee 'B'. The melting points of these samples are 39.1°C, 23.8°C, 25.3°C, solidifying points -18°, 39.5° and 40.2° centigrade respectively. The iodine values of these samples are 36.64, 25.87, 9.96, 141.5, 65.49 and 57.94 respectively. (The melting points and iodine values of safflower oil and cocoanut oil are quoted here with the kind permission of Miss Naegamvala and those of vegetable ghees are quoted with the kind permission of Mr. V. S. Kulkarni who worked in the Nowrosjee Wadia College Biochemistry

Laboratory). The results show that the percentage hydrolysis of oils and fats does not depend only on melting point and iodine value. But if the same sample is divided into different layers according to their liquidity then the percentage hydrolysis depends upon, but not proportional to, the melting point and iodine value.

These results show that the vegetable ghees are less hydrolysed than the butter fats and edible oils. Dr. V. N. Patwardhan (Lecture at Science Congress at Bangalore, 1946) revealed that vegetable ghee adversely affected growth and reproductive function of animals. Of the fats he investigated that butter fat stood out as the fat par excellence viewed from any angle—growth, maintenance, reproduction and lactation. He said, "Consume butter if you can afford, otherwise take any of the ordinary edible oils, without any treatment, as the next best".

## SECTION II

**The effect of thermal treatment on the hydrolysis of goat-ghee and cow-ghee samples by castor-seed lipase.**

Sudborough (Jour. Ind. Inst. Sci. 1918 and 1922, 2, 213; 5, 119) observed that certain crude oils after refining treatment with alkali did not undergo appreciable hydrolysis when acted upon by castor-seed lipase in absence of an activator. Addition of volatile fatty acid (which had been removed during the neutralisation and washing) as an activator was found to restore the hydrolysis of these oils. It was therefore assumed that volatile free acids present in crude oil act as a natural activator to castor-seed lipase.

Menezes and B. N. Banerjee (J. Ind. Inst. Sci. 1939) heated the crude oil and then subjected it to hydrolysis with pancreatic lipase both with and without an activator. The heated oil when hydrolysed in the absence of an activator, undergoes very poor hydrolysis. When however the activator (0.5 c. c. of 2% CaCl<sub>2</sub> plus 0.5 c. c. of 3% albumin solution) is added then the hydrolysis is restored to the original rate. This

clearly demonstrates that on heating, some substance which acts as an activator to the pancreatic lipase is rendered inactive. They also found the same effects in gingili, ground-nut and cotton - seed oils. Their results indicate a gradual fall in the hydrolysis with rise in the temperature and also with the duration of heating. The ground-nut oil was also hydrogenated and it was found that the process of hydrogenation retards the hydrolysis.

We studied the effects of thermal treatment on hydrolysis of goat - ghee and cow - ghee.

*Goat - ghee* :—A sample of genuine goat-ghee was divided into three portions. One portion was kept as it was. Another portion was heated at 160°C for half an hour over a spirit lamp. The third portion was boiled for half a minute on a stove. The free acidity and the saponification value of each of these portions were found out after one day after thermal treatment. The free acidities of the unheated sample, heated sample and boiled sample were 0.38, 0.28 and 0.14 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of each sample. The saponification values of these samples were 77.36, 77.62, 77.62 respectively in terms of c. c. N/10 NaOH for 2 c. c. of the sample. These three portions were subjected to hydrolysis under similar conditions. The results are given in the following table.

TABLE No. 4.

**Effect of thermal treatment on hydrolysis of goat-ghee**

Name of sample	Percentage hydrolysis after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
Unheated ...	3.853	6.649	18.52	22.41
Heated at 160°C for half an hour.	3.650	5.444	15.83	21.52
Boiled for half a minute	3.398	4.943	11.49	17.55

The figures in table No. 4 show that :-

The rate and also the degree of hydrolysis are the highest in the unheated sample. Then there is a fall in these values in the heated sample and in the boiled sample the rate and the degree of hydrolysis are the lowest. The explanation for the fall of the acid value can be attributed to certain volatile acids which might have been present in the ghee samples before heating or boiling and which on heating or boiling the ghee samples were driven off. On account of the fall in the acid value the hydrolysis in the heated sample or the boiled sample might have suffered.

*Cow-ghee*:-A sample of genuine cow-ghee was divided into three portions. As in the goat-ghee samples two of the three portions were subjected to thermal treatment. The free acidity and the saponification value of each of these three portions were found out after one day after thermal treatment. The free acidities of the unheated sample, the heated sample and the boiled sample were 0.909, 0.813 and 0.622 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of the sample. The saponification values were 76.05, 76.33 and 76.58 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of the sample. These three samples were subjected to hydrolysis under similar conditions. The results are given in the following table :-

TABLE No. 5.

**Effect of thermal treatment on the hydrolysis of cow-ghee**

Name of the sample	Percentage hydrolysis after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
Unheated ...	4.392	7.193	19.86	24.37
Heated at 160°C for half an hour	3.865	6.463	16.15	19.70
Boiled for half a minute	2.897	4.409	11.47	16.75

These results show that the rate and also the degree of hydrolysis are the highest in the unheated sample. There is a fall in these values in the heated sample and in the boiled sample the rate and the degree of hydrolysis are the lowest.

In the unheated goat-ghee sample the hydrolysis in the first 24 hours is 4.73 times the hydrolysis in the next 24 hours and that in the unheated sample of cow-ghee is 4.403. But in the boiled goat-ghee sample the hydrolysis in the first 24 hours is 1.89 times the hydrolysis in the next 24 hours and that in the boiled cow-ghee sample is 2.17. These figures indicate that in the unheated samples the hydrolysis is rapid in the beginning and then falls down. But in the boiled samples the hydrolysis is not so rapid in the beginning and proceeds further without much fall in its rate of hydrolysis. In the boiled goat-ghee sample the degree of hydrolysis is less by 4.89 than that in the unheated goat-ghee sample. In the boiled cow-ghee sample the degree of hydrolysis is less by 7.62 than that in the unheated cow-ghee sample.

The effect of thermal treatment on the hydrolysis of rancid ghee was studied with a view to see whether it has got the same or different effect in rancid ghee. The cow-ghee sample selected for the experiment had become rancid by keeping for two and a half years. Some quantity of rancid ghee was boiled for half a minute on a stove and cooled again. The free acidity and also the saponification value of the rancid sample and those of the boiled rancid sample were found out separately. The free acidity and the saponification value of the original rancid ghee were 9.90 and 74.74 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of the sample. These values of the boiled rancid ghee were 7.56 and 72.38 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of the sample. The difference in the free acidities and in the saponification values is appreciable. These samples were subjected to hydrolysis and the results are shown in table No. 6.

TABLE No. 6

**Effect of boiling on the hydrolysis of rancid cow-ghee.**

Name of sample	Percentage hydrolysis after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
Unboiled ...	5.165	8.191	14.53	15.57
Boiled ...	5.389	8.702	21.41	27.16

From the above figures it clearly shows that if the rancid ghee is boiled even for half a minute, the free acidity is appreciably decreased and the percentage hydrolysis is considerably increased. But Table No. 5 shows that in the case of non-rancid sample, the hydrolysis is decreased if the sample is heated or boiled. The probable explanation that can be given is that in rancid ghee there may be some volatile compounds present which may affect the hydrolysis. On boiling the sample these volatile compounds may be partially driven off and then there may not be any specific influence of these compounds on hydrolysis or on boiling, a substance which acts as a deterrent to the working of castor seed lipase is rendered active.

Another sample of rancid cow-ghee was selected for the sake of confirmation. The sample chosen was a seven year old sample and had gone rancid. The rancid sample was divided into three portions. One portion was kept as it was. The second portion was boiled for about half a minute. The third portion was boiled for about one minute. The free acidity and the saponification values of these three portions were found out separately after one day after thermal treatment. The free acidity and the saponification value of the original rancid-ghee sample were 3.640 and 74.98 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of the sample. These values of the rancid ghee boiled for about half a minute were 3.024 and 74.25 respectively in terms of c.c. of N/10 NaOH for 2 c.c. of the sample and those of the rancid ghee boiled for about one minute were 1.750 and 73.77 respectively, in terms of terms of c.c. of N/10 NaOH for 2 c.c. of the sample. The free acidity present in the sample which had become rancid by keeping for seven years was less than that present in a sample which had become rancid by keeping only for two and a half years. The above three portions were subjected to hydrolysis and the results are shown in table No. 7.



TABLE No. 7

**Effect of boiling on hydrolysis of rancid cow-ghee**

Sample	Percentage hydrolysis after		
	3 hrs.	24 hrs.	48 hrs.
Unboiled ...	4.174	10.86	11.86
Boiled for half a minute.	4.711	15.39	21.23
Boiled for one minute	4.527	16.69	23.16

Here again it is found that if rancid ghee is boiled, the free acidity is decreased and the hydrolysis is considerably increased. Figures in table No. 6 and No. 7 show that in the boiled rancid ghee sample the percentage hydrolysis even after 24 hours is greater than that after 48 hours in the unboiled sample. Also these tables show that in the unboiled samples the hydrolysis is stopped after 24 hours. But in the boiled samples it does not stop after 24 hours and continues further without much fall in the rate of hydrolysis.

## SECTION III

**The effect of certain substances on the enzymic hydrolysis of fat.**

Common salt is one of the most essential requirements of living tissues. It is mostly ingested with every kind of food-stuff in order to make the stuff tasty and palatable. Common salt, as we know, causes an increased secretion of saliva and gastric juice thereby bringing into action more quantity of enzyme to react with food stuff. Naturally there is bound to be an increased hydrolysis of food constituents. Leaving aside this property it is worthwhile to study whether the addition of salt itself has any effect on the hydrolysis of ghee keeping the quantity of enzyme lipase fixed.

Narayanmurti (Jour. Ind. Inst. Sci. 1930, 13A, 63) found that sodium chloride was inhibitive to purified cholam-malt

amylase when its concentration exceeded 0.0000166 N. Loevenhart and Pierce (Jour. Biol. Chem. 2,397, 1917) while working on animal lipase noted that dilute solution of sodium chloride had accelerating effect but retarding effect with more concentrated solution. In our experiments we used pure common salt, cow-ghee and castor-seed lipase. 200 mgms. 400 m.gms. and 600 m.gms. were weighed accurately and dissolved in 10 c.c. of distilled water separately. 0.1 gm. of castor seed lipase and 2 c.c. of ghee were well mixed. To this mixture 5 c.c. of distilled water and 1 c.c. from the above 10 c.c. solution of sodium chloride were added and the whole mixture was shaken vigorously for about one minute. Three such flasks were arranged containing different quantities of sodium chloride. Side by side a blank experiment was also run containing 0.1 gm. of lipase, 2 c.c. of ghee and 6 c.c. of distilled water. These four flasks were incubated at 37°C for 24 hours. Thus the concentrations of sodium chloride in the three flasks were 20 mgms., 40 mgms. and 60 mgms. respectively.

After 24 hours 10 c.c. of neutral absolute alcohol were added to each flask and after heating the flasks for one minute at 60 to 70 degrees centigrade the contents of the flasks were filtered using Bucher funnel and suction pump. The flasks and the funnels were washed three times using 10 c.c. of water every time. The filtrate and the washings were titrated against N/10 NaOH. The end point was sharp. The results obtained are given in table No. 8.

TABLE No. 8

**Effect of NaCl on the enzymic hydrolysis of fat.**

Salt	20 mgms.	40 mgms.	60 mgms.	Blank
c.c. of N/10 NaOH	1.93	1.61	1.06	3.31
Difference ...	-1.38	-1.70	-2.25	...

The figures in the above table clearly show that common salt has retarding effect on the enzymic hydrolysis of fat. This retarding effect goes on increasing as the concentration of the salt goes on increasing but this effect is not proportional to the quantity of the salt used.

Another substance which is frequently used up in our daily diet in an appreciable amount is sugar. Sugar as such is not eaten frequently but in solution everybody takes it.

The effect of glucose, milk-sugar and cane-sugar on enzymic hydrolysis of fat was studied. The procedure adopted was the same as in the case of common salt experiment except that instead of common salt a necessary quantity of sugar was added. The concentrations of sugar were 20 mgms., 40 mgms. and 60 mgms. and cow-ghee was used as a substrate. The flasks were incubated at 37°C for 24 hours. The results obtained are given in Table No. 9.

TABLE No. 9

## Effect of sugars on the enzymic hydrolysis of fat.

Sugar	20 mgms.	40 mgms.	60 mgms.
Glucose c.c. of N/10 NaOH.	3.76	2.71	2.71
Difference ...	+0.45	-0.60	-0.60
Milk-sugar-c.c. N/10 NaOH.	2.89	3.03	3.03
Difference ...	-0.42	-0.28	-0.28
Cane-sugar-c.c. of N1/0 NaOH.	3.40	3.67	3.31
Difference ...	+0.09	+0.36	nil
Blank - c.c. of N/10 NaOH.	3.31	3.31	3.31

From the figures in the above table we find that lower concentration of glucose has accelerating effect while higher concentration of it has retarding effect on the enzymic hydrolysis of fat. When the concentrations of glucose were 40 and 60 mgms. the retarding effect was to the same extent.

In case of milk-sugar in all types of concentrations there is retardation in hydrolysis. In case of cane-sugar when the concentration is 20 mgms. there is an acceleration in the rate of hydrolysis and when the concentration is 40 mgms. then this accelerating effect is more pronounced. But at a still higher concentration there is no effect whatsoever on the enzymic hydrolysis of fat.

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## PAPER XVI

### "A STUDY OF ASH OF MILK AND THAT OF MILK - FAT OF ASS"

By

RAJADHYAKSHYA, M.Sc.

*(With the kind permission of the authorities of the Bombay University)*

#### SECTION I

**Analysis of the ash of ass's milk and its comparison with the ash of the milk of other animals.**

A study of the ash obtained by incinerating the solids and thus removing the organic matter is very interesting. Salts of the metals like calcium, magnesium, potassium, sodium, iron, containing chlorine, phosphorus and sulphur in a combined form are found to occur in milks according to the nature of the animal and the food on which it is fed. Sulphur has been found only in the milks of goat and cow. Again

iron has been found only in the case of cow's milk while the traces of iodine have been observed in both the milks from the cow and the goat. Physiologically speaking these salts, though present in very minute quantities, play a very important part in resisting a number of diseases like T.B. and development of Tonsillitis. In this connection it should be remembered that due to the presence of these various salts of organic nature the normal milk is amphoteric in reaction i.e. it behaves both as an acid and also as an alkali towards an indicator.

Zindabad has recently observed during a thorough spectroscopic examination of milk, traces of the following metals:—

Aluminium, chromium, copper, lead, manganese, tin, titanium, zinc, vanadium. In the opinion of the investigator even traces of these metals play a very important part in imparting to the milk the best nutritive and medicinal properties.

#### **Analytical results of the ash of ass's milk.**

As previously said analysis of five samples of the ash of ass's milk was done and table number 1 will show the percentage composition of the various constituents of the ash of ass's milk.

TABLE No. 1

Sample No.	CaO	P <sub>2</sub> O <sub>5</sub>	Cl	MgO	SO <sub>3</sub>	Na <sub>2</sub> O	K.
1	32.76	41.32	17.50	2.80	3.40	1.30	trace.
2	40.18	41.05	12.20	0.79	3.62	2.88	do.
3	40.80	38.79	9.70	0.55	5.40	3.12	do.
4	36.40	44.50	11.80	0.30	2.83	4.13	do.
5	30.18	47.99	13.00	0.30	2.48	5.15	do.

As can be seen from the above table, calcium and phosphoric acid contain about 80% of the total constituent of the ash.

A comparison between the constituents of the ash of the ass's milk and that of buffalo's and cow's milk will be very interesting. Table Nos. 2 and 3 show the constituents of the ash of buffalo's and cow's milk as recorded by various authors.

TABLE No. 2

**Composition of the ash of buffalo's milk.**

Authority	K	Na <sub>2</sub> O	CaO	MgO	Fe <sub>2</sub> O <sub>3</sub>	SO <sub>2</sub>	P <sub>2</sub> O <sub>5</sub>	Cl.
Dr. Sahasra- budhe	22.5- 26.9	10.3- 11.9	19.7- 23.6	1.8- 3.2	trace- 0.2	3.2- 4.3	22.4- 26.5	13.1- 16.2

TABLE No. 3

**Composition of the ash of cow's milk.**

Authority	K <sub>2</sub> O	Na <sub>2</sub> O	CaO	MgO	Fe <sub>2</sub> O <sub>3</sub>	SO <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	Cl.
Leach	25.02	10.01	20.01	2.42	0.13	3.84	24.89	14.28

Table No. 4 shows the composition of the ash of human milk as noted by Harrington and Kinnicutt.

TABLE No. 4

K	Na <sub>2</sub> O	CaO	MgO	FeAl <sub>2</sub> O <sub>3</sub>	SO <sub>2</sub>	P <sub>2</sub> O <sub>5</sub>	Cl.
29.84	12.39	15.69	1.92	0.40	4.38	10.73	20.11

Table No. 5, shows the mean composition of the ash of the ass's milk obtained by taking the average figures for the various constituents of the ash of ass's milk analysed by us.

TABLE No. 5

K	Na <sub>2</sub> O	CaO	MgO	Fe <sub>2</sub> O <sub>3</sub>	SO <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	Cl.
Trace	3.33	36.19	0.95	...	3.55	42.73	12.84

As can be seen from the above tables there is a marked difference in the composition of the ash of ass's milk and that of buffalo's and cow's milk. The composition of the ash of buffalo's, cow's and human milk shows a close resemblance to one another, the different constituents differing slightly from animal to animal. But the composition of the ash of the ass's milk is quite different from the above mentioned animals. The former contains about 40% each of calcium and phosphoric acid i.e. approximately double the amount found in the case of ash of cow's, buffalo's and human milk. It is very interesting to note that while in the case of the three animals, percentage of potassium amounts to about 30, in the ass's milk it is found in traces only. The other constituents of the ash of the ass's milk will naturally show low percentage figures as compared to other animals. The chlorine content of the ash of ass's milk is approximately the same compared to that of other animals.

C. P. Anthrkrishnan has obtained the following percentage figures for lime, phosphoric acid, and chlorine in the ash of ass milk he has analysed.

CaO	.	...	∴	25.1	—	31.8
P <sub>2</sub> O <sub>5</sub>	.	...	...	26.5	—	32.3
Cl	.	...	...	8.1	—	10.5

The above figures differ considerably from the corresponding figures obtained by us especially in the contents of CaO and  $P_2O_5$ . As regards the mineral contents therefore there is also a great variation from animal to animal.

Taking the mean percentage of ash of ass's milk as 0.45 grams table No. 6 shows the amounts in grams of the different constituents of the ash in the ass's milk.

TABLE No. 6

K	Na <sub>2</sub> O	CaO	MgO	Fe <sub>2</sub> O <sub>3</sub>	SO <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	Cl.
Trace	0.015	0.163	0.004	...	0.016	0.194	0.058

Table No. 7 shows the corresponding values for the buffalo's milk as given by Leach in his book "Food Inspection and Analysis, Fourth Edition, Page 111," where the percentage of ash is 0.70.

TABLE No. 7

K <sub>2</sub> O	Na <sub>2</sub> O	CaO	MgO	Fe <sub>2</sub> O <sub>3</sub>	SO <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	Cl.
0.175	0.070	0.140	0.017	0.001	0.027	0.170	0.100

From table Nos. 6 and 7, it can be seen that ass's milk contains a greater portion of calcium and phosphoric acid than the buffalo's milk. While the latter contains an appreciable amount of potassium, ass's milk contains only traces.

From the large percentages of calcium and phosphoric acid found in the ass's milk, it can now be easily understood as to why it is given to children suffering from rickets.



## SECTION II

**(A) A study of milk fat of ass for its preliminary values.**

The problem of tackling the ass's milk is not complete without the study of its fat. We thought that by the study of the milk fat of ass more information would be obtained regarding the utility of ass's milk. We have, therefore, made an attempt to study its fat in detail.

Analysis for the preliminary values was carried out on four samples of ass's milk fat, particular care being taken to obtain representative sample in each case. Table No. 8, shows the preliminary values obtained on four samples of ass's milk fat.

TABLE No. 8

**Preliminary values of four samples of ass's milk fat.**

Obs. No.	Analytical Value.	First Sample.	Second Sample	Third Sample.	Fourth Sample.
1	Butyro-refractometer @ 40°C.	49.2	46.4	43.0	49.3
2	Reichert Meisel Value.	4.78	6.71	7.88	6.54
3	Polenske Value.	6.30	6.80	10.8	9.70
4	Iodine Value.	63.7	67.8	52.8	41.6
5	Saponification Value.	220.16	233.35	222.60	237.10
6	Kirschner Value.	3.28	5.26	4.80	5.26
7	Unsaponifiable matter %	0.72	0.84	0.94	0.90

Table No. 9 shows the same values obtained by C. P. Anthrkrishnan, as given by him in the Journal of Dairy Research, 1941, Volume 12, Page 190.

TABLE No. 9

Observation No.	Analytical Value.	First Sample.	Second Sample.
1	Butyro-refractometer reading @ 40°C.	54.5	54.7
2	Reichert Meisel Value.	9.6	9.5
3	Polenske Value ...	8.2	7.6
4	Iodine Value ...	81.4	90.0
5	Saponification Value.	258.5	261.2
6	Kirschner Value ...	5.9	5.5

As can be seen from Table Nos. 8 and 9, the samples analysed by us are not in close agreement with those analysed by Anthrkrishnan. The butyro-refractometer reading which we have obtained is too low compared with the corresponding figures obtained by him. In general figures for all the values obtained by Anthrkrishnan show a tendency to be a little higher than the corresponding figures obtained by us. As the milk fat was being collected every day we were taking the butyro-refractometer reading of the fat and mean value of the butyro-refractometer reading which we have obtained is 48.5, the minimum and the maximum being 43.0 and 52.5 respectively. In all about 25 butyro refractometer readings were taken for one sample and only on four occasions did the reading show above 50. We have thus never obtained 54.5 as the butyro-refractometer reading for the fat of ass's milk.

It appears that not much work is done abroad on ass's milk fat. The only reference available is about the Reichert

Meisel Value. T. P. Hilditch in his book on "The Chemical Constitution of Natural Fats" has given 13.1 as the Reichert Meisel value, while Richmond states that the Reichert value of the milk fat of ass is only half that of cow's buffer fat. We have obtained 7.88 as the maximum R.M. value.

**(B) A comparison of the preliminary values for the milk fat of ass with the corresponding values obtained for the common milking animals.**

In respect of all the values, the fat of the ass's milk shows no resemblance to any of the other common milking animals except perhaps in the case of R.M. value where it has some resemblance with the fat of human milk. The R.M. value obtained for the fat of human milk varies from 1.4 to 3.4 and the Polenske from 1.9 to 2.2. Ass's milk, therefore, resembles more closely to human milk, in that both contain low percentage of volatile fatty acids. Polenske value is generally high in the case of ass's milk fat, differing considerably from that of cow's and buffalo's milk fats but resembling closely with that of goat's milk fat, where the Polenske figure is obtained as high as 9.5. The chief characteristic of ass's milk fat is its higher iodine value. Here also the iodine value has the tendency to differ considerably from animal to animal. We have obtained iodine value for ass's milk fat ranging between 41.6 — 67.8, whereas Anthrakraishnan gives the figures for iodine value as high as 81.4 — 90. The high value obtained for the ass's milk fat shows that the percentage of the unsaturated acids i.e. oleic, linoleic and linolenic (if any) is more than in the case of any other milk fat of the common milking animals, chiefly cow and buffalo. In respect of the iodine value of the ass's milk fat the ass's milk represents more closely to that of mare's milk where the iodine value for the latter has been given as 61. (Reference Dairy Chemistry Richmond, Elsdon and Walker, Page 78). The iodine value in the case of cow's and buffalo's milk fat does not exceed 45. We have obtained saponification value for the ass's milk fat varying from 220 — 237. These figures do not vary much from the corresponding figures obtained from

other animals. In general ass's milk fat has its own characteristics and can readily be distinguished from the milk fat of other animals, namely by its peculiar unpleasant odour, high iodine value, and very low Reichert Meisel value.

**(C) Determination of the fully saturated glycerides content of the milk fat of ass and its comparison with the milk fat of buffalo and cow.**

In 1927 Hilditch and Lea showed that when a fat is oxidised in acetone solution with powdered potassium permanganate all glycerides containing one or more unsaturated acyl radicals are ultimately converted into the corresponding azelao-glycerides (in the case of oleo-, linoleo-, linolen- or elaeostearo-glycerides), whilst the completely saturated glycerides remain unattacked. The acid azelaic glycerides usually form a complex mixture which is somewhat difficult to separate from the unchanged fully saturated glycerides, because the alkali salts of the azelao-glycerides are very strong emulsifying agents (especially those of the monoazelao-derivatives, which in addition are soluble in ether as well as in water). By taking suitable precautions during the removal of the acid azelao-glycerides by washing with alkali, it is, however, quite possible to recover quantitatively the unchanged fully saturated glycerides.

The following method as described in the Analyst 1931, volume LVI, page 361 and modified by Hilditch was employed to determine the fully saturated glycerides content of the milk fat of ass.

5 grams of the sample were accurately weighed in a 1000 mls. conical flask and 300 mls. of acetone were added and the flask was refluxed. Powdered potassium permanganate (20 grams) was added in small portions at a time and the flask was gently and constantly boiled. After the whole addition of potassium permanganate, the flask was vigorously boiled for half an hour and set aside over night. Next day a fresh quantity (7 grams) of powdered potassium permanganate was added and the flask was refluxed in the similar manner for half

an hour. The acetone was then carefully distilled over till the last drop of acetone was completely removed. The residue was then decolourised by sodium sulphite and 10% sulphuric acid. The contents were then thoroughly extracted by means of petroleum ether, the extraction being repeated three times to ensure complete recovery of organic compounds from the decolourised aqueous solution of mineral acids and salts, and the acidic products were extracted therefrom with a solution of ammonia in 50% aqueous alcohol. This removed much of the acidic compounds, but the ammonium salts of some of the latter are partly soluble in light petroleum. The latter solution was therefore shaken with calcium chloride (10%) solution and washed with water. The light petroleum was then removed by distillation and the residue extracted with ethyl acetate which dissolved neutral fatty matters but left the calcium salts of the acidic products. Evaporation of the ethyl acetate solution yielded the fully saturated glycerides free from acidic products.

Determination of the percentage of fully saturated glycerides was done on three samples, as shown below:-

Sample No.	Fully saturated glycerides (per cent.)		
1.	...	...	30.66
2.	...	...	37.72
3.	...	...	26.68

As can be seen from above, the percentage of fully saturated glycerides varies from animal to animal. This can easily be understood if we take into consideration the variability exhibited by the iodine value and the butyro-refractometer reading of the milk fat of different asses. Due to the scarcity of the ass's milk it is very much regretted that a series of determination of the fully saturated glycerides from milk fat of many asses could not be carried out.

If the three determinations of the percentage of fully saturated glycerides are typical for ass's milk fat we can take 31.7 as the percentage of fully saturated glycerides in the ass's milk fat.

The percentage of fully saturated glycerides in the case of milk fats of Indian camel, sheep and goat have been studied by Dhingra and that of the Indian buffalo and cow by Bhattacharya and Hilditch. (Reference "The Chemical Constitution of Natural Fats" by T. P. Hilditch, Page 259.)

Table No. 10, shows the percentage of fully saturated glycerides in the case of the whole milk fats of various above mentioned animals. (Reference "Ibid, Page 256 and 260").

TABLE No. 10

Cow	Buffalo	Camel	Sheep	Goat
31.7	32.3	24.2	33.7	36.3

As can be seen from the table the percentage of fully saturated glycerides in the case of milk fat of cow and ass is the same.

The percentage of the fully saturated glycerides in the case of buffalo ghee was also determined. For this purpose two samples of genuine buffalo ghee received in the laboratory were taken for analysis. The percentage of the fully saturated glycerides obtained for the buffalo's milk fat was 38.8.

Table No. 11, shows the percentage of saturated glycerides determined by us together with the saponification value and the melting points in the case of milk fats of ass and buffalo.

TABLE No. 11

Sample.	Fully Saturated Glycerides %.	Saponification Value.	Melting Point.
Ass ...	37.72	302.65	15.8° C.
Ass ...	33.66	312.78	14.0° C.
Ass ...	26.68	261.38	23.0° C.
Buffalo ...	34.8	261.5	23.5° C.
Buffalo ...	35.1	245.1	25.0° C.

Very interesting facts are revealed by the study of the saturated glycerides and their saponification value and the melting points. Table No. 11 shows that the saponification value of the fully saturated glycerides is more or less inversely proportional to its melting point. Same observation is also made by Christian and Hilditch (Reference "Dairy Chemistry", by Richmond, Elsdon and Walker, Fourth Edition, Page 195). T. P. Hilditch has also made the same observation. (Reference "Chemical Constitution of Natural Fats", by T. P. Hilditch, Page 256, Table No. 92). The unsaturated acids were determined by difference. Table No. 12 shows the percentage of volatile saturated, non-volatile saturated and unsaturated acids found in the case of ass's fat.

TABLE No. 12

## Ass's milk fat.

Sample	Volatile saturated acids.	Non-volatile saturated acids.	Unsaturated acids (by difference).
1	9.6	33.6	56.8
2	10.2	34.1	55.7

Determination of solid (mostly saturated) and liquid (mostly unsaturated) acids was done on one sample of buffalo's milk fat taken from the laboratory. The method followed was that described in "Analyst" 1931, Volume LVI, Page 376 (A new method for the determination of solid unsaturated fatty acids by Cocks, Christian and Harding). Here also the solid acids were found to possess a small but definite iodine value (3.04). The iodine value was wholly supposed to be due to oleic acid and therefore the percentage of solid acids was calculated after duly making a correction for the iodine value. The percentage of liquid acids was calculated by difference. The percentage of solid and liquid acids obtained was 60.81 and 39.19 respectively.

Though the total number of milk fats examined is not large enough to justify definite generalisations useful indications were obtained.

Table No. 13, shows the iodine value and the corresponding thiocyanogen value determined on four samples of ass's milk fat.

TABLE No. 13

Sample No.	Iodine Value .	Thiocyanogen Value
1	46.1	38.8
2	49.9	39.6
3	62.8	38.9
4	59.4	51.1

**(D) Nutritive value and the digestibility of ass's milk fat.**

It has been proved by experiments that the digestibility of fat depends upon the nature of the acids of which the fat is composed. The higher the proportion of unsaturated acids present in the fat the more is the digestibility. Again it has also been proved that the fats with higher melting points are less digestible than those with lower melting points.



Accordingly it is found that oils and fats whose melting points lie below the normal temperature of the body (about 36°C.) are absorbed and digested in the intestines almost upto 97% - 98%, whereas the fat which has got a higher melting point (say 71°C.) it is found that only 9% - 14% is used i.e. remaining 91% - 86% of the fat only helps in loading the system without in any way benefiting it. The assimilability of free acids is in general, a function of its molecular weight, i.e. the lower ones are more easily absorbed than the higher ones although in the case of unsaturated acids like oleic, they are more easily absorbed than the similar but saturated acids both having the same number of carbon atoms, not merely because of their low melting points but also because of their unsaturated character which is responsible for their greater chemical activity.

The milk fat like all other fats consists of mixture of glycerides i.e. esters of glycerol. These mixed glycerides consist of acids of low and high molecular weight. In these again the glycerides of high molecular weights are of either saturated or unsaturated acids. Whenever the oils and fats are introduced into the human system their assimilability and digestibility as previously said are dependent upon the nature of acids. The glycerides of saturated acids of high molecular weights are not digestible and therefore load the human system and pass off undigested and unassimilated. They are likely to prove therefore some harm to the system and serve no useful purpose. This is mainly due to the fact that the glycerides of saturated acids of high molecular weights have melting points higher than that of the body temperature itself. The liquid glycerides of unsaturated acids of high molecular weights, particularly those of oleic and linoleic acids, on the contrary are not harmful as those of the corresponding stearic glycerides of about the same molecular weights. They are easily absorbed and are known to be beneficial. The glycerides of saturated acids of low molecular weights ranging from those of butyric to those of myristic acids are known to be easily assimilable by the system because of their varying low melting points and easy digestibility and assimilability. The remarks made above for the non-assimilability of solid glycerides of the saturated acids of high molecular weights are

modified to a certain extent when the glycerides of either unsaturated acids or acids of low molecular weights are mixed with them. The proportionate percentage of the glycerides of saturated acids of high molecular weights on one side and that of the glycerides of either unsaturated acids or acids of low molecular weights on the other, decides the comparative assimilability and digestibility of any given oil or fat. (Extracts from the book "Butter fat" by Godbole and Sadgopal).

From the above discussion we can form our own conclusions as to the assimilability and digestibility of the ass's milk. The milk fat of ass is liquid at the room temperature (28°C). It is therefore below the normal temperature of the body and should therefore be easily absorbed and digested. We have seen that the proportion of unsaturated acids present in the milk fat of ass is considerably more than in the milk fat of any of the common milking animals. Ass's milk should therefore be even more superior to buffalo's and cow's milk as regards its digestibility and assimilability.

### Conclusions :—

Analysis of the ash of ass's milk reveals about 80% each of calcium and phosphoric acid with potassium as a trace. This proves why ass's milk is recommended as a medicine for rickety children.

The fat of ass's milk has a penetrating odour and is coloured orange yellow. It has a low R. M. value and high iodine value though the latter differs considerably from animal to animal. The milk fat shows a close resemblance to human and mare's milk fat.

The percentage of saturated glycerides obtained for ass's milk fat is 31.7 and the percentage of saturated acids 44.

Due to the greater proportion of unsaturated acids present in the milk fat of ass the latter should be easily assimilated and digested in the human system.

The thiocyanogen value for the ass's milk fat ranges from 38.8 - 51.1.

## PAPER XVII

"A COMPARATIVE STUDY OF SOME  
VEGETABLE GHEES"

BY

V. S. KULKARNI, M. SC.

*(With the kind permission of the authorities of the Bombay University)*

**General Introduction**

Hydrogenated oils and fats have been of great use in industries since their invention. Since the second world war broke out there has been scarcity of milk and milk products such as butter and ghee, the partially hydrogenated oils known as vegetable ghees have been extensively used for household purposes, such as frying or eating. It was thought, therefore, worthwhile studying how far such vegetable ghees differed from genuine ones and amongst themselves in various respects such as the general analysis giving preliminary values, the extent of saturation and unsaturation, the hydrolytic disintegration and keeping power. For the study three common vegetable ghees available in the market were employed. They were named as 'A', 'B' and 'C' to avoid ourselves from being involved legally in any way. The whole study was done in three respects and hence was divided in three sections :-

**Section I:**—A comparative estimation of the solid saturated, the solid unsaturated, and liquid unsaturated acids from the vegetable ghees ;

**Section II:**—A study of the comparative hydrolytic disintegration of the vegetable ghees ;

**Section III:**—A comparative study of the keeping power-hydrolytic rancidity - of the vegetable ghees.

The work will be described now section by section,

## SECTION I

**A comparative estimation of the solid saturated, the solid unsaturated and the liquid unsaturated acids from the vegetable Ghees :**

Fats and oils form an essential part of the diet of men and animals besides their important role in many industries. They serve as an important source of energy for the body and in this respect are more effective than proteins or carbohydrates. Consequently the life and progress of a nation depend much on the supply of fats and oils, more so with the discovery of new uses they are put to.

At the outset it should be made clear that the distinction of oils and fats is only a physical one depending only on the melting point and so oil in one country may be fat in another one.

Fats and oils are tri-glycerides formed by the combination of higher fatty acids and glycerine.

The fats with a few exceptions of those from tropical plants are characterised by the presence of notable quantities of saturated acids whereas those from plants growing in cold countries contain large quantities of unsaturated acids—(The Taxonomic and Climatic Distribution of oils, Fats and Waxes; by J. B. Mc. Nair *Am. J. Bot.* 16, 832—841—1929).

With the advent of the oil chemistry the vegetable oils are used for hydrogenation. The process consists in passing hydrogen gas under pressure at about 150° to 180°C temperature, into the oils in the presence of finely divided nickel serving as catalyst.

Hydrogenation converts liquid oils into solid fats which are used for edible purposes with the addition of a few vitamins. The main indications of the stage of hydrogenation are—(i) the melting point which is raised and (ii) the iodine value which is lowered. Saponification value remains the same.

The recognition of hydrogenated oils as a class is not always easy when they are present in admixture with other fats. When the use of hydrogenated oils became general the most useful method was the one based on the detection of traces of the catalyst nickel which remained in the oil on completion of hydrogenation. A 0.02% solution of  $\alpha$ -benzildioxime in alcohol which gives a rose red colour even with traces of nickel was the reagent employed (F. W. Atack, *Analyst*, 1913, 38, 316). There have been suggestions for detection of the hydrogenated oils by means of those glycerides which are less soluble in other organic solvents. The method consists in the isolation of glycerides containing stearic and other solid acids—(J. Leimdorfer, *F. S. C. I.*, 1914, 33, 206), so that they will differentiate between hydrogenated oils and tallow particularly in mixtures (cf. *A.O.A.C. Analyst* 1923, 48, 225; K. Amberger, *F. S. C. I.* 1916, 35, 1077). Normal and Hugel depend on the isolation of arachidic acid from alcohol, the proportion of stearic and arachidic acids being determined by neutralisation value (*ibid.* 1917, 36, 658). A. Grun (*ibid.* 1919, 38, 729 A) suggests the method of alcoholysis which is not suitable for routine work. The unsaponifiable matter has also been suggested for the same.

The most useful method for the detection of hydrogenated oils in mixtures appears to be that which is based on the detection of solid unsaturated acids e. g. isomers of oleic acid—produced during hydrogenation. It is well known that during hydrogenation certain isomers of oleic acid are formed, the properties of which are different from normal oleic acid. These are called—“New acids of hydrogenation” by Middleton and Barry in their book ‘Fats, natural and synthetic’ and by other observers as “iso-oleic acid”. This new isomer has been isolated along with solid acids by the usual method for separating solid and liquid acids (William and Botton, *Analyst*, 1924, 49, 460). If the iodine value of the solid fraction is less than 5 units there is no hydrogenated oil but if it is more, hydrogenated oils are present. The iodine value of the solid acids is due to the iso-oleic acid whose percentage is calculated as follows:—

Percentage of iso-oleic acid :-

$$\frac{95}{100} \times \frac{s}{90} \times \frac{(I-5)}{1}$$

I = Iodine value of the solid fraction

s = Percentage of total solid acids

This equation allows 5 units of iodine value for incomplete washing. Most hydrogenated oils (i. v. 50 to 70) contain 30-35% iso-oleic acid.

For the determination of the saturated and unsaturated acids Twitchell's method (J. Ind. Eng. Chem. 1921, 13, 806) is in general use and is undoubtedly superior to the lead-salt-ether method of Gusserow and Varrentrop (J.S.C.I., 1925, 63, T; J. A. C. S. Jan. 1923, 45, 113). Twitchell's method when applied to hydrogenated oils, however, shows low values for the percentage of solid unsaturated acids. The marked extent to which Twitchell's method underestimates the solid unsaturated acids in partially hydrogenated oils has been shown by Cocks, Christian, and Harding (Analyst, 1931, pages 368-380) firstly by the separation of solid acids which escape determination by this method and secondly by applying the method to artificial mixtures of known compositions. The underestimation has been attributed by them to the solubility of the lead salts of solid unsaturated acids into 95% alcohol used for precipitation, crystallisation and washing.

The method has been shown to give satisfactory results for the separation of solid and liquid acids by controlling the quantity of alcohol used for precipitation in relation to the amount of acid employed. The loss of solid unsaturated acids through the solubility of their lead salts in alcohol is reduced to a minimum value and washing of the precipitated acids with petroleum spirit removes the liquid acid and their lead salts without dissolving out more than mere traces of the solid unsaturated acids which can also be recovered by removing petroleum spirit from the washings and dissolving the residue in boiling alcohol containing 0.5% glacial acetic acid from the

solution of which the lead salts of the solid acids precipitate almost immediately on cooling and can be worked up with the main bulk of the soaps of the solid acids.

The methods employed for the general analysis are mostly taken from standard books on this subject, viz:-

- (1) Vegetable fats and Oils by Jamieson.
- (2) Chemical Technology and analysis of Oils, Fats and Waxes by Lewkowitsch.
- (3) Industrial Chemistry of Fats and Waxes by Hilditch.

TABLE No. 1

**Results of General Analysis of the Vegetable Ghees.**

	A	B	C
1. Melting point ...	39.5° C	40.2°	39.2° C
2. Acid value ...	2.894	1.467	4.357
3. Saponification value ...	189.5	191.1	186.1
4. Percentage of the unsaponifiable matter	2.012	5.936	3.622
5. Percentage of total mixed acids after removal of unsaponifiable matter	56.19	75.51	76.63
6. Iodine value of ghee itself	65.49	57.94	61.10
7. Iodine value of mixed fatty acids.	68.19	59.55	62.19
8. Mean molecular weight of the mixed fatty acids	290.0	292.4	285.6
9. Melting point of mixed fatty acids after removal of unsaponifiable matter	38.7° C	39.5° C	37.6° C

### Experimental:—

As already stated in the introduction a correct estimation of solid unsaturated acids forms the most useful method to detect hydrogenated oils in admixtures with other fats. The method of Cocks and coworkers (Analyst, 1931, 368-380) gives a correct estimation of them. It was thought worth while investigating how the method works in the case of the vegetable ghees which are mainly partially hydrogenated oils and so a comparative estimation by the method of Cocks and coworkers and the one similar to that of Twitchell was undertaken.

#### Method I:—(Cocks and Coworkers - Analyst. l.c.)

3.5 grams of the freshly prepared fatty acids were dissolved in 50 c.c. of 92-93% (by weight) alcohol and (except with mixtures containing less than 25% solid acids in which case 1 gm. of lead acetate was employed) 3.45 gms. lead acetate were dissolved in 50 c.c. of similar alcohol. Both the solutions were heated to boiling and the lead acetate solution was poured into the acid solution. After mixing well and again heating to boiling the solution was allowed to cool slowly and evenly for about half an hour in air and then for about 4 hours at 15° - 20°C.

The next morning the mixture was stirred and filtered from alcohol as completely as possible on a 10 c.m. Buchner funnel and any remaining in the precipitating vessel were washed with 100 c.c. of petroleum spirit (40°-60°) using 20 c.c. at a time and the washings were then distilled from water bath to remove petroleum spirit completely. The residue was dissolved by boiling under reflux condenser with 20 c.c. of 92-93% alcohol containing a drop (0.05 gm) of glacial acetic acid. The solution was allowed to stand at 15° - 20°C for three hours. The lead salts which precipitated under these conditions were filtered, washed with 20 c.c. of cold 92 - 93% alcohol and the solid remaining behind on the filter paper was worked up with the main bulk on the Buchner funnel.



The lead soaps of the solid acids were then decomposed by hot dilute nitric acid. The liberated free fatty acids were taken up with ether, the ethereal solution was washed with warm water twice to remove the mineral acids and dried over anhydrous magnesium sulphate and the acids were then obtained by evaporating ether. The yield, iodine value, m.p. and the mean molecular weight of the acids were then determined. From the iodine value the solid unsaturated acids were calculated on the basis that the latter would have a mean i.v. of 90 units - no allowance of 5 units was made for incomplete washing as stated in the introduction.

For the liberation of liquid acids alcohol was distilled off completely from the filtrates and washings, the residue was then dissolved in ether and water was added when the acetic acid produced by decomposition of some of the lead acetate decomposed the lead soaps of the liquid acids (cf. Hilditch, chemical constitution of natural fats). The lead acetate formed went into the aqueous layer while the acids liberated were taken up by ether. The aqueous layer was reextracted with ether. The ethereal solutions were washed twice by ice cold nitric acid to ensure complete decomposition and then with distilled water to remove the mineral acid. The ethereal layer was then dried over anhydrous magnesium sulphate and the liquid acid obtained by removing ether. The liquid acids were pale yellow and viscous. The yield, i.v. and the mean molecular weight were then determined quickly.

**Method II:**—(Twitchell, *J. Ind. Eng. chem.* 1921, 13, 806).

The essential details employed in this method were kept the same as in method I for making the comparison, only that instead of washing the precipitated lead soaps with 100 c.c. of petroleum spirit, they were recrystallised from 100 c.c. 92-93% alcohol containing 0.5 gm. glacial acetic acid.

The other details for obtaining the solid and liquid acids were exactly the same.

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TABLE No. 2.

Physical and chemical constants of the acids separated by the two methods.

Sample of the vegetable ghee	A	B	C
1. i.v. of solid acids separated by			
Method I ...	30.55	40.26	41.42
Method II ...	17.25	23.56	37.87
2. m.p. of solid acids separated by			
Method I ...	48.7°	47.2°	44.8°
Method II ...	52.25°	51.6°	46.8°
3. Mean molecular wt. of solid acids separated by			
Method I ...	288.9	291.6	294.6
Method II ...	292.6	306.8	295.4
4. i.v. of liquid acids separated by			
Method I ...	98.65	82.15	84.48
Method II ...	84.10	74.96	84.80
5. Mean molecular wt. of liquid acids separated by			
Method I ...	277.00	303.6	298.0
Method II ...	315.1	301.6	298.4
6. Yield of acids by Method I			
wt. of mixed acids ...	3.2709	2.4599	3.1292
wt. of solid acids ...	1.9890	1.4569	1.7903
wt. of liquid acids ...	1.2819	1.0030	1.3389
7. Yield of acids by Method II			
wt. of mixed acids ...	3.3724	2.6114	2.9826
wt. of solid acids ...	0.7590	0.7814	1.6207
wt. of liquid acids ...	2.6134	1.8300	1.3619

TABLE No. 3  
**Percentages of solid saturated, solid unsaturated and liquid unsaturated acids  
 obtained by the two methods.**

Method I.

Sample of the vegetable ghee	A		B		C	
	found	calculated	found	calculated	found	calculated
Solid saturated ... ..	40.22	36.44	33.16	29.82	30.89	27.94
Solid unsaturated (iso-oleic acid).	20.62	18.69	26.84	24.13	26.33	23.82
Liquid unsaturated ...	39.16	44.87	40.00	46.05	42.78	48.24

Method II.

Solid saturated ... ..	18.20	19.24	22.07	22.12	31.47	27.90
Solid unsaturated (iso-oleic acid).	4.31	4.56	7.83	7.85	22.86	20.27
Liquid unsaturated ...	77.49	76.20	70.10	70.03	45.67	51.83

Percentages of total solid acids (saturated and unsaturated) were first calculated from (1) the actual weights given in table No. 2 and also from (2) the iodine values of (i) the mixed fatty acids (ii) the solid acids and (iii) the liquid acids. Calculations of the solid unsaturated acids were done, from the iodine values of the total solid acids, on the basis that the mean iodine value of the solid unsaturated (iso-oleic) acid would be 90. The values calculated from actual weights are entered in the columns under the heading, 'found' and those calculated from the iodine values in the columns under the heading 'calculated'.

The calculations from the iodine values were done as follows:- (cf. rapid method of examination of hydrogenated products in the book "Fats, natural and Synthetic", by Mydleton and Barry - page 114).

1. Iodine value of the mixed fatty acids from the ghee 'A'	.....	68.19
2. Iodine value of the solid acids - separated by Method II.	.....	17.25
3. Iodine value of the liquid acids - separated by Method II.	.....	84.1

Let 'x' be the percentage of the solid acids then 100-x will be that of the liquid acids, then

$$\frac{17.25 \times 'x'}{100} + \frac{(84.1)(100-'x')}{100} = \frac{68.19 \times 100}{100}$$

From this equation  $x = 23.8$  is the percentage of solid acids. The solid unsaturated acid in this was further calculated as follows:-

$$\frac{17.25 \times 100}{90} = 19.17\% \text{ solid unsaturated in total } 100 \text{ of the solid acids.}$$

hence in 23.8,

$$\frac{23.8 \times 19.17}{100} = 4.56 \text{ is the amount of solid unsaturated and hence } (23.80 - 4.56) \text{ is the solid saturated.}$$

The percentages of all the acids would be

solid saturated	19.24
solid unsaturated	4.56
liquid unsaturated	76.20

From table No. 3, it is evident that the Method II, which is similar to that of Twitchell gives a considerable underestimation of the solid unsaturated acids in the case of vegetable ghees. In the case of 'A' this underestimation is  $18.69 - 4.56 = 14.13$  as calculated from i.v. and  $20.62 - 4.31 = 16.31$  as calculated from actual weights.

In the case of 'B' it is—

$24.13 - 7.85 = 16.28$  as calculated from i.v. and

$26.84 - 7.83 = 19.01$  as calculated from actual wts.

In the case of 'C' it is—

$23.82 - 20.27 = 3.55$  as calculated from i.v. and

$26.33 - 22.86 = 3.47$  as calculated from actual wts.

This observation is in keeping with the one made by Cocks, Christian and Harding in the case of other hydrogenated oils (Analyst. 1931, pp. 368-380). The method of Cocks and coworkers i.e. Method No. I. here can be said to be more suitable for detecting vegetable ghees in adulterations.

## SECTION II

**A study of the comparative hydrolytic disintegration of the vegetable ghees amongst themselves and with cow-ghee.**

It has been observed and proved that seeds such as linseed, poppy, sesame, ground nut, sweet and bitter almonds contain an enzyme-lipase which is capable of bringing about hydrolysis of the oils on keeping. The first observation in this connection was done by J. Pelouze (C. R. 1855, 40, 605).

J. Reynolds Green (Proc. R. S. 1890, 48, 370) described a series of experiments on the enzyme in castor seed. He discovered the "Ricinus lipase" from castor bean seed (*Ricinus Communis*) and found that the plant enzyme was a typical lipase hydrolysing fats into fatty acids and glycerol.

Since that time the observations of Green were confirmed by various workers. Its optimum pH was found to be between 4.7 and 5.0 (Willstatter and Waldschmidt-Lietz E. "Über Ricinus lipase"-Z. physiol. chem. 134, 161, 1923-24) varying slightly with the buffer employed.

Castor beans contain two distinct lipolytic enzymes one of which is soluble in water and has greater hydrolysing effect on esters, like ethyl butyrate and hence is known as esterase while the other is insoluble in water and is more active towards glycerides and is known as lipase. The lipase proper is soluble to a certain extent in common salt solution, especially in 1.5 N solution.

According to Constein, Hoyer and Wartenberg 35°C is the optimum temperature for the lipase but for liquid oils 15°-20° is better. At 50° the reaction is slow while at 100°C it is nil.

Sahasrabuddhe and Lilaowalla (N. Wadia College Lab.) have done a work on "study on the enzyme lipase from the ground nut and linseed oil and its action on ground nut, linseed and sesame oils" (M.Sc. thesis Lilaowalla, Bomb. Univ. 1944).

Kale and Sahasrabuddhe have studied the 'disintegration of fats and enzymes in the dry fruits—cashew nut, almond and pistachio nut—together with the effect of common salt and vitamins on enzyme hydrolysis'.

When fats form part of the diet of an animal, they are dealt with not in the liver but in the epithelial cell (villi) of the small intestine where the glycerides undergo hydrolysis by the catalytic agency of a fat-splitting enzyme 'lipase' secreted by pancreas. The results of investigation of Munk go to show that the fats are not directly absorbed but are first hydrolysed

and then resynthesised into glycerides (cf. Moore, Proc. Roy. Soc. A, 1903, 72, 134)

The breaking down of fats is greatly accelerated by bile salts such as sodium glycocholate, sodium taurocholate and bile pigments - bilirubin and biliverdin. These tend to increase the dispersion of the oily droplets by their marked power of lowering the surface tension and give rise to emulsion. The hydrolysed fats pass as free acids (or soaps) into the chyle or lymph (which flow through the lacteals of the small intestine) and are resynthesised into glycerides which pass on to thoracic duct and finally emerge into the blood system. Frazer (Analyst. 1938, 63, 308) modifies this and states that the hydrolysis is only partial and the unhydrolysed fat passes directly by the lacteal-lymphatic system and then to fat deposits while the fatty acids pass by the capillaries and the portal vein to the liver where they may be metabolised.

The absorption of the majority of the edible oils and fats is almost quantitative-digestibility coeffs. 95-100. (Langworthy, Ind. Eng. chem. 1923 - pp. 276-278). Lower values have been recorded for some fats, more particularly in case where the melting point is higher than the temperature of the body i.e. 37°C, so that the emulsification of the fatty globules in the intestinal contents may be less satisfactory (oils, fats and fatty food - by J.O. Drumonds). The optimum temperature for the pancreatic lipase is 40°C while at 65°-70° the enzyme is destroyed (Kastle and Lovenhart l.c.).

### **Experimental :-**

In this section it is intended to study the hydrolytic disintegration of the vegetable ghees and compare the amount and rate of hydrolysis of the vegetable ghees amongst themselves and with a sample of genuine ghee - cow ghee. The hydrolysis was brought about by castor seed lipase prepared by the method already stated before.

### **Procedure :-**

Lipase (0.1 gm.) was weighed in five small (100 c.c. cap.) conical flasks and phosphate buffer of 4.8 pH (6 c.c.) and finally

the melted ghee (2 c.c.) were added to each. The flasks were kept in an incubator at 37°C for varying periods i.e. 2, 4, 24, and 48 hours. A blank experiment was also run side by side. The fatty acids resulting from the hydrolysis of the ghees by lipase after each interval were determined by titration with N/10 NaOH using phenolphthalein as an indicator, after adding 10 c.c. of neutral alcohol to stop the reaction in the small flasks the contents of which were then transferred to a 300 c.c. conical flask and washed with 20 c.c. of neutral alcohol. The saponification value of each sample was determined by saponifying 2 c.c. of each sample with excess i.e. 25 c. c. of alcoholic KOH and titrating the excess KOH with N/2 HO1. The Sap. value was expressed in terms of c.c. of N/10 NaOH for complete saponification of 2 c.c. of each sample and the percentages of hydrolysis after each interval were calculated on the basis that after complete hydrolysis the reading of N/10 NaOH for neutralisation of the acids produced would be the same as the saponification value thus expressed. The free acidity of each sample (2 c.c.) was also determined and also expressed in terms of c.es. of N/10 NaOH.

TABLE No. 4  
Hydrolysis of the Ghees in terms of c.cs. of NaOH.

Sample	Free acidity @	Hydrolysis in terms of N/10 NaOH in hours.*			
		2	4	24	48
A	0.0934	1.9606	3.8586	7.2376	10.8266
B	0.0467	1.2133	2.1953	7.3783	10.7330
C	0.1401	2.4749	5.1359	12.0399	17.8899
Cow-ghee solid- layer.	0.3538	3.6892	5.8642	22.7962	29.3062

\* calculated c.es. of N/10 NaOH after subtracting the reading for the blank (10.4 c.c.) and the free acidity of 2 c.c. of each sample.

@ c.es. of N/10 NaOH for neutralisation of the acidity of 2 c.c. of each sample.



TABLE No. 5.

## Percentage hydrolysis of the Ghees :-

Sample	Sap. value *	Free acidity @	Percentage hydrolysis during hours			
			2	4	24	48
A	60.2366	0.0934	3.255	6.406	12.01	17.97
B	60.7933	0.0467	1.995	3.611	12.14	17.65
C	59.6999	0.1401	4.146	8.602	20.17	29.96
Cow- ghee	69.9462	0.3538	5.274	8.385	32.58	41.90

\* Sap. value :- c.cs. of N/10 NaOH for complete saponification after deducting the free acidity for 2 c.c. of the sample.

@ Free acidity :- c.cs. of N/10 NaOH for the neutralisation of free acids in 2 c.c. of the sample.

## Interpretation and comments on the results of hydrolysis :-

From the values given in Table No. 4 and Table No. 5, it is evident that for the sample 'A' the velocities of hydrolysis are :-

0-2 hours	2 to 4 hours	0-24 hours	24-48 hours
3.255	3.151	12.01	5.96

During the interval of the first two hours the hydrolysis is 50.82% of the total hydrolysis in 4 hours while during the interval of the first 24 hours the hydrolysis is 66.82% or approximately 2/3rd the hydrolysis in 48 hours.

## For the sample 'B'

The velocities of hydrolysis are :-

0-2 hours	2-4 hours	0-24 hours	24-48 hours
1.995	1.616	12.14	5.57

The hydrolysis in the first interval of 2 hours is 55.26% of the total hydrolysis in 4 hours while the hydrolysis in the first interval of 24 hours is 68.79% of the total hydrolysis in 48 hours.

### For the sample 'C'

The velocities of hydrolysis are :-

0-2 hours	2-4 hours	0-24 hours	24-48 hours
4.416	4.457	20.17	9.79

The hydrolysis in the first interval of 2 hours is 48.19% of the total hydrolysis during the first four hours while the hydrolysis in the first interval of 24 hours is 67.32% of the total hydrolysis in 48 hours.

For the sample of the cow-ghee the velocities of hydrolysis are :-

0-2 hours	2-4 hours	0-24 hours	24-48 hours
5.274	3.111	32.58	9.32

The hydrolysis in the first interval of 2 hours is 62.9% of the total hydrolysis in the first 4 hours while the hydrolysis in the first interval of 24 hours is 77.76% of the total hydrolysis in 48 hours.

**Comparison of the vegetable ghees amongst themselves :-**

From table No. 5, it is evident that (i) of the three vegetable ghees 'C' is attacked the most easily giving the maximum amount of hydrolysis. 'A' stands second and then comes 'B'. So the order is (1) 'C' (2) 'A' (3) 'B'.

**Comparison of the vegetable ghees with the sample of cow-ghees -**

The sample of cow-ghee gives the highest amount of hydrolysis. The rate of hydrolysis is also more either in the first short interval of 2 hours (62.9%) or in the first long interval of 24 hours (77.76%).

## SECTION III

**A comparative study of the keeping power-hydrolytic rancidity - of the vegetable ghees.**

**Introduction :**

Oils and fats when freshly extracted or when refined contain very small quantities of free acids but if, however, they are kept or exposed to the atmosphere the formation of notable quantities of volatile and non-volatile fatty acids takes place gradually. The changes which they undergo are of great importance both from scientific and commercial view points. Sometimes they develop an objectional odour and taste on keeping. This phenomenon is known as rancidity.

Rancidity is of various types:—(i) more especially simple hydrolysis of the glycerides into free fatty acids (ii) secondly oxidation—*b*-oxidation—of the saturated fatty acids with the production of ketones, and (iii) the type which is common—oxidation at the double bonds of unsaturated glycerides or unsaturated fatty acids (Hilditch: chemical constitution of natural fats). The first two of these three types, are almost exclusively the result of enzyme action and the last is usually due to the action of air, heat, light etc., and may also be influenced by the presence of enzymes.

The first type of rancidity which may be called hydrolytic is caused by exposure of fats to moisture in presence of lipolytic enzymes. In seeds lipase is always present and hence seed fats are prone to develop acidity if kept under damp conditions. Fruit flesh fats such as palm or olive oil rapidly undergo partial hydrolysis unless the non-fatty pulp is removed or the enzyme destroyed by heating with steam. Animal fats on the other hand are less liable to become hydrolysed and the hydrolytic rancidity of such fats, if it occurs, is usually due to the growth of moulds like “*Penicillium Aspergillus*” on the surface, which contain lipolytic enzymes in abundance. In the marine oils - liver oils etc. - there is a risk of contamination with intestinal bacteria some of

which are rich in lipase and hence are liable to undergo hydrolysis.

### Experimental.

In this section it is intended to study the hydrolytic rancidity of the three vegetable ghees and compare the amount and rate of rancidity amongst themselves and with a sample of cow-ghee. The following method was employed.

#### The Method:

100 gms. of each of the vegetable ghees were weighed separately in three bottles and 1 gm of lipase was added to each of them. Equal quantities of ghees were also kept in three other bottles without lipase in it. All the bottles were tightly corked by rubber bungs and kept in an incubator at 40°C. The acids produced by the hydrolytic action of lipase or by merely keeping were determined by titration with N/10 NaOH. For each titration, about 5 gms. of the sample were weighed accurately in the titration flask, mixed with 50 c. c. of neutral alcohol to dissolve the free acids, the mixture was warmed slightly and then titrated with N/10 NaOH using phenolphthalein as indicator. The amount of hydrolysis in terms of c.c. of N/10 NaOH per gram was then calculated. The results obtained are given in a tabular form:—(in table No. 6 and No. 7).

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TABLE No. 6.

Hydrolytic rancidity of cow-ghee in terms of c. cs. of N/10 NaOH per gm. \*

Days	with lipase @	without lipase.
0	—	0.2887
59	2.5610	0.3718
93	3.5580	0.4057
124	4.2050	0.4096

\* By kind permission from the work of Mr. M. M. Thatte, M. Sc., who also worked in the Nowrosjee Wadia College, Poona.

@ For these readings 2 gms. of the lipase for 100 gms. of ghee were added, but this does not affect much as the hydrolysis is not directly proportional to the amount of lipase added as found in a previous experiment.

TABLE No. 7.

Hydrolytic rancidity of the three vegetable ghees in terms of c.cs. of N/10 NaOH per gram.

Days	A with lipase	A without lipase	B with lipase	B without lipase	C with lipase	C without lipase
0	—	0.0515	—	0.0261	—	0.0777
43	0.6956	0.0115	0.6618	0.0089	0.7693	0.1390
58	0.9482	0.0318	0.7971	0.0206	0.9114	0.1093
75	1.0080	0.0381	0.9354	0.0173	1.1270	0.0842
89	1.1290	0.0344	1.0640	0.0200	1.2260	0.0971
116	1.4930	0.0325	1.1720	0.0206	1.3820	0.1110
134	1.6020	0.0388	1.3290	0.0213	1.5470	0.1087

TABLE No. 8.

**Amount of hydrolysis with lipase in the intervals used  
in the case of the three vegetable ghees.**

Period or interval in days	Amount of hydrolysis in terms of c.cs of N/10 NaOH per gram.		
	A	B	C
0 - 43 ...	0.6440	0.6357	0.6916
43 to 58 = 15 ...	0.2526	0.1353	0.1421
58 to 75 = 17 ...	0.0598	0.1383	0.2156
75 to 89 = 14 ...	0.1210	0.1286	0.0990
89 to 116 = 27 ...	0.3640	0.1080	0.1560
116 to 134 = 18 ...	0.1090	0.1570	0.1650

The amount of hydrolysis with lipase in the interval used  
in the case of cow-ghee :-

Period or interval in days	Amount of hydrolysis in c.cs. of N/10 NaOH per gm.
0 to 59 ...	2.2723
59 to 93 = 34 ...	0.9970
93 to 124 = 31 ...	0.6470

It can be said from the above two tables that both in the  
case of vegetable ghees and the cow-ghee the hydrolysis is  
rapid in the beginning but the rate falls afterwards.

### **Comparison of the vegetable ghees among themselves**

If we compare the vegetable ghees amongst themselves we find that without lipase there is no appreciable hydrolysis, the slight apparent fluctuations in the acidity may be due to experimental errors the original acidity being very low. With lipasē, however, all of them are slowly hydrolysed. The hydrolysis does not proceed regularly.

### **Comparison of the vegetable ghees with cow-ghee**

The sample of the cow-ghee under study has initially more free acidity than any of the vegetable ghees and the free acidity increases gradually without lipase whereas with lipase the acidity increases more rapidly than any of the vegetable ghees and so it can be said that cow ghee is more prone to become rancid than any other vegetable ghees.

### **The nutritive value of the vegetable ghees**

Special significance attaches to the nutritive value of the vegetable ghees in view of the unwelcomed popularity as articles of diet in these days and the increasing number of their factories which are encouraged and brought into being by the Central Government. It is curious to note as Prof. Damodaran of Madras pointed out recently that in spite of the increasing use of vegetable ghees in diet and the growth of the industry very little information regarding their nutritive value was available until recent times. Under no circumstances such artificial material should be introduced into commerce as articles of diet without due notice being given to the purchaser of the character of the material being sold.

The food value of hydrogenated oils or vegetable ghees will depend on (i) the digestibility (ii) contents of growth promoting factors (iii) foreign materials present in the ghees.

(1). The digestibility of a fat in general depends upon a number of factors one of which is its m. p. For easy assimilation of a fat its m. p. should be below the normal body temperature i.e.  $37^{\circ}$ , for otherwise the intestinal secretions will have less

effect on a fat if it remains solid in the intestines. Materials of high m. p. are not therefore quite easily broken down and assimilated (Cf. Holmes and Deuel, *Analyst*, 1921, 46, 503; Langsworthy C. F. *Ind. Eng. chem.* 1923, Vol. 15, No. 3 pp. 276-278). The following table shows the m.p. of some fats.

TABLE No. 9

Name of the fat		Melting point in degrees C
Butter	...	32.0°
Ghee from Buffalo milk	...	37.5°
Cocoonut oil	...	25.0°
Ground-nut oil	...	27.0°
Vegetable ghee 'A'	...	39.5°
Vegetable ghee 'B'	...	40.2°
Vegetable ghee 'C'	...	39.2°

From the above table and discussion it is evident that vegetable ghees due to their higher melting points are inferior in digestibility to butter or ghee and also to oils which are liquid at ordinary temperature.

• The digestibility of a fat also depends on its rate and amount of hydrolysis by pancreatic lipase, as has already been stated in section II. From the results of hydrolysis, interpretations thereof and discussions thereon as given there, it can be said in conclusion that even with lipase the vegetable ghees are much less hydrolysed than genuine ghee and hence are more difficult to be digested than ghee.

(2). The food value also depends on the contents of the growth promoting factors, mainly vitamins and unsaturated acids. The two essential processes viz. refining and hydrogenation deprive the oils of all the vitamins (the vitamins are destroyed at a very high temperature - 180° - used during



hydrogenation) and most of the unsaturated acids. And even if the vitamins are supplemented from outside into the vegetable ghees, the former do not serve as equally good substitutes.

(3). The vegetable ghees may contain only traces of the catalyst nickel as foreign materials which are not likely to be harmful. (Bordas F.S C.I. 1919, 38, 787A ; K. B. Lehmann, *ibid*, 1914, 33, 763).

In conclusion it can be said that for all the reasons stated above, it is not advisable to recommend vegetable ghees in place of genuine ones or even in place of vegetable oils for food purposes.

This conclusion is supported by the observations of Dr. Patwardhan of Bombay who has proved by experiments on rats fed only on vegetable ghees that the vegetable ghees adversely affect growth and reproductive functions of animals (Current Science Vol. 15, No. 4, April 1946, page 94).

Dr. N. N. Godbole from Benares Hindu University, Miss D'Souza from Bangalore and Professor Damodaran from Madras have also come to the same conclusion from their own experiments.

## PAPER XVIII

**“A COMPARATIVE STUDY IN THE HYDRO-  
LYSIS OF A FEW EDIBLE OILS WITH  
ENZYME LIPASE”**

BY

**(MISS) SILLOO P. NAEGANVALA, M. Sc.***(With the kind permission of the authorities of the  
Bombay University)***Introduction**

Realising the importance of the problem of nutrition many research workers all over the world in the fields of bio-chemistry have tackled the subject of nutrition with success and thousands are continuing the work of analysing and assaying the different values of various food grains, vegetables and edible oils for human and animal nutrition.

In India also a lot of work is being done at various places viz. in Conoor, Madras, Bangalore, Calcutta and Poona.

The digestibility of any foodstuff is generally found by studying the limits of disintegration of its complex constituents during the course of its passage through the alimentary canal. The usual laboratory practice of studying the comparative utility and digestibility of foodstuffs is to try them on animals like guinea pigs, rats etc. that is in vivo experiments, while the other method is resorting to experiments in vitro.

The comparative values of different foodstuffs obtained by the in-vitro experiments, may not refer to any particular species yet they may be at least strictly comparable with each other.

Since in in-vitro experiments the work can be carried out under controlled conditions, as well as any variation in experimental condition required may be carefully introduced which cannot be done in the case of in-vivo experiments.

Man lives on a varied diet but the essential constituents of all food-stuffs as found by chemical analysis are (1) Carbohydrates, (2) Proteins, (3) Fats (4) Mineral matter and (5) Vitamins. Carbohydrates and fats are the main sources of energy for the body and therefore the digestibility of the fats and oils is of great importance, and a study of this hydrolysis with regard to the refining, thermal treatment and hydrogenated oils and fats is chosen for this paper, for during the processes of refining and heating the oil is subjected to several changes of temperature for varying periods of time, to see whether this change effects the process of hydrolysis.

The oils chosen for this work were linseed, safflower and cocoanut oils, belonging to the drying, semi-drying and non-drying variety of oils.

The oils have been studied by different workers in India and foreign countries from various points of view, but here the main object is to study the hydrolysis of these oils under various conditions, as well as the comparative utility of these oils with the natural ghees on the one hand and on the other with the hydrogenated vegetable products which are so commonly used now-a-days as substitutes for ghees.

Since it is well known that in the alimentary tract of the higher animals fats and oils are hydrolysed by the catalytic agency of the fat splitting enzyme 'lipase' secreted by the digestive gland, the pancreas and the intestines and then absorbed in the form of fine emulsion in which the bile salts—sodium glycocholate, sodium taurocholate and bile pigments—bilirubin and biliverdin play an important part. These by their marked power of lowering surface tension tend to increase the dispersion of the oily droplets and to give rise to emulsions. The hydrolysis of the glycerides starts as soon as the fatty acid molecule enters the lining cells of the adsorbing surface.

Studies on different animal species including human beings with a variety of food fats have presented ample proof that majority of edible oils and fats are readily digestible (Langworthy Ind, Eng. Chem. 1923 15,276),

Recent studies indicate that fat absorption and digestion is influenced by the composition of the diet or by the balance of constituents that affect the processes of digestion and absorption. Lyman (J. Biol. Chem. 1917, 32, 7) fed dogs with glyceryl stearate and palmitate and observed great differences in absorption. He doubts whether absorption is dependent on M. P. of fats as suggested by Langworthy. Mecay and Paul (J. Nutrition, 1938, 15, 377) experimented on guinea pigs and found that fats and oils of low M. P. were utilised better than those of high M. P. and suggested that this might be due to the inability to absorb to any great extent the higher M. P. fatty acids. Hol et al (J. Pediatrics 1935, 6, 427) have reported that volatile fatty acids and glycerides were well absorbed, short chain fatty acid glycerides were completely absorbed than longer chain fatty acid glycerides. They suggested that chemical composition rather than M. P. is an important factor in absorption. (Oils, Fats and Fatty Foods by J. C. Drummond).

It has been observed and proved that oil seeds contain an enzyme 'lipase' which by its lipolytic activity brings about hydrolysis of the oil on keeping. This observation was first made by J. Pelouze in 1855 (C. R. 1855, 40, 605) that oil seeds such as linseed, poppy, sesame, groundnut and almond contain a substance capable of hydrolysing the oil present in the seed when they are crushed.

The oil extracted from freshly crushed seeds contains negligible amount of acid, but on keeping the oil for some days after extraction, appreciable quantities of free acid can be detected, and the amount tends to increase as the period increases.

The above decomposition was attributed to the presence of a ferment which has since been termed 'lipase'.

Since lipase is the agent which brings about the hydrolysis of oils, therefore for the study of linseed, safflower and cocoanut oils, lipase from the seeds of castor bean and *Butea Frondosa* were chosen, and it being an essential agent

in hydrolysis of oils, the two lipase actions have also been studied side by side.

'Lipase' from castor seed (*Ricinus Communis*) was first discovered in 1890 by J. Reynolds Green (Proc. R.S. 1890, 48, 370). He found that the plant enzyme is a typical lipase hydrolysing true fats, i.e. the oil is split into fatty acids and glycerol.

The optimum pH for the 'Ricin Lipase' is between 4.7 & 5.0. (Willstatter and Waldschmidt-Lietz. E. *Über Ricinus lipase* Z. Physiol. Chem. 134, 161-1923-24).

Constein, Hoyer and Wartenberg (Ber. 1902, 35, 3989) were the first to recognise the technical importance of vegetable Lipases from a chemical point of view. Their first experiments consisted of grinding together a given weight of castor seed with its own or twice its weight of a 1% chloral hydrate solution. The results proved that in the latter case 95% of the oil present in the seed was hydrolysed within four days at 35 degrees centigrade.

In their latter experiments they added small amounts of acid to the mixture of water, oil and ground seed, and found that reaction was much accelerated. They also showed that many other oils besides castor oil can be saponified by the lipase present in the castor seed, but that some oils are not readily hydrolysed.

So far a good amount of work has been done in studying the 'Ricin Lipase' by J.J. Sudborough, H.E. Watson and P. S. Varma (Jr. Ind. Inst. Sc. Vol. 2 part XV Page 213-273, 1919).

The hydrolysing action of the lipase on oils is being studied at the Nowrosjee Wadia College, Biochemistry Department, Poona.

Glick D & King C. G. have done investigations on pancreatic lipase (Jr. Am. Chem. Sc. 55, 2445, 1933). Sobotka & Glick have worked on the mechanism of the lyolytic enzyme

actions (J. Biol. Chem. 105, 199, 1934). Falk K. G. has tried extraction experiments with castor bean lipase (J. Am. Chem. Sc. 35, 1904, 1913). J. A. Hartwell (Bio Chem. Jr. XXXII, 4667) has published a note on the digestion of fats by pancreatic lipase, using cocoanut oil, castor oil, palm kernel oil, palm oil, bacon fat, beef fat, cod liver oil etc. as substrates.

## SECTION I

### Extraction of the Enzyme Lipase from the Castor Seed

Numerous methods have been found for obtaining lipase from the crushed seeds. All these consist of removing from the seeds the materials such as oil, albuminoid substances devoid of hydrolysing properties.

For the present investigation Tanaka's method has been utilised.

#### Tanaka's Method:

The oil seeds were first decorticated and crushed to a coarse powder, packed in paper bags and dipped into ether for 3 days to remove all the oil, alurenol etc.

The powder was further pounded and the remaining oil was extracted with the help of the Soxhlet apparatus. It was dried and passed through a 100 mesh sieve to separate any coarse particles, then 100 grams of this fine powder was triturated with 600-700 c.cs. of 0.1 N acetic acid at 37°C. for 30 minutes. After which the milky liquid was filtered and the powder washed free from acetic acid and dried at a temperature not exceeding 40°C. The temperature was an important factor as the enzyme becomes inactive at a higher temperature at about 60°C. Therefore a second sample of the lipase was extracted exactly as mentioned above but dried at a temperature of about 45°C.

These two different lipase powders obtained were preserved in glass bottles with the addition of a few drops of toluene.

A comparative study of these two lipase powders are carried out side by side with the study of the hydrolysing action of lipase on oils in the next section.

Castor beans contain two distinct lipolytic enzymes as stated by Falk and Sugnira (J. Am. Chem. Soc. 1912, 1913) one of these is soluble in water and has a comparatively greater hydrolysing effect on esters like ethyl-butyrate than on glycerides like triacetin. The other is insoluble and more active towards triacetin than towards ethyl-butyrate. The soluble enzyme is termed "Esterase" and the insoluble "Lipase".

The optimum pH of the castor bean lipase is between 4.7 and 5 (Uber Ricinus lipase by Willstatter and Waldschmidt-Leitz-physiol. Chem. 134, 161, 1923-24).

J. J. Sudborough, H. E. Watson and P. S. Varma (Jr. Ind. Inst. Sc. Vol. 2 Part XV P.P. 213-273) have done a good amount of work on the 'Oil splitting by Castor Lipase'.

## SECTION II

### **A comparative study of the effect of refining and heating on the hydrolysis of the edible oils in-vitro:**

- 1) Linseed Oil,
- 3) Safflower Oil,
- 4) Cocoanut Oil,

With the enzyme lipase from castor seeds.

In this section it is intended to study the effect of refining and heating on the hydrolysis of the linseed, safflower and cocoanut oils by the action of castor seed lipase. As well as inactivation of the lipase with rise of temperature, lipase powder marked A dried below 40°C. and lipase powder marked B dried at about 45°C.

The following three oils linseed, safflower and cocoanut were pressed in the Agriculture College Ghanny in order to be sure that oils were pure, this oil was termed as "Crude Oil".

Now this "crude oil" was refined as follows:-

The acid value of the oil was first determined and on the basis of this value, the free fatty acids were neutralised by exact amount of N/10 NaOH diluted considerably and added in a fine stream to the oil, the mixture was heated on a water bath to 100°F and gradually to 120°F. the whole being stirred continuously for 15 minutes and then allowed to cool, the soap and other impurities were made to settle down by the addition of common salt, the oil was washed and dried by shaking with anhydrous  $\text{Na}_2\text{SO}_4$  and filtered. This oil is termed as "Neutralised". Next the crude oil was heated for  $\frac{1}{2}$  hour at 160°C. and also heated to its boiling point which was over 350°C. This was termed as "Boiled Oil".

Each of the 4 samples of the three oils crude oil, neutralised oil, heated oil and boiled oil were hydrolysed with the castor seed lipase.

### Method of Hydrolysis

For getting the optimum hydrolysis of the oils under consideration, trial experiments were carried out in the beginning to determine the factors such as substrate concentration, buffer used and the quantity of the enzyme lipase needed.

It was found that the following quantities acted the best:— 2 c. c. of the oil (or its equal weight), 2 c. c. of an acid buffer of 4.8 pH (of 0.2M disodium phosphate and 0.1 M citric acid) and 100 mg. of the enzyme lipase added and this reaction mixture was incubated for varying time from 3 to 48 hours at a constant temperature of 37° C.

A blank experiment was also conducted side by side using all the above factors except the oil. The difference between the blank and the real experiment minus the acidity (if any) originally present in the oil, gave the amount of hydrolysis that had taken place in the same time.



10 c. c. of alcohol were added to dissolve the fatty acids resulting from the hydrolysis of the fats by lipase and then the solution was titrated against N/10 NaOH, using phenolphthalein as the indicator.

The results are expressed as the percentage of the total oil hydrolysed at different periods.

The saponification values and the acid values of the oils in question were determined in terms of c.cs. of N/10 alkali, before starting actual experiments.

### (1) Hydrolysis of Linseed Oil.

TABLE No. 1

Linseed Oil	Sap. Value c.cs.	Acid Value c.cs.	% Hydrolysis hrs.			
			3 hrs.	24 hrs.	48 hrs.	
1 Crude	34.43	1.23	A	11.206	19.030	32.840
			B	2.238	4.824	7.898
2 Neutralised	34.10	Nil	A	5.561	8.108	13.155
			B	0.667	1.540	3.150
3 Heated for $\frac{1}{2}$ hr. at 160°C	34.43	1.10	A	9.990	17.928	30.46
			B	1.824	4.476	7.461
4 Boiled	34.43	0.77	A	2.133	6.208	10.785
			B	Nil	0.663	1.410

In the table No. 1 the results of the hydrolysis of the linseed oil with castor seed lipase A i.e. dried below 40°C and lipase B dried at about 45°C are tabulated.

From the figures in Table No. 1 we find that :

In the "crude" linseed oil :—

The velocities of reaction in the three intervals are—

11.206; 7.824; 13.810 with lipase A  
and 2.238; 2.586; 3.074 with lipase B

Out of the total fats hydrolysed at the end of 48 hours 34.2%, 23.8% and 42% are hydrolysed at the end of 1st, 2nd and 3rd intervals of 3, 24 and 48 hours with lipase A.

While with lipase B the corresponding figures are 23.3%, 32.7% and 39% respectively.

In "neutralised" linseed oil :—

The velocities of reaction in the three intervals are—

5.561; 2.547; 5.047 with lipase A.  
and 0.667; 0.873; 1.610 with lipase B.

Out of all the total fats hydrolysed at the end of 48 hours, 42.4%; 19.3%; 33.3% are hydrolysed at the end of 1st, 2nd and 3rd intervals by lipase A.

While 21.2%; 27.7%; 51.1% are the corresponding figures with lipase B.

In the oil "heated" at 160°C. for  $\frac{1}{2}$  hour. :—

The velocities of reaction in the three intervals are—

9.990; 7.938; 12.532 with lipase A.  
and 1.824; 2.652; 2.985 with lipase B.

Out of the total fats hydrolysed at the end of the third interval 32.8%; 26.0% and 41.2% are hydrolysed by lipase A at the end of 1st, 2nd and 3rd intervals.

While 24.4%; 35.6% and 40.0% are the corresponding figures with lipase B.

**In "boiled" linseed oil:—**

The velocities of reaction in the three intervals are—

2.133; 4.075; 4.577 with lipase A.

and Nil; 0.663; 0.747 with lipase B.

Out of the total fats hydrolysed at the end of the third interval 19.8%; 37.8% and 42.4% are hydrolysed at the end of the three intervals with lipase A.

While the corresponding figures for the hydrolysis with the lipase B are — Nil: 47% and 53% respectively.

On comparing the results of percentage hydrolysis of crude oil at the end of 48 hours, a retardation of 76% occurs in the case of lipase B.

We find that the crude linseed oil is very easily attacked by castor bean lipase and also to a maximum limit, then comes in order "heated at 160°C for ½ hour, "neutralised" and "boiled" linseed oil. Thus we see that on the whole the percentage hydrolysis falls. By comparing the maximum hydrolysis result of 'crude' oil at the end of 48 hours with the others, we find a loss of 7%; 59% and 67% respectively for "heated", "neutralised" and "boiled" oils with lipase A.

Similarly on comparing the result obtained for maximum hydrolysis of crude linseed oil with lipase B with the others, a loss of 6%; 60%; 82% occurs for "heated", "neutralised" and "boiled" oils respectively.

**(2) Hydrolysis of Safflower Oil—**

TABLE No. 2

Safflower Oil.	Sap. Value c.cs.	Acid Value c.cs.	hydrolysis after (hours) %			
			3 hrs.	24 hrs.	48 hrs.	
1 Crude	34.45	0.368	A	6.355	11.412	18.505
			B	1.663	2.874	5.187

2 Neutralised	34.45	Nil	A	1.316	4.525	8.570
			B	0.316	1.663	3.598
3 Heated for $\frac{1}{2}$ hour. at 160°C	34.45	0.23	A	5.115	10.620	17.72
			B	1.060	2.009	4.47
4 Boiled	34.45	Nil	A	1.323	4.313	7.64
			B	Nil	1.130	2.11

Table No. II contains the results of the hydrolysis of safflower oil.

The conditions of experiment are similar to the previous one.

From the results tabulated in Table II we find that

In the "crude" safflower oil:—

The velocities of reaction in the three intervals are—

6.355; 5.057; 7.093 with lipase A.

and 1.663; 1.211; 2.313 with lipase B.

Out of the total fat split up at the end of 48 hours. 34.4%; 27.3%; 38.3% are split up at the end of 1st, 2nd and 3rd period of 3, 24, and 48 hours respectively with lipase A.

While 32.1%; 23.3%; 44.6% are the corresponding figures with the lipase B.

In the "neutralised" safflower oil:—

The velocities of reaction in the three intervals are —

1.316; 3.209; 4.045 with lipase A

and 0.316; 1.347; 1.935 with lipase B.

Out of the total fat split up at the end of 48 hours 15.3%; 37.5%; 47.2% are split up at the end of the 1st, 2nd and 3rd period with lipase A.

While the corresponding figures with lipase B are 8.8% ; 37.4% ; 53.8%.

**In the "heated" safflower oil:—**

The velocities of reaction in the three intervals are -

5.115 ; 5.505 ; 7.10 with lipase A

and 1.060 ; 0.949 ; 2.461 with lipase B.

Out of the total fat split up at the end of 48 hours 29% ; 31% ; 40% are split up at the end of the 1st, 2nd and 3rd periods with lipase A.

While with the lipase B the corresponding figures are 23.7% ; 21.3% ; 55.0% respectively.

**In "boiled" safflower oil:—**

The velocities of reaction in the three intervals are -

1.323 ; 2.990 ; 3.327 with lipase A

and Nil 1.13 ; 0.98 with lipase B.

Out of the total fat split up at the end of the 3rd period 17.3% ; 39.1% ; 43.6% are split up at the end of 1st, 2nd and 3rd periods by lipase A.

While the corresponding figures with lipase B are - Nil, 53.5% and 46.5% respectively.

Here again on comparing the results of hydrolysis obtained with lipase A and B we find that the percentage hydrolysis of the "crude oil" falls to the extent of 72% with the lipase B.

Next we find that the crude safflower oil is disintegrated to a maximum extent by castor bean lipase and then comes in order "heated", "neutralised" and "boiled" safflower oils.

Now by comparing the maximum hydrolysis percentage result of "crude" safflower oil at the end of 48 hours with the others, we find a loss of 4% ; 53% and 58% respectively for "heated", "neutralised" and "boiled" safflower oils with the enzyme lipase A.

Similarly the corresponding results obtained by comparing the maximum hydrolysis of 'crude' safflower oil with lipase B and the others, a percentage fall of 13%; 30%; 59% occurs for "heated", "neutralised" and "boiled" safflower oils.

(3) Hydrolysis of Coconut Oil:—

TABLE No. III

Cocoanut oil.	Sap. Value. e. cs.	Acid Value. e. cs.	hydrolysis after (hours) %			
			3 hrs.	24 hrs.	48 hrs.	
1 Crude	45.88	0.34	A	5.182	11.130	16.26
			B	1.313	2.681	4.73
2 Neutralised	45.18	Nil	A	3.432	8.99	12.932
			B	0.516	1.77	3.370
3 Heated for $\frac{1}{2}$ hour. @ 160°C.	45.88	0.31	A	4.646	10.720	15.606
			B	0.818	2.299	4.365
4 Boiled	45.88	Nil	A	1.894	6.190	9.586
			B	0.283	0.823	1.088

Table No. III records the values obtained for the percentage hydrolysis of the cocoanut oil with the castor seed lipases A & B.

The conditions of experiments are exactly similar to the previous one.

In "crude" cocoanut oil:—

The velocities of reaction in the three intervals are:—  
5.182; 5.948 5.130 with lipase A.  
and 1.313; 1.368; 2.049 with lipase B.

Out of all the total fats hydrolysed at the end of 48 hours, 32.0%; 36.5% and 31.5% takes place at the end of 1st, 2nd and 3rd intervals with lipase A.

While with the lipase B the corresponding figures are 27.7% 29.3% and 43.0%.

**In 'neutralised' cocoanut oil:—**

The velocities of reaction in the three intervals are - 3.432; 5.558; 3.942 with lipase A

and 0.516; 1.254; 1.600 with lipase B.

Out of all the total fats hydrolysed at the end of 48 hours, 26.5%; 43.0% and 30.5%; takes place at the end of the 1st, 2nd and 3rd intervals with lipase A.

Similarly with the lipase B the corresponding figures are 15.3; 37.2% and 47.5% respectively.

**In 'heated' cocoanut oil:—**

The velocities of reaction in the three intervals are - 4.646; 6.074; 4.886 with lipase A

and 0.918; 1.481; 2.066 with lipase B.

Out of all the total fats hydrolysed at the end of 3rd interval, 29.8%; 33.9% and 31.3% are hydrolysed at the end of 1st, 2nd and 3rd intervals with lipase A.

While 18.7%; 33.9% and 47.4% are the corresponding figures with the lipase B.

**In 'boiled' cocoanut oil:—**

The velocities of reaction in the three intervals are - 1.894; 4.296; 3.396 with lipase A

and 0.283; 0.540; 0.265 with lipase B.

Out of the total fats hydrolysed at the end of the 3rd interval, 19.7%, 44.8% and 35.5% are hydrolysed at the end of the 1st, 2nd and 3rd intervals with lipase A.

While with lipase B the corresponding figures are 26.0%; 49.6% and 24.4% respectively.

The fall in hydrolysis of the crude cocoanut oil with the lipase B is 70.9% on comparing the hydrolysis of crude cocoanut oil with the lipase A.

The crude cocoanut oil is easily split up by castor bean lipase and to a maximum limit, then comes the "heated at 160° C. for  $\frac{1}{2}$  hr., 'neutralised' and 'boiled' cocoanut oils.

Now by comparing maximum hydrolysis result of 'crude' cocoanut oil at the end of 48 hours with the others we find a retardation in the percentage hydrolysis to the extent of 4%; 20%; and 41% respectively with "heated" "neutralised" and "boiled" cocoanut oils with the lipase A.

The corresponding figures on comparing the result obtained for maximum hydrolysis of crude cocoanut oil with the lipase B with the others, a retardation in percentage hydrolysis to the extent of 7%, 28.7% and 77% occurs for 'heated', 'neutralised' and 'boiled' oils respectively.

Now comparing the three oils among themselves and the two lipases also, we find as follows :-

Linseed oil out of the three is most easily attacked and to the highest limit also. Then comes in order, the safflower oil and cocoanut oil. Comparing on the basis of maximum hydrolysis of linseed oil at the end of 48 hours, the safflower oil is hydrolysed 43% less than the linseed and cocoanut oil 50% less than the linseed oil. Such a difference can be attributed to the linseed oil being a drying oil with an iodine value of 165.54, and M.P.-19°C (Lewkowitsch), and the percentage of unsaturated or liquid acids being 91.54% and the saturated or solid acids being 8.3%.

Whilst the Safflower oil is a semi-drying oil with an iodine value of 141.5 and M.P.-16 to -20 (Lew.) the percentage of unsaturated or liquid acids being 94.76% and the saturated or solid acids being 5.23%.

The cocoanut oil is a non-drying oil with an iodine value of 9.96 and M.P. 25.3°C. and its unsaturated or liquid acids



being 17.39, and the saturated or solid acids being 82.71%. Therefore chemical composition and perhaps the M.P. is an important factor in the disintegration of fat by lipase.

Now comparing the results of 'neutralised' linseed, safflower and cocoanut oils with its 'crude' oil respectively, it is observed that there is an appreciable retardation in hydrolysis, showing that the oil refined by alkali treatment retards hydrolysis. J. J. Sudborough and H. E. Watson *J. Ind. Inst. Sc.* (1918, 1922, 2,213, 5,119) observed that certain crude oils after refining treatment with alkali did not undergo appreciable hydrolysis when acted upon by castor seed lipase in the absence of an activator, but addition of the volatile fatty acids (which had been removed during neutralisation and washing) as activator was found to restore digestibility of these oils. It was therefore assumed that the volatile acids present in the crude oil act as a natural activator on castor seed lipase.

Menezes and Banerjee (*Ind. Inst. of Sc.* 1939) noted that the rates of digestion of different oils are different after refining treatment and that the digestibility of gingili oil improves on neutralisation while the effect of refining does not cause an appreciable change in the digestibility of cocoanut oil either with or without activator using pancreatic lipase.

On comparing the maximum hydrolysis results after 48 hours of each of the three crude oils with that of its heated oils respectively, we find a retardation of 7.2%; 4.2% and 4.0% with the linseed, safflower and cocoanut oils with lipase A.

Similarly for each of the 'boiled' oils, the fall in percentage hydrolysis amounts to 67%; 58% and 41% on the basis of maximum hydrolysis of the crude oils.

Thus we see that on the whole by 'heating' and 'boiling' the oils a retardation in percentage hydrolysis occurs, and the fall is in keeping with the order of the oil, thus linseed (being a drying oil) is effected to a maximum limit, then comes safflower and lastly the cocoanut oil.

Tanaka's experiments (J. Coll. Eng. Imp. Univ. Tokyo, 1912, 5, 152, J. S. C. I. 1912, 31, 1084) shows that polymerised oils obtained by heating oils in a current of nitrogen are more slowly hydrolysed than the original oils from which they are derived and the effect is most marked with drying and the least marked with non-drying oils.

The effect of thermal treatment and hydrogenation on the absorption of a few vegetable oils has been studied by Roy (Ann. Biochem & exp. Med. 4.17, 1944). The author states that the change in viscosity of oils due to thermal treatment reduced the activity of emulsifying agents, thereby retarding lipase activity and absorption.

It is noticed that on heating, the linseed oil becomes more viscid than the other oils.

On comparing the activity of the two lipases A and B, A, dried below 40°C. is far more active than the lipase B dried at a temperature of about 45°C; on comparing the results of percentage hydrolysis of the three crude oils at the end of 48 hours, a retardation of over 70% occurs in the case of the lipase B.

Therefore the lipase is very susceptible to heat and tends to be inactive as the temperature increases even though the rise is only 7 to 8°C.

### SECTION III

#### A Study of the rate of fall of hydrolysis on heating of

- (1) linseed oil,
- (2) safflower oil, and
- (3) cocoanut oil.

Having found that on heating the oils a remarkable retardation in its rate of hydrolysis was observed, it was thought desirable to study the course of change i.e. inactivation of natural activator.

Samples of crude oil were heated at a constant temperature of 160°C. for 3 hours and test samples were taken out at an interval of 1, 2 and 3 hours each, and these samples were hydrolysed, keeping all the other factors, such as buffer used, substrate concentration, enzyme concentration, etc. constant as stated in the previous section, but only lipase A which was found to be far more active than lipase B was used.

TABLE No. 4  
Rate of fall of hydrolysis on heating  
(1) linseed oil.

Linseed oil.	Sap. value	Acid value.	hydrolysis after (hours) %.		
			3 hrs.	24 hrs.	48 hrs.
1 Crude (unheated)	c.cs. 34.43	c.cs. 1.23	11.206	19.03	32.84
2 Heated at 160°C. for 1 hour.	34.43	1.1	7.863	15.58	28.72
3 Heated at 160°C. for 2 hours.	34.43	1	5.742	13.71	22.49
4 Heated at 160°C. for 3 hours.	34.43	0.94	3.987	9.514	16.92

In the table No. 4 the results of linseed oil heated for 1, 2, 3 hours are recorded, now considering the figures for -

“linseed” oil heated at 160°C. for 1 hour -

The velocities of reaction in the three intervals are -  
7.863; 7.717; 13.140.

Out of the total fats split up at the end of the third interval 27.0%; 26.8% and 46.2% are hydrolysed by the lipase at the end of the 1st, 2nd and 3rd intervals.

"linseed" oil heated at 160°C. for 2 hours. -

The velocities of reaction in the three intervals are - 5.742; 7.968; 8.780.

Out of the total fats hydrolysed at the end of the third interval 25.5%; 35.5%; and 39.0% are hydrolysed by the lipase at the end of the 1st, 2nd and 3rd intervals.

"linseed" oil heated at 160°C. for 3 hours. -

The velocities of reaction in the three intervals are - 3.987; 5.527; 7.406.

Out of the total fats split up at the end of the third interval by the lipase 23.6%; 32.7%; 43.7% are split up at the end of 1st, 2nd and 3rd intervals respectively.

The retardation in percentage hydrolysis, comparing on the basis of total hydrolysis of crude linseed oil at the end of 48 hours is 12.5%, 31% and 48% respectively for heating the oil at 160°C. for 1, 2 and 3 hours.

**Rate of fall of hydrolysis on heating  
(2) safflower oil.**

TABLE No. 5

Safflower oil	Sap. value c.es.	Acid value c.es.	hydrolysis after (hours) %.		
			3 hrs.	24 hrs.	48 hrs.
1 Crude (unheated)	34.45	0.368	6.355	11.412	18.505
2 Heated at 160°C. for 1 hour.	34.45	0.318	4.161	9.361	16.131

3 Heated at 160°C. for . 2 hours.	34.45	0.26	2.990	7.713	14.630
4 Heated at 160°C. for 3 hours.	34.45	0.21	1.715	6.454	11.260

The table No. 5 contains the results of the percentage hydrolysis of safflower oil on heating for 1, 2 and 3 hours. The conditions of experiment are identical to the previous one.

**In "safflower" oil heated at 160°C. for 1 hour.**

The velocities of reaction at the end of the third interval are - 4.16; 5.20; 6.77.

Out of all the total fats split up at the end of the third interval 25.8%; 32.4%; 41.8% are split up by the lipase at the end of the 1st, 2nd and 3rd intervals.

**In "safflower" oil heated at 160°C. for 2 hours.**

The velocities of reaction at the end of 3rd interval are - 2.99; 4.72; 6.92.

Out of the total fats split up at the end of 3rd interval 20.4%; 32.3%; and 47.3% are split up at the end of 1st, 2nd and 3rd intervals.

**In "safflower" oil heated at 160°C. for 3 hours.**

The velocities of reaction at the end of the third interval are 1.71; 4.74 and 4.81.

Out of all the total fats split up at the end of the third interval 15.2%; 42.1% and 42.7% are split up by the lipase at the end of 1st, 2nd and 3rd intervals.

The fall in percentage hydrolysis of the heated oils is 12.8%; 20.9% and 39.0% for the oils heated at 160°C. for 1, 2 and 3 hours, comparing on the basis of maximum hydrolysis of the crude safflower oil.

**Rate of fall of hydrolysis on heating  
(3) cocoanut oil.**

TABLE No. 6

Cocoanut oil.	Sap. value c.c.s.	Acid value c.c.	hydrolysis after (hours.) %		
			3 hrs.	24 hrs.	48 hrs.
1 Crude (unheated)	45.88	0.34	5.182	11.13	16.26
2 Heated at 160°C. for 1 hour.	45.88	0.32	3.812	10.12	14.65
3 Heated at 160°C. for 2 hours.	45.88	0.26	3.017	8.751	12.96
4 Heated at 160°C. for 3 hours.	45.88	0.24	2.258	7.98	11.23

Table No. 6 records the values obtained for the hydrolysis of the heated cocoanut oil for a varying period of 1, 2, and 3 hours at a constant temperature of 160°C. The conditions of experiments are exactly similarly to the previous one.

In 'cocoanut' oil heated 160°C. for 1 hour.

We find that the velocities of reaction at the end of the third interval of 48 hours are — 3.812; 6.308; 4.530.

Out of the total fats hydrolysed at the end of the third interval 26%; 43% and 31% are hydrolysed at the end of the 3 intervals of 3, 24 and 48 hours.

In 'cocoanut' oil heated at 160°C. for 2 hours.

The velocities of reaction at the end of the third interval are 3.017; 5.734; 4.209.

Out of the total fats hydrolysed at the end of the third interval 23.2%; .442% and 32.6% are hydrolysed at the end of the 1st, 2nd and 3rd intervals.

**In 'cocoanut' oil heated at 160°C. for 3 hours.**

The velocities of reaction at the end of the third interval are 2.258; 5.724; 3.248.

Out of the total fats hydrolysed at the end of third interval 20.1%; 50.9%; 29.0% are hydrolysed at the end of the 1st, 2nd and 3rd intervals.

A retardation in percentage hydrolysis amounting to 9.9%, 20.3%, and 30.9% respectively are obtained for heated oils at an interval of 1, 2 and 3 hours, on the basis of maximum hydrolysis of the crude cocoanut oil at the end of 48 hours.

Taking into consideration all the facts as stated above, which clearly indicate that on heating the oils some substance which acts as a natural activator to the castor seed lipase is rendered inactive.

The next explanation for the fall in percentage hydrolysis is perhaps the loss of free fatty acids and volatile acids, which must be lost on heating and retard hydrolysis; for, in the case of cocoanut oil, illipe oil and mohua oil (J. J. Sudborough, H. E. Watson, Part V, Vol. 5 Part IX pp. 120-122-J. Ind. Inst. Sc. 1922) they found possible to isolate the volatile acids and solutions of such acids were used as activator instead of acetic acid and it was found that crushed castor seeds produced appreciable hydrolysis.

It is also interesting to note from the tables Nos. (1 to 6) that the acid values of the oils falls on heating and boiling as for e.g. the acid value of linseed oil is 1.23 c.cs. while for the boiled oil is 0.768 c.cs.

Besides, in a hot country like India, oils and fats are liable to go rancid if kept for a long period and again in the process of frying, roasting, and baking, oils and fats are exposed to

high temperatures, as well as to air and are liable to oxidation which is a serious drawback for it does effect the process of digestion.

While this lower digestibility may not matter much in the case of healthy person whose system is strong and normal and whose bile and other secretions are regular and sufficient, it will not be the same in the case of a weak or a sick person, if the natural activators are lost or the process of digestion delayed then it is a strain on the system. Menezes and Banerjee (J. Ind. Inst. Sc. 1938) also found that on heating the cocoanut, cotton-seed, gingilli oils a gradual fall in digestibility occurs, working with the pancreatic lipase.

#### SECTION IV

**A comparative study of the hydrolysis of (1) oils (2) hydrogenated products and (3) ghees.**

TABLE No. 7

Substrate used.	Sap. value.	Acid value.	hydrolysis after (hours.) %.		
			3 hrs.	24 hrs.	48 hrs.
	c. cs.	c. cs.			
1 Linseed oil.	34.42	1.23	11.206	19.030	32.840
2 Safflower oil.	34.45	0.368	6.355	11.412	18.505
3 Cocoanut oil.	45.88	0.34	5.182	11.130	16.260
4 Hydrogenated product "X"	34.43	Nil	2.030	7.530	10.670



5 Hydrogenated product "Y"	47.27	0.096	2.072	8.480	13.280
6 Ghee (Cow's Milk).	41.17	0.380	4.236	10.790	15.586
7 Ghee (Buffalo's Milk).	40.80	0.239	4.728	13.600	21.540

In table No. 7 the results of the comparative study of the three oils with hydrogenated vegetable products "X" and "Y" available in the market as well as pure home made ghee from cow's milk and ghee from buffalo's milk are recorded.

The velocities of reaction and the percentage of total fats hydrolysed at the end of 1st, 2nd and 3rd intervals for linseed, safflower and cocoanut oils are recorded in section II pages 16, 19 and 22.

Now considering the figures for:—

The "X" hydrogenated product:—

We find that the velocities of reaction in the three intervals are 2.03; 5.50; 3.14.

Out of the total fats split up at the end of the 3rd interval by the lipase 19.0%; 51.6% and 29.4% are split up at the end of 1st, 2nd and 3rd intervals of 3, 24 and 48 hours.

In "Y" hydrogenated product:—

The velocities of reaction in the three intervals are 2.072; 6.408; 4.800.

Out of the total fats hydrolysed at the end of the 3rd interval 15.6%; 43.3% and 36.1% are hydrolysed at the end of the 1st, 2nd and 3rd intervals.

### In the ghee (cow's milk)-

The velocities of reaction in the three intervals are 4.236; 6.554; 4.796.

Out of the total fats hydrolysed at the end of 3rd interval 27.2%, 42.0% and 30.8% are hydrolysed by the lipase at the end of the 1st, 2nd and 3rd intervals.

### In the ghee (buffalo's milk)-

The velocities of reaction in the three intervals are 4.728; 8.872; 7.940.

Out of the total fats hydrolysed at the end of 3rd interval 21.9% 41.2% and 36.9% are hydrolysed at the end of 1st, 2nd and 3rd intervals.

On comparing the results obtained for the maximum hydrolysis of each of the different oils, ghees and the hydrogenated products, at the end of 48 hours it is found that linseed oil is hydrolysed to the maximum limit, then comes in order the buffalo ghee, safflower oil, cocoanut oil, cow's ghee, "Y" and "X" hydrogenated vegetable products respectively.

Now comparing the results of percentage hydrolysis on the basis of maximum hydrolysis of the linseed oil at the end of 48 hrs., the descending order for the loss in percentage hydrolysis is 34%; 43%; 52%; 60% and 67% respectively with the buffalo ghee, safflower oil, cocoanut oil, cow ghee, hydrogenated products "Y" and "X".

Out of the two "X" and "Y" hydrogenated products selected we find that they are the least hydrolysed on comparing with oils and ghees.

The low percentage hydrolysis is always mainly attributed to the M. P. now in the case of hydrogenated product "X" the M. P. is 39.5°C. while the M. P. of 3 oils are very low, the highest being 25.3°C. for cocoanut oil; for if the M. P. of any fat is higher than the body temperature 37.5°C, it will remain

in a solid form in the incubator and the enzyme lipase may not come into contact as easily as in the case of liquid fats and hinder the process of emulsion thereby retarding lipase action.

Some workers state that perhaps the degree of unsaturation may effect hydrolysis but Roy (Annual Review of Biochem. and Allied Research in India 1944) studied the effect of thermal treatment and hydrogenation on the absorption of a few vegetable oils and found that hydrogenation of groundnut oil and cocoanut oil to different degrees does not effect absorption or the saponification value of these oils; therefore it is evident that the degree of unsaturation does not affect the absorption of oils. The author remarks that the oils which are emulsified to a lesser extent on any treatment lowers the rate of hydrolysis.

Perhaps there may be some other factor accounting for this low hydrolysis, may be "X" and "Y" are the hydrogenated products of mixed oils or fats, or different treatment in the process of hydrogenation itself may account for the lower digestibility of the two products. "Y" is hydrolysed slightly more than "X".

Now between the two ghees the buffalo ghee is split up more than the cow ghee, on comparing the percentage hydrolysis of these two ghees with the three oils, we find the buffalo ghee is less hydrolysed than linseed and more hydrolysed than cocoanut or safflower oil, while the three oils are more hydrolysed than the cow ghee.

The values for cocoanut oil and cow's milk ghee lie very close together, only a difference of 0.7% between them. Dastur and Giri (Proc. Soc. Biol. chem. Ind. 1936, 1,40) also studied the invitro digestion of butter fat and other edible oils and found that butter fat and cocoanut oil are almost equally digestible while sesame and groundnut oil are much less hydrolysed by the pancreatic lipase.

## SECTION V

The effect of -

(1) NaCl, (2) Cane Sugar, (3) Vitamins, on the Hydrolysis of Oils by Castor Seed Lipase.

(1) Effect of NaCl:—

No diet is complete without the addition of common salt, besides improving the taste of foodstuffs, it was realised that sodium chloride is one of the most essential requirements of living tissues. Common salt, as we know, causes an increased secretion of saliva and gastric juice thereby increasing the quantity of enzyme to react with the foodstuffs, naturally there is bound to be an increased hydrolysis of the food constituents, therefore it is intended to study in this section, whether the addition of salt, has any influence on the digestibility of the oils.

K. G. Falk (J. Am. Chem. Soc. 35, 601, 1913) has studied the action of neutral salts of the uni-uni, uni-bi, bi-bi valent types on the activity of castor bean lipase; decreased activity was shown by all the uni-univalent salts, by the chlorides and nitrates of Ba and Ca (except for the most dilute solutions) and magnesium, by NaCl and dilute solutions of sodium sulphate.

Increased activity was shown by dilute solutions of chlorides of Ba and Ca and more concentrated solutions of  $\text{Na}_2\text{SO}_4$ ,  $\text{MgSO}_4$ , and  $\text{MnSO}_4$ . Glick and King investigated the relationship between the activation of pancreatic lipase and the surface effects of the compounds involved the mechanism of inhibition and activation (Jr. Biol. Chem. 97, 675, 1932).

Parfentjev and Devrient have studied the influence of sodium taurocholate and copper sulphate on lipase.

Dastnr and Giri (Proc. Soc. Biol. Chem. Ind. 1936, 1, 40) working on the in-vitro digestion of butter fat and edible oils found that sodium taurocholate exerts a selective action in the digestion of fats, because it activates the digestion of butter-

fat and cocoanut oil by pancreatic lipase, while the digestion of sesame and groundnut oil is not affected by the salt

Jerroine (Biochem. Z. 23, 429, 1910) found that the dilute solutions of NaCl, bromide and iodide increased the percentage hydrolysis of olive oil by pancreatic lipase, while concentrated solution decreases the activity. Similar effects were noted by Loevenhart and Pierce on the animal lipase (J. Biol. Chem. 2, 397, 1917) while Hansik (Z. Physiol. Chem. 71, 246, 1911) noticed retarding action of sodium chloride with pancreatic lipase and olive oil.

(1) Effect of NaCl on the hydrolysis of oils.-

TABLE No. 8

Each set of experiments contains 10, 20, 40 mgms. of NaCl, in addition to 2 c.cs. of the oil, 2 c.cs. of the buffer of 4.8 pH and 100 mg. of castor seed lipase and incubated for a period of 24 hrs. and 48 hrs. at 37.5°C.

The extent of hydrolysis is expressed in terms of c.cs. of 0.1 N NaOH.

Substrate	NaCl mgms.	Hydrolysis in terms of c.cs. of N/10 NaOH	
		24 hrs.	48 hrs.
1 Linseed oil	0	13.39	21.70
	10	12.28	16.15
	20	10.35	15.19
	40	8.90	14.50
2 Safflower oil	0	7.64	11.99
	10	6.96	9.00
	20	5.80	8.32
	40	5.80	7.45
3 Cocoanut oil	0	9.57	13.73
	10	8.51	11.99
	20	7.54	11.32
	40	7.00	10.83

It is observed from the above table that NaCl retards hydrolysis of oil with the castor seed lipase as concentration increases but the retardation is not proportional to the increase in concentration of NaCl. The decrease is more marked in linseed than in safflower and cocoanut oils.

**(2) Effect of cane sugar on the hydrolysis of oils :-**

Next experiments were carried out exactly as stated above but using 10, 20, 40 mgms. of cane sugar, to see whether it affects the hydrolysis being the next common item in the diet.

**TABLE No. 9**

Substrate.	Sugar mgms.	Hydrolysis in terms of N/10 NaOH.	
		24 hrs. c.cs.	48 hrs. c.cs.
1. Linseed oil	0	13.39	21.70
	10	13.15	21.19
	20	12.96	20.90
	40	12.19	19.90
2. Safflower oil	0	7.64	11.99
	10	7.64	11.99
	20	7.25	11.70
	40	6.86	11.22
3. Cocoanut oil	0	9.57	13.73
	10	9.57	13.54
	20	9.48	13.44
	40	8.50	13.25

It is seen from the above table that there is no action of the sugar at a small concentration and there is a slight retardation at higher concentrations on the hydrolysis of oils with castor seed lipase.

The retarding effect of sugar is more marked in the case of linseed oil than safflower and cocoanut oils.

### (3) The Effect of vitamins on the hydrolysis of oils:-

It is well known that vitamins are an essential factor in nutrition, and normal life is not possible in the absence of these from the diet.

However none of the vitamins are affected by digestion, they are absorbed as such from the intestinal canal and utilised in the system for growth and repair of tissues. Their presence affects the digestibility of other food constituents. Thus Martin E.E. (Biol. Abs. Vol. 13, 9095, 1939) studied the effect of carotene and ascorbic acid on the hydrolytic and synthetic activity of lipase and found that carotene inhibited fat hydrolysis while ascorbic acid stimulated hydrolysis of fats by pancreatic lipase.

While Mikhlin (Chem. Abstracts 1943 pp. 922) studied the influence of ascorbic acid and other oxidising substances on the pancreatic lipase. The auto-oxidation of ascorbic acid increased appreciably the activity of pancreatic lipase.

Arnulf Purr (Biochem. Jr. 1934, 28, 1141) has studied the influence of vitamin C (ascorbic acid) on plant and animal lipase. He concluded that vitamin C has no action on the lipase.

The vitamins themselves do not undergo any material change during the process of digestion still we see that they effect the digestibility of other food constituents more or less.

The vitamin products used in the present investigations were bought from Indo-Pharma, Pharmaceutical works, Bombay.

The effect of the following vitamins on the hydrolysis of oils by castor seed lipase is studied :—

(1) Vitamin A.

(2) Thiamin Chloride ... Vitamin B<sub>1</sub> - Antineuratic or Anti-beri-beri vitamin

(3) Nicotinic acid ... Vitamin B (complex P. P. Factor Antipellagra vitamin factor.

- (4) Riboflavin ... Vitamin B<sub>2</sub> indicated, in growth disturbances, and gastro intestinal diseases.
- (5) Ascorbic Acid ... Vitamin C-Antiscorbutic vitamin.
- (6) Vitamin K ... Antihemorrhagic or coagulation vitamin.

#### Effect of vitamins on the hydrolysis of oils :-

Procedure: 2 c.cs. of the oil, 100 mgms. of castor seed lipase, 2 c.cs. of buffer solution of 4.8 pH and 10 mgms. and 30 mgms. of vitamins kept in an incubator at 37.5°C. for 24 hrs. The extent of hydrolysis is expressed as c.cs. of N/10 NaOH.

Initial acidity of the vitamin products were determined by dissolving them either in water or neutral alcohol. Strength of vitamins used were 10 mgms. and 30 mgms.

TABLE No. 10

Blank experiment (without vitamins).	—	13.35 c.cs
With vitamins (after deducting the initial acidity):	10 mgms.	30 mgms.
(1) Thiamin chloride-Vit. B <sub>1</sub> ...	13.84 c.cs.	13.86 c.cs.
(2) Nicotinic Acid-B. comp. ...	13.38	13.44
(3) Riboflavin - Vit. B <sub>2</sub> ...	12.77	13.06
(4) B. Complex (10 mgs. of Vit. B <sub>1</sub> ; B <sub>2</sub> ; Nic. A).	13.06	
(5) Ascorbic acid (Vit. C) ...	12.01	11.41
(6) Vitamin K ...	13.35	13.35
(7) Vitamin A -	12.96 c.cs.	

From the above table we find that thiamin chloride and to a slight extent nicotinic acid has an accelerating effect on hydrolysis of linseed oil with castor bean lipase, while



vitamin C, riboflavin vitamins B<sub>1</sub>, B<sub>2</sub>, Nic. acid used in a syngenous condition retard hydrolysis.

Vitamin A as such cannot be compared with the above vitamins as the quantity used was much greater than 30 mgms. This vitamin A was a highly active standardised solution of vitamin A (British Drug Houses Ltd. London - A Physician's sample) was also found to retard hydrolysis.

Similar effects were also noted in the safflower oil.

Blank experiment (without vit.) - 7.64 c.es.

	10 mgms.	30 mgms.
With vitamins (after deducting initial acidity).		
(1) Thiamin chloride	7.94 c.es.	8.06 c.es.
(2) Nicotinic acid	7.76	7.56
(3) Riboflavin	7.25	7.06
(4) Mixed (Vit. B <sub>1</sub> , B <sub>2</sub> and Nic. A, 10 mgms. each).	7.41	
(5) Ascorbic acid	6.59	6.62
(6) Vitamin K	7.51	7.64
(7) Vitamin A	7.06 c.es.	

## SECTION VI

### A study of the hydrolysis of oils by the lipase from the seeds of *Butea Frondosa*.

Besides the lipase from the castor seeds numerous other seeds also contain lipolytic enzymes.

At the Nowrosjee Wadia College, Sahasrabudhe and Lilaovala studied the enzyme lipase from the groundnut and linseed with groundnut, linseed and sesame oils as the substrate.

N. R. Chatterjee, S. Gosh and R. N. Chopra (J. Ind. Chem, Soc. 1938 Pages 101, 107) were the first to investigate and confirm the presence of both proteolytic and lipolytic enzymes

from the seeds of *Butea Frondosa*. Its lipolytic action was studied in substrates like olive oil, rape seed oil and castor oil and was found to act equally well on all these.

*Butea Frondosa* is a common Indian plant and a fair amount of chemical work has been carried out on all the constituents, flowers, gum and seeds.

There being no other work on the enzyme from the seeds of *Butea Frondosa* in the literature after 1938, the present section deals with the lipase action on the oils, linseed, safflower and cocconut and also a comparative study of the lipase action from the *Butea Frondosa* and castor seed.

#### **Extraction of the enzyme from seeds of *Butea Frondosa* :—**

The seeds were powdered well and six times the weight of carbon tetrachloride was added and vigorously shaken in a flask for some time. The solid material was filtered by means of the suction pump and washed first with carbon tetrachloride and then with ether. It was then dried and stored in a bottle with the addition of a few drops of toluene.

N. R. Chatterjee, S. Gosh and R. N. Chopra (J. Ind. Chem. Soc. 1938, 107) found that the defatted seeds showed strong lipolytic action but the results obtained were not uniform and therefore they used an enzyme extract with success, their method is followed in the present investigation.

#### **Preparation of Aqueous Emulsion of enzyme :—**

10 gms. of the defatted seeds were treated with 60 c. cs. of distilled water, added in small portions and rubbed intimately in a mortar, the cream was pressed out through a muslin cloth and the residue again treated in the same manner with 60 c. cs. of distilled water and pressed out. The total extract amounted to 100 c. cs; therefore 10 c. cs. of this enzyme extract amounted to 1 gm. of the enzyme. The enzyme extract was always prepared fresh each time, as a sample of the extract was kept for 48 hours and then used for the hydrolysis of the oil in one of the trial experiments and it was found that the lipase activity decreased to the extent of 60%.

Trial experiments were first carried out to determine the factors, such as buffer used, substrate concentration and enzyme concentration.

#### Determination of the pH for the enzyme lipase :—

According to Chatterjee, S. Gosh and R. N. Chopra (J. In. Chem. Soc. 1938, 107) the lipase seems to act best in slightly alkaline medium, the optimum pH being near about 8 varying slightly according to the buffer employed. Buffer solutions of 0.2 M disodium phosphate and 0.1 M citric acid were prepared, a range of buffer mixtures from acidic to slightly alkaline i.e. from 6 pH onwards to 8 pH were made and the following experiments carried out to determine the right pH value for the enzyme lipase prepared from the seeds of *Butea Frondosa*.

Each set of experiments contains 1 c. c. of cocoanut oil, 2.5 c.cs. of the enzyme extract equivalent to 0.25 gm. of the lipase and 2 c.cs. of varying pH value and incubated for a fixed period of 24 hours at 37.5°C.

TABLE No. 11.

The saponification value of the oil = 45.88 c.cs.  
of N/10 alkali for 1 gm. oil.

Acid value = 0.34 c.cs. of N/10 alkali for 1 gm. of oil.

Set.	Oil in c. cs.	pH value.	hydrolysis (in 24 hours).
1	1	6	0.708%
2	1	6.5	1.170%
3	1	7	1.510%
4	1	7.5	2.360%
5	1	8	3.977%

The maximum pH required for hydrolysis was found out for the enzyme lipase as shown in table No. 11 was 8 pH,

**Effect of Buffer concentrations on the rate of hydrolysis :-**

**TABLE No. 12**

Each set of experiments contains 1 c.c. of cocoanut oil, 2.5 c.cs. enzyme extract equivalent to 0.25 gm. of lipase and varying quantities of the buffer solution of 8 pH and incubated for a fixed period of 24 hours at 37.5°C.

Saponification value of the oil = 45.88 c.cs.

Acid value ... .. = 0.34 ,,

Set	Oil in c.cs.	Quantity of buffer added c.cs.	hydrolysis (in 24 hrs.) %
1	1	1	2.36
2	1	2	3.31
3	1	3	2.59
4	1	4	1.65
5	1	5	1.17

It will be observed from the table No. 12 that the optimum hydrolysis occurs at the concentration of 2 c.cs. it being 3.31 and subsequently falling to a minimum 1.17%, on increasing the concentrations to 5 c.cs. hence buffer concentrations also influences in the course of hydrolysis of oils.

**Effects of variation in the substrate concentration on the course of hydrolysis :-**

**TABLE No. 13**

Each set of experiments contains varying quantities of cocoanut oil, 2 c.cs. of buffer of 8 pH 2.5 c.cs. of the enzyme extract and incubated for a fixed period of 24 hours at 37.5°C.

Saponification value of the oil = 45.88 c.cs.

Acid value ... .. = 0.34 ,,

Set.	Oil in c.cs.	hydrolysis (in 24 hrs.)
1	1	3.077 %
2	2	3.533 %
3	3	3.140 %
4	4	2.650 %

From table No. 13 it will be seen that a maximum hydrolysis of the oil occurs when the substrate concentration is 2 c.cs. of oil or its equal weight.

TABLE No. 14

Each set of experiments contains 2 c.cs. of cocoanut oil, 2 c.cs. of activator or buffer of '8 pH, and varying quantities of the enzyme extract and incubated for a fixed period of 24 hours at 37.5°C.

Sap. value of the oil = 45.88 c.cs.

Acid value ... .. = 0.34 "

Set	Oil in c.cs.	Enzyme extract. c.cs.	hydrolysis (in 24 hrs.)
1	2	2.5	3.53 %
2	2	5	4.36 %
3	2	7.5	4.71 %
4	2	10	4.94 %

It is observed that for an increased quantity of enzyme extract the hydrolysis is increased, the difference in the figures for the percentage hydrolysis of 2.5 enzyme extract cream with the others is 0.827, 0.35, 0.23 with 5 c.cs., 7.5 c.cs. and 10 c.cs. of the enzyme extract, therefore with the increase in the strength of the hydrolysing agent, there is a slight increase though not proportional to the substrates hydrolysed.

For the present investigation 5 c.cs. enzyme extract was chosen for greater increase was noted here than in 7.5 c.cs. or 10 c.cs.

## SECTION VII

**A study in the hydrolysis of (1) linseed oil, (2) safflower oil, (3) cocoanut oil with the enzyme lipase from *Butea Frondosa* seeds.**

A. In this section the study of the effects of refining and heating on the hydrolysis of the linseed, safflower and cocoanut oils with the enzyme lipase from the seeds of *Butea Frondosa* is carried out to compare the two lipase actions i.e. with the castor seed.

The oils were refined and heated exactly as done before.

Each of the 4 samples of the three oils - crude, neutralised, heated for  $\frac{1}{2}$  hr. at  $160^{\circ}\text{C}$ . and boiled were hydrolysed with lipase from *Butea Frondosa*.

### **Method of hydrolysis :-**

The quantity of the oil used was 2c.cs. and its weight determined, Buffer 2 c.cs. of 8pH, and the enzyme added was 5 c.cs. of the enzyme extract equivalent to 0.5 gm. of the defatted seeds and incubated for varying time from 3 to 24 hours at  $37.5^{\circ}\text{C}$ ,

The above factors were determined as will be seen from the previous section, for getting the maximum hydrolysis of the oils under consideration.

At the end the mixture was diluted with 20 c.cs. of water and 80 c.cs. of alcohol and titrated with N/10 NaOH using phenolphthalein as indicator. A control experiment without the enzyme extract was done in every case. The difference in the amount of N/10 NaOH required gave a measure of the lipase action and the results are expressed as the percentage of the total oil hydrolysed at the different periods.

The saponification values of the oils in question were determined in terms of c.es. of N/10 alkali.

**Hydrolysis of the linseed oil with the *Butea Frondosa* lipase.**

TABLE No. 15.

Linseed Oil.	Sap. value c.es.	Acid value c.es.	hydrolysis after (hrs.)%	
			3 hrs.	24 hrs.
1 Crude ...	34.43	1.230	3.014	6.822
2 Neutralised ...	34.10	nil	1.853	4.261
3 Heated @ 160°C for ½ hr.	34.43	1.100	2.847	6.346
4 Boiled ...	34.43	0.768	2.190	4.380

Considering the time factor from the table No. 15 we find that the maximum hydrolysis is attained at the end of 24 hours and no further hydrolysis takes place even at the end of 48 hours as recorded with the castor seed lipase.

From the figures in the table No. 15 we find that

**In the "crude" linseed oil**

The velocities of reaction in the two intervals are - 3.014 and 3.808 with *Butea Frondosa* lipase.

Out of the total fats hydrolysed at the end of 24 hours 44.2% and 55.8% are hydrolysed at the end of 1st and 2nd intervals of 3 and 24 hours each with the lipase from *Butea Frondosa*.

**In "neutralised" linseed oil**

The velocities of reaction in the two intervals are - 1.843 and 2.418.

Out of the total fats hydrolysed at the end of the 2nd interval of 24 hours 43.2% and 56.8% are hydrolysed by the lipase at the end of the 1st and 2nd intervals.

In the linseed oil "heated" at 160°C. for  $\frac{1}{2}$  hour.

The velocities of reaction in the two intervals are - 2.847; 3.499.

Out of the total fats hydrolysed at the end of the 2nd interval 44.8% and 56.2% are hydrolysed by the lipase at the end of the 1st and 2nd intervals.

In "boiled" linseed oil

The velocities of reaction in the two intervals are - 2.19; 2.19 respectively.

Out of the total fats hydrolysed at the end of the 2nd interval of 24 hours 50% and 50% are hydrolysed by the lipase at the end of the 1st and 2nd intervals.

By comparing the maximum hydrolysis result of the crude linseed oil at the end of 24 hours with the others, a retardation of 6.9%; 35.8% and 37.5% occurs with "heated", "boiled" and "neutralised" linseed oils with lipase from *Butea Frondosa*.

#### Hydrolysis of the safflower oil with *Butea Frondosa* lipase

TABLE No. 16

Safflower oil	Sap. value c.cs.	Acid value c.cs.	percentage hydrolysis after (hours)	
			3 hrs.	24 hrs.
1 Crude ...	34.45	0.368	2.621	5.394
2 Neutralised	34.45	nil	1.830	3.400
3 Heated at 160°C. for $\frac{1}{2}$ hour.	34.45	0.23	2.457	5.130
4 Boiled ...	34.45	nil	1.880	3.584



Table No. 16 contains the results of the hydrolysis of safflower oil. The conditions of experiment are similar to the previous ones.

From the above results we find that:—

**In the "crude" safflower oil**

The velocities of reaction in the two intervals are—2.621; 2.773.

Out of the total fats split up at the end of 24 hours, 48.6% and 51.4% are split up at the end of the 1st and 2nd intervals.

**In the "neutralised" safflower oil**

The velocities of reaction in the two intervals are—1.83; 1.57.

Out of the total fats split up at the end of 24 hours 53.8% and 46.2% are split up at the end the 1st and 2nd intervals.

**In the "heated" safflower oil**

The velocities of reaction in the two intervals — 2.457; 2.673.

Out of the total fats split up at the end of 24 hours 47.8% and 52.2% are split up by the lipase as the end of the 1st and 2nd intervals.

**In the "boiled" safflower oil**

The velocities of reaction in the two intervals are - 1.880; 1.704.

Out of the total fats split up at the end of the 2nd interval 52.5% and 47.5% are split up at the end of the 1st and 2nd intervals.

Comparing the loss percentages of heated and refined oils with the maximum hydrolysis at the end of 24 hours of crude safflower oil and the others the results obtained are 4.8%; 33.5% and 36.9% retardation for "heated", "boiled" and "neutralised" safflower oil.

Hydrolysis of cocoanut oil with *Butea Frondosa* lipase

TABLE No. 17

Linseed oil	Sap. value c.cs.	Acid value c.cs.	hydrolysis after hours-%	
			3 hrs.	24 hrs.
1 Crude	45.88	0.34	2.119	4.230
2 Neutralised	45.18	nil	0.962	2.910
3 Heated at 160°C for $\frac{1}{2}$ hour.	45.88	0.31	1.982	4.00
4 Boiled	45.88	nil	1.078	3.040

## In "crude" cocoanut oil

The velocities of reaction in the two intervals are — 2.119 and 2.111 with *Butea Frondosa* lipase.

Out of the total fats hydrolysed at the end of the 2nd interval of 24 hours 50.1% and 49.9% are hydrolysed by the lipase at the end of the 1st and 2nd intervals.

## In "neutralised" cocoanut oil

The velocities of reaction in the two intervals are — 0.962; 1.948.

Out of the total fats split up at the end of the two intervals 33% and 67% are split up at the end of the 1st and 2nd intervals of 3 and 24 hours.

## In "heated" cocoanut oil

The velocities of the reaction in the two intervals are — 1.982 and 2.018.

Out of the total fats split up at the end of two intervals 49.5% and 50.5% are split up at the end of the 1st and 2nd intervals by the lipase.

### In "boiled" cocoanut oil

The velocities of reaction in the two intervals are — 1.078 and 1.962.

Out of the total fats hydrolysed at the end of 48 hours 35.5% and 64.5% are hydrolysed at the end of the 1st and 2nd intervals by the lipase.

Now by comparing on the basis of maximum hydrolysis of the crude oil at the end of 24 hours the retardation is as follows:—5.4% ; 28.2% ; and 31.2% with "heated", "boiled" and "neutralised" cocoanut oil.

Now comparing the three oils among themselves, we find that the linseed oil out of the three is attacked to the highest limit by the *Butea Frondosa* lipase, then come in order the safflower oil and cocoanut oil. By comparing on the basis of maximum hydrolysis for linseed oil at the end of 24 hours, the safflower oil is 20.9% less than the linseed and cocoanut oil 37.9% less than the linseed oil.

Then comparing the two lipase actions from the Section II Table Nos. 1, 2 and 3 for castor seed lipase on the three oils and the results obtained in the above tables Nos. 15, 16 and 17, for the hydrolysis of the three oils with *Butea Frondosa* lipase the first thing noticed is that the hydrolysing activity of *Butea Frondosa* reaches its maximum after 24 hours and stops at this stage, there being no further oil split up after this period of 24 hours, while in the case of castor seed lipase, the results of hydrolysis are recorded even for 48 hours.

Both *Butea Frondosa* and castor seed lipases split the linseed oil to the maximum limit then come in order the safflower and cocoanut oils.

The descending order of reaction in the three oils refined and heated, hydrolysed by *Butea Frondosa* lipase is "crude", "heated", "boiled", and "neutralised", "linseed", "safflower" and "cocoanut" oils respectively.

While the descending order of reaction with castor seed lipase is "crude", "heated", "neutralised" and "boiled", "linseed", "safflower" and "cocoanut" oils.

The maximum activity of *Butea Frondosa* lipase is near about pH 8 i.e. towards the alkaline side of neutrality, while the castor seed lipase has an optimum pH of 4.8 i.e. acidic, comparing the activities at the optimum pH it can be noticed that castor seed lipase is 5 times as much powerful as the *Butea Frondosa* lipase.

Then comparing just the maximum hydrolysis of the three crude oils with *Butea Frondosa* lipase and castor seed lipase it is seen that at the end of 24 hours the retardation in percentage hydrolysis is 64% while comparing on the basis of maximum hydrolysis of crude linseed oil at the end of 48 hours, the retarding effect is 80% with crude linseed oil and *Butea Frondosa* lipase.

Now comparing on the basis of maximum hydrolysis of the crude safflower oil split up by castor seed at the end of 24 hours and that split up by *Butea Frondosa* lipase, a retardation of 52% occurs and on comparing the maximum hydrolysis at the end of 48 hours with castor seed and the maximum hydrolysis at the end of 24 hours with *Butea Frondosa* a loss of 71% occurs.

Similarly for the crude cocoanut oil hydrolysed by castor seed at the end of 24 hours and that by *Butea Frondosa* in the same period we find a retardation of 62%, but comparing on the basis of maximum hydrolysis of the crude cocoanut oil at the end of 48 hours with castor seed lipase and maximum hydrolysis at the end of 24 hours with *Butea Frondosa* lipase a loss of 74% occurs.

For the castor seed lipase comparing on the basis of maximum hydrolysis of linseed oil at the end of 48 hours, the safflower oil is hydrolysed 43% less than the linseed and cocoanut oil 50% less than the linseed oil. While similar results for the *Butea Frondosa* lipase at the end of 24 hours for the safflower oil is 20.9% less than the linseed and cocoanut

oil 37.9% less than the linseed oil. Therefore the safflower and cocoanut oils are split up to a greater extent by *Butea Frondosa* lipase than with castor seed lipase on the basis of maximum hydrolysis of linseed oil.

Both the lipases retard the percentage hydrolysis of refined and heated oils to a more or less extent.

### SECTION VIII

**Effect of a few salts on the hydrolysis of the three oils with *Butea Frondosa* lipase.**

**Action of a few Salts on:—**

- (a) Linseed oil; Sap. Value = 34.43 c.cs.;  
Acid Value = 1.23 c.c.
- (b) Safflower oil; ,, ,, = 34.45 c.cs.;  
Acid Value = 0.368 c.c.
- (c) Cocoanut oil; ,, ,, = 45.88 ,, ,, = 0.34 c.c.

TABLE No. 18

Each set of experiments contains 2 c.cs. of the oil under consideration, 5 c.cs. of enzyme extract equivalent to 0.5 gm. of the lipase, 10 mgms. each of various salts and no buffer solution added. The mixture was incubated for varying periods at 37.5°C.

Set.	Salts added 10 mgms.	percentage hydrolysis after (hours.)		
		3 hrs.	24 hrs.	
1	Nil	a	2.22	5.390
		b	1.85	3.392
		c	1.53	3.062
2	MnSO <sub>4</sub>	a	2.23	5.39
		b	1.80	3.29
		c	1.53	2.95

3	CaCl <sub>2</sub>	a	1.90	3.11
		b	1.80	2.80
		c	1.41	2.12
4	NaCl	a	2.06	3.65
		b	1.70	3.00
		c	1.29	2.47
5	K <sub>2</sub> CrO <sub>4</sub>	a	2.22	4.44
		b	1.82	2.83
		c	1.46	2.36
6	Na <sub>2</sub> CO <sub>3</sub>	a	2.30	6.34
		b	1.88	3.61
		c	1.76	3.17
7	KCN	a	2.69	6.817
		b	2.25	4.360
		c	2.00	3.536

Table No. 18 contains the results of the hydrolysis of the three oils with the action of salts on the lipase. Buffer solution was not used in this experiment so that the actual effect of salts on the gain or loss percentage in hydrolysis is noted. First, MnSO<sub>4</sub>, was used to see whether it has any effect on percentage hydrolysis because as it acts as an activator to the castor seed lipase (Falk, Hoyer, Nicloux etc.). From the table it is seen that MnSO<sub>4</sub> has no action on linseed oil with the *Butea Frondosa* lipase at the end of 24 hours; for coconut and safflower oils a slight retardation occurs.

Similarly retardation in percentage hydrolysis for the three oils occurs with CaCl<sub>2</sub> and *Butea Frondosa* lipase, while CaCl<sub>2</sub> is known to activate castor seed lipase.

Since NaCl was found to inhibit the activity of castor seed lipase, from Section V, its action was tried on *Butea Frondosa* lipase and as it can be seen from the Table No. 18 common salt also retards hydrolysis with *Butea Frondosa* lipase.

Mastbaum states that in the case of Kolanut lipase KCN,  $\text{CaCl}_2$ , bismuth nitrate etc. have an inhibiting effect on the activity, but that potassium chromate, salicylic acid have a stimulating effect. Van den Driesser Mareeuw (J. S. C. I. 1909, 23, 612) on the other hand states that KCN has no action and that potassium chromate, mercury chloride have a deleterious action and potassium ferrocyanide, sodium carbonate a favourable one.

Different substances act differently on lipases from different sources. As castor seed lipase is activated by acids while that from *Chelidonium Majus* is not. Since *Butea Frondosa* lipase shows optimum activity on the alkaline side, salts like  $\text{K}_2\text{CrO}_4$ , KCN and  $\text{Na}_2\text{CO}_3$  were next tried on the lipase, and it was found that  $\text{K}_2\text{CrO}_4$  retards hydrolysis while  $\text{Na}_2\text{CO}_3$  and KCN accelerates hydrolysis, the effect is more marked in the case of KCN after 24 hours.

### Conclusions :-

From the hydrolysis of the oils with the castor seed lipase it is noticed that:-

I (a) linseed, safflower and cocoanut oils stand in the descending order of susceptibility towards the hydrolysing action of the castor seed lipase.

(b) Refining treatment with alkali, heating at  $160^\circ\text{C}$  for half an hour and boiling the oils retards hydrolysis, the descending order of reaction is 'crude', 'heated', 'neutralised' and 'boiled' linseed, safflower and cocoanut oils.

(c) Castor seed lipase is susceptible to heat and tends to be inactive as the temperature increases even though the rise is only about  $7^\circ$  to  $8^\circ\text{C}$ .

II A gradual fall in digestibility of linseed, safflower and cocoanut oils occurs with the duration of heating and also with rise of temperature.

III (a) From the hydrolysis of the oils, hydrogenated products and pure ghees with castor seed lipase, the descending order of reaction is, linseed oil, buffalo ghee, safflower oil,

cocoanut oil, cow ohee, "Y" and "X" hydrogenated products.

(b) The two hydrogenated products "X" and "Y" selected at random undergo very poor digestion on comparing with that of the linseed, safflower and cocoanut oils, also they are less easily hydrolysed than the two ghees.

IV (a) NaCl has an inhibiting effect on the hydrolysis of oils by castor seed lipase at the optimum pH.

As the concentration of salt increases the percentage hydrolysis decreases though the fall is not proportional to the concentration of salt.

(b) Cane sugar has no effect in small quantity but a slight retardation occurs at higher concentrations on the percentage hydrolysis of linseed, safflower and cocoanut oils at the optimum pH. The retarding effect of sugar is marked in the case of linseed oil than in the safflower and cocoanut oils.

(c) At the optimum pH, vitamin B<sub>1</sub> (thiamin chloride) and nicotinic acid has a slight accelerating effect on the percentage hydrolysis of the oils and vitamin B<sub>2</sub>, vitamin C (ascorbic acid) and vitamin A a retarding effect while vitamin K has no action.

V The linseed oil is split up to a maximum limit then come the safflower and cocoanut oils with both *Butea Frondosa* and castor seed lipases. Similarly the refining and thermal treatment of the three oils retard hydrolysis with both the lipases.

However the castor seed lipase is 5 times as much powerful as the *Butea Frondosa* one.

Safflower and cocoanut oils are split up to a greater extent by *Butea Frondosa* lipase than with castor seed lipase on the basis of maximum hydrolysis of linseed oil.

On comparing the two lipases we find that *Butea Frondosa* lipase acts best in an alkaline medium while the optimum pH of castor seed lipase is acidic.



The hydrolysing activity of *Butea Frondosa* lipase stops after 24 hours while for castor seed lipase the results of hydrolysis are recorded even for 48 hours.

VI KCN and  $\text{Na}_2\text{CO}_3$  have an accelerating effect on the hydrolysis of linseed, safflower and cocoanut oils with *Butea Frondosa* lipase, while  $\text{MnSO}_4$ ,  $\text{CaCl}_2$ ,  $\text{K}_2\text{CrO}_4$  and NaCl retards hydrolysis.

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## PAPER XIX

### "A STUDY OF COMPARATIVE DISINTEGRATION OF FATS FROM THE DRY FRUITS"

BY

Dr. R. S. KALE, Ph. D.

*(With the kind permission of the authorities of the Bombay University)*

#### Introduction :

It has been observed and proved that oil-seeds contain an enzyme 'lipase' which by its virtue of lipolytic activity brings about hydrolysis of the oils on keeping. This was first observed by J. Pelouze in 1885 (C.R. 1855, 40, 605). He pointed out that seeds such as linseed, poppy, mustard and ground nut contain a substance which is capable of bringing about the hydrolysis of the oils present in the seeds when these are crushed.

The freshly extracted oils from the crushed seeds contain very negligible amount of acid, but on keeping for some days appreciable quantities of free acid can be detected and the amount tends to increase gradually as the period increases. The decomposition has been attributed to the presence of a ferment which has been termed 'lipase'.

J. Reynolds Green (Proc. R.S. 1890, 48, 370) was the first person to discover the "Ricinus lipase" from castor bean seed (*Ricinus Communis*). Green found that the plant enzyme is a typical lipase hydrolysing true fats to fatty acids and glycerol. Since that time the observations of Green have been confirmed many a time. Its optimum pH is between 4.7 and 5.0 (Willstatter and Waldschmidt-Lietz E. "Über Ricinus Lipase" -Z. Physiol. Chem. 134, 161, 1923-24). It varies slightly according to the buffer employed.

Numerous other seeds also contain lypolytic enzymes, (J. Pelouze C.R. 1855, 40, 605). The well-known fact that the ground nut oil, cocoanut oil and palm kernel oil rapidly develop acidity when kept, unless they are carefully refined, indicates the presence of small amounts of enzymes in these oils derived from the meal from which they are extracted.

It is well known that in the alimentary canal of the higher animals, glycerides undergo hydrolysis by the catalytic agency of a fat-splitting enzyme 'lipase' secreted by pancreas. The breaking down of the fats by this agent is greatly accelerated by the physical action of bile salts—sodium glycocholate, sodium taurocholate and bile pigments—bilirubin and biliverdin. These by their marked power of lowering surface tension tend to increase the dispersion of the oily droplets and to give rise to emulsions.

#### **Extraction of the enzyme lipase from the castor seed :**

Numerous methods have been devised for obtaining a product with a hydrolysing power greater than that of the seeds only crushed. All these consist in removing from the seeds the materials such as oils, albuminoid substances devoid of hydrolysing properties.

For the present investigation we have adopted two methods (i) Taylor's method and (ii) Tanaka's method and have tried to compare the two with each other.

## SECTION I

**A Study of the comparative disintegration of the  
fats from dry fruits with castor bean lipase**

In this section it is intended to study the disintegration of the fatty contents of the dry fruits by the action of castor seed lipase. The castor bean lipase was prepared in two different ways as stated before.

**Procedure :-**

3 grams of each sample were taken. To this the following were added in order:-5 c.c. of phosphate buffer (4.8 pH), 10 c.c. of distilled water, 25 c.c. of neutral rectified spirit and lastly the enzyme lipase (50, 100, 150 mgms.) as the case may be. The reaction mixture was incubated for varying periods i.e. one hour, three hours and five hours. A blank experiment was also conducted side by side. The difference between blank and the experimental sample gave the amount of hydrolysis that had taken place in the same time. The fatty acids resulting from the hydrolysis of the fats by lipase were titrated against N/10 NaOH, using phenolphthalein as the indicator. The results are expressed as the percentage of the total fats in the sample hydrolysed in the time of reaction.

Before starting the actual work the acid value, saponification value, specific gravity and refractive index of oil from each of the dry fruits were determined.

**Hydrolysis with 50 mgms. of castor bean lipase prepared  
by (i) Taylor's method**

TABLE No. 1

Name of the sample	1 hour	3 hours	5 hours
Cashew nut ...	1.33	1.77	2.00
Almond ...	4.12	5.25	6.19
Pistachio nut ...	3.58	4.58	5.17

## by (ii) Tanaka's Method

TABLE No. 2

Name of the sample	1 hour	3 hours	5 hours
Cashew nut ...	1.55	2.45	2.89
Almond ...	5.63	6.75	7.50
Pistachio ...	4.18	5.57	6.77

Hydrolysis of the fats in the fruits with 100 mgms. of castor bean lipase prepared by (1) Taylor's method

TABLE No. 3

Name of the sample	1 hour	3 hours	5 hours
Cashew nut ...	1.56	2.67	3.34
Almonds ...	5.44	6.75	7.69
Pistachio nut ...	4.78	5.97	6.77

## 2. Tanaka's Method

TABLE No. 4

Name of the sample	1 hour	3 hours	5 hours
Cashew nut ...	2.68	4.01	5.12
Almond ...	6.56	7.88	8.81
Pistachio nut ...	4.98	6.57	7.37

**Hydrolysis of the fats in the dry fruits with 150 mgms.  
of lipase prepared by 1 Taylor's Method**

TABLE No. 5

Name of the sample	1 hour	3 hours	5 hours
Cashew nut ...	2.23	3.56	4.23
Almond ...	6.19	7.13	7.88
Pistachio nut ...	5.37	6.57	7.57

**2. Tanaka's Method**

TABLE No. 6

Name of the sample	1 hour	3 hours	5 hours
Cashew nut ...	3.12	4.47	5.57
Almond ...	7.31	9.00	9.94
Pistachio nut ...	5.57	6.95	8.16

Now comparing the three fruits among themselves and the two lipases also, we find that :-

1. Almond out of the three is most easily attacked and to the highest limit also. Then come in order pistachio nut and cashew nut.
2. The Tanaka's lipase is more powerful than the Taylor's lipase, as it hydrolysis more fats from the dry fruits in the same period.
3. More than 60% of the total fats are hydrolysed in the first interval. The rate then falls gradually as the hydrolysis proceeds.
4. The velocities of reaction in the three intervals in the case of Tanaka's lipase are greater than the corresponding velocities in the case of Taylor's lipase except in the case of almonds in the 2nd and 3rd interval.

## SECTION II

**The effect of non-fatty material on the hydrolysis of fats by the castor bean lipase :**

In the former sections of this work the effect of removing the fatty contents of the dry fruits on the hydrolysis of the carbohydrate and protein material by the enzymes was noticed. In the present section the effect of non-fatty material on the hydrolysis of fats by the castor bean lipase is studied.

For this purpose the non-fatty material was eliminated by extracting pure oils from the dry fruits by ether in Soxhlet. Traces of ether were then finally removed from the oils by heating in hot air oven and then exposing to the sun. Before starting with the work the necessary physical and chemical properties of these oils were investigated which are given below:-

1. Cashew nut oil...	Specific gravity	0.915
	Acid value.....	1.68
	Sap. value.....	189.62
	Refractive index	1.462
2. Almond oil ...	Specific gravity	0.917
	Acid value .....	4.49
	Sap. value.....	191.30
	Refractive index	1.463
3. Pistachio oil ...	Specific gravity	0.919
	Acid value .....	5.61
	Sap. value.....	190.74
	Refractive index	1.461

To find out the effect of "Non-fatty material" on the hydrolysis of the fatty material, the oils extracted from the dry fruits were alone subjected to the enzymic action.

**Procedure :**

2 c.c.s. of each oil were taken in a flask and to it were added the following - phosphate buffer (pH 4.8) 2 c. c., distilled water 2 c. c., neutral rectified spirit 25 c. c. and the required amount of lipase. This reaction mixture was kept in an incubater at 37.5°C and the reaction was allowed to proceed for three different periods- 1, 3, and 5 hours. The fatty acids resulting from the hydrolysis of fats were estimated by titrating against 0.1 N NaOH. The results in the following tables are expressed as the percentage of the total oil hydrolysed at different periods.

**Hydrolysis of the oils with 50 mgms. of Taylor's lipase:**

TABLE No. 7.

Name of the sample.	1 hour	3 hours	5 hours
Cashew nut oil ...	0.81	1.14	1.36
Almond oil ...	3.28	4.10	4.76
Pistachio nut oil ...	2.81	3.47	4.14

**Tanaka's lipase :**

TABLE No. 8.

Name of the sample	1 hour	3 hours	5 hours
Cashew nut oil ...	0.98	1.31	1.95
Almond oil ...	4.59	5.74	6.22
Pistachio nut oil ...	3.31	4.47	5.30

**Hydrolysis of the oils with 100 mgms. of lipase:-****Taylor's lipase:-**

TABLE No. 9.

Name of the sample	1 hour	3 hours	5 hours
Cashew nut oil	... 0.97	1.46	1.79
Almond oil	... 4.43	5.58	6.40
Pistachio nut oil	... 3.64	4.47	5.13

**Tanaka's lipase :-**

TABLE No. 10.

Name of the sample	1 hour	3 hours	5 hours
Cashew nut oil	... 1.63	2.61	3.59
Almond oil	... 5.41	6.72	7.22
Pistachio nut oil	... 3.80	4.80	5.63

**Hydrolysis of the oils with 150 mgms. of lipase :-****Taylor's lipase :-**

TABLE No. 11.

Name of the sample	1 hour	3 hours	5 hours
Cashew nut oil	... 1.36	1.95	2.44
Almond oil	... 5.09	5.91	6.56
Pistachio nut oil	... 3.97	4.96	5.63



## Tanaka's lipase:—

TABLE No. 12.

Name of the sample		1 hour	3 hours	5 hours
Cashew nut oil	...	2.12	3.10	3.90
Almond oil	...	6.07	7.55	8.54
Pistachio nut oil	...	4.30	5.46	6.13

Taking into consideration all the facts stated above it can be concluded that—

1. The 'non-fatty' contents of the dry fruits have an accelerating effect on the hydrolysis of fats by castor bean lipase.
2. Lipase obtained by Tanaka's method is more powerful than that obtained by Taylor's method.
3. The descending serial order may be stated as almond, pistachio nut and cashew nut as far as the disintegration of the fatty constituents is concerned.
4. With the increase in the strength of the hydrolysing agent, there is also an increase — though not proportional—in the amounts of substrates hydrolysed.

## PAPER XX

## "KEEPING POWER OF LINSEED, SAFFLOWER AND COCOANUT OILS."

BY

(MISS) SILLOO P. NAEGANVALA, M. Sc.

*(With the kind permission of the authorities of the  
Bombay University)*

## SECTION I

**General Analysis of Linseed, Safflower and Coconut Oils**

Extensive researches show that the estimation of various values can only be approximate as the characteristics of an oil vary to a certain extent depending on the source as well as on the conditions of soil, climate etc., and therefore a general analysis as well as the keeping power on storage of the linseed, safflower and coconut oils is carried out in this part.

Linseed oil (*Linum usitatissimum*) when expressed in the cold, is a golden yellow oil with a pleasant taste, so that it can be used as an edible oil and considerable quantities are being expressed for this purpose in India, particularly in C.P., Berar and Northern parts of India. It is a drying oil.

Eibner and Schmidinger (J. S. C. I. 1924, 43, 13, 10) examined the composition of the fatty acids from an oil of iodine value 173.5 and obtained the following results:—  
*a* - linolenic acid 20.1%, isolinolenic acid 2.7%, *a* - linoleic acid 17%, *b* - linoleic acid 41.8%, oleic acid 4.5%, oxyacids 0.5%, glycerol 4.1%, saturated acids 8.3%; however, results differing considerably have been recorded by various observers depending on the oil.

Safflower oil (*Carthamus tinctorius*) is a pale yellow coloured, semi-drying oil and in the Deccan it is one of the most valued of edible oils. The component glycerides of Indian safflower oil have been studied by N. L. Vidyarthi (J. Ind. Chem. Soc. 1943, 45) who found that the oil contains

myristic acid (along with lauric and other lower acids), 1.5%, palmitic acid 3%, stearic acid 1%, arachidic with a trace of lignoceric acid (0.5%), oleic acid 33%, linoleic acid with a trace of linolenic acid 61 %.

Cocoonut oil is obtained from the kernels of the nuts of the Palm (*Cocos nucifera*), which grows along the coast lines of practically all of the tropical regions. It is a non-drying oil. The fatty acid constituents have been studied by Allen, (Analyst 1925, 50, 16), Armstrong and others-(*J. S. C. I.* 1925, 44, 63 T). Allen finds the percentage of acids as caproic 0.2%, caprylic 7%, capric 7%, lauric 50%, myristic 18%, palmitic 6%, stearic 3%, oleic 8%, linoleic 1%. While Armstrong and others find caprylic acid 9.5%, capric 4.5%, lauric 51%, myristic 18.5%, palmitic 7.5%; stearic 3%, oleic 5% and linoleic 1%.

The general analysis of the samples of linseed, safflower and cocoonut oils for determination of various values was carried out according to the official methods laid down by the Association of Official Agricultural Chemists (U.S.A. 1926) and from Technology of Oils, Fats and Waxes by Lewkowitsch.

### General Analysis of the Linseed, Safflower and Cocoonut oils:

TABLE No. 1

	Linseed oil	Safflower oil	Cocoonut oil
Sap. value	191.8	193.0	256.8
Iodine	165.54	141.50	9.96
Acid	6.30	1.48	1.39
Höhner	94.70%	95.90%	89.44%
R. M.	nil	1.25	7.14
Polenske No.	nil	nil	18.06
M. P.	-16 to -20 (Lewkowitsch)	-13 to -18 (Lewkowitsch)	25.8°C.
Unsaponifiable matter	2.340%	1.260%	0.435%
Titre test	21.6°C.	-16. (Lew)	22.2°C.
Percentage of liquid acid...	91.50%	94.76%	17.39%
Percentage of solid acid	8.30%	5.23%	82.71%

The above values obtained for different constants of the oils agree well with the range of values obtained by various observers such as Lewkowitsch, and other workers. The only value which is rather low is the iodine value of linseed oil; for, Lewkowitsch examined a number of seeds and obtained a range of iodine values for linseed oil as lying between 170-201 (Wij's), while the one obtained above by Wij's method is 165.54, but this low value can be attributed to; for, in general, the composition of fats and fatty oils varies according to the environment under which the seeds are grown. Ivanov, who has studied the effect of climate upon the composition of vegetable fats for a considerable period of years, with particular reference to drying oils has formulated (Chem. Timchan 1929, 36, 40) that the climate of southern lands favours the formation of linoleic acids. The investigations have indicated that the linseed and soya beans grown in temperate climates have higher iodine values than those grown in the tropics and on the basis of this finding, the linseed grown in India must have a different composition than grown elsewhere under different climatic conditions.

Besides the source and other factors such as soil, climate, diet-etc., lower iodine values for the linseed have been found occasionally in "genuine" linseed but the adulteration may have taken place in the seed itself as it were, the oil having been produced from impure seed itself. In this case the seeds were bought from the local market, so that there is every likelihood of seeds being adulterated.

Hehner and Mitchell record the I. V. as low as 160.7 for one sample. Similarly Hubl found the I. V. of 5 linseed oils of different origin to be from 156-160, the saturated acids of linseed oil appear to vary from about 6 to 11%. Morrell (J.Soc. Chem. Ind. 32, 109, 1913).

Due to wide range of the iodine numbers and the comparatively small differences in the quantity of saturated acids in oils from various sources it is evident that the proportion of the saturated acids are subject to notable variation.

Extensive researches are being conducted at the H. B. Technological Institute, Cawnpore, for the standardisation of oil seeds and oils.

## SECTION II

**1. Keeping Power of the Linseed, Safflower and Coconut oils on storage :—**

Vegetable and animal oils and fats deteriorate on keeping, resulting in disagreeable odour and acrid taste, in which state they are commonly described as having developed rancidity. No industry in which oils and fats are used can afford to overlook the factors which contribute to the development of rancidity, particularly some vegetable oils are in enormous demand as the basis of edible products as 'vegetable butter' etc. and therefore it is very important to be able to produce an oil which as nearly as possible is free from fatty acids, rancid odour or taste and which at the same time may be stored or exported without the fear of deterioration.

Most investigators agree that decomposition or rancidity proceeds in two stages (1) hydrolysis of the glycerides and consequent formation of free fatty acids and glycerol, (2) and atmospheric oxidation of the acid and glycerol thus formed and interaction of the oxidation products, the resulting compounds being responsible for the unpleasant taste and odour of rancid fat. The change towards rancidity varies with the type of fat and the conditions of storage.

The tendency of an oil to become rancid depends upon the proportion of volatile and unsaturated fatty acids, molecular structure of the glycerides it contains as well as a number of external agencies influence the atmospheric oxidation such as heat, light, moisture, micro-organisms and the action of metals.

Rancidity is accompanied by an increase in the saponification value and acid value and by a decrease in the iodine value which is due to the absorption of oxygen by the unsaturated bonds of the liquid fatty acids.

Oils and fats are solvents and carriers of vitamins besides the Margarine and vegetable products are fortified with vitamins, and it is known that rancid fat destroys vitamin A

(Lease et al. J. Nutrition 1939, 16, 571), therefore, if the conditions of storage and the factors that influences rancidity are known and controlled, fats and oils can be stored without deterioration for longer periods.

Three samples of linseed oil, unfiltered, filtered and neutralised were filled in glass bottles with stoppers and kept in a cupboard for a period of 6 months. Readings of acid value, iodine and saponification values were taken every month to find if any change in free fatty acids, unsaturated acids or combined acids occurs on storage.

The unfiltered oil contained mucilage, moisture etc. and was therefore foggy in appearance, the filtered oil was clearer and brighter while on neutralisation the oil becomes clearer and lighter in colour. On neutralising the initial acidity, the saponification and iodine values fall from the original.

Similar samples were also kept for safflower and cocoanut oils.

All these samples were filled in glass bottles with stoppers and kept in a cupboard so that they were protected from aerial oxidation, light, moisture etc.

The determination of the sap., iodine and acid values is not a criterion for rancidity; for, changes may occur in these values without the oils becoming rancid due to a number of factors.

### Keeping Power of Linseed oil on Storage.

TABLE No. 2

#### Linseed oil (Unfiltered):

Period in months	0	1	2	3	4	5	6
Acid value	6.30	6.32	6.40	6.52	6.67	6.69	7.05
Iodine value	165.54	165.01	164.63	164.10	163.52	163.06	162.64
Sap. value	191.80	192.10	193.50	192.70	193.11	193.54	193.95

**Linseed oil (Filtered):**

Period in months	0	1	2	3	4	5	6
Acid value	5.95	6.09	6.09	6.11	6.14	6.14	6.17
Iodine value	165.54	165.28	164.93	164.37	163.99	163.68	163.58
Sap. value	191.80	191.88	192.09	192.36	192.70	193.10	193.46

**Linseed oil (Neutralised):**

Period in months	0	1	2	3	4	5	6
Acid value	nil	nil	nil	0.25	0.54	0.61	0.74
Iodine value	157.60	157.28	157.20	157.11	156.91	156.54	156.34
Sap. value	190.47	190.50	190.50	190.82	191.10	191.31	191.55

From the Table No. 2 we find that in the unfiltered linseed oil after six months storage the acid value increases by 0.75 and saponification value by 2.15 while the iodine value decreases by 2.9 from the initial values. The oil becomes much darker in colour, reddish yellow and also becomes slightly viscous from the original, as well as dries and thickens on the sides of the bottles. The odour is more pronounced resembling that of fresh paint.

In the filtered oil the acid value increases only by 0.22 and saponification value by 1.66 while iodine value decreases by 1.96. The colour, odour and thickness of oil intensifies though not as much as in the case of unfiltered oil.

In the neutralised oil the acid value is nil for the first two months and then increases gradually till 0.75 at the end of six months, the saponification value increases by 1.08 while the iodine value decreases by 1.26. The colour and smell is slightly developed.

Here we find that unfiltered oil is more susceptible to change on storage, and filtered and neutralised oil keep better on storage.

**Keeping Power of Safflower oil on Storage.**

**TABLE No. 3**

**Safflower oil (Unfiltered):**

Period in months	0	1	2	3	4	5	6
Acid value	1.48	1.51	1.58	1.64	1.68	1.71	1.78
Iodine value	141.50	141.38	141.08	140.92	140.76	140.56	140.34
Sap. value	193.00	193.16	193.80	193.72	193.78	194.08	194.29

**Safflower oil (Filtered):**

Period in months	0	1	2	3	4	5	6
Acid value	1.46	1.49	1.54	1.59	1.62	1.62	1.62
Iodine value	141.50	141.50	141.32	141.29	141.18	141.00	140.96
Sap. value	193.00	193.22	193.40	193.47	193.51	193.50	193.80

**Safflower oil (Neutralised):**

Period in months	0	1	2	3	4	5	6
Acid value	nil	nil	nil	nil	nil	nil	nil.
Iodine value	141.50	141.40	141.32	141.32	141.26	141.30	141.24
Sap. value	190.50	190.50	190.50	190.60	190.76	190.70	190.80

In the unfiltered safflower oil after six months storage the acid value increases by 0.3 and saponification value by 1.29 and the iodine value decreases by 1.16 from the original values.



The odour is not too fresh and rather rancid, while the oil dries in a thin film and thickens on the sides of the bottle, the thickness of the oil as such is not visibly altered.

In the filtered safflower oil the acid value increases very slightly only by 0.16 and saponification value by 0.8 while iodine value decreases by 0.54.

Now in the neutralised oil the acid value is nil throughout and the increase in saponification value is only 0.3 while decrease in iodine value is 0.26 so that the removal of original free fatty acids and other impurities improve the keeping power of the oil on storage, while filtered oil keeps better than the unfiltered safflower oil.

#### Keeping power of Coconut Oil on storage.

TABLE No. 4

#### Cocoanut oil (Unfiltered):

Period in months	0	1	2	3	4	5	6
Acid value	1.39	1.46	1.44	1.50	1.57	1.71	1.80
Iodine value	9.96	9.82	9.80	9.67	9.58	9.49	9.47
Sap. value	256.80	257.00	257.30	257.63	257.85	258.32	258.53

#### Cocoanut oil (Filtered):

Period in months	0	1	2	3	4	5	6
Acid value	1.32	1.35	1.34	1.34	1.35	1.37	1.40
Iodine value	9.96	9.83	9.81	9.77	9.74	9.73	9.73
Sap. value	256.80	256.90	257.09	257.41	257.65	257.89	258.11

**Cocconut oil (Neutralised) :**

Period in months	0	1	2	3	4	5	6
Acid value	nil	nil	nil	nil	nil	nil	nil
Iodine value	9.05	9.06	9.06	9.06	9.06	9.06	9.03
Sap. value	254.00	254.00	254.00	254.15	254.29	254.34	254.50

In the unfiltered cocconut oil after six months storage the acid value increases by 0.41 and saponification value by 1.73, while the iodine value decreases by 0.49. The odour intensifies and gives the peculiar smell of copra but not a bad smell as is emitted by rancid copra, therefore apparently the cocconut oil after six months storage keeps fresh and does not give the usual rancid smell.

Now in the filtered cocconut oil the acid value increases only by 0.08 while the saponification value by 1.31 and the iodine value decreases by 0.23. Walker also (1906, J. Phil. Sc.I.117 and 142) observed that a sample of unfiltered cocconut oil increased in acid value from 2.6 to 3.4% in two months while the same sample filtered shows no change. However he found, contrary to expectation that the difference between filtered and unfiltered oil is most pronounced during first few months, while on longer standing over a year the acid value tends to approach each other.

In the neutralised cocconut oil the acid value is nil throughout, while the iodine value remains constant, only in the last month a decrease of 0.03 is observed, while the sap. value increases slightly by 0.5 during six months storage, therefore the neutralised oil keeps by far the best on storage and next comes the filtered and then unfiltered oils.

We find from the tables Nos. 2, 3, and 4, that unfiltered oils containing mucilage, moisture etc. does not keep well on storage and even a simple process of purification like filtration improves the keeping quality, while removing the initial

acidity and other impurities improves to a marked degree the keeping qualities of all the three oils.

The slight increase or decrease in the above values which a pure oil suffers on long standing is probably due to simple hydrolysis, air, heat, moisture, light, enzymes already present in the oil and the amount of surface exposed. Although the above oils were stored in glass bottles with stoppers and kept in a cupboard, the oils were exposed every month whilst taking the readings to air, light etc. as well as the quantity of oil decreased in the bottles after taking readings, so that the amount of surface exposed increased and more air came in contact with the oil. So the above changes in the values can be attributed to these causes, for, Walker (1906, J. Phil. Sc. I. 117, 142) working on rancidity of cocoanut oil, sealed one sample and took care that it was not exposed to any of the factors such as air, light etc. and kept for a period of one year and found that the acid value did not change at all from the original, while another sample exposed to air was found to develop rancidity rapidly. Therefore it seems that air is necessary for rancidity to be brought about.

It is generally believed that cocoanut oil has a great tendency to become rancid, but above experiment shows that its keeping qualities are quite good. Similarly Walker states that the keeping qualities of cocoanut oil prepared in a pure state are equally good if not superior, to those of most vegetable oils. While commercial products having high acid value and bad odour deteriorate rapidly, this change being greater as a rule the greater the initial acidity of the oil.

Most of the free acid accompanying bad odour and taste is produced in the copra itself before the oil is expressed for the oil from a sample of copra which had been cut and exposed to moist air for a month increased in acidity from 1.5 to 23.3%.

Fresh oils when kept always thicken and develop some peculiar smell, which is ascribed to rancidity (W. C. Powick, R. S. Morrell, and H. R. Wood - "The Chemistry of Drying Oils"). The odour of oils and fats are due to the presence of small quantity of volatile substances either derived from

vegetable substances or formed by the decomposition of the oil itself. As according to the researches of Haller and Lassieurs (Comptes Rend. 1910, 150, 1013) the unpleasant odour of cocoanut oil must be due partly to a process of decomposition of the glycerides with the liberation of free fatty acids with a pronounced odour and partly to the presence of certain substances which include methyl-heptyl-ketone and methyl-nonyl-ketone, traces of these may be detected in the refined fat and account for its odour. The odour of rancid fats that do not (like cocoanut oil) contain volatile fatty acids is due to the formation of aldehydes or of esters.

In the above experiments it was found that the odour of particularly unfiltered oils was quite pronounced from the original though not rancid.

The increase in thickness of linseed oil and to a slight extent of the safflower oil may probably be due to the oxidation of the unsaturated acids.

**(2) Effect of addition of castor seed lipase on the keeping power of the linseed, safflower and cocoanut oils on storage.**

About 1 gm. of the castor seed lipase was added to about 100 grms. of each oil and bottled in glass bottles with stoppers and kept for a period of 6 months. Readings of acid value and iodine value were taken every month.

TABLE No. 5

**Linseed oil + Lipase**

Period in months ...	0	1	2	3	4	5	6
Acid value	6.30	10.61	11.86	13.20	17.06	22.69	39.82
Iodine value	165.54	164.61	163.88	162.66	160.80	159.26	157.41

**Safflower oil + Lipase**

Period in months ...	0	1	2	3	4	5	6
Acid value	1.48	6.32	6.95	8.15	10.07	19.44	29.82
Iodine value	141.50	140.83	140.67	140.14	139.44	138.89	137.93

**Cocoanut oil + Lipase**

Period in months ...	0	1	2	3	4	5	6
Acid value	1.37	6.82	8.25	10.42	16.09	36.27	56.66
Iodine value	9.96	9.87	9.87	9.62	9.46	9.22	8.98

It is seen that the acid value increases a great deal it being 33.52 while the iodine value decreases by 8.13 after keeping the linseed oil for six months, the lipase accelerates the hydrolysis of the oil and then by the action of light, air, etc. (to which the oil is exposed every month at the time of taking readings) further changes towards rancidity occur, the odour of linseed oil on storage is rather strong though not quite unpleasant. It is noticed that the acid value increases by 10.76 in the first 4 months and then the increase is rapid being 22.76 for the next two months, this rapid increase at the end may perhaps be due to more oil coming in contact with the lipase as the quantity decreased every time when readings were taken.

Now in the safflower oil the acid value increases by 28.34 and the iodine value by 3.57 at the end of six months. The odour is extremely rancid and not pleasant, therefore the lipase has a deteriorating effect on the safflower oil on storage. The acid value increases by 55.29 and the iodine value decreases by 0.98 only in the case of cocoanut oil + lipase after keeping for six months, here too the odour of cocoanut oil is rather rancid.

The unpleasant odour or the rancidity in the case of safflower and cocoanut oils is an extreme case of lipolytic hydrolysis and subsequent oxidation of the free fatty acid thus formed.

If rancidity is defined as the formation of high percentage of free acids then the smell need not be always unpleasant but it generally happens that oils which are polymerised and are acted upon by lipolytic activity generally develop aldehydic acids having a bad smell but this is not a general rule, as Kale (Thesis submitted to Bom. Univ. 1928) exposed Niger seed oil to the action of these enzymes developed high percentage of acidity, the viscosity is increased and peroxides are formed but the smell is not unpleasant, therefore the rancid and unpleasant odour of cocoanut and safflower oil and not quite a bad odour of linseed oil may be accounted for from the above discussion (Salway A. H. studies on the Oxidation of unsaturated fatty oils and acids, J. C. S., Tran. U. 109, P. 138-145).

### (3) Aeration of Oils.

The effect of atmosphere on oils and fats varies with the nature of the oil. As a general rule, it may be stated that greater the proportion of unsaturated acids in the oil or fat the greater its liability to change.

About 50 grms. of the linseed, safflower and cocoanut oils were kept in a conical flask with an arrangement of two glass tubes as is done in wash bottles, the shorter tube is connected to the water pump so that air is sucked out, while the other longer tube sucks in air which bubbles through the oil. In this way air was passed through each of the samples every day for 15 minutes and then the flasks were kept in a cupboard.

Table No. 6 records the acid values at an interval of 15 days of the three oils in which air is passed for 15 minutes every day. The acid value is measured in terms of number of milligrams of KOH required to neutralize the free fatty acids in 1 gm. of the oil.

TABLE No. 6

Acid Value	Linseed oil	Safflower oil	Cocoanut oil
Initial ...	6.30	1.48	1.39
1st & 2nd week	6.30	1.48	1.84
3rd & 4th ,,	6.33	1.64	1.84
5th & 6th ,,	6.64	1.69	1.84
7th & 8th ,,	6.66	1.81	1.85
9th & 10th ,,	7.03	1.84	1.88
11th & 12th ,,	7.33	1.86	1.92
13th & 14th ,,	8.38	1.91	1.92
15th & 16th ,,	11.00	2.00	1.96
17th & 18th ,,	13.84	2.03	1.96
19th & 20th ,,	Viscous Mass	2.70	1.96
21st & 22nd ,,	,, ,,	3.50	2.01
23rd & 24th ,,	,, ,,	6.62 (Viscous)	2.07

In the aeration of linseed oil we see that the acid value increases rather slowly for the first two to three months, then there is a rapid increase till at the end of 4½ months the acid value has increased by 7.54 and the oil had become quite viscous so that no more air could be passed. The colour of the oil lightens to a great extent. Linseed oil being oil with the greatest unsaturation absorbs oxygen from air and becomes viscous.

In the case of aerated safflower oil for the first five months the increase is quite gradual, being 0.54 but thereafter there is a sudden increase and in a month more the increase comes to

be 5.14 and the oil becomes quite viscous at this stage. One thing noticed about this oil is that it suddenly dried and became viscous when the quantity of oil was very little in the flask, as compared to the linseed which set to a resinous mass even when the quantity was much greater.

Now in the coconut oil we find that the acid value increases rather rapidly in the beginning and then continues to increase gradually till at the end of 6 months the increase is 0.68 so that the action of air is of relatively little importance on coconut oil than on linseed and safflower oils, so that atmospheric oxidation varies with the type of fat.

Various theories have been put forward to explain the process of oxidation of oils and fats. The first being the absorption of oxygen followed by the formation of a loose addition compound and thereafter various chemical changes occur, the initial being the formation of oxides or peroxides which either break down directly into aldehydes and acids of lower molecular weights or react with water to form substances containing hydroxyl and keto groups. Powick (*J. Agri. Res.* 1925, 58 B, 1075), Browne (*Ind. Eng. Chem.* 1925, 17, 44) and Ellis (*J. Soc. Chem. Ind.* 1925, 1926, 44, 601T, 45, 193T).

Acid values of all the three oils increase with time more or less because organic acids are the final auto-oxidation products of oils and fats. Joti Sarup Aggarval (*J. In. Ch. Soc.* 1946, V. 5, P. 121-33) studying the effects of storage on Indian vegetable oils for lubricating purposes found that two fats may have the same acid values, but one may be more oxidised than the other, depending upon the amount of the intermediate oxidation products present; for, it is well known that the unsaturated nature of the oils is to a great measure the cause of their susceptibility to autooxidation, thus the tendency of linseed oil to oxidation (this oil having the greatest unsaturation of all the oil) is so great that it sets to a resinous mass in all the containers exposed to air, even after only six months storage.

We find that acid values of safflower and coconut oils are practically the same in the beginning and both gradually increase upto the 5th month, and then the safflower oil on the



basis of its iodine value being more oxidised than cocoanut sets to a resinous mass, the acid value also increasing rapidly at this stage.

Aggarval also states that the fact that some oils oxidised more on the basis of iodine value shows that besides unsaturation the inherent constituents of the fatty acids forming the oil also influence their autooxidation thus the comparatively high resistance to oxidation of castor and mustard oils is due to the presence of ricinoleic and erucic acids.

The increase in viscosity and setting to a resinous mass in the case of linseed and safflower oils may probably be due to the oxidation of the unsaturated acids — not mainly to lower acids but oxygen added acids which with internal condensation of molecules form bigger molecules, joined with oxygen atoms.

(a) From the keeping power of the linseed, safflower, and cocoanut oils — a noticeable change in linseed oil occurs on storage, it being a drying oil absorbs oxygen readily with the decrease in iodine value, then comes the safflower and cocoanut oils.

(b) The neutralised oils keep by far the best on storage and then come the filtered and unfiltered oils, therefore the keeping power of oil improves on simple purification.

(c) Out of the three oils kept for a period of 6 months with the addition of lipase, cocoanut oil develops rancidity with the highest percentage of free fatty acids then come the linseed and safflower oils, while the decrease in iodine value is in keeping with the unsaturated nature of the linseed oil.

(d) From the aeration of oils, linseed oil is affected to a maximum limit, setting to a resinous mass after 4 months storage, next comes the safflower oil, while aeration has relatively little importance on cocoanut oil. The absorption of oxygen is in keeping with the unsaturated nature of the oil. Therefore the keeping power of the oil depends on the conditions of storage.

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## PAPER XXI

KEEPING POWER OF GROUNDNUT  
OIL-CAKE

By

Dr. N. V. BHIDE, M. Sc., Ph. D.

*(With the kind permission of the authorities of the Bombay University and also of the Magazine, Agr. College, Poona)*

Groundnut is a very common crop in the Bombay Deccan. The groundnut is chiefly used for extracting oil, but it is also used as food for human consumption. The analysis of the groundnut seed shows that it is rich in proteins.

The oil-cake is the residue which remains after the extraction of oil from oil seeds. The oil-cake, therefore, is naturally richer in albuminoids and mineral matter than the seeds. The actual average composition of groundnut oil-cakes has been found to be as given below :—

	Per cent.
Water	8.0
Albuminoids	49.0
Fat	10.0
Sol. carbohydrates	24.0
Fibre	4.0
Mineral matter	5.0

The amino acids in the albuminoids are of greater importance than even the total albuminoids. And the comparative value of the groundnut oil-cake will be seen from the follow-

ing Table, which compares amino acids from four important food stuffs.

Amino Acids	Groundnut Cake	Milk	Soyabean	Gram
Arginine ...	13.26	4.84	5.12	11.85
Histidine ...	1.58	2.59	1.39	1.42
Lysine ...	4.69	5.95	2.71	7.42
Cystine ...	1.42	1.20	...	2.02
Tyrosine ...	4.80	4.50	1.86	2.95
Tryptophane ...	0.66	1.50	...	0.46

These figures indicate that the groundnut oil-cake is very rich in albuminoids and mineral matter which are valuable constituents of human food. It may be stated that the figures given here are for an oil-cake from an expeller. Care must be taken to remove the shells completely. If the shells are not removed the cake will be unfit for human consumption.

This valuable food in the form of an oil-cake is allowed to go out of India or fed to cattle or used only as manure. When there is so much demand for food material we can ill-afford to waste the groundnut oil-cake. As an oil cake, people have their prejudices against using it as human food. This prejudice can be removed by putting correct information before the public. There are, however, two items which must be taken care of. The cake must be absolutely free from shells and secondly the cake should be kept properly, so that it will not go rancid. The groundnut cake especially if it contains much water or is kept in contact with moisture soon gets spoiled and rancid. It is necessary to ascertain how to keep groundnut cake from getting rancid.

The Hon. Mr. A. B. Lathe, who was the Minister of Finance for Bombay under Congress Government wanted to

know exactly what the position was with regard to keeping power of groundnut cake. He asked the Chemistry Department of the Nowrosjee Wadia College to actually arrange for experiments in this connection and give him the results of the experiments.

At the suggestion of the Hon. Mr. Lathe a bag of oil-cake free from shells was obtained from Belgaum Oil Mills. The manager of the Mills deserves our best thanks for giving the cakes free of cost.

Samples were prepared both for flakes and flours and kept under different conditions. The samples were kept in paper packets, in tins with covers and in closed bottles. For comparison samples were kept exposed to air and also in humid air in closed vessels. The samples in paper packets were in thick brown paper, thick white paper, thin transparent paper and semi-transparent paper.

The samples were kept under observation on the first of February 1939 and were tested for the development of acidity at intervals of one month and the experiment was continued for six months which included a period of rainy days. The last determinations of acidity were done at the beginning of August 1939. The acidity in each case is expressed in terms of acetic acid per cent. and the following Table gives the figures for acidity per cent. to start with and at the end of six months. The flakes and flours showed the increase in acidity percentage as follows:—

**Acidity expressed in terms of acetic acid per cent.**

Packet.	February for flakes and flours.	August for flakes.	August for flours.
1. Semi-transparent ...	0.17 %	0.20 %	0.39 %
2. Thin transparent ...	0.17 %	0.22 %	0.36 %
3. Brown paper ...	0.17 %	0.23 %	0.30 %
4. Ordinary thick white paper.	0.17 %	0.24 %	0.39 %

All the samples started with 0.17 p.c. of acidity in February and increased in different proportions by August but none of them went so bad as to be called rancid or became useless for food purpose. The rise was least in flakes kept in semi-transparent paper packet and the highest was in flour kept in ordinary thick white paper.

In every case the flakes showed a better keeping capacity than the flours. For samples in closed tins and bottles as well as for those kept exposed to air in glass cupboards the results were as follows:—

**Acidity expressed in terms of acetic acid per cent.**

Container.	February flakes and flours.	August flakes.	August flours.
1. Closed tin ..	0.17 %	0.21 %	0.34 %
2. Closed bottle ...	0.17 %	0.22 %	0.27 %
3. Exposed to air ...	0.17 %	0.25 %	spoiled

In closed tins and bottles samples keep quite well as in paper packets. Here also in both the tins and bottles the flours show more acidity than flakes.

Samples exposed to humid air were spoiled very quickly. The sample of flour exposed to air was spoiled in about three months. The conclusion that can be drawn from experiments done is that if the oil-cake contains very little moisture as in the expeller cake and is kept closed in paper or tins with as little contact with moisture as possible the cake can easily keep well at least for six months and perhaps much longer. It is always better to keep the cake in flakes condition than in flour condition.

In the ordinary *ghani* the cake contains much moisture. The best way to improve this is to dry it quickly in the sun in the form of small pieces.

## PAPER XXII

## STUDY IN GERMINATION OF OIL SEEDS

BY

JASSIE S. LILAOOWALLA, M. SC.

*(With the kind permission of the authorities of the Bombay University)*

## SECTION I

**Introduction**

The object of the investigation was to study the chemical changes during the complete germination of oil seeds. Several investigators have worked on the chemical changes taking place during germination of oil seeds. H. T. Brown and G. H. Morris (Jr. Ch. Soc. Part I, Vol. LVII. 1890) have done much work on the germination of grass seeds. A study of the chemical changes during the formation of the rice grain is done by D. L. Sahasrabuddhe and M. M. Kibe (Jr. of U.B. Vol. III. Part II. Sep. 1934 p.p. 121-134).

Shinkichi Suski (Agric. Expt. Stu. Bull. No. 124 of 1917, Formosa) of Japan in his publication "The growth of Rice Plant" and Sahasrabuddhe D. L. (Bom. Bull. No. 154 of 1928) in his publication "Assimilation of Nutrients" by rice plant have given indication of the way in which translocation takes place during the formation of rice grain along with the study of other parts of the rice plant.

The present investigation is restricted here only to the oil seeds, to the study of chemical changes taking place during the period of its complete germination. By complete germination is meant the stage of the plant development at which the new seedlings are capable of nourishing themselves with food material from the soil and that they can manufacture from the air by the help of chlorophyll, even in the absence of any such supply in the seed themselves.

For the above work the following two oil seeds were selected :- (i) Linseed (*Linum usitatissimum*), (ii) Groundnut (*Arachis hypogaea*)—the first drying and the second non-drying variety. The linseed seeds were bought from the Poona market, while the groundnut seeds were bought from the Agricultural College, Poona. First their general analysis was carried out to determine the following constituents :- moisture, ash, ether extract, proteins, crude fibre and carbohydrates (by difference).

The seeds were cleaned and made free from dust particles and sand and then ground to a coarse powder with the help of a pestle and mortar. Then the powder was duly bottled after addition of a little toluene to preserve it against any bacterial action.

The table No. 1 shows the general analysis of the linseed seed and groundnut seed before germination.

TABLE No. 1.

**General Analysis of the Linseed and the Groundnut Seeds.**

	Moisture	Ash	Ether extract	Proteins	Crude Fibre	Carbohydrates (by difference)
Linseed seed ...	5.5%	2.85%	47.61%	27.17%	5.17%	11.70%
Groundnut seed ...	4.9%	3.15%	49.52%	36.34%	1.57%	4.62%

**Germination of Linseed**

The actual work of germination of linseed was started sometime in the month of September 1942. The seeds were bought from the Poona market, and were spread in troughs for germination. But they were attacked almost very next day by fungus and the material was lost. So next time the sand which

was used as a substratum was sterilised, and the seeds were spread again after rinsing them with a little dilute alcohol. The germination was quite alright for first three days, but again the seeds were attacked by fungus in part and the growth was rather arrested; besides it was not so luxuriant as it is found in the natural condition. The sand in the sand trough was again re-sterilised, this time by boiling the sand in water and taking all the necessary precautions to prevent the infection of fungus on the germinating material. Thus the linseed was germinated with all the necessary precautions and samples were removed every twenty-four hours from the starting day of germination. The last sample was collected when the green of the plant began to show its appearance. The whole process of germination took five days, from the day of its sowing till it was removed. The samples that were removed every day were first washed to remove the sticking sand particles, and then dried in the sun. Finally they were dried in an oven at 98 degrees centigrade, to drive away the moisture and remaining water. They were then duly bottled after crushing them to powder with pestle and mortar. A little toluene was added to each sample as a preservative. The original seed before germination was also crushed side by side and bottled in the same way with the rest of germinated material. All the samples were studied by analysing them completely for moisture, ash, ether extract, proteins, crude fibre, acidity, sugar, reducing and non-reducing, as will be seen in the next section.

### **Germination of Groundnut**

An attempt was made to germinate the groundnut using sand as substratum in troughs. It was found that the germination was very slow, besides that it was attacked in most of the cases by fungus inspite of all the precautions taken. In order to avoid unnecessary delay and inconvenience, the seeds were finally germinated in the college garden. The seeds were sown in nursery beds four feet by three feet of the college garden. The groundnut received the same treatment as was given to the linseed, but they were sown under natural conditions. The germinated seeds were removed every forty-



eight-hours from the starting day of germination. The last sample was collected as in the case of the linseed, when the germinated seeds had a tinge of green colour in them, i.e. just as soon as chlorophyll in the plant began to show its appearance. The process of germination took six complete days.

The samples that were removed every second day were first washed under tap water to remove the mud and soil. Then they were first dried in the sun and then finally in the oven at 98 degrees centigrade to drive away the remaining water before bottling them. Each sample of seeds was then crushed to a coarse powder with the help of a pestle and mortar and then bottled after addition of a few drops of toluene. The original sample before germination was also crushed and bottled along with the others.

All the above samples were studied by analysing them as in the case of linseed.

## SECTION II

One of the most important manifestations of the vital activity of plants is their growth, their continuous increase in size. Growth is connected with the formation of new cells, tissues and organs of plants as well as with the increase in volume of the cells. Growth is usually combined with a corresponding increase in the total mass of the plant, but there are cases where growth is accompanied by an expenditure of substance. For instance, during the first stages of germination of seeds or utilisation of reserves from storage organs when the leaves have not developed, there may be observed considerable losses of organic substances due to respiration, but new cell formation occurs.

The growth of seedlings is combined not with an accumulation but with redistribution of organic substances. Organic substances that have been stored during ripening of the seed are partly used for the creation of protoplasm as well as for laying down the walls of newly formed cells; they are consumed also, to obtain the energy necessary for growth.

These two opposite functions, the consumption of reserves and creation of new tissues, which characterize germination and growth are localised in different regions of most seeds. From a physiological point of view, there may be distinguished three main parts of the seed: (i) the seed-coat covering the seeds from outside and serve as a protection from various injuries and unfavourable conditions; (ii) the embryo consisting of rudimentary rootlet, leaves and stem uniting them; and (iii) reserve tissues, occupying usually the greatest part of volume of exalbuminous seed, when the reserves are stored mainly outside of the embryo itself in the endosperm. The endosperm may be small in the seeds whose reserves are stored in the cotyledons (exalbuminous seeds).

The germination of seed begins with swelling. With the development of the embryo, the seed coat bursts, ceasing to impede further growth. The growth of embryonic parts of seeds proceeds at the expense of reserve substances, which are decomposed by enzymes in the process of hydrolysis. The depletion of reserve organs is usually accompanied by their shrivelling and desiccation and finally their detachment from the plantlet after the embryo has attained complete independence. With the endosperm this is always the case. Frequently it is entirely dissolved and absorbed by the seeds so that only the empty seed coat remains. When these reserve organs are cotyledons and are carried above the ground, they usually become green and begin to function like real leaves, simultaneously increasing in size.

This is the stage where the seedling has completed its germination, that stage of the plant development at which the new seedlings are capable of nourishing themselves with food material that is present in the soil or with food material that they can manufacture from the air by the help of chlorophyll even in the absence of any such supply in the seed themselves.

Chemical analysis of the seeds was done to determine the chemical changes that an oil seed undergoes during the process of germination. We have already discussed the physiological

side of germination. The chemical changes were actually determined in the laboratory after germinating the two oil-seeds namely linseed and ground-nut.

In the table No. 2. analysis of the linseed showing changes during germination is considered. All the figures excepting moisture are on oven dry basis. The general analysis is carried on the lines as laid down by A.I.O.C. (U.S.A. 1926) Moisture, ash, ether extract, proteins, crude fibre, and carbohydrates (by difference) are calculated.

### **Moisture**

The figures for moisture during the first two days of germination remain the same, but at the end of the third day the moisture falls subsequently rising for the fourth day and again falling a little for the fifth day.

### **Ash**

The percentage of ash remains almost constant for the germinated seeds as well as the non-germinated seeds. It varies between 2.85% to 2.94% in all the cases.

### **Ether extract**

The ether extract or the percentage of oil at the end of the first day germination falls by 1%, but on the third day it falls by about 3% from its original value. At the end of the fourth day the percentage fall of the oil is about 11%, and at the end of the fifth day it is 18%. We see from the above figures that as the germination proceeds the oil contents of the seedling goes on decreasing gradually for the first two days but very rapidly thereafter.

### **Proteins**

As far as the proteins are concerned they seem to remain constant throughout the process of germination. In this particular case the protein percentage varies between 27.17% to 27.95%.

### **Crude Fibre**

The percentage of crude fibre goes on gradually increasing on the whole. For the first two days there is hardly any

appreciable increase, but from the third day onwards the increase is almost 1%. Before the germination was started it was 5.17%, but at the end of the fifth day of germination it had reached to 8.48%.

### **Carbohydrates (by difference)**

The total digestible carbohydrates were calculated by subtracting the moisture, ash, ether extract, and crude fibre from hundred. The difference gave the carbohydrates. It is observed from the table that the carbohydrates show a general tendency of increase during the process of germination. Before the germination of the seed the carbohydrates were almost 11%, but at the end of complete germination they increased to about 27%. A total increase of 16% during germination. It is observed as in the case of oil, crude fibre, that a definite change is brought about on the third day of germination. The carbohydrates also show this change.

Side by side with the other constituents in table No. 2, figures for sugars-reducing and non-reducing have been given. The sugars are calculated by Bertrand's method. Acidity is also determined in terms of milligrams of acetic acid present.

### **Reducing sugars**

The reducing sugars were found to be absent in the case of linseed seed either before or after germination.

### **Non-Reducing sugars**

In the case of linseed seed there is hardly any non-reducing sugar in the non-germinated material, as well as for the first three days after germination. It is present in very minute traces, but at the end of the fourth day germination there is sudden increase of the same. It is found that the non-reducing sugar is 4.05 % and it again increases to 4.90 % on the fifth day of germination. Excepting moisture, all the figures for sugars, in the table, are on oven dry basis.

TABLE No. 2  
**Analysis of linseed seed showing changes during germination.**  
**Excepting moisture, all figures are on oven-dry basis.**

Stage	Moisture	Ash	Ether-extract	Proteins	Crude-fibre	Carbohydrates by difference	Acidity	Reducing sugars	Non-reducing sugars
Before germination	5.5	2.85	47.61	27.17	5.17	11.70	0.36	nil	trace
1st day germination	5.0	2.94	46.52	27.74	5.26	12.60	0.54	nil	trace
2nd day germination	5.0	2.94	46.10	27.95	5.78	12.23	0.98	nil	trace
3rd day germination	3.9	2.91	44.53	27.23	6.45	14.98	1.37	nil	trace
4th day germination	4.6	2.93	36.16	27.25	7.44	21.62	1.69	nil	4.05
5th day germination	4.3	2.89	29.16	27.94	8.48	27.23	1.90	nil	4.90

## Acidity

The acidity of all the samples was calculated side by side with the sugars. It was measured in terms of milligrams of acetic acid present per gram of the substance. The acidity during germination gradually goes on increasing from day to day germination. As it will be seen from the table on the fifth day it is maximum, being 1.90 %.

A similar chemical analysis of the ground-nut seed was done to determine the chemical changes that the seed undergoes during the process of germination. As said before the seeds were germinated under natural conditions.

In the table No. 3, analysis of the ground-nut showing changes during germination is considered. All the figures excepting moisture are on oven dry basis. The general analysis is carried on the lines as laid down by A. L. O. C. (U. S. A. 1926). Moisture, ash, ether extract, proteins, crude fibre, and carbohydrates (by difference) are determined.

## Moisture

The moisture contents of the seeds decrease during the second day of germination; and still falls further on the third day as will be seen from the table, but again on the sixth day of germination the values increase and remain almost the same as those at the end of the second day of germination.

## Ash

The percentage of ash contents remains almost constant as in the case of linseed, for the germinated seeds as well as the non-germinated seeds. It varies between 3.04 % to 3.26 % in all the cases.

## Ether extract

The ether extract or the percentage of oil at the end of the second day germination falls by 3 %, but on the fourth day it falls by about 9 % from its original value. At the end of the sixth day germination the percentage fall of the oil is about 16 %. Comparing the figures in table No. 2, with those of the ground-nut in table No. 3, we see that the percentage fall

of the oil contents in the case of ground-nut seeds is more for the first four days of germination. But at the end of germination the total percentage fall of the oil is very nearly in the neighbourhood with those of the linseed at the end of complete germination. We see from the figures for the oil, that as the germination proceeds the oil contents go on decreasing gradually for the first two days, but very rapidly thereafter for the subsequent days.

### **Proteins**

As far as the proteins are concerned they seem to remain constant throughout the process of germination. In the case of ground-nut the protein percentage though higher than in the case of linseed behaves in the same way as far as germination is concerned. The protein percentage varies between 36.34 % to 36.73 %.

### **Crude fibre**

The percentage of crude fibre goes on increasing from day to day germination. On the second day of germination it is 2 % more than its original value. On the fourth day it is 5 % more and goes on increasing as the germination proceeds, reaching to 8.52 % on the sixth day. The figures for the crude-fibre for the linseed and ground-nut are very nearly the same for the last day of germination, but to start with the ground-nut has very small percentage of crude fibre before the germination, as compared to the linseed.

### **Carbohydrates (by difference) :**

The total digestible carbohydrates were calculated by subtracting moisture, ash, ether extract, and crude fibre from hundred. The difference gave the carbohydrates. It is observed as in the case of linseed, that the carbohydrates show a general tendency of increase from day to day germination. Before the germination of the seed had started, the carbohydrates were about 4 % in the resting condition of the seed. At the end of second germination they increase to about 8 %. At the end of fourth day the increase was maximum being about 14 %, which increased to about 16 % at the end of complete germination. It is observed from all the above

figures that a definite change is brought about at the end of fourth day germination in the percentage of oil crude fibre, as well as total digestible carbohydrates.

Side by side with the other figures in table No. 3, figures for sugars, reducing and non-reducing, are also given. The sugars were calculated by Bertrand's method, in terms of glucose. Acidity too is determined in terms of milligrams of acetic acid present.

#### **Reducing sugars**

Reducing sugars were found to be absent in the case of ground-nut seed either before or after germination.

#### **Non-reducing sugars**

The non-reducing sugars were determined by Bertrand's method. Before the germination had started the percentage of non-reducing sugar was about 2 % in the case of the ground-nut seed, but as the germination proceeded the quantity of non-reducing sugar went on increasing gradually. At the end of sixth day germination it was 6.55 %. Comparing the figures for the linseed the ground-nut contains higher percentage of non-reducing sugars. All the figures for sugars are on oven dry basis.

#### **Acidity**

The acidity of all the samples were calculated side by side with the sugars. It was measured in terms of milligrams of acetic acid present per gram of the substance. The acidity during germination gradually goes on increasing from day to day germination. It is maximum on the sixth day of germination being 1.46 %.



TABLE No. 8

Analysis of ground-nut seed showing changes during germination.  
 Excepting moisture, all figures are on oven-dry basis.

Stage	Moisture	Ash	Ether-extract	Proteins	Crude-fibre	Carbohydrates by difference	Acidity	Reducing sugars	Non-reducing sugars
Before germination	4.9	8.15	49.42	36.84	1.57	4.62	0.63	nil	2.81
2nd day germination	2.0	8.15	45.91	36.73	3.57	8.64	0.79	nil	3.06
4th day germination	1.4	8.04	39.55	36.51	6.79	14.11	1.09	nil	5.50
6th day germination	2.0	8.26	32.65	36.73	8.52	16.84	1.46	nil	6.55

### **Loss or gain in the various constituents of the ground-nut seeds before and after germination**

The table No. 4, shows the changes that occur during germination of ground-nut seeds. In one column figures for various constituents of the ground-nut seeds, before germination are given. The adjoining column contains the figures for analysis of various constituents for the same seeds, but the seedlings were analysed after their complete germination. In the extreme right column figures for gain or loss of each constituent after completion of germination are given. All the figures excepting moisture are calculated on oven dry basis.

#### **Moisture**

Moisture or loss in dry weight, for the ground-nut seeds is -2.90 after germination.

#### **Ash**

The ash contents remain very nearly the same, but here they show an increase of 0.11 after germination.

#### **Fats or oils**

The stored fats decrease during the process of germination, here in this particular case the decrease of fat contents after germination is + 16.77.

#### **Proteins**

The proteins are almost constant but there is an increase of 0.39 in the protein contents after germination.

#### **Crude fibre**

The crude fibre too shows an increase of 6.95 after germination.

#### **Carbohydrates**

Total digestible carbohydrates also show a gain of 12.22. The gain is quite appreciable comparing the figures for carbohydrates before germination,

**Acidity**

Acidity too shows a general tendency of increase. The increase is 0.83 after germination.

**Sugars: Reducing and Non-reducing :**

Non-reducing sugars show an increase of 3.74 after germination.

Reducing sugars are absent in the seeds either before or after germination.

TABLE No. 4

**Loss or gain in the various constituents of the ground-nut seed, before and after germination.  
Excepting moisture, all figures are on oven-dry basis.**

		Seeds before germina- tion.	Seeds after germina- tion.	Gain or loss during germina- tion.
Moisture	...	4.90	2.00	-2.90
Ash	...	3.15	3.26	0.11
Fats or oil	...	49.42	32.65	-16.77
Proteins	...	36.34	36.73	0.39
Crude fibre	...	1.57	8.52	6.95
Carbohydrates (by difference).	(by diff- erence).	4.62	16.84	12.22
Acidity	...	0.63	1.46	0.83
Sugars - reducing	...	nil	nil	nil
Sugars - non-reducing	...	2.81	6.55	3.74

### **Loss or gain in the various constituents of the linseed seeds before and after germination.**

The table No. 5, shows the changes that occur during the germination of linseed seeds. As in the case of the ground-nut seeds, the table for linseed too is prepared to give a comparative idea of the changes that occur during the germination of oil seeds. We shall now compare the results for both ground-nut and linseed.

#### **Moisture**

Moisture or loss in dry weight for the linseed seeds is -1.2 after germination. Comparing the loss of dry weight with that of the ground-nut seeds after germination, it is slightly less.

#### **Ash**

The ash contents also increase as in the case of the ground-nut but the increase of ash content is only 0.04, while in the case of the ground-nut it was 0.11. In the case of linseed the ash content remains very nearly constant before and after germination.

#### **Fats or oil**

The stored fats decrease during germination as in the case of the ground-nut seed. But the decrease of oil contents or fats is more in the case of linseed seeds being -18.45 comparing the figures for the decrease of oil in the case of ground-nut seeds, which only show a decrease of -16.77.

#### **Proteins**

The proteins show an increase of 0.77 after germination. The increase is almost double that for the ground-nut seeds.

#### **Crude fibre**

The crude fibre shows an increase of 3.31 after germination while in the case of ground-nut it was 6.95. Actually the increase of crude fibre material is less in the case of linseed as compared to that of ground-nut after germination.

## Carbohydrates

Total digestible carbohydrates show an increase of 15.53 after germination in the case of linseed seeds, while in the case of ground-nut seeds the increase was only 12.22

## Acidity

Acidity too shows an increase of 0.64, but the increase is less when compared with the ground-nut.

TABLE No. 5

Loss or gain in the various constituents of the linseed seeds before and after germination.

Excepting moisture, all figures are on oven-dry basis.

	Seeds before germination	Seeds after germination	loss or gain during germination
Moisture ...	5.50	4.30	- 1.20
Ash ...	2.85	2.89	0.04
Fats or oil ...	47.61	29.16	- 18.45
Proteins ...	27.17	27.94	0.77
Crude fibre ...	5.17	8.48	3.31
Carbohydrates by difference).	11.70	27.23	15.53
Acidity ...	0.36	1.90	0.64
Sugars reducing ...	nil	nil	nil
Sugars — Non-reducing.	trace	4.90	4.90

**Sugars: Reducing**

Reducing sugars are absent here as in the case of the ground-nut seeds, either before or after germination of the seeds.

**Sugars: Non-reducing**

The non-reducing sugars were in traces before germination, but at the end of complete germination they seem to have developed rapidly, being 4.90, even more than the ground-nut seeds.

We see in the case of linseed that upto the end of third day germination there is a slight decrease of oil and slight increase of carbohydrates. The same thing is observed in the case of the ground-nut up to the end of second day germination.

The minimum of the oil and the maximum of the total digestible carbohydrates is precisely reached at the same stage of germination. The corresponding decrease in the oil content of the seeds is balanced by a corresponding increase of total digestible carbohydrates, simultaneously, during the period of germination.

The above fact leads us to conclude that the carbohydrates are formed from the fatty acids or fats in the seeds.

Le clere du Sablon (1893) has shown that in almonds carbohydrates are first brought in the seed and subsequently transformed into oil. Another important observer is Eyre (1924). He has shown that in the flax seed the accumulation of oil is as rapid as three per cent. of the dry seed per day. The unsaturation of the oil increases only at the end of the development of the seed. Similarly D.L. Sahasrabudde and N. P. Kale, in their "The formation of the oil in the Niger seed", have indicated that the fatty acids or fats are formed from carbohydrates in the seed. In all the above cases it has been shown that an enzyme is present in the developing seed which has the property of bringing about synthesis of fats from the free acid and glyceride.

As regards the nature of the agents which are responsible for the changes that the seed material undergoes during germination only few facts are known. The resting seed especially rich in oil contains an enzyme 'lipase' which is supposed to bring about the hydrolysis of the fat so as to liberate the free fatty acids and these in their turn are broken down into still smaller units of carbohydrates which are necessary for the newly developing plant.

With a view to solve the above problem "Study in Germination of Oil Seeds" has been considered in the preceding pages. The composition of the seed from day to day germination has already been determined.

Looking to the figures of chemical changes during germination of the oil seeds studied for this paper it is clearly observed that during germination, when calculated on dry matter, the ash and protein constituents remain fairly constant, but there is a regular fall in the percentage of oil throughout the period of germination and this is shown by both the seeds under study. It will also be observed that corresponding to the percentage fall of oil there is a rise in the percentage of carbohydrates. The carbohydrates include crude fibre and other carbohydrates put together. There is a small amount of acidity developed, which perhaps comes during the change of the oil into carbohydrates.

Ordinarily during germination there is a loss in the total weight, calculated on dry matter, this is due to respiration of the germinating seeds. It will, therefore, be proved that during germination there is some necessary loss which will be found in all germinating experiments. In proteinaceous and starchy seeds the change is by converting 'glucose, but in the case of the oil seeds as has already been proved by so many workers the most important change is conversion of oil into carbohydrates. The formation of acidity and its increase is perhaps an intermediate change from the oil to carbohydrates.

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## PAPER XXIII

SYNTHESIS AND HYDROLYSIS OF ESTERS BY  
RICINUS LIPASE

BY

C. V. RAMKRISHNAN, M.Sc.

*(With the kind permission of the authorities of the Bombay University)*

## SECTION I

**General study of the Ricinus Lipase from Castor seed.****Introduction**

In this twentieth century which is an enzymic age, enormous amount of work is carried out throughout the world to investigate the great role which enzymes play theoretically as well as practically. Therefore the present work has been undertaken to investigate the general nature of the enzyme 'lipase' from castor seed.

J. Pelouze (C. R. 1855, 40, 605), W. Sigmund (Monats : 1890, 11, 272) had found that several seeds contain the active part called as enzyme. J. Reynolds Green (Proc. R. S. 1890, 48, 370) discovered the enzyme 'ricinus lipase' present in castor seed. Willstatter and Waldschmidt had found that the optimum pH for the ricinus lipase from castor seed is between 4.7 and 5.0 (Willstatter and Waldschmidt-Lietz. E. Uber ricinus lipase Z. Physiol. Chem. 134, 161-1923-24) varying slightly according to the buffer used.

So far Tanaka's method (eighth int. Cong. of applied chemistry, 1912, sec. Vd, 11, 37; J.S. C.I-192, 31, 884) has been used by all the workers to get a powdered form of the ricinus lipase from castor seed. But for the present work Longnecker. H. E. and Haly's D. E. (J. Am. Chem. Soc. 57, 2019, 1935) method has been used since it is the most modern method and the lipase obtained is the most active one.



## Experimental

The fresh well ripened castor seeds are purchased from the local market in large quantities. The outer skin of the seeds is removed, the decorticated seeds were ground well in a mortar and packed in a filter paper. The packet is kept in a Soxhlet and extracted with low boiling petroleum ether for about 3 hours i.e., till it is completely free from oil and other fatty materials. Then the packet is opened, the extracted mass is spread on a long sheet of filter paper and dried completely from petroleum ether at room temperature. When it is completely dried, it is powdered and sieved through a 60 mesh sieve and the fine powder obtained is preserved in a bottle after adding few drops of toluene to prevent bacterial action and used for the present work.

It is found that the lipase is acidic which may be due to the presence of 9-10 dihydroxy succinic acid and the acidity goes on increasing with the period of incubation but it is independent of the aging of the lipase.

J. J. Sudborough, H. E. Watson, P. S. Varma (Jr. In. Int. Sc.; Vol. 2, Part XV, pp. 213-273), Hoyer (Ber 1904, 37, 1436), Nielloux (1906) and Falk (J. Am. Ch. Soc. 1923, 35, 601) have studied the action of different substances on the activity of lipase. In the present work, the study of different factors which control the activity of the lipase has been made by studying the hydrolysis of olive oil by ricinus lipase from castor seed under different conditions.

### Effect of the nature of the buffer on the optimum pH of the ricinus lipase:—

Disodium phosphate-citric acid buffer mixtures of different pH are prepared according to MCILVAINE'S method (J. Biol. Chem. 1921, 49, 183), Sodium acetate-hydrochloric acid and sodium acetate-acetic acid buffer mixtures of different pH are prepared according to Walpole's method (Biochem. Jour. 1914, 105, 105, 2501, 2521). The hydrolysis of olive oil by ricinus lipase is carried out at different pH using the above prepared buffer mixtures.

Each set of experiments contains 1 c.c. of olive oil, 2 c.c. of water, 0.1 gram of lipase, 5 c.c. of buffer of varying pH value and a few drops of toluene in a conical flask. It is corked well and incubated for 24 hours at 37°C. Always a blank accompanied each sample. After the period of incubation, each flask is taken out and the contents are titrated against N/10 sodium hydroxide. The results are given in the following tables:—

TABLE No. 1

Determination of optimum pH for lipase using disodium phosphate-citric acid buffer.

Set No.	Oil in c.cs.	pH value	c.c. of N/10 NaOH for sample	c.c. of N/10 NaOH for blank	c.c. of N/10 NaOH for free acid
1	1 c.c.	2.4	19.0	15.9	3.1
2	"	2.8	18.8	14.0	4.8
3	"	3.1	15.5	10.6	4.9
4	"	4.8	10.2	5.2	5.0
5	"	5.2	4.9	4.2	0.7
6	"	5.5	3.8	3.3	0.5
7	"	5.7	7.1	6.1	1.0
8	"	6.2	9.2	9.0	0.2

TABLE No. 2

Determination of optimum pH for lipase using sodium acetate-hydrochloric acid buffer.

Set No.	Oil in c.cs.	pH value	c.cs. of N/10 NaOH for sample	c.cs. of N/10 NaOH for blank	Free acid
1	1 c.c.	1.5	13.8	12.9	0.9
2	"	2.9	18.2	10.3	7.9
3	"	3.8	25.3	11.5	13.8
4	"	4.3	25.2	9.9	15.3
5	"	4.8	31.0	10.4	19.6
6	"	5.2	10.8	8.3	2.5
7	"	5.5	9.5	7.3	2.2
8	"	5.7	8.6	7.2	1.6
9	"	6.0	7.2	6.1	1.1

TABLE No. 3

**Determination of optimum pH for lipase using sodium acetate — acetic acid buffer**

Set No.	Oil in c.cs.	pH value	c.cs. of N/10 NaOH for sample	c cs. of N/10 NaOH for blank	Free acid
1	1 c.c.	3.8	16.2	9.1	7.1
2	„	4.1	19.4	8.2	11.2
3	„	4.8	23.1	7.3	15.8
4	„	4.9	37.8	6.1	31.7
5	„	5.5	35.7	5.2	10.5
6	„	5.7	11.6	4.3	7.3
7	„	5.9	9.6	3.8	5.8
8	„	6.0	6.9	2.6	4.3

From the above tables, it is found that in disodium phosphate-citric acid buffer, the optimum pH is 4.8 since the hydrolysis is maximum i.e. equal to 5.0 c. e. in terms of N/10 NaOH. In sodium acetate-hydrochloric acid buffer, the optimum pH is 4.8 where the maximum hydrolysis is 19.6 c.c. in terms of N/10 NaOH. In sodium acetate-acetic acid buffer, optimum pH is 4.9 where the maximum hydrolysis is 31.7 c.c. of N/10 NaOH. It is inferred that the optimum pH for the hydrolysis of the olive oil by ricinus lipase from castor seed is constant i.e. 4.8 to 4.9 pH varying slightly according to the buffer used. But at the same time the percentage hydrolysis of olive oil is the greatest in sodium acetate-acetic acid buffer and the least in phosphate buffer. Therefore the percentage hydrolysis depends upon the nature of the buffer.

**Effect of buffer concentration on the hydrolysis of olive oil by lipase.**

Each set of the experiments consists of 1 c. c. of olive oil, 2 c.cs. of water, 0.1 gm of lipase and varying quantities of acetate buffer of 4.9 pH and incubated for 24 hours at 37°C. Always the blank experiment is carried out in each case.

After the period of incubation, the contents of each flask are titrated against N/10 NaOH after adding 25 c. cs. of neutral alcohol. The results are given in table No. 4.

TABLE No. 4

Set No.	Oil in c.cs.	c. cs. of buffer added.	c.cs. of N/10 NaOH for the sample	c.cs. of N/10 NaOH for the blank	free acid
1	1 c.c.	1 c.c.	30.3	1.2	29.1
2	"	2 "	25.0	1.8	23.2
3	"	3 "	20.5	2.1	18.4
4	"	4 "	18.3	2.3	16.0
5	"	5 "	17.4	2.5	14.9
6	"	6 "	15.7	3.4	12.3
7	"	7 "	14.8	3.8	11.0

From the above table it is found that maximum hydrolysis is obtained at the buffer concentration of 1 c.c. and falls gradually as the concentration increases which definitely shows that the percentage hydrolysis goes on decreasing with the increase of buffer concentration.

#### Effect of substrate concentration on the hydrolysis of olive oil by lipase:-

Each set of experiments consists of varying quantities of olive oil, 2 c.c. of water, 0.1 gm. of lipase and 1 c.c. of acetate buffer of 4.9 pH, incubated for 24 hours at 37° C. After the incubation, the contents are titrated against N/10 NaOH after addition of 25 c.c. of neutral alcohol. Each set is accompanied by a blank. The results are given in table No. 5.

TABLE No. 5

Set No.	Oil in c cs.	c.cs. of N/10 NaOH for the sample	c.cs. of N/10 NaOH required for the blank	free acid
1	1 c.c.	43.0	1.2	41.8
2	2 "	50.4	1.2	49.2
3	3 "	57.3	1.2	56.1
4	4 "	54.4	1.2	53.2
5	5 "	48.2	1.2	47.0
6	6 "	42.5	1.2	41.3

From table No. 5 it is found that the hydrolysis increases from the concentration of the substrate one c.c. to 3 c.c. and then gradually decreases as the concentration of the oil increases. Therefore the maximum hydrolysis is obtained when the concentration of the oil is 3 c.c.

#### Effect of enzyme concentration on the hydrolysis of olive oil by lipase:-

Each set of the experiments consists of 1 c.c. of olive oil, 2 c.cs. of water, 1 c.c. of acetate buffer and varying quantities of the enzyme lipase and incubated at 37° C. for 24 hours. After the period of incubation the contents are titrated against N/10 NaOH. Always a blank experiment is carried out in each case. The results are given in table No. 6.

TABLE No. 6

Set No.	Oil in c.cs.	Enzyme in gms.	c.cs. of N/10 NaOH for the sample.	c.cs. of N/10 NaOH required for the blank.	free acid.
1	1 c.c.	0.1 gram	38.1	1.6	36.7
2	"	0.2 "	38.3	1.6	36.7
3	"	0.3 "	39.1	1.7	37.4
4	"	0.4 "	40.3	2.0	38.3
5	"	0.5 "	46.2	2.0	44.2
6	"	0.6 "	48.9	2.1	46.8
7	"	0.7 "	52.1	2.3	49.8

From the above table, it is found that the percentage hydrolysis of olive oil increases with the increase in the concentration of the enzyme. It appears that it is better to use a concentrated form of lipase. This agrees with the statement of Constain, Hoyer and Wartenberg stating that increase in the amount of seed accelerates the hydrolysis.

**Effect of aging on the activity of the lipase:—**

There are so many factors which affect the activity of the enzymes e. g., heat, light, time, chemicals etc. out of that almost all other factors can be prevented except the time factor. So an investigation has been carried out to study the effect of aging on the activity of the lipase.

The hydrolysis of olive oil by ricinus lipase is studied from the time of preparation of the lipase upto a period of 4½ months. Each set of the experiments consists of 1 c. c. of olive oil, 2 c. c. of water, 0.1 gm. of lipase and 1 c. c. of acetate buffer. It is kept in the incubator at 37°C. for 20 minutes and then titrated against N/10 NaOH. The results are given in Table No. 7.

TABLE No. 7.

Set No.	Time	c.cs. of oil	Activity in terms of N/10 NaOH
1	0 minute	1 c.c.	7.5 c.c.
2	20 minutes	1 "	7.9 "
3	40 "	1 "	7.9 "
4	1 hour	1 "	8.4 "
5	24 hours	1 "	13.7 "
6	2 days	1 "	23.2 "
7	3 days	1 "	23.3 "
8	4 days	1 "	23.4 "
9	5 days	1 "	23.5 "
10	6 days	1 "	24.4 "
11	8 days	1 "	24.8 "
12	11 days	1 "	20.5 "
13	15 days	1 "	20.2 "
14	23 days	1 "	19.9 "
15	71 days	1 "	17.9 "
16	78 days	1 "	10.7 "
17	85 days	1 "	10.2 "
18	93 days	1 "	9.8 "
19	100 days	1 "	8.9 "
20	114 days	1 "	8.6 "
21	121 days	1 "	7.8 "
22	134 days	1 "	6.5 "
23	142 days	1 "	5.1 "
24	150 days	1 "	3.9 "
25	155 days	1 "	2.8 "
26	160 days	1 "	0.5 "

From the above table, it is found that the activity increases upto 8th day and then slowly decreases and on 160th day, it is almost lost. The reason may be due to a little amount of petroleum ether which adheres to the lipase or due to some physical change which takes place in the enzyme after some time.

### **Preparation of different samples of lipase :—**

As it is found that lipase dried by petroleum ether is not keeping its activity constant, several preparations of lipase have been prepared and the effect of aging on their activities has been studied to select a sample which will keep its activity fairly constant for a long time.

So first petroleum ether extract of the lipase is prepared by usual method and then three samples are prepared by draining for 2 hours one with petroleum ether, the other with ethyl ether and the third with boiling acetone. All the three samples are completely dried, sieved through a 60 mesh sieve and used for the experiment.

It is found that acidity of lipase is the same in case of all samples and it also remains constant with change of time. Then the activities of three samples are determined at different intervals.

Each set of experiments consists of 1 c. c. of olive oil, 2 c. c. of water, 1 c. c. of acetate buffer and 0.1 gm. of lipase of each variety at different intervals of time kept in the incubator at 37°C. for 20 minutes and immediately titrated against N/10 NaOH. The results are given in table No. 8.

From table No. 8, it is found that in case of petroleum ether dried and ether dried samples, the activity first increases and then falls. In case of petroleum ether samples, it is increased upto the 8th day and then falls gradually and on 160th day it is almost nil. In case of ether dried sample it increases upto the 5th day and then decreases and on 142nd day it is almost zero. In case of acetone dried sample, it is almost constant throughout even though it is very low. So acetone dried sample is a modified sample of lipase which

retains its activity fairly constant for a long time even though the activity is less and it can be used for all the synthesis and hydrolysis experiments.

TABLE No. 8

Set No.	Time	Gms. of Lipase	c.cs. of N/10 NaOH required for		
			P. E. sample	E. sample	A. sample
1	0 time	0.1 gm.	7.5	5.2	1.4
2	20 minutes	„	7.9	5.8	„
3	40 minutes	„	7.9	5.9	„
4	1 hour	„	8.4	6.1	„
5	24 hours	„	13.7	10.2	„
6	2 days	„	23.2	11.5	„
7	3 days	„	23.3	12.1	„
8	4 days	„	23.4	14.8	„
9	5 days	„	23.5	18.2	„
10	6 days	„	24.4	16.5	„
11	8 days	„	24.8	15.8	„
12	11 days	„	20.5	10.2	„
13	15 days	„	20.2	9.2	„
14	23 days	„	19.9	8.4	„
15	71 days	„	17.9	5.3	„
16	73 days	„	10.7	4.6	„
17	85 days	„	10.2	3.7	„
18	93 days	„	9.8	2.9	„
19	100 days	„	8.9	2.7	„
20	114 days	„	8.6	1.2	„
21	120 days	„	7.8	1.1	„
22	134 days	„	6.5	0.8	„
23	142 days	„	5.1	0.4	„
24	150 days	„	3.9	...	„
25	155 days	„	2.8	...	„
26	160 days	„	0.5	...	„

### Summary

(1) The optimum pH of the lipase is independent of the nature of the buffer mixture used but the percentage hydrolysis depends upon the nature of the buffer mixture and sodium



acetate-acetic acid-buffer is the best activator for the hydrolysis of oils by ricinus lipase.

(2) The hydrolysis of olive oil decreases as the buffer concentration goes on increasing reaching a maximum value at concentration equal to 6 c.cs.

(3) The hydrolysis of olive oil increases as the concentration of the substrate increases upto 3 c.cs. and then gradually decreases as concentration of substrate increases.

(4) The hydrolysis of olive oil increases as the concentration of enzyme increases reaching maximum value of 49.8 at concentration 0.7 gm. of lipase.

(5) On aging, the activity of petroleum ether dried lipase goes on decreasing and after 160th day almost becomes zero. In case of acetone dried sample, the activity remains fairly constant even though it is low and therefore it is used for the work to be described in synthesis and hydrolysis of esters.

## SECTION II

### Synthesis of some aliphatic esters by ricinus lipase.

#### Introduction

Bodenstein and Dietz and Dietz (Zeitschr. F. - Electrochem. 12, 605, 1906; Zeitschr. F, Physical. Chem. 52. 279, 1907; Siehe such euler, diese Zeitschr. 52, 146, 1907) investigated in the initial experiments the formation and the splitting of isoamyl butyrate catalysed by pancreatic powder. In the year 1931, P. Rona and R. Ammon (Vel. Rona Ammonu: Fischgold, diese Zeitschr. 241, 460, 1931) synthesised normal butyl butyrate by pig's pancreatic lipase. They found that it was possible to synthesise and hydrolyses that ester by pig's pancreatic lipase and further it worked in greater excess of alcohol. Afterwards the syntheses of butyl and phenyl-ethyl oleates were carried out using ricinus lipase and the percentage reached is 85. (Bull Sochin—1939).

In 1939, Leon Velluz (loc. cit) synthesised butyl oleate using 17 organic solvents to study the effect of the solvent on the synthesising action of the lipase.† He found that in petroleum ether the synthesis is maximum i.e. 85 per cent.

In 1942, fat was synthesised from glycerol, oleic acid and lipase obtained from 21 seeds at 30 degrees centigrade and it was inferred that the synthesis was much appreciable in case of *ricinus communis* (effect of glycerol concentration on enzymic synthesis of fats by seed lipases - Z. Physiol. Chem. 1942-G.A. 6706, 1943).

From the above data it is seen that only small amount of work has been done on the synthesising action of the lipase. In the present work the synthesis of eleven aliphatic esters has been carried out to study the effect of change of number of carbon atoms in the alcohols and acids on the synthesis of esters by *ricinus* lipase.

#### **Synthesis of butyl oleate using different samples of lipase :**

Leon Velluz (Loc. cit.) synthesised butyl oleate using petroleum ether dried lipase in 17 organic solvents. In the present work, the synthesis of butyl oleate is carried out with different solvents such as petroleum ether, carbon tetrachloride, acetone, ether, benzene and chloroform, and it is observed that the mixture is homogeneous only in ether solvent. As all the work is to be carried out with a view to find out the effect of number of carbon atoms in the alcohol and the acid on the synthesis and hydrolysis of esters, ether is chosen as a solvent for all the experiments.

Here the results of the synthesis of butyl oleate by petroleum ether dried lipase are compared with the results obtained by Leon Velluz. The results obtained in case of petroleum ether dried lipase agree with that of the French author within the limits of the experimental error whereas when the synthesis is carried out using acetone dried lipase, the percentage synthesis is always low which is due to the less activity of the acetone dried sample.

### Synthesis of butyl oleate by petroleum ether dried lipase:

The chemicals used are :-

- (1) Butyl alcohol (normal) — Density-0.809; mol. wt=74.1  
B.P. = 117°c
- (2) Oleic Acid — B.P. 286°C—Density-0.895; mol. wt.=282.5

In different conical flasks, 5 c.c. of acid and 3 c.c. of alcohol are added. One gram of lipase and 10 c.c. of different solvents are added to each flask, which was shaken well and kept in the incubator at 37°C after corking them well. At different intervals of time, 1 c.c. from each flask is taken, 25 c.c. of neutral alcohol added, warmed for some time and titrated against N/10 NaOH. Always the blank is carried out in case of each experiment.

**The same experimental procedure is followed for the synthesis of the remaining esters in this part of the work.**

The comparison of the percentage synthesis of butyl oleate by petroleum ether dried lipase with the results of the French paper is given in table No. 1.

It is found that the results of the synthesis of butyl oleate by petroleum ether dried lipase is almost the same as that obtained by the French author, the small difference existing may be due to difference in the castor seeds used by the authors. So after verifying the results, the same synthesis has been carried out using acetone dried lipase and the results are given in table No. 2.

From the results, it is inferred that the synthesis is always less in case of acetone dried lipase due to its less activity than in case of P. E. dried lipase. So the synthesis of all the esters is carried out using acetone dried lipase in the following paragraphs since the activity of the acetone dried lipase is fairly constant even though it is less.

### Synthesis of some aliphatic esters :—

Study of effect of change of number of carbon atoms in alcohols and in acids on the synthesis of esters by ricinus lipase:—

The synthesis of the following esters has been carried out in ethyl ether solvent to see whether the synthesis of those

TABLE No. 1.

Solvent	Number of days													
	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Petroleum ether.	32.4	35.5	54.9	69.0	70.8	82.3	80.7	85.6	77.4	85.6	...	...	...	...
Carbon tetra-chloride.	27.3	36.7	34.2	54.5	60.9	67.8	69.6	75.6	68.4	...	...	82.3	...	...
Acetone ...	24.3	26.7	33.3	35.5	45.6	46.7	47.4	53.4	52.5	55.6	59.1	56.7	...	...
Ether ...	16.5	17.8	29.7	31.2	40.2	42.7	50.7	53.4	52.5	56.7	62.7	66.7	...	...
Benzene ...	13.5	15.4	21.9	25.6	31.2	33.4	46.5	48.9	52.5	...	65.7	62.3	...	...
Chloroform ...	1.2	2.3	1.8	2.3	9.3	11.2	11.4	...	12.0	11.2	12.3	...	...	...

TABLE No. 2.

	A	C	A	C	A	C	A	C	A	C	A	C	A	C
Petroleum ether.	32.4	25.2	54.9	37.5	70.8	42.3	80.7	51.9	77.4	60.6	...	59.7	...	...
Carbon tetra-chloride.	27.3	17.7	34.2	21.9	60.9	35.1	69.6	41.1	68.4	47.7	...	50.7	...	47.4
Acetone ...	24.3	13.5	33.3	20.1	45.6	28.5	47.4	32.1	52.5	37.5	59.1	39.6	54.6	38.7
Ether ...	16.5	16.5	29.7	19.5	40.2	27.0	50.7	30.3	52.5	39.6	52.7	42.6	58.5	41.1
Benzene ...	13.5	6.9	21.9	8.4	31.2	11.7	46.5	15.6	52.5	25.2	66.7	32.1	61.8	30.3
Chloroform ...	1.2	0.0	1.8	1.2	9.3	5.4	11.4	8.4	12.0	8.4	12.3	12.3	13.5	10.1

- A. The percentage synthesis using petroleum dried lipase.  
 B. The percentage synthesis according to the French paper.  
 C. The percentage synthesis using acetone dried lipase.

esters is possible:—methyl, ethyl, n-propyl, iso-propyl, normal-butyl, iso-butyl, normal amyl, iso-amyl butyrates, amyl-formate, acetate and propionate.

In the present experiments, equimolecular quantities of alcohols and acids are taken (0.054 gm. molecule) and the percentage synthesis is calculated. In the first 8 syntheses, butyric acid is common and only different alcohols are used. In the second case, amyl alcohol is common and different acids are used. From the results, conclusions regarding the effect of change of number of carbon atoms in the alcoholic and acidic groups on the synthesis of esters have been drawn.

For expt.      Acid — 0.054 gm. mol.  
                   Alcohol — 0.054    „  
                   Ether solvent — 10 c.c.  
                   Lipase — 1 gm.

### Discussion of the Results

The synthesis is maximum in case of n-amyl butyrate on 5th day i.e. 78.1% and minimum in case of methyl butyrate on 1st day i.e. 3.28%.

From the first 8 syntheses, it is found that the percentage synthesis steadily increases from methyl to amyl butyrate. It shows clearly that as the number of carbon atoms goes on increasing in the alcohol group, the synthesis also increases up to amyl butyrate.

Secondly the synthesis is greater in the iso forms of the alcohol than in the normal one.

The difference in percentage synthesis in case of normal propyl and iso-propyl butyrate is due to the difference in structure (n-propyl alcohol is primary where as iso-propyl alcohol is secondary). In case of normal and iso butyl esters, difference in percentage is not due to the difference in structure because both are primary, but it may be due to the difference in densities.

(Density of n-Butyl alcohol is 0.809 gm/ml.)

(Density of iso-    „    „    is 0.802 gm/ml.)

But however in iso variety of amyl alcohol, the percentage is always less which can be only stated as unexplained.

Thus these results give in general the effect of change of number of carbon atoms in alcohols in synthesis of esters and of structure and density of alcohol on the synthesis of these esters.

By comparing last four syntheses, it is found that the percentage synthesis is almost the same in case of these esters, the difference being very small. So it can be concluded that the synthesis of esters is independent of number of carbon atoms in the acids and only depends upon the number of carbon atoms in the alcohol.

### I. Percentage Synthesis of Different Esters in *Ether Solvent*

Lipase - 1 gm. Acid Butyric :-0.054 gm. mol.

Ether Solvent - 10 c.c. Alcohols : -0.054 gm. mol.

Always Blank is accompanied

Name of the Ester	Percentage Synthesis					
	Days					
	1	2	3	4	5	6
(1) Methyl Butyrate ...	3.28	4.51	6.97	9.02	11.48	...
(2) Ethyl Butyrate ...	3.20	4.21	11.60	16.80	16.5	...
(3) n-Propyl Butyrate...	3.25	4.6	8.2	9.2	15.8	16.9
(4) iso-Propyl Butyrate	11.44	18.04	21.76	22.8	26.8	...
(5) n-Butyl Butyrate ...	11.20	13.3	14.8	20.5	24.5	28.3
(6) iso-Butyl Butyrate...	14.70	23.4	33.3	42.9	68.4	...
(7) n-Amyl Cutyrate ...	30.80	55.0	64.9	69.3	78.1	...
(8) iso-Amyl Butyrate...	6.6	12.1	26.4	31.9	47.3	...
n-Amyl Formate ...	32.4	40.8	63.6	75.8	78.2	...
„ Acetate ...	26.1	32.1	38.7	74.5	82.9	...
„ Propionate ...	45.1	55.7	71.2	77.2	76.8	...
„ Butyrate ...	30.8	55.0	64.9	69.3	78.1	...

## II. Synthesis of Propionates

Name of the Ester	Percentage Synthesis Days					
	1	2	3	4	5	6
(1) Methyl Propionate...	4.2	4.9	6.2	10.1	11.6	...
(2) Ethyl Propionate ...	5.1	6.3	10.8	15.4	17.2	...
(3) n-Propyl Propionate	5.5	6.8	9.1	14.8	16.8	18.2
(4) iso-propyl Propionate	12.3	19.1	22.8	25.4	27.6	...
(5) n-Butyl Propionate...	12.5	15.6	21.7	26.2	28.4	29.2
(6) iso-Butyl Propionate	15.2	25.3	31.8	39.5	43.8	65.4
(7) n-Amyl Propionate..	45.1	55.7	71.2	77.2	76.8	...
(8) iso-Amyl Propionate.	9.2	12.3	20.5	30.8	41.2	...

## III. Synthesis of n-Butyl - Formate, Acetate, Propionate and Butyrate

N-Butyl Formate ...	12.8	16.2	22.5	25.6	27.2	...
.. Acetate ...	11.8	14.5	18.2	22.6	25.4	...
.. Propionate ...	12.5	15.6	21.7	26.2	28.4	29.2
.. Butyrate ...	11.2	13.3	14.8	20.5	24.5	28.3

### Summary :-

(1) Synthesis of butyl oleate has been carried out using P. E. dried lipase and acetone dried lipase. The results in case of P. E. dried lipase agree with that of the results obtained by Leon Velluz. In case of acetone dried lipase, the synthesis is less due to less activity of the lipase. Still the acetone dried lipase only is used for further experiments since its activity remains constant for a fairly long time.



(2) The synthesis of eleven aliphatic esters has been carried out by taking equimolecular quantities of alcohol and acid. The synthesis of methyl, ether n-propyl, iso propyl, n-butyl, iso-butyl, n-amyl, iso-amyl butyrates, amyl formate, acetate and propionates has been carried out in ether solvent.

(3) From the first eight synthesis experiments, it is found that the percentage synthesis steadily increases from methyl to amyl butyrate showing that as the number of the carbon atoms goes on increasing in the alcohol, the percentage synthesis also increases.

(4) Further, a clear distinction is shown between primary and secondary alcohols due to difference in structures. Difference in case of n-and iso-propyl butyrate is due to the difference in structures (n-propyl alcohols is primary whereas iso propyl alcohol is secondary). In case of n-and iso-butyl esters, the difference is not due to the difference in structure because both are primary, but it may be due to the difference in densities (Density of n-butyl alcohol is 0.809 gm/c.c.)

( iso ,, 0.802 ,, )

In case of iso-amyl ester, the percentage is always low but the results obtained remain unexplained.

(5) From the last four syntheses, it is found that the percentage is always the same showing that the percentage synthesis of the ester is independent of the number of carbon atoms in the acid group but always depends upon the number of carbon atoms in the alcohol.

### SECTION III

#### Hydrolysis of some aliphatic esters by ricinus lipase:—

##### Introduction

In 1932, P. Rona and R. Ammon (Versuche uber enzymatische ester hydrolyse und synthese - 1932) carried out the hydrolysis of normal butyl butyrate by pig's pancreatic lipase and obtained valuable results. The hydrolysis of benzy-

stearate and butyrate were carried out at 40°C by pancreatic lipase and it was found out that the hydrolysis is independent of the substrate concentration (C. A. 9109-3-1938). Another investigator has found that triacetin is rapidly hydrolysed by pancreatic lipase in presence of calcium chloride (C. A. 1287-10, 1938).

F. E. Kelsey has found out that the hydrolysis of olive oil, beef fat, lard, tristearin and tripalmitin by castor bean lipase goes to completion ("use of lipase in lipid analysis"—F. E. Kelsey—J. Biol. chem.—130, 1939). Also the hydrolysis of butter fat by pancreatic lipase has been studied by Erkki Peltola - Suomen Kemistilehti (C. A. 1213-5-6, 1939).

F. P. Mazza and C. Mala Guzzi. Valeri have studied the hydrolysis of 2-3 dimethyl, 1-stearyl glycerol and 1-3 dimethyl 2-stearyl glycerol by pancreatic lipase and concluded that lipase splits up ester linkage of primary and secondary alcoholic groups (Arch. Sci. Biol. Italy, 1939).

But so far nobody has studied the hydrolysis of esters of a homologues series. So in the present work, the hydrolysis of the following esters by ricinus lipase has been investigated to study the effect of change of alkyl and carboxyl groups on the ester hydrolysis:- methyl, ethyl, n and iso propyl, normal and iso butyl, normal and iso amyl butyrates, amyl formate, acetate and propionate. As all the esters were not available, they were prepared in the laboratory according to the standard methods and the physical constants were found out.

#### **Hydrolysis of butyl oleate by different samples of lipase:-**

As it is compared in case of synthesis experiments, here also hydrolysis of butyl oleate is carried out using acetone dried lipase as well as petroleum ether dried lipase and the results are compared in table No. 1.

Each set of the experiments consists of 2 c.c. of ester solution, 10 c.c. of the solvent, 1 gm of lipase and a few drops of toluene; the mixture was shaken well and 1 c.c. of it is taken and immediately titrated against N/10 NaOH, which gives the

reading for the blank. Then the flask is corked well, incubated at 37°C and at different intervals of time 1 c.c. of it is taken, 25 c.c. of neutral alcohol added, warmed and titrated against N/10 NaOH. This gives the reading for the sample. From the difference in reading between the sample and the blank, the percentage hydrolysis is calculated. The same experimental procedure is followed for the hydrolysis of other esters also in this part.

From table No. 1, it found that the percentage hydrolysis is always greater in case of petroleum ether dried lipase than in case of acetone dried lipase. But only acetone dried lipase is used for further experiments since its activity remains fairly constant.

### Hydrolysis of some aliphatic esters

(To study the effect of change of number of carbon atoms in the alcohol and acid on the hydrolysis of esters).

As it is done in synthesis experiments, the hydrolysis of the following esters has been carried out by taking the same molecular quantity of ester in each case (0.018 gm. mol):— methyl, ethyl, n-propyl, iso-propyl, n and iso butyl, n and iso amyl butyrates, amyl formate, acetate and propionate in ether solvent.

For each experiment, Ester sol. — 2 c.c.	}
Ether solvent — 10 c.c.	
Lipase — 1 gm	

The results are tabulated.

### Discussion of the Results :—

The hydrolysis is maximum in case of methyl butyrate on 3rd day and minimum in case of iso propyl butyrate on 1st day.

The percentage hydrolysis goes on decreasing from methyl to iso-amyl butyrate and so it can be inferred that the number of carbon atoms in the alcohol group decreases the percentage hydrolysis. In other words hydrolysis is difficult in case of higher esters. This is quite in agreement with the results of the Japanese investigator who has found that increase of carbon atoms in alcohol group decreases the hydrolysis by studying the different esters of valeric acid (Jour. of Agri Soc. Japan-1940).

TABLE No. 1

## Percentage Hydrolysis

Solvent	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Petroleum ether.	22.0	20.0	32.0	26.0	48.0	42.0	56.0	50.0	68.0	56.0	78.0	68.0	76.0	66.0
Carbon tetra- chloride.	16.0	12.0	22.0	20.0	32.0	26.0	42.0	32.0	38.0	40.0	...	38.0	...	...
Acetone ...	12.0	10.0	18.0	16.0	36.0	22.0	50.0	36.0	62.0	42.0	70.0	40.0	66.0	...
Ether ...	28.0	8.0	32.0	14.0	42.0	20.0	54.0	32.0	66.0	50.0	70.0	54.0	68.0	...
Benzene ...	30.0	16.0	36.0	20.0	48.0	38.0	48.0	34.0	...	...	...	...	...	...
Chloroform ...	12.0	6.0	20.0	10.0	24.0	16.0	26.0	20.0	24.0	18.0	...	...	...	...

A — Hydrolysis of Butyl oleate by petroleum ether dried Lipase.

B — Hydrolysis of Butyl oleate by acetone dried Lipase.

The percentage hydrolysis is always greater in case of ester obtained from primary alcohol than in the one obtained from secondary alcohol. In case of n and iso propyl esters, the difference in percentage hydrolysis is due to the difference in structure of alcohol used for preparing ester (normal is primary whereas iso is secondary). In case of n and iso butyl butyrates and n and iso-amyl butyrates, the difference in percentage is due to the difference in densities

(n. B. ester - 0.872

I. B. ester - 0.861

n. A. ester - 0.871

I. A. ester - 0.862)

From the last four hydrolyses, it is found that the percentage hydrolysis is almost the same in all cases. So it can be inferred that the change of number of carbon atoms in acid group does not affect the percentage hydrolysis of esters. In other words the hydrolysis of esters is independent of the number of the carbon atoms in acid but depends upon the number of carbon atoms in alcoholic group.

### Summary.

(1) The hydrolysis of butyl oleate has been carried out using both petroleum ether dried lipase and acetone dried lipase. The hydrolysis is less in case of acetone dried lipase due to its less activity but since it retains its activity for a fairly long time, it is only used for other experiments.

(2) The hydrolysis of the following esters has been carried out in ether solvent by taking the same molecular quantity of different esters. (0.018 gm molecule):—methyl, ethyl, n and iso propyl, n and iso butyl, n and iso amyl butyrates, amyl formate, acetate and propionate.

(3) From the hydrolysis of first eight esters, it is found that the percentage hydrolysis decreases from methyl to amyl butyrate showing that increase in the number of carbon atoms in the alcoholic group decreases the hydrolysis.

(4) The hydrolysis is always greater in ester got from normal alcohol than in one got from iso-alcohol. The difference in percentage hydrolysis in case of normal and

iso propyl esters is due to the difference in structure whereas in case of n and iso butyl as well as n and iso amyl esters, it is due to difference in densities.

(5) From the last four hydrolyses, it is found that the percentage hydrolysis is the same in case of all the esters which clearly shows that the percentage hydrolysis is independent of the number of carbon atoms in the acid group but always depends upon the number of carbon atoms in alcoholic group.

#### L Hydrolysis of some aliphatic esters by ricinus lipase :-

Lipase - 1 g.m.

Ester Sol. = 0.018 gm. mol.

Solvent—Ether - 10 c.c.

Name of the Ester	Percentage Hydrolysis					
	Days					
	1	2	3	4	5	6
Methyl butyrate ...	44.0	46.0	51.5	50.5	...	...
Ethyl butyrate ...	45.5	51.5	43.5	...	...	...
n-Propyl butyrate ...	38.2	40.9	43.7	45.9	43.7	...
iso-Propyl butyrate ...	15.5	18.8	22.6	29.2	36.7	34.8
n-Butyl butyrate ...	21.2	23.8	30.9	33.7	32.3	...
iso-Butyl butyrate ...	22.6	25.8	33.4	33.7	31.8	...
n-Amyl butyrate ...	19.8	28.1	29.2	31.2	30.5	...
iso-Amyl butyrate ...	21.1	24.9	29.2	30.8	28.7	...
N-Amyl formate ...	10.8	19.2	23.8	28.2	30.9	...
„ acetate ...	16.1	20.8	31.8	30.4	...	...
„ propionate ...	14.9	25.8	33.7	32.5	...	...
„ butyrate ...	19.8	28.1	29.2	31.2	30.5	...

## II. Hydrolysis of Propionates:-

Name of the Ester	Percentage Hydrolysis					
	Days					
	1	2	3	4	5	6
Methyl propionate ...	40.1	44.8	49.2	50.8	48.5	...
Ethyl propionate ...	45.0	48.5	50.7	51.9	51.5	...
n-Propyl propionate ...	37.0	39.2	42.5	46.7	45.8	...
iso-Propyl propionate ...	17.2	19.1	24.8	31.6	35.8	34.5
n-Butyl propionate ...	20.4	24.7	31.2	34.5	33.5	...
iso-Butyl propionate ...	21.6	25.0	32.6	33.9	32.5	...
n-Amyl propionate ...	14.9	25.8	33.7	32.5	...	...
iso-Amyl propionate ...	15.5	26.0	32.8	32.1	...	...

## III.

n-Butyl formate ...	20.9	22.9	29.8	34.0	33.2	...
„ acetate ...	19.8	25.7	32.4	35.3	35.0	...
„ propionate ...	20.4	24.7	31.2	34.5	33.6	...
„ butyrate ...	21.2	23.8	30.9	33.7	32.3	...

## SECTION IV

**Effect of salts on the synthesis and hydrolysis of some aliphatic esters by lipase.**

### Introduction

Constain, Hoyer and Wartenberg found that the hydrolysis of oils is accelerated by the addition of chloral hydrate. Armstrong (Proc. R.S., 1905, 76 B, 606) has found that the accelerating action is due to the liberation of small amount of hydrochloric acid. It was found by the same investigators that the acids accelerate the hydrolysing action of the lipase.

Hoyer (1907), Nicoloux (1906) and Tanaka (1912) found that manganese sulphate in dilute form can be used as a cofe-ment. Falk and Hamblim (J. Am. Chem. Soc. 1910, 35, 210) investigated the reaction and found that manganese sulphate in dilute form oxidises inactive Zymogen of castor seed into active enzyme.

It was also found that alcohol, formaldehyde act as poisons to castor seed lipase whereas NaCl, FeSO<sub>4</sub> have no action (Constein, Hoyer and Wartenberg-1902).

Van den Driessen Marecuw (Pharm. Week blad, 1909, 46, 346, J.S.C.I. 1909, 28, 612) stated that potassium cyanide has no action in case of kolanut lipase and potassium chromate, mercuric chloride have deleterious action and potassium ferrocyanide, a favourable effect. Falk (J. Am. Chem. Soc. 1913, 35, 601) has also studied the effect of various salts on the activity of the lipase.

In 1932, P. Rona and R. Ammon (Loc. cit.) studied the action of calcium chloride, chinin chloride and strychnine sulphate on the synthesis and hydrolysis of normal butyl normal butyrate by pancreatic lipase. Calcium chloride acts strongly in hydrolysis and the others have no action.

In 1934, D. E. Haley has found that chlorides and acetates retard or inhibit the action of enzymes, salts of cobalt, copper and mercury showing great inhibiting action. Iron, lead, nickel and zinc salts definitely retard the reaction, calcium and cerium salts to less extent (C.A. 2161, 2156-D. E. Haley-Jour. Am. Chem. Soc. 1934).

A mixture of albumin and calcium chloride was found to be superior to sodium oleate in the hydrolysis by pancreatic lipase (J. Agr. Chem. Soc. Japan-1939) whereas Ed. Keeser has found that acetone activates the lipase (C.A. 4779/8-1943). The effect of 36 salts on the activity of the ricinus lipase is studied and two lipolytic series, one for cation and one for anion, are reported. (Effect of salts on ricinus lipase-C.A. 3299-1944-J. Chinese Chem. Soc.-1943).



In this paper, the action of the following eleven substances has been studied on the synthesis and hydrolysis of all aliphatic esters, carried out in Paper II and Paper III:—acetic acid, potassium cyanide, sodium chloride, calcium chloride, aluminium chloride, sodium sulphate, sodium phosphate, magnesium sulphate, manganese sulphate, barium chloride and potassium dichromate. The ether solvent has been chosen in case of each ester and in that solvent, the action of the above eleven substances has been studied and the results are tabulated.

### Experimental

Decinormal solutions of the above eleven substances are prepared and used for the experiments.

For synthesis experiments, each set of the experiments consists of 5 c.c. of acid, 3 c.c. of alcohol, 1 gram of lipase, 10 c.c. of the best solvent and 1 c.c. of the salt solution. The blank experiment is also carried out for reference. After thorough shaking, all are kept in the incubator at 37°C and at different intervals of time, 1 c.c. of each is taken, 25 c.c. of neutral alcohol added, warmed and titrated against N/10 NaOH. The results, in the form of difference in reading between the sample and the blank, in case of each salt are given in tabular form. The difference between the two readings show whether a particular salt is an accelerator inhibitor depending upon whether the difference is a positive one (showing the accelerating effect) or a negative one (showing the inhibiting action). The same experimental procedure is followed in case of all the esters.

For hydrolysis experiments each set of the experiment consists of 2 c.c. of ester solution, 1 gram of lipase, 10 c.c. of the best solvent and one c.c. of salt solution. The blank is carried out without salt solution. All are kept in the incubator at 37°C and at different intervals of time, 1 c.c. of each is taken, 25 c.c. of neutral alcohol and titrated against N/10 NaOH. The difference in reading between the sample and the blank shows whether particular salt is an accelerator or retarder depending upon whether the difference is a +ve one (showing accelerating effect) or a negative one (showing the retarding effect).

Results :—

**Synthesis of methyl butyrate**

**TABLE No. 1**

**Solvent - Ether**

Days	Difference in c.cs. of N/10 NaOH for the sample and the blank										
	A	B	C	D	E	F	G	H	I	J	K
1	2.0	4.7	5.0	6.7	- 6.6	- 31.2	5.7	2.8	3.4	5.2	5.4
2	- 0.3	- 3.3	1.2	4.1	- 11.0	- 35.5	1.5	- 1.0	3.8	4.8	4.4
3	- 0.5	- 4.2	0.9	3.9	- 15.2	- 36.8	- 20.8	- 1.2	3.8	3.2	3.8
4	- 0.7	- 6.6	0.7	3.2	- 19.4	- 38.5	- 35.3	- 1.5	3.9	1.8	2.2
5	- 1.1	- 13.5	0.4	3.0	- 18.6	- 39.5	- 44.1	- 1.3	4.1	1.7	2.0
6	- 1.2	- 11.0	- 0.4	4.4	- 8.5	- 40.0	- 47.0	- 2.0	4.2	- 0.9	- 0.2

**Synthesis of ethyl butyrate**

**TABLE No. 2**

**Solvent—Ether**

1	-0.7	0.0	-2.2	-1.3	-1.1	-2.2	-1.4	-1.5	0.5	0.7	-0.9
2	-0.4	-0.7	-1.7	-0.9	0.1	-1.4	-0.8	-0.9	0.7	0.9	0.1
3	0.6	-0.3	0.3	2.2	2.0	-0.4	-0.3	1.3	0.7	-0.4	5.3
4	1.4	0.9	1.0	2.4	-4.5	0.6	2.4	2.1	0.5	-1.3	4.1
5	-1.8	1.3	1.5	2.5	-4.8	2.3	3.0	2.0	-1.3	-1.8	1.5
6	-0.7	-1.8	-3.2	-1.3	-5.1	-2.3	1.7	0.8	0.0	-1.9	-3.2

**Synthesis of normal propyl butyrate**

**TABLE No. 3**

**Solvent—Ether**

1	-2.0	-2.6	-2.5	-4.5	-1.8	0.4	-2.8	0.5	-0.4	7.3	-2.9
2	-1.9	-2.2	-2.0	-3.9	-2.1	-0.5	-2.2	0.8	0.4	7.5	-2.9
3	-1.8	-1.7	-0.4	-2.2	0.0	-3.3	-2.1	1.2	1.8	8.3	-1.6
4	1.2	1.2	1.0	0.5	0.0	-3.7	-2.3	4.8	3.1	10.5	3.5
5	-1.3	2.6	4.6	1.0	0.0	-4.9	-3.0	2.5	1.2	13.6	0.4
6	-3.4	3.2	-2.8	1.8	0.0	-5.2	-3.8	1.8	0.8	12.1	0.1

Synthesis of iso-propyl butyrate

TABLE No. 4

Solvent--Ether

Difference in c.cs. of N/10 NaOH for the sample and the blank

Days	A	B	C	D	E	F	G	H	I	J	K
1	3.9	2.8	-2.6	-1.2	-1.8	-5.2	-2.9	-2.1	-1.8	-9.0	-3.5
2	5.1	6.1	-3.8	-0.6	-2.0	-4.1	-1.7	-1.0	-1.4	-7.0	-4.6
3	6.3	8.8	2.9	2.8	8.2	8.2	5.0	-1.0	1.4	1.6	-3.2
4	4.4	5.7	-0.2	-3.0	0.4	1.2	0.6	0.2	3.2	-7.7	-3.4
5	3.1	4.9	-1.8	-3.9	-0.1	-2.6	0.4	-5.2	-1.2	-8.9	-5.4

Synthesis of normal butyl butyrate

TABLE No. 5

Solvent-Ether.

1	-1.2	-0.9	-0.8	2.7	2.4	4.0	2.2	-4.1	4.9	0.9	-0.8
2	-6.2	-1.1	-1.8	2.6	2.8	2.9	-10.1	-3.8	3.2	3.9	-0.4
3	-8.7	-1.4	-2.7	2.5	2.9	0.4	-17.9	-3.6	2.1	4.3	0.4
4	-12.3	-2.2	-3.6	0.8	2.6	-5.4	-23.2	-5.9	0.5	-0.9	-3.9
5	-15.3	-5.1	-4.1	-4.2	2.2	-3.9	-23.3	-3.9	-3.8	-2.1	-4.1

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Synthesis of iso-butyl butyrate

TABLE No. 6

Solvent-Ether.

1	2.3	0.9	-1.3	2.8	2.3	-0.6	2.5	1.4	2.3	3.4	0.5
2	3.1	1.2	2.2	4.1	3.6	3.8	5.2	5.6	2.4	4.2	0.8
3	3.7	1.4	2.7	2.5	3.9	3.4	7.9	5.8	3.1	4.3	1.4
4	4.7	2.4	4.7	2.2	5.4	2.9	4.0	5.6	3.8	5.1	1.7
5	4.9	3.9	4.5	1.8	4.1	1.8	2.2	6.4	2.9	4.2	2.9

**Synthesis of n-amyl butyrate**

1	1.9	3.2	1.2	2.9
2	2.5	4.5	1.6	4.1
3	3.2	5.3	-5.3	5.2
4	4.2	5.6	-5.8	3.3
5	3.6	4.3	-6.2	2.5

**TABLE No. 7**

-1.8	3.9	1.7	2.2	-1.9
0.2	4.4	2.5	2.8	-1.7
1.3	0.4	2.1	0.6	0.0
-2.3	-1.8	-0.8	-1.3	0.7
-2.7	-2.6	-2.5	-1.5	2.1

**Solvent-Ether.**

2.5	1.2
3.0	1.5
-0.6	-2.7
-1.7	-2.8
-2.0	-3.5

**Synthesis of iso-amyl Butyrate**

1	3.2	1.1	2.8	1.5
2	4.5	2.0	3.3	2.7
3	6.3	4.2	4.5	3.8
4	-3.0	3.7	2.2	2.2
5	-4.1	3.5	1.2	1.3

**TABLE No. 8**

3.9	3.5	-4.1	-1.7	-2.2
4.2	4.0	-3.9	-2.7	-3.1
4.8	4.9	-2.6	3.4	-2.4
3.6	3.8	-3.4	-3.9	-6.4
3.2	3.3	-3.8	-4.4	-7.5

**Solvent-Ether.**

3.9	0.8
4.1	1.0
3.7	-2.7
5.3	-2.8
4.2	-3.5

**Synthesis of normal amyl formate**

1	0.3	-2.3	-2.1	1.3
2	0.8	-2.0	-3.4	1.8
3	2.1	1.3	-5.2	1.3
4	3.2	1.6	2.1	...
5	-1.6	1.4	-1.2	...

**TABLE No. 9**

-1.3	-3.8	-4.5	3.2	2.8
-2.2	-1.9	-4.2	4.1	3.1
-3.1	2.4	-2.7	3.6	4.3
...	2.9	0.0	...	5.8
...	0.0	...	...	5.4

**Solvent-Ether.**

2.5	0.6
3.1	1.0
4.2	2.4
5.1	2.2
4.9	...

**Synthesis of n-amyl acetate**

1	1.7	-0.8	1.3	2.5
2	1.9	-1.3	2.5	3.4
3	2.3	-2.1	2.9	4.2
4	-0.5	0.0	3.6	5.1
5	-1.9	-1.2	3.2	4.6

**TABLE No. 10**

-0.9	1.4	2.3	-0.4	2.1
-1.5	2.3	2.3	1.6	2.2
-2.9	1.9	2.1	2.1	3.9
...	...	...	3.2	4.2
...	...	...	2.9	3.7

**Solvent-Ether.**

3.2	-0.4
6.5	-0.6
7.2	-1.2
6.9	...
...	...

**Synthesis of n-amyl propionate**

**TABLE No. 11**

**Solvent-Ether.**

Difference in c.cs. N/10 NaOH for the sample and the blank

Days	A	B	C	D	E	F	G	H	I	J	K
1	0.2	1.2	-0.5	3.1	-1.2	0.8	3.1	-1.9	1.6	4.5	-1.2
2	0.4	2.3	-1.2	4.6	-1.8	0.4	1.2	2.8	1.5	5.0	-1.8
3	0.8	3.1	-2.6	3.9	-3.5	-2.3	...	3.3	...	5.3	-2.5
4	1.2	4.5	-2.3	...	...	...	...	4.2	...	5.0	...
5	0.0	3.8	...	...	...	...	...	...	...	...	...

**Hydrolysis of methyl butyrate**

**TABLE No. 12**

**Solvent-Ether.**

1	3.7	2.0	4.6	5.2	3.2	3.2	0.9	4.3	6.3	4.0	3.0
2	4.9	4.9	7.8	5.3	6.3	3.9	2.0	5.5	6.9	4.9	3.2
3	4.5	6.3	4.3	5.8	5.6	5.2	3.5	7.0	9.4	6.8	4.5
4	3.7	10.2	...	5.3	...	5.9	1.0	4.7	9.3	7.0	4.4
5	...	10.1	...	...	...	5.7	...	...	...	6.5	...

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**Hydrolysis of ethyl butyrate**

**TABLE No. 13**

**Solvent-Ether.**

1	3.1	0.0	1.3	0.6	1.6	0.4	0.7	0.9	1.4	1.0	0.9
2	3.7	0.0	1.3	1.4	1.6	3.7	1.9	3.1	2.6	2.7	0.9
3	3.6	0.0	1.6	0.9	2.7	2.6	2.3	3.1	3.9	4.0	0.0
4	...	0.0	2.7	...	2.9	...	2.5	3.0	3.7	4.5	...
5	...	...	2.7	...	3.1	...	2.7	...	...	3.8	...

**Hydrolysis of n-propyl butyrate**

TABLE No. 14

Solvent—Ether

1	5.7	4.4	6.1	6.4	6.9	7.6	6.1	8.3	6.0	6.3	4.4
2	8.7	6.0	7.7	8.1	7.3	6.9	5.9	7.2	8.7	7.7	5.7
3	8.9	8.5	8.2	8.5	7.5	...	5.7	6.4	8.5	8.2	6.2
4	8.8	10.2	7.9	8.9	8.5	...	...	...	7.5	9.5	7.3

**Hydrolysis of iso propyl butyrate**

TABLE No. 15

Solvent—Ether

1	2.3	3.8	5.2	7.2	3.1	2.2	6.5	5.1	6.3	2.2	1.2
2	2.5	5.2	5.9	8.1	4.2	2.8	7.2	5.4	7.2	3.4	1.8
3	3.4	6.1	6.5	9.5	4.5	2.8	7.4	5.8	8.1	4.1	2.1
4	4.2	7.9	6.4	9.5	4.4	2.5	6.9	6.1	9.6	5.2	1.9
5	5.2	6.9	...	8.9	...	...	...	6.4	9.9	4.8	...

**Hydrolysis of n - butyl butyrate**

TABLE No. 16

Solvent—Ether

1	-0.7	1.2	0.3	0.6	-1.7	-2.1	-1.7	0.5	-0.7	-2.3	-1.8
2	1.2	2.2	1.1	1.8	-0.8	-3.2	1.5	1.2	1.5	1.2	1.1
3	2.3	3.8	2.4	3.1	1.9	0.8	2.2	2.8	0.4	3.4	0.8
4	2.1	3.4	2.0	2.6	1.4	1.1	2.0	3.1	1.2	3.2	...

**Hydrolysis of iso - butyl butyrate**

TABLE No. 17

Solvent—Ether

1	1.8	2.1	-1.8	1.2	0.4	-1.8	0.0	0.2	0.4	-1.8	0.5
2	2.4	3.2	2.2	1.9	1.5	0.3	1.2	1.8	2.2	0.9	1.2
3	3.2	4.5	3.1	2.1	2.3	1.5	2.4	3.1	3.8	1.6	2.1
4	3.8	3.9	4.5	2.8	2.9	1.2	2.3	3.8	4.1	2.1	1.9
5	3.5	...	4.5	2.6	2.4	...	...	3.5	3.9	2.0	...

## Hydrolysis of n - amyl butyrate

TABLE No. 18

Solvent—Ether

Difference in c.cs. of N/10 NaOH for the sample and the blank

Days	A	B	C	D	E	F	G	H	I	J	K
1	2.6	1.2	0.6	1.1	1.0	2.5	2.7	1.2	2.9	1.2	1.8
2	2.8	3.8	1.1	1.2	1.0	2.8	1.9	1.8	3.1	1.5	3.4
3	2.2	5.7	2.2	2.8	1.4	2.1	1.5	2.5	3.6	2.5	4.1
4	-0.8	3.9	1.9	3.1	1.6	1.8	...	2.2	11.2	2.4	2.0
5	...	10.7	...	3.0	1.4	...	...	...	13.0	...	...

## Hydrolysis of iso - amyl butyrate

TABLE No. 19

Solvent—Ether

1	3.1	0.5	0.4	0.8	1.0	2.5	1.8	1.9	2.8	1.6	0.5
2	3.8	1.2	1.0	1.5	1.5	3.2	2.1	2.2	4.1	2.1	1.2
3	4.2	2.6	1.8	2.6	1.3	3.8	2.6	3.5	10.6	3.2	1.8
4	4.6	3.1	1.5	3.4	...	3.6	2.5	4.1	11.8	3.2	1.5
5	4.4	4.9	...	3.1	...	...	...	3.9	11.5	2.9	...

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## Hydrolysis of n amyl formate

TABLE No. 20

Solvent—Ether

1	-1.5	0.6	-1.2	1.6	0.0	1.4	1.2	0.8	2.2	0.8	0.8
2	-2.1	0.9	0.8	2.2	0.3	2.3	1.8	1.2	3.4	1.1	0.8
3	-3.2	1.2	1.5	3.1	0.6	2.6	2.1	2.3	4.6	2.2	1.2
4	-3.4	1.5	1.8	4.2	0.5	2.5	2.4	3.1	4.5	3.9	1.5
5	-3.3	1.4	1.5	3.9	...	...	2.0	3.0	...	3.6	1.3

## Hydrolysis of amyl acetate

TABLE No. 21

Solvent—Ether

1	2.3	1.2	-0.3	1.5	1.4	2.5	1.2	0.5	2.9	1.1	1.8
2	3.4	1.8	0.8	2.1	1.4	3.1	2.7	1.2	3.9	1.3	2.8
3	4.2	2.1	1.8	2.8	1.8	3.8	2.9	2.3	8.2	1.5	3.2
4	5.3	2.5	2.3	3.1	1.6	4.2	2.6	3.8	10.6	2.8	3.4
5	4.8	2.6	2.1	3.0	...	3.9	...	3.6	9.5	2.4	3.0

## Hydrolysis of amyl propionate

TABLE No. 22

Solvent—Ether

1	3.1	0.8	0.4	2.2	0.5	1.9	2.1	1.5	3.8	1.9	0.5
2	3.8	2.3	1.2	3.1	1.2	2.3	2.9	2.5	5.2	2.3	0.8
3	4.1	3.1	2.6	4.2	2.1	3.1	3.2	3.1	7.5	3.1	1.2
4	4.8	2.8	2.9	3.9	1.9	2.6	3.6	4.8	11.2	4.2	0.9
5	4.6	2.5	2.3	...	...	...	3.4	4.5	10.8	3.9	...

A = Acetic acid; B = KCN; C = NaCl; D = CaCl<sub>2</sub>; E = AlCl<sub>3</sub>; F = Na<sub>2</sub>SO<sub>4</sub>;  
 G = Na<sub>2</sub>HPO<sub>4</sub>; H = MgSO<sub>4</sub>; I = MnSO<sub>4</sub>; J = BaCl<sub>2</sub>; K = K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>.



## Results

Even though a clear distinction between the accelerators and retarders is not possible in general for the synthesis of all esters, still it is found that  $\text{CaCl}_2$ ,  $\text{BaCl}_2$  and  $\text{MnSO}_4$  are the best accelerators for most of the synthesis and  $\text{AlCl}_3$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaCl}$  and  $\text{K}_2\text{Cr}_2\text{O}_7$  are poisons for most of the syntheses.

Further since the same salt appears for one ester as the best accelerator and for some other ester as a poison (i.e.  $\text{MnSO}_4$  is accelerator for methyl butyrate and poison for amyl formate), it may be concluded that the accelerating or the retarding effect may also be depending upon the substances used for synthesis.

As regards the hydrolysis of the esters in general  $\text{MnSO}_4$  and  $\text{KCN}$  are the best salts for most of the esters.  $\text{K}_2\text{Cr}_2\text{O}_7$  in general has got least accelerating power.

## Summary

(1) Action of 11 substances has been studied on the synthesis and hydrolysis of esters.

(2) Salts can be classified as accelerators, retarders and poisons for the synthesis as well as the hydrolysis from the results given in tabular form.

(3) In synthesis experiments,  $\text{CaCl}_2$ ,  $\text{MnSO}_4$ ,  $\text{BaCl}_2$  are best salts and  $\text{AlCl}_3$ ,  $\text{NaCl}$  and  $\text{K}_2\text{Cr}_2\text{O}_7$  are poisons for most of the syntheses.

(4) In hydrolysis experiments,  $\text{KCN}$  and  $\text{MnSO}_4$  are best salts and  $\text{K}_2\text{Cr}_2\text{O}_7$  retards the reaction.

(5) In synthesis as well as in hydrolysis,  $\text{MnSO}_4$  appears to be the best accelerator and  $\text{K}_2\text{Cr}_2\text{O}_7$  appears to be a retarder.

(6) The accelerating or the retarding effect depends upon the nature of the salt as well as the ester.

(7) Finally it appears to be difficult to conclude anything definite as regards the effect of the nature of ions on the synthesis and hydrolysis of esters.

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## PAPER XXIV

A STUDY OF THE ENZYMES PRESENT IN  
GERMINATING SEEDS

By

Dr. N. V. BHIDE, M. SC , Ph. D.

(With the kind permission of the authorities of the Bombay University and also of the Agri. College, Poona, Magazine)

Since 1842 the study of the germinated grains has been carried out by a number of workers, many of whom have concentrated their attention mainly on oil-seeds. Some of the workers in this field are De Saussure (Uber. Des. Chemen. Der. Oeesam Escieen--Poripp Notium, 1842, pp. 24.), Helriegel (Bot. Ztg., 1859, pp. 187), Brown and Morris (Jour. Chem. Sec. ,1895, LVII, pp. 485), and Sahasrabuddhe and Kibe (J. Uni. Bom., 1934, Vol. III, Part 2). These workers have studied the enzymes in the extract of the seeds by using either pure fat or pure starch as the substrate. It is proposed to study the amylase and proteinase activities of the extract of the germinated grains employing the flours of the same grains as substrate. The flours of the following grains were used:

1. *Bajri* (*Pennisetum typhoideum*)
2. *Vari* (*Panicum milaceum*)
3. Gram (*Cicer arietinum*)
4. Black-gram (*Phaseolus radiatus*).

**Preparation of the Enzyme Extract**

Twenty grams of *bajri* seeds were soaked in water for three hours and kept aside for germination with usual precautions. Samples were removed after every twenty-four hours, crushed and extracted with 250 c.c. of water, filtered and the extract thus obtained was subsequently used for its amylase and proteinase activity.

### Amylase Activity of the Enzyme Extract

1 gram of each of the flours was gelatinised in 50 c.c. of water in boiling water bath for ten minutes and on cooling to the room temperature, 10 c.c. of the enzyme extract were added together with 30 c.c. of the acetate buffer solution of pH 4.8. After the intervals of 1, 2 and 3 hours the enzyme was inactivated by 5 drops of NaOH (10%), clarified with ZnO, the volume was made (100 c.c.), filtered and 10 c.c. of the filtrate were used for the determination of the reducing sugar by Bertrand's method. The percentage hydrolysis was expressed on the basis of the total hydrolysable starch as determined by the method given in the methods of analysis of A.O.A.-C, 1926, p 119, (oven-dry). The results have been given in Table 1.

TABLE No. 1

Percentage hydrolysis of the flours with enzyme extract (*bajri* amylase) expressed on the basis of the total hydrolysable starch

Name	Extract after 24 hours			Extract after 48 hours			Extract after 72 hours			Total hydrolysable starch
	1 hr.	2 hrs.	3 hrs.	1 hr.	2 hrs	3 hrs.	1 hr.	2 hrs.	3 hrs.	
<i>Bajri</i> flour ...	5.05	8.16	10.88	11.39	22.27	24.99	16.15	26.01	28.73	59.82
<i>Vari</i> flour ...	4.37	7.69	10.70	10.26	16.15	21.47	15.58	23.18	25.27	56.23
Gram flour ...	6.44	7.59	12.42	9.89	18.66	21.16	14.26	20.08	25.74	42.32
Black-gram flour ...	4.00	6.20	8.22	8.20	24.60	27.00	11.60	14.80	18.00	50.87

It is seen that the extract of the germinated *bajri* seeds contained an active amylase which can hydrolyse the starches from the flours of different grains which include both the cereal and pulse grains. Although the enzyme is obtained from *bajri* seeds it is not behaving in any special way towards *bajri* flour. The activity of the amylase goes on increasing for the first 72 hours, then it begins to decrease (Table 2).

TABLE No. 2

The activity of the *bajri* amylase from day to day of the germination expressed on the total hydrolysable starch from *bajri*

Period of germination	1 hour	2 hours	3 hours	% of Total hydrolysable starch
24 hours ...	5.01	8.16	10.88	59.82
48 hours ...	11.89	22.27	24.99	59.82
72 hours ...	16.15	26.01	28.73	59.82
96 hours ...	7.82	15.47	23.46	59.82

(B)

### The Proteolytic Enzymes from *Bajri* Seed Extract

For studying the proteolytic activity the enzymes extract was prepared in exactly the same way as above. The activity of the enzyme was expressed in mgm. of amino-nitrogen as determined by the Van Slykes method.

#### Experimental

1 gram of the flour was suspended in 10 c.c. of the phosphate buffer of pH 9.6 (which was found to be the optimum) together with 10 c.c. of the enzyme extract and kept at 37°C. The activity of the enzyme was killed with dialysed iron 5 c.c. followed by magnesium sulphate (50%) solution, the volume was made up (100 c.c.).

It was filtered and 5 c.c. of the filtrate were used for the determination of the amino-nitrogen by Van Slyke's method. The results are given in Table 3.

TABLE No. 3

**Hydrolysis of the flours with enzyme extract (proteolytic from *bajri* seeds) expressed as mgms. of amino-nitrogen per 100 gms. of the flour**

Name	Extract after 24 hours			Extract after 48 hours			Extract after 72 hours			% of Total proteins in the flours
	1 hr.	2 hrs	3 hrs.	1 hr.	2 hrs.	3 hrs.	1 hr.	2 hrs.	3 hrs.	
<i>Bajri</i> flour ...	188.0	227.8	269.8	269.2	310.6	331.3	310.5	352.4	372.7	9.6
<i>Vari</i> flour ...	20.70	82.8	168.0	40.3	145.0	207.3	103.4	186.2	227.8	7.53
Gram flour ...	209.13	316.2	385.2	315.2	420.8	492.3	517.6	579.8	579.8	20.03
Black-gram flour ...	242.80	367.9	420.2	375.9	490.2	520.6	641.2	724.4	744.5	23.21

The proteolytic enzymes from *bajri* seeds act well on all the flours. The activity of the enzymes goes on increasing as in the case of the amylase. The pulse flours give higher values of hydrolysis than the cereal flours, as they contain a higher percentage of proteins. The order of the susceptibility was found to be as *udid* (black-gram), gram, *bajri* and *vari*. It may be stated that the comparison is made on the basis of the weights of the flour without any consideration of their protein contents. The activity of the enzyme was maximum at the end of the 72 hours. It then began to decrease.

## II

### Enzymes From Gram Seed

**Gram-amylase:**—The enzyme was not obtained in the form of the extract as above as the process was found to be impracticable due to the difficulties in filtration, adjusting the equality of the number of seeds, etc. The plumules of the germinated seeds were cut and dried at 55°C, powdered and used as such. The changes in the activity of the enzyme along with the advancing period of germination could not obviously be studied as before. The experimental procedure was exactly

the same as in the previous case except that the buffer used was of pH 5.2 instead of 4.8 as it was found to be the optimum. The results are given in Table 4.

TABLE No. 4

**Hydrolysis of the flours with gram-amylase expressed on the basis of the total hydrolysable starch**

Name	1 hour	2 hours	3 hours	% of Total hydrolysable starch
<i>Bajri</i> flour ...	2.38	5.37	13.74	59.82
<i>Vari</i> flour ...	2.47	4.94	14.13	50.23
Gram flour ...	4.6	5.98	17.11	42.32
Black-gram flour	4.0	5.30	161.8	50.37

It is seen that the enzyme has a preferential action on the flours. The flour of the seed from which the enzyme is obtained is more easily hydrolysed than any other flours.

#### Gram-Proteinase

The experimental procedure was the same as in the case of the *bajri* seed except that the buffer solution used was of pH 9.0. The results are given in Table 5.

TABLE No. 5

**Hydrolysis of the flours with gram-proteinase expressed as mgms. of amino-nitrogen per 100 gms. of the flour**

Name	2 hours	3 hours	% of Total proteins
<i>Bajri</i> flour ...	182.3	217.4	9.6
<i>Vari</i> flour ...	90.4	123.5	7.53
Gram flour ...	253.3	333.3	20.03
Black-gram flour ...	294.0	430.8	23.21

### Summary

(i) The activity of the amylase and proteinase from the extracts of the germinated *bajri* and gram seeds was studied by using the flours of *bajri*, *vani*, gram and black-gram as substrates.

(ii) The extract of the germinated seeds was found to contain both amylase and proteinase which act equally well on the flours of all the grains studied.

(iii) The activity of the enzymes goes on increasing for the first 72 hours, it then begins to decrease.

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## PAPER XXV

### A STUDY OF THE ENZYMES PRESENT IN GERMINATING SEEDS

By

DR. N. V. BHIDE, M.Sc., Ph.D.

(With the kind permission of the authorities of the Bombay University and also of the Agri. College, Poona, Magazine)

In part I, (J. Uni. Bom., 1943, 12, 5A, 81) an account of the simultaneous presence of amylase and proteinase in germinating *bajri* (*Pennisetum typhoideum*) and gram (*Cicer arietinum*) seeds is given and some experiments on the activities of these enzymes on different flours are also described.

The importance of the *pH* for the activity of the enzyme has been recognized since long and a large amount of work has been done in this connection. The specific effect of the different buffers on the action of the enzymes was studied by Sherman (J. A. C. S., 1925, 47, 28). The optimum *pH* values for some enzymes were found by K. S. Murti. (J.I.C.S., 1940,

17, 578). In the present article the optimum pH for the amylase and proteinase from the germinating *bajri* and gram seeds are described.

### Experimental

*Bajri* seeds were germinated for two days and were dried with hot air at 55°C, powdered and passed through 100 mesh sieve. 25 c.cs. of 1% starch solution were mixed with 0.2 gm. of *bajri* powder together with 25 c.cs. of the buffer solutions of different pH values, in separate flasks. The mixtures were then incubated at 37°C for two hours, clarified with ZnO and filtered. Ten c. cs. of the filtrate were used for the determination of the reducing sugars by Bertrand's method. The following buffers were used :—

(i) 0.5 M borax and 0.05 M sodium carbonate for pH. 9.2 to 11 (Kolthoff and Vlecolhower, *Biochem., Z.*, 1927, 189, 191).

(ii) 0.1 M citric and 0.2 M disodium phosphate for pH 2.2 to 8 (McIlvaine, *J. Bio. Chem.*, 1921, 49, 183).

The optimum pH for the *bajri* amylase lies between 4.7 and 4.9. This value is practically the same as that for the diastase viz. 4.8. It therefore seems that the extract from the germinated seeds of *bajri* contains diastase to a large extent.

pH	c.cs. of KMnO <sub>4</sub>	pH	c.cs. of KMnO <sub>4</sub>
2.6	0.4	5.0	3.8
3.2	1.2	5.2	3.5
3.5	1.9	5.9	2.8
3.6	2.7	7.0	1.7
3.9	3.0	8.1	1.7
4.2	3.9	9.0	0.4
4.7	4.0	9.6	0.4
4.9	4.0	10.7	0.2

For the determination of the optimum pH for the *bajri* proteinase 0.2 gm. of casein (which was specially prepared for



the present work from milk) was thoroughly mixed with 25 c.cs. of different buffer solutions and 0.2 gm. of the powder of *bajri* germinated for three days was then added to it together with some quantity of toluene. The reaction was allowed to proceed for two hours at 37°C. The enzyme activity was killed by boiling the solution. It was then treated with dialysed iron followed by 1 c. c. of 50% magnesium sulphate solution. The volume was made to 100 c. cs., filtered and 5 c. cs. of the filtrate were used for the determination of amino nitrogen by Van Slyke's method.

pH	c.cs. of amino nitrogen at N. T. P.	pH	c.cs. of amino nitrogen at N. T. P.
3.3	0.33	7.0	0.30
4.2	0.33	8.1	0.40
4.9	0.41	9.0	0.50
5.2	0.35	9.6	0.54
5.9	0.33	10.7	0.45

Here we find that there are two values for the optimum pH, 4.9 and 9.6, with a minimum at pH 7. The first optimum pH corresponds to the optimum pH for the *bajri* amylase. The second value is a little more than that for trypsin. It may also be noted that one value is on the acidic side like that for pepsin while the other is on the alkaline side like that for trypsin. It therefore appears that the extract contains both pepsin and trypsin, each acting according to its respective pH range.

An observation of two optima in the case of emulsin has already been made by Bruns Wike (Oester. Bot. Z., 1923, Nr. 105, 58). This has been explained as due to the simultaneous presence of amygdalase and prunase in the enzyme preparation. So the two optima observed in the present case may be due to the simultaneous existence of two enzymes as mentioned above.

For the determination of the optimum pH for gram amylase the experimental details are exactly the same as those

for *bajri* except that the quantity of the enzyme powder was 0.1 gm.

pH	c. cs. of $\text{KMnO}_4$	pH	c. cs. of $\text{KMnO}_4$
2.6	0.40	5.2	2.00
3.5	0.40	5.9	1.20
3.6	0.60	7.0	0.80
3.9	0.80	8.1	0.40
4.2	1.80	9.0	0.40
4.7	1.90	9.6	0.30
4.9	2.00	10.7	0.20

Here the optimum value lies between 4.9 and 5.2. This value also is not much different from the optimum value for the diastase viz. 4.8. The enzyme therefore appears to be diastase. It may be noted that in *bajri* which is a typical cereal and in gram which is a pulse, the amylase is found to be of the same type.

The optimum pH for the gram protein was determined as in the case of *bajri*, taking 0.1 gm. of the enzyme powder.

pH	c. cs. of amino-nitrogen at N. T. P.	pH	c. cs. of amino-nitrogen at N. T. P.
3.2	0.36	7.0	0.45
4.2	0.37	8.0	0.53
4.9	0.47	9.0	0.56
5.2	0.47	9.6	0.55
5.9	0.50	10.7	0.53

Here also there are two optima, one at pH 5.9 and the other at pH 9.0.

## PAPER XXVI

STUDIES IN THE ENZYMES DURING THE  
PERIOD OF GERMINATION OF THE SEEDS OF  
*RALA AND HARIK*

BY

MR. M. V. SATHE, M. Sc.

*(With the kind permission of the authorities of the  
Bombay University)*

## SECTION I

**(A) Introduction**

The work in this paper is restricted to the amylolytic activity of the enzymes only. During some preliminary trials it was observed that the enzyme extract of the *rala* and *harik* seeds contain the amylase with varying degree of concentration. It was also observed that the enzyme activity changes with change in the period of germination.

During the previous chapters we have studied the comparative disintegration of the different flours alone and their isolated starches. Hence we have obtained the comparative idea of their disintegrations with hydrochloric acid and a number of regular enzymes like diastase, pancreatin, pepsin alone and pepsin superimposed by trypsin. All these ferments were obtained from the bodies other than the grains, the flours of which were used.

It was thought interesting to study the comparative disintegration of the flours by hydrolysis with enzyme extracts of germinated seeds of *rala* and *harik*. A study of this type would be interesting in two ways. In the first place it would give some idea about the ability of the enzyme for hydrolysis of the other flours and if it is able to do so, it would also be interesting to see whether it behaves equally well with the grains from which it is prepared; secondly side by side the information about the activity of the enzyme with advancing period of germination may be obtained.

**(B) (a) Determination of the optimum pH for *Rala* and *harik* amylases**

**1. *Rala* amylase : Experimental :-**

The *Rala* seeds were germinated for four days and the germinated seeds were dried, powdered and passed through a 100 mesh sieve. The powder thus obtained was used for the determination of optimum pH, keeping powers and susceptibility to different temperatures.

25 c.c. of one per cent. starch solution were mixed with 0.2 grams of the *Rala* enzyme powder together with 20 c.c. of buffer solutions of different pH values in separate flasks. The mixtures were then incubated for two hours at the end of which the activity of enzyme was killed with a few drops of 10 per cent. sodium hydroxide solution. Then it was clarified and the volume was made to 100 c.c., and filtered through a dry filtered paper. 10 c.c. of the filtrate were used for the determination of reducing sugars by Bertrand's method. The following buffers were used for various ranges of pH values. (i) 0.05 M borax and 0.05 M sodium carbonate for pH 9.2 to 11 (Kohlthoff and Velcochhower, Biochem. Z. 1927, 189, 191). (ii) 0.1 M citric acid and 0.2 M sodium hydrogen phosphate for pH 2.2 to 8 (McIlvaine, Jour. Biol. Chem. 1921, 49, 183, 191); Snell's calorimetric estimations 1939.

TABLE No. 1

***Rala* amylase**

c.cs. of decinormal  $\text{KMnO}_4$  for c.c. of the filtrate for various pH values.

Observation No.	pH value.	c.cs. of $\text{KMnO}_4$
1	2.4	0.9
2	2.8	1.6
3	3.2	2.0
4	3.6	2.2
5	4.0	2.6
6	4.2	2.6
7	4.4	2.5
8	4.6	2.3
9	4.8	2.2
10	5.2	1.8
11	5.6	1.5
12	6.0	1.1
13	7.1	0.9
14	8.0	0.6
15	9.1	0.25

From the above table it is observed that the optimum pH for the *rala* amylase is between 4 and 4.2, as the values of  $\text{KMnO}_4$  are maximum at this range of pH values. If we consider this optimum pH value with that of pure diastase this value is slightly low.

TABLE No. 2

***Harik* amylase**

c.cs. of decinormal  $\text{KMnO}_4$  for 10 c.cs. of the filtrate for various pH values.

Observation No.	pH values.	c.cs. of $\text{KMnO}_4$
1	2.2	0.4
2	3.4	0.6
3	4.0	0.9
4	4.8	1.25
5	5.6	1.5
6	5.8	1.8
7	6.0	1.8
8	6.2	1.6
9	6.8	1.4
10	7.2	1.1
11	8.0	0.7
12	8.4	0.5
13	9.4	0.25

From the above table it is observed that the optimum pH value for the *harik* amylase is between 5.8 and 6 as the values of decinormal  $\text{KMnO}_4$  are maximum for this range. Even though *rala* and *harik* are cereals, the amylases obtained from their germinated seeds have different optimum pH values.

## SECTION II

## THE EFFECT OF AMYLASE EXTRACTS FROM GERMINATED SEEDS OF *RALA* AND *HARIK* ON THE FLOURS AS SUCH.

### Effect of *harik* amylase on flours

**Experimental :-** One gram of each of the flours (oven dry) was weighed in a conical flask and 50 c.c. of water were added to make a thin paste. The flasks were heated in a boiling water bath for 5 minutes. It was then cooled to room temperature and 10 c.c. of the enzyme extracts were added together

with 20 c.c. of phosphate buffer of pH 5.8 which is the optimum pH value for *harik* amylase. At the end of each period the enzyme was killed by a few drops of 10 per cent. sodium hydroxide solution. It was then clarified with zinc oxide and the volume was made exactly 100 c.c. It was filtered through a dry filter paper and 10 c.c. of the filtrate were used for the estimation of reducing sugars by Bertrand's method. The percentage hydrolysis was expressed on total hydrolysable starch basis (oven dry). Periods of hydrolysis were 1, 2 and 3 hours. The seeds were germinated for 1, 2, 3, 4, and 5 days. The extracts at the end of each day were used for the hydrolysis of the flours.

Percentage hydrolysis of the flours expressed on total hydrolysable starch basis (oven dry) with enzyme extracts from *harik* seeds after 1, 2, 3, 4 and 5 days germination.

TABLE No. 3.

Period of germination	Name of the flour	Hour 1	Hours 2	Hours 3	Total hydrolysable Starch
1 day	<i>Rala</i>	0.93 %	1.43 %	1.77 %	66.83 %
1 day	<i>Harik</i>	1.21 %	1.87 %	2.63 %	70.52 %
2 days	<i>Rala</i>	1.85 %	3.24 %	3.87 %	66.83 %
2 days	<i>Harik</i>	2.34 %	3.86 %	4.37 %	70.52 %
3 days	<i>Rala</i>	3.81 %	7.13 %	9.15 %	66.83 %
3 days	<i>Harik</i>	4.73 %	8.31 %	10.43 %	70.52 %
4 days	<i>Rala</i>	5.83 %	8.15 %	10.46 %	66.83 %
4 days	<i>Harik</i>	6.61 %	9.52 %	11.53 %	70.52 %
5 days	<i>Rala</i>	4.11 %	6.47 %	7.93 %	66.83 %
5 days	<i>Harik</i>	4.77 %	6.98 %	8.72 %	70.52 %

From the above considerations it is observed that the extract of germinated *harik* seeds contain an active amylase. The diastatic power of the amylase goes on changing from day

to day of germination. The enzyme obtained from *harik* seeds has some more affinity for *harik* flour. Hence it seems that the enzyme from the same seeds has more action on the starchy material of its grain than that of the other grains. The extracts after three days and four days germination contain a more concentrated and active form of amylase as the values in these cases are higher than those in the other cases.

#### Effect of *rala* amylase on the flours of *rala* and *harik*

**Experimental :—**The experimental procedure is similar to that in the case of the *harik* amylase. The only change is that of the optimum pH value for the *rala* amylase which is found to be 4.2. All the reactions for *rala* amylase are carried out at this pH value 4.2.

TABLE No. 4

Percentage hydrolysis of the flours expressed on total hydrolysable starch basis (oven dry) with enzyme extracts obtained from *rala* seeds after 1, 2, 3, 4 and 5 days germination.

Period of germination	Name of flour	Hour 1	Hours 2	Hours 3	Total Hydrolysable starch
1 day	... <i>Rala</i>	1.83 %	4.38 %	6.42 %	66.83 %
1 day	... <i>Harik</i>	1.12 %	2.81 %	4.21 %	70.52 %
2 days	... <i>Rala</i>	6.23 %	10.31 %	12.57 %	66.83 %
2 days	... <i>Harik</i>	4.82 %	7.73 %	8.93 %	70.52 %
3 days	... <i>Rala</i>	11.27 %	13.83 %	14.26 %	66.83 %
3 days	... <i>Harik</i>	13.44 %	15.34 %	16.87 %	70.52 %
4 days	... <i>Rala</i>	23.37 %	26.29 %	30.87 %	66.83 %
4 days	... <i>Harik</i>	19.51 %	22.35 %	25.92 %	70.52 %
5 days	... <i>Rala</i>	20.83 %	22.18 %	24.53 %	66.83 %
5 days	... <i>Harik</i>	18.57 %	21.31 %	23.31 %	70.52 %

From the above results if we consider the activity of the *rala* amylase of different periods of germination with different periods of hydrolysis expressed on total hydrolysable starch basis it is observed that the activity goes on increasing from day to day up to four days germination. After this the activity decreases which is shown by the hydrolysis by enzyme extract after five days germination.

### SECTION III

## A STUDY OF SUSCEPTIBILITIES OF THE AMYLASES FROM RALA AND HARIK SEEDS AT DIFFERENT TEMPERATURES AND THEIR KEEPING POWER.

### Susceptibilities of the enzymes at different temperatures

It is a well known fact that the enzymes lose their activity when they are subjected to higher temperatures. In order to get some idea as regards the stability of the enzymes at different temperatures their activity was studied at different temperatures.

**Experimental:**—0.5 gram of the enzyme was taken in a conical flask. The enzymes were kept at different temperatures for half an hour. To these flasks were added 26 c. c. of 1 per cent. potato—starch solution. Then to the *rala* enzyme powder 25 c. c. of buffer solution of pH 4.2 and to the *harik* enzyme powder 25 c. c. of buffer solution of pH 5.8 were added. The reaction was carried out for one hour at the end of which the enzyme was killed by a few drops of 10 % sodium hydroxide solution. It was then clarified by zinc oxide and the volume was made to 100 c. c. The mixture was then filtered through a dry filter paper and 10 c. c. of the filtrate were used for the determination of the reducing sugars by Bertrand's method.

Percentage hydrolysis of the starch by the *rala* and *harik* amylase at different temperatures.



TABLE No. 5

Temperature in centigrade.	<i>Rala</i> enzyme powder	<i>Harik</i> enzyme powder
27	21.68%	16.23%
37	29.87	20.93%
40	...	20.93%
42	31.12%	...
45	31.12%	...
47	...	13.83%
52	21.32%	...
57	...	3.67%
67	10.2%	0.0%
77	6.93%	...
87	0.0%	...

From the above table it is observed that the optimum temperature range for *harik* amylase is 37°C-40°C, whereas for the *rala* amylase it is 42°C-45°C. As the temperatures increase more than the room temperature the activity increases upto a certain temperature and falls down beyond the optimum temperature. The *harik* enzyme loses its activity at 67°C. whereas the *rala* amylase loses its activity at 87°C. Hence it seems that the *rala* enzyme is more stable at higher temperatures than the *harik* enzyme.

#### Keeping Powers of the Enzymes

It is a well known fact that the enzymes lose their activity due to keeping. In the preliminary trials with the enzymes used in the previous sections it was observed that their activity did not remain constant. In order to get the idea about the stability their keeping power was studied. The study was restricted to the amylolytic activity.

**Experimental**— 0.5 gram of the enzyme powder was taken and 25 c.c. of 1 per cent. potato starch were added to it. 25 c.c. of buffer solutions of pH 4.2 were added to *rala* enzyme powder and of pH 5.8 to *harik* enzyme powder. The flasks were incubated at 37 degrees centigrade for two hours at the end of which the enzymes were inactivated by two drops of 10 per cent. sodium hydroxide solution. It was then clarified by zinc oxide and the volume was made to 100 c.c. It was then filtered through dry filter paper and 10 c.c. of the filtrate were used for the determination of the reducing sugars by Bertrand's method.

Percentage hydrolysis of the starch by the enzymes at different periods (expressed in days).

TABLE No. 6

Observation No.	Days	<i>Rala</i> enzyme powder	<i>Harik</i> enzyme powder
1	0	29.12 %	23.32 %
2	30	14.62 %	13.64 %
3	45	11.73 %	12.41 %
4	60	10.42 %	7.83 %
5	80	9.13 %	5.31 %
6	100	6.26 %	2.92 %
7	130	4.73 %	2.23 %

In both the cases there is rapid fall in the activity of the enzymes during the first month. The fall is about 50 per cent. in the first month in case of *rala* amylase and 34 per cent. in case of *harik* amylase. The rate falls down slowly after 30 days in both the cases. After four months the *rala* amylase loses activity to the extent of about 80 % and *harik* amylase to about 90 per cent. It seems that the *rala* enzyme keeps slightly better than the *harik* enzyme.

### Conclusions

1. The extracts from the germinated seeds of both *rala* and *harik* contain an active amylase.
  2. The enzyme extracts act upon both the flours. However the enzyme extracts from the same seeds have more action on the starchy material of its own grain than the starchy material of the other grains.
  3. The activity goes on increasing from the first day of germination up to the fourth day of germination. Then it decreases gradually in the case of both the enzyme extracts.
  4. The optimum pH for the *rala* amylase is between 4 and 4.2, whereas for *harik* amylase it is between 5.8 to 6.
  5. Both the enzymes lose their activity at higher temperatures. The *rala* amylase loses its activity at 87°C. whereas *harik* amylase loses activity at 67°C.
  6. Both the enzymes lose their activity after the first month of their preparation. The activity slows down at the end of the fourth month. It is observed that the *rala* amylase keeps slightly better than the *harik* amylase.
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## PAPER XXVII

## STUDY OF THE ENZYME 'LIPASE'

By

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(With the kind permission of the authorities of the Bombay University).

## SECTION I

## Introduction

It has been observed and proved that 'Oil-seeds' contain an enzyme 'Lipase' which by its virtue of lipolytic activity brings about hydrolysis of the oils on keeping.

The above observations were first made by J. Pelouze in 1855 (C.R. 1855, 40, 605). He drew attention to the fact that seeds, such as linseed, poppy, mustard, sesame, ground-nut and almonds contain a substance which is capable of producing comparatively rapid hydrolysis of the oil present in the seeds when these are crushed.

The oil extracted from freshly crushed seeds contains very negligible amount of acid, but if the oil is kept for some days after extraction, appreciable quantities of free acid can be detected, and the amount tends to increase gradually as the period increases.

The above decomposition has been attributed to the presence of a ferment which has been termed 'lipase'. Similar observations have been made by Chevreul (loc. cit. 611) in 1853 when working on an African seed, *Pentadesma*.

J. Reynolds Green (Proc. R. S. 1890, 48, 370) was the first person to discover the '*ricinus* lipase' from castor bean seed (*Ricinus communis*). Green found that the plant enzyme is a typical lipase hydrolysing true fats. Since that time the observations of Green have been confirmed many a time.

Its optimum pH is between 4.7 and 5.0 (Willstatter and Waldschmidt-Lietz. E. Uber Ricinus lipase Z. Physiol. Chem., 134, 161-1923-24). It varies slightly according to the buffer employed.

Constein, Hoyer and Wartenberg (Ber , 1902, 35, 3839) were the first to recognise the technical importance of vegetable lipases from a chemical point of view. Their first experiments consisted in grinding together a given weight of castor seed with its own or twice its weight of a one per cent. chloral hydrate solution. The results proved that in the latter case 95% of the oil present in the seed was hydrolysed within four days at 35 degrees centigrade. In their later experiments they added small amounts of acid to the mixture of water, oil and ground seed, and found that the reaction was much accelerated. They also showed that many other oils besides castor oil can be saponified by the lipase present in the castor seed, but that some oils are not readily hydrolysed.

Numerous other seeds also contain lipolytic enzymes (J. Pelouze C. R. 1855, 40, 605). The well known fact that the ground-nut oil, cocoanut oil, and palm kernel oil rapidly develop acidity when kept, unless they are carefully refined, indicates the presence in these oils of small amounts of enzymes derived from the meal from which they are extracted.

So far a good amount of work has been done in studying the 'Ricinush Lipase' from the castor beans seed by (J. J. Sudborough, H. E. Watson and P. S. Varma. Jr. Ind. Inst. Sc., Vol. 2, Part XV pp. 213-273, 1919).

Among the recent workers in this field the following may be mentioned. Falk 'Effect of proteases on lipase action' (J. Biol. Chem. Vol. 108, 1935, pp. 421) Bregman and Frusten have studied the 'Proteolytic enzymes' (J. Biol. Chem., Vol. 118, 1937, pp. 715). Kelsey has given his contribution to the 'Action of castor bean and pancreatic lipase on blood neutral fat'. (J. Biol. Chem. Vol. 130, 1939, pp. 199). Kelsey has also studied 'The use of lipase' in lipid analysis

(J. Biol. Chem. Vol. 180, 1939, pp. 187). Hellburman, Lestie, and Perkins "Activation of enzymes" (J. Biol. Chem. Vol. 112, 1935-36, page 175). Greenstein and Jessie 'The action of proteolytic enzymes on synthetic substances' (J. Biol. Chem. Vol. 112, 1935-36, Page 517). Weinstein, "Studies on pancreatic lipase (J. Biol. Chem. Vol. 112, 1935-36, page 641-648). Mulin H. Peterson, Mavin J. Johnson and W. V. Price "Determination of milk lipase" (J. Biol. Chem. Vol. 37, 1943). I. A. Could, 'Influence of pH, type of fat and pancreatic extract on lypolysis in homogenised milk. (J. Dairy Sci. 25, 1942, 869-75). Longnecker, H. E. and Haley D. E. "Ricinus Lipase and its nature and specificity". (J. American Che. Soc. 57, 2019, 1935).

This paper contains the work done on the 'Study of the enzyme lipase from the ground-nut and linseed'. It also deals with the hydrolysis of the following oils, ground-nut, linseed, and sesame with enzyme 'lipase' extracted from their corresponding meals.

It is well known that in the alimentary tract of the higher animals, glycerides undergo hydrolysis by the catalytic agency of fat splitting enzyme 'lipase' secreted by one of the digestive glands, the pancreas. The breaking down of the fats by this agent is greatly accelerated by the physical action of the salts of the bile acids, which by their marked power of lowering surface tension tend to increase the dispersion of the oil droplets and to give rise to emulsions.

The adsorption of the majority of the edible oils and fats is almost quantitative, that is, values ranging from 95 % to 100% are obtained for the digestibility coefficients. Lower values have been recorded for some fats, more particularly in cases where the melting point approaches, or is higher than the body temperature, so that the emulsification of the fatty globules in the intestinal contents may possibly be less satisfactory than in the case of oil. (Oils, Fats and Fatty foods, by J. C. Drumond, Professor of Biochemistry, University of London).

## Extraction of the Enzyme 'Lipase' from the Oil Seeds

The enzyme 'lipase' from the oil seeds ground-nut and linseed - was extracted in the three following ways :—

- (A) From the resting seeds.
- (B) From the resting seeds, but with Tanaka's dry powder method.
- (C) From the germinated seeds.

## SECTION II

### Determination of the pH for the enzyme 'lipase'

The pH for the enzyme lipase was determined as follows. Since it has been stated before that acids accelerate the hydrolysis, an acid buffer of 0.2 M disodium phosphate and 0.1 M citric acid was made to be used as a buffer solution. A range of buffer mixtures was made from 2.2 pH onwards to 4.6 pH. The maximum pH required for hydrolysis was found out for the enzyme lipase was 4.2 pH. Thus we see that the optimum pH for the 'lipase' lies in the neighbourhood of 4.2 pH for the above acid buffer and the lipase. According to Willstatter and Waldschmidt-Lietz E. (Uber Ricinus Lipase Z. Physiol. Chem. 134, 161, 1923-24). The optimum pH for the 'Ricinus Lipase' lies between 4.7 and 5.0 pH, varying slightly according to the buffer employed.

It is observed at first that the hydrolysis of the oil goes on increasing slowly from 2.2 pH to 3.4 pH. Here the increase is very gradual, but there is an abrupt rise in the hydrolysis of the oil from 3.8 pH and it reaches its maximum at 4.2 pH, while again it falls abruptly at 4.8 pH, the hydrolysis falling from 3.359% to 2.239%. It is quite evident that the optimum pH for the lipase is in the neighbourhood of 4.2 pH.

TABLE No. 1

**Determination of the pH for the Enzyme Lipase**

Buffer used—0.2 M disodium phosphate and 0.1 M citric acid.

A range of activators from 2.2 onwards to 4.6 pH was prepared and the following experiments were carried out to determine the optimum pH value for the lipase enzyme prepared from the ground-nut and linseed.

Each set of experiment contains 1 c.c. of sesame oil, 2 c.cs. water, and 200 mg. of enzyme lipase, 2 c.cs. of buffer of varying pH value, and the mixture was incubated for a fixed time of three hours at 37.5°C.

Saponification value of the oil = 45.36

Acid value of the oil. = 1.53

Set.	Oil in c.cs.	pH value	C.Cs. of N/10 NaOH required	Percentage hydrolysis.
1	1 c.c.	2.2	0.3 c.c.	0.840 %
2	1 „	2.6	0.4 „	1.1189 %
3	1 „	3.0	0.5 „	1.402 %
4	1 „	3.4	0.6 „	1.682 %
5	1 „	3.8	0.9 „	2.519 %
6	1 „	4.2	1.2 „	3.359 %
7	1 „	4.8	0.8 „	2.239 %

**Effects of Buffer Concentrations on the Rate of Hydrolysis**

It will be seen from the table No. 2, that buffer concentration plays a very important part in the course of hydrolysis of oils. An increase in the quantity of the buffer brings about a shift in the percentage hydrolysis,



A number of control experiments was carried along as shown in the table No. 2, keeping other factors constant. A buffer of 4.2 pH was added from 0.3 c.c. to 1.0 c.c. as shown in the table, to study the rate of hydrolysis. It is observed that the maximum hydrolysis is attained at the concentration 0.5 c.c. it being 3.359% and subsequently falling to a minimum 1.959% when the concentration is gradually increased to 1.0 c.c. Thus we see that on doubling the concentration of the buffer solution the hydrolysis does not increase but it drops.

TABLE No. 2

**Effect of buffer concentrations on the rate of hydrolysis**

Each of the experiments contains 1 c.c. of sesame oil, 2 c.c.s. of water, 100 mg. enzyme lipase, and varying quantities of the activator or buffer of 4.2 pH and incubated for a fixed period of 3 hours at 37.5°C.

Saponification value of the oil = 45.36  
Acid value ... = 1.53

Set	Oil in c.c.s.	Quantity of buffer added	C.Cs. of N/10 NaOH required	Percentage hydrolysis.
1	1 c.c.	0.3 c.c.s.	1.0 c.c.	2.799 %
2	1 ,,	0.5 ,,	1.2 ,,	3.359 %
3	1 ,,	0.6 ,,	1.1 ,,	3.107 %
4	1 ,,	0.9 ,,	0.8 ,,	2.239 %
5	1 ,,	1.0 ,,	0.7 ,,	1.959 %

**Effects of variation in the substrate concentration on the course of hydrolysis**

A further study was done by changing the substrate concentrations and keeping other factors constant, to see whether the variations in the substrate concentration influences the rate of hydrolysis.

From the table No. 3, it is seen that a change of substrate influences the hydrolysis of oil to a greater extent. It is obviously seen that a maximum hydrolysis of the oil takes place when substrate concentration is 2.0 c.cs. of oil or its equal weight. At 3.0 c.cs. it falls slightly but when the quantity of the oil is increased to 4.0 c.cs. it falls still further.

Thus we see from the table No. 3 that the substrate concentration plays a very important part in influencing the rate of hydrolysis.

TABLE No. 3

**Effect of variation in the substrate concentrations on the course of hydrolysis**

Each set of experiments contains varying quantities of sesame oil, 2 c.cs. of water, 100 mg. of the enzyme lipase, 0.5 c.cs. of activator or buffer of 4.2 pH, and incubated for a fixed period of 3 hours at 37.5°C.

Saponification value of the oil = 45.36  
Acid value = 1.53

Set.	Oil in c.cs.	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	1 c. cs.	0.5 c. cs.	1.402 %
2	2 „	1.3 „	1.820 %
3	3 „	1.7 „	1.586 %
4	4 „	2.6 „	1.092 %

**Effects of variations in the enzyme concentration on the amount of hydrolysis**

Study of enzyme concentration was next taken to see whether a variation in the enzyme concentration brings about any change in the rate of hydrolysis of the oil.

Three varying quantities of enzymes were used as shown in table No. 4. The factors, that is, oil, water, buffer solution added, temperature 37.5 degrees centigrade and time were kept constant as in the previous cases. It is observed from the table that for 50 milligrams of enzyme and 100 milligrams of enzyme the hydrolysis carried out is the same. While for 200 milligrams the hydrolysis is increased, but the increase is from 2.519 % to 2.659 % - a very negligible one. It appears that 50.0 milligrams of the enzyme 'lipase' work best. According to Constein Hoyer, and Wartenberg the proportion of seed or enzyme to oil is important. On the whole an increase in the amount of seed accelerates the hydrolysis, but the velocity of the decomposition is by no means proportional to the amount used, as small quantities act relatively more energetically than larger ones. (cf. Fokin J.S.C.I. 1904, 23, 1152).

TABLE No. 4

**Effect of variations in the enzyme concentrations on the course of hydrolysis**

Each set of experiments contains 2 c.cs. of sesame oil, 2 c.cs. of water, 0.5 c.cs. of activator or buffer of 4.2 pH, and varying quantities of the enzyme lipase and incubated for a fixed period of 3 hours, at 37.5 degrees centigrade.

Saponification value of the oil = 45.36

Acid value = 1.53

Set	Oil.	Enzyme in mgm.	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	2 c.cs.	50 mg.	1.8 c.c.	2.519 %
2	2 ,,	100 ,,	1.8 ,,	2.519 %
3	2 ,,	200 ,,	1.9 ,,	2.659 %

## SECTION III

**Study of the Hydrolysis of the Edible Oils In-Vitro**

A study of hydrolysis of the following two oils was done in-vitro. The oils—ground-nut and linseed—were preserved after extracting their corresponding meals, which were used as enzyme 'lipase'. The oils were purified by filtering and the ether driven off by keeping them in oven at 98 degrees centigrade. Then they were duly bottled after cooling and filtering them. The filtering was done to remove any suspended material.

A comparative study of hydrolysis of the above oils was then done in-vitro, keeping them at 37.5 degrees centigrade, and incubating them from three hours to twenty one hours, to study their hydrolysis.

The enzyme lipase at first used was from its own oil meal but treated in three different ways as given previously. The other factors such as substrate concentrations, buffer used, water added, and the temperature under consideration were the same in all cases. The above factors were determined as will be seen from the description already given, for getting the maximum hydrolysis of the oils under consideration.

In the first instance three different preparations of the enzyme lipase from the ground-nut meal were used on the original oil. The enzymes were respectively named A, B, and C, in order of their extraction. The quantity of the oil used was 2 c.cs. and its weight determined, buffer 0.5 c.c. of 4.2 pH, water added 2 c.cs., and the enzyme added 50 milligrams and incubated for varying time from 3 hours to 21 hours at 37.5 degrees centigrade. A blank experiment was also conducted side by side using all the above factors except the oil. The difference between the blank and the real experiment gave the amount of hydrolysis that had taken place in the same time. The hydrolysis was measured in terms of N/10 NaOH used up. Before starting the actual work the acid value and the saponification value of the oil in question were determined in terms of N/10 NaOH.

### Study of hydrolysis of the ground-nut oil with the enzyme 'lipase' as present in the ground-nut meal

In the table No. 5, three different preparations of the enzyme from the ground-nut meal, that is, ground-nut enzyme meal on the ground-nut oil are used. The enzymes are named A, B and C, respectively in order of their extraction. As stated above the other factors were kept constant except the time factor. It will be seen from the table that the enzyme 'lipase' B, which has been treated with Tanaka's dry powder method is more active than the enzyme 'lipase' from A, which has been extracted from the resting seeds of the ground-nut. While on the other hand the enzyme 'lipase' C from the same table is almost as active as enzyme A. Sigmund (Sitz. Kais. Akad. Wiss. 1892, 100, 328) states that germinating seeds are more active. Constein and co-workers deny this and Walker and Bourne (Tech. Quart., 1904, 17, 284) claim that germination lessens the activity of the seed. According to Hoyer (Ber., 1904, 37, 1436) seeds in process of germination, the enzymatic activity is weaker in the portions of seed adjoining the germ than in those further removed. K. G. Liawala (Jr. Ind. Inst. Sc., Vol. 2, Part XV. pp. 226 - 267, 1922) states that germinated seeds on the whole are not as efficient as resting seeds from the point of view of lipolytic activity. Our experiments show that the lipolytic activity of the resting ground-nut seed and germinated ground-nut seed is nearly equal. But at the end of 21 hours we find that the percentage hydrolysis is greater in the case of enzyme 'lipase' C, which is extracted from the germinated seeds. Thus we see that the germination does bring about the activation of the enzyme 'lipase', to a certain extent.

Now we shall consider the time factor from table No. 5. It is evident in all cases that as the time is increased from three hours to 21 hours, the hydrolysis of the oil gradually increases. It is almost one and a half times in all the three cases. On the whole the hydrolysis is less than even one per cent. The maximum hydrolysis is attained in the table at the end of twentyone hours with enzyme B, being 0.533 %.

TABLE No. 5

**Study of hydrolysis of the ground-nut oil with enzyme 'lipase' as present in the ground-nut meal**

Each set of experiments contains 2 c.cs. of ground-nut oil, 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated at 37.5° C.

Saponification value of oil = 42.5

Acid value = nil

Set	Enzyme in mgs.	Oil in c.cs.	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	A 50	2 c.cs.	3	0.20	0.266
	A 50	2 ,,	21	0.35	0.354
2	B 50	2 c.cs.	3	0.30	0.400
	B 50	2 ,,	21	0.40	0.533
3	C 50	2 c.cs.	3	0.20	0.266
	C 50	2 ,,	21	0.40	0.467

**Study of hydrolysis of the linseed oil, with the enzyme 'lipase' as present in the linseed meal**

Now, we come to the study of the hydrolysis of the linseed oil, with the enzyme 'lipase' as present in the linseed meal. The table No. 6 shows all the details. Here too we have got three preparations of the enzyme 'lipase' namely, A, B, and C, in order of their extraction. It is seen from the table that the enzyme 'lipase' from the linseed acts on linseed oil better than the ground-nut 'lipase' on ground-nut oil. Comparing the figures for hydrolysis in table No. 5, with those of table No. 6, it is seen that the percentage hydrolysis of linseed oil is more than the hydrolysis of the ground-nut oil, while the enzyme extraction behaves in the same order as far as their lipolytic character is concerned. The enzyme B,

treated with Tanaka's method is more active than the enzyme A or C. The maximum hydrolysis attained in the table with enzyme B is 0.769%. The other conditions remain the same as in the previous enzyme from the ground-nut.

Here also we observe that the hydrolysis of the oil is greater with enzyme 'lipase' C, at the end of 21 hours, while at the end of three hours the figures for hydrolysis for the enzyme 'lipase' A and C are just the same.

Further study in 'hydrolysis' of the oils was done by the same enzyme 'lipase' from the ground-nut and linseed meals, but here the substrates used were interchanged to see whether interchanging of substrates effects the rate of hydrolysis of the oils in any way. The other factors were kept constant as in the case of tables No. 5 and 6.

TABLE No. 6

**Study of hydrolysis of the linseed oil with the enzyme 'lipase' as present in the linseed meal**

Each set of experiments contains 2 c.cs. of linseed oil, 2 c.cs. of water, activator or buffer 0.5 c.cs. of 4.2 pH and incubated at 37.5°C.

Saponification value 40.3  
Acid value nil

Set	Enzyme in mgs.	Oil in c.cs.	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	A-50	2 c.cs.	3	0.30	0.419
	A 50	2 ,,	21	0.40	0.559
2	B 50	2 c.cs.	3	0.35	0.489
	B 50	2 ,,	21	0.55	0.769
3	C 50	2 c.cs.	3	0.30	0.419
	C 50	2 ,,	21	0.50	0.699

**Study of hydrolysis of the linseed oil with the enzyme as present in the ground-nut meal**

In the table No. 7, the results of hydrolysis of the linseed oil, with the enzyme 'lipase' as present in the ground-nut meal are tabulated. As in the previous cases three different preparations of the enzyme 'lipase' A, B and C are respectively used. Comparing figures in table No. 6 with those of table No. 7, we see in the first instance that the activity with the enzyme 'lipase' at the end of three hours is just the same, while at the end of 21 hours, it is less in the former case being 0.559%, while in the latter case it is 0.769%. Same observation is met with in the enzyme 'lipase' B, the activity increasing at the end of 21 hours. While in the case of enzyme C, there is no change at all, the activity remaining constant.

**TABLE No. 7**

**Study of hydrolysis of the linseed oil with the enzyme 'lipase' as present in the ground-nut meal**

Each set of experiments contains 2 ccs. of linseed oil, 2 c.cs. of water, activator or buffer 0.5 c.cs. of 4.2 pH and incubated at 37.5°C.

Saponification value = 40.4

Acid value = nil

Set	Enzyme in mgs.	Oil in c.cs.	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis.
1	A 50	2 c.cs.	3	0.30	0.419
	A 50	2 c.cs.	21	0.55	0.769
2	B 50	2 c.cs.	3	0.35	0.489
	B 50	2 c.cs.	21	0.65	0.909
3	C 50	2 c.cs.	3	0.30	0.419
	C 50	2 c.cs.	21	0.50	0.699



One important observation that is made here is that as in the previous tables No. 5, and No. 6, the figures for hydrolysis of the oil with the enzyme 'lipase' C—which has been extracted from the germinated material—is just the same at end of three hours hydrolysis. But at the end of 21 hours the hydrolysis is a little less, when compared to the hydrolysis with the enzyme 'lipase' A, extracted from the resting seeds, in the same table No. 7. Thus we observe that the percentage hydrolysis with enzyme 'lipase' A, and C is just the same, but in the latter case it is even less as the hydrolysis proceeds for 21 hours.

#### **Study of hydrolysis of the ground-nut oil with the enzyme 'lipase' as present in the linseed meal**

In the following table No. 8, we observe that a change of substrate with respect to the enzyme 'lipase' brings about an accelerated hydrolysis on the whole. Comparing figures in table No. 5, with those in table No. 8, we observe that in all the three cases of the enzyme A, B, and C, greater percentage of oil is hydrolysed at the end of 21 hours, and the percentage hydrolysis is almost doubled in the latter table. The figures of the above two tables could be easily compared to see the acceleration in hydrolysis. The hydrolysis actually increasing after three hours and reaching its maximum at the end of twenty-one hours.

From the tables No. 7, and 8, we conclude that a change of substrate with respect to the enzyme 'lipase' used influences the hydrolysis of the oil to a greater or lesser extent. Each substrate responding in its own way towards the action of the enzyme 'lipase', from different sources. By using the ground-nut oil and the enzyme 'lipase' from linseed meal, and vice versa, the hydrolysis is increased on the whole in both the cases, as it is evident from table No. 7 and table No. 8. Compare figures in table No. 5 with those of table No. 8, for the ground-nut oil and tables No. 6 and 7, for the linseed oil.

TABLE No. 8

**Study of hydrolysis of the ground-nut oil with the enzyme 'lipase' as present in the linseed meal**

Set	Enzyme in mgs.	Oil in c.cs	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	A 50	2 c.cs	3	0.25	0.311
	A 50	2 „	21	0.50	0.666
2	B 50	2 „	3	0.35	0.466
	B 50	2 „	21	0.70	0.936
3	C 50	2 „	3	0.20	0.266
	C 50	2 „	21	0.40	0.533

Each set of experiments contains 2 c.cs. of ground-nut oil, 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated at 37.5 degrees centigrade.

Saponification value : 42.5

Acid value ... : nil

#### SECTION IV

**Study of Hydrolysis of Sesame and Ground-nut Oil extracted from Ghanny**

A comparative study of the hydrolysis of the above two oils was done by using three different preparations of the enzyme 'lipase' - which were extracted from ground-nut and linseed meal - as shown in section II.

The oils were locally bought from Agricultural College, *ghanny* (country oil press), in order to be sure that the oils were pure and unmixed. The above study was specially done to see the difference between *ghanny* expelled oil, and ether expelled oil, as shown in the previous section III.

The oils were duly filtered after buying, to remove suspended material that might have been there and then bottled. The acid and saponification values of both the oils were noted before starting the actual work. The method of hydrolysis of both the oils was the same as that of the ground-nut and linseed oil in section III.

### **Study of hydrolysis of the sesame oil (*ghanny* pressed) with the enzyme 'lipase' as present in the ground-nut meal**

In table No. 9, three different preparations of the enzyme 'lipase' from the ground-nut meal were used to hydrolyse the oil. As before the other factors were kept constant throughout the experiment.

We see from the table No. 9, that the hydrolysis is maximum with the enzyme B, i.e. the enzyme 'lipase' which is treated with Tanaka's method is more active than the enzyme 'lipase' A, or C, the former is extracted from the resting seeds of ground-nut while latter is from the germinated seeds of ground-nut. On the whole the hydrolysis is more than 3% in all the cases. One important fact that we come across in the table is that the hydrolysis at the end of three hours and twenty-one hours is just the same. It is evident from the table that the oil is completely hydrolysed within a period of three hours. This rapid hydrolysis may be attributed to the higher acid value of the oil, which in the case of sesame oil as used is 1.53 in terms of N/10 sodium hydroxide, while in the case of ground-nut and linseed oil in section III, the acid value is nil for both the oils.

### **Study of hydrolysis of the sesame oil-*ghanny* pressed with the enzyme 'lipase' as present in linseed meal**

In table No. 10, three different preparations of the enzyme 'lipase' extracted from the linseed meal were used to hydrolyse the sesame oil.

Comparing figures in table No. 9 with those in table No. 10, we see that the 'lipase' extracted from ground-nut meal is more active than the 'lipase' in the linseed meal, as

far as its lipolytic activity is concerned, while in other respects the enzyme 'lipase' behaves in the same way as in table No. 9. The sesame oil being completely hydrolysed as in table No. 9, at the end of three hours, the respective enzymes A, B, and C behaving in the same manner as in the previous cases. The rapid hydrolysis may be again attributed to the higher acid value of the oil in question. The maximum hydrolysis attained is 3.560 % while in the case of ground-nut enzyme 'lipase' it is 3.90 % (refer table No. 9).

TABLE No. 9

**Study of hydrolysis of the sesame oil with the enzyme 'lipase' as present in the ground-nut meal**

Each set of experiments contains 2 c.c.s of sesame oil, 2 c.c.s. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated at 37.5°C.

Saponification value	...	45.86.
Acid value	...	1.53.

Set	Enzyme in mgs.	Oil in c.c.s.	Time in hours	C.Cs. of N/10 <sup>1</sup> NaOH required	Percentage hydrolysis
1	A 50	2 c.c.s.	3	2.6	3.644
	A 50	2 c.c.s.	21	2.6	3.644
2	B 50	2 c.c.s.	3	2.8	3.910
	B 50	2 c.c.s.	21	2.8	3.910
3	O 50	2 c.c.s.	3	2.4	3.359
	C 50	2 c.c.s.	21	2.4	3.359

Thus keeping the same substrates i.e. sesame oil, constant and using enzymes from two different sources, one from ground-nut meal and the other from the linseed meal, we observe that the enzyme from the ground-nut meal hydro-

lyses the oil more than the enzyme from the linseed meal. Thus we observe that the activity of the enzyme 'lipase' is in keeping with the substrate under consideration.

TABLE No. 10

**Study of hydrolysis of sesame oil with the enzyme 'lipase' as present in the linseed meal**

Each set of experiments contains 2 c.cs. of sesame oil, 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated at 37.5°C.

Saponification value 45.36

Acid value 1.53

Set	Enzyme in mgs.	Oil in c.cs.	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	A 50	2 c.cs.	3	2.3	3.211
	A 50	2 ,,	21	2.3	3.211
2	B 50	5 c.cs.	3	2.55	3.560
	B 50	2 ,,	21	2.55	3.560
3	C 50	2 c.cs.	3	2.2	3.072
	C 50	2 ,,	21	2.2	3.072

**Study of hydrolysis of the ground-nut oil (ghanny pressed) with the enzyme 'lipase' as present in the ground-nut meal**

In the table No. 11, a study of hydrolysis of the ground-nut oil - ghanny pressed - is undertaken. The oil was bought from Agricultural College, Poona, and the method of hydrolysis was the same as in the previous cases.

It is seen from table No. 11, that the total hydrolysis of the oil varies from 0.743% to 1.487% depending upon the 'lipase' used. The lipolytic activity of the enzyme 'lipase' being invariably constant for the 'lipase' A and C.

Comparing table No. 5, in section III, in which case the substrate ground-nut oil is extracted by the help of chemical means, i.e. dissolving the oil in ether, with table No. 11, in which the same substrate ground-nut oil is extracted with the help of a country oil press - commonly known as *ghanny* - in the Bombay Deccan areas, we see in the latter case that the hydrolysis is one and half times more than in the former case, where the oil has been extracted by chemical means. We also observe that the acid value of the oil is nil in case of table No. 5, while in the case of table No. 11 the oil has an acid value of 0.28 in terms of N/10 sodium hydroxide. It seems that the acid value of the oil plays an important part in accelerating the lipolytic activity of the enzyme 'lipase' indirectly or directly. As in the previous cases the hydrolysis is maximum at the end of 21 hours and does not stop after three hours as we have seen in the case of sesame oil (table No. 9 and 10).

TABLE No. 11

**Study of hydrolysis of the ground-nut oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the ground-nut meal**

Each set of experiments contains 2 c.cs. of ground-nut oil, 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated at 37.5°C.

Saponification value ... 42.23  
Acid value ... 0.28

Set	Enzyme in mgs.	Oil in c.cs.	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	A 50	2 c.cs.	3	0.5	0.743
	A 50	2 "	21	0.7	1.041
2	B 50	2 "	3	0.7	1.041
	B 50	2 "	21	0.9	1.487
3	C 50	2 "	3	0.5	0.743
	C 50	2 "	21	0.7	1.041

**Study of hydrolysis of the ground-nut oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the linseed meal**

In the table No. 12, a study of hydrolysis of the ground-nut oil (*ghanny*-pressed) is done. The oil was hydrolysed as before, keeping the various factors constant, and using the enzyme 'lipase' from the linseed meal.

It is seen from table No. 12, that the hydrolysis of the oil varies according to the preparation of the enzyme used, as well as time, the minimum hydrolysis being 0.593 % in the case of enzyme C, at the end of three hours, and the maximum being 1.338 % in the case of the enzyme 'lipase' B, at the end of 21 hours. One important point to be noted is that the hydrolysis with enzyme 'lipase' A, and C is not the same as we have seen in table No. 11, and other foregoing tables where the activity of the enzyme 'lipase' A and C is very nearly the same. The activity of the 'lipase C is much less than either A or B.

TABLE No. 12

**Study of hydrolysis of the ground-nut oil (*ghanny* pressed) with the enzyme 'lipase' as present in the linseed meal**

Each set of experiments contains 2 c.cs. of ground-nut oil, 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated at 37.5°C.

Saponification value	...	42.23
Acid value	...	0.28

Set	Enzyme in mgs.	Oil in c.cs.	Time in hours	C.Cs. of N/10 NaOH required	Percentage hydrolysis.
1	A 50	2 c.cs.	3	0.7	1.041
	A 50	"	21	0.8	1.189
2	B 50	"	3	0.8	1.189
	B 50	"	21	0.9	1.338
3	C 50	"	3	0.4	0.593
	C 50	"	21	0.6	0.892

Comparing figures in table No. 8, and those in table No. 12, in the former case the substrate is ground-nut oil, but is extracted by a different process (ether extracted oil). The similar observation is met with the enzyme 'lipase' C, in table No. 8, where the activity of the lipase is minimum as in the case of table No. 12. Thus we come to a conclusion that the activity of the 'lipase' is in keeping with the substrate under consideration, it being different for two different substrates, but agreeing with the same substrate.

## SECTION V

### Effect of heating the oil on the course of its hydrolysis

A comparative study of heating the oil was done to see whether heating the oil brings about any change in the rate of its hydrolysis.

The oils that were selected for the experimental work were sesame and ground-nut, which were bought from the Agricultural College, Poona. The oils used were first heated on a stove till they started boiling. They were then duly cooled and bottled. The saponification value and the acid values were once again determined to see whether heating the oil brings about any difference in their values. The saponification values of both the oils, sesame and ground-nut remained constant—for the former it was 45.36 in terms of N/10 NaOH and for the latter it was 42.23.

However a change in the acid values was observed. The acid value of the sesame oil fell from its original value 1.53 to 0.18 in terms of N/10 NaOH and for the ground-nut oil the acid value fell from its original 0.28 to 0.12 both measured in terms of N/10 NaOH. The only explanation for the fall of the acid value can be attributed to certain volatile acids which might have been present in the oils before heating and which on heating the oil were driven off.



Subsequently the hydrolysis of the heated oils was done with the three preparations of the enzyme 'lipase' to see whether heating of the oil brings about any change in the rate of hydrolysis.

**A study of the sesame oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the ground-nut meal, before and after heating the oil**

Two sets of experiments were conducted side by side, one with the ordinary *ghanny*-pressed oil cold, and the same after heating.

In the table No. 13, three enzyme preparations from the ground-nut meal, extracted in three different ways A, B, and C were used as in the previous cases. From the table we observe that in all the three cases the percentage hydrolysis falls in the case of the oil which has been pre-heated, though the same oil has been used. The percentage hydrolysis of the sesame oil, with the ground-nut enzyme meal falls from 3.910% to 1.219% in the case of enzyme 'lipase' B. Similarly it falls in the case of the enzyme 'lipase' A, as well as for C. The total percentage fall of the hydrolysis in all the three cases is near about 70%, after heating the oil. The only explanation for the fall of hydrolysis is due to the low acid value of the oil, which after heating the oil stands at 0.18 in the case of sesame oil. The acid values of the crude oils are much higher than those of the corresponding refined oils which are not appreciably hydrolysed under similar conditions, and hence it is presumably the free fatty acids and volatile acids present that act as co-enzymes and perhaps help the hydrolysis.

In the case of cocoanut oil and mohua oil, it was found possible to isolate volatile acids, and aqueous solutions of such acids were used in place of acetic acid it was found that crushed castor seeds produced appreciable hydrolysis. (J. J. Sudborough and H. E. Watson, Part V, Vol. 5. Part IX pp. 120 - 122, Jr. Ind. Inst., Sc. 1922).

TABLE No. 13.

**Effect of heating sesame oil on the course of its hydrolysis**

A study of hydrolysis of the sesame oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the ground-nut meal, before and after heating the oil.

Each set of experiments contains 2 c.cs. of sesame oil (*ghanny*-pressed) 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated for a fixed period of three hours at 37.5 degrees centigrade.

Set	Enzyme in mgs.	Oil in c.cs.	Sap. value of oil	Acid value	Heating	C.Cs. of N/10 NaOH Required	Percentage hydrolysis.
1	A 50	2 c.cs.	45.36	1.53	Before	2.6 c.cs.	3.641
	A 50	2 ..	45.36	0.18	After	0.8 ..	1.083
2	B 50	2 ..	45.36	1.53	Before	2.8 ..	3.910
	B 50	2 ..	45.36	0.18	After	0.9 ..	1.219
3	C 50	2 ..	45.36	1.53	Before	2.4 ..	3.351
	C 50	2 ..	45.36	0.18	After	0.8 ..	1.083

**A study of hydrolysis of the sesame oil, (*ghanny*-pressed) with the enzyme 'lipase' as present in the linseed meal, before and after heating the oil**

In the table No. 14, three preparations of the enzyme from the linseed meal were used on the 'sesame oil'. As in the case of table No. 13, the effect of heating the oil on the course of its hydrolysis was the main object of investigation. We observe from the table, that in all the three sets of enzymes, the percentage hydrolysis of the sesame oil, with the enzyme 'lipase' as present in the linseed meal, falls from 3.560 % to 1.151 % in the case of the enzyme 'lipase' B. Similarly in the case of the enzyme 'lipase' A, as well as C, the percentage hydrolysis falls. The total percentage fall of the hydrolysis in all the three cases is more than 70 % after heating the oil. While in the case of table No. 13, the total percentage fall of the hydrolysis is near about 70%, after

heating the oil. The above fall can be attributed to the weaker activity of the enzyme 'lipase' in the linseed meal. Hence the enzyme 'lipase' as present in the linseed meal is not able to hydrolyse the oil which has been pre-heated, to the same extent as the enzyme 'lipase' present in the ground-nut meal. Comparing the figures in table No. 13, with those of table No. 14, we see that the lipolytic activity of the enzyme 'lipase' from the ground-nut meal is greater than that of the enzyme 'lipase' as present in the linseed meal when the substrate used is the same. This also confirms our observations in the previous tables No. 9 and No. 10.

TABLE No. 14.

**Effect of heating the sesame oil on the course of its hydrolysis**

A study of hydrolysis of the sesame oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the linseed meal, before and after heating.

Each set of experiments contains 2 c.cs. of sesame oil (*ghanny*-pressed) 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated for a fixed period of three hours at 37.5 degrees centigrade.

Set	Enzyme in mgs.	Oil in c.cs.	Sap. value of oil	Acid value	Heat-ing	C. Cs. of N/10 NaOH required	Percentage hydrolysis
1	A 50	2 c.cs.	45.36	1.53	Before	2.3 c.cs.	3.211
	A 50	2 ,,	45.36	0.18	After	0.7 ,,	0.948
2	B 50	2 ,,	45.36	1.53	Before	2.55 ,,	3.560
	B 50	2 ,,	45.36	0.18	After	0.85 ,,	1.151
3	C 50	2 ,,	45.36	1.53	Before	2.2 ,,	3.072
	C 50	2 ,,	45.36	0.18	After	0.6 ,,	0.812

**A Study of hydrolysis of the ground-nut oil - (*ghanny* pressed) - with the enzyme 'lipase' as present in the ground-nut meal before and after heating**

In the table No. 15 we have studied the effect of heating the ground-nut oil on the course of its hydrolysis

Two samples of the ground-nut oil were used, but one was heated to boiling temperature and then cooled, while the other was taken as it was, from the *ghanny*-press. All the factors were kept constant as before including temperature and the samples were incubated for a fixed period of three hours, after adding the respective enzyme preparations A, B and C as present in the ground-nut meal. We observe from the table that in all the three sets of enzymes, the percentage hydrolysis of the ground-nut oil falls, in the case of the oil which has been heated to the boiling temperature. The fall of hydrolysis varies from 20 % to 28 % according to the enzyme preparation used, it being maximum with the enzyme 'lipase' B being 28 % while in the case of the 'lipase' A and C, the fall of hydrolysis is 20 %. The acid value of the oil falls from 0.28 to 0.12 after heating the oil.

TABLE No. 15.

**Effect of heating the ground-nut oil on the course of its hydrolysis**

A study of hydrolysis of the ground-nut oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the ground-nut meal, before and after heating.

Each set of experiments contains 2 c. cs. of ground-nut oil (*ghanny* pressed) 2 c. cs. of water, activator or buffer 0.5 c. c. of 4.2 pH and incubated at 37.5 degrees centigrade, for a fixed period of three hours.

Set	Enzyme in mgs.	Oil in c. cs.	Sap. value of oil	Acid value	Heat- ing	C.Cs. of N/10 NaOH required	Percentage hydrolysis
1	A 50	2 c. cs.	42.23	0.28	Before	0.5 c. cs.	0.743
	A 50	2 c. cs.	42.23	0.12	After	0.4 c. cs.	0.592
2	B 50	2 c. cs.	42.23	0.28	Before	0.7 c. cs.	1.041
	B 50	2 c. cs.	42.23	0.12	After	0.5 c. cs.	0.740
3	C 50	2 c. cs.	42.23	0.28	Before	0.5 c. cs.	0.743
	C 50	2 c. cs.	42.23	0.12	After	0.4 c. cs.	0.592

**A study of hydrolysis of the ground-nut oil - (*ghanny* pressed) - with the enzyme 'lipase' as present in the linseed meal, before and after heating the oil.**

In the table No. 16, we have studied the effect of heating the ground-nut oil on the course of its hydrolysis. But instead of the enzyme 'lipase' as present in the ground-nut meal, enzyme 'lipase' as present in the linseed meal is used. All the other factors have been kept constant as in table No. 15. As before three enzyme préparations A, B and C as present in the linseed meal were used. The samples were incubated for a fixed period of three hours, after adding the respective enzymes and the hydrolysis of the oil calculated as usual. We observe in general from the table, that in all the three sets of enzyme 'lipase' A, B, and C the percentage hydrolysis of the oil in question falls. In the case of oil which has been pre-heated to boiling temperature the fall of hydrolysis varies from 50% to 57%. The maximum fall of hydrolysis is noted here in the case of enzyme 'lipase' A, being 57%, while in the case of enzyme 'lipase' B and C it is only 50%. Thus we see that in the case of oils, which have been pre-heated the hydrolysis is retarded. The retardation in all the cases, though varying for different enzyme préparations is quite marked in all the cases. It is in keeping with the oil and enzyme used. The retardation in the hydrolysis of the oils might be due to the distribution of the 'lipase' initially present in the oils, which gets destroyed when the oil is heated to the boiling temperature.

TABLE No. 16

**Effect of heating the ground-nut oil on the course of its hydrolysis**

A study of hydrolysis of the ground-nut oil (*ghanny*-pressed) with the enzyme 'lipase' as present in the linseed meal, before and after heating the oil.

Each set of experiments contains 2 c.cs. of ground-nut oil, (*ghanny*-pressed) 2 c.cs. of water, activator or buffer 0.5 c.c. of 4.2 pH and incubated for a fixed period of three hours at 37.5 degrees centigrade.

Set	Enzyme in mgs.	Oil in c.cs.	Sap. value of oil	Acid value	Heating	U.Cs. of N/10 NaOH reqd.	Percentage hydrolysis
1	A 50	2 c.cs.	42.23	0.23	Before	0.7 c.cs.	1.041
	A 50	2 „	42.23	0.12	After	0.3 c.cs.	0.444
2	B 50	2 c.cs.	42.23	0.28	Before	0.8 c.cs.	1.169
	B 50	2 „	42.23	0.12	After	0.4 c.cs.	0.592
3	C 50	2 c.cs.	42.23	0.28	Before	0.4 c.cs.	0.593
	C 50	2 „	42.23	0.12	After	0.2 c.cs.	0.296

## PAPER XXVIII

### STUDIES OF THE ENZYMES OF THE DRY FRUITS

BY

DR. R. S. KALE, Ph. D.

(With the kind permission of the authorities of the Bombay University)

#### SECTION I

#### Lipolytic enzyme of the dry fruit

Numerous seeds have been examined to see whether they contain any lipolytic enzyme. In this field mention may be made of the original researches of Pelouze (C.R. 1855, 40, 605); S. Fokin Chem. Rev. Fett. Ind. Harz. Ind. 1904, 11, 30, 48, 69).

In the present section it is intended to study the lipolytic activity of the three dry fruits. Amongst all, pancreatic lipase of animal origin and castor bean lipase of plant origin have been the favourites for many research workers.

Lipases from all the three fruits are prepared according to Tanaka's method as given before. Olive oil and castor oil are used as substrates. The following properties of these oils were determined before starting the actual work.

1. Olive oil	Specific gravity.....	0.916
	Sap. value .....	188.49
	Refractive index .....	2.462
	Acid value.....	1.68
2. Castor oil	Specific gravity.....	0.96
	Sap. value .....	180.1
	Acid value .....	2.244
	Refractive index .....	1.471

### Preparation of the aqueous emulsion of enzyme

This was prepared always by treating the lipase powder (10 grams) with distilled water 60 c.c., added in small portions and rubbed intimately in a mortar. The cream was pressed out through muslin and the residue again treated in the same manner. The total extract was made to 100 c.cs. which was shaken every now and then vigorously before use.

### Procedure

2 c.cs. of the substrate oil were taken in a conical flask and mixed with 10 c.cs. of enzyme cream (equivalent to 1 gm. of dry lipase powder) with addition of 5 c.cs. of a phosphate buffer. The contents of the flask were thoroughly shaken and kept in an incubator at 37°C for 24 hours. The mixture was then treated with neutral absolute alcohol and titrated with N/10 NaOH using phenolphthalein as the indicator.

The end point was noted immediately when the colour of the mixture became distinctly red. A control experiment without the enzyme extract was done in every case. The difference in the amount of N/10 NaOH required, gave a measure of the lipase action.

In the table No. 1, hydrolysis is expressed in terms of c.cs. of NaOH N/10.

TABLE No. 1.

		Hydrolysis with lipase after 18 hours						
		pH	3	4	5	6	7	8
Cashew nut lipase	Substrate							
	Castor oil		0.6	0.9	0.8	1	1.1	1.4
	Olive oil		0.5	0.8	0.6	0.7	0.8	1.1
Almond lipase	Castor oil		0.3	0.8	0.7	1.1	1.4	1.3
	Olive oil		0.2	0.7	0.6	0.9	1.1	1.4
Fistichio nut lipase	Castor oil		0.9	1.4	1.3	1.4	1.6	2.1
	Olive oil		0.6	1.0	1.2	1.1	1.5	1.9
Castor seed lipase	Castor oil		7.6	10.4	12.2	11.4	10.8	10.0
	Olive oil		7.2	8.4	10.2	9.6	9.0	8.2

From the above table it is noticed that every one of the three dry fruits does contain a lipolytic enzyme though very weak in action. The maximum activity is shown by all of them near about pH "8" i.e. towards the alkaline side of neutrality. Comparing all of them with the castor seed lipase we find that the optimum pH for castor bean lipase is near about "5" i.e. acidic, while the optimum pH for the dry fruit lipases is about "8" i.e. alkaline. Comparing the activities at the optimum pH, it can be noticed that the Cashew nut



lipase is not even one-ninth as much powerful as the castor bean lipase while the almond lipase is not even one-eighth and the pistachio lipase not even one-sixth as much powerful. pistachio nut, almond, and cashew nut stand in the descending serial order as far as the lipolytic activity is concerned.

## SECTION II

### Study of the proteolytic enzyme from the dry fruits

As in the last case our next intention is to seek for the existence of any protease in the dry fruits by which they could bring about the proteolysis of some of the common proteins. Amongst proteases - pepsin, trypsin, erepsin, pepsin and renin have been the favourites for the research workers all peptone and casein are used as substrates and aqueous extracts of the fruits as the hydrolysing agents. In all the experiments Sorenson Formol titration method is used and the results are expressed in terms of N/10 caustic soda. Toluene is used as a preservative in all experiments.

**Substrates**—20 c.c. of a 5 per cent. solution of peptone were used as the substrate in the first case and 0.5 gram of casein which were thoroughly mixed with 5 c.cs. of buffer and 10 c.cs. water in the 2nd case.

**Enzyme cream**—5 grams of the prepared sample were treated with 30 c.cs. of distilled water and rubbed in a mortar for some time. The extract was squeezed through muslin cloth and the residue was again treated with water, and the total extract measured 50 c.cs. 10 c.cs. being equivalent to 1 gram of the sample.

**Procedure**—To the substrate (20 c.cs. of peptone solution or 0.5 grams of casein) 10 c.cs. of the enzyme cream were added together with 5 c.cs. of phosphate buffer. The mixture was kept in an incubator at 37°C and the reaction was allowed to proceed for 24 hours. The extent of hydrolysis was then finally determined with the Sorensen's formaldehyde titration method and the results are expressed in terms of c.cs. of N/10 NaOH.

TABLE No. 2

**Hydrolysis with the protease in the dry fruits after 24 hours**

	pH	3	4	5	6	7	8
Cashew nut protease	Substrate						
	Peptone	3.5	4.2	4.5	4.3	4.6	5.0
	Casein	0.4	0.5	0.8	0.7	0.9	0.9
Almond protease	Peptone	2.6	2.7	2.9	2.8	3.0	3.5
	Casein	0.3	0.4	0.5	0.4	0.6	0.8
Pistachio nut protease	Peptone	2.1	2.4	2.8	2.6	2.9	3.1
	Casein	0.2	0.3	0.6	0.4	0.5	0.7

From the figures in table No. 14, we find that the proteolytic activity of the dry fruits is maximum at pH "8" i.e. towards the alkaline side of neutrality though there is a slight tendency towards increasing hydrolysis at pH "5", while in the case of pepsin the optimum pH is 1.8 i.e. towards the acidic side of neutrality. The results show that casein is very little acted upon by the fruit enzymes. This is in accordance with the observation made previously by various workers (Chopra, Ghosh and Chatterjee-Jr. Ind. Chem. Soc. 1938), that plant erepsins are not usually capable of hydrolysis as casein like animal erepsins. These results should be more or less looked upon as qualitative rather than quantitative.

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## PAPER XXIX

STUDY OF THE AMYLASES FROM SOME  
EDIBLE ROOTS AND TUBERS

By

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*(With the kind permission of the authorities of the Bombay University and also of the Agri. College, Poona, Magazine)*

**Introduction**

The work described in this paper forms a part of the investigation undertaken by the author to elucidate the nature of the amylase. This enzyme was the first to be discovered by Irvine in 1785 having observed that malt extract produced sugar from starch. Payen and Peroze (Ann. 1834, 53, 73) obtained the enzyme by precipitation with alcohol.

More recently amylases that were prepared from ungerminated grain were termed as Saccharogenic or "b" amylases; while germinated grain is supposed to contain both "a" and "b" amylases, from which "a" amylase could be prepared according to methods suggested in literature for preparing them.

The enzyme had attracted the attention of numerous investigators. Isolation and study of the same was mainly undertaken by the earlier investigators, till Grutzner (Pfluger's Archiv. 12, 1876, 285) found that heating the enzyme for a short period at 80°C inhibited the saccharifying activity considerably without affecting its power of liquefying starch. Wijsman (Rec. Trav. Chem. 9, 1890, 1) was the first to express the two enzyme theory in a definite form. From evidence based on some diffusion experiments, he concluded that diastase is a mixture of two enzymes, one hydrolysing starch to maltose and erythrogranulose and another which converts this erythrogranulose to leuco dextrin and starch to malto dextrin.

Pottenvin (*Ann. Inst. Pastens.* 13, 1899, 665) also believed that the hydrolysis proceeds in two successive phases. He assumed the existence of two different enzymes, one transforming starch to dextrin, and another which effects the further cleavage to maltose. He found like Grutzner (*Loc. cit.*) that heating the enzyme at 80°C affected the saccharifying power to a far greater extent than the liquefying capacity. This has recently been confirmed by E. Ohlsson (*Compt. rend. Soc. Biol.* 87, 1922, 1183). Frankel and Hamburg (*Hofm. Beitr.* 8, 1906, 389) claimed that they had separated malt diastase into two components by dialysis, the sugar producing enzyme diffusing out and the liquefying component remaining behind in the dialyser. On the other hand, Chrzaszcz - (*Biochem. Z.* 142, 1923, 417; *ibid* 150, 1924. 60) also recognised three distinct stages in starch hydrolysis namely liquifaction, dextrin formation, and sugar production. He could effect a selective alteration of any of these processes by precipitation, application of high temperature, or aging of the enzyme. He claims to have effected a partial separation of the enzyme by fractional precipitation with ammonium sulphate. He also observed that the relation between saccharification, and iodine colouration varied with the origin of the malt. Fricke and Kaja (*Ber.* 57, 1924, 313) found that during electro-dialysis of barley malt diastase with increasing purification, the liquifying power decreased with an increase in the saccharifying power. It was observed by Norris and Vishwanath (*Agri. J. Ind.* 18, 1923, 362) that cholam malt diastase is a more powerful liquifier and a weaker saccharifier than the corresponding barley enzyme. Their experiments lend further support to the two enzyme theory and indicated the presence of liquifying component in greater proportion than the saccharifying component in cholam. Patwardhan, D. Narayanamurti and Norris (*J. Ind. Inst. Sc.* Vol. 11 A 1928, Part XI) gave further support to the two enzyme theory.

Oppenheimer (*Die Fermente*, Band 1, Page 665) had drawn attention to the fact that it was highly improbable that the various phases of starch hydrolysis were catalysed by one and the same enzyme. According to him three quite different processes, were to be recognised; the dissolution of the crystal lattice,

the liquifaction of the starch, the depolymerization, usually measured by the vanishing of the iodine reaction, and the cleavage of the dihexosan to maltose and all sorts of transformations, in addition to the above in the case of amylopectin and glycogen the splitting of trihexosan. The existence of two enzymes seems most probable one which disaggregates the starch molat, and another which attacks the anhydride ring. Karrer—(Helv. Chem. Acta, 4, 1921, 678) also inclines to the same view, while he postulates as a logical consequence of the present conception of starch constitution, the existence of two enzymes, one depolymerising and the other anhydride splitting.

According to Ohlsson (Z. Physiol. Chem. 221, 1933, 174) malt amylase of cereals consists of an "a" and "b" amylase. Myrback (Woch. Brau. 49, 1932, 246) have shown that ungerminated barley contains mostly only "b" amylase, whilst after germination considerable amounts of "a" amylase is formed. In malt according to researches of Ohlsson (Z. Physiol. Chem. 189, 1930, 70) there are to be distinguished a starch liquifying enzyme and one which converts starch into sugar, that is, a dextrinogen and saccharogen amylase. Since then the separation of the amylatic components in malt extracts has been accomplished by help of adsorbents. Thus Waldschmidt Leitz and Purr—(Z. Phy. Chem. 203 : 1931 : 117) have separated amylase from maltase. Contrary to Ohlsson's view Waldschmidt, Leitz and Purr (Natur Weise 20, 1932, 253) have come to the conclusion that ungerminated barley contained both "a" and "b" amylases and that the amylase was present in an inactive form, and required amylokinase for activation. This kinase was developed during germination. In question of the formation of amylases during the germination of seeds, as well as in the question of the proportion at any time between the liquifying and saccharifying powers, the coaction of specific natural activators of starch degradation has to be taken into account. Waldschmidt Leitz and Purr (Loc. cit.). Borchardt and Pringsheim (Biochem. Z. 239, 1931, 193) working on the amylase prepared from pressed juice of the potato, which was deproteinised with colloidal ferric hydroxide had shown that the potato amylase occupied

an intermediate position between the malt amylase particularly rich in "b" amylase, and the taka amylase which was pure "a" amylase as it contained less "b" than "a" amylase. Willstatter and Rohdewald (Z. Phy. Chem. 221, 1933, 13) have shown that the amylases of leucocytes are dextrinogenic in character, thereby resembling pancreatic amylase and differing from malt amylase. The foregoing observations bring out a distinct difference between the two amylases, the former belonging to the class of "a" amylase and the latter to that of "b" amylase. According to Borchartd and Pringsheim (Loc. cit.) the saccharogen amylase is a "b" amylase, "a" and "b" amylases are present in different proportions in malt.

## SECTION I

### Methods of Preparation of Amylases

Several methods have been used in separating the amylases. Among plant amylases those from leaves and grains have been mostly studied, but only comparatively meagre information is available regarding the nature and behaviour.

So far for the investigation in hand the work done by various workers on amylases has been given. It was therefore decided to carry on the work on roots and tubers such as sweet potato, yam, suran, and carrot.

Stamberg and Bailey (J. Biol. Chem. 126, 1938, 479) prepared amylases from ungerminated wheat, which they termed as "b" amylase and "a" amylase which were also prepared by them from the germinated wheat. Sandstedt, Kneen. Blish, (Cereal Chem. 16, 1939, 712); Van Klinkenberg (Z. Physiol. Chem. 209, 1932, 253); Hanes and Cattle (Proc. Roy. Soc. Lond. Series B, 125, 1938, 387); Ballow and Luck (J. Biol. Chem. 139, 1941) prepared "b" amylase from wheat; Ford and Peat (J. C. S. 858: 1941) prepared "b" amylase from wheat. According to investigators amylases prepared from germinated material is supposed to contain "a" plus "b"

amylases together, from which "a" amylase could be separated according to methods suggested by them. Ungerminated material is supposed to contain "b" amylase only. So in the present investigation amylases prepared from sweet potato, carrot, yam and suran are termed as "b" amylases. Amylase prepared from germinated sweet potato will be termed as "a" plus "b" amylase.

Among the tubers selected for preparing the amylases, Giri (J. Ind. Chem. Soc. 1934, 339) had also prepared "b" amylase from sweet potato. But we wanted to find out whether the sweet potato had the same strength of activation at different stages of growth. In addition to the preparation of the amylases from two different sets, in the present investigation, experiments were also carried out to germinate the full developed sweet potato, and then to prepare amylase from germinated sweet potato. The activity in this case was judged by studying the action of the amylase so prepared on starch, and was also compared with the activity of the "b" amylase prepared from ungerminated one. Just as grains were germinated and the amylase activity was then studied from the amylases so prepared, among the tubers selected by us for preparing the amylases, sweet potato was also germinated, and the amylase was prepared from the same. Study of the amylase from sweet potato does not seem to have been carried out so far. Hence the preparation of the amylase from germinated one will give additional information regarding the tuber like the sweet potato.

## SECTION II

### Study of the "b" Amylase from Sweet Potato

"b" amylase from sweet potato was prepared by Giri (Loc. Cit.). But we wanted to find out whether the sweet potato (red variety) had the same strength of activation at different stages of growth. In one case "b" amylase was extracted from sweet potato when its size was 5" in length, approximately and 4" in circumference; and in the other case when the size was 7" to 8" in circumference. The object was

to judge whether the activity of the amylases remained practically the same, or went on increasing or diminishing in the case of these two sets.

### Method of Preparation

After mincing the tuber in a chopper, the ground tissue was spread on trays and kept for drying, and the dried material was then ground to a fine powder. The powdered material was preserved in bottles with a few drops of toluene. The method of preparing the "b" amylase was according to the method carried out by Ford and Peat (Loc. Cit.) for preparing "b" amylase from wheat, with a slight modification.

100 gms. of the powdered meal was extracted with approximately 4.5 times volume of distilled water containing 20% for twelve hours in the presence of toluene. It was then centrifuged, and 90% alcohol was added to the supernatant liquid which contained the colloidal material, so that the total volume of alcohol was 80%. Usually the precipitate requires two to three hours to settle down the fine enzyme material. But in order to make sure that most of it settled down, the material was allowed to stand for three hours, supernatant liquid was siphoned off. The sediment thus obtained was treated with absolute alcohol and finally dried in a desiccator over anhydrous calcium chloride to remove last traces of moisture. The resultant powder is light brown in color.

### Effect of H ion concentration

The first experiment was tried to determine the Optimum pH. Acetate buffer mixtures of varying pH were prepared. "b" amylase used in this case was from sweet potato.

### Experimental

Four different conical flasks (200 c.c. capacity) were taken and to these were added 20 c.c. of distilled water, and 10 mgms. of the amylase powder was also added to each of these four flasks. To one flask was added 10 c.c. of buffer solution (pH 4.8). To the second flask was added acetate buffer



(*pH* 5.3) 10 c.c.; to the usual 10 c.c. of the buffer solution (*pH* 6.0); and to the fourth flasks 10 c.c. having *pH* 6.8 were added. When the required temperature was reached, to these four flasks were then added 10 c.c. of 1% soluble starch solution which had attained the required temperature. The reaction mixture was kept at 29°C in an incubator for twenty-four hours. A control was also run by side. After the reaction was allowed to proceed for twenty-four hours, the reaction was stopped by adding a few drops of alkali, and the sugar formed was then estimated. The object of performing this experiment was to obtain the optimum *pH* in the case of sweet potato. The results obtained are given below :—

TABLE No. 1

**Hydrolysis of starch with "b" amylase from sweet potato at varying *pH***

Temperature (°C)	Varying <i>pH</i> used.	Time in hours	Starch hydrolysed %
29	4.8	24	36.4
"	5.3	"	37.3
"	6.0	"	37.2
"	6.8	"	36.5

After determining the optimum *pH* the experiment was carried out to the effect that could be produced by the addition of the different quantities of the amylase to the fixed quantity of the substrate. In other words in order to see whether the addition of the greater quantity of the amylase had any influence upon the saccharifying action, experiments were then carried out in the case of sweet potatoes, which were smaller in size,

Three flasks were taken which contained 20 c.c. of distilled water. To one of these flasks were added 10 mg. of amylase powder, to another was added 15 mg. of the powder, and to the third was added 20 mg. of the amylase powder. These flasks were kept in an incubator to attain the required temperature. 10 c.c. of the buffer solutions were also added as usual (pH 5.3). When the reaction mixture had reached the desired temperature, 10 c.c. of the starch solution which was kept in the incubator were added to each of these flasks. The reaction was allowed to proceed for twenty four hours. A control was also carried out. After twentyfour hours the reaction was stopped by adding a few drops of alkali, and the sugar formed was estimated as usual according to the method of Bertrand. The results obtained with amylase from sweet potato (younger in age) are given in table No. 2.

TABLE No. 2

**Hydrolysis of starch with "b" amylase from sweet potato  
(small size)**

Temperature (°C)	Quantity of amylase added in mgms.	Time in hours	starch hydrolysed %
29	10	24	37.3
"	15	"	45.8
"	20	"	54.7

From the values of hydrolysis obtained it will be observed that when 10 mgms. of the amylase was used, the value of hydrolysis is 37.3%; while when 20 mgms. of the amylase is used, the value of hydrolysis is 54.7%. That is, by doubling the quantity in this case in particular, one and half times hydrolysis is produced. The data obtained shows the existence

of a sharp saccharification, the values being dependent upon the concentration of the amylase used or added. The results indicate that with greater quantity of the amylase, we get more hydrolysis,

After determining the extent to which starch is hydrolysed by using different quantities of amylase, the next point was to determine whether fully developed sweet potatoes of large size contain more active amylase, or whether it is less active than the one given above, experiments were carried out side by side with the above by using flasks containing amylase from larger size, keeping the conditions exactly identical, but in this case the sample of the amylase was different, that is "b" amylase was prepared from sweet potato of large size, according to the procedure already given, with a view to compare the saccharogenic activity.

In table No. 3 are given the results obtained when "b" amylase was prepared from sweet potatoes of large size, and the changes occurred in starch hydrolysis due to variation in quantities of the amylase.

TABLE No. 3

**Hydrolysis of starch with "b" amylase from sweet potato  
(large size)**

Temperature (°C)	Quantity of amylase added in mgms.	Time in hours	starch hydrolysed %
29	10	24	32.5
"	20	"	35.0
"	30	"	39.4
"	40	"	45.8

From the results it is observed that by using 10 and 20 mgms. of the amylasé, the difference in the percentage of hydrolysis produced is 2.5% indicating thereby that upto this much quantity, the hydrolysis does not vary appreciably; while with 30 or 40 mgms. greater hydrolysis is produced. However, from the values of saccharification obtained, it is clearly seen that with the addition of more quantity of the amylase to the substrate, the values of hydrolysis increase, but more readily with 30 and 40 mgms. than with 10 and 20 mgms.

The values of hydrolysis obtained in the case of large size do not show any increase very rapidly as is found in the case of sweet potato younger in age. The results obtained in the case of the two different sizes of sweet potatoes, some of them are given in table No. 4 for the sake of comparison. When 10 and 20 mgms. were used, the values of hydrolysis obtained were 37.3 and 54.7; while in the other case the values obtained were 32.5 and 35. In the latter case the difference in these values is practically very small.

TABLE No. 4

**Comparison of "b" Amylase from sweet potato (having two different sizes).**

Mgms. added	Small size	mgms. added	Large size
	starch hydrolysed %		starch hydrolysed %
10	37.3	10	32.5
20	54.7	20	35.0

From the results obtained it could be observed that the saccharogenic values are higher in the case of small size indicating thereby that "b" amylase is more active when extracted from the younger one than in the case of fully developed one. Falk, McGuire and Blount (J. Biol. Chem. 38,

1919, 229) studied the enzyme action by extracting juice from carrot. He states. "In considering the enzyme results, it may be pointed out that the state of ripeness, and the age of the vegetable undoubtedly influence the activities". When the proportion of the enzyme concentration and the substrate concentration were maintained 1:10, this difference is not very markedly observed. With still lower quantity than 10 mgms., that is, with the proportion more than 1:10, this difference may not be much. When the proportion of the enzyme to the substrate concentration is maintained 1: 5, the difference is distinctly observed as 54.7 and 35.0 being the values of respective hydrolysis.

It was found that in the case of sweet potato, the activity of the "b" amylase was more during growth than during the fully developed stage, and the activity of the amylase rapidly increases in the case of the younger one.

### SECTION III

#### Study of the amylase from germinated sweet potato

Preparation of the amylase from germinated sweet potato is more important as the amylase will contain both "a" and "b" amylases together. Study of the germinated grains such as wheat, barley, jowar etc. has been done, but in the case of tuber like sweet potato, study of the germinated sweet potato does not seem to have been done. According to Borchardt and Pringsheim (Loc.Cit.) potato amylase occupies an intermediate position between the malt amylase which is particularly rich in "b" amylase and the taka amylase which is a pure "a" amylase.

Before giving the detailed procedure of the preparation of the amylase, it is necessary first to give the procedure regarding the germination of sweet potato, as the process of germination and period of growth are quite different from what is generally observed in the case of grains such as wheat, jowar, barley etc.

Developed sweet potatoes were kept in the wet soil for a period of fifteen days, when they germinated. They got

plumules, the plumules produced measured about  $1\frac{1}{2}$ . They were then washed with water. That end of sweet potato which had these plumules, was cut from the rest of the portion. These were then chopped into fine pieces, dried and powdered. The powdered flour was preserved with a few drops of toluene. The amylase ("a" and "b") was prepared from the dried flour.

The method of preparing the amylase from the flour of the germinated sweet potato was the same as was followed in the case of ungerminated sweet potato.

The Optimum pH of the amylase so prepared was the same as was obtained in the case of ungerminated one. The activity of the amylase was judged by studying its action on soluble starch as usual.

Different flasks were taken. To these were added 20 c.c. of distilled water and different amounts of the amylase powder was added to each of these flasks. The flasks were kept in an incubator. Buffer solution was also added, and when the required temperature was reached, 10 c.c. of 1 per cent. starch solution were also added, which had also attained the required temperature. The reaction was allowed to proceed for twenty-four hours. After twenty-four hours, the reaction was stopped by adding a few drops of alkali and the sugar formed was estimated, and the amount of starch hydrolysed was calculated. The results obtained by using amylase from germinated sweet potato are given in table No. 5.

TABLE No. 5.

**Hydrolysis of starch with amylase from germinated sweet potato**

Temperature (°C)	Quantity of amylase added in mgms.	Time in hours	starch hydrolysed %
29	10	24	34.9
"	20	"	39.6
"	30	"	47.5
"	40	"	54.7

From the results obtained it is found that with increase in quantity of the amylase, the amount of hydrolysis also increases. When the amylase concentration of 10 mgs. is used, the hydrolysis obtained is 34.9%; while with 40 mgs. the value is 54.7%.

It is found that the values of starch hydrolysed are not independent of the amylase concentration used. It will be seen that with the addition of more amylase, the degree of hydrolysis is also more. From the results of saccharogenic activity obtained it is seen that an active amylase is present in germinated sweet potato. The results obtained in both the cases are given in table No. 6 for the sake of comparison.

TABLE No. 6

**Comparison of the activity of the amylase from germinated and ungerminated sweet potato**

Germinated sweet potato		Ungerminated sweet potato	
Quantity of amylase added in mgs.	starch hydrolysed %	Quantity of amylase added in mgs.	starch hydrolysed %
10	34.9	10	32.5
20	39.6	20	35.0
30	47.5	30	39.4
40	54.7	40	45.8

From the results obtained it is clearly seen that the values of the germinated amylase from sweet potato are higher than those of amylase from ungerminated one. Perhaps, in the case of germinated one "a" amylase is present in combination with "b" amylase. On comparing the results of germinated with ungerminated sweet potato, it is observed that if 10 mgs. of the amylase are used, in both the cases, the difference in the saccharogenic values is small. But if higher concentration of the amylase is used, the values show a marked difference. Thus when 40 mgs. of the concentration of the amylase is

used, the difference in the values is more, as the values obtained are 54.7 and 45.8 respectively. From these values it is clear that germinated sweet potato contains an amylase which becomes more active on germination as it contains both "a" and "b" amylases together. According to Myrback (Loc. Cit.) germinated grain contains active amylases namely "a" plus "b", while ungerminated seed contains "b" amylase only. Hence on germination the amylase is more active than before germination. According to Waldschmidt Leitz and Purr (Loc. Cit.) ungerminated seed contains both "a" and "b" amylases, but "a" amylase is present in inactive form, and requires amylokinase for activation. This kinase is developed during germination. Hence the amylase becomes more active.

It is observed that in both the cases hydrolysis increases by increasing amylase concentration, but the values of hydrolysis due to ungerminated one are always lower than those of germinated one.

#### SECTION IV

#### Study of the "a" amylase from grains such as *jowar*, and comparison of the activity of the same with that of "b" amylase from sweet potato

As the results obtained from germinated sweet potato were higher than those of ungerminated one, because germinated one contained both "a" and "b" amylases together, while ungerminated one contained "b" amylase only, it was thought that it would be interesting to prepare an "a" amylase from sweet potato and to compare its activity with an amylase from germinated tuber such as potato. But according to Borchardt and Pringsheim (Loc. Cit.) potato amylase occupies an intermediate position between the malt amylase which is particularly rich in "b" amylase, and the taka amylase, which is pure "a" amylase. Hence "a" amylase, was prepared from germinated grain such as *jowar*. As detailed study of the amylase containing both "a" and "b" has been already done in the case of germinated sweet potato,



and also detailed study of the "b" amylase from sweet potato has been done, "a" amylase from sweet potato was prepared with a view to compare its activity only with that of "a" amylase from *jowar* grain.

Before preparing "a" amylase from *jowar* grain, conditions of germinating *jowar* grain were followed according to Patwardhan and Narayanamurti (J. Ind. Inst. Sc. Vol. XI, Part XII, 1928). *Jowar* grains were soaked in water, for 24 hours. The grains were then spread on a moist cloth over a bed of sand. Grains were kept moist by frequent supply of water. In about three days the seeds had nearly an inch of plumule, when germination was stopped. The grains were dried in the sun for a day and the dried material was then powdered.

Among the various methods suggested in literature for preparing "a" amylase by various investigators - Hanes, Bailey, Giri (Loc. Cit.) etc., "a" amylase was prepared from *jowar* grain as follows:—

To 100 gms. of the germinated *jowar* flour was added 400 c.c. of distilled water, containing 0.025 % of potassium dihydrogen phosphate. It was kept for eighteen hours. It was then centrifuged and supernatant liquid was heated to 70°C for fifteen minutes, which inactivated most of the "b" amylase present. It was then heated in nine minutes, heated at 70°C for fifteen minutes, and cooled to the room temperature in eleven minutes. It was centrifuged, and then 60% of alcohol by volume was added. When the precipitate settled down, after three hours, the precipitate was washed with 90% alcohol and finally with absolute alcohol and dried in vacuo over calcium chloride.

## Experimental

To three different flasks containing 20 c.c. of distilled water were added different quantities of the amylase powder, namely to one were added 20 mgms., to another 30 mgms, and to the third were added 40 mgms. These flasks were kept in incubator till the required temperature was reached. As

the amylase activity was not affected appreciably by using buffers between pH 5.3 and 6.0, and as the amylase activity was to be compared, buffer solution having 5.3 as pH was used. Buffer solutions and starch solutions were added as in last experiments to these flasks. A control was also run. The flasks were kept for twenty-four hours and the sugar formed was estimated and the amount of starch hydrolysed was calculated as usual. The object of this experiment was to ascertain whether by varying the quantity of the "a" amylase there was any variation in starch hydrolysis as was observed in the case of "b" amylase. The results obtained are given in the following table:—

TABLE No. 7

#### Hydrolysis of starch with "a" amylase from *jowar* grain

Temperature (°C)	Quantity of amylase added in mgms.	Time in hours	starch hydrolysed %
29	20	24	24.05
"	30	"	26.80
"	40	"	30.60

By varying the quality of "a" amylase it was observed that even by doubling the quantity of the amylase, starch is hydrolysed by about 6% more.

#### Study of the "a" amylase from germinated sweet potato

"a" amylase was also prepared according to the procedure followed in the case of *jowar* grain.

In order to find out the saccharogenic activity of the "a" amylase from sweet potato, the following experiment was performed.

To two flasks were added 20 c.c. of distilled water. Then 20 and 30 mgms. of "a" amylase from sweet potato were added. The flasks were kept as usual in an incubator, and buffer solutions and starch solutions were added. A control was also run and the amount of starch hydrolysed was determined as usual. The results obtained are given in table No. 8.

TABLE No. 8

**Hydrolysis of starch with "a" amylase from sweet potato**

Temperature (°C)	Quantity of amylase added in mgms.	Time in hours	starch hydrolysed %
29	20	24	28.7
"	30	"	30.6

From the results obtained in tables No. 7 and 8 it will be seen that the activity in both the cases differs but slightly by increase in quantity of the amylase. The results obtained from the cereal amylase is not stronger than the material obtained from sweet potato.

## SECTION V

**Study of the Characters of the "a" and "b" Amylases**

From what has been studied so far it is observed that "b" amylase prepared from developed sweet potato is more saccharogenic in character than "a" amylase. "a" amylase is a weaker saccharifier.

"b" amylase in the case of sweet potato in a large size is less active than the "b" amylase prepared from small size.

In the case of "b" amylase by adding greater quantity of the "b" amylase to the substrate concentration, the values of hydrolysis increase while in the case of "a" amylase with the

addition of more quantity of "a" amylase to the substrate, the values of hydrolysis increase very slowly.

In the case of germinated sweet potato, (which contains both "a" and "b" amylase) the values of hydrolysis are higher than those obtained in the case of "b" amylase from sweet potato of large size.

In order to study the characteristics of the "a" and "b" amylases, experiments were tried, which are described further.

It is widely accepted that starch can be divided into two fractions: amylose, which is water soluble and amylopectin a mucilaginous substance with the characteristic paste forming properties. Their relative proportions vary considerably. Samec - (Trans. Farad. Soc. 31, 1935, 395). Ling and Nanji (J.C.S. 123, 1923, 2666) determined the ratio of amylose to amylopectin in the molecule of starch, and found it to be 1:2. According to them when starch is kept at a point below gelatinising point, thus in the case of potato starch say the temperature should be 60°. The mass will dissolve in water and amylose will pass into solution.

### Experimental

To 5 gms. of *suran* flour were added 100 c.c. of distilled water. It was heated at 60°C for fifteen minutes. It was supposed that amylose will pass into solution. The solution was then cooled and kept for half an hour and then centrifuged.

To two different flasks containing 20 c.c. of distilled water were added 20 mgms. of "a" amylase and 20 mgms. of "b" amylase and 10 c.c. of the buffer solutions as usual. When the temperature had attained 37°C, 20 c.c. of the amylose solution prepared as described above were added to each of these flasks. It had already attained 37°C temperature. The reaction was then carried out for 4 hours. Estimations were carried out according to method of Willstatter and Schudel. N/100 sodium thiosulphate solution was used for estimation. The results obtained were as follows:-

"a" amylase required 2.0 c.c. of sodium thiosulphate solution.

"b" amylase required 7.0 c.c. of sodium thiosulphate solution.

Although the amylose prepared is not pure, yet, this experiment shows that the two amylases are different in character.

According to Ohlsson (Compt. rend. Trav. Lab. Carlsberg, 16, 1926, 7) "a" amylase is prepared by heating an extract to 70° for fifteen minutes, thus inactivating most of the "b" amylase present in germinated seed. It has also been stated by Myrback (Loc. Cit.) that ungerminated seed contains only "b" amylase. An experiment was arranged to see whether in the case of sweet potato (ungerminated) "b" amylase becomes completely inactive at 70°C by heating for fifteen minutes.

Two flasks were taken. To these 20 c.c. of distilled water were added and 20 mgms. of amylase powder were also added. One of these flasks was kept in a waterbath and the solution was heated to 70°C for fifteen minutes. It was then cooled to the room temperature and the two flasks were kept in an incubator. When the temperature was 29°C, buffer solutions and starch solutions which were kept in an incubator were taken out and 10 c.c. of each of these solutions were added. The reaction was allowed to proceed for twenty-four hours. After twenty-four hours the reaction was stopped by adding a few drops of alkali and the sugar formed was estimated and the percentage of starch hydrolysed was calculated. The results obtained are given in table No. 9.

TABLE No. 9

**Effect of heat on the hydrolysis of starch with amylase**

Quantity of amylase used	Action of heat	starch hydrolysed %
20	without heating	34.9
20	Heated to 70°C for 15 minutes	5.9

This result shows that by heating "b" amylase to 70°C for fifteen minutes, the hydrolysing action is not completely stopped.

It has already been stated that the germinated material contains both "a" and "b" amylases together, and the activity of the amylase remaining after heating to 70°C for 15 minutes is due to "a" alone. The results obtained in the case of germinated and ungerminated sweet potato by heating to 70°C for 15 minutes and without heating, which are given in the preceding tables are mentioned below :—

TABLE No. 10

**Hydrolysis of starch with amylase from germinated and ungerminated sweet potato**

Amylase used	starch hydrolysed %
20 mgms. of the amylase from germinated one ("a" and "b" together).	39.6
20 mgms. of the amylase from germinated one after heating to 70°C for fifteen minutes ("a" only).	28.7
20 mgms. from ungerminated one ("b" only)	35.0
20 mgms. from ungerminated one heated to 70°C for 15 minutes.	5.9

Amylase obtained from germinated sweet potato is taken to contain both "a" and "b" amylases, while ungerminated one is supposed to contain "b" amylase only. Germinated sweet potato which was heated to 70°C for fifteen minutes which can be taken to contain originally "a" and "b", but after heating only "a" amylase. From the results obtained it is clearly observed that "b" amylase alone hydrolyses starch to 35%; while "a" amylase hydrolyses to 28.7; while the combination of the two gives us 39.6%. This means that there is some overlapping in their hydrolysis, and that is why we get 39.6% in the mixture; while separately when their figures are added together they give us a total of 63.7% .

Study of the "a" and "b" amylases has been done from grains such as wheat or barley by several investigators. Study of the germinated grain practically amounts to the study of the "a" and "b" amylases together. It was thought that it would also be interesting to study the combination of the "a" amylase from grain such as *jowar* and the "b" amylase from tuber such as sweet potato, with a view to study the saccharogenic activity as a result of their combination. The object was to see whether a combination of this type also gives the same types of results as are usually obtained when "a" and "b" amylases from grains are studied in combination.

Kuhn (Annalen, 1925, 443, 1) (abstr. from Giri's paper J. Ind. Chem. Soc. 15, 1938) has made the discovery that there are two different types of amylases which are separable under certain conditions. One of these amylases converts starch into *a*-maltose, the other produces only *b*-maltose under similar conditions. The existence of two different specific amylases is a stronger point in favour of the existence of correspondingly different components in starch molecule. More recently Van Klinkenberg (Proc. Acad. Sc. Amsterdam, 1931, 34, 893; Z. Physiol. Chem. 1932; 209, 253; *ibid* 1932, 212: 173) has put forward the view that the liberation of *a* and *b* maltose by "a" and "b" amylases is due to the selective hydrolysis of two components of starch, which he designated as *a* and *b* starch. The hypothesis developed by Van Klinkenberg is based on the following points:—

(1) When soluble starch is hydrolysed to completion by "b" amylase, the reducing power of the products corresponds to 64% of the theoretical maltose. The residual fraction is designated as *a* starch and constitutes about 36% of the starch.

(2) When soluble starch is hydrolysed by "a" amylase the reducing power rises rapidly to a value that corresponds to 36% of the theoretical maltose, after which it continues to rise slowly. The initial rapid rise is interpreted as being due to the selective hydrolysis of *a* starch (36% of the total substrate) to maltose. The slow hydrolysis beyond 36% in presence of

“a” amylase was explained by the supposed slow transformation of *b* into *a* starch. The converse transformation (*a* starch into *b* starch) does not occur because a sharp “Saccharification limit” is always observed in the hydrolysis of starch by “b” amylase.

According to Freeman and Hopkins (Biochem. J. 1936, 30, 446) (1) Both “a” and “b” amylases readily attack and hydrolyse completely amyloamylose component (about 20%) of starch, and a portion of the erythroamylose component. (2) Both enzymes hydrolyse much the same fraction: 60% of the starch; but whereas the remainder (*a* amylopectin) is almost if not completely, resistant to the “b” amylase, it can be with difficulty hydrolysed by the “a” enzyme. (3) *a*-amylopectin, the hydrolysis of which has been commenced by the “a” amylase can be further degraded to a certain extent by the “b” amylase. The “a” enzyme holds the key to further hydrolysis. (4) In degrading starch to the 50% stage, the “a” enzyme does so mainly at the expense of the portion which is other than amylopectin, since action on the residue by the “b” still leaves about 30% of the original starch unattacked.

It is well known that when starch is decomposed by amylases (Abstr. Giri's Paper J. Ind. C. S. 1938) inhibition zones and saccharification limits are formed, which are found to vary with different types of amylases and the results obtained by several workers are found to be of varying magnitude. The following table represents the values given by several workers.

Author	Reference	Inhibition zones
	“a” amylase	
Syniewski	Annalen, 1925, 441, 288, 214	about 34%
Polak & Tychowski	Biochem. Z. 1929, 214, 216	36%
Van Klinkenberg	Z. Physiol. Chem. 1932, 209, 253	36%
Hanes	Canad. J. Res. 1935, 138, 185	34-38%
Blom, Bak & Brae	Z. Physiol. Chem. 1936, 241, 273	33-40%



Author	Reference	"b" amylase	Sacchari- fication limit
Baker	J. C. S. 1902, 81, 1177		60-65%
Ling & Nanji	J. C. S. 1923, 123, 2666		67%
Polak & Tychowski	(Loc. Cit.)		66%
Van Klinkenberg	(Loc. Cit.)		64%
Hopkins, Cope & Gres	J. Inst. of Brew, 1933, 39, 487		64-65%
Hanes	(Loc. Cit.)		61%
Blom, Bak & Brae	(Loc. Cit.)		53.1-0.5%

The whole problem of the hydrolysis of starch seems to have been found to be a difficult one and every worker has arrived at his own conclusion. It was therefore thought necessary not to enter into the controversies, which are prevalent, but to take an independent line to see how this problem could be attacked. The procedure that was followed can be said to be based on determining whether the starch actually contains two parts of which one is attacked by "a" amylase, (to the exclusion of the other part) and the other by "b" amylase (to the exclusion of the other part). Experiments were arranged where equal quantities of starch solutions were taken in two parts, to one "a" amylase was added, and to the other "b" amylase was added.

### Experimental Procedure

To one of the two different flasks containing 20 c.c. of distilled water were added 20 mgms. of "a" amylase and to the other 20 mgms. of "b" amylase. Then to each 10 c.c. of buffer solution and 10 c.c. of starch solution were added. The procedure followed as usual. They were kept in an incubator at 29°C and after the fixed period, sugar formed was estimated. Another experiment:- In one flask 20 mgms. of "a" amylase was allowed to complete its action on starch for 24 hours, and then 20 mgms. of "b" amylase were added to it. Another flask was taken in which 20 mgms. of "a" and "b" each were added together, other procedure followed being similar as above. The results obtained are as follows:-

Amylase used	starch hydrolysed %
"a" amylase alone	24.1
"b" amylase alone	35.4
"a" amylase superimposed by "b" amylase	57.0
"a" amylase plus "b" amylase together	56.0

When "a" amylase was allowed to react first for twenty-four hours, and then "b" amylase was added next day, the value of hydrolysis obtained on the third day was 57%. This distinctly shows that after the attack of "a" amylase, some starch remains, which is then hydrolysed by "b" amylase.

Similarly when "a" and "b" amylases were allowed to react in combination, after 48 hours, 56% starch was hydrolysed. This makes it clear that there are two types of starchy materials, one attacked by "a" amylase, and the other attacked by "b" amylase, whether experiments were tried separately or in combination.

Experiments were carried out further with 30 and 40 mgms. when "a" and "b" amylases were allowed to act separately and in combination as described above, and the results obtained are as follows :-

#### Results with 30 mgms. amylases

	Starch hydrolysed %
"a" amylase alone	26.9
"b" amylase alone	39.3
"a" amylase and "b" amylase together in combination	61.2
If the value of "a" and "b" amylases obtained are added, the total is	66.2

#### Results with 40 mgms.

"a" amylase alone	30.5
"b" amylase alone	45.6

Amylase used	Starch hydrolysed %
"a" amylase and "b" amylase together in combination	70.0
If the values of "a" and "b" amylases obtained are added, the total is	76.1

In every case with different strengths of the amylases, we find that the two working separately hydrolyse more starch than when they are added together in one flask. This is found to be true with 20, 30 and 40 mgms.

In the case of 20 mgms. "a" and "b" amylases together in combination gave 56% hydrolysis, but the two working separately, when added, give the total of 59.5%. Similarly in the case of 30 and 40 mgms. the results obtained with "a" and "b" amylases in combination are 61.2% and 70%. But the two working separately when added together give the total of 66.2% and 76.1%, the difference in the two cases being 5% and 6.1% respectively. Similarly with 20 mgms. the difference is 3.5%. In every case we find some difference. It means, therefore, that although "a" amylase attacks one part and "b" amylase attacks another part, yet there is a certain part, which can be attacked by "a" amylase or by "b" amylase. Freeman and Hopkins (Biochem. J. 30, 1936, 442, 446, 451) have also arrived at a similar conclusion. Blom, Bak and Brae (Z. Phy. Che. 241, 1936, 273) made an extensive study of the separate and simultaneous actions of different purified "a" and "b" amylase -preparations on starch from potatoes. On the basis of a rather single conception of the simultaneous action of two enzymes upon the same substrate. they came to an interesting and somewhat astonishing conclusion that 1/4 to 1/5 of the glucosidic linkages in the starch molecules, are split by "a" as also by "b" amylase.

The limit of temperature for the inactivation of "b" amylase in case of grain such as wheat or barley has been found to be 70°C, when "b" amylase becomes inactive. Andrews and Bailey (Cereal Chem. 11, 1934, 551); Stamberg and Bailey (J. Biol. Chem, 126, 1938, 479) etc. There is,

nowever, one difficulty in as much as when the "b" amylase is superimposed by "a" amylase or vice versa, the full quantity hydrolysed is less than when "a" and "b" amylases are made to act separately, and the figures for hydrolysis are added together, indicating as we have already said that there is some overlapping in this hydrolysis where "a" and "b" amylases are in a mixed condition. This means that there is a part of starch which is acted upon by "a" and "b" both, and that is why there is some overlapping and difference in the total hydrolysed starch. In order to make sure that there is no ambiguity left, an experiment was arranged to see whether the "b" amylase is completely made inactive at 70°C. In the experiment conducted, the solution containing "b" amylase was heated at 50°, 60°, and 70°C. and made to act on equal amount of the starch solution.

To three different flasks were added 20 c.c. of distilled water, and 20 mgms. of the amylase powder were added to each of these flasks. One of the flasks containing the amylase solution was heated to 50°C., the other was heated to 60°C, and the remaining flask was heated to 70°C. These flasks were heated at the respective temperatures for exactly fifteen minutes. They were then cooled to the room temperature, and then they were kept in an incubator to attain the required temperature of 29°C. Buffer solution and starch solutions were then added as usual. The reaction mixtures were kept for 24 hours and the sugars formed were estimated as usual. The results obtained are given below :

Temperature (°C)	starch hydrolysed %
50	9.8
60	7.1
70	5.8

This experiment clearly shows that at 70°C. "b" amylase is not completely destroyed by heating for fifteen minutes, but a certain amount of activity is still left there. From this experiment it is observed that when "a" amylase is allowed to react on starch, the value of hydrolysis obtained is not due to

“a” amylase alone, but also due to some proportion of “b” amylase, which remains along with “a” amylase. Hence “a” amylase being not sufficiently pure, value of hydrolysis of “a” amylase, will depend on its freedom from “b” amylase. That is why the anomalous position pointed out above occurs. The experiment that the “b” amylase is not completely destroyed at 70°C. in the case of tuber like sweet potato, or *jowar* grain was repeated and it was found that a small proportion of the activity took place by “b” amylase which had been heated to 70°C.

It was also considered necessary to find out the extent to which starch is hydrolysed by these amylases. From experiments carried out with 20, 30 and 40 mgms., it was found that “a” amylase hydrolyses soluble starch to about 40-41 %; and “b” amylase from fully developed sweet potato, hydrolyses soluble starch to about 59-60 %.

From experiments given in this section, it was observed that—

“b” amylase prepared from ungerminated sweet potato is saccharogenic in character.

“a” amylase is a weaker saccharifier.

Germinated sweet potato containing both “a” and “b” amylases is of course a better saccharifier, but the total values of hydrolysis obtained in combination are, however, less than when added together additively, when their reactions take place separately.

It seems that there are two types of starchy materials, one attacked by “a” amylase and the other by “b” amylase, but there is a certain part which is attacked by “a” as well as by “b” amylases.

In the case of “a” and “b” amylases we find that the two working separately hydrolyse more starch than when they are in combination.

When "a" amylase was allowed to act first followed by "b" amylase, about the same amount of hydrolysis had taken place, as when both the amylases acted together.

In the case of the "a" amylase as it is to be prepared from germinated material, if "b" amylase is not destroyed at 70°C. by heating for fifteen minutes, value of hydrolysis due to "a" amylase, will depend upon its freedom from "b" amylase.

It was found that "a" amylase hydrolyses soluble starch to about 40-41%.

"b" amylase from fully developed sweet potato hydrolyses soluble starch to about 59-60%

## SECTION VI

### (A) Determination of sugar from sweet potato during growth

The object was to find out whether both reducing and non-reducing sugars are present during growth of sweet potato. Hasselbring and Hawkins (J. Agri. Res. 5,1915,331,543) investigated the general course of carbohydrate transformation in sweet potato during storage and observed that in the stored sweet potato, starch is first converted into reducing sugars and finally cane sugar is synthesised from reducing sugar.

Cuttings of sweet potatoes were put in the soil. After the period of five, six and seven months, newly formed sweet potatoes were taken out from the soil. They were divided into three sections. Transverse sections were taken. First section was near vine, second section was the middle portion, and the third section was the end portion remaining, the object being to determine in which portion the sugar was more. These different portions were dried and flours were preserved,

for sugar determination. The results obtained are given below.

TABLE No. 11

**Determination of reducing sugar during growth**

Section. (No.)	sugar after five months %	sugar after six months %	sugar after seven months. %
1	0.551	1.525	4.252
2	0.998	2.150	4.488
3	1.020	2.154	4.251

From the results obtained it will be seen that the middle portion finally contained more sugar than the other two portions, and that no non-reducing sugar was found during the period of five, six and seven months. Starch percentage was found to be approximately the same in different portions.

**(B) Study of the "b" amylase from yam and suran**

*Suran* crop when taken out from the soil was two years old. Yam crop was one year old. For preparation of the dried flours of these materials, the outer skin was removed. They were then cut into very thin slices. Both these materials were sticky, but among them yam was more sticky than *suran*. Hence dried material was obtained after about four days. It was then powdered, and the amylases were prepared according to the method followed in the case of sweet potato.

The experimental procedure and method of estimation was the same as described in the case of sweet potato.

TABLE No. 12

**Hydrolysis of starch with "b" amylase from yam and suran**

Name of the flour	Quantity of the amylase added in mgms.	Time in hours	Percentage of starch hydrolysed.
Yam	20	24	18.1
<i>Suran</i>	20	24	15.6

If these values of hydrolysis obtained in the case of yam and *suran* are compared with that of sweet potato, the activity of these two amylases is very low.

**(C) Study of the "b" amylase from carrot**

After the preparation of the "b" amylase from sweet potato, yam and *suran*, and their actions on starch carrot was the next that was selected. As already described, like the sweet potato, carrots were cut into very thin slices, and were dried. The dried material was then powdered, and flour was preserved.

Experimental procedure for preparing the amylase was the same as that followed in the case of sweet potato, with slight modification. In order to prepare an amylase in solid form, 50 gms. of the flour were mixed with 300 c.c. of water containing 20% alcohol as by adding 200 c.c. of distilled water. The mixture became rather thick. Other procedure followed was the same as described in the case of sweet potato.

**Experimental**

To the flask containing 20 c.c. of distilled water were added 20 mgms. of the amylase powder, and after adding the required amount, it was kept in an incubator to attain the required temperature. When the temperature had been reached, 10 c.c. of the buffer solution and 10 c.c. of the starch solutions



were added as usual. A blank experiment was also run. The mixture was kept for 24 hours, and the sugar formed was estimated as usual. Similar experiment was also carried out by using 40 mgms.

The results obtained are given below :—

TABLE No. 13

**Hydrolysis of starch "b" amylase from carrot**

Temperature (°C)	Quantity of amylase added in mgms.	Time in hours	Percentage of starch hydrolysed
29	20	24	38.6
29	40	24	46.1

From the results obtained it will be observed that when 20 mgms. of the amylase are used, starch is hydrolysed to the extent of 38.6%; while when 40 mgms. are used, it is hydrolysed to the extent of 46.1%. It will be seen that with increase in concentration of the amylase, the values of hydrolysis also increase. The values of hydrolysis vary according to the concentration of the amylase used.

For the sake of comparison values of hydrolysis obtained in the case of sweet potato and carrot are given below:-

TABLE No. 14

**Comparison of "b" amylase from sweet potato and carrot**

Quantity of amylase added in mgms.	starch hydrolysed in case of sweet potato %	starch hydrolysed in case of carrot %
20	35.0	38.6
40	45.8	46.1

From the results obtained it will be seen that "b" amylase in the case of carrot is slightly more active than "b" amylase from sweet potato.

In order to see whether "b" amylase from carrot also becomes inactive by heating to 70°C for fifteen minutes, the following experiment was carried out.

To one flask was added 20 c.c. of distilled water, and 20 mgms. of the "b" amylase from carrot was added. The flask was then heated to 70°C for fifteen minutes. It was then cooled and kept in an incubator to attain the required temperature (29°C). Then 10 c.c. of the buffer solution and 10 c.c. of the starch solution were added as usual. Similar experiment was carried out simultaneously without heating. After twenty four hours, the amount of sugar formed was estimated and the percentage of starch hydrolysed was calculated.

TABLE No. 15

Action of heat	starch hydrolysed
(Amylase) Without heating	%
(Amylase) Heated to 70°C	38.5
	7.1

This result shows that even by heating at 70°C for 15 minutes, hydrolysing action is not completely stopped in the case of "b" amylase. Similar observation was made in the case of "b" amylase from sweet potato. As in the case of sweet potato, starch hydrolysed if calculated theoretically, is hydrolysed to the extent of 59-60%, similarly in the case of carrot, if "a" and "b" amylases are allowed to act on starch, starch is hydrolysed to the extent of 60-62%.

It is found that "b" amylase from carrot as well as from sweet potato is saccharogenic in character. However, "b" amylase from carrot is slightly more active than "b" amylase from sweet potato.

In both the cases with increase in concentration of the amylases, values of hydrolysis also increase.

By heating "b" amylase to 70°C it does not become completely inactive in both the cases.

In the case of "b" amylase from sweet potato, starch is hydrolysed to the extent of 59-60%; while in the case of "b" amylase from carrot, starch is hydrolysed to the extent of 60-62%.

### Summary and Conclusions

For studying amylase activity, amylases were prepared from roots and tubers such as sweet potato, yam, *suran* and carrot. They were termed as "b" amylases. Sweet potato was also germinated, and amylase ("a" plus "b") was also prepared from the same. "b" amylase from two different sizes of sweet potatoes was also prepared in order to determine whether activity remains the same or not at different stages of growth. In order to study the characteristics of the "a" and "b" amylases experiments were tried with two types of amylases that were prepared, namely "a" amylase from grain such as *jowar*, and "b" amylase from sweet potato. Another object in trying these experiments was to see whether combination of this type produces similar types of reactions, as when "a" and "b" amylases behave in combination when extracted from grains. From the results it was observed that:—

In the case of "b" amylase from two different sizes of sweet potatoes, the saccharogenic values were higher in the case of small size indicating thereby that "b" amylase was more active when extracted from the younger one than in the case of fully developed one. When the proportion of the amylase concentration and the substrate concentration was maintained 1:10, this difference was not very markedly observed. When the proportion was maintained 1:5, this difference could be distinguished.

In the case of "b" amylase from carrot, the values of saccharification do not differ much from the values obtained in the case of "b" amylase from sweet potato. The values of saccharification in the case of "b" amylase from yam and *suran* are low. "b" Amylase is saccharogenic in character. In the case of sweet potato and carrot by adding greater quantity of amylase to the substrate concentration, hydrolysis is also increased. In the case of "a" amylase with the addition

of more quantity of the amylase to the substrate concentration, the values of hydrolysis do not show much variation "a" amylase is a weaker saccharifier.

In the case of amylase from germinated sweet potato, the values of hydrolysis were higher than those obtained in the case of "b" amylase. The values of hydrolysis were not independent of the fixed concentration of the substrate.

In the case of "b" amylase from carrot or sweet potato it was observed that by heating to 70°C for fifteen minutes, it did not become completely inactive.

Germinated sweet potato containing both "a" and "b" amylases together, is of course a better saccharifier, but the total values of hydrolysis obtained in combination, are however, less than when added together additively when their reactions take place separately.

In the case of "a" amylase as it is to be prepared from germinated material, if "b" amylase is not completely destroyed, by heating to 70°C for fifteen minutes, the value of hydrolysis due to "a" amylase will depend on its freedom from "b" amylase. "b" amylase from developed sweet potato hydrolyses soluble starch to about 59-60%, and "b" amylase from carrot hydrolyses starch to about 60-62%.

When "a" amylase was allowed to act first followed by "b" amylase about the same amount of hydrolysis was obtained as when both the amylases acted together.

When experiments with "a" and "b" amylases were tried in combination and separately, it was found that the two working separately hydrolyse more starch than when they are in combination. From the results it was observed that although a part of starch is attacked by "a" and the other by "b", yet there is a certain part of starch, which is attacked by both "a" as well as by "b" amylases, whether experiments are tried separately or in combination.

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## PAPER XXX

PHOSPHATE SUPPLYING POWER OF SOME  
TYPICAL SOILS FROM WESTERN INDIA

By

DR. V. K. LELEY, M. Sc. Ph. D.

*(With kind permission of the authorities of the Bombay University)***Introduction**

Phosphates present in soils and their supply to plants, are two questions of long standing interest, before the soil chemist. Voluminous work done in past many years, has thrown much light on the nature of soil phosphates and the mode of their supply to plants. Yet a satisfactory method of universal application to diagnose phosphatic status of a soil, is still not available.

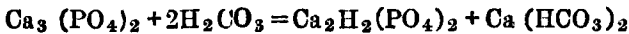
Soil phosphates which are insoluble in water are not directly useful to plants. It was originally believed that plant roots produce acidic exudations, which bring in solution, some of these insoluble phosphates, before they are absorbed by plants. Phosphates which are soluble in weak acidic solutions, are, therefore, considered to be available to plants. Phosphates of Fe, Al, Ca, Mg, etc., fall in this category. Recently phosphates adsorbed on soil colloids, and the organic phosphorus, are also considered to be available to plants.

A number of acidic extractors have been suggested from time to time to remove these phosphates from soils for estimation. (5) This figure is taken to represent the total quantity of soluble phosphates in the soil, which plants can utilize for absorption.

A comparison of available phosphate figures with the phosphatic status of the soil, as judged from the plant growth, is many a time not borne out. In calcareous or alkaline soils, acid extraction methods very often give misleading results (1, 2, 3, 4). Plants, grown on a soil containing fairly good amount of available phosphates, often show symptoms of phosphates-deficiency and respond vigorously to the application of a comparatively small amount of superphosphate.

The fact that, in spite of a good reserve of available phosphates, plants fail to get an adequate supply of phosphorus, appears to indicate that, all stock of these phosphates are not, all at a time, at the disposal of plants, but they are fed at a definite rate which is not necessarily related to the available  $\text{PO}_4$  content alone. If this rate is high enough to meet the plant requirements, the plants would grow satisfactorily, otherwise they would show deficiency symptoms, inspite of the fairly high concentration of available phosphates in the soil. Soil factors other than the available phosphates, seem to control this rate of phosphate supply. These factors being in integral part of the soil, this rate of supply is a characteristic of the soil rather than a function of the total quantity or the nature of the available phosphates. This rate may, therefore, be designated as the Phosphate Supplying Power of the soil.

Carbon dioxide also appears to exert considerable influence on the rate of phosphate supply to plants. It has been observed that increase in  $\text{CO}_2$  production in soil favours the phosphate absorption by plants. Baure (6) has also observed that when rock phosphate is supplied to fields with organic matter, a marked increase in yield is obtained. Trong (quoted) by Cook (10) attributed this to the carbon dioxide produced in the decomposition of organic matter. He suggested the following reaction :-



and stated that the amount of calcium biphosphate in solution depends on the rate of removal of calcium ions from soil solution. McGeorge et. al. (7) have similarly observed in the case of calcareous soils, a better absorption of phosphates when the soil is treated with acidic water. They considered this being due to the production of  $\text{CO}_2$  in the decomposition of carbonates. Carbon dioxide should, therefore, be considered as a major external factor in controlling the rate of phosphate supply, and consequently the phosphorus supplying power of a soil (18).

It is evident that, the amount of phosphates retained in soil solution, in the presence of excess of carbonic acid,

may be used as a measure of the phosphate supplying power of the soil. The following method was found convenient in the present study to determine chemically the phosphate supplying power of soils.

10 gms. of soil passing through 1 mm. sieve were mixed with 200 ml. of distilled water and saturated with carbon dioxide under a pressure of 5 cm. of mercury. It was shaken at room-temperature for an hour under the same pressure and kept overnight. It was then filtered and phosphates were estimated colorimetrically from the filtrate. The Phosphate Supplying Power is expressed as p.p.m. (parts per million) of  $P_2O_5$ .

In this method, carbonic acid, the only variable factor in controlling the Phosphate Supplying Power of a soil, is kept in excess. The results will, therefore, be proportional to the Phosphate Supplying Power of the soil under optimum conditions.

### **Scope of Work**

Phosphate supplying powers of some typical soils of the Bombay Province are reported here. The effects of some of the factors influencing this property and also that of addition of non-phosphatic substances to the soil have also been included.

### **Physical & Chemical Characteristics of Soils Studied**

A few typical soils of the Bombay Province were selected for this study. The samples were taken from the first 9" layer and after passing through 1 mm. sieve they were stored in closed tins. Particulars regarding the locality, crops grown, etc., are given in table 1.

TABLE No. 1

Particulars of the soils		
Locality	Crops grown	Average yield per acre in lbs.
Arbhavi	Sugarcane	8836
	Cotton	978
	Maize	2233
Belgaum	Paddy	1980
	<i>Watana</i>	389
Broach	Cotton	511
	<i>Jowar</i>	---
Dharwar	Groundnut	749
	<i>Jowar</i>	1410
	Cotton	517
Jalgaon	Cotton	587
	Groundnut	1929
Nadiad	<i>Bajri</i>	2000
Poona	Tobacco	1000

(dry leaf)

Table 2 below shows the important chemical constituents of the soils.

TABLE No. 2

Chemical analysis of the soil  
(Percentage of dry fine matter)

Soils	Sesqui oxides	Cal- cium Ca	Magne- sium Mg	Potas- sium K	Phospho- ric acid P <sub>2</sub> O <sub>5</sub>	Carbo- nates CaCO <sub>3</sub>	Humus
Arbhavi	8.40	3.32	0.20	0.80	0.09	7.88	2.48
Belgaum	26.80	0.49	0.28	0.71	0.22	Trace	2.58
Broach	10.30	0.77	0.32	0.60	0.09	0.17	1.56
Dharwar	11.80	0.81	0.36	0.64	0.06	0.19	6.35
Jalgaon	12.40	2.71	0.24	0.63	0.11	4.33	4.16
Nadiad	6.75	0.72	0.13	0.94	0.21	1.14	0.69
Poona	11.70	1.10	0.34	0.52	0.09	0.51	2.76



Mechanical analysis and other important physical properties of the soil samples are given in table 3.

TABLE No. 3

**Mechanical analysis of soils**

Soil	Coarse sand	Fine sand	Silt	Clay	Water holding capacity	Moisture
	%	%	%	%	%	%
Arbhavi	38.22	14.12	5.50	29.00	54.46	3.34
Belgaum	2.68	10.94	3.75	75.25	57.85	4.29
Broach	0.26	31.35	16.00	45 50	55.91	5.16
Dharwar	6.03	13.24	20.50	47.00	65.10	6.69
Jalgaon	0.21	29.11	22.50	32.50	76.07	7.19
Nadiad	2.38	76.95	5.75	11.75	40.63	1.34
Poona	3.51	38.18	21.50	27.75	75.53	5.79

From the results of mechanical analysis the soils may be divided into three groups. The first group comprises sandy soils from Nadiad and Arbhavi. These contain more than 50 per cent. sand (coarse and fine together). Soils from Poona and Jalgaon containing sand, silt and clay more or less in the same proportion, form the second group of medium soils, while soils from Belgaum, Broach, and Dharwar, forming the third group, are decidedly clayey in nature. Belgaum soil again contains a high percentage of sesquioxides in which iron oxide predominates. This gives it a distinct red colour. Percentage of sesquioxides, in the soils, amounts to about 10 per cent. Arbhavi and Jalgaon soils contain a comparatively higher percentage of calcium, most of which is in the form of carbonates. These soils may be taken as representatives of calcareous type. Phosphoric acid content of all the soils, varies from 0.06 to 0.1 per cent. ( $P_2O_5$ ) except those from Belgaum and Nadiad which contain 0.2 per cent. phosphoric acid.

As regards their general fertility nothing definitely can be said. In the first place weather conditions are different at the various places, and the crops grown are also different. If compared roughly, Nadiad soil is very fertile, followed by Arbhavi, and then by the rest, which are more or less of the same quality.

### Available Phosphates

Available phosphate contents of these soils were estimated by the method of extraction with one per cent. citric acid solution (Dyre's method). The extracted  $\text{PO}_4$  was determined colorimetrically, by reducing the phospho-molibdate compound with stannous-chloride solution (8). Results are recorded in table No. 4.

TABLE No. 4

### Available phosphate contents of the different soils

Soil	Available phosphates $\text{P}_2\text{O}_5$
	p.p.m.
Arbhavi	66.50
Belgaum	37.81
Broach	57.64
Dharwar	19.21
Jalgaon	67.72
Nadiad	80.31
Poona	28.11

Compared with the total phosphate contents of the soils (table No. 2) Nadiad soil, rich in total phosphates also stands first in the available phosphate content. This soil is very sandy in character. It contains only 11.75 per cent. clay. The sesquioxides are also very low (6.75 per cent.). The fixation of  $\text{PO}_4$  is, therefore, only slight.

Belgaum soil containing an equal quantity of total phosphates, possesses only half the quantity of available phosphates, indicating a high phosphate fixation. This is to be expected from the very clayey character of the soil and the high sesquioxide content.

Jalgaon, Arbhavi and Broach soils, though they contain a much less quantity of total  $\text{PO}_4$  compared with the Nadiad or Belgaum soils, possess almost the same quantity of available phosphates as the Nadiad soil. Arbhavi soil which is poor in clay and sesquioxides, is not expected to show much  $\text{PO}_4$  fixation. But Jalgaon and Broach soils, containing a pretty high percentage of clay and sesquioxides, still show a fairly high content of available phosphates. Poona soil, though containing the same quantity of total  $\text{PO}_4$  and much less clay shows a very high  $\text{PO}_4$  fixation resulting in a low available  $\text{PO}_4$  content. Dharwar soil, is poor in total and so also in available  $\text{PO}_4$  content. Arbhavi and Jalgaon soils are calcareous in nature, but the calcium carbonate appears to have no significant effect on their available  $\text{PO}_4$  content. The percentage of the total phosphates that are in the available form are shown in table No. 5.

TABLE No. 5

**Percentage of total  $\text{PO}_4$  in the available form**

Soil	Available phosphates as per cent. of total $\text{P}_2\text{O}_5$	In the soils from Arbhavi, Broach, and Jalgaon about 6 to 7 per cent. of the total $\text{PO}_4$ is in the available form. Arbhavi is a sandy soil, Jalgaon is of medium type, while Broach is clayey in character.
Arbhavi	7.4	
Belgaum	1.7	
Broach	6.4	
Dharwar	3.2	
Jalgaon	6.2	
Nadiad	3.8	
Poona	3.1	

Again Arbhavi, Jalgaon both contain appreciable quantity of  $\text{CaCO}_3$ , while Broach soil is almost free of that. Similarly, soils from Dharwar, Nadiad and Poona which represent the above three types again contain almost equal per cent. of total  $\text{PO}_4$  in the available form. Sesquioxide content of all these six soils is almost the same.

This is in agreement with the finding of Murphy (9) who has shown that, citrate ion replaces  $\text{PO}_4$  adsorbed on soil colloids. Similarly, citric acid dissolves  $\text{PO}_4$  in combination with sesquioxides, thus rendering both these ingredients, ineffective in  $\text{PO}_4$  fixation. Available  $\text{PO}_4$ , estimated by extraction with citric acid solution will naturally not bear any correlation with these soil factors.

It is also interesting to know whether the above figures of available  $\text{PO}_4$  represent the total quantity of available  $\text{PO}_4$  in the soils, and soils are completely deprived of their available  $\text{PO}_4$  after extraction with the citric acid solution. Walkoh (15) has found that when soils, which had been previously treated with phosphatic fertilizers, were extracted with 0.2N nitric acid, about 50 per cent. of the added phosphates were recorded at the first extraction, while the subsequent extractions failed to remove appreciable quantities of  $\text{PO}_4$ . This is verified by extracting once more, the same soil sample with one per cent. citric acid solution. The quantity dissolved out in the second extract was almost equal to the first. The same soil sample was, therefore, repeatedly extracted with the citric acid solution and  $\text{PO}_4$  dissolved out in each extract was determined separately. Seven such extractions were possible. Afterwards, it became very difficult to filter the soil suspension. The quantity of  $\text{PO}_4$  dissolved out in the seventh extract had also appreciably reduced. It had, however, not completely disappeared in the case of any of these soils. Results of these repeated extractions are given in table No. 6.

TABLE No. 6

## Repeated extractions of the different soils with 1 per cent.

## Citric acid solution

(expressed as  $P_2O_5$  p.p.m. of soils)

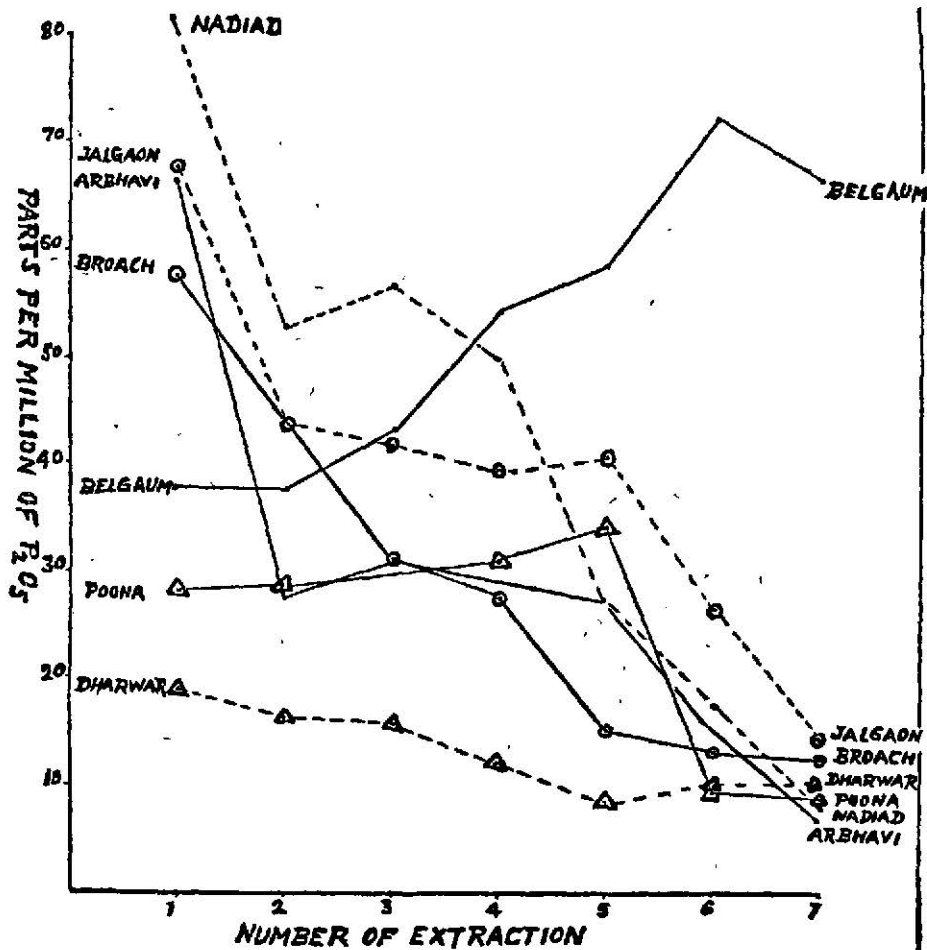
Soil	Number of extractions							Total quantity extracted
	1	2	3	4	5	6	7	
Arbhavi	66.50	27.80	30.91	29.83	27.13	15.11	7.14	204.42
Belgaum	37.81	37.63	43.31	54.30	58.80	72.18	66.77	370.80
Broach	57.64	30.97	31.00	27.73	15.31	13.40	13.00	189.05
Dharwar	19.21	16.63	16.01	12.32	8.93	10.97	11.03	95.00
Jalgaon	67.72	43.75	41.70	39.44	40.48	26.31	14.71	274.11
Nadiad	80.31	52.41	56.33	49.72	27.00	18.05	8.63	282.45
Poona	28.11	28.84	43.21	31.17	34.13	10.10	9.11	184.67

It is evident that, the results of the first extraction with the citric acid solution do not represent all the available (easily soluble)  $PO_4$  in the soils. Almost equal quantities of  $PO_4$ , in some cases even greater quantities, can be removed at subsequent extractions, and it may take a pretty large number of extractions to completely free the soils of the available phosphates.

The last column of Table 6 shows the total quantity of  $PO_4$  dissolved from each soil sample, in the seven extractions. This may be taken to mean the total quantity of easily soluble (available) phosphates in the soils, as the further addition to this quantity will be comparatively small, except in the Belgaum soil. The amount of  $PO_4$  removed from this soil is already much greater than any of the other soils. It is evident that available  $PO_4$  as shown by the first extraction is not in any way related to the total quantity of easily soluble phosphates present in the soils.

It becomes, therefore, apparent that the figures of available phosphates, as determined by extraction with one per cent. citric acid solution do not represent the total quantity of

(Read the following after the first para on page 468)



Repeated extractions of the soils with one per cent.  
Citric acid solution.

Results when plotted graphically show that the curves  
obtained for the Nadiad and Broach soils are of the same

type. The quantities of  $PO_4$  extracted are continuously decreasing and roughly they are inversely as the number of extractions. Soils from Arbhavi, Jalgaon show a sudden drop at the second extraction, similar to the first two, but later, almost equal quantities of  $PO_4$  are given out at the next four extractions and then the proportionate fall commences. The curves for the soils from Poona and Dharwar are also similar to the above curves except that the first drop at the second extraction is absent.

The first sudden drop at the second extraction may be due to the removal of all the easily soluble phosphates at the first extraction. Flattening of the curve at the subsequent extractions indicates the presence of sparingly soluble phosphates, which are removed at each extraction, in quantities sufficient to saturate the solution. The gradual fall in the end, appears to indicate an extraction of the adsorbed phosphates from soil colloids. At each stage an equilibrium seems to be reached between the solid and the liquid phases, and the quantities at equilibrium with the solid phase are removed. As the soil colloids get progressively unsaturated less and less quantities in the liquid phase get at equilibrium with the solid phase.

Behaviour of the Belgaum soils under the repeated extractions, is very peculiar. The quantity of  $PO_4$  dissolved out, rapidly increases from extraction to extraction, and only at the last extraction a slight fall is recorded. This soil contains a very high percentage of sesquioxides which may be responsible for this unique quality.

*(Continue on page 468 para two)*

the easily soluble phosphate present in the soil and is also not in any way related to it. It is representative of a rate at which  $\text{PO}_4$  is brought into solution from the soil under the conditions of extraction. This rate alters when  $\text{PO}_4$  is removed from the solution. The nature of these alterations differs from soil to soil and appears to be a characteristic of the soil.

This rate, however, cannot be taken to indicate the actual rate of  $\text{PO}_4$  supply to plants i. e. the phosphate supplying power of the soil. In the first place the acidity of the extractant was very high. Under normal condition of cultivation soil never shows such a high acid reaction. The phosphates which are soluble at this acidity will not be in solution in the same amount at the usual soil reaction. Secondly, the citrate ion is strongly adsorbed by soil colloids with a displacement of  $\text{PO}_4$  ion. A plant cannot do this unless it exudes citrate ions.

### Phosphate Supplying Power of the Soils

This was determined by the method outlined previously. Results are recorded in table No. 7.

TABLE No. 7

#### Phosphate supplying powers of soils

Soil	P.S.P. ( $\text{P}_2\text{O}_5$ ) (p.p.m.)	Available phosphate ( $\text{P}_2\text{O}_5$ )	Clay per cent.	Easily soluble phosphates ( $\text{P}_2\text{O}_5$ p.p.m.)
Arbhavi	8.81	66.50	29.00	38.70
Belgaum	1.42	37.81	75.25	0.18
Broach	2.46	57.64	45.50	26.67
Dharwar	Trace	19.21	47.00	2.58
Jalgaon	Trace	67.72	32.50	23.97
Nadiad	74.87	80.31	11.75	27.90
Poona	5.98	28.11	27.75	...



The figures of available phosphate contents, clay per cent. and the easily soluble phosphates are also included in the table for comparison. The figures in the last column are based on the inference drawn previously (pp. 468) and show difference between the first and the second extractions of the soils with 1 per cent. citric acid solution.

No correlation between the Phosphate Supplying Power of these soils and their available phosphate contents or the easily soluble  $PO_4$ , is traceable. It is, therefore, apparent that, the Phosphate Supplying Power of a soil is independent of the available phosphate content. When compared with the clay percentages of the different soils, an inverse correspondence is evident. Higher the clay per cent. lower is the Phosphate Supplying Power of the soil. Belgaum soil for instance contains 75.25 per cent. clay and has Phosphate Supplying Power. 1.42. On the other hand Nadiad soil, containing 11.75 per cent. clay, has Phosphate Supplying Power 74.87 units. Other soils also show a similar relation. However, since the nature of the clays, their degree of saturation, vary from soil to soil no quantitative relation between the clay per cent. and the Phosphate Supplying Power of the soils is to be expected. Soils from Dharwar and Jalgaon, though they contain much less clay than the Belgaum soil, still have only a negligible Phosphate Supplying Power.

The changes in the rate of extraction of  $PO_4$  from these soils, with 1 per cent. citric acid solution have been very characteristic of the soils. Changes in the Phosphate Supplying Power when dissolved phosphates are continuously removed from solution, have also been studied by repeatedly extracting the same soil sample with the carbonic acid solution. The results are tabulated in table No. 8.

TABLE No. 8

Repeated extraction with the carbonic acid solution  
(Expressed as  $P_2O_5$  p.p.m. of Soils)

Soil	Number of extractions						
	1	2	3	4	5	6	7
Arbhavi	8.81	8.33	7.39	9.89	9.81	10.68	11.50
Belgaum	1.42	1.31	1.41	0.70	0.72	Traces	
Broach	2.462	2.90	2.33	3.89	3.99	3.90	4.07
Dharwar	Traces		Traces			Traces	
Jalgaon	Traces		2.81	2.68	3.87	8.63	9.12
Nadiad	74.87	81.45	64.83	66.22	67.56	66.49	61.12
Poona	5.98	6.57	8.98	8.53	5.42	3.36	3.18

No very sudden changes occur in the P.S.P. (Phosphate Supplying Power) during seven repeated extractions, as happened in the case of citric acid extractions. In soils from Belgaum, Nadiad and Poona, P.S.P. slightly decreases at latter extractions while in soils from Arbhavi, Broach and Jalgaon it shows a gradual increase. The increase is very distinct in the case of Jalgaon soil only. This trend of changes appears to be no way related to the clay content of the soils. When compared with carbonate percentages in these soils (table No. 2) it appears that P. S. P. shows an increasing trend in soils containing calcium carbonate, while in soils, containing little or no calcium carbonate, the trend is decreasing.

Carbonic acid, besides dissolving insoluble phosphates such as those of calcium and magnesium also acts on calcium carbonate bringing it in solution as bicarbonate. This increases the Ca-ion concentration in solution, which has been shown to have a depressing effect on the solubility of phosphates (9,10,11). In the repeated extractions, the calcium carbonate is gradually leached out from the soils, and hence more and more  $PO_4$  is brought in solution. This may explain the increasing P.S.P. of Arbhavi and Jalgaon soils. Broach soil contains much less  $CaCO_3$ , but the percentage of clay is

higher, which seems to be the cause of the very gradual rise in the P.S.P. Nadiad and Poona soils also contain  $\text{CaCO}_3$  but it appears to be in a finer state of division and is leached out within the first or the second extraction. This results in a momentary rise in the P.S.P. and then the decline commences. The degree of saturation of the  $\text{PO}_4$  adsorbing complexes, also appears to influence the P.S.P. of the soil. Dharwar soil contains almost the same quantity of  $\text{CaCO}_3$  as the Broach soil, but its P.S.P. is almost nil at the first extraction and does not show any increase at the subsequent extraction similar to the Broach soil. This may indicate a very unsaturated state of the colloids with respect to the  $\text{PO}_4$  ion.

The gradual decline of the P. S. P. under the conditions of repeated extractions appears to suggest a partition of  $\text{PO}_4$  between the solid and the liquid phases as has been inferred in the case of similar extractions with one per cent. citric acid solution.

The depressing effect of  $\text{CaCO}_3$  on the P.S.P. is further confirmed by adding precipitated chalk to some of the soils and extracting them as above. The results are tabulated in table No. 9.

TABLE No. 9

**The depressing effect of the increasing quantities of  $\text{CaCO}_3$  on the P. S. P. of the Poona soil**

(Expressed as  $\text{P}_2\text{O}_5$  p.p.m. of the soil)

Calcium carbonate added per cent.	Number of extractions						
	1	2	3	4	5	6	7
1	2.84	2.84	8.14	8.63	5.45	4.19	3.27
3	Trace	1.39	1.93	2.82	4.94	5.87	5.56
5	Traces	Traces		0.73	2.11	3.56	3.23
Blank	5.98	6.57	8.97	8.53	5.42	3.36	3.18

Next Arbhavi soil which contains considerable quantity of  $\text{CaCO}_3$  (7.88%) and the Nadiad soil contains only 1.14 per cent.  $\text{CaCO}_3$  but with very high P. S. P. (74.87) are mixed with precipitated chalk and repeatedly extracted with the carbonic acid solution. Results are recorded in table No. 10.

TABLE No. 10

**Depressing effect of  $\text{CaCO}_3$  on the P. S. P. of the Nadiad and Arbhavi soils**

No. of extractions	Arbhavi Soil		Nadiad Soil	
	1% $\text{CaCO}_3$	0% $\text{CaCO}_3$	1% $\text{CaCO}_3$	0% $\text{CaCO}_3$
	$\text{P}_2\text{O}_5$ p.p.m. of the soils			
1	0.092	8.810	32.60	74.87
2	0.070	8.330	51.98	69.45
3	0.98	7.93	62.04	64.83
4	1.49	9.89	62.92	66.22
5	2.67	9.81	63.05	67.56
6	2.86	10.68	69.69	66.49
7	4.14	11.55	71.71	61.12

The effect of the particle size of calcium carbonate is very clearly brought out in the above experiments. The original P. S. P. of the Arbhavi soil, in presence of 7.88 per cent.  $\text{CaCO}_3$  was as high as 8.81 units but after addition of only 1 per cent. finely powdered  $\text{CaCO}_3$  it is reduced to a very insignificant figure.

The presence of calcium ion has also been found to depress the absorption of  $\text{PO}_4$  by plants (19), Cook (10) has also observed that when bentonite, which has been previously

leached with ammonium oxalate, is added to culture pots to adsorb Ca ion, the phosphate content of plants increased manifold. This effect of  $\text{CaCO}_3$ , which is not shown by the citric acid extractions, is clearly brought out by the study of the P. S. P. of the soils.

### Additions of non-phosphatic salts

Sodium nitrate and ammonium sulphate are used as nitrogenous manures. Addition of gypsum is often recommended to improve the calcium status of soils. Their effect on the P.S.P. of the Poona and Nadiad soils was studied, by adding 1 per cent. of these salts separately and extracting the soils with the carbonic acid solution. Results are tabulated in table No. 11.

TABLE No. 11.

### Effects of additions of non-phosphatic salts on the P. S. P. of the Poona and Nadiad soils

(Expressed as  $\text{P}_2\text{O}_5$  p.p.m. of the soils)

No. of extractions	Poona soils			Nadiad soil	
	1% $\text{NaNO}_3$	1% $(\text{NH}_4)_2\text{SO}_4$	1% $\text{CaSO}_4$	1% $\text{NaNO}_3$	1% $(\text{NH}_4)_2\text{SO}_4$
1	6.50	6.03	4.87	81.98	74.13
2	8.58	8.67	5.61	89.10	82.76
3	9.78	11.35	8.26	68.38	63.17
4	7.87	9.68	7.12	62.94	67.78
5	6.00	5.42	5.50	60.53	67.14
6	3.32	3.23	3.54	57.31	61.34
7	2.92	Traces	3.27	56.39	56.13

## Humus

Humus, the organic colloids of soils, is believed to play an important role, in making soil phosphates available to plants. It is usually attributed to the strong adsorbing properties possessed by the colloid. Peterson (12) has observed a considerable increase in soluble phosphates when organic matter in the soil was destroyed by hydrogen peroxide. Humus contents of the soils under investigations were estimated colorimetrically by the method described by Eden (13). Results are shown in table No. 2.

No correlation exists between the P.S.P. of the soils and their humus contents. Next, to confirm the results of Peterson (loc. cit.) humus from the Poona soil was oxidised with hydrogen peroxide. Latter contained large quantity of soluble phosphates. It was first removed by adsorptions on freshly precipitated aluminium hydroxide at pH 4.8. When the treated soil was next extracted with the carbonic acid solution, no phosphate was dissolved out by the extractor. Presence of humus thus appears to favour the solution of phosphates. This was further confirmed by adding different quantities of humic acid to the Poona soil, and measuring the P. S. P. by seven repeated extractions. The results are tabulated below.

TABLE No. 12

### Effect of humus on the phosphate supplying power of the Poona soil

(Expressed as  $P_2O_5$  p.p.m. of the soil)

Humic Acid added	No. of extractions.						
	1	2	3	4	5	6	7
2 per cent.	9.60	8.38	7.85	6.90	6.29	5.15	4.87
4 per cent.	11.52	9.04	6.61	6.31	5.90	5.87	4.85
Blank	5.98	6.57	8.97	8.53	5.42	3.36	8.18

The gradual rise of the P. S. P. of the Poona soil has been attributed to the washing down of Ca ion during the course of repeated extractions. When humus was added to the soil this tendency disappeared. The P. S. P. increased at the first extraction, and then declined continuously. This appears to suggest that the added humic acid has removed calcium ion from the solution, relieving its depressing effect, on the P. S. P. To verify this, humic acid was added to the Poona soil along with precipitated chalk, which alone has been found to depress the P. S. P. to a considerable extent (table 9). The quantities of  $PO_4$  obtained at the different extractions with the carbonic acid solution are shown in table No. 13.

TABLE No. 13

**Changes in the phosphate supplying power of the Poona soil mixed with humus (2 per cent.) and calcium carbonate (1 per cent.)**

(Expressed as  $P_2O_5$  p.-p. m. of the soil)

Treatment	No. of extractions						
	1	2	3	4	5	6	7
Soil + Humus 2% + Calcium Carbonate (1%)	4.38	4.87	7.22	6.90	6.30	5.19	5.41
Soil + Calcium Carbonate (1%)	2.84	2.84	8.14	8.63	5.45	4.19	3.27
Soil + Humus (2%)	9.60	8.38	7.85	6.90	6.26	5.15	4.87
Soil only	5.98	6.57	8.97	8.53	5.42	3.36	4.87

This effect of humic acid appears to be very similar to that of leached bentonite described previously.

## Conclusions

Available phosphate contents of the soils, as estimated by a single extraction with one per cent. citric acid solution do not bear any relation either, to total  $\text{PO}_4$  contents of the soils or to other soil characteristics such as clay per cent. etc. The figure of the available  $\text{PO}_4$  content, also does not indicate the total quantity of easily soluble phosphates, present in the soil, but it represents a rate of dissolving phosphates, from the soil, under the conditions of extraction. The nature of fluctuation in this rate, under repeated extractions, appears to be regulated by the nature and the amounts of phosphates present in the soil. A continuously decreasing rate may be indicative of extraction of adsorbed  $\text{PO}_4$  from soil colloids.

For reasons, discussed previously this rate may not be representative of the actual rate of supply of  $\text{PO}_4$  to growing plants. In addition, the presence of calcium ion which has been shown to have a depressing effect on the absorption of phosphates by growing plants, does not seem to influence this rate. Secondly, phosphates of iron and aluminium which are believed to be not readily available to plants are removed in appreciable quantities in the citric acid solution.

There is also not sufficient evidence to justify the use of such organic acids to determine the available  $\text{PO}_4$  content, or to assay the rate of its supply to plants. Parker et. al., (14,17) on the other hand conclude that carbonic acid is the only acid, exuded from plant-roots in any significant amounts. Carbon dioxide is also produced in soils by a variety of agencies, keeping soil atmosphere usually rich with the gas. Insoluble phosphates are also dissolved in carbonic acid producing phosphate ions (16). Even though adsorbed phosphates may be available to plants by contact reactions, phosphates in solution are much more readily absorbed by plants and must be considered as extremely available.



The rate of extraction with the carbonic acid solution shows an inverse proportion to the clay content of a soil. The carbonic acid also seems to extract adsorbed  $\text{PO}_4$  from the colloidal complex as evidenced by a gradually decreasing rate of extraction. This phosphate may thus be available to plants but its rate of supply would be controlled by the degree of saturation of the colloids.

Phosphate absorption by plants is also known to bear a similar type of relation with the soil colloids.

Carbonic acid extractions are considerably reduced by the presence of calcium carbonate. The extent of this reduction is proportional to state of division of the carbonate. This brings out very clearly the unavailability of soluble phosphates in calcareous soils. The repeated extraction of the soils sample again brings out the fact that if soluble salts, especially calcium ions are removed from the soil by plant absorption or otherwise, phosphates are supplied to the plants at a higher and higher rate from this type of soils. The favourable effect of addition of humic acid to such soils, appears to be limited to the removal of calcium ion from solution, which increases the  $\text{PO}_4$  solubility.

Thus some of the factors which are known to influence adversely or otherwise, the  $\text{PO}_4$  absorption by plants, also shows a similar effect on the rate of extraction of  $\text{PO}_4$  with the carbonic acid solution. This, to some extent, justifies the designation of this rate as the phosphate supplying power of the soil.

### Summary

(1) A single extraction of soil with one per cent. citric acid does not remove all easily soluble phosphates.

(2) The citric acid extractions show a rate of dissolving soil phosphates. Fluctuations in this rate of extractions are dependent on the nature and amount of the easily soluble phosphates and do not bear any any relation to other soil constituents.

(3) Rate of extraction of  $\text{PO}_4$  with the carbonic acid solution, that is the P.S.P. of the soils shows an inverse proportion to the clay content of the soils, and also to the percentage of calcium carbonate.

(4) When calcium carbonate is leached out in the repeated extractions, the P.S.P. of the soils gradually increases.

(5) Addition of calcium carbonate considerably lowers the P.S.P. of the soil. The amount of lowering depends on the degree of fineness of calcium carbonate.

(6) Addition of humic acid has a favourable effect on the P.S.P. of a soil.

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## PAPER XXXI

A STUDY OF NITRIFICATION OF RAW BONE  
MEAL, BONE GRIT, DRIED BLOOD, HORN  
SHAVINGS AND GROUNDNUT CAKE  
IN DIFFERENT SOILS OF BOMBAY  
PRESIDENCY

BY

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*(With the kind permission of the University of Bombay)*

## SECTION I

**Introduction**

Nitrogenous manures are important and their value is usually judged by the percentage of total nitrogen they contain but the real value of such a manure should be judged by the rate at which the nitrogen from that particular manure becomes available to the plant. The availability of various manures is a very important problem as all the plants depend upon available food materials for their nutrition. Nitrogen becomes available only when it is soluble in soil water and then it is taken up by the plants. Thus the real value of a nitrogenous organic manure depends more on its available nitrogen than on the amount of total nitrogen present.

According to Waksman (1927), nitrogen added as manure is not assimilated by higher plants as such but has to be first broken down into simpler compounds. This process is carried out in the soil through the agency of micro-organisms, the final product of hydrolysis being chiefly ammonia. The latter is either used up by the plant as such or is oxidised further to nitrates. Nitrates are either assimilated by plants or washed out in the drainage waters. Sahasrabudde and Gokhale (1933), remark that nitrogen is generally taken up by plants in the form of nitrates. The rate of nitrification may therefore be taken as a measure to determine the value of

nitrogenous manure. Plyman and Bal (1919) suggest that organic manures undergo physical, chemical and biological changes before they reach the plants and that the last of the changes is probably the most important.

According to Rubins and Bear (1942), "The nitrogen of fertilizer materials of animal or vegetable origin is, for the greater part, combined in complex proteinaceous compounds many of which are largely insoluble in water. If such nitrogen is to be converted into soluble forms that are utilised by plants, the parent materials must be subjected to agencies of decomposition in the soil." Thus in order to convert organic matter into humus and the nitrogenous material into nitrate, it is necessary that the manure should first be decomposed. Waksman (1924), explains how the decomposition takes place as follows, "After organic substance is added to the soil, it is acted upon by various groups of micro-organisms, including the fungi, actinomycetes, and bacteria and possibly also the protozoa. A part of the organic matter is completely decomposed with the formation of  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{S}$  etc. Another part is reassimilated by the organisms and synthesised into protoplasm; a part is left undecomposed being more resistant to the action of micro-organisms; a part is left in the form of intermediate products, due either to the greater resistance of these to the action of the micro-organisms in general and in certain specific groups in particular, or to the fact that products are formed hindering the further development of the organisms."

Thus during this process of changes by-products important to plant life are formed. Some of those by-products such as  $\text{NH}_3$ ,  $\text{CO}_2$  and  $\text{H}_2$  play a very important part in the nutrition of the plant through micro-organisms.  $\text{CO}_2$  is taken up by plant leaves to form carbohydrates by photo-synthesis, water acts as a solvent for so many important minerals which are thus absorbed by the root hairs of the plant and ammonia is either taken up as such or is further oxidised to nitrite and nitrate and absorbed by the same root hairs to supply the nitrogen in the synthesis of protoplasm. This oxidation of

$\text{NH}_3$  to nitrite and ultimately to nitrate by means of micro-organisms is commonly known as nitrification. This work of nitrification is done by special micro-organisms and it will be seen in this work through what stages it proceeds and to what extent nitrification takes place and how long it takes to nitrify such nitrogenous organic matters as raw bone meal, bone grit, dried blood, horn shavings and groundnut cake in different soils. As Waksman (1923), says, "Either the low grade nitrogenous materials should be used or a low concentration of high grade materials for the study of nitrification of organic materials in the soil", we have made use of a low concentration of high grade nitrogenous materials.

The chief aim is to study the different phases of nitrification and to find out which manure is suitable for which particular soil. So a study was made of the various changes in amounts of ammonia, nitrite, nitrate and total nitrogen in about five months, when the above mentioned manures are added separately in each of the three soils. The three soils chosen were the garden soil of Poona collected from the compound of Poona Water Works, the laterite soil from Belgaum and the sandy soil (alluvial) from Nadiabad.

Since the first inorganic decomposition product in any manure is ammonia, its changes were first observed and later on those of nitrites and nitrates. According to Waksman, (1923), the fact that ammonia is the final product of decomposition of nitrogenous organic materials was known as far back as 1893. He says, "The fact that soil organisms both bacteria and fungi, are active in the breaking down of various proteins with the formation of ammonia was pointed out in 1893 by Muntz, Condon and Marshal, and soon afterwards by other investigators". About how ammonia is formed he writes, "Ammonia is formed by the various groups of soil micro-organisms chiefly as a waste product in the utilization of proteins as a source of energy. It is also produced, of course, in the nitrogen metabolism of the organisms, in the hydrolysis of proteins and their degradation products by enzymes and in the autolysis of microbic cells, but to a comparatively less extent".

Next the studies of how the changes in amounts of nitrites and nitrates take place were taken during these five months of experiments, in these soils containing added nitrogen in the form of manures as shown above. This is the real study of nitrification. Gopal Rao (1934), says, "This oxidation of ammonia to nitrite and nitrate is technically known as "nitrification" and is one of the most important of soil chemical processes from the point of soil fertility". To show the importance of nitrification, Stephenson (1936), writes, "There is good evidence that nitrification is of more than passive significance in plant nutrition. Nitrates are absorbed and renewed in the soil many times throughout the growing season". He further adds, "with nitrification eliminated there are no adequate means for dissolving a sufficient quantity of the cations of the mineral soil and for getting sufficient quantities of cations into the plant."

Then a study was made as to how the total nitrogen was changed in the same pots of soil during these five months of experiments, to get some knowledge as to what is most responsible for the loss of total nitrogen from soil during this most important biological process known as nitrification.

The material used was finely powdered bone (raw bone meal) passed through 100 mesh sieve and bone grit of 60 mesh sieve to compare how the particles of the same material but different in size nitrify.

The pots were kept at room temperature and the water added was 50% of its maximum water holding capacity. As regards the temperature and moisture for nitrification A. M. Smith (1927), says "Nitrification takes place at all temperatures between 15° and 40°C. Optimum temperature is nearly 35°C. Seasonal variations in the rate of nitrification are largely due to temperature, moisture and other seasonal changes in soil". He further adds, "Harris and Butt found that soil moisture contents of 23 to 28% was optimum for nitrification. Coleman, Jacobs, Allison and Braham's work indicates that 50 to 60% of the water holding capacity is the optimum."

### **The methods employed to determine various constituents of the manures and the soils**

First the analysis of manures were taken up. Each was analysed for its total nitrogen and total phosphoric acid contents. Duplicate samples were taken for this analysis.

The percentage of total nitrogen was determined by Kjeldahl method given in "Methods of Analysis A. O. A. C." p. 21 (1930).

The phosphoric acid determinations were done from the ash of the manures by the volumetric method of Pemberton given in "Volumetric Analysis" by Sutton, p. 134, 2nd method (1925), and "Methods of Analysis A. O. A. C." (1930), pp. 15-16.

### **Mechanical Analysis of Soils**

For the mechanical analysis of soils; "Soil Analysis" by C. H. Wright, p. 33, (1934), was followed throughout the analysis. For dispersion the Agricultural Education Association's method was used.

### **Chemical Analysis of the Acid Extract of Soil**

The soil was extracted with HCl by the method given in "Soil Analysis" by C. H. Wright, p. 170, (1934).

The mixed oxides of iron, aluminium, titanium and phosphorus were determined by the method given in "Soil Analysis" by C. H. Wright, p. 170, (1934).

Calcium, magnesium, potassium and phosphoric acid were determined by the methods given in "Soil Analysis" pp. 76-77 method (iii), p. 79, p. 171 and pp. 102-103 respectively.

### **The methods employed to determine the changes brought about every fortnight in soils as regards their Ammonia, Nitrites, Nitrates and Total Nitrogen Contents**

As all the determinations were calculated on overdry matter it was found necessary to determine the moisture



contents of the soil samples as well as with the other determinations.

As the moisture contents of soil sample had to be kept constant it was first necessary to determine how much and how many times per fortnight water was to be added. For the former the maximum water holding capacity of each soil sample was determined.

Ammonia was determined by MacClean and Robinson's method given in "Soil Analysis" by C. H. Wright, (1934).

For nitrite Griess-Ilosva colofimeter method given in Treadwell and Hall, "Analytical Chemistry", Vol. ii, pp. 306-307 (1928) was used.

Devarda's alloy was used to determine nitrate and the usual Kjeldahl method for determination of total nitrogen was made use of.

To determine organic carbon, wet combustion method was employed. There the  $\text{CO}_2$  evolved was allowed to be absorbed by KOH in potash bulb and the increase in its weight gave the weight of  $\text{CO}_2$ . From the weight of  $\text{CO}_2$  percentage of carbon was calculated.

### **Results of Mechanical and Chemical Analysis of the Soil**

According to the methods given above the different proportions of coarse sand, clay, silt, fine sand and sesquioxide and silica were estimated mechanically in the soil; as well as moisture, carbonates and organic matter. Here carbonates were calculated from the figures of CaO obtained in chemical analysis and organic matter was calculated from the figures of organic carbon determined by the wet combustion method as described before. The figure for carbon was multiplied by Van Bemmelen factor 1.724 to give organic matter as given by Leighty and Shorey, (1930).

## Mechanical analysis

	Garden soil of Poona	Laterite soil of Belgaum	Sandy alluvial soil of Nadiad
Moisture ...	5.460 %	4.200 %	0.960 %
Carbonates (CaCO <sub>3</sub> ) ...	8.000 ,,	0.312 ,,	0.750 ,,
Sesquioxide and silica	4.110 ,,	0.370 ,,	0.810 ,,
Organic matter ...	3.641 ,,	2.638 ,,	1.514 ,,
Coarse sand ...	15.320 ,,	8.090 ,,	1.645 ,,
Clay ...	16.650 ,,	30.860 ,,	10.705 ,,
Silt ...	10.420 ,,	23.670 ,,	1.845 ,,
Fine sand ...	37.040 ,,	20.885 ,,	82.245 ,,

## Maximum water holding capacity

	69.15 %	61.47 %	44.06 %
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## Chemical analysis

Fe <sub>2</sub> O <sub>3</sub> , Al <sub>2</sub> O <sub>3</sub> etc. ...	15.138 %	35.500 %	5.630 %
CaO ...	4.460 ,,	0.175 ,,	0.420 ,,
MgO ...	1.230 ,,	0.310 ,,	0.620 ,,
K <sub>2</sub> O ...	0.214 ,,	0.268 ,,	0.179 ,,
P <sub>2</sub> O <sub>5</sub> ...	0.085 ,,	0.139 ,,	0.078 ,,
N <sub>2</sub> ...	0.2156 ,,	0.147 ,,	0.078 ,,

## SECTION II

### A Study of Nitrification in the Garden Soil of Poona

The Poona garden soil was first taken for experiments, after passing through 2 mm. sieve. Five samples of 2000 grams of soil were put separately in five different earthen pots. Now finely powdered and weighed raw bone meal, bone grit (which was not powdered very fine), dried blood and very small thin scrapings of horn were sterilized at 15 lbs. pressure for one hour. Care was taken that no moisture could come in contact with the organic matter, for which they were put separately in air tight wide-mouthed glass bottles having glass stoppers. These organic materials were weighed in such a way that each pot would receive approximately 0.10% of nitrogen with the addition of each manure to different pots. They were separately examined for their total nitrogen and then weighed in such a manner that the weight of total nitrogen in each sample was equal to 2 grams. Table No. 1 gives the amount of total nitrogen present in different organic manures.

TABLE No. 1

	Raw bone meal.	Bone grit.	Dried blood.	Horn shavings.
Total nitrogen ...	4.00%	4.29%	12.47%	13.00%

Therefore 50 grams of raw bone meal, 46.62 grams of powdered bone grit, 16.04 grams of dried blood and 15.38 grams of horn shavings in separate glass bottles were taken and sterilized as stated above.

The 50 grams of raw bone meal was first mixed in 2000 grams of soil thoroughly and its moisture, ammonia, nitrite, nitrate and total nitrogen were determined on the first day.

For each determination duplicate samples were taken. These results were noted down as determinations at start. Now water was added to the extent of 50 per cent. of its maximum water holding capacity and kept for further determinations of its nitrogen changes after 14 days. Thus each sample was analysed after every fortnight.

The experiment was so arranged as to get the turn of analysis of each pot every alternate day. Thus pot No. 1 was taken for analysis on Monday, pot No. 2 on Wednesday, pot No. 3 on Friday, pot No. 4 on next Monday and pot No. 5 on the next Wednesday. To each was added the manure on the day of first analysis except in control which was without any manure. The experiments were carried on for nearly five months i.e. 154 days, as it was found that the time taken for nitrifying these organic substances was very long.

The figures in table No. 2 give an idea as to how the ammonia changes every fortnight.

TABLE No. 2

Mgs. of ammonia (NH<sub>3</sub>) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings.	Control
At start	0.994	0.876	1.066	0.840	0.875
14 days	1.066	1.064	1.105	1.008	0.920
28 days	1.156	1.113	1.126	1.048	0.980
42 days	1.245	1.152	1.352	1.050	1.006
56 days	1.257	1.240	1.371	1.081	1.346 <sup>v</sup>
70 days	1.442	1.375	1.423	1.116	1.440
84 days	1.473	1.518	1.451	1.394	1.488

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Control
98 days	1.581	1.523	1.810	1.407	1.586
112 days	1.625	1.575	2.013	1.527	1.664
126 days	1.613	1.657	2.223	1.480	1.664
140 days	1.562	2.130	1.596	1.580	1.480
154 days	1.168	1.386	1.089	1.380	0.505

From the above figures of ammonia in the garden soil of Poona containing about 0.10 % of nitrogen in the form of raw bone meal, bone grit, dried blood and horn shavings it could be seen that there is very little ammonification as compared to the control soil i.e. the soil receiving no form of manure. There is gradual increase of ammonia in all the five pots of soils; pots containing raw bone meal, dried blood and control show maximum figures on the 126th day and later on there is a marked decline in the figures; this indicates that there were set up certain forces which retard further ammonification or the organic matter is no longer present, all being used up in nitrification. Bone grit and horn shavings show maximum ammonia on the 140th day indicating it takes a longer time to ammonify bigger particles.

These figures of ammonia indicating low ammonia in the soil of all the pots lead us to believe that ammonia is not accumulated in the soil and whatever organic nitrogen was ammonified is at once oxidised to nitrite and nitrate or is lost in the atmosphere. Further Puri, Rai and Kapur, (1946), suggest that the rate of nitrification is considered to be greater than that of ammonia production. Ammonia, therefore, does not tend to accumulate, as it is converted into nitrite and nitrate as soon as it is produced from organic matter.

Now the study of the figures of nitrite in the soil of different pots will be taken up separately.

TABLE No. 3

Mgs. of nitrite ( $N_2O_3$ ) per 100 grams of oven dry soil

Time.	Raw bone meal	Bone grit	Dried blood	Horn shavings	Control
At Start ...	0.0100	0.0180	0.0148	0.0129	0.0105
14 days ...	0.0149	0.0140	0.0170	0.0157	0.0119
28 days ...	0.0192	0.0145	0.0200	0.0253	0.0185
42 days ...	0.0201	0.0238	0.0248	0.0362	0.0154
56 days ...	0.0204	0.0333	0.0264	0.0408	0.0157
70 days ...	0.0281	0.0434	0.0274	0.0412	0.0200
84 days ...	0.0300	0.0477	0.0318	0.0421	0.0207
98 days ...	0.0348	0.0506	0.0411	0.0424	0.0217
112 days ...	0.0394	0.0518	0.0428	0.0514	0.0268
126 days ...	0.0405	0.0538	0.0490	0.0551	0.0274
140 days ...	0.0444	0.0817	0.0500	0.0810	0.0320
154 days ...	0.0462	0.0900	0.0700	0.0870	0.0326

From table No. 3 it will be seen that all the four pots containing added manure show higher amounts of nitrite than the control, which indicates that the organic manures themselves were decomposed first to ammonia then to nitrite and later on to nitrate. Bone grit shows 0.0900 mg. of nitrite in 100 grams of oven dry soil on the 154th day whilst raw bone meal shows only 0.0462 mgs. This discrepancy may be due to varying sizes of bone pieces. Raw bone meal being a very finely powdered form must be getting oxidised almost immediately to nitrate thus keeping the amount of nitrite low. Raw bone meal being in finer form than bone grit has a greater

chance of coming in contact with oxidising agents. Dry blood and horn shavings also show nitrites as high as 0.0709 mg. and 0.0870 mg. respectively on the 154th day. As horn shavings could not be powdered to very minute particles, here again the oxidation seems to be slow and similar to that of bone grit. Here nitrite is accumulated to some extent and then oxidised to nitrate. On the whole we can safely say that the organic matter is first converted to ammonia by certain micro-organisms, this ammonia is further oxidised by certain oxidising micro-organisms to nitrite and at once again further oxidised to nitrate, i.e. the state of nitrite is very unstable as can be seen from the figures in the table No. 3, where it is shown that the highest figure of nitrite is never higher than 0.090 mgs. per 100 grams of oven dry soil. So there seems to be no chance of accumulation.

Now from the figures of nitrate in the table No. 5 it could be seen that nitrate is allowed to accumulate indicating that it is the final product of nitrification and both the oxidising micro-organisms must be living together. In this connection Dr. G. J. Fowler (1934) says, "These two organisms the nitric and nitrous, work together in nature and neither can do its work without the help of the other; the nitric organism is incapable of directly oxidising ammonia and the nitrous organism cannot carry out oxidation of ammonia further than the stage of nitrite". He further proves by pointing out the figures as a result of the experiments carried out by Dr. W. E. Adeney, where the nitrate forming bacteria are destroyed or rendered inactive. In table No. 4 is shown the results of spontaneous oxidation of sewage and comparative mixtures of sewage and fresh water and sewage and sea water respectively.

TABLE No. 4  
Parts per 100,000

At commencement	Sewage	Sea water mixture	Fresh water mixture
Nitrogen as ammonia ...	0.825	0.165	0.165
Nitrogen as nitrites ...	0.0	0.0	0.0
Nitrogen as nitrates ...	0.0	0.01	0.01
Organic nitrogen ...	0.675	0.135	0.135

## At conclusion

Nitrogen as ammonia	...	0.02	0.0	0.0
Nitrogen as nitrites	...	0.0	0.14	0.0
Nitrogen as nitrates	...	0.5	0.0	0.142
Organic nitrogen	...	0.5	0.072	0.076

Thus Dr. Adeney showed from the above figures that the ultimate product of oxidation of nitrogen was nitrite and not nitrate in sea water which seems to inhibit the action of nitrate forming bacteria.

Also the same fact gets further proof through Gopal Rao and Dhar, (1931), who say in their article in Soil Science, "The biological character of nitrification was first indicated by Muller and by Soyka. From the painstaking researches of Warrington, Frankland and Winogradsky, we now know that this nitrification is really due to the joint action of two organisms, one of which converts ammonium salts to nitrites and the other brings about the oxidation of nitrites to nitrates but has no effect on ammonium salts".

## Study of Nitrate Changes

Nitrate changes in the soils after every fortnight will be next discussed. Below is given the table showing change of nitrate in different pots at different times.

TABLE No. 5

Mgs. of nitrate ( $N_2O_5$ ) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Control
At start	2.659	2.758	2.731	2.557	2.544
14 days	2.683	3.501	3.180	4.691	2.571



Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Control
28 days ...	3.795	4.261	3.988	4.894	3.021
42 days ...	4.050	4.650	4.130	5.116	3.035
56 days ...	5.666	4.791	4.233	6.610	3.052
70 days ...	7.307	6.105	6.401	6.818	3.154
84 days ...	7.707	6.435	6.411	8.788	3.180
98 days ...	7.950	7.560	6.762	8.815	3.433
112 days ...	9.270	8.466	7.281	8.971	3.544
126 days ...	8.630	8.653	8.450	9.116	3.795
140 days ...	8.550	9.747	7.080	10.116	2.035
154 days ...	3.598	9.912	7.428	10.397	0.592

From the above table it will be seen that there is continuous increase of nitrate in all the samples up to the 112th day, there is slight decrease of nitrate in raw bone meal sample from the 126th day onwards, while dried blood shows maximum nitrate on the 126th day and all others go on increasing and the maximum amount of nitrate is shown on the 154th day. In other words nitrification of raw bone meal and dried blood stopped at about the 112th day and the 126th day respectively, after they were mixed with soil, while of bone grit and horn shavings continued even up to the 154th day. Control shows maximum nitrate on the 126th day. It could be seen that it takes about 112 days and 128 days to completely nitrify raw bone meal and dried blood respectively, yet it takes perhaps more than 154 days to completely nitrify bone grit and horn shavings or in other words nitrate is retained for a longer time in the last two samples.

The percentages of maximum nitrates formed is 11.59, 12.39, 12.25 and 12.23 in raw bone meal, bone grit, dried

blood and horn shavings respectively. Here raw bone meal shows the least amount of nitrate formation while bone grit shows the maximum. As the raw bone meal powder was very fine it nitrified quickly and lost to the soil also quickly as could be seen from the figures on the 154th day, where it is only 3.598 mgs. per 100 grams of oven dry soil. Bone grit decomposes slowly but retains whatever nitrate is formed for a longer time than raw bone meal. This is advantageous to the soil. Dried blood also forms nitrates gradually and gives out also gradually, while horn shavings show good results. Gradually the nitrates increase here and are retained for a longer time. Even control shows slight increment in its nitrates content up to the 126th day and then loses it and on the 154th day it is lost to the extent of about 5/6 of the maximum. Thus the soil itself shows the tendency of losing nitrates after the 126th day. So it is seen that nitrate never remains constant in soil but as soon as it is formed it is changed into some other form or is quite likely to be lost by solution (or is lost in the atmosphere in the form of nitrogen) or is changed to organic nitrogen by bacteria.

In the beginning the loss of nitrate is slow or the nitrification is fast, so actually we see nitrate accumulating but later on when nitrification slows down perhaps due to the fact that the percentage of organic matter was reduced to some small fraction, so there was no further scope for them to find more nitrogen to nitrify, there is decrease in total nitrates. Here nitrates may be decomposed and form  $\text{NO}_2$  and thus be lost to the soil or by addition of water may be going at the bottom of the pot and thus going out with some water in soluble forms or taken by small organisms to form their own body.

While calculating on the basis of manures we have added, it is seen that the amounts of raw bone meal, bone grit, dried blood and horn shavings nitrified are 7.16%, 10.50%, 6.74%, and 11.54% respectively, thus showing the greatest amount of nitrification in horn shavings; second comes bone grit; third raw bone meal and last dried blood. Now raw bone meal and dried blood were in very fine powdered forms which indicates that when manure is in very fine particles its end

nitrifying product is soon lost to the soil, while bigger particles are able to retain the end product for a longer time.

### A Study of Total Nitrogen Changes

The changes that take place in total nitrogen during the period of experiments will be next discussed.

TABLE No. 6

**Mgs. of total nitrogen (N<sub>2</sub>) per 100 grams of oven dry soil**

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Control
At Start	296	297	285	301	215.6
14 days	294	296	282	294	215
28 days	282	293	279	287	215
42 days	273	282	276	240	215
56 days	271	265	251	221	215
70 days	265	264	229	212	205
84 days	262	263	227	206	205.2
98 days	250	263	225	204	202
112 days	241	216	224.5	201	201
126 days	235	244	219	199	193.5
140 days	234	241	218	199	190.7
154 days	229	239	217	194	186.5
Loss of total nitrogen	67	58	68	107	29.1

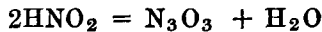
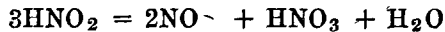
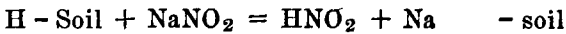
Table No. 6 shows how the total nitrogen changes in all the five pots of soil during the experiments. From the figures of total nitrogen at start it is seen that the amount of total nitrogen added was not constant in all the samples. Table No. 7 shows how much of it was actually added in the form of raw bone meal, bone grit, dried blood and horn shavings and how much per cent. of it is lost after 154 days, as well as how much per cent. of the total nitrogen in soil itself (including the manural nitrogen) is lost in 154 days.

TABLE No. 7

Pots containing	Raw bone meal	Bone grit	Dried blood	Horn shavings	Control
Total nitrogen added ...	0.080%	0.081%	0.069%	0.085%	—
Total nitrogen lost from the organic matter added ...	47.38%	35.68%	56.38%	91.65%	—
Total nitrogen lost from the soil (including manural nitrogen) ...	22.64%	19.53%	23.86%	35.55%	13.49%

Thus it could be seen that horn shavings are almost completely decomposed in 154 days. Next in succession comes dried blood, raw bone meal and bone grit. Bone grit is least decomposed proving that the bigger and hard particles take more times to decompose while acted on by micro-organisms. All these losses of nitrogen must be due to loss through unstable nitrogen compounds like ammonia and nitrite, which are constantly changing by oxidation, while nitrate being a more stable compound is not lost so easily which is also shown by the table No. 5 where we can actually

see the accumulation of nitrate, while table No. 2 and 3 showing the figures of ammonia and nitrite respectively, show practically no accumulation. These unstable natures must be the cause of loss of total nitrogen in soil while in the process of nitrification. Of course, little nitrogen may be lost due to the decomposition of nitrates and loss through solution, but it may be very little. Ammonia being a light gas may escape into the atmosphere by itself and thus be lost to the soil, while nitrites may be lost due to its further oxidation to oxides like NO and  $N_2O_3$  as is shown by Fraps and Sterges, (1932), giving the equation of Turtshchin as



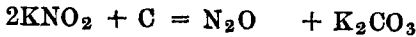
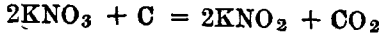
**Conclusion:**—From the above facts we can say that there is always some nitrogen present in soil which tries to remain more or less constant, that is it could not be further lost in any form if natural conditions are maintained. It may be in different forms but still it will be there.

Secondly it may be noted that ammonia shows very unstable nature in this particular soil. It was at once changed to some other form of nitrogen thus keeping the figures of ammonia very low.

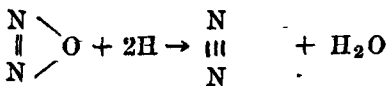
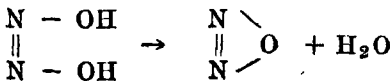
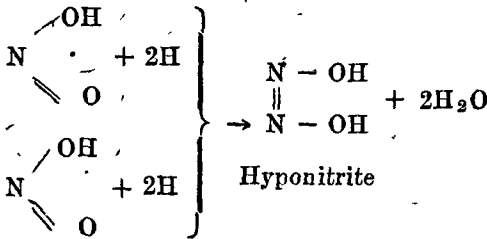
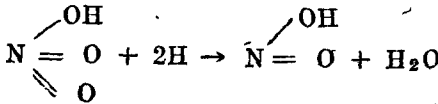
Thirdly, even nitrites were unstable and as soon as they were formed, they were changed to some other product of nitrogen giving no time for accumulation, indicating that there must be two types of activities, one in which ammonia was changed to nitrite and the other in which nitrite was changed to nitrate showing the presence of two types of micro-organisms working simultaneously. The experiments of Dr. Adeney also support this view.

Fourthly, it is observed that nitrate is also changing further to some other form of compound, some of which is lost to the soil. According to Waksman (1932) the loss of nitrogen

through nitrate may be due to presence of a carbohydrate, the carbon of which is used up to reduce the nitrogen thus :-



$2\text{N}_2\text{O} + \text{C} = 2\text{N}_2 + \text{CO}_2$  or by the reduction due to hydrogen from the carbohydrates as :-



We have seen in this soil that when nitrate formation is faster than nitrate decomposition or nitrate loss due to its solubility in water, it accumulates; but as soon as nitrification slows down it is lost gradually to the soil. Only two assumptions are there that the loss might be due to water or some further oxidation of nitrate as shown above.

In the end we can say that some of the nitrogen is lost as free ammonia, the remaining changes to nitrite, some of which on further oxidation is lost as  $\text{N}_2\text{O}_3$  in the atmosphere, as we have seen nitrites are unstable compounds. Thus most of the

nitrogen is lost after nitrite formation. Rane and Varma in their book, "Inorganic Chemistry", (1935), say while giving the properties of nitrous acid as follows, "Nitrous acid is unstable and decomposes even in dilute solutions at ordinary temperature thus :  $3\text{HNO}_2 = \text{HNO}_3 + 2\text{NO} + \text{H}_2\text{O}$ ". We can see from the above equation that nitrous acid formed is at once changed to nitric oxide which is lost to the soil being a gas and very slightly soluble in water.

Nitrates are lost mostly through water when excess water filters out of the pots, while some may be used up in oxidising other organic matter, itself being reduced to free nitrogen as shown in the equation by Waksman:

### SECTION III

#### The Study of Nitrification in the Laterite Soil

Here sampling and mixing was done as in the case of the Garden Soil of Poona.

#### The Study of Changes in Ammonia at Different Intervals

As before the changes of ammonia in different pots at different intervals will be first taken up. The table No. 8 gives us some idea as to how ammonia changes in all the six pots at different intervals of times.

TABLE No. 8

Mgs. of ammonia ( $\text{NH}_3$ ) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
At start	7.350	7.125	7.830	10.460	7.301	7.030
14 days	9.000	8.470	15.938	12.360	14.142	7.394
28 days	9.348	10.114	26.444	26.229	23.272	8.750

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
42 days	10.111	11.690	27.520	37.056	35.868	8.955
70 days	14.540	13.073	44.800	40.018	57.920	10.472
98 days	6.404	4.488	12.540	42.192	8.918	5.895
126 days	3.690	1.639	10.450	9.127	4.424	4.000
154 days	1.850	1.523	1.524	1.499	1.033	1.800

From the table No. 8 it could be seen that in this particular soil ammonification is quite marked and figures of ammonia are high showing that nitrous bacteria are not very active here, thus allowing some ammonia to be accumulated before it is changed to nitrite. Further we see that raw bone meal and bone grit are ammonified to a very small extent when compared with the ammonification of the other manures. The table No. 9 gives us a fairly good idea of the ammonification of different manures in the laterite soil.

TABLE No. 9

	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
	%	%	%	%	%	%
Manural nitrogen ammonified	6.30	2.89	37.07	40.33	52.72	...
Total nitrogen ammonified	6.86	5.51	18.82	17.80	24.34	7.12



Ammonification is highest in ground-nut cake while second highest comes horn shavings, then dried blood. Raw bone meal and lastly bone grit where the total nitrogen is least ammonified as shown in the table. Now from the table No. 8 it is seen that the maximum ammonia is developed in 70 days in all the samples except horn shavings where the maximum is on 98th day showing that in the soil horn shavings some time to reach the maximum. The other very obvious fact is that the ground-nut cake, dried blood and horn shavings are decomposed very readily and all these three manures show very little of phosphate as compared with raw bone meal and bone grit. This is seen in the table No. 10 where percentages of phosphates for all are given.

TABLE No. 10.

**Percentages of phosphates ( $P_2O_5$ ) in different manures**

Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake
23.03	25.96	1.01	0.09	1.24

Thus it is seen that the manure containing more of phosphorus is ammonified with difficulty and to a small extent, which indicates that phosphorus with nitrogen must be forming a very inactive, stable and hard compound, not easily acted upon by the nitrifying organism. Waksman (1932) says that phosphorus compounds greatly accelerate activities of nitrogen fixing organisms since they play an important part in the metabolism of *Azotobacter*, which requires large quantities of the mineral for the synthesis of its cells. From our figures also we conclude that phosphorus does not help in breaking up of nitrogen but must be fixing it, as where there is more phosphorus there is little ammonification as observed through our experiments.

### The Study of Changes in Nitrite and Nitrate at Different Intervals

In laterite soil there was no accumulation of nitrites to any extent, and throughout the experiments only a trace was found. Thus here nitrate forming organisms seem to be more active than in the previous soil, where nitrites were seen accumulating to some extent. So it is found, here also, that the nitrite state is very unstable and in acidic soil like the laterite it is the most unstable, which goes to prove that in alkaline soils nitrous acid must be neutralising the alkali and forming a salt; thus making the nitrite a little more stable. In acidic soil nitrous acid must be at once oxidised to nitric acid and some nitric oxides (NO or  $N_2O_3$ ).

Now the nitrate changes brought about in the soil in 154 days will be discussed. Below is given table No. 11 showing the nitrate changes at different intervals.

TABLE No. 11

Mgs. of nitrate ( $N_2O_5$ ) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Groundnut cake	Control.
At start	4.095	4.125	5.502	1.777	4.068	1.653
14 days	4.305	5.779	6.208	1.841	15.789	1.707
28 days	4.765	6.943	8.374	2.142	17.137	1.756
42 days	5.042	7.141	9.503	3.575	21.243	1.812
70 days	5.354	9.750	13.680	4.997	31.053	2.705
98 days	7.420	9.928	9.123	6.773	5.728	1.875
126 days	7.151	8.715	6.872	4.775	3.565	1.653
154 days	6.825	7.518	1.717	3.872	3.651	1.800

The highest amount of nitrification as seen from the above table takes place in about 98 days in raw bone meal, bone grit, and horn shavings, while dried blood and control show it in about 70 days.

From the same table we observe a very strange fact, that while compared with other manures, horn shavings show the least amount of nitrate formation i. e. up to the extent of 5.44% only, while the same manure shows much higher percentage of ammonification viz. 40.33%, which means that ammonia does not change quickly to nitrate but is allowed to remain as ammonia, thus showing always more of ammonia and less of nitrate. It may be true that in soil both the reactions nitrification and denitrification or oxidation and reduction, may be occurring. Joshi (1919), when working on green manures says that true denitrification sets in simultaneously with nitrate formation. When the process of oxidation is faster we see more of its products, i.e. nitrate; if it is not so active we see more of ammonia, so perhaps here the activity of denitrification is more, thus giving less of nitrate and more of ammonia according to P. H. De and S. N. Sarkar (1936), "In the presence of added organic materials, whether rich or poor in nitrogen, nitrate disappears very quickly." Table No. 11A shows the maximum nitrate formed in different manures.

TABLE No. 11A

**Percentages of nitrified ( $N_2O_5$ ) total nitrogen added as manure.**

Raw bone meal	Bone grit	Dried blood	Horn shavings	Groundnut cake.
8.57	8.95	12.06	5.44	31.15

The ammonia formed is used up regularly through a channel giving out nitrate, this reaction allows regular decomposition of ground-nut cake; but in horn shavings these regular changes do not seem to take place and ammonia is

only accumulated, thus retarding further progress. It is not exactly understood why this reaction does not continue to proceed further and thus use up the whole amount of horn shavings in the process. Perhaps much accumulation of ammonia might have made nitrite forming organisms inactive as also there was no carbonaceous matter present there, to provide the energy requirements of those organisms. Niklewski (1934), states that there is no loss of nitrogen if an ample supply of ammoniacal nitrogen is present as urine, so that the process of nitrification is checked, which indicates that ample ammonia checks nitrification when allowed to accumulate. Raw bone meal and bone grit show a little more nitrate formation than horn shavings but the nitrogen loss is nearly 50% in raw bone meal while bone grit and horn shavings show only 21 to 22%. Dried blood shows higher figures of nitrate than all the above three mentioned manures and highest total nitrogen loss.

**The Study of total nitrogen loss during the period of experiments**

TABLE No. 12

**Mgs. of total nitrogen per 100 grams of oven dry soil**

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
At start	211.7	237	238	237	238	147
14 days	207.6	236	235	236	237	146
28 days	201.6	234	230	235	232	145
42 days	195	228	220	235	226	144
70 days	194	222	209	234	209	144
93 days	194.3	216	204	232	199	144
126 days	194	211	178	231	178	142
154 days	174	213	173	212	173	142
Loss of total nitrogen	37.7	24	65	25	65	5

From the table No. 12 it is seen how the total nitrogen changes during the experiments. From the figures of total nitrogen at start it could be seen that the amount of nitrogen added was not constant. Table No. 13 shows how much nitrogen had been added as well as percentage lost from manure and from the soil including the added nitrogen.

TABLE No. 13

Pots containing	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
	%	%	%	%	%	%
Total nitrogen added ...	0.0647	0.090	0.091	0.090	0.091	—
Total nitrogen lost from the organic manure added ...	49.45	21.11	66.00	22.22	66.00	—
Total nitrogen lost from the soil (including organic manure) ...	17.80	10.13	27.31	10.55	27.31	3.42

Thus it could be seen here that dried blood and ground-nut cake were lost to a large extent, then next came raw bone meal, horn shavings, and lastly bone grit. These results also indicate that bigger particles of bone are lost to a very small extent, when compared to the fine particles of bone powder.

**Conclusion:**— It may be concluded from the above given facts that in laterite soil also, some nitrogen present always tries to remain constant and only the nitrogen added as manure is lost, i.e., there is a certain amount of nitrogen in each soil which is a constant factor for that particular soil.

In this soil it is seen that an appreciable amount of ammonification takes place in all the manures, thus proving that this soil contains efficient ammonifying agents and according to Buswell (1928) perhaps very little non-nitrogenous organic matter. This is really found to be the case as it is seen there is very little carbon in this soil. Buswell has stated thus, "The bacteria attack compounds from which they obtain energy, if suitable non nitrogenous compounds are present, proteins will be attacked but slightly, or not at all, and consequently little or no ammonia will be found". Thus we may say that the soil containing little of organic carbon has more nitrifying power than the one with more carbon, if all the other factors are constant.

Here nitrate formation was very little as can be seen from the above; only a trace was detected in all the samples throughout the experiments.

In this particular soil nitrate forming bacteria showed a slight discrepancy in nitrifying the manures. Nitrification of dried blood and groundnut cake occurred to the extent of 12.06 and 31.15 percentage respectively, but nitrified horn shavings to the extent of 5.44% only. Even raw bone meal and bone grit showed nitrate formation to the extent of 8.57 and 8.95 percentage respectively. It has been seen that horn shavings showed maximum ammonification viz. to the extent of 40.33% which is the second highest figure in the table No. 9 showing the maximum ammonification in all the manures. Thus we may say that there is no connection between the nitrogen ammonified and the nitrogen nitrified in an organic substance, these ammonifications and nitrifications are independent of each other. Thus Lipman and Burgen, (1917), rightly say, "It, appears, therefore, that a good ammonifying power of a soil is no criterion as to its nitrifying power" and concludes by saying, "The ammonifiability data of fertilizer nitrogen in soils are not useful indicators of the nitrifiability data on the same fertilizers in the same soil."

Lastly we observe that bigger and harder particles of organic substances do not show as great a nitrogen loss as

finer and softer particles. The concrete example of such a loss can be found when we compare the nitrogen losses in all the manure samples. We see that dried blood and groundnut cake show the highest loss, which is 66.00% in both the cases. Raw bone meal shows nitrogen loss in the same period to the extent of 49.45%, while harder and bigger particles like bone grit and horn shavings show the total nitrogen loss only to the extent of 21.11% and 22.22% respectively. Perhaps the smaller particles having a bigger surface exposed to soil organisms are used up more by the same to build up their body substance, and later on liberate the same nitrogen in the form of  $N_2$  gas or some gaseous oxides of nitrogen as waste matters which are lost to the soil.

#### SECTION IV

### The Study of Nitrification in the Sandy Soil

The sampling and mixing of manures in this soil was exactly the same as in the other two soils mentioned above.

It should be mentioned here that in this particular soil moisture loss was very rapid, perhaps due to the fact that it contained a very large proportion of fine sand and that was why the addition of water was done more often than in the other two soils, to keep the moisture contents constant.

### The Study of Changes in Ammonia at Different Intervals

TABLE No. 14

Mgs. of ammonia ( $NH_3$ ) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Groundnut cake	Control
At start	2.573	2.550	4.541	2.573	4.727	2.522
28 days	2.033	2.094	16.440	28.701	15.600	1.300

Time	Raw bone Meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
56 days	2.229	2.284	3.840	4.956	3.910	1.340
70 days	2.573	2.554	3.474	3.445	3.678	1.360
84 days	3.741	3.386	2.803	3.421	3.218	1.479
112 days	1.435	1.733	2.625	3.198	2.206	1.821
126 days	1.425	1.500	2.280	3.008	2.003	2.012
154 days	1.400	1.300	1.779	1.804	1.308	2.316

From the table No. 14 it could be seen clearly that in this particular soil ammonia formation is peculiar. At start the ammonia readings in all the pots show the figures higher than 2.000 mgs. per 100 grams of oven dry soil. On the 28th day the ammonia figures for the pots containing dried blood, horn shavings and ground-nut cake powder in soil are the highest, i.e. the decomposition is very quick, yet in raw bone meal, bone grit and control the same figures show a slight decrease than at start. Thus it appears from the figures of ammonia at start and after 28 days that water plays an important part in the biological actions taking place in this soil. On the 28th day the figures of ammonia show a decrease in raw bone meal and bone grit the same figures rise again up to the 24th day in both the pots, and later on show a decrease again, whilst the soil in the pots containing dried blood, horn shavings and ground-nut cake behaves quite peculiarly and shows a sudden rise in ammonia on the 28th day and it is the highest, then there is a sudden drop on the 56th day and from then onwards there is a gradual fall. This clearly and definitely shows that the ammonifying power in this soil is very high when dried blood, horn shavings and ground-nut cake are used as manures. It ammonifies all the three in a very short time, thus decomposing most of the organic matter in a very short time. Later on when dealing with nitrates it will be seen



that there was no particular rise in the figures of nitrate on the 28th day, thus the conclusion is that, this soil is lacking in nitrifying power and ammonia is allowed to accumulate, which in other words is a loss of nitrogen to the soil, because when ammonia is allowed to accumulate it is either lost to atmosphere by itself or is further oxidised to nitrite and is later on lost as  $\text{NO}$ , or  $\text{N}_2\text{O}_3$  or as  $\text{N}_2\text{O}$  or  $\text{N}_2$  when there is no further nitrification. Actually it will be seen afterwards from the figures of nitrite and total nitrogen that the deductions are true, that nitrogen is lost to a great extent and nitrite formation is also very high. Of course, it is not definite as to which particular form of nitrogen was lost to the soil out of  $\text{NH}_3$ ,  $\text{NO}$ ,  $\text{N}_2\text{O}_3$ ,  $\text{N}_2\text{O}$  or  $\text{N}_2$  or in some proportion by all the five forms, but these are the only forms in which nitrogen must have escaped, as the figures of nitrate do not show any high amount of it, to be lost by solution or any other method. As recently as 1948, H. Lees also formed the same opinion about nitrogen loss. He said, "The lysis of the dead cells produces ammonium ions, which are nitrified by the soil to nitrate ions, and the nitrate -N-concentration begins to rise again. Not all the N initially immobilized is released again, because a certain amount is lost as a result of its conversion to gaseous nitrogen by the denitrifying bacteria, some of which can function under aerobic conditions." Here nitrification seems to have stopped after nitrate formation, which again points to the fact that there are distinctly two separate classes of micro-organisms, one changing ammonia to nitrite and the other changing nitrite to nitrate. There is strong evidence to show that whilst the organisms which acted so vigorously in changing the nitrogenous organic matter like dried blood, horn shavings and groundnut cake to ammonia, did not show any of the same activity to convert the nitrogenous organic matter of raw bone meal and bone grit to ammonia. Thus it may be rightly concluded that there may be some selective power in the same type of organisms whilst making use of nitrogenous organic matter, i.e. on some organic matter it shows more activity, while in others it works very slowly.

The figures of ammonia for the control soil show gradual increase up to the last day indicating that the soil itself accumulates ammonia and very little of it must be nitrifying.

### The Study of Changes of Nitrite and Nitrate at Different Intervals of Time

TABLE No. 15

Mgs. of nitrite ( $N_2O_3$ ) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Groundnut cake	Control
At start	a trace	a trace	a trace	a trace	a trace	a trace
28 days	a trace	a trace	1.620	0.0108	1.0755	a trace
56 days	a trace	a trace	0.013	a trace	0.0151	a trace
7 <sup>th</sup> days	a trace	a trace	0.025	a trace	a trace	a trace
84 days	a trace	a trace	a trace	a trace	a trace	a trace
112 days	a trace	a trace	a trace	a trace	a trace	a trace
126 days	a trace	a trace	a trace	a trace	a trace	a trace
154 days	a trace	a trace	a trace	a trace	a trace	a trace

From the table No. 15 it can be seen that there is only a trace of nitrite developed throughout in the three pots containing raw bone meal, bone grit and control, whilst in the other three pots in which ammonia was formed in comparatively large quantities on the 28th day, the figures of nitrite show high especially in the pots containing dried blood and groundnut cake, which were 1.620 mgs. and 1.755 mgs. res-

pectively. In the pot containing horn shavings on the 28th day the amount of nitrite is 0.0108. Thus it will be seen here that the amount of nitrite was allowed to accumulate and it will be seen later that there was no appreciable increase of nitrate in the pots containing dried blood and groundnut cake. The only way of escape open to this unstable nitrogen compound was the atmosphere through the volatile gases, which the nitrites give out on decomposing. Due to this fact it will be seen that there was much loss of total nitrogen in both these samples. Horn shavings also show some loss of total nitrogen but comparatively it is not much and they show slight increase in nitrate also. Thus the possible conclusion is that where nitrite changes to nitrate there is not much loss, but if this change over is stopped or retarded for any reason, the loss in total nitrogen is great. In other words total nitrogen is lost to a great extent when a soil possesses only ammonia and nitrite forming organisms. There must be present nitrate forming organisms as well to make the soil fertile in the real sense of the word. Without nitrate forming organisms, no soil is of any use to the plant, which takes up nitrogen only in the form of nitrate. The above facts are in agreement with those of Lipman and Burges (1917), when they conclude from their experiments, "It, appears, therefore that a good ammonifying power of a soil is no criterion as to its nitrifying power" and "the ammonifiability data of fertilizer nitrogen in soils are not useful indicators of the nitrifiability data on the same fertilizer in the same soils". Further how nitrification fails, when only the nitrite forming organisms are present is also noted by J. W. Mellor (1928), in his book, "A Comprehensive Treatise on Inorganic and Theoretical Chemistry, P. 357" as follows: "R. Warington then showed that nitrification is really the joint effect of two organisms: one converts ammonium salts into nitrites, but fails to change nitrite into nitrate even after a lapse of several years. The subsequent conversion of nitrite to nitrate is the work of another organism.

Now the changes that took place in the amount of nitrate during 154 days will be taken up.

TABLE No. 16

Mgs. of nitrate (N<sub>2</sub>O<sub>5</sub>) per 100 grams of oven dry soil

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Groundnut cake	Control
At start	1.703	1.597	1.644	1.604	1.606	1.603
28 days	1.749	1.752	2.280	5.578	3.886	0.497
56 days	2.331	2.180	2.625	2.391	2.653	0.546
70 days	2.975	2.489	2.803	2.496	1.549	1.776
84 days	4.251	2.528	3.840	2.642	1.883	2.244
112 days	3.311	3.657	4.541	4.589	1.776	1.789
126 days	3.138	3.397	4.474	3.657	1.754	1.524
154 days	1.392	2.556	1.779	1.901	0.492	1.381

From the table No. 16 it can be seen that there is very little nitrate formation in this particular soil. Only horn shavings show 5.578 mgs. of nitrate per 100 grams of oven dry soil on the 28th day; suggesting that in this pot there was some nitrification, thus accumulated nitrites were given a way of ready conversion into the form and that is why, it will be seen, only slight accumulation of nitrite in the same pot and it will be seen that there is only a slight loss of total nitrogen on the 28th day, as compared to the loss of total nitrogen on the same day from dried blood and groundnut cake. On the 56th day the soil pot containing horn shavings showed a decrease in nitrate which later on increased but slightly and continued till it reached 4.589 mgs. on the 112th day. Raw bone meal and bone grit also show increase in nitrate as weeks pass, but the increment is very small. Raw bone meal showed the highest amount on the 84th day being 4.251 mgs. and bone grit showed that on the 112th day being 3.657 mgs. Then both decreased later on. Dried blood and

groundnut cake show the highest amount of nitrate on the 112th day and the 28th day respectively. There was comparatively very little nitrification in the soil containing groundnut cake, except on the 28th day, on which day as already noticed there was a comparatively high accumulation of ammonia and nitrite. According to Waksman (1927), "The free ammonia, especially in a medium of high alkalinity is also injurious to the nitrite forming bacteria", so it is quite possible that high ammonification may be the cause of little nitrification.

The control soil showed a slight amount of nitrification, the highest amount being 2.244 mgs. per 100 grams of oven dry soil on the 84th day. So from all the above facts it can be said that in this soil, nitrification is not satisfactory, though ammonification is rapid. Waksman in his "Methods for the Study of Nitrification," Soil Science, (1923), writes, "The rapid decomposition of dried blood brings about an abundant formation of ammonia, which is not sufficiently neutralized by acids or buffer agents in alkaline and poorly buffered soils. The free ammonia has an injurious action upon the activities of the nitrifying bacteria".

#### The study of changes in total nitrogen at different intervals of time

The comparison of the loss of total nitrogen from different manures in the sandy soil from Nadiad will be next discussed.

TABLE No. 17

**Mgs. of total nitrogen (N<sub>2</sub>) per 100 grams of oven dry soil**

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Groundnut cake	Control
At start	168	165	157	156	160	78
28 days	163	164	117	141	113	78
56 days	160	162	107	128	109	77

Time	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
70 days	153	160	105	125	101	76
84 days	151	158	100	119	101	76
112 days	146	156	96	103	98	75
126 days	144	153	95	103	97	75
154 days	142	152	95	95	93	74
Loss of total nitrogen	26	13	62	61	67	4

From the above table it is clear that the total nitrogen added at start is not constant in all the samples. Table No. 18 shows the quantity of nitrogen that was actually added, the percentage lost and the percentage of the total nitrogen from the soil itself, (including the manural nitrogen) that is lost in 154 days.

TABLE No. 18

Pots containing	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-nut cake	Control
Total nitrogen added ...	0.090%	0.087%	0.079%	0.078%	0.082%	—
Total nitrogen lost from the organic matter added ...	24.44%	10.35%	73.42%	73.08%	76.83%	—
Total nitrogen lost from the soil (including manural nitrogen) ...	15.47%	7.88	39.49%	39.10%	41.88%	5.13%

It could be seen here that the loss of total nitrogen in raw bone meal and bone grit is gradual, but the three other manures viz. Dried blood, horn shavings and groundnut cake show a sudden drop on the 28th day. Dried blood and groundnut cake show as much drop as 40 mgs. and 47 mgs. per 100 grams of oven dry soil respectively, while horn shavings show that of 15 mgs. only. If the figures of nitrite were examined again in table No. 15 it can be seen that some relationship exists between nitrite and total nitrogen loss, where there is more accumulation of nitrite, there is a great loss of total nitrogen. Dried blood, horn shavings and groundnut cake show nitrite as much as 1.620, 0.0108 and 1.755 mgs. per 100 grams of oven dry soil respectively and at the same time the total nitrogen loss of the same is 40, 15 and 47 mgs. respectively. This relationship clearly indicates that much of the total loss of total nitrogen in the soil is greatly due to its nitrite accumulation. Thus most of the nitrite accumulated is lost to the soil by further oxidation to various volatile nitrogen oxides, as we have seen before.

**Conclusion:** Here also the control soil shows very little loss of total nitrogen, again pointing to the fact that there is some minimum constant amount of nitrogen in the soil. If some organic manures are added to the soil, they later on decompose and the added nitrogen escapes slowly in various forms, as decomposition progresses, and till finally only the original constant amount of total nitrogen remains and this is not further reduced to any significant amount. Harder particles like bone pieces show very little total nitrogen loss as compared to softer particles. It might be due to the fact that in harder particles of manure ammonification is little as compared to softer particles of the same. Thus there is not much accumulation of ammonia or nitrite.

Further if nitrification is faster there will be no nitrite accumulation and thus hardly any loss of total nitrogen, which means nitrate forming organisms also play an important part in saving total nitrogen loss from the soil.

So it will be seen that if the organic manure is very fine and soft and the soils have plenty of ammonia nitrite and nitrate forming organisms, nitrification is very rapid. Further the size of the particles also plays some part in connection with total nitrogen loss. Bone grit (i.e. bigger particles) show less loss of total nitrogen than the very fine powdered form of raw bone meal and also the nitrate formation is a little more in the powdered form. Total nitrogen loss of dried blood and horn shavings is practically equal, whilst ground-nut cake shows a little more. If there was any crop growing in the soil, it would have gained that nitrogen, in the form of ammonia and nitrite. So it may be stated that where there is considerable total loss of nitrogen from the soil, there is much total gain of nitrogen by the crop and thus this soil can be said to be more fertile.

## SECTION V

### THE COMPARATIVE STUDY OF THE THREE SOILS AS REGARDS THEIR AMMONIFYING AND NITRIFYING POWERS AS WELL AS THE LOSS OF TOTAL NITROGEN

#### **The comparison of changes in ammonia in the three soils**

On examining the tables showing changes in ammonia, it will be noticed that the garden soil of Poona shows negligible ammonification as compared to the laterite and the sandy soils. The latter two soils show a distinctly high ammonification. The nitrifying power shown by the laterite soil is comparatively high. In the same soil the ground-nut cake powder is highly ammonified, while in the sandy soil, horn shavings show the highest amount of ammonification. Again the highest amount of ammonia in the laterite soil is 57,920 mgs. per 100 grams of oven dry soil, while that in the sandy soil is 28,701 mgs. per 100 grams of oven dry soil. Thus when the same organic manures are added in the same proportion in different soils, the manures are not ammonified equally in all the different soils, but each soil selects a particular manure and decomposes it to ammonia and the degree of



ammonification is also varied in different soils. Dry blood and ground-nut cake are almost equally ammonified in the sandy soil, whilst in the laterite soil, ground-nut cake is highly ammonified, then comes dried blood and horn shavings.

In the garden soil ammonification is negligible and that is why its ammonifying powers could not be compared.

In sandy soil manures like groundnut cake, dried blood, and horn shavings are very rapidly ammonified. On the 28th day they show the highest figures of ammonia and the same figures show a drop on the 56th day, i.e. after another 28 days the the same figures are very low, indicating that here these manures ammonified very rapidly, showing very high ammonifying power, whilst in the same soil raw bone meal and bone grit show very little ammonification. Thus it will be seen that a hard substance when used as manure takes a long time to ammonify and the degree of ammonification is also very low here.

In the laterite soil ammonification is gradual and the highest figures of ammonia in groundnut cake, dried blood, raw bone meal and bone grit are on the 70th day, whilst that of horn shavings is on the 98th. Here it will be seen that the ammonia formed is retained for a longer time and is not changed into nitrite or nitrate or even lost as rapidly as in the sandy soil, where nitrite forming bacteria seemed to be very active and changed the ammonia formed very readily to nitrite; thus showing nitrite, specially in the case of dried blood and groundnut cake. The laterite soil never showed nitrite higher than a trace, i.e. there is continuous change of ammonia to nitrite and nitrite to nitrate.

### **The Comparison of Changes in Nitrite and Nitrate in the Three Soils**

When the nitrates are compared in all the three soils, it will be noticed that there is a sudden rise of nitrogen in the sandy soil, showing that some of the ammonia suddenly developed had changed to nitrite and nitrate also. The highest

amount of nitrate was in the soil pot containing horn shavings, which showed only 0.0108 mgs. of nitrite per 100 grams of oven dry soil on the 28th day. The nitrite formed is not accumulated here but is changed into nitrate. Dried blood and groundnut cake showed accumulation of nitrites as high as 1.620 mgs, and 1.755 mgs. respectively on the 28th day and that is why the nitrate figures are less for these latter two manures after the same interval.

In the laterite soil the amount of nitrate as rises gradually up to the 70th day then falls gradually in all the pots, except in that containing groundnut cake, where the amount of nitrate accumulated was comparatively very high. It will be seen here that when the concentration of nitrate in the soil, reaches its maximum, and falls again. It is denitrified or decomposed into some other form and once more forms nitrates which accumulate if there be any more organic matter left in the soil.

In the garden soil of Poonn nitrification is gradual and reaches a maximum up to the 126th day in all the samples of manures except raw bone meal where a slight decrease is observed on the 126th day, after which it begins to drop. The figures of nitrite also show a gradual rise.

Thus from the above mentioned facts it will be seen that the garden soil shows stability in ammonia, nitrite and nitrate formation and accumulation. Laterite soil shows very high ammonification and also high nitrification as compared to the other two soils, but the fall is very rapid in both the figures of ammonia and nitrate. The sandy soil rapidly forms high ammonia and nitrate and loses nitrogen very rapidly. Its figures for ammonia, nitrite and nitrate are not steady.

One point that is very clear when comparing the ammonification in all the three soils is that in all the pots containing raw bone meal and bone grit, ammonification is very low and never more than 15 mgs. per 100 grams of oven dry soil. dried blood, horn shavings, and groundnut cake show high amount of ammonification in laterite and sandy soils. The garden soil does not show much difference in its ammonia

contents in all the different pots during the period of experiments. In all the three controls there is very little ammonification and nitrification is at a standstill. Also there is no decomposable nitrogenous organic matter present. Whatever nitrogenous matter remaining there, is not allowed to decompose further, thus showing that in all the three soils there remains some constant amount of nitrogen and that amount differs in different soils. If at all there is a change it is negligible.

From the figures of nitrate conclusion arrived at is that the laterite soil shows the highest amount of nitrate forming power, next comes the garden soil of Poona and lastly the sandy soil, in which the maximum figure of nitrate formation is below 6.0 mgs. per 100 grams of oven dry soil. In all the three soils, the control never shows more than 2.5 mgs. of nitrate per 100 grams of oven dry soil, thus proving that where there is no foreign organic matter present, is no nitrate formation in soil. The nitrogen present in soil itself is a constant factor and is stable and never changes into another form, although the amount of total nitrogen is different in different control soils, i.e. in one garden, the laterite and the sandy soils it is 215.6, 147 and 78 mgs. respectively.

### The Comparison of Changes in Total Nitrogen in the Three Soils

TABLE No. 19

**Percent of total nitrogen lost in 100 grams of oven dry  
soil in a period of 154 days**

Soils	Raw bone meal	Bone grit	Dried blood	Horn shavings	Ground-unt cake
The Garden ...	47.38	35.68	56.38	91.65	—
The Laterite ...	49.45	21.11	66.00	22.22	66.00
The Sandy ...	24.44	10.35	73.42	73.08	76.83

As regards the total nitrogen loss in all the different pots, it will be noticed from table No. 19, that there is always, the least amount of total nitrogen loss in bone grit, where the pieces are of a larger size than that of the raw bone meal, which is a fine powder. That is to say bigger particles of one and the same manure take more time to decompose and remain unchanged for a longer time in the soil. In the case of horn shavings most of it is lost in the garden Soil (91.65%), while in the laterite soil its loss of total nitrogen is only 22.22%, then again in the sandy soil the loss of total nitrogen is as high as 73.08%. It will be seen from the table that the maximum nitrate formation of the horn shavings in the laterite soil is very low even lower than that in raw bone meal and bone grit, which means that there is something in horn shavings which prevents nitrification, although ammonia formed in the same showed as high figures as 42.192 mgs. per 100 grams of oven dry soil. Hence it will be seen that high ammonification has no connection with nitrification. Thus as before we see that there are distinctly three separate types of micro-organisms which do not work equally quickly and that is why we find that ammonification or nitrification does not proceed with equal rapidity and there is accumulation of ammonia or nitrous nitrogen.

The total loss of nitrogen is much due to nitrite accumulation as can be seen from the figures of nitrite in the sandy soil, where the figures of nitrite for dried blood and groundnut cake are as high as 1.626 mgs. and 1.755 mgs. respectively on the 28th day, so also the total nitrogen loss is high on the same day; it is 40 mgs. and 47 mgs. for dried blood and groundnut cake respectively, while Horn Shavings show only 0.0108 of nitrite and thus comparatively less total nitrogen loss also, which is 15 mgs. Raw bone meal and bone grit show a trace of nitrite and the same show the total nitrogen loss as 5 mgs. and 1 mg. respectively on the same day. Thus when nitrites are accumulated they change to  $\text{NO}_2$ ,  $\text{N}_2$  or  $\text{N}_2\text{O}_3$  as the case may be and are lost to the soil. If they are allowed to oxidise to nitrate at once much waste of total nitrogen is prevented.

### Conclusions

(1) From the above facts it can be said that in every soil there is a particular amount of total nitrogen which has a tendency to remain constant and that amount is different for each soil.

(2) Much of total nitrogen is lost after ammonia formation and before nitrate formation i. e. ammonia on oxidation to nitrite loses some nitrogen and nitrite on oxidation to nitrate loses some more nitrogen. If nitrites are allowed to accumulate there is a tremendous loss of total nitrogen as nitrites are unstable compounds and decompose on standing to different gaseous nitrogen oxides.

(3) Every soil has some affinity for certain nitrogenous organic matter only and decomposes only that nitrogenous organic matter before any other. It selects the manure to nitrify. Thus in different soils different manures are nitrified first and there is not one particular manure which is best nitrified in all the soils.

(4) Bigger and harder particles of manure are nitrified after a long time and to a very little extent as compared to the other softer and finer particles.

(5) In any soil good ammonifying power is in no way an indication that it has also a high nitrifying power.

(6) The accumulation of nitrate is also limited in every soil. After reaching its highest limit the figures of nitrate fall again due to decomposition by denitrifying bacteria and never remains constant.

(7) For a fast growing crop horn shavings are the best in the garden soil of Poona. For quick action in the laterite soil, dried blood and ground-nut cake are the best. Though horn shavings show good and quick ammonia formation here, its nitrite formation is not much. The sandy soil shows good reaction with dried blood, horn shavings and ground-nut cake, which are at once decomposed here, thus supplying assimilable nitrogen to a fast growing crop if present.

(8) When a crop does not require nitrogen quickly at start but takes up gradually, bone grit is the best manure to be used in all the three soils. Raw bone meal is advantageous in all the three soils when a medium growing crop is present.

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