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PERMANENT COMMISSION ON STANDARDISATION  
OF SERA, SEROLOGICAL REACTIONS  
AND BIOLOGICAL PRODUCTS

MEMORANDA

ON

Cardiac Drugs, Thyroid Preparations,  
Ergot Preparations, Filix Mas,  
Suprarenal Preparations, Vitamins,  
Pituitary, Salvarsan, Oil of Chenopodium,  
Insulin

By

Professor E. KNAFFL-LENZ

(VIENNA)

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## INTRODUCTION

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The Memorandum by Professor Knaffl-Lenz deals with the results of two International Conferences on the biological standardisation of certain remedies, held in July 1923 at Edinburgh and in September 1925 at Geneva; and also of a subsidiary Conference, with more restricted membership, held in Frankfurt in May 1928, with the object of reviewing the principal recommendations made by the Geneva Conference, in the light of the further experience which had accumulated in the interval, and of presenting them in suitable form for adoption by the Permanent Standards Commission, then meeting at Frankfurt. Professor Knaffl-Lenz reviews in some detail the course of the discussions at the different Conferences, the data obtained in the various investigations undertaken in different countries as the outcome of these discussions, and the recommendations which finally resulted. It may be appropriate to use the opportunity afforded by the publication of his memoranda for a more general survey of the principles which seem to have emerged from the discussions, and from the experience of those who have participated during the past five years in this international work.

When the Health Committee of the League of Nations decided to summon the Conference which met at Edinburgh in 1923, they acted on experience, already gained, as to the possibility and value of international agreement with regard to the standardisation, by biological methods, of certain sera and bacteriological re-agents. Historically, also, it was to certain antitoxic sera that the measurement of potency by biological methods had first been successfully applied. The success and the general acceptance of such methods, for the assay of the diphtheria and tetanus antitoxins in particular, had naturally given rise to the suggestion that certain potent drugs and organ extracts, of which the active principles, for one reason or another, were not accessible to chemical determination, should similarly be assayed by biological tests. Practical effect had been given to this suggestion in many different directions, and the results of a large volume of ingenious investigation, by numerous workers in different countries, was available for study. The problems, however, which faced the members of the first Conference when they assembled at Edinburgh, appeared to be at once more numerous, more varied and more indefinite than those which had been presented to the Serological Conferences previously held under the same auspices.

It was necessary, in the first place, to recognise clearly the fact that two separate problems are involved in the construction of a method of real standardisation: (1) the definition and preparation of a stable standard of reference, and (2) the discovery of a specific and accurate biological method of comparison with that standard. In the case of sera, such as the diphtheria and tetanus antitoxins, this dual requirement had early been appreciated. Stable, arbitrary standards of such sera had been prepared and preserved in suitable centres, and methods of sufficient accuracy had been devised for comparative testing, so that the potency of any preparation could be indicated in units, defined in terms of the relevant permanent standard. From time to time, methods differing in detail, and even in principle, had been introduced, but the standards had remained permanent. The first and most obvious task of international action with regard to these sera had accordingly been to compare the different national standards which had come into existence, and to secure for each serum the general adoption and international recognition of one common standard, and of a common unit defined in terms thereof.

In the work which had been done on drugs and hormones, on the other hand, there had been no clear recognition of these two separate factors of successful standardisation. The principle of using stable standards of reference had not been generally accepted. Various efforts had, indeed, been made to introduce a notation of units of activity; but these, in the case of such drugs as digitalis, or, to take a more recent example, of the hormone insulin, had actually been defined in terms of some animal reaction. Accordingly, the large volume of writings on the standardisation of substance of this mixed group had concerned itself chiefly with the rival merits, whether of ease or of accuracy, of the different biological methods which various investigators had devised and advocated. This material formed the basis of the initial discussions of the Edinburgh Conference. Even in the case of a remedy of such recent introduction at that date as insulin, the members of the Conference found themselves involved in a consideration of the respective merits of two different suggested units, one based on the reaction of rabbits, the other on that of mice, without any common basis of reference which would enable the relation of the two suggested units to one another to be expressed with any precision. The difficulty of securing agreement on such questions of method soon became obvious by the attempt to persuade an investigator, with long experience and confidence in one technique, to abandon it in favour of another which he knew and trusted less. It further became clear that, in many cases, the definition of a unit in absolute terms of some animal reaction would give no sufficient guarantee of its uniform measurement in different countries. Even if such

difficulties could have been overcome, there would have remained the doubt whether an attempt to secure uniformity, by adopting and stereotyping a method, would have been justifiable on scientific grounds. By such considerations the Edinburgh Conference and its successors were led, in dealing with each of the more important of the remedies which have come under their notice, to regard it as their primary task to define a stable standard of activity; to make arrangements for the standard to be prepared and to be kept in some suitable central institute on behalf of the League of Nations Health Organisation; and to define, where necessary, a suitable unit of activity, in terms of a weight of the standard.

When, as the result of the discussions at Edinburgh, this general principle of action became clear, arrangements were made for the experimental production of stable standards for digitalis, insulin, and the pituitary posterior lobe. In the following two years, these provisional standards were circulated among investigators in numerous countries, with a view to collecting evidence, from as wide a range of experience as possible, as to the acceptability of these standards for international adoption. At this stage, the investigations necessarily involved the trial of different methods of biological comparison with the standard. The use of a common standard by so many independent workers provided, indeed, an opportunity, previously lacking, of direct comparison between the results obtained by the application of different methods of biological measurement to the same remedial agent. Such comparisons, again, gave rise to useful and interesting subsidiary discussions on points of detail. These investigations served the further purpose of emphasising the conditions which a method must satisfy, in order to be regarded as appropriate for biological measurement; namely, that it shall be capable of giving a sufficient degree of quantitative accuracy and that the active constituent, which it measures with such adequate precision, shall be the therapeutically important one. Such conditions, as thus stated, appear self-evident; but the wide literature of the subject gives abundant evidence of the possibility that they may be forgotten. The mode of their application, and their relative importance, show interesting differences in the case of the three important remedies above mentioned.

In the case of insulin, no doubt arose as to the specific nature of the effect, which was measured by the different methods under investigation. On the other hand, it was a practical therapeutic necessity that this specific action should be determined with the greatest possible accuracy. The result was to show that several of the methods proposed would give identical results, within satisfactorily small limits of error, when applied in relation to the common standard. The needs

of the situation were therefore met by the adoption, by general agreement, of the provisional standard and the definition of one common unit in terms of it, in place of the various units previously current and qualified as "physiological", "clinical", "rabbit", or "mouse" units. Several of the existing methods of measurement were recognised as suitable, and the opportunity for the introduction of new or improved methods was left freely open.

? In the case of digitalis, on the other hand, there was uncertainty as to whether the active principles measured by the different methods were the same and, further, as to which, if any, of the methods measured that active constituent which was therapeutically desirable and important. Much work was accordingly devoted to the evaluation of different samples by several methods in comparison with a common standard, and, at a later stage, to the endeavour to ascertain whether the relations of activity displayed by any of the methods would hold good for the relative therapeutic activities as determined by the use of the same specimens of the drug in the treatment of selected human patients. To this last question no clear and decisive answer was obtained; but the failure was attributable, not to the inaccuracy of the laboratory methods of assay — any one of which, indeed, would give reasonably concordant results in the hands of different investigators — but to the highly variable results obtained in the clinical trials. From the point of view of a scientific decision, such an outcome was unsatisfactory. From another point of view, however, it was reassuring, since it indicated that the requirements of clinical practice for uniformity of dosage could be adequately met by the use, in conjunction with the standard, of any of the principal biological methods of assay which had been brought under this organised investigation. Again, therefore, the principle emerged that the proper aim of international action was the acceptance of a common, stable standard, with provision for the central preservation and ultimate renewal of the same. As regards the methods of assay, on the other hand, approval could be recorded of those which the organised international trials had shown to be adequate, without compromising in any way the freedom of scientific work directed to the improvement of these, or the development of new and better ones.

The methods used for measuring the activity of pituitary (posterior lobe) preparations, in comparison with the suggested standard, presented again a different problem. Three methods were advocated, based on three quite different physiological reactions, and there was evidence that any of them, in expert hands, could be made to give sufficiently accurate and self-consistent measurements. Each of them, moreover, was clearly and directly related to one of the three important, and

equally distinct, therapeutic uses of the extract. On the other hand, the Conferences were faced with a conflict of evidence on the important question as to whether the three types of activity represented three distinct active principles of three aspects of the action of one common principle. The Geneva Conference of 1925 therefore recommended the international adoption of the proposed stable standard, which had been widely tested in the interval and found satisfactory, and defined a unit of activity in terms of the standard ; on the other hand, they recognised any of the proposed methods of biological measurement as satisfactorily equivalent. Since that date, the position has been cleared by the production of definite evidence that two, at any rate, of the chosen physiological actions are due to distinct and chemically separable principles. As regards these two principles, there is satisfactory evidence that they maintain their normal relative proportions in different samples of the standard, prepared in accordance with the accepted process. Any preparation, therefore, can be assayed by comparison with the standard, using the biological method appropriate to its therapeutic purpose ; and its activity in the chosen direction can still be expressed in units corresponding to the activity of the chosen, arbitrary weight of the standard. With this new evidence before them, the members of the Technical Committee, which met in May 1928 at Frankfurt, were able to confirm the recommendations of the Geneva Conference with regard to the adoption for international use of the proposed stable standard, and of the unit defined in terms thereof. As regards methods, on the other hand, uniformity was not merely difficult, but inadmissible : it had to be recognised that different methods of assay must be used in relation to the different therapeutic uses of this potent extract.

The case of the arsenobenzene group (salvarsan and its analogues and derivatives) first came under consideration at the Geneva Conference of 1925. The problem was again a different one. There was no question here of measuring a variable activity and expressing it in units. What was needed was a guarantee that no sample of a particular preparation should have a toxicity significantly greater, or a therapeutic potency significantly less, than that of a good, average preparation of the compound which it claimed to represent. After prolonged discussions of the possibility of laying down standard methods of testing and securing their international adoption, the Conference found themselves again driven back upon the principle of setting up stable standards of reference for the various compounds included in the group, and leaving latitude of choice with regard to the methods of comparison used to determine the conformity of samples with these, both as regards toxicity and potency. The subsequent meeting at Frankfurt reaffirmed these recommendations on the basis of

experience, with such modifications as would define more precisely the permissible limits of variation, while widening yet further the range of acceptable modifications in the methods used.

In the case of some other remedies, such as *Filix mas*, it was not considered practicable, or necessary, to prepare a basic standard of reference, and the different Conferences contented themselves with recognising, as an adequate guarantee of activity, the determination of the dilution in which the preparation produced a characteristic biological effect. In general, however, it may be said that the decisions with regard to remedies of major importance, requiring biological standardisation, resulted in an ever clearer recognition of the fact that the principal aim of international action in this connection should be the creation of permanent stable standards of reference, and that the main object of international agreements should be the acceptance of such standards and of arbitrary units defined in terms of them. Standards, in the nature of the case, should be permanent, and the object of further action with regard to them should be, not to change them, but to ensure that they do not change. Methods, on the other hand, must be open to progressive modification and improvement, in accordance with the teaching of experience and research. If the International Conferences on the biological standardisation of drugs and hormones had had no other result than to bring the practice with regard to these remedies thus into line with the traditions long established in the standardisation of sera, they would have justified all the work which so many experienced investigators in different countries have contributed to the common stock and have put before their colleagues in a series of fruitful discussions.

I may take this opportunity, as Chairman of the more recent meetings, of expressing my personal thanks to all who have so generously co-operated in this important international work. Those who still remain would, I am sure, desire that I should also express, on behalf of us all, our deep sense of the irreparable loss to science, and to our circle of international friendship, occasioned by the deaths of two of those who were among the most prominent and effective in our earlier discussions — Professor Cushny, of Edinburgh, who presided with genial tact over the technical part of our Conference at Edinburgh, and Professor Magnus, of Utrecht, who undertook the heavy work and responsibility involved in the preparation of one of our most important standards. None contributed more than they to whatever of solid worth has resulted from our meetings and our investigations, or to that atmosphere of genuine and friendly co-operation that has happily attended our activities.

H. H. DALE.

# I. CONFERENCES HELD AT EDINBURGH 1923 AND AT GENEVA 1925.

## CARDIAC DRUGS

(DIGITALIS, STROPHANTHUS, SCILLA).

### DISCUSSION AT THE EDINBURGH CONFERENCE

On the question of the assay of digitalis, which, in spite of a decade of work, has not yet been satisfactorily settled, reports were received at the Conference at Edinburgh from Professor CUSHNY and Professor MAGNUS.

Professor CUSHNY first dealt with the methods for which frogs, or the isolated hearts of frogs, are used. The isolated heart reacted not only to the digitalis glucosides but also to the therapeutically inactive saponins, and it was preferable to use a method which determined the minimal lethal dose for an intact frog. As a standard he suggested dried digitalis leaves instead of the usual strophanthin.

Professor MAGNUS dealt with the use of cats for the assay of digitalis according to a modification of the method of HATCHER and BRODY. The original method depended on the observation that, if a given quantity of a solution of *g. strophanthin* were slowly infused intravenously into a narcotised cat, in the course of 90 minutes, the cat's heart stopped beating. If digitalis were infused first, less strophanthus was necessary. From the deficit, the strength of the digitalis preparation could be calculated in strophanthus units.

STORM VAN LEEUWEN, SLUYTERS, and particularly DE LIND VON WYNGAARDEN, have shown in a series of detailed investigations from the Pharmacological Institute in Utrecht that the modification of ROWNTREE and MACHT (in which they determined the quantity of digitalis necessary to stop the heart unassisted by strophanthus) allows greater accuracy to be attained.



Professor MAGNUS discussed the method used in the Institute at Utrecht and the results obtained with some hundreds of cats and showed that with three or four cats the mean error amounted to only 6.5 per cent, and that the error was seldom more than 10 per cent.

According to Professor MAGNUS, this method had the great advantages over the various frog methods that the result was independent of the time of year, that the experiment was quickly performed without the use of a standard, and that an unconcentrated 0.5 per cent watery infusion could be used. Finally, it did not necessitate the assumption that the mammalian heart responded to digitalis in the same way as the heart of a frog.

After Professor KROGH had spoken of the researches of Mrs. Marie KROGH in which an isolated frog's heart was used, the Conference acknowledged the advantages of the modification of HATCHER'S method, but expressed the view that this method was then unpracticable in many countries because of the cost and that the frog methods provided a sufficiently accurate test.

Dr. DALE stated that, according to the experience of Dr. BURN and Sir THOMAS LEWIS, it appeared that the cat method and the frog method measured different active principles in digitalis, and Professor MEYER proposed that the values obtained by the two methods should be compared with values obtained by the colorimetric method of KNUDSON and DRESBACH. After further discussion, the Conference agreed to the following proposal :

“ The Conference holds the opinion that the activity of digitalis, strophanthus, scilla and apocynum, and of other heart tonics of this group and of their preparations, can be determined with reasonable accuracy, either by the lethal dose method on frogs, with injection into the lymph-sac, or by the lethal dose method on the cat, with slow intravenous infusion. The members of the Conference have not sufficient evidence before them with regard to the relation between the activities determined for the same preparation by the two methods, or as to the relation between the activity determined by either method and the potency of the preparation as a therapeutic agent in human medicine. The Conference accordingly holds the opinion that for the present the choice of method should be determined by the experience of the individual worker or by the variation of local conditions. It considers, however, that further investigation should be promoted, with a view to determining the relation between values obtained by the two methods, and of both to therapeutic activity.

“ The Conference recommends the establishment of a unit of activity, for each of the remedies of this class,

as highly desirable. In view of the multiplicity of methods and the possible variation of the experimental animals, it regards it as desirable that the unit should be defined in terms of a definite weight of some standard preparation, and not in terms of lethal effect upon a species of animal. It recommends the investigation of the possibility of using, as a standard for digitalis, a specially prepared sample of dried digitalis leaves, preserved with precautions against access of moisture or oxygen, and accepts the offer of Professor Magnus to prepare such an experimental standard in Utrecht. By comparison of the determinations made on this sample by different methods and in different centres, it hopes that a basis may ultimately be provided for the definition of an international unit, in terms of a definite weight of such a standard preparation.

“As standard for strophanthus and its preparations, the Conference recommends the adoption of the pure crystalline *Gratus-strophanthin* (*g. strophanthin, ouabain*).”

#### ARRANGEMENTS FOR THE COMBINED TEST.

To enable the resolutions of the Conference to be carried out, Professor MAGNUS made and assayed three different preparations of dried digitalis leaves by his method, using cats, and placed the samples in their order of potency.

After these preparations had again been tested in London by DR. BURN and DR. DALE they were sent out to a number of investigators without any information as to the results of the tests already completed.

These investigators were also given directions for the making of the tests, together with the detailed memorandum of DR. DALE and DR. KNAFFL-LENZ, which contained a review of all the work which had so far appeared on the biological testing of digitalis, and a critical discussion of the methods of preparing extracts and making the tests. The directions which were intended to enable comparable results to be obtained in the different institutes were as follows :

1. The three samples of dry digitalis leaves, labelled “Standard”, “A” and “B” respectively, should be standardised by the cat method in institutes having the requisite facilities, the method described by MAGNUS being used, with the watery infusions recommended by him, or with extracts made by exhaustion with absolute alcohol.

2. For any of the frog methods, it is recommended that only complete extracts, made with absolute alcohol, should

be used. These should be suitable, after replacement of alcohol by saline solution, for either the lethal dose, injection methods, or perfusion methods.

3. For the frog methods based on determination of the lethal dose, with injection into the lymph-sac, it is suggested that, in addition to determinations by the one-hour and twelve-hour methods, which will be made in institutes favouring the one or the other limit, some workers should provide, for comparison, data obtained with an intermediate three-hour limit.

4. It is suggested that all results obtained on frogs by the minimal lethal dose methods should, to facilitate comparison, be expressed in milligrammes of drug, represented in the extract, per gramme of frog.

5. It is requested that full details, such as species of frog, temperature, date, etc., may be given in all records of experiments on frogs.

6. It is suggested that as many investigators as possible shall make, with each extract used for biological assay, a parallel colorimetric determination by the picrate method, following the indications given by KNUDSON and DRESBACH.

#### SUMMARY OF THE RESULTS.

The method using cats and the method using frogs both gave results which agree well among themselves. While the test on frogs showed (taking the mean of all the results) that powder A has only 50 per cent of the activity of the Standard, and that powder B has the same activity as the Standard or slightly less, the tests on cats and guinea-pigs gave the result that B had slightly more activity than the Standard, and that A had about 60 per cent of the activity of the Standard. The results obtained by different workers with the proposed and other methods are, on the whole, quite satisfactory. They have shown that concordant results can be obtained in different countries both with cats and with frogs. But the relative values obtained by the two methods are not quite identical. When cats were used, preparation B appeared, on the average, about 15 per cent stronger than the Standard, while the experiments with frogs showed, with one exception, that it was weaker. Also preparation A appeared weaker on frogs than on cats.

RESULTS OF TESTS ON FROGS.

Considering that the conditions of the tests on frogs, and particularly the methods of preparing the extracts, were quite uniform, the differences in the results are not great.

Table I. — Ordinary Test.

Investigator	Minimal lethal dose per gramme of frog.			Relative potency		
	Standard (mg.)	A (mg.)	B (mg.)	Standard	A	B
JOACHIMOGLU	0.55	1.15	0.60	100	47.8	91.6
STRAUB	0.51	1.12	0.52	100	45.5	98.0
(Soxhlet)	[0.58]					
ROST	0.64	1.08	0.68	100	59	94
DALE and BURN	0.70 1.25	1.80	1.00 1.25	100 100	38.8	70.0
PICK	0.66	1.15	0.60	100	57.4	110.0
Mean	0.72	1.26	0.73		49.9	93.9
Mean deviation from the mean	±0.16	±0.21	±0.22		± 6.8	±7.3
Mean deviation per cent.	±22.2	±16.7	± 28		±13.6	±7.8

In this connection it should be noted that the tests in which extracts were injected into the lymph-sac of *R. temporaria* were carried out in the spring. With the exception of PICK (who used the two-hour method), all the investigators used the so-called "timeless" method. As regards the preparation of the extracts, DALE and BURN and JOACHIMOGLU extracted the preparations with ethyl alcohol for 24 hours in a soxhlet and, after boiling off the alcohol, took up the residue in Ringer solution. STRAUB and ROST, on the other hand, digested the powders for 24 hours with 96 per cent alcohol and then heated them for half-an-hour with a reflux condenser, boiled off most of the alcohol, and, by adding Ringer's solution, prepared an emulsion containing 20-25 per cent alcohol. PICK digested the powders for 24 hours with 25 per cent alcohol in the cold.

Neglecting the divergent results obtained by DALE and BURN, the average deviation is 10-20 per cent. The mean values for the lethal doses in mg. per gramme of frog would then be : for the Standard, 0.61 ; for A, 1.12 ; and for B, 0.68. From the protocols it was evident that the method of ROST, who used

for each test six frogs, of which at least five must die, gave the most regular results. The values he gives are almost identical with the means for the whole series. The assays of CUSHNY are not included in the table, because they must have been carried out on unsuitable frogs. This explains also the divergent results he obtained (Standard, 2 mg. ; A, 2.5-3 mg. ; B, 0.8 mg.).

Table II. — Some Special Tests.

Investigator	Minimal lethal dose per gramme of frog			Relative strength		
	Standard (mg.)	A (mg.)	B (mg.)	Standard	A	B
GARCIA, GUEVARA and JIMINEZ (Manila)	0.26	0.56	0.24	100	46.5	108.3
HEYMANS ( <i>Rana esculenta</i> )	1.51	1.72	1.05	100	87.9	144.8
SMITH and McCLOSKEY	0.48	0.90	0.50	100	52.8	96
KNAFFL-LENZ and STERN ( <i>Rana esculenta</i> )	0.80	1.80	0.90	100	44.5	88.9
KROGH, isolated heart of <i>Rana esculenta</i> .				100	41.0	96.0

In this table it is to be noted that GARCIA and his co-workers extracted the leaves for 60 hours in Soxhlet's apparatus and, after boiling off the alcohol, took up the residue in Ringer's solution. About 200 frogs (*Rana tigrina*) were used for the three tests at a temperature of 27°C. The high temperature would account for the low lethal doses observed. SMITH and McCLOSKEY have shown that increase of the temperature greatly increases the toxicity of digitalis glucosides, as it does that of strophanthin (SOLLMANN, MENDELHALL, STINGLE and BAKER). When the temperature is increased by 10°, the toxicity is about doubled. The results recorded in Table I, which were obtained with temperatures of 15-20°, are thus in good agreement with GARCIA's results, as regards the absolute toxicity of each of the samples.

SMITH and McCLOSKEY injected into the skin vein of *Rana pipiens*, which leads directly to the heart, solutions made up

with pure water, since solutions containing alcohol were in themselves more toxic by this method. As the minimal lethal dose, they took the dose which stopped the heart of a decerebrate frog in one hour. KNAFFL-LENZ and STERN also employed this method, but used *R. esculenta*, since in this species the skin vein is well adapted for injections. The method of SMITH and McCLOSKEY had the advantage over the other methods employing frogs that the seasonal variation in the sensibility of the frogs is less. Errors due to differences in rate of absorption are eliminated. Hence the one-hour method gives reliable values after intravenous injection.

Since the values obtained by HEYMANS with *Rana esculenta* for A and B are almost identical with those of KNAFFL-LENZ and STERN, while the lethal dose observed by HEYMANS for the standard is abnormally high, it is assumed that some mistake has occurred.

M. KROGH has shown that the isolated heart of *R. temporaria* is unsuitable for assays, because the digitalis glucosides cannot be washed out of it, and the effect of a solution of given concentration is therefore dependent on the time for which it acts. On the other hand, digitalis can be washed out of the heart of *R. esculenta*, and the action is accordingly independent of the time and is directly proportional to the concentration, so that STRAUB'S preparation of the heart of this frog may be used for assays. The relative values obtained are about the same as those obtained by SMITH and McCLOSKEY with *R. esculenta*.

#### RESULTS OF TESTS ON CATS.

As has been mentioned already, the relative values obtained with the modification of HATCHER'S method used in the Utrecht Institute were not identical with those established by the use of frogs. Powder B appeared to have a stronger effect than the Standard on cats when a watery infusion was used, but the same effect when the solutions were prepared from alcoholic extracts. The difference in the method of preparation appears to be responsible for the difference in the relative activities. The results obtained on cats are given below :

Table III.

Investigator	Minimal lethal dose, c.cm. per kg. of cat			Relative potency		
	Standard	A	B	Standard	A	B
<i>With 0.5 Per Cent Watery Infusion</i>						
HEYMANS (Gand)	18.02 18.86	31.88 28.58	17.11 14.8	100 100	57.0 65.7	105.0 127.4
GARCIA, GUEVARA and JIMINEZ (Manila)	20.90	39.00	19.20	100	53.6	108.8
DALE and BURN (London)	(18.11 20.40)	34.50	16.80 15.45	100 100	52.5	107.8 132.0
JOACHIMOGLU (Berlin)	22.34 20.51	32.40 36.01	19.50 18.86	100	68.9	111.5
McFARLANE and MASSON (Edinburgh and Lon- don)	21.34 20.80					
MAGNUS (Utrecht)	18.11 18.65 17.99 17.06 17.61	29.43 30.54	15.41 16.02	100 100	61.2 61.1	112.0 116.4
Mean	19.33	32.78	17.04		60.00	115.1
Mean deviation from the mean	+1.39	+2.78	+1.47		+4.8	+7.5
Mean deviation per cent	+6.97%	+8.68%	+8.63%		8.0%	6.5%
CUSHNY (Edinburgh)	23.5 +3.3	36.7 +1.4	21.8 +0.6	100	64	108
<i>With Alcoholic Extracts</i>						
SMITH and McCLOSKEY (Washington)	18.80	25.50	19.4	100	73.7	96.9
DALE	18.50	30.90	17.74	100	59.8	104.5
GARCIA	16.30	34.40	14.60	100	47.5	111.6
JOACHIMOGLU	19.39	28.46	21.74	100	68.1	89.2
Mean	18.25	29.81	18.37	100	62.3	100.5
Mean deviation from the mean	+0.97	+2.89	+2.37		+8.7	+7.5
Mean deviation per cent	+5.31%	+9.69%	+12.90%		+14.1	+7.4

RESULTS OF TESTS ON GUINEA-PIGS.

Table IV.

Investigator	Minimal lethal dose, mg. of drug per 100 gm. of guinea-pig.			Relative potency		
	Standard	A	B	Standard	A	B
<i>With Watery Infusions</i>						
KNAFFL-LENZ	19.0	39.9	17.5	100	49.9	108.6
	±0.8	±0.2	±0.3			
	±4.4%	±0.5%	±1.7%			
<i>With Alcoholic Extracts</i>						
KNAFFL-LENZ	19.8	37.2	18.8	100	53.2	105.4
	±0.7	±1.8	±0.5			
	±3.6%	±4.9%	±2.7%			
Mean	19.4	38.6	18.2	100	51.5	107.0

From this summary it is evident that MAGNUS' modification of HATCHER's method gives very consistent results in different countries. When a 0.5 per cent watery infusion is used, the mean deviations from the mean value do not exceed 10 per cent. DE LIND VAN WYNGAARDEN has shown, on the basis of experiments on 573 cats, that in 95 per cent of cases the deviation from the mean is less than 10 per cent, and that, in general, the strength may be measured by using three or four cats. If the mean error is not to exceed 6 per cent, the number of estimations must be such that the mean of the percentage deviations from the mean of the results is less than  $6.67 \sqrt{n-1}$  ( $n$  is the number of cats used), results being neglected which differ from the mean of all the results by more than 20 per cent. An exception to the consistency of these results is provided by those obtained in Edinburgh by CUSHNY, and later by MCFARLAN and MASSON. The absolute values obtained by CUSHNY (the values given in the table are the arithmetic mean of 10 results with the Standard, 4 with A, and 3 with B) are,



on the average, higher, and the differences between the individual tests are actually greater than those obtained in other places. In the case of the Standard, they exceed 70 per cent (the volume injected was in one case 29.7 c.cm. and in another 17.5 c.cm.). Further tests showed that, after atropinisation of the cats, the results were lower and more concordant. MCFARLANE and MASSON, in detailed investigations with the standard preparation on 55 cats, showed that the differences in sensitivity of the Edinburgh cats do not depend on the sex, or on the diet, or on differences in the tone of the vagus, but that there are two classes of cat, one class being more sensitive to digitalis than the other. These differences were lessened, but not abolished, by atropine. On the basis of observations made by these authors on 16 London cats, they suggest that the class of cat which is more resistant to digitalis is rarer in London than in Edinburgh.

KNAFFL-LENZ has carried out investigations to determine whether the cheaper and more easily procurable guinea-pig was not as suitable as the cat for assays by HATCHER'S method. He found that by using guinea-pigs weighing between 550 and 800 grammes, which had been kept on a constant diet, the assay could be made as effectively as by the use of cats. As the heart of the guinea-pig is about twice as resistant to digitalis as the heart of the cat, the 0.5 per cent watery infusion is too weak. 1.5 to 2 per cent infusions are used, so that not more than 1.5 c.cm. per 100 grammes of guinea-pig is infused. The lethal dose is taken as that which renders the heart unexcitable by mechanical means after opening the thorax. Especial care must be given to the artificial respiration and to the amount of ether administered, since guinea-pigs are easily killed by an excess of ether.

As can be seen from the table, the observed relation between the strengths of the standard and preparation B is about the same as that found when cats are used, but preparation A, with which in nearly all the investigations divergent results were obtained, appeared stronger than in the tests on cats.

Further investigations on guinea-pigs in Vienna have shown that the deviations of individual determinations from the mean are seldom as large as 10 per cent, and that three guinea-pigs are usually sufficient for a test. In experiments on 21 guinea-pigs by J. H. Gaddum (National Institute for Medical Research, Hampstead, London) the standard deviation of a single observation from the mean was 12.5 per cent, so that the amount of variation was about the same as that of the 573 cats used by DE LIND VON WYNGAARDEN.

## MRS. KROGH'S TWO METHODS.

### 1. *Isolated Heart.*

In addition to these biological tests, there is the test of MRS. KROGH, who, as already stated, used the heart of *Rana esculenta*, with which the action is proportional to the concentration, since there is no accumulation of the glucosides in the heart such as occurs with *R. temporaria*. With the heart of the latter species the action depends not only on the concentration but also on the total quantity present and the time for which it is allowed to act.

The test is carried out as follows :

Two isolated esculenta hearts (STRAUB'S preparation) were tested with solutions, one of which was presumably too strong and the other too weak. This was repeated with pairs of hearts until a concentration was found which was just sufficient to stop the ventricular contraction for at least a minute, at a temperature of 18-20°, within 1½ hours. An "esculenta unit" was defined as the activity contained in 1 c.cm. of this solution. By the use of 8 to 12 hearts, the assay can be carried out with an accuracy of 10 per cent.

### 2. *Mice.*

M. KROGH also investigated the use of young mice weighing from 18 to 20 grammes for the assay of digitalis — and strophanthus — preparations.

As mice are particularly resistant to cardiac drugs, very concentrated solutions are necessary for the production of marked toxic symptoms or death. According to M. KROGH, after adequate doses, death occurs in from one to three days with increasing ventricular spasm and not through paralysis of the respiration. With sub-lethal doses, toxic symptoms appear more slowly, persist for a day or more, and then gradually disappear.

In carrying out a test, concentrated solutions were injected into several series of mice to determine : (1) the largest dose which produced no effect ; (2) that which produced slight symptoms — disturbances of gait ; (3) that which produced marked symptoms — the inability to run or to rise from the recumbent position ; (4) the smallest dose which produced death.

By comparing the quantities of the different preparations required to produce these effects, their relative potencies are estimated. With these methods, MRS. KROGH has found that the Standard and preparation B have equal potency and that preparation A has 45 per cent of the potency of the Standard.

Both methods, however, give significant results only if qualitatively similar solutions are used.

KNAFFL-LENZ and STERN, as the result of their investigations, came to the conclusion that the minimal lethal dose of digitalis preparations for mice cannot be determined. With preparations of average strength the lethal dose by intraperitoneal or intravenous injection (2-6 mg. of leaf per gramme of mouse) is so large that it cannot be attained without exceeding the permissible volumes of fluid. The low solubility of digitoxin makes it undesirable to use more concentrated infusions or solutions. The quantity necessary to kill mice of identical weight within one or two hours varies by 100 per cent. In general, it has been observed that those mice which do not die in the first two hours after intravenous injection will, in spite of the appearance of severe toxic symptoms, generally recover after a few days if they are kept warm. The deaths which occur within one or two hours are due, however, not to the action of digitalis on the heart but to asphyxia following complete paralysis of the body musculature. The breathing becomes severely affected in the later stages and ceases without the appearance of appreciable asphyxial convulsions, while the heart continues to beat for some time longer. Late deaths (after two or three days) are the result of general weakness — the cold paralysed animals decline to take food.

#### PROFESSOR MANSFELD'S METHOD.

Finally the method proposed by Professor MANSFELD (Pécs, Hungary) must be mentioned. The suitability of this method can only be gauged when further comparative tests have been made. The assay is carried out as follows :

Strips of the sinus of *R. Esculenta* are suspended in small vessels filled with Ringer's solution saturated with oxygen. Every ten minutes the number of contractions is counted and the solution changed. The lowest concentration of strophanthin which will arrest four such strips in 60 minutes is determined. The same method is then applied to the digitalis preparation and the potency expressed in milligrammes of strophanthin.

#### EXTRACTION.

To the old question whether the extraction of the active glucosides is best effected by water or alcohol, the above-mentioned researches give no conclusive answer.

STRAUB has shown that a cold-water extract contains gitalin and digitalin, but no digitoxin. On the other hand, hot-water extracts contain digitoxin, but only a part of the gitalin, which is destroyed by boiling.

JOACHIMOGLU showed that with a Soxhlet apparatus (and alcohol) about 25 per cent more of active substance can be extracted than with water alone — a result which should be confirmed.

Professor MAGNUS, on the other hand, as a result of investigations carried out in his institute, held the view that a 0.5 per cent watery infusion carried out according to the directions in the Dutch pharmacopœia is exhaustive; all but 0.4 to 1 per cent of the active principles are extracted, and the method gives constant results. On the other hand, the action of alcohol is said to cause an inconstant and often considerable increase in activity, for which the digitalin fraction (STRAUB) is said to be responsible.

DE LIND VAN WYNGAARDEN has shown that, when powdered leaves, dried at 15° C., are treated with hot alcohol vapour, the increase in activity may be 58 per cent, but that this increase does not occur with all leaves, and is not constant in amount with any one sample. Professor MAGNUS therefore rejected extraction by alcohol, because it is subject to the influence of inconstant factors. This increase of activity is suggested as the reason for the fact that the Soxhlet extracts prove to be stronger. Thus, for example, the activity of the powder B showed the following changes;

1. Normal . . . . .	12.1
2. Treated with alcohol vapour . . . .	14.6
3. Alcohol, Soxhlet extract of, 1. . . .	14.6
4.       "       "       "       " 2. . . .	14.2

The treatment with alcohol vapour produces the same increase of activity as does Soxhlet extraction. If a comparison is made of the results obtained in tests on the cat by JOACHIMOGLU, on the one hand, with Soxhlet extracts, and, on the other hand, with watery infusions, it is found that the former were 11-15 per cent more potent. The mean activities obtained in all the investigations with alcoholic extracts of the Standard and A were about 9 per cent greater than those obtained with watery infusions. In the case of B, on the contrary, they were 9 per cent weaker. These differences are of the order of the mean deviation from the mean. In the experiments with guinea-pigs, the difference is less than the error of the method. The deviations from the mean shown by the results of the different workers with alcoholic extracts is about the same as that shown with watery infusions, so that the inconstancy of the action of alcohol appears to be absent in the case of these three preparations. In general, although the results which seem to indicate a greater strength for the alcoholic than for the watery extract are in the majority, there are examples in the other direction, and the significance of the

apparent difference is in any case open to doubt. The stabilisation of the leaves with alcohol vapour producing irregular increments of activity, which DE LIND has investigated, is carried out as follows :

Ten grammes of powdered leaf is spread evenly on linen stretched over a porcelain jar on the bottom of which is a beaker with 50 c.m. of 96 per cent alcohol. The jar is not completely sealed by its lid, and it is put in a water bath and the drug is thus exposed for two hours to the hot alcohol vapour. If there is some liquid left and it is stained green, the alcohol is evaporated off and the residue rubbed up with the leaves. If the linen is stained green, it is extracted with alcohol and the extract is similarly treated.

This process of "stabilisation" is really an incomplete extraction with alcohol. If the treated leaves are extracted with water, the effect must be similar to that of extracting the leaves first with alcohol and then with water, and then taking up the alcoholic residue with water. Further investigation should show whether the leaves whose potency is increased by treatment with alcohol can be completely extracted with water alone without this treatment, and whether the double extraction with alcohol and water is not the cause of the increase in potency.

The question whether alcoholic extraction or watery infusion is the more suitable method is thus still undecided. The still unexplained increase in potency described by MAGNUS may be urged against the former; the destruction of gitalin (as described by STRAUB) against the latter.

From the collected results of the assays on cats, it may be concluded that the difference between the figures obtained by different methods of extraction has no practical importance.

#### COLORIMETRIC METHOD.

KNUDSON and DRESBACH's method of assay in which the brown colour given by digitalis infusions on the addition of basic picrate solution is compared with that given by an ouabain or potassium-chromate standard gives the relative potencies fairly well. The Standard and preparation B appeared of about equal strength, while A appeared definitely more potent than would have been expected from the results of the biological tests. On the other hand, all three preparations appeared about 2 to 2.5 times as potent as they did on cats. As the colour is produced by the sugar in the glucoside, it may be assumed that it is given not only by the intact glucosides but also by the sugar, which is always split off from the glucosides by fermentation before the leaves are dried. Thus, the colorimetric method gives a measure of the original content of glucoside and not of the quantity actually present when the estimation is made. It would then be necessary to

assume that preparation A had once contained a larger quantity of active glucosides, but that these had been partly destroyed by storing the leaves.

The following table gives the results obtained by the chemical method :

Table IV.

Investigator	Mg. per "cat unit" = 0.1 mg. of ouabain			Relative strength		
	Standard	A	B	Standard	A	B
SMITH and McCLOSKEY	44.8	57.0	46.8	100	78.6	95.7
KNUDSON, the same alcoholic extracts as the above	46.8	63.7	52.5	100	78.5	95.2
DALE and BURN, alco- holic extract	69.0	108.0	65.0	100	63.9	106.1
Watery infusion	46.3	51.5	47.0	100	89.0	98.4
GARCIA, Soxhlet	41.5	43.0	32.5	100	96.5	127.6
Infusion	36.5		34.0	100		107.3
EDMUNDS (compared with potassium chro- mate)				(100)	(104)	(140)
Mean	47.5	64.6	46.3	100	81.3	105.05
				(100)	(85.1)	(110.0)
	<i>Comparisons with the standard.</i>					
GARCIA, Soxhlet	100	110	76.5	100	90.4	130.7
Infusion	100	108	89.5	100	92.1	111.7
DE LIND, 0.5 % infusion				100	70	120.0
Mean of all cat results	19.94 18.25	32.44 29.81	16.81 18.37		60.48 62.3	116.9 100.5

KNUDSON and DRESBACH's assumption that it is possible, knowing the results of the biological assay of digitalis and ouabain on cats, to use the same ouabain as a standard for the colorimetric assay of digitalis and to express the result in cat-units, appears to be erroneous, since the potencies as measured by KNUDSON and DRESBACH's method differ very widely from the results of the biological tests. Thus, the colorimetric method appears to be unsuitable for measuring the potency in

absolute units, but to give a good indication of the relative strengths of leaves collected and stored under identical conditions.

### THE VALUE OF DIFFERENT METHODS.

Professor EDMUNDS sent some very instructive information on the subject of the relative values of the different methods. In order to determine which of the biological methods should be adopted in the new edition of the *American Pharmacopœia*, three tinctures of digitalis were sent out to nine different laboratories, and the different methods gave the following divergencies :

	Test 2	Test 3
Guinea-pigs M.L.D. (subcutaneous injection)	2.8-5%	5-5.5%
"Timeless" frog method . . . . .	9%	4%
One-hour frog method . . . . .	13-30%	3-21.8%
Hatcher method with cats . . . . .	5.1-13%	5.7-32.6%

From these results it was concluded that the tests using guinea-pigs and frogs were superior to that using cats, and that the "timeless" frog method gave more satisfactory results than the one-hour method. Nevertheless, for practical reasons, the one-hour method was proposed for the *Pharmacopœia*, and ouabain was chosen as a standard.

### DISCUSSION AT THE CONFERENCE AT GENEVA.

On the basis of these results, Professor MAGNUS read a paper at the Second International Conference in Geneva in which he concluded that the tests on cats had given in different laboratories very consistent relative and absolute values which, on the average, did not differ from the mean by more than 10 per cent. The only exception was the assay carried out by Professor CUSHNY, who found the three preparations less toxic than did the other laboratories. It was finally shown that the cause of this was that there are two kinds of cat in Edinburgh of which one is more than usually resistant to digitalis. Where this difference did not occur, the use of a standard was unnecessary as the tests had given (the same) absolute values in other countries.

On the proposed tests with other warm-blood animals, no final judgment can yet be passed, as the material is inadequate. In any case it is risky to use concentrated alcoholic solutions, as MRS. KROGH proposes to do on mice.

A systematic trial with more material must also be made before judgment can finally be passed on the test worked out by KNAFFL-LENZ on guinea-pigs; in particular, it should be determined whether infusions stronger than 0.5 per cent contain all the active principles of the leaves.

Should these methods survive the test, they would replace the cat method where it is difficult to obtain cats.

Against the frog method it may be objected that the sensitivity of frogs varies, and is different in summer from what it is in winter, and that the relative effects of the individual glucosides is not the same on frogs as on men; that, for most frog methods, an alcoholic extract is necessary and that this introduces uncontrolled sources of error. The results of the other assays on frogs showed large variations.

As regards the preparation of the extract, alcohol is to be avoided, as DE LIND VAN WYNGAARDEN has shown that the treatment of digitalis leaves with alcohol introduces irregular uncontrollable increases in activity, while the 0.5 per cent watery infusion of the *Dutch Pharmacopœia* gives regular results and is practically a complete extract.

The chemical method gives qualitative agreement with the cat method, but quantitatively the agreement is poor and the method should only be used in preliminary work.

The clinical results obtained on patients by DR. KENNEDY with the three preparations are not numerous enough for any conclusions to be drawn from them.

Professor MAGNUS was therefore of the opinion that the cat method is superior to the other methods, and that they should be removed from the pharmacopœias, and that digitalis leaves dried at 55-60° and kept under conditions suitable to the preservation of their potency should be used as a standard instead of ouabain. Such a standard powder might be one 17.5-20 c.cm. of which would contain the lethal dose for a kilogramme of cat by slow intravenous injection in 0.5 per cent watery infusion.

In the discussion which followed, Professor CUSHNY said that justice had not been done to the result of the tests on frogs, and that, as the researches of Professor ROST had shown, by standardising the conditions, it was possible to obtain very reliable results with frogs.

After Professor KROGH had remarked in this connection that the relative toxicity of the isolated glucosides was different for different species of animals and that this made it difficult to compare preparations which differ qualitatively from one another, a sub-committee was appointed to study the different proposals and report to the general meeting.

The Sub-Committee, after a thorough consideration of the foregoing facts, came to the conclusion that even now it was not possible to decide whether the cat method or the frog



method, or this or that method of extraction was preferable, but that good comparative results could be obtained with any well-worked-out method, provided that the standard and the tested preparation received the same treatment.

The Sub-Committee formulated the following proposals, which were adopted unanimously by the Conference :

“1. That, as an international standard, a dry, powdered preparation of the leaves of *digitalis purpurea* shall be made by Professor Magnus, on behalf of the Health Organisation of the League of Nations, of the same strength ( $\pm 10\%$ ) as the experimental standard powder, prepared in accordance with the decision of the First International Conference on Biological Standardisation (Edinburgh 1923), and forming the basis of the various reports presented to this Conference. This standard shall be prepared by the mixture of ten different powders, made from leaves properly dried at  $55-60^{\circ}$  C., shall be adjusted by biological assays, carried out by Professor Magnus (who will use the method of assay on cats), and shall be distributed for international use. The permanence of its activity shall be annually controlled by Professor Magnus. If it should deteriorate, or if the supply should be nearly exhausted, a new standard preparation shall be prepared by the same method, and of exactly equal activity.

“The preparation shall be distributed in sealed ampules of brown glass. These shall be placed at the disposal of the different countries, for the assay of their own national standard preparations.

“2. That, according to present knowledge, no particular method of extraction (infusion, cold alcohol, warm alcohol) can be recommended as the only correct one. It is necessary, however, for the purpose of assay, that the preparation to be tested and the standard preparation shall be extracted by the same method.

“3. As methods of biological assay, the following can at present be recommended as sufficiently accurate :

“(1) *The frog method, with a period of observation of at least 4 hours.*

“A. *Preparation of an extract of digitalis leaves with absolute alcohol.*

“One gramme of digitalis leaves, coarsely powdered (B. 20 = mesh of about 0.75 mm.) and dried to constant weight over sulphuric acid, is allowed to stand for 24 hours at room temperature with 25 c.cm. of absolute alcohol, with occasional shaking in a closed spherical flask of about

100 c.cm. content. The mixture is then boiled for 30 minutes with a reflux condenser on a sand-bath over the smallest possible flame, and, while still hot, is filtered through a plain filter of about 9 cm. diameter. The residue is washed with absolute alcohol on the filter until the filtrate becomes colourless. The combined filtrates are slowly evaporated in a thin-walled tared watch-glass on a boiling-water bath to 5 c.cm. (about 4.5 gm.), the drying of any portion being carefully avoided.

"The concentrated extract, while still hot, is transferred with the aid of distilled water to a graduated flask, and made up to 25 c.cm. with distilled water. By this procedure one obtains an emulsiform greenish solution in weak watery alcohol. This must be used immediately for the test.

"B. Assay of the extract, obtained as described under (a), on frogs, by determination of the minimal lethal dose by the so-called unlimited-time method.

"For the test only healthy male frogs must be used (grass frogs, *Rana temporaria* or *Rana pipiens*), kept under constant conditions and weighing up to 40 gm. each. The body-weight of the frogs, kept for several hours in the laboratory in a moist glass case, is determined immediately before the injection to an accuracy of 0.5 gm., after drying the skin and expressing the urine.

"The extract prepared as above described is injected into frogs, through the mouth, into the breast lymph-sac, with a syringe graduated in hundredths of a c.cm. Larger quantities than 0.3 c.cm., or with weakly active preparations 0.5 c.cm., should not be injected into the breast lymph-sac; if necessary, the injections are to be made, in such cases, also into one or both of the lymph-sacs of the thighs.

"The following signs of intoxication appear: within  $\frac{1}{2}$  to 2 hours after the injection, restlessness, air-hunger, formation of froth, paralysis and, in the course of four hours, stoppage of the heart. The criterion for the determination is that the stoppage is either systolic or rapidly transformed into systole.

"The orientating tests are carried out as follows: doses differing by 20 per cent per gramme of frog are injected, one or two frogs being used for each dose.

"The final determination can be made by the following procedure:

"The mean between the smallest active and the greatest inactive dose is the first approximation. By further more exact determination, with four to six frogs on each dose, the final value can be obtained with an accuracy of 10 per cent. The determination is completed when, of two doses

differing by 10 per cent, the higher kills a majority of the frogs injected, the lower a smaller number.

“ The value is expressed as a percentage of the standard preparation, which is tested at the same time and in the same manner. Only such leaves shall be passed for issue as differ from the standard preparation by not more than 25 per cent.

“ The assay of digitalis tinctures is made in the following manner :

“ 10 c.cm. of the official tincture (=1 grm. of leaves) are concentrated on the water bath at temperatures not above 60°C. to 5 c.cm. volume, washed into a measuring flask with distilled water and made up to 25 c.cm. The assay is made according to the same method as described above for digitalis leaves.

“ (2) *The cat method, as modified by Magnus from that of Hatcher.*

“ For biological standardisation on the cat the  $\frac{1}{2}$  per cent infusion of the digitalis leaves is used, prepared according to the indications of the *Dutch Pharmacopœia*, and then made isotonic by the addition of NaCl; in preparing this infusion, the temperature of 90°C. is not to be exceeded, and the extraction is to be continued for 15 minutes after this temperature has been attained. Cats are used with a body-weight between 1.7 and 2.7 kilograms. The cat is anæsthetised with ether, a tracheal canula is inserted, and, with the help of artificial respiration, a moderate anæsthesia with ether is maintained. The infusion runs at a regular rate from a graduated burette, arranged as a Mariotte's bottle, through a wide canula into the femoral vein. The rate of infusion is so adjusted that the duration of the experiment amounts to about forty minutes; minimum 30 minutes, maximum 55 minutes. If, as a result of the first determination, it appears that the preparation is especially potent, the infusion is suitably diluted, and the first experiment is not included in the calculation.

“ The dose is determined which is necessary to produce stoppage of the heart; this is recognised by inspection and palpation of the thorax, by the asphyxial convulsions, and often also by the interrupted flow of the fluid into the vein: it is further confirmed by opening the chest. If the animal is found to be ill (pneumonia) or pregnant, the result obtained with it is rejected.

“ In this manner one determines the lethal dose of the 0.5 per cent infusion “on  $n$  cats” and continues the determination until the mean percentage deviation of the

single results, from the mean value of the whole series, is smaller than  $6.67 \sqrt{n-1}$ . The average of the volumes infused per kilogramme of animal gives the true "assay value" of the preparation. The lethal dose of the digitalis powder, in milligrammes per kilogramme of cat, is obtained by multiplying this number by 5. The number of lethal cat doses contained in 1 gramme of digitalis powder is obtained by dividing 200 by the assay value.

"For the assay of digitalis tinctures, these are diluted 20 times with physiological salt solution.

"An exact description of the method, and details of the method of calculation, has been published by Dr. C. de Lind van Wyngaarden (*De betrouwbaarheid van physiologische ijkingen, uitgewerkt voor Digitalis, Proefschrift, Utrecht, 1925*).

"4. Other digitalis preparations and strophanthus tinctures can be assayed by corresponding methods, using as a standard for strophanthus tinctures *G. strophanthin* (ouabain), as recommended by the First Conference on Biological Standardisation (Edinburgh 1923).

"5. That no definite conclusions can be based on the clinical reports presented to the Conference concerning the activity of the three digitalis powders which were distributed for comparison. It is necessary that these important observations should be continued on a very large number of cases by different methods.

"6. That the methods of biological assay presented to the Conference other than those above recommended for acceptance should be the subject of further co-ordinated investigations."

#### PREPARATION OF A NEW STANDARD.

In accordance with the decision of the Conference, Professor MAGNUS prepared a new standard by mixing ten different samples of powdered leaf tested by the cat method. The source and nature of these samples is shown in the following note by Professor MAGNUS.

Table V.

No.	Source	Harvest and preparation	Temperature used for drying	Subsequently dried at	Colour	Lethal dose per kg. cat determined at Utrecht.	
						Lethal dose in mg.	1 grm powder contains lethal doses
1	CÆSAR and LORETZ (Halle)	1923. Powder A	50—55°		Yellowish green	156.5	6.4
2	"	1923. Powder B	50—55°		Light bronze-green	80.8	12.4
3	"	1923. Standard (Edinburgh Conference)	50—55°		"	91.2	10.9
4	"	1925. Activity 4 (Focke)	50—55°		"		
5	Upsher SMITH (U.S.A.)	1923.	55—60°		Light green	79.6	12.6
6	"	1925. Minnesota	55—60°		"	81.0	12.4
7	"	1925. Canadian	55—60°		"	88.5	11.3
8	Onderlinge Pharmaceutische Groothandel (Utrecht)	1925. Activity 4 (Storm v. Leeuwen).	50—60°		Light bronze-green	(93.8)	(10.6) *
9	"	1925. Activity 3.8	50—60°		"	(98.8)	(10.1) *
10	Kon. Pharmaceutische Handelsvereniging (Amsterdam)	1925.	at a low temperature	60°	"	102.6	9.8

\* The figures in parentheses were supplied by the manufacturers.

From each of these ten samples 500 grm. were taken and the whole quantity carefully mixed. The standardisation carried out on the cat gave a lethal dose of 84.8 mg. per kg. of cat. The mixture was therefore 7.5 per cent stronger than the old standard of the Edinburgh Conference. Accordingly 500 grm. more of the weak digitalis powder A were added to 4,900 grm. of the mixture, and the determination now gave a direct value 91.7 mg. of the powder per kilogram of cat. The standardisation was made on 14 cats. The mean error of the determination

amounted to 13.4 per cent ; the mean error of the series to 3.6 per cent.

The powder was then, after careful mixing, sealed into ampoules of brown Jena glass in quantities of about 3 grm. in each, and thereafter a further standardisation was carried out. This gave as the lethal dose 86.5 mg. per kg. of cat. The difference from the former value was 6 per cent. The standardisation was carried out on nine cats, the mean error of a single determination being 8.9 per cent, and the mean error of the series 2.9 per cent.

From the total of 23 single determinations may be deduced as the fatal dose of the new standard powder (1926), 89.7 mg. per kg. cat, so that 1 grm. of the powder contains 11.1 fatal cat doses. The mean error of a single determination amounted to 12 per cent ; the mean error of the standardisation was 2.5 per cent.

The water content of the preparation amounts to 8 per cent. If calculated as *dry substance*, the activity is therefore such that 82.5 mg. contain a fatal dose for 1 kg. cat, or 1 gramme of the powder contains 12.1 fatal cat doses.

#### THE QUESTION OF A UNIT OF DIGITALIS.

Since no single method of assay was proposed, and since the relation between the methods using cats and frogs was not determined, the Conference did not define an international unit for digitalis as it had for other biologically standardised therapeutic substances, but it proposed that digitalis leaves ought not to differ from the Standard by more than  $\pm 25$  per cent. This proposal has the disadvantage that active digitalis leaves must be mixed with less active preparations to bring their potency to the required level, but, on the other hand, it has the great advantage that a doctor can always obtain digitalis of constant strength and need not estimate the strength in cat units or frog units, about which he probably knows little.

#### CLINICAL TESTS.

The results of the investigations arranged by the Conference of the activity of the three leaves on patients suffering from cardiac disease showed that a quantitative evaluation on man is not possible by the existing clinical methods, but they did show that preparation A, which the experiments on animals proved to be about half as active as the Standard, also had less effect on man.

DR. GILCHRIST and DR. LYON, of the University of Edinburgh, have undertaken the testing of the leaves by

EGGLESTON'S method of "massive doses", using the alteration of pulse rate as an indicator of the effect. 122 patients—none of whom were feverish or hyperthyroid—received 219 doses. The pulse was counted four hours before and after the dose of digitalis. In spite of the use of similar doses throughout the work, individuals showed great variety of response. The degree of slowing appears to be dependent on the original rate. If the law governing this dependence could be determined, it would be possible to compare the three leaves directly. The greatest slowing was observed in cases of auricular fibrillation, and, in the absence of this condition, the effect was erratic.

The authors have sought to determine the relative potencies graphically, assuming formulæ obtained empirically, and have reckoned that if the strength of the Standard is 100, that of A is 64.3 and of B, 88.9.

Another clinical test by DR. MARTIN, of the Johns Hopkins University in Baltimore, on 19 patients gave the following relation :

Standard 100	A 75.8	B 141.5
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For this purpose DR. MARTIN used patients with marked weakness of the heart muscle (some with a normal rhythm and some with auricular fibrillation), and, after keeping them for five days under observation in hospital, he gave them on the first day six doses of 0.2 grammes and then 0.8 grm. a day until the effect appeared. As an indication of the effect, he took : (1) improvement of the respiration (disappearance of orthopnoea, cyanosis, Cheyne Stokes breathing and lessening of the congestion of the lungs) ; (2) diuresis, disappearance of œdema and fall in weight ; (3) slowing of the pulse and, in the cases of fibrillation, disappearance of the pulse deficit, and shortening of the P-R interval. The therapeutic effect always appeared before any toxic symptoms.

The quantity of digitalis leaf required to produce these effects was divided by the lowest weight of the patient and the quotient taken as the therapeutically active dose.

The mean deviations from the mean were :

Standard	$\pm 10.7$ per cent.
A	$\pm 21.6$ per cent.
B	$\pm 17.8$ per cent.

If the relative values obtained in these clinical researches are compared, it is seen that in both cases powder A was found to be 25-35 per cent weaker than the Standard — which is not very different from the result obtained biologically. On

the other hand, powder B appeared in one case 10 per cent weaker, and in the other 40 per cent stronger than the Standard.

The investigations show that from the clinical point of view it is unnecessary to assay digitalis preparations with great exactitude, and also that preparation A, which appeared in the biological tests to be very inactive, was nevertheless therapeutically effective. From the clinical point of view it is therefore superfluous to give activity in units. The proposal that only those digitalis leaves should be used which differ in potency from the international standard by less than 25 per cent would clearly meet the practical needs of the situation.

### STROPHANTHUS AND SQUILLS.

#### *Biological Testing of Strophanthus and Scilla Preparations.*

DR. BURN, in collaboration with DR. TREVAN, has shown that the assay of strophanthus tinctures on cats gives the same result as on frogs if ouabain is used as a standard. It has been found that the ouabain used as a standard in the United States differs materially in strength from MERCK'S preparation. While the lethal dose of the American preparation is 0.0612 mg. per kg. of cat, that of MERCK'S preparation is 0.0818.

DR. LINNELL found that this was due to differences in amount of water of crystallisation. If the crystallisation is carried out at the usual temperature, the preparation contains 9 molecules of water, but if at 30° there are four, and if at 60° only three molecules. Thus, if ouabain is to be used as the international standard for the testing of strophanthus preparations, it must have a specified rotation and water content.

DR. BURN proposes that 1 c. cm. of strophanthus tincture should be equivalent to 3.5 mg. of ouabain (American preparation) with an allowable deviation of  $\pm$  25 per cent.

As regards the assay of scilla preparations, according to BURN, the cat method and the frog method give widely different results, although good agreement may be attained by the use of scillaren. But, as he was of the opinion that this latter substance could not be proposed as an international standard, DR. BURN recommended that a standard tincture should be prepared by mixing several tinctures, of such strength that a one-in-forty dilution would contain the lethal dose for 1 kg. of cat in 18.1 c.cm. 100 c.cm. of such a tincture would correspond to 25.8 mg. of scillaren.

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## THYROID PREPARATIONS.

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At the Edinburgh Conference, Dr. DALE discussed the biological assay of thyroid preparations (by their effect on the resistance of mice to acetonitrile and on the metamorphosis of frog larvæ) and proposed that the potency of preparations should be so determined, together with their content of combined iodine, and that the results should be compared with their efficacy in the treatment of thyroidless patients, so as to determine the value of these methods.

The Conference agreed to this proposal, and asked Professors REID HUNT and STRAUB, respectively, to undertake these investigations.

Professor STRAUB reported to the Conference at Geneva that HAFFNER and KOMIYAMA had clearly shown in his Institute that the method proposed by REID HUNT in 1905, using acetonitrile, could be used with certain modifications, for the assay of thyroid preparations.

REID HUNT'S investigations had shown that the detoxication of the prussic acid split-off from acetonitrile in the organism was more complete if the metabolism had been raised by the administration of thyroid, but that the increase in resistance was not strictly proportional to the quantity of thyroid given, and was influenced by the diet, the season and the temperature.

HAFFNER and KOMIYAMA succeeded in showing that a maximal increase of 100 per cent in the minimal lethal dose was regularly attained 24 hours after the administration of 10 mg. of thyroid to a fasting mouse, so long as the animals were kept at a constant temperature (18-19°). The strength of thyroid preparations can thus be measured to within 30 per cent. The assay of thyroid preparations by HAFFNER, and KOMIYAMA'S modification of REID HUNT'S method is carried out as follows :

### 1. *Estimation of the Minimal Lethal Dose of Acetonitrile.*

Male mice of 15-20 grm. from as uniform a stock as possible, kept at a constant temperature (18-20°) were weighed to within 0.1 grm. after fasting for four hours, and a freshly prepared 9 per cent solution of acetonitrile in physiological saline was injected intravenously (the lethal dose of such a solution is usually about 0.01 c.cm. = 0.9 mg. per gramme of mouse).

## 2. *Estimation of the Increased Resistance due to Thyroid Feeding.*

Six male mice kept under the above conditions receive 0.2 c.cm. per 20 gm. of body weight of a 5 per cent suspension of the thyroid preparation by means of a stomach tube. Two other groups of six mice receive the same volume of a 3.3 per cent, and a 2.2 per cent suspension respectively. After 24 hours, the mice are deprived of food for four hours. They are then again weighed, and receive an intravenous injection of ~~an~~ acetonitrile solution, which contains in 0.01 c.cm., the lethal dose per gm. of mouse.

Mice which have received the 5 per cent or the 3.3 per cent suspension of thyroid are given 5/3, 6/3 and 7/3 of the lethal dose—two mice being injected with each dose. Mice which receive the 2.2 per cent suspension are given 4/3, 5/3, and 6/3 of the lethal dose. Untreated control mice are given 3/4, 4/4 and 5/4 of the lethal dose.

The results are read after 24 hours, during which the mice must be kept in vessels containing wood-wool, at a temperature of 18°-20°.

If after 24 hours all the mice injected with the highest concentration survive, and if, of those injected with the middle concentration, only those which have received 5/3 of the lethal dose of acetonitrile, and, of those injected with the lowest concentration, only those which have received 4/3 of the dose survive, then 0.2 c.cm. (10 mg. per 20 gm. of mouse) of the suspension of dried thyroid corresponds to one acetonitrile unit.

It is proposed that 10 mg. of dried thyroid should correspond to such an acetonitrile unit. The strength of the preparation would thus be such that, under the given conditions, the resistance to acetonitrile would be increased by 100 per cent.

Professor REID HUNT confirmed the adequacy of the method and proposed some modifications in it. Since, according to his investigations, the potency of thyroid preparations was proportional to their iodine content, it would, he said, be more convenient to define the standard in terms of its iodine content instead of in acetonitrile units. For this purpose, he proposed as the standard a dried and powdered preparation of thyroid, which would contain 0.2 per cent of iodine in specific combination. 10 mg. (*i.e.* 0.2 c.cm. of a 5 per cent suspension) of such a preparation usually gave, under the above conditions, an optimum resistance to acetonitrile, which was neither maximal nor minimal, so that it would be possible to compare both weaker and stronger preparations with the standard.

Since, however, the resistance of mice to acetonitrile was not constant (but dependent on the time of year, diet, etc.), he proposed that the resistance of the breed of mice

which would be used for the test should be tested with the standard preparation. For this purpose, it would usually be sufficient if groups of mice were tested with 5 mg. and with 10 mg. Further, only preparations with similar iodine contents should be compared. If it should occur that a preparation with the same iodine content as the standard should show definitely less protection against acetonitrile, then it would be assumed that it contained iodine which was not in the specific combination.

A biological test would thus only be necessary if the suspicion arose that the iodine was not combined in the specific way. The chemical determination of iodine content gave a measure of the therapeutic value of thyroid preparations. When Professor Straub had expressed these views, the Conference passed the following resolutions unanimously :

“1. That a biological method for the standardisation of thyroid gland substance is not necessary for routine application, the determination of the iodine in natural combination, as thyroid active principle, being a sufficient indication of the specific therapeutic activity. Where a biological method is needed, as, for example, for the detection of preparations which have been artificially enriched with iodide, they recommend the adoption of the acetonitrile test recommended by Professors Reid Hunt and Straub, as described in the publications of Drs. Haffner and Komiyama and Professor Reid Hunt. As a standard of activity, they recommend the activity of a dried preparation of healthy thyroid gland with a natural iodine content of 0.2 per cent.

“2. That Professor Reid Hunt be invited to obtain and keep as an international standard on behalf of the Health Organisation of the League of Nations a sufficient sample of dried thyroid gland substance corresponding to the above definition.”

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## SECALE CORNUTUM. — ERGOT PREPARATIONS.

As Professor TIFFENEAU said at the Edinburgh Conference, the estimation of the therapeutic activity of preparations of ergot presents especial difficulties, as there are in ergot two groups of substances which have a marked effect on the uterus. These are, on the one hand, the unspecific amines (histamine, tyramine) which are formed from proteins by bacterial action, and, on the other hand, the specific alkaloids (ergotoxin, ergotamine). Since the concentrations and proportions of these active substances are very variable, there is great need for an unexceptionable method of assay.

The chemical methods were, he said, too inaccurate, the colorimetric too insensitive and the biological methods which had been used up to that time, which depended on the measurement of the action on the uterus, either isolated or *in situ*, of the effect on the blood pressure, or of the necrotic action on a cock's comb, had given unsatisfactory results, so that none of the methods in use at that time could be recommended.

In the discussion which followed, the view was universally expressed that in the assay of ergot the content of specific alkaloid should be the decisive factor, and that for this purpose only those methods by which the concentration of these alkaloids was determined came into consideration. The Conference was further of the opinion that only such ergot preparations should be adopted in any pharmacopœia as contained the whole of the ergotoxine or ergotamine.

Professor CUSHNY then communicated the fact that Professor CLARK (London) had worked out, but not yet published, a method which would possibly meet these demands. It depends on the action of ergotoxine, discovered by BARGER and DALE, in reversing the action of adrenalin, and was carried out on the isolated uterine muscle of the rabbit.

The members of the Conference were invited to test the adequacy of this method.

At the Second International Conference at Geneva, Professor TRENDELENBURG expressed the opinion that the question of the assay of ergot was not yet satisfactorily solved.

A suitable method would, on the one hand, allow the estimation of the total effect on the uterus, and, on the other, it would give information on the subject of what proportion of this effect was due to the specific alkaloids and what proportion to the amines (histamine, tyramine).

The methods using the isolated uterus would, he said, be suitable for the estimation of the total activity, if a standard ergot preparation were available. Histamine was unsuitable

as a standard, because the sensitivity of the isolated uterus to histamine did not vary in the same way as its sensitivity to ergot. A further difficulty depended on the fact that the effect of ergotamine was only washed out with great difficulty, and that therefore the repetition of the experiment on the same strip of uterus was not possible. Further, there was a lack of investigations of the questions whether the pregnant and the non-pregnant uterus were equally sensitive, on the one hand, to the specific alkaloids, and, on the other, to the amines, and as to the conclusions which could properly be drawn with regard to the therapeutic effect on the pregnant human uterus.

As regards the estimation of the specific alkaloids, according to BROOM and CLARK the method recommended by EDMUNDS and HALE, using cocks, is unsuitable. Only those methods come into consideration which depend on the characteristic property of ergotoxine and ergotamine of annulling and reversing the action of adrenaline.

The method recommended by BARGER and DALE, which depended on testing the effect of adrenaline on the blood pressure of a cat before and after the action of ergot, had the disadvantage that many animals would be needed and that, because of their wide variation in sensitivity, the errors would be large.

On the other hand, BROOM and CLARK'S method, in which the inhibiting action of ergotoxine on the stimulating action of adrenaline on the non-pregnant rabbit's uterus was employed, had the advantage that several strips of uterus from the same animal could be used, so that the difficulties caused by the irreversibility of the action of ergotoxine appeared to be avoided. The authors gave the precision of their method as  $\pm 30$  per cent. Against this method SCHEGG raised the objection that it is not strictly specific for ergot.

PLANELES (STRAUB'S Institute) had proposed the use of the guinea-pig's small intestine (the peristalsis of which is inhibited by adrenaline) in place of the rabbit's uterus. This inhibition was inhibited by the specific ergot alkaloids. The investigations that had been carried out with this method were not sufficiently numerous for the formation of an estimate of the usefulness of the method, which had the advantage that very many similar preparations could be made from the same animal.

Finally, MASUDA, acting on the suggestion of TRENDELENBURG, had used the weakening or annulling of the vasoconstrictor action of adrenaline on the well-known LÄWEN-TRENDELENBURG preparation of a frog as an index of the concentration of the specific alkaloids. The accuracy of this method appeared, however, to be less than that of BROOM and CLARK. But since histamine caused a slight weakening

of the stimulating effect of adrenaline, the above-mentioned methods were only of value if methods were also available by which the concentration of the associated amines could be estimated.

PROFESSOR TRENDELENBURG was thus of the opinion that the work that had been done was not sufficient to enable a method of assay to be proposed. Since the above investigations had shown that the activity of different appropriately stored ergot preparations did not alter, it was doubtful whether further biological tests of the drug were necessary. But, on the other hand, a biological assay of the official preparation and of preparations obtained from the industry was absolutely essential, as their content of specific alkaloids varied within wide limits. Prof. STRAUB stated that the search in his Institute for a practical biological method had been fruitless, but that it appeared probable that it would be possible to extract the whole of the specific alkaloids from the drug with 50 per cent acetone, and after purification to obtain them in the form of a white powder.

In view of these considerations, the Conference arrived at the conclusion that the question of the biological tests was not yet so thoroughly investigated that final proposals could be made; but that it was desirable that further investigations should be carried out with the already known methods, or with any possible new methods, and that the results should be compared with those given by the chemical method devised by STRAUB.

Recently J. H. BURN and J. M. ELLIS have retested the method of BROOM and CLARK and shown that under the conditions of their experiments the assay of the specific alkaloids would be carried out with an accuracy of 10-25 per cent, according to the sensitivity of the uterus.

According to BURN and ELLIS, the test is carried out as follows :

1 *Apparatus*. — This consists of a copper water-bath, which can be maintained at 37° either electrically or by means of a micro burner, and two glass cylinders, each of 100 c.cm. capacity, to take the uterine strips. The levers are so arranged that the contractions of both strips are recorded with a magnification of 6.

2 *The Preparation of the Uterus*. — Fully-grown female rabbits were kept 30 days in the Institute in order to make certain that they were not pregnant. From full-grown animals 10 to 13 strips of muscle can be obtained, but from young animals weighing about 2 kg. not more than three or four. In young virgins the uterus is often so thin that it cannot be divided longitudinally into strips. In this case both horns must be used. If the uterine horns are kept at 0° from the first they can be used 7 to 10 days after the death of the animal.

3. *Carrying out the Test.* — A piece of uterine horn about 1 cm. long is placed on the convex side of a watch glass and divided along the attachment of the broad-ligament. The part lying next the attachment is then removed and the remaining position divided into two longitudinal strips 3 to 4 mm. wide; these are fixed to the levers in the glass cylinders. The levers are weighted sufficiently to stretch the muscle. 0.02 mg. of adrenaline per 100 c.cm. of Ringer's solution, when added to the bath, will cause a definite contraction. Usually the contractions given by the two strips are unequal; in this case the weight on the lever is altered till the excursions are equal.

Since the spontaneous contractions are at first slight, but increase later, the weight must be greater than appears necessary at first. The quantity of adrenaline which is necessary to produce a suitable contraction depends on the sensitivity of the uterus. In many cases 0.2 mg. are needed. In any case, the adrenaline concentration must be the same for both strips. The outcome of the test depends essentially on the choice of a suitable concentration of adrenaline and a suitable weight on the lever. As a rule, three or four changes of the adrenaline solution are necessary before the right concentration is found.

When this is done, ergotamine tartrate is added to one strip, and after 30 seconds some of the solution to be tested to the other. Usually, 0.4 c.c. of a 1/30,000 ergotamine tartrate solution is sufficient. The concentration depends on the sensitivity of the muscle and on the length of time for which it is allowed to act.

Exactly ten minutes after the ergotamine the chosen quantity of adrenaline is added without changing the Ringer's solution. If the inhibition of the two strips is equal, it may be concluded that the volume of the tested solution used contains the same quantity of specific alkaloid as the standard. If this is not the case, the test must be repeated, on fresh strips of muscle, with suitable alterations of the volumes of the alkaloid-containing solutions, until the inhibition of the adrenaline-contraction is equal in the two strips. Since the specific alkaloids are only washed out with great difficulty, the inhibition of the action of adrenaline in any one pair of strips may be measured several times by changing the adrenaline-containing Ringer's solution.

BURN and ELLIS, in their investigation, confirmed the finding of DALE and SPIRO that the action of ergotoxine and ergotamine is qualitatively and quantitatively identical, so that either ergotoxine phosphate or ergotamine bitartrate may be used as a standard if allowance is made for the fact that the latter corresponds to 85 per cent of the former in alkaloidal content.



The American Pharmacopœia has adopted ergotoxine phosphate as a standard and demands of the liquid extract of ergot a content of 0.1 per cent of the specific alkaloid with an allowable range of variation of  $\pm 25$  per cent.

It follows from the investigations of BOURNE and BURN in which the intra-uterine pressure was recorded during labour, that with good preparations of ergot it is only the ergotoxine or ergotamine content which has important therapeutic action; while that of the amines is too slight to have any action on the uterus worth speaking about.

The intravenous injection of 10 mg. of tyramine caused only a very weak response lasting scarcely five minutes. Histamine administered subcutaneously had no action on the pains in doses under 1 mg.; 2 mg. increased the pains for 50 minutes. On the other hand, the typical effect of ergot was produced by ergotamine tartrate in doses of 0.25, 0.75 and 1 mg. The highest dose produced a contraction which could be recorded for three and a half hours and could still be detected after 16 hours.

In the usual therapeutic doses good preparations of ergot should not contain more than 0.2 mg. of histamine. According to the above evidence the therapeutic effect of this quantity is not significant.

Since to-day the purified specific alkaloids of ergot are on the market it may be asked whether the use of preparations with a varying content of active principles is still justifiable, and whether they should not be replaced by the pure alkaloids with or without the addition of histamine. Since the action of ergotoxine is slow in onset and may last many hours, and since the temporary effect of histamine (2 mg.) has a rapid onset, a combination of the two drugs would perhaps be the ideal substitute for ergot.

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## FILIX MAS.

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Professor W. STRAUB'S proposal at the Edinburgh Conference, in 1923, that earth worms (*Lumbricus terrestris*, *Allolobophora fetida*) should be used for the assay of *filix mas* preparations was extended at the Conference at Geneva in 1925. It had been found that small fish (*Godio*, *Carassius*, *Scardinius*) could equally well be used for this purpose, and, according to KNAFFL-LENZ, the lethal concentration is the same as for worms.

The following method of testing was proposed for the pharmacopœias.

The ordinary ethereal extract, or the ethereal extract prepared from the rhizome of *filix mas* according to the directions of the pharmacopœia, is dissolved in spring-water saturated with magnesium oxide so that concentrations of .004, .002 and .001 per cent are obtained. Either average-sized specimens of the above worms, or fishes 5 to 10 cm. long, are placed in 100 c.c. of these solutions each. After some hours all the animals in the concentrated solution, and most of the animals in the middle concentration, will be dead, while those in the weak concentration will survive.

The new *German Pharmacopœia* demands that the *extractum filicis* shall contain at least 25 per cent of crude filicin, determined chemically.

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## SUPRARENAL PREPARATIONS.

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Professor ABEL reported in Edinburgh on the different chemical and biological methods of estimating epinephrin (adrenaline, suprarenin). Although the active principle of suprarenals can either be isolated from the glands as a crystalline chemically pure body, or prepared synthetically, the Conference proposed a biological method of assay, since it is easily carried out with an accuracy of 5 per cent, and far exceeds the chemical methods in sensitivity.

The following proposal was carried unanimously :

“The Conference proposes as a suitable standard for the assay of solutions or pharmaceutical preparations containing the active principle of the suprarenals, pure crystalline lævo-rotatory dioxyphenyl-methylamino-ethanol (epinephrin, adrenaline, suprarenin) either obtained from the suprarenals or prepared synthetically.

“As a method for the biological comparison of preparations with this standard, it proposes the determination of the quantities necessary to produce equal submaximal increases of blood pressure in an anæsthetised and atropinised or decapitated mammal.”

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## VITAMINS.

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The great therapeutic importance of the vitamin content of certain drugs made it appear legitimate for the Second International Conference to consider the question of the assay of vitamins, and Professor POULSSON undertook to report on the position of the question at that time.

Professor POULSSON submitted the usual methods of biological testing for the therapeutically important vitamins to a detailed critical review, and came to the conclusion that the method given in the tenth edition of the *United States Pharmacopœia* for ascertaining the concentration in cod-liver oil of the therapeutically important Vitamin A (growth-promoting) could be recommended for universal adoption, but that the methods of estimating Vitamin D (anti-rachitic) were still too complicated for general use.

DR. DALE expressed doubt as to whether the Conference was competent, as regards its composition, profitably to discuss the question of vitamins. In his opinion a special conference, composed of known authorities on this subject, should deal with these important questions. As regards the estimation of the growth-promoting vitamins, he recommended the retesting of the colorimetric method recently described by DRUMMOND and ROSENHEIM, which, according to these authors, was specific and quantitative. For this purpose, he suggested that Professor POULSSON might supply oils with different vitamin contents, with which similar tests could be made in London and in the United States, both by the biological and by the colorimetric method. This proposal was adopted and the following recommendations were made :

“1. That, in the opinion of this Conference, it is of great importance that the preparations used in therapeutics to supply vitamins to the patient should be standardised as accurately as possible, each for its content of its characteristic vitamin or vitamins.

“2. That the preparation for which such standardisation appears at present to be most important and most practicable is cod-liver oil, Vitamin A (growth-promoting factor) being the constituent of this oil which can be most accurately assayed.

“3. That the general question of the accuracy and usefulness of methods for the standardisation of all vitamins

could be more suitably considered by a special conference of experts, appointed for the purpose.

"4. That this Conference should limit its present activity to the initiation of a comparative test, designed to determine the accuracy and specificity of the colour-reaction for Vitamin A, recently described by Drummond and Rosenheim.

"5. That, for the purpose of this investigation, the Conference invite Professor Poulsson, Professor Voegtlin and Dr. Dale to act as a Sub-Committee."

In accordance with these recommendations Professor POULSSON obtained a series of samples of cod-liver oil of varying value, submitted them to a preliminary biological examination for estimation of their Vitamin A content, and forwarded them to DR. DALE for distribution to other biological centres. The proposal to enlist the co-operation of workers in the United States of America proved to be impracticable, and the oils were ultimately submitted, on the one hand, to the British Committee on Accessory Food Factors, jointly appointed by the Lister Institute and the Medical Research Council, who arranged for independent biological estimations of their Vitamin A contents in four English centres, and also to DR. ROSENHEIM, who made estimations by the colorimetric method without knowledge of the biological findings. The results were finally collected for comparison, and a report by the Committee, presenting and commenting on the results, was published in the *Lancet* (4) as a report to the League of Nations Health Organisation. The following were the conclusions which the Committee drew from their consideration of the results :

"In the examination of cod-liver oils for Vitamin A the colorimetric method of ROSENHEIM and DRUMMOND afforded information consistent with that derived from the biological tests :

"In recording this conclusion the Accessory Food Factors Committee, however, considers that before a conclusion is drawn as to the general validity of the colorimetric method for the assay of Vitamin A, tests should be made with substances containing Vitamin A, other than cod-liver oil, such as butter, palm oil, cereal oils, etc."

Meanwhile, the investigations of ROSENHEIM and WEBSTER with liver fats and body fats from various mammals, birds and fishes, showed that generally concordant results were obtained biologically and colorimetrically, and strengthened the evidence in favour of the view that the colour reaction is specific for Vitamin A.

If this specificity is finally established by further work at present in progress, it should ultimately be possible to replace the tedious and difficult biological test for Vitamin A by an equally accurate colorimetric one. A problem for further investigation will then be the preparation of a stable standard.

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## PITUITARY (POSTERIOR LOBE).

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At the Edinburgh Conference, Professor VOEGTLIN discussed the methods of estimating extracts of the posterior lobe of the pituitary. He gave a critical discussion of the usual methods (effect on the blood pressure and on the isolated uterus) and the application of artificial standards such as potassium chloride or histamine for determining the variations in excitability of the uterus. He concluded that the best and most sensitive method was that which had been worked out by BURN and DALE in which the isolated uterus of the virgin guinea-pig is used and in which a fresh watery extract made from fresh glands is used as a standard.

In place of this extract, Professor VOEGTLIN recommended, however, an extract prepared from a dry powder according to the procedure of SMITH and McCLOSKEY in which the fresh gland is defatted and dehydrated by the use of acetone. So far as his experience had gone, such a preparation lost none of its activity when suitably preserved and contained the whole active principles of the posterior lobe. The Conference unanimously accepted this suggestion, but was also concerned for the measurement of the other physiological properties, such as the effect on the blood pressure and on diuresis, since there was no proof at present that these physiological actions were produced through the same active principle.

Professor MAGNUS stated, on the basis of investigations carried out by Dr. BIJLSMA in his laboratory, that there is no proportionality between oxytocic and pressor constituents in commercial preparations. This conclusion was confirmed by Dr. DALE and Professor DIXON.

The Conference therefore thought it would be valuable in addition to the testing of the oxytocic action, that the pressor effect and the therapeutic action on the kidney secretion should be determined, at least qualitatively, and that methods suitable for this should be devised. Professor VOEGTLIN expressed himself as ready, with his collaborators, to supply a quantity of the acetone extract for investigation which he had suggested as a standard.

On the basis of these conclusions, the Secretary of the Conference has since that time requested a series of investigators to direct their work in the first place to the following problems :

- (1) To devise a method which shall measure the diuretic action of pituitary extract quantitatively.

(2) To determine whether the diuretic and anti-diuretic action of the extract is to be attributed to different substances or, on the other hand, to one and the same substance which acts differently under different conditions.

(3) To improve the method for the quantitative determination of the extract on the blood pressure.

(4) To compare the physiological activity on the uterus, on the blood pressure and on the kidney secretion of :

(a) Extracts prepared from the acetone preparation sent for investigation by Professor VOEGTLIN ;

(b) Extracts prepared from fresh gland according to BURN and DALE ;

(c) All commercial preparations which are readily obtainable in the different countries.

(5) To determine whether the three physiological actions named are produced through one and the same principle, or through different ones, and in the latter case, through how many.

Since, however, histamine (which can arise on account of putrefaction or which might be added artificially) can simulate in the uterus test a very active preparation, it is absolutely necessary to make sure of the absence of histamine. This can be done from the effect of the extract on the blood pressure of the cat or dog, and also determined by following the proposal of DALE. This was that an extract found active should have sodium hydroxide added to normality, and then be allowed to stand at room temperature for one hour. It should then be neutralised with hydrochloric acid and tested on the uterus again. Since the active principles are completely destroyed in the cold by alkali, as GUGGENHEIM has shown, the extract when so treated should, in the absence of histamine or other non-specific stimulant, have completely lost its effect on the uterus.

If all the outstanding questions were not answered by the investigations, at least a basis would be provided for a readily prepared stable international standard preparation and for a satisfactory method of estimation. The results of the investigations of the different workers were to be compiled in two memoranda by Dr. DALE and distributed for the information of members of the Conference.

From what has gone before, it follows that the dry pituitary preparation proposed by VOEGTLIN was considered quite suitable as an international standard. BURN and DALE had recommended a freshly prepared extract as a standard, but further investigations had shown that this was often of varying



strength and was weaker than the extract made from the dry powder. Moreover, a dry powder prepared by BURN according to the directions of SMITH and McCLOSKEY was found to have the same strength as that supplied by Professor VOEGTLIN. From seven parts of fresh gland, one part of dry powder was obtained. BURN had found that one extract of the dry powder was eleven times as strong as one from fresh glands measured both on the blood pressure and on the isolated uterus. On the basis of this finding, BURN and DALE admitted that the acetone preparation was to be preferred as a standard to the watery extract recommended by them.

All were agreed that the method of estimation proposed by BURN and DALE was the most sensitive and the best.

Professor PICK recommended his method of estimating the anti-diuretic activity, which he considered was proportional to the oxytocic activity. Professor KNAFFEL-LENZ said that difficulties arose with the method of BURN and DALE because suitable guinea-pigs were not easily obtained. He said that the methods could only be considered equally trustworthy if absolute proof were first obtained that the anti-diuretic activity and the oxytocic activity were proportional to one another.

Views were expressed on the necessity of further tests beside that on the isolated uterus. CUSHNY and CLARK thought that the determinations on the blood pressure and on the kidney were superfluous, but Professor KROGH thought it desirable, on the basis of the work of his colleagues, that further tests should be made. Professors STRAUB and MAGNUS thought it more important to have a measurement of the anti-diuretic activity where this was possible, because of the greater importance of this therapeutic action than of the effect on the blood pressure. MACKERSIE, from the laboratory of Professor HENDERSON, proposed as a method the determination of the smallest dose which had some anti-diuretic effect, made in comparison with the effect of the standard preparation, because, in carrying out determinations on the blood pressure, false conclusions were easily reached on account of a typical effects.

Professor HEYMANS (Jr.) had worked out a method for the measurement of the pressor activity in which he perfused the isolated head of a rabbit with warm Ringer's solution.

Professors TRENDELENBURG and STRAUB considered, however, that the method first applied by SCHLIMPERT and RIESCHRIETER for the perfusion of the isolated rabbit's ear was much more practical.

Professors REID HUNT, MAGNUS, MACLEOD and ABEL preferred the measurement of the pressor activity on the anaesthetised or decapitated cat. Professor ABEL drew particular attention to the fact that one and the same

substance can exert on the blood pressure of the decapitated cat the opposite effect to that on the anæsthetised cat. Thus, for example, extracts treated with acid cause a fall of blood pressure on the anæsthetised cat, and a rise on the decapitated cat. Dr. DALE, who was able to confirm these observations, believed them to be due to the presence of histamine-like products of protein breakdown, and recommended for the avoidance of errors in determinations of pressor activity, whether on the whole animal or on an isolated organ, that the extracts be tested both before and after treatment with alkali; as between the different methods of testing which were offered, individual workers should be free to choose.

In order to harmonise so far as possible the different recommendations concerning the strength of extracts, Dr. DALE proposed that a watery extract corresponding to 0.4 per cent of the standard preparation be adopted in the different pharmacopœias for obstetric use, and a second, five times as strong, for surgical purposes, or for the treatment of severe post-partum hæmorrhage. Professor STRAUB said that the strength which German doctors were accustomed to apply for obstetric purposes was at most only half as strong as that proposed.

When, however, it became known that the American Pharmacopœia was adopting in its next edition a 0.5 per cent watery extract made from the standard powder (corresponding to a 3.5 per cent extract of fresh glands), Dr. DALE recommended this strength for general acceptance.

Finally, Professors MAGNUS and TRENDELENBURG considered the question of the stability of the watery extract. The latter proposed that the extract should be of such an acidity that it should remain colourless on the addition of p-nitro-phenol.

On the basis of the results of his collaborators, Professor VOEGTLIN suggested to the Second International Standard Conference at Geneva that the chief difficulty in the way of a satisfactory solution of the standardisation problem lay in the fact that the different physiological activities of pituitary extract did not exist in the same proportions in different commercial preparations, and that therefore one could say nothing concerning the strength of the anti-diuretic and pressor action merely from a knowledge of the oxytocic action. The determination of all these properties, however, would make the assay too complicated.

These difficulties could, however, be overcome if commercial preparations were prepared according to the prescription of SMITH and McCLOSKEY from the preparation of dry gland. Investigations have shown that this dry powder could be obtained without difficulty in the same strength in all countries, containing the oxytocic and pressor

properties of the fresh gland in undiminished amount, as DALE and BURN, HEYMANS and also BIJLSMA had shown.

If the Conference should conclude that the extracts were only to be prepared from the standard preparation, then the method of testing worked out by BURN and DALE on the isolated guinea-pig uterus would be sufficient, but if the Conference did not agree to this proposal, then a further measurement of the potency of the extract in pressor activity on the anæsthetised and atropinised cat would, in his opinion, be necessary.

For recognition of the presence of misleading quantities of histamine, he recommended that the procedure suggested by DALE for the destruction of the specific activity through alkali be adopted, in such form that the largest permissible amount of histamine would correspond to 0.01 mgm. per c.c.

Finally, he supported the proposal of DALE for the adoption of a strong and a weak watery extract in the Pharmacopœia, and the proposal of TRENDELENBURG to recommend a hydrogen-ion concentration corresponding to pH. 4.2 in order to preserve the stability.

In the discussion which followed, Professor ROST referred specially to the difficulty of pituitary estimation on the virgin guinea-pig uterus. This method in Germany gave such unsatisfactory results that at that time it could not be recommended. In reply to this, Professors MAGNUS and VOEGTLIN said that they also at the beginning had had to deal with these difficulties, but that since they had used guinea-pigs of a single breed, which were separated at birth from the males, the method had given very satisfactory results.

Against the general adoption of a 0.5 per cent extract as was described in the American Pharmacopœia, difficulties were raised by the German representatives<sup>1</sup>.

According to the view of the German gynæcologists, these extracts would be too strong and by careless application not without danger for the life of the child; moreover, the increase of the usual strength in their country would certainly have undesirable consequences.

Dr. DALE therefore proposed that it should be left to each country to decide what strength it thought was most prac-

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<sup>1</sup> N.B. : In the course of work appearing in the *Journal of Obstetrics and Gynæcology of the British Empire*, BOURNE and BURN state, on the basis of their measurements of the intra-uterine pressure in women in labour, that a dose of 2 international units (corresponding to 1 mgm. of standard powder) increases the response of the muscle to a sufficient extent and that this dose, in all cases where there is no mechanical obstruction, can be applied before delivery without danger. They recommend, therefore, a strength of 2 units for commercial preparations.

licable, but the activity of all extracts should be indicated in units of the international standard, one unit to be the amount of activity contained in 0.5 mgms. of the dry preparation prepared by SMITH and McCLOSKEY. According to this, the extract of the American Pharmacopœia would contain 10 units.

The Conference took the view that the introduction of a preparation four times as strong for special purposes would cause danger, since it might accidentally be used for obstetrical purposes and would lead to the death of the child. But in order not to deny doctors the possibility of using stronger extracts, the dry acetone preparation should be kept in the pharmacy, so that, according to the procedure of the American Pharmacopœia, extracts could be made of whatever strength might be desired.

Following is the text of the conclusions, which were agreed to unanimously.

“ 1. That the dry (acetone) extracted substance of the fresh posterior lobe of the pituitary gland, which was recommended by Professor VOEGTLIN to the Edinburgh Conference as suitable for adoption as a standard of activity for pituitary extracts, and which has since been adopted as the standard for this purpose in the United States Pharmacopœia, Edition X, be now definitely accepted as the International Standard.

“ 2. That, since the evidence before the Conference indicates that, by strict adhesion to the instructions for its preparation, as given in the United States Pharmacopœia, Edition X, a sample of this powder of standard strength can be prepared at any time and in any country, the authority responsible for biological standardisation, in each country concerned, should prepare such quantities of the standard as are needed for distribution in its own country. That Professor VOEGTLIN be requested to furnish, on behalf of the Health Organisation of the League of Nations, a small sample of the standard, as originally prepared for examination by the Edinburgh Conference, to any authority which may need it for confirmation of its own national standard.

“ 3. That it be recommended to the authorities responsible in the different countries for the pharmacopœias that a dry preparation of the pituitary posterior lobe, prepared in exact accordance with the method indicated for preparing the standard powder, should be included in each pharmacopœia, to serve as the official raw material for the preparation of the official watery extract.

“ 4. That in order to ensure the stability of the liquid extract prepared from such a powder, the hydrogen-ion concentration should be adjusted to within the limits represented by pH. 4 and pH. 5. The extract should be sterilised and sealed in ampules of non-alkaline resistance glass.

“ 5. That the pharmacopœial dried preparation and the extracts prepared therefrom should be biologically assayed in comparison with the standard, the extracts from the standard powder, and from the pharmacopœial dried preparation, being prepared for biological comparison according to the method indicated in the United States Pharmacopœia, Edition X. For the purpose of the biological assay, the test on the isolated uterus of the virgin guinea-pig, as described in the United States Pharmacopœia, Edition X, is recommended, as giving the most accurate quantitative results, among the available methods. As additional methods may be recognised the test for pressor activity on the anæsthetised dog or the decapitated cat, and the test for anti-diuretic action on the unanæsthetised dog.

“ 6. That in making the assay by the action on the guinea-pig's uterus, it is recommended that a test for non-specific, stimulant activity on that organ should be applied. This can be done by treating the extract under examination with normal NaOH for one hour at the ordinary temperature (20°C.), neutralising to litmus paper, and re-testing. Not more than 5 per cent of the activity on the uterus should survive this treatment.

“ 7. That the strength of all pituitary extracts should be expressed in *units of activity, the activity corresponding to 0.5 mgm. of the standard powder being defined as one unit*, so that, for example, the official liquid extract of the United States Pharmacopœia, Edition X, would contain 10 international units of activity per c.c. ”

The following are technical details of the methods for preparing the standard powder and carrying out certain of the recognised tests therewith :

(1) *Preparation of dry powder according to Smith and McClosky.* The carefully separated-posterior lobes of at least twenty-five absolutely fresh ox pituitaries are put into 150 c.c. of acetone, and, after standing for three hours, are cut into small pieces with scissors, and then allowed to stand overnight in fresh acetone. The pieces are then dried in a vacuum desiccator during five hours over calcium chloride, at a temperature below 50°. After this, the material is finally

powdered and put through a sieve, when it is again dried for twelve hours in the desiccator; it is then extracted with acetone in a small Soxhlet for three hours. After once more standing for twelve hours in a desiccator, the powder is suitable for use. This standard powder should be distributed in sealed phials of brown glass.

(2) *Preparation of the extract.* A suitable quantity of this powder is accurately weighed out, and is rubbed up in an agate mortar with a few drops of 0.25 per cent acetic acid to a smooth paste; with the addition of more acetic acid it is then transferred to a beaker, and brought to such a volume that 1 c.c. corresponds to 1 mgm. of the dry powder. The mixture is then brought to a boil and filtered. The extract so prepared can then be preserved in hard-glass ampules, after sterilisation at a temperature not exceeding 100°.

(3) *Test of the extract on the guinea-pig uterus.* Non-pregnant female guinea-pigs are used, weighing between 175-350 gms., which since the time of their separation from their mother at about two weeks old have been kept in a pen in which no males are allowed. The guinea-pigs are killed by a blow on the head. The whole uterus is removed, and a portion or the whole of one horn is suspended in warm Ringer-Locke solution at 37°, and the contractions of the uterus are registered in the usual way. The bath should contain 100 c.c. of the solution and during an experiment must be kept at constant temperature. When the muscle is fully relaxed, alternating doses of a standard preparation and of the unknown preparation are added, and the doses are determined which give equal sub-maximal contractions in at least two experiments. The strengths of the standard and of the test solution then stand in inverse relation to the quantities which are necessary to produce equally large contractions.

(4) Composition of Ringer-Locke solution :

Na Cl . . . . .	0.9	gm.
KCl . . . . .	0.042	„
CaCl <sub>2</sub> . . . . .	0.024	„
MgCl <sub>2</sub> . . . . .	0.0005	„
NaHCO <sub>3</sub> . . . . .	0.05	„
Dextrose . . . . .	0.05	„
Fresh glass-distilled water	1000	c.c.

(5) *Estimation of anti-diuretic action on dogs according to Molitor and Pick.* Female dogs weighing from 8-12 kgms. are prepared according to the method of SCHWARZ and WIECHOWSKI so as to have a permanent bladder fistula. The animals are suitable for the testing of the anti-diuretic activity eight days after the operation. The test is carried out in the

following way. Dogs which have had neither food nor water for five hours are given 250 c.c. of water by stomach tube and the preparation to be tested is injected subcutaneously. The quantities of urine excreted are collected every ten minutes during 1½-2 hours and are measured. The anti-diuretic activity of different preparations can only be compared on one and the same dog. MOLITOR proposed the adoption as a unit of anti-diuretic potency the activity contained in a quantity of extract which would inhibit the normal water diuresis during the first two hours by 80 per cent. Such a unit corresponds to 0.5 mgm. of the international standard powder.

(6) *Testing of the anti-diuretic activity according to BIJLSMA.* The method is based on the observation that the water excretion of fasting dogs which have received 800-1,000 c.c. of water reaches a maximum in the second hour, but by simultaneous administration of extracts of pituitary, follows so much later according to the strength of the extract. The urine is measured hourly either by removal through a catheter or through a bladder fistula cannula according to SCHARWZ and WIECHOWSKI.

Preparations which delay the maximal excretion to the third hour can be considered as equivalent.

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## SALVARSAN.

On the motion of Professor VOEGTLIN and Professor ROST, the Edinburgh Conference unanimously expressed the wish that an international agreement should be obtained, as soon as possible, with regard to the biological methods used for controlling the toxicity and efficacy of those organic arsenical remedies which require this kind of control.

The second Conference, held at Geneva in 1925, having considered a report by Professor KOLLE, agreed to the principle of the standardisation of salvarsan and its derivatives in relation to permanent standard preparations, and passed the following resolutions :

“ I. That the internationally recognised biological standardisation of remedies of the arsenobenzene group should be made with a series of standard preparations, one for each of the compounds in question.

“ II. That the following are the remedies which at present should be the subject of internationally recognised standardisation :

“ (1) Dioxydiamino-arsenobenzene dihydrochloride (syn. salvarsan, arsphenamine, arsenobenzol, etc.); and

“ (2) its metallic derivatives (silver-salvarsan); and

“ (3) its sodium salt (sodium salvarsan);

“ (4) Dioxydiamino-arsenobenzene sulphoxylate of sodium (syn. neosalvarsan, neoarsphenamine; novarsenobenzol, etc.);

“ (5) Neosilver-salvarsan;

“ (6) Sulpharsphenamine (syn. sulfarsenol).

“ III. That Professor KOLLE of the Georg-Speyer Haus, Frankfurt-on-Main, be requested to accept the responsibility for preparing, maintaining and distributing the standard preparations (1) to (5) on behalf of the Health Organisation of the League of Nations, and that Professor VOEGTLIN, of the Hygienic Laboratory, Washington, be invited similarly to be responsible for the standard preparation of (6).

“ IV. That every batch of the remedies in question, before issue for therapeutic use on human patients, should be tested on normal animals for toxicity and on animals infected with a suitable strain of pathogenic trypanosomes (*T. brucei*, *T. equiperdum*, etc.) for therapeutic potency.



“ V. That samples from every batch should be tested for toxicity on at least ten mice or five rats, or on both, material from several separate ampoules of each batch being separately tested, and that only such preparations should be passed for issue as exhibit, under identical conditions of experiment, a toxicity not greater than that of the corresponding standard sample.

“ VI. That samples of each batch should be tested for therapeutic potency on mice or rats infected with a suitable strain of pathogenic trypanosomes (*T. brucei*, *T. equiperdum*, etc.) in accordance with the following principles :

“ (1) A series of mice or rats is to be taken, having the same degree of infection with the trypanosome employed, as determined by some method of enumeration per unit volume of blood.

“ (2) That, on such a series of animals with a uniform degree of infection, each batch shall be tested for therapeutic action in several (*e.g.*, two to four) doses, with at least three animals on each dose, and the result shall be evaluated by comparison with the effects of the standard preparation, administered to animals of the same species, with the same degree of infection.

“ VII. That it is further recommended that, before a batch of one of the remedies in question is certified for general issue, samples of it shall have been used on a series of human patients, under the supervision of a qualified expert. ”

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## **OIL OF CHENOPODIUM.**

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In view of the extensive use of oil of chenopodium as an anthelmintic and the frequency of poisoning, it appeared desirable to devise a method of biological assay for these remedies as well, since a chemical determination of the content of active principle, *i. e.*, ascaridol, was not possible at the time. Professor MAGNUS therefore urged, at the Edinburgh Conference, that this question should be included in the proposed programme of research.

At the Geneva Conference, Professor KNAFFL-LENZ reported that the tests on earthworms and fishes, which had been found suitable for the assay of preparations of Filix MAS, could not be used without modification for oil of chenopodium, and that this question must be decided by testing a series of oils obtained from different sources.

The tests subsequently made by Professor KNAFFL-LENZ on a series of oils obtained through Professor VOEGTLIN, of the Rockefeller Foundation showed that neither earthworms nor fishes, nor mammals, were suitable for testing purposes; that a biological method of assay did not therefore appear to be possible, but that the ascaridol content could be ascertained with sufficient accuracy by colorimetric determination.

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## INSULIN.

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At the Edinburgh Conference, Professor MACLEOD presented a report on methods of assaying insulin, based respectively on the determination of the blood-sugar and on the occurrence or absence of hypoglycæmic convulsions. He proposed that one unit of insulin should be defined as one-third of the quantity which lowers the blood-sugar to the convulsant level of 0.045% in a normal rabbit of approximately 2 kilograms body-weight, which has been starved for a period of twenty-four hours. The proposal was unanimously accepted.

In the view of the Conference, the determination of the dextrose equivalent in depancreatized dogs could also be used in fixing the unit. Professor KROGH stated that white mice were also suitable for the assay of insulin preparations, provided the minimum convulsive dose were ascertained under certain conditions.

Furthermore, the Conference unanimously accepted Dr. DALE's offer to have prepared in the National Institute for Medical Research, Hampstead, a sufficient quantity of a dry, stable sample of insulin, for examination in different centres with a view to the definition of the unit in terms of milligrams of such a standard sample.

The comparative tests to which this dry sample was subjected showed, in all cases, that one milligram corresponds to eight insulin units as defined by the Toronto Committee. Professor MACLEOD therefore proposed, at the Second International Conference at Geneva, that one international unit should be the quantity producing the same effect as 0.125 milligrams of the aforesaid standard preparation.

The following resolutions were unanimously adopted by the Conference :

“ 1. That the dry preparation of insulin hydrochloride, prepared by the Medical Research Council of Great Britain at the request of the Edinburgh Conference, should be accepted as the international standard preparation of insulin. That 1 milligram of this standard contains 8 units of insulin (or 1 unit = 0.125 milligrams), as provisionally defined by the Insulin Committee of the University of Toronto.

“ 2. That this standard preparation be kept, on behalf of the Health Organisation of the League of Nations,

by the Medical Research Council, who will undertake to test the permanence of its potency from time to time.

“ 3. That\* samples of this preparation, weighing 0.100 gramme each, be sent to some responsible organisation in each country (such as an Insulin Committee or a Government institution) which will undertake further distribution to testing laboratories. In those countries in which no suitable organisation for this purpose exists, samples of the standard will be distributed by the Medical Research Council after consultation with the Insulin Committee of the University of Toronto or, in case this Committee be discontinued, with one appointed by the Health Committee of the League of Nations.

“ 4. That each testing laboratory should prepare a standard of its own and should compare the potency of this with the sample of the international standard placed in its hands for this purpose. When the latter is exhausted, further comparisons with the international standard should, where possible, be undertaken by the responsible authority for the particular country.

“ 5. That either of the following methods be considered as suitable for the bio-assay of insulin :

“ (a) *Methods depending on the effect on blood-sugar.*

“ *First method.* — Varying quantities of insulin that are less than the convulsive dose are injected subcutaneously into rabbits of about two kilograms body-weight, from which food has been withheld for 18-24 hours, and the average of the blood-sugar percentages over a period of five hours after the injection is subtracted from the blood-sugar percentage immediately preceding the injection. The number of units of insulin present in each cubic centimetre of the preparation is then calculated by use of a formula\* (see report). Each rabbit

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(\* ) The formula is as follows :

$$\text{Units per c.c.} = \frac{a}{b} \times \frac{w}{c} \times 1.5$$

Where —

a = percentage of blood-sugar before injection minus the average of the percentages of blood-sugar found in the samples taken one and a-half, three and five hours following the injection ;

b = percentage of blood-sugar before injection minus 0.045 per cent ;

w = weight of the rabbit in kilograms ;

c = number of c.c. of the original (undiluted) insulin.

used in the assays is tested at suitable intervals with a standard preparation which is periodically compared with the international standard.

\* “ *Second method.* — Alternatively, one-half of a series of rabbits receives, in each case, an injection of half a unit of the standard preparation per kilogram, and the other half receives, on the same day, the dose supposed to be equivalent of the sample under test. The percentage fall of the blood-sugar content over a period of five hours is determined as above. A few days later the determinations are repeated on the same series of rabbits in this way, that the rabbits previously receiving the standard preparation now receive that under test and *vice versa*.

“ From the relation between the falls of blood-sugar content produced, on the one hand, by the standard preparation, and on the other hand by the sample under test, the true activity of the latter in units per c.c. can be calculated (see separate report).

“ (b) *Method depending on the incidence of symptoms in white mice.*

“ The assay is carried out by comparison with a standard preparation injected simultaneously with the unknown sample on an equal number of mice from a common stock. The onset of convulsions or collapse is used as the end-point of the reaction, and a mouse dose is the quantity producing convulsions (or collapse) in half the number of mice injected. During the test the mice are kept in an incubator at a uniform temperature of not less than 30°C. (For details of these methods see separate report.)

“ 6. That the Conference appoint a Sub-Committee, which shall submit recommendations with regard to the permissible content of organic solid matter per unit in preparations of insulin and with regard to tests for the stability of such preparations.

“ 7. That, in future, the term ‘unit of insulin’ or ‘insulin unit’ should only be used in the sense indicated above.”

With regard to the preparation of the international standard, the definition of the international unit and the detailed description of the various tests, reference should be made to the League of Nations publication “The Biological Standardisation of Insulin” (document III, Health 1926.III.7 — C.H. 398).

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## II. CONFERENCE AT FRANKFURT-ON-MAIN 1928.

On the occasion of the meeting of the Permanent Commission on Standardisation of Sera, Serological Reactions and Biological Products, consisting of Dr. MADSEN, Dr. H.H. DALE, and Professors KOLLE and Louis MARTIN, which was held at Frankfurt-on-Main, from April 25th to 28th, 1928, a Sub-Commission was appointed to revise the resolutions of the 1925 Geneva Conference in the light of subsequent experience, and to make any changes which might be desirable.

This Sub-Commission consisted of Dr. BIJLSMA, Dr. H.H. DALE, Professor HATA, Professor HIRSZFELD, Professor KNAFFL-LENZ, Professor ROST and Professor TIFENEAU.

The Sub-Commission came to the conclusion that the Geneva proposals relating to thyroid preparations and *filix mas* should remain unchanged, that a biological standardisation of oil of chenopodium was unnecessary, and finally that the method of assaying ergot, which was proposed by BROOM and CLARK at the Edinburgh Conference could be recommended as convenient and accurate.

The methods of testing insulin, which were proposed in 1925, the proposed unit and the standard sample prepared by the National Institute for Medical Research, London, had proved generally satisfactory, and were in use in all countries. Accordingly, the previous recommendations on the subject were left unchanged.

In view of the general improvement in the manufacture of preparations of insulin, recommendation No. 6 to the effect that a Sub-Committee should submit recommendations with regard to the permissible content of organic solid matter per unit and with regard to tests for the stability of such preparations, appeared to be unnecessary until there was some indication of the need for international action.

It was decided that the standard should be preserved and distributed by the National Institute for Medical Research, London, acting on behalf of the League of Nations.

With regard to the other preparations, however, it appeared desirable to supplement the recommendations of the Geneva Conference.

### SALVARSAN

Points I, II, III, VII were endorsed without modification, but the following was added with reference to neosalvarsan and sulpharsphenamine (syn. sulfarsenol) :

“ (1) That the standard samples for neosalvarsan and sulpharsphenamine provided by Professors KOLLE and VOGTLIN respectively for trial are suitable, as regards toxicity and experimental therapeutic activity, for adoption as the basis for the international standards ;

“ (2) That, when a new preparation of neosalvarsan is required, it shall be tested in the first instance in the Frankfurt Institute of Experimental Therapy, and there found to be equivalent in toxicity and experimental therapeutic activity to the existing standard ; and that its equivalence to the existing standard in these respects shall be confirmed by tests carried out in the National Institute for Medical Research, London, before its adoption for use as an international standard.

“ Similarly, when a new standard of sulpharsphenamine (sulpharsenobenzene, sulpharsenol, myosalvarsan) is required, this shall similarly be tested and found equivalent to the existing standard in the Hygienic Laboratory, Washington, and this finding shall be similarly subject to international confirmation, before the new standard is adopted for international use.

“ With a view to ensuring the greatest possible stability for the standards of these two salvarsan derivatives, the Commission considered that the following precautions should be adopted. The material intended for use as a standard should be tested for its stability by heating to 60°C. for several days ; it should be dried to constant weight *in vacuo* over P<sub>2</sub>O<sub>5</sub> ; it should be hermetically sealed in glass ampoules filled with a dry, neutral gas ; and it should thereafter be kept at a temperature not higher than 0°C. before issue, and subsequently in the official institute in each country to which it is distributed. ”

The earlier recommendation that only animals infected with trypanosomes should be used for tests was amended to read that both animals infected with trypanosomes and those infected with spirochaetes could be used for tests.

Point IV was accordingly modified to read as follows :—

“ That every batch of the remedies in question, before issue for therapeutic use on human patients, should be tested on normal animals for toxicity and on animals infected with a suitable strain of pathogenic trypanosomes or spirochaetes for experimental therapeutic activity. ”

The extensive investigations into the question of toxicity determination, on which Dr. DALE submitted a detailed report, led the Conference to conclude that a 20 per cent excess of toxicity above that of the standard would be a suitable limit of tolerance. Point V was accordingly modified to read as follows :

“ That samples of every batch should be tested for toxicity on at least ten mice or five rats, or on both, material from several separate ampoules of each batch being separately tested, and that only such preparations should be passed for issue as exhibit, under identical conditions of experiment, a toxicity not exceeding that of the corresponding standard sample by more than 20 per cent. ”

The modifications made in Points IV and V entailed a corresponding alteration of paragraph 1 of Point III and the addition of a paragraph 3, as shown below :

“ (1) A series of mice or rats is to be taken, having the same degree of infection with trypanosomes or spirochaetes, as determined by some method of enumeration per unit volume of blood. ”

“ (3) Only such preparations should be passed for issue as exhibit, under identical conditions of experiment, an experimental therapeutic activity not less than that of the corresponding standard sample by more than 20 per cent. ”

Finally, at the desire of Professor Tiffeneau, the following was added :

“ VIII. It was agreed that any individual State could modify its national standard on the condition that its toxicity and its experimental therapeutic activity are determined by comparison with those of the International Standard. ”

## DIGITALIS

Professor ROST reported that, in Germany, a national standard preparation of approximately the same strength as that prepared by Professor MAGNUS was officially produced, and that there had been no difficulty in preparing the whole of the supply of *Folia digitalis* to this strength. Since, in other countries as well in which Professor MAGNUS' standard preparation was used, it had been found suitable, the Commission decided that the standard preparation in question should now be definitely adopted for international use.

One detail of Point I of the Geneva recommendations required modification. Owing to the lamented death of Professor MAGNUS, it was necessary to decide who should in future undertake the preparation and standardisation of a standard leaf powder of the same composition and strength when the present supply should be nearly exhausted or should show signs of deterioration. The Commission proposed the



Pharmacological Laboratory of the University of Utrecht, Holland, and recommended that Dr. RIJLSMA should be asked to undertake this work in future. It was further proposed that the standard, measured by the cat method, should, before its adoption as an international standard, be tested in other countries by other methods. It should be tested by determination of the minimum lethal dose in intravenous infusion, using guinea-pigs as recommended by Professor KNAFFL-LENZ, or dogs as recommended by Professor TIFFENEAU, and particularly by the lethal dose method on the frog. Only when the various tests showed it to be practically identical in activity with the existing standard should it be used as an international standard, and be kept and distributed by the National Institute for Medical Research, London.

The Commission was further of opinion that more stringent precautions should be used than in the past in regard to the water-content of the international standard preparation. In future, the water-content of the leaf powder, as distributed in the sealed glass ampoules for preservation should not be more than 3 per cent in place of the 7 to 8 per cent water-content of the existing standard preparation.

Point II was endorsed without modification, as no facts had come to light which would justify any change. The Commission considered, however, that they had sufficient evidence before them to justify a somewhat wider recommendation with regard to the methods of comparative testing which had previously been adopted. Accordingly, they modified Point III to read as follows :

“(a) The frog method in the form recommended by the Geneva Conference, or in its other modifications.

“(b) The method using intravenous infusion in the mammal, as described by HATSCHER and modified by MAGNUS and his colleagues for the cat, by KNAFFL-LENZ for the guinea-pig, or by TIFFENEAU for the dog.

“(c) The Commission considered that the methods described by Mansfeld, using portions of the isolated *sinus venosus* of the frog, and by TREVAN, using the isolated auricle of the rabbit, merited further investigation, with a view to consideration on a future occasion.”

With regard to the recommendation made by the Geneva Conference in connection with the frog test, as described in detail in their report, the Commission considered, in the light of experience since obtained, that the tolerance of  $\pm 25\%$  in relation to the standard was unduly generous. It therefore proposed a tolerance of  $\pm 15\%$ .

With regard to the preparation and standardisation of the tincture of *digitalis*, the Commission took the view that it was

desirable to have regard to the conditions of its preparation, whether in small quantities, as by the individual pharmacist, or on a large scale, as in pharmaceutical factories. In the former case, no further prescription was necessary than the use of standardised leaves. In the latter case, it was regarded as unnecessary to require the use of leaves of standard strength, provided the activity of the tincture is controlled by biological assay and adjusted to identity in activity with the tincture prepared from the standard leaves. This provision applied also to the manufacture of special preparations of digitalis containing the whole of the active principles of the drug.

For the convenience of notation, when indicating the degree of activity of such preparations, it was considered that the said activity should be expressed in international units. The Commission accordingly made the following recommendation :

“ That, when the dosage of digitalis or its preparations is expressed in units of activity, the unit employed for any preparation and in any country should be an international unit, which should be defined as the specific activity contained in 0.1 gramme of the international standard powder. ”

With regard to the recommendation of the Geneva Conference for the use of ouabain as a standard for preparations of strophanthus, the Commission, considering the variable activity of different crystallised preparations on the market, due to varying percentage of water of crystallisation, recommended that the activity should be expressed in terms of water-free ouabain.

To facilitate this, they recommended that a sample of ouabain should be prepared and kept as an international standard for the biological assay of strophanthus and its preparations, and that this standard should have an accurately determined content of water-free ouabain.

Professor Tiffeneau will prepare such a standard and place it at the disposal of the League of Nations, the International Institute for Medical Research, London, keeping and distributing it on behalf of the Health Organisation.

## SCILLA

The Commission considered the possibility of establishing an international standard for scilla and its preparations and recommended that the possibility of preparing a stable standard from a suitable dried sample of the drug should be the subject of experimental enquiry.

## PITUITARY EXTRACT

In view of the favourable results obtained at various centres with the standard material prepared by Professor Voegtlin, the Commission felt that recommendations Nos 1 and 2 could be endorsed without modification. With regard to recommendations Nos. 3 and 4, however, the Commission regarded these as no longer necessary and as possibly undesirable in view of the doubt expressed by certain members of the Conference as to whether the dry powder prepared by extraction with acetone retained the anti-diuretic activity of the original gland without loss. It accordingly decided to delete recommendations Nos 3 and 4.

Recommendation No. 5 was discussed in detail and the Commission decided that it should be altered to read as follows :

“ That the extracts from the standard powder should be prepared for the purposes of the comparative biological tests according to the method indicated in the United States Pharmacopœia, Edition X ; for the purposes of biological assay, the test on the isolated uterus of the virgin guinea-pig as described in the United States Pharmacopœia, Edition X, is recommended for estimation of the oxytocic activity ; for estimation of the pressor activity, comparative tests on the anæsthetised dog or the decapitated cat are recommended ; for estimation of the anti-diuretic action, tests on the unanæsthetised dog, on the normal man, or on the patient with diabetes insipidus are regarded as suitable. ”

### NOTE

The Commission took into consideration the effect on the recommendations of the 1925 Geneva Conference of recent evidence proving that the pressor and oxytocic activities of the pituitary extract are due to different active principles, and of indications that the anti-diuretic activity may be due to yet another principle. It noted that the discoverers of the method of separating the pressor and oxytocic principles had found no difficulty in expressing the activity of their univalent preparations in pressor and oxytocic units of the international standard powder. It was accordingly of opinion that, if the anti-diuretic action should prove to be due to yet another principle, it might be possible to express its activity in units of anti-diuretic action of the same standard powder, but considered that further evidence of the uniformity and stability was necessary as regards this action of different preparations of the standard.

The Commission accordingly recommended, in the light of the uniformly favourable results obtained since the Geneva

Conference, that the dry preparation of the acetone-extracted fresh posterior lobe substance of ox pituitary be now definitely adopted as the international standard preparation for the biological evaluation of preparations of the posterior lobe of the pituitary body, whether containing all the active principles of the lobe, or the pressor or oxytocic principle only, in separate solution.

It further recommended that the activity of any such preparation should be expressed in relation to the international standard in units of pressor or oxytocic activity and that, in each case, the unit of such activity should be that of 0.5 milligram of the standard powder.

Concerning the suitability of the standard for assay of the anti-diuretic action, the Commission expressed no final opinion.

With regard to recommendation No. 6 of the Geneva Conference, the following amended draft was adopted:—

“ That, in making the assay for oxytocic action on the guinea-pig's uterus, it is recommended that a test for non-specific, stimulant activity on that organ should be applied. This can be done by treating the extract under examination with normal NaOH for one hour at the ordinary temperature, neutralising to litmus paper, and re-testing on the guinea-pig's uterus; or the untreated extract may be tested for such non-specific, stimulant action on the isolated guinea-pig's intestine in comparison with histamine. Neither method should show an activity corresponding to that of more than 0.1 milligram of histamine (base) per 10 units of specific oxytocic activity. ”

Recommendation No. 7. In view of the evidence before them that the methods of indicating the strength of extracts in the units recommended by the Geneva Conference had been found of practical value in different countries and particularly in the United States of America, where the standard, but not the unit, has already been adopted in the Pharmacopœia, the Commission decided to endorse recommendation No. 7 of the Geneva Conference of 1925, with modifications, as follows:

“ That the strength of all extracts of the pituitary posterior lobes should be expressed in units of activity, the activity extracted from 0.5 milligram of the standard powder under the conditions above indicated being defined as one unit. ”

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