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**A Study of Variation of Salmonella  
Pullorum**

By H. Van Roekel

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Bacterial variation has played an important role in bacteriology and related fields of science. From a practical point of view, and particularly in the diagnosis, control and eradication of human and animal diseases, it has presented problems of great importance. Since bacterial variation has received little attention in the study of pullorum disease, it was the purpose of this investigation to add to the knowledge on this subject.

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MASSACHUSETTS STATE COLLEGE  
AMHERST, MASS.

47605

## A STUDY OF VARIATION OF SALMONELLA PULLORUM<sup>1</sup>

By H. Van Roekel, Chief of Laboratory  
Poultry Disease Control

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### INTRODUCTION

Bacterial variation has been observed in numerous species of microorganisms, according to Hadley, Löhnis and others. It is of interest to note that this phenomenon or group of phenomena was recognized by some of the early investigators in bacteriology, contrary to the generally accepted view that individual species maintain their morphological and other characteristics, with great constancy.

Bacterial variation has received little attention in the study of pullorum disease. From the many reports that have been published since the isolation of the etiological agent by Rettger in 1899, we were until quite recently led to believe that the organism is very stable in its morphological, cultural, pathogenic, and serological properties. Variations in carbohydrate fermentations have been reported, but the differences have been attributable in most instances to such factors as culture media, incubation temperature, observation time, etc.

Eporititics due to *S. pullorum* have been found to occur in varying degrees of intensity, showing that the organism may fluctuate widely in pathogenicity. A most striking case may be cited in the fatal outbreak observed by Plastridge and Rettger (1930) among young and adult domestic fowl. In this instance the adult stock suffered rather heavy losses, which is, indeed, very uncommon in natural outbreaks. Further details of this outbreak will be discussed later.

Dearstyne, Kaupp, and Wilfong (1929) detected intermittent and low titre reacting birds, but the relation of such reactions to the infecting organism was not investigated.

The employment of the macroscopic agglutination test as a diagnostic means of detecting "carriers" of the causative organism has been and is recognized as a progressive step in the control and eradication of the disease. While the testing method has been improved and standardized to a high degree of efficiency, yet certain important and perplexing problems still remain to be solved. In the routine testing of flocks, doubtful or non-specific reacting birds may be encountered in small numbers in most flocks and in large numbers in occasional flocks. Whether or not this problem will prove to be an everpresent one appears dependent upon the insight that may be gained concerning the reagents and the factors involved in antibody production and the agglutination phenomenon. To what extent bacterial variation enters into this problem in nature is a question.

It has been recognized for several decades that suitable strains are necessary to produce a satisfactory antigen for the agglutination test. While attempts have been made to designate certain strains and methods as standard in order to overcome serological discrepancies and difficulties, certain problems still remain. This fact has been observed in the eradication of pullorum disease where the macroscopic agglutination test was employed. Further knowledge concerning bacterial variation of *S. pullorum* which will, in a measure at least, solve our present problems may be anticipated.

This investigation was stimulated by the fact that our present understanding

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with regard to variation of *S. pullorum* is, indeed, very limited, as has been shown by Mallman (1932). It was deemed advisable to determine to what extent variants of this organism occur in nature, as well as to what extent they may be present under certain laboratory conditions; and also to study some of the morphological, cultural, pathogenic, and serological properties of the variants, with the purpose of offering an explanation for problems encountered in our present diagnostic methods.

### HISTORICAL REVIEW

The field of bacterial variation has attracted considerable interest and caused much controversy among investigators since the early days of bacteriology. Apparently two strongly opposing schools on the subject of variability and mutation existed. The one subscribed to the monomorphic view, while the other supported the pleomorphic concept that microorganisms are extremely variable in their characteristics, especially when subjected to changed external conditions. The monomorphists maintained that bacterial species preserve their morphological and other characters with great constancy, and that all deviations from the normal type are to be considered as contaminations or so-called involution forms.

Since the literature on all phases of variation, dissociation and mutation has been so voluminous, the reader is referred to the reviews published by Löhnis (1922), Hadley (1927) and Arkwright (1930). In the résumé of the literature cited here it is deemed advisable and expedient to present some of the outstanding observations of general interest, as well as those contributions which are related to the phases included in the problem under investigation.

The extreme views of Nägeli (1877) concerning variability are of interest. He believed that all species of bacteria are highly variable and capable of passing from one morphological state to another, as well as undergoing profound physiological changes. Furthermore, he claimed that one species can be the etiological agent in various forms of disease.

For a decade or so after the views of Nägeli were presented very little was published on the subject of variation.

Chamberlain and Roux (1883) observed that anthrax cultures became asporogenic when subjected to phenol and other antiseptics. This characteristic was retained even upon passage through guinea pigs, rabbits, and sheep. Later Roux (1890) reported that the virulence of the asporogenic type was reduced, but that it could be raised by passage through susceptible animals. In cultural and physiological properties this type resembled the sporogenic form. The author suggested that the loss and gain in virulence may occur under natural conditions, and that the so-called saprophytes may progressively adapt themselves to a parasitic mode of living.

Bacteria cultivated in the presence of small amounts of agglutinating serum were reported by Durham (1898) to exhibit departures from their normal habit of growth. Morphologically the organisms revealed a tendency to grow in chains or threads, which were normally absent.

Nicolle (1898) observed spontaneous clumping of broth cultures of *Bact. typhosum* to the extent that they were unsuitable for the agglutination test. The phenomenon was similar to that observed when the culture was placed in contact with immune serum. Cultures exhibiting spontaneous agglutination possessed slight sensitivity toward serum agglutination. Repeated inoculation into serum-broth medium failed to eliminate the property described.

Horton-Smith (1900) encountered considerable variation in the agglutinability

of some typhoid strains. He found that a broth culture may manifest inagglutinability, while a suspension of the same strain prepared from an agar slant is agglutinable.

Spontaneous clumping was also observed among typhoid strains by Savage (1901), who emphasized the fact that this phenomenon may be of great practical importance, since it may lead to errors of diagnosis in routine work. An investigation of the conditions necessary for the formation of these clumps revealed that the phenomenon occurred within 24 hours, independent of age of cultures and of scum formation. Organisms suspended in saline solution, peptone-water, and broth exhibited clumping only in the broth.

Preisz (1904) found that *B. anthracis*, when cultivated on slightly alkalized agar, developed papillae or secondary colonies, which appeared as contaminants, and after several weeks might give rise to tertiary colonies. Marked differences in the morphology of the bacilli belonging to the primary and secondary colonies were observed.

Hiss (1904) observed that *Bact. dysenteriae* (type "Y"), when subjected to frequent transfer in maltose medium, acquired the property of attacking that carbohydrate more rapidly.

Steinhardt (1904) was able to demonstrate the influence of serum on the virulence of paradysentery and typhoid organisms. Normal horse serum exhibited little influence, while inactive and immune sera reduced the virulence. He isolated a spontaneously agglutinating strain from a typhoid stock culture. The colonies of the agglutinating type possessed a more irregular surface and greater opacity than the stable strain.

Eisenberg (1906) observed granulation formation in typhoid and other colonies after the addition of egg albumin to the medium. This appearance was obtained only in old cultures. He believed that this type of development was due to the detachment from the colonies of bacilli which penetrated more deeply into the medium and gave rise to small adjacent colonies.

The investigation of Massini (1907) definitely revealed that a lactose-fermenting organism was derived from a non-lactose-fermenting strain, and that it was absolutely stable. On lactose-fuchsin agar it formed white colonies, but after a short incubation period red papillae developed. This occurred only in the presence of lactose, and the new characters could be removed by subjecting the organism to phenol medium. An homologous antiserum for the white strain agglutinated both the white and red strains to the same degree. Likewise, an antiserum prepared against the red organism agglutinated both types. Histological sections of the papillated colonies revealed light and dark streaks which extended throughout the colony mass. Certain layers in the colony stained with different intensities.

Peckham (1907) was successful in accentuating the proteolytic activity of the typical colon bacillus, and in stimulating indol formation by atypical colon organisms that had been devoid of it, and by typical typhoid bacilli. He also stated that *Bact. coli-communis* at times possesses pathogenic properties, and that by artificial methods of treatment it may often be brought from a condition of benignity to one of virulence.

Twort (1907) was able to induce *Bact. typhosum* to ferment dulcitol and lactose by cultivating the organism in the respective media and transferring at bi-weekly intervals over a long period of time. Likewise, *Bact. paratyphosum B* acquired the power of fermenting saccharose. He concluded that by this means a pathogenic organism may be altered until it gives fermentative reactions characteristic of a non-pathogenic member of its group. Furthermore, he suggested that this process may occur in nature.

Goodman (1908), by artificial selection, obtained a strain of *C. diphtheriae* which had moderate power of producing acid in dextrose broth, and two strains possessing respectively a greatly augmented and greatly diminished power of acid production. He suggested that fermentative properties are not species characters, since the zymogenic power exhibited can be readily and markedly altered at will by artificial selection.

Buchanan and Truax (1910) attempted to select high and low acid races of *Strept. lacticus*, but found no evidence of a divergence of high and low races among the cultures studied. They concluded that impressed variations did not appear to be heritable and that continued growth of *Strept. lacticus* under favorable conditions seemed to render the organism less variable.

Jacobsen (1910) isolated a typhoid strain which he designated typhoid mutabile, since it was characterized by a restricted colony type possessing slight agglutinability and yielding secondary colonies which resembled typical typhoid in morphology and serological behavior.

Burri (1910) encountered an organism belonging to the coli-typhoid group which he designated *Bact. imperfectum*, since it was unable to ferment saccharose, but, when cultivated in the presence of this carbohydrate, certain colonies acquired this property. The latter he named *Bact. perfectum*. Since the ability to ferment saccharose was acquired gradually, he concluded that this property appeared to be latent in the *imperfectum* type.

Typhoid strains could be trained to attack dulcitol, according to Penfold (1911). Non-fermenting colonies on dulcitol agar plates revealed after five days or more secondary colonies as papillae which might or might not possess an acid reaction. He further observed that *Bact. typhosum* could be trained to ferment lactose only with very great difficulty, and that the newly acquired property was very unstable and transient. He concluded that papillae formation indicates that variation affects only very few individuals of a colony to a great extent, and does not guarantee permanency in the character.

Rettger and Sherrick (1911) stated that bacterial variation may be brought about by artificial selection. They maintained that variation does not depend solely upon changes in nutriment, environmental conditions, etc., but that it is frequently brought about through inherent properties within the organism itself. By artificial selection they obtained from a slightly pigmented strain of *B. prodigiosus* two strains showing in one case brilliant coloration and in the other complete absence of color. Also, they observed the return of the pigment-producing property in a strain of *Ps. pyocyanea* which had previously been very nearly colorless.

Müller (1911), working with typhoid and known pseudo-dysentery bacteria, observed secondary colonies when growing these organisms on rhamnose agar. He regarded this as a new characteristic comparable to that of *Bacterium coli mutabile*, and further observed the same forms on old gelatin cultures of typhoid.

Dobell (1912) concluded that bacteria are subject to mutation in that a given race may give rise to a new race which differs from the parent strain in its genetic make-up. The new characters of the race may be transmitted for many generations.

Revis (1912) subjected a typical strain of *Bact. coli* to peptone broth which contained malachite green. Subsequent culture on ordinary agar yielded large, viscous, circular masses, which consisted of a mixture of very long filaments and short bacilli, together with a gummy cementing substance. He found this strain to differ physiologically, morphologically and culturally from the typical *Bact. coli*.

A temporary alteration in cultural and antigenic properties of *Staphylococcus aureus* was demonstrated by Abbot (1912). Pigment formation and proteolytic action were increased, while the agglutinability was decreased. No modification was observed in its pathogenicity, carbohydrate-splitting power and ability to clot milk.

Baerthlein (1912) isolated several coli-like organisms from the intestinal tract which appeared colorless on Endo-agar, but formed red secondary colonies. These mutants gave rise to further mutants which were characterized by their colonial, cellular, and serological differences. Two colony types were detected: the one a transparent type containing long, slender filaments, and the other opaque containing short, plump bacilli. The opaque type appeared to be antigenic but inagglutinable with homologous and heterologous antisera.

From studies on strains isolated from excreta of suspected typhoid carriers Penfold (1912) concluded that lactose mutation appears to be a common property of many bacterial species, and that it has no specific differentiating value. The power to vary quickly in respect to dulcitol fermentation appears to belong to every strain of *Bact. typhosum*. Furthermore, the inability to vary quickly in respect to certain carbohydrates would appear to be as characteristic of certain species as the power to vary.

Eisenberg (1912) observed numerous colonial and cellular types in his study of the cholera vibrio.

Rowland (1914) also demonstrated variation in cellular forms of the plague bacillus. He further observed that the virulence and immunizing power of this organism can be greatly modified when cultivated in various culture media.

Cole and Wright (1916) concluded that most of the variation observed in ordinary cultures is due to selection of pre-existent biotypes by the investigator or by the environment. Certain biotypes may be entirely lost if the process of selection is complete, and the culture will not return to the original type, even when placed again under normal conditions. If the selection process is incomplete, certain biotypes become predominant and others become suppressed, so that the return to normal conditions brings out the dormant types adapted to those conditions, and the culture reveals its original characteristics.

In 1917 Jordan made a comparative study of biochemical and agglutinative properties of some members of the *Salmonella* group. He offered the following conclusion: "Old stock cultures that have been in laboratory collections for some years often show variations, irregularities, and departures from type. Some give reactions, biochemical and agglutinative, that they did not give when first isolated. On the other hand, some strains that apparently possessed unusual fermentative reactions when first isolated later show conformity to type".

Eisenberg (1918), in one of his series of papers on variation reported a large number of strains in the coli-paratyphoid-dysentery group. He observed variation in size, transparency and structure of colony, mucoid property, biochemical activity, gelatin liquefaction, pigment production, and reducing power. The coli-mutable strains possessed the power to change suddenly. Dwarf colonies were detected among typhoid, paratyphoid B, and dysentery strains, as well as secondary colonies among the latter.

A classical work on variation appeared in 1918 by Baerthlein, which included an extensive study of colony variation and other characteristics in fourteen bacterial species. He demonstrated numerous colony types and correlated other important characteristics, such as cell morphology, slime and pigment production, biochemical and serological reactions, and virulence. His observations greatly added to the knowledge of bacterial variation.

An inagglutinable strain of the Shiga dysentery bacillus was experimentally produced by Benians (1919) from an agglutinable strain, by subcutaneous inoculation into a guinea pig of the agglutinable bacteria suspended in mucilage of tragacanth.

Dawson (1919) in an extensive study of bacterial variation induced by changes in the composition of culture media observed that constituents in the medium stimulated variation from the simplest to the most complex degree, which in some cases practically amounted to the production of a new strain.

Mackie (1920) reported variants obtained from *B. vesiculosus* when growing the original type on brilliant green agar. The variants exhibited differences in colonial and cellular morphology and in agglutinating ability. One variant strain possessed a higher degree of agglutinability than its parent strain.

Arkwright in 1920 introduced the terms "S" and "R" to designate so-called "smooth" and "rough" colonies. He obtained from a single strain two forms, one a "smooth" which formed a stable suspension in physiological saline, and the other a "rough" which agglutinated in saline solution without the addition of serum. These forms were derived from *Bact. dysenteriae* (Shiga), *Bact. typhosum*, *Bact. paratyphosum B*, and *Bact. dysenteriae* (Flexner). Other distinguishing characteristics, such as behavior in broth, and antigenic and agglutinative properties, were ascribed to the two forms. In 1921 Arkwright further reported that "S" and "R" forms, as well as intermediate forms of *Bact. dysenteriae* (Shiga) proved to be virulent for rabbits. It was further stated that to change one form into the other, after they were apparently quite pure, was more difficult than when unselected cultures were used which had shown "S" and "R" characters.

In 1921 De Kruif reported two types of organisms as existing in a culture of *Past. leipseptica*, recently isolated from a spontaneous infection. The two organisms differed greatly in their degree of virulence, as well as in other characteristics. The features of each type were constant through many passages in artificial media.

Non-specificity of agglutination with "rough" variants was reported by Schütze in 1921. He defined a substrain variant as one which contained less agglutinating antigen than another of the same type. It absorbed and agglutinated the serum of a superstrain, but not to the extent that the homologous strain did. On the other hand, the superstrain agglutinated and absorbed the serum of the substrain to its titre limit. The author also referred to "serological cosmopolitanism" among rough cultures, which signified that two rough members derived from unrelated sources possessed serological relationships.

In 1922 Löhnis presented an extensive review and discussion on the subject of variation and life cycles of bacteria, with special reference to the different phases in the life history of microorganisms. He maintained that all the bacteria studied live alternately in an organized and in an amorphous or symplastic stage, and that the life cycle of each species is composed of several subcycles showing wide morphological and physiological differences.

Arkwright (1924) observed that variants sensitive to bacteriophage arose spontaneously in old normal cultures from resistant strains; also, that sensitive variants which were studied had always been "rough", that is, were agglutinated by salt (0.85%). However, not all "rough" variants had been sensitive to phage action, although all of the "smooth" cultures examined were resistant to phage. The variants obtained by phage action and without the addition of phage were very similar.

In 1924 Arkwright and Goyle attempted to determine whether the "S" and "R" forms correspond in any way to the "O" and "H" forms of Weil and Felix. The analogies that the authors have advanced do not appear justified from the

standpoint of the organism and tests employed.

Savage and White (1925) stated that in direct platings from pathological material, colonies of *Salmonella* bacilli and other intestinal pathogens were, as a rule, wholly smooth. Occasionally, however, roughness might be detected in the primary cultures. They demonstrated in a few experiments that "rough" forms possess a lower pathogenic and invasive power than the smooth type. Also, they inferred that in nature a process of natural selection tending to smoothness doubtless occurs, but that this presumably consists in the weeding out of "rough" variants which may have low pathogenicity and penetrating power. They suggested maintaining smooth cultures either by animal passage or by using unheated culture medium.

In 1925 Enderlein described in great detail the phenomenon of cyclogeny. He defined cyclogeny of bacteria as a cycle of morphological development commencing with the simplest morphological unit, "mychit", passing through the highest morphological structure, and then returning to the "mychit" stage. The different stages of morphological structure were designated as cyclostages, and a single passage through a cyclogenic development process was called a cyclode, which had a peak in its development that was designated as "Kulmination". Virulence and pathogenicity were associated with the parasitic cyclostage, which was the virostage.

Jordan (1926) demonstrated that single cell strains of paratyphoid bacilli of the "R" and "S" types could more or less regularly be made to yield cells of the opposite type by appropriate treatment.

White (1926) called attention to the effect of alcohol on rough variants. Rough forms were extracted with 96 per cent alcohol, which conferred upon the most salt-sensitive strains a degree of suspension stability at least equal to that of the normal smooth races. Among the 21 rough strains examined in the various *Salmonella* types, he did not encounter an exception or partial exception to this rule. Agglutination tests were performed satisfactorily with 0.85 per cent NaCl, and even at twice this concentration.

In 1927 Webster and Pritchett reported that smooth colony types of paratyphoid-enteritidis bacilli prevailed throughout the various stages of rodent typhoid infection. In the same year Webster and Burn reported the effects of external conditions in the occurrence of smooth, mucoid, and rough colony types in *Bact. enteritidis*.

Goyle (1927) was successful in deriving two forms (N and R) from *Bact. typhosum* and *Bact. enteritidis* by plating old broth cultures. The colonies of the "R" form differed from those of the "N" form in being larger and in having a surface which was markedly granular. An "S" form was obtained by growing the "N" type on phenol-agar, but it did not remain as a permanent variant. He observed that the "N", "S", and "R" forms differed in their antigenic, absorbing, and agglutinating properties.

In 1927 Arkwright reported that variants may be distinguished by a great variety of characters, some of which vary quite independently, while others are closely linked or correlated. Virulence of a culture and its value as a prophylactic agent may often be foretold, without animal inoculation, from purely cultural or cultural and serological features. He found that vaccines prepared from "R" forms gave comparatively slight protection, while "S" forms made efficient vaccines.

In 1927 Gwatkin reported the isolation of a weak pullorum phage from an abscess in a rabbit inoculated with *Bact. pullorum*. Pullorum cultures placed in contact with this phage yielded two types of colonies. The individual bacteria



in one type of colony were coccus-like and were not agglutinated by pullorum serum, but gave the same fermentation reactions as the normal type.

It was observed by White (1928) that rough organisms which were agglutinated in the presence of NaCl (0.85%), were inagglutinable in the presence of the same salt concentration after being extracted with alcohol and chloroform. However, if the rough suspension was heated to 100°C. after treatment with alcohol and chloroform, it was again rendered unstable in saline (0.85%). Smooth strains treated in the same manner remained unchanged. The author concluded that the reaction of denatured rough protein to electrolytes afforded a final test for roughness. It was further observed that the most salt-sensitive races were by no means of necessity those which were the most serum-sensitive. It was suggested that the serum-sensitive reaction was due to a special substance or group of substances which appeared to be restricted to certain strains or types. The relation to normal agglutinins was also brought out. The isolation of a soluble specific substance from the smooth races and not the rough was recorded. He regarded the soluble specific substance as the specific element of the smooth body complex of the *Salmonellas*. Circumstantial evidence pointed to a relation between soluble specific substance and salt-sensitiveness manifested by certain strains.

In variation studies with *B. subtilis*, Soule (1928) found that normal rabbit serum enhanced variation of this organism, while serum homologous to the variant stimulated reversion to the normal type.

Wilson (1928) reported that daily subculture in broth of *Bact. aertrycke*, (Mutton type), led in about four weeks to a marked change in the composition of the culture, the great majority of the virulent organisms present at the start being replaced by completely avirulent ones.

Lancefield and Todd (1928) showed that rabbit sera prepared against "Matt" hemolytic streptococci, whether virulent or avirulent for mice, contained type-specific antibody, while sera prepared against completely degraded glossy organisms contained no type-specific antibody.

Two serologically active protein substances and another that was non-protein were isolated by Furth and Landsteiner (1928) from *Bact. typhosum*. Cross-testing of non-protein substances of various species with the corresponding sera revealed a strong group reaction between *Bact. typhosum* and *Bact. enteritidis*.

Li (1929) reported that "S" forms of the hog cholera bacillus were virulent, while the "R" forms were not. Certain types of variants remained unchanged by serial animal passage. Successful results were obtained in attempting to produce a definite immunity against the virulent "S" forms with the living "R" forms.

Koser and Styron (1930) demonstrated that smooth forms of *Bacterium dysenteriae*, Sonne, could be formed from rough forms quite regularly by rapid, successive transfers in dextrose broth incubated at 37°C. In some cases the reversion appeared to be complete, and the rough forms totally disappeared. Rough cultures held on agar slants at room temperature were much more stable than when subjected to daily transfers on the same medium. A tendency toward spontaneous clumping in 0.85 per cent saline solution was observed in the relatively smooth cultures derived from the rough forms.

In 1930 Plastridge and Rettger reported a most unusual outbreak of pullorum disease. The nature of this outbreak appeared to be an acute septicemia among chicks and mature fowls which manifested marked pathological changes and suffered a heavy mortality. The causative organism isolated from the infected birds presented characteristics which had not been reported for *S. pullorum*. It failed to grow on nutrient agar containing meat extract, and grew indifferently

on meat infusion agar. On pneumo-agar the organism developed reasonably well, while abundant growth was observed on liver infusion agar. Two types of colonies were observed on pneumo-agar which did not lend themselves to further propagation by transfer, except in one or two instances. The organism exhibited marked pleomorphism. The pleomorphic forms did not stain uniformly, and in some cases the violet color was retained in the Gram stain. Small cells resembling typical *S. pullorum* were observed among the larger pleomorphic forms. Visibly infected birds tested with known pullorum antigen possessed a high agglutinating titre. The organism recovered from these birds was agglutinable, and capable of attacking the same fermentable substances as *S. pullorum*.

White (1932) stated that, in addition to the carbohydrate haptenes which characterize the "S" and "R" forms of *Salmonella* bacilli, an alcohol-soluble protein which he termed "Q" was concerned in the somatic serology of these organisms. This substance was readily extracted by warm acid-alcohol and was a full antigen which stimulated the development of potent precipitating antibodies. It occurred in all of the serological types and variant forms of the *Salmonella* group. Besides the ordinary "S" and "R" forms of *Salmonella*, he referred to rough races derived from strains long cultured in the laboratory, which lacked the "S" and "R" carbohydrate haptenes, and in which no corresponding constituent had yet been demonstrated. These races he designated "p"-forms which agglutinated much more vigorously than did ordinary "R"-forms. The removal of the "Q" fraction markedly reduced the somatic agglutinability of the treated bacilli.

In 1932 Mallmann reported on dissociation of *S. pullorum* and related species. His studies were entirely confined to old stock cultures of *S. pullorum*. He classified his strains according to colony form into three groups: smooth, intermediate, and rough. Other colony types, such as mucoid and opaque, were completely ignored. In the investigation he observed two types of variation, the one involving the intermediate form, and the other the smooth and rough forms. The intermediate type was found to fluctuate in the direction of either "S" or "R" or both when subjected to different environments, but when the incitants were removed they would return to the intermediate type. In the study of the second type of variation, rough and smooth immune serum broth was seeded with rough and smooth strains, respectively, and incubated at optimum temperatures. Only temporary changes in colony forms were observed. Smooth cultures were changed to partially rough types by exposing them to bacteriophage, and rough types were converted to partially rough types by animal passage. When cultures were maintained in stock on liver infusion agar or gelatin-agar, the various strains tended to return to their normal type. Intermediate strains varied at times from one type to another. Liver infusion agar frequently caused stable smooth types to appear as rough types, but the condition was regarded as purely transitory. Electrophoretic measurements failed to show any significant changes due to animal passage.

Plastridge and Rettger (1932) reported different colony types of *S. pullorum* observed on various agar media. Meat and liver infusion agars produced more pronounced rough types than plain meat extract agar. Pleomorphic cellular forms, varying greatly in size, were encountered. Phage-treated strains yielded various colonial and cellular types. The phage action in some instances markedly affected the agglutinating properties of *S. pullorum*. A bacteriophage that was highly active against *S. pullorum* cells was obtained in extracts of livers and intestinal contents of adult birds surviving an acute attack of the disease.

White (1933) reported the isolation of a "T" substance from *Salmonella* bacilli by means of an extracting fluid containing 75 per cent alcohol, sodium chloride,

and hydrochloric acid. This fraction, protein in nature, was extracted from "S", "R", and "p" races of several *Salmonella* types. It was concluded that the "T" fraction was more deeply situated in the organism than were the "S", "R", and "p" receptors, and could engage with its universal antibody only when the bacterial surface had been partly cleared of impediments as a result of washing, or during autolysis.

Henry (1933) observed that the "R" forms of *Br. abortus* exhibited less invasive power for the natural host than the "S" type. This relation likewise was true for agglutinogenic properties. Cultural examination of milk from reacting cows in various stages of lactation revealed no "R" types.

MacKenzie and Fitzgerald (1933) demonstrated qualitative and quantitative alterations in agglutinative, absorptive, and agglutinogenic properties of *Salmonella* and *Shigella* strains. The antigenic behavior was found to vary independently of colonial morphology and mode of growth in fluid media. The convergence phenomenon was observed in which variants, derived from different species, manifested serological identity.

Rettger and Gillespie (1933) found that cellular morphology varied with the medium employed and with the temperature and duration of incubation, in working with *B. megatherium*.

Stearn and Stearn (1933) in a series of papers referred to the fact that different H-ion concentrations may cause various types of growth with the same organism.

Murphy<sup>2</sup> in an attempt to induce variation in *S. pullorum* strains, observed that the variants failed to remain stable and that reversion to the so-called "normal" occurred on subsequent transfers.

In a recent paper by Plastridge and Rettger (1934) virulence studies on *S. pullorum* were reported. The strains employed were variants isolated from natural outbreaks or derived in the laboratory. The test animals included chicks, adult hens, rabbits and guinea pigs. Marked differences were observed in the virulence of the individual strains of *S. pullorum* for these animals. Some strains were found to be highly virulent for both chicks and adults; others possessed a low degree of pathogenicity for chicks, but were highly virulent for adult birds. Some strains were relatively avirulent for both young and adult chickens. Pronounced changes in virulence of some strains of *S. pullorum* were induced by the action of bacteriophage. The passage of strains through the animals had, in general, no appreciable effect on morphological, colonial, and agglutinative characteristics of the variants studied.

#### THE INCIDENCE OF VARIANTS AMONG NEWLY ISOLATED STRAINS

In the identification of *S. pullorum* strains several properties such as colonial and cellular morphology, Gram stain reaction, biochemical reactions, and serological response are studied. While this organism possesses certain features in common with some of the other members of the *Salmonella* group, yet as a rule it can be isolated and differentiated with ease. Associated with its cultural habits is a considerable tolerance to fluctuation in environmental conditions without leading to death of the microorganism. In spite of the fact that *S. pullorum* is not as selective in its growth environment as some organisms, certain conditions may bring about profound changes in one or more of its characters, as will be discussed later.

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<sup>2</sup>Unpublished data (1933).

In order to detect variants among any strain of bacteria, it is necessary to establish a criterion for a so-called "normal" or typical strain. Rettger (1900) first described the organism as a Gram-negative, actively motile, non-liquefying, non-chromogenic, facultatively anaerobic bacillus with slightly rounded ends. When grown on agar the bacilli vary in size from 0.3—0.5 micron in breadth and from one to two microns in length. On agar plates the colonies are small (one-fifth of a millimeter) and white after twenty-four hours of incubation, but they may attain a diameter of one millimeter after several days of incubation. The colonies vary in form from oval, kidney-shaped, or even spindle-shaped, to round. In almost every case the colony surface is marked by a peculiar rosette figure.

Rettger and Harvey in 1908, after a more extensive study of the disease, concluded that the organism is a long, slender bacillus (.3—5  $\times$  1—2.5 microns) with slightly rounded ends. It usually occurs single, chains of more than two bacilli being rarely found. It is Gram-negative and stains readily by the ordinary basic anilin dyes. It is non-motile, non-liquefying, non-chromogenic, non-sporogenic, and facultatively anaerobic. On agar plates the colonies appear white and small at the end of twenty-four hours. They increase in size slowly and seldom attain more than one millimeter in diameter, even after a few days incubation. Under the microscope they appear yellow and vary in shape from oval and spindle-shaped to round. A rosette surface is usually demonstrable. On slant agar the growth resembled that of the typhoid bacillus.

Rettger and Plastringe (1932) in their recent monograph on pullorum disease state that in heavily seeded meat extract slants or plates the colonies are discrete and appear more or less dewdrop-like. When isolated on meat infusion or liver infusion agar the growth is more luxuriant, with characteristics approaching those of the coli-typhi group. Rosette figures are usually present on the colony surface.

In the examination of primary isolations the author accepted the aforementioned characteristics as a criterion for normal or typical *S. pullorum*. However, among the strains investigated it was observed that certain characteristic features may be added to those described above. On plain meat extract agar (pH 7.0—7.2) heavily seeded with inoculum, the colonies appear discrete, smooth, glistening, homogeneous, entire, dome-shaped, transparent, and varying in form from round to angular. On chicken infusion agar the growth is slightly more luxuriant, with colonies possessing a lesser degree of transparency. On liver infusion agar the growth is even more luxuriant than on meat infusion medium. The colonies are smooth, round, entire, glistening, homogeneous, markedly translucent, and extremely dome-shaped. Congested colonies remain small (1 mm. or less), but isolated colonies may attain a diameter of three to four millimeters or more. Surface markings may appear as the colony increases in size and age, but as a rule the young colony on a heavily seeded plate changes little with age.

The cellular morphology of the organisms taken from growth on meat extract agar resembled closely the description cited above. In most smears an occasional filament and large cell was observed. Similar observations were made in smears prepared from growth on meat infusion agar. However, a most striking picture was noted in smears prepared from colonies on liver infusion agar. The cells were considerably larger, stained with different degrees of intensity, and varied quite markedly in their shapes. This cellular response will be discussed further in another section.

In the fermentative studies the following substances were employed to identify *S. pullorum*: dextrose, maltose, lactose, sucrose, and dulcitol. The production of acid and gas or acid only in dextrose was considered typical of the organism.

Antigens were prepared by removing the growth from young cultures with 0.9

per cent NaCl solution which contained 0.5 per cent phenol. These antigens were tested with negative and positive pullorum sera in dilutions of 1:10 and higher. The results of the test were recorded after 48 hours incubation.

All strains were stained by the Gram method.

The cultures were obtained from several different sources, including eggs, chicks, and mature birds. A large number of cultures was received from my associates in the Department of Veterinary Science. The original cultures received from Amherst were isolated on chicken meat infusion agar plates. The age of the culture at the time of the examination varied from three to seven days. In the majority of cases plain meat extract agar plates were streaked with the primary cultures, in order to determine the character of the growth on meat extract agar during the various incubation periods.

Primary isolations of *S. pullorum* from chicks and adult birds were also received from various members of the Yale Department of Bacteriology.

The Department of Veterinary Science also furnished fresh eggs laid by hens whose sera reacted with pullorum antigen. Among 176 eggs cultured, 39 yielded *S. pullorum*. As a matter of interest concerning the bacteriological examinations of the eggs, it may be stated that the percentage of isolation is markedly raised by increasing the incubation period from two to six days. No definite explanation is offered as to why the presence of the organism is not detected by streaking on agar plate with egg yolk-broth mixture which has been incubated only 48 hours. In every instance the eggs were incubated in the shell for a period of seven days before being cultured. It was also observed that occasionally eggs contain particles of necrotic tissue which have been picked up in the abdominal cavity by the oviduct and expelled as part of the egg. Such bodies may be found in the albuminous portion. By careful technique the yolk and the foreign body may be cultured separately. In such cases it was observed that the yolk may be sterile, while the necrotic tissue yields *S. pullorum*. Hence, it appears important, in the bacteriological examination of fresh eggs, to recognize the fact that the organism may exist in other portions of the egg aside from the yolk. Also this observation suggests that the disease may be transmitted from adult to chick by means of such infective foreign bodies, instead of the yolk in every instance, as is the common belief.

A total of 163 strains was examined as to colonial morphology, Gram stain reactions, carbohydrate fermentations, and agglutinability. During a period of approximately fourteen months, 69 strains were isolated from maturing and adult birds, 55 strains from chicks and 39 strains from fresh eggs. The organism was recovered from various organs, tissues and diseased processes.

Thirteen of the 163 strains did not resemble typical *S. pullorum* in every respect. Table 1 shows that the majority of these 13 strains were salt sensitive, exhibiting a tendency to settle out in normal NaCl solution.

In the original isolation the colonial and cellular morphology, carbohydrate reaction and Gram-staining properties resembled those of *S. pullorum*, except in two strains, 3 and 12. The latter exhibited a rough type of colony, but on subsequent transfers to meat extract agar and beef and liver infusion agar the growth was smooth and typical. No explanation is offered for this marked temporary roughness as manifested in the primary culture.

The different atypical strains were tested on several occasions with positive and negative pullorum sera. The results of a few of the tests are shown in Tables 2 and 3.

The antigens listed in Table 2 were prepared from meat extract agar cultures which had been incubated at 37° C. for 48 hours. The cells were suspended in

TABLE I. HISTORY OF THE NEWLY ISOLATED VARIANTS

Strain No.	Date Isolated	Source	Remarks
1	10/4/32	Hen (ovary)	Salt sensitive
2	11/21/32	Hen (ovary)	Salt sensitive
3	11/22/32	Pullet (liver)	Grew with some difficulty on plain agar, and in sugar media. Revealed a tendency to settle out in broth, colony outline irregular when small, filamentous forms numerous.
4	12/1/32	Hen (hematoma)	Salt sensitive
5	12/19/32	Hen (ovary)	Salt sensitive
6	2/3/33	Hen (peritoneum)	Salt sensitive
7	2/3/33	Hen (ovule)	Salt sensitive
8	5/26/33	Chick	Salt sensitive
9	10/20/33	Chick (spleen)	Salt sensitive
10	12/29/33	Chick (yolk)	Salt sensitive
11	1/7/34	Hen (ovule)	Salt sensitive
12	2/15/34	Hen	Rough colony on primary culture
13	11/24/33	Egg	Salt sensitive

0.9 per cent NaCl solution which contained 0.5 per cent phenol. In three instances the salt concentration was lowered to 0.22 per cent, in order to reduce the instability of the strain in saline solution. It had been observed previously that these three strains (9, 11, and 13) were salt sensitive. The concentration of the antigens was adjusted to the desired turbidity, which in the final serum-antigen mixture was equivalent to tube number 1.0 of McFarland's nephelometer scale. All strains produced a uniform, homogeneous turbidity, with the exception of No. 8, the suspensions of which appeared slightly clumpy.

The results in Table 2 show that the different strains do not behave alike as antigens in the presence of positive and negative sera and in 0.9 per cent saline solution. In the majority of the antigens it was difficult to determine whether the cells were agglutinated. Stable, well-defined clumps of cells were not visible to the naked eye, as one ordinarily observes in typical agglutination with *S. pullorum*. However, even with a so-called typical pullorum antigen, one may observe more than one type of agglutination. This fact is well brought to light when numerous agglutination tests are recorded in the routine testing of fowl sera. Arkwright (1930) stated that specific and non-specific agglutination of bacteria is a phenomenon governed by conditions which belong to the realm of colloidal physics and which are imperfectly understood at the present time. When old cultures containing autolyzed or fragmented bacterial cells are suspended in salt solution they may cause a precipitate. Under certain conditions the precipitating substance may entangle the bacterial bodies, producing a reaction that can not in reality be considered identical with true agglutination brought about by properly sensitized intact bacterial cells suspended in a suitable electrolyte solution. A comparable phenomenon is frequently observed when substances of a lipid and protein nature are precipitated under certain conditions, bringing down the bacterial cells with the precipitating material.

According to the results tabulated in Table 2, it is apparent that certain factors are operating which are difficult to explain. The character of the agglutination or settling differed among certain antigens. Some antigens appeared unstable in 0.9 per cent saline solution, but in the presence of negative serum the cells remained in suspension. In one instance (antigen 5) agglutination occurred in a dilution of 1:640, but not in 1:1280. In the latter dilution the cells remained in suspension, but settled out in the antigen control. With antigen 6, it also appears as if the serum had a stabilizing effect in maintaining the cells in suspension. On the other hand, in some cases the serum definitely appears to have an opposite effect. Furthermore, antigens may vary to such an extent in their mosaic that they may react differently when placed under certain uniform conditions.

TABLE 2.—REACTIONS OF VARIANTS WITH POSITIVE AND NEGATIVE PULLORUM SERA.

Strain No.	Sera	Agglutination Titre								Antigen Control	Remarks
		10	20	40	80	160	320	640	1280		
1	{P {N	4?	4?	4?	4?	4?	4?	PS	PS	PS	Muddy type of agglutination
2	{P {N	4	4	4	4	4	4	3	1	SS	
3	{P {N	4	4	4	4	4	4?	4?	3?	PS	
4	{P {N	4	4	4	4	4	4	3	2	SS	
5	{P {N	4	4	4	4	4	4	3	0	SS	
6	{P {N	4?	4?	4?	4?	2?	0	0	0	CS	Fluffy precipitate in first 5 tubes
7	{P {N	4?	4?	4?	4?	0	0	0	0	0	Muddy type of agglutination
8	{P {N	3	3	3	3	2	1	0	0	0	Antigen appeared clumpy
9	{P {N	4?	4?	4?	3?	1?	0	0	0	0	Cells suspended in .22% saline solution
10	{P {N	4?	3?	2?	0	0	0	0	0	0	
11	{P {N	4?	4?	4?	4?	3?	2?	2?	2?	0	Cells suspended in .22% saline solution
13	{P {N	3?	3?	3?	2?	2?	SS	SS	SS	0	Cells suspended in .22% saline solution

Legend: P—positive serum  
 N—negative serum  
 4—complete agglutination  
 3—incomplete agglutination  
 2—partial agglutination  
 1—slight agglutination  
 0—no agglutination  
 ?—questionable agglutination  
 CS—complete settling of cells  
 PS—partial settling of cells  
 SS—slight settling of cells

Because of the influence of salt concentration upon the ability of a strain to remain in suspension, the twelve strains were suspended in different salt concentrations (0.11, 0.45 and 0.90 per cent) and tested against positive and negative pullorum sera. The method of preparing the antigens and the technique for conducting the agglutination test were the same as employed previously.

The data presented in Table 3 confirm the observations recorded in Table 2 in many respects, and show beyond a doubt that a decrease in electrolyte will increase the stability of the organism when in suspension. In no case was settling

of cells observed in 0.11 per cent saline solution in the absence of serum. One antigen, No. 6, settled out in this salt concentration after negative serum was added, but then only in the lower serum dilutions. Antigens 6, 7, and 8 exhibited no clumping in the lower saline concentrations in the presence of positive serum, while in the higher saline concentrations fine clumps were visible. However, this clumping of cells was observed only in the first two serum dilutions. In the higher dilutions the cells had settled out and appeared similar to the antigen control. With antigen 7 definite clumping was observed in the first two dilutions, no clumping or settling in the four succeeding dilutions, but slight settling in the last and partial settling in the antigen control. It is evident that antigens 6, 7, and 8 were highly sensitive to salt and practically inagglutinable in the saline concentrations employed. Antigens 2, 4, 5, and 13 exhibited typical agglutination in the 0.9 per cent saline solution and remained in suspension in the presence of negative serum and in the antigen control. However, in the weaker salt concentrations the clumping of cells was not as marked and well defined as in a stronger concentration. Also the titre was reduced with the weaker saline concentration. It was also observed that the clumps in the lower dilutions were as a rule larger than in the higher dilutions. Furthermore, in some instances the suspension appeared more turbid after agitation in the lower dilutions than in the higher dilutions and in the antigen control. This indicated that other substances aside from the bacteria or bacterial fragments had precipitated. This phenomenon was observed even after the H-ion concentration was lowered to pH 8.4-8.6.

TABLE 3.—REACTIONS OF VARIANTS WITH POSITIVE AND NEGATIVE PULLORUM SERA IN DIFFERENT CONCENTRATIONS OF NaCl.

Strain No.	Sera	Per cent Salt	Agglutination Titre						Antigen Control	Remarks*	
			20	40	80	160	320	640			1280
1	P	.11	4	4	4	4	2	0	0	Muddy type Muddy type Fine agglutination, difficult to distinguish from settling	
	P	.45	4	4	4	4	4	0	0		
	P	.9	4	4	4	4	4	4	PS		
	N	.11	0	0	0	0	0		0	PS	
	N	.45	0	0	0	0	0		0		
	N	.9	SS	SS	SS	SS	SS	SS	0		
	2	P	.11	4	4	4	3	1	0	0	Very fine agglutination Fine type of agglutination
		P	.45	4	4	4	3	1	0	0	
		P	.9	4	4	4	4	2	1	0	
N		.11	0	0	0	0	0		0	0	
N		.45	0	0	0	0	0		0		
N		.9	0	0	0	0	0		0		
3		P	.11	4	4	4	4	2	0	0	Muddy type of agglutination Muddy type of agglutination Definite clumps in first 5 tubes
		P	.45	4	4	4	4	4	0	0	
		P	.9	4	4	4	4	4	PS	SS	
	N	.11	0	0	0	0	0		0	0	
	N	.45	0	0	0	0	0		0		
	N	.9	CSCS	CSCS	CSCS	CSCS	CSCS	CSCS	CS		
	4	P	.11	4	4	4	4	2	0	0	Very fine agglutination Very fine agglutination Fine agglutination
		P	.45	4	4	4	4	4	1	0	
		P	.9	4	4	4	4	4	2	0	
N		.11	0	0	0	0	0		0	0	
N		.45	0	0	0	0	0		0		
N		.9	0	0	0	0	0		0		
5		P	.11	4	4	4	4	3	0	0	Very fine agglutination Fine agglutination Fine agglutination
		P	.45	4	4	4	4	3	0	0	
		P	.9	4	4	4	4	4	1	0	
	N	.11	0	0	0	0	0		0	0	
	N	.45	0	0	0	0	0		0		
	N	.9	0	0	0	0	0		0		



TABLE 3.—REACTIONS OF VARIANTS WITH POSITIVE AND NEGATIVE PULLORUM SERA IN DIFFERENT CONCENTRATIONS OF NaCl.—Continued.

Strain No.	Sera	Per cent Salt	Agglutination Titre								Antigen Control	Remarks*
			20	40	80	160	320	640	1280			
6	P	.11	3	2	1	1	0	0	0		Muddy type of agglutination	
	P	.45	3	2	0	0	0	0	0		Muddy type of agglutination	
	P	.9	2	1	PS	PS	PS	PS	PS		Fine clumping in first tube	
	N	.11	PS	PS	PS	0	0			0		
	N	.45	PS	PS	0	0	0			CS		
7	P	.11	PS	PS	PS	PS	PS	0	0		No agglutination	
	P	.45	CS	PS	PS	0	0	0	0		No agglutination	
	P	.9	3	1	0	0	0	0	SS		Definite clumping in first 2 tubes	
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			PS		
8	P	.11	SS	0	0	0	0	0	0			
	P	.45	PS	SS	SS	0	0	0	0			
	P	.9	4	2	PS	PS	PS	PS	PS		Very fine clumps in tubes 1 and 2	
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			PS		
9	P	.11	4	4	4	4	1	0	0		Muddy type of agglutination	
	P	.45	4	4	4	4	4	4	0		Very fine agglutination	
	P	.9	4	4	4	4	4	4	PS		Fine agglutination in first 5 tubes, muddy in the sixth	
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			0		
10	P	.11	4	4	4	4	0	0	0		Muddy type of agglutination	
	P	.45	4	4	4	PS	PS	SS	0		Very fine agglutination in first 3 tubes	
	P	.9	4	4	4	4	4	CS	0		Clumps decrease in size with dilution	
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			0		
11	P	.11	4	4	4	2	1	0	0		Muddy type of agglutination plus settling	
	P	.45	4	4	4	4	4	4	0		Very fine agglutination, difficult to distinguish from true settling	
	P	.9	4	4	4	4	4	CS	PS		Difficult to determine amount of settling and agglutination	
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			0		
13	P	.11	4	4	4	3	2	0	0		Loose type of clumping	
	P	.45	4	4	4	4	2	0	0		Fine clumps	
	P	.9	4	4	4	4	2	0	0			
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			0		
Typical Pullorum Antigen	P	.11	4	4	4	4	1	0	0		Very fine agglutination	
	P	.45	4	4	4	3	1	0	0		Fine agglutination	
	P	.9	4	4	4	4	3	0	0			
	N	.11	0	0	0	0	0			0		
	N	.45	0	0	0	0	0			0		

\*Refer to legend of Table 2 for explanation of symbols.

The agglutinating response of the four antigens approached the normal more closely in Table 3 than in Table 2. It is likely that the organisms may have been altered slightly in composition during the interval between the two tests. Organisms which exhibit variation, especially in their antigenic structure, apparently may vary in the degree and type of reactions from time to time.

The agglutination titres were determined in the host in five cases from which the organisms were isolated. In every instance the organism was capable of

stimulating the production of agglutinins, as will be seen in the following data:—

<i>Strain Number</i>	<i>Titre of the Host</i>
2	1—160
4	1—640
6	1— 80
7	1— 80
11	1—160

No significance can be attached directly to the variation in titres, since other factors aside from the organism play a role in producing agglutinins.

In a brief summary of the data presented in this section it may be stated that variants among *S. pullorum* may be isolated from naturally infected cases. The incidence of variants as observed in this investigation amounted to 13 out of 163 cases, or 7.97 per cent. While this may be considered a significant percentage, yet from the diagnostic point of view, this figure would have been reduced to zero, since these strains were identified by tests for properties other than agglutinability and colony morphology. Of course, one is unable to state to what extent such organisms may vary further under natural conditions. The variants detected in this investigation were isolated from naturally infected eggs, chicks, and adults. The behavior of the variants in NaCl solution of various concentrations and in the presence of positive and negative sera differed greatly.

#### VARIATION INDUCED BY ARTIFICIAL METHODS

According to Hadley, Arkwright, and others, many species of bacteria, if not all, are subject to variation. Variability in one or more characters of an organism may be the result of natural environment or artificial conditions set up in the laboratory. In certain species which have been carefully studied the majority of characters have been found liable to variation. Some investigators have ventured so far as to claim the establishment of new species. These claims, however, are few in number and since such observations cannot be corroborated at will, one feels reluctant to entertain a view of this nature.

Different methods of studying variation have been described. In this investigation attention was directed chiefly towards deviations from the normal colony type (See Figures 1 and 2). A change in colony form was selected as a starting point to study the degree and type of variation. After having ascertained variation in one character, changes in other characters such as cellular morphology, biochemical and tinctorial reactions, pathogenicity, antigenicity, and agglutinability were studied. The author is cognizant of the fact that variation may occur in certain properties such as antigenic structure and virulence, without the strain undergoing macroscopic or even microscopic changes in colony form. To detect strains that show a variation in antigenic structure and virulence would of necessity involve a large number of experimental animals. Hence, it appeared expedient to select any change in colony type as a starting point for investigation, since the main objective was to determine to what extent *S. pullorum* would lend itself to variation in some of its properties under certain conditions.

The isolation of variants was accomplished by repeated selection of single colonies. While this method may have its shortcomings, success was attained in isolating variants that appeared markedly, if not entirely, stable.

In the historical résumé it is shown that variation may be induced through several means, such as nutriment, chemical agents, aging in broth, growth in serum, bacteriophage action, etc. In this investigation repeated transfer and

aging in broth were found to bring about variation in the majority of strains examined. Other methods employed proved to be less successful.

### Influence of Frequent Transfer and Aging in Beef Infusion Broth

*Experiment I.* In this experiment four strains were employed. The source and history of the different strains are as follows:—

Strain I—Received from Miss Murphy, Department of Bacteriology, Yale University,—a sub-strain of a culture isolated from the ovary of a hen in 1930.

Strain II—Received from Miss Murphy,—a sub-strain of a culture isolated from a chick in 1929.

Strain III—Received from the Department of Veterinary Science, Massachusetts State College,—isolated from a chick in November, 1932.

Strain IV—Received from the Department of Veterinary Science, Massachusetts State College,—isolated from the ovary of a hen in October, 1932.

Strains I and II were regarded as partial variants, as the result of treatment applied by Miss Murphy to bring about variation. In her attempts she was not successful in establishing and maintaining a rough type. On plain meat extract agar these two strains formed smooth and rough colonies, the latter varying in degree of roughness. In broth, Strain I produced a flaky type of sediment, with the supernatant fluid partially turbid; while Strain II gave a uniform turbidity, with moderate sedimentation. Strains III and IV resembled a typical *S. pullorum* strain in all respects.

The strains were cultivated in 10 cc. of beef infusion broth containing 1 per cent peptone, pH 6.8–7.0. Transfers were made daily for a period of two weeks. After 24 hours incubation at 37° C., the broth cultures were held at room temperature. The character of growth in all broth tubes appeared to be similar to that described above for Strains I and II. Likewise, Strains III and IV exhibited no deviation from the normal type in broth.

Meat extract agar plates were streaked with broth cultures inoculated prior to the sixth transfer. Strains I and II revealed smooth and rough colonies. Both strains produced a larger cell than did typical strains of *S. pullorum*. Cultures III and IV exhibited no change.

Each set of broth cultures, after being incubated for 24 hours at 37° C., was streaked on plain meat extract agar plates. The colony type of the different strains was little altered by frequent passage through beef infusion broth.

Sixteen days after the first set of broth tubes was inoculated all broth cultures were streaked on meat infusion agar (pH 7.0). Strain I produced a rough type of colony, with a dense center surrounded by a granular outer area which was veined. Some colonies resembled the characteristic grape-leaf type of the typhoid colony. A few smooth type colonies were also observed. Colonies approaching the rough type were more abundant in the older cultures. Strain II produced a dull, dense, granular, and entire colony which was slightly irregular in outline and raised in the center. The diameter varied from two to three millimeters. Strains III and IV exhibited colony forms which appeared to be identical with the original type.

Slight variations in colonial morphology on the different plates were observed

for each strain. However, it was apparent that daily transfer for a period of two weeks, and also aging in broth for this period, did not markedly alter the colonial appearance of strains that appeared typical at the outset.

After further aging of these cultures for a period of two months, Strains I and II had partially lost their rough colonial characteristics, while Strain IV exhibited a typical smooth colony and numerous extremely small colonies which appeared very irregular in shape. Meat extract broth tubes which were inoculated with the small colonies did not reveal as heavy a turbidity of growth as is usually observed with typical *S. pullorum*. Stained smears prepared from the small colonies contained cells that were highly pleomorphic. A peculiar characteristic of this variant (designated IV-a), was the production of very small "pin-point" colonies. The size of these colonies increased but little with prolonged incubation. Some of the widely separated colonies attained a size of one millimeter, and after selection and further cultivation of these larger colonies it was observed that they retained the peculiar characteristic small size. The pleomorphic cellular material consisted mostly of thick, short rods which appeared almost coccoid in shape. Filaments, large rods, club-forms and other "aberrant" types were observed.

As a matter of interest, the small type colony was transferred to hormone agar. The response of the organism led to the formation of a very irregular colony which possessed numerous projections and resembled the appearance of a tick. The structure of the colony appeared partly granular and partly or entirely striated. Stained smears from the striated portions showed mostly a mass of filamentous forms, whereas smears from the granular portion revealed a mixed pleomorphic picture.

In an attempt to develop a larger type of colony by artificial selection it was found that the majority of the colonies that developed resembled the small type in both colonial and cellular form. This process of colony selection was carried on for a period of two years. At the present writing both variants (IV-a—small type and IV-b—large type) exhibit characteristics which are essentially the same, but appear different from the original isolated variants. While the characteristic small size of the colony has been retained, the peculiar irregular colony shape of the original variant has been lost to a very marked degree. The colony appears almost typical, except for its extremely small size. The cellular morphology has been altered slightly in that the filamentous forms are not as numerous as in the original variant.

Other properties of these variants will be discussed in the latter part of this section (See Table 4).

*Experiment II.*—In this experiment the procedure was identical with that of Experiment I, except that 15 different strains of *S. pullorum* were employed. The history of the strains is as follows:—

<i>Strain No.</i>	<i>History</i>
I } II } III }	..... Isolated from a chick in 1933
IV } V }	..... Isolated from a hen in 1932 (Same as Strain IV in Exp. I)
VI } VII }	..... Isolated from a chick in 1933
VIII } IX }	..... Old laboratory strain which fermented maltose
	..... Isolated from a chick in 1933

- X } .....
- XI } .....
- XII } .....
- XIII ..... Isolated from a chick in 1933
- XIV ..... Isolated from a young pullet in 1932
- XV ..... Isolated from a chick in 1933

All but two of the strains exhibited characteristics typical of *S. pullorum*. Number VII was an old strain which had acquired the property of fermenting maltose since it was first isolated. In other respects it appeared to be typical. Strain XIV, which was isolated from a young pullet in 1932, revealed cultural characteristics different from *S. pullorum* in that it grew with difficulty in different media, settled out in broth culture, and produced some atypical colonies and cell forms.

The growth appearance of the different strains in broth from day to day resembled that of *S. pullorum*, with the exception of Strain XIV, which settled out in every instance. After the fifth transfer this strain was streaked on plain meat extract agar; this yielded colonies which possessed the features of the "normal" type.

Meat extract agar plates were streaked from the last set of broth cultures of the 14 inoculated sets. The character of the growth on the plates after an incubation period of 24 hours and longer at 37° C. was, in every instance, that of typical strains.

One month after the last set of broth tubes was inoculated all of the broth cultures were streaked on agar plates. All strains exhibited some degree of colony variation from the normal. However, some strains varied more than others. This was also true of a single strain which was inoculated into different broth tubes. In the majority of the broth tubes little or no variation was observed. In those instances where colony variation was most evident agar plates were again streaked. The results revealed no sign of colony variation in some of the strains, while others produced colonies that in some instances were markedly different from the typical.

Through a process of further selection of the colonies that varied from the normal, and by repeated transfer, three definite variants were obtained. Variants VIII-a and IX-a were cultivated on meat extract agar and transferred at frequent intervals, always selecting the roughest colony type. These two types appeared to have undergone a profound change in their colonial morphology; they appeared very stable. Variants VI-a and IX-a were placed on liver infusion agar and transferred at frequent intervals. Variant VI-a exhibited a colony type that was not greatly different from the typical on this medium. The degree of colonial roughness varied from time to time, but it never approached the marked roughness of Strain IX-a. The properties of the three variants will be described later in this section. The remaining strains, while they did exhibit slight variation in colony morphology, reverted to the normal type after a few transfers on agar.

Four sets of broth cultures were streaked on meat extract agar plates two and one-half months after the last set of broth tubes was inoculated. Negative results were obtained in attempts to isolate definite colony variants.

At five and one-half months the same broth cultures were again streaked on agar plates. Three variants were isolated from Strains I, II, and XV. The variant from Strain XV gradually reverted to the parent type. The other two variants (designated as I-a and II-a) were maintained on liver infusion agar through frequent transfer.

Six months after the last set of broth tubes was inoculated three additional variants, of Strains XII and XIII, were isolated from the broth culture. One variant of Strain XIII was placed on liver infusion agar, one type of variant (XII-a) of Strain XII was cultivated on meat extract agar, and the other (XII-b) was grown on liver infusion agar. The different variants will be described more fully in another part of this section. (See Table 4.)

*Experiment III.* According to the results obtained thus far, the author was inclined to believe that, while only six of fifteen strains had yielded variants, the remaining strains would also produce variants as a result of transfer and aging in beef broth, if conditions were suitable and if the platings were made at the proper time. With this view in mind, 13 of the strains employed in Experiment II (I, II, III, IV, V, VII, VIII, X, XI, XII, XIII, XIV, and XV) and three additional strains (XVI, XVII, and XVIII), were subjected to the same treatment that was employed in the second experiment. The history of the three new strains is as follows:—

- Strain XVI—Isolated from the ovary of a hen in 1911  
by Doctor Rettger
- Strain XVII—Isolated from the liver of a chick in 1917  
by Doctor Rettger
- Strain XVIII—Isolated from the liver of a chick in 1916  
by Doctor Rettger

During the process of transferring from day to day it was observed that Strain XIV did not appear as turbid as the other cultures. The same characteristic had been noted in Experiment II. Ten days after the last transfer in broth three sets of the broth cultures were examined and plated on plain agar. The three sets included the first, seventh, and fourteenth broth culture transfers.

An examination of the bouillon cultures in the three sets revealed that Strains III, IV, V, VII, X, XIII, and XV produced a ring on the side of the tube at the surface of the broth, and also formed a heavy flaky sediment in the bottom of the tube. The remaining strains did not show these characteristics.

The agar plates that were streaked with the broth culture sets revealed definite colony variants of Strains III, V, X, XV, XVI, and XVIII. Three colony types of one strain were observed. In a few instances the colonies varied slightly in their appearance, but not sufficiently to designate them as definite types. Such colonies apparently had deviated from the normal type, and when placed under suitable environment the original characteristics re-appeared. This may also be true of certain types that seem rather remote from the normal in colonial features. Colony selection may be of no avail in stabilizing such types. The different variants isolated in this experiment will be discussed in the latter part of this section. The broth cultures were placed at room temperature and retained for further examination. The pH was determined approximately two months after the cultures had been placed in storage. Several cultures in different series were examined, and in every instance a pH of 8.8 was observed, which meant that the medium had become more alkaline (7.0 to 8.8). Uninoculated control broth tubes possessed a pH of 7.0.

#### **Influence of Frequent Transfer and Aging in Liver Infusion Broth**

*Experiment IV.* Since beef infusion broth appeared to be a suitable medium for producing variants by rapid transfer and aging, it seemed plausible that a more complex and favorable medium might exert an even greater influence. The liver infusion broth was prepared as follows:—

Beef liver,	500 grams
Peptone (Difco)	10 "
Tap water	1000 cc.
(pH 7.0—7.2)	

The beef liver was ground fine and added to the desired quantity of water. This was placed in the refrigerator over-night and then slowly brought to a boil. The extract was decanted and filtered off, then adjusted to pH 7.2. After the peptone was added the pH was checked before and after heating at 15 pounds steam pressure. The broth was tubed in 10 cc. quantities and sterilized.

The cultures inoculated into the liver infusion broth were the same as those which were employed in beef broth in Experiment II. The strains were transferred daily for a period of two weeks, and at different intervals liver infusion agar plates were streaked to detect any colony variation. An examination of the broth tubes from day to day revealed that a slight amount of gas was being liberated. All tubes appeared uniformly turbid except those which were inoculated with Strain XIV. This strain settled out as it did in the beef infusion broth. Liver infusion agar plates were streaked with broth cultures transferred on the first, seventh, and fourteenth days.

No definite colony variation was observed on the plates inoculated with the broth cultures. Two months after the last transfer in broth, liver infusion agar plates were inoculated with ten of the fourteen broth sets. Only a few strains in the last three inoculated sets showed growth on the plates. The hydrogen-ion concentration of the medium had changed from 7.0-7.2 to 4.5-5.8. This marked acid production, in the presence of other metabolic products, may have been responsible for the sterility of most of the cultures. Among the strains that survived none exhibited definite variation in colony form. Six strains (II, III, VIII, IX, and two cultures of Strain X, designated b and c) were transferred at three- to four-day intervals on liver infusion agar. Strains III, VIII, and IX died off after four weeks of frequent transfer. It was observed that certain strains, including all roughs and certain so-called typical strains, appeared to be rather sensitive to liver infusion agar, and that they required frequent transfer in order to maintain their viability. If the cultures are placed at a lower temperature, the viability period may be increased, but even then such cultures do not remain alive as long as on plain meat extract agar. Strain X-b during the course of frequent transfer revealed a temporary change in colony appearance which resembled some of the more stable forms. This variation in colony type reverted to the normal after two transfers. The temporary alteration can not be attributed to any known cause, since this strain had been subjected to different environments. However, it appears that the conditions in the liver infusion agar were particularly suitable for stimulating a certain type of colony which had not been observed before in this strain. Failure to stabilize this type cannot be accounted for in this instance. Unfortunately, this strain also failed to survive after it had been transferred for a period of four months. Strains II and X-c will be discussed later with the variants previously reported. (See Table 4.)

#### **Influence of Frequent Transfer and Colony Selection on the Enhancement of Variation**

Selection of variant colonies on solid medium has been demonstrated to be a valuable means of isolating types that differ from the normal parent type. Baerthlein (1912, 1918), Hadley (1927) and Arkwright (1930) have reported that selection of variants by means of colony appearance may lead to the stabilization of

well-defined types. Also by frequent transfer of such variants and by selection of colonies which reveal the greatest degree of variation, certain definite forms can be established.

*Experiment V.* Two old strains which revealed some degree of variation in colony type were selected for this experiment. These strains were the same as Strains I and II employed in Experiment I. Both strains produced smooth and rough colonies as well as intermediate colonies. An attempt was made to select and isolate both the roughest and the smoothest types of colonies. At each transfer each type was selected, and by this procedure it was possible to isolate a rough type which appeared stable and a smooth type which at times would exhibit a certain degree of roughness. At first transfers were made every 48 hours, but later the interval was extended to from four to five days.

The rough and smooth colony types isolated from Strain I were designated A and A-1, respectively. The two types, rough and smooth, isolated from Strain II were designated B and B-1, respectively. These different colony types will be described in another part of this section. The rough types resembled each other very closely on plain meat extract agar, although slight differences could be detected at times. The rough forms from both Strains I and II appeared to be stable, since little variation was observed in the numerous transfers of these types. The smooth types did not resemble a typical smooth *S. pullorum* colony in every respect, as will be brought out later. Also type B-1 very infrequently produced a markedly rough type of colony. On one occasion during the period of 14 months of frequent transfer a papillated colony was detected in this culture. The appearance of this type was rather sudden and it disappeared in the same manner. An attempt to propagate and stabilize the papillated variant met with failure. The cellular morphology was not as striking as the colony forms, and little variation could be detected in the cell types during the period under observation. (See Table 4.)

*Experiment VI.* The purpose of this experiment was to determine the influence on variants and on normal *S. pullorum* strains of frequent transfer on liver infusion agar. Since it is well recognized that liver infusion agar yields a heavier bacterial growth than plain meat extract medium, the possibility suggested itself that the former might be more suitable for the isolation and stabilization of variants. Liver infusion agar is employed in some laboratories for the cultivation of strains for antigen production, and by some even for the maintaining of stock cultures. Hence, it seemed all the more important to employ this medium to determine its influence on variants and normal strains. The strains used in this experiment were:

- Strain I—Same as Strain I in Experiments I and V
- Strain IV-a—Variant isolated from Strain IV in Experiment I
- Strain XII—Same as Strain XII in Experiments II, III, and IV
- Strain XIV—Same as Strain XIV in Experiments II, III, and IV

The liver infusion agar was prepared in the same manner as in previous experiments. The strains were transferred on plates at intervals of 48 hours. Later in the experiment the interval between transfers was increased to four or five days. The cultures were incubated at 37° C. When more than one type of colony appeared on the plate, only the roughest were selected for transfer. The colonial response of Strains I, IV-a, and XIV was impressive. A striking difference was observed between these three strains and the so-called normal Strain XII. The last appeared as typical *S. pullorum* on liver infusion agar. These strains were cultivated on this medium and transferred at frequent intervals for a period of a



year. During this period the colony type varied but slightly for Strains I and XII. Strain IV-a reverted partly to the normal type. The original variant type of colony was observed for four months before it reverted to the normal. Occasionally, definite rough features were observed. At the time of reversion to the normal, the original parent Strain IV-a was again placed on liver infusion agar. The growth which resulted contained colonies that were identical with those observed in the first instance. However, this last variant also exhibited a marked tendency to revert to the normal type. After eight months of frequent transfer, colony types appeared that did not resemble typical *S. pullorum*. Strain XIV also yielded a type of colony that did not remain stable. While fluctuation in colony type was observed it never approached the normal type. The different variants isolated in this experiment are designated as follows:—

- C —Variant derived from Strain I
- D —Variant derived from Strain IV-a (first trial)
- D-1—Variant derived from Strain IV-a (second trial)
- E —Variant derived from Strain XIV

Strain XII remained typical in its colonial features; variation was observed at no time in this strain (See Table 4).

#### Miscellaneous Attempts to Induce Variation

When this investigation was undertaken, little knowledge was available as to what methods would be most effective in bringing about variation in *S. pullorum*. Among some of the successful methods applied to other microorganisms are (a) alternate transfer in liquid and solid media, (b) cultivation in medium with increased concentration of peptone, (c) growing in deep liquid medium, (d) cultivation in meat extract solution. The author was especially interested in the influences that such methods might exert on *S. pullorum*, since similar conditions are often found in the isolation and study of this organism.

*Experiment VII.* An effort was made to determine the effect of alternate transfer from liver infusion broth onto beef infusion agar. Four strains employed in Experiment I were selected. The liver infusion broth was prepared in the same manner as in Experiment IV. The beef infusion agar contained 1 per cent peptone and 1.5 per cent agar (pH 7.2). The organisms were incubated in the broth for 24 hours at 37° C. and then streaked on agar plates. After incubation for 24 hours at 37° C., the roughest colonies were selected for transfer to fresh liver infusion broth. Each strain was cultivated in broth and on agar plates six times. At the termination of the transfer period no definite variation was observed in the strains treated. After a period of seven weeks several broth cultures were streaked on plates. Only two strains revealed growth but no variation. This experiment was not repeated since some of the previously mentioned experiments proved more successful in bringing about variation. It is interesting to note that the organisms in this experiment, as well as in those previously reported, grew with difficulty in liver infusion broth, and in the majority of cases died within two months.

*Experiment VIII.* In this experiment a study was made of the influence of peptone concentration on colony form. Five per cent peptone solution, pH 7.2, was placed in flasks in 250 cc. quantities. Meat extract broth (1 per cent peptone, 0.3 per cent meat extract, pH 7.2) was also prepared in 250 cc. quantities. Strains I, II, and IV employed in Experiment I were selected. Each strain was inoculated into a flask of peptone solution and of meat extract peptone broth. The flasks

were incubated at 37° C., and at frequent intervals meat extract peptone agar plates were streaked. The flasks were incubated at 37° C. for three weeks and at room temperature for 11 weeks. While incubating at the higher temperature a heavy scum formation was observed in the flask inoculated with Strain I. Upon agitation of the flask the scum would gravitate to the bottom in flocules. Ring formation was observed in the flask containing Strain II. Heavier growth occurred in the 5 per cent peptone solution than in the meat extract peptone broth. Streaked plates made from the peptone and the broth cultures did not reveal a colony picture which differed from the original. Strains I and II, which are older than Strain IV and are not entirely normal, seemed to thrive better in the peptone solution than Strain IV. The pH increased in each instance during the 14 weeks period.

TABLE 4.—REMARKS CONCERNING COLONIAL AND CELLULAR MORPHOLOGY OF ISOLATED VARIANTS.

Original Strain No.	Variant No.	Culture Medium*	Colonial and Cellular Morphology
<b>Experiment No. I</b>			
IV	IV-a	P. A.	Small irregular colonies at 24 hours of age; older colonies appear more round and entire; average size of former approximately .1 mm., larger colonies .5—.75 mm. in diameter. Surface smooth, slightly convex and glistening. Structure coarsely granular to filamentous. Cells—many bizarre forms including thick filamentous, coccoid and club-shaped. Some contained granules. Typical cells were present. See Figures 3, 4, 5, 6, and 7. After frequent transfer, a colony type was produced as shown in Figure 8, with a less pleomorphic cellular morphology.
IV	IV-b	P. A.	Colonial and cellular morphology at first similar to IV-a. Later a colony type developed which moved in mass, exhibited small projections, was dome-shaped and opaque. See Figure 9. Cellular forms slightly pleomorphic.
<b>Experiment No. II</b>			
I	I-a	L. A.	Colony has crenated edge, thin periphery, and is almost transparent; markedly convoluted. The interior is raised, level, slightly rough, dull, and opaque. Cellular forms highly pleomorphic. See Figures 10 and 11.
II	II-a	L. A.	Colonial and cellular features similar to I-a.
VI	VI-a	L. A.	Colony with undulating edge, semi-dome shape, and prominent central portion with shallow grooves winding outward. Dull in appearance. See Figure 12. Cells markedly pleomorphic, including cells of various lengths, club-shaped and dumb-bell forms, and some filamentous types.
VIII	VIII-a	P. A.	Colony irregular in outline, periphery flat and thin; central portion raised and opaque; surface rough, due to numerous shallow valleys and ridges; brittle consistency. Size may vary from 7 mm. to less. See Figure 13. Cells slightly pleomorphic, short thick rods, approaching coccoid type.
IX	IX-a	P. A.	Colony type similar to VIII-a, but cells slightly more pleomorphic.
IX	IX-b	L. A.	Colony has undulating edge, flat surface varying in roughness and dull in appearance. Some colonies are markedly convoluted with thin periphery. Isolated colonies may attain a diameter of 2 cm. See Figures 14, 15, and 16. Cells are markedly pleomorphic, long and thick filaments. Some cells are short.
XII	XII-a	P. A.	Colony is smooth, mound-shaped, undulating in outline, opaque, and moves in mass. See Figure 17. Cells resemble normal, but a few odd shapes and filamentous forms are present.
XII	XII-b	L. A.	Colony has dentated edge and very thin periphery which is markedly convoluted, striated and tenacious. Central portion is raised, dense, and faintly convoluted. See Figure 18. Cells in the outer portion entirely filamentous, while cells from central portion approach the normal with slight variation. See Figures 19 and 20.
XIII	XIII-a	L. A.	Colony has irregular edge and periphery is thin and prominently convoluted. Inner portion raised, dense, and marked with faint convolutions. Parallel striations are also very prominent at times. See Figure 21. Cells are very pleomorphic.

TABLE 4.—REMARKS CONCERNING COLONIAL AND CELLULAR MORPHOLOGY OF ISOLATED VARIANTS.—*continued.*

Original Strain No.	Variant No.	Culture Medium*	Colonial and Cellular Morphology
<b>Experiment No. III</b>			
III	III-a	P. A.	Colony similar to VIII-a, Expt. II. See Figure 13.
V	V-a	P. A.	Colony has granular surface, appendages projecting from edge, moves partly in mass; cells are long and filamentous.
X	X-a	P. A.	
XV	XV-a	P. A.	Colony type similar to IV-b of Expt. I. Cells — a few bizarre forms observed.
XVI	XVI-a	P. A.	
XVIII	XVIII-a	P. A.	Colony type similar to VIII-a of Expt. II. See Figure 13.
<b>Experiment No. IV</b>			
II	II-b	L. A.	Colony morphology similar to normal type. Occasionally a rough beveled edge is observed. Interior is smooth and homogeneous. Cells—bizarre forms numerous.
X	X-c	L. A.	
<b>Experiment No. V</b>			
I	A	P. A.	Colony type similar to VIII-a of Expt. II. Cell morphology slightly pleomorphic.
I	A-1	P. A.	
II	B	P. A.	Colony similar to VIII-a of Expt. II, but appears to be somewhat butyrous in consistency and more dense. Cells long, approaching short filaments.
II	B-1	P. A.	
<b>Experiment No. VI</b>			
I	C	L. A.	Colony has irregular edge, may appear curled; peripheral portion thin and frequently markedly convoluted and filamentous. Central portion appears raised and dense, at times filamentous. Surface appears dull, consistency butyrous. Young colonies appear very irregular, filamentous, and granular. Older colonies may resemble the Medusa-head colony and attain a diameter of 1.5 cm. Cells vary in length from the normal to long filamentous types. See Figures 22, 23, 24, and 25.
IV-a	D-1	L. A.	
IV-a	D-2	L. A.	The variant when first placed on liver infusion agar produced a very irregular colony possessing numerous projections. The structure might be entirely granular, semi-granular, or entirely filamentous. As the colony grew larger the angular portions were filled in. Many cells of these colonies were thick filaments of various lengths. Other bizarre forms of cells were also observed. Granular types were seen frequently, especially in older cultures. The colony appeared dull and could be removed in mass. See Figures 26, 27, and 28. This colony type has reverted almost completely to smooth form.
XIV	E	L. A.	
This variant followed practically the same course of reversion, but not as completely, as D-1 of Expt. VI.			
Young colonies exhibit numerous projections. The structure of the projections and interior of the colony appears striated. Older colonies appear less angular, but have an irregular outline, convoluted and striated periphery with a raised dense center. Isolated colonies may attain a diameter of 1 cm. The cellular types in the small striated colonies consisted largely of filamentous forms. Older colonies contain cells of various sizes. See Figures 29 and 30.			

\*P. A.—Plain meat extract agar.  
L. A.—Liver infusion agar.

*Experiment IX.* In this experiment the influence of the quantity of medium on colony and cellular form was studied. Meat infusion broth prepared as mentioned above was used in flasks in 300 cc. quantities and in tubes in 10 cc. amounts. Strains I, II, and IV, employed in Experiment I, and Strain VII, used in Experiment II, were studied. The flasks and tubes, after being inoculated, were incubated at 37° C. Meat infusion peptone agar plates were streaked at frequent intervals. As in the previous experiments, a heavy scum formation was observed in all strains, especially I and II. The turbidity of the broth was approximately the same in every case, except in the flask and tube inoculated with Strain I. The cultures were examined for a period of one month, with the result that no definite colony variants were detected. While Strains I and II did reveal tendencies toward colony variation, no definite variants were established. The larger quantity of broth apparently had no different effect on the strains than did the smaller quantity.

*Experiment X.* An attempt was made to determine the influence of meat extract solution on colony and cellular form. A 3 per cent meat extract (Difco) solution (pH 7.2) was prepared. The medium was flaked in 125 cc. quantities. Strains I, II, and IV listed in Experiment I were studied. Ordinary meat extract broth, as employed in Experiment VIII, was used as a control for determining the influence on the growth characteristics of *S. pullorum*. In the meat extract flasks it was difficult to ascertain the presence of growth by macroscopic examination. At the end of the first week of incubation agar plates were streaked from both the meat extract solution flasks and meat extract broth flasks. No growth was obtained from the meat extract solution. Apparently a 3 per cent solution of meat extract exerted a toxic effect that was detrimental to the growth of *S. pullorum*. Further investigation of the influence of meat extract as such was not continued. Lower concentrations of meat extract would probably permit growth of this organism. The flasks containing the meat extract peptone broth revealed growth, but no definite colony variants were isolated.

### Summary of Results

While variants of *S. pullorum* have been reported by a few investigators to occur spontaneously, the foregoing data show that variants of this organism may be induced by artificial conditions. These results should be of much interest, since most investigators and diagnosticians in animal diseases regard *S. pullorum* as exceedingly stable in its various characteristics. Like other species of microorganisms, it will respond to certain environmental influences. Under some conditions extreme variation may be observed in which the variant does not resemble the parent strain in any of its colonial and cellular features. It will change also in other characters, as will be brought out later. Different degrees of variation may be observed in cultures that are subjected to certain influences. These changes may be temporary or the new types may be stabilized by various methods, such as frequent transfer, colony selection, and growing on special media. In this section 28 variants are reported. Several different types are included in this number, as may be seen in Table 4.

The extremely rough colony type which was stabilized on plain meat extract agar has undergone a marked change in colony appearance. As will be pointed out later, profound alterations were also observed in virulence, antigenicity, and ability to remain in suspension in saline solution. The cellular morphology of this type, while exhibiting slight pleomorphism at times, appeared on the whole to be quite stable and typical.

Another type observed on plain agar is that exhibited by variant XII-a. This type apparently has not digressed from the normal to the extent that some others have, and also shows a greater tendency to revert to the typical form. A characteristic peculiar to this type is that the colony moves in mass when disturbed with a needle. This characteristic may be due to the fact that the colony is small and mound-shaped and that its internal structure contains filamentous forms. These forms may serve as a reinforcement in retaining the colony in its entirety. An apparently similar action by these filamentous forms may be seen in XII-b, in which the periphery is largely composed of filaments which exhibit a tenacious property. Also in small colonies composed largely of filaments the entire mass moves in toto.

The different types of variants established on liver infusion agar show colonial and cellular characteristics different from those observed on plain meat extract agar. The liver agar apparently contains certain constituents that have an influence on the colony appearance and cell types. It has been observed that friable, fatty, yellowish-brown liver tissue will produce an agar that is lighter in color than that obtained from a firm, dark brown liver. The growth on agar medium prepared with the latter is not as abundant and *butyrous* in character as on the other medium. The liver tissue may vary in composition and thus affect the character of the growth.

In two instances during this investigation a mucoid type of colony was observed on liver infusion agar. Attempts to stabilize this type failed.

Certain variants may develop to a very impressive size on liver infusion agar. This has not been observed on plain agar, although the colony size may be markedly larger than that of the normal type. It was also observed in most instances that liver infusion agar greatly exaggerated any unusual features that were slightly noticeable in colonies growing on plain agar. Hence, liver infusion agar may serve in some instances to determine the normalcy of a strain, or at least bring out certain characteristics that may not be demonstrable with the ordinary media. The continuous cultivation of *S. pullorum* in liver infusion media may in some cases lead to temporary variation and possibly permanent change in one or more characteristics. A temporary variation was observed in one strain after having been cultivated on liver infusion agar for a period of some months. The mucoid type of colony also occurred spontaneously on liver infusion agar. Cellular types produced on this medium do not resemble the normal types, especially in the first transfer. With the organism capable of producing such responses on liver infusion agar, it appears that an antigen prepared from liver cultures may be expected not to react in the same manner as that prepared from a meat extract agar growth.

#### BEHAVIOR OF VARIANTS IN DIFFERENT ENVIRONMENTS

Numerous investigations, reviewed by Hadley (1927), show that organisms may lose or gain more than one property when undergoing variation. This process does not necessarily involve certain characteristics, but it appears that some properties are more frequently altered than others. Colonial and cellular morphology, and pathogenic and serologic properties seem to be most susceptible to change. Fermentative and other biochemical characteristics do not change so readily.

The variants in this investigation were studied as to their behavior in (1) broth, (2) NaCl solution, (3) fermentable substances, (4) fresh whole egg and egg yolk, and (5) the natural host.

## Behavior in Broth

Arkwright (1921) and others have demonstrated that the rough colony variants may form a pellicle and yield a sediment in the bottom of the tube, with the supernatant fluid almost or entirely clear. Also, the growth of the rough type is usually retarded.

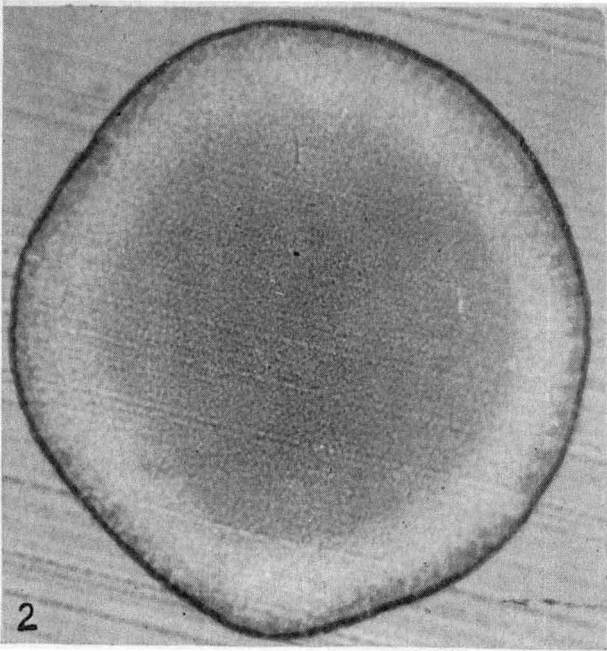
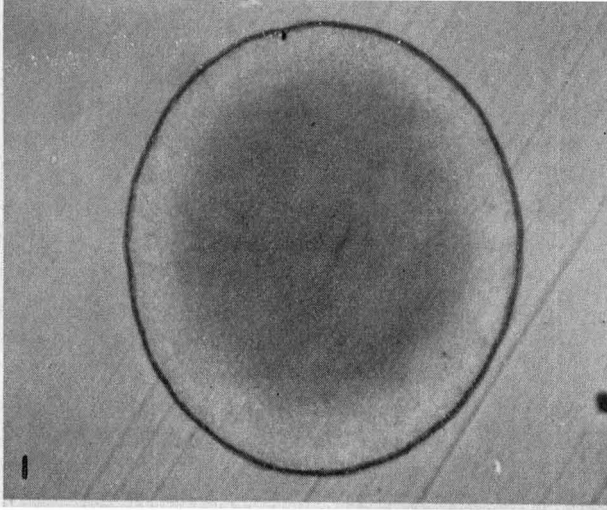
Table 5 shows the growth characteristics of the different variants in meat extract broth (1 per cent Bacto peptone and .3 per cent Liebig's meat extract, pH 7.0) at different incubation periods. The data show that 13 strains produced normal growth in broth, while 14 variants exhibited modified growth characteristics in this medium. It is interesting to note that strains D-1, D-2, IV-a, IV-b, V-a, X-a, XII-a, and XV-a are characterized by a similar type of growth in broth as well as on solid medium. Strains I-a and II-a produced growth that was slightly different in its nature from that of the aforementioned strains. The sediment was more abundant and not so compact. It is of interest to note that the variants which produced the so-called "roughest" colony produced growth in nutrient broth that appeared quite typical or normal. These results show that not all colony variant strains tend to settle out in meat extract broth. The characteristic of settling in broth apparently cannot always be associated with any definite type of colony or cellular form. Obviously, this property may vary from time to time in a given type, since earlier in this investigation variants A and B revealed a tendency to grow with difficulty and to settle out in broth. The colonial and cellular features were not observed to have changed in these two instances.

TABLE 5.—BEHAVIOR OF VARIANTS IN BROTH.

Variant No.*	Observations at End of Various Incubation Periods			
	24 hours	48 hours	72 hours	96 hours
A	1+(g)	1+(g)	N	N
A-1	N	N	N	N
B	N	N	2+	1+
B-1	N	N	N	N
C	N	N	N	N
D-1	1+	2+(g) (r)	2+(g) (r)	2+(g) (r)
D-2	—	—	2+(g) (r)	2+(g) (r)
F	N	N	N	N
I-a	3+(g)	2+(g)	2+(g) (r)	2+(g) (r)
II-a	4+(g)	3+(g)	2+(g) (r)	2+(g) (r)
II-b	N	N	N	N
III-a	2+(g)	2+(g)	2+(g)	1+(g)
IV-a	N	1+(g) (r)	1+(g) (r)	1+(g) (r)
IV-b	3+	3+(g) (r)	3+(g) (r)	3+(g) (r)
V-a	N	1+(g) (r)	1+(g) (r)	1+(g) (r)
VI-a	N	N	N	N
VIII-a	N	N	N	N
IX-a	N	N	N	N
IX-b	N	N	N	N
X-a	4+(f)	4+(f) (r)	4+(f) (r)	4+(f) (r)
X-c	N	N	N	N
XII-a	3+(f)	3+(f)	3+(f)	3+(f)
XII-b	N	N	N	N
XIII-a	2+(g)	1+	1+	1+
XV-a	N	1+(g) (r)	1+(g) (r)	1+(g) (r)
XVI-a	N	N	N	N
XVII-a	N	N	N	N

\*Refer to Table 4 for history of variants.

Legend: N—uniform heavy turbidity  
 1+—slight clearing  
 2+—partial clearing  
 3+—marked clearing  
 4+—complete clearing  
 (g)—granular precipitate  
 (f)—flaky precipitate  
 (r)—ring formation



**Figure 1.** Typical *S. pullorum* colony on meat extract agar. 24 hours incubation.  $\times 125$  diameters.

**Figure 2.** Typical *S. pullorum* colony on liver infusion agar. 24 hours old.  $\times 100$  diameters.

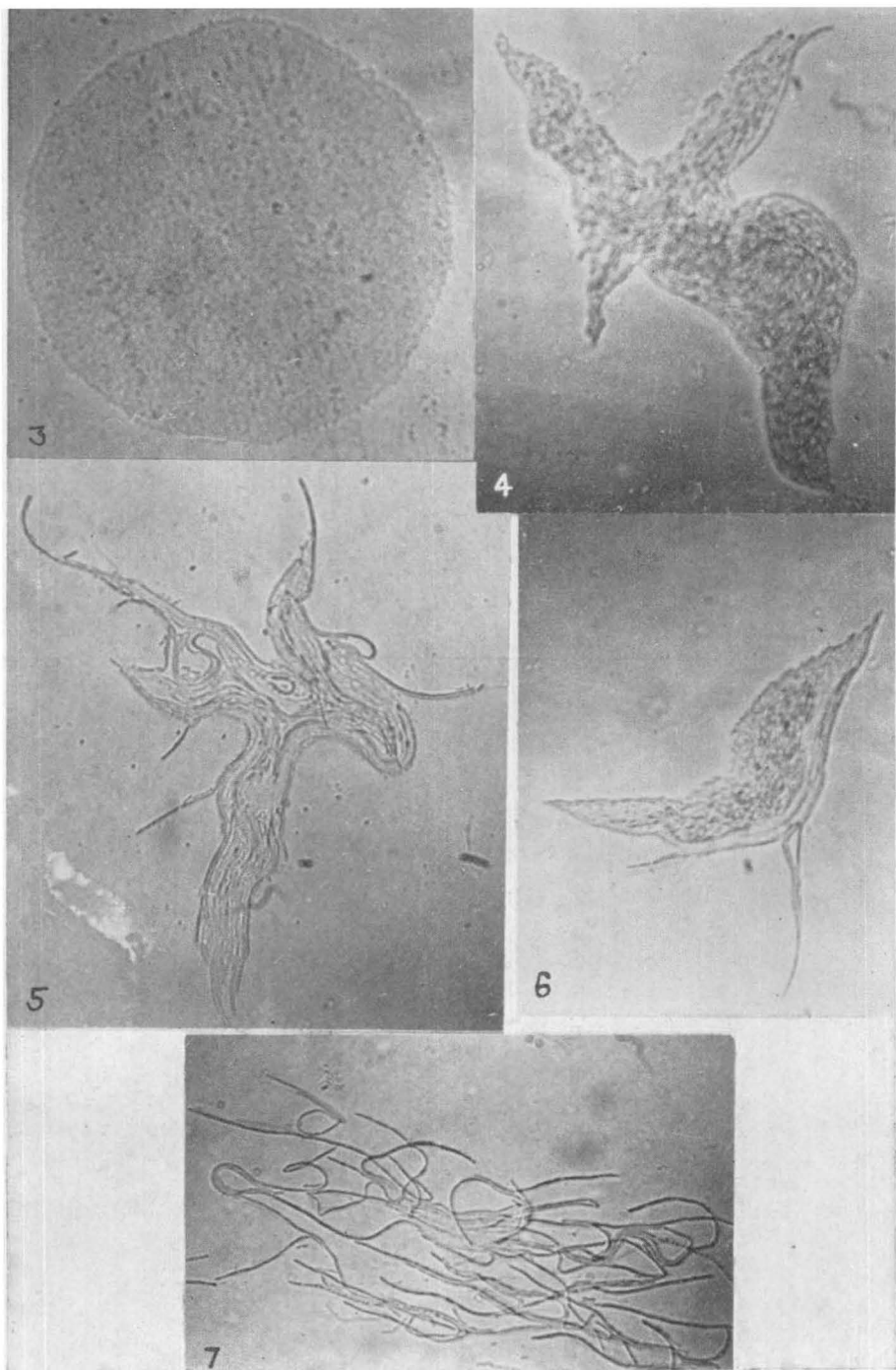


Figure 3. Strain IV. Typical colony on hormone agar. 20 hours old.  $\times 340$  diameters. (See page 20 and Table 4.)

Figure 4. Variant IV-a. A granular, irregular-shaped colony on meat extract agar. 20 hours old.  $\times 340$  diameters. (See Table 4.)

Figure 5. Variant IV-a. An irregular-shaped striated colony on meat extract agar. 20 hours old.  $\times 340$  diameters.

Figure 6. Variant IV-a. An irregular-shaped colony, partly granular and partly striated. Meat extract agar. 20 hours old.  $\times 340$  diameters.

Figure 7. Variant IV-a. Scattered and grouped threads of growth on meat extract agar. 20 hours old.  $\times 340$  diameters.



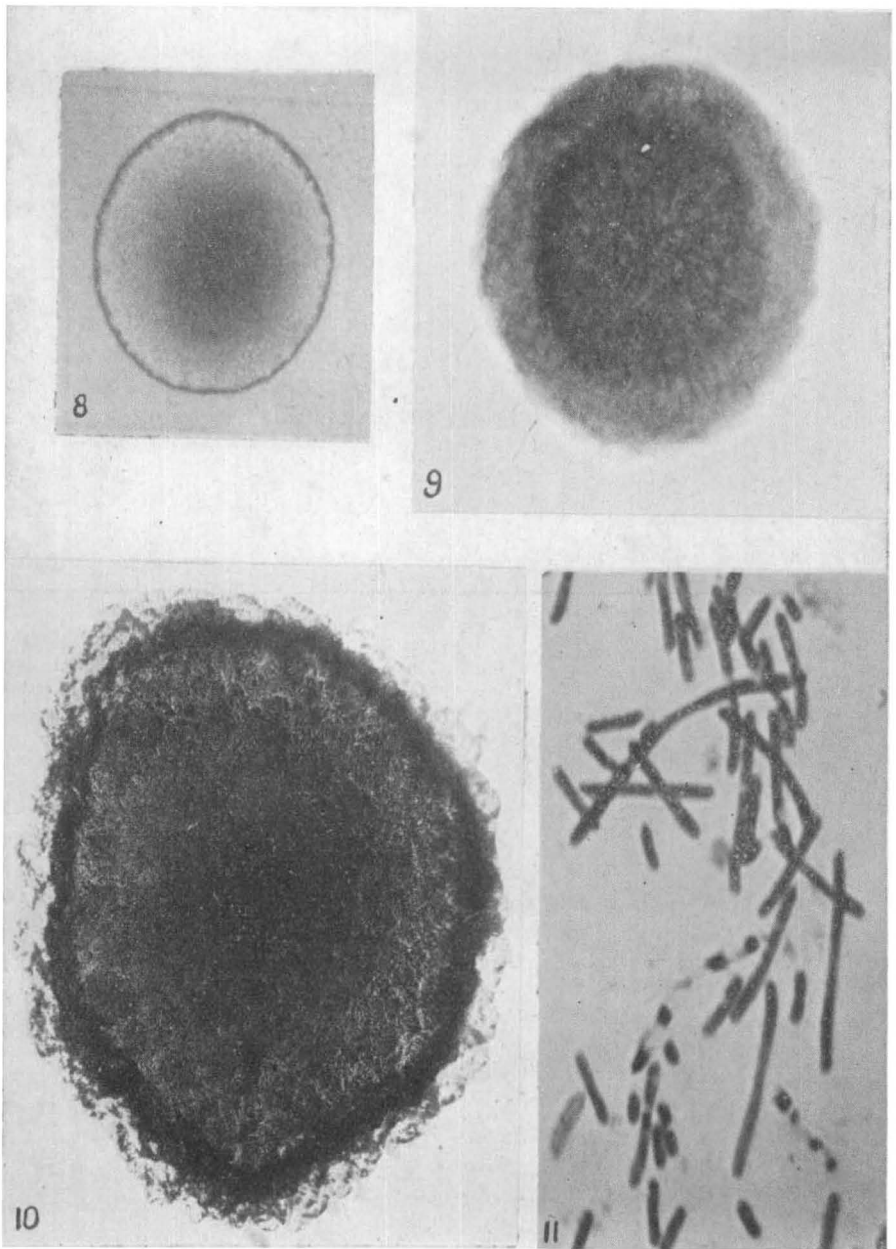
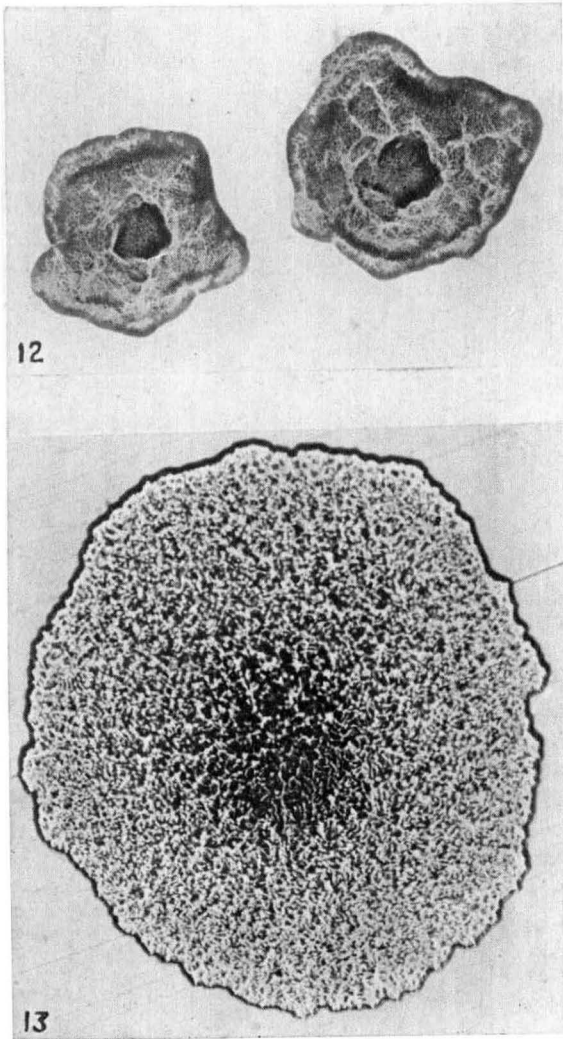


Figure 8. Variant IV-a. Two-day-old colony on meat extract agar.  $\times 50$  diameters. (See Table 4.)

Figure 9. Variant IV-b. Two-day-old colony on meat extract agar.  $\times 125$  diameters. Colony exhibited marked opaqueness and projections. (See Table 4.)

Figure 10. Variant I-a. Two-day-old colony on liver infusion agar.  $\times 25$  diameters. (See Table 4.)

Figure 11. Variant I-a. Cells in a smear prepared from the colony illustrated in Figure 10.  $\times 1500$  diameters.



**Figure 12. Variant VI-a. Two-day-old colony on liver infusion agar.  $\times 11$  diameters.**

**Figure 13. Variant XVIII-a. Two-day-old colony on meal extract agar.  $\times 25$  diameters.**  
(See Table 4.)

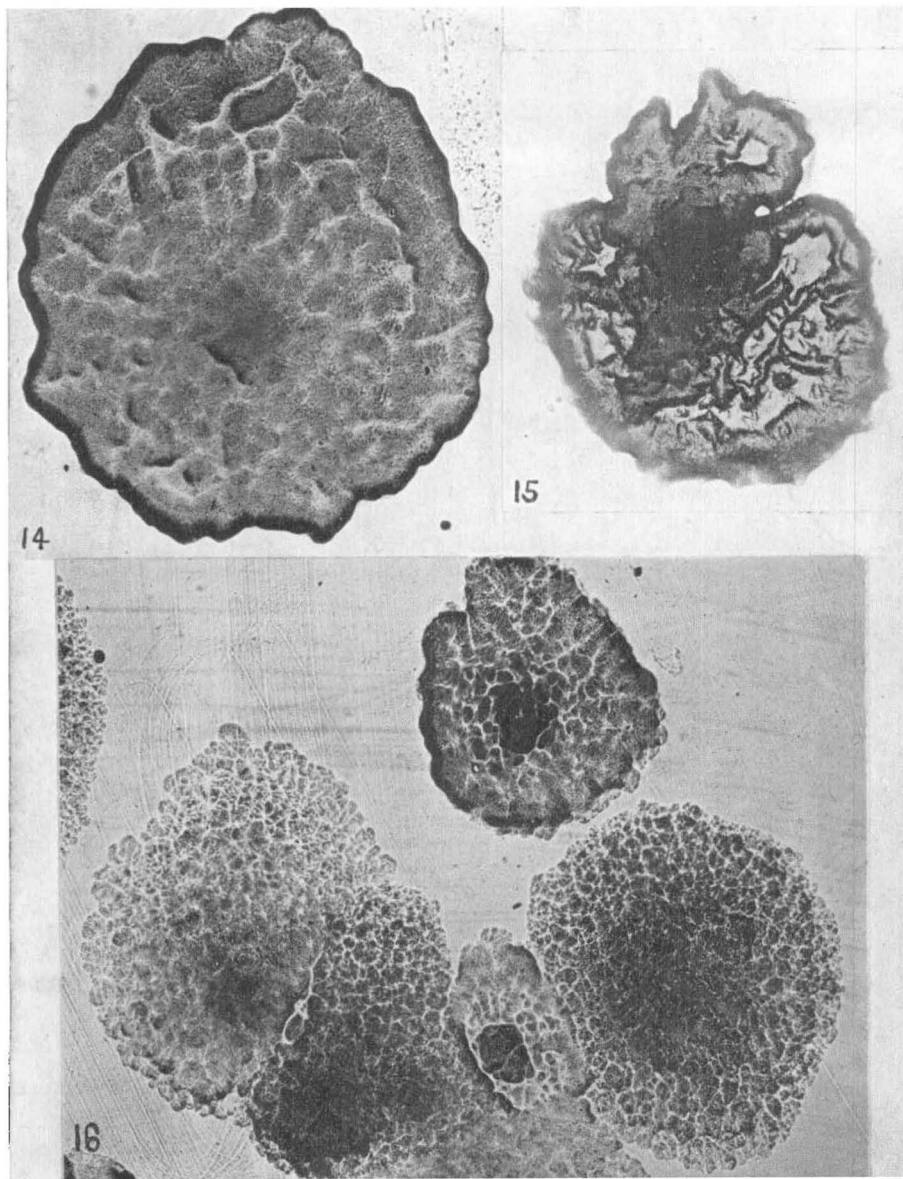


Figure 14. Variant IX-b. Five-day-old colony on liver infusion agar. Culture subjected to frequent transfer.  $\times 11$  diameters. (See Table 4.)

Figure 15. Variant IX-b. Colony type produced after passage through natural host. Liver infusion agar. Six days old.  $\times 11$  diameters. (See Table 4 and page 38.)

Figure 16. Variant IX-b. Slight variation in colony types. Liver infusion agar. 48 hours old. Culture maintained in whole fresh eggs.  $\times 11$  diameters. (See Table 4 and page 34.)

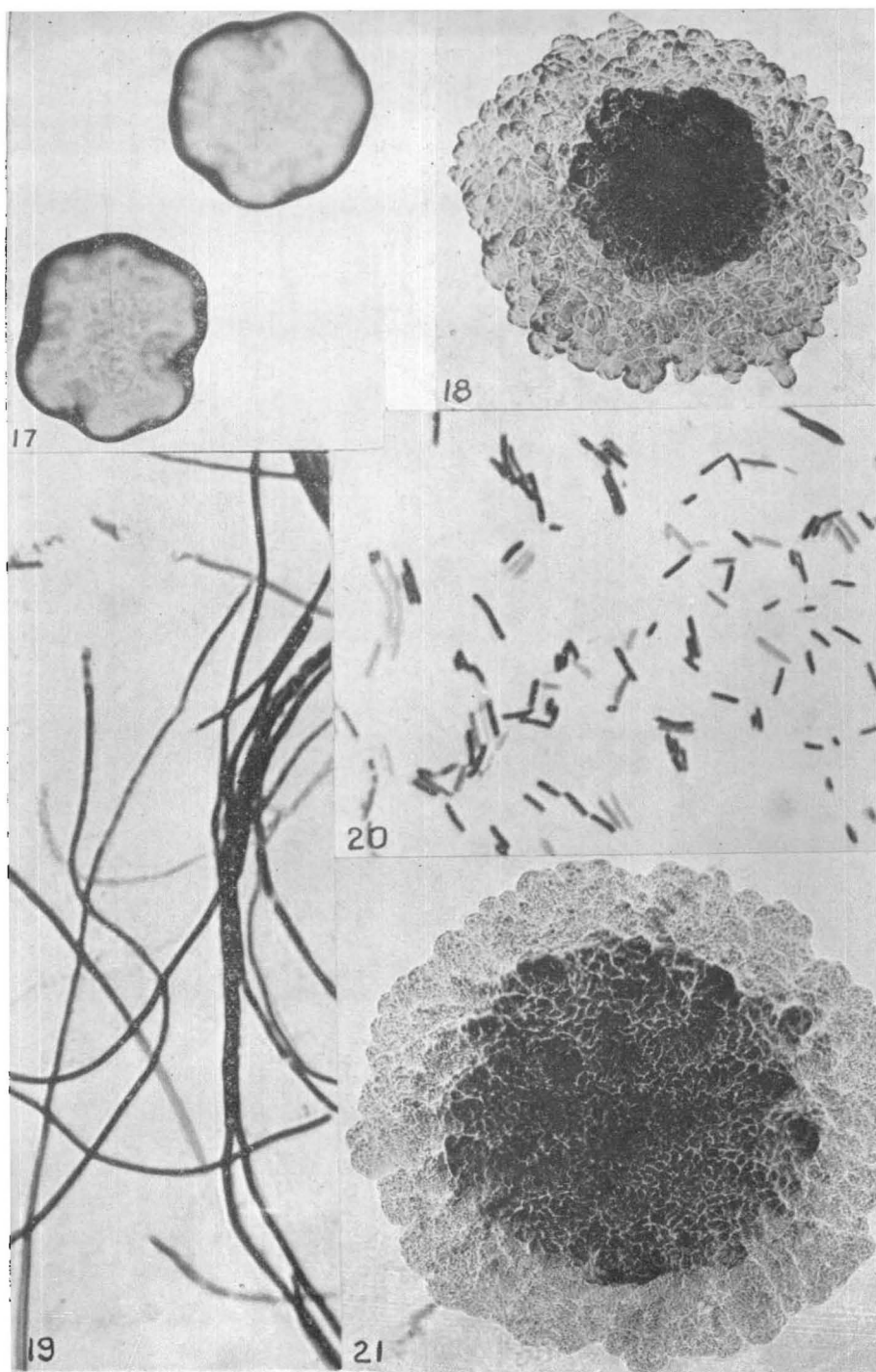


Figure 17. Variant XII-a. Two-day-old colonies on meat extract agar.  $\times 50$  diameters. (See Table 4.)

Figure 18. Variant XII-b. Three-day-old colony on liver infusion agar.  $\times 10$  diameters. (See Table 4.)

Figure 19. Variant XII-b. Filamentous forms in a smear prepared from the peripheral portion of the colony illustrated in Figure 18.  $\times 1200$  diameters.

Figure 20. Variant XII-b. Types of cells in a smear prepared from the central portion of the colony illustrated in Figure 18.  $\times 1200$  diameters.

Figure 21. Variant XIII-a. Two-day-old colony on liver infusion agar.  $\times 20$  diameters. (See Table 4.)

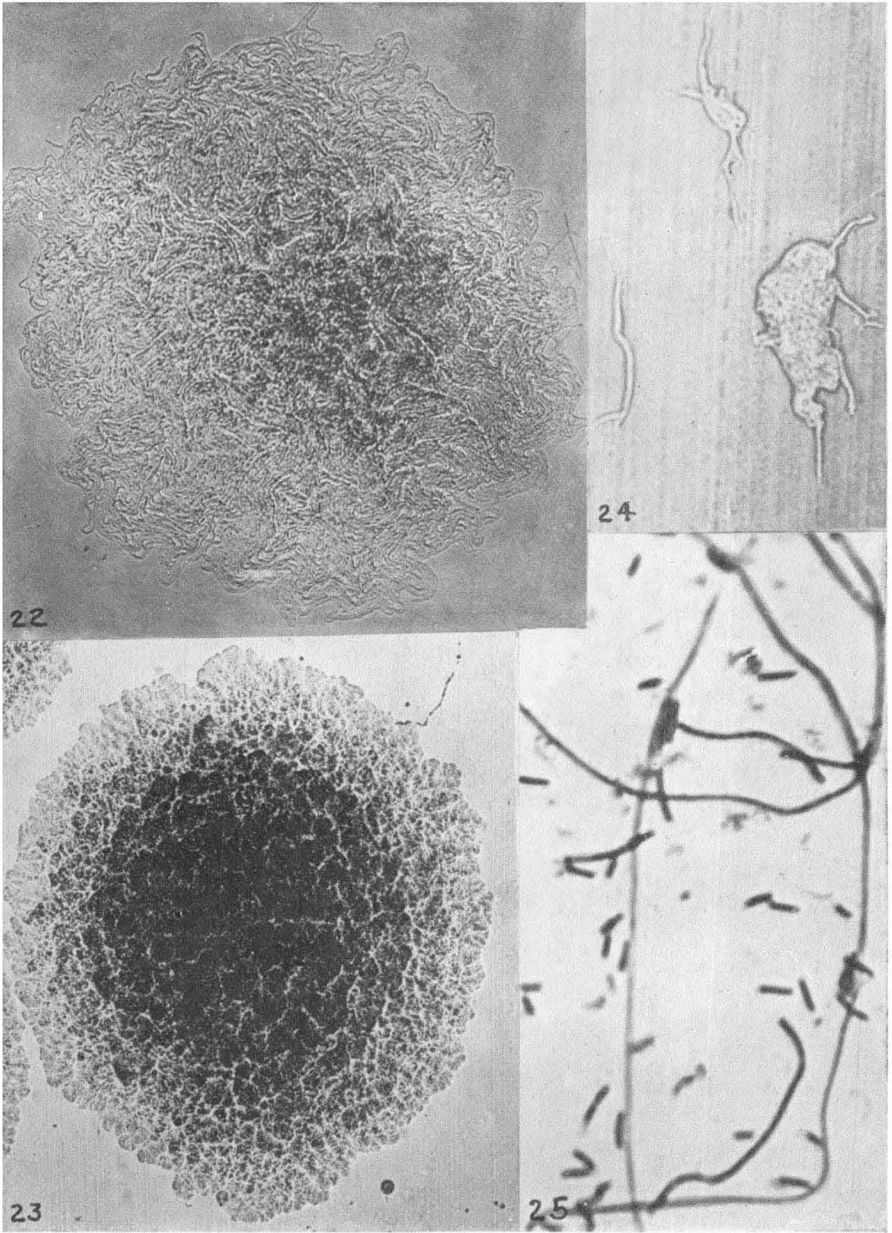
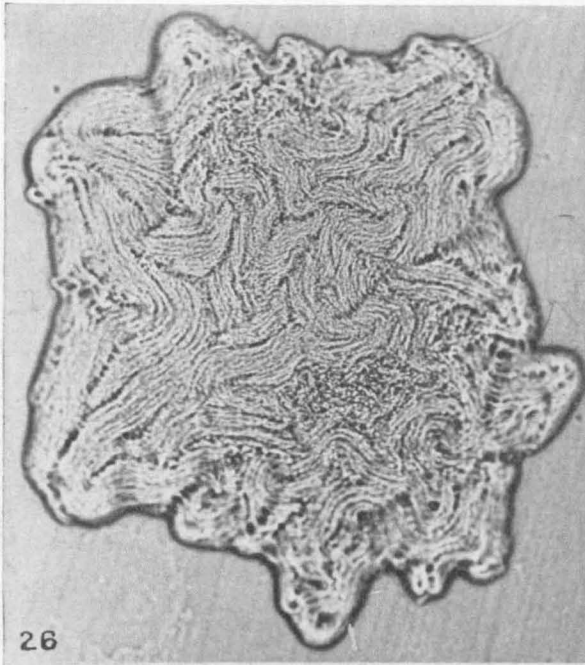


Figure 22. Variant C. Three-day-old colony on liver infusion agar.  $\times 125$  diameters. (See Table 4.)

Figure 23. Variant C. Two-day-old colony on liver infusion agar.  $\times 11$  diameters. Culture maintained in whole fresh eggs. (See Table 4 and page 34.)

Figure 24. Variant C. One-day-old colonies on liver infusion agar.  $\times 125$  diameters. (See Table 4.)

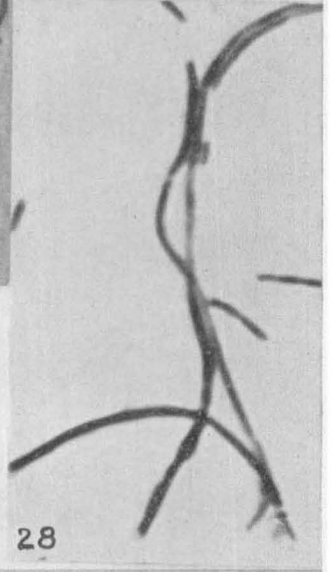
Figure 25. Variant C. Cells and filamentous forms in a smear prepared from colonies illustrated in Figure 24.  $\times 1500$  diameters.



26



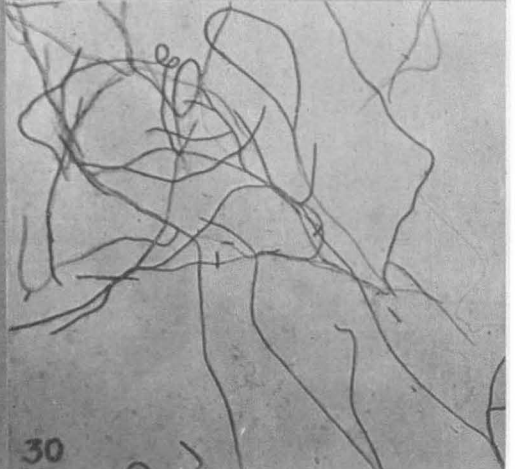
27



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29



30

Figure 26. Variant D-1. Six-day-old colony on liver infusion agar.  $\times 125$  diameters. (See Table 4.)

Figure 27. Variant D-1. One-day-old colony on liver infusion agar.  $\times 125$  diameters.

Figure 28. Variant D-1. Cells in a smear prepared from colony type illustrated in Figure 27.  $\times 1500$  diameters.

Figure 29. Variant E. One-day-old colony on liver infusion agar.  $\times 125$  diameters. (See Table 4.)

Figure 30. Variant E. Scattered filamentous forms in a smear prepared from colony type illustrated in Figure 29.  $\times 375$  diameters.

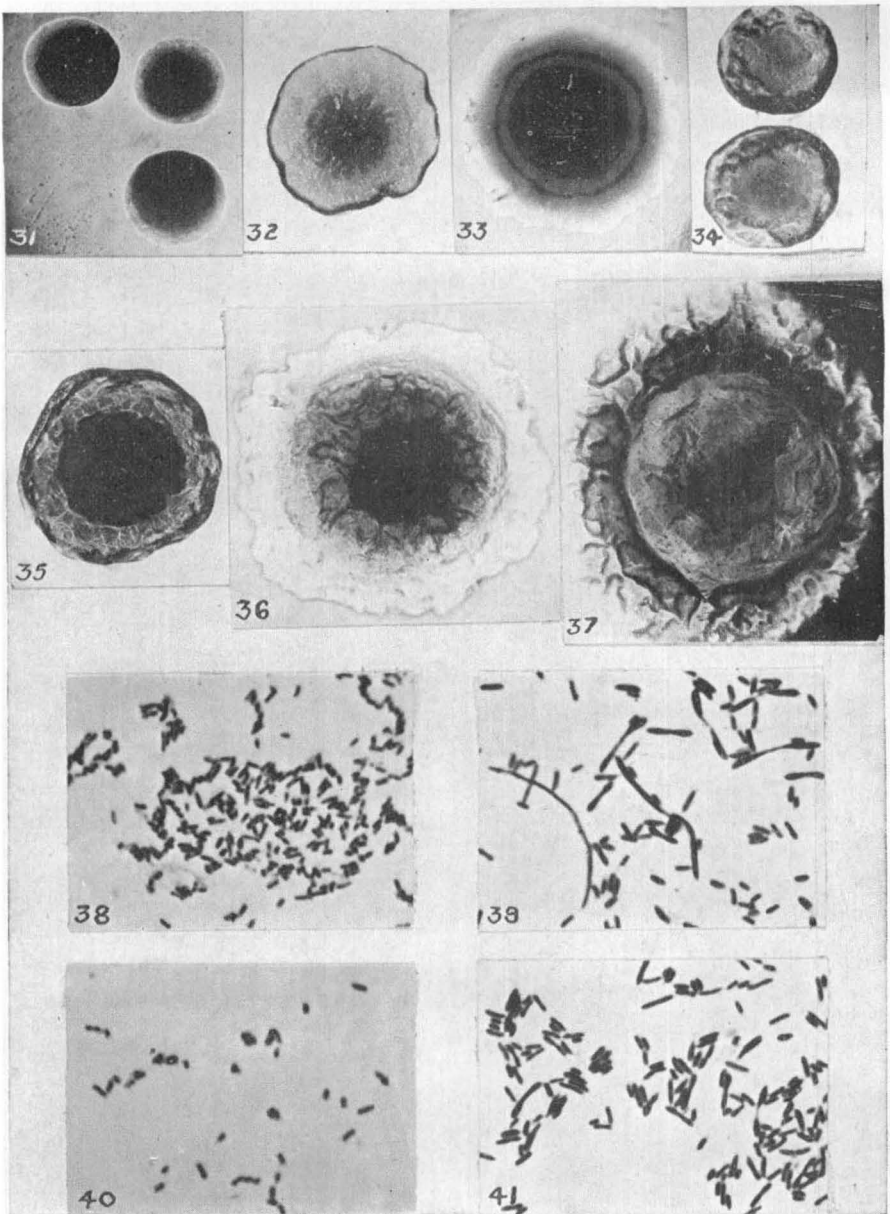


Figure 31. Variant B. Colonies of primary culture. Meat extract agar. 40 hours old.  $\times 15$  diameters. (See page 36.)

Figure 32. Variant B. Isolated colony in primary culture from spleen. Reveals an undulating edge due to the debris on the agar surface. Meat extract agar. 40 hours old.  $\times 15$  diameters.

Figure 33. Variant B. Isolated colony in primary culture from spleen. Meat extract agar. Six days old.  $\times 15$  diameters.

Figure 34. Variant B. Congested colonies in primary culture from spleen. Liver infusion agar. Two days old.  $\times 15$  diameters.

Figure 35. Variant B. Isolated colony in primary culture from spleen. Liver infusion agar. Two days old.  $\times 15$  diameters.

Figure 36. Variant B. Isolated colony in primary culture from spleen. Liver infusion agar. Six days old.  $\times 25$  diameters.

Figure 37. Variant B. Isolated colony in primary culture from liver. Liver infusion agar. Six days old.  $\times 15$  diameters.

Figure 38. Variant B. Cells in a smear prepared from colony illustrated in Figure 32.  $\times 1200$  diameters.

Figure 39. Variant B. Cells in a smear prepared from colony type illustrated in Figure 35.  $\times 1200$  diameters.

Figure 40. Variant B. Cells in a smear prepared from colony type illustrated in Figure 31.  $\times 1200$  diameters.

Figure 41. Variant B. Cells in a smear prepared from colony type illustrated in Figure 34.  $\times 1200$  diameters.

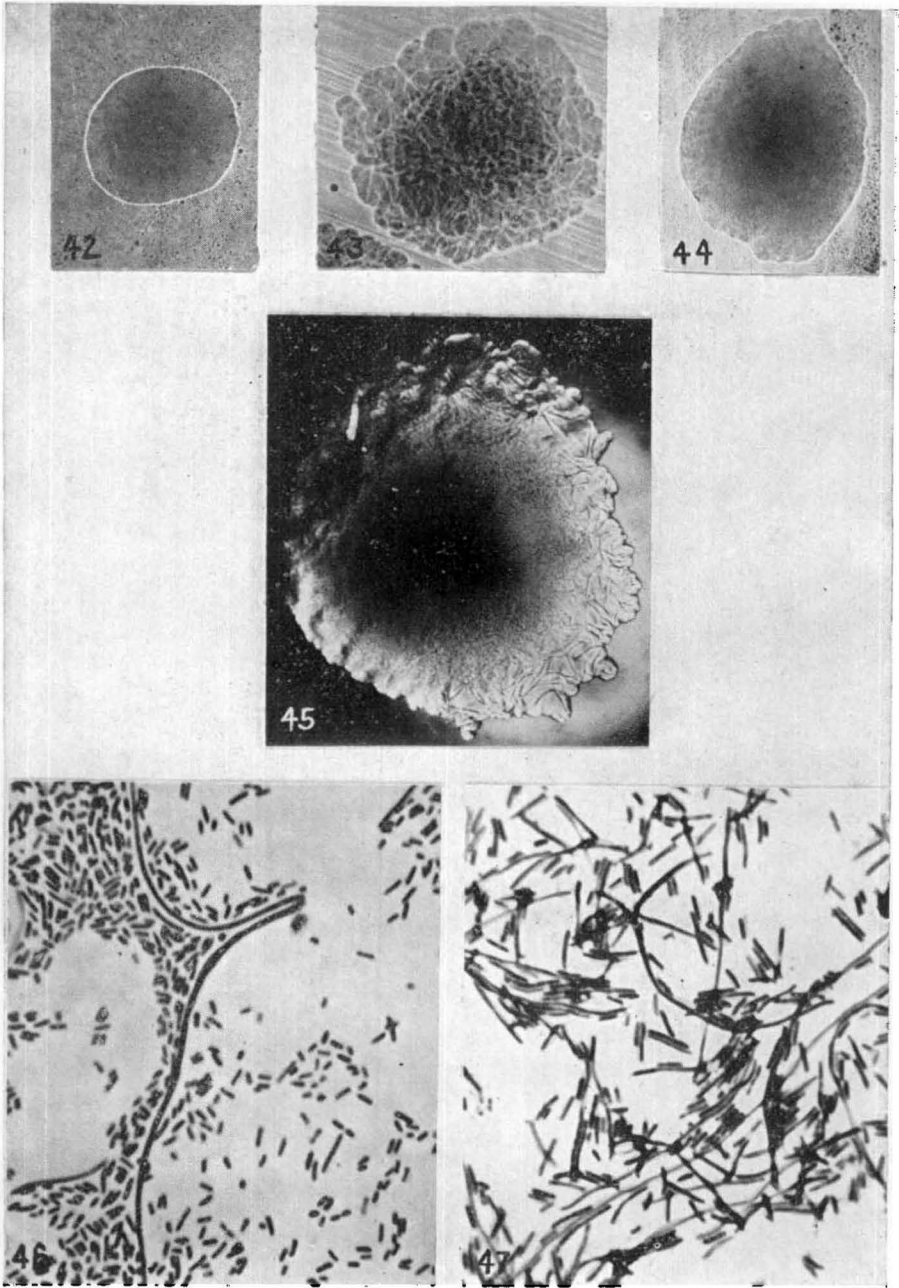


Figure 42. Variant E. Isolated colony on primary culture. Meat extract agar. 96 hours old.  $\times 17$  diameters. (See page 36.)

Figure 43. Variant E. Colonies on primary culture. Liver infusion agar. 72 hours old.  $\times 17$  diameters.

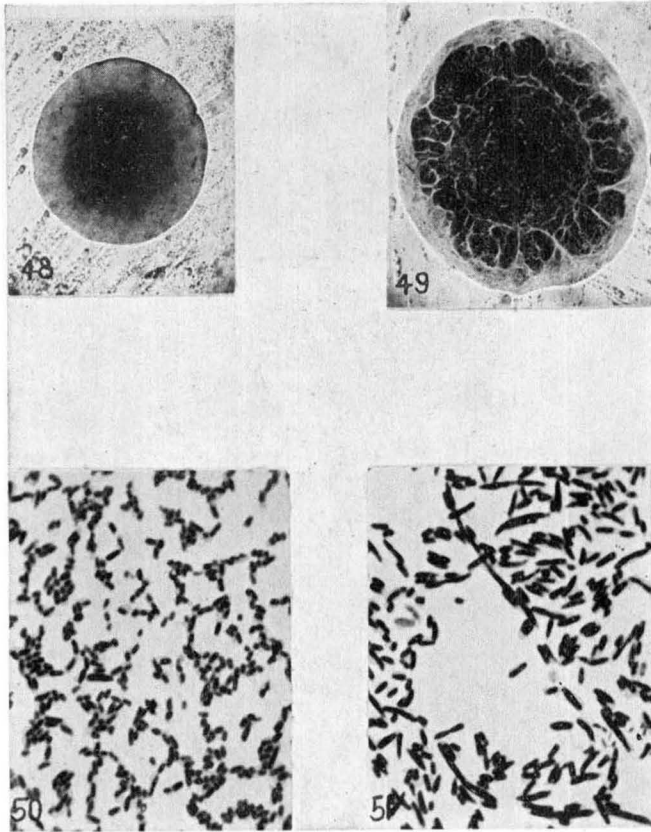
Figure 44. Variant E. Isolated colony on primary culture. Faint convolutions are visible. Meat extract agar. 48 hours old.  $\times 15$  diameters. (See page 37.)

Figure 45. Variant E. Isolated colony on primary culture. Irregular edge and numerous and prominent convolutions. Liver infusion agar. 48 hours old.  $\times 15$  diameters.

Figure 46. Variant E. Cells in a smear prepared from colony illustrated in Figure 44. Long filaments occasionally present.  $\times 1200$  diameters.

Figure 47. Variant E. Cells in a smear prepared from colony illustrated in Figure 45. Numerous short and long filamentous forms.  $\times 1200$  diameters.





**Figure 48.** Strain IV. Isolated colony on primary culture. Meat extract agar. 40 hours old.  $\times 15$  diameters. (See page 37.)

**Figure 49.** Strain IV. Isolated colony on primary culture. Liver infusion agar. 40 hours old.  $\times 15$  diameters.

**Figure 50.** Strain IV. Cells in a smear prepared from colony illustrated in Figure 48.  $\times 1200$  diameters.

**Figure 51.** Strain IV. Cells in a smear prepared from colony illustrated in Figure 49.  $\times 1200$  diameters.

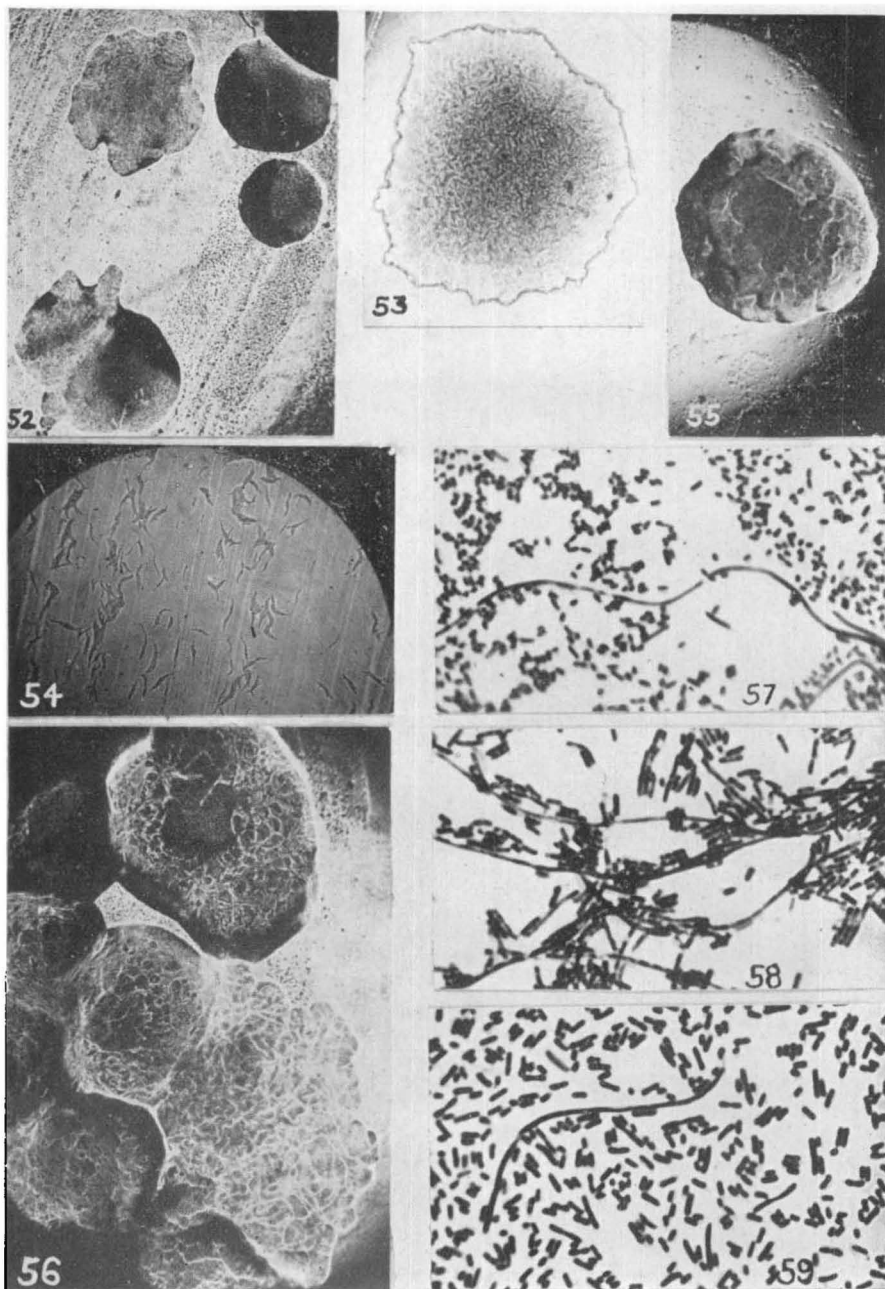


Figure 52. Strain XIV. Rough and smooth colonies on primary culture. Meat extract agar. 24 hours old.  $\times 20$  diameters. (See page 37.)

Figure 53. Strain XIV. Rough isolated colony on meat extract agar. Primary culture. 72 hours old.  $\times 15$  diameters.

Figure 54. Strain XIV. Small irregular-shaped colonies on meat extract agar. Transfer from colony illustrated in Figure 53. 18 hours old.  $\times 35$  diameters.

Figure 55. Strain XIV. Colonies on liver infusion agar. 24 hours old.  $\times 15$  diameters.

Figure 56. Strain XIV. Congested colonies on liver infusion agar. Periphery of the growth exhibits numerous convolutions. 40 hours old.  $\times 20$  diameters.

Figure 57. Strain XIV. Cells in a smear prepared from rough colonies illustrated in Figure 52.  $\times 1200$  diameters.

Figure 58. Strain XIV. Cells in a smear prepared from colonies illustrated in Figure 56.  $\times 1200$  diameters.

Figure 59. Strain XIV. Cells in a smear prepared from growth on meat extract agar.  $\times 1200$  diameters.

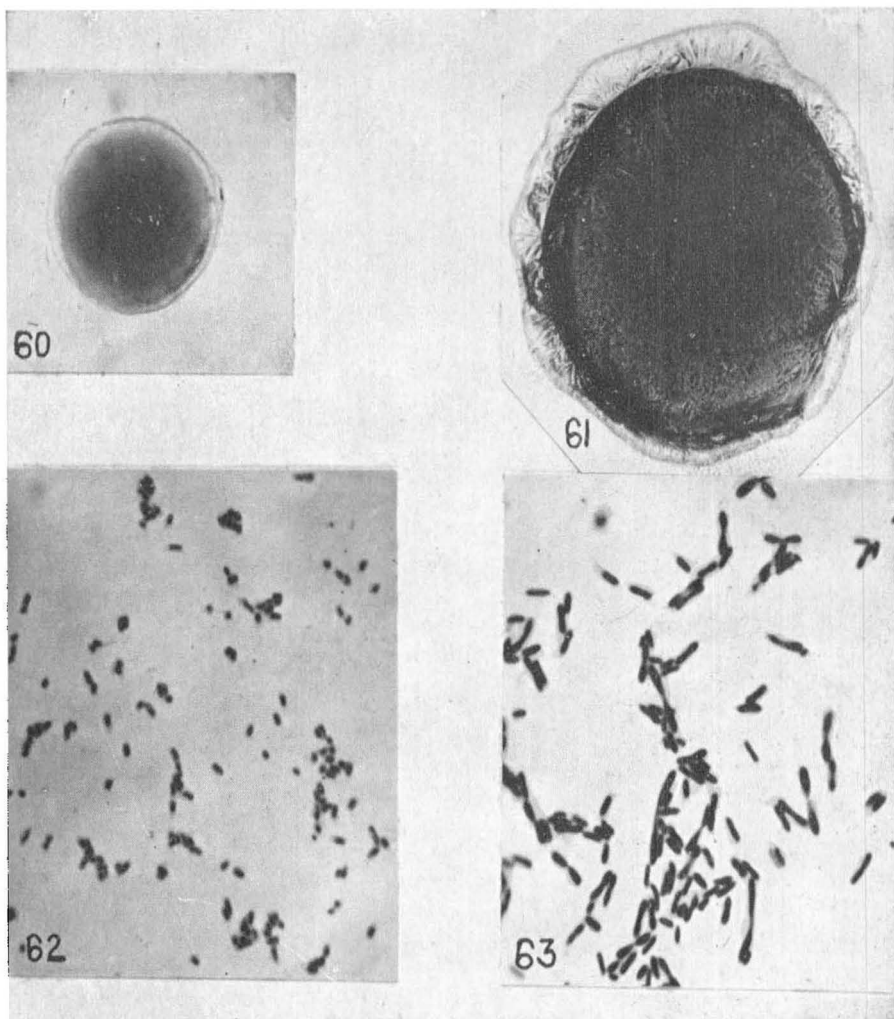
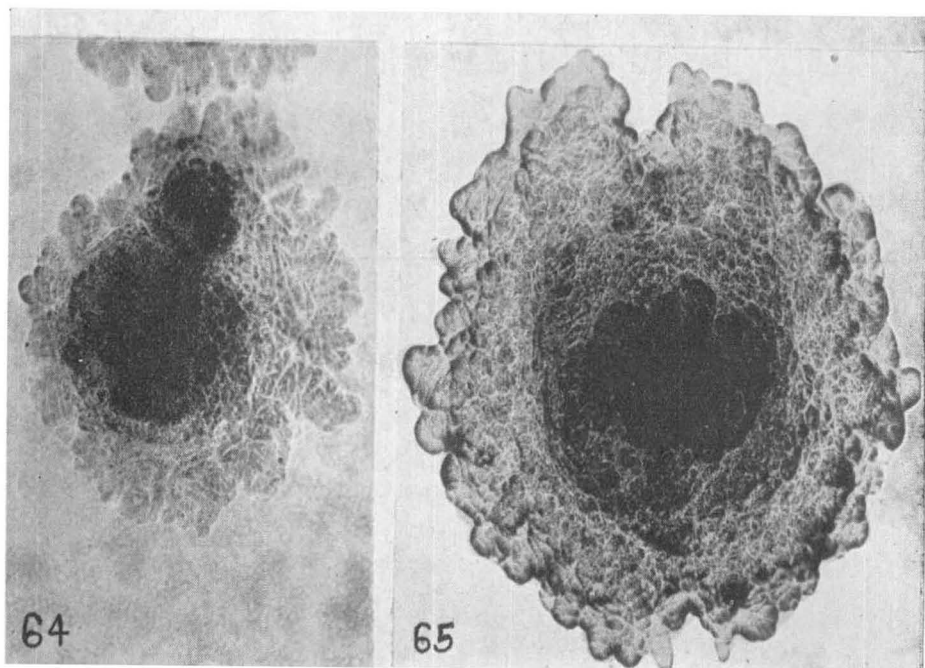


Figure 60. Strain 11706. Isolated colony on meat extract agar. Primary culture. 48 hours old.  $\times 15$  diameters.

Figure 61. Strain 11706. Isolated colony on liver infusion agar. Primary culture. 48 hours old.  $\times 35$  diameters.

Figure 62. Strain 11706. Cells in a smear prepared from growth on meat extract agar. Primary culture.  $\times 1200$  diameters.

Figure 63. Strain 11706. Cells in a smear prepared from growth on liver infusion agar. Primary culture.  $\times 1200$  diameters.



**Figure 64. Variant XII-b. Colony type on primary culture isolated from yolk of chick. General features similar to those of the colony illustrated in Figure 18. Liver infusion agar. Five days old.  $\times 11$  diameters. (See page 39.)**

**Figure 65. Variant XII-b. Colony type on primary culture isolated from necrotic tissue at site of inoculation. General features resemble those of colony type illustrated in Figure 18. Liver infusion agar. Five days old.  $\times 11$  diameters. (See page 39.)**

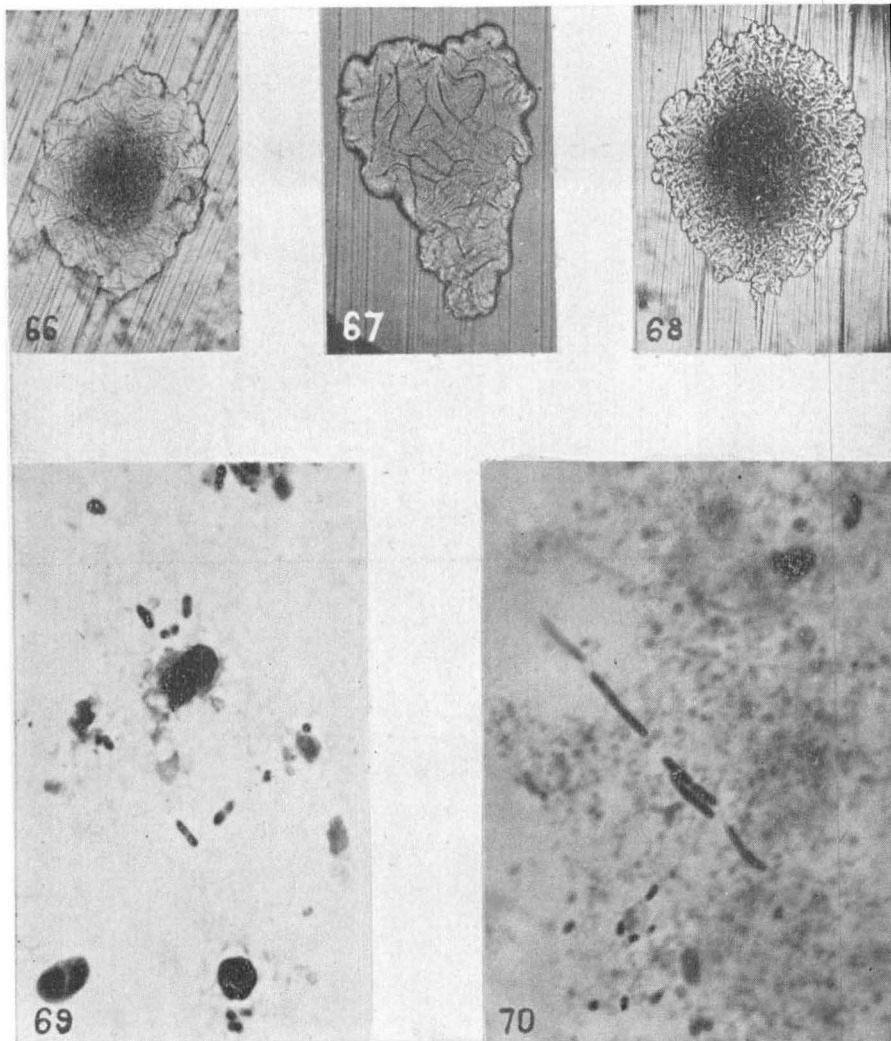


Figure 66. Variant C. A colony type with an irregular edge and markedly convoluted surface. Primary culture on liver infusion agar. Colony is isolated. 24 hours old.  $\times 11$  diameters. (See Table 4 and page 39.)

Figure 67. Variant D-1. A colony type with a very irregular outline. Surface prominently convoluted and striated. Primary isolation from necrotic tissue. Liver infusion agar, 24 hours old.  $\times 40$  diameters. (See Table 4 and page 39.)

Figure 68. Variant E. Colony type isolated from necrotic tissue. Primary isolation on liver infusion agar. Colony with an irregular edge and finely convoluted surface. Four days old.  $\times 15$  diameters. (See page 39.)

Figure 69. Strain 11706. Cells in a tissue smear prepared from an abscess in an infected chick which yielded pure cultures of *S. pullorum*.  $\times 1200$  diameters. (See page 39.)

Figure 70. Strain XIV. Cellular types in a smear prepared from unabsorbed yolk of a chick. Typical cells and short filaments may be seen.  $\times 1200$  diameters. (See page 39.)

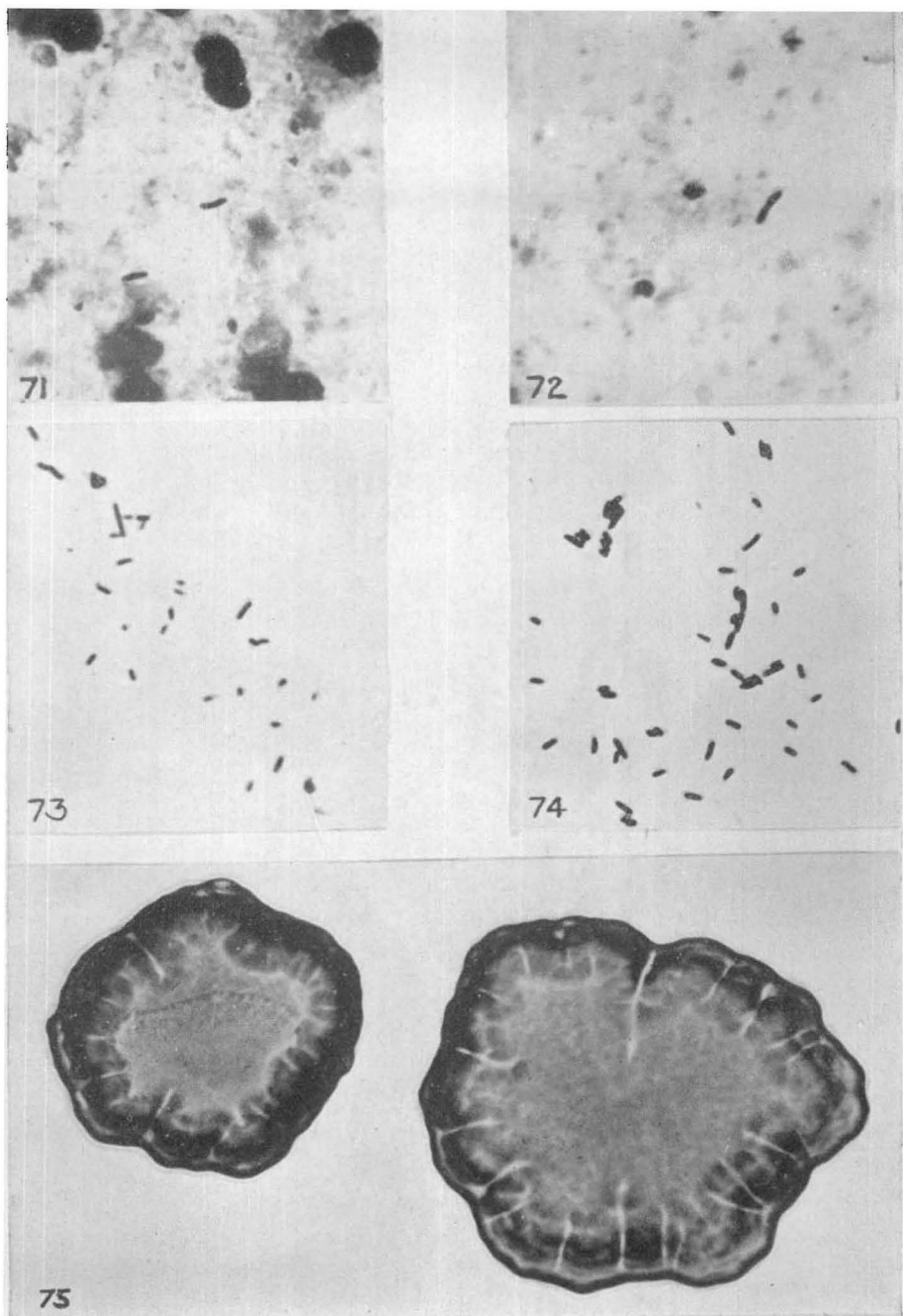


Figure 71. Variant IX-a. Cells in a tissue smear prepared from the spleen of an adult bird that died from a septicemic form of the disease. Cells, while slightly larger, resemble typical *S. pullorum*.  $\times 1200$  diameters. (See page 39 and Figures 73 and 74.)

Figure 72. Variant IX-a. Cells occurring in pairs. Same preparation as represented in Figure 71.  $\times 1200$  diameters.

Figure 73. Variant IX-a. Cells in a smear prepared from a meat extract agar culture isolated from the spleen tissue represented in Figures 71 and 72.  $\times 1200$  diameters.

Figure 74. Variant IX-a. Cells in a smear prepared from a liver infusion agar culture isolated from the spleen tissue represented in Figures 71 and 72.  $\times 1200$  diameters.

Figure 75. Variant XII-a. Colony type on primary isolation from yolk of chick. Meat extract agar. 96 hours old.  $\times 50$  diameters. (See Table 4 and page 44.)

### Behavior in Saline Solution

The property of auto-flocculation or agglutination of cultures was first reported by Nicolle, Steinhardt, Savage, and others. This phenomenon was looked upon as being similar to the agglutination reaction which occurs between a saline emulsion of cells and agglutinating serum. Furthermore, it was claimed that the end result in the two phenomena cannot be differentiated. This statement cannot be applied to the reaction of all *S. pullorum* variants, since the type of reaction can readily be distinguished from the true typical agglutination in the majority of cases. It is also recognized by some investigators that the so-called true "R" variant is characterized by its instability in saline solution. In order to determine whether this applies also to *S. pullorum* variants, the various types that have been isolated were placed in different concentrations of NaCl solution. The suspensions were prepared from cultures incubated at 37° C. for 48 hours. Table 6 shows the reactions of different variants. The results in this table reveal that different types of variants may exhibit the same type and degree of reaction. For instance, Variants D-1, D-2, IV-a, IV-b, V-a, X-a, and XII-a do not exhibit so much settling as do some other variants which are characterized by a colony form that is more remote from the normal type. However, some very rough colony types do appear more stable in saline solution than variants that are less rough in nature. The precipitate in some cases may be organized in clumps, and in others it may be re-suspended in a homogeneous emulsion. The extremely rough colonies developing on plain agar are characterized by their brittleness, which thus far has made it impossible to produce a uniform homogeneous suspension. Grinding of such cultures in a mortar greatly reduces the size of the clumps, but does not prevent the cells from settling. The rough variants on liver infusion agar produce a suspension in saline solution which is practically free from clumps which are visible to the naked eye. The process of settling may occur within a few minutes after the cells are suspended in the salt. The degree of clearing may be as complete as in true "four plus" agglutination. Partial or complete clearing may baffle one in the routine testing of fowl for pullorum disease, especially when slight clumping is also present. Such a reaction may in certain respects resemble that of a doubtful or non-specific reacting bird. Hence it appears that strains employed for the preparation of antigen which is to be used for serological purposes may cause considerable trouble if their stability in salt solution is decreased through certain environmental influences.

### Behavior in Fermentable Substances

In this experiment an effort was made to determine the ability of variants to attack different fermentable substances, and to observe the character of growth. The different variants (A, A-1, B, B-1, C, D-1, D-2, E, I-a, II-a, II-b, III-a, IV-a, IV-b, V-a, VI-a, VIII-a, IX-a, IX-b, X-a, X-c, XII-a, XII-b, XIII-a, XV-a, XVI-a, XVII-a) were tested in the following fermentable substances: Arabinose, dextrine, dextrose, dulcitol, galactose, inositol, inulin, lactose, levulose, maltose, mannitol, raffinose, rhamnose, salacin, sucrose and xylose. It may be stated that the variants attacked the same carbohydrates as did the typical *S. pullorum* strains or types. However, retarded growth and delayed fermentation were exhibited by some variants, particularly variants B and B-1. Levulose was not attacked by all of the variants, and most strains which acted upon it showed late acid production. Few produced gas with this carbohydrate. Some strains exhibited a settling of cells and clearing of the supernatant fluid, which was especially true when acid produc-

TABLE 6.—BEHAVIOR OF VARIANTS IN DIFFERENT CONCENTRATIONS  
OF SODIUM CHLORIDE.

Variant No.*	Concentrated Suspension of Cells .9% Salt	Dilute Suspension of Cells**			Observation Period Hours
		.9% Salt	.45% Salt	.11% Salt	
A	{CS CS	PS PS	SS SS	SS SS	24 48
A-1	{0 0	0 0	0 0	0 0	24 48
B	{CS CS	PS PS	SS SS	SS SS	24 48
B-1	{CS CS	PS PS	SS SS	0 0	24 48
C	{PS PS	PS PS	0 SS	0 0	24 48
D-1	{SS SS	0 SS	0 SS	0 SS	24 48
D-2	{PS PS	PS PS	PS PS	PS PS	24 48
E	{CS CS	CS CS	CS CS	0 SS	24 48
I-a	{CS CS	CS CS	CS CS	CS CS	24 48
II-a	{CS CS	CS CS	CS CS	PS PS	24 48
II-b	{PS PS	PS PS	PS PS	PS PS	24 48
III-a	{CS PS	PS PS	PS PS	SS SS	24 48
IV-a	{0 SS	0 SS	0 SS	0 SS	24 48
IV-b	{PS PS	SS SS	SS SS	SS SS	24 48
V-a	{SS SS	0 0	0 0	0 0	24 48
VI-a	{CS CS	CS CS	CS CS	CS CS	24 48
VIII-a	{CS CS	PS PS	PS PS	SS SS	24 48
IX-a	{CS CS	PS PS	SS SS	SS SS	24 48
IX-b	{CS CS	CS CS	0 0	0 0	24 48
X-a	{PS PS	SS SS	SS SS	SS SS	24 48
X-c	{SS SS	SS SS	SS SS	SS SS	24 48
XII-a	{SS SS	0 0	0 0	0 0	24 48
XII-b	{PS PS	SS SS	SS SS	SS SS	24 48
XIII-a	{CS CS	CS CS	PS PS	SS SS	24 48
XV-a	{0 0	0 0	0 0	0 0	24 48
XVI-a	{CS CS	PS PS	PS PS	SS SS	24 48
XVIII-a	{PS PS	PS PS	SS SS	SS SS	24 48

\*Refer to Table 4 for history of variants.

\*\*Turbidity equal to tube 1 of McFarland nephelometer scale.

Legend: CS—complete settling  
PS—partial settling  
SS—slight settling  
0—no settling



tion occurred. These results seem to show that, on the whole, the variants have undergone very little or no change in their fermentative powers.

**Behavior in Fresh Whole Egg**

In this experiment seven variants (C, D-2, E, IV-a, IV-b, VI-a, and IX-b) and their parent strains were cultivated in fresh whole eggs. The purpose of this experiment was to determine whether a natural environment, such as is found in the whole egg, would bring about a change in the colonial morphology of the variants and their parent strains. The fresh eggs were obtained from flocks that had revealed no signs of pullorum infection for at least two years. The cultures were suspended in 0.9 per cent saline solution and injected into the yolk through a small opening in the shell with a hypodermic syringe. The opening was sealed with hot paraffin after the inoculation. Measures for insuring sterility were observed in every step of this process. The eggs were incubated at 37° C. At each transfer tubes containing sterile saline solution were inoculated with yolk from inoculated eggs. The saline-yolk mixture was then injected into fresh eggs. Agar plates were also streaked with the saline yolk mixture, in order to check for purity and to study colony morphology. During the first part of this experiment transfers were made approximately once a week, while later the interval between transfers was extended to two weeks.

TABLE 7.—INFLUENCE OF FREQUENT TRANSFER IN FRESH EGGS ON THE COLONY MORPHOLOGY OF *S. pullorum*.

Transfer No.	Days After Previous Transfer	Colony Morphology of Strains											
		Variant Strains						Normal Strains					
		C	D-2	E	IV-a	IV-b	VI-a	IX-b	I	IV	VI	IX	XIV
1	0	R	RS	R	SR	SR	RS	R	SR	S	S	S	S
2	8	R	RS	R	SR	SR	RS	R	SR	S	S	S	S
3	8	R	SR	R	S	SR	RS	R	SR	S	S	S	S
4	6	R	SR	R	S	SR	RS	R	SR	S	S	S	S
5	7	R	SR	R	S	SR	R	R	S	S	S	S	S
6	7	R	RS	R	SR	SR	RS	R	SR	S	S	S	S
7	7	R	SR	R	S	SR	RS	R	S	S	S	S	S
8	15	R	SR	R	S	SR	RS	R	SR	S	S	S	S
9	15	R	SR	R	S	SR	RS	R	SR	S	S	S	S
10	13	R	RS	R	S	SR	R	R	S	S	S	S	S
11	14	R	RS	R	S	RS	RS	R	SR	S	S	S	S
12	12	R	RS	R	S	RS	RS	R	SR	S	S	S	S
13	21	R	RS	R	S	RS	RS	R	SR	S	S	S	SR
14	15	R	RS	R	S	RS	RS	R	SR	S	S	S	S
15	17	R	SR	R	S	SR	R	R	SR	S	S	S	S

Legend: R—rough type  
 RS—rough type predominating  
 SR—smooth type predominating  
 S—smooth type

Table 7 gives the number of passages in whole fresh eggs, the number of days between transfers, and the colony type for each strain. The terms "S" and "R" were selected to describe the type of colony produced. However, in this instance these terms are employed in a relative sense. The appearance of a rough colony may vary considerably, yet fall into the category of roughness; likewise, a culture may resemble the "S" type more closely than the "R" form, yet not be identical

with the normal smooth type. In Table 7 it may be observed that three variants with a rough colony type did not change at any time to the extent that the colony type resembled the smooth form. The four remaining variants exhibited some degree of fluctuation in their colony morphology. (See Figures 12, 16, and 23.) Two parent strains (I and XIV) also manifested a variation in colony type. However, as mentioned earlier, these two strains cannot be considered as stable, normal types. According to the results obtained, one is led to believe that the colonial morphology of variants and typical, smooth strains seems to be affected but slightly when cultivated in whole fresh eggs. This observation may have a very important bearing upon current methods of preserving antigen stock cultures. It would seem that the change in environmental conditions for this organism may not be so great in the egg as in agar slope cultures maintained at different temperatures.

At the time of three different transfers (tenth, eleventh, and fifteenth), the growth from the streaked plates was placed in different concentrations of NaCl solution in order to determine the stability of the organism in suspension. Table 8 shows that all variants exhibited some degree of salt sensitiveness. Three strains among the typical group exhibited a slight degree of instability. However, two of these strains (I and XIV) are erratic in their behavior and almost always will pass for typical, smooth types. From the results given in Table 8, it appears that little or no change occurred in the behavior of any of the strains in NaCl solution following frequent transfer and cultivation in whole egg.

TABLE 8.—INFLUENCE OF FREQUENT TRANSFER IN FRESH EGGS ON THE CELLULAR STABILITY OF *S. pullorum* AS REVEALED IN SALINE SOLUTIONS

Transfer No.	Salt Solution	Cellular Stability of Strains											
		Variant Strains						Normal Strains					
		C	D-2	E	IV-a	IV-b	VI-a	IX-b	I	IV	VI	IX	XIV
10.....	.9%	PS	SS	PS	PS	SS	CS	PS	PS	SS	0	0	PS
	.45%	0	0	0	SS	SS	0	0	0	0	0	0	0
	.22%	0	0	0	SS	SS	0	0	0	0	0	0	0
11.....	.9%	PS	0	PS	PS	0	PS	CS	SS	SS	0	0	SS
	.45%	0	0	PS	SS	0	PS	PS	—	0	0	0	0
	.22%	0	0	SS	SS	0	SS	0	0	0	0	0	0
15.....	.9%	PS	0	SS	SS	0	CS	0	0	0	0	0	SS
	.22%	0	0	0	SS	0	SS	0	0	0	0	0	0
	.11%	0	0	0	SS	0	0	0	0	0	0	0	0

Legend: CS—complete settling  
 PS—partial settling  
 SS—slight settling  
 0—no settling  
 (—)—no suspension

The cultivation of variants in fresh egg yolk placed in sterile tubes did not prove as satisfactory as the whole-egg method for maintaining the different types. The egg yolk-tube method is objected to for the reason that the yolk undergoes desiccation, a change in composition, and, in all probability, an alteration in appearance. Environmental factors seem to exert a greater influence in the egg-yolk than in the whole-egg method. Considerable difficulty was encountered in keeping the variants viable during their sojourn in the yolk. In a few instances an incubation period of two weeks was sufficient to cause death. Also, this method involves a greater risk of contamination. The organism apparently attacks the components of the yolk material, since the inoculated tubes differed

materially in appearance from the uninoculated. In some instances the contents, after an incubation period of two weeks, resembled the interior of diseased ova in adults and unabsorbed yolk in young stock.

The observation that variants may pass through the egg unchanged in their colony form is substantiated by the above findings.

### Behavior of Variants in the Natural Animal Host

In this experiment the behavior of the variants in chicks and adult fowl was studied. Special attention was directed toward determining the stability of the types in the living host. Pathogenic and serologic characters were studied in greater detail in another series of experiments. Pullorum disease-free stock, including chicks and mature fowl, was employed in this experiment. Both variants and typical normal strains were investigated, the latter being regarded chiefly as controls. Saline suspensions were prepared from 24-hour agar cultures. The concentration was adjusted to a turbidity of tube 2 of McFarland's nephelometer scale in all instances except one, in which the turbidity was equal to tube 1.25.

Four different lots of chicks were fed or inoculated with suspensions of the organism. The chicks exposed to the different strains were confined in separate cages and cared for in such a manner as to minimize the possible hazard of cross infection. In a few instances the chicks were intentionally chilled in order to reduce their resistance to the invasive power of the organism. A period of approximately two weeks elapsed between the time of inoculation or feeding and the necropsy. Chicks that succumbed during the observation period were necropsied within a short time after death. Cultures were made from the liver, spleen, yolk, and in some instances other organs or tissues, depending upon the pathological picture encountered. Tissue impression smears were made from chicks in the first few inoculated series for the purpose of determining the presence and morphology of the organism in the host. Only those smears prepared from tissues which yielded *S. pullorum* in pure culture were stained with a dilute solution of Hucker's gentian violet, and examined. The isolated cultures were checked for type in broth containing dextrose, maltose, lactose, sucrose, and in some instances dulcitol; by the Gram staining method; also in a number of cases by the agglutination test.

No. 1. In this lot, 26 chicks were fed with three variants (C, D-1, and E) and one typical strain designated 11,706, which had been recently isolated from the ovary of a hen. The dose was 0.03 cc. The results obtained from feeding the different organisms are presented in Table 9, and show that the percentage of infected chicks was very low. Three of the four chicks which revealed *S. pullorum* had been inoculated with the typical strain, 11,706. The recovered organism resembled the original strain in colonial and cellular morphology. The strain recovered from the group infected with Variant E also appeared identical with the original, and was agglutinated by positive pullorum serum. The cells settled out in the presence of negative serum and in the antigen control. A few of the cultures were isolated in duplicate on meat extract and liver infusion agars. Infrequently growth occurred on one medium and not on the other, especially when but few colonies were isolated from an organ. Occasionally only one colony was observed on a plate, which suggests that the amount of tissue used for inoculating is an important factor in obtaining positive isolations.

No. 2. Eighty-two chicks obtained from the same source as those in No. 1 were fed and inoculated with six variants (B, C, D-1, E, VI-a, and IX-b) and four so-called smooth types (IV, XIV, 11,706, and 18,292). For a description of

the variants the reader should refer to Table 4. Saline suspensions were prepared from 24-hour cultures, and the turbidity was standardized to tube 2 of McFarland's nephelometer. Each chick was given 0.1 cc. of the bacterial suspension per orem. During the first seven days after exposure no symptoms were observed, and there was no mortality. It was deemed advisable at this time to expose the chicks a second time in order that positive isolation might be obtained. Hence, a second dose (0.5 cc.) of a suspension similar to the first was administered subcutaneously. In Table 10 are given the results obtained from the two exposures to infection.

TABLE 9.—RESULTS OF FEEDING *S. pullorum* VARIANTS TO YOUNG CHICKS.

Strain No.	Number of Chicks	Number Died	Number Yielding <i>S. pullorum</i>	Number Killed	Number Yielding <i>S. pullorum</i>	Remarks
11706*	5	2	2	2**	2	Symptoms and lesions observed Typical <i>S. pullorum</i> recovered
C	5	0	—	5	0	No lesions observed
D-1	5	1	0	5	1	Few lesions observed
E	5	0	—	5	1	Few lesions observed. The recovered organism resembled the original variant
Uninoculated controls	6	2	0	4	0	No lesions

\*Represents a normal strain.

\*\*Unfortunately, one chick from group 11706 escaped to cage D-1 on the day the chicks were necropsied. The organism isolated from Group D-1 resembled 11706 more closely than D-1; hence it may be that the escaped bird was infected with strain 11706 and that all birds in Group D-1 were negative.

It is apparent that a greater mortality was encountered from the smooth typical strains than from the variants. Likewise, the percentage of positive isolations of *S. pullorum* was far greater among the typical strains than among the variants. Among the latter, three strains (C, D-1, and IX-b) failed to produce infection.

Variant B (Table 10) established itself in six of seven chicks. In most instances necrotic foci were observed in the liver of these chicks. Prominent fibrous nodules were detected in the myocardium, wall of the proventriculus, gizzard, and intestine. This type of lesion was observed in one or more organs in every chick inoculated with this strain. *S. pullorum* was isolated from the majority of tissues cultured. In a few instances the colony form on plain meat extract agar revealed a slight tendency toward roughness, but these rough features were more pronounced in cultures isolated on liver infusion agar. (See Figures 31-37, inclusive.) Stained smears prepared from colonies on plain meat extract agar revealed typical cells, while those prepared from liver infusion agar contained markedly pleomorphic types. (See Figures 38-41, inclusive.) The cells were also larger in smears prepared from colonies on liver infusion agar. The results obtained with Variant B clearly show that the organism had lost some of its original features during its passage through the host. However, it also is apparent that the recovered organism is not typical in every respect, as shown by the colonial and cellular morphology.

Variant E (Table 10) was recovered from two chicks. One of these died within twelve hours after inoculation. Upon necropsy no significant gross lesions were observed. The organism was recovered from the liver and spleen. On plain meat extract agar the growth resembled that produced by this organism before injection. On liver infusion agar typical rough growth was detected. (See Figures 42 and 43.)

TABLE 10.—RESULTS OBTAINED WITH CHICKS EXPOSED TO NORMAL AND VARIANT STRAINS OF *S. pullorum*.

Strain No.	Number of Chicks	Chicks Died		Number Yielding <i>S. pullorum</i>	Number Killed	Number Yielding <i>S. pullorum</i>	Total Per cent Positive
		Number	Days After Exposure				
Variants:							
B	7	0	—	—	7	6	85.71
C	7	1	9	0	6	0	0
D-1	7	0	—	—	7	0	0
E	7	1	8	1	6	1	28.57
VI-a	7	0	—	—	7	1	14.28
IX-b	7	0	—	—	7	0	0
Normal Strains:							
IV	7	0	—	—	7	4	57.12
XIV	7	{1 1	{10 16	{1 1	5	3	71.42
11706	7	{1 1 1 2	{11 15 16 18	{1 1 1 2	2	1	85.71
18292	7	1	15	1	6	6	100.00
Uninoculated controls	12	0	—	—	6*	0	0

\*Six control chicks were not destroyed.

Few colonies were isolated from the second chick, all of which appeared atypical in colonial and cellular morphology. (See Figures 44-47, inclusive.)

Variant VI-a was recovered on meat extract agar, and resembled the normal type. Liver infusion agar plates were negative. As stated earlier, this variant does not present a very marked colonial difference from the normal.

Strain IV was recovered from four out of seven chicks. Few gross lesions were observed. In every case the organism was isolated from the spleen, while cultural tests with other tissues were negative in most instances. The colonial and cellular morphology on plain extract agar appeared typical, while on liver infusion agar a rough colony type which contained pleomorphic cells was observed. (See Figures 48-51, inclusive.)

Strain XIV was recovered from five out of seven chicks. Gross lesions were observed in most instances, involving one or more of the following organs:—liver, spleen, and heart. The recovered organism exhibited a smooth colony type on meat extract and liver infusion agars in most instances. This strain, while exhibiting a tendency to vary, generally appears quite typical. (See Figures 52-59, inclusive.)

The chicks infected with Strain 11,706 manifested clinical symptoms of the disease. Upon necropsy extensive lesions were observed, and the organism was recovered from six of the seven chicks. This apparently typical strain seemed to be more pathogenic than the other variants employed. Meat extract agar plates yielded typical growth, while on liver infusion agar a few colonies exhibited surface

markings. Smears from the different colonies showed the cells from liver infusion agar cultures to be much larger than those taken from meat extract agar. In some cases the difference was so great that the two cultures might be considered different species. (See Figures 60-63, inclusive.)

Strain 18,292 was recovered from all of the seven infected chicks. Gross lesions, which were quite extensive in some cases, were present in all chicks. The colonial and cellular types were similar to those observed with Strain 11,706.

Antigens prepared from the isolated strains were agglutinated by positive pullorum serum, but not by negative serum. However, four antigens (one prepared from Strain VI-a, and three from Strain XIV) exhibited atypical agglutination.

Blood samples were collected from all killed chicks. Agglutination tests with pullorum antigen produced complete agglutination in dilution of 1:40, and a trace in 1:80.

No. 3. Variant IX-b was passed through five different groups of chicks by serial passage. In the first passage a suspension of the organism with a turbidity equal to tube 2 of McFarland's nephelometer scale was administered per orem on three successive days in the following doses:—0.2 cc., 0.5 cc., and 0.5 cc. The remaining groups of chicks were inoculated subcutaneously with 0.4 cc. of a suspension having the same concentration. The time during which the organism remained in artificial cultivation varied between passages. Between the first and second passages the organism was maintained in fresh egg yolk for twenty days before being re-inoculated. In the remaining passages the primary culture was two to five days old when inoculated. Table 11 shows the results of the serial passage of this strain through chicks. The percentage of recovery of the organism is low in every group except No. III. The organism recovered resembled the original inoculated variant in every case. (See Figures 14 and 15.) Scattered isolated colonies attained a diameter of approximately two centimeters. It appeared as if the freshly isolated culture possessed some growth property which appeared absent in the same variant maintained under continual artificial cultivation. The cellular morphology did not vary to any degree during this series of passages.

TABLE 11.—RESULTS OF SERIAL PASSAGE OF VARIANT IX-B THROUGH CHICKS.

Group	Number of Chicks	Number Killed	Number Showing Lesions	Number Yielding <i>S. pullorum</i>	Tissues Yielding <i>S. pullorum</i>
I	4	4	2	1	Liver
II	10	10	6	1	Yolk
III	5	5	3	3	Subcutaneous inoculation necrotic tissue at site of
IV	5	5	2	1	Subcutaneous inoculation necrotic tissue at site of
V	4	4	4	1	Subcutaneous inoculation necrotic tissue at site of

It will also be noted in Table 11 that the majority of isolations were made from necrotic tissue located at or near the site of inoculation. The amount of necrotic tissue and local tissue reaction varied greatly in the different chicks. In some instances a proliferation of connective tissue, greatly congested and surrounding the leathery yellowish necrotic material, was observed. In two instances the organism invaded the tissues of the host beyond the point of inoculation. However, it was apparent that the invasive power of the organism was not great, although it

evidently possessed some power of stimulating a local tissue reaction, which was entirely absent in chicks inoculated with other variants.

These results definitely show that Variant IX-b can invade the natural host beyond the point of inoculation, as well as cause local tissue response without undergoing an alteration in colonial and cellular morphology. Furthermore, little or no increase in virulence was observed. However, this conclusion may not be justified since the experiment was not designed for such a determination.

No. 4. Ten chicks were inoculated subcutaneously with 0.4 cc. of a suspension of Variant XII-b. No mortality resulted from the injection. The variant strain was recovered from three of the killed chicks. In two instances the organism was isolated from the yolk, and in the third, from the spleen, which indicates that the organism had definitely invaded the host and established itself. A marked thickening of the yolk capsule, due to infection, was observed. The isolated organism resembled the original variant in colonial and cellular morphology. (See Figures 18, 64 and 65.) In only one instance was local tissue response evident at the time of necropsy. Variant XII-b is characterized by the filamentous portion of its colony, and this feature apparently is little affected through animal passage.

No. 5. Twelve pullets which were approximately four months of age were inoculated subcutaneously with suspensions of eight variants (A, B, C, D-1, E, VI-a, VIII-a, and IX-a) and four so-called normal strains (IV, XIV, 11,706, and 18,292). Three injections of 2 cc. each were administered on alternate days. Approximately three weeks after the last injection, all birds were necropsied. No clinical manifestations were observed as the result of the inoculation. Table 12 shows the response of the different strains in the living host. Five birds inoculated with variants failed to produce agglutinins. Also, the organism was not recovered at necropsy. Two birds inoculated with typical strains did not yield *S. pullorum* at necropsy, but their sera agglutinated pullorum antigen. Three variants (C, D-1, and E) and two so-called normal strains (XIV and 18,292) were recovered from the birds. The different variants did not lose their characteristic features to any extent, as the result of passage through the host. (See Figures 66-68, inclusive.) Strain XIV exhibited typical colonial features, but the cells appeared pleomorphic in smears prepared from meat extract and liver infusion agar. Antigens prepared from the isolated organisms all exhibited atypical reactions, with the exception of that prepared from Strain 18,292.

*S. pullorum* was observed in stained tissue impression smears from chicks and adults that yielded pure cultures of the organism on necropsy. The morphology of the organism in a few instances appeared atypical and slightly larger than the normal type, but as a rule a very close resemblance was seen. (See Figures 69-74, inclusive.) Extreme pleomorphic forms were never detected, and it is doubtful whether such forms ever occur in the natural host. However, organisms which possess the potentialities of reproducing pleomorphic types on artificial medium do exist, as has been reported.

In a brief summary concerning the data presented in the different experiments of this part, it may be stated that variants of *S. pullorum* may settle out in broth cultures, but that the degree of settling will vary among the different variants. Likewise, the stability in NaCl solution will vary among different types, and even fluctuate within the same type. The ability to attack different fermentable substances appears to be changed but little, if any, for all variants. Sojourn in fresh whole eggs and in the natural host apparently had little or no influence on the general features of the different variants.

TABLE 12.—RESULTS OBTAINED WITH PULLETS INOCULATED WITH NORMAL AND VARIANT STRAINS OF *S. pullorum*.

Strain No	Bird No.	Days Between Last Injection and Test	Agglutination Titre								Tissues Yielding <i>S. pullorum</i>
			10	20	40	80	160	320	640	1280	
Variant:											
A	39391	{ 6	0	0	0	0	0	0	0	0	None
		{ 21	0	0	0	0	0	0	0	0	
B	39386	{ 6	0	0	0	0	0	0	0	0	None
		{ 22	0	0	0	0	0	0	0	0	
D-1	39394	{ 7	4	4	4	2	1	0	0	0	Necrotic cyst in abdominal (wall)
		{ 20	4	3	1	0	0	0	0	0	
C	39389	{ 7	0	0	0	0	0	0	0	0	Ovary
		{ 20	4	4	4	4	3	2	1	0	
E	39374	{ 7	4	4	4	2	1	0	0	0	Necrotic tissue in abdominal (nal fat)
		{ 23	3	2	0	0	0	0	0	0	
VI-a	39390	{ 7	0	0	0	0	0	0	0	0	None
		{ 20	0	0	0	0	0	0	0	0	
VIII-a	39387	{ 7	0	0	0	0	0	0	0	0	None
		{ 20	0	0	0	0	0	0	0	0	
IX-a	39371	{ 7	2	0	0	0	0	0	0	0	None
		{ 20	0	0	0	0	0	0	0	0	
Normal:											
IV	39380	{ 4	4	4	4	4	4	2	1	0	None
		{ 21	4	4	4	4	2	0	0	0	
XIV	39383	{ 6	4	4	2	0	0	0	0	0	Ovary and spleen
		{ 21	3	2	1	0	0	0	0	0	
18292	39381	{ 5	4	4	4	4	4	2	1	0	Heart, spleen and ovary
		{ 21	4	4	4	4	3	1	0	0	
11706	39373	{ 4	4	4	4	4	4	2	0	0	None
		{ 20	4	4	4	4	3	0	0	0	

Legend: 4—complete agglutination  
 3—incomplete agglutination  
 2—partial agglutination  
 1—slight agglutination  
 0—no agglutination

### VIRULENCE STUDIES

The virulence of pathogenic bacteria has been known to fluctuate since the discovery of human and animal pathogens. A decrease or loss of virulence is frequently observed among laboratory strains that are cultivated on artificial media. Also, it has been claimed that fluctuations in epidemics and epizootics are in part due to a variation in the virulence of the causative agent. However, direct and conclusive evidence to support such a statement is wanting at the present time. It is accepted that avirulent strains may be isolated simultaneously with highly virulent types from the same natural outbreak. The virulence of pathogens may be attenuated by various means such as the application of anti-septics, drugs, heat, body fluids, etc. Furthermore, diminished or complete loss of virulence is frequently associated with a variation of the other properties of a microorganism, such as colony and cellular form, antigenicity, and agglutinability. Arkwright (1921), De Kruif (1921), and others have demonstrated that certain types may possess different degrees of pathogenicity. In order to determine the virulence possessed by the different variants described in this investigation, some of the types were studied in the natural host and in an alien host (guinea pig).



For convenience in the exposition of the results obtained in this phase of the investigation, the virulence studies concerning the natural host are presented first.

### Pathogenicity for the Natural Host

A total of 312 day-old chicks and 19 different strains of *S. pullorum* (including variants and their parent strains) were employed. The various strains were studied in small lots of chicks. Comparative virulence tests were made with the parent strain and its variant in every lot of chicks except one. The chicks were maintained under the best possible sanitary conditions and were given ample food and water. Necessary heat was provided by improvised means. Every possible precaution was taken to eliminate cross-infection, and proved effective, as will be seen later. The procedure of infecting the chicks was in general the same throughout this investigation. Previous experiments made it seem advisable to select an inoculating dose that would produce positive results with the parent strains. Hence, a dose of 0.4 cc. of a saline suspension of the organism, with a turbidity equal to tube 2 of McFarland's nephelometer scale, was inoculated subcutaneously in the region of the loose skin between the thigh and abdomen. The suspended organisms were prepared from 24-hour agar cultures. Under natural conditions it has been observed that the onset and course of a disease outbreak may be greatly influenced if the resistance of the host is lowered by overheating, chilling, or improper feeding. In order to further insure the establishment of infection, the different lots of chicks were chilled immediately after inoculation. The chilling temperature and the time of exposure were not the same for all groups of chicks. However, the chicks within the same lot were subjected to the same treatment, unless otherwise stated. Clinical manifestations were recorded and all dead chicks were necropsied. *S. pullorum* cultures were identified by the cultural, tinctorial, and biochemical properties. Two weeks after the inoculation the surviving chicks were destroyed and necropsied. The results obtained are presented in Table 13.

Brief supplementary remarks may be made for each lot of chicks as follows:—

*Lot I.* The chicks in this group were not chilled. No clinical manifestations were observed. A similarity in the degree of virulence between Variant C and its parent Strain I may in part be accounted for by Strain I having been subjected to various treatments since the time of isolation, and having at times exhibited characteristics remote from the typical smooth type.

*Lot II.* Ten chicks, five of which were chilled, were inoculated with parent Strain IX. Symptoms were observed in both the chilled and unchilled chicks. Extensive gross lesions were observed in most cases, and a marked local tissue response was seen at the site of inoculation. The latter was absent in the chicks inoculated with variant IX-a.

*Lot III.* The same parent strain as in Lot II, and its variant IX-b, which was maintained on liver infusion agar, were employed. Also, a strain (Stellar) which was freshly isolated from naturally infected chicks was included. During the chilling process two chicks were lost from each of the groups inoculated with Strains IX and IX-b. No clinical response was seen in chicks inoculated with the variant, while in the other two groups marked reactions were observed in some chicks. The lesions in the latter two groups were more extensive than in the group inoculated with the variant. The organisms recovered resembled the original inoculated strains. The variant grew profusely and the colonies attained a diameter of one centimeter.

TABLE 13.—RESULTS OBTAINED WITH CHICKS INOCULATED WITH THE NORMAL PARENT STRAINS OF *S. pullorum* AND THEIR VARIANTS.

Lot No.	Strain No.*	Number of Chicks	Number Died	Number Yielding <i>S. pullorum</i>	Number Killed	Number Yielding <i>S. pullorum</i>	Total Per cent Positive	Remarks
I	**I (not chilled)	10	0	—	10	0	0	No significant gross lesions
	C (not chilled)	10	0	—	10	0	0	No significant gross lesions
	Controls (not chilled)	6	0	—	6	0	0	No gross lesions observed
II	**IX (not chilled)	5	1	1	4	4	100.00	All chicks revealed gross lesions
	**IX	5	0	—	5	5	100.00	All chicks revealed gross lesions
	IX-a	10	0	—	10	0	0	No gross lesions observed
	Controls	6	0	—	6	0	0	No gross lesions observed
III	**IX	10	3	3	5	5	100.00	All chicks revealed gross lesions
	IX-b	10	0	—	8	4	50.00	Five chicks revealed gross lesions
	Stellar	10	3	2	7	7	90.00	All chicks revealed gross lesions
	Controls	6	0	—	6	0	0	No gross lesions observed
IV	**I	5	0	—	5	0	0	No gross lesions observed
	C	5	0	—	5	0	0	No gross lesions observed
	**XIV	5	1	1	4	4	100.00	All revealed gross lesions
	E	5	2	2	3	3	100.00	All revealed gross lesions
	Controls	5	0	—	5	0	0	No gross lesions observed
V	B	10	0	—	10	0	0	No gross lesions observed
	B-1	10	0	—	10	0	0	No gross lesions observed
	Controls	7	0	—	7	0	0	No gross lesions observed
VI	**VIII	10	10	10	0	—	100.00	All chicks revealed extensive gross lesions
	VIII-a	10	2	—	8	0	0	One chick revealed gross lesions
	Controls	6	0	—	6	0	0	No gross lesions observed
VII	**III	10	10	9	0	—	90.00	All chicks revealed gross lesions
	III-a	10	1	0	9	0	0	One chick revealed gross lesions
	III-S	10	8	8	2	2	100.00	All chicks revealed gross lesions
	Controls	5	0	—	5	0	0	No gross lesions observed
	**VIII	5	2	2	3	2	80.00	All chicks revealed gross lesions
VIII	**VIII (diluted)	5	2	2	3	3	100.00	All chicks revealed gross lesions
	VIII-a	10	0	—	10	0	0	No gross lesions observed
	VIII-a†	5	0	—	5	0	0	No significant gross lesions observed
	III-a†	5	0	—	5	0	0	One chick revealed gross lesions
	Controls	6	1	0	5	0	0	No gross lesions observed
	**XII	10	6	6	4	4	100.00	All chicks revealed gross lesions
IX	XII-a	10	0	—	10	3	30.00	Four chicks revealed gross lesions
	XII-b	10	0	—	10	3	30.00	Two chicks revealed gross lesions
	Controls	5	1	0	4	0	0	No significant gross lesions observed
	**XII	10	10	10	0	—	100.00	All chicks revealed gross lesions
X	XII-a	10	0	—	10	2	20.00	Three chicks revealed gross lesions
	XII-S	10	0	—	10	4	40.00	Three chicks revealed gross lesions
	VIII-a††	10	0	—	10	2	20.00	Three chicks revealed gross lesions

\*Refer to Table 4 for history of strains.

\*\*Represents parent strain.

†Chicks received five daily inoculations.

††Chicks received fourteen daily inoculations.

*Lot IV.* The chicks inoculated with Strain I and its variant C responded in the same manner as those in Lot I, in spite of the fact that they had been chilled while those in Lot I had not. It appears that these two strains, as employed here, might be considered avirulent. Strain XIV and its variant E behaved similarly, although the parent strain appeared slightly more virulent. However, this difference may not be significant, since the clinical and pathological manifestations may be due in part to other factors, aside from virulence. The strains isolated resembled the original inoculated type.

*Lot V.* The strains employed in this lot produced negative results in every respect. As a matter of interest, ten chicks from this lot were inoculated with variant XII-b. Positive isolations of this variant were obtained. (These results are reported in Experiment 4, page 39.)

The chicks employed in subsequent lots were received from a different source. Unfortunately, one infected adult bird was detected in the parent stock from which the chicks in the previous experiments were obtained. This flock had not harbored birds reacting to the macroscopic agglutination test, for three years. However, the chicks received from this flock did not represent the progeny of the infected bird or birds that were in contact with the latter, and it appears from the results that the chicks employed undoubtedly were free from the disease.

*Lot VI.* Most striking results were obtained with the strains in this group. Strain VIII was observed to be markedly virulent, as compared to its variant and other strains employed previously. All chicks died within two days after inoculation. No symptoms were observed in the chicks that were inoculated with the variant.

*Lot VII.* In this group the type of variant was similar to VIII-a used in Lot VI. Strain III-S represents a typically smooth strain selected from the same "SR" culture from which III-a was derived. Strain III-S had been treated in the same manner as III-a and was included in this lot to determine whether the decrease or loss in virulence might be due to the treatment of the organism which affected virulence only, or whether the difference in colonial appearance represented certain profound changes in the organism which also influence virulence. It was observed that the symptoms, mortality rate, and lesions were quite similar for Strains III and III-S. One chick inoculated with III-a died from overcrowding.

*Lot VIII.* In this lot an effort was made to determine the influence of diluting the concentration of the dose used for inoculation. The parent strain was prepared in two different concentrations, the one equal to the usual turbidity employed, and the other diluted three times. It may be observed that there were no noticeable differences in the results of the inoculations. Also, two groups of five chicks each were inoculated with Variants VIII-a and III-a, respectively, for five consecutive days. No symptoms were detected in the chicks inoculated with the variants.

*Lot IX.* Two variants derived from one and the same strain were employed in this lot. The parent strain caused mortality, and the number of positive isolations of this strain was greater than that of the variants. Variant XII-a was isolated as typical *S. pullorum*, but XII-b was recovered with apparently little or no change in colonial and cellular morphology. (See Figures 18, 64, and 65.)

*Lot X.* In this group XII-S was a smooth strain which was derived from XII-a ten days prior to inoculation. While the difference in symptoms, mortality, and lesions between the parent strain and the two variants is marked, the two variants behaved quite similarly. It appears that, although the property of smoothness had returned, the virulent property of the parent strain had not been restored.

Strain XII-a was recovered, and the growth resembled that of the original variant type. (See Figures 17 and 75.)

*Lot XI.* The chicks in this group were inoculated with variant VIII-a. A dose of 0.4 cc. was administered daily for the first seven days and 0.5 cc. for the next seven days. No apparent symptoms were observed. Necropsies were conducted one week after the last injection. Two chicks yielded *S. pullorum*. The organism appeared typical (normal) and in no way resembled the variant originally inoculated. In one instance the organism was isolated from the spleen and necrotic tissue at the site of inoculation, while in the other it was isolated only from the necrotic tissue. Lesions in the remaining chicks were of no significance. The recovered organism underwent characteristic agglutination with positive pullorum serum, while with negative serum no agglutination or settling was seen. The serum of the chicks was tested with the homologous antigen and with typical pullorum antigen. The homologous antigen (VIII-a) revealed a tendency to settle out in the first few dilutions, while no agglutination or settling was observed with the pullorum antigen.

TABLE 14.—COMPARISON OF RESULTS OF INOCULATION WITH PARENT AND VARIANT STRAINS OF *S. pullorum*.

Treatment	Number of Chicks	Chicks Died		Chicks Yielding <i>S. pullorum</i>	
		Number	Per cent	Number	Per cent
Inoculated with Parent Strain ..	120	48	40.00	90	75.00
Inoculated with Variant.....	130	5	3.85	21	16.15
Controls, not inoculated.....	52	2	3.84	0	0

Table 14 gives a comparison of the results obtained with parent strains and their variants, when inoculated into chicks. Among the 120 chicks inoculated with the parent strains, 48, or 40 per cent, died. Among the 130 inoculated with the variants, 5, or 3.85 per cent, died. *S. pullorum* was isolated from 90, or 75 per cent, of the chicks inoculated with the parent strains as compared with 21, or 16.15 per cent, of those injected with the variants. The differences between the two groups in the percentage of mortality and positive isolations are very significant.

Beyond a doubt, some of the variants studied here definitely appear less pathogenic for chicks than their parent strains. Variants III-a and VIII-a, which were administered in frequent doses without producing symptoms, must have undergone a profound change in their structure, since their parent strains were among the most pathogenic of the strains studied. Relative to the two positive isolations from chicks in Lot XI, one may question whether some of the cell variants reverted to the normal type, or whether the strain was not a pure variant, and contained a few normal cells which, upon repeated injection, gradually established themselves as a so-called typical strain. The author is inclined to subscribe to the latter view, although this is still a matter of speculation, since it is impossible to follow the cell progeny of a mass culture.

It appears that repeated injection of a variant is a means of determining the purity of such a type. As is shown in Table 13, types III-a and VIII-a failed to establish themselves in chicks after five inoculations, but after 14 inoculations of Strain VIII-a positive results were obtained. The fact that this variant was inoculated into the natural host may also account in part for the positive results. Thus, before designating a strain as a pure variant, it would seem advisable to

subject the variant to such a selective test or process as would eliminate the weaker organism (the variant) and permit the stronger organism (the normal) to survive.

The results also suggest that all true variants possess a low degree of virulence, or none at all. Strain E apparently seems to be as pathogenic as its parent Strain XIV, which at times was in all probability a variant. Plastringer and Rettger (1930) (1934) have clearly demonstrated that variants may be extremely virulent. Such types, although apparently rare, are very baffling to the diagnostician and difficult to identify, especially to one who has not before encountered such types.

### Pathogenicity for an Alien Host (Guinea Pig)

In the study of the behavior of *S. pullorum* in the guinea pig, 10 strains (four parent, five variants, and one apparently normal strain derived from a variant) and 32 guinea pigs were employed. Each strain was inoculated intraperitoneally into three pigs, with the exception of Strain I, which was given to five pigs. All pigs received 0.5 cc. of a suspension of the organism having a turbidity equal to that of tube 2 of the McFarland nephelometer, except animals 43, 44, and 45, which received a concentration equal in turbidity to that of tube 1. The pigs inoculated with the same strain were confined in one cage.

Preliminary experiments revealed that typical normal strains of *S. pullorum* would produce marked symptoms and even immediate death after one or more inoculations, while the variants produced no ill effects. Therefore, antigens with a greater concentration of organisms than previously employed were inoculated. A series of six daily doses was administered to each pig, unless otherwise stated.

The results of this experiment are in part briefly summarized in Table 15. It may be seen that all of the normal strains and one variant produced a symptomatology which was quite similar for all groups. However, the time of response to the inoculation varied greatly, as is revealed by pig 40, which appeared sick on the second day after the first inoculation, and pig 39, which exhibited clinical manifestations on the tenth day. The number of inoculations was guided by the condition of the animal. Among the animals exhibiting symptoms only a few did not succumb. Three pigs (numbers 16, 17, and 43) died following cardiac bleeding, on the twelfth day. *S. pullorum* was recovered from 12 of the 17 pigs (one not cultured) inoculated with apparently typical strains, while no positive isolations were obtained from the pigs inoculated with the variants. Sera were tested for the presence of agglutinins in only 21 animals, since the remainder of the group died as the result of the infection. Agglutinins were detected in two sera, both obtained from pigs inoculated with a normal strain. It is of considerable interest to note that the strain recovered from chicks in Lot XI and designated as VIII-c in Table 15 behaved in a manner similar to the original normal type Strain VIII when inoculated into guinea pigs. These observations point out that virulence can be grossly masked or hidden in a culture that may be a variant with respect to one or more of its characters, but through proper treatment of the variant the virulent types may manifest themselves.

Recognizing the fact that the number of pigs employed for each strain was small, a second experiment was conducted by inoculating one parent strain (VIII) and its variant (VIII-a) each into 14 guinea pigs. The procedure was quite similar to that of the previous experiment. Table 16 shows that the results in the main substantiated previous observations. It is clearly evident that the variants used in the guinea pig inoculations did not possess the invasive power of their parent strains.

TABLE 15.—BEHAVIOR OF NORMAL PARENT STRAINS AND THEIR VARIANTS IN GUINEA PIGS.

Strain No.	Pig Number of Injections	Symptomatology	Necropsy Findings			
			Days after First Injection	Isolation of <i>S. pullorum</i>	Agglutinin Titre	
I	43	5	Inactive, weak and loss of appetite on the 5th day	12	Not cultured	1:80
	44	4	Inactive, weak and loss of appetite on the 4th day; evidence of purulent conjunctivitis	7	Positive	N. T.**
	45	5	Inactive, weak and loss of appetite on the 5th day; evidence of purulent conjunctivitis	9	Positive	N. T.
	46	3	Inactive, weak and loss of appetite on the 8th day; evidence of purulent conjunctivitis	9	Positive	N. T.
	47	3	Inactive, weak and loss of appetite	21	Negative	1:1280
I-a	16	6	{ No symptoms	12	Not cultured	None
	17	6		12	Negative	None
	18	6		22	Negative	None
VIII	34	4	{ Inactive, weak and loss of appetite on the 4th day; evidence of purulent conjunctivitis	7	Positive	N. T.
	35	4		6	Positive	N. T.
	36	4		7	Positive	N. T.
VIII-a	4	6	{ No symptoms	21	Negative	None
	5	6		21	Negative	None
	6	6		21	Negative	None
VIII-c*	40	1	Inactive, weak and loss of appetite on the 2d day	5	Positive	N. T.
	41	4	Inactive, weak and loss of appetite on the 4th day	5	Positive	N. T.
	42	4	Inactive, weak and loss of appetite on the 4th day	7	Positive	N. T.
	37	4	Inactive, weak and loss of appetite on the 4th day; evidence of purulent conjunctivitis	7	Positive	N. T.
IX	38	5	Inactive, weak and loss of appetite on the 9th day	10	Positive	N. T.
	39	5	Inactive, weak and loss of appetite on the 10th day	12	Positive	N. T.
IX-a	7	6	{ Inactive on the 5th day	21	Negative	None
	8	6		21	Negative	None
	9	6		21	Negative	None
IX-b	10	6	{ No symptoms	21	Negative	None
	11	6		21	Negative	None
	12	6		21	Negative	None
XVIII	22	6	{ Inactive on the 6th day	21	Negative	None
	23	6		21	Negative	None
	24	6		21	Negative	None
XVIII-a	1	6	{ No symptoms	21	Negative	None
	2	6		21	Negative	None
	3	6		21	Negative	None

\*Apparently normal strain derived from VIII-a.

\*\*N. T.—no test.

Summarizing briefly, it may be stated that the so-called normal type of *S. pullorum*, as described previously, possesses certain characters that have been lost or modified in the variants derived from the normal parent strains. The marked difference in virulence between the normal and the variant, as revealed in the chick experiment, was also confirmed in the studies with the guinea pigs. The variant was not only less invasive, but also less capable of establishing itself locally in the host than was the parent strain.

TABLE 16.—BEHAVIOR OF STRAIN VIII AND ITS VARIANT VIII-A IN GUINEA PIGS.

Strain No.	Cage No.	Pig No.	Sex	Weight (Grams)	Number of Injections*	Symptomatology	Necropsy Findings			
							Days after First Injection	Lesions	Isolation of <i>S. pullorum</i>	Agglutinin Titre
VIII	I	1	F	729	5	Inactive and gaunt 3 days after the first injection. Inactivity and weakness progressive, resulting in death 8 days after the first inoculation.	8	Acute peritonitis	Positive	N. T.**
		2	F	723	5	Inactivity and dullness during the course of inoculation. Marked weakness and inactivity the 1st day after the first inoculation; slight conjunctivitis; died 2 days after the initial inoculation.	28	None	Negative	1:40
	II	3	F	627	1	No symptoms	2	Transudative peritonitis; liver and spleen markedly congested.	Positive	N. T.
		4	F	782	5	No symptoms	28	None	Negative	1:40
	III	5	F	680	5	No symptoms	25	Slight peritonitis and congestion of the intestines.	Negative	N. T.
		6	F	689	5	Inactivity and general weakness, associated with purulent conjunctivitis; gradually improved after the 10th day. Marked weakness and inactivity after the first injection, associated with purulent conjunctivitis on the 2d day. Death occurred on the 4th day.	28	None	Negative	1:160
	IV	7	M	772	3	Very weak and dull expression on the 1st day, followed by death on the 2d day.	4	Fibrinous peritonitis and congestion of the liver and spleen.	Positive	N. T.
		8	M	817	1	Slight dullness on the 3d day.	2	Transudative peritonitis; liver and spleen markedly congested.	Positive	N. T.
	V	9	M	665	5	No symptoms	28	None	Negative	None
		10	M	847	5	No symptoms	28	None	Negative	1:40
	VI	11	M	592	5	No symptoms	28	None	Negative	None
		12	M	627	5	No symptoms	28	None	Negative	1:10
	VII	13	F	1012	5	No symptoms	29	None	Negative	1:40
		14	F	712	5	No symptoms	29	None	Negative	None
VIII-a	VIII	15	F	729	5	No symptoms	30	None	Negative	None
		16	F	754	5	No symptoms	30	None	Negative	None
	IX	17	F	622	5	No symptoms	31	None	Negative	None
		18	F	789	5	No symptoms	31	None	Negative	None
	X	19	F	689	5	No symptoms	30	None	Negative	None
		20	F	682	5	No symptoms	30	None	Negative	None
	XI	21	M	772	5	No symptoms	31	None	Negative	None
		22	M	804	5	No symptoms	31	None	Negative	None
	XII	23	M	784	5	No symptoms	29	None	Negative	None
		24	M	665	5	No symptoms	29	None	Negative	None
	XIII	25	M	622	5	No symptoms	31	None	Negative	None
		26	M	614	5	No symptoms	31	None	Negative	None
	XIV	27	F	1005	5	No symptoms	31	None	Negative	None
		28	F	730	5	No symptoms	31	None	Negative	None

\*Injection—0.5 cc.

\*\*N. T.—no test

VARIATION OF SALMONELLA PULLORUM

## SEROLOGICAL STUDIES

In the preceding sections it was shown that variation of *S. pullorum* may occur in colonial and cellular morphology, macroscopic appearance in broth medium, stability in saline solution, and virulence. Similar observations have been reported by other investigators for different species of bacteria. Arkwright (1920, 1921), in a study of some of the members of the colon-typhoid-dysentery group, observed that changes in colony form, salt-sensitiveness, and growth in broth may be associated with profound alteration in antigenic structure. Specificity in agglutination was observed for the normal strain, while non-specific agglutination occurred with the variant. The variant might be altered to the extent that it would be agglutinated by immune serum homologous for variants of unrelated species. Peculiar immunizing and agglutinogenic properties have been ascribed to variants by such investigators as Schütze (1921), De Kruif (1921), and Arkwright (1921.).

According to the classification of the antigenic structure of the typhoid-paratyphoid group by Kauffmann (1930) and White (1926), *S. pullorum* contains only a somatic antigen. White (1932, 1933), in his studies concerning different antigenic fractions of the *Salmonella* group, observed that different surface antigens may be lost through bacterial variation.

In these experiments several normal strains and variants were studied as to their serological behavior. This phase of the investigation bears a direct relationship to the routine serological diagnosis of pullorum disease because, as will be pointed out, *S. pullorum* strains can be so altered in their antigenic structure that they lose their power to produce agglutinins in the host and fail to absorb the specific agglutinins.

Realizing that a salt-sensitive variant could not be employed as a satisfactory antigen strain, an effort was made to stabilize it and yet maintain optimum conditions for agglutination. From the literature one may gain the impression that the problem of maintaining variants suspended in saline solution is of minor importance. It is possible that the variants reported by others were of a different character from those dealt with here. Moreover, while variant types that are different as to their salt-sensitiveness do occur, it is also possible that variation may exist in the interpretation of the behavior of variants in salt solution. In the tests conducted with the variants in this study, the author is inclined to believe that in certain instances a combination of agglutination and settling of cells has occurred. It has been reported by some that the phenomenon of self-clumping in salt solution is identical with that of true agglutination which occurs in an agglutinating serum-antigen-saline mixture. It is apparent that some variants will form perceptible clumps in salt solution, but others may settle without perceptible clumping. With either type of variant, when it is placed in contact with agglutinating serum, the reaction may be quite complex and the end result baffling. The question is to what extent the two phenomena are represented.

White (1926) reported that variants can be made stable in salt solution by treating the culture with 95 per cent alcohol. He also stated that the alcoholic extract contains an antigenic substance, which suggests that the variant itself has been deprived of some of its chemical components by the alcohol treatment. To what extent one can proceed in subjecting the organism to extracting agents without depriving it of the essential constituents for true agglutination remains a question.

Four pullorum-disease-free birds which were negative to the macroscopic agglutination test with pullorum antigen were inoculated, one with normal Strain VIII and the three others with Variants C, VIII-a, and IX-b, respectively.



The birds were maintained in separate cages. Each was inoculated intraperitoneally with 0.5 cc. of a saline suspension of the organism having a turbidity equal to tube 2 of McFarland's nephelometer. Their sera were tested at frequent intervals with known pullorum antigen, and in some instances with an antigen prepared from Strain VIII, which represents the parent strain of variant VIII-a. (See Table 4.) In Table 17 are given the reactions of the different sera with known pullorum antigen and with their homologous antigens nine days after the first injection of the different strains. It will be seen that only one serum (Bird 3) produced with the typical strain reacted with pullorum antigen. The reaction appeared similar to that produced with the homologous antigen. The reaction between the serum of Bird 1 and its homologous antigen (C) did not appear as typical agglutination in the lower dilutions, and in the higher dilutions the character of the settling was the same as in the antigen control. A similar observation was made with the serum of Bird 4, while the serum of Bird 2 did not react with its homologous antigen. It appears from these results that the three variants employed do not possess antigenic power equal to that of the typical normal strain.

TABLE 17.—AGGLUTINATION TITRES OF SERA TESTED WITH HOMOLOGOUS AND HETEROLOGOUS *S. pullorum* ANTIGEN.

Bird No.	Antigen	Agglutination Titre								Antigen Control	Serum Control
		10	20	40	80	160	320	640	1280		
1	{ Pullorum	0	0	0	0	0	0	0	0	0	0
	{ C*	?	?	?	?	?	S	S	S	S	
2	{ Pullorum	?	?	0	0	0	0	0	0	0	0
	{ IX-b*	S	S	S	S	S	S	S	S	S	
3	{ Pullorum	4	4	4	4	4	4	4	2	0	0
	{ VIII**	4	4	4	4	4	4	4	3	0	
4	{ Pullorum	0	0	0	0	0	0	0	0	0	0
	{ VIII-a*	?	?	?	S	S	S	S	S	S	

\*Variants

\*\*Normal strain

Legend: 4—complete agglutination  
 3—incomplete agglutination  
 2—partial agglutination  
 1—slight agglutination  
 0—no agglutination  
 ?—not typical agglutination  
 S—settling of cells

In a second experiment Strain VIII and its variant VIII-a were inoculated into three and five mature birds, respectively. Each bird was inoculated intraperitoneally with 2 cc. of live antigen, with a turbidity equal to that of tube 2 of McFarland's nephelometer. Six consecutive daily doses were administered to each bird, except the birds inoculated with the variant, which received seven consecutive daily doses approximately one month after the first inoculation. Table 18 shows that the three birds injected with the normal strain developed a titre within eight days after the first inoculation, while the birds receiving the variant gave no agglutinin response until after the second series of inoculations. Furthermore, the agglutinin response observed in the birds receiving the variant was extremely feeble. All birds were necropsied at the end of a period of 190 days. While the titres at necropsy were considerably lower than those observed earlier, the decrease in titre does not necessarily mean freedom from infection, since *S. pullorum* was recovered from Bird 18950, which possessed a titre of 1:20.

TABLE 18.—BEHAVIOR OF A PARENT NORMAL STRAIN (VIII) AND ITS VARIANT (VIII-A) IN THE NATURAL ADULT HOST.

Strain No.	Bird No.	Number of Injections	Agglutinin Titre (Days after First Injection)													Necropsy Findings	Isolation of <i>S. pullorum</i>	
			8	12	19	28	33	40	47	54	69	82	97	124	190			
VIII	18950	6	80	1280	2560	—	—	640	—	320	320	160	160	80	20	Neoplastic invasion (sarcomatous in nature) of the heart muscle, kidney, liver, and spleen. A cyst (2 cm. in size) was attached to the gizzard. Contents consisted of yellow cheesy material and amber oily fluid. <i>S. pullorum</i> isolated only from the cyst contents.	Positive	
	19018	6	320	1280+	5120	—	—	2560	—	640	640	320	320	320	160		No gross lesions	Negative
	19050	6	5120	2560	1280	—	—	160	—	40	20	40	20	20	40		No gross lesions	Negative
VIII-a	18882	13	0	0	0	0	20	20	10	0	0	0	0	10	0	Adhesions of the visceral peritoneum. Few small cysts embedded in abdominal fat.	Negative	
	18989	13	0	0	0	0	20	10	10	0	0	0	0	10	0		No gross lesions	Negative
	19002	13	0	0	0	0	20	40	10	10	0	10	0	10	20	Cyst (2 mm. in size) free in abdominal fat. One misshapen cystic ovum (1.5 cm. in size).	Negative	
	19042	13	0	0	0	0	10	10	10	10	0	10	0	10	20		No gross lesions	Negative
	19049	13	0	0	0	0	40	0	10	10	0	0	0	0	10	No gross lesions	Negative	

These results conclusively show that the variant VIII-a does not possess the power to stimulate agglutinin production in the living natural host to the extent that its parent strain does. Also, it failed to establish itself in the natural host.

The absorptive capacities of several normal strains and their variants were determined. In preliminary studies natural, positive pullorum serum and a homologous chicken serum for Strain VIII were employed. Strain VIII and its variant VIII-a were used for the absorbing organisms. The unabsorbed and absorbed sera were tested with known pullorum antigen and with antigen prepared for Strain VIII. The results in Table 19 show that the typical strain, VIII, was capable of absorbing all of the agglutinins from its homologous serum and from the natural serum, while variant VIII-a reduced the titre to a lesser degree.

TABLE 19.—RESULTS OF AGGLUTININ ABSORPTION.

Serum	Serum Titre					
	Before Absorption		After Absorption with VIII		After Absorption with VIII-a	
	Antigen (Pullorum)	Antigen VIII	Antigen (Pullorum)	Antigen VIII	Antigen (Pullorum)	Antigen VIII
Pullorum	640	1280	0	0	640	160
No. 3	1280	2560	0	0	640	640

In the determination of the absorptive capacities of the different parent strain and their variants, five sera which varied in titre were employed. The sera were tested with a stock antigen before and after absorption to determine the reduction in titre. The absorbing antigens were prepared from either a 24- or 48-hour growth, depending upon the yield obtained. The different sera were absorbed with a live antigen which had a turbidity equal to tube 8 of McFarland's nephelometer. However, in some cases a less dense suspension was found satisfactory to bring about complete absorption. Some sera were absorbed three times, others twice, depending upon the agglutinin titre and the absorbing power of the strain. The serum and antigen dilution employed during absorption was 1:10. Twelve original parent strains (I, II, III, VI, VIII, IX, XII, XIII, XIV, XVI, XVIII, and I (Experiment I)) and 16 variants (I-a, II-a, III-a, VI-a, VIII-a, IX-a, IX-b, XII-a, XII-b, XIII-a, XVI-a, XVIII-a, A, A-1, C and E, and four miscellaneous strains) were studied. Since the results in general are very much the same in the two groups (parent and variant) only one set of data is presented in Table 20. It may be seen in the table that the variant does not possess the absorptive capacity of its parent strain.

TABLE 20.—COMPARATIVE RESULTS IN AGGLUTININ ABSORPTION WITH PARENT STRAIN IX AND ITS VARIANT IX-A.

Test Antigen	Sera	Titre before Absorption										Titre after Absorption	
		20	40	80	160	320	640	1280	2560	5120	10,240	With Strain IX	With Variant IX-a
Regular Stock Antigen	A	4	4	4	4	3	1	0				0	640
	B	4	4	4	4	4	4	3	2	1	0	0	10,240
	C	4	4	4	2	1	0					0	320
	D	4	4	4	4	4	4	3	2	1	0	0	5,120
	E	4	4	4	4	4	4	4	3	2	1	0	10,240

Strain XIV exhibited only slight absorption, while Strain I, Experiment I, caused no reduction in agglutinin titre. The variants derived from these strains behaved in a like manner. Variants XII-a and XII-b exhibited absorptive ability, but not to the same degree; only the latter completely absorbed the agglutinin content. No definite explanation is offered for this difference in action, except that the one variant possessed a sufficient number of the cell type which was capable of absorbing the agglutinins. The fact that this variant produced two types of cells in its colony structure (See Figures 18, 19, and 20), makes it quite possible that the more typical appearing cell was present in sufficient quantity to bring about complete absorption.

The four miscellaneous strains, which appeared typical, had been subjected to treatment similar to that of the variants, but did not change in colonial and cellular morphology. Likewise, their capacity to absorb agglutinins was as great as that of the original parent strain.

These results show that variants which yield different colonial and cellular forms, which are different from the original strain, also possess little or no power for absorbing specific agglutinins.

#### DISCUSSION

A review of the literature shows that bacterial variation of *S. pullorum* has been observed and studied by only a few investigators. Mallmann (1932) worked principally with strains that had been maintained under laboratory conditions for a period of time varying from four to nine years. The impression is given that variants are seldom isolated from natural outbreaks of the disease. The criterion used for the variant study seemed to include only a change in colony morphology. On the other hand, Plastridge and Rettger (1930) reported a natural outbreak of the disease among young and adult stock from which pleomorphic types were isolated. These types appeared strikingly different from the normal type in several respects.

Since so few observations have been reported concerning the incidence of *S. pullorum* variants among freshly isolated strains, the writer believed that an examination of a large number of recently isolated strains might yield valuable additional information. It was realized from the first that a change in colony form was not the only feature that would classify a strain as a variant or normal type. If the colony type had been selected as the only means of identifying variants, all but two of the strains studied by the writer would have been designated as typical. Likewise, if these strains had been identified only by the Gram-stain method and biochemical reactions, all would have been considered typical. Hence, to classify an organism as typical, its various properties must be studied. Furthermore, only slight deviations, which might be overlooked by one not familiar with the characteristics of variants, may be observed at times. The property of salt-sensitiveness would in all probability be overlooked when the rapid serum agglutination method is employed to determine the agglutinability of a freshly isolated strain. While this method may have its merits in the diagnosis of pullorum disease, it should not be abused by being employed for the typing of the organism unless every step is carefully controlled.

The source of the strain apparently cannot be correlated with the incidence of variants, since the different types were isolated from eggs, chicks, and adult fowl. However, if a large number of cultures from one source were examined, it might be possible to show such a correlation. Strange as it may seem, in the routine testing of domestic fowl re-infected flocks generally show a very small number

of infected birds, often as few as one. Cultures obtained from adult birds and studied in this investigation represent positive isolations from flocks revealing only one infected bird. Some of these infected individuals possessed very low agglutinin titres, which may be due in part to the organism. In order to obtain more evidence which might serve to explain the relation of bacterial variation to flocks revealing one or more infected birds, a specially designed project seems necessary.

### Environment and Bacteria

The placing of bacteria under different environmental conditions may bring about many types of variation in the organism. The observations reported in this investigation are in general agreement with those reported for other organisms. While it may be true that *S. pullorum* is quite stable when maintained under uniform and constant laboratory conditions, under certain influences such as described earlier, the organism may undergo profound changes. The majority of strains studied were susceptible to marked variation. It was impossible to study slight variation simultaneously with extreme changes, but the author feels that this phase is probably of great practical importance. Slight variations from the normal type are more difficult to detect, and they may be induced by unfavorable environment. Various strains will respond differently to the same environment. This has been observed when strains were streaked on liver infusion agar. Differences in colony and cellular morphology may be noted. This also holds true for agar medium prepared from different livers. Liver medium may serve to bring out variation tendencies that otherwise might remain obscure. The results suggest that continuous cultivation of *S. pullorum* on liver infusion medium is more liable to bring about a change in the organism than growth on plain meat extract agar.

The relation of one colony type to another cannot be explained with any degree of satisfaction. It appears that certain types are more remote from the parent strain than others, but this may be applicable only to one or more features, such as colony form, virulence, and antigenic structure. To classify all of the different variants as to their order of development or degree of variation does not seem possible at this time. The realm of variation may possess great latitude. The border between normal and variant types should not be looked upon as a sharp line of demarcation. A normal type may vary in some of its aspects, but the changes may not be perceptible. Furthermore, a normal type may lose one property which is replaced by another characteristic as, for example, smoothness for roughness in colony surface. The change from the smooth to the rough character may extend over a wide range of colony types. Also, a character may completely disappear without the acquisition of a new property, as, for example, the loss of ability to ferment a certain carbohydrate. Additional properties may be acquired without previously having lost a character, as is the case with the maltose fermenting strain included in this investigation. One and the same type of variant may appear different on two kinds of media. Variants may fluctuate in their colonial and cellular types. Colonies that consist largely of the filamentous forms are the most difficult to propagate and to maintain as stable. However, Variant XII-b, which produces a filamentous type of colony, has remained very stable and uniform in its features.

The terms "S" and "R" introduced by Arkwright for smooth and rough forms seem most inappropriate, because variants may be observed which have a smooth surface and yet are far remote from the parent strains in antigenic

structure. The correlation between colonial form and antigenic structure is by no means constant. This also applies to other features.

Since our knowledge concerning the underlying causes that bring about these changes from the normal to the variant is meagre, it is apparent that a greater insight with respect to the physiology and chemistry of the microorganism is needed.

### Behavior of Variants

According to the results obtained in the preceding experiments, variants may differ greatly when placed under certain conditions. The phenomenon of clumping, settling, and clearing of broth cultures was reported by Nicolle in 1898. Other investigators have also made this observation, but Arkwright (1920) associated this feature with other distinguishing characters, such as change in colony form and serological properties. While this view may be correct for certain variants, for others it does not seem to hold. The mechanism that is operating when organisms settle out in broth is little understood. The fact that variants may or may not exhibit this phenomenon indicates that it cannot be employed as a general criterion for all variants.

Salt-sensitiveness has also been designated as a means of detecting variants. Certain variants studied in this investigation exhibited this property, but the degree of instability in salt-solution did not appear to be constant. The degree of roughness of the colony type could not be correlated with the degree of salt-sensitivity. The reaction of auto-flocculating or settling was always completed within 24 hours. As the salt concentration was reduced the stability of the organism was increased. However, not all variants responded alike to diminished salt concentration.

The ability of the different variants to attack various fermentable substances was practically comparable to that of normal types.

In some cases the character of the growth was markedly influenced by the acidity and possibly other environmental factors. The extreme variation in some of the characteristics had little or no influence on the fermentative property. That *S. pullorum* may acquire or reveal the property to ferment substances in addition to those that are attacked by the normal type was observed by the author previously. A maltose-fermenting property was either acquired or revealed which has remained stable for several years. This property may be looked upon as a very unusual characteristic for *S. pullorum*. Investigational results pertaining to this maltose-fermenting strain will appear in detail in another publication.

The cultivation of pathogens in an environment which approaches the natural was undertaken. Beyond a doubt, the most frequent mode of transmission of *S. pullorum* from adult to chick is by means of the egg. Since the egg is frequently a part of the natural environment of the organism, it appeared that it might exert a definite influence on the variants. The egg medium employed in this investigation caused little or no change in the character of the variants. It appears to the author, therefore, that fresh eggs might prove more satisfactory in retaining strains in their original state than the media ordinarily employed.

In the experiment dealing with the passage of the parent type and the variants through the natural host, it was demonstrated that the variants are less invasive than the normal strains. Larger infecting doses than are ordinarily employed with normal strains were necessary to obtain positive infection. Even then some variants failed to establish themselves in the chick or adult bird. In one instance (Variant B) it was demonstrated that partial reversion to the normal type had

occurred. This variant was similar in colony morphology to Variant VIII-a which yielded smooth and apparently typical colonies only after a series of injections. With both variants the chicks exhibited no clinical manifestations, but the pathological picture was more extensive in the chicks injected with Variant B than with Variant VIII-a. The smooth type strain (VIII-c) derived from Variant VIII-a through a series of inoculations into baby chicks apparently exhibited the same degree of virulence for guinea pigs as the original parent Strain VIII. That some variants may pass through the host unaltered in some of their characteristics was demonstrated with Strains IX-b and XII-b. It is extremely interesting to note that one may inoculate the natural host with a variant as remote in colony form from the normal type as Variant IX-b and recover the same apparently unchanged.

The presence of the organism in the tissues was detected by tissue smears. The observation of different pleomorphic types of cells outside of the host raised the question of the morphology of the cells when present in the host. It was demonstrated that tissue smears from organs which yielded pure growth on primary culture might not reveal the organism. It was found that the organism most frequently appeared in smears from the yolks of chicks. In this tissue some atypical forms were observed, but for the most part the organisms appeared slightly larger than the normal type isolated on meat extract agar. It does not seem likely that extremely pleomorphic types would be found in body tissues except possibly in tissues such as unabsorbed yolk, where there is a different histological and physiological environment than is found in other parts of the host. Whether the pathological ovary of an adult fowl is conducive to variation is not known, except that the majority of positive isolations originating from this organ seem typical.

The experimental evidence concerning the pathogenicity of the variants definitely suggests that the organisms have become void of some constituent or physiological property that renders them avirulent. Some variants (III-a and VIII-a) that appear markedly different from the normal type on plain agar, seem to possess no invasive power and induce no local tissue response at the site of inoculation. It appears that the defensive power, as well as the aggressive property, has been lost. Such types are not expected to survive in the host for any length of time, even when injected in large doses. Variants that have been developed on liver infusion agar appear to be slightly more aggressive and offer a greater resistance to the defense mechanism of the host than the type represented by III-a and VIII-a. Such types may be encountered under natural conditions. However, the type represented by Variant E appeared the most virulent of all types isolated. The fact that its parent strain XIV at times revealed tendencies to vary may account for its high degree of virulence. The pleomorphic types described by Plastridge and Rettger possessed a marked invasive and infective power. None of the strains described in this investigation, with the possible exception of strain XIV, approached these types in virulence.

The serological investigations show that the variants underwent a profound change in the antigenic structure, since they were unable to stimulate the production of agglutinins, and also were unable to absorb agglutinins from natural and artificial immune sera. These observations appear very significant. One must assume from them that such profound changes, may occur among stock cultures that are employed for the preparation of antigens used in the macroscopic agglutination test. The degree of sensitivity of an antigen and the type of agglutination may be greatly influenced by the ratio of the variant to the normal type in a mixed population of cells.

## SUMMARY AND CONCLUSIONS

From the experimental evidence obtained in the investigation discussed here the following conclusions seem justified.

Variation of characters of *S. pullorum* may be detected among freshly isolated strains, as revealed by 13 of 163 strains studied.

The behavior of the recently isolated variants in sodium chloride solution of different concentrations and in the presence of positive and negative sera differed greatly.

Such properties as colonial and cellular morphology, Gram-staining, and biochemical reactions revealed little or no variation in the majority of the derived variants.

Many strains of *S. pullorum* readily underwent variation when subjected to frequent transfer and storage in meat infusion broth.

Variants were isolated and stabilized on solid media by colony selection and by frequent transfer.

When variants were cultivated on both meat extract agar and liver infusion agar the colonial and cellular features often appeared markedly different. Some strains that appeared typical on meat extract agar exhibited pleomorphic tendencies when placed on liver infusion agar.

Variation of *S. pullorum* was not observed when the organism was subjected to alternate transfer in liquid and solid media, or cultivated in 5 per cent peptone solution, in deep meat infusion broth, or in plain 3 per cent meat extract solution.

*S. pullorum* variants may settle out in broth cultures, the degree of settling varying with the different types.

The ability to remain suspended in sodium chloride solution varies with the different variants, and it may fluctuate even within the same type.

The power of the variants to attack the different fermentable substances was practically the same as that of the normal type.

Passage of the variants through whole fresh eggs and through the natural host (chicks and mature fowl) had little or no influence on the general features of the different types.

Most variants exhibited a lesser degree of virulence than their parent strains, and in some instances appeared avirulent.

Repeated subcutaneous inoculations of massive doses of variants may serve as a means of detecting organisms that resemble the normal type in colonial and cellular morphology, Gram stain and biochemical reactions, and in agglutinability.

A menstruum which maintained the variants in suspension and which was also satisfactory for conducting the agglutination tests was not discovered.

The agglutinogenic power and the absorptive capacity of the variants tested were not equal to those of the normal type.



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