

A STUDY OF THE DISSOCIATION OF CERTAIN
SALMONELLA ORGANISMS

by

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INTRODUCTION

Since Arkwright (1921) and DeKruif (1921) have shown from their experimental data that rough (R) and smooth (S) forms may be obtained from the same pure culture of bacteria, increasing attention has been given to studies dealing with variations within a bacterial species. The studies dealing with bacterial variations have, for the most part, been made of the physiological and serological properties of the R and S form and more particularly with such variation as affects colony form.

It seems quite unnecessary to give a complete review of the literature on this subject since Hadley, in 1927, published a thorough and excellent review dealing with all phases of the subject up to that time. In brief, one is able to conclude that nearly all bacterial species may be dissociated into variants and that this dissociation may be made reversible by subjecting the variant to different environmental conditions either in vivo or in vitro.

The significance which has been attached to variation in properties of bacteria is widely different. Some investigators believe bacterial variation to be an hereditary characteristic inherent within all bacterial cultures; while others believe that variation is due to environmental con-

ditions resulting from the effect of different chemicals, or the accumulation of metabolic products in the medium; to prolonged incubation at a temperature above or below the optimum for the species, or to the action of a bacteriophage specific for the species. Hadley believes the variation to be a characteristic inherent within the bacterial cell and that a dissociant manifests itself only with a change in environmental conditions.

No consensus of opinion exists as to the exact significance to be attached to such occurrences. At the present time, the principles in the dissociation phenomena cannot be discussed with any degree of finality. Frequent revision and reconstruction of ideas in the light of new experience will be necessary. The appearance of the variant is regarded as a fact to be explained. Why does a smooth variant become rough and vice versa? Eastwood (1932) has attempted to explain the fact on the basis of certain peculiar characteristics of growth. He stated that there are reasons to believe that growth depends on a rhythmic cycle of synthesis peculiar to living protein whereby at each successive stage one particular "building stone" is selected for synthesis with the rejection of all others. The enzymes are considered as centers of "Chemico-Physical" activity and the emergence of variants may be explained by the liability of these "centers" to change under "Chemico-Physical" influences.

This new conception of bacterial variation has upset the theory of Cohn and Koch who claimed that bacteria were characterized by rather rigid fixity to their specific type as pertains both to morphology and to physiology. This theory was formerly so widely accepted that all deviations from the normal type were considered as contaminations. However, before Cohn and Koch advanced the monomorphic theory, Nägeli (1877) had advanced the view that the fission fungi were all of one type that had marked ability to vary. This view was long disregarded in favor of the former.

Dissociation studies on the S. pullorum group of organisms have been very meagre. Mallmann (1932) published a paper dealing with dissociation of S. pullorum organisms and related species. He concluded that dissociation changes occurred only in the intermediate type culture and that dissociation incitants were without permanent effect upon pure "rough" and "smooth" types of Salmonella organisms, although S to R variation in the colon-typhoid group has been produced with little or no difficulty. With this fact in mind studies were made on the S. pullorum organisms in order to compare their ability to dissociate with that of the colon-typhoid group, and to find whether the S and R variants of S. pullorum exhibit the same properties as S and R variants of other dissociated species.

Rettger and Plastridge (1932) were concerned in a study

of the effect of tissue extracts, obtained from adult birds infected with pleomorphic organisms, on ordinary strains of S. pullorum. They concluded that the conditions necessary for the development of these pleomorphic cells arise when the cells in question exist in a certain phase of their life cycle, and when a definite balance between bacteriophage and development of cells is established.

In much of the work on dissociation, rough colonies or other variants have been obtained more or less at random by plating out old stock cultures, etc. Studies of the environmental conditions that might stimulate or incite dissociation have been made, for the most part, only recently and on comparatively few forms of bacteria. Because of the prevalence of S. pullorum in nature, its great economic importance, and the fact that the dissociation into "smooth" and "rough" variants changes its antigenic nature, led to this study.

GENERAL METHODS

The methods used in this investigation have been to modify in various ways the media on which the organisms were grown, and then, having streaked the culture over nutrient agar plates, to compare the colonies that developed. Some observations were also made regarding the comparative morphology of the individual cells, the comparative size of

rough and smooth colonies, their physiological behavior, their serological properties, and the filtrability of the R and S variant.

Liquid culture media were used more frequently throughout this investigation because there seems to be a general agreement among investigators who have been working on this phenomenon that the change may be induced most readily in liquid cultures. This fact was mentioned by Hadley (1927) in his article reviewing dissociation and also observed by Arkwright (1921), DeKruif (1922), Soule (1928) and others.

The strains of S. pullorum organisms (No's 137, 144, 40, 49, 50, 51, and 16) used throughout this investigation were obtained from Dr. C. A. Brandly who is in charge of the Poultry Disease investigations at the Kansas Agricultural Experiment Station. These strains were selected because they are used in the making of pullorum antigens used for the testing of poultry for pullorum disease. Because it has been shown by Andrewes (1922), White (1929), Krumwiede (1923), and others, that the rough forms of closely related species of organisms lose some of their antigenic properties on dissociation, we were interested in determining the tendency to variation of the S. pullorum and the possibility of the variant to produce an antiserum of effective titer. The remainder of the cultures used in this investigation were stock cultures obtained in our laboratory.

In some cases the culture was allowed to age many days before being streaked on nutrient agar plates to determine the value of aging to incite dissociation. At appropriate intervals during the history of the culture, nutrient agar plates were streaked and examined for colony types after 24 hours and 48 hours incubation at 37°C. This procedure permitted one to follow the changes in an aging culture.

Throughout the investigation the cultures which were used to obtain variants were streaked on ordinary nutrient agar consisting of 1.0 per cent peptone, 1.5 per cent agar, 0.3 per cent beef extract and 0.5 per cent salt.

The purity of the particular stock cultures with which this work was started was determined by removing a portion with a loop and diluting it in broth. This was well mixed and then immediately streaked on agar plates. The agar plates were incubated at 37°C. for 24 hours after which time examinations were made and a typical colony was removed and again streaked on an agar slant. The slant was allowed to incubate at 37°C. for 24 hours and the above process repeated. This process was repeated on ten different days. At the end of that period, fermentation tubes containing xylose, dextrose, maltose, and mannite were inoculated with a culture of each strain to doubly insure purity. The reaction was read at the end of 48 and again at 96 hours incubation at 37°C.

The change from one form of S. pullorum to another was determined by streaking agar plates from cultures to be examined. After incubating such plates the number of each type of colony was estimated and recorded as a per cent of all colonies present.

DESCRIPTION OF COLONIES

The terms "smooth", "intermediate", and "rough" (S, I, and R) or in some cases merely "smooth" and "rough" have been used throughout this discussion to designate the changes produced by cultivation in varying media. These forms are referred to as "S", "I", and "R". This is an arbitrary classification and does not make clear the true course of events. The experiences encountered throughout this investigation point to the fact that the change from smooth to rough is one which is gradual but continuous and is reversible; it is not abrupt and does not appear to occur in several distinct steps.

The results of the different workers on dissociation are comparable only in a general way because what one investigator may designate as an I type, another may designate as an R type.

Smooth Forms of S. pullorum

The typical "smooth" colony of S. pullorum on an agar plate appears small, smooth, round, soft, glistening, and

slightly raised, and with an even and entire margin. When touched with an inoculating needle the colonies have a soft, moist, and butyrous consistency.

On an agar slant the growth is moderate, slightly spreading, glistening, smooth, and translucent.

In broth the growth gives a moderate clouding with no surface growth and very little granular sediment. In physiological salt solution it forms a uniform turbid suspension.

The individual cells of the smooth colony form are Gram negative rods with rounded ends, non-motile and are arranged singly. They possess no spores or capsules.

Rough Form of S. pullorum

The "rough" form of S. pullorum on an agar plate presents an irregular, rough, granular, or wrinkled surface, which is flat and dull luster. The rough colonies are usually larger than the smooth colonies and have irregular broken margins. When touched with an inoculating needle they appear dry and brittle and may be pushed over the surface of the medium.

On an agar slant the growth is more profuse than the smooth form, spreading, dull, and flat.

In broth an agglutinative growth appears at the bottom and sides of the tube. There is no surface growth and the upper portion of the broth tube is clear as if it had not

been inoculated. In physiological salt solution it is impossible to obtain a uniform suspension of the organism. According to Arkwright this is due to the concentration of salt in the solution.

The individual cells of the rough colony form are Gram negative rods with rounded ends, arranged in chain formation or in clumps instead of singly. Some cells are larger and other cells are smaller than those seen in the smooth forms.

Intermediate Form of S. pullorum

In this investigation many colonies of different appearances are included under this heading. Such colonies frequently occurred as one of the first notifications of a change and developed before rough forms occurred. At other times, the intermediate type would not give rise to a rough colony but would revert to the smooth colony while retaining the sedimenting characteristic of the rough form when grown in broth.

On an agar plate the intermediate type of colony is larger than the smooth form and with an uneven margin, while the surface is smooth to slightly rough, and the colonies are slightly raised and less glistening than the smooth forms.

On agar slants the growth is moderate, slightly raised, a little spreading, glistening, and translucent.

In broth the intermediate form gives rise to no surface growth, but to a uniform turbidity with a moderate granular sediment in some cases and a large amount of granular sediment in others.

In physiological salt solution the intermediate forms produce a uniform turbidity with a moderately granular sediment.

The individual cells of the intermediate colony form are Gram negative rods with rounded ends, occasionally appearing in chain formation or clumps instead of appearing singly. However, many of the cells appeared singly in all of the intermediate type cultures. The size of the cells was variable, some were slightly larger than the cells of the smooth colony form and others were of the same size or smaller than the cells of the smooth colony form.

At times the surface of the intermediate forms appeared quite wrinkled, but not rough enough to be classed as a distinctly rough form. At other times the margin of the colony was quite irregular, but the colony had a smooth surface which removed the colony form from the smooth class.

If the intermediate forms were not inoculated into media which would incite dissociation they would revert to the smooth type in a few days.

EXPERIMENTAL METHODS USED TO INDUCE
S TO R DISSOCIATION

The Effect of 2 Per Cent Beef Extract Upon Dissociation

The beef extract was prepared as follows: 20 grams of Liebig's Extract of Beef was placed in a 2 liter flask containing 1000 cc. of distilled water. After heating until all ingredients were thoroughly dissolved the loss of weight due to evaporation was made up and the solution adjusted to a pH of 8.0 by the colorimetric method. The broth was added to flasks in 100 cc. amounts and to test tubes in 10 cc. amounts and autoclaved at 20 pounds pressure for 15 minutes. After sterilization the reaction was again determined colorimetrically.

The broth was seeded with seven smooth strains of S. pullorum, three smooth strains of E. typhi, and one smooth strain of S. schottmülleri. The broth cultures were incubated at 37°C. for 24 hours and during the remainder of the experiment at room temperature. At various intervals during the experiment a loopful of the material from each flask and tube was streaked on nutrient agar. The plates were incubated at 37°C. for 24 hours after which time they were examined for typical colonies.

Summary. An examination of Table I will show that all of the cultures were free from R forms during the first 7 days of the experiment. On later examination only a few R forms appeared in the 10 cc. tubes of the beef extract. However, the flask cultures presented a striking contrast showing a much greater production of R forms than did the tube cultures. Of the various cultures in 10 cc. and 100 cc. amounts some exhibited only intermediate colonies even after 116 days of incubation while others gave a small percentage of R forms and in other cultures no intermediate or R forms were noticed throughout the entire experiment. A greater percentage of R forms were produced in the typhoid-paratyphoid cultures than in the S. pullorum cultures under the same condition.

A greater percentage of R forms were obtained in the beef extract media which was contained in flasks in 100 cc. amounts. The greatest number of R variants were obtained at the end of 60 days incubation at room temperature after which time there was a decrease in the number of R variants. In the beef extract cultures incubated in 10 cc. amounts at the end of 60 days five per cent R colonies were obtained while in the larger volumes of media an average of 25 per cent R colonies were obtained.

The reversion in the pullorum strains as well as in the typhoid-paratyphoid strains appeared to be S to I to R. After the organisms were grown in the medium used to incite dissociation for a long period of time there was a R to I to S reversion. R colonies could be obtained only after a certain period of incubation of the culture in the inciting medium after which time they again gradually reverted to the S form. This was true throughout the entire investigation. In no case were 100 per cent R colonies formed by the methods used in this study to incite dissociation.

These results are in accord with those of Soule (1928) who has shown the influence of large volumes of media upon the dissociation of B. subtilis. This was thought to be due to the fact that the growth period has been lengthened.

Koser and Stryon (1930) working with B. dysenteriae, have shown that the use of 3 per cent meat extract induced some change in colony form and concluded that meat extract alone, in the concentration used in nutrient broth, supplied little impetus to changes of a dissociative nature. They found that ten times the concentration used in broth produced intermediate and rough colonies on aging the cultures as determined by streaking on nutrient agar.

Table I. A Comparison of the Effect of 2 Per Cent Beef Extract (pH 7.6) in 10 cc. and 100 cc. Amounts to Incite Dissociation of Salmonella Pullorum, E. typhi, and S. schottmulleri

Strain	Amount of broth (cc.)	7 days	50 days	60 days	77 days	113 days	152 days
Period of Incubation		Percentage of R Forms Obtained					
144 Pullorum	10 100	100S 100S	100S 2I	100S 100S	100S 100S	100S 100S	100S 100S
137 Pullorum	10 100	100S 100S	9R 32R	5R 40R	10I 20R	100S 100S	100S 100S
40 Pullorum	10 100	100S 100S	25I 20R	10I 25R	100S 10R	100S 100S	100S 8R
49 Pullorum	10 100	100S 100S	2R 15R	5R 23R	10I 20R	100S 5I	100S 100S
51 Pullorum	10 100	100S 100S	100S 10R	5R 15R	17I 25I	100S 100S	100S 100S
50 Pullorum	10 100	100S 100S	100S 13R	100S 4R	100S 100S	100S 100S	100S 100S
18 Pullorum	10 100	100S 100S	100S 100S	2R 100S	100S 100S	100S 100S	100S 2R
4 Typhi	10 100	100S 100S	20I 40R	2R 50R	5I 10R	100S 5I	100S 100S

The Effect of Aging Smooth Bacterial Cultures
in Nutrient Broth

Arkwright and Pitt (1929) employing the typhoid and paratyphoid A organisms confirmed earlier observations by Arkwright (1921) that R forms can usually be found in old broth cultures originally inoculated with the S form. The time required to produce the change varied from 2 or 3 weeks to 2 or 3 months depending upon the organism and the strain used.

Accordingly, this experiment was started to determine the effect of aging S. pullorum cultures in nutrient broth on the development of R variants.

The nutrient broth was prepared as follows: 10 grams peptone, 3 grams beef extract, and 5 grams of salt were placed in a two liter flask containing 1000 cc. of distilled water. After heating until the ingredients were thoroughly dissolved the loss of weight due to evaporation was made up and the solution adjusted to a reaction of a pH 7.0 by the colorimetric method. The broth was placed in 125 cc. Erlenmeyer flasks in 80 cc. amounts and in test tubes in 10 cc. amounts, and autoclaved at 20 pounds pressure for 15 minutes. The reaction was not adjusted after autoclaving.

The broth was seeded with seven smooth strains of S. pullorum, three smooth strains of E. typhi, and one smooth strain of S. schottmülleri. The broth cultures were incubated at 37°C. for 24 hours and during the remainder of the experiment at room temperature. Each week during this experiment a loopful of the material from each flask and each tube was streaked on nutrient agar plates.

Summary. At the end of 78 days no R variants were noticed in any of the S. pullorum cultures. At the end of 42 days intermediate forms were noticed but these soon reverted to the S type. With the typhoid and paratyphoid organisms, however, the results were different. A very small percentage of R forms were produced both in the flask culture and in the tube culture.

Aging in a medium which is favorable for the growth of the S form has no apparent effect upon the S form of S. pullorum whether the culture be cultivated in a large volume or a small volume of medium. The I forms which appeared reverted to the S form possibly because there was no radical change in environment. Dissociation is usually most marked when conditions are unfavorable to the growth of the organism, or when an organism is grown in a medium different than that to which it is accustomed.

The Effect of 5 Per Cent Oxgall Containing 5 Per Cent
and 10 Per Cent Bacto-peptone Upon Dissociation

Koser and Styron (1930) reported that the addition of 5 per cent peptone to 5 per cent bile greatly favored the production of I and R forms of B. dysenteriae, but that the bile itself provided no decided stimulus to such dissociative changes with their strains.

Accordingly, experiments were started in which the medium consisted of peptone alone and of bile to which peptone had been added. Fifty grams of oxgall were placed in a 2 liter flask containing 1000 cc. of distilled water and 50 grams of peptone added. The solution was dissolved by heating and adjusted to a pH 7.0. Fifty cc. amounts of the medium were placed in 125 cc. Erlenmeyer flasks and autoclaved at 20 pounds pressure for 15 minutes. A solution containing 5 per cent oxgall and 10 per cent peptone was prepared in the same manner.

After preparation of the culture medium each series of flasks were inoculated from a 24 hour slant culture of the organisms and the cultures were incubated at 37°C. throughout the entire experiment. At intervals plates of nutrient agar were streaked from the culture flasks and then examined for types of colonies after 24 hours incubation at 37°C. The results of this experiment are tabulated in Table II.

Summary. Growth in 5 per cent oxgall containing 5 per cent peptone resulted in much greater variation in the types of colonies found on nutrient agar plates. In some instances the change appeared to be largely to the I form with no R forms appearing. Growth in 5 per cent oxgall containing 10 per cent peptone offered less stimulus to change, R colonies never appearing.

The greatest number of variants was obtained after 34 days incubation. On strain # 137-pullorum at the end of 34 days incubation 22 per cent R colonies were obtained in the five per cent peptone medium containing five per cent bile whereas no R colonies were obtained in the ten per cent peptone medium containing five per cent bile.

With the typhoid and paratyphoid organisms dissociation occurred equally as well in 5 per cent oxgall containing 5 per cent peptone as in 5 per cent oxgall to which 10 per cent peptone had been added.

The Effect of Various Concentrations of Peptone and Variation in Temperature as the Inciting Factors in Producing S to R Reversion With S. pullorum.

Two per cent, five per cent, and ten per cent Bactopeptone solutions were prepared, and adjusted to pH 6.6, 7.2, and 7.8 by the colorimetric method either by the addition of N/10 HCl or N/10 NaOH. The media were tubed in

Table II. A Comparison of the Number of R Forms of *Salmonella Pullorum* Produced by the Cultivation of the S or Normal Form in 5 per Cent Oxgall Containing Different Concentrations of Peptone

Period of Incubation		12 days	18 days	34 days	68 days	116 days
Strain	Per Cent Peptone	Percentage of R Forms				
144 Pullorum	5 10	1003 1003	1003 1003	101 71	1003 1003	1003 1003
137 Pullorum	5 10	1003 1003	1003 1003	22R 1003	101 1003	1003 1003
40 Pullorum	5 10	20R 1003	36R 1003	701 101	231 1003	1003 1003
49 Pullorum	5 10	1003 1003	2R 1003	17R 1003	401 31	101 1003
51 Pullorum	5 10	1003 1003	1003 1003	20R 171	401 1003	1003 1003
50 Pullorum	5 10	1003 1003	101 1003	32R 1003	601 1003	1003 1003
18 Pullorum	5 10	1003 1003	1003 1003	301 1003	1003 1003	1003 1003

(Table II continued)

4 Typh1	5	25R	40R	20R	40I	20I
	10	40R	45R	70I	10I	100S
5 Typh1	5	100S	30R	10R	15I	10I
	10	10I	21R	5I	100S	100S
13 Para. B	5	5R	10R	22R	10I	100S
	10	21R	13R	34R	20I	10I

Only the percentage of H forms are recorded and on plates from which no R forms were noted the percentage of I forms were recorded.

10 cc. amounts and autoclaved at 20 pounds pressure for 15 minutes.

The tubes of each concentration and each pH value were seeded with seven smooth strains of S. pullorum. One series of tubes was incubated at 21.5°C. and another series of the same concentration at 37°C. At various intervals the cultures were mixed and streaked on nutrient agar plates and examined for the various types of colonies.

The results of this experiment are shown in Table III. The dissociation of organisms grown on 2 per cent peptone solution was variable, the greatest dissociation occurring in pH 7.6. That higher temperatures offer a greater stimulus to incite dissociation is shown by the fact that many more R variants were produced in the cultures incubated at 37°C. than in those incubated at 21.5°C. At 21.5°C. very little dissociation occurred.

Likewise, dissociation in 5 per cent Bacto-peptone was variable, depending upon the strain used. Again a hydrogen-ion concentration of pH 7.6 appeared to exert a somewhat more favorable influence on dissociation. A greater degree of dissociation was effected in the 5 per cent peptone solution.

In 10 per cent peptone solution little S to R reversion occurred, and the dissociants which did appear were variable, occurring equally as well in any of the different

hydrogen-ion concentrations. Soule (1928) has shown that in the case of B. subtilis, no diasociation occurred at 5°C. and very little at a temperature of less than 25°C. He obtained his maximum dissociation at 45°C. Nungester (1929) found, while working with B. anthracis, that a marked decrease in dissociation activity occurred at 26°C. and 37°C., while at 42°C. he found dissociating activity to be greatest.

Koser and Stryon (1930) working with B. dysenteriae found that organisms grown in 1 per cent peptone, irrespective of pH, formed smooth colonies throughout the entire course of their experiment, while those grown in 5 per cent peptone sooner or later formed intermediate and rough colonies. They also found that in 10 per cent peptone solution there was less stimulus to change, in some cases K colonies never appeared.

DeKruif (1922) found that increasing concentrations of peptone accelerated the appearance of the granular form of B. leptisepticum and that a 20 per cent solution was nearly as effective as the 5 and 10 per cent solutions.

Coyle (1926) working with B. typhoeum and B. enteriditis stated that 20 per cent peptone offered no advantage in obtaining K variants.

Summary. We notice from the results obtained in this experiment that the greatest variation in type of colony,

and the greatest proportion of R colonies, for S. pullorum and E. typhi were produced in 5 per cent peptone. Concentrations on either side of this appeared to give less stimulus to dissociation. Usually the proportion of R forms was not large though many intermediates were found. The S, or S-like colonies, usually persisted throughout the entire period of incubation, but only in small numbers.

The greatest dissociation in two per cent Bacto-peptone occurred in the medium adjusted to a pH of 7.6 at the end of 18 days incubation at 37°C. In strain # 137-pullorum group, 23 per cent R colonies were obtained at the end of 18 days, while at the end of 55 days 10 per cent R colonies were noticed. On five per cent peptone the greatest dissociation occurred in the media adjusted to pH 7.6. In strain # 40-pullorum group, 62 per cent R variants were present at the end of 18 days and 10 per cent R variants were present at the end of 55 days. In ten per cent peptone the dissociation which occurred in the medium adjusted to the different pH values was variable. Media adjusted to pH 6.6 offered as great an impetus to dissociation as media adjusted to pH 7.6. In strain # 40-pullorum group, 25 per cent R colonies were obtained at the end of 18 days incubation and 2 per cent R colonies after 55 days incubation at pH 6.6, while at pH 7.6 after 18 days 3 per cent R colonies were obtained, and at the end of 55 days 30 per cent I

Table III. A Comparison of the Number of R Forms of Salmonella Pullorum Produced by the Cultivation of the S, or Normal Form, in Different Concentrations of Peptone Adjusted to Varying Hydrogen-ion Concentrations

		Initial pH of peptone solutions from which strains were plated		
Strain Days at 37°C.		pH 6.6	pH 7.2	pH 7.6
		Bacteria grown in 2 per cent peptone solution		
144	5	100S	100S	100S
	18	100S	100S	10I
	55	100S	100S	100S
137	5	100S	100S	70I
	18	100S	10R	23R
	55	100S	5I	10R
40	5	100S	100S	100S
	18	10I	100S	20R
	55	13R	5I	15R
49	5	21I	100S	10I
	18	9R	100S	15R
	55	10I	7I	50I
51	5	10I	100S	10I
	18	5R	100S	12R
	55	2I	2I	25R
50	5	100S	100S	100S
	18	10I	100S	100S
	55	100S	100S	5I
18	5	10I	100S	10I
	18	12R	100S	24R
	55	3I	100S	17R

(Table III continued)

		Bacteria grown in 5 per cent peptone solution		
		<hr/>		
144	5	1003	1003	1003
	13	1003	1003	1003
	55	1003	1003	1003
137	5	1003	1003	101
	13	10R	22R	25R
	55	3I	5I	20R
40	5	1003	1003	101
	13	21R	40R	62R
	55	10R	25R	10R
49	5	1003	1003	25I
	13	5R	10I	43R
	55	3I	13I	27R
51	5	1003	1003	1003
	13	1003	10I	25I
	55	1003	30I	46I
50	5	1003	1003	1003
	13	5I	3R	10R
	55	1003	1003	2I
18	5	1003	1003	1003
	13	2I	10R	25R
	55	1003	1003	6I
		Bacteria grown in 10 per cent peptone solution		
		<hr/>		
144	5	1003	1003	1003
	13	25I	1003	1003
	55	1003	1003	1003
137	5	50I	1003	1003
	13	1003	1003	101
	55	1003	1003	1003
40	5	17I	1003	101
	13	25R	1003	3R
	55	2R	10I	50I
49	5	1003	10R	1003
	13	25R	5R	1003
	55	6I	25I	1003

(Table III concluded)

51	5	100S	5I	100S
	18	10I	2R	100S
	55	100S	25I	10I
50	5	100S	20I	100S
	18	100S	2R	100S
	55	100S	30I	2I
18	5	10I	100S	10I
	18	25R	10I	15R
	55	2R	6R	30I

colonies were obtained.

The Effect of Various Concentrations of Peptone to Which
Brilliant Green has been Added as the Inciting
Factors in S to R Reversion of S. pullorum

Brilliant green medium was prepared by adding 3.5 cc. of a 1 per cent aqueous solution of brilliant green to 500 cc. of 2 per cent, 5 per cent, and 10 per cent solutions of peptone respectively. The pH of each solution was adjusted colorimetrically before brilliant green was added. The brilliant green peptone solution was tubed in 10 cc. amounts and autoclaved at 15 pounds pressure for 15 minutes and each tube of varying hydrogen-ion concentration and varying peptone concentration was inoculated with a smooth strain of S. pullorum. Incubation was accomplished at 21.5°C. and 37°C. throughout the entire experiment.

Brilliant green had little or no effect in producing dissociation of the S. pullorum organism in any concentration of peptone used. With some strains dissociation occurred to a greater extent in an alkaline reaction, while in other strains dissociation occurred to a greater degree on the acid side. In 2 per cent and 10 per cent solutions to which brilliant green had been added the dissociation which occurred was of the S to I to R type. The R forms were produced on the easily dissociating strains only. More S to R dissociation occurred among the bacteria grown in 5 per cent peptone solution than in any of the other concentrations. Likewise, more R variants were produced in the cultures incubated at 37°C. than those incubated at 21.5°C.

Brilliant green added to a medium is favorable for the growth of pullorum organisms. It is used in many laboratories as a selective medium for the growth of pullorum organisms and thus one would expect brilliant green to have little effect in producing dissociation of the S form of S. pullorum.

Mallmann (1932) added brilliant green to nutrient broth in order to obtain variants of the S form of S. pullorum. The R forms which he obtained were very unstable and soon reverted to the S type.

The Effect of Alkaline Nutrient Broth as the Inciting
Factor in Producing S to R Reversion of S. pullorum

Many workers have reported on the favoring influence of an alkaline over an acid reaction in effecting the S to R reversion. DeKruif (1922) claims a favoring influence of an alkaline reaction (pH 8.5) over an acid reaction (pH 6.0) in producing variation of B. leptisepticum. Hadley (1924) likewise reports the favoring influence of a medium with a pH of 7.8 on the dissociation of B. pyocyaneus and in the colon-typhoid-dysentery group. Dulaney (1928) reported that alkaline nutrient broth promoted the S to R change in a strain of B. coli while acid completely inhibited it. Dutton (1928) believed alkalinity to be an essential factor in the dissociation of streptococci. On the other hand, Soule (1928) has shown the favoring influence of an acid over an alkaline reaction in producing dissociation in the case of B. subtilus. Nungester (1929) reported that an acid condition in the dissociation of B. anthracis showed somewhat the greater dissociating influence.

Nutrient broth was prepared as in the previous experiment. The broth was divided into two parts and adjusted to a reaction of pH 7.6 and pH 8.0, respectively. The colorimetric method of determining hydrogen-ion concentration was used and N/10 NaOH was added to obtain the desired alkali-

inity. The media was seeded with seven smooth strains of S. pullorum and incubated at 37°C. At intervals the cultures were removed from the incubator, mixed well to insure a thorough distribution, and streaked on nutrient agar plates. After 24 and 48 hours of incubation the plates were examined for typical colonies.

The results of this experiment are shown in Table IV. One can see by inspection of the table that the results are variable. With some strains the variants were produced in a larger degree in pH 7.6 while in other strains nutrient broth adjusted to a pH 8.0 had more effect upon dissociation. In every case an I or R variant was obtained after four days incubation, more R variants being obtained in the nutrient broth adjusted to a pH of 8.0. In no case were 100 per cent R forms obtained and, with the exception of one strain at the end of 40 days incubation, only typical S forms were noted which indicates a R to I to S reversion as was noted in preceding experiments.

At the same time, five intermediate cultures obtained from a preceding experiment were inoculated into alkaline broth pH 7.6 and pH 8.0, respectively. The results of the experiment are recorded in Table IV.

Summary. At the end of a four day period of incubation many R and I colonies were observed in each culture. At the end of 17 days incubation the number of R and I colonies

present in the cultures were diminishing and at the end of 33 days of incubation only I and S forms were noted, which again indicates a R to I to S reversion.

A larger number of R variants were obtained in the nutrient broth adjusted to pH 8.0. In strain # 137-pullorum group at the end of 17 days incubation, 24 per cent R forms were obtained in the media at pH 8.0 while no R forms were noticed in the media adjusted to pH 7.6 and incubated for the same length of time.

A greater percentage of R forms could be obtained in a very short time if I forms were used for inoculation. I forms as well as R forms occurred and the number of S forms increased as the period of incubation lengthened.

At the end of a four day incubation period in strain # 137-pullorum group, 80 per cent R forms were obtained in the nutrient broth adjusted to pH 8.0, while 75 per cent R forms were obtained in nutrient broth adjusted to pH 7.6. The R forms gradually reverted to the S and at the end of 33 days the colonies observed in the media adjusted to pH 7.6 and pH 8.0 were 100 per cent S.

Table IV. A Comparison of the Effect of Alkaline Nutrient Broth to Inocite Dissociation on the Smooth and Intermediate Form of Salmonella pullorum

Strain	pH	Period of Incubation	4 days	17 days	33 days	40 days	76 days
			Percentage of R Forms Obtained				
144	7.8 8.0		20I 40I	100S 100S	100S 100S	100S 100S	100S 100S
137	7.6 8.0		40I 30R	100S 24R	100S 15R	100S 10I	100S 100S
40	7.6 8.0		40I 50I	10I 30I	50I 100S	4R 100S	2I 100S
49	7.6 8.0		50I 15R	100S 10R	100S 2I	100S 100S	100S 2R
51	7.6 8.0		50I 45I	4R 10I	7I 100S	100S 100S	100S 100S
50	7.6 8.0		100S 25I	100S 100S	100S 100S	100S 100S	100S 100S
18	7.6 8.0		15R 100S	10I 20R	17I 30I	100S 100S	100S 100S

(Table IV continued)

		Intermediate form inoculated into alkaline broth					
		75R	25R	100G	100S	100B	100I
157	7.6 8.0	80R	10I	100G	100S	100I	100I
40	7.6 8.0	80I 87R	75I 90R	10I 25I	18I 2I	100B 100I	100B 100I
49	7.6 8.0	80I 90I	50I 73I	10I 30I	3I 2I	100B 100I	100B 100I
51	7.6 8.0	70I 100B	100B 100B	100B 100B	10I 100B	18I 100B	18I 100B
18	7.6 8.0	71R 50R	10R 100B	15I 2I	60I 30I	25R 75I	25R 75I

The Effect of 1 Per Cent Bacto-peptone Containing 3
Per Cent Beef Extract on S to R Dissociation

Test tubes were prepared containing 10 cc. amounts of 1 per cent Bacto-peptone and 0.3 per cent beef extract. To each 100 cc. of 1 per cent Bacto-peptone was added 0.3 grams of beef extract. The peptone - beef extract solution was heated until the substances were thoroughly dissolved and the loss of weight due to evaporation was adjusted. The reaction was adjusted to a pH 7.0 by the colorimetric method and the medium tubed in 10 cc. amounts and autoclaved at 20 pounds pressure for 15 minutes.

Each culture tube was seeded with seven smooth strains of S. pullorum, two smooth strains of E. typhi, and one smooth strain of S. schottmülleri. The cultures were incubated at 37°C. throughout the entire course of the experiment. At various intervals the cultures were removed from the incubator, mixed thoroughly, and streaked on nutrient agar. The agar plates were examined after 24 hours and 48 hours incubation for the various types of colonies.

Summary. The results of the experiment are recorded in Table V. This medium proved very effective in inciting S to R dissociation.

The greatest number of R variants were obtained in most cases at the end of 34 days incubation. In strain # 40-pullorum group 36 per cent R colonies were obtained at the end of 34 days incubation and at the end of 65 days incubation 10 per cent I colonies were noticed, and when the experiment was terminated at 116 days the colonies noticed on the plates were 100 per cent S. In the case of the typhoid-paratyphoid organisms the greatest number of variants were obtained in this medium at the end of 18 days incubation. In strain #3-typhi group, 50 per cent R colonies were obtained, and at the end of 116 days 10 per cent I colonies were noticed.

This medium caused an S to R reversion in a shorter length of time in the typhoid-paratyphoid group than it did with the pullorum organisms.

The Effect of Glycerol in Inciting S to R Dissociation

Nutrient broth was prepared as described in a preceding experiment, tubed in 100 cc. amounts and 0.1 cc. of glycerol added to each tube containing 10 cc. of nutrient broth. The medium was then sterilized at 15 pounds pressure for 15 minutes.

Each culture was seeded with seven smooth strains of S. pullorum, two smooth strains of E. typhi, and one smooth strain of S. schottmüller. The cultures were incubated at 37°C. throughout the entire experiment and at various inter-

vals the culture tubes were removed from the incubator, mixed thoroughly, and streaked on nutrient agar. The agar plates were examined after 24 and 48 hours incubation at 37°C. and examined for the various types of colonies.

Summary. The results of this experiment are recorded in Table V. One per cent Bacto-peptone containing 0.3 per cent beef extract is able to incite S to R dissociation to a greater degree among the pullorum organisms than nutrient broth to which glycerol has been added. This may be explained by the fact that the addition of glycerol to 10 cc. amounts of nutrient broth causes the medium to become too acid for the growth of S. pullorum before the bacteria are able to produce R variants. In most cases dissociation occurred to the greatest extent between the 18th and 34th day of incubation.

No R forms were produced in strain #137-pullorum group at the end of 34 days incubation while in strain #40-pullorum group 43 per cent R variants had developed at the end of 34 days incubation. Here again is indicated the variability of each strain and the production of variants in the same medium.

With the typhoid and paratyphoid organisms S to R dissociation occurred equally as well on nutrient broth to which glycerol had been added as it did in 1 per cent peptone and 0.3 per cent beef extract. However, the typhoid and paratyphoid organisms dissociated in 12 to 18 days when grown in nutrient broth to which glycerol was added as compared

with 34 days when grown in the peptone beef extract medium.

Ninety per cent R variants were obtained in a strain of E. typhi at the end of 18 days incubation while in a peptone beef extract medium 50 per cent R variants were obtained in the same length of time. Nutrient broth containing glycerol was very effective in inciting S to R dissociation in the typhoid and paratyphoid organisms in a comparatively short length of time probably due to the fact that the typhoid-paratyphoid organisms are dissociated with little difficulty and the acidity of the media may thus aid in the S to R reversion.

The Influence of a Medium Containing 1 Per Cent Bacto-
Peptone and 0.3 Per Cent Beef Extract Adjusted to
Varying Hydrogen-ion Concentrations to Incite
Variation of the Normal or S Form of
S. pullorum

The medium was made as described in a preceding experiment and adjusted by the colormetric method to pH 6.6, 7.2, and 7.8 by adding N/10 HCl or N/10 NaOH tubed in 10 cc. amounts, and autoclaved at 15 pounds pressure for 15 minutes.

The medium was seeded with seven smooth strains of S. pullorum and the cultures were incubated at 21.5°C. throughout the entire experiment. At various intervals the

Table V. A Comparison of the Number of R Forms of *Salmonella Pullorum* Produced by the Cultivation of the S. or Normal Form, in 1 per Cent Peptone Plus 0.3 per Cent Beef Extract and in Nutrient Broth Plus Glycerol

Strain	Kind of Media	Period of Incubation						Percentage of R Forms Obtained					
		12 days	18 days	24 days	56 days	65 days	116 days						
144	Peptone-Beef extract N.B. Gly.	100S 10I	100S 25I	72I 100S	53I 100S	100S -	100S						
137	Peptone-Beef extract N.B. Gly.	1R 100S	90I 100S	72R 100S	10R -	10I -	100S						
40	Peptone-Beef extract N.B. Gly.	10I 43I	21R 34R	36R 42R	25R -	10I -	100S						
49	Peptone-Beef extract N.B. Gly.	25R 43I	26I 82I	17R 15R	50R -	25I -	100S						
51	Peptone-Beef extract N.B. Gly.	100S 100S	100S 100S	32I 100S	25I 100S	100S -	100S						
50	Peptone-Beef extract N.B. Gly.	100S 100S	100S 100S	39I 100S	10I 100S	100S -	100S						
18	Peptone-Beef extract N.B. Gly.	50I 12I	20R 85I	60I 16R	100S 10I	100S -	100S						

(Table V continued)

3	Peptone-Beef extract N.B. Gly.	25I 26R	50R 90R	47R 73R	40R -	30I -	10I -
4	Peptone-Beef extract N.B. Gly.	100S 18R	25R 63R	14R 26R	3I 10I	- -	- -
5	Peptone-Beef extract N.B. Gly.	50I 18I	24R 30R	17R 45R	8I -	- -	- -
13	Peptone-Beef extract N.B. Gly.	20R 90R	100S 30R	100S 10R	100S -	100S -	10I -

N.B. = Nutrient broth.
Gly. = Glycerol.

cultures were removed from the incubator, mixed thoroughly, and streaked on agar plates to determine the type of colonies present.

Summary. One per cent Bacto-peptone plus 0.3 per cent beef extract had little effect in inciting dissociation at a temperature of 21.5°C. regardless of the pH. At pH 7.2 the dissociation was quite uniform in all strains while at pH 7.8 and pH 6.6 the dissociation was variable with I and R forms observed on the plate.

Another series of tubes were prepared to which brilliant green had been added, the tubes seeded and the experiment conducted as the one preceding. The colonies obtained throughout were uniformly of the S type regardless of the pH. In a few strains I forms appeared, but no complete S to R dissociation occurred with the exception of strain # 40 in which R forms appeared after 45 days incubation. (However, strain # 40 has been very easy to dissociate throughout the entire investigation).

The Effect of Immune Serum-broth in Inciting Dissociation

Griffith (1923) was the first to intentionally use antiserum prepared by the immunization with homologous antigens, for dissociating S. pneumococcus into its related R types. However, Stryker (1916) grew pneumococci in

homologous immune serum and produced a rough strain which was quite stable. Later similar results were obtained by Arkwright and Pitt (1921) working with Eberthella typhi, by Dulaney (1926) who worked with E. coli, and by Soule (1928), Li (1929), and others who worked with the paratyphoids. Soule (1928) also obtained the same results with B. subtilus.

Contrary to these results Mallmann (1932) working with S. pullorum found no indication of roughness when the organisms were grown in immune-serum broth. Likewise Koser and Stryon (1930) working with B. dysenteriae were not able to produce any true R forms of that organism when grown in immune-serum broth.

In this investigation the immune serum used for one experiment was obtained from a chicken whose serum agglutinated pullorum organisms at a high titer, and the immune serum used in another experiment was obtained by inoculating rabbits with heat killed S and R pullorum organisms until a high titer serum was obtained.

One cubic centimeter of immune serum obtained from a positive pullorum chicken was added to 9 cc. of sterile nutrient broth to make a 10 per cent immune-serum broth by volume. After 48 hours incubation at 37°C. to test for sterility, the serum broth was inoculated with the S form of the pullorum organisms. The serum broth cultures were incubated at 37°C. and at intervals were removed from the

incubator, mixed thoroughly, streaked on agar plates, and the types of colonies present were observed after the plates were incubated 24 hours and 48 hours at 37°C.

The immune serum used gave complete agglutination of a pullorum antigen at a titer of 1:1280.

Summary. The results indicated that immune serum has no effect in accelerating the S to R change of the S. pullorum organisms. From the seventh to the twenty-second day of incubation the greater percentage of I forms were obtained, and after 51 days of incubation the I forms had entirely disappeared. No true R forms were found at any time.

Rabbits were inoculated at 3 to 5 day intervals with 0.5 to 5.0 cc. amounts of heat killed S and R pullorum organisms. The heat killed antigens were standardized to tubes 1, 2, and 3 of McFarland's nephelometer depending upon the number of doses which each rabbit had received. The rabbits were given 15 inoculations (subcutaneously), and after inoculation had been discontinued for one week, the rabbits were bled from the heart. Agglutination tests were run to determine the titer of the serum. The serums were added to nutrient broth to give concentrations of 10 per cent by volume. The serum broth was incubated 48 hours to test for sterility and inoculated with the S strain of the pullorum organism. After incubating at 37°C. for var-

ious intervals the cultures were removed from the incubator, mixed well, and streaked on agar plates and types of colonies looked for. No change was noted until the end of the seventh day when in culture 51S, 10I forms were noted and at the end of 19 days culture 18S contained SI forms and the remainder S. At the end of 35 days no I forms were noticed in any culture, all the forms were of the S type.

Likewise, the R serum obtained from rabbits inoculated with the R form of the pullorum organism was added to sterile broth to give a 10 per cent R-immune serum broth. This broth was incubated 48 hours to test for sterility after which time the tubes were inoculated with 144S, 51S, and 18S cultures of pullorum. The cultures were incubated at 37°C. and removed from the incubator at various intervals and streaked on agar plates.

Summary. At no time during the course of the experiment were any I or R forms noted in the medium to which R-immune sera had been added. The cultures remained 100 per cent S. Rough-immune-serum has no apparent effect on S to R dissociation.

On the other hand, R forms inoculated into rough-immune-serum broth soon reverted to the S form.

S-immune serum as well as R-immune serum do not accelerate the S to R reversion of S. pullorum organisms.

The Effect of 4.5 Per Cent Agar and 3 Per Cent CaCl_2
Agar in Producing S to R Dissociation of
S. pullorum

Hunter (1931) reported that R variants are produced when a culture is grown on 4.5 per cent agar. Accordingly, 4.5 per cent agar was prepared with and without dextrose. S forms were streaked on the 4.5 per cent agar and after 24 hours incubation the colonies were examined and all were found to appear rough. However, the roughness proved to be a temporary state because when the cultures grown on rough agar were again streaked on 2 per cent agar the colonies appeared entirely smooth. Aging a smooth culture on the 4.5 per cent agar had no permanent effect on the culture, because as soon as the culture was again placed on 2 per cent agar all the colonies were smooth. Antigens were made of the cultures when grown on 4.5 per cent agar and agglutination tests were run. No serological differences were noted between the cultures grown on 2 per cent agar and those grown on 4.5 per cent agar. Variations in colony form which occur when this organism is grown on 4.5 per cent agar were only temporary variants or perhaps not variants at all, since four and one-half per cent agar had no effect in inciting permanent dissociation. Whatever roughness resulted from this treatment should undoubtedly be consider-

ed merely as a peculiarity of growth on that particular medium.

Summary. When an R variant was streaked on four and one half per cent agar no difference could be noted between the S and R form. However, when the R colony was again streaked on two per cent agar it still retained the rough characteristics of the original culture while the S colony retained the smooth characteristics. In one experiment one series of the smooth forms were allowed to age on the thick agar and another series transferred weekly on thick agar. When the cultures were later streaked on two per cent agar no rough colonies were noted. The roughness obtained on the thick agar is only a pseudo-roughness on that agar.

The same type of variation was produced when 3 per cent CaCl_2 was added to nutrient agar. Strains of S. pullorum and E. typhi were allowed to remain on this agar at room temperature and at ice box temperature for three months and when the cultures were suspended in salt solution and streaked on nutrient agar 100 per cent S colonies were obtained, while the same colonies appeared 100 per cent R when grown on 3 per cent CaCl_2 agar.

Three per cent CaCl_2 incited a temporary type of variation, but when the variants were again subjected to normal conditions the typical S variants were again obtained.

R TO S REVERSION

At one time it was believed that the rough (R) form of the colonies of a number of different bacterial types could not be made to revert to the smooth (S) form. At the present time, it is known that R to S reversion may be accomplished by a variety of different methods.

Dulaney (1928) reported that the "early" R form of B. coli could be changed back to the S type readily, but when the R form had become "stabilized" the R to S change could be brought about only by cultivation in lactose broth or in nutrient broth containing homologous R-immune-serum. Soule (1928) accomplished the R to S change with the same organism by serial transfers in R-immune-serum broth. Jordan (1926) obtained S colonies from several single cell isolations from R colonies of B. paratyphosum B by twice daily transplanting them on beef-infusion broth. Dawson (1928) emphasized the use of R-immune-serum and of passage through the mouse on the R to S change of the pneumococcus.

Arkwright (1921) showed that R forms of typhoid and of paratyphoid B were made to yield an almost uniform turbidity by subculture in broth, indicating the loss of R characteristics and reversion toward the S form. Webster and Burn (1927) reported a rapid change of R to S in a mouse strain of B. enteriditis; reversion to the S type occurred after

the first or second passage in broth.

Nungester (1929) reported the production of the S forms from the R in B. anthracis, and Koser and Stryon (1930) produced smooth forms of B. dysenteriae from the rough form by successive transfers in dextrose broth made daily or twice daily. However, the S-like cultures derived from the R forms showed some tendency to spontaneous clumping in 0.85 per cent salt solution.

It is now recognized that many of the R forms which were once thought to be stable and irreversible can be made to yield colonies which again regain the smooth characteristics in their proper environment.

The question has been raised, however, as to whether the S type colony obtained from the R form are really identical with the original S type colony in antigenic and other properties. White (1929) believed that the S-like culture which he obtained from the R form of B. aertrycke and B. paratyphosus B had not regained the normal stable antigen of the primary smooth cultures even though the colony formation resembled the smooth type. Arkwright and Pitt (1929) also failed to obtain the true S forms. The S colonies which they obtained from the R were agglutinated by an R but not by an S anti-serum. Koser and Stryon (1930) found that the S cultures of B. dysenteriae obtained from the R form were agglutinated by the S-rabbit serum, apparent-

ly in as high dilution as the original S cultures.

MacKenzie, Fitzgerald, and Irons (1933) demonstrated, through their studies on S. schottröller, S. morgani, and S. paradysenteriae (Fleiner), that many of the characters of these cultures serological, morphological, and physiological, are capable of independent variation during the dissociative and revertive processes. In studying these cultures they found no evidence that during the dissociative or revertive process the characters of a culture change hand in hand. Each character appears to be capable of varying independently of the others, and in no instance did they notice complete reversion of all the characteristics.

In this investigation the attempted conversion of the S form was made when R forms were transferred daily in dextrose and mannite broth; when the R forms were aged in nutrient broth, and in salts broth at room temperature; and when the cultures were held on nutrient agar slants without transfer at room temperature and at ice box temperature.

Rough variants of six strains of S. pullorum were obtained from peptone solutions, beef extract cultures, or alkaline nutrient broth. Well isolated colonies secured on agar plates were removed, placed in nutrient broth, immediately restreaked on fresh plates, and the process repeated a number of times to insure a stable culture of the R forms.

Conversion From R to S Through Aging on
Nutrient Agar Slants

A series of agar slants inoculated with the R and S forms of the pullorum, typhoid, and paratyphoid organisms were held at room temperature 83 days without transplanting. At the end of that time a loopful of each culture was suspended on nutrient broth and immediately streaked on nutrient agar plates. At the end of 48 hours incubation at 37°C. the plates were examined for typical R and S colonies.

Summary. In no case did the R cultures of S. pullorum revert to the original S type completely, but in every instance a few I forms were noted. In the case of typhoid and paratyphoid organisms, however, the R cultures were still 100 per cent R. The S cultures which were allowed to age on nutrient agar slants for the same length of time as the R cultures did not show any indication of change. Due to the desiccation of the culture this experiment was discontinued.

Another series of agar slants inoculated with the R and S forms of S. pullorum, R. typhi, and S. schottmülleri were incubated at ice box temperature without transplanting. At the end of 60 days a loopful of each culture was placed on nutrient broth and immediately streaked on nutrient agar

plates. The plates were incubated for 48 hours at 37°C. after which time they were examined for typical colonies.

In the S. pullorum cultures at the end of 48 days, 100 per cent R colonies were not noted, but in each case only a few intermediate forms were observed and no S forms were present. The cultures were again removed from the ice box after 124 days incubation at that temperature. Here again no S forms were observed but a larger percentage of I forms were observed than when the plates were examined at the end of 60 days incubation. However, the typhoid and paratyphoid organisms which were held under the same conditions were still 100 per cent R at the end of 124 days incubation.

The S forms of S. pullorum were still 100 per cent S at the end of 124 days incubation at ice box temperatures while a few R variants were noticed in the S cultures of E. typhi and S. schottmülleri at the end of 124 days incubation.

The R variants seem to be more stable at room temperature than when they are incubated at ice box temperatures. Aging S cultures of S. pullorum on nutrient agar slants is not effective in inciting S to R dissociation in a short length of time because growth is retarded.

The Effect of Aging R Cultures in Nutrient Broth
and in Salts Broth in Causing R to S Reversion

Many investigators have claimed that aging on S culture on nutrient broth is effective in producing R variants. However, with S. pullorum it was found that nutrient broth had no effect in producing R variants. With this fact in mind it was decided to observe the effect of nutrient broth upon R variants which had been obtained in previous experiments. Accordingly, nutrient broth was prepared as in a preceding experiment and tubed in 10 cc. amounts. After the broth was autoclaved and tested for sterility it was inoculated with R variants of S. pullorum, E. typhi, and S. schottmülleri. The cultures were incubated at 37°C. for 24 hours and during the remainder of the experiment at room temperature. At the end of 32 days and again at the end of 48 days the broth cultures were well mixed and streaked on nutrient agar plates. At the end of 48 hours incubation at 37°C. the plates were examined for typical colonies. The results of this experiment are tabulated in Table VI.

Summary. At the end of 32 days incubation in strain #137 R-pullorum 10 per cent I forms were observed, while in strain #40 R-pullorum 50 per cent I forms were noticed, and at the end of 48 days incubation #137 R-pullorum strain had completely reverted to the S and in strain #40 R-pullorum

10 per cent I forms were noted.

Salts broth has been found to very effective in obtaining a very profuse growth of S. pullorum organisms and is used quite extensively in this laboratory. In a previous experiment it was found that salts broth was not effective in producing R variants of S. pullorum.

The salts broth was made as described by Scott (1930) as follows:

Peptone	20.00 grams.
Potassium citrate	2.00 grams.
Potassium bicarbonate	0.50 grams.
Ammonium dihydrogen phosphate	0.60 grams.
Ferrie ammonium citrate	2.50 grams.
Glucose, not more than	.50 grams.
Distilled H ₂ O to make	1000.00 cc.

The medium was dissolved thoroughly and the reaction adjusted to pH 7.2 after which it was tubed in 10 cc. amounts and autoclaved.

The salts broth was inoculated with R variants of S. pullorum, E. typhi, and S. schottmülleri organisms. The cultures were treated in the same manner as above described. The results are recorded in Table VI.

Summary. The results obtained indicate that R to S reversion in salts broth cultures is less complete than in nutrient broth cultures. The added minerals in the salts broth appear to inhibit a complete R to S dissociation.

At the end of 32 days incubation in strain #137 R-

Table VI. The Effect of Salts and Nutrient Broth on
the R Variants of S. pullorum, E. typhi,
and S. schottmulleri.

Period of Incubation		32 days	48 days
Strain	Type of Media		
144I	Salts broth	100S	100S
	Nutrient broth	100S	100S
137R	Salts broth	72R	50R
	Nutrient broth	10I	100S
40R	Salts broth	43R	25R
	Nutrient broth	50I	10I
49R	Salts broth	53R	35R
	Nutrient broth	25I	100S
51R	Salts broth	23R	10I
	Nutrient broth	100S	100S
50R	Salts broth	48R	25R
	Nutrient broth	100S	100S
18R	Salts broth	18R	10R
	Nutrient broth	25R	5R
3 Typhi R	Salts broth	13R	5R
	Nutrient broth	2R	10I
13 Schottmulleri R	Salts broth	48R	32R
	Nutrient broth	4R	25I

pullorum, 72 per cent R forms were observed and at the end of 48 days 50 per cent R forms were observed, while in strain #40 R-pullorum, 43 per cent R forms were noticed at the end of 32 days and 25 per cent R forms were noted at the end of 48 days incubation.

The Effect of Alkaline Nutrient Broth in Causing R to S Reversion

Since it has been found that the R variants will revert to the S form with little difficulty through the use of synthetic media, we were interested in determining if an R variant could be placed in a medium and remain 100 per cent R without transfer or attempts to keep the culture rough.

Alkaline broth proved very effective in inciting dissociation resulting in R variants in a very short period of time. Accordingly, R forms of S. pullorum, E. typhi, and S. schottmülleri were placed in alkaline broth (pH 7.8). When the cultures were streaked after ten days incubation I forms were noted in every culture indicating the instability of R cultures in alkaline broth.

The Effect of Successive Daily Transplants of the R Variant in Dextrose and Mannite Broth in the Production of the S Form

To one thousand cubic centimeters of nutrient broth prepared as in a previous experiment, 20 grams of mannite were added and to another 1000 cc. of nutrient broth 20 grams of dextrose were added. Each mixture was heated until the ingredients were thoroughly dissolved and then tubed in 10 cc.

amounts and autoclaved at 15 pounds pressure for 15 minutes.

Each sugar broth tube was inoculated with the R variants of S. pullorum, L. typhi, and S. schottmülleri. The tubes were incubated for 24 hours at 37°C., after which time they were removed from the incubator and a loopful from each culture was transferred to another tube of sterile sugar broth. The original culture was streaked on nutrient agar plates which were examined for typical colonies after 24 and 48 hours incubation at 37°C. This process was repeated for a period of 25 days.

Summary. From the results obtained it is concluded that the R colonies of S. pullorum are quite stable in dextrose and mannite broth, a greater percentage of I forms occurring on the mannite broth. At the end of 25 days incubation in the sugar broth only a few S forms of S. pullorum were noted. Mannite and dextrose broth is not effective in causing a R to S reversion in the case of S. pullorum. However, the typhoid-paratyphoid strains acted differently. At the end of 18 days incubation on sugar broth the greater percentage of the forms noted were I and S indicating a greater reversion than with the S. pullorum organisms.

In Nutrient Broth Containing Glycerol

R cultures were inoculated into tubes containing 10 cc. nutrient broth to which 0.1, 0.2, and 0.3 cc. glycerol were added. At the end of 24 days incubation at 37°C. the cultures were examined, and in every case regardless of the concentration of glycerol, 100 per cent R colonies were obtained. The same results were obtained at the end of 68 days incubation. This experiment was discontinued at this time, due to the production of so much acidity in the presence of glycerol, that growth was inhibited. Glycerol added to nutrient broth proved very effective in maintaining the stability of the variant.

In "K" Media

K-media prepared from pig intestine and chicken intestine containing Tyrode's solution was adjusted to a reaction pH 7.2 and after autoclaving, each was inoculated with R variants of S. pullorum, E. typhi, and S. schottmüller. The cultures were incubated at 37°C. for 24 hours after which time they were removed from the incubator and sealed with a mixture containing two-thirds vaseline and one-third paraffin. The cultures were streaked from time to time and at the end of one years incubation at room temperature the

cultures were again streaked and all were found to be 100 per cent R.

In "K" Media Containing Peptone

One per cent peptone was added to K-media prepared from chicken and pig intestine. The tubes thus prepared were inoculated with R variants and sealed as in the preceding case. However, at the end of 30 days incubation S, I, and R variants were present in each culture indicating that peptone is one factor which causes a R to I to S reversion.

Summary. In no case were 100 per cent R colonies obtained in the methods used to incite dissociation. R colonies could be secured only at certain stages of the incubation period after which time they soon reverted to the S. This may be due to the fact that the organisms became accustomed to the media in which they were growing and again assumed their normal form or it may indicate that a cyclic change occurs. At any rate, the R form was not stable in the medium in which they were produced because after a prolonged period of incubation the cultures appeared 100 per cent S.

Pure strains of the R form of pullorum cultures obtained in various ways were inoculated into nutrient broth containing glycerol, into K medium, and into K medium to which peptone had been added. At the end of 68 days incu-

bation on the R cultures in nutrient broth containing glycerol the colonies remained 100 per cent R. This indicates that an acid medium is favorable to the stability of the R form.

R cultures inoculated into K-medium prepared from pig and chicken intestine remained 100 per cent R after one year's incubation at room temperature. However, the R strains inoculated into K-media to which peptone had been added reverted to the I and S types at the end of 30 days incubation at room temperature.

AGGLUTINATION REACTIONS

Preparation of Antigens

Before taking up the preparation of antigen, a statement should be made as to the stability of the S and R forms of S. pullorum used in these tests when grown on slanted agar. While grown at room temperature the cultures remain quite stable and both S and R forms retain their characteristics to a great degree. The R cultures allowed to remain at ice box temperatures retain their characteristic roughness, but not for as great a time as similar cultures held at room temperature. However, the cultures which were once R and had again regained the smooth colony characteristic still retained certain of the other properties peculiar to

the R form, such as flocculating (agglutinative growth) in broth and in physiological salt solutions. Purified strains of the organisms, obtained by the various methods used to incite dissociation had been carried through an additional 30 serial subcultures at the time of the preparation of the antigens, with alternate streaking on agar plates and the subsequent selection of typical colonies for transference to slanted agar. Physiological salt solution was used in the preparation of the suspensions previous to each streaking. No R colonies were observed on plates streaked with S material and no S colonies were present on any of the R plates.

The antigens used in the agglutination tests were prepared in the following manner: 24 hour agar cultures of the organisms were obtained and the growth suspended in 10 cc. of 0.45 per cent saline. The growth was mixed with the saline by using a sterile loop and 2 cc. of the saline suspension was placed in a Kille flask containing nutrient agar. The Kille flasks were incubated at 37°C. for 48 hours and the growth was washed off with 0.45 per cent saline. The suspensions were then centrifuged for 15 minutes. The supernatant liquid was decanted and the sediment resuspended in 10 cc. of 0.45 per cent saline. The suspensions were again suspended in 0.45 per cent saline and again centrifuged, this time for 45 minutes. This process was repeated

four times and the washed antigens were phenolized with 0.5 per cent phenol and allowed to remain in the ice box until ready for use with the agglutination test.

The antisera were developed in rabbits in the following way: 24 hour cultures of the organisms were obtained and the growth suspended in 5 cc. of 0.45 per cent saline. The growth was mixed with a loop and the cultures placed in a centrifuge tube and centrifuged 5 minutes. The supernatant liquid was decanted and the sediment suspended in 0.45 per cent saline. The suspensions were first adjusted to tube 1 of McFarland's nephelometer and heated at 55°C. for one hour. The heated cultures were streaked on nutrient agar slants to test for sterility and the antigens placed in the ice box until inoculated into rabbits. Two cc. of such suspensions were introduced subcutaneously into rabbits every 3 to 5 days. Fresh antigen was prepared for each inoculation, the turbidity being adjusted to tubes 2 and 3 of McFarland's nephelometer as the immunization progressed. A total of 20 cc. of the material was introduced into each rabbit. Fourteen days after the last inoculation the animals were bled from the heart, the blood allowed to clot, and the serum removed with pipette and kept in the ice box until ready for use. Several series of immune sera were prepared in this manner.

It is very difficult to prepare a satisfactory homo-

ogenous suspension of the R organism for agglutination tests in 0.8 per cent salt solution due to the fact that this form tends to settle out leaving a clear supernatant fluid. This is not the case when 0.45 per cent salt solutions are used. Thus 0.45 per cent salt solutions were used throughout all the tests.

All the tests were incubated at 21°C. for 24 hours before they were read macroscopically.

The results in Tables VII, VIII, IX, and X indicate, in every case, that the S type antigens are agglutinated in a higher titer with S-immune serum than they are with R-immune serum. The R type antigens, as a rule, are agglutinated in the same titer with R-immune serum as with S-immune serum. # 137S-pullorum anti-sera agglutinated #137S-organisms in a titer of 1:640 and R organisms in a titer of 1:160. The # 137 pullorum R-sera agglutinated # 137R and #137S organisms both in a titer of 1:160. 40S-sera agglutinated # 40S organisms in a titer of 1:640, and # 40R organisms in a titer of 1:40. The 137-pullorum R-sera agglutinated # 40R-organisms in a titer of 1:40 and # 40S-organisms in a titer of 1:80. The 7643 serum which was obtained from a field case agglutinated 137S-pullorum organisms in a titer of 1:640 and # 137R-pullorum organisms in a titer of 1:80.

Soule (1928) found that Paratyphoid B S-sera agglutin-

ated S-organisms in a titer of 1:2560 and R-organisms in a titer of 1:640. Arkwright (1921) found that B. dysenteriae agglutinated S organisms up to a titer of 1:320 and R-organisms to a titer of 1:80. He found that R-sera agglutinated R-and S-organisms both in a titer of 1:640. The R-serum agglutinated R-organisms in a dilution of 1:320 and did not effect the S-organisms above a titer of 1:80. He found very little cross agglutination between the S-and R-forms. Andrewes (1922) stated in his work on the Salmonella organisms that two antigens are present which are not distributed throughout the culture as a whole, but are attributes of separate bacilli. He stated that two well defined types are present in every culture and either may undergo transformation into the other.

The Filtrability of R-and S-Variants Grown on K-Media

The work of Kendall (1932) prompted us to duplicate his experiments on inducing filtrable stages of common known pathogens by cultivation on K-media.

Fresh intestines of 100 small chickens were washed in water, cleaned, and ground in a fine meat chopper. The ground intestines were extracted with four volumes of 95 per cent ethyl alcohol for three subsequent 36 hour periods at 37°C. after which time the alcohol was poured off and

Table VII. The Maximum Dilutions of Homologous Immune Serums in Which a Positive Agglutination of Variants Occurred

Titers of Agglutination of Variants			Titers of Agglutination of Variants			
S antigen	Anti-S-serum	Anti-R-serum	Titer	R antigen	Anti-S-serum	Anti-R-serum
<u>E. coli</u>	<u>Coll-S</u>	<u>Coll-R</u>	<u>1:640</u>	<u>26 E-coli</u>	<u>Coll-S</u>	<u>Coll-R</u>
<u>S. schott-mulleri</u>	<u>Schott-mulleri-S</u>	<u>Schott-mulleri-R</u>	<u>1:2560</u>	<u>S. schott-mulleri</u>	<u>Schott-mulleri-S</u>	<u>Schott-mulleri-R</u>
<u>E. typhi</u>	<u>E. typhi-S</u>	<u>E. typhi-R</u>	<u>1:1280</u>	<u>E. typhi</u>	<u>E. typhi-S</u>	<u>E. typhi-R</u>
<u>137 S. pullorum</u>	<u>137 S</u>	<u>137 R</u>	<u>1:640</u>	<u>137 R-pullorum</u>	<u>137 S</u>	<u>137 R</u>
<u>40S pullorum</u>	<u>40S</u>	<u>40R</u>	<u>1:640</u>	<u>40 R-pullorum</u>	<u>40S</u>	<u>40R</u>

Table VIII. The Maximum Agglutination of S and R Variants in Immune Serums Obtained From Biological Companies

Antigen	Anti-serum	Titer	Antigen	Anti-serum	Titer
<u>S. schott-</u> <u>mulleri-9</u>	<u>S. schott-</u> <u>mulleri</u>	1:12800	<u>S. schott-</u> <u>mulleri-R</u>	<u>S. schott-</u> <u>mulleri</u>	1:6400
<u>2 E. typhi-3</u>	<u>E. typhi</u>	1:12800	<u>2 E. typhi-</u> <u>R</u>	<u>E. typhi</u>	1:3200

Table IX. Cross Agglutination of S. pullorum and E. typhi

Antigen	Anti-S serum	Titer	Antigen	Anti-R serum	Titer
4 <u>E. typhi-S</u>	137S	1:320	4 <u>E. typhi-S</u>	137R	0
4 <u>E. typhi-R</u>	137S	1:80	4 <u>E. typhi-R</u>	137R	0
137 <u>S. pullorum-S</u>	4S	1:100	137 <u>S. pullorum-S</u>	4R	0
137 <u>S. pullorum-R</u>	4S	1:80	137 <u>S. pullorum-R</u>	4R	0

Table X. Maximum Agglutinations of R and S Antigens Using Positive Pullorum Serum Obtained From Field Cases

Titer of Agglutination of Variants			Titer of Agglutination of Variants		
S. antigen S. pullorum strain	Serum 7643	Serum 7613	R. antigen S. pullorum strain	Serum 7643	Serum 7613
40	1:640	1:1280	40	1:320	1:320
49	1:640	1:640	49	1:100	1:160
50	1:640	1:1280	50	1:320	1:40
18	1:640	1:640	18	1:160	1:60
137	1:640	1:1280	137	1:80	1:80

benzol (Merck) added. The benzol was allowed to remain on the extracted intestine for 36 hours at 37°C. before it was removed. The supernatant liquid was poured off and the residue was dried with the aid of an electric fan. The dried material was ground in a large mortar until fine commutation was affected and the ground material was placed in a glass stoppered bottle until ready for use.

Fresh intestines of one hog were likewise washed in water, cleaned, and ground in a fine meat chopper and treated in the same manner as the chicken intestines.

One gram of powdered chicken intestine and one gram powdered pig intestine was each placed in 150 cc. Erlenmeyer flasks and 50 cc. of Tyrode's solution added to each. Two hundred milligrams of powdered K chicken intestine and K pig intestine were placed in large sterile test tubes and 10 cc. Tyrode's solution was added. The flasks and tubes were autoclaved at 15 pounds pressure for 20 minutes. The reaction of pH 6.8 was adjusted to pH 7.6 after autoclaving, by the addition of 0.5 cc. of a 5 per cent solution of sodium bicarbonate (NaHCO_3) to each 150 cc. flask and 0.1 cc. of sodium bicarbonate to each tube containing 10 cc. of the media. The media was then incubated for 24 hours at 37°C. to test for sterility.

S. pullorum cultures 137S and 137R were each inoculated into a tube of K-chicken intestine and K-pig intestine

and incubated at 37°C. for seven days. At the end of that time each culture was streaked on an agar plate and examined for typical R and S colonies to ascertain the type of culture which was to be filtered. One-tenth cc. of the first K-chicken and K-pig cultures were transferred to new K-chicken and K-pig intestine and these were called the second K-chicken and K-pig cultures. The first "K"-cultures were filtered through Berkefeld "W" filters and 0.5 cc. of each of the R and S filtrates was placed in nutrient broth, on agar slants, in salts broth, in "K"-chicken and "K"-pig (pH 7.6 and 6.4) to which Tyrode's solution was added, in "K"-chicken and "K"-pig (pH 7.6 and 6.4) to which physiological saline (0.85 per cent) was added, and in "K"-chicken and "K"-pig, to which besides the addition of physiological salt solution, 1 per cent peptone was added. The cultures containing the filtrate from the first "K" cultures were incubated at 37°C. and each week the cultures were stained and examined for visible growth. At the end of one month's incubation no visible growth was noted, however, the cultures were sealed with vaseline, which consisted of two-thirds vaseline and one-third paraffin, to prevent evaporation and they were again examined at the end of nine months incubation at room temperature showing no growth either macroscopically or microscopically. The period of

incubation for these cultures was not extended any further.

The "K"-chicken and "K"-pig intestine was inoculated with S and R variants of several dissociated strains of S. pullorum and E. typhi. The cultures were incubated at 37°C. for varying lengths of time to determine if the organism might be filtrable only at a definite time during its growth on K-media. Each culture was treated as in the preceding experiment with S-and R-pullorum, and the S-and R-strain of each culture was filtered through the same Berkfeld "W" filter and the filtrate treated as in the preceding experiment with 137S-and 137R-pullorum. Likewise, no growth has ever been noticed in the filtrates of the cultures thus treated.

Summary. A filtrable stage of S. pullorum and E. typhi could not be produced in K media whether the S-or R-variant was used.

Hadley (1931), as well as others, have found that the S or R form are not filtrable, but according to Hadley (1931) in his recent work on "Filtrable Bacteria" claims that he has cultivated in pure lines the filtrable virus-like stage of the Shiga culture which they called the "C" type. This "C" type culture was produced by forcing the dissociation reaction on the S-or R-type culture.

Seastone and Lawrence (1933) attempted to confirm the existence of a filtrable cycle of bacteria by use of "X"

medium, but thus far they have had no success in repeating the experiments of Kendall. Bronfenbrenner (1932) finds that "K" medium renders bacterial suspensions more easily filtrable than do other media and he finds that turbid "K" medium is especially effective in coating the filter bed and rendering the filters more permeable. Carpenter and Perrin (1933) were not able to produce filtrable forms of B. typhosus and Beta hemolytic streptococci although Kendall's technique was followed minutely.

Kendall's results are probably due to the passage through his filters of bacteria or bacterial fragments because the filter permeability is increased when suspended on "K" medium. Bronfenbrenner (1932), and Ward and Tang (1929) demonstrated that many of the filtrable viruses passed through filters if suspended in certain types of broth, but were held back if suspended in water or salt solutions.

COMPARATIVE STUDIES OF SMOOTH AND ROUGH FORMS

Colony size. Agar plates were streaked with the R and S form of each strain and the plates were incubated at 37°C. for 24 hours. At the end of that time the plates were removed from the incubator and well isolated variants of the smooth and rough forms were measured with a micrometer.

The colonies were again measured at the end of 36, 48, and 96 hours incubation. The average size of the S colony was 0.869 mm. and of the R colony 1.325 mm.

Generation time. S and R cultures were streaked on nutrient agar and incubated at 37°C. At the end of 12 hours incubation the plates were removed from the incubator and an entire colony of a well isolated S and an R form were removed and each placed in a 750 cc. Erlenmeyer flask containing 100 cc. of nutrient broth. The flasks were thoroughly mixed and dilutions were made in nutrient broth. Each dilution was plated and the colonies on the plates were allowed to incubate 48 hours after which time they were counted. The experiment was repeated using 24 hour, 36 hour, 48 hour, and 96 hour colonies which had developed on plates. It was found from these experiments that no clear cut results could be obtained. In most cases the number of cells of the R colony was less than that of the S colony probably due to the fact that the individual cells of the R colony tend to adhere and remain in clumps which could not be separated by shaking and thus the colonies which appeared on the plates did not represent a single cell, but a group of cells. In observing the plates the R colony appeared to grow more slowly than the S during the first 24 hours, after which time it grew more rapidly.

The S colony did not become much larger after 48 hours incubation while the R colony continued to increase in diameter and become very large after 96 hours incubation. From this it may indicate that the R colonies were slower in starting to grow and continued the slow growth over a long period of time while the S colonies grew more rapidly and their growth ceased in a shorter length of time.

The generation time in minutes of the S and the R forms were so nearly the same that their difference would be of little consequence.

Cell morphology. In S. pullorum no constant correlation could be made between the type of colony and the morphology of the individual cells composing the colony. The cells of the smooth colonies of the various strains appeared slightly longer than the cells of the rough colonies when stained by the Grams method. All of the cells were Gram negative rods. The most noticeable feature of the preparations was the clumping of the R variant after it was stained. The individual cells of the R colonies tended to adhere to each other and appear in clumps and in chain formation instead of appearing singly as is the case with the S form. Several types of stain were used, but none were effective in bringing out a mucoid substance which was thought to cause the adhering of the cells.

Table XI. A Comparison of the Number of Cells in a Single Colony of the S and H Variant after Varying Periods of Incubation

Strain	Period of Incubation			
	12 hr.	24 hr.	36 hr.	48 hr.
	Number of Cells			
183	96,000	500,000	1,800,000	2,000,000
18R	39,000	60,000	200,000	500,000

Table XII. A Comparison of the Generation Time of R and S Variants

Time	S-strain	Generation time	R-strain	Generation time
6:30- 8:30 A.M.	40S	81.7 min.	40R	69.8 min.
8:30-10:30 A.M.		40.0 min.		38.8 min.
10:30-12:30 A.M.		38.3 min.		37.1 min.
12:30- 1:30 A.M.		63.5 min.		38.6 min.
1:30- 2:30 P.M.		15.3 min.		16.6 min.
2:30- 3:30 P.M.		141.5 min.		41.5 min.
3:30- 4:30 P.M.		51.6 min.		61.7 min.
4:30- 5:30 P.M.		55.6 min.		45.3 min.
5:30- 6:30 P.M.		42.9 min.		67.0 min.
6:30- 7:00 P.M.		64.5 min.		35.8 min.
8:00- 9:00 A.M.	18S	248.9 min.	18R	108.5 min.
9:00-11:00 A.M.		57.4 min.		82.9 min.
11:00- 1:00 P.M.		38.7 min.		41.3 min.
1:00- 2:30 P.M.		44.1 min.		33.0 min.
2:30- 7:30 P.M.		48.4 min.		76.4 min.
7:30- 7:30 A.M.		284.7 min.		574.5 min.

Sugar fermentation. The variant colonies were tested in nine sugars - lactose, maltose, levulose, saccharose, xylose, dextrose, mannite, salicin, and rhamnose. No difference in their reaction on these sugars was noted by the usual qualitative fermentation tests.

Production of acid. Dextrose and mannite broth was prepared by adding 20 grams of dextrose and 20 grams mannite to 1 liter of nutrient broth made as described in a previous experiment. The sugar broths were placed in 125 cc.

Erlenmeyer flasks in 50 cc. amounts and were autoclaved at 15 pounds pressure for 15 minutes. The culture flasks were incubated at 37°C. for 24 hours to test for sterility. At the end of that time the initial pH of each sugar broth was determined by the use of the potentiometer. The dextrose and mannite broth cultural flasks were inoculated each with an S and R variant of S. pullorum. The cultures were incubated at 37°C. At the end of 12 hours incubation the cultures were removed from the incubator and mixed thoroughly after which time the pH of each culture was determined on the potentiometer. This process was repeated again for each culture at the end of 24, 36, 48, and 96 hours incubation. From the results recorded the difference in the production of acid by the S and R variant is so small that one may conclude the R and S variant have not lost any of their ability to produce acid.

Production of gas. Smith Fermentation tubes were prepared containing dextrose and mannite broth. The tubes were sterilized and inoculated with the S and R variants of S. pullorum, S. typhi, and S. schottmülleri organisms. The fermentation tubes were incubated at 37°C. and at the end of 24, 48, and 96 hours, 1 week and 2 weeks incubation, the production of gas by each variant was measured by means of a Frost gasometer. In every case the S variant produced a greater amount of gas than the R variant even after 2 weeks

incubation. In some cases the R variant did not produce any gas, but with the paratyphoid organisms in every case the R variants produced a small amount of gas.

Nungester and Jung (1932) working with S. schottmülleri obtained R variants which produced gas and others which did not produce gas. They showed that an S culture may lose its capacity to form gas without change in colony form. They concluded that these observations were in accord with the concept of independent variation of bacterial properties.

The typhoid carrier and the R form. Several investigators have advanced the theory that a carrier may carry an R form which changes to an S form under different conditions. In some cases this may be true but in this particular case it did not prove to be an R form.

While this investigation was in progress a typhoid epidemic arose which was later proved to be due to a typhoid carrier. We were very fortunate in that we had the opportunity to determine the cause of the epidemic. Many plates of course, were necessarily streaked with feces and urine of many individuals. Each plate was noted carefully and in no instance were rough colony forms observed. Also the typhoid strain isolated from the carrier showed no rough characteristics, and after it has been carried one year on nutrient agar slants incubated at ice box and at room

temperature no R forms were noticed.

INFLUENCE OF BACTERIOPHAGE ON DISSOCIATION

Many investigators have succeeded in producing bacterial variants with the aid of bacteriophage and many workers have noticed the action of bacteriophage in dissociating cultures.

There have been several theories proposed regarding the nature of bacteriophage and the mechanism of the lytic action. The conception of d'Herelle (1921) regarding the nature of the lytic action is that it concerns a foreign, filtrable virus which parasitizes bacteria and causes their destruction by lysis. The infectious unit is an ultra microscopic and filtrable corpuscle, multiplying only at the expense of young living bacteria, some of which may acquire an immunity to the parasite and thus become resistant to the lytic action and thus secondary colonies are produced. He stated that bacteriophage is the sole cause of bacterial "mutations" and when a bacteriophage is obtained it may possess the power to instigate, as well as to further, a certain sort of dissociative action in bacterial cultures, whether they are the R or the S form.

Bordet and Ciucu (1921) interpreted the bacteriophagic reaction as an autolysis or bacteriophagy resulting from the rupture of the equilibrium existing between assimilation

and metabolism. They have come to regard bacteriophage as an autolysin arising in the bacteria themselves. This theory is the one which is accepted by the majority of workers.

Zinsser (1927) believes that the lytic substance is an enzyme probably active upon the lipodial constituents of the bacterial limiting membranes and the structure that holds them together. No proteolytic action is evident in this reaction. He believes that this enzyme is liberated by all bacteria but inhibited from acting under normal conditions until after death, but in the lytic culture either a catalytic action starts the process or an anti-enzyme is liberated.

Hadley (1928) has proposed a theory of transmissible autolysis based on the dissociative reaction which he calls the "Homogamic Theory" (Homo-self, gamic-marriage) in which he postulated that the bacteriophagic corpuscles represent either stages in the cyclogeny of the species of the substratum (or of a closely related species, containing at least some of the common O or H antigens or units of accessory physiologic significance, such as fecundating elements of the same sort). This theory is based on facts relating to the dissociative reaction.

Many other theories have been proposed, but the theory of Bordet and Gijca is the most widely accepted either as the original or modified to a certain extent. Much will necessarily need to be done on this subject before any theory can be accepted wholly or in part.

Undoubtedly there is some relationship which exists between filtrability, dissociation, and bacteriophage as has recently been pointed out by Hadley (1931) working with his C form of Shiga bacillus. At the present time, many studies are in progress dealing with these three great problems in bacteriology and when their relationship is determined a great forward step in bacteriology will have been made.

In this investigation the presence of bacteriophage was not noted by any of the usual tests. S and R variants of the same culture may be obtained without the aid of bacteriophage as has been found in this investigation. Bacteriophage may be one incitant to produce S and R variants in a very short time, but it is not an essential factor.

In this laboratory a more recent study has been made in an attempt to isolate several strains of colon from the intestines of chickens. The plates obtained in this study were also examined for the presence of bacteriophage, but in this investigation the evidence of colonies which have

been attacked by phage is lacking.

DISCUSSION

A study was made of the influence of certain modifications in the culture medium in enforcing dissociative changes. After the variants of S. pullorum were obtained studies were made on their serological and physiological properties as well as on their comparative morphology. Studies were also made on the influence of certain modifications of the media in producing an R to S reversion and experiments were run to determine the filtrability of the R and S form and the effect of K media upon the filtrate.

Smooth forms of seven strains of S. pullorum, two strains of E. typhi, and one strain of S. schottmülleri were used for the inoculation of various types of media. The typhoid-paratyphoid organisms were used as a check to determine if the S. pullorum organisms dissociated as easily as the typhoid-paratyphoid group. The cultures were held at 21.5 °C. and 37°C. for one to two months and longer, and at intervals they were plated on nutrient agar to note any change in colony form.

Variant colonies frequently made their appearance and they were designated as smooth (S), intermediate (I), and rough (R). Considerable irregularity in the time of occurrence and in the preparation of different variants was al-

ways evident.

The type of medium used played a part in the form of the colony. Four and one-half per cent agar, and three per cent CaCl_2 agar, frequently caused smooth types to appear as rough types. This condition was confined to colony appearance on this medium only, the cultures again reverting to the original form on 2 per cent agar.

Alkaline broth as well as peptone solutions proved quite effective in inducing rough dissociants. Five per cent peptone was more effective in inciting dissociation than any concentration on either side. Dissociants were produced in alkaline broth in a comparatively short length of time as compared with other media which were used to incite dissociation.

Brilliant-green-peptone-broth and brilliant-green-beef-extract media had no effect in inducing rough dissociants.

No stable rough strains of S. pullorum were obtained when the smooth strain was grown in immune-serum broth.

Rapid transferring of the rough form in sugar broths had no effect in producing smooth colonies from rough colonies, but aging the culture on nutrient broth caused a smooth colonial appearance in many of the rough strains. However, many of the cultures which had reverted to the smooth forms, at least in appearance, still exhibited their agglutinative growth in broth. When rough cultures were allowed to re-

main on stock agar slants at room and at ice box temperatures the various strains tended to return to their normal type. The tendencies were more pronounced at ice box temperatures.

In every case the R-immune-serum agglutinated the R-antigen in a lower titer than the S-immune-serum agglutinated the S-antigen. In an earlier experiment some cross agglutination was noticed, but in a later experiment no cross agglutination was evident.

The S and R variant reacted similarly in sugars in the qualitative reaction. However, the S variant produced a greater amount of gas than the R variant and in some instances the R variant did not produce any gas.

The S colony was always smaller than the R colony even after 2 weeks incubation. The individual cells of the S colony appeared singly while those of the R colonies appeared in masses and in chain formation. No appreciable difference was noted in the size of the cells of the R and S colonies.

No clear cut results could be obtained in the quantitative determination of the number of cells contained in a colony of the R and S form. Even though the R colony appeared larger, the S colony always yielded the greater number of cells on quantitative determination probably due

to the fact of the tendency of the R form to adhere in clumps. The generation time was practically the same.

Many plates were examined for the presence of bacteriophage but no indications of its presence was evident. Plates were streaked from human feces and urine, and from the intestinal tract of chickens.

The rough colonies remained very stable in K media containing no peptone and in media slightly acid in reaction.

K media had no effect in producing a filtrable stage in the S or R variant and the S or R variant of S. pullorum did not show any signs of filtrability.

It appears that as more work is done upon the study of dissociation more confusion results in an already confused state of affairs. The results obtained by different investigators and in many cases the same investigators are quite variable even with the same species of bacteria. The same experiment repeated at another time may give entirely different results although the same conditions are closely adhered to.

Throughout this investigation an attempt was made to keep the experiments as simple as possible, hence the limitation to only smooth, intermediate, and rough type colonies. Other types of colonies were observed, but their study was not included in the report.

In the experimental work the types of variants were observed when dissociant incitants were applied to smooth (S) cultures as well as to intermediate (I) cultures. Observations were also made on the length of time this induced property would remain after the specific incitants were removed before reverting to the mixed colonies again.

Smooth cultures were changed to intermediate and rough types by exposing them to various environmental conditions. Attempts were likewise made to convert rough types to intermediate and smooth types in the same manner.

From the data presented it would seem that the type of colony form depended upon environmental factors, because dissociation was most marked in media on which the organism was not accustomed to being cultivated. The change from one colony form to another seemed to be a constant gradual one with the occurrence of many intermediate forms before the appearance of the rough colony. It appears that the dissociation tendencies are reversible with intermediate colonies appearing in the reversion before the appearance of the smooth form. Mungester (1929) working with B. anthracis and Koser and Stryon (1930) working with B. dysenteriae also found the R to I to S, and the S to I to R relationship true in their dissociation experiments.

Whether the smooth colonies which result from the reversion of the R form retain all their smooth character-

istics is a fact to be determined. Due to the independent variation of colonies some may again acquire the original smooth characteristics while others may retain some of the rough characteristics even if they have all the appearances of a smooth colony. On this point there is little agreement. Jordan (1926) working with B. paratyphosus B has reported a return of virulence following a reversion from R to S while Griffith (1928) working with pneumococcus has reported a reversion as far as colony form was concerned, but the reverted cultures lacked virulence.

The results obtained in this study are not in disagreement with data presented by others. There is a generally accepted idea that dissociation is easily produced and that rough and smooth types can be changed back and forth at will as reported by Stryker (1916), Arkwright (1921), Jordan (1926), Dawson and Orcutt (1923), Webster and Burn (1927), Soule (1927), Dawson (1928), Griffith (1928), Dulaney (1928), Arkwright and Pitt (1929), and others.

There is no set method of excitation which will induce certain changes in the organism and in many cases the different strains of the same species require different incitants before variants are produced and the same media will not incite dissociation in every strain of the same species.

The R and S colonies of S. pullorum appear to be

analogous to the R and S colonies of other members of the genus *Salmonella* as reported by Li (1929), Wilson (1930), Goyle (1926), White (1929), Kettger and Pastridge (1932), and others.

CONCLUSIONS

1. S, I, and R variants of *S. pullorum* may be obtained.
2. *S. pullorum* was more difficult to dissociate than *E. typhi* and *S. schottmülleri*.
3. The dissociated forms of *S. pullorum* are analogous to dissociated forms of other species.
4. Large volumes of culture medium are more effective in inciting S to R dissociation than small volumes.
5. Aging smooth cultures of *S. pullorum* in nutrient broth had no effect in producing dissociation.
6. Five per cent peptone produced a greater number of R variants than any concentration on either side.
7. Five per cent oxgall in peptone solutions had no effect in producing S to R variants because variants were produced equally as well in peptone solutions which did not contain oxgall.
8. Brilliant green had no effect in inciting dissociation.
9. Alkaline media proved to be a greater incitant

to dissociation than acid media.

10. One per cent Bacto-peptone to which 0.3 per cent beef extract had been added was effective in producing R variants in certain strains of S. pullorum, if incubated at 37°C.

11. Nutrient broth to which 0.1 cc. glycerol was added was a very good medium to obtain R variants of the typhoid-paratyphoid group as well as variants in some strains of S. pullorum.

12. Neither S nor R-immune-serum-broth had any effect in causing S to R dissociation of S. pullorum.

13. Four and one-half per cent agar as well as 3 per cent CaCl_2 agar produced colonies which resembled the R form, but the variants immediately reverted to the S type when placed on 2 per cent agar.

14. R to S reversion is accomplished more readily in nutrient broth than in broth containing sugars.

15. R colonies held on nutrient agar slants at ice box temperatures tend to revert to the S form more readily than R colonies aged at room temperatures.

16. R variants of S. pullorum tend to remain quite stable in dextrose and mannite broth even when transplanted daily. On the contrary R colonies of E. typhi and S. schottmülleri revert to the S form in a very short time when placed in sugar broth.

17. The R variants of S. pullorum and of the typhoid-paratyphoid organisms remained stable in K medium, and in nutrient broth containing glycerol.

18. The R cultures which regained the S characteristics in appearance still retained the agglutinative growth in broth and saline which is peculiar to the R form.

19. The production of variants in different media was variable, one strain would be easily dissociated in a medium which would have no effect upon another strain.

20. S antigens were agglutinated by S sera in a higher titer than R antigens were agglutinated by R sera.

21. In high titer serum no cross agglutination was evident.

22. The R forms remained stable in K-pig intestine and K-chicken intestine if no peptone was added.

23. K-media had no effect in producing a filtrable stage in the R or S variant.

24. The individual cells of the R culture were somewhat longer than the individual cells of the S variants. The cells of the R variant appeared in chain formation while those of the S variant appeared singly.

25. The R colonies were larger than the S colonies.

26. The generation time of the R and S cells was nearly the same.

27. In the quantitative determination of the number of cells in an S or R colony the S colony appeared to contain more cells probably due to the clumping of the R cells.

28. The S variant produced a greater amount of gas than the R variant. In some cases the R culture produced no gas at all.

29. An S form was isolated from a typhoid carrier and in no instance was any rough characteristic noted.

30. The presence of bacteriophage was not noted throughout this investigation.

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LITERATURE CITED

- Andrewes, F. W. Studies on group agglutination. I. The Salmonella group and its antigenic structure. Jour. Path. and Bact., 25:505-521. 1922.
- Arkwright, J. A. Variation in bacteria in relation to agglutination both by salts and by specific serum. Jour. Path. and Bact., 24:36-60. 1921.
- _____, and Pitt, R. M. The effect of growing smooth and rough cultures in serum. Jour. Path. and Bact., 32:229-246. 1929.
- Bordet, J., and Ciuca, M. Cited by Hadley, P. The Twort d'Herelle phenomenon. Jour. Infect. Dis., 42:263-434. 1928.
- Bronfenbrenner, J. Studies on the bacteriophage of d'Herelle. Jour. Exper. Med., 45:873-886. 1927.
- _____, and Varney, P. L. The effect of "K" medium on the filtrability of bacteria. Proc. Soc. Exper. Biol. and Med., 29:7. 1932.
- Carpenter, H. M., and Perrin, H. L. Experiments with Kendall's medium. Jour. Bact., 25:241-244. 1933.
- Craig, C. F., and Johns, F. M. Failure to produce filtrable forms of common pathogens in Kendall's media. Proc. Soc. Exper. Biol. and Med., 29:661-662. 1932.
- Dawson, Martin, H. The interconvertibility of "R" and "S" forms of pneumococcus. Jour. Exp. Med., 47:577-591. 1928.
- d'Herelle, F. The bacteriophage and its behavior. Translated by Geo. H. Smith. Williams and Wilkins Co. 629 p. 1926.
- DeKruif, P. H. Dissociation of microbic species. I. Co-existence of individuals of different degrees of virulence in cultures of the bacillus of rabbit septicemia. Jour. Exp. Med., 33:773-789. 1921.

- _____. Mutation of the *Bacillus* of rabbit septicemia.
 Jour. Exp. Med., 35:561-574. 1922.
- Dulaney, A. D. Microbic dissociation of *B. coli-communis*.
 Jour. Infect. Dis., 42:575-588. 1928.
- Dutton, L. C. Microbic dissociation in streptococci.
 Jour. Bact., 16:1-16. 1928.
- Eastwood, Arthur. Combining affinities in bacterial variation.
 Jour. Hyg., 32:301-330. 1932.
- Frobisher, Martin. On the action of bacteriophage in producing filtrable forms and mutations of bacteria.
 Jour. Infect. Dis., 42:461-472. 1928.
- Coyle, A. M. On bacterial variation with special reference to the alleged convergent phenomena exhibited by certain distinct pathogenic species. (*B. typhosus* and *B. enteriditis*, Gaertner).
 Jour. Path. and Bact., 29:149-166. 1926.
- Griffith, F. The influence of immune serum on the biological properties of pneumococcus.
 Rpt. 18 on Public Health and Medical Subjects.
 Ministry of Health. 1923.
- _____. The significance of pneumococcal types.
 Jour. Hyg., 27:113-159. 1928.
- Grinnell, F. B. Observations on some of the factors involved in filtration experiments.
 Jour. Bact., 18:175-180. 1929.
- Hedley, P. Transmissible lysis of *Bacillus pyocyaneus*.
 Jour. Infect. Dis., 34:260-304. 1924.
- _____. Microbic dissociation.
 Ibid. 40:1-312. 1927.
- _____, Delves, E., and Klimek, John. The filtrable forms of bacteria. I. A filtrable stage in the life history of the Shiga dysentery bacillus.
 Ibid. 48:1-159. 1931.
- _____, and Jiménez, Beunavéntura. Production of bacteriophage by enforced dissociation.
 Ibid., 48:176-182. 1931.

- _____. The Twort - d'Herelle phenomenon.
Ibid. 42:263-434. 1928.
- Hunter, C. A. Colonial variation of E. coli on solid media with especial reference to the production of rough colonies.
Jour. Bact., 21:13. 1931.
- Jordan, E. O. Intersconvertibility of "rough" and "smooth" bacterial types.
Jour. Amer. Med. Assoc., 86:177-178. 1926.
- Kendall, A. I. The filtration of bacteria.
Science 75: 1942:295-301. 1932.
- Koser, S. A., and Stryon, H. C. The production of smooth from rough forms of B. dysenteriae, (Sonne).
Jour. Infect. Dis., 47:443-452. 1930.
- _____. Dissociation of B. dysenteriae, (Sonne) as influenced by variation in culture medium.
Ibid. 47:453-477. 1930.
- Krumwiede, Ch., Mishulow, L., and Oldenbuch, C. The existence of more than one immunological type of B. pertussis.
Ibid. 52:22-32. 1923.
- Li, C. P. Studies on dissociation of the hog cholera bacillus. II. Serological reactions, virulence, and stability of the variant forms.
Jour. Exp. Med., 50:255-262. 1929.
- Mac Kenzie, G. M., Fitzgerald, Helen, and Irons, Vernal. Independent variation of biological characters of bacteria as a result of dissociation.
Proc. Sec. Exp. Biol. and Med., 30:536-538. 1933.
- Mallmann, W. L. The dissociation of Salmonella pullorum and related species.
Unpublished thesis, Univ. of Chicago. 40 pp. 1932.
- "
Nagelli, C. V. Cited by Hadley, P. Microbio dissociation.
Jour. Infect. Dis., 40:1-312. 1927.
- Rungester, W. J. Dissociation of B. anthracis.
Jour. Infect. Dis., 44:73-125. 1929.

- Greutt, M. L. Mutation among hog cholera bacilli.
 Jour. Exp. Med., 38:9-15. 1923.
- Plastringe, W. M., and Rettger, Leo, F. Variants of
Salmonella pullorum.
 Jour. Infect. Dis., 50:146-161. 1932.
- Pyle, N. J. The bacteriophage in relation to S. pullorum
 infection in the domestic fowl.
 Jour. Bact., 12:245-253. 1926.
- Schutze, H. The prominence of the serological paratyphoid
 B. types, with observations on the non-specificity of
 agglutination with "Rough" variants.
 Jour. Hyg., 20:330-341. 1921.
- Scott, J. P. A simple mineral mixture agar for the culti-
 vation of Pasteurella bovisepctica.
 Jour. Bact., 20:9-14. 1930.
- Seastone, C. V., and Lawrence, Mary B. Attempt to confirm
 the existence of a filtrable cycle of bacteria by use
 of "E" medium.
 Jour. Infect. Dis., 52:20-26. 1933.
- Soule, M. H. Microbic dissociation.
 Jour. Infect. Dis., 42:93-148. 1928.
- . The interconvertibility of R and S bacterial types
 of B. paratyphosus B by homologous immune sera.
 Jour. Bact., 15:39-40. 1928.
- Stryker, L. M. Variations in pneumococcus induced by growth
 in immune serum.
 Jour. Exp. Med., 24:49-68. 1916.
- Thjotta, Th., and Eide, O. K. A mutating mucoid para-
 typhoid bacillus isolated from the urine of a carrier.
 Jour. Bact., 5:501-510. 1920.
- Ward, H. K., and Tang, F. F. A note on the filtration of
 the virus of Herpetic Encephalitis of vaccina.
 Jour. Exp. Med., 49:1-4. 1929.
- Webster, L. T., and Burn, C. Studies on the mode of spread
 of B. enteritidis mouse typhoid infection. II. Ef-
 fects of external conditions on the occurrence of
 smooth, mucoid, and rough colony types.
 Jour. Exp. Med., 46:855-870. 1927.

White, P. B. Notes on intestinal bacilli with special reference to smooth and rough races.

Jour. Path. and Bact., 32:85-94. 1929.

Wilson, G. S. Discontinuous variation in the virulence of B. aertrycke, mutton.

Jour. Hyg., 28:295-317. 1928.

_____. The relationship between morphology, colonial appearance, agglutinability, and virulence to mice of certain variants of B. aertrycke.

Ibid. 30:40-54. 1930.

Wilson, W. J. Pleomorphism as exhibited by bacteria growing in media containing urea.

Jour. Path. and Bact., 11:394-404. 1906.

Zinnser, Hans. Textbook of Bact.

6th Ed. New York and London. 1053 pp.

D. Appelton and Co., 57-62 pp. 1927.