A STUDY ON THE DISSOCIATION OF SALMONELLA PULLORUM AND THE SENSITIVITY OF THE VARIANTS TO A QUATERNARY AMMONIUM COMPOUND

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by

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INTRODUCTION

The phenomenon of bacterial dissociation or variability is a subject of both fundamental and practical significance. As a result, this phenomenon in all its diversity has been intensively studied for the last thirty years; however, much of the evidence which has accumulated has been just inconclusive enough to warrant widely different interpretations and theories. Thus, the various changes in colonial and cellular morphology, biochemical behaviour, antigenicity, and virulence exhibited by the bacterial cell may be possible expressions of one of several fundamental conceptions. Such concepts include, (1) bacteria vary in definite trends or patterns, (2) the environmental conditions imposed select variants or may directly result in variation, (3) bacteria just as other organisms are subject to mutations, and finally, (4) the various forms expressed by a bacterial species represent progressive ontogenetic transformation, commonly referred to as life cycles. Regardless of the theory one may adopt, the evidence to date indicates that dissociation occurs in virtually all bacterial species, and changes may be induced under suitable environmental conditions.

With regard to the practical significance of bacterial dissociation, direct consideration can be made to the anomalies that frequently occur in the phenol coefficient results of quaternary ammonium germicides. Maier and Muller in 1936 emphasized that dissociation of test organisms may result in a increase or decrease in sensitiveness of the bacterial to the test solutions. It seems

apparent then, that the stability of the test organism in regard to dissociation must be considered if more accurate results are to be expected in the phenol coefficient tests.

Mallmann in 1952 reported finding three stable colonial types in the Salmonella group. These three strains of Sal. pullorum were found to be (1) a stable smooth type, (2) a stable rough type, and (3) a variable rough-smooth type. Dissociation incitants were without permanent effect upon the pure rough and smooth types, while true dissociation changes occured only in the variable type cultures.

In view of these findings and other evidence presented in the literature, this study was undertaken with a twofold purpose in mind: to attempt to isolate a stable smooth strain of Sal. pullorum, and to determine the sensitivity of smooth and variant forms of this organism to a quaternary ammonium compound.

HISTORICAL RESUME'

Before pure culture techniques were developed, bacteria were regarded as belonging to an homogenous group, possibly of but a single species. In fact, in 1877, Nägeli propounded the doctrine of pleomorphism wherein all fission fungi were one type of cell, highly variable and capable of passing from one morphological type to another, as well as being capable of undergoing profound alterations in biochemical behaviour.

The opposite extreme of monomorphism became predominant with the subsequent development of pure culture methods by Koch and others. This doctrine proclaimed a constancy of species and form in bacterial morphology. Despite the adoption of monomorphism by the great majority of bacteriologists, data accumulated which indicated that though most bacteria showed a remarkable constancy of form, morphological and physiological variation is not uncommon.

In 1918, Baerthlein demonstrated that colony variability may serve as a criterion of cultural variability, for closely correlated with colony types are other important characteristics of the organism including cellular morphology, pigment and slime production, fermentation reactions, serological reactions and virulence. Arkwright, 1921, recognized the significance of Baerthlein's work and described smooth and rough colony forms of the colon-typhoid-dysentery group. Subsequent work by many others followed in voluminous proportion regarding the organisms belonging to this group and many others. Hadley published extensive literature reviews in 1927 and 1937 citing numerous instances of dissociation changes in various organisms.

The colonial forms described fall into six general categories; namely, smooth (S), rough (R), intermediate (SR), mucoid (M), dwarf (D), and gonidial (G). The smooth colony is characterized by a round, even margin, and a convex smooth and glistening surface; the organisms are uniformly turbid in broth and produce a stable suspension in 0.85% Nacl solution. The rough colony is described as follows: the margin is irregular; the surface is flat, uneven, and granular; the organisms produce a granular sediment in broth and clump spontaneously in physiological saline. The intermediate colonies are those that appear intermediate in form between S and R colonies and usually change into one or the other. The mucoid forms have a moist, glistening, mucous-like consistency; the colonies tend to coalesce. The dwarf forms are characteristically very small measuring less than 1 mm. in diameter and differing in respect to larger colonies of the same organism only in size. Finally, the gonidial forms are minute colonies representing growth from filterable forms of bacteria; however, much disagreement still prevails as to the actual existance of these so-called filterable forms.

While dissociation of various members of the Salmonella group has been widely studied, comparatively little work has been forthcoming with Sal. pullorum. In 1930, Plastridge and Rettger isolated, from all appearances, a rough strain of Sal. pullorum from infected adult fowl and young chicks. These same authors described in 1932 three principle colony types of this organism including the smooth, rough, and a somewhat intermediate form. Gauger (1936) reported the isolation of two rough strains of Sal. pullorum from baby chicks; however, though these strains varied morphologically from typical

smooth cultures, no autoflocculation in saline was exhibited. In 1939 Morgan and Beckwith observed that Sal. pullorum temporarily exhibited a mucoid type when grown at temperatures between 16° and 25° C., but when cultured at 37° C. the loss of the mucoid character or reversion to the smooth type immediately occured.

The most extensive work on the dissociative character of Sal. pullorum was reported in 1932 by Mallmann. Although it is generally believed that the chief culture types (S and R) are easily convertible one into the other, Mallmann presented evidence to the contrary. Working with stock cultures that had been kept in culture for many years, a total of 27 of these were found to be characterized by their smooth colony formation, 15 were markedly rough and at no time showed smooth characteristics, while 16 of the organisms were variable R to S and S to R types.

Employing numerous well recognized dissociation incitants for considerable time periods including brilliant green beef extract broth as a chemical incitant, aging at various temperatures, rapid transferring, immune smooth and rough serum, bacteriophage, and animal passage, it was found that there were several strains which were unaffected by these methods, maintaining the original colony appearance of either smooth or rough. The SR strains found variable as stock cultures were, on the other hand, easily changed to either S or R colonies depending on the type of incitant used.

In support of this evidence, it was indicated that a large number of workers previous to 1930 found refractory organisms that did not respond to inciting agents, although in many cases they were disregarded

in the final conclusions.

Barber (1907) obtained abnormal strains of Esch. coli that never reverted to the parent strain.

Clark (1910) was unable to convert avirulent diptheria bacilli to virulent types by means of animal passage or by sensitization with homologous immune serum.

Buchanan and Truax (1910) in a study analogous to S and R types were unable to secure high and low acid producers of Strep. lacticus. They attributed their lack of success to the fact that a pure-line culture has invariable characteristics and therefore variations, in their estimation, will occur in cultures of mixed lines only.

Schutze (1921) was unable to convert R type paratyphoids to the S form.

In 50 rapid transfers DeKruif (1922) found a strain of the bacillus of rabbit septicemia that would not revert to the normal type.

Reiman (1925) could not revert R type paratyphoids to the S form by rapid transferring or 105 passages through mice. He believed that the change to an R type was characterized by a loss of certain properties in the cell that cannot be regained.

Jordon (1926) could not induce smooth types in two strains after 72 rapid transplants.

In a study of pneumococci, Dawson and Avery (1927) found one R strain that could not be reverted to the parent S type by animal passage.

In the previous year Bronfenbrenner, Muckenfuss, and Korb reported an avirulent strain of B. pestis caviae which remained avirulent even

after 200 rapid transfers, and concluded that this irreversible reaction is not a phenomenon of active hereditary immunization but rather a result of irreversible variation.

Julianelle (1928) employing rapid transferring in plain broth and broth containing supernatant serum did not convert R type strains of Friedlander's bacillus to their S types. Animal passage was likewise unsuccessful.

In the same year Dulaney found that the reversal of stabilized rough forms of E. coli was difficult. Todd (1928) reported that glossy colonies (S) of haemolytic streptococci derived from matt colonies (R types from fresh isolated cultures) were permanently attenuated and could not be made virulent by growing in the presence of undiluted normal human serum. The attenuated R colonies, furthermore, returned to virulent cultures upon growth in serum.

Later publications also contributed significant data to the existence of highly stable forms. Leslie and Gardner (1931) working with four phases of B. pertussis (two S and two R) reported that "phase IV", which appeared to be the most advanced R, became "firmly and irrevocably established" in some strains. All other strains were interconvertible.

Kun and Fenyvessy in 1932 found extreme rough cultures of the influenza bacillus, characterized by thread forms and twisted mycelium that remained unchanged for years. Their reversion to smooth colonies could not be accomplished by the use of any of the media most favorable for propogation of smooth phase cultures. Only transient success was obtained by animal passage - reversal to R from the S form being accomplished after a few passages on

chocolate agar.

Further evidence was presented by T'ung in 1940. By irradiation with gamma rays obtained from radon, a change of S to R forms of pneumococcus was regularly induced, the change being so complete that it was "impossible to revert the dissociants to the primary state." The R form appeared suddenly, becoming totally avirulent and irreversible. Animal passage was unsuccessful in reverting the organisms.

Later in 1947, Kelner found a strain of Chromobacterium violaceum, derived from a secondary colony formation on LiCl agar, that was stable for 33 months undergoing at least forty transfers on LiCl free medium.

Won (1950) produced "giant" cells of Pasteurella pestis by treatment with camphor that neither reverted nor dissociated after ten animal passages or fifteen medium passages.

As previously mentioned, in testing quaternary ammonium compounds using the regular F.D.A. phenol coefficient, fluctuations in end titers frequently occur from day to day, even when the same individual performs the test. During the past several years many investigators have studied the quaternary ammonium germicides in this light, and the variations have been attributed to one or more factors related to the compounds or to the test procedure itself. Inconstancy of the peptone composition of the culture media, unequal distribution of the bacteria in the test medium, the "carry-over" of bacteriostatic amounts of the quaternary ammonium compound, and bacterial dissociation of the test organisms have been considered the more important factors.

Numerous modifications of the phenol coefficient method have been devised to overcome the first three factors mentioned, and vigorous controversy has arisen over the results obtained and theories proposed. The factor of dissociation, on the other hand, has received comparatively little attention. Klimek and Umbreit in 1947 stated that variations in the susceptibility of the individuals comprising the population of the inoculum may account for viable organisms after short exposures to the quaternary ammonium germicide. In connection with this proposition is the reference to bacterial dissociation made by Maier and Muller discussed above.

BACTERIOLOGICAL STUDY OF THE CULTURES

A total of 48 stock cultures of Sal. pullorum was received for study. Three of the strains, originally isolated in 1942, were obtained from the culture collection of the Department of Bacteriology and Public Health, Michigan State College. These cultures had been freshly transferred to tryptose agar slants. The remaining 45 strains were obtained from the Poultry Diagnostic Laboratory, Department of Bacteriology and Public Health, Michigan State College. These cultures had been originally isolated from infected chickens or turkeys in July of 1950 and were received freshly transferred on nutrient agar slants. Immediately upon receipt, all the cultures were incubated for 48 hours at 37° C. and subsequently stored at 8-10° C. for future reference.

A preliminary survey of all the stock cultures was made to determine the particular colony characteristic of each strain. The media utilized consisted of tryptose broth and agar and nutrient broth and agar. The tryptose broth was composed of 2% Bacto-tryptose and 0.5% NaCl, while the formula for the nutrient broth was 0.5% Bacto-Beef Extract and 0.5% Bacto-Peptone. For the preparation of the agar, 1.5% plain agar was added to the respective broth formula. All media were adjusted to a pH of 6.9 before sterilization. The broth was dispensed in ten ml. quantities and then autoclaved. The agar plates were prepared in the usual manner using 15 to 18 ml. of agar per plate.

Tryptose broth was seeded first with each stock culture and the tubes were incubated for 24 hours at 37° C. Tryptose agar plates

were streaked from the broth cultures employing a needle streak technique in which approximately 5 mm. of the tip of the inoculating needle, bent at a 30° angle, was dipped into the broth culture and then lightly streaked over the surface of the agar plate making ten to twelve individual streaks. All plates were incubated for 48 hours at 37° C. and subsequently observed as to colony appearance.

Observations of the agar plates were made using a dissecting microscope with a total magnification of 10X. The colonies were illuminated with oblique transmitted light.

To insure against any contamination and eliminate, if possible, any subordinate types, each culture was repeatedly plated by picking representative colonies, subculturing in broth for 24 hours, and subsequently plating as described above. When cultured in tryptose media it was found that 39 of the stock cultures demonstrated typical smooth colonies. The remaining nine cultures were intermediate in form. When representative colonies of each smooth culture were subcultured in nutrient media it was found that all the strains except Nos. 795, 856, and 878 showed a definite change in colonial appearance which persisted on successive platings. Generally the surface of the colonies became granular and wrinkled, taking on a somewhat opaque cast. When these colonies were subcultured again on tryptose media the typical smooth forms reappeared. All nine intermediate cultures produced the same forms on nutrient agar as on tryptose agar. The data are presented in Table I.

It thus appeared that a temporary environmental effect had been

produced with the smooth cultures, and in actuality, the changed forms found on the nutrient agar were not true variants. In any event, the three strains which remained the same on both media were considered to be more suitable for further study. Mallmann (1932) found such strains of Sal. pullorum to be characteristically more stable to various inducement methods.

Prior to the dissociation studies, cultures Nos. 795, 856, and 878 were checked culturally, morphologically, and physiologically to insure their species type. Smears were stained according to Gram's method and fresh nutrient agar stock slants were made from the final pure-line strains. The data are presented in Table 2.

The following studies represent an attempt to evaluate the influence of dissociating agents on these three strains.

METHODS USED TO INDUCE BACTERIAL DISSOCIATION

Experiment I

Various inorganic salts have been frequently reported in the literature as successful agents for producing rough forms in numerous bacterial species. Hadley (1927) demonstrated R forms in members of the paratyphoid group by using lithium chloride.

In this experiment a plain nutrient broth was prepared to which was added lithium chloride in a concentration of one percent. The pH was adjusted to 6.9 and the broth was tubed in ten ml. quantities and autoclaved. Each strain was inoculated into the broth tubes, which were then incubated at 37° C. At various intervals, nutrient agar plates were streaked from each culture and the plates were observed after 48 hours incubation. Weekly transfers were made using one loopful of culture. The experiment was carried through a period of 80 days. The data are presented in Table 3.

Initial platings were not accepted as indicative of a true variant. Typical colonies were picked and subcultured in nutrient broth and replated. If the colony type remained true to form as first observed, the variant was recorded. The results indicate that variants appeared in each strain by 24 days. Several variant forms from strain No. 795 appeared on the agar plates after 20 days and reoccurred intermittently from that time on. All of the forms were generally intermediate, having surfaces that were either granular and slightly veined or coarsely granular to somewhat wrinkled. The margins remained circular and entire.

Strains Nos. 856 and 878, on the other hand, exhibited a different

variant. After 24 days exposure to lithium chloride, colonies which were smaller than the typical smooth forms, decidedly darker, raised, and granular became evident on plates of both strains. By 48 days this variant disappeared on plates from No. 856 and in another eight days the same thing occurred with culture No. 878. Instead, a new form considerably rough in character, appeared in both instances. Refer to figure 1.

It may be noted that typical smooth colonies for each strain persisted throughout the entire 80 day period. Refer to figure 2.

Experiment 2

Various toxic substances and dyes when used in sublethal concentrations have been reported as successful dissociation incitants. Mallmann (1932) utilized the dye brilliant green obtaining R variants with several Sal. pullorum strains. This method was followed in this experiment.

The medium was prepared by adding seven ml. of a one percent aqueous solution of the dye to one liter of nutrient broth. The pH was adjusted to 6.9, the medium was dispensed in ten ml. quantities and sterilized in the autoclave. Strains Nos. 795, 856, and 878 were seeded into the broth and the tubes were then incubated at 37° C. Nutrient agar plates were streaked at various intervals. The data are presented in Table 4. Weekly transfers were made in fresh broth. The experiment was carried on for 81 days.

Intermediate variants appeared from each strain after 12 and 18 days and persisted throughout the rest of the experiment. Typical smooth colonies were observed in each strain up to 32 days, after

which they were no longer noted. Figure 5 is a photograph of the intermediate strain derived from culture No. 795.

With cultures Nos. 856 and 878, the same type of intermediate variant that appeared after exposure to LiCl also appeared in this experiment. However, at no time were any rough colonies observed. It may be noted that with brilliant green as the inciting agent, a shorter time interval elapsed before variants were observed.

Experiment 5

Many workers have commonly observed variants in old stock cultures. Thus, methods of aging have been utilized to recover these forms. Three different broth media were employed in this experiment at incubation temperatures of 20° - 25° C. Nutrient, tryptose, and liver infusion broths were prepared in the usual manner. Tubes were seeded with strains of Sal. pullorum being studied. Nutrient agar streak plates were made at various intervals; the total incubation period being a month. The data are presented in Table 5.

Strain No. 795 exhibited intermediate colonies after 16 days in nutrient broth, eight days in tryptose broth, and 30 days in liver infusion broth. Strain No. 878 demonstrated intermediate colonies after a month on nutrient broth, 16 days in tryptose broth, and a month in liver infusion broth. On the other hand, strain No. 856 remained smooth after a month's incubation in each medium, and also typical smooth colonies prevailed in each culture for the entire length of the experiment. One defined rough form was observed with strain No. 795 after a month in tryptose broth. This colony closely resembled those rough colonial types exhibited by the spore-forming bacilli. The surface was very coarsely granular and the edges were

irregular. However, an interesting phenomenon was noted when such a colony was subcultured in plain nutrient media. It was found that after 48 hours on the agar plate both typical smooth colonies (although in the minority) and the rough variants described appeared. Several successive platings revealed the same thing. Whether these colonies were unstable variants as described by Deskowitz (1936) with Sal. aertrycke, or merely mixed strains, could not be determined.

Experiment 4

Irradiations by X-rays, gamma rays, and ultra-violet light have been reported as successful dissociating incitants. Haberman and Ellsworth (1940) demonstrated the dissociative effects of X-rays on Staphylococcus aureus and Serratia marcescens, producing many variant forms. Grainger (1947), also using X-rays, produced intermediate colony forms from smooth cultures of Sal. typhosa. Ultra-violet light has been shown to produce biochemical variants. This type of irradiation was used here in an attempt to induce changes in the colonial morphology of the selected cultures.

Twenty-four hour nutrient broth cultures of strains Nos. 795, 856, and 878 were smeared on a four square inch surface of a nutrient agar plate with a sterile cotton swab. Two plates were made for each strain in the manner described. The first plate, with the cover removed, was exposed at a distance of four inches from a 15 watt GE germicidal lamp for a period of 30 seconds. The second plate was exposed in the same manner for a period of 60 seconds. A third plate was streaked by the needle technique and left unexposed as the control. The plates were incubated and then observed.

Two variants that were selected for the germicidal sensitivity

tests described below were also exposed to the ultra-violet light. The experiment was repeated twice for all strains with the same results. The data are presented in Table 6. No colonial variants or reverted forms were produced.

GERMICIDAL SENSITIVITY DETERMINATIONS OF SELECTED VARIANTS OF SAL. PULLORUM

Culture Study:

Two cultures of Sal. pullorum were selected for this study. Culture No. 795 demonstrated both typical smooth and intermediate colonies on nutrient agar after 32 days exposure to brilliant green nutrient broth. Culture No. 856 exhibited smooth and rough colonies after 48 days exposure to one percent lithium chloride.

Representative colonies of each form were picked and subcultured in nutrient broth. Successive platings were made to insure the initial stability and purity of each colony type designated. Smears were made and stained according to Gram's method. The biochemical behavior, motility, agglutinability with Sal. pullorum smooth immune serum, and reactivity in physiological saline were tested for each culture. Data are presented in Tables 7 and 8.

It should be emphasized that this preliminary culture study was undertaken to make sure that the smooth organisms isolated were identical not only in colony form, but also physiologically and serologically with the original cultures and species. The variant forms were studied to determine any differences in the nature of the organisms besides the colony appearance.

Cultures Nos. 795 S, 795 SR, and 856 S gave results representative of Sal. pullorum organisms; i.e., dextrose and mannitol were fermented with acid and gas while lactose, dulcitol, sucrose, and maltose were not. Furthermore, the cells were gram negative, non-spore forming rods, and morphologically typical of what is most frequently described. Each culture gave a strong positive reaction with smooth immune serum

and formed stable suspensions in physiological saline.

Culture No. 856 R, on the other hand, differed. While acid and gas were produced in mannitol, only acid was produced in dextrose. Maltose was also reacted upon with acid resulting. The organism appeared to have lost the typical gram's staining characteristic, i.e., while most of the cells were gram negative, some were tending to become gram positive. Morphologically, the cells were uniformily short rods arranged in packets. A granular suspension and sediment were apparent in 0.85% NaCl. When the salt concentration was reduced to 0.21%, a more stable suspension was apparent, although some fine suspended granules were still present. Furthermore, in dilutions of 1-100 and 1-200 of positive smooth immune serum, the agglutinability was somewhat less. It may be noted here that the antigenic suspension of this culture was prepared using a 0.21% NaCl solution. Arkwright (1921) found that rough organisms sometimes formed more stable suspensions when the salt concentration was reduced.

An attempt at reverting the two variants was also undertaken to determine whether the forms typical of the parent strains could be recovered. Nutrient broth cultures of Nos. 795 SR and 856 R were made and at 18 to 20 hours, transfers were made into fresh broth. This method of rapid transferring was continued and nutrient agar streak plates were made from each tube. After four such transfers, strain No. 856 R reverted completely to typical smooth forms. Culture No. 795 SR reverted after nine rapid transfers.

As described in Experiment 4, ultra-violet light had no effect on these cultures. Unfortunately, time was not available for making

a complete study of the reverted forms, although gram's stains demonstrated typical gram-negative rods.

Sensitivity Tests:

The procedure utilized in testing the germicidal sensitivity of the selected cultures consisted of a modification of the F.D.A. phenol coefficient method.

Ten ml. of nutrient broth was seeded from the stock slant and incubated for 24 hours at 37° C. The broth culture was then centrifuged at 2200 R.P.M. for 20 minutes. The supernatant broth was discarded and the cells were suspended in an equal quantity of saline and recentrifuged. This washing procedure was repeated, after which the cells were finally suspended in ten ml. of saline. In the case of culture No. 856 R, a 0.21% NaCl solution was used at all times in this procedure.

The final suspension was shaken for 10 to 15 minutes to insure the dispersal of any clumps. Since some fine granules persisted with culture No. 856 R, this suspension was filtered through sterile Whatman filter paper No. 1 into a sterile test tube. Total cell counts were made of each cell suspension with a Petrauf-Hausser cell counter. Each suspension was adjusted in volume with the appropriate sodium chloride diluent to give a count of 320 million cells per ml. One-half ml. was used as the inoculum for each medication tube.

Nutrient agar plates were streaked from each cell suspension and incubated for 48 hours. These plates served as a control for the colony stability.

The cresolic compound employed and sold under the trade name Lysol,

consists of soap, cresylic acid, and ortho hydroxydiphenyl as the active ingredients in a total concentration of 59%. The Lysol was used in seven aqueous dilutions ranging from 1-300 to 1-600.

The quaternary ammonium compound, sold under the trade name of Phemerol Chloride, consists of an aqueous solution of a para-tertiary-octyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium-chloride-monohydrate. It was used in dilutions ranging from 1-10,000 to 1-70,000 and 1-30,000 to 1-90,000.

Dilutions of phenol (1-90, 1-100, 1-110) served as the standard control. The test was run as otherwise prescribed by the F.D.A. method.

Results:

The data are presented in Tables 9 and 10, and represent results repeatedly obtained for each culture tested against each compound. Using Lysol a phenol coefficient of 5.0 was obtained with each smooth and variant culture, indicating no difference in sensitivity to this cresolic compound.

Considerably different results were obtained with the quaternary ammonium compound. First of all, each culture was more highly sensitive to this compound than to Lysol. Secondly, each strain and its variant differed as to their resistance to Phemerol Chloride. Culture No. 795 S gave a phenol coefficient of 400. Culture No. 795 SR showed a phenol coefficient of 500. Culture No. 856 S showed a phenol coefficient of 650, while culture No. 856 R gave a coefficient of 550. It appeared then that strain No. 856 was more sensitive than strain No. 795.

It may be noted that the resistance to the phenol standard controls was quite constant, as was the case with the expressed resistence of the test organisms to the cresolic germicide Lysol.

DISCUSSION

The first objective of this study was to attempt an isolation of a stable smooth strain of Sal. pullorum, or in other words, an organism which maintained its smooth colonial characteristics despite being exposed to various dissociation methods. Forty-eight stock cultures of Sal. pullorum were initially studied, and of these, three were found to produce the same smooth type of colony when grown on tryptose and nutrient agar. While ultra-violet light had no effect upon these cultures, variants were produced by exposure to lithium chloride and brilliant green. One strain remained stable when aged in different media at room temperature for a period of a month. The other two strains exhibited several variants.

In reality, the problem of stability is of a relative nature. Thus, one can present a new environment to an organism and observe a change in the colonial morphology upon initial plating, while another culture would not show an observable change until after a month's exposure, or still another would not vary at all for a year or more. Wolfram (1951) using Sal. pullorum cultures also obtained from the Poultry Diagnostic Laboratory of Michigan State College, found a number of variants after only three days incubation in plain tryptose broth. On the other hand, the strains this writer used were considerably more stable, although, eventually variants were produced with various incitants. It was further found that by a method of rapid transferring in broth, a typical intermediate and rough variant previously induced reverted to colonies identical in morphology to the original smooth forms. Thus, the variants produced were not of a very stable nature, although they did maintain

their particular characteristics in stock culture and when grown in preparation for the germicidal sensitivity tests.

The second objective of the study was, as mentioned in the Introduction, to determine the sensitivity of the variants to a quaternary ammonium compound. In these determinations the experiments were designed so that a comparison in sensitivity could be drawn between the variants and the original forms to a cresolic as well as a quaternary ammonium compound. The inoculum was standardized in each instance as to the number of cells introduced into each medication tube, and sodium chloride suspensions of the organisms were used to discount any possible interference by the peptone content of the culture medium. Brewer (1943) and others considered the phospholipid content of peptones to be an important factor in interferring with the germicidal action of quaternary ammonium compounds.

In the case of the phenol standards and the cresolic compound, a constant resistance on the part of all the test organisms was apparent. However, this was not the case with the quaternary ammonium germicide. The intermediate variant of one strain, from the results obtained, was more sensitive to the germicidal action of the quaternary ammonium compound than the original smooth culture of the same strain. The rough variant of the second strain, however, was more resistant than the smooth culture of the same strain. Thus, both an increase and decrease in sensitiveness was observed by different variants of the same organism. These results serve to emphasize the possible action of bacterial dissociation in producing

fluctuating and titers when quaternary ammonium compounds are tested according to the phenol coefficient method.

Whether a truly stable organism would show a consistant level of sensitiveness to a quaternary ammonium compound, while reasonable to expect, could not be determined in this study since such an organism was not isolated.

SUMMARY

1. A dissociation study was made of three strains of Sal. pullorum that were originally smooth in colonial morphology on stock culture.

2. Intermediate and rough variants were found to occur with the application of certain dissociation incitants.

3. Ultra-violet light had no effect on the colonial morphology of smooth, intermediate, or rough cultures of the same organism.

4. No observable differences were found to exist in the sensitivity of smooth, intermediate, and rough cultures of Sal. pullorum to phenol and the cresolic compound Lysol.

5. Both an increase and decrease in the resistance to a quaternary ammonium compound Phemerol Chloride was observed to occur with variants of Sal. pullorum, i.e., the intermediate form was less resistant and the rough form was more resistant than the typical smooth culture.

TABLE 1: Colony Appearance of Stock Cultures of Sal.pullorum on Tryptose and Nutrient Agar

Culture Number	Tryptose Agar	Nutrient Agar
13-1	SR	SR
13-2	S	SR
13-3	SR	SR
770	S	SR
775	S	SR
776	S	SR
781	S	SR
793	S	SR
795	S	S
822	SR	SR
827	S	SR
829	S	SR
830	S	SR
843	S	SR
856	S	S
860	SR	SR
866	SR	SR
867	SR	SR
869	S	SR
873	S	SR
874	S	SR
878	S	S
879	SR	SR
880	S	SR
892	S	SR
895	S	SR
897	S	SR
909	SR	SR
911	SR	SR
937	S	SR
940	S	SR
942	S	SR
945	S	SR
949	S	SR
951	S	SR
960	S	SR
962	S	SR
963	S	SR
969	S	SR
980	S	SR
990	S	SR
1005	S	SR
1008	S	SR
1033-2	S	SR
1035-I	S	SR
1040-3	S	SR
1049-2	S	SR
1028	S	SR

S - Smooth
SR - Intermediate

TABLE 2: Morphological and Physiological Characteristics of Smooth Cultures of *Sal. pullorum*

Culture Number	Original Colony	Manni-tol	Lac-tose	Dulci-tol	Suc-rose	Mal-tose	Dex-trose	H_2S	Indol	Motil-ity	Gram's Stain	Morph-ology
795	S	AG	-	-	-	-	AG	+	-	-	-	Typi-cal
856	S	AG	-	-	-	-	AG	+	-	-	-	Typi-cal
878	S	AG	-	-	-	-	AG	+	-	-	-	Typi-cal

AG - Acid and Gas

A - Acid only

Incubation: 14 days at 37° C.

TABLE 3: Effect of One Percent Lithium Chloride on Selected Smooth Cultures of *Sal. pullorum*

Culture Number	Original Colony	Days of Exposure *														
		2	4	8	12	16	20	24	28	32	40	48	56	64	72	80
795	S	S	S	S	S	S	S,SR	S,SR	S,SR	S,SR	S	S	-	S,SR	S	S,SR
856	S	S	S	S	S	S	S	S,SR	S,SR	S,SR	S,SR	S,R	S,R	S,R	S,R	S,R
878	S	S	S	S	S	S	S	S,SR	S,SR	S,SR	S,SR	S,SR	S,R	S,R	S,R	S,R

S - Smooth

SR - Intermediate

R - Rough

* Weekly transfers made in fresh LiCl broth.

TABLE 4: Effect of Brilliant Green on Selected Smooth Cultures of Sal.pullorum

Culture Number	Original Colony	Days of Exposure *													
		2	4	6	12	18	25	32	39	46	53	60	67	74	81
795	S	S	S	S	S,SR	S,SR	S,SR	S,SR	SR	SR	SR	SR	SR	SR	SR
856	S	S	S	S	S	S,SR	S,SR	S,SR	SR	SR	SR	SR	SR	SR	SR
878	S	S	S	S	S	S,SR	S,SR	S,SR	SR	SR	SR	SR	SR	SR	SR

S - Smooth

SR - Intermediate

* - Weekly transfers made in fresh brilliant green broth.

TABLE 5: Effect of Aging on Smooth Cultures of Sal.pullorum in Several Media at 20° - 23° C.

Culture Number	Original Colony	Broth Medium	Days of Aging			
			4	8	16	30
795	S	Nutrient	S	S	S,SR	S,SR
856	S	Nutrient	S	S	S	S
878	S	Nutrient	S	S	S	S,SR
795	S	Tryptose	S	S,SR	S,SR	S,SR,R*
856	S	Tryptose	S	S	S	S
878	S	Tryptose	S	S	S,SR	S,SR
795	S	Liver Infusion	S	S	S	S,SR
856	S	Liver Infusion	S	S	S	S
878	S	Liver Infusion	S	S	S	S,SR

S - Smooth

SR - Intermediate

*R - Rough colony which produced R and S colonies on subculture.

TABLE 6: Effect of Ultra-violet Light on Smooth and Variant Cultures of *Sal. pullorum*

Culture Number	Original Colony	30 sec. Exposure	60 sec. Exposure	Control
795	S	S	S	S
856	S	S	S	S
878	S	S	S	S
795	SR	SR	Slight SR	SR
856	R	R	R	R

S - Smooth

SR - Intermediate

R - Rough

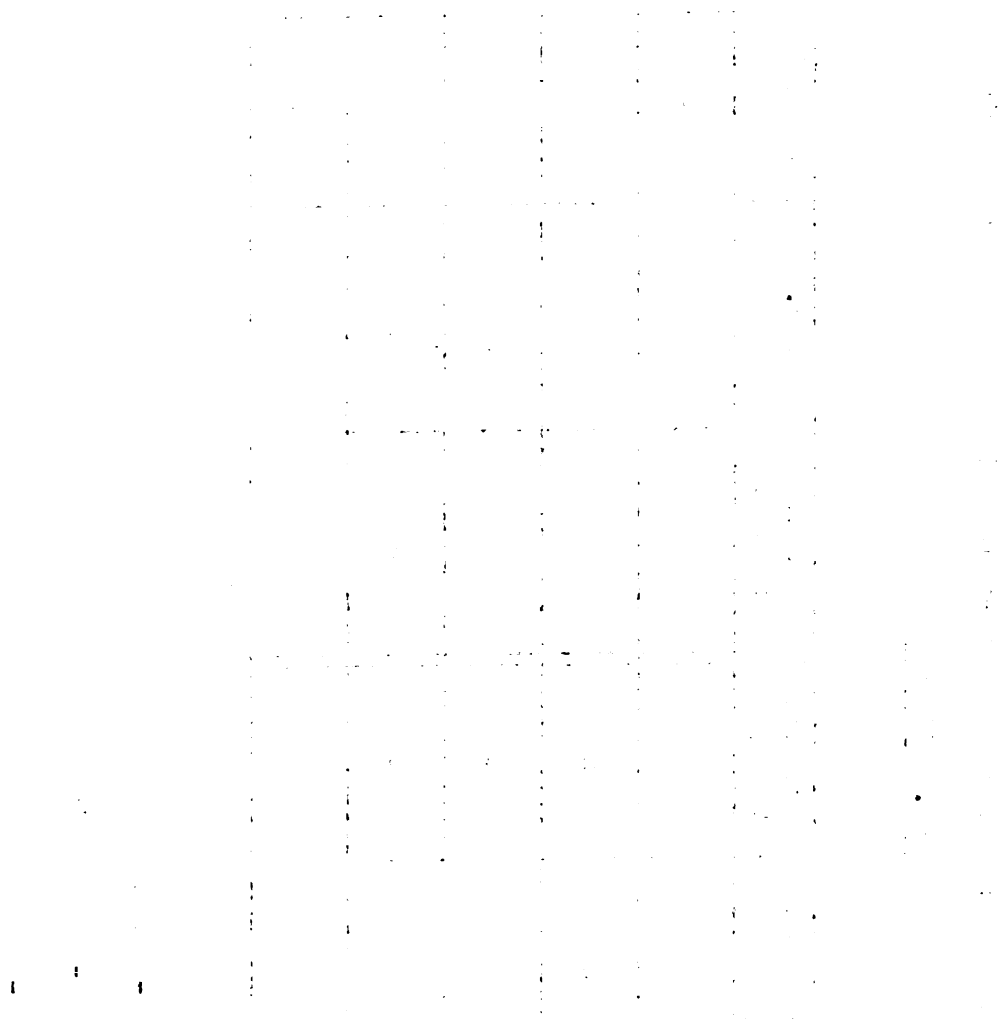

TABLE 7: Morphological and Biochemical Characteristics of Smooth and Variant Cultures of Sal. pullorum Selected for Sensitivity Studies

Culture Number	Original Colony	Manni-tol	Lac-tose	Dulci-tol	Suc-rose	Mal-tose	Dex-trose	H_2S	Indol	Motil-ity	Gram's Stain	Morph-ology	Auto Floccu-lation with 0.85% NaCl
795	S	AG	-	-	-	-	AG	+	-	-	-	Typi-cal	-
795	SR	AG	-	-	-	-	AG	+	-	-	-	Typi-cal	-
856	S	AG	-	-	-	-	AG	+	-	-	-	Typi-cal	-
856	R	AG	-	-	-	A	A	+	-	-	+	Uniform rods in packets	+

AG - Acid and Gas

A - Acid only

Incubation: 14 days at 37° C.

TABLE 8: Agglutinability of Sal. pullorum Cultures with Smooth Immune Serum

Culture Number	Original Colony	1/25	1/50	1/100	1/200	Control
795	S	++++	++++	++++	++++	-
795	SR	++++	++++	++++	++++	-
856	S	++++	++++	++++	++++	-
856	R	++++	++++	+++	+++	-

TABLE 9: The Sensitivity of Selected Cultures of *Sal. pullorum* to Lysol

Culture	Colony	Dilution	Transfer Minutes (5)	(10)	(15)	Phenol Coefficient
795	S	1-300	-	-	-	
		1-350	-	-	-	
		1-400	-	-	-	$\frac{500}{100} = 5$
		1-450	+	-	-	
		1-500	+	-	-	
		1-550	+	+	+	
		1-600	+	+	+	
795	SR	1-300	-	-	-	
		1-350	-	-	-	
		1-400	-	-	-	$\frac{500}{100} = 5$
		1-450	-	-	-	
		1-500	+	-	-	
		1-550	+	+	-	
		1-600	+	+	-	
856	S	1-300	-	-	-	
		1-350	-	-	-	
		1-400	-	-	-	$\frac{500}{100} = 5$
		1-450	+	-	-	
		1-500	+	-	-	
		1-550	+	+	-	
		1-600	+	+	-	
856	SR	1-300	-	-	-	
		1-350	-	-	-	
		1-400	-	-	-	$\frac{500}{100} = 5$
		1-450	-	-	-	
		1-500	+	-	-	
		1-550	+	+	-	
		1-600	+	+	+	
Phenol Standard		1-90	-	-	-	
		1-100	+	-	-	
		1-110	+	+	-	

TABLE 10: Sensitivity of Selected Cultures of Sal. pullorum to Phemerol

Culture	Colony	Dilution	Transfer Minutes (5)	(10)	(15)	Phenol Coefficient
795	S	1-10,000	-	-	-	
		1-20,000	-	-	-	
		1-30,000	-	-	-	$\frac{40,000}{100} = 400$
		1-40,000	≠	-	-	
		1-50,000	≠	≠	≠	
		1-60,000	≠	≠	≠	
		1-70,000	≠	≠	≠	
795	SR	1-30,000	-	-	-	
		1-40,000	-	-	-	
		1-50,000	≠	-	-	$\frac{50,000}{100} = 500$
		1-60,000	≠	≠	-	
		1-70,000	≠	≠	≠	
		1-80,000	≠	≠	≠	
		1-90,000	≠	≠	≠	
856	S	1-30,000	-	-	-	
		1-40,000	-	-	-	
		1-50,000	-	-	-	$\frac{65,000}{100} = 650$
		1-60,000	-	-	-	
		1-70,000	≠	≠	≠	
		1-80,000	≠	≠	≠	
		1-90,000	≠	≠	≠	
856	SR	1-10,000	-	-	-	
		1-20,000	-	-	-	
		1-30,000	-	-	-	$\frac{55,000}{100} = 550$
		1-40,000	-	-	-	
		1-50,000	-	-	-	
		1-60,000	≠	≠	-	
		1-70,000	≠	≠	-	
Phenol Standard		1-90	-	-	-	
		1-100	≠	-	-	
		1-110	≠	≠	-	

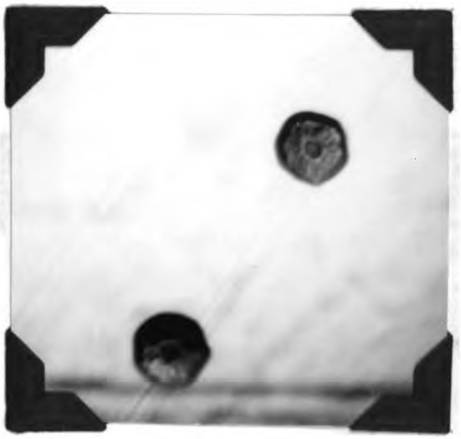

Figure 1. Rough colonies of Sal. pullorum on nutrient agar after 48 hours. Culture No. 856 at a magnification of 3.3.

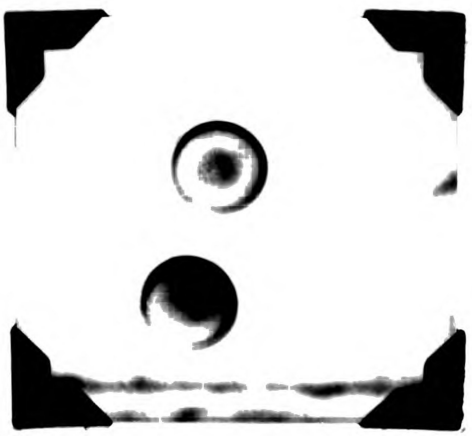

Figure 2. Typical smooth colonies of Sal. pullorum on nutrient agar after 48 hours. Culture No. 856 at a magnification of 3.3.

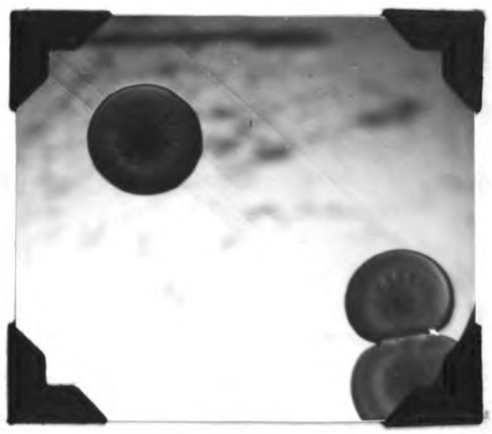

Figure 3. Intermediate colonies of Sal. pullorum on nutrient agar after 48 hours. Culture No. 795 at a magnification of 3.3.

LITERATURE CITED

1. Arkwright, J. A., "Variation in Bacteria in Relation to Agglutination Both By Salts and By Specific Serum", J. Path. and Bact., 24: 36,1921.

2. Baerthlein, K., "Ueber bakterielle Variabilität, inbesondere sogenannte Bakterienmutationen", Centralbe. f. Bakt. u. Parasitenk., Bd. Ixxxi, H.6, S. 369. (As cited by Hadley (1927).)

3. Barber, M.A., "On Heredity in Certain Microorganisms", Kansas Univ. Sc. Bull., 4. : 1,1907.

4. Bronfenbrenner, J., Muckenfuss, R.S., and Korb, C., "Studies on Bacteriophage of d'Herelle. VI. On the Virulence of the Overgrowth in the Lyzed Cultures of B. pestis caviae", J. Exp. Med., 44: 607,1926.

5. Buchanan, R.E., and Traux, R., "Non-inheritance of Impressed Variation in Strep. lactieus", J. Infect. Dis., 7: 680, 1910.

6. Clark, P.F., "The Pseudo-diptheria and Diptheria Bacillus", J. Infect. Dis., 7: 335, 1910.

7. Dawson, M. H., and Avery, O. T., "Reversion of Avirulent "Rough" Forms of Pneumococcus to Virulent "Smooth" Types", Proc. Soc. Exp. Biol. and Med. , 24: 943, 1927.

8. Deskowitz, M. W., "Bacterial Variation As Studied in Certain Unstable Variants", J. Bact. , 33: 349, 1937.

9. DeKruif, P. H., "Dissociation of Microbic Species", Proc. Soc. Exp. Biol. and Med., 19: 34, 1922.

10. Dulaney, A. D., "Microbic Dissociation of B. Coli communis", J. Infect. Dis. , 42: 575, 1928.

11. Gauger, H. C., "Rough Salmonella Pullorum Variants from Chickens", J. Ag. Res., 53: 235, 1936.

12. Grainger, T. H., "The Effects of X-Rays on a Strain of Eberthella Typhosa", J. Bact., 53: 165, 1947.

13. Hadley, P., "Microbic Dissociation", J. Infect. Dis., 40: 1,1927.

14. Hadley, P., "Further Advances in The Study of Microbic Dissociation", J. Infect. Dis. , 60: 129, 1937.

15. Haberman, S., and Ellsworth, L. D., "Lethal and Dissociative Effects of X-Rays on Bacteria", J. Bact. 40: 485, 1940.

16. Jordon, E. O., "The Interconvertibility of R and S Bacterial Types", J. Am. Med. Assc., 86: 177, 1926.

17. Jordon, E. O., and Burrows, W., "Textbook of Bacteriology", 14th Edition, W. B. Saunders Co., Philadelphia, 1945.

18. Julianelle, L. A., "Variations in Cultures of Friedlander's Bacillus", J. Exp. Med. , 47: 889, 1928.

19. Kelner, A., "Secondary Colonies of Bacteria Induced by Salts of Alkali Metals", Am. J. Bot. , 34: 105, 1947.

20. Klimek, J. W., and Umbreit, L. E., "On The Germicidal Behaviour of a Quaternary Ammonium Compound", Soap Sanit. Chemicals, 23: 125, 1947.

21. Kun and Fenyvessy, Zentralbe. f. Bakt., 124: 485, 1932, as cited by Hadley (1937).

22. Lawrence, C. A., "Surface-Active Quaternary Ammonium Germicides", Academic Press Inc., New York, 1950.

23. Leslie, P. H., and Gardner, "The Phases of Haemophilus Pertussis", J. Hyg., 31: 423, 1931.

24. Maier, E. and Muller, E., "Die Wirksamkeit der geleräuchlichen Desinfectionsmittel", Fortschr. Therap., 12: 204, 1936. (As cited by Lawrence (1950).)

25. Mallman, W. L., "The Dissociation of Salmonella Pullorum and Related Species", Agricultural Experiment Station, Michigan State College, Technical Bulletin No. 122, 1932.

26. Morgan, H. R., and Beckwith, T. D., "Mucoid Dissociation in the Colon-Typhoid-Salmonella Group", J. Inf. Dis. , 65: 113, 1939.

27. Nägeli, "Die niederen Pilze in ihren Beziehungen Zu den Infectionskrankheiten und der Gesundheitspflege", Munich, 1877. (As cited by Jordon and Burrows (1945).)

28. Plastridge, W. N., and Rettger, L. F., "Variants of Sallmonella Pullorum", J. Inf. Dis. 50: 146, 1932.

29. Plastridge, W. N., and Rettger, L. F., "An Epidemic Disease of Domestic Fowl caused by a Hitherto Undescribed Organism of Salmonella Pullorum Type", J. Inf. Dis., 47: 334, 1930.

30. Reiman, W. A., "Variations in Specificity and Virulence of Pneumococci During Growth in Vitro", J. Exp. Med. 41: 587, 1925.

31. Ruehle, G. L. A., and Brewer, C. M., U. S. Food and Drug Administration; "Methods of Testing Antiseptics and Disinfectants", U.S. Dept. of Agr. Circ. 198, 1931.

32. Schutze, M., "The Permanence of the Serological Paratyphoid B. with Observations on the Non-Specificity of Agglutination with Rough Variants", J. Hyg. 20: 330, 1921.

33. Todd, E. W., "Further Observations on Virulence of Haemolytic Streptococci with Special Reference to Morphology of the Colonies", Brit. Journ. Exp. Path., 9: 1, 1928.

34. T'ung, T., "Dissociation of Pneumococcus by Radon Irradiation", Proc. Soc. Exp. Bio. and Med., 43: 744, 1940.

35. Wolfram, B. S., "The Dissociation Pattern of Salmonella Pullorum", Thesis for the degree of M.S., Michigan State College, 1951.

36. Won, W. D., "Production of "Giant" Cells of P. pestis by Treatment with Camphor", J. Bact., 60: 102, 1950.

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