AGGLUTINATION STUDIES OF PROTEUS, AND THE EFFECT OF CHEMICAL AND PHYSICAL TREATMENT ON THE ANTIGENIC FACTORS OF REGULAR PULLORUM, VARIANT PULLORUM, AND PROTEUS ANTIGENS

by

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B. A., University of Missouri, 1948

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1951

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INTRODUCTION

Some importance has been assigned in the past few years to the fact that certain organisms possess a common antigenic fraction with Salmonella pullorum. This antigenic relationship causes a certain amount of trouble in routine pullorum testing in that these organisms can cause a false positive pullorum reaction to occur. The first phase of this problem was concerned with a group of organisms that has been reported as having exhibited a cross antigenic relationship with Salmonella pullorum. In this particular problem, the interest was wholly applied to the relationships existing between the Proteus organism and S. pullorum in turkeys. The first experiment dealt with the possible connection between Proteus antigens and sera from turkeys reacting both positively and negatively to the pullorum antigen. The second experiment had to do with the problem of trying to demonstrate a cross antigenic relationship between Proteus and Salmonella pullorum.

In studying the various aspects concerning the antigenic structure of many organisms, it is found that some work has been done in showing the effects of altering the environmental conditions of an organism on its antigenicity. The second phase of this problem was devoted to studying the effects of various chemical and physical treatments on the antigenic fractions of regular and variant pullorum and Proteus organisms, as shown in tube agglutination tests of these organisms with specific antisera.

PART I. AGGLUTINATION STUDIES OF *PROTEUS*

Review of Literature

Among the first to study the serological aspects of the genus *Proteus* were Renner and Rettger (1919) who were attempting to use agglutination as one of the means of classification of *Proteus*. They immunized rabbits with several strains of *Proteus mirabilis* and *Proteus vulgaris*. Using the sera collected from the rabbits, they ran agglutination tests with 73 strains of *Proteus*. They found all but one serum agglutinated the other strains besides those used in their preparation, and some strains were agglutinated by more than one serum.

Thornton (1944) made a group of serological studies of this genus, and discovered that reciprocal cross agglutination tests on flagellar antigens divided 27 *Proteus mirabilis* strains into three groups. Two subgroups within one of the cross agglutination groups were detected by flagellar agglutinin absorption. In testing 35 somatic antigens in their respective sera by reciprocal cross agglutination, six groups were detected. Thornton found it impossible to group all *Proteus mirabilis* strains on the basis of somatic antigens.

Rustigian and Stuart (1945) made a quite detailed study of *Proteus*, both from a biochemical and serological standpoint. Their work revealed a marked antigenic continuity, showing a much more compact picture antigenically than do the members of the

coliform group. They stated, however, that this continuity was not indicative of great numbers of antigenically identical strains within the different species. That except for "X" strains, antigenically identical strains are relatively infrequent within each species. They also found common antigens to occur quite frequently among the different species. West, Scherazo, and Weaver (1946) used 18 strains of Proteus mirabilis to compare their flagellar antigens and determine whether there is any relationship with their flagellar antigens and those of the genus Salmonella. By means of cross agglutination reactions in 10 antisera and agglutinin absorption tests, the 18 strains were placed in six flagellar groups. They could demonstrate, by tube agglutination, no relationship between the flagellar antigens of Proteus mirabilis strains and members of the genus Salmonella. Cole and Kasper (1949) studied in detail the serological relationships of Proteus rettgeri. In their study they used five type strains for each of which they prepared antisera and tested these antisera against 134 biochemically similar strains isolated from fecal specimens. They prepared L, H, and O antigens for each of the strains and 111 were found to possess antigenic components similar to those in the type strains.

From this very brief survey of the serology of Proteus as a group, it is to be observed that, like other members of the enteric group, this genus is fairly diversified antigenically speaking, and that classification from this standpoint would be difficult. The purpose of the first part of this problem is not

concerned with the antigenic relationships of the group as a whole, but with the relationships that might exist between Proteus and Salmonella pullorum. The primary interest in this problem was in the part Proteus plays in nonspecific agglutination in routine testing for pullorum disease in turkeys. In the last 10 years there has been much interest displayed in the study of those organisms, other than S. pullorum, that can cause positive agglutination in pullorum agglutination.

It has been shown by Garrard, McDermott, Burton, and Carpenter (1946) that strains of enterococci and coliform can exhibit nonspecific agglutination reactions with variant and standard pullorum antigens.

Burton and Garrard (1948), in further observations of this problem, inoculated 18 pullets with two Colobactrum and three Paracolobactrum strains isolated from non-pullorum reacting fowl. All the inoculated pullets reacted with variant pullorum antigen. One Paracolobactrum strain reacted with the standard pullorum antigen. These men postulated that organisms common to the intestinal tract of fowl are more commonly being found in other organs where they may cause low grade infection and induce the production of agglutinins strong enough to cause cross reactions with the pullorum antigen.

Carpenter, Burton, and Garrard (1947) inoculated intravenously into a group of fowl an Enterococcus isolated from the liver of a hen exhibiting non-pullorum reactions with the pullorum antigen. Agglutination tests on the various bird's sera showed

that reactions occurred with both the standard and variant pullorum antigens, the majority of reactions being with the variant type antigen, however.

In our work we were more interested in seeing if this nonspecific agglutination occurred in turkeys, as we were to be working with turkey serum. An effort was then made to try to find work done with turkeys in relation to nonspecific agglutination in pullorum testing. Johnson and Pollard (1940) worked with a Gram-positive organism that had been isolated from a turkey whose cultural and morphological characteristics resembled those of lactobacilli in a study of this sort. They showed that agglutinins for Salmonella pullorum were developed on inoculation in rabbits and turkeys.

Sanders, Pomeroy, and Fenstermacher (1943) undertook a study to determine why it is not possible to consistently isolate Salmonella pullorum from turkeys reacting positive to agglutination for pullorum disease. They used five organisms isolated from turkeys, three Salmonella, a Proteus, and Paracolon species for preparing antigens and for inoculation into turkeys. They did cross agglutination tests using S. pullorum serum and antigen, with antigen and antiserum prepared from the five organisms. It was found by them that in every case cross agglutination did take place and to high enough titer as to be called a positive pullorum reaction.

Agglutination Titration of Turkey Sera With *Proteus* Species

Materials and Methods. Four cultures of *Proteus* were selected for use in this study to see whether there exists any antigenic relationship between *Proteus* species and turkey serum. Of the four strains used, *Proteus morgani*, *Proteus rettgeri* were obtained from the American Type Culture Collection. The strain 5473, which was identified as being a *Proteus mirabilis*, was obtained from Dr. L. E. Erwin[1] who isolated it from the liver of a turkey. The strain designated *Proteus* I was obtained from the late Dr. L. D. Bushnell's[2] stock collection. It was originally obtained from Dr. R. Gwatkin[3], and was isolated from the ovary of a chicken. Gram's stain reaction, microscopic examination, and biochemical tests were utilized in testing the purity and identification of each culture. Some of the culture, that was kept on nutrient agar slants, was streaked onto plates of nutrient agar. These agar plates were incubated for 48 hours at 37° C. Smooth colonies were picked from the plates and transferred to tubes of nutrient broth. These transplants were incubated for 24 hours at 37° C. One cc of the suspensions of organisms were used for seeding Blake bottles containing 100 cc of T. G. medium containing

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20 grams peptone, 20 cc glycerine, 10 cc sodium thiosulfate, 10 cc ammonium sulfate, 30 grams agar, and 3 grams beef extract per liter of medium. The seeded Blake bottles were incubated at 37° C. for five days. At the end of the incubation period, the Blake bottles were removed from the incubator, examined for any contamination both macroscopically and microscopically. To those bottles that showed no evidence of contamination were added 20 cc of 0.5 percent phenol in saline by flaming it over the surface of the agar. The bottles were rotated until all growth had been removed from the surface of the agar, and the liquid contents were decanted into sterile 6 oz. bottles. The suspensions of cells were transferred from the 6 oz. bottle to centrifuge tubes and centrifuged for 10 minutes at 3500 rpm in a Serval angle centrifuge. The supernatant from these tubes was removed by a pipette, sterile saline added, and the cells resuspended. These cells were again centrifuged for the same length of time at the same speed as before. The supernatant again removed, sterile saline added to resuspend the cells, and the same centrifugation operation was done. The supernatant was removed and the cells were resuspended with saline with 0.5 percent phenol added as a preservative and kept at refrigerator temperature. These cell suspensions were standardized by the MacFarland nephelometer method to a concentration of 100 times nephelometer No. 1. The pH of the suspension was adjusted to pH 7.5 by the electrometric method.

In the protocol for the agglutination test, tubes were set up as in Table 1 using the four antigens with each sample of

Table 1. Protocol for agglutination tests.

	Tube							
	1	2	3	4	5	6	7	8
Dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Control
Antigen	0.9 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Serum	0.1 ml							

turkey serum. The first tube contained antigen and serum to give a dilution of 1:10 and each of the following tubes contained 0.5 ml of antigen. The two-fold dilution method was carried out in obtaining the above dilutions.

The turkey sera used in the tests were supplied by Dr. L. E. Erwin. These sera were collected from samples of turkey blood sent into the poultry clinic for routine pullorum testing. The sera was stored at 0° C. Sera reacting both positive and negative to pullorum antigen were used.

Infectivity of Turkey Poults with a *Proteus mirabilis* strain

In view of the fact that the Proteus strain designated 5473 showed somewhat of an antigenic relationship with the turkey sera, both negative and positive to pullorum, an additional study was undertaken using this organism with turkey poults to observe if there might be any additional relationships existing between this strain of *Proteus* and *Salmonella pullorum*. The main idea in this study was to determine if, by intramuscular, intravenous, and oral inoculation of this strain of *Proteus* into turkey poults, one could observe an antigenic relationship of *Proteus* with pullorum antigens as shown by cross agglutination reactions. In this study 19 poults were used. Ten of them were used for intramuscular and intravenous inoculation, and nine for oral administration of the organisms. There was a control bird used for each

Table 2. Agglutination of turkey sera with Proteus antigen.

Turkey serum	Antigens			
	Prot. morgani	Prot. rettgeri	5473	Proteus I
	Negative pullorum serum			
1	00000000	00000000	42000000	00000000
2			33300000	10000000
3			33200000	00000000
4			30000000	00000000
5			42000000	31000000
6			31000000	00000000
7			43100000	00000000
8			43000000	00000000
9			30000000	10000000
10			43300000	00000000
11			33100000	10000000
12			30000000	10000000
13			31000000	00000000
14			21000000	00000000
15			32000000	31000000
16			33000000	00000000
17			43000000	00000000
18			43100000	10000000
19			33000000	00000000
20			43200000	00000000
21			32000000	00000000
22			43100000	00000000
23			30000000	10000000
24			10000000	00000000
25			43300000	10000000
26			32000000	21000000
27			22000000	00000000
28			21000000	00000000
29			43300000	10000000
30			10000000	33100000
31			43300000	00000000
32			33200000	10000000
33			43220000	00000000
34			33200000	00000000
35			33000000	10000000
36			10000000	00000000
37			43330000	31000000
38			43000000	10000000
39			43220000	00000000
40			20000000	10000000

Table 2 (concl.).

Turkey serum	Antigens			
	Prot. morgani	Prot. rettgeri	5473	Proteus I
	Positive pullorum serum			
1	00000000	00000000	44330000	00000000
2			44430000	
3			44443300	
4			44300000	
5			43300000	
6			44330000	

4 = Complete agglutination
3 = Almost complete agglutination
2 = Less complete agglutination
1 = Trace agglutination
0 = No agglutination

group. The birds were tested for the presence of titer to pullorum by the rapid plate method, using both a regular and variant antigen. This test was done by placing a drop of the serum and antigen side by side on a piece of glass plate. The serum and antigen were mixed together, the plate warmed slightly, and the reaction observed. A plate agglutination test for the presence of agglutinins to the Proteus strain was done. All birds used showed a negative response both to the pullorum and Proteus antigens. The strain 5473 organisms were grown on nutrient agar slants for 24 hours at 37° C. Gram stains were done on the slants to assure purity of the cultures. The cells were washed from the slants with sterile physiological saline, the suspensions poured

into sterile 15 ml bacterin bottles. The concentration of the suspensions was adjusted to 100 times nephelometer No. 1. Ten turkey poults were inoculated intramuscularly with 1.5 cc of the cell suspension. After a three-day interval, another 1.5 cc of the cells was inoculated intramuscularly into the poults. At the end of a second three-day interval, the poults were inoculated intravenously with 0.5 ml of the organisms. The birds were killed two days after this final inoculation. The blood was collected aseptically as possible, allowed to clot, centrifuged, and the serum collected and used for the agglutination tests. To the nine birds used for oral administration of the organisms, 1 cc of the cell suspension was given by forcing a 1 cc pipette down their esophagus. One cc amounts were given every day for three days. Twenty-four hours after the last administration, the birds were bled, the serum prepared, and agglutination tests performed. Post mortem examinations were done on the birds to note any changes that might have occurred. The control birds were also killed and examined. The results of the agglutination tests on the serum from the 10 poults inoculated intramuscularly and intravenously are shown in Table 3. The agglutination tests on the control bird were negative. The only pathological changes noted in the birds were at the sites of inoculation in the muscle tissue of the legs. There occurred at these sites extensive tissue destruction. This is not surprising when one considers the fact that the Proteus organism is fairly proteolytic. In all cases of the birds inoculated intramuscularly, the organism was recovered from the liver.

Table 3. Intramuscular and intravenous inoculation of turkey poults with *Proteus* strain 5473.

Bird no. serum	Antigen: Homologous	Variant	Regular
252	444444430	42100000	21100000
274	44444330	33200000	11000000
279	444444430	22000000	10000000
287	4444333O	20000000	11000000
271	4444321O	21000000	11000000
241	44444320	32100000	11000000
244	44300000	10000000	00000000
247	44444330	33200000	11000000
251	44444330	22100000	10000000
280	444444430	31000000	11000000

4 = Complete agglutination
3 = Almost complete agglutination
2 = Less complete agglutination
1 = Trace agglutination
0 = No agglutination

The tube agglutination tests on the sera of the poults, which were given the Proteus organisms orally, with the regular variant pullorum antigen and homologous antigen, were negative. The direct smears from the liver tissue on E.M.B., S S, and bismuth sulfite agar plates failed to show the presence of the organisms. The agglutination test and liver smear on the control bird were both negative.

Discussion

In noting the results of the agglutination tests of the 46 samples of turkey serum, with the four Proteus antigens, it is seen that the strain 5473 agglutinated in all the samples of sera, the titer being higher in the case of the serum positive for pullorum. Proteus I, the strain isolated from the ovary of a chicken, agglutinated 17 sera, but in most cases only a trace of agglutination was noted. The antigens of Proteus morgani and Proteus rettgeri failed to agglutinate in any of the sera. These results do not in any way indicate that any definite antigenic relationship exists between this Proteus strain and Salmonella pullorum but that there is some sort of common relationship existing between the turkey sera tested and this strain (5473) of Proteus. The results of the agglutination of the Proteus I strain and the turkey sera are so slight that little importance can be attached to them. The relationship of strain 5473 and the turkey sera is probably a minor one, as indicated by the rather weak

titers obtained in the agglutination tests. Whether this relationship is due to the actual presence of a Proteus organism in the turkeys at one time or another would be difficult to say definitely. This idea would not be too unreasonable to imagine, however, when one realizes that Proteus is fairly widespread in nature and is found in the intestinal contents of many animals. In the poultry clinic at Kansas State College it has been found present in turkeys and in one case was the only organism that could be isolated. Using the idea that the Proteus organism might be fairly widespread in turkeys led the author to believe that if a cross-relationship with pullorum could be demonstrated, a little more evidence might be brought forth in showing the part this organism could play in false positive pullorum reactions. The results of the experiment, shown in Table 3, in which the turkey poults were inoculated intramuscularly with strain 5473, show indication that a slight cross-relationship might exist between this strain of Proteus and the variant form of Salmonella pullorum. The agglutinations of the sera with regular pullorum antigen were so slight as to be disregarded. The attempts to infect the poults by oral administration of the organism were not successful. One might then ask the question as to how the organism would gain entrance into the turkeys if not through the digestive tract. The fact that the poults could not be infected by oral administration artificially would not necessarily eliminate the possibility of their being infected this way under natural conditions existing in the field. One might also suppose that the

Proteus organism exists as a part of the normal intestinal flora and causes infection, and perhaps development of agglutinins which would react with S. pullorum antigens when the bird is suffering from some disturbance, possibly an enteritis, in which the bird's resistance might be lowered somewhat. From the few results obtained here, coupled with results of other workers, it might be said that certain Proteus species have the potentiality of causing false positive pullorum reactions to occur. However, much more work is needed to determine more closely the extent, the part Proteus plays in nonspecific pullorum agglutinations.

PART II. THE EFFECT OF CHEMICAL AND PHYSICAL TREATMENT ON THE ANTIGENIC FACTORS OF REGULAR PULLORUM, VARIANT PULLORUM, AND PROTEUS ANTIGENS

Review of Literature

Ten Broeck (1914) was one of the first to show that chemical alteration of proteins affects their antigenic and immunologic properties. He used racemized egg albumin in his experiments and showed that the albumin thus treated was unable to sensitize guinea pigs, precipitate in homologous egg albumin serum, or to fix complement.

Hsien Wu, Ten Broeck, Chien-Pien Li (1926) using dilute acids, alkalies, alcohol and heat as denaturing agents found that denatured albumin is immunologically different from natural albumin. The denatured albumins were found to be closely related,

although not identical.

Wormall (1930) studied the effects of iodinated, brominated, nitrated, and diazotized proteins and showed that these reactions cause the proteins to lose, more or less completely, the species specificity, and gain a new specificity for the antiserum that has had a similar treatment as the antigen. Johnson and Wormall (1932) were interested in the fact that alkali has more effect on protein antigen than does acid, and led them to try and restore the antigenicity of a protein that had been treated with alkali. They found that iodination did effect a regeneration of the alkali treated protein and that nitration was even more effective in this action.

Horsfall (1934) found that the changes produced in the protein molecule by the action of formaldehyde were sufficient to modify very considerably the immunological characteristics of such proteins.

Much more literature could be cited concerning the effects of chemical treatments on protein antigens. In this problem, the concern is more to see the effects of chemical treatment on bacterial antigens. The literature concerned in this particular phase is not as voluminous, in fact is quite restricted. One of the workers who has been concerned with this type of work was Holmes (1941) who was interested in ascertaining the possible relationships existing between surface tension and the agglutination of antigen particles. She used three classes of surface active agents; alcohols, soaps, and esters. Using suspensions of typhoid

and Staphlococcus organisms as antigens, she found that the soaps and all of the esters but one did inhibit the immunological reaction. The one ester, cetyl pyridinium bromide, exhibited an opposite behavior by decreasing the stability of the antigen and causing spontaneous agglutination in the serums. The alcohols did not alter the serological reactions in any detectable manner. She discovered by detailed studies, including electrophoretic determinations, that the inhibition of serological reactions by surface active agents is due to the increasing of the negative charge on the antigen and not by denaturation or any action on the interfacial tension.

Miller, Becker, Schad, and Robbins (1943) studied the action of heat on the endotoxin of the meningococcus and its destructive effects on the antigenic properties of the cell. They determined these reactions by heating the cell suspensions to temperatures of 50°, 80°, and 100° C. for various lengths of time and using these heated suspensions as antigens in the agglutination tests. They found that heating the cell suspensions markedly reduced the titer of the antigen as far as the agglutination reaction was concerned. They also noted an increase in cloudiness in the heated suspensions which suggested to them that precipitation might have resulted from the interaction of antibody with dissolved antigen which had separated from the cell during heating. They confirmed this idea by doing precipitin tests on the supernatant from the heated cells' suspensions and found a positive precipitin reaction in a dilution of 1:100. These results indi-

cated that some of the loss of agglutinability of the organisms was due to the binding of antibody by dissolved antigen which had gone into solution during heating. There was some reduction of titer due to the heating process, because they found the heated and resuspended meningococci were agglutinated less well than were the controls.

Hayes (1947) was studying the behavior of Salmonella typhi in agglutination reactions and subjected suspensions of cells of this organism to heat, 5 percent HCl, dilute alkali, various concentrations of alcohol including absolute, acetone and chloroform saline. The treated suspensions were tested against serum obtained from a rabbit which had been immunized with a Salmonella typhi strain. He found that heating at a temperature of 56° C. and over qualitatively affected the agglutination by the rabbit serum until, at 75° C., the reaction is completely destroyed at one hour. He found, also, that agglutinability was unaffected by treatment with 5 percent HCl, chloroform, saline, acetone, absolute and 30 percent alcohol. Treatment with 75 percent alcohol reduced the agglutination titer to one-quarter that of an untreated suspension, and treatment with alkali produced variable results, as some suspensions were reduced in agglutinability and others were not.

Orland (1950), in attempting to clarify the subgeneric relationships of lactobacilli, studied the antigenic pattern of this group. He included, besides identification of innumerable antigenic components, the effects of environmental conditions on the

antigen. He subjected cell suspensions of lactobacilli to boiling for two hours, steaming at 15 pounds pressure for 15 minutes and freezing. He also treated the cells with 50 percent and absolute alcohol, N/16 and N/1 HCl, N/10 NaOH for different periods of time and at different temperatures. He found that boiling the cells' suspensions for two hours had no effect on the agglutination of these cells. Likewise, steaming at 15 pounds pressure for 15 minutes or freezing at -7° C. for several days had no effect on agglutinability of the suspensions. He treated the antigen with 50 percent and absolute alcohol for 24 hours at 37° C., and the antigens ability to agglutinate in homologous serum was unaffected. Subjecting the cells to boiling in N/16 HCl for ten minutes did not influence the antigenicity, but he treated them with 1/N HCl for 24 hours at 37° C. and this completely destroyed the antigen. Ten minutes boiling in N/10 NaOH apparently lysed about 60 percent of the cells, but those remaining still manifested their usual antigen as before, although he found there was a slight tendency toward spontaneous agglutination.

The Effects of Treatment on the Antigenic Factors of Regular Pullorum, Variant Pullorum, and Proteus Antigens

Materials and Methods. It has been shown in preceding studies, (Luzzio, 1950), that the antigenic properties of regular and variant forms of Salmonella pullorum can be altered somewhat

by the addition of certain chemical substances to the media upon which the organisms are growing. In this study the author was interested in seeing how the antigenicity of these organisms might be changed if the antigens were subjected to various chemical and physical treatments.

The organisms used for antigens were: a strain of regular pullorum, variant pullorum, and Proteus mirabilis. The cultures of the pullorum organisms were obtained from the Kansas State College Department of Bacteriology collection. The strain of Proteus mirabilis was obtained from a stock collection of Dr. T. H. Lord[1]. The procedures followed in the preparation of the antigens in experiment one were followed in preparing the antigens for this study. However, in arriving at the proper pH of the antigens in this case, phosphate buffer was added to the cell suspensions to give a resulting pH of about 7.5. The three sera used for the antigens included a regular serum, obtained by inoculating a rabbit with killed cell suspensions of a regular strain of Salmonella pullorum, an absorbed and a Proteus antiserum. The absorbed and Proteus antisera were obtained from Dr. Erwin. The absorbed serum had been prepared by absorbing S. paratyphi A var. Durazzo antiserum with S. reading antigen and was specific for the regular strains of pullorum. The Proteus antiserum was prepared by inoculating a horse with the Proteus I strain and was specific for the variant forms of pullorum and Proteus species. The serum

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was stored at 0° C. Each treatment included all three antigens, thus avoiding any differences in time that might affect the comparisons. The treatments of the cell suspensions included steaming with dilute acid (0.01N $HC_2H_3O_2$) for one, three, and six hours; steaming with dilute alkali (0.01N K_2HPO_4) and also untreated, unbuffered cell suspensions for the same time intervals. Extraction of the cells with various concentrations of ethyl, methyl, and normal butyl alcohol, acetone, phenol, urea, trichloracetic acid and 0.01N sodium hydroxide and 0.01N hydrochloric acid. In the steaming treatments, to 5 cc of unbuffered antigen were added 50 cc of dilute $HC_2H_3O_2$ or K_2HPO_4. The suspensions were then subjected to steam for the various time intervals. Upon completion of the treatment, the cell suspensions were removed from the steamer, allowed to cool, centrifuged, washed twice in saline, and resuspended in buffered phenolized saline. The suspensions were then diluted to nephelometer tube No. 1 and agglutination tests done. In using sodium hydroxide and hydrochloric acid, the same procedures were followed, with the exception that unbuffered cells were used. In the treatments using acetone and the various alcohols, the unbuffered suspensions were first centrifuged, then resuspended with the reagent used. These suspensions were allowed to stand for 30 minutes, centrifuged, washed in saline, and resuspended in buffered 0.5 percent phenolized saline. The remaining procedures were the same as were used in the other treatments. The protocol followed in doing the agglutination tests was the same as followed in the first experi-

ment (Table 1). The tubes in the agglutination test were incubated at 37° C. and read at 24 and 48 hours. The titer shown in the tables represents the highest dilution showing complete agglutination.

The steaming of the antigens with 0.01N $HC_2H_3O_2$ for three and six hours completely destroyed the cells. The effects of steaming the suspensions with the dilute acid for one hour are shown in Table 4. The steaming treatment with K_2HPO_4 for one, three, and six hours are shown in Tables 4 and 5. The changes in agglutinability of the steaming treatment of the untreated, unbuffered cells for three and six hours are shown in Table 6. There was no appreciable reduction in reactivity of the unbuffered antigens on steaming them for one hour. Concentrated acetone caused destruction of the cell suspensions, as did treatment with 95 percent ethyl alcohol, 100 percent methyl alcohol, and concentrated N. butyl alcohol. Treatment of the antigens with 25 percent acetone, 20 percent urea, 10 percent trichloracetic acid, and 5 percent phenol caused no reduction in the agglutinability of the cells. The effects of treatment of the antigens with 0.01N HCl and NaOH, and 50 percent methyl and ethyl alcohol are shown in Tables 7 and 8.

Discussion

The bacterial cell functioning as an antigen has been described as having many chemical groups existing in a sort of

Table 4. Comparisons of treated and untreated antigens.

	Antigens untreated			Antigens steamed 1 hour with 0.01N K_2HPO_4		
	Serum			Serum		
	Regular	Absorbed	Proteus	Regular	Absorbed	Proteus
Jones	5120	320	0	5120	160	10
4803	5120	0	320	5120	0	160
Prot. mirab.	0	20	80	0	20	40
	Antigens untreated			Antigens steamed 1 hour with 0.01N $HC_2H_3O_2$		
Jones	5120	320	0	2560	80	10
4803	5120	0	320	2560	0	160
Prot. mirab.	0	20	80	Treatment destroyed antigen		

Table 5. Comparisons of treated and untreated antigens.

	Antigens untreated			Antigens steamed 6 hours with 0.01N K_2HPO_4		
	Serum			Serum		
	Regular	Absorbed	Proteus	Regular	Absorbed	Proteus
Jones	5120	320	0	1280	80	40
4803	5120	0	320	1280	20	80
Prot. mirab.	0	20	80	10	0	20
	Antigens untreated			Antigens steamed 3 hours with 0.01N K_2HPO_4		
Jones	5120	320	0	2560	160	20
4803	5120	0	320	2560	10	160
Prot. mirab.	0	20	80	0	10	40

Table 6. Comparisons of treated and untreated antigens.

	Antigens untreated			Antigens (unbuffered) steamed 6 hours		
	Serum			Serum		
	Regular	Absorbed	Proteus	Regular	Absorbed	Proteus
Jones	5120	320	0	2560	80	0
4803	5120	0	320	2560	0	160
Prot. mirab.	0	20	80	0	10	40
	Antigens untreated			Antigens (unbuffered) steamed 3 hours		
Jones	5120	320	0	5120	160	0
4803	5120	0	320	5120	0	320
Prot. mirab.	0	20	30	0	20	80

Table 7. Comparisons of treated and untreated antigens.

	Antigens untreated			Antigens treated with 0.01N NaOH		
	Regular	Serum Absorbed	Proteus	Regular	Serum Absorbed	Proteus
Jones	5120	320	0	2560	160	10
4803	5120	0	320	2560	0	160
Prot. mirab.	0	20	80	0	20	40
	Antigens Untreated			Antigens treated with 0.01N HCl		
Jones	5120	320	0	2560	160	10
4803	5120	0	320	5120	0	320
Prot. mirab.	0	20	80	0	20	40

Table 8. Comparisons of treated and untreated antigens.

	Antigens untreated			Antigens treated with 50 percent methyl alcohol		
	Serum					
	Regular	Absorbed	Proteus	Regular	Absorbed	Proteus
Jones	5120	320	0	5120	160	0
4803	5120	0	320	5120	0	160
Prot. mirab.	0	20	80	0	20	40
	Antigens untreated			Antigens treated with 50 percent ethyl alcohol		
Jones	5120	320	0	5120	160	0
4803	5120	0	320	5120	0	320
Prot. mirab.	0	20	80	0	20	40

mosaic pattern on the cell. Kabat and Mayer (1948) state that in many Gram-negative organisms are found antigens composed of complex carbohydrates, lipid, protein, or polypeptide-like compounds, known collectively as the Baivin antigen. Kabat and Mayer also indicate that these complex compounds are found in the "O" or somatic fraction of the cell, the same fraction that was considered in this study. In view of the complexity of the known fractions of the cell surface, it is apparent that the simple physical and chemical treatments used in the present study might affect the cells in a variety of ways to cause alterations in their sensitivity as antigens. However, since proteins and carbohydrates differ in the ease with which they are altered or destroyed by treatment, the results obtained allow certain speculations as to the nature of the substance affected.

The steaming treatment with 0.01N $HC_2H_3O_2$ for three and six hours caused a destruction of the cells. It is probable that an actual disintegration of the cell membrane took place due to the combination of the acidity and the steaming on the protein fraction of the cell surface. Steaming the suspensions for one hour in 0.01N $HC_2H_3O_2$ destroyed the Proteus antigen but not the salmonellae; however, a decrease in agglutinability of the salmonellae was noted. The steaming treatments with 0.01N K_2HPO_4 were not as destructive to the cell suspensions as was steaming with the acid. A definite decrease in agglutinability and also in specificity was seen to result from the treatments. The stability of the suspensions when subjected to heat for extended

periods of time might well be attributed to the complex polysaccharide fractions of the cell. The decrease in agglutinability and specificity is consistent with the findings of other workers in studies such as this one. The treatments with concentrated alcohols caused a disintegration of the cells. Alcohols are often used in the extraction of protein compounds; probably the same sort of reaction occurred in this case. Concentrated acetone caused disintegration of the cells, acting perhaps in the lipid fraction of the cells. In more dilute concentrations of the acetone no effects on the agglutinability of the cells were noted.

The treatments using 0.01N NaOH and HCl did cause a decrease in agglutinability of the cells. The NaOH had a more decided effect on the agglutinatility than did the acid. In noting the results of past work, a comparison of the effects of NaOH and HCl on antigens does not show consistent results as to which compound has more effect on the cells. The treatments involving 50 percent methyl and 50 percent ethyl alcohol caused a slight diminution in agglutinability of the antigens. As was stated before concerning the more concentrated solutions of the alcohols, the idea that the protein fraction of the cells being involved in the reaction is offered as a possibility of explanation. In order to be able to study more accurately and explain more fully the changes that take place with the various chemical and physical treatments of the antigens, it would be necessary to actually separate these various fractions of the cell that are responsible for its antigenicity. In this way a study and explanation of the actual re-

actions that occur in these treatments could be made.

SUMMARY

1. In running tube agglutination tests, using four species of Proteus with 46 turkey antisera, both positive and negative for pullorum, it was found that the antigen of a Proteus mirabilis isolated from a turkey agglutinated in all sera, higher titers occurring in the sera positive for pullorum. The antigens from the species of Proteus morgani and Proteus rettgeri failed to agglutinate in any of the sera. Agglutination occurred in 17 of the sera with the antigen of a species of Proteus isolated from a chicken. Positive agglutinations for this organism were, in 13 of the sera, only a trace.

2. Inoculation of 10 turkey poults, intramuscularly and intravenously, using the strain of Proteus isolated from a turkey, caused development in the sera of these poults, agglutinins for variant pullorum antigen in all 10 poults. The sera of 9 poults agglutinated, in a trace, the regular pullorum antigen. A high titer was developed by the sera for the homologous antigen. Oral administration of 9 turkey poults with the Proteus organism failed to cause development of titer against either the homologous organism or the variant or regular pullorum organisms.

3. Steaming, for three and six hours, the antigens of regular and variant pullorum and Proteus mirabilis in 0.01N $HC_2H_3O_2$ destroyed them as did their treatment with concentrated acetone,

95 percent ethyl alcohol, concentrated methyl and N butyl alcohol. Steaming the antigens for one hour in 0.01N $HC_2H_3O_2$, and for one, three, and six hours in 0.01N K_2HPO_4, and three and six hours using untreated and unbuffered cells, caused a decrease in agglutinability of the antigens and in a few cases a decrease in specificity. Treatment of the antigens with 0.01N NaOH and 0.01N HCl, 50 percent methyl and ethyl alcohol for 30 minutes caused a decrease in agglutinability to occur in some of the antigens. Treatment with 10 percent trichloracetic acid, 20 percent urea solution, and 5 percent phenol, had no effect on the agglutinability of the three antigens.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Drs. L. E. Erwin and J. O. Harris for their helpful suggestions and guidance throughout this problem. Grateful thanks are extended, also, to Dr. P. L. Gainey for his assistance in the preparation of this manuscript.

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AGGLUTINATION STUDIES OF PROTEUS, AND THE EFFECT OF CHEMICAL AND PHYSICAL TREATMENT ON THE ANTIGENIC FACTORS OF REGULAR PULLORUM, VARIANT PULLORUM, AND PROTEUS ANTIGENS

by

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B. A., University of Missouri, 1948

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1951

The first phase of this problem was undertaken in an effort to determine the extent of the antigenic relationships that might exist between Salmonella pullorum and Proteus organisms, more specifically as they might exist in routine pullorum testing in turkeys. The first experiment dealt with detection of the antigenic relationships existing between the serum of turkeys which tested both negatively and positively to pullorum. Four strains of Proteus, a P. rettgeri, a P. morgani, and two strains of P. mirabilis, one of which was isolated from a turkey, the other isolated from a chicken, were used as antigens, with the turkey sera, for tube agglutination tests. The strain of P. mirabilis isolated from a turkey agglutinated in all 46 of the sera, the titers being higher in the sera which were positive to pullorum. The antigens of P. rettgeri and P. morgani failed to agglutinate in any of the sera. The strain of P. mirabilis isolated from a chicken agglutinated in 17 of the 46 samples of sera, 13 of these agglutinations found to be present in only a trace. The second experiment of this phase of the problem dealt with an attempt to demonstrate cross reactions between the strain of Proteus isolated from a turkey and regular and variant strains of S. pullorum, by inoculating turkey poults with the Proteus organisms and observing, by tube agglutination tests, cross reactions of the sera from the poults with the S. pullorum antigens. Nineteen poults were used, 10 of which were inoculated intramuscularly and intravenously, and 9 inoculated orally. The sera of the 10 poults inoculated intramuscularly and intravenously with Proteus aggluti-

nated, in all cases, the variant pullorum antigen, and in 9 cases the regular pullorum antigen, the titer being decidedly higher with the variant antigen. There was in all 10 sera a high titer developed against the homologous antigen. Oral administration of the poults with the Proteus organisms failed to cause development, in the sera, of a titer against the homologous antigen as well as for the regular and variant pullorum antigens.

The second phase of this problem was devoted to a study of the effects of treating the antigens of regular and variant pullorum and Proteus organisms in various chemical and physical ways, the results of which are shown in tube agglutination tests with specific antisera. Steaming the antigens for three and six hours in 0.01N $HC_2H_3O_2$ destroyed them as did their treatment with concentrated acetone, 95 percent ethyl alcohol, concentrated methyl and N butyl alcohol. Steaming the antigens one hour in 0.01N $HC_2H_3O_2$, one, three, and six hours in 0.01N K_2HPO_4, and three and six hours using untreated and unbuffered cell suspensions caused a decrease in their agglutinability and in a few cases a decrease in specificity. Treatment of the antigens with 0.01N NaOH, and 0.01N HCl, 50 percent methyl and ethyl alcohol for 30 minutes resulted in a decrease in agglutinability of the antigens. Treatment with 10 percent trichloracetic acid, 20 percent urea solution, and 5 percent phenol had no effect on the antigens as far as their reactivity in tube agglutination tests was concerned.