THE SPECIFIC ANTIGENS OF VARIANTS OF SHIGELLA SONNEI

By EDGAR E. BAKER, PH.D., WALTHER F. GOEBEL, PH.D., AND ELY PERLMAN,* M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 13

(Received for publication, November 29, 1948)

The microorganism which has come to be known as *Shigella sonnei* was probably first isolated by Duval (1) in the United States. Some years later Kruse and his collaborators (2) and Baerthlein (3) independently isolated the same microorganism. It remained for Sonne (4), however, to demonstrate the etiological significance of this member of the dysentery group. The investigations of Sonne were confirmed and extended by Thjøtta (5) and Öhnell (6). The early history of this microorganism has indeed been confused, as emphasized by Bojlén (7) who states: "Certainly no other pathogenic microbe has been 'discovered' so many times as Sonne's bacillus."

Sh. sonnei was early recognized as readily undergoing variation. Baerthlein (3) as well as Sonne (4) and Öhnell (6) observed dimorphism of growth. However, Thjøtta (5) was the first to point out the existence of two characteristic colony types, one of which did not agglutinate with the usual diagnostic antisera. Others (8–18) have also observed this characteristic variation and noted serological differences between the two colony types. Many investigators (11, 12, 15) have found that primary stool cultures are often mixtures of the two, and that the sera of patients suffering from Sonne dysentery infections frequently agglutinate both forms. Koser and Styron (14) were the first to apply the classical Smooth-Rough terminology to these variants of *Sh. sonnei.* The small, raised colony with regular edges described by Thjøtta was termed the S variant, whereas the flat, irregular colony was termed the R variant.

Waaler (17) has made the most extensive study of the dissociation of dysentery bacilli. He described four variants of *Sh. sonnei.* No. 1 was the typical S form; No. 2 grew in rough colonies but was antigenically identical with No. 1. The change from No. 1 to No. 2 appeared to be reversible. From No. 1 and No. 2 there could be obtained a third form, No. 3, which was antigenically different and which was believed to be an R variant. Still a fourth variant, No. 4, was described which was also rough but was antigenically feeble. Waaler also noted the hair tuft colonies described by Thjøtta (5) and questioned whether these colonies were in the process of dissociating from S to R.

Our understanding of the relationship among variants of *Sh. sonnei* has been considerably clarified by the recent work of Wheeler and Mickle (18). These investigators have emphasized that there are probably but three culturally and antigenically distinct types of *Sh. sonnei.* These they termed Phase I (smooth), Phase II, and

* Present address: Mt. Sinai Hospital, New York.

Rough. In the present communication this terminology will be used, with some modification. It should be pointed out, however, that the term *phase* does not have the same significance as in the case of phase variation of the flagellar antigens of the Salmonella group.

Very little is known regarding the chemical nature of the antigens of *Sh. sonnei*. Haas (19) prepared a trichloracetic acid extract of agar-grown bacilli and isolated a toxic substance found only in the smooth form. This substance was capable of eliciting antibodies in dogs which neutralized the toxin and agglutinated smooth bacilli. It gave negative biuret and sulfosalicylic acid, phloroglucinol and orcinol tests, but positive Molisch and ninhydrin tests. The material was not fully characterized.

In the present report the phase variation exhibited by *Sh. sonnei* will be scrutinized. It will be shown that, in the instance studied, variation did not involve a loss in ability of the microorganism to synthesize a somatic antigen, but was associated with a change in the immunological specificity of the latter.

EXPERIMENTAL

Methods

Cultivation of Bacteria.—The cultures used in this study were obtained from the United States Army Medical School and will be described later. The microorganisms were grown in a medium devised by Dole (20). Fifteen liters of the medium (containing 0.05 per cent glucose and 0.05 per cent phenol red) in a 5 gallon pyrex bottle was inoculated in the evening. The following morning 600 ml. of sterile 50 per cent glucose solution was added and the culture stirred mechanically. One ml. of tributyl phosphate was added to prevent foaming. Sterile air, dispersed by passage through a sintered glass disc, was bubbled through the culture at the rate of 500 ml. per minute. Five normal sodium hydroxide was added from a dropping funnel when necessary to maintain neutrality in the culture. Six to seven hours after the addition of glucose growth ceased, though acid production continued. Formalin was then added to a concentration of 1 per cent and the culture allowed to stand overnight at room temperature. The bacilli were then collected in a Sharples centrifuge and dried from the frozen state. The yield was usually about 35 gm. of dry microorganisms per 15 liters of culture.

The use of formaldehyde as a killing agent is open to criticism but it is known that treatment of other dysentery bacilli with this reagent does not alter the toxic or immunological properties of their somatic antigens.

Analytical Methods.—Nitrogen determinations were performed by the usual micro-Kjeldahl method. Phosphorus determinations were carried out according to the procedure of Allen (21). Reducing sugar was determined by the Shaffer-Somogyi method (22). Glucosamine was determined on acid hydrolysates by the method of Sørensen (23). Turbidimetric determinations of serologic activity of antigen preparations were made by means of the turbidimeter devised by Libby (24).

Serological Methods.—Antibacterial sera were prepared by injecting rabbits intravenously with graded doses of formalin-killed bacilli totaling 4.75 ml. of a 24 hour broth culture. Six injections in all were given at intervals of 3 to 4 days. The rabbits were bled on the 7th day after the last injection. Antisera to the somatic antigen preparations were obtained in a similar manner. A total of 850 micrograms was given.

The bacterial suspensions used in the agglutination tests were prepared by killing 18 hour broth cultures with 1 per cent formalin. The sedimented bacteria were washed twice and resuspended in saline to the desired concentration. Occasionally preparations of the rough strain could not be used because of spontaneous agglutination, but most preparations were fairly stable after two saline washings.

The agglutination tests were performed by mixing equal volumes of the serum dilutions and the antigen suspension. The mixtures were incubated for 2 hours at 37°C. and allowed to stand overnight in the ice box. Readings were made the following morning.

Variants of Sh. sonnei

The Phase I culture as received from the United States Army Medical School proved to be a mixture of Phase I and II bacilli.

The Phase I colonies (Fig. 1) are round, raised, with entire edges and have a relatively smooth glistening surface. The colonies attain a diameter of 2 to 3 mm. after 18 to 24 hours incubation. Upon longer incubation many colonies develop irregular outgrowths,—the "hair tufts" of Thjøtta (5). These latter consist of a mixture of Phase I and II bacilli. Growth of the Phase I microorganism in broth is uniformly turbid with relatively little tendency to settle. Agglutination with specific antiserum occurs as coarse clumps which do not break up upon shaking.

Under proper growth conditions, there may be obtained from the Phase I culture two variants which differ markedly in gross colony morphology but which are antigenically identical. The first of these, which we have termed Phase II_R, is identical with the Phase II variant of Wheeler and Mickle (18). When a Phase I culture is streaked on agar, most of the colonies are identical with the parent, but usually a few characteristic Phase II_R colonies are found. This same variant is observed on subculturing the "hair tuft" outgrowth from Phase I colonies. The Phase II_R colonies (Fig. 3) are flat, with an irregular outline and a rather granular although slightly glistening surface. The colonies attain a diameter of 5 to 6 mm. after 18 to 24 hours incubation at 37°C. They may become 1 to 2 cm. in diameter after longer incubation. Growth in broth is uniformly turbid but there will be some settling after 24 hours incubation. Agglutination with specific antiserum occurs as fine clumps which are easily dispersed upon shaking.

The second culture obtained from the Army Medical School proved to be Phase II, but because of its characteristically smooth colony morphology we have named it Phase II_S. Variants of this type probably develop spontaneously by mutation in Phase I cultures but because their colony morphology is so nearly like that of the Phase I variant they cannot be recognized. However, if a Phase I culture be grown in broth containing a small amount of chloroform, colonies of the Phase II_S variant will usually predominate when the culture is plated.

Phase II_S colonies (Fig. 2) are round, raised, with entire edges and a smooth glistening surface. The colonies are somewhat smaller than those of Phase I and attain a diameter of 1.5 to 2 mm. after 18 to 24 hours incubation at 37°C. Growth in broth is uniformly turbid with relatively little tendency to settle. Agglutination with specific antiserum occurs as fine clumps which are easily dispersed upon shaking.

The Rough variant was isolated by streaking on agar the unagglutinated bacilli in the supernatant of a Phase II_S culture which had previously been grown in the presence of homologous antiserum. The Rough colonies (Fig. 4) are similar to those of Phase II_R although the surface is somewhat more granular in appearance. Growth in broth occurs as fine clumps easily broken up upon shaking. There is a tendency for some preparations to show spontaneous agglutination in physiological salt solution.

TABLE I

The Quantitative Estimation of Phase Variation in Cultures of Shigella sonnei Phase I

Mutants found in a series of cultures				Mutants found in samples of a single culture			
Culture No.	Total No. of colonies counted	No. of mutant colonies counted	Per cent mutants	Sample No.	Total No. of bacteria counted	No. of mutants counted	Per cent mutants
1	509	8	1.6	1	541	4	0.74
2	531	10	1.9	2	545	3	0.55
3	536	5	0.93	3	550	4	0.73
4	500	13	2.6	4	539	5	0.92
5	505	7	1.4	5	515	4	0.77
6	540	5	0.93	6	513	2	0.39
7	501	71	14.0	7	534	7	1.3
8	541	4	0.74	8	531	6	1.1

Quantitative Aspects of Phase I to Phase II_R Variation

As pointed out previously, the Phase I culture used in these experiments was unstable; during growth in liquid or on solid media, Phase II_R variants were constantly being formed and possibly Phase II_S variants as well. No satisfactory methods have been developed for quantitative study of the phenomenon. In view of the presence of small amounts of Phase II antibody in all Phase I antisera it was thought desirable to obtain some information concerning the amount of Phase II contamination in Phase I cultures. Luria and Delbrück (25) have proposed the "fluctuation test" for detecting spontaneous mutation. According to this hypothesis, when mutations are rare, the frequency of mutation in each of a series of cultures should vary widely, while the number of mutants in several samples taken from the same culture should be identical within the counting error. This hypothesis is now widely accepted (26).

Observations of this type were made with the Phase I culture. Twenty-five tubes containing 5 ml. of neopeptone-beef heart infusion broth were inoculated with 0.1 ml. of a 10^8 dilution of an 18 hour broth culture inoculated from a single typical Phase I colony. This inoculum, according to a plate count, approximated one organism. After 18 hours incubation eight of these tubes showed growth. Appropriate dilutions of these cultures were prepared in sterile distilled water and 1 ml. distributed over the surface of five neopeptone agar plates (0.2 ml. per plate). The plates were incubated for 18 hours and the total number of colonies and of Phase II_R mutants were then counted. The results of this experiment are presented in Table I.

These and other data not tabulated indicated that almost all the Phase I cultures contained approximately 1 to 2 per cent of Phase II_R mutants. The greater variation in the number of mutants in samples taken from each of several cultures as compared with those removed from a single culture indicated the random distribution of mutation which occurs during growth of the microorganism. Since the original inoculum was so small it is improbable that mutants were present when growth was initiated. In view of the high apparent

TABLE II

Agglutination Reactions of Variants of Shigella sonnei in Homologous and Heterologous Antisera

Antiserum prepared by immunization with:	Antigen	Final dilution of antiserum						
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Phase I bacilli	Phase I bacilli	++++	++++	++++	++++	+	0	0
	" II_S "	++	0	0	0	0	0	0
	" II_R "	+++	0	0	0	0	0	0
	R "	0	0	0	0	0	0	0
Phase II_S bacilli	" I "	0	0	0	0	0	0	0
	" II_S "	++++	++++	++++	++++	+++	0	0
	" II_R "	++++	++++	++++	++++	+++	0	0
	R "	+++	+++	+	0	0	0	0
Phase II_R bacilli	" I "	0	0	0	0	0	0	0
	" II_S "	++++	++++	++++	++++	+++	±	0
	" II_R "	++++	++++	++++	++++	+++	+	0
	R "	++++	++++	+++	+	0	0	0
Rough bacilli	" I "	0	0	0	0	0	0	0
	" II_S "	+++	+	0	0	0	0	0
	" II_R "	+++	0	0	0	0	0	0
	R "	++++	++++	++++	++++	++++	+++	+++

0 = no agglutination.

++++ = complete agglutination with clear supernatant.

mutation rate no attempt was made to apply the statistical computations developed by Luria and Delbrück (25).

Serological Properties of the Variants

The results of agglutination tests with the four variants in homologous and heterologous antisera are presented in Table II.

It will be noted that the Phase I variant agglutinated only in homologous antiserum. The Phase I antiserum used in these experiments was found to contain a small amount of antibody for the two Phase II but not for the Rough variant. It is probable that the presence of Phase II antibody in the Phase I antiserum was the result of the contamination of the Phase I vaccine with Phase II_R mutants, as shown above. This question will be discussed in greater detail later.

The agglutination tests also indicated that the Rough variant was to some extent contaminated with Phase II bacilli. Miller (27) has obtained evidence which substantiates this observation, for she has found that the R culture contained variants resistant to certain of the T series of bacteriophages. Some of these phage-resistant variants agglutinated only with Rough antiserum, whereas others agglutinated only with the Phase II antiserum. These data indicated that the Rough culture was a mixture rather than a single variant bearing a common antigenic component. Whether these variants were present because of the failure to purify the Rough culture by streaking or whether they

TABLE III

Toxicity in Mice of Variants of Shigella sonnei

Variant tested	Mg. bacteria injected		
	20	10	5
Phase I	DDD_{18}	DDD_{18}	$DD_{18}S$
Phase II_S	DDD_{18}	$DD_{18}S$	DDD_{18}
Phase II_R	DDD_{18}	$DD_{18}S$	—
Rough	DDD_{18}	$D_{18}D_{48}S$	$D_{18}SS$

D = death, the numerals representing the hours before death of the animal occurred.
S = survived. All animals observed for 3 days.

arose as a result of reverse mutation from Rough to Phase II is not yet established.

Toxic Properties of the Variants

Because of the difficulty in producing experimental dysentery in laboratory animals, there is but little information concerning the relative pathogenicity of the variants of *Sh. sonnei*. Some information concerning the relative toxicity of the variants for mice has, however, been obtained. These data are presented in Table III.

The results show that all the variants studied had approximately the same toxicity; a finding which differs from those of Hilgers (13) and Weil (28) who found the Phase I organism to be considerably more toxic than the other variants.

The Isolation and Characterization of the Specific Antigens

Isolation of the Phase I Antigen.—The use of 50 per cent aqueous pyridine for the extraction of the somatic antigens of the Flexner dysentery bacilli (29) was unsuccessful when applied to *Sh. sonnei*. Preliminary experiments showed that only traces of Phase I antigen were obtained when the corresponding microorganisms were extracted with this solvent. Good yields of the Phase I antigen

could be obtained, however, by extracting the bacilli with 50 per cent aqueous glycerol. Since preextraction of the microorganisms with 50 per cent pyridine removed considerable amounts of serologically inactive material, together with small amounts of a substance having Phase II activity, it was considered advisable to treat the Phase I organisms with aqueous pyridine prior to this extraction with 50 per cent glycerol.

128 gm. of dry bacilli were extracted twice for 24 hour periods and at 37°C. with 1200 and 800 ml. portions of 50 per cent aqueous pyridine. The bacilli were removed by centrifugation. The wet microorganisms were then extracted twice for 24 hours at 37°C. with 800 ml. portions of 50 per cent aqueous glycerol. After removal of the bacilli by centrifugation at 16,000 R.P.M., the combined glycerol extracts were filtered through a Berkefeld N filter. The filtrate was then dialyzed free of glycerol, concentrated *in vacuo*, and dried from the frozen state. 4.8 gm. of crude antigen was obtained. This material contained 5.11 per cent nitrogen and 3.25 per cent phosphorus.

4.7 gm. of the crude antigen was dissolved in 470 ml. of water and, after chilling, 0.5 volume of cold acetone was added with stirring. After standing overnight in the cold, the precipitate was removed. Acetone was added to the supernatant to give a concentration of 66 per cent. After standing 24 hours in the cold, the precipitate was collected by centrifugation, dissolved in 400 ml. of water, and 1 volume of cold acetone added. After 24 hours in the cold the small amount of precipitate which formed was removed by centrifugation, discarded, and to the supernatant cold acetone was again added to give a final concentration of 66 per cent. This precipitate, containing the active material, was collected by centrifugation, dialyzed, and dried from the frozen state. 1.95 gm. of substance was obtained which contained 6.2 per cent nitrogen, 3.9 per cent phosphorus, and some ribonucleic acid.

1.9 gm. of the above material was dissolved in 75 ml. of 0.01 M borate buffer at pH 7.8 and 4 mg. of crystalline ribonuclease added. The mixture was dialyzed against 2 liters of the 0.01 M borate buffer for 2 days at 37°C. in the presence of toluene. This process rendered most of the nucleic acid dialyzable. After a final dialysis against distilled water for 2 days the contents of the bag were concentrated to 75 cc. by pervaporation. Sodium acetate was then added to a concentration of 0.015 M, the pH adjusted to 7.0, and 1 volume of cold acetone added. After standing in the cold for 24 hours, a small amount of precipitate was removed by centrifugation, discarded, and cold acetone added to the supernatant to give a final concentration of 66 per cent. After 24 hours in the cold this precipitate was collected. The process of purification was repeated. The final precipitate of the Phase I antigen was dialyzed against distilled water, electrodialyzed, and dried from the frozen state. 1.2 gm. of the product was isolated. Several lots of antigen were prepared in the above manner and all had essentially the same chemical and biological properties.

Isolation of the Phase II_S Antigen.—Preliminary experiments showed that some of the type-specific somatic antigen could be extracted from Phase II_S bacilli with 50 per cent aqueous pyridine, but the major part remained within the cell. Fifty per cent aqueous glycerol did not extract any of the antigen. However, it was found that a 7 M solution of urea would extract most of the antigen from bacilli which had previously been treated with aqueous pyridine.

110 gm. of dry Phase II_S bacilli were preextracted with aqueous pyridine as described above. The microorganisms were then suspended in sufficient water so that after the addition of 504 gm. of urea the total volume reached 1200 ml. (7 M urea concentration). The extraction was

continued for 24 hours at 5°C. Higher temperatures did not extract greater amounts of antigen and resulted in the liberation of large amounts of ribonucleic acid. The microorganisms were removed from the urea solution by centrifugation at 16,000 R.P.M. for 30 minutes. The urea extract was filtered through a Berkefeld N filter, dialyzed free of urea, concentrated by pervaporation, and the solution dried from the frozen state. 1.57 gm. of crude antigen containing 6.0 per cent nitrogen, 3.3 per cent phosphorus, and considerable ribonucleic acid was obtained. The ribonucleic acid was eliminated by digestion and dialysis as previously described. From the dialysis sac 1.2 gm. of partially purified substance was obtained. The material contained 4.75 per cent nitrogen and 3.4 per cent phosphorus and a small amount of nucleic acid.

1.1 gm. of the partially purified antigen was dissolved in 110 ml. of 0.05 M sodium acetate and 0.5 volume of cold acetone added. After standing for 1 hour, the precipitate was removed by centrifugation at 15,000 R.P.M. for 30 minutes. This precipitated material, having very little serologic activity, was discarded. Sufficient cold acetone was added to the supernatant to give a concentration of 50 per cent. The precipitate was collected by centrifugation after

TABLE IV

Toxicity in Mice of the Specific Antigens of Phase I and II_S Shigella sonnei

Antigen tested	Micrograms of antigen injected				
	2000	1000	500	250	125
Phase I	DDD_{18}	$DD_{18}D_{24}$	$D_{18}D_{24}S$	$D_{18}SS$	SSS
Phase II_S	DDD_{18}	DDD_{18}	$D_{18}SS$	$DD_{18}S$	$D_{18}SS$

standing overnight in the cold. The material was dissolved in water and again precipitated at 50 per cent acetone concentration. The final precipitate was collected by centrifugation, dialyzed against distilled water, electrodialyzed, and dried from the frozen state. 0.56 gm. of purified antigen was obtained. Various preparations have been made by the above procedure and all have essentially the same properties; these will be described below.

The Toxic and Serologic Properties of Phase I and II_S Antigens.—That the specific antigens of Phase I and II_S, *Sh. sonnei* had toxic properties comparable to those of the somatic antigens isolated from Flexner dysentery bacilli (30) is evident from the experiments recorded in Table IV. Inspection of the data presented in Table IV shows that the LD_{50} for mice lay between 250 and 500 micrograms. The antigens of both variants were about equally toxic.

Antisera obtained from rabbits injected with the two purified antigens showed a high degree of specificity as can be seen from Table V. It will be noted, with one exception, that there was no evidence that the antisera obtained from rabbits injected with the purified Phase I and II antigens exhibited cross-reactions. The Phase II_S antiserum, at a dilution of 1:10, gave partial agglutination of the Rough variant, which may not, however, have been specific.

In Table VI are presented data on precipitin tests conducted with the Phase I and II_S antigens and the antisera of rabbits immunized with whole bacilli and with the two purified antigens. These tests show that in each instance the

purified antigens reacted specifically in the homologous immune sera, whether the serum was obtained from animals injected with whole bacilli, or with the highly purified lipocarbohydrate-protein complex. It is noteworthy that no cross-precipitin reactions were observed.

The Chemical and Physical Properties of Phase I and Phase II_S Antigens.— The antigens obtained from Phase I and II_S *Sh. sonnei* both gave positive biuret and Molisch tests. They were soluble in hydrochloric, acetic, and trichloracetic acids, as are the somatic antigens of the other dysentery bacilli.

TABLE V

Agglutinins in Sera of Rabbits Immunized with Specific Antigens of Shigella sonnei Phase I and II_S

Antiserum prepared by immunization with:	Micro-organism tested	Final dilution of antiserum									
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Phase I antigen	Phase I	+++	++++	++++	++++	++++	++++	+++	±	0	0
	Phase II_S	0	0	0	0	0	0	0	0	0	0
	Phase II_R	0	0	0	0	0	0	0	0	0	0
	Rough	0	0	0	0	0	0	0	0	0	0
Phase II_S antigen	Phase I	0	0	0	0	0	0	0	0	0	0
	Phase II_S	++++	++++	++++	++++	++++	++++	++++	++++	+++	0
	Phase II_R	++++	++++	++++	++++	++++	++++	++++	++++	+++	0
	Rough	++	0	0	0	0	0	0	0	0	0

The gross analytical properties of the two antigens were not strikingly different as shown by the data presented in Table VII.

The Phase I and II_S antigens had similar nitrogen and phosphorus contents. The reducing sugar values, calculated as glucose, are maximum values obtained after 4 hours hydrolysis with 2 N HCl at 100°C. Longer heating or higher acid concentrations resulted in considerably lower reducing sugar values. The analytical values presented for glucosamine were obtained by colorimetric analysis (23) after hydrolysis of the antigens for 18 hours at 100°C. with 6 N HCl. In view of the low values for glucosamine, it is possible that this saccharide was actually not present in the original substances and that the color developed was due to some other substance in the hydrolysis mixture. Actual isolation of glucosamine hydrochloride or a derivative is necessary in order to establish the presence of this sugar.

When the antigens were hydrolyzed with 0.1 N acetic acid for 4 hours at 100°C. (31), they split into an acid-insoluble protein and an acid-soluble carbohydrate fraction. In this respect they resemble the lipocarbohydrate-protein complexes obtained from other dysentery bacilli. There has not been sufficient material available for the direct isolation of the lipid fraction of these antigens, but their physical and chemical properties were so characteristic that they can probably be safely classified as typical lipocarbohydrate-protein complexes.

The ultraviolet absorption spectra of the two antigens were measured with the Beckman model DU quartz spectrophotometer using solutions of 0.02 per cent concentration and an optical depth of 1 cm. The fact that neither prepa-

TABLE VI

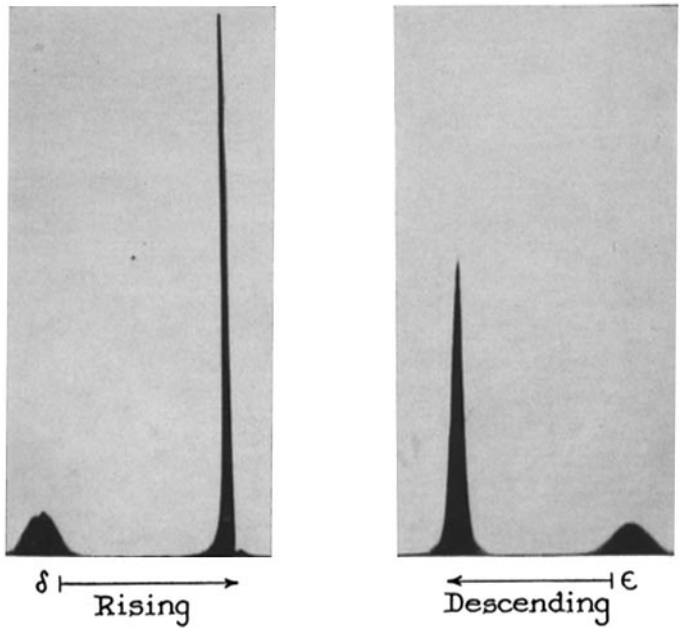
Precipitins in Sera of Rabbits Immunized with the Specific Antigens of Shigella sonnei Phase I and II$_S$

Antiserum prepared by immunization with:	Antigen tested	Final dilution of antigen			
		1:2000	1:10,000	1:50,000	1:250,000
Phase I antigen	Phase I	+++	++++	++++	++
	Phase II$_S$	0	0	0	0
Phase II$_S$ antigen	Phase I	0	0	0	0
	Phase II$_S$	++++	++++	++	±

TABLE VII

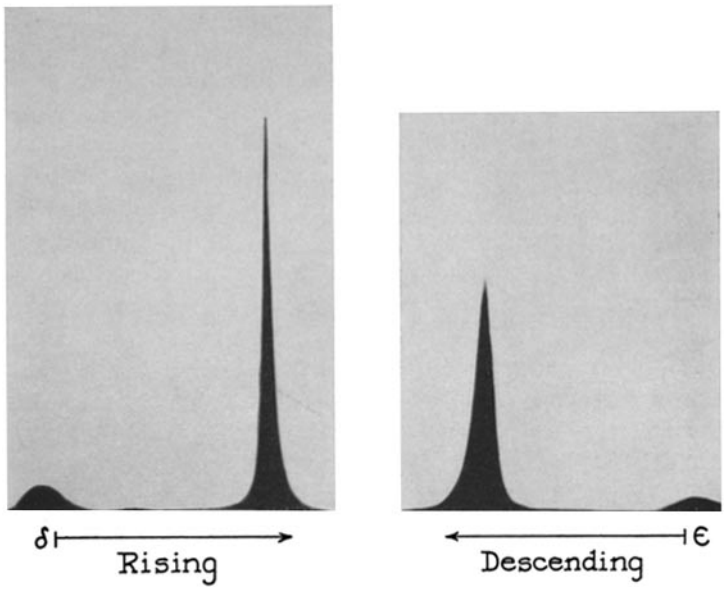
Analyses of the Specific Antigens of Phase I and II$_S$ Shigella sonnei

Antigen	N	P	Reducing sugar after hydrolysis	Glucosamine
	per cent	*per cent*	*per cent*	*per cent*
Phase I	6.0	2.1	14.5	3.04
Phase II$_S$	5.45	3.1	23.4	4.19



TEXT-FIG. 1. Electrophoretic pattern of purified antigen obtained from *Shigella sonnei* Phase I.

ration showed a definite peak at a wave length of 260 millimicrons indicated that the substances were uncontaminated with nucleic acid. Electrophoretic analyses of the two antigens were made in the Tiselius apparatus using the Longsworth scanning method. One per cent solutions of the antigens in veronal buffer at pH 8.6 and an ionic concentration of 0.1 were employed. The patterns obtained are shown in Text-figs. 1 and 2. It is evident from the accompanying figures that both antigens were homogeneous at this pH value. The mobilities were 3.2 $\times$ 10^{-6} cm.^{2}/volt sec. for the Phase I antigen and 7.7



TEXT-FIG. 2. Electrophoretic pattern of purified antigen obtained from *Shigella sonnei* Phase II_S.

$\times$ 10^{-6} cm.^{2}/volt sec. for the Phase II_S antigen. While it would be desirable to make determinations at other values of pH, these data are considered sufficient to show the electrical properties of the two antigens.

DISCUSSION

That *Sh. sonnei* undergoes variation in a predictable manner is apparent from the data presented in this communication. The Smooth or Phase I microorganism readily dissociated to yield two well defined variants which have been termed Phase II_S and II_R. The latter were quite stable and were serologically identical. They differed from one another in their colonial form and from the parent microorganism in their immunological specificity. Prior to the re-

searches of Wheeler and Mickle (18), the Phase II_R variant was considered to be a Rough form of *Sh. sonnei*, but these investigators found that still another form could, under certain conditions, be obtained from their Phase II variant. Because of its cultural characteristics, the term Rough was assigned to this variant. The relatively rare occurrence of the Rough microorganism accounts for the failure of its earlier recognition. Although other colony forms of *Sh. sonnei* have been described (16, 17), they have not been encountered in this laboratory. In general, it would appear that the variants of *Sh. sonnei* most frequently encountered are those which have been termed Phases I, II (II_S and II_R), and Rough.

The data presented show that when *Sh. sonnei* undergoes variation the immunological change which occurs does not involve the loss of ability of the microorganism to elaborate a somatic antigen. The variant has acquired a new specificity yet it is still capable of carrying out the biochemical synthesis of a substance comparable in its gross chemical composition to that characteristic of the parent cell. In fact, the variation of *Sh. sonnei* may well involve only changes in the enzyme systems responsible for the synthesis of the somatic antigen. This change is reflected in a new and distinct serological specificity of the latter. It is probable that a detailed chemical study of the two antigens will reveal the nature of the differences responsible for the change in serological specificity. That these differences may well reside only in the chemical structure of the hapten components is not beyond the realm of possibility.

It will be observed from Table II that the antisera of rabbits injected with cultures of Phase I bacilli invariably showed some serological crossing with Phase II microorganisms. It is our opinion that this phenomenon occurred not because the Phase II antigen is an integral constituent of Phase I bacilli, but because the cultivation of the latter is accompanied by the production of small numbers of Phase II bacilli which arise through mutation. It has been clearly demonstrated that such mutations occur and hence the evidence is strong in support of this view. If, as it exists in the bacterial cell, the Phase I antigen contains a group which showed serological crossing in Phase II antisera, then the purified antigen should likewise show serological cross-reactions. This is not the case, however, for it has been demonstrated that the purified Phase I antigen is immunologically specific and gives rise to antibodies which show no serological crossing with Phase II antigen.

Phase variation has been shown to occur among bacilli of the dysentery group other than *Sh. sonnei*. Takita (32) studied a strain of Flexner Type V in detail, and found that two types of colonies could be isolated which were identical in gross morphology but were serologically distinct. One type, V_a, invariably bred true. The other, V_b, upon subsequent cultivation gave rise both to V_a and V_b variants. Serological tests showed that antisera prepared by injecting rabbits with cultures of the V_b variant contained antibodies which

agglutinated both V_a and V_b organisms. Antisera to the V_a variant, on the other hand, contained only homologous antibody. Boyd (33) and Weil, Farsetta, and Knaub (34) have confirmed and extended Takita's observations with other Flexner types.

All these investigators believed that the *a* variant represented a "degraded" Type V which had lost the *b* antigen, whereas the *b* variant contained both antigens. In view of the data presented in the present report, it is suggested that V_a represents a stable variant comparable to *Sh. sonnei* Phase II_S, and that V_b represents the smooth variant, cultures of which, due to mutation, contain both V_b and V_a variants. An antiserum prepared by immunization of animals with V_b bacilli would contain both antibodies, not because of the presence of a common antigen but because the V_b vaccine used would always contain some V_a bacilli arising through mutation.

Whether or not this phase variation of the Flexner bacilli is similar in nature to that of *Sh. sonnei* requires further investigation. The data presented here emphasize the need to establish the composition of vaccines used for the preparation of antisera. If a Smooth culture is undergoing mutation, a vaccine prepared from it will contain bacilli of the variant. The resulting antiserum, in addition to the type-specific antibody, will contain antibodies directed against the variant. The interpretation of data based upon the use of such antisera may lead to unwarranted assumptions.

SUMMARY

It has been shown that phase variation of *Sh. sonnei* is accompanied by changes in morphology and antigenic structure. Two mutants of the Phase I organism (II_S and II_R), which were studied, elaborate somatic antigens which are immunologically identical. The purified lipocarbohydrate-protein antigens of the Phase I and II_S microorganisms are chemically similar yet immunologically distinct and specific. By inference the same should hold true of the antigen of II_R, but it has not yet been investigated in this relation.

It is suggested that when *Sh. sonnei* undergoes variation from Phase I to II_S the immunological changes occurring are dependent upon a change in the enzyme systems responsible for the synthesis of the lipocarbohydrate-protein constituent.

BIBLIOGRAPHY

1. Duval, C. W., *J. Am. Med. Assn.*, 1904, **43**, 381.
2. Kruse, Rittershaus, Kemp, and Metz, *Z. Hyg. u. Infektionskrankh.*, 1907, **57**, 417.
3. Baerthlein, *Berl. klin. Woch.*, 1912, No. 1, 735.
4. Sonne, C., *Centr. Bakt., 1. Abt., Orig.*, 1915, **75**, 408.
5. Thjøtta, T., *J. Bact.*, 1919, **4**, 355.
6. Öhnell, H., Kliniska och Bakteriologiska Bidrag till Kannedom om Dysenterien i Sverige, Dissertation, Stockholm, 1918, cited by Bojlén (7).

7. Bojlén, K., *Comm. Inst. Serotherap. État Danois*, 1934, **24,** 1.
8. Øerskov, J., and Larsen, A., *J. Bact.*, 1925, **10,** 473.
9. Elkeles, G., and Schneider, A., *Centr. Bakt., 1. Abt., Orig.*, 1927, **103,** 48.
10. Leuchs, J., and Plochmann, E., *Centr. Bakt., 1. Abt., Orig.*, 1927, **104,** 347.
11. Braun, H., and Weil, A. J., *Centr. Bakt., 1. Abt., Orig.*, 1928, **109,** 16.
12. Braun, H., and Baake, F., *Centr. Bakt., 1. Abt., Orig.*, 1930, **116,** 462.
13. Hilgers, P., *Centr. Bakt., 1. Abt., Orig.*, 1929, **114,** 320.
14. Koser, S. A., and Styron, N. C., *J. Infect. Dis.*, 1930, **47,** 443, 453.
15. Thjøtta, T., and Waaler, E., *J. Bact.*, 1932, **24,** 301.
16. Dienst, R. B., *J. Bact.*, 1933, **26,** 489.
17. Waaler, E., Studies on the dissociation of the dysentery bacilli, *Skrifter Norske Videnskaps-Akad. Oslo, I. Mat. Naturv. Kl.*, 1935, No. 2.
18. Wheeler, K., and Mickle, F. L., *J. Immunol.*, 1945, **51,** 257.
19. Haas, R., *Z. Immunitätsforsch.*, 1938, **94,** 239.
20. Dole, V. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **43,** 122.
21. Allen, R. J. L., *Biochem. J.*, 1940, **34,** 858.
22. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100,** 695.
23. Sørensen, M., *Compt.-rend. trav. Lab. Carlsberg, série chimique*, 1938, **22,** 487.
24. Libby, R. L., *J. Immunol.*, 1938, **34,** 71, 269; 1938, **35,** 289.
25. Luria, S. E., and Delbrück, M., *Genetics*, 1943, **28,** 491.
26. Luria, S. E., *Bact. Rev.*, 1947, **11,** 1.
27. Miller, E., unpublished data, 1948.
28. Weil, A. J., *J. Immunol.*, 1947, **55,** 363.
29. Goebel, W. F., Binkley, F., and Perlman, E., *J. Exp. Med.*, 1945, **81,** 315.
30. Perlman, E., and Goebel, W. F., *J. Exp. Med.*, 1946, **84,** 223.
31. Boivin, A., and Mesrobeanu, L., *Rev. Immunol.*, 1935, **1,** 553.
32. Takita, J., *J. Hyg.*, Cambridge, Eng., 1937, **37,** 271.
33. Boyd, J. S. K., *J. Hyg.*, Cambridge, Eng., 1938, **38,** 477.
34. Weil, A. J., Farsetta, K., and Knaub, V., *J. Immunol.*, 1946, **52,** 221.

EXPLANATION OF PLATE 13

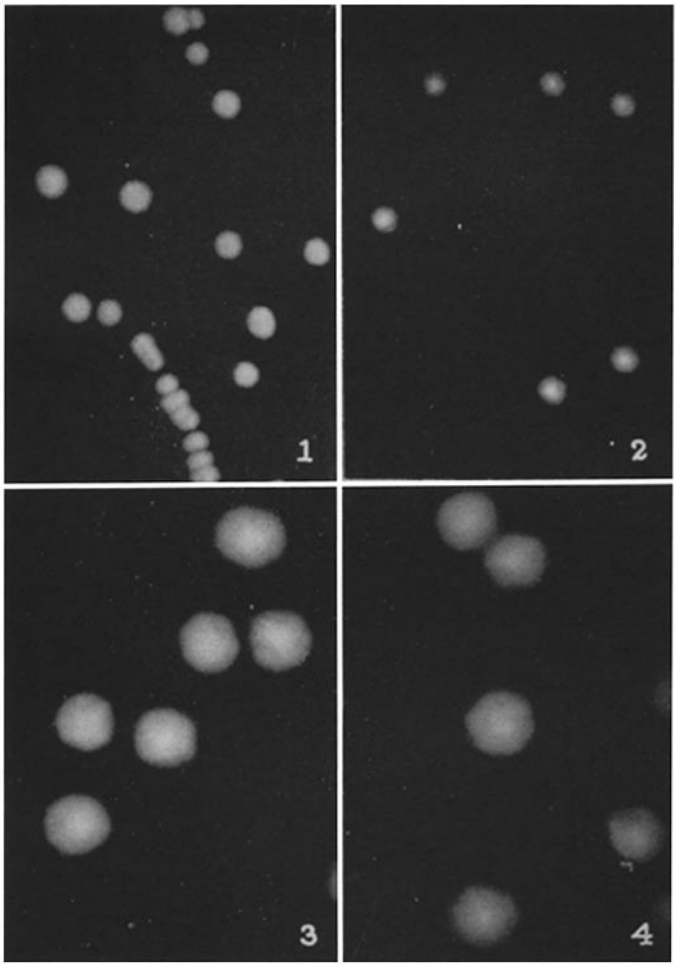
Colony morphology of the variants of *Shigella sonnei*. Cultures were grown for 24 hours on neopeptone-beef heart agar. × 3.25.

FIG. 1. *Sh. sonnei*, Phase I—round, raised glistening colonies, 2 to 3 mm. in diameter.

FIG. 2. *Sh. sonnei*, Phase II_S—colony size slightly smaller than Phase I and more translucent.

FIG. 3. *Sh. sonnei*, Phase II_R—flat, irregular colonies with granular but slightly glistening surface, 5 to 6 mm. in diameter.

FIG. 4. *Sh. sonnei*, Rough—similar to Phase II_R but with more granular surface.



(Baker *et al.*: Specific antigens of *Shigella sonnei* variants)