THE SOMATIC ANTIGEN OF A PHAGE-RESISTANT VARIANT OF PHASE II SHIGELLA SONNEI

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The type-specific somatic antigen of Phase II *Shigella sonnei* is a lipomucoprotein complex which has the ability to inhibit *in vitro* all the T *coli*-dysentery phages to which this microorganism is known to be susceptible (1). Because of this it has been suggested that the antigen, which is present on the cell surface of the Phase II bacillus, serves as the receptor for the T viruses which attack this particular dysentery bacillus (2).

In order that a phage may combine specifically with a susceptible bacterial cell it is essential that sufficient binding forces be available between the two structures. Since the geometric pattern of these structures is determined by the chemical nature of the substances distributed on the surface of both the virus and the cell, it is reasonable to assume that combination can occur only if these active groups possess a precise spatial relationship. If the chemical nature of the cell surface were to undergo change, the relationship of these forces would be disturbed and the virus might no longer be capable of combining with the cell in question.

If this hypothesis has any basis in fact, it should be possible to demonstrate differences, both chemical and immunological, between the antigen of the Phase II organism and that of a phage-resistant variant. Such a variant has been isolated; it was obtained by plating a large population of susceptible Phase II bacilli with a mixture of T_3, T_4, and T_7 phages and selecting a resistant colony. This variant, designated as II/3, 4, 7 because of its resistance to these three phages, is the subject of the study which follows. It will be seen that this bacillus, like the cell from which it was derived, also elaborates a lipomucoprotein, but that this latter differs from that of the parent in serological specificity, antiviral properties, and chemical make-up.

Materials and Methods

Strains of Microorganisms.—The Phase II bacillus used was obtained some years ago from the United States Army Medical School collection. It has been kept in a frozen state (−78°C.) in 50 per cent horse serum. The phage-resistant variant, II/3, 4, 7, was isolated as described in the experimental procedure.

Bacteriophages and Phage Assays.—The bacteriophages employed were obtained from Dr. Mark Adams of New York University. Freshly prepared stocks of the various viruses

were used throughout this work and were obtained in the usual manner by passage on *Escherichia coli* B. Phage assays were conducted according to the technique of Hershey (3).

Media.—Both Difco nutrient broth and 1 per cent neopeptone–meat extract media were used. Solid media were prepared by the addition of 1.5 per cent Difco agar. The somatic antigen of the variant II/3, 4, 7 was obtained from organisms grown at pH 7.0 in a medium containing 1 per cent casamino acids and dialyzed meat extract (4).

Inhibition Tests.—Viral inhibition tests were conducted as previously described (2). Platings of the T_7 virus were made on neopeptone–meat infusion agar because this medium contains cofactors of unknown nature which give a somewhat larger number of plaques than when platings are made on nutrient broth agar.

Antisera.—Antibacterial sera were obtained from rabbits which had received multiple intravenous injections of living Phase II bacilli, or the variant II/3, 4, 7. In order to minimize the toxic effects of the microorganisms, it was advantageous to administer the first injection intradermally; 0.2 ml. of living bacilli containing 2×10^{10} micoorganisms per ml. was therefore given as the first dose. 5 days later, 0.1 ml. of 1×10^8 bacilli per ml. was injected intravenously; the injections were made on alternate days thereafter, and the dose was doubled each time, until 1.6 ml. had been given. Two series of injections, with a rest period of 1 week between courses, were administered. Bleedings were made 8 days after the last injection.

Antisera to the two somatic antigens, that from the Phase II bacillus, and from the variant II/3, 4, 7, were obtained by injecting two separate groups of rabbits with sterile saline solutions of these substances. Here again it was desirable to administer as the initial dose 0.2 ml. of the antigen solution containing 5 mg. per ml., intradermally. The first intravenous dose, containing 0.05 mg. of antigen, was given 5 days later. After administering a total of some 2.0 mg. of antigen intravenously, the animals were bled. The anti-sera thus obtained, agglutinated, in high dilution, the homologous microorganisms, and gave potent precipitin reactions with the respective antigens.

Somatic Antigens.—The somatic antigen of Phase II *Sh. sonnei* was prepared as outlined in the previous communication (1). The preparation of the antigen of the variant, II/3, 4, 7, will be described in the experimental procedure which follows.

Agglutination and Precipitin Tests.—Agglutination tests were conducted in the ordinary manner; it was necessary to use young living cultures of II/3, 4, 7 because the organism has a tendency to agglutinate spontaneously if the cultures are more than 4 to 5 hours old. The vaccines contained about 2.5×10^8 organisms per ml. A broth suspension of the bacteria was used and the antisera were also diluted in nutrient broth. The tubes were read after incubation at 37°C. for 2 hours. In all instances control experiments utilizing similar dilutions of the corresponding normal sera of the animals, were included in the tests. Any rabbit whose normal serum spontaneously agglutinated the phage-resistant variant II/3, 4, 7, was discarded before the immunization schedule was begun.

Analytical Methods.—Nitrogen determinations were performed by the procedure of Koch and McMeekin (5), phosphorus by the method of Allen (6), and glucosamine by the technique of Sørensen (7). Quantitative turbidimetric serological assays were performed as described by Libby (8). Reducing sugars were determined by the method of Shaffer and Somogyi (9).

EXPERIMENTAL

Isolation of the Phage-Resistant Variant II/3, 4, 7.—Phase II *Sh. sonnei* is susceptible to bacteriophages T_2, T_6, T_3, T_4, and T_7; it is resistant to T_1 and T_5. In order to procure a variant organism resistant to T_3, T_4, and T_7, the parent Phase II bacillus was plated with a mixture of these three phages, and

several resistant colonies which appeared on the plate were picked and freed of virus by repeated streaking and selection.

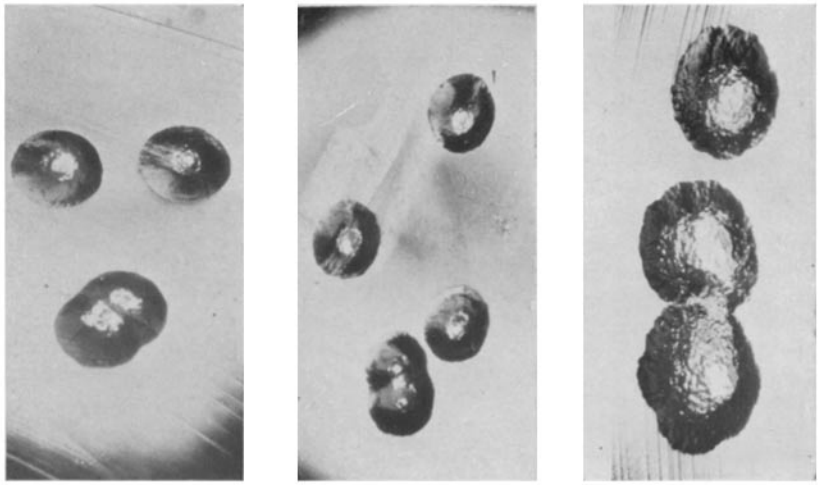
Fresh stocks of T_3, T_4, and T_7 were prepared, and a mixture of the three phages made so that the final concentration was 1×10^7 particles of T_3, 7×10^9 particles of T_4, and 1×10^5 particles of T_7 per ml. It is desirable to choose these lower concentrations of the T_3 and T_7 phages in order to eliminate lysis of any II/3, 4, 7 variants in the Phase II cultures by host-range mutants. In freshly prepared stocks of T_3 and T_7 there is approximately one host-range mutant virus particle in every 10^5 particles of T_3, and one in every 10^4 particles of T_7. T_4 contains no host-range mutants which attack II/3, 4, 7 and can therefore be used in higher concentrations.

To 0.9 ml. of a 3 1/2 hour culture of the Phase II organism containing 1×10^9 bacteria per ml. was added 0.1 ml. of the mixture of T_3, T_4, and T_7 phages. After a few minutes, 0.1 ml. was spread on a neopeptone–agar plate. After 24 hours incubation, some 32 colonies of the resistant variant had grown out. This represents approximately 1 phage-resistant variant in every 3×10^6 cells. Several of these resistant colonies were picked and freed of phage by emulsifying in sterile broth, streaking, and again picking a fresh colony. The process was repeated three times.

The phage-resistant mutant II/3, 4, 7 forms two types of colonies which have been termed large and small. They cannot be distinguished readily when plated on Difco nutrient broth-agar, or on casamino acid–meat extract agar, but on neopeptone (1 per cent)–meat infusion–agar they are readily recognizable. On this medium, the parent Phase II bacillus forms smooth glistening colonies 2 to 3 mm. in diameter with slightly irregular borders. They show only very slight stippling. The small colonies of II/3, 4, 7 on this same medium are 1.5 to 2.5 mm. in diameter, have slightly crenated edges, are raised, and show moderate roughening or stippling of the surface. The large colonies of the variant organism are from 2.5 to 3.5 mm. in diameter. They are flatter, not as shiny, and are considerably rougher than the small variant colonies. These differences are revealed to some extent in the accompanying photographs (Fig. 1).

The large and small colonies of the II/3, 4, 7 variant are serologically indistinguishable. Organisms from small colonies appear to undergo mutation to yield bacilli which form large colonies, but the degree of mutation is not great, and probably does not occur with a frequency greater than 1 in 10^4. Organisms which form the large colonies do not back-mutate to the small-colony producer.

Extensive studies were made which establish the serological identity of these two organisms but no protocols will be given here. The sera of rabbits immunized with II/3, 4, 7 (small) are completely exhausted of agglutinating antibodies when absorbed with II/3, 4, 7 (large). Furthermore, the agglutinating antibodies are completely removed when such sera are absorbed with the somatic antigen prepared either from small or large colony-forming organisms. In the work which is about to be described, the small colony-forming variant was used. This organism will be referred to as II/3, 4, 7.



(*a*) (*b*) (*c*)

FIG. 1. Colony morphology of Phase II *Sh. sonnei* and the phage-resistant variants II/3, 4, 7. (*a*) Phase II *Sh. sonnei*; (*b*) II/3, 4, 7 (small); (*c*) II/3, 4, 7 (large). All × 5.

Cultivation of II/3, 4, 7.—Mass cultures of the phage-resistant variant, II/3, 4, 7, were obtained as follows:—

Fifteen liters of 1 per cent casamino acid medium containing the dialyzed extract of 1.5 lb. of ground beef heart (4), 7.5 gm. of glucose, and 0.75 gm. of $MgSO_4$ were seeded with a culture of the variant II/3, 4, 7. After incubation for 12 hours the culture contained approximately 1×10^8 bacilli per ml. At this point 300 ml. of a sterile solution of 50 per cent glucose was added, and sterile air was bubbled through the 5 gallon pyrex glass container. After some 60 to 90 minutes, rapid growth ensued. The pH of the medium was maintained at 7.2 ± 0.02 by means of an electronic device[1] which automatically added a sterile solution of molar Na_2CO_3. After some 5 hours, the bacterial population had reached approximately 3×10^9 organisms per ml. and growth had stopped. The culture was killed by the addition of a 50 per cent alcoholic solution of phenol so that the final concentration was 0.5 per cent. After standing at 5°C. for 15 hours, the organisms were separated in a Sharples centrifuge and dried from the frozen state. Each batch of the variant so prepared was tested for the presence of Phase II bacilli, which might have arisen by back-mutation, in the following manner:—

To 0.9 ml. of culture was added 0.1 ml. of T_4 phage containing 2×10^8 particles per ml. After standing for 5 minutes at 37°C., 0.1 ml. of this mixture was added to 0.9 ml. of a dilution of T_4 antiserum, sufficiently concentrated to neutralize all virus, *i.e.* a serum, the *K* value of which had been accurately determined. After an additional 7 minutes at 37°C., 0.1 ml. of this mixture was added to 9.9 ml. of nutrient broth and 0.1 ml. of the dilution was plated, using the Phase II organism as host. An appropriate virus assay, and colony count of the culture were likewise made. Any back-mutation of the variant II/3, 4, 7 to the parent Phase II organism could in this manner be detected and quantitatively assayed. In one or two instances it was found that back-mutation to the parent had indeed taken place, but never to an extent

[1] To be described in a separate communication.

greater than 0.3 per cent. These cultures of the II/3, 4, 7 variant were discarded. It was later found that the addition of $MgSO_4$ apparently supressed back-mutation, for none of the batches grown in media containing this substance showed the presence of Phase II bacilli. By following the above procedure, several hundred grams of the dried microorganisms, II/3, 4, 7, were isolated. Yields of approximately 20 gm. of dried bacilli per 15 liters of culture medium were obtained.

Isolation of the Purified Somatic Antigen of II/3, 4, 7.—

100 gm. of lyophilized II/3, 4, 7 bacilli was extracted with 0.5 liters of water at pH 7.2 for 1 hour at 60°C. The bacteria were separated by centrifugation, the opalescent aqueous extract was filtered through a Berkefeld filter, and dialyzed for 48 hours against 15 liters of distilled water. Seven such extracts were made, and treated in this manner. Each was concentrated *in vacuo* to approximately 100 ml. and then dried from the frozen state. The serological activity of each extract was determined by turbidimetric titrations against a standard antibacterial serum. The absorption spectrum of the material from each extraction was likewise measured. It was found that the third, fourth, and fifth extracts yielded material which had essentially the same absorption spectra and serological activity. The first two extracts were low in activity and were also contaminated with ribonucleic acid; they were discarded. The sixth and seventh extracts, though containing no nucleic acid, had only a quarter as much substance which precipitated specifically in antiserum, and were likewise discarded.

The material from the third, fourth, and fifth extracts, weighing approximately 9 gm., was combined, suspended in distilled water, and adjusted cautiously to pH 8.4 with normal sodium hydroxide. After stirring for an hour, the insoluble residue was removed by centrifugation, and the pH of the supernatant liquid, which was opalescent but free from particulate matter, was adjusted to pH 4.5. A heavy precipitate formed and was centrifuged off, and the supernate, which was serologically inert, was discarded.

The precipitated antigen was again suspended in several hundred ml. of water and again brought to pH 8.4. Recentrifugation at 15,000 *g* for 30 minutes separated a small amount of insoluble material which was discarded. The active material was again precipitated at pH 4.5, centrifuged, then redissolved at pH 8.4, and the solution finally adjusted to pH 7.2 and dialyzed. The antigen was now dried from the frozen state. 6 gm. of material was recovered.

The substance isolated in the above manner is a phosphorylated lipomucoprotein which contains 11 per cent of nitrogen and 1.0 per cent of phosphorus. Its lipid and reducing sugar contents are 10.7 and 6.4 per cent respectively, when determined after hydrolysis for 6 hours at 100°C. in 1 normal HCl. The antigen gives the usual color reactions for protein, and a positive Molisch reaction, indicating the presence of carbohydrate. Further isoelectric precipitations failed to enhance the serological activity of the antigen, nor did they alter materially its chemical composition. The antigen appears to be heterodispersed for on centrifugation at 15,000 *g* a small amount of material is sedimented and separates as an opalescent jelly. Neither the chemical properties nor the biological activity of the sedimented material shows any great differences from those of the major portion of the substance in the supernatant.

Enzymatic Degradation of the Purified Antigen.—The serological activity of the antigen prepared in the above manner can be considerably enhanced by digesting with pancreatin.

8 gm. of the antigen was dissolved in 300 ml. of 0.02 M phosphate buffer at pH 8.0; to this was added 0.5 gm. of pancreatin which had previously been dissolved in water and centrifuged to remove insoluble residue. The mixture was placed in a cellophane bag together with a few milliliters of chloroform, and this in turn has placed in a cylinder of the same buffer saturated with toluene. The mixture was incubated for 3 days at 37°C., the buffer being changed each day. The material in the membrane was then dialyzed thoroughly against distilled water, and the substance isolated by drying from the frozen state. 4.7 gm. was recovered. The material gave both a biuret reaction and a positive Molisch test. The serological activity of this material was nearly four times that of the undegraded antigen.

The enzymatically degraded antigen was further fractionated by dissolving 2.8 gm. in 100 ml. of 0.01 M sodium acetate, cooling to 0°C., and adding 100 ml. of acetone. After standing for 3 days at −10°C. the precipitated material was centrifuged off at 0°C., dissolved in water, dialyzed, and then frozen and dried. This yielded 2.3 gm. of the purified, enzymatically degraded antigen. The relatively inert material in the supernate, which showed marked absorption in the ultraviolet, was discarded.

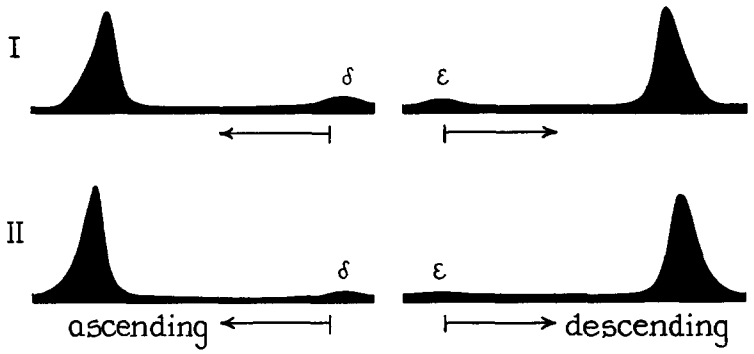


FIG. 2. Electrophoretic patterns of the enzymatically degraded antigen of Phase II/3, 4, 7 *Sh. sonnei*. I, pattern at pH 8.6; II, pattern at pH 6.1.

The enzymatically degraded antigen is a water-soluble amorphous powder containing 6.3 per cent nitrogen and 2.0 per cent phosphorus. The specific optical rotation of this material is low, $[\alpha]_D = +3.4°$. On electrophoresis it shows a considerable degree of homogeneity and migrates as a single boundary (Fig. 2). The mobility of $u = -9.86 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$, was determined at pH 8.6 in veronal buffer of ionic strength 0.1; the mobility, $u = -11.23 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$, at pH 6.1 in phosphate buffer of ionic strength of 0.1, was likewise determined. The degraded antigen liberated 42 per cent of lipids on hydrolysis in 1 N HCl and 10.7 per cent of reducing sugars, calculated as glucose. The serological and antiviral properties of this substance will be discussed later.

Isolation of the Lipocarbohydrate.—When the enzymatically degraded antigen is treated with phenol it dissociates into its protein and lipocarbohydrate components.

393 mg. of degraded antigen was suspended in 20 ml. of 88 per cent phenol and stirred for 1/2 hour at room temperature. The mixture was transferred to a cellophane membrane and

dialyzed against successive changes of distilled water for 3 days at 5°C. After removal of the phenol there could be seen within the bag three distinct substances, a protein conglomerate or cake, a small amount of a finely divided precipitate, and of course the aqueous solution containing the lipocarbohydrate. The cake was separated from the finely divided precipitate by filtration through a layer of kleenex tissue. The finely divided precipitate was in turn separated from the solution by high speed centrifugation. The latter was again dialyzed, pervaporated, and dried from the frozen state. This material still contained a small amount of free lipid which was removed by extraction with ether. 185 mg. of substance was recovered.

The finely divided precipitate, obtained by centrifugation, was dissolved in 5 ml. of water by the addition of 0.1 N sodium hydroxide, dialyzed and likewise frozen and dried. 47 mg. was recovered. This substance contained 1.8 per cent nitrogen and 2.7 per cent phosphorus; its nature has not yet been ascertained.

The protein cake was dissolved by adjusting the pH of a suspension to 8.4 with dilute NaOH. The substance was reprecipitated at pH 4, redissolved and finally dialyzed, frozen, and dried. 120 mg. of material containing 13.5 per cent nitrogen and 0.64 per cent phosphorus was obtained. This component had but slight serological and antiviral activity and was not further investigated.

Chemical Properties of the Lipocarbohydrate.—The lipocarbohydrate isolated from the aqueous phase was readily soluble in water, it was serologically active, and could be precipitated from solution by the addition of hydrochloric acid. The complex contained 2.0 per cent nitrogen and 2.4 per cent phosphorus and its specific optical rotation was $[\alpha]_D^{24^\circ} = +21.2^\circ$. The lipid content of the substance proved to be higher than that of the degraded antigen; on acid hydrolysis some 51 per cent of lipid was liberated and the reducing sugar content, 10.0 per cent calculated as glucose, proved to be slightly lower than that of the degraded antigen. This fact indicates that some hydrolysis of the polysaccharide moiety of the antigen had taken place during treatment with concentrated phenol. Color reactions performed on solutions of the lipocarbohydrate revealed no pentoses, desoxypentoses, uronic acids, or ketohexoses. A determination of the hexose content of the lipocarbohydrate, performed on unhydrolyzed samples by the micro method of Dische (10), revealed that the material contained only traces of these sugars. The hexosamine content was also determined (7) and found to be 7.7 per cent after 10 hours hydrolysis in 4 N HCl, calculated as glucosamine.

In an attempt to identify the hexosamine component of the lipocarbohydrate, a hydrolyzed sample was analyzed on a paper chromatogram as previously described (1).

14.3 mg. of lipocarbohydrate was hydrolyzed in 5 ml. of 1 N H_2SO_4 for 10 hours. After removal of the liberated lipid by filtration, the hydrolysate was neutralized with barium hydroxide to pH 5.0 and the $BaSO_4$ was separated by centrifugation. The supernatant was evaporated to dryness. 1.0 mg. of the residue was dissolved in 10 μl. of water and placed on the chromatogram. Samples of glucosamine and chondrosamine (50 μg.) were likewise placed on the paper. The chromatogram was developed for 24 hours in ethyl acetate–pyridine–water mixture (5:3.5:1.5), then dried, and stained with ammoniacal silver nitrate.

From Fig. 3 it can be seen that the lipocarbohydrate contains but one saccharide, for only one spot was observed on the chromatogram developed with

silver nitrate. Since this same spot became pink when the chromatogram was sprayed with the Elsen-Morgan (11) reagent, it is evident that the unknown saccharide is a hexosamine. It should be observed, however, that the location of the spot formed by the unknown sugar is neither identical with that of glucosamine nor with that of chondrosamine. This fact indicates that the hexosamine of the lipocarbohydrate is different from either of these two known amino sugars. Although the exact chemical nature of the hexosamine of the lipocarbohydrate has not yet been determined, the results of these experiments

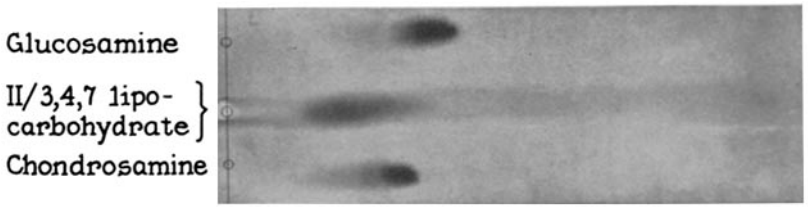


FIG. 3. Chromatogram of acid hydrolysate of lipocarbohydrate derived from variant antigen.

TABLE I

Agglutination Reactions of Phase II Sh. sonnei and of the Phage-Resistant Variant II/3, 4, 7 in Antibacterial Immune Sera

Serum of rabbit immunized with	Microorganism tested	Final dilution of antiserum						
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
II bacilli	II	+++	++++	++++	++++	+++	++±	+
	II/3, 4, 7	0	0	0	0	0	0	0
II/3, 4, 7 bacilli	II	+++	++++	++++	++±	++	0	0
	II/3, 4, 7	+++	++++	++++	++++	+++	+	0

indicate clearly that the chemical composition of the antigen of the variant bacillus differs radically from that of the parent Phase II organism.

Agglutination Reactions of Phase II Sh. sonnei and Its Phage-Resistant Variant, II/3, 4, 7, in Antibacterial Immune Sera.—In order to learn something of the serological relationship of these two microorganisms, extensive studies were carried out on the agglutination reactions of Phase II *Sh. sonnei* and its variant, II/3, 4, 7. Since the specificity of these reactions is determined by the lipomucoprotein distributed on the cell surface of each, any differences which they might show, in so far as their immunological reactions are concerned, must be related to differences in this antigenic component. The sera of rabbits which had been immunized with Phase II *Sh. sonnei* will, of course, agglutinate the homologous microorganism. When the phage-resistant variant, II/3, 4, 7,

was tested in these same sera, however, it can be seen from Table I that no detectable agglutination occurred.

The cultures of the two microorganisms tested were grown with aeration in casamino acid–meat extract medium until the bacterial population had reached approximately 2.5×10^8 cells per ml. In all cases the sera to be tested were diluted in nutrient broth medium. The variant microorganism, II/3, 4, 7, has a tendency to aggregate as the culture ages, or as it stands. Since it also tends to aggregate when diluted in physiological salt solution, it was necessary to use young cultures, to carry out the serum dilutions in broth, and to read the agglutination reactions after 2 hours incubation at 37°C.

The results presented in the first half of Table I appear to have but one interpretation; namely, that the antigen distributed on the surface of the parent cell differs from that on the cell surface of the variant, and that this difference is sharply reflected in the immune response elicited in animals immunized with the Phase II organism. If one now examines the agglutination reactions of

TABLE II

Agglutination Reactions of Phase II Sh. sonnei and of the Phage-Resistant Variant II/3, 4, 7 in Anti-Antigen Immune Sera

Serum of rabbit immunized with	Microorganism tested	Final dilution of antiserum						
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
II antigen	II	+++	+++	+++	++	+	±	0
	II/3, 4, 7	0	0	0	0	0	0	0
II/3, 4, 7 antigen	II	+++	+++	+++	++	+	0	0
	II/3, 4, 7	++++	++++	++++	+++	++	+	±

these two bacilli in the sera of animals immunized with the variant II/3, 4, 7 (Table I), it is seen that both microorganisms are agglutinated. The significance of these observations, which at first sight appear to be paradoxical, will be discussed later.

Agglutination Reactions of Phase II Sh. sonnei and the Phage-Resistant Variant, II/3, 4, 7, in Somatic Antigen Antisera.—Because the somatic antigens of the two microorganisms under consideration were obtained in a highly purified form, it was possible to investigate the specificity of the antibodies which each elicits in experimental animals. These antisera are presumably monovalent and contain antibodies directed against only the two somatic antigens themselves. We have at hand two such sera, one from animals immunized with the Phase II antigen, the other from rabbits injected with the antigen of the variant II/3, 4, 7.

In Table II are presented the results of the agglutination of the Phase II organism, and of the phage-resistant variant, II/3, 4, 7, in Phase II antigen antiserum. Here it can be seen that the agglutination reactions follow a pattern

identical with that shown by the corresponding Phase II antibacterial serum. The mutant cell is not agglutinated by the antiserum elicited by the Phase II antigen. When similar tests are made using the sera of rabbits immunized with the enzymatically degraded antigen derived from the mutant cell, II/3, 4, 7, it can be seen that these sera also agglutinate the homologous microorganism, and show crossing with the heterologous Phase II cell. Again the pattern shown by the antibacterial sera is duplicated. In this instance, however, it should be pointed out that the cross-agglutination of the Phase II organism

TABLE III

Precipitin Reactions of the Somatic Antigens of Phase II Sh. sonnei and of the Phage-Resistant Variant II/3, 4, 7 in Antibacterial Immune Sera

Serum of rabbit immunized with	Somatic antigen tested	Final dilution of antigen				
		1:2,000	1:10,000	1:50,000	1:250,000	1:1,250,000
II bacilli	II	+++	+++±	++	+	±
	II/3, 4, 7	0	0	0	0	0
II/3, 4, 7 bacilli	II	+	++±	++	±	0
	II/3, 4, 7	++	+++	+++	+	±

TABLE IV

Precipitin Reactions of the Somatic Antigens of Phase II Sh. sonnei and of the Phage-Resistant Variant II/3, 4, 7 in Anti-Antigen Immune Sera

Serum of rabbit immunized with	Somatic antigen tested	Final dilution of antigen				
		1:2:000	1:10:000	1:50,000	1:250,000	1:1,250,000
II antigen	II	++±	++++	+++	+	±
	II/3, 4, 7	0	0	0	0	0
II/3, 4, 7 antigen	II	++	++±	+±	±	0
	II/3, 4, 7	++++	++++	++	+	±

in anti-antigen immune serum does not appear to be as vigorous as in the corresponding antibacterial sera.

Precipitin Reactions.—The precipitin reactions of the two purified antigens derived from the Phase II bacillus and from the variant II/3, 4, 7 were next investigated. These results are presented in Tables III and IV. It can be seen that the specificity of these reactions parallels those shown by the agglutination reactions of the two microorganisms. The antibodies elicited by the Phase II bacillus, and by its purified somatic antigen, show a high degree of specificity. These antisera precipitate only the antigen of the parent Phase II bacillus and not that of the variant II/3, 4, 7. On the other hand, the immune sera of rab-

bits injected with living II/3, 4, 7 bacilli, or with the purified antigen, give rise to immune bodies which precipitate the homologous II/3, 4, 7 antigen, as one would of course expect. Yet these same sera, unlike those evoked by the parent cell or its somatic antigen, readily cross-react.

Antiviral Properties of the II/3, 4, 7 Antigen.—Phase II *Sh. sonnei* is susceptible to lysis by five of the T series of *coli*-dysentery phages, T_2 and T_6, and T_3, T_4, and T_7, whereas the variant, II/3, 4, 7, is attacked only by T_2 and T_6.

TABLE V

Inhibition of T Phages by the Antigen of Sh. sonnei II/3, 4, 7

Substance tested	Plating microorganism	Virus tested	Final concentration of antigen in virus-antigen mixture*				
			1.0 mg.	0.1 mg.	0.01 mg.	0.001 mg.	0.0001 mg.
Purified antigen	II/3, 4, 7	T_2	74	51	47		
		T_6	12	0	0		
	Phase II	T_2	86	74	57		
		T_6	0	0	0		
		T_3	0	0	0		
		T_4	0	0	0		
		T_7	0	0	0		
Purified antigen after enzymatic degradation	II/3, 4, 7	T_2	100	100	100	96	77
		T_6	0	0	0		
	Phase II	T_2	100	99	100	98	80
		T_6	0	0	0		
		T_3	0	0	0		
		T_4	0	0	0		
		T_7	49	0	0		

* The figures represent per cent of total phage inactivated.

As pointed out in the previous communication (1), the Phase II antigen inactivated *in vitro* all the T phages to which this bacillus is susceptible.

It has also been seen from the foregoing that the somatic antigens derived from the Phase II bacillus and from its variant, II/3, 4, 7, are endowed with distinct and individual serological specificities. It is of great interest, therefore, to determine whether the somatic antigen of the phage-resistant variant is capable of inhibiting the T_2 and T_6 viruses to which the II/3, 4, 7 organism is susceptible, and also to ascertain whether this antigen will inhibit the phages which attack the parent Phase II bacillus.

Freshly prepared stocks of the virus under study were so diluted as to contain 2×10^3 particles per ml. 0.5 ml. of varying concentrations of the antigen were placed into each of four tubes, and to these was added 0.5 ml. of the virus dilution. A fifth tube containing 0.5

ml. of virus and 0.5 ml. of broth served as control. After 18 hours incubation at 37°C., 0.1 ml. of each virus-inhibitor mixture was plated, using the Phase II bacillus in one instance, and the II/3, 4, 7 variant in the other, as the host. Plaque counts were made after incubation at 37°C. for 18 hours. In the case of the T_7 phage, the platings were made on neopeptone–meat infusion–agar. The results of these experiments are recorded in Table V.

The results of the specific inhibition tests presented in Table V reveal certain salient facts. First, the antigen derived from the variant microorganism is a powerful antiviral agent, but its activity is confined to the inhibition of T_2, whether the host be the parent or the variant microorganism. Unlike the antigen of the Phase II bacillus, that of the mutant, either before or after enzymatic degradation, fails to inhibit T_6—the only other phage of the T series to which the mutant cell is susceptible. Second, the antigen, as well as its products of degradation, is incapable of inhibiting T_3, T_4, and T_7. This observation is quite in accord with the anticipated result, for the variant itself is resistant to these three phages and hence its surface antigen, the lipomucoprotein, cannot function as a receptor for them. It cannot be said at the present time just why the antigen of the mutant cell is devoid of activity against T_6, whereas that of the parent Phase II organism, as was shown in the preceding communication, is active against this particular phage. This may be due to the chemical treatment which the antigen receives during its purification. This suggestion has some basis in fact because it was observed that one or two preparations of the mutant antigen, although it is not indicated in Table V, actually showed some activity against T_6, though never against T_3, T_4, or T_7.

In sum it can be stated that the differences in chemical nature of the antigens of Phase II *Sh. sonnei* and its variant, II/3, 4, 7, are reflected not only in their diverse serological specificities, but also in dissimilarities in their specific antiviral properties as well.

DISCUSSION

A population of *E. Coli* B is by no means homogeneous but contains a number of different mutants, the presence of which can be demonstrated by means of a series of bacteriophages. While Demerec and Fano (12) were concerned with the classification of these variants and the genetic factors involved in their differentiation other investigators have studied the relationship between phage sensitivity and antigenic structure; it has been repeatedly demonstrated, even among microorganisms otherwise unrelated, that a common susceptibility to a given phage may be correlated with similarities in antigenic structure. The earlier literature in this field, and that dealing with attempts to isolate the socalled phage receptor substances from susceptible organisms have been reviewed in a previous communication (2). In addition, it is well to make mention of the important and more recent work of Beumer (13), Weidel (14), and that of Mondolfo and Hounie (15).

The loss of susceptibility of certain Gram-negative bacilli to phages has also been correlated with changes from the smooth to the rough form, and it has been pointed out that these changes are frequently, though not always, accompanied by alteration in their serological specificity (16). In certain instances this change has been attributed to the loss in function of the cell to elaborate its type-specific agglutinogen. It should be pointed out, however, that the interpretation of serological evidence *per se* is by no means certain, for it has been seen from the foregoing that Phase II *Sh. sonnei* can undergo variation to yield a rough form which fails to agglutinate in antisera evoked by the parent cell. On the basis of this evidence it would appear at first sight that the variant II/3, 4, 7 has indeed lost the function of elaborating a type-specific antigen, yet chemical scrutiny has revealed that this is not the case. It is still endowed with enzyme systems capable of synthesizing a lipocarbohydrate protein complex, though to be sure it is different, both chemically and serologically, from that of the parent. In addition, this newly constituted complex is incapable of inhibiting *in vitro* three of the phages to which the parent is susceptible.

The fact that the antisera elicited in rabbits immunized with Phase II *Sh. sonnei* are specific and fail to agglutinate the variant II/3, 4, 7, while those evoked by the latter microorganism cross-react with the parent cell, at first appears paradoxical. It was demonstrated in these laboratories many years ago, however, that it is possible for azoprotein antigens containing disaccharides of known chemical structure, to evoke in experimental animals antibodies which show a non-reciprocal immunological relationship (17). It is suggested that the serological relationship between the antigen of the parent Phase II bacillus and that of the variant II/3, 4, 7 is likewise governed by the chemical configuration of their respective carbohydrate moieties, and that this phenomenon is comparable to that exhibited by the azoprotein antigens referred to.

In the previous communication (1) evidence was presented which left little doubt that the antiviral properties of the Phase II antigen, in so far as T_3, T_4, and T_7 are concerned, are a function of the carbohydrate moiety of the molecular complex. In the present study it has been shown that the polysaccharide of the variant antigen differs radically from that of the parent. In view of this it is suggested that, not only is the serological specificity of each antigen governed by its carbohydrate component, but that its ability to inhibit the T_3, T_4, and T_7 phages is likewise determined by this same constituent. In the one instance, the structure of the biologically active polysaccharide appears to be such that it is fully capable of combining with and inactivating these phages *in vitro*. In the other, failure of the antigen to interact with the phage must mean that the appropriate geometric pattern of the forces required for specific combination is no longer present.

In conclusion it should be emphasized that variation, as exemplified by Phase II *Sh. sonnei* and its variant, II/3, 4, 7, does not necessarily involve a

complete loss in ability to synthesize a lipocarbohydrate protein complex, but that this function is altered and appears to involve changes in the enzyme system which is responsible for the synthesis of the carbohydrate component of the thermostable agglutinogen.

During the course of this work the authors engaged in a number of stimulating conversations with Dr. Theodore Shedlovsky. For his interest and his suggestions we are deeply grateful.

SUMMARY

1. A phage-resistant variant of Phase II *Shigella sonnei* has been isolated and the type-specific antigen from it has been obtained.

2. The antigen of the variant microorganism, II/3, 4, 7, differs from that of the parent Phase II bacillus in serological specificity and in chemical make-up.

3. Although the antigen of Phase II *Sh. sonnei* inhibits all T phages to which the bacillus is susceptible, that of the variant fails to inhibit T_3, T_4, and T_7.

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