THE CHEMICAL AND ANTIVIRAL PROPERTIES OF THE SOMATIC ANTIGEN OF PHASE II SHIGELLA SONNEI

By MARGERIS A. JESAITIS, Sc.D., and WALTHER F. GOEBEL, Ph.D.

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

(Received for publication, June 2, 1952)

In an earlier communication it was demonstrated that the type-specific somatic antigen derived from Phase II *Shigella sonnei* previously killed with formalin, was capable of inactivating *in vitro* three of the five T *coli*-dysentery phages to which this bacillus is susceptible (1). Although the antigen inactivated T_3, T_4, and T_7, it was without effect upon T_2 and T_6. Chemical agents appear to have a profound effect upon the phage receptors of this bacillus (2) and because of this it was thought that the techniques used in earlier work might well have impaired or destroyed certain, though not all, of the antiviral properties of this serologically active substance. A different method of isolating the Phase II antigen has therefore been sought.

In the present communication it will be shown that the somatic antigen of Phase II *Sh. sonnei*, obtained by extracting phenol-killed bacilli with water, inhibits all the T phages to which the Phase II bacillus is susceptible, and that chemical degradation of this substance is accompanied by a selective loss in its ability to inhibit these viruses. The chemical, immunological, and antiviral properties of this antigen and its degradation products will also be described.

Materials and Methods

Bacteria.—The culture of Phase II *Sh. sonnei* used in this study was obtained from the United States Army Medical School. The bacteria were grown in a medium devised by Dole (3) using techniques previously described (4). At the termination of growth the microorganisms were killed by adding a solution of 100 ml. of 88 per cent phenol in 200 ml. of 50 per cent alcohol. The culture was kept overnight at 5°C., the bacilli were collected in a Sharples centrifuge and dried from the frozen state. The yield was usually 40 to 50 gm. of dry cells from 15 liters of culture.

Antisera.—Antisera to the type-specific antigen were obtained from rabbits injected intravenously with graded doses of the Phase II antigen as previously described (4).

Quantitative Precipitin Reactions.—The immunological activity of the various substances studied was determined turbidimetrically by the method of Libby (5).

Bacteriophages.—The bacteriophages used for the experiments on inhibition were originally obtained from Dr. Mark Adams of New York University. They have been maintained in this laboratory for some years by transferring periodically on *Escherichia coli* B in nutrient broth.

Inhibition Tests.—These were performed as described in earlier work (6, 7). Phase II *Sh. sonnei* was used as the host.

Analytical Methods.—The carbon and hydrogen content of the materials studied were estimated by the micro method of Pregl; nitrogen was determined colorimetrically by the technique of Koch and McMeekin (8) and phosphorus by the procedure of Allen (9). The amino nitrogen content of the substances studied was estimated by the colorimetric procedure of Moore and Stein (10) on samples hydrolyzed in 4 N HCl for 24 hours at 100°C. The determination of reducing sugars was made by the method of Shaffer and Somogyi (11) after 6 hours of hydrolysis at 100°C. in 1 N HCl; the results presented in the tables are expressed in terms of the reducing value of glucose. The estimation of amino sugars was made by the technique of Sørensen (12) after 10 hours of hydrolysis of the substances in 4 N HCl at 100°C. The percentage of lipids was determined by weighing the chloroform-soluble material liberated after hydrolysis for 6 hours at 100°C. in 1 N HCl. The determination of volatile organic acid, calculated as acetyl, was made by the method of Elek and Harte (13).

The absorption spectra of the materials studied were observed in a Beckman spectrophotometer (model DU). Electrophoretic analyses were made in a Tiselius apparatus, using the scanning method of Longsworth (14).

EXPERIMENTAL

The Purified Antigen of Phase II Sh. sonnei.—The antigen of Phase II *Sh. sonnei* can be readily separated from phenol-killed microorganisms by repeated infusion with warm distilled water. It can be purified by isoelectric precipitation followed by fractionation at low ionic strength with alcohol. The immunologically active material thus obtained has a relatively high nitrogen content and is essentially free from nucleic acid.

140 gm. of phenol-killed lyophilized Phase II *Sh. sonnei* was extracted with 2 liters of distilled water for 1 hour at 65°C. The cells were separated in a Sharples centrifuge and reextracted. The combined extracts were passed through a Berkefeld filter, acidified with acetic acid to pH 4.0 and allowed to stand overnight at 5°C. The main portion of the clear supernatant liquid was decanted and the precipitate separated from the remaining solvent by centrifugation at 0°C. The deposit was suspended in 300 ml. of water and dissolved by the cautious addition of alkali to pH 7.5. The solution was dialyzed against distilled water at 5°C. for 2 days, then concentrated by pervaporation, and dried from the frozen state. 12.75 gm. of crude antigen was obtained. This substance contained 12.1 per cent of N and 1.1 per cent of P.

The material was now purified by precipitation with alcohol at low ionic strength as follows: 10.0 gm. of crude antigen was dissolved in 700 ml. of 0.02 N sodium acetate and adjusted to pH 6.4 with acetic acid. The solution was cooled to 0°C. and precipitated by the slow addition of 700 ml. of cold 50 per cent alcohol. The mixture stood overnight at 0°C. The finely dispersed precipitate which formed was separated by centrifugation at 10,000 *g* for 30 minutes, dissolved in 100 ml. of water by the addition of alkali to pH 7.6, dialyzed, pervaporated, and dried from frozen state. 2.0 gm. of material containing 12.6 per cent nitrogen and 0.8 per cent phosphorus was recovered (fraction 1).

To the supernatant liquid of fraction 1 was added 500 ml. of cold absolute alcohol. After standing overnight at 0°C. the precipitate (fraction 2) was collected by centrifugation, redissolved, dialyzed, and dried from the frozen state. 6.5 gm. of a material was obtained. This material, referred to as the purified antigen, contained 11.7 per cent N and 1.2 per cent P. The supernatant liquid from fraction 2 yielded 0.8 gm. of substance which contained 13.1 per cent nitrogen. It had low serological activity and was discarded.

Digestion of the Purified Antigen with Pancreatin.—A considerable increase in the serological activity of the purified antigen (fraction 2) as determined

by the turbidimetric method was achieved by digestion with pancreatin. It was necessary to repeat this procedure several times before obtaining material with a low nitrogen content.

6.2 gm. of the purified antigen was dissolved in 200 ml. of 0.05 M phosphate buffer at pH 8.0. To this was added a solution of 0.2 gm. of pancreatin. The mixture was placed in a cellophane membrane and dialyzed at 37°C. against three successive changes of the same buffer. Sterility was maintained by the addition of chloroform. The solution was treated twice more at 24-hour intervals with fresh 0.1 gm. portions of pancreatin. The digest was finally dialyzed against running distilled water, pervaporated, and dried from the frozen state. 1.57 gm. of material having a nitrogen content of 5.1 per cent and a phosphorus content of 2.8 per cent was finally obtained.

Isolation of the Lipocarbohydrate.—The enzymatically degraded antigen still contained bound protein which could be removed by dissociation in 90 per cent phenol (15).

1.2 gm. of enzymatically degraded antigen was added to 50 ml. of 90 per cent phenol. The mixture was stirred at room temperature for 1/2 hour, and then placed in a cellophane membrane and dialyzed against running distilled water at 5°C. After 2 days, the precipitated protein was separated by filtration. The precipitate was washed, dissolved in 20 ml. of water by the addition of 1 N NaOH, dialyzed, and dried from the frozen state. 0.33 gm. of substance was obtained which contained 10.9 per cent of nitrogen and 0.5 per cent of phosphorus. The filtrate from the protein was concentrated by pervaporation, dialyzed, and also dried from the frozen state. 0.76 gm. of a substance, the lipocarbohydrate, was recovered, which contained 2.9 per cent nitrogen and 3.8 per cent phosphorus.

Further purification of the lipocarbohydrate was achieved as follows:—

0.70 gm. was dissolved in 10 ml. of distilled water. To the viscous solution was added 200 ml. of chloroform-methyl alcohol (2:1) and the mixture stirred vigorously. The precipitate which formed was first separated by centrifugation, then dissolved in 20 ml. of water, and finally dialyzed and the solution dried from the frozen state. 0.61 gm. of lipocarbohydrate was thus obtained. This material contained 2.8 per cent nitrogen and 3.9 per cent phosphorus.

Physical Properties of the Purified Antigen and Its Products of Degradation.—The purified antigen (PA), the enzymatically degraded antigen (DA), and the lipocarbohydrate (LC) are amorphous substances of high molecular weight, which are readily soluble in water. The specific optical rotation of the purified antigen is $-21.8°$, that of the enzymatically degraded antigen $+55.0°$ whereas the rotation of the lipocarbohydrate was found to be $+90.0°$. The ultraviolet absorption spectra of these substances, measured on 0.05 per cent solutions in 0.05 molar phosphate buffer at pH 7.0, are shown in Fig. 1. The absorption curves of PA(I) and of DA(II) are comparable to those of proteins; the maximum at 275 mμ (curve I) is due to the presence of aromatic amino acids. The absence of maxima at 260 mμ indicates that the materials are free from nucleic acid. The low extinction values shown by the spectrum of LC(III) indicate that this substance has but a slight content of amino acids.

Electrophoretic analyses of PA, DA, and LC were performed on 1.5 per

cent solutions with a potential gradient of 4.4 volts/cm. in veronal buffer of pH 8.6 at an ionic strength of 0.1. Under these conditions the substances form but a single boundary. The mobilities were calculated from the descending patterns and the following values were observed: for PA, $u = -5.36 \times 10^{-5}$; for DA, $u = -6.27 \times 10^{-5}$; and for LC, $u = -5.68 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$. It can be seen from Fig. 2 that the shapes of the peaks are slightly assymetrical, especially in the case of PA. This fact indicates some heterogeneity of the materials and that they consist of molecules with closely similar electrical charges.

Chemical Properties of the Purified Antigen and Its Products of Degradation.—The purified antigen is a complex substance which can be classified as a phosphorylated lipomucoprotein. It is precipitated from solution at pH 4–5 and redissolves on the further addition of acid. The complex gives the usual color

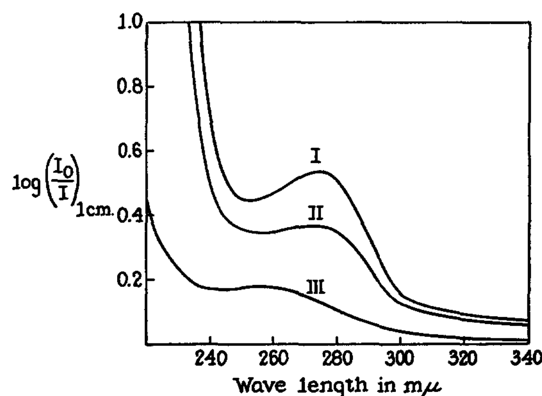

FIG. 1. Absorption spectra of the purified antigen of Phase II *Sh. sonnei* and its degradation products. I, purified antigen; II, enzymatically degraded antigen; III, lipocarbohydrate.

reactions characteristic for proteins and a positive Molisch test, which indicates the presence of a polysaccharide. It also forms precipitates with the salts of Ba, Zn, Cd, Cu, and Pb, and is precipitated by picric and trichloracetic acids. As can be seen from the analytical data given in Table I, the substance has a high nitrogen content (11.7 per cent), nearly all of which is liberated as amino nitrogen on hydrolysis. From these values it can be estimated that the substance contains some 65 to 70 per cent of protein. The reducing sugar value indicates that it also contains approximately 8 per cent of a polysaccharide. In addition it yields on hydrolysis some 12 per cent of lipid.

The enzymatically degraded antigen, in contrast to the native antigen, is not precipitated by the salts or by the acids listed above; however, it still gives a positive biuret reaction. As can be seen from the data presented in Table I, the degraded antigen still contains some 25 per cent of amino acids, and its reducing sugar and lipid contents are 20.6 and 23.4 per cent, respectively.

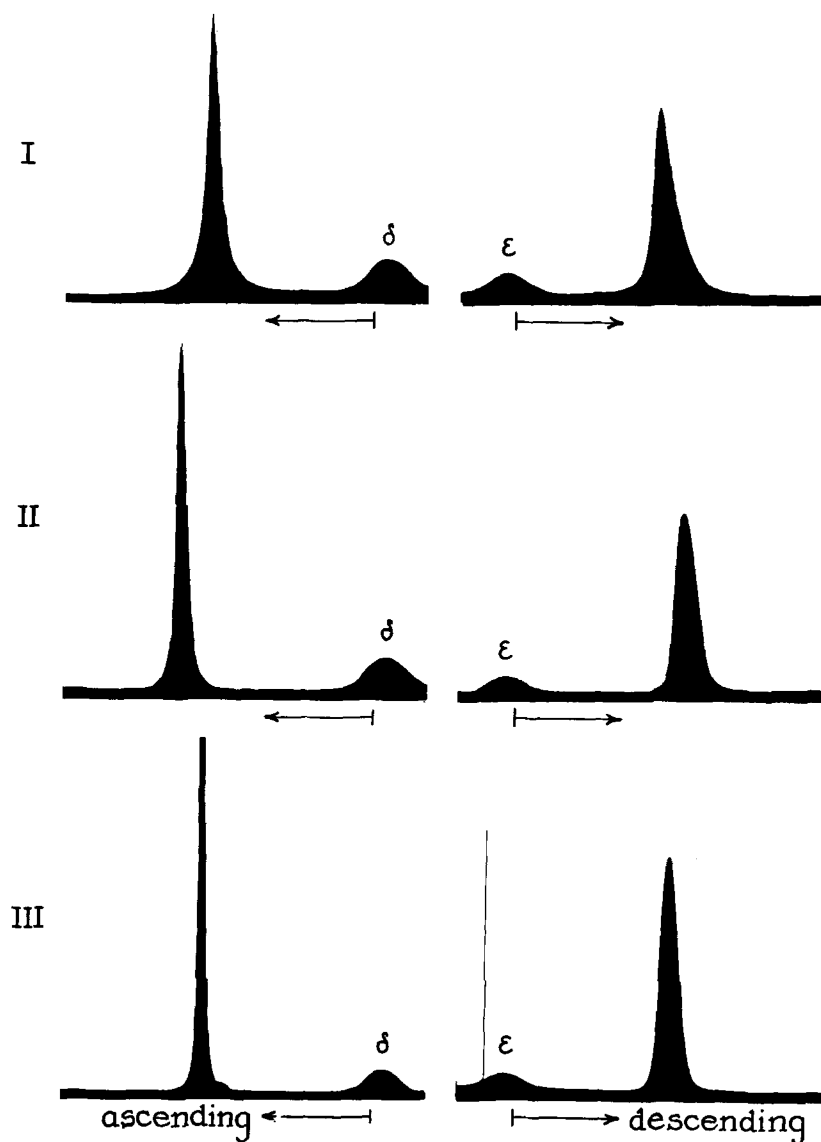

FIG. 2. Electrophoretic patterns of the purified antigen of Phase II *Sh. sonnei* and its degradation products. I, purified antigen; II, enzymatically degraded antigen; III, lipocarbohydrate.

The lipocarbohydrate (LC), obtained by dissociating the degraded antigen with phenol, is soluble both in acid and in alkali. Its alkaline solutions are viscous. Aqueous solutions of LC give a strong Molisch test as well as a positive

reaction for hexoses (16) and hexosamines (12). The color reactions for pentoses and methyl pentoses (17), desoxypentoses (18), uronic acids (19), ketohexoses (20), and mannose (21) are negative. Hydrolysis of LC with 1 N acid results in the liberation of lipid. The lipid fraction, which accounts for 29.2 per cent of the total weight of LC, contains 1.4 per cent of nitrogen and 1.3 per cent of phosphorus. The lipid itself contains no cholesterol as indicated by a negative Liebermann-Burchard reaction. The other components of LC, both reducing sugars and amino acids, are present to the extent of 29.8 and some 8 per cent, respectively. The substance also contains 3.9 per cent of bound acetyl groups (*cf.* Table I).

TABLE 1

Chemical Properties of the Purified Antigen of Phase II Sh. sonnei and Its Degradation Products

	Purified antigen	Enzymatically degraded antigen	Lipocarbohydrate
	per cent	*per cent*	*per cent*
Carbon	52.8	49.8	45.4
Hydrogen	7.2	7.2	7.1
Nitrogen	11.7	5.1	2.8
Phosphorus	1.2	2.8	3.9
Amino nitrogen	10.2	4.3	1.8
Lipids	12.2	23.4	29.2
Reducing sugars	8.2	20.6	29.8
Glucosamine	1.9	5.2	8.3
Acetyl	1.1	3.1	3.9
Ash	4.2	10.9	12.5

Identification of the Monosaccharide Components of the Lipocarbohydrate.—The identification of the sugars from which the polysaccharide moiety of the lipocarbohydrate is constituted was achieved by means of paper chromatography and by employing the cysteine color reaction of Dische (21).

The latter procedure is based upon the fact that many saccharides, upon heating with H_2SO_4, give decomposition products which show absorption in the ultraviolet (22). These products react with cysteine and form colored compounds. By performing this reaction under strictly standardized conditions and by comparing the absorption spectra of solutions of unknown material with those of pure sugars, it is possible, even in the presence of other saccharides, to identify and to quantitate the individual hexoses. Since many organic substances produce color when dissolved in H_2SO_4 in the absence of cysteine, it is necessary to run appropriate controls under identical conditions. For the full details of this method the reader is referred to the original communications (21)

To each of two tubes containing 4.5 ml. of 87 per cent H_2SO_4 at 0°C. was added 1 ml. of an aqueous solution of the material under investigation. The tubes were warmed to 24°C. for 5 minutes, then heated to 100°C. for 3 minutes, and finally cooled to 24°C. by immersion in cold water. To one was now added 0.1 ml. of water, to the other 0.1 ml. of 3 per cent cysteine hydrochloride solution. After standing for 1.5 hours, and again after 48 hours, the absorption spectra of the two solutions were measured in a Carey recording spectrophotometer. In each instance, the spectrometer was balanced against a sample of the appropriate reaction mixture to which an equal volume of water had been added in place of the solution of saccharide. Four substances were examined, the lipocarbohydrate derived from the Phase II antigen, two hexoses, glucose and galactose, and the seven-carbon-atom sugar, galaheptose (*d*-α-gala-l-mannoheptose). The solutions employed contained 500, 50, 50, and 100 μg. per ml. of the respective saccharides.

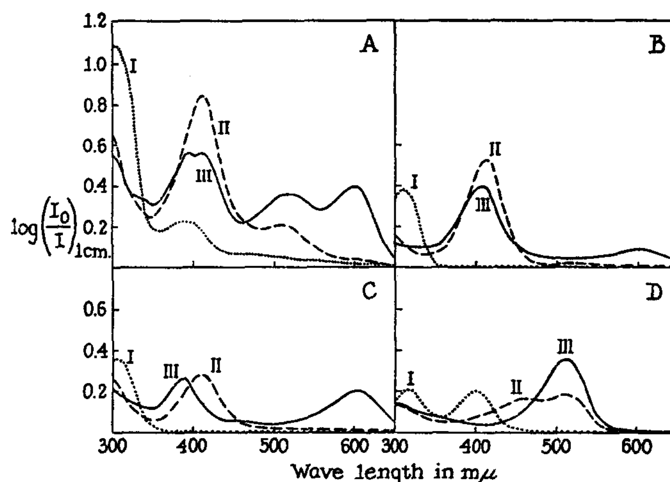

FIG. 3. Absorption spectra of Phase II lipocarbohydrate and of glucose, galactose, and galaheptose. *A*, 500 μg. of lipocarbohydrate; *B*, 50 μg. of glucose; *C*, 50 μg. of galactose; *D*, 100 μg. of galaheptose.

In each instance, curve I represents absorption spectrum of control sample, curve II absorption spectrum of primary cysteine reaction, and curve III absorption spectrum of secondary cysteine reaction.

The absorption spectra of the four substances are given in Fig. 3, in which curve I is, in each instance, that of the substance without addition of cysteine. Curves II are those of the substance to which cysteine had been added; the spectra were measured 1.5 hours after the addition of the reagent. This is termed the "primary cysteine reaction." Curves III, those of the "secondary cysteine reaction," represent the spectra of the latter after standing 48 hours. It should be noted that the spectra of the solutions without cysteine (curves I) showed but little change on standing and are therefore measured only after 1.5 hours.

A comparison of the absorption spectra of the three monosaccharides with the spectrum of the lipocarbohydrate (curves I) reveals that they have certain

characteristics in common. The spectra of glucose and galactose show absorption maxima at 308 and at 298 mμ respectively, whereas the spectrum of galaheptose has two maxima, one at 318 and the other at 400 mμ. The absorption spectrum of the lipocarbohydrate also shows maxima at 300 and at 380 to 400 mμ. It is apparent, therefore, that the polysaccharide contains all three of these monosaccharides. It should be noted, however, that the maximum shown by galaheptose is located sharply at 400 mμ, whereas that of the polysaccharide is broader and appears between 380 and 400 mμ.

Corroborative evidence for the presence of these sugars is again had by comparing the curves of the primary cysteine reactions (curves II). Here it can be seen that the lipocarbohydrate shows two maxima which now appear at 410 and at 500 to 520 mμ. The first maximum agrees quite precisely with the maxima of the two hexoses (curves II *B* and II *C*), whereas the second maximum coincides approximately with that of galaheptose (curve II *D*).

Examination of the curves of the secondary cysteine reactions (curves III) yields still additional information. The maximum which is shown by the lipocarbohydrate at 410 mμ (curve II *A*) has become a double maximum with peaks at 390 and at 410 mμ (curve III *A*). Furthermore, the maximum of the galactose curve, which appeared in the primary cysteine reaction at 410 mμ (curve II *C*) has also shifted to 385 to 390 mμ (curve III *C*), whereas that of glucose is located at 400 to 410 mμ (curves II *B* and III *B*). On the basis of these observations it now becomes apparent that both glucose and galactose are present in the bacterial polysaccharide.

It will be noted that both hexoses also show maxima at 600 mμ (curves III *B* and III *C*), but that the extinction coefficient of galactose at this wavelength is some 2.5 times greater than that of glucose. Because the optical density of the polysaccharide at 600 mμ is high, it can be concluded that galactose must be a constituent of the polysaccharide. Finally, it can be seen that the maximum shown by the curve of the lipocarbohydrate at 510 mμ (curve III *A*) corresponds to that of galaheptose (curve III *D*). On the basis of this evidence it can be stated with reasonable certainty that glucose, galactose, and a heptose form part of the polysaccharide moiety of the lipocarbohydrate complex. Still further confirmation of the presence of glucose and galactose was had by evaluating quantitatively the spectral data by the method of Dische (21). The results of these calculations, although not presented here, indicated clearly that these two hexoses are constituents of the lipocarbohydrate.

It should be pointed out that the characterization of the heptose component of the lipocarbohydrate could not be fully achieved on the basis of spectral data, for it was observed that three other heptoses, glucoheptose, glucoheptulose, and mannoheptulose,[1] also gave absorption spectra similar to the spectrum

[1] The authors are greatly indebted to Dr. C. S. Hudson of the National Institutes of Health, for his kindness in supplying the samples of heptoses used in this study.

of galaheptose.[2] Although the absorption maxima shown by these three heptoses lie approximately at the same wavelengths as those of galaheptose, they differ in that their extinction coefficients are considerably higher. It is also worthy of note that, because the lipocarboyhdrate failed to give a positive test for ketoheptoses (orcinol test (23)), it was possible to conclude that the heptose present could only be an aldoheptose.

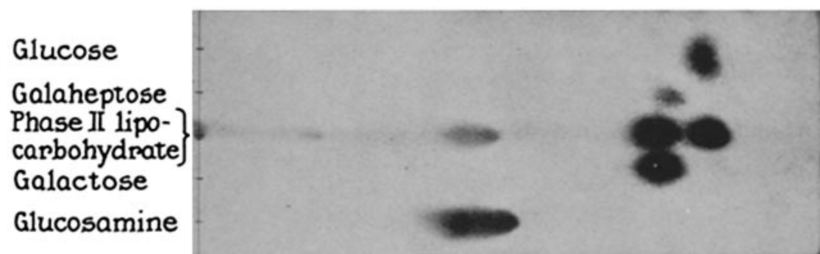

FIG. 4. Chromatogram of hydrolysate of Phase II liopcarbohydrate.

By means of chromatographic studies, it was possible to show that the unknown heptose present in hydrolysates of the lipocarbohydrate migrated at the same rate as did galaheptose. Additional evidence confirming the presence of glucose, galactose, and glucosamine was likewise obtained by this technique.

50 mg. of lipocarbohydrate was hydrolyzed for 6 hours at 100°C. in 5 ml. of 1 N H_2SO_4. The precipitated lipid was removed by filtration and the solution neutralized to pH 5.0 with $Ba(OH)_2$. After separation of the $BaSO_4$ by centrifugation, the supernatant liquid was evaporated in a desiccator. 23.2 mg. of residue was obtained. This material, which contained the saccharides liberated by hydrolysis of the lipocarbohydrate, was suitably diluted in water, and used for chromatography. For purposes of comparison solutions of pure glucose, galactose, glucosamine, and galaheptose were placed on the chromatogram. The latter was developed for 40 hours by the ascending method (24), (using No. 1 Whatman paper) with a mixture of 5 parts of ethyl acetate, 3.5 parts of pyridine, and 1.5 parts of water (25). All solvents were redistilled and mixed prior to their use. The developed chromatogram was dried and stained with ammoniacal $AgNO_3$ (26).

As can be seen from Fig. 4, the hydrolysate of LC produces three distinct spots which have R_{Gl}[3] values of 1.0, 0.91, and 0.55, respectively. The positions of these spots correspond to those of glucose, galactose, and glucosamine. In the presence of hexoses, galaheptose and glucoheptose do not appear as separate spots on a chromatogram developed with the solvent employed, because their R_{Gl} values (0.93 and 0.98) are very similar to those of glucose and

[2] The authors wish to express their thanks to Dr. Z. Dische, who in a private communicaion has informed them that he has made similar observations concerning the cysteine reactions of other heptoses.

[3] The ratio of the distance travelled by a given sugar to that travelled by glucose, is defined as its R_{Gl} value.

galactose (R_{Gl} = 1.0 and 0.91). It was possible, however, to locate the heptose by spectrophotometric analyses of eluates obtained from sections of the chromatogram.

Thus, 40 μl. of a solution containing 5 mg. of the hydrolysate of LC was distributed on the base line of a sheet of filter paper 18 cm. wide and 46 cm. long. The sheet was developed for 40 hours with ethyl acetate-pyridine-water mixture. After drying, two narrow strips were cut vertically from both sides of the sheet and the location of the hexoses was determined by staining with ammoniacal silver nitrate. The unstained portion of the chromatogram containing the hexoses was cut out parallel to the base line of the sheet and divided into 6 sections of equal width. The strips were numbered 1 to 6 in the direction of flow of the solvent. They were dried *in vacuo*, suspended from a wire in large reagent tubes, and each was eluted (27) with 3 ml. of water. The cysteine reaction was performed on two 1 ml. samples of each eluate, and the absorption spectra were measured after 1 hour, and again after 48 hours. The spectrograms revealed that the unknown heptose was present in eluates 2 and 3; galactose was found in eluates 1, 2, and 3; whereas glucose appeared in eluates 4, 5, and 6.

From these observations it is apparent that the heptose migrates at a rate only slightly higher than that of galactose, on chromatograms developed with ethyl acetate-pyridine-water solvent. It can also be seen from Fig. 4 that the R_{Gl} of a known specimen of galaheptose is nearly identical with that of the heptose present in the lipocarbohydrate. On the basis of the data presented, there can be little doubt that the hexose components of the lipocarbohydrate are glucose, galactose, and glucosamine, and that the fourth constituent is a heptose which shows properties very similar to those of galaheptose.

Since the monosaccharide components of the lipocarbohydrate have now been identified, it is possible to estimate their content approximately from spectrophotometric data. By comparing, at appropriate wavelengths, the extinction values shown by known amounts of the saccharides in question with those of LC, it was found that the lipocarbohydrate contains approximately 9 per cent each of glucose and of galactose, and 20 per cent of heptose, calculated as galaheptose. Because glucosamine does not produce a color reaction with cysteïne, its content in the lipocarbohydrate was estimated by the method of Sørensen (12) and was found to be 8.3 per cent.

Finally, it should be noted that the total monosaccharide content of the lipocarbohydrate, determined by the spectrophotometric method (46 per cent) is considerably higher than is the reducing sugar value determined by the copper reduction method (29 per cent) on acid hydrolysates. This discrepancy is due to the fact that both galactose and galaheptose have considerably lower reducing values than does glucose. In addition, not all of the glucosamine is liberated under the conditions employed for the hydrolysis. When the reducing value of the hydrolysates was determined by an alkaline ferricyanide method (28), excellent agreement (44 per cent) was had.

Serological Properties of the Phase II Antigen and Its Products of Degradation.—The Phase II antigen prepared in the manner described differs in its chemical

composition from that obtained from formol-killed bacilli (4). The chief difference resides in the higher nitrogen and hence protein content of the antigen prepared from phenol-killed bacilli. In order to compare the serological properties of the latter and of its products of degradation with those of the antigen derived from formol-killed microorganisms, quantitative turbidimetric precipitin tests were performed (5). The immune serum employed was that of a rabbit immunized with the purified antigen derived from phenol-killed bacilli.

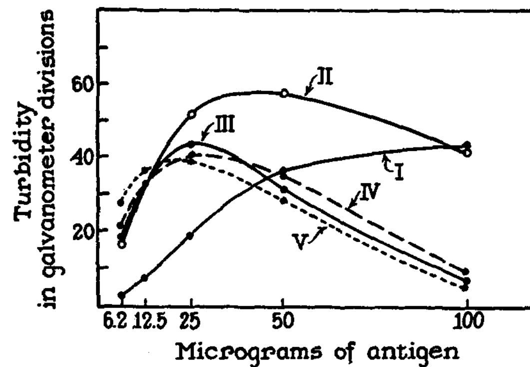

FIG. 5. Turbidimetric precipitin reactions of Phase II antigen and its degradation products. I, purified antigen; II, enzymatically degraded antigen; III, lipocarbohydrate; IV, antigen derived from formol-killed bacilli; V, lipocarbohydrate derived from IV.

Various concentrations of the material under study were added to standard homologous antigen antiserum diluted 1:7.5. After standing for 30 minutes the turbidity which developed was determined by means of a photoelectric turbidimeter. The turbidity, which is proportional to the precipitated antibody nitrogen, was recorded in arbitrary galvanometer units. The results of these tests are presented in Fig. 5.

Inspection of the data shows that the differences in the chemical composition of the material studied are reflected in the shape of the turbidity curves. It can also be seen that the activity of the purified antigen derived from phenol-killed bacilli is not great; only slight turbidity is produced by 10 μg. of this antigen (curve I). The removal of the protein component enhances its serological activity (curve II); for the enzymatically degraded antigen precipitates approximately twice as much antibody as does the same quantity on undegraded material. From this it appears that the digestible portion of the protein component of the antigen contributes but little to its ability to function as a precipitinogen. The lipocarbohydrate, at high dilutions, reacts as vigorously as does the enzymatically degraded antigen; however, it forms little precipitate in the region of antigen excess, as can be seen from curve III. The shapes of the precipitin curves given by the antigen and by the lipocarbohydrate obtained from formol-killed bacilli are nearly identical with the shape of the curve

given by the lipocarbohydrate derived from phenol-killed microorganisms (curves IV and V). From this it is apparent that the antigen prepared from formol-killed bacilli by extraction with 7 M urea (4) has undergone considerable chemical degradation by the method employed.

Antiviral Properties of the Phase II Antigen and Its Product of Degradation.—It will be recalled that although the antigen derived from formol-killed Phase II bacilli exhibited marked antiviral properties against certain of the T *coli*-dysentery phages, it was active against only three of the five phages to which this microorganism is susceptible. Our belief is that the antigen derived from phenol-killed bacilli represents the substance more closely as it occurs in its native state. A comparison of the antiviral properties of these two immunologically active materials and their products of degradation has therefore been made. The antiviral tests were performed as follows:—

0.5 ml. portions of a dilution of phage in nutrient broth, containing approximately 2×10^3 particles per ml., were placed in each of five tubes. To the first four tubes was added 0.5 ml. of the appropriate dilution of the substance to be tested. The material was first sterilized as previously described (1), dissolved in 0.1 M phosphate buffer, and then diluted in increments of 10 in nutrient broth. To the fifth tube, the virus control, was added 0.5 ml. of nutrient broth. The tubes were incubated for 18 hours at 37°C., and finally, 0.1 ml. portions of the inhibitor-virus mixture were plated with Phase II *Sh. sonnei* (6). In the case of T_3 and T_7, plaques were counted after 8 hours of incubation at 37°C., and after 18 hours in the case of T_2, T_4, and T_6. The plating of T_7 was made on neopeptone–meat infusion–agar, whereas the other phages were assayed on nutrient broth–agar.

From the results of these tests, which are presented in Table II, it can be seen that the purified antigen derived from phenol-killed bacilli inhibits all five T phages, although its antiviral activity is not great. Concentrations ranging between 100 and 10 μg. per ml. were necessary to obtain inactivation of 50 per cent of the T_2, T_4, T_6, and T_7 phages. The T_3 phage was inhibited even less; only 43 per cent was inactivated by 1 mg. of antigen. After digesting the antigen with pancreatin, however, much smaller quantities were required to achieve this effect. The enzymatically degraded antigen, even at concentrations as low as 1 μg. per ml., inactivated some 80 to 90 per cent of the T_2, T_4, and T_7 phages, and the inhibition of T_3 was increased nearly tenfold. The T_6 phage, on the other hand, was inhibited less vigorously by the degraded antigen than by the material before treatment with the enzyme.

The tests reveal that the antigen isolated from formol-killed bacilli inhibits only T_4, T_7, and to some extent T_3, and is without effect upon T_2 and T_6. Furthermore, its activity against T_7 is considerably lower than that of the enzymatically degraded antigen derived from phenol-killed organisms.

In order to learn why the antigen prepared from formol-killed bacilli does not inactivate T_2 and T_6, a study was made of the effect of the chemical reagents employed in its isolation, on the fully active inhibitor. Treatment of

the enzymatically degraded antigen with 1 per cent formalin or with 7 M urea solution in the cold failed to diminish its antiviral activity. However, exposure of this substance to 50 per cent pyridine for 24 hours at 37°C. destroyed completely its activity for T_2 and T_6, but had no effect in so far as its activity against T_3, T_4, and T_7 was concerned. Since pyridine is used in the preparation of the antigen from formol-killed bacilli, it is undoubtedly this step which causes partial inactivation of the antigenic complex.

TABLE II

Inactivation of T-Phages by Different Preparations of the Somatic Antigen of Phase II Sh. sonnei

Substance tested	Phage tested	Final concentration of antigen in virus-antigen mixture*			
		1 mg.	0.1 mg.	0.01 mg.	0.001 mg.
Purified antigen from phenol-killed bacteria	T_2	87	72	46	0
	T_3	43	14	6	4
	T_4	97	96	60	2
	T_6	100	93	31	0
	T_7	98	68	10	0
Enzymatically degraded antigen	T_2	96	95	97	92
	T_3	72	52	26	0
	T_4	100	100	98	88
	T_6	47	34	10	2
	T_7	98	95	95	84
Purified antigen from formol-killed bacilli	T_2	0	0	0	0
	T_3	57	14	0	0
	T_4	98	97	92	91
	T_6	0	0	0	0
	T_7	92	54	34	9

* The figures represent per cent of total phage inactivated.

The treatment of the enzymatically degraded antigen with phenol also destroys the activity against T_2 and T_6, for the lipocarbohydrate obtained by this procedure inhibits only the T_3, T_4, and T_7 phages. The degree of inhibition however, is somewhat lower than that shown by the material from which it was derived. The protein fractions obtained by dissociation of the degraded antigens in phenol, show no appreciable antiphage activity.

During the course of this study a number of preparations were isolated by the methods described, and tested for antiviral activity. In general, all preparations, regardless of the method of isolation, inhibited strongly T_4 and T_7, whereas the inhibition of T_3 was usually not great. T_2 and T_6 were inhibited only by antigens obtained from phenol-killed microorganisms. A few exceptions were encountered, however; one preparation from phenol-killed bacilli showed

low activity against T_4, whereas another failed to inhibit T_6. On the other hand, two preparations of the somatic antigen prepared in this laboratory in 1948 (1) inhibited the T_3 virus vigorously and maintained this property for several years. The reasons for the occasional variation in activity of these antigen preparations are not fully understood.

DISCUSSION

Miles and Pirie (29) showed that the native antigen of *Brucella melitensis* could be extracted from bacteria which had been killed with dilute phenol, acetic acid, or chloroform; when other bactericidal agents were employed, only degradation products of the native antigen could be isolated. Analogous findings have now been obtained with Phase II *Sh. sonnei*. When this microorganism is killed by heat or by dilute formalin, only small amounts of material, having low serological activity, can be isolated by extraction with distilled water at 65°C. In order to obtain the antigen it is necessary to employ potent agents, for example 50 per cent pyridine and concentrated urea solutions. When the bacilli are killed by dilute phenol, on the other hand, some 10 per cent of bacterial substance is released when the cells are extracted with warm distilled water. The somatic antigen thus obtained differs in its protein content from that isolated from formol-killed bacilli (4). The substance isolated by the first mentioned procedure has a nitrogen content of 12 per cent, whereas that of the antigen isolated from formol-killed microorganisms is much lower (4 to 5 per cent).

The Phase II somatic antigen is a complex substance which can be designated as a phosphorylated lipomucoprotein constituted from a large protein moiety firmly linked to a phosphorylated lipocarbohydrate. A number of fractionation procedures were employed in an attempt to learn whether the substance is a true chemical complex and not a mixture of protein, lipid, and polysaccharide. In no case was it possible to obtain fractions having a nitrogen content of less than 11 per cent. This fact, together with the observation that the antigen shows a high degree of homogeneity on electrophoresis, leaves little doubt that the protein and lipocarbohydrate moieties form a single macromolecule. In view of this and because of the mild procedures whereby the substance itself was isolated, it is believed that the material closely resembles the antigen as it occurs *in situ*.

This concept is substantiated by the fact that the antigen has marked biological properties; it inactivates *in vitro* all five phages which attack the parent microorganism. It is suggested that its antiviral activity is an attribute of the molecule as a whole, and that the presence of certain specific groupings is essential in order that it can function in its entirety. By chemical treatment it is possible to deprive the molecular complex of one or more of its antiviral functions. Thus, degradation by proteolytic enzymes destroys for the greater part

its ability to inhibit T_6, but leaves intact its capacity to inactivate T_2, T_3, T_4, and T_7. Similarly, its antiviral activity in so far as both T_2 and T_6 are concerned, can in turn be eliminated by subjecting the substance to the action of phenol or aqueous pyridine, again leaving intact its affinity for T_3, T_4, and T_7. This observation indicates that the capacity to combine with these three phages and inactivate them is a function of the lipocarbohydrate portion of the lipomucoprotein, a concept which is supported in part by the work in the accompanying paper (30). It is important to bear in mind that those groupings which determine the serological specificity of the intact antigen are not identical with those which endow it with antiviral properties for T_2 and T_6. On the other hand, since the specific immunological properties of the antigen are determined predominantly by its carbohydrate moiety, its antiviral activity against T_3, T_4, and T_7 would appear to be related very intimately to the structure of this portion of the complex.

SUMMARY

1. The somatic antigen of Phase II *Shigella sonnei* can be isolated by extracting phenol-killed microorganisms with water. This substance inactivates all T phages to which this bacillus is susceptible.

2. The Phase II antigen is constituted from a protein and a phosphorylated lipocarbohydrate. The major portion of the protein component can be removed by digestion with pancreatin. The enzymatically degraded antigen thus obtained can be further dissociated into its protein and lipocarbohydrate components by treatment with 90 per cent phenol. Glucose, galactose, glucosamine, and an aldoheptose have been identified as the monosaccharide constituents of the lipocarbohydrate.

BIBLIOGRAPHY

1. Miller, E. M., and Goebel, W. F., *J. Exp. Med.*, 1949, **18,** 255.
2. Barry, G. T., and Goebel, W. F., *J. Exp. Med.*, 1951, **94,** 387.
3. Dole, V. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **43,** 122.
4. Baker, E. E., Goebel, W. F., and Perlman, E., *J. Exp. Med.*, 1949, **89,** 325.
5. Libby, R. L., *J. Immunol.*, 1938, **34,** 269; **35,** 289.
6. Hershey, A. D., Kalmanson, G. M., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46,** 267.
7. Goebel, W. F., *J. Exp. Med.*, 1950, **92,** 527.
8. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46,** 2066.
9. Allen, R. J. L., *Biochem. J.*, 1940, **34,** 858.
10. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1948, **176,** 367.
11. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100,** 695.
12. Sørensen, M., *Compt.-rend. trav. Lab. Carlsberg*, 1938, **22,** 487.
13. Elek, A., and Harte, R. A., *Ind. and Eng. Chem., Anal. Ed.*, 1936, **8,** 267.
14. Tiselius, A., *Nova Acta Regiae Soc. Sc. Upsala*, series 4, 1930, **7,** No. 4. Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, **61,** 523.

15. Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, 1941, **35,** 1140.
16. Dische, Z., *Mikrochemie*, 1929, **1,** 33. Holzmann, G., McAllister, R. V., and Nieman, C., *J. Biol. Chem.*, 1947, **171,** 27.
17. Dische, Z., *J. Biol. Chem.*, 1949, **181,** 379. Hahn, L., and von Euler, H., *Ark. Kemi Mineral. och Geol.*, 1946, **22A,** No. 23, 6.
18. Dische, Z., *Mikrochemie*, 1930, **8,** 4.
19. Dische, Z., *J. Biol. Chem.*, 1947, **167,** 189.
20. Dische, Z., and Borenfreund, E., *J. Biol. Chem.*, 1951, **192,** 583.
21. Dische, Z., Shettles, L. B., and Osnos, M., *Arch. Biochem.*, 1949, **22,** 169.
22. Ikawa, M., and Nieman, C., *J. Biol. Chem.*, 1949, **180,** 923.
23. Benevenue, A., and Williams, K. T., *Arch. Biochem. Biophysic.*, 1951, **34,** 225.
24. Williams, R. J., and Kirby, H., *Science*, 1948, **107,** 481.
25. Malyoth, G., and Stein H. W., *Biochem. Z.*, 1951, **165,** 322.
26. Partridge, S. M., *Biochem. J.*, 1948, **42,** 238, 251.
27. Flood, A. E., Hirst, E. L., and Jones, J. K. N., *Nature*, 1947, **160,** 86.
28. Todd, W. R., Vreeland, J., Myers, J., and West, E. S., *J. Biol. Chem.*, 1939, **127,** 269.
29. Miles, A. A., and Pirie, N. W., *Brit. J. Exp. Path.*, 1939, **20,** 83, 109, 278.
30. Goebel, W. F., and Jesaitis, M. A., *J. Exp. Med.*, 1952, **96,** 425.