

STUDIES ON THE FLEXNER GROUP OF DYSENTERY BACILLI

I. THE SPECIFIC ANTIGENS OF *SHIGELLA PARADYSENTERIAE* (FLEXNER)*

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(Received for publication, January 16, 1945)

Microorganisms belonging to the group *Shigella paradysenteriae* (Flexner) are of considerable importance from an epidemiological point of view, and hence we have undertaken a study of their specific antigens. In this and subsequent reports the isolation and properties of the antigens of several types of Flexner dysentery bacilli are given together with an account of their immunological characteristics.

The isolation of cell-free antigenic constituents from Gram-negative microorganisms has been reported by a number of investigators; most notable among these are Boivin (1) and his collaborators who have studied the specific antigens of *Salmonella typhimurium*, *Salmonella enteritidis*, *Eberthella typhosa*, *Escherichia coli*, etc., and Topley (2) who has described the specific H and O antigens obtained from enzymatic breakdown products of *E. typhosa*. Perhaps the most comprehensive study regarding the nature of antigenic fractions from Gram-negative bacilli is that of Morgan and Partridge who have studied in detail the specific antigens of the Shiga (3) and typhoid (4) bacillus and have investigated their chemical and immunological properties.

The chemical nature of antigens derived from Gram-negative bacilli is not yet fully understood. Boivin regarded these substances as belonging to a class of compounds known as lipocarbohydrates. The early investigators failed, however, to characterize a protein or protein-like constituent which was shown by later workers to form a part of the type specific antigens derived from the Shiga and typhoid bacillus. It remained for Morgan and Partridge (3) to demonstrate that this protein forms an important constituent of the antigenic complex of both organisms and it has many unusual chemical and immunological properties.

EXPERIMENTAL

Cultivation of Bacteria.—The types V, W, Z, *Shigella paradysenteriae* (Flexner) used in this study were obtained from the U. S. Army Medical Center. The Newcastle strain was sent us by Dr. Carl TenBroeck of Princeton. The organisms were grown in a broth consisting of 50 gm. of bactotryptone, 100 cc. of blood extract (5), 400 gm. $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, 100 gm. KH_2PO_4 , and 25 liters of tap water. Copious growth was obtained in 18 hours. As a rule, 50 liters of culture medium yielded 25 gm. of dry organisms. The living bacteria were col-

* The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

lected in a Sharples centrifuge, killed, and desiccated by pouring a thick aqueous suspension into 10 volumes of acetone.¹

Quantitative Precipitin Reactions.—The immunological activity of the various fractions isolated during the purification of the specific antigens was determined by means of the quantitative precipitin reaction. Varying concentrations of the fraction under study were added to a constant amount of a standard homologous antiserum diluted 1:10. After standing 30 minutes, the turbidity which developed was determined by means of a photoelectric turbidimeter (6). The turbidity, which is proportional to precipitated antibody nitrogen, was recorded in arbitrary galvanometer units, as will be seen in the accompanying figures. This procedure enabled one to ascertain both qualitative and quantitative differences in the immunological activity of the fractions under study and was of great assistance in following the purification of the specific antigens and their separation from inert contaminants.

Electrophoresis.—Purification of the antigenic fraction was also followed by frequent electrophoretic analyses using the standard Tiselius apparatus (7) and the Longworth (8) schlieren scanning device. The procedure is well known and will not be described here.

Preparation of the Type V Specific Antigen

1. Extraction with Diethylene Glycol.—280 gm. of acetone-killed desiccated Type V organisms were extracted with 2.8 liters of diethylene glycol for 3 days at 37°C. according to the procedure of Morgan (3). The cells were centrifuged from the solvent and reextracted twice more. The combined extracts were passed through a Berkefeld filter and poured into 5 volumes of chilled acetone. The precipitated crude antigen was collected by centrifugation, washed with acetone, and dried. 15.5 gm. were recovered (Fraction 15).

The material was dissolved in 200 cc. of water and dialyzed. A considerable quantity of yellow pigment and diffusible extractives passed through the membrane. The material in the sac was concentrated to 100 cc. *in vacuo* and centrifuged for 15 minutes at 17,000 r.p.m. to remove a small amount of insoluble residue (0.2 gm.) which was discarded (Fr. 15B). The active material was recovered from the supernatant liquid by freezing and desiccation (Fr. 15A). 7.88 gm. were obtained. This substance contained 4.1 per cent nitrogen and yielded 55 per cent reducing sugars (calculated as glucose) on hydrolysis. Fraction 15A was dissolved in 100 cc. of water and shaken with octyl alcohol and chloroform (1:4) followed by centrifugation. This process was repeated several times. The emulsion layer was carefully washed and the washing added to the main fraction. The latter was again thoroughly dialyzed and yielded 4.84 gm. of active material (Fr. 15C). The emulsion layers on evaporating the solvent yielded 0.65 gm. of substance (Fr. 15D). The latter had a higher nitrogen content than Fr. 15A and its serological activity was approximately the same. Since this procedure resulted in considerable loss of active material, it was not used in subsequent preparations.

Fraction 15C was further purified as follows: 4.5 gm. of material were dissolved in 200 cc. of H₂O and chilled to 0°C. One volume of cold acetone was added and the precipitate (Fr. 15E, 0.3 gm.) separated. A second volume of acetone yielded 2.05 gm. of substance (Fr. 15F), whereas the supernatant liquid on evaporating the solvent and freezing and drying gave 1.8 gm. (Fr. 15G). Refractionation of 15F with acetone gave 1.6 gm. of active material (Fr. 15F₂) which was precipitated between 50 and 66 per cent acetone concentration.

During the course of the purification of the original glycol extract frequent determinations of the activity of the material were made by means of the

¹ We are indebted to Dr. W. A. Jamieson, of the Eli Lilly Company, Indianapolis, for his generous cooperation in furnishing us with dysentery bacilli.

quantitative precipitin reaction, the results of which are given in Fig. 1. Here it is seen that activity of the product is greatly increased during the purification process. Analyses revealed that acetone fractionation eliminated contaminants of higher nitrogen and lower activity than the end product. The analytical constants of the purified product (Fr. 15F₂) are given in Table I.

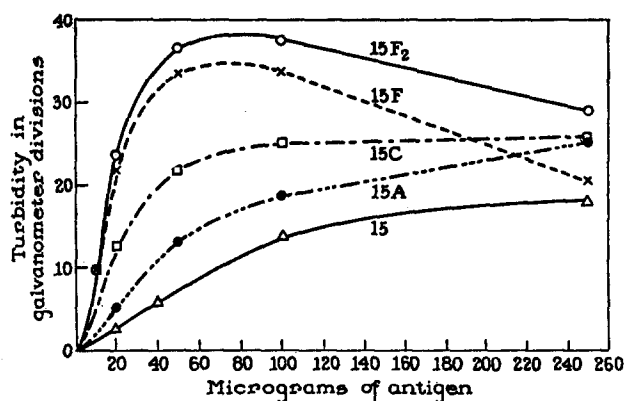


FIG. 1. Turbidometric titration of active fractions obtained during the purification of Type V antigen.

TABLE I
Specific Antigens of Shigella paradysenteriae (Flexner)

Specific antigen	$[\alpha]_D$	C	H	N	P	Lipid	Acetyl	Reducing sugars after hydrolysis
<i>type</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
V	+22.0°	47.00	6.89	6.16	1.50	12.0	5.95	40.0
W		44.13	7.09	5.67	1.91	8.3	6.00	39.4
Z	+32.8°	44.32	6.79	5.64	1.91	10.5	6.54	49.0
Sp. Newcastle	+32.8°	46.11	6.82	6.49	1.80	15.0	5.91	31.0

2. *Extraction with Aqueous Pyridine.*—Extraction of Type V organisms with diethylene glycol fails to remove all of the specific antigen. It is apparent, furthermore, that the method is not selective for it removes a certain amount of dialyzable and inert material. We have found considerable variation in the efficacy of the method depending upon the organism studied. For example, practically all of the specific antigen (together with extraneous and inert material) can be extracted from Type Z organisms with glycol. On the other hand, the strain of Newcastle organism studied yields no specific antigen whatsoever when extracted with this substance.

A more effective solvent for extracting the specific antigens from the Flexner organisms is 50 per cent pyridine. This solvent is less specific than is diethylene

glycol for it extracts not only the antigen but some nucleic acid and an immunologically inert polysaccharide as well. However, by making use of the fact that the specific lipocarbohydrate-protein complexes are soluble in 50 per cent acetone (3) it is possible to eliminate both nucleic acid and the inert carbohydrate by subsequent acetone fractionation of the pyridine extract. The method used is as follows:

120 gm. of Type V organisms, which had been previously extracted three times with diethylene glycol, were suspended in 1 liter of 50 per cent pyridine. After standing at 37° for 24 hours the bacteria were separated by centrifugation. The extraction was repeated and the combined supernatant liquids were passed through a Berkefeld filter. The pyridine was distilled *in vacuo*, and the opalescent solution was thoroughly dialyzed. The solution, at a volume of 300 cc., was cooled to 0° and a half-volume of chilled acetone was added. A precipitate of nucleic acid and inert polysaccharide was separated by centrifugation and the pale yellow supernatant liquid containing the antigen was brought to 66 per cent acetone concentration by the addition of 1½ volumes of acetone. The precipitated antigen was separated and the supernatant liquid containing the hapten was saved.

The precipitate was dissolved in 100 cc. of water, chilled, and an equal volume of acetone added. After standing 24 hours at 0° a small amount of precipitate was separated by centrifugation and discarded. The addition of one more volume of acetone precipitated the antigen. The latter was dissolved in water, electro-dialyzed, then frozen and desiccated, 4.9 gm. of the purified antigen were recovered.

The supernatant solution containing the hapten was evaporated, electro-dialyzed, and isolated as above. 0.4 gm. was recovered. Both the antigen and the hapten isolated from the bacteria previously extracted with glycol showed the same analytical constants as the products obtained by the initial glycol extraction. Furthermore, the pyridine-extracted antigen had the same toxicity and precipitability as the glycol-prepared antigen.

The Immunologically Inert Polysaccharide.—During the purification of the Type V specific antigen, as was pointed out above, a fraction is obtained which is insoluble in 33 per cent acetone. This material consists largely of nucleoprotein, nucleic acid, and an immunologically inert polysaccharide. The latter was separated and purified as follows:—

The dialyzed pyridine extract from Type V organisms was treated with ½ volume of chilled acetone, as previously described. The precipitate was separated by centrifugation. This material was dissolved in 100 cc. of water and sufficient normal alkali added to render everything soluble (about pH 8.0). The solution was now adjusted to pH 5.10 and an excess of saturated cupric acetate added. After standing overnight, the precipitated material was separated by centrifugation and discarded. To the supernatant liquid was added some 10 gm. of sodium acetate, and the inert polysaccharide precipitated by the addition of 4 volumes of alcohol. After several reprecipitations the polysaccharide was dissolved in water and the solution electro-dialyzed, then frozen and dried.

The inert polysaccharide is obtained as a snow white, amorphous powder readily soluble in water to yield a clear solution which neither foams nor is viscous. The polysaccharide has a specific rotation of +185° and contains no nitrogen or phosphorus. On hydrolysis it yields approximately 98 to 100 per

cent reducing sugars calculated as glucose. Glucosazone can be readily prepared from the hydrolysis mixture, and when the latter is oxidized with nitric acid, potassium acid saccharate can also be separated. The specific rotation of a hydrolyzed solution of the polysaccharide gives a value nearly identical with that of pure glucose. It is our belief that this polysaccharide is constituted solely from glucose molecules. The substance is not identical with either glycogen or starch; it gives no color with iodine, and is not hydrolyzed by takadiastase, ptyalin, or by a very active fungus amylase. This would indicate that the glucose molecules are coupled in a position other than 4. The electrophoretic properties of this material will be discussed later.

Specific Antigens of Other Types.—The specific antigens of Types W, Z, and the Newcastle strain of *Shigella paradysenteriae* were prepared by both of the above procedures. In general, we prefer the use of aqueous pyridine, for not only is the yield better, but two extractions suffice to remove the major portion of the antigen. We have found the yield of antigen to vary considerably depending upon the type of organism employed and no doubt upon the strain as well. The chemical properties of these substances are given in Table I.

Chemical Properties of the Shigella paradysenteriae Antigens.—The specific antigen of Type V *Shigella paradysenteriae* is a highly purified product which has been separated from accompanying impurities by mild chemical procedures. The complete antigen is a protein-phospholipid-carbohydrate complex resembling in its gross chemical properties the antigens of the Shiga and typhoid bacillus described by Morgan (3, 4). The substance after desiccation is difficultly soluble but dissolves readily in 0.05 molar NaHCO_3 to give slightly opalescent solutions which foam on shaking. A 1 per cent solution of the antigen gives strong positive tests both for carbohydrate (Molisch) and for protein (biuret). The material has a specific rotation of $[\alpha]_D = +22^\circ$. The substance contains 6.5 per cent nitrogen and 1.5 per cent phosphorus (9). On prolonged alkaline hydrolysis at 100° followed by acidification and extraction with ether, the material yields some 12 per cent of lipid (10). The antigen yields 40 per cent reducing sugars (calculated as glucose) on hydrolysis (11). The antigen is not precipitated from solution by CuSO_4 , BaCl_2 , HgCl_2 , or AgNO_3 nor does picric or trichloroacetic acid cause precipitation. Heating with 1 per cent acetic acid destroys the antigen and yields a mixture of an insoluble protein (11 per cent N), a phospholipid, and an immunologically active carbohydrate. In general the physical and gross chemical properties of the antigens derived from Types W and Z *Shigella paradysenteriae* and from the Newcastle strain are essentially the same as those of the Type V.

The Type V Antigen Prepared by Tryptic Digestion.—The extraction of young smooth Type V cells with diethylene glycol by no means removes all the type-specific antigen. By subjecting the extracted cells to digestion with Fairchild's trypsin a goodly quantity of antigenic material is liberated together

with a large quantity of an inert polysaccharide and nucleic acid. The immunologically active fraction can be obtained from the digest by further fractionation with acetone.

Cells which had been thoroughly extracted with glycol were suspended in $\mu/20$ phosphate buffer at pH 8.1 and digested with Fairchild's trypsin until the mixture became fairly clear (2 to 3 days). The digest was dialyzed for several days against successive changes of distilled water. The insoluble residue, largely cellular debris, was removed by centrifugation, the supernatant liquid concentrated to small volume *in vacuo*, and the free protein and nucleic acid precipitated with copper acetate at pH 5.1. After electro dialysis the solid material which consisted of an inert polysaccharide and specific antigen was obtained by freezing and desiccation. The specific antigen was separated from the inert polysaccharide by extraction with diethylene glycol. Following dialysis the antigen was separated from solution by repeated fractionation between 50 and 60 per cent acetone concentration. The yield of purified product averaged about 3 gm. per 100 gm. of organisms employed.

The antigen obtained by enzymatic degradation of extracted cells differs in its chemical properties from that secured by glycol extraction, and, in our opinion, represents a degradation product of the latter. The substance has a specific rotation of $[\alpha]_D = +60^\circ$, a nitrogen content of about 3.0 per cent, and yields 60 per cent reducing sugars (calculated as glucose) on hydrolysis. The product contains 11 per cent fat and 12.0 per cent glucosamine (12). When examined by electrophoresis the material appears to be homogeneous.

If unextracted whole cells are subjected to the same treatment, an identical product is obtained but in greater yield. The "tryptic" antigen has about the same toxicity as the glycol-extracted antigen, will precipitate almost as much type-specific antibody from a given quantity of serum, and gives rise to antibodies when injected into rabbits.

Electrophoretic Properties of the Type V Antigen and Specific Hapten.—An electrophoretic analysis of the purified Type V antigen prepared by glycol extraction was performed with a 1.3 per cent solution in barbital buffer of ionic strength 0.05 and a pH of 8.65. It was found that the material is not altered in any way when exposed to this pH. The pattern in the descending limb is shown in Fig. 2. The migration of the material is anodic and its mobility under these conditions is 3.8×10^{-6} cm.²/volt sec. The arrow in the figure indicates the position of the initial boundary. Although the ϵ boundary is due to known salt concentration gradients, its size and mobility made it necessary to rule out the possibility of another active component. Accordingly the material causing the ϵ boundary and that forming the major boundary were recovered and tested by means of the quantitative precipitin reaction. The activity was found to be associated only with the single major component. From the pattern in Fig. 2 it can be seen that the material is essentially homogeneous electrophoretically. The leading edge of the electrophoretic pattern shows a slight asymmetry which may be due either to

contamination with the inert polysaccharide to be described later or to the viscosity of the solution.

In order to determine whether the homogeneity of the material was due to the fortuitous selection of a pH value at 8.65, patterns were obtained over a range of pH values at a constant ionic strength of 0.05 using monovalent buffers. The mobility of the antigen at these various values of pH is represented graphically in Fig. 3. In all instances the patterns obtained were homogeneous and essentially symmetrical. From the pH-mobility curve

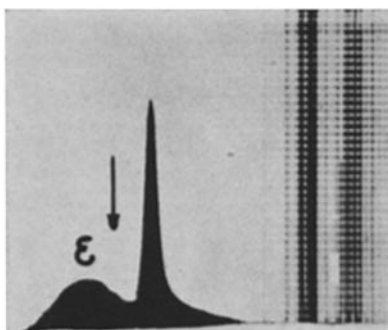


FIG. 2. Electrophoretic pattern of purified Type V antigen.

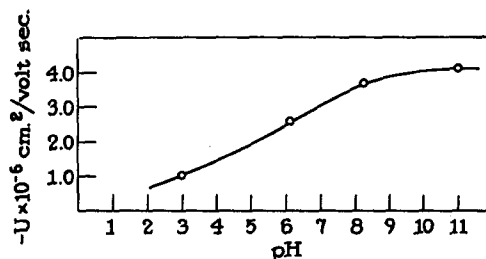


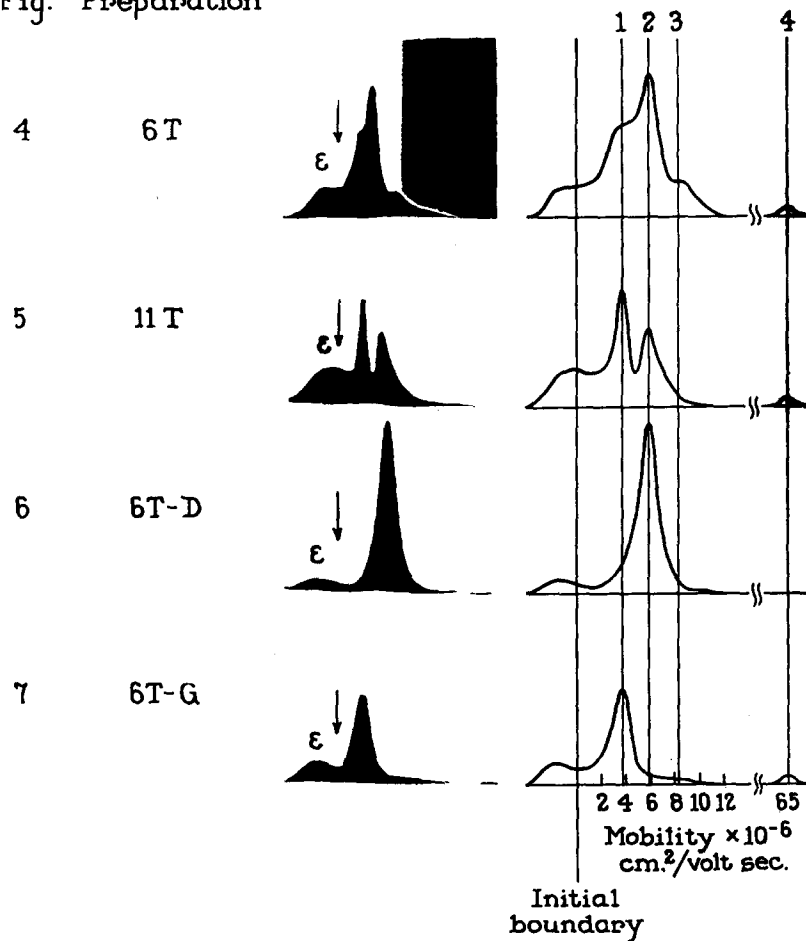
FIG. 3. The mobility-pH curve of Type V antigen.

shown in Fig. 3 it is apparent that the antigen does not have an isoelectric point over the pH range studied.

The fractionation of the Type V antigen from material prepared by tryptic digestion of Type V organisms was followed by frequent electrophoretic analyses at pH 8.65. A detailed study of the tryptic preparation revealed several points of interest. The patterns obtained from two different tryptic preparations are shown in Figs. 4 and 5. Here it can be seen that the material contains at least four components. Quantitative tests of the toxicity and precipitability in immune serum of these two preparations revealed that their activity is proportional to the relative concentration of the component having a mobility of $3.8 \pm 0.2 \times 10^{-6} \text{ cm.}^2/\text{volt sec.}$ and signified as number 1 in the diagram.

Component number 4 was shown to be identical with the toxic protein constituent of the antigen which appears to be uncombined with the hapten and phospholipid. This material will be described in greater detail in a subsequent communication.

Fig. Preparation



FIGS. 4 to 7. Electrophoretic patterns of fractions obtained from Type V *Shigella paradyserteriae* by enzymatic degradation.

A 2 per cent solution of one of the tryptic preparations (6T) was made in anhydrous formamide. A small amount of insoluble material was separated by centrifugation. The addition of one volume of alcohol to this solution gave a precipitate (6T-D) the electrophoretic pattern of which is shown in Fig. 6. The material soluble in 50 per cent formamide (6T-G) was recovered and its electrophoretic pattern is given in Fig. 7. It can be seen that Fr.

6T-D shows electrophoretic homogeneity and the material was found to be identical with the immunologically inert polysaccharide described above. The mobility of this material is 5.9×10^{-6} cm.²/volt sec. and is the same as that of component number 2 in Figs. 4 and 5. The pattern of Fr. 6T-G given in Fig. 7 apparently represents the residual material in which the concentration of immunologically active material is predominant.

The electrophoretic analyses of several preparations of the specific hapten reveal that the mobility of the latter ($3.3 \pm 0.3 \times 10^{-6}$ cm.²/volt sec.) is but slightly less than that of the complete antigen. The pattern of the descending boundary of one of these preparations shown in Fig. 8 reveals that the material is essentially homogeneous.

It has been seen that the toxic protein constituent has a mobility of 65×10^{-6} cm.²/volt sec., or some 17 times as great as that of the complete antigen.

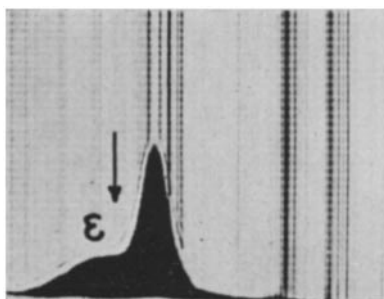


FIG. 8. Electrophoretic pattern of Type V specific hapten.

Furthermore, the toxic protein is insoluble at its isoelectric point (approximately pH 4.0). Since neither the hapten nor the complete antigen precipitates at any pH value it is suggested that the mobility and perhaps some of the chemical properties of the complete antigen are determined by the polysaccharide moiety.

The Type Specific Haptens.—During the preparation of the antigens from the different types of organisms by glycol extraction there remained a fraction which is not precipitated by 2 volumes of acetone. The material can be isolated by evaporation of the solvent. In each instance this fraction was found to be an immunologically active polysaccharide. The respective substances precipitate in dilutions of 1×10^{-6} in antisera prepared by immunization of rabbits with organisms of the homologous type. The polysaccharides, regardless of the type from which they were derived, contained neither protein nor phospholipid, and, in our opinion, represent the carbohydrate hapten of the antigenic complex. The polysaccharides differ from one another in their specific rotations and nitrogen content. Since these carbohydrates are free of proteins and protein degradation products, the nitrogenous constituent is believed to be an amino hexose. On acid hydrolysis all the polysaccharides

yielded reducing sugars. Quantitative determinations for glucosamine (12) made on the hydrolysis mixture reveal that the nitrogen constituent cannot in all instances be accounted for as hexosamine. This discrepancy is due, in our opinion, to differences in the intramolecular linkages of the constituent saccharide units of the various haptens. It is obvious from Table II that practically all the hexosamine is liberated on acid hydrolysis of the Type V hapten and that the nitrogenous constituent is fully accounted for. In the case of the Types W and Z haptens, however, only part of the nitrogen appears as hexosamine, the aldehydic groups of the remaining amino sugar must necessarily remain in glycosidic union resistant to acid hydrolysis.

The haptens differ in their serological properties from the respective protein-carbohydrate antigens in that they precipitate less antibody nitrogen from homologous antiserum and are neither toxic nor antigenic when injected into

TABLE II
Specific Haptens of Shigella paradysenteriae (Flexner)

Specific hapten	$[\alpha]_D$	C	H	N	Acetyl	Glucosamine	Reducing sugars after hydrolysis
<i>type</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
V	+21.0°	44.02	6.74	2.06	13.85	21.5	79.0
W*	+32.7°	38.49	6.66	3.10	7.03	12.2	65.6
Z	+26.0°	44.53	6.74	3.37	12.14	18.6	68.1

* This preparation was not electrolyzed and had an ash content of 7.77 per cent.

rabbits. The analyses of specific haptens from three Flexner types are given in Table II.

Amino Acid Analyses.—The purified Type V antigen was analyzed for certain amino acids. Various preparations were all found to contain tyrosine, tryptophane, histidine, and arginine, whereas methionine and cystine were absent. Quantitative analyses revealed that the antigen prepared by glycol extraction contained 4.3, 0.9, and 0.6 tyrosine, arginine, and histidine, respectively. The percentages of these amino acids in the antigens prepared by tryptic digestion were 1.4, 0.4, and 0.2, respectively. The difference in these analyses lends support to the view that the antigen prepared by tryptic hydrolysis represents a degradation product of the native, or glycol-prepared antigen.

Immunological and Toxic Properties of the Specific Antigens.—The antigens isolated from Types V, W, and Z *Shigella paradysenteriae* and from the Newcastle strain are toxic and highly antigenic. Rabbits injected intravenously with 10, 40, and 100 μ g. on alternate days and bled 7 days later yield antisera which in high dilution agglutinate the organism from which the antigen is derived. The results of such experiments are given in Table III. The type-

specific haptens in all instances fail to evoke antibodies in rabbits, even after prolonged immunization. The intradermal administration of the antigens in rabbits in quantities of 100 to 500 μg . causes severe local and general reactions. The temperature remains elevated for 24 to 72 hours, and the site of injection becomes inflamed, edematous, and eventually an area of necrosis develops which may be several centimeters in diameter. Animals thus treated usually survive and their sera, a week to 10 days following the injection, agglutinate in high dilutions the organism from which the antigen is derived. Animals which have received one intradermal injection appear to be sensitized to subsequent intradermal injections of the antigen for they give rise to reactions which are even more extensive than the original.

TABLE III
Homologous Agglutinins in Sera of Rabbits Immunized with Specific Antigens of Shigella paradysenteriae (Flexner)

Antiserum	Final dilution of serum					
	1:200	1:400	1:800	1:1600	1:3200	1:6400
<i>type</i>						
V	4+	4+	4+	4+	3+	1+
Z	4+	4+	4+	3+	2+	1+
Sp. Newcastle	4+	4+	4+	4+	2+	0

Tubes incubated at 56° for 2 hours. Readings made after standing overnight at 0°.

4+ = Complete agglutination of microorganisms, clear supernatant liquid.

0 = no agglutination.

The specific antigens of the *Shigella paradysenteriae* are toxic both to mice and rabbits. Intraperitoneal injections in mice cause death when given in quantities of 500 μg . 20 gm. mice were injected intraperitoneally with a sterile saline solution of antigen. The animals were observed for 5 days. The results of such tests are given in Table IV where it is seen that the antigens of Types Z, W, and Newcastle are more toxic than the antigen derived from Type V. The toxicity of the various antigens in rabbits has not been ascertained, but in general it can be said that the rabbit is more susceptible than the mouse. Frequently an initial dose as small as 20 μg . given intravenously was found to kill rabbits.

Detoxification Studies.—As pointed out above, one of the important properties of antigens derived from Gram-negative organisms is their toxicity. Unfortunately, no detailed studies have been reported either as to the nature of the toxic constituent or in regard to detoxification of the antigen. Numerous and varied attempts have been made by us to detoxify the Type V antigen. These experiments have been singularly unsuccessful and have revealed the remarkable stability of the toxic component to ordinary chemical manipulation.

Experimental details need not be given. Let it suffice to say that treatment of the antigen with oxidizing agents such as N/10 sodium hypiodite or performic acid fails to impair its toxic properties. Acetylation of the antigen with ketene is without effect. Deaminization with nitrous acid likewise causes no diminution in toxicity. When the Type V antigen is treated with proteolytic enzymes such as trypsin or pepsin at the optimal pH, neither its toxicity nor its capacity to evoke antibodies is lost. Treatment with 2.0 per cent formalin at pH 8.5 over a period of 2 weeks fails to destroy its toxic properties.

TABLE IV
Toxicity of Specific Antigens of Shigella paradysenteriae (Flexner) in Mice

Antigen tested	Mg. of antigen injected			
	0.2	0.5	1.0	2.0
<i>type</i>				
V	S S S	D ₂₀ D ₃₆ S	D ₂₀ D ₂₀ D ₂₀	D ₂₀ D ₂₀ D ₂₀
W	D ₁₄ D ₃₈ S	D ₁₄ D ₁₄ D ₃₈	D ₁₄ D ₁₄ D ₁₄	D ₁₄ D ₁₄ D ₁₄
Z	D ₁₆ D ₂₂ S	D ₄₈ D ₄₈ S	D ₁₆ D ₁₆ D ₁₆	D ₁₆ D ₁₆ D ₂₂
Sp. Newcastle	D _{5 days} SS	D ₁₈ D ₁₈ D _{5 days}	D ₁₈ D ₁₈ D ₁₈	D ₁₈ D ₁₈ D ₁₈

D = death (subscript denotes hours survival after injection of material).
S = survival.

TABLE V
Toxicity of Type V Antigen before and after Irradiation

Time of irradiation	Mg. of material injected			
	0.62	1.25	2.50	5.00
<i>hrs.</i>				
0	D ₁₈ D ₁₈ S	D ₁₈ D ₁₈ D ₁₈	D ₁₈ D ₁₈ D ₁₈	— — —
1.5	S S S	D ₁₈ S S	D ₁₈ D ₃₆ S	D ₁₈ D ₁₈ D ₂₄
3.0	S S S	S S S	S S S	D ₂₀ D ₄₂ S
6.0	S S S	S S S	S S S	S S S

Coupling the diazonium salt of *p*-aminobenzoic acid to the tyrosine rest in the antigen yields a colored product the toxicity and immunological activity of which are essentially unaltered. In these important respects the antigen of Type V *Shigella paradysenteriae* differs markedly from the classical toxins of the diphtheria, tetanus, and gas gangrene bacilli.

Ultraviolet Irradiation.—The chemical procedures employed in the detoxification studies either failed to detoxify the antigens or were so drastic (acid hydrolysis) as to cause complete destruction of the antigenic complex. Since the specific antigens all show distinct absorption in the ultraviolet,² attempts

² The authors are greatly indebted to Dr. George Lavin for his kind assistance in conducting this part of the experimental work.

were made to detoxify the antigenic complex by irradiation with ultraviolet light.

An aqueous solution of the antigen (5 mg. per cc.) was placed in a quartz test tube 200 x 20 mm. which in turn was suspended in the center of a quartz spiral (13). The material was constantly stirred during the irradiation and samples were removed at various time intervals. Quantitative precipitin tests were made on the material after $1\frac{1}{2}$, 3, and 6 hours irradiation. Toxicity tests were likewise performed. 22 gm. mice were injected intraperitoneally and observed for 5 days. The results of these tests are given in Fig. 9 and Table V.

From the results presented in Table V it can be seen that after 3 hours irradiation the material has lost some 90 per cent of its toxicity. In this

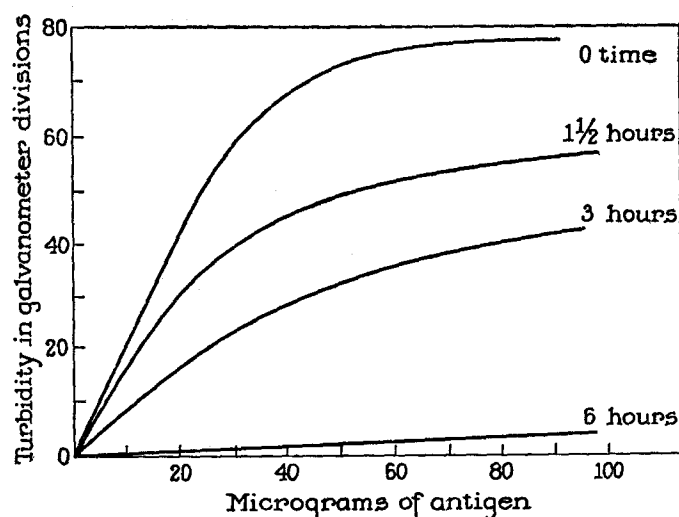


FIG. 9. Effect of ultraviolet irradiation on the precipitability of Type V antigen.

same time interval, however, the precipitability of the antigen (Fig. 9) in homologous antiserum is likewise impaired and the loss in activity closely parallels loss in toxicity. At the end of 6 hours irradiation the toxicity of the antigen is destroyed and its precipitability is likewise gone. Rabbits were immunized with the irradiated material. Although no protocols are given it may be said that those animals injected with a total of 150 μ g. of unirradiated material developed excellent precipitating and agglutinating antisera. Rabbits injected with the material irradiated for 6 hours showed no antibody response, whereas animals injected with the substance irradiated for 3 hours showed a weak antibody response. From this experiment it can be concluded that irradiation under the conditions described does not favorably affect the toxicity/antigenicity ratio.

DISCUSSION

The specific antigens of the *Shigella paradysenteriae* group of bacilli fall into that group of complex organic substances, the lipocarbohydrate proteins, to which so many antigens of the Gram-negative microorganisms are now known to belong. These substances can be extracted from the bacterial cell, presumably in an unaltered condition, by a variety of procedures. None of the methods can be considered ideal, but from the point of view of minimal chemical injury, the technique of Morgan seems most desirable. In our hands this procedure has not always been successful for in some instances the yield of antigen was low and from the Newcastle strain no antigen whatsoever was obtained. On the other hand, the use of aqueous pyridine gave good yields of antigen from strains of organisms which resisted glycol extraction. The method has the drawback, however, of extracting in some instances a considerable quantity of inert material; yet the fact that the lipocarbohydrate protein complexes are soluble in 50 per cent acetone permits them to be fractionated readily enough without incurring much loss. Whether obtained by glycol or pyridine extraction, the various preparations compare remarkably well in their properties and apparently no gross chemical alteration takes place during contact with the pyridine solvent.

The specific antigens of the Flexner organisms are intimately related chemically and, as will be shown in a later communication, immunologically as well. They are resistant to certain chemical manipulations but, like the antigens of other Gram-negative organisms studied by Boivin and by Morgan, they can be readily broken down into their constituents by acid hydrolysis, or by the action of alkaline alcohol.

The appearance of considerable quantities of specific hapten in the extracts of the various paradysentery types and the fact that a protein-like constituent, higher in nitrogen content than the complex itself, is obtained by fractionation of crude antigen extracts, suggests that the organisms contain enzymes which hydrolyze the specific complexes. Mesrobeanu (14) has evidence for such an enzyme in the *B. aertrycke* which hydrolyzes its specific antigen, but we have made no direct experimentation to prove this point in the case of the paradysentery microorganisms. That the toxic constituent of the antigenic complexes is also remarkably resistant to chemical manipulation is evident from the foregoing experimental account. We have as yet found no method for detoxifying the antigens without destroying their immunological efficaciousness. Ultraviolet irradiation which inactivates certain viruses and bacterial toxins, destroys the antigenicity of the complex as effectively as it destroys toxicity. To be sure, the conditions of these experiments, which we consider preliminary in nature, are not ideal, for no effort was made to exclude oxygen, but it is apparent that both an intense and prolonged irradiation is necessary to bring about this simultaneous change.

The tryptic digestion of Type V organisms yields an immunologically active product which is quite different from the complex obtained by direct solvent extraction. It is significant that this product retains its toxicity, and that its capacity to incite specific antibodies, though impaired, is by no means lost. A comparison of the nitrogen and amino acid analyses of the complete antigen and of the antigen prepared by tryptic digestion, reveals that a considerable portion of the protein moiety is hydrolyzed by enzymatic digestion. Even prolonged digestion of the complete antigen fails to destroy its toxicity and antigenicity. It appears, therefore, that a portion of the protein of the complex is resistant to the action of the enzymes of pancreatic trypsin, and it is not out of the question that this resistant nucleus represents the toxic component which at the same time renders the degraded complex antigenic.

A more detailed study of the dissociation of the specific antigen of the Type Z organism, and the immunological relationship of the components of the complex molecule will be presented later. That the antigen of Type V organisms can be used successfully for the production of antibodies in man will also be described in a separate communication. In addition it is hoped that a study of the serological interrelationship of the purified specific antigens of the Flexner organisms will dispel, to some extent at least, the confusion that exists regarding the antigenic mosaic of the *Shigella paradysenteriae* group. The present account records methods for the isolation of these substances, and some of their physical and chemical properties. To these we shall have occasion to refer later.

SUMMARY

1. Methods for the isolation of the specific antigens of Types V, W, Z, and Newcastle *Shigella paradysenteriae* are given.
2. The physical, chemical, toxic, and immunological properties of these substances are described.

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