STUDIES ON THE FLEXNER GROUP OF DYSENTERY BACILLI

II. The Chemical Degradation of the Specific Antigen of Type Z Shigella paradysenteriae (Flexner)*

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Boivin and his collaborators have investigated in some detail the decomposition of the specific antigens of Gram-negative microorganisms by hydrogen and hydroxyl ions (1). Under conditions of relatively mild acid treatment it was found that antigens derived from a variety of unrelated microorganisms were dissociated into their constituent components, a phospholipid and a serologically active polysaccharide. In addition, these investigators state that the antigens were rapidly destroyed by the action of dilute alkali. Our knowledge concerning the nature of antigens derived from Gram-negative bacilli was greatly advanced when Morgan and Partridge (2) found that, in addition to the phospholipid and polysaccharide components, a third constituent was liberated on acid hydrolysis. This substance proved to be a protein and was shown to be an important component not only of the specific antigen of the Shiga bacillus, but of the typhoid bacillus as well (3).

In our studies on the antigens of the Flexner group of dysentery bacilli we too have found protein constituents analogous to those described by Morgan and Partridge (2, 3). It has been observed that a protein-like moiety forms an integral part of the antigenic complex of each specific type of organism thus far investigated. In reviewing the work of other investigators it can be said in general that emphasis has been placed upon the chemical composition of the antigenic complexes, and but few data regarding the toxicity of the dissociation products have been given.

The isolation and chemical characterization of specific antigens from various types of *Shigella paradysenteriae* have been described in the preceding report (4). During the course of this investigation it became increasingly evident that the toxicity of the antigens is neither completely destroyed nor dissociated from the products of hydrolysis by certain chemical treatments. The toxic component was found to be quite resistant to a variety of chemical manipulations and was entirely destroyed only by methods which brought about destruction of the serological and antigenic properties of the antigens themselves. In the report which follows, our efforts have been directed toward a study of the dissociation of the antigenic complexes derived from Flexner microorganisms, paying particular attention to the fate of the toxic component. Although

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a pure toxic component has not yet been obtained, the data have enabled us to draw certain conclusions regarding its nature and have perhaps pointed the way toward its eventual chemical characterization.

EXPERIMENTAL

In the following account the results of a detailed study of the chemical degradation of the antigenic complex derived from Type Z *Shigella paradysenteriae* will be given. In carrying out this study we have made use of a nephelometric method for determining the precipitin reaction quantitatively (5). In ascertaining the action of hydrogen and hydroxyl ions upon the serological activity of the antigenic complex this procedure has been most helpful.

Measurement of Toxicity.—In order to test the toxicity of the various fractions under investigation a sterile solution containing from 1 to 10 mg. of material per cc. was injected intraperitoneally into mice 4 weeks old and weighing approximately 20 gm. The animals were observed for 5 days and their deaths were recorded. Each dilution of the material to be tested was injected into 3 or 4 mice, and the highest dilution which killed all animals in the group was considered as the lethal dose. Due to the scarcity of animals it was not possible to use greater numbers in the toxicity tests.

In order to simplify the presentation of results, the following symbols have been used to represent the constituent parts of the antigenic complex:

L— lipid component.
P— protein component.
C_{Ac}—carbohydrate hapten component, where Ac signifies labile acetyl groups.
C— carbohydrate component devoid of alkali-labile acetyl groups.
T— toxic component.

According to these symbols the antigenic complex as obtained in purified form from the bacterial cells is represented as $LC_{Ac}TP$.

Stability of the Type Z Antigen at Various Values of pH.—In order to determine the stability of the specific antigen derived from Type Z *Shigella paradysenteriae* to hydrogen and hydroxyl ions, solutions of the complex were heated to 56° in 0.1 molar buffer solutions at various pH values and the serological activity of the material was determined by means of the nephelometric precipitin reaction.

A solution of the Type Z antigen containing 1 mg. per cc. was made by dissolving a weighed sample in the appropriate volume of 0.1 molar buffer at pH 1.3, 3.0, 5.6, 6.8, 8.2, and 9.8. The solutions were allowed to stand at 56° and samples removed at the end of 24 hours. The serological activity of the solutions in a standard homologous antiserum was determined by means of the quantitative precipitin reaction as previously described. The results of these experiments are presented graphically in Fig. 1, where the turbidity developing from the interaction between antigen and antibody is expressed in terms of arbitrary galvanometer readings.

From the results shown in Fig. 1, it is evident that but little loss in serological activity takes place when the antigen is heated at pH values lying between 3.0 and 8.3, and that considerable loss of activity occurs at pH values beyond these limits.

Stability of the Type Z Antigen in 0.1 N NaOH.—From the preceding experiment it is seen that the greatest destruction of the antigen as measured by the loss of serological reactivity takes place at pH 9.8. In order to determine the effect of still stronger alkali, a solution of the antigen was treated as follows:—

33.8 mg. of the Type Z antigen were dissolved in 20 cc. of 0.1 N NaOH and the solution allowed to stand at 0°. Samples were removed at stated time intervals, neutralized, and their activities determined as above. The data are presented graphically in Fig. 2.

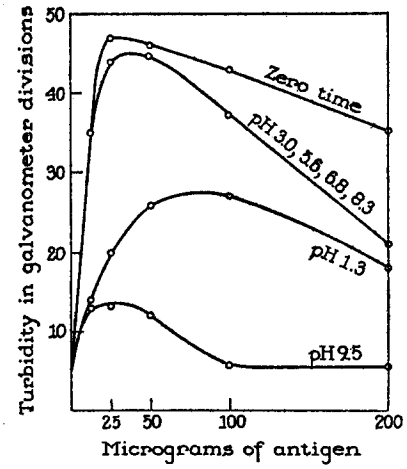

FIG. 1. Stability of the Type Z antigen at various values of pH.

It is seen from Fig. 2 that the serological activity of the antigen decreases rapidly on brief contact with alkali at 0° and that the reaction is complete within the first 40 minutes.

Degradation of the Type Z Antigen with Alkali.—Since alkali brings about a decided change in the serological activity of the specific antigen, it was considered of interest to determine the chemical nature of the changes involved.

1 gm. of Type Z antigen was dissolved in 30 cc. of water and cooled to 0°. 3 cc. of 20 per cent sodium hydroxide were added and after standing for 2 hours the solution was neutralized with glacial acetic acid. 66 cc. of saturated ammonium sulfate were then added with stirring. The mixture was allowed to stand for 1 hour and the precipitate removed by centrifugation. Treatment with alkali and ammonium sulfate was twice repeated. The combined supernatants and the precipitate were dialyzed to remove electrolytes and the non-dialyzable material was in each instance obtained by drying from the frozen state. Less than 50 mg. of material was found in the supernatant solution. The precipitate amounted

to 840 mg. This material was then fractionated with acetone as in the isolation procedure (4). 770 mg. of material were recovered.

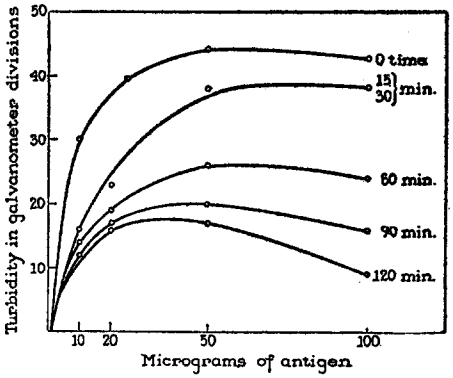

FIG. 2. Effect of 0.1 N NaOH on the precipitability of the Type Z antigen in homologous antiserum.

TABLE I

Properties of the Degradation Products Derived from the Specific Antigen of Type Z Shigella paradysenteriae

Substance	$[\alpha]_D$	Nitrogen	Phosphorus	Acetyl	Glucosamine	Reducing sugar on hydrolysis	L.D. for mice
	degrees	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*mg.*
Complete antigen, $LC_{Ac}TP$	—	6.79	0.99	5.05	10.8	35.5	0.25
Alkali-treated antigen, LCTP	—	6.71	0.81	3.07	—	32.4	0.25
Hapten (natural), C_{Ac}	+26	3.15	0.27	12.4	18.6	68.1	>20
Hapten (by acid degradation), C_{Ac}	+26	2.88	0.25	10.3	18.6	63.5	>20
Hapten (by alkaline degradation), CT	+35	2.72	1.72	4.5	16.4	62.6	0.5
Hapten (from LCTP by acid), C	—	2.20	0.16	5.83	—	62.5	>20
Degraded hapten (alkali), C	—	2.20	0.32	5.94	—	63.0	>20
Degraded hapten (acid), C	—	2.20	0.67	5.63	—	61.5	>20
Toxic protein, TP	−52	11.8	1.27	—	—	0.0	0.5
Non-toxic protein, P	−65	13.9	0.18	—	—	0.0	>20
Phospholipid, L	—	1.4	2.90	—	—	—	>20

The analyses and toxic properties of this material, designated as LCTP, are given in Table I. Except for a lower acetyl content and a greatly diminished serological activity, the material appears to be essentially unchanged. That the acetyl comes from the carbohydrate hapten is suggested by the change in serological activity. More direct evidence in support of this view will be presented later. The chemical reaction involved can be represented as:

$$LC_{Ac}TP \rightarrow LCTP + Ac$$

Degradation of the Type Z Antigen with Alkaline Alcohol.—It was shown by Morgan and Partridge that the specific antigen of the Shiga bacillus can be dissociated into a polysaccharide hapten and an amphoteric protein by treatment with alkaline alcohol (2). In order to ascertain whether the specific antigen of the Type Z Flexner organism can be dissociated in a similar manner, and whether the toxic component can be separated from the constituents, a sample of the antigen was treated with alkaline alcohol as follows:—

1 gm. of the Type Z antigen was dissolved in 50 cc. of water and the mixture cooled to 0°. 3 cc. of 20 per cent sodium hydroxide were added followed within 5 minutes by the addition of 100 cc. of chilled ethyl alcohol. After standing for 30 minutes the precipitate was removed by centrifugation. The precipitate was redissolved in 50 cc. of water and the alkaline alcohol treatment repeated twice more. The final precipitate was dissolved in water, dialyzed thoroughly, and isolated as before. 600 mg. of a substance, largely carbohydrate in nature, were obtained. The analytical constants and toxic properties of this fraction, designated as CT, are given in Table I.

The combined alkali-alcohol supernatant solution obtained in the manner described above was neutralized with glacial acetic acid, concentrated *in vacuo*, and adjusted to pH 4.0. A protein fraction was removed by centrifugation. The remaining material was dissolved in dilute alkali and reprecipitated by the addition of sodium acetate and acetic acid. After three isoelectric precipitations the material was suspended in water, dialyzed thoroughly, and isolated by freezing and drying. 210 mg. of a protein, designated as P, and having the analytical constants given in Table I were isolated.

The supernatant liquid remaining after the isolation of the protein by isoelectric precipitation was extracted with three 50 cc. portions of ether. The extracts were combined, the resulting solution washed with water, dried, and evaporated to dryness. 50 mg. of a lipid were obtained.

These experiments demonstrate that treatment of the Type Z antigen with alkaline alcohol dissociates the complex into a phospholipid, an amphoteric protein, and a carbohydrate hapten in which labile acetyl groups are removed. When the complex is dissociated by this procedure the toxic component remains attached to the carbohydrate. The dissociation can be represented as follows:—

$$LC_{Ac}TP \rightarrow L + Ac + P + CT$$

Degradation of the Type Z Antigen with Acid and Heat.—Degradation of the specific antigen with acid and heat was carried out by two methods. The first of these procedures, introduced by Boivin (1) involves heating a 1 per cent acetic acid solution of the material for 4 hours at 100°. A second procedure which we have found useful consists in heating a solution of the material previously saturated with picric acid. In both cases identical carbohydrate and protein fractions are obtained. Heating with picric acid is simpler because, after a short period, the picrate of the dissociated protein precipitates and can be readily removed by centrifugation.

1 gm. of the Type Z antigen was dissolved in 30 cc. of saturated picric acid and the solution heated on a boiling water bath for 30 minutes. The precipitated protein picrate was removed by centrifugation and washed with saturated picric acid. The precipitate was then washed with acidified acetone, dissolved in dilute alkali, and freed of picric acid by isoelectric precipitation and dialysis. The non-dialyzable material was recovered by freezing and drying. 290 mg. of a toxic protein, designated as TP, were obtained. The analytical constants of this material are given in Table I.

The supernatant picric acid solution was extracted with ether to remove the dissociated lipid and then dialyzed to remove the picric acid. The dialyzed material was shaken with a mixture of chloroform and octyl alcohol to remove traces of protein, again dialyzed, and finally dried from the frozen state. 420 mg. of a carbohydrate which proved to be identical with the specific hapten, C_{Ac}, were isolated.

45 mg. of lipid were recovered from the ether extract. The lipid fractions from several acid degradations were combined and aliquot samples taken for analysis. The material had the analytical constants given in Table I for a phospholipid, L. The nitrogen to phosphorus ratio which approximates 1 indicates that this material is indeed a phospholipid.

From the foregoing experiment it is evident that the antigenic complex of Type Z *Shigella paradysenteriae* is dissociated by treatment with acid and heat into a carbohydrate hapten, a phospholipid, and a protein constituent. The protein constituent was found to be toxic, thus indicating that treatment of the antigen with acid cleaves the complex in such a manner that the residual toxicity remains associated with the protein constituent. The hapten component appears to be identical with that isolated during the purification of the specific antigen (4). The acid dissociation of the Type Z antigen can be represented as follows:—

$$LC_{Ac}TP \rightarrow L + C_{Ac} + TP$$

Further Dissociation of the Products of Degradation of the Type Z Antigen.—Once it was established that the alkaline and acid degradation of the Type Z antigen yielded degradation products with definite chemical and physiological characteristics, it became of interest to establish the interrelationships of these different substances. For example, it was believed that an acid degradation of the toxic carbohydrate, CT, obtained by alkaline dissociation of the complex $LC_{Ac}TP$, would yield a non-toxic carbohydrate and the toxic component T. Such treatment of the toxic carbohydrate CT yielded a third carbohydrate, C, which proved to be non-toxic and nearly devoid of serological activity. This material will be referred to in the text as the "inert" carbohydrate. This same carbohydrate could be prepared from the hapten, C_{Ac}, by the direct action of alkali. Similarly, when the alkali-treated, partially degraded antigen, LCTP, was further dissociated with acid and heat, again the same carbohydrate, C, was obtained. A detailed account of these studies follows.

A. Dissociation of the Complex LCTP.—300 mg. of the alkali-treated antigen, LCTP, were dissolved in 25 cc. of saturated picric acid solution, heated, and the products of degradation separated as described above. 125 mg. of a serological inactive carbohydrate, C, and 90 mg

of a toxic protein, TP, were obtained. As seen in Table I, the acetyl content of the carbohydrate, C., was near 6 per cent in contrast to 10 per cent found for the true hapten, C_{Ac}. The toxic protein appeared to be identical with that isolated by acid degradation of the complete antigen, $LC_{Ac}TP$.

Since the complex, LCTP, yielded a toxic protein and a carbohydrate of very low serological activity, the dissociation can be represented by the following equation:

$$LCTP \rightarrow L + C + TP$$

B. Dissociation of the Hapten, C_{Ac}.—The following experiment shows that the same serologically inactive carbohydrate, C, is obtained from the hapten, C_{Ac}, by treatment with dilute alkali.

300 mg. of the hapten, C_{Ac}, from the acid degradation of the complete antigen were dissolved in 20 cc. of water, cooled to 0°, and 1 cc. of 40 per cent sodium hydroxide was added. After standing 1 hour the mixture was neutralized with acetic acid and thoroughly dialyzed. 230 mg. of a carbohydrate were isolated. This material had an acetyl content of 5.83 per cent and was nearly inert serologically.

The loss in acetyl groups may be represented by the equation:

$$C_{Ac} \rightarrow C + Ac$$

C. Dissociation of the Toxic Carbohydrate CT.—The toxic carbohydrate resulting from the alkaline degradation of the intact complex, $LC_{Ac}TP$ may be further dissociated by acid treatment to yield the same serologically "inert" and non-toxic carbohydrate C as that just described.

300 mg. of toxic carbohydrate CT were dissolved in 45 cc. of 1 per cent acetic acid and heated at 100° for 4 hours. The small amount of precipitate which formed was removed by centrifugation and the supernatant solution was repeatedly shaken with a mixture of chloroform and octyl alcohol until no more emulsion formed. The aqueous layer was then dialyzed thoroughly and the non-dialyzable material isolated. 170 mg. of a carbohydrate having an acetyl content of 5.63 per cent were obtained.

This material was no longer toxic and was identical with the partially deacetylated, serologically "inert" carbohydrates obtained as described above. The toxin was apparently destroyed in part or in entirety, for the substance which precipitated during the acid treatment was found to be non-toxic for mice in amounts up to 5 mg. The dissociation of the toxic carbohydrate is represented as follows, where [T] indicates destruction of the toxin:

$$CT \rightarrow C + [T]$$

D. Dissociation of the Toxic Protein TP.—The toxic protein TP obtained by the acid dissociation of the complex $LC_{Ac}TP$ may be further dissociated by treatment with alkaline alcohol, and converted to the non-toxic, amphoteric protein P. In the following experiment the isolation of the non-toxic protein is described.

120 mg. of the protein TP were dissolved in 20 cc. of 1 N NaOH at 0° and allowed to stand 30 minutes. 60 cc. of chilled alcohol were added and after standing 2 hours a small amount of precipitate was removed by centrifugation. When the supernatant alkaline-alcohol solution was acidified, a precipitate appeared. This precipitate was removed by centrifugation and dissolved in 0.1 N HCl. A small amount of insoluble material (probably unaltered TP) was removed by centrifugation. The clear supernatant liquid was treated with 2 volumes of ethyl alcohol; the precipitate was transferred to a cellophane bag, dialyzed, and finally isolated by freezing and drying. 65 mg. of a protein having a nitrogen content of 14.5 per cent and a phosphorus content of 0.12 per cent were isolated. The protein had a specific rotation of $[\alpha]_D = -66°$ for a 1 per cent solution in 0.1 N sodium hydroxide.

The data given above indicate that this amphoteric protein P obtained from the toxic protein TP is identical with that obtained from the alkaline-alcohol degradation of the intact antigen $LC_{Ac}TP$. In this instance too it should be noted that the dissociation, represented by the equation TP $\rightarrow$ P + [T], is accompanied by the destruction of the toxic component T.

Degradation of the Type Z Antigen Prepared by Tryptic Digestion.—Morgan and Partridge (2) have reported that the specific complex from the Shiga bacillus is resistant to the action of trypsin. It is obvious from the experiments detailed below that the antigen of the Type Z Flexner microorganism and the protein derived from it are both readily attacked by trypsin. The antigen prepared from the Type Z organism by a procedure involving tryptic digestion (4) has a lower nitrogen and higher carbohydrate content than that prepared by extraction with diethylene glycol or aqueous pyridine. It is obvious, therefore, that the "tryptic" antigen has a lower protein content. It was of interest to determine whether the protein of this partially degraded antigen is identical with that of the complete antigen prepared by direct extraction methods.

1 gm. of Type Z "tryptic" antigen was dissociated by heating in 1 per cent acetic acid for 4 hours at 100° as previously described. 600 mg. of carbohydrate, identical with the hapten C_{Ac}, were isolated. 55 mg. of phospholipid were also obtained and at the same time 170 mg. of a fraction which was insoluble in the acetic acid were isolated. This material had a nitrogen content of 7.3 per cent and a phosphorus content of 1.9 per cent. 0.5 mg. sufficed to kill mice when given intraperitoneally.

The isolation from the complete antigen of a protein higher in nitrogen than the constituent obtained by dissociation of the "tryptic" antigen, indicates that the enzyme is capable of hydrolyzing a considerable part of the protein moiety of the former substance. That these two nitrogenous constituents are related is evident from the fact that both have a high phosphorous content, are insoluble in dilute mineral acid, and are toxic when administered to mice.

From the following experiment it will be seen that a nitrogenous constituent identical with that obtained from the "tryptic" antigen is also obtained by the enzymatic degradation of the toxic protein, TP.

300 mg. of the toxic protein, TP, were digested with 30 mg. of Fairchild's trypsin at pH 8.0 at 37° for 3 days. The digest was then dialyzed at 0° and the dialysate collected. The latter was mainly phosphate derived from the buffer and was not toxic for mice. 125 mg. of the non-dialyzable material were recovered from the cellophane membrane. This substance was found to have a nitrogen content of 6.8 per cent and a phosphorus content of 2.1 per cent. These values are in close agreement with those of the material described above. 0.5 mg. of the substance sufficed to kill mice.

The fact that tryptic digestion of the intact antigen $LC_{Ac}TP$ and of the toxic protein TP fails to destroy the toxic component as far as can be determined

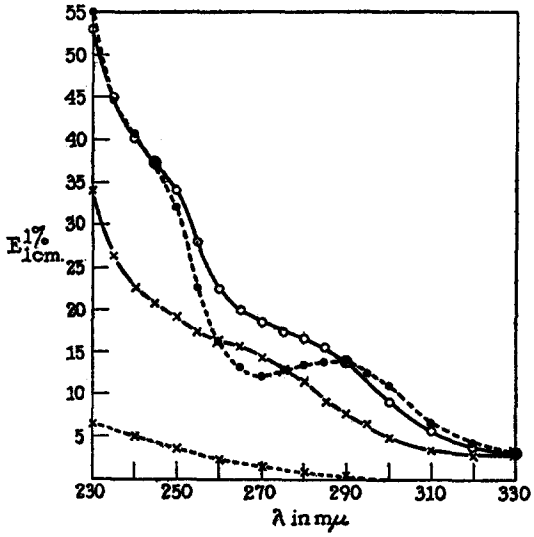

FIG. 3. Ultraviolet absorption spectra of degradation products derived from the Type Z antigen.

○——○ toxic protein, TP; ●- - -● non-toxic protein, P; ×——× toxic carbohydrate, CT; ×- - -× non-toxic carbohydrate, C_{Ac}.

by mouse toxicity tests suggests either that the toxin is not protein in nature or that it is a peculiar protein fraction resistant to the action of the enzymes of pancreatic trypsin. In this regard it should be borne in mind that the toxic carbohydrate CT obtained from the alkaline alcohol degradation of the antigen was also found to contain little or no protein.

Ultraviolet Absorption Spectra.—The absorption spectra of various fractions obtained from the degradation of the Type Z antigen were determined in a Beckmann Model DU quartz spectrophotometer. The concentrations of the various solutions were in each instance 0.2 mg. per cc. in 0.01 N NaOH. The observations were made in a cell 1 cm. in thickness. The absorption curves are given in Fig. 3. It is evident that the antigen, the toxic carbohydrate, and the toxic protein contain some grouping which absorbs in the region of

260 mμ and which is not present in the non-toxic protein or in the non-toxic carbohydrate. Whether or not the absorption at 260 mμ is due to the toxin cannot be definitely stated at this time. It is interesting, however, that the toxic materials all have a significant absorption at 260 mμ and that their phosphorus content is significantly higher than that of the non-toxic materials.

Serological Activity of Various Fractions.—For the purposes of this study the precipitin reactions as given in Table II will suffice to demonstrate the serological activity of the various fractions obtained by degradation of the specific antigen, $LC_{Ac}TP$. The serum employed was prepared by the immunization of rabbits with heat-killed Type Z *Shigella paradysenteriae.* From the data presented in Table II it can be seen that the two haptens, one obtained

TABLE II

Precipitin Titrations of the Degradation Products in Type Z Antiserum

Precipitinogen	1:2,000	1:10,000	1:50,000	1:250,000	1:1,000,000
Complete antigen, $LC_{Ac}TP$	4+	4+	3+	2+	2+
Natural hapten, C_{Ac}	0	1+	4+	3+	2+
Acid hapten, C_{Ac}	0	1+	4+	3+	2+
Alkali hapten, CT	1+	2+	4+	3+	2+
Degraded hapten, C	0	1+	0	0	0
Acid protein, TP	2+	1+	0	0	0
Non-toxic protein, P	1+	1+	0	0	0
Alkali antigen, LCTP	0	2+	2+	1+	0

0.5 cc. of pooled serum (diluted 2 + 3 with saline) mixed with 0.5 cc. of the precipitinogen in saline. Incubated 2 hours at 37° and read after 12 hours at 0°.

4 = heavy precipitation, clear supernatant liquid.

0 = no precipitation.

directly from the organisms and the other by acid degradation of the complete antigen, appear to be closely related in their serological behavior as well as in their chemical composition. Both are found to inhibit in the region of antigen excess. The toxic carbohydrate, CT, in contrast to the true hapten, precipitates in the region of antigen excess although it does show some inhibition. The degraded hapten, C, as previously pointed out, has a lowered acetyl content and is almost inert serologically. The toxic protein, TP, and the non-toxic protein, P, are serologically similar and both react weakly in an antiserum to the whole organism. The alkali-treated antigen LCTP resembles the hapten in its serological activity inasmuch as it exhibits inhibition in the region of antigen excess.

Immunization with the Products of Degradation of the Type Z Antigen.—Rabbits were immunized with the various degradation products of the Type Z antigen. Three intravenous injections of 50 μg. of each fraction under investigation were administered to 3 or more

rabbits and the animals bled 10 days after the final injection. The results given in Table III are the precipitin reactions of the different antisera when tested with the various fractions.

It is apparent that none of the fractions possesses the antigenic properties of the original complex $LC_{Ac}TP$. The latter gives rise in rabbits to antibodies which react not only with the intact complex but with all the fractions derived from it save the deacetylated carbohydrate, C. The material produced by brief treatment of the complete antigen with alkali, LCTP, is only weakly antigenic and produces antibodies which react more vigorously with the

TABLE III

Precipitin Titrations of Antisera Prepared by Immunization of Rabbits with Fractions Derived from the Type Z Antigen

Antiserum prepared by immunization with	Homologous				Complete antigen, $LC_{Ac}TP$				Hapten, C_{Ac}			
	1:2,000	1:10,000	1:50,000	1:250,000	1:2,000	1:10,000	1:50,000	1:250,000	1:2,000	1:10,000	1:50,000	1:250,000
Antigen, $LC_{Ac}TP$....	—	—	—	—	4+	3+	2+	1+	0	0	2+	1+
Hapten, C_{Ac}..	—	—	—	—	0	0	0	0	0	0	0	0
Toxic carbohydrate CT	0	1+	2+	1+	2+	1+	0	0	0	0	0	0
Toxic protein, TP.....	3+	2+	1+	1+	2+	3+	3+	2+	0	0	0	0
Non-toxic protein, P..	2+	1+	1+	0	2+	1+	1+	0	0	0	0	0
Alkali-treated antigen, LCPT.....	1+	1+	0	0	2+	1+	0	0	0	0	0	0

untreated complex than with the homologous antigen itself. The hapten, C_{Ac}, fails to evoke antibodies when administered to rabbits. The toxic carbohydrate, CT, on the other hand, is weakly antigenic and gives rise to antibodies which react with the homologous toxic carbohydrate and to some extent with the complete antigen as well. The toxic protein, TP, gives rise to an antiserum containing antibodies which react with the complex and with the homologous antigen to about the same titer. The non-toxic protein, P, is weakly antigenic and evokes antibodies which react with the intact complex as well as with the homologous antigen. From these experiments it appears that all the products of degradation of the specific antigen save the hapten C_{Ac} are antigenic in rabbits and that even the toxic carbohydrate is still capable of evoking precipitins.

Degradation of the Antigenic Complexes from Other Types of Shigella paradysen-

teriae.—The specific antigens of Types V and W were degraded in much the same manner as that of the Type Z. Although no analytical data will be given, let it suffice to say that the protein constituents, by elementary chemical analysis and specific rotation, appear to be closely related to that derived from the Type Z complex. Preliminary data indicate that the amino acid composition of the proteins varies with the type of organism. Furthermore it was found that each of the specific antigens, regardless of the type from which it was derived, could be degraded by mild acid hydrolysis into a carbohydrate hapten, a phospholipid, and a toxic protein. The fact that similar degradation products are obtained from Types V, W, and Z specific complexes indicates that the results of the more detailed studies on the Z substance may be considered applicable to the antigens of the Flexner group as a whole, and that in general these substances are chemically very closely related.

Demonstration of a Bacterial Growth Principle in the Specific Antigen of Type Z Shigella paradysenteriae and Its Toxic Degradation Products.—Early in the course of these studies attempts were made to find a more convenient and rapid method of assay for the toxicity of the substances under investigation. It was hoped that a bacterial species might be found which would be susceptible to the action of the toxin. The latter was found to have little or no growth inhibitory or stimulative properties for *S. lactis* or *L. casei* in amounts as great as 1 mg. per cc. of medium. However, the addition of minute amounts of the Type Z antigen to the medium markedly stimulated the growth of *E. coli.* The toxic carbohydrate, the toxic protein as well as the intact complex were stimulative in action whereas the non-toxic degradation products stimulated growth but slightly.

By repeated transfer a sulfanilamide-susceptible strain of *E. coli* was adapted to grow in a medium consisting of inorganic salts and glucose. The organism grew through as many as 10 transfers when 1 drop of an undiluted culture was added to 5 cc. of medium. When, however, small amounts of the Type Z antigen were added to the basal medium, growth was obtained with an inoculum of 1 drop of a 10^{-4} dilution of original culture.

The basal medium consisted of $(NH_4)_2SO_4$, 2 gm.; glucose, 4 gm.; Na_2HPO_4, 6.64 gm.; KH_2PO_4, 2.72 gm.; $MgSO_4 \cdot 7H_2O$, 0.05 gm.; and NaCl, 0.10 gm. in 1 liter of solution. Amounts of antigen or its degradation product ranging from 0.001 mg. to 1 mg. per cc. or greater were added to the medium. The tubes were autoclaved and inoculated with 1 drop of a 10^{-4} dilution of a 6 hour culture in 5 cc. of the medium. At the end of a 24 hour period at 37° the turbidity of the solutions was determined in a photoelectric colorimeter. The amounts of material necessary to produce half-maximal growths are recorded in Table IV.

It is evident from the results given in Table IV that of the various fractions tested, the intact antigen possesses the greatest stimulative property, followed in order by the toxic protein and the toxic carbohydrate. The non-toxic protein and the non-toxic carbohydrate were without significantly stimulative properties.

Once it was established that the antigen possessed bacterial growth-stimulating properties for *E. coli* it became of interest to determine whether the action of this microorganism upon the antigen altered either its serological or its toxic properties.

The microorganisms from 200 cc. of a 24 hour culture of *E. coli* were removed by centrifugation, washed 3 times, and finally suspended in 10 cc. of normal saline. Equal volumes of the suspension were mixed with a solution of the antigen containing 4 mg. per cc. dissolved in 0.006 M $NaHCO_3$. A control experiment was performed in which a portion of the suspension of organisms was boiled and incubated in the presence of the antigen at 37°. At the end of 24 hours the quantitative precipitin curve and toxicity of both materials were determined.

TABLE IV

Growth-Stimulating Properties of the Specific Antigen of Type Z Shigella paradysenteriae and Its Products of Degradation

Material tested	Amount necessary for half-maximal growth in 24 hrs.
	μg. per cc. of medium
Complete antigen, $LC_{Ac}TP$	50
Toxic protein, TP	125
Toxic carbohydrate, CT	280
Non-toxic protein, P	5,000
Hapten, C_{Ac}	>10,000

It was found by quantitative measurement that the serological activity and toxicity of the two samples remained unchanged by contact with the bacteria. It is not to be assumed that the antigen was unattacked, however, for the determination of inorganic and total phosphorus in the supernatant solution after the removal of the microorganisms by centrifugation revealed that about 80 per cent of the organically bound phosphorus of the antigen had been converted to the inorganic form. It would appear, therefore, that organically bound phosphorus is not essential to the serological activity or toxicity of the Type Z antigen.

DISCUSSION

From the foregoing experimental account it is evident that the specific antigenic complexes of the *Shigella paradysenteriae* are phospholipid-acetylated carbohydrate-protein complexes, grossly similar in their chemical make-up to the specific antigens of other Gram-negative microorganisms. These substances all contain a toxic component, and from evidence derived from dissociation studies it would appear that the toxin is a distinct chemical moiety.

Degradation of the Type Z complex in acid solution is effected by heating

in 1 per cent acetic acid or in the presence of saturated picric acid solution. In either case, dissociation occurs and a phospholipid, a carbohydrate, and a protein fraction can be isolated. Under the conditions of these experiments the carbohydrate and the phospholipid were found to be non-toxic when administered to experimental animals. The protein fraction, on the other hand, is almost as toxic as the original complex. The degradation may be represented by the equation $LC_{Ac}TP \rightarrow L + C_{Ac} + TP$.

Dissociation of the antigenic complex in alkaline solution can be carried out in two ways. The first involves treatment of the complex with strong alkali at 0°. Under these conditions the only product recovered is a complex with unaltered toxicity and with greatly impaired serological and antigenic activity. The only chemical change which can be correlated with loss in serological activity is a loss in acetyl groupings. It is quite possible, however, that more subtle alterations of the antigenic molecule have occurred. This is expecially likely in the light of the observation that brief contact with strong alkali at room temperature destroys the toxicity as well as the serological and antigenic activity. If the loss of acetyl groupings is the only change involved the degradation my be represented as $LC_{Ac}TP \rightarrow LCTP + A_c$.

The second method of alkaline degradation used by Morgan in his studies on the Shiga antigen involves repeated fractionation with alkaline alcohol at 0°. Under these conditions a phospholipid, a carbohydrate, and a protein fraction are also obtained. The phospholipid and the protein are not toxic, whereas the carbohydrate possesses a toxicity of the same order as that of the protein obtained by acid degradation. The degradation under these conditions can be represented as follows: $LC_{Ac}TP \rightarrow L + CT + A_c + P$.

The two carbohydrates, one obtained by alkaline degradation, CT, and the other by acid degradation, C_{Ac}, differ in that the former is toxic and has a lower percentage of acetyl groupings. In addition, the toxic carbohydrate, CT, contains considerably more phosphorus and slightly more nitrogen than the carbohydrate, C_{Ac}, and shows an appreciable absorption in the ultraviolet. This absorption is apparently not due to protein since the biuret test is essentially negative and amino acids are not released during hydrolysis. Both carbohydrates are serologically active.

The toxic carbohydrate, CT, may be still further degraded by the action of acid to a non-toxic carbohydrate, C, but in so doing the toxic component, T, is destroyed. The carbohydrate, C, has practically no serological activity and is identical with that obtained by the alkaline treatment of the hapten, C_{Ac}. These reactions can be represented by the equations $CT \xrightarrow{H^+} C + [T]$ and $C_{Ac} \xrightarrow{OH^-} C + A_c$, respectively.

The proteins obtained by the two methods of degradation differ as markedly as do the carbohydrates. The protein from the alkaline degradation, P, is

not toxic whereas that from the acid degradation, TP, is toxic. The former has a higher nitrogen and a lower phosphorus content than has the toxic protein obtained by acid degradation. In addition, the non-toxic protein is soluble both in acid and in alkaline solutions whereas the toxic protein, TP, is insoluble in all concentrations of acid. When the ultraviolet absorption spectra of the two proteins are compared it is seen that the toxic protein, TP, in addition to an absorption analogous to that shown by the non-toxic protein, P, has a zone of absorption near 260 mμ. This latter zone of absorption corresponds exactly with that found for the toxic carbohydrate, CT.

Precipitation of the antigen from a formamide solution with varying concentrations of ethyl alcohol produced no fractionation of the material. The lipid content remained constant during repeated treatment with formamide and ethyl alcohol. Morgan and Partridge (2) have reported that the lipid content of the Shiga complex is reduced by such treatment.

When the antigenic properties of the various degradation products are compared, marked contrasts in behavior are noted. The intact complex $LC_{A_c}TP$ is highly antigenic when administered to rabbits; as few as 3 injections of 50 μg. each produce an antiserum possessing powerful precipitins. The alkali-degraded antigen, LCTP, on the other hand, is only weakly antigenic. The hapten from the acid degradation, C_{A_c}, does not incite the formation of antibodies in rabbits. The toxic carbohydrate, CT, obtained by alkaline degradation, is weakly antigenic, however, and evokes an antiserum which reacts with the intact antigen as well as with the homologous carbohydrate.

Both proteins, P and TP, are weakly antigenic and when administered to rabbits lead to the production of antisera which react with the intact antigen and with both proteins as well. A detailed study of the antibody composition of these antisera has been made and will be reported in a later paper.

The degradation studies can be summarized as follows:—

Complete antigen $LC_{A_c}TP$ (Phospholipid-acetylated carbohydrate-toxin-protein)
Acid degradation:

$$LC_{A_c}TP \xrightarrow{\text{acid and heat}} L + C_{A_c} + TP$$

Alkaline degradations:

$$LC_{A_c}TP \xrightarrow{\text{alkali at } 0^\circ} LCTP + A_c$$

$$LC_{A_c}TP \xrightarrow{\text{alkaline alcohol}} L + CT + A_c + P$$

Further degradation of constituents of the antigen:

$$LCTP \xrightarrow{\text{acid and heat}} L + C + TP$$

$$CT \xrightarrow{\text{acid and heat}} C + [T]$$

$$C_{A_c} \xrightarrow{\text{alkali at } 0^\circ} C + A_c$$

$$TP \xrightarrow{\text{alkaline alcohol}} P + [T]$$

The fact that the toxic constituent is found associated either with the protein or the carbohydrate component, depending upon the mode of degradation, is of special interest. One point which needs further elucidation before this schematic presentation can be accepted is the quantitative aspect of the toxin dissociation. The degradation products are less toxic than the original material, rather than more so. At least half the total toxicity is lost during the chemical degradation of the specific antigen. Further degradation of the toxic dissociation products brings about a still further loss in activity. The nature of the toxic component has not been determined but certain facts are known relating to its stability which may be useful in its ultimate identification. The toxin is labile when subjected to prolonged contact with dilute alkali. It is reasonably thermostable in neutral solution and is slowly destroyed when heated in the presence of dilute mineral acid. It is not destroyed by prolonged contact with the enzymes present in a commercial preparation of pancreatic trypsin. The antigenic complex in which it is found may be degraded so that the toxin remains associated with a moiety which is predominantly carbohydrate or protein in nature depending upon the method of dissociation. From the results of these dissociation studies the toxin should not be regarded as a typical protein. It is a non-diffusible substance which appears to be associated with organically bound phosphorus and which has a significant absorption band in the ultraviolet near 260 mμ. The substance probably has a nitrogen content of between 6 and 10 per cent. Sulfur is apparently absent. On the basis of the above considerations, which are admittedly incomplete, it is suggested that the toxin may be associated with a purine or a pyrimidine-like derivative.

A possible relationship of the toxin to a growth principle for *E. coli* is indicated by the experiments detailed in this report. The material, however, does not replace the known growth factors for *S. lactis* or for *L. casei*, nor is it antagonistic to them. Treatment of the toxin with a resting suspension of *E. coli* does not destroy the toxin but does remove phosphorus from the complex with which the toxin is combined. It is obvious that further experiments are necessary before the nature of the toxic moiety can be established.

SUMMARY

The chemical and enzymatic dissociation of the specific antigen of Type Z *Shigella paradysenteriae* has been studied. The chemical, toxic, and serological properties of the products of degradation have been investigated. The nature of the toxic component has been discussed.

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