ON THE NATURE OF THE TOXIC COMPONENT OF THE SOMATIC ANTIGEN OF SHIGELLA PARADYSENTERIAE TYPE Z (FLEXNER)

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During the past two decades (1) many new and significant data have been accumulated concerning the chemical make-up of the microorganisms which cause bacillary dysentery.

It has long been known (1, 2) that when Shiga bacilli were injected into experimental animals degenerative changes in the brain and intestines resulted which were ascribed to toxins present in the bacterial cells. It was subsequently found (3) that animals treated with other Gram-negative bacilli, or with crude extracts obtained from them, showed a marked increase in plasma glucose and that this phenomenon was followed by hyperglycemia and a diminution of inorganic phosphorus. These observations were later confirmed by others (4). Boivin (5) and Haas (6) found that certain fractions obtained from other members of the colon-salmonella-dysentery-proteus group, all showed primary toxicity in experimental animals and that the lethal dose varied for different species. Death of the animals was accompanied by characteristic symptoms: diarrhea, loss of weight, leucopenia followed by leucocytosis, and a marked influence on the nervous centers which regulate body temperature. Today it is believed that many Gram-negative bacteria possess a similar toxic component which is responsible for the symptoms elicited in experimental animals. Hutner and Zahl (7) have demonstrated that the somatic antigens of Gram-negative bacteria are capable of causing severe damage to malignant tumors. Fractions obtained from *B. prodigiosus* can likewise produce hemorrhages in tumors (8), and these substances have also been shown to be antigenic in experimental animals (9).

It was Boivin (10, 5) who first established the chemical nature of the somatic antigens of Gram-negative bacteria; these substances were believed by him to be carbohydrate-lipid complexes. The somatic antigen of *Bact. aertrycke* isolated by Raistrick and Topley (11) was also regarded as belonging to this class of compounds. Our knowledge concerning the somatic antigens of Gram-negative bacilli was greatly advanced by the investigations of Morgan (12), who studied in detail the specific antigens of the Shiga and typhoid bacillus (12, 13). After extensive chemical investigation, these substances were shown to be complexes of a carbohydrate, a protein, and a phospholipid, and not merely lipocarbohydrates as postulated by Boivin. By means of acid and heat it was possible to split the antigen into its component parts and it was observed, furthermore, that the carbohydrate determined the specificity of the antigen, though it was not in itself antigenic.

The somatic antigens from the Flexner group of dysentery bacilli have likewise been studied in some detail (14). The antigenic complexes, extracted from these organisms by aqueous pyridine, also appeared to be lipocarbohydrate-protein complexes (15) which were

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highly antigenic and toxic for mice and rabbits. Although attempts were made to identify the toxic component, this was not achieved. Tal and Olitzki (16) have shown that the conjugated protein prepared from Shiga bacilli evoked most of the lethal and hemorrhagic effects exhibited by the whole antigenic complex.

In spite of the extensive investigations in this field, the chemical identification of the toxic component of the somatic antigens of dysentery bacilli has not been accomplished. Achievement of this goal should be of value not only in gaining an understanding of the mechanism of its action, but it might conceivably enable one to devise procedures to prevent its harmful effect in the infected host.

EXPERIMENTAL

Methods

Cultivation of Bacteria.—The culture of the Type Z (Flexner) *Sh. paradysenteriae* used in this study was obtained from the United States Army Medical Center. The microorganisms were grown in a medium devised by Dole (17). Fifteen liters of the medium containing 0.05 per cent phenol red, in a 5 gallon pyrex bottle was inoculated in the evening. The following morning 500 cc. of sterile 50 per cent glucose solution was added and the culture vigorously stirred under aseptic conditions. Two ml. of tributyl phosphate was added to prevent foaming. Sterile air was bubbled through the culture at a rate of 500 cc. per minute. As the medium became acid, 10 N sodium hydroxide was added from a dropping funnel to maintain neutrality. Six to 7 hours after the addition of glucose, the culture was killed by adding 75 gm. of phenol dissolved in 300 cc. of 30 per cent alcohol and left to stand overnight at 4°C. The bacilli were then collected in a Sharples centrifuge and dried from the frozen state. The yield was usually about 45 gm. of dry microorganisms per 15 liters of culture.

Analytical Methods.—Nitrogen determinations were performed by the usual micro-Kjeldahl method; phosphorus, according to the procedure of Allen (18), and reducing sugars by the Shaffer-Somogyi method (19). Glucosamine was measured after acid hydrolysis in 2 N HCl for 18 hours at 100°C. in a sealed tube by the method of Sørensen (20). The serologic activity of the whole antigen and the various fractions was studied by means of the turbidimeter devised by Libby (21).

Serological Methods.—Antisera were prepared by injecting rabbits intravenously with graded amounts of the fraction under investigation, sterilized with 1:10,000 merthiolate. The bacterial suspensions used for agglutination tests were prepared from twice washed 18 hour cultures of the microorganism, resuspended to the desired concentration. The test mixtures were incubated 2 hours at 37°C. and left overnight in the ice box.[1] Readings were made on the following morning. The precipitin tests were performed by mixing 0.5 cc. of dilutions of the antigen and 0.2 cc. serum (2:3) and 0.3 cc. saline, incubating at 37°C. for 2 hours, then leaving overnight in an ice box and reading the next morning.

Measurement of Toxicity.—In order to test the toxicity of the various fractions under investigation, a sterile solution in varying concentrations was injected intraperitoneally into 6-week-old mice (Rockefeller Institute strain). The animals weighed approximately 30 gm. The mice were observed for 5 days and their deaths recorded. Each dilution of the material tested was injected into 6 mice and the highest dilution which killed all animals in the group was considered as the lethal dose.

Ultraviolet Absorption Spectra.—The absorption spectra of the materials investigated were observed in a Beckmann Model DU quartz spectrophotometer using 1 cm. quartz cells. The

absorption spectra were determined on solutions all made to a concentration of 1 mg. per cc. and in 0.1 M phosphate buffer at pH 7.2.

Preparation of the Specific Antigen.—The specific antigen of *Sh. paradysenteriae* Type Z used in this study was prepared from phenol-killed organisms by extraction with 50 per cent pyridine as previously described (14). From 140 gm. of bacteria 8.5 gm. of purified antigen was obtained. The product contained 5.4 per cent nitrogen, 1.44 per cent phosphorus, and 11 per cent lipid.

Degradation of the Antigen

It has been shown by Boivin (22) that the lipocarbohydrate-protein complexes of various Gram-negative bacilli can be broken down by heating with

TABLE I

The Effect of Hydrolysis upon the Toxicity of the Specific Antigen of Type Z Sh. paradysenteriae

Time of heating	Mg. of material injected			
	4	2	1	0.5
min.				
0	$DDDDDD_{18}$	$DDDDDD_{18}$	$DDDDDD_{18}$	$DD_{18}D_{24}SS$
30	$DDDDDD_{18}$	$DDDDD_{24}S$	$D_{24}SSSSS$	SSSSSS
45	$DDDDDD_{24}$	$D_{24}SSSSS$	SSSSSS	SSSSSS
60	$D_{36}SSSSS$	SSSSSS	SSSSSS	SSSSSS

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

1 per cent acetic acid for 4 hours at 100°C. During this procedure a precipitate appears, which has been found to be protein in nature (12). Subsequent investigation (15) has shown that a fraction prepared in this manner from the somatic antigen of Type Z *Sh. paradysenteriae* is toxic. It can now be reported that hydrolysis by acid and heat not only splits the protein fraction from the whole antigenic complex, but if the process goes on long enough, toxicity is gradually destroyed as shown in Table I. In order to prepare the protein constituent in a toxic state it is necessary to limit the period of heating to 35 minutes, and to facilitate precipitation, to carry out the hydrolysis in dilute solution (4 mg. per cc.) in the presence of 1 per cent sodium chloride. Even under these conditions the results are not always reproducible. In general, however, it may be said that when the protein was prepared in this manner it was toxic, though less so than the original antigen. The material contained 10.4 per cent nitrogen, 1.46 per cent phosphorus, and showed an absorption peak at 2600 Å, as seen in Fig. 1 where α is the absorption curve of a 0.1 per cent solution. When hydrolysis was prolonged (4 hours) the precipitated

protein fraction was no longer toxic, contained little or no phosphorus, and did not show a peak at 2600 Å.

On digesting the toxic protein with crystalline ribonuclease most but not all of the toxicity was lost and the residual protein no longer showed a maximum at 2600 Å. In order to establish whether the residual toxicity was due to small amounts of nucleic acid components which could not be removed by enzymatic digestion, the protein, both before and after enzymatic digestion, was treated

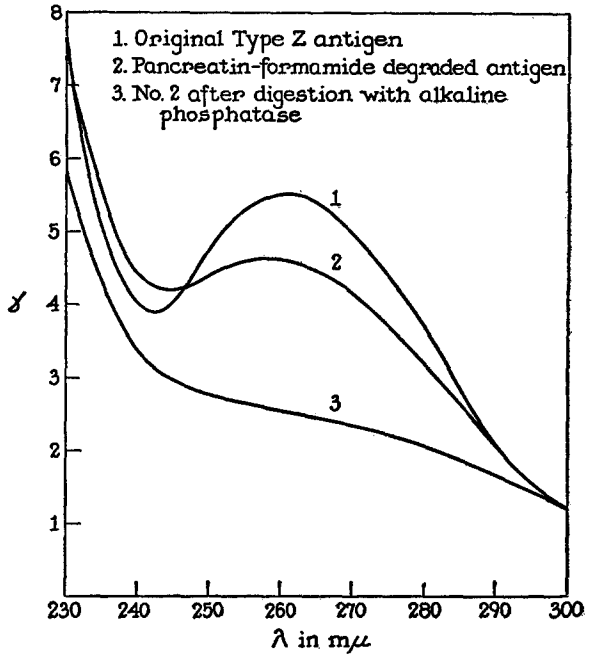

FIG. 1. Absorption spectrum of the Type Z antigen and its degradation products.

with 0.36 M trichloracetic acid at 90°C. for 15 minutes. In both instances the precipitated material showed no toxicity, and the supernatants showed the presence of a substance which had an absorption peak at 2600 Å. When the complete antigen was treated in a similar manner a precipitate was likewise obtained. This substance was without toxicity, nor did it show an absorption peak at 2600 Å.

In contrast to these observations, if the undegraded antigen is digested with ribonuclease, the peak at 2600 Å remains, though the extinction coefficient is lowered. The toxicity is somewhat diminished, though by no means completely lost. The protein component prepared from this material showed no peak at 2600 Å, and had a low order of toxicity, yet on subsequent treatment

with trichloracetic acid, the supernatant showed the presence of a substance which showed maximum absorption at 2600 Å.

These observations indicate that the toxic component appears to be associated with some substance which shows an absorption peak at 2600 Å and that toxicity is manifested only when this material is in chemical combination with something else in the intact antigenic complex. This manner of degradation is open to criticism, however, not only because considerable destruction of the toxic component occurs during the process, but also because hydrolysis is not complete, for the so called protein component still contains a considerable amount of polysaccharide. It will be shown later, however, that if the protein component of the intact antigen is first digested with pancreatin, it is possible to eliminate the component which shows maximum absorption at 2600 Å and the product reveals a degree of toxicity similar to that of the original material.

Enzymatic Degradation of the Somatic Antigen

It has previously been reported (15) that the toxic protein of the Type Z antigen is still toxic even after tryptic digestion. It was decided therefore to attempt to degrade the whole antigen by means of proteolytic enzymes.

Five gm. of antigen containing 6.2 per cent nitrogen and 1.5 per cent phosphorus was dissolved in 200 cc. of M/10 borate buffer pH 7.9. To this was added 100 mg. of pancreatin. The mixture of antigen and enzyme was placed in a cellophane bag and dialyzed against 800 cc. of the same borate buffer saturated with toluene for 6 days at 37°C. The buffer was changed daily. The digested material was finally shaken three times with chloroform–octyl alcohol (4:1) and then thoroughly dialyzed. The material (3.7 gm.) was finally isolated from the frozen state. This substance contained 2.4 per cent nitrogen, 1.04 per cent phosphorus, and showed an absorption peak at 2600 Å and compares in its analytical and toxic properties with that prepared by degradation of the antigen with alkaline alcohol (15). The material gave a positive ninhydrin test and yielded 58.5 per cent of reducing sugars (calculated as glucose) after hydrolysis in a sealed tube at 100°C.

If the pancreatin-digested material is heated in 1 per cent acetic acid at 100°C. for varying periods of time, a phosphorus-containing component is gradually liberated. This component is dialyzable, and shows a specific absorption peak at 2600 Å. If the heating is continued for 2 hours, the phosphorus liberated equals 51 per cent of the total and all of the material absorbing at 2600 Å becomes dialyzable. As stated above, during acid hydrolysis the toxicity is destroyed. This is not the case, however, if the complex is heated in a borate buffer at pH 9.2 and at 56°C. Under these conditions an almost complete loss in serological activity occurs within 6 hours, but there is no diminution in toxicity, nor is there any release either of dialyzable phosphorus or nitrogen or of a material absorbing at 2600 Å. From these experiments it can be concluded that the protein component of the somatic antigen does not appear to be associated with the toxicity.

Further Dissociation by Acid Formamide

Morgan has shown that the somatic antigen of the Shiga bacillus can be dissociated by treatment with neutral formamide (12), but this procedure is without effect on the antigen of Type Z *Sh. paradysenteriae*. The addition of 3 per cent formic acid to the formamide made it possible, however, to bring about the dissociation of the enzymatically degraded Type Z antigen in the following manner:—

One gm. of the pancreatin-digested somatic antigen was dissolved in 50 cc. of neutral formamide and to this was added 3 per cent of formic acid. The solution was kept at room temperature for 24 hours; then 0.5 gm. of sodium chloride dissolved in a few cubic centimeters of water was added, followed by the addition of 3.5 volumes of alcohol. After standing overnight in an ice box, a precipitate settled out and was collected by centrifugation. The procedure was repeated three times. The final product was thoroughly dialyzed, concentrated, and dried from the frozen state. From 1 gm. of antigen there was obtained 0.8 gm. of substance having the following analysis: N = 1.9 per cent, P = 1.1 per cent. The absorption spectrum of a 0.1 per cent solution of this material in 0.1 M phosphate buffer at pH 7.2 is given in Fig. 1. The toxicity of this substance was similar to that of the original material.

In order to determine whether the phosphorus-containing component could be removed enzymatically, the material was now treated with alkaline phosphatase from the intestine (obtained from Armour and Company, Chicago).

0.5 gm. of the material was dissolved in 10 cc. of M/10 borate buffer at pH 8.4. One mg. of alkaline phosphatase was added. The mixture was placed in a cellophane bag[1] and the latter suspended in 25 cc. of the same buffer saturated with chloroform and kept for 24 hours at 37°C. It was observed that a material which absorbed at 2600 Å diffused through the membrane. In addition, part of the phosphorus, some 10 per cent of the total, likewise diffused. After enzymatic digestion the material within the bag was shaken repeatedly (18 times) with fresh portions of chloroform–octyl alcohol mixture. The substance remaining in the aqueous phase was finally dialyzed and dried from the frozen state. The yield was 0.4 gm.

This substance, designated as TM, no longer showed an absorption peak at 2600 Å, yet it was as toxic as the original antigen. From the results of this experiment it is apparent that contrary to previous beliefs (15) the presence of a substance which shows a maximum absorption at 2600 Å is not associated with the toxicity.

Properties of the Toxic Material (TM)

From the foregoing it is apparent that it has been possible to obtain from the Type Z antigen of *Sh. paradysenteriae* a product of degradation fully as toxic as the native antigen, yet quite different in its chemical make-up. This substance has been designated as TM. The material showed no absorption

[1] It should be emphasized that it is important to wash the dialyzing membrane thoroughly; for membranes from the Visking Company contain a soluble material which not only fluoresces but which shows distinct absorption bands at 2500, 2600, and 2630 Å.

peak at 2600 Å (Fig. 1), and had the following chemical analysis, in which the figures are expressed in terms of percentage: C 47.0, H 7.2, N 2.2, P 1.1, glucosamine 22.7, and NH_2 (calculated as glucosamine) 22.6, total lipids 5.0. The substance TM precipitates in an antiserum prepared by the immunization of rabbits with whole type Z bacilli. Furthermore, the substance itself elicited in rabbits both agglutinating and precipitating antibodies. It was not known whether the lipid component of the product TM was responsible for its toxicity; in order to ascertain this point it was necessary to free it from the lipid component and this was accomplished in the following manner:—

500 mg. of the material TM was dissolved in 5 cc. of distilled water, and 100 cc. of chloroform–methyl alcohol (2:1) was added. The precipitated material was collected by centrifugation, thoroughly dialyzed, and dried from the frozen state.

The final product was found to have lost some 10 per cent of the total phosphorus and was as toxic as the substance from which it was derived. It was also found to contain no lipid, for after acid hydrolysis there was no material soluble either in chloroform, in alcohol-ether, or in ether. On heating the material with 1 per cent acetic acid for 60 minutes the solution remained clear, but toxicity was destroyed, and in this respect the substance behaved like the so called "toxic carbohydrate" previously described (15).

Beyond the fact that after acid hydrolysis some 40 per cent of the total phosphorus was liberated in a dialyzable form, it was not possible to demonstrate any striking difference between the parent material (TM) and the detoxified product. It should be further noted that half of the dialyzable phosphorus was in the form of inorganic phosphorus, whereas the remainder was organically bound.

When the material was heated in 1 per cent acetic acid over a longer period of time (2 hours or longer), a precipitate appeared. This material represented about 10 per cent by weight of the starting material. The precipitate could be separated by centrifugation and when dried gave the following analysis, C 63.3, H 9.5, P 3.3, N 2.96, whereas the substance remaining in the supernate, on freezing and drying, had the composition C 48.3, H 6.5, P 0.7, N 2.0. The nature and significance of this insoluble material will be discussed later.

DISCUSSION

Previous studies on the somatic antigen of Type Z *Sh. paradysenteriae* have suggested that its toxicity might be due to the presence of a distinct chemical component (15). At that time attempts were made to isolate and identify this substance but without success. It was found that fractionation with a variety of organic solvents failed to separate the toxin from the antigen itself, and chemical manipulation destroyed the toxic component. The toxin appeared to be firmly bound to the other constituents of the bacterial antigen.

It was observed that the antigen could be readily dissociated by hydrolysis either with acid or with alkali. The former procedure yielded three distinct fractions, a protein, a phospholipid, and an immunologically active polysaccharide. Neither the polysaccharide nor the phospholipid was toxic, whereas the protein component still appeared to be bound to the toxin. Dissociation of the antigenic complex with alkaline alcohol likewise yielded the three components, but under these conditions the toxin remained bound to the polysaccharide, and the protein component was then devoid of toxicity. Although toxic fractions were obtained by these two methods, the toxic component had been impaired, for the fractions were considerably less toxic than was the original material. Separation of the toxin was not achieved because further hydrolysis of the fractions resulted in the destruction of the toxin itself. It was further observed that there was always associated with these fractions a substance which showed a specific absorption peak in the ultraviolet region at approximately 2600 Å, and it was suggested that this might be related to the toxic component.

In the present account it has been shown that by acid hydrolysis for a short time it has been possible to dissociate the antigen of Type Z *Sh. paradysenteriae* in such a manner as to obtain a protein component fully as toxic as the original antigen. Under these conditions, however, dissociation is not complete for the protein still contains bound carbohydrate. In view of these results, it was felt that dissociation could be achieved by milder procedures. It was found that the protein component could be eliminated almost completely by digestion with pancreatin. Subsequent fractionation with acid formamide further eliminated some 50 per cent of the total lipids and small amounts of unidentified nitrogenous material without impairing either the serological activity, the toxicity, or the antigenic efficacy of the resulting product. Furthermore, through the action of alkaline phosphatase it was possible to eliminate the component which showed a specific absorption peak at 2600 Å and which, up to this point, had been believed to be associated with the toxin. Finally, it was possible to remove from this degraded product the remaining lipid by extraction with chloroform–methyl alcohol.

Thus a product has been obtained which is both toxic and antigenic and which appears to be predominantly polysaccharide in nature. This substance contains 2.1 per cent nitrogen which is only 0.2 per cent more than the nitrogen calculated for the glucosamine content of the carbohydrate (1.9 per cent). This polysaccharide is phosphorylated and contains approximately 1 per cent of organically bound phosphorus. When this substance, designated as TM, is further hydrolyzed with 1 per cent acetic acid for a period of several hours, toxicity is destroyed, and there appears, as an insoluble precipitate, a small amount of a component high in its content of carbon and phosphorus. At the same time a polysaccharide, no longer toxic, but still serologically active, is

liberated. This polysaccharide differs from the toxic polysaccharide only in that it contains 1 per cent less carbon, and only 0.3 per cent less phosphorus. Whether the insoluble fraction represents the component responsible for toxicity, when combined with the polysaccharide, cannot be said with certainty.

It appears that the toxin is bound to the polysaccharide and that mild acid hydrolysis severs this union and concurrently toxicity is lost. Because of its high carbon (63 per cent) and low nitrogen content, the insoluble precipitate cannot be characterized as a protein. Nor can it be identified as a lipid because the substance is quite soluble in water and because its nitrogen content (2.7 per cent) is too high. It is conceivable that this material might be a complex of a peptide and lipid, but as yet the experimental evidence is not sufficient to characterize the substance definitely. The chemical nature of the toxic component has still not been elucidated, but that it cannot be classified with either the protein or lipid component of the antigen, or with that component showing maximum absorption at 2600 Å, is evident.

SUMMARY

From the chemical evidence presented it appears that the toxicity associated with the somatic antigen of Type Z *Sh. paradysenteriae* may be attributed to an entity distinct from the known components of the complex. That this substance is neither the protein, the lipid, nor the carbohydrate component of the antigen is evident. Because of its lability to chemical manipulation, the nature of the toxic component has not yet been ascertained.

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