ON PRECIPITABLE SUBSTANCES DERIVED FROM BACILLUS TYPHOSUS AND BACILLUS PARA-TYPHOSUS B.

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The present study was primarily undertaken in order to verify certain findings reported on the extraction of precipitable substances of *B. typhosus* with alcohol and ether, but it was extended later to a more general investigation of the precipitable substances of this and allied microorganisms (1).

A number of papers have dealt with the question of lipoids or alcohol-soluble specific substances of typhoid bacilli.

Pick (2) investigating chemically the nature of the specific substances of the *B. typhosus* described one soluble in alcohol which gave no protein and no Molisch reaction and resisted digestion. In concentrated solution it was precipitated by immune sera. Nicolle (3) reported that the agglutinogens of typhoid bacilli are soluble in alcohol and ether but Winterberg (4) was unable to confirm these results. That the lipoids of typhoid bacilli, or even the saponified fats, possess antigenic activity was claimed by Schachenmeier (5) and Stuber (6). Borcic (7), H. Schmidt (8), and Weil and Felix (9), however, failed in their attempts to use alcohol or ether extracts of the typhoid bacilli for immunization. Similar negative results were recorded by Zurugzoglu (10). Very recently Przesmycki (11) has reported that ordinary antibacterial immune sera react specifically with alcoholic extracts of homologous bacilli. Definite statements that bactericidal immune sera develop after the injection of alcohol and ether extracts of typhoid bacilli have been made by Schlemmer (12). So far as we are aware Schlemmer's work has not been repeated as yet.

EXPERIMENTAL.

Bacillus typhosus.—Immune sera were produced by injections of ether extracts of typhoid bacilli according to the directions of Schlemmer. The sera obtained had very weak agglutinating and bactericidal activity when compared with typhoid immune sera obtained in the usual way with whole bacilli.

Attempts to prepare alcohol-soluble precipitable substances from typhoid bacilli by the method of Pick gave no clear-cut results with our material. A saline extract of agar cultures of *B. typhosus* was precipitated with alcohol and both the centrifuged sediment and the supernatant fluid were tested with a precipitating immune serum. It was found that the bulk of the precipitinogen was carried down in the precipitate while the solution contained only a small fraction of the active substance.

Definite results were secured when in place of ether or absolute alcohol 75 per cent alcohol was used for the extraction, in the manner already described for *Vibrio choleræ* (13).

B. typhosus was grown on agar for 24–48 hours at 37°C. The bacilli were centrifuged, washed once with saline and twice with 95 per cent alcohol, and heated for about 2 hours with absolute alcohol (10 cc. per Blake bottle) on the steam bath. The bacilli were collected by filtration on a hot water funnel and extracted twice with boiling 75 per cent alcohol for about 2 hours, the suspension filtered hot, and the filtrate chilled in the ice box. The precipitate formed was dried after washing with 95 per cent alcohol and absolute alcohol and ether. For the precipitin and chemical tests the substance was dissolved in weak alkali and precipitated by acidifying with acetic acid. The procedure was repeated two to three times. This preparation will be referred to as 75 per cent alcohol extract or P1.

When the substance was tested with ordinary typhoid immune sera obtained from rabbits injected with typhoid bacilli heated to 60–62°C. for about 40 minutes, faint reactions only were noticed. We tried therefore to prepare immune sera with the product itself. It was found to possess strong antigenic activity. Two to three injections of 0.2–2 mg. each were sufficient for the production of immune sera which, in contrast to the ordinary typhoid immune sera, precipitated strongly the extracted substance, but agglutinated only feebly suspensions of *B. typhosus* (Table I).

The substance is insoluble in water but soluble in weak alkali. It precipitates out of its solution on acidifying. It gives strong protein reactions. After digestion with trypsin or treatment with antiformin the substance is no longer precipitated by immune sera. On hydrolysis for 5 hours only slight or no reduction of Fehling's reagent was found. The product contains only traces of phosphorus

and traces of lead-blackening sulfur. The N content of the ash-free preparation was 15.7 per cent.

Since the alcohol extract gave definite reactions only with a special immune serum the attempt was made to prepare solutions which would react with the ordinary immune sera. Such active solutions have been obtained previously by various means of extraction, as with saline solution (Pick (2), Weil and Felix (9)), dilute alkali (Zinsser and Parker (14)), antiformin (Altmann and Schultz (15), Krumwiede and Nobel (16)) etc.

We extracted active substance in the following manner:

Washed bacilli previously treated with 95 per cent, hot absolute, and hot 75 per cent alcohol, as described above, were extracted for 2 hours on the steam bath with 2 cc. of .9 per cent saline solution per Blake bottle. After centrifugalization the solution was precipitated with about half the volume 95 per cent alcohol. This crude material was purified to a certain extent by repeated precipitations with acid, and by extraction with 75 per cent alcohol as described below. The product obtained is designated as P2.

Immune sera active for P2 were got by immunization of rabbits with the bacillary residue remaining after the preparation of P1. These sera had a considerable agglutinin titer for live typhoid bacilli.

The product P2 dissolves readily in water and is precipitated by acidulating. It has the reactions and composition of a protein (N 17.2 per cent) and contains only faint traces of phosphorus and traces of lead-blackening sulfur. After hydrolysis for 5 hours only a slight or no reduction of Fehling's reagent was found.

By digestion with trypsin or treatment with antiformin the serological activity, tested by precipitation, was destroyed like that of P1.

That specific substances differing from those just described are present in typhoid bacilli seemed to follow from the important study of Douglas and Fleming (17, *cf.* 11). These authors digested with trypsin typhoid bacilli previously extracted with acetone. Using this material they produced in rabbits immune sera which reacted with the solution resulting from the digestion after removal of an undigested part. The sera had a high bactericidal activity but a weaker agglutinating activity than ordinary immune sera and precipitated more intensely the soluble digestion product. The authors

seem inclined to believe that this precipitable substance is a lower degradation product of proteins.

On repeating[1] the experiments of Douglas and Fleming which deal with the precipitinogen we arrived at confirmatory results. The immune sera obtained had moderate agglutinating power (2000–5000) and only a slight precipitating action for the substances P1 and P2. In conformity with these facts the immune sera for P1 and P2 did not react with the solution resulting from digestion.

When the solution resulting from digestion was made strongly alkaline and precipitated with alcohol a material was obtained with a high content in carbohydrates. Substances with similar properties could be prepared by various methods, *e.g.* as follows:

Typhoid bacilli grown on agar were taken up in N/2 sodium hydroxide solution and kept for about 1½ hours at 37°C. After adding hydrochloric acid till only a slight alkalinity remained and centrifuging, the fluid was precipitated with 1½ volumes of alcohol, the precipitate was redissolved, and after removing some insoluble material and adding hydrochloric acid to make the solution N/10 it was again precipitated with 1½ volumes of alcohol.

This crude product, designated as $C_{typh.}$, gave negative or faint protein reactions, yielded 39.4 per cent reducing sugars after hydrolysis for 5 hours with N/2 hydrochloric acid, and contained N 4.05 per cent (ash-free). Qualitative tests for phosphorous were strongly positive. (A sample obtained after preciptation with barium hydroxide gave, on hydrolysis, about 46 per cent reducing sugars and had a N content of 3.9 per cent.)

Apparently the same substance can be extracted by dissolving the typhoid bacilli with alkaline hypochlorite solution as suggested by Altmann and Schultz, Krumwiede and Nobel.

Typhoid bacilli grown on agar were suspended in saline, and antiformin was added in sufficient amount to cause solution of the bacteria at 55°C. When to this unneutralized solution alcohol was added an active substance separated out. This was suspended in water and after removal of some insoluble material again precipitated with alcohol.

The preparation $C_{typh.}$, exhibits serological reactions similar to those of the substance of Douglas and Fleming. Its serological activity,

[1] In place of acetone, alcohol was used for the extraction.

unlike that of P1 and P2, is not affected by tryptic digestion and treatment with antiformin (1–10 per cent solution for 30 minutes at 37°C.). The preparation was not precipitated in as high dilutions as apparently similar substances from other bacilli.

The precipitation tests performed with the preparations of *B. typhosus* described are summarized in Table I.

TABLE I.

Precipitation Tests with Various Preparations of B. typhosus.

Preparation	Dilution of antigen	Immune sera prepared with					Normal serum
		Whole bacilli		P1	Bacilli after extraction of P1	Digested bacilli	
		96	31				
P1	1,000	+±	tr.	+++	f.tr.	+	f.tr.
	5,000	tr.	0	++±	tr.	±	0
	25,000	0	0	+	0	0	0
	100,000	0	0	±	0	0	0
	500,000	−	−	0	−	−	−
P2	1,000	+		±	++	tr.	0
	5,000	+		tr.	+++	0	0
	25,000	±		0	++	0	0
	100,000	tr.		0	+	0	0
	500,000	0		−	tr.	−	−
$C_{typh.}$	1,000	++		0	0	+++	0
	5,000	+		0	0	++	0
	25,000	tr.		0	0	±	0
	100,000	f.tr.		0	0	tr.	0
	500,000	0		−	−	f.tr.	−
Agglutinin titer for live bacilli		50,000	20,000	100	10,000	2,000	100 neg.

In this and the following experiments 1 drop of immune serum was added to 0.2 cc. of the diluted antigen and the reactions were read after 2 hours incubation at room temperature.

It seems from these experiments that the three preparations tested are distinctly different as to their serological reactions. This holds particularly for substance C. Between P1 and P2 group reactions occur to a slight degree and both react weakly with an immune serum for C. Since this latter reaction did not disappear after treatment

with antiformin it is very probably due to the presence of C in P1 and P2. With several other preparations the group reactions were more pronounced.

When preparing P1 the bacilli were extracted several times with 75 per cent alcohol. The first and second extracts reacted mainly with sera for P1 whereas later extracts gave also considerable reactions with sera for C. In preparing P2 a similar behaviour was noted. The second saline extract gave a stronger reaction for C than the first. The content in C of the protein preparations could be reduced by repeated precipitations with acid. P2 could be freed to a certain extent from P1 by dissolving in 1 per cent saline solution, adding alcohol to a concentration of 75 per cent, boiling, and filtering hot. P2 was isolated from the insoluble part by dissolving in slightly alkaline water and precipitating with acid.

Bacillus paratyphosus B.—From *Bacillus paratyphosus* B a specific substance was prepared by extraction with 75 per cent alcohol as described above. This preparation contained proteins and a considerable amount of carbohydrates. It was precipitated strongly by the ordinary paratyphoid B immune sera and induced the formation of agglutinins and precipitins when injected into rabbits. It was precipitated to a certain extent by immune sera for the substance P1 of typhoid bacilli, but this reaction disappeared after digestion with trypsin or treatment with antiformin while the reaction with paratyphoid immune sera persisted after such treatment. This behaviour suggested the presence in the alcoholic extract of two specific substances analogous to the preparations P_1 and C from *B. typhosus*. In order to prepare the latter the following method was used.

Bacilli previously extracted with hot absolute alcohol were heated on the steam bath with saline solution for about 1½ hours. The suspension was centrifuged, acidulated, and after the removal of the precipitate by centrifugalization the supernatant liquid was precipitated with alcohol. The precipitation with alcohol was repeated 2–4 times in acid and in alkaline solution.

The substance which will be designated as $C_{\text{paratyph. B}}$ gave only faint protein reactions. After hydrolysis with N/2 hydrochloric acid for 5 hours 63.8 per cent reducing sugar was found, calculated as glucose. An analysis gave the following figures for ash-free substance: C 43.8 per cent, H 6.5 per cent, N 1.86 per cent, P 2.06 per cent, ash 7.16 per cent. The yield was considerable and amounted to 10–20 mg. per Blake bottle.

Tests for Species Specificity.

The precipitable substances derived from typhoid and paratyphoid bacilli and the corresponding immune sera were tested with a number of other immune sera and antigens respectively (Tables II, *a*, *b*, and III).

TABLE II, *a*.

Precipitation Tests with P1 from B. typhosus and Various Immune Sera.

Dilutions of antigens	Immune sera obtained with									
	B. typhosus		*B. enteritidis*		*B. paratyphosus* B		*V. choleræ*		*Proteus* HX19
	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli
1,000	+ tr.	−	f.tr.	−	±	−	tr.	0	0
5,000	tr. 0	+++	0	++	f.tr.	+++	f.tr.	0	0
50,000	0 0	+	0	+	0	+	0	0	0
500,000	0 0	0	−	0	−	0	−	−	−

TABLE II, *b*.

Precipitation Test of an Immune Serum for P1 and Crude 75 Per Cent Alcohol Extracts from Various Organisms.

Dilutions of antigens	75 per cent alcohol extract obtained from					
	B. typhosus	*B. enteritidis*	*B. paratyphosus* B	*B. coli*	*V. choleræ*	*Proteus* HX19
1,000	−	−	−	+	0	0
5,000	+++	++	+	+	0	0
50,000	+	±	tr.	tr.	0	0
500,000	0	0	0	−	−	−

It appears from Table II, *a* and *b*, that the serum against the substance $P1_{typh.}$ gives marked group reactions with the analogous substances of *B. enteritidis*, *B. paratyphosus* B, and *B. coli*, and not with the preparations of the more distant organisms, *V. choleræ* and *Proteus* HX19. This result was confirmed by tests with various immune sera.

There were less pronounced group reactions with the preparation

TABLE III.

Precipitation Tests for Specificity with the Preparations C.

Substances obtained from	Dilutions of antigens	Immune sera prepared with									
		B. typhosus digested bacilli		*B. enteritidis* G whole bacilli		*B. paratyphosus* B whole bacilli		*Proteus* HX19 whole bacilli		*V. choleræ* whole bacilli	
B. typhosus	1,000	++±	++++	+	++±	0	0	0	0	0	0
	5,000	+±	+++	±	+±	0	0	0	0	0	0
	25,000	±	++	f.tr.	tr.	0	0	0	0	0	0
	100,000	tr.	+±	0	0	0	0	0	0	0	0
	500,000	0	tr.								
		(5,000)		(10,000)		(1,000)					
B. enteritidis G	1,000	++	++++	±	++±	0	0	0	0	0	0
	5,000	+±	+++	+	++±	0	0	0	0	0	0
	25,000	±	++±	+±	+++	0	0	0	0	0	0
	100,000	0	f.tr.	+	+++	0	0	0	0	0	0
	500,000	0	0	±	++						
				(5,000)		(100)					
B. paratyphosus B	1,000	+±	++	0	tr.	+±	++±	0	0	0	0
	5,000	tr.	+	tr.	±	+±	++±	0	0	0	0
	25,000	0	tr.	±	+	++	+++	0	0	0	0
	100,000	0	0	tr.	+	+±	++++	0	0	0	0
	500,000			f.tr.	±	tr.	++±				
		(1,000)*		(1,000)		(50,000)					

Proteus OX19	1,000	0	tr.	0	0	0	0	++	+++	0	0
	5,000	0	0	0	0	0	0	++++	+++	0	0
	25,000	0	0	0	0	0	0	++++	++++	0	0
	100,000	0	0	0	0	0	0	++±	++++	0	0
	500,000							+	++		
								(2,000)			
V. choleræ	1,000	tr.	tr.	0	0	0	0	0	0	++++	++++
	5,000	0	0	0	0	0	0	0	0	++++	++++
	25,000	0	0	0	0	0	0	0	0	+++	+++
	100,000	0	0	0	0	0	0	0	0	+±	++±
	500,000									tr.	+
										(20,000)	

First reading after 2 hours room temperature, the second after standing overnight in the ice box. The figures indicate the agglutinin titre.

* Very weak reactions also in high concentrations of serum.

P2. $P2_{typh.}$ was not acted upon in the dilutions tested by two common immune sera against *B. enteritidis* and gave a weak reaction with only one of two common immune sera against *B. paratyphosus* B. A moderate reaction became evident when a serum very active against $P2_{typh.}$ was tested with a crude P2 preparation of *B. paratyphosus* B, and a faint reaction occurred with a similar preparation of *Proteus* HX19.

The reactions involving the species specificity of the substances C are summarized in Table III. The substance C of *V. choleræ* has already been described (13). C_{ox19} was obtained in a similar manner from *Proteus* OX19.[2] This latter product gave negative or faint protein reactions and had a carbohydrate content of 57.3 per cent after hydrolysis for 5 hours. A substance apparently with the same serological and chemical properties could be prepared from *B. proteus* HX19 with about the same yield as that got from *Proteus* OX19. C of *B. enteritidis* was prepared in the same manner as $C_{typh.}$ The yield of reducing sugars after hydrolysis of this substance was 56.89 per cent (calculated as glucose for ash-free substance).[3]

In tests with the substances C showing strong reactions, the precipitates appear generally in heavy flakes or membranes unlike the more fluffy precipitates of proteins as has already been observed by Avery and his coworkers.

DISCUSSION.

The investigations reported may be regarded as an initial step in the study of the antigens of typhoid bacilli and related organisms. The work should be extended in various directions with special reference to the purification of the isolated products, and the rôle they play in the group reactions of the related species. Methods of preparation should be studied, a search made for additional active substances, and various strains of the same organism examined.

The active substances thus far prepared fall into two groups differentiated sharply by their behaviour towards trypsin and alkaline

[2] A report on specific polysaccharides in *Proteus* HX19 has been made recently by Przesmycki (18).

[3] An antigenic solution of *B. enteritidis*, containing traces of proteins and much carbohydrate was dealt with recently by Branham and Humphreys (19).

hypochlorite solution (antiformin). Those of one group (P) are easily destroyed by these agents and behave in general like proteins, while the others (C) are resistant to trypsin and considerably so to antiformin and yield a large quantity of reducing sugar on hydrolysis. There was no definite evidence in our studies with *B. typhosus* of the existence of lipoids as assumed by Schlemmer and others.

The presence in bacilli in general and in typhoid bacilli especially of specific non-protein substances which do not induce antibody formation has been emphasized by Zinsser and Parker (14). An indication of the existence of such products can be found in the paper of Pick although he considered the possibility that his substances were disintegration products of proteins. A similar view was held by Douglas and Fleming (17) concerning their substances obtained by digestion.

In the light of the well known studies of Avery and Heidelberger it seemed likely that the specific component in the products of the above authors may belong to the group of specific carbohydrates, a view substantiated by our findings. The immune sera obtained by us following the directions of Douglas and Fleming reacted on our substance C which is almost free from proteins and rich in carbohydrates. This preparation C will probably prove to be a hapten in our terminology, *i.e.*, a specific substance devoid of antigenic activity.

Substances belonging apparently to the same group, that is to say yielding much sugar on hydrolysis and practically free from protein, were also found in *B. paratyphosus* B, *B. enteritidis*, and *Proteus* HX19 and OX19.[2]

On cross-testing the various substances of the group C with the corresponding immune sera the strongest group reaction occurred between *B. typhosus* and *B. enteritidis*, the homologous reactions being more intense. The substance of *B. paratyphosus* B was precipitated by the sera for *B. typhosus* and *B. enteritidis* though not very intensely. It is remarkable that the reciprocal reactions, namely those of the paratyphoid B serum with the substances from *B. typhosus* and *B. enteritidis* were entirely negative. For an understanding of these relations further investigations are required, involving also absorption tests. With the substances and sera of microorganisms not belonging to the typhoid groups negative or only faint reactions took place.

Observations similar to ours were made by Krumwiede and Nobel who found strong reactions of typhoid immune sera with antiformin extracts of *B. pullorum* and *B. sanguinarium* and faint reactions with extracts of *B. paratyphosus* B. Probably the active substances in the antiformin extracts correspond to our preparations C.

The tests with the preparations P1 and P2 show that the proteins of a given bacillus may be sharply differentiated by serological reactions in the same way as the proteins of an animal species (20) or those of yeasts (21). No effort has yet been made to fractionate our active protein preparations or to isolate other precipitable proteins.

The high antigenic power of the bacterial proteins is remarkable (22) and it may serve to explain some of the statements published on the antigenic activity of preparations from bacilli that are apparently protein-free. In view of the antigenic capacity of P1 it seems significant that the immune sera formed after injection of whole bacilli heated to 60 or 100°C. have ordinarily only a weak action on P1, so that it is necessary to immunize with the substance itself if one desires to obtain potent precipitins for P1. This phenomenon could be explained either by a change of the precipitable substance through the action of alcohol, or more readily by the masking of its antigenic activity in the original complex. To such an effect one may ascribe also the fact that the sera active for the various precipitable fractions, especially the P1 serum, are considerably less agglutinative than the common immune sera. Examples of both phenomena, namely of the failure of an immune serum for a complex antigen to react with the components of that antigen and of the failure of a serum for one component to react with the complex were encountered in the study of blood antigens (23). Avery and his coworkers (24) have described similar findings in their work on pneumococci.

By absorbing a common typhoid immune serum with bacilli treated with alcohol a fluid was procured which is supposed to contain the flagellar agglutinins (Smith and Reagh (25)) and indeed agglutinated live typhoid bacilli to a high titer and also suspensions containing flagellæ (Orcutt (26)). The fluid failed to precipitate any of our precipitable substances. Similarly an immune serum for *Proteus*

HX19 after absorption with *Proteus* OX19 did not precipitate the substance C_{x19} although it agglutinated intensely *B. proteus* HX19 (not *Proteus* OX19). Thus there is no proof as yet of a connection of these precipitable substances with the so called "labile" agglutinogen.

SUMMARY.

Attempts to confirm certain statements that ether-soluble specific substances can be obtained from *B. typhosus* have lead to negative results.

Two serologically active protein substances and another that was non-protein have been separated from *B. typhosus*. The first two are not resistant to tryptic digestion or to treatment with alkaline hypochlorite solution whereas the third resists both. One of the proteins could be extracted with 75 per cent alcohol.

Specific precipitable substances reacting like the non-protein substance of *B. typhosus* and containing large amounts of carbohydrates have been prepared from *B. paratyphosus* B, *B. enteritidis*, and *Proteus* HX19 and OX19. Observations on the serological behaviour of these preparations are described.

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