STUDIES ON THE PRECIPITABLE SUBSTANCES OF BACILLI OF THE SALMONELLA GROUP.

By J. FURTH, M.D., and K. LANDSTEINER, M.D.

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

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In earlier communications we (1) have described precipitable substances, probably of carbohydrate nature, isolated from *B. typhosus*, *paratyphosus* B, and *enteritidis*, and two antigenic protein preparations obtained from *B. typhosus*. These studies have also been extended to the main serological types of the Salmonella group of organisms.

For a general review of the subject we refer to the communications by P. Bruce White (2), Krumwiede, Cooper, and Provost (3), Tulloch (4), and to the references given in our earlier papers.

We would however like to mention several recent publications. Thus Happold (5) described a precipitable substance obtained from *B. aertrycke* which he considered to be of protein nature and "to be identical with the antigen which stimulates the production of agglutinins to a heat-stable antigenic form of the organism." Ecker and Rimington (6) report obtaining from *B. aertrycke* a carbohydrate containing material possessing toxic properties; while White (7) states that he has extracted a soluble specific substance from *B. aertrycke* similar to the specific soluble substances of Avery and Heidelberger. The precipitable solution obtained was acted upon by the sera of *B. aertrycke* and *paratyphosus* B but not by those of *B. Newport* and *suipestifer*. White's paper contains a full discussion of the serological properties of the rough *B. aertrycke* strains. Casper (8) prepared from *B. paratyphosus* B a carbohydrate containing substance which reacted with sera for *B. paratyphosus* B, *B. typhosus*, and *B. typhi murium*, to a lesser degree with sera for *B. enteritidis* Gärtner, but not with *suipestifer* serum. A report on the carbohydrate and protein fractions of *B. typhosus* was made by Heidelberger, Shwartzman, and Cohn (9).

Preparation of the Crude Precipitable Carbohydrate Substances.—Three methods were employed for the preparation of the crude substances:

1. Extraction by alkaline hypochlorite solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, and alkaline hypochlorite solution was added in a quantity sufficient to dissolve the bacteria at about 50°C. The solution was chilled and cold 95 per cent alcohol added until a heavy precipitate was formed carrying down most of the active substance. This

TABLE I.

Precipitation Tests.

Carbohydrates from *Bacillus*	Antigen dilutions	Immune sera obtained with *Bacillus*											
		Typhosus		*Enteritidis*		*Paratyphosus* B		*Derby*		*Newport*		*Hog cholera*	
Typhosus	5,000	+++	+++	++	++++	+	+±*	++	+++	0	0	0	0
	50,000	+±	++±	+	++	±	+	+	+	0	0	0	0
	500,000	±	±	f. tr.	±	0	0	0	tr.	0	0	0	0
Enteritidis	5,000	+±	+++	++	++++	tr.	±	+	+	0	0	0	0
	50,000	±	±	+	++	0	0	0	tr.	0	0	0	0
	500,000	0	tr.	f. tr.	±	0	0	0	0	0	0	0	0
Paratyphosus B	5,000	tr.	±	+±	++	+±	++	+++	++++	0	0	0	0
	50,000	0	0	±	+	+	+++	++	++±	0	0	0	0
	500,000	0	0	0	±	±	+	±	±	0	0	0	0
Derby	5,000	±	+	+	++	+±	+++	++++	++++	0	0	0	0
	50,000	f. tr.	±	±	+	+	+++	++	++±	0	0	0	0
	500,000	0	0	0	tr.	±	+	±	±	0	0	0	0
Newport	5,000	0	0	0	0	0	0†	0	0	++	++++	tr.	±†
	50,000	0	0	0	0	0	0	0	0	+	+++	0	0
	500,000	0	0	0	0	0	0	0	0	±	+	0	0
Hog cholera	5,000	0	0	0	0	0	0	0	0	+	+++	+++	++++
	50,000	0	0	0	0	0	0	0	0	±	++	++	++++
	500,000	0	0	0	0	0	0	0	0	tr.	++	+	+±

To 0.1 cc. of the antigen solution 0.05 cc. immune serum and 0.05 cc. physiological salt solution were added. The reactions were read after 2 hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

The immune sera were obtained by injections of rabbits with heated bacilli, as stated above.

* With other sera of the same type the reactions were negative with the substance of *B. typhosus*.

† With some other sera of the same type marked reactions were obtained with the substance of *B. Newport*.

precipitate was dissolved in water, and, after removal of some insoluble material, was reprecipitated with 95 per cent alcohol. After washing with 95 per cent alcohol, absolute alcohol, and ether, it was dried *in vacuo*.

2. Extraction with saline solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, centrifuged,[1] the sediment washed with 95 per cent alcohol, and extracted with boiling 95 per cent, and absolute, alcohol. After filtration on a hot water funnel the bacillary mass was heated in the steam bath two or three times with 0.9 per cent sodium chloride solution for 1 to 2 hours and the extract separated each time by centrifuging. Much of the protein was removed by addition of hydrochloric acid in an amount sufficient to produce maximum precipitation and the fraction was reprecipitated to recover some of the active non-protein substance; the joint mother liquids were then precipitated with alcohol after addition of 1/20 volume of normal sodium hydroxide. The further redissolving, removal of insoluble material, reprecipitation, and drying of the preparation were done as described above.

3. Extraction by autoclaving and subsequent tryptic digestion (*cf.* Goebel and Avery (10)): Agar cultures were washed off and were autoclaved for 20 minutes and digested overnight with purified trypsin solution (Goebel and Avery) at 37°C. The subsequent alcohol precipitation, removal of insoluble material, reprecipitation, and drying of the substance were carried out in the manner given.

Cultures.—Besides our laboratory strains the following cultures obtained through the courtesy of the National Collection of Type Cultures, London,[2] were used: *B. paratyphosus* B, *Tidy No. 14;* type *Stanley, No. 92;* type *Reading, No. 72;* type *Derby, No. 1729;* type *Newport, No. 129;* type *Hog cholera, No. 356; B. enteritidis, Gärtner, No. 127; B. abortivo-equinus, No. 766;* and *B. aertrycke.*[3] They were plated on agar and colonies showing homogeneous growth in broth were selected.

Immune Sera.—Unless otherwise stated, immune sera were prepared by weekly injections of rabbits with about 1/10 to 1/20 of 24 hour agar slant cultures heated to 62–65°C. for 40 minutes. Immune sera prepared with various strains of the same type, *e.g. B. typhosus*, showed considerable variations in their capacity to precipitate the carbohydrate preparations.

Precipitin Reactions of the Carbohydrates and Their Parallelism to the "Small Flaking" Agglutination.—Precipitin reactions with the purified specific substances (see page 737) are presented in Table I. The crude substances had a lower titer but showed in cross-tests almost the

[1] In a control experiment the bacillary mass was washed several times with saline solution.

[2] We are indebted to Dr. R. St. John-Brooks, Curator of the National Collection of Type Cultures, London, for supplying us with the cultures mentioned.

[3] The numbers correspond to those given in the catalogue of the collection.

same range of specificity as purified preparations, no matter which of the methods described was employed.

It is seen that the strains can be divided roughly into three groups within which the strongest cross-reactions occur. The first group consists of *B. typhosus* and *B. enteritidis*, the second of *B. paratyphosus*

TABLE II.*

Typhosus	*Enteritidis*	*Paratyphosus* B	*Derby, Reading, abortivo-equinus*	*Newport*	*Hog cholera*
III, (X), 8	III, 8	I, II, 7, 8	II, 7, 8	IV, VI, 7	V, VI

* Roman numerals are used by White to designate salient components, Arabic numerals for those of minor development.

TABLE III.

Absorption Tests with Immune Serum for B. typhosus.

Dilution of the carbohydrates	Serum, unabsorbed		Serum absorbed with alcohol-treated *B. enteritidis*	
	Precipitation tests with the carbohydrate of *B. typhosus*			
5,000	++	+++	0	0
50,000	++	++	0	0
500,000	f. tr.	tr.	0	0
Dilution of the immune serum	Agglutination tests with *B. typhosus* (suspension preserved with chloroform)			
300	+++	+++	+++	+++
900	+++	+++	++	++
2,700	++	++	+±	+±
8,100	+	+	±	tr.
24,300	0	tr.	0	0

The reactions were read after 1½ hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

B, and *B. Derby*, and the third of *B. Newport* and *B. hog cholera*. Additional tests with crude preparations, not included in Table I, showed that the substances and sera of *B. Stanley*, *B. Reading*, and *B. abortivo-equinus* reacted like those of *B. paratyphosus* B.

A comparison of these results with the agglutinin reactions of the "stable agglutinogens" suggested itself. The distribution of the

agglutinable factors underlying the "small flaking" agglutination is summarized, according to White, in the scheme shown in Table II.

On comparing this scheme with the precipitin reactions an approximate correspondence between the stable agglutinogens and the precipitable carbohydrates appears (see White).

This relation is also illustrated by the following observations (Table III): An immune serum for *B. typhosus* was absorbed with alcohol-treated *B. enteritidis* and tested for precipitins and agglutinins to *B. typhosus*. Since the "stable" alcohol-resistant agglutinogens of *B.*

TABLE IV.

Absorption Experiments.

Dilution of the precipitable substance	Precipitable substance	Immune serum for *B. enteritidis*		Precipitable substance	Immune serum for *B. Newport*		Precipitable substance	Immune serum for *B. hog cholera*	
		Unabsorbed	Absorbed with *B. paratyphosus* B		Unabsorbed	Absorbed with *B. hog cholera*		Unabsorbed	Absorbed with *B. Newport*
2,000	*Enteritidis*	+++	+++	*Newport*	+++	+++	*Hog*	+++	+++
20,000		+++	+++		++±	++±	*cholera*	+++	+++
200,000		±	±		+	+		++±	++±
2,000	*Paraty-*	+++	0	*Hog*	++	0	*Newport*	+	0
20,000	*phosus* B	+++	0	*cholera*	+++	0		±	0
200,000		+±	0		++±	0		tr.	0

The immune sera were absorbed with bacillary suspensions kept in alcohol and washed once with saline solution.

typhosus and *B. enteritidis* are supposed to be very similar it was to be expected that by the exhaustion with alcohol-treated *B. enteritidis* the precipitins would be removed but not the "large flaking" agglutinins. The result of the experiment bore out this assumption.

In contrast with the absence of precipitating action of fluids with large flaking agglutinins of high titer (*cf.* Heidelberger, Shwartzman, and Cohn) are instances in which immune sera with a relatively low agglutinin content, mainly of the small flaking type, precipitated intensely the carbohydrate solutions. Such sera were prepared by the method of Douglas and Fleming (1).

Some discrepancies will be noted such as the absence of precipitin reactions of certain *B. paratyphosus* B sera on the substances of *B. Newport* (factor 7) and *B. typhosus* (factor 8), and the lack of precipitating capacity of most of the hog cholera sera for the substance derived from the strain *Newport* (factor VI). Since in these combinations some of the sera give positive reactions one may assume that the precipitable substances do not lack the properties in question. An explanation may perhaps be seen in the observation that the agglutinin reactions with alcohol-treated bacilli were comparatively weak in

TABLE V.

Precipitable substance	Dilution	Immune serum for *paratyphosus* B				
		Unabsorbed, diluted			Absorbed with *B. Reading*, diluted	
		1:2	1:8	1:32	1:2	1:8
Paratyphosus B, not treated with alkali	5,000	+++	+++	+	++++	+++
	50,000	+±	++	++	++	++
	500,000	±	±	tr.	±	−
Paratyphosus B, treated with alkali	5,000	++	+	tr.	0	−
	50,000	+	+	0	0	−
	500,000	f. tr.	0	0	0	−
Reading (treated with alkali)	1,000	+++	++	tr.	0	−
	5,000	++	+±	f. tr.	0	−
	25,000	+	+	f. tr.	0	−

those instances in which precipitation was lacking. However, a thorough study of this question was not made.

In order to determine whether the multiplicity of precipitins corresponds to that of the agglutinins, absorption experiments were made in several cases (Table IV).

On the whole the tests were in agreement with the idea of a multiplicity of precipitins, corresponding to that of agglutinins. A striking exception was the following:

Immune sera for *B. paratyphosus* B or *B. Stanley*, when absorbed with *B. Reading* or *B. abortivo-equinus* did not precipitate the substances prepared from the homologous organisms, although they agglutinated the alcohol-treated bacillary suspensions. Search for

the missing precipitable property (attributable to factor I) revealed that it was present in the crude saline extracts, but disappeared after treatment with alkali. Substances exhibiting the property were prepared as follows:

Saline extracts obtained from alcohol-extracted bacilli, as described above, were precipitated with alcohol (without the addition of alkali), after previously removing the substances precipitable by dilute acid. The precipitate was dissolved in a small volume of water, some insoluble material discarded, and 50 per cent trichloracetic acid added to cause optimal precipitation. The unneutralized mother liquid containing most of the active substance was then precipitated with an excess of 95 per cent alcohol and dried after washing several times with 95 per cent alcohol, absolute alcohol, and ether.

Table V indicates that immune sera for *B. paratyphosus* B (or *B. Stanley*) contain a fraction of antibodies directed towards the alkali-labile property I. After absorption with *B. Reading* the immune serum for *B. paratyphosus* B still precipitates the substances derived from this bacillus but no longer acts on the substance from *B. paratyphosus* B after treatment with alkali. That this effect is not due to a diminution of one single antibody was shown by tests with diluted immune serum.

The reactivity of the substance was not altered by peptic and tryptic digestion at a pH not in itself injurious. It was not destroyed by treatment for 1 hour at room temperature, with normal hydrochloric acid, but it did not withstand under the same conditions the action of 0.01 normal sodium hydroxide (Table VI).

A preliminary investigation was made of the carbohydrates of "rough" strains of *B. paratyphosus* B and *B. aertrycke*. According to several authors such strains may contain special antigenic components. In view of these statements we prepared precipitable substances from "rough" strains by the method of dissolving the bacilli in alkaline hypochlorite solution (see page 727). The substance prepared from *B. aertrycke* "R" and purified as described below proved to contain large amounts of carbohydrates not further investigated. In precipitation tests (Table VII) the "R" substances reacted specifically with the immune serum to the "R" strains whereas the corresponding "S" preparations reacted also to "R" immune sera. It is uncertain whether the interaction can be explained by the presence of "R" forms in the "S" strains.

Attempts to Fractionate the Precipitable Substances by Means of Precipitins.—The resistance of the specific precipitable substances to the action of alkali seemed to offer a way of ascertaining whether the various serological properties of these substances are due to different

TABLE VI.

Precipitin Tests Showing the Effect of Acid, Alkali, Peptic, and Tryptic Digestion on Factor I of B. paratyphosus B.

Dilution of the precipitable substance	Control I	0.01 N NaOH	N HCl	Peptic digestion	Control II	Tryptic digestion I	Tryptic digestion II	Control III
5,000	++++	0	+++	+++	+++	+++	0	0
100,000	+	0	±	+	+±	+	0	0

To 0.1 cc. of the precipitable substance, 0.1 cc. of an antibody solution for factor I (immune serum to *B. paratyphosus* B absorbed with suspensions of *B. Reading*) was added.

Control I: untreated precipitable substance.

Control II: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.45 cc. water; kept at 37° for 24 hours.

Control III: 0.5 cc. of a 1 per cent solution of the substance, 0.2 cc. of a 1 per cent solution of Na_2CO_3, 0.8 cc. water; kept at 37° for 24 hours.

Peptic digestion: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.1 cc. of a 0.2 per cent pepsin solution, 0.35 cc. water; kept at 37° for 24 hours.

Tryptic digestion I: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.05 cc. 1 per cent Na_2CO_3, 0.45 cc. water; kept at 37° for 24 hours.

Tryptic digestion II: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.2 cc. 1 per cent Na_2CO_3, 0.3 cc. water; kept at 37° for 24 hours.

For the treatment with acid and alkali a 2 per cent solution of the substance was mixed with an equal volume of 0.02 N NaOH or 2 N HCl respectively and the tests kept for 1 hour at room temperature.

separable portions. Accordingly attempts were made to fractionate the precipitable substances in a manner analogous to the usual absorption experiments with immune sera.

By suitable exhaustion with heterologous bacilli fractions of immune sera were prepared acting only on part of the supposed precipitable factors. The precipitates produced were washed twice with saline

solution and boiled for a few seconds with a small quantity of 0.1 normal sodium hydroxide solution, the solutions obtained being neutralized and centrifuged.

When such solutions were tested with various antibodies they behaved like the original precipitable substance and not like qualitatively different fractions thereof.[4] With the procedure described no difficulty was encountered in separating precipitable substances of two different strains of bacilli after their solution had been mixed. It was

TABLE VII.

Precipitation Test.

Immune serum of *Bacillus*.....		*Aertrycke* "S"		*Aertrycke* "R"		*Paratyphosus* B "S"		*Paratyphosus* B "R"	
Precipitable substance	Dilutions								
Aertrycke "S"	2,000	+++	+++	+±	++±	+±	+±	+±	+++
	20,000	++	++	±	++	+	++	tr.	+
	100,000	±	+	f. tr.	+	±	+±	0	tr.
	500,000	0	0	0	0	0	0	0	0
Aertrycke "R"	2,000	0	0	+±	++±	0	0	++	++±
	20,000	0	0	0	0	0	0	tr.	+
Paratyphosus B "S"	2,000	+++	+++	++	++±	+	++	++	++
	20,000	++	++±	+	++	±	++	tr.	+±
Paratyphosus B "R"	2,000	0	0	++	+++	0	0	+++	+++
	20,000	0	0	++	+++	0	0	++	++±

Reactions read after 2 hours (Column 1) and after standing overnight (Column 2).

thought however that in these control experiments the mixture of the substances might not have been as intimate as that of the hypothetical fractions in the precipitable carbohydrates derived from one organism. We attempted therefore to separate by the same method precipitable substances after their solutions had been mixed, boiled, precipitated with alcohol, and dried in the usual manner.

[4] The results were the same in experiments aiming at the separation of Factors I and II of the precipitable substances of *B. paratyphosus* B. In this instance, instead of alkali, dilute acid was used for decomposing the precipitate.

TABLE VIII.

Precipitable substance	Dilution	Immune sera for:							
		B. paratyphosus B absorbed with *B. enteritidis* (II-7)		*B. enteritidis* (8)		*B. hog cholera* absorbed with *B. Newport* (V)		*B. Newport* (VI)	
Mixture of the carbohydrates of *B. paratyphosus* B and *B. hog cholera*	1,000	+±	++++	+	++++	++	++++	++	++++
	10,000	++	++++	±	+±	+±	+++	+±	++++
	100,000	tr.	+	f. tr.	±	f. tr.	tr.	f. tr.	tr.
Factor II, 7	1,000	+±	+++	+	+±	0	0	0	0
	10,000	±	+±	tr.	±	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor 8	1,000	+±	+++	+	++	0	0	0	0
	10,000	tr.	tr.	0	f. tr.	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor V	1,000	0	tr.	0	0	+++	++++	++±	++++
	10,000	0	0	0	0	±	+	±	+±
	100,000	0	0	0	0	0	tr.	0	tr.
Factor VI	1,000	0	f. tr.	0	0	++	++±	+±	++±
	10,000	0	0	0	0	±	+	±	++
	100,000	0	0	0	0	0	0	0	0

To a 0.1 per cent solution of the mixture of *B. paratyphosus* B and *B. hog cholera* carbohydrates an equal volume of the immune sera (diluted 1:2) was added. After keeping the tubes for 2 hours at room temperature and overnight in the ice box, the precipitate formed was dissolved by sodium hydroxide, as described, and brought with saline solution to half of the original volume. (This dilution was arbitrarily designated 1:1000.)

Factor II, 7: precipitate formed by *paratyphosus* B immune serum absorbed with *B. enteritidis*.

Factor 8: precipitate formed by immune serum of *B. enteritidis*.

Factor V: precipitate formed by hog cholera serum absorbed with *B. Newport*.

Factor VI: precipitate formed by immune serum of *B. Newport*.

In these tests a considerable amount of carbohydrate was carried down non-specifically by the heterologous serum. The possibility that this effect was caused by imperfect solution of the mixture of precipitable substances led to the following modification of the experiment. The dried mixture obtained as before was dissolved in 0.1 normal sodium hydroxide, boiled for 1 hour, and neutralized. In this way again by specific precipitation an almost complete separation could be brought about.

In the following experiment this method was applied to the separation of fractions of single precipitable substances (Table VIII).

The experiment shows that while the two substances mixed together could be separated, apparently no fractionation of either of them was accomplished.

An analogous experiment was carried out with the substance of *B. Newport* and with the sera to *B. suipestifer* (factor VI), *B. paratyphosus* B (factor 7), and *B. Newport*, after exhaustion with *B. suipestifer* and *B. paratyphosus* B (factor IV). Comparing factors IV and VI the results resembled those just reported; the substance however precipitated by *paratyphosus* B serum gave a considerably stronger reaction with this serum than with the hog cholera serum. Conversely the solution obtained from the precipitate caused by the hog cholera serum reacted, like the original substance, more intensely with this serum than with *B. paratyphosus* B serum.

Chemical Data on the Precipitable Substances.—Several of the substances described were purified to a certain extent by a procedure very similar to that used for pneumococcus polysaccharides by Avery, Heidelberger, and Goebel (11).

The crude preparations were dissolved in water, some insoluble matter removed, and, after addition of normal sodium hydroxide to a concentration of about N/20, the active substance was precipitated with alcohol. This precipitation was repeated 4 to 6 times. Usually sodium acetate was added to aid flocculation. Then the solution was acidified with hydrochloric acid and precipitated with acidulated alcohol. When possible the precipitate was made in two steps, the first precipitate, containing most of the proteins and much carbohydrate, was removed by centrifugalizing and the supernatant fluid poured into an excess of acidulated alcohol. From the first pre-

cipitate, rich in proteins, part of the carbohydrate could be recovered by repeating the procedure.

The carbohydrate substance was reprecipitated in acid solution and dried after washing with alcohol and ether. The solutions were kept at low temperature while in acid solution.

These fractions were soluble in water, strongly reduced Fehling's solution after hydrolysis, and gave faint or negative protein reactions, particularly no precipitate with tannic acid or uranyl nitrate. The purified substance of *B. typhosus*, unlike the crude preparation, was not precipitated by barium hydroxide.

On analysis, the figures for the N content of the substances prepared from *B. typhosus*, *B. enteritidis*, *B. paratyphosus* B, *B. Derby*, *B. Newport*, and *B. hog cholera*, varied from 0.5 per cent to 1.4 per cent. The values for $[\alpha]^D$ were as follows: *B. typhosus*, +103; *B. enteritidis*, +95; *B. paratyphosus* B, +94; *B. Derby*, +76; *B. Newport*, +75; *B. hog cholera*, +48. For the sugar analysis the substances were hydrolyzed by heating 1 per cent solutions with an equal volume of normal hydrochloric acid in the steam bath for 5 hours, and the sugar content was determined by reduction of Fehling's solution. It was not established whether the time chosen was sufficient for complete hydrolysis. The values obtained were between 63 per cent and 74 per cent (calculated as glucose) for the six strains mentioned. During hydrolysis with acid some insoluble material, partly soluble in alcohol and of acid character, separated from the solutions as in the case of the specific substance from *V. choleræ* (12). No conclusion can be reached as yet whether these products form a part of the precipitable substances or are impurities.

Since it seemed possible that the precipitable substances contained carbohydrates derived from the agar used for cultivating the bacteria, two preparations were made from gelatin cultures of *B. typhosus* and *B. paratyphosus* grown in Blake bottles. They gave the following figures: *B. typhosus*, reducing sugar 69.5 per cent, $[\alpha]^D + 98°$; *B. paratyphosus*, reducing sugar 67.3 per cent, $[\alpha]^D + 99°$. It cannot be claimed that the substances were obtained in a state of purity and therefore all of the figures given are to be considered as preliminary.

On testing the action of alkali and acid it was found that the precipitable substances were remarkably resistant to alkali but readily destroyed by acids.

TABLE IX.

Precipitin Tests with the Carbohydrates after Treatment with Acid and Alkali.

Carbohydrates		Heating with N HCl for				Heating with N NaOH for				Control untreated	
From *Bacillus*	Dilution	3 min.		15 min.		½ hr.		2 hrs.			
Proteus O_{X19}	10,000	0	f. tr.	0	f. tr.	0	0	0	0	+++	+++
	100,000	0	0	0	0	0	0	0	0	+	+±
V. choleræ	10,000	±	+++	0	0	0	+++	0	+	++	+++
	100,000	0	++	0	0	0	±	0	0	±	+
Pneumococcus Type III	10,000	++	++	+±	+±	++	++	++	++	++±	++±
	100,000	+	+±	±	+	±	+	±	+	+	+±
B. typhosus	10,000	0	0	0	0		−	++	+++	+++	+++
	100,000	0	0	0	0		−	±	++	+	++
B. enteritidis	10,000	0	0	0	0		−	++±	++±	++±	+++
	100,000	0	0	0	0		−	±	+±	+	+±
B. paratyphosus B	10,000	0	f. tr.	0	0		−	+±	++±	++	++±
	100,000	0	0	0	0		−	++	++±	++	++
B. Derby	10,000	0	0	0	0		−	+++	+++	+++	+++
	100,000	0	0	0	0		−	+	+±	+	+
B. Newport	10,000	0	0	0	0		−	++	+++	++	+++
	100,000	0	0	0	0		−	+	++	+	++
B. hog cholera	10,000	+±	++±	0	f. tr.		−	++±	+++	+++	+++
	100,000	0	+±	0	0		−	+±	++	+±	++

The substances of *Proteus* O_{X19} and cholera were prepared by extraction with boiling 75 per cent alcohol (1,10). The specific polysaccharide of Pneumococcus III was obtained through the courtesy of Dr. Avery.

The first reading of the precipitin tests was made after 2 hours at room temperature, the second reading after the tests were kept overnight in the ice box.

To a 2 per cent solution of the various preparations an equal volume of 2 normal sodium hydroxide or 2 normal hydrochloric acid was added and the solutions were kept in boiling water.

It is seen from the experiment (Table IX) that the preparations of the *typhosus-paratyphosus* group were similar as regards their resistance to acid and alkali, with the exception of the hog cholera substance which was somewhat more resistant to acid than the other preparations. The substances from the other organisms tested, behaved differently. Thus the preparation obtained from *Proteus* O_{X19} was destroyed by alkali as well as by acid, under the conditions of the experiment; the Pneumococcus III preparation was resistant to both, whereas the substance derived from *V. choleræ* was less resistant to alkali than the Salmonella carbohydrates. The differences found would seem to be significant even if one takes in account the variation in the method of preparation.

Observations on the Precipitable Proteins of B. typhosus.—A precipitable protein was prepared in a similar manner as the preparation P_2 *typhosus* described previously (1). The precipitation with alcohol of the extracted substance was omitted but after removal of the suspended bacilli by centrifugalizing, the saline extract was precipitated with dilute hydrochloric acid, redissolved by addition of a small quantity of alkali, filtered through a Berkefeld filter, and reprecipitated with acid. The precipitate was dried after washing with alcohol and ether.

This substance when injected into rabbits induced the formation of "large" and "small" flaking agglutinins aside from precipitins. Although this would seem to point to a relation between the substance P and "large flaking" agglutinogens there are observations which do not agree with this assumption. In the first place the titer of the large flaking agglutinins was relatively low in comparison with sera obtained with bacillary suspensions, and on prolonged immunization the increase of precipitins was not accompanied by a corresponding rise in the agglutinin titer. Furthermore in an absorption experiment the large flaking agglutinins of the sera for P_2 were apparently absorbed to a greater extent by typhoid bacilli treated with alcohol than the large flaking agglutinins of common typhoid sera.

It is as yet difficult to interpret these observations. They may possibly be ascribed to the presence in the P_2 preparations of a special substance responsible for the production of flagellar agglutinins, or to some flagellar material perhaps in an altered state which passed the filter candles. In this respect attention may be called to the observation that bacilli treated with alcohol are no longer agglutinable by large

flaking sera although they give rise to the formation of large flaking agglutinins (13).

The toxic action of the preparation P_2, mentioned previously, showed considerable variation. One preparation was toxic for rabbits in a dose of 0.5 mg. given intravenously. On repeated injections the animals tolerated doses up to about 20 mg. These animals as well as those immunized with digested bacilli (14) exhibited a typical Arthus phenomenon on intradermal injections of about 1 mg. of P_2 or the carbohydrate respectively. In cross-tests the reactions with the homologous substances were more pronounced. (*Cf.* the experiments on anaphylaxis by Tomcsik (15) and Avery and Tillett (16).) These tests were made on a small number of animals and therefore should be considered as preliminary.

DISCUSSION.

The carbohydrate-containing preparations isolated from the main serological types of the Salmonella group gave on analysis figures for nitrogen of 0.5 to 1.4 per cent, but they showed only weak or negative reactions for proteins. One may assume that their serological activity is due to specific carbohydrates, for during purification there was a diminution of the nitrogen content and of the protein reactions along with an increase in the amount of sugar liberated by hydrolysis and an increase of the serological activity. The specific reactivity of the preparations to immune sera remained almost unimpaired after heating to about 100° with normal alkali for 2 hours but was quickly destroyed by boiling with normal hydrochloric acid; parallel with the disappearance of the serological activity reducing sugar and some insoluble material were set free. Aside from these observations the assumption that the substances are carbohydrates rests on the analogy of the results with those of Avery, Heidelberger, and Goebel (*cf.* 11).

The present studies support the view that the specific carbohydrates form an essential part of the "stable" agglutinogens of the bacilli (White (7)). We failed to establish a relationship to the phenomenon of large flaking agglutination.

The similarity of agglutination and precipitation is also shown by absorption experiments, which demonstrate that from one immune serum precipitins can be separated which correspond to agglutinin fractions (*cf.* Krumwiede (17)).

The explanation of these phenomena so far as the antigens are concerned is still an open one. The fact that certain antibodies are removed from an immune serum through successive absorptions and others are left behind gives evidence for a multiplicity of antibodies; but when the conclusion is drawn from the reaction of certain antibody fractions upon several antigens, that all of the positively reacting antigens contain a definite common substance or one clearly defined chemical group, it remains hypothetical so long as the assumed different elements have not been separated or established as individual structures by chemical methods (see 18). It could also be assumed that the phenomena are at least in part brought about by the action of one antibody on several antigens whose specific groups are similar but not identical (see 19, 4).

In order to examine the question raised, an attempt was made by specific precipitation to separate the carbohydrates into their hypothetical units. On the whole these experiments did not lead to obtaining fractions with different properties; and therefore the results did not support the idea of the existence of separable units in the single antigens. Indeed it would be desirable also to apply chemical methods for the purpose of fractionation of the specific carbohydrates.

A noteworthy difference between the observations reported and the results obtained with the carbohydrates of pneumococci by Heidelberger, Avery, and Goebel, is the following: The carbohydrates of the three fixed types of pneumococci exhibit very marked chemical differences in correspondence with their serological diversity. Such conspicuous chemical differences have not been found among the carbohydrate preparations from the Salmonella group, which, although serologically different, showed no very striking variation in sugar content and optical rotation, with perhaps one exception (*B. hog cholera*).

SUMMARY.

Specific precipitable substances rich in carbohydrates, containing very little protein and small amounts of a material apparently of fatty nature, have been prepared from the main serological types of the typhoid-paratyphoid groups. The preparations in their present

state of purity do not exhibit very pronounced chemical differences in spite of serological dissimilarity. In this respect the results differ from those observed with the polysaccharides of pneumococci.

The specificity of the precipitin reactions of these substances parallels in a general way the so called small flaking agglutination.

Attempts to separate different fractions from the active substance serologically by means of precipitation with antibody solutions were on the whole unsuccessful.

The differences in resistance to the action of acid and alkali were found to be characteristic for various specific carbohydrates.

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