A TYPE SPECIFIC SUBSTANCE DISTINCT FROM THE SPECIFIC CARBOHYDRATE IN PNEUMOCOCCUS TYPE I

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The facts first determined by Avery and Heidelberger (1) and Zinsser and Tamiya (2) concerning the species specific nucleoprotein fraction of the pneumococcus as well as the type specific carbohydrate substance are at present so widely known and generally recognized that their recapitulation here would be superfluous. Aside from these fractions, other workers have described antigenic substances obtained by various chemical manipulations. Perlzweig and Steffen (3) in 1923 reported the finding of a material derived from the pneumococcus which was capable of active immunization upon injection into rabbits but which survived digestion with trypsin, was non-lipoidal, and gave Millon, xanthroproteic and ninhydrin reactions. Perlzweig and Keefer (4) in 1925 described an actively immunizing substance of a protein nature isolated from broth culture filtrates of Pneumococcus Type I by ultrafiltration, precipitation at a definite hydrogen ion concentration and the separation of a soluble picrate fraction. Jungeblut (5) has described an alcohol soluble substance which under certain conditions reacts specifically by precipitation with type sera. The latest studies of Julianelle (6) on skin and conjunctival hypersensitiveness in rabbits treated with products of the pneumococcus suggest the presence of a third non-type specific substance in that organism.

In this paper evidence will be presented which points to the presence of a substance other than the specific carbohydrate in the autolytic products of Pneumococci Type I which reacts specifically with immune serum as determined either by the precipitin reaction, or, *in vivo*, by the anaphylactic behavior of guinea pigs appropriately sensitized.

Primary Observation

The possibility of the existence of a substance quite distinct from the type specific carbohydrate was suggested by the behavior of the

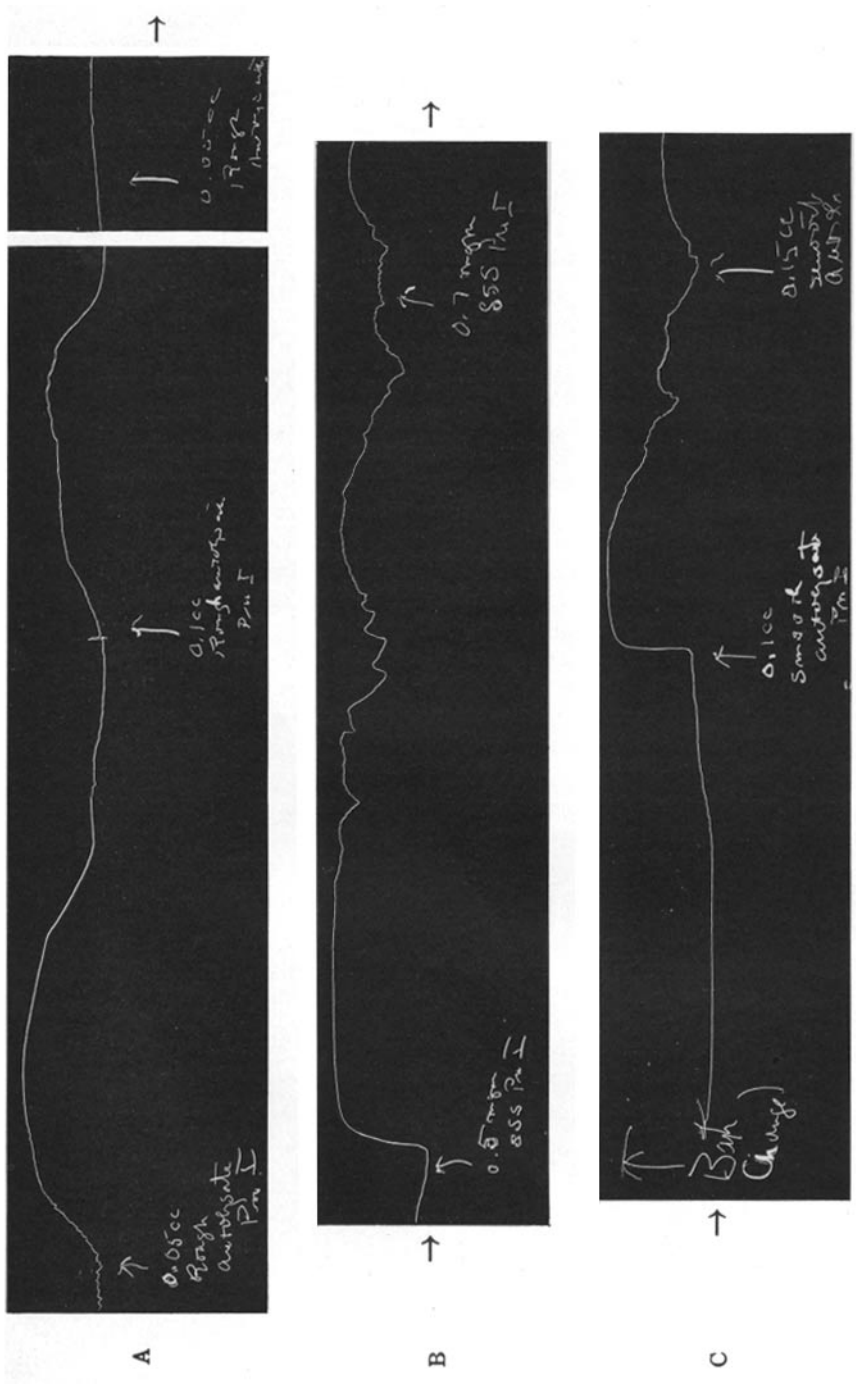

FIG. 1. The sections above represent the continuous tracing of one uterine horn of a guinea pig sensitized 24 hours before the experiment was performed by the intraperitoneal injection of 1 cc. of antipneumococcus Type I rabbit serum. In (A) is shown the effect of the autolysates from a "rough" strain of Pneumococcus Type I, in (B) that of Type I specific soluble substance, and in (C) the reaction caused by the autolysate from a "smooth" strain of Pneumococcus Type I. The volume of Ringer's solution in the bath was 25 cc.

uterine horns of guinea pigs which had been passively sensitized with antipneumococcus Type I rabbit serum. It was found that the uterine strip when treated with the following materials in the order named responded typically to each in turn:

1. Autolysate from a "rough" strain of pneumococcus.
2. The homologous specific carbohydrate purified according to the method of Heidelberger and Avery.
3. The autolysate obtained from a "smooth" strain of Pneumococcus Type I. (A representative tracing is reproduced in Fig. 1).

Desensitization of the uterus to each substance was demonstrated before the addition of a different material. It was also shown that in the quantities used, these materials had no comparable effect upon the uterus of a normal guinea pig. This result indicated that the autolysate obtained from the "smooth" or virulent Pneumococci Type I contained a substance capable of causing a typical contraction of the uterine muscle after it had been shown to be completely unresponsive to the nucleoprotein fraction (as represented by R autolysate) and to the purified specific carbohydrate. This substance has provisionally been designated the "A" substance.

The remainder of this communication consists in a description of the methods and results employed in the confirmation of the significance of this primary observation.

EXPERIMENTAL

In every instance the autolysates were produced by centrifuging, in large bottles, cultures of pneumococci grown for 24 hours in 0.1 per cent dextrose hormone broth. The sediment from about 250 cc. broth culture was taken up in 1 cc. of saline. The suspensions were collected and adjusted to contain 0.5 per cent phenol. The mixture was allowed to stand for about 72 hours at 37°C. and then kept in the ice box. The undissolved débris was centrifuged off before the clear supernatant autolysate was used in any test.

The antipneumococcus Type I rabbit serum hereafter to be designated as "normal" antipneumococcus rabbit serum was obtained in the usual manner by treating rabbits intravenously with cultures of Pneumococci Type I grown in broth containing 1 per cent rabbit's blood and killed by the addition of 0.3 per cent formalin.

The antipneumococcus Type I rabbit serum hereafter to be designated as anti-A Pneumococcus Type I rabbit serum was produced by the following procedure:

Heavy suspensions of pneumococci obtained by centrifuging 0.1 per cent dextrose hormone broth cultures, and taking up the sediment of 1 cc. saline, were treated with sufficient formalin in 10 per cent solution to give a final concentration of 0.2 per cent. After 24 to 48 hours at 37°C. tests for sterility were made. Rabbits were treated intravenously at suitable intervals with rather large amounts of the formalinized suspension.

It will be particularly noted that when heavy suspensions such as those employed are treated in the manner described, what may be spoken of as a "partial" autolysis appears to take place, *i.e.*, the organisms become Gram negative and seem to become somewhat smaller. In the case of two of the sera (228 and 230), doses of formalinized broth culture of pneumococci were given to the rabbits subsequently to the large amounts of partially autolyzed organisms. The details concerning the manner in which anti-A pneumococcus rabbit Sera 228 and 230 were prepared are given in Table I. It must be admitted that the immunological principles responsible for the production of sera which do not develop the S antibody but which do contain an antibody against the A substance are entirely unknown. Accordingly the presentation of a reliable technique for obtaining such sera cannot be given. Subsequently it will be shown that "normal" antipneumococcus Type I sera contain the A antibody which remains in the serum after the antibody reacting with the specific carbohydrate has been removed by precipitation. The most convenient and readily available method for obtaining an antiserum against the A substance depends upon this fact.

The nucleoprotein was obtained by precipitation with dilute acetic acid solution derived by dissolving pneumococci with bile according to the method of Avery and Heidelberger (7).

The specific carbohydrate was produced from pneumococci grown on 0.1 per cent dextrose hormone broth according to the methods described by Heidelberger and his associates. Owing, probably, to a slight amount of $BaSO_4$ in the Type I carbohydrate, the nitrogen present was equivalent to only 3.7 per cent.

Demonstration of the Presence of the A Substance in Antipneumococcus Type I Sera by Means of the Precipitin Test.—It was found that antipneumococcus rabbit sera produced by injecting the so-called "partially" autolyzed organisms gave no precipitation upon the addition of the specific carbohydrate. On the other hand, when the homologous autolysate was added, an abundant flocculent precipitate formed immediately. That this precipitate was only in a minor proportion due to the presence in the serum of a nucleoprotein antibody is shown by the results recorded in Tables II and III, which also demonstrate the absence of an antibody capable of reacting with the specific soluble substance.

A further indication that the species specific nucleoprotein is not responsible for the bulk of the precipitate obtained by adding the

TABLE I

Immunization of Rabbits Producing Anti-A Pneumococcus Serum

Serum	Days after first injection of vaccine							
	0	3	6	18	21	23	29	36
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
228	0.5 F	0.5 F	0.5 F	0.5 BC	1.0 BC	2.0 BC	5.0 BC	1.0 HB
230	1.0 F	1.0 F	0.5 F	0.5 BC	1.0 BC	2.0 BC	5.0 BC	—

F = Centrifugate from 250 cc. 0.1 per cent dextrose hormone broth culture Pneumococcus Type I taken up in 1 cc. saline. 0.2 per cent formalin. Culture grown for 16 hours.

BC = 16 hour hormone broth culture containing 1 per cent rabbit blood. 0.2 per cent formalin.

HB = 1/10 dilution of vaccine prepared by washing growth from horse blood agar pie plate in 5 cc. 0.2 per cent formalin solution in saline.

All vaccines were injected intravenously.

TABLE II

Titration of Anti-A Pneumococcus I Rabbit Sera with the Specific Soluble Substance and the Homologous Autolysate

Antigen		1/1	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/2,000,000	1/4,000,000
S S S Pn. I	Serum 228	Not done	Not done	−	−	−	−	−	−	−
	Serum 230	Not done	Not done	−	−	−	−	−	−	−
Autolysate Pn. I (smooth)	Serum 228	++++	++++	+++±	+++	±	−	−	−	−
	Serum 230	+++±	+++	++±	++	±	−	−	−	−

++++ = very heavy precipitate falling to bottom of tube. +++ = precipitate falling to bottom of tube. ++ = heavy ring—stays up. + = definite ring. ± = ring just discernible.

The ring test technique was employed in this and the following precipitin tests in which the antigen was diluted. Readings taken after 2 hours at room temperature.

autolysate of Pneumococcus Type I to this serum is offered by the experiment recorded in Table IV. Type I autolysate was treated with 10 per cent acetic acid in the cold until test of a portion of the fluid indicated a pH of about 4. After standing over night in the cold, the acid-precipitable material was removed by centrifugation. The supernatant fluid was tested with acetic acid. Any further precipitate which formed after 2 hours in the ice box was removed and the supernatant fluid neutralized with sodium hydroxide. When this material was tested against an anti-"rough" Pneumococcus I rabbit serum

TABLE III

Titration of an Anti-A Pneumococcus Rabbit Serum, Employing Various Pneumococcus Autolysates as Antigens

Antigen	Dilution of serum						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Pn. I autolysate (smooth)	++++	+++±	++±	++	+	±	−
Pn. II autolysate (smooth)	−	−	−	−	−	−	−
Pn. II autolysate (rough)	±	−	−	−	−	−	−
Pn. III autolysate (smooth)	±	−	−	−	−	−	−
Pn. I S S S	−	−	−	−	−	−	−

In this experiment the antiserum was diluted while the amount of antigen was kept constant. 1 drop of undiluted autolysate was added to 0.25 cc. of the serum dilution. 1 drop of the specific soluble substance diluted 1/10,000 was added to the same volume of diluted serum. Readings taken after 2 hours at room temperature.

which had a precipitin titre of 1:3200 (dilution of antigen) against the chemically isolated nucleoprotein, it produced no precipitate in the dilution employed. When, however, it was titrated with an anti-A serum, containing no precipitating antibody for the specific soluble substance, it showed only a slight diminution in precipitating capacity as compared with the untreated autolysate against the same serum. This diminution may in large part be accounted for by the loss of the nucleoprotein.

It may also be mentioned in this connection, although a discussion of the chemical aspects of the A substance will be temporarily deferred,

that it is possible to obtain by acid and alcohol precipitation a material which fails to give any precipitin reaction with the anti-"rough" pneumococcus serum mentioned above, but which reacts strongly with anti-A serum. This fact, taken with the evidence already presented, together with that which is to follow, makes it quite clear that the nucleoprotein fraction cannot be identified with the A substance, while the complete failure of the purified specific soluble substance to produce precipitation in these sera conclusively indicates that this substance is not the precipitating antigen concerned in the reactions.

In the light of the foregoing facts and the evidence afforded by the

TABLE IV

Titration of the A Substance before and after Precipitation with Acetic Acid of the Nucleoprotein from the Homologous Autolysate

Antigen		Dilution of antigen					
		1/8	1/32	1/128	1/512	1/2048	1/8192
Autolysate Pn. I (smooth)	Anti-R Pn. serum	++	+	−	−	−	−
	Anti-A Pn. I serum	+++±	+++	++±	++	+	−
Supernatant from acetic acid precipitation of autolysate Pn. I (smooth)	Anti-R Pn. serum	−	−	−	−	−	−
	Anti-A Pn. I serum	+++	++±	++	+±	+	−

Readings taken after 2 hours at room temperature.

isolated uterus of a guinea pig sensitized with antipneumococcus Type I rabbit serum prepared in the customary manner, it became of interest to determine, if possible, by the method of specific precipitation whether or not "normal" antipneumococcus rabbit serum contains the A antibody.

A technique was devised* which enabled us to demonstrate the presence of this antibody in serum which originally contained a large amount of antibody reacting

* Dr. Hugh K. Ward in this laboratory devised this technique for eliminating a precipitating antibody from an antiserum.

with the specific carbohydrate. By titration the quantity of the purified specific soluble substance was found which caused the optimum precipitate in a "normal" antipneumococcus Type I rabbit serum. The calculated amount of carbohydrate was then added to any desired volume of the antiserum and, after 2 hours at 37°C., the precipitate was removed by centrifugation. A portion of the supernatant fluid was again titrated against varying quantities of the specific soluble substance and the optimum precipitate recorded. The calculated amount of carbohydrate causing this precipitate was added to the bulk of the supernatant fluid and after 2 hours at 37°C. the precipitate was removed. The supernatant from this precipitation was then tested with varying dilutions of the carbohydrate.

Usually no visible precipitation occurred with any dilution of the specific soluble substance employed in the tests. When tested, however, with the homologous autolysate, an abundant flocculent precipitate was observed. These results, together with those obtained with the "rough" Pneumococcus I autolysate are summarized in Table V. The autolysates of heterologous types freed from the acid precipitable nucleoprotein failed to give definite precipitates with these sera. In addition data are included which were recorded in an experiment with a therapeutic Type I antipneumococcus horse serum treated in an identical manner which was produced at the Massachusetts State Antitoxin Laboratory.

The precipitin tests with "normal" Pneumococcus Type I horse and rabbit sera lend strong support to the evidence presented by the conduct of the isolated uterus for the presence of a type specific substance in the homologous autolysate which is distinct from the specific carbohydrate.

Passive Anaphylaxis in Guinea Pigs Sensitized with Anti-A Pneumococcus Rabbit Serum.—Further proof of the existence of the A substance was sought in experiments which employed the systemic anaphylactic reaction in guinea pigs passively sensitized with anti-A rabbit serum or with a "normal" antipneumococcus rabbit serum from which the S antibody had been removed by means of the procedure described above.

It was found that guinea pigs injected intraperitoneally with 1 to 2 cc. of both anti-A rabbit sera used in the precipitin tests failed to develop symptoms of anaphylaxis upon intravenous injection of varying quantities of the purified specific carbohydrate. Nor was the antibody against the nucleoprotein or "rough" autolysate present in

sufficient concentration in these sera to confer upon guinea pigs anaphylactic sensitivity to these substances. Again, no anaphylaxis developed in animals treated with such sera when autolysates derived from either Type II or Type III virulent strains of pneumococci were introduced intravenously. The Type I anti-A sera did, however,

TABLE V

Demonstration of the A Precipitating Antibody in "Normal" Antipneumococcus Type I Sera from Which the Antibody Reacting with the Specific Substance Had Been Removed by Precipitation

Serum	Antigen	Dilution of antigen					
		1/2	1/8	1/32	1/128	1/512	1/2048
Antipneumococcus I rabbit serum	S I A	+++±	+++	++±	++	++	+±
	S II A	+	−	−	−	−	−
	S III A	+±	±	−	−	−	−
	R I A	+±	−	−	−	−	−
Antipneumococcus I horse serum	S I A (ppt.)	+++±	+++	++±	+±	+	±
	S III A (ppt.)	−	−	−	−	−	−
	R I A	++	++	+	±	−	−

S I A = Pneumococcus I autolysate (smooth).

R I A = Pneumococcus I autolysate (rough).

S II A = Pneumococcus II autolysate (smooth).

S III A = Pneumococcus III autolysate (smooth).

ppt. = Autolysate treated with dilute acetic acid in the cold to remove nucleoprotein. This was done in the case of the horse antiserum because it contained a large amount of "P" antibody.

Both horse and rabbit antisera after removal of the S S S antibody showed no precipitation with the specific soluble substance in dilutions of the latter ranging from 1/1000 to 1/4,000,000.

Ring tests—readings after 2 hours at room temperature.

regularly confer upon guinea pigs a very high degree of anaphylactic sensitivity to the autolysate derived from Type I pneumococcus. The "normal" antipneumococcus Type I rabbit serum from which the S antibody had been removed also rendered guinea pigs anaphylactically hypersensitive to the A substance in the homologous autolysate. Before the removal of the S antibody by precipitation *in vitro*, this serum

sensitized guinea pigs anaphylactically to the type specific soluble substance. After the S antibody had been eliminated, animals sensitized with this serum failed to react anaphylactically upon the injection of the carbohydrate, but did respond typically upon being injected with the autolysate from Type I virulent organisms. In addition, they showed no symptoms upon intravenous treatment with autolysate derived from a "rough" strain of Type I or from autolysates of smooth strains of Types II and III. The results of these experiments are summarized in Table VI.

Although not relevant to this subject, it is of interest to note that in these experiments with a rabbit serum which at first contained two different antibodies capable of anaphylactic sensitization, but which was deprived of one of these antibodies by precipitation with the specific antigen, the complete dependence of the anaphylactogenic antibody upon the precipitating antibody is made very clear. That these two antibodies are distinct immunological entities is still held by certain workers. There is, of course, no final proof of their identity in these experiments—a proof that can only be obtained when a chemical isolation of an antibody is accomplished. Yet the fact that the sensitizing antibody may be completely removed *in vitro* by precipitation with the specific antigen from a serum in which a second sensitizing antibody remains undisturbed is additional evidence for regarding them as one.

These anaphylaxis experiments show that in the sera of rabbits which have received injections of formalinized pneumococci an antibody develops which reacts specifically with an antigen found in the autolytic products of Type I pneumococcus. Similar materials obtained from the other two types of organism, as well as from "rough" strains of the homologous and heterologous types cause no anaphylactic symptoms. The chemically prepared nucleoprotein is likewise incapable of eliciting anaphylaxis. That the antigen in the Type I autolysate responsible for the anaphylactic symptoms is not the specific carbohydrate appears to be definitely shown not only by the failure of the latter material when pure to produce shock in guinea pigs sensitized with anti-A rabbit sera, but also by the experiments with the rabbit serum from which the S antibody originally capable of sensitizing had been removed, leaving unimpaired the serum's power to sensitize to the A substance.

TABLE VI

Anaphylaxis Experiments with Guinea Pigs Passively Sensitized with Anti-A Rabbit Sera

Guinea pig	Amount serum injected I. P.	S S S	S I A	R I A	S II A	R II A	S III A	N P	Results
Anti-A Pneumococcus I Rabbit Serum 228									
1	2 cc.	2 mg.	—	—	—	—	—	—	No symptoms
2	2 cc.	1 mg.	0.4 cc.	—	—	—	—	—	S S S = no symptoms S I A = death in 3 minutes. Autopsy typical
3	1 cc.	1 mg.	0.4 cc.	—	—	—	—	—	S S S = no symptoms S I A = death in 3 minutes. Autopsy typical
4	1 cc.	—	0.2 cc.	—	—	0.2 cc.	—	—	R II A = no symptoms S I A = death in 4 minutes. Autopsy typical
5	1 cc.	—	0.2 cc.	—	—	0.2 cc.	—	—	R II A = no symptoms S I A = death in 4 minutes. Autopsy typical
6	1 cc.	—	0.2 cc.	—	0.2 cc.	—	—	—	S II A = no symptoms S I A = death in 3 minutes. Autopsy typical
7	1 cc.	—	0.1 cc.	0.2 cc.	—	—	—	—	R I A = no symptoms S I A = death in 3 minutes. Autopsy typical
8	1 cc.	1 mg.	0.2 cc.	0.2 cc.	—	—	—	—	S S S = no symptoms R I A = no symptoms S I A = death in 3 minutes. Autopsy typical
9	1 cc.	2 mg.	0.05 cc.	—	—	—	0.2 cc.	—	S S S = no symptoms S III A = no symptoms S I A = death in 4 minutes. Autopsy typical

TABLE VI—*Continued*

Guinea pig	Amount serum injected I. P.	S S S	S I A	R I A	S II A	R II A	S III A	N P	Results
					Anti-A Pneumococcus I Rabbit Serum 230				
10	1 cc.	1 mg.	0.1 cc.	0.2 cc.	—	—	—	—	S S S = no symptoms R I A = no symptoms S I A = death in 3 minutes. Autopsy typical
11	1 cc.	1 mg.	0.1 cc.	—	0.2 cc.	—	—	—	S S S = no symptoms S II A = no symptoms S I A = death in 3 minutes. Autopsy typical
12	1 cc.	1 mg.	0.1 cc.	—	—	—	0.2 cc.	—	S S S = no symptoms S III A = no definite symptoms* S I A = death in 5 minutes. Autopsy typical
13	1 cc.	—	0.2 cc.	—	—	—	—	3 mg.	N P = no symptoms S I A = death in 3 minutes. Autopsy typical
					Anti-Pneumococcus I Rabbit Serum without S S S Antibody				
14	1 cc.	2 mg.	0.2 cc.	0.2 cc.	—	—	—	—	S S S = no symptoms R I A = no symptoms S I A = death in 3 minutes. Autopsy typical
15	1 cc.	1 mg.	0.1 cc.	—	—	—	—	—	S S S = no symptoms S III A = no symptoms S I A = death in 3 minutes. Autopsy typical
16	1 cc.	1 mg.	0.1 cc.	—	0.2 cc.	—	—	—	S S S = no symptoms S II A = no symptoms S I A = death in 6 minutes. Autopsy typical

S S S = Pneumococcus I specific soluble substance.
S I A = Pneumococcus I (smooth) autolysate.
R I A = Pneumococcus I (rough) autolysate.
S II A = Pneumococcus II (smooth) autolysate.
R II A = Pneumococcus II (rough) autolysate.
S III A = Pneumococcus III (smooth) autolysate.
N P = Pneumococcus I nucleoprotein.

The sensitizing serum was injected intraperitoneally 24 hours before the test substances were administered intravenously (ear vein). An interval of about 10 minutes was allowed to elapse between the intravenous injection of the various test materials. In every case the specific soluble substance was injected first. This was followed by the non-specific autolysate or nucleoprotein. The Pneumococcus I (smooth) autolysate was injected last. The Pneumococcus I (smooth) autolysate in the amounts used in these experiments produced no symptoms in normal guinea pigs.

* Guinea pig 12 after the injection of Type III autolysate showed weakness and rapid respiration. Recovery was immediate. These symptoms were not interpreted as being anaphylactic.

The autolysate prepared from a different strain of virulent Type I pneumococcus was tested both by the methods of precipitation and of anaphylaxis for the A substance, which was found to be present.

TABLE VII

Effect of Heat on the A Substance

		Dilution of antigen					
		1/8	1/32	1/128	1/512	1/2048	1/8192
Anti-A Pneumococcus Type I rabbit serum 228	Untreated phenolized S I A	+++±	+++	++±	++	+	+
	Supernatant after treatment with HAc	+++	++±	++	+±	+	−
	HAc supernatant boiled at pH 9	±	−	−	−	−	−
	HAc supernatant boiled at pH 4	+++	++±	++	+±	+	−
	HAc supernatant autoclaved at pH 9	−	−	−	−	−	−
	HAc supernatant autoclaved at pH 4	+*	±*	−	−	−	−
Anti-R Pneumococcus Type I rabbit serum	Untreated phenolized S I A	++	+	−	−	−	−
	Supernatant after treatment with HAc	−	−	−	−	−	−
	HAc supernatant boiled at pH 9	−	−	−	−	−	−
	HAc supernatant boiled at pH 4	−	−	−	−	−	−
	HAc supernatent autoclaved at pH 9	−	−	−	−	−	−
	HAc supernatant autoclaved at pH 4	±*	−	−	−	−	−

* These reactions may probably be interpreted as non-specific, since the HAc supernatant did not give this reaction with anti-R serum before autoclaving at pH 4.

S I A = Pneumococcus Type I (smooth) autolysate.

HAc = Acetic acid.

Solutions boiled for ½ hour.

Solutions autoclaved for 1 hour at 15 pounds pressure.

The ring test was employed in this experiment. Readings after 2 hours at room temperature.

Physical and Chemical Characteristics of the A Substance

Effect of Heat.—A characteristic of the A substance which sharply distinguishes it from the soluble specific substance is the destructive

effect of heat upon it when in weakly alkaline solution. If boiled for ½ hour at pH 9, its effectiveness as a precipitating antigen is reduced at least one thousand fold, while the specific carbohydrate remains unchanged. On the other hand, when the reaction is adjusted to pH 4 with 10 per cent acetic acid, a solution containing the A substance may be boiled for the same period of time without losing its activity. In both acid and alkaline solution, autoclaving for 1 hour at 15 pounds pressure practically destroys its effectiveness. In the experimental results recorded in Table VII, the evidence for these statements is presented. To eliminate in so far as is possible the nucleoprotein fraction, the autolysate used in these experiments was

TABLE VIII

Effect of Peptic Digestion on A Substance

Serum		Dilution of the antigen				
		1/1	1/8	1/32	1/128	1/152
Anti-A Pneumococcus I rabbit serum 228	After digestion	+++	++	+±	+	−
	Control (undigested)	+++	++	+±	+	−
Anti-R Pneumococcus rabbit serum	After digestion	−	−	−	−	−
	Control (undigested)	++	±	−	−	−

Readings taken after 2 hours at room temperature.

first treated with acetic acid and the resulting precipitate discarded before the supernatant fluid containing the A substance was either boiled or autoclaved.

Effect of Peptic and Tryptic Digestion.—Digestion for 72 hours in solution containing 1 per cent pepsin at 37.5°C. does not decrease the precipitating power of the A substance. To 1 cc. of Pneumococcus I autolysate, 1 cc. of 2 per cent solution of pepsin in 0.1 N hydrochloric acid was added. The mixture was then placed in a "celofane" dialyzing bag and immersed in a solution of 0.1 N hydrochloric acid. The liquid outside the bag was changed twice each day. As a control a similar mixture of autolysate and boiled pepsin solution was dialyzed under the same conditions. The enzyme in a concentration of 1 per

cent readily digested coagulated egg white (Mett tube). Its activity was also indicated by the reduction in the precipitating strength of the digest when added to an anti-R pneumococcus rabbit serum. This loss of precipitating capacity is, no doubt, to be attributed to the digestion of the nucleoprotein by the enzyme. In Table VIII are presented the results of an experiment to determine the effect of pepsin on the A substance.

Because of the fact that in weakly alkaline solution the activity of the A substance seems to be markedly impaired by prolonged exposure to a temperature of 37.5°C., it was not possible to determine so clearly the effect of tryptic digestion on it. It may be stated, however, that a solution of the A substance exposed to the action of this enzyme loses no more of its capacity to react with an A antiserum than does a control solution to which no trypsin has been added but which has been subjected to identical conditions.

Chemistry of the A Substance.—Until the completion of a study now being purused which is concerned with the isolation and properties of this substance, little can be definitely stated in respect to its chemistry. In many of its reactions it resembles the specific soluble substance. Precipitated by the same volume of alcohol, traces of it are to be found in that material until very near the end of the purifying process. It is precipitated by phosphotungstic acid and by silver nitrate. It is is not precipitated either by acetic or hydrochloric acid. Until isolated in a state at least approaching purity, the tests for protein have, of course, no meaning.

DISCUSSION

In the preceding sections experimental evidence has been presented which leads to the conclusion that in the autolytic products of Pneumococcus Type I there is to be found a substance of unknown chemical composition reacting specifically both in precipitin and anaphylactic reactions with sera produced by injecting rabbits with formalinized pneumococci. This substance is distinguished from the specific carbohydrate not only by its specific precipitating and anaphylactic action with such sera, but also by its instability when boiled in weakly alkaline solution. Although this last characteristic is analogous to the conduct of certain proteins which undergo racemization under similar

conditions, the fact that pepsin and, less clearly, trypsin do not digest the A substance renders exceedingly doubtful its ultimate identification as a protein. At present the chemical aspects of the problem are being investigated.

The hypothesis of Landsteiner concerning the "haptene" or partial antigen so conclusively confirmed experimentally in the case of the lipoidal antigen of Forssmann and the bacterial carbohydrates, must inevitably be considered in relation to any substance which reacts *in vitro* with an antiserum. Since the chemical isolation of the A substance has not yet been accomplished, it is obviously impossible to arrive at any definite conception in respect to its function as a complete or partial antigen. *A priori,* however, the facts mentioned above, which suggest a substance of non-protein nature, also make it unlikely that the A antigen *per se* possesses the capacity to stimulate the production of antibody when injected into an animal.

The work of Reimann (9) and others has shown that immunization with the autolytic products of the pneumococcus produces an antibody reacting only with the nucleoprotein fraction. Although their results do not definitely preclude the possibility that an A antibody was also present in small concentration, they render it improbable. Had an A antibody been present in a serum along with the nucleoprotein antibody, that serum would have exhibited a higher precipitin titre against the homologous autolysate than against heterologous autolysates. Actually, it was found that in the sera of an animal immunized with the autolysate from any pneumococcus or with the nucleoprotein fraction the precipitin titre was the same whether the homologous or heterologous autolysate was employed as antigen.

We have not attempted an extensive series of experiments to determine whether or not an antibody against the A substance developed in the sera of rabbits immunized with autolysate derived from Pneumococcus Type I. In the sera of three animals treated in this way, however, no antibody against the A substance was demonstrated. Thus, until further data are available, the preponderance of evidence is in favor of regarding the A substance as belonging to the order of haptenes.

Whether or not materials analogous to the A substance in Pneumo-

coccus Type I exist in the autolytic products of Pneumococci Type II and Type III has not yet been definitely shown.

CONCLUSIONS

1. Evidence has been presented for the existence of a substance distinct from the specific carbohydrate in the autolytic products of Pneumococcus Type I.

2. The substance reacts specifically by precipitating homologous antiserum which either occurs naturally without antibody against the specific carbohydrate or has been deprived of that antibody artificially.

3. In guinea pigs passively sensitized with such antisera the homologous autolysate containing the substance alone produces typical lethal anaphylactic shock.

4. In weakly alkaline solution the substance is destroyed by boiling. In weakly acid solution it resists a temperature of 100°C. for at least ½ hour. Autoclaving for 1 hour at 15 pounds pressure in either acid or alkaline solution destroys its activity as precipitinogen.

5. The substance is resistant to peptic digestion.

6. The chemical nature and the possible identification of the substance as a haptene have been discussed.

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