STUDIES ON MENINGOCOCCUS INFECTION

III. The Antigenic Complex of the Meningococcus—A Type-Specific Substance

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In the two preceding papers, it has been shown that freshly isolated strains of meningococcus differ not inconsiderably from those strains maintained in the laboratory on artificial media over long periods, and known as stock strains. Amongst the observations which have been recorded is the ability of fresh strains to evoke the production of precipitin-containing sera (1) which will react specifically with the type-specific substances occurring in these same fresh strains (2). This and other related observations pointing to the probability that the meningococcus has an antigenic complex comparable, in its essentials, to that described for other organisms—especially those of the non-encapsulated group (3)—are described in some detail in this and the following paper which deal with the isolation and analysis of some of the antigenic components.

That precipitation reactions between soluble extracts of the meningococcus and antimeningococcal sera occur and are of some importance diagnostically is no new observation. As long ago as 1906, Bruckner and Cristeanu (4) showed that, while extracts in 0.75 per cent NaCl are but feeble precipitinogens, extracts of meningococci prepared in 0.15 per cent NaOH give good precipitates on the addition of antimeningococcal serum, but not with normal horse serum. They showed, moreover, that the meningococcal extract precipitates with antigonococcal serum and that the same cross-precipitation occurs when an alkaline extract of gonococci is set up with antimeningococcal and antigonococcal sera. Dopter and Koch (5) confirmed these results and introduced the conception of co-precipitins to account for the cross-reactions. They showed that when the antimeningococcal serum is absorbed with whole meningococci both the specific meningococcus precipitins and also the group precipitins, which react with the gonococcus extract, are removed; on the other hand, when the serum is absorbed with whole gonococci,

only the group precipitins are removed and the absorbed serum will still react with the meningococcus extract. In the same paper they showed that the reverse is true and that whole gonococci remove both specific and group precipitins from antigonococcal serum, while whole meningococci remove only the group precipitins. In a subsequent paper (6), Dopter extended these observations. He stated that precipitating sera can be obtained by means of intravenous injection of either living organisms or meningococcus extract, and that the former are often the more efficacious. The titre obtained varies with the animal immunized and the strain used for vaccination, and is not always highest when a readily agglutinable strain is used. The precipitin usually parallels the agglutinin titre, but this is not invariably the case. He showed that cross-precipitation of antimeningococcal serum, often in high dilution, occurs with all members of the Gram-negative pharyngeal micrococcus group but that, as is the case with the gonococcus, the cross-reaction is due to the presence of group precipitins which can be removed by absorption with any member of the group, while the meningococcus alone can remove the specific antibodies from the serum. He pointed out that precipitation occurs even at room temperature. Still later, Dopter (7) showed that, in general, the extract of each type (A, B, C and D) reacts only with its homologous antiserum. He found, however, not infrequent exceptions in which cross-precipitation occurred, though the titre in such cases is higher in homologous than in heterologous sera. Absorption of anti-A serum with either Type A organisms or extract removed the homologous precipitins and the same is true for the absorption of other type sera by their homologous strains.

Zinsser and Parker (8) obtained a residue antigen from the meningococcus by alkaline extraction of young and old broth cultures. Przesmycki, in a brief communication (9), showed that specific residue antigens can be obtained for each of the four types by precipitation with five volumes of absolute alcohol. He found that these specific antigens react most strongly with their homologous antisera, but do show cross-precipitation with heterologous sera at lower titre—results which correspond closely with those of Dopter. Przesmycki made but few tests and himself stated that no definite conclusions could be drawn from his results. Recently Zozaya (10) has studied polysaccharide and protein fractions obtained from the different types of meningococci and compared them with fractions obtained from pneumococci and Gram-negative cocci. The polysaccharide is obtained from alkaline extracts of the organisms by precipitation with four to five volumes of ethyl alcohol, while the nucleoprotein is isolated by 10 per cent acetic acid precipitation of a watery solution of organisms which have been frozen and thawed. He found cross-precipitation of the polysaccharides of meningococcus, gonococcus and *Micrococcus catarrhalis* with polyvalent antimeningococcal serum and showed that these group precipitins could be removed by absorption of the serum with any one of the three polysaccharides. There was no precipitation of the polysaccharides of pneumococcus and of Bargen's organism with antimeningococcal serum, nor did the meningococcus polysaccharide react with antipneumococcal and anti-Bargen sera. Polysaccharides from each of the meningococcus

types showed equal precipitation with polyvalent antimeningococcal serum, and furthermore, absorption with Type I polysaccharide removed polysaccharide precipitins for all types. This polysaccharide is, therefore, in no way type-specific. The nucleoprotein was also found to be common to the group rather than type-specific. It does not precipitate with sera of organisms outside the group of Gram-negative cocci and absorption with nucleoprotein of Type I removes the nucleoprotein precipitins for all the types. In a further paper (11), Zozaya found similarities in the polysaccharides of the meningococcus and certain Gram-positive bacilli (anthrax, *proteus*, *mesentericus* and *subtilus*). Absorption of polyvalent antimeningococcal serum with the polysaccharide of any one of these organisms removes the precipitins for the meningococcus polysaccharide. On the other hand, only absorption with anthrax polysaccharide will remove the anthrax precipitins from anti-anthrax serum, while the other polysaccharides are unable to do this. The presence of at least two carbohydrate antibodies is presumed, one species-specific and the other group- or non-specific. It is of interest to note that in no case was the agglutination titre of the absorbed serum altered from that of the unabsorbed control and this even when the absorption of precipitin was successful.

As this brief review of the previous studies on precipitable substances of the meningococcus indicates, the findings may be divided conveniently into two periods. In the first, no attention was paid to the chemical complex of the various extracts. The earlier investigators showed that alkaline extracts of the meningococcus contain precipitinogens which react with both antimeningococcus serum and the serum prepared against others of the Gram-negative cocci (4–6); that the antisera contain both species-specific precipitins which can be removed only by the homologous organism or its extract, and group precipitins which can be removed by any of the organisms or their extracts (5, 6), and finally, that there is evidence of the presence of type-specific precipitinogens as contrasted with those which are only species-specific and common to all the meningococcus types (7). In the later period, commencing with the work of Zinsser and Parker (8), endeavors have been made to isolate and identify the different fractions of the meningococcus extract which are responsible for the varying reactions observed. It has been shown that precipitation with 10 per cent acetic acid will give a nucleoprotein fraction which is species-specific (2, 10), while precipitation with ethyl alcohol gives at least two fractions of which one is type-specific (2, 9) while the other is related to similar substances present in and obtained from other microorganisms, especially those of the Gram-negative coccus group (2, 10, 11).

Material and Technique

Several methods of extracting the meningococci were tried. These included extraction with normal saline and also with N/20 HCl. The most satisfactory, as far as yield of the various fractions and comparative ease of separation are concerned, was that of autolysis.

The organisms are planted in 9 cc. of beef heart infusion broth at pH 7.6. At the end of 24 hours, or as soon as good growth is apparent, the whole 9 cc. are planted in a liter flask containing from 700 to 900 cc. of hormone broth. If growth takes place, a pellicle becomes obvious on the surface of the broth in 2 or 3 days, which breaks up into fine granules and gradually settles to the bottom of the medium when the flask is disturbed. A new pellicle now forms on the surface and by daily shaking of the flask a constant new growth may be obtained at the surface, while those organisms which have settled out undergo gradual autolysis at the bottom. Growth is maintained at 37°C. for about 2 weeks.

The broth culture is now centrifuged at high speed to remove all debris. The supernatant, slightly cloudy fluid is measured in a graduated cylinder and then placed in a liter flask. 10 cc. are taken in a test-tube, and to this is added at room temperature from a burette 10 per cent acetic acid drop by drop until no more precipitation occurs. A simple reckoning will permit the adding of sufficient 10 per cent acetic acid to the bulk of the autolysate to throw out of solution the acetic acid-precipitable fraction. This appears as a greyish white deposit. The mixture is centrifuged, the clear supernatant fluid decanted and the precipitate, labelled P, is preserved.

A strong solution of sodium hydroxide is added to the supernatant fluid until it is neutral to litmus. It is now treated with 95 per cent ethyl alcohol. When an equal volume of alcohol is added, a slight precipitate appears and this becomes heavy when the amount of alcohol reaches two to two and a half volumes. This amount of alcohol is allowed to stand in contact with the broth solution for 24 hours at room temperature. During this time a white flocculent precipitate settles out completely. It is packed by centrifugation at high speed and the supernatant fluid decanted. This fraction, for reasons to be shown later, is termed the specific substance. It is dried *in vacuo* over phosphorus pentoxide, when it forms a brownish white material somewhat like gelatin in appearance. Most of the first precipitate is insoluble in distilled water or saline, but all or apparently all of the active material goes into solution after standing in contact with distilled water or saline for a day or two. The clear, somewhat brownish solution is reprecipitated with two and a half volumes of 95 per cent ethyl alcohol. The yield of this precipitate varies between about 250 and 500 mg. of dried material from 900 cc. of broth. It is dried *in vacuo* as before and redissolved in saline or distilled water. On this occasion about 90 per cent of the precipitate goes into solution; *i.e.*, in one case 390 mg. out of 436 mg. and in another 209 mg. out of 224 mg. The substance may be further purified by repeated alcohol precipitation. A few crystals

of sodium acetate should be added to every alternate solution-alcohol mixture in order to facilitate, indeed to ensure, complete precipitation. A standard solution containing 10 mg. of specific substance in 1 cc. of solution, *i.e.* 1/100, is made up, a few drops of toluol are added and it is stored in the ice box to be used as required.

To the supernatant fluid decanted from the specific substance precipitate are added another six volumes of 95 per cent ethyl alcohol. A heavy white precipitate settles out and is allowed to stand in contact with the supernatant alcohol mixture for at least 24 hours at room temperature. It is now centrifuged and the supernatant fluid is decanted. The precipitate is dried over phosphorus pentoxide *in vacuo* and forms a white, caked powder. Most of this redissolves readily in water. It is further purified by repeated alcohol precipitation followed by resolution, sodium acetate being added as above. After the first two occasions all but the merest trace goes back into solution. This fraction is labelled C.

The Type-Specific Substances

The fraction precipitable by means of two to two and a half volumes of ethyl alcohol has been obtained from Type I, Type II and Type III strains. Difficulty was experienced in obtaining a strain of Type III organisms. At the present time, the great majority of strains which occur are either Type II or belong to what may be termed the I-III group. The members of this latter group agglutinate equally well with both Type I and Type III monovalent antisera and, as will be demonstrated below, show even closer relationships when examined by serum absorption experiments. There is general agreement among those working with this organism that, whatever may have been the case some years ago, at the moment great difficulty exists in separating out two types from the I-III group, and it has been conceded that the strains which occur at the present time correspond most closely to what was formerly termed Type I. Some laboratories continue to report Type III strains, but the writer has not found such a strain among the freshly isolated spinal fluid and throat strains which have been received. Through the kindness of Dr. Sara Branham of Washington, a Type III strain (No. 302), isolated in 1930, was obtained. This is said to be the most narrowly specific Type III strain at present in the United States Public Health Service Department. It has been used for the preparation of the Type III specific substance and is fully discussed below. No freshly isolated strain of Type IV meningococcus has been available up to the present time and, apparently, few, if any cases of meningitis due to this organism are occurring at the moment.

Type I

The type-specific substance has been obtained from several Type I strains. Most of the work, however, has been carried out on the fraction obtained from a hormone-broth autolysate of Strain 428 which had grown in 900 cc. of the broth for 2 weeks. The method of preparation has been described above.

This Strain 428 showed the characteristic agglutination of a freshly isolated Type I strain.[1]

The agglutination test is carried out for 2 hours at 37°C. and overnight in the ice box. The readings given are the final ones. All the monovalent sera are

TABLE I

Dilutions	Serum			
	I	II	III	IV
	No. 578	No. 551	No. 556	No. 535
1/10	+++	0	+++	0
1/25	+++	0	++	0
1/50	+++	0	++	0
1/75	++	0	+	0
1/100	++	0	+	0
Saline—negative				

prepared from freshly isolated spinal fluid strains (1) with the exception of the Type IV serum which, in the absence of any fresh strains of this type, is prepared from a stock strain isolated in March, 1929.

The fraction gives a good precipitin reaction with homologous antisera. Little or no difference can be found between the reaction with Type I and that with Type III sera, either in the amount or the type

[1] All precipitin tests have been carried out in the same way. Equal parts—0.1 cc.—of serum and solution of the fraction to be investigated have been placed with a capillary pipette in a small precipitin tube 4 to 7 mm. in diameter. The serum is placed at the bottom of the tube and the solution carefully layered on top to form a clear line of junction. The reading is made immediately and then after the tube has stood for 1 hour in the 37°C. water bath. This constitutes the ring test. The two fluids are then mixed and returned to the bath for 1 hour, when they are read again and then placed in the ice box overnight for a final reading.

of precipitate formed. On the other hand, there is little cross-precipitation with Type II and Type IV antisera, and whatever cross-precipitation exists can be removed by appropriate absorption of the sera.

Experiment 1.—A purified specimen of Type I specific substance is made up in a dilution of 1 in 200. This is then set up against specimens of the four monovalent antisera.[1] In the first case, the antisera have not been treated. In the second case, 0.5 cc. of each of the monovalent sera has been absorbed (1) with 0.15 cc. of packed Type II organisms, and (2) with 5 mg. of dried C substance (prepared from Strain 23, Type I meningococcus). These absorptions were carried out on separate occasions. The organisms stood in contact with the serum for 18 hours at 37°C., the C substance for 12 hours at 37°C. and 12 hours in the ice box.[2] The results of the precipitin experiments with both unabsorbed and absorbed sera are shown in Table II.

It will be seen that the fraction obtained from Type I organisms gives a good precipitate with homologous unabsorbed Type I or Type III sera and also a slight precipitate with unabsorbed Type II or Type IV sera. Absorption of the sera in the manner indicated produces a much clearer result in that it removes all the precipitins in the heterologous sera but leaves those in the homologous I and III sera unaffected. In this particular experiment the Type I serum used had a rather low precipitin titre which accounts for the difference in the amount of precipitate formed with this serum as compared to that of Type III.

It will also be noted that the type of precipitate formed with the homologous sera is different after absorption, being now in the form of a partial disc whereas formerly it was finely granular. The exact significance of this cannot be determined at present, but it can be said that the purer the specimen of specific substance the more liability there is for the formation of a partial disc or even a true disc in the

[2] As has been the experience of other workers with other sera, it has been found difficult to absorb any antimeningococcal sera completely, no matter what the method employed or the number of times it is repeated. Theoretically, it should be sufficient in this particular case to absorb the sera with the heterologous Type II whole organisms. In practice, however, even repeated absorption with these organisms fails to remove all the non-specific precipitins in the sera and the additional absorption with C substance has been found greatly to improve the results.

precipitin reaction. It would seem that the presence of impurities being precipitated with the type-specific substance changes the character of the precipitate and renders it granular.

It might be supposed that the cross-reactions which occur between the type-specific substance and heterologous unabsorbed antisera (not including under this term the Type III antiserum) are due to the presence in the purified fraction of small amounts of non-type-specific carbohydrate C and nucleoprotein P fractions, and the presence in the monovalent antisera of their corresponding antibodies. This is no doubt the case to some degree, yet experiments such as Experiment 2 have shown that the monovalent antisera contain such insignificant amounts of the anti-C and anti-P antibodies that one must doubt whether these are altogether responsible for the cross-reactions.

TABLE II

Sera unabsorbed				Sera absorbed				
I	II	III	IV	I	II	III	IV	
No. 578	No. 551	No. 266	No. 535	No. 578	No. 551	No. 266	No. 535	
$+^{r}$	$\pm^{r}$	$+^{r}$	$\mp^{r}$	$+^{r}$	0	$+^{r}$	0	Immediate } Ring test
$+\pm^{p}$	$+^{r}$	$+++^{p}$	$\pm^{r}$	$^{p}++^{r}$	0	$^{p}+++^{r}$	0	1 hr. } Ring test
$+\pm^{p}$	$+$	$+++^{p}$	$\pm$	$++^{p}$	0	$+++^{pd}$	0	2 hrs.
$++^{p}$	$+\pm^{p}$	$++++^{p}$	$\pm$	$++^{pd}$	0	$++++^{pd}$	$\mp$	Ice box

r = ring.
p = granular precipitate.
pd = disc broken up on agitation.
$\mp$, $\pm$, $+$, $+\pm$, $++$, $+++$, $++++$ = increasing amounts of precipitate.

Experiment 2.—The nucleoprotein fraction (of Strain 31, Type II, purified by three precipitations with 10 per cent acetic acid) was made up in a standard 1/100 solution. A specimen of C substance (from Strain 7, Type I-III, purified by three precipitations with 95 per cent ethyl alcohol) was also prepared in a dilution of

1/100. These two were set up with the four monovalent sera and with a specimen of polyvalent serum. The results are shown in Table III.

It will be seen from the table that although these group-specific fractions react strongly with the polyvalent serum they give only very slight reactions with the monovalent sera. With the P fraction indeed the reaction is only with the homologous serum and this, as will be shown later, is due to impurities of the type-specific substance, great difficulty being experienced in freeing the P fraction altogether from this component. These facts suggest that the cross-precipitation seen with some specimens of type-specific substances may be due

TABLE III

P fraction 31P[3] (Type II)					C fraction 7C[3] (Type I-III)					
I	II	III	IV	Polyvalent	I	II	III	IV	Polyvalent	
No. 578	No. 551	No. 556	No. 535	No. 2	No. 578	No. 551	No. 556	No. 535	No. 2	
0	$\pm^{cr}$	0	0	$\pm^{cr}$	$\pm^{r}$	$\pm^{cr}$	0	$\mp^{r}$	$++^{r}$	Immediate } Ring test
0	$\pm^{cr}$	0	0	$\pm^{cr}$	$\pm^{r}$	$\pm^{r}$	0	$\pm^{r}$	$^{d}+++^{r}$	1 hr. } Ring test
0	$\pm$	0	0	$\pm$	$\pm$	$\pm$	$\mp$	$\pm$	$+++^{d}$	2 hrs.
0	$\pm$	0	0	$++++^{p}$	$\pm$	$\pm$	$\mp$	$\pm$	$++++^{d}$	Ice box

c = cloudiness.
r = ring.
p = granular precipitate.
d = firm disc.

to some antigen other than those as yet isolated. The antibodies for this antigen can be absorbed by using whole organisms, as was shown in Experiment 1.

The specificity of the precipitin reaction between the type-specific substance and the monovalent sera can be brought out by a method other than that of absorption; namely, by dilution.

Dilutions may be made either of the solution of type-specific substance with the serum remaining constant, or of the serum with the dilution of type-specific substance remaining constant. Under both of these circumstances the titre is higher with the homologous sera (including Type III serum) and the amount of precipitate formed is greater with such sera.

Experiment 3.—Dilutions of the type-specific substance of Type I (Strain 428), which had been purified by reprecipitation, were made from 1/100 to 1/10,000. These were set up against undiluted serum. Serum dilutions were made from undiluted serum to 1 part in 32. All dilutions were made in normal saline. The serum dilutions were set up against a 1/1,000 solution of type-specific substance. The results are shown in Table IV.

It will be noted that the specific precipitation occurs with dilutions of specific substance of 1/10,000 and undiluted serum, or with dilutions of serum of 1/32 and specific substance 1/1,000. On the other hand, the heterologous reaction occurs only at 1/1,000 of specific substance dilutions and 1/8 of serum dilution. Moreover, the amount of precipitate formed in the cross-precipitation is throughout more scanty. It will further be seen that the Type III antiserum gives a higher titre in both parts of the experiment than the Type I serum. The specimen of Type III antiserum used in this experiment had the highest titre of any rabbit monovalent serum prepared up to the time the experiment was carried out.[3]

The Type I specific substance precipitates readily with some specimens of polyvalent antimeningococcal horse serum. The titre reached is often higher than that obtained with the homologous monovalent rabbit serum.

Experiment 4.—Dilutions of the type-specific substance from 1/100 to 1/10-000,000 were set up against undiluted polyvalent antimeningococcal horse serum No. 3. Dilutions of the serum from 1/1 to 1/32 were set up against type-specific substance diluted 1/1,000. The precipitin test was carried out in the usual way. Table IV shows the final readings.

It will be seen from Table IV that the type-specific substance precipitates well with the particular sample of polyvalent antimeningococcal horse serum used in this experiment. With dilutions of the specific substance itself, a higher titre is reached with the polyvalent serum than with the homologous antisera, while in the serum dilutions the titre is the same in both instances. From this it might appear that polyvalent sera contain more type-specific precipitins than do the specifically prepared monovalent sera. Absorption experiments, how-

[3] Specimens of Type I specific substance prepared more recently by improved chemical methods give a good precipitate in homologous sera at 1/1,000,000 and fail to precipitate with Type II or Type IV sera at 1/1,000.

TABLE IV

Serum		Dilutions of type-specific substance						Serum		Dilutions of serum					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}			1/1	1/2	1/4	1/8	1/16	1/32
Type I serum	Serum 1/1	$+++^{p}$	$+++^{p}$	$+^{p}$	0	0	0	Type I serum	Type-specific substance 10^{-3}	$+++^{pd}$	$++^{p}$	$\pm$	$\pm$	0	0
Type II serum	Serum 1/1	$++^{p}$	$+^{p}$	0	0	0	0	Type II serum	Type-specific substance 10^{-3}	$\pm$	$\mp$	$\mp$	$\mp$	0	0
Type III serum	Serum 1/1	$++++^{pd}$	$++++^{pd}$	$++^{p}$	0	0	0	Type III serum	Type-specific substance 10^{-3}	$+++^{pd}$	$+++^{pd}$	$++^{p}$	$+^{p}$	$\pm$	$\mp$
Type IV serum	Serum 1/1	$+^{p}$	$\pm$	0	0	0	0	Type IV serum	Type-specific substance 10^{-3}	$\pm$	$\pm$	$\mp$	$\mp$	0	0
Polyvalent serum	Serum 1/1	$++++^{pd}$	$++^{p}$	$++^{p}$	$\pm$	0	0	Polyvalent serum	Type-specific substance 10^{-3}	$++^{p}$	$++^{p}$	$++^{p}$	$+^{p}$	$\mp$	$\mp$

Only the ice box readings are given.
p = granular precipitate.

ever, prove that such is not the case, for heterologous strains or specimens of non-type-specific fractions will remove a larger part of these precipitins. They are, therefore, not type-specific and the precipitates formed represent in large degree reactions with impurities remaining in the sample of type-specific substance.

Two samples of polyvalent serum No. 3 were absorbed twice with large amounts of homologous (Type I, Strain 454) and heterologous (Type II, Strain 442) whole organisms for $2\frac{1}{2}$ hours at 37°C. and overnight in the ice box. The polyvalent absorbed sera were set up against dilutions of the type-specific substance. Not even the homologous absorption removed all the precipitins from the serum. The heterologous strain removed a large part of the precipitins, showing that they were group-specific rather than type-specific; yet the absorption with homologous organisms was even more complete, showing that a part of the precipitins in the polyvalent antimeningococcal horse serum are, as might be expected, type-specific.

The precipitin reaction between the type-specific substance and the monovalent rabbit antiserum shows an inhibition zone in the lower dilutions. This phenomenon is brought out very clearly by the method of precipitin test that has been used—namely, first a ring test and then mixing of the sera. No zone phenomenon is shown by the ring test, but when lower dilutions of the type-specific substance have been used, reabsorption of the precipitate occurs in quite a dramatic manner directly the solution of specific substance and serum are mixed intimately.

Experiment 5.—Dilutions of Type I specific substance were made from 1/40 to 1/125. These were set up against undiluted Type I monovalent serum and the precipitin test was carried out in the usual way.

At the end of 1 hour at 37°C., there is no inhibition zone demonstrated by the ring precipitin test. Directly on mixing, however, in the three lower dilutions all precipitate formed disappears completely, the precipitate in the fourth tube is decreased slightly, while that in the fifth tube, *i.e.*, the highest dilution, remains unchanged. At the end of the 2nd hour at 37°C., the first three tubes show very little precipitate. The precipitate in the fourth and fifth tubes is about equal. After standing overnight in the ice box the amounts of precipitate in all tubes are about equal.

The type-specific substance in a dilution of 1/100 gives a slight precipitate with both antigonococcal and antipneumococcal Type III sera. There is no reaction at 1/1,000 or higher dilutions. The reaction is without doubt due to the incomplete purification of the fraction.

Chemical Analysis.—A more detailed chemical study of the various fractions thus far isolated is being undertaken at present and the results will be presented in a later paper (12). Crude specimens of Type I specific substance give a positive biuret test, a precipitate with 20 per cent trichloracetic acid and a Molisch reaction. The most purified specimen yet obtained, giving a precipitin reaction out to 1/10,000 with homologous rabbit serum and 1/100,000 with polyvalent horse

TABLE V

Dilutions	Serum			
	I	II	III	IV
	No. 578	No. 551	No. 556	No. 535
1/10	0	+++	0	0
1/25	0	+++	0	0
1/50	0	++	0	0
1/75	0	++	0	0
1/100	0	++	0	0

Saline—negative

serum, showed the following on analysis. A Molisch reaction could be obtained in a dilution of 1/100; and a strong biuret test and a marked precipitate with trichloracetic acid were obtained in a dilution of 1/1,000, though both tests were negative at 1/10,000.

Type II

In this case also, most of the work has been carried out with a single specimen of type-specific substance obtained from Strain 31 in the manner already described. This strain showed a characteristic agglutination (Table V) in monovalent sera prepared with freshly isolated strains (1).

The fraction gives a good precipitin reaction with homologous Type II monovalent rabbit antiserum with rather less cross-precipitation

than is obtained with the specimen of type-specific substance obtained from Type I. A noticeable feature is a quite marked cross-precipitation with Type IV antiserum—more marked than is the case with Type I specific substance. This suggestion of relationship of Type II to Type IV has been noted several times throughout the investigations; owing, however, to the absence at the present time of a freshly isolated Type IV strain, the serum used is not of the same value as that for Types I, II and III, nor can the problem be investigated further at the moment. Absorption of the monovalent sera removes all the group precipitins and, it must be noted, removes the precipitins of the Type IV serum.

TABLE VI

Sera unabsorbed				Sera absorbed				
I	II	III	IV	I	II	III	IV	
No. 578	No. 551	No. 556	No. 535	No. 578	No. 551	No. 556	No. 535	
0	$+^{cr}$	0	0	0	$+^{cr}$	0	0	Immediate } Ring test
$+^{r}$	$^{p}++++^{r}$	$+^{r}$	$+\pm^{r}$	0	$++^{cr}$	0	0	1 hr. } Ring test
$+$	$+++^{p}$	$+$	$++^{p}$	0	$++$	0	0	2 hrs.
$+$	$++++^{pd}$	$+$	$++^{pd}$	0	$+++^{p}$	0	0	Ice box

Experiment 6.—A purified specimen of Type II specific substance is made up in a dilution of 1 part in 100 of saline. This is set up against unabsorbed and absorbed monovalent rabbit sera and the precipitin test carried out in the usual manner. As in Experiment 1, the absorption was carried out both with dried C substance and with whole organisms, on this occasion of Type I (Table VI).

As with Type I specific substance, dilution of either the solution of specific substance or of the serum serves to bring out the specificity of the reaction.

Experiment 7.—Dilutions of a purified specimen of Type II specific substance were made, ranging from 1/100 to 1/10,000,000. These were set up against undiluted serum. Serum dilutions were made from undiluted serum to 1 part in 32 and these were set up against a 1/1,000 solution of the specific substance (Table VII).

It will be noted that the type-specific substance reacts with the homologous serum in a dilution of 1/100,000 albeit slightly. This is

TABLE VII

Serum		Dilutions of type-specific substance						Serum		Dilutions of serum					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}			1/1	1/2	1/4	1/8	1/16	1/32
Type I serum	Serum 1/1	$+\pm$	$+$	$\mp$	0	0	0	Type I serum	Type-specific substance 10^{-3}	$+^{p}$	$+^{p}$	$\pm$	$\mp$	0	0
Type II serum	Serum 1/1	$++++^{pd}$	$+++^{p}$	$\pm$	$\mp$	0	0	Type II serum	Type-specific substance 10^{-3}	$+++^{p}$	$++^{p}$	$++^{p}$	$+$	$\pm$	$\mp$
Type III serum	Serum 1/1	$++^{p}$	$+^{p}$	0	0	0	0	Type III serum	Type-specific substance 10^{-3}	$+^{p}$	$++^{p}$	$+^{p}$	$\pm$	$\mp$	0
Type IV serum	Serum 1/1	$++^{p}$	$+\pm^{p}$	$\mp$	0	0	0	Type IV serum	Type-specific substance 10^{-3}	$++^{p}$	$++^{p}$	$+^{p}$	$+$	$\pm$	0
Polyvalent serum	Serum 1/1	$+++^{pd}$	$++^{p}$	$\mp$	0	0	0	Polyvalent serum	Type-specific substance 10^{-3}	$++^{p}$	$++^{p}$	$++^{p}$	$+^{p}$	$\pm$	$\pm$

Final readings shown.

to be compared with the high dilutions in which this same specific substance gives protein reactions (see below). In the serum dilution protocol, the relationship of the Type IV to the Type II already mentioned above is brought out quite sharply.

As with the Type I specific substance, so the Type II reacts with some specimens of polyvalent antimeningococcal horse serum. This reaction is due in part to type-specific and in part to group-specific precipitins in the serum, as can be shown by absorption.

Experiment 8.—Dilutions of Type II specific substance from 1/100 to 1/10-000,000 were set up against undiluted polyvalent serum No. 3. Dilutions of serum from undiluted up to 1/32 were set up against Type II specific substance diluted 1/1,000 (Table VII).

As with the Type I specific substance and polyvalent serum (see Experiment 4) so here also the precipitin reaction between Type II specific substance and the particular specimen of polyvalent serum used reaches a high titre, slightly higher in fact in the serum dilutions for the polyvalent than for the homologous Type II serum. Here again, however, it can be shown by absorption that the reaction is due in large part to the non-specific precipitins in the polyvalent serum precipitating impurities, including the non-specific polysaccharide C, remaining in the type-specific substance.

Two samples of polyvalent serum were absorbed with homologous (Type II, Strain 442) and heterologous (Type I, Strain 454) whole organisms and set up against the dilutions of type-specific substance. Absorption with heterologous organisms removed a large amount of the antibodies from the serum, showing them to be group-specific; homologous absorption removed even more of the antibodies, these obviously being those against the type-specific substance.

Type II specific substance in a dilution of 1/100 gives a slight precipitate with antigonococcal serum and a still slighter reaction with antipneumococcal Type III serum. No reaction occurs in a dilution of 1/1,000. The reaction is due to the presence in the specimen of type-specific substance of some group- or non-specific fractions.

Chemical Analysis.—Crude specimens of Type II specific substance give a positive biuret test, a precipitate with 20 per cent trichloracetic acid and a weak Molisch reaction. The most purified specimen yet

obtained, which gives a precipitin reaction with homologous serum in a dilution of 1/100,000, gives a positive biuret test and a marked precipitate with trichloracetic acid at 1/1,000 and a slight precipitate with trichloracetic acid at 1/10,000. The Molisch reaction is positive at 1/100.

Type III

As has been mentioned above, the strain used in obtaining the Type III specific substance has been No. 302, secured from Dr. Sara Branham of the Public Health Service, Washington, as their most narrowly specific Type III strain. Tested with the monovalent sera in

TABLE VIII

Dilutions	Serum			
	I	II	III	IV
	No. 578	No. 551	No. 556	No. 535
1/10	++++	0	+++	0
1/25	++++	0	++	0
1/50	+++	0	++	0
1/75	++	0	+	0
1/100	+	0	+	0

Saline—negative

use in this laboratory and using low dilutions in a 37°C. water bath, the strain gave an agglutination very similar to that given by any other Type I or Group I-III strain which has been isolated for some months and subcultured on artificial media (Table VIII).

Since this strain was not freshly isolated, it was to be expected that the yield of type-specific substance would be small, for this has been found to decrease in amount with the length of time the organism has been grown on artificial media (13). This fact explains, no doubt, some of the morphological changes which are characteristic of the transformation from "fresh" to "stock" cultures (14), and also the well recognized fact that stock strains usually show a loss of their type specificity as judged by the agglutination test (especially when that test is carried out at 55°C.). The total yield from this strain was 75

mg. of dried purified material. Of this, 55 mg. were obtained in the usual manner described above for isolation of type-specific substance. This specimen contained other substances which gave cross-precipitation with Type II and Type IV serum in the same way as the specimens of Type I and Type II specific substance gave cross-precipitation. The other 20 mg. were obtained from the nucleoprotein fraction or P which had been precipitated with 10 per cent acetic acid. This P fraction was found to contain not inconspicuous amounts of the type-specific fraction when it was tested with monovalent sera. It has been noted throughout the work that the nucleoprotein fraction contained varying amounts of type-specific substance which could be removed only with great difficulty. As a rule the amount was very

TABLE IX

	Serum			
	I	II	III	IV
	No. 578	No. 592[2]	No. 266	No. 577
No. 302 specific substance 1/250	+++^{d}	0	+++^{d}	0

TABLE X

Serum	Dilutions of type-specific substance						Serum	Dilutions of serum					
	1/250	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		1/1	1/2	1/4	1/8	1/16	1/32
Type I serum	+++^{p}	+++^{p}	+^{p}	0	0	0	Type I serum	+++^{p}	+++^{p}	++^{p}	+^{p}	0	0
Type III serum	+++^{p}	+++^{p}	++^{p}	0	0	0	Type III serum	+++^{p}	+++^{p}	+++^{p}	++^{p}	+^{p}	∓

TABLE XI

Serum...........................	I	I	III	III	I	III
Absorbed with...................	I	III	I	III	–	–
Type I specific substance 1/200	0	0	0	0	++^{pd}	+++^{pd}
Type III specific substance 1/250	0	0	0	0	+++^{d}	+++^{d}

small but in the present case it was quite considerable. As a consequence, it was precipitated with two volumes of ethyl alcohol and a scanty yield of type-specific substance obtained. The important feature about the type-specific substance secured in this way was the absence of impurities giving any significant cross-precipitations with Type II and Type IV sera (Table IX).

Titrations of the specific substance show that it behaves in a manner almost identical with that of the Type I specific substance. It gives a positive reaction with both Type I and Type III serum out to 1/-10,000 but not to 1/100,000, and in dilutions of 1/1,000 it reacts with Type I serum diluted 1/8 and Type III serum diluted 1/32 (Table X).

Chemical analysis of the Type III specific substance fails to show any differences from the analysis of the Type I fraction. Thus, a purified specimen gives a positive Molisch reaction at 1/100, and a strong biuret test and a good precipitate with 20 per cent trichloracetic acid are demonstrable in a dilution of 1/1,000 though both are negative at 1/10,000.

An even more intimate relationship between the specific fractions is brought out by means of absorption experiments.

Experiment 9.—Samples of Type I and Type III serum were absorbed with whole organisms of both Type I and Type III strains. The organisms were left in contact with the serum for 2½ hours at 37°C. and 18 hours at the temperature of the ice box. These absorbed sera together with unabsorbed controls were then set up against specimens of specific substance from Strains 428 (Type I) and 302 (Type III). The results are shown in Table XI.

It will be seen that the absorption of either Type I or Type III serum by either Type I or Type III whole organisms removes completely the precipitins in these sera for both Type I and Type III specific substances. It has been demonstrated elsewhere (see Experiments 1 and 6) that, when strain of organisms and serum are heterologous, absorption with whole organisms helps to remove the non-specific antibodies in the serum but leaves untouched the heterologous precipitins for the type-specific substance. One can only conclude, therefore, from the above experiment (Experiment 9) that the type-specific substances isolated from the Type I and the Type III strains must be very similar in constitution if they be not identical.

The result of the absorption test agrees with the results obtained by other means which, as Tables I, II, IV, VIII, IX, X and XI have shown, tend to prove the identity of the two substances.

SUMMARY

Three fractions have been isolated from autolysates of the meningococcus. Of these, one, the type-specific substance, has been described in detail. The same type-specific substance appears to be present in Type I and Type III organisms, but a substance differing at least serologically has been obtained from Type II strains. Detailed chemical analysis of both of the type-specific substances thus far isolated is being carried out and will be the subject of a later paper.

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