STUDIES ON MENINGOCOCCUS INFECTION

VIII. The Type I Specific Substance

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In a previous paper (1) the preparation of fractions from the meningococcus with marked type specificity toward antimeningococcal monovalent serum was described. For the most part these fractions were obtained from autolysates of the meningococci growing in hormone broth. They were not pure chemical entities and, besides being rarely able to evoke the precipitin reaction in dilutions higher than 1:10,000, gave cross-reactions with heterologous serum. An additional fact noted was that, as far as the type-specific substances were concerned, Types I and III were serologically identical. Subsequently (2) a method was described briefly for the preparation of a type-specific substance from Type I meningococcus which was pure and highly active serologically. In the present communication a more detailed account of the preparation and properties of this substance, to be referred to as S I, will be given.

Material and Technique of Preparation

Strains of Type I meningococci were obtained from sources previously acknowledged (3). For the most part they were used shortly after isolation, that is within 10 weeks, though in one case a strain was used after it had been maintained on artificial media for more than 12 months. In this case the yield of type-specific substance was poor, and in general it has been found that the growth characteristics of the strains might become unsatisfactory in a very short period. Thus Strain 503, which gave a good yield of S I on Apr. 23, 1934, (preparation 19), being then 23 days old, gave only a small yield when inoculated on May 4 and again on May 10, 1934. This loss in the power to produce the type-specific substance is in keeping with the observations of Petrie (4) and the present authors (3). Some strains, as for example 502, continued to give an excellent yield after 12 weeks but in general the sooner the strain was utilized after isolation the better the results.

For the preparation of S I, autolyzed cultures were found most convenient. This fact has been mentioned before (1, 5) and it is only necessary to repeat here that the S I can be obtained by the various methods of extracting young, active cultures washed off solid media. However, these methods are more time-consuming and give a much smaller yield and for these reasons autolysis in broth has been the method adopted.

The organisms were grown in 1 liter or 2 liter Florence flasks containing 600 cc. or 1200 cc. of hormone broth, respectively, at pH 7.0 to 7.2 and containing 0.3 per cent glucose. The glucose increased the amount of growth and it was found that the substitution of this pH for the usual one of 7.6 caused growth to begin more readily and proceed more rapidly. The inoculum was 5 to 10 cc. of an actively growing culture in hormone broth. The flasks were capped with lead foil and incubated at 37°C. After pellicle formation began they were agitated daily to break this pellicle and allow new growth to occur at the surface. Growth ceased within 10 to 18 days. Each flask was then examined for the presence of contaminants in stained smears and if these were found, that flask was discarded.

Isolation of the Type-Specific Substance

The fact has already been noted (1, 2) that the protein precipitable from Type I meningococcus autolysates by dilute acetic acid, the so called nucleoprotein P fraction, carries with it appreciable amounts of S I. This reaction has been utilized as the first step in isolating purified S I. It was first observed that when the P fraction was precipitated by acidifying the broth to pH 4.0 with acetic acid in the presence of 1.5 volumes of ethyl alcohol, the S I present was removed almost quantitatively from solution (as shown by the precipitin test with Type I monovalent antimeningococcal serum[1]). Since the large

[1] The precipitin test now used shows slight modifications from the method previously described (1). Tubes of about 3.5 mm. internal diameter are used. The serum is introduced first and the solution carefully layered on top. The difference lies in the fact that the overnight reading is usually omitted and the immediate, 1 hour, and 2 hour tests are all made by the ring method; *i.e.*, without mixing the two fluids. The use of tubes of smaller diameter facilitates this, as appreciable mixing of the fluids does not occur even after 2 hours at 37°C. provided agitation of the tubes be carefully avoided. More sensitive readings can be obtained by this method. It is, however, unsuitable for comparative estimation of the amount of precipitate which occurs, and for this the method previously described is still used. It should be emphasized that the use of a strong beam of light and a dull black background against which to observe the tubes not only facilitates all readings but is essential for reading finer degrees of precipitin reaction.

scale precipitation of unconcentrated broth with ethyl alcohol was not practical, experiments were carried out starting with broth which had been concentrated *in vacuo* to 1/10 or 1/15 the original volume. When this material was acidified to pH 4.0 with acetic or hydrochloric acid in the presence of as much as 1.75 volumes of ethyl alcohol, only about one-half of the S I appeared in the precipitate. The use of larger amounts of alcohol was precluded because this caused the constituents of the broth to precipitate out as a gum.

Since concentration of the broth before precipitation rendered the adsorption of the S I on the P fraction less efficient than when unconcentrated broth was used, it seemed possible that preliminary dilution of the broth might prove more satisfactory and it was soon shown that the adsorption could be made nearly quantitative (as shown by the precipitin test), without the use of ethyl alcohol, if the broth was diluted with water before acidification.

The optimal dilution was found to vary from 1:1 to 1:4 with different batches of broth. It should, ideally, be determined for each preparation. However, the increased efficiency of the adsorption at higher dilutions is offset by the more cumbersome manipulation entailed and by the presence of serologically inactive polysaccharides in the final product, the proportion of which increases with increasing dilution. In one preparation in which the broth autolysate was diluted with 9 volumes of water before acidification, the inactive polysaccharides formed the bulk of the product.

These considerations led to the adoption of the following procedure which proves satisfactory with 18 liter quantities of broth.

The autolysate is divided equally between two 5 gallon bottles, diluted with an equal volume of cold tap water and allowed to stand at 4°C. overnight. The next morning it is stirred and acetic acid is slowly added in the proportion of 10 cc. of acid per liter of original broth autolysate. The resultant pH, usually 4.0, is equal to or more acid than the isoelectric point of the P fraction which begins to flocculate out. The bottles are returned to the refrigerator for 24 hours[2] when the bulk of the precipitate is found to have settled to a fairly compact layer several centimeters in thickness. The clear or slightly cloudy supernatant is siphoned off as completely as possible and the precipitate collected by centrifugal-

[2] The S I is sensitive even to weak acid if the temperature is sufficiently high. Hence, as a matter of precaution, solutions were chilled whenever it was necessary to expose the preparation to acid conditions for more than a few minutes.

ization, preferably in the cold.[3] The precipitate is then homogenized in 1 liter of distilled water very slightly acidulated with acetic acid and the suspension filtered on hard filter paper sheets 38 cm. in diameter in the refrigerator. The filtration is complete in 24 to 48 hours when the filtrate is discarded since it contains little or no S I.

Having determined the optimal conditions for the primary adsorption, the problem remains to separate the S I from the relatively enormous amounts of protein which accompany it (20 to 100 times the amount of S I) and to which it seemed to be firmly attached. The combination proved, however, to be a loose one and it was possible to dissociate the complex and remove about 90 per cent of the protein by reprecipitation at pH 4.8 to 5.0 in the following manner. The precipitate of crude P fraction is readily scraped off the filter paper and is homogenized in a volume of distilled water not greater than 900 cc. in an Erlenmeyer flask. Normal sodium hydroxide is added slowly with stirring until pH 7.0 is reached and the stirring is continued until all lumps disappear. 10 gm. of sodium acetate are then added (in the absence of this electrolyte reprecipitation of the P fraction at pH 4.8 or 5.0 does not occur readily) and dissolved, followed by the calculated amount of normal acetic acid (for 10 gm. of sodium acetate [Green (6)]) necessary to produce pH 5.0, namely 35 cc. After the solution has stood for 10 to 15 minutes the precipitate may begin to separate leaving a narrow ring of clear fluid at the top. If this does not happen, 5 cc. portions of normal acetic acid are added with stirring until precipitation does occur. The amount of acid needed varies from preparation to preparation and indeed is not critical since there is little danger of making the solution too acid by using the procedure described. The precipitation mixture is filtered on hard paper in the refrigerator for 24 to 48 hours. The clear, slightly yellow filtrate (pH 4.8 to 5.0; *gamma* dinitrophenol indicator) is neutralized with normal sodium hydroxide and set aside in the refrigerator with chloroform as a preservative. The precipitate is scraped off the filter paper and subjected once more to the process outlined above of solution followed by reprecipitation at pH 4.8 to 5.0. This repetition usually calls for more acetic acid than at first. The mixture is now filtered on soft paper in the refrigerator and at the end of 24 to 48 hours transferred to a large Buchner funnel and sucked dry. It is possible to dissociate a small additional amount of S I from the P fraction by a third solution and reprecipitation, but this is usually not profitable. The combined neutralized filtrates contain the bulk of the S I accompanied by a very considerable amount of protein, colored material and sodium acetate. They are concentrated *in vacuo* to a volume of 150 to 200 cc. The S I and protein are precipitated by the addition of 5 volumes of ethyl alcohol, the mixture standing overnight in the refrigerator. The precipitate is centrifuged

[3] This, and the succeeding precipitates of the P fraction, are bulky, gelatinous and do not pack well when centrifuged. For this reason, filtration has been the method generally chosen for collection of this material. It is a slower technique but is less laborious and gives more nearly complete separation.

off and the supernatant, which contains much pigment and most of the sodium acetate, is discarded. The precipitate is dissolved in 150 to 200 cc. of distilled water and concentrated *in vacuo* to a volume of 50 to 70 cc. in order to remove the alcohol. Most of the remaining protein is now precipitated by adding the calculated amount of ammonium sulfate necessary to produce a 0.7 saturated solution; namely, 0.5 gm. per cc. of solution.[4] The precipitate, which contains very little S I, is collected on soft paper in a Buchner funnel and discarded. For removal of the ammonium sulfate the clear, light yellow filtrate is neutralized with sodium hydroxide and dialyzed through cellophane[5] in a bath of running tap water until it gives only a slight test for the sulfate ion. Chloroform is added as a preservative. It is usually necessary to concentrate the solution *in vacuo* once during the course of the dialysis. The last of the sulfate is removed by adding 10 per cent barium acetate solution to the dialysate until further increments produce no additional precipitate. The excess barium is removed by adding enough sodium carbonate to make a 5 per cent solution and the precipitate of barium carbonate and barium sulfate is centrifuged off. The clear, often colorless supernatant is then acidified with acetic acid to pH 4.5, concentrated *in vacuo* to 40 cc. and transferred to a 250 cc. centrifuge bottle. The S I is precipitated by adding 4 volumes of ethyl alcohol and centrifuged off after standing in the refrigerator 5 or 6 hours or preferably overnight. In the presence of an excess of sodium acetate 4 volumes of ethyl alcohol precipitate the S I almost quantitatively. On centrifuging, the S I forms a compact button of yellowish gum. This material is pure enough for serological purposes and if desired may be rinsed off with ethyl alcohol and dried in a vacuum desiccator.

For further purification the gum is dissolved in 20 cc. of 5 per cent copper acetate solution. The resultant precipitate, usually small in amount, contains the remaining protein and most of the pigment. It is centrifuged off at high speed leaving a clear or slightly opalescent supernatant. To this are added 10 cc. of distilled water, 2 gm. of sodium acetate and 3.5 cc. of acetic acid which ensure the copper remaining in solution when the S I is precipitated with 4 volumes of ethyl alcohol. The S I is dissolved in 3 or 4 cc. of distilled water and if there is much

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[4] Since these experiments were completed, it has been found that copper acetate solution, buffered at pH 5.0 or slightly higher (just yellow to methyl red indicator) by the addition of sodium hydroxide, is much more satisfactory and efficient than is ammonium sulfate for the removal of protein at this stage of the preparation. The copper acetate is removed by repeated precipitation of the S I with 4 volumes of ethyl alcohol from a solution containing 10 per cent sodium acetate and sufficient acetic acid to maintain pH 4.0. Optimal conditions for this technique have not been completely determined, so that details of procedure cannot be given at this time.

[5] Collodion membranes are not suitable. When prepared in the customary manner, they were found to be permeable to the S I. Parchment may be substituted for cellophane but is apt to introduce calcium into the product.

insoluble matter the copper acetate treatment is repeated. The S I can be freed of all traces of copper by two or more precipitations from acid solution as follows: The S I is dissolved in 40 cc. of 10 per cent aqueous sodium acetate and freed of insoluble matter by centrifuging; 6 cc. of acetic acid is added and precipitation is brought about by the addition of 4 volumes of ethyl alcohol. The product now contains S I and varying amounts of a serologically inactive polysaccharide. The latter has usually been present in only small amounts and can be removed by dissolving the S I in not more than 10 cc. of 20 per cent aqueous sodium acetate, chilling the solution in an ice bath and adding 4 volumes of cold acetic acid. After 1 to 2 hours at 0°C. the inactive material appears as a small amount of gelatinous precipitate which is removed either by centrifuging or filtering in the cold.

In certain cases, notably those in which the amount of S I in the original broth was scant and in one case where the broth was originally diluted tenfold, 50 per cent or more of the product at this stage consisted of this inactive fraction. Its presence in large amounts was shown by the fact that the product, separated out from the sodium acetate solution by means of ethyl alcohol, appeared almost immediately as a flocculent precipitate, whereas under these conditions the purified S I always separated out slowly (6 hours or overnight at 0–4°C.) as a gum. When present in such large amounts complete separation of this inactive fraction from the S I was not possible. Fairly good results might be obtained by the acetic acid precipitation outlined above, using, however, 40 cc. of 10 per cent sodium acetate as solvent and 4 to 10 volumes of acetic acid as precipitating agent. On account of the bulky gelatinous nature of the precipitated inactive fraction it was necessary to repeat the process.

The S I is now precipitated in a white, rather gelatinous form by addition of 4 volumes of ethyl alcohol to the supernatant from the precipitation of the inactive fraction with acetic acid. The precipitate forms almost immediately and is centrifuged off after 15 to 30 minutes. It is dissolved in 40 cc. of aqueous 10 per cent sodium acetate and the solution is freed of any insoluble material by centrifuging. The S I is precipitated with 4 volumes of ethyl alcohol as a colorless or slightly yellow gum and this last procedure is repeated to ensure complete removal of free acetic acid. Finally, the S I is taken up in 10 cc. of neutralized 20 per cent aqueous sodium acetate and precipitated by the addition of 200 cc. of absolute ethyl alcohol as a white, finely divided product. The precipitate is collected by centrifuging and dried in a vacuum desiccator. The yield from 18 liters of broth varies from 0.3 to 0.8 gm.

The product in the finely divided state described is unsuitable for analytical work, since it cannot be satisfactorily freed from sodium acetate by washing, owing to its tendency to form colloidal suspensions in alcohol when an excess of sodium acetate is not present. In preparing samples for analysis the following method has been used, and while this procedure is not entirely satisfactory, nevertheless, owing to

the difficulty experienced in precipitating the S I from pure solvents, it has proved to be the best method available.

A suitable amount of the S I (0.1 to 0.25 gm.) is dissolved in 5 to 10 cc. of a neutralized 10 per cent aqueous solution of sodium acetate. The solution is centrifuged at high speed and transferred to a weighed 50 cc. centrifuge tube. The S I is then precipitated in the gummy state by adding 4 volumes of redistilled ethyl alcohol and allowing the mixture to stand overnight in the refrigerator. The precipitate is centrifuged off and the tube is allowed to drain for several minutes by resting it in the inverted position on a piece of filter paper. The tube is then rinsed out with several portions of 10 to 20 cc. of redistilled ethyl alcohol allowing the gum to remain in contact with each portion for at least 2 hours. Finally, after draining it thoroughly, the tube is dried to constant weight under a high vacuum in the presence of calcium chloride and sodium hydroxide. The S I is then dissolved in distilled water and made up to such a volume as to contain 10 mg. per cc. The solutions are stored in the refrigerator with a little chloroform as preservative and portions are withdrawn as required.

Analysis

All the analytical data given represent an average of two or more determinations.

Carbon, hydrogen and micro Dumas nitrogen determinations were carried out[6] in the customary manner, using solid samples prepared by precipitation with 20 volumes of absolute ethyl alcohol. It should be noted that the figure given for carbon is doubtless low due to the great difficulty experienced in burning the ash free of carbon. Phosphorus was determined by Elek's modification of the micro method of Lieb (7). Aliquot portions of the standard solutions were evaporated to dryness at 100°C. in the silver crucible used for digestion. For the ash determinations aliquot portions were evaporated to dryness at 100°C. in a small platinum crucible which was then ignited to constant weight. No sulfuric acid was added since the phosphorus content of the S I is more than large enough to convert all the sodium to sodium phosphate. Calculation of the sodium was made on the assumption that the ash was $Na_4P_2O_7$. Qualitative tests on the ash were carried out according to Noyes (8). Nitrogen was determined by a slight modification of the Pregl micro Kjeldahl procedure (9). Reducing sugars were determined by the Shaffer-Hartmann micro method (10) on samples which had been subjected to the following method of acid hydrolysis.

The aliquot portion (about 2 mg.) of a standard solution of S I was pipetted into the tube in which the determination was to be made; sufficient hydrochloric

[6] The determinations were carried out by Mr. D. R. Rigakos to whom our thanks are due.

acid was added to make a final concentration of 1.0 normal; and hydrolysis was carried out at 100°C. for ½ hour. Longer heating did not increase the yield of reducing sugars. The hydrolysate was always neutralized with normal sodium hydroxide and diluted to the proper volume before the Shaffer-Hartmann reagent was added.

The ash left after ignition of samples of S I has been identified as a sodium phosphate, probably sodium pyrophosphate. It forms a clear melt in the crucible at medium red heat, and is readily soluble in water giving a solution strongly alkaline to phenol red. No gas is evolved when the ash is treated with dilute acid. Solutions of 3 mg. of the ash give an immediate reaction with ammonium molybdate reagent. Careful analyses of 20 mg. samples have shown the absence of all cations up to the alkali group. For the positive identification of sodium, 53 mg. of preparation 17 was ashed in a platinum crucible and ignited until all the carbon was burned. The ash was dissolved in 1 cc. of distilled water and to this solution in a small test tube was added 2 cc. of potassium pyroantimonate solution (8). After several minutes the typical heavy crystalline precipitate of sodium pyroantimonate began to settle rapidly. There was no evidence of non-crystalline precipitate.

The following experiment showed that the phosphorus is firmly held in organic combination. To 2 cc. of 0.1 per cent solution of S I (2 mg.) was added 5 cc. of dilute nitric acid and 15 cc. of ammonium nitrate solution (all solutions being prepared according to the directions given by Treadwell and Hall (11)). The solution was warmed to 50° or 60°C. and 10 cc. of ammonium molybdate solution at the boiling point was added. A yellow color developed but only a slight precipitate of ammonium phosphomolybdate appeared even on standing overnight at room temperature. As a control a similar amount of S I was tested after it had been boiled for 15 minutes in 50 per cent nitric acid. The test was strongly positive as soon as the various reagents had been added.

Analytical data of several preparations are summarized in Table I. Nos. 8 and 9 were early preparations and were not subjected to either the copper acetate treatment or the precipitation with 4 volumes of acetic acid. Instead they were purified by repeated precipitation from a solution of the substance in 10 per cent aqueous sodium acetate by 4 volumes of ethyl alcohol. It is interesting to note that the properties of preparation 9, made from a stock strain, were not significantly different from the properties of the other preparations all of which were from freshly isolated strains. The yield in this case, however, was very small, being only 0.12 gm. from 40 liters of broth. If the early preparations are disregarded it will be seen that, in the last four preparations (including 15 B which was not subjected to the barium acetate and sodium carbonate treatment) made from three

different strains, a product has been obtained with a nearly constant composition and an average content of 4.36 per cent nitrogen, 8.91 per cent phosphorus and 9.59 per cent sodium. On acid hydrolysis an average of 45.2 per cent reducing sugars calculated as glucose is formed. The average optical rotation is +56.8° for the sodium D line.

The biuret and trichloracetic acid tests have been negative when carried out on 10 mg. samples of S I, indicating absence of appreciable quantities of protein. Tryptic digestion does not destroy the sero-

TABLE I

Summary of Analytical Data. Type I Meningococcus Specific Substance

Preparation No.	Strain No.	C*	H*	N*	Ash as Na	P*	Reducing sugars on acid hydrolysis as glucose*	$[\alpha]_D$*	Precipitin titer†
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*degrees*	
8	484 + 489	30.37	6.0	4.90‡	10.2		46.6	+56.8	1:8,000,000
9	455			4.70	12.7		44.5	+55.5	1:8,000,000
15 B	497			4.53	9.55	8.83	44.7	+55.9	1:8,000,000
17	497			4.44	9.80	9.01	47.0	+56.0	1:8,000,000
18	502			4.20	9.74	8.93	44.3	+57.0	1:8,000,000
19	503			4.28	9.27	8.88	44.8	+58.4	1:8,000,000
Average of 15 B, 17, 18, 19.....				4.36	9.59	8.91	45.2	+56.8	

* Ash-free.

† *Vs.* Type I antimeningococcal horse serum.

‡ Micro Dumas.

logical activity of the preparations. Addition of a little iodine and potassium iodide solution to a 5 mg. sample produces no red or blue coloration indicating an absence of glycogen and starch. The Molisch test has been positive in dilutions as high as 1:100,000. The orcinol-hydrochloric acid test was negative when carried out on a 5 mg. sample indicating the absence of pentoses.

Characteristics and Properties of the Type-Specific Polysaccharide

The purified S I gives precipitates with uranyl nitrate, basic lead acetate and safranin but not with copper acetate, mercuric chloride,

saturated barium hydroxide, saturated ammonium sulfate, saturated calcium chloride or saturated magnesium sulfate. Samples of S I have been purified by precipitation with basic lead acetate or uranyl nitrate and eluting respectively with sodium carbonate or disodium phosphate. However, the yields were poor and the properties of the product not different from those of the original material.

The S I in the form isolated has no acidic properties. When 100 mg. samples are dissolved in distilled water with a drop of phenol red added, the addition of 0.06 cc. of N/10 sodium hydroxide produces the end-point. The S I is characterized by its high solubility in distilled water and by the difficulty with which it is precipitated. 20 per cent aqueous solutions of S I are readily obtained. In the absence of an excess of electrolyte such as sodium acetate, the S I is not precipitated from aqueous solutions by 10 volumes of absolute alcohol, by 20 volumes of acetone or by 10 volumes of acetic acid. It is not precipitated by 10 volumes of acetic acid even in the presence of sodium acetate. It cannot be precipitated from a solution in 50 per cent acetic acid by adding 8 volumes of ethyl alcohol unless sodium acetate is added also. These facts have made impossible the isolation of an ash-free product.

Attempts have been made to secure an ash-free product by dialyzing solutions of S I in cellophane bags against distilled water. The dialyzed solution was afterwards evaporated to dryness at room temperature and the residue, having been dried to constant weight in a high vacuum in the presence of calcium chloride or sulfuric acid, was analyzed for ash. The recovery of S I was generally poor, possibly due to its adsorption on the surface of the cellophane. The ash content was the same as that of the original material. In one experiment a solution of S I was subjected to electrodialysis using parchment membranes, but since the combined diffusates gave a positive precipitin test with Type I antimeningococcal serum, showing that the S I had diffused through the membranes, the experiment was abandoned.

The S I remains stable at temperatures around 0°C. at least for many months. Thus, preparation 8 was made from broth harvested May 16 and 23, 1933. When the preparation was finished, namely in September, 1933, the titer was 1:8,000,000. On January 8, 1934, a 1:10,000 saline solution was heated for 15 minutes at 100°C. for

sterilization. The product after this treatment still titered 1:8,000,000.

The effect of exposure to various conditions on the precipitin titer of S I is summarized in Table II. In a 1:5,000 solution in M/15 phosphate buffer at pH 7.0, the S I withstood a temperature of 100° for 15 minutes. The S I, however, is very sensitive to acid conditions.

Thus, in a 1:1,000 solution in normal hydrochloric acid the precipitin titer had dropped to 1:10,000 after 3 hours at room temperature. In a similar experiment with 0.5 normal HCl, carried out for 1 hour at room temperature, the titer dropped to about one-fifth the value for untreated S I. In a 1:1,000 solution of S I which stood for 23 hours at room temperature in 0.01 normal HCl the effect was not as great but there was a perceptible loss of serological activity. A 1:5,000 solution of S I in 0.2 normal acetic acid-sodium acetate buffer, pH 4.0, was heated for ½ hour at 100°C. and almost all of the serological activity was lost. Accompanying this loss there is an appearance of varying amounts of reducing sugars (7 to 15 per cent calculated as glucose). These appear rapidly (in 15 minutes in the case of the experiment in pH 4.0 buffer heated to 100°C.) and then increase slowly and in 24 to 48 hours may not reach the maximum value of 45 per cent obtained by hydrolysis with strong acid. S I which has not been exposed to strong acid shows no reducing action with the Shaffer-Hartmann reagent.

The exact relationships involved in the acid hydrolysis of S I have not been studied, but preliminary rate measurements have been made, the results of which indicate that a labile prosthetic group is first split off, whereas the bulk of the molecule undergoes a slower degradation. It is hoped to carry out a detailed study of this phenomenon, in order to determine what grouping or configuration is responsible for the serological activity of the S I.

The S I is only slowly broken down by alkali.

The test was a severe one since the dilution of S I was 1:20,000. Heating this in N/140 sodium hydroxide at 100°C. for 15 minutes did not affect the serological activity while the use of 0.5 normal sodium hydroxide for 1 hour at 100°C. and of 10 per cent sodium hydroxide for 3 hours at room temperature caused only slight degradation.

The serological activity of the S I is not affected by exposure to nitrous acid at room temperature.

To 1 cc. of a 1:10,000 saline solution of S I were added 0.5 cc. of 30 per cent aqueous sodium nitrite and 0.3 cc. of acetic acid (2.4 times the amount equivalent to the sodium nitrite). A control of 1 cc. of 1:10,000 saline solution of S I with

0.3 cc. of acetic acid was set up. After standing for 3 hours at room temperature with occasional shaking, the solutions were chilled and neutralized with strong sodium hydroxide solution. The results of the precipitin tests showed no dis-

TABLE II

Effect of Exposure to Various Conditions on the Precipitin Titer of S I

Conditions of experiment	Dilution for precipitin test	Precipitin titer with Type I antimeningococcal horse serum
100°C. for 15 min. at pH 7.0	1:50,000	+++pd
	1:500,000	+±
	1:5,000,000	±
1.0 N HCl for 3 hrs. at 23°C.	1:10,000	∓
0.5 N HCl for 1 hr. at 23°C.	1:100,000	+
	1:1,000,000	∓
0.1 N HCl for 23 hrs. at 23°C.	1:100,000	+
	1:1,000,000	±
100°C. for 0.5 hr. in pH 4.0 buffer	1:50,000	∓
3 hrs. at 20°C. in $NaNO_2$ and CH_3COOH	1:50,000	+++pd
3 hrs. at 20°C. in 25 per cent CH_3COOH	1:50,000	+++pd
3 hrs. at 20°C. in 10 per cent NaOH	1:50,000	++pd
1 hr. at 100°C. in 0.5 N NaOH	1:50,000	++
0.25 hr. at 100°C. in N/140 NaOH	1:50,000	+++pd
Control, untreated S I	1:50,000	+++pd
	1:100,000	++pd
	1:1,000,000	+
	1:5,000,000	±

∓, ±, +, +±, ++, +++ } increasing amounts of precipitate.

pd indicates the formation of a disc-like precipitate which can be broken by agitation.

cernible difference between the two solutions and a control of 1:50,000 S I containing the same amount of sodium acetate.

On the basis of the evidence presented, the type-specific substance isolated by the described methods seems to be the sodium salt of a polysaccharide acid composed of a nitrogen-containing sugar and phosphoric acid units. Different lots have uniform composition. The ratio of sodium (calculated on the basis that the ash left upon ignition of S I is $Na_4P_2O_7$) to phosphorus is 1.6 to 1 or nearly 3 Na to 2 P. The ratio of sodium to nitrogen is 3 to 2. The equivalent weights calculated for 1.5 Na, 1 N or 1 P are respectively 325, 321 and 348, corrected to the ash-free basis. The fact that the ash content is not reduced by prolonged dialysis indicates that the sodium is not present as an impurity in the form of salts which might have been carried over in the purification process. The phosphorus is firmly held in organic combination since the molybdate test with S I is negative until the S I has been subjected to vigorous hydrolysis. This conclusion is supported by the fact that the S I gives no precipitate with cupric acetate, barium hydroxide, calcium chloride or magnesium sulfate. The exact form of linkage of the nitrogen has not been determined. It is not present in a condition suitable to give a positive biuret test.

In order to determine whether part or all of the nitrogen contained in S I was present in the form of amino groups, a sample of S I was treated with nitrous acid as follows:

To 2 cc. of a standard solution of preparation 18 (35 to 36 mg. S I) in a 50 cc. centrifuge tube was added 0.3 cc. of a 30 per cent solution of sodium nitrite and 1 cc. of acetic acid. These quantities provided 13 times the amount of sodium nitrite theoretically necessary to liberate the nitrogen present (assuming that all of the latter was in the form of amino groups) and 3 times the amount of acetic acid equivalent to the sodium nitrite. The solution was allowed to stand for a short time and then a slow current of air was drawn through it for 2½ hours at room temperature in order to remove the gaseous decomposition products. The solution was then worked up according to the method described for preparing analytical samples, the product being subjected to two precipitations from its solution in 10 per cent aqueous sodium acetate by the addition of 4 volumes of redistilled ethyl alcohol. The yield was 28.9 mg. (80 per cent recovery).

Analysis showed 9.9 per cent of ash, calculated as sodium, and 4.27 per cent of nitrogen, calculated on the ash-free basis. These

figures are the same, within the experimental error, as those for the starting material (Table I). Consequently, it was concluded that the S I contained no amino nitrogen. Since it has been shown that nitrous acid does not destroy the ability of S I to give precipitates with homologous antiserum, it seems probable that amino groups play no vital part in the grouping responsible for the serological activity of S I.

In view of the findings with respect to the effect of alkali on the specific polysaccharide of the Type I pneumococcus (12), it is realized that the use of barium acetate and sodium carbonate for the removal of sulfate, as described, constitutes a possible weak point in the method of preparation of the S I, although there is some evidence that Na_2CO_3 does not alter the polysaccharide. This procedure may, however, be avoided by the use of prolonged dialysis, and it is thus possible to prepare the S I by mild methods, performing all operations at or below room temperature and never on the alkaline side or more acid than pH 4.0. We have preparations which have not been exposed to alkaline conditions. It is hoped to make a study of these by means of the quantitative precipitin reaction to determine what, if any, changes take place in the S I during the course of purification.

Serological Reactions

All specimens of S I prepared in the method described had a high precipitin titer with monovalent Type I antimeningococcal horse serum. Tables III and IV give typical protocols obtained with the ring test and by the orthodox precipitin test. Specimens react only in very low dilutions with heterologous sera as is shown in Table V. Here both rabbit and horse monovalent sera of Types I and II were used. Salt controls were consistently negative.

That there was complete immunological identity between the different specimens of S I is shown by the following experiment on cross-absorption.

Equal quantities of the various bleedings of Type I antimeningococcal horse serum, which had been absorbed with 0.3 cc. of a 1:1,000 saline solution of S I, preparation 18, per cc. of serum, were pooled. Precipitin tests were set up as usual with this pooled absorbed serum and 1:10,000 saline solutions of preparations 8, 9, 15 B, 17, 18 and 19 of S I. The tubes were allowed to stand for 2 hours at

37°C. and were then mixed and placed in the refrigerator overnight. All were negative. Controls of the S I solutions at 1:10,000 gave a $++++^{d}$ reaction against pooled unabsorbed serum diluted with saline to the same strength as the unabsorbed serum.

TABLE III

Serum dilutions	1:1,000,000	1:4,000,000	1:8,000,000	Saline
Immediate	$\mp^{R}$	0	0	0
1 hr. at 37°C.	$+^{R}$	$\pm^{R}$	$\mp^{R}$	0
2 hrs. at 37°C.	$++^{R}$	$\pm^{R}$	$\mp^{R}$	0

Precipitin test with Lot 15 B against third bleeding Type I horse serum using the ring test.

^{R} indicates the formation of a precipitate at the line of junction of the fluids.

TABLE IV

Serum dilutions	1:100,000	1:1,000,000	1:2,000,000	1:4,000,000	Saline
	++	+	±	∓	0

Precipitin test carried out with 0.5 cc. portions of antigen and serum in the orthodox method of precipitin tests. The mixture stood for 2 hours at 37°C. and overnight in the refrigerator.

Lot 17 of S I was used and seventh bleeding Type I horse serum.

TABLE V

Serum dilutions	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	1:2,000,000	1:4,000,000	Saline
Rabbit Type I	$++^{d}$	$++^{d}$	$++^{d}$	$++^{d}$	$+^{R}$	$+^{R}$	0	Negative
Rabbit Type II	$\pm^{R}$	0	0	0				
Horse Type I			$+++^{pd}$	$++^{pd}$	$++^{R}$	$+^{R}$	$\pm^{R}$	
Horse Type II		0	0					

^{d} indicates the formation of a disc-like precipitate not broken by agitation.

RÉSUMÉ

The isolation and preparation of the type-specific polysaccharide (S I) of the Type I meningococcus from hormone broth autolysates is described. As had been noted before, the S I is carried down with the so called nucleoprotein or P fraction at pH 4.0, a reaction which is specific since attempts to repeat it with, for example Type II menin-

gococcus material, have yielded very indifferent results. This reaction has been utilized. Using untreated broth only about half of the S I was precipitated in this way. More could be obtained by additional ethyl alcohol precipitation but this was unsatisfactory as was, also, the method of concentrating the broth *in vacuo*. However, it was found that preliminary dilution of the broth with an equal volume of tap water, followed by pH 4.0 precipitation, gave good results. The S I was separated from the relatively large amounts of P (20 to 100 times the amount of S I) by repeated fractional precipitation at pH 4.8 or 5.0 in the presence of sodium acetate. Even at this stage the S I shows little cross-reaction with heterologous sera. Additional P was removed with 0.7 saturated ammonium sulfate. The latter can be removed by dialysis through cellophane and precipitation with 10 per cent barium acetate which in turn is removed with sodium carbonate. The S I is obtained from solution by precipitation with 4 volumes of ethyl alcohol and is pure enough for serological purposes. For analysis the last remnant of P is removed with 5 per cent copper acetate and the S I freed of copper by precipitation with alcohol from acid solution. It contains some inactive polysaccharides which may be precipitated out with 4 to 10 volumes of acetic acid.

Analysis of different samples of S I from different Type I strains gave a nearly constant composition with 4.4 per cent nitrogen, 8.9 per cent phosphorus and 9.6 per cent sodium. On acid hydrolysis 45 per cent reducing sugars, calculated as glucose, were liberated. The optical rotation was $+56.8°$ for the sodium D line. Qualitative tests for protein, pentoses, glycogen and starch were negative. The substance was not inactivated by tryptic digestion. The Molisch test was strongly positive in 1:100,000 solutions.

The S I in the form isolated is not acidic. It is highly soluble and precipitable only with some difficulty; is highly sensitive to acid even at room temperature but is unaffected by nitrous acid; and shows but little sensitivity to alkali.

The chemical evidence presented leads to the belief that the type-specific substance is a sodium salt of a polysaccharide acid composed of a nitrogen-containing sugar and phosphoric acid units. The phosphorus is firmly held in an organic combination. The nature of the nitrogen linkage has not been determined. It has been shown that

the S I is unaffected by tryptic digestion and fails to give a biuret test. If the nitrogen is present as amino nitrogen it is not vitally concerned with the serological activity for the latter is unaffected by treatment of the S I with nitrous acid.

Specimens of S I react in dilutions of 1:8,000,000 with Type I monovalent antimeningococcus serum but not higher than 1:100 with heterologous serum. Cross-absorption tests show the immunological identity of the various specimens of S I which have been obtained.

SUMMARY

The Type I meningococcus specific substance has been isolated and purified. It appears to be a sodium salt of a polysaccharide acid.

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