THE ISOLATION OF THE BLOOD GROUP A SPECIFIC SUBSTANCE FROM COMMERCIAL PEPTONE

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As part of an investigation on the chemical constitution of the capsular polysaccharide of Type III Pneumococcus we have been engaged during the past year in the preparation of large quantities of the specific carbohydrate. This substance has been isolated from autolysates of Type III pneumococci grown in meat infusion broth containing 1 per cent commercial peptone. The method of purification was that described by Heidelberger, Kendall, and Scherp (1). When precipitation of the specific carbohydrate as the copper salt was omitted, it was found that the Type III polysaccharide isolated was contaminated in the gross, in some instances with as much as 60 per cent of a substance having many characteristics of a polysaccharide, serologically inert in Type III antipneumococcus horse serum. It was considered of interest, therefore, to separate and identify this substance and to trace its origin.

The polysaccharide inert in antipneumococcus serum can be separated from the type specific pneumococcus carbohydrate either by precipitating the latter as the insoluble copper or barium salt, or by direct precipitation of the specific substance in the cold at pH 1.5 (2). After removal of the specific bacterial carbohydrate considerable quantities of a polysaccharide rich in glucosamine and having a low specific rotation could be separated by alcoholic precipitation from the supernatant liquid. The similarity in chemical properties of this substance which appears to be polysaccharide in nature, to the blood group A specific substance described by Landsteiner (3) seemed so striking as to suggest that it might be identical with the latter. Serological tests and chemical analysis proved this to be the case. Since it is unlikely that the Type III Pneumococcus itself produced

this substance in such large quantity, we presumed that it came from the culture medium in which the organisms were grown. On concentrating some 50 liters of sterile broth *in vacuo* and working up the concentrate according to the method outlined (1), 3 gm. of the blood group A specific substance were obtained. At this juncture it was thought that the true source of the latter was not the meat infusion but the commercial peptone employed in preparing the medium. This proved to be the case since a substance having the same chemical properties was readily isolated from commercial peptone as described below.

EXPERIMENTAL

Preparation of the Blood Group A Specific Substance from Commercial Peptone.—500 gm. of commercial peptone[1] and 200 gm. of sodium acetate were dissolved in 1500 cc. of warm water and a crude polysaccharide-like material precipitated by the addition of 3750 cc. of 95 per cent ethyl alcohol. After standing overnight the clear supernatant liquid was decanted, the precipitate centrifuged, and then dissolved as completely as possible in 300 cc. of water. Insoluble material was removed by centrifugation and discarded. The substance was again precipitated from the supernatant liquid by the addition of 2.5 volumes of alcohol after first adding 50 gm. of sodium acetate. The precipitate was redissolved and reprecipitated. The crude substance was once more dissolved in 200 cc. of water, 30 gm. of sodium acetate were added, and the pH of the solution adjusted to 4.8 by the addition of 25 per cent acetic acid. The solution was then deproteinized by the method of Sevag (4), using 50 cc. of chloroform and 10 cc. of butyl or amyl alcohol until a portion of the supernatant liquid no longer gave a protein test on saturation with picric acid. In the case of the neopeptone, only two treatments were necessary, whereas the Pfanstiehl peptone required five treatments. The material was finally precipitated from the deproteinized solution by the addition of 2.5 volumes of alcohol. The precipitate was dissolved in 100 cc. of water, passed through a Berkefeld V filter, and the filtrate dialyzed for several days against successive changes of distilled water. The clear colorless solution (faintly yellow in the case of Pfanstiehl peptone) was now poured, with stirring, into 10 volumes of acetone. The precipitated substance was collected on a hardened paper, washed with fresh acetone, and finally dried over sulfuric acid *in vacuo*. 500 gm. of neopeptone yielded 14 gm. of a substance having many of the properties of a polysaccharide. An equal quantity of Pfanstiehl peptone yielded only 4 gm. of crude carbohydrate.

[1] The two preparations of peptone studied were Pfanstiehl peptone, manufactured by the Pfanstiehl Chemical Company, Waukegan, Illinois, and neopeptone, prepared by the Difco Laboratories, Detroit, Michigan.

Chemical Properties of the Group A Specific Substance from Peptone.—The chemical properties of the two preparations, one from neopeptone and the other from Pfanstiehl peptone, are given in Table I, and compared with the properties of the group A specific substance isolated from commercial pepsin and from horse saliva by Landsteiner (3, 5). The material obtained by fractional precipitation with alcohol from a solution of neopeptone was isolated as a white amorphous powder, readily soluble in water to give a viscous faintly opalescent solution which foamed readily on shaking. A 2 per cent solution of the sub-

TABLE I

Analyses of Blood Group A Specific Substance Derived from Various Sources

Blood group A specific substance derived from	$[\alpha]_D$	Ash	C	H	N	Total S	S as SO_4	P	Acetyl	Reducing sugars after hydrolysis
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Horse saliva*	+10°	1.20	44.56	6.91	7.08	1.78	0.05	0.23	9.40	57.6
Fairchild pepsin†	+16°	0.0	46.88	6.62	6.16	0.08	—	0.10	9.95	7.07
Difco neopeptone	+11.5°	1.86	46.68	6.53	5.85	0.46	0.00	0.00	9.56	73.0
Pfanstiehl peptone	+9.6°	4.72	44.86	6.23	5.48	1.96	0.00	0.71	8.90	62.0

All analyses given in this table have been calculated on an ash-free basis of the various preparations.

* Reference 5.

† Reference 3.

stance gave a weakly positive biuret reaction, a weak xanthoproteic reaction, and a negative ninhydrin test. The solution gave a positive Hopkins-Cole test, indicating the presence of tryptophane, and a weak Sakaguchi test for arginine. The Millon test was negative as was the Tollens test for uronic acids and the Bial test for pentose. The solution gave a strongly positive Molisch test, and a strong test for glucosamine (6). The substance was not precipitated from solution by copper sulfate, lead acetate, uranium nitrate, or by barium chloride or hydroxide. Saturation with ammonium sulfate precipitated the material from solution, and high concentrations of tannic acid caused partial precipitation. In solution the substance when

strongly acidified with hydrochloric acid, was precipitated on the addition of phosphotungstic acid. Neither picric, trichloroacetic, nor sulfosalicylic acid caused precipitation. A similar solution of the blood group A substance isolated from Pfanstiehl peptone behaved as did that from neopeptone except that the former gave a positive Tollens test for uronic acid. In their qualitative reactions the two preparations appear to be very closely related. Because of the

TABLE II

Inhibition of Hemolysis of Sheep Cells in Anti-A Rabbit Serum by Blood Group A Specific Substance Derived from Commercial Peptone

Blood group A specific substance derived from	Dilution of substances tested					
	1:1,000,000	1:2,000,000	1:4,000,000	1:8,000,000	1:16,000,000	1:32,000,000
Mucin (Landsteiner)	0	0	W	S	AC	C
Difco neopeptone	0	0	0	Dis.	S	C
Pfanstiehl peptone	0	Dis.	AC	C	C	C

A control test, containing immune serum in dilution employed in above tests, complement and washed sheep cells, gave complete hemolysis.

C = complete hemolysis.
AC = almost complete hemolysis.
S = strong hemolysis.
Dis. = distinct hemolysis.
W = weak hemolysis.

fact that the two materials yield such a high percentage of reducing sugars on hydrolysis, they appear to be essentially carbohydrate in nature. The two substances have nearly the same specific rotations and their chemical analyses agree rather closely, though the Pfanstiehl peptone carbohydrate contains less carbon and more sulfur and phosphorus. Since the latter preparation gives a positive test for uronic acid, and because it has a relatively higher sulfur content, it may possibly be contaminated with chondroitin-sulfuric acid.

Serological Properties of the Blood Group A Specific Substance from Peptone.—Although the two preparations derived from peptone are

probably not pure chemical entities, they appear to be essentially free from contaminating proteins. They possess a marked inhibitory action on the hemolysis of sheep cells when added in high dilution to a system containing group A antiserum and complement, as may be seen from Table II. The titrations of activity of the two preparations were performed by Dr. Landsteiner according to the method described by him (5), and the results compared with those obtained with a partially purified preparation of the group A substance isolated by him from commercial mucin. It may be seen from Table II that the serological activity of the group A substance from neopeptone is greater than is that derived from the Pfanstiehl peptone, a fact which likewise indicates the greater purity of the former substance.

DISCUSSION

The occurrence in commercial peptone of a substance which inhibits the hemolysis of sheep cells by an antiserum to blood group A human erythrocytes has been observed by Schiff (7). Landsteiner (8) has also noted that a large number of commercial peptones are rich in this substance. The isolation and identification of this substance during the preparation of the specific polysaccharide of Type III Pneumococcus is therefore not surprising. Since the majority of peptones are prepared by the peptic hydrolysis of animal proteins, the true source of the blood group A specific substance probably resides in the commercial pepsin (3) used in the manufacturing process. Although the method of isolation of bacterial polysaccharides from cultures grown in peptone broth usually involves a selective precipitation of the specific bacterial carbohydrate with a heavy metal salt, it is not unlikely that some of our preparations have been contaminated with small amounts of the blood group A specific substance.

Witebsky, Neter, and Sobotka (9), using a preparation of Type I pneumococcus polysaccharide prepared in this laboratory, showed that this substance markedly inhibited the hemolysis of sheep cells by blood group A antiserum in the presence of complement. Samples of Types II and III pneumococcus polysaccharides, not prepared in this laboratory, likewise exhibited this property, though to a considerably less degree. The possibility that the Type I pneumococcus polysaccharide possesses this activity by virtue of a similarity in

chemical constitution to the blood group A substance was suggested by these authors, though they were well aware of the possibility that this common activity might be due to an accompanying contaminant. In fact it has recently been shown by Sobotka, Witebsky, Neter, and Schwarz (10) that the Type I pneumococcus polysaccharide, prepared from a medium without peptone and free from blood group A substance, though still retaining the property of inhibiting lysis, was greatly diminished in activity. The specific bacterial polysaccharides isolated in this laboratory are in most instances prepared from peptone broth. The solubility properties of the blood group A substance in aqueous alcohol closely resemble those of the pneumococcus polysaccharides. It is, therefore, necessary to observe great precaution in the fractionation of the pneumococcus polysaccharides from the constituents of the medium in which the microorganisms are grown in order to effect a complete separation.

Some years ago Heidelberger and Kendall (11) described a polysaccharide derived from autolysates of Type IV pneumococci grown in meat infusion broth. This material was found to have serological activity and to be intimately associated with a second serologically inert polysaccharide, a "chitin-like substance." The specific rotation carbon, hydrogen, nitrogen, and acetyl content of the latter substance resembles very closely that of the blood group A specific substance. It is suggested, therefore, that the chitin-like substance described by these investigators may be identical with the blood group A substance derived from the media employed.

Recently there has been described by Bliss (12) a substance in Difco neopeptone which appears to account for certain of the cross serological reactions exhibited by strains of group C streptococci. Preliminary tests conducted by Dr. R. C. Lancefield indicate that the group A specific substance isolated by the author from neopeptone, precipitates in antistreptococcus sera of various groups not only in those of group C streptococci, but in the antisera of other groups as well. It appears, therefore, that the group A specific substance or a material intimately associated with it may be the substance accounting for the phenomena observed by Bliss.

In conclusion it should be pointed out that cognizance of the blood group A specific substance in many brands of commercial peptone

makes it imperative that this substance be eliminated during the fractionation and purification of the constituents of microorganisms grown in a peptone-containing medium before attempting to characterize such fractions either chemically or immunologically. This is true also in the preparation of bacterial polysaccharides of encapsulated microorganisms by methods now commonly employed (1, 4, 13, 14, 15). Whether the blood group A substance is adsorbed upon the bacteria themselves and not readily eluted even by repeated washing of the cells is not as yet known. Nevertheless this possibility should be considered in describing the antigenic and serological relationships of various microorganisms.

SUMMARY

The isolation of the blood group A specific substance from commercial peptone has been described. The chemical and serological properties of the material from that source have been defined.

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