ON GROUP SPECIFIC A SUBSTANCES*

III. The Substance in Commercial Pepsin

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As mentioned in a previous paper (2), in the study of the substances underlying the serological groups of human beings the comparison of preparations from various sources appears desirable. In the present paper there is reported an investigation of the substance present in commercial pepsin which as first shown by Schiff and his colleagues (4–6) gives intense reactions with immune sera for human A erythrocytes. Indeed by this finding Schiff made available a source which permits obtaining without difficulty large amounts of a serologically highly active material. The literature has been reviewed before (2); it should be supplemented by mention of the investigation of Hallauer (3) on water soluble group specific substances from human erythrocytes or stromata, resulting in preparations containing carbohydrate and probably lipoid.

Preparation of the Substance.—A 2 per cent solution of pepsin (Fairchild Bros. and Foster, 1:15,000, made from pig stomachs) in 1 per cent saline, about pH 6 in reaction, was heated to 95° and kept in the steam bath for 10 minutes, then cooled and freed of coagulum by centrifugation. The supernatant fluid was acidified with 50 per cent acetic acid, 6 cc. per 100 cc. solution, and after being brought to 95°C. was heated for 5 minutes in the steam bath; it was then filtered hot through fluted paper. The filtrate was neutralized with NaOH and an equal volume of 95 per cent alcohol was added with stirring. After an hour or so at room temperature, with occasional stirring, the precipitate was removed by filtration.

The active substance was precipitated from the filtrate by adding one-quarter its volume of alcohol. The gummous precipitate either can be collected by centrifugation or can be allowed to settle. The sediment was rubbed up with water, 100 cc. for each 10 gm. of pepsin employed, and insoluble material was removed

* Previous communications (1, 2).

by spinning. To 100 cc. of this solution 1 gm. of sodium acetate and 100 cc. of 95 per cent alcohol were added, and the fluid was passed through paper pulp to yield a perfectly clear filtrate, a new paper pulp bed being used for each 100 cc. portion. The precipitate separating upon addition to the filtrate of one-quarter its volume of alcohol was sedimented by centrifugation, washed with absolute alcohol, and dried.

The substance, which represented about 16 per cent of the starting material, still gave distinct xanthoprotein and ninhydrin reactions. To 100 cc. of a 2 per cent solution of this substance, which dissolves slowly in hot water, were added 1 gm. of sodium acetate and 110 cc. of 95 per cent alcohol. The turbid fluid was filtered through a large Berkefeld V candle, and if necessary refiltered until the solution was clear. Sufficient alcohol was added to raise the alcohol concentration to 57–60 per cent, and the precipitate was sedimented by centrifugation, washed with absolute alcohol and ether, and dried.

The material was then precipitated from 5 per cent solution by addition of glacial acetic acid until flocculation occurred; about 18 volumes were necessary. The precipitate, washed with absolute alcohol, dried, and finely ground, was freed of acetic acid by soaking in 70 per cent alcohol with frequent changes over a period of several days and was dried again. The yield was about 8–10 per cent of the commercial pepsin employed.

The substance dissolves slowly in water, yielding a slightly turbid, viscous solution which becomes clear when neutralized by a trace of alkali. Upon analysis the preparation was found to have: C 46.88 per cent, H 6.62 per cent, N 6.16 per cent; S 0.08 per cent; P 0.10 per cent; acetyl 9.95 per cent; ash, nil; reducing sugar following acid hydrolysis, as glucose, 70.7 per cent (mean value). The optical rotation determined from a 2.5 per cent aqueous solution was $[\alpha]_D^{30^\circ} = +16^\circ$.

At this stage of purification the preparation still gives, in 2 per cent solution, some weakly positive protein tests, as the biuret and xanthoprotein, and a faint turbidity with trichloroacetic acid. A distinct color reaction was obtained with diazotized sulfanilic acid.[1] The substance even in 5 per cent solution failed to give reactions with picric acid, lead acetate, sulfosalicylic acid, and uranyl nitrate, and the Millon and ninhydrin tests also were negative. As seen from the analysis, the preparation is practically free from sulfur.

[1] A similar instance is that reported by Rimington (7) concerning the isolation of the carbohydrate in horse serum where histidine causing a positive diazo reaction could be removed only by prolonged treatment with alkali.

The substance was precipitable by tannic acid, by mercuric chloride, and by ammonium sulfate (4 volumes of saturated aqueous solution). Like the horse saliva substance, when the solution was strongly acidified it gave a heavy precipitate with phosphotungstic acid.

The preparation showed a strongly positive Elson and Morgan reaction. Colorimetrically, the content of amino sugar was determined[2] as 27 per cent; it was identified as glucosamine.

For identification of glucosamine, material was taken at a stage of purification prior to the Berkefeld filtration. It was sealed in a tube with N HCl and hydrolyzed in the steam bath, and the solution evaporated to dryness *in vacuo*; recrystallization was carried out in aqueous alcohol containing a small amount of HCl. From 500 mg. of substance, 140 mg. of hexosamine hydrochloride were obtained. It was converted into the anisal derivative (8), which melted at 165–166°C. (melting point of *p*-methoxybenzylidene *d*-glucosamine); there was no depression of the melting point on mixing with a known sample. Additional confirmation was obtained by determination of the initial and equilibrium rotations.

In testing for mucic acid by HNO_3 oxidation, 250 mg. of substance yielded 26.7 mg. of an insoluble acid melting at 220–221°C.; the presence of galactose was further confirmed by the isolation of the galactose-*o*-tolyl hydrazone from the products of H_2SO_4 hydrolysis.

In Tollens' test for uronic acids, the ether extract was colorless. Bial's orcin test for pentoses was negative, and the present substance in contrast to preparations from other sources gives no violet color (2) nor indeed any characteristic color with the reagent. The preparation gave a faintly positive reaction in the Sakaguchi test.

As regards elementary composition and the demonstration, after hydrolysis, of glucosamine and galactose, as well as the presence of acetyl groups (*cf.* Schiff, Freudenberg), the results reported are in conformity with the findings on the water soluble group specific A preparations from human urine[3] and horse saliva (9, 10, 2). A further point of agreement is the fact that the substance from pepsin, like the A substance in human A saliva and that separated from horse saliva, is decomposed by a carbohydrate-splitting bacterium (11).

[2] We are indebted to Dr. Karl Meyer of Columbia University for this determination.

[3] It may be interesting to note that at an early date a carbohydrate containing nitrogen was described in human urine by Salkowski (16).

Of the substances named that from pepsin is peculiar in that it is precipitated by tannic acid and by mercuric chloride. Also it may be noted that the value for reducing sugar after hydrolysis is distinctly higher.

In hemolysis tests (2) the present substance was about as active as that from horse saliva, while it gave an even stronger inhibition of the isoagglutination of A cells.

A preparation very similar to that from commercial pepsin was obtained from a commercially available crude gastric mucin (pig)[4] which is rich in A substance, by removing insoluble material and fractionating with alcohol.

From the data at hand, it would appear that the A substances from human urine, horse saliva, and pig stomach, and the acetyl polysaccharide of Pneumococcus I which likewise reacts with A immune sera (13), all agree in that they possess amino sugar and galactose, and acetyl groups most probably on the amino group of the hexosamine. Whether both acetylated hexosamine and galactose are essential for the specificity of the reaction remains to be determined. However, in view of the observations of Freudenberg on human urine of group O (and group B) it seems that polysaccharides which contain these constituents need not react with anti-A sera.

Although the A preparations so far examined cannot with certainty be regarded as chemical individuals, yet on account of the differences observed, particularly the apparent differences in serological activity, it is possible that the active substances are not identical, and that the reaction with A immune serum is a group reaction exhibited by various substances which have certain structural similarities, as doubtless is the case with the Forssman antigens.

Substances reacting to a greater or lesser degree with A immune sera seem to be rather widely distributed; such reactions we found with gastric juice of the dog, with rabbit stomach mucosa, with a substance found in commercial beef pepsin (Armour and Company), and with extracts of *N. catarrhalis*,[5] although here the effect of constituents of the medium is not entirely excluded. In this connection the observations on anti-A agglutinins in antibacterial sera (paratyphoid B (14, 15)) may be mentioned.

[4] Gastric mucin, No. 1701-W (Wilson and Co.); see Miller (12).

[5] This culture was supplied through the courtesy of Dr. G. Howard Bailey.

A crude preparation of the A substance in beef pepsin was secured by heat coagulation, alcohol fractionation, and discarding inactive material by changes in hydrogen ion concentration; it was found to have 33 per cent reducing sugar, as glucose, after acid hydrolysis. The material proved to be strongly inhibitory in the customary hemolysis and isoagglutination tests (2) and also showed some degree of inhibition in the isoagglutination of human B cells.

The continuation of the investigation will chiefly involve attempts at further purification and more thorough chemical examination of the active substances.

The experiments were carried out with the assistance of Mr. Robert A. Harte.

SUMMARY

A method of preparation and a preliminary chemical investigation of the substance present in commercial (pig) pepsin which reacts with human A antiserum are presented. The material offers especial advantage in securing in quantity a serologically highly active preparation suitable for further studies. Active preparations were isolated moreover from commercial (pig) gastric mucin. Some other materials showing group specific reactions are mentioned.

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