

## ON THE GROUP SPECIFIC A SUBSTANCE IN HORSE SALIVA. II\*

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In the present paper, additional data will be given on the separation and properties of the substance contained in horse saliva that reacts with group specific immune sera produced by injection of human blood of group A.

The results gathered so far in regard to the substances characterizing the human blood groups appear to prove that their specificity is due to carbohydrate groupings, and thus relate haptens of animal origin to those existing in bacteria. The first experimental evidence along this line was obtained in the examination of the so called Forssman substance which is serologically allied to the group A substance (2). Thus, from horse organs in which Forssman antigen is present, highly active preparations were separated that on hydrolysis yielded fatty acids and a considerably higher percentage of sugar than the known lipoids (3).

The next significant information was gained when the Forssman substances as found in certain bacteria were recovered in polysaccharide preparations and were shown to be intimately connected and probably identical with the specific precipitable substance (4-7). Then there followed studies of Schiff and Brahn (8, 9) on the A substance of human origin and in commercial pepsin which led to carbohydrate preparations with about 5 per cent nitrogen, possibly in an amino sugar, and containing galactose.

The group substances in red blood cells which can be extracted by alcohol represent, according to Schiff, probably a combination of a water soluble form with lipoids, as may be assumed also for the Forssman hapten mentioned above and as has been fairly well demonstrated in the case of several bacterial substances.

Freudenberg and his coworkers (10-12) in the study of the group A substance in human urine obtained it in a polysaccharide preparation containing about 5 per cent N and 9-10 per cent N-acetyl. After hydrolysis 45-50 per cent reducing sugar was found, and galactose and glucosamine were identified. An active substance of similar elementary composition and properties, yielding 48.5 per cent sugar on hydrolysis, was prepared by the writer from horse saliva (1).

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\* First communication (1).

Additional evidence as regards the nature of the specific substance was afforded by enzyme and serological reactions. Thus it was shown that the activity of A haptens is destroyed by Morgan's *Myxobacterium* and *Saccharobacterium ovale* (13), microorganisms which have the characteristic property of decomposing bacterial polysaccharides (14, 15); the specific substance is likewise split by snail enzymes (12). Further, Witebsky, Neter, and Sobotka (16) found that A immune sera react with the acetyl polysaccharide of pneumococci of Type I, a result stressed by the significant observation of Freudenberg and Eichel who reported that they were able to restore the activity of the substance by reacetylation after it had been inactivated by treatment with alkali. Finally papers by Jorpes (17) are to be mentioned in which he suggests the protein nature of the substance from human urine which neutralizes anti-A agglutinins. Possibly his observations could be explained on the assumption that the A hapten occurs in a combination with protein of higher anti-agglutinating activity as compared with the free hapten.

Notwithstanding the importance of the results already achieved it does not seem superfluous to continue these chemical investigations. It is not certain whether the substances prepared represent approximately pure chemical individuals, and, although acetyl groups seem to play an important rôle, the structure essential for the specificity is not yet defined. This is emphasized by the chemical similarity in the substances which were obtained from urine of individuals belonging to groups A, B, or O (11). Furthermore, the examination of A haptens from various sources is of interest because as in the case of Forssman haptens, it is possible and indeed is indicated by experimental results that the sundry substances reacting with A antibodies are not identical.

#### EXPERIMENTAL

Highly active preparations, similar in composition, of the A substance in horse saliva were separated in several ways, *e.g.* preliminary purification could be effected by precipitating the active substance along with mucin with acetic acid, and removing the mucin through adsorption with Fuller's earth, or by removing proteins from the saliva by means of charcoal and kaolin. The following method gave satisfactory results.

Saliva was obtained from a horse salivated by injection of 100 mg. of arecoline hydrobromide in four portions at intervals of 20 minutes. 1 liter portions of saliva were evaporated to dryness on the steam bath, the reaction becoming quite alkaline. The dry residue was taken up in 50 cc. of water; a considerable amount

of insoluble material, after soaking for about 15 minutes, was removed by centrifuging. The supernatant fluid gave a strongly positive picric acid test. After neutralization with 50 per cent acetic acid the solution (40 cc.) was acidified with 6 cc. of 10 per cent acetic acid and the heavy precipitate removed by centrifuging. The supernatant fluid was neutralized with sodium carbonate; it was clear and no longer gave a precipitate with picric acid. The active material was precipitated by 1.5 volumes of acetone. After standing overnight the precipitate was collected and dissolved in 5 cc. of water, and a slight sediment centrifuged off. The solution in 10 cc. portions was brought to a boil with 1 gm. of animal charcoal at a reaction slightly alkaline to phenolphthalein. This adsorption was repeated once, or again with a smaller quantity of charcoal if the xanthoprotein reaction was not yet negative. Addition of 4 volumes of alcohol and some solid sodium acetate caused the formation of a flocculent precipitate which was centrifuged down and redissolved in 2 cc. of water. The procedures following were carried out with 50 cc. of solution representing 50 liters of saliva. Addition of 6 volumes of glacial acetic acid yielded an inert sediment which was removed by spinning; on further addition of 14 volumes of glacial acetic acid the active material precipitated. It was washed with alcohol and dissolved in 20 cc. of water. A sediment formed on adding 100 cc. of glacial acetic acid was discarded, and the substance precipitated by 220 cc. of glacial acetic acid. The material was washed with alcohol, dried, and dissolved in 20 cc. of water to form a viscous solution which no longer gave a xanthoprotein reaction. The solution was diluted with 1.5 volumes of alcohol and 1 gm. of sodium acetate was added. A sediment was separated by spinning. When a total of 2.5 volumes of alcohol had been added a gummy, sticky precipitate formed which was centrifuged down, redissolved, and refractionated with alcohol and sodium acetate. The substance was dissolved in 20 cc. of water and hydrochloric acid was added to N/10 concentration. Addition of 3 volumes of alcohol gave a slight precipitate which was removed and the remainder of the substance was precipitated with an excess of alcohol. To get rid of the last traces of charcoal the solution of the substance in 30 cc. of water was centrifuged at 18,000 R.P.M. After dialysis in cellophane bags, the substance was precipitated by the addition of 4 volumes of alcohol and a small amount of concentrated hydrochloric acid. After washing with alcohol and ether a white powder was obtained, the yield amounting to about 5 mg. per liter of saliva.

The various steps during the separation of the substance were controlled serologically. The activity was tested by means of immune sera hemolytic for sheep blood, prepared by injection into rabbits of human red blood cells of group A. To 0.5 cc. of progressively doubled dilutions of the solutions was added 0.5 cc. of 1:10 normal guinea pig serum and 0.5 cc. of diluted lysin containing 2 hemolytic units, determined after 15 minutes incubation. After 1 hour at 37°C. 1 drop of 50 per cent blood was added. The highest dilution still showing complete inhibition of lysis was taken as the titer; the readings were made when a control tube showed complete hemolysis.

In addition determinations of the inhibitory effect of the substance on the isoagglutination of A blood were made by mixing 1 volume each of a solution of the substance and human serum of group B, and adding 1 volume of a 1 per cent suspension of washed A cells after  $\frac{1}{2}$  hour at room temperature; after a short time the tubes were centrifuged and readings were made.

The substance analyzed as follows (calculated for ash-free substance): C 44.56 per cent; H 6.91 per cent; N 7.08 per cent; total S 1.78 per cent; sulfate S 0.05 per cent; P 0.23 per cent; acetyl<sup>1</sup> 9.40, 9.47 per cent; ash 1.20 per cent; reducing sugar after acid hydrolysis, calculated as glucose, 56.2, 57.6, 58.2 per cent.

The rotation in a 2.5 per cent aqueous solution at 30°C. was  $[\alpha]_D = +10.0^\circ$ . Another preparation under the same conditions gave a specific rotation of  $+10.8^\circ$ .

The substance in 1 per cent solution gave negative reactions with picric acid, lead acetate, basic lead acetate, trichloroacetic acid, sulfosalicylic acid, tannic acid, ammonium sulfate, safranine, mercuric chloride, hydroferrocyanic acid, uranyl nitrate, and picrolonic acid. It gave a weak biuret reaction, negative or very faint xanthoprotein test, negative Millon test and ninhydrin reaction, a faint Sakaguchi test (18), and no coupling with diazonium solutions. There was no formation of lead sulfide on heating with lead acetate and alkali. A strongly acidified solution gave a heavy precipitate with phosphotungstic acid.

Fehling's solution was not reduced before hydrolysis. The Molisch reaction was intense. A sample after acid hydrolysis gave a strongly positive test for hexosamine according to Elson and Morgan's technique (19). The non-hydrolyzed material gave no reaction for hexosamine. The test for galactose by the formation of mucic acid through oxidation with nitric acid yielded 12.8 mg. of an insoluble acid, m.p. 213°, from 100 mg. of substance (obtained by a method of preparation other than the one described in detail). Tollens' reaction (20) for uronic acids was negative (light green ether solution), in Bial's orcin test (21) an intense violet color developed.

In the tests for inhibition of hemolysis the preparation proved to be of high activity, a 1 per cent solution being about 500 times as active

<sup>1</sup>The micro acetyl determinations were made through the kindness of Dr. W. F. Goebel.

as the original saliva. By inhibition of isoagglutination about 1/1000  $\gamma$  of the substance could be detected. Quantitative tests were also made with the technique of hemolysis inhibition used by Freudenberg and his coworkers (22) which yields considerably higher absolute titers than the method mentioned above. In this way 1/2000–1/4000  $\gamma$  of the substance was detectable. Since this value is ten to twenty times greater than that given for the preparations from human urine, comparative tests were made with the saliva substance and samples of the substance from human urine,<sup>2</sup> and in these experiments<sup>3</sup> the difference in activity was confirmed but was found to be considerably greater, likewise in tests of the inhibiting action of the substances on the isoagglutination of A blood.

#### COMMENT

From the data presented it is seen that in chemical composition and properties the horse saliva preparation is essentially, at least, carbohydrate in nature and is very similar to the preparations separated by Freudenberg from human urine. Thus the substance gives negative reactions with almost all protein reagents, yields on hydrolysis a high percentage of reducing sugar, contains about 10 per cent acetyl, and gives positive reactions for glucosamine and galactose. There were, however, the following differences as compared with the urine substance: the negative Tollens' reaction for uronic acids, the failure to give a precipitate with basic lead acetate, and, notably, the considerably higher serological activity. To explain this discrepancy one could assume that the urine substance contains a large amount of serologically inactive polysaccharides—in tests with A immune sera—or else that each of the two materials contains a different substance, both of them reacting with A antibodies, but unlike in reactivity. Preparations, still under investigation, made from commercial pepsin were found to be not less active than the one obtained from horse saliva.

The substance described gave a weak biuret test; whether this is

<sup>2</sup> These preparations were obtained from Professor Freudenberg and Dr. Jorpes to whom the author is greatly indebted.

<sup>3</sup> These tests were in part conducted by Dr. Witebsky with the method of the Institut für Experimentelle Krebsforschung at Heidelberg.

due to an impurity has still to be determined. As to the violet color in the orcin test it is doubtful whether it is connected with the active substance since with other preparations possessing high serological activity the color developed was distinctly less intense. This color reaction was shown also by the substance from urine, which likewise gave a precipitate with phosphotungstic acid in strongly acid solution.

The experiments were carried out with the technical assistance of Mr. Jack Black.

#### SUMMARY

A method is described for the purification of the A substance in horse saliva, and additional observations on the chemical properties of the preparation are reported. The preparation isolated was found to be highly active serologically and it appears to be polysaccharide in nature.

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