Corresponding Member of the Academy of Sciences of the USSR N. K. KOCHETKOV, S. G. KARA-MURZA,

V. A. DEREVITSKAYA

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Abstract

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Corresponding Member of the Academy of Sciences of the USSR N. K. KOCHETKOV, S. G. KARA-MURZA,
V. A. DEREVITSKAYA

THE STRUCTURE OF BLOOD-GROUP SUBSTANCES

HYDROXYLAMINOLYSIS OF BLOOD-GROUP SUBSTANCE A AND THE GENERAL STRUCTURE OF THE BIOPOLYMER

The systematic study, begun in our laboratory, of the structure of blood-group substances—the most important mixed biopolymers ([1]), which perform highly responsible biological functions—requires, above all, the search for methods for cleaving the polymer into large fragments. Such fragmentation would then make it possible to study the structure of these fragments and, by comparing data on the destruction of the biopolymer according to different types of bonds, to reconstruct a picture of the general structure of blood-group substances. Recently, in our laboratory, the destruction of blood-group substance A was carried out by the action of a new proteolytic enzyme isolated from the Asian influenza virus ([2]). The data obtained in this way made it possible to draw certain conclusions about the structure of blood-group substance A, in particular, about the presence in it of a long polysaccharide chain and about the branched character of the biopolymer as a whole. The present work presents data on the study of hydroxylaminolysis of blood-group substance A, which makes it possible to obtain further valuable information about the structure of this biopolymer, in agreement with the previous data ([2]).

Hydroxylaminolysis is a widely used technique in the study of ester and O-peptide bonds in proteins and peptides ([3,4]), and has also been applied in the study of more complex biopolymers (see, for example, ([5])). It is well known that hydroxylamine readily cleaves an ester bond (see, for example, ([6])). In our laboratory this question, as applied to O-aminoacyl derivatives of carbohydrates, was studied on specially synthesized model compounds. At the same time, it is known that under more severe conditions hydroxylamine can also affect certain other bonds ([3,7]). Therefore, under the conditions used by us, the stability of glycosidic and amide bonds toward the action of hydroxylamine was checked. It was shown that simple glycosides, di- and oligosaccharides, as well as N-aminoacyl derivatives of amino sugars, are not cleaved by hydroxylamine. It should, however, be borne in mind that if, under the action of hydroxylamine on so complex a biopolymer as a group substance, even a small number of any bonds other than ester bonds are cleaved, this will not substantially affect the

Fig. 1 diagram

Figure 1: Fig. 1 diagram

results obtained.

Blood-group substance A was isolated from the mucous membranes of pig stomachs by Kabat' s method ([8])*.

Hydroxylamine in the form of the free base was obtained from hydroxylamine hydrochloride by the action of sodium ethylate in absolute alcohol. A 4% solution of the polymer in a 5 M aqueous solution of hydroxylamine was left for 24 hours at room temperature and pH 8.6, after which the solution was lyophilized and dried until the excess hydroxylamine had been removed. The dry residue was dissolved in water and dialyzed against distilled water.

* Data on the isolation and composition of blood-group substance A are being published by us separately in one of the articles in the journal *Izvestiya AN SSSR*.

The diffusate was evaporated in vacuo at 30° to a small volume and lyophilized. The cleavage products that had passed through the membrane amounted, by weight, to about 30% of the initial polymer. The composition of the mixture obtained was then studied by chromatography and electrophoresis on paper. The most complete separation was achieved by using a two-dimensional variant, electrophoresis–chromatography: first electrophoresis was carried out (pyridine –acetate buffer, pH 4.2, 25 V/cm, 3 hours), then the electropherogram was dried and chromatography was performed in the perpendicular direction in the solvent system C_4H_9OH–H_2O–CH_3COOH (4 : 1 : 1) (system A). For different batches of group substance, the number and relative position of these spots varied somewhat, but the general picture, shown in Fig. 1, was preserved on repeated performance of two-dimensional electrophoresis with chromatography. It is seen from Fig. 1 that hydroxylaminolysis of blood group substance A gives a large number of low-molecular fragments, since the number of spots on the two-dimensional electropherogram–chromatogram reaches 20-22. All these spots may be roughly divided into three groups: spots detected by the metaperiodate reagent and ammoniacal silver; spots developed by ninhydrin; and spots developed by ninhydrin and metaperiodate. The first obviously correspond to osazones of monosaccharides or small oligosaccharides, the second to small peptides, and the third group should correspond to substances containing amino acids and monosaccharides and thus constituting small glycopeptides.

Fig. 1. Separation of low-molecular products of hydroxylaminolysis of blood group substance A by electrophoresis with chromatography:
a –spots detected by the metaperiodate reagent; *b* –spots detected by ninhydrin; *c* –spots detected by ninhydrin and metaperiodate reagent.

For obtaining more detailed information on the nature of certain products of

Fig. 2 diagram

Figure 2: Fig. 2 diagram

hydroxylaminolysis, the mixture was further preparatively separated by electrophoresis on paper from the Leningrad factory "Goznak." Electrophoresis was carried out for 3 hours; the electropherogram (Fig. 2) was dried; a narrow strip was cut from it, on which the arrangement of zones was detected with ninhydrin; then the electropherogram was cut into zones and the separated substances were eluted with 25% alcohol. Among the neutral substances, by paper chromatography in the solvent systems C_5H_5N–C_4H_9OH–C_6H_6–H_2O (3 : 5 : 1 : 3) and system A, osazones of galactose, fucose, and N-acetylhexosamines were detected (detection with $AgNO_3$ and $FeCl_3$ upon co-chromatography with authentic samples, the most intense spot corresponding to the osazone of fucose). Upon hydrolysis of the mixture of diffusate products with 0.5 N HCl for 8 hours at 100°, free monosaccharides were detected on the chromatograms (by the action of $AgNO_3$ and metaperiodate): fucose, galactose, small amounts of glucosamine and galactosamine, and a spot of an unknown substance having, in system A, $R_{\text{galactose}} = 2$. Some of the zones containing substances mobile during electrophoresis were subjected to acid hydrolysis under the same conditions,

Fig. 2. Separation of low-molecular products of hydroxylaminolysis of blood group substance A by electrophoresis.

after which monosaccharides were detected in the hydrolysate, which once again indicates that the products of hydroxylaminolysis, along with mono- and oligosaccharides and peptides, contain glycopeptides.

The nondialyzable part of the hydroxylaminolysis products is a high-molecular-weight fragment which, upon fractionation of the cleavage products on a column with Sephadex G-50 (medium), emerges as a fairly narrow peak. This fragment, which does not pass through the membrane during dialysis, is apparently a glycopeptide strongly depleted in amino acids in comparison with the original one. The amino-acid content, determined by the Lowry method ([9]), decreases during hydroxylaminolysis from $\sim$ 16% to 8–9%, i.e., about 50% of all amino acids are split off. Although the qualitative monosaccharide composition of the fragment obtained did not change, it is noteworthy that under the action of hydroxylamine mainly galactose and fucose are split off. Very characteristic is the change during hydroxylaminolysis in the content of certain amino acids and their relative content in the diffusate and dialysate. Since, according to our previous data ([2]), it is very probable that aspartic acid links the carbohydrate and peptide parts of the biopolymer, its content in the glycopeptide should change greatly during hydroxylaminolysis. This indeed occurs. The high-molecular-weight fragment was subjected to hydrolysis with 6*N* HCl for 24 hours at 100°. The hydrolysate was evaporated several times with water in vacuo to remove HCl and was separated by paper electrophoresis in the indicated buffer. It turned out that the high-molecular-weight fragment contained only traces of

Fig. 3. General scheme of the structure of the molecule of the blood-group substance

Figure 3: Fig. 3. General scheme of the structure of the molecule of the blood-group substance

aspartic acid and, consequently, it was almost completely split off under the action of hydroxylamine. This indicates, first, that aspartic acid is linked to the polysaccharide chain by a hydroxamic-ester bond and, apparently, second, that the greater part of it is located at the sites of linkage of peptides with the carbohydrate chain. It is interesting that no significant change in the content of glutamic acid was detected, although it also could have participated in the formation of a hydroxamic-ester bond.

Fig. 3. General scheme of the structure of the molecule of the blood-group substance

The data presented show that the blood-group substance A is apparently based on a fairly high-molecular-weight polysaccharide chain, to which peptide chains are attached, which is fully consistent with the point of view expressed in the preceding communication ([2]).

Analyzing the data obtained by us in the proteolysis ([2]) and hydroxylaminolysis of blood-group substance A, one may put forward initial considerations regarding the general principle of the structure of this important biopolymer. A schematic representation of this structure is given in Fig. 3. Evidently, the basis of the biopolymer is a polysaccharide chain, which plays the role of the molecular skeleton. This central polysaccharide part is almost completely released from the biopolymer during proteolysis ([2]), whereas during hydroxylaminolysis it is separated in the form of a fragment that still bears a considerable number of peptide chains. This central part of the biopolymer molecule includes a large proportion of the residues of *N*-acetylhexosamines, along with relatively

with a smaller amount of galactose and fucose, which is in complete agreement with the data on periodate oxidation of blood-group substances ([10]). The question of the degree of branching of this central polysaccharide chain can be resolved only after a more detailed study of it. The data from proteolysis ([2]) and hydroxylaminolysis indicate that there are several, or even many, peptide chains attached to the polysaccharide skeleton, and thus that the polymer as a whole is branched. Some of these peptide chains are bound to the polysaccharide through aspartic acid residues, and the almost complete cleavage of the latter as a result of hydroxylaminolysis indicates that it is linked to the polysaccharide chain by an ester bond.

The cleavage during proteolysis and hydroxylaminolysis of a considerable number of carbohydrate residues, especially fucose and galactose, indicates that some portion of the monosaccharides is located in the peripheral part of the molecule and is apparently bound to peptide chains. Direct confirmation of this

is the cleavage of small glycopeptides during hydroxylaminolysis. The nature of the linkage of these peripheral carbohydrate residues to the peptides may be clarified, at least in part, by studying the fragments obtained upon proteolysis and hydroxylaminolysis.

The views expressed concerning the general structure of group substances are consistent with certain data on their immunological determinants. As was shown by Morgan and Kabat, terminal oligosaccharides determine the group specificity of these biopolymers (see, for example, ([11])), and, by carrying out selective cleavage of the terminal carbohydrate residues, one can alter the specificity of the polymer, for example, convert group substance A into group substance H, etc. Comparing the literature data with those obtained by us, one may suggest that the specific substances of different blood groups that have the same origin possess a similar polysaccharide skeleton and differ in their external framework.

Institute of the Chemistry of Natural Compounds
Academy of Sciences of the USSR

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Note: Figure translations are in progress. See original paper for figures.