Structure of Human Skeletal Keratosulfate

THE LINKAGE REGION*

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SUMMARY

Keratosulfate from old human rib cartilage (KS II) was fractionated into two fractions on Bio-Gel P-6 resin. Glutamic acid or glutamine, serine, proline, and galactosamine were concentrated in the higher molecular weight fraction, whereas aspartic acid or asparagine was concentrated in the retarded peak. During alkaline treatment the higher molecular weight fraction became polydisperse through breaking of bonds involving N-acetylgalactosamine as well as serine and threonine.

Studies of the action of alkali on KS II in the presence and absence of borohydride have led to the following conclusions: (a) The predominant O-glycosyl group linked to the hydroxyamino acids appears to be N-acetylgalactosamine. (b) A direct Ehrlich's chromogen is produced during the alkaline elimination, which parallels the decrease in seryl and threonyl groups. (c) When KS II or a product of KS II produced by partial hydrolysis with N-acetic acid is treated with alkali in the presence of borohydride at 24°, a new ninhydrin-positive fraction is produced after hydrolysis in HCl. This is presumably derived from a 3-substituted N-acetylgalactosamine.

The methylpentose of KS II was isolated and characterized as fucose by its methylphenylhydrazone.

EXPERIMENTAL PROCEDURE

Preparation and Analysis of Mucopolysaccharides—Procedures for the preparation of mucopolysaccharides and for their analysis have been reported (6, 1). One of the fractions was further purified by digestion with testicular hyaluronidase and refractionation. The preparation had the following analysis: hexosamine, 26.1%; anthrone (as galactose), 28.9%; SO₄, 18.2%; sialic acid (direct Ehrlich), 2.0%; methylpentose, 2.6%; protein (by the Lowry method) 6.0%; molar ratio of galactosamine to glucosamine (by amino acid analyzer) 1:10; total amino acid 851.2 μmoles per g; [α]D° = −12. In some cases the method of Antonopoulos (7) was used to determine ester sulfate.

The methylpentose of KS II was isolated and characterized as fucose by its methylphenylhydrazone.
and Blue Dextran 2000 were obtained from Pharmacia.

Fractions have been numbered Fraction 1, Fraction 2, etc. Sephadex G-25 and Blue Dextran 2000 were obtained from Pharmacia.

Preparation of Glycopeptide Mixture—Keratosulfate was hydrolyzed in batches of 200 mg in 20 ml of N acetic acid at 105° for 4 hours, conditions under which glycosidic bonds are cleaved but amide bonds, including N-acetyl, are not hydrolyzed. Each batch was concentrated repeatedly with water on a flash evaporator and lyophilized, to yield 185 mg. The fraction was then dissolved in 2 ml of N acetic acid and was chromatographed on a column, 1.2 x 54 cm, of Sephadex G-25 equilibrated with N acetic acid at 4 ml per hour. A gray band appeared in the first 25 ml and was taken as the glycopeptide mixture (Fraction A). The column was further eluted with 200 ml of N acetic acid (Fraction B). From 1 g of KS II 325 mg of Fraction A and 508 mg of Fraction B were obtained (92% yield). On paper in Solvent 1 for 18 hours all the material in Fraction A staining with aniline hydrogen phthalate remained at the origin, but with ninhydrin there appeared a trace amount of an additional spot in the glucosamine area. In order to permit conclusion of the presence of neutral sugars the glycopeptide mixture was hydrolyzed at 10 mg per ml in N HSO4 for 8 hours at 100°. The hydrolysate was neutralized with BaCO3 and 500 mg of the supernatant were chromatographed in Solvent 3 and stained with aniline hydrogen phthalate.

Isolation of Fucose—Fraction B was put onto a carbon Celite (1:1) column. The water eluate was resolved by preparative chromatography on washed Whatman No. 3MM in Solvent 1. One of the products was characterized as fucose by its methylphenylhydrazone (11), and the rotation was measured in a Bendix automatic polarimeter. It was also reduced with NaBH4 and the product was chromatographed beside known fucitol.

Preparation of N-Acetylchondrosine—Chondrosine was prepared according to the method of Davidson and Meyer (12), except that N acetic acid was necessary to elute chondrosine from the column. The isolated chondrosine was crystallized, \([\alpha]_D^{25} = 43 (1\% \text{ in } 0.05 \text{ N HCl})\), and gave one spot with a tail when chromatographed in Solvent 1, with only faint traces of galactosamine and a trace of material at the origin. The chondrosine was N-acetylated by treating it with magnetic stirring at room temperature with acetic anhydride in the presence of methanol and an excess of BaCO3 at pH 5. Addition of acetic anhydride and BaCO3 was repeated four times. The completeness of the reaction was tested by spotting the product with ninhydrin on paper and by electrophoresis at pH 5.6, the approximate isoelectric point of chondrosine. At the end of the reaction no product remained at the origin. Barium was removed in part as BaSO4 by titrating to pH 3.0 with N HSO4. Since some of the barium sulfate remained in colloidal suspension after centrifugation, it was removed by passing the solution through Bio-Rad AG 50 W-X8 (H+), 100 to 200 mesh. The yield was quantitative. After hydrolysis by 4 N HCl galactosamine, ammonia, and only traces of other ninhydrin-positive peaks appeared in the amino acid analyzer.

N-Acetylation of chondrosine by another method gave low yields. The tetrasaccharides from hyaluronic acid and chondroitin sulfate were obtained by testicular hyaluronidase digestion of the respective polymers. The tetrasaccharide from hyaluronic acid was crystalline and had been previously well characterized in this laboratory (13).

Effect of Alkali on Keratosulfate—Keratosulfate (201 mg) was chromatographed on a column, 1.46 x 105 cm, of Bio-Gel P-6, 50 to 100 mesh. Three fractions were combined and lyophilized on the basis of hexose screening. The leading fraction, which appeared mostly in the void volume, presumably the material with the higher molecular weight, was then permitted to react with 0.48 N NaOH at a concentration of 10 mg per ml. Half of the sample was neutralized with dry Dowex 50 (H+) immediately after mixing with alkali. The other half was flushed with nitrogen, stopped, incubated at 24° in a constant temperature bath for 48 hours, and then neutralized. In both cases the resin was removed by filtration, and the filtrate and washes were concentrated on a flash evaporator and lyophilized. Both experimental and control samples were sealed in a vacuum and hydrolyzed for amino acid analyses (6 N HCl, 22 hours, 110°) and separately for hexosamines (4 N HCl, 12 hours, 100°). Both the experimental sample and the control (15 mg each) were chromatographed separately on the same F-4 column from which the starting material was obtained. The fractions were screened by the anthrone method for hexoses (10) and by a modified Reissig, Strominger, and Leloir test (14) for preformed chromogen (15). The modification consisted in not heating the samples after the addition of potassium tetraborate. As standards, N-acetylgalactosamine and N-acetylglucosamine were carried through the standard test as described (14). An absorption spectrum between 450 and 650 mp of the chromogen formed from keratosulfate was identical with that of the product from the standard N-acetylgalactosamine.

The formation of chromogen with time was investigated for keratosulfate, keratosulfate glycopeptides, N-acetylcchondrosine, N-acetylgalactosaminose, and N-acetylgalactosamine. The reaction was carried out in 0.48 N NaOH in a water bath regulated to 24° or in an ice bath at 0°. Keratosulfate and the glycopeptide mixture were allowed to react at a concentration of 10 mg per ml and the others at 1 mg per ml. Immediately after mixing, zero time samples were taken and frozen. For the longer reaction times the tubes were flushed with nitrogen and tightly stoppered after each sample was taken and frozen. When studying the relationship of formation of chromogen with the disappearance of serine and threonine, samples were taken for amino acid analysis at the same time as for color development. These were immediately adjusted to 2 ml of 6 N HCl and were frozen until all samples were taken, whereupon they were hydrolyzed simultaneously. Before the glycopeptide mixture was used in the chromogen experiments it was first treated (at a concentration of 20 mg per ml) with NaBH4 for 20 min at 24° to reduce any reducing groups formed during the acid hydrolysis. It was adjusted to pH 2 with dry Dowex 50 (H+). The resin was removed by filtration, and the filtrate and washes were taken to dryness on a flash evaporator and treated four times with absolute methanol to remove borate. The reduced sample was lyophilized.

Formation of Unknown (Peak 5)—In the presence of NaBH4 during the alkaline elimination reaction an unknown ninhydrin-positive peak (Peak 3) was produced, which appeared in the
A mixture was treated at a concentration of 20 mg per ml with the destruction of hydroxyamino acids, the glycopeptide mixture was treated at 20 min at 24° with NaBH₄. An equal volume of 0.972 N NaOH was added and the reaction was continued for 42 hours. In order to study the nature of the unknown peak, N-acetylhydroxamine and enzymatically produced tetrasaccharide from hyaluronate, at a concentration of 10 mg per ml, were allowed to react with 0.5 M NaBH₄ and 0.48 N NaOH simultaneously for 20 min at 24°. Enzymatically produced tetrasaccharide from chondroitin 6-sulfate was treated for 20 min at 24° with NaBH₄ in the absence of alkali. All samples were neutralized with dry Dowex 50 (H⁺), filtered, dried, and treated with methanol to remove borate. Samples were hydrolyzed for 22 hours at 110° in 2 ml of 6 N HCl, evaporated to dryness in the presence of NaOH, and dissolved in pH 2.2 buffer. The chromatograms were developed in pH 5.28 buffer at 40 ml per hour on 57-cm columns of resin in the Spinco amino acid analyzer.

In an attempt to correlate the rate of formation of Peak 3 with the destruction of hydroxyamino acids, the glycopeptide mixture was treated at a concentration of 20 mg per ml with NaBH₄ for 20 min. An equal volume of 0.972 N NaOH was added, and, immediately after mixing, a zero time sample (0.5 ml) was taken. The main body of the solution was flushed with nitrogen, stoppered, and set in a 25° water bath. Samples (0.5 ml) were taken at various times up to 72 hours. To each, 1 drop of caprylic alcohol, 0.5 ml of water, and 1 ml of concentrated HCl were added, and the flask was flushed with nitrogen, stoppered, and set in a 25° water bath. Samples (0.5 ml) were taken for amino acid analysis. The main body of the solution was flushed with nitrogen, stoppered, and set in a 25° water bath. Samples (0.5 ml) were taken at various times up to 72 hours. To each, 1 drop of caprylic alcohol, 0.5 ml of water, and 1 ml of concentrated HCl were added. At the end of the experiment all samples were sealed in a vacuum and hydrolyzed for 22 hours at 110°. Aliquots were run in the analyzer for neutral and acidic amino acids and for hexosamine and Peak 3 in pH 5.28 buffer.

Use of Tritiated Sodium Borohydride—The sodium borohydride-T was obtained from Nuclear-Chicago, 102 mCi per mmole. Sodium borohydride-T was obtained from Metal Hydrides, Inc., Beverly, Massachusetts.

KS II (400 mg) was dissolved in 20 ml of water. Sodium borohydride (767 mg) was added and the solution was left for 20 min at 24° in a constant temperature bath. At this point two (0.5-ml) samples were taken for amino acid analysis. NaOH (19 ml of 0.972 N) containing 10 mCi of NaBT, was added, and the flask was flushed with nitrogen, loosely stoppered, and placed in the constant temperature bath (24°) for 40 hours. Then a few drops of caprylic alcohol were added, and two (1.0-ml) samples were taken for amino acid analysis. The flask containing the remainder of the sample was placed in an ice bath, and dry Dowex 50, (H⁺) was added until the pH was 3.5 to 4. The resin was removed by filtration, and the filtrate and washes were treated repeatedly with absolute methanol in a flash evaporator and treated with methanol to remove borate. The sample was lyophilized. The entire sample was chromatographed sequentially on Bio-Gel resins P-2, P-4, and P-6, the leading fraction from P-2 being put onto P-4 and the leading fraction from P-4 being put onto P-6. As control, untreated KS equal in amount to the sample of treated KS was chromatographed on the respective column. The columns were screened for hexose as described above, and for radioactivity in Bray's solution (16) in a Packard scintillation counter.

RESULTS AND DISCUSSION

Keratosulfate from old human rib cartilage (KS II) is polysaccharide on Bio-Gel resins P-4, P-6, and P-10. When 50 mg of KS II were chromatographed on Bio-Gel P-10, only 4% of the recovered material was excluded (Fig. 1, Fraction 1), indicating an apparent molecular weight of <10,000 for most of the material. When 172 mg were chromatographed on Bio-Gel P-4, 67% of the material was in a broad peak which included the excluded volume (Fig. 2, Fraction 4) and behaved, therefore, as if it had a molecular weight ≥3,600. On a column of the P-6 resin the broad peak was separated into two fractions (Fraction 3). When 201 mg of KS II were chromatographed (Fig. 3), 21% of the recovered material appeared in the partially excluded peak. Of material partially excluded by P-4 (Fraction 4), 38% was in the leading peak from P-6 (Fig. 3). Therefore, 21 to 38% of KS II has an apparent molecular weight of 4,500 to 10,000; for a large part

It is not known whether these polysaccharides behave in the same way as the proteins used for standardizing the resins.
of the remainder the apparent molecular weight is between 3,600 and 4,500 (Fig. 2).

In general, the higher molecular weight fractions from the P-6 gel contained increased quantities of amino acids. The leading peaks in both experiments (Fractions 8 and 11) concentrated serine, glutamic acid (or glutamine), and proline but had a decreased amount of aspartic acid (or asparagine) (Fig. 4). The ratio of galactosamine to glucosamine was increased in the larger molecules: Fraction 11 had a ratio of 1 galactosamine to 7 glucoseamine; Fraction 12, 1:11; and Fraction 13, 1:14. This fact is of special interest since, as will be shown below, N-acetylgalactosamine is in the alkali-labile linkage region of the hydroxyamino acids.

Fraction 8, the higher molecular weight fraction from P-6 (Fig. 3), was treated at 24° with 0.48 N NaOH, and aliquots were taken at zero time and at 48 hours. When each was rechromatographed (after neutralization and lyophilization on the same P-6 column, the result seen in Fig. 5 was obtained. The alkaline treatment obviously caused a spread to lower molecular weight values. Fractions from both chromatograms were also screened for chromogen (15) in the modified N-acetylhexosamine test (see "Experimental Procedure"). The zero time fraction showed no chromogen formation. The

![CHROMATOGRAPHY OF KS II ON BIO-GEL P-6](image)

**Fig. 3.** Chromatography of 201 mg of KS II (O—O) on a column, 1.5 X 105 cm, of Bio-Gel P-6. Fractions were combined as follows: Fraction 8, 53 to 80 ml; Fraction 9, 81 to 101 ml; Fraction 10, 102 to 225 ml. BD indicates a Blue Dextran 2000 peak.

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount lost (μmole/g)</th>
<th>Loss of original (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine</td>
<td>89</td>
<td>87</td>
</tr>
<tr>
<td>Serine</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>88</td>
<td>8</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>101</td>
<td>72</td>
</tr>
</tbody>
</table>

48-hour sample showed chromogen in both the excluded and the retarded fractions (Fig. 5). Therefore, some of the N-acetylhexasamines involved in the alkali-labile bonds remained attached to longer chains and did not occur only as side chains, as Mathews and Cifonelli (2) have suggested. When the samples were analyzed in the amino acid analyzer for hexosamines (4 N HCl hydrolysis for 12 hours, 100°) and for neutral and acidic amino acids (6 N HCl hydrolysis, 22 hours, 110°), the losses seen in Table I were observed in the alkali-treated sample. Whereas the 8% loss of glucosamine may not be significant, the loss of galactosamine was real. This loss of galactosamine after alkali treatment was also seen in other experiments in which the KS II was hydrolyzed to obtain amino acid data, although in the latter case the destruction by the 6 N HCl was too excessive to give an accurate hexosamine value. The preferential loss of galactosamine was also reported by Mathews and Cifonelli (2).
The second indication of the involvement of the N-acetyl-
galactosamine in an alkali-labile linkage is the formation from it of a new basic amino compound in the presence of alkali and borohydride. The same compound results from similar treatment of KS, KS-glycopeptides, N-acetylchondrosine, and a tetrasaccharide from chondroitin 6-sulfate but not from the same treatment of a tetrasaccharide from hyaluronic acid (containing N-acetylglucosamine). This is illustrated for KS-glycopeptides, N-acetylchondrosine, and the tetrasaccharide from hyaluronic acid in Fig. 6, where the new compound is designated Peak 3.

Since the new basic amino compound appeared to be derived from N-acetylgalactosamine but not from N-acetylglucosamine, studies were undertaken to elucidate the mechanism of its formation. The compound survived strong acid hydrolysis (6 n HCl, 110°, 22 hours), indicating it to be a saturated compound. It was, therefore, postulated to be saturated, reduced Kuhn's chromogen I (15). One formulation of this chromogen (17), with the double bond between C-2 and C-3, presents a system analogous to that in dehydroalanyl and α-amino-α-amino-κ-oxotetrahydropeptides. It has been reported (18, 19) that these dehydro compounds become saturated in the presence of borohydride, as confirmed in this laboratory. One possible mechanism for this reaction is by way of enolization to a C=C bond which is easily reduced by borohydride. However, a recent report (20) related that borohydride also reduces C=C double bonds in several situations where there is a delocalization of electrons by conjugation with ester, cyanide, phenyl, or amide leading to the formation of an electrophilic center which is attacked by the borohydride ion. Indeed, the formation of Peak 3 (Fig. 6) depended on the presence of borohydride. When N-acetylchondrosine was treated at a concentration of 10 mg per ml with 0.48 n NaOH without borohydride for only 20 min at room temperature, chromogen was formed, as was evidenced by the appearance of a purple spot when 60 µg of the reaction mixture on paper were sprayed with p-dimethylaminobenzaldehyde-HCl. The solution was then adjusted to 6 n HCl in a volume of 2 ml and was hydrolyzed for 22 hours at 110°. On the analyzer chromatogram it was observed that nearly half of the hexosamine had reacted, but only a trace of material was seen in the region of Peak 3, in contrast to that seen when the hydrolysis with n NaOH for 20 min at 24°. The samples were neutralized with dry Dowex 50 (H+) filtered, dried, and sampled. The samples were hydrolyzed for 22 hours at 110° in 2 ml of 6 n HCl, evaporated to dryness in the presence of NaOH, and dissolved in pH 2.2 buffer. The chromatograms were developed in pH 5.25 buffer at 40 ml per hour on 37-cm columns of the Spinco amino acid analyzer.
borohydride was present (Fig. 6). Kabat et al. (19) observed on the Technicon analyzer a new basic compound near valine after treatment of blood group substances with alkaline borohydride, but not in the absence of borohydride. This unknown may be identical with Peak 3 described here, but the two have not been compared directly.

**Linkages to Hydroxyamino Acids**—No pentoses were detected in a hydrolysate of a KS II glycopeptide mixture which on the basis of serine and threonine destroyed by alkalii would have allowed detection, whereas both xylose and galactose were detected in a hydrolysate of chondroitin sulfate glycopeptides (6, 21). Therefore, it was concluded that the monosaccharide linked to serine is different from that in chondroitin sulfate. Another possibility investigated was that the hydroxyl groups of serine or threonine (or both) were esterified with sulfate. In the borotritiide experiment (see below) the total tritiated product was chromatographed on Bio-Gel P-2. The sulfate content of the larger molecular weight fractions was unchanged from that of the starting material. Also, the amount of sulfate in the fraction where inorganic sulfate should have been was too little to account for the destruction by alkalii of either hydroxyamino acid. For these reasons and because an N-acetylgalactosamine reducing group is liberated by NaOH, as evidenced by the formation of chromogen and also Peak 3 (in the presence of alkalii borohydride), the N-acetylgalactosamine was postulated to be linked to one or both of the hydroxyamino acids.

In order to study the possible involvement of N-acetylgalactosamine in the linkages, the formation of chromogen

Peak 3 is not talosaminitol, which could possibly arise by epimerization from galactosamine. Talosaminitol appears in the chromatogram close to galactosaminitol.

In the acid hydrolysate of KS II one unknown spot was seen which stains brown with aniline hydrogen phthalate and has an R_d value of 1.3 in Solvent 3 (mannotose-arabinose area) and R_g value of 1.4 in Solvent 1. Further experiments established this unknown as mannose, present in both KS I and KS II in about 2% of the carbohydrate (V. P. Bhavanandan and K. Meyer, unpublished observation).

![Graph: Concentration of amino acids and galactosamine in the larger glycopeptides obtained by chromatography on Sephadex G-25 of an n-acetic acid hydrolysate of KS II. The open bar represents untreated KS II; the solid bar represents the glycopeptide mixture.](http://www.jbc.org/)

**Fig. 7.** Concentration of amino acids and galactosamine in the larger glycopeptides obtained by chromatography on Sephadex G-25 of an n-acetic acid hydrolysate of KS II. The open bar represents untreated KS II; the solid bar represents the glycopeptide mixture.

As can be seen in Fig. 8, where it can be seen that chromogen formation occurs at the same rate as the destruction of the hydroxyamino acids. The results are shown in Fig. 8, where the rate of formation of Kuhn's chromogen with the rate of destruction of serine and threonine when a reduced KS II-glycopeptide mixture was treated with alkalii at 24°.

![Graph: Comparison of the rate of formation of Kuhn's chromogen with the rate of destruction of serine and threonine.](http://www.jbc.org/)

**Fig. 8.** Comparison of the rate of formation of Kuhn's chromogen with the rate of destruction of serine and threonine. The data are not accurate enough to distinguish between the rates for serine and threonine. At the same alkalii concentration and temperature, no color was given by N-acetylglactosamine (linked in position 4) up to 4 hours; 3% was given by N-acetylgalactosamine and 1% by N-acetylgalactosamine, whereas N-acetylgalactosamine gave 34% of that given in the Reissig, Strominger, Leloir test (14). The reaction with N-acetylgalactosamine is due to the greater facility with which an -OR group at C-3 (the O-glucuronosyl group) is eliminated relative to an -OH group. The positive reaction with the glycopeptide mixture, therefore, strongly suggests the appearance during alkalii treatment of an N-acetylgalactosamine reducing group which carries a substituent at C-3. The fact that the higher molecular weight fractions after 48 hours in 0.48 N NaOH also show chromogen formation indicated that the chromogen was still linked even after elimination of the C-3 substituent. If the N-acetylgalactosamine is present in the pyranose form, the only possible position for the alkalii-stable linkage is C-6, since if it carried a substituent at C-4, little or no chromogen would be formed. From these considerations it is postulated that at least some of the N-acetylgalactosamine residues in KS II are linked in three positions; C-1, C-3, and C-6. Since, after elimination with alkalii borotritiide, the substituent at C-3 gave no radioactive spot proportional to the amount of chromogen formed, it cannot be a short chain carbohydrate.

Since Peak 3 (Fig. 6) was postulated to result by the reduction and saturation of the chromogen formed from the N-acetylgalactosamine residues, a time study at 24° in 0.48 N NaOH was made in the presence of 0.5 M borohydride. As can be seen in Fig. 9, the rate of formation of Peak 3 was parallel with the rate

When boiled with tetraborate, N-acetylgalactosamine gave a value 1.76 times the theoretical, presumably by elimination of the glucuronic acid moiety linked in position 3.

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of destruction of the hydroxyamino acids, at least up to 30 hours. It appears that the reduction of the chromogen by borohydride is slower than its formation.

The rate study on chromogen formation was repeated at 0°, where the actual destruction of hexosamines was also studied; i.e. a separate sample at each time point was hydrolyzed in 4 N HCl for 12 hours at 100° and run on the amino acid analyzer in pH 5.28 buffer. The surprising result shown in Fig. 10 was obtained. Even though serine and threonine were destroyed, no hexosamines were destroyed, and no chromogen was formed up to 80 hours. The formation of chromogen was repeated, the samples being held at 24° for 13 min just before the addition of borate (conditions under which N-acetylgalactosamine gave maximum color). Again no chromogen was detected in the modified N-acetylgalactosamine test. Therefore, the substituent on C-3 of the N-acetylgalactosamine must be a weaker leaving group than the glucuronyl moiety of N-acetylcysteine because N-acetylgalactosamine immediately gave color even at 0°.

N-Acetylgalactosamine has been found linked to serine in ovine submaxillary mucin (22). The situation in this complex is simpler than in KS II because the N-acetylgalactosamine is part of a disaccharide and carries a C-6 substituent. In the case of the ABH(0) blood group glycoproteins (9) and the NN and Me-Vg antigens (23), the dilemma is similar to that described here for KS II. N-Acetylgalactosamine is one of the components destroyed by alkali at room temperature, as are serine and threonine, and the evidence strongly suggests it to be linked to the hydroxyamino acids. Whereas in blood group substances evidence for peeling was observed, in KS II the quantity of small radioactive fragments was insignificant. This finding appears to exclude the formation of chromogen by a peeling reaction.

Fig. 10. Lack of destruction of galactosamine and, therefore, lack of formation of Kuhn's chromogen when reduced KS II-glycopeptide mixture was treated with alkaline borohydride at 0°. Since the glycopeptide mixture was previously reduced with borohydride, the galactosamine values include galactosaminitol and glucosaminitol, which appear at the same place on the chromatogram.

The isolation of the intact linkage region including substituent 3 on the N-acetylgalactosamine group remains to be accomplished.

Characterization of Fucose—The methylphenylhydrazone had a melting point (uncorrected) of 184–187°, which was unchanged when mixed with the methylphenylhydrazone of crystalline L-fucose (Mann) prepared in the same way. The melting point was high compared to 173° reported by Black et al. (11). However, there is another report in the literature of this derivative having a melting point and mixed melting point of 182–184° (24). The isolated fucose was not crystalline and it appeared to be contaminated with material from the paper. The optical rotation was negative and corresponded with the analysis of 73% methyl pentose. The reduced compound had the same Rf as fucitol in Solvent 3.

Incorporation of Tritium into KS II—No significant destruction of serine and threonine occurred in the 20-min prior treatment with unlabeled borohydride. Therefore, any sugar linked glycosidically to either of them should have been reduced during the 48 hours in 0.48 N NaOH-0.5 N NaBT4. A variety of labeled compounds resulted from such a borotritiide experiment, and work is under way to characterize them. Tritium incorporated into the reaction mixture on the basis of serine and threonine destroyed was 7 times the calculated value, assuming that all the dehydroalanyl and α-amincrotonyl peptides were reduced. This is in keeping with the supposition that Peak 3 is a reduced saturated chromogen, since the saturation of the double bond would account for extra incorporation. The variety of products would also be expected in a reaction sequence involving chromogen formation and saturation.

As stated in the introduction, KS II is heterogeneous and polydisperse. It is not known at present whether the conclusions as to the structure of such compounds are justified, since they necessarily are based on statistical data, assuming identity in the

* A characteristic crystalline derivative of L-fucose has been obtained after methylation of KS II (V. P. Bhavanandan and K. Meyer, J. Biol. Chem., in press).
essential structures of the different chains. Since the starting material consists of a pool of cartilage from more than a dozen individuals, it might be suspected that some of the heterogeneity is due to individual genetic variation. However, this does not seem to be the case since KS II from one individual with Marfan’s syndrome showed the same behavior with respect to loss of serine, threonine, and galactosamine in alkali and formation of Peak 3 in alkaline borohydride.

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