The Structure of a Sulfated Glycoprotein of Chick Allantoic Fluid*

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SUMMARY

The molecular structure of a sulfated glycoprotein from chick chorioallantoic fluid has been investigated. The effect of alkali and alkali-borohydride under various conditions has been shown to lead to quantitatively and qualitatively different products of elimination and reduction. The carbohydrate chains of the products, by gel filtration, were heterogeneous, ranging from an average molecular weight of 1,100 to 2,600. The purified glycoprotein (mol wt 26,000 by gel filtration) contained a series of fatty acids bound to carbohydrate in ester linkage. The sialic acid of the glycoprotein was characterized as N-acetylneuraminic acid.

It has been shown that the host factor of influenza virus grown in chick embryos is an integral part of the viral particle (1, 2). The HF of influenza virus was isolated from the supernatant fluid obtained in the commercial preparation of influenza vaccine by Haukenes et al. (3). The preparation of chorioallantoic fluid saccharides of our laboratory (4, 5) differed significantly in some respects from the HF. The final yield of our preparation, in dry weight per liter of fluid, was 6 to 7 times greater than that reported by the Norwegian workers. The sialic acid in our preparation was much higher than in their product, and immunologically the HF had a higher potency than the CAFS as measured in both the capacity to block inhibition of influenza virus hemagglutination and to precipitate antibody to HF and CAFS (5) of noninfected chicks. It was also shown previously (4) that the carbohydrate chains were linked to a peptide backbone via galactosamine to threonine and serine by 0-glycosidic linkages. Mild acid hydrolysis followed by paper chromatography showed, among other spots, N-acetylglucosamine 6-sulfate as one of the main spots (4). This report deals with more detailed investigation of the CAFS with particular attention to the heterogeneity and size of the carbohydrate chains. One of the surprising findings of this investigation was that the CAFS contained several fatty acids covalently linked to the carbohydrates as an integral part of the macromolecule.

Preparation of Glycoprotein from Chick Allantoic Fluid Injected with Influenza Virus (Japan 170/62A)—The method was essentially similar to those used in this laboratory for isolation of keratan sulfate (6). After removal of the virus, the supernatant was precipitated with acetone. The acetone powder (300 g) was suspended in 2 liters of distilled water and dialyzed against running tap water for 4 days, followed by digestion with activated papain for 2 days. After alcohol precipitation, the product was further purified by gel filtration on a Bio-Gel P-100 column (2.50 X 230 cm) with 0.5 M NaCl as eluent (yield 1.38 mg per g of acetone powder). In another preparation, the CAFS was digested further with pronase for 3 days with no apparent change in the size of the molecule and amino acid composition. The products were homogeneous in ion exchange chromatography on a DEAE-Sephadex column in pH 6.2 acetate buffer with a linear gradient of 0.3 to 1.5 M NaCl, electrophoresis on Sephaphore at pH 5.0 and 1.8, and gel filtration chromatography on Sephadex column (1.04 X 90 cm) with 0.025 M NaCl solution as eluant, as well as on a Bio-Gel P-200 column (1.70 X 230 cm) with 0.5 M NaCl as eluant. Table I shows some analyses of the CAFS.

Analytical Procedures—Hexosamine and reduced Kuhn's chromogen (6) were determined on an improved method (7) on the amino acid analyzer and by gas-liquid chromatography. For amino acid analysis, samples were hydrolyzed in 6 M HCl at 110° for 22 hours and for hexosamine by acid hydrolysis in 4 M HCl at 100° for 18 hours, both in a nitrogen atmosphere. Descending paper chromatography on Whatman No. 1 paper was carried out in the following solvent systems (in volume ratios): Solvent A, ethanol-acetic acid-water, 6:3:2; Solvent B, 1-butanol-acetic acid-water 4:1:5; Solvent C, 1-butanol-pyridine-water, 6:4:3. Sugars were detected on paper chromatograms with alkaline silver nitrate reagent (8).

Gas-liquid chromatography was carried out on a Hewlett-Packard No. 5750 instrument with flame ionization detector. For compositional analysis of the glycoprotein (9, 10) a 6-foot stainless steel column containing 3% (w/w) ECNSS-M on Gas-chrom Q (100 to 200 mesh, Applied Science Laboratories, Inc.)

1 The abbreviations used are: HF, host factor; CAFS, chorioallantoic fluid saccharide.

2 We are greatly indebted to the Eli Lilly Company of Indianapolis for the supply of the acetone powder of chick allantoic fluid.

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MATERIAL AND METHODS
was used with a gas flow rate of 70 ml of helium per min. Neutral sugar alditol acetates were analyzed isothermally at 180°. Amino sugar alditol acetates were determined by temperature programming from 180° to 220° at 2° per min with postinjection time of 10 min. Long chain fatty acid esters were analyzed with a 6-foot stainless steel column containing 3% SE-30 on Gas-chrom Q and programmed from 120° to 220° at 4° per min at a gas flow rate of 70 ml per min (11). Short chain fatty acid esters were analyzed essentially as described by Nagai and Watanabe (12) on a 6-foot stainless steel column (5/8 inch) packed with 17% FFAP and 2% phosphoric acid on non-acid-washed 60/80 mesh Chromosorb (Applied Science Laboratories, Inc.) at 120° with a helium gas flow rate of 30 ml per min.

**Estimation of Molecular Weight of Intact CAFS and its Alkaline-Borohydride-treated Products by Gel Filtration—**For determination of the molecular weight of intact CAFS, elution diagrams of mucopolysaccharides of known molecular weights were prepared with a Sephadex G-200 column (1.04 x 90 cm) (13). Some of the standard mucopolysaccharides, whose molecular weights were determined by a sedimentation method, were kindly supplied by Dr. M. B. Mathews. The mucopolysaccharide markers were chondroitin sulfate A (mol wt 12,000), a mixture of chondroitin sulfate A and C (mol wt 22,000), and human cartilage keratan sulfate (mol wt 9,500). The molecular weight of chondroitin sulfate A (22,000) was determined in our laboratory by the ratio of xylose to galactosamine. A small column (1.06 x 90 cm) of Sephadex G-200 was packed after swelling the gel at 70° for 5 hours (according to Pharmacia Technical Data Sheet No. 11) in 0.025 N NaCl solution. The packing was supported by glass wool and a layer of glass beads. The column was stabilized by eluting with 0.025 N NaCl solution for 2 days. Standards and samples were applied and eluted with the same solution. The elution pattern of CAFS and calibration curve (inset) is shown in Fig. 4.

For estimation of molecular weights of alkaline borohydride treated CAFS, calibrated columns of Sephadex G-25 (fine, 1.40 x 116 cm) and Sephadex G-50 (fine, 1.00 x 120 cm) were used. Gels were swelled and packed as indicated in the Pharmacia Fine Chemical literature. The qualities of the packing, and the void volumes as well as the salt regions were ascertained with blue dextran 2000 and NaSO₄. The columns were stabilized by eluting for 24 hours with 0.1 N NaCl solution for the Sephadex G-50 column and 0.5 N NaCl solution for the Sephadex G-25 column, and they were eluted continuously with the respective salt solutions when not in use. Calibration standards were prepared by digestion of chondroitin sulfate C isolated from pig nucleus pulposus with testicular hyaluronidase (Sigma, H2376) in 0.1 N sodium acetate buffer (pH 5.0) containing 0.15 N NaCl at 50°. The Sephadex G-50 column was calibrated with a 20-hour and the Sephadex G-25 column with a 3-day digest. Purified N-acetylclyehondrosine was used as an additional calibration standard with the Sephadex G-25 column. The effluents were analyzed by the carbazole method for uronic acid (14), Elson-Morgan reaction for total hexosamine, Park-Johnson's reducing sugar method (15) and Morgan-Elson reaction (16) with N-acetylclyehondrosine as standard. The molar ratio of each fraction was calculated and expressed in uronic acids (or total hexosamines) per N-acetylgalactosamine at the reducing end (reducing sugar value and Morgan-Elson reaction). Molecular weights of each fraction were then calculated with the assumption that all the oligosaccharides in the calibration mixtures were made of repeating disaccharide units composed of one equivalent of uronic acid, N-acetylgalactosamine, and sulfate.

Calibration curves of log molecular weight against elution volume were plotted (see insets in Fig. 5, b and c).

**RESULTS**

**Identification of Sialic Acid—**Sialic acid was released from CAFS by both acid hydrolysis and neuraminidase digestion. For acid hydrolysis, CAFS (3 mg) was treated with 0.05 N HCl (0.2 ml) at 80° for 1 hour in a sealed tube. The solution was neutralized with an equivalent amount of NaOH and chromatographed on paper as described below. For enzymatic release of sialic acid, CAFS (2 mg) was digested with neuraminidase (0.1 mg) Clostridium perfringens (Sigma, N29576) in 0.1 M potassium acetate buffer (pH 5.0) at 37° for 24 hours, which released all of the sialic acid. The macromolecule was isolated from the mixture by gel filtration on a Bio-Gel P-100 column. The sialic acid eluted along with salt was desalted on a Sephadex G-10 column. The sialic acid was applied to Whatman No. 1 paper and chromatographed with Solvents A and C. Visualization by dipping the paper into silver nitrate solution showed that the sialic acid was N-acetylnemaminic acid. This was confirmed by gas-liquid chromatography, which showed neither O-acetyl nor glycolyl groups were present in the CAFS.

**Fatty Acid Content in Glycoprotein—**The purified glycoprotein was subjected three times to Folch extraction (17) to remove any extraneous lipids. The CAFS (5 mg) was mixed with 25 ml of chloroform-methanol (2:1, v/v), stirred with a magnetic bar for 24 hours at room temperature, and filtered through sintered glass of medium porosity. The residue was twice reextracted for 4 hours. The residue was then suspended in a solution made of 5 ml of chloroform and 10 ml of freshly prepared 0.6 M methanolic NaOH and stirred constantly at room temperature for 1 hour (11, 18). The solution was neutralized with 5 ml of 0.6 M methanolic HCl followed by addition of water (5 ml) and chloroform (10 ml). After mixing thoroughly, the layers were separated by centrifugation. The chloroform layer was washed twice with equal volumes of water, dried over anhydrous sodium sulfate, and solvents removed in vacuo below 25°. The dried residue (2.8% of the original glycoprotein) was dissolved in a small volume of chloroform and subjected to gas-liquid chromatography. The fatty acids were identified by comparison with standard mixtures under the same conditions. To determine whether unsaturated fatty acids were present, a portion of the fatty acid ester was hydrogenated (11). A small portion of the extract was dissolved in 10 ml of methanol and hydrogenated under 1 atmosphere of hydrogen in the presence of 10% palladium-charcoal catalyst (10 mg) at room temperature for 30 min. The catalyst was removed by filtration on celite and the methanol removed by a stream of nitrogen at 30°. The residue was analyzed by gas-liquid chromatography in a small volume of chloroform. For analysis of total short chain fatty acids (including the N-acetyl groups) the glycoprotein (0.5 mg) was hydrolyzed in 2 N HCl at 100° for 2 hours in a small sealed tube and directly analyzed by gas-liquid chromatography (12).

Alkali-labile short chain fatty acids (below C₄ acids) were also analyzed by gas-liquid chromatography after saponification with 0.1 N NaOH at 24° for 1 hour followed by conversion to the free acids. The gas-liquid chromatographic analysis of fatty acids showed the absence of short chain fatty acids except acetic acid, which all could be accounted for as N-acetyl groups of hexosamines and neuraminic acid. The long chain fatty acids as shown in Table I are heterogeneous. The five fatty acids (C₁₆ to C₂₉) constituted nearly all of the fatty acid (97%), with palmitic acid in the highest quantity (37.8%). The dry weight of
After Folch extraction three times, the fatty acids were released from CAFS by mild alkali-catalyzed methanolysis (11, 18). The fatty acids were analyzed by gas-liquid chromatography (see text for details). In addition to the fatty acids below, there were trace amounts of C11:0, C14:0, CALM (Iso branched), C17:0 and C18:0 acids.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>C12:0</th>
<th>C14:0 (Iso br)</th>
<th>C15:0</th>
<th>C16:0 (Alic br)</th>
<th>C17:0</th>
<th>C18:0</th>
<th>C18:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>37.6k</td>
<td>12.6k</td>
<td>18k</td>
<td>10.8k</td>
<td>0.7k</td>
<td>1.3k</td>
<td>2.1k</td>
</tr>
</tbody>
</table>

* br, branched.

a Percentage of total fatty acid.

These fatty acids, as methyl esters, was 2.8\% of the intact glycoprotein. Analysis of the glycoprotein for phosphate (19) was negative.

**Progressive Acid Hydrolysis of CAFS**—CAFS (3 mg) was heated in 0.5 x HCl (0.5 ml) at 80° in a tube with Teflon-lined screw cap, and aliquots were removed at various intervals and neutralized with NaOH. These were applied to Whatman No. 1 paper developed in Solvents A and 13. Visualization in the AgNO₃ solution showed that the sialic acid was hydrolyzed quantitatively within 15 min. A light galactose spot started to appear at 15 min followed by fucose, N-acetylglucosamine-6-sulfate, and N-acetylgalactosamine in that order.

**Action of Galactosidases on CAFS**—Enzyme activities with enzymes from three different sources were studied with intact CAFS, CAFS after removal of sialic acid, and CAFS after removal of fatty acids. β-Galactosidase from *Escherichia coli* (Worthington, code BO) was incubated with the glycoproteins according to Method II (Worthington Enzyme Manual) in pH 7.0 phosphate buffer at 37°. β-Galactosidase from jack bean meal (20) and α-galactosidase from ficin (21) were incubated with the glycoproteins in citrate buffer at pH 4.5 and at pH 3.5 at 37°. The enzyme activities of the β-galactosidases were assayed with lactose and α-galactosidase with p-nitrophenyl-α-D-galactopyranoside as substrates. The enzyme reactions were followed by Schale's reducing sugar and gas-liquid chromatographic analysis. In all cases, even up to 7 days of incubation, no significant amount of galactose was released.

**Anhydrosugar Formation**—It has been shown in our laboratory (22) that mucopolysaccharides (keratan sulfate, heparin, and heparitin sulfate) with sulfate on C6 and a free hydroxy group on C3 of the hexosamine moiety when treated with alkali form 3,6-anhydro-α-glucosamine. This reaction was applied to the CAFS. CAFS (3 mg per ml) was reduced with 1.0 M NaBH₄ at 45° for 16 hours and NaBH₄ removed as methyl borate. The reduced CAFS was treated in 1.0 N NaOH (1.0 ml) containing 20 mg of NaBH₄ at 80° for 7 hours and neutralized with AG 50 W-X8 (H⁺) cation resin. After removal of the resin the product was hydrolyzed in 4.0 N HCl (1.0 ml) at 100° for 1 hour, evaporated at 30° in vacuo, dissolved in pH 2.2 citrate buffer, and assayed on an amino acid analyzer with pH 5.28 buffer.

Analysis showed that 90% of glucosamine was destroyed by the alkali, and 11% of the glucosamine loss was as 3,6-anhydro-α-glucosamine. 3,6-Anhydrogalactose as tested by the Selivanoff reaction (23) was absent. Since lysine had the same retention time as 3,6-anhydroglucosamine in the pH 5.28 buffer run on the amino acid analyzer, the original CAFS was deaminated (24). CAFS (3 mg) was dissolved in 5% NaNO₂ (1.0 ml), mixed with 33% acetic acid (1.0 ml), and left at room temperature for 20 hours. Deaminated CAFS was recovered by precipitation with 3 volumes of alcohol. One-half of the material was treated with alkali and anhydrosugar formation followed by acid hydrolysis in 4 N HCl for 1 hour as before, and the other half was hydrolyzed in 6 N HCl at 110° for 22 hours for routine amino acid analysis. Analysis with the pH 5.28 buffer showed that a peak, whose retention was the same as 3,6-anhydroglucosamine, appeared only with the alkali-treated sample.

**Time Course of Chromogen Formation by Alkali**—This was studied under three different conditions as follows: (a) 0.05 N NaOH at 37°; (b) 0.05 N NaOH at 50°; and (c) 0.5 N NaOH at 24°. In all cases, samples of CAFS (2 mg) were dissolved in the respective NaOH solutions (2 ml) in test tubes with Teflon-lined caps, flushed with nitrogen, and incubated at the indicated temperatures. Aliquots were taken at various intervals and assayed by the modified Morgan-Elson reaction (6) with N-acetylgalactosamine as standard.

As shown in Fig. 1, condition b (as stated above) gave the fastest initial rate, with a rapid increase of chromogen formation and reaching a maximum in 20 hours, followed by rapid destruction of chromogen. Condition c gave the slowest initial rate, leveling off after 48 hours but still increasing slowly even up to 5 days. Condition c fell between the two reaching a maximum at 48 hours followed by rapid decrease of chromogen.

**Alkaline and Gel Filtration**—Samples of CAFS (10 mg each) were treated under the three different alkali conditions described above (conditions a and b for 48 hours; and condition c for 24 hours). After neutralization with HCl they were fractionated on a Bio-Gel P-100 column (1.70 x 230 cm) eluting with 0.5 N NaCl and assayed for hexose and preformed Kuhn's chromogen. A typical chromatogram is shown in Fig. 2 with condition c. The hexosamine peak, which was not drawn in this figure, coincided with the hexose peak. Fractions were cut as shown in the figure. Fraction A was 35% and Fraction B was 65% of isolated total dry weight. The condition b gave approximately the same weight ratio as c, while condition a yielded 70% of total dry weight in Fraction A. Kuhn's chromogen was eluted as a single peak at the
salt region in the Bio-Gel P-100 column. When the B fractions were rechromatographed on a Bio-Gel P-2 column with 10% ethanol as eluent, almost all of the hexose-containing peaks were eluted at the void volume, while more than 70% of preformed Kuhn's chromogen was retarded.

**Effect of Alkali-Borohydride on CAFS**—Samples of CAFS (2 mg each) were treated under four different conditions in a nitrogen atmosphere as follows: (d) CAFS was first treated with 0.05 N NaOH at 37° for 48 hours, neutralized, and reduced with 1.0 M NaBH₄ for 24 hours; (e) CAFS was treated with 0.5 N NaOH at 24° for 24 hours, neutralized and reduced with 0.5 M NaBH₄ at 24° for 16 hours, (f) CAFS was treated with 0.5 M NaOH at 37° for 48 hours, neutralized and reduced with 0.5 M NaBH₄ at 24° for 24 hours. (g) CAFS was treated with 0.5 N NaOH in presence of 0.5 M NaBH₄ at 24° for 24 hours. NaBH₄ was destroyed by addition of glacial acetic acid and removed as methyl borate by repeated evaporation with anhydrous methanol. The residue was hydrolyzed in 4 N HCl and analyzed by the amino acid analyzer for hexosamine and reduced Kuhn's chromogen in pH 5.28 buffer and hexosaminitol in borate buffer (7).

As shown in Table II, both of the sequential treatments (conditions d and e) gave only Peak 3 material, while the simultaneous treatments (conditions f and g) produced only Peak 2. In the hexosaminitol analysis, glucosaminitol was formed only when the CAFS was treated sequentially (conditions d and e), which indicated the treatment with alkali alone resulted in "peeling," while NaBH₄ prevented this. The amount of glucosaminitol represented 73 to 82% of total glucosamine loss, indicating that most of the loss of glucosamine was in the form of glucosaminitol but not as chromogen, i.e., the glucosamine did not "peel." The galactosaminitol represents only 15 to 20% of the total galactosamine lost except when the CAFS was treated under condition f, where galactosaminitol represented almost 40% of the galactosamine loss. The over-all loss of galactosamine and glucosamine was higher in simultaneous treatment probably due to its higher over-all alkalinity.

**Alkali-Borohydride and Gel Filtration**—In order to fractionate the carbohydrate chains and estimate the chain length, preliminary experiments were first carried out to find an optimum condition for complete elimination of the chains with a minimum of "peeling." CAFS (3 mg each) was treated in a nitrogen atmosphere under the following conditions: (h) 0.05 N NaOH at 37° for 48 hours, neutralized, and reduced with 1.0 M NaBH₄ at 37° for 48 hours; (i) 0.5 N NaOH containing 0.5 M NaBH₄ at 24° for 24 hours, (j) 0.05 N NaOH containing 1.0 M NaBH₄ at 37° for 48 hours. After removal of NaBH₄ as methyl borate, each sample was subjected to gel filtration through a Bio-Gel P-100 column (1.70 x 230 cm), eluting with 0.5 N NaCl solution. Effluents were assayed for hexose by the anthrone method. The intact CAFS eluted as a sharp peak at the void volume on the Bio-Gel P-100 column. The borohydride treatment alone (Fig. 3a) produced considerable cleavage of carbohydrate chains, resulting in the spreading of the main peak and the appearance of a second peak close to the salt region. These small chains represented 24% of total recovered dry material. Even

### Table II

**Effect of alkali-borohydride on hexosamine in CAFS**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Loss</th>
<th>Gain</th>
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<tbody>
<tr>
<td>CAFS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlCN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GalNtol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FT⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlCN-NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GalNtol-NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% umoles/g</td>
<td>uW/g</td>
</tr>
<tr>
<td>d. Sequential treatment (0.05 N NaOH at 37° for 48 hours, neutralized, reduced with 1.0 M NaBH₄)</td>
<td>6.9</td>
<td>49.8</td>
</tr>
<tr>
<td>e. Sequential treatment (0.5 N NaOH at 24° for 24 hours, neutralized, reduced with 0.5 M NaBH₄)</td>
<td>10.9</td>
<td>65.9</td>
</tr>
<tr>
<td>f. Simultaneous treatment (0.05 N NaOH in presence of 1.0 M NaBH₄ at 37° for 48 hours)</td>
<td>15.3</td>
<td>75.5</td>
</tr>
<tr>
<td>g. Simultaneous treatment (0.5 N NaOH in presence of 0.5 M NaBH₄ at 24° for 24 hours)</td>
<td>21.8</td>
<td>89.2</td>
</tr>
</tbody>
</table>

* P₁ and P₂ were peaks which appeared in the hexosamine analysis on an amino acid analyzer with pH 5.28 buffer, and their relative retention times to galactosamine were 1.18 and 1.24, respectively. These two peaks were also obtained from N-acetylgalactosamine under the same conditions. P₃ has been postulated as a reduced Kuhn's chromogen (6), and P₄ is also most likely one of the reduced Kuhn's chromogens. In addition, another peak at 1.61 (relative retention time to galactosamine) was produced but only with condition f and N-acetylgalactosamine.

GlNtol, glucosaminitol.

GalNtol, galactosaminitol.

GlCN-NS, galactosaminitol.

GlCN, glucosamine.

FT, trace.
larger amounts were eliminated under condition \( k \), producing nearly complete elimination (Fig. 3d) was accomplished under condition \( l \) causing most of the material to shift close to the salt region (91.4% of total dry weight). A treatment of CAFS with condition \( l \) at 46° and subsequent gel filtration on the same Bio-Gel P-100 column produced very little change. The condition \( l \) therefore was chosen for fractionation and molecular weight estimation of the carbohydrate chains.

Fractionation of CAFS and Estimation of Molecular Weights by Gel Filtration—The calibration curve of the Sephadex G-200 column, as seen in the inset in Fig. 4, demonstrates a linear relationship between elution volume and log of respective molecular weight of mucopolysaccharide markers. It was, however, necessary, in the case of keratan sulfate, to use the average value of the elution volume due to the broadness of the peak. The gel filtration of the intact CAFS through the Sephadex G-200 column with aqueous 0.025 N NaCl as eluent yielded a symmetrical peak (Fig. 4). A similar elution pattern was observed with a Bio-Gel P-200 column with aqueous 0.5 N NaCl as eluent. A plot of the midpoint of the elution volume of the intact CAFS on the calibration curve gave an average molecular weight of 26,000.

The exhaustive digestion of CAFS with Pronase (Calbiochem, 53702), before and after the removal of fatty acids by mild alkali-catalyzed methanolysis from the glycoprotein, did not change the elution diagram and thus did not significantly affect the molecular weight. As indicated previously, the treatment of alkali-borohydride on CAFS (0.5 N NaOH in presence of 1.0 M NaBH₄ at 37° for 48 hours) followed by gel filtration on Bio-Gel P-100 caused almost complete elimination of the chains, resulting in a shift of the elution diagram to smaller molecular weight (Fig. 3d). The fractions were pooled as indicated in the figure and Fractions A and B were recovered by desalting through a Bio-Gel P-2 column. The combined recovery of the Fractions A and B was 92.2% of initial starting CAFS material, and Fraction B represented 91.4% of the total recovered material. The chemical analyses are presented in Table III. Fraction A had lower, while Fraction B had a slightly higher sulfate content compared to that of intact CAFS. It is of interest to note that there was a sharp drop in N-acetylneuraminic acid in Fraction A (to 4.2%), while Fraction B virtually remained unchanged. Another point

![Fig. 3. Alkali-borohydride treatment of CAFS and gel filtration on Bio-Gel P-100 column (1.70 x 230 cm). Elution with 0.5 N NaCl solution. Eluent was assayed for hexose by the anthrone method. a, intact CAFS; b, 1.0 M NaBH₄ at 37° for 48 hours (condition h); c, 0.5 N NaOH in presence of 1.0 M NaBH₄ at 24° for 24 hours (condition k); d, 0.05 N NaOH in presence of 1.0 M NaBH₄ at 37° for 48 hours (condition l). Fractions (A and B) were pooled as indicated in the figures. Fraction B in Fig. 3d was rechromatographed on a Sephadex G-25 column (Fig. 5a).](image)

![Fig. 4. Elution diagram of intact CAFS on a Sephadex G-200 column (1.04 x 90 cm). The inset is a calibration curve made by plotting the logarithm of the molecular weights of marker mucopolysaccharides versus the elution volumes. The CAFS and markers were eluted with 0.025 N NaCl and eluents were assayed by anthrone for hexose and carbazole for uronic acid. Mucopolysaccharide markers: A, a mixture of chondroitin sulfate A and C, mol wt (MW) 29,000; B, chondroitin sulfate A, mol wt 22,000; C, chondroitin sulfate A, mol wt 12,000; D, keratan sulfate, mol wt 9,500. The molecular weights of mucopolysaccharide markers were determined by other physical and chemical methods (see text).](image)
of interest is that the hexosamine content in Fraction A remained practically unchanged from the intact CAFS, while most of the galactosamine in Fraction B was destroyed, appearing mainly as galactosaminitol and the rest as Peak 2 material. There was no Peak 3 material in either fractions. The conservation of galactosamine in Fraction A could occur for two possible reasons. (a) Some of the carbohydrate chains are linked to the peptide backbone through an alkali-stable linkage, such as the N-acyl-glycosylamine type; (b) the elimination reaction simply did not go to completion.

Refractionation of Fraction B of Fig. 3d on the Sephadex G-25 column with 0.5 N NaCl as eluent gave two fractions as shown in Fig. 5a. Fraction A was eluted at the void volume, and weighed 88.5% of total dry weight, while Fraction B was retarded. Chemical analysis of the two fractions are shown in Table III. The sulfate value is relatively high in Fraction B of Fig. 5a. This fraction did not contain galactosamine, which apparently had been converted into either galactosaminitol or Peak 2 material, while Fraction A of Fig. 5a still retained approximately one-half of the original galactosamine and some galactosaminitol, but did not contain reduced Kuhn's chromogen. It should be noted that Fraction B of Fig. 5a contained the Peak 2 material,

### Table III

**Compositional analysis of CAFS fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Galactose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Protein&lt;sup&gt;c&lt;/sup&gt;</th>
<th>L-Fucose</th>
<th>SO₄</th>
<th>Sialic acid</th>
<th>Hexosamine</th>
<th>P&lt;sub&gt;i&lt;/sub&gt;</th>
<th>M.W.&lt;sup&gt;g&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Intact CAFS</td>
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<tr>
<td>Fig. 3d</td>
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<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td>8.6</td>
<td>30.8</td>
<td>3.1</td>
<td>1.8</td>
<td>4.5</td>
<td>4.2</td>
<td>50.2</td>
<td>49.8</td>
<td>nil</td>
</tr>
<tr>
<td>Fraction B</td>
<td>91.4</td>
<td>32.7</td>
<td>3.8</td>
<td>1.4</td>
<td>8.3</td>
<td>12.7</td>
<td>64.2</td>
<td>13.2</td>
<td>22.6</td>
</tr>
<tr>
<td>Fig. 5a</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Fraction A</td>
<td>58.5</td>
<td>29.4</td>
<td>10.1</td>
<td>1.2</td>
<td>7.3</td>
<td>12.6</td>
<td>69.0</td>
<td>22.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Fraction B</td>
<td>41.5</td>
<td>33.6</td>
<td>2.7</td>
<td>0.4</td>
<td>12.8</td>
<td>13.5</td>
<td>57.3</td>
<td>nil</td>
<td>42.3</td>
</tr>
</tbody>
</table>
| *Percentage of total dry weight of recovered material.*
| *Galactose and fucose were only neutral sugars present.*
| *Average molecular weight by gel filtration.*
| *Percentage of total dry weight.*
| *Percentage of total hexosamines and hexosaminitol.*
| *Height times width at half-height in 1/100 of square inches per g.*
| *ND, not determined.*
| *Value in parentheses is molecular weight obtained from elution volume at peak point (see Fig. 5a).*

**Fig. 5.** Fractionation of alkali-borohydride-treated CAFS and estimation of chain length using Sephax columns. Fractionation of the alkali-borohydride-treated CAFS (a) was carried out on a Sephadex G-25 column (1.40 X 116 cm) and eluted with 0.5 N NaCl. Fractions from the peak were pooled into two fractions as indicated in the figure and Fraction B was redchromatographed with the same solution on the same Sephadex G-25 column (b), which was calibrated with a 3-day digest of chondroitin sulfate C by testicular hyaluronidase. The inset in the figure is the calibration curve. Fraction A was redchromatographed on a precalibrated Sephadex G-50 column (1.00 X 120 cm) with 0.1 N NaCl as eluent (c). The calibration of this column was carried out the same way with a 20-hour digest of chondroitin sulfate C by testicular hyaluronidase. MW, molecular weight.
which is believed to be one of the reduced Kuhn's chromogens, indicating that the chromogen was still linked to the carbohydrate chain after alkali-borohydride treatment. The molecular weights of both fractions were calculated by plotting the elution volumes relative to the respective calibration curves (insets). The rechromatography of Fraction A of Fig. 5a on the calibrated Sephadex G-50 column produced an asymmetrical peak, as shown in Fig. 5b. The plot of the elution volume at the peak point to the calibration curve gave an apparent molecular weight of 2000, while the average volume of the peak gave a molecular weight of 2600. An exhaustive digestion of Fraction A with Pronase did not change the elution diagram. The rechromatography of the Fraction B of Fig. 5a on the calibrated Sephadex G-25 column (Fig. 5c) gave a fairly symmetrical elution diagram, and the elution volume at the peak point corresponded to a molecular weight of 1100, approximately one-half of that of Fraction A.

**DISCUSSION**

The homogeneity of the purified CAFS (papain digested and gel filtered on a Bio-Gel P-100 column) was based on the following results. The gel filtration on a Sephadex G-200 column yielded a symmetrical elution diagram (Fig. 4). The same result was obtained on a Bio-Gel P-200 column. Pooling the peak material into three equal volumes and subsequent analyses, including amino acid composition, of the products revealed no significant difference between the fractions. Ion exchange chromatography on a DEAE-Sephadex column, which gave a symmetrical peak, also produced the same result. Electrophoresis on Sephaphore and Whatman No. 3 paper in various buffers at different pH values yielded a single spot.

Further digestion of the CAFS preparation with pronase did not change the amino acid composition nor the molecular size of the macromolecule. The removal of the fatty acids (see below) from the macromolecule did not improve the digestibility by this enzyme. Attempts made to digest the macromolecule with α- and β-galactosidases from different sources before and after removal of sialic acid as well as of the fatty acids failed to release galactose. In addition, the lability of the galactosyl group to mild acid hydrolysis suggests that galactose in the macromolecule is present, at least to some extent, as fumaroside. This was confirmed by the observation that one of the oxidation products of CAFS by metaperiodate under a mild condition was arabinose (5% of total glucose). A similar result from the host factor was reported by How and Higginbotham (26).

The sialic acid, which is one of the major components in the CAFS, was opposed to a minor component in the HP preparation of the Norwegian authors, was identified as N-acetylneuraminic acid by paper chromatography as well as by gas-liquid chromatography. O-Acetyl and N-glycolyl groups were absent. The digestibility by neuraminidase (C. perfringens) and progressive acid hydrolysis results indicated that sialic acid is located at nonreducing ends.

One of the most surprising results of this investigation is the presence of fatty acids in the CAFS preparation covalently bound to the carbohydrate moieties apparently via ester linkages. Gas-liquid chromatographic analysis of the fatty acids showed that they were a heterogeneous mixture, C14 to C2 fatty acids constituting 97% of total fatty acid, with palmitic acid in the highest quantity (37.8%). The dry weight of the fatty acids, as methyl esters, was 2.8% of the CAFS preparation. Analysis for short chain fatty acid by gas-liquid chromatography (12) did not reveal any other acid except acetic acid, all of which could be accounted for as N-acetyl in hexosamines as well as in neuraminic acid. In double diffusion analysis (Ouchterlony) with antiserum to HF, the reactivity was not significantly altered by prior removal of the fatty acids. Removal of the fatty acids, however, caused a marked increase in oxidation of the CAFS by metaperiodate. Fatty acids esterified with carbohydrates have been demonstrated in microorganisms, but to our knowledge have heretofore not been known to occur in higher animals. Thus far we have been unable to obtain similar esters in mucopolysaccharide or sulfated glycoprotein preparations.

The action of alkali on the glycoprotein was examined under various conditions. In all cases described in Table II, the loss of threonine and serine was approximately equivalent to the loss of galactosamine with concomitant gains of α-amino butyric acid and alanine, respectively. As seen in the table, only the sequential treatments (conditions d and e) produced Peak 3, a reduced Kuhn's chromogen, while the simultaneous treatments (conditions f and g) yielded only Peak 2, apparently another reduced Kuhn's chromogen. (The structure and mechanism of formation of Peaks 2 and 3 material is being investigated.) Although not to the same extent, a similar pattern was obtained with N-acetylchondrosine. Condition d produced the Peak 3 material in an amount approximately three times that of that produced by condition e, while condition f yielded one-third more Peak 2 than that produced by condition g. Thus, for production of the reduced Kuhn's chromogens, lower NaOH concentration is preferred. Another point of interest is that the glucosamininitol is formed only when the glycoprotein was treated sequentially and the glucosamininitol formed represented almost all of the glucosamine lost. Analysis of neutral sugar of the glycoprotein treated under condition e showed that the loss of galactose was approximately half of the amount of glucosamine lost. Although other mechanisms cannot be ruled out completely, the losses in both glucosamine and galactose could be due to “peeling.” The quantitative and qualitative differences of the products of alkaline elimination emphasize the unexpected and underestimated complexity of the reaction.

The study of the rate of the chromogen formation in different alkaline solutions (Fig. 1) demonstrated the lability of the chromogen in alkaline solution. For the preparation of the chromogen condition a was the preferred method. The subsequent gel filtration of the alkali-treated CAFS on the Bio-Gel P-100 column showed that the elimination reactions under the conditions described were not complete.

Almost complete β elimination was accomplished by treating the CAFS under condition l (0.05 N NaOH in presence of 1.0 M NaBII at 37° for 48 hours), and gel filtration of the product on Bio-Gel P-100 column (Fig. 3d) showed a complete shift of hexose-containing peaks, the majoritly eluting close to the salt region. Fig. 3b shows that borohydride alone at 37° degraded a considerable amount of the macromolecule. Another point of interest is that condition l released carbohydrate chains more extensively than 0.5 N NaOH in presence of 0.5 M NaBII at 24° for 24 hours. The chemical analyses of the gel filtration products (Table III) shows that nearly all of the loss of galactosamine was in the small molecular size fraction (Fraction B of Fig. 3d), most of which appeared as glucosamininitol as well as Peak 2. Fraction A of Fig. 3d, which obviously had a bigger molecular weight than Fraction B, differed little in hexosamine and amino acid content from CAFS (Table III). Fraction A, however, had a low sialic

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5 We thank Dr. B. L. Slomiany for his help in this analysis.
acid content. The explanation for these facts may be (a) that the chain was of higher molecular weight (assuming that sialic acid terminates the chains), or (b) that the carbohydrate chains were not eliminated if linked to terminal hydroxyamino acids. The rechromatography of the short chain fraction (Fraction B of Fig. 3d) on a Sephadex G-25 (Fig. 5a) further reveals the heterogeneity of the carbohydrate chains. It was fractionated into two fractions (A and B of Fig. 5a). The sulfate content was relatively high in Fraction B compared to Fraction A, which was changed only slightly. Fraction B had lost all the galactosamine which apparently was converted to either galactosaminitol or Peak 2 material and Fraction A retained approximately one-half of the original galactosamine and a small amount of galactosaminitol. Kuhn's chromogen and Peak 2 were still linked to the C4 as well as C2 of the linkage region galactosamine.

The average molecular weights of the CAFS and its alkali-borohydride-treated fractions (Fractions A and B of Fig. 5a) were 26,000, 2,600, and 1,100, respectively. The intact CAFS were 26,000, 2,600, and 1,100, respectively. The intact CAFS was a composite of Fraction A of Fig. 3d (260), Fraction A (53.4%) and Fraction B (38.0%) of Fig. 5a, and it contained 12.7% protein by amino acid analysis. Simple calculation from these figures indicates that each macromolecule of the CAFS is mainly composed of two different chain sizes, i.e. eight short chains with an average molecular weight of 1,100 and five longer chains with an average molecular weight of 2,600. A more detailed structural analysis of the CAFS by metaperiodate oxidation and methylation will be reported in a subsequent communication.

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