

with water and dried (yield 70 mg). Sublimation (110—120°/0.05 mm) gave 47 mg (2 %) of *trans*-2-furanacrylic acid (white crystals, m. p. 140—142°, mixed m. p. with an authentic specimen of *trans*-2-furanacrylic acid 141—143°). [Found: C 60.9; H 4.6. Neut.equiv. 137.8 (phenolphthalein indicator). Calc. for $C_7H_6O_3$ (138.1): C 60.9; H 4.4].

The filtrate from the crude furanacrylic acid was continuously extracted with ether (2 h), the ethereal extract dried with magnesium sulfate and the ether evaporated in a vacuum. 1.45 g of a semisolid residue remained. Crystallization from water followed by sublimation (130—140°/0.05 mm) gave 0.53 g (26 %) of III (white crystals, m. p. 197—200°, mixed m. p. with an authentic specimen of *m*-hydroxybenzoic acid 198—200°). [Found: C 61.0; H 4.5. Neut.equiv. 137.8 (phenolphthalein indicator) Calc. for $C_7H_6O_3$ (138.1); C 60.9; H 4.4].

In another experiment the acid solution was not refluxed. After standing for 6 days at room temperature a 6 % yield of *trans*-2-furanacrylic acid was obtained, but no III could be isolated.

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On the Carbohydrate Components of the α_1 -Acid Glycoprotein of Human Plasma

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α_1 -Acid glycoprotein of human plasma was first isolated in a pure state by Weimer, Mehl and Winzler¹ in 1950. They used ammonium sulphate fractionation under pH control. In 1953, Schmid² independently isolated a glycoprotein, called α_1 -acid glycoprotein, from the supernatant of fraction V of Cohn's fractionation procedure. Judging from the physicochemical and analytical figures, this substance was considered to be the same one as Weimer *et al.* described.

The α_1 -acid glycoprotein is one of the best characterized substances among the numerous plasma proteins. The carbohydrate content is extremely high, amounting to nearly 40 %. Neutral sugars, amino sugars and sialic acids were found by Winzler¹, Schmid² and Odin³.

The present author prepared a certain amount of this glycoprotein for study. After hydrolysis, the substance was analyzed for neutral sugars, amino sugars and sialic acids by means of chromatographic methods. Galactose, mannose, fucose, glucosamine, ovine-type sialic acid and its derivative were determined quantitatively.

In order to investigate the chemical structure proteolytic degradation of the glycoprotein was attempted. At first, the glycoprotein was submitted to a mild hydrolysis in order to remove sialic acids because the intact glycoprotein, was hardly digested by trypsin or papain. The degraded sialic acid free glycoprotein, however, could be digested by trypsin. Actually, a peptide carrying carbohydrate was obtained.

Experimental: Preparation. The electrophoretically pure glycoprotein was prepared principally according to Schmid's method⁴. However, the ethanol concentration was raised to 25 % instead of 20 at the final stage of the fractionation. In this way, the pure substance was regularly obtained, although the yield was somewhat low. Thus, 1 gm of glycoprotein was usually obtained from 50

liters of pooled supernatant of fraction V of Cohn's fractionation procedure (Method 6).

Analytical methods. Total neutral sugars were determined both by the orcinol reaction according to Hewitt⁵ and by the cysteine reaction according to Dische⁶. For the chromatographic analysis, Gardell's method⁷ was used after hydrolysis with 2 N sulphuric acid for 12 h at 100°C. Amino sugars were determined also according to Gardell's method⁸ after hydrolysis with 6 N hydrochloric acid for 8 h at 100°C. For the sialic acid determination, Bial's reagent⁹ was used on the intact glycoprotein and Ehrlich's reagent⁹ on the fractions of the column chromatography. Paper chromatographic analysis of sialic acid was performed with butanol-acetic acid-water (4:1:5) and butanol-pyridine-water (6:4:3) as solvents according to the directions of Blix, Lindberg, Odin and Werner¹⁰. The sialic acids were detected by spraying the paper with the orcinol-trichloroacetic acid reagent of Klevstrand and Nordal¹¹.

Digestion by proteolytic enzymes. For the tryptic digestion, the autoactivation product of trypsinogen was used which was prepared by keeping trypsinogen in borate buffer (0.1 M, pH 8.0) containing 0.1 M CaCl₂ for 24 h at 0°C. Trypsinogen was prepared according to Northrop and Kunitz¹² through crystallization and further purification by trichloroacetic acid.

The papain used in this experiment was the product of Worthington (Freehold, New Jersey, U.S.A.).

The digestion was followed by measuring the ninhydrin reaction¹³ on the whole reaction mixture.

Results and Discussion. Analytical values on sugar components are given in Table 1 together with the figures given by the earlier workers. As shown in the table, chromatographic values are consistent with the values obtained by direct determination.

From the X-ray diffraction patterns of the isolated and crystallized sialic acid, Odin³ concluded that the glycoprotein contains sialic acid having N-acetyl group (ovine-type sialic acid). However, two types of sialic acid were found by the present author when the glycoprotein was submitted to a mild hydrolysis (0.01 N H₂SO₄, 100°C, 1 h).

The sialic acids liberated were chromatographed on a Dowex 2 column (formate form) according to the directions of Svennerholm¹⁴. Thus, the hydrolysate was treated by Dowex

Table 1. Comparison of analytical values.

	Winzler	Odin	Schmid	Yama-shina
Hexose	16.4*%	13.5*%	17.2*%	14.2* %
Galactose	—	—	—	6.5 ⁷
Mannose	—	—	—	4.8 ⁷
Hexosamine	11.9	12.2	11.5	12.3
Glucosamine	—	12.2 ⁸	—	12.3 ⁸
Fucose	—	1.4 ⁶	—	1.1 ⁶ , 0.9 ⁷
Sialic acid	—	10.6 ⁹	—	10.8 ⁹

* Orcinol reaction.

50 (H⁺ form) in order to remove the excess amount of Ba(OH)₂ used for the neutralization, and the solution was freeze-dried. The residue was dissolved in 0.1 N formic acid and put on a column of Dowex 2 which had been equilibrated by 0.1 N formic acid. A stepwise elution was carried out. A chromatogram on Dowex 2 is shown in Fig. 1.

The sialic acid which was eluted by 0.1 N formic acid was identified as O-sialic acid by means of paper chromatography. The other sialic acid, tentatively named sialic acid II, whose structure is not elucidated yet, migrates faster than the first one on paper chromatography using the aforementioned solvents.

The amount of both types of sialic acid depends on length of hydrolysis time, the total amount being constant and about

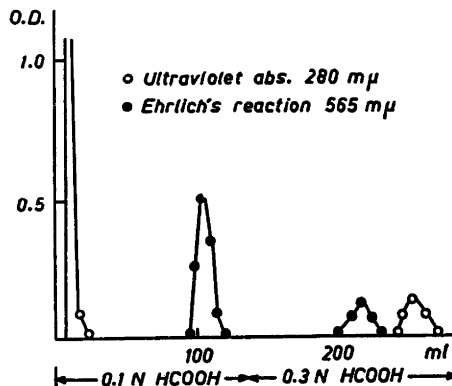


Fig. 1. Chromatogram on Dowex 2 (200—400 mesh), 0.9 × 32 cm. 67 mg of α-acid glycoprotein was hydrolyzed for 40 min. Elution rate: 1 ml per 10 min.

Table 2. Relation between the amount of sialic acids (I and II) and the time of hydrolysis.

Time (Min)	Sialic acid I	Sialic acid II (μg per mg glycoprotein)	Total
30	58	31	89
60	37	43	80
90	27	54	81
120	18	70	88

80–90 % of sialic acid present in the original glycoprotein. The relation between the amount of both sialic acids and the time of hydrolysis is shown in Table 2.

As could be expected, the isolated N-acetyl sialic acid can be converted to sialic acid II by heating in 0.01 N sulphuric acid.

While the structural studies on sialic acid II will be performed in due time, the present findings would provide a definite method for the isolation of sialic acids from glycoproteins. Actually, similar findings, the presence of two transformable sialic acids, were also observed on prothrombin which was prepared according to Seegers¹⁵ and supposed to be a glycoprotein (Magnusson¹⁶).

After the removal of sialic acid by means of mild hydrolysis as mentioned above, the remaining product was dialyzed against distilled water and freeze-dried. This sialic acid free glycoprotein contained practically the whole amount of sugar present in the original glycoprotein. It behaved as a homogeneous and rather neutral substance, the isoelectric point being higher than 4. Thus, the acidic nature of the original glycoprotein is mainly due to the presence of sialic acid.

While the original glycoprotein is essentially resistant to proteolytic digestion both by trypsin and by papain, the sialic acid free glycoprotein could be digested by trypsin. Thus, to the 0.5 % solution of the sialic acid free glycoprotein in 0.1 M borate buffer (pH 8.0), trypsin was added to a final concentration of 0.25 mg per ml. The digestion was carried out at 25° C for 24 h. The digestion product was separated into two groups, one being dialyzable and the

other non-dialyzable. The latter is quite resistant to the further addition of trypsin. The dialyzable part comprises 45 % of the total neutral sugars as determined by the orcinol reaction.

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