The Metabolism of Sialic Acid in Cystic Fibrosis

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Summary

The activities of plasma and fibroblast cytidine 5′-monophosphate-sialic acid:glycoprotein sialyltransferases of patients with cystic fibrosis have been found to be within the range of activities of age- and sex-matched normal controls when asialofetuin served as the sialic acid acceptor. The use of desialylated preparations of purified human plasma α_2-macroglobulin, as an acceptor, demonstrated 35 to 52% reduction in the incorporation of sialic acid into the α_2-macroglobulin from patients with cystic fibrosis as compared to that of α_2-macroglobulin from normal controls. The reduced sialylation was dependent upon the source of the α_2-macroglobulin acceptor but independent of the source (cystic fibrosis or normal) of the sialyltransferase enzyme. Using radiolabeled precursors, the rates of the synthesis of N-acetylneuraminic acid from N-acetyl-D-mannosamine, the release of sialic acid from glycoproteins and the conversion of free sialic acid into CMP-sialic acid have been determined in cultured skin fibroblasts from patients with cystic fibrosis and found to be not significantly different from those of normal controls.

Speculation

The reduced sialylation of desialylated preparations of purified α_2-macroglobulin from patients with cystic fibrosis as compared to that of asialo-α_2-macroglobulin from age- and sex-matched normal controls indicates a possible alteration in the carbohydrate moiety of glycoproteins in cystic fibrosis. The metabolism of sialic acid appears, however, to be normal in this disease and could not account for the observed differences. Additional glycoproteins and glycosyltransferases should be examined to find out if a general defect in glycosylation is involved in cystic fibrosis.

Cystic fibrosis (CF) is an autosomal recessive disorder, one of the most common genetic diseases among Caucasian populations (13). The disease involves abnormality of all exocrine glands, associated with highly viscous mucus secretions (9). The major manifestations are pancreatic exocrine deficiency, chronic pulmonary disease, and abnormally high sodium and chloride concentrations in sweat (13). The disease is lethal, and death usually occurs before or in early adulthood. Numerous biochemical abnormalities were reported in patients with CF but the basic biochemical defect has not been as yet elucidated.

Many studies have found altered glycoproteins in CF patients (1) and these abnormal glycoproteins are thought to contribute to the pulmonary and gastrointestinal pathology of the disease. Carbohydrate analyses of duodenal fluid (10), sweat (14), and rectal mucus (16) fractions from patients with CF have shown an increased ratio of L-fucose to sialic acid as compared to normal controls. Altered carbohydrate composition was also reported in the glycoprotein α-L-fucosidase, purified to homogeneity from liver of CF patients (2). The amounts of D-mannose, N-acetyl-D-glucosamine, and sialic acid were reduced to 30 to 50% of their content in normal liver α-L-fucosidase. Comparative studies on the enzymes involved in the metabolism of the carbohydrate moiety of glycoproteins in CF patients and normal controls have been reported for several glycosidases and glycosyltransferases (1, 13). Although altered levels of activity were found for some of the

enzymes studies, the results were inconsistent in the case of lysosomal hydrolases and highly dependent on the source of the enzyme and the type of the acceptor substrate in the case of glycosyltransferases.

Previous studies in our laboratory have documented that an altered α_2-macroglobulin (α_2M) is present in CF plasma. The α_2M from patients with CF demonstrated an abnormal interaction with various proteolytic enzymes (17) but was indistinguishable from normal α_2M by its antigenicity and ultraviolet absorbance spectra (18). Two-dimensional electrophoretic analysis of purified α_2M preparations from CF and normal did not reveal any significant differences between the two species (8). However, the content of sialic acid was found to be reduced in the CF α_2M and its binding to the lectins concanavalin A and wheat germ agglutinin was lower than that of the normal (5).

The present study was undertaken to find out if a defect in the metabolism of glycoprotein-sialic acid is involved in cystic fibrosis. The enzymic activities leading to the *de novo* synthesis of sialic acid, the recycling of sialic acid from glycoconjugates by neuraminidases, the formation of the nucleotide-sugar, cytidine 5′-monophosphate (CMP)-sialic acid, and the sialylation process were studied in plasma and cultured skin fibroblasts from CF patients and normal controls.

MATERIALS AND METHODS

[6-^{3}H]-N-Acetyl-D-mannosamine (ManNAc), 31.5 Ci/mmole, [4-^{14}C]-N-acetylneuraminic acid (NeuNAc), 49 mCi/mmole, and cytidine monophosphate-[9-^{3}H]-N-acetylneuraminic acid (CMP-NeuNAc), 18.9 Ci/mmole were from New England Nuclear (Boston, MA). Adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate (CTP), nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, phenylmethyl sulfonylfluoride, agarose-bound neuraminidase (*Clostridium perfringens*), soybean trypsin inhibitor (type 1-S), and phosphoenol pyruvic acid (PEP) were from Sigma Chemical (St. Louis, MO). ManNAc, NeuNAc, neuramine lactose (beef colostrum), and fetuin (fetal calf serum) were from Calbiochem-Behring (LaJolla, CA).

Skin fibroblasts were obtained by punch biopsies from three patients with CF and three normal controls. All were males 13 to 14 years of age. The cells at their third to fifth passage were cultured and harvested as previously described (4). Fibroblast lysates were prepared in five volumes of distilled water by five consecutive cycles of freezing and thawing. Whole homogenates were used for enzyme assays, and boiled cell lysates served as blanks. Plasma was separated from fresh venous blood obtained from three patients with CF and three normal controls. All were 12- to 14-year-old males. Blood was collected on 3.8 mg/ml sodium citrate, and the protease inhibitors phenylmethyl sulfonylfluoride and soybean trypsin inhibitor were added at final concentrations of 0.1 and 0.16 mg/ml, respectively. Protein was determined by the dye binding assay (6) using bovine γ-globulin as a standard. Absorbance measurements were made on a Beckman D.B. spectrophotometer, and radioactivity of ^{3}H and ^{14}C was monitored by a Tricarb scintillation spectrometer.

Synthesis of sialic acid from N-acetyl-D-mannosamine (Fig. 1, reactions 1 to 3) was assayed according to Warren and Glick (20) using fibroblast lysate as the source of enzymes. The reaction

N-ACETYL-D-MANNOSAMINE

1. N-ACYL-D-MANNOSAMINE KINASE, EC 2.7.1.60
— ATP
→ ADP

N-ACETYL-D-MANNOSAMINE-6-PHOSPHATE

2. N-ACYLNEURAMINATE-9-PHOSPHATE PYRUVATE-
LYASE (N-Acylneuraminate-9-phosphate synthase)
EC 4.1.3.20
— PEP
→ P_i

N-ACETYLNEURAMINATE-9-PHOSPHATE

3. N-ACYLNEURAMINATE-9-PHOSPHATE PHOSPHO-
HYDROLASE (N-Acylneuraminate-9-phosphatase)
EC 3.1.3.29
→ P_i

N-ACETYLNEURAMINATE ←

4. CTP: N-ACYLNEURAMINATE CYTIDYLYLTRANSFE-
RASE (Acylneuraminate cytidylyltransferase)
EC 2.7.7.43
— CTP
→ PPi

CMP-N-ACETYLNEURAMINATE

5. CMP-N-ACETYLNEURAMINATE : GLYCOPROTEIN
N-ACETYLNEURAMINYLTRANSFERASE (Sialyl-
transferase) EC 2.4.99.1
Asialo-glycoproteins ←
→ CMP

GLYCOPROTEINS

6. ACYLNEURAMINYL HYDROLASE (α-N-Acetyl-
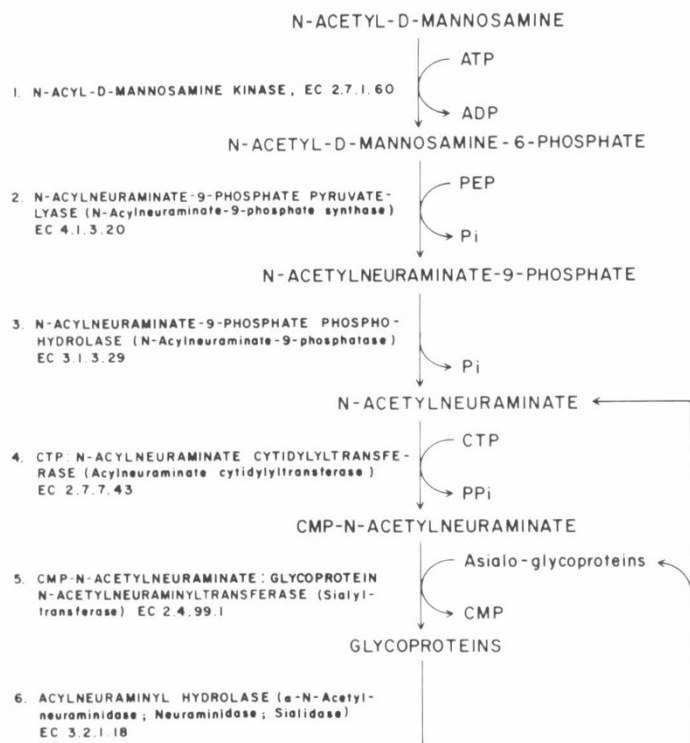neuraminidase ; Neuraminidase ; Sialidase)
EC 3.2.1.18

Fig. 1. The metabolism of glycoprotein-sialic acid. Synthesis, reactions 1 to 3; activation reaction 4; sialylation, reaction 5; and desialylation, reaction 6. *ADP*, adenosine 5'-diphosphate; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate.

mixture contained 100 μg cell protein, 100 nmoles [6-³H]ManNAc (6,800 dpm/nmole), 400 nmoles ATP, 2400 nmoles $MgCl_2$, 1600 nmoles PEP, 20 nmoles nicotinamide adenine dinucleotide and 20 nmoles nicotinamide adenine dinucleotide phosphate in a final volume of 0.2 ml of 0.1 M Tris-acetate buffer, pH 7.6. After incubation for 3 hr at 37°C, the sialic acid formed was either quantitated chemically by the thiobarbituric acid assay (3) or radioactively after separation from the radiolabeled precursor by descending paper chromatography (Whatman No. 3, 50 x 20 cm) in *n*-butanol/pyridine/water, 6/4/3 (by volume). The relative mobilities (R_f values) were 0.07 and 0.53 for NeuNAc and ManNAc, respectively. The corresponding areas were cut out from the chromatogram and placed in scintillation vials containing 10 ml Bray's solution (7), and the radioactivity was measured.

The activity of fibroblast CTP:*N*-acylneuraminate cytidyltransferase (EC 2.7.7.43; Fig. 1, reaction 4) was assayed according to Kean and Roseman (12) by incubating 100 μg cell lysate 4000 nmoles $MgCl_2$, 1000 nmoles CTP, and 1000 nmoles [4-¹⁴C] NeuNAc (10,800 dpm/nmole) for 2 hr at 37°C in final volume of 0.2 ml of 0.1 M Tris-HCl buffer, pH 8.2. The product, CMP-NeuNAc, was isolated from the reaction mixture by descending paper chromatography (Whatman No. 3, 50 x 20 cm) in 0.1 M ammonium acetate buffer, pH 7.5, containing 70% ethanol. The R_f values were 0.22 for CMP-NeuNAc and 0.61 for the free sialic acid.

CMP-NeuNAc:glycoprotein *N*-acetylneuraminyltransferase (EC 2.4.99.1; Fig. 1, reaction 5) activity was measured in plasma and fibroblast lysates using desialylated preparations of fetal calf serum fetuin and human plasma α_2M as the glycoprotein acceptors. Purification of α_2M from the three cystic fibrosis and three normal plasma samples was carried out as previously described (5). Greater than 95% sialic acid (3) was removed by hydrolyzing the glycoprotein (5 mg/ml) for 1 hr at 80°C in 0.1 N H_2SO_4. Neuraminidase treatment was used as an alternative method, and 55 to 65% of the initial sialic acid (3) was removed by this procedure. Agarose-bound neuraminidase was shaken for 24 hr at 37°C with 2.5 mg glycoprotein in 0.5 ml of 0.1 M sodium acetate

buffer, pH 5.0. Aliquots containing 0.1 unit enzyme each, were added initially and after 6 and 12 hr incubation. The insoluble neuraminidase was spun down, and sialic acid was discarded from the desialylated preparations by dialysis against 0.1 M Tris-HCl buffer, pH 7.5. The sialylation mixture contained 50 μl plasma or 100 μg fibroblast lysate protein, 0.2 mg fetuin or 1 mg α_2M, and 150 nmoles CMP-[9-³H]NeuNAc (41.8×10^6 dpm/nmole) in 0.2 ml of 0.1 M Tris-HCl buffer, pH 7.5. The reaction mixture was incubated for 2 hr at 37°C and terminated by adding 0.8 ml of 3% phosphotungstic acid in 0.5 N HCl. After 16 hr at 4°C, the glycoprotein precipitate was spun down ($12,000 \times g$ for 20 min) and washed twice in 1 ml of 2.4% phosphotungstic acid in 0.4 N HCl. The washed precipitate was dissolved in 1 ml of 1 N NaOH and the radioactivity was measured.

The activity of α-*N*-acetylneuraminidase (EC 3.2.1.18; Fig. 1, reaction 6) toward neuramine lactose, α_2M, and fetuin was assayed using fibroblast lysate prepared by 1- to 2-sec sonication of freshly harvested cells in five volumes of distilled water. The reaction mixture contained 100 μg fibroblast protein and 2000 nmoles neuramine lactose, 1 nmole α_2M or 50 nmoles fetuin in 0.2 ml of 0.1 M sodium acetate buffer, pH 4.3. The sialic acid released was determined by the thiobarbituric acid assay (3).

RESULTS

The rate of synthesis of sialic acid from *N*-acetyl-D-mannosamine (Fig. 1, reactions 1 to 3) in cultured skin fibroblast of three patients with CF and three age- and sex-matched normal controls is shown in Table 1. The assays were performed in duplicate that agreed within 5%. The production of sialic acid by fibroblasts of the CF patients was within the range obtained for the normal controls. The direct chemical determination of the sialic acid formed agreed with the determination of its radioactivity after chromatographic separation from the radiolabeled precursor. Omission of ATP or PEP from the reaction mixture resulted in poor synthesis of sialic acid (less than 5%) by both the CF and the normal fibroblast lysates. All assays used boiled cell lysates as enzyme blanks.

The activity of CTP:NeuNAc cytidylyltransferase (Fig. 1, reaction 4) in fibroblast lysates from three CF patients (0.417 ± 0.117 nmoles/hr/mg protein) did not differ significantly from that of three normal controls (0.470 ± 0.110 nmoles/hr/mg protein). Addition of asialofetuin to the reaction mixture (Fig. 1, reaction 5) resulted in a slight decrease in CMP-NeuNAc formed and an increased incorporation of sialic acid into the high-molecular-weight fraction which does not migrate in the chromatography system. Figure 2 illustrates similar chromatographic patterns of radioactivity obtained with CF and normal fibroblast lysates after incubation with CTP, [4-¹⁴C]NeuNAc, and asialofetuin. Incubation of either type of cell lysate with radiolabeled sialic acid and asialofetuin but no CTP did not show any significant incorporation into the glycoprotein fraction.

Table 1. *Synthesis of sialic acid from N-acetyl-D-mannosamine and phosphoenol pyruvic acid in cultured skin fibroblasts*

Cell line	*N*-Acetylneuraminate formed (nmoles/hr/mg protein)	
	Thiobarbiturate assay	Paper chromatography
Normal controls		
1	27.3	25.0
2	28.4	26.2
3	24.0	23.8
CF		
1	26.2	23.8
2	30.6	25.6
3	35.0	28.2

Direct determination of glycoprotein:CMP-NeuNAc sialyl-transferase (Fig. 1, reaction 5) activity was carried out in plasma and cultured skin fibroblasts from patients with CF and normal controls, three of each using CMP-[9-^{3}H]NeuNAc as the sialic acid donor and acid or neuraminidase desialylated preparations of fetal calf serum fetuin or human plasma α_2M as the glycoprotein acceptors. The results obtained for CF and normal plasma and fibroblast samples with and without acid-treated exogenous glycoproteins are summarized in Table 2. Regardless of the enzyme source (CF or normal plasma), similar incorporation of sialic acid into each of the glycoproteins examined (fetuin, CF-α_2M, or normal α_2M) was determined. In contrast, the sialylation reaction was found to depend upon the source of the glycoprotein acceptor. Acid desialylated preparations of α_2M purified from three CF plasma samples were poor acceptors for CF as well as normal sialyltransferase, incorporating only 48 to 65% of that observed for three normal α_2M preparations. Similar differences were observed when neuraminidase-treated α_2M preparations were used as the acceptor substrate (not shown). Using either normal or CF enzyme source, the incorporation of sialic acid into neuraminidase-desialylated CF α_2M was 54 to 68% of that found for neuraminidase-desialylated normal α_2M.

The activity of α-N-acetylneuraminidase (Fig. 1, reaction 6) toward neuramine lactose and fetuin in cultured skin fibroblast of three CF patients and three normal controls is shown in Table 3. Wide range of activity was found for each cell type, but no significant differences were found between the two groups. The

Table 2. *Incorporation of sialic acid into exogenous asialoglycoproteins in plasma and fibroblasts*

Acceptor substrate	No.	Plasma		Fibroblasts	
		Normal (3)	CF (3)	Normal (3)	CF (3)
None		84 ± 6.7[1]	78 ± 7.1	153 ± 13	135 ± 13
Fetuin		1940 ± 168	1980 ± 181	2820 ± 198	2950 ± 218
Normal α_2M	3	476 ± 34	494 ± 40	800 ± 67	789 ± 74
CF α_2M	3	270 ± 28[2]	302 ± 21[2]	575 ± 62[2]	522 ± 58[2]

[1] Mean cpm/mg glycoprotein ± S.D.

[2] $P < 0.01$ as compared to normal α_2M.

Table 3. *N-Acetylneuraminidase activity in cultured skin fibroblasts*

	Enzyme activity (nmoles/hr/mg protein)	
Cell line	Neuramine lactose	Fetuin
Normal controls		
1	19.8	2.92
2	30.0	4.35
3	38.7	5.14
CF		
1	28.8	3.73
2	45.0	5.85
3	32.6	4.71

ratio of the rate of hydrolysis of the low-molecular-weight substrate (neuramine lactose) to that of the high-molecular-weight substrate (fetuin) in the CF fibroblast lysates was similar to the ratio found for the corresponding activities of the normal controls. Neuraminidase activity of either CF or normal fibroblasts toward CF-α_2M preparations (three of each), 3.96 ± 0.19, did not differ significantly from the activity toward normal-α_2M preparations, 3.35 ± 0.62.

DISCUSSION

The possible involvement of an abnormal sialic acid metabolism in cystic fibrosis has been indicated by many reports (1, 13) describing an altered carbohydrate moiety in CF glycoproteins. The altered binding of CF glycoproteins to lectins (5, 19) and especially to wheat germ agglutinin which was shown to interact specifically with N-acetyl-neuraminic acid (15), provides an indirect evidence for an abnormal number or accessibility of sialic acid residues. The most consistent finding of altered carbohydrate composition in crude glycoprotein fractions from CF patients is a reduced ratio of sialic acid to L-fucose (1). Decreased content of sialic acid was also observed in purified glycoprotein preparations from CF patients as compared to that of normal controls (2, 5). In view of all these findings, we have undertaken the present study to compare the metabolism of sialic acid in CF patients and normal controls.

Glycoprotein sialic acid originates from two sources: (1) the synthesis de novo from N-acetylmannosamine and phosphoenol pyruvic acid (ManNAc + ATP + PEP + 2H$_2$O→NeuNAc + adenosine 5'-diphosphate + 2-phosphoric acid; (2) the recycling of sialic acid from sialic acid-containing glycoconjugates, by α-N-acetyl-neuraminidases (glycoconjugates $\rightarrow$ asialo-glycoconjugates + NeuNAc). The synthesis of sialic acid represents the overall reaction of three enzymes: ManNAc kinase, NeuNAc-9-P synthase, and NeuNAc-9-P phosphatase. The normal rate of synthesis determined in fibroblasts from CF patients and the similar residual activity when ATP or PEP were excluded from the reaction mixture, indicate that neither of these enzymes is deficient in CF. Sialidase activity in CF, as determined using a glycoprotein (fetuin) or an oligosaccharide (neuramine lactose) substrate, was also within the range of activity of normal controls.
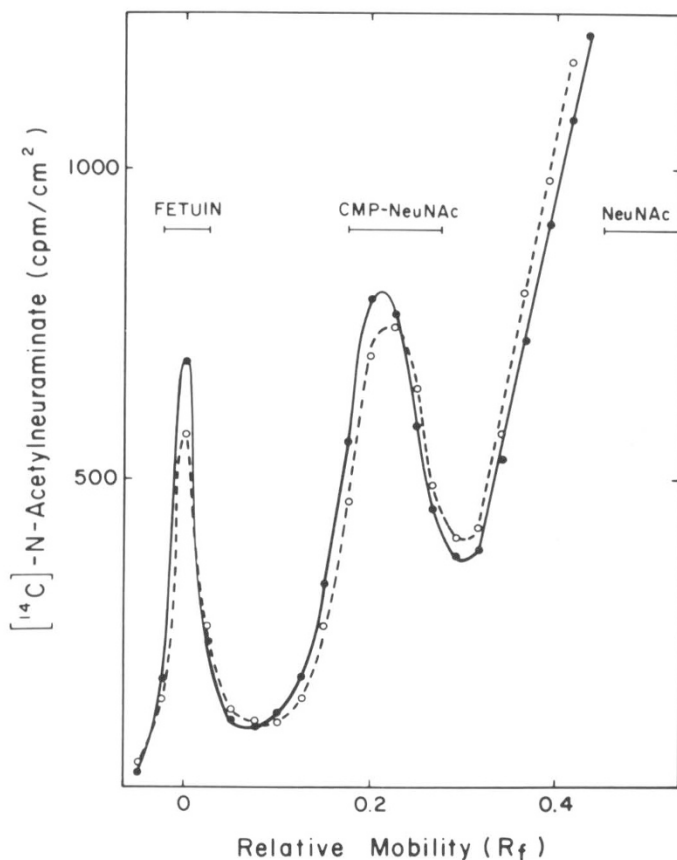

Fig. 2. Paper chromatography of the products of sialic acid activation and fetuin sialylation in homogenates of cultured skin fibroblasts from normal control (●) and a patient with CF (○). The reaction mixture contained 100 μg cell lysate protein, 1 μmole [4-^{14}C]N-acetylneuraminic acid (10,800 dpm/nmole), 1 μmole CTP, 0.2 mg asialofetuin, and 4 μmoles MgCl$_2$ in 0.2 ml of 0.1 M Tris-HCl buffer, pH 8.2. After 2 hr incubation at 37°C, the reaction mixture was chromatographed on Whatman No. 3 paper in 0.1 M ammonium acetate buffer, pH 7.5, containing 70% (v/v) ethanol. The relative mobilities (R$_f$ values) were 0 for fetuin, 0.22 for CMP-NeuNAc, and 0.61 for free sialic acid.

Y-axis: [^{14}C]-N-Acetylneuraminate (cpm/cm^2); X-axis: Relative Mobility (R$_f$). Labels: FETUIN, CMP-NeuNAc, NeuNAc.

The activation of the free sialic acid by formation of CMP-sialic acid is a prerequisite for the sialylation step in which sialic acid is transferred from the nucleotide sugar donor into a glycoprotein acceptor. The activity of CTP:sialic acid cytidylyltransferase in CF fibroblasts did not differ significantly from that of normal cells, and addition of asialofetuin into the reaction mixture resulted in similar pattern of incorporation for both cell types. In contrast to asialofetuin, the sialylation of desialylated preparations of purified $\alpha_2 M$ from CF plasma was poor and found to be 48 to 65% of that determined for desialylated preparations of normal $\alpha_2 M$. However, the reduced sialylation of CF $\alpha_2 M$ could not be attributed to a defective sialyltransferase because similar results were observed using plasma or fibroblast from either CF patients or normal controls, as the enzyme source. Both the CF and the normal enzyme possessed relatively high activity toward normal $\alpha_2 M$, whereas the CF $\alpha_2 M$ species was a poor glycoprotein substrate. The decreased content of sialic acid, previously found (5) for CF $\alpha_2 M$, may account for the reduced sialylation of this glycoprotein species. An opposite pattern has been reported (11) for the fucosylation reaction in CF, namely, using fucosyltransferase from either CF or normal plasma, the incorporation of L-fucose into a glycoprotein acceptor from CF saliva was significantly higher than the incorporation into the glycoprotein acceptor from control. The decreased sialylation and increased fucosylation of CF glycoproteins are in agreement with the previously reported (1) decreased ratio of sialic acid to L-fucose in glycoprotein fractions from CF patients.

The enzymes involved in the metabolism of sialic acid in CF patients appear to be normal and could not account for the observed differences. The altered carbohydrate composition and the altered glycosylation reactions of CF glycoproteins are probably the result of a defect prior to the sialylation of the oligosaccharide portion. The metabolism of additional glycoprotein sugars should be examined to find out if a general defect in the glycosylation process might be involved in CF.

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21. Appropriate informed consents were obtained for this study.
22. Two cultured skin fibroblast lines from patients with cystic fibrosis, GM 142 and GM 768, were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ.
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