COMPARISON OF THE CARBOHYDRATE COMPOSITION OF α_2-MACROGLOBULIN FROM CONTROLS AND PATIENTS WITH CYSTIC FIBROSIS

By

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ABSTRACT

The putative involvement of α_2-macroglobulin (α_2M) in the pathogenesis of Cystic Fibrosis (CF) has long been a subject of controversy. Previous reports have indicated that there are alterations in the carbohydrate moieties of α_2M. The objective of the present study was to compare the carbohydrate composition of α_2M isolated from the plasma of patients with CF and from the plasma of age- and sex-matched normal controls. The gas-liquid chromatographic procedure of Lehnhardt and Winzler was investigated using both α_2M and α_1-acid glycoprotein and modified to obtain the required sensitivity, precision and accuracy for these analyses. Investigation of the hydrolysis of both sugar mixtures and glycoproteins showed that significant losses of the released sugars occurred with extended hydrolysis and that use of the recommended hydrolysis times led to underestimation of the absolute carbohydrate composition. Analysis of the rates of degradation of mixtures of free sugars indicated that not all are degraded at the same rate. This implies that estimates of glycoprotein composition based on molar ratios are also in error. Therefore, hydrolysis times were individually determined for the two glycoproteins studied; those chosen were a compromise between maximal release and minimal destruction of the various carbohydrate components. In contrast to the hydrolysis time recommended by Lehnhardt and Winzler (40 hr for α_1-acid glycoprotein), the hydrolysis times selected for α_1-acid glycoprotein and α_2M, were 30 hr and 35 hr, respectively.

The analysis of α_2M from six CF patients and their age- and sex-matched normal controls using the modified gas-liquid chromatographic procedure indicated that there were no significant differences between the carbohydrate compositions of the glycoproteins. The carbohydrate composition of CF α_2M expressed as mean $\pm$ S.D. μmole carbohydrate per 100 mg protein was: fucose, 0.70 $\pm$ 0.12; mannose, 14.07 $\pm$ 1.31; galactose, 6.72 $\pm$ 0.65; glucosamine, 15.38 $\pm$ 1.59; sialic acid, 5.52 $\pm$ 0.33 and that of normal control α_2M was: fucose, 0.69 $\pm$ 0.11; mannose, 14.42 $\pm$ 1.21; galactose, 6.91 $\pm$ 0.52; glucosamine, 16.13 $\pm$ 1.77; sialic acid, 5.58 $\pm$ 0.31. Therefore, contrary to previous reports, this thesis demonstrates that there is no difference in the carbohydrate composition of α_2M in CF.

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LIST OF ABBREVIATIONS

α_1AG	α_1-acid glycoprotein
α_2M	α_2-macroglobulin
Asn	asparagine
BSA	bovine serum albumin
CBS	Cibacron Blue Sepharose
CF	cystic fibrosis
cm	centimeter
Con A	concanavalin A
conc.	concentrated
C.V.	coefficient of variation
Fuc	fucose
g	gram
Gal	galactose
glc	gas-liquid chromatography
GlcNAc	N-acetylglucosamine
hr	hour
ID	internal diameter
Ig	immmunoglobulin
L	liter
M	molar
ma	milliampere
Man	mannose

$MeCl_2$	methylene chloride
MeOH	methanol
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MRF	molar response factor
mv	millivolt
MW	molecular weight
µl	microliter
µmol	micromole
n	number in sample
nmol	nanomole
nm	nanometer
p	statistical probability
PAGE	polyacrylamide gel electrophoresis
psi	pounds per square inch
r	correlation coefficient
RID	radial immunodiffusion
S.D.	standard deviation
SDS	sodium dodecyl sulfate
Ser	serine
Thr	Threonine
TMS	trimethylsilyl

Tris	tris-hydroxymethylaminomethylmethane
v	volt
WGA	wheat germ agglutinin
w/w	weight per weight
w/v	weight per volume
y	year
$<$	less than
$\leq$	less than or equal to
^{o}C	degrees Celsius
σ	standard deviation

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"Learning is acquired by reading books; but the much more necessary learning, the knowledge of the world, is only to be acquired by reading men, and studying all the various editions of them."

Lord Chesterfield: Letters to His Son, March 16, 1752.

INTRODUCTION

I. Cystic Fibrosis: Clinical and Pathological Aspects

With an estimated incidence of 1 in 2000 live births, Cystic Fibrosis (CF) is the most common life-threatening inherited disorder of Caucasian populations (Wood et al., 1976). Pancreatic insufficiency, elevated sweat sodium chloride concentrations and chronic pulmonary disease are the major clinical findings in CF (Di Sant'Agnese and Davis, 1976). Genetic studies suggest an autosomal mode of inheritance for the CF trait(s) (Thompson, 1980) and heterozygote advantage has been proposed as a mechanism for the maintenance of CF genes at a high level in the Caucasian population (Danks et al., 1965; Hirschhorn, 1973).

Cystic fibrosis affects the body's exocrine glands. The abnormally viscid mucous secretions characteristic of this disorder cause obstruction of a number of ducts and passageways including the respiratory tract, small intestine, mucus-secreting salivary glands, pancreas, biliary tract and genital tract (Wood et al., 1976). Obstruction and resulting cystic dilation of the pancreatic ducts leads to fibrosis of the pancreas hence the original name for this disorder, cystic fibrosis of the pancreas (Anderson, 1938). Another consequence of this obstruction is the deficiency of enzymes required for digestion which leads to malabsorption, steatorrhea and malnutrition (Park and Grand, 1981). Inspissation of mucus in the bile ducts may lead to biliary cirrhosis (Park and Grand, 1981) while obstruction of the ducts of the genitourinary tracts results in reduced fertility and often sterility, especially in male patients

(Wood et al., 1976). Chronic pulmonary infection as a result of inspissation of mucus secretions in the lung airways and subsequent colonization by Staphylococcus aureus and Pseudomonas aeruginosa is one of the major complications of CF (Talamo et al., 1983). Death, often as a result of progressive lung damage, usually occurs before or in early adulthood. Although the serous glands are morphologically and physiologically normal, there is an elevation in the sodium and chloride content of eccrine sweat. The detection of elevated sweat chloride by the "sweat test" is used in the diagnosis of CF (Davis and Di Sant'Agnese, 1980).

Despite advances in the clinical management of CF patients in recent years, the basic biochemical defect in CF remains unknown. Research into the nature of the basic defect has proceeded in many different directions. One hypothesis maintains that abnormalities in glycoprotein metabolism give rise to glycoproteins with altered physicochemical properties which are thought to contribute to the pulmonary and gastrointestinal pathology in CF patients. However, many of the studies on mucus glycoproteins, glycosidases and glycosyltransferases in CF have yielded contradictory results and no consistent finding has emerged in support of this hypothesis (Alhadeff, 1978).

Since the exocrine glands are associated with autonomic nerve fibers, dysfunction of the autonomic nervous system is another possible explanation for the observed exocrinopathy in CF. While autonomic drugs such as isoproterenol and pilocarpine induce changes in the mucous glands of animals similar to those found in CF, studies

by other investigators have failed to demonstrate any ultrastructural or histochemical abnormalities in the autonomic nerve fibers of CF patients (Di Sant'Agnese and Davis, 1976).

The presence of pathogenic polypeptide factors ("CF factors") in the serum, saliva and sweat of patients with CF has been reported. Spock et al. (1967) detected a factor in CF serum which caused ciliary dyskinesis in rabbit tracheal explants. A second type of factor present in the sweat and saliva of CF patients was shown to inhibit sodium reabsorption in rat parotid and human sweat glands (Mangos and McSherry, 1967; Mangos et al., 1967; Mangos, 1973). CF factors are reported to be cationic polypeptides rich in arginine and lysine residues (Mangos and McSherry, 1968; Bowman et al., 1969) and some investigators have postulated that these small peptides are responsible for the defective mucociliary transport in the respiratory tract and for the elevation of sodium chloride in the sweat of CF patients (Wilson, 1979). These factors are not specific to CF, however, (Conover et al., 1973; Caplin and Haynes, 1973; Schmoyer et al., 1972) and their nature and mechanism of action remains unknown.

One area which has been extensively investigated in the past few years is the involvement of proteases. Following reports of a deficiency in arginine esterase activity in CF serum, several workers studied the interaction of serum proteases with the protease modulator, α_2-macroglobulin (α_2M). The involvement of α_2M in CF is discussed on p. 10 .

II. α_2-Macroglobulin

α_2-Macroglobulin (α_2M) is a high molecular weight glycoprotein (MW = 725,000) (Hall and Roberts, 1978) found in high concentrations in human plasma (265 mg/100 ml) (James et al., 1966). The major site of synthesis of α_2M is the liver but fibroblasts and lymphocytes may also produce this glycoprotein (Starkey and Barrett, 1977).

A. α_2M as a Glycoprotein

In common with a great majority of plasma glycoproteins α_2M is believed to have oligosaccharide units N-glycosidically linked to asparagine residues in the protein core (Sottrup-Jensen et al., 1983). A common structural feature of the oligosaccharide units is the inner core branched pentasaccharide shown in Fig. 1A. In various glycoproteins the peripheral mannose residues of this core region are substituted by either additional mannose residues resulting in the "simple" high mannose type of oligosaccharide unit (Fig. 1B) or by two to four units to N-acetyllactosamine (Galβ 1,4 GlcNAC) bearing terminal sialic acid residues to form the "complex" N-acetyllactosamine type of unit (Fig. 1C). Oligosaccharide units belonging to this latter group may be further classified as bi-, tri- and tetraantennary structures (Fig. 1C). Small amounts of fucose may be present in complex type units (Montreuil, 1980).

The biosynthesis of both simple and complex types of oligosaccharide units occurs via a lipid-linked intermediate. As shown in Fig. 2A, the oligosaccharide precursor consists of three glucose, nine mannose and two N-acetylglucosamine residues linked by a

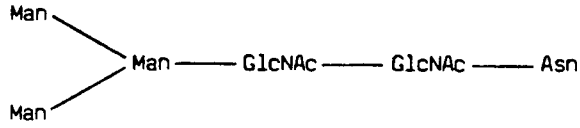
pyrophosphate bridge to the polyisoprenoid lipid, dolichol (Fig. 2B). The oligosaccharide is transferred en bloc to an asparginyl residue in the sequence Asn - X - Ser/Thr (where X = one of a variety of amino acids) on the nascent, ribosome-bound polypeptide chain. An oligosaccharide transferase in the rough endoplasmic reticulum is believed to be responsible for this process (Struck and Lennarz, 1980). The next step in the sequence involves processing of the protein-bound oligosaccharide in the Golgi apparatus. This begins with the removal of three glucose and zero to six of the nine mannose residues by specific glycosidases (Sharon and Lis, 1982). Removal of zero to four of these mannose residues results in a high-mannose type of structure (Fig. 1B). The heptasaccharide, $Man_5GlcNAc_2$ resulting from the removal of four mannose residues may then undergo a series of elongation reactions mediated by specific glycosyltransferases located in the Golgi apparatus leading to the formation of complex type structures (Fig. 1C). The biosynthetic pathway for these complex oligosaccharides has been extensively reviewed by Schachter (1984).

It has been demonstrated that the carbohydrate moieties of plasma glycoproteins serve as recognition markers and play a role in controlling their lifetime in the circulatory system. Morell et al., (1971), using animal models, showed that removal of sialic acid residues from various plasma glycoproteins including α_2M, enhances their rate of clearance from circulation and that the uptake of asialoglycoproteins depends on the recognition by the hepatocytes of galactose residues exposed after removal of sialic acid (Ashwell and Morell, 1974).

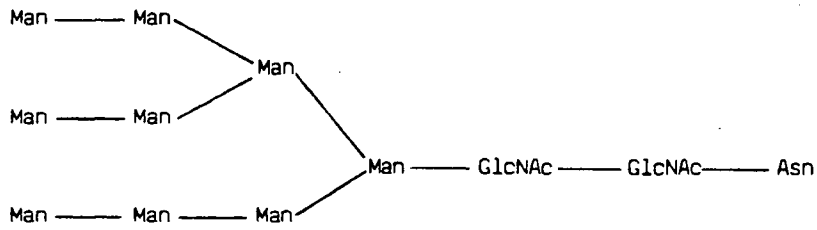
Fig. 1 Structural features of oligosaccharide units of glycoproteins (Montreuil, 1980)

SA, sialic acid; Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose; Asn, asparagine

A. CORE STRUCTURE

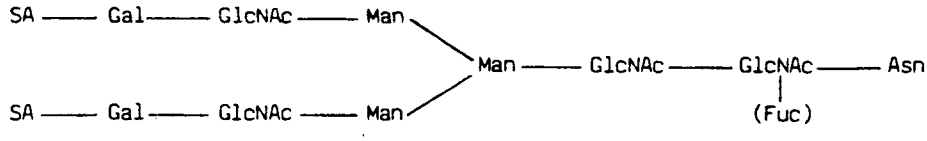


B. SIMPLE HIGH-MANNOSE TYPE

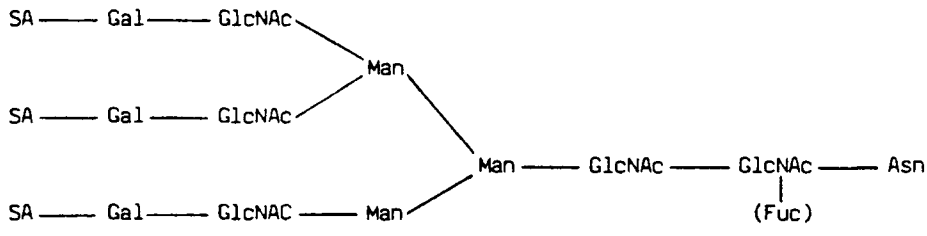


C. COMPLEX N-ACETYLLACTOSAMINE TYPE

a) BIANTENNARY



b) TRIANTENNARY



C) TETRAANTENNARY

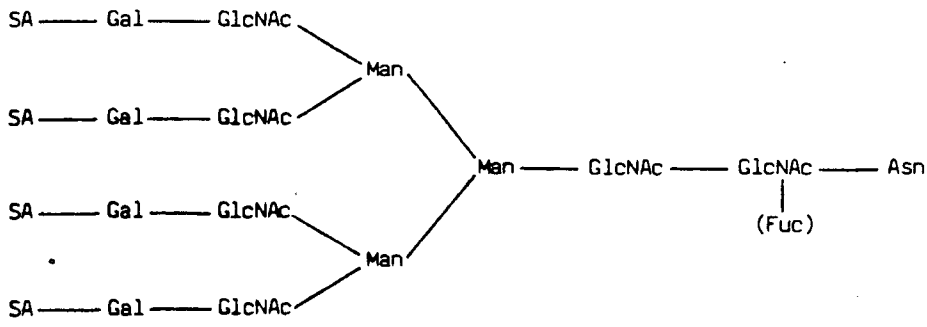
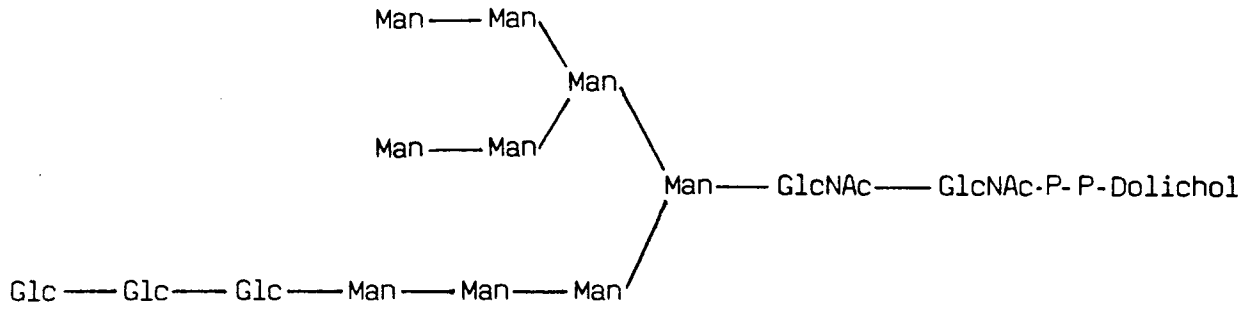


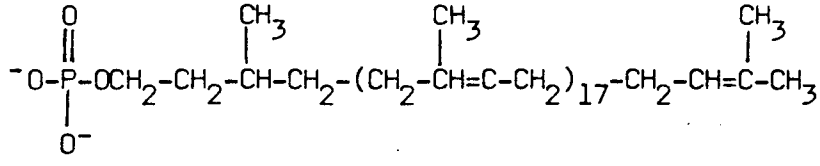
Fig. 2 A. Oligosaccharide precursor of simple and complex carbohydrate moieties.

B. Dolichol phosphate

A.


Man
Man
Man
Man
Man
Man
GlcNAc
GlcNAc-P-P-Dolichol
Glc
Glc
Glc
Man
Man
Man

B.


O
CH3
CH3
CH3
-O-P-OCH2-CH2-CH-CH2-(CH2-CH=C-CH2)17-CH2-CH=C-CH3
O-

B. α_2M as a Protease Modulator

A unique feature of α_2M is its property of acting as a "molecular trap" for several proteases. Once the protease is enclosed within the α_2M molecule, its activity towards high molecular substrates is inhibited while activity towards low molecular weight substrates is retained; in this way α_2M is able to modulate proteolytic activity. α_2M-protease complexes are rapidly cleared from the circulation by the reticulo-endothelial system (Barrett, 1981).

A hypothesis for the mechanism of "trapping" of proteases by α_2M was formulated by Barrett and Starkey and can be summarized as follows (Barrett, 1981): α_2M consists of four identical subunits each bearing a region which is susceptible to limited proteolysis (now known as the "bait" region). Cleavage of this region in one or more of the subunits leads to a conformational change in the α_2M molecule such that it physically encloses the protease. Only molecules of MW < 10,000 are able to diffuse through the trap thus the protease retains its activity towards small molecular weight substrates while interaction with larger substrates and inhibitors is sterically hindered.

C. α_2M in Cystic Fibrosis

A proposed role for α_2M in the pathogenesis of CF arose from the observation that the body fluids of CF patients are deficient in arginine-esterase activity (Rao and Nadler, 1972; Rao et al., 1972; Rao and Nadler, 1974; Chan et al., 1977). When CF and control plasma was analyzed for arginine esterases using immunoelectrophoretic techniques,

Shapira et al. (1976) found that the fraction of arginine esterase activity usually associated in complex with α_2M was missing. In order to examine the possibility that CF α_2M may have failed to complex arginine esterases, these investigators measured the trypsin binding capacity of CF α_2M and found it to be significantly depressed. This suggested that the deficiency of α_2M-complexed arginine esterase activity might be a reflection of a molecular defect in CF α_2M rather than a deficiency of protease molecules.

Further studies with α_2M isolated from CF and control plasma indicated that there were differences in: i) the molar binding ratios of proteases to α_2M (Shapira et al, 1976; Shapira et al, 1977a), ii) the kinetic interaction of purified α_2M-bovine trypsin complexes with the low molecular substrate benzoyl arginine ethyl ester (Shapira et al., 1977a), iii) the stability of the complexes (Shapira et al., 1977b), and iv) the gel electrophoretic behaviour of the complexes in the presence of SDS.

It was therefore hypothesized that there is a molecular defect in CF α_2M which impairs its ability to regulate proteolytic enzyme activity leading to a deficiency of arginine esterase-type activity in CF plasma and exocrine glands. This would result in the accumulation of the lysine- and arginine-rich CF factors giving rise to disturbed mucociliary transport and increased excretion of sodium chloride in sweat (Wilson, 1979).

Several studies suggested that the altered functional properties of CF α_2M might be secondary to abnormalities in its carbohydrate composition. CF α_2M was shown by Ben-Yoseph et al. (1979) to have a 40% decreased sialic acid content when compared with normal control α_2M. This decrease in sialic acid content would be expected to significantly alter the electrophoretic mobility of the α_2M molecule. Two-dimensional gel electrophoretic analyses, however, did not reveal any significant differences between preparations from controls and CF patients (Comings et al., 1980).

Studies comparing the binding of CF and normal α_2M to various lectins indicate possible differences in the carbohydrate composition of the two species may exist. The binding of CF α_2M to the lectins concanavalin A (Con A) and wheat germ agglutinin (WGA) was lower than normal (Ben-Yoseph et al., 1979) and these authors suggest that this finding reflects a decrease in the number or the accessibility of D-mannose, N-acetyl-D-glucosamine and possibly sialic acid residues in CF α_2M. However Shapira and Menendez (1980) showed that, as compared to normal, CF α_2M displayed significantly increased Con A binding.

More recently, Ben-Yoseph et al. (1981) investigated the incorporation of sialic acid into asialoglycoproteins by the sialytransferases found in the plasma and fibroblasts of normal controls and CF patients. Desialylated preparations of fetuin, control and CF α_2M were used as acceptors. Sialylation was reduced when CF α_2M was employed as the sialic acid acceptor but not when fetuin or control α_2M was used; sialylation was also independent of the source

(CF or normal) of the sialyltransferase. These data provide additional evidence for a carbohydrate alteration in CF α_2M.

In contrast to the studies discussed above, other investigators have shown that CF and normal α_2M are identical with regard to gel electrophoretic patterns, subunit molecular weight and trypsin cleavage products (Burdon et al., 1980; Parsons and Romeo, 1980). Following a careful investigation, Bridges et al., (1982b) concluded that there were no differences between CF and normal control α_2M with repect to: i) molar protease binding, ii) interaction of bovine cationic trypsin-α_2M complexes with benzoyl arginine ethyl ester, iii) stability of α_2M-trypsin complexes, and iv) subunit structure.

While it is doubtful that functional differences between CF and normal α_2M exist, it is evident that the issue of carbohydrate alterations in CF α_2M warrants further investigation. This thesis therefore addresses the question: Is there an alteration in the carbohydrate composition of plasma α_2-macroglobulin in cystic fibrosis?

III. Analysis of the Carbohydrate Composition of Glycoproteins

The carbohydrate content of α_2M is low (8-10%) (Barrett, 1981) and only limited amounts are available from pediatric patients. It was therefore necessary to select a highly sensitive, precise and accurate method for the analysis of the carbohydrate composition of α_2M from CF patients and controls. As has been extensively discussed by Dutton

(1973) and Clamp et al., (1972), gas-liquid chromatography (glc) is a technique which fulfills these requirements and has been used extensively for the quantitation of the carbohydrate components of glycoproteins. It affords greater sensitivity and specificity than colorimetric methods and the separation, identification and quantitation of submicrogram amounts of carbohydrate can be achieved in a single procedure. Three main steps are involved in the glc analysis of the carbohydrate moieties of glycoproteins: the glycoprotein sample is first hydrolyzed, a suitable derivative of the released monosaccharides is prepared and these compounds are then analyzed by glc. A variety of carbohydrate derivatives may be employed, of which the trimethylsilyl ether (TMS) and alditol acetate derivatives have been most commonly used. A disadvantage of the TMS derivatization procedure is that several glycosides can be formed per monosaccharide resulting in a complex chromatogram. Identification and quantitation of the sugar components may therefore be difficult (Sweeley et al., 1963; Clamp et al., 1967). A more satisfactory method employs the alditol acetate derivatives. Reduction of the monosaccharides to their alditols prior to derivatization eliminates the problem of anomerization and a single acetylated derivative is formed for each sugar (Sawardeker et al., 1965). However, reduction of the ketone group of sialic acids would be expected to yield two compounds and thus it is simpler to quantitate sialic acid by an alternate method. Of the colorimetric assays available for the estimation of sialic acids those most commonly used are the methods developed by Warren (1959), Aminoff (1961) and Jourdian et al. (1971).

A variety of glc procedures have been developed for the estimation of neutral and amino sugars in glycoproteins as their alditol acetates. The procedure for the quantitation of the neutral sugars in glycoproteins described by Lehnhardt and Winzler (1968) involves hydrolysis of the glycoprotein sample in the presence of Dowex 50 resin (H+ form), addition of an internal standard, reduction of the released sugars with sodium borohydride, acetylation of the resulting alcohols with acetic anhydride and pyridine, and glc of the resulting alditol acetates. Hydrolysis in the presence of a cation exchange resin (Kim et al., 1967) has the advantage that amino acids are bound to the resin and hence do not interact with and destroy the neutral sugars. Another advantage of the method is that it employs an internal standard. Addition of an internal standard allows not only identification of the various components but also accounts for any losses of the sugars during subsequent steps in the procedure.

Simultaneous estimation of neutral and amino sugars in glycoproteins can be carried out by the method of Niedermeier (1971); this procedure does not, however, utilize resin hydrolysis and temperature programming of the chromatograph is required in order to obtain adequate separation of the components. Alternatively, by including a nitrous acid deamination step, it is possible to convert the hexosamines to their corresponding 2,5-anhydro sugars and analyze the derived polyol acetates together with those of the neutral sugars (Niedermeier, 1971; Porter, 1975).

Reid et al. (unpubl.), using the procedure of Lehnhardt and Winzler, were able to quantitate the neutral and amino sugars present in a single hydrolysate. Resin-bound hexosamines were eluted with hydrochloric acid and the derived acetates were analyzed by glc. This modified method retains the advantages of the original resin hydrolysis method as it employs an internal standard and the hydrolysis conditions are identical to those of Lehnhardt and Winzler (1968) and much milder than those used by Niedermeier (1971). A further advantage is that excellent separation of the alditol acetate derivatives can be obtained under isothermal conditions. Therefore, this method was chosen for the analysis of the neutral and amino sugar content of α_2M. In addition, the method of Jourdian et al. (1971) was selected for the quantitation of sialic acid, as it is a sensitive and easily performed method which does not require hydrolytic or enzymatic release of sialic acid from the glycoprotein. As none of these methods had previously been applied to α_2M, it was necessary to first evaluate their sensitivity, precision and accuracy and, if required, modify them in order to obtain the best possible analytical conditions for the study.

The objectives of this thesis were therefore to:

A. evaluate and, if necessary, modify the analytical procedures selected to allow quantitation of the small amounts of carbohydrate present in the α_2M samples available from pediatric patients.

B. use these methods to determine whether or not the carbohydrate composition of α_2M isolated from CF patients differs from that of normal controls.

MATERIALS AND METHODS

I. Materials

The chemicals and materials used in the isolation of α_2M were obtained from the following sources:

Blue Sepharose CL-6B, CNBr-activated Sepharose 4B (Pharmacia); NOR-Partigen α_2-macroglobulin radial immunodiffusion plates, protein standard serum (human) for NOR-partigen, rabbit antisera against human haptoglobin α_2M, IgA, IgG, IgM, transferrin, albumin, α_1-antitrypsin, ceruloplasmin and whole serum (Behring/Hoechst Canada); soybean trypsin inhibitor No. T-9003, polybrene, bovine serum albumin No. A-7638 (Sigma); Aquasil (Pierce); Tris barbital-sodium barbital high resolution buffer pH 8.8 No. 51104, human control serum for electrophoresis (Gelman); electrophoresis-grade acrylamide, N,N-methylene bisacrylamide, N,N,N',N'-tetramethylethylenediamine and ammonium persulfate (Bio-Rad); YM 30 ultrafiltration membranes (Amicon).

For the chemical analyses of α_2M isolates and model glycoproteins, the chemicals and supplies listed below were used:

D-Mannose, D-galactose, L-arabinose, N-acetyl-D-glucosamine, D-glucosamine hydrochloride (Calbiochem A grade); D-galactitol, D-mannitol (Eastman); α-lactose monohydrate (Fisher); L-fucose, D-mannosamine hydrochloride (Sigma); GP 3% SP-2340 on 100/120 mesh Supelcoport, Thermogreen LB-1 septa (Supelco); dimethyldichlorosilane, silicone rubber septa,

Reacti-vials (Pierce); A.C.S. Spectranalyzed methanol, A.C.S. certified pyridine and acetic anhydride (Fisher); anion exchange resin AG 1-X8 200-400 mesh [Cl^- form] and cation exchange resin AG 50W-X8 200-400 mesh [H^+ form] (Bio-Rad); gas chromatographic syringes (Hamilton). The carbohydrates listed above were used without further purification with the exception of lactose which was recrystallized once according to the method of Hudson (1902).

All other chemicals employed were of reagent grade or better.

A preparation of human seromucoid (0.6M perchloric acid soluble fraction of human serum) of known sialic acid content was generously supplied by Dr. P.E. Reid, Dept. of Pathology, U.B.C. A gift of pure lyophilized α_1-acid glycoprotein from Drs. G. Strecker and B. Fournet, Laboratoire de chimie biologique, Université des sciences et techniques de Lille, France is gratefully acknowledged.

II. Subjects

Non-fasting blood samples were obtained from six CF patients and six age- and sex-matched normal controls.

Control subjects were apparently healthy individuals with no family history of CF.

III. Methods

A. General Methods

A Büchi Rotavap rotary evaporator with a 37°C water bath was

used for all evaporations at reduced pressure. Melting points were determined with a Drechsel melting point apparatus. A Beckman Model 25 spectrophotometer was used for all spectrophotometric measurements and densitometric analysis of tube gels was performed with a Helena Auto-Scan densitometer.

B. Isolation of α_2M from Plasma

1) Isolation Procedure

α_2-Macroglobulin was isolated from 5 ml plasma samples by dye affinity chromatography on Cibacron Blue Sepharose followed by immunoadsorption chromatography directed against the major protein contaminants. Procedures for blood collection, harvesting plasma and column chromatography were those described by Bridges et al. (1982a). Following immunoadsorption chromatography, fractions containing α_2M were pooled, the protein content was determined by extinction at 280 nm (Dunn and Spiro, 1967) and samples were concentrated to approximately 2 mg/ml on an ultrafiltration apparatus fitted with YM 30 membranes (MW retention limit = 30,000). The concentrate was then subjected to exchange dialysis against 5 volumes of 25 mM sodium bicarbonate approximately pH 8.3. After determination of the protein content of the α_2M isolates by colorimetric assay (see below p.21), aliquots of known volume were dispensed as required for the various analyses and stored at -20^{o}C for up to 6 months.

2) Radial Immunodiffusion

The α_2M content of 5 µl aliquots of plasma and purified α_2M

was measured by single radial immunodiffusion using NOR-Partigen α_2-macroglobulin immunodiffusion plates and the appropriate protein standard serum. Immunodiffusion and evaluation of the plates was carried out according to the manufacturer's directions.

3) Quantitation of Protein

The protein content of the α_2M isolates was determined according to the method of Lowry et al. (1951). The procedure for the analysis of proteins in solution was miniaturized to give a final assay volume of 650 µl. Results are expressed as mg Lowry protein using bovine serum albumin as standard.

IV. Assessment of Purity of α_2M Isolates

The purity of α_2M isolates was assessed by immunoelectrophoresis with polyspecific and monospecific antisera and by SDS-PAGE.

1) Immunoelectrophoresis

Immunoelectrophoresis was performed by a micro-method on glass microscope slides coated with 1% (w/v) agar in Tris barbital-sodium barbital buffer pH 8.8 essentially as described by Bridges (1981). Samples containing 10 µg protein were applied to the lower wells cut in the agar and human control serum (2 µl) was applied to the upper wells. Electrophoresis was performed at 250 v for 2 hr at room temperature. Following electrophoresis, a longitudinal trough was cut between the two wells in each gel and polyspecific or monospecific antiserum (25 µl) was added. Immunodiffusion was carried out in a humid chamber at room temperature for 18 hr. The gels were then

soaked in 0.9% saline for 2 hr, rinsed with distilled water and air-dried overnight. Staining was accomplished by immersing the gels in 0.25% (w/v) Coomassie blue R-250 in 45% (v/v) aqueous methanol containing 9% acetic acid for 1 hr; gels were subsequently destained in a solution of 30% (v/v) aqueous methanol containing 5% acetic acid. All α_2M isolates were tested with rabbit antisera against human serum, α_2M, IgG, IgA, IgM, α_1-antitrypsin, ceruloplasmin, transferrin, albumin and haptoglobin. Previous work by Bridges et al. (1982a) had confirmed that these were the contaminants found in the α_2M obtained by the above isolation procedure.

2) SDS-PAGE

Polyacrylamide tube gels (5% total acrylamide, 2.6% of this as methylene bisacrylamide) were prepared according to the method of Weber and Osborn (1972). Samples (10 µl) containing approximately 20 µg protein were mixed with 90 µl of sample buffer (0.01 M sodium phosphate pH 7.2, 1% SDS, 10% glycerol, 0.002% bromophenol blue) and electrophoresis was carried out at room temperature at 3 ma per tube for 30 min. The current was increased to 5 ma per tube for the remainder of the run (5-6 hr). Proteins were visualized by staining with 0.25% (w/v) Coomassie blue R-250 in 45% (v/v) aqueous methanol containing 9% acetic acid. The gels were then destained in a solution of 5% (v/v) aqueous methanol containing 7.5% acetic acid for 2-3 days. The gels were evaluated by densitometry at 595 nm.

V. Carbohydrate Analysis of α_2M

1) Preparation of Alditol Acetate Standards for Gas-liquid Chromatography (glc)

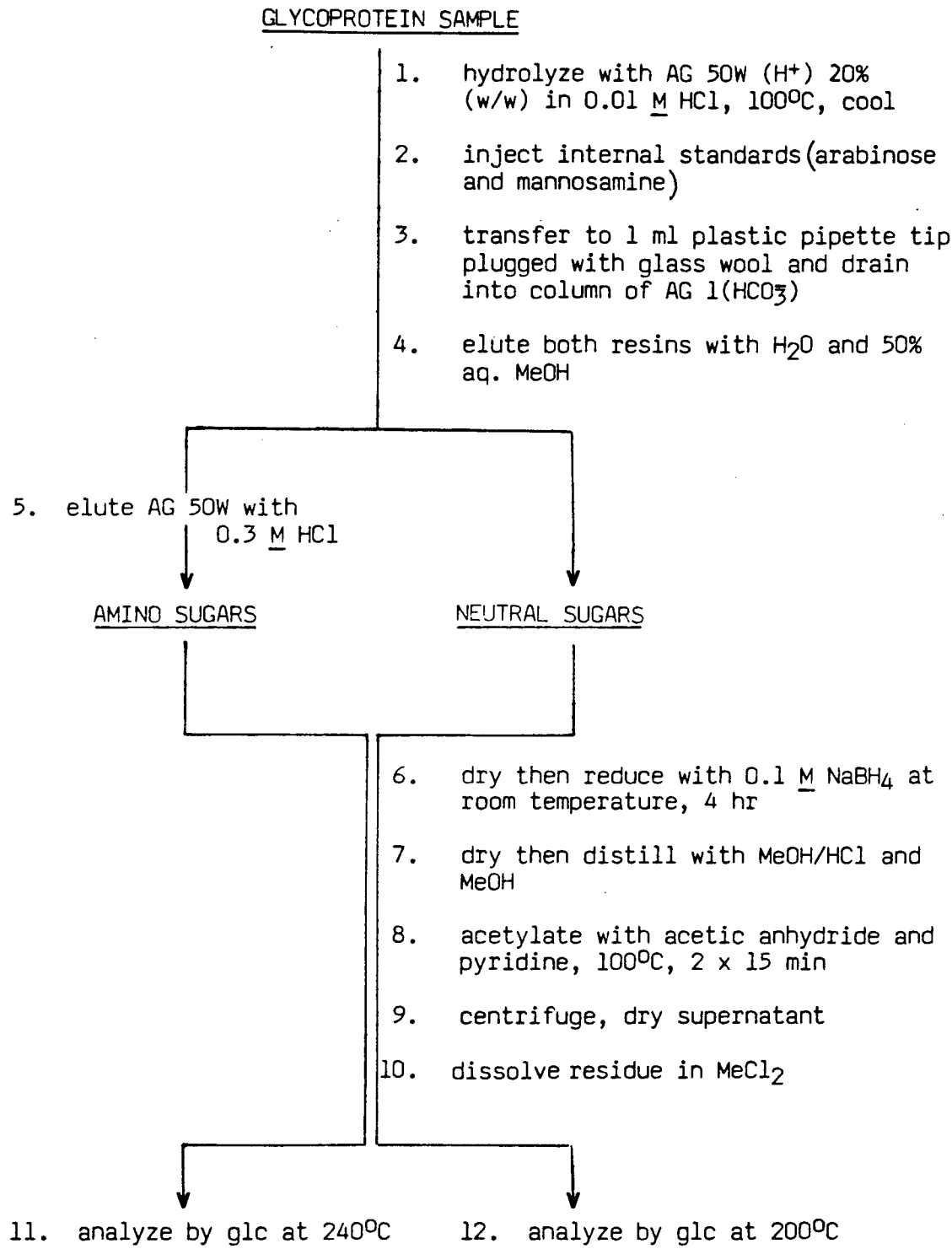
The alditol acetates of D-galactose, L-fucose, D-mannose, and L-arabinose, were synthesized and recrystallized to constant melting point. The melting points of these compounds were as follows: arabinitol pentaacetate, 72-74°C; fucitol pentaacetate, 119-120°C; mannitol hexaacetate, 119-121°C; and galactitol hexaacetate, 158-160°C. The acetates of D-glucosamine and D-mannosamine were obtained as syrups. (This procedure is described in detail in Appendix B.)

2) Quantitation of Neutral Sugars and Hexosamines by glc

The methodology described below and outlined in Fig. 3 was developed from procedures described by Lehnhardt and Winzler (1968) and Niedermeier and Tomana (1974).

All glassware was washed with chromic/sulfuric acid cleaning solution (Fisher) at 80°C, rinsed with distilled water and oven-dried overnight at 100°C to ensure removal of any contaminating material. Glycoprotein samples (180 µl) containing a minimum of 20-50 µg total carbohydrate were pipetted and accurately weighed into 0.3 ml Reacti-vials and equal volumes of a 40% (w/v) suspension of AG 50W-X8 (H^+) 200-400 mesh in 0.02 M HCl were added (see Appendix A for resin preparation.) After sealing the vials with silicone rubber septa, hydrolysis was carried out in an oven at 100°C for an appropriate period of time. (The selection of suitable hydrolysis times for α_2M and other glycoproteins is discussed in the Results and Discussion sections. For α_2M, a hydrolysis time of 35 hr was

Fig. 3 Outline of the procedure for the estimation of neutral and amino sugars from glycoproteins by glc


GLYCOPROTEIN SAMPLE
1. hydrolyze with AG 50W (H^+) 20% (w/w) in 0.01 M HCl, 100°C, cool
2. inject internal standards (arabinose and mannosamine)
3. transfer to 1 ml plastic pipette tip plugged with glass wool and drain into column of AG 1(HCO_3^-)
4. elute both resins with H_2O and 50% aq. MeOH
5. elute AG 50W with 0.3 M HCl
AMINO SUGARS
NEUTRAL SUGARS
6. dry then reduce with 0.1 M $NaBH_4$ at room temperature, 4 hr
7. dry then distill with MeOH/HCl and MeOH
8. acetylate with acetic anhydride and pyridine, 100°C, 2 x 15 min
9. centrifuge, dry supernatant
10. dissolve residue in $MeCl_2$
11. analyze by glc at 240°C
12. analyze by glc at 200°C

used.) A description of the apparatus used to mix and heat samples during hydrolysis is included as Appendix C. Following hydrolysis, the samples were cooled to room temperature and aliquots of both arabinose (25 µl, 0.03 µmol) and mannosamine HCl (50 µl, 0.06 µmol) in distilled water were injected through each septum with a Hamilton syringe. Each sample was mixed thoroughly (to allow binding of mannosamine to the resin) and the contents of the vial were then transferred to a 1 ml plastic disposable pipette tip plugged with glass wool which was stacked on top of a second pipette tip containing a bed (300 µl) of AG 1-X8 (HCO_3^-) 200-400 mesh resin (Appendix A) on a glass wool plug support. The eluate from this series of "columns" was collected in a 15 ml Pyrex conical centrifuge tube fitted with a female ℔ 13 ground glass joint. Portions (6 x 300 µl) of distilled water were used to rinse the vial and these washings were transferred to the columns. The columns were then eluted with 50% (v/v) aqueous methanol (2 x 1 ml) and allowed to drain. The neutral sugars were contained in the eluates plus washings. The two columns were separated and that containing the AG 50W resin was eluted with 0.3 M HCl (2 x 1 ml). The acidic eluate containing the amino sugars was collected in a separate centrifuge tube. Neutral and acidic eluates were evaporated to dryness under reduced pressure at 37°C.

Reduction was carried out by adding 0.1 M sodium borohydride (1 ml) to each dried sample. After 4 hr at room temperature, glacial acetic acid (60 µl) was added to each sample to terminate the reaction

and all fractions were subsequently evaporated to dryness. Excess borate was removed as volatile trimethyl borate by distilling with portions of 0.1% (v/v) HCl in methanol (4 x 1 ml) followed by methanol (2 x 1 ml).

The dry residue was triturated with a mixture of pyridine (200 µl) and acetic anhydride (200 µl), and then the centrifuge tubes were fitted with glass stoppers and heated for 15 min in an oven at 100°C. The samples were mixed well, reheated at 100°C for 15 min, cooled and then centrifuged to pellet the insoluble inorganic salts. The supernatants were transferred to 3 ml glass conical centrifuge tubes using glass Pasteur pipettes and dried in vacuo over fresh sodium hydroxide and sulfuric acid for a minimum of 6 hr. Samples were removed from the dessicator and stored at room temperature until analyzed (within one week). Derivatives are reported to be stable for up to 3 months (Lehnhardt and Winzler, 1968). The residue from each sample was dissolved in methylene chloride (10 µl) and an aliquot (3 µl) was analyzed on a Hewlett Packard 7610A gas chromatograph fitted with a flame ionization detector and on-column injection. Chromatography was carried out isothermally at 200°C (for neutral sugars) or 240°C (for hexosamines) on 183 cm x 2 mm ID siliconized glass columns packed with GP 3% SP-2340 on 100/120 mesh Supelcoport using nitrogen as the carrier gas. Peak areas were recorded with a 3370B electronic integrator. Details of column preparation and glc operating conditions are given in Appendices D,E and F, respectively.

3) Quantitation of Sialic Acid by Colorimetric Assay

Total sialic acid was estimated by the method of Jourdian et al. (1971) with the following modifications (Reid et al., unpubl.) Samples (27 µl) containing 0.2-4.0 µg sialic acid were saponified by treatment with 1 M potassium hydroxide (3 µl) for 30 min at room temperature then neutralized with 0.5 M sulfuric acid (3 µl). After addition of 0.04 M sodium metaperiodate (7 µl), the samples were mixed well and placed in an ice bath for 35 min. Resorcinol reagent (80 µl) was added, the samples were mixed well and, after standing 5 min, heated in a boiling water bath for 8 min. After cooling to room temperature, t-butanol (80 µl) was added and the samples were reheated at 37°C for 5 min and read at 630 nm. Human seromucoid was employed as a standard and was used without saponification.

RESULTS

I. Isolation of α_2M from CF and Control Plasma

A. Plasma α_2M Concentrations

As shown in Table I, the plasma α_2M levels of the CF patients were not significantly different from those of the controls. As previously observed (Ganrot and Schersten, 1967; Tunstall et al., 1975; Wagner et al., 1982), there was a general decrease in plasma α_2M concentration with increasing age. The values obtained were similar to those reported for this age group by Wagner et al. (1982).

B. Column Chromatography of CF and Control Plasma

Fig. 4 shows the typical elution profile obtained when plasma was subjected to Cibacron Blue Sepharose column chromatography. The first peak (CBS pool 1, Fig. 4) contained 80 percent of the α_2M applied. The fractions constituting this peak were pooled and further fractionated using an immunoadsorption column to remove the major plasma protein contaminants. As shown in Fig. 5, the α_2M was obtained as a single peak. No differences were observed in the behaviour of CF and control plasma during column chromatography.

C. Problems Caused by Tris Buffer

The procedure for the isolation of α_2M (Bridges et al., 1982a) employs 50 mM Tris-HCl pH 7.5 as a buffer. The sialic acid content of glycoprotein samples in Tris could not be determined by the modified periodate-resorcinol assay as Tris suppressed the formation of the chromagen in this assay. The problem could be solved by carrying out the periodate oxidation in 0.125 N H_2SO_4. At this pH Tris is protonated and presumably does not undergo periodate oxidation. Tris

Table I — Plasma α_2M levels in CF patients and normal controls as measured by radial immunodiffusion (p.20).

Age (yr)	Sex	Plasma α_2M (mg/100 ml)	
		Normal	CF[1]
7	M	406	375
10	F	404	343
12	M	406	438
13	M	511	544
14	F	358	300
18	M	331	253

[1]Differences between normal and CF plasma α_2M concentrations were not significant when evaluated by paired t test.

Fig. 4 Cibacron Blue Sepharose chromatography of normal human plasma.

Five ml of harvested plasma (see p.20) was applied to a siliconized column (1.5 x 90 cm) containing Cibacron Blue Sepharose CL-6B. The column was eluted with 50 mM Tris HCl, pH 8.0 containing 3 mM sodium azide at a rate of 1.5-2.0 ml/hr. Two ml fractions were collected and monitored for absorbance at 280 nm. All steps were carried out at 4°C. The fractions constistuting the first peak were then monitored for α_2M and plasma protein contaminants by RID before being pooled (CBS pool).

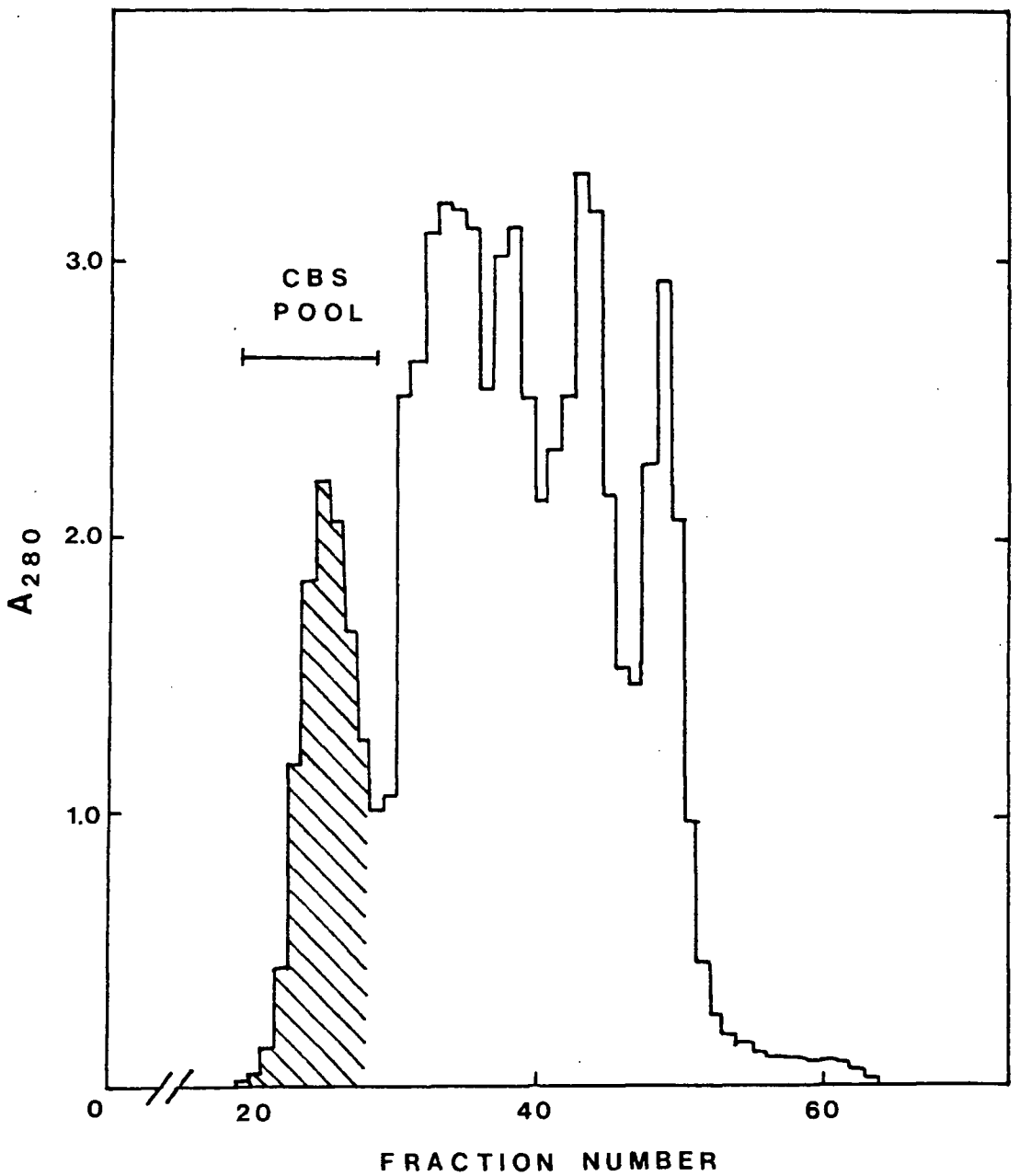
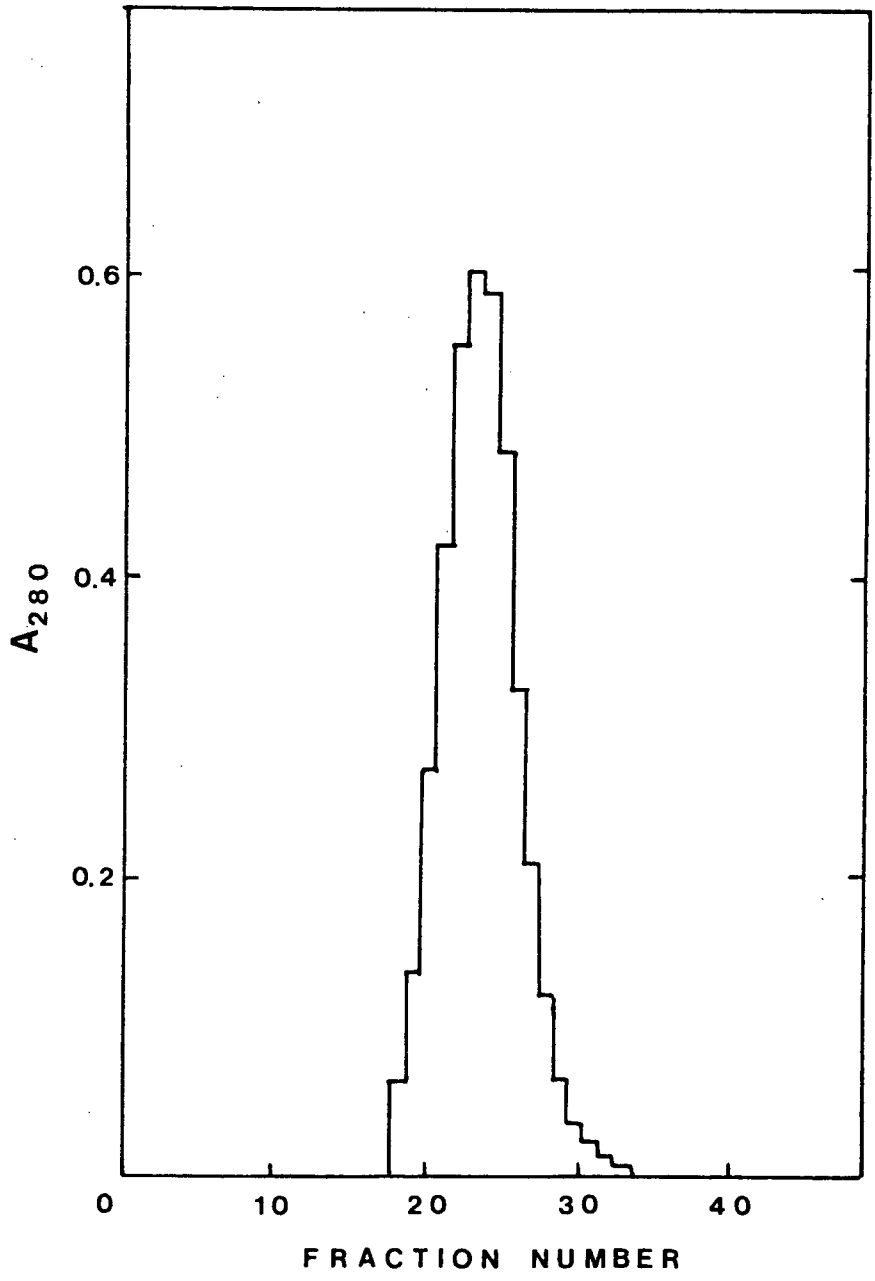

CBS
POOL
3.0
2.0
1.0
0
A_{280}
20
40
60
FRACTION NUMBER

Fig. 5 Immunoadsorption chromatography of CBS pool.

The CBS pool (Fig. 4) was applied to a siliconized column (1.6 x 28 cm) containing an immunoadsorbent prepared by binding antibodies against contaminating protein species to Sepharose 4B (Bridges et al., 1982a). α_2M was eluted with 50 mM Tris HCl, pH 7.5 containing 250 mM sodium chloride and 3 mM sodium azide at a rate of 15 ml/hr. Two ml fractions were collected and monitored for absorbance at 280 nm. All steps were carried out at 4°C.


0.6
0.4
0.2
A_{280}
0
10
20
30
40
FRACTION NUMBER

also interfered with the quantitation of neutral and amino sugars by glc (see Discussion section) and since it is known to interfere in the Lowry protein assay (Rej and Richards, 1974; Peterson, 1979), exchange dialysis against 25 mM sodium bicarbonate was carried out, prior to concentration of the material obtained after immunoadsorption chromatography, in order to remove Tris buffer. Sodium bicarbonate was an appropriate substitute for Tris as the pH of a 25 mM solution (pH = 8.3) exceeds the isoelectric point of α_2M (pI = 5.0 - 5.5) thus α_2M remains in solution (Starkey and Barrett, 1977). Furthermore, as shown in Table II analysis of a model glycoprotein, α_1-acid glycoprotein demonstrated that 25 mM sodium bicarbonate does not interfere with either the Lowry protein or periodate-resorcinol assays. Quantitation of neutral and amino sugars by glc could also be carried out in the presence of sodium bicarbonate as discussed below (p. 68).

II Assessment of Purity of α_2M Isolates

Two methods were used to assess the purity of the α_2M isolates: immunoelectrophoresis and SDS-PAGE.

A. Immunoelectrophoresis

Immunoelectrophoresis of α_2M isolates against antiserum to whole human serum yielded a single precipitin band (Fig. 6). Repetition of the analysis using rabbit antiserum to human α_2M positively identified the material in the arc as α_2M. None of the potential plasma protein contaminants were detected in the final α_2M preparations; approximately 5 percent (w/w) contamination would have been detected (Bridges et al., 1982a).

Table II Determination of the sialic acid and protein content of α_1-acid glycoprotein (α_1AG) in water and in 25 m<u>M</u> sodium bicarbonate (n = 4).

	α_1AG in water	α_1AG in 25 mM $NaHCO_3$
Protein (mg/ml)	0.81 ± 0.02	0.80 ± 0.04[1]
Sialic acid (μmol/100 mg protein)	29.4 ± 1.8	28.2 ± 0.9[1]

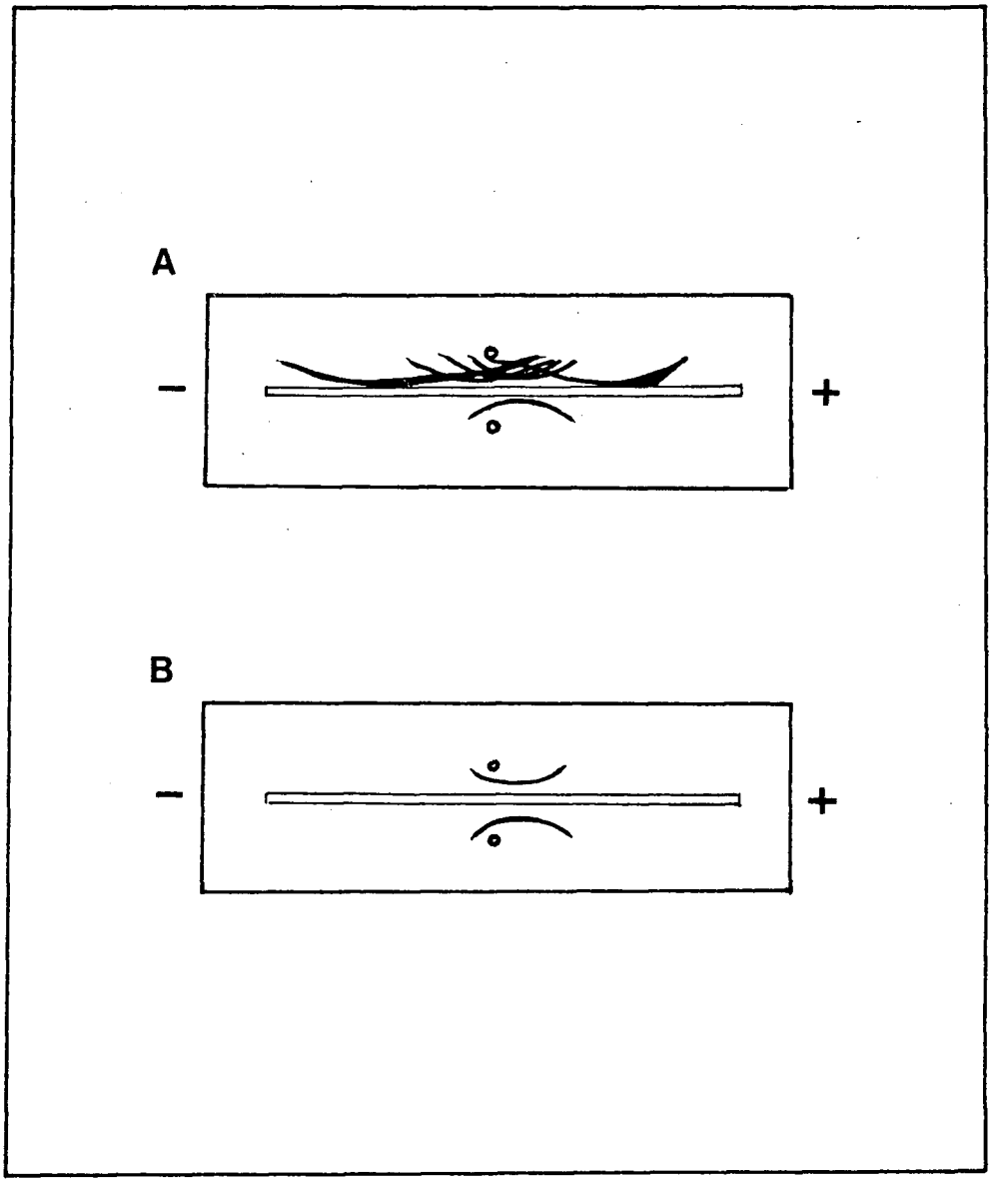
1 Differences between the results of analyses performed in water and in sodium bicarbonate were not significant when evaluated by the t statistic for the comparison of two means.

Fig. 6 Immunoelectrophoresis of α_2M isolates

The upper well of each slide contained human serum (2 µl); α_2M isolated from normal plasma (10 µg) was added to the lower well.

A. Diffusion against rabbit antiserum to whole human serum

B. Diffusion against rabbit antiserum to human α_2M


A
−
+
B
−
+

B. SDS-PAGE

A single high molecular weight band was observed when α_2M (10 µg) was analyzed by SDS-PAGE. Densitometric analysis of the SDS-PAGE tube gels showed that all CF and normal preparations were free of detectable contaminants (Fig. 7).

III. Quantitation of Neutral Sugars and Hexosamines by glc

A. Glc of Alditol Acetates

Solutions in methylene chloride of mixtures of the alditol acetates of fucose, arabinose, mannose and galactose (10 µmol/ml) and of the alditol acetates of mannosamine and glucosamine (10 µmol/ml) yielded well-resolved symmetrical peaks when aliquots (3 µl) were analyzed by glc. Excellent results were obtained under isothermal conditions. Fig. 8 shows typical chromatograms of the alditol acetates of neutral and amino sugars.

B. Determination of Molar Response Factors and Relative Retention Times of the Alditol Acetates

The detector responses and relative retention times of the alditol acetates of neutral and amino sugars were calculated relative to arabinitol and mannosaminitol acetates, respectively. The results of the glc analysis of three mixtures containing equimolar amounts of either the alditol acetates of fucose, arabinose, mannose and galactose (see Appendix B for synthesis) or the alditol acetates of mannosamine and glucosamine (synthesis is outlined in Appendix B) prepared in methylene chloride (10 µmol/ml of each sugar) and analyzed in triplicate were used to calculate the relative molar response factors from the following equation.

Fig. 7 Densitometric scans of control (A) and CF (B) α_2M (20 µg) analyzed on 5% polyacrylamide tube gels in the presence of SDS

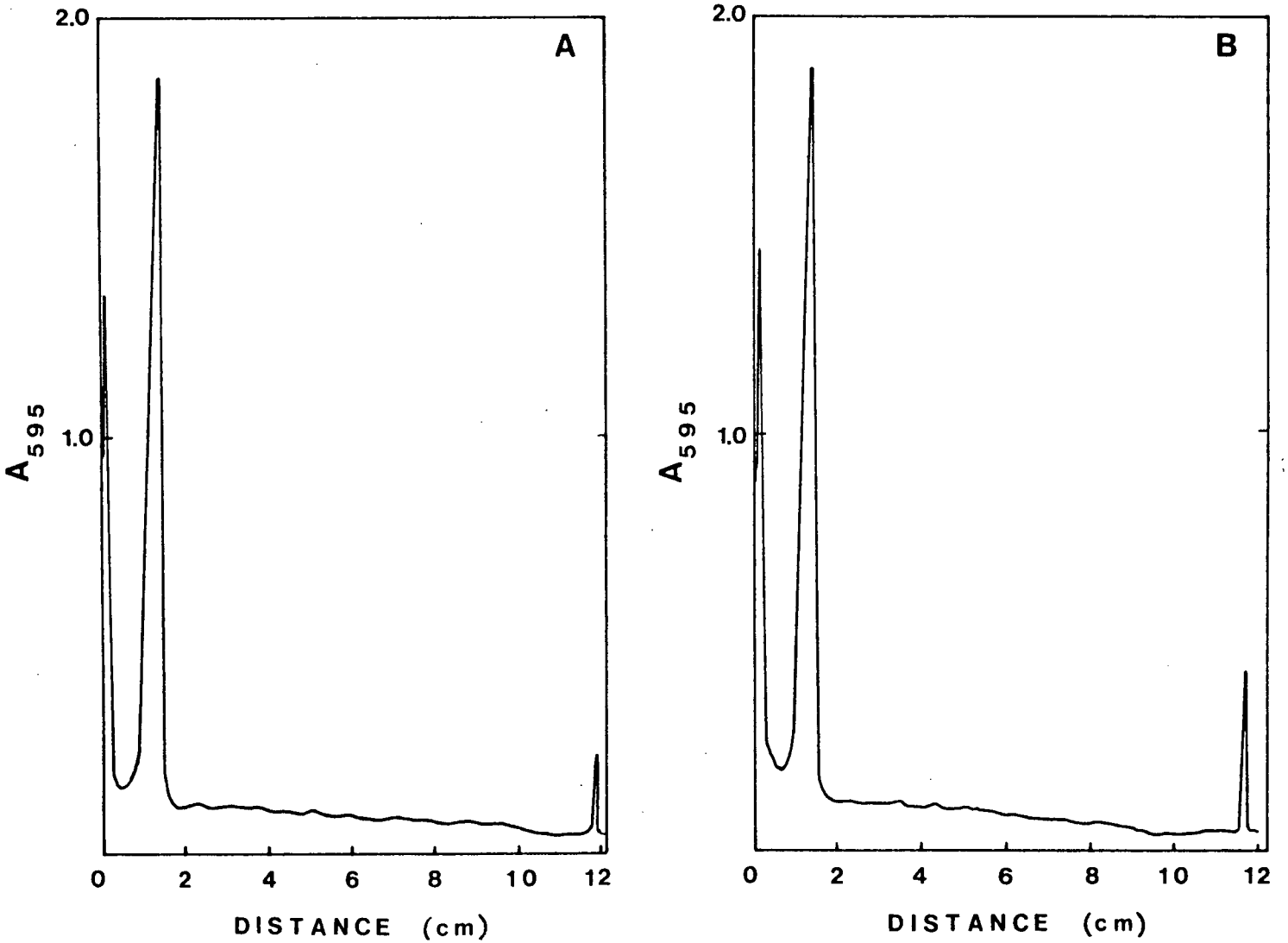
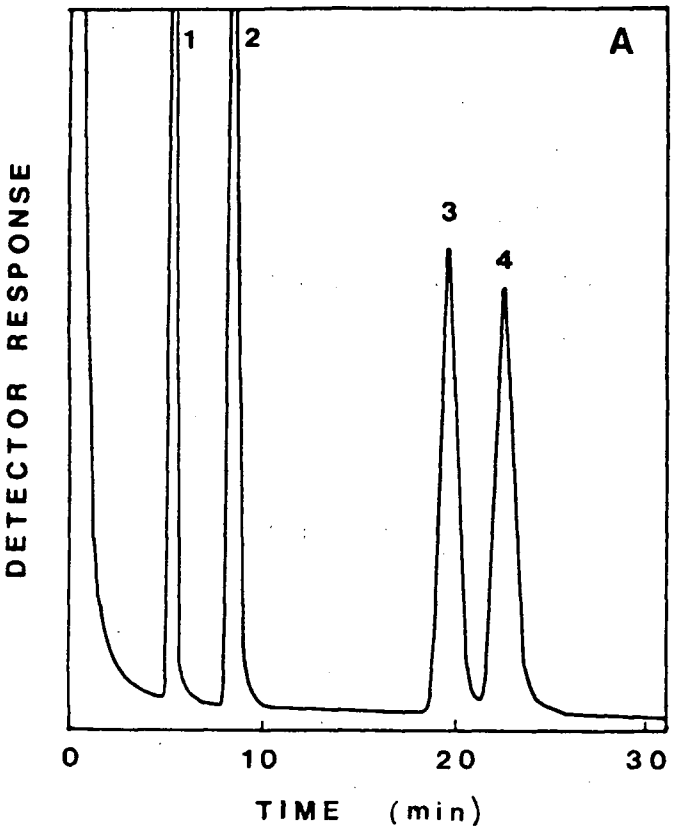
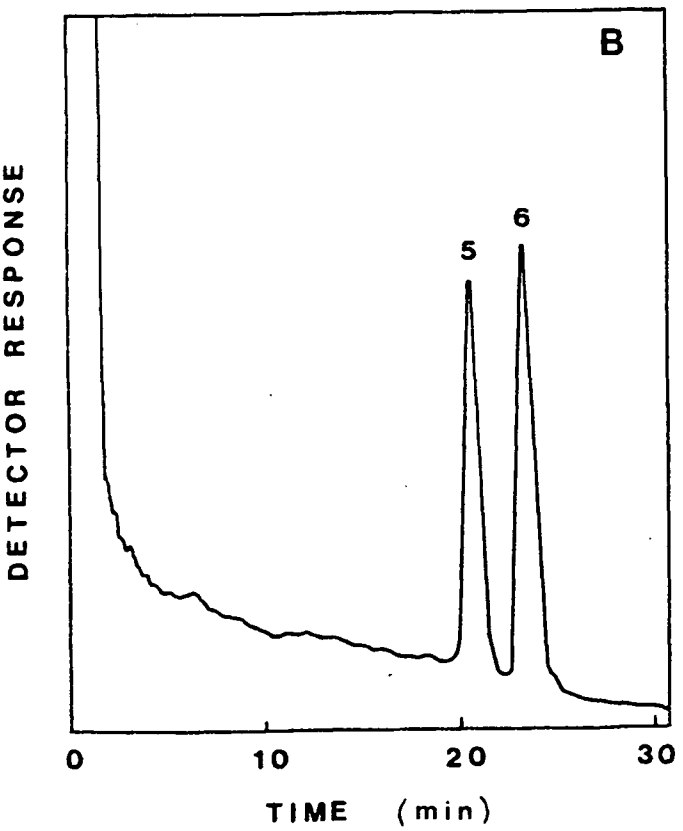

A
B
2.0
1.0
A_{595}
0
2
4
6
8
10
12
DISTANCE (cm)

Fig. 8 Gas-liquid chromatography of the alditol acetates on GP 3% SP-2340 on 100/120 mesh Supelcoport

A. neutral monosaccharides at 200°C
B. hexosamines at 240°C

1 = fucose; 2 = arabinose; 3 = mannose; 4 = galactose;
5 = glucosamine; 6 = mannosamine


A
1
2
3
4
DETECTOR RESPONSE
0
10
20
30
TIME (min)


B
5
6
DETECTOR RESPONSE
0
10
20
30
TIME (min)

$$MRF_u = \frac{\text{peak area}}{\text{peak area standard}} \times \frac{\text{moles standard}}{\text{moles u}}$$

where MRF_u = molar response factor of u and standard = arabinitol pentaacetate (for neutral sugars) or mannosaminitol hexacetate (for amino sugars)

The results obtained are shown in Table III.

C. Materials Interfering with the Quantitation of Neutral Sugars and Hexosamines by glc

Due to the limited amounts of α_2M available for the analyses and its relatively low carbohydrate content, it was necessary to operate the gas chromatograph at levels of high sensitivity. Under these circumstances, contaminants which have not previously been reported to interfere with the quantitaton of the alditol acetate derivatives of the carbohydrate exponents were detected in significant amounts. Material which co-eluted with fucitol acetate (Fig. 9) presented a major problem and was eliminated by ensuring that the acetates were dried *in vacuo* over fresh drying agents. Additional contaminants which interfered with the quantitation of the alditol acetates arose from the use of reagent grade methanol and could be eliminated by using spectro A.C.S. grade methanol (Fig. 10). In addition, it was imperative to wash glassware with a chromic/sulfuric acid mixture in order to remove traces of detergent which also gave rise to interfering peaks.

Table III Means ± S.D. of the relative retention times and molar response factors for the alditol acetates

The values shown represent the average of three replicate preparations analyzed in triplicate. The retention time for arabinitol pentacetate was 8 min while mannosaminitol hexacetate had a retention time of 22 min under the operating conditions described (see Appendix F). The retention times varied between runs depending upon the exact column conditions employed. It should be noted that with the column packing used the absolute retention times decreased with the age of the column.

Parent monosaccharide	Relative retention	Molar response factor
Neutral sugars		
Fucose	0.63 ± 0.01	1.14 ± 0.03
Arabinose	1.00	1.00
Mannose	2.28 ± 0.04	1.13 ± 0.03
Galactose	2.62 ± 0.05	1.15 ± 0.03
Amino sugars		
Glucosamine	0.88 ± 0.00	0.85 ± 0.02
Mannosamine	1.00	1.00

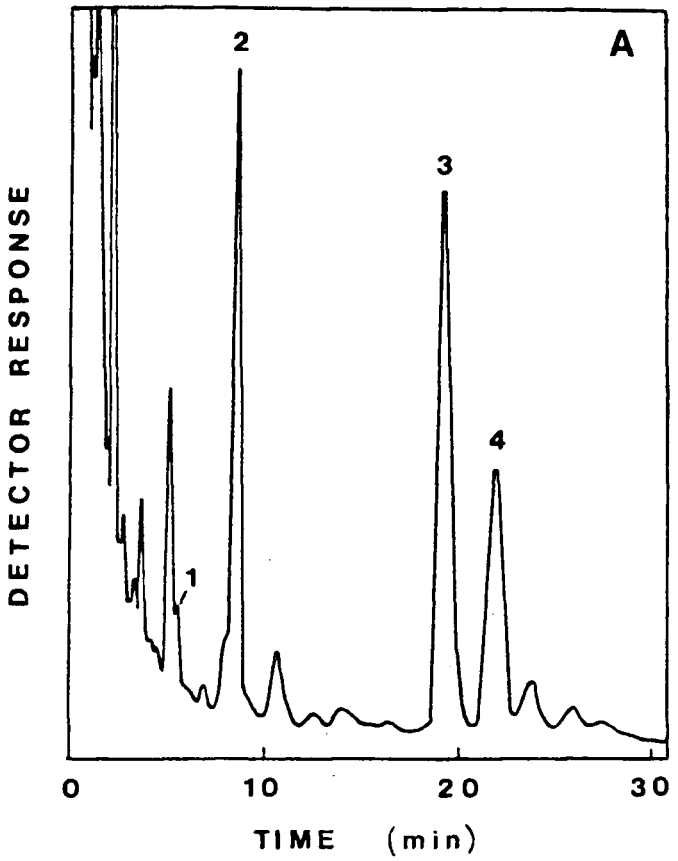
Fig. 9 Analysis of the neutral sugars of α_2 M

A preparation of α_2 M in water was analyzed according to the procedure outline on p. 23 .

A. Acetylated sugars were dried in vacuo over NaOH/conc. H_2SO_4 which had previously been used to dry acetylation mixtures.

B. Acetylation mixture was dried in vacuo over fresh NaOH/conc. H_2SO_4.

1 = fucose; 2 = arabinose (internal standard); 3 = mannose; 4 = galactose


A
2
3
4
1
DETECTOR RESPONSE
0
10
20
30
TIME (min)

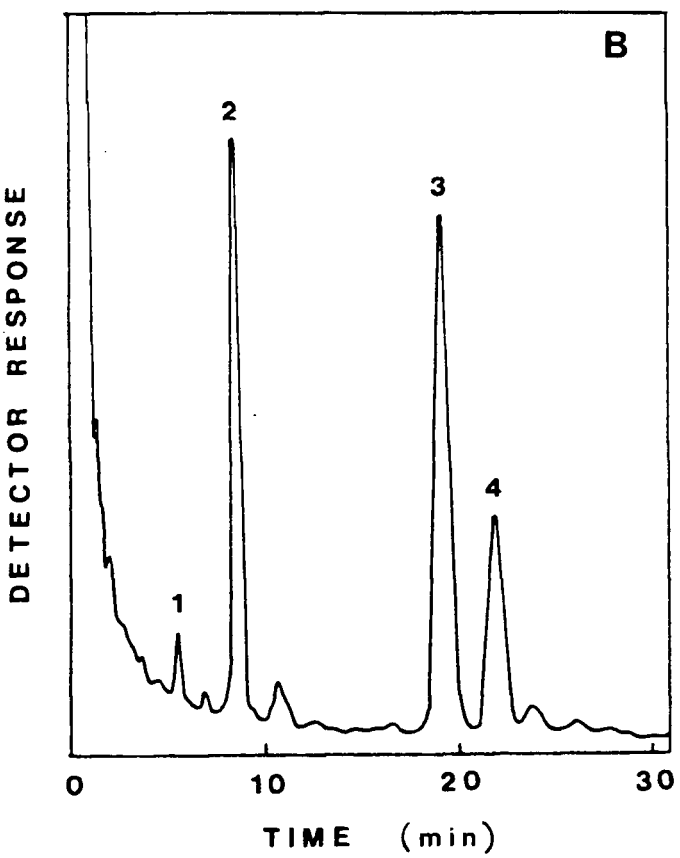

B
2
3
4
1
DETECTOR RESPONSE
0
10
20
30
TIME (min)

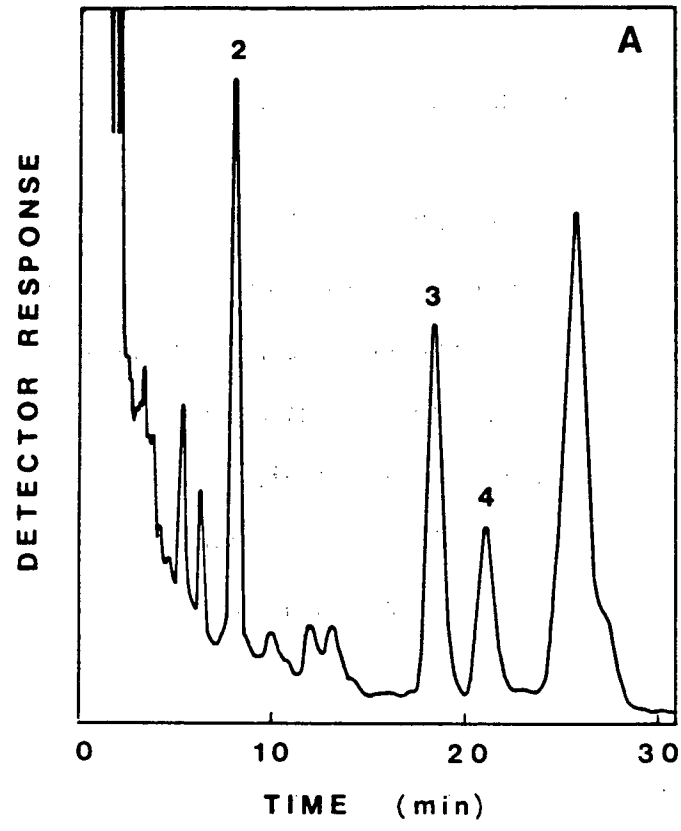
Fig. 10 Analysis of the neutral sugars of α_2M by glc.

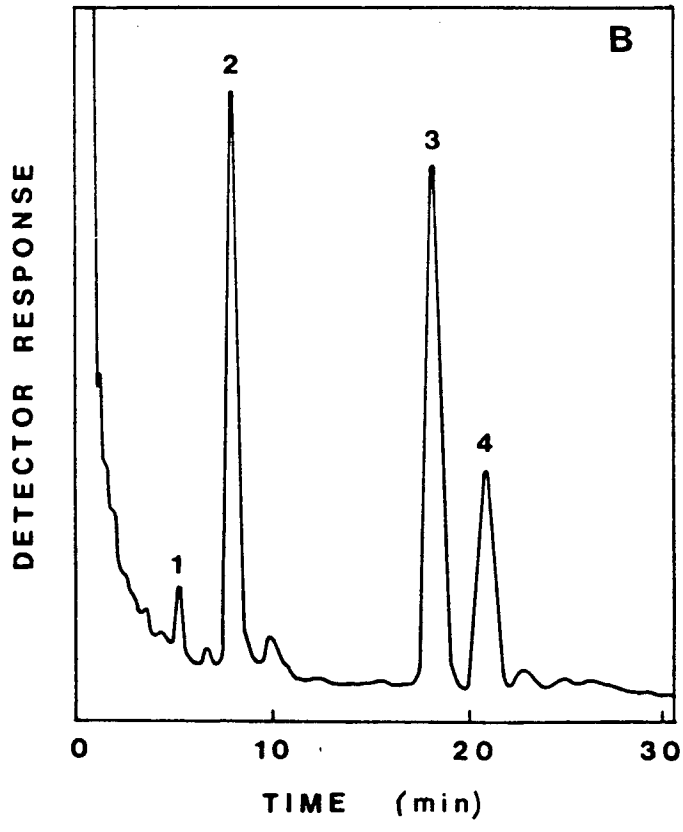
α_2M in water was analyzed according to the standard procedure.

A. Reagent grade methanol was used to remove excess borate.

B. Spectranalyzed ACS grade methanol was employed.

1 = fucuse; 2 = arabinose (internal standard)
2 = mannose; 4 = galactose


A
2
3
4
DETECTOR RESPONSE
0
10
20
30
TIME (min)


B
1
2
3
4
DETECTOR RESPONSE
0
10
20
30
TIME (min)

D. Assessment of the Recovery of Sugars Assayed by the glc Procedure

In order to determine the recoveries of monosaccharides assayed by the glc procedure, aliquots (180 µl) each containing accurately weighed amounts of L-fucose, D-mannose, D-galactose and N-acetyl-D-glucosamine (0.5 - 80 nmol) plus the appropriate internal standard were mixed with AG 50W-X8 [H^+] resin 40% (w/w) in 0.02 M HCl (180 µl) and subjected to the standard procedure (p. 23) omitting the hydrolysis step. The quantity of each sugar was calculated from the following equation:

$$\text{moles sugar} = \frac{\text{Peak area of sugar}}{\text{Peak area of standard}} \times \frac{\text{Moles standard}}{\text{MRF*}}$$

As shown in Table IV, the amounts of neutral and amino sugars recovered (expressed as percent) were statistically different from the amounts of the sugars added. However, there was no significant difference between the recoveries of fucose, mannose and galactose.

In view of this, response factors were calculated from an analysis of an unhydrolyzed mixture of sugars of known composition. Other investigators have used a similar method and express their data as molar response factors. As shown in Table V, when the data were

*MRF calculated from a weighed quantity of alditol acetate (see p.41).

Table IV Mean recoveries of the components of unhydrolyzed mixtures of monosaccharides.

Monosaccharide	% recovery[1] (mean ± S.D.)
Neutral sugars[2]	
Fucose	91 ± 11 (18)
Mannose	93 ± 12 (18)
Galactose	89 ± 7 (18)
Amino sugars	
Glucosamine	79 ± 8 (15)

1 The values for all sugars were significantly different from 100% when analysed by t statistic for comparison of two means

2 The values for neutral sugars did not differ from one another using the t statistic for the comparison of two means

Table V Response factors of the alditol acetates prepared from mixtures of unhydrolyzed monosaccharides.

	This study	Porter (1975)	Niedermeier & Tomana (1974)	Metz et al. (1971)	Neidermeier (1971)	Shaw & Moss (1969)	Lehnhardt & Winzler (1968)
Neutral sugars							
Fucose	1.018	1.05		1.10	1.07	0.99	1.089
Arabinose	1.000	1.00		1.00	1.00	1.00	1.000
Galactose	1.048	1.21		1.15	1.14	1.09	1.211
Mannose	1.014	1.33		1.15	1.25	1.18	1.182
Amino sugars							
Glucosamine	0.688		1.24				
Mannosamine	1.000		1.00				

expressed in this way, they fell within the scatter of values reported by these investigators although the value obtained for glucosamine differed radically from that reported by Niedermeier and Tomana (1974).

E. Time Required for Hydrolysis

As α_2M had not previously been analyzed using the resin hydrolysis method, it was considered necessary to establish the optimal time for hydrolysis. This was determined by hydrolyzing aliquots of normal α_2M (180 µl) containing 300-400 µg protein in the presence of AG 50W-X8 (H^+) ion exchange resin in 0.01 M HCl at 100°C for the following times: 3,6,10,20,30,40,50,60 and 70 hr; two separate aliquots were hydrolyzed at each time interval. In addition, the hydrolysis of the following models was also investigated over a similar time period: α_1-acid glycoprotein (90 µg), a mixture of neutral and amino sugars in approximately the same total amounts as expected in 300-400 µg α_2M (fucose, 2 nmol; mannose 40 nmol; galactose 20 nmol; glucosamine 40 nmol) and lactose (100 nmol) in the presence and absence of bovine serum albumin (100 µg).

On hydrolysis of α_2M, fucose and galactose were released most rapidly as shown in Fig. 11; the maximum amounts of these sugars were obtained after 6 hr of hydrolysis. Mannose and glucosamine were released at somewhat slower rates with maximum amounts of mannose and glucosamine detected after approximately 20 hr. As shown, there was considerable degradation of the released sugars with extended hydrolysis times. On the basis of this study, the hydrolysis time selected for α_2M was 35 hr; this ensured maximal release of mannose and

glucosamine while minimizing the acid degradation of the more rapidly released galactose and fucose. The release of sugars from α_1-acid glycoprotein (Fig. 12) followed a similar pattern to that found with α_2M. Fucose was released most rapidly (maximal release at 6 hr), followed by release of galactose and glucosamine (10 hr) and mannose (20 hr). The relative rates of release of the various sugars are similar to those observed by Lehnhardt and Winzler (1968). A hydrolysis time of 30 hr was selected for α_1-acid glycoprotein rather than the 40 hr reported previously (Lehnhardt and Winzler, 1968).

Figs. 11 and 12 demonstrate that, as shown previously (Lehnhardt and Winzler, 1968; Porter, 1975), sugars were released from glycoproteins at different rates. However, in contrast to these reports, loss of the released sugars was observed with extended hydrolysis times. This was apparently not due to interaction of the free sugars with amino acids as studies on hydrolysis of lactose demonstrated that the release and subsequent loss of galactose was not affected by the presence of bovine serum albumin (Fig. 13). Presumably, the observed loss of the released sugars was a consequence of acid degradation since it was also observed when mixtures of free sugars were hydrolyzed (Fig. 14).

Analysis of the data obtained from the hydrolysis of the free sugars (Fig. 14) indicated that there were significant differences in the rates of degradation of the various sugars. The data shown were re-analyzed by expressing them as the slopes of the regression lines

Fig. 11 Release of sugars from normal α_2M by acid hydrolysis at 100°C in 0.01 M HCl in the presence of 20% (w/v) AG 50W-X8 [H^+] 200-400 mesh resin

(-□-) , glucosamine; (-■-) , mannose;

(-◇-) , galactose ; (-◆-) , fucose.

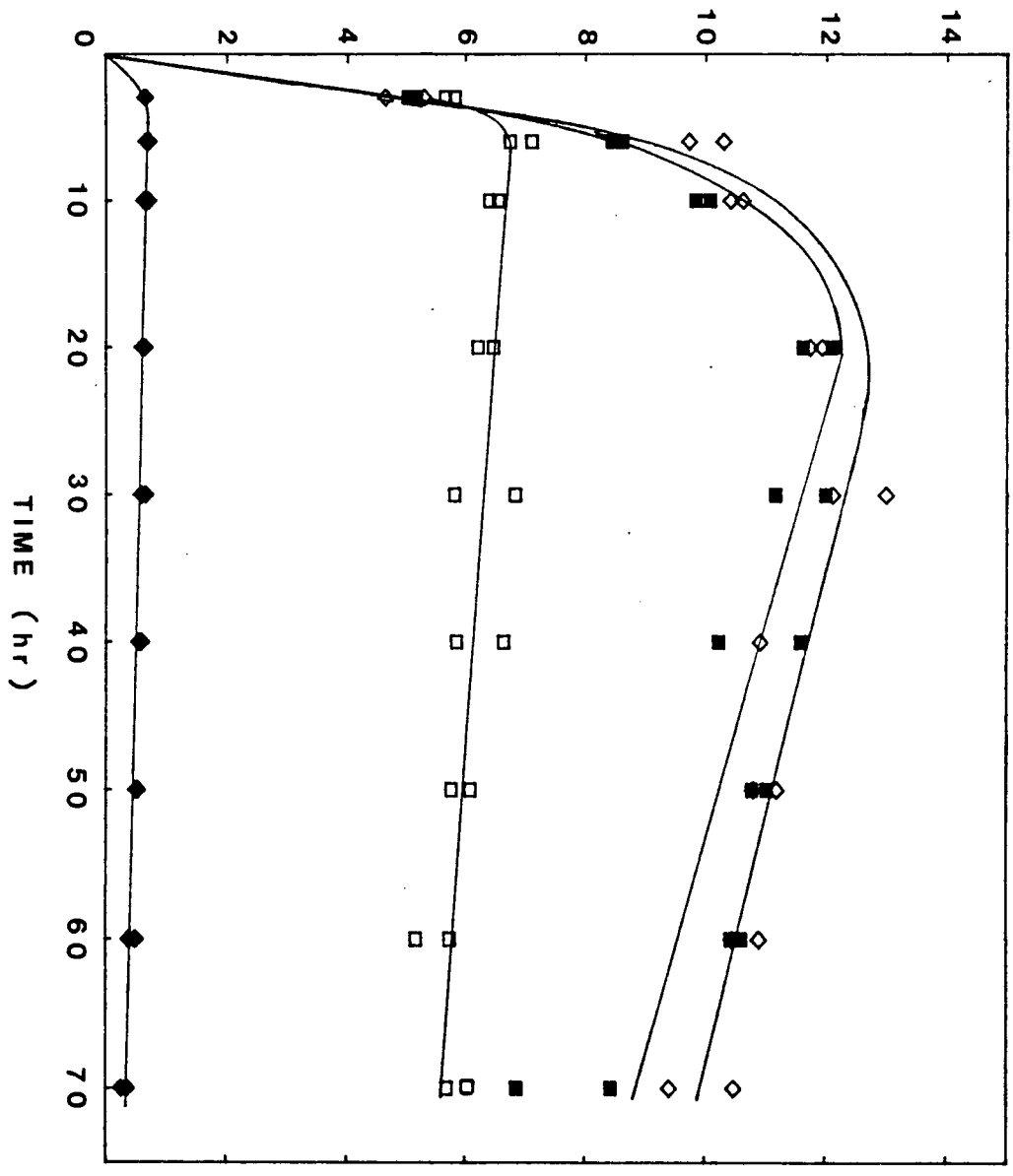

µmol CARBOHYDRATE
100 mg PROTEIN
0
2
4
6
8
10
12
14
TIME (hr)
0
10
20
30
40
50
60
70

Fig. 12 Release of sugars from α_1-acid glycoprotein by aid hydroloysis at 100°C in 0.01 M HCl in the presence of 20% (w/v) AG 50W-X 8 [H^+] 200-400 mesh resin

(-□-) , glucosamine; (-■-) , mannose;
(-◇-) , galactose ; (-◆-) , fucose.

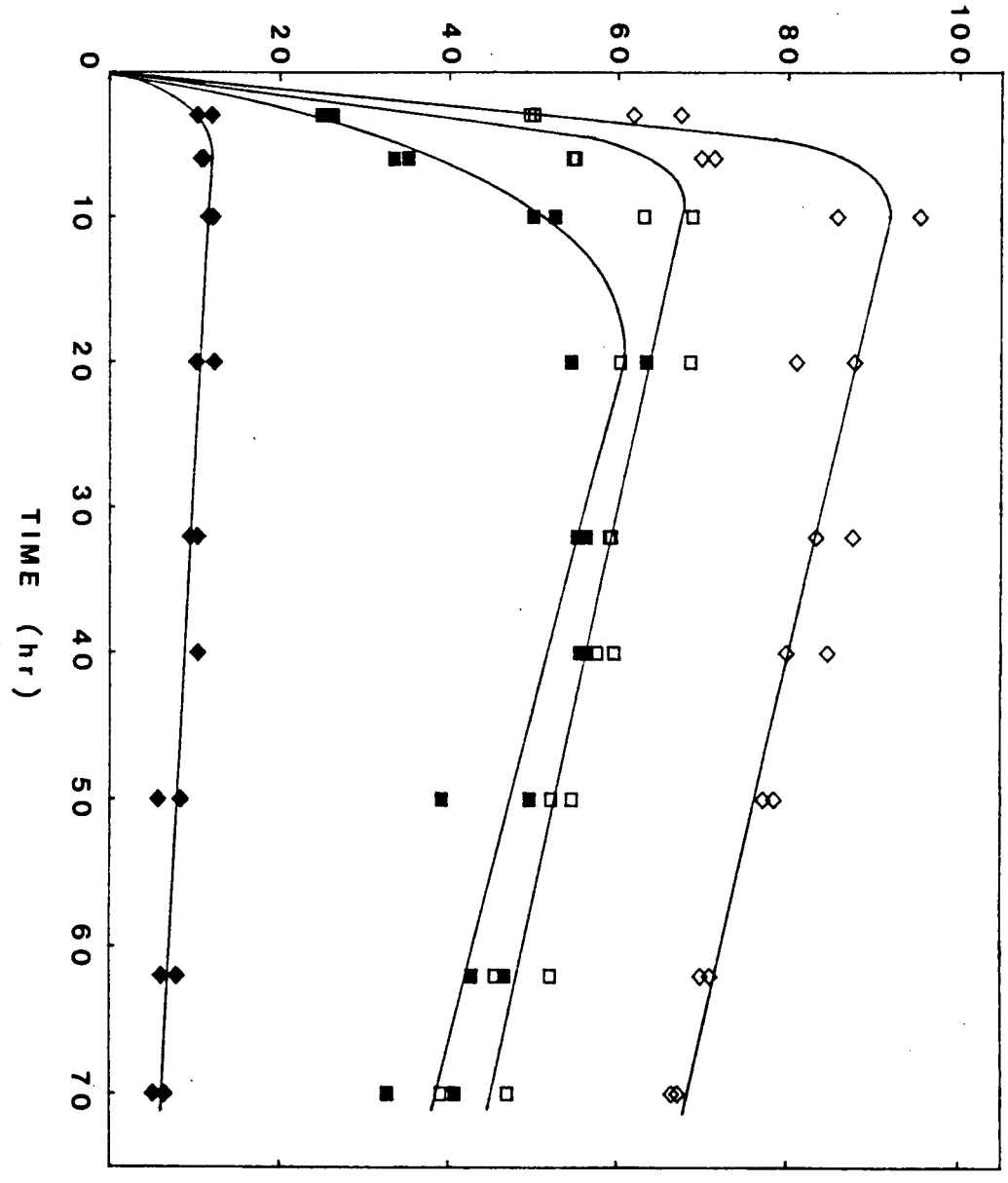

µmol CARBOHYDRATE
100 mg PROTEIN
0
20
40
60
80
100
TIME (hr)
0
10
20
30
40
50
60
70

Fig. 13 Release of galactose from lactose by acid hydrolysis at 100°C in 0.01 M HCl in the presence of 20% (w/v) AG 50W-X 8 [H^+] 200-400 mesh resin.

Lactose (10 µmol) was hydrolyzed in the presence and absence of bovine serum albumin (100 µg).

(-□-) , galactose released from lactose
(-◇-) , galactose released from lactose in the presence of bovine serum albumin.

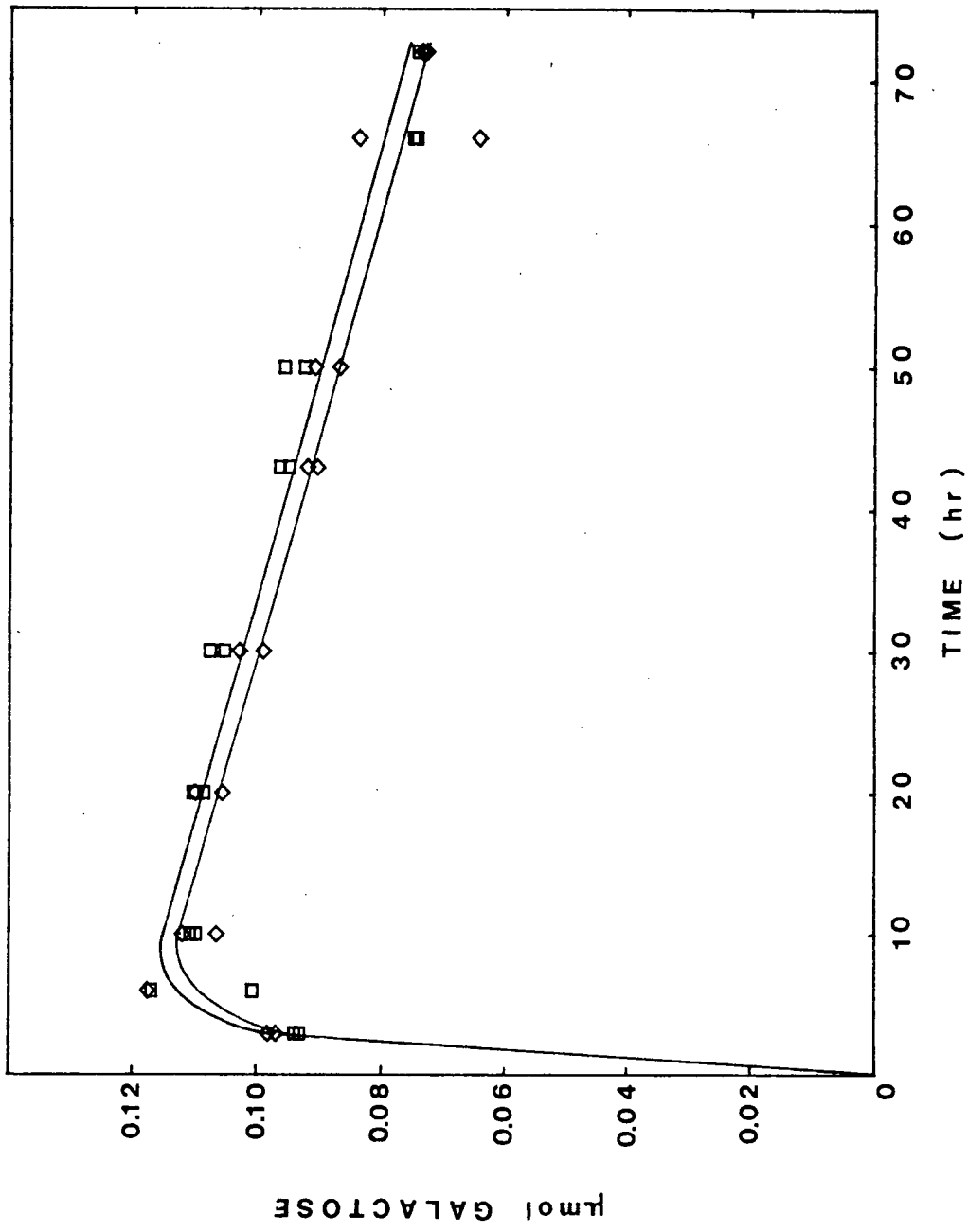
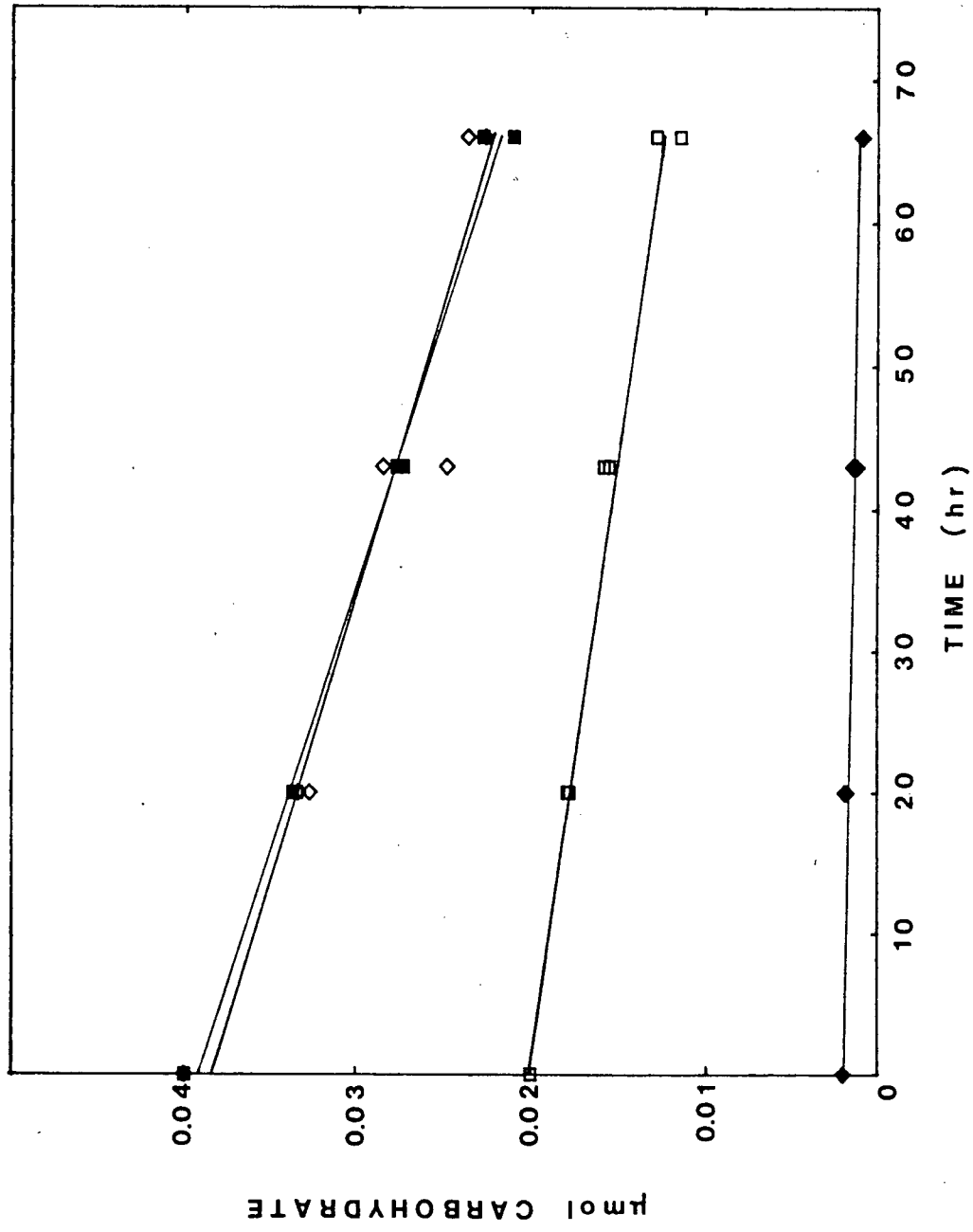

μmol GALACTOSE
0.12
0.10
0.08
0.06
0.04
0.02
0
10
20
30
40
50
60
70
TIME (hr)

Fig. 14 Loss of free sugars during acid hydrolysis at 100°C in 0.01 M HCl in the presence of 20% (w/v) AG 50W-X 8 [H^+] 200-400 mesh resin.

A mixture containing glucosamine, mannose, galactose and fucose was analyzed according to the standard procedure.

(-□-) , glucosamine; (-■-) , mannose;
(-◇-) , galactose ; (-◆-) , fucose.


μmol CARBOHYDRATE
0.04
0.03
0.02
0.01
0
10
20
30
40
50
60
70
TIME (hr)

(Mendenhall et al., 1981). This analysis (Table VI) indicated that fucose was degraded at a significantly different rate to mannose ($P < 0.001$), galactose ($P < .001$) and glucosamine ($P < 0.001$). In addition, mannose was degraded at a different rate to galactose ($P < 0.01$).

F. Effect of Sodium Bicarbonate on the Quantitation of Neutral and Amino Sugars by glc.

In order to determine whether or not sodium bicarbonate interfered with the glc procedure, preparations of α_1-acid glycoprotein (α_1AG) in water and in 25 mM sodium bicarbonate were hydrolyzed for 35 hr and carried through the standard procedure as α_1-acid glycoprotein was used as a model for α_2M. The hydrolysis time used for this and subsequent studies was that selected for α_2M. There was no significant difference between the results obtained from analyses performed in sodium bicarbonate and those obtained when the analyses were carried out in water (Table VII).

Table VI Comparison of the rates of loss of free sugars during acid hydrolysis.

The data shown in Fig. 14 were expressed as a percentage of the amount of each monosaccharide detected at zero time. The regression lines through these data points were determined and the slopes were compared using a t statistic for two means (Mendenhall *et al.*, 1981).

Monosaccharide	Slope	S.D.
Fucose[1]	-0.91	0.09
Mannose[2]	-0.67	0.03
Galactose	-0.60	0.05
Glucosamine	-0.62	0.07

1 The slope estimated from the fucose data was significantly different from those obtained from the mannose, galactose and glucosamine data ($P < 0.001$).

2 The slope estimated from the mannose data was significantly different from those obtained using fucose and galactose data ($P < 0.001$).

Table VII Carbohydrate composition of α_1-acid glycoprotein (α_1AG) in water and in 25 mM sodium bicarbonate.

A solution of α_1AG (1.64 mg/ml in water) was diluted 1:1 with either water or 50 mM $NaHCO_3$ and the sialic acid and protein content of each was analyzed in quadruplicate by the Lowry and periodate resorcinol assays.

µmol/100 mg protein	α_1AG in water (n=3)	α_1AG in 25 mM sodium bicarbonate (n=3)
Fucose	8.8 ± 0.5	8.5 ± 0.4[1]
Mannose	44.8 ± 1.8	44.1 ± 1.2[1]
Galactose	56.3 ± 3.2	56.3 ± 2.7[1]
Glucosamine	102.8 ± 2.8	103.2 ± 3.8[1]

1 t statistic for the comparison of two means. Results for samples analyzed in sodium bicarbonate were not significantly different from those for samples analyzed in water.

G. Precision of the Analytical Methods

The precision of an analytical method as defined by Reed and Henry (1974) is "the variation of results obtained by a method when the same sample is run repeatedly" and is expressed as a coefficient of variation, C.V.:

$$C.V. = \sigma/x \times 100\%$$

where σ = standard deviation

x = mean

The precision of the glc method (n=6) and the periodate resorcinol assay (n=9) was determined by analysis of a preparation of α_1-acid glycoprotein in water. The coefficients of variation shown in Table VIII fell within the range of values (C.V. = 2-10%) reported by other investigators (Niedermeier, 1971; Niedermeier and Tomana, 1974).

H. Proportionality of Response with Amount of Glycoprotein

In order to demonstrate that the amounts of carbohydrate estimated by the glc procedure were proportional to the amount of glycoprotein analyzed, 90,180 and 270 µg of α_1-acid glycoprotein were hydrolyzed for 35 hr according the outlined glc procedure (p.23). The amounts of fucose, mannose, galactose and glucosamine released relative to the amount of α_1-acid glycoprotein hydrolyzed are shown in Fig. 15. These results demonstrate that there is a linear relationship between the amount of carbohydrate estimated by the glc method and the amount of glycoprotein hydrolyzed.

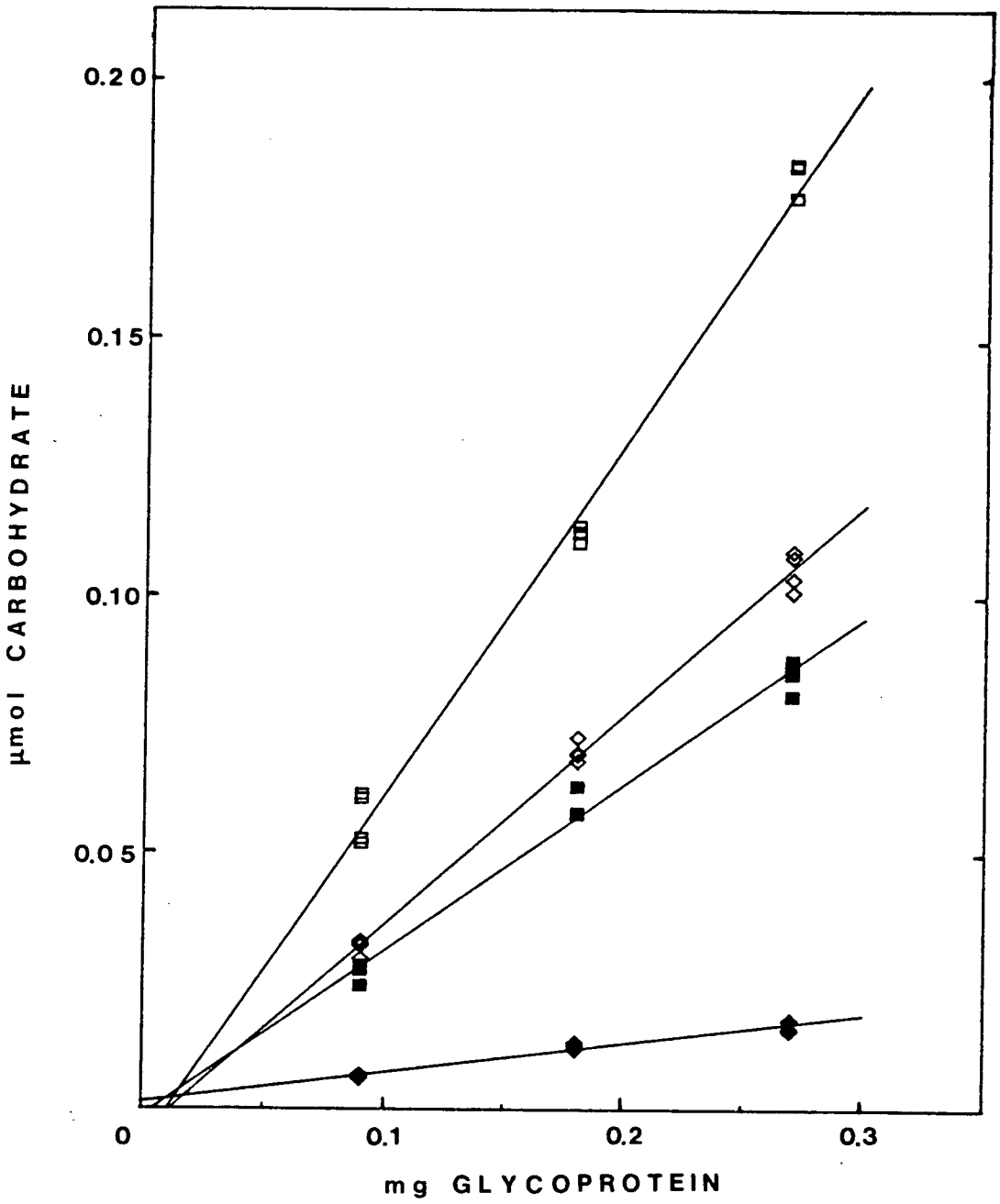
Table VIII Precision of the analytical method on α_1-acid glycoprotein

Following hydrolysis for 35 hr, the neutral and amino sugar content of a preparation of α_1-acid glycoprotein in water was estimated according to the glc procedure described on p. 23 (n=6). The sialic acid content of the unhydrolyzed preparation was determined by periodate resorcinol assay (n=9). The coefficients of variation (C.V.) were calculated from the equation given in the text.

Monosaccharide	Mean Concentration (μmol/100 mg protein)	σ	C.V. (%)
Fucose	8.63	0.43	5.0
Mannose	44.43	1.43	3.2
Galactose	56.30	2.64	4.7
Glucosamine	102.99	2.99	2.9
Sialic Acid	27.63	1.39	4.8

Fig. 15 Proportionality of the quantity of carbohydrate found to quantity of glycoprotein (α_1-acid glycoprotein) hydrolyzed for 35 hr by the standard method.

(-□-) glucosamine, (-◇-) galactose; (-■-) mannose; (-◇-) fucose.


0.20
0.15
0.10
0.05
0
0.1
0.2
0.3
µmol CARBOHYDRATE
mg GLYCOPROTEIN

IV. Analysis of the Carbohydrate Composition of α_2M from CF Patients and Normal Controls.

The carbohydrate composition of α_2M isolated from six CF patients and six age- and sex-matched normal controls is shown in Table IX. There was no significant difference in the neutral sugar, hexosamine, sialic acid or total carbohydrate content of CF and control α_2M. When monosaccharides were expressed as mole % of total carbohydrate (Table X), again no significant differences between CF and control α_2M were observed.

Table IX Carbohydrate composition of α_2M from CF patients and controls.

Neutral and amino sugars were estimated by glc; samples were analyzed simultaneously in duplicate on two separate occasions. Estimation of sialic acid by periodate resorcinol assay was carried out in triplicate twice. The values shown are the mean $\pm$ S.D. of all analyses. (For neutral sugars and hexosamines n=4 and for sialic acid n=6).

Age/Sex	μmol carbohydrate/100 mg protein[1]											
	Fucose		Mannose		Galactose		Glucosamine		Sialic Acid		Total Carbohydrate	
	N	CF	N	CF	N	CF	N	CF	N	CF	N	CF
7y M	0.72±0.11	0.71±0.13	15.17±1.35	14.41±0.86	6.73±0.39	6.66±0.46	15.87±0.33	15.88±1.04	5.50±0.58	5.70±0.47	43.99	43.36
10y F	0.61±0.04	0.67±0.10	13.89±0.79	13.93±1.94	7.11±0.40	7.17±0.52	15.72±1.50	16.00±1.10	5.59±0.19	5.61±0.09	42.92	43.38
12y M	0.73±0.09	0.60±0.09	14.97±0.84	14.10±0.85	6.80±0.53	6.66±0.44	16.49±0.93	15.09±2.08	5.47±0.26	5.61±0.32	44.46	42.06
13y M	0.62±0.09	0.62±0.07	13.83±1.77	15.18±2.02	7.18±0.70	6.68±1.23	17.61±2.17	15.11±1.40	5.66±0.13	5.22±0.15	44.90	42.81
14y F	0.78±0.08	0.74±0.09	15.15±0.96	13.38±0.71	7.02±0.67	6.65±0.24	17.15±1.90	16.28±1.39	5.49±0.37	5.39±0.48	45.59	42.44
18y M	0.68±0.07	0.87±0.03	13.49±0.42	13.41±0.63	6.60±0.44	6.51±0.78	14.14±1.44	14.08±2.02	5.86±0.01	5.66±0.42	40.77	40.53
Mean	0.69±0.11	0.70±0.12	14.42±1.21	14.07±1.31	6.91±0.52	6.72±0.65	16.13±1.77	15.38±1.59	5.58±0.31	5.52±0.33		

[1] No significant differences were observed in the carbohydrate composition of CF and normal α_2^M when data was analysed by paired t-statistic

Table X Carbohydrate composition of α_2M from CF patients and controls expressed as mean mole % calculated from data in Table IX.

	mole % carbohydrate[1]									
	Fucose		Mannose		Galactose		Glucosamine		Sialic Acid	
	N	CF	N	CF	N	CF	N	CF	N	CF
7y M	1.6	1.6	34.5	33.2	15.3	15.4	36.1	36.6	12.5	13.2
10y F	1.4	1.6	32.4	32.1	16.6	16.5	36.6	36.9	13.0	12.9
12y M	1.6	1.4	33.7	33.5	15.3	15.8	37.1	35.9	12.3	13.4
13y M	1.4	1.4	30.8	35.5	16.0	15.6	39.2	35.3	12.6	12.2
14y F	1.7	1.7	33.2	31.5	15.4	15.7	37.6	38.4	12.1	12.7
18y M	1.6	2.1	33.1	33.1	16.2	16.1	34.7	34.7	14.4	14.0

[1]No significant differences were observed in the carbohydrate composition of CF and normal α_2M when data was analyzed by paired t-statistic

DISCUSSION

The aim of this thesis was to compare the carbohydrate composition of CF and control α_2M. To accomplish this, a method which was highly sensitive, precise and accurate was required. The glc method used, a further development of the procedures used by Lehnhardt & Winzler (1968) and Reid et al. (unpubl.), met these criteria. In order to achieve the required degree of precision and accuracy it was found necessary to use highly purified solvents, especially methanol, to avoid the accumulation of contaminants which interfered with quantitation. Arabinose was selected as an internal standard since its alditol acetate derivative separated well from the alditol acetate derivatives of the neutral sugars found in α_2M and in addition, it is not a component of either α_2M or other plasma glycoproteins. It was also a more appropriate standard than the alditol, arabinitol, employed by Lehnhardt and Winzler (1968), as it is chemically similar to the compounds being analyzed. Furthermore, arabinose is subjected to all reactions of the derivatization procedure including reduction. Arabinose has been successfully utilized as an internal standard by Niedermeier (1971).

The amino sugars were quantitated relative to the internal standard mannosamine as first described by Niedermeier and Tomana (1974). The use of a hexosamine as an internal standard (rather than a neutral compound such as inositol, for example), accounts more closely for any losses incurred during the elution of hexosamines from the AG 50W resin. Mannosamine is a suitable internal standard for the

quantitation of hexosamines in glycoproteins such as α_2M which contain only glucosamine. However, application of this method to glycoproteins containing galactosamine or galactosamine plus glucosamine would require the use of a column packing capable of resolving the alditol acetates of mannosamine and galactosamine, for example, Poly A 103 (Niedermeier, 1971).

The excellent separation of the alditol acetates under isothermal conditions eliminated the need for temperature programming and the balanced dual columns used in other procedures. The columns proved sufficiently stable for 200-300 determinations, thus the complete analysis of normal and CF α_2M could easily be performed on one column. It was possible to accurately analyze with a high degree of precision as little as 0.3 mg of α_2M which represents 0.03 mg total carbohydrate. Of the neutral sugar components it was possible to analyze approximately 0.3 μg of fucose. The method also gave a linear relationship between the amount of carbohydrate estimated and the amount of glycoprotein hydrolyzed and the precision of the method as measured by the coefficients of variation for the various sugars was similar to that reported by other investigators (Niedermeier, 1971; Niedermeier and Tomana, 1974; Gehrke et al., 1979). The coefficient of variation obtained for the estimation of sialic acid by periodate resorcinol assay (4.8%) was comparable to the coefficients of variation obtained for the other sugars estimated by glc.

Careful investigation of the hydrolyses of α_1AG and α_2M revealed that there was significant loss of the released sugars with

extended hydrolysis times. This has not apparently been observed previously. Lehnhardt and Winzler (1968) and Porter (1975) found that the release of sugars reached a plateau and concluded that there was no loss of released sugars when hydrolysis was performed for periods of up to 48 hr. Our investigations indicated that a suitable hydrolysis time for α_1AG was 30 hr while that for α_2M was 35 hr Lehnhardt and Winzler (1968) and Porter (1975) reported optimum α_1AG hydrolysis times of 40 and 48 hr, respectively. Our studies showed however, that when the hydrolysis of α_1AG was extended beyond 40 hr there was a significant reduction in the quantity of carbohydrate detected.

The degradation of released sugars was also demonstrated, using as models, lactose, lactose plus BSA and a mixture of monosaccharides. The hydrolysis of lactose was not affected by the presence of BSA indicating that the observed loss of free sugars was not a function of the presence of amino acids; presumably, therefore, the sugars are degraded by the acid. The data suggests that the acid degradation of the sugars occurs continuously and therefore the degradation of sugars released from glycoproteins would be expected to begin immediately. Hence the maximum quantity of carbohydrate detected in a glycoprotein hydrolysate will be less than the actual quantity of carbohydrate present in the glycoprotein. Further, as the free sugars are degraded at different rates, it is clear that data based upon molar ratios obtained at any single hydrolysis time must also be in error to some extent.

A potential method for obtaining values closer the actual values would be to conduct a time course of hydrolysis for each glycoprotein sample. A maximal value for each sugar is obtained at the point at which the rate of release of the sugar equals the rate of its degradation. This does not, however represent the actual amount of carbohydrate present in the glycoprotein. Further, in many cases it would be difficult to perform such an experiment as large quantities of material would be required. An alternative approach is to conduct one hydrolysis time course study using a sample of the glycoprotein under investigation. (Use of the actual glycoprotein is necessary because as shown in Figs. 11 and 12, glycoproteins may behave differently during hydrolysis. Similarly, free sugars are not a suitable model (Fig. 14) because degradation of sugars which are not glycosidically bound begins immmediately.) Selection of a hydrolysis time which guarantees that complete hydrolysis has occurred is then made on the basis of the data. A correction factor is obtained by estimating the percentage loss of sugar that occurs between the time at which a maximum is reached and the selected hydrolysis time. This is then used to correct the data obtained from other samples of the glycoprotein analyzed at the selected time. For example, analysis of the data in Fig. 11 by this method shows that the potential losses of sugars released from α_2M after 35 hr of hydrolysis are as follows: fucose, 23%; galactose, 8%; mannose, 9%; glucosamine, 2%. However, with the exception of fucose, application of such correction factors would alter the values by less than 10%. Furthermore, the values

would be increased by the same percentage for each of the samples analyzed and thus when comparing the carbohydrate analyses of α_2M from CF patients and controls, the interpretation of the corrected and uncorrected data would be identical. The values reported in Table IX are therefore left as the uncorrected values obtained after 35 hr of hydrolysis. No attempt was made to compare hydrolysis behaviours of CF and control samples. The assumption made is that all α_2-macroglobulins behave similarly during hydrolysis.

Previous analysis of normal α_2M by other investigators (Table XI) established the presence of fucose, mannose, galactose, glucosamine and sialic acid. Colormetric methods were used to quantitate these components. The values for the quantities of the various sugars presented in this thesis fall within the scatter of the literature values (see Table XI) although these values and those of other investigators are considerably lower than the data reported by Bottiger and Norberg (1964). The value for sialic acid content obtained by Hall and Roberts (1978) is significantly lower than that presented here or reported by others. Calculations based upon their data give a molar hexose to sialic acid ratio of 21:1. Assuming that the oligosaccharide moieties of α_2M are of the complex type and that the ratios of mannose to galactose reported here and by Dunn and Spiro (1967) are correct, one may calculate the number of sialic acid residues per galactose residue for bi-, tri- and tetraantennary structures (Fig. 1). Calculations of this type indicate that in the best possible case (biantennary structure) which minimizes the number

Table XI Comparison of literature values for the carbohydrate composition of normal α_2M with values obtained in this study. Values in brackets represent the range.

mg/100 mg protein

	This study	Hall and Roberts (1978)	Bourillon and Razafimahaleo (1972)	Dunn and Spiro (1967b)	Bottiger Norberg (1964)	Heimburger et al. (1964)	Muller-Eberhard (1956)
Fucose	0.12 (0.10-0.14)		0.71	0.24		0.1	
Mannose	2.56 (2.41-2.73)			1.86			
Galactose	1.23 (1.17-1.29)			1.22			
Hexose	2.79	2.97	4.5	3.08	5.6	3.6	4.55
Glucosamine	3.49 (3.11-3.89)	2.47	3.95	3.62	4.2	2.9	2.68
Sialic acid	1.72 (1.61-1.81)	0.32	1.83	1.7	2.8	1.8	2.3
Total Carbohydrate	9.12		10.99	8.64		8.40	

of galactose residues, the galactose to sialic acid ratio is still 8:1. This implies that such a glycoprotein would be rapidly cleared from the circulation (Ashwell and Morell, 1974) and it therefore seems likely that the value reported by Hall and Roberts represents analytical error or post-isolational loss of sialic acid.

The only data which can be readily compared with that obtained by the glc method used in this thesis is that of Dunn and Spiro (1967a,b) who identified the hexoses and hexosamines present and were able to estimate by colorimetry the relative proportions of mannose and galactose. While their values for glucosamine and sialic acid content were similar to those obtained here in (Table XI), they obtained less total hexose and a different molar ratio of mannose to galactose. These investigators found that maximal release of mannose and galactose during hydrolysis in 2 $\underline{N}$ H_2SO_4 at 100°C occurred after 4 hr. Degradation of sugars has been shown to occur under less vigorous conditions (1 $\underline{N}$ H_2SO_4 100°C, Lehnhardt and Winzler, 1968). Therefore the mannose and galactose values reported by Dunn and Spiro (1967b) are likely underestimates. While the hydrolysis conditions employed in the present study are somewhat milder, degradation of the released sugars still occurs and the hydrolysis times selected represent a compromise between maximizing the release of mannose and glucosamine while minimizing the degradation of fucose and galactose. However as discussed in a previous section, this must also represent an underestimate of the actual carbohydrate content.

Analysis of the glycopeptides isolated from normal α_2M suggested

that there is considerable microheterogeneity of the oligosaccharide moieties and that the most complete structure is the tetraantennary type shown in Fig. 1 (Dunn and Spiro, 1967b). Insufficient data is available to allow further comments to be made on the structure of the oligosaccharide moieties of α_2M. The latter would require detailed structural analyses of purified glycopeptides obtained from α_2M.

Ben Yoseph et al. (1979) compared the sialic acid content of α_2M from three CF patients and three normal controls and found a 40% decrease in the amount of sialic acid present in CF α_2M. Significant differences in the binding of CF α_2M to the lectins Con A and WGA were also reported (Ben-Yoseph et al., 1979; Shapira and Menendez, 1980) although no differences in the total hexose content of CF and control α_2M were found (Ben-Yoseph et al., 1979). These studies raised the possibility of alterations in the carbohydrate composition and/or structure of CF α_2M.

In contradiction, in the present study, no significant differences were found in the overall carbohydrate content or proportions of the carbohydrate components of α_2M isolated from six CF patients and their age- and sex-matched normal controls. Although small differences in carbohydrate composition would have been masked by either the inherent analytical uncertainty of the methods and/or biological variability in the carbohydrate composition of α_2M, only small analytical and within the population variations were observed.

In view of the fact that the values for the sialic acid content of normal α_2M obtained by Ben-Yoseph et al., (1979) are identical to

those reported herein, it is difficult to explain the significantly reduced sialic acid content of CF α_2M found by these investigators. Further, Comings et al. (1980) were unable to detect any differences in the two-dimensional gel electrophoretic patterns of control and CF α_2M; this technique would be expected to reveal any charge differences due to differences in sialic acid content.

Previous studies on the role of α_2M in the pathogenesis of CF have indicated that there are no functional differences in CF α_2M but did not address the question of whether or not there are differences in its carbohydrate composition. In conclusion, the data presented in this thesis demonstrates that no such differences exist and therefore, if α_2M is involved in the pathogenesis of CF then differences in the α_2M molecule must be at a structural level not yet investigated.

APPENDIX A

Preparation of Ion Exchange Resins

AG 50W-X8 [H^+] resin was prepared as follows. The resin was stirred with excess 1 M NaOH, filtered under vacuum using a glass fiber filter and washed with distilled water until the eluate was neutral. The resin was then stirred with excess 1 M HCl, filtered and washed with distilled water until neutral. The resin was prepared as a 40% (w/v) suspension in 0.02 M HCl.

AG 1-X8 [Cl^-] resin was converted to its bicarbonate form by the following procedure. The resin was treated with excess 1 M HCl, washed with water, then treated with excess 1 M NaOH and washed with water until neutral as described above. This was followed by addition of excess 1 M $NaHCO_3$ and the resin was washed with water until neutral. A 20% (w/v) suspension of the treated resin was prepared in distilled water. Both resins were prepared immediately before required.

APPENDIX B

Preparation of Alditol Acetate Standards

The hexitol acetate derivatives of D-galactose and D-mannose were prepared from the corresponding hexitols according to the procedure of Abdel-Akher et al. (1951) with the following modifications. To the hexitol (10 g) was added pyridine (133 ml) followed by acetic anhydride (78 ml); the mixture was stirred and, in the case of mannitol heated gently, in order to dissolve the hexitol. The acetylation mixture was left at room temperature overnight after which it was poured into a stirred mixture of ice and water (1 L). The precipitate was collected by filtration and washed with ice-cold water until the odor of pyridine could no longer be detected. Galactitol and mannitol hexaacetates were recrystallized from absolute ethanol to constant melting point.

L-Fucitol and L-arabinitol pentaacetates were prepared from their respective parent carbohydrates as follows. To fucose or arabinose (2 g) dissolved in distilled water (20 ml), was added an equal weight of sodium borohydride in 20 ml of distilled water. After 24 hr at room temperature excess sodium borohydride was destroyed by addition of acetone (10 ml) and the reduced product was stirred for 1 hr with an excess of AG 50W-X2 (H^+) ion exchange resin. The resin was removed by filtration on a Buchner funnel fitted with a glass fiber filter and the filtrate was evaporated to dryness. Boric acid was removed by distillation with 0.1% HCl in methanol (4 x 40 ml) followed by methanol (2 x 40 ml). The flask containing the syrup was stoppered,

placed on its side and left at -20°C until the syrup had frozen. The flask, still on its side, was rotated 180° and a few ml of methanol was added such that the syrup adhering to the side of the flask was suspended above a pool of methanol. Crystallization of the product was effected by leaving the stoppered flask in this position at -20°C overnight. The crude product was collected by suction on a Buchner funnel and dried in vacuo before being acetylated according to the procedure outlined above. Fucitol and arabinitol pentaacetates were recrystallized from absolute ethanol to constant melting point.

The polyol acetates of D-glucosamine and D-mannosamine could not be obtained in crystalline form by the above methods and were consequently prepared by reduction and acetylation of accurately weighed amounts of the corresponding parent carbohydrates according to the method outlined in Section Vii. On gas chromatography, all of the polyol acetates eluted as single symmetrical peaks.

APPENDIX C

Apparatus for Mixing and Heating Samples During Hydrolysis

Reacti-vials were enclosed in a wire mesh basket and fastened to the bar of a tumbler mixing apparatus in such a manner as to provide end-over-end mixing of samples; this apparatus was secured to the bottom of a thermostatically controlled oven set at 100^{o}C. The shaft of the mixing bar extended to the outside through a small hole drilled in one side of the oven and connected by flexible mechanical drive to a 1/20 HP electrical motor.

APPENDIX D

Preparation of GP 3% SP-2340 Columns for Gas-liquid Chromatography

An empty U-shaped glass column (183 cm x 2 mm ID) was filled with 2% (v/v) dimethyldichlorosilane in toluene using a 30 ml syringe and allowed to stand at room temperature for 15 min. After silylation, the column was rinsed successively with ten 10 ml portions of toluene and methanol and was air-dried for 24 hr.

A small plug of glass wool was inserted into one end of the column (detector end). This end of the column was connected to a vacuum line and moderate suction was applied. Column packing was added to the other end of the column via a small funnel and even settling of the packing was achieved by vibrating the column with the chuck of an electric engraving tool. The column was packed to within 5 cm of the open end and then plugged with glass wool. This end was connected to the injection port of the gas chromatograph.

APPENDIX E

Column Conditioning Procedure

The packed column was fitted with 1/4" Vespel ferrules, installed in the oven of the gas chromatograph and purged at ambient temperature for 30 min with the carrier gas flowing (N_2 = 25 ml/min, 40 psi). The oven temperature was then programmed from 50°C to 250°C at 2°C/min and maintained at 250°C overnight (approximately 16 hr). The detector assembly was disconnected during the conditioning procedure to prevent deposition of contaminants on the collecting ring.

APPENDIX F

Operating Conditions for Gas Chromatography

Column temperature:	$200^{o}C$ (neutral sugars)
	$240^{o}C$ (amino sugars)
Injection port temperature:	set 25^{o} above column temperature
Detector temperature:	set 25^{o} above column temperature
Carrier gas:	N_2 = 25 ml/min, 40 psi with
	auxiliary flow = 35 ml/min
Air:	500 ml/min, 40 psi
Hydrogen:	40 ml/min, 20 psi
Program mode:	isothermal
Range:	10^{3} (signal fed through integrator)

Integrator settings

Noise suppression:	maximum
Slope sensitivity up:	0.03 mv/min
down:	0.03 mv/min
Baseline reset delay:	zero
Area threshold:	1000
Shoulder control front:	on
rear:	1000 mv

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