Isolation and Characterization of a Glycoprotein from Human Colostrum*

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(Received 11 May 1973)

A glycoprotein was isolated from the M-1 acid glycoprotein fraction of human colostrum. It had a molecular weight of 31 200 and contained 27% galactose, 21.7% hexosamine, 8.0% fucose and 10.8% sialic acid by weight. The glycoprotein had no absorption maxima in the 240–300nm region, and was virtually free of ABH(O) and M and N blood-group activity. Alkaline borohydride cleavage of the glycoprotein resulted predominantly in the destruction of threonine and galactosamine.

The acid glycoprotein fraction of human colostrum may play a significant role in the health and well-being of the newborn infant (György *et al.*, 1954*a,b*; Hirano *et al.*, 1968). Partial purification of human colostrum acid glycoproteins by DEAE-cellulose and ethanol-precipitation techniques has been achieved (Got *et al.*, 1963; Hirano *et al.*, 1968). The present paper describes the isolation and characterization of one of several acid glycoproteins present in human colostrum.

Experimental

Materials

Colostrum. Human colostrum was collected from individual mothers no more than 4 days after parturition and was de-fatted and frozen immediately. The samples were thawed overnight at 4°C, pooled and merthiolate (1 mg/100ml) was added as preservative and clear whey was prepared as described previously (Bezkorovainy, 1965).

Proteins. Human serum orosomucoid and transferrin (Bezkorovainy, 1965, 1966) were reference compounds in ultracentrifugal and polyacrylamide-gel-electrophoresis analyses respectively. Human serum albumin was purchased from Dade Corp., Miami, Fla., U.S.A., and was the standard in the Lowry protein test (Lowry *et al.*, 1951).

Chromatographic media. DEAE- and CM-cellulose (coarse mesh), with exchange capacities of 0.91 and 0.70 mequiv./g respectively, were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Chemicals. Aniline was purchased from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A., and was redistilled from zinc (Wilson, 1959); *o*-phthalic acid was obtained from Eastman-Kodak Co., Rochester, N.Y., U.S.A., and 98% pure NaBH₄ from

Metal Hydrides Inc., Beverly, Mass., U.S.A. All other chemicals were of reagent-grade quality and were purchased from Fisher Scientific Co., Chicago, Ill., U.S.A.

Methods

Fractionation procedures. DEAE-cellulose chromatography was carried out in two parts and utilized the discontinuous-gradient elution method essentially as detailed elsewhere (Bezkorovainy, 1965), except that it was extended to include 1.0M-sodium acetate buffer, pH 5.0, and 10ml effluent volumes were collected at an average flow rate of 30ml/h. A column (2cm × 40cm) was employed to accommodate 1 litre of fluid. Fractions were monitored for protein by absorption measurements at 280nm and for hexose by the phenol-H₂SO₄ method (Dubois *et al.*, 1956). The appropriate effluent fractions were pooled, dialysed against three changes of 100vol. of distilled water at 4°C over 72h and freeze-dried. Corresponding peaks from each DEAE-cellulose fractionation column were combined, and 70–150mg of each were dissolved in 0.03M-sodium acetate buffer, pH 4.1, and applied as such to CM-cellulose columns (1.1cm × 20cm) equilibrated with the same buffer. The M-1 fractions were eluted at the void volume and were rechromatographed in the same medium on a column (1.5cm × 35cm). Their homogeneity was judged by ultracentrifugal, polyacrylamide-gel- and moving-boundary-electrophoresis criteria. The M-2 fractions were eluted from the column with the same buffer having an increased ionic strength (*I* = 0.5) and were not further purified at this stage.

Physical measurements. Most of the techniques employed herein have been described in detail elsewhere (Bezkorovainy, 1967; Bezkorovainy & Grohlich, 1969). Ultracentrifugal analyses were performed in a Spinco model E apparatus at 20°C and

* This paper is dedicated to the memory of Richard J. Winzler.

52000 rev./min at four different protein concentrations, over the range of 0.375–1.0%, and sedimentation constants were calculated from concentration–sedimentation plots (Schachman, 1957). Diffusion-constant measurements were made in a Spinco model H apparatus at 2.7°C at several protein concentrations, and the diffusion coefficient, evaluated from interference-fringe patterns (Svensson, 1951), was reported as an average of these values. Molecular weights were calculated from the sedimentation and diffusion results by the Svedberg equation. Partial specific volumes were calculated (Schachman, 1957). The buffer used in the physical studies was 0.05M-sodium cacodylate–0.1M-NaCl, pH 7.0. It had a density and relative viscosity of 1.0057 g/ml and 1.030 respectively at 25°C (Bezkorovainy & Grohlich, 1969).

U.v. absorption spectra, in addition to single wavelength measurements, were made in a Zeiss PMQ-II spectrophotometer in both the u.v. and visible ranges. Where column effluents were assayed for hexose, a Coleman 6/20 Jr. II spectrophotometer was used instead. Tryptophan content was estimated by the method of Bencze & Schmid (1957). Optical rotations were measured at 20°C in a Perkin–Elmer 141 polarimeter by using the sodium light source (589 nm). A standard cell of 1 ml capacity was used and the protein concentration was 1%.

Moving-boundary electrophoresis was performed at 4°C in a Perkin–Elmer model 238 apparatus in 0.1M-sodium barbital buffer, pH 8.6 ($I=0.1$). The 9 mm cell was used and the current was 6 mA. Electrophoretic mobilities were determined by the current–cross sectional area–buffer resistance method of Longworth (1959). Polyacrylamide-gel electrophoresis (Davis, 1964) was carried out at room temperature in a Bio-Rad model 150 vertical cell for 3 h at 40 V and 3 mA/tube. The buffer was 0.05M-sodium barbital, pH 8.6, and the polyacrylamide concentration was 7.5% (w/v).

Analytical procedures. Hexose was determined by the cysteine–H₂SO₄ assay (Dische *et al.*, 1949) and a solution of galactose was used as standard. Methylpentose was measured as fucose by the method of Dische & Shettles (1948). Hexosamines were assayed as their hydrochloride derivatives by the Gatt & Berman (1966) modification of the Elson–Morgan reaction and as their acetyl derivatives by the method of Reissig *et al.* (1955) after acetylation by the method of Roseman & Daffner (1956). Samples were hydrolysed in 2M-HCl for 10 h at 100°C before being assayed for amino sugars. Standards were solutions containing different molar ratios of glucosamine/galactosamine hydrochloride (1:1–3:1). Sialic acid was measured by the thiobarbituric acid method (Warren, 1959). Amino acid analyses were done in a Spinco model 120 apparatus by the method of Moore *et al.* (1958), essentially as described by Bezkorovainy & Grohlich (1969), except that 2 mg of protein in 1 ml of constant

boiling HCl was used. Amino acid values were corrected for losses owing to acid hydrolysis in the presence of substantial quantities of carbohydrate by the method of Anderson *et al.* (1965). Amino sugars were also quantitated in the same apparatus after acid hydrolysis under the conditions described by Gatt & Berman (1966) for the colorimetric determination of hexosamines. These analyses were done on three columns: ‘long’ (0.9 cm × 69 cm); ‘medium’ (0.9 cm × 17 cm); and ‘short’ (0.9 cm × 5 cm). The absorbance value of glucosamine was used in the calculations.

Both the semi-quantitative and qualitative assays of neutral sugars employed the paper-chromatographic technique of Wilson (1959), after prior hydrolysis in 1M-HCl under N₂ for 4 h at 100°C and freeze-drying to remove the acid. No corrections for losses of sugars under these conditions have been applied. The molar ratio of glucosamine/galactosamine was determined by two additional procedures. First, by quantitating the arabinose and lyxose formed (Wilson, 1959) after ninhydrin degradation as applied by Bezkorovainy & Grohlich (1969) and, secondly, by assaying, at two concentrations, portions of the hydrolysate of the glycoprotein, along with the hexosamine standards in both the modified Elson–Morgan and Morgan–Elson reactions, and matching the values in the former reaction with those in the latter reaction.

Materials to be assayed were dried to constant weight at 13.3–1.33 Pa in a desiccator over P₂O₅.

Serological tests. ABH(O) and M and N hemagglutination–inhibition assays were performed by Mrs. H. Tegtmeyer in Professor G. F. Springer’s laboratory by established techniques (cf. Springer, 1956; Springer *et al.*, 1966). The volume of all reagents used was 0.02 ml and the M-1 glycoprotein concentration was 0.5%.

Alkaline borohydride treatment of proteins. The conditions of alkaline reductive cleavage were modified from those of Donald *et al.* (1969). Protein (2 mg in 1 ml of 0.2M-NaOH solution containing 0.5M-NaBH₄) was kept under N₂ in the dark for 46 h at 37°C. Excess of borohydride was destroyed by the addition of 0.5M-HCl to pH 5–6. The reaction mixture was freeze-dried and excess of boric acid removed as trimethylborate by repeated addition of HCl–methanol (1:1000, v/v) and evaporation *in vacuo*. Amino acid and amino sugar analyses were then performed in parallel with untreated protein as described above.

Results

Isolation of the colostrum M-1 glycoprotein

The isolation of the colostrum glycoprotein is depicted in Fig. 1, where human colostrum whey, at pH 4.5, was first passed through a DEAE-cellulose

Fig. 1. Isolation of the human colostrum M-1 glycoprotein

Dialysed colostrum whey (0.99 litre; $\Omega = 1600$), adjusted to pH 4.5 with acetic acid, was passed over a column (2 cm $\times$ 40 cm) of DEAE-cellulose equilibrated with 0.03 M-sodium acetate buffer at pH 4.5 and was washed with the same buffer until the column was cleared of non-absorbed protein. Fractions A and B were eluted with 0.1 M-sodium acetate buffer and fractions C–F were successively eluted with 0.2 M-, 0.3 M-, 0.5 M- and 1.0 M-sodium acetate buffers, pH 5.0 (shown by arrows), yielding 254.7, 77.4, 175.4, 33.1, 30.1 and 41.0 mg of dry material respectively. Fraction C of column in 1(a) was subfractionated by elution from CM-cellulose columns in 1(b) and 1(c). The amount applied to column in 1(b) was 150 mg and the quantities recovered were 89.1 and 31.1 mg for fractions CI and CII respectively. All of fraction CI was applied to column in 1(c) and 75.0 mg of purified material (CIA) was recovered. —, E_{280} ; ----, E_{490} (hexose-phenol chromogen).

column, and the M-1 fraction retained (Fig. 1a, fraction C) was rechromatographed on a CM-cellulose column. The glycoprotein so obtained (Fig. 1b, fraction CI) accounted for 50%, by weight, of fraction C. It gave a single, faint band in polyacrylamide-gel electrophoresis on staining with Amido Black. It migrated faster than human serum transferrin but more slowly than human serum albumin.

Properties of the glycoprotein

Physical parameters. The homogeneity of the M-1 glycoprotein was confirmed by ultracentrifugal and moving-boundary-electrophoresis criteria. A single component was observed in the ultracentrifuge at a relative position that indicated its molecular weight to be less than that of human orosomucoid when tested at the same concentration.

The physical parameters of the M-1 glycoprotein are listed in Table 1. Its molecular weight was less than that of orosomucoid.

The u.v. absorption spectrum of the glycoprotein was virtually flat between 240 and 300 nm, thus demonstrating little absorption in the u.v. range. The $E_{280}^{1\%}$ was 1.27 at pH 7.0.

Composition of the colostrum M-1 glycoprotein

The carbohydrate composition of the glycoprotein is listed in Table 2.

Acid hydrolysis with subsequent paper-chromatographic analysis revealed that galactose was the only hexose present. In addition, it was confirmed that fucose was the only methylpentose present.

The molar ratio of the hexosamines, as determined in the amino acid analyser, was in good agreement with the results obtained by the direct colorimetric procedures. The use of three columns for the analysis of these sugars, which gave identical results, assured that no peptides were eluted with the hexosamines to give artificially high values. The quantities of hexosamine and *N*-acetylhexosamine, as determined in the

Table 1. *Physical properties of the human colostrum M-1 glycoprotein and a bovine colostrum M-1 preparation*

Ultracentrifugal, diffusion and optical-rotation experiments were done in 0.05M-cacodylate-0.1M-NaCl buffer, pH7.0, at 20°, 2.7° and 20°C in the Spinco model E, Spinco model H and the Perkin-Elmer model 141 apparatus respectively. The partial specific volume was obtained from composition data and the molecular weight was calculated by the sedimentation-diffusion method.

	Colostrum glycoproteins	
	Human	Bovine*
Sedimentation constant ($s_{20,w}^0$)	2.45	1.43
$\frac{ds}{dc}$ (S/mg)	0.051	—
$10^{-5} \times$ Electrophoretic mobility at pH8.6 ($\text{cm}^2/\text{s per V}$)	-3.27	-5.75
Molecular weight	31 200	12 100
Specific rotation ($[\alpha]^{20}$)	-48	—
Partial specific volume (ml/g)	0.663	0.685

* Values from Bezkorovainy & Grohlich (1969).

modified Elson-Morgan and Morgan-Elson reactions respectively, matched well when read from the standard curves of both reactions in which the standard solution containing a glucosamine/galactosamine ratio of 3:1 was used. The arabinose and lyxose found on ninhydrin degradation of the hexosamines contained in the M-1 glycoprotein hydrolysate also were in agreement with a 3:1 ratio, thus again confirming the results from the amino acid analyser.

The protein contains large amounts of threonine, proline, serine, glutamic acid, alanine and valine (Table 2). There are small amounts of the basic amino acids, no cystine, tyrosine, methionine and tryptophan, and only contaminating traces of arginine. The small absorption observed in the u.v. was probably due to phenylalanine and the small (probably contaminating) amounts of tryptophan and tyrosine.

Alkaline borohydride cleavage of the colostrum M-1 glycoprotein

Table 3 lists the amino acid and amino sugar composition of the glycoprotein before and after treatment with alkaline borohydride under conditions favorable to β -elimination (Anderson *et al.*, 1965). The results revealed substantial losses of threonine and galactosamine and, to a lesser extent, serine with a

Table 2. *Amino acid and carbohydrate composition of the human colostrum M-1 glycoprotein*

For details see under 'Methods'. NeuAc is *N*-acetylneuraminic acid.

Residue	Content (g/100g)	Residues/molecule (nearest integer)
Lys	0.4	1
His	0.3	1
Arg	0.2	0
Asp	1.7	4
Thr	6.0	17
Ser	2.1	7
Glu	3.9	8
Pro	5.6	16
Gly	0.5	2
Ala	2.2	8
Cys	0.0	0
Val	2.8	8
Met	0.0	0
Ile	2.0	5
Leu	0.3	1
Tyr	0.1	0
Phe	0.8	2
Trp	0.1	0
NeuAc	10.8	12
Gal	27.0	52
Fuc	8.0	15
GlcN*	16.6	32
GalN*	5.1	10

* Corrected for 6% loss owing to destruction by acid hydrolysis (2M-HCl, 100°C, 10h). Acetyl groups were not determined.

concomitant increase in alanine. However, the overall loss of hydroxyamino acids was matched by only a 62% loss of galactosamine. The concomitant appearance of α -aminobutyric acid accounted for 72% of the threonine destroyed and the increase in alanine accounted for 81% of serine destroyed. The increase in glycine was of relatively lesser significance since a smaller concentration of this constituent (0.53%) is present in the glycoprotein. There was no significant change in the amounts of the other amino acids or in glucosamine. No attempt was made to measure the galactosaminitol produced on reduction of cleaved galactosamine.

Biological properties of the glycoprotein

The results of the haemagglutination-inhibition assays showed that the M-1 glycoprotein preparation possessed only traces of blood-group A, B and H(O) activities and no M and N activity.

Table 3. *Effect of alkaline borohydride on the human colostrum M-1 glycoprotein*

The protein (0.2% in 0.2M-NaOH-0.5M-NaBH₄) was incubated under N₂ in the dark for 46 h at 37°C, and excess of borohydride destroyed with 0.5M-HCl. After freeze-drying, excess of boric acid was removed by the addition of HCl-methanol (1:1000, v/v) and evaporation *in vacuo*. The protein was then analysed for hexosamine and amino acids in the amino acid analyser. Values given are the averages of two analyses.

	Original composition (mol/mol of protein)	Amount present after alkaline borohydride treatment (mol/mol of protein)	% change
Asp	4.1	4.4	+7
Thr	15.7	5.8	-63
Ser	6.2	4.6	-26
Glu	8.3	7.4	-11
Pro	15.1	15.7	+4
Gly	2.2	2.8	+27
Ala	7.7	9.0	+17
Val	7.4	6.6	-11
α -Aminobutyric acid	0	7.1	-
GlcN	36.1	36.8	+2
GalN	11.0	4.2	-62

Discussion

The human colostrum M-1 acid glycoprotein was apparently homogeneous by ultracentrifugation and by polyacrylamide-gel- and moving-boundary-electrophoresis criteria. It had the following features in common with the bovine colostrum M-1 acid glycoprotein previously characterized (Bezkorovainy & Grohlich, 1969; Bezkorovainy *et al.*, 1970): (1) no u.v. absorption maxima at 275 nm; (2) high contents of threonine, glutamic acid and proline; (3) low contents of basic amino acids; and (4) absence of tryptophan, tyrosine and cystine. The human M-1 glycoprotein was about 2½ times as large as the bovine M-1 glycoprotein and contained about twice as much carbohydrate. Unlike the bovine fraction, the human M-1 glycoprotein contained fucose and more glucosamine than galactosamine.

Alkaline borohydride cleavage (β -elimination) of the M-1 glycoprotein clearly established that threonine and galactosamine were involved in the glycopeptide linkages. It seems likely from the results that serine also played a similar, but minor role. The incomplete conversion of galactosamine indicates that the β -elimination reaction did not proceed to completion, assuming, of course, that all of the carbohydrate chains commence with galactosamine.

The lack of a stoichiometric recovery of α -aminobutyric acid (73% of that expected from the destruction of threonine) and alanine (84% of that expected from the destruction of serine) indicated that a portion of the hydroxyamino acids, over and above that accounted for as degradation products of β -elimination, had been destroyed. This phenomenon has also been

observed by others (McGuire & Roseman, 1967; Weber & Winzler, 1969) and is believed to be due to the greater specific destruction of the hydroxyamino acids by alkali (McGuire & Roseman, 1967). An increase in glycine after alkaline borohydride treatment, similar to the one reported here, has also been observed by others in work on acidic glycoproteins from breast carcinoma mucin (Adams, 1965) and from bovine vitreous humour (Allen & Wardi, 1973).

It would then be reasonable to propose that all the carbohydrate chains present in the glycoprotein commence with galactosamine and are attached to threonine, and, to a minor extent, serine *via* an *O*-glycosidic bond. On the basis of this assumption, the glycoprotein might contain an average of 10 carbohydrate chains, each consisting of 1-2 sialic acid, 1-2 fucose, 5 galactose, 3 glucosamine and 1 galactosamine residues with a total molecular weight of approximately 2200 each.

This work was supported by Grant no. HD-05657-01 from the National Institutes of Health, U.S. Public Health Service. Professor G. F. Springer's help in assaying for blood-group activities is also gratefully acknowledged.

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