Isolation, Composition and Reactivity of the Neutral Glycoproteins from Human Meconiums with Specificities of the ABO and Lewis Systems

By RAYMOND H. CÔTÉ and JEAN-PAUL VALET*

Département de Biochimie, Université Laval, Québec, Qué. G1K 7P4, Canada

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Blood-group-specific A₁, B, AB, H and Le^a neutral glycoproteins were isolated from suitable pools of human normal meconiums by a preliminary fractionation with a cationic detergent at pH 5 and 9 (borate), followed by ion-exchange and gel chromatography. The ABH materials have sedimentation coefficients of about 10S–11S, whereas the Le^a preparation, not strictly homogeneous, shows a coefficient of 7S. From the detailed analytical data collected, the following relations are deduced between these various substances: they all possess a common peptide core; there are stable ratios of *N*-acetylglucosamine/*N*-acetylgalactosamine in the B, H and Le^a materials and of *N*-acetylglucosamine/galactose in A, H and Le^a materials, from which the numbers of A and B determinants are estimated. In the ABH substances, the ratio of glucosamine to the sum of threonine and serine is stable. Presumably because of genetic factors, the amount of fucose varies among the different glycoproteins, but it is always definitely lower than in the average cyst substances. Various serological tests and precipitin methods were used to measure the potency, purity and integrity of the preparations, including comparisons between A₁ and A₂ substances from this source. The Le^a activity did not appear as high as it is in glycoproteins from adults and a possible interpretation would be the immature Lewis system as observed on erythrocytes; this could explain their very strong inhibiting power towards iso-agglutinins. This family of substances with various specificities has common features with that prepared from ovarian cysts, but differs clearly on some points.

There is a similarity in the qualitative composition of the water-soluble ABO blood-group substances from meconium and from ovarian cysts. They are abundant in normal meconiums (Buchanan & Rapoport, 1951), which are practically devoid of proteins (Schachter & Dixon, 1965). Partially purified blood-group substances from this source inhibit influenza virus haemagglutination (Curtain *et al.*, 1953) and a possible explanation of this finding might be based on the sialic acid content (Odin, 1955).

The results of an experiment in which partial acid hydrolysis was used suggested that purified blood-group substance from meconium was heterogeneous (Kuhn & Kirschenlohr, 1954). Not only glycoproteins, but active glycolipids are present (Côté, 1963, 1970). Meconium also contains a glycoprotein (s) that have binding sites for myxoviruses, for an anti-*N*-reagent (*Vicia graminea*) as well as A-active structures (Springer *et al.*, 1966).

The present paper deals with the isolation from meconiums of the neutral glycoproteins with A, B, AB, O and Lewis activities and their chemical and immunological properties. Their detailed physico-

chemical properties will be described elsewhere. A preliminary report has appeared (Côté, 1970).

Experimental

Isolation of the specific neutral glycoproteins

Normal meconiums (10–60g) were collected over a period of 48 h for each newborn infant at l'Hôpital de l'Enfant-Jésus, Québec, Qué. G1J 1Z4, Canada. All specimens were classified by the haemagglutination-inhibition technique towards anti-A, anti-B and anti-H reagents (inactive samples were considered as Le^a materials) and specific pools were made. Each pool (300g wet wt.) was first treated as described by Côté (1970), which was partially based on the work by Kuhn & Kirschenlohr (1954) and Barker *et al.* (1957). The crude neutral material was fractionated through Sepharose 4B (Pharmacia of Canada Ltd., Dorval, Qué. H9P 1H6, Canada), 1–1.5g portions in 40 ml of a 0.05M-sodium phosphate buffer, pH 7.5 (0.042M-Na₂HPO₄/0.008M-NaH₂PO₄) being applied to a column (5cm×55cm; V₀, 300ml) equilibrated with the same solvent as that used for elution. The fractions (15ml) were assayed for serological activity, for carbohydrate by the anthrone reaction (Scott & Melvin, 1953) and for absorbance at 260

* Present address: Laboratoire d'Hématologie, C.H.U.L. Québec, Qué. G1V 4G2, Canada.

and 280nm. The pool of fractions containing the peak of activity (Fig. 1a) was concentrated by ultrafiltration (XM-50 membrane; Amicon Corp., Lexington, Mass., U.S.A.) and recycled through the same column to yield material F2 (Fig. 1b). Further purification was achieved by chromatography on DEAE-cellulose (Whatman DE-52; Reeve Angel, Clifton, N.J. 07014, U.S.A.) by a procedure similar to that used by Pusztai & Morgan (1964). A solution (0.4g/20ml) of material F2 after dialysis against 0.005M-sodium phosphate buffer, pH8, (0.047M- Na_2HPO_4 /0.003M- NaH_2PO_4 , diluted tenfold) was percolated through a column (1.5cm $\times$ 30cm) of the ion-exchanger equilibrated with the same buffer. The system was monitored as before and washed with the solvent until the neutral fraction had been completely removed; it was then treated with a linear gradient of NaCl (0–0.2M) in the presence of the same buffer. The non-absorbed material (Fig. 2) was recovered as a fluffy white hygroscopic powder by freeze-drying after dialysis of the combined fractions against water. The moisture content (up to 10%) was determined by drying *in vacuo* for 24h over P_2O_5 at room temperature (25°C).

Carbohydrate analysis

Reducing sugars were determined by the method of Somogyi (1945) after hydrolysis of the substances as 0.1% solutions in 1M-HCl at 100°C for 6h in sealed ampoules. Galactose was determined by the same method in eluates from paper chromatograms (Schleicher 2043b, acid-washed; Carl Schleicher and Schuell Co., Keene, N.H., U.S.A.) developed in butanol/pyridine/0.1M-HCl (5:3:2, by vol.) (Bourrillon & Michon, 1959). Samples of the hydrolysates were neutralized with Amberlite resin CG-45 (OH^- form; Rohm and Haas Co., Philadelphia, Pa. 19105, U.S.A.) and used for this purpose. Fucose was determined as described by Gibbons (1955). Neuraminic acid was measured by the procedure of Svennerholm (1958) with the solvent proposed by Miettinen & Takki-Luukkainen (1959); *N*-acetylneuraminic acid was used as standard. Total hexosamines were determined (Belcher *et al.*, 1954) after hydrolysis of samples (0.2% solutions) in 4M-HCl for 8h at 100°C in sealed ampoules; the excess of acid was removed *in vacuo* over NaOH at room temperature. Hexosamines were also identified and measured in a Beckman model 120B analyser by a procedure similar to that of Lee *et al.* (1969): 1ml portions of the hydrolysates dissolved in citrate buffer, pH2.2 (0.2M- Na^+), were chromatographed on column no. 3 (PA-28 resin height of 58cm) at 55°C in the presence of citrate buffer, pH5.25 (0.35M- Na^+), and the effluents were examined with ninhydrin as usual.

Measurement of proteins and amino acids

Total proteins in specific precipitates were determined by the method of Lowry *et al.* (1951) with rabbit γ -globulins as standard. For quantitative amino acid analyses, samples were treated as 0.1% solutions in 6M-HCl at 100°C for 24h in sealed ampoules; the hydrolysates were dried *in vacuo* over solid NaOH and the residues taken up in citrate buffer, pH2.2 (0.2M- Na^+), and the solutions were applied to a Beckman analyser model 120B [resin PA-35 in citrate buffer, pH5.28 (0.2M- Na^+) and resin PA-28 in citrate buffers at pH3.25 and 4.25 (0.2M- Na^+) all at 52°C].

Nitrogen and acetyl-group determination

The micro-Kjeldahl method was used for the determination of nitrogen with mercuric selenite as catalyst (Dumazert & Marcelet, 1938); the distillate obtained in the presence of sodium thiosulphate through a modified Markham still (one-piece model, A.S.T.M. type; Arthur H. Thomas Co., Philadelphia, Pa. 19105, U.S.A.) was titrated with a mixed indicator (Sher, 1955). *N*-Acetyl groups were measured as described by Ludowieg & Dorfman (1960). *O*-Acetyl group assay was done as described by Alcino (1948) on a 100mg sample of glycoprotein as occurring before the final purifications.

Haemagglutination-inhibition assays

The haemagglutinating reagents used for the ABO system were human anti-A and anti-B sera (Dade Reagents Inc., Miami, Fla., U.S.A.), anti-A from lima beans (*Phaseolus limensis*) as a purified form [precipitate recovered by treatment of the NaCl extract with 12–20 (v/v) ethanol], anti- A_1 as a NaCl extract of *Dolichos biflorus* seeds (gift from Dr. J. A. Albrey, Australian Red Cross Society, Queensland, Australia) and anti-H(O), as a crude NaCl extract of *Ulex europaeus* seeds (Herbst Brothers Inc., Brewster, N.Y., U.S.A.). Samples of purified ovarian-cyst substances 240 (A_1), 376 (B) and 370 (H) were a gift from Professor W. T. J. Morgan (The Lister Institute, London SW1W 8RH, U.K.). Takátsy's (1955) microtechnical equipment, as available commercially (Cooke Eng. Co., Alexandria, Va., U.S.A.) was used throughout, the unit volume being 25 μl . All assays were performed in duplicate at room temperature (25°C). In the ABH system, 1 vol. of the serially diluted sample and 1 vol. of antiserum or equivalent were first mixed and, 15min later, 1 vol. of a 2% suspension of erythrocytes was added and the total volume was made up to 100 μl with 0.85% NaCl, which was always used as solvent. The strength of anti-A, anti-B or anti-H reagent was adjusted to four haemagglutinating doses, as evaluated in the same total volume (one dose

being the minimum amount of reagent for a complete haemagglutination). Readings were made after 30 min. The subtyping of A glycoproteins into A₁ and A₂ was done by the following procedure (R. H. Côté, P. Gadbois & J.-P. Valet, unpublished results), verified by Gadbois (1971) on salivas from a large number of adults of known phenotype. To two rows of serial dilutions of A material (1 vol.) were added 1 vol. each of human anti-A serum (12 doses) and of anti-H (four doses towards O cells); after 15 min, 1 vol. of a 2% suspension of A₁ cells was dispensed into one row of cups and a similar amount of A₂ cells into the other row. In the presence of the glycoprotein from an A₂ secretor, the inhibition was stronger in the system with A₂ cells than in the one with A₁ cells; in the presence of the glycoprotein from an A₁ secretor, the reverse pattern was obtained. There were a few specimens that gave equivocal results (probably from A₁A₂ donors) and these were not used in further studies. Human anti-Le^a and anti-Le^b sera were kindly provided by Dr. B. P. L. Moore, (National Reference Laboratory, the Canadian Red Cross Society, Toronto, Ont., Canada). In this system, the first step was performed as above, then the mixtures were evaluated near 20°C by the capillary technique (Chown & Lewis, 1946) with O erythrocytes treated with papain (Goldsmith, 1955) in the presence of cysteine. The anti-Le^b serum used was considered to be bL type, since, according to our tests, it was not neutralized by the saliva of a Le (a-b-)B(H) secretor (Race & Sanger, 1968); it could be inhibited to some degree by the saliva from an adult of group OLe(a+b-), which is not unusual. The viral haemagglutination-inhibition tests were done as described by Stone (1949), except that human O erythrocytes were used and the microtechnique referred to was adapted. The influenza virus was the strain Montréal 68A₂, kindly supplied by Dr. A. Boudreault (Service des Virus, Institut de Microbiologie et d'Hygiène, Ville de Laval, Qué. H7N 4Z3, Canada).

Forssman activity

A rabbit antiserum was raised by intravenous injection of human A₁ erythrocytes (Kabat, 1956). After 8 weeks, the serum was collected, inactivated at 56°C and absorbed by O erythrocytes before the γ -globulins were separated by (NH₄)₂SO₄ fractionation (Campbell *et al.*, 1964). Haemolysis-inhibition tests were done as described by Kabat (1956) but adapted to a micro-scale, in the presence of a 2% suspension of sheep erythrocytes.

Quantitative precipitation

This was done as described by Kabat (1956). The cross-reaction between horse serum anti-(type

XIV pneumococcus, H635, 1939) (samples given by Professor E. A. Kabat, Columbia University, N.Y., U.S.A., and Professor G. F. Springer, Northwestern University, Evanston, Ill., U.S.A.) and the specific substances were studied as follows. A sample (0.05 or 0.10 ml) of 0.1% (w/v) specific substance in 0.85% NaCl was added to 0.20 ml of antiserum and the total volume was made up to 0.40 ml. Quantitative precipitin curves for A₁, A₂ and AB materials towards rabbit serum anti-(A₁ erythrocytes) were established in the following way: each substance (up to 60 μ g) was added to 0.1 ml of antiserum in a final volume of 0.20 ml. A₁ and AB substances (100 μ g) were also treated with a large excess of the same serum (1.50 ml) and the precipitates were analysed for proteins and fucose, the latter component being evaluated by the general protocol described by Kabat (1956) in similar cases.

Results

Isolation of the neutral specific glycoproteins

Out of more than 500 meconiums tested, 19% were inactive towards the ABH system. The preliminary treatment of the defatted and water-soluble extracts of meconiums with the cationic detergent at pH 5 eliminated most of the acidic glycoproteins (about 10 g per 300 g pool) containing up to 11% sialic acid and yielded about 6 g of crude neutral glycoproteins (approx. 3% sialic acid). Dissociation of the detergent-borate complex was regularly achieved quickly in 2M-acetic acid; parallel dissociation under milder conditions (0.1M-sodium acetate buffer, pH 3.6, 24 h at 4°C) did not reveal any loss of fucose by the former way. At that stage, all the extracts were heterogeneous with respect to sedimentation analysis and electrophoresis (Tiselius cell), but their inhibiting power had increased fourfold. The first sieving showed that at least three families of molecules were present (Fig. 1a). At the end, there was a group of smaller molecules, also reactive with anthrone, but devoid of strong activity; they could sometimes reach 40%, but they were not further investigated. After recycling, the elution pattern revealed the presence of residual large molecules (fraction F1), having a lower activity but a higher viscosity (Fig. 1b). Each pool yielded about 1.5 g of the main fraction F2 where the three peaks had become superimposed. The homogeneity, as to size, of this type of fraction was checked on one sample which, percolated through a long and narrow column of Sepharose 4B, gave rise to a single symmetrical peak. During the last process (Fig. 2), most of the material applied to the ion-exchanger (50–70%) emerged without appreciable retention and was most active (fraction F'1). A greenish coloured product, present in small amount and inactive towards anthrone, could be eluted by

Fig. 1. Typical molecular sieving on Sepharose 4B of a crude neutral glycoprotein

(a) Portions (about 1 g) of the neutral glycoprotein in 40 ml of 0.05 M-sodium phosphate buffer, pH 7.5, were applied to a column (5 cm $\times$ 55 cm) of Sepharose 4B equilibrated with the same buffer. (b) The active product collected between about 400 and 750 ml in (a) was recycled, after concentration, on the same column in the same conditions. Undiluted effluent was used for absorbance at 280 nm ($\circ$) and the haemagglutination-inhibition titres ($\square$), and diluted samples were used for the carbohydrate assay with anthrone (Δ). Examples of the relative characteristics of fractions F1, F2 and F3, on a weight basis: amount (90% recovery) $\approx$ 1, 3.5, 0.5; titre ($\log_2$) $\approx$ 2, 3, 1; $E_{1\text{cm}}^{0.1\%}$ at 625 nm (B substance) $\approx$ 4.4, 4.4, 3.8.

Fig. 2. Fractionation on DEAE-cellulose of a glycoprotein collected after sieving through Sepharose 4B

About 0.4 g of fraction F2 produced by chromatography on Sepharose 4B was applied as a 2% solution in 0.005 M-sodium phosphate buffer, pH 8.0, to a column (1.5 cm $\times$ 30 cm) of DE-52 DEAE-cellulose equilibrated with the same buffer; elution with the buffer alone and, later, in the presence of a gradient of NaCl (0–0.2 M). Undiluted effluent was used for absorbance at 280 nm ($\circ$) and the haemagglutination-inhibition titres ($\square$), and diluted samples were used for the carbohydrate assay with anthrone (Δ); ----, NaCl. Examples of the relative characteristics of fractions F'1 and F'2, on a weight basis: amount (90% total recovery) $\approx$ 1, 0.5; titre ($\log_2$) $\approx$ 4, 2; $E_{1\text{cm}}^{0.1\%}$ at 625 nm (B substance) $\approx$ 4.3, 4.4.

1 M-NaCl. Some of the physicochemical properties (Valet, 1972) of the fractions F'1 may be resumed as follows. No heterogeneity was detectable in the sedimentation-velocity patterns obtained at several concentrations for the ABH materials ($s_{20,w}^0$ approx. 10S–11S); in the Le^a preparation ($s_{20,w}^0$ approx. 7S),

a minor (10%?) but heavier component was detectable (skew curves) at concentration of 1% or more. Free-boundary electrophoresis over long periods of time was also used. At pH 8, all materials appeared to be homogeneous (except Le^a with a slight distortion); at pH 4, A₁, AB and B substances remained homogeneous, whereas H material developed a skew boundary (20% having a higher charge) and the Le^a preparation displayed two unresolved peaks (1:1). They dissolve easily in water and have similar spectra and specific extinction coefficients as observed for cyst substances ($E_{1\text{cm}}^{1\%}$ approx. 1.3 at 280 nm, measured at 0.1% in phosphate buffer, pH 8, $I = 0.2$), which shows the absence of the detergent used ($E_{1\text{cm}}^{0.1\%}$ approx. 2.3 at 280 nm) and of any usual protein or ring-containing contaminant, whereas the absorbance ratios at 260 and 280 nm remain at about 1. At a minimum concentration of 1%, the watery solutions appear slightly yellow, but there is no detectable peak in the visible spectrum and no change in absorbance by variation of the pH (3–9). All attempts to split off the pigment without damage to the molecule (e.g. strong cationic exchanger in H⁺ form) were unsuccessful. Because of their high activity, and low net charge (m approx. $-1.2 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ at pH 8 and $-0.4 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ at pH 4, both at 4.5°C), these materials were considered typical of the neutral specific glycoproteins (with respect to the active sialomucoproteins) in meconiums and were used throughout for all assays.

Amino acid compositions of the neutral glycoproteins

The details results are listed in Table 1. No special treatment was applied to evaluate precisely

Table 1. *Amino acid compositions of the neutral blood-group glycoproteins from meconium*

Results are expressed (a) as amino acid residues in g/100g of glycoprotein and (b) as μmol of amino acid/100 μmol of total amino acids.

	A ₁		B		AB		H		Le ^a	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Lys	0.3	1.4	0.2	1.0	0.2	1.0	0.2	1.2	0.2	0.9
His	0.5	2.7	0.5	2.2	0.4	2.0	0.4	2.0	0.5	2.1
Arg	0.2	1.0	0.3	1.4	0.2	1.1	0.3	1.4	0.4	1.2
Asp	0.3	2.0	0.3	1.7	0.3	1.9	0.4	2.6	0.5	2.4
Thr*	4.7	32.1	5.2	34.1	4.7	33.6	4.8	32.8	6.1	31.9
Ser*	2.8	22.8	3.0	23.0	2.8	23.4	2.7	22.0	4.1	24.9
Glu	0.7	3.6	0.5	2.7	0.5	2.8	0.6	3.4	0.9	3.6
Pro	1.7	11.9	1.9	13.1	1.7	12.8	1.8	12.6	2.1	11.1
Gly	0.3	3.9	0.4	4.2	0.3	4.1	0.4	4.4	0.4	4.0
Ala	0.7	6.5	0.8	7.4	0.8	7.9	0.8	7.7	0.9	6.7
Val	0.6	4.2	0.6	3.9	0.6	4.1	0.6	4.1	0.8	4.3
Ile	0.5	2.9	0.4	2.3	0.4	2.5	0.4	2.4	0.5	2.5
Leu	0.5	3.2	0.4	2.4	0.4	2.4	0.5	2.9	0.6	2.9
Tyr†	0.1	0.5	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0	0.1	0.3
Phe	0.3	1.5	0.1	0.6	0.1	0.4	0.2	0.7	0.4	
Total residues	14.2		14.6		13.4		14.1		18.5	
N content (calc.) (%)	15.6		15.9		15.7		15.8		15.6	
Calc. mol.wt./100g of residues	9843		9666		9671		9713		9769	

* Corrected values (Ser, 0.95; Thr, 0.97).

† Uncorrected values.

Table 2. *Carbohydrate compositions of the neutral blood-group glycoproteins from meconium*

Components are expressed as residues in g/100g of glycoprotein. Full experimental details are given in the text

	A ₁	B	AB	H	Le ^a
Fuc	9.9	9.3	11.4	12.2	4.8
Gal*†	26.4	3 μ .6	30.3	28.4	30.9
GlcN*†	16.8	17.1	15.8	17.8	17.9
GalN*†	16.6	13.1	16.3	13.1	13.7
Neu	0.8	0.8	0.8	1.3	1.3
Ac groups	8.6	7.4	8.2	8.0	8.4
Amino acids	14.2	14.6	13.4	14.1	18.5
Total residues	93.3	93.9	96.2	94.9	95.5
Kjeldahl N	5.6	5.4	5.3	5.4	6.1
Carbohydrate N (calc.)	2.97	2.69	2.86	2.77	2.84
Amino acid N (calc.)	2.22	2.32	2.10	2.23	2.89
Total N (calc.)	5.2	5.0	5.0	5.0	5.7
Ac (calc.)	9.1	8.3	8.8	8.5	8.7
Hexosamines§	34.9	30.8	34.0	31.7	33.5
Reducing (as free Glu)	59	59	58	59	52
Reducing (calc.)	58.2	59.3	60.7	58.8	57.7

* Corrected values (Gal, 0.92; GlcN, 0.94; GalN, 0.91).

† After hydrolysis in 1M-HCl at 100°C for 5–7h and chromatography. Nearly similar results were obtained if the contribution due to fucose was subtracted from the value obtained for carbohydrate by the anthrone method.

‡ After hydrolysis in 4M-HCl at 100°C for 8h. Lower values (85–90%) were obtained if hydrolysis were done in 1M-HCl for 5–7h. The direct Elson–Morgan method gave values for total hexosamines about 10% higher.

§ Colorimetric Elson–Morgan.

a few minor amino acids, such as tryptophan and tyrosine (weak u.v. absorbance) or methionine and cysteine (very low sulphur content). Serine and threonine residues were determined in the same chromatographic runs as the other amino acids, but their measured quantities were corrected by 0.95 and 0.97 respectively, as is usual under the experimental conditions used. The notable features are the high serine, threonine and proline contents and the similarity of their overall composition, which suggests that the peptide core is uniformly structured and homogeneous among the group substances.

Carbohydrate compositions of the neutral glycoproteins

The carbohydrate compositions of the specific preparations are given in Table 2. The glycoproteins differ slightly from each other with respect to their fucose, galactose and hexosamine contents, as might be expected from their various specificities. The neuraminic acid concentration was uniformly low and the test for uronic acids was negative in the purified materials.

Reducing values

The evaluation of the reducing sugars in all the group substances gave nearly similar results (Table 2), except for the Le^a product, which must contain less side-chain units and more hexosamines with resistant bonds.

Other components

No phosphorus was present. No trace of *O*-acetyl group was detected. Neither mannose nor glucose were detectable by paper chromatography in the hydrolysates used for galactose determinations. From the summations made, the yellowish pigment must be present in a very small amount, as well as lipid, if any.

Precipitin reactions

Treatment of the preparations with horse serum anti-(type XIV pneumococcus) gave the following results (expressed as μg of antibody N precipitated by 50 and 100 μg of specific substances): A₁, 0.0; B, 6.7; H, 5.5; Le^a, 20.25. The behaviour of the ABH materials is similar to that of the substances prepared from other human sources (Kabat, 1956), whereas the Le^a preparation reacts more strongly. Only traces of B activity were detected by testing of the supernatant after precipitation of the AB substance by anti-A lectin (*Phaseolus limensis*). The insoluble complexes formed by A₁ and AB materials in the presence of an excess of rabbit serum anti-A₁ contained 96 and 92% respectively of the

Fig. 3. Quantitative precipitin curves established with rabbit serum anti-A₁ erythrocytes and specific glycoproteins from meconium

○, A₁; △, A₂; □, AB. Full experimental details are given in the text.

fucose present in the antigens (no correction for solubility of the complexes), which parallels an amino sugar recovery of about 95% (Elson-Morgan) in the precipitate produced by interaction of A₁ substance with anti-A lectin. No appreciable amount of large molecules had been found (Gadbois, 1971) in the ABH materials prepared this way by the analysis, through a column of Sepharose 4B, of the supernatants from the precipitation at the equivalence point by the lectin *Phaseolus limensis* of A₁ and A₂ substances which, after recovery, exhibited the same characteristics as before the treatment. These results suggest the absence of any fucose and hexosamine contaminants, including free H and B substances. Fig. 3 shows the precipitation curves established towards the rabbit serum anti-A₁ by A₁, A₂ and AB substances. The same plateau was reached by A₁ and A₂ glycoproteins; AB material fell short of the maximum value by 10% and the addition to the supernatant of A₁ substance precipitated six-tenths of the remaining antibodies (no correction for solubility).

Haemagglutination-inhibition assays

The properties of the glycoproteins in the inhibition tests are recorded in Table 3. Because of the presence of sialic acid, the acidic glycoproteins possess a lower specific activity in the ABH system than the neutral ones and their elimination from each pool has contributed to the high potency of the final products. In addition, the inhibition power of each group product towards influenza virus haemagglutination has decreased in parallel with its sialic acid content, as was shown by Springer *et al.* (1969) for various sialomucopolysaccharides. A few residual H structures were detected in A₁, B and AB substances, whereas A₂ glycoprotein

Table 3. *Properties in inhibition tests of the neutral glycoproteins from meconium*

Results are expressed as minimum amounts (μg) for complete inhibition of the reactions. The values in parentheses relate to A₁, B and H substances from ovarian-cyst fluids. Full experimental details are given in the text.

	A ₁	A ₂	B	AB	H	Le ^a
Human anti-A/A ₁ cells*	0.03 (0.2)	0.06	>20	0.03	>20	>20
Human anti-A/A ₂ cells	0.006 (0.03)	0.01	>20	0.02	>20	>20
<i>Dolichos biflorus</i> /A ₁ cells	1 (1.5)	1	>20	2	>20	>20
<i>Phaseolus limensis</i> /A ₁ cells	0.1 (0.1)	0.1	>20	0.1	>20	>20
Anti-H (<i>Ulex europaeus</i>)/O cells*	3 (3)	0.5	6 (>12)	6	0.2 (0.2)	>20
Human anti-B/B cells*	>20 (>20)	>20	0.02 (0.04)	0.08	>20	>20
Forssman haemolysis	0.001 (0.001)	0.002	>20	0.002	>20	>20
Influenza virus haemagglutination						
Montreal 68A ₂ /O cells†	≥40	≥40	≥40	≥40	≥40	≥40
Human anti-Le ^a /OLe ^a cells	0.02	0.02	0.02	0.02	0.02	0.002

* The ABH materials separated at pH 5 and 9 during the preliminary treatment had specific activities amounting to about one-sixteenth and one-quarter respectively of those of the final products in the systems indicated.

† Approx. 0.4 μg for the acidic glycoproteins eliminated at pH 5 and approx. 3 μg for the products obtained before the final purifications.

appeared to be quite rich in this respect. The Forssman activity in substances from meconiums with A determinant is not greater than that from the cyst products (Watkins, 1972). Each of the ABH glycoproteins appeared quite potent when compared with the specific products from cyst fluids, especially towards iso-agglutinins. In addition, they showed a substantial Le^a activity. These findings complete qualitative reports on the presence of this determinant in mucoids before or at birth (Race & Sanger, 1968; Szulman & Marcus, 1973). It could be deduced that the Le^a product was as active as that isolated from adults, from comparisons of haemagglutination-inhibition assays made with the saliva from a few Le^a adults, as already done by Bianco *et al.* (1960) with Le^a material from cyst fluid. Because of the cross-reaction with the anti-Le^b serum shown in haemagglutination-inhibition tests by the Le^a sites of the materials, the results in this system were appraised by comparison with those produced by the same substances towards the anti-Le^a serum. The tests included as controls salivas from OLe^a and A₁Le^b adults. Under the conditions used, on the one hand, towards the anti-Le^a serum, the following ratios of inhibition titres were obtained: Le^a substance/Le^a saliva, 60; Le^a/Le^b saliva, 600; Le^a/A₁, A₂, AB, B and H, 6. On the other hand, towards the anti-Le^b serum, the ratios were: Le^a/Le^b saliva, 50; Le^a/Le^b saliva, 3; Le^a/A₁, A₂, AB and B, 10 and Le^a/H, 1. The Le^b activity shows clearly in the Le^b saliva, if one compares the ratios obtained, for both antisera (anti-Le^a/anti-Le^b). The mediocre Le^b activity of the substances from meconiums contrasts with the potency of the products elaborated in adults.

Discussion

The proportion of non-secretors in the meconiums tested (19%) shows that, as to the specific glycoproteins, this source reflects the same status as that detected in saliva or ovarian cyst, whereas the secretor gene could be sometimes uniformly expressed in the body (Szulman & Marcus, 1973). In practice, each secretion was not assayed before pooling for the presence of the Lewis activity, which should not have introduced an appreciable variation in the properties of the specific pools, as the incidence of *lele* individuals was assumed to be small according to ethnicity (Race & Sanger, 1968). The presence of large glycoproteins accompanying each main active fraction (Fig. 1b) is also evident in other sources, such as salivas (Fiori *et al.*, 1971a) and purified cyst substances (Donald, 1973). The final yields (1g/100g of a dried pool) of the neutral substances from meconiums compare well with those obtained from other sources. These neutral glycoproteins differ strikingly from the lipoglycopeptides occurring on erythrocyte membranes from secretors only (Gardas & Kościelak, 1973) in their substantial galactosamine and amino acid contents. On the other hand, they resemble on some points the active gastroferrins (Multani *et al.*, 1970) but, according to the information available, more closely the blood-group-specific substances isolated from ovarian cysts (Watkins, 1972), except for the yellowish pigment which, although present in small amount, seems to typify all glycoproteins (including the acidic ones) from meconiums and could reflect a subtle character related to the elaborating organ. It

may be related structurally to conjugates of bilirubin or its derivatives, which are largely represented in this source. The occurrence in meconium of hexosamines is about 1.3 times that found in cyst materials, both values being compared without correction. On the other hand, alanine and glycine appear less often in meconium than in the average cyst substances, and are compensated for by larger amounts (approx. 1.3 times) of the predominant threonine and serine, which are present in the ratio of 1.5:1. The other amino acids occur to the same extent as in the average glycoproteins from cysts, which were shown to have a common peptide backbone. It should be noted that the quantitative differences in some components between materials from these two sources are less when the cyst materials have been treated with Pronase (Donald, 1973).

The analytical data on fractions obtained by solvent fractionation (Creeth *et al.*, 1974), as well as those prepared by density-gradient ultracentrifugation (Bhaskar & Creeth, 1974), make one cautious about comparisons and the use of the term homogeneity in this field. However, it should be pointed out that in the above experiments no preliminary treatment by molecular sieving and ion-exchange had been made, which might have decreased the range of heterogeneity. The new and mild fractionation procedure used in the present work on this hitherto unexplored source has selected a family of molecules different as to their specificity, but uniform as to their contents in some components, and their apparent homogeneity (except for Le^a) is also shown by physicochemical criteria and the immunological behaviour. Table 4 lists, on an arbitrary basis, the molar ratios of the main constituents in our preparations along with those deduced from the model megalosaccharide proposed by Lloyd & Kabat (1968) for the average cyst substances. In the ABH materials, the rather constant ratio of glucosamine/threonine plus serine (24:18–19) suggests that the synthesis, if incomplete, is nevertheless orderly to some degree. In the B, H and Le^a glycoproteins, the ratio of glucosamine/galactosamine is

around 4:3, and there is a 1:1 ratio of galactosamine/threonine plus serine, as also found in Pronase-treated cyst materials (Donald, 1973) (except in Le^a). With respect to the B, H and Le^a materials, the A₁ substance contains excess of galactosamine, which should represent the immunodominant sites (about 125 determinants for a molecule of 520000 daltons, *M_{s,D}*). From partial results obtained in our laboratory (Gadbois, 1971), A₁ and A₂ substances (purified and checked as described above) had the following respective properties and composition (as residues): *s*_{20,w} at 9mg/ml, 4.8S and 4.5S; [α _D²⁵], +3.5° and -5.1° (c 1.0 in water); fucose, 10.3 and 11.7%; hexosamines (Elson-Morgan), 36 and 33%, and the amino acids were present in similar amounts. The constant ratio of glucosamine/threonine plus serine in the other preparations suggests that A₂ glycoprotein contains less galactosaminyl determinants than the A₁ glycoprotein, approximately one-half the number deduced for glycoprotein A₁, which means about 65 for a particle molecular weight assumed to be around 470000. Except for the H activity, the tests reported (Table 3 and Fig. 3) revealed only slight differences between these two subgroup substances, which is at variance with some of the results published by Moreno *et al.* (1971). However, our findings are not contrary with the suggestion of these authors about the absence of type 1A determinant from A₂ individuals, this probably being due to different galactosaminyl-transferases (Schachter *et al.*, 1973). The molar ratios of antibody (7S)/antigen along the initial segment of each precipitin curve (Fig. 3) are around 80 and 60 for A₁ and A₂ substances respectively and are compatible with the calculated numbers of determinants. On a weight basis, the ratios of antibody/antigen are much higher than some obtained with cyst substances (Moreno *et al.*, 1971), but remain comparable with those published by other workers (Gardas & Kościelak, 1973; Donald, 1973). The presence of aggregates in the antiserum might explain to a limited extent these high ratios, which may be possible only with antigens having very elongated structure. Inspection of the precipitin

Table 4. Molar proportions of the main constituents of the neutral glycoproteins from meconium

The analytical data presented in Tables 1 and 2 within fixed limits of deviation (fucose, ± 0.4 ; galactose, ± 0.8 ; glucosamine or galactosamine, ± 0.4 ; sum of threonine and serine, ± 0.4) were used to deduce the molar proportions shown below. The appropriate values derived from Lloyd & Kabat's (1968) model are given in parentheses.

	A ₁	B	AB	H	Le ^a
Fuc	15–16 (24)	14–15 (24)	18–19	18–19 (24)	7–8 (12)
Gal	37–39 (36)	43–45 (48)	43–45	38–40 (36)	39–41 (36)
GlcNAc	24 (24)	24 (24)	24	24 (24)	24 (24)
GalNAc	24 (24)	18–19 (12)	23–24	18–19 (12)	18 (12)
Thr plus Ser	18–19	18–19	18–19	18	22
10 ⁻⁴ × Partial weight	2.3	2.2	2.4	2.2	2.1

curves also suggests that on an average one-tenth of the sites on such antigens have to be bound to an antibody molecule for precipitation, which would explain some results about the A or B substance produced by the transformation of an H material through biosynthesis (Poretz & Watkins, 1972; Baker *et al.*, 1973).

In A₁, H and Le^a substances, the ratio of glucosamine/galactose is approximately 24:39 and the excess of galactose in the B material would mean the presence of around 165 determinants for a molecular weight of 620000. As to the AB glycoprotein, from the analytical values, it would not have much less A or B determinants than either A₁ or B material. Nevertheless, this unexpectedly high degree of substitution agrees with the observations of Greenbury *et al.* (1963), about the A₁B erythrocytes. In quantitative precipitation (Fig. 3), some A sites on this antigen also remain inaccessible (approx. 60 sites occupied for a molecule of about 640000 daltons), as was also found for the A₁ molecule. As for the non-precipitable antibodies towards this material, they may have been raised against a topography including more than one A determinant and can bind firmly enough in the vicinity of an H determinant (as in A₂ substance) but not of a B determinant. This would imply that every accessible A determinant on most AB molecules is similarly surrounded which, along with our other results, hardly supports the existence of many molecules with A or B specificity alone among AB substances (Popivanov *et al.*, 1969; Fiori *et al.*, 1971b). Interference between A and B sites has been noted by Gibbons *et al.* (1955), though in a different pattern since normal reaction could be restored by heating the substances (Gibbons & Morgan, 1954). A group AB preparation was then showing a weak action towards natural human α -agglutinins (in competition with A₁ erythrocytes) and β -agglutinins, but a strong reaction towards natural human α -agglutinins (A₂ cells) and rabbit immune β -agglutinins.

The glycoproteins with blood-group activity are assumed to derive from a precursor material (Vicari & Kabat, 1969; Watkins, 1972) and are at some stage subjected to the action of Le and ABH gene products. Fucose, which is involved in different specificities, is present in a low amount in the precursor molecule, so that its subsequent transfer must depend on the competition between variously inherited systems and a few relevant reports have been made. Thus, in saliva, there is a relation in the intensity of H activity with respect to the ABO phenotype, which holds in the present case (Table 3), and also on that of Le^a to H substance according to the zygotic character of group O secretors (Race & Sanger, 1968). Arcilla & Sturgeon (1973) reported a higher concentration than usual of A or B substance

but not of H material in the saliva from adult secretors of cell type Le (a-b-x). These results can also be interpreted as a higher specific activity developed by a larger number of A or B sites appearing without competition from the Lewis system, since an H site but not a Le^b one is the only acceptor for the A or B determinants (Watkins, 1972). So, a certain degree of variation is to be expected in the proportions of fucose to the other components in the specific pools of meconiums and even more in the individual cyst products, where there are appreciable deviations within the same group or subgroup specificity. Notwithstanding these irregularities, it can be deduced that there is definitely a lower degree of substitution by fucose in meconium than in all cyst glycoproteins and this is also a characteristic of the source, as already pointed out (Côté, 1970), as it does not result from degradation before or during the isolation as shown by the Forssman activity and the type XIV cross-reactivity. The specific glycoproteins isolated from gastric juice or erythrocyte membranes are also clearly different from the cyst products with respect to fucose. The A₁, B and AB substances from meconium should include at least as many fucosyl substituents as specific sites and they do. Part of the excess of fucose should be linked $\alpha(1\rightarrow3)$ GlcNAc on the widespread type 2 sequences, as its absence from there would cause an increase of the type XIV cross-reactivity (Lloyd *et al.*, 1968) and of the reaction with *Dolichos biflorus* with respect to the cyst materials (Lundblad & Kabat, 1971). Finally, there must be a few fucose residues linked $\alpha(1\rightarrow4)$ GlcNAc on type 1 sequences to account for the Lewis activity detected.

The pool of meconiums classified as Le^a because of its inactivity towards the ABH system does not follow closely the common pattern. Its molar ratio of glucosamine/galactosamine or galactose remains unchanged, as well as the relative distribution of the amino acids, this excluding the hypothesis of a two-region peptide (Donald, 1973). However, it differs from the other glycoproteins isolated in the same way, by the ratio of glucosamine/threonine plus serine, which suggests the presence of a contaminant (as shown by the physicochemical observations) of the same structural type with some free threonine and serine residues. It might be an incomplete precursor molecule (according to the model) derived from a higher percentage of samples than usual (in weight or number) babies H, *sese*, *lele*. Its sedimentation coefficient is much smaller than that expected from the higher peptide content, on the basis of the work of Bhaskar & Creeth (1974); however, such anomalies were encountered on some glycoprotein fractions (e.g. AmSsol, 43-50) investigated by Creeth *et al.* (1974). The low fucose content may not be significant as regard the Le^a activity

(as measured), since two cyst materials with 8.5 and 17% fucose were found to be approximately equivalent in precipitation (Lloyd *et al.*, 1968). Finally, the higher reactivity towards type XIV antiserum, although not unusual (Watkins, 1972), might be ascribed to the degree of fucose substitution and/or to the action of precursor (?) molecules as shown by Vicari & Kabat (1969). The poor reactivity of the glycoproteins from meconium in the anti-Le^b system indicates a low concentration of the type 1 difucosyl determinant, which might arise from the immature Lewis system in the secreting apparatus of the fetuses, paralleling the phenomenon observed on erythrocytes that gradually change from Le^a to Le^b (Race & Sanger, 1968). The findings of Gardas & Kościelak (1973) about the specific glycoproteins present on secretors' erythrocyte membranes, which would be devoid of Lewis activity, lend support to the hypothesis that this system is derived from the serum, but the negative tests for these determinants do not necessarily mean the absence of type 1 sequences on these materials, as suggested by these authors. In Le^b adults, a fucosyltransferase, besides transforming H sites on secretions into Le^b ones and competing with the A and B transferases, would also change some AH or BH sites into A-Le^b or B-Le^b structures (Watkins, 1972). Our observations on the Le^b activity in meconium and the transition observed with babies' erythrocytes lead us to think that the first fucosyltransferase, $\alpha(1 \rightarrow 4)$ to N-acetylglucosamine, to appear can operate only on unsubstituted type I sequences with competition from the ABH system. The maturation of the Lewis system might be expressed by the appearance of the type of enzyme proposed by Watkins (1972), giving rise to bulky structures, probably unable to bind firmly many anti-A or anti-B iso-agglutinins. It would then explain the weaker reactivity of the cyst materials towards these reagents with respect to that of the meconium products.

In conclusion, it may be said that the family of neutral glycoproteins isolated from human meconiums differs clearly on some points from the corresponding ones occurring in cyst fluids. This may be attributed primarily to the source itself, as exemplified in the porcine species (Watkins, 1972), and partly to the evolution of the integrated Lewis system. It is evident that the model megalosaccharide suggested from data on average cyst substances does not apply here, though it may be representative of a certain proportion of molecules from that particular source.

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