The Macromolecular Properties of Blood-Group-Specific Glycoproteins

CHARACTERIZATION OF A SERIES OF FRACTIONS OBTAINED BY DENSITY-GRADIENT ULTRACENTRIFUGATION

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1. Equilibrium density-gradient ultracentrifugation in caesium salts was used in two stages in the isolation and subfractionation of the glycoprotein component from a human ovarian-cyst fluid. The eight main subfractions thus obtained were the subject of detailed physicochemical characterization. 2. The fractions were unimodal in buoyant-density distribution, but had discrete ρ^0 values ranging from 1.31 to 1.35. 3. Weight-average molecular weights and sedimentation coefficients decreased regularly with decreasing density of the fraction, whereas the partial specific volumes and selective solvation parameters increased. The latter behaviour correlates well with the increasing peptide content of the lighter fractions. 4. The fractions exhibited a range of analytical composition, although all were within the limits previously observed for blood-group substances of Le^a specificity. All fractions had approximately equal Le^a activity. The peptide content varied systematically from 7% for the densest fraction to 15% for the lightest, but the relative distributions of the amino acids remained essentially constant throughout the series. In particular, serine plus threonine plus proline made up about 50% of the peptide content of all the fractions. Fucose, galactose and *N*-acetylglucosamine contents decreased with increasing peptide content of the fractions, but *N*-acetylgalactosamine and sialic acid exhibited the opposite trend. Molar ratios of *N*-acetylglucosamine to the sum of serine and threonine remained essentially constant at 0.8–0.9, implying a high degree of glycosylation of all the molecules, but the ratio of *N*-acetylglucosamine to *N*-acetylgalactosamine decreased steadily with increasing peptide content, suggesting the presence of oligosaccharide side chains of various lengths. The results are discussed in terms of the accepted structure of glycoprotein molecules. 5. Experiments on the glycoproteins extracted with phenol from the same cyst fluid have confirmed that equilibrium centrifugation in caesium salts does not remove any non-covalently bound protein nor cause any changes in the tertiary structures of these glycoprotein molecules.

The previous paper in this series (Creeth *et al.*, 1974) was concerned with the analytical and physicochemical characterization of a series of glycoprotein fractions obtained from a human ovarian-cyst fluid by solvent fractionation (Morgan, 1967). Equilibrium ultracentrifugation in a gradient of CsCl provides an alternative means of isolating the glycoprotein fraction of cyst fluids (Creeth & Denborough, 1970; Denborough & Creeth, 1970), and also, at least in principle, a means of subfractionation. The avoidance of steps such as extraction with phenol and fractional precipitation with ethanol lends particular interest to the properties of the resulting fractions and in the comparison with similar fractions obtained by the phenol-(NH₄)₂SO₄-ethanol method. The series of glycoprotein fractions referred to, obtained by the latter method, was derived from a Le^a cyst fluid, no.

603. Accordingly we now describe a two-stage density-gradient fractionation procedure applied to this fluid, the properties of the fractions obtained, and the comparison of these fractions with those obtained previously (Creeth *et al.*, 1974).

Experimental

Reagents were of analytical grade, where obtainable, and were used without further purification. The analytical methods for the determination of amino acids, *N*-acetylglucosamine and *N*-acetylgalactosamine, fucose, galactose and sialic acid were as described previously (Creeth *et al.*, 1974). The cyst fluid (no. 603) had been preserved under toluene at 2–4°C for 3–4 years.

Preparation of whole glycoprotein component

The procedure was based on that of Creeth & Denborough (1970). Solid CsCl was added to the cyst fluid (approx. 100ml) to give a weight fraction of 0.42, the solution density being about 1.45g/ml. Twelve 13.5ml cellulose nitrate centrifuge tubes were completely filled with this solution, and centrifuged in the 50T rotor of a Beckman model L preparative ultracentrifuge for 48h at 40000rev./min at about 5°C (105000g, r_{av} . approx. 5.9cm). After removal from the rotor the tubes were sliced just below the mid-point and the contents of the halves were pooled. The pooled solution from the lower corresponding halves, containing all of the glycoprotein of the original cyst fluid, was then dialysed against glass-distilled water until free of CsCl; it is referred to subsequently as the whole glycoprotein component.

Subfractionation of the whole glycoprotein component

The high concentration of serum protein in the cyst fluid restricts the choice of a dense electrolyte to CsCl for the isolation of glycoprotein; however, for the second stage other options are open. For a subfractionation based on buoyant-density differences, the resolving power of the salt is a factor of prime consideration. The relevant properties of the salt are determined by the thermodynamic parameter, β , which is defined by Vinograd & Hearst (1962): in brief, the density gradient is proportional to β^{-1} , and accordingly the separation of two species of different density is proportional to β . Since the standard deviation of the distribution is proportional to $\sqrt{\beta}$, the resolution parameter λ , as defined by Ift *et al.* (1961) (eqn. 1; see also Hu *et al.*, 1962) is also proportional to $\sqrt{\beta}$:

$$\lambda \equiv \frac{\Delta r}{\sigma_1 + \sigma_2} = \Delta \rho \left(\frac{M}{RT} \right)^{1/2} \left(\frac{\beta}{\rho} \right)^{1/2}$$

Here Δr is the separation between modes with standard deviations of σ_1 and σ_2 . At first sight therefore it appears desirable to use salts with β values as high as possible. For CsCl $\beta = 1.25 \times 10^9$ at the appropriate density (Vinograd & Hearst, 1962), whereas for Cs₂SO₄ it is about 0.74×10^9 (Ludlum & Warner, 1965). However, the buoyant densities are approx. 1.5 and 1.3g/ml, respectively, a difference which arises from an increase in the value of the selective solvation parameter, Γ' , from approx. 0.1g of water/g of glycoprotein in CsCl to approx. 0.5g/g in Cs₂SO₄ (Creeth & Denborough, 1970). Because the appropriate factor has the form $(1 + \Gamma'_2)/(1 + \Gamma'_1)$, the apparent solvated molecular weight of a glycoprotein in Cs₂SO₄ is about 36% higher than in CsCl. This factor largely negates the advantage of the higher (β/ρ) ratio of CsCl: if the buoyant-density difference between two macromolecules is conserved in changing media, $\lambda_{corr.}(\text{CsCl})/\lambda_{corr.}(\text{Cs}_2\text{SO}_4) = 1.04$.

A practical advantage of Cs₂SO₄ is that the smaller value of β allows a lower speed to be used to attain the same gradient.

Another consideration may, however, have some bearing on the choice. For a useful fractionation, the macromolecules must be distributed in measurable concentration over some 70% of the tube length to avoid excessive difficulties in pooling corresponding fractions from parallel tubes. Where density heterogeneity occurs, conditions leading to such a distribution also bring about end effects, which distort the distribution at the top and bottom of the tube. These effects appear to be less with Cs₂SO₄ than with CsCl (Creeth & Denborough, 1970). Accordingly Cs₂SO₄ was selected as the appropriate medium for the subfractionation. The speed variable, which does not affect resolution, was selected partly empirically at a value where the equilibrium concentration of glycoprotein at the top and bottom of the tube was low. Also some guidance was available from the density-gradient pattern for the whole glycoprotein component (see Fig. 1b), where two components were visible, of buoyant-density difference 0.033g/ml. Taking the molecular weight of each as 4×10^5 , one finds for Cs₂SO₄ a separation between the modes of the distributions of 0.55cm at $\lambda = 2$, at 25000rev./min, the standard deviations being 0.14cm. The value of λ quoted corresponds to 95% separation of the species; the total spread ($3 \times \text{s.d.}$) would be about 1.4cm. The speed of 25000rev./min (40000g at r_{av} . 5.9cm) was therefore selected.

The whole glycoprotein was diluted to its original volume with phosphate-chloride buffer (16.7mM-Na₂HPO₄–16.7mM-NaH₂PO₄–33mM-NaCl–1mM-NaN₃), I 0.10M, pH 6.8, and solid Cs₂SO₄ added to a weight fraction of 0.31, giving a density of 1.33g/ml. Ultracentrifugation was carried out in the same rotor and with similar tubes as before, with a prior period of 18h at 37000rev./min (90000g) to accelerate the attainment of equilibrium at the lower speed (25000rev./min, 40000g), which was maintained for 4 days.

At the end of this period the centrifuge tubes were fractionated by bottom puncture and 20–23 fractions of approx. 0.6ml were collected from each. The densities and glycoprotein contents of all the fractions from one tube were determined as before (Creeth & Denborough, 1970), to define the distribution; similar measurements were made on selected fractions from other tubes, to confirm the identity of the distributions. Corresponding fractions from all the centrifuge tubes were pooled, dialysed against water until free of salts and freeze-dried.

Physicochemical characterization

This followed the general scheme used for the solvent-fractionated samples (Creeth *et al.*, 1974). Measurements of sedimentation velocity were made

Fig. 1. Characterization of whole glycoprotein component

(a) Sedimentation-velocity pattern at 56100 rev./min, 40 min. Initial concentration 10 mg/ml. (b) Equilibrium density-gradient pattern in Cs_2SO_4 , initial density $\rho_e = 1.333$, at 39460 rev./min; components have ρ^0 1.311, 1.344 g/ml. (c) As in (b) but with added protein (ex cyst) to 10% of glycoprotein concentration.

at two concentrations (approx. 9 mg/ml and 1 mg/ml), 12 mm single-sector cells being used for the higher concentration and 12 mm double-sector cells and Rayleigh optics for the lower. Buoyant densities in Cs_2SO_4 and apparent molecular weights derived from the distribution in the gradient were determined in pairs, in double-sector cells with appropriate wedge windows. A constant speed (39460 rev./min) was used for all fractions, minor adjustments in the proportion of Cs_2SO_4 being made to ensure that all fractions banded close to the centre of the cells. The establishment of equilibrium was verified in each case by constancy of the radial separation of the peaks of the lobes.

Intermediate-speed sedimentation equilibrium experiments were made at low concentration (approx. 0.3 mg/ml) to define the weight-average molecular weight M_w^0 . Some experiments were done in pairs, a procedure which was made possible with two 30 mm cells by the use of a set of special window masks (Beckman Instrument Corp., Palo Alto, Calif., U.S.A.) to avoid superposition of the Rayleigh images. One cell is fitted with a mask obscuring the upper two-thirds of its windows. This cell is filled normally, when the image is produced in the region corresponding to the bottom third of the cell: the second cell, which is masked at its upper and lower thirds, is loaded with the same quantities of solution and reference solvent, but with sufficient inert liquid (Fluorocarbon oil FC43) to displace the solution column to occupy the middle third of the cell. Its image is therefore recorded in the region immediately above that of the first cell. A third mask, obscuring the lower two-thirds of the cell, enables an additional cell to be run simultaneously, if required. All experiments were done at the same speed, 2378 rev./min, after an initial overspeed of 2695 rev./min for 18 h. These conditions are approximately equivalent to those used in the other series, the values of A running from 0.55 to 0.81 [$A = M(1 - \bar{v}\rho)\omega^2/2RT$, where the symbols have their usual significance].

Partial specific volumes and specific refraction increments were determined as before, the measurements referring to the freeze-dried samples. The phosphate-chloride buffer specified above was used throughout the physical measurements. The absorption spectra of representative fractions were recorded over the wavelength region 220–350 nm with a double-beam scanning spectrophotometer (Perkin Elmer Ltd., Beaconsfield, Bucks., U.K.; model 402). Solutions of approx. 2 mg/ml in water were used, with 10 mm cells.

Results

First-stage separation

The whole glycoprotein component prepared in the first centrifugation (with CsCl) was examined in sedimentation-velocity and analytical density-gradient experiments, giving the results shown in Figs. 1(a) and 1(b). The velocity experiment gives a single, fairly sharp but slightly skewed peak, whereas the density-gradient experiment shows an unsymmetrical pattern indicative of the presence of two components differing slightly in buoyant density; no material is evident near the meniscus nor at the base of the cell. As a check on sensitivity of detection of protein by this method, protein from the cyst fluid obtained in the same preparative step was added to the extent of 10% and the mixture was also examined in an analytical density-gradient experiment. The resulting pattern (Fig. 1c) shows a very marked accumulation of material at the meniscus and it is therefore concluded that the protein content of the glycoprotein solution is indeed negligible.

Second-stage fractionation

The distribution of the glycoprotein at the end of the relatively low-speed centrifugation in Cs_2SO_4 is shown in Fig. 2. It is seen that the glycoprotein is concentrated into a band, with very little material at the base (high-density) or at the top of the centrifuge

Fig. 2. Distribution of glycoprotein in the second-stage density-gradient experiment

Volumes of 0.6ml were collected starting from the bottom of the centrifuge tubes. ●, Glycoprotein concentration (mg/ml); ○, density of solution (g/ml). Tube number in the Figure refers to the sequential order of tubes in which the 0.6ml volumes were collected. 0-9, positions of fractions 0-9 respectively.

tube (low-density). The band is steeper on the more dense side, and in general shows the shape to be expected from the derivative curve recorded in the analytical experiment. The eight fractions in the central section of the centrifuge tube contain about 75% of the total glycoprotein. These were therefore regarded as the main fractions and collected individually with the designation 1-8 in order of decreasing density. The high-density tail occurring in the bottom of the centrifuge tube was collected as a single fraction and designated 0. Fraction 9 similarly comprised the low-density tail in the top of the centrifuge tube.

Immunodiffusion against anti-(whole human serum) gave no precipitin lines for the whole glycoprotein or fractions 1-8 at concentrations of 10mg/ml. Fraction 9, however, gave indication of a trace of albumin; this fraction will contain the whole of any lighter impurity present in the 'whole glycoprotein' from the first stage.

Characterization of the fractions

The sedimentation-velocity and density-gradient patterns obtained for all the fractions are shown in Fig. 3. Their analytical composition in terms of the five carbohydrate constituents and the total peptide is given in Table 1, and the detailed amino acid

compositions are given in Table 2. Table 3 summarizes the physicochemical characterization; of the quantities measured, the buoyant density is the most immediately relevant.

It is at once apparent that the sedimentation-velocity patterns show a marked change in character from one end of the series to the other. The most-dense fractions give rapidly moving but very spread peaks with the suggestion of the presence of a trace of material of low sedimentation coefficient. As the density decreases, the peak becomes progressively sharper, and the velocity less, until the least-dense fractions show the typical behaviour observed with other glycoproteins (Creeth & Knight, 1967) of a peak which is hypersharp in the early stages of the experiment, but which retains a slight asymmetry throughout. The sedimentation coefficients recorded in Table 3 show a systematic decrease.

The density-gradient patterns also show clearly defined changes over the series and the buoyant-density values decrease systematically, except that fractions 3 and 4 have essentially identical values. Although all the main fractions are roughly similar in total degree of spreading, the negative lobes of the more-dense fractions are sharper than the positive lobes, whereas the reverse is the case with the less-

Fig. 3. Ultracentrifuge patterns of the glycoprotein fractions

(a) For each of the fractions designated, the sedimentation-velocity pattern is in the upper row and the density-gradient pattern in the lower row. Velocity patterns were obtained after 40 min at 56 100 rev./min. Initial concentration about 9 mg/ml. Speed for density-gradient experiments 39 460 rev./min; glycoprotein concentration about 1.4 mg/ml. (b) Equilibrium density-gradient patterns of the high- and low-density 'tails' (fractions 0 and 9) under the same conditions as for 3(a).

dense fractions. The apparent molecular weights in Table 3 give a quantitative indication of these effects; also Fig. 4 shows two of the logarithmic concentration distributions from which the apparent molecular-weight values are calculated. It is evident that not only are there differences in slope between the two lobes, but that the sharper lobe in each case gives a

linear plot within the limits of error of the measurement. This behaviour follows expectation from the density-gradient analysis of the whole glycoprotein: two relatively homogeneous species, giving well-defined but overlapping distributions, would be expected to yield a series of fractions the lighter of which will be relatively less contaminated with the

Table 1. *Percentage composition (by weight) of glycoprotein fractions from cyst no. 603*

Fractions are numbered as shown in Fig. 2.

Fraction no. and wt. (mg)	Fucose	Galactose	N-Acetyl- glucosamine	N-Acetyl- galactosamine	N-Acetyl- neuraminic acid	Peptide	Total
0 (30)	12	35	27	3.2	5.3	9.5	92
1 (32)	15	34	27	3.4	5.7	7.0	92
2 (50)	14	29	30	4.2	5.8	8.6	92
3 (57)	13	31	31	5.3	6.1	8.4	95
4 (40)	13	28	28	5.8	7.0	10.4	92
5 (57)	12	30	27	7.7	7.9	11.5	96
6 (43)	11	31	24	8.3	8.5	13.4	96
7 (36)	10	29	24	9.7	9.2	15.5	97
8 (27)	11	28	18	8.5	10.3	15.2	91
9 (30)	9	28	20	7.7	8.7	17.7	91
Glycoprotein whole	12	33	29	7.0	6.5	14.1	102

Fig. 4. *Logarithmic concentration distributions in analytical density-gradient experiments*

Fractions 1 (○) and 8 (●), both in Cs_2SO_4 at 39460 rev./min. Divisions on the ordinate axis represent equal increments in log (concentration), in arbitrary units; $(r-r_0)$ is radial displacement from band centre. Values from negative lobes plotted on left of ordinate axis (see Creeth & Denborough, 1970).

heavier, and vice versa. Thus a light fraction would contain only a small proportion of the dense component, and when it is itself subjected to density-gradient analysis, would yield a pattern with a positive lobe (less-dense region) corresponding almost exclusively to the lighter component. Similarly the denser fractions would give negative lobes corresponding largely to the denser component of the original.

In spite of these significant differences in shape of the distributions, it is evident that all the main fractions are very clean in terms of buoyant density,

there being no trace of any discrete second component. Only in fraction 9, the least-dense tail, is there any evidence of heterogeneity of this kind. The middle fractions 3–6 give nearly symmetrical patterns, the curves qualitatively resembling the Gaussian derivative curves expected for a single molecular species of constant density (Vinograd & Hearst, 1962). This was confirmed by the nearly linear form of the logarithmic concentration distributions. It is concluded from these results that the two-stage fractionation has been successful in its main purpose of preparing subfractions of more limited density range than the original, and which show distinct and systematic differences in their mean density. Accordingly, the distribution of the isolated fractions was compared with that indicated by the density-gradient analysis of the whole glycoprotein (Fig. 1b), the starting point of the second stage.

The curve of Fig. 1(b) was integrated, and the resultant curve resolved into two components, on the assumption that the major, more-dense component ($\rho^0 = 1.34 \text{ g/ml}$) had a symmetrical distribution. The results of this analysis gave a nearly symmetrical distribution for the less-dense component also ($\rho^0 = 1.31 \text{ g/ml}$) and the ratio 1:2 for their proportions. The weights of the individual fractions, reported in Table 1, are generally compatible with this analysis, although the total weight of the fractions whose buoyant density falls in the range 1.33–1.36 g/ml is approx. 3 times the total for those in the range 1.30–1.31 g/ml. The absence of material of mean buoyant density 1.32 g/ml may be taken as provisional further evidence of a good separation.

The partial specific volumes observed for the fractions are shown in Table 3; they show the small but systematic increase with proportion of peptide that is to be expected in glycoproteins (Creeth & Denborough, 1970; Creeth *et al.*, 1974).

The molecular-weight values shown in Table 3 are

Table 2. Amino acid composition of glycoprotein fractions from cyst no. 603

Fractions are numbered as shown in Fig. 2; the amino acid composition is expressed as μmol of amino acid/100 μmol of total amino acids.

Fraction	Buoyant density	Asp	Thr	Ser	Glu	Pro	Gly	Ala	γCys	Val	Met	Ileu	Leu	Tyr	Phe	His	Lys	Arg	Pro	Peptide (%)
0	1.361	12.1	15.4	13.0	10.5	10.6	6.8	9.1	1.2	4.5	0.5	1.7	3.8	1.0	1.8	2.6	3.0	2.6	39.0	9.5
1	1.353	7.7	19.0	14.1	7.2	12.9	7.0	10.9	0.6	4.8	0.3	1.9	3.2	0.9	1.6	2.8	2.2	2.9	46.1	7.0
2	1.351	6.1	19.6	14.2	6.0	14.5	6.9	11.2	0.9	4.8	0.3	1.9	3.1	1.1	1.7	2.8	1.6	3.3	48.3	8.6
3	1.341	5.5	21.5	15.5	4.9	15.0	7.2	11.6	0.1	4.9	0.2	1.8	3.0	0.8	1.3	2.6	1.4	2.8	52.0	8.4
4	1.342	5.4	22.5	15.6	5.8	13.8	6.7	10.3	0.8	5.0	0.2	1.8	3.1	0.7	1.2	2.2	1.8	3.3	51.8	10.4
5	1.332	5.5	21.9	15.2	6.8	13.6	6.1	9.6	1.8	4.9	0.2	2.0	3.1	0.7	1.1	1.9	2.4	3.2	50.7	11.5
6	1.327	5.3	22.3	15.5	6.8	13.7	5.9	8.7	1.8	4.8	0.2	2.0	3.1	1.0	1.5	1.7	2.4	3.2	51.6	13.4
7	1.310	5.5	22.7	15.7	6.9	12.9	5.8	8.7	1.8	5.0	0.2	2.2	3.2	1.1	1.6	1.7	2.3	2.8	51.2	15.5
8	1.307	5.9	22.7	15.4	7.4	12.2	5.6	8.0	2.0	4.9	0.2	2.2	3.4	1.3	1.7	1.7	2.5	2.9	50.3	15.2
9	1.311	8.0	18.4	13.3	9.1	11.5	6.0	8.2	2.1	5.0	0.4	2.4	4.4	1.4	2.0	1.7	3.1	3.0	43.2	17.7
Glycoprotein whole		6.8	19.8	14.5	7.6	12.3	5.8	9.5	1.7	4.5	0.6	2.2	3.5	0.8	1.4	2.3	3.0	3.4	46.6	14.1

all in the range 1×10^6 – 2×10^6 , indicating the large size of these glycoprotein molecules: they show an inverse correlation with peptide content, but rather less systematically than do the sedimentation coefficients measured at approx. 9 mg/ml. The latter values are not only a function of size and shape, but are also influenced strongly by concentration-dependence, which may not be identical in effect on substances of possibly differing conformation (Creeth & Knight, 1965). The values of the limiting sedimentation coefficient at zero concentration are not affected by this restriction, but owing to the very diffuse boundaries in the experiments at 1 mg/ml, the values of s^0 are unreliable for fractions 1 and 2. For fractions 3–8, however, the s^0 values are fairly well established, and allow the calculation of the concentration-dependence parameter K_s :

$$K_s = s^0 d(1/s)/dc$$

K_s values of approx. 0.12 ml/mg were observed, there being no systematic variation. Also values of the frictional ratio f/f^0 (Svedberg & Pedersen, 1940) were determined for fractions 3–8, giving values in the range 5–6.

Absorption spectra of fractions 1 and 8 recorded between 200 and 350 nm are shown in Fig. 5. Fraction 1 shows a small absorption in the region of 280 nm, whereas fraction 8 shows a distinct peak as would be expected from the higher peptide content of the latter fraction.

All the main fractions were of high and approximately equal serological activity, inhibiting the agglutination of Le^a cells by an anti-Le^a serum at dilutions of 0.75×10^6 – 1.5×10^6 from an initial solution of 2.5 mg/ml. In terms of both physicochemical and serological

Fig. 5. U.v.-absorption spectra of glycoprotein fractions Fractions 1 (full line) and 8 (broken line). Concentration 2.0 mg/ml; solvent, water; 1 cm cell.

Table 3. *Physical parameters of glycoprotein fractions from cyst no. 603*

$\bar{v}$, partial specific volume; Γ' , selective solvation parameter; M^{app} (d.g.), apparent weight-average molecular weight from distribution in the gradient, corrected for solvation; M_w^0 , weight-average molecular weight of whole distribution. $s_{25,w}$ is at c about 9 mg/ml. n.d., Not determined.

Fraction	Peptide content (%)	$\bar{v}$	Buoyant density, ρ^0	Γ'	$10^{-3} \times M^{\text{app}}$ (d.g.)		$10^{-3} \times M_w^0$	$s_{25,w}$ (S)	$s_{25,w}^0$ (S)
					+ve lobe	-ve lobe			
0	9.5	n.d.	1.361	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1	7.0	0.621	1.353	0.452	189	344	1680	7.30	13.0
2	8.6	0.621	1.351	0.461	204	329	1490	6.89	15.0
3	8.4	0.623	1.341	0.482	181	233	1500	7.01	13.9
4	10.4	0.624	1.342	0.476	157	222	1350	6.43	13.7
5	11.5	0.634	1.332	0.468	114	151	1220	6.37	12.7
6	13.4	0.629	1.327	0.506	154	117	1290	5.84	12.3
7	15.5	0.641	1.310	0.516	187	111	1420	5.78	11.8
8	15.2	0.639	1.307	0.537	208	103	1290	5.58	11.1
9	17.7	0.635	1.311	0.538	n.d.	n.d.	n.d.	5.58	n.d.

criteria, therefore, the fractions represent well-defined preparations, and the details of their analytical composition become of interest accordingly.

Restricting attention to fractions 1–8, it is seen from Table 1 that the proportion of peptide rises steadily through the series, the most-dense fraction having only half the peptide content of the least dense. The data presented in Table 2, however, show that there is little variation in the amino acid profiles, all of which are characterized by a content of (serine plus threonine plus proline) of approx. 50%. The values for aspartic acid and glutamic acid total about 12% in the middle fractions and are marginally higher in both extremes. The content of sulphur-containing and aromatic amino acids is very low throughout, and the content of arginine, lysine and histidine is also fairly low and essentially constant. All the fractions therefore have an amino acid proportion and profile similar to those of other blood-group-specific glycoproteins, irrespective of specificity (Pusztai & Morgan, 1963; Watkins, 1972), and fractions 3–8 in particular (where the proportion of serine plus threonine plus proline exceeds 50% and aspartic acid plus glutamic acid are relatively low) are very similar to the Le^a substances prepared by solvent fractionation (Morgan, 1967; Creeth *et al.*, 1974).

The remaining data of Table 1, referring to the carbohydrate constituents, also show well-marked trends. The galactose, *N*-acetylglucosamine and fucose values all decrease with increasing peptide content, whereas *N*-acetylgalactosamine and sialic acid increase. The significance of these observations is discussed below; here we note only that the composition of all fractions is within the range observed for blood-group-specific glycoproteins of Le^a activity (see Watkins, 1972).

Control experiments on effect of CsCl and guanidinium chloride

On the basis of observations made with pig gastric

mucoprotein, Starkey *et al.* (1972) and Snary *et al.* (1972b) suggested that CsCl may cause large and irreversible changes in the tertiary structure of the mucoprotein, with the release of protein. Concentrated solutions of KCl were also shown to cause irreversible effects (Snary *et al.*, 1971, 1972a). Also, Sajdera & Hascall (1969) and Hascall & Sajdera (1969) showed that the protein–polysaccharide complex of nasal cartilage was dissociated by 4M-guanidinium chloride in the presence of CsCl to a carbohydrate-rich proteoglycan and a relatively peptide-rich glycoprotein.

It has already been established that soluble blood-group-specific glycoproteins from ovarian-cyst fluids, not previously exposed to CsCl, are unaffected by this medium at least with respect to the liberation of protein: inspection of the density-gradient patterns for these materials in CsCl or Cs_2SO_4 (see for example Creeth & Denborough, 1970; Creeth *et al.*, 1974) shows that the main fractions from many different cysts are free of material of lower density than the glycoprotein.

Ionic strength variation in the range 0.05–0.5, and pH variation in the range 3–10, had been shown previously to be without effect on one well-characterized sample (Creeth & Knight, 1967), but the effect of higher salt concentrations, particularly on the less-soluble, peptide-rich, fractions had not been so critically examined. Denaturants other than sodium dodecyl sulphate had not been investigated. Accordingly appropriate control experiments were devised, based partly on materials prepared by the solvent extraction procedure from cyst fluid no. 603 (Creeth *et al.*, 1974), which therefore had not been previously exposed to CsCl. Two of these fractions were chosen: (i) fraction 0–45, which had a high proportion of peptide-rich incompletely soluble component, and had the highest molecular weight, and (ii) fraction 43–50, which was fully soluble, had a more typical proportion of peptide, and was physically the best-defined fraction. Fraction 0–45, when suspended in

Fig. 6. Equilibrium density-gradient patterns of the phenol-extracted fraction 0-45 from the same cyst fluid

In CsCl, at 50740 rev./min. (a) $\rho_e = 1.55$; (b) $\rho_e = 1.45$; (c) $\rho_e = 1.29$ g/ml.

Cs₂SO₄, gave density-gradient patterns showing two soluble components of discrete buoyant densities, and an insoluble component, the buoyant densities being approx. 1.33, 1.354 and 1.308 g/ml respectively (Creeth *et al.*, 1974). Similar effects are observed in CsCl; however, only one very widely spread soluble component, of buoyant density 1.58 g/ml, is seen (Fig. 6a), even though the density gradient was approximately the same as in the experiment in Cs₂SO₄. The insoluble component, of buoyant density 1.472 g/ml, forms a sharp band, seen as a dark zone near the meniscus in Fig. 6(a) and close to the cell centre in Fig. 6(b). The latter experiment, which was performed at a lower initial density, indicates also the presence of a small proportion of less-dense material. This component, presumably protein, although revealed by the curvature near the meniscus in Fig. 6(b), was present in too small a proportion to be visible under conditions in which protein is banded (Fig. 6c). The density differences between the components in the two media are similar to those reported by Creeth & Denborough (1970). It is concluded that CsCl extracts only a trace of protein from this relatively peptide-rich glycoprotein, and that it does not solubilize appreciably more of the insoluble component.

Fraction 43-50 yielded no trace of protein when banded in Cs₂SO₄ (Creeth *et al.*, 1974); accordingly it was used to explore the reversibility of the effects of CsCl. The substance was dissolved at a concentration of 10 mg/ml in the phosphate-chloride buffer specified, and divided into two portions. CsCl was added to one portion, giving a density of 1.6 g/ml at a concentration of approx. 5.5 molal; after 5 days at 4°C the CsCl was removed by dialysis against the same buffer, and the glycoprotein concentration was measured refractometrically. The other portion of the solution was then diluted to give the same glycoprotein concentration (approx. 2 mg/ml) and the relative viscosities and sedimentation coefficients of both solutions were determined by using the same viscometer. The values of $(\eta_{rel.}^{-1})/c$ so found were 88 and 82 ml/g, for the treated and control solutions respectively, and the sedimentation coefficients were 10.2S and 10.3S. Bearing in mind the limitations of the refractometric

Fig. 7. Effect of guanidinium chloride on fraction 6

Equilibrium density-gradient patterns in CsCl at 44770 rev./min. (a) $\rho_e = 1.48$; (b) with added guanidinium chloride (2.8 M), $\rho_e = 1.48$ g/ml.

method of concentration determination, it appears that CsCl has had no significant action on the glycoprotein. Accordingly the use of CsCl as a fractionating medium for these glycoproteins is justified.

The effect of guanidinium chloride was determined by using one of the peptide-rich fractions prepared in the present study (fraction 6, see above). In CsCl its buoyant density was 1.516 g/ml, the pattern showing a wide spread of density (Fig. 7a). In a CsCl-guanidinium chloride mixture, of weight fractions 0.37 and 0.18, respectively, a slightly less-spread pattern was observed (Fig. 7b), of buoyant density 1.48 g/ml, with no trace of dissociated material of lower density. The guanidinium chloride concentration represents a value of 4.0 M in the aqueous solution before addition of CsCl, as used by Sajdera & Hascall (1969). It is concluded that guanidinium chloride at nearly maximum concentration has no effect on these glycoproteins.

Discussion

Comparison of the fractions obtained by the density-gradient procedure with those obtained by solvent extraction and fractionation shows that those

Table 4. *Molar ratios of selected constituents in the glycoprotein fractions from cyst no. 603*

For details see the text.

Fraction	Peptide (%)	Molar ratios			
		GalNAc/(Ser+Thr)	GlcNAc/GalNAc	Fuc/GalNAc	Fuc/GlcNAc
1	7.0	0.8	8.1	6.0	0.7
2	8.6	0.8	7.1	4.5	0.6
3	8.4	0.9	5.9	3.3	0.6
4	10.4	0.8	4.8	3.0	0.6
5	11.5	0.9	3.5	2.1	0.6
6	13.4	0.9	2.9	1.8	0.6
7	15.5	0.9	2.4	1.4	0.6
8	15.2	0.8	2.1	1.7	0.8

obtained by the density-gradient method are much more homogeneous in terms of the physical methods used. The discrete additional components evident in all but one of the solvent-extracted fractions (Creeth *et al.*, 1974) are lacking in the present preparations. Although the weight-average molecular weights of the serologically most active solvent-extracted fractions are very similar to those recorded in Table 3 above, the apparent molecular weights in the salt gradient are much lower than those of the density-gradient prepared materials.

The sedimentation-velocity analysis is less sensitive to small differences in density, and confirms that the fractions prepared here are free from gross heterogeneity. Thus the selection of the apparently low-resolving salt Cs_2SO_4 for the second fractionation stage is validated; RbCl , at a higher speed, would doubtless have been yet more efficient.

Two other aspects of the density-gradient method are relevant. First the yield is essentially quantitative. Secondly, the fractions obtained give optically clear solutions after freeze-drying and no insoluble or sparingly soluble material is obtained: for the particular cyst fluid examined some 30% of the recovered glycoprotein obtained by solvent fractionation came into this category. It should be emphasized, however, that the best fractions obtained by solvent extraction are large in quantity, of the highest serological activity and often fairly homogeneous by physical criteria.

The molecular parameters of the fractions show that they conform to the pattern previously established for blood-group-specific glycoproteins (Creeth & Knight, 1967; Creeth & Denborough, 1970; Creeth *et al.*, 1974). The values of the frictional ratio indicate, in conjunction with other evidence (Creeth & Knight, 1965), that the conformation resembles that of random-coil polymers in a good solvent.

Since GalNAc is thought to occur only in the *O*-glycosidic linkage to the hydroxy amino acids in H, B and Le^a substances (Watkins, 1972; Rovis *et al.*, 1973*a,b*), the ratio GalNAc/(serine+threonine) is a measure of the extent of glycosylation of the hydroxy

amino acids. Similarly, the ratio GlcNAc/GalNAc may give an indication of the average length of the oligosaccharide chains, since GlcNAc may occur several times in a chain. These, and other ratios, are shown in Table 4. The molar ratio of GalNAc to (serine+threonine) is constant at 0.8–0.9 throughout the series, implying that most of the serine and threonine residues are glycosylated (cf. Donald, 1973); the mean oligosaccharide chain length, however, varies significantly, the GlcNAc/GalNAc ratio falling steadily from 8.1 for the heaviest fraction to 2.1 for the lightest. The Fuc/GalNAc ratio changes rather similarly. Le^a specificity is associated with the terminal sequence $\text{Gal}\beta(1-3)[\text{Fuc}\alpha(4)\text{GlcNAc}]$ - (see Watkins, 1972), so the invariance of the ratio Fuc/GlcNAc suggests that most sub-terminal GlcNAc residues are substituted also by fucose, irrespective of chain length.

The findings summarized in Table 4 provide a satisfactory interpretation of the density-gradient distribution of the whole glycoprotein and the differences in the physical properties of the fractions. The wide spread in buoyant density arises primarily through variation in the proportion of carbohydrate, a variation which is correlated with changes in the mean oligosaccharide chain length rather than variations in the extent of glycosylation of the hydroxy amino acids. Heterogeneity of the non-specific parts of the chain is recognized in these macromolecules, as is variable chain-branching (e.g. Rovis *et al.*, 1973*b*); accordingly the more heavily substituted molecules can attain a given buoyant density in an increasing variety of ways, depending on the particular substituents incorporated. This accounts for the striking increase in spreading in sedimentation-velocity experiments as the proportion of carbohydrate rises. The two incompletely resolved components seen in the whole glycoprotein thus represent essentially similar families of molecules, whose differences lie primarily in the mean oligosaccharide chain length, although the reason for the earlier chain-termination in the lighter series is not apparent. It must be remembered that ovarian cysts are multilocular.

The experiments on the effect of CsCl on the phenol-extracted fractions have confirmed that equilibrium centrifugation in this medium does not remove appreciable amounts of protein from these glycoproteins or change their conformation from the highly expanded form. This observation may be compared with the findings of Starkey *et al.* (1972) and Snary *et al.* (1972a) on a gastric mucoprotein preparation. It may be inferred that the chromatographic procedure by which the mucoprotein was isolated does not break down all those non-covalent interactions between protein and glycoprotein which exist in the native gelatinous secretion. Treatment with CsCl, or the more strongly dissociative reagent of Sajdera & Hascall (1969), may therefore be considered desirable when a study of the glycoprotein component is intended, but undesirable when a protein-glycoprotein complex is to be examined. The absence of the sparingly soluble components from the series of fractions prepared in the present investigation, referred to above, is presumably related to the use of CsCl to separate the glycoprotein and protein components of the cyst fluid.

Note Added in Proof (Received 3 October 1974)

The recent publications of Snary *et al.* (1974) and Starkey *et al.* (1974) give full details of, among other things, the conformational transitions of the gastric mucoprotein on exposure to CsCl. The authors state that the large and irreversible effect of CsCl is accompanied by the separation of 18% of the total protein of the mucoprotein preparation, although they infer that the changes in physical properties are not directly related to this change in composition. Concerning the desirability or otherwise of exposure to CsCl in the extraction of a glycoprotein, discussed above, we should add the qualification: whether the glycoprotein, after exposure to CsCl, has the same conformation as existed in the secretion can be established only by methods applicable to both secretion and product; however, a dissociative density-gradient step should lead to the isolation of a species containing only covalently bound structural peptide. With cyst glycoproteins, CsCl induces no conformational change that is not induced identically by the alternative procedure based on solvent fractionation.

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