Biochem. J. (1970) **117**, 879–891
Printed in Great Britain

The Use of Equilibrium-Density-Gradient Methods for the Preparation and Characterization of Blood-Group-Specific Glycoproteins

By J. M. CREETH and M. A. DENBOROUGH*
The Lister Institute of Preventive Medicine, London S.W.1, U.K.

(*Received* 12 *December* 1969)

1. The method of sedimentation equilibrium in a gradient of caesium chloride has been applied to the preparation of blood-group-specific glycoproteins from human ovarian-cyst fluids: it is shown that virtually complete separation from contaminating protein is easily accomplished in a single step. 2. The glycoproteins isolated in this way have been characterized by analytical density-gradient experiments in both caesium chloride and caesium sulphate and values of the buoyant density, selective solvation and apparent molecular weight have been obtained. 3. In some cases, materials prepared from the same cysts by solvent extraction methods have also been characterized in these terms. 4. The selective solvation values are about 0.1 and 0.5 g of water/g of glycoprotein in caesium chloride and caesium sulphate respectively. 5. The apparent molecular-weight values are much lower than the weight-average molecular weights, and it is shown that the origin of the discrepancy is heterogeneity in density of the glycoproteins. 6. Some sources of error in the interpretation of density-gradient schlieren patterns are examined.

Blood-group-specific substances occur in water-soluble form in many secretions; of these, human ovarian cyst fluid has been the source of some of the best-characterized materials (see, e.g., Morgan, 1960*a*,*b*; Watkins, 1966*a*,*b*; Creeth & Knight, 1967, 1968). These blood-group substances are glycoproteins, and typically contain about 15% peptide and 85% carbohydrate; considerable variation occurs, however, between materials from different cysts. In addition, from a single cyst, fractions may be prepared showing a wide variation in the proportions of the two constituents. The cyst fluids generally contain much serum protein and some insoluble material: the amount of blood-group substance is variable in proportion to the total protein, but is rarely in excess of 10%.

The buoyant density of proteins in aqueous caesium chloride is approx. 1.3 g/ml (Cox & Schumaker, 1961; Ifft & Vinograd, 1962), whereas that of carbohydrates is approximately 1.6 g/ml (Erikson & Szybalski, 1964). Accordingly, isopycnic equilibrium methods (see, e.g., Vinograd & Hearst, 1962) should be particularly useful for the preparation and characterization of blood-group-specific glycoproteins, where a buoyant density intermediate between the extremes quoted may be expected. In the general field of glycoprotein fractionation the value of the method has been demonstrated by the applications to protein–polysaccharide complexes from aortic tissue and nasal cartilage (Franek & Dunstone, 1966, 1967), to hyaluronic acid (Silpananta, Dunstone & Ogston, 1967, 1968) and to the blood-group-specific substances derived from water-insoluble cyst glycoproteins (Dunstone, 1969).

In the present paper, we report on the application of the density gradient method in the preparation of the water-soluble glycoproteins from the cyst fluids and in their characterization in terms of buoyant density, selective solvation, apparent molecular weight and behaviour in the approach to equilibrium. The relative advantages of caesium chloride and caesium sulphate as gradient-forming materials have been examined. A preliminary account of some parts of this work has been published (Creeth & Denborough, 1970).

* Present address: University of Melbourne Department of Medicine, Royal Melbourne Hospital, Melbourne, Vic. 3050, Australia.

EXPERIMENTAL

Reagents. Analytical grade CsCl and Cs_2SO_4 (British Drug Houses Ltd., Poole, Dorset, U.K.) were used without further purification. Analytical experiments were performed in a phosphate buffer composed of 33 mM-NaCl, 16.7 mM-Na_2HPO_4, 16.7 mM-NaH_2PO_4, I 0.10, pH 6.8 and the appropriate amount of CsCl or Cs_2SO_4. The pH was essentially unchanged in the presence of the caesium salts.

Cyst fluids. Samples of cyst fluids were obtained through the courtesy of Professor W. T. J. Morgan; they had been preserved at 4°C, for various lengths of time, by the addition of a small quantity of toluene, but were otherwise untreated.

Analytical methods. The hexose measurement of Dubois, Gilles, Hamilton, Rebers & Smith (1956) was used to determine the concentrations of blood-group-specific glycoprotein in the fractions prepared from the cyst fluids. The method was calibrated on the basis of a galactose standard and the empirical finding that the apparent galactose content of typical purified preparations is 33% by this test. The CsCl was dialysed out of all samples, and, in addition, those rich in protein were treated with 10% trichloroacetic acid to precipitate the protein, before the analyses were carried out.

The haemagglutination-inhibition method of Morgan & King (1943) was used to assay the group-specific activity.

Density measurements. These were performed by weighing a calibrated 0.20 ml micro-pipette at room temperature (Vinograd & Hearst, 1962).

Control blood-group substance preparations. These materials had been prepared by aqueous extraction of the phenol-insoluble residue from freeze-dried cyst fluid (the 'phenol-extraction' method; see, e.g., Morgan, 1965) and were supplied by Professor Morgan in the form of freeze-dried salt-free powders. They are identified subsequently by the designation 'ϕ' after the number of the originating cyst. For density-gradient experiments, solutions of concentration 2–4 mg/ml were prepared with the phosphate buffer and clarified, if necessary, by prior centrifugation at 600*g* for 30 min.

Preparation of solutions. For CsCl, densities were predicted with the aid of a large-scale graph prepared from the published values of density versus weight fraction (*International Critical Tables*; New York: McGraw–Hill Publishing Co., 1932). For Cs_2SO_4, the equation of Ludlum & Warner (1965) was used. It was assumed that the density increment for the phosphate buffer (4.8 mg/ml) applied also to the caesium salt solutions. Solutions were prepared by weight from the solid salts.

Preparative ultracentrifugation experiments. In the preparation of the glycoprotein from the cyst fluid, a concentration of caesium chloride is selected to give an initial density of 1.40–1.45 g/ml; with the proper choice of experimental conditions the proteins are then concentrated at the meniscus, the glycoproteins form a band in the lower half of the tube and a small pellet of cell debris is found at the bottom. Virtually complete separation is therefore possible, the glycoproteins being obtained in a single-step operation and at a concentration considerably higher than that existing in the cyst fluid.

The experiments were conducted in a Beckman Spinco model L preparative ultracentrifuge, with a no. 50 Ti rotor; the tubes ($\frac{5}{8}$ in × 3 in) were filled completely with the cyst fluid–CsCl solution. In one series of experiments (with cyst no. 603) a weight-fraction of CsCl of 0.42 was used, giving an initial density of 1.45 g/ml. The rotor was maintained at 42000 rev./min for 48 h at a temperature of 12°C, when two tubes were removed for analysis: the rotor was then accelerated again to the same speed for a further 3 days. The tubes were pierced at the bottom and the contents collected in approx. 0.7 ml fractions. The densities of the top and bottom fractions were 1.38 and 1.56 g/ml, respectively, for the 48 h period and 1.37 and 1.59 g/ml for the 5-day period. The values expected for the equilibrium distribution in a sector-shaped cell at this speed are approximately 1.29 and 1.60 g/ml, respectively, as determined from the equations of Jeffrey (1968) and taking r_a and r_b to be 4.2 and 7.7 cm. Considering the cylindrical shape of the tube, the unavoidable delay in sampling and the contamination of the lighter fractions by the heavier, the agreement is as good as can be expected (cf. Ifft, Voet & Vinograd, 1961). Thus equilibrium is shown to be attained in 5 days, whereas the 2 day period gives a distribution that is slightly but significantly displaced from equilibrium.

After being pooled to approx. 2 ml volumes, the fractions collected in the above manner were analysed for glycoprotein as described above and titrated for group-specific activity; also, their densities and extinctions at 280 nm were determined. Sedimentation velocity and analytical density-gradient experiments were made on selected fractions. These experiments (see the Results section) indicated that the 48 h centrifugation period removed all the protein from the lower half of the tube, and showed also that the blood-group substance was distributed in a zone a little above the bottom. In view of this finding, and for other reasons to be discussed, subsequent preparative experiments were conducted for the 48 h period only, and the tubes were fractionated by slicing. The lowermost 40% of the solution was then removed without disturbing the small pellet. In general, the weight fraction of CsCl in these experiments was 0.40, giving an initial density of 1.42 g/ml. Blood-group-specific glycoproteins obtained in this manner are designated by the number of the cyst-fluid sample followed by the letters d.g.

Analytical density-gradient experiments. These were conducted in a Beckman Spinco model E analytical ultracentrifuge, with either a 12 mm cell with a double-sector Kel-F coated aluminium centrepiece, or two 12 mm single-sector cells (also with Kel-F coated centrepieces), one with a plain window and the other with a 1° negative-wedge window. Runs were made at 25°C and were usually followed to equilibrium, which was found to be attained in 30–40 h. In general, two-cell operation was restricted to Cs_2SO_4 solutions. The solutions for the reference compartment of the double-sector cell contained caesium salt in the same weight proportion as in the glycoprotein solution.

Photographic recording and measurement of the records of runs in the double-sector cell were performed by the general procedures of Creeth (1964). In those runs where two cells were used and therefore no base-line was recorded, measurements were made on a ten-fold enlargement traced on graph paper; the base-line could then be drawn in without much difficulty, as the gradient of Cs_2SO_4 is very nearly linear in the conditions employed. Concentration determination from these experiments (see below) was, however, less satisfactory than when the double-sector cell was used.

Calculations of buoyant density, selective solvation and apparent molecular weight. These followed the procedures described by Hearst & Vinograd (1961*b*,*c*), Hearst, Ifft & Vinograd (1961) and Ifft & Vinograd (1962) and will accordingly be summarized only briefly.

The apparent iso-concentration point, r'_e, defined as the

root mean square value of the upper and lower solution limits r_a and r_b, differed from the true value, r_e, by approx. 4×10^{-3} cm in the conditions employed. This produces an error in the buoyant density of 3×10^{-4} g/ml. Accordingly, the distinction was ignored and buoyant densities at atmospheric pressure, ρ_0^0, were calculated from the equation

$$\rho_0^0 = \rho_e^0 + \omega^2 r_0(r_0 - r_e)/\beta^0 \quad (1)$$

Here r_0 is the radial position of the band centre, ρ_e^0 the density at atmospheric pressure at the iso-concentration point and therefore equal to the initial solution density, ω the angular velocity and β^0 the salt-distribution parameter tabulated for CsCl by Vinograd & Hearst (1962) and for Cs_2SO_4 by Ludlum & Warner (1965); $\beta^0 = \beta(\rho_e^0)$.

Pressure corrections were applied to experiments performed in CsCl, by using the expressions

$$\rho_0 = \rho_0^0(1 + \kappa P_0) \quad (2)$$

$$P_0 = \rho_\alpha \omega^2 (r_0^2 - r_a^2)/2 \quad (3)$$

Here ρ_0 is the buoyant density at the pressure P_0 developed at the band centre in the centrifugal field, κ is the specific compressibility of the CsCl solution, ρ_α is the density at the point r_α (the root mean square value of r_0 and r_e):

$$\rho_\alpha = \rho_e + \omega^2(r_\alpha^2 - r_e^2)/2\beta^0 \quad (4)$$

This formulation ignores the contribution of the macromolecule, for which no compressibility data are available. No correction could be made to experiments performed in Cs_2SO_4, for which κ has not been established.

The selective solvation parameter, Γ', was then determined from the relation:

$$\Gamma' = (\rho_0\bar{v}_3 - 1)/(1 - \rho_0\bar{v}_1) \quad (5)$$

where $\bar{v}_1$ and $\bar{v}_3$ are respectively the partial specific volumes of water and glycoprotein. Strictly, the $\bar{v}$ values required are those existing in the ternary solution at the appropriate pressure: these were taken to be 1.00 and 0.633 ml/g, respectively, in both solvents and for all glycoprotein samples. The validity of this procedure is discussed below.

The density gradient required in the expression for calculating molecular weight is the effective value, containing terms relating to the salt distribution at atmospheric pressure and the compressibility of solution and macromolecular component. As before, the full calculation was not possible and therefore the value of the physical density gradient was substituted in experiments performed in CsCl:

$$(d\rho/dr) = [(1/\beta_0) + \kappa\rho_0^2]\omega^2 r \quad (6)$$

No pressure correction could be applied to the experiments in Cs_2SO_4.

The concentration distribution was determined from the measurements of the schlieren patterns, which gave Y, the displacement of the gradient curve from the base line, at regular intervals of r. Trapezoidal approximation was employed to give the refraction increment n_c:

$$n_c(r) = \int_a^r (dn/dr)\,dr = k\Delta r\left(\sum_1^{m-1} Y_i + Y_m/2\right) \quad (7)$$

Here k is the appropriate constant of the optical system and the summation runs from 1, the first measurable value, to $m-1$, the mth value being $Y(r)$.

Apparent values of the solvated molecular weight M_s were calculated from the expression:

$$M_s = \frac{2RT(d\ln n_c/d\delta^2)\rho_0}{\omega^2 r_0(d\rho/dr)} \quad (8)$$

where $\delta = r - r_0$: the unsolvated molecular weight M was then determined from:

$$M = M_s/(1 + \Gamma') \quad (9)$$

In most cases, the original concentration of glycoprotein, c^0, was not known precisely, and accordingly values were determined from the patterns, by using the conservation law:

$$c^0(r_b^2 - r_a^2)/2 = \int_a^b rc\,dr \quad (10)$$

The double integral implicit in the use of eqn. (10) may be evaluated simply in terms of a modified running variable i':

$$i' = q + 1 - i \quad (11)$$

where i runs from 1 to q, the last measurable value. With the approximation that the single value r_0 may be substituted for the variable r, we obtain:

$$c^0 = \frac{2k(\Delta r)^2 r_0}{R_s(r_b^2 - r_a^2)} \sum_{i=1}^{q} i'\,Y_i \quad (12)$$

Here R_s is the differential refraction increment for the solvated macromolecule.

The use of eqns. (7) and (8) entails the implicit assumptions that n_c is linearly related to the macromolecule concentration c and that R_s is invariant over the salt concentration range in which the macromolecule is distributed. The use of eqn. (12) demands a value for R_s: it may be shown simply that R_s is related to R_3, the corresponding value for ordinary aqueous solutions, by the expression

$$R_s = R_3 + \Gamma'_{32} R_2 \quad (13)$$

Here Γ'_{32} is the selective solvation parameter expressed in terms of the interaction between macromolecule and salt: it is obtainable from eqn. (5) on substituting $\bar{v}_2$ for $\bar{v}_1$. The values of R_2 appropriate to this work are 6.76×10^{-5} l/g for 4.6 molal CsCl and 6.29×10^{-5} l/g for 1.18 molal Cs_2SO_4.

RESULTS

Analysis of cyst fluids

The behaviour of two typical cyst fluids in sedimentation-velocity experiments is illustrated in Fig. 1. As expected from the low proportion of specific glycoprotein only serum components are revealed, the main peak in each case being almost symmetrical and having a sedimentation coefficient of about 4S. The behaviour of these materials in analytical density-gradient experiments in caesium chloride is shown in Fig. 2. With fluid 376, most

of the refracting material has risen to the meniscus, but a band is visible near the middle of the cell, indicating the concentration of a denser component into a zone almost completely resolved from the protein. With fluid 603, however, no conditions were found in which the denser component formed a clearly defined band. Fig. 2(*b*) shows typical separation conditions (ρ_e^0 1.51g/ml) the protein being concentrated at the meniscus and the denser component at the base. Fig. 2(*c*) (ρ_e^0 1.56g/ml) shows a partially resolved band, the positive lobe of which is lost because of the proximity to the meniscus. Clearly, the spread of the denser component is greater in this fluid than in fluid 376.

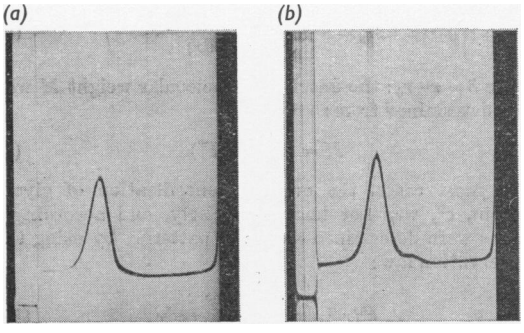

Fig. 1. Sedimentation-velocity experiments on cyst fluids. (*a*) Cyst 376, fluid diluted threefold with phosphate–chloride buffer, *I* 0.10, pH6.8. (*b*) Cyst 603, undiluted. Frames recorded after (*a*) 66min and (*b*) 69min at 59780rev./min. Temperature: 25°C.

Preparation and characterization of glycoprotein fractions

Fluid 603 was subjected to preparative ultracentrifugation under the equilibrium conditions (5 day period) described in the Experimental section. The results of the fractionation of the preparative tubes are shown in Fig. 3: it is evident that the protein has been separated almost completely from the glycoprotein.

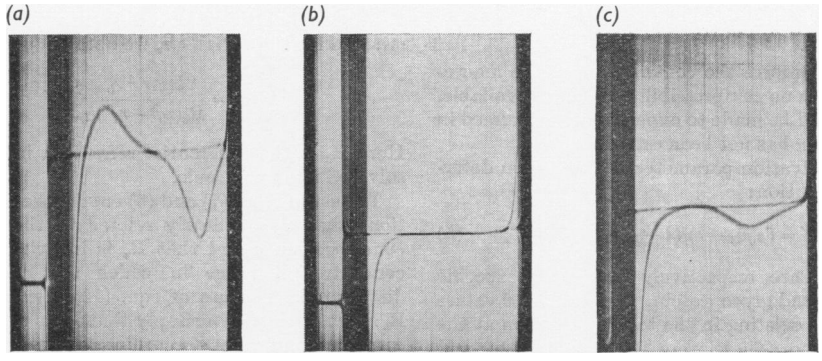

Fig. 2. Density-gradient experiments on cyst fluids containing CsCl. (*a*) Cyst 376, diluted threefold as in Fig. 1(*a*), initial density 1.47g/ml, after 17h at 39460rev./min. (*b*) Cyst 603, undiluted, initial density 1.51g/ml, after 23h at 39460rev./min. (*c*) Cyst 603, undiluted, initial density 1.56g/ml, after 29h at 44770rev./min.

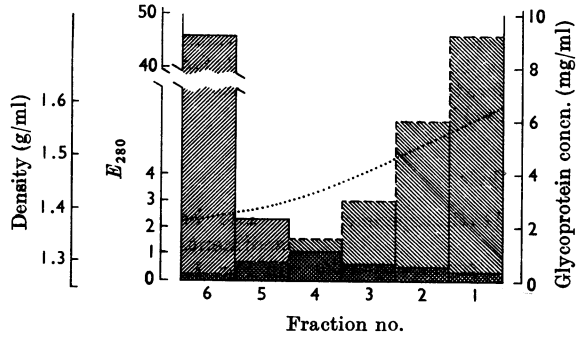

Fig. 3. Preparative density-gradient experiment on cyst fluid 603. The tube was fractionated into six samples of equal volume, whose density was determined. After removal of the CsCl by dialysis, the samples (still of approximately equal volume) were analysed for glycoprotein (as described in the Experimental section) and protein (E_{280}). ·····, Density; —— and //////, E_{280}; ----- and \\\\\\, glycoprotein concentration.

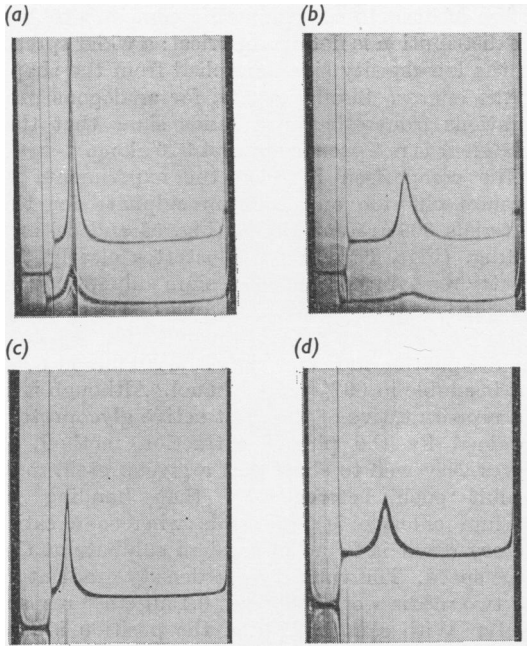

Fig. 4. Sedimentation-velocity experiments on glycoprotein fractions obtained in the preparative experiment on cyst 603 (Fig. 3): (*a*) and (*b*) fractions 1 (upper curve) and 3 (lower curve), 56100 rev./min; (*c*) and (*d*) fraction 2, 52640 rev./min. Both sets of pictures were obtained at 9 (*a* and *c*) and 33 (*b* and *d*) min after the set speed was reached.

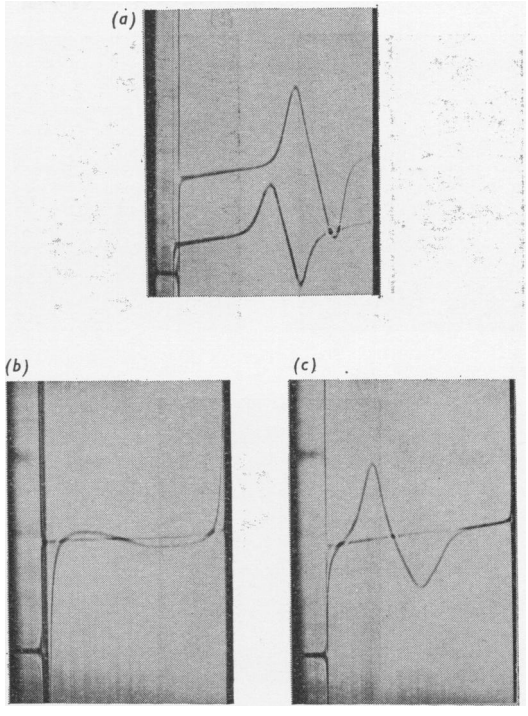

Fig. 5. Analytical density-gradient experiments on glycoprotein fractions from cyst 603. (*a*) Fraction 1 (upper curve) and 2 (lower curve) from the preparative experiment (Figs. 3 and 4). Both solutions contained Cs_2SO_4 at an initial density of 1.32 g/ml; patterns obtained after 47 h at 37020 rev./min. (*b*) Preparation 603ϕ: 50/60 EtOH (phenol-extracted) in CsCl, initial density 1.54 g/ml, 21 h at 39460 rev./min. (*c*) Preparation 603ϕ: 50/60 EtOH in Cs_2SO_4, initial density 1.35 g/ml, 27 h at 39460 rev./min.

In haemagglutination-inhibition tests, all the lower fractions (fractions 1–4) showed specific activities of the same order of magnitude as the standard test blood-group substance, there being no marked differences between the fractions. This lack of variation between the fractions probably reflects the contamination of the upper by the lower fractions, as well as the relative insensitivity of the test to changes in the concentration of the blood-group substance.

Analytical sedimentation-velocity experiments were performed on the three fractions comprising the lower half of the preparative tube, giving the results shown in Fig. 4: the lowermost two fractions (1 and 2) migrate as single peaks. The sedimentation coefficients, $s_{25,w}$, observed were 7.42S (fraction 1, 6.5 mg/ml) and 7.87S (fraction 2, 3.8 mg/ml); the corresponding limiting values, obtained from measurements at lower concentrations and extrapolation of $1/s$ versus c were 15.1 and 12.0S, respectively. The concentration-dependence parameters, s^0 d$(1/s)$/dc, were 0.18 and 0.15 ml/mg respectively.

Fractions 1 and 2 were examined in analytical density-gradient experiments, with caesium sulphate, when the results shown in Fig. 5(*a*) were obtained. One of the fractions obtained by the phenol-extraction procedure was also examined, giving the results shown in Figs. 5(*b*) and 5(*c*). The curves in Fig. 5(*a*) are almost symmetrical, and qualitatively resemble the Gaussian derivative curve expected for a single molecular species behaving ideally (cf. the results for bovine plasma albumin found by Ifft & Vinograd, 1962). However, it is also clear that these fractions, obtained from adjacent zones in the preparative tubes, have different buoyant densities: this is confirmed by the results of the detailed measurements (see below and Table 1). At this stage of the work, it was desired to avoid subfractionation of the glycoprotein but rather to characterize the whole glycoprotein component: therefore, in subsequent preparative experiments on the cyst fluids, the shorter procedure described in the Experimental section was employed.

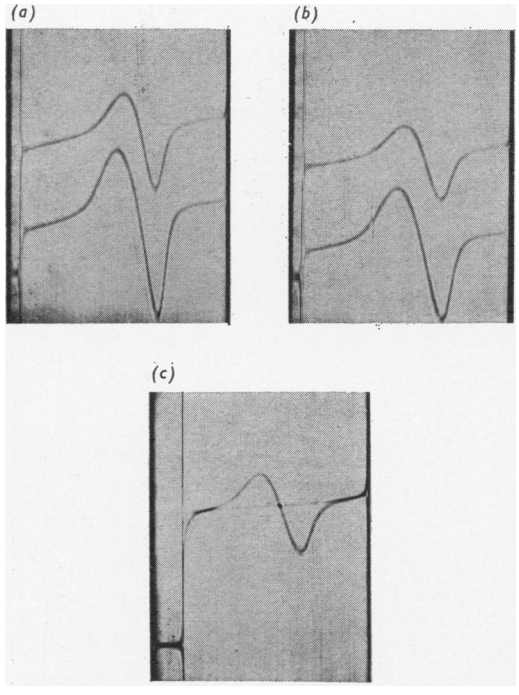

Fig. 6. Analytical density-gradient experiments on whole glycoprotein fraction from cyst 376, separated by preparative density-gradient experiment. Experiments in Cs_2SO_4, initial density 1.30g/ml, at speeds (*a*) 39460rev./min and (*b*) 35600rev./min. The upper patterns represent a 16-fold, and the lower pattern an eightfold, dilution of the material from the preparative experiment. (*c*) Experiment in CsCl, initial density 1.48g/ml, 44770 rev./min.

Haemagglutination-inhibition tests on these whole glycoprotein fractions from cysts 376 and 531 confirmed the location of the blood-group activity within the density range 1.45–1.55g/ml.

As before, the glycoprotein fractions were examined in sedimentation-velocity and analytical density-gradient experiments: single peaks were found in the velocity experiments, very similar to those found with fractions 1 and 2 from fluid 603 (Fig. 4). The analytical density-gradient experiments, again in caesium sulphate, revealed two components in the fraction from fluid 531 and this material was not further investigated at this stage (see below, and Fig. 8*f*). The analytical density-gradient experiments on the whole glycoprotein fraction from cyst 376 were conducted both in caesium chloride and caesium sulphate. In the latter reagent, two concentrations of glycoprotein were examined, and the effects of differences in equilibrium speed were determined. The results are shown in Fig. 6; it is evident that the preparation is free of discrete contaminating components, but the distribution is not symmetrical: a wider spread on the low-density side is implied from the shape of the curves. Similar results for analogous preparations from other cyst fluids show that this behaviour is not peculiar to cyst 376 alone.

The comparison between the experiments in caesium chloride and caesium sulphate for the materials 603ϕ:50/60 EtOH (Fig. 5*b* and 5*c*) and 376d.g. (Figs. 6*c* and 6*a*) illustrates clearly the greater resolving power of caesium sulphate. The fraction 603ϕ:50/60 EtOH is one of the tail-end fractions from an ethanol-precipitation scheme; the designation indicates that it is soluble in 50% but insoluble in 60% (v/v) ethanol. Although it is not representative of the most active glycoprotein obtained by the phenol extraction method, it serves very well to show that a glycoprotein may exhibit such heterogeneity that banding in caesium chloride is impossible whereas a satisfactory result is found in caesium sulphate at the same speed. The values of the density gradient in the two media were 0.090 and 0.150gcm^{-4} respectively. With sample 376d.g. the position is less extreme, but the caesium chloride experiment shows some material remaining at the meniscus even though the band centre is below the middle of the cell. It is noteworthy that the gradient has the same value (0.125gcm^{-4}) in both the caesium sulphate experiment (35600rev./min) and the caesium chloride experiment (44770rev./min).

A comparison may be made between the preparation 376d.g. and a sample prepared from the same cyst by the phenol-extraction method. The fraction examined and characterized by Creeth & Knight (1967) is suitable: the material was a narrow cut from an ethanol-fractionation scheme, and was shown to give only a single peak in sedimentation-velocity experiments under a wide range of conditions. Analytical density-gradient experiments were therefore performed on this material, after the solutions had been subjected to a prior low-speed centrifugation to remove the small proportion of very rapidly-sedimenting component. The results are shown in Fig. 7; it is seen that, in spite of the attempted removal of the rapidly-sedimenting material, an additional component is revealed in the density-gradient patterns. This component, of lower buoyant density than the main fraction, is most clearly resolved in the early stages of the experiment: at full equilibrium (not shown), it is superimposed on the positive lobe of the main pattern, where it produces a very sharp spike, not fully resolved by the optical system. A longer period of prior centrifugation removes this component almost completely (Fig. 7*c*). The pattern in Fig. 7(*a*) provides a striking example of the power of the density-gradient method in

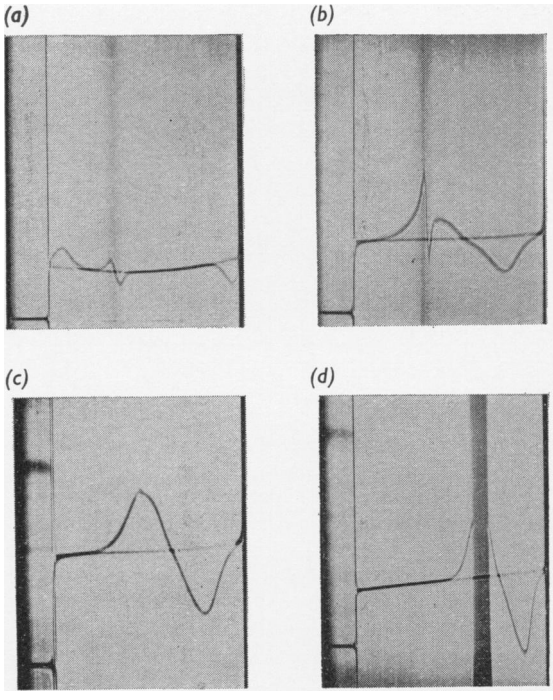

Fig. 7. Analytical density-gradient experiments on glycoprotein 376ϕ, prepared by phenol-extraction procedure. Stages in the approach to equilibrium in CsCl, initial density 1.47g/ml, (*a*) after 200min; (*b*) after 21h at 39460rev./min. The original solution of glycoprotein had been pre-centrifuged at 600*g* for 30min; (*c*) equilibrium pattern (42h), under the same conditions, of a solution pre-centrifuged at 2000*g* for 50min; (*d*) equilibrium pattern (34h) of experiment in Cs_2SO_4, 39460rev./min, initial density 1.28g/ml, glycoprotein solution as in (*c*).

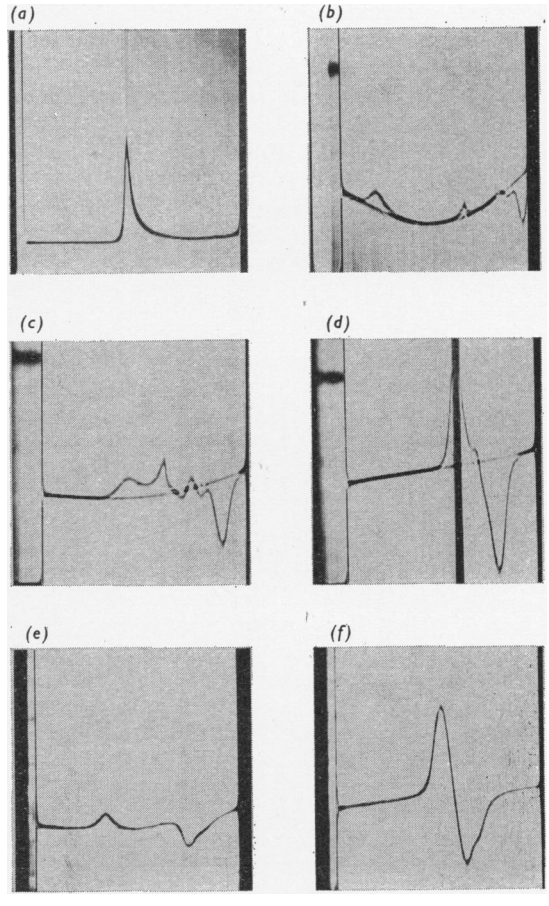

Fig. 8. Experiments on glycoprotein fractions from cyst 531. (*a*) Sedimentation-velocity experiment on preparation 531ϕ: frame recorded after 51min at 56100 rev./min. (*b*), (*c*) and (*d*), Stages in the approach to density-gradient equilibrium of preparation 531ϕ in Cs_2SO_4, initial density 1.32g/ml: frames recorded after 3, 8 and 20h respectively at 39460rev./min. (*e*) Approach to equilibrium of preparation 531d.g. in Cs_2SO_4, initial density 1.32g/ml, frame recorded after 6h at 37020 rev./min. (*f*) Full equilibrium (40h) of preparation 531d.g. in Cs_2SO_4, final stages of experiment referred to in (*e*).

resolving small proportions of high-molecular-weight components. The concentration of the minor component is accurately determinable from the pattern, and was found to be only 1.6% of the total; it is readily visible because it is concentrated into a narrow zone at a time when the main component still exhibits a plateau region. The molecular weight of the minor component cannot be measured precisely; nevertheless, the non-equilibrium pattern in Fig. 7(*a*) has a standard deviation which, if observed at equilibrium, would indicate a molecular weight of about 20×10^6.

The pattern in Fig. 7(*d*) shows the preparation at the same initial concentration, but in a gradient of caesium sulphate; although the speed is identical, the density gradient is higher, and has increased the concentration gradient of the positive lobe to a value higher than can be resolved by the optical system. Nevertheless, comparison of the negative lobe with the corresponding negative lobe in caesium chloride (Fig. 7*c*) demonstrates once more the higher resolving power of caesium sulphate gradients.

Figs. 6(*c*) and 7(*c*), referring to caesium chloride, and Figs. 6(*a*) and 7(*d*), referring to caesium sulphate, show the comparative properties of the preparations 376d.g. and 376ϕ. The material prepared by the density-gradient method gives a more continuous pattern, but the spread is greater, particularly on the low density side.

A further example of the value of the density-gradient method in the approach to equilibrium is afforded by the results obtained with another phenol-extracted glycoprotein, 531ϕ, shown in Fig. 8. The sedimentation-velocity pattern (Fig. 8*a*) reveals only a single peak, where the enhanced spreading of the leading edge indicates polydispersity but does not suggest more than one discrete group of components. However, in the early stages of the density-gradient experiment at least two major and one minor groups of components are clearly displayed (Figs. 8*b* and 8*c*). In a later pattern, this heterogeneity is only barely discernible (Fig. 8*d*). The corresponding material, 531d.g., prepared directly from the cyst fluid, gives no evidence of similar heterogeneity in the approach to equilibrium (Fig. 8*e*), although the equilibrium pattern (Fig. 8*f*) shows a small proportion of a component denser than the main component.

Numerical calculations

Buoyant densities and selective solvation. In these calculations, the band centre position was taken to be the cross-over point, where dn/dr is zero. Even with unsymmetrical distributions the error so introduced is very small, and was therefore neglected. The buoyant densities are obtained rather directly from the experimental data (eqns. 1–5) and are consequently reasonably precise. The selective solvation parameters, Γ', however, may be less precise, since they were calculated on the assumption that the value for $\bar{v}$ of 0.633ml/g found for the substance 376ϕ (Creeth & Knight, 1967) applies to the other substances also. The results are summarized in Table 1.

Inspection of the Table shows that all the glycoproteins here examined band at approximately 1.48g/ml (extremes 1.45 and 1.53) in caesium chloride. A similar average value was found by Dunstone (1969) for the products obtained by sulphite reduction of insoluble blood-group-specific glycoproteins. In caesium sulphate, however, the much lower value of 1.33 (1.31, 1.34)g/ml is found. The minor differences exhibited by substances 376ϕ and 376d.g. in caesium chloride are faithfully reproduced in caesium sulphate, the density difference between the two salts being 0.155g/ml; however, substance 603ϕ bands at a higher density in caesium chloride and a lower density in caesium sulphate, the difference here being 0.210g/ml.

All the values of Γ' cluster round the figure of 0.1g of water/g of glycoprotein in caesium chloride and 0.5g/g in caesium sulphate, which are reliable values for substance 376ϕ. The values thus imply a small positive preferential interaction between water and glycoprotein in 4.6 molal caesium chloride and a much larger interaction in 1.18 molal caesium sulphate. In terms of the interaction between glycoprotein and salt, these values become −0.15 and −0.26g of salt/g of glycoprotein for caesium chloride and caesium sulphate respectively.

Concentration distributions and apparent molecular weights. The logarithmic concentration distributions were calculated, from eqns. (7) and (8), and some typical examples of the results are shown in

Table 1. *Buoyant densities and solvation parameters of blood-group specific glycoproteins*

Density corrections (CsCl only) were made by using eqns. (2)–(4); values of Γ' are quoted on the basis of a constant value of 0.633ml/g for the partial specific volumes of the glycoproteins. Speeds were in the range 35600–39460rev./min.

Glycoprotein no.	Solvent	ρ_0^0 (uncorr.)	ρ_0 (corr.)	Γ'
376cyst fluid	CsCl	1.472	—	—
376d.g.	CsCl	1.483	1.489	0.109
376d.g.*	Cs_2SO_4	1.327	—	0.477
376d.g.†	Cs_2SO_4	1.329	—	0.443
376ϕ	CsCl	1.473	1.480	0.123
376ϕ	Cs_2SO_4	1.318	—	0.509
603cyst fluid	CsCl	1.526	—	—
603d.g. fraction 1	Cs_2SO_4	1.344	—	0.423
603d.g. fraction 2	Cs_2SO_4	1.322	—	0.495
603ϕ	CsCl	1.526	1.530	0.051
603ϕ	Cs_2SO_4	1.316	—	
241ϕ	Cs_2SO_4	1.307	—	0.550
500ϕ	CsCl	1.452	1.459	0.159
531ϕ	Cs_2SO_4	1.337	—	0.445
531d.g.	Cs_2SO_4	1.328	—	0.486

* Initial concentration 3.01mg/ml.
† Initial concentration 1.54mg/ml.

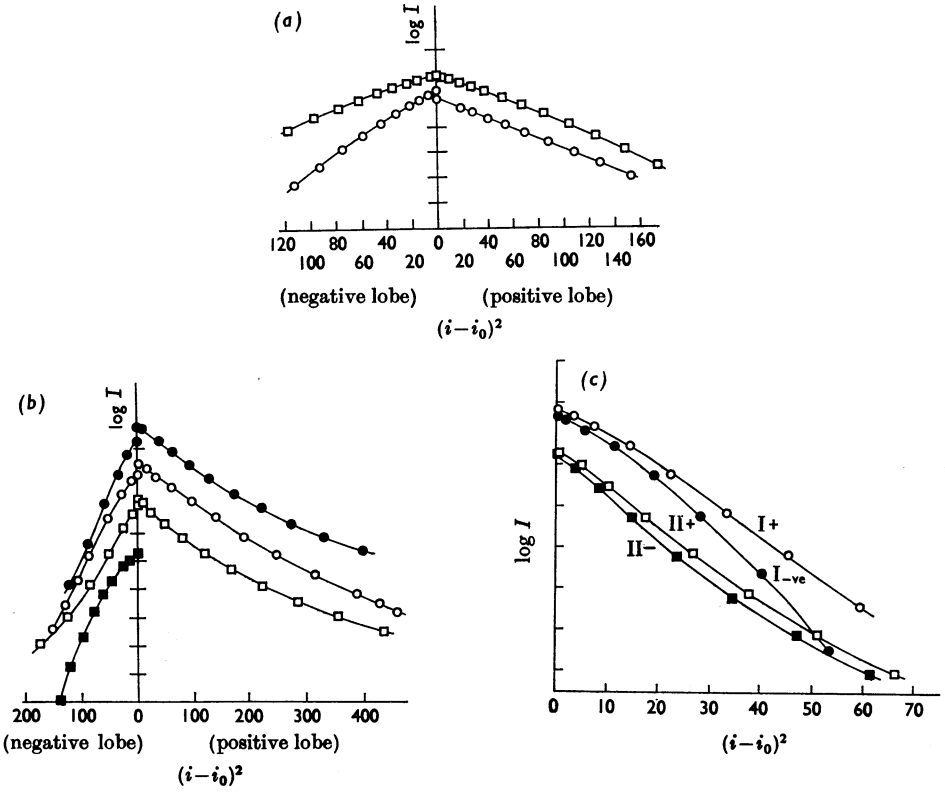

Fig. 9. Logarithmic concentration (I) distributions found for glycoproteins in analytical density-gradient experiments. (a) Curve I, □, preparation 376ϕ, c^0 2.5 mg/ml; curve II, ○, preparation 376 d.g., c^0 1.1 mg/ml. Both were in CsCl at 39460 rev./min. (b) Curve I, ●, preparation 376 d.g., c^0 3.0 mg/ml.; curve II, ○, preparation 376 d.g., c^0 1.5 mg/ml; curve III, □, preparation 376ϕ, c^0 3.3 mg/ml. All were in Cs_2SO_4 at 35600 rev./min. Curve IV, ■, preparation 376 d.g., c^0 1.5 mg/ml in Cs_2SO_4 at 39460 rev./min after correction for higher speed. (c) Curve I, ●, ○, preparation 603 d.g. fraction 1, c^0 2.0 mg/ml; curve II, ■, □, preparation 603 d.g. fraction 2, c^0 1.2 mg/ml. Both were in Cs_2SO_4 at 37020 rev./min. Curves representing different conditions have been separated by addition of a constant to each ordinate; the ordinate scale is therefore arbitrary, each division representing 0.2 in $\log_{10}$. The abscissae are expressed in terms of the running variable, i (eqn. 7); $(i-i_0)^2 = k(r-r_0)^2$, where $k = 2774$ (a); 2796 (b); 1242 (c).

Fig. 9. Three general types of behaviour are evident: (i) a linear or only slightly curved distribution (Fig. 9*a*, for the 376 d.g. and 376ϕ materials in caesium chloride), (ii) a concave downward form (Fig. 9*b*, 376ϕ, at relatively high concentration in caesium sulphate; Fig. 9*c*, 603 d.g. fraction 1, negative lobe) and (iii) a concave upward form (Fig. 9*b*, 376 d.g. in caesium sulphate; Fig. 9*c*, 603 d.g. fraction 2). In Figs. 9(a) and 9(b), the abscissa scale has been reflected about the origin, and the negative-lobe distributions plotted on the left-hand side of the figure. This was done to avoid confusion from intersecting curves, and also to demonstrate two important points about experiments in caesium sulphate. First, the negative-lobe distributions are often steeper than the corresponding positive-lobe distributions in a single experiment, and secondly, experiments done at different speeds do not yield strictly comparable results (Fig. 9*b*, curves I and II). The small displacement sometimes visible between the intercepts on the ordinate axis, corresponding to the positive and negative lobes, is an indication of the magnitude of an error arising from a minor modification of the integration procedure described in the Experimental section. In general, the patterns obtained from experiments in double-sector cells gave a satisfactory integration from the meniscus: that is, the summation commencing at r_a returned to zero as r_b was approached. Where measurements were made from enlargements, this

Table 2. *Apparent molecular weight values for blood-group-specific glycoproteins found from density-gradient experiments, after correction for solvation*

The c^0 values were obtained from eqn. (12), by using $R_s = 1.54 \times 10^{-4}$ l/g in CsCl and 1.48×10^{-4} l/g in Cs_2SO_4, these values following from the quoted values for Γ'_{32} and $R_3 = 1.64 \times 10^{-4}$ l/g found for glycoprotein 376ϕ (Creeth & Knight, 1967).

Glycoprotein no.	Solvent	Speed (rev./min)	c^0 (mg/ml)	$10^{-3} \times M^{app}$ +ve lobe	$10^{-3} \times M^{app}$ −ve lobe	$1+\Gamma'$	$10^{-3} \times M_w^0$
376ϕ	CsCl	39460	2.45	114	108	1.12	1140
	Cs_2SO_4	39460	3.27	—	133	1.51	
376d.g.	CsCl	44770	1.08	107	153	1.11	—
	Cs_2SO_4	39460	3.01	81	185	1.48	
	Cs_2SO_4	35600	1.54	79	185	1.44	
	Cs_2SO_4	39460	1.54	79	233	1.44	
603d.g. fraction 1	Cs_2SO_4	37020	2.04	156	204	1.42	—
603d.g. fraction 2	Cs_2SO_4	37020	1.15	185	193	1.49	—
241ϕ	Cs_2SO_4	35600	1.72	181	186	1.55	1900

was often not the case, and rather than making repeated adjustments to a single base-line, the quicker procedure was adopted of integrating the positive lobe from the meniscus and the negative lobe from the base, the integrals being curtailed at r_0 in each case. Since base-line errors have a serious effect only on the concentration values found from the second lobe of an integration, this procedure minimized the effect of small uncertainties in the base-line position, but led to slightly different values of $c(r_0)$.

The slopes of the logarithmic plots define apparent molecular-weight values through application of eqns. (8) and (9): only in the few cases where a straight-line relationship was found can an unambiguous value of $M^{app.}$ be obtained, and it is noteworthy that even here the values for positive and negative lobes may well differ significantly. In the other cases, where a curved or slightly sigmoidal logarithmic plot was found, the slope was found at points corresponding approximately to 1 S.D. from r_0, i.e. at c/c_0 values of 0.6; inspection of Fig. 9 shows that these are reasonably representative values. The apparent molecular-weight values found in this manner are shown in Table 2, together with the weight-average values found from conventional sedimentation equilibrium experiments (376: Creeth & Knight, 1968; 241: J. M. Creeth, unpublished work), where these were known. The column headed $1+\Gamma'$ gives the magnitude of the correction for solvation: all values are close either to 1.1 for caesium chloride or to 1.5 for caesium sulphate, and although some uncertainty attaches to the values quoted, the error is relatively much smaller than for Γ' alone.

Several noteworthy points are evident from Table 2. First, whereas three of the five materials reported show symmetry between positive and negative lobes, the other two (376d.g., 603d.g. fraction 1) show higher values for the negative lobe, which is on the high-density side of the distribution. Secondly, in the limited range studied, the concentration of the macromolecular species has only a slight influence on the shape of the distribution, the two experiments on material 376d.g. in caesium sulphate giving, in fact, indistinguishable values of $M^{app.}$, even though the radial separation of the peaks appears to differ. Thirdly, a given material exhibits greater asymmetry in caesium sulphate than in caesium chloride. Fourthly, the asymmetry effects are significantly increased at higher speed (cf. material 376d.g. at speeds of 35600 and 39460 rev./min). Fifthly, although it is obvious that the molecular-weight values found from non-linear logarithmic distributions are at best ill-defined averages, there is no doubt that they are significantly lower than the true weight-average molecular weights, the latter being greater by a factor of about ten in the examples shown.

DISCUSSION

The results obtained in this work illustrate further the value of the density-gradient equilibrium method in investigating high-molecular-weight glycoproteins. From the preparative aspect, since glycoproteins must frequently be separated from a source material containing much protein, the procedure based on long-column sedimentation equilibrium in caesium chloride has many advantages. The shorter method described here, which is essentially that of Franek & Dunstone (1966), seems to be quite adequate to make a quantitative separation of protein and glycoprotein, a step which may very often be difficult and incomplete by precipitation or chromatographic methods. The

concomitant increase in concentration, by a factor of up to ten, is also an obvious advantage.

The properties of the glycoprotein isolated from the cyst by the density-gradient method are very similar to those of well-fractionated materials prepared by the phenol-extraction method, particularly with respect to serological activity, sedimentation-velocity characteristics and buoyant density.

Dunstone and co-workers (Franek & Dunstone, 1966, 1967; Silpananta *et al.* 1967, 1968; Dunstone, 1969) have emphasized the applicability of the density-gradient method to the separation and characterization of the different components of glycoprotein mixtures and complexes. We have shown, similarly, that the glycoprotein fraction of cyst fluids is composed of a range of materials of differing buoyant density, and that a partial fractionation of these substances is possible during the experiment in which they are separated from protein. In particular, the results on the fractions from cyst 603 strongly imply that the glycoproteins in the cyst fluid have a continuous range of densities, as would indeed be expected from the analytical results (W. T. J. Morgan, unpublished work) on the fractions derived from this fluid by the phenol-extraction method. The density-gradient experiment shows that these components exist in the cyst fluid, and are not artifacts of the phenol-extraction procedure.

From the analytical aspect, the procedures have proved to be very sensitive, in addition to being experimentally simple and convenient. In the approach to equilibrium, indications of heterogeneity may be observed which supplement information obtained from sedimentation-velocity analysis or the pattern obtained at full equilibrium; this constitutes a useful additional advantage of the method.

On the attainment of equilibrium, the distributions are of interest with respect to their position and shape. Thus the buoyant density, and the selective solvation derived from it, should be unique properties of the glycoprotein and the solvent system: the results, with their minor but undoubtedly significant differences between preparations, confirm that ρ_0 at least is a useful empirical characteristic. The values of Γ', however, are based on the assumptions that the partial specific volumes of water and glycoprotein retain, in the three-component systems, the values they have when the caesium salt is omitted; and, in addition, that all glycoproteins have the same value of $\bar{v}$. For water, the assumption is well founded, both for caesium chloride solutions (Hearst & Vinograd, 1961*c*) and for caesium sulphate solutions (results of Robinson & Stokes, 1955). For the glycoproteins, the assumption is less secure, as no measurements have been made in either caesium chloride or caesium sulphate; however, $\bar{v}$ for bovine serum albumin in caesium chloride is very close to its value in water (Ifft & Vinograd, 1962), and the same is probably true for glycoproteins. Several other blood-group-specific glycoproteins have $\bar{v}$ values close to that quoted (e.g., Pusztai & Morgan, 1961). For these reasons, therefore, the values of Γ' given above are unlikely to be much in error.

For globular proteins, the values of Γ' in 2.5 molal caesium chloride are between 0.1 and 0.2g of water/g of protein (Cox & Schumaker, 1961; Ifft & Vinograd, 1962; Wagman, Edwards & Schantz, 1965; Hade & Tanford, 1967). The Γ' values themselves, therefore, do not appear to be a rewarding source of information, the macromolecular conformation of blood-group-specific glycoproteins being so different from that of globular proteins (Creeth & Knight, 1967). No directly comparable values for proteins in caesium sulphate are available, but Cox & Schumaker (1961) reported much larger values for precipitated proteins in caesium chloride–ammonium sulphate mixtures than in caesium chloride.

The difference in glycoprotein hydration between caesium chloride and caesium sulphate follows that observed for DNA by Hearst & Vinograd (1961*a*,*c*); here large variations in Γ' were satisfactorily correlated with the differences in the water activity for a wide range of dense salts. From the results given by Robinson & Stokes (1955), the values of the water activity, a_w, are approximately 0.86 in 4.6 molal caesium chloride and 0.96 in 1.18 molal caesium sulphate (densities 1.473 and 1.318 respectively). It seems unlikely that such a small difference in a_w can be the sole cause of the difference in solvation of the glycoproteins in caesium chloride and caesium sulphate, and salt-exclusion interactions, related to ion size, are probably implicated (cf. Schumaker & Cox, 1961; Wagman *et al.* 1965).

The other main notable point concerns the significance of the apparent molecular-weight values found from the density-gradient distributions. From eqns. (7)–(9) and (13) it is seen that no error in $M^{app.}$ arises from solvation effects unless Γ'_{32} varies (Fujita, 1962). Since this parameter is independent of macromolecule concentration (Hearst & Vinograd, 1961*b*), its dependence on salt concentration should not introduce major errors over the comparatively small range spanned by the glycoprotein zone. Similarly, although the zones are broad, the generally satisfactory results for proteins in caesium chloride imply that neglect of the non-linear terms in the expansions about r_0 introduces no serious error. It is also assumed in the derivation of eqn. (7) that the macromolecule is electrically neutral and exhibits thermodynamically

ideal behaviour. These particular glycoproteins possess few titratable groups, so that charge effects (Daniel, 1969) will be trivial; the second assumption, however, is questionable. For substance 376ϕ, for example, the second virial coefficient is 0.3–0.4mmol ml g^{-2} in dilute aqueous solution (Creeth & Knight, 1968). This value, if applicable in the dense electrolytes, would lead to significant broadening of the zones, on the basis of the theoretical treatment of Silpananta *et al.* (1968). Although the present results do not extend over a sufficient concentration range for final conclusions to be drawn, it appears that errors arising from thermodynamic non-ideality are relatively minor, particularly when the log $c-\delta^2$ plot in the central region is used to display the distributions.

Heterogeneity of molecular weight of a substance of constant $\bar{v}$ broadens the distribution relative to that for a homogeneous species; moreover, blood-group-specific glycoproteins are typically very polydisperse in M values (e.g. Creeth & Knight, 1968). However, such polydispersity cannot account for the low values of $M^{app.}$ found in the density-gradient experiments, because the logarithmic plot gives the weight-average M value (Meselson, Stahl & Vinograd, 1957). The value found would therefore be comparable, for a substance of constant $\bar{v}$ value, with that determined from regular sedimentation equilibrium.

It therefore follows that the main cause of the low values of $M^{app.}$ must be heterogeneity in $\bar{v}$, a conclusion in general agreement with expectation from the known properties of these glycoproteins, and the known sensitivity of the density-gradient method to density heterogeneity (Baldwin, 1959). The method thereby provides a quantitative index of analytical variations; clearly, a study of a range of closely-fractionated materials, and correlation with their analytical values, will be necessary before the full value of the method can be realized.

A preparation homogeneous in M but with a Gaussian distribution of $\bar{v}$ gives a Gaussian distribution at density-gradient equilibrium (Baldwin, 1959): thus, heterogeneity in $\bar{v}$, although accounting satisfactorily for low values of $M^{app.}$, is not a sufficient explanation for the asymmetry found in some preparations. Accordingly, it follows that this asymmetry represents a genuine fractionation, and that the denser components are also of higher molecular weight. The effect of increased speed, whereby the asymmetry is increased, is in accordance with this conclusion, the fractionation being relatively greater at the higher speed.

When proper allowance is made for the effects of pressure, values of ρ_0 and Γ' are obtained that differ slightly but significantly from those found if the pressure correction is ignored; correspondingly different values of $M^{app.}$ are found also, because these depend on ρ_0, $d\rho/dr$ and Γ'. However, in a given solvent, uncorrected values of ρ_0 and the derived quantities are quite consistent, provided experiments are conducted at constant speed. Accordingly, there seems to be little point in applying the correction, because quantitative interpretation of the $M^{app.}$ values is not yet possible.

Finally, caesium sulphate is seen to have several advantages over caesium chloride as a gradient-forming material for glycoproteins, as it has with nucleic acids (Wake & Baldwin, 1962; Ludlum & Warner, 1965; Szybalski, 1968). The gradient at the same field strength is about 50% greater, which may enable a preparation to be banded completely where caesium chloride is ineffective. Alternatively, one may use lower speeds for the same gradient, thereby minimizing the non-linearity of the gradient. In either case, the zone is more compressed than it would be in caesium chloride, which facilitates drawing in base-lines when single-sector cells are run in pairs: the ability to double output in this way is particularly important with experiments of 40h duration. The lower precision of measurements made from enlargements is quite adequate for revealing the essentials.

The second main advantage of caesium sulphate arises because the glycoproteins are preferentially solvated by water to a greater extent than they are in caesium chloride: thus the buoyant densities are lower, and the distributions are more compressed, the solvated molecular weight being increased. There is, correspondingly, a greater variation in solvation across the zone, and this, together with the higher value of $d\beta/dr$ in the region of ρ 1.3g/ml, are possible disadvantages of the salt. Whereas the inference is that the disadvantages are trivial in their effect, some caution should be exercised in interpreting highly-spread distributions, particularly if they are asymmetric.

We are very grateful to Professor W. T. J. Morgan and Professor W. M. Watkins for supplying the cyst fluids and phenol-extracted glycoproteins, for carrying out the hexose determinations and many haemagglutination-inhibition tests, and for their advice and comments and criticism of this manuscript. We are grateful also to Dr R. R. Race and Dr R. Sanger who carried out some of the haemagglutination-inhibition tests.

REFERENCES

Baldwin, R. L. (1959). *Proc. natn. Acad. Sci. U.S.A.* **45**, 939.

Cox, D. J. & Schumaker, V. N. (1961). *J. Am. chem. Soc.* **83**, 2439.

Creeth, J. M. (1964). *Proc. R. Soc.* A, **282**, 403.

Creeth, J. M. & Denborough, M. A. (1970). *FEBS Lett.* **6**, 117.

Creeth, J. M. & Knight, C. G. (1967). *Biochem. J.* **105**, 1135.
Creeth, J. M. & Knight, C. G. (1968). *Spec. Publs chem. Soc.* no. 23, p. 303.
Daniel, E. (1969). *Biopolymers*, **7**, 359.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Dunstone, J. R. (1969). *Eur. J. Biochem.* **9**, 128.
Erikson, R. L. & Szybalski, W. (1964). *Virology*, **22**, 111.
Franek, M. D. & Dunstone, J. R. (1966). *Biochim. biophys. Acta*, **127**, 213.
Franek, M. D. & Dunstone, J. R. (1967). *J. biol. Chem.* **242**, 3460.
Fujita, H. (1962). *Mathematical Theory of Sedimentation Analysis*, p. 262. New York and London: Academic Press.
Hade, E. P. K. & Tanford, C. (1967). *J. Am. chem. Soc.* **89**, 5034.
Hearst, J. E., Ifft, J. B. & Vinograd, J. (1961). *Proc. natn. Acad. Sci. U.S.A.* **47**, 1015.
Hearst, J. E. & Vinograd, J. (1961*a*). *Proc. natn. Acad. Sci. U.S.A.* **47**, 825.
Hearst, J. E. & Vinograd, J. (1961*b*). *Proc. natn. Acad. Sci. U.S.A.* **47**, 999.
Hearst, J. E. & Vinograd, J. (1961*c*). *Proc. natn. Acad. Sci. U.S.A.* **47**, 1005.
Ifft, J. B. & Vinograd, J. (1962). *J. phys. Chem., Ithaca*, **66**, 1990.
Ifft, J. B., Voet, D. H. & Vinograd, J. (1961). *J. phys. Chem. Ithaca*, **65**, 1138.
Jeffrey, P. D. (1968). *Biochim. biophys. Acta*, **158**, 295.
Ludlum, D. B. & Warner, R. C. (1965). *J. biol. Chem.* **240**, 2961.
Meselson, M., Stahl, F. W. & Vinograd, J. (1957). *Proc. natn. Acad. Sci. U.S.A.* **43**, 581.
Morgan, W. T. J. (1960*a*). *Proc. R. Soc.* B, **151**, 308.
Morgan, W. T. J. (1960*b*). *Bull. Soc. Chim. biol.* **42**, 1591.
Morgan, W. T. J. (1965). In *Methods in Carbohydrate Chemistry*, vol. 5, p. 95. Ed. by Whistler, R. L. & Wolfrom, M. New York: Academic Press Inc.
Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
Pusztai, A. & Morgan, W. T. J. (1961). *Biochem. J.* **78**, 135.
Robinson, R. A. & Stokes, R. H. (1955). *Electrolyte Solutions*. London: Butterworths Scientific Publications.
Schumaker, V. N. & Cox, D. J. (1961). *J. Am. chem. Soc.* **83**, 2445.
Silpananta, P., Dunstone, J. R. & Ogston, A. G. (1967). *Biochem. J.* **104**, 404.
Silpananta, P., Dunstone, J. R. & Ogston, A. G. (1968). *Biochem. J.* **109**, 43.
Szybalski, W. (1968). In *Methods in Enzymology*, vol. 12, part B, p. 330. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Vinograd, J. & Hearst, J. E. (1962). *Fortschr. Chem. org. NatStoffe*, **20**, 372.
Wagman, J., Edwards, R. C. & Schantz, E. J. (1965). *Biochemistry, Easton*, **4**, 1017.
Wake, R. G. & Baldwin, R. L. (1962). *J. molec. Biol.* **5**, 201.
Watkins, W. M. (1966*a*). *Science, N.Y.*, **152**, 172.
Watkins, W. M. (1966*b*). In *Glycoproteins*, p. 462. Ed. by Gottschalk, A. Amsterdam, London and New York: Elsevier Publishing Co.