DENSITY GRADIENT EQUILIBRIUM METHODS APPLIED TO BLOOD-GROUP SPECIFIC GLYCOPROTEINS

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The blood-group specific glycoproteins of human ovarian cyst fluids have been isolated by equilibrium density gradient centrifugation in CsCl; they have been characterised in terms of buoyant density, selective solvation and apparent molecular weight, both in CsCl and Cs_2SO_4.

1. Introduction

Density gradient equilibrium methods owe their widespread application in the nucleic acid field to their very high resolving power and the fact that small constitutional differences in the macromolecules are reflected in variations in the buoyant density (e.g. [1]). An analogous situation exists for glycoproteins, at least for those of high molecular weight, because the individual constituents have widely differing buoyant densities: in CsCl, proteins and carbohydrates band respectively at about 1.3 g/ml [2,3] and 1.6 g/ml [4]. A corresponding role may therefore be expected for the methods; many of the possibilities are illustrated by the work of Dunstone and his colleagues (e.g. [5, 6]). Here we report on the use of density gradient methods in the isolation and characterization of the water-soluble, blood-group specific glycoproteins from human ovarian cyst fluids. These glycoproteins, prepared by a phenol-extraction procedure from freeze-dried fluid, have been extensively investigated (e.g. [7]).

Cyst fluids generally contain much serum protein and some insoluble material. Because the proportion of specific glycoprotein is usually low, sedimentation velocity experiments on the fluid generally detect only serum components (fig. 1a). In equilibrium in a gradient of CsCl, however, the protein components may be concentrated at the meniscus while the glycoprotein forms a band near the middle of the cell (e.g. fig. 1 b).

As expected from the separation shown in fig. 1b, preparative ultracentrifugation in a similar gradient of CsCl provides a satisfactory means of isolating the glycoprotein component of cyst fluids: the experiments were performed in a No. 50Ti rotor of the Beckman Model L ultracentrifuge. To maximise the separation, an initial density was selected approximately 0.05 g/ml lower than the buoyant density of the glycoprotein, CsCl in the appropriate amount was added directly to the cyst fluid and the tubes (3″ × 5/8″)

Fig. 1. (a) Sedimentation velocity pattern for cyst fluid 376, diluted 3 x with phosphate buffer (μ0.1 pH 6.8) after 66 min at 59,780 rev/min (b) Density gradient pattern on diluted cyst fluid 376 in CsCl of initial density 1.47 g/ml, after 17 hr at 39,460 rev/min.

filled completely. Centrifuging at 40,000 rev/min and 12° was then carried out: in the first trial, tubes were sampled after periods of 2–5 days. Analyses for hexose and determinations of density and OD_{280} showed that the shorter period gives an adequate separation of protein from glycoprotein, although the CsCl distribution is slightly displaced from equilibrium. When divided into six fractions of equal volume, the protein was confined to Fraction 6 (top) and the glycoprotein to Fractions 1 and 2. The glycoprotein possessed similar activity in haemagglutination inhibition tests to that of authentic phenol-extracted materials. After dialysis, the fractions were examined in analytical sedimentation velocity experiments: the patterns for the bottom 3 fractions are shown in fig. 2, a. and b. After dilution to 2–3 mg/ml, Fractions 1 and 2 were examined in an analytical density gradient experiment, using Cs_2SO_4 as the dense salt, giving the results shown in fig. 2c.

It is evident from these figures that the separation yields glycoprotein fractions which are free from contamination by protein or carbohydrate components. The velocity patterns show almost symmetrical peaks, free of discrete faster or slower components. Nevertheless, the difference in banding position of Fractions 1 and 2 indicates a significant difference in buoyant density (see table 1) and accordingly the glycoprotein in the cyst cannot be homogeneous in density. This finding is not unexpected from the known variable composition of the phenol-extracted glycoproteins, and the ability to separate fractions on the basis of small density differences is the kind of advantage that it was hoped the method would yield. However, subfractionation was not desired at this stage, and the remaining fluids were fractionated by tube-slicing, the lowermost third containing essentially all the glycoprotein. The density gradient equilibrium patterns for a glycoprotein obtained in this manner are shown in fig. 3: in (a) CsCl is used as the dense medium, whereas in (b) Cs_2SO_4 was employed. These patterns illustrate the greater resolving power of Cs_2SO_4 [8–10]: some material remains at the meniscus in CsCl, whereas it is all banded in Cs_2SO_4. The numerical value of the density gradient was 0.125 g cm^{-4} in both solutes.

The gradient curves were analysed [3], giving values for the buoyant density, ρ_0, at the cross-over point, r_0, the selective solvation parameter, Γ', in terms of g H_2O per g glycoprotein, and the apparent molecular weights for each lobe of the distribution, M^{app}, obtained from the slopes of the plots of log c vs $(r\text{-}r_0)^2$. Typical results for the two glycoproteins described here are given in table 1, together with the corresponding values for the glycoprotein (376ϕ) prepared by phenol extraction from cyst 376.

The ρ_0 values in CsCl are similar to those found for insoluble cyst glycoproteins after reduction by sulphite [5]. All values of Γ' are based on the figure of $\bar{v}$ = 0.633 ml/g measured for 376ϕ in aqueous solution [11]: thus the figures for Γ' for the other preparations

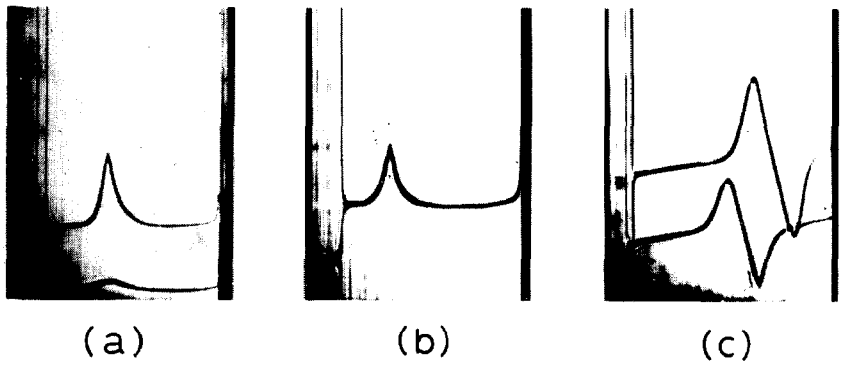

Fig. 2. Sedimentation velocity and density gradient patterns on glycoprotein fractions prepared by density gradient method from cyst fluid 603. (a) Fractions 1 (upper curve) and 3 (lower curve) after 33 min at 56,100 rev/min. (b) Fraction 2 after 33 min at 52,640 rev/min. (c) Density gradient patterns on Fraction 1 (upper curve) and Fraction 2 (lower curve). Both solutions contained Cs_2SO_4 of initial density 1.32 g/ml. Patterns obtained after 47 hr at 37,020 rev/min.

Table 1

Glycoprotein	Salt	ρ_o	Γ'	10^{-3} x M^{app}	
				+ve lobe	-ve lobe
376	CsCl	1.483	0.11	107	153
376	Cs_2SO_4	1.327	0.48	79	185
376ϕ	CsCl	1.473	0.12	114	108
376ϕ	Cs_2SO_4	1.318	0.51	–	133
603 fr 1	Cs_2SO_4	1.344	0.42	156	204
603 fr 2	Cs_2SO_4	1.322	0.49	185	193

may be slightly in error. However, all values cluster around the figures 0.1 in CsCl and 0.5 in Cs_2SO_4, the buoyant densities in these salts showing the same qualitative differences as are observed with nucleic acids.

The molecular weight values are seen to be greater in the negative lobe for two of the three materials prepared by the density gradient method, and the asymmetry is increased in Cs_2SO_4 relative to CsCl. This probably indicates a genuine fractionation in the analytical gradient. The most striking feature of the M^{app} values, however, is revealed only on comparison with the known weight-average molecular weights: e.g. for 376ϕ, MW is 1.14×10^6 [12]. Thus the values obtained from the density gradient experiments are lower by a factor of 10 in this case, and similar factors have been found with other preparations. Thermodynamic non-ideality is not a major effect in these density-gradient experiments (cf. 6), nor are charge effects important. Accordingly, the discrepancy must be due to heterogeneity in density [13]. The comparison therefore appears to offer a first approximation to a quantitative index of density heterogeneity.

We conclude that CsCl density gradient experiments are very useful in the preparation and characterisation of blood-group specific glycoproteins, while for analytical purpose, Cs_2SO_4 has many advantages. Some mucous secretions in which the glycoprotein is an important constituent cannot be dissolved in CsCl, and for these CsBr may be suitable; this reagent has been used successfully with saliva. Detailed reports on these studies will be published elsewhere.

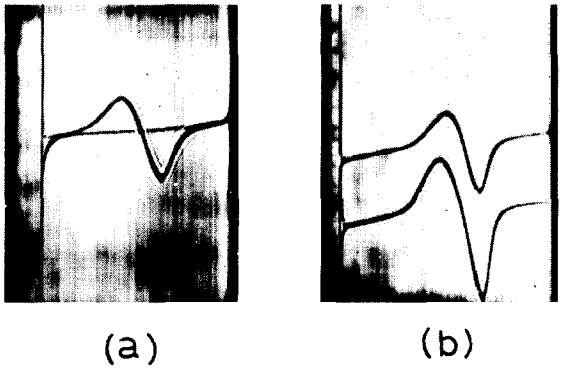

Fig. 3. Density gradient patterns for glycoprotein from cyst 376. (a) in CsCl initial density 1.48 g/ml; 30 hr at 39,460 rev/min. Solution was a 16 x dilution of fraction from lowermost third of preparative tube. (b) In Cs_2SO_4, initial density 1.30 g/ml, 30 hr at 35,600 rev/min. Solutions were 16 x dilution (upper pattern) and 8 x dilution (lower pattern).

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