An Interaction between Lysozyme and Mucus Glycoproteins

IMPLICATIONS FOR DENSITY-GRADIENT SEPARATIONS

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1. Some mucus glycoproteins form soluble complexes with lysozyme at neutral pH values. 2. The extent of complex-formation was determined, by an ultracentrifugal difference method, for a range of glycoproteins covering the common blood-group specificities. 3. Interaction was strongest with those glycoproteins of blood-group Le^a specificity; these were also richest in sialic acid. 4. Interaction diminished with increase of ionic strength, and was not detectable at I 0.50; however, an asialoglycoprotein was found to retain some activity. The interaction is accordingly primarily, but probably not exclusively, coulombic in origin. 5. The buoyant density of lysozyme in CsCl, CsBr, CsI and Cs₂SO₄ was determined; the values in the last three salts are anomalously high. This finding accounts for the previously noted difficulty of separating free protein from glycoproteins by single-stage centrifugation in CsBr. 6. Conditions for effective separation of glycoproteins from secretions containing lysozyme by density-gradient centrifugation are reported.

Isopycnic centrifugation in caesium salts is generally a convenient, non-destructive method for the preparation of the glycoproteins of mucous secretions (Creeth & Denborough, 1970). The typical separation conditions in these experiments are a 48 h period of centrifugation at an initial density of about 1.4 g/ml in either CsCl or CsBr; both practice and theory (Creeth & Horton, 1977) suggest that separation of protein and glycoprotein into the upper and lower halves of the tubes should then be virtually complete. However, in a recent application of the method to bronchial secretions (Creeth *et al.*, 1977), attention was drawn to the unexpectedly difficult removal of free protein from the glycoprotein when using CsBr; with several samples, the glycoprotein fractions so obtained still contained a significant proportion of free protein. Further centrifugations in CsCl were necessary for the elimination of this contamination, detracting from the convenience of the method.

The reasons for this behaviour have now been investigated. The contaminating protein was identified as lysozyme, which is a well-recognized component of bronchial secretions (e.g. Lorenz *et al.*, 1957; Ryley & Brogan, 1968). Its presence in the glycoprotein fraction could have been caused by a strong interaction between the lysozyme and the mucus glycoprotein, similar to that between the enzyme and other negatively charged macromolecules (e.g. Imoto *et al.*, 1972), or by the failure of one of the assumptions on which the density-gradient separation was based. Both these possibilities have been explored.

First, the interaction between lysozyme and a model series of mucus glycoproteins has been investigated, both under approximately physiological conditions, and at higher salt concentrations. Second, the buoyant-density values of lysozyme in a range of caesium salts have been determined, so that its degree of spreading under the separation conditions can be predicted. The results described here show that strong interactions may indeed occur between lysozyme and the glycoproteins; although these interactions are of interest in their own right, they are eliminated at high salt concentrations. On the other hand, the buoyant density of lysozyme in CsBr turns out to be unexpectedly high, this fact alone accounting for the decreased efficiency of the separation. The present paper deals with both aspects of the investigation.

Experimental

Materials

Bronchial glycoproteins were prepared as described previously (Creeth *et al.*, 1977), by suspension and first-stage centrifugation in CsBr; however, 10 ml tubes were used in preference to the 13 ml tubes used previously, the shorter column giving a closer approach to equilibrium in the 48 h period. Further centrifugations for sample CF/LJ were in CsCl at ρ 1.6 and 1.4 g/ml respectively, the former serving to separate the glycoproteins from DNA, and the latter to remove free protein not separated by the initial CsBr step. The whole glycoprotein was recovered

from the bottom halves of the tubes in the final step. With sample BM/Gr, which contained very little DNA, the second centrifugation was in CsCl at 1.40 g/ml, the glycoprotein recovered from the bottom halves of the tubes being resuspended in CsCl at 1.47 g/ml for a third centrifugation. Narrow cuts were made at this stage to obtain a series of four more closely fractionated samples, identified as 5-8, 9-12, 13-15 and 16-19 in order of increasing density.

Ovarian-cyst-fluid glycoproteins, prepared by the phenol-extraction/ethanol-fractionation method of Morgan (1967) were donated by Professor W. M. Watkins (MRC Clinical Research Centre, Harrow, Middx., U.K.). They are designated 'OC', followed by the number of the specimen and the percentage of ethanol in which they were precipitated. Analytical data for these materials have been published previously (Watkins, 1972; Puzsai & Morgan, 1960; Donald, 1973; Creeth *et al.*, 1974).

The human-erythrocyte-membrane glycoprotein, of blood-group-MN specificity, had been prepared by the method of Marchesi & Andrews (1971) and was the gift of Professor J. R. Clamp (Clinical Research Laboratories, Department of Medicine, University of Bristol Medical School, Bristol, U.K.).

The glycoprotein fractions for which binding results are reported gave negative tests for lysozyme by immunodiffusion against anti-(human lysozyme) sera produced in rabbits (Dako-Immunoglobulins, Copenhagen, Denmark). An authentic preparation of human lysozyme, the gift of Professor Elliott Osserman (Columbia University, New York, NY, U.S.A.), was used as a control. The glycoproteins were also examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber *et al.*, 1972). These methods would have detected contamination by lysozyme at the concentration of 1% of the glycoprotein.

An asialoglycoprotein was prepared enzymically from one ovarian-cyst glycoprotein. Glycoprotein OC 350/48-55 (4 mg) was dissolved in 1.1 ml of 0.05 M-sodium acetate/0.01 M-acetic acid/0.002 M-CaCl₂ buffer, pH 5.5, and 0.10 ml (100 units) of a neuraminidase preparation from *Vibrio cholerae* (Behringwerke A.G., Marburg-Lahn, Germany) added. The reaction was monitored viscometrically at 25°C. Approx. 80% of the total change in viscosity occurred within 2 h, but the reaction was allowed to proceed for 48 h; at this time, determination of free sialic acid (NeuNAc) by the method of Warren (1963) gave the same value as that obtained with the original glycoprotein after hydrolysis with H₂SO₄. The asialoglycoprotein was separated from the enzyme by density-gradient centrifugation in CsCl (Creeth & Denborough, 1970).

A crystalline preparation of hen's-egg lysozyme (Sigma, Poole, Dorset, U.K.; lot no. L6876) was used

throughout. In some experiments the human lysozyme preparation described above additionally was used. Both glycoprotein and enzyme solutions were separately dialysed against a phosphate/chloride buffer, pH 6.8, usually at *I* 0.10 (0.0167 M-NaH₂PO₄/0.0167 M-Na₂HPO₄/0.033 M-NaCl/0.001 M-Na₂CO₃/0.002 M-EDTA) before use.

Methods

Buoyant-density determinations were made in either the Centriscan (MSE, Crawley, Sussex, U.K.) or Beckman (Palo Alto, CA, U.S.A.) ultracentrifuges, by using absorption optics at 280 nm or schlieren optics at 546 nm respectively. Previously described procedures were used in the calculation of buoyant-density values from the experimental records (Creeth & Denborough, 1970; Creeth *et al.*, 1974; Creeth & Horton, 1977). Beckman 12 mm 2° or 4° Kel-F cells were used throughout, in both machines; blank corrections, involving the determination of absorbance differences when a pair of cells contained identical non-absorbing solutions, were determined frequently. The blank corrections were usually small and nearly constant for a given assembly, provided organic solvents were not used in cleaning procedures. Where calculations were made on the absorbance values recorded, these were corrected for a substantial non-linearity in the response of the optical system.

Interactions between the glycoproteins and lysozyme were measured by difference experiments in the Centriscan ultracentrifuge, by either sedimentation velocity or sedimentation equilibrium, by using procedures generally similar to those of Richards & Schachman (1959), Kirschner & Schachman (1971) and Steinberg & Schachman (1966). Full cells (approx. 12 mm column) were used for the velocity experiments, but 4 mm columns sufficed for sedimentation-equilibrium studies. Cells were loaded to give identical meniscus positions with a solution of lysozyme (lz), a solution of glycoprotein (gp), or a mixture of the two in which the concentration of each species was the same as in the individual solutions (lz+gp). Since the molecular weight of the glycoprotein is much greater than that of the lysozyme, the difference pattern exhibited by the combination [lz-(lz+gp)] is close to zero over the upper part of the cell if no interaction occurs between lysozyme and glycoprotein, but becomes positive if interaction occurs, since the speed chosen removes the glycoprotein virtually completely from this part of the cell. In a sedimentation-velocity run the effect is observed as a peak, diminishing almost to zero in the plateau region, whose area grows with time (Kirschner & Schachman, 1971). In a sedimentation-equilibrium experiment, the pattern observed is essentially an equilibrium distribution for lysozyme alone, the difference between two distributions being itself an equilibrium distribution, albeit for a lower initial concentration.

The method, although giving less information than the classical lower-speed, procedure of Steinberg & Schachman (1966), is rapid, simple and economical, and demands only the one experiment on the three solutions.

Quantitative estimates of the binding, expressed as weight of lysozyme bound per unit weight of glycoprotein and denoted r_w , were obtained from those equilibrium experiments where binding was obviously substantial. The method chosen depends on the fact that uncombined lysozyme will follow, throughout the cell, a distribution characteristic of the enzyme alone; accordingly, a measurement at one point suffices to define the whole distribution.

The integrated form of the equation of sedimentation equilibrium (Rinde, 1928) is written for each of two solutions of lysozyme; subtraction then gives an expression for the difference in initial concentrations, Δc^0 , in terms of the difference in concentration at some convenient point in the cell. Taking this point as the meniscus, denoted a , one has:

$$\Delta c^0 = \frac{\Delta c(a) \{ \exp [A(b^2 - a^2)] - 1 \}}{A(b^2 - a^2)} \quad (1)$$

Here $A = M(1 - \bar{v}\rho)\omega^2/2RT$, the symbols having their usual significance (Creeth & Horton, 1977). A is determined from the slope of the logarithmic plot for the cell containing lysozyme alone, referred to buffer solvent. $\Delta c(a)$ is obtained by extrapolation of the corrected difference plot $[\text{Iz} - (\text{Iz} + \text{gp}) + \text{gp}]$; in agreement with expectation, the slope of this plot was usually identical, within experimental error, with those of the solutions of lysozyme alone. The correction of the enzyme-mixture difference plot in this way makes allowance for the small, but usually finite, residual absorbance of the glycoprotein, and allows the extrapolation to find $\Delta c(a)$ to be based on the whole pattern recorded.

At high lysozyme concentrations and strong interactions, the difference recording specified is 'noisy'; however, the alternative function $[(\text{Iz} + \text{gp}) - \text{gp}]$ could then be recorded satisfactorily. When analysed similarly, this trace gives the concentration of uncombined lysozyme so that Δc^0 of eqn. (1) could be obtained by difference from the known initial concentration. By definition:

$$r_w = \frac{\Delta c^0 (\text{Iz})}{c^0 (\text{gp})} \quad (2)$$

Results and Discussion

(i) Lysozyme-glycoprotein interactions

Glycoproteins covering the range of common blood-group specificities were examined. Since previous physico-chemical characterization of the glycoproteins has mostly been at neutral pH and

moderate ionic strength (e.g. Creeth & Knight, 1967) Creeth *et al.*, 1974, 1977), the main survey for the detection of interaction was performed at pH 6.8, I 0.10. The results obtained are summarized in Table 1. Several of the samples showed no interaction with lysozyme, the difference curves in sedimentation equilibrium being essentially zero. With some others, although the difference curves were unmistakably positive, quantitative treatment was not attempted because the derived value of r_w would have been unduly influenced by the blank correction. These cases have been designated '+' or '++' to indicate 'faint' or 'distinct' interaction.

The behaviour where interaction was strong is exemplified in Figs. 1 and 2, referring respectively to the velocity and equilibrium methods. The growth in area of the difference boundary (Fig. 1a) is unmistakable. Lysozyme alone (Fig. 1b) yields a classical ideal profile, with an essentially flat plateau region, whereas the mixture (Fig. 1c), although evidently of lower absorbance near the meniscus, lacks an authentic plateau and reaches higher absorbances near the base. The lack of a plateau is characteristic of these glycoproteins (Creeth & Knight, 1967).

The attainment of equilibrium in a difference experiment with short columns (Fig. 2a) shows the development of a typical nearly ideal distribution; the corresponding curves for lysozyme alone and the glycoprotein alone (Fig. 2b, curves III and I respectively) show the similarities between the difference distribution and the lysozyme distribution, and the almost total sedimentation of the glycoprotein.

Even where the numerical values of r_w are small, binding can nevertheless be very significant: for example, the value of 0.033 g/g for glycoprotein OC 603/43-50 implies that 30% of the lysozyme in the initial mixture was bound to the glycoprotein.

The results at I 0.10 show that binding occurs chiefly with glycoproteins of blood-group-Le^a specificity; others, apart from the blood-group-MN-active erythrocyte-membrane glycoprotein, are mostly inactive. However, the Le substances show the greatest variation in content of sialic acid; this component is the major determinant of the charge on these glycoproteins, the other sugars being neutral and the proportion of charged amino acids in the peptide core quite small.

The most active glycoprotein, OC 350/48-55, was therefore examined in greater detail. Table 2 shows the results obtained when temperature, ionic strength and lysozyme concentration were varied in turn, and also includes a value for the asialoglycoprotein. The binding of lysozyme by the native glycoprotein is seen to be little affected by temperature, but to increase with concentration of lysozyme. The influence of ionic strength, however, is most striking: the contrasting appearance of the patterns in Fig. 2(c)

Table 1. *Interactions between hen's-egg lysozyme and various mucus glycoproteins*

Interaction was determined by the equilibrium method described in the text; the initial concentrations of glycoprotein and lysozyme were 1.5 mg/ml and 0.22–0.26 mg/ml respectively; the temperature was 20°C and the medium was at pH 6.8, I 0.10 (see the Experimental section). Abbreviations used: OC, human ovarian cyst; BM, human bronchial mucus; CF, bronchial mucus from cystic-fibrotic patient; EMG, erythrocyte membrane. Human enzyme (see the Experimental section) was substituted for hen's-egg lysozyme with glycoproteins OC 445 and BM/Gr 13–15 and 16–19, yielding qualitatively identical results.

Glycoprotein	Blood-group specificity	NeuNAc (%)	Degree of interaction	r_w
OC 186/45–50	A ₂ B	1.5	0	0.105
OC 238	La ^a	4.5	+	
OC 350/48–55	Le ^a	18.3	+++	
OC 370/45–50	H	1.7	+	
OC 376/50–56	B	1.1	0	
OC 445	Le ^a	3.4	0	0.033
OC 485/48–52	A	2.9	0	
OC 500/50–55	H	2.7	0	
OC 603/43–50	Le ^a	7.9	+++	
BM/Gr 13–15*	Le ^a	8.7	+++	0.063
BM/Gr 16–19	Le ^a	11.6	+++	0.042
CF/LJ	A	7.1	0	0.042
EMG	M,N	20.9	++	

* The two less-dense fractions referred to in the Experimental section gave positive tests for lysozyme and were not examined for interactions.

shows how a substantial interaction at I 0.05 is eliminated at I 0.5. The asialoglycoprotein, which was shown by direct determination to contain not more than 2% of sialic acid (cf. the indirect result quoted under 'Materials'), retained about half the activity of the parent substance, and was thus significantly more active than other Le substances of higher content of sialic acid.

Further evidence for the elimination of interaction at high ionic strength is provided by the results of a density-gradient experiment on a mixture of the moderately active glycoprotein OC 603/43–50 and lysozyme in Cs_2SO_4 at $\rho = 1.34 \text{ g/ml}$ (I 3.5) (Fig. 3). The buoyant density of the glycoprotein is almost unaffected by the lysozyme, and there is virtually no change in the value of the peak A_{280} .

These observations show that the interaction is

Fig. 1. *Recordings of difference sedimentation-velocity experiments*

(a) Mixture of lysozyme and Le^a cyst glycoprotein OC 603 (see Table 1). The ordinate is the difference in A_{280} between the cell containing lysozyme alone and that containing the mixture. Successive scans were at 10 min intervals, starting 11 min after attaining the operating speed (49000 rev./min). (b) Scan of cell containing lysozyme alone, after 88 min at operating speed; c^0 was 0.3 mg/ml (referred to solvent). (c) Scan of cell containing lysozyme (c^0 0.3 mg/ml) and glycoprotein (3.0 mg/ml), after 83 min at speed (referred to solvent). Note: because of stray light and scatter, the response above 0.5A is markedly non-linear with respect to concentration (see the text).

Fig. 2. Recordings of a difference sedimentation-equilibrium experiment on a mixture of lysozyme and *Le^a* cyst glycoprotein OC 350 (see Tables 1 and 2, and Fig. 1)

(a) Scans of difference between lysozyme and (lysozyme + glycoprotein) at I 0.05; first scan (I) after 1 h at 25000 rev./min, others at 30 min intervals. The speed was decreased to 18000 rev./min between the third and fourth scans, leading to a small shift in the meniscus position. Recording was continued for 24 h, but scans after the sixth are not resolved. (b) Scans of individual cells (versus solvent) in difference equilibrium experiment: I, glycoprotein; II, lysozyme/glycoprotein mixture I 0.05; III, lysozyme, I 0.05; IV, lysozyme, I 0.50; V, lysozyme/glycoprotein mixture, I 0.50. (c) Scans of differences between two mixtures of lysozyme and glycoprotein, differing only in ionic strength: I, I 0.50; II, I 0.05. The absorbance scale is amplified by nominal factor of 2, relative to scans in (a) and (b). See also the note on the legend to Fig. 1.

Table 2. Effect of temperature (T), ionic strength (I) and lysozyme concentrations on interaction between glycoprotein OC 350/48–55 and lysozyme

$T(^{\circ}\text{C})$	I	[Lysozyme] (mg/ml)	r_w
3	0.10	0.144	0.071
		0.289	0.132
25	0.10	0.144	0.073
		0.289	0.119
20	0.05	0.231	0.105
	0.50		0
20*	0.05	0.250	0.057

* After removal of NeuNAc with neuraminidase; analysis of the reaction mixture showed essentially quantitative removal of NeuNAc but direct determination on the purified asialoglycoprotein (see the Experimental section) gave a value of 2.0%.

Fig. 3. Distributions of glycoprotein OC 603/43–50 at equilibrium in Cs_2SO_4 of initial density 1.343 g/ml, with and without added lysozyme

Curve I, glycoprotein ($c^0 = 1.5$ mg/ml) alone, $\rho_0 = 1.324$ g/ml. Curve II, lysozyme ($c^0 = 0.07$ mg/ml) alone, $\rho_0 = 1.281$ g/ml. Curve III, glycoprotein/lysozyme mixture, ρ_0 of peak = 1.321 g/ml. Note the small difference in meniscus positions between the two cells. See also the note on the legend to Fig. 1.

probably coulombic in origin, being at least partially correlated with the content of sialic acid, and sensitive to the shielding brought about at high ionic strengths.

The existence of electrostatic interactions between lysozyme and the glycoproteins is not unexpected, because the macromolecules are oppositely charged at this pH; the isoelectric point of hen's-egg lysozyme is 11.2 (Sophianopoulos & Sasse, 1965). Interactions between lysozyme and many negatively charged macromolecules are well known [see Table 18 of

Imoto *et al.* (1972) and the reviews of Osserman *et al.* (1974) and Kuettner *et al.* (1975)]. At low ionic

strengths, soluble complexes may be formed with negatively charged proteins: for example, with ovalbumin at pH 6.8 and I 0.02 (Nichol & Winzor, 1964).

It is somewhat surprising, however, to find an interaction so strongly manifested at I 0.10, and one must conclude that the remainder of the glycoprotein molecule has a modifying effect, as witnessed by the relatively weak interaction shown by the sialic acid-rich membrane glycoprotein and the fairly strong interaction of the asialoglycoprotein referred to above. It may be significant that the oligosaccharide chains of blood-group-Le^a-specific glycoproteins lack the terminal sugars found in those of blood-group-A, -B or -H specificity (e.g. Watkins, 1972) and thus

expose the α -L-fucosyl-(1 $\rightarrow$ 4)-*N*-acetyl- β -D-glucosaminyl groupings to a greater extent. A specific interaction between lysozyme and dextran was suggested by Whitaker (1963) to account for the anomalous behaviour of lysozyme on Sephadex columns at high ionic strength, where charge cannot be a primary effect. Whatever the origin of the interaction, it is clear that much of the lysozyme in many secretions must be in the bound form.

(ii) Buoyant densities of lysozyme in caesium salts

The prediction of conditions for effective separation of proteins and glycoproteins depends on knowledge of their respective apparent buoyant densities, ρ_0 , and effective molecular weights, M_s^{app} (e.g. Creeth & Horton, 1977). Accordingly, the buoyant densities of lysozyme in four caesium salts in common use were determined, giving the results shown in Table 3. Buoyant densities for lysozyme at near-neutral pH have not been previously reported; however, the value in CsCl is in good agreement with that of Ifft (1976), measured at pH 10.5, if allowance is made for the difference in pH.

The values of ρ_0 in bromide and iodide are noticeably higher, and we decided to calculate how much of the increase can be ascribed directly to the higher density of the counterions. The net charge of lysozyme at pH 6.8 expected from the amino acid analysis (Imoto *et al.*, 1972) is about +8; the partial specific volume is 0.726 ml/g (Deonier & Williams, 1970; Millero *et al.*, 1976). If the selective solvation by water (see below) is unchanged, then the differences in the partial molar volumes of the anions (Millero, 1971) would lead to increases in ρ_0 , relative to CsCl, of

Table 3. Buoyant densities and selective solvations of lysozyme at pH 6.8 and 25°C

Values were determined in the Centriscan ultracentrifuge, by using absorption optics, at initial lysozyme concentrations of approx. 0.15 mg/ml, except where shown otherwise; ρ_0^0 is the uncorrected, and ρ_0 the pressure-corrected, buoyant density.

Salt	ρ_0^0 (g/ml)	ρ_0 (g/ml)	Γ' (g/g)
CsCl	1.294	1.299	0.190
CsBr	1.356	1.363	0.029
CsBr*	1.351	1.357	0.042
CsI*	1.397	1.406	-0.051
Cs ₂ SO ₄	1.280	1.281	0.254

* Value determined in the Beckman ultracentrifuge by schlieren optics; initial lysozyme concentration approx. 8 mg/ml.

Fig. 4. Simulated distributions of lysozyme and glycoprotein in density gradients in caesium salts

Conditions were chosen to represent those in typical separation experiments on preparative scale. (a) CsBr, initial density 1.40 g/ml, speed 42 000 rev./min, solution limits $a = 4.4$ cm, $b = 7.0$ cm. Initial concentrations: lysozyme 0.11 mg/ml, glycoprotein 1.0 mg/ml; apparent solvated molecular weights: lysozyme 14 360, glycoprotein 110 000; buoyant densities 1.356 and 1.510 g/ml (see the text). (b) As (a), but in CsCl at 45 000 rev./min. The buoyant densities and apparent solvated molecular weights have been modified to take into account the different properties of the two salts. Here $\rho_0 = 1.294$ g/ml (lysozyme) and 1.480 g/ml (glycoprotein) and $M_s^{app} = 17 250$ (lysozyme) and 78 000 (glycoprotein).

0.021 g/ml for bromide and 0.040 g/ml for iodide. These are smaller than the observed increases, particularly for iodide.

The selective solvation parameter, Γ' (Ifft & Vinograd, 1962), takes no account of ion binding, but merely expresses, when positive, the mass of water necessary to bring the density of the protein from $(1/\bar{v})$ to ρ_0 . The values found here, calculated from the pressure-corrected buoyant densities, are shown in Table 3. For comparison, the values of Γ' found by Ifft & Vinograd (1966) for bovine plasma albumin in CsCl, CsBr, CsI and Cs₂SO₄ were 0.20, 0.10, 0.025 and 0.36 respectively. Lysozyme is thus seen to behave normally in CsCl, but to be exceptional in the other salts. This could be regarded as a consequence of abnormally low solvation by water, but more probably indicates greater anion binding than would be expected from the net charge of the protein (cf. Ifft & Vinograd, 1966).

These findings have several implications relating to protein-glycoprotein separations. In previous work (Creeth & Horton, 1977), it was shown that CsBr was an effective medium for the removal of free protein from protein/glycoprotein mixtures when the buoyant-density difference between the two species had the same value, 0.20 g/ml, as occurs in CsCl. Comparative measurements in the two salts with bovine albumin and several glycoproteins had shown that the buoyant densities of both species were about 0.03 g/ml greater in CsBr than in CsCl. The higher value of ρ_0^0 for lysozyme in CsBr now reported (1.356 g/ml) implies that separations will be less efficient, as shown in Fig. 4(a), where the simulation refers to a mixture of a density-disperse glycoprotein with 10% of its weight of lysozyme. Although segregation of the two species into the upper and lower halves of the preparative tube is very largely achieved, a single-stage preparation must leave some lysozyme as contaminant in the glycoprotein fraction. Thus the original observation of difficulty in removing protein when using CsBr (Creeth *et al.*, 1977) is accounted for on the basis of the abnormally high buoyant density of lysozyme in this medium, although the low molecular weight (14 300) is also a factor. In CsCl, at a suitably higher speed (Fig. 4b), the separation is quite good, mainly because of the lower buoyant density of lysozyme. As recorded by u.v. optics at 280 nm, the displacements due to lysozyme in Figs. 4(a) and 4(b) would be multiplied by about 20 relative to those of the glycoprotein, because of the large differences in absorption coefficients (respectively 2.6 and about 0.1 litre·g⁻¹·cm⁻¹).

Thus a two-stage preparation, entailing the consecutive use of CsBr and CsCl, both at about 1.4 g/ml, to separate protein from glycoprotein, should be effective, provided care is exercised in slicing the tubes at the end of the centrifugation period. Any attempt to subfractionate the glycoprotein component by

banding it centrally in the second stage, as previously suggested, should be postponed to a third stage.

Some of the time-dependent phenomena previously reported (Creeth *et al.*, 1977), in which changes in density-gradient pattern were observed after prolonged (48 h+) periods of centrifugation, may have their origin in the slow release of lysozyme from a complex with glycoprotein, the delay arising from factors yet unknown. We could find no evidence for complex-formation at I 0.5, which is considerably lower than exists in isopycnic experiments with caesium salts.

In conclusion, it must be pointed out that the present findings illustrate the better resolution obtainable with CsCl rather than with CsBr for separating lysozyme from glycoproteins; regrettably, CsBr appears to be essential for the dispersal of gelatinous mucus, CsCl being ineffective with native secretions (Creeth *et al.*, 1977). For the detection of lysozyme as a contaminant in glycoprotein solutions, the u.v.-absorption system has considerable advantages over the schlieren system. CsCl is more effective than CsBr as a medium, whereas Cs₂SO₄ is essentially useless because of the small difference in ρ_0 between lysozyme and glycoproteins. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is more sensitive than either ultracentrifugal method.

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