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REVIEW

Application of Equilibrium Density Gradient Sedimentation to the Separation and Purification of Proteinpolysaccharides

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Summary

Mixtures of proteinpolysaccharides can be effectively separated by equilibrium sedimentation of solutions containing high concentrations of salts such as cesium chloride.

The method is most effective when the buoyant densities of the constituent molecules differ widely. Degradation of the proteinpolysaccharide macromolecules is minimized because degradative enzymes readily sediment away from the denser carbohydrate-containing substances. The macromolecules are subjected to conditions no more severe than exposure to high salt concentrations. The recovery of material is always quantitative and all components are subjected to identical treatments. By sedimenting dilute solutions of proteinpolysaccharides to equilibrium in cesium salt density gradients, separation and concentration can often be effected in a single step.

The method is less suitable when the constituent proteinpolysaccharides are polydisperse or of small molecular weight. In the case of very complex mixtures of proteinpolysaccharides, i.e., extracts from arterial tissue, the method may be useful for preliminary separations only.

The method is time-consuming and can be expensive with regard to materials and equipment. However, as most laboratories possess high-speed preparative centrifuges and cesium salts can be recovered after use, these problems are not insurmountable.

INTRODUCTION

Studies of proteinpolysaccharides* have been directed toward the development of methods for their quantitative isolation from tissues

* The nomenclature recommended by Jeanloz (*1*) has been followed.

and secretions followed by extensive fractionation to obtain material adequate for identification. As the functions of the various tissue proteinpolysaccharides are probably due, in part, to their macromolecular properties, isolation procedures which cause little or no degradation must be used to obtain representative preparations of all the components.

Glycosaminoglycuronoglycans have partial specific volumes in the range 0.5–0.65 ml/g (*2–7*) and thus should have buoyant densities of about 1.6–2 g/ml depending on the nature of the supporting solvent. Proteins, which usually have partial specific volumes above 0.7 ml/g, should have buoyant densities less than about 1.4 g/ml (*8–10*), and glycoproteins, with partial specific volumes between those of pure protein and glycosaminoglycuronoglycan (*11*), should have buoyant densities in the range 1.4–1.6 g/ml. Because of these differences in density, isopycnic density gradient sedimentation (*12,13*) should allow efficient separations.

This method has been used extensively in other fields and excellent reviews are available (*14,15*). As it is the purpose of this article to describe the practical application of the method to proteinpolysaccharide systems, the following sections deal only with experimental procedures, examples of the application of the method to specific systems, and a discussion of advantages and disadvantages.

MATERIALS AND METHODS

Extraction

The most common method of extracting proteinpolysaccharides from tissue is by mechanical disintegration of the tissue in water or dilute salt solutions; not all the carbohydrate-containing material can be extracted in this way (*16,17*), but increased yields may be obtained by repeated treatments and by varying the composition of the extracting solvents (*16–18*). Gentle agitation of thin tissue slices with solvents containing high concentrations of salts (3 *M* magnesium chloride, 4 *M* guanidinium chloride) gives high yields; this "dissociative" process avoids high shear treatment that may cause degradation (*18*). Extracts are usually centrifuged to remove tissue residues.

Gradient Forming Materials

The group of highly soluble low molecular weight salts (CsCl, RbCl, RbBr, Cs_2SO_4, Cs acetate, Cs formate) studied by Vinograd and

his co-workers (*10,12,14,15*) have proved ideal for the study of carbohydrate systems since high densities (up to 2.2 g/ml) and steep density gradients (up to approximately 0.08 g/cm^4 in the rotor no. 40 of the Beckman model L centrifuge) can be obtained. These materials do not affect the solvent viscosity or pH greatly, do not interfere seriously with many of the analytical methods used for carbohydrates, and are separated easily by dialysis from the macromolecular constituents of the equilibrium gradients. Further, the macromolecules are subjected to conditions no more severe than exposure to high salt concentrations, and thus degradation should be minimal only.

Solution Preparation

Solutions for centrifugation are prepared by dissolving the required solid salt in solutions of the proteinpolysaccharides to give the required densities. Relationships between weight composition and density and between density and refractive index are given by Vinograd and Hearst (*14*). As cesium chloride is used most often, the relationship between weight concentration and density for this salt is given below (*14,15*).

$$\text{Wt } \% = 137.48 - 138.11/\rho_{25^\circ}$$

where ρ_{25° is the required density at 25°. This relationship is valid over the density range 1.2–1.9 g/ml.

The densities of all solutions, including those obtained by fractionating the equilibrium gradients, are determined by using 0.2-ml constriction pipettes as pycnometers. The densities obtained in this way are accurate to about ±0.001 g/ml (*14,15*).

Analytical Sedimentation

This is performed in a Beckman model E analytical ultracentrifuge in cells fitted with either single-sector (Kel-F, Minnesota Mining and Manufacturing Co.) or double-sector (filled Epon, Shell Chemical Co.) centerpieces. When double-sector centerpieces are used, one sector contains the solution and the other equilibrium diffusate with both sectors filled identically. A bottom layer of Kel-F fluid, approximately 1 mm deep, is used when possible. Cell leaks can be avoided in experiments at high speed by tightening the cells slightly more than is usual, i.e., up to about 140 inch-pounds. Sometimes it is necessary to use a negative wedge window in the centrifuge cell to compensate for the high refractive indices of the gradient solutions (*15*).

Sedimentation is observed with schlieren optics and the equilibrium gradient distributions are calculated from the data of Ifft, Voet, and Vinograd (*19*) and checked by measurement of the schlieren photographs as described by these authors.

Preparative Sedimentation

Centrifuges and Rotors. Preparative isopycnic density gradient sedimentation is often performed in swinging bucket rotors (Beckman rotors nos. SW25.2 and SW39). These swinging bucket rotors have some disadvantages; the SW39 rotor, while capable of producing sufficiently high gravitational fields, has only a very small capacity (15 ml); the SW25.2 rotor has a large capacity (180 ml) but can be run at relatively slow speeds only. Further, the difference between maximum and minimum radii of the rotors is large and this results in excessively long equilibrium times. This also applies to some of the high-capacity zonal rotors (B-XIV, B-XV; see (*13*)), which are capable of high angular velocities.

Angle rotors (Beckman rotors nos. 40 and 50) have a high capacity, can be run at high angular velocities, and, most important, allow rapid attainment of equilibrium (*20*). When density gradients are produced in these rotors, a reorientation of the gradient occurs during deceleration but with little mixing. However, a change in the physical widths of the solute bands in the gradient results from the geometry of the tubes and rotor (*22*). With the proteinpolysaccharide systems so far studied, this effect does not seem to be important.

Because of the advantages of the angle rotors over the swing-out types, most experiments with proteinpolysaccharides can best be carried out in either a no. 40 or no. 50 rotor of a Beckman preparative ultracentrifuge, at speeds close to the maximum allowable.

Unloading Tubes. Rotors are allowed to come to rest without braking; the tubes are removed and carefully placed in a vertical position. Fractions are then collected either by the use of a tube-slicer (Beckman) or by using one of the many devices designed to collect fractions serially from the top or bottom of the tube. Fractionations can be carried out easily and accurately by expelling the tube contents through a hole in the bottom of the tube by forcing kerosine in through the hole in the tube cap with a micrometer syringe burette (*23*).

Determination of the Density Gradient. This is done by direct measurement of the density of the several fractions obtained from the equi-

librium gradient (see Preparation of Solutions). An approximate calculation of the density gradient can be made as described by Ifft et al. (*19*) for cylindrical tubes (rotors nos. SW39, SW25.2) or as described by Fisher, Cline, and Anderson (*21*) for angle rotors (nos. 40, 50) using the radial center of the cell for the isoconcentration position (i.e., the radial position at which the loading density occurs). As the true isoconcentration position is slightly nearer (0.1–0.2 cm) to the cell bottom than the radial center (*19*), the calculated densities will be slightly too high. However, all experiments show reasonable agreement between the measured and calculated gradients. This is illustrated in Fig. 1, for an experiment at 36,000 rpm in the no. 40 rotor with a cesium chloride solution of loading density 1.66 g/ml.

Time Required for Equilibrium. From the time taken to establish equilibrium in an analytical experiment, an estimate of the time required to reach equilibrium in a preparative angle rotor can be made by using the relation

$$\frac{t_P}{t_A} = \frac{\omega_A^2}{\omega_P^2} \times \frac{r_A}{r_P} \times \frac{d_P}{d_A}$$

where the subscripts A and P refer to analytical and preparative ex-

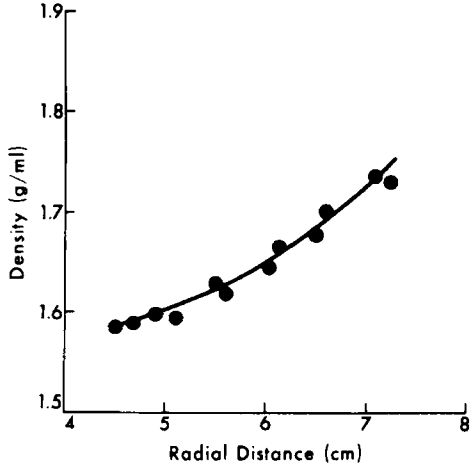

FIG. 1. Comparison of calculated and experimentally determined density gradients. The points represent measured densities from two experiments with cesium chloride solution carried out in the no. 40 rotor of a Beckman model L centrifuge. The loading density was 1.66 g/ml, the speed 36,000 rpm, and the equilibrium time 48 hr. The line represents the calculated density gradient.

periments respectively, t is the equilibrium time, ω the angular velocity, r the mean radius of rotation, and d the effective distance through which sedimentation occurs. The value of d for rotors no. 40 and no. 50 is assumed to be equal to the diameter of the tube (1.6 cm). Times computed in this way have proved to be more than adequate for experiments carried out in these rotors.

APPLICATION OF THE DENSITY GRADIENT METHOD TO SOME PROTEINPOLYSACCHARIDE SYSTEMS

Separation of Fetuin from a Chondroitin Sulfate-Protein (*24*)

Fetuin is a well-characterized glycoprotein consisting of about 80% by weight amino acids and about 20% by weight carbohydrate; it has a partial specific volume near 0.7 ml/g (*11,25*). The chondroitin sulfate-protein consists of about 15% by weight amino acids and about 85% by weight chondroitin sulfate; it has a partial specific volume of about 0.55–0.6 ml/g (*2–7*). The material used was prepared as described by Buddecke, Kröz, and Lanka (*3*).

A mixture of the two substances is a simplified model of a connec-

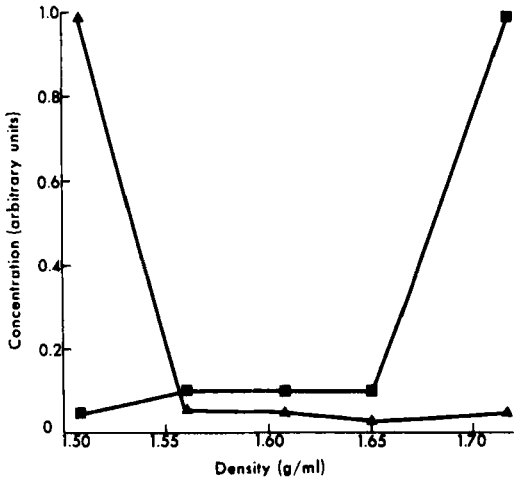

FIG. 2. The separation of fetuin from a chondroitin sulfate-protein. 5 ml of a solution containing 3 mg fetuin and 3 mg chondroitin sulfate-protein was adjusted to density 1.63 g/ml with CsCl and sedimented to equilibrium in the SW39 rotor of a Beckman model L centrifuge at 36,000 rpm, for 72 hr. ▲, fetuin, from the determination of sialic acid (*46*); ■, chondroitin sulfate-protein, from the determination of uronic acid (*47*).

tive tissue extract. A solution containing 3 mg fetuin and 3 mg chondroitin sulfate-protein in 5 ml was prepared and cesium chloride added to give a density of 1.63 g/ml. The solutions were then sedimented to equilibrium in the SW39 rotor of a Beckman Model L centrifuge (72 hr, 36,000 rpm). Figure 2 shows that the fetuin (indicated by the sialic acid values) accumulated in the low-density region and the chondroitin sulfate-protein (indicated by the uronic acid values) accumulated in the high-density region. Thus a clean separation of the two substances was effected.

Separation of the Components of an Ultrafilter Residue (UFR) of Ox Synovial Fluid (*23,26,27*)

From the form of the equilibrium sedimentation distribution of UFR prepared from ox synovial fluid (*28*), Nichol, Ogston, and Preston (*29*) concluded that this material may contain a fraction with density higher than that of the main bulk of the material. The main components of UFR are hyaluronic acid (about 75% by weight) and

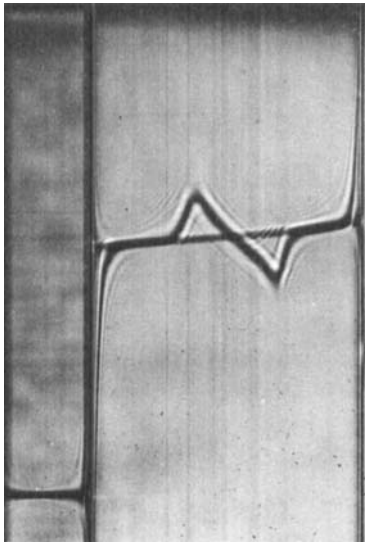

FIG. 3. Analytical density gradient separation in cesium chloride solution of the components of UFR. The experimental conditions were: Beckman model E analytical centrifuge, rotor An-D; concentration of UFR, 1.1 mg/ml; loading density, 1.66 g/ml; schlieren phase plate angle, 60°; time, 69 hr; speed, 44,770 rpm.

protein (about 25% by weight); these components cannot be easily separated (*28*). A partial specific volume of 0.65 ml/g has been assumed for a hyaluronic acid protein complex of the above composition (*29*), and thus it would be expected to have a buoyant density near 1.6 g/ml. A schlieren photograph from the analytical equilibrium sedimentation of UFR in a cesium chloride gradient (loading density 1.66 g/ml, Beckman model E centrifuge, 44,770 rpm, 69 hr) is shown in Fig. 3. The UFR was cleanly separated into three fractions with apparent densities less than 1.58 g/ml, 1.65–1.66 g/ml, and greater than 1.72 g/ml, respectively. The results of a preparative experiment, modeled on the analytical experiment, are shown in Fig. 4 (loading density 1.67 g/ml, rotor no. 50, Beckman model L2 centrifuge, 42,000 rpm, 6 days). The conditions seen in the analytical cell were almost exactly reproduced in the preparative experiment. The least dense fraction was identified as protein, the middle fraction as hyaluronic acid, almost free from protein, and the densest fraction as a chondroitin 6-sulfate protein. Subsequent equilibrium density gradient sedimentation of the protein fraction showed that it contained at least

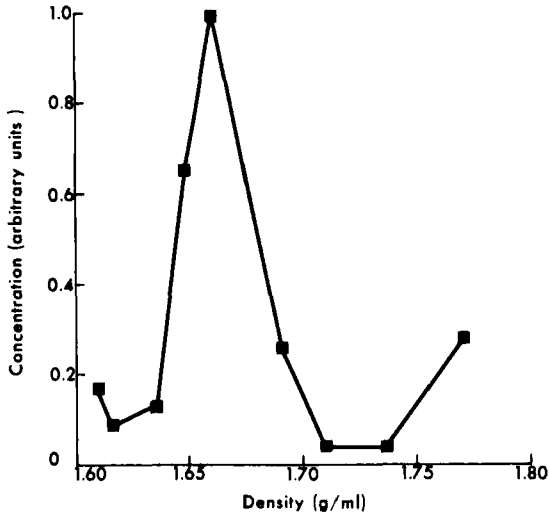

FIG. 4. Preparative density gradient separation in cesium chloride solution of the components of UFR. The experimental conditions were: Beckman model L2 centrifuge, rotor no. 50; concentration of UFR, 3 mg/ml; loading density, 1.67 g/ml; time, 6 days; speed, 42,000 rpm. The experimental points are uronic acid values (*47*).

two protein components with buoyant densities near 1.30 g/ml and 1.32 g/ml respectively (*27*).

This example illustrates the effectiveness of the method in the separation of a naturally occurring proteinpolysaccharide system that contains three major components with widely differing buoyant densities. Further, it shows the close similarity between analytical and preparative experiments and suggests that analytical experiments should be performed first to allow the proper planning of any large scale preparative experiment.

Separation of the Proteinpolysaccharides from Aqueous Extracts of Cartilage (*30–32*)

Aqueous extracts of nasal cartilage contain glycoprotein and several chondroitin sulfate-containing proteinpolysaccharides (*30,31*). Equilibrium sedimentation in a cesium chloride density gradient (loading density 1.63 g/ml, rotor no. 40, Beckman model L centrifuge, 35,000 rpm, 48 hr) separated the proteinpolysaccharides into two fractions

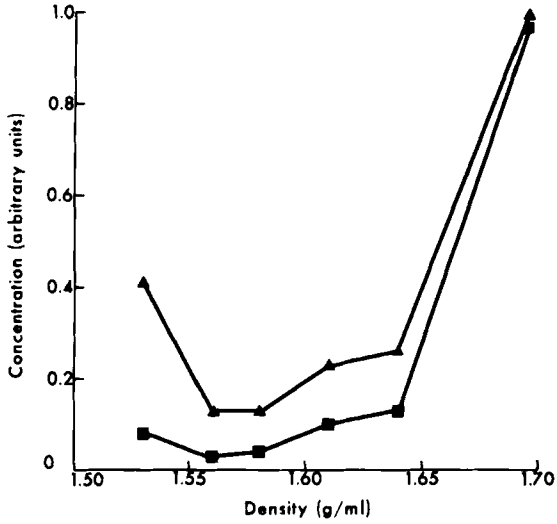

FIG. 5. Preparative density gradient separation in cesium chloride solution of proteinpolysaccharides from aqueous extracts of nasal cartilage. The experimental conditions were: Beckman model L centrifuge, rotor no. 40; loading density, 1.63 g/ml; time, 48 hr; speed, 35,000 rpm. ▲, protein, from the difference between absorbances at 215 and 225 mμ (*48*). ■, glycosaminoglycuronoglycan, from uronic acid (*47*).

with densities less than 1.55 g/ml and greater than 1.65 g/ml respectively (Fig. 5). The denser fraction contained the greater proportion of the chondroitin sulfate (indicated by the uronic acid values) while the less dense fraction contained glycoprotein (indicated by the A_{215}–A_{225} values) and the remainder of the chondroitin sulfate.

The denser fraction consisted of two chondroitin sulfate–containing components that differed only in their proportions of peptide (*31*). Equilibrium density gradient sedimentation in cesium formate solutions (loading densities 2.12 g/ml and 1.90 g/ml, rotor no. 50, Beckman model L2 centrifuge, 42,000 rpm, 60 hr) showed that the bulk of the material comprising this fraction was in the density range 1.92–2.10 g/ml (Fig. 6). However, material with density above 2.10 g/ml and below 1.92 g/ml was also present. The method is therefore unsatisfactory for the separation of these high-density polydisperse proteinpolysaccharides.

The glycoprotein and chondroitin sulfate-protein in the less dense fraction were easily separated by equilibrium density gradient sedi-

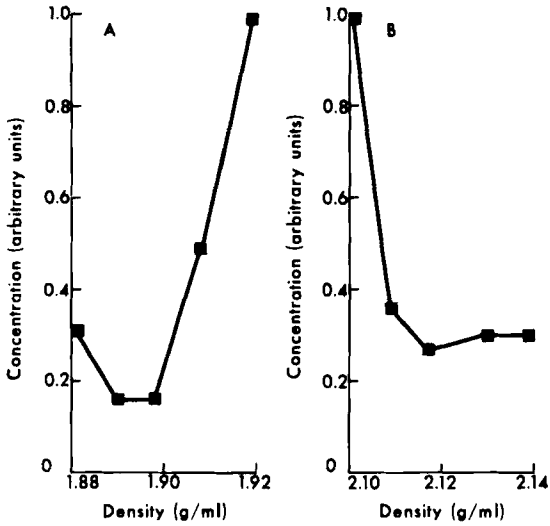

FIG. 6. Preparative density gradient sedimentation in cesium formate solutions of the denser fraction (density greater than 1.65 g/ml) obtained by sedimenting cartilage extracts to equilibrium in a cesium chloride gradient (Fig. 5). The experimental conditions were: Beckman model L2 centrifuge, rotor no. 50; loading density, A—1.90 g/ml, B—2.12 g/ml; time, 60 hr; speed, 42,000 rpm. The experimental points are uronic acid values (*47*).

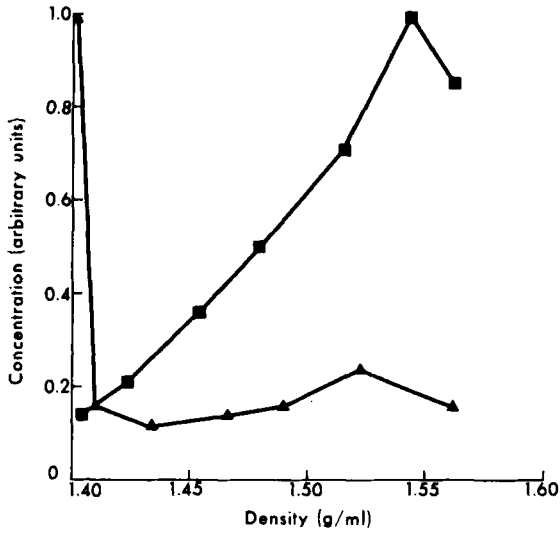

FIG. 7. Preparative density gradient separation in cesium chloride solution of the least dense material (density less than 1.55 g/ml) obtained by sedimenting cartilage extracts to equilibrium in a cesium chloride gradient (Fig. 5). The experimental conditions were: Beckman model L centrifuge, rotor no. 40; loading density, 1.47 g/ml; time, 48 hr; speed, 35,000 rpm. ▲, protein, from the absorbance at 215 mμ (*49*). ■, glycosaminoglycuronoglycan, from the determination of uronic acid (*47*).

mentation at a lower density (Fig. 7; loading density 1.47 g/ml, rotor no. 40, Beckman model L centrifuge, 35,000 rpm, 48 hr). An equally satisfactory separation can also be effected by using Sephadex G-200 (*31*) or Sepharose 4B (Dr. M. Janado, personal communication).

These experiments further demonstrate the effective separation of materials with large density differences (Figs. 5 and 7) and that these separations are comparable with those obtained by other methods (i.e., gel filtration). However, the results show that the method cannot be used successfully with systems that contain molecules whose densities are broadly distributed (Fig. 6).

Separation of Glycoproteins from Ovarian Cyst Fluids (*33*)

The sparingly soluble gel-like glycoproteins prepared from the human ovarian cyst fluids by phenol extraction (*34–36*) are thought to be large macromolecules composed of at least two types of peptide-containing structure associated through disulfide bonds (*33,37*). Solu-

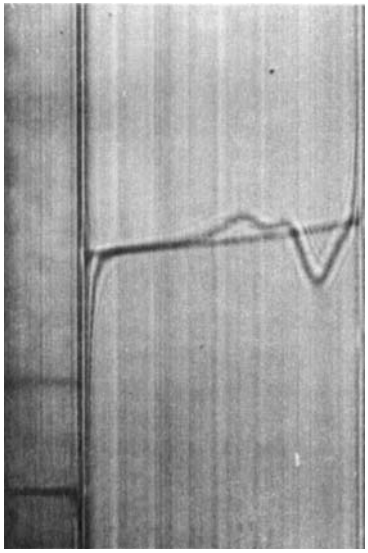

FIG. 8. Analytical density gradient separation in cesium chloride solution of glycoproteins from an ovarian cyst fluid. The experimental conditions were: Beckman model E analytical centrifuge, rotor An-D; concentration, 1.5 mg/ml; loading density, 1.45 g/ml; schlieren phase plate angle, 60°; time, 24 hr; speed, 44,770 rpm.

tions of these materials were obtained by reduction with thioglycolate or sulfite and fractions with differing chemical compositions were obtained by chromatography on DEAE-cellulose columns (*37*). As the yields were not quantitative, conclusions concerning the structures of the sparingly soluble materials were considered to be tentative only.

Analytical density gradient sedimentation of the solubilized blood group substances (Fig. 8; loading density 1.45 g/ml, Beckman model E ultracentrifuge, 44,770 rpm, 24 hr) showed heterogeneous material banded over the density range 1.44–1.48 g/ml and also material with density below about 1.43 g/ml. The result of a preparative experiment (Fig. 9; loading density 1.40 g/ml, rotor no. 40, Beckman model L centrifuge, 34,000 rpm, 72 hr), based on the analytical experiment, showed that the conditions seen in the analytical cell were satisfactorily reproduced in the preparative centrifuge. Fractions were collected and analysed. Quantitative recoveries of carbohydrate and protein were obtained and the different chemical compositions of the fractions (see Table in (*33*)), while confirming the heterogeneity of

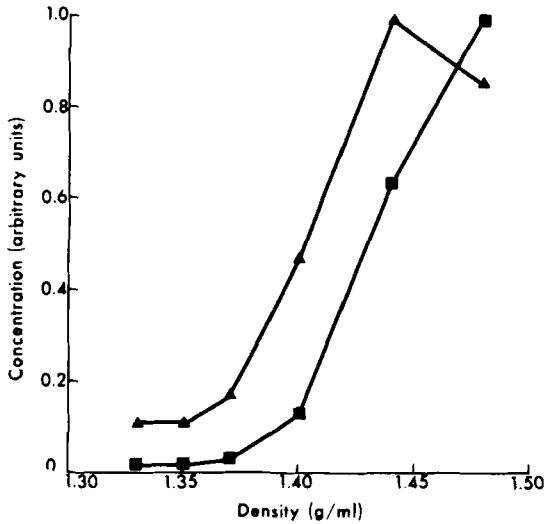

FIG. 9. Preparative density gradient separation in cesium chloride solution of glycoproteins from an ovarian cyst fluid. The experimental conditions were: Beckman model L centrifuge, rotor no. 40; loading density, 1.40 g/ml; time, 72 hr; speed, 34,000 rpm. ▲, protein, from the absorbance at 215 mμ (*49*). ■, carbohydrate, by the phenol-sulfuric acid method (*50*).

the banded glycoproteins, allowed more definite conclusions concerning the structures of these molecules to be made (*33*).

This example shows the value of the method in allowing the quantitative recovery of material and further illustrates the usefulness of preliminary experiments in the analytical ultracentrifuge.

Separation of the Proteinpolysaccharides in Aqueous Extracts of Aorta *(24,38)*

Aqueous extracts of aortic tissue contain glycoproteins and a complex spectrum of glycosaminoglycuronoglycan protein complexes (*24*, *38–40*). Because of this, these extracts contain molecules with densities spread over a wide range. Thus, the equilibrium density gradient method will only provide an arbitrary fractionation in the first instance. Sedimentation to equilibrium in cesium chloride solution (loading density 1.63 g/ml, rotor no. 40, Beckman model L centrifuge, 35,000 rpm, 48 hr) allowed two major proteinpolysaccharide fractions

(density less than 1.55 g/ml and greater than 1.65 g/ml, respectively) to be obtained (Fig. 10).

The densest material contained protein complexes of heparan sulfate and chondroitin 6-sulfate (*38,41*) with some nucleic acid. While these materials could not be separated by density gradient sedimentation, an efficient separation was obtained by fractional precipitation with quaternary ammonium ions (*38,41*).

The least dense material contained glycoproteins and glycosaminoglycuronoglycan proteins. Equilibrium density gradient sedimentation of this material at a lower density (loading density 1.55 g/ml, rotor no. 40, Beckman model L centrifuge, 35,000 rpm, 48 hr) allowed further separation (Fig. 11). The least dense material contained most of the glycoprotein while the glycosaminoglycuronoglycans were distributed in the regions of higher density (*38*).

In this example the equilibrium density gradient method only effected the partial separation of the components of a complex protein-polysaccharide system. These components, while not being separable

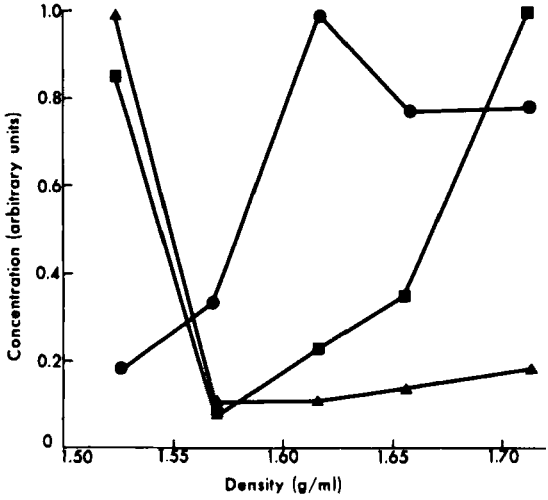

FIG. 10. Preparative density gradient separation in cesium chloride solution of the proteinpolysaccharides in an aqueous extract of aortic tissue. The experimental conditions were: Beckman model L centrifuge, rotor no. 40; loading density, 1.63 g/ml; time, 48 hr; speed, 35,000 rpm. ▲, glycoprotein, from the determination of sialic acid (*46*). ■, glycosaminoglycuronoglycan, from the determination of uronic acid (*47*). ●, absorbance at 260 mμ.

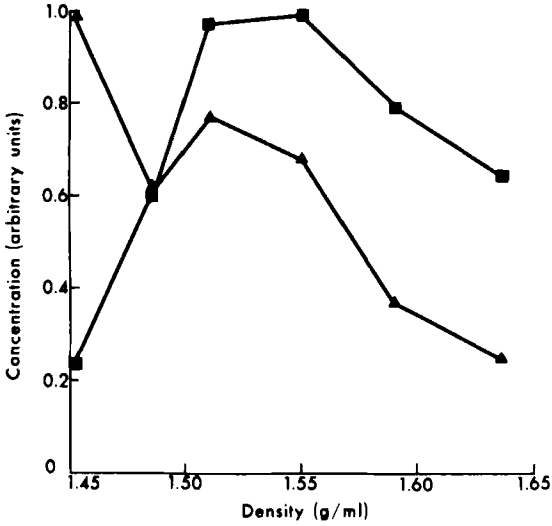

FIG. 11. Preparative density gradient separation in cesium chloride solution of the least dense material (density less than 1.55 g/ml) obtained by sedimenting cartilage extracts to equilibrium in a cesium chloride gradient (Fig. 10). The experimental conditions were: Beckman model L centrifuge, rotor no. 40; loading density, 1.55 g/ml; time, 48 hr; speed, 35,000 rpm. ▲, sialic acid. ■, uronic acid.

by further gradient sedimentation, can be separated by other methods, which normally could not be applied successfully to the crude extracts.

DISCUSSION

The examples considered above show that equilibrium sedimentation of proteinpolysaccharide mixtures in the presence of high concentrations of cesium salts is a useful separation method. It is most effective when the densities of the components of the mixture differ widely, and is least effective when the constituent molecules are polydisperse and of high density. Degradation of proteinpolysaccharide macromolecules is minimized because degradative enzymes, which are often present in tissue extracts or secretions (*42–44*), readily sediment away from the more dense carbohydrate-containing substances. The constituent macromolecules are subjected to conditions no more severe than exposure to high salt concentrations. Indeed, the high ionic strength of the supporting solvent may be of considerable significance in effecting the separation of components loosely bound by salt linkages (cf. (*18*)

where 3 *M* magnesium chloride and 4 *M* guanidinium chloride are used to extract the proteinpolysaccharides from cartilage).

Because of the very large molecular weights of many proteinpolysaccharides, it is often possible to work only with relatively dilute solutions. Concentration of these solutions by conventional methods, i.e. ultrafiltration, rotary film evaporation, freeze concentration, etc., sometimes leads to degradation of the molecules and usually gives very viscous solutions from which it is difficult to separate the various components. In these circumstances equilibrium density gradient sedimentation of dilute solutions can effect separation and concentration in a single step.

Some proteinpolysaccharides have densities in excess of the maximum attainable in solutions of cesium ions; successful separations of these materials from other components can be performed by sedimenting them to the bottom and banding the less dense components within or at the top of a gradient. Similarly, the method can be used to remove unwanted material of low density, i.e., nonspecific protein, lipoprotein, etc., by sedimenting it to the top of a gradient of high density. Where a particular proteinpolysaccharide in a mixture has a density very different from those of the other components, the method may be used as a single-step procedure for its isolation in relatively pure form; this avoids many of the complex isolation and purification methods that are currently in use. For example, the extraction of cartilage with cesium chloride solution followed by sedimentation to equilibrium (conditions similar to those in Fig. 5) allows the high-density chondroitin sulfate-protein to be obtained directly. Second, the glycoproteins present in various secretions, such as cyst fluids and submaxillary gland secretions, have densities in the range 1.45–1.55 g/ml (*11*) while the other components of these fluids probably have somewhat lower densities; the addition of cesium chloride to the native secretion to give a suitable density, followed by sedimentation to equilibrium, should allow relatively uncontaminated glycoproteins to be obtained. Finally, hyaluronic acid, with only a small proportion of protein, can be prepared directly by equilibrium density gradient sedimentation of synovial fluid in cesium chloride.

The method is unsuitable for separations of mixtures containing low molecular weight substances because these materials form broad bands when sedimented isopycnically in density gradients (*10,12,14, 15*). Some experiments in the analytical ultracentrifuge with protein-free chondroitin sulfate and keratan sulfate showed a spread of ma-

terial over the density range 1.8 g/ml to over 2.0 g/ml (J. R. Dunstone, unpublished experiments); this spread was most likely due to the small molecular weights of these substances.

The method is time-consuming and can be expensive with respect to materials (cesium salts) and equipment (centrifuges, centrifuge drive units, and rotors). However, most laboratories have preparative centrifuges of the Beckman model L type with angle rotors, and cesium salts can be recovered and purified after use (*45*). The present availability of large-capacity, high-speed rotors with short sedimentation path lengths makes the method more useful as a large-scale preparative technique.

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REFERENCES

1. R. W. Jeanloz, *Arthritis Rheumat.*, **3,** 233 (1960).
2. M. B. Mathews and I. Losaityte, *Arch. Biochem. Biophys.*, **61,** 367 (1956).
3. E. Buddecke, W. Kröz, and E. Lanka, *Z. Physiol. Chem.*, **331,** 196 (1963).
4. C. Tanford, E. Marler, E. Jury, and E. A. Davidson, *J. Biol. Chem.*, **239,** 4034 (1964).
5. M. Luscombe and C. F. Phelps, *Biochem. J.*, **102,** 110 (1967).
6. E. J. Eyring and J. T. Yang, *J. Biol. Chem.*, **243,** 1306 (1968).
7. M. Luscombe and C. F. Phelps, *Biochem. J.*, **103,** 103 (1967).
8. A. Siegel and W. Hudson, *Biochim. Biophys. Acta,* **34,** 254 (1959).
9. D. J. Cox and V. N. Schumaker, *J. Am. Chem. Soc.*, **83,** 2439 (1961).
10. J. B. Ifft and J. Vinograd, *J. Phys. Chem.*, **66,** 1990 (1962).
11. R. A. Gibbons, in *The Glycoproteins* (A. Gottschalk, ed.), Elsevier, Amsterdam, 1966, p. 62.
12. M. S. Meselson, F. W. Stahl, and J. Vinograd, *Proc. Natl. Acad. Sci. U.S.*, **43,** 581 (1957).
13. N. G. Anderson, in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (N. G. Anderson, ed.), National Cancer Institute Monograph No. **21,** U.S. Government Printing Office, Washington, D.C., 1966, pp. 15–16.
14. J. Vinograd and J. E. Hearst, *Progr. in Chem. Org. Nat. Prods.*, **20,** 372 (1962).

15. J. Vinograd, in *Methods in Enzymology* (S. P. Colowick and N. O. Kaplan, eds.), Vol. 6, 1st ed., Academic Press, New York, 1963, p. 854.
16. M. Schubert, *Federation Proc.*, **17**, 1099 (1958).
17. D. A. Lowther, B. P. Toole, and F. A. Meyer, *Arch. Biochem. Biophys.*, **118**, 1 (1967).
18. S. W. Sajdera and V. C. Hascall, *J. Biol. Chem.*, **244**, 77 (1969).
19. J. B. Ifft, D. H. Voet and J. Vinograd, *J. Phys. Chem.*, **65**, 1138 (1961).
20. E. G. Pickels, *J. Gen. Physiol.*, **26**, 341 (1943).
21. W. D. Fisher, G. B. Cline, and N. G. Anderson, *Analyt. Biochem.*, **9**, 477 (1964).
22. W. G. Flamm, H. E. Bond, and H. E. Burr, *Biochim. Biophys. Acta*, **129**, 310 (1966).
23. P. Silpananta, J. R. Dunstone, and A. G. Ogston, *Biochem. J.*, **104**, 404 (1967).
24. M. D. Franek and J. R. Dunstone, *Biochim. Biophys. Acta*, **127**, 213 (1966).
25. R. G. Spiro, *J. Biol. Chem.*, **235**, 2860 (1960).
26. P. Silpananta, J. R. Dunstone, and A. G. Ogston, *Biochem. J.*, **109**, 43 (1968).
27. P. Silpananta, J. R. Dunstone, and A. G. Ogston, *Australian J. Biol. Sci.*, **22**, 1031 (1969).
28. B. N. Preston, M. Davies, and A. G. Ogston, *Biochem. J.*, **96**, 449 (1965).
29. L. W. Nichol, A. G. Ogston, and B. N. Preston, *Biochem. J.*, **102**, 407 (1967).
30. J. R. Dunstone and M. D. Franek, *Biochem. Biophys. Res. Commun.*, **27**, 39 (1967).
31. M. D. Franek and J. R. Dunstone, *J. Biol. Chem.*, **242**, 3461 (1967).
32. J. R. Dunstone and M. D. Franek, *J. Biol. Chem.*, **244**, 3654 (1969).
33. J. R. Dunstone, *European J. Biochem.*, **9**, 128 (1969).
34. D. Aminoff, W. T. J. Morgan, and W. M. Watkins, *Biochem. J.*, **46**, 426 (1950).
35. R. A. Gibbons, W. T. J. Morgan, and M. N. Gibbons, *Biochem. J.*, **60**, 428 (1955).
36. A. Pusztai and W. T. J. Morgan, *Biochem. J.*, **80**, 107 (1961).
37. J. R. Dunstone and W. T. J. Morgan, *Biochim. Biophys. Acta*, **101**, 300 (1965).
38. M. D. Franek, Ph.D. thesis, Australian National University, Canberra, A.C.T., Australia, 1968.
39. C. A. Antonopoulos, S. Gardell, and B. Hamström, *J. Atheroscler. Res.*, **5**, 9 (1965).
40. B. Radhakrishnamurthy, A. F. Fishkin, G. J. Hubbell, and G. S. Berenson, *Arch. Biochem. Biophys.*, **104**, 19 (1964).
41. M. D. Franek and J. R. Dunstone, *Biochim. Biophys. Acta*, **165**, 555 (1968).
42. S. M. Partridge, A. H. Whiting, and H. F. Davis, in *Structure and Function of Connective and Skeletal Tissue* (S. F. Jackson, R. D. Harkness, S. M. Partridge, and G. R. Tristram, eds.), Butterworth, London, 1965, p. 160.
43. E. Buddecke, in *Biochemie et Physologie du Tissu Conjonctif*, International Symposium, Lyon, France, 1965, p. 59.
44. E. Buddecke and D. Platt, *Z. Physiol. Chem.*, **343**, 61 (1966).
45. R. R. Wright, W. S. Pappas, J. A. Charter, and C. W. Weber, in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (N. G. Anderson, ed.), National Cancer Institute Monograph No. 21, U.S. Government Printing Office, Washington, D. C., 1966, p. 241.

46. L. Warren, *J. Biol. Chem.*, **234,** 1971 (1959).
47. Z. Dische, *J. Biol. Chem.*, **167,** 189 (1947).
48. J. B. Murphy and M. W. Kies, *Biochim. Biophys. Acta,* **45,** 382 (1960).
49. M. P. Tombs, F. Souter, and N. F. MacLagen, *Biochem. J.*, **73,** 167 (1959).
50. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28,** 350 (1956).

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