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Ultracentrifugation Studies of Polyelectrolytes in Polyglucose Density Gradient

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SUMMARY

An ideal solute for density gradient ultracentrifugation of polymers in aqueous solution should be inert and readily soluble in water to form an extended range of solution densities of low viscosity. High molecular weight is an added attraction because osmotic effects are minimized. Highly branched spherical synthetic polysaccharides fulfill these requirements. High degree of branching is a consequence of the condensation of polyfunctional monomers. Density and relative viscosity of solutions of polyglucose, sucrose, and of a natural sucrose polymer, Ficoll, are compared. The behavior of various polyelectrolytes was studied in low viscosity polyglucose density gradients in equilibrium buoyant density measurement in the ultracentrifuge. Macromolecules or macromolecular complexes attain low apparent equilibrium buoyant density, probably because of an excluded volume effect of the solute. This allows sedimentation to isopycnic position of complex biopolymers in inert polyglucose solutions, which otherwise can be attempted only in concentrated solutions of heavy salts (such as CsCl or Cs_2SO_4). Such salts, however, may salt out, or through osmotic effects degrade or alter the properties of certain biologic macromolecular complexes.

INTRODUCTION

Equilibrium density gradient centrifugation is a useful technique often employed both to characterize and to separate polymers with respect to their apparent buoyant densities in various solutions. An ideal solute for this purpose should be neutral, inert, and readily soluble in water (or other solvent) to form an extended range of solution densities. These should exhibit a low viscosity to permit the rapid attainment of equilibrium both with respect to the density gradient and with respect to the sedimentation of the macrospecies to its equilibrium position within the gradient. Moreover, low viscosity simplifies the preparation of preformed gradients by simple gravity feed techniques [1]. It is an additional advantage if this solute has a high molecular weight when it is important to minimize osmotic effects on the polymers or polymer complexes to be examined.

This communication presents experiments employing a density gradient of water-soluble synthetic polyglucose of relatively low viscosity, in which the equilibrium buoyant densities of various large particles were determined in the preparative ultracentrifuge. In addition, polyglucose is compared with various other solutes commonly used in density gradient centrifugation.

Polyglucose is a neutral, inert, highly branched spherical polymer prepared by the polycondensation of glucose [2]. It is highly soluble in water or in buffers, and aqueous solutions up to 60% concentration can be obtained readily. Polyglucose does not form insoluble cross-linked gels because the polymerization conditions are carefully controlled so that only one functional group, the glucosidic hydroxyl, is always a partner to each polycondensation step and polymers contain only one such group [3, 4]. The polymer, however, is highly branched, since unreacted glucose molecules can condense through their glucosidic hydroxyls with any and up to all four of the other hydroxyls of the polymeric glucose residues [3]. Thus, these glucose polymers possess a relatively low intrinsic viscosity commensurate with approximately spherical over-all shape in aqueous solutions [5].

MATERIALS AND METHODS

Materials

Polyglucose was a sample (#L 524023-0-2) prepared for investigational use for the National Cancer Chemotherapy Service Center, NCI, NIH, by

Merck & Co., employing methods similar to those published by this laboratory [2, 3]. Sucrose, density gradient grade, Mann Research Laboratories, Inc.; Ficoll, a sucrose polymer of MW = 400,000, Pharmacia, Uppsala, Sweden; and cesium chloride, optical grade for density gradient centrifugation, Harshaw Chemical Co., were obtained commercially. Sperm whale myoglobin and horse heart cytochrome c were obtained from Mann; human hemoglobin, from Calbiochem; and Blue Dextran 2000, from Pharmacia. Various other complex biopolymers (viruses, membrane vesicles from cell homogenates, etc.) were purified in this laboratory, as will be indicated at the appropriate places in the text.

Methods

Linear density gradients of polyglucose, Ficoll, or cesium chloride were prepared as indicated in the legends to the appropriate figures. Solutions of proteins, dextran, membrane vesicles, etc., were layered on the top of the density gradients in a small volume (0.2-0.5 ml) of buffered aqueous solutions and centrifuged in the Spinco Model L-2 Preparative Ultracentrifuge. Equilibrium time varied considerably, depending on size of the test particle, and is indicated in the appropriate figure legends. The tubes were punctured at the bottom and 30-40 fractions of equal volume were collected.

Densities (ρ^t) of the fractions were determined by reading the refractive index (n_D^t) of a drop of solution using an Abbe refractometer. The relations between density and refractive index were determined to be, for polyglucose [6],

$$\rho^{30} = 2.5426 n_D^{30} - 2.3668,$$

for $1.00 < \rho < 1.22$; and for Ficoll,

$$\rho^{30} = 2.2405 n_D^{30} - 1.9682$$

for $1.00 < \rho < 1.20$. For a 60% (w/v) solution of polyglucose, ρ^{30} was 1.208; of Ficoll, it was 1.189. Similar data are available in the literature for CsCl solutions [7] and for the other supporting solutes cited for comparison. The distribution of macromolecules was determined with another aliquot of each fraction by measuring absorbancy, radioactivity, or biological activity.

RESULTS

Figure 1 compares the relative viscosity and density of polyglucose, Ficoll, and sucrose solutions. It can be seen that while sucrose at any particular solution density has the lowest relative viscosity, polyglucose has lower relative viscosity than Ficoll when the carbohydrate polymers are contrasted. In the density range up to about 1.15 g/ml, polyglucose is not too

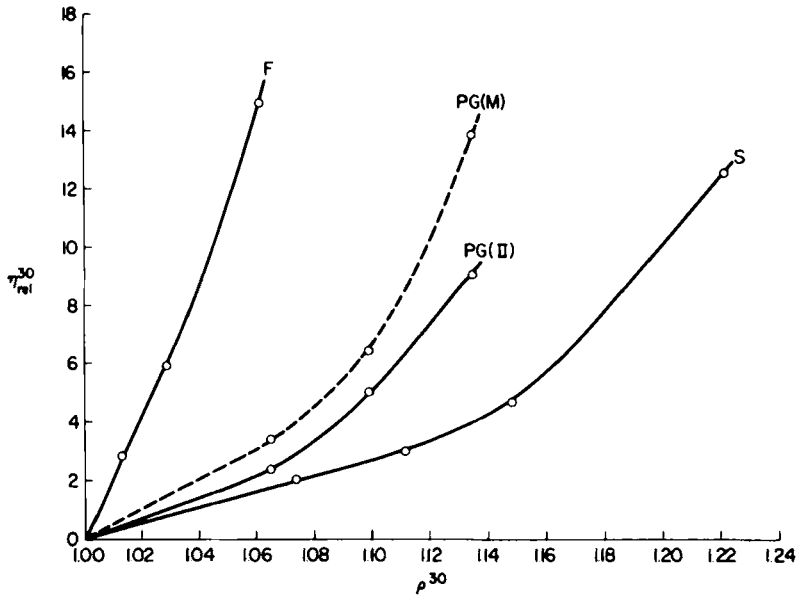

Fig. 1. Viscosity as a function of density for various nonionic density gradient solutes. S, sucrose; PG(II), polyglucose, fractionated by alcohol precipitation [3]; PG(M), polyglucose, Merck & Co., commercial sample; F. Ficoll, Pharmacia. The densities of the PG(M) sample were not measured as precisely as the others.

viscous to prolong equilibration times. It can therefore be conveniently used as a solute for equilibrium density gradient centrifugation, particularly through preformed density gradients. For example, the equilibrium position of a spherical lipoprotein cell membrane vesicle fraction (Fig. 2) was reached after 3 hr of ultracentrifugation. The radioactive label incorporated into the

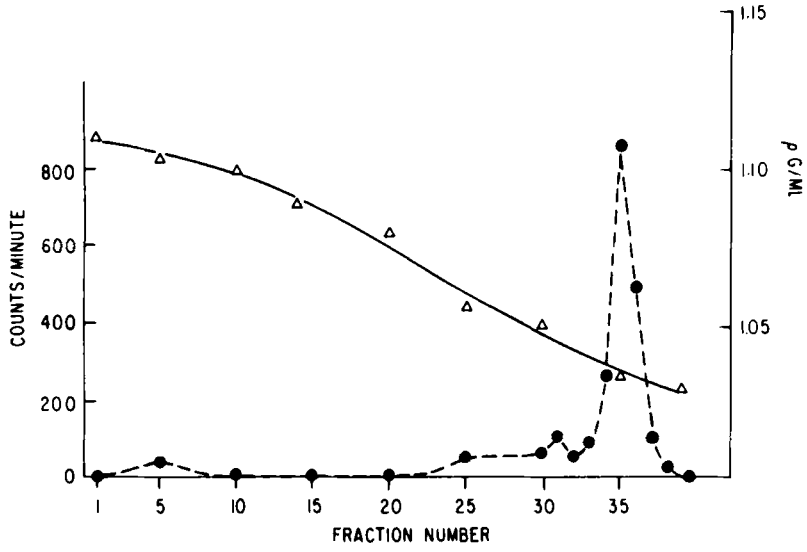

Fig. 2. Equilibrium buoyant density distribution of a spherical cell membrane vesicle fraction in polyglucose gradient. An established mouse cell line (designated TAL/N) was grown in tissue culture [8] in a medium which included ^{3}H-N-acetyl mannosamine. The radioactive label was incorporated into sialic acid, a characteristic cell membrane component. The cells were disrupted by rapid decompression from high pressure, which resulted in the formation of small closed spherical vesicles from the membrane component [9]. The labeled surface membranes were then purified from other components [9] suspended in 0.5 ml 0.25 M sucrose, and layered on top of a preformed 10-30% (w/v) polyglucose density gradient in a tube of the Spinco SW41 rotor. Centrifugation in the Model L-2 preparative ultracentrifuge was at 40°C for 3 hr at 39,000 rpm, which was shown in earlier experiments to permit the band to attain its equilibrium position. Fractions were collected by bottom puncture, and the radioactivity (●) and the refractive index (△) was determined.

cell membrane material was used to locate the position of the membrane vesicles at a density of 1.03 g/ml in the polyglucose gradient.

Table 1 gives comparisons of the same membrane vesicles centrifuged similarly to equilibrium in three nonionic carbohydrate gradient solutes: sucrose, Ficoll, and polyglucose. It can be seen that the lowest buoyant density is attained in the high molecular weight spherical polymer,

polyglucose; a somewhat higher density, in the linear polymer, Ficoll; and the highest density, in the low molecular weight solute, sucrose.

Table 1. Apparent Buoyant Density in Preformed Gradients of Sucrose, Ficoll, and Polyglucose

Solute	Density of a spherical cell membrane vesicle[a] (g/ml)
Sucrose	1.08
Ficoll	1.06
Polyglucose	1.04

[a]Determined as described in legend to Fig. 2.

Similar differences in buoyant density values of a spherical virus particle are shown in Table 2, which compares polyglucose with the low molecular weight solutes, sucrose, cesium chloride, and potassium citrate, commonly used in virus purification. When polyglucose was used it was found that the biologic activity (virus infectivity) was preserved, while there was considerable loss of infectivity after ultracentrifugation in the salt gradients [12]. Note again that the lowest apparent buoyant density was observed in the polyglucose solution.

It was of interest to compare buoyant densities obtained in polyglucose with those obtained in CsCl, which is one of the most frequently used density gradient solutes in biochemistry. Figure 3 and 4 show examples of the kind of distributions obtained in these two gradient solutes. Table 3 gives a tabulation of apparent buoyant densities obtained by this method for a variety of particles. It can be seen that the buoyant densities observed in polyglucose for all macrospecies examined are much lower than those observed in CsCl gradients. Note the especially large difference in the case of the Blue Dextran.

DISCUSSION

The data presented above compare the apparent buoyant density of

Table 2. Apparent Buoyant Density of a Spherical Virus Particle[a] in Various Solutes

Solute	Density (g/ml)
Cesium chloride [11, 12]	1.18-1.21
Potassium citrate [11, 12]	1.16
Sucrose [11, 13-15]	1.16
Polyglucose [12]	1.08-1.12

[a]Murine leukemia virus (Rauscher) particle, approx diam = 1000 Å, S = 640s [10].

various macromolecules in polyglucose and in other supporting solutes. Polyglucose fulfills most of the requirements for an ideal gradient solute. It is neutral, inert, and highly water soluble to form an extended range of solution densities with relatively low viscosity. For any procedure it is necessary to weigh the advantages and disadvantages for the experiment of any particular gradient solute. Polyglucose has two main disadvantages for some purposes, and three particular advantages over other gradient solutes.

A limitation to its use is its high absorbancy out to wavelengths near 400 mμ, which includes the most useful wavelength regions for determining the distribution of proteins and nucleic acids. Thus, only particles which have chromophores can be assayed spectrophotometrically in polyglucose gradients, as shown in Fig. 4. Even in this case, however, it was necessary to use higher protein concentrations than usual (Fig. 3) to minimize the high absorbancy background of the polyglucose. Other methods of observation must be used if the macrospecies lacks a chromophore. These include, for example, radioactive labeling, shown in Fig. 2, or biological activity (cf. Refs. 6 and 12).

The second limitation, particularly for preparative work, is the difficulty of removing the high molecular weight polyglucose from the high molecular weight macromolecule of interest. For many purposes, however, this difficulty poses no problem. Depending upon the difference in molecular weights, differential sedimentation may effectively remove much of the polyglucose. The murine leukemia virus purified in a polyglucose gradient

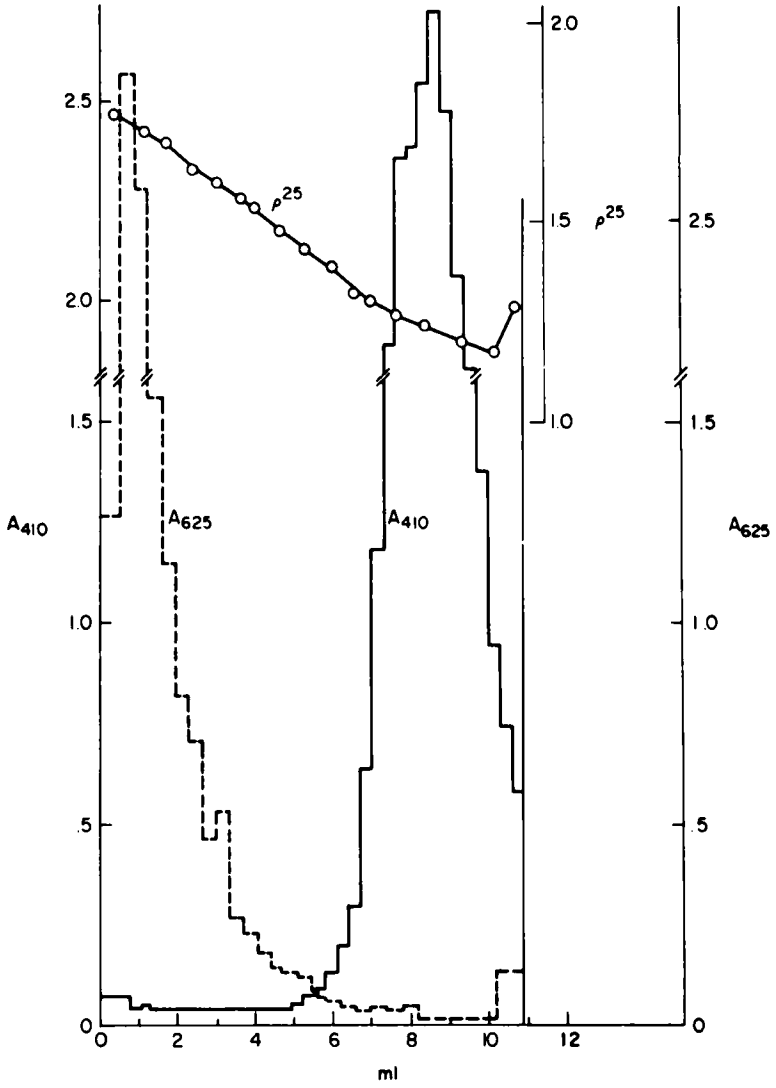

Fig. 3. Equilibrium density gradient sedimentation of myoglobin and of Blue Dextran in two CsCl gradients here plotted together. The Spinco SW41 rotor was centrifuged at 35,000 rpm, 25°C, for 36 hr. The gradient was preformed [1] to save equilibration time from 5 ml each of solutions of density 1.80 and 1.02 g/ml over 1 ml of high density solution in the tubes. Overlay solutions of 0.2 ml each contained 5 mg/ml myoglobin or 15 mg/ml Blue Dextran. Myoglobin was detected by absorbancy at 410 mμ, Blue Dextran by A_{625}.

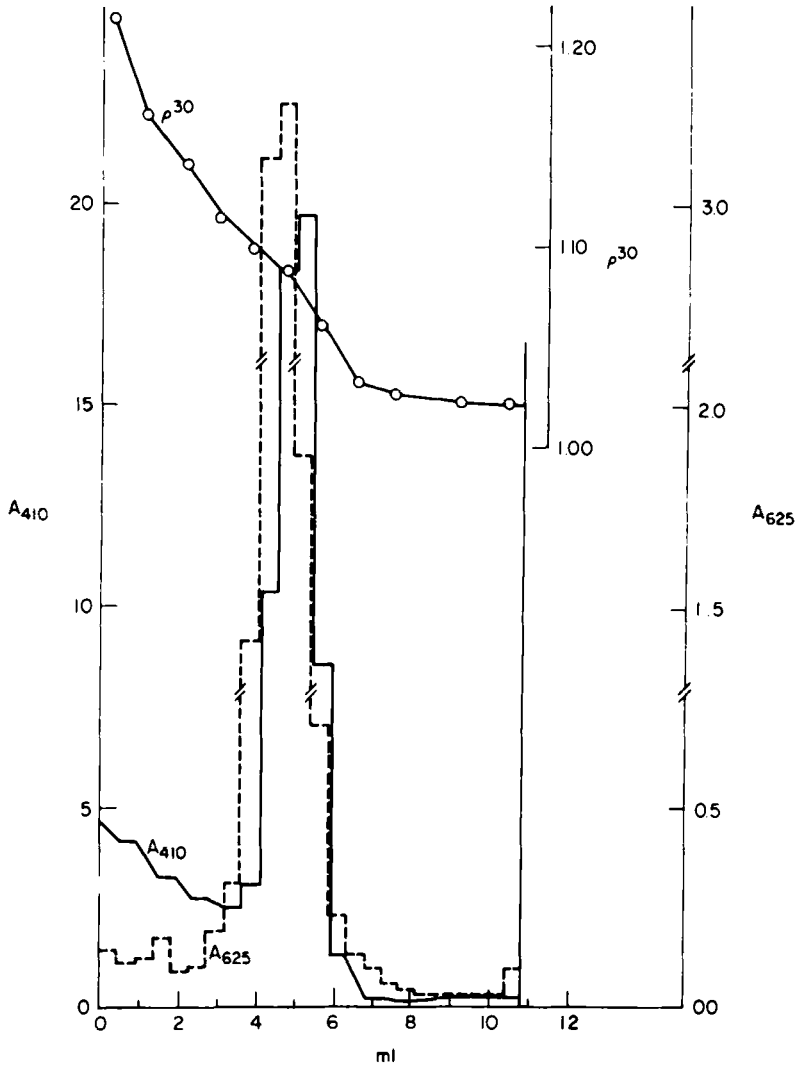

Fig. 4. Equilibrium density gradient sedimentation of myoglobin and of Blue Dextran in two polyglucose gradients here plotted together. The Spinco SW41 rotor was centrifuged at 35,000 rpm, 25°C, for 60 hr. Similar results were obtained after 48 hr of centrifugation. The gradient was prepared [1] from 5 ml each of 2 and 30% polyglucose over 1 ml of 30% polyglucose in the tubes. Sample solutions (0.2 ml overlay) contained 24 mg/ml myoglobin or 15 mg/ml Blue Dextran. Myoglobin: A_{410}; Blue Dextran: A_{625}.

Table 3. Apparent Buoyant Densities of Macromolecules and Macromolecular Complexes in Cesium Chloride and in Polyglucose

	Buoyant density (g/ml)	
	CsCl	Polyglucose
Dextran[a] (Blue)	1.75	1.09
Hemoglobin[a]	1.25	1.09
Myoglobin[a]	1.23	1.08
Cytochrome c[a]	1.23	1.08
ϕX 174 Bacteriophage	1.43[b]	1.18[c]
Murine leukemia virus[d]	1.18	1.10

[a]From measurements similar to those shown in Figs. 3 and 4.
[b]From Ref. 16.
[c]From Ref. 6.
[d]Table 2.

(Table 2) was simply diluted and injected into mice without any interference from the residual biologically inert polyglucose [12].

Two advantages to the use of polyglucose as a supporting solute for density gradient centrifugation derive from the lower buoyant density of various macromolecules in this solute as compared to the others studied. Membrane vesicles (Table 1), viruses (Tables 2 and 3), as well as proteins and dextran polymers (Table 3), all exhibit lower buoyant densities in polyglucose solutions. The first advantage is that particles, which in other solutes sediment to the bottom of the tube because of density (or solubility) limitations, can be supported at an easily attainable solution density. Proteins, for example, sediment to the bottom of gradients formed of lower molecular weight solutes, such as sucrose, in a reasonable (<60%) concentration (and viscosity) range. The second advantage is that the average viscosity of the solution through which the particles must sediment is lower even than suggested by the direct comparison of η_{rel} for equivalent densities (Fig. 1). Consequently, the somewhat higher viscosity of polyglucose solutions seen in Fig. 1 is not translated into longer equilibration times.

Another useful property of polyglucose as a density gradient solute derives from the low osmolarity of its solutions, even at high concentrations. Polyglucose is therefore especially useful when osmotic effects must be minimized; for example, when it is important to avoid damage to sensitive macromolecules or macromolecular compelxes, such as whole cells, viruses, or protein complexes [6]. Such an effect was apparently partly the basis for the good recovery of virus infectivity after purification in polyglucose (Table 2) as compared to other gradient solutes [12].

Since no ion binding occurs in the neutral polyglucose, various ion effects, such as the influence of ion binding on the buoyant density of polyelectrolytes, salting out of macromolecules, and inactivating effects of ions on biologic macromolecules and viruses [12], are not encountered. Whether the absence of ion binding or changes in hydration is the basis of the lower buoyant density of proteins, dextran, etc., observed in polyglucose as compared to cesium chloride, or the cause is partly due to the difference in excluded volume [17] in high and low molecular weight solutes, one cannot differentiate on the basis of experiments shown in Fig. 3 and 4 and Table 3. It should be pointed out, however, that in Tables 1 and 2 the buoyant densities of the particles were higher in the other nonionic carbohydrate solutes, both the low molecular weight sucrose and the high molecular weight linear polymer, Ficoll. Moreover, separate experiments showed that neither 0.1 M CsCl or KI affected the buoyant behavior of myoglobin when added to the polyglucose gradient solutions. It appears then that the excluded volume effect of the highly branched, essentially spherical, polyglucose polymer is a primary factor producing the decrease in apparent buoyant densities.

Thus, the low buoyant densities observed in polyglucose solutions and the low osmolarity of these solutions make polyglucose a useful density gradient solute for many macromolecular separation and characterization procedures.

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