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STUDIES OF A SERUM ALBUMIN–LIPOSOME COMPLEX AS A MODEL LIPOPROTEIN MEMBRANE

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SUMMARY

Electron microscopy shows that the lipoprotein dispersions formed from the interaction of negatively charged liposomes with bovine serum albumin contain closed, vesicular, multilamellar structures. Discontinuous density gradient studies indicate that the lipoprotein suspensions are vesicles in which bovine serum albumin homogenously associates with lipid.

Low angle X-ray diffraction results show that all the systems, positively and negatively charged, with and without protein, have the characteristic lamellar structure observed in biological membranes. The lamellar spacing (bilayer plus water layer) of negatively charged liposomes without bovine serum albumin is 55 Å. The same lamellar separation in the positively charged system is 108 Å. The lamellar spacing corresponding to bilayer, water, and protein for the negatively charged lipoprotein system is 93 Å while that for the positively charged lipoprotein system is 91 Å. These dimensions suggest that a layer of protein one molecule thick is incorporated between the lamellae bound to the surface of the bilayer.

Wide angle X-ray diffraction results indicate no major effect of the protein on the 4.1 Å spacing, characteristic of hexagonal packing of the hydrocarbon chains.

A classical light scattering technique is used to show that the lipoprotein systems are osmotically active. The solute permeability exhibited by these lipoprotein systems follows the sequence (glucose $<$ arabinose $<$ malonamide $<$ glycerol). K^+ diffusion from negatively charged lipoprotein systems is greater than that found for positively charged lipoprotein systems.

INTRODUCTION

In 1969, we described a model system for cellular membranes, formed by the interaction of bovine serum albumin with phospholipid liposomes [1]. In this and a subsequent report [2], we presented evidence which suggested that this model

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system involved interaction of the protein with lipid in a fashion which was sensitive to the protein conformation, and that the nature of the lipoprotein interaction could change from electrostatic to hydrophobic as a function of pH. The apolar interaction was believed to give rise to an increased permeability of liposomes to solute molecules such as glucose.

However, these interpretations were based on the assumption that the liposome lamellae retain their membrane, or barrier, qualities during this interaction. In order to test this assumption more rigorously we have conducted further studies on the structure and osmotic properties of the bovine serum albumin–liposome complex utilizing electron microscopy, density gradient centrifugation, X-ray diffraction and light scattering techniques. The studies reported here confirm the above assumption and further support the notion that this lipoprotein system is an interesting model system for cellular membranes.

MATERIALS AND METHODS

Chemicals

All chemicals other than the ones specifically listed were analytical grade obtained from Matheson, Coleman and Bell and were used without further purification. Deionized, distilled water was used. Purified egg lecithin purchased from Nutritional Biochemicals Corporation was further purified by passage through an activated alumina column by the method of Singleton et al. [3]. The purified lecithin migrated as a single spot when analyzed by thin-layer chromatography with chloroform–methanol–water (65 : 35 : 5, by vol.) used as the developer. It was stored as a benzene solution at −20 °C. The benzene solution was flushed with N_2 prior to storage to retard oxidation. Dicetyl phosphate was obtained from Sigma Chemical Co, and stearylamine from K and K Laboratories. Crystalline bovine serum albumin (Pentex) was obtained from Miles Laboratories.

Liposomes and lipoprotein complexes

The lipid and lipoprotein suspensions which were used throughout this work were prepared in an identical manner. The technique is similar to that originally used by Bangham and Horne [4]. An aliquot of a stock chloroform solution containing the desired lipid constituents was pipetted into a 50-ml round-bottomed flask. The solvent was rapidly removed on a rotor evaporator under vacuum. The flask containing the lipid film coated on the sides was flushed with N_2 and stoppered. The appropriate aqueous medium saturated with N_2 was then added to give the desired final lipid (i.e. liposome) concentration. Several glass beads were added and the mixture was shaken vigorously on a mechanical shaker at room temperature for 1–2 h in a N_2 atmosphere. After the suspension was formed, identical aliquots were removed. To one aliquot was added a specific amount of an aqueous bovine serum albumin solution. To the other was added an equivalent amount of water. The pH of both solutions was adjusted with the appropriate acid or base.

Three types of liposomal systems were employed: (1) a negatively charged system of lecithin, cholesterol and dicetyl phosphate in molar proportions of 70 : 10 : 20; (2) a positively charged system consisting of lecithin, cholesterol and stearylamine in 60 : 10 : 30 proportions; (3) a negatively charged system consisting of lecithin and

dicetyl phosphate (96 : 4). The amount of albumin necessary to give the specifically desired percentage saturation was based on the total amount of protein which is bound by the liposome suspension in the presence of an excess of protein. The amount of protein necessary to saturate the particular systems was as follows: The negative system (70 : 10 : 20) requires 1.232 mg albumin/ml suspension (6.16 mg total lipid/ml suspension). The positive system (60 : 10 : 30) requires 1.268 mg albumin/ml suspension (5.21 mg total lipid/ml suspension). These values were determined for suspensions formed in 19 mM KCl.

Electron microscopy

A negative staining technique analogous to that employed by Papahadjopoulos and Miller [5] was used. The suspensions were prepared in a medium composed of ammonium acetate and ammonium molybdate (145 mM). The pH was adjusted with acetic acid or NH_4OH.

X-ray diffraction

KCl solutions, preadjusted to the desired pH, were used as the medium for the suspensions studied by this technique. Each suspension was centrifuged at 40 000 rev./min for 1 h. The clear supernatant was discarded and a sample of the remaining pellet was sealed between mylar windows (1 mm wide) and used at room temperature for the X-ray diffraction measurements.

Two different cameras were employed for obtaining X-ray data. A Frank camera

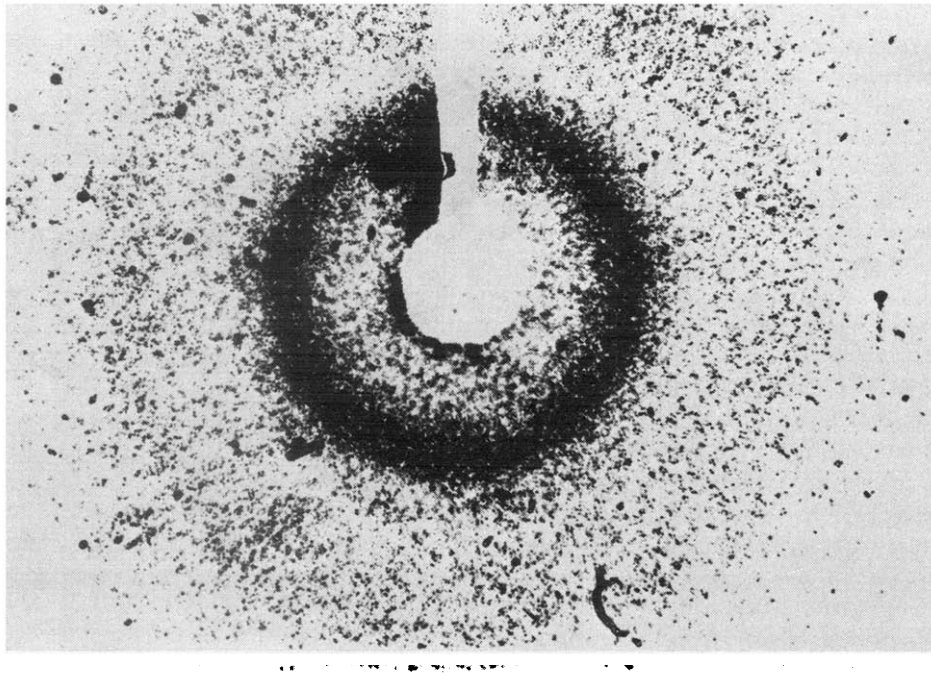

Fig. 1. Densitometer trace of low angle X-ray patterns of negatively charged lipoprotein system (molar ratio 70 : 10 : 20, lecithin : cholesterol : dicetylphosphate) at 50 % satn with bovine serum albumin. Magnification 7.5×.

was used for low angle scattering. Nickel-filtered copper radiation ($\lambda = 1.542$ Å) giving a 40 μm beam was used with a sample to film distance, $m = 15$ cm. Exposure times were usually 70–100 h. Spacings were calculated from Bragg's law. The X-ray diffraction patterns given by the suspensions usually consisted of at least two concentric diffraction rings having diameters in a 1 : 2 ratio, characteristic of lamellar structure [6]. An EDP scanning microscope with plotter made by Photometrics, was used to obtain density plots for analysis of these data. Fig. 1 shows a typical densitometer trace obtained in this manner. The trace shows the major diffraction ring; the unexposed lollipop-shaped region is due to the beam stop.

Wide angle diffraction data were obtained using a Guinier–DeWolf camera. The X-ray beam is monochromatized and focused by a bent quartz crystal which isolates the CuKα radiation ($\lambda = 1.540$ Å). A line focus was used. Exposure time was 24–30 h. This particular camera was designed so that four samples could be run simultaneously using a four-compartment cell. Low angle information obtained from this camera could not be used because the bands in this region were too diffuse to be accurately measured.

Light scattering studies

Absorption measurements were made on a Beckman Model DU spectrophotometer with a Gilford electronics package operating at maximum sensitivity with a digital readout from which could be obtained absorbance values to four digits. The instrument was fitted with thermospacers on either side of the cell compartment through which water was circulated to maintain the temperature of the cell compartment at constant temperature to $\pm$ 0.5 °C during a complete experiment. All determinations were made at 450 nm. A cell containing the appropriate solute solution was used as a blank.

EXPERIMENT AND RESULTS

Electron microscopy

In order to test whether bovine serum albumin causes major disruption and disorganization of the lipid lamellae we initially investigated the appearance of the lipoprotein complex by electron microscopy. The micrographs shown in Fig. 2 are of dispersions formed by the negative system alone and containing bound albumin at 45 % satn. It is evident that the dispersions consist of numerous particles which are vesicular and multilamellar, analogous to the model membranes formed in the absence of protein [4, 5]. The vesicular nature of these lipoprotein particles is similar to that reported by Papahadjopoulos and Watkins [7] for "closed", diffusionally discriminating particles. The results suggest that the presence of the protein in the liposomes has not caused a major disruption of the basic structural characteristic required of a model membrane system. However, we did note a small apparent decrease in the average size of the vesicles formed in the presence of the protein.

Discontinuous density gradient experiments

While the microscope data establish that vesicles are indeed present in the lipoprotein suspension, they do not provide proof that all the lipid is in vesicular form, or that the vesicles observed do, in fact, contain bound protein. We, therefore,

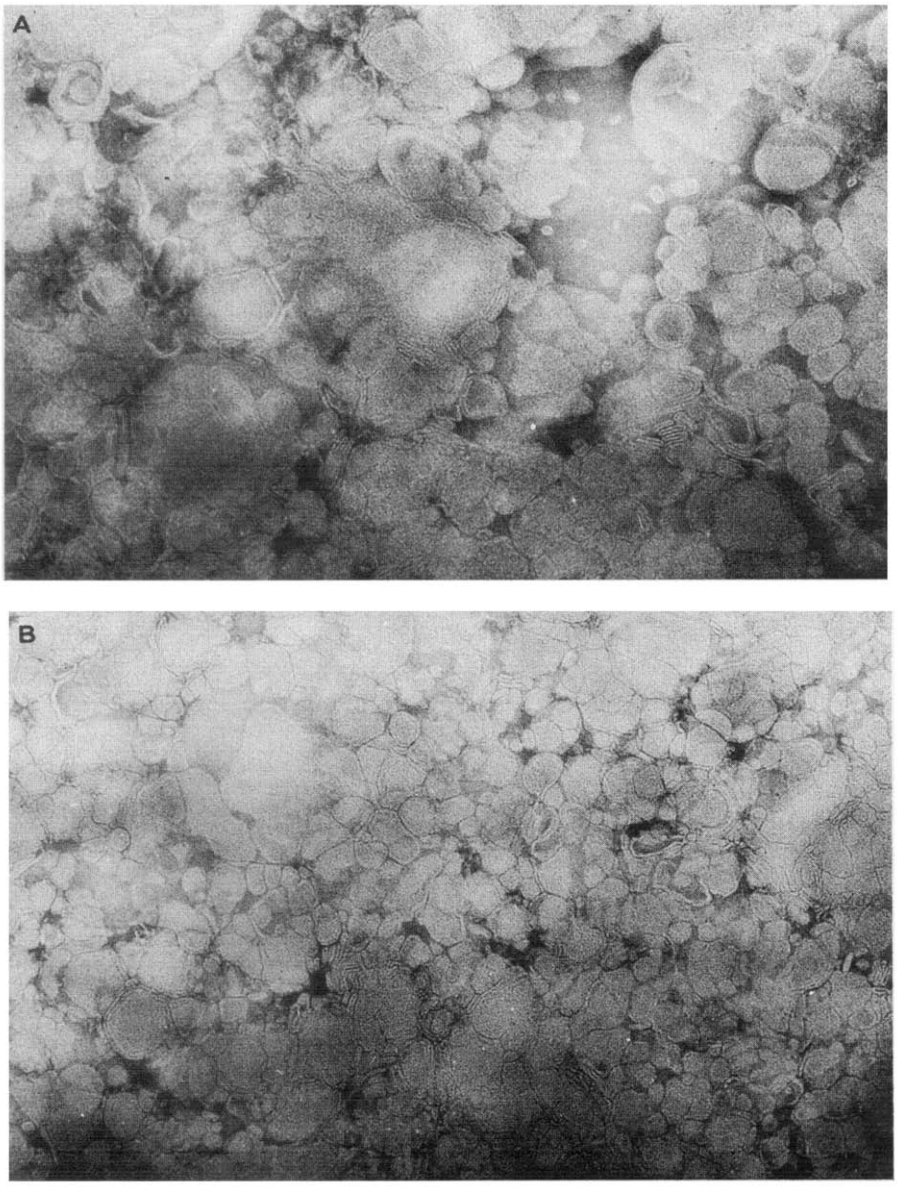

Fig. 2. Negatively stained phospholipid dispersions. (A) Negatively charged (20 molar %, dicetyl phosphate) liposomes. (B) Negatively charged liposomes pre-incubated with albumin at protein concentration of 0.4 mg/ml. Magnification 70 000 ×.

investigated the homogeneity of the lipoprotein complex with regard to density. If a significant number of protein-free liposomes are present it would be expected that such particles would be less dense than lipoprotein particles. Thus, for a suspension containing particles heterogenous with respect to density, density gradient centrifugation should result in a separation of the initial suspension into two or more

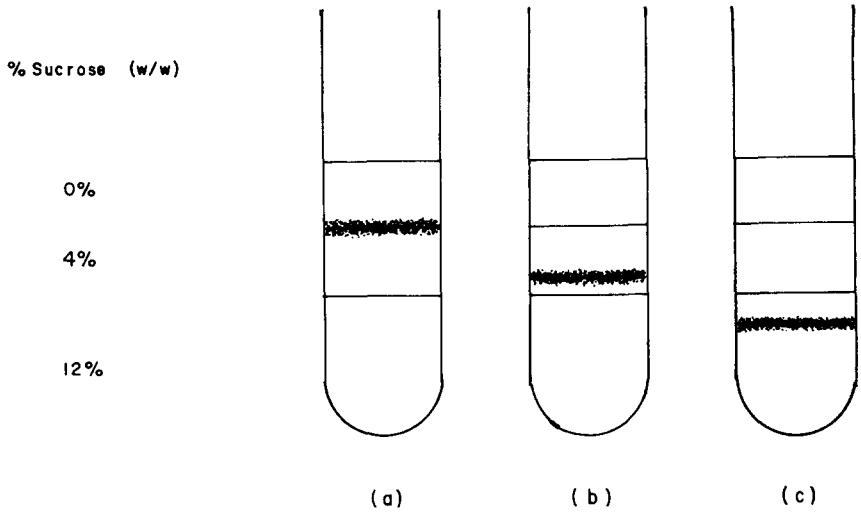

Fig. 3. A diagrammatic representation of the bands obtained by discontinuous density gradient centrifugation of the negatively charged lipoprotein system (molar ratio 70 : 10 : 20, lecithin : cholesterol : dicetylphosphate) at pH 3.45 with varying amounts of protein. a, liposomes; b, lipoprotein system at 34 % satn with albumin; c, lipoprotein system at 100 % saturation with albumin.

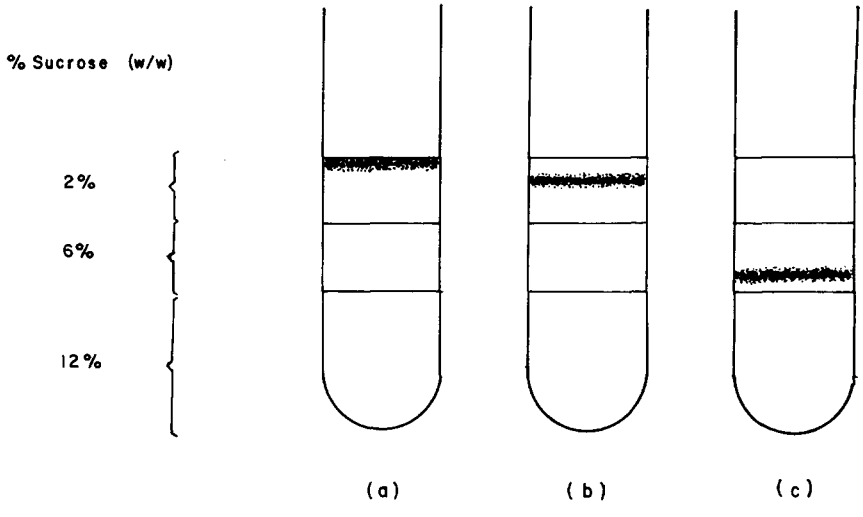

Fig. 4. A diagrammatic representation of the bands obtained by discontinuous density gradient centrifugation of the negatively charged lipoprotein system (molar ratio 96 : 4, lecithin : dicetylphosphate) at pH 3.45 with varying amounts of protein.

bands, one composed of less dense liposomes and the other(s) of lipoprotein particles. It might also be expected that for a given suspension, heterogenous in nature, the size of the less dense band would decrease and the other band increase as the amount of protein initially added is increased and the population shifts in favor of lipoprotein particles.

As shown by the diagrams presented in Figs 3 and 4, it was found that a single band is formed from every preparation used. In the lipoprotein systems no free liposome band was ever observed. Furthermore, the relative densities are qualitatively as would be predicted with the free liposomes being less dense. These results indicate that the protein is associating with the liposomes to form a population of particles of uniform density. The observation that the bands shift toward higher densities as the concentration of protein is increased suggests homogenous association. The results are the same whether the system contains cholesterol, as in Fig. 3, or not, as in Fig. 4.

X-ray diffraction data

In Table I is presented the data obtained from the X-ray diffraction of the various lipid and lipoprotein suspensions. Low angle diffraction measurements on the positive system (60 : 10 : 30, pH 8.0) without albumin give a lamellar spacing of 108 Å. With the protein present, the spacing decreased to 91 Å. The fundamental long spacing for the negative (70 : 10 : 20, pH 3.45) system without protein was found to be 55 Å. This value compares well with those reported earlier for the negative phospholipid model systems [5, 8]. With the inclusion of the bovine serum albumin, the total spacing increased to 93 Å.

On the right side of Table I are listed the observed wide angle spacings for the dispersions. The major phospholipid constituent of the dispersions which were ex-

TABLE I

X-RAY DIFFRACTION DATA FROM DIFFERENT LIPID AND LIPOPROTEIN SYSTEMS

System	Amount of albumin (%)	First order low angle spacings (Å)	Wide angle spacings (Å)		
Lecithin	0		20.1	4.4	
	0		19.6	4.5	
96 : 4 (negative)	0		20.4	4.4	
70 : 10 : 20 (negative)	0	56			
	0		20.2	4.4	4.1
	0		19.5	4.4	4.1
	0	54	19.4	4.5	4.1
	11.8		19.7	4.4	4.1
	11.5		18.8	4.4	4.1
	23.4		19.5	4.5	4.1
	52.7	93			
	52.8		19.8	4.4	4.1
	97.0		20.3	4.4	4.2
60 : 10 : 30 (positive)	0	108			
	0		20.9	4.4	4.1
	0		19.7	4.3	4.1
	0		18.2	4.5	4.2
	12.4		19.3	4.4	4.2
	37.4		20.2	4.4	4.1
	46.2		20.2	4.4	4.1
	49.7	91			
	50.6		19.2	4.4	4.1
	50.6		20.6	4.4	4.1

amined is egg lecithin. Since the data were obtained at room temperature (25 °C), the hydrocarbon chains of the lecithin are expected to be in the liquid crystalline phase, and consequently, to give a characteristic short spacing for the disordered conformation. For the first two systems listed in the table, a broad band around 4.4 Å (4.3–4.5 Å) was indeed found. For all the other systems a band near 4.5 Å was also evident, although it was very weak, indicating that these systems also contain regions in which the hydrocarbon tails are disordered. An interesting aspect of the wide angle results is evident when the systems without cholesterol are compared to those containing cholesterol (no protein). A sharp band near 4.1 Å appears with the inclusion of cholesterol. It appears to be specifically related to the presence of the cholesterol since the variation in the nature of the charged component did not result in its disappearance.

Another important aspect of the wide angle results pertains specifically to the lipoprotein systems. It is evident from the data in Table I that the sharp band of 4.1 Å has remained for both lipoprotein systems at high protein concentrations. This result has particular significance for the negative (70 : 10 : 20) lipoprotein case (see Discussion).

Light scattering and the osmotic properties of the albumin–liposome complex

Since no previous work using light scattering has been done on lipoprotein model systems, it was necessary to confirm the relationship between volume and absorbance as has been described by Bangham [8] directly in the albumin–liposome system. A plot of the pellet volume versus $1/A_{450\,nm}$ for the negative lipoprotein system

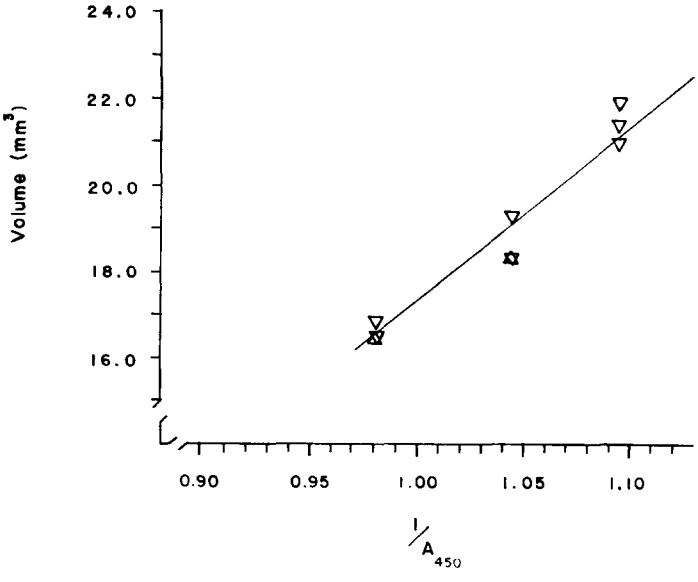

Fig. 5. Pellet volumes (mm^3/1.363 μmoles of phospholipid) plotted against the reciprocal of absorbance at 450 nm for negatively charged lipoprotein dispersions at 25.4 % satn with bovine serum albumin. The lipid composition of the dispersion was lecithin : cholesterol : dicetylphosphate (molar ratio 70 : 10 : 20) at a phospholipid concentration, 1.363 μmoles/ml.

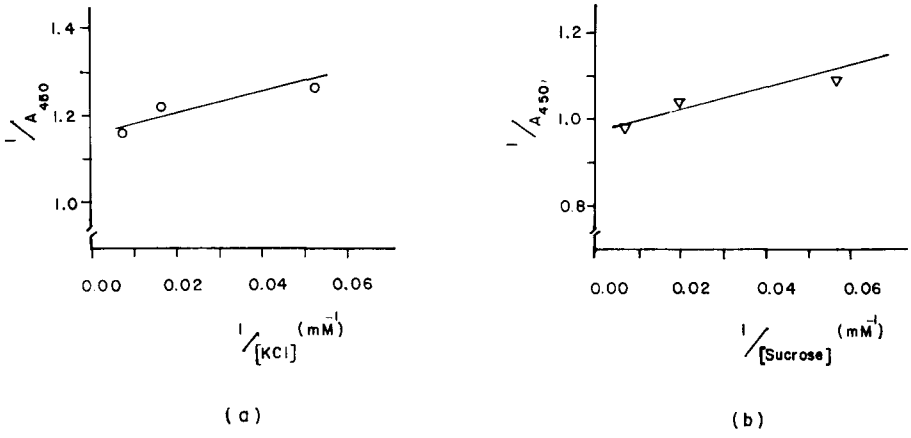

Fig. 6. Relationship, at equilibrium, between the reciprocal of absorbance at 450 nm and the reciprocal of solute concentration for negatively charged lipoprotein dispersions. 0.2 ml of dispersion was diluted into 2.7 ml of the appropriate solute concentration and allowed to equilibrate 4 h prior to reading absorbance. (a) Negatively charged lipoprotein system at 45.6 % satn with albumin. Phospholipid concentration in cuvette, 0.413 mM. (b) Negatively charged lipoprotein system at 25.4 % satn with albumin. Phospholipid concentration in cuvette, 1.363 mM.

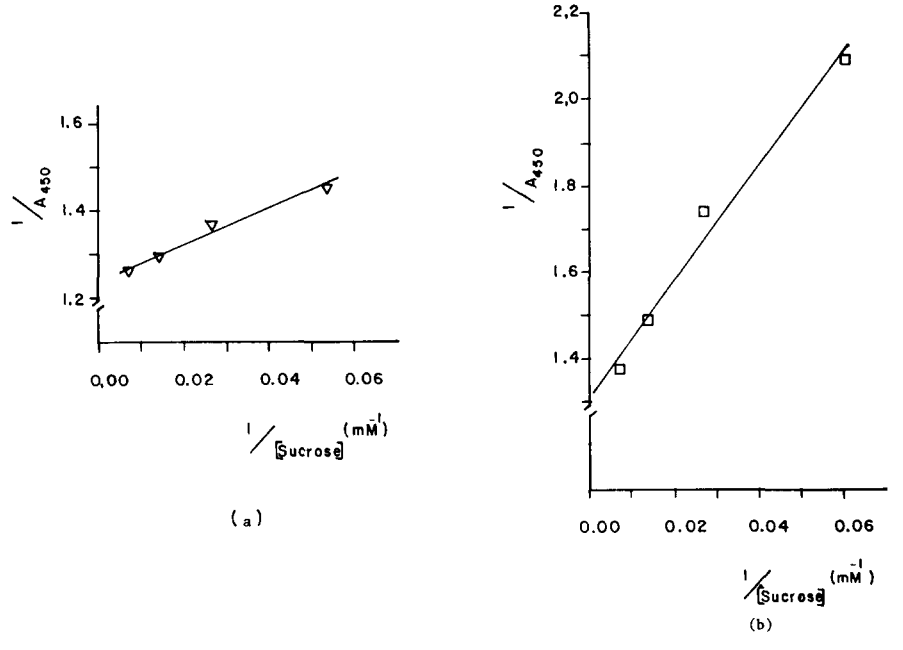

Fig. 7. (a) Reciprocal of absorbance at 450 nm versus reciprocal of sucrose concentration for the negatively charged lipoprotein system at 9.6 % satn with albumin. Phospholipid concentration in cuvette, 1.051 mM. Dilution was described in Fig. 6. (b) Reciprocal of absorbance at 450 nm versus reciprocal of sucrose concentration for the positively charged lipoprotein system at 9.3 % satn with albumin. Phospholipid concentration in cuvette, 1.009 mM. Dilution as described in Fig. 6.

in which bovine serum albumin was bound at 25 % satn is shown in Fig. 5. The expected linear reciprocal relationship indicates that the light scattering behavior is a measure of volume as has been reported for other similar systems in the absence of protein [8–10]. Thus for the lipoprotein systems it appears that changes in $1/A$ can also be used as an indication of the change in volume.

In Figs 6 and 7 are presented graphs of $1/A$ vs 1/[solute] for the negative and positive systems for both ionic and non-ionic solutes at several protein concentrations. These data were obtained by prior formation of liposomes in a given solute at a given concentration and then subsequently diluting them into the final solute concentration indicated in the figures. After equilibration for 4 h, the absorbance at 450 nm was measured. It can be seen that the theoretical linear relationship between $1/A$ and $1/S$ exists for both ionic and non-ionic solutes (Fig. 6), and for both positively and negatively charged liposome systems (Fig. 7). In addition, these relationships hold over a range of albumin concentration from 9.3 % of saturation (Figs 6 and 7) to 46 % of saturation (Fig. 6a). Thus, these data further support the interpretation that liposome–protein complexes are osmotically active.

Another experiment which tested the retention of osmotic activity by the lipoprotein system yielded the results presented in Fig. 8. It can be seen from the curve that the volume ($1/A$) increases rapidly in response to the osmotic gradient created by the diffusion of the highly permeating solute, ethylene glycol. When an opposing osmotic gradient is created by the addition of the much less permeable solute, KCl, to the external solution, a sharp reversal of swelling occurs as water leaves the vesicles in response to the gradient. The osmotic reversibility exhibited by the lipoprotein system further supports the idea that the bovine serum albumin–liposome complex is membranous and vesicular in nature.

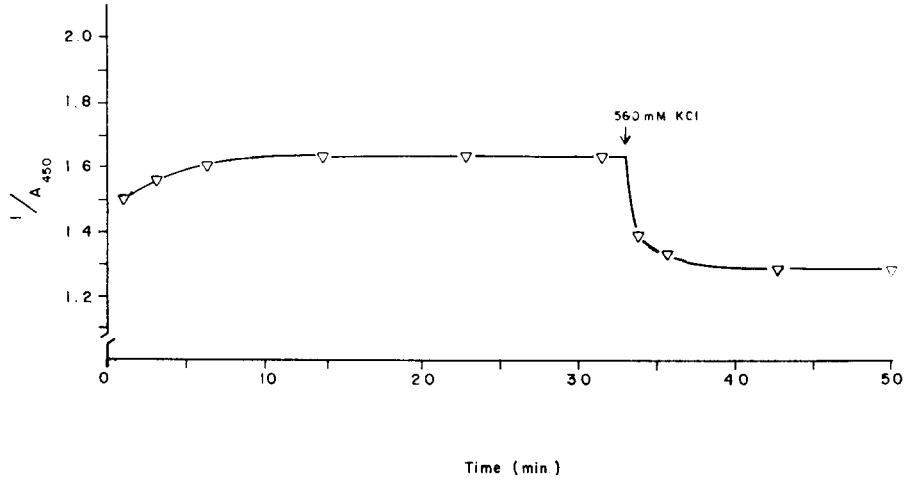

Fig. 8. Reversibility of osmotic swelling ($V \propto 1/A$) for the negatively charged lipoprotein system (molar ratio 70 : 10 : 20, lecithin : cholesterol : dicetylphosphate) at 9 % satn with albumin at pH 3.65. (Phospholipid concentration in the cuvette, 1.089 mM.) At zero time, 0.1 ml of liposome suspended in 19 mM KCl, was added to a cuvette containing 2.7 ml of isotonic ethylene glycol (38 mM). After the absorbance changes induced by swelling of the liposomes had reached a maximum, 0.1 ml of 560 mM KCl was added as indicated.

Relative solute permeability

Utilizing the approach of Katchalsky and Curran [11] and the reciprocal relationship between A and V, it can be shown that

$$\frac{d(1/A)}{d(t)} = \frac{-BL_p RTc_2^0}{z}(\sigma_2 - \sigma_1).$$

where B is the membrane area, L_p is the hydraulic conductivity coefficient, σ is the reflection coefficient for the indicated solute (1 and 2), and z is the proportionality constant between A and V. Thus, when the liposomes are suspended in Solute 1 and subsequently diluted into an isotonic solution of Solute 2, the initial value of $d(1/A)/dt$ will be proportional to the permeability of Solute 2.

Following this theory studies of the relative permeability of different solutes in the liposome-bovine serum albumin complex were conducted as follows: aliquots of liposome suspensions in Solute 1 (19 mM KCl) were added to cuvettes containing isotonic aliquots of various solutes (Solute 2), and absorbance measurements made as a function of time. Duplicate diffusion experiments were done for each solute. Graphs of the raw data are shown in Figs 9–12. For the positive systems in Figs 9 and

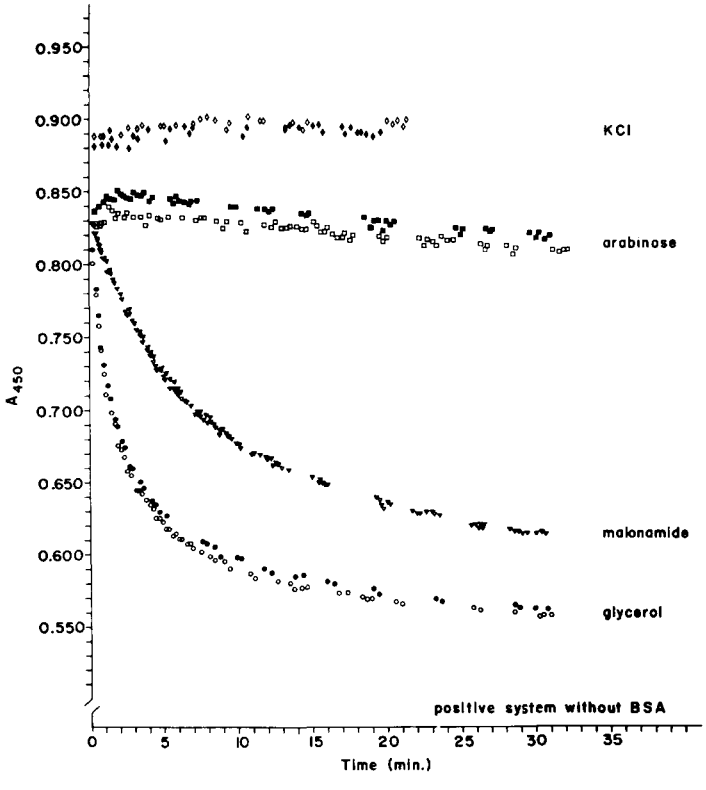

Fig. 9. Time course of absorbance changes for the positively charged liposome system (molar ratio 60 : 10 : 30, lecithin : cholesterol : stearylamine) in the presence of various solutes at pH 8.0. Phospholipid concentration in cuvettes, 0.957 mM.

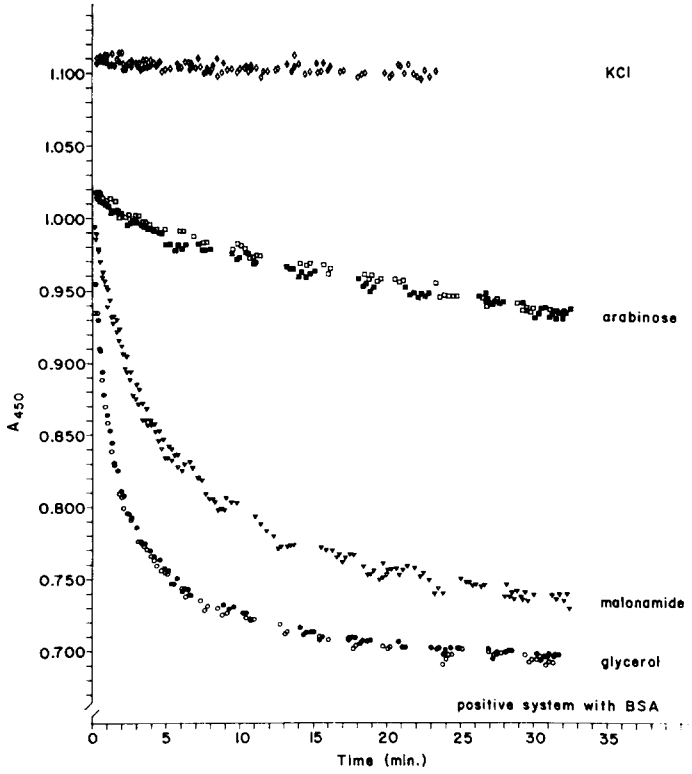

Fig. 10. Time course of absorbance changes for the positively charged lipoprotein system (molar ratio 60 : 10 : 30, lecithin : cholesterol : stearylamine) at 9.3 % satn with albumin in the presence of various solutes at pH 8.0. Phospholipid concentration in cuvettes, 0.957 mM.

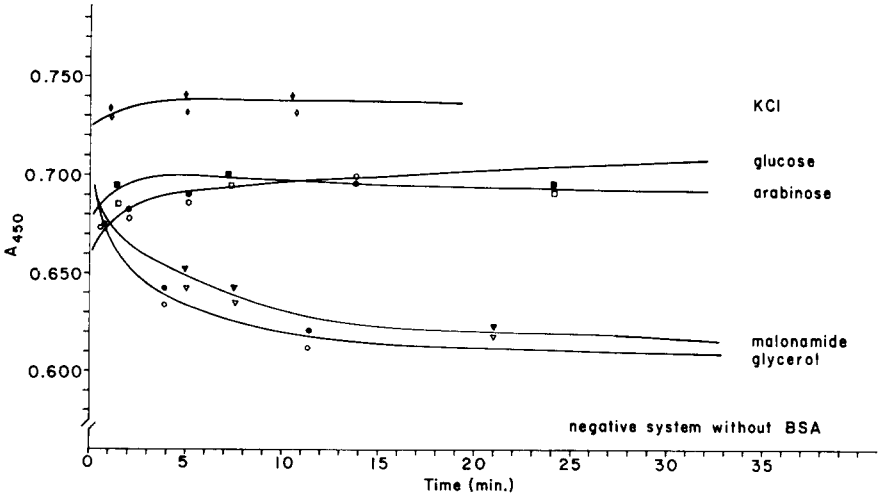

Fig. 11. Time course of absorbance changes for the negatively charged liposome system (molar ratio 70 : 10 : 20, lecithin : cholesterol : dicetyl phosphate) in the presence of various solutes at pH 3.45. Phospholipid concentration in cuvettes, 1.650 mM.

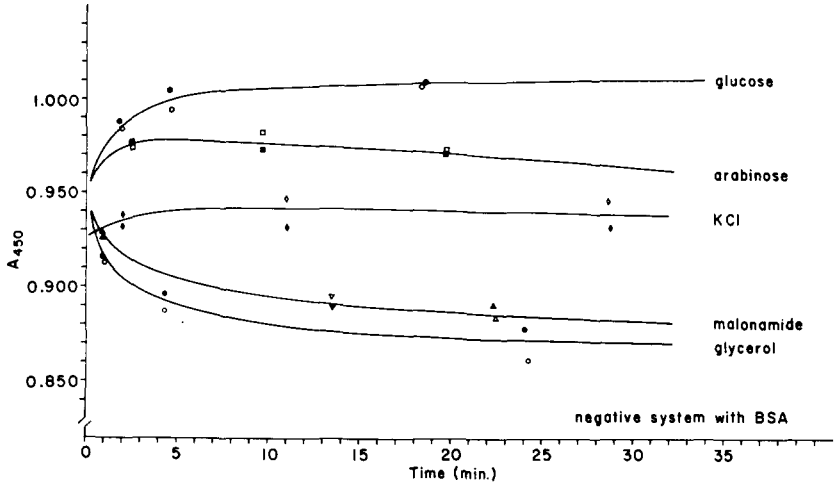

Fig. 12. Time course of absorbance changes for the negatively charged lipoprotein system (molar ratio 70 : 10 : 20, lecithin : cholesterol : dicetylphosphate) at 10.6 % satn with albumin in the presence of various solutes at pH 8.0. Phospholipid concentration in cuvette, 1.650 mM.

10, all experimental points are indicated. For the data in Figs 11 and 12, an identical number of points were obtained. However, in these figures fewer points are shown since the permeability changes for these systems were small for all solutes and thus the cluster of data points, especially at early times, proved to be visually confusing. The initial slope determination from each permeability experiment was calculated using a least squares analysis. An average error ± 0.003 min^{-1} was determined for the $d(1/A)/dt$ values.

The values of $d(1/A)/dt$ for several solutes in the different lipid and lipoprotein systems are listed in Tables II and III. It is evident from the data in these tables that the permeabilities of the various solutes are different. This discrimination among the solutes is given by all systems, including the lipoprotein ones. Furthermore, the order

TABLE II

INITIAL RATE OF CHANGE IN $1/A$ FOR NEGATIVELY CHARGED LIPOSOMES WITH AND WITHOUT BOUND ALBUMIN (SOLUTE 1 = KCl)

pH is 3.45, with albumin at 10 % satn.

Solute 2	$\frac{d\,1/A}{dt}$ (min^{-1})	
	+bovine serum albumin	−bovine serum albumin
Glucose	−0.023	−0.030
Arabinose	−0.010	−0.028
Malonamide	0.016	0.023
Glycerol	0.036	0.059
KCl	−0.008	0.009

TABLE III

INITIAL RATE OF CHANGE IN $1/A$ FOR POSITIVELY CHARGED LIPOSOMES WITH AND WITHOUT ALBUMIN (SOLUTE 1 = KCl)

pH is 8.0, with albumin at 10 % satn.

Solute 2	$\frac{d\ 1/A}{dt}$ (min^{-1})	
	+bovine serum albumin	−bovine serum albumin
Glucose	−0.005	−0.007
Arabinose	0.007	−0.007
Malonamide	0.062	0.047
Glycerol	0.016	0.186
KCl	−0.007	−0.003

of the solute permeabilities follows that predicted in 1899 by Overton [12] and found to be the general order in many natural membranes (e.g. *Nitella micronata* [13], alga; *Chara ceratophylla* [14]; and rabbit gallbladder epithelium [15]).

From the data in Tables II and III it is clear that while the glucose permeability of positively charged membrane is very similar to that for KCl, in the case of the negatively charged membrane it appears that KCl may be considerably more permeable than glucose. It has been previously reported by Bangham et al. [16] as well as Papahadjopoulos and Watkins [7] that the diffusion of K^+ from similar liposome systems (protein free) markedly increased with an increase in the negative charge on the membrane while it decreased to essentially zero when the membrane was positively charged. Cl^- permeability seemed little affected by membrane charge. Thus the permeability to KCl might be expected to be higher in the negative membrane, giving a greater $d(1/A)/dt$ value than the positive membrane where KCl cannot permeate as readily and thus has a σ value nearer to the poorly permeating glucose.

In order to test this interpretation, the experiment reported in Table IV was performed using the negative (70 : 10 : 20) system. In this case the non-electrolyte, sucrose (38 mM, pH 3.45), was used as Solute 1, in place of KCl. Not only should

TABLE IV

INITIAL RATE OF CHANGE IN $1/A$ FOR NEGATIVELY CHARGED LIPOSOMES WITH AND WITHOUT ALBUMIN, WITH SOLUTE 1 = SUCROSE

pH is 3.45, with albumin at 10.0 % satn.

Solute 2	$\frac{d\ 1/A}{dt}$ (min^{-1})	
	+bovine serum albumin	−bovine serum albumin
Glucose	−0.004	−0.003
Glycerol	0.035	0.081
Sucrose	−0.005	−0.005

this solute have a very high σ, close to or equal to 1; but, also its permeability should not be influenced by the membrane charge. The resulting situation is one in which the negative system should be analogous to a positive membrane with KCl as solute 1 for which $\sigma \sim 1$. As indicated in Table IV, the experimental $d(1/A)/dt$ value for glucose is insignificantly different from the control for both lipid and lipoprotein systems. The results are similar to the observations reported from the positively charged system in which the permeability of KCl was near zero and not very different from that of glucose. Thus the charge effect interpretation for the anamolous permeability of glucose in the negative systems is very probably correct.

DISCUSSION

These data strongly confirm the interpretation that the liposome–bovine serum albumin complex originally described by Sweet and Zull [1], is a vesicular system homogenous with respect to density and with discrete membrane features found in biological membranes. Furthermore, the lipoprotein vesicles show behavior observable only in membrane systems which are osmotically sensitive and possess differential permeability for various solutes. In addition, the lipoprotein model membrane shows charge sensitive control of cation diffusion as indicated by the low permeability to K^+ when the membranes carry a net positive charge. The order of solute permeability is consistent with that expected from the partition coefficients and is similar to that found for many natural membranes and other liposome systems.

The X-ray diffraction studies were conducted primarily to confirm the lamellar nature of the lipoprotein vesicle. However, some additional suggestions concerning the lipoprotein membrane structure can be made from the data. First, the results are consistent with the idea that a single layer of albumin molecules may be inserted between the lipid lamellae in the liposome. In both the positive and negative systems, if one assumes the reported bilayer thickness for the lipid lamellae (39 Å) and subtracts this value from the observed spacing one is left with an approximate difference of 50 Å. Assuming no major change in the albumin upon binding to the liposome, this is adequate space between the bilayers for a single molecule of protein but not for two. Luzzati et al. [17] suggest a minor axis of 48 Å for native bovine serum albumin and 54 Å for the expanded form, while Bloomfield [18] suggested a minor axis dimension of approx. 53 Å in both the expanded and native conformation. Since titration of the rather high number of albumin carboxyls does not appear to lead to major alteration in this dimension, there is no apparent basis for suggesting that interaction of the albumin with the lipid lamellae would drastically alter the dimensions of the protein molecule.

Furthermore, since upon addition of bovine serum albumin both lipid systems alter their major long spacing toward the same point, i.e. approx. 90 Å, albeit in opposite directions, it is possible that the insertion of a single protein layer between lipid lamellae is in fact the limiting factor in the final dimensions of the lipoprotein complex; charge–charge repulsions between the membranes are probably reduced and diffused by binding of the protein, and the ultimate spacing is a function of the size of the molucules involved in the interaction. This hypothesis differs from the case for the phosphatidylserine–cytochrome *c* system reported earlier where it is believed that two layers of protein are incorporated between lamellae [5].

Sweet and Zull [1] reported a substantial effect on glucose diffusion from the negative liposomes when albumin at low concentrations was added. The effect has been explained on the basis of a model in which the protein molecule is incorporated into the phospholipid bilayer. From such a model, significant disordering of the hydrocarbon chains might be expected [19] which could result in a decrease and possible disappearance of the 4.1-Å reflection as the amount of protein associated with the system is increased, analogous to the effect of temperature on this band noted in other systems [20]. The wide angle X-ray results reported here suggest that this model may be incorrect since the albumin had no obvious effect on the 4.1-Å spacing. However, a conclusive statement on the presence or absence of a protein effect on wide angle X-ray reflection cannot be made with certainty without a study of the intensities of both bands over the entire range of bovine serum albumin concentrations.

The effect of cholesterol on wide angle X-ray reflections is interesting since it has not been previously reported for unsaturated lecithin–cholesterol systems in the presence of excess water (i.e. dispersions). Earlier X-ray data on similar systems were derived from partially hydrated bilayers only. In the systems which we utilized, 10 mole % cholesterol appears to enhance the "crystallinity" of the hydrocarbon lipid side chains as indicated by the appearance of the characteristic hexagonal 4.1-Å band. This observation correlates with data from other physical techniques regarding the role of cholesterol. Thermal analysis on dispersions of unsaturated lecithin systems show that the chains are less mobil above the transition temperature in the presence of cholesterol [21]. Results from laser raman spectroscopy [22] and electron spin resonance studies [23] also indicate an increase in the rigidity of the hydrocarbon chains due to the incorporation of cholesterol. Our results, however, were obtained with lipid dispersions containing 20–30 mole % of non-physiological components (either stearylamine or dicetyl phosphate). These components conceivably could interact with cholesterol in such a fashion as to produce the observed results independently of the physiological components. However, recently, Hui and Parsons [24] have reported that this spacing can also be observed in systems containing only physiological lipids, consequently a unique role for the non-physiological lipids in our systems seems unlikely.

The question concerning the effect of albumin on the permeability of the liposomes to solute is difficult to answer from the present data since the relationship between A and V is a complex one. Koch [25], using various approximations, has derived an analytical expression relating absorbance changes to the volume of the suspended particles, which he found adequate for interpreting the osmotic behavior of mitochondria and bacteria:

$$A = \frac{27}{(4)(2.3)} \sqrt[3]{\frac{\pi}{6}} \left(\frac{\frac{dn}{dc}}{n_0} \right)^2 \frac{q^2 v}{V^{\frac{4}{3}} \lambda'^2}$$

Where A = absorbance due to light scattering;
dn/dc = specific refraction index increment for the particle
n_0 = index of refraction of the medium
q = anhydrous mass of material in the single particle;

v = number of particles per unit volume;
V = volume of the particle
λ' = wavelength of light in the medium.

Thus A is a function not only of V but of the square of particle mass (q^2), the number of particles per unit volume (v), and the square of the refractive index increment dn/dc.

However, although the data do not allow us to evaluate q, v and dn/dc so that quantitative comparison may be made of dV/dt for the protein-free and protein-bound systems, a qualitative comparison is possible. It is likely that dn/dc for both systems is small and positive. Both Koch [25] and Reeves and Dowben [10] draw similar conclusions from their data, and it appears unlikely that addition of protein equivalent to 10 % of saturation will significantly alter this factor. On the other hand, electron microscopy indicates that it is likely that particle size is smaller in the presence of the protein so that v most probably is larger with albumin bound to the liposomes. Finally, from the amount of albumin present in the suspensions utilized in Tables II–IV it can be calculated that q for the protein-bound liposomes is about 2 % greater than for the protein-free system.

Thus, the two most significant factors influencing the quantitative relationship between $d(1/A)/dt$ and dV/dt both produce an increase in dV/dt for a given value of $d(1/A)/dt$ when protein is bound to the liposome. This suggests that if one wishes to compare dV/dt for the protein-bound and the protein-free systems, the actual rates dV/dt for the albumin–lyposomes are all somewhat larger than indicated by the measured $d(1/A)/dt$ values. Thus, although the data for the negative membranes (Table I) show that $d(1/A)/dt$ for the albumin–liposome complex is somewhat smaller for each solute than the comparable protein-free liposome system, in fact it is possible that the protein containing system may have true dV/dt values equal to or possibly slightly greater than the free liposome systems.

It therefore appears that our data do not support the earlier suggestion that albumin greatly alters the negative membrane permeability to glucose. If there are effects of the protein on solute permeability, they must be rather small and certainly not equal to the 3–4-fold increase suggested in our earlier work. Rather, it seems likely that the effects of the protein on liposome permeability [1] probably represents a transitory disruption of the vesicles by the protein as it becomes associated with the lipid. In our earlier experiments, diffusion was measured from the time of addition of the protein, whereas in the present work lipoprotein vesicles are allowed to form and equilibrate prior to the osmotic manipulation. Thus, while our belief that the albumin associated with liposomes to form lipoprotein vesicles which possess similar permeability properties to cell membranes appears substantiated, the protein probably does not effect solute permeability of negatively charged membranes to the extent that we previously concluded.

However, for the positively charged membranes (Table II), the protein appears to enhance the diffusion of all solutes except glycerol. Correction for q, v and dn/dt will tend to increase these effects, and therefore they appear to be significant and real. In the case of glycerol, Figs 10 and 11 indicate that the very rapid changes in A occurring with this solute makes accurate measurement of $d(1/A)/dt$ difficult with the manual method of mixing and reading absorbancies utilized here. Thus, it may well be that the permeabilities for glycerol are not as accurate as those for the other

solutes. It is quite clear from visual inspection of the raw data (Figs 10 and 11) that bovine serum albumin does enhance the permeability of positively charged liposomes to, e.g. arabinose. The mechanism for the effect of albumin on positively charged liposomes is not presently known.

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