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SPECIFIC INTERACTION OF CENTRAL NERVOUS SYSTEM MYELIN BASIC PROTEIN WITH LIPIDS

EFFECTS OF BASIC PROTEIN ON GLUCOSE LEAKAGE FROM LIPOSOMES

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SUMMARY

The leakage from liposomes preloaded with glucose was continuously monitored in a Perkin–Elmer Model 356 dual beam spectrophotometer using an enzyme-linked assay system. The central nervous system myelin basic protein (A_1 protein) caused a 3–4-fold increase in the rate of leakage from liposomes prepared from either central or peripheral nervous system lipids and also from an egg lecithin–5 % phosphatidic acid mixture. Some basic proteins, lysozyme, cytochrome *c*, two peripheral nerve myelin basic proteins, trypsin and the tryptic peptides derived from the A_1 protein did not alter glucose leakage from the central nervous system lipid mixture. Other basic proteins, poly L-lysine, protamine sulphate and a reduced, "arginine-rich" histone from bull sperm had a "lytic action" on these liposomes

Basic proteins derived from both central and peripheral nervous myelin reduced the poly L-lysine-stimulated leak of the central nervous system liposomes whereas lysozyme had no effect. Additional evidence suggesting a strong interaction of the A_1 protein with liposomes was provided by studies of the action of trypsin on the liposome–protein complexes. The glucose leak of central and peripheral nervous liposomes complexed with A_1 protein was reduced by incubation with trypsin, but was still significantly higher than leakage of these liposomes before the addition of the A_1 protein.

INTRODUCTION

The highly organized nerve myelin sheath has been a most useful preparation for the study of the structure of membranes[1,2]. Unfortunately, many of the investigations have been utilized to develop generalized models for all biological membranes, and have come under sharp criticism owing to the atypical nature of myelin[3]. Nevertheless, these investigations have given us a clear picture of the arrangement of lipids and proteins in the myelin sheath[4–7]

The purification[8,9] and characterization[10,11] of one of the two major protein constituents of central nervous system myelin, the basic protein or A_1 protein[8], and the purification of two similar basic proteins, the P1 and P2 proteins of ox intra-

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dural roots[12], prompted us to study the interaction of these proteins with nervous tissue lipids. Kimelberg and Papahadjopoulos[13,14] have investigated the nature of the interaction of other non-membrane basic proteins with liposomes and found that permeability studies can be used to classify various proteins on their ability to interact with the lipid preparations.

In the present study lipid mixtures were used in conjunction with a variety of basic proteins to test if there is a specificity of interaction of the myelin-derived proteins for myelin lipids.

METHODS

Purification of peripheral and central nerve myelin basic proteins

The procedure for purification of ox peripheral nervous system myelin basic proteins (P1 and P2) from the acid-extractable proteins of intradural roots has recently been described[12].

Central nervous system myelin basic protein (A_1 protein) was isolated from ox spinal cords. Lipid depletion of the spinal cords was performed according to the method of Oshiro and Eylar[15] within 30 min after the death of the animals. Purification of the basic protein was carried out on the lipid-depleted residue isolated by centrifugation by a modification of the procedure of Eylar *et al.*[9,10]. The lipid-depleted spinal cord residue was homogenized and extracted overnight at 4 °C with 0.06 M HCl containing 0.2 M NaCl. The acidity was checked periodically during the first few hours of extraction and kept at pH 2.0 by additions of concentrated HCl.

The residue was removed by centrifugation and the supernatant was brought to pH 3.0 by addition of glycine to give a final concentration of 3 mM. The NaCl concentration was increased to 0.5 M and this protein solution (12.5 g) was applied to an SP-Sephadex A25 column (50 cm × 5 cm) which had been previously prepared and washed in 3 mM glycine–HCl, 0.5 M NaCl buffer. The unbound proteins were removed by elution with the same buffer at a rate of 240 ml/h. The elution pattern was determined with a Unicam ultraviolet recorder at 280 nm. The bound proteins were then eluted with a linear gradient of NaCl ranging from 0.5 M to 1.2 M in the presence of 3 mM glycine–HCl buffer at pH 3.0 The A_1 protein was further purified by gel filtration on Sephadex G-50 and G-75 columns eluted with 0.01 M HCl[16]. Purity of the A_1 protein was confirmed by acrylamide disc electrophoresis[17].

Preparation of lipids

Central nervous system (bovine spinal cord) total lipids were extracted with chloroform–methanol (2:1, v/v) as described by Folch *et al.*[18]. The solvent was removed under reduced pressure by means of a rotary evaporator. The dried lipids were suspended in chloroform–methanol (19:1, v/v) saturated with water and, after filtration to remove insoluble material, were further purified by Sephadex G-25 column chromatography according to the method of Rouser *et al* [19]. The procedure separated gangliosides and the Folch–Lees proteolipid from the extract. The lipids were stored dry under N_2 at −20 °C. Peripheral nerve total lipids (from bovine intradural roots) were extracted and purified according to the same procedure.

Fractionation of central nervous system total lipids was carried out by DEAE cellulose column chromatography according to the procedure of Rouser *et al.*[19]. An

acidic lipid fraction was prepared according to Sequence 1 (ref. 19) and the ammonium acetate was removed by repeated washes with chloroform and removal of the organic phase under reduced pressure. Remaining salt was removed by passage of the lipids over a Sephadex column[19]

Phosphatidylserine, prepared from the central nervous system lipids by DEAE column chromatography according to the procedure of Sanders[20] was found to be more than 99% pure as determined by thin-layer chromatography[21]. The acidic lipid was converted to the salt form by a Folch wash procedure[18] with 0.2 volumes of 0.1 M sodium–potassium phosphate buffer, pH 7.4.

The lipid compositions were determined for all fractions used in this study. Phospholipid phosphorus was determined by the method of Fiske and SubbaRow (see ref. 22). Phospholipid compositions were determined after separation of the lipids by two-dimensional thin-layer chromatography[21]. Cholesterol was determined according to the method of Huang *et al.*[23].

Measurement of liposomal swelling

Liposomes were prepared in 50 mM KCl at room temperature and swelling rates determined according to the procedure of de Gier *et al.*[24,25] Experiments to test the effect of the A_1 protein on the swelling of the liposomes were performed by addition of the liposomes to 100 mM glycerol solutions containing the protein. Liposomes (20–50 μl) were added to give a final absorbance at 450 nm of 0.3 to 0.4 absorbance unit Control experiments to test whether possible aggregation of the liposomes by the A_1 protein affected the absorbance were performed by substitution of isotonic sucrose (a non-permeant, non-electrolyte) for the isotonic glycerol

Measurement of glucose trap and glucose leak

Preparation of liposomes. Liposomes were prepared by a modification of the procedure of Demel *et al.*[26] in which 10 mg of dried lipid was suspended in a 1-ml solution containing 0.285 M glucose, 3.75 mM NaCl–KCl and 0.5 mM Tris–HCl, pH 8.0, by agitation on a Vortex mixer under a N_2 atmosphere. Untrapped glucose was removed by Sephadex G-50 gel filtration chromatography A column (1.5 cm × 29 cm) was prepared and eluted with 0.075 M KCl and 0.075 M NaCl buffered with 5 mM Tris–HCl, pH 8.0 The liposomes were eluted in the void volume (approx. 20 ml) and the column was washed with five additional column volumes before reuse. The liposomes were usually allowed to stand for 1 h prior to use. Because of high leakage rates liposomes prepared from an acidic lipid fraction and from phosphatidylserine were used immediately after preparation. Fresh liposomes were prepared daily.

Assay of glucose trap and glucose leak. The amount of glucose trapped in the liposomes was determined as reported previously[26]. The complete system contained, in cuvettes with a 10-mm light path, the following reagents (in order of addition): 0.40 ml of 0.1 M Tris buffer (pH 8), 0.50 ml of "double strength" isotonic salt mixture (0.15 M KCl and 0.15 M NaCl, prepared in Tris buffer), 0.10 ml of 0.02 M magnesium acetate, 0 05 ml of 0.02 M ATP, and 0 05 ml of 0.01 M $NADP^+$.

5 μl of hexokinase (6 7 mg protein/ml) and 5 μl of glucose-6-phosphate dehydrogenase (3,4 mg protein/ml) The reaction was started by the addition of

the appropriate liposome preparation. External glucose was measured by the $NADP^+$-linked enzyme assay system and after addition of Triton X-100 the total glucose (external *plus* trapped) was measured. From these measurements the trapped glucose was calculated. The amount trapped was measured within 1 h after separation of the liposomes from the untrapped glucose.

The glucose leak was monitored with a Perkin–Elmer Model 356 used in the dual mode, $\lambda_1 = 338$ nm, the maximum absorption of the product NADPH, $\lambda_2 = 388$ nm, an isosbestic point minimum absorption wavelength for NADPH. The single quartz cuvette was jacketed and the circulating water from a constant temperature bath was maintained at 25 °C.

The components used for measurement of trapped glucose[26] were used at the same concentrations for the glucose leak studies. The concentration of the enzymes and co-factors were varied separately, or together over a 4-fold range, without noticeable change in leakage. The leakage rates were determined for 50 μl of liposomes (approx. 75 nmoles lipid), and, after a linear leak was obtained (5–10 min), aliquots of protein dissolved in 0.075 M NaCl, 0.075 M KCl, 5 mM Tris–HCl, pH 8.0 were added. The solution was stirred with a small plastic rod, and this stirring procedure caused some rupture of the liposomes (0.05–0.15 %). Linear leakage rates were observed; the order of addition of liposomes and protein had no effect on leakage rate.

Measurements of the dependence of the absorbance increase on added glucose were made with both a conventional spectrophotometer (Unicam SP 600) and with the double beam spectrophotometer (Perkin–Elmer 356) set in the dual mode (Fig. 1). A linear response was observed in both systems over the range of 0–70 μM glucose. When the double beam was used at 30 times sensitivity, a linear response having the same dependence on glucose was also observed.

In order to reduce the light scattering the following precautions were taken: (1) The cuvettes were placed close to the photomultiplier, reducing light scattering by 90 %. (2) Possible changes in light scattering caused by protein–lipid aggregation were eliminated by operation of the instrument in the dual mode (single cuvette).

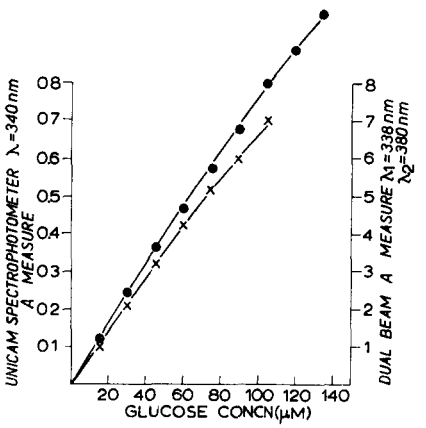


Fig. 1. Glucose enzyme response curves Aliquots of glucose were added to a cuvette containing the enzyme assay components The change in absorbance was recorded at 340 nm with a Unicam SP-600 spectrophotometer Similar recordings were made with the Perkin–Elmer Model 356, set in the dual mode (see Methods). Absorbance measurements (○—○); dual beam absorbance measurements (×—×).

Aggregation phenomena occurred at both the sample and reference wavelengths and were therefore eliminated. Evidence for this conclusion comes from control experiments run without $NADP^+$, the precursor of the absorbing product NADPH. Without $NADP^+$ no change in absorbance was observed when various basic proteins were mixed with liposome suspensions

Electron microscopy

Liposomes were prepared from the central nervous system lipid extract and the acidic lipid fraction derived from this extract by mechanical dispersion in isotonic veronal–acetate buffer, pH 8.0. These liposomes (4 mg/ml) were added with stirring to an equal volume of 1 mg/ml protein solution prepared in the same buffer. The resulting precipitate was recovered after centrifugation at 3000 × *g* for 5 min in a Sorvall RC-2 rotor at 4 °C

These lipoprotein complexes and samples of the liposomes from which they were made were fixed with 1 % OsO_4, dehydrated with acetone and embedded in araldite. The thin sections were stained with uranyl acetate followed by lead citrate[27] The specimens were examined in a Siemens Emicon electron microscope, with initial magnifications of 20000 × and 40000 ×.

Materials

ATP and $NADP^+$ were from Sigma Chemical Co , St. Louis, Mo. Hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer–Mannheim Corp. New York, N. Y. Egg lysozyme (crystalline) and horse cytochrome *c* and trypsin (three times crystallized) from Fluka. Protamine sulphate from Koch–Light Laboratory, England. Poly L-lysine hydrobromide (molecular weight 50000–100000) from Pierce Chemicals.

Arginine-rich histone from bull sperm was kindly provided by Dr C H. Monfoort, Laboratory of Physiological Chemistry, University of Utrecht. Sephadex G-75 and SE-Sephadex A-25 were purchased from Pharmacia. The solvents and reagents were all of analytical grade

RESULTS

Lipid composition of central and peripheral nervous system lipids

Lipid composition of the ox spinal cord and intradural roots were determined and these results compare favorably with values reported by Rouser and Fleischer[28] and O'Brien[29] (Table I). The composition of the spinal cord total lipid and intradural root extracts are also quite similar to the lipid compositions of myelin isolated from these structures. Also presented is the lipid composition of an acidic lipid fraction derived from the spinal cord extract.

Characterization of the lipid dispersions

(A) Permeability to glycerol and erythritol: Bilayers prepared from total lipid extract of bovine spinal cord and phosphatidylserine purified from this extract, were tested for their osmotic behaviour in the presence of non-electrolytes (erythritol and glycerol). Liposomes, shaken by hand, prepared from these lipids in 50 mM KCl were found to act as nearly ideal osmometers when suspended in solutions of higher

TABLE I

COMPARISON OF LIPID COMPOSITIONS OF CENTRAL AND PERIPHERAL NERVOUS TISSUE FRACTIONS WITH MYELIN

Central nervous system lipids were prepared from ox spinal cord and peripheral nervous system lipids from ox intradural roots (A) Phospholipid and cholesterol levels were determined as described in Methods and are expressed as percentage dry weight for lipid species, and (B) percentage of lipid phosphorus as recovered from thin-layer plates Phospholipid values are calculated assuming a mol wt 750, and glycolipid values were calculated as material not present as phospholipid or cholesterol Comparison of phospholipid compositions are made with values reported by Rouser and Fleischer[28] CNS, central nervous system, PNS, peripheral nervous system

A Lipid species	*CNS lipids*	*CNS lipids*[33]	*CNS myelin*[33]	*CNS acidic lipids*	*PNS lipids*	*PNS lipids*[33]	*PNS myelin*[33]
Cholesterol	29 3						
Phospholipid	42 2						
(Glycolipid)	(28 5)						
B Phospholipids·							
Serine phosphoglycerides	18 0 ± 0 7	15 3	16 7	63 0	12 5	16 5	15 5
Ethanolamine phosphoglycerides	37 0 ± 3 0	38 4	38 6		20 3	30 9	29 2
Inositol phosphoglycerides	1 7 ± 0 1	1 7	1 7	4 7	2 6		
Choline phosphoglycerides	21 0 ± 3 2	19 7	19 5		17 1	27.0	24 2
Sphingomyelin	19 2 ± 2 8	17 6	21 7		35 6	25 5	30.5
Phosphatidic acid	1 2 ± 0 2	1 3	1 5	19 6	2 8		
Poly phosphoinositides	0 7 ± 0 1			7 9	2 2		
Cardiolipin				6 1			
Unknown	1 0 ± 0 2			3 5			

and lower ionic strength KCl The permeability to the non-electrolytes of liposomes prepared from phosphatidylserine was greater than for the spinal cord lipid extract

(B) Glucose trap and glucose leakage Liposomes were prepared in a glucose solution from the various lipid fractions. The amount of glucose trapped in the liposomes and rate of glucose leakage from the liposomes were determined (Table II and Fig 2)

As may be seen, both central and peripheral nervous system liposomes trapped about 3.0 nmoles of glucose per nmole of phospholipid, and since these lipid mixtures contain about 40 % (by weight) phospholipid, the trapping is roughly 1.0 nmole of glucose per nmole of lipid This is comparable to that of liposomes prepared from egg lecithin containing 5 % phosphatidic acid. Liposomes prepared from phosphatidylserine trapped a significantly higher amount of glucose, whereas an acidic lipid fraction containing mainly a high proportion of phosphatidylserine (Table I) trapped somewhat less glucose Liposomes of phosphatidylserine and the acidic lipid extract prepared with 20 % cholesterol by weight were found to trap similar amounts of glucose, and higher amounts than liposomes prepared without added cholesterol

The glucose leakage of the liposomes was studied as a function of liposome concentration (Fig. 2). The rate of glucose leakage from liposomes of the acidic lipid fraction and phosphatidylserine was 10–25 times greater than from liposomes of the central and peripheral nervous system lipids or liposomes of egg lecithin containing 5 % phosphatidic acid This is probably because of the high content of cholesterol in the liposomes prepared from central and peripheral nervous system lipids. In agreement with this observation the swelling rate of liposomes of phosphatidylserine in isotonic glycerol and erythritol at a temperature of 25 °C was much higher than for liposomes formed from the central nervous system lipids.

The glucose leak was linearly dependent upon the concentration of liposomes from either central nervous system lipids, peripheral nervous system lipids or egg lecithin (Fig. 2B). Although a linear leakage rate was sometimes observed for acidic lipids over a 30-min period, increasing amounts of liposomes did not give linearly

TABLE II

GLUCOSE TRAP AND GLUCOSE RELEASE

Liposomes were prepared in buffered glucose solution as described in Methods Trapped glucose was determined from enzymic measurements of external glucose and total glucose associated with the liposomes[25] (see Methods) Lipid phosphate measurements were made on each liposome preparation Glucose release was monitored with the double beam spectrophotometer as described in Methods CNS, central nervous system, PNS, peripheral nervous system

Lipid	*Glucose trap (nmoles glucose/nmole lipid phosphorus)*	*Glucose release (pmole glucose/nmole lipid phosphorus/min)*	*Glucose release (% off trap/min)*
PNS lipids	3 0	0 05	0 00167
CNS lipids	2 5 ± 0 5	0 044	0 00176
CNS acidic lipid fraction	0 75	1 5	0 2
CNS acidic lipids + 20% cholesterol	3 0	5 0	0 167
CNS phosphatidylserine	1 8	1 2	0 067
CNS phosphatidylserine + 20% cholesterol	2 7	1 0	0 037
Egg lecithin + 5% phosphatidic acid	0 775	0 037	0 0048
CNS neutral lipids ± endogenous cholesterol	0 0	0 0	0 0

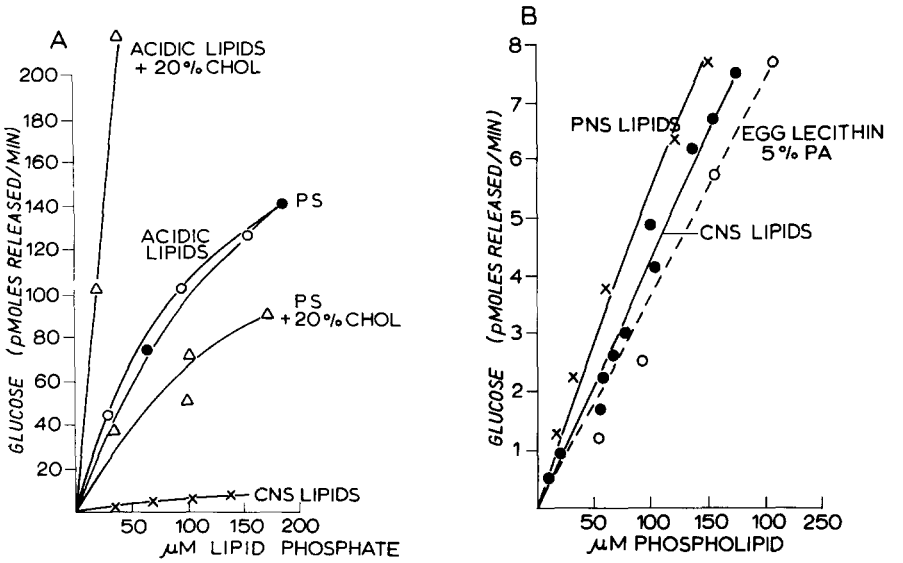


Fig 2 Variation in glucose leak with liposome concentration Various liposome concentrations found to trap glucose were added sequentially to a cuvette containing the glucose enzyme assay components Changes in leak rate were monitored for 5–15 min prior to further addition of liposomes (A) Comparison of the glucose leak from various acidic lipid preparations and total central nervous system (CNS) lipids. (B) Comparison of central and peripheral nervous system (PNS) liposomes with liposomes of egg lecithin containing 5% phosphatidic acid PS, phosphatidylserine; PA, phosphatidic acid

increasing rates of leakage (Fig. 2A). Alterations in the concentration gradients of glucose in the outer lamellae of the liposomes may account for these discrepancies.

Electron microscopy

Electron microscopy was performed on lipoprotein complexes formed from mixtures of aqueous lipid dispersions with the A_1 protein and poly L-lysine. Liposomes formed from both the central nervous system lipid extract and acidic lipid fractions exhibited the concentrically enclosed vesicular structures common to liposomes (Fig. 3A and 3C). Aqueous dispersions of the acidic lipid fraction contained a reduced number of bilayer lamellae and a higher electron density of the aqueous compartments compared with the central nervous system lipid liposomes.

Interaction of the central nervous system liposomes with the A_1 protein vesicles appeared not to alter the concentric organization of the liposomal structure, although the electron density of the aqueous compartments was significantly increased (Fig 3B). The liposomal structure of the acidic lipids was lost upon interaction with the A_1 protein and the resulting multilamellar sheets showed a 30–40% increase in spacing between the electron-dense region (Fig 3D).

Poly L-lysine–central nervous system liposome complexes appeared as a variety of structures ranging from small amorphous material to multilamellar structures (Fig. 4A). Liposomes of acidic lipids complexed with poly L-lysine exhibited a uniform, amorphous, highly aggregated phase with faintly visible closely packed lamellar sheets (Fig. 4B).

Effect of basic proteins on glucose leakage

A number of basic proteins were tested to see if their presence would alter the glucose leakage of liposomes prepared from the central nervous system lipids The nine proteins tested fell into three classes (Fig 5) Proteins such as lysozyme did not alter the linear leakage rate Slight decreases noted at the time of addition resulted from dilution of the NADPH as the volume was increased by 5–10 % upon addition of protein The rise observed in the first minute is a mixing artifact caused by the rupture of a small percentage (approx. 0.05–0.15 %) of the liposomes. Similar increases were observed when the liposomal solution was stirred without addition

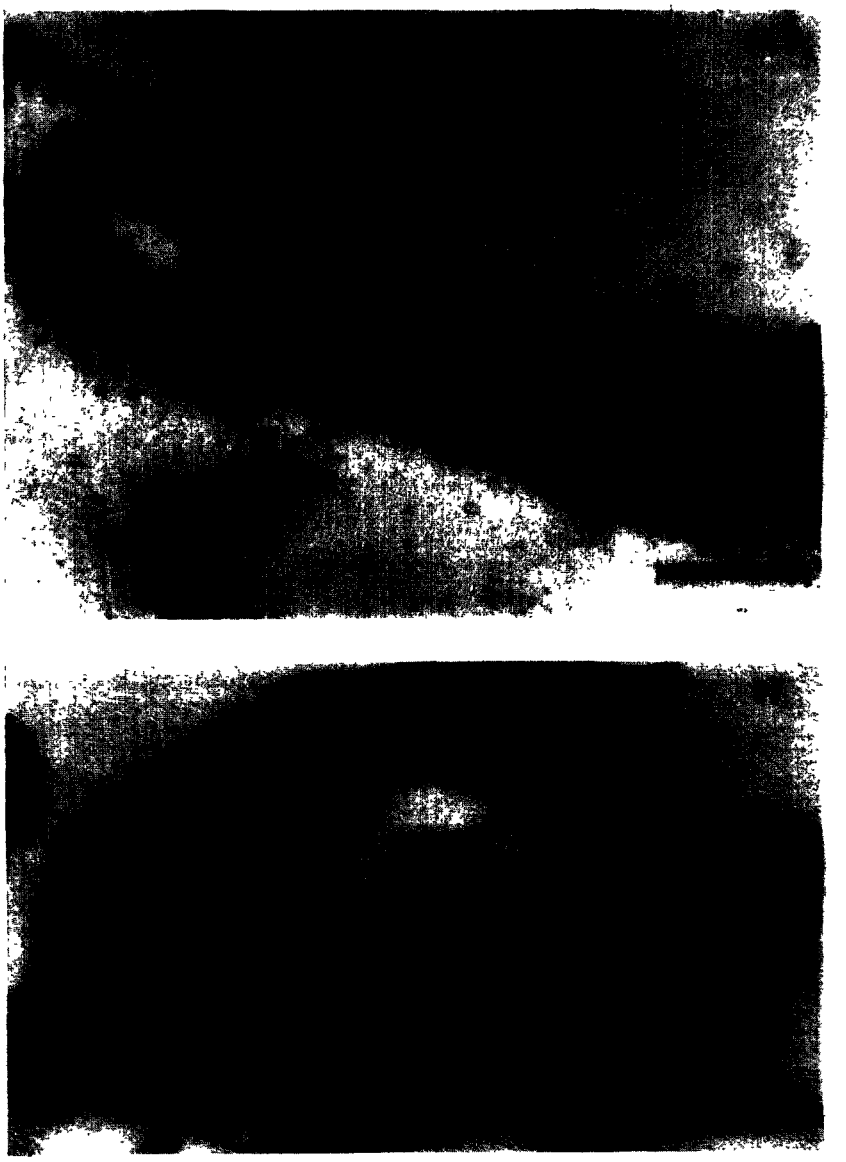


Fig. 3 (A) Liposomes prepared from central nervous system lipid extract Magnification with all samples is 175000 × Samples were prepared in isotonic veronal–acetate buffer (pH 8 0), and, after centrifugation, fixed with OsO_4 and stained with uranyl acetate and lead citrate (B) Recombination of central nervous system liposomes with A_1 protein (C) Liposomes prepared from the acidic lipid fraction (D) Recombination of liposomes of the acidic lipid fraction with A_1 protein

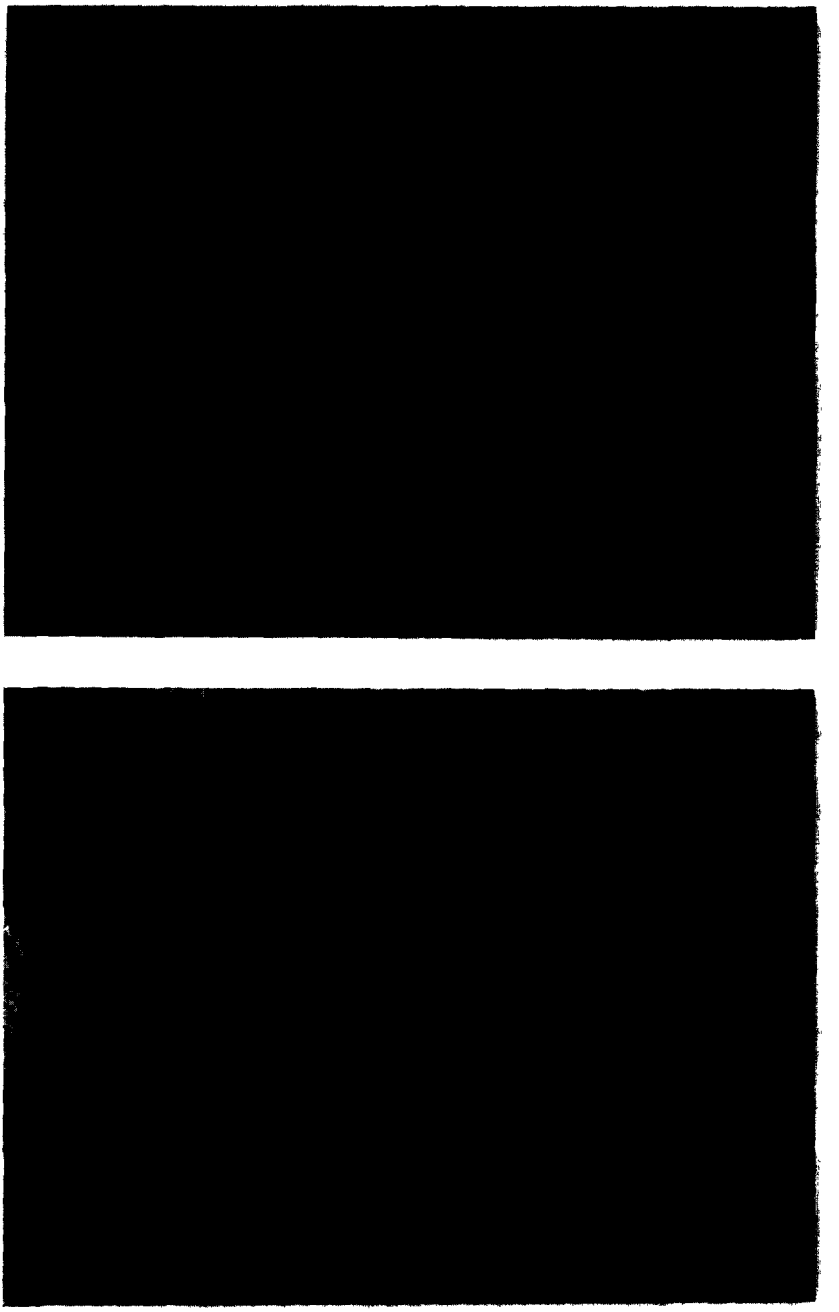
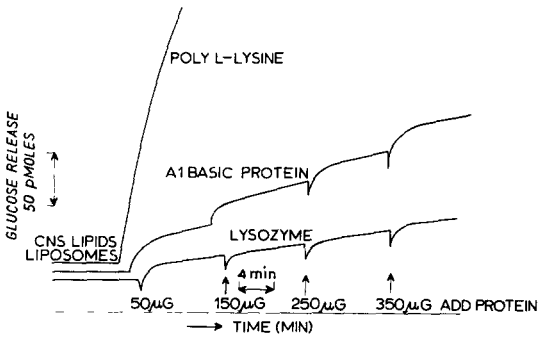


Fig 4 (A) Recombination of central nervous system liposomes with poly L-lysine (B) Recombination of liposomes of acidic lipid fraction with poly L-lysine Samples were prepared as described in Fig 3 and Methods Magnification is 280000 ×

of protein. The second type of response is elicited solely by the A_1 protein. The leakage rate is increased by a factor of 4–5 after about 50 μg of this is added. The third type of response is a "lytic effect" produced by proteins such as poly L-lysine. A rapid release of glucose greater than 10 times the rate from liposomes alone was produced by as little as 1 μg of these proteins.



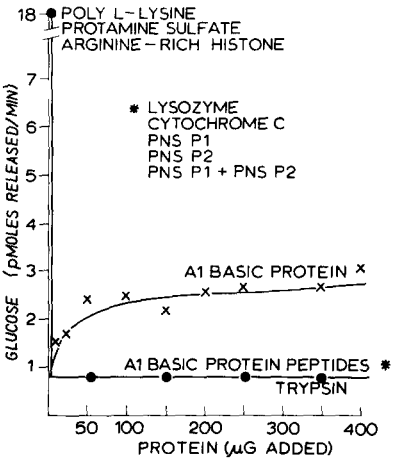


Fig 5 Tracings showing effect of indicated basic proteins on glucose leak. Tracings were taken from three separate experiments with a dual beam recorder Increasing absorbance, resulting from NADPH production, is in the upward direction. CNS, central nervous system

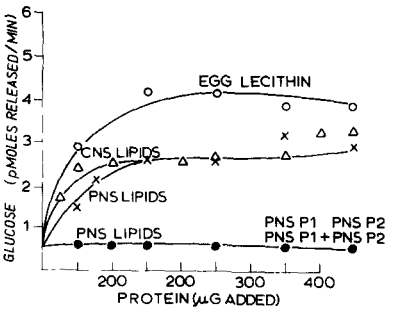
Fig 6 Concentration dependence of indicated basic proteins and A_1 peptide mixture on glucose leakage from central nervous system liposomes Leakage rates were calculated from regions of tracings (see Fig 5) PNS, peripheral nervous system

The different responses to the A_1 basic protein and the poly L-lysine could be explained by the fact that A_1 protein or certain areas of the protein molecule penetrate the lipid matrix and thus increase the permeability of the liposomes to glucose. The poly L-lysine may interact electrostatically with the charged lipids and produce a concentration of charge at the site of interaction, causing rupture of the liposomes and fast release of glucose. Fig. 6 shows the concentration dependence of these changes, and also the effect of a tryptic digest of the A_1 protein.

The A_1 protein shows a unique interaction with liposomes prepared from the central nervous system lipids. When this protein is added to liposomes at a lipid phosphorus to protein molar ratio of 10:1, there is an increase in the leakage rate by a factor of 3–4. A further increase in the protein concentration to a 1:1 molar ratio resulted in no further increase in the leakage rate. The A_1 protein also increased the glucose leakage from peripheral nervous system lipid liposomes and liposomes of egg lecithin containing 5% of phosphatidic acid (Fig. 7). The two peripheral myelin basic proteins P1 and P2, either alone or in combination, did not alter the glucose leakage. This finding is in agreement with the investigation on the effect of the A_1 protein on glycerol-induced swelling (Fig. 9). The glucose leakage as a function of protein concentration was similar for all three liposome preparations.

The influence of lysozyme, the A_1 protein and poly L-lysine on glucose leakage from liposomes prepared from the acidic lipid fraction containing 20% cholesterol

(by weight) is shown in Fig. 8. Both poly L-lysine and the A_1 protein have a "lytic action" on the liposomes for upon addition of 50 μg of protein there is an immediate release of 15–20 % of the trapped glucose by A_1 protein and 5–10 % by poly L-lysine.



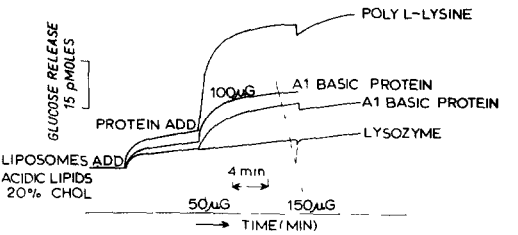
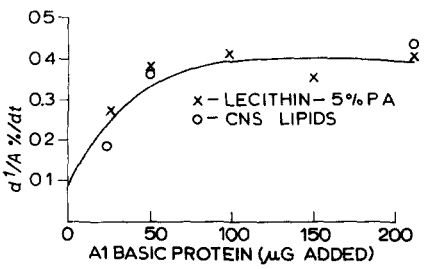


Fig 7 Concentration dependence of indicated myelin basic proteins on glucose leak from various liposome preparations Leak changes caused by A_1 protein on liposomes of O, egg lecithin containing 5% phosphatidic acid, △, central nervous system (CNS) lipids, ×, peripheral nervous system (PNS) lipids Leak changes caused by P1 or P2 protein or equimolar mixture of the two proteins on liposomes of peripheral nervous system lipids, (●)

Fig 8 Tracings showing perturbation by basic proteins of glucose leak from liposomes of acidic lipid fraction containing 20% cholesterol by weight



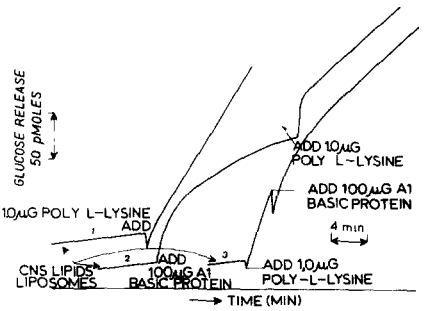


Fig 9 Effect of various concentrations of A_1 protein on glycerol-induced swelling Liposomes of egg lecithin containing 5% phosphatidic acid or central nervous system (CNS) lipids were added to cuvette containing 100 mM glycerol equilibrated with A_1 protein Initial swelling rates were measured as described by de Gier *et al* [25], by measurement of absorbance at 450 nm PA, phosphatidic acid

Fig 10 Tracings showing combined activities of poly L-lysine and A_1 protein on glucose leak of central nervous system (CNS) liposomes (1) Poly L-lysine alone (2) 100 μg A_1 protein followed by 10 μg poly L-lysine (3) 10 μg of poly L-lysine followed by 100 μg A_1 protein

Further addition of either polypeptide causes little additional glucose leakage, probably because of the formation of ordered lipoprotein complexes which still contain trapped glucose but are not affected by added protein. Other basic proteins, the P1 protein of peripheral nerve myelin and lysozyme had no lytic effect and furthermore did not alter the leakage rate of these liposomes. Similar results were obtained when the A_1 and P1 proteins, lysozyme and poly L-lysine were tested on the acidic lipid fraction prepared without cholesterol or phosphatidylserine liposomes preparated both with and without added cholesterol (20 %).

Effect of central nervous system myelin basic protein (A_1 protein) on glycerol-induced swelling

Liposomes prepared in 50 mM KCl were added to 100 mM glycerol solutions containing varying amounts of A_1 protein. Turbidity changes were followed and initial swelling rates were calculated as a percentage of initial turbidity. The effects of A_1 protein concentration on the swelling of liposomes of central nervous system lipids and egg lecithin containing 5 % phosphatidic acid were determined (Fig. 9). The A_1 protein caused similar increases in the swelling rates of both liposome preparations. Increases of protein concentration above 100 μg/10 ml caused no further increase in the swelling rate.

Stabilizing effect of myelin basic proteins on liposomes

When low levels of the extended, highly charged basic proteins such as poly L-lysine were added to liposomes prepared from central nervous system lipid a rapid, linear release of glucose could be measured for 5–10 min. When 100 μg of A_1 protein were added to the liposomes the release of glucose upon subsequent addition of 1.0 μg poly L-lysine was greatly reduced (Fig. 10). Increasing concentrations of the A_1 protein caused a more pronounced reduction in glucose release rate upon subsequent poly L-lysine addition than did the P1 protein or lysozyme (Table III). The leak produced by poly L-lysine was also reduced when A_1 protein was added after the poly L-lysine. This result implies specific interaction of these polypeptides with a limited number of sites on the bilayer surface.

The P1 protein, which was not able to alter glucose leakage from liposomes, did alter the liposomes by reducing the rapid leakage induced by poly L-lysine, although much less effectively than the A_1 protein. On the other hand, lysozyme added in very high quantities did not protect the liposomes against the action of poly L-lysine.

Effect of trypsin on leakage of A_1 protein–central or peripheral nervous liposomes

To test whether regions of the A_1 basic protein were penetrating into the lipid

TABLE III

EFFECT OF OTHER BASIC PROTEINS ON LEAKAGE BY POLY L-LYSINE

Glucose leakage rates were measured for central nervous system liposomes alone, and after subsequent additions of proteins (as indicated) and 1 0 μg poly L-lysine In one experiment the order of addition of the proteins was reversed so that the A_1 protein was added after poly L-lysine See also Fig 10

Leak without protein (pmoles glucose released/min)	*Protein added*	*Amount protein added (μg)*	*Leak with protein (pmoles/min)*	*Leak after addition of 1 μg poly L-lysine*
1 3				30 6
1 2	Lysozyme	500	1 6	30 0
1 2	A1 basic protein	50	4 15	10 0
1 2	A1 basic protein	100	4 0	9 1
1 2	A1 basic protein	100	—	8 7
1 2	A1 basic protein	250	4 2	6 4
1 3	P1 basic protein	200	1 43	24 3
1 0	P1 basic protein	400	1 6	17 0

matrix, and thus increasing the leakage, the A_1 protein–central nervous system lipid complexes were treated with trypsin. It was felt that regions of the protein buried in the lipid would be protected from this digestion and perhaps still enhance the leakage.

After obtaining leakage rates for liposome of central nervous system lipid alone and in the presence of 100 μg A_1 protein, 10 μg of trypsin were added to the liposomes (Fig. 11A) The leakage rate was followed for an additional 20 min and was compared with a blank run to which buffer without trypsin was added. Trypsin addition resulted in a slight decrease in the leakage rate from 4.75 to 3.9 nmoles glucose per min, a rate that was significantly higher than glucose leakage of the liposomes alone (1.9 nmoles glucose per min)

Control experiments (Fig. 6) have shown that neither the A_1 tryptic peptide mixture nor trypsin influence glucose leakage. When 100 μg A_1 protein and 10 μg trypsin were pre-incubated in the cuvette for 5 min prior to the addition of liposomes (Fig. 11B) The glucose leakage rate observed was identical to that in the absence of basic protein. Thus, trypsin is capable of quickly breaking down the A_1 protein to small peptides which are ineffective in stimulating the glucose leakage.

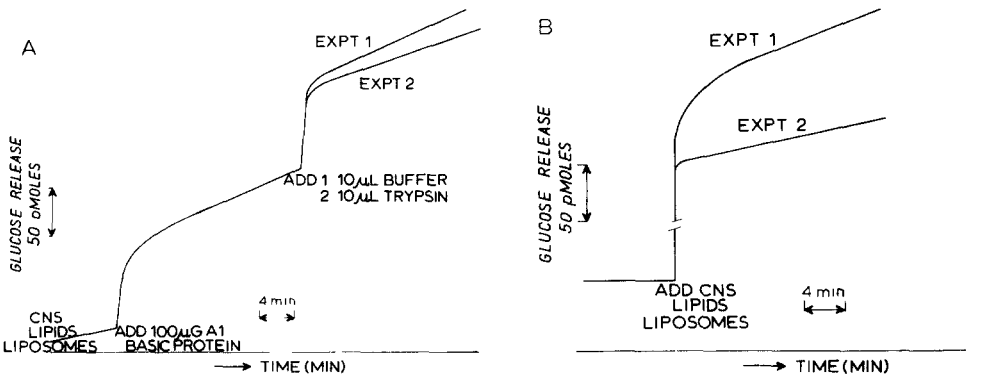


Fig 11. Effect of trypsin on glucose leakage from central nervous system (CNS) lipid A_1 protein complex (A) Tracings showing leakage rate of liposomes, alone, after addition of 100 μg of A_1 protein, and (Expt 2) 10 μg of trypsin A control (Expt 1) was run in which buffer and not trypsin was added to the cuvette (B) Tracings showing leakage of central nervous system liposomes added to (1) 100 μg of A_1 protein alone (2) 100 μg of A_1 protein preincubated 5 min with 10 μg of trypsin.

TABLE IV

EFFECT OF TRYPSIN ON A1 PROTEIN-INDUCED LEAK

Glucose leakage rates were measured with central and peripheral nervous system liposomes as indicated, after addition of 100 μg A_1 protein and after subsequent addition of 10 μg trypsin. The response was followed over 20–40-min periods Leak was also measured for liposomes added to a cuvette containing 100 μg A_1 protein alone, and 100 μg A_1 protein, preincubated 5 min with 10 μg trypsin CNS, central nervous system; PNS, peripheral nervous system

Experiment	*Lipid*	*Normal leak*	*Leak with protein (100 µg)*	*Leak with protein measured after 10% (w/w) trypsin was added*	*Leak of liposomes added to protein preincubated 5 min with 10% trypsin*
1	CNS	1 0	3 7	1 7	1 0
2	CNS	1 9	4 75	3 9	1 9
3	PNS	0 2	1 45	0 75	0 1

The results of the above experiments and parallel experiments performed with liposomes prepared from peripheral nervous system lipids are summarized in Table IV.

DISCUSSION

The method of Demel *et al.*[26] for measuring glucose leak from liposomes has been adapted for continuous monitoring of the leak using a dual beam spectrophotometer This modification has several advantages. (1) Detection of glucose release is rapid and sensitive, so that rates of 0.001 % of glucose trapped (0.5 nmole/min) can be measured. (2) Due to the slow rates of leakage for liposomes of egg lecithin and total nervous tissue lipid mixtures at room temperature, changes in the glucose concentration gradient are small. Therefore, liposomes prepared in the morning can be used all day without significant change in the leakage rates. (3) Once a linear leak is obtained, the addition of possible effector molecules (*e.g.* proteins, hormones, *etc.*) can be made directly to the cuvette and the new leak rate can be compared directly to the control (liposomes without effector). (4) Several additions may be made to the same cuvette to study the dependence of leak on concentration of effector, or possibly synergistic or competitive effects between various types of effectors. Leakage rate has not been found to be dependent on the order of addition, *e.g.* whether protein is added to liposomes or *vice versa*. (5) Unlike permeability studies which measure turbidity changes induced by the substrate, the glucose-linked enzyme assay is not subject to artifacts caused by protein-induced aggregation of liposomes.

In order to exclude the possibility that the assay components alter the leak, a number of control experiments were performed. Variation in the concentration (0.25 to 3.0 times listed values) of the enzyme (glucose-6-phosphate dehydrogenase, and hexokinase) and co-factor (ATP and $NADP^+$) did not disturb the glucose leakage rate of central nervous system liposomes. Other globular protein in far greater concentration than the assay enzymes, likewise did not impair glucose leakage.

Another way to test if the components interact with the liposomes was to vary the concentration of liposomes. The glucose leakages from liposomes of central and peripheral nervous system lipid fractions and egg lecithin *plus* 5 % phosphatidic acid were directly proportional to the concentration of liposomes. Even at the highest leakage rates, the enzyme system was not limiting, addition of glucose to the cuvette gave an immediate increase in absorbance roughly proportional to the amount added. These experiments also suggest that the components of the enzyme assay system are not inactivated by the lipid. Possible effects of Mg^{2+}, required for the hexokinase reaction, were not investigated

Prior to utilization of glucose leak, glycerol swelling experiments showed that the A_1 protein enhanced liposome swelling of both central nervous system lipid and egg lecithin liposomes. These observations were confirmed by the glucose leak studies. The maximum increase in both swelling and leakage rates of central nervous system liposomes occurred in the presence of 10–15 mole % of A_1 protein, a value very close to that found in native myelin. Electron microscopy confirmed that the A_1 protein does not damage the structure of these liposomes. Further studies are in progress to determine how the protein may cause an increase in the electron density of the aqueous spaces between the lipid bilayers.

The A_1 protein stimulation of glucose leakage could not be mimicked by any

one of a variety of other basic proteins, including the P1 and P2 proteins of peripheral nerve myelin. According to the criterion of glucose permeability the interaction is not specific for central nervous system lipids, similar increases in leakage rates were observed with peripheral nervous system liposomes and even with liposomes composed mainly of egg lecithin

The highly charged basic proteins, poly L-lysine, protamine sulphate and an arginine-rich histone, markedly enhanced glucose leak of central nervous system liposomes. Furthermore, poly L-lysine caused damage to the structure of some of the liposomes as observed by electron microscopy Both Hammes and Schullery[30] and Kimelberg and Papahadjopoulos[13] have reported that this polypeptide increases the permeability of phosphatidylserine liposomes. Hammes and Schullery[30] further presented electron microscopic evidence that poly L-lysine causes aggregations and disruption of these acidic liposomes.

Both the A_1 protein of central nervous system myelin and the P1 protein of peripheral nervous system myelin reduce the poly L-lysine stimulated leak of central nervous system liposomes. The stabilizing effect of these proteins is dependent upon their concentration. The other basic protein tested, lysozyme, does not reduce the poly L-lysine induced leak. Although insufficient data are available to describe fully the mechanism of the competition, these experiments show increased sensitivity over simple leak studies in the investigation of the interaction of protein with liposomes. For example, since the P1 protein does not alter the glucose leak of central and peripheral nervous system lipid liposomes, it was believed that the P1 protein did not interact with the liposome The finding that the P1 protein stabilizes the liposomes against the poly L-lysine-stimulated leak, however, reveals that it does bind to the liposomes Another interesting point is that the order of addition of basic proteins and poly L-lysine has no effect on the final leakage rate

Kimelberg and Papahadjopoulos[13,14] have found that lysozyme and cytochrome *c* enhance Na^+ leak from phosphatidylserine liposomes These stimulating effects are greatly diminished when the salt concentration is increased to 0.15 M (the concentration in the present series of experiments), or when some of the acidic lipid is replaced by non-acidic lipids. Both central and peripheral nervous system lipid liposomes, having glucose leaks which are not stimulated by lysozyme and cytochrome *c*, contain high levels of non-acidic lipids.

In addition, lysozyme does not stimulate glucose leakage from phosphatidylserine or central nervous system acidic lipid liposomes A distinction between permeability with respect to ion leak and glucose leak was recently made by Calissamo and Bangham[31]. They found that brain-specific proteins, in the presence of Ca^{2+}, greatly increased Na^+ leak but did not alter glucose leak.

The possibility that the A_1 protein interacts with central nervous system lipids at specific sites in the polypeptide chain is now being explored with trypsin digestion. Such a study is appealing, for the amino acid sequence of this protein is known[32], and certain limited regions of the protein have been shown to provoke an autoimmune response[33,34]. Certain peptide linkages of the A_1 protein are protected from trypsin digestion when associated with lipid (London and Gould, unpublished)

If regions of the protein associated with the lipid are completely responsible for the glucose leak, digestion of the lipoprotein complex of A_1 protein–central nervous system lipids by trypsin should not reduce this leak. Addition of trypsin

(10 % w/w) to the liposome–A_1 protein complex resulted in some decrease in glucose leakage but the leakage remained high relative to liposomes alone. When the A_1 protein and trypsin were preincubated for a short period prior to addition of the liposomes there was no enhancement of leakage. These experiments indicate that regions of the protein presumably penetrate the liposomal structure and are, at least in part, responsible for the enhanced leak. The fact that the leak remains at a high level indicates that these regions of the A_1 protein may remain in the lipid matrix after digestion. This result is in agreement with the peptide mapping studies which reveal that portions of the protein are protected from tryptic digestion by association with liposomes

With complementary studies of X-ray diffraction, tryptic digestion, monolayer and bilayer systems, we hope to achieve a better understanding of the role of the A_1 protein in central nervous system myelin and the P1 and P2 proteins of the peripheral nervous system myelin structure.

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