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ACTIVATION OF GLUCOSE DIFFUSION FROM EGG LECITHIN LIQUID CRYSTALS BY SERUM ALBUMIN

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SUMMARY

The diffusion of [^{14}C]glucose from phospholipid model membrane systems has been measured in the presence and absence of bovine serum albumin under various conditions.

At acid pH, bovine serum albumin enhances the rate at which glucose diffuses from lecithin–cholesterol–dicetyl phosphate micelles. The enhancement effect has a pK of 4.3. This is close to the pH at which the well-known acid conformation change occurs in this protein. The activation of diffusion only takes place with negatively charged micelles, and is not disrupted by high salt concentrations. The effects of temperature and protein concentration on the diffusion rate were also studied.

It is suggested that the protein binds through electrostatic interactions initially, but that the subsequent formation of apolar bonds brings about the activation of glucose diffusion.

INTRODUCTION

An understanding of lipid–protein interactions in cellular membranes is a central problem in molecular biology. Although the subject of much speculation[1–4], this area is, as yet, only poorly understood. It seems clear that both lipids and protein are intimately involved in structure and function of biological membrane systems, and the problem becomes one of attempting to define how these components of the membrane relate to one another in such a fashion as to form a membrane matrix of specific functional capability.

In recent years, one of the more fruitful approaches in the field of membrane study has been the use of simple model systems. In 1964 BANGHAM and co-workers[5,6] reported experiments with a model membrane system which appeared to possess many of the properties of its natural counterpart. When shaken with water or salt solutions, dry lecithin forms a liquid–crystalline suspension, which on electron microscopic examination can be shown to consist of intact spherules with diameters of up to 2000 Å. Optical birefringence studies and other considerations suggest that these spherules consist of concentric shells of bimolecular leaflets. Other experiments[6,7] indicated that these artificial membranes possess certain physical and permeability characteristics of biological membranes. This system thus provides an opportunity

to study the process of diffusion of small molecules and ions across a relatively simple membrane system, whose structure and composition reflects that of cellular membranes.

Work in our laboratory has centered on the concept that alteration of a functional aspect of such a model system (*e.g.*, diffusion) by protein can serve as a measure of interaction of lipids in a membrane with that protein. A study of the structural aspects of such a system in different functional states should then be of considerable assistance in elucidation of the biomembrane problem.

The work reported here is an investigation of the interaction of bovine serum albumin with such a suspension of lecithin spherules. It was found that under certain conditions, small amounts of this protein greatly enhance the efflux of glucose trapped inside the spherules. This effect was studied as a function of pH, temperature, ionic strength, and membrane charge.

MATERIALS AND METHODS

All reagents were A.R. grade and were used without further purification. Distilled, deionized water was used. Egg lecithin was prepared by the method of SINGLETON *et al.*[8], bovine serum albumin (crystallized) was obtained from General Biochemicals, dicetyl phosphate and stearyl amine from K and K Laboratories and cholesterol from Sigma Co. D-[^{14}C]Glucose was obtained from Tracerlab. Paper chromatography done in our laboratory indicated that this material did not contain any significant radioactive impurities.

Radioactivity determinations were done with a Picker Ansitron liquid scintillation counter, using 10 ml of a counting solution consisting of 300 g naphthalene, 14 g 2,5-diphenyloxazole (PPO), 0.600 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 3000 ml of dioxane.

Preparation of the lecithin dispersions

The method used to disperse the lecithin was essentially that of BANGHAM, STANDISH AND WATKINS[6]. The swelling solution contained 1 μC/ml of isotopically labeled glucose made up to a concentration of 0.145 M. In addition, the solution was buffered with 1 mM tartaric acid–NaH_2PO_4 at a specified pH in 0.0725 M NaCl. The lipid components were dissolved in chloroform and thinly coated on the walls of a round-bottom flask by rotary evaporation. The glucose buffer solution was then added together with a few glass beads and the mixture was shaken vigorously with a mechanical shaker for 45 min. In the final suspension there were 15 μmoles of lecithin per ml, and the molar ratio of lecithin to the other constituents was 70:10:20 (lecithin: cholesterol:dicetyl phosphate or stearyl amine).

Measurement of glucose diffusion

The lipid spherules were separated from the external solution by passage through a column of Sephadex G-75 which was equilibrated with a solution containing buffer, but no glucose. Diffusion of glucose from the lipid spherules was measured with the use of a diffusion cell consisting of two compartments separated by a dialysis tubing membrane. The membrane was supported between the ground glass surfaces of two female 28/15 pyrex socket joints which were sealed off at the end.

One side of the cell held 10 ml of buffer solution, while the other contained 5 ml of the lipid suspension. The whole apparatus was placed on a mechanical shaker to assure efficient mixing, and samples were taken from the buffer solution at various intervals depending on the diffusion rate for a particular experiment.

A sample of the lipid suspension was always counted directly after passage through the Sephadex column to determine the amount of glucose trapped by the spherules.

RESULTS

In the following experiments, the lipid suspensions were all prepared in the same way, *i.e.*, by mechanical agitation for 45 min. It has been shown[9] that the size of phospholipid spherules and the rate of efflux of trapped molecules depend on the type of phospholipid involved, the method of dispersion and the environment in the solution. We have held the first two of these factors constant in order to examine the effect of changing environmental factors on the diffusion rate.

Separation of the spherules from untrapped glucose

After swelling and equilibration, the lipid suspension was layered onto a column of Sephadex G-75. The large lipid aggregates pass through this column rapidly and are easily separated from the unassociated glucose. Fig. 1 shows radioactivity and $A_{520\,m\mu}$ (light scattering by micelle particles) plotted as a function of the column effluent volume. The untrapped and trapped radioactivity are readily separated, and about 11% of the total radioactivity and all of the absorbance is associated with the lipid spherules. This value compares favorably with that of WEISSMANN, SESSA AND BERNHEIMER[10] and it is assumed to represent the amount of glucose mechanically trapped in the lipid spherules.

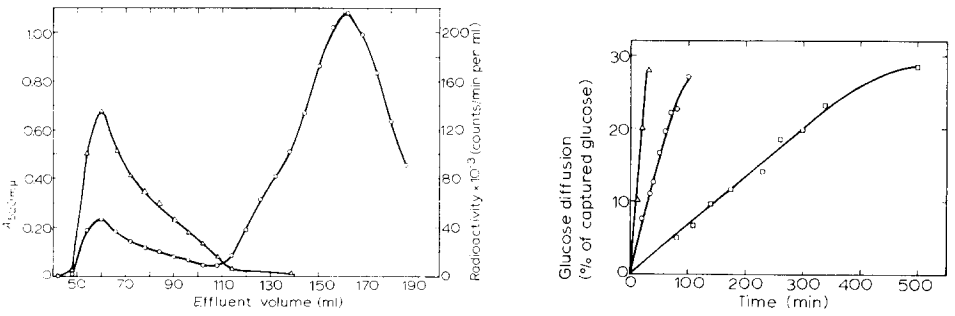

Fig. 1. Separation of phospholipid micelles and trapped [^{14}C]glucose from the untrapped solution on Sephadex G-75. Six ml of suspended phospholipid were placed on a column, 22 cm × 2.5 cm. △—△, $A_{520\,m\mu}$; ○—○, radioactivity as counts/min per ml of effluent.

Fig. 2. The diffusion of sequestered [^{14}C]glucose from the phospholipid micelles. Zero time corresponds to the addition of the micelles to one side of the cell. The amount of radioactivity appearing on the buffer side of the cell is expressed as the percent of total capture. Temp., 22°; pH, 3.4. △—△, diffusion across dialysis tubing only; ○—○, diffusion from micelles activated by bovine serum albumin; □—□, diffusion from micelles only.

Diffusion of glucose

The initial observation was that although bovine serum albumin had no effect on the movement of glucose at neutral pH, diffusion was greatly enhanced at lower pH values. Fig. 2 summarizes the data obtained from these experiments. The glucose appearing across the dialysis membrane is expressed as the percent of the amount captured, *i.e.*, the amount of radioactivity trapped inside the lipid spherules.

Unhindered diffusion (movement across the dialysis tubing only) was, as expected, considerably faster than either leakage of trapped glucose from the lipid spherules or protein-enhanced leakage. The introduction of bovine serum albumin at neutral pH had little or no effect on the diffusion rate. However, when the system was at approx. pH 3.5, more than a 4-fold increase in the diffusion rate was observed. Over the same pH range, the rate of glucose efflux from the lipid spherules in the absence of protein increased only about 25%.

Effect of pH on the diffusion rate

Since the enhancement effect of the protein on glucose diffusion was only observed in acidic solutions, a study of the effect of pH on diffusion activation was conducted. The results are shown in Fig. 3. Here the difference between the rate of glucose diffusion in the presence and absence of bovine serum albumin is plotted against pH. In the region between pH's 5.5 and 2.5, the activation of diffusion takes place in two phases. A gradual increase in the rate of glucose diffusion begins at pH 5.5 and reaches a plateau between pH's 3.5 and 3.0; the midpoint of this portion of the curve is at pH 4.3. It was of interest that this effect occurs in the same pH region as the "N–F" transition studied by AOKI AND FOSTER[11], which has been shown to

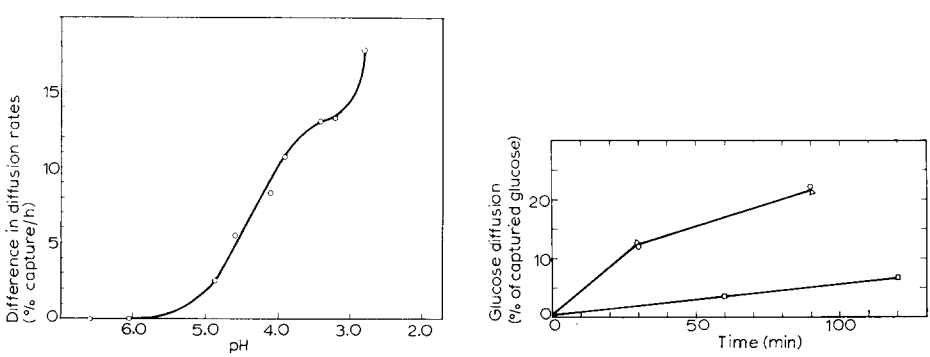

Fig. 3. Titration curve for the enhancement of glucose diffusion. The pH of the buffer was varied by the addition of NaOH or HCl. The magnitude of the enhancement effect is expressed as the difference between the diffusion rates (% per h of total capture) with and without protein present. (There was a slight increase in the diffusion rate of control micelles with pH. Using a difference scale eliminates this effect.) Temp., 22°; bovine serum albumin concn., $4 \cdot 10^{-4}$ mole protein per mole of lecithin.

Fig. 4. The effect of increased I on enhanced diffusion. Initially, the same experiment measuring the effect of bovine serum albumin in Fig. 2 was performed. △—△, at 30 min these samples were made 1 M in NaCl; ○—○, these samples were allowed to diffuse normally and made 1 M in NaCl after 90 min just prior to taking a sample. (This high concentration of salt lowered the efficiency with which the radioactivity was measured.) □—□, diffusion from micelles without protein. Temp., 25°; pH 3.4.

involve a conformation change in the protein. A second phase in the activation process is indicated by a point at pH 2.8 which shows a further increase in the diffusion rate in this area.

Effect of membrane charge and ionic strength

The isoelectric point of bovine serum albumin is about 4.8 (ref. 12); at pH values below this point the protein molecule will carry a net positive charge. Since the spherules which contain dicetyl phosphate will have a net negative charge under these conditions[6], it seemed possible that electrostatic forces are involved in the interaction.

To test this hypothesis, the charge on the membrane was changed from negative to positive by the substitution of an equimolar amount of stearyl amine for dicetyl phosphate[6]. In this system, there will be a repulsion between the positive membrane and the protein molecule, which should disrupt the interaction. At neutral pH, on the other hand, the protein is negatively charged and an attractive force would be expected. The spherules were also made uncharged by inclusion of only lecithin and cholesterol. As indicated in Table I, it was found that the membrane must be negatively charged for any enhancement to occur.

TABLE I

EFFECT OF MEMBRANE CHARGE ON BOVINE SERUM ALBUMIN-ENHANCED DIFFUSION

Temp., 22°; concn. of bovine serum albumin, $4 \cdot 10^{-4}$ mole protein per mole of lecithin. The membrane was given a positive charge by substitution of an equimolar amount of stearyl amine for dicetyl phosphate. The neutral membrane contained only lecithin and cholesterol.

Membrane charge	*Number of experiments**	*Diffusion rate (% capture per h) at*	
		pH 7	*pH 3.5*
Positive	2	3–4	3–4
Negative	5	3–4	18
Neutral	1	—	4

* Each experiment involves 5 replicates.

Since high salt concentrations will disrupt charge–charge interactions, the effect of ionic strength on the activation of glucose diffusion was studied. The protein was allowed to interact with the spherules at a low salt concentration for 30 min, at which time enough NaCl was added to the cell to make the final concentration 1 M. Diffusion was allowed to continue for another 90 min. As indicated in Fig. 4, the rate of diffusion at high ionic strength was the same as the rate for diffusion at low ionic strength.

If the salt concentration of the buffer was increased to one molar before the addition of bovine serum albumin, there was no activation of diffusion. This may be due to the fact that bovine serum albumin has a strong tendency so aggregate at low pH and high ionic strength[13].

Effect of temperature

The above experiments were all done at room temperature (22°). However, it was of interest to see how the rates of diffusion would vary with temperature. Samples

were either shaken in a constant temperature bath or in a cold room to obtain the data presented in Fig. 5. The initial rate was determined for each temperature and is expressed as the percent of the total capture per h. The logarithm of this rate is plotted against reciprocal temperature to obtain the activation energy from the Arrhenius equation. The activation energies for the albumin-enhanced diffusion and for diffusion in the absence of protein are -10 kcal·mole^{-1} and -11 kcal·mole^{-1}, respectively.

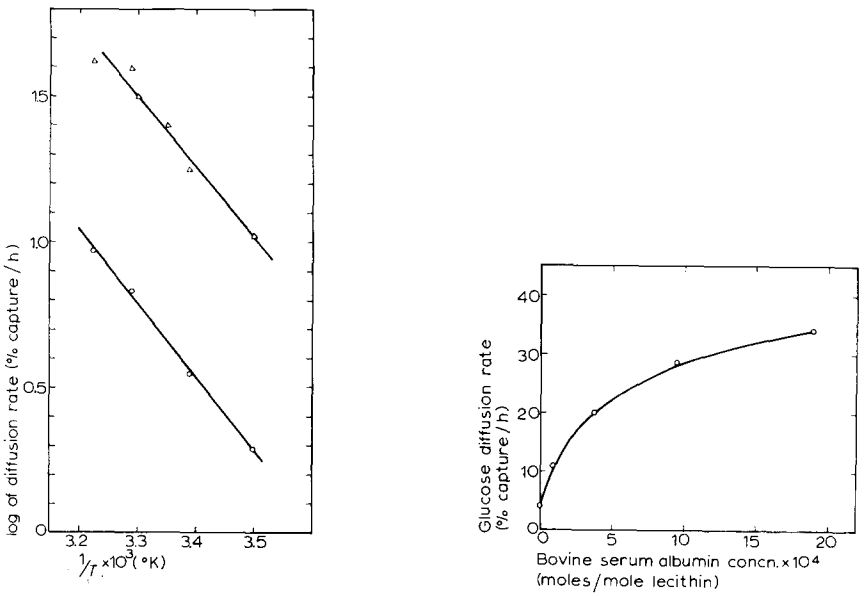

Fig. 5. Arrhenius plot for diffusion of glucose from phospholipid micelles. The log rate term is the $\log_{10}$ of % per h of total capture. O—O, diffusion rate without bovine serum albumin; △—△, diffusion rate in the presence of bovine serum albumin. pH, 3.4; protein concentration, $4 \cdot 10^{-4}$ mole protein per mole of lecithin.

Fig. 6. The effect of bovine serum albumin concentration on diffusion. The diffusion rate is expressed as % of total capture per h and the bovine serum albumin concentration as moles of protein per mole of lecithin. (The rate with no bovine serum albumin present is 4% per h.) Temp., 23°; pH, 3.4.

Removal of free fatty acid

Studies of crystalline bovine serum albumin[14] have indicated that the protein has several strong binding sites for free fatty acids. The fatty acid may be removed by treatment with activated charcoal in acid solution. Upon such treatment, we observed no detectable difference between the treated bovine serum albumin and the natural material. Both enhanced the diffusion of glucose in the same manner.

Effect of bovine serum albumin concentration

In all the previous experiments, the same concentration of bovine serum albumin was used, $4 \cdot 10^{-4}$ mole of protein per mole of lecithin. Fig. 6 shows the dependence of the diffusion rate on protein concentration. It can be seen that the phenomenon is saturable and a point can be reached where added protein does not increase the enhancement effect. In fact, the data fit the classical Michaelis–Menten expression for enzyme kinetics and a Lineweaver–Burke plot gives a very good

straight line. However, the v_{max} obtained in this manner approaches v_{max} for simple diffusion across the dialysis membrane. Therefore, interpretation of these data must await further study.

DISCUSSION

Diffusion of glucose

BANGHAM, STANDISH AND WATKINS[6] have shown previously thas it is possible to separate the lipid spherules containing sequestered solute molecules from the rest of the solution. Our data confirm these observations. To be able to measure the subsequent diffusion of captured molecules, the limiting step must be the actual movement across the lipid barrier. In our experiments as in those of BANGHAM, STANDISH AND WATKINS[6], the movement of solute across the dialysis tubing barrier was found to be many times more rapid than the movement across the lipid lamellae of the phospholipid spherules.

The rate of diffusion depends on the area of the membrane involved and the concentration gradient driving the process. Assuming that the area of the membranes does not change, the initial linearity of the curves in Fig. 2 indicates that the gradient is constant during the time course of the experiments reported here. In other words, the lamellar structure of the spherules and rapid dispersal of diffused molecules appears to provide for the maintenance of nearly constant gradient during the initial stages of diffusion.

Temperature studies

In experiments with similar model membranes, PAPAHADJOPOULOS AND WATKINS[9] calculated the activation energies for the diffusion of cations. Their values are somewhat greater than the 11 kcal·mole^{-1} we calculated for glucose diffusion (15 kcal·mole^{-1} for K^+), but this is perhaps not too surprising in view of the fact that we are dealing with diffusion of an uncharged molecule. In fact, it is of interest that the values for cation diffusion and those for glucose diffusion are so similar.

Another important aspect of the temperature study is the observation that enhancement by bovine serum albumin involves only a small change in the slope of the Arrhenius plot, and that the major change is in the intercept. Apparently the entropy of activation plays a large part in determining the rate increase because this factor is reflected in the intercept and not the slope of the Arrhenius plot. A more detailed interpretation is difficult at the moment because of the many factors that may be involved. However, this observation does suggest that the active conformation of bovine serum albumin is able to reduce the entropy requirements for the transition state in glucose passage through the membrane, while the enthalpy of activation remains nearly the same. This observation is presently under more intensive investigation.

The pH effect

Between pH's 5.5 and 2.0, several changes occur in the conformation and electrostatic properties of bovine serum albumin. The most obvious change occurs at the isoelectric point (pH 4.9–4.7) where the net charge on the protein molecule goes from negative at higher pH values to positive at pH values below the isoelectric point.

We have already mentioned the conformation change studied by electrophoresis experiments[11] which takes place between pH's 4.5 and 3.5 with a pK of 4.1. Measurements of intrinsic viscosity[15] have also indicated that the protein conformation changes at this point. These studies as well as optical rotation measurements[16,17] and increases in the radius of the protein[11] indicate that further alteration in the protein occurs between pH's 3.5 and 2.5. Through this pH range there seems to be a general expansion of the bovine serum albumin molecule which is distinct from the "N–F" conformation change.

It is obvious from Fig. 3 that the effect of bovine serum albumin on the lecithin spherules cannot be directly correlated with any of the effects mentioned above. Between pH's 5.5 and 2.5, bovine serum albumin goes through a complex series of transformations, and it would seem from the width, position and biphasic nature of the curve in Fig. 3 that many of these changes are involved in the enhancement of glucose diffusion.

Ionic strength and membrane charge effects

Initially, we considered the activation of diffusion at lower pH values (Fig. 4) to be due to electrostatic interactions between the increasingly positive protein molecules (pI, 4.8) and the negatively charged lipid spherules. However, if these interactions were the only requirement for activation of diffusion, bovine serum albumin would affect positively charged spherules at pH values above its isoelectric point. This was not the case; when stearyl amine was substituted for dicetyl phosphate in the lipid spherules, the addition of bovine serum albumin at neutral pH had no effect on the diffusion rate (Table I). It thus appears that other factors are involved in the activation process. This conclusion is reinforced by the observation that sharply increasing the ionic strength does not alter the rate of glucose diffusion. Simple electrostatic interactions should be disrupted in 1 M NaCl (ref. 18); however, the bovine serum albumin activation occurs even at this high salt concentration.

FOSTER[19] has reviewed the known properties of bovine serum albumin; and on the basis of titration behavior, the binding of ionic detergents and other factors, has proposed a model for the tertiary and quaternary structure of this molecule. The model consists of four subunits, all connected in the same protein backbone. These subunits are associated with one another through both hydrophobic and electrostatic interactions. Upon acidification of 3 crucial carboxyl groups per subunit, the subunits unfold, exposing hydrophobic areas of the protein. Subsequent experimental studies of digestion of the protein by pepsin by WEBER AND YOUNG[20] and of the binding of alkanes to bovine serum albumin by WISHINA AND PINDER[21] support FOSTER'S conclusions.

Our results appear to suggest that the "F" or unfolded conformation is involved in the activation of glucose diffusion in the model membrane system. Opposite charge on the protein and the lipid spherules appears to be necessary, but not sufficient to activate diffusion. Since the interaction is not disrupted by high salt concentration, and appears to involve protein with exposed hydrophobic sites, the existence of apolar interactions is implied.

Thus, our picture of this protein–lipid interaction involves the initial binding of the protein to the surface of the spherule due to electrostatic interactions. Activation of diffusion may then be caused through disruption of the lipid barrier brought about

by the penetration of apolar portions of the protein molecule into the hydrocarbon matrix of the membranes.

There are other conceivable explanations for the enhancement of glucose diffusion by bovine serum albumin. In our studies, it was assumed that the model membranes remain essentially intact during the activation process. However, if the lamellar membrane systems of some of the spherules were grossly disrupted, it would lead to the release of trapped glucose into the surrounding solution. As a result, the apparent leakage rate would be greatly enhanced. Such an effect is seen with certain non-ionic detergents and globular proteins[10]. However, under circumstances where the radioactive marker is free in solution, one would expect the activation energy for the diffusion process to be the same as that for free diffusion across the dialysis membrane (3–4 kcal). In fact, in our system the activation energy is about the same for both normal and enhanced diffusion (10–11 kcal). This indicates that the barrier to glucose diffusion is not destroyed in the activation process. Electron microscopic studies now under way may also help resolve this question.

Another alternative explanation might be the selective solubilization of a component of the lipid lamellar system; bovine serum albumin is known to bind long-chain organic ions similar to dicetyl phosphate[19]. We have found, however, that on high-speed centrifugation in acid solution, bovine serum albumin sediments with the lipid*. One would expect bovine serum albumin to remain in the supernatant if the effect were due to removal of the organic anion by this protein.

Obviously, more work needs to be done to fully substantiate our hypothesis, but in any case, it seems clear that both polar and apolar forces are important in this lipid–protein interaction. This appears to be the first example of a system where both types of interactions are involved in a lipoprotein system, although GREEN[18] has demonstrated both for the association of different proteins with lipids in mitochrondria.

In conclusion, it appears that further study of the bovine serum albumin interaction with model phospholipid membranes may be of assistance in understanding protein–lipid interactions in cell membranes.

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