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Adsorption of Glyceraldehyde 3-Phosphate Dehydrogenase on Condensed Monolayers of Phospholipid

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1. The adsorption of [^{14}C]alkylated glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle to condensed monolayers of phosphatidic acid was investigated under a variety of conditions. 2. The rate constant for association at 20°C depended on ionic strength. At $I/2 = 60$mM the rate constant was $0.39\,min^{-1}$. At $I/2 = 260$mM it decreased to $0.27\,min^{-1}$. 3. The apparent association constant ($K_{ass.}$) for adsorption at $I/2 = 60$mM was $1.06\times10^{6}\,M^{-1}$ and was strongly influenced by subphase changes in pH and ionic strength. Measurements of $K_{ass.}$ at 20° and 5°C gave a value for the apparent enthalpy change on adsorption of $-33\,kJ\cdot mol^{-1}$. Calculations of the apparent change in free energy and apparent entropy change for the adsorption process gave values of $-34\,kJ\cdot mol^{-1}$ and $+2\,J\cdot K^{-1}\cdot mol^{-1}$ respectively. 4. Decreasing the amount of phosphatidic acid in the monolayer by replacement with phosphatidylcholine caused the shape of the adsorption isotherm to change from apparent hyperbolic to sigmoid. Subphase changes in pH or ionic strength did not affect the shape of the adsorption isotherm. However, adsorption of enzyme on monolayers of 100% phosphatidic acid in the presence of 1mM-$CaCl_2$ was sigmoid in nature. 5. It is concluded that glyceraldehyde 3-phosphate dehydrogenase binds to condensed charged monolayers by multiple electrostatic interactions. At low concentrations of phosphatidic acid in the monolayer or in the presence of Ca^{2+}, this occurs in a two-step process and depends on lateral diffusion of phosphatidic acid for strong binding to take place.

A monolayer of phospholipid provides a useful model system for characterizing lipid–protein interactions. For protein-adsorption studies it has the special advantages of a defined surface area, pressure and potential. With this system it is possible to follow the adsorption of labelled protein from the bulk aqueous phase on to the phospholipid surface, and to obtain information on the lipid–protein interactions involved. Quinn & Dawson (1969*a*, 1970) have used cytochrome *c* and serum albumin to demonstrate that at low film pressures these proteins can penetrate into a phospholipid monolayer, but that at higher film pressures adsorption without penetration occurs. The nature of the interactions involved in protein adsorption at high film pressures is, as yet, unknown. Initial attraction appears to be diffusion-limited (Quinn & Dawson, 1970) and influenced by electrostatic interactions between protein and phospholipid. Once bound, however, the adsorbed protein does not appear to dissociate easily from the lipid–protein complex. Quinn & Dawson (1970) found that adsorbed cytochrome *c* could not be removed by transferring the film containing adsorbed protein to a fresh subphase containing no protein. Apolar interactions, which might be thought to contribute to strong binding, do not appear to be involved. Apolar interactions between apolar amino acid side chains with fatty acyl residues on the phospholipid would be expected to be accompanied by changes in surface pressure and also by some degree of unfolding of the protein molecules. Neither of these effects was apparent with cytochrome *c* binding to condensed monolayers (Quinn & Dawson, 1969*b*), nor were they apparent when haemoglobin was adsorbed on the hydrophilic side of an arachidate film (Fromherz *et al.*, 1972) or when ribonuclease was adsorbed on a condensed phosphatidylcholine monolayer (Khaiat & Miller, 1969). Gonzalez & MacRitchie (1970) have postulated a multiplicity of binding sites on the protein and suggested that the large resistance to desorption for adsorbed proteins is related to a co-operative step in which all segments of the molecule must desorb simultaneously for the compete molecule to desorb. This results in a large energy barrier to desorption and an apparent irreversibility to the interaction. In an attempt to clarify further the details of interaction between a water-soluble protein and a condensed charged matrix we have investigated the changes in various parameters associated with the adsorption of glyceraldehyde 3-phosphate dehydrogenase on condensed monolayers of phosphatidic acid. Several workers (Mitchell *et al.*, 1965; Green *et al.*, 1965; Nilsson & Ronquist, 1969; Tanner & Gray, 1971; Duchon & Collier, 1971) have reported very firm

retention of this glycolytic enzyme in the isolated membrane 'ghosts' of mammalian erythrocytes haemolysed under various conditions of pH and ionic strength. Arnold & Pette (1968) have also reported that glyceraldehyde 3-phosphate dehydrogenase is bound to actin in rat and rabbit muscle homogenates but can be solubilized by 0.1 M-EDTA. These workers have suggested that the binding of enzymes to subcellular structures could be important in the organization of the glycolytic system and that specific influences of metabolites on the binding phenomenon could play a role in functional control. We describe here the characteristics of adsorption of glyceraldehyde 3-phosphate dehydrogenase on condensed phospholipid monolayers and report several thermodynamic values for the adsorption process. The presence of a co-operative step in desorption has been tested by following protein interaction with mixed monolayers of phosphatidic acid and phosphatidylcholine.

Materials and Methods

The subphase for the monolayer binding studies was 0.01 M-Pipes [piperazine-*N*,*N*'-bis-(2-ethanesulphonic acid)] buffer, pH 6.0, 1 mM-cysteine, 1 mM-EDTA and KCl to the concentrations indicated in the Figure legends. For studies at pH 7.6, 0.01 M-Tris/HCl buffer was used. Phospholipids from egg yolk (Lipid Products, Nutfield Nurseries, Nutfield, Surrey, U.K.) were tested for purity by t.l.c.

Preparation of labelled protein

Rabbit skeletal-muscle glyceraldehyde 3-phosphate dehydrogenase (Boehringer Co., London W5 2TZ, U.K.) was labelled with iodo[2-^{14}C]acetate of specific radioactivity 22.6 or 51 mCi/mmol (The Radiochemical Centre, Amersham, Bucks., U.K.) as described by MacQuarrie & Bernhard (1971) to the degree of one thiol group per enzyme molecule. Labelled protein was separated on Sephadex G-25 and specific radioactivity was determined by liquid-scintillation counting and absorbance at 276 nm. Specific enzyme activity was found to be diminished by approx. 25% after labelling.

Monolayer techniques

Details of the monolayer apparatus and surface-pressure measurements were as described by Bromfield *et al.* (1974). Monolayers were formed by successive additions of 0.1 μl of a solution of the lipids in chloroform/methanol (9:1, v/v) to the surface of the slowly stirred subphase solution at 20°C, 15 s being allowed for evaporation of solvent. Surface pressures of 35 ± 1 mN·m^{-1} were used for all experiments. No change in surface pressure was detected on addition of protein to the subphase. Surface radioactivity was monitored by a solid scintillator probe (*p*-terphenyl in polyvinyltoluene with an end-window thickness of approx. 10^{-2} kg·m^{-2}) connected to a scalar-amplifier (Nuclear Enterprises model SR5). The output, integrated over 40 s, was fed into a pen recorder. At equilibrium, radioactivity counts were measured over intervals of 1–2 h. A solid ^{14}C source of labelled polymethacrylate was used to calibrate the counter. Enzyme binding is generally expressed in terms of mol of adsorbed protein per mol of phosphatidic acid in the monolayer (*R*). In this way, slight differences in the amount of phospholipid spread on the monolayer in different experiments can be taken into account.

Results

Adsorption of enzyme on 100% phosphatidic acid monolayers

The rate of adsorption of labelled enzyme on a compressed monolayer of phosphatidic acid under various conditions is shown in Fig. 1(*a*). After the

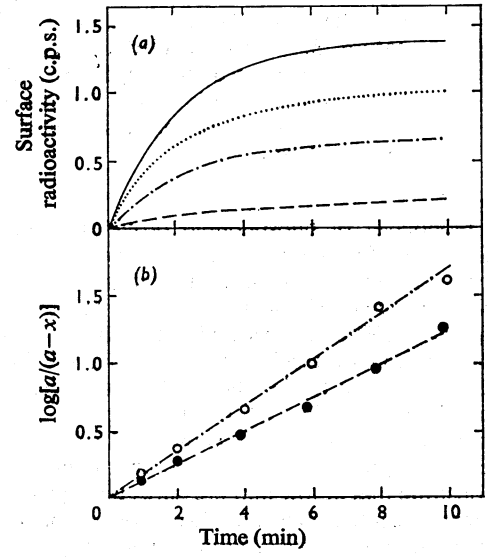

Fig. 1. *Changes in surface radioactivity with time on adding ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase below a monolayer of phosphatidic acid at 35 mN·m^{-1}*

The subphase contained 10 mM-Pipes or 10 mM-Tris buffer at the required pH, 1 mM-cysteine, 1 mM-EDTA and KCl at the required concentration. Pipes buffer at pH 7.6 gave the same amount of protein adsorption as Tris buffer at pH 7.6. In (*b*) the data for two of the curves have been plotted in a logarithmic form, where *a* is the initial concentration of free enzyme and *x* the amount adsorbed at any time. ——, 0.058 μM-protein, 50 mM-KCl, 10 mM-Pipes buffer, pH 6.0; ····, 0.025 μM-protein, 50 mM-KCl, 10 mM-Tris buffer, pH 7.6; ·–·–·, 0.025 μM-protein, 50 mM-KCl, 10 mM-Pipes buffer, pH 6.0; – – – –, 0.025 μM-protein, 250 mM-KCl, 10 mM-Pipes buffer, pH 6.0.

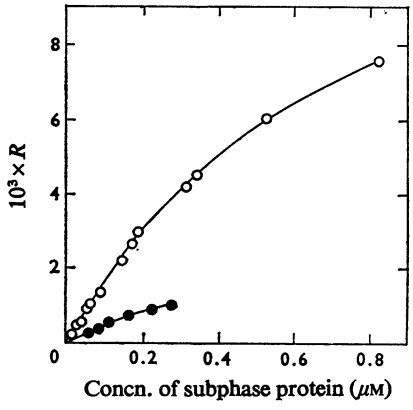

Fig. 2. *Surface radioactivity of ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added at different concentrations below a monolayer of phosphatidic acid at 35 mN·m^{-1}*

Surface radioactivity (R) is expressed as the molar ratio of adsorbed protein to phospholipid in the monolayer at equilibrium. The subphase contained 10mM-Pipes buffer, pH6.0, 1mM-cysteine, 1mM-EDTA and (○) 50mM-KCl or (●) 250mM-KCl.

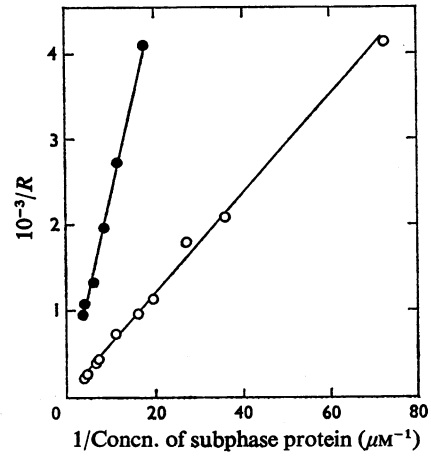

Fig. 3. *Double-reciprocal plot of changes in surface radioactivity of ^{14}C-labelled glyceraldehyde phosphate dehydrogenase added at different concentrations below a monolayer of phosphatidic acid at 35 mN·m^{-1}*

Conditions were as given in Fig. 2. ○, 50mM-KCl; ●, 250mM-KCl.

initial rise in the amount of protein interacting with the monolayer the surface concentration of protein remained constant up to several hours. A plot of $\log[a/(a-x)]$ against time, where a is the initial concentration of free enzyme and x the amount adsorbed at any time, gives a straight line (Fig. 1*b*), indicating simple first-order kinetics with respect to enzyme concentration for these particular protein concentrations. The rate constant for association of 0.39min^{-1} at $I/2 = 0.06$M decreases to 0.27min^{-1} at $I/2 = 0.26$M.

Measurement of the equilibrium plateau of Fig. 1(*a*) was made for different amounts of enzyme in the subphase and shows (Fig. 2) that enzyme adsorption increases with increasing concentration of free enzyme. Raising the ionic strength of the subphase by addition of KCl decreases the extent of binding. An apparent association constant ($K_{ass.}$) of $(1.06\pm 0.03)\times 10^6$M^{-1} at $I/2 = 0.06$M is obtained from a double-reciprocal plot of the data (Fig. 3). At the higher ionic strength of $I/2 = 0.26$M, the binding of protein is significantly diminished. The number of mol of protein bound per mol of phospholipid at saturation can be estimated from the double-reciprocal plot, but a more accurate method is to plot the data by the method of Scatchard (1949). Extrapolation to conditions of saturation binding in Fig. 4 gives this value as $(17\pm 1)\times 10^{-3}$. The area per phospholipid molecule in the monolayer was kept constant at 0.56nm^2 for these experiments, and therefore the area occupied by each enzyme molecule

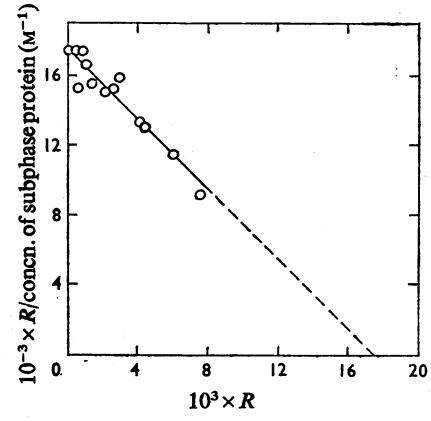

Fig. 4. *Scatchard plot for data in Fig. 2 with 50mM-KCl in the subphase*

at saturation can be calculated as 32.8 ± 1.3nm^2. Assuming a spherical conformation for the protein, this gives a radius of 3.2 ± 0.1nm for the bound enzyme, which is close to the value of 3.3nm calculated from the partial specific volume of the enzyme in free solution.

From the results, the concentrations of enzyme used in these association experiments appear to be well below theoretical saturation binding values. The linearity of the Scatchard plot over the range used also indicates that there is little effect on binding by any

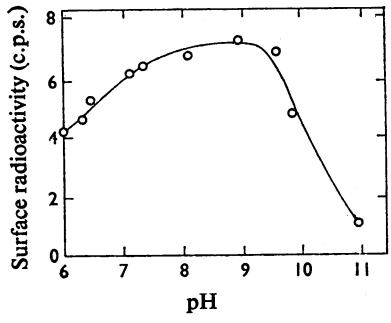

Fig. 5. *Effect of pH on the surface radioactivity (expressed in c.p.s.) of ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added below a monolayer of phosphatidic acid at 35 mN·m^{-1} with subphase maintained at different pH values*

The subphase contained 10mM-Pipes buffer and 10mM-Tris buffer (adjusted to the required pH by HCl or KOH), 1mM-cysteine, 1mM-EDTA and 50mM-KCl.

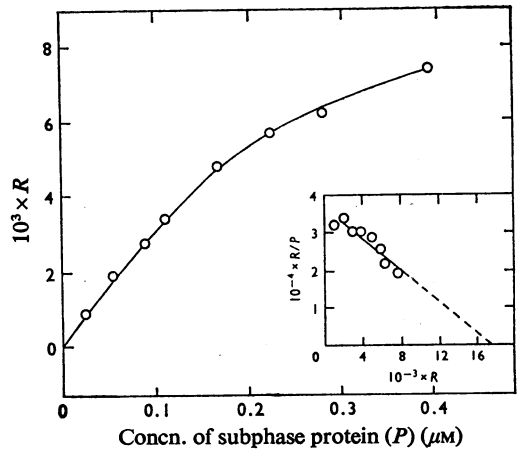

Fig. 6. *Surface radioactivity of ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added at different concentrations below a monolayer of phosphatidic acid at 35 mN·m^{-1}*

The subphase contained 50mM-KCl and 10mM-Tris buffer, pH7.6. Other conditions were as given in Fig. 2. Inset: Scatchard plot of the data.

electrostatic interaction between binding sites (Scatchard, 1949).

Effect of temperature on adsorption

A fall in temperature from 20°C to 5°C resulted in $K_{ass.}$ increasing from 1.06×10^6M^{-1} to $(2.1\pm0.5)\times10^6$M^{-1}. From this rise in $K_{ass.}$ it is possible to obtain an estimate of the enthalpy change ($\Delta H'$) on binding (assuming that $\Delta H'$ is independent of temperature) of -33kJ·mol^{-1}. The apparent change in free energy ($\Delta G'$) on binding, calculated from the values of $K_{ass.}$, is -34kJ·mol^{-1} and is independent of temperature over the range used. (For calculation of the thermodynamic parameters the activity of water is taken as unity.) From these values for $\Delta H'$ and $\Delta G'$, an estimate of the entropy change ($\Delta S'$) for the adsorption process can be made from the relation $\Delta G' = \Delta H' - T\Delta S'$. $\Delta S'$ turns out to be positive, with a value of 2 J·K^{-1}·mol^{-1}.

Changing the temperature also altered the value for the number of mol of protein bound per mol of phospholipid under conditions of saturation binding. This changed from 17×10^{-3} at 20°C to $(11\pm2)\times10^{-3}$ at 5°C, giving an apparent radius for the bound enzyme of 4.1nm at the lower temperature.

Effect of pH of adsorption

Adsorption of protein on the condensed monolayer of 100% phosphatidic acid increases as the pH of the bulk phase is increased from pH6 to pH9 (Fig. 5). Above pH9.5, the amount adsorbed decreases rapidly with increasing pH. Between pH6 and pH7.6, the value of $K_{ass.}$ changes from 1.06×10^6M^{-1} to $(2.15\pm0.15)\times10^6$M^{-1} ($I/2 = 0.06$M) without any significant change in the number of mol of protein bound per mol of phospholipid at saturation (Fig. 6).

Adsorption of enzyme on mixed monolayers of phosphatidic acid and phosphatidylcholine

The effect of decreasing the surface-charge density of the monolayer on the adsorption process was followed by using monolayers in which the amount of phosphatidic acid was diminished by replacement with phosphatidylcholine. The surface pressure was kept constant at 35mN·m^{-1} as before. The area occupied by each adsorbed protein molecule under conditions of saturation binding, calculated from double-reciprocal plots, is plotted against the phospholipid composition of the monolayer in Fig. 7. The area occupied by each protein molecule under conditions of saturation binding is relatively constant until concentrations of phosphatidic acid reach approx. 30% or less. The number of binding sites for protein on the monolayer then falls as the phosphatidic acid concentration is further lowered. No binding of protein to a 100% phosphatidylcholine monolayer could be detected.

At low concentrations of phosphatidic acid in the monolayer the adsorption curve of the protein has a sigmoid shape (Fig. 8), with little adsorption taking place at low protein concentrations. The shape of the curve is not significantly affected by changes in ionic strength, although the amount of protein adsorbed is

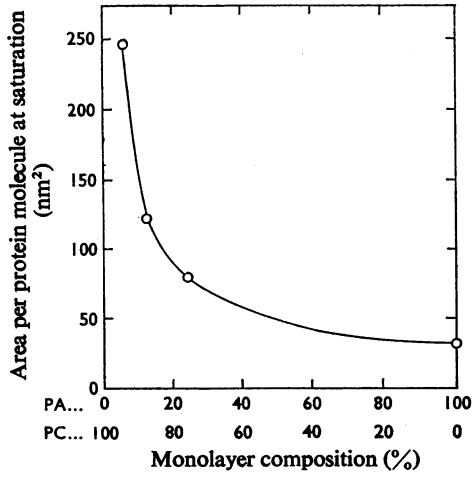

Fig. 7. *Changes in area per adsorbed protein molecule at saturation for ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added below mixed monolayers of phosphatidic acid (PA) and phosphatidylcholine (PC) at 35 $mN \cdot m^{-1}$*

The subphase contained 10mM-Pipes buffer, pH6.0, 1mM-cysteine, 1mM-EDTA and 50mM-KCl.

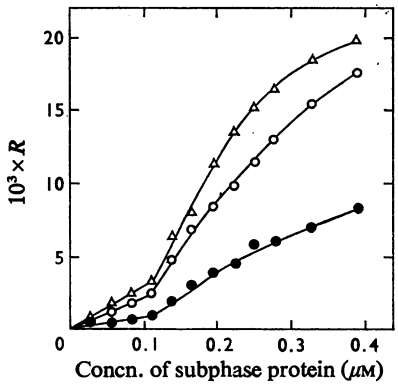

Fig. 8. *Effect of ionic strength on the surface radioactivity of ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added at different protein concentrations below a mixed monolayer of 12.5% phosphatidic acid and 87.5% phosphatidylcholine at 35 $mN \cdot m^{-1}$*

The subphase contained: △, 10mM-KCl; ○, 50mM-KCl; ●, 250mM-KCl. Other conditions were as given in Fig. 2.

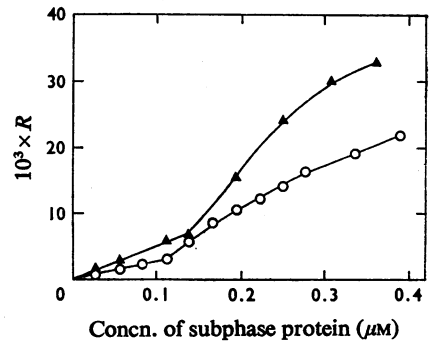

Fig. 9. *Effect of pH on surface radioactivity of ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added at different protein concentrations below a mixed monolayer of 12.5% phosphatidic acid and 87.5% phosphatidylcholine at 35 $mN \cdot m^{-1}$*

The subphase contained 50mM-KCl and ▲, 10mM-Tris buffer, pH7.6, or ○, 10mM-Pipes buffer, pH6.0. Other conditions were as given in Fig. 2.

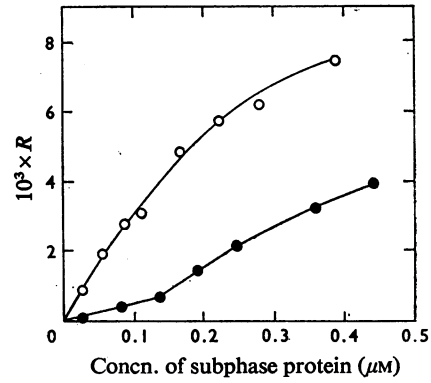

Fig. 10. *Effect of 1 mM-Ca^{2+} on surface radioactivity of ^{14}C-labelled glyceraldehyde 3-phosphate dehydrogenase added at different concentrations below a monolayer of phosphatidic acid at 35 $mN \cdot m^{-1}$*

The subphase contained: ○, 10mM-Tris buffer, pH7.6; ●, 10mM-Tris buffer, pH7.6, plus 1mM-$CaCl_2$. Other conditions were as given in Fig. 2.

decreased. Changing the pH of the subphase to pH7.6 increases the extent of protein binding (Fig. 9) without removing the sigmoid nature of the adsorption curve. As the percentage of phosphatidic acid in the monolayer increases the adsorption curve shifts from sigmoid to apparent hyperbolic. However, a sigmoid adsorption curve can be seen with monolayers of 100% phosphatidic acid in the presence of 1mM-$CaCl_2$ (Fig. 10), although in this case the molar ratio of adsorbed protein to phospholipid is greatly decreased.

A Hill plot (not shown) of the adsorption results for mixed monolayers gave values of the Hill coefficient greater than unity. For 12.5% phosphatidic

acid in the monolayer the Hill coefficient was 2.1 ($I/2 = 0.06$M). The values of the Hill coefficient for adsorption to 100% phosphatidic acid monolayers were unity except when Ca^{2+} ions were present.

Discussion

The present results confirm previous observations (Quinn & Dawson, 1969*a*) that the rate of adsorption of soluble protein on a condensed monolayer of charged phospholipid is diffusion-limited and depends on favourable electrostatic interactions between protein and phospholipid. The rate constant for adsorption at 20°C, pH6 and $I/2 = 0.06$M is approx. $0.39\,min^{-1}$, which gives a half-time for the adsorption process of 1.8min. The value for the apparent association constant under the same conditions is $1.06 \times 10^6\,M^{-1}$. This means that the desorption step must be very slow. Under the conditions used, this turns out to be 1.3×10^3 days, which is consistent with the apparent irreversibility of the adsorption process reported for other water-soluble protein (Quinn & Dawson, 1970). It is also possible to calculate the apparent association constant for the competing K^+ ions as $12\,M^{-1}$, which is higher than that obtained by Abramson *et al.* (1966) with phospholipid dispersions but too low to have any significant effect, by direct competition, on electrostatic interactions between lipid and protein.

The affinity of glyceraldehyde 3-phosphate dehydrogenase for the monolayer over the range pH6–9 appears to be more influenced by the surface charge on the monolayer than by changes in net charge on the enzyme. Over this range there would be little change in the net charge on the enzyme (Velick & Furfine, 1963), and its increased affinity for the monolayer may be attributed to an increased negative surface charge density resulting from secondary ionization of the phosphate group on phosphatidic acid. The maximum rate of change of affinity between pH6 and pH9 occurs at about pH7. This could correspond to the second pK_a of ionization of the phosphatidic acid, and is a value similar to that reported for phosphatidic acid in monolayers in the presence of Ca^{2+} ions (Papahadjopoulos, 1968). The close agreement between the values calculated for the area per protein molecule at saturation binding at pH6 and pH7.6 shows that no gross denaturation of the protein occurs in this range. Above pH9 the ε-amino groups on the protein would start to titrate and the net negative charge on the protein would increase. This would decrease its affinity for the negatively charged monolayer and binding would be inhibited, as observed.

The low rate of the desorption process is calculated from the apparent association constant and the rate of the adsorption reaction. This calculation assumes that an equilibrium state has been achieved when the final amount of adsorbed protein is measured. There was no indication that this was not so. The amount of protein interacting with the monolayer clearly relates to the concentration of protein in the subphase and, after the initial rise, remains constant. The calculated area occupied by each protein molecule under saturation binding conditions corresponds to a radius for the bound enzyme similar to that in free solution, and suggests, in line with other reports (Quinn & Dawson, 1969*b*; Fromherz *et al.*, 1972; Khaiat & Miller, 1969), that protein denaturation is not a major factor in binding when condensed monolayers are used. The low rate of desorption does suggest, however, that the final complex formed between protein and monolayer is very stable. Some indication of the nature of the interactions involved can be gained from the present measurements of the apparent association constant at different temperatures. The value of this parameter approximately doubles as the temperature is lowered from 20° to 5°C, corresponding to an apparent enthalpy change for the reaction of $-30\,kJ \cdot mol^{-1}$. The apparent change in entropy is $+2\,J \cdot K^{-1} \cdot mol^{-1}$. The results contrast with the change in apparent association constant with temperature reported for the binding of a solubilized membrane adenosine triphosphatase to phospholipid bilayers (Redwood & Patel, 1974). For this enzyme, the apparent association constant did not significantly change with temperature. The apparent enthalpy change for the reaction was therefore small, whereas the corresponding entropy contribution was $+125\,J \cdot K^{-1} \cdot mol^{-1}$. Redwood & Patel (1974) concluded that the binding of this membrane protein predominantly involved apolar interactions between the protein and the hydrocarbon interior of the bilayer. In the present case the change in apparent association constant with temperature and the relatively low change in entropy suggest that apolar interactions are not involved. This is also suggested by the lack of any surface-pressure change on binding. Surface-pressure changes would be expected to occur if the protein penetrated into a condensed monolayer to interact with the hydrocarbon side chains on the phospholipid (Phillips *et al.*, 1975). Any positive entropy changes that do occur could easily arise from displacement and reorganization of bound water at the interface when protein is adsorbed. The change in apparent radius of the adsorbed protein molecule with temperature could similarly arise from an increased hydration shell at the lower temperature. It is possible to estimate the number of phosphatidic acid molecules per molecule of protein for 'optimum' binding from the data in Fig. 7. This turns out to be about 25, and is not an unreasonable value for electrostatic interaction between single oppositely charged groups on the monolayer and protein, given that the number of free positively charged groups on the protein would be about 130 at this pH (Velick & Furfine, 1963).

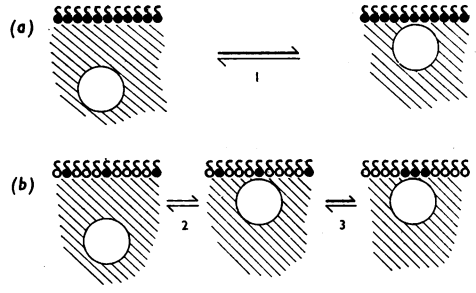

Fig. 11. *Kinetics of multi-point binding of glyceraldehyde 3-phosphate dehydrogenase to condensed monolayers of (a) phosphatidic acid and (b) phosphatidic acid plus phosphatidyl-choline*

Strong binding by multi-point attachment in (*a*) occurs as soon as protein is adsorbed on the monolayer (step 1) and is relatively irreversible. In the mixed monolayer of phosphatidic acid and phosphatidylcholine (*b*) an initial 'nucleation' complex forms (step 2), which can readily dissociate. Strong binding occurs when lateral diffusion of phosphatidic acid in the monolayer causes multipoint attachment (step 3) and leads to the formation of a relatively stable complex.

When the amount of phosphatidic acid on the monolayer is diminished by substitution with phosphatidylcholine, adsorption is decreased and the shape of the adsorption curve changes from apparent hyperbolic to sigmoid. These results are consistent with multiple binding between protein and monolayer involving electrostatic interactions. At high phosphatidic acid concentrations sufficient favourable interactions between each segment of the protein and the corresponding reacting sites on the monolayer can be satisfied simultaneously for strong binding to occur (Fig. 11). At low phosphatidic acid concentrations, however, an initial 'nucleation' complex would be formed by interaction of a single segment of the protein with one, or a few, phosphatidic acid molecules, followed by lateral diffusion of the phosphatidic acid on the monolayer leading to binding of the remaining segments on the protein. The strength of binding in the initial complex will be much less than in the final 'multi-bound' complex, and the initial complex can dissociate relatively easily. Desorption of protein from the final complex, however, is slow, since all bound segments of the protein must desorb simultaneously for the complete molecule to desorb (Burgen *et al.*, 1975). For strong multiple binding to occur, the lifetime of the initial complex must be long enough to allow sufficient lateral diffusion of phosphatidic acid necessary for further interaction. At low phosphatidic acid concentrations and low concentrations of protein in the subphase, the time average for interaction of protein with the monolayer will also be low. Increasing either the concentration of protein in the subphase or the concentration of phosphatidic acid in the monolayer will increase the average interaction time between the two and allow multiple binding to occur. These effects will result in a sigmoid-shaped adsorption curve. The diffusion constant for lateral motion of phospholipid molecules in phospholipid bilayers is of the order of $10^{-12}m^2 \cdot s^{-1}$ to $10^{-11}m^2 \cdot s^{-1}$ at 37°C (Scandella *et al.*, 1972) and compares with a value of approx. $5 \times 10^{-11}m^2 \cdot s^{-1}$ for glyceraldehyde 3-phosphate dehydrogenase in free solution at 20°C (Velick & Furfine, 1963). It is therefore not unlikely that lateral diffusion of phosphatidic acid in the monolayer would be rate-limiting to the kinetics of multiple binding, especially at low phosphatidic acid concentrations on the monolayer where relatively large distances would be involved.

Changes in ionic strength of the subphase or an alteration in the net charge on the phosphatidic acid molecule would not be expected to alter significantly the lateral movement of phosphatidic acid in the monolayer and would not therefore be expected to alter the shape of the adsorption curve. On the other hand, it is known that Ca^{2+} ions can, by a process of aggregation, considerably decrease the lateral motion of negatively charged phospholipids on a monolayer (Ohnishi & Ito, 1974). The present results indicate that this can occur to a sufficient extent on 100% phosphatidic acid monolayers to inhibit multi-point binding to protein at low protein concentrations, resulting in a sigmoid-shaped adsorption curve. A similar effect by Ca^{2+} ions or any 'diffusion-inhibiting' molecules could be important in the binding mechanism of many other water-soluble proteins to biological membranes.

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