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INTERACTION OF ELECTRICALLY CHARGED LIPID MONOLAYERS WITH MALATE DEHYDROGENASE*

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SUMMARY

Malate dehydrogenase was adsorbed onto monomolecular lipid films, using a multicompartment trough. The quantity of adsorbed protein and its enzymatic activity were studied with monolayers of various electrical charge densities and subphases of various electrolyte compositions. A closely packed layer of enzyme molecules was adsorbed onto negatively charged films, whereas considerably less protein was adsorbed onto neutral and positively charged monolayers. Electrolytes reduce the quantity of adsorbed protein. The adsorption was found to be irreversible even at high ionic strength. When adsorbed to uncharged lipid films the enzyme is nearly inactive, whereas negatively charged lipid headgroups enhance the specific activity of the enzyme.

INTRODUCTION

Condensed lipid monolayers at the air-water interface exhibit a plane of tightly packed lipid headgroups facing the liquid phase. The adsorption of water-soluble proteins onto these headgroups has been investigated in some detail in the case of hemoglobin [2, 3] and cytochrome *c* [4–6]. The physical properties of the adsorbed protein molecules have been characterized by penetration experiments [3] and infrared spectroscopy [7]. The enzymatic properties of the adsorbed proteins have been investigated by preliminary experiments only in the cases of catalase, trypsin and malate dehydrogenase [2, 3]****.

In this study, the interaction of malate dehydrogenase with various lipid monolayers is investigated in some detail. The headgroup plane of the lipid films was systematically modified by adding electrically charged lipids to a neutral lipid film. The

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**** For studies, however, on the interaction of glutamate dehydrogenase and malate dehydrogenase with phospholipid micelles see refs 8 and 9.

quantity of adsorbed enzyme and its enzymatic activity were used as indicators for the interaction of the lipids, the enzyme and the electrolytes in the subphase. In order to avoid any ambiguities on the dissociation and conformation of lipid headgroups, long-chain sulfates and quaternary ammonium ions were used as charged lipids, whereas the neutral matrix was provided by methylstearate. In addition, monolayers of lecithin and arachidic acid were studied.

MATERIALS AND METHODS

The lipid monolayers were spread at the air-water interface from 5 mM solutions of methylstearate (Merck), eicosylsodiumsulfate (Schuchardt), eicosyltrimethylammoniumbromide (Schuchardt), arachidic acid (Merck), and L-α-dipalmitoyllecithin (a gift from Dr H. J. Eibl of this institute) in chloroform. The lipids were recrystallized twice and the chloroform was freshly distilled and stabilized with 1 % ethanol. In order to dissolve the sulfate, 20 % dimethylsulfoxide was added to the solvent. The aqueous subphase contained varying concentrations of NaCl and $CaCl_2$ (Merck). Mitochondrial pig heart malate dehydrogenase (in 3.2 M $(NH_4)_2SO_4$, Boehringer) was dialyzed against 100 mM sodium phosphate buffer, pH 7.4. Water was distilled twice, once from alkaline permanganate [10].

Using a multicompartment trough [2,3], lipid monolayers were spread on a protein-free compartment (volume 50 ml) and compressed to 30 dynes/cm. These films (area 110 cm^2) were shifted to another compartment containing a homogeneous solution of malate dehydrogenase (concentration e.g. 25 μg/ml), dialysed and diluted immediately before the experiments. After a certain adsorption time, the films were separated from the protein solution by enclosing them between two movable barriers and shifting them to a protein-free compartment. During the shifting procedure at least two other protein-free compartments were passed in order to wash out unadsorbed protein present in the boundary layer. Details of the technique are found elsewhere [2, 3].

The quantity of the adsorbed protein was determined by the method of Lowry et al. [11] using serum albumin as a standard. For this purpose a double layer of the lipid-protein film (13 cm^2) was transferred onto a hydrophobic glass slide and placed into the test tubes, in which the analysis was performed. The protein is resolubilized under Lowry-test conditions. Five samples from each lipid-protein film prepared were tested in this manner.

The enzymatic activity of adsorbed malate dehydrogenase was determined by measuring the decreasing NADH absorbance at 340 nm in a Gilford 2400-S spectrophotometer. For this purpose a slide was covered with a lipid-protein double layer and dropped immediately after preparation (before the double layer had dried) into a cuvette containing the test solution (100 mM sodium phosphate buffer, pH 7.4, 12 mM NADH, 12 mM oxalacetate, in a total volume of 3.25 ml, at 25 °C [12]). During this procedure the lipid-protein double layer is not detached from the slide and the protein is not resolubilized. The activity was calculated from the absorbance change within the first 20 s. Two samples were tested from each film prepared.

RESULTS

The quantity of malate dehydrogenase which is adsorbed onto a lipid monolayer depends on the protein concentration in the subphase. This is shown in Fig. 1 for the case

of an arachidic acid film. The adsorption is found to be irreversible, i.e. no protein is desorbed on a subphase of lowered protein concentration or even a protein-free subphase within 1h. The maximum quantity of malate dehydrogenase adsorbed to an arachidic acid and to a methylstearate/eicosylsulfate film (Figs 1, 2) is the same. The adsorption rate, however, is considerably higher in the latter case.

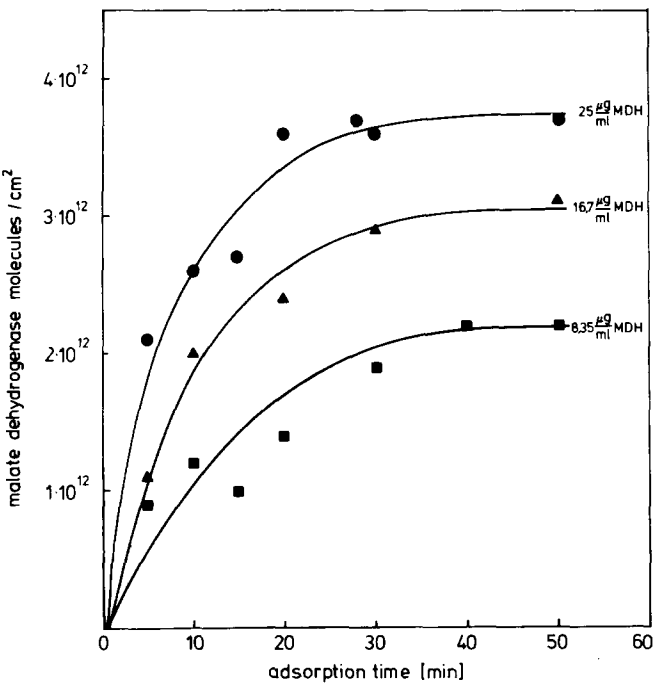

Fig. 1. Quantity of malate dehydrogenase adsorbed to a monolayer of arachidic acid versus adsorption time. Adsorption conditions: pH 6.5, no electrolyte, protein concentration as indicated in the figure. The standard deviations (approx. $0.3 \cdot 10^{12}$) are omitted for clarity. (In this figure, as well as in Figs 2, 3a, 4a and 5a, the quantity of adsorbed malate dehydrogenase is given in molecules/cm^2. This was calculated from the weight of the adsorbed protein and a molecular weight of 70 000 [12]. $1 \cdot 10^{12}$ malate dehydrogenase molecules/cm^2 correspond to 0.116 μg protein/cm^2.)

The quantity of malate dehydrogenase adsorbed to a methylstearate/eicosylsulfate film decreases with increasing concentration of NaCl present in the subphase during adsorption (Fig. 2). However, protein adsorbed at low ionic strength does not desorb when brought into contact with a protein-free subphase of high ionic strength (e.g. 1 M NaCl solution). Similar results were found using $CaCl_2$ instead of NaCl.

The quantity of adsorbed malate dehydrogenase increases with an increasing number of negatively charged sulfate groups in the lipid layer (Fig. 3a) : addition of 10 % sulfate to methylstearate doubles the quantity of adsorbed protein. When arachidic acid is added to methylstearate or lecithin films the adsorption is enhanced significantly only at percentages greater than 50 % (Fig. 4a).

The specific activity of adsorbed malate dehydrogenase increases with increasing molar fraction of eicosylsulfate in methylstearate/eicosylsulfate films* (Fig. 3b): addition of only 1 % sulfate causes a strong enhancement of activity as compared to the pure methylstearate film. Arachidic acid, when added to methylstearate, enhances the activity as well. However, its activation effect differs from that of eicosylsulfate. The activation effect of the carboxylgroups also looks different in the neutral film of lecithin compared to the methylstearate film (Figs 3b, 4b). In all these experiments the enzyme was adsorbed without electrolyte in the subphase, since the activity of the adsorbed malate dehydrogenase was found to be lowered if salts (NaCl, $CaCl_2$) were present in the subphase during adsorption.

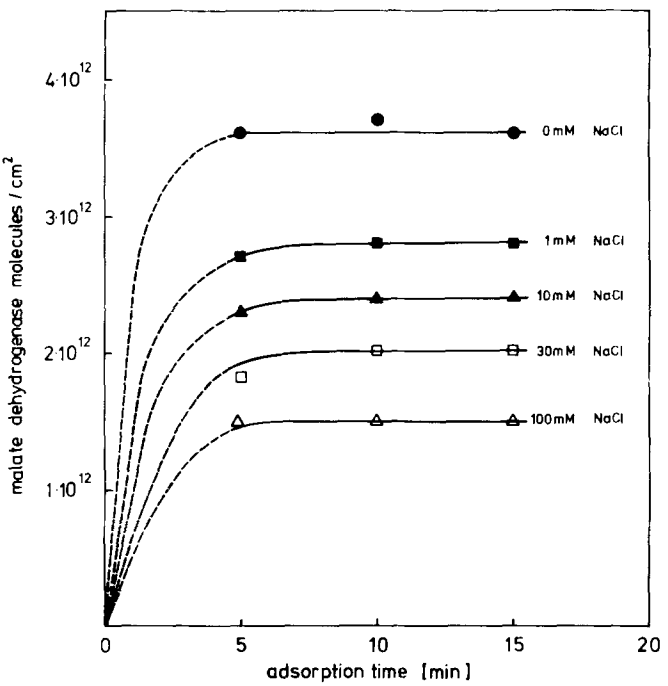

Fig. 2. Quantity of malate dehydrogenase adsorbed to a monolayer of methylstearate/eicosylsulfate (molar ratio 9 : 1) versus adsorption time. Adsorption conditions: pH 6.5, NaCl concentration as indicated in the figure, protein concentration 25 μg/ml. The standard deviations (approx. $0.3 \cdot 10^{12}$) are omitted for clarity.

Addition of eicosyltrimethylammonium ions to a methylstearate film does not have any influence either on the quantity of adsorbed malate dehydrogenase or on its activity (Figs 5a, b). However, in a methylstearate film containing eicosylsulfate and

* These results were obtained with films that were kept under high surface pressure (30–40 dynes/cm). If the surface pressure on such lipid-protein films was lowered allowing the enzyme to penetrate into the lipid monolayer, then, after recompression to the original surface pressure, the specific activity was found to be reduced proportionally to the degree of film expansion.

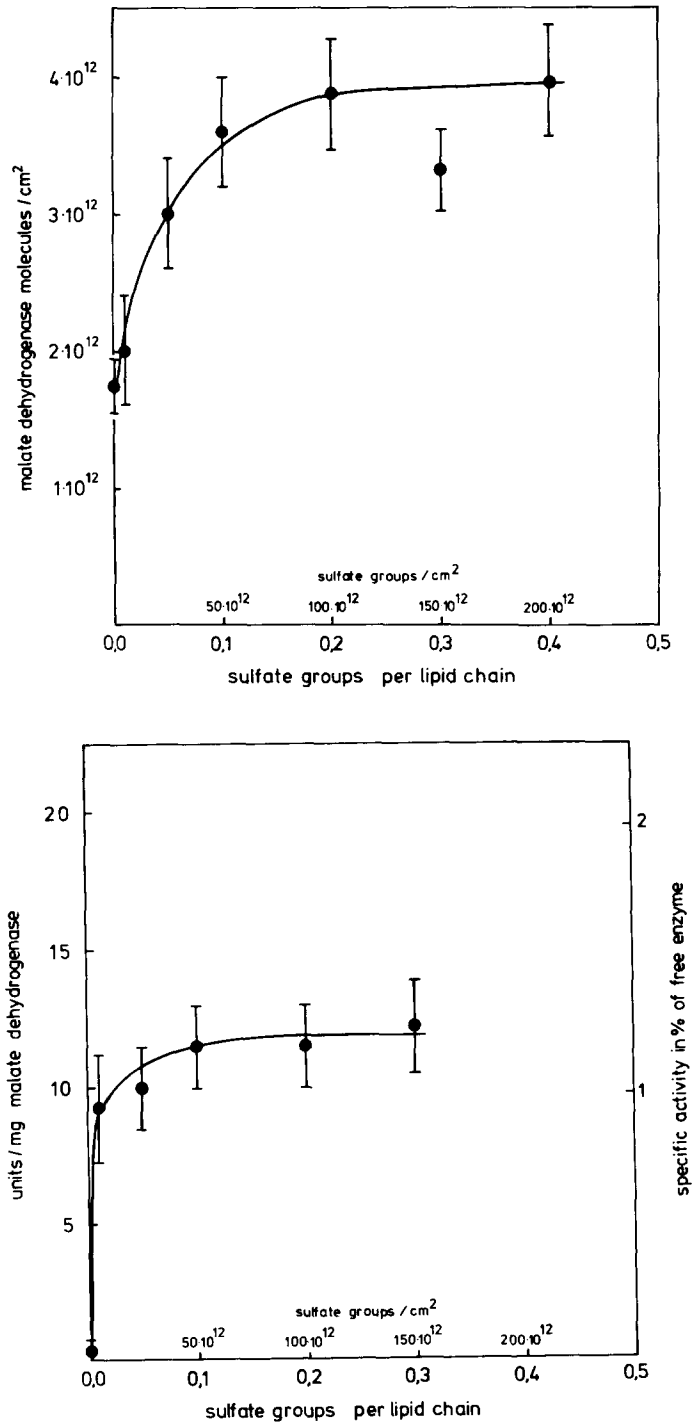

Fig. 3. Quantity (a) and specific activity (b) of malate dehydrogenase adsorbed onto monolayers of methylstearate/eicosylsulfate versus number of sulfate groups per lipid chain (i.e. molar fraction of eicosylsulfate). Adsorption conditions: pH 6.5, no electrolyte, protein concentration 25 μg/ml, adsorption time 15 min.

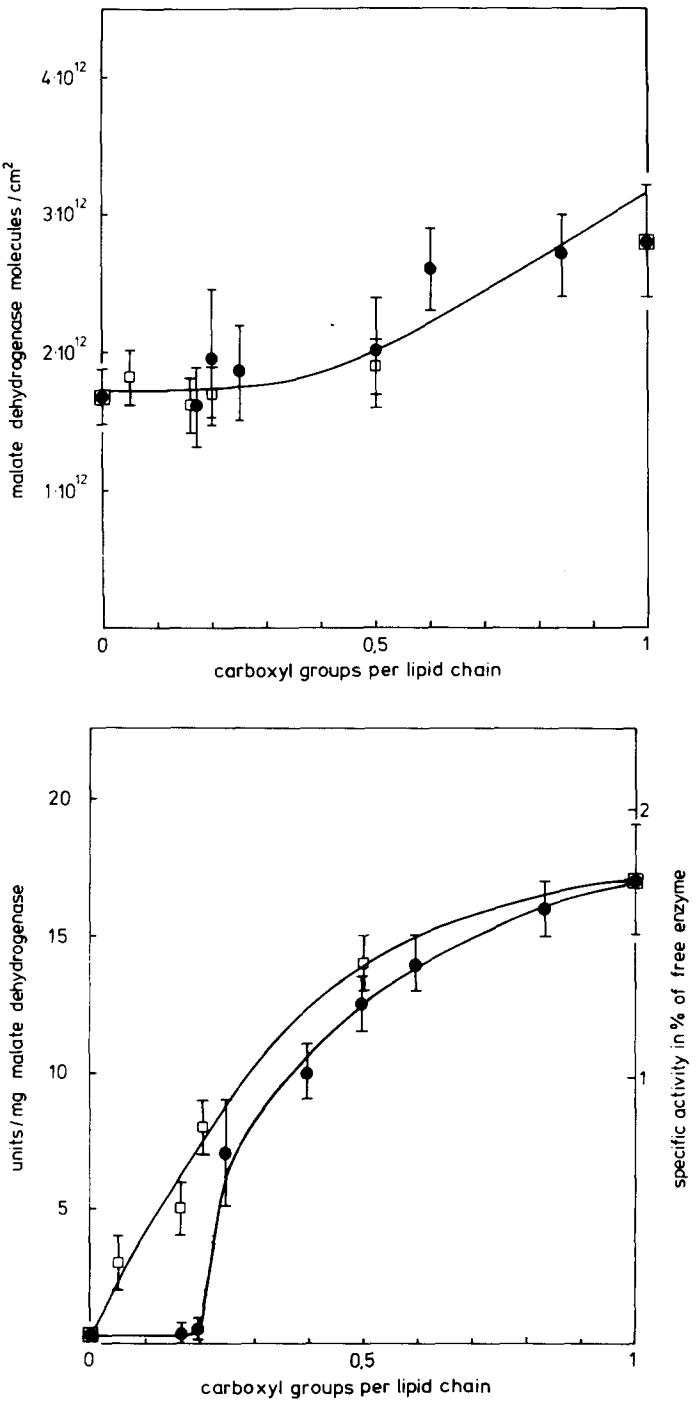

Fig. 4. Quantity (a) and specific activity (b) of malate dehydrogenase adsorbed onto monolayers of methylstearate/arachidic acid (●) and of L-α-dipalmitoyllecithin/arachidic acid (□) versus number of carboxylgroups per lipid chain (i.e. molar fraction x_A of arachidic acid for methylstearate film and $x_A/(2-x_A)$ for the lecithin film). Adsorption conditions: pH 6.5, no electrolyte, protein concentration 25 μg/ml, adsorption time 15 min.

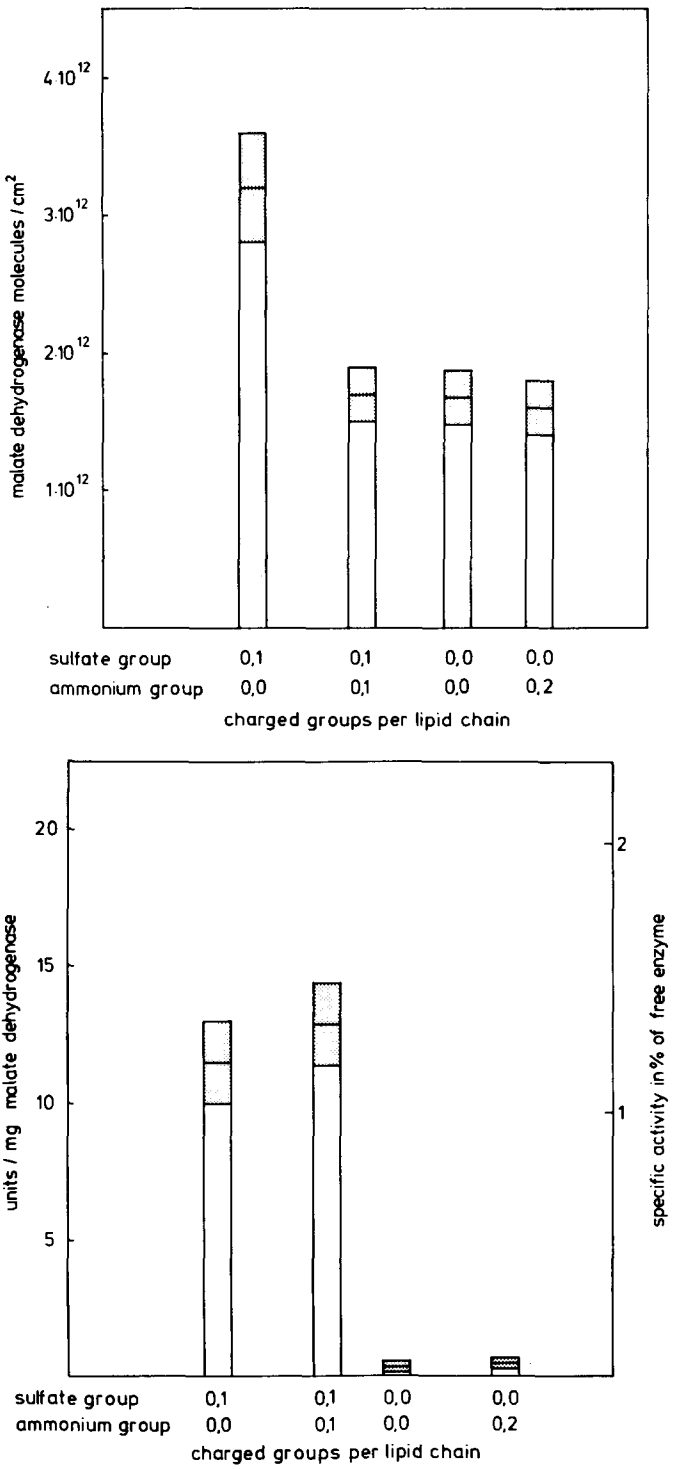

Fig. 5. Quantity (a) and specific activity (b) of malate dehydrogenase adsorbed onto monolayers of methylstearate mixed with eicosyltrimethylammonium and/or eicosylsulfate ions. The number of charged groups per lipid chain corresponds to their molar fraction in the monolayer. Adsorption conditions: pH 6.5, no electrolyte, protein concentration 25 μg/ml, adsorption time 15 min.

eicosyltrimethylammonium ions, the positively charged groups are able to compensate the effect of the negatively charged groups on the quantity of adsorbed malate dehydrogenase (Fig. 5a). The enhancement of activity caused by the sulfate, however, is not affected by the addition of ammonium groups (Fig. 5b).

DISCUSSION

The maximum quantity of malate dehydrogenase that is adsorbed to the monolayers of arachidic acid and methylstearate/eicosylsulfate corresponds to a closely packed layer of protein molecules, if the mean diameter of the malate dehydrogenase (mol. wt 70 000 [12]) is assumed to be 60 Å. The adsorbed quantity is lowered by decreasing the protein concentration* and increasing the ionic strength in the subsolution, whereas desorption does not occur under these conditions. A similar metastable behaviour has been observed with adsorbed hemoglobin [2]. An explanation of this phenomenon cannot be given as yet.

Since the pH of the subphase (6.0–6.5) was chosen to be near the isoelectric point of malate dehydrogenase (6.1–6.4, ref. 13), the protein molecules in the bulk aqueous phase are without net charge. In the neighbourhood of the charged lipid film, however, the protein molecules become electrically charged, the sign being opposite to that of the monolayer. This is the result of the interfacial pH shift in the diffuse electrical double layer (e.g. Δ pH = 3–4 with 10^{-3} M NaCl [14]). Thus, an increase of the charge density of the lipid film and a decrease of the ionic strength should enhance the attraction between protein and the lipid fiilm, because of the increasing pH shift. Indeed, the quantity of adsorbed malate dehydrogenase was found to depend on the the density of sulfate groups in the film (Fig. 3a) and on the NaCl concentration in the subphase (Fig. 2); this relationship closely resembles the changes in the interfacial pH shifts (i.e. interfacial potentials) near the same type of lipid films [14]).

This observation and the fact that the quaternary ammonim ions are able to compensate the enhancing effect of the sulfate groups on adsorption indicate that the negative net charge of the monolayers is responsible for the enhancement. The more gradual increase of the adsorbed quantity of malate dehydrogenase upon adding arachidic acid to a methylstearate film (Fig. 4a) may be attributed to the incomplete dissociation of the carboxyl groups [15], resulting in a considerably lower charge density than indicated by the molar fraction of the acid.

Once adsorbed to the lipid film, the enzyme does not desorb when exposed to a high ionic strength. This, together with the fact that the positively charged film does not affect the adsorption, suggests that the interaction of malate dehydrogenase with charged and uncharged lipid films may not be explained satisfactorily in terms of an electrostatic interaction of charged colloids as in the case of cytochrome *c* [6]. Electrostatic interaction in that sense seems to be important for the adsorption step, but it does not explain all the features of adsorption and not the impeded desorption. Other types of lipid-protein and protein-protein interaction must be involved.

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* Due to the absorption of the enzyme to the lipid film and to the walls of the trough, its final concentration is somewhat reduced (by 10–20 %). Still, there is always a great surplus of malate dehydrogenase present in the subphase. The adsorption time (15 min) is chosen to be appreciably longer than the time necessary for the diffusion of the protein molecules to the interface (1–5 min).

The enzymatic activity of malate dehydrogenase adsorbed to the hydrophobic headgroup plane of methylstearate is negligible (Figs 4b, 5b). This corresponds to the pronounced alterations of protein structure found with hemoglobin adsorbed to the same film [2, 7].

The presence of approximately two sulfate groups per adsorbed malate dehydrogenase molecule causes an activation of the enzyme which cannot be enhanced by further addition of sulfate groups (Fig. 3b). Together with the fact that the positively charged quaternary ammonium groups are not able to compensate this activation (Fig. 5b), the observation suggests that enzymatic activity is not directly related to the average negative charge density of the lipid film, but influenced by the interaction of single sulfate groups with the enzyme molecules. A similar activating effect of carboxyl groups is different in a surrounding of methylstearate and lecithin molecules. This indicates, as well, that more specific interactions of the enzyme and the polar headgroups considered are involved than merely the influence of the net charge of the film.

The relationship found between adsorbed quantity of protein, its activity and the lipid film composition may not be explained by the assumption that the lipids in the monolayers are immiscible. This can be proved by calculating the hypothetical quantity of adsorbed malate dehydrogenase and its activity for immiscible films with some immiscibility gaps conceivable, using the results for the films of appropriate composition of the coexisting phases.

As compared to the specific activity in bulk solution [12] the activity of the enzyme adsorbed to the lipid films amounts to only a few percent. Thus a large deactivation is connected to the location of the enzyme molecule within the lipid-protein double layer. This can be due to a change of the intrinsic catalytic properties of the enzyme molecules or to restraints of transport processes. In order to distinguish between these possibilities the kinetics of an enzyme at an interface have to be analyzed in more detail (cf. ref. 16).

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