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IMMOBILIZED ELECTRIC EEL ACETYLCHOLINESTERASE

I. KINETICS OF ACETYLCHOLINESTERASE TRAPPED IN POLYACRYLAMIDE MEMBRANES

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Summary

Techniques are described for the trapping of electric eel acetylcholinesterase in polyacrylamide gel. The activity of the trapped enzyme was substantially reduced, the effect being due to inhibition by acrylamide, but the enzyme immobilized in polyacrylamide was considerable more stable than that in free solution. A kinetic study was made of the hydrolysis of acetylthiocholine, covering a range of membrane thicknesses, enzyme concentrations, substrate concentrations and temperatures. The results were interpreted with reference to the theoretical treatment of Sundaram, Tweedale and Laidler, and of Kobayashi and Laidler, and provided support for those treatments. Clear evidence was obtained for diffusion control with the thicker membranes. An activation energy was obtained for the diffusion of the substrate within the membrane, by combining the temperature results for thick and thin membranes, at low substrate concentrations. The results lead to the conclusion that the in vivo kinetics of acetylcholinesterase are largely diffusion-free in muscle filaments, but are substantially diffusion-controlled in fibrils and fibers.

Introduction

Apart from enzymes in the digestive system, most enzymes in vivo occur not free but associated with highly organized cellular material. Even the so-called "soluble" glycolytic enzymes are in fact immobilized, either physically or chemically, in a cytoplasmic matrix. This has been demonstrated by Kempner and Miller [1] who used centrifugal stratification to show that all of the intracellular enzymes of the viable alga *Euglena gracilis* (about 30 μm in length) are associated with particulate cell fractions, no enzymic activity or macromolecules being found in the soluble cytoplasmic fraction. In view of this

widespread association in vivo of enzymes with cellular material, it is important to pursue investigations of the kinetics of action of enzymes when they are attached to solid supports. The present investigation is concerned with acetylcholinesterase, which has been immobilized by trapping it in polyacrylamide, the resulting gel being sliced into membranes of varying thicknesses.

The kinetic behavior of an immobilized enzyme may differ from that of the free enzyme for several reasons: (a) Immobilized enzyme may be conformationally different from the enzyme in free solution. (b) The interaction between immobilized enzyme and substrate takes place in a different environment from that existing in free solution. (c) There will be partitioning of the substrate between the matrix on which the enzyme is immobilized and the free solution. (d) The reaction catalyzed by immobilized enzyme may be to some extent diffusion controlled.

The theoretical treatments of Sundaram et al. [2] and of Goldman et al. [3] predict that at low substrate concentrations ($[S] \ll K_m$) the kinetics for an enzyme immobilized in a membrane differ substantially from those when the enzyme is in free solution. The theory also predicts that at low substrate concentrations the rate of reaction is directly proportional to the square root of the enzyme concentration rather than to its first power. The experimental work of Bunting and Laidler [4] using β-galactosidase immobilized in polyacrylamide gel has confirmed the correctness of this theory. Kobayashi and Laidler [5] have outlined methods which allow the intrinsic parameters, k_c' and K_m', relating to the behavior of immobilized enzyme, to be derived from the experimental results. Recently Kobayashi and Laidler [6] have further elaborated on the kinetics of membrane-bound enzyme under different kinetic conditions.

The experimental work presented here was done with membrance of polyacrylamide in which acetylcholinesterase was uniformly distributed. These membranes were immersed in solutions of acetylthiocholine. Kinetic studies were carried out over a range of enzyme concentrations, substrate concentrations and membrane thicknesses. The results are in excellent agreement with the theory. Reaction rates were also measured at low substrate concentration over a range of temperature, with thin and thick membranes. A method will be outlined for obtaining the activation energy for the diffusion of the substrate within the membrane, the procedure being to combine the results of temperature studies with thin and thick membranes. The present investigation not only serves to test and confirm the theories, but also serves as a model for enzyme systems in vivo. Electric eel acetylcholinesterase is membrane-bound in situ [7—9].

Materials

The enzyme, acetylcholinesterase from electric eel (Type III), was obtained from Sigma Chemical Co. The substrate acetylthiocholine and the chromogenic agent 5,5′-dithiobisnitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. *N,N,N′,N′*-tetramethyl-ethylene diamine (TEMED), *N,N′*-methylene bisacrylamide (BIS) and acrylamide were obtained from Eastman Organic Chemicals. The ammonium persulfate used in the polymerizations, and

dipotassium hydrogen phosphate, were obtained from Fisher Scientific Co. Phthalic acid and boric acid were obtained from British Drug Houses, and sodium chloride from Canadian Laboratory Supplies Ltd.

Method

All solutions used were made up in Silman and Karlin buffer [8], which consists of 0.15 M NaCl, 2 mM phthalate, 2 mM phosphate and 2 mM borate, pH 7.0.

Immobilization of acetylcholinesterase in polyacrylamide

The procedure follows essentially that described by Bunting and Laidler [4]. The polyacrylamide gels were made by polymerization of acrylamide solution [10]; BIS was used as cross-linking agent, ammonium persulfate as catalyst, and TEMED as catalyst and cross-linking agent. Since other work [11] had shown that the use of 5% BIS produces the smallest pore size in the gel, this percentage was used in the present experiments. In all experiments 15% acrylamide solution was used, and the following indicates typical concentrations used to make the mixture: acrylamide (+5% BIS), 1.5 ml of 60%; TEMED, 0.5 ml of 2%; persulfate, 0.1 ml of 2.8%; and enzyme and buffer 3.9 ml; the total is 6.0 ml.

The polymerizations were carried out in glass tubes 1.0 cm in diameter. The tubes were first dipped into Fotoflow solution (Eastman—Kodak) diluted 1 : 20 with distilled water; this facilitated subsequent removal of the gel from the tube. Irradiation was done using a fluorescent lamp about 1 m away from the tube at room temperature ($\approx 27^\circ$ C); complete gelation took about 30 min.

Membrane slices were cut from the gels by use of a Model 880 American Optical Co. microtome [12]. The gel was first frozen by CO_2, cooled by allowing it to escape from a cylinder through a nozzle. The slice thickness was varied by manual adjustment of the blade-to-sample distance. The membrane slices were stirred in buffer for a few minutes to remove adsorbed enzyme; this procedure was repeated between runs to remove substrate and product.

The membrane thicknesses were measured using a Shimadzu light microscope with a calibrated × 10 magnification eyepiece and × 4 and × 10 magnification objectives. The slices were mounted on their sides between cover slips and observed immediately after removal from the solution.

Kinetic procedure

The details of the flow technique used to obtain the kinetic parameters of the immobilized enzymes have been described by Ngo and Laidler [13]. By the use of Watson—Marlow MHRE 200 flow inducer, 10 ml of reaction mixture (substrate and immobilized enzyme) in a thermostatted vessel was caused to flow through Tygon tubing into a microvolume flow cell (1 cm pathlength), and back into the vessel. The absorbance at 412 nm was monitored spectrophotometrically on an SP1800 Pye Unicam recording spectrophotometer.

Results

Entrapment of acetylcholinesterase in polyacrylamide under the conditions described by Bunting and Laidler [4], resulted in a loss of more than 90% of the original enzyme activity. A series of experiments were therefore carried out to determine the effects, on the activity, of the chemical reagents used to bring about polymerization. The results of such studies are shown in Fig. 1. The enzyme retains its full catalytic activity in the presence of 0.05% (w/v) ammonium persulfate, the free radical initiator. Bunting [14] found that ammonium persulfate decreases the activity of β-galactosidase by approximately 35%. Fifteen percent (w/v) acrylamide, with or without TEMED, causes about 80% decrease from the original acetylcholinesterase activity (Fig. 1). It is clear that the major factor that causes the decrease in the enzymatic activity is acrylamide. The effect of acrylamide on the enzyme activity was further investigated. The enzymic activities were measured at six different acrylamide concentrations from 5% to 30% (w/v); rapid decreases in enzymic activities were observed, only 5% of the original activity remaining at 30% acrylamide. The enzyme lost its activity rapidly, when incubated at 25° C in 15% (w/v) acrylamide. It lost approximately 70% of its original activity during 30 min incubation in 15% (w/v) acrylamide solution. Because of this loss of activity with time, and the relatively heat-stable nature of the enzyme, it was decided to carry out the polymerization at room temperature rather than at 4° C and with 1 m between the fluorescent lamp and the polymerizing gel, in order to achieve complete polymerization of the acrylamide monomers in a shorter time. A further investigation was carried out in an attempt to gain some insight into the inhibitory nature of acrylamide. The rates of acetylcholinesterase-catalyzed reactions, in acrylamide solutions of different concentrations, were measured

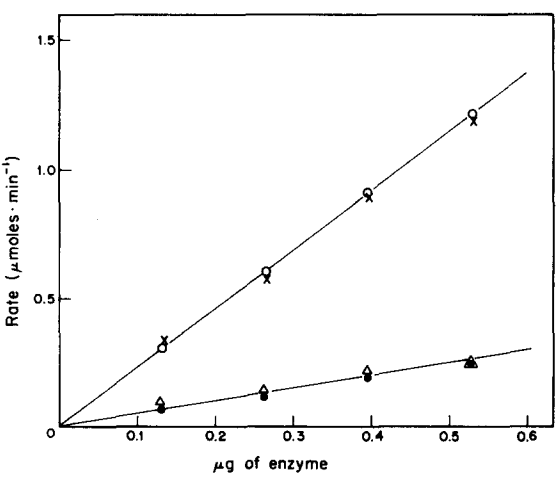

Fig. 1. The effects of ammonium persulfate, acrylamide and TEMED on the activities of acetylcholinesterase at various enzyme concentrations. T = 25° C, [acetylcholine] = 0.5 mM, [DTNB] = 0.5 mM , pH 7.0. ○, no addition; ×, 0.05% (w/v) ammonium persulfate added; △, 15% (w/v) acrylamide added; ●, 15% (w/v) acrylamide + 0.15% (v/v) TEMED added.

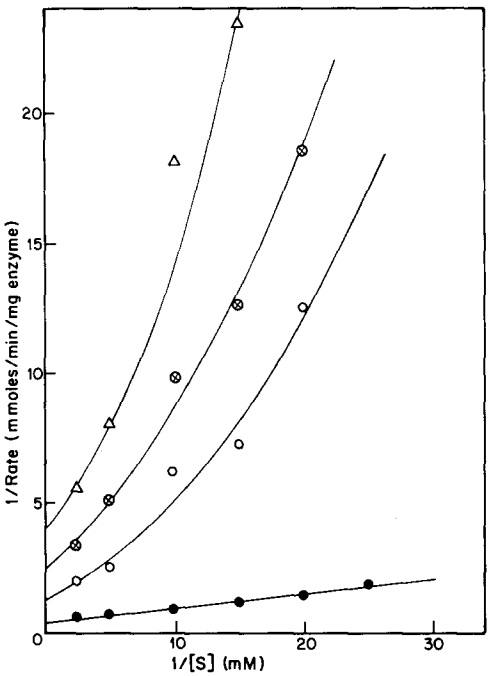

Fig. 2. Lineweaver—Burk plots for acetylcholinesterase in the absence and presence of acrylamide monomers (●, 0%; ○, 5%; X, 10%; △, 15%). No free radicals were added.

over a range of substrate concentrations. The results are shown as Lineweaver—Burk plots in Fig. 2.

Both V and K_m are altered by acrylamide. The increase in the K_m values may be due to: (1) changes in the intrinsic properties of the enzyme in the presence of acrylamide monomer, (2) an environmental effect, or (3) a diffusional effect. It is possible that under the experimental conditions used in the present study a certain amount of polyacrylamide may exist in solution. This could lead to the restricted enzyme mobility which, according to Laurent [15], gives rise to an increase in K_m. The V decreases as the concentration of acrylamide increases, perhaps as a result of denaturation of enzyme by acrylamide, which can act as a denaturing agent in a manner similar to that observed with urea; this is understandable, since both substances possess similar amide functional groups. Degani and Miron [11] have observed similar inhibitory effects of acrylamide on the activity of horse serum butyrylcholinesterase. The combined diffusion control and denaturating effect of acrylamide solution may give rise to the observed (Fig. 2) kinetic pattern with increase in K_m values and decrease in V values.

The enzyme immobilized in polyacrylamide retains about 70% of its original activity even after being stored at 40°C for 150 h; under the same conditions the free enzyme has virtually lost all of its activity. The stabilities of immobilized e zymes to heat and storage depend on the nature of the surface, to which the enzyme is bound. Hydrophilic surfaces, such as are found with polyacrylamide, generally tend to confer stability [16]. However, even when a surface is hydrophilic overall, hydrophobic features, such as the hydrocarbon

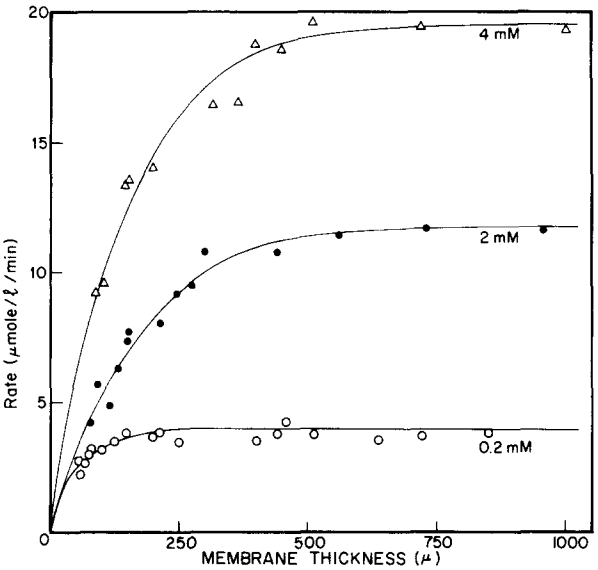

Fig. 3. Influence of membrane thickness on rate, at three substrate concentrations; pH 7.0; 25°C; $[E]_m$ = 90 μg enzyme per ml of gel.

backbone of the polyacrylamide, may interact with similar features on the enzyme. thereby leading to denaturation and detracting from the overall stabilizing effect. β-Galactosidase is less stable when immobilized in polyacrylamide as compared with the enzyme in free solution [17]. The stabilizing effect upon immobilization may be due to at least two factors: (a) immobilization contributes to stabilization of the tertiary and quaternary structure of enzyme, (b) immobilization creates local high protein concentrations; many enzymes in free solution require the presence of other protein in order to preserve their activity [18,19].

The influence of membrane thickness on the rate of reaction, at constant enzyme concentration within the gel, was determined at three different substrate concentrations. The results are shown in Fig. 3. At all substrate concentrations, there is an initial linear increase in reaction rate with increasing membrane thickness. The rate ceases to increase, however, when the membrane is thick enough for the reaction to become diffusion controlled; the higher the substrate concentration, the larger is the thickness at which diffusion becomes rate limiting. Bunting and Laidler [4] have found a similar kinetic pattern with three different concentrations of β-galactosidase immobilized in polyacrylamide membrane, all reactions being assayed at one substrate concentration. In this case, the higher the enzyme concentration, the smaller is the thickness at which diffusion becomes rate limiting.

Fig. 4 shows the variation of rate with enzyme concentration within the gel. Curves C and D in Fig. 4 were obtained by plotting the enzyme activity against the first power of the enzyme concentration; the relationship between the rate and enzyme concentration is not linear. However, when the rate is plotted against the square-root of the enzyme concentration, better straight lines are obtained; this is clearly shown by line B in Fig. 4, which indicates that

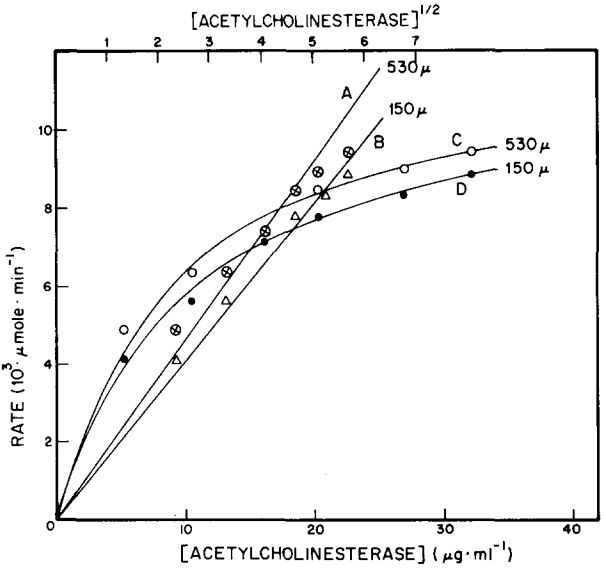

Fig. 4. Plots of rate against square root of enzyme concentration (lines A and B) and of rate against enzyme concentration (curves C and D); pH 7.0, T = 25° C and [Acetylthiocholine] = 0.1 mM.

the rate is directly proportional to the square-root of the enzyme concentration. The dependence of rate on the square-root of enzyme concentration for a thicker membrane (530 μm; line A in Fig. 4) is not as linear as that of a thinner membrane (150 μm; line B in Fig. 4). This is because the rates obtained with thick membranes are not steady-state values. Fig. 1 shows that for the free enzyme in the presence of 15% acrylamide, the rates were directly proportional to enzyme concentration. The square-root dependence is thus not due to the effect of acrylamide on the enzyme properties.

Lineweaver—Burk plots of 1/rate against 1/[acetylthiocholine] are shown in Fig. 5 for various membrane thicknesses. It is to be noted that only the results with membrane of 70 μm thickness yield a straight line; thicker membranes appear to give nonlinear Lineweaver—Burk plots. The theory of Kobayashi and Laidler [6] predicts that Lineweaver-Burk plots for immobilized enzymes should, when there is diffusion control, be convex to the 1/[S] axis; this is observed for thicker membranes (Fig. 5). The results with the 767 μm membrane, probably do not correspond to an attainment of the steady state. The time (τ) required to come within 10% of the steady-state substrate concentration can be estimated using the equation [2]

$$\tau = 0.257 \frac{l^2}{D} \tag{1}$$

where l is the membrane thickness in cm and D (in cm^2 · s^{-1}) the diffusion coefficient for the substrate. Thus it takes approximately 6 min for the establishment of the steady state with a 767 μm membrane. For the 225 μm membrane only about 30 seconds are needed to establish the steady state, so that the results are reliable as representing steady-state conditions. For the 70

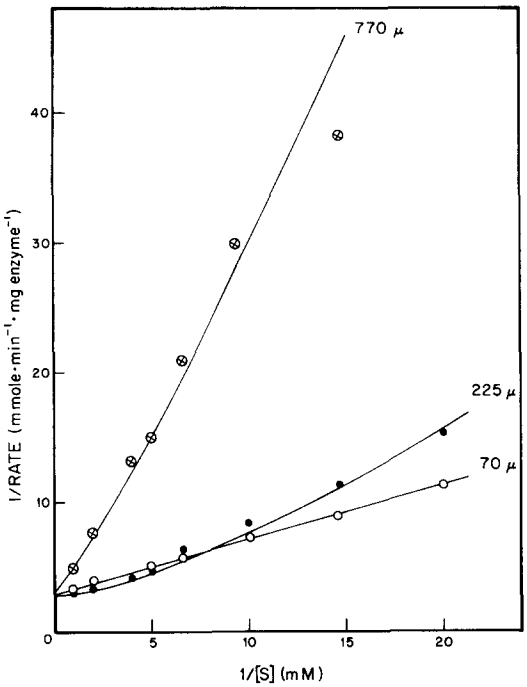

Fig. 5. Lineweaver—Burk plots for the immobilized enzyme; pH 7.0, T = 25°C, $[E]_m$ = 2.7 μg enzyme per ml of gel.

μm membrane, steady state is established within 3 s. Regardless of the thickness (varied from 70 to 770 μm) of the membranes used, the data can be interpreted as giving the same value of V, which is in accord with the theoretical predictions of Kobayashi and Laidler [6].

Discussion

The theoretical treatment of Sundaram et al. [2] shows that the reaction catalyzed by immobilized enzyme can be approximated by an equation of the Michaelis—Menten form:

$$v = \frac{k_c(\text{app})\,[E]_m\,[S]}{K_m(\text{app}) + [S]} \tag{2}$$

where [S] is the concentration of the substrate in the surrounding solution, $[E]_m$ the concentration of enzyme in the membrane, and k_c(app) and K_m(app) are given by

$$k_c(\text{app}) = k_c' \tag{3}$$

$$K_m(\text{app}) = \frac{K_m'}{PF} \tag{4}$$

k_c' and K_m' are the intrinsic kinetic parameters of the immobilized enzyme and P is the partition coefficient. The function F relates to the extent to which the reaction in the membrane is diffusion controlled. The theories (2,3) give the following expression for F

$$F = \frac{\tanh \gamma l}{\gamma l} \tag{5}$$

where l is the thickness of the membrane and $\gamma(\mathrm{cm}^{-1})$ is given by

$$\gamma = \frac{1}{2}\left(\frac{k_c' [E]_m}{D \cdot K_m'}\right)^{\frac{1}{2}} \tag{6}$$

The values of F can be obtained from experimental data. For a given enzyme-membrane, $[E]_m$ is known, and D can be determined experimentally using the technique developed by Bunting and Laidler [4]. The k_c and K_m can be obtained from Lineweaver—Burk plots using thin membranes (70—100 μm). From these parameters (k_c', $[E]_m$, D and K_m') the values of γ can be calculated using Eqn 6, and from the thickness of the membrane and the γ value the value of F can be calculated using Eqn 5. The F values shown in Table I and Fig. 6 are calculated in this way. $[E]_m$ is the molar concentration of the enzyme in the membrane and D is the diffusion coefficient ($\mathrm{cm}^2 \cdot \mathrm{s}^{-1}$). The

TABLE I

THE VALUES OF F FOR THREE CONCENTRATIONS OF ENZYME IN THE MEMBRANE

The enzyme concentration was calculated using a molecular weight of 107 000 [36] · k'_c = 595 s^{-1}, K'_m = $1.6 \cdot 10^{-4}$ M.

Membrane thickness (μM)	$[E]_m \cdot 10^8$ (moles·l^{-1} of gel)	γ	F
70	2.7	0.276	0.975
70	5.4	0.390	0.952
77	2.7	0.304	0.973
144	2.7	0.568	0.907
144	16.1	1.392	0.635
168	2.7	0.663	0.872
230	2.7	0.908	0.794
230	16.1	2.225	0.439
303	2.7	1.196	0.696
303	5.4	1.699	0.560
336	5.4	1.699	0.560
336	5.4	1.885	0.507
394	2.7	1.555	0.588
394	16.1	3.808	0.262
398	2.7	1.571	0.584
398	16.1	3.847	0.259
578	2.7	2.281	0.429
578	5.4	3.242	0.308
763	2.7	3.011	0.331
927	2.7	3.658	0.273

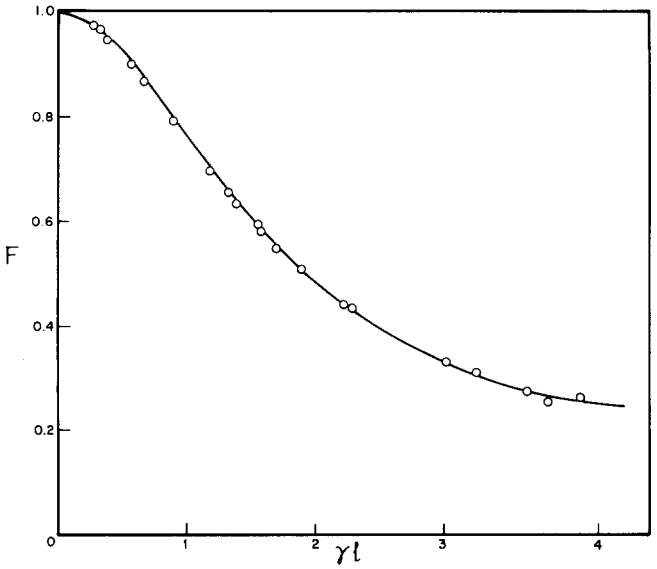

Fig. 6. Plot of the function F against γl. The solid line is the theoretical line and the circles are experimental points.

curve in Fig. 6 shows the function F plotted against γl. For γl values up to about 0.2, F, is close to unity, which means that diffusional effects are unimportant. The product γl tends to be small, and $F \approx 1$, under the following conditions:

(1) When l is small, which means that the substrate has ready access to the enzyme.

(2) When D is large, so that again the substrate can easily reach the enzyme molecules.

(3) When the enzyme concentration $[E]_m$ within the membrane is small, which means that the catalysis is slow so that diffusion can keep up with it.

(4) When k_c'/K_m' is small; again, this means slow catalysis, the chemical process, rather than the rate of diffusion, being rate-limiting.

At higher γl values, the function F becomes significantly less than unity. For γl values higher than 2, F is given quite accurately by $1/\gamma l$; this region of higher γl values, where F is significantly less than unity, corresponds to some measure of diffusion control of the reactions.

Fig. 4 shows that at low substrate concentrations, where there is diffusion control, the reaction rate is directly proportional to the square-root of the enzyme concentration, rather than to the first power of the enzyme concentration. This is readily explained by the theory; at low substrate concentrations Eqn 2 becomes

$$v = \frac{k_c' [E]_m}{K_m'} [S] \cdot F \cdot P \tag{7}$$

When thick membranes are used $F \approx 1/\gamma l$, and since γ is proportional to $([E]_m)^{1/2}$ as shown the overall rate of reaction is predicted to be proportional

to $([E]_m)^{1/2}$ as shown in Fig. 4. This interpretation of the square-root dependence on enzyme concentration is of particular interest in view of an empirical result obtained in 1885 by Schütz [20], and commonly known as Schüt's law. Schütz found a square-root dependence on enzyme concentration for the hydrolysis of egg albumin by pepsin, a result that was later confirmed by Schütz [21] and by Arrhenius [22]. Evidence of the same kind was also obtained by a number of workers, including Arrhenius, for several other enzyme systems. During more recent years Schütz's law was either discounted or forgotten, since much work with pure enzyme preparations has shown a linear dependence of rate on enzyme concentration. Moelwyn-Hughes [23] suggested that the effect was due to diffusion control in the concentrated solutions and with the impure preparations employed by the early workers. It seems likely, in view of the results described above, with acetylcholinesterase and the results of Bunting and Laidler [4] with β-galactosidase, that this explanation is quite correct.

At low values of γl, i.e. thin membranes, K_m (app) becomes very much smaller. If the rate is measured at high substrate concentration ($[S] > K_m$ (app)) Eqn 2 becomes

$$v = k_c' [E]_m \tag{8}$$

The rate is therefore directly proportional to the first power of the enzyme concentration within the membrane. This in fact has been found with β-galactosidase immobilized in thin polyacrylamide membranes [4].

The K_m' value for the immobilized enzyme (cf. Eqn 4) is of considerable importance in comparing the membrane theory with experiment, and corresponds to the case where F is unity, i.e. where diffusion control is not evident. The straight-line Lineweaver—Burk plot (Fig. 5) obtained with the 70 μm membrane preparation indicates that the rates obtained with this membrane are practically free of diffusion-control. On the assumption that little diffusion control is involved at low enzyme concentrations and thin membrane preparations, a value of K_m' can be estimated from Eqn 6 as $K_m' = K_m(\text{app})/PF = 1.6 \cdot 10^{-4}$ M with $F = 1$ and $P = 1$. With the use of this value, it was then possible to calculate F values over the range of enzyme concentrations; the values obtained in this way are shown plotted as open circles (Fig. 6). The agreement is very satisfactory. There is very little diffusion control at the lowest γl values, the highest value of F being 0.975 (Table I).

A number of temperature studies on immobilized enzymes have been made [24—30], the general conclusion being that immobilized enzymes have similar activation energies to those of the corresponding free enzymes. However, in all of the previous investigations, the rates were measured at high substrate concentrations. Under these conditions the theory [2] predicts that the behavior of the immobilized enzyme will be similar to that of the enzyme in free solution; no activation-energy difference is therefore to be expected. However, when rates for the immobilized enzyme are measured at low substrate concentration and at different membrane thicknesses, it is possible to deduce a value for the activation energy corresponding to the diffusion of the substrate.

The theory of this is as follows. The rate v_l at low substrate concentration and thin membrane ($F \approx 1$) is given by

$$v_l = \frac{k_c'}{K_m'} P\,[E]_m\,[S] \tag{9}$$

(cf. Eqn 7). The rate constant k_c' can be expressed in terms of a frequency factor A_c' and an activation energy E_c',

$$k_c' = A_c' e^{-E_c'/RT} \tag{10}$$

and similarly the Michaelis constant K_m' can be expressed in terms of a pre-exponential factor A_m' and an energy term E_m':

$$K_m' = A_m' e^{-E_m'/RT} \tag{11}$$

where R is the gas constant and T is the absolute temperature. Eqn 9 therefore becomes

$$v_l \propto \frac{e^{-E_c'/RT}}{e^{-E_m'/RT}} = e^{-(E_c' - E_m')/RT} \tag{12}$$

If on the other hand the rate (v_h) is measured with thicker membranes, such that $F \approx 1/\gamma 1$, the corresponding relationships are

$$v_h = \frac{k_c'}{K_m'\gamma} \cdot \frac{[E]_m\,[S]}{l} \propto \left(\frac{k_c' D}{K_m'}\right)^{\frac{1}{2}} \tag{13}$$

(cf. Eqn 6 for γ), and it follows that

$$v_h \propto \frac{e^{-E_c'/2RT}\, e^{-E_D/2RT}}{e^{-E_m'/2RT}} \tag{14}$$

where E_D is the activation energy for the diffusion of the substrate within the membrane.

Combining Eqns 12 and 14 then gives

$$\ln \frac{v_h^2}{v_l} = \text{constant} - \frac{E_D}{RT} \tag{15}$$

An Arrhenius plot of v_h^2/v_l thus provides a value for E_D, the activation energy for diffusion.

Temperature studies were carried out using two different thicknesses of membrane. The rates with thin membrane (v_l) were obtained by using mem-

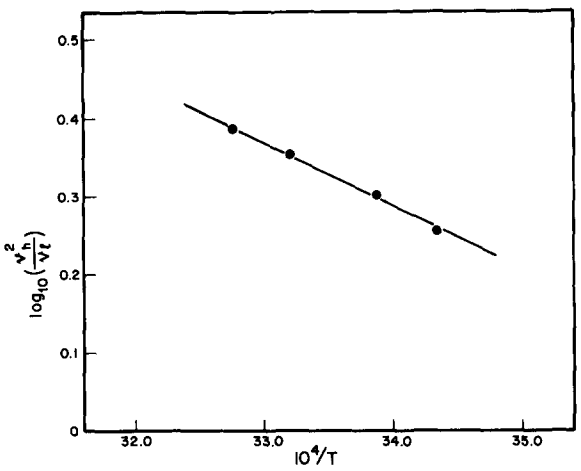

Fig. 7. Arrhenius plot of $\log_{10}(v_h^2/v_l)$ against $1/T$, to obtain the activation energy for diffusion, E_D. The rate v_l was obtained using membrane of 190 μm thicknesses and v_h using membrane of 1025 μm thickness.

brane of 190 μm thickness; a membrane of 1025 μm thickness was used to obtain v_h. The results, plotted as $\log_{10} v_h^2/v_l$ against $1/T$, are shown in Fig. 7, and the value of E_D is 3.7 kcal. This is of the magnitude expected for the activation energy for the diffusion of small molecules [31].

The results presented in Figs 2—4 strongly support the theoretical treatments [2,6]. The temperature studies provide an additional test of the theory and also provide a simple method of obtaining E_D for the substrate.

Atkinson [32] has pointed out that the existence of thousands of macromolecules and smaller molecules within a cell is possible, only if the solvent capacity of water is conserved by maintaining the concentrations of all the solutes at a low level. A major function of the extensive membrane systems that permeate the whole cell may be to provide a very large surface area, on which enzymes may be immobilized, thus preventing their precipitation as agglomerates, in which most of the active sites would be covered and thus inactive. For small molecules, no expedient is available except that of holding their concentration low so that they will not be precipitated. For more chemically reactive metabolites, it is even more important that their concentrations do not rise to a level where nonenzymic and hence uncontrolled reactions might occur. The concept that cytoplasm (the largest cellular compartment) is not a dilute water solution but a gel was advanced as early as 1835 by Dujardin [33]. There is definite evidence for the existence of a structural organization for the enzymes involved in oxidative phosphorylation on the mitochondrial membrane [34]; these enzymes are essentially an immobilized multienzyme system. Coupled with low metabolite concentrations within the mitochondrion, the rate of oxidative phosphorylation is very likely to be diffusion-controlled. For muscle ATPase the γ value is $3.5 \cdot 10^4$ cm^{-1} [4]. The diameters of muscle filaments, fibril and fiber are approximately 0.1 μm, 2μm and 5.0 μm, respectively [35], and the corresponding F values for muscle filament, fibril and fiber are 0.96, 0.14 and 0.006 respectively. The rates for the ATPase of muscle filament are therefore largely diffusion-free, while those of muscle fibril and muscle fiber are to a large extent, diffusion-controlled.

The results of the present investigation and of that of Bunting and Laidler [4] show that diffusional effects are often important at low substrate concentrations, such as are commonly found under physiological conditions. It follows that in dealing with the in vivo behavior of an enzyme, it is essential to take into account such diffusional effects, as well as the conformational, environmental and partitioning effects that were mentioned in the introduction to this paper.

Acknowledgement

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