Invited Discussions

Microenvironmental Effects on Enzyme Activity

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One of the problems to be considered in evaluating the results of experiments involving fragmentation of cells and subcellular organelles and fractionation of the particles and supernatants obtained, is that enzymic activities measured by a standard assay at various stages of the procedure may not provide a true measure of the amount of a given enzyme which is present. In some cases the reason for this may be that the enzyme is localized inside a compartment, for example within a vesicular organelle, which the substrate cannot penetrate. However, more subtle microenvironmental effects may also play a role. An enzyme which is bound to or embedded in a lipoprotein membrane is situated in a milieu different from that of the same enzyme in aqueous solution. The bound enzyme may, therefore, display an activity different from that of the soluble enzyme, as a result of local charge effects or of the lipid character of the membrane. Moreover, diffusion of substrates and products to and from a membrane-bound enzyme may be affected owing to the presence of unstirred layers around the membrane fragment. At the Weizmann Institute we have been engaged in studying enzymes bound to insoluble polymeric carriers, and to synthetic membranes, as simple model systems for native particulate enzymes (see, for example, reference 1). In the following, I want to describe briefly one type of anomalous enzymic activity observed in a synthetic membrane, and to show how the explanation for the behavior of this system could, in its turn, be used to explain the behavior of a natural membrane-bound enzyme.

The synthetic enzyme membrane system under consideration was prepared by impregnating a highly porous collodion membrane with a solution of crystalline papain, and then chemically cross-linking the absorbed enzyme with the bifunctional reagent bisdiazobenzidine disulfonic acid (2, 3). The membranes used in the experiments I will describe were about 400 μ thick and contained layers of enzyme 70 μ thick on each side, colored by reaction with the cross-linking agent (Fig. 1). The papain-collodion membrane contained about 1 mg of enzyme per cm^2. These membranes were very

permeable to low molecular weight solutes such as sucrose or synthetic papain substrates such as benzoyl-L-arginine ethyl ester (BAEE). At pH 6 the papain membrane displayed about 5 % of the activity of an equivalent amount of the crystalline enzyme on BAEE, and about 40 % of the activity of the crystalline enzyme on benzoyl-L-arginine amide (BAA). When the pH dependence of enzymic activity was studied (Fig. 2), the native enzyme dis-

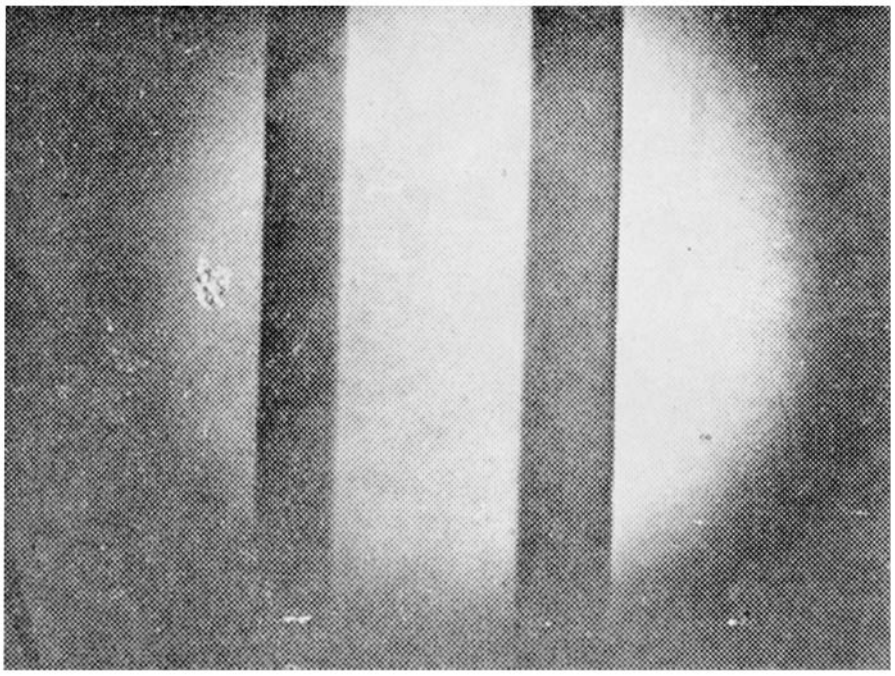

FIGURE 1. Paraffin-embedded section (5 μ thickness) of a papain-collodion membrane stained with hematoxylin-eosin.

played a normal bell-shaped curve for both BAEE and BAA, while the membrane-bound enzyme gave a similar curve on BAA, although somewhat displaced. However, the pH dependence of the esterolytic activity of the membrane-bound enzyme is markedly different. The curve is s-shaped, flattening out in the neutral pH region and then rising sharply at alkaline pH values, up to, and above pH 9.6; alkaline hydrolysis of the substrate interferes with measurements at higher pH values. It was postulated that the changed pH dependence resulted from a lowering of the local pH in the vicinity of the enzyme, caused by generation of hydrogen ions within the membrane by enzymic ester hydrolysis. Thus, as the external pH was raised, the increased rate of enzymic activity would cause a decrease in local pH which would tend to decrease the observed rate. This is a form of negative

feedback and leads to the flattening of the pH activity curve in the neutral pH range. BAA would not be expected to produce such an anomalous pH activity curve since the carboxyl group proton generated would be taken up by the ammonia released.

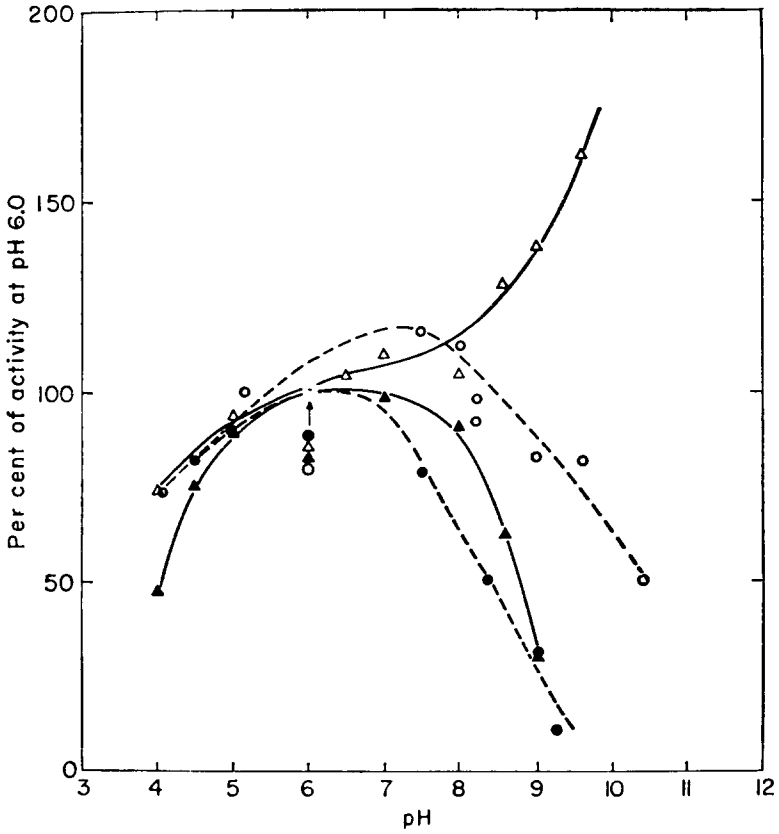

FIGURE 2. Activities of crystalline papain and of the papain-collodion membrane on benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-L-arginine amide (BAA). △, membrane-bound papain acting on BAEE; ▲, crystalline papain acting on BAEE; ○, membrane-bound papain acting on BAA; and ●, crystalline papain acting on BAA.

Additional evidence supporting the contention that changes in local pH were producing the behavior observed was obtained in three ways:

1. If the reaction mixture contained a high concentration of buffer (such as 0.4 M Tris) the pH dependence of activity on BAEE became more like that of the soluble enzyme (Fig. 3).

2. If the papain membrane was frozen in liquid air and then powdered, optimum activity was observed at pH 8, the activity at pH 9.6 being only 35 % of that at pH 6.0.

3. Papain membranes which had not been treated with the cross-linking

reagent retained part of the adsorbed active enzyme. The fact that these membranes were colorless allowed comparison of the color, inside and outside the membrane, of an acid-base indicator. When the indicator, neutral red, was added to a solution of BAEE containing an unactivated papain membrane, both membrane and solution were yellow at all pH values above pH 7.

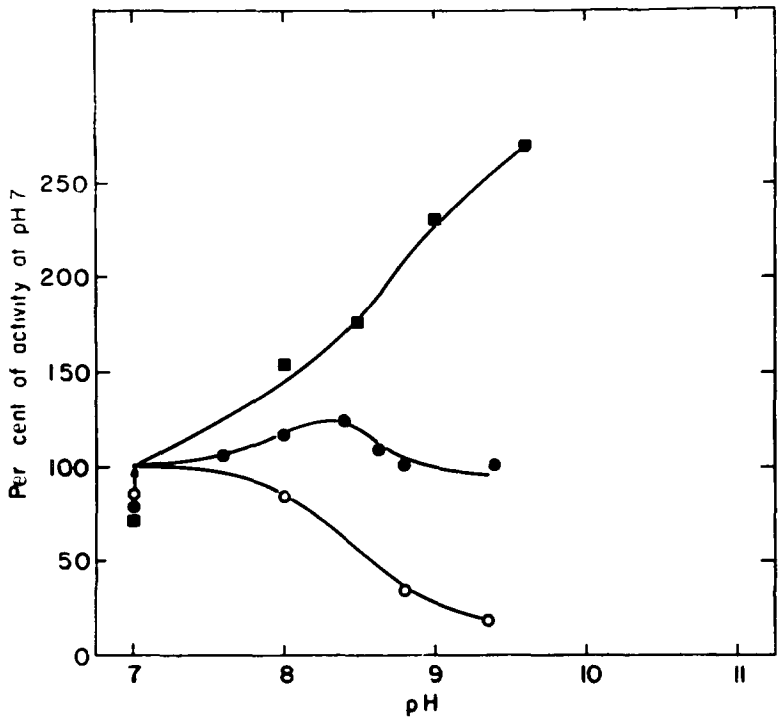

FIGURE 3. Activity of membrane-bound papain on benzoyl-L-arginine ethyl ester (BAEE) in 0.4 M tris buffer. ■, membrane-bound papain in the absence of buffer; ●, membrane-bound enzyme in the presence of 0.4 M Tris; and ○, crystalline papain in the presence of 0.4 M Tris.

On activation of the enzyme by addition of a sulfhydryl reagent, the membrane immediately became red at all external pH values up to pH 10, although the indicator in solution remained yellow. Moreover, when BAEE was replaced by BAA, or if the BAEE reaction mixture contained 0.4 M Tris, the membrane had a yellow color at all alkaline pH values.

An estimate of the magnitude of the pH to be expected within the membrane could be made by taking into account the observed overall enzymic activity, and the diffusion constant and dissociation constant of benzoyl-L-arginine. Such an estimate showed that it was reasonable to expect differences of several pH units between solution and membrane, as indicated by the experimental findings (2).

In studies on the enzymic activity of a membranous fraction rich in acetylcholinesterase prepared by Karlin from the electric organ of *Electrophorus electricus* (4), it was observed that the specific activity of the membrane-bound enzyme (M-AChE) assayed by the hydroxamic acid method was threefold greater than when the enzyme was assayed by measuring acid liberation in the pH stat. No such discrepancy was observed in assaying the soluble crystalline enzyme (S-AChE). When the pH dependence of enzymic activity was

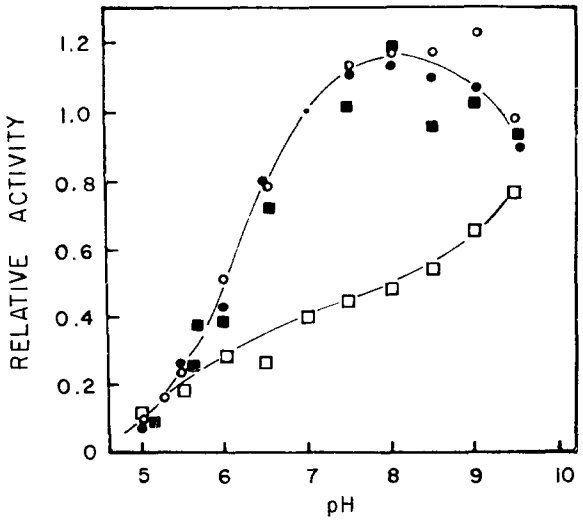

FIGURE 4. pH dependence of activity of M-AChE and S-AChE in the presence and absence of buffer. Activity in the presence of buffer was determined using a reaction mixture which contained 2 mM phthalate, 2 mM phosphate, and 2 mM borate. □, M-AChE in the absence of buffer; ■, M-AChE in the presence of buffer; ○, S-AChE in the absence of buffer; and ●, S-AChE in the presence of buffer.

compared for M-AChE and S-AChE(5), it was realized that the pH dependence of M-AChE was altered by addition of buffer to the reaction mixture, whereas S-AChE was not affected, thus explaining the discrepancy between the two assay methods. In Fig. 4 it can be seen that S-AChE, in the presence and absence of buffer, displays a bell-shaped pH activity curve, as does M-AChE in the presence of buffer. However, in the absence of buffer the pH dependence of activity of M-AChE resembles that of the papain membrane, being relatively flat in the neutral pH region, and rising more steeply at alkaline pH values where the activity of the soluble enzyme is decreasing. It thus appears that the acetic acid released from acetylcholine by M-AChE lowers the local pH in unstirred regions in or around the membrane, just as was observed for the synthetic system. It is reasonable to assume that for a given rate of reaction of M-AChE in the absence of buffer, the local pH is the same as the bulk pH at which M-AChE in the presence of

buffer reacts at the same rate; e.g., at pH 7.0 in the bulk solution, the local pH of M-AChE in the absence of buffer appears to be about pH 5.8, and at an external pH of 9.0 the local pH is near 6.3 (Fig. 4). As Fig. 5 shows, very low concentrations of buffer were needed, saturation occurring at about 2 mM. Moreover, any species which buffered well at the pH tested would produce the same effect. Thus, imidazole could replace phosphate at pH 7 and Tris was very effective at pH 8.

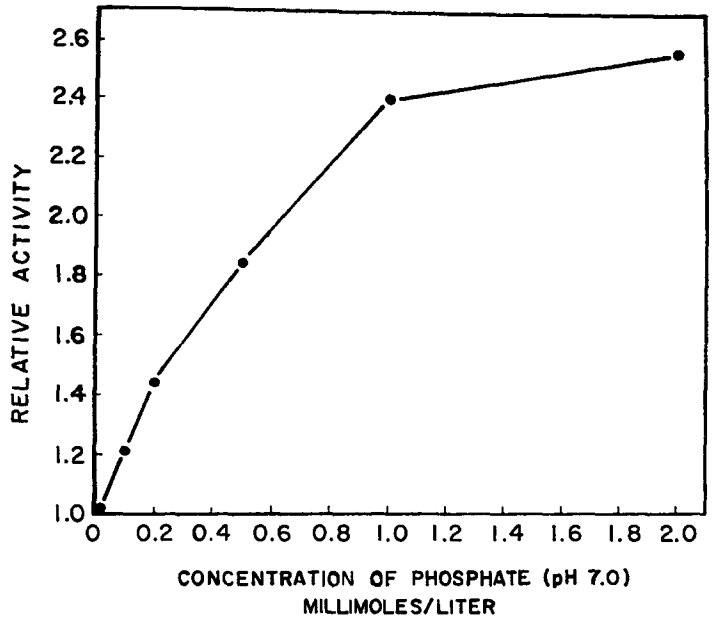

FIGURE 5. Activity of M-AChE as a function of phosphate concentration at pH 7.0.

Two additional lines of evidence can be offered for the contention that the behavior of M-AChE resembles that of the papain membrane:

1. Complete solubilization of M-AChE could be achieved by suspending the membrane fraction in 1 M NaCl. This treatment activated the enzyme fourfold, but its activity was not further increased or modified by addition of buffer to the reaction mixture.

2. It can be predicted that if the effects observed arise as a result of the formation of local pH gradients, reduction of the rate of reaction, by use of a poor substrate, or by addition of a competitive inhibitor to the reaction mixture, should reduce the anomalous behavior, because the slower the rate of reaction the smaller the steady-state gradients created. Thus, when M-AChE was inhibited 80 % by addition of the competitive inhibitor phenyltrimethylammonium, addition of buffer to the reaction mixture increased activity by only 5 %. The effect of the competitive inhibitor is expressed in

another way in Fig. 6, where the reciprocal relative rate is plotted as a function of the concentration of inhibitor. If the normal behavior of a competitive inhibitor is observed, a straight line should be obtained (6), as is indeed observed for S-AChE and for M-AChE in the presence of buffer. However, the curve for M-AChE in the absence of buffer is anomalous, the apparent inhibition being less than expected. This is because slowing down the rate of enzymic hydrolysis by addition of inhibitor brings the local pH closer to that of the reaction mixture, so that negative feedback of the type observed for the pH activity curve is obtained.

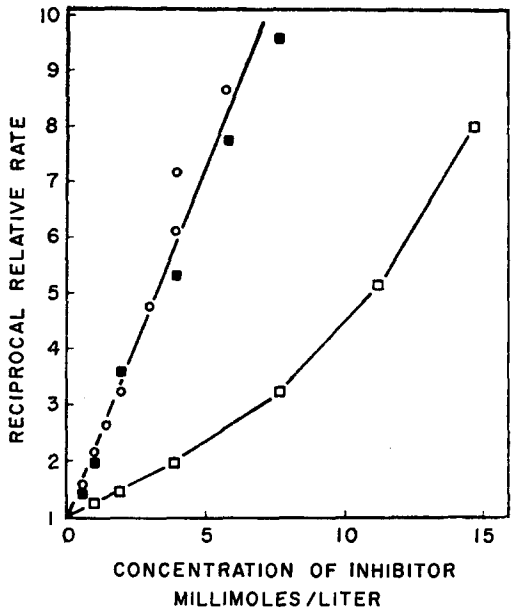

FIGURE 6. Inhibition of M-AChE and S-AChE by phenyltrimethylammonium chloride at pH 7.0. ○, S-AChE; □, M-AChE in the absence of buffer; and ■, M-AChE in the presence of 2 mM phosphate.

As pointed out, 2 mM buffer suffices to make the pH dependence of the activity of M-AChE identical with that of S-AChE, while in the case of the papain membrane, even in the presence of 0.4 M Tris, differences between bound enzyme and soluble enzyme still existed. Undoubtedly, differences in the thickness of the membranes and in the distributions of the associated enzymes contribute to this difference in sensitivity to buffer. In the case of the papain membrane the enzyme was about 7 % by weight in the papain layer, which was 70 μ thick. In M-AChE about 1 % of the protein is AChE, and in this case the membrane, including extracellular material, is in the range of 10–100 mμ thick. That the pH effects are observed at all in M-AChE is probably due to the high turnover number of AChE, which is about 500 times as active as papain.

A variety of reactions, catalyzed by both hydrolytic and oxidation-reduc-

tion enzymes, result in the liberation or uptake of protons. Thus, the possible occurrence of substantial pH gradients should be borne in mind both in assaying subcellular particles and in considering intracellular control mechanisms. Podleski and Changeux (7) have shown that in a modified electroplax cell derived from *Electrophorus electricus* changes in the membrane potential of the innervated membrane on addition of acetylcholine to the perfusing medium can be best explained by changes in local pH resulting from enzymic hydrolysis of the acetylcholine. Whether significant local pH gradients are created in the functioning electroplax, and whether they play any role in controlling the function of the AChE-acetylcholine receptor-acetylcholine system remains an open question.

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