*EFFECT OF LOCAL PH CHANGES CAUSED BY SUBSTRATE HYDROLYSIS ON THE ACTIVITY OF MEMBRANE-BOUND ACETYLCHOLINESTERASE**

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In the living cell many enzymes are bound to membranous structures (e.g., see ref. 1) and function, therefore, in an environment which may differ from that which obtains in solution. By comparing membrane-bound enzymes prepared by cell fractionation with their solubilized and purified counterparts, we may infer the effect of the membrane on the function of the enzymes and thereby better understand their mode of action *in situ*. One such enzyme, acetylcholinesterase, appears to be localized in the cell membrane of many cells (for literature see refs. 2 and 2*a*), a localization consistent, at least in the case of excitable cells, with its probable physiological function.[3]

A subcellular fraction rich in membrane-bound acetylcholinesterase (M-AChE) has been prepared from electric tissue of *Electrophorus electricus*.[2] The acetylcholinesterase activity of this fraction shows an anomalous dependence on pH, similar to that described for a model system in which papain is bound to a collodion membrane.[4] In both cases, this effect can be explained as due to local pH changes in the vicinity of the membrane-bound enzyme consequent to the hydrolysis of substrate.

Materials and Methods.—M-AChE was fraction 4A prepared as previously described.[2] It was assayed in an automatic titrator (Radiometer, Copenhagen, Model TTT1a), operated as a pH-stat, at 30°C, under nitrogen, with 0.01 *M* NaOH as the titrant. The basic reaction mixture (10 ml) contained 0.15 *M* NaCl, 0.004 *M* acetylcholine bromide (ACh), and 0.01% gelatin added to stabilize the enzyme.[5] Various additions were made as indicated below. At pH 7.0, with no additions to the reaction mixture, M-AChE had a specific activity of 2.0 mmoles substrate hydrolyzed per mg protein per hour. Solubilized and purified acetylcholinesterase[6] (S-AChE) was a gift from Dr. W. Leuzinger; it had a specific activity, assayed as above, of 500 mmoles substrate hydrolyzed per mg protein per hour.

Results.—The rates of hydrolysis of ACh by S-AChE and M-AChE as a function of pH were determined in the pH-stat in the presence and absence of buffer. The reaction mixture was buffered over the range pH 4–10 by a mixture of phthalate, phosphate, and borate, all at a concentration of 2 mM. The pH-dependence of the activity of S-AChE in the presence and absence of buffer, and of M-AChE in the presence of buffer was similar, whereas that of M-AChE in the absence of buffer was very different (Fig. 1). It had been previously observed that when M-AChE was assayed by the hydroxamic acid method,[7] it appeared approximately three times more active than when assayed in the pH-stat in the absence of buffer. S-AChE had the same activity by both methods. It was found that the presence of phosphate buffer in the former assay was responsible for the higher activity.[8] A low concentration of phosphate suffices to produce a maximum apparent activation

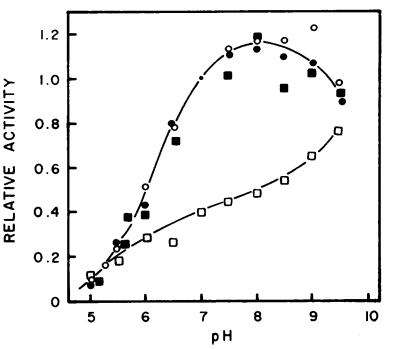

FIG. 1.—pH-dependence of activity of M-AChE and S-AChE in the presence and absence of buffer. Reaction mixtures (10 ml) contained 0.15 *M* NaCl, 0.004 *M* acetylcholine, and 0.01% gelatin. Activity in the presence of buffer was determined using a reaction mixture which contained, in addition to the above components, 2 mM phthalate, 2 mM phosphate, and 2 mM borate. Determinations were performed at 30°. For M-AChE, activities are expressed relative to the activity of M-AChE in the presence of a saturating concentration of buffer at pH 7.0. For S-AChE, activities are expressed relative to the activity of S-AChE at pH 7.0 (which is unchanged by buffer). The actual rate at pH 7.0 for S-AChE, and for M-AChE in the presence of buffer, was in the range of 0.25–0.50 μmoles substrate hydrolyzed/min.

□ M-AChE in the absence of buffer;
■ M-AChE in the presence of buffer;
○ S-AChE in the absence of buffer;
● S-AChE in the presence of buffer.

(Fig. 2). Similar concentrations of imidazole or of a variety of other buffering species at pH 7, and of Tris, for example, at pH 8, yield the same kind of result.

Reducing the rate of enzymic hydrolysis, either by using a poorer substrate than ACh, or by adding a competitive inhibitor of ACh, resulted in a smaller difference between the rates observed in the presence and absence of buffer for M-AChE. Acetyl-β-methylcholine is a stereospecific substrate for AChE, the D isomer being preferentially hydrolyzed.[9, 10] The maximum rate of hydrolysis of the racemic mixture is approximately 30 per cent of that of ACh. With 0.01 *M* acetyl-DL-β-methylcholine as a substrate, the ratio of the rate of hydrolysis in the presence to that in the absence of 2 mM phosphate at pH 7.0 was 1.84 ± 0.05 ($n = 6$) compared with 2.50 ± 0.03 ($n = 5$) for ACh (for the difference $P < 0.001$).

Addition of the competitive inhibitor, phenyltrimethylammonium chloride,[11] at a concentration of 0.011 *M*, reduced the rate of hydrolysis of ACh by S-AChE to 7 per cent of its control value. M-AChE in the presence of buffer is similarly inhibited. However, the activity of M-AChE in the absence of buffer is only reduced to 20 per cent of its control value. The apparent activation of M-AChE by phosphate at the same concentration of inhibitor is only 5 per cent. The plot of the reciprocal rate *versus* the concentration of inhibitor is straight for M-AChE in the

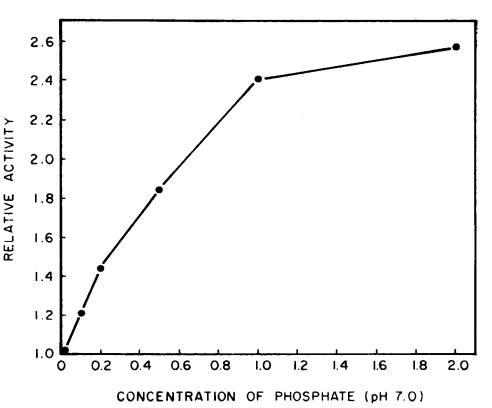

FIG. 2.—Activity of M-AChE as a function of phosphate concentration. The reaction mixture was the usual one described in *Materials and Methods*, and contained in addition phosphate at the concentrations indicated on the abscissa. Determinations were performed at pH 7.0 and 30°.

presence of buffer and for S-AChE, as is expected for a competitive inhibitor,[12] and has the same slope in these two cases (Fig. 3). In the case of M-AChE in the absence of buffer the plot is not straight; in fact, inhibition increases quite slowly at first, and remains lower than that obtained in the presence of buffer at all inhibitor concentrations tested.

Preincubation of M-AChE in 1 *M* NaCl for 5 minutes at room temperature and then assaying as usual in 0.15 *M* NaCl, or adding the fraction directly to an assay medium containing 1 *M* NaCl, results in a four-fold increase in specific activity at pH 7.0. The subsequent addition of phosphate increased this activity further by not more than 15 per cent. (The activity of S-AChE did not vary significantly in the range 0.15–1 *M* NaCl.) The activation and loss of the phosphate effect are due to the solubilization of the enzyme by 1 *M* NaCl. In one experiment, 6 ml of a solution of M-AChE containing 0.6 mg protein was diluted to 12 ml so that the final mixture contained either 1 *M* NaCl or 0.15 *M* NaCl (and also 0.01% gelatin). After 5 minutes' incubation at room temperature, the mixtures were centrifuged at 105,000 × *g* for 60 minutes (Spinco model L centrifuge, no. 40 rotor). The supernatants and sediments were collected and assayed in 1 *M* NaCl–0.01% gelatin. After incubation and centrifugation in 1 *M* NaCl–0.01% gelatin, the supernatant contained 104 per cent of the initial activity and the sediment 18 per cent. After incubation and centrifugation in 0.15 *M* NaCl–0.01% gelatin, the supernatant contained 8 per cent and the sediment 101 per cent of the initial activity.

Discussion.—The anomalous behavior of M-AChE assayed in the absence of buffer in the pH-stat is believed to result, as in the case of the synthetic papain membrane,[4] from the following conditions: (1) The hydrolysis of substrate by the enzyme generates H^+. (2) Due to an unstirred region in or around the membrane, a steady state is reached in which the pH of this region is substantially lower than that of the bulk solution. (3) The intrinsic pH-dependence of the enzyme[13, 13a] is such that the rate of hydrolysis decreases with decreasing pH in the pertinent range. As a consequence of conditions (1) and (2), the local pH in the vicinity of the membrane-bound enzyme will depend on the rate of enzymic reaction. Furthermore, because of condition (3), any change in the rate of reaction due to a change in the reaction conditions will be retarded; e.g., an increase in rate will cause a decrease

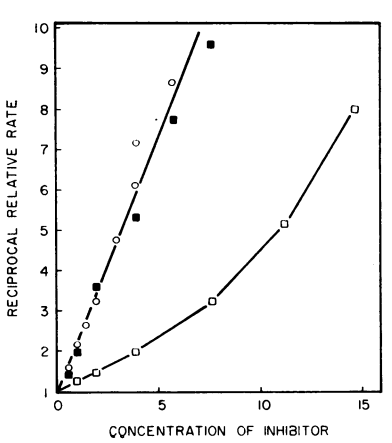

FIG. 3.—Inhibition of M-AChE and S-AChE by phenyltrimethylammonium chloride. To the usual reaction mixture was added phenyltrimethylammonium chloride at the concentrations indicated on the abscissa. Determinations were performed at pH 7.0 and 30°. The reciprocal of the relative reaction rate is plotted versus the inhibitor concentration.

- ○ S-AChE;
- □ M-AChE in the absence of buffer;
- ■ M-AChE in the presence of 2 mM phosphate.

in local pH which in turn will tend to decrease the rate. This is a form of negative feedback and is evident in the pH-dependence of M-AChE in the absence of buffer (Fig. 1) and in the effect of the competitive inhibitor phenyltrimethylammonium ion on M-AChE (Fig. 3). In the former case the increase in activity with increasing pH and in the latter case the decrease in activity with increasing concentration of inhibitor are suppressed relative to the effects of similar changes in the reaction conditions on S-AChE, or on M-AChE in the presence of buffer.

The effect of adding buffer to M-AChE in the pH-stat is to reduce the deviation of the local pH from the bulk solution pH; i.e., the local pH is higher in the presence than in the absence of buffer. The resulting increase in the activity of M-AChE reaches a maximum at a relatively low concentration of buffer, e.g., at 2 mM phosphate (pH 7.0) with an increase of approximately 2.5-fold (Fig. 2). Consistent with this explanation is the identity of the pH-dependence of M-AChE in the presence of sufficient buffer with that of S-AChE (Fig. 1). Slowing down the reaction, either by using a poorer substrate such as acetyl-β-methylcholine or by adding a competitive inhibitor, should also reduce the deviation of the local pH from that of the bulk solution and, therefore, reduce the apparent activation by buffer, as is indeed observed.

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 on the basis of the measured activity to be about pH 5.8; and at pH 9.0 in the bulk solution, where the curve for S-AChE and for M-AChE with buffer is falling, the curve for M-AChE without buffer is still rising, the local pH being near 6.3 (Fig. 1).

The hypothesis that the effect of buffer on the activity of M-AChE is due to the association of the enzyme with the membrane is supported by the effect of 1 *M* NaCl. The enzyme is solubilized and activated, and the effect of buffer is almost completely eliminated. The activation by 1 *M* NaCl is four-fold, whereas the maximum activation by buffer at pH 7.0 is 2.5-fold (1 *M* NaCl does not activate S-AChE). The additional activity obtained suggests that a considerable part of the enzyme activity is masked in M-AChE. This effect could arise either through some of the enzyme being buried in the membrane or through effective sequestration of the enzyme in vesicles or long tubules. In this connection, the appearance in the electron microscope of the fraction used here is mainly that of large and extensively folded fragments of the cell membrane.[2]

As we have pointed out, 2 mM buffer suffices to make the pH-dependence of the activity of M-AChE identical with that of S-AChE. In the case of the artificial papain-collodion membrane, 0.4 *M* Tris did not quite suffice to eliminate differences between the soluble and the bound enzyme. Undoubtedly, the 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 papain-collodion membrane the enzyme was about 7 per cent by weight in the papain layer, which was approximately 70 μ thick. In M-AChE, it can be estimated that one per cent 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 very high turnover number of AChE, which is about

7×10^5 per minute per active site.[14, 15, 15a] It is also possible that the enzyme is not uniformly distributed, but rather is concentrated in discrete regions of the membrane. This would contribute to the effects described.

Given that the local pH in the vicinity of M-AChE is different from that in the bulk solution, it would also be expected that the local substrate concentration might differ from that in the bulk solution. Such an effect becomes apparent at low substrate concentrations. It was previously reported that the K_m of M-AChE in the absence of buffer was close to that of S-AChE.[16] However, this result appears to be due to the fortuitous cancelling of two effects, a substrate effect at lower concentrations of substrate tending to increase the apparent K_m, and a pH effect at higher substrate concentrations tending to decrease the apparent K_m. These effects will be dealt with in a subsequent paper.[17]

Summary.—A subcellular fraction containing membrane-bound acetylcholinesterase shows an anomalous pH-dependence relative to soluble acetylcholinesterase when assayed in the absence of buffer in the pH-stat. When assayed in the presence of buffer, the membrane preparation is apparently activated and the pH-dependence becomes similar to that of the soluble enzyme. The membrane-bound enzyme is solubilized by 1 *M* NaCl, and this treatment also causes activation and eliminates the effect of buffer. The anomalous pH-dependence can be explained as due to local pH changes in the vicinity of the membrane-bound enzyme consequent to the hydrolysis of substrate. The effects of buffer and of solubilization by 1 *M* NaCl are seen to be due to their elimination of the difference between local pH and the pH of the bulk solution.

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