ENZYME ACTIVITIES IN POLARIZED CELL MEMBRANES

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ABSTRACT The theoretical pH dependence of enzyme activities in membranes of low dielectric constant is estimated. It is shown that in biological membranes some types of enzymes may attain a limiting pH sensitivity such that an increment of only 0.2 pH unit (sufficient to induce action potentials in squid axons) causes a relative activity change of over 25%. The transients of enzyme activity generated by membrane depolarization and by pH increments in the bathing solution are discussed in relation to the transients of nervous excitation.

1. INTRODUCTION

Several enzymes appear to play an essential part in some transient and steady-state properties of biological membranes, particularly of cell membranes responsible for nervous excitation and transmission. Acetylcholine esterase is involved in the operation of cholinergic synapses (Eccles, 1964) and perhaps in the action potential in axonal membranes (Nachmansohn, 1959). Adenosine triphosphatase is essential in the active transport of alkali ions across axonal membranes (Hodgkin, 1965). On the other hand, neurophysiological properties of cell membranes are very sensitive to changes in pH. Effects of pH changes are well known in the phenomena of acidosis and Moore (1967, 1968) suggested that an electrically induced pH increase of similar repetitive firing of squid axons perfused by solutions of slightly increased pH. Bass and Moore (1967) suggested that an electrically induced pH increase of similar magnitude (0.2–0.3 pH unit) may be the universal link between depolarization and permeability change in excitable membranes.

We are thus led to consider how enzyme activities may vary with pH in biological membranes. Nearly all such membranes have a capacitance C of 1 $\mu F/cm^2$ and thickness δ of 50–70 A (Cole, 1962) and therefore the relatively low dielectric constant ϵ of 5–8 ($C = \epsilon/4\pi\delta$). Now, most enzymes are not soluble in vitro in solvents of such low dielectric constant (Laidler, 1958). Enzymes are probably "dissolved" in the membranes in vivo in the course of gradual growth and maintenance of the cell. Studies of the pH dependence of activities of certain enzymes in vitro in mixtures of water and at the most 50% of less ionizing solvents (Findlay, Mathias, and

Rabin, 1962) have yielded qualitative results consistent with the theoretical analysis given below.

We show in section 2 how the pH dependence of enzyme activity (on the Michaelis Davidsohn model) is transformed when the enzyme is transferred to a medium of different dielectric constant. We show especially that such an ϵ transformation, as we shall call it, to a medium of a *low* dielectric constant may, in some cases, bring about the greatest possible variation of enzyme activity with pH. In section 3 we show how pH increments can be generated in cell membranes by electrical and other means. In section 4 we outline some transients of enzyme activity arising from the different ways of generating pH increments, and we consider one aspect of Nachmansohn's theory of nervous excitation in the light of the preceding results.

2. THE ϵ TRANSFORMATION OF ENZYME ACTIVITY

Consider the activity of an enzyme, as a function of pH, in a medium which has a dielectric constant ϵ sufficiently high for the dissociation constants of the active groups on the enzyme to be inferred from empirical work in vitro. When the enzyme is transferred to another medium of dielectric constant ϵ', a new pH dependence of its activity is obtained. We shall refer to the resulting change in the pH dependence as the change under an ϵ transformation. Such a transformation with $\epsilon \gg \epsilon'$ may not be possible in vitro by reason of the low solubility of the enzyme in the ϵ' medium, yet may be accomplished in vivo. The ϵ transformation must then include a theoretical estimate of the change of the dissociation constants of the active groups on the enzyme with the change in the dielectric constant.

We adopt the Michaelis-Davidsohn model (Laidler, 1958), in which the enzyme is affected by pH (before combination with substrate) through having two active base groups, each of which can accept at the most one proton. The groups are spatially separated so that they form distinct moieties and, we suppose, have separate energies of solvation. Furthermore, let the one base be neutral, combining with a proton to form a cationic acid (such as NH_3^+) with the dissociation constant K_c, and the other base be singly charged, combining with its proton to form a neutral acid (such as COOH) with the dissociation constant K_n. The enzyme then appears in four distinct arrangements $E_{p_c p_n}$, where the subscripts denote the proton occupation numbers on the base of the cationic acid ($p_c = 0$ or 1) and on the base of the neutral acid ($p_n = 0$ or 1). Thus E_{00} is, over all, a negative ion, E_{01} is neutral, E_{11} is positive, and E_{10} is dipolar (zwitterion). We finally make the common assumption that the dipolar form E_{10} is the only active one (with respect to some substrates).

Denoting concentrations (in mol/l) of protons and enzyme forms by $[H]$ and $[E_{p_c p_n}]$, respectively, we obtain from the chemical independence of the two groups the four equilibria

$$\left.\begin{aligned} [E_{0p_n}][H] &= K_c[E_{1p_n}], \qquad p_n = 0, 1 \\ [E_{p_c 0}][H] &= K_n[E_{p_c 1}], \qquad p_c = 0, 1 \end{aligned}\right\}, \tag{1}$$

of which three are independent. Expressing $[E_{p_c p_n}]$ in terms of the active $[E_{10}]$, we obtain from conservation of enzyme

$$[E_{10}]\left(1 + \frac{K_c}{K_n} + \frac{[H]}{K_n} + \frac{K_c}{[H]}\right) = [E_{\text{tot}}] - [C],$$

where $[E_{\text{tot}}]$ refers to all enzyme forms, including the complex C with the substrate. If C has a short life, or if the substrate is relatively dilute, we may neglect $[C]$ as compared with $[E_{\text{tot}}]$, obtaining for the active fraction f of the enzyme

$$f = \frac{[E_{10}]}{[E_{\text{tot}}]} = \left(1 + \frac{K_c}{K_n} + \frac{[H]}{K_n} + \frac{K_c}{[H]}\right)^{-1}. \tag{2}$$

None of the concentrations $[E_{p_c p_n}]$ may be neglected, as compared with $[E_{\text{tot}}]$, for *all* values of ϵ.

The maximum value $f_{\max}$ of f is at the isoelectric point $[E_{11}] = [E_{00}]$, $[H] = (K_c K_n)^{1/2}$. We transform (2) to the pH scale by writing the expression in parentheses in the form

$$1 + \frac{K_c}{K_n} + \left(\frac{K_c}{K_n}\right)^{1/2} (10^{-(\text{pH}-\text{pH}_{\max})} + 10^{(\text{pH}-\text{pH}_{\max})})$$

and hence

$$\left.\begin{aligned} f &= \left(1 + \frac{\alpha^2}{4} + \alpha \cosh y\right)^{-1} \\ y &= (\text{pH} - \text{pH}_{\max}) \ln 10, \qquad \alpha = 2\left(\frac{K_c}{K_n}\right)^{1/2} \end{aligned}\right\}; \tag{3}$$

y is a measure of the ratio of proton concentration to that at which $f = f_{\max}$. The symmetry of cosh y with respect to the position of $f_{\max}$ makes the symmetry of f(pH) explicit. The absolute values of K_c, K_n are involved only in the position of $f_{\max}$ on the pH scale; the width and height of the well known bell-shaped curve (3) are determined entirely by their *ratio* determining α. Now, $f_{\max}$ falls monotonically with increasing α, while the half maxima are at values of y given by

$$\cosh y = 2 + \frac{1}{\alpha} + \frac{\alpha}{4},$$

so that the width of the curve, as a function of α, goes through only one minimum at $\alpha = 2$, $(K_c = K_n)$. In this limiting case the pH dependence of the active fraction f, and hence of enzyme activity, is the strongest possible on our model; $f_{\max} = \frac{1}{4}$ (all $[E_{p_c p_n}]$ are equal to one another) and the pH interval between the half maxima (cosh y = 3) is approximately 1.53 pH units. At the inflections of the limiting curve (cosh $y = 2$) a change of 0.2 pH unit generates a relative change $\Delta f/f$ of more than 25%; the pH interval between the inflections is approximately 1.15 pH units. We

note that when pH is varied in the perfusion fluid of squid giant axons, action potentials are induced by increments of 0.2–0.3 pH unit (Tasaki, Singer, and Takenaka, 1965) and the pH interval in which conduction is possible at all is about 1.2 units (Tasaki, Watanabe, and Takenaka, 1962).

Can this limiting case be approached or actually attained in biological membranes? Starting from values of K_c/K_n known in aqueous solution, $\epsilon \approx 80$, we estimate the effect of the ϵ transformation to the membrane, $\epsilon' \approx 8$. For acetylcholine esterase (Nachmansohn, 1959) and several amino acids (Kortüm, 1957) the two dissociation constants differ in aqueous solutions by 4–6 pK units, $K_n \gg K_c$, so that $\alpha \approx 10^{-2}$–10^{-3}. The corresponding pH dependence of f is slight in terms of pH increments of the order of 0.2, the pH interval between the half maxima being 4–6 units.

The main effect of the reduction in ϵ is to increase coulombic interactions; K_n will clearly be reduced, contributing to an increase in α. To obtain a semiquantitative estimate of the increase in α, we suppose first that the dielectric constant of the *entire* surroundings of the two enzyme acid groups is varied in the ϵ transformation. The change in the acid dissociation constants can then be estimated from the change in the Born solvation energies involved in the dissociation (Kortüm, 1957). We envisage each acid removed from the medium to vacuum, dissociated, and the resulting components returned to appropriate positions in the medium. In the dissociation of the neutral acid group two solvation energies are thus *gained*, so that, on changing the medium,

$$\Delta \ln K_n = -\frac{e^2}{2kT}\left(\frac{1}{b_{H^+}} + \frac{1}{b_-}\right)\Delta\frac{1}{\epsilon},$$

where e is the elementary charge and b_{H^+}, b_- are the effective Born radii of the dissociated proton and base, respectively; $\Delta(1/\epsilon) = 1/\epsilon' - 1/\epsilon$, k is Boltzmann's constant, and T is absolute temperature (300°K). In the dissociation of the cationic acid the solvation energy of the undissociated acid (effective radius b_+) must be *supplied;*

$$\Delta \ln K_c = -\frac{e^2}{2kT}\left(\frac{1}{b_{H^+}} - \frac{1}{b_+}\right)\Delta\frac{1}{\epsilon}.$$

The unreliable Born estimate of the change in proton solvation energy is fortunately not involved in the relative change of the ratio K_c/K_n, and hence in the relative change of α:

$$\Delta \ln \frac{K_c}{K_n} = \frac{e^2}{2kT}\left(\frac{1}{b_+} + \frac{1}{b_-}\right)\Delta\frac{1}{\epsilon}. \tag{4}$$

However, only the dissociated protons, not contributing to (4), are fully surrounded by the medium subjected to the increment $\Delta(1/\epsilon)$. Both the undissociated cationic

acid and the dissociated negative base are on the surface of a protein of fixed dielectric properties. Hence only parts of their surroundings change in the ϵ transformation and the corresponding changes in their solvation energies fall short of those given by the Born model. Let the *correct* change of solvation energies give a fraction, $\beta < 1$, of the right-hand side of (4). Taking $\Delta\,(1/\epsilon) \approx \frac{1}{8}$ and $b_+ \approx b_- \approx 2$ A, we obtain an increase in α by a factor $\approx 10^2$ for $\beta = \frac{1}{3}$, and by $\approx 10^3$ for $\beta = \frac{1}{2}$. Starting from the observed values of α in aqueous solution, the value $\alpha \approx 2$ may therefore be attained by an ϵ transformation to $\epsilon' \approx 8$. Thus the feasibility of the limiting pH dependence of some enzyme activities in cell membranes is demonstrated.

3. GENERATION OF pH CHANGES IN MEMBRANES

Let the membrane be immersed in a solution in which the proton concentration $[H]_S$ may be varied at will, as in perfusion of axons, by varying the solution buffer. If the work of transferring a proton from the solution to the membrane is W, we have in equilibrium

$$[H]/[H]_S = e^{-W/kT} \equiv K_d\,, \tag{5}$$

K_d being the distribution coefficient of protons between the solution and the membrane. W is scarcely calculable outright, but it may be supposed independent of the proton concentrations. Then the *increments* of pH are equal inside and outside the membrane. The low value of the dielectric constant in the membrane indicates the presence of few water molecules, particularly since the membrane contains dipoles other than water. Even if protons contributing to membrane pH exist as H_3O^+ ions, they need not be coupled to OH^- ions as in aqueous solutions (Bass and Moore, 1968).

Let the membrane contain a buffer (such as the substituted phosphoric acids of the phospholipids) confined to the membrane. Then, with acid and base concentrations $[A]$, $[B]$ and the dissociation constant K,

$$[H] = K\frac{[A]}{[B]}\,, \qquad [A] \text{ and } [B] \gg [H]. \tag{6}$$

In equilibrium $[A]/[B]$ is determined by $[H]_S$ through (5) and (6), the membrane buffer having to adjust itself to the large proton reservoir of the bathing solution. When a positive or negative pH increment (membrane alkalosis or acidosis) is to be induced by the equal pH increment in the bathing solution, the membrane buffer ratio $[A]/[B]$ must first be adjusted by exchange of protons between the membrane and the solution. We note that whereas conduction phenomena in the actual axon are radically affected by pH increments of 0.2–0.3 in the axoplasm (Tasaki et al., 1965), they are nearly insensitive to ten times larger pH increments in the external

solution (Lorente de Nó, 1947). These observations are consistent with the view that the axonal membrane is strongly buffered, and that pH-sensitive structural elements are located close to its inner surface. The latter assumption has already yielded a natural interpretation of some remarkable effects of nonelectrolyte perfusion of axons (Bass and Moore, 1967).

pH changes in polarized membranes can also be induced electrically (Bass and Moore, 1968). In the medium of the membrane ($\epsilon' \lesssim 8$) an appreciable Wien effect on dissociation must occur in a field E of 10^5 v cm^{-1}, such as exists in resting axonal membranes (with 70 mv across 70 A). Here we assume that the resting potential difference is distributed across the whole thickness of the membrane as a uniform field, in accord with both the Planck and the Goldman models (Bass and Moore, 1967). For the buffer system (6) a change in the field from E to E' gives a pH increment equal to the pK increment of the buffer dissociation constant:

$$\Delta \text{ pH} \approx -\log \frac{K(E')}{K(E)} = -\log \frac{K(E')}{K(0)} + \log \frac{K(E)}{K(0)} \tag{7}$$

($[A]/[B]$ remains practically unchanged because of the inequalities in (6) expressing the buffer character of the system). The only existing calculation of $K(E)/K(0)$ (Onsager, 1934) is based on the weakening of coulombic attraction between point ions by an external field E. On this model (which, when applicable, is in good agreement with experiments) a general expression in Bessel functions is obtained, with the notable limiting cases

$$\ln \frac{K(E)}{K(0)} \approx \begin{cases} b & \text{for } b \ll 1 \\ (8b)^{\frac{1}{2}} & \text{for } b \gtrsim 1 \end{cases} \tag{8}$$

$$b = \frac{z^3 e^3 |E|}{2\epsilon k^2 T^2}$$

for a symmetric electrolyte of valency z. For example, a reduction by 30% of a field of 10^5 v cm^{-1} (such as a critical depolarization of a resting axonal field) would yield Δ pH $\approx$ 0.2–0.3, depending on the valency of the buffer.

It remains to consider the direct field dissociation effect on the enzyme acid dissociation constants K_c, K_n. Though there exists no quantitative theory of the Wien effect on K_c (representing a purely noncoulombic bond), it is clear that the effect must be less than on K_n for all values of the field: the external field enhances dissociation by doing work on charges moving across the range of the binding interaction, which is longer for the coulombic part of the bond represented by K_n. We now show that $\Delta pK_n < \Delta$ pH for a given field change (from which $\Delta pK_c < \Delta$ pH follows).

In Onsager's model the external field aids only the dissociation of suitably aligned molecules (associated ion pairs). To obtain the full dissociation effect, free angular

redistribution of the ion pairs is necessary. Such redistribution is hindered for acid groups lodged in the surfaces of the large enzyme molecules in the semisolid membrane; the external field will therefore leave the bonds of a fraction of the acid groups unaffected, and may even strengthen the bonds of others. The average field dissociation effect on enzyme groups will therefore fall short (increasingly so with increasing field) of Onsager's value, which, however, may remain applicable to the dissociation constant of the smaller buffer molecules. For a given field change, ΔpK_n is reduced further relative to Δ pH if the buffer ions are polyvalent. From (8), ΔpH is then between $z^{3/2}$ and z times greater than the ΔpK_n already overestimated by the use of the Onsager model. Qualitatively similar results (involving ionic mobilities) are obtained for nonsymmetric buffer electrolytes (Onsager, 1934).

For these reasons the Wien coupling between field changes and enzyme activities may, in the lowest approximation, be ascribed to pH changes induced by the Wien effect on the buffer. The theory of the direct Wien effect on enzyme groups, necessary for closer approximations to the coupling, remains to be constructed.

4. SOME ACTIVITY TRANSIENTS

Ionic diffusion coefficients in aqueous solutions are 10^4–10^5 times higher than in axonal membranes (Cole, 1965). When the pH of the bathing solution is changed, the membrane pH rapidly attains the new equilibrium value ($[H]_{eq} = K_d[H]_S$) on the very surface of the membrane. We estimate the time of propagation of the pH change through the buffered membrane.

Let $\bar{A}$, $\bar{B}$, and $\bar{H}$ be average concentrations of buffer acid, of base, and of protons in a surface layer of thickness x of the membrane. Since the equilibrium value $\bar{H}_{eq}$ is attained at the membrane surface, the concentration gradient in the surface layer is approximately $(\bar{H} - \bar{H}_{eq})/x$. Since each proton transported across the membrane surface converts one buffer acid molecule to base (or vice versa),

$$\frac{d(\bar{A}x)}{dt} \approx - D \frac{\bar{H} - \bar{H}_{\mathrm{eq}}}{x}, \tag{9}$$

where D is the proton diffusion coefficient in the membrane. On the other hand, with the buffer confined to the membrane, $\bar{A} + \bar{B} = N = \text{const.}$ and

$$\bar{H} = K \frac{\bar{A}}{N - \bar{A}}, \qquad \frac{d\bar{A}}{dt} = \frac{NK}{(K + \bar{H})^2} \frac{d\bar{H}}{dt},$$

so that

$$\frac{x^2}{D} \frac{NK}{(K + \bar{H})^2} \frac{d\bar{H}}{dt} \approx \bar{H}_{\mathrm{eq}} - \bar{H}. \tag{10}$$

Suppose that $K > \bar{H}$ and $\bar{H}$ is decreasing (alkalosis). Then the coefficient of $d\bar{H}/dt$ in (10) is the nearly constant *relaxation time* τ_{buff} of the pH distribution in the layer

of thickness x:

$$\tau_{\text{buff}} = \frac{x^2}{D} \frac{NK}{(K + \bar{H})^2} \approx \frac{x^2}{D} \frac{N}{K}. \tag{11}$$

This is the product of the ordinary diffusion relaxation time x^2/D with a term which is very large in a buffer. As an example, suppose $K = 10^{-7}$ mol/l for a membrane buffer giving pH = 7 at its maximum capacity ($\bar{A} = \bar{B}$). With $D = 10^{-9}$ cm^2/sec and $x = 10^{-7}$ cm, we obtain $\tau_{\text{buff}} = 10^{-3}$ sec for $N = 10^{-5}$ mol/l. At the other extreme, $x = 10^{-6}$ cm and $N = 10^{-1}$ mol/l yields $\tau_{\text{buff}} = 10^{3}$ sec. Equation (11) follows also from the more exact mathematical treatment of $[H]$ as a function of space as well as of time.

If the equilibria (1) of the enzyme forms $E_{p_c p_n}$ are established rapidly (on the scale of τ_{buff}), then the solution of equation (10) may be substituted in (2) or (3) and τ_{buff} becomes the characteristic time constant also of the change in enzyme activity in the membrane layer of thickness x.

A different set of transients is obtained if the membrane pH is changed by a rapid change in the polarization of the membrane in accordance with (7). Now, the establishment of the new pH and of the consequent new enzyme activity does not require the slow conversion of the membrane buffer described by (9) and (10). However, the new state is transient even if the polarization change is held fixed (voltage clamp), because the resulting pH is not in equilibrium with the bathing solution: $\bar{H} = K(E')\bar{A}/\bar{B} \neq K_d[H]_S$. The ultimate equilibrium under voltage clamp, $K(E')\bar{A}'/\bar{B}' = K_d[H]_S$, is approached by conversion of the membran buffer from $\bar{A}/\bar{B}$ to $\bar{A}'/\bar{B}'$ according to (10). Thus τ_{buff} becomes the time constant of the *reversal* of the change in enzyme activity engendered by the polarization change. (A transient of this type recurs on restoring the initial state of polarization.) Depending on values of x, N, K, and D, the transient change in enzyme activity may last milliseconds or hours.

Suppose that an enzyme E_a is distributed near a membrane surface which is sensitive to changes in $[H]_S$, and an enzyme E_b in the well buffered interior of the membrane ($x_a \ll x_b$, $N_a \ll N_b$); let the enzymes have the limiting pH sensitivity in the membrane. If the maxima of the active enzyme fraction f_a, f_b are suitably placed on the alkaline side of the resting membrane—pH, then the time course of the active fractions under voltage-clamped depolarizations will be qualitatively similar to the time course of the generalized ionic conductances g_{Na}, g_K in axonal membranes (Hodgkin, 1965). The correspondence between f_a and g_{Na} will also fit the observed firing of axons under alkaline perfusion. Furthermore, if the depolarization is slow on the scale of τ_{buff},

$$\frac{dE}{dt} \tau_{\text{buff}} \ll |E - E'|,$$

the proton equilibrium $\bar{H} = K_d[H]_S$ remains undisturbed and we obtain *accommoda-*

tion of the enzyme activity. These similarities suggest that if a physical connection between enzyme activities and ion transport in membranes can be postulated, a quantitative enzymatic theory of nervous excitation and transmission might be constructed.

A qualitative enzymatic theory of the nerve impulse based on acetylcholine esterase, acetylcholine, and its receptor (all contained in the membrane) has been proposed by Nachmansohn (1959) and discussed in terms of the Hodgkin-Huxley parameters by Eyring (1960). We conclude with a remark on one of several objections to this theory (Katz, 1960). The theory postulates that enough acetylcholine for some 10^5 impulses is stored in the membrane in lipid-bound form (immune from hydrolysis by the esterase) and replenished by constant slow synthesis. Depolarization is supposed to induce combination of acetylcholine with its (as yet unidentified) protein receptors, which then undergo conformational changes in turn leading to the changes in membrane permeability responsible for the rising part of the action potential. Inactivation is ascribed to hydrolysis of the acetylcholine interacting with the receptors. However, neither intracellular nor extracellular application of acetylcholine alone can depolarize axonal membranes (Katz, 1960), whereas extracellular application of acetylcholine depolarizes some postsynaptic membranes (Eccles, 1964). These observations have been ascribed to hypothetical barriers to acetylcholine at both axonal membrane surfaces (Nachmansohn, 1959).

The common view that, in the absence of such barriers, application of acetylcholine should have the same effect as depolarization presupposes that depolarization acts by making stored acetylcholine available to receptors which are themselves not directly affected by the depolarization. Our preceding considerations suggest an alternative view. The active groups of the receptor are likely to have a structure and arrangement similar to those of acetylcholine esterase (Wilson, 1960), though $(K_cK_n)^{1/2}$ need not be the same for both. The increase of the active (dipolar) fraction f_a of the receptor by depolarization or alkaline perfusion could then be a necessary condition for firing even when acetylcholine is available to receptors: since the acetylcholine interacting with receptors is being hydrolyzed, f_a would have to exceed some threshold value depending on esterase activity. Application of acetylcholine would then be ineffective in the resting state and unnecessary in the activated state. No hypotheses about barriers would be needed.

The cholinergic post synaptic membrane could be pictured as a patch of the axonal membrane turned to face the synaptic cleft (rather than the axoplasm) with its pH-sensitive side. The pH in the cleft is higher by some 0.3–0.4 unit than in the axoplasm (Ruch et al., 1961), so that the active fraction f_a of the receptor would be permanently high, as in alkaline perfusion of axons. In the steady state the acetylcholine store in the membrane would therefore become empty or thoroughly depleted by continued hydrolysis. A supply of acetylcholine, arriving as transmitter across the cleft, would then be necessary to depolarize the membrane by interaction with the permanently active receptors.

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