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THEORETICAL PAPER

The kinetics of enzyme action and inhibition in intact tissues and tissue slices, with special reference to cholinesterase

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A major problem in biochemical methodology is the meaningful assessment of the effect of drugs, hormones or other environmental or physiological stimuli on enzyme activity *in vivo*. The common practice of excising appropriate tissues, homogenizing them, and assaying the enzyme in the homogenate, with or without some prior purification and under arbitrary conditions, has at least two major drawbacks. Firstly, the activity *in vivo* is dependent on the local concentration of the substrate, which may differ enormously from that used in the assay; and, secondly, enzymes in intact tissues may be compartmentalized, and the only relevant changes in enzyme activity are those in the compartments accessible to the substrate. Homogenization generally destroys this compartmentation. To overcome these problems, many workers have advocated assaying enzymes in intact tissues or tissue slices, rather than in homogenates, and conducting the assay at a substrate concentration (usually low) comparable with the likely concentration of substrate in the *in vivo* situation, rather than at the optimal concentration for *in vitro* assay. As shown below, on purely kinetic considerations, this approach to *in vivo* enzyme assay may be totally erroneous. The general kinetic principles involved in enzyme assay in intact tissues, and some of the difficulties arising in interpretation are illustrated by reference to experimental results reported in the literature for the physiologically important enzyme acetylcholinesterase.

The cholinesterase activity of tissues is normally determined from the rate of hydrolysis of a suitable ester substrate when incubated with a known amount of tissue. For brain slices (Strickland & Thompson, 1955; Hobbiger & Lancaster, 1971), intact nerve (Berkowitz, 1955; Dettbarn & Rosenberg, 1962), rat diaphragm (Fleisher, Hansa & others, 1960; Welsch & Dettbarn, 1972; Heffron, 1972), frog rectus abdominis muscles (Fleisher, Corrigan & Howard, 1958), electroplax from electric eels (Rosenberg & Dettbarn, 1963), guinea-pig retinas (Harris, Fleisher & Yamamura, 1971) and chick biventer cervicis muscles (Baldwin & Lesser, 1971) it has been found that the activity is lower when the assay is conducted using intact tissues than it is if the tissue is first homogenized. With many of these tissues it has also been observed that the activity of the intact tissue relative to that of the homogenate increases when the substrate concentration is raised, and that at sufficiently high substrate concentration the activity of the intact tissue may approach or equal the V_{max} value found for the homogenate (Dettbarn & Rosenberg, 1962; Rosenberg & Dettbarn, 1963; Baldwin & Lesser, 1971; Hobbiger & Lancaster, 1971; Welsch & Dettbarn, 1972). The reduced activity of the intact tissue is usually ascribed to sequestration of part of the enzyme behind a lipid membrane barrier which is impermeable to the quaternary acetylcholine ion. If this were true, the V_{max} value for cholinesterase in intact tissues should be lower than that in the homogenate, but the K_m value should be the same, whereas experimentally this is not so. The main kinetic difference between intact tissues and homogenates lies in a different dependence of the hydrolysis rate on substrate concentration, leading to a much higher apparent K_m value (defined as the substrate concentration at which the velocity is half-maximal) for the intact tissue than for the homogenate.

Several factors could cause an increase in apparent K_m for an enzyme in an intact tissue. Firstly, the conformation of the enzyme protein may change when the tissue is homogenized, but this is unlikely for cholinesterase since even in the homogenate the bulk of the enzyme protein is still bound to fragments of the same membranous structures to which

it is attached in the intact tissue. Secondly, the local environment of the enzyme active centres within the intact tissue may differ from that in the surrounding medium. The K_m value for acetylcholinesterase is particularly susceptible to changes in ionic strength of the medium (Brestkin, Brik & others, 1970; Dawson & Crone, 1973), ranging from less than 10 μM at very low ionic strengths to 500 μM at high ionic strength. However, since the media ordinarily used for cholinesterase assay are usually of high ionic strength, it is improbable that the ionic environment around the active centres in intact tissues could be substantially more concentrated than that in the assay medium. The third, and most likely, explanation for the rise in apparent K_m is that the average concentration of substrate within intact tissues is lower than that prevailing in the medium, whereas with homogenates, all the active sites are exposed to the same substrate concentration (Hobbiger & Lancaster, 1971). This is qualitatively reasonable, since in order to reach those active sites located near the centre of the intact tissue, the substrate must first diffuse through a mesh of the same enzyme that destroys it. Provided diffusion is the rate-limiting process, a substrate gradient will develop within the tissue such that most of the enzyme active sites are exposed to a substrate concentration below that in the medium. Eventually, a steady-state will be attained, in which the rate of diffusion of substrate into the tissue exactly balances the rate of substrate destruction within the tissue. Experimentally, a constant rate of substrate destruction by the intact tissue indicates that such a steady-state has been reached. The kinetics of diffusion-controlled enzyme catalysed reactions have been reported by numerous workers recently in connection with purified enzymes immobilized by being bound to solid supports. In the present review this analysis has been extended to enzymes immobilized in intact tissues. The conditions are summarized under which substrate gradients might exist within intact tissues and the equations are given which describe these gradients and which permit calculation of the net rates of substrate hydrolysis. The relevance of these equations to cholinesterase assay, and especially to the assessment of the degree of cholinesterase inhibition *in vivo*, is then discussed.

THEORY

Diffusion-controlled processes obey Fick's Second Law of Diffusion, which states that the rate of accumulation of solute (which in the present case is the enzyme substrate) at any cross section in the tissue (dS/dt) is related to the substrate concentration (S) by the equation $dS/dt = D\nabla^2 S$, where D is the diffusion coefficient for the substrate in the tissue, and $\nabla^2 S$ is the second derivative of S with respect to the appropriate spatial co-ordinates. In the steady state, dS/dt must also equal the rate of substrate destruction at the same cross section. If the enzyme obeys a Michaelis–Menten type rate dependence on S, then $dS/dt = V_{max}S/(K_m + S)$. Thus, in the steady state, the substrate concentration at any point within the tissue can be obtained by solving the differential equation $D\nabla^2 S = V_{max}S/(K_m + S)$. This differential equation can only be solved in analytical form when the substrate concentration is very low [if $S \ll K_m$, then $D\nabla^2 S = (V_{max}/K_m)S$], or very high [if $S \gg K_m$, then $D\nabla^2 S = V_{max}$], but as shown below, solution of even these two simpler equations permits many useful deductions to be made about the behaviour of enzymes in intact tissues.

The solutions to the above equations differ for differing tissue shapes, and three types must be considered, namely the tissue slice in which all diffusion takes place perpendicular to the parallel faces of the slice, the long cylinder in which diffusion only occurs perpendicular to the cylindrical surface towards the central axis, and the sphere in which the substrate diffuses from the spherical surface towards the centre point.

For a disc of constant thickness (equivalent to a tissue slice) and for a sphere, solutions of the above differential equations have already been given in connection with the kinetics of enzymes bound to solid supports (Sundaram, Tweedale & Laidler, 1970; Katchalski, Silman & Goldman, 1971; Kasche, Lundqvist & others, 1971). For the long cylinder (and for the sphere) solutions may be obtained from the general analysis by Danckwerts (1951) of the mathematics of diffusion coupled with chemical reaction. The relevant solutions for all three systems are summarized below. Details of the mathematical procedures may be found in the references quoted.

(i) *Substrate concentration high* ($S \gg K_m$)

The substrate concentration (S) at any point within the tissue is given by

$$S = S_o - (V_{max}/nD)(l^2 - r^2) \quad .. \quad .. \quad (1)$$

where S_o is the substrate concentration in the assay medium, $n = 2$, 4 or 6 depending on whether the tissue is a slice, cylinder or sphere, l is half the thickness of the slice, or radius of the cylinder or

sphere, and r is the distance from the centre plane, axis or point.

(ii) *Substrate concentration low* ($S \ll K_m$)

The dependence of substrate concentration on position within the tissue obeys the following relations for the slice, cylinder and sphere respectively:

slice, $S = S_o \cosh(\alpha r)/\cosh(\alpha l)$ .. (2)

cylinder, $S = S_o I_o(\alpha r)/I_o(\alpha l)$ .. .. (3)

sphere, $S = S_o l \sinh(\alpha r)/r \sinh(\alpha l)$ .. (4)

where S, S_o, r and l have the same meaning as above, $I_o(x)$ is the hyperbolic Bessel function, and $\alpha = (V_{max}/K_m D)^{\frac{1}{2}}$.

The nature of the substrate gradients at low substrate concentration is illustrated in Fig. 1. Fig. 1A shows that for a constant value of α(100 cm^{-1}) and l (0·04 cm), the gradient is steepest for the slice, and least steep for the sphere. However, the influence of tissue shape is not great, and it becomes less with increasing values of α and l. Fig. 1B shows that for a tissue of fixed shape (the slice is used as an example but the same holds for the cylinder or sphere) the substrate gradient becomes significant for values of αl around 1, and its steepness increases rapidly as the value of αl increases. Thus if l and D are fixed, the gradient increases with increasing enzyme activity (V_{max}/K_m), and if the enzyme activity and D are constant, the relative gradient increases rapidly with increasing thickness of the tissue. For tissues with large values of α or l, the substrate concentration towards the centre of the tissue will be negligible and all the enzyme activity is contributed by those active sites closest to the surface. Thus as l increases, the measured total activity will not increase in proportion to the mass of the tissue, but only in proportion to the increase in surface area. For thick slices, in which the surface area does not change as l increases, the total activity for large values of l will ultimately become constant and independent of l. As will be seen later, this point has a considerable bearing on the assessment of cholinesterase activity and inhibition in thick tissues.

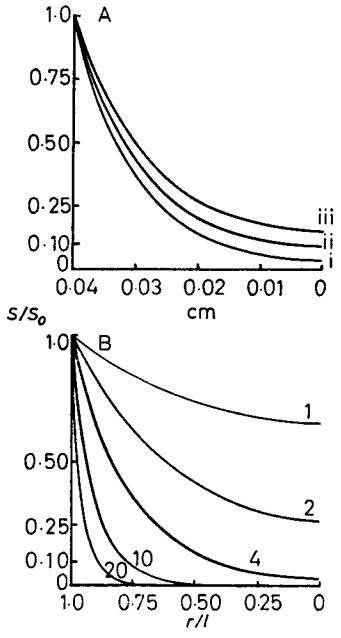

FIG. 1A. Theoretical substrate gradients (S/S_o) for low substrate concentrations ($S_o \ll K_m$) in an intact tissue slice (i), cylinder (ii) or sphere (iii), for which l = 0·04 cm and α = 100 cm^{-1}. x axis—distance (cm) from centre of tissue (r).

FIG. 1B. Theoretical substrate gradients (S/S_o) for low substrate concentrations ($S_o \ll K_m$) in an intact tissue slice for the values of αl indicated against each curve. x axis–fraction of distance between centre and surface of slice (r/l).

Under conditions where $S \ll K_m$, the substrate gradient will markedly affect the rate of the enzyme-catalysed reaction. In the steady state, the rate of substrate destruction per unit mass of tissue equals the rate of diffusion of substrate into the tissue across the whole tissue surface (which, by Fick's First Law of Diffusion, equals $DA(dS/dr)_{r=l}$, where A is the surface area) divided by the mass of the tissue. The value of $(dS/dr)_{r=l}$ can readily be obtained from (2)–(4) and, since tissue densities are about 1 kg litre^{-1} the mass of the tissue can be equated to its volume. This gives the following rates of substrate destruction per unit mass of tissue:

slice, $v = (\alpha D S_o/l) \tanh(\alpha l)$ .. .. (5)

cylinder, $v = (2\alpha D S_o/l)\,[I_1(\alpha l)/I_o(\alpha l)]$ .. (6)

sphere, $v = (3\alpha D S_o/l)\,[\coth(\alpha l) - 1/\alpha l]$ .. (7)

If αl is large (>1·5 for the slice, >5 for the cylinder, and >10 for the sphere) the hyperbolic term in equations (5)–(7) approaches 1, hence the rate of substrate destruction for all three tissues is encompassed by the general equation:

$$v = n\alpha D S_o/l = (nS_o/l)\,(V_{max} D/K_m)^{\frac{1}{2}} \quad (8)$$

where n = 1, 2 or 3 for the slice, cylinder or sphere respectively.

If αl is small, namely for a very thin tissue, or one possessing only low enzyme activity, diffusion ceases to be rate-limiting, and in all three cases, $v = V_{max} S_o/K_m$, the same as the value of v for the homogenate at low substrate concentration.

Although if $S \gg K_m$ there will still be a substrate gradient present, all the enzyme active sites will be saturated with substrate, even at the centre of the tissue. Consequently, the existence of the gradient will not affect the rate of the reaction, which will simply be V_{max}, the same as the rate for the homogenate at saturating substrate concentration.

Since, when αl is large, the rate depends on V_{max} at high substrate concentration, but on $(V_{max})^{\frac{1}{2}}$ at low substrate concentration, the dependence of v on S in intact tissues cannot adhere strictly to the simple Michaelis–Menten relationship. Some model solutions of the general equation $D\nabla^2 S = V_{max}S/(K_m + S)$ obtained by numerical integration using arbitrary kinetic constants for an enzyme in disc form, have confirmed that Michaelis–Menten kinetics are not obeyed by this system (Goldman, Kedem & Katchalski, 1968).

Equations (1) to (8) are derived on the assumptions (a) that the tissue contains a single enzyme with fixed values of K_m and V_{max}, (b) that this enzyme is uniformly distributed throughout the tissue, and (c) that the diffusion coefficient D of the substrate is constant throughout the tissue, so that the enzyme in all parts of the tissue is equally accessible to the substrate. For all real tissues, none of these assumptions will be completely correct. Thus, besides acetylcholinesterase, small amounts of butyrylcholinesterase, with different values of V_{max} and K_m, are likely to be present in all intact tissues, and it is extremely difficult to determine the precise proportion of the two. Both soluble and membrane-bound cholinesterases are found in many tissues, and these too may differ in K_m and V_{max} values. Tissues are also not homogeneous as far as acetylcholinesterase distribution is concerned. The bulk of the acetylcholinesterase in cholinergically-innervated tissues is associated with the neuro-effector junctions, and although most appears to be localized on external membranes, some is also present within muscle and nerve fibres, where it is probably inaccessible to exogenous substrate. A further problem with acetylcholinesterase is that at high substrate concentration it is subject to substrate inhibition, which means that the Michaelis–Menten equation cannot be applied at very high values of S. In these circumstances it is not expected that equations (1) to (8) will give an exact description of the kinetic properties of cholinesterase (or any other enzyme) in intact tissues, but, as discussed below, they do allow a meaningful interpretation of many of the more puzzling properties which cholinesterase displays when it is measured on intact tissues.

DISCUSSION

Application to cholinesterase assay

Although substrate gradients can exist within intact tissues at both high and low external substrate concentration, these gradients most affect the rate of the enzyme-catalysed reaction when the substrate concentration is low. At low external substrate concentration, the factor which determines whether diffusion control is significant is the magnitude of αl, where $\alpha = (V_{max}/K_m D)^{\frac{1}{2}}$.

For acetylcholinesterase and acetylcholine, the experimental value of K_m for tissue homogenates in assay media of moderate ionic strength is largely independent of the nature of the tissue and has a value around 100–200 μM (Rosenberg & Dettbarn, 1963; Brestkin, Brik & others, 1970; Baldwin & Lesser, 1971; Dawson & Crone, 1973). V_{max} for a wide range of tissue homogenates varies between about 10 and 200 μmol kg^{-1} s^{-1}, being relatively low for rat diaphragm (Berry & Rutland, 1971; Mayer & Michalek, 1971; Welsch & Dettbarn, 1972) and high for brain (Hobbiger & Lancaster, 1971) and electroplax (Rosenberg & Dettbarn, 1963). The diffusion coefficient, D, for acetylcholine in free solution is similar to that for other small ions, namely about 10^{-5} $cm^2 s^{-1}$, but in tissues, such as rat diaphragm or other muscle cells, D may be lower (Krnjevic & Mitchell, 1960; Kushmerick & Podolsky, 1969; Brookes & Mackay, 1971). Typical values of l for brain slices range from 0·01 to 0·05 cm (Hobbiger & Lancaster, 1971), while rat diaphragm may also be regarded as a slice with l between 0·02 and 0·05 cm, depending on the weight of the rat (Krnjevic & Mitchell, 1960; Brookes & Mackay, 1971). Chick biventer cervicis muscles may be regarded as typical of cylindrical tissues, with l about 0·04 cm (Baldwin & Lesser, 1971). Electroplax has a prismatic structure between a slice and a cylinder, with a minimum value of l of about 0·02 cm (Nachmansohn, 1959). For all these tissues, α has a value of 10^2 to 10^3 cm^{-1}, and αl is substantially greater than 1. Thus diffusion control would be expected to operate, and, initially at least, it would seem valid to apply equation (8) to the assay of cholinesterase in these intact tissues at low substrate concentration.

The most extensive studies of cholinesterase in intact tissues at very low substrate concentrations are those of Ehrenpreis, Mittag and their co-workers who used a radiometric cholinesterase assay with acetylcholine present at micromolar concentrations or less (Ehrenpreis, Mittag & Patrick, 1970). For intact rat diaphragm (Mittag, Ehrenpreis & Hehir, 1971b; Mittag, Ehrenpreis &

others, 1971a), or guinea-pig ileum (Mittag, Ehrenpreis & Patrick, 1971c) the apparent K_m value was about 1–2 mM, which is ten times higher than the value normally found for acetylcholine in homogenates. At very low substrate concentration where $S \ll K_m$, the rate of hydrolysis (v) is directly proportional to the external substrate concentration (S_o), so that $v = kS_o$. Experimentally, the rate constant k was about 0·2–0·4 min^{-1} for diaphragm and 0·4–1 min^{-1} for guinea-pig ileum. For a rat diaphragm, treated as a slice with $n = 1$, $l = 0{\cdot}04$ cm, $V_{max} = 10\,\mu mol\,kg^{-1}\,s^{-1}$, $K_m = 200\,\mu mol\,litre^{-1}$ and D assumed to be $10^{-6}\,cm^2\,s^{-1}$, the rate constant calculated from equation (8) is 0·34 min^{-1}. In view of the many assumptions involved in this theoretical calculation, this value is in excellent agreement with the above experimental values. The experimental rate constant for the rat diaphragm fell from 0·39 min^{-1} to 0·245 min^{-1} when older rats were used, with diaphragms of twice the original weight (Mittag & others, 1971b). With increasing age, the diaphragm is likely to get thicker, with an increase in l. Since the calculated rate constant is proportional to $1/l$, an increase in thickness from 0·5 to 0·8 mm would account for this decrease in rate constant. Another interesting observation (Mittag & others, 1971c) was that with guinea-pig ileum, stretching the intact tissue by about 25% caused up to a four-fold increase in activity. Stretching the tissue would be expected to have two consequences; firstly, the tissue sheet would become thinner (i.e. reduce l), which would cause a small increase in activity, and secondly, it might markedly increase the diffusion coefficient D. Physical changes have been shown to affect the diffusion coefficient of quaternary ions through rat diaphragm (Brookes & Mackay, 1971). An increase in diffusion coefficient from 10^{-6} to 10^{-5} $cm^2\,s^{-1}$ coupled with a 25% decrease in l, would lead to a four-fold increase in the value of v calculated from equation (8). Direct evidence that there is a linear inverse relation between the activity of intact tissues and their thickness ($2l$) has been obtained for the hydrolysis of 0·5 mM acetylthiocholine by slices of rat brain of various thicknesses (Hobbiger & Lancaster, 1971).

Ehrenpreis (1967) and Mittag & Patrick (1968) reported that at the ED50 (10^{-8}—10^{-7} M) for contraction of several tissues, such as rabbit atria, rat stomach, frog rectus abdominis muscles and guinea-pig ileum, the rate of acetylcholine hydrolysis by these tissues in a gut bath was very low, and that even after 1 h, the concentration of acetylcholine in the bath did not change to any significant extent. They concluded that the action of exogenous acetylcholine on these tissues was not influenced by enzymic hydrolysis. If the ileum is considered as a slice with a thickness of about 0·1 cm, and D is $10^{-6}\,cm^2\,s^{-1}$, by substituting the measured first order rate constant for hydrolysis (0·6 min^{-1}) (Mittag & others, 1971c), into (8) it can be calculated that αl has a value of about 25. Reference to Fig. 1B will show that under these conditions there will be a very marked acetylcholine gradient within the tissue. Since the acetylcholine receptor occupancy in the tissue is determined by the acetylcholine concentration in the immediate vicinity of each receptor, the fractional receptor occupancy will be much less at the centre of the tissue than at the surface. Thus the total fractional receptor occupancy, and hence the response, will be determined not only by the external acetylcholine concentration, but also by the acetylcholine gradient within the tissue, which, in turn, depends on the rate of hydrolysis by cholinesterase. Hence, even though the destruction of acetylcholine in the organ bath is negligible, it is wrong to conclude that the intensity of the tissue response to acetylcholine does not depend on the rate of hydrolysis.

For tissue homogenates at low substrate concentration (i.e. where $S \ll K_m$), the rate of hydrolysis is given by $v = V_{max}S_o/K_m$. This expression combined with (8) leads to the following ratio for the relative activities of intact tissue and homogenate at the same substrate concentration:

$$v_{intact}/v_{homogenate} = (n/l)\,(K_m D/V_{max})^{\frac{1}{2}} \qquad (9)$$

If the same values of l, K_m, D and V_{max} for rat diaphragm as used earlier (above), are inserted into (9) the calculated value of $v_{intact}/v_{homogenate}$ is 0·11 Experimentally, values of about 0·03 (Fleisher & others, 1960) and 0·08 (Heffron, 1972) have been obtained by bioassay of the acetylcholine remaining in contact with intact and homogenized diaphragm after various periods of time. Cholinesterase in brain is rather more active than in diaphragm, with V_{max} about $100\,\mu mol\,kg^{-1}\,s^{-1}$ (Hobbiger & Lancaster, 1971). An increase in V_{max} from 10 to $100\,\mu mol\,kg^{-1}\,s^{-1}$ reduces $v_{intact}/v_{homogenate}$ from 0·11 to about 0·03, which is in excellent agreement with the value (0·025) found by Hobbiger & Lancaster (1971) for the hydrolysis of acetylthiocholine (0·5 mM) by brain slices. For a typical cylindrical tissue, namely chick biventer cervicis muscles, where $n = 2$, $l = 0{\cdot}04$ cm, $K_m = 200\,\mu M$, $V_{max} = 50\,\mu mol\,kg^{-1}\,s^{-1}$ and D is assumed to be $10^{-6}\,cm^2\,s^{-1}$, the calculated ratio is 0·10 which is in close agreement with the value obtained manometrically at a substrate concentration of 73 μM (Baldwin & Lesser, 1971).

Because of the experimental difficulty in obtaining accurate rate measurements of cholinesterase at low substrate concentration, most of the earlier comparisons between intact tissue and homogenate, as well as some of the more recent ones, were made at high substrate concentrations. From equation (1), the concentration of substrate attained at the centre of the intact tissue at high substrate concentration, is given by $S_{centre} = S_o - V_{max}l^2/nD$. Assuming no serious permeability barriers in the intact tissue, the activity of the intact tissue will equal that of the homogenate when all the active sites in the intact tissue are effectively saturated with substrate. This position will be reached when $S_{centre} \geqslant 10K_m$, thus S_o must be $\geqslant 10K_m + V_{max}l^2/nD$. If $K_m = 200$ μM, $D = 10^{-6}$ cm^2 s^{-1} and V_{max} and l have values of the order previously discussed, S_o must be > 4 mM for a thin ($l = 0{\cdot}02$ cm), weakly active ($V_{max} = 10\,\mu$mol kg^{-1} s^{-1}) slice, and >250 mM for a thick ($l = 0{\cdot}05$ cm), active ($V_{max} = 100\,\mu$mol kg^{-1} s^{-1}) slice. Experimentally, many intact tissues at substrate concentrations of 30–100 mM have been found to have cholinesterase activities approaching or equalling V_{max} for the homogenate (Berkowitz, 1955; Strickland & Thompson, 1955; Dettbarn & Rosenberg, 1962; Rosenberg & Dettbarn, 1963; Baldwin & Lesser, 1971; Hobbiger & Lancaster, 1971; Welsch & Dettbarn, 1972). Identity of the maximum activities of intact tissue and homogenate indicates that there is no absolute permeability barrier to the substrate in the intact tissue (Rosenberg & Dettbarn, 1963).

It is clear from the above analysis, that comparison of the enzyme activities of intact tissue and homogenate can only be used to determine the fraction of enzyme active sites which are accessible to substrate in the intact tissue (i.e. the proportion of the enzyme which is 'external' or 'functional') if it is first verified that the substrate concentrations used give maximal activity in both cases. Assays under any other conditions, particularly assays at low substrate concentration, will give an erroneous estimate of this fraction of the enzyme.

Application to cholinesterase inhibition

A major pharmacological interest in cholinesterase assay has been to relate the degree of cholinesterase inhibition in cholinergically-innervated tissues to disturbance of function, either facilitation or block of cholinergic transmission, or the potentiation of exogenous acetylcholine. Koelle (1957) and McIsaac & Koelle (1959) showed that the pharmacological effects of anticholinesterases at autonomic ganglia or at neuromuscular junctions were related to the degree of inhibition of that fraction of the total enzyme in the tissue which is accessible to extracellular acetylcholine ('surface', 'external' or 'functional' cholinesterase). Almost complete inhibition of the intracellular or 'reserve' enzyme caused no functional disturbance. Subsequently, it has been repeatedly argued that the degree of enzyme inhibition found in tissue homogenates (in which the substrate has access to both 'functional' and 'reserve' enzyme) may be misleading, and that more meaningful measurements can be made using intact tissues in which the exogenous substrate has access only to the 'functional' enzyme (Fleisher & others, 1958; Fleisher & others, 1960; Baldwin & Lesser, 1971; Harris & others, 1971; Mittag & others, 1971b). It has been further argued that since the pharmacological effects of acetylcholine are normally elicited at low concentration, cholinesterase assays intended to relate enzyme inhibition to alteration in function should also be conducted at low concentrations of substrate.

In a typical experiment, an animal is killed some time after injection of the inhibitor, and a suitable tissue (such as the diaphragm-phrenic nerve) is excised and set up in an organ bath. The degree of facilitation (or block) of responses to cholinergic nerve stimulation can be assessed, as can any potentiation of exogenous acetylcholine. The tissue may then be assayed for residual cholinesterase activity, with or without prior homogenization, and a relation established between functional disturbance and degree of inhibition. The organophosphate anticholinesterases are ideally suited to such studies, as they act by phosphorylating the enzyme active centres to give a stable, inactive product, and enzyme activity recovers only very slowly when the free inhibitor is removed by dilution, dialysis or washing. Unfortunately, the extent of cholinesterase inhibition required to produce a given degree of functional disturbance differs when measured on homogenates from that observed with intact tissue. For example, for marked potentiation by di-isopropylphosphorofluoridate (DFP) of the action of acetylcholine on rat diaphragm, 80–90% inhibition was required when assayed on the diaphragm homogenate (Barstad, 1960), but only about 20% when measured on the intact tissue at low substrate concentration (Ehrenpreis, Chiesa & others, 1967; Hazra, 1967). Ehrenpreis (1967) reported that DFP and eserine cause marked potentiation of the actions of acetylcholine on a number of tissues, such as rat stomach and diaphragm and rabbit atria, at doses which caused relatively little cholinesterase inhibi-

tion when assayed on the intact tissues, although the doses of these inhibitors (0·05 μg ml^{-1} DFP, 0·1 μg ml^{-1} eserine) were such as would normally be expected to give substantial inhibition in tissue homogenates. On the basis of these observations Ehrenpreis argued that the acetylcholine potentiation must be due to an effect on the receptors and not to prevention of acetylcholine destruction.

At a fixed, low ($S \ll K_m$) substrate concentration, the activity of an enzyme in a tissue homogenate is proportional to V_{max}/K_m, whereas, from (8), that in the intact tissue is proportional to $(V_{max}/K_m)^{\frac{1}{2}}$. Since enzyme inhibitors decrease V_{max}, increase K_m, or both, the measured degree of inhibition resulting from a given change in V_{max} or K_m will be less when the enzyme is assayed in an intact tissue than when assayed as a tissue homogenate. The difference will exist irrespective of any differences arising from selective inhibition of 'functional' or 'reserve' enzyme. Since organophosphates inhibit cholinesterase irreversibly, their kinetic effect is to reduce the value of V_{max} by that fraction of the active centres of the accessible enzyme with which they combine. If y is the fraction of active sites remaining uninhibited, then if the tissue is homogenized before enzyme assay, the activity measured on the homogenate at high substrate concentration will be yV_{max}, and at low substrate concentration $yV_{max}S/K_m$. The percentage inhibition in both cases is 100 $(1-y)$ and is independent of the substrate concentration used for the assay. On the other hand, if the inhibited enzyme is assayed directly using the intact tissue, at a high enough substrate concentration the measured activity will still be yV_{max} and the apparent percentage inhibition will still be 100 $(1-y)$, the same as for the homogenate, but, at low substrate concentration the measured activity will be $(nS_0/l)\,(yV_{max}D/K_m)^{\frac{1}{2}}$, and the apparent degree of inhibition 100 $(1-y^{\frac{1}{2}})$. Thus the degree of inhibition in the intact tissue will appear to decrease as the substrate concentration is reduced. This phenomenon results from the fact that when the enzyme is assayed using intact tissue and low substrate concentrations, the substrate is able to penetrate further into the tissue as some of the enzyme active sites become inhibited, thus reducing the steepness of the substrate gradient within the tissue. The measured enzyme activity will consequently be greater than that expected from the fraction of active sites inhibited.

These conclusions are supported by two studies in which cholinesterase inhibition was measured over a range of substrate concentrations on intact tissues and on homogenates made from them. Hobbiger & Lancaster (1971) injected rats with DFP, then assayed the residual acetylcholinesterase in brain slices, before and after homogenization. They found that the apparent degree of inhibition in the slices was invariably less than that in the homogenates, and that whereas the degree of inhibition produced by a given dose of DFP was independent of substrate concentration when assayed on the homogenate, the degree of inhibition when assayed on the intact slices fell as the substrate concentration was lowered. After 2·5, 5, 10 or 20 μmol kg^{-1} of DFP, the percentage inhibition [$= 100\,(1-y)$] in the homogenates, assayed at a substrate concentration of 0·5 mM, was 23, 43, 86 and 92% respectively, whereas in the intact slices it was 12, 22, 44 and 61%. The corresponding values of $100\,(1-y^{\frac{1}{2}})$ are 12, 25, 63 and 72% which are in fair agreement with the experimental values for the intact slices. Baldwin & Lesser (1971) carried out similar experiments with chick biventer cervicis muscles and eserine as inhibitor. They also found that the apparent degree of inhibition of the enzyme in the intact tissue unexpectedly increased with increasing substrate concentration, but was less than that in the homogenate at all substrate concentrations below 0·1M. Eserine is less satisfactory than DFP for this type of experiment since the inhibited enzyme recovers its activity (by spontaneous decarbamylation of the carbamylated active centre) at a moderate rate (Winteringham & Fowler, 1966), hence cholinesterase assays made after removal of free inhibitor probably underrate the true degree of inhibition.

The quantitative relationships just derived, depend on the assumption that the inhibitor acts uniformly throughout the intact tissue, so that the residual active centres after treatment with the inhibitor are distributed throughout the tissue in the same pattern as the total enzyme. With relatively labile inhibitors, such as the organophosphates, this will probably not be true. The inhibitor will tend to react preferentially with the enzyme active sites that it encounters first, hence the enzyme active centres close to the surface of the tissue will tend to be inhibited before those nearest the centre. As far as assays with the homogenate are concerned this is immaterial, but with intact tissues it could be very important, especially with thicker tissues. As can be seen from Fig. 1B, a steep substrate gradient will mean that the only enzyme active sites which make a significant contribution to the total activity at low substrate concentration are those very near the surface. The complete inhibition of these sites will merely serve to allow the substrate to penetrate

quantitatively to the uninhibited enzyme nearer the centre of the tissue, so that the substrate may still be broken down at much the same rate as in the uninhibited tissue. Thus, a substantial degree of inhibition of enzyme active sites in the peripheral regions of the tissue which would be readily measureable when the enzyme is assayed in the tissue homogenate, may go largely undetected in assays on intact tissue. This phenomenon probably accounts for the discrepancy between the results obtained by Barstad (1960) and by Ehrenpreis (1967) described earlier. As the peripheral enzyme becomes inhibited, the substrate (acetylcholine) will obviously be able to reach a much larger fraction of the available acetylcholine receptors in the intact tissue, resulting in a substantial potentiation of its pharmacological effects, but this will not be accompanied by an apparent corresponding inhibition of cholinesterase if this is assayed on the intact tissue at the low acetylcholine concentrations which are all that are needed to elicit the pharmacological responses.

Fleisher and co-workers (1958, 1960) have attempted to relate the degree of oxime-induced recovery of cholinesterase inhibited by administration of near-lethal doses of organophosphates to the restoration of neuromuscular function. The enzyme activity was measured at moderately low substrate concentration on both intact tissues and homogenates using either frog rectus abdominis muscles or rat diaphragm. The intact tissue was always found to recover its activity faster than the homogenate. Since the reactivators used were of the quaternary type (such as 2-PAM), the difference was attributed to greater recovery of the 'external' or 'functional' enzyme, which is probably the only fraction accessible to quaternary compounds. On the assumption that what was actually being measured in the intact tissue was exclusively this 'functional' enzyme (estimated at 2·6–5% of the total, based on the activity of the intact tissue relative to that of the homogenate), it was argued that the intact tissue measurements gave the best guide to the amount of cholinesterase required to maintain neuromuscular function. As already pointed out, the activity of intact tissue relative to homogenate at low substrate concentration is largely determined by the relative magnitudes of V_{max}, K_m, D and l, and does not measure the 'functional' enzyme. The relative recoveries reported in intact tissue and homogenate are roughly consistent with the $y^{\frac{1}{2}}$ rule (p. 271). For example, in frog rectus abdominis muscles inhibited with sarin and then treated with 2-PAM, 35% recovery of cholinesterase was found in the muscles after homogenization (i.e. $y = 0{\cdot}35$) and 71% in the intact muscles ($100\,y^{\frac{1}{2}} = 59\%$) (Fleisher & others, 1958). In rat diaphragm inhibited with sarin and reactivated with the quaternary oxime TMB4, the activity of the diaphragm after homogenization was 10% of normal ($y = 0{\cdot}10$) and that of the intact diaphragm 28% of normal ($100\,y^{\frac{1}{2}} = 32\%$) (Fleisher & others, 1960). In similar experiments with rat diaphragm using DFP as inhibitor and 2-PAM as reactivator (Mittag & others, 1971b), the activity of the homogenate was 32% of normal ($y = 0{\cdot}32$) and that of the intact tissue 67% ($100\,y^{\frac{1}{2}} = 57\%$). Harris & others (1971) studied the reactivation of sarin-inhibited cholinesterase in intact guinea-pig retinas. In the absence of inhibitor, and assayed with 3 mM acetyl-β-methylcholine as substrate, the intact retina had only 10% of the activity obtained after disruption of the retinas by sonication. In this tissue, the cholinesterase activity recovers slowly after inhibition with sarin without the aid of any external reactivator, and 24 h after complete inhibition, the activity of the retinas after sonication was 20% of normal ($y = 0{\cdot}20$) and that of the intact retinas 35% of normal ($100\,y^{\frac{1}{2}} = 45\%$). With either quaternary (2-PAM or toxogonin) or non-quaternary (mono-isonitrosoacetone) reactivators, the rate of recovery was faster when the enzyme was assayed on intact retinas than when assayed on retinal sonicates. This consistent difference is unlikely to be due to faster recovery of the 'functional' enzyme compared with the intracellular enzyme.

Although the correspondence between the observed recoveries of the enzyme when assayed on the intact tissue and that calculated by applying the $y^{\frac{1}{2}}$ rule to the recovery assayed on the homogenate is not exact, the agreement in all the above examples is close enough to suggest that the measured activity of the intact tissue at low substrate concentration is no guide to the degree of recovery of the 'functional' enzyme, but is largely determined by the changed substrate gradient within the tissue.

Irreversible inhibitors, and non-competitive reversible inhibitors exert their effects in kinetic terms by decreasing V_{max}. Competitive reversible inhibitors do not change V_{max}, but increase K_m by a factor of $(1 + I/K_I)$. However, as can be seen from (9), an increase in K_m has precisely the same effect on the ratio $v_{intact}/v_{homogenate}$ as a decrease in V_{max}. Thus for all types of inhibitor, the degree of inhibition when the enzyme is assayed on an intact tissue at low substrate concentration would be less than that produced at the same concentrations of inhibitor and substrate on the homogenate, and to achieve the

same apparent degree of inhibition in both preparations a higher concentration of inhibitor would be needed when the enzyme is assayed on the intact tissue. Webb & Johnson (1969) reported that a range of quaternary (and presumably reversible) inhibitors were all less active on cholinesterase in intact electroplax than they were on the purified soluble electric eel enzyme, but the relative order of potency was unchanged. They also found that whereas with the purified enzyme plots of $1/v$ against inhibitor concentration (I) were linear, these plots were non-linear for the intact electroplax. This may be readily understood, since on the soluble enzyme the activity is related to $1/K_m\ (1 + I/K_I)$, but, from (8), the activity of the intact tissue at low substrate concentration is related to $[1/K_m\,(1 + I/K_I)]^{\frac{1}{2}}$. With either acetylcholine or ethyl chloroacetate as substrate, the V_{max} values for intact electroplax and for the homogenate made from it are identical, indicating that there is no absolute permeability barrier in the intact electroplax to the penetration of quaternary ions to all the enzyme active centres (Rosenberg & Dettbarn, 1963).

General conclusions

If diffusion of substrate is a rate-limiting step in enzyme-catalysed reactions in intact tissues or tissue slices, a substrate gradient will develop within the tissue when the enzyme is assayed by immersion of the intact tissue in a solution of substrate. The existence of such substrate gradients does not alter the apparent V_{max} for the enzyme, but does cause deviation from normal Michaelis-Menten kinetics, and raises the apparent K_m (defined as the substrate concentration at which the rate is half-maximal). Provided no complications are introduced as a result of substrate inhibition, the activity of the intact tissue at sufficiently high substrate concentration will equal that of the homogenate made from it, unless some of the enzyme active sites are inaccessible to the substrate in the intact tissue due to permeability barriers. However, at low substrate concentration, the activity of the intact tissue will always be less than that of the homogenate and the ratio of the two will depend on the kinetic parameters (V_{max} and K_m) of the enzyme in the homogenate, the diffusion coefficient of the substrate in the tissue, and the shape and size of the intact tissue. Consequently, the activity of the intact tissue relative to that of the homogenate at low substrate concentration cannot be used to determine the fraction of enzyme active sites in the intact tissue which are accessible to the substrate ('surface', 'external', 'extracellular' or 'functional' enzyme).

The altered kinetic behaviour of enzymes in intact tissues may also have a profound effect on the apparent degree of inhibition produced by enzyme inhibitors. At low substrate concentration, inhibitors will be apparently less effective when the enzyme is assayed on an intact tissue than when it is assayed on the tissue homogenate. Thus, the degree of enzyme inhibition measured on an intact tissue at low substrate concentration is not a suitable parameter for correlating enzyme inhibition in a tissue with change in physiological function.

Although the equations developed in this paper to describe enzyme kinetics in intact tissues have been exemplified with particular reference to cholinesterase and acetylcholine, they have a potentially far wider biochemical and pharmacological significance. The substrate gradients which may develop when a tissue is bathed with an exogenous substrate which is a neurotransmitter would not exist when that transmitter is liberated locally in synaptic regions by nerve stimulation. This may provide a basis for understanding the differences which are sometimes found between the effect of drugs on responses to nerve stimulation and on responses to exogenously applied neurotransmitter. Other inactivation processes (such as tissue uptake) may also cause substrate gradients to exist, and some of the confused literature in the field of adrenergic transmission may be amenable to re-interpretation in these terms. Superfusion and perfusion of low concentrations of drugs in intact tissues could likewise result in the development of concentration gradients within the tissue with a consequent effect on the quantitative interpretation of the results of such experiments.

Finally, since the nature of substrate gradients may be calculated quantitatively in specific instances, it would be possible to calculate the fractional receptor occupancy in a tissue bathed with a substrate which is an agonist on that receptor. The effect of enzyme inhibition on this fractional occupancy could also be calculated, and thus permit a quantitative assessment of the effect of enzyme inhibition on the pharmacological responses of the tissue to the substrate concerned.

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