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A Determination of K_s , the Substrate Constant, for Serum Cholinesterase

By A. R. MAIN

Occupational Health Division, Department of National Health and Welfare,
45 Spencer Street, Ottawa, Ontario, Canada

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K_s , the substrate constant (Dixon & Webb, 1958), has not previously been determined for esterases. Slater (1955) has suggested that if the constant k_3 , governing the rate of breakdown of the enzyme-substrate complex, can be varied independently of K_s , then the plot of K_m against V , the maximum reaction velocity, will give K_s on extrapolation to the K_m axis. In this paper the alcohol-

activation and reversible inhibition of the hydrolysis of certain nitrophenyl carboxylic esters by human serum cholinesterase have been employed to evaluate K_s by the use of the suggestions, but not the procedure, of Slater (1955).

Knowledge of K_s is particularly important when evaluating the substrate-specificity pattern of esterases since it is K_s , and not K_m , that is a

measure of the affinity of the enzyme for the substrate. Knowledge of K_s is also desirable when determining activation energies, since K_s is a true equilibrium constant whereas K_m includes a rate constant k_3 as well as the equilibrium constant K_s .

EXPERIMENTAL

Activities were measured in most instances with a Beckman DK-2 recording spectrophotometer equipped with a time drive and a temperature-regulated cell-holder. Usually, human serum was used as a source of cholinesterase. As observed by Aldridge (1953), human serum does not contain measurable B- or A-esterase activity. The only esterases hydrolysing *o*-nitrophenyl butyrate, *p*-nitrophenyl butyrate and similar esters were therefore cholinesterase and A-esterases.

The DK-2 can be made to record the difference in extinction between the contents of a reference cell and an unknown cell as a function of time. The reference solution contained 0.1 mM-diisopropyl phosphorofluoridate (DFP) but otherwise was identical with the unknown solution. The DFP completely and quickly inhibited the cholinesterase, but left the A-esterases unaffected. The rate of change of extinction between the reference and unknown cells was therefore a measure of the cholinesterase activity. The A-esterase activity towards *o*-nitrophenyl butyrate was negligible (Main, Miles & Braid, 1961), but was of the same order as the cholinesterase activity towards *p*-nitrophenyl butyrate, *p*-nitrophenyl acetate and *o*-nitrophenyl acetate.

o-Nitrophenyl butyrate was prepared and purified according to the method of Main *et al.* (1961). *o*-Nitrophenyl acetate was purchased from the Eastman Kodak Co. (Rochester, N.Y., U.S.A.). *p*-Nitrophenyl acetate and *p*-nitrophenyl butyrate were prepared according to the method of Aldridge (1953). The *p*-nitrophenyl butyrate was purified in a manner similar to that used for *o*-nitrophenyl butyrate.

Stock solutions (0.5M) of these esters were prepared by weighing out the appropriate amount and dissolving in slightly acid methanol (e.g. 1.046 g. of *o*-nitrophenyl butyrate to 10 ml. with methanol).

Substrate solutions were prepared by pipetting appropriate volumes of stock solution into 50 mM-sodium phosphate buffers, pH 7.6. The solubility of *o*-nitrophenyl butyrate at 25° under these conditions was about 1.5 mM. A mM-*o*-nitrophenyl butyrate solution was prepared by pipetting 0.20 ml. of stock into 100 ml. of buffer. Lower concentrations were prepared by diluting the mM-*o*-nitrophenyl butyrate solution.

The alcohols were reagent-grade commercial products. Appropriate volumes were diluted with phosphate buffer to obtain the final alcohol concentration (v/v) desired.

Human serum was obtained from the blood of volunteers. No anticoagulants were used. To obtain comparable results it was important to use fresh serum which had not been frozen nor stored for periods longer than a week. The K_m with *o*-nitrophenyl butyrate decreased gradually from about 0.14 mM for fresh serum to values as low as 0.06 mM. This storage effect had no relevance to the effect of alcohols, which will be discussed. However, the results obtained from studies with alcohols and serum of differing ages suggested that the shift in K_m was associated with K_s . The

possibility is suggested that a naturally occurring inhibitor is associated with cholinesterase in fresh serum and that this inhibitor gradually falls away during storage.

The serum was diluted with buffer containing the appropriate alcohol, 0.1% in acacia, so that the concentration of the alcohol in the enzyme solution was identical with that of the substrate solution. Typically 1.0 ml. of serum was diluted to 100 ml. with appropriate buffer and 1 ml. of this dilution containing 0.01 ml. of serum was used for an activity determination. The dilution was stored continuously in crushed ice.

The reference enzyme dilution was prepared by taking 25 ml. of the initial dilution and adding 0.10 ml. of a 25 mM-DFP solution made with propan-2-ol.

A typical determination was performed as follows: 1.0 ml. of a suitable serum dilution was pipetted into a clean, dry 25 ml. conical flask (A). Similarly, 1.0 ml. of the corresponding reference dilution (0.1 mM in DFP) was pipetted into flask (B). Buffered substrate (5.0 ml.) was pipetted simultaneously into each of the two flasks. After mixing, and as quickly as possible, a suitable amount of A was poured into the 'unknown' cell and a suitable amount of B into the 'reference' cell. The rate of change of extinction was plotted for at least 10 min. A straight line, whose slope was a measure of the activity, could usually be obtained over the major part of this interval for each substrate concentration with suitable variations of enzyme concentration. Just before every determination, the ratio-recording spectrophotometer was calibrated with respect to 0 and 100% transmittance with a freshly prepared reaction mixture corresponding to the final contents of flask B.

The extinction of *o*-nitrophenol was measured at 414 m μ (Main *et al.* 1961) and that of *p*-nitrophenol at 400 m μ .

The temperature was controlled by circulating the water from a temperature-controlled bath through the cell-holder. The constant-temperature bath was kept at 24.5° to maintain the thermometer in the regulator at 25.1°.

The activity was calculated in terms of μ moles of nitrophenol liberated/ml. of serum/min.

Occasionally activities were determined with the Hilger Uvispek spectrophotometer as follows. Buffered substrate (5.0 ml.) was pipetted into two or more volumetric flasks A and B. To B was added 0.05 ml. of 25 mM-DFP. The flasks were placed in a bath at 25° and 0.2–1.0 ml. of enzyme dilution containing a suitable amount of serum (0.004–0.05 ml., depending on the substrate and alcohol concentration) was pipetted into both flasks A and B and the time was noted. After a suitable digestion time (10–30 min., again depending on conditions) 0.05 ml. of DFP was added to flask A. Flasks A and B were then immediately made up to the 10 ml. mark with 40% (v/v) ethanol solution. The ethanol terminated the A-esterase activity. The colour was stable for at least 1 hr. and could be read at any convenient time on the Hilger Uvispek instrument.

RESULTS

The activities at five different concentrations of *o*-nitrophenyl butyrate were determined in 0, 0.5, 1, 2.5 and 3.5% butanol. The results were plotted according to the Dixon (1953) modification of the Lineweaver & Burk (1934) plot. As shown in Fig. 1, the extrapolations of the experimental

points were carried below the $1/s$ axis into the lower left-hand quadrant, where the system of lines obtained approximated a common point of intersection (P). Interpretation of the point P led to a new method for evaluating K_s .

Each line corresponded to the activities at a single butanol concentration. The intercept on the vertical axis gave the reciprocal of V , the maximum velocity. The intercept on the horizontal axis gave the negative reciprocal of K_m , where K_m has the Briggs & Haldane (1925) meaning:

$$K_m = (k_2 + k_3)/k_1 = K_s + k_3/k_1. \quad (1)$$

The individual rate constants are those used to describe the reaction

where E is the enzyme, S is the substrate and ES is the enzyme-substrate complex.

The point P represents the average $-1/s$ values of the points of intersection. If a line is drawn from P perpendicular to the $1/s$ axis, the intercept is equal to $-1/K_s$.

This conclusion was reached by considering the limiting value of $-1/K_m$ when $1/V$ is equal to infinity.

If P is joined to the $1/v$ axis at infinity, the line will be parallel to the $1/v$ axis since parallel lines meet at infinity.

On the $1/v$ axis, $1/s = 0$, and by substituting into

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{s} + \frac{1}{V} \quad (2)$$

then $1/v = 1/V$ and, at infinity, $V = 0$. Similarly, on the $1/s$ axis, $1/s = -1/K_m$. The intercept on the $1/s$ axis of the line joining P to infinity on the $1/v$ axis is therefore $-1/K_m$. But $V = 0$ for this line, and since e , the enzyme concentration, is constant, it follows from the relationship

$$V = k_3 \cdot e \quad (3)$$

that $k_3 = 0$. Substituting into (1), $K_m = K_s$ and $-1/K_m = -1/K_s$. Similarly it can be shown that the intercept on the $1/v$ axis of the line joining P to $1/s$ at infinity is $-1/k_3 \cdot e$. The slope of the line joining P to the origin is then $1/k_1 \cdot e$.

The five lines of Fig. 1 intersect at ten different points. The deviation from a common point of intersection appears to be random, suggesting the limits of experimental precision, rather than a progressive shift in K_s with increasing butanol concentration. Summing the values at each intersection and dividing by 10 gave K_s 0.072 ± 0.026 mM. But this method did not appear to be entirely satisfactory since equal weight was given to the value at each intersection regardless of the angle of intersection. The precision appears to depend on the angle of intersection, with the wider angle giving the higher precision. This was shown when K_s values were grouped in descending order of intersecting angles as in Table 1.

Fig. 1. k_3 -Activation plot. The Lineweaver & Burk (1934) plot of reciprocal activities against corresponding reciprocal o -nitrophenyl butyrate concentrations. Each line is the plot at the given butanol concentration. Intercepts on the $1/s$ -activity axis give $1/V$ and on the $1/\text{mM-}o$ -nitrophenyl butyrate axis, $-1/K_m$. The values of these intercepts obtained from a plot on 16 in. $\times$ 10 in. graph paper are given in Table 2.

It is evident that more confidence may be placed in the K_s values obtained at the wider angles of intersection. These observations suggest that the precision of the method may be somewhat greater than the standard deviation ± 0.026 mm implies.

K_s may also be evaluated by plotting V against K_m by the method of Slater (1955). Each butanol concentration was associated with characteristic

and particular values of K_m and V . These were calculated from the intercepts on the $1/v$ and $1/s$ axis as suggested by Fig. 1. In practice K_m and V were obtained from a plot on 10 in. $\times$ 16 in. graph paper and are given in Table 2.

If the plot of V against K_m gives a straight line, then according to Slater's (1955) equation

$$K_m = V \frac{1}{k_1 e} + K_s$$

(4)

K_m will equal K_s when V is equal to 0. As shown in Fig. 2, the plot of V versus K_m did approximate a straight line, from which K_s could be evaluated.

Evaluation of K_s from the point of intersection (P), Fig. 1, has advantages over the use of Slater's (1955) K_m versus V plot. Evaluation from the point of intersection is more direct, making use of primary activity data, whereas the Slater (1955) method uses secondary data. The intersection method also suggests the experimental conditions which will give the most precise K_s evaluation, whereas this is not readily apparent from the plot of K_m against V .

Table 1. K_s values grouped in descending order of intersecting angles

No. of points of intersection	Average angle of intersection (degrees)	$K_s \pm$ deviation (mm)
1	14.2	0.072
2	8.3 $\pm$ 2.3	0.069 $\pm$ 0.003
3	5.5 $\pm$ 2.0	0.073 $\pm$ 0.021
4	3.4 $\pm$ 2.1	0.081 $\pm$ 0.040

K_m and V were obtained by extrapolation according to the Dixon (1953) modification of the Lineweaver & Burk (1934) plot. For each butanol concentration the activities were determined at 1, 0.33, 0.2, 0.15 and 0.1 mm-*o*-nitrophenyl butyrate concentrations, with 1.0 ml. of diluted serum containing 0.01 ml. of the pooled serum from four individuals.

Butanol (% v/v)	V_{max} (μ moles of <i>o</i> -nitrophenol/ml. of serum/min.)	K_m (mm)
0	3.01	0.139
0.5	5.18	0.173
1.0	7.64	0.250
2.5	11.1	0.318
3.5	18.2	0.455

Fig. 2. Plot of V against K_m with extrapolation to the K_m axis giving K_s . (For conditions see Table 2.)

Fig. 3. Effect of butanol on V , showing the linearity of the relationship. (For conditions see Table 2.)

Table 3. *Effect of butanol on the p-nitrophenyl butyrate K_m and V of human serum cholinesterase*

K_m and V were obtained by extrapolation according to the Dixon (1953) modification of the Lineweaver & Burk (1934) plot. The activities were determined with the Beckman DK-2 recording spectrophotometer at 1, 0.33 and 0.2 mM *p*-nitrophenyl butyrate concentrations in duplicate for each butanol concentration. With water, 0.02 ml. of the pooled serum from four individuals was used for each determination but this was increased to 0.05 ml. for the activities determined in butanol.

Butanol (%, v/v)	V (μ moles of <i>p</i> -nitrophenol/ ml. of serum/min.)	K_m (mM)
0	1.06	0.80
0.2	0.83	0.72
0.5	0.63	0.63
1.0	0.40	0.50

Table 4. *Effect of alcohol chain-length on the activation of o-nitrophenyl butyrate hydrolysis by human serum cholinesterase*

Substrate solutions were 0.20M with respect to each alcohol. Activities were determined at pH 7.6 and 25° in the DK-2 recording spectrophotometer. K_m and V were determined with 1, 0.33 and 0.2 mM *o*-nitrophenyl butyrate concentrations and 0.010 ml. of serum from four individuals in a suitable dilution.

	V_{max} (μ moles of <i>o</i> -nitrophenol/ml. of serum/min.)	K_m (mM)
Water	3.28	0.135
Methanol (0.74M)	3.60	0.141
Ethanol	4.00	0.151
Propan-1-ol	5.88	0.202
Butan-1-ol	8.50	0.244

Activation of enzyme-catalysed ester hydrolysis by alcohols is not common, but inhibition by alcohols has been widely observed (e.g. Huggins & Lapidus, 1947). It was therefore of interest to determine whether alcohol inhibition of enzyme-catalysed ester hydrolysis was also a k_3 -dependent effect.

Butanol inhibited the hydrolysis of *p*-nitrophenyl butyrate by cholinesterase. Both K_m and V decreased, as shown in Table 3. Competitive inhibition typically results in an increased K_m . With non-competitive inhibition K_m remains unchanged whereas V decreases with decreasing effective enzyme concentration, k_3 remaining unchanged. Mixed competitive-non-competitive inhibition results in increased K_m and decreased V . The butanol inhibition of *p*-nitrophenyl butyrate hydrolysis by cholinesterase where both V and K_m decrease is therefore unique and characteristic of k_2 inhibition. The Lineweaver-Burk plots approxi-

mated a common point of intersection when extrapolated to the lower left-hand quadrant, from which the *p*-nitrophenyl butyrate K_s was 0.38 mM. Butanol inhibition of cholinesterase *p*-nitrophenyl butyrate hydrolysis appeared therefore to be a k_2 -dependent effect similar to *o*-nitrophenyl butyrate activation, but in reverse. A comparison of the K_s for *o*-nitrophenyl butyrate with the K_s for *p*-nitrophenyl butyrate suggested that the affinity of serum cholinesterase for *o*-nitrophenyl butyrate was five times as great as for *p*-nitrophenyl butyrate.

The effect of butanol on the hydrolysis of *o*-nitrophenyl acetate and *p*-nitrophenyl acetate catalysed by human serum cholinesterase was also examined. With *o*-nitrophenyl acetate an activation similar to that with *o*-nitrophenyl butyrate was observed, whereas with *p*-nitrophenyl acetate an inhibition corresponding to that of *p*-nitrophenyl butyrate occurred.

The effect of butanol on the hydrolysis of *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate by a Strelitz (1944) Stage-2 purified horse-serum cholinesterase preparation was also investigated briefly. Butanol activated the *o*-nitrophenyl butyrate hydrolysis strongly. However, the *p*-nitrophenyl acetate hydrolysis results were difficult to interpret because the *p*-nitrophenyl acetate K_m was in the order of 60 mM whereas the activities were determined at substrate concentrations of 1 mM and less. The accuracy of K_m and V determined by extrapolation under these conditions is necessarily too low to make measurement of differences meaningful. This impasse may be

Table 5. *Effect of the isomeric configuration of butanol and of acetone on the activation of o-nitrophenyl butyrate hydrolysis by human serum cholinesterase*

Activities were determined with mM *o*-nitrophenyl butyrate in 5% (v/v) solutions of butanol at pH 7.4, 25°; 0.20 ml. of a 1:20 serum dilution, equivalent to 0.01 ml. of serum from one individual, was added to 5 ml. of buffered substrate containing the butanol, according to the method described in the Experimental section with the Hilger Uvispek.

Isomer	Activity (μ moles of <i>o</i> -nitrophenol/ml. of serum/min.)	Percentage of control
Control (no butanol)	2.82	100
Butan-1-ol	8.45	298
2-Methylpropan-1-ol	4.81	171
2-Methylpropan-2-ol	4.64	164
Butan-2-ol	3.15	112
Butyric acid	2.90	103
Acetone*	4.26	151

* 3% (v/v) Acetone by DK-2 ratio-recording spectrophotometer.

overcome by using higher alcohol concentrations, which will permit the use of higher substrate concentrations.

Investigation of the effect of other alcohols on the hydrolysis of *o*-nitrophenyl butyrate showed that each alcohol activated. At equivalent concentrations, the activation increased with increasing chain length (Table 4). A common point of intersection was approximated when the activities were plotted by the method used in Fig. 1. The K_s value calculated was 0.077 ± 0.010 mM and was thus within the experimental limits of the K_s value obtained by varying the butanol concentration (0.072 ± 0.026 mM).

Pentan-1-ol was also tested and activated more than butanol at equivalent concentrations. These observations suggested that the activating effect of alcohols is through k_3 .

The effect of isomeric configuration and of acetone is given in Table 5. Butan-1-ol activated more than its isomers, but all isomers and acetone did activate. Butyrate ion did not activate.

DISCUSSION

The results described have been interpreted to suggest that K_s was constant whereas k_3 varied. Variation in k_3 has been inferred from the experimentally observed simultaneous increase or decrease in K_m and V . The approximation to a common point of intersection of the Lineweaver-Burk plots suggested that K_s was constant.

The case of simultaneous competitive inhibition and k_3 activation or inhibition has been investigated by M. Dixon & E. C. Webb (personal communication). Analysis of their hypothesis by the author suggested that K_m would not be a linear function of V should simultaneous variation of K_s and k_3 occur. The plot of K_m against V in Fig. 2 would be a curved line corresponding to a progressive shift in the lines of intersection. The observation that different alcohols yielded a similar K_s value was consistent with the interpretation that simultaneous competitive inhibition and k_3 activation had not occurred. This followed from the likely assumption that different alcohols would have different hypothetical K_s values.

There is at least one hypothetical case where both K_s and k_3 could vary simultaneously and give a straight-line plot of K_m against V . This line would pass through the origin, implying that k_2 is 0. But this would not necessarily be true since the plot would be obtained by a simultaneous variation of K_s (k_2/k_1) with k_3 . The Lineweaver-Burk plots for this would result in a series of parallel straight lines. Since no point of intersection would result no K_s could be derived.

The characteristics of the activation and inhibi-

tion described in this work are not readily interpreted by the usual concepts of specific interaction between the enzyme active centre and the activator or inhibitor.

The variety of solvents which activated, ranging from methanol to acetone, does not suggest a particular affinity for the active centre.

If it is postulated that butanol might have an affinity for the active centre on the basis of its similarity to the substrate, it must also be remembered that butyrate ion had no effect. Moreover butanol has a dual role depending on the substrate, activating *o*-nitrophenyl butyrate hydrolysis and inhibiting *p*-nitrophenyl butyrate hydrolysis. Finally when it is remembered that other quite different alcohols and acetone also activated, the concept of direct interaction appears more unlikely.

This is not to say that these solvents do not exert their influence in the region of the active centre. The enzyme protein is normally surrounded by water, which plays two roles during hydrolysis. It is the supporting medium and it is a reactant. It is suggested that the properties of this environment affect k_3 , governing the rate at which the enzyme-substrate complex breaks down. Addition of solvents displaces water and thus may alter the properties of the environment on which k_3 is dependent.

This k_3 effect may be termed an 'environmental' effect to distinguish it from the specific-interaction effects which are characteristic of competitive and non-competitive inhibitions and activation.

The property of the environment which the alcohols change and on which k_3 is dependent is not known. However, it seems possible that the dielectric constant may be concerned. Laidler (1955) has stated that: 'A reaction in which the activated complex is more polar than the reactants will be accelerated by an increase in the dielectric constant; conversely one in which the complex is less polar will be slowed down'. Addition of alcohols to water decreases the dielectric strength, and the decrease is proportional to the chain length of the alcohol. However, at the alcohol concentrations used, the change in dielectric strength does not appear to be sufficiently large to account for activating and inhibiting effects. The activation of *ortho*-orientated nitrophenol esters and the inhibition of *para*-orientated nitrophenol esters may be related to the relative polarity of these compounds. *ortho*-Compounds may form rings with adjacent groups, thus making them less polar than *para*-compounds.

The experimental method devised by Slater depended on varying the rate of breakdown of the enzyme-substrate complex into products by varying the concentration of a second substrate

necessary for this breakdown. Consequently hydrolytic enzymes could not be studied since the reactions could not be carried out in the absence of water. It seems most unlikely that the effect of alcohols is in any way related to the conception of a second substrate. Consequently Slater's (1955) experimental treatment is related to the present procedure only by the fact that both methods depend on varying k_3 independently of K_s . Slater's (1955) method appears to apply only when k_3 is a first-order reaction constant, dependent only on the concentration of ES. This condition is satisfied in the present procedure since butanol (etc.) evidently does not act as a second substrate, nor does it appear to interact directly with the active centre.

If the reversible effect of alcohols on other hydrolytic enzymes is the same as that for cholinesterase, then this effect may provide a useful means of determining the K_s for these enzymes. When the concentration of the enzyme is also known, then the individual rate constants k_1 , k_2 and k_3 may also be calculated. The relative magnitudes of the individual reaction constants can be estimated and this information may provide valuable insights into the reaction mechanism. It seems likely that alcohols will also affect K_s , but evidently the effect on k_3 is much greater than the effect they might have on K_s .

SUMMARY

1. The hydrolysis of *o*-nitrophenyl butyrate by human serum cholinesterase was activated by alcohols and acetone.

2. Lineweaver-Burk plots of the activities at a number of butanol concentrations gave lines, which, on extrapolation into the lower left-hand quadrant, approximated a common point of intersection. The point of intersection could be used to determine K_s , k_2e and k_1e directly from the plot. It is suggested that this plot is typical of k_3 activation.

3. The activation was dependent on the chain length, methanol activating least and pentan-1-ol most. Butan-1-ol activated more than its stereoisomers.

4. The hydrolysis of *p*-nitrophenyl butyrate was inhibited by butan-1-ol. Lineweaver-Burk plots of

the activities at a number of butan-1-ol concentrations approximated a common point of intersection in the lower left-hand quadrant in a manner similar to the activation plot. This plot is probably typical of k_3 inhibition.

5. The human serum cholinesterase *o*-nitrophenyl butyrate K_s was 0.072 mM, compared with K_m 0.139 mM, and the *p*-nitrophenyl butyrate K_s was 0.38 mM, compared with K_m 0.80 mM. The serum cholinesterase K_m of these substrates has therefore a mixed meaning, being both k_3 - and K_s -dependent.

6. *o*-Nitrophenyl butyrate hydrolysis by a partially purified horse-serum cholinesterase preparation was also activated by butan-1-ol.

7. *o*-Nitrophenyl acetate hydrolysis by human serum cholinesterase was activated by butan-1-ol and *p*-nitrophenyl acetate hydrolysis was inhibited.

8. It is suggested that alcohols affect the activity of hydrolytic enzymes by changing some aspect of the environment on which k_3 is dependent. The effect has been called 'environmental' to distinguish it from specific interaction. On the basis of an environmental effect alcohols may provide a useful method for determining the K_s of many hydrolytic enzymes.

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