the optimum substrate concentration, where linear relationships are a good first approximation. This note mentions some deductions from the higher-order equations which do not appear to have been published.

Lineweaver & Burk (1934) have recapitulated the argument by which it was shown that the relationship between velocity (v) and substrate concentration $[S]$, derived from the simultaneous equations,

$$\begin{aligned} E + S &\rightleftharpoons ES \text{ (active),} \\ ES + (n-1) S &\rightleftharpoons ES_n \text{ (inactive),} \end{aligned}$$

is

$$v = \frac{V[S]}{K_s + [S] + \frac{[S]^n}{K_s}} \quad (1)$$

(V =hypothetical maximum velocity if ES_n were not formed.) The optimum substrate concentration is found when $dv/d[S]=0$. By differentiating equation 1 it is found that

$$[S]_{\text{opt.}} = \left(\frac{K_s K_s}{n-1} \right)^{1/n} \quad (1a)$$

For non-competitive inhibition the simplest treatment involves considering only the equilibrium

whence the velocity in the presence of inhibitor (v') is

$$v' = v \frac{K_t}{K_t + [I]} \quad (2)$$

It is clear that $dv'/d[S]=0$ at the same value of $[S]$ that makes $dv/d[S]=0$; i.e. in the presence of a non-competitive inhibitor the optimum substrate concentration is unaltered.

For competitive inhibition there are three simultaneous equations to solve:

The velocity in presence of inhibitor is now

$$v' = \frac{V[S]}{K_s \left(\frac{K_t + [I]}{K_t} \right) + [S] + \frac{[S]^n}{K_s}} \quad (3)$$

and by differentiating as before, the optimum substrate concentration is

$$[S']_{\text{opt.}} = \left(\frac{K_s K_s K_t + [I]}{n-1} \right)^{1/n} \quad (3a)$$

By comparing equations (1a) and (3a) it is seen that in the presence of a competitive inhibitor the optimum substrate concentration is increased by the factor

$$\left(\frac{K_t + [I]}{K_t} \right)^{1/n}$$

The application of these findings is discussed in the accompanying paper (Austin & Berry, 1953).

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Hydrolysis of Benzolcholine Derivatives by Cholinesterase in Serum

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A series of derivatives of benzoylcholine (I) with substituents in ortho, meta, and para positions has been synthesized, and velocity constants of hydrolysis by horse-serum cholinesterase determined; the object of this has been to investigate the influence which the chemical reactivity of a substrate might have on its rate of reaction catalysed by an enzyme. There is already much quantitative information on changes in reactivity that can be expected from the

introduction of substituents into meta and para positions in the benzene ring; the data have been summarized by Hammett (1940), who expressed these changes by the substituent constant S , where $S = \log K - \log K_0$, K being the dissociation constant of the corresponding substituted benzoic acid and K_0 the dissociation constant of unsubstituted benzoic acid. S is positive for an electrophilic substituent, negative for a nucleophilic substituent,

and zero for the unsubstituted compound. Work which is most relevant for comparison is by Tommila & Hinshelwood (1938), who studied alkaline hydrolysis of substituted derivatives of ethyl benzoate (II).

EXPERIMENTAL

Analyses were performed by Miss V. Pickerden of the National Institute for Medical Research. Melting and boiling points are uncorrected.

Materials

The method of preparing benzoylcholine iodide illustrates the general method employed for all compounds unless otherwise stated. Benzoic acid was esterified with ethylene chlorohydrin in the presence of H_2SO_4 . The product distilled at 258° at atmospheric pressure and 103–108°/3 mm. Hg, and was converted by NaI in ethylmethylketone into β -iodoethyl benzoate, b.p. 135–140°/3 mm. Hg. This was dissolved at room temperature in acetone with excess trimethylamine from which the product crystallized. Recrystallized from ethanol, it had m.p. 243° (Ford-Moore, 1950).

p-Toluoylecholine iodide, crystallized from ethanol, had m.p. 197–198. (Found: C, 44.9; H, 5.81. $\text{C}_{13}\text{H}_{20}\text{O}_2\text{NI}$ requires C, 44.7; H, 5.73%.)

m-Toluoylecholine iodide, crystallized from ethanol, had m.p. 110–112°. (Found: C, 44.65; H, 5.8. $\text{C}_{13}\text{H}_{20}\text{O}_2\text{NI}$ requires C, 44.7; H, 5.7%.)

p-Methoxybenzoylcholine iodide, crystallized from ethanol, had m.p. 201–204°. (Found: C, 42.6; H, 5.9. $\text{C}_{13}\text{H}_{20}\text{O}_3\text{NI}$ requires C, 42.7; H, 5.5%.)

m-Nitrobenzoylcholine iodide, crystallized from ethanol, had m.p. 183–186° (decomp.). (Found: C, 37.95, H, 4.40. $\text{C}_{12}\text{H}_{11}\text{O}_4\text{N}_2\text{I}$ requires C, 37.9; H, 4.5%.)

p-Fluorobenzoylcholine iodide, crystallized from ethanol, had m.p. 173–175°. (Found: C, 40.7; H, 4.8. $\text{C}_{13}\text{H}_{17}\text{O}_2\text{NFI}$ requires C, 40.8; H, 4.8%.)

m-Fluorobenzoic acid was prepared from *m*-toluidine which was converted by the method of Balz & Schieman (1927) to *m*-fluorotoluene; this was oxidized with permanganate to *m*-fluorobenzoic acid, which was recrystallized from water, m.p. 119° (decomp.). *m*-Fluorobenzoylcholine iodide, recrystallized from ethanol, had m.p. 206–209°. (Found: C, 40.4; H, 5.2. $\text{C}_{13}\text{H}_{17}\text{O}_2\text{NFI}$ requires C, 40.8; H, 4.8%.) (Owing to shortage of material the intermediate compounds were not isolated and purified.)

m-Chlorobenzoylcholine iodide recrystallized from ethanol, m.p. 192–195°. (Found: C, 39.1; H, 4.5. $\text{C}_{13}\text{H}_{17}\text{O}_2\text{NCII}$ requires C, 39.0; H, 4.6%.)

o-Chlorobenzoic acid with thionyl chloride yielded *o*-chlorobenzoyl chloride. This reacted readily with ethylene

chlorohydrin in the presence of pyridine. *o*-Chlorobenzoylcholine iodide, prepared from this in the usual way and recrystallized from ethanol, had m.p. 166–168°. (Found: C, 39.3; H, 4.7. $\text{C}_{13}\text{H}_{17}\text{O}_2\text{NCII}$ requires C, 39.0; H, 4.6%.)

Methods

The rates of hydrolysis were studied at 25, 30 and 37° in the usual type of Warburg apparatus. The main compartment of each manometer flask contained 2.8 ml. of 0.1 M-NaHCO₃ in which was dissolved 2×10^{-6} mole of the substance to undergo hydrolysis (final concentration 6.67×10^{-3} M). CO₂ liberated from the bicarbonate solution by the free acid was measured in the manometers. The side arm contained 0.2 ml. of enzyme solution which was crude horse serum.

Experiments were performed also (at 37°) using 1×10^{-5} mole substrate and, where the reaction at this concentration was completed too rapidly for accurate determination, using 3×10^{-6} mole. As there was no significant change in rate of reaction within this range, it was assumed that the enzyme was saturated in each instance by 2×10^{-5} mole, the amount used throughout these experiments relative to 0.2 ml. of crude enzyme preparation. No determinations could be made with 5×10^{-6} mole of any of the substrates.

Two samples of horse serum were used; these were taken at different times from the same animal and gave different results in that sample 2 was more potent than sample 1. It is probable that they represent different concentrations of the same enzyme, but this assumption is in no way vital to the results reported, which are based on work with sample 2. Observations with sample 1 (see Table 1) have been repeated using sample 2.

The degree of confidence that can be placed in results is shown by the standard errors of the mean values which are included where multiple determinations were made. An approximate value only is given for the *p*-methoxy derivative as this was hydrolysed too slowly for accurate determination. Non-enzymic hydrolysis was slow compared with enzymic hydrolysis, and the errors in determination correspondingly large, and it also tended to deviate from a zero-order course. The rate of enzymic hydrolysis has therefore not been corrected for non-enzymic hydrolysis.

Further determinations were limited by the amount of the compounds available.

RESULTS

In Table 1 the zero-order velocity constants for the hydrolysis of the compounds by the two samples of horse serum enzyme are given, together with the constants for alkaline non-enzymic hydrolysis and substituent constant (*S*). Fig. 1 shows the correlation between reactivity of the ester linkage and rate of reaction by plotting $\log_{10} k_{37}$ against *S*. A linear relationship would be expected if the changes in reaction rate could be ascribed solely to the changes in reactivity caused by the substituents. Such deviations as occur can be accounted for by alterations in the 'fit' of enzyme and substrate.

Previous work, summarized by Hinshelwood (1945), has shown that the inductive effect of substituents in the benzene ring affects *E*, the energy of activation, and that factors concerned with molecular collision and the probability of a reaction

Table 1. *Velocity constants for substituted benzoylcholine derivatives for enzymic and non-enzymic hydrolysis and their substituent constant S which gives an estimate of the reactivity of the ester linkage*

(The figures in parentheses are the numbers of experiments performed. The standard error is given where more than two determinations were made.)

Derivative	Enzyme sample 1, k_{37}	Enzyme sample 2			Non-enzymic alkaline hydrolysis k_{37}	S
		k_{37}	k_{30}	k_{35}		
<i>m</i> -Nitro	3.53 ± 0.15 (4)	4.50 (1)	2.64 (1)	1.87 (1)	0.23 ± 0.17 (3)	+0.71
<i>m</i> -Chloro	3.61 ± 0.09 (4)	—	3.13 (2)	2.20 (1)	0.13 ± 0.10 (3)	+0.37
<i>m</i> -Fluoro	2.39 ± 0.03 (3)	3.12 (1)	2.70 (1)	2.39 (2)	0.22 (1)	+0.34
<i>p</i> -Fluoro	1.71 ± 0.03 (4)	2.58 (1)	1.70 (1)	1.22 (2)	0.13 ± 0.02 (3)	+0.06
Unsubstituted	1.79 ± 0.08 (7)	2.95 ± 0.08 (4)	1.90 ± 0.05 (6)	1.59 ± 0.03 (5)	0.075 ± 0.007 (3)	0.00
<i>m</i> -Methyl	0.66 ± 0.09 (4)	0.811 (2)	0.642 (1)	0.577 (1)	0.10 ± 0.018 (3)	-0.07
<i>p</i> -Methyl	—	0.531 (2)	0.404 (2)	0.325 (2)	0.038 (1)	-0.17
<i>p</i> -Methoxy	0.1 (approx.)	—	—	—	0.065 (1)	-0.27
<i>o</i> -Chloro	—	2.73 (1)	1.95 (1)	—	0.189 (1)	—

Fig. 1. Graph showing the $\log_{10}$ rates of hydrolysis, k_{37} , of the derivatives of benzoylcholine correlated with the reactivity (given by the substituent constant S). ●, Enzyme sample 1; ○, enzyme sample 2.

Fig. 3. Correlation of energy of activation (E) with substituent constant (S) for derivatives of benzoylcholine in enzyme-catalysed hydrolysis. The trend shown in this figure is in the opposite sense from that shown in Fig. 2.

Fig. 2. Data from Tommila & Hinshelwood (1938). Correlation of energy of activation (E), with substituent constant (S) for derivatives of ethylbenzoate in alkali-catalysed hydrolysis.

Table 2. *Energy of activation (E) (affected by chemical reactivity of compounds) in the Arrhenius expression for hydrolysis of the ester linkage by serum cholinesterase*

Derivative of benzoylcholine	$E \times 10^4$ (cals.)	S
<i>m</i> -Nitro	13.3	+0.71
<i>m</i> -Chloro	12.6	+0.37
<i>m</i> -Fluoro	7.8	+0.34
<i>p</i> -Fluoro	11.7	+0.06
Unsubstituted	6.3	0.00
<i>m</i> -Methyl	5.1	-0.07
<i>p</i> -Methyl	7.8	-0.17

occurring affect the non-exponential term A in the Arrhenius expression as

$$\log_{10} k = A - \frac{E}{2.303 RT},$$

where R is the gas constant and T the absolute temperature. The values of E were estimated by a

graphical method from the data in Table 1, and the results are shown in Table 2. The correlation between E and the substituent constant (S) is shown in Fig. 3. The figure shows, in spite of the scatter of points, that the increase in the energy of activation tends to occur as the ester linkage becomes more reactive. This is the opposite to what might be expected and to the findings of Tommila & Hinshelwood (1938) whose results, plotted in the same way, are shown in Fig. 2.

The experimental values were not sufficiently accurate to determine values of A .

DISCUSSION

It is usually supposed that alterations in the structure of a substrate will affect the rate of an enzyme-catalysed reaction by altering the degree of contact between the enzyme and the substrate on which interaction depends; the possibility that structural alteration may change the intrinsic reactivity of the substrate is frequently overlooked. This work shows that the reactivity of a compound can be at least as important as the 'goodness of fit' between it and the enzyme.

It is at first sight anomalous that in a reaction involving the cleavage of a bond to give an overall reaction of type (i), E , the energy of activation, should tend to increase as the bond becomes more reactive. This anomaly may indicate that the temperature coefficient of the reaction is controlled by the formation of an intermediate compound between the enzyme and some activated part of the substrate molecule such as the carbonyl group.

The formation of such an intermediate compound is no new hypothesis. Wilson, Bergman & Nachmansohn (1950) have produced extensive evidence of the formation of compounds between esters and the

cholinesterase from the electric organ of *Electrophorus*. The evidence given above favours the type of bond which would require greater energy of activation as the carbonyl group becomes activated by an electrophilic substituent in the benzene ring. This condition might best be fulfilled by the enzyme itself acting as an electrophilic reagent on the carbonyl oxygen which carries a partial negative charge giving a reaction mechanism according to scheme (ii).

Further light might have been thrown on this problem by a determination of Michaelis constants but, as has been shown above, this was not possible with the methods available.

SUMMARY

1. A series of derivatives of benzoylcholine has been prepared with substituents in the benzene ring, and the rate of hydrolysis of these compounds with cholinesterase of horse serum has been determined.
2. For this series it is concluded that the innate reactivity of the ester linkage is as important as the 'goodness of fit' between enzyme and substrate in determining the rate of reaction.
3. Solution of the Arrhenius equation suggests that greater energy of activation is required when the carbonyl group is the more activated. This condition could be fulfilled by supposing a positive or partially positive charge on the enzyme to react with the carbonyl oxygen of the ester to form an intermediate compound.

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