we have investigated is the case of potassium iodate, which reacts with the ceric sulphate-arsenious acid mixture, but does not yield any iodine with ceric sulphate alone (Table 3). It has been shown by adding arsenious acid before ceric sulphate, that this anomaly is due to preliminary reduction of potassium iodate by the arsenious acid.

The method as described is relatively insensitive to aliphatic iodine-containing compounds such as iodoacetic acid, but we are informed by Dr I. R. Bush (St Mary's Hospital, London) that spots of such compounds are detectable if the chromatograms are previously treated with alkali. This causes hydrolysis of the compound with the liberation of iodide. Since alkaline solutions of thyroxine contain iodide after prolonged storage, we tried the effect of alkali on chromatograms bearing spots of this substance, but were unable to demonstrate any increased sensitivity.

SUMMARY

1. Methods are described for the detection of inorganic and organic iodine-containing compounds in solution and on paper chromatograms using ceric sulphate and arsenious acid.

2. In solution 0.2 μ g. potassium iodide and 2.0 μ g. thyroxine are detectable at room temperature. On paper chromatograms the method is sensitive to 0.01 μ g. potassium iodide and 0.1 μ g. thyroxine.

3. Methods for making permanent records of the chromatograms are described. The general procedure can be adapted for the semi-quantitative estimation of thyroxine in the range 0.1–0.4 μ g.

4. The mechanism of the reaction has been studied and the conclusion reached that the method depends upon the liberation of iodine from the compounds by oxidation with ceric sulphate. With

one exception (potassium iodate), only those compounds which readily yield iodine when treated with ceric sulphate can be detected on paper with this reagent.

5. The reaction is given by all the aromatic compounds studied which contain one or more iodine atoms in positions *ortho* to an electron-donating substituent such as the hydroxyl or amino group.

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REFERENCES

- Bowden, C. H. & MacLagan, N. F. (1954). *Biochem. J.* **56**, vii.
 Foote, J. B., Mackenzie, D. H. & MacLagan, N. F. (1952). *Lancet*, **1**, 486.
 Fraser, T. R. & MacLagan, N. F. (1953). *J. Endocrin.* **9**, 301.
 Gross, J., Leblond, C. P., Franklin, A. E. & Quastel, J. H. (1950). *Science*, **111**, 605.
 Harington, C. R. & Barger, G. (1927). *Biochem. J.* **21**, 169.
 Kolthoff, I. M. & Sandell, E. B. (1934). *J. Amer. chem. Soc.* **56**, 1426.
 MacLagan, N. F., Sprott, W. E. & Wilkinson, J. H. (1952). *Lancet*, **2**, 915.
 MacLagan, N. F. & Wilkinson, J. H. (1951). *Nature, Lond.*, **168**, 251.
 MacLagan, N. F. & Wilkinson, J. H. (1954). *Biochem. J.* **56**, 211.
 Nanninger, L. & Bink, B. (1951). *Nature, Lond.*, **168**, 389.
 Riggs, D. S. & Man, E. B. (1940). *J. biol. Chem.* **134**, 193.
 Sheahan, M. M., Wilkinson, J. H. & MacLagan, N. F. (1951). *Biochem. J.* **48**, 188.
 Wilkinson, J. H., Sheahan, M. M. & MacLagan, N. F. (1951a). *Biochem. J.* **49**, 714.
 Wilkinson, J. H., Sheahan, M. M. & MacLagan, N. F. (1951b). *Biochem. J.* **49**, 710.

The Inhibition of Horse-Liver Esterase by Rhodamine B

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Horse-liver esterase is inhibited by low concentrations of several fluorescent dyes (Burch, 1954). It was considered that this inhibition might be of special interest for the theory of enzyme action, since the combination of small fluorescent molecules with proteins may be studied by an independent

physical method: that of the polarization of fluorescence, introduced by Weber (1952a, b), and applied by Laurence (1952) to the study of the reversible adsorption of dyes by serum albumin. Combination between enzyme and substrate or inhibitor is fundamental to the accepted mechanisms of enzyme action and its reversible inhibition; but, except in the important case of the haem

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enzymes, evidence for rapidly reversible combination is provided by kinetic data only. Extra-kinetic evidence for such combination, and its quantitative role in enzyme action and inhibition, would be very desirable for the hydrolytic enzymes in which no active centres have yet been identified.

Rapid-flow techniques would be needed to observe the combination of enzyme with fluorescent substrate; inhibitors may, however, be studied by ordinary methods, and the theory of competitive inhibition is very closely linked with the theory of normal enzyme action. Preliminary experiments indicated that the inhibition of horse-liver esterase by rhodamine B is competitive in type; and it was hoped to obtain a quantitative comparison between dissociation constants determined kinetically and by fluorescence studies, and to find the number of combination sites per unit weight of protein in the enzyme preparation.

It will be shown, however, that on further study the inhibition proved to be kinetically much more complex than the usual competitive pattern. Combination between dye and enzyme has been demonstrated by the fluorescence method, but the quantitative connexion between the results of the two types of investigation has not been established. A possible mechanism for the inhibition is put forward.

THEORY OF THE FLUORESCENCE METHOD

The polarization of the fluorescent light from molecules in solution depends on the relation between the lifetime of the excited state and the rotational relaxation time. Rhodamine B in aqueous solution gives a very low polarization p_p : in solvents of higher viscosity the relaxation time is increased and the polarization rises to a limiting value p_p' ; in glycerol this value is attained. In aqueous solution, combination of the dye with protein increases the relaxation time, and the polarization of fluorescence is raised to a value p_B (Weber, 1952*a*). The proportion of dye in the free and bound forms, in any solution, may be calculated from the polarization p of the fluorescence of the solution if p_B , p_p and R , the ratio of the fluorescent intensity of the bound dye to that of the free, have been determined. If the molar concentrations of bound and free dye are C_B and C_F respectively, and we put $3p/(3+p) = p'$, Weber's (1952*a*) eqn. 8 shows that

$$C_F/C_B = R(p'_B - p')/(p' - p'_p), \quad (1)$$

p'_p may be measured directly, and p'_B found as the limiting value of p' when sufficient enzyme is present to bind all the dye.

To obtain R , comparison is made between the intensities of fluorescence of two identical cells, containing dye in equal concentrations. Cell x contains dye free in solution, and cell y contains enzyme in addition, so that the dye is almost wholly bound. Let the observed intensity ratio, (cell y)/(cell x), be R_1 . To obtain the true relation between the fluorescent intensities of the molecular species present in the two cells, two corrections are required. (i) A difference between the absorption spectra of the two solutions will

lead to a difference in the proportions of both exciting and emitted light which are absorbed. It may readily be shown that R_2 , the intensity ratio corrected for this effect, is given by

$$R_2 = R_1 a_x A_y (1 - e^{-a_x l}) (1 - e^{-A_x L}) / a_x A_x (1 - e^{-a_x l}) (1 - e^{-A_y L}), \quad (2)$$

where the suffixes refer to solutions x and y , and a =absorption coefficient at the wavelength of the exciting light; A =absorption coefficient for the observed emitted light; l =cell depth in the direction of the exciting beam; L =cell depth in the direction of observation of the emitted light. When al and AL are small, $R_2 = R_1$. (ii) The proportion of the total fluorescent intensity which is observed depends also on the polarization: Weber's (1952*a*) analysis shows that if

$$R_3 = R_2(1 + p_y/3)/(1 + p_x/3), \quad (3)$$

R_3 represents the true ratio between the fluorescent intensities of solutions y and x . If cell y contains bound dye only, $R = R_3$, but in practice this may not be possible, since the high enzyme concentrations necessary may introduce such large absorption coefficients that the calculation of R_3 from R_1 (eqn. 2) becomes inaccurate. In this case, a simple proportion argument, together with eqn. 1, shows that

$$R = R_3 \{1 - (R_3 - 1)(p'_B - p'_y)/(p'_x - p'_y)\}, \quad (4)$$

and R is determined by measurements of intensities, absorption coefficients and polarizations. Then in any solution C_F/C_B may be found from eqn. 1 by measurement of p' .

For a simple equilibrium combination between dye and enzyme, $(e - C_B) C_F/C_B = K$, where e is the total molar concentration of binding centres present, and K is the dissociation constant. Hence, if $e = c[e_a]$, where $[e_a]$ is the enzyme concentration in arbitrary units and c is a constant,

$$[e_a] C_F/C_B = K/c + C_F/c. \quad (5)$$

This equation may be used to find K and C from the experimental observations.

EXPERIMENTAL

Enzyme. The purified horse-liver esterase preparation previously described (Burch, 1954) was used throughout.

Rhodamine B. A commercial sample of the dye was recrystallized 4 times: twice as the hydrated base, which separated slowly in green plates, at room temperature, after addition of dilute NaOH to aqueous solutions of the hydrochloride (Noelting & Dziewonsky, 1905); and twice as the hydrochloride from very small volumes of hot water. The hydrochloride was finally dried *in vacuo* over H_2SO_4 . Stock solutions used for kinetic and polarization work were stored in dark bottles.

Dolinsky (1949) reports extinction coefficients for pure rhodamine B hydrochloride in dil. NH_3 solutions. $E_{1cm}^{1mg/l.}$ is given as 0.233 ± 0.001 at the wavelength of the maximum (553 ± 2 m μ .); and E_{580}/E_{553} as 0.36 ± 0.01 . The present sample in the specified solvent, examined in a Beckman spectrophotometer gave a maximum extinction at $554-555$ m μ ., where $E_{1cm}^{1mg/l.}$ was 0.234 ; E_{580}/E_{554} was 0.35 . Chromatography in water on alumina columns showed that the commercial dye contained a component which remained stationary at the top of the column. In the purified sample this could not be detected, and the coloured material travelled as a single band.

Anhydrous rhodamine B base, for the freezing-point measurements, was prepared from the purified hydrochloride by crystallization of the hydrated base in the previous manner. The washed crystals were dried to constant weight *in vacuo* over P_2O_5 .

Depressions of the freezing point were determined in the usual way (Findlay, 1941) using a Beckman thermometer. Successive additions of the anhydrous base were made to benzene of a grade supplied commercially for this purpose.

Inhibition kinetics. The rates of ester hydrolysis were measured by the continuous potentiometric titration method, using the apparatus and detailed procedures previously described (Burch, 1954). Unless otherwise indicated, initial reaction velocities were used and reaction mixtures were made up containing substrate, phosphate buffer, KCl, rhodamine B hydrochloride and nearly one equivalent of NaOH/g. mol. dye. Titration to the standard pH of 7.4 was completed as usual after equilibration in the reaction vessel, and the reaction was started by addition of 0.5 ml. enzyme solution to the 50 ml. of mixture. The accuracy of measurement of each reaction rate depended on the individual conditions of the run, and was estimated as described previously (Burch, 1954). All error limits given are estimated in this way, unless otherwise stated.

Fluorescence studies. Solutions, unless otherwise indicated, contained 0.01 M phosphate buffer (0.008 M Na_2HPO_4 –0.002 M KH_2PO_4 , pH 7.4) and 0.1 M-KCl. Reagents had been previously boiled to remove CO_2 . A solution of the enzyme preparation, dialysed against 0.01 M phosphate, pH 7.4, and containing 1660 esterase units and 3.56 mg. N/ml. was freeze-dried and stored in the cold. Samples of the powder were dissolved in 0.1 M-KCl to the original volume, and very small amounts of insoluble matter removed by high-speed centrifuging. These stock solutions showed an esterase activity identical with that before freeze-drying. In the fluorescence work, enzyme concentrations were expressed in arbitrary units, as fractions of the concentration of these stock solutions, and denoted by $[e_0]$.

In polarization and intensity measurements, the Hg line at 546 m μ . was used for excitation. Light from a mercury discharge was filtered through a cell of $NaNO_2$ solution, a didymium filter Corning no. 5120, and a green filter Corning no. 401. The emitted light was observed through a red filter complementary to the exciting light. No emission could be detected from the enzyme preparation alone. Solutions were contained in closed optical cells, of depth 1 cm. in the direction of the exciting beam, and thickness 2 cm. in the direction of observed emission. Polarizations were measured by the method of Weber (1952a), using his calibrated apparatus at 25°. Fluorescent intensity comparisons between two cells were made visually at room temperature, using a fluorimeter with a divided incident beam and calibrated adjustable diaphragms in the observation pathways. Unpolarized exciting light was used in all measurements except those of p_p at high dye concentrations, when a Nicol prism was placed in the incident beam. This increased the measured polarization value, permitting more accurate observations on solutions of very low polarization; but it seriously diminished the intensity of illumination, and was therefore only useful at high dye concentrations. The relation $p = P/(2 - P)$ (Perrin, 1929), where P and p are the polarizations observed with polarized and natural exciting light respectively, was used to calculate p when P was measured.

RESULTS

Kinetic characteristics of the inhibition

Inhibitor and substrate concentrations. Fig. 1 shows the results of four experiments, each at a constant concentration of enzyme and of methyl butyrate. The rhodamine concentration was varied, and the reciprocal of the hydrolysis rate, $1/v$, plotted against the dye concentration $[I]$. In each case the velocity decreased steadily as $[I]$ rose; and, at any fixed value of $[I]$, the percentage inhibition became greater as the substrate concentration fell, indicating a competitive type of inhibition. In normal competitive inhibition, eqn. 11 of Lineweaver & Burk (1934) shows that the apparent value of the Michaelis constant, K_A , is linear with $[I]$, and at constant substrate concentration $1/v$ is linear with $[I]$; but in Fig. 1 each experiment gave a curve concave downwards.

Fig. 2 shows the results of a series of experiments at constant enzyme concentration. To allow for small differences in the active enzyme concentrations used on different days, a velocity determination with no inhibitor was made each day, and all measured velocities were multiplied by a factor referring them to a standard enzyme concentration which gave a limiting velocity V of 25.0 $\mu M/min$. The resulting values of $1/v$ were plotted against $1/[S]$, and each series of points at a given dye concentration lay reasonably closely about a straight line of intercept $1/V = 0.04 \text{ min. } \mu M^{-1}$. Thus the inhibition was truly competitive with respect to substrate: the presence of inhibitor altered only the apparent value of the Michaelis

Fig. 1. Inhibition: effect of rhodamine at various concentrations of substrate. Phosphate concn. $1.0 \times 10^{-3} M$. Methyl butyrate concn. ($M \times 10^{-3}$): A, 2.27; B, 5.68; C, 14.2; D, 56.8.

constant, leaving the maximum velocity unchanged. But the influence of $[I]$ was not that of simple competitive inhibition; in Fig. 3 the K_A values obtained from the gradients of the straight lines of Fig. 2 are plotted against the rhodamine concentrations, and the result is a continuous curve rather than a straight line.

Reversibility. Three types of evidence showed that the inhibition was rapidly established and reversible, so that the experimental results referred to an equilibrium condition.

(i) The reaction mixtures were made up containing inhibitor but no substrate. After equilibration and titration to the standard pH, either the substrate was added and followed immediately by enzyme, or enzyme was added first and allowed to remain with the inhibitor for varying periods before the addition of 2 ml. of substrate solution started the reaction. There was no detectable acid liberation or consumption when enzyme and inhibitor were together without substrate. Table 1 shows the results of two experiments. Some decrease in reaction rate resulted from leaving enzyme with inhibitor before substrate addition; but this decrease was no greater than that caused by leaving the enzyme under the same conditions (of pH, temperature, and concentrations of KCl, phosphate and enzyme) with no inhibitor present (Burch, 1954). In both cases the loss in activity was progressive, and was about 10 % per 20 min., so that the percentage inhibition was unaffected.

(ii) The enzyme was added as usual to a mixture of inhibitor and substrate. After the reaction had proceeded for 10 min., the mixture was suddenly diluted by the addition of an equal volume of a solution containing substrate, KCl and phosphate. The inhibitor concentration was thereby halved, while the total amount of enzyme, and the concentrations of all other components, were unchanged. The reaction rate immediately increased, becoming equal to that in a control experiment at the lower dye concentration. The progress curves are shown in Fig. 4. The solutions added at the dilution points were previously warmed to 25°, and brought approximately to the standard pH by addition of the quantity of alkali judged necessary. This quantity

was slightly underestimated, as shown by the discontinuities in the curves; but the pH of the reaction mixture fell by less than 0.15 unit during the dilution of each run. The reaction rates obtained from the gradients of the curves are given in the

Fig. 2. Variation of hydrolysis rate with methyl butyrate concentration at various concentrations of inhibitor. Phosphate concn. $1.0 \times 10^{-3} M$. Rhodamine B concn. ($M \times 10^{-4}$): $\blacksquare$ — $\blacksquare$, 0.25; $\bullet$ — $\bullet$, 1.0; $\blacktriangledown$ — $\blacktriangledown$, 2.5; $\blacktriangle$ — $\blacktriangle$, 12.5. Estimated error limits shown as vertical lines through the points. Results with no inhibitor taken from Burch (1954).

Fig. 3. Effect of rhodamine concentration on the apparent value of the Michaelis constant for methyl butyrate. Results from Fig. 2.

Table 1. *Speed of the inhibitory action of rhodamine B on the esterase*

Mixtures containing $1.0 \times 10^{-3} M$ phosphate, $0.1 M$ KCl, and $1.0 \times 10^{-4} M$ rhodamine B were equilibrated in the reaction vessel and titrated to pH 7.4. Either enzyme (E) or methyl butyrate solution (S) was introduced; and after the time interval shown, the last component was added and the reaction rate measured by the continuous potentiometric titration method described in the text, p. 99. Final substrate concn. $= 4.55 \times 10^{-3} M$. Two separate experiments are shown.

Experiment	...	...	1	1	2	2	2
1st addition			S	E	S	E	E
Time interval (min.)			2	19½	3	23½	41
2nd addition			E	S	E	S	S
Reaction rate ($\mu M/\text{min.}$)			9.6	8.95	9.45	8.2	7.85

(Estimated error in all reaction rates, $\pm 0.3 \mu M/\text{min.}$)

figure. On dilution the rate of *A* (Fig. 4) rose to $1.10 \times$ its initial value, and became equal (within less than 1%) to the rate in the corresponding period of reaction *B*. Larger increases in the rate of *A* could not be obtained in dilution experiments, because of the insensitivity of the rates to changes in dye concentration (Fig. 3) and because the maximum and minimum volumes which could be used in the reaction vessel prevented dilutions greater than twofold.

Fig. 4. Dilution experiment showing reversibility of inhibition. $\times \times$, experimental null-points in the continuous potentiometric titration of reaction mixtures. Initial solutions, volume 40 ml., contained 0.1M-KCl , $0.667 \times 10^{-3}\text{M}$ phosphate, $14.2 \times 10^{-3}\text{M}$ methyl butyrate, and $12.5 \times 10^{-4}\text{M}$ rhodamine (reaction *A*) or $6.25 \times 10^{-4}\text{M}$ rhodamine (reaction *B*). After equilibration, enzyme was added and the reactions followed. At $\uparrow$ each mixture was diluted by the addition of 40 ml. solution containing 0.1M-KCl , $0.667 \times 10^{-3}\text{M}$ phosphate, $14.2 \times 10^{-3}\text{M}$ methyl butyrate, and no rhodamine (reaction *A*) or $6.25 \times 10^{-4}\text{M}$ rhodamine (reaction *B*). Reaction rates (mm. burette divisions/min.) were: *A*, initially 0.305, after dilution 0.337; *B*, initially 0.346, after dilution 0.335. 1 mm. micrometer burette division = 0.0359 ml. of 0.0896M-NaOH .

(iii) Evidence for the speed with which the equilibrium degree of inhibition was established was obtained from the linearity of the progress curves of ester hydrolysis. If the inhibitor acted slowly on the enzyme, a progressive decrease in reaction rate with time would be expected when enzyme was added to a mixture of inhibitor and substrate; while if the inhibitor was only slowly displaced from the enzyme by the substrate, a progressive increase in reaction rate would have occurred when substrate was added to a mixture of enzyme and inhibitor. The progress curves of the reactions of Table 1 showed no such difference in form depending on the order of addition of components, and the progress curves of the reactions of Figs. 2 and 4 were substantially linear for periods of about 10 min.

Enzyme concentration. In normal competitive inhibition the percentage inhibition at constant values of $[S]$ and $[I]$ is independent of enzyme concentration. The observed dependence of K_A on $[I]$ (Fig. 3), in which K_A is approximately linear with the square root of $[I]$, could result if an equilibrium of the form $2E + I \rightleftharpoons E_2I$ was responsible for the inhibition. But in this case the percentage inhibition at constant $[S]$ and $[I]$ would necessarily increase with rising enzyme concentration. The results of an experiment testing this point are given in Table 2. The reactions were carried out at room temperature, using a Marconi glass electrode pH meter to record null points at pH 7.40 during the continuous titration. Reaction mixture (25 ml.), in an open vessel, contained substrate and inhibitor. After titration to pH 7.40, different volumes of an enzyme solution were added to start the reaction. Thereafter additions were made from a 2 ml. burette, at intervals of about 90 sec., of a sodium hydroxide solution whose concentration was varied according to the amount of enzyme used. The phosphate concentration was varied similarly, so that each addition of alkali gave a pH increment of about 0.18 unit in every run. The reaction velocity was proportional to enzyme concentration over a tenfold range, both with and without inhibitor. The percentage inhibition was thus independent of enzyme concentration.

Phosphate concentration. In the absence of inhibitor, the rate of hydrolysis of methyl butyrate by the enzyme is independent of phosphate

Table 2. Influence of enzyme concentration on reaction rates with and without inhibitor

All reaction mixtures contained 0.1M-KCl and $14 \times 10^{-3}\text{M}$ methyl butyrate. Rates were measured at room temperature by a continuous titration procedure using an ordinary pH meter; for details see text, this page.

	No rhodamine		$12.4 \times 10^{-4}\text{M}$ rhodamine			
	1.07	10.7	0.53	1.07	4.27	6.40
Phosphate (10^{-3}M)	0.3	3.0	0.3	0.44	1.5	3.0
Enzyme (ml.)	41.4	406	29	42	144	273
Reaction rate ($\mu\text{M}/\text{min.}$)	138	135	97	95	96	91
Rate/ml. enzyme						

concentration. This has been shown both at high and low substrate concentrations (Burch, 1954). The inhibition by rhodamine B was also found to be independent of phosphate concentration from $0.5 \times 10^{-3} \text{ M}$ to $2.0 \times 10^{-3} \text{ M}$.

Inhibition of ethyl butyrate hydrolysis. When ethyl butyrate was used as substrate instead of the methyl ester, the inhibition by rhodamine B showed the same characteristics, in that it was competitive with respect to substrate, and the graph of K_A against $[I]$ was concave towards the $[I]$ axis. In normal competitive inhibition K_A/K_m is independent of the substrate used, being a function of inhibitor concentration only. This is also true for certain other possible inhibition mechanisms based on the competitive pattern. However, Table 3 shows that K_A/K_m was greater with ethyl butyrate than with the methyl ester, at equal dye concentrations.

Inhibition with $50 \times 10^{-4} \text{ M}$ rhodamine. Segal, Kackmar & Boyer (1952) have postulated a type of inhibition in which the inhibitor combines with the enzyme at a point different from the substrate-binding centre, and the resulting enzyme-inhibitor compound has enzymic activity with kinetic constants different from those of the uninhibited enzyme. If the result of the combination with inhibitor is a reduction in the affinity of the enzyme for the substrate, with no change in the rate of breakdown of the enzyme-substrate complex, the inhibition is termed 'apparent competitive', since the apparent value of the Michaelis constant is altered, while the maximum velocity is unchanged. It differs from normal competitive inhibition in giving a graph of K_A against $[I]$ which is concave towards the $[I]$ axis, obeying the equation

$$K_A = K_m(1 + [I]/K_1)/(1 + [I]/K_1')$$

(This may be seen from eqn. 6 below, when K_1 and K_2 are very large so that the truly competitive component of the inhibition is absent.)

Thus, putting $f = [I]/(K_A/K_m - 1)$,

$$f = K_1 K_1' / (K_1' - K_1) + K_1 [I] / (K_1' - K_1)$$

and K_A increases from K_m , at $[I] = 0$, towards the asymptotic value $K_m K_1' / K_1$, when $[I] \gg K_1'$; while f increases linearly with $[I]$.

In order to show whether this mechanism accounted for the inhibition results, the data of Fig. 3 were replotted in Fig. 5 as f against $[I]$. The result appeared to be a curve concave downwards, indicating that as $[I]$ increased, K_A continued to rise steadily without approaching an asymptotic value. Estimation of the accuracy of the K_A determinations is difficult: in this figure the vertical line through each point represents a deviation of $\pm 10\%$ in the K_A value. It was considered that a definite con-

clusion as to the form of this graph required another point at a higher value of $[I]$. K_A for methyl butyrate hydrolysis was therefore determined in the presence of $50 \times 10^{-4} \text{ M}$ rhodamine, by the method

Table 3. Comparison of the effects of rhodamine B on the hydrolysis of two separate substrates

The inhibition was in each case competitive with respect to substrate: K_A denotes the apparent value of the Michaelis constant in the presence of inhibitor.

Rhodamine (10^{-4} M)	Methyl butyrate		Ethyl butyrate	
	K_A (mM)	K_A/K_m	K_A (mM)	K_A/K_m
0	2.32	—	0.62	—
1.0	6.5	2.8	2.5	4.0
12.5	17.9	7.7	9.9	16.0

Fig. 5. Effect of rhodamine concentration on the inhibition of methyl butyrate hydrolysis. Results from Fig. 3. $f = [I]/(K_A/K_m - 1)$. Vertical lines show deviations of $\pm 10\%$ in K_A values.

Fig. 6. Effect of rhodamine concentration on the inhibition of methyl butyrate hydrolysis. Results from Fig. 5. $f_0 =$ value of f when $[I] \rightarrow 0 = 0.3 \times 10^{-4} \text{ M}$. Vertical lines show deviations of $\pm 10\%$ in K_A values.

used in the experiments of Table 2, except that the reaction volume was 30 ml., and the temperature was maintained at 25°. This simple technique was adequate, since the high value of K_A allowed high substrate concentrations and reaction rates to be employed. On including this point in Fig. 5 it became clear that the graph was curved, so that the results did not conform with 'apparent competitive' inhibition.

A more complex inhibition mechanism is discussed below: 'apparent competitive' and true competitive inhibitions are assumed to be acting simultaneously, and it is shown that in this case $[I]/(f-f_0)$ should be linear with $[I]$, where f_0 is the value of f when $[I] \rightarrow 0$. In Fig. 6 f_0 is taken as $0.30 \times 10^{-4}M$, and the inhibition results of Fig. 5 are plotted in this form. As before, vertical lines through the points represent deviations of $\pm 10\%$ in the K_A values.

Properties of rhodamine B in solution

The possibility presented itself that rhodamine B might reversibly form a dimer in aqueous solution. If the monomeric form inhibited competitively while the dimer was inert towards the enzyme, the increase in K_A would be nearly proportional to the square root of the total dye concentration. Ghosh & Sengupta (1938) have postulated such association for rhodamine B in dilute aqueous solution, but alternative explanations of their experimental results are available (Wawilow, 1942), and in all these studies solutions of the hydrochloride of rhodamine B were used. No data are available on the relevant properties of the uncharged base, which is the form present at pH 7.4. Experiments were therefore carried out to investigate the behaviour of the dye in solution under conditions closely comparable with those of the inhibition work.

Partition coefficients. The distribution of rhodamine B between benzene and an aqueous phase of pH 7.4, containing $0.1M$ -KCl and $4 \times 10^{-3}M$ phosphate at 25°, was examined. Dye concentrations in the separated layers were determined colorimetrically: in benzene, rhodamine B is colourless, but the addition of an equal volume of ethanol was

found to give deeply coloured solutions. The partition coefficient (concn. in benzene/concn. in aqueous layer) was found to have a constant value of 8.5×10^2 over a 40000-fold range of dye concentrations, from $7 \times 10^{-9}M$ to $3 \times 10^{-4}M$ in the aqueous phase. Thus the rhodamine could only dimerize in the aqueous medium if it also did so in the benzene layer.

Depression of the freezing point. Rhodamine B base in benzene was found to give depressions of the freezing point in agreement with those calculated for monomeric dye, at concentrations up to $0.082M$. At $0.147M$ the depression was 91% of the calculated value: since this solution contained over 7% of solute, a slight deviation from ideal behaviour is not unexpected. All the observed depressions were much greater than those calculated assuming a degree of dimerization in the aqueous medium sufficient to account for the inhibition data of Fig. 3.

Effect of benzene on the inhibition. It remained possible that the results of the distribution experiments did not truly represent the relation between the benzene solutions of rhodamine, used for freezing-point measurements, and the aqueous solutions of the kinetic work, since in the partition experiments each phase was necessarily saturated with the other. At 25° the benzene layer thus contained about 0.03 g. mol. water/l. and since this represents less than one molecule of water per dye molecule (at the upper dye concentrations used), it is very improbable that it fundamentally altered the state of aggregation of rhodamine in benzene. But the solubility of benzene in water is rather greater, and the dye concentrations in this phase were much lower so that a change in the molecular state of the dye due to the presence of benzene was possible. In this case the inhibitory action of the dye on liver esterase should have been changed markedly by the addition of benzene to the reaction mixture. Table 4 shows the results of experiments in which the effect of benzene on the esterase reaction rates was tested, by the method used to investigate the inhibition at $50 \times 10^{-4}M$ rhodamine. In the reactions with benzene present, the mixtures contained water previously saturated with benzene and carefully

Table 4. *Effect of benzene on the enzymic hydrolysis of methyl butyrate, in the presence and absence of rhodamine B*

Reaction mixtures contained $0.1M$ -KCl. Benzene was added as a saturated solution in water, its final concn. being approximately half the saturation value. Hydrolysis rates were measured at 25° by a continuous titration procedure using an ordinary pH meter: for details see text, this page.

Methyl butyrate (mm)	17.15	17.15	17.15	2.34	2.34
Phosphate (mm)	3.3	3.3	5.6	0.87	0.67
Rhodamine B (mm)	1.25	1.25	0	0	0
Enzyme (ml.)	1.0	1.0	1.0	0.25	0.25
Reaction rate ($\mu M/min.$)	no benzene (v_1)	152	153	211	29.1
	benzene present (v_2)	158	160	199	34.2
v_2/v_1		1.04	1.05	0.94	1.17
					1.13

Table 5. *Polarization of fluorescence of rhodamine B bound to esterase*

Solutions contained 0.01M phosphate buffer pH 7.4 (0.008M- Na_2HPO_4 -0.002M- KH_2PO_4) and 0.1M-KCl, at 25°. High enzyme concentrations and low dye concentrations were used, in order to determine p_B . For method of measuring polarizations see text, p. 99.

Enzyme concn. $[e_a]^*$	0.1	0.1	0.1	0.375	0.375	0.375
Rhodamine ($\text{M} \times 10^{-7}$)	2.0	4.0	6.0	2.0	4.0	6.0
p	0.262	0.255	0.248	0.270	0.270	0.270
$p' = 3p/(3+p)$	0.241	0.235	0.229	0.248	0.248	0.248

* Arbitrary units.

Table 6. *Polarization of fluorescence of free rhodamine B*

Solutions contained 0.01M phosphate buffer pH 7.4 (0.008M- Na_2HPO_4 -0.002M- KH_2PO_4) and 0.1M-KCl, at 25°. For method of measuring polarizations see text. P = polarization of fluorescence with polarized exciting light; p = polarization of fluorescence with natural exciting light.

Rhodamine concn. ($\text{M} \times 10^{-7}$)	5	50	100	200	400
P	—	—	0.07	0.06	<0.035
$\frac{P}{2-P} = p$	0.045	0.045	0.036	0.031	<0.018
$\frac{p}{1+\frac{1}{2}p} = p'$	0.045	0.045	0.036	0.031	<0.018

freed from liquid droplets. The final benzene concentration was about half the saturation value. Benzene had small effects on the reaction rates, both with rhodamine present and with no inhibitor at high and low substrate concentrations. The kinetic constants were thus altered somewhat by the change in medium, but the very large effect, which would be expected if the dye associated in the purely aqueous solution but not in the presence of benzene, was not observed.

Fluorescence studies

Polarization experiments. Preliminary observations showed that successive additions of enzyme to a solution of rhodamine B increased the polarization steadily towards a limiting value; and at a fixed enzyme concentration the polarization fell as the dye concentration increased. A series of experiments was carried out, in each of which the enzyme concentration was constant, while successive additions of a concentrated rhodamine B solution were made from an 'Agla' micrometer syringe. The polarization results were shown as follows to relate to a reversible equilibrium state: at the end of an experiment the mixture was diluted with a solution of dye in buffer, to reduce the enzyme concentration only. The resulting lower polarization value was always equal to that of a solution of the same final composition freshly prepared with no intermediate dilution step.

Polarizations at high enzyme and low dye concentrations (Table 5) indicated that $p_B = 0.270$, $p'_B = 0.248$. p_F was determined at a series of concentrations as shown in Table 6. At the higher concentrations polarized exciting light was used so that P_F was measured and p_F calculated. At concentra-

Fig. 7. Absorption spectra (determined in the Beckman spectrophotometer) of the following solutions:

Solution	...	...	...	A	B	C
Rhodamine B concn. ($\text{M} \times 10^{-6}$)	1.99	1.99	0			
Enzyme concn. $[e_a]$	0	0.374	0.374			

All solutions contained 0.1M-KCl and 0.01M phosphate buffer, pH 7.4. Curve D is the difference between curves B and C, and represents bound rhodamine. Polarization p' of solution B = 0.234. Enzyme concn. $[e_a]$ in arbitrary units.

tions below $5 \times 10^{-6}\text{M}$, p_F was constant at 0.045; and at higher concentrations depolarization occurred and p_F fell. A solution of rhodamine B in glycerol gave a polarization p_F^g of 0.270. Thus p_B was equal to p_F^g , indicating that the dye was bound by molecules or particles too large to permit an estimate of their size from the value of p_B .

Table 7. *Effect of concentration on the fluorescent intensity of free rhodamine B*

The fluorescent intensity of each solution, relative to that of an arbitrary standard solution of rhodamine, was measured as described in the text. All solutions contained 0.01M phosphate buffer pH 7.4 (0.008M- Na_2HPO_4 -0.002M- KH_2PO_4) and 0.1M-KCl. Absorption coefficients a and A , for exciting and observed emitted wavelengths respectively, were determined in a Beckman spectrophotometer and used to calculate the correction terms for light absorption in each solution. l =Cell depth in the direction of the exciting beam = 1 cm.; L =cell depth in the direction of observation of the fluorescent light = 2 cm.

Rhodamine (M $\times 10^{-7}$)	3.99	5.99	9.95	13.9	19.8	48.8
Observed relative intensity	0.891	1.43	2.11	2.90	4.14	7.07
$\frac{1 - e^{-al}}{al}$ (correction factor for absorption of exciting light)	0.955	0.94	0.90	0.865	0.812	0.62
$\frac{1 - e^{-AL}}{AL}$ (correction factor for absorption of observed emitted light)	0.99	0.985	0.975	0.965	0.955	0.90
Observed relative intensity divided by						
$\frac{1 - e^{-al}}{al} \times \frac{1 - e^{-AL}}{AL} \times \text{concn.}$	0.24	0.26	0.24	0.25	0.27	0.26

Absorption spectrum of bound dye. Fig. 7 shows the absorption spectrum of a solution of dye and enzyme, in which the dye was almost all bound, as shown by the polarization. The spectrum of the enzyme alone in the same concentration is shown, and the difference between the curves gives the spectrum of the bound dye. In relation to that of the free dye, it was shifted towards the red, and its maximum was lower.

Fluorescent intensities. (i) *Free dye.* Intensity comparisons were made between each of a series of rhodamine B solutions and an arbitrary standard solution. The observed relative intensities were corrected for absorption, as in eqn. 2, and Table 7 shows that they were then proportional to concentration, between 4×10^{-7} M and 4.9×10^{-6} M. Higher concentrations were not used because of the uncertainty in the large absorption corrections involved.

(ii) *Bound dye.* Table 8 shows two experiments to find R (see the theoretical section). In Expt. 2, the absorption of fluorescent light by the enzyme was too great for accurate determination of the absorption correction: maximum and minimum probable values are given. The maximum figure gave a value for R agreeing with the more reliable Expt. 1.

Displacement by substrate. Both competitive and 'apparent competitive' inhibitors should be displaced from combination with the enzyme by sufficiently high concentrations of substrate. In Table 9, comparison of solutions 2 and 4 shows the large fall in polarization which was caused by the presence of ethyl acetate. The fall in pH, due to substrate hydrolysis by the enzyme, was minimized by using this slowly hydrolysed substrate, a temperature of 20°, and concentrated buffer. At the end of the experiment the pH of solution 4, quickly measured with a glass electrode, was found

Table 8. *Ratio of fluorescent intensities, bound/free rhodamine*

The fluorescent intensity of solution y was compared with that of solution x by the method described in the text. All solutions contained 0.01M phosphate buffer pH 7.4 (0.008M- Na_2HPO_4 -0.002M- KH_2PO_4) and 0.1M-KCl. R , the ratio of the fluorescent intensity of the bound dye to that of the free, was then calculated by the steps explained in the theoretical section. The necessary measurements of absorption coefficients were made in a Beckman spectrophotometer; and the polarization of solution y was determined as described in the text, p. 99.

Experiment	...	...	...	1	2
Solution y					
Rhodamine B concn., M $\times 10^{-7}$				6.0	19.9
Enzyme concn., $[e_a]$				0.10	0.374
Solution x					
Rhodamine B concn., M $\times 10^{-7}$				6.0	19.9
Observed intensity ratio, R_1				1.77	1.48
R_2 (from eqn. 2)				1.92	1.73-1.97
p_y				0.248	0.254
R_2 (from eqn. 3)				2.05	1.85-2.11
p_y				0.229	0.234
R (from eqn. 4)				2.30	1.98-2.30

Table 9. *Displacement of dye by substrate*

The polarization of fluorescence of each solution, at 20°, was determined as described in the text, p. 99.

Soln. no.	Soln. composition	Polarization (p')
1	0.05M phosphate, pH 7.4, 0.1M-KCl, 5×10^{-7} M rhodamine B	0.045
2	4 ml. solution (1) + 0.1 ml. stock enzyme solution	0.187
3	4 ml. solution (1) + 0.2 ml. ethyl acetate	0.045
4	Solution (3) + 0.1 ml. stock enzyme solution	0.055
5	Solution (2) + 0.06 ml. N-HCl	0.176

to be 6.8. Hydrochloric acid was added to solution 2, bringing its pH to 6.7: little or no change in polarization resulted (solution 5).

The effect of ethyl acetate was not due to inactivation of the enzyme. Immediately after the experiment, the esterase activity of solution 4 was measured, by adding 0.04 ml. of it to 10 ml. of the standard reaction mixture of the drop-titration test (Burch, 1954) containing ethyl butyrate and bromthymol blue. The resulting reaction rate showed the enzyme to be fully active. The concentrations of rhodamine, ethyl acetate and hydrolysis products introduced were too small to interfere with this activity test.

Comparison of the fluorescent intensities of solutions 1 and 3 showed that the presence of ethyl acetate increased the intensity of fluorescence of the free dye by a factor of 1.3, with no change in absorption spectrum. By this intensity change alone, the presence of the ester would have caused a fall in the polarization of fluorescence of the mixture of free and bound dye; but the following calculation shows that this effect was small compared with the observed difference between solutions 2 and 4. By eqn. 1, at constant C_F/C_B , the reduction in R would have raised the term $(p'_B - p')/(p' - p'_F)$ by a factor of 1.3. Taking p' initially as 0.18 it would thereby have become 0.17. Since the observed final value was only 0.055, C_F/C_B cannot have remained constant: that is, displacement of the rhodamine by the ethyl acetate must have been nearly complete. The only alternative explanation of the polarization change would be an almost complete quenching of the fluorescence of the bound dye by the ester; but such an extreme difference between its effects on the bound and the free rhodamine seems very unlikely.

Equilibrium between dye and enzyme. Using the measured values $p'_B = 0.248$, $p'_F = 0.045$, and $R = 2.30$ in eqn. 1, C_F and C_B were calculated for each mixture of enzyme and dye in the polarization experiments. At dye concentrations above 10^{-5} M, values for p'_F were obtained from Table 6 by interpolation. The resulting values of $[e_a] C_F/C_B$ were plotted against C_F ; Fig. 8 shows the results at low dye concentrations and Fig. 9 those at high; and the same straight line is drawn in both figures.

DISCUSSION

Inhibition kinetics

The assumption that rhodamine B inhibits liver esterase by interaction with the enzyme, rather than with the substrate, is justified by the low molar concentrations of inhibitor used, compared with the concentrations of substrate. It has been assumed that the concentration of dye free in solution was not appreciably reduced by the presence of the

enzyme, so that the quantity $[I]$ in all equations was equal to the total concentration of rhodamine added. The number of enzyme active centres present was almost certainly much too small to bind an appreciable fraction of the dye, by any ordinary inhibition

Fig. 8. The equilibrium between rhodamine B and esterase, as shown by the polarization of fluorescence. Enzyme concn. $[e_a]$: $\bullet$, 0.0125; $\blacklozenge$, 0.0175; $\blacksquare$, 0.05; $\blacktriangledown$, 0.10. C_F = concn. of free dye, C_B = concn. of bound dye. C_F and C_B in each solution were calculated from the measured polarization of fluorescence, as described in the text. All solutions contained 0.1M-KCl and 0.01M phosphate buffer pH 7.4 (0.008M- Na_2HPO_4 -0.002M- KH_2PO_4) at 25°. For method of measuring polarizations, see text, p. 99. —, straight line identical with that of Fig. 9.

Fig. 9. The equilibrium between rhodamine B and esterase, as shown by the polarization of fluorescence. Enzyme concn. $[e_a]$: $\bullet$, 0.0125; $\blacklozenge$, 0.0175; $\blacksquare$, 0.05; $\blacktriangledown$, 0.10; $\blacktriangle$, 0.25. C_F = concn. of free dye, C_B = concn. of bound dye. C_F and C_B in each solution were calculated from the measured polarization of fluorescence, as described in the text, p. 98. All solutions contained 0.1M-KCl and 0.01M phosphate buffer pH 7.4 (0.008M- Na_2HPO_4 -0.002M- KH_2PO_4) at 25°. For method of measuring polarizations see text. —, straight line identical with that of Fig. 8.

mechanism. Dye concentrations were not less than 2.5×10^{-5} M, and active centre concentrations of this order would have entailed a turnover number for each centre of the order of 0.8 min.^{-1} (for ethyl butyrate hydrolysis at 20°). This value seems unreasonably low. Moreover, Bournsall & Webb (1949) have inhibited this esterase preparation with diisopropyl phosphorofluoridate containing ^{32}P and found 1 g. atom of phosphorus to be bound per 1×10^5 g. protein. If this is taken to indicate the number of esterase centres present, the concentration of active centres in the inhibition experiments with rhodamine B was only about 5×10^{-9} M.

Binding of the dye by adsorption at less specific sites on the protein is also possible, but two types of evidence show that it is very unlikely to have reduced the free-dye concentration appreciably. The percentage inhibition, at constant rhodamine and substrate concentrations, was independent of enzyme concentration, instead of falling when more enzyme was added; and extrapolation of the measurements of the polarization of fluorescence shows clearly that, at the low enzyme and high dye concentrations used in the kinetic work, the polarization was equal to that of the free dye, suggesting that a negligible fraction was bound to protein.

The inhibition has been shown to be rapidly established and reversible, to be competitive with respect to substrate, but to yield graphs of K_A against $[I]$ which can be explained neither by the normal competitive mechanism, nor by the 'apparent competitive' inhibition of Segal *et al.* (1952). Inhibition mechanisms involving a complex of the type E_2I are eliminated by the proportionality of the inhibited reaction rate to enzyme concentration, while association of the dye in solution is ruled out by measurements of freezing-point depressions and partition coefficients, together with the small effect of benzene on the reaction rates.

The possibility that the esterase preparation may consist of a mixture of enzymes must be considered, since its activity has not definitely been shown to be due to a single type of active centre (Burch, 1954). A mixture of two independent types of centre, acting on a single substrate, gives a graph of $1/v$ against $1/[S]$ which is concave downwards; and the curvature depends on the square of the difference between the K_m values of the two types of centre (Burch, 1954). The activity of the present enzyme preparation could thus be due to several esterases if their Michaelis constants were effectively equal, since straight-line graphs were obtained both with methyl butyrate and with the ethyl ester (Burch, 1954). Inhibition by rhodamine B does not affect the observed maximum velocity, therefore it can act only on the apparent values of the individual Michaelis constants. If these are changed in the

same ratio, and thus remain equal at all values of $[I]$, the system behaves as a single enzyme, and a mechanism still remains to be found to account for the results. If there is any quantitative difference between the actions of the inhibitor on the separate components of the system, the two K_A values will differ and the graphs of $1/v$ against $1/[S]$ in the presence of inhibitor must then be concave downwards rather than straight. Since the observed graphs (Fig. 2) were not significantly curved, it is clear that the existence of more than one type of active centre in the preparation cannot in itself account for the form of the K_A - $[I]$ relationship; and the straight lines of Fig. 2 are evidence that the esterase activity of the preparation is uniform.

An inhibition mechanism which accounts for the observed kinetics will now be proposed. It is assumed that the inhibitor combines with the enzyme at two separate points. When it combines at the substrate-binding centre, as in normal competitive inhibition, the resulting complex, EI , is enzymically inactive. The inhibitor may also combine at a different point, and this complex, EI' , has enzymic activity with changed kinetic constants. Its affinity for the substrate is reduced, while the rate of breakdown of the complex EIS is equal to that of ES . It may also be completely inhibited by further combination of inhibitor at the substrate-binding centre. Thus competitive and 'apparent competitive' inhibitions are acting simultaneously; and the hypothesis may be formulated as follows:

As pointed out by Segal *et al.* (1952), the generalized stationary state treatment of such a reaction scheme leads to very complex expressions; a special case in which each individual equilibrium is maintained, however, gives simpler results. The graphs of $1/v$ against $1/[S]$ at constant $[I]$ are linear only in this special case, so that the straight lines of Fig. 2 justify the equilibrium treatment for the present system. We may therefore put:

$$\begin{aligned} [E][S]/[ES] &= K_m, & [E][I]/[EI] &= K_1, \\ [E][I]/[EI'] &= K_1, & [EI'] [S]/[EIS] &= K'_m, \\ [EI'] [I]/[EI_2] &= K_2, \end{aligned}$$

and it follows that

$$[ES][I]/[EIS] = K_1 K'_m / K_m$$

which may be termed K'_1 . Manipulation of these equations in the ordinary way, assuming

$$v = k_3[\text{ES}] + k'_3[\text{EIS}],$$

shows that v is related to $[\text{S}]$ by the ordinary Michaelis expression; that if $k'_3 = k_3$ the maximum velocity is unchanged by the presence of inhibitor, and that

$$K_A = K_m \frac{1 + [\text{I}]/K_1 + [\text{I}]/K_i + [\text{I}]^2/K_1 K_2}{1 + [\text{I}]/K'_1}. \quad (6)$$

Putting $f = \frac{[\text{I}]}{K_A/K_m - 1}$, eqn. 6 gives

$$f = \frac{1 + [\text{I}]/K'_1}{1/K_i + 1/K_1 - 1/K'_1 + [\text{I}]/K_1 K_2}, \quad (7)$$

and when $[\text{I}] \rightarrow 0, f \rightarrow f_0$, where

$$f_0 = \frac{1}{1/K_i + 1/K_1 - 1/K'_1}. \quad (8)$$

Combination of eqns. 7 and 8 gives

$$\frac{[\text{I}]}{f - f_0} = \frac{K_1 K_2 / f_0}{K_1 K_2 / K'_1 - f_0} + \frac{[\text{I}]}{K_1 K_2 / K'_1 - f_0}. \quad (9)$$

The graph of $[\text{I}]/(f - f_0)$ against $[\text{I}]$ is thus linear, and its slope and intercept, together with the value of f_0 , determine the several equilibrium constants. The straight line of Fig. 6 yields the following values, for the inhibition of methyl butyrate hydrolysis:

$$K_1 K_2 = 5.3 \times 10^{-8} \text{ M}^2,$$

$$K'_1 = 1.2 \times 10^{-4} \text{ M},$$

$$1/(1/K_1 + 1/K_i) = 0.24 \times 10^{-4} \text{ M}.$$

Since K_i , K_1 , and K_2 refer to reactions between enzyme and inhibitor only, they must be independent of the substrate used. But K'_1 may differ for different substrates, giving a change in the value of K_A/K_m at a given value of $[\text{I}]$, as found in Table 3. For ethyl butyrate hydrolysis in the presence of $12.5 \times 10^{-4} \text{ M}$ rhodamine, K_A/K_m was found to be 16.0. Hence K'_1 for this substrate may be calculated (from eqn. 6) to be $3.0 \times 10^{-4} \text{ M}$. In $1.0 \times 10^{-4} \text{ M}$ rhodamine, K_A/K_m should then be 4.04, which compares well with the observed value of 4.0. This agreement provides independent evidence for the proposed inhibition mechanism, since the constants derived from the inhibition of methyl butyrate hydrolysis account for the relation between $[\text{I}]$ and K_A/K_m for the ethyl ester.

It must be emphasized that this hypothesis of the mechanism of inhibition can only be regarded as tentative; in common with all reaction mechanisms based on kinetic data, it can only be shown to fit the observations, and cannot be demonstrated as a unique solution to the problem. Furthermore, it involves three independent constants whose values can only be obtained experimentally. Simpler mechanisms, such as the ordinary competitive or the 'apparent competitive' modes of inhibition, with one or two such constants respectively, are

capable of more satisfactory verification. It is, however, believed to be the most economical hypothesis to explain the results, whose simplicity in many respects seems to justify the search for a reasonably simple inhibition mechanism.

Fluorescence studies

On addition of the enzyme to solutions of rhodamine B, the polarization of fluorescence becomes equal to the limiting value for the dye in very viscous media. This demonstrates that physical interaction occurs, such that the dye loses its freedom to rotate independently as small molecules, and becomes bound to large molecules in the esterase preparation in the rigorous sense that it shares their rotational Brownian movement. Physical combination is also indicated by a change in absorption spectrum.

The quantitative effect of dilution on the polarization verifies that the results refer to a reversible equilibrium; and the observed displacement of rhodamine from combination by ethyl acetate provides evidence that the binding is specifically related to the inhibition of the esterase activity.

The small change of absorption spectrum which results from the combination of rhodamine B with esterase is in contrast with the large change on absorption to serum albumin (Laurence, 1952), and suggests a different mechanism of binding.

The fluorescence data are analysed quantitatively in Figs. 8 and 9. For a simple combination between dye and enzyme eqn. 5 shows that these graphs should be linear, with slope $1/c$, and intercept K/c when $C_F = 0$. (K is the dissociation constant of the combination, and c the molar concentration of binding centres in the stock enzyme solution.) Results of an identical form would be expected from the proposed inhibition mechanism. Although the dye combines at two points on the enzyme, with different dissociation constants, the two compounds EI and EI' must always be present in a constant concentration ratio, $[\text{EI}]/[\text{EI}'] = K_1/K_i$, and the observed values of p_B and R will refer to this equilibrium mixture of EI and EI'. Eqn. 5 will therefore apply, with $K = 1/(1/K_1 + 1/K_i)$. The further combination $\text{EI}' + \text{I} \rightleftharpoons \text{EI}_2$, will not be observed under the conditions of the polarization experiments, because dye concentrations are here very low. Since

$$[\text{E}][\text{I}]^2/[\text{EI}_2] = K_1 K_2, \quad \text{or} \quad [\text{EI}_2]/[\text{E}] = C_F^2/K_1 K_2,$$

and $K_1 K_2$ was found to be $5.3 \times 10^{-8} \text{ M}^2$ while C_F was not greater than $3 \times 10^{-5} \text{ M}$, $[\text{EI}_2]/[\text{E}] < 0.017$. That is, the concentration of EI_2 must always have been less than 1.7% of that of the free enzyme, and an even smaller proportion of the total enzyme present, so that eqn. 5 cannot have been appreciably disturbed by formation of EI_2 .

Figs. 8 and 9 show that, in experiments with different enzyme concentrations, $[e_a] C_F/C_B$ was approximately independent of $[e_a]$ and was a function of C_F only, as in eqn. 5. The results are also consistent with a linear relation between $[e_a] C_F/C_B$ and C_F . But the straight line drawn gives constants very different in magnitude from those expected. Its slope is $3.6 \times 10^4 \text{ M}^{-1}$, giving $c = 0.28 \times 10^{-4} \text{ M}$; and its intercept at $C_F = 0$ is 0.027, so that

$$K = 0.075 \times 10^{-5} \text{ M},$$

which is only $0.031 \times$ the value of

$$K = 1/(1/K_1 + 1/K_i) = 2.4 \times 10^{-5} \text{ M}$$

found from the inhibition data.

In the inhibition by diisopropyl phosphorofluoridate (Boursonell & Webb, 1949), 1 g. atom of phosphorus was bound/ 10^5 g. protein, corresponding to a value of $2.2 \times 10^{-4} \text{ M}$ for the concentration of phosphorus-binding centres in the stock enzyme solution. This is 8 times the concentration of rhodamine-binding centres found above, while it might be expected that either one or two molecules of dye would be bound for every phosphorus atom. These discrepancies are difficult to interpret. It would be possible to explain the fluorescence results without conflict with the kinetic data if, in addition to the enzymic component of K value $2.4 \times 10^{-5} \text{ M}$, there was also present in the enzyme preparation a small concentration of an impurity which adsorbed the dye very strongly. Then at low values of C_F , the impurity would be responsible for almost all the observed binding, while at higher C_F values the impurity would become saturated with the dye and a progressively larger fraction of the bound rhodamine would be in combination with the enzymic component.

This hypothesis is made improbable, however, by the experiment which demonstrated the displacement of the dye from combination by ethyl acetate. In this experiment C_F was initially only $2.5 \times 10^{-7} \text{ M}$; nearly all the bound dye should therefore have been in combination with the impurity, and the ester should not have had any marked effect. In fact it displaced the dye almost completely, suggesting that the dye was combined at the esterase centres even at the lowest C_F values used.

It has been assumed throughout the analysis of the fluorescence results that the dynamic inter-conversion between bound and free dye was not rapid enough to interfere with their fluorescence. Weber (1948), in an analysis of the quenching of fluorescence by collision and by complex formation, shows that, unless the mean life of a fluorescent species is much longer than the lifetime of its excited state, the polarization of its fluorescence is not constant, but depends on its mean life and hence on its concentration and that of other species present.

The assumption has thus been made that the mean life of both bound and free dye was much greater than 2×10^{-9} sec., the lifetime of the excited state of rhodamine B (Weber, 1948). The following argument shows that the assumption was justified, so that no explanation of the fluorescence results is provided by this consideration.

In an equilibrium of the type, $E + I \xrightleftharpoons[k_2]{k_1} EI$, the mean life of the species EI is $1/k_2$, and that of the species I is $1/k_1[E]$, which at equilibrium is equal to $[I]/[EI] k_2$. Since C_F/C_B was never much less than 1, the assumption may be expressed as

$$1/k_2 \gg 2 \times 10^{-9} \text{ sec.},$$

or since the dissociation constant K is equal to k_2/k_1 , $k_1 \leq 0.5 \times 10^9/K \text{ sec.}^{-1}$. Now k_1 is the velocity constant of a bimolecular reaction, which cannot be larger than a reasonable collision frequency; so that if K is much less than about 0.01 M, the assumption is certainly valid. Since appreciable inhibition was observed with $2.5 \times 10^{-5} \text{ M}$ rhodamine, at least one K value for this system must be much lower than 0.01 M, whatever the detailed mechanism of the inhibition. If several types of combination occurred simultaneously, the assumption might break down for one with a very high K value; but the proportion of bound dye present in this form would necessarily be extremely small, so that no disturbance of the observed polarization would result.

It remains possible that the interactions between rhodamine and esterase which occurred in the fluorescence studies were quantitatively different from those taking place in the inhibition work, due to the differences between the experimental conditions in the two types of experiment. While temperature and potassium chloride concentrations were identical, and phosphate concentrations differed only slightly, the enzyme concentrations of the fluorescence work were about $1000 \times$ greater than those of the kinetic studies, and dye concentrations about $100 \times$ smaller. For instance, a complex of the form E_2I could have been formed under the conditions of the fluorescence work, although it was not apparent in the inhibition studies.

SUMMARY

1. The inhibition of horse-liver esterase by the fluorescent dye rhodamine B has been studied kinetically; and the interaction between enzyme and dye has been investigated by measurements of the intensity and polarization of fluorescence.

2. The inhibition is rapidly established and reversible, and competitive with respect to substrate. At constant substrate and dye concentrations, the percentage inhibition is independent of enzyme and phosphate concentrations.

3. The relation between the dye concentration and the apparent value of the Michaelis constant is not linear. It resembles the function entailed by the 'apparent competitive' inhibition mechanism of Segal *et al.* (1952), but on analysis is found to deviate significantly from it.

4. An inhibition mechanism is proposed which accounts for the experimental results, including the relation between the inhibitions of the hydrolyses of two different substrates.

5. Distribution experiments and freezing-point measurements show that rhodamine B is monomeric in the aqueous solutions of pH 7.4 used for the enzyme studies.

6. Measurements of the polarization of fluorescence demonstrate the reversible combination of rhodamine with large molecules in the enzyme preparation. The combination is accompanied by a change in absorption spectrum and an increase in fluorescent intensity.

7. Displacement of the dye from combination by ethyl acetate gives evidence that the binding is specifically related to the esterase inhibition.

8. The fluorescence data are analysed quantitatively, and their relation to the inhibition results is discussed.

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REFERENCES

- Boursnell, J. C. & Webb, E. C. (1949). *Nature, Lond.*, **164**, 875.
 Burch, J. (1954). *Biochem. J.* **58**, 415.
 Dolinsky, M. (1949). *J. Ass. off. agric. Chem. Wash.* **32**, 130.
 Findlay, A. (1941). *Practical Physical Chemistry*, 7th ed. London: Longmans Green and Co.
 Ghosh, B. N. & Sengupta, S. B. (1938). *Z. phys. Chem.* **B 41**, 117.
 Laurence, D. J. R. (1952). *Biochem. J.* **51**, 168.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Noelting, E. & Dzierzowsky, K. (1905). *Ber. dtsch. chem. Ges.* **38**, 3516.
 Perrin, F. (1929). *Ann. Phys., Paris*, **12**, 169.
 Segal, H. L., Kackmar, J. F. & Boyer, P. D. (1952). *Enzymologia*, **15**, 187.
 Wawilow, S. I. (1942). *C.R. Acad. Sci. U.R.S.S.* **35**, 100.
 Weber, G. (1948). *Trans. Faraday Soc.* **44**, 185.
 Weber, G. (1952a). *Biochem. J.* **51**, 145.
 Weber, G. (1952b). *Biochem. J.* **51**, 155.

The Oxidation of Indolyl-3-Acetic Acid by Waxpod Bean Root Sap and Peroxidase Systems

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The presence in extracts of certain plant tissues of systems which oxidize indolyl-3-acetic acid (IAA) is well established (e.g. by Tang & Bonner, 1947, 1948; Wagenknecht & Burris, 1950; Gortner & Kent, 1953). The enzyme system present in extracts of etiolated pea epicotyls has been most studied, and it has been suggested that the system consists of a flavoprotein which gives rise to hydrogen peroxide and a peroxidase which catalyses the oxidation of IAA by hydrogen peroxide (cf. Galston, Bonner & Baker, 1953). The enzyme in extracts of waxpod bean roots has received little attention, although according to Wagenknecht & Burris (1950) such extracts show greater activity towards IAA than extracts of etiolated pea epicotyls. Wagenknecht & Burris (1950) have shown that the rate of oxidation of IAA by dialysed extracts of waxpod bean roots is increased in the presence of suitable amounts of Mn^{2+} . It has been shown by Galston *et al.* (1953) that horseradish peroxidase preparations catalyse

the oxidation of IAA in the absence of added hydrogen peroxide, and Kenten (1953) has reported that the rate of this oxidation is stimulated in the presence of Mn^{2+} . These facts suggested that the system peroxidase- Mn^{2+} might be responsible for the activity of extracts of waxpod bean roots towards IAA, and the present work was undertaken to investigate this possibility.

MATERIALS AND METHODS

Plant sap. The waxpod beans (*Phaseolus vulgaris*, L., var. Sutton's Golden Waxpod) were glasshouse-grown in a John Innes potting compost. They were harvested 4–8 weeks after sowing. The rest of the plants were found growing wild or in gardens in the neighbourhood of Harpenden. The plant material was washed free from soil, and either ground in a mortar with sand or passed through a domestic meat mincer. The pulp was squeezed by hand through strong cotton cloth, the sap centrifuged for 15–20 min. at 6000 rev./min., the precipitate discarded and the supernatant stored at 0°.