J. Chem. Soc., Faraday Trans. 1, 1985, **81**, 2667–2679

Activity of Lipase in Water-in-oil Microemulsions

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Received 24*th September*, 1984

The lipase-catalysed hydrolysis rates of several nitrophenyl alkanoate esters of varying alkyl chain length (C_4–C_{16}) have been measured both in aqueous solution and in water-in-oil (w/o) microemulsions (which are known to contain discrete droplets). Lipase retains its activity in w/o microemulsions of water, heptane and sodium bis-2-ethylhexyl sulphosuccinate (AOT); the observed rates are consistent with the intrinsic activity of the enzyme (*i.e.* k_{cat}/K_m) being the same as in water. However, the observed conversion rates for C_4 and C_6 substrates are slower in the microemulsion system because of substrate partitioning to the oil-continuous phase, which results in a reduced concentration in the aqueous pseudophase. This conclusion is reached by comparing lipase and non-enzymic-(*i.e.* buffer) catalysed rates in both solution media. Again for the C_4 and C_6 substrate, partition coefficients for the substrates in the limit of high molar ratio of H_2O:AOT, as determined from the kinetic results, show good agreement with measured values in heptane + water mixtures. This suggests that lipase functions effectively in the water pseudophase of the microemulsion. Lipase in the microemulsion can also catalyse the hydrolysis of longer chain alkanoates (up to C_{16}). It can be inferred from the kinetics that such substrates partition to the interface where the lipase must also be active. In the case of AOT microemulsions, the pH profile of enzyme activity is not significantly altered compared with bulk water. The lipase retains > 60% activity in the microemulsion after incubation at 35 °C for 6 days. In w/o microemulsions of water, heptane, chloroform and cetyltrimethylammonium bromide (CTAB), the observed hydrolysis rates are significantly reduced and the intrinsic activity is reduced by a factor of twenty as compared with the AOT system. This is thought to be caused by inhibitory binding of CTAB to the protein.

There is current interest in the use of water-in-oil (w/o) microemulsions as a medium for the study of enzyme properties. When solubilised in the small water droplets of the microemulsion, enzymes are afforded some protection from the denaturing effect of the oil solvent. There are novel synthetic possibilities for such systems. Enzymes studied to date include α-chymotrypsin, liver alcohol dehydrogenase and lysozyme.[1–7]

This paper, which describes the activity of a lipase in w/o microemulsion systems, has novel aspects. Lipases in general catalyse the hydrolysis of fatty acyl esters and are known to act at oil/water interfaces.[8] Previous work on enzyme activity in microemulsions has been largely concerned with substrates and enzymes located in the water phase of the microemulsion. The substrates used in this work partition strongly to the oil domain of the microemulsion system.

The microemulsions used in this study are (*a*) water + AOT + heptane and (*b*) water dispersed in CTAB solutions in a 50-vol % heptane + chloroform mixture. Microemulsions are simply prepared; on mixing the reagents clear thermodynamically stable microemulsions are formed on gentle shaking. The AOT system has been extensively characterised using a variety of techniques including PCS (photon-

correlation spectroscopy),[9,10] ultracentrifugation,[11] SANS (small-angle neutron scattering)[12] and a fluorescence technique.[13] For compositions used in this study, the optically-clear microemulsions consist of small (similar sized) water droplets surrounded by a surfactant shell and dispersed in a continuous oil solvent. The droplet size increases linearly with the mole (or molar) ratio [water]/[surfactant] ($= R$) and can be varied over the radius range 1–20 nm.[9–13] Increasing the water volume fraction at constant R proportionally increases the concentration of droplets without significantly changing their size. We have recently studied the CTAB system using PCS, SANS and viscosity and observed qualitatively similar behaviour.[14]

In this paper kinetic data are presented for the lipase-catalysed hydrolysis of *p*-nitrophenylesters of fatty acids (C_4–C_{16}) in the two microemulsion systems. The effect on the kinetics of altering droplet size (by varying R), droplet concentration, the oil solvent, temperature and pH of the dispersed water is discussed. The time course of enzyme inactivation in the microemulsion is also reported. In addition to lipase-catalysed reactions, non-enzymic-catalysed reactions of the same substrates in water and microemulsions have been studied.

The subsequent data analysis allows rate effects due to (*a*) changes in substrate partitioning and (*b*) changes in intrinsic activity of the lipase in the microemulsion media to be identified.

EXPERIMENTAL

The lipase used was an extract from *Chromobacterium viscosum*. It was purchased from Genzyme Corporation and used without further purification. In a series of papers Isobe and coworkers [15–19] have described the purification and properties of two lipases from *Chromobacterium viscosum*. They identify lipase A (mol. wt. $= 1.2 \times 10^5$, 2 subunits, isoelectric point 4.7) and lipase B (mol. wt. $= 2.7 \times 10^4$, single unit, isoelectric point 6.9). Both proteins have intrinsic viscosity values of *ca.* 3 $cm^3\ g^{-1}$ in water, which indicates they are close to spherical in shape. The lipase preparation used in this study has a molecular weight of 3.3×10^4 by sodium dodecylsulphate polyacrylamide gel electrophoresis. Iso-electric focussing showed a major band at pH 7 ± 0.2 and three minor bands in the pH range 4–6. A further band was observed at pH 6.5. Lowry analysis showed that 60 wt % of the commercial material is protein. It is concluded that the predominant lipase species present is the Lipase B of Isobe and coworkers.

Substrates [*p*-nitrophenyl alkanoates (C_4–C_{16})] were purchased from Sigma. Substrate purity was determined by complete hydrolysis and measurement of the liberated *p*-nitrophenol spectrum. AOT may contain an acidic impurity (resulting from surfactant hydrolysis) which can seriously distort kinetic results.[20] For these measurements, AOT was purchased from Sigma and was shown to contain negligible quantities of the acidic impurity.[20] CTAB was a Sigma product. n-Heptane (Fisons) was distilled over sodium metal, stored over type 4A molecular sieve and filtered prior to use. Chloroform (May and Baker, analytical reagent) contains 1.5 vol % ethanol as stabiliser and was not purified further. Various buffers (indicated in fig. 3) were freshly prepared and used within 1–2 days.

Rate measurements were performed using a Unicam SP-8 200 spectrophotometer. Temperature control was to ± 0.2 °C. Rates were monitored by means of the absorbance of the *p*-nitrophenol liberated. Since *p*-nitrophenol has $pK_a \approx 7$, the spectrum is a sensitive function of pH. The ratio of nitrophenol/nitrophenolate species is also sensitive to the microemulsion composition as microemulsification causes a pK_a change. To obviate these difficulties, the monitoring wavelength was selected as the isosbestic point (λ_{iso}) of the nitrophenol/nitrophenolate couple. (For measurements in aqueous solution, it was necessary to add 4% ethanol to dissolve the substrates.) The relevant spectroscopic parameters were found to be: 4 vol % ethanol + water $\lambda_{iso} = 346$ nm and $\varepsilon = 4.8 \times 10^3$ $dm^3\ mol^{-1}\ cm^{-1}$; AOT system, $\lambda_{iso} = 333$ nm and $\varepsilon = 4.42 \times 10^3$ $dm^3\ mol^{-1}\ cm^1$; CTAB system, $\lambda_{iso} = 347$ nm and $\varepsilon = 4.80 \times 10^3$ $dm^3\ mol^{-1}\ cm^{-1}$.

A typical experiment was performed as follows. Aqueous solutions of lipase and buffer were added to a concentrated solution of AOT in heptane in a 10 cm^3 volumetric flask. A solution

of the substrate in n-heptane was added and the solution made up to the mark with heptane. Gentle shaking produced clarification within 1 or 2 min. (Clarification time depends on the buffer used, the R values and the temperature.) The initial linear rate of increase of absorbance with time was then recorded at λ_{iso}. Measured rates were independent of the order of addition of reactants. Where necessary, small corrections were made for the non-enzymic-catalysed rates of hydrolysis. Partition coefficients for the C_4–C_8 substrates between n-heptane and water were measured using a u.v. spectrophotometric method, with allowance for spontaneous hydrolysis in the water phase.[21]

RESULTS AND DISCUSSION

THEORETICAL BASIS FOR THE INTERPRETATION OF THE KINETICS

There was no significant solubility of active lipase in n-heptane. Therefore, in the microemulsion, the lipase is assumed to be exclusively associated with the droplets. The ester substrates, however, preferentially partition to the oil-continuous phase. The reaction scheme may be modelled, to a first approximation, as follows:

$$\text{enzyme } (E)_w + \text{substrate } (S)_w \underset{(K_m)_w}{\overset{\text{fast}}{\rightleftharpoons}} \text{enzyme–substrate complex (Michaelis complex)} \quad (ES)_w \xrightarrow[k_{cat}]{\text{slow}} \text{products (P)}$$

$$P_s \Big\| \Big\uparrow \text{fast}$$

$$\text{substrate } (S)_o$$

The subscripts w and o indicate whether the species is located within the water droplet pseudophase or oil-continuous phase, respectively, $(K_m)_w$ is the Michaelis constant for the formation of the enzyme–substrate complex (ES) in the water droplet pseudophase and k_{cat} is the first-order rate constant for reaction within the Michaelis complex. The substrate partition coefficient P_s is defined as:

$$P_s = \frac{[S]_w}{[S]_0}. \tag{1}$$

Concentrations are defined per unit volume of the water and oil phases.

Using the analysis of Berezin,[22] the following expression is derived for the initial velocity of reaction (v):

$$v = \frac{d[P]}{dt} = \frac{k_{cat}[E]_T[S]_T}{(K_m)_{app} + [S]_T} \tag{2}$$

where the subscript T indicates that the concentration is expressed as number of moles per unit of total volume of the microemulsion medium (as opposed to per unit volume of one of the pseudophases).

The apparent reaction parameter $(K_m)_{app}$ is related to the 'true' parameter $(K_m)_w$ in the water droplet pseudophase (where reaction occurs) as follows:

$$(K_m)_{app} = \frac{(K_m)_w[1+\theta(P_s-1)]}{P_s} \tag{3}$$

where θ is the volume fraction of the water droplet pseudophase. For a water-soluble substrate, *i.e.* $P_s \gg 1$, then $(K_m)_{app} = (K_m)_w\theta$. For an oil-soluble substrate ($P_s \ll 1$)

$$(K_m)_{app} = \frac{(K_m)_w(1-\theta)}{P_s} \tag{4}$$

$$= (K_m)_w/P_s \quad \text{when} \quad \theta \ll 1. \tag{5}$$

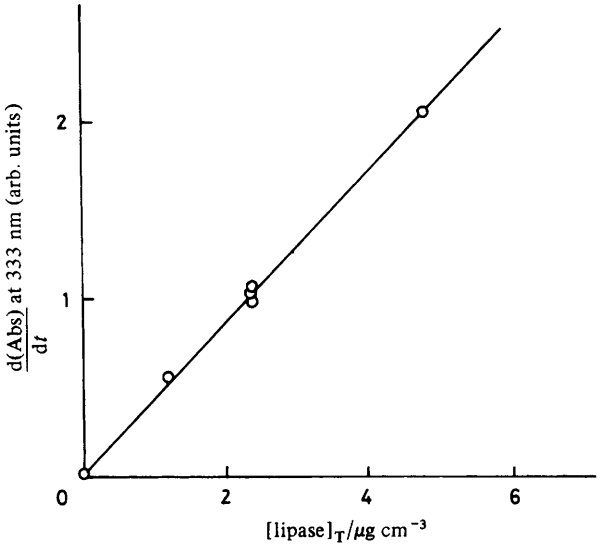

Fig. 1. Dependence of initial rate of lipase-catalysed hydrolysis of *p*-nitrophenyloctanoate in 0.1 mol dm^{-3} AOT ($R = 11.1$) at 25.0 °C.

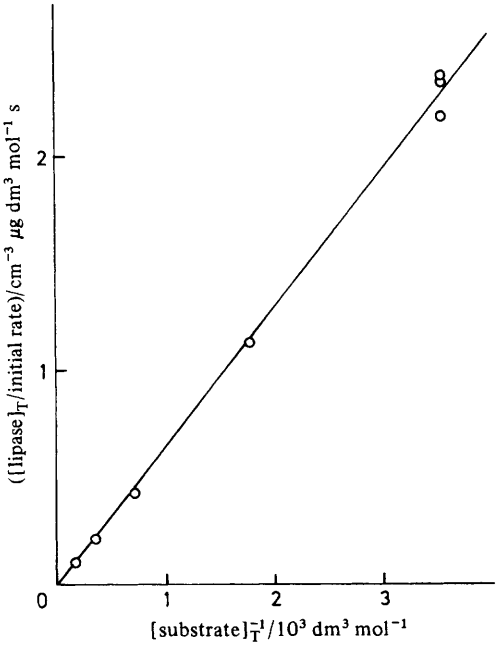

Fig. 2. Lineweaver–Burk plot for the lipase-catalysed hydrolysis of *p*-nitrophenyloctanoate in 0.1 mol dm^{-3} AOT ($R = 11.1$) in heptane at 25.0 °C. The pH is 8.15 (diglycine).

For these experiments, θ was typically in the range 0.01–0.15.

Fig. 1 and 2 shows the rate behaviour for the lipase-catalysed hydrolysis of *p*-nitrophenyloctanoate. The rate of reaction is linearly dependent on enzyme concentration (fig. 1). The Lineweaver–Burk plot (fig. 2) (from which K_m and k_{cat} are generally obtained from the slope and intercept) shows a zero intercept, indicating that $(K_m)_{app}$ is too large to be separated from k_{cat} in this system. Hence $(K_m)_{app}$, which is a dissociation constant, is much greater than $[S]_T$. Eqn (2) can then be expressed in the form

$$v = \frac{k_{cat}[E]_T[S]_T}{(K_m)_{app}}. \quad (6)$$

It is simpler, in practice, to derive a second-order rate constant, k_2, defined as follows:[21]

$$v = k_2[E][S]_T \quad (7)$$

where $$k_2 = \text{constant } k_{cat}/(K_m)_{app} = \text{constant } k_{cat} P_s/(K_m)_w. \quad (8)$$

The constant term is necessary because the enzyme concentration [E] is here expressed in g cm^{-3} of total solution, since the molecular weight of the enzyme is unknown. Hence the units of k_2 are $cm^3\ g^{-1}\ s^{-1}$ [from eqn (7)].

FACTORS INFLUENCING THE RATE OF LIPASE-CATALYSED REACTIONS IN AOT MICROEMULSIONS

The influence of various experimental parameters on k_2 was examined. For a constant R value ($R = [H_2O]/[AOT] = 11.1$) the rate constant k_2 was independent of [AOT] over the range 0.05–0.4 mol dm^{-3}. In addition, k_2 is independent of the buffer (diglycine) concentration up to 2×10^{-3} mol dm^{-3} diglycine (expressed as an overall concentration). This independence of the rate on buffer concentration is an important experimental check that sufficient buffering capacity is present in solution. The effect of pH on the rate constant is shown in fig. 3. Different buffers were used near their pK_a values to ensure sufficient buffering capacity. A maximum is found in the range 6.0–7.5, as observed for the same reaction in bulk water.[21] Since the pH cannot be measured absolutely in microemulsions, the pH refers to that of the water before solubilization. However, we estimate from the buffer range used that the pH is precise to < 1 pH units in the microemulsion. The effect of changing droplet size is shown in fig. 4. Droplet size was varied by changing R. Fig. 4 shows results obtained with three different buffers. The data show that k_2 is indeed constant in large droplets (high R), but increases as the droplet size is reduced, going through a maximum at $R \approx 11$. The radius of the water core of an $R = 11$ water droplet is *ca.* 2 nm.

The temperature dependence of k_2 is shown in fig. 5. For all R values, the rate constant goes through a maximum at *ca.* 30 °C. The decrease in rate at high temperature is not the result of an irreversible loss of enzyme activity, since when the solubilised enzyme was incubated at 55 °C for 15 min and subsequently assayed at 25 °C, the observed rate was only 10% lower than that in a control that had not been incubated. This compares with 50% lower activity noted in fig. 5 when the assay is carried out at 55 °C. From eqn (8) it is clear that the temperature dependence of k_2 is a composite of the dependences of k_{cat}, $(K_m)_w$, P_s and of any effect of temperature on the microemulsion structure (*e.g.* size of water droplets). No simple interpretation of the temperature dependence of k_2 is therefore to be expected. However, note that the low-temperature data of fig. 5 for $R = 11.1$, when plotted as an Arrhenius plot, yield an apparent activation energy of 44 kJ mol^{-1} compared with the value of 43 kJ mol^{-1} observed in bulk water.[21]

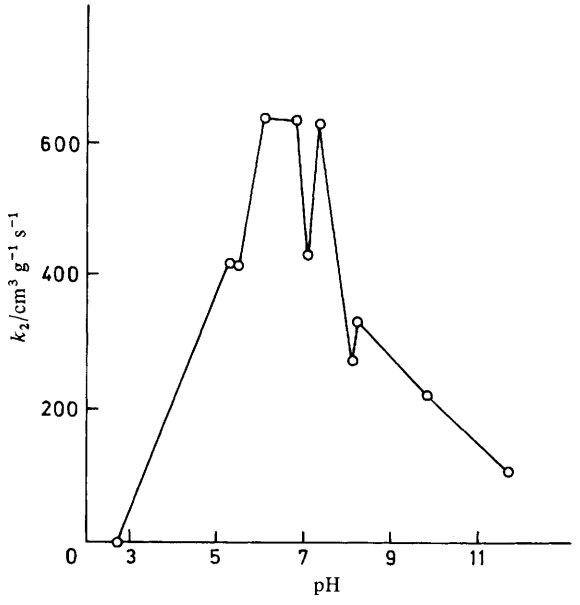

Fig. 3. Variation of k_2 with aqueous pH in 0.1 mol dm^{-3} AOT ($R = 11.1$) in heptane. The substrate is *p*-nitrophenyloctanoate, the temperature is 25 °C and $[\text{buffer}]_T = 10^{-3}$ mol dm^{-3}. In ascending order of pH the buffers used were citrate, maleate, citrate, cacodylate, phosphate, imidazole, collidine, tris, diglycine, glycine and phosphate.

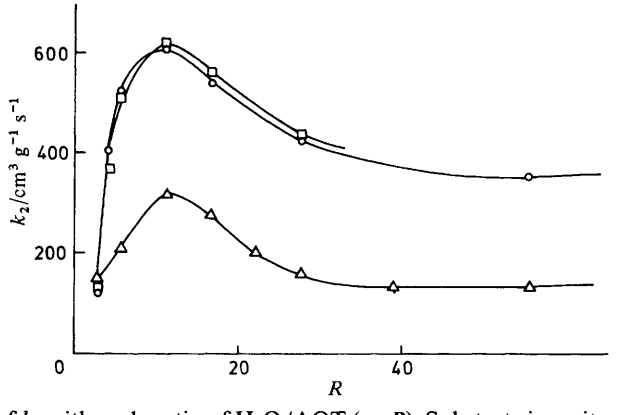

Fig 4. Variation of k_2 with mole ratio of H_2O/AOT ($= R$). Substrate is *p*-nitrophenyloctanoate in 0.1 mol dm^{-3} AOT in heptane at 25.2 °C. The buffers used were diglycine (△), cacodylate (○) and phosphate (□).

The stability of the enzyme in microemulsions was investigated over extended periods at 35 °C. Fig. 6 shows that the enzyme is very stable in microemulsions of all R values, with 60–85% activity remaining after 6 days. The most rapid drop in activity is observed for the largest R values (largest water droplets); in microemulsions of $R = 2.8$ an initial increase in activity is observed, followed by a relatively rapid decrease. There is no obvious explanation for this latter effect.

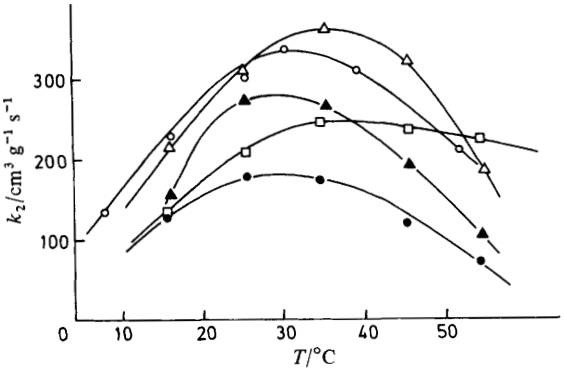

Fig. 5. Variation of k_2 with temperature for the reaction of C_8 in 0.1 mol dm^{-3} AOT at R values of 5.6 (□), 8.3 (△), 11.1 (○), 16.7 (▲) and 27.8 (●).

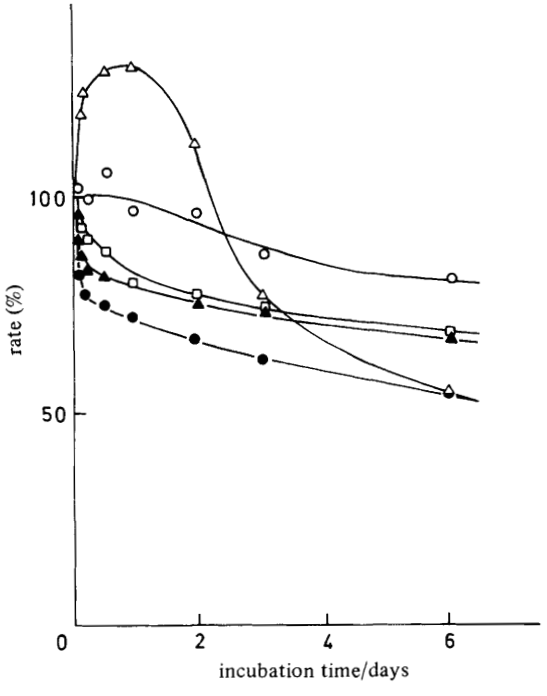

Fig. 6. Activity of lipase in 0.1 mol dm^{-3} AOT microemulsions as a function of time. The solutions were incubated at 35.0 °C and assayed at 25.0 °C using the C_8 substrate. R values are 2.8 (△), 5.6 (○), 11.1 (□), 27.8 (▲) and 55.6 (●).

A comparison of the rates observed with *p*-nitrophenylalkanoates of different chain lengths provides some interesting data. Fig. 7 shows the variation of k_2 with R for the different substrates and the effect of substituting dodecane for heptane as the oil-phase solvent. The value of k_2 for the C_{16} substrate is only four-fold lower than that for the C_4 homologue. From eqn (7), k_2 is linearly dependent on the partition

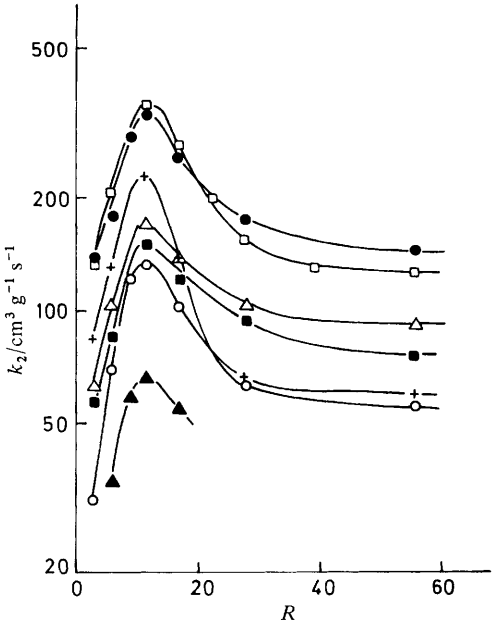

Fig. 7. Variation of k_2 with R for different *p*-nitrophenylalkanoates (C_4–C_{16}). The solutions contain 0.1 mol dm^{-3} AOT at 25.3 °C with diglycine buffer. The substrates are C_4 (●), C_6 (+), C_8 (□), C_{10} (△), C_{12} (■), C_{16} (○) and C_8 in dodecane (as opposed to heptane) oil solvent (▲).

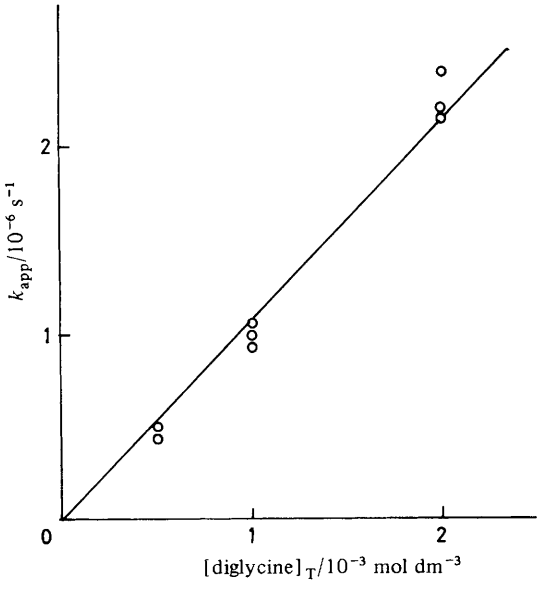

Fig. 8. Variation of k_{app} for C_4 hydrolysis in 0.1 mol dm^{-3} AOT (R = 11.1) at 25.2 °C.

coefficient P_s, and for homologous compounds P_s would be expected to decrease by a factor of four for each additional —CH_2— group.[23] Hence P_s for these substrates would be expected to vary by over a 10^7-fold range. The fact that the values of k_2 for all the substrates fall within a relatively narrow range indicates that some other factor is also varying. A decrease in $(K_m)_w$ with increasing chain length, in parallel with compensatory variations in partition behaviour, may offer an explanation, but it is also likely that for the longer chain substrates a build up of substrate in the interface region can occur.

STUDIES OF NON-ENZYMIC HYDROLYSIS RATES IN MICROEMULSIONS

In order to resolve effects on partition behaviour from effects arising from intrinsic properties of the enzyme, studies were carried out on related non-enzymic hydrolysis reactions in microemulsions since the results provide an alternative route to P_s. This approach is in many ways preferable to a direct determination of the partition coefficients since it is difficult to study the partition behaviour of the substrates in the microemulsions, and partition coefficients obtained from studies using bulk water and heptane phases may not necessarily be appropriate.

Esterolysis reactions are subject to nucleophilic catalysis. The non-enzymic rates of hydrolysis of the *p*-nitrophenyl esters were determined in water and in AOT microemulsions as a function of R and of buffer, substrate and AOT concentration. Reactions in phosphate and cacodylate buffers showed a spontaneous rate unaffected by buffer concentration. However, at high concentrations of diglycine buffer, the rate showed a first-order dependence on diglycine concentration both in water an in microemulsions, and hence the reaction is catalysed by diglycine (fig. 8). The reaction also showed first-order dependence on substrate concentration. Since diglycine is charged and hence confined to the water-droplet pseudophase, the reaction scheme shown for the enzyme-catalysed reaction is applicable. The observed second-order rate constant k_2(obs.) for buffer catalysis is then given by

$$k_2(\text{obs.}) = (k_2)_w P_s. \tag{8}$$

This provides a route for the indirect determination of P_s in the microemulsion system itself, by comparison of rates of diglycine-catalysed hydrolysis in microemulsions and in bulk water. The assumption is made that $(k_2)_w$, the rate constant in the microemulsion water droplets, is the same as the rate constant measured in bulk water. This assumption is most likely to hold at high R.[24, 25]

Data for a comparison of reaction rates in water and in high-R microemulsions are collected in table 1. The data are for C_4 and C_6 substrates and include second-order rate constants for both lipase- and buffer-catalysed reactions and first-order rate constants for the spontaneous hydrolysis in phosphate buffer. For non-enzymic reactions the ratio of rates in the microemulsion to rates in water gives an estimate of P_s. For the C_4 substrate this estimate is in the range $(5.0–6.6) \times 10^{-3}$, whereas the partition coefficient directly determined for partition between water and heptane is 4.7×10^{-3}. For the C_6 substrate the rate ratios are an order of magnitude lower, as is the directly determined partition coefficient. The self-consistency of this set of data suggests that the partitioning of these short-chain substrates between the water and heptane pseudophases of AOT microemulsions roughly corresponds to that between bulk water and heptane. Furthermore, the trend in partition coefficients with substrates of varying chain length has been found[21] to agree with that predicted from the theory of Tanford.[23]

Most importantly, this set of data shows that incorporation of the lipase into AOT microemulsions has very little effect on its catalytic properties. The difference in

Table 1. Comparison of rates in aqueous solution and AOT microemulsion for buffer- and lipase-catalysed reactions

system	rate in 4% EtOH + water	rate in AOT microemulsion (high R)	rate (micro-emulsion) /rate (water)
C_4/diglycine	$(3.0 \pm 0.3) \times 10^{-2}$ dm^3 mol^{-1} s^{-1}	$(1.5 \pm 0.3) \times 10^{-4}$ dm^3 mol^{-1} s^{-1} [a]	5.0×10^{-3}
C_6/diglycine	$(2.2 \pm 0.5) \times 10^{-2}$ dm^3 mol^{-1} s^{-1}	$(1.7 \pm 0.7) \times 10^{-5}$ dm^3 mol^{-1} s^{-1} [a]	7.7×10^{-4}
C_4/phosphate	$(3.5 \pm 0.7) \times 10^{-5}$ s^{-1}	$(2.3 \pm 0.5) \times 10^{-7}$ s^{-1}	6.6×10^{-3}
C_4/lipase	$(6.5 \pm 0.5) \times 10^{4}$ cm^3 g^{-1} s^{-1}	145 ± 15 cm^3 g^{-1} s^{-1}	2.2×10^{-3}
C_6/lipase	$(5.0 \pm 1.0) \times 10^{5}$ cm g^{-1} s^{-1}	60 ± 6 cm^3 g^{-1} s^{-1}	1.2×10^{-4}

[a] These values are extrapolated values corresponding to $R = \infty$, otherwise $R = 55$. Values of P_s observed experimentally between bulk heptane and water were $(2.5 \pm 0.2) \times 10^{-3}$ for the C_4 ester and $(1.4 \pm 0.2) \times 10^{-4}$ for the C_6 ester.[21] A comparison should be made of P_s with data in the last column.

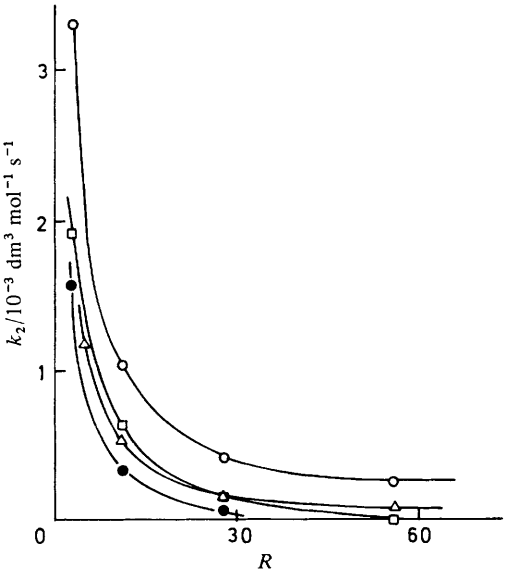

Fig. 9. Variation of k_2 with R for the diglycine-catalysed hydrolysis of various alkanoate substrates in 0.1 mol dm^{-3} AOT at 25.3 °C. Substrates are C_4 (○), C_6 (△), C_8 (□) and C_{16} (●).

observed rates of lipase-catalysed reactions in water and in the microemulsion can be entirely explained by effects of substrate partitioning, since the rate ratios for the lipase catalysed reaction are close to those for the non-enzymic reactions and so there can be little change in the enzyme's catalytic parameters (*i.e.* k_{cat}/K_m) on incorporation into the microemulsions. This conclusion, together with the data of fig. 3 and 5 on pH and temperature dependence, implies that the enzyme in AOT microemulsions is comparable in catalytic behaviour to the enzyme in bulk water. This behaviour is in contrast to that observed for α-chymotrypsin in AOT microemulsions, where K_m is increased one-hundred fold[1] as compared with bulk water.

However, reactions in microemulsions show complexities which indicate that the

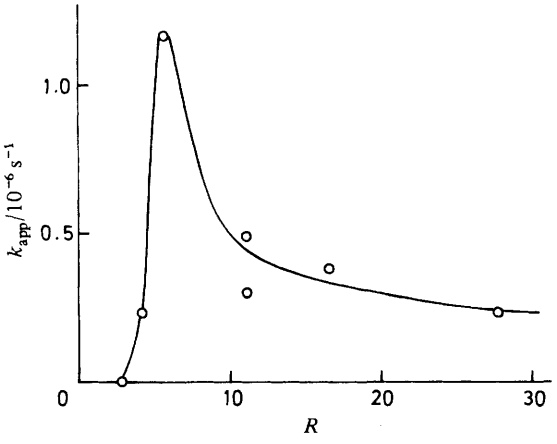

Fig. 10. Variation of first-order rate constant k_{app} with R for C_4 hydrolysis in 0.1 mol dm^{-3} AOT in heptane at 25.3 °C.

initial model is a considerable oversimplification. Fig. 9 shows the variation of k_2(obs.) for the buffer-catalysed ester hydrolysis as a function of R and of substrate chain length. The rate constants increase with decreasing R. This may be explained, in part, by the fact that the solubilised water in droplets of low R is known to be of lower polarity than that of bulk water.[25] Hence the substrates may partition into water droplets of low R more favourably than they do into droplets of high R, leading to increased rates of hydrolysis.

The complexity of microemulsions as a reaction medium is also demonstrated by the data of fig. 10, which shows the variation with R of the spontaneous hydrolysis rate in phosphate buffer. The rate goes through a maximum at $R = 8$ and decreases sharply at low R, in contrast to the situation for the diglycine-catalysed rate (fig. 9). With diglycine acting as a nucleophilic catalyst, water is not directly involved in the rate-determining step. For the spontaneous reaction in phosphate buffer, the rate-determining step presumably involves attack by H_2O or OH^-. Thus the two reactions will be influenced differently by effects on the activity of water, which is thought to drop rapidly[26] with R as R decreases below 10. Hence the decrease in rate at low R in fig. 10 is mainly caused by the decrease in water activity, whereas the increase in rate at low R observed in fig. 9 is mainly determined by the decrease in water polarity and consequent increased partitioning. The combination of these effects may explain the variation in lipase-catalysed rates with R (fig. 4).

The range of rates from C_4 to C_{16} substrates is much less than that expected if substrate partitioning alone is significant. In other words the rate of buffer-catalysed hydrolysis of the C_{16} substrate is much higher than would be expected on the basis of the simple partition model used so far. This is due to the fact that the long-chain substrates are surface-active and will partition into the surfactant interface between the water and heptane pseudophases. For the long-chain alkanoates, the concentration at the interface will exceed that in the water droplet. It would appear that the substrate at the interface is able to react and so the observed rate will be higher than that predicted on the basis of the heptane/water partition coefficient. The model initially proposed neglects this interface entirely. Thus the apparently anomalously high reaction rates for the long-chain substrates are readily explained.

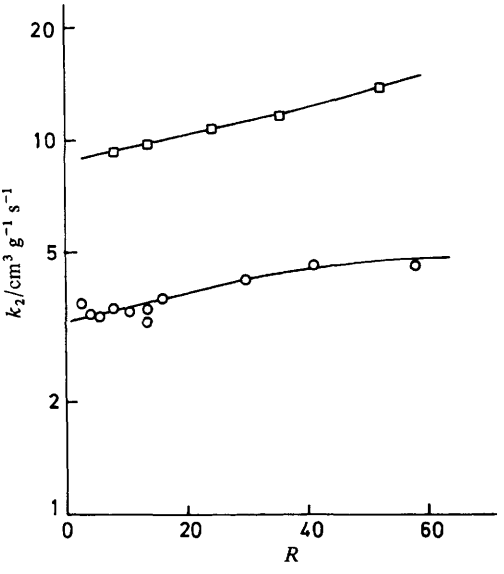

Fig. 11. Variation of k_2 with R for the lipase-catalysed hydrolysis of C_4 (□) and C_8 (○) in 0.1 mol dm^{-3} CTAB at 25.3 °C. The oil solvent is 50 vol % heptane + chloroform.

Table 2. Comparison of rates in aqueous and CTAB microemulsion for buffer- and lipase-catalysed reactions

system	rate in 4% EtOH + water	rate in CTAB (high R) microemulsion	rate (microemulsion) /rate (water)
C_4/diglycine	$(3.0 \pm 0.3) \times 10^{-2}$ dm^3 mol^{-1} s^{-1}	$(1.2 \pm 0.4) \times 10^{-4}$ dm^3 mol^{-1} s^{-1}	4.0×10^{-3}
C_4/lipase	$(6.5 \pm 0.5) \times 10^4$ cm^3 g^{-1} s^{-1}	12 ± 1.5 cm^3 g^{-1} s^{-1}	1.8×10^{-4}

STUDIES IN CTAB MICROEMULSIONS

For comparison, some studies of lipase-catalysed hydrolyses were also carried out in CTAB + heptane + chloroform + water microemulsions. As with the AOT system, the observed initial rates were first order with respect both to enzyme and to substrate. Fig. 11 shows the second-order rate constant k_2 as a function of R in these CTAB microemulsions. Very significantly, the rate constants are approximately one–two orders of magnitude lower than in AOT microemulsions. The variation with R is also qualitatively different in that the rate is only slightly dependent on R. A further difference between CTAB microemulsions and AOT microemulsions is noted when the pH is varied; in CTAB microemulsions, the rate is virtually independent of the pH of the solubilised water over the range 5–11. Results obtained with the enzyme α-chymotrypsin in CTAB microemulsions showed normal pH behaviour,[1] so this effect is peculiar to the lipase and is not a property of these pH buffers in CTAB microemulsions.

The apparent independence of the second-order rate constant on R and on microemulsion pH suggests that the lipase in CTAB microemulsions does not 'see'

the water droplets. Some specific interaction between lipase and CTAB may occur, for example the formation of an oil-soluble complex of lipase and a few molecules of CTAB. The lipase in such an aggregate would have little or no interaction with the buffered water pseudophase and would be unaffected by its pH or other properties. This proposal is supported by our observation that lipases in aqueous solution interact with low concentrations of CTAB, suggesting very strong interactions between the enzyme and this particular surfactant.

Table 2 shows the rates of diglycine-catalysed ester hydrolysis in CTAB microemulsions and a comparison with corresponding rates in bulk water. In contrast to the situation with AOT microemulsions (table 1), where the ratios of rate in microemulsion to rate in water were the same for lipase- and buffer-catalysed reactions, the rate ratio for lipase in CTAB microemulsions is 20-fold lower than that for the buffer-catalysed reaction. This again suggests that incorporation of the lipase into CTAB microemulsions produces some specific effect on the lipase reducing its catalytic activity.

We thank the S.E.R.C. (Biotechnology Directorate) and Tate and Lyle Research for financial support.

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