Comparison of Hydrolysis and Esterification Behavior of *Humicola lanuginosa* and *Rhizomucor miehei* Lipases in AOT-Stabilized Water-in-Oil Microemulsions: I. Effect of pH and Water Content on Reaction Kinetics

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Lipolase and Lipozyme are produced in large quantities (as a result of genetic engineering and overexpression) for the detergents market and provide a cheap source of highly active biocatalysts. *Humicola lanuginosa* lipase (HIL) and *Rhizomucor miehei* lipase (RmL) have been isolated in partially purified form from commercial preparations of Lipolase and Lipozyme, respectively. These lipases were solubilized in Aerosol-OT (AOT)-stabilized water-in-oil (w/o) microemulsions in *n*-heptane. HIL and RmL activity in these microemulsions was assayed by spectrophotometric measurement of the initial rate of *p*-nitrophenyl butyrate hydrolysis, and by chromatographic determination of the initial rate of octyl decanoate synthesis from 1-octanol and decanoic acid. The hydrolytic activity of HIL in microemulsions measured as a function of buffer pH prior to dispersal, followed a sigmoidal profile with the highest activities observed at alkaline pHs. This broadly matches the pH-activity profile for tributyrin hydrolysis by Lipolase in an aqueous emulsion assay. The hydrolytic activity of RmL in the same microemulsions, measured as a function of pH, gave a bell-shaped profile with a maximum activity at pH 7.5. Again, the observed pH-activity profile was similar to that reported for a purified RmL in a tributyrin-based aqueous emulsion assay. In contrast, the esterification activity exhibited by both HIL and RmL in AOT microemulsions over the available range pH 6.1 to 10.4, decreases as the pH increases, most likely reflecting the effect of substrate ionization. The dependence of the hydrolytic and condensation activity of HIL on *R*, the mole ratio of water to surfactant, were similar with both profiles exhibiting a maximum at $R = 5$. The hydrolytic and esterification activities of RmL followed similar *R*-dependent profiles, but the profiles in this case exhibited a maximum at $R = 10$. The water activities at these *R* values were directly measured as 0.78 and 0.9, respectively. Measured water activities were unperturbed by the presence of lipase at the concentrations used in these studies.

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INTRODUCTION

Water-in-oil (w/o) microemulsions have been widely employed over the last 10 to 15 years as a means of solubilizing enzymes in what is essentially an apolar organic reaction medium.[28,29,42,45] While there are a variety of alternative methods for using biocatalysts in organic media[8,13,23,25,51] w/o microemulsions possess particular physicochemical properties which make them an attractive option. Those microemulsions which consist of a solution of nanometer-sized surfactant-stabilized water droplets in an apolar solvent, provide a unique microenvironment in which many enzymes can be solubilized at the molecular level with retention of catalytic activity. Such single-phase optically transparent low viscosity media are well suited to spectrometric study, and the presence of microdomains of differing polarity in this thermodynamically stable solution facilitates the uptake of both polar and apolar materials. Biotransformations can be successfully carried out using water-insoluble substrates, because the very large interfacial area created by millimolar concentrations of droplets results in rapid mass transfer of substrate/product in the oil to or from the droplet where the enzyme is confined.

In terms of synthetic utility, the most widely exploited biocatalysts are the proteases, lipases, and esterases. Their wide availability, general robustness under a range of operating conditions, and their modest cost has resulted in an increase in their use as selective synthetic catalysts. A number of different lipases have been incorporated into w/o microemulsions and aspects of their behavior studied.[1,4,11,16–21,19,35,37,39] In particular, the use of 1,3-regioselective lipases for fat modification[31] has received considerable attention with glyceride interesterification,

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synthesis, and hydrolysis having been demonstrated using w/o microemulsion media.[7,9,12,16,20,32,33,36,46,48] Many scientists are uncomfortable with using enzymes in an apolar reaction medium containing surfactants, particularly synthetic ionic surfactants such as sodium bis-2-(ethylhexyl)sulphosuccinate (AOT) or cetyltrimethylammonium bromide (CTAB). Concerns regarding ease of product isolation, the achievement of high substrate and enzyme loadings, and satisfactory enzyme reuse are commonly encountered.

In practice, however, many of these problems have already been addressed.[27,30,41] Separation of products from the microemulsion may be achieved via a temperature-induced phase separation to yield an appropriate Winsor system,[27] or through the use of semipermeable membrane technology.[9,30,38] It is also possible to immobilize the lipase in a microemulsion-based organogel (MBG), which in granulated form can be used as a filterable solid phase catalyst in apolar organic solvents.[41,42]

Because w/o microemulsions are "low-water" systems, the hydrolysis/synthesis equilibrium can be shifted to favor the condensate.[12,19,40] However, despite the advantages offered by microemulsions and microemulsion-based media, their use for preparative scale synthesis has, with a few exceptions,[40,41] received little attention. In contrast to some of the procedures for using lipases and esterases in organic solvents, w/o microemulsions (if they are to be used for preparative scale synthesis) generally require enzyme preparations which are at least partially purified and of high specific activity. This is because the enzyme is generally introduced as a concentrated aqueous stock solution, usually to a reverse micellar solution of the surfactant in oil. The enzyme loading is therefore limited by the total amount of water present in the microemulsion (typically 1% to 10% v/v), although factors such as the solubility of the enzyme in water as well as its effect on phase behavior are also important.

As a consequence of the need to use more highly purified enzyme preparations in w/o microemulsion media, the overall cost of the synthesis is increased. In recent years, however, the widespread use of lipases and proteases in household detergent products has resulted in a demand for low cost enzyme preparations of high specific activity. This demand has been met principally as a result of advances in genetic engineering skills, and two commercially available, genetically engineered lipase preparations that meet these requirements are Lipolase™ and Lipozyme™ which contain the enzymes *Humicola lanuginosa* lipase (HlL) and *Rhizomucor miehei* lipase (RmL), respectively.[2] Both HlL and RmL are 1,3-regioselective.[2,3,22] Previous X-ray crystallographic studies have shown *Rhizomucor miehei* lipase to be a single domain protein with a molecular weight of 29,500 and possessing a catalytic triad similar to that found in serine proteases.[3] An α-helix lid on the surface of the protein has been observed which can expose a large hydrophobic surface (≈7.5 nm^2) when inhibitors are bound to the serine residue of the catalytic triad.[5,10] The enzyme in this "activated" state is likely to be preferentially located in the interfacial domain of a w/o microemulsion droplet.

In this and the accompanying article we describe our findings regarding the catalytic behavior of HlL and RmL in w/o microemulsions based on sodium bis-2-(ethylhexyl)sulphosuccinate (AOT) in *n*-heptane. Their hydrolytic activity as well as their condensation activity has been investigated as a function of incubation temperature, pH, and the water content, or more specifically the R value, defined as the mole ratio of water to surfactant, i.e., $[H_2O]$/[surfactant] and often referred to as ω_o.

MATERIALS AND METHODS

Materials

AOT, *p*-nitrophenol (pNP), and the synthetic lipase substrate *p*-nitrophenyl butyrate ($pNPC_4$) were obtained from Sigma and used without further purification. The following buffers were also purchased from Sigma and used at or very close to their aqueous pK_a values, given in parentheses: citrate (4.7), succinate (5.6), MES (6.1), ACES (6.8), BES (7.1), MOPS (7.2), HEPES (7.5), EPPS (8.0), diglycine (8.0), TAPS (8.4), CHES (9.3), and CAPS (10.4). 1-Octanol and decanoic acid were supplied by Aldrich. Solvents were of AR or HPLC grade and supplied by Fisons.

The octyl decanoate standard (≈6 g) was synthesized enzymatically using 2.5 mg of *Chromobacterium viscosum* (CV) lipase immobilized in an AOT-based MBG. The synthesis was performed overnight using 0.020 mol each of octanol and decanoic acid in 25 mL of *n*-heptane at 25°C. Lipase from *Humicola lanuginosa* was supplied by Novo Nordisk A/S (Bagsvaerd, Denmark) in the form of a straw-colored aqueous solution under the trade name Lipolase. Lipase from *Rhizomucor miehei* in the form of a dark brown aqueous solution was also supplied by Novo, under the trade name Lipozyme.

Both the HlL and RmL preparations were subjected to a short clean-up procedure before being freeze-dried to maximize the potential for employing microemulsion-based systems containing high enzyme concentrations. The Lipolase and Lipozyme preparations as supplied had quoted activities of 50,000 lipase units (LU) mL^{-1} and 20,000 lipase units (LU) mL^{-1}, respectively. They were first passed through a 0.22-μm Millex-GS filter to remove large colloidal material before being dialyzed at 5°C against distilled water using tubing with an approximate molecular weight cut-off of 12,000. The dialyzed enzyme solutions were then subjected to ultrafiltration and further concentration using a Centriprep 30 on a Mistral 3000i centrifuge at 4°C and 2000 rpm.

The resulting retentates were freeze-dried overnight to yield, in the case of HlL, a light fluffy beige-colored powder with an extrapolated activity of 3,500,000 LU g^{-1}. In the case of RmL, a beige-colored powder was isolated with an extrapolated activity of 1,000,000 LU g^{-1}. Calculation

of the purification factor (PF) was based on a series of $pNPC_4$ hydrolysis assays performed in 0.050 mol dm^{-3} MES buffer and on a second series of $pNPC_4$ hydrolysis assays performed in a 0.20 mol dm^{-3} AOT (R = 10) microemulsion containing MES at an aqueous phase concentration of 0.050 mol dm^{-3}.

Both assay series were performed at 25°C and gave a reproducible value for the PF of 70 ± 4 for HlL and 50 ± 6 for RmL. Stain densities in the SDS-PAGE gels indicated that HlL and RmL represented >80% of the total protein in the isolates. In the case of HlL, further purification to apparent homogeneity was carried out using ion-exchange chromatography on DE52. The molecular weight was estimated at ≈30,000 using an SDS-PAGE gel against a series of molecular weight markers. This value is in good agreement with the value of ≈32,000 previously reported.[2] However, the improvement in specific activity (≈10%) was considered insufficient to warrant the additional step and, consequently, the partially purified preparation was used throughout these studies. No attempt was made to purify RmL to apparent homogeneity. It should be noted that, where concentrations of microemulsion components are expressed in terms of an aqueous phase concentration, the square brackets denoting concentration are followed by the subscript "aq." When the subscript is absent, the concentration referred to is expressed in mol dm^{-3} of microemulsion.

Preparation of Microemulsions: Assay Compositions

Microemulsions were prepared by addition of known volumes of the aqueous component to a reverse micellar solution of AOT in n-heptane at 25°C. The mixture was briefly shaken until an optically clear single-phase solution was formed. Assays designed to measure condensation activity employed microemulsions into which the octanol and decanoic acid had been preweighed. Assays designed to measure hydrolytic activity employed microemulsions in which $pNPC_4$ was introduced as a stock solution in n-heptane. After addition of any necessary buffers, a stock aqueous solution of HlL or RmL was added to initiate the reaction.

The hydrolysis assay was generally carried out in microemulsions containing $[buffer]_{aq}$ = 0.050 mol dm^{-3} and $[pNPC_4]$ = 1.0×10^{-3} mol dm^{-3}. The release of p-nitrophenol was followed at the appropriate isosbestic wavelength for the p-nitrophenol/p-nitrophenolate couple using a Kontron Uvikon 860 spectrophotometer. The extinction coefficients of the p-nitrophenol/p-nitrophenolate couple (ϵ^1) were calculated from the absorption spectra of various AOT-stabilized microemulsions containing p-nitrophenol, and were corrected to take into account the extinction coefficient (ϵ^2) of the substrate $pNPC_4$ at the same wavelength to give the extinction coefficient (ϵ) used to calculate the initial rate. The relevant data are shown in Table I. The treatment of initial rate data is based on the calculation of the second-order rate constant k_2 having units of dm^3 per gram per second as indicated in Eq. (1),[11] with the enzyme concentration expressed in units of grams per litre.

$$d[pNP]/dt = \text{Initial rate} = k_2[\text{Enzyme}][\text{Substrate}] \quad (1)$$

Initial rates are corrected where necessary to take into account any nonenzymic hydrolysis. These rates were separately measured, but were not significant below pH 9 and below R = 10 (data not shown).

Lipase activity in aqueous solution was measured by following the hydrolysis of $pNPC_4$ in 0.050 mol dm^{-3} MES buffer at pH 6.1 and 25°C. The absorbance was monitored at 346 nm and a value of ϵ = 4800 $dm^3\ mol^{-1}\ cm^{-1}$ was used to calculate the initial rate. The $pNPC_4$ was introduced as a solution in acetone to give an assay concentration of 0.32×10^{-3} mol dm^{-3}; then, the volume of acetone in the assay was 4%. The reproducibility of the aqueous assay was much less than the microemulsion-based assay, apparently due to strong adsorption of the enzyme to the surface of the cuvettes in the case of the aqueous assay, and to problems with slight substrate precipitation on contacting the stock $pNPC_4$ solution in acetone with the MES buffer. Microemulsion-based lipase assays were therefore a much preferred option.

The condensation activities of HlL and RmL were monitored by measuring the initial rate of octyl decanoate synthesis using a chromatographic procedure. The rate was

Table I. R- and pH-independent isosbestic wavelengths for the p-nitrophenol/p-nitrophenolate couple in 0.20 mol dm^{-3} AOT microemulsions in n-heptane at 25°C.[a]

	Isosbestic wavelength (/nm)	Extinction coefficient (/$dm^3\ mol^{-1}\ cm^{-1}$)		
		ϵ^1	ϵ^2	ϵ
pH independent (R = 10)	335	4750	200	4550
R independent				
pH 6.1	316	8850	430	8420
pH 9.3	333	4770	200	4570
Unbuffered	325	7500	240	7260

[a]$[Buffer]_{aq}$ = 0.050 mol dm^{-3}.

generally measured in unbuffered microemulsions using 0.010 mol dm^{-3} each of the substrates 1-octanol and decanoic acid. Where the effect of pH was under consideration, the substrate concentrations were reduced to 0.0050 mol dm^{-3} to minimize the self-buffering capacity of the system.

It was convenient to include hexadecane as an internal standard in the assay microemulsions and the octyl decanoate concentration was calculated by GC, based on response factors obtained from microemulsions standards injected on a daily basis. Both hexadecane and octyl decanoate give fully resolved symmetrical peaks using a 30-m DB5 column installed in an HP5890 Series II gas chromatograph with flame ionization detection and using H_2 as carrier. An initial temperature of 80°C with a 2-min hold was employed before ramping at 15°C min^{-1} to a final temperature of 240°C. The assay may also be performed, again with hexadecane as the internal standard, by HPLC using a C_{18} reverse phase column with isocratic elution with acetonitrile. In this case, refractive index detection is required to monitor the peaks of interest. The sampled microemulsion must be mixed with 1:1 acetone:acetonitrile prior to injection to obtain a solution miscible with the mobile phase.

Water Content and Water Activity

The concentration of water in the microemulsions was measured using a Metrohm 684 Karl-Fischer coulometer. The water activity (a_w) in the microemulsions was controlled using vapor phase equilibration with saturated salt solutions of known a_w as indicated in Table II, different phases in equilibrium having the same water activities.[47,49,50] The procedure involved taking 50 mL of 0.20 mol dm^{-3} AOT in an $R = 10$ microemulsion in the bottom of a 500 mL jar and placing a second, smaller open jar containing 20 mL of a saturated salt solution, in the presence of a significant excess of solid, inside the first. The 500 mL jar was then sealed and the system allowed to come to equilibrium.

Water activities were monitored by measuring the humidity of the vapor phase in equilibrium with the microemulsion. Relative humidities were measured with a Vaisala humidity and temperature indicator (HMI 31). This was done by removing 3 mL of the microemulsion into a 25 mm × 150 mm boiling tube. The probe of the humidity meter extended about 110 mm into the tube, leaving a headspace of approximately 33 mL. The sealed tube was then fully immersed in a water bath at 25°C. Probe equilibration times were typically 1 to 2 h.

Table II. Literature values for the water activities of saturated salt solutions.[15]

Salt	a_w
Lithium chloride	0.13
Potassium acetate	0.23
Magnesium chloride	0.33
Magnesium nitrate	0.53
Sodium chloride	0.75
Potassium chloride	0.97

RESULTS

Control and Measurement of a_w in w/o Microemulsions

Many enzymes in w/o microemulsions exhibit a dependence of catalytic activity on R value so it is useful to be aware of the parameters that change as the R value is changed. There is, for example, a clear distinction between water that is essentially "bound," that is, participating directly in the hydration of surfactant headgroups, counterions or charged amino acid residues, and water that is essentially "free" (i.e., behaves as if it were bulk water). In AOT w/o microemulsions, the hydration requirements of the surfactant and counterions are thought generally not to be exceeded until an R value of ≈10 is reached. Above this R value, the additional water is "free" and at R values of 40 to 50 the properties of the water in the droplets closely match those of bulk water. Below $R = 10$, all the water is "bound" and, as R is further decreased, there is a marked decrease in a_w. For any enzyme a decrease in a_w is significant because the intrinsic enzyme activity is to some extent dependent on the level of hydration of the enzyme. Also, of course, it might be expected that the equilibrium position of a condensation reaction would be affected.

In w/o microemulsions, there is the additional consideration that, at very low R values, surfactant/protein interactions may play a specific role in modifying the intrinsic activity. For hydrolases in low R-value microemulsions, the situation is further complicated by the fact that water is involved in the reaction and we would therefore expect some dependence of the reaction rate on a_w. This behavior was indeed observed when nonenzymic hydrolysis of $pNPC_4$ was examined as a function of the R value and there is almost a tenfold decrease in the nonenzymic rate in going from $R = 10$ to $R = 2.5$.

Control of the water content and a_w in w/o microemulsions by vapor phase equilibration with saturated salt solutions is demonstrated in Figure 1 using a saturated solution of potassium acetate ($a_w = 0.23$). The water content of a 0.20 mol dm^{-3} AOT microemulsion was monitored coulometrically, as a function of time, under the experimental conditions described in the previous section. Water is clearly removed from the microemulsion, which had an initial R value of 10. The equilibration process is relatively slow under the conditions employed with the water content leveling off after ≈50 h leaving an $R = 1$ microemulsion with an $a_w = 0.23$.

The humidity meter was calibrated with saturated salt solutions and with microemulsions pre-equilibrated with the same saturated salt solutions. Figure 2 shows the calibration curve obtained. There was a difference of approximately

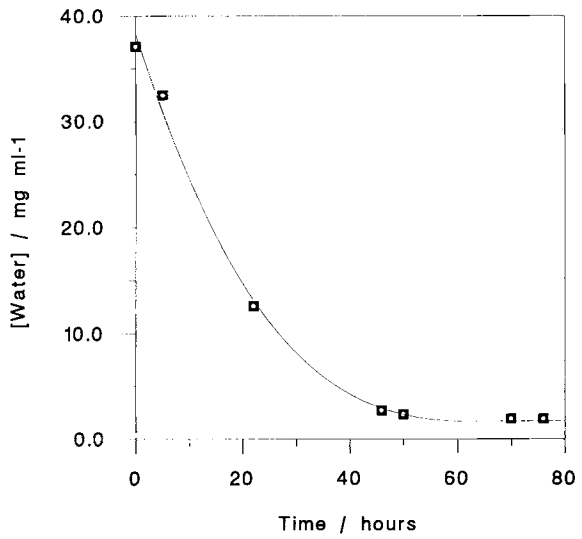


Figure 1. Change in water content with time for an $R = 10$, 0.20 mol dm^{-3} AOT microemulsion equilibrated through the vapor phase with a saturated solution of potassium acetate ($a_w = 0.23$).

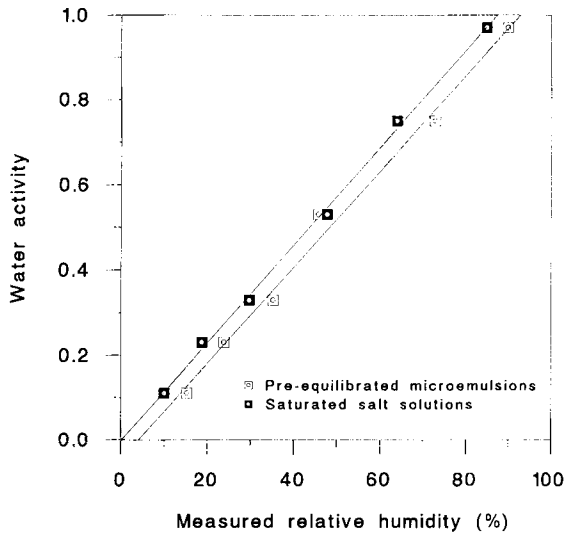


Figure 3. Variation of water activity (a_w) as a function of R for unbuffered 0.20 mol dm^{-3} AOT microemulsions in n-heptane.

5% between the measured relative humidities of the saturated salt solutions and microemulsions pre-equilibrated with saturated salt solutions. It is possible that this difference is caused by n-heptane vapor perturbing the measurement. As the water activity of microemulsions was of principal interest, the calibration curve obtained from the pre-equilibrated microemulsions was used.

Figure 3 shows a_w measured using the HMI 31 meter, as a function of the R value for 0.20 mol dm^{-3} AOT stabilized n-heptane microemulsions. Two series of microemulsions were analyzed; the first series comprised microemulsions of known R value prepared by injection of measured amounts of water to a reverse micellar solution of AOT in n-heptane.

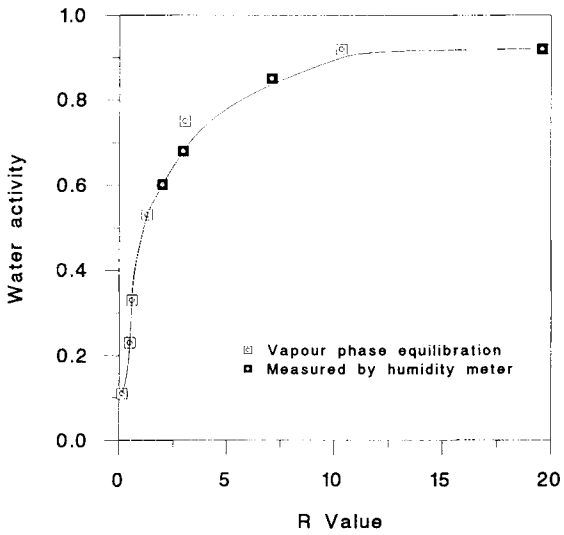


Figure 2. Calibration data for Vaisala humidity indicator (HMI 31) at 25°C. Microemulsion composition prior to vapor phase equilibration was 0.20 mol dm^{-3} AOT, $R = 10$.

The second series of analyses were carried out on microemulsions which had been equilibrated through the vapor phase and their water contents measured by Karl-Fischer coulometry.

There is a clear correlation between those samples prepared by addition of known volumes of water and those whose water contents and a_w have been adjusted by vapor phase equilibration. The data agree well with results using more indirect methods to calculate the water activity in similar microemulsion systems,[14] and also with data obtained using a humidity meter by investigators elsewhere (P. J. Halling, private communication). Above $R = 10$, the water activity is relatively constant, at about 0.92. Below $R = 10$, the water activity drops rapidly. There was no significant difference in a_w between microemulsions containing enzyme at the concentrations used in this work (up to 15.6 μg mL^{-1}) and those which did not contain enzyme.

The $pNPC_4$ Hydrolysis Assay

The dependence of the rate of $pNPC_4$ hydrolysis on both the enzyme concentration and substrate concentration is a crucial test of Eq. (1), which applies when [substrate] $\ll K_M$. As the studies reported here depend on systematic changes in the microemulsion system, Eq. (1) should ideally be shown to hold for all systems under study. However, this is clearly impractical and, consequently, the enzyme and substrate concentration dependencies have been investigated under the most commonly employed experimental conditions. HIL assays were therefore performed at 25°C in 0.20 mol dm^{-3} AOT ($R = 10$) microemulsions containing $[\text{CHES buffer, pH } 9.3]_{aq} = 0.050$ mol dm^{-3}. The variation of the initial rate of $pNPC_4$ hydrolysis as a function of the HIL and $pNPC_4$ concentration, where concentrations are expressed in moles per liter of microemulsion, are shown in Figures 4 and 5, respectively. RmL assays, however, were

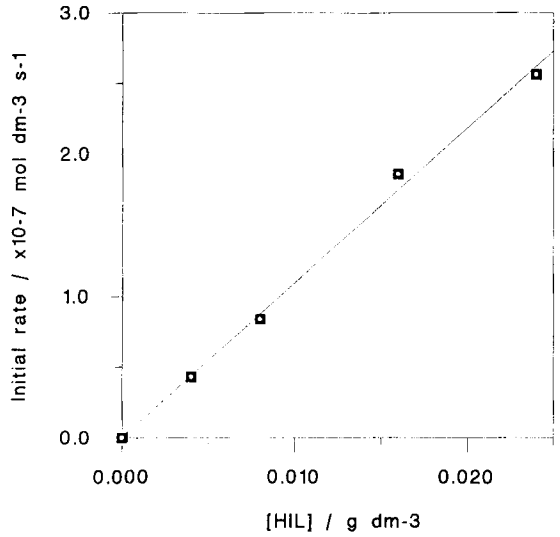


Figure 4. Dependence of the rate of $pNPC_4$ hydrolysis by HIL as a function of enzyme concentration in 0.20 mol dm^{-3} AOT, R = 10 microemulsions at 25°C. Assay conditions: [CHES buffer, pH 9.3] = 0.0018 mol dm^{-3}; [$pNPC_4$] = 0.0010 mol dm^{-3}.

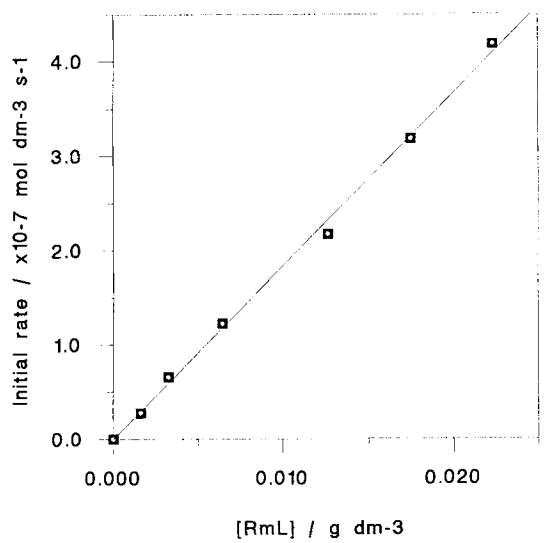


Figure 6. Dependence of the rate of $pNPC_4$ hydrolysis by RmL as a function of enzyme concentration in 0.20 mol dm^{-3} AOT (R = 10) microemulsions at 25°C. Assay conditions: [MES buffer, pH 6.1] = 0.0018 mol dm^{-3}; [$pNPC_4$] = 0.0010 mol dm^{-3}.

performed at 25°C in 0.20 mol dm^{-3} AOT (R = 10) microemulsions containing [MES buffer, pH 6.1]$_{aq}$ = 0.050 mol dm^{-3}.

The variation of the initial rate of $pNPC_4$ hydrolysis as a function of the RmL and $pNPC_4$ concentration are shown in Figures 6 and 7, respectively. All plots exhibit excellent linearity over the range studied, which is predicted by Eq. (1); there is no evidence for the onset of saturation kinetics, even at the highest $pNPC_4$ concentration. A value of k_2 can be easily obtained either individually from each assay or from the slopes of these figures. If this is done, a value of k_2 = 0.0133 $dm^3\ g^{-1}\ s^{-1}$ is obtained from Figure 4 and a value of k_2 = 0.0124 $dm^3\ g^{-1}\ s^{-1}$ is obtained from Figure 5. Similarly, Figure 6 yields a value of k_2 = 0.020 $dm^3\ g^{-1}\ s^{-1}$ while a value of k_2 = 0.022 $dm^3\ g^{-1}\ s^{-1}$ is obtained from the slope of Figure 7. As predicted by Eq. (1), each pair of values are in excellent agreement.

Effect of pH on HIL and RmL Activity

The effect of pH on HlL and RmL activity was investigated at 25°C using R = 10 microemulsions containing 0.20 mol dm^{-3} AOT for the hydrolysis assays, and R = 10, 0.40 mol dm^{-3} AOT microemulsions for the condensation as-

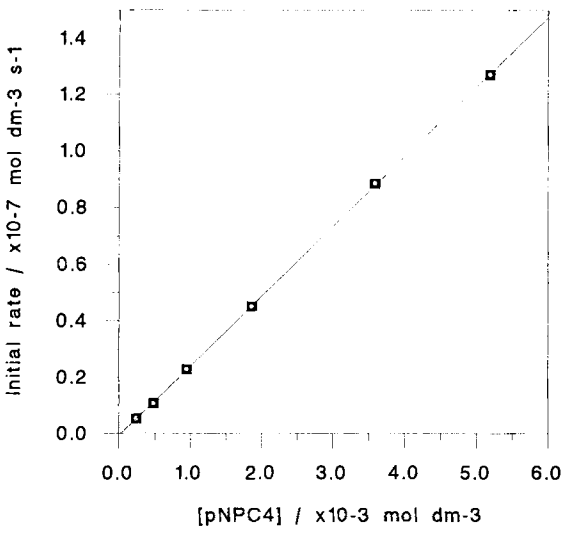


Figure 5. Dependence of the rate of $pNPC_4$ hydrolysis by HIL as a function of substrate concentration in 0.20 mol dm^{-3} (R = 10) microemulsions at 25°C. Assay conditions: [CHES buffer, pH 9.3] = 0.0018 mol dm^{-3}; [HIL] = 0.0040 g dm^{-3} (14,000 LU dm^{-3}).

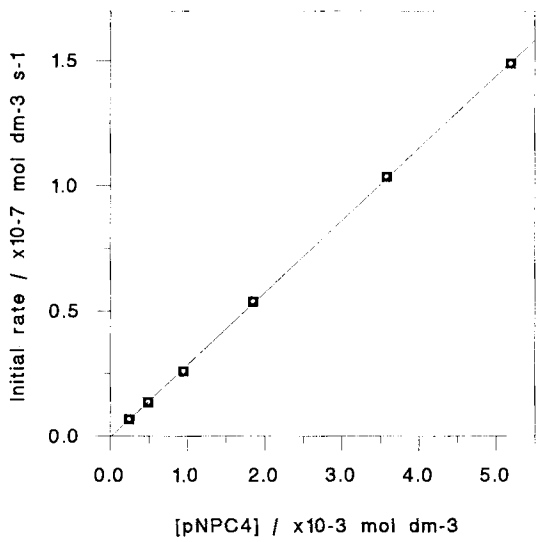


Figure 7. Dependence of the rate of $pNPC_4$ hydrolysis by RmL as a function of substrate concentration in 0.20 mol dm^{-3} AOT, R = 10 microemulsions at 25°C. Assay conditions: [MES buffer, pH 6.1] = 0.0018 mol dm^{-3}; [RmL] = 0.0016 g dm^{-3} (1600 LU dm^{-3}).

says. A series of aqueous buffers were prepared at pH levels close to their pK_a and solubilized in the microemulsion prior to dispersal of the stock HlL or RmL solution in distilled water. For the hydrolysis assay an overall buffer concentration of 0.0018 mol dm^{-3} was employed, while in the condensation assays the buffer concentration was increased to 0.012 mol dm^{-3} overall. The increased loading of buffer, equivalent to an aqueous phase concentration of 0.167 mol dm^{-3}, was necessary to buffer the 0.0050 mol dm^{-3} decanoic acid present in the assay microemulsion.

To demonstrate that these conditions were sufficient to satisfactorily buffer the systems, a series of assay microemulsions were prepared containing decanoic acid at 0.0050 mol dm^{-3} but also including *p*-nitrophenol at 5.0×10^{-4} mol dm^{-3} as a probe molecule whose UV/visible spectrum is sensitive to pH. The incubations contained MES, ACES, HEPES, TAPS, and CAPS which, prior to dispersal, had pH levels of 6.1, 6.8, 7.5, 9.3, and 10.4, respectively. Absorbance spectra were run on each incubation in the wavelength range 250 to 500 nm and comparison of the spectra clearly demonstrate that the *p*-nitrophenol experiences a pH close to that expected if the decanoic acid is not perturbing the buffering. The AOT concentration in the microemulsion selected for this condensation assay is twice that used in the hydrolysis assays and in the condensation assays described later. This was done to extend the pH range available for study because very high dispersed phase concentrations of buffer led to phase instability, particularly at lower pH. The substrate concentrations were also reduced for the same reason. The behavior of HlL and RmL in 0.40 mol dm^{-3} AOT ($R = 10$) microemulsions should be similar to their behavior in 0.20 mol dm^{-3} AOT ($R = 10$) microemulsions, because it is the droplet concentration that is affected by the change in AOT concentration, rather than the droplet radii which are fixed for a given R value.

The dependence of HlL and RmL activity on pH for $pNPC_4$ hydrolysis is shown in Figures 8 and 9, respectively. For HlL, the pH optimum occurs around pH 9.3 to pH 10.4 and the assigned value of 100% activity corresponds to a value of $k_2 = 0.0132$ dm^3 g^{-1} s^{-1}. It is interesting to compare this result with the pH dependence supplied by Novo (product information leaflet) of the Lipolase preparation itself. This is included in Figure 8 and is based on the hydrolysis of tributyrin at 30°C in an aqueous emulsion assay using a pH-stat method to monitor the liberation of free fatty acid (Novo Publication AF 95.4/1). The enzyme activity follows the same general trend, increasing with increasing pH, although the low pH end of the profile was not available. There is no evidence, however, that the emulsion assay data follow the sigmoidal profile observed for $pNPC_4$ hydrolysis in the microemulsion.

The dependence of RmL activity on pH for $pNPC_4$ hydrolysis is shown in Figure 9. In contrast to HlL, the RmL exhibits a clear bell-shaped dependence in these AOT microemulsions with a maximum at around pH 7.5. The pH-

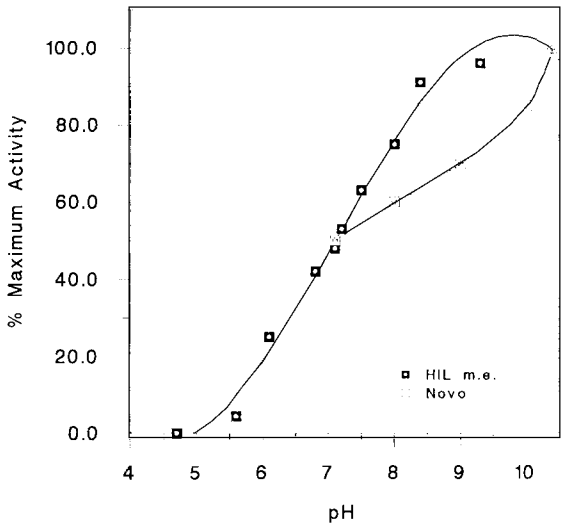


Figure 8. pH dependence of HlL hydrolysis activity in 0.20 mol dm^{-3} AOT ($R = 10$) microemulsions at 25°C. Assay conditions: [HlL] = 0.0080 g dm^{-3}; [buffer] = 0.0018 mol dm^{-3}; [$pNPC_4$] = 0.0016 mol dm^{-3}. Lipolase pH dependence acquired at 30°C in tributyrin emulsion assay (from Novo).

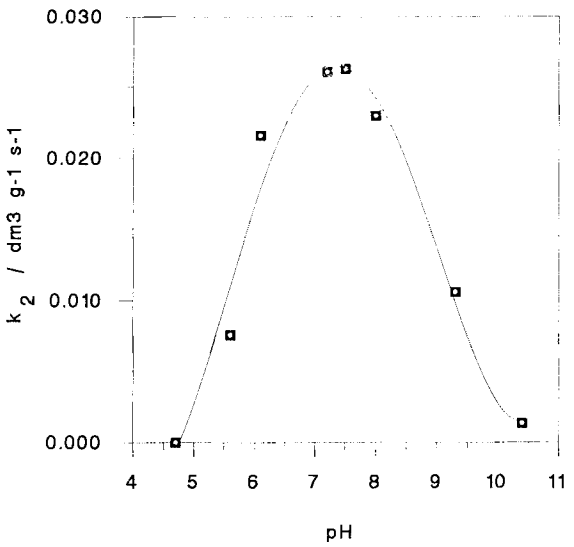
activity profile for purified RmL obtained using a tributyrin aqueous emulsion assay[22] is shown in Figure 9(b). The trend is clearly similar with the maximum activity occurring around pH 7.0. It is noticeable, however, that the activity does not drop as rapidly at the low pH end.

The dependence of HlL and RmL condensation activity on pH is shown in Figures 10 and 11, respectively. The behavior is similar in both cases with a steady and progressive decrease in activity observed as the pH increases, and contrasts markedly with the hydrolysis behavior shown in Figures 8 and 9. The decrease cannot be attributed to enzyme inactivation, because all initial rate plots of octyl decanoate concentration versus time were linear over the assay period (typically 1 to 5 h). The decrease in condensation activity at higher pH is most likely due to ionization of the decanoic acid which, although more surface active than the nonionized form, is presumably a much poorer substrate.

Effect of *R* Value

The effect on HlL and RmL activity of changing the R value while keeping the AOT concentration constant at 0.20 mol dm^{-3} was examined at 25°C. For an ionic surfactant like AOT, virtually all the amphiphile is resident at the interface of the droplets. As a consequence, changing R in this manner has the effect of creating larger droplets with reduced interfacial curvature and in lower concentration because more surfactant molecules are needed to stabilize each droplet. The total interfacial area, however, remains approximately constant, assuming that there are no significant changes in the average area of interface occupied per surfactant molecule.

The activity of HlL toward the hydrolysis of $pNPC_4$ was measured as a function of the R value in unbuffered microemulsions and in microemulsions buffered to pH 9.3 with CHES buffer. The hydrolysis activity of RmL was mea-



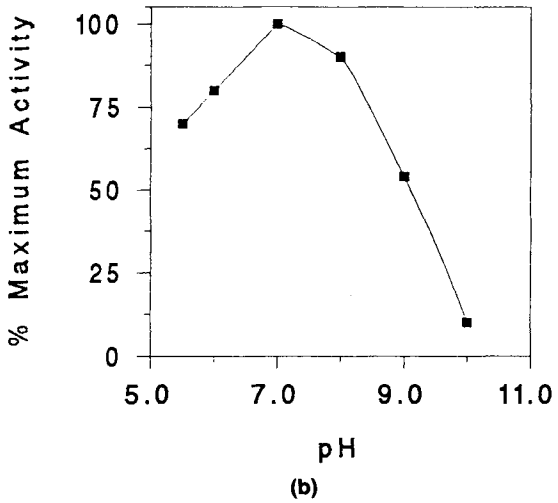


Figure 9. (a) pH dependence of RmL hydrolysis activity in 0.20 mol dm^{-3} AOT (R = 10) microemulsions at 25°C. Assay conditions: [RmL] = 0.0016 g dm^{-3} (1600 LU dm^{-3}); [buffer] = 0.0018 mol dm^{-3}; [$pNPC_4$] = 0.0010 mol dm^{-3}. (b) pH dependence of RmL at 30°C in tributyrin emulsion assay (from ref. 22).

sured as a function of R in microemulsions buffered to pH 6.1 with MES buffer. The R dependencies of HIL and RmL condensation activity were investigated in unbuffered microemulsions containing 0.010 mol dm^{-3} each of octanol and decanoic acid. In this case, although no "formal" buffer has been introduced, it is assumed that the decanoic acid itself buffers the system.

The results of these studies are summarized in Figures 12 through 14, respectively. All three figures show plots following a similar trend. In the case of HIL, both the hydrolysis and activity profiles exhibit a maximum at around R = 5, but the activity drops off rapidly at low R values. At higher R values, the HIL activity drops a little less dramatically, tending to level off above R = 10 in unbuffered microemulsions for both $pNPC_4$ hydrolysis and for octyl decanoate synthesis. In the buffered microemulsions, HIL activity toward $pNPC_4$ hydrolysis shows a 40%

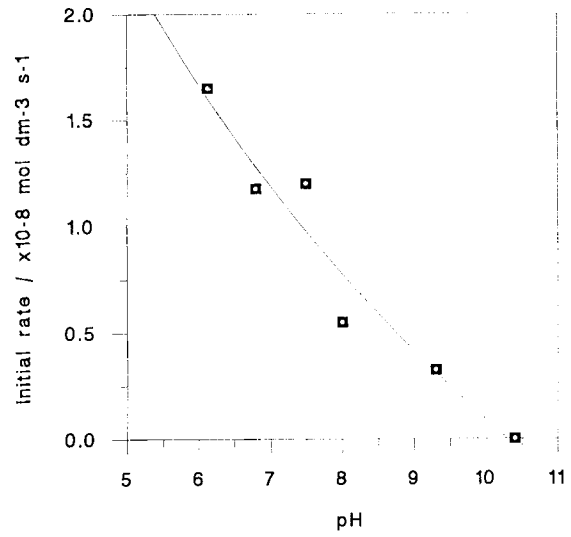


Figure 10. pH dependence of HIL esterification activity in 0.40 mol dm^{-3} AOT (R = 10) microemulsions at 25°C. Assay conditions: [lipase] = 0.0156 g dm^{-3} (54,600 LU dm^{-3}); [buffer] = 0.012 mol dm^{-3}; [l-octanol] = 0.0050 mol dm^{-3}; [decanoic acid] = 0.0050 mol dm^{-3}.

fall in activity between R = 10 and the activity maximum at $R \approx 5$. Above R = 15, the activity is more or less constant. In the case of RmL, like HIL, both the hydrolysis and condensation activity profiles are closely matched, but in this case the optimum activities are expressed at R = 10. At R values above the optimum R value, RmL activity falls much less dramatically than it does for HIL, and clearly plateaus out at the higher R values.

As the R value is decreased below R = 10 the water activity also decreases and, as we have demonstrated in Figures 2 and 3, can be directly measured using a suitable humidity probe. A decrease in the hydrolytic activity was

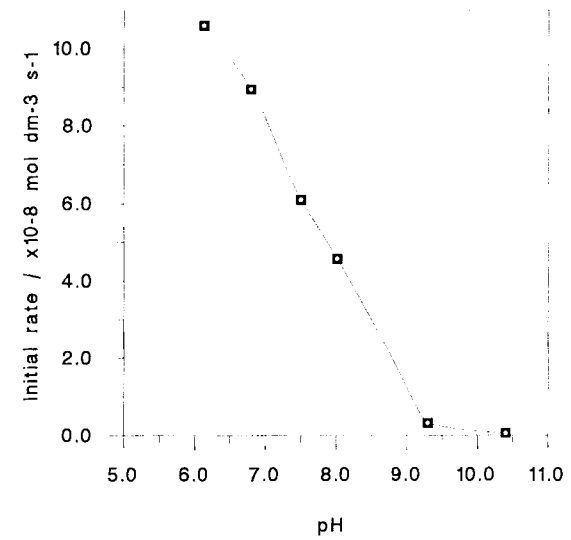


Figure 11. pH dependence of RmL esterification activity in 0.40 mol dm^{-3} AOT (R = 10) microemulsions at 25°C. Assay conditions: [RmL] = 0.0072 g dm^{-3} (7200 LU dm^{-3}); [buffer] = 0.012 mol dm^{-3}; [l-octanol] = 0.0050 mol dm^{-3}; [decanoic acid] = 0.0050 mol dm^{-3}.

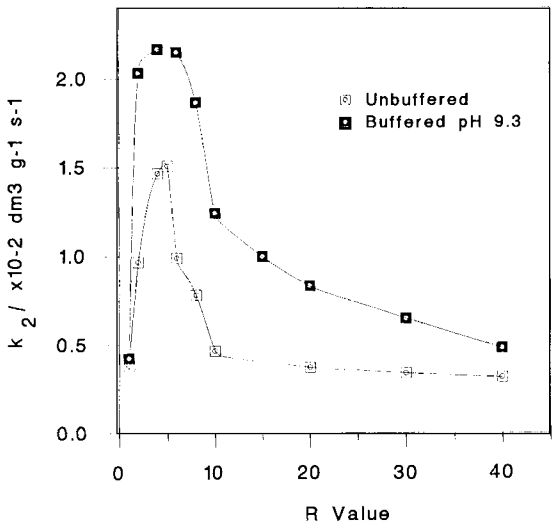


Figure 12. R dependence of HIL hydrolysis activity in 0.20 mol dm^{-3} AOT (R = 10) microemulsions at 25°C. Assay conditions: [HIL] = 0.008 g dm^{-3} (28,000 LU dm^{-3}); [CHES buffer, pH 9.3] = 0.0018 mol dm^{-3}; or unbuffered, [$pNPC_4$] = 0.0016 mol dm^{-3}.

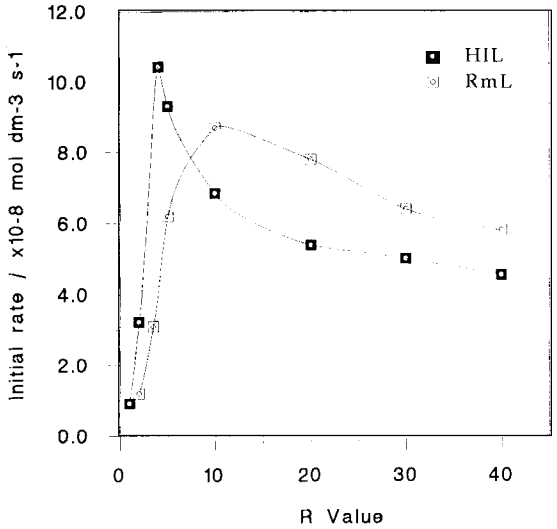


Figure 14. R dependence of HIL and RmL esterification activity in 0.20 mol dm^{-3} AOT, (R = 10) microemulsions at 25°C. Assay conditions: [1-octanol] = 0.010 mol dm^{-3}; [decanoic acid] = 0.010 mol dm^{-3}; [HIL] = 0.0156 g dm^{-3} (54,600 LU dm^{-3}) or [RmL] = 0.0072 g dm^{-3} (7200 LU dm^{-3}).

therefore expected at low R values and is indeed observed. However, for HIL as well as RmL, the esterification activity also decreased markedly at low R values. For many synthetic applications it is desirable to reduce the water content and a_w of the microemulsion as much as possible, for example, to maximize the equilibrium shift to the condensate or to reduce hydrolysis products in an interesterification reaction. The fact that RmL esterification activity falls so dramatically at low R values is particularly disappointing because RmL has already been shown to retain 30% of its maximum condensation activity in hexane when the water activity was apparently less than 0.0001.[49] This result suggests that, for RmL at least, specific surfactant/protein interactions might be a major factor influencing its behavior in low R value microemulsions.

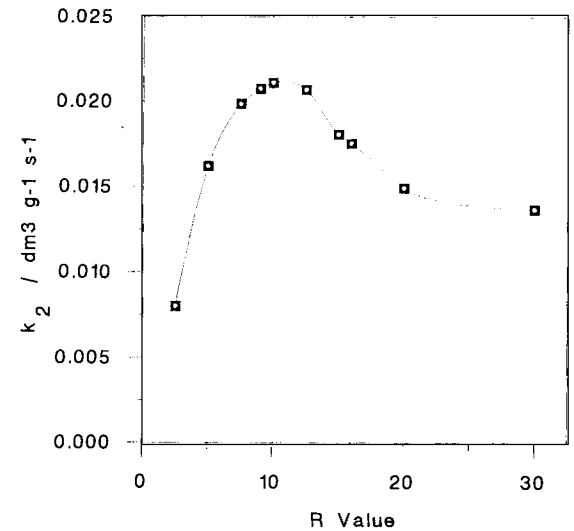


Figure 13. R dependence of RmL hydrolysis activity in 0.20 mol dm^{-3} AOT (R = 10) microemulsions at 25°C. Assay conditions: [RmL] = 0.0016 g dm^{-3} (1600 LU dm^{-3}); [MES buffer, pH 6.1]$_{aq}$ = 0.050 mol dm^{-3}; [$pNPC_4$] = 0.0010 mol dm^{-3}.

The bell-shaped curves that are observed for the R dependence of HIL and RmL activity have been reported on many occasions for other enzymes in a variety of microemulsion systems. However, although a number of quite different explanations have been proposed,[6,24,26,44] a satisfactory rationalization of this characteristic trend has yet to find general acceptance. This is principally because changing the R value also has the effect of changing several other system parameters. It is therefore difficult to extract out the relevant information which might otherwise allow the activity behavior to be correlated with a single systematic change in the microemulsion. For lipase-catalyzed hydrolysis of $pNPC_4$, reaction rates might be predicted to slow at low R values as there is little "free" water available and the hydrolysis reaction requires the presence of water to regenerate free enzyme from the acyl-enzyme intermediate.

In addition, Figure 3 clearly shows that the water activity (a measure of the amount of "available" water) drops rapidly as R is reduced below 10. In the case of octyl decanoate synthesis, however, the free enzyme can be regenerated by attack on the acyl-enzyme intermediate by the alcoholic substrate. Water is not essential for the reaction to occur, provided the enzyme exists in an appropriate "active" conformation. A fall-off in activity at low R value is nevertheless observed for the HIL- and RmL-catalyzed synthesis of octyl decanoate and it would be reasonable to conclude that whatever is responsible for the observed reduction of enzyme activity in Figure 14 also operates in the microemulsion systems containing $pNPC_4$. Above R = 10, a_w is essentially constant (~1) and its effect on enzyme activity is therefore minimal.

Comparison of the HlL-catalyzed reaction rates in unbuffered and buffered microemulsions allows an estimate of the pH of the water domain of an unbuffered microemulsion to be made. The measured rate of $pNPC_4$ hydrolysis by HlL in an $R = 10$ unbuffered microemulsion was 0.00469 $dm^3 g^{-1} s^{-1}$ (from Fig. 12). Cross-referencing this value with the pH dependence shown in Figure 8 for buffered $R = 10$ microemulsions allowed the pH of the unbuffered microemulsions to be estimated as approximately 6.5. The estimated value of pH 6.5 for the unbuffered $R = 10$ microemulsion is supported by UV/Vis absorption spectra of the *p*-nitrophenol/nitrophenylate couple in buffered and unbuffered microemulsions. The extinction coefficient of the *p*-nitrophenol absorption peak at 315 nm follows the sequence: buffered, pH 6.1 > unbuffered > buffered, pH 7.2.

DISCUSSION

The studies reported here describe aspects of the catalytic behavior of HlL and RmL in AOT microemulsions ascertained using complementary assay procedures based on nitrophenyl ester hydrolysis and octyl decanoate synthesis. It was first established that, in relation to $pNPC_4$ hydrolysis, both HlL and RmL exhibited first order kinetics with respect to both the enzyme and substrate concentrations over the studied range. The activity of the enzyme could therefore be reliably characterized by the second order rate constant k_2 using the procedures described in the Materials and Methods section.

The dependence of HlL and RmL hydrolytic activity in AOT microemulsions on buffer pH was examined and was found to be broadly similar to the behavior observed for tributyrin hydrolysis by Lipolase and a purified *Rhizomucor miehei* lipase in an aqueous emulsion assay. HlL was found to be most active at alkaline pH in both the microemulsion and the aqueous emulsion assay, although the pH-activity profile appears to be sigmoidal in AOT w/o microemulsions. The pH dependence of RmL hydrolysis activity in AOT microemulsions was clearly bell-shaped, although this profile was not as evident in the pH-activity dependence observed for tributyrin hydrolysis at 30°C in an aqueous emulsion assay.[22] The pH optima for RmL in the microemulsion and aqueous emulsion assays were found to be pH 7.5 and pH 7, respectively. In the absence of additional data points for the latter pH-activity profile, there is no reason to suppose that any shift in the pH maximum has occurred. Both HlL and RmL esterification activity fall as the pH is increased. The pH-activity profile in this case is a composite of the effects of substrate ionization and partitioning, as well as changes in the intrinsic activity of the enzyme.

HlL and RmL exhibited a classic bell-shaped dependence for both hydrolysis and condensation activity when measured as a function of R value, the maximum activities being observed at $R = 5$ for HlL and $R = 10$ for RmL. The measured specific activity of HlL toward $pNPC_4$ hydrolysis in AOT microemulsions buffered with pH 9.3 CHES buffer was higher than that obtained in the unbuffered microemulsion reflecting our estimate of pH 6.5 for the unbuffered system at $R = 10$.

Experiments performed using the HMI 31 humidity meter demonstrated that the water activity of a microemulsion can be directly measured and correlated with the R value. Further, the a_w of any microemulsion can be simply controlled through vapor phase equilibration with an appropriate saturated salt solution. A decreased rate of hydrolysis was predicted on the basis of the lowered a_w values measured for 0.20 mol dm^{-3} AOT microemulsions with R values below 10. Large decreases in the esterification rate resulting from low a_w values were not, however, expected for RmL as it had previously been shown to be active at very low water activities. Nevertheless, poor esterification activity was observed at low R value, which indicates that specific effects such as unfavorable surfactant/protein interactions and/or inadequate levels of protein hydration dominate the contribution to the catalytic activity at these R values.

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