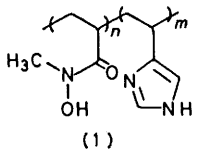
Acyl Transfer Reactions in Functional Micelles studied by Proton Magnetic Resonance at 270 MHz

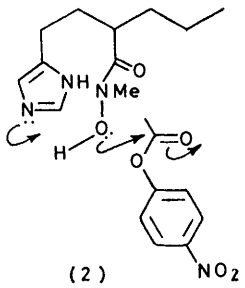
By **John M. Brown,* Penny A. Chaloner,** and **Alain Colens,** The Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY

The course of micelle-catalysed reactions at millimolar concentrations in aqueous solution may be followed by Fourier transform n.m.r. Micelles of the histidine-bearing surfactant (4a) react rapidly with *p*-nitrophenyl acetate in phosphate-buffered media between pD 7 and 8, entirely by acyl transfer to the imidazole with no concurrent general-base catalysis. The resulting acylimidazole breaks down by a pH-independent route between pD 7.0 and and 7.9 forming both acetate ion and monoacetyl phosphate, the latter by reaction with the counter-ion of the buffer. In similar reactions of *p*-nitrophenyl *N*-acetylphenylalanine with (4a) no acylimidazole intermediate is observed. The surfactant hydroxamic acid (5a) is also readily acylated by *p*-nitrophenyl acetate in the same pH region, and the resulting acylhydroxamate is unstable to hydrolysis. This latter reaction is pH-dependent and exhibits a solvent isotope effect of 2.7 at pH(pD) 7.9 .Addition of (5a) to the acylimidazole intermediate derived from (4a) causes rapid acyl transfer, although the latter does not affect the rate of acylhydroxamate hydrolysis.

CATALYSIS of *p*-nitrophenyl ester hydrolysis by micelles derived from functional surfactants [1-3] normally occurs by a nucleophilic mechanism, with subsequent breakdown of an acylated micellar intermediate. This mechanism has been most frequently postulated in the case of imidazole or histidine bearing surfactants although there has been much recent interest by Japanese workers in the formation and breakdown of micellar acylhydroxamates.[2] A particular reason for interest in this problem has been the search for co-operativity in catalysis of ester hydrolysis by mixed micelles, in an attempt to mimic the charge-relay mechanism [4] by which serine proteases operate. True bifunctional catalysis has never been observed in micelles although in the case of copolymer (1) it has been claimed that both the acylation and deacylation steps in hydrolysis of *p*-nitrophenyl acetate are enhanced relative to reaction rates for related polymers containing only one of the two reactive functionalities and that the former step involves a concerted mechanism of the type shown in



(1)



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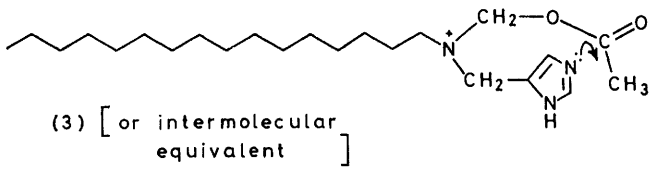
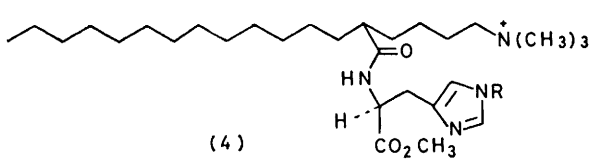
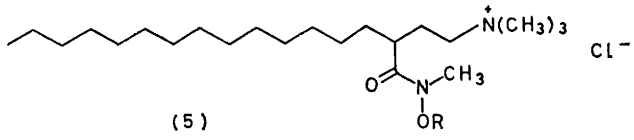


(3) [or intermolecular equivalent]



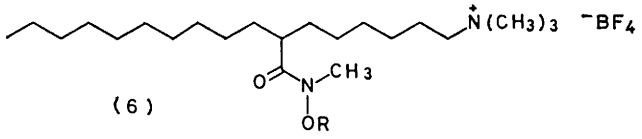
(4)

a; R = H
b; R = COCH
c; R = Ph



(5)

a; R = H
b; R = $COCH_3$



(6)

a; R = H
b; R = $COCH_3$

(2). Although the evidence is strongly indicative of this and is perhaps supported by the fact that the acylation step has a solvent isotope effect of 1.5 the modest rate enhancement observed makes further work desirable.[5] This contrasts with the position of co-operative catalysis in deacylation of intermediates in ester hydrolysis by functional micelles, for which a number of examples [*e.g.* (3)] [1c,d] are known, and the phenomenon quite well established.

We had considered [6] the possibility of co-operative catalysis by two-component micelles of functional surfactants, using (4a) together with (5a) or (6a) as catalyst for the hydrolysis of *p*-nitrophenyl esters in buffered solution at pH 7.4. Since the kinetic results obtained were equivocal, but did not encourage an interpretation based on co-operative catalysis, it was felt desirable to probe the system more thoroughly. In mixed micelles where both components absorb in a similar region of the u.v. spectrum, it is difficult to delineate the formation and breakdown of acylated intermediates, or to define in the present case the relative proportions of acylhistidine and acylhydroxamate formed at any given time in the course of reaction. The availability of 270 MHz Fourier transform n.m.r. encouraged us to examine a number of aspects of functional micellar catalysis by this technique. The present paper describes (a) the reaction of micelles of (4a) or (5a) with *p*-nitrophenyl acetate, (b) the reaction of mixed micelles of (4a) and (5a) with *p*-nitrophenyl acetate, and (c) the reaction of micelles of (4a) with *p*-nitrophenyl *N*-acetylphenylalanine. Concentrations of 5mM-surfactant and 2mM-ester were normally employed, and satisfactory spectra could be accumulated in less than 100 s. These concentrations ensure that surfactants are well above their respective critical micelle concentrations and the ester is completely soluble.

Reaction of (4) *with* p-*Nitrophenyl Acetate.*—The

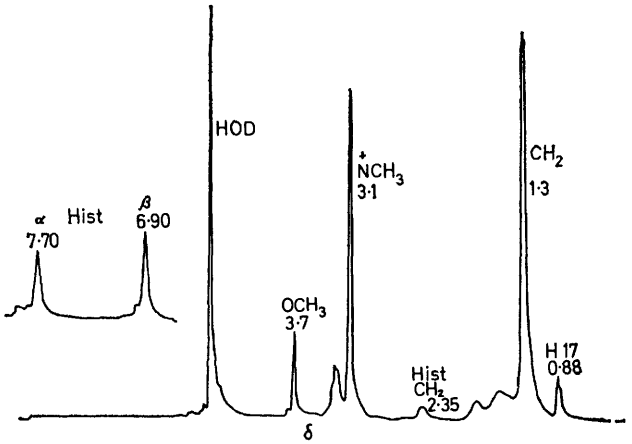


FIGURE 1 Proton magnetic resonance spectrum of histidine-derived sufactant (4a) in D_2O at pD 7.1 and 313 K, 5mM solution, 270 MHz, accumulation of 200 scans

spectrum of (4) [D_2O (0.5 ml), 5mM, 313 K, pD 7.1] is recorded in Figure 1. The general features of this spectrum are a resolved methyl group at δ 0.88, a methylene envelope due to the long alkyl chain at δ 1.3, a single methylene group (histidine C_α) at δ 2.3, the quaternary *N*-methyl protons at δ 3.1, the ester methyl group

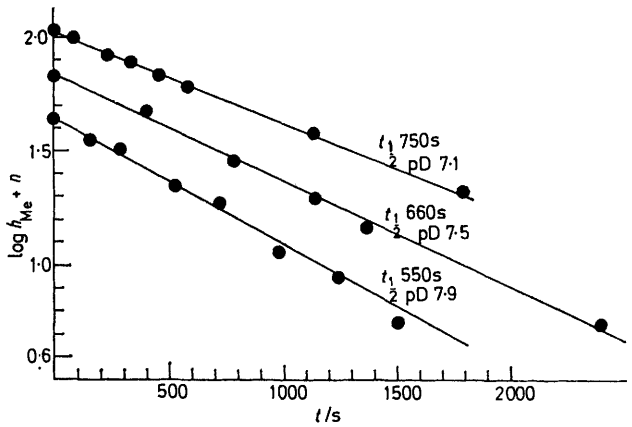


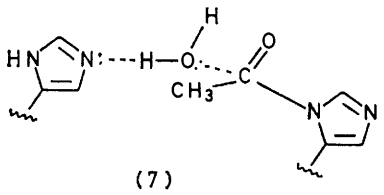
FIGURE 2 Hydrolysis of acetylhistidine (4b) prepared by adding *p*-nitrophenyl acetate (1mM in [$^2H_6$]acetone) to a solution of (4a) [5mM in buffer (0.5 ml)], 313 K. Reaction was monitored by recording the n.m.r. spectrum at intervals and measuring the height of the acetylmethyl signal at δ 2.57. The first-order plots shown contain an arbitrary constant *n*

at δ 3.7, and histidine 1- and 2-H at δ 7.70 and 6.90 respectively. Under the normal conditions of spectral acquisition, with 3.5 Hz line broadening added to the time-domain spectrum before processing, spin–spin couplings were not resolved. Addition of *p*-nitrophenyl acetate [16 μl, 60mM in $(CD_3)_2CO$] led to the rapid formation of a new species identified as the acylimidazole (4b). New n.m.r. adsorptions were observed at δ 2.57, due to the acylmethyl group, at δ 3.77, since the methoxycarbonyl group is appreciably shielded on acylation, and at δ 7.43 and 8.07, due respectively to 2- and 4-H of the acylimidazole ring.

Changes in the n.m.r. spectrum become apparent on further standing at 313 K, consisting with hydrolysis of the acylimidazole (4b) and reformation of (4a). There are two new methyl peaks, demonstrating that the acyl group is transferred in part to water and in part to phosphate buffer, forming acetyl phosphate monoanion. This latter species was identified by n.m.r. comparison with an authentic sample, prepared by addition of acetic anhydride to a surfactant-free buffer solution. The rate of hydrolysis of (4b) was followed by n.m.r. at various pH values from the decay of the peak at δ 2.57 using the chain-terminal methyl group at δ 0.88 as an internal standard (Figure 2). Given the approximations and limitations of n.m.r. as a kinetic tool, the reaction was found to be first-order and only slightly dependent on the acidity of the solution. In an additional experiment, sufficient *p*-nitrophenyl acetate [40 μl, 60mM in $(CD_3)_2CO$] was added to acylate all the available imidazole rings. Hydrolysis was appreciably slower, with an apparent induction period. This suggests that free histidines catalyse the breakdown of (4b) according to the mechanism depicted in (7), which is in line with observations on micellar [7] and non-micellar [8] systems. Only one acylated species (4b) is

observed during the course of acylation and hydrolysis, and if the isomer (8) is formed it is not detected by n.m.r. This is presumably because steric effects are important in acylation, for 2-picoline reacts [9] 0.12 times as fast as pyridine with acetic anhydride, despite being 0.7 pK_a units the more basic. It was already known [10] on the basis of u.v. studies that micellar imidazoles operate as nucleophilic rather than general-base catalysts in the hydrolysis of *p*-nitrophenyl esters (as do [11] protease enzymes!) The present study directly confirms this, and places an upper limit of 2% on the proportion of reaction proceeding by a general-base mechanism with direct formation of acetate ion.

Reaction of (5a) *with* p-*Nitrophenyl Acetate.*—Less structural information is available from the 270 MHz



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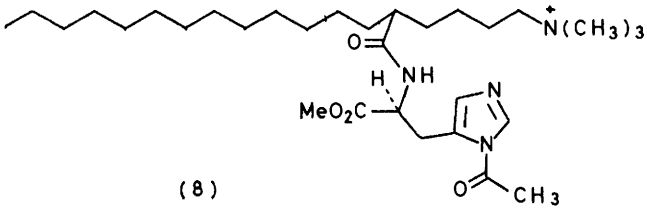

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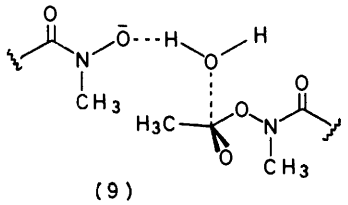


(9)

n.m.r. spectrum of (5a) in buffered D_2O, the long alkyl chain giving rise to absorptions at δ 0.88 and 1.3 respectively with a methine 3-H signal at δ 2.3. The quaternary ammonium group and hydroxamate *N*-methyl group are in similar environments at δ 3.2. When a solution of (5a) (5mM, 0.5 ml in 0.05M-phosphate buffer, D_2O, 313 K, pD 7.9) is treated with *p*-nitrophenyl acetate [16 μl, 60mM in $(CD_3)_2CO$] rapid acylation occurs, the resulting acylhydroxamate (5b) exhibiting a new, slightly broadened n.m.r. absorption at δ 2.32. In previous work,[2] surfactant acylhydroxamates have proved to be rather stable towards hydrolysis at near-neutral pH in the absence of added nucleophiles. The intermediate (5b) is degraded rather rapidly, however, and its hydrolysis could readily be followed by n.m.r. in the pH range 7—8. There is an apparent non-stoicheiometric dependence on hydroxide ion concentration (Figure 4), this being a frequent observation in micellar reactions.[12] In the particular case of (5a), ionisation of the hydroxamic acid does not follow typical Henderson–Hasselbach plots

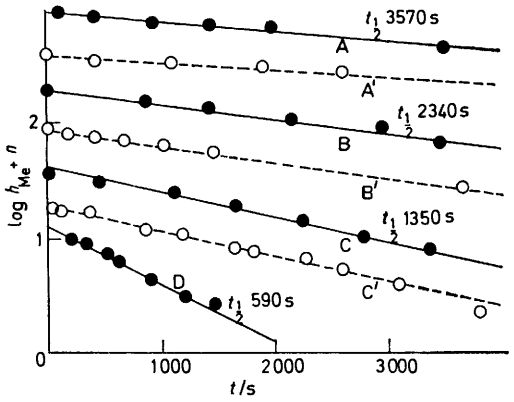


FIGURE 3 Hydrolysis of acetyl hydroxamate (5b) formed in the presence or absence of histidine-surfactant (4a). Runs A—C at pD 7.1, 7.5, and 7.9 respectively were conducted by adding *p*-nitrophenyl acetate (1mM in [$^2H_6$]acetone) to a solution of (5a) [5mM in buffer (0.5 ml)]. Runs A'—C', at the same pD values, were conducted by carrying out the procedure described for the data of Figure 2 and adding (5a) [5mM in buffer (0.5)ml] when hydrolysis of the *p*-nitrophenyl ester was complete. Run D was conducted in the same way as run C, but in 85 : 15 H_2O–D_2O solution

since the micelle behaves as a polyelectrolyte whose surface potential changes with the degree of ionisation.[6] Plotting the rate data against the spectrophotometrically determined degree of ionisation in buffered solutions (Figure 5) corresponding to the n.m.r. reaction media still gives a line of slope <1. This suggests that there may be a pH-independent component of reaction, although the major mechanism must be direct nucleophilic attack at acyl carbon. The solvent isotope effect of 2.7, observed when reaction was carried out at pH 7.9 in 85 : 15 H_2O–D_2O * is consistent with general-base catalysis by hydroxamate as in (9). This is similar to

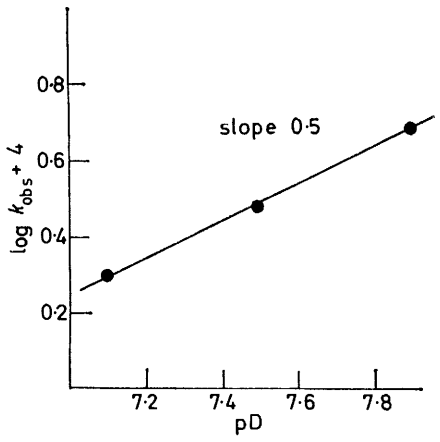


FIGURE 4 Non-integral rate–pH dependence for hydrolysis of acetylhydroxamate (5b) under conditions described for the data of Figure 3.

mechanisms previously proposed for imidazole-catalysed acylhydroxamate hydrolysis.[13] The higher lability of

* This concentration of D_2O is the minimum requisite for satisfactory spectrometer locking.

(5b) when compared to related compounds such as (10) is presumably due to an anion-stabilising field effect of the quaternary ammonium residue, which labilises the acyl group towards nucleophilic attack.

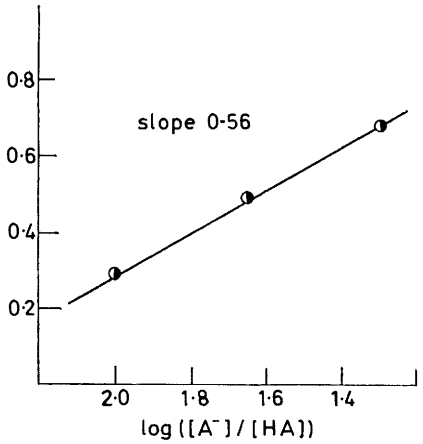


FIGURE 5 As Figure 4, the abscissa now referring to the extent of ionisation of hydroxamate (5a)

Reaction of (4a)–*Hydroxamic Acid Mixtures with* p-*Nitrophenyl Acetate.*—As discussed earlier, a primary aim of the work was to determine whether mixed micelles of (4a) and (5a) are capable of bifunctional catalysis in the hydrolysis of *p*-nitrophenyl esters. Kinetic work is reported separately.[6] When *p*-nitrophenyl acetate was added to a mixture of (4a) and (5a), both 5mM in buffered D_2O at pD 7.5, and the n.m.r. spectrum recorded immediately, then (5b) was the only observed acyl species. Since it is known that acyl transfer between imidazole and hydroxamic acid groups is rapid in dilute aqueous solution, this experimental result might reflect initial formation of (4b), at least in part, and subsequent rapid conversion into the thermodynamically favoured *O*-acyl compound (5b) by the pH-independent mechanism[13] (11). The point was proved (Figure 6) in a separate experiment whereby (4a) was acylated under standard conditions at pD 7.5, followed by rapid monitoring of the n.m.r. spectrum after addition of (5a) as a concentrated aqueous solution. Acyl transfer to give (5b) was essentially complete after 75 s, with an estimated half-life of 20 s or less. The rate constant for the micellar reaction appears to be quite similar to that observed by Gerstein and Jencks[13] in the simple case. This result emphasises the random character of mixed micelles of (4a) and (5a), for if they formed separate aggregates (or separate domains within a common aggregate) this would tend to arrest the rate of acyl transfer. Even when a similar experiment was conducted with (4b) and the 7-hydroxamic acid derivative (6a), whose functional group will be more deeply buried in the micelle, acyl transfer occurred too rapidly for n.m.r. monitoring at

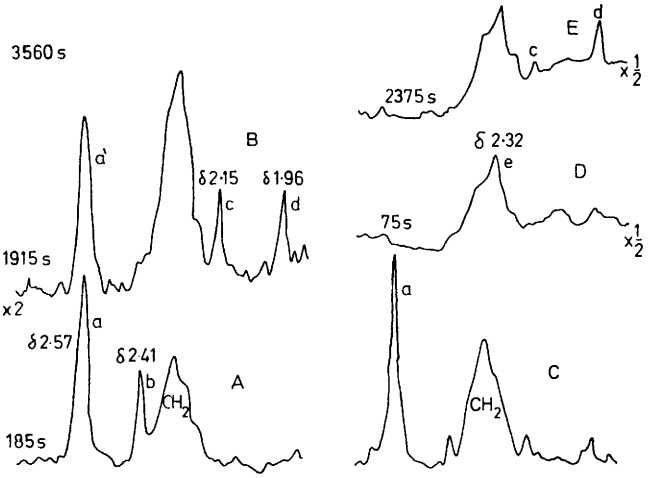
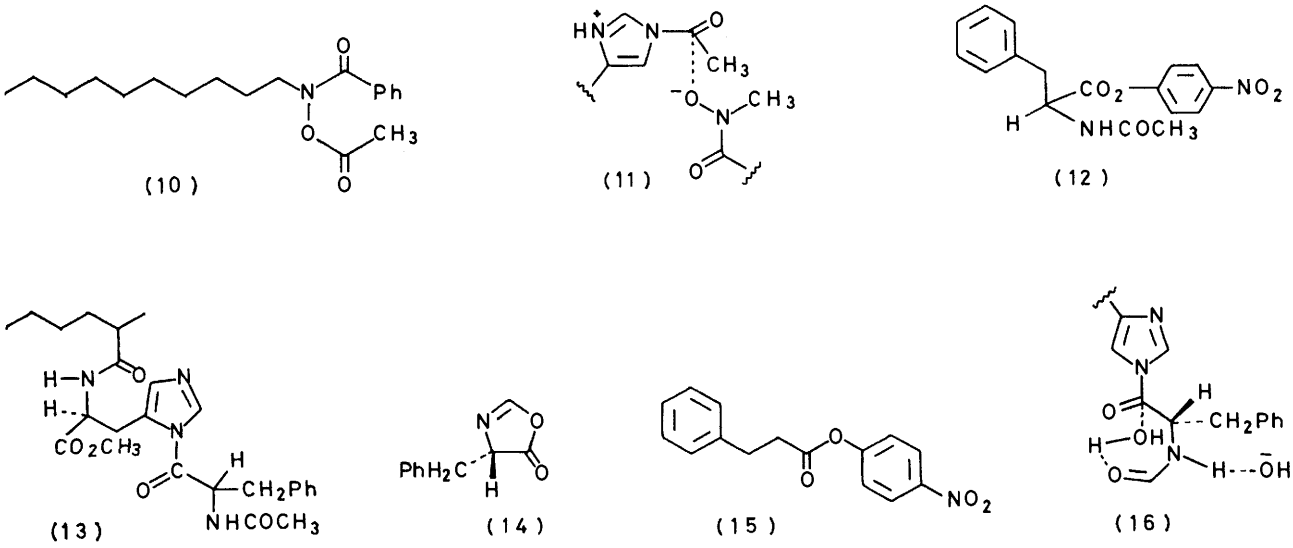


FIGURE 6 N.m.r. spectra of acyl transfer reactions at 270 MHz: A,B, addition of *p*-nitrophenyl acetate to (4a) under standard conditions as described for the data of Figure 2; C—E, histidine hydroxamic acid acyl transfer as described for the data of Figure 3; 313 K, pD 7.1. a, Histidine $NCOCH_3$; b, CH_3COpnp; c, $CH_3CO_2PO_3^{2-}$; d, $CH_3CO_2^-$; e, hydroxamate $NCOCH_3$



313 K, * although the first spectrum obtained after 69 s reaction did show a small quantity of residual (4b) which subsequently disappeared rapidly.

Previous studies of the hydrolysis of micellar acylhydroxamates have established general-base catalysis by imidazoles, the ester normally being hydrolytically stable at neutral pH in its absence. Compound (5b) is exceptional in this regard as it undergoes rapid unassisted hydrolysis at pD 7.9. The reaction rate is unaffected, within experimental error, by added (4a) (Figure 4). General-base catalysis by (4a) does not therefore contribute significantly, and neither is there an appreciable contribution to hydrolysis from reversible formation of (4b) at low concentration. Hydrolysis of (6b) proceeded much more slowly [in the presence of (4b)] than that of (5b) but was not investigated in detail.

Reaction of (4a) *with* p-*Nitrophenyl* N-*Acetylphenylalanine*.—Once it had been established that the hydrolysis of *p*-nitrophenyl acetate catalysed by micelles of (4a) proceeded entirely by a nucleophilic mechanism, it was of interest to apply the same criteria to (12). The micelle-catalysed hydrolysis of (*R*)-(12) by (4a) occurred three times more slowly than that of (*S*)-(12) in buffered solution at pH 7.3.[1b,14] This was considered to be a consequence of nucleophilic catalysis, with less favourable non-bonded interactions in the transition state for nucleophilic attack of imidazole nitrogen on the ester carbonyl group of (*R*)-(12). A solvent isotope effect of 1.3 is additional evidence for a nucleophilic rather than a general-base hydrolytic mechanism.[14]

Addition of either (*R*)- or (*S*)-(12) to a solution of (4a) (5mM in buffered D_2O, pD 7.1) led to rapid formation of a new species, reaction being essentially complete by the time of the first spectroscopic observations (<100 s). The resulting spectrum was unchanged over the course of a further hour. This new species, characterised by an acetyl-methyl resonance at δ 1.96, was *not* the acyl imidazole (13) since no changes occurred in the histidine region of the n.m.r. spectrum during the course of reaction. Nor was it the benzyloxazoline (14), whose *N*-methyl group resonated at δ *ca.* 1.7 in micelles of (4a) being partly obscured by the methylene envelope of the detergent. Addition of an authentic sample of (*S*)-*N*-acetylphenylalanine to a solution of (4a) in buffered D_2O gave a spectrum which was superimposable, aside from *p*-nitrophenolate resonances, with that observed in hydrolysis of (12). It therefore seems that there is no stable acyl intermediate (13) in amino-acid ester hydrolysis.

Notwithstanding this result, it is unlikely that hydrolysis of amino-acid *p*-nitrophenyl esters in micelles of (4a) occurs by a general-base catalysed mechanism, since the observed solvent isotope effect is too low and the dependence of rate on pH above pH 8, when nucleophilic attack by imidazolide anion (4c) becomes kinetically important,[14b] is similar for (*R*)-(12) and the simple *p*-nitrophenyl ester (15). It is most probably the case that (13) is formed in the micelle-catalysed hydrolysis of (12) but breaks down sufficiently rapidly that it remains undetected. Mechanisms involving the oxazoline (14) formed on intramolecular nucleophilic displacement of the acylimidazole (13) are precluded since the former is stable under the reaction conditions. Intramolecular general-base catalysis according to (16) is unlikely [16] to be an efficient mechanism. The remaining possibility is simple, or imidazole general-base catalysed, hydrolysis of (13) within the micelle with a reaction half-life of 30—40 s or less. This is at least 20 times faster than the corresponding hydrolysis of (4b) and requires some rationalisation. A Hammett ρ value of 2.44 was obtained for general-base catalysed hydrolysis of benzoylimidazoles,[17] and thus a ρ* value of *ca.* 2.5 may be assumed for the like reaction of aliphatic acylimidazoles. Given that the δ_I constant for $NHCOCH_3$ (0.27) is similar to that of $COCH_3$ (0.28) then δ* values for CH_2COCH_3 (0.60) and $CH(CH_2Ph)NHCOCH_3$ are likely to be of similar magnitude.[18] Neglecting steric effects, then it might be expected that (13) would hydrolyse *ca.* 30 times faster than (4b). A rate difference of this magnitude would be quite sufficient to make (13) unobservable under the conditions of the acylation experiments.

An attempt was made to prepare (13) under anhydrous conditions. The surfactant (4a) in [$^2H_6$]acetone was treated with (*S*)-(12) and two equivalents of triethylamine at 0°. The resulting solution was syringed into buffered D_2O and an n.m.r. spectrum recorded immediately. The only species observed derived from (12) was *N*-acetylphenylalanine. This lends weight to the supposition that (13) is indeed formed in the micelle-promoted hydrolysis of (12) but has very limited lifetime under the experimental conditions.

EXPERIMENTAL

Fourier transform n.m.r. spectra were recorded, unless otherwise stated, at 270 MHz and 313 K on 0.05M-phosphate buffer solutions in D_2O. The pD (pH + 0.4) of each buffer used was measured on a Pye–Unicam 297 pH meter. Synthesis of the surfactants used has been described elsewhere.[14] *p*-Nitrophenyl esters were prepared by literature procedures [19] as was *N*-acetylphenylalanine. The standard technique used for recording of n.m.r. spectra was as follows. The surfactant solution, usually 5mM in buffer (0.5 ml), was pre-warmed to 313 K in the spectrometer probe and its n.m.r. spectrum recorded and stored on disc. The sample tube was then removed from the probe, substrate added as a 60mM solution in [$^2H_6$]acetone, the resulting solution quickly shaken, the stopclock started, and sample returned to the probe. Spectral accumulation was commenced immediately, points being recorded and stored until no further changes were apparent. Processing of the resulting time-domain spectra was normally effected with 3.5 Hz line-broadening function to improve the signal to noise ratio. Each kinetic point was the accumulation of 100 scans, total acquisition time 61 s.

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* Below this temperature the microviscosity of the solution is sufficient to cause appreciable line broadening.

REFERENCES

[1] (*a*) P. Heitmann, R. Husung-Bublitz, and H. J. Zunft, *Tetrahedron*, 1974, **30**, 4137; (*b*) J. M. Brown and C. A. Bunton, *J.C.S. Chem. Comm.*, 1974, 969: (*c*) U. Tonellato, *J.C.S. Perkin II*, 1977, 821; (*d*) R. A. Moss, R. C. Nahas, and S. Ramaswami, *J. Amer. Chem. Soc.*, 1977, **99**, 627; (*e*) .W Tagaki, S. Kobayashi, and D. Fukushima, *J.C.S. Chem. Comm.*, 1977, 29.

[2] (*a*) T. Kunitake, S. Shinkai, and Y. Okahata, *Bull. Chem. Soc. Japan*, 1976, **49**, 540; (*b*) I. Tabushi and Y. Koruda, *Tetrahedron Letters*, 1974, 3613 and earlier papers by these workers; (*c*) R. Ueoka, M. Kato, and K. Ohkubo, *Tetrahedron Letters*, 1977, 2163.

[3] (*a*) J. H. Fendler and E. J. Fendler, ' Catalysis in Micellar and Macromolecular Systems ', Academic Press, New York, 1975, ch. 5; (*b*) R. Shiffman, M. Chevion, J. Katzhendler, C. Rav-Acha, and S. Sarel, *J. Org. Chem.*, 1977, **42**, 856; (*c*) C. A. Bunton and M. McAnemy *ibid.*, p. 475.

[4] D. M. Blow, *Accounts. Chem. Res.*, 1976, **9**, 145.

[5] T. Kunitake and Y. Okahata, *J. Amer. Chem. Soc.*, 1976, **98**, 7793.

[6] J. M. Brown and J. L. Lynn, jun., *Ber. Bunsengesellschaft Phys. Chem.*, submitted for publication.

[7] *Cf.* C. G. Overberger and R. C. Glowaky, *J. Amer. Chem. Soc.*, 1973, **95**, 6014.

[8] (*a*) J. L. Hogg, M. K. Phillips, and D. E. Jergens, *J. Org. Chem.*, 1977, **42**, 2459; (*b*) J. L. Hogg and M. K. Phillips, *Tetrahedron Letters* 1977, 3011 and earlier references cited therein.

[9] V. Gold and E. G. Jefferson, *J. Chem. Soc.*, 1953, 1409.

[10] C. Gilter and A. Ochao-Solano, *J. Amer. Chem. Soc.*, 1968, **90**, 5001.

[11] C. D. Hubbard and J. F. Kirsch, *Biochemistry*, 1972, **11**, 2483.

[12] T. Kunitake, Y. Okahata, and T. Sakamoto, *J. Amer. Chem. Soc.*, 1976, **98**, 7799.

[13] J. Gerstein and W. P. Jencks, *J. Amer. Chem. Soc.*, 1964, **86**, 4655.

[14] J. M. Brown, C. A. Bunton, S. Diaz, A. R. Ferwanah, and P. E. Rivers, to be published.

[15] J. P. Greenstein and M. Winitz ' Chemistry of the Amino-acids ', Wiley, New York, 1961, vol. 3, p. 2173.

[16] A. J. Kirby and G. J. Lloyd, *J.C.S. Perkin II*, 1976, 1948, 1953, 1962.

[17] (*a*) M. Choi and E. R. Thornton, *J. Amer. Chem. Soc.*, 1974, **96**, 1928; (*b*) W. Palaitis and E. R. Thornton *ibid.*, 1975, **97**, 1193.

[18] P. R. Wells, *Chem. Rev.*, 1963, 171.

[19] D. W. Ingles and J. R. Knowles, *Biochem. J.*, 1967, **104**, 369.