Reactions of *p*-Nitrophenyl Dodecanoate in Normal and Modified Cholic Acid Micelles

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The mild catalysis by cholic acid micelles of *p*-nitrophenyl dodecanoate hydrolysis, and of *p*-nitrophenoxide release in the presence of imidazole groups, provide useful probes of micellar growth and structure. We have made a molecule consisting of two cholic acid residues joined by a flexible hydrophilic oligomer. This molecule shows a small catalysis on its own at low concentrations in the presence of 40% dioxan, and it can halve the critical micelle concentration of cholic acid when present in a proportion of one part in 50.

MENGER and MCCREERY [1] found that cholic acid (CA) micelles catalyse the hydrolysis of *p*-nitrophenyl dodecanoate (*p*-NPD) under alkaline conditions. This is contrary to the usual inhibition of alkaline hydrolysis of *p*-nitrophenyl esters by negatively charged micelles [1,2] and was seen as reflecting a difference between the molecular environment within the micelles and that created

[1] F. M. Menger and M. J. McCreery, *J. Amer. Chem. Soc.*, 1974, **96**, 121.
[2] C. A. Bunton, *Progr. Solid State Chem.*, 1973, **8**, 239.

by self-aggregation of *p*-NPD. (Such aggregation had already been shown strongly to inhibit hydrolysis.[3])

We have studied both the hydrolysis of *p*-NPD, and its reaction with imidazole, in CA micelles. We also report some effects of a new molecule consisting of two cholyl groups joined by a flexible hydrophilic ' string '.

EXPERIMENTAL

Materials.—*p*-NPD, CA, and L-histidine were used as obtained from Sigma Chemical Co. Hydrolysis of *p*-NPD, m.p. 44.5—46°, gave the expected yield of *p*-nitrophenoxide ions. Crystallisation of CA twice from absolute ethanol did not affect its m.p., 196—197.5°. Spectrosil grade dioxan and AnalaR dioxan, acetonitrile, ethyl acetate, and CPR phthalimide were obtained from Hopkin and Williams. Buffer components and imidazole were from B.D.H. and purest grade tetraethylene glycol from Fluka A.G. Sachs S.G. A conjugate of CA and L-histidine, presumably the N^{α}-conjugate,[4] was prepared according to the literature,[4,5] m.p. 185—188° (from ethanol–ethyl acetate). The homogeneity of all compounds used in the kinetics was tested by t.l.c. on silica plates. The water was glass distilled.

Kinetics.—Buffers and stock solutions were prepared as described by Menger and McCreery.[1] The pH of solutions made from these were checked before each kinetic run, using sample solution (3 ml) in a Unicam SP 1800 spectrophotometer set at 400 nm and fitted with an automatic absorbance recorder. After 15 min a solution (50 μl) of *p*-NPD in acetonitrile or dioxan was added with a microsyringe and the solution stirred with a glass rod. The absorbance, due to *p*-nitrophenoxide ions, was recorded as a function of time. Half times for the reaction were determined by plotting $A_{\infty} - A_t$ against time on semi-log graph paper. Pseudo-first-order rate constants, $k_{obs.}$, were then obtained from $k_{obs.} = 0.693/t_{\frac{1}{2}}$.

Scattering curves were obtained at 500 nm with the Unicam SP 1800.[6]

Bis-2-[2-(3α,7α,12α-trihydroxy-5β-cholanamidoethoxy]ethyl Ether.—Bis-2-(2-aminoethoxy)ethyl ether was prepared by a Gabriel synthesis from the corresponding dibromo compound [7] which had been prepared by the action of PBr_3 on tetraethylene glycol.[8] CA (10 mM) and tri-n-butylamine (10 mM) in dioxan (30 ml) was cooled to 10° and ethyl chloroformate (10 mM) added with stirring. After 20 min at that temperature bis-2-(2-aminoethoxy)ethyl ether in 10N-NaOH (10 ml) was added. Stirring was continued for a further 1 h at 10°. The mixture was left overnight to attain room temperature. Three volumes of water were then added to give a sticky precipitate which was washed twice with water and dissolved in dioxan (25 ml). This solution was acidified to pH 4 with HCl and the product reprecipitated with three volumes of water. After washing with water until the washings were neutral to litmus the product was dried and crystallised from ethyl acetate giving hygroscopic *crystals* (60%), m.p. 125—126° (Found: C, 69.2; H, 10.05; N, 2.6. $C_{56}H_{96}O_{11}N_2$ requires C, 69.15; H, 9.9; N, 2.9%).

[3] F. M. Menger and C. E. Portnoy, *J. Amer. Chem. Soc.*, 1968, **90**, 1875.
[4] J. J. Myher, L. Marai, A. Kuksis, I. M. Yousef, and M. M. Fisher, *Canad. J. Biochem.*, 1975, **53** 583.
[5] S. Bergstrom and A. Norman, *Acta Chem. Scand.*, 1953, **7**, 1126.
[6] Pye Unicam Technical Manual for SP1800 Spectrophotometer, section 10.6.

RESULTS AND DISCUSSION

Figure 1 shows that imidazole and histidine can increase the CA assisted release of *p*-nitrophenoxide ions from *p*-NPD. This does not seem to be a direct action on *p*-NPD aggregates since imidazole and histidine show little activity by themselves: also the activity is much greater when histidine is covalently linked to CA [Figure 1(e)]. The somewhat greater effect of imidazole over histidine may reflect a stronger binding of the more

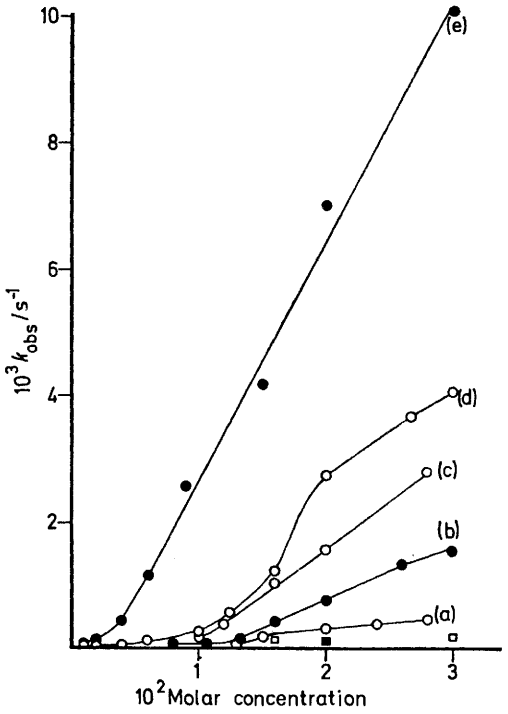

FIGURE 1 Changes in first-order rate constants for the release of *p*-nitrophenoxide from *p*-NPD against concentrations of (a) CA; (b) equimolar CA and histidine; (c) CA and 0.013M-imidazole; (d) equimolar CA and imidazole; (e) CA–histidine conjugate. Open and closed squares refer to the effects of imidazole and histidine respectively in the absence of CA. [*p*-NPD] = 4×10^{-5}M; pH = 10.5 in 0.025M-phosphate buffer, 0.1M-NaCl and 3.3% acetonitrile at 33 °C

hydrophobic imidazole by CA micelles. We found some evidence in any case for hydrophobic binding of imidazole: its u.v. absorption at 207 nm in water showed a bathochromic shift of 2 nm, indicative of a non-polar environment,[9] in the presence of CA.

Effect of *p*-NPD concentrations on the release of phenoxide from *p*-NPD in the presence of 0.03M-CA and 0.021M-imidazole at pH 10.5 in a 0.025M-phosphate buffer and 0.1M-NaCl. Temperature 34 °C

10^5[*p*-NPD]/M	0.5	1.7	4.2	6.2	8.3	12.5	16.6	25.0
$10^4 k_{obs}/s^{-1}$	37.3	36.0	34.4	35.6	35.1	19.0	11.4	5.6

The Table shows the effect of increasing *p*-NPD concentrations on its reaction with imidazole in the presence

[7] B. Dietrich, J. M. Lehn, J. P. Sauvage, and J. Blanzat, *Tetrahedron*, 1973, **29**, 1629.
[8] J. R. Dann, P. P. Chiesa, and J. W. Gates, jun., *J. Org. Chem.*, 1961, **26**, 1991.
[9] S. Riegelman, N. A. Allawala, M. K. Hrenoff, and L. A. Strait, *J. Colloid Sci.*, 1958, **13**, 208.

of CA micelles. The break at *ca.* 10^{-4}M is consistent with the idea that, as with simple alkaline hydrolysis, CA micelles catalyse reaction with imidazole by suppressing aggregation of the *p*-NPD molecules. Aggregation can be expected when the free *p*-NPD concentration exceeds *ca.* 10^{-7}M.[10] For this to happen at a total *p*-NPD concentration of 10^{-4}M would imply a binding constant for *p*-NPD in CA micelles of *ca.* 10^5 l mol^{-1} on the assumption [11] that each micelle contains four CA molecules. This is a reasonable order of magnitude for the binding constant in view of the published estimates of 1.1×10^2 and 2.2×10^3 l mol^{-1} for those of the acetate and octanoate esters respectively.[1] Suppression of aggregation of *p*-NPD does not seem to be the only catalytic effect of CA, however, since beyond the point at which CA micelles have apparently destroyed *p*-NPD aggregation the catalytic effect is still rising, even where the imidazole concentration is held constant [Figure 1(c)] and where

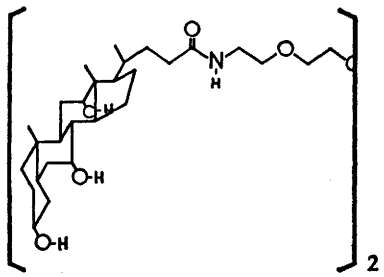

FIGURE 2 Molecule consisting of two cholyl residues joined by an amido-oxyethylene oligomer

one might have expected a decline in activity as further addition of CA diluted both the reactants in the micellar phase. It would seem that with increasing CA concentration the micelles change in a way that makes them more efficient catalysts. This may reflect a more gradual build up of CA micelles compared with the micelles of simple surfactants.[12,13] A similar but much smaller effect is seen in the profiles for the simple hydrolysis of *p*-NPD at pH 10.5 [Figure 1(a)] and 10.9 [Figure 5(a) (ii)].

The double molecule shown in Figure 2 was found to be barely soluble in aqueous buffers so we used 40 : 60 dioxan–phosphate buffer mixtures to study its kinetic effects. Under these conditions CA itself gives a different kind of rate profile for *p*-NPD hydrolysis [Figure 3(a)]. Since dioxan is known to catalyse *p*-NPD hydrolysis by breaking up *p*-NPD aggregates [3] this profile can perhaps be seen as resulting from CA micelles binding *p*-NPD and hence *reducing* the pseudo-first-order rate constants from an initial high level towards values more typical in CA micelles. With the double molecule, on the other hand, there is a small but clear initial increase before a decline due, presumably, to aggregation [Figure 3(b)].

[10] J. P. Guthrie, *Canad. J. Chem.*, 1973, **51**, 3494.
[11] D. M. Small, S. A. Penkett, and D. Chapman, *Biochim. Biophys. Acta*, 1969, **176**, 178.
[12] B. Lindman, N. Kamenka, and B. Brun, *J. Colloid Interface Sci.*, 1976, **56**, 328.

At a lower pH [Figure 4(a)] this initial effect is stronger. The scattering curve [Figure 4(b)] indicates that catalysis is indeed interrupted by aggregation of some sort.

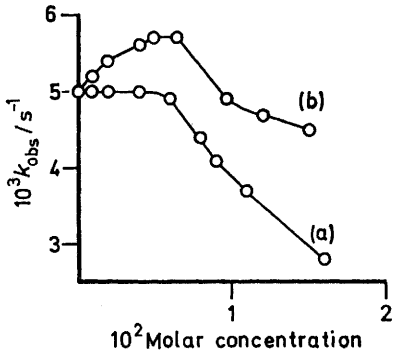

FIGURE 3 Changes in first-order rate constants for the release of *p*-nitrophenoxide from *p*-NPD against concentration of (a) CA; (b) the compound shown in Figure 2. [*p*-NPD] = 4×10^{-5}M; pH = 10.9 in 40 : 60 v/v dioxan–0.025M-phosphate buffer containing 0.1M-NaCl. Temperature 30 °C

It seems possible that the unassociated double molecules could bind *p*-NPD rather as in CA micelles. The connecting chain is long enough and its trioxyethylene section probably flexible enough [14] for the double molecule to ' clamp ' the dodecyl group neatly between the hydrophobic β surfaces of its pair of cholyl groups. This can be readily done with CPK models. With such an arrangement the ester linkages should be more acces-

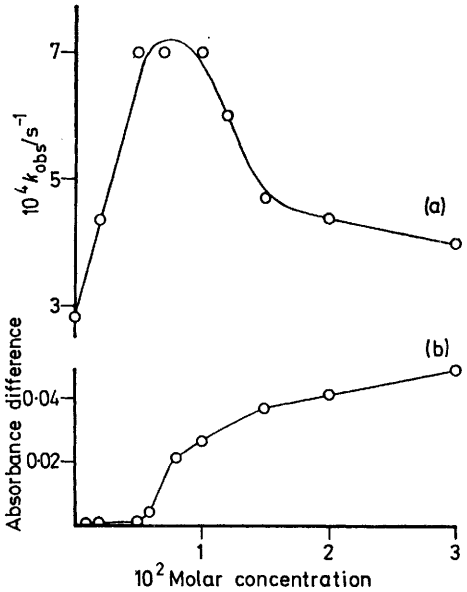

FIGURE 4 (a) First order rate constants for the release of *p*-nitrophenoxide from *p*-NPD and (b) measure of light scattering at 500 nm against concentration of compound shown in Figure 2. pH 9.5, otherwise conditions as for Figure 3

sible to the water than in CA micelles, at least so long as the double molecules remain unassociated.

[13] A. Djavanbakht, K. M. Kale, and R. Zana, *J. Colloid Interface Sci.*, 1977, **59**, 139.
[14] C. C. Price, in ' The Chemistry of the Ether Linkage,' ed. S. Patai, Interscience, London, 1967, p. 499; J. Conrad, ' Encyclopedia of Polymer Science and Technology,' Interscience, New York, 1967, vol. 6, p. 121.

In another series of experiments we used much lower dioxan concentrations at which CA rate profiles are not greatly affected. Here one part in 50 of the double compound had a very noticeable effect on CA rate profiles (Figure 5). We might consider two possible explanations for this. Conceivably the earlier rise in the curves in the presence of the double molecule might be due to its micellising on its own at a critical micelle concentration (c.m.c.) of *ca.* 100 times less than the c.m.c. for

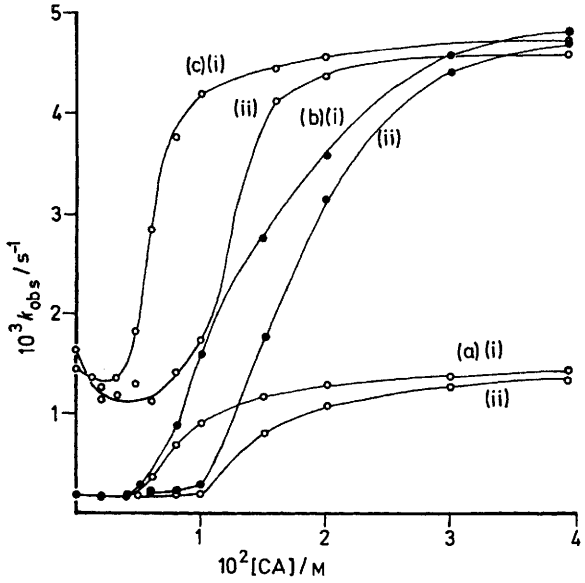

FIGURE 5 Changes in first-order rate constants for the release of phenoxide from *p*-NPD (a) at pH 10.9; (b) with imidazole at pH 10.9; (c) at pH 12.05, against CA concentrations. The imidazole was equimolar with the CA for curves (b). For curves (i) the compound shown in Figure 2 was present in a proportion of 1 : 50 of CA. [*p*-NPD] = 5×10^{-6}M in 4 : 100 v/v dioxan–0.025M-phosphate buffer containing 0.1M-NaCl. Temperature 33 °C

CA. To account for the otherwise similar shapes of the members of each pair of curves we would have to suppose that the double compound micelles were suitably more catalytically active than CA micelles, initially by a factor of *ca.* 50 but reducing towards a factor of one as the CA micelle population built up. This seems a rather contrived explanation particularly as members of three different pairs of profiles are so similarly related. The alternative is to suppose that the double molecule somehow reduces the c.m.c. of CA to about half the usual value. On this view the members of each pair of profiles are similar to each other because in each case they reflect a build up of micelles that are always predominantly of CA. There remains the problem of how such a small proportion of double molecule could produce this effect.

The CPK model of the double molecule suggests that the hydrophobic β surfaces of its cholyl groups could associate with each other as is presumed to happen in normal CA micelles.[1,11,15] The maintained proximity of the two groups would be an additional factor in favour of such association. We might thus expect that the double molecule would spend much of its time in a conformation resembling the initial two-molecule nucleus from which CA micelles must presumably grow, through tetramers [11] and possibly higher structures,[12,13] and we might explain the lowering of the c.m.c. of CA by supposing that the double molecules form nuclei to which CA molecules can add at a concentration of about half that required for net formation of two-molecule nuclei by CA molecules themselves.

On the basis of this picture the data in Figure 5 suggest that one double molecule can induce the aggregation of at least 30 CA molecules. Consider the curves (a)(i) and (b)(i). At a CA concentration of *ca.* 4mM micelles begin to form. At *ca.* 10mM, 6mM of CA will be in micelles that would not have formed without the double molecules [*cf.* curves(a)(ii) and (b)(ii)]. The double molecule concentration is then 0.2mM. If each micelle contains only one double molecule this implies *ca.* 6/0.20 = *ca.* 30 CA molecules per nucleated micelle at pH 10.9. Curves (C)(i) and (ii) imply a similar number (*ca.* 4/0.14) for pH 12.05. Although large for a CA micelle,[11] this number is perhaps reasonable if, as we have suggested, micellar growth can here take place spontaneously while the number of nuclei available is limited.

Comment.—Surfactant micelles are well known simple models for globular protein molecules. Perhaps their main weakness is that they generally fail to mirror the more definite solid-like structure of globular protein interiors. Micelles of large molecules are thus particularly interesting. But the solid-like structure of proteins arises less from the inherent strength of the forces between its aggregating groups than from the fact that these groups are tied together, thus reducing their degrees of freedom. ' Tied micelles ' in which surfactant units are similarly controlled, may prove to be better protein models: our double molecule is an essay in this direction even if the catalytic and micelle inducing effects that we found are, so far, modest.

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[7/1949 *Received*, 7*th November*, 1977]

[15] M. C. Carey and D. M. Small, *Arch. Internat. Medicin.*, 1972, **130**, 506.