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Effects of Cationic Micelles on the Rate of Reaction of *p*-Nitrophenyl Esters with Dihydrolipoic Acid and Dihydrolipoylamido Derivatives

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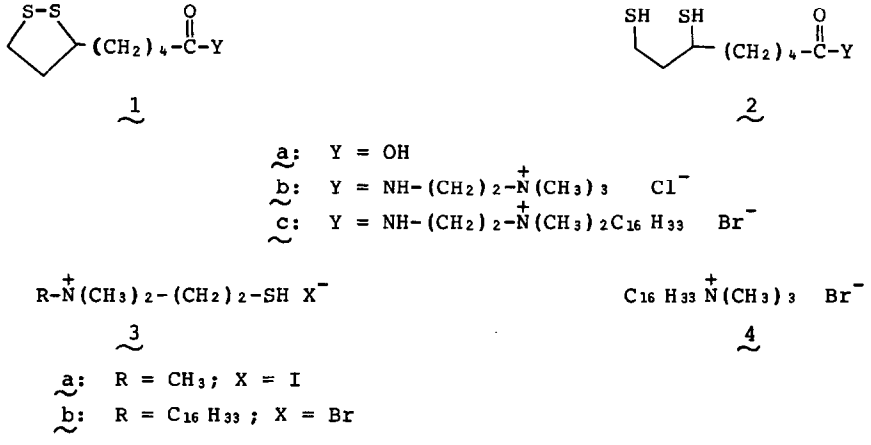
Dihydrolipoic acid (DHLA) **2a** and its surfactant derivatives, trialkyl(2-lipoylamidoethyl) ammonium salts **2b–c**, have been investigated, mainly in micellar solutions of cetyltrimethylammonium bromide (CTABr), as esterolytic reagents toward *p*-nitrophenyl esters. The origins of the observed kinetic effects are discussed, and the reactivity of these reagents are compared with that of other thiolytic systems. The results indicate that DHLA, although not a surfactant, is effectively comicellized by CTABr, and micelles of CTABr and DHLA are among the most effective esterolytic systems, at moderately alkaline *p*Hs, so far reported.

INTRODUCTION

Lipoic acid (LA), **1a**, dihydrolipoic acid (DHLA), **2a**, and *S*-acetyl derivatives of DHLA are involved in acyl transfer processes at the active site of α-*ketoacid dehydrogenases* (*1*) (Scheme 1). Although considerable information is available (*2*) concerning the mode of action of lipoyl derivatives *in vivo*, very little is known



SCHEME 1

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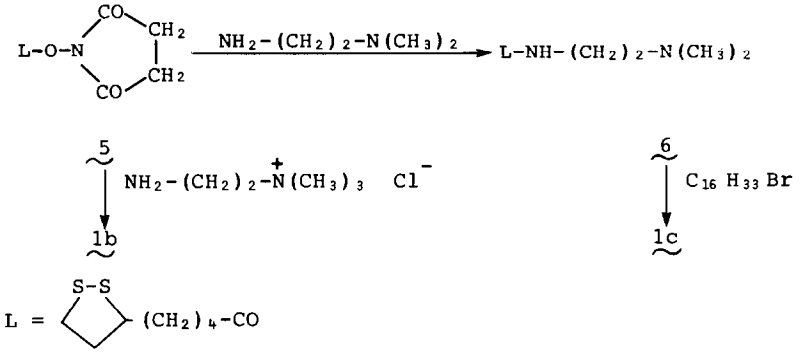
as regards the reactivity of DHLA in acyl transfer processes *in vitro*, such as in simple esterolytic reactions.

Other thiol cofactors have attracted more attention and have also been investigated as micellar esterolytic reagents. Micelles or comicelles of surfactant derivatives of cysteine (*3*) and thiocholine **3a** (*4*) comicelles containing coenzyme A (CoASH) glutathione (GSH) (*5*) and simple thiols (*6*) have been reported to be very effective nucleophilic reagents in the cleavage of activated esters, the thiocholine **3b** micellar system being near to the enzyme (ficin) (*7*) reaction in giving a high rate enhancement at neutral *p*Hs.

We herein report the esterolytic properties of DHLA and its derivatives **2b** and **2c** where the lipoyl moiety is bound to an ammonium salt structure through an amide linkage.[1] We have investigated the cleavage of *p*-nitrophenyl acetate (PNPA) and hexanoate (PNPH) by dihydrolipoyl reagents **2** mainly in micellar solutions of cetyltrimethylammonium bromide (CTABr, **4**) in borate buffer, pH 8.7, and the reactivity is compared to that of other micellar and nonmicellar thiol cofactors.

RESULTS AND DISCUSSION

Lipoic acid derivatives **1b** and **1c** were synthesized following the sequence shown in Scheme 2. These salts are waxy hygroscopic substances routinely stored under nitrogen in the dark. The critical micelle concentration, cmc, in borate buffer is about 3.5×10^{-4} *M* (**1c**) and 3.0×10^{-3} *M* (**1b**), as determined by surface tension measurements.



SCHEME 2

Reduction by $NaBH_4$ of lipoyl to dihydrolipoyl derivatives is easily achieved (*8*); however, attempts to isolate **2b** and **2c** failed to give materials with a reasonable free SH titre by the Ellman assay (*9*). The reduction was carried out in

[1] The lipoyl moiety is similarly linked to a lysine residue in *dihydrolipoyl transacetylase*.

aqueous solutions, under rigorous anaerobic conditions, immediately before use; the free *SH* activity toward Ellman's reagent was 90–100% and decreased on standing (~10% in 1 hr). The oxidative degradation of dihydrolipoyl derivatives complicated the kinetic measurements; rate data were usually obtained during the first 10–15 min after reduction, before substantial degradation of the reagents.

The cleavage of PNP esters was followed by monitoring the appearance of *p*-nitrophenol at 400 nm for (0.020–0.025) *M* borate buffers adjusted with KCl to μ = 0.07, pH 8.7, at 25°C. The limited solubility in water of the dihydrolipoyl derivatives and their easy degradation allowed only a limited set of kinetic measurements for nonmicellar solutions of DHLA. The second-order rate constant shown in Table 1 as k_w was obtained from (initial-rate) data measured for solutions of [DHLA] in the range $(0.8–4.0) \times 10^{-4}$ *M* and [ester] = $(0.5–0.9) \times 10^{-5}$ *M*. For solutions containing CTABr, the esterolysis by dihydrolipoyl derivatives is much faster and, in most cases, the kinetic runs were completed in a few minutes. Measurements were made for solutions of CTABr and each of the reagents, *R*: LA, DHLA, **2b**, and **2c**, in the molar ratio (8–9):1.

For micellar solutions, under conditions where [ester] $\ll$ [*R*], the pseudo-first-order rate constants k_ψ (see Fig. 1 for rate-concentration prophiles in the hydrolysis of PNPA) were found to obey reasonably the rate equation

$$k_\psi = \frac{(k_m/\bar{V})K_s K_R[R][D]_m + k_w[R]}{(1 + K_s[D]_m)(1 + K_R[D]_m)} \quad [1]$$

or

$$k_\psi = \frac{(k_m/\bar{V})K_s[R]_m + k_0}{(1 + K_s[D]_m)} \quad [2]$$

derived and discussed by Berezin *et al.* (*10*) (Eq. [1] for second-order reactions in micelles) and ourselves (*11*) (Eq. [2] for functional comicelles). In these equations,[2] k_m is the second-order rate constant in the micellar pseudophase, $\bar{V}$ is the molar volume of the micelles, K_s and K_R are the association constants of the substrate (ester) and reagent *R*, to the micellized detergent *D*, $[D]_m = [D] - \text{cmc}$ the concentration of micellized detergent and k_0 approximates the pseudo-first-order rate constant measured for micellar solutions of CTABr alone, in the absence of *R*. The quantity $(k_m/\bar{V})K_s$ is usually referred to as k_c, the apparent catalytic rate constant (*11*). Rate data for micellar solutions of DHLA and **2c** could be handled by the use of Eq. [2]; those for **2b** using Eq. [1].

The rate parameters k_c, k_m and K_R, K_S obtained from raw data following previously described methods (*10, 11*) and affected by a rather large error, particularly in the case of **2b**, are shown in Table 1.

The apparent catalytic rate constants k_c for CTABr solutions of DHLA and **2c** are very close. Since **2c** is a surfactant and forms comicelles with CTABr, the rate

[2] Equation [1] becomes Eq. [2] when K_R is very large and conditions are such that $K_R[D]_m \gg 1$ and when $k_\psi \gg k_w[R]$ or k_0.

TABLE 1

KINETIC PARAMETERS (see text) FOR CLEAVAGE OF PNPA AND PNPH BY DIHYDROLIPOYL DERIVATIVES, AT 25°C

R	Ester[a]	k_w (M^{-1} sec^{-1})	k_c (M^{-1} sec^{-1})	K_S (M^{-1})	K_R (M^{-1})	k_m (M^{-1} sec^{-1})
DHLA (**2a**)	A	0.49	105	65	[b]	0.6
	H	0.43	1080	1600	[b]	0.25
2b	A		8.8	35	1.7×10^4	0.10
	H		55	750	1.3×10^4	0.04
2c	A		135	95	[b]	0.55
	H		1350	1300	[b]	0.40

[a] A, PNPA; H, PNPH.
[b] $K_R > 2 \times 10^5$.

parameters of Table 1 indicate that DHLA is just as effectively comicellized as **2c** to the cationic micelles due to hydrophobic and electrostatic (through the CO_2^- function) attractive effects. The hydrophobic effects in the case of **2b** are instead counterbalanced by electrostatic repulsion; as a result, **2b** is not effectively comicellized, and its k_c value is much lower than that of DHLA and **2c**.

The reactivity of DHLA depends entirely on its thiol functions and not on the CO_2^- group. In fact, solutions of CTABr and lipoic acid are less reactive than those of CTABr alone (see Fig. 1); i.e., lipoic acid acts as a weak inhibitor of the CTABr micellar system due probably to partial charge neutralization of the positive charge of the micelles. From Table 1 it appears that the k_w and k_m are remarkably similar; and, since the pK_a of the thiol function of DHLA does not appreciably change (see below) in the presence of CTABr, this correspondence indicates that the second-order rate constant in bulk water and in micellar phase are very close and that the micellar kinetic benefits are entirely accounted for in terms of substrate and reagent binding as observed for other micellar systems (*6, 11, 12*).

Under conditions [PNPH] > [CTABr] > [*R*], we observed "burst" kinetics (*11*) for all dihydrolipoyl derivatives. These experiments indicated that the amount of *p*-nitrophenol liberated during the "burst" phase equals the free *SH* (Ellman) of the reagent and that the turnover rate is rather low, in the range (2–4) $\times 10^{-4}$ mol of *p*-nitrophenol liberated per mole equivalent of *SH* per second. These findings clearly suggest that the esterolysis process involves fast acylation of both thiol functions of the dihydrolipoyl reagents followed by a slow deacylation to water, a mode of action common to other micellar thiol cofactors (*4, 5*). There is no evidence, under our conditions, of any difference in reactivity between the 6-*SH* and the 8-*SH* groups, at variance with enzymatic (*13*) reactions where the 6-*S* acetyl dihydrolipoyl derivative is formed and the 8-*SH* group is apparently not involved in acylation processes.

Quite likely, the effective nucleophilic species are the thiolate anions S^- (*2, 4*). We measured an apparent pK_a value of 10.6 for DHLA in the presence of CTABr ([CTABr]:[DHLA] = 6:1) by observing the S^- concentration at 240 nm following

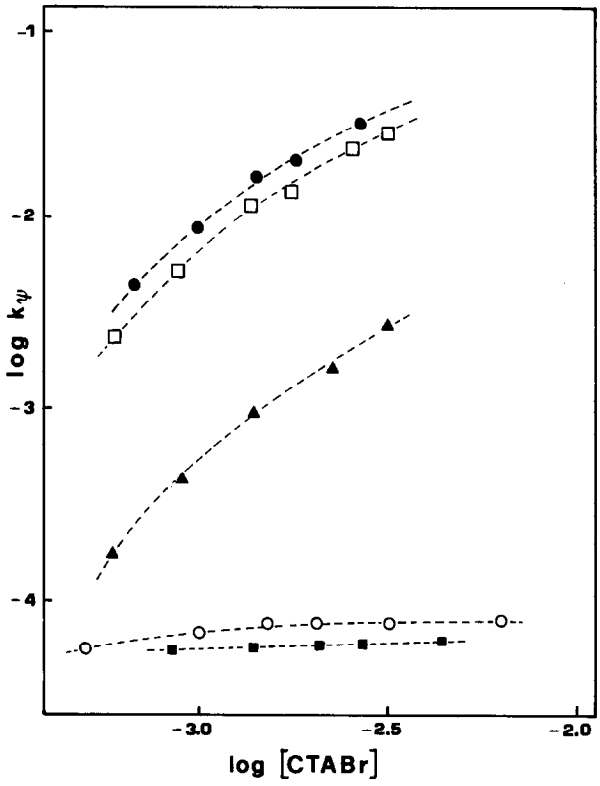


FIG. 1. Log k_ψ versus log[CTABr] for the cleavage of PNPA for micellar solutions of: ■, CTABr and LA, **1a** (8.1); ○, CTABr alone; ▲, CTABr and **2b** (8.3); □, CTABr and DHLA, **2a** (8.9); ●, CTABr and **2c** (8.9). The ratio [CTABr]:[*R*] is shown in parentheses. Log(cmc) = −(3.41 − 3.37).

standard procedures. Such a value is very close to that (10.7) reported in the literature (*14*) for DHLA in the absence of micelles.[3]

The rate constants obtained at a given pH may, therefore, be corrected for the fraction of thiolate anion to give k^-, being $k^- = k(1 + [H^+]/K_a)$ (*11*). The k^- values for DHLA and **2c** are in Table 2, together with those reported or evaluated for other thiol cofactors in nonmicellar and micellar systems for the esterolysis of PNPA. As expected for the cleavage of activated esters (*2*), the S^- reactivity increases with increasing pK_a of the *SH* function; and dihydrolipoyl derivatives, being also dithiols, are the most reactive of the series.

In conclusion, the micellar systems investigated here rank among the most effective esterolytic systems at moderately alkaline pHs so far investigated. It is notable that such an effective system is simply obtained by mixing lipoic acid and CTABr, triggered by a fast $NaBH_4$ reduction and does not require, as in other

[3] The apparent pK_a of a given acidic function at the head of cationic micelles is usually lower by 0.3 to 1.5 units than in bulk water (see also Table 2). The pK_a invariance in the case of DHLA associated to CTABr may indicate that the thiol functions mainly resides in a largely neutralized (by the CO_2^- group) environment at the surface of the micelles.

TABLE 2

pK_a (*SH*) CORRECTED RATE CONSTANTS[a] (M^{-1} sec^{-1}) FOR THE ESTEROLYSIS OF PNPA WITH THIOL COFACTORS *R*

Nonmicellar systems *R*	pK_a	k_w^-	Micellar systems *R* + CTABr	$pK_{a_{app}}$	k_c^-	Ref.
3a	7.8	2.8	**3b**	7.4	820	(*4c*)
Cys	8.4	7.1[b]				(*7*)
GSH	9.1	16	GSH	9.2	1,600	(*5*)
CoASH	9.8	11	CoASH	9.3	3,200	(*5*)
DHLA	10.7	45	DHLA	10.6	11,000	tw[d]
			2c		14,000	tw[d]

[a] At 25.0°C, unless otherwise specified.
[b] At 29.6°C, phosphate buffers, 5.0% (v/v) ethanol.
[c] At 30.0°C, borate buffers, 30% (v/v) ethanol.
[d] This work.

cases, the often troublesome synthesis and handling of functional surfactant derivatives such as **2c.**

EXPERIMENTAL

D,L-α-Lipoic acid, *p*-nitrophenylacetate and hexanoate, cetyltrimethylammonium bromide, and 2-dimethylaminoethylamine were obtained from commercial sources. Trimethyl(2-aminoethyl)ammonium chloride was prepared as described by Price *et al.* (*15*). Lipoyl succinimide ester **5** was obtained according to Gorecki and Patchornik (*8*), nmr (δ): 1.65 (8H, br m), 2.8 (4H, m), 3.1 (2H, t), 3.5 (1H, m).

Synthesis of Cetyldimethyl(2-lipoylamidoethyl)ammonium Bromide (**1c**)

Lipoyl succinimide ester **5** (1.41 g) and 2-dimethylaminoethylamine (0.42 g) in $CHCl_3$ (30 ml) were allowed to react for 4 hr at room temperature. The solvent was removed under reduced pressure and the residue washed with ether and crystallized from methanol–ether to yield *N-(2-dimethylaminoethyl)lipoylamide* **6** (yield 45%); nmr (δ): 1.53 (8H, m), 2.3 (6H, m), 2.6 (4H, s), 3.06 (2H, t), 3.27 (1H, m). This product, **6,** (0.70 g) and cetylbromide (0.76 g) dissolved in ethanol (20 ml) were allowed to react at 45° for 24 hr. The solvent was then removed under reduced pressure and the residue repeatedly dissolved in methanol and precipitated with ether to yield the moderately hygroscopic waxy *product* **1c** (yield 32%); nmr (δ): 1.25 (20H, m), 2.3 (6H, m), 3.3 (2H, t), 3.58 (1H, m).

Synthesis of Trimethyl(2-lipoylamidoethyl)ammonium Chloride (**1b**)

Lipoyl succinimidoester (0.93 g) dissolved in $CHCl_3$ was added to a mixture of trimethyl(2-aminoethyl)ammonium chloride (0.54 g) and Na_2CO_3 (0.8 g) in $CHCl_3$

(25 ml). Stirring was continued overnight at room temperature. After removal of the salts by filtration and of the solvent under reduced pressure, the residue was repeatedly crystallized from $CHCl_3$/ether to yield **1b** as a hygroscopic waxy solid (yield 82%); nmr (δ): 1.55 (8H, m), 2.28 (2H, m), 3.40 (9H, s), 3.73 (3H, m).

The products described above had correct elemental analyses and nmr spectra were taken for $CDCl_3$ solutions.

Reduction of Lipoyl to Dihydrolipoyl Derivatives

The $NaBH_4$ reduction of lipoyl derivatives was carried out in a dry-box apparatus in a nitrogen atmosphere to obtain stock solutions of dihydrolipoyl derivatives to be used immediately for the kinetic measurements. To an aqueous solution of a given lipoyl derivative at 0 to 5°C, a (5–6 *M*) excess of $NaBH_4$ was added under stirring. After 30 min, the excess $NaBH_4$ was destroyed by adding 1 *N* HCl until the pH of the solution was about 3. The pH was then raised to 8 by the addition of 1 *N* NaOH and the free *SH* titre of the solution determined following the Ellman assay (*9*).

Kinetic Measurements

Buffer and stock solutions were prepared using deaerated bidistilled water flushed with nitrogen and all manipulations were made in a dry-box. A kinetic run was started by injecting a 20-μl aliquot of ester solution in CH_3CN (50 μl in the case of "burst" kinetics) into a cuvette containing 2.00 ml of the proper buffered solution thermostatted at 25.0°. Absorbance changes were monitored using a Varian Cary 219 spectrophotometer.

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