thioredoxin-S_2 have a strongly quenched fluorescence; the active center disulfide has been strongly implicated as the quencher (Holmgren, 1972b). Upon reduction of the disulfide to the dithiol in thioredoxin-$(SH)_2$, a 3-fold increase in fluorescence per tryptophan takes place. The similar tryptophan emission of 1-NBS-thioredoxin-$(SH)_2$ and thioredoxin-$(SH)_2$ (Holmgren, 1973) is consistent with a 6-fold increase in quantum yield for Trp-28. Thus, Trp-28 signals the conformational change in thioredoxin accompanying reduction of the active-center disulfide.

The environments of Trp-28 and Trp-31 are quite different, as seen from the X-ray structure of thioredoxin-S_2, although both are located on the surface of the protein (Holmgren et al., 1975). As shown in Figure 1, Trp-31 is located in the active-site protrusion; its side chain appears free and has no intramolecular interactions. In contrast, Trp-28 is part of a β-pleated sheet (β_2) and is partly shielded. These locations agree with the initial modification of Trp-31 and the apparently small effects on the structure of thioredoxin-S_2 given by modification of Trp-31 to oxindolylalanine. Proton nuclear magnetic resonance spectra of thioredoxin in H_2O led to the identification of two resonance peaks from the indole NH proton of the two tryptophan residues of the molecule (Holmgren & Roberts, 1976). Upon reduction of oxidized thioredoxin, only one of these two peaks showed a major change in chemical shift while both peaks shifted as a function of pH.

The fluorescence quantum yield of *E. coli* thioredoxin-$(SH)_2$ is strongly pH dependent with a maximum at pH 5 (Holmgren, 1972b). The increase follows an apparent titration curve for a group with a pK of ~6.75 (Holmgren, 1972b), suggested to be a cysteinyl residue. The two cysteines in thioredoxin-$(SH)_2$, Cys-32 and Cys-35, have different reactivities toward iodoacetic acid (Kallis & Holmgren, 1980); only Cys-32 is modified at pH 7, and its pK value has been determined to be 6.7 (Kallis & Holmgren, 1980). Thus, an interaction of Cys-32 and Trp-28 is suggested as an explanation of the fluorescence behavior of thioredoxin-$(SH)_2$.

References

Hall, D. E., Baldesten, A., & Reichard, P. (1971) *Eur. J. Biochem. 23*, 328–335.

Holmgren, A. (1968) *Eur. J. Biochem. 6*, 475–484.

Holmgren, A. (1972a) *Eur. J. Biochem. 26*, 528–534.

Holmgren, A. (1972b) *J. Biol. Chem. 247*, 1992–1998.

Holmgren, A. (1973) *J. Biol. Chem. 248*, 4106–4111.

Holmgren, A. (1979a) *J. Biol. Chem. 254*, 9113–9119.

Holmgren, A. (1979b) *J. Biol. Chem. 254*, 9627–9632.

Holmgren, A., & Reichard, P. (1967) *Eur. J. Biochem. 2*, 187–196.

Holmgren, A., & Roberts, G. (1976) *FEBS Lett. 71*, 261–265.

Holmgren, A., Perham, R. N., & Baldesten, A. (1968) *Eur. J. Biochem. 5*, 352–358.

Holmgren, A., Söderberg, B.-O., Eklund, H., & Brändén, C.-I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2305–2309.

Holt, L. A., Milligan, B., & Rivett, D. E. (1971) *Biochemistry 10*, 3559–3564.

Kallis, G.-B., & Holmgren, A. (1980) *J. Biol. Chem.* (in press).

Moore, E. C., Reichard, P., & Thelander, L. (1964) *J. Biol. Chem. 239*, 3445–3452.

Slabý, I., & Holmgren, A. (1975) *J. Biol. Chem. 250*, 1340–1347.

Stryer, L., Holmgren, A., & Reichard, P. (1967) *Biochemistry 6*, 1016–1020.

Thelander, L. (1967) *J. Biol. Chem. 242*, 852–856.

Selective Inactivation of Lactate Dehydrogenase Isoenzymes with Ionic Surfactants†

Karl J. Sanford,* Donna J. Meyer, Michael J. Mathison, and John Figueras

ABSTRACT: Ionic surfactants selectively inactivate porcine lactate dehydrogenase (LDH) isoenzymes in 30 mM phosphate buffer, pH 7.4. The cationic surfactants hexadecylpyridinium bromide and hexadecyltrimethylammonium bromide rapidly inactivate LDH isoenzymes containing the B subunit; inactivation of LDH-A_4 is slower and also retarded by the cofactor reduced nicotinamide adenine dinucleotide. The anionic surfactants sodium decyl sulfate and sodium dodecyl sulfate rapidly inactivate LDH isoenzymes containing the A subunit; inactivation of LDH-B_4 is much slower and retarded by the cofactor. The selectivity of the inactivation process correlates with electrostatic interactions: positively charged surfactants preferentially inactivate isoenzymes containing a subunit of net negative charge, and negatively charged surfactants preferentially inactivate isoenzymes containing a subunit of net positive charge. Inactivation takes place near the critical micelle concentration for the cationic surfactants. Inactivation with anionic surfactants occurs above the critical micelle concentration. The cationic surfactants show little discrimination among LDH-B_4 and the hybrid isoenzymes, AB_3, A_2B_2, and A_3B, inactivating all at approximately the same surfactant concentration. The anionic surfactants, however, show a more graded inactivation–concentration profile with discrete differences in threshold surfactant concentrations required for complete inactivation of the four A subunit containing isoenzymes. At a particular surfactant concentration, loss in activity can be correlated with the percent A- or B-subunit composition of the isoenzyme.

The interaction of surfactants with proteins has been the subject of numerous investigations over the past several decades, and the topic has been reviewed by Steinhardt & Reynolds (1969), Tanford (1973), Waehneldt (1975), and Steinhardt (1975). Much of the interest in this area was generated because protein–surfactant systems can serve as models for the study of interactions between membrane proteins and

† From the Research Laboratories, Eastman Kodak Company, Rochester, New York 14650. *Received May 14, 1980.*

0006-2960/81/0420-3207$01.25/0

lipids. Also, widespread use of surfactants, especially anionic surfactants such as sodium *n*-dodecyl sulfate, for the solubilization of membrane proteins and in polyacrylamide electrophoresis has provided impetus for investigation into the nature of surfactant–protein complexes (Reynolds & Tanford, 1970a,b; Nelson, 1971; Weber & Kuter, 1971). The binding forces involved in the formation of the complexes are not fully understood, but both ionic and hydrophobic interactions have been suggested (Nakaya et al., 1971; Jones et al., 1973; Jones & Wilkinson, 1976; Ashoka et al., 1966).

Nelson (1971) reported that some proteins, i.e., pepsin and glucose oxidase, do not bind sodium dodecyl sulfate ($NaDodSO_4$)[1] or lose enzymatic activity in the presence of high $NaDodSO_4$ concentrations. It was argued in the case of pepsin (isoelectric point <1) that the high negative charge density of the protein prevented binding of the anionic surfactant, whereas for glucose oxidase its tightly knit structure prevented the initiation of chain unfolding which precedes large-scale $NaDodSO_4$ binding. Jones (1977) has shown that $NaDodSO_4$ at low concentrations can bind to discrete ionic sites on trypsin without inducing loss of enzymatic activity, but at higher $NaDodSO_4$ concentrations cooperative binding results in extensive protein chain unfolding, and denaturation takes place. There is evidence that cationic surfactants can have dramatically different effects on protein structure and activity relative to anionic surfactants (Blinkhorn & Jones, 1973; Tanaka et al., 1973; Wojtczak & Nalecz, 1979; Nozaki et al., 1974).

In this initial paper, we report the results of the interaction of several anionic and cationic surfactants with LDH isoenzymes and the subsequent rapid and selective inactivation. The inactivation is correlated with the electrostatic charges on the ionic surfactants and isoenzymes. The purpose of the work reported here was to investigate the relative stabilities of the isoenzymes in the presence of ionic surfactants and the effects of surfactant charge, surfactant concentration, buffer, and ionic strength on the inactivation.

Experimental Section

Materials. Porcine LDH isoenzymes were obtained from Boehringer Mannheim GmbH as ammonium sulfate suspensions and used without further purification. Cellulose acetate electrophoresis showed isoenzyme purity >95% of the total LDH activity present. The enzymes were stored at 4 °C.

Surfactants were Eastman reagent-grade chemicals and were recrystallized twice from ethanol, acetone, or mixtures of these solvents. The recrystallized surfactants were dried under vacuum and stored in amber bottles at ambient conditions.

Reduced nicotinamide adenine dinucleotide (NADH) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) were purchased from P-L Biochemicals, Inc. The NADH was stored desiccated in the dark. All other reagents were reagent-grade Eastman organic chemicals.

Methods. Enzyme activity was measured in the absence and presence of surfactant by the following procedure. Stock solutions of NADH (2 mM) and pyruvate (10 mM) were prepared daily in 30 mM sodium phosphate buffer, pH 7.4. Enzyme solutions were prepared daily by withdrawing an aliquot from the stock enzyme solutions and diluting with phosphate buffer to give solutions containing ~1 unit of enzyme/mL. For enzyme assay, 0.1 mL each of NADH and pyruvate stock solutions was added to 2.7 mL of buffer (or buffer plus surfactant) in a 3.5-mL quartz cuvette (1-mm path length). The cuvette was placed in a thermostated cell holder in a Cary 118 spectrophotometer and allowed to equilibrate to 25 ± 0.2 °C. Enzyme in buffer solution, 0.1 mL, was added with mixing, and the absorbance decrease at 340 nm was measured as a function of time. The rate was determined by visual fit of the best straight line to the initial part of the progress curve, i.e., within 50 s of the start of the reaction.

Enzyme stability was studied by incubating the enzyme in buffered surfactant solution contained in several cuvettes. At various time intervals, substrate and NADH were added to a cuvette, and the enzyme activity was measured. Suitable controls, i.e., without surfactant, were also run to ensure that loss of activity was due to surfactant–enzyme interactions and not due to nonspecific effects.

Surface tension measurements required for critical micelle determinations were made with a DuNouy tensiometer (Osipow, 1962) with the assistance of the Industrial Laboratories at Eastman Kodak Company. Surfactant solutions were prepared at 25 °C in 30 mM sodium phosphate or 30 mM Tes buffer, pH 7.4.

Electrophoresis experiments were done by using a Beckman Microzone electrophoresis system and cellulose acetate membranes. LDH-A_2B_2 was exposed to either CTAB or $NaDecSO_4$ solution and immediately electrophoresed by using Beckman B-2 barbital buffer, pH 8.6 and ionic strength 0.05. Staining for LDH activity was done by the method of Gebott (1977).

Difference spectra were recorded on a Cary 219 spectrophotometer at 25 °C. The spectra were obtained on 1.5 mg/mL solutions of either LDH-A_4 or LDH-B_4 in 30 mM sodium phosphate buffer, pH 7.4. Ionic surfactant was added to the sample cell, and an equivalent amount of buffer was added to the reference cell. Spectra were recorded within 2 min of mixing. Controls were also run in which the time dependency of the spectra was checked. Except for the anionic surfactant concentrations in the transition region, the spectra were stable enough to permit completion of the experiment.

Results

Stability of LDH-A_4 and LDH-B_4 to Cationic Surfactants. Lactate dehydrogenase (LDH; L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) is a tetrameric enzyme of molecular weight ca. 140 000. The subunits of the enzyme exist as two major structural forms, usually referred to as M (muscle) and H (heart) or A and B, respectively. The combination of these two subunits to form the active tetramer can give rise to five different isoenzymes. The differences in the properties of these LDH isoenzymes are dependent on their subunit composition and are most pronounced between the homotetramers A_4 (LDH-5) and B_4 (LDH-1) (Wieland & Pfleiderer, 1957; Markert & Møller, 1959; Kaplan, 1965; Nisselbaum & Bodansky, 1963). The stability of LDH-A_4 and LDH-B_4 in the presence of hexadecyltrimethylammonium bromide (CTAB) and hexadecylpyridinium bromide (CP) is shown in Figure 1a. A rapid loss in activity of LDH-B_4 in the presence of either cationic surfactant is noted. The elapsed time between mixing enzyme with surfactant and beginning the measurement of the resulting enzyme activity is less than 15 s. The LDH-A_4 isoenzyme is considerably more stable to the cationic surfactants, with loss of activity occurring over 30 min or more. The CTAB surfactant shows less effect on LDH-A_4 than the CP surfactant, indicating that the structure of the ionic head

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate (SDS in figures); LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; CTAB, hexadecyltrimethylammonium bromide; CP, hexadecylpyridinium bromide; $NaDecSO_4$, sodium decyl sulfate (DSS in figures).

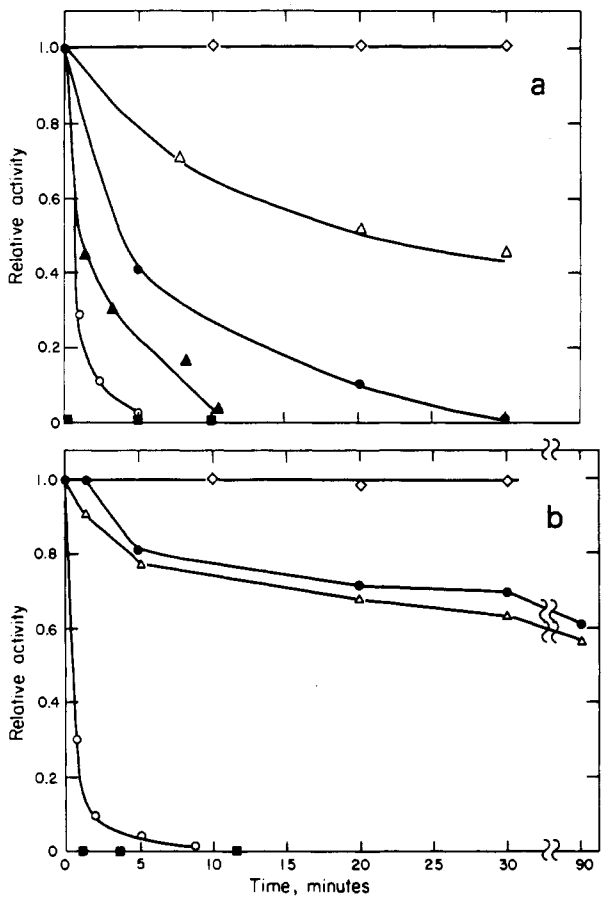

FIGURE 1: Inactivation of porcine LDH-A_4 and -B_4 at 25 °C. (a) LDH-A_4 incubated with CP (○), CTAB (▲), CP + 0.06 mM NADH (●), and CTAB + 0.06 mM NADH (△); LDH-B_4 incubated with CP or CTAB (■) and 20 mM *N*-ethylpyridinium bromide (◊). Surfactant concentrations were 0.25 mM CTAB or 0.23 mM CP in 30 mM sodium phosphate buffer, pH 7.4. (b) LDH-A_4 incubated with 20 mM sodium sulfate (◊) and $NaDecSO_4$ or $NaDodSO_4$ + 0.06 mM NADH (■); LDH-B_4 incubated with $NaDecSO_4$ (○), $NaDecSO_4$ + 0.06 mM NADH (●), and $NaDodSO_4$ + 0.06 mM NADH (△). Surfactant concentrations were 8.3 mM $NaDecSO_4$ or 2.0 mM $NaDodSO_4$ in 30 mM sodium phosphate buffer, pH 7.4.

group is important in the interaction of cationic surfactant with the A_4 isoenzyme. The presence of NADH protects the isoenzyme from surfactant inactivation: in the absence of NADH, complete inactivation is observed within 5 min with CP, whereas complete inactivation of A_4 occurs within about 10 min with CTAB. The presence of 1 mM pyruvate, rather than NADH, does not offer increased stability to surfactant inactivation of A_4 as might be expected from the obligatory binding order of coenzyme followed by substrate to LDH (Novoa & Schwert, 1961; Gutfreund et al., 1968). The analogue of the CP ionic head group, *N*-ethylpyridinium bromide, does not affect the activity of either A_4 or B_4 isoenzyme.

Stability of LDH-A_4 and LDH-B_4 to Anionic Surfactants. Incubation of LDH-A_4 with anionic surfactants sodium decyl sulfate ($NaDecSO_4$) or sodium dodecyl sulfate ($NaDodSO_4$) in the presence of 0.06 mM NADH results in rapid loss in activity. Complete inactivation occurs within 15 s of mixing enzyme with surfactants (see Figure 1b). The isoenzyme B_4 is much more stable to the anionic surfactants, with >50% of the original activity remaining after 90 min of incubation. In the absence of NADH, loss in B_4 activity is more rapid, with complete inactivation within 10 min of incubation. Sodium sulfate, 20 mM, does not noticeably affect the activity of either isoenzyme. Comparison with the results obtained with cationic

Table I: Selective Inactivation of LDH-A_4 and -B_4 by Ionic Surfactants

solution	activity (units/L) LDH-A_4	LDH-B_4
buffer	405	464
CP (0.12 mM)	324	0
CTAB (0.25 mM)	370	0
$NaDodSO_4$ [a] (2 mM)	0	394
$NaDecSO_4$ (8.7 mM)	0	345

[a] Activity measurements were made by visually fitting the best straight line along the progress curve between 0 and 50 s elapsed assay time. Assays were run at 25 °C in 30 mM sodium phosphate buffer, pH 7.4, with 0.06 mM NADH and 0.42 mM sodium pyruvate. The assay using $NaDodSO_4$ was identical with the others, except the buffer was 30 mM Tes, pH 7.4.

surfactants (see Figure 1a) shows several interesting differences: (1) LDH-A_4 is rapidly inactivated by anionic surfactants but not by cationic surfactants in the presence of NADH. (2) LDH-B_4 is rapidly inactivated by cationic surfactants but not by anionic surfactants in the presence of NADH. (3) At pH 7.4, LDH-A_4 has a net positive charge (p*I* 8.5), whereas LDH-B_4 has a net negative charge (p*I* 5.2) (Malamud & Drysdale, 1978). Therefore, the negatively charged surfactants preferentially inactivate the positively charged isoenzyme, whereas the positively charged surfactants preferentially inactivate the negatively charged isoenzyme. It appears that electrostatic forces play a significant role in influencing the selectivity observed in the inactivation process. NADH, which induces considerable conformational changes in the active site area of LDH upon binding (Eventoff et al., 1977), enhances the selectivity of the surfactant inactivation by preferentially stabilizing one of the isoenzymes.

LDH Isoenzyme Assay in the Presence and Absence of Ionic Surfactants. If the kinetic determination of LDH activity is made shortly after mixing enzyme with surfactant, the selectivity of the inactivation increases, owing to the extremely rapid inactivation of the susceptible isoenzyme. The respective isoenzyme was added to buffer–substrate or buffer–substrate–surfactant solution, and the rate of NADH oxidation was determined within 50 s of mixing. The results (Table I) show that cationic surfactants, i.e., CTAB and CP, completely inactivate LDH-B_4 while minimally affecting LDH-A_4 activity. On the other hand, anionic surfactants, i.e., $NaDecSO_4$ and $NaDodSO_4$, completely inactivate LDH-A_4 and only minimally affect LDH-B_4 activity. If the activity determination is not made within 100 s of mixing, significant inactivation of the less susceptible isoenzyme may occur, as indicated in Figure 2a,b. This result agrees with the stability data previously discussed.

Influence of Surfactant Concentration on the Selective Isoenzyme Inactivation. The influence of CP and CTAB concentrations on the inactivation of LDH isoenzymes is shown in Figure 3a,b, respectively. The following observations stand out: (1) the onset of inactivation of isoenzymes containing the B subunit occurs over a very narrow range of surfactant concentrations for both CP and CTAB, suggesting a highly cooperative process. Below this critical cationic surfactant concentration, which is approximately the same for CTAB and CP, isoenzymes containing the B subunit are relatively stable in the presence of cationic surfactant. (2) Isoenzymes containing various B-subunit compositions are inactivated at approximately the same CP concentration, with little discrimination observed among B_4, B_3A, B_2A_2, and BA_3. (3) CTAB, however, shows some selectivity in preferentially inactivating isoenzymes of higher B-subunit composition. These results

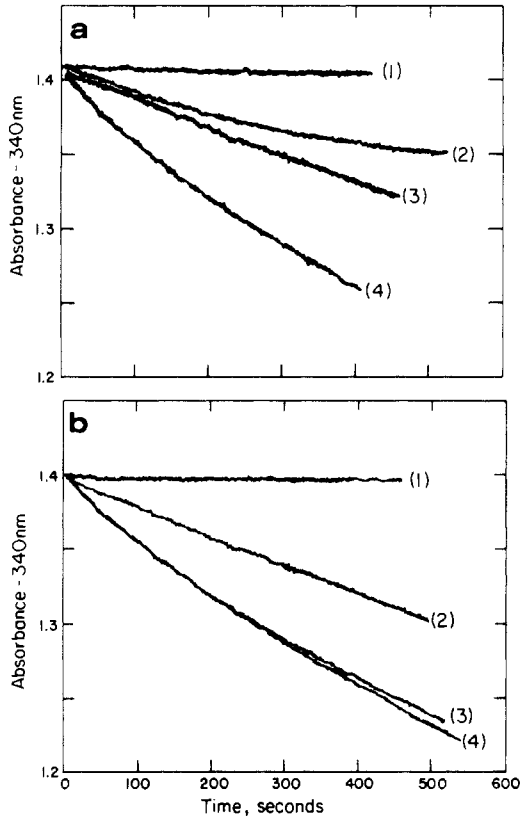

FIGURE 2: Absorbance–time profiles for LDH-A_4 and -B_4 in the presence of 0.3 mM CTAB or 8.6 mM $NaDecSO_4$. The assay was run at 25 °C in 30 mM sodium phosphate buffer, pH 7.4, with 0.23 mM NADH and 2.2 mM sodium pyruvate. (a) LDH-B_4 with CTAB (1), LDH-A_4 with CTAB (2), LDH-A_4 with buffer (3), LDH-B_4 with buffer (4). (b) LDH-A_4 with $NaDecSO_4$ (1), LDH-A_4 with buffer (2), LDH-B_4 with $NaDecSO_4$ (3), LDH-B_4 with buffer (4).

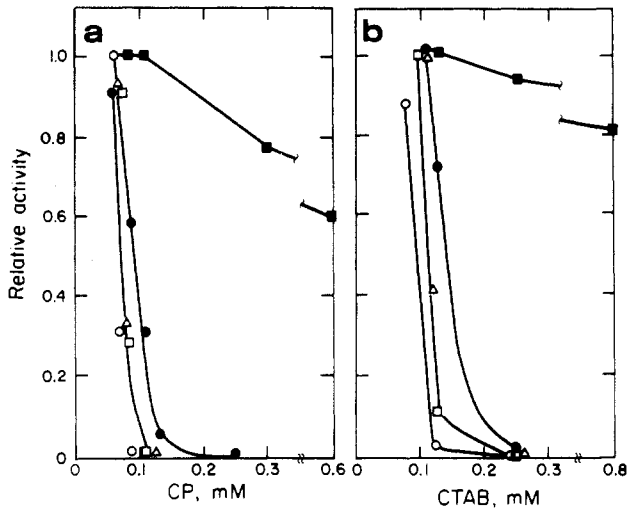

FIGURE 3: Inactivation of porcine LDH isoenzymes at 25 °C by different surfactant concentrations in 30 mM sodium phosphate buffer, pH 7.4, with 0.06 mM NADH and 0.42 mM sodium pyruvate. Activities were measured within 50 s of mixing enzyme with surfactant solution and are relative to the isoenzyme activity in the absence of surfactant: (○) B_4; (△) AB_3; (□) A_2B_2; (●) A_3B; (■) A_4. (a) Inactivation with CP; (b) inactivation with CTAB.

suggest that CTAB preferentially binds to and inactivates the B subunit of the LDH isoenzymes. (4) For isoenzymes containing more B subunits, a lower CTAB concentration is required to completely inactivate the isoenzyme. This finding suggests that surfactant binding to B subunits can affect the kinetic behavior of neighboring A subunits and perhaps sensitize it to surfactant inactivation.

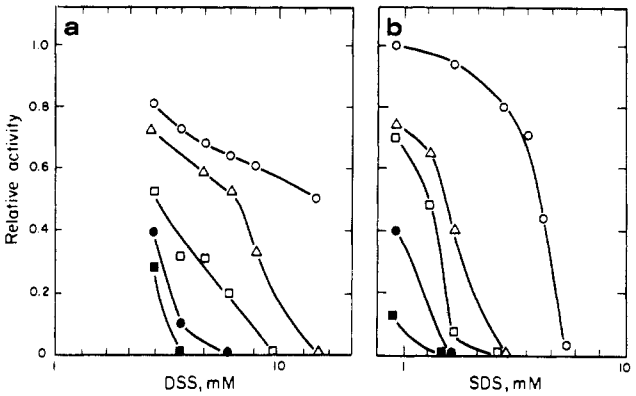

FIGURE 4: Inactivation of porcine LDH isoenzymes by different surfactant concentrations of $NaDecSO_4$ or $NaDodSO_4$. With $NaDecSO_4$, 30 mM sodium phosphate buffer, pH 7.4, was used, whereas with $NaDodSO_4$, 30 mM Tes buffer, pH 7.4, was used. Concentration of NADH was 0.07 mM, and sodium pyruvate concentration was 0.42 mM. Assays were run at 25 °C, and rates were measured within 50 s of mixing surfactant and enzyme. The rates are relative to isoenzyme activity in the absence of surfactant: (○) B_4; (△) AB_3; (□) A_2B_2; (●) A_3B; (■) A_4. (a) Inactivation with $NaDecSO_4$; (b) inactivation with $NaDodSO_4$.

Inactivation of LDH isoenzymes by various anionic surfactant concentrations of $NaDecSO_4$ and $NaDodSO_4$ is illustrated in Figure 4a,b, respectively. Several observations can be made: (1) Both anionic surfactants preferentially inactivate the A subunit. For isoenzymes containing more A subunits, a lower surfactant concentration is required to completely inactivate the isoenzyme. This result is similar to that observed for CTAB, but greater discrimination among the isoenzymes is observed with the anionic surfactants. (2) At a given surfactant concentration, i.e., 6 mM $NaDecSO_4$ or 2 mM $NaDodSO_4$, the percent inactivation of a given isoenzyme is approximately inversely proportional to the percentage of A subunits comprising the isoenzyme. (3) Inactivation occurs at lower surfactant concentrations of $NaDodSO_4$ than $NaDecSO_4$. Since their ionic head groups are identical, the difference must be attributable to the length of the aliphatic side chain of the surfactants. (4) The inactivation of a given isoenzyme occurs over a narrow range of anionic surfactant concentrations, indicating a cooperative process. A similar phenomenon was reported for the inactivation of trypsin by $NaDodSO_4$ (Jones, 1977).

Irreversible Inactivation of LDH by Ionic Surfactants. The inactivation by ionic surfactants appears irreversible. This conclusion is reached from the following experiment. LDH-B_4 (6000 units/L) was mixed at 22 °C with CTAB (final concentration 0.12 mM) in the presence of 0.06 mM NADH in 30 mM phosphate buffer, pH 7.4. Under these conditions, B_4 activity is rapidly lost. After 30 s, an aliquot of this solution was diluted 60-fold with a solution of NADH and pyruvate in phosphate buffer and assayed for LDH activity. This dilution reduces the CTAB concentration to a point where B_4 activity is not affected (see Figure 3b). There is no reappearance of LDH activity during several hours of incubation, at 25 °C, of the diluted LDH solution. Dialysis against a large volume of phosphate buffer also failed to restore activity. A similar experiment with anionic surfactant, $NaDecSO_4$ (8.3 mM), and LDH-A_4 gave similar results. The reversibility of inactivation of several enzymes by $NaDodSO_4$ has been reported (Weber & Kuter, 1971; Liao, 1975), but these studies did not include LDH. Also, these enzymes were reversibly denatured by the use of denaturants such as urea or guanidine hydrochloride followed by dilution or anion-exchange chromatography. These techniques were not used in attempting

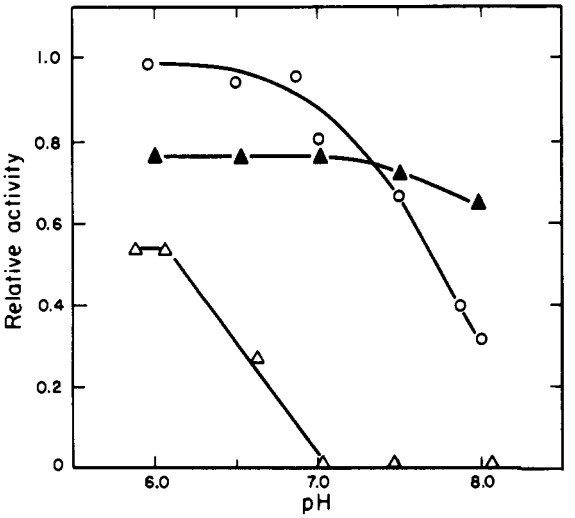

FIGURE 5: Effect of pH on surfactant inactivation of LDH-A_4 (○) and LDH-B_4 (△, ▲). Enzymes were incubated at 25 °C in 30 mM sodium phosphate buffer containing 0.06 mM NADH and surfactant at pH 7.4. Aliquots were taken after 1-min incubation and assayed at 25 °C, pH 7.4, in 30 mM phosphate buffer containing 0.06 mM NADH and 0.42 mM sodium pyruvate. Open symbols denote 0.12 mM CP was present in the incubation solution. Solid symbols denote 8.6 mM $NaDecSO_4$ was present in the incubation solution.

Table II: Critical Micelle Concentrations (cmc)[a] for Ionic Surfactants

surfactant	cmc (mM)
CP	0.1
CTAB	0.1
$NaDecSO_4$	1.5
$NaDodSO_4$	0.9

[a] Determined at 25 °C in 30 mM sodium phosphate buffer, pH 7.4, by using a DuNouy tensiometer.

to reactivate LDH after exposure to cationic or anionic surfactants. Thus, irreversibility of LDH inactivation by ionic surfactants is restricted to a limited set of experimental conditions.

Effect of pH on Surfactant Inactivation. The selective inactivation of LDH isoenzymes by ionic surfactants appears to involve electrostatic interactions. That is, positively charged surfactants selectively inactivate the more negatively charged isoenzyme and vice versa for anionic surfactants. The importance of net charge on the enzyme surface is further illustrated by the effect of pH on the inactivation of LDH-A_4 and -B_4 by CP. The results (Figure 5) show that as pH is decreased the ability of the cationic surfactant to inactivate LDH isoenzymes also decreases. Below pH 7.0, complete surfactant inactivation of B_4 is no longer observed, and A_4 is also less susceptible to inactivation by cationic surfactant. In fact, the stability of LDH-A_4 to inactivation by cationic surfactant at pH 5.5, in the presence of 0.06 mM NADH, is markedly increased relative to that at pH 7.4 (see Figure 1a), and no loss in enzyme activity is observed with up to 1 h of incubation at 25 °C. These observations suggest that the increase in net positive charge on the enzyme surface at lower pH reduces the interaction with the positively charged surfactant and retards or prevents enzyme inactivation.

A similar pH study with 8.6 mM $NaDecSO_4$ shows different results (Figure 5). The A_4 isoenzyme is completely inactivated at pH 5.5–8.5, but the B_4 isoenzyme is not noticeably affected.

Critical Micelle Concentration and Effect on Enzyme Inactivation. Table II gives the critical micelle concentration (cmc) for the ionic surfactants, as determined by surface tension measurements at 25 °C in 30 mM sodium phosphate buffer, pH 7.4. The cationic surfactant cmc values and the surfactant concentrations at which selective inactivation of LDH-B_4 begins (ca. 0.1–0.2 mM, see Figure 3a,b) are almost identical. This observation suggests that inactivation of LDH-B_4 by cationic surfactant may involve micelles.

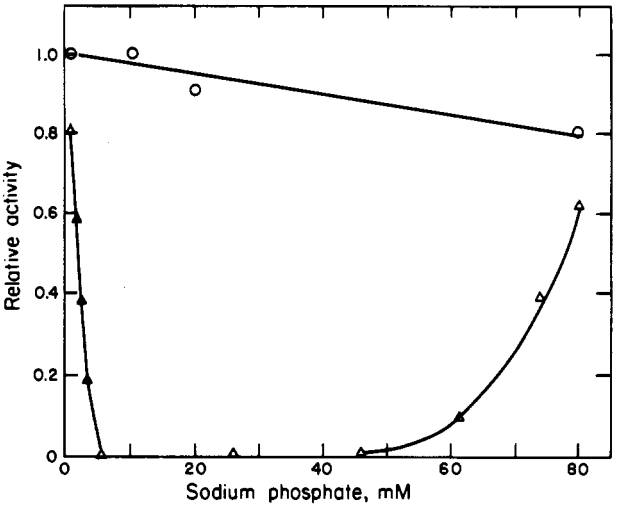

FIGURE 6: Effect of ionic strength on the inactivation of LDH-A_4 (○) and LDH-B_4 (△). Activities were measured at 25 °C in 30 mM phosphate buffer, pH 7.4, with 0.07 mM NADH and 0.42 mM sodium pyruvate. Rates were measured within 50 s of mixing enzyme and surfactant solutions (0.12 mM CP).

The anionic surfactants initiate selective inactivation of LDH-A_4 at surfactant concentrations above the cmc value (see Figure 4a,b), suggesting that inactivation of the A_4 subunit may involve micelles.

Effect of Ionic Strength on Surfactant Inactivation of LDH. From the CP concentration–inactivation profiles depicted in Figure 3a, a 0.12 mM CP solution is near the minimum surfactant concentration (cmc) which will completely inactivate LDH-B_4. Altering the ionic strength of the surfactant solution has a dramatic effect on B_4 inactivation. Below 2 mM and above 60 mM sodium phosphate, 0.12 mM CP does not inactivate the LDH-B_4 isoenzyme (Figure 6). A similar result is obtained by using sodium chloride to change ionic strength; thus, phosphate is not unique. If the surfactant concentration is increased, complete inactivation is again achieved even at the higher ionic strength. However, at 0.15 M phosphate, even a 15 mM CP solution failed to inactivate LDH-B_4. Similar results are obtained for CTAB.

Increases in the ionic strength of an ionic surfactant solution can decrease the cmc (Mukerjee & Mysels, 1971). This effect could be responsible for the increased inactivation of the B_4 isoenzyme as the phosphate concentration increases from 2 to 30 mM. Makino & Niki (1977) reported a similar observation for $NaDodSO_4$ binding to K-casein. The failure to achieve inactivation at phosphate concentrations above 60 mM, at 0.12 mM surfactant, is possibly the result of decreased electrostatic interaction of surfactant or micelles and enzyme at higher ionic strength. With increased surfactant concentration, alteration in size, shape, and concentration of micelle or increased surfactant monomer concentration could overcome this ionic strength limitation.

The anionic surfactants do not appear to show this sensitivity to ionic strength. For example, $NaDecSO_4$ (8.6 mM) still effectively inactivates LDH-A_4 in 0.15 M phosphate buffer.

Effect of Nonionic Surfactants on LDH-A_4 and LDH-B_4. Both Tween-20 surfactant (2% w/v) and Triton X-100 surfactant (2% w/v) in 30 mM phosphate buffer, pH 7.4, failed to selectively inactivate the isoenzymes. During 10 min of

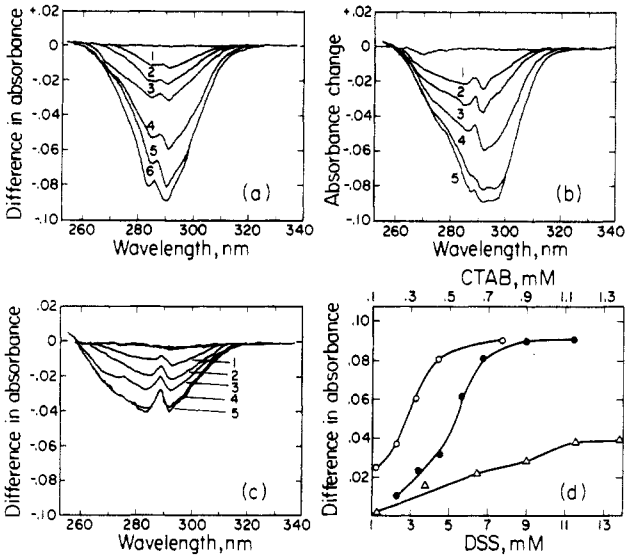

FIGURE 7: Effect of CTAB and $NaDecSO_4$ on the difference spectra of LDH-A_4 and LDH-B_4. Spectra were recorded on 1.5 mg/mL enzyme in 30 mM sodium phosphate buffer, pH 7.4, at 25 °C. (a) LDH-B_4 (1.5 mg/mL) with $NaDecSO_4$ concentrations of (1) 2.3, (2) 3.4, (3) 4.5, (4) 5.6, (5) 6.7, and (6) 8.8 mM. (b) LDH-A_4 (1.5 mg/mL) with $NaDecSO_4$ concentrations of (1) 1.1, (2) 2.3, (3) 3.4, (4) 4.5, and (5) 7.8 mM. (c) LDH-A_4 (1.5 mg/mL) with CTAB concentrations of (1) 0.38, (2) 0.64, (3) 0.90, (4) 1.1, and (5) 1.4 mM. (d) Plot of absorbance change against surfactant concentrations: (○) LDH-A_4 vs. $NaDecSO_4$; (●) LDH-B_4 vs. $NaDecSO_4$; (△) LDH-A_4 vs. CTAB.

incubation, less than 10% LDH-A_4 activity and less than 5% LDH-B_4 activity were lost. The slightly increased sensitivity of A_4 to the nonionic surfactants is consistent with previous studies showing increased sensitivity of LDH-A_4 to a number of protein denaturants such as heat, urea, and guanidine hydrochloride (Wroblewski & Gregory, 1961; Emerson & Wilkinson, 1965; Wilkinson, 1970). The effects of the nonionic surfactants on LDH activity are negligible compared to those of the ionic surfactants.

Effect of Ionic Surfactants on the Difference Spectra of LDH-A_4 and LDH-B_4. Porcine LDH-A_4 and LDH-B_4 contain the same numbers of tyrosine (28) and tryptophan (24) groups (Eventoff et al., 1977). The influence of $NaDecSO_4$ on the difference spectra of both isoenzymes is shown in Figure 7a,b for LDH-B_4 and LDH-A_4, respectively. As the anionic surfactant concentration is increased, both isoenzymes show similar perturbations in the tryptophan and tyrosine regions of the difference spectra. However, the positively charged LDH-A_4 undergoes the transition at lower surfactant concentrations than the negatively charged LDH-B_4. This effect is shown in Figure 7d. In addition, the $NaDecSO_4$ concentrations at which the perturbations occur are similar to the surfactant concentrations which affect enzyme activity (see Figure 4a). The magnitude of the spectral change was similar for both isoenzymes and reached an end point where further increases in $NaDecSO_4$ concentration caused no further spectral changes. The spectral effects for both isoenzymes at low and high $NaDecSO_4$ concentrations were produced rapidly (<1 min) for both isoenzymes and were stable, i.e., no change over 10 min. However, for $NaDecSO_4$ concentrations in the steep transition portion of the curve, there was a slow time-dependent change that approached the end-point value over the course of a 20-min incubation. The curves shown in Figure 7 were recorded within 2 min after mixing.

The effect of the cationic surfactant CTAB on both isoenzymes is also interesting. The addition of CTAB in even low (0.05 mM) concentrations to LDH-B_4 resulted in a turbid solution that prevented the determination of the difference spectrum. This effect could be prevented by increasing the ionic strength to 0.5 M sodium chloride, which suggests that electrostatic interactions are important in the aggregation process. No such effects were observed with LDH-A_4; the spectrum is shown in Figure 7c. As in the interaction with anionic surfactant, a transition and an end point are observed (Figure 7d). The transition is not as sharp as with anionic surfactant, and the magnitude of the change is about half that with $NaDecSO_4$.

Discussion

Earlier work (Blinkhorn & Jones, 1973; Jones et al., 1973; Dessert, 1949; Saraswat, 1953; McLoughlin et al., 1978) has shown that anionic and cationic surfactants can have different effects on enzyme activity. For example, ribonuclease A is inactivated by $NaDodSO_4$ but not by CTAB or CP (Blinkhorn & Jones, 1973). DiSabato & Kaplan (1964) showed that LDH-B_4, isolated from chicken or beef heart, was inactivated by $NaDodSO_4$, but the enzymes could be stabilized to surfactant inactivation by the presence of cofactor or cofactor analogues. Surfactant inactivation was correlated with possible dissociation of the native tetrameric structure of LDH into subunits, and binding of cofactor stabilized the oligomer, thwarting inactivation. No work was reported on the comparative sensitivity of LDH-A_4 to $NaDodSO_4$.

Lehnert & Berlet (1979) have shown that the anionic bile salt sodium deoxycholate preferentially inactivates the A subunit of LDH isolated from rat tissues. No work was reported on the effect of cationic surfactants on LDH-B_4.

Our work has shown that the nonionic surfactants Tween-20 and Triton X-100, which associate primarily hydrophobically with hydrophobic domains of proteins (Helenius & Simons, 1972), are not effective as inactivation agents of LDH isoenzymes, suggesting that hydrophobic interactions alone may not be sufficient to explain LDH inactivation by ionic surfactants. That the ionic head groups, *N*-ethylpyridinium bromide and sodium sulfate, do not significantly alter LDH activity suggests that combined ionic and hydrophobic interactions are responsible for the observed inactivation. The ionic surfactants selectively inhibit LDH isoenzymes according to charge type. That is, LDH-A_4, which has a net positive charge at pH 7.4, is rapidly inactivated by anionic surfactants, whereas it is only minimally inactivated by cationic surfactants. On the other hand, LDH-B_4, which has a net negative charge at pH 7.4, is rapidly inactivated by cationic surfactants, but only slightly by anionic surfactants. The electrostatic forces impart selectivity to the surfactant-mediated inactivation. This selectivity can be increased by adding cofactor to the enzyme, which stabilizes the isoenzyme and minimizes inactivation by surfactant of like charge; the cofactor does not noticeably improve the stability of the isoenzyme exposed to surfactant of opposite charge.

The inactivation of LDH isoenzymes by both anionic and cationic surfactants occurs at or above the critical micelle concentration. Previous work (Jones & Wilkinson, 1976; Makino & Niki, 1977; Satake & Yang, 1976) has shown that, for a number of proteins, cooperative binding of anionic surfactants takes place at or above the cmc of the surfactants. Hill & Briggs (1956) showed that binding of *n*-octylbenzene-*p*-sulfonate anions to β-lactoglobulin occurs in three stages: a first stage in which two surfactant anions are bound, a second stage where ~22 anions are bound cooperatively, and a third micellar-like binding stage. The mechanism of surfactant denaturation of protein involves the binding of surfactant ions to sites on the protein molecule, which results in

unfolding and further binding, often cooperatively (Jones & Wilkinson, 1976; Nelson, 1971; Jones et al., 1973; Stauffer & Treptow, 1973; Steinhardt et al., 1977). A similar mechanism may be involved in the inactivation of LDH isoenzymes by ionic surfactants. Maley & Guarino (1977) showed that $NaDodSO_4$ can form micelles or aggregates with amino acids, and the size of the complex varies with the nature of the amino acid. Thus, the selectivity of the LDH interactions with ionic surfactant could be influenced by the variation in amino acid composition of the A and B subunits of the LDH isoenzymes (Eventoff et al., 1977).

The complete loss of LDH-B activity brought about by cationic surfactants occurs over a very narrow range of cationic surfactant concentrations, which coincides with the cmc values of the surfactants. Little discrimination is observed among the homotetramer, B_4, and the three hybrid isoenzymes. Thus, the presence of a B subunit in the isoenzyme is sufficient to cause complete loss of activity at similar cationic surfactant concentrations. This result is in contrast to what is observed for the anionic surfactants, where a more graded loss in LDH-A subunit activity is observed. At 6 mM $NaDecSO_4$, the following relative activities are noted (Figure 5a): A_4 (0%), A_3B (15%), A_2B_2 (35%), AB_3 (65%), and B_4 (75%). For 2 mM $NaDodSO_4$, the following relative activities are noted: A_4 (0%), A_3B (15%), A_2B_2 (50%), AB_3 (65%), and B_4 (100%). Thus, loss of enzymatic activity correlates reasonably well with the A-subunit composition of the isoenzyme, and there are relatively large anionic surfactant concentration changes required for complete inactivation of the A_4 and hybrid enzymes. The greater susceptibility of LDH isoenzymes to complete inactivation by cationic surfactants may be due to the increased instability of the A subunit to cationic surfactants relative to the stability of the B subunit to anionic surfactants (compare Figure 1a,b). Hence, binding of positively charged surfactants to a hybrid isoenzyme induces more rapid activity loss in neighboring A subunits compared to the analogous situation with anionic surfactants.

At first glance, the greater sensitivity of LDH to cationic surfactants is at variance with the work of Nozaki et al. (1974), who showed that the cationic surfactant tetradecyltrimethylammonium chloride had fewer binding sites with lower affinities to bovine serum albumin than anionic surfactants of similar alkyl chain length. Moreover, the cationic surfactant was less effective in producing conformational changes in the protein structure. From the difference spectra shown in Figure 7a–d, it is seen that CTAB induces less spectral change in LDH-A_4 than $NaDecSO_4$, which is consistent with the results of Nozaki et al. (1974). Thus, the inactivation of LDH isoenzymes by cationic surfactant requires only subtle conformational changes in the enzyme.

The residual activity of the hybrid isoenzymes argues against the dissociation of the native tetrameric structure of the enzyme (Markert & Massaro, 1968; Chan & Mosbach, 1976; Chan & Shanks, 1977) by surfactant and indicates that conformational and/or partial chain unfolding of the subunits is responsible for the inactivation.

A possible explanation of the residual activity is dissociation of the hybrid forms and reassociation to form LDH-A_4 or LDH-B_4. However, electrophoresis of LDH-A_2B_2 exposed to CTAB (0.12 mM) or $NaDecSO_4$ (6 mM), which inactivates about 50% of the initial activity, resulted in only one band of enzyme activity having the same mobility as LDH-A_2B_2 not exposed to surfactant. If the homotetramers were produced, an additional band of activity should have been observed.

The anionic surfactants $NaDecSO_4$ and $NaDodSO_4$ differ in the length of the aliphatic chain by only two methylene groups. However, studies have shown (Tanford, 1973) that $NaDodSO_4$ binding to bovine serum albumin causes drastic conformational changes, whereas $NaDecSO_4$ binding does not. $NaDecSO_4$ and $NaDodSO_4$ are similar in their inactivation of LDH isoenzymes (Figure 1b); the only apparent difference is that $NaDodSO_4$ initiates inactivation at a lower surfactant concentration than does $NaDecSO_4$ (Figure 4a,b). Thus, the results suggest that inactivation of LDH by anionic surfactants may not require extensive conformational changes in the enzyme structure.

The cationic surfactants differ from one another only in the ionic head group. Yet, CP inactivates LDH-A_4 to a greater extent than CTAB, indicating that the structure of the ionic head group can induce changes in the interaction and inactivation of the isoenzymes. Thus, both chain length and structure of the ionic head group can influence the interaction of surfactants with the LDH isoenzymes.

The preferential inactivation of LDH-B_4 relative to LDH-A_4 by cationic surfactants is a unique observation. Denaturants such as heat, urea, and guanidine hydrochloride (Wroblewski & Gregory, 1961; Emerson & Wilkinson, 1965; Wilkinson, 1970) preferentially inactivate the LDH-A_4, suggesting that the A subunit is less stable than the B subunit. This finding has been used in developing isoenzyme assays for clinical use. Use of ionic surfactants to this end is under investigation.

References

Ashoka, R., Reynolds, J. A., Polet, H., & Steinhardt, J. (1966) *Biochemistry 5*, 2606–2616.

Blinkhorn, C., & Jones, M. N. (1973) *Biochem. J. 135*, 547–549.

Chan, W. C., & Mosbach, K. (1976) *Biochemistry 15*, 4215–4222.

Chan, W. C., & Shanks, K. E. (1977) *J. Biol. Chem. 252*, 6163–6168.

Dessert, A. M. (1949) *Ann. N.Y. Acad. Sci. 52*, 167.

DiSabato, G., & Kaplan, N. O. (1964) *J. Biol. Chem. 239*, 438–443.

Emerson, P. M., & Wilkinson, J. H. (1965) *J. Clin. Pathol. 18*, 803.

Eventoff, W., Rossmann, M. G., Taylor, S. S., Toriff, H. J., Meyer, H., Keil, W., & Kiltz, H. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2677–2681.

Gebott, M. D. (1977) *Beckman Microzone Electrophoresis Manual*, Beckman Instruments, Inc., Fullerton, CA.

Gutfreund, H., Cantwell, R., McMurray, C. H., Criddle, R. S., & Hathaway, G. (1968) *Biochem. J. 106*, 683.

Helenius, A., & Simons, K. (1972) *J. Biol. Chem. 247*, 3656–3661.

Hill, R. M., & Briggs, D. R. (1956) *J. Am. Chem. Soc. 78*, 1590–1597.

Jones, M. N. (1977) *Biochim. Biophys. Acta 491*, 121–128.

Jones, M. N., & Wilkinson, A. (1976) *Biochem. J. 153*, 713–718.

Jones, M. N., Skinner, H. A., Tripping, E., & Wilkinson, A. (1973) *Biochem. J. 135*, 231–236.

Kaplan, N. O. (1965) in *Evolving Genes and Proteins* (Bryson, V., & Vogel, H. J., Eds.) pp 243–277, Academic Press, New York.

Lehnert, T., & Berlet, H. (1979) *Biochem. J. 177*, 813–818.

Lia, T. H. (1975) *J. Biol. Chem. 250*, 3831–3836.

Makino, S., & Niki, R. (1977) *Biochim. Biophys. Acta 495*, 99–109.

Malamud, D., & Drysdale, J. W. (1978) *Anal. Biochem. 86*, 620–647.

Maley, F., & Guarino, D. V. (1977) *Biochem. Biophys. Res. Commun. 77*, 1425-1430.
Markert, C., & Møller, F. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 753-763.
Markert, C. L., & Massaro, E. J. (1968) *Science (Washington, D.C.) 162*, 695.
McLoughlin, D. J., Shahied, I. I., & MacQuarrie, R. (1978) *Arch. Biochem. Biophys. 188*, 78-82.
Mukerjee, P., & Mysels, K. J. (1971) *Critical Micelle Concentrations of Aqueous Surfactant Systems*, NSRDS-NBS36 Superintendant of Documents, U.S. Government Printing Office, Washington, DC.
Nakaya, K., Yamada, K., Onozawa, Y., & Nakamura, Y. (1971) *Biochim. Biophys. Acta 251*, 7-13.
Nelson, C. A. A. (1971) *J. Biol. Chem. 246*, 3895-3901.
Nisselbaum, J. S., & Bodansky, O. (1963) *J. Biol. Chem. 238*, 969-974.
Novoa, W. B., & Schwert, G. W. (1961) *J. Biol. Chem. 236*, 2150-2153.
Nozaki, Y., Reynolds, J. A., & Tanford, C. (1974) *J. Biol. Chem. 249*, 4452-4459.
Osipow, L. I. (1962) *Surface Chemistry*, p 18, Reinhold, New York.
Reynolds, J. A., & Tanford, C. (1970a) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002-1007.
Reynolds, J. A., & Tanford, C. (1970b) *J. Biol. Chem. 245*, 5161-5165.
Saraswat, H. C. (1953) *Proc.—Indian Acad. Sci., Sect. A 38A*, 220.
Satake, I., & Yang, J. T. (1976) *Biopolymers 15*, 2263-2275.
Stauffer, C. E., & Treptow, R. S. (1973) *Biochim. Biophys. Acta 295*, 457-466.
Steinhardt, J. (1975) *Protein-Ligand Interact., Proc. Symp.*, 412-426.
Steinhardt, J., & Reynolds, J. A. (1969) *Multiple Equilibria in Proteins*, Academic Press, New York.
Steinhardt, J., Scott, J. R., & Birdi, K. S. (1977) *Biochemistry 16*, 718-725.
Tanaka, H., Horiachi, Y., & Konishi, K. (1973) *Anal. Biochem. 66*, 489-491.
Tanford, C. (1973) *The Hydrophobic Effect*, Wiley, New York.
Waehneldt, T. V. (1975) *BioSystems 6*, 176.
Weber, K., & Kuter, D. J. (1971) *J. Biol. Chem. 246*, 4505-4509.
Wieland, T. H., & Pfleiderer, G. (1957) *Biochem. Z. 329*, 112-116.
Wilkinson, J. H. (1970) *Isoenzymes*, 2nd ed., Lippincott, Philadelphia.
Wojtczak, L., & Nalecz, M. H. (1979) *Eur. J. Biochem. 94*, 99-107.
Wroblewski, F., & Gregory, K. F. (1961) *Ann. N.Y. Acad. Sci. 94*, 912.

Inactivation of RTEM β-Lactamase from *Escherichia coli* by Clavulanic Acid and 9-Deoxyclavulanic Acid†

Robert L. Charnas and Jeremy R. Knowles*

ABSTRACT: The interaction of the TEM-2 β-lactamase with 9-deoxyclavulanic acid (**3**) and with both extensively labeled (**2**) and specifically labeled (**1**) clavulanic acid has been studied. The close similarity between 9-deoxyclavulanate and clavulanate in kinetic, spectroscopic, and protein chemical terms shows that the allyl alcohol group of clavulanate is irrelevant to its action as a β-lactamase inactivator. Use of the radiolabeled samples of clavulanate shows that, of three irreversibly inactivated forms of the enzyme, two contain the whole clavulanate skeleton and the third only retains the carbon atoms of the original β-lactam ring. These findings allow the complex interaction between clavulanic acid and the β-lactamase to be defined more narrowly in chemical terms.

β-Lactam antibiotics comprise one of the major classes of antibacterial agents, exerting their lethal effects by interfering with the metabolic machinery responsible for the normal growth and development of the bacterial cell wall (Blumberg & Strominger, 1974; Tomasz, 1979). Bacterial resistance to these lethal effects poses an obvious clinical problem, and this resistance has a number of different origins. The most prevalent cause of resistance derives from the production by the bacterium of a β-lactamase, which catalyzes the hydrolysis of the β-lactam in penicillins and cephalosporins to yield the innocuous penicilloic or cephalosporoic acids. Although the β-lactamase presents a ready target for the design of a drug that would overcome the defense mechanism of the bacterium, the problem was originally attacked by the development of β-lactam antibiotics that were less susceptible to the action of the β-lactamase. This situation changed with the discovery of clavulanic acid (Brown et al., 1976), a potent inhibitor and inactivator of β-lactamases. The announcement of clavulanic acid in 1976 heralded the discovery and synthesis of a number of other β-lactamase inactivators (English et al., 1978; Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979; Cartwright & Coulson, 1979; Gordon et al., 1980; Fisher & Knowles, 1980), as well as the more intense scrutiny of the mechanism of action of the enzyme. These studies have not only clarified the normal pathway of β-lactam hydrolysis by the enzyme but have also elucidated some of the details of the reactions that lead to enzyme inactivation.

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received November 14, 1980.* This work was supported by the National Institutes of Health and the National Science Foundation. R.L.C. was a National Science Foundation Predoctoral Fellow.

0006-2960/81/0420-3214$01.25/0