Selective Inactivation of Lactate Dehydrogenase of Rat Tissues by Sodium Deoxycholate*

By THOMAS LEHNERT and HANS H. BERLET†

*Institute of Pathochemistry and General Neurochemistry, University of Heidelberg,
D-6900 Heidelberg, German Federal Republic*

(Received 7 August 1978)

Soluble lactate dehydrogenase (EC 1.1.1.27) extracted from brain, skeletal and cardiac muscle and liver of rats, and purified isoenzymes LDH-1 and LDH-5, were incubated with sodium deoxycholate. Deoxycholate almost totally inactivated isoenzyme LDH-5 (A_4), whereas it left isoenzyme LDH-1 (B_4) unaffected. Tissue lactate dehydrogenase was inactivated to different degrees depending on the origin of the enzyme. Electrophoretic isoenzyme studies of tissue lactate dehydrogenase showed the loss of activity to be quantitatively related to the overall percentage of subunit A distributed among the homotetramer LDH-5 and the heterotetramers LDH-2, LDH-3 and LDH-4. It was concluded that subunit A of lactate dehydrogenase interacts selectively with deoxycholate, irrespective of its association with subunit B. Distinct changes in electrophoretic mobilities of deoxycholate-treated isoenzymes strongly indicated an indiscriminate binding of deoxycholate by all LDH isoenzymes, probably through hydrophobic interactions. The results suggest that the inactivation of the enzyme is non-competitive, but the basis of the selectivity of deoxycholate towards subunit A is not known at present.

Anionic bile salts do not usually denature proteins, unlike strong ionic detergents such as sodium dodecyl sulphate and others. Because of their 'mild' action, preserving the functional and structural integrity of proteins, they are now widely used to solubilize both hydrophilic and hydrophobic membrane-bound proteins (Tzagoloff & Penefsky, 1971; Helenius & Simons, 1975). Neutral detergents may serve the same purpose, and Triton X-100 was used to release membrane-bound creatine kinase (Berlet *et al.*, 1977) and lactate dehydrogenase (EC 1.1.1.27) (Berlet & Lehnert, 1978) from rat brain particles without concomitant loss of enzyme activity. Subsequent comparative studies with sodium deoxycholate resulted, however, in a considerable loss of soluble lactate dehydrogenase activity at higher concentrations of deoxycholate, whereas low concentrations of the detergent solubilized the membrane-bound enzyme intact (T. Lehnert, unpublished work).

Because the loss of lactate dehydrogenase activity of brain extracts was only partial, it was considered that deoxycholate might interact with individual isoenzymes in a selective way, all five molecular forms of the enzyme being present in brain (Wieland *et al.*, 1959). To verify this assumption advantage was taken of the fact that other rat tissues exhibit different iso-

enzyme patterns (cf. Wilkinson, 1970), so that a specific interaction of deoxycholate with a particular isoenzyme might be recognized easily. Soluble lactate dehydrogenase of several rat tissues was therefore treated with deoxycholate and the degree of inhibition was related to results of electrophoretic isoenzyme studies.

Materials and Methods

Chemicals

All chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany. NAD^+ and NADH were supplied by Boehringer, Mannheim, Germany, as were preparations of purified isoenzymes LDH-1 and LDH-5. On electrophoresis the isoenzyme preparations proved to be homogeneous when stained for the enzyme activity. Triton X-100 was purchased from Merck, and sodium deoxycholate from Serva, Heidelberg, Germany. Materials for electrophoresis were also supplied by Serva.

Analytical

Assay of lactate dehydrogenase. Enzyme activity was determined spectrophotometrically at 25°C and 366 nm (Bergmeyer & Bernt, 1970). The final assay mixture contained 0.62 mM-pyruvate and 0.20 mM-NADH in 0.05 M-phosphate buffer, pH 7.5. Enzyme units are μmol of substrate transformed/min.

* This paper is dedicated to Professor Volker Klingmüller on his 70th birthday. To whom reprint
† To whom reprint requests should be addressed.

Protein determination. The protein concentration of tissue extracts was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Electrophoresis. Separation of LDH isoenzymes was performed by standard methods. Briefly, 1% agar-gel slides were run at a constant 200 V and 8°C, in 43 mM-sodium acetate/43 mM-sodium diethylbarbital buffer, pH 8.2 ($I = 0.1$). Slides were stained as described elsewhere (Berlet & Lehnert, 1978) with NAD⁺, lactate, phenazine methosulphate and Nitro Blue Tetrazolium chloride at 37°C for 30 min, or up to 120 min if weak bands were to be detected. After fixing the gels in 2% (v/v) acetic acid they were dried at about 37°C and scanned densitometrically at 578 nm.

Preparation of tissue extracts. Soluble lactate dehydrogenase was extracted from brain hemispheres, heart, skeletal muscle and liver of adult Wistar rats weighing 180–305 g. Animals were lightly anaesthetized with diethyl ether, decapitated and bled. Tissues were removed quickly, rinsed and used immediately to prepare 10% homogenates in ice-cold 2.5 mM-Tris buffer adjusted to pH 7.5 with 1 M-HCl, containing 1.0 mM-EDTA, by means of a motor-driven Potter-Elvehjem glass/Teflon homogenizer for brain, minced cardiac muscle and liver tissue, and by means of a mechanical homogenizer (Ultra-Turrax, Jahnke and Kunkel) for skeletal-muscle tissue. After the homogenates had been rapidly frozen and thawed at room temperature (25°C) to ensure a thorough release of all soluble components from particulate matter they were centrifuged at 100000g for 60 min. The clear supernatants were decanted and either analysed at once or stored at 4°C. All procedures except thawing of extracts were carried out at 0–4°C.

Incubation of extracts and purified lactate dehydrogenase. Samples of tissue extracts were added to 10 vol. of 1.0 mM-EDTA/2.5 mM-Tris/HCl buffer, pH 7.5, containing detergent. Incubation was carried out at 25°C for 60 min. Conditions for incubation were derived from previous experiments with membrane-bound enzymes (Berlet *et al.*, 1977; Berlet & Lehnert, 1978).

Isoenzyme suspensions containing (NH₄)₂SO₄ were freshly diluted (1:200) in 0.154 M-NaCl to give a protein concentration of 25 mg/litre and incubated as described above.

Results

The activities of total soluble lactate dehydrogenase of brain, cardiac and skeletal muscle and liver are listed in Table 1. Samples of these extracts were incubated with various concentrations of deoxycholate or Triton X-100 at 25°C for 1 h throughout, since this time period proved to be adequate for the full effect of deoxycholate to develop (Fig. 1).

Fig. 1. Time course of inactivation of soluble lactate dehydrogenase by sodium deoxycholate

Samples of tissue extracts (see the Materials and Methods section) were incubated with 10 vol. of 1.0 mM-EDTA/2.5 mM-Tris buffer, pH 7.5, containing 2.5 g of deoxycholate/litre. Incubation was carried out at 25°C for 60 min. Δ , Brain; $\square$, cardiac muscle; $\triangle$, skeletal muscle; $\blacksquare$, liver.

Apart from a slight initial increase in activity at low concentrations of deoxycholate higher concentrations caused the enzyme activity to fall distinctly to different values depending on the origin of the enzyme (Table 1). Extracts of skeletal muscle and liver showed the most marked effects, their activities disappearing almost completely at concentrations above 1.0 g of deoxycholate/litre. In brain, enzymic activity was only diminished at 10 g of deoxycholate/litre, and with cardiac muscle changes were smaller still. Quite consistently, the inactivation occurred suddenly rather than gradually within the range 1.0–2.5 g of deoxycholate/litre with little change below and above this range.

Neither different protein concentrations of the extracts ranging from 2.02 g/litre (skeletal muscle) to 2.56 g/litre (liver) nor initial activity of lactate dehydrogenase (Table 1) could be held responsible for the differential inhibition of the enzyme. Rather, the degree of inactivation appeared to depend on the LDH isoenzyme composition of the individual extracts (Table 2). In extracts of brain tissue deoxycholate had caused a loss of activity of 45.9%. This value came close to 41.4% calculated for subunit A present in brain (Table 2). Lactate dehydrogenase of cardiac muscle exhibited a similar close relationship between the total amount of subunit A and the loss of activity, the values being 17.1 and 23.8% respectively. Lactate dehydrogenase of skeletal muscle and liver is almost entirely accounted for by the homotetramer A₄ and accordingly the activities still measurable at

Table 1. *Effect of sodium deoxycholate on the activity of soluble lactate dehydrogenase of rat tissues*

For full details see the Materials and Methods section: 1 vol. of tissue extract was incubated with 10 vol. of 1.0 mM-EDTA/2.5 mM-Tris buffer, pH 7.5, containing deoxycholate as listed. Incubation was carried out at 25°C for 60 min. Values for absolute enzyme activity of freshly prepared extracts are means $\pm$ range of duplicate experiments. Values for the incubation experiments are means $\pm$ S.D. of three (for cardiac muscle: five) experiments and are expressed as percentages of controls.

Control lactate dehydrogenase activity (units/g wet wt.) ... Concn. of deoxycholate (g/litre)	Lactate dehydrogenase (% of control activity)			
	Brain 156 $\pm$ 12	Cardiac muscle 431 $\pm$ 22	Skeletal muscle 784 $\pm$ 29	Liver 615 $\pm$ 15
0.1	100.2 $\pm$ 6.5	99.8 $\pm$ 3.6	94.7 $\pm$ 4.7	91.8 $\pm$ 8.4
0.25	102.7 $\pm$ 4.0	106.6 $\pm$ 4.5	93.3 $\pm$ 3.8	102.5 $\pm$ 12.4
0.5	110.4 $\pm$ 5.0	98.6 $\pm$ 5.6	91.1 $\pm$ 13.6	102.2 $\pm$ 8.8
1.0	106.7 $\pm$ 4.3	97.7 $\pm$ 6.6	90.4 $\pm$ 1.3	100.6 $\pm$ 10.8
2.5	54.1 $\pm$ 10.0	76.2 $\pm$ 10.5	5.2 $\pm$ 5.2	7.9 $\pm$ 5.3
5.0	61.1 $\pm$ 9.2	79.2 $\pm$ 11.8	2.0 $\pm$ 0.6	3.6 $\pm$ 1.8
10.0	59.9 $\pm$ 11.1	87.2 $\pm$ 7.4	2.6 $\pm$ 0.4	4.7 $\pm$ 3.6

Table 2. *Isoenzyme distribution of soluble lactate dehydrogenase of rat tissue*

Values for the relative distribution of isoenzymes are based on the densitometric evaluation at 578 nm of electrophoretograms stained with Nitro Blue Tetrazolium chloride for enzyme activity (see the Materials and Methods section). Values are means $\pm$ range of duplicate experiments. For skeletal muscle it should be noted that fast-migrating isoenzymes LDH-1, LDH-2 and LDH-3 could only be detected when staining time was prolonged up to 90 min. In liver extract no staining of these isoenzymes was observed. Total percentage of subunit A was calculated on the assumption that heterotetramers LDH-2, LDH-3 and LDH-4 contain 25, 50 and 75% of subunit A respectively.

Isoenzyme ... Subunit compositions ... Tissue	Relative distribution of lactate dehydrogenase isoenzyme activity (%)					Total percentage of subunit A
	LDH-1 B ₄	LDH-2 B ₃ A	LDH-3 B ₂ A ₂	LDH-4 B A ₃	LDH-5 A ₄	
Brain	37.7 $\pm$ 0.3	11.3 $\pm$ 3.7	7.3 $\pm$ 2.7	35.2 $\pm$ 5.1	8.5 $\pm$ 2.5	41.4
Cardiac muscle	53.7 $\pm$ 5.7	25.5 $\pm$ 2.5	13.0 $\pm$ 1.0	4.3 $\pm$ 0.3	1.0 $\pm$ 0.0	17.1
Skeletal muscle	1.0	1.0	1.0	5.0	95.0	98.8
Liver	—	—	—	5.0	95.0	98.8

deoxycholate concentrations in excess of 1.0 g/litre were minimal only.

Experiments with purified lactate dehydrogenase confirmed the particular lability of the homotetramer A₄ towards deoxycholate. When either isoenzyme LDH-1 from pig heart or isoenzyme LDH-5 from rabbit muscle was incubated with deoxycholate the latter lost its activity almost entirely, whereas the former remained unaffected (Table 3). The course of inactivation of isoenzyme LDH-5 was similar to that found with tissue extracts, although somewhat lower concentrations of deoxycholate were sufficient to accomplish full inactivation. Control experiments with Triton X-100 failed to elicit any inhibitory or differential effect on both homotetramers, nor was lactate dehydrogenase of tissue extracts affected by Triton X-100 at all even though the incubation time was prolonged to 90 min.

The enzyme activity was compared with the elec-

trophoretic isoenzyme patterns of deoxycholate-treated tissue extracts (Fig. 2). The bands attributable to isoenzymes LDH-5 and LDH-4 disappeared completely at detergent concentrations at which losses of activity had been observed spectrophotometrically. However, isoenzymes LDH-3 and LDH-2 were still detectable, though staining was less intense, and the band representing isoenzyme LDH-1 appeared to become even stronger at high detergent concentrations.

Since equal sample volumes were applied to the gels and run together under identical conditions, increased peak areas on scanning indicated an activation of the isoenzyme by about 60% at maximum deoxycholate concentrations compared with the peak area at 1.0 g of deoxycholate/litre (Fig. 3). Fig. 3 also shows that deoxycholate increased the mobility of enzymes migrating towards the anode, but decreased that of isoenzymes migrating to the cathode.

Fig. 2. Electrophoretic patterns of lactate dehydrogenase from rat tissues after treatment with sodium deoxycholate. Samples were incubated with the detergent (see the Materials and Methods section) at concentrations given in the Figure. For electrophoresis preincubated samples were mixed with 1 vol. of 1% agar at approx. 45°C, and 30 μ l of the mixture was applied to the gel. The gels were run at 8°C for 100 min at a constant 200 V. Arrow indicates the origin.

Table 3. Effect of sodium deoxycholate and Triton X-100 on purified lactate dehydrogenase isoenzymes B₄ and A₄

Electrophoretically pure isoenzymes LDH-1 and LDH-5 were diluted with 0.154 M NaCl to give a protein concentration of 25 mg/litre. To a sample 10 vol. of 1.0 mM-EDTA/2.5 mM-Tris buffer, pH 7.5, containing detergent was added and the mixture incubated for 60 min at 25°C. Enzyme activity is expressed as percentage of that of incubated detergent-free controls.

Subunit composition Concn. of detergent (mg/ml)	Enzyme activity (% of that of detergent-free controls) after incubation with			
	Deoxycholate		Triton X-100	
	B ₄	A ₄	B ₄	A ₄
0.1	104	90	81	80
0.25	102	92	107	100
0.5	113	15	99	88
1.0	95	16	96	90
2.5	96	18	98	92
5.0	94	14	92	95
10.0	94	10	98	100

Discussion

Selective inactivation of lactate dehydrogenase

The experiments with pure homotetramers of lactate dehydrogenase have shown that deoxycholate exclusively interferes with the catalytic activity of isoenzyme LDH-5. In keeping with these results was the complete inactivation of lactate dehydrogenase of skeletal muscle and liver respectively, known to consist of isoenzyme LDH-5 predominantly, if not exclusively (Wieland *et al.*, 1959). The selectivity of deoxycholate towards isoenzyme LDH-5 was also evident from the results obtained with extracts of

Fig. 3. Densitometric tracings of electrophoretograms of rat brain lactate dehydrogenase treated with sodium deoxycholate

Samples of soluble lactate dehydrogenase extracted from rat brain (see the Materials and Methods section) were treated with 10 vol. of 1.0 mM-EDTA/2.5 mM-Tris buffer, pH 7.5, containing three different concentrations of deoxycholate: —, 1.0 g/litre, ····, 2.5 g/litre; ----, 5.0 g/litre. After incubation a set of samples of identical volume was simultaneously submitted to electrophoresis and staining. Electrophoretograms were scanned at 578 nm and electrophoretic mobility was determined by measuring the distance between the origin (arrow) and absorbance maximum of the respective bands.

cardiac muscle and brain tissue whose lactate dehydrogenase activity arises from the combined action of several isoenzymes. Since the degree of inactivation distinctly exceeded their percentages of isoenzyme LDH-5, a selective interaction of the detergent with

subunit A was suggested, no matter whether it occurs as a component of isoenzyme LDH-5 or the heterotetramers isoenzymes LDH-2, LDH-3 and LDH-4. Typically, lactate dehydrogenase of brain and cardiac muscle was inactivated to degrees closely commensurate with their respective contents of subunit A. Further support for this assumption came from the electrophoretic patterns of brain lactate dehydrogenase preincubated with deoxycholate (Figs. 2 and 3), when isoenzymes LDH-2 and LDH-3 were much less intensely stained than the corresponding bands of untreated controls.

Interaction of deoxycholate with protein

The interaction of detergents with protein has been reviewed by Makino *et al.* (1973) and Tanford (1973). Generally hydrophilic proteins do not bind deoxycholate. In our study, however, changes in electrophoretic migration rates (Fig. 3) of preincubated tissue lactate dehydrogenase strongly suggest that the different enzyme forms bind deoxycholate. Since both deoxycholate and Triton X-100 are believed to associate primarily hydrophobically with hydrophobic domains of proteins (Helenius & Simons, 1972), free carboxy groups of deoxycholate, which are predominantly ionized at the electrophoretic pH of 8.2 (pK_a of deoxycholate 6.58), are likely to confer a higher overall negative charge on the enzyme molecule.

Hydrophilic membrane proteins may contain specific hydrophobic segments (Spatz & Strittmatter, 1971), besides hydrophobic residues on the surface (Klotz, 1970). The latter may arise from non-polar side chains of the amino acids alanine and valine as well as from the aromatic moiety of phenylalanine and tryptophan, possibly centred around crevices at subunit junctions (Yon, 1978). The amino acid analysis of lactate dehydrogenase isoenzymes has indeed shown previously that the percentage of hydrophobic amino acids is higher in subunit A than in subunit B (Wachsmuth *et al.*, 1964). The association of isoenzyme LDH-5 with structural elements of rat brain and its particular resistance to the solubilizing effect of Triton X-100 (Berlet & Lehnert, 1978) are also consistent with the more hydrophobic nature of isoenzyme LDH-5 than of LDH-1.

Mechanism of action of deoxycholate

At present it is not clear how deoxycholate interferes with subunit A. Since even a sixfold increase in concentration of pyruvate in the assay mixture failed to counteract deoxycholate (T. Lehnert & H. H. Berlet, unpublished work), a competitive inhibition does not appear likely. Neither would this notion be supported by the abrupt transition from full activity to the final degree of inactivation.

The dissimilarity of the structure of deoxycholate to that of any of the known substrates or coenzymes of lactate dehydrogenase suggests that deoxycholate does not react with the active centre itself, but still the fairly rigid deoxycholate molecule (Carey & Small, 1972) may force a possibly slight conformational change on subunit A sufficient to affect the active centres of the enzyme. This effect could be enhanced by repulsive forces of the charged groups of bound deoxycholate, facing away from the protein surface towards each other and pushing apart adjacent hydrophobic segments of subunit A. In turn bulkiness and lack of polarity would render Triton X-100 ineffective compared with deoxycholate.

A complete loss of activity of lactate dehydrogenase brought about by a detergent was previously observed when sodium dodecyl sulphate dissociated the enzyme into its subunits (Di Sabato & Kaplan, 1964). However, in view of the residual activity of the heterotetrameric isoenzymes in this study, such a mechanism is to be disregarded, for neither monomers nor dimers of lactate dehydrogenase retain catalytic activity (Markert & Massaro, 1968; Chan & Mosbach, 1976).

The effect of bile salts on isoenzyme LDH-5 may have pathophysiological implications in disease conditions associated with cholestasis. As bile salts accumulate, they may eventually interfere with the isoenzyme LDH-5 of liver tissue and hence upset the hepatic carbohydrate metabolism. Deoxycholate may also prove a useful tool in chemical pathology to assay the percentages of subunits A and B of serum lactate dehydrogenase spectrophotometrically.

This study was in part supported by the Deutsche Forschungsgemeinschaft.

References

- Bergmeyer, H. U. & Bernt, E. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), 2nd edn., vol. 1, pp. 533–538, Verlag Chemie, Weinheim
- Berlet, H. H. & Lehnert, T. (1978) *FEBS Lett.* **45**, 48
- Berlet, H. H., Birringer, H. & Volk, B. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 214
- Carey, M. C. & Small, D. M. (1972) *Arch. Intern. Med.* **130**, 506–527
- Chan, W. W.-C. & Mosbach, K. (1976) *Biochemistry* **15**, 4215–4222
- Di Sabato, G. & Kaplan, N. O. (1964) *J. Biol. Chem.* **239**, 438–443
- Helenius, A. & Simons, K. (1972) *J. Biol. Chem.* **247**, 3656–3661
- Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415**, 29–79
- Klotz, I. M. (1970) *Arch. Biochem. Biophys.* **138**, 704–706
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Makino, S., Reynolds, J. A. & Tanford, C. (1973) *J. Biol. Chem.* **248**, 4926–4932

- Markert, C. L. & Massaro, E. J. (1968) *Science* **162**, 695–697
- Spatz, L. & Strittmatter, P. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1042–1046
- Tanford, C. (1973) *The Hydrophobic Effect*, pp. 120–142, John Wiley and Sons, London
- Tzagoloff, A. & Penefsky, H. S. (1971) *Methods Enzymol.* **22**, 219–230
- Wachsmuth, E. D., Pfeleiderer, G. & Wieland, T. (1964) *Biochem. Z.* **340**, 80–94
- Wieland, R., Pfeleiderer, G., Haupt, I. & Wörner, W. (1959) *Biochem. Z.* **332**, 1–10
- Wilkinson, J. H. (1970) *Isoenzymes*, 2nd edn., p. 137, Chapman and Hall, London
- Yon, R. J. (1978) *Int. J. Biochem.* **9**, 373–379