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THE EFFECT OF β-MERCAPTOETHANOL ON LACTATE DEHYDROGENASE FROM RAT BRAIN*

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Abstract—Catalytic studies on lactate dehydrogenase from rat brain in the presence of β-mercaptoethanol have been performed. It has been found that pyruvate reduction and lactate dehydrogenation were differently affected by the addition of the sulfhydryl compound to the reaction mixture. The effect of β-mercaptoethanol on the catalyzed reaction has been shown to be dependent on the hydrogen ion concentration. When lactate dehydrogenation is measured, β-mercaptoethanol causes a net enhancement of the reaction rate at the more acidic pH values.

A FEW years ago, Brachet[1] and Brachet and Delange-Cornil[2] have shown that β-mercaptoethanol strongly inhibits morphogenetic movements at the gastrulation and neurulation of amphibian embryos. As reported by these authors, if late gastrulae are treated with 0·01 M solutions of the compound, the medullary plate begins to form, but it remains flat for a long time.

Since β-mercaptoethanol is very effective in maintaining protein —SH groups in the reduced state, it was supposed at once that the compound inhibits neurulation because —SH groups in the embryo are kept in the reduced form. Subsequent studies by Brachet *et al.*[3] and by Schram and Brachet[4] have shown that β-mercaptoethanol also produces a significant inhibition of the incorporation *in vivo* or *in vitro* of ^{14}C-phenylalanine and ^{14}C-leucine into the proteins of mouse liver.

A possible effect of β-mercaptoethanol on pyridine nucleotides and related enzymes has not been taken into consideration by chemical embryologists, although van Eys and Kaplan[5] have described the addition of sulphydryl compounds to diphosphopyridine nucleotide and its analogues.

It has been the purpose of this investigation to obtain preliminary data on the kinetic behaviour of a pyridine nucleotide-dependent enzyme, such as lactate dehydrogenase† of the adult rat brain, in the presence of β-mercaptoethanol. Lactate dehydrogenase has been recently shown by Bonavita *et al.*[6, 7] to undergo conspicuous changes in its kinetic features as well as in the isozymatic composition during the post-natal neurogenesis of the rat. It has been thought, therefore, that an investigation on the possible

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† The abbreviations used are the following: DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; DeDPN; deamino-DPN; Py3AlDPN, pyridine-3-aldehyde-DPN, TNDPN, thionicotinamide-DPN; LDH, lactate dehydrogenase.

effects of β-mercaptoethanol on this enzyme could be of some relevance to chemical embryology.

EXPERIMENTAL

β-Mercaptoethanol was a product from the California Corporation for Biochemical Research (Los Angeles, Calif., U.S.A.). Diphosphopyridine and triphosphopyridine nucleotide were purchased from Sigma Company (St. Louis, Mo., U.S.A.), while pyridine-3-aldehyde-DPN, thionicotinamide-DPN and deamino-DPN were prepared by Pabst Brewing Company (Milwaukee, Wisconsin, U.S.A.). Sodium pyruvate and L(+)lactic acid *purum* (40%) were products from Fluka (Basel, Switzerland).

The reaction of β-mercaptoethanol with DPN and the other nucleotides was studied under different conditions, as indicated below. DPN was measured by the cyanide addition reaction, as reported by Colowick *et al.*[8] Absorption spectra were taken with a Beckman DB spectrophotometer connected to a Sargent recording unit (mod. SR.).

Lactate dehydrogenase was extracted with two volumes of glass distilled water from the brain of adult albino rats (Wistar strain) by using an Omni-Mixer Servall (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) at 9,000 rev/min. The homogenate was centrifuged at 32,000 × *g* for 30 min in a refrigerated Lourdes centrifuge, mod. LRA, and the supernate used as such after an appropriate dilution.

Pyruvate reduction was measured spectrophotometrically, at the pH values indicated below, in a reaction mixture containing the enzyme plus 2·5 ml 0·1 M sodium phosphate buffer, 0·1 ml 0·01 M sodium pyruvate, 0·1 ml 2×10^{-3} M DPNH, and 0·2 ml 12 M β-mercaptoethanol or 0·2 ml of the phosphate buffer mentioned above. It was demonstrated at each pH that DPNH oxidation was entirely pyruvate dependent. For this purpose, were prepared reaction mixtures from which only sodium pyruvate was omitted.

Lactate dehydrogenation was also measured spectrophotometrically, and evidence was similarly obtained that DPN reduction was entirely lactate dependent even at pH 8 and below, thus ruling out that it could have been partially mediated by some other enzyme supplied in the crude preparation of LDH. In a typical experiment, the reaction mixture for the assay of lactate dehydrogenation contained the enzyme plus 2·5 ml 0·1 M glycine–NaOH at the pH indicated, 0·1 ml 0·222 M sodium L(+)lactate, 0·1 ml 7×10^{-3} M DPN, and 0·2 ml 12 M β-mercaptoethanol or 0·2 ml of the glycine–NaOH buffer mentioned above.

Also other concentrations of mercaptoethanol were used and they are indicated below.

RESULTS

The reaction of β-mercaptoethanol with pyridine nucleotides

As reported by van Eys and Kaplan,[5] DPN reacts with several mercaptans. Figure 1 shows the absorption spectrum of DPN reacted with excess β-mercaptoethanol at pH 10·6 in a glycine–NaOH buffer. Since the rate of the reaction is extremely rapid, it may be assumed that the spectrum in Fig. 1 represents the equilibrium point, which has been found to be very sensitive to the hydrogen concentration.

The chemical interaction of sulphydryl reagents and pyridine nucleotides has been reinvestigated with the use of β-mercaptoethanol. van Eys and Kaplan[5] employed various dinucleotides. The kinetic observations made by these authors with ethyl

mercaptan have all been duplicated with mercaptoethanol, exception made for minor details. In fact, Py3AlDPN gives the absorption maximum with mercaptoethanol at a slightly higher wavelength (350 mμ) than with ethyl mercaptan (345 mμ). Moreover, the pH-interaction curve reported in Fig. 2 does not repeat the curve obtained by van Eys and Kaplan[5] with the same nucleotide and ethyl mercaptan. It has been found

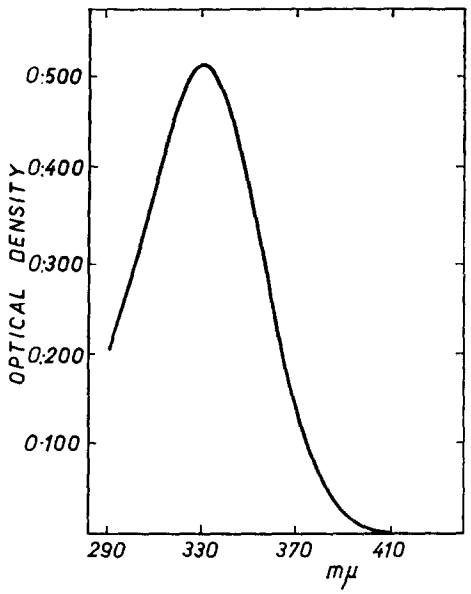

FIG. 1. Absorption spectrum of DPN reacted with β-mercaptoethanol. The reaction mixture contained 2·6 ml glycine–NaOH buffer, 1 M, pH 10·6, 0·2 ml alkalinized 12 M-mercaptoethanol, and 0·1 ml DPN, $5{\cdot}25 \times 10^{-3}$ M.

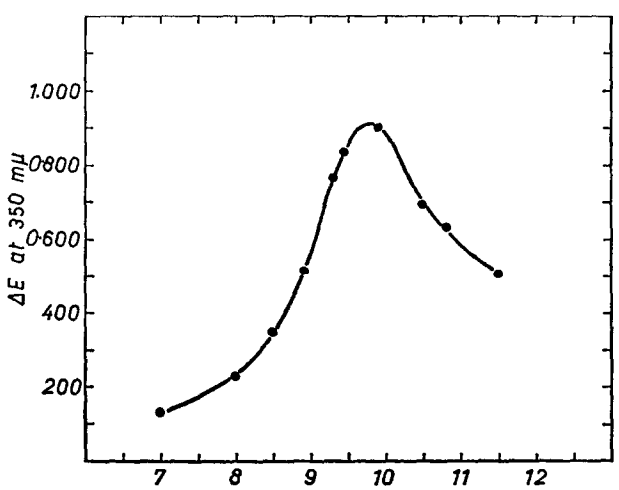

FIG. 2. The effect of the hydrogen ion concentration on the interaction between β-mercaptoethanol and Py3AlDPN. β-mercaptoethanol, 0·8 M, in 1 M glycine buffer of the pH indicated plus Py3AlDPN, $0{\cdot}87 \times 10^{-4}$ M. Values are corrected for the dinucleotide in buffer alone.

that at a pH higher than 10·5 the equilibrium is still favourable to the addition of ethyl mercaptan and not of mercaptoethanol to Py3AlDPN. Conversely, at this same pH the equilibrium is still favourable to the addition of mercaptoethanol to DPN, as measured at 330 mμ (Fig. 1). It is worthy of mention in this connection that the highest extinction at 330 mμ is observed when the molar ratio between DPN and mercaptoethanol is not lower than 1 :13,500. Under these conditions and when the pH is between 10·0 and 10·5, the millimolar extinction coefficient of the DPN complex at 330 mμ is 4·46.

Kinetics of lactate dehydrogenase in the presence of β-mercaptoethanol

The finding of a reaction *in vitro* between DPN or its derivatives and β-mercaptoethanol has suggested an investigation of the possible effects of this sulphydryl compound on the kinetic behaviour of a pyridine nucleotide-dependent enzyme. Thus, preliminary experiments have been performed on LDH from adult rat brain. Figure 3

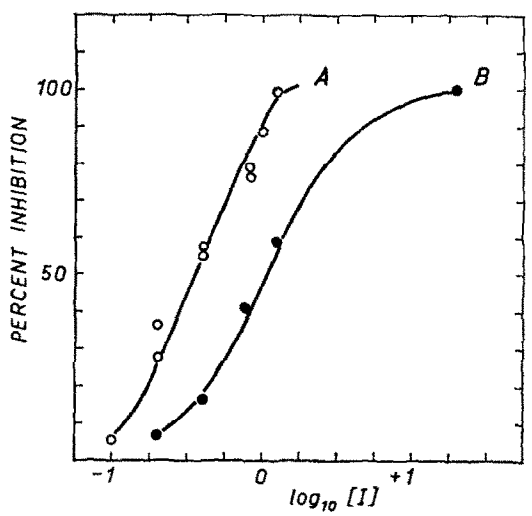

FIG. 3. Percent inhibition of lactate dehydrogenation (A) and pyruvate reduction (B) as a function of β-mercaptoethanol concentration. The two reactions were measured under their optimal conditions. Lactate dehydrogenation was followed at pH 9·6 in a reaction mixture containing 2·5 ml glycine-NaOH buffer, 0·1 ml 0·222 M sodium L(+)lactate, 0·1 ml 7×10^{-3} M DPN and 50 μl. of rat brain extract (1 to 30, w/v). Pyruvate reduction was measured at pH 7·4 in a reaction mixture containing 2·5 ml 0·1 M sodium phosphate buffer, 0·1 ml of 0·01 M sodium pyruvate, 0·1 ml DPNH 2×10^{-3} M and the same amount of the enzyme.

summarizes an experiment in which the percent inhibition of the enzyme by increasing amounts of β-mercaptoethanol was determined. It can be readily seen that pyruvate reduction and lactate dehydrogenation were affected at different degrees, and, as one would have expected, complete inhibition was obtained with a lower amount of β-mercaptoethanol when DPN was the coenzyme added (see Discussion). This was not, however, the only difference between the effects of β-mercaptoethanol on the two reactions catalyzed by LDH.

Figure 4 shows pH-activity curves for lactate dehydrogenation and pyruvate oxidation both in the absence and the presence of mercaptoethanol. At pH 9·6, where lactate dehydrogenation exhibits its maximal rate, the apparent inhibition is definitely greater than at a lower pH. Moreover, a shift of the pH optimum from 9·6 to 8·6 is noticed, when β-mercaptoethanol is added to the reaction mixture. It is also shown in Fig. 4A that at a pH lower than 8·5 the rate of reaction is clearly greater in the presence of mercaptoethanol. This is not so when pyruvate reduction is measured, although even in this case the greater inhibition at the pH optimum and the shift of the maximal rate to a more acidic pH are observed. The two peaks of the control curve in Fig. 4B are no more found when mercaptoethanol is added to the enzyme.* Finally, it will be noted that the pH-activity curve for pyruvate in the presence of β-mercaptoethanol is quite similar to the curve observed with brain LDH from newborn rat. [7]

In some experiments DeDPN and TNDPN have been used as coenzymes in the presence of β-mercaptoethanol. Table 1 shows the differential kinetic behaviour of the

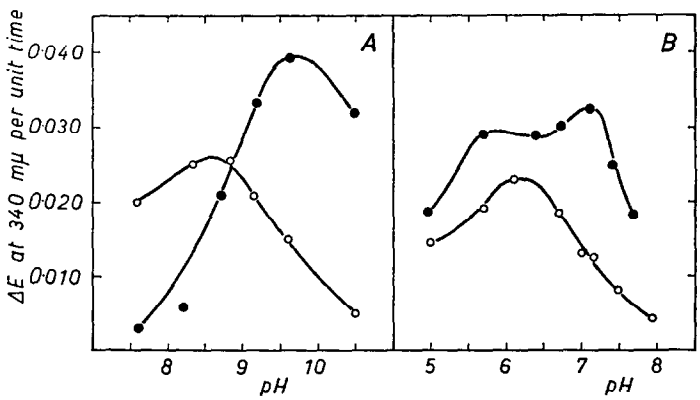

FIG. 4. pH-Activity curves in the absence (●) and presence (○) of 0·8 M mercaptoethanol. Lactate dehydrogenation (A) was measured using twice the amount of enzyme used when measuring pyruvate reduction (B).

rat brain enzyme in the presence of β-mercaptoethanol according to the coenzyme used, and it is of interest that the inhibition observed with TNDPN, the thionicotinamide analogue, was much higher than with DPN.

DISCUSSION

The findings described in the present article do not allow consideration of the embryological effects of β-mercaptoethanol as due to LDH inhibition. As reported above, 0·01 M solutions of β-mercaptoethanol were found to cause abnormal neurulation.[1,2] Similarly, the incorporation *in vitro* of ^{14}C-phenylalanine or ^{14}C-leucine into proteins was significantly inhibited by this same concentration of the compound.[4]

* At variance with reactions catalyzed by LDH, the dehydrogenation of glucose-6-phosphate is almost uniformly inhibited in the pH range 6 to 8. (V. Bonavita and R. Guarneri, unpublished results.)

This investigation has shown no effect at the enzyme level and the absence of any appreciable interaction with pyridine nucleotides, when low quantities of mercaptoethanol are used. The data on enzyme catalyzed reactions deserve, however, some comment.

As shown in Fig. 3, β-mercaptoethanol is not a strong inhibitor of LDH, but the kinetics of the enzyme can be greatly altered by the addition of conspicuous amounts of the compound. According to Thompson and O'Donnell,[9] it may well be that 4 M (and more concentrated) solutions of β-mercaptoethanol are effective denaturants, but also in the opinion of these authors further study is required to establish the equivalence in the effect on protein configuration of treatments with 8 M urea and aqueous mercaptoethanol. Since the enzyme studies described herein were performed with thiol solutions not higher than 0·8 M (final), the possibility that β-mercaptoethanol has acted as a true denaturing agent is quite remote. One would think, therefore, that the changes in the kinetic behaviour of LDH are to be referred to a reduction of essential disulphide bonds in the enzymic protein[10] and/or to the maintainance of —SH groups in the reduced state. Such an effect, however, seems to be clearly dependent on the hydrogen ion concentration, as shown by the peculiar shape of the pH-activity curves. It seems worth mentioning in this connection that for sulfhydryl groups pK values equal to 8·3–8·6 have been reported.[11]

The most interesting finding is that shown in Fig. 4, i.e. the possibility of enhancing lactate dehydrogenation by addition of β-mercaptoethanol when the pH is lower than

TABLE 1. PERCENT RESIDUAL ACTIVITY OF LDH FROM RAT BRAIN IN THE PRESENCE OF DIFFERENT PYRIDINE NUCLEOTIDES AND TWO LACTATE CONCENTRATIONS

(L) symbolizes a final concentration of L(+) lactate equal to 0·0074 M, while (H) symbolizes a tenfold concentration of the substrate. Assay conditions: 0·7 micromoles of dinucleotide plus lactate as mentioned above plus glycine–NaOH buffer, 0·1 M, pH 9·6, up to a final volume of 3 ml. When DeDPN and TNDPN were used, a five-fold amount of the enzyme was added. The final concentration of β-mercaptoethanol was 0·8 M.

Coenzyme	Lactate concentration	Percent residual activity
DPN	(H)	70·0
	(L)	23·0
DeDPN	(H)	55·0
	(L)	10·0
TNDPN	(H)	17·0
	(L)	3·5

8·5. Since the same phenomenon has not been observed in the measurement of pyruvate reduction in the range of pH where an increase of lactate dehydrogenation is found, one would conclude that the same conformational change of the enzymic protein causes a different kinetic modification whether pyruvate reduction or lactate dehydrogenation is measured. This finding may be considered as support for the view of two different binding sites for lactate and pyruvate.

It is obvious that β-mercaptoethanol may affect the kinetics of lactate dehydrogenation by two main mechanisms:

(i) by reacting with DPN, which, as mentioned before, occurs at a greater extent when the pH is alkaline; (ii) by a possible action on the apoenzyme. The importance of the first mechanism cannot be evaluated directly, i.e. by measuring the amount of free DPN after addition of β-mercaptoethanol, since any method for DPN determination requires a preliminary dilution which causes a dissociation of the complex. However, that the inhibition shown by the curves in Fig. 4A does not originate only from the decrease of free DPN due to complexing can be readily seen from the following.

Let us take into consideration the experimental data obtained at pH 10·4, i.e. where the equilibrium is most favourable to the complex formation of DPN plus β-mercaptoethanol and where the stronger inhibition of lactate dehydrogenation is observed. On the basis of the assumption that the DPN-β-mercaptoethanol complex has a molar extinction coefficient at 330 mμ equal to 4·46, when the pH is 10·4, it can be easily calculated that at this pH, in the experimental conditions of Fig. 4A, about 34 per cent DPN is still free (see Fig. 1). On the other hand, the decrease of free DPN to 1/3 (from $2{\cdot}33 \times 10^{-4}$ M to $0{\cdot}77 \times 10^{-4}$ M) has been found to cause, at pH 10·4, a decrease of the rate of reaction of only 50 per cent. Thus, the inhibition of about 70 per cent observed in the presence of β-mercaptoethanol (Fig. 4A) must be accounted for, at least partially, by an action of β-mercaptoethanol on the enzyme protein. This same disagreement between the observed inhibition and the decrease of the rate of reaction due to complexing of DPN with mercaptoethanol has been noticed also at a less alkaline pH (pH 9·75).

The second mechanism is the only one through which the kinetics of pyruvate reduction may be altered, since DPNH cannot react with β-mercaptoethanol. It is very likely, however, that the two mechanisms mentioned in connection with lactate dehydrogenation are not independent. Also Winer and Schwert[12] have suggested, in fact, that the unionized sulfhydryl group of LDH is important in coenzyme binding to the apo-protein, and the data of experiments with DPN analogues give support to this hypothesis.

Note added in proof—While this paper was in press, Fritz and Jacobson (*Science* **140**, 64 (1963)) have reported that β-mercaptoethanol can dissociate the coenzyme from lactate dehydrogenase and that the splitting concerns preferentially the A subunits (muscle type). Thus, while the kinetic studies described in the present communication do not allow consideration of the blocking effect of β-mercaptoethanol on neurulation as due to LDH inhibition, the evidence derived from the electrophoretic studies of Fritz and Jacobsen give some support to the hypothesis. This is particularly true when considering that, at least in the rat brain[6,7], muscle type subunits prevail over the heart type subunits in the early developmental stages.

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