Biochemical Pharmacology, Vol. 20, pp. 3331–3339. Pergamon Press, 1971. Printed in Great Britain

# DIFFERENT EFFECT OF CHLORPROMAZINE ON THE ACTIVITY OF CRYSTALLINE LACTIC DEHYDROGENASE ISOENZYMES

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(*Received* 21 *December* 1970; *accepted* 16 *June* 1971)

**Abstract**—Chlorpromazine (CPZ)* inhibits the H lactic dehydrogenase activity in both directions in a purely noncompetitive type with the substrate. The inhibition is apparently uncompetitive with NADH.

The effects of CPZ resulted in "competitive activation" with NADH, pyruvate and lactate using M lactic dehydrogenase as enzyme.

Irradiated CPZ acted similarly to CPZ but was more active without preincubation.

Using various coenzyme analogues (ADP-ribose, ADP and ATP) for inhibition of lactic dehydrogenase activity, preincubation of the enzyme with CPZ suspended the inhibitory action of ADP and ATP, but was without effect on ADP-ribose inhibition.

IN PREVIOUS publications,[1,2] it was reported that crystalline lactic dehydrogenase (LDH) from pig muscle was inhibited by $10^{-4}$ M CPZ using high concentrations of pyruvate $10^{-3}$ M. Heart (H) LDH was found to be more sensitive to inhibition than muscle (M) LDH using the same concentration of CPZ. After demonstrating the occurrence of LDH isoenzymes by starch gel electrophoresis, CPZ inhibition was demonstrated only in $H_4$, $H_3$ M and $H_2M_2$ isoenzymes, whereas $HM_3$ and $M_4$ were activated by CPZ treatment. Preincubation with NADH suspended the action of CPZ on the M LDH activity, although CPZ did not influence the fluorescence spectra of the M LDH–NADH complex.

In order to investigate further the mechanism of CPZ action on the different types of LDH, extensive kinetical experiments were performed.

## MATERIALS AND METHODS

Pig muscle lactate dehydrogenase was prepared according to the method described by Jécsay[3] and three times recrystallized. The pig heart LDH was obtained by a modification of the original method of Straub[4] by Südi[5] and twice recrystallized.

The protein content was determined spectrophotometrically in 0·1 N NaOH by measuring the absorbance at 280 nm. The optical density of 1 mg/ml solutions for both type of LDH was found to be 1·29, with 1 cm optical path and under the above conditions in agreement with Jécsay.[3]

LDH activity measurements were performed according to the method of Kubowitz and Ott[6] with a "Spectromom 201" spectrophotometer at room temperature. The

* Abbreviations used: CPZ, chlorpromazine; LDH M and H, lactic dehydrogenase of muscle and heart; GAPDH, phosphoglyceraldehyde dehydrogenase.

assay mixtures were buffered to pH 7·4 with 0·05 M phosphate buffer and when the oxidation of lactate was studied 0·1 M glycocoll buffer (pH 8·5) was applied. The initial reaction velocities ($v_0$) of the oxidation of NADH and that of the reduction of NAD respectively were calculated for 1 min from the differences of extinctions ($\Delta E$) measured at 340 nm. Readings were made at 15-sec intervals after initiating the reaction by the addition of the substrates.

The enzyme was preincubated at 37° with the effectors for 20 min.

The apparent $K_i$ value was calculated by plotting the reciprocal of the reaction velocity against the inhibitor concentrations as introduced by Dixon.[7]

NAD, ATP and ADP were preparations of Reanal, Budapest. NADH was purchased from Kyowa Hakko Kogyo Co., Tokyo, ADP-ribose from Sigma, St. Louis. CPZ was a kind gift of EGYTGY, Budapest. The free radical of CPZ was produced by ultraviolet irradiation during 8–10 hr from a distance of 1 m with a mercury-vapour lamp.

## RESULTS

### *Effect of the concentrations of pyruvate, lactate and CPZ*

Lineweaver–Burk plots are represented in Fig. 1 using pyruvate as substrate. In the case of H LDH, the inhibition is noncompetitive at $10^{-4}$ M and $10^{-3}$ M CPZ. The inhibitory action of CPZ is the most expressed at high substrate concentrations in accordance with previous observations.[1]

Figure 2 illustrates the reciprocal of the reaction velocity plotted against the inhibitor concentrations. The lines intersect each other on the abscissa, therefore the inhibition

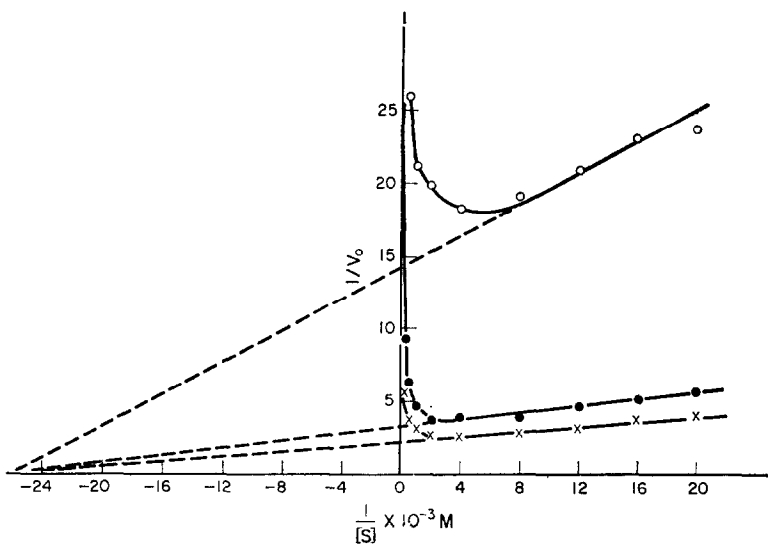


FIG. 1. Lineweaver–Burk plot of the effect of CPZ on the H LDH activity. $1/S$ is given as $1/M \times 10^{-3}$; $1/v_0$ is the reciprocal of the initial reaction velocity of the oxidation of NADH, calculated for 1 min from the decrease in absorbancy at 340 nm. Solutions of the enzyme were incubated with the CPZ at 37°, 20 min. The enzyme activity was assayed with $1{\cdot}4 \times 10^{-4}$ M NADH. The lactic dehydrogenase concentration was $2{\cdot}8 \times 10^{-9}$ M. The CPZ concentrations were $1 \times 10^{-3}$ M O——O; $1 \times 10^{-4}$ M ●——● and without CPZ ×——×.

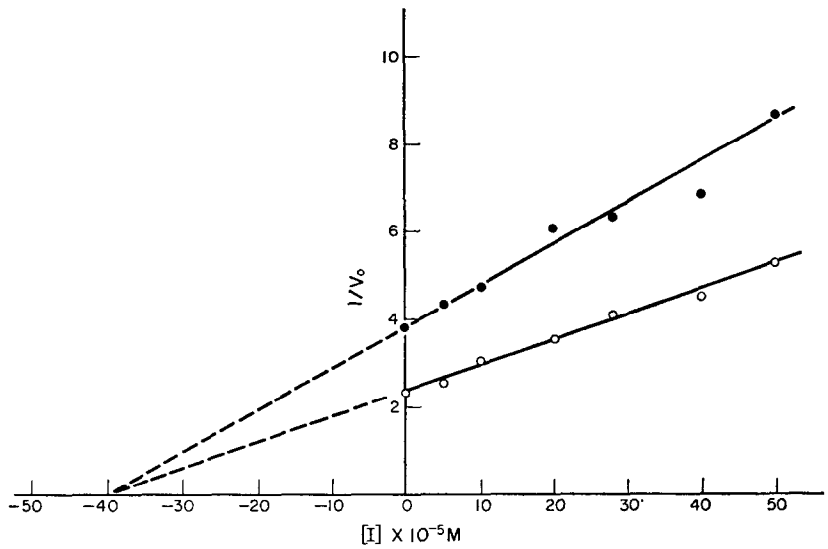


FIG. 2. Dixon plot of the effect of CPZ on the H LDH activity. $I$ is given as $10^{-5}$ M. The lines intersect the abscissa at a point which indicates the apparent $K_i$ in the presence of $1{\cdot}4 \times 10^{-4}$ M NADH. The pyruvate concentrations were ○——○ $5 \times 10^{-4}$ M and $5 \times 10^{-5}$ M ●——●. (Further data can be seen Fig. 1.)

is noncompetitive. The apparent $K_i = 3{\cdot}7 \times 10^{-4}$ M using $1{\cdot}4 \times 10^{-4}$ M NADH concentration. This value agrees fairly well with the 50 per cent inhibitory concentration which is approximately $4 \times 10^{-4}$ M.

Figure 3 shows the Lineweaver–Burk plots in the case of M LDH at two different CPZ concentrations. The intersection point of the straight lines is laying on the

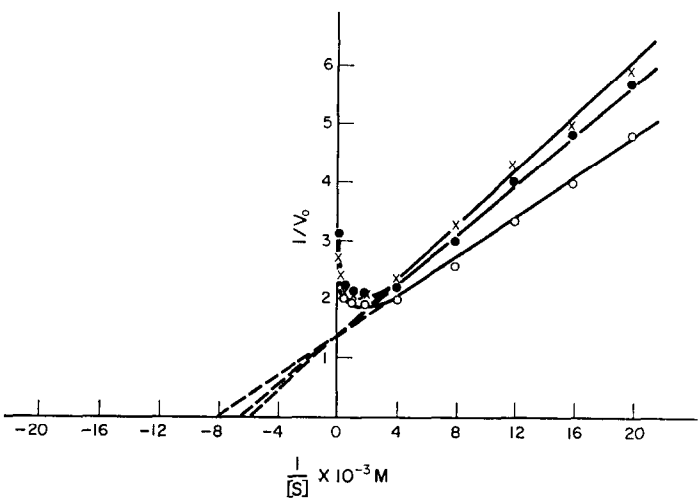


FIG. 3. Lineweaver–Burk plot of the effect of CPZ on the M LDH activity. (Data can be seen Fig. 1.)

ordinate. The activating effect shifts toward slight inhibition at high substrate concentrations.

Investigating the reaction catalyzed by LDH in the reversed direction, thus starting from NAD and lactate, the effect of CPZ is the same, i.e. inhibition using H LDH and activation with M LDH. CPZ was applied in a concentration $1 \times 10^{-4}$ M with H and M LDH because, at the pH optimum of the reaction, CPZ is precipitated in higher concentrations. At concentrations higher than the substrate optimum, in addition to the inhibitory effect of CPZ, substrate inhibition occurs too; therefore analysis of the two different inhibitions is more difficult. The intersection point of the graphs is on the abscissa. Plotting the curves according to Lineweaver and Burk the inhibition is non competitive (Fig. 4).

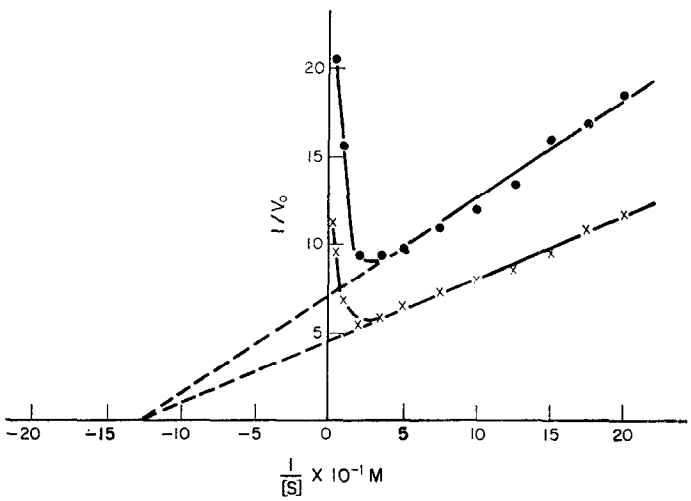


FIG. 4. Lineweaver–Burk plot of the effect of CPZ on the H LDH activity. $I/S$ is given as $1/M \times 10^{-1}$ M; $1/v_0$ is the reciprocal of the initial reaction velocity of the reduction of NAD, calculated for 1 min from the increase in absorbancy at 340 nm. Solutions of the enzyme were incubated with the CPZ at 37°, 20 min. The enzyme activity was assayed with $4{\cdot}2 \times 10^{-4}$ M NAD. The lactic dehydrogenase concentration was $5{\cdot}6 \times 10^{-9}$ M. The CPZ concentration was $1 \times 10^{-4}$ M ●——● and without CPZ ×——×.

The activating effect of CPZ on the M LDH activity decreases significantly by increasing the lactate concentration (Fig. 5). The intersection point of the lines is on the ordinate.

The action of CPZ is more intensive for both types of LDH using NAD lactate as substrate. This could be explained also by the different pH of the reaction mixture.

*The relation of CPZ and NADH*

Since the binding of coenzyme to the LDH precedes the substrate binding, it seemed to be of interest to investigate the effect of CPZ on the NADH binding to the enzyme. From previous work, it was known[1] that addition of NADH to the enzyme before the incubation with CPZ prevented its effect on M LDH.

The inhibitions of CPZ on H LDH activity was also suspended on the previous addition of NADH (Fig. 6).

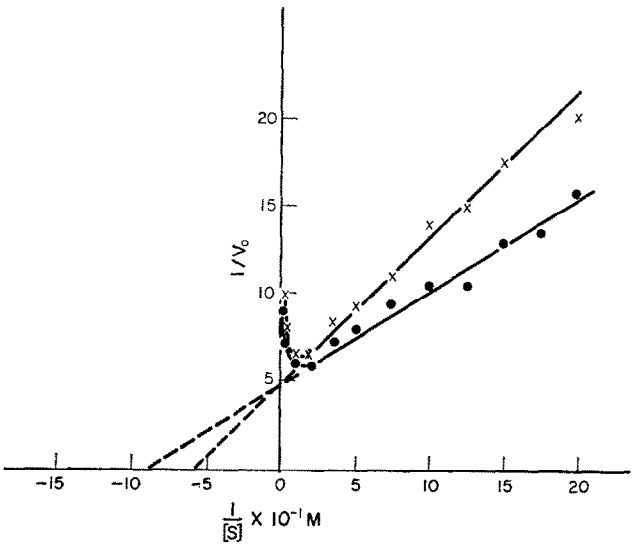


FIG. 5. Lineweaver–Burk plot of the effect of CPZ on the M LDH activity. (Further data can be seen Fig. 4.)

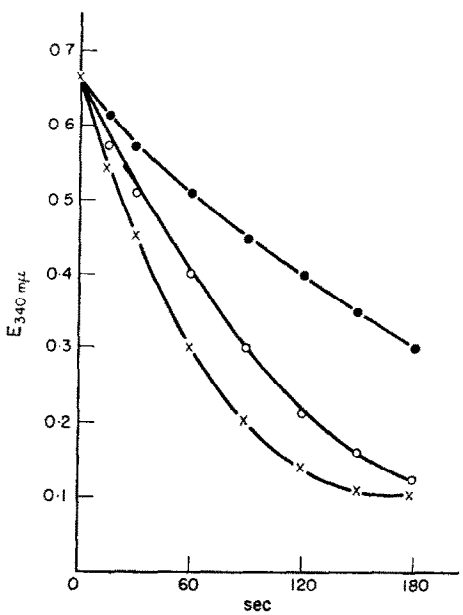


FIG. 6. Reversibility of CPZ action on previous addition of NADH with H LDH. Solutions of the enzyme were incubated with NADH and CPZ at 37°, 20 min. The enzyme concentration was $2{\cdot}8 \times 10^{-9}$ M, the pyruvate concentration was $5 \times 10^{-4}$ M, the NADH concentration was $1{\cdot}4 \times 10^{-4}$ M and the CPZ concentration was $1 \times 10^{-4}$ M in all curves. ×——× without CPZ; ●——● preincubated with CPZ; ○——○ preincubated with NADH and CPZ.

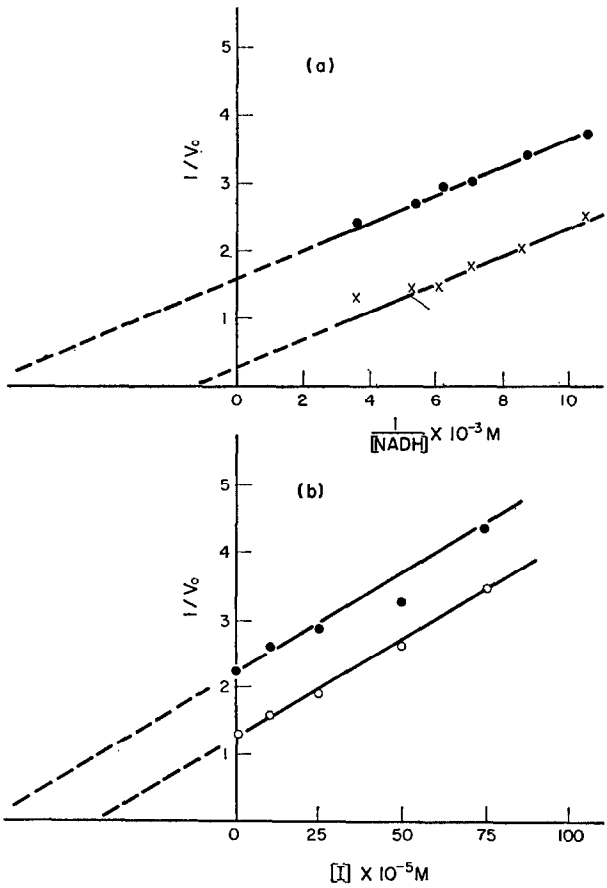


FIG. 7. Effects of varying NADH concentrations on the H LDH activity inhibited by CPZ. In both cases the pyruvate concentration was $5 \times 10^{-4}$ M and the lactic dehydrogenase concentration was $2{\cdot}8 \times 10^{-9}$ M. Solutions of the enzyme were incubated with the NADH and the CPZ at 37°, 20 min. (a) Lineweaver–Burk plots. The CPZ concentration was $5 \times 10^{-4}$ M ●——● and without CPZ ×——×. (b) Dixon plots. The NADH concentrations were $1{\cdot}4 \times 10^{-4}$ M ●——● and $2{\cdot}8 \times 10^{-4}$ M ○——○.

In order to elucidate the mechanism of these effects, Lineweaver–Burk and Dixon plots were calculated and illustrated in Figs. 7 and 8.

From Fig. 7, it can be concluded that using pyruvate as substrate and H LDH in the presence of different NADH (a) resp. CPZ (b) concentrations, the lines are parallel; thus it means that the inhibition is of the uncompetitive or coupling type.

Plotting the same curves using M LDH as enzyme, it is obvious that the increase of the coenzyme concentration diminishes the activating effect of CPZ on the M LDH activity; thus the activation is apparently of the "competitive" type (Fig. 8).

According to the investigations of Pfleiderer *et al.*,[8] the coenzyme is bound through the adenine and pyrophosphate groups to the enzyme. Forlano[9] stated that components of the coenzyme could act as competitive inhibitors. In our experiments ADP-ribose, ATP and ADP inhibited the H and M LDH activity in decreasing order.

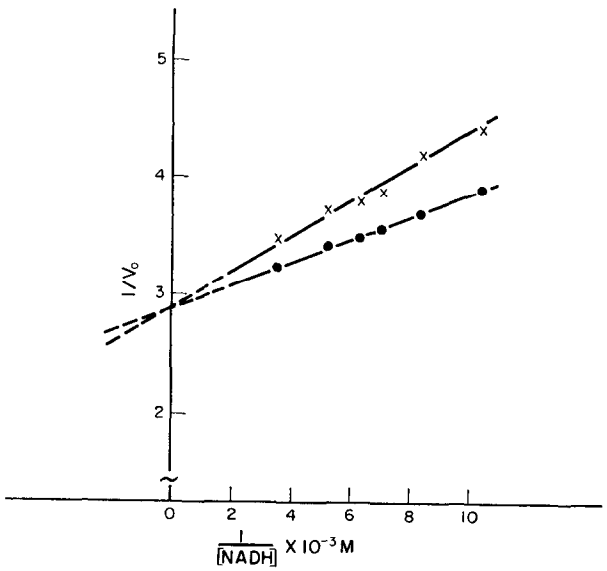


FIG. 8. Lineweaver–Burk plot of the effect of varying NADH concentrations on the M LDH activity activated by CPZ. The CPZ concentration was $5 \times 10^{-4}$ M ●——● and without CPZ ×——×. (Further data can be seen Fig. 7.)

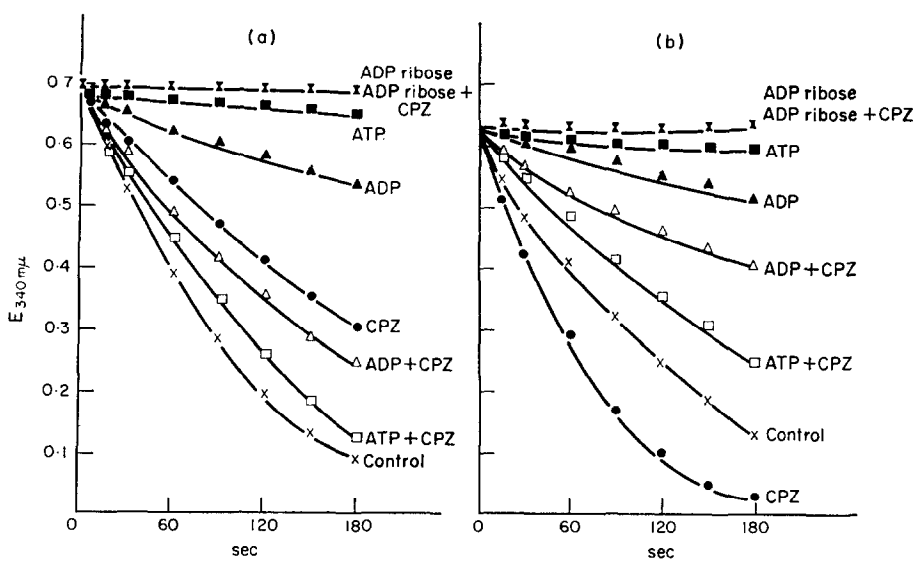


FIG. 9. Effects of CPZ on the H (a) and M (b) LDH activity inhibited by different nucleotides. Solutions of the enzyme were incubated with the nucleotides and the CPZ at 37°, 20 min. The enzyme concentration was $2{\cdot}8 \times 10^{-9}$ M, the pyruvate concentration was $5 \times 10^{-4}$ M, the NADH concentration was $1{\cdot}4 \times 10^{-4}$ M and the CPZ concentration was $1 \times 10^{-4}$ M in all curves. The concentration was $1 \times 10^{-4}$ M for all nucleotides.

The inhibitory action of ATP and ADP could be partially reversed by the addition of CPZ using H LDH as enzyme.

Using M LDH as enzyme, CPZ did not suspend the ADP inhibition on LDH activity. In contrast the inhibitory effect of ATP on M LDH activity could be reversed by the addition of CPZ. The inhibitory action of ADP-ribose could not be reversed by CPZ neither with H nor with M LDH (Fig. 9). The effect of CPZ on both types of enzyme could not be influenced by the addition of cysteine.

*The action of CPZ free radical on the H and M LDH*

From previous investigations[10,11] it was known that after irradiation of CPZ the free radical acted without preincubation on the GAPDH activity. In principle the the same was also valid for LDH. The inhibitory effect on H LDH was observed immediately with $CPZ^+$ without any incubation and with preincubation. In fact preincubation with the enzyme diminished the effect of $CPZ^+$ which could be explained by further oxidation of the free radical on addition of the enzymes.[10] The activating effect of CPZ and $CPZ^+$ on the M LDH activity does not need preincubation with the enzyme (Table 1).

TABLE 1. EFFECT OF CPZ AND CPZ FREE RADICAL ON THE LACTIC DEHYDROGENASE ACTIVITY

| | Increase in absorbancy after 60 sec | | | |
|---|---|---|---|---|
| | Without preincubation | | With preincubation 20 min | |
| | H | M | H | M |
| Control | 0·25 | 0·17 | 0·24 | 0·18 |
| CPZ, $5 \times 10^{-4}$ M | 0·24 | 0·33 | 0·16 | 0·31 |
| $CPZ^+$, $5 \times 10^{-4}$ M | 0·18 | 0·34 | 0·19 | 0·29 |

Absorbancy measured at 340 m; light path, 1 cm.

## DISCUSSION

The results obtained with the H LDH using different pyruvate and CPZ concentrations indicate an inhibition of the purely non competitive type, which means that the inhibitor is bound to the enzyme (*E*) as well as to the enzyme–substrate complex (*ES*). The inhibitor does not influence the formation of the *ES* but the enzyme complexes containing the inhibitor are completely inactive. Varying the lactate concentration in the presence of H LDH the CPZ inhibition is also of the non competitive type.

Varying the concentration of NADH and CPZ the Lineweaver–Burk and Dixon plots revealed, using H LDH as enzyme, parallel straight lines, thus the inhibition is uncompetitive, which means that the inhibitor is not bound to the free enzyme but only to the ECo complex. However the addition of NADH before incubating the enzyme with CPZ suspend the action of CPZ on the H LDH activity (Fig. 6); therefore the inhibition is only apparently uncompetitive. That means that the coenzyme is bound only to the EI complex.

Using the M LDH and varying the lactate, NADH and CPZ concentrations "competitive activation" resulted in the Lineweaver–Burk plots; using different pyruvate concentrations, the activation was also competitive.

The kinetic results obtained with M and H LDH as well as the results with adenine nucleotide and previous fluorescence measurements[2] on the binding of NADH to M LDH in the presence of CPZ all suggest and fit only with an allosteric action of CPZ on LDH. This might be a conformation change of the LDH subunit during previous incubation of CPZ with the enzyme, which is confirmed by the apparent uncompetitive and non competitive inhibitory mechanisms.

The results obtained with $CPZ^+$ indicate that for inhibition it is necessary that CPZ should be transformed to $CPZ^+$ for being active as suggested also for other dehydrogenases.[12,13] This transformation proceeds during the preincubation with the H LDH. The activating effect using M LDH is, however, instantaneous. The effects were similar in the reversed direction starting from lactate and NAD—although a direct reduction of NADH by $CPZ^+$ occurs instantaneously also[14]—the reversed action of $CPZ^+$ on the two types of LDH indicates a direct effect on the enzymes.

Summarizing the different actions of CPZ on the two types of LDH (M and H) both actions are minimal at substrate optimum. Decreasing the substrate concentration, inhibition or activation increases; increasing the substrate concentration higher than optimal concentration, inhibitory action only remains. This could be explained by a facilitating effect of CPZ on the formation of ternary LDH–NAD–pyruvate complexes.[15] The kinetics data obtained with coenzymes reveal that CPZ is apparently not fixed to the coenzyme binding site but influences the binding of ligands as an allosteric effector. The differences between H and M LDH could be explained by different bindings of CPZ to the enzymes. CPZ as a base forms salt complexes with acidic amino acids, which are more abundant in the H form of LDH. Thus the conformation change caused by CPZ which favors the activity of M LDH might act in the reverse direction using H LDH.

A possible physiological and pharmacological role of this regulatory action of CPZ in different tissues is discussed elsewhere.[16]

*Acknowledgements*—We gratefully acknowledge Drs P. Elődi and J. Südi for crystalline LDH preparations, Dr. T. Keleti for valuable discussion of the manuscript and Miss E. Róna for technical assistance.

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