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ALTERATION OF LIVER GLUTAMIC DEHYDROGENASE BY CHLORPROMAZINE

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Abstract—The effects of chlorpromazine on purified bovine liver glutamic dehydrogenase were studied. The amine at 5×10^{-5}M inhibited the reductive amination of α-ketoglutarate and the oxidation of glutamate, and stimulated the reductive amination of pyruvate catalyzed by the enzyme. These results are comparable to the effects of other modifiers of the enzyme described by other workers and apparently are due to an allosteric effect. The greatest change occurred around pH 8·5. With α-ketoglutarate as substrate the apparent K_m for NADH was decreased, whereas with pyruvate it was increased by chlorpromazine. The K_m for α-ketoglutarate was not affected by the amine. Chlorpromazine also appeared to inhibit the glutamate dehydrogenase activity of isolated rat liver mitochondria at levels that caused some uncoupling of oxidative phosphorylation.

CHLORPROMAZINE[1–5] or other phenothiazines[6,7] have been reported to inhibit several dehydrogenases. It seemed possible that the amine might also affect glutamic dehydrogenase, since this enzyme is influenced by a number of aromatic and other compounds, including thyroxin,[8] 1,10-phenanthroline,[9–11] and diethylstilbesterol,[11] which appear to act as allosteric modifiers, according to Frieden[12] and Tomkins *et al.*[13] The results show that chlorpromazine inhibits the glutamate dehydrogenase and stimulates the alanine dehydrogenase activity of the enzyme in a manner that is indeed analogous to the effects of these compounds.[11]

METHODS

Crystalline bovine liver glutamic dehydrogenase in 50 per cent glycerol and phosphate buffer, obtained from Sigma Chemical Company, was diluted for each experiment in 0·05 M potassium phosphate buffer, pH 7·4, containing 1×10^{-4} M EDTA. The enzyme concentration was measured spectrophotometrically using a value of 9·7 for $E_{280}^{1\%}$.[14] Enzyme activity was determined at room temperature by measuring the change in absorbance of NADH at 340 mμ (molar absorbance: $6{\cdot}22 \times 10^3$ $M^{-1}cm^{-1}$) with either a Beckman DU spectrophotometer coupled to a Sargent model SRLG recorder or a Cary model 14 recording spectrophotometer; quartz cuvettes with a 1·0 cm light path were used. The enzyme was added to the medium and preincubated for 3–4 min; then the reaction was initiated with the addition of NADH (or NAD). With α-ketoglutarate as a substrate, the rate measured between 5 and 15 sec, which

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was linear, was taken to represent the initial velocity. With pyruvate, the rate was linear over a longer period of time and was generally measured between 5 sec and 1 min.

The experiment with mitochondria was carried out by measuring oxygen uptake polarographically as described previously.[15] The vibrating reed, platinum electrode was lightly coated with collodion. ADP was added about 2 min after the mitochondria. With the amine, the initial rates tended to be erratic and also decreased with time. Therefore, the rates obtained between 1 and 2 min after the addition of ADP, rather than the initial values, are reported.

The four amines were kindly donated: chlorpromazine-HCl was obtained from Smith Kline & French Laboratories; imipramine-HCl from Geigy Pharmaceuticals; 1-(1-phenylcyclohexyl)piperidine-HCl (Sernyl, phencyclidine) from Parke, Davis & Company; and laurylamine-HCl from General Mills Chemical Division. The α-ketoglutarate was obtained from Calbiochem; pyruvate, glutamate, NAD and NADH were from Sigma Chemical Company.

RESULTS AND DISCUSSION

The effects of chlorpromazine on glutamic dehydrogenase varied with the substrate. With α-ketoglutarate the reductive amination reaction was inhibited, whereas with pyruvate it was stimulated (Fig. 1). The per cent inhibition was approximately equivalent to the per cent stimulation, 35 and 31 per cent, respectively, with 0·08 mM

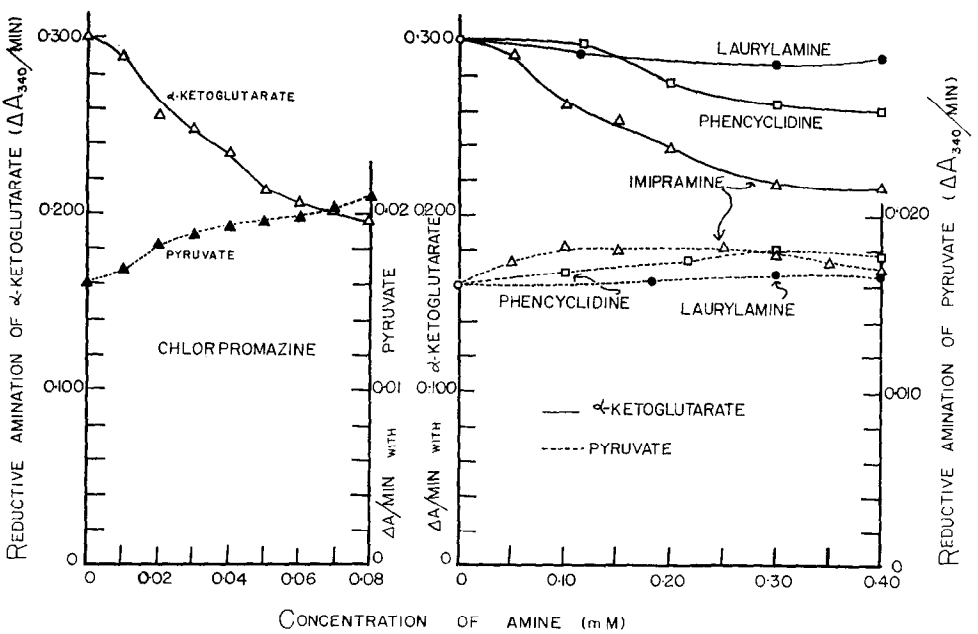

FIG. 1. Effects of varying concentrations of chlorpromazine, imipramine, phencyclidine, and laurylamine on the reductive amination of α-ketoglutarate and pyruvate by glutamic dehydrogenase. The medium contained 0·1 M NH_4Cl, 25 mM tris-HCl buffer, 0·1 mM EDTA, and 0·06 mM NADH, and either 20 mM α-ketoglutarate or 16 mM pyruvate. The enzyme concentration was 0·0019 mg protein/ml for experiments with α-ketoglutarate and 0·019 mg/ml with pyruvate. The pH was 8·4.

chlorpromazine. The concentration of the amine was limited to 0·08 mM, since higher levels produced a turbidity due to insolubility at the alkaline pH. A preincubation period of 3–4 min between the addition of enzyme and coenzyme was employed to assure sufficient time for the change in activity, although it was essentially complete within 1 min (data not shown). Three other amines, which have been compared in previous studies in this laboratory,[16,17] gave similar results (Fig. 1), but were less effective, even at 0·1–0·4 mM concentrations. Of the three, imipramine caused the greatest change in activity, which interestingly was more marked with α-ketoglutarate than with pyruvate. Phencyclidine was somewhat less effective. Laurylamine, which has about the same effect as chlorpromazine on oxidative phosphorylation,[15,16] caused only a small but consistent change, even at 0·4 mM concentration. Similar inverse effects of other compounds, including diethylstilbesterol, 1,10-phenanthroline and estrogenic steroids, on activity with these two substrates and also the corresponding amino acids, glutamate and alanine, were described by Tomkins *et al.*[11] Such changes are characteristic of this enzyme. Purine nucleotides also affect the enzyme; guanosine triphosphate gives similar results, whereas ADP stimulates the glutamate dehydrogenase and inhibits the alanine dehydrogenase activity.[18] All of these appear to act as allosteric modifiers of the enzyme.[12,13] Two monomeric forms of the enzyme have been postulated. One catalyzes the α-ketoglutarate–glutamate interconversion and has minimal alanine dehydrogenase activity; also it tends to associate to a polymer. The other form catalyzes the pyruvate–L-alanine interconversion and a corresponding reaction for other monocarboxylic L-amino acids.[18]

The influence of pH on the effects of chlorpromazine on these reactions was determined. With pyruvate as the substrate (Fig. 2), the increase in activity with the amine at 0·05 mM was greatest in the range of the optimal pH (around 8·8) and was least as

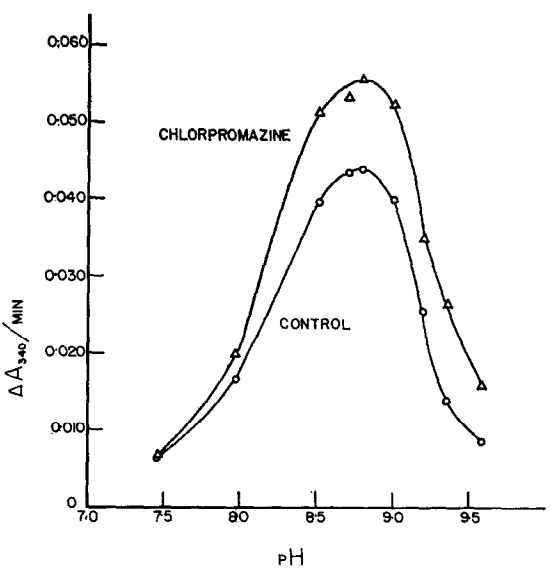

FIG. 2. Effect of chlorpromazine on the reductive amination of pyruvate at varying pH. The medium contained 0·1 M NH_4Cl, 50 mM tris-HCl buffer, 0·1 mM EDTA, 20 mM sodium pyruvate, 0·1 mM NADH, and the enzyme concentration was 0·027 mg protein/ml. Chlorpromazine was 0·05 mM.

the pH was decreased to 7·5. With α-ketoglutarate (Fig. 3), the amine had little effect at pH 6·8 and appeared to cause the greatest decrease in activity around pH 8·5. (For this reason the other experiments were carried out near pH 8·5.) It appeared that the pK value of the group (or groups) that controls the enzyme activity on the alkaline side of the pH activity curve may be shifted from pH 8·8 (the apparent pK for the control

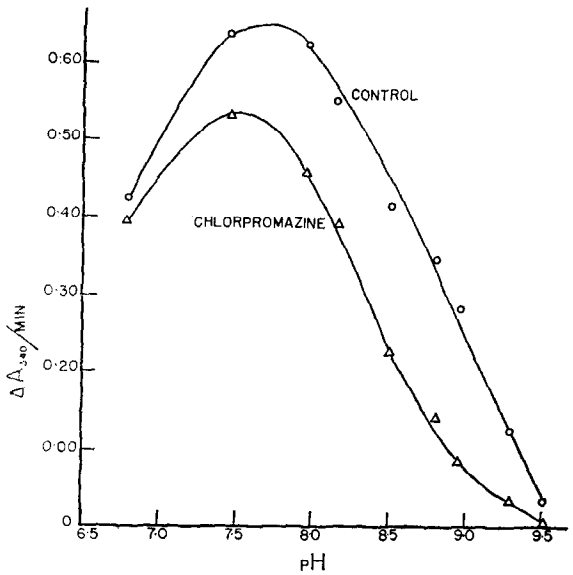

FIG. 3. Effect of chlorpromazine on the reductive amination of α-ketoglutarate at varying pH. The medium contained 0·1 M NH_4Cl, 50 mM tris-HCl buffer or 50 mM aminopropanediol-HCl buffer for pH 8·8–9·5 or higher, 0·1 mM EDTA, 25 mM α-ketoglutarate, 0·1 mM NADH, and the enzyme concentration was 0·0018 mg protein/ml. Chlorpromazine was 0·05 mM.

curve with α-ketoglutarate) to 8·4 by chlorpromazine. However, the enzyme is very complex and Bitensky *et al.*[19] have suggested that the allosteric effect may also be influenced by the pH *per se* (without a modifying compound present), to account in part for the greater activity with pyruvate and the decreased activity with α-ketoglutarate that occur as the pH is increased in the alkaline region (above pH 8).

Inhibition of the reductive amination of α-ketoglutarate by chlorpromazine was influenced by the concentration of NADH (Fig. 4). At high concentrations NADH *per se* tends to be inhibitory.[13,20,21] With chlorpromazine, the inhibitory effect at higher levels of NADH was much greater than with the coenzyme alone; in fact, the curve for the Lineweaver–Burk plot became hyperbolic as the concentration of NADH was increased above 0·1 mM. NADH has been shown to increase markedly the inhibitory effect of diethylstilbesterol[22] and of GTP and GDP[23] on the activity with this substrate. Yielding *et al.*[20] have concluded that this inhibition is not related to the conformational changes that shift activities for the different substrates.

With pyruvate (Fig. 5), the stimulatory effect of chlorpromazine on the reductive amination occurred at all concentrations of NADH employed, and the double reciprocal plot appeared to be a straight line throughout, in contrast to the curve obtained with α-ketoglutarate. A straight line was also obtained with the control data. However, sufficiently high levels of NADH do inhibit activity with pyruvate.[13,20]

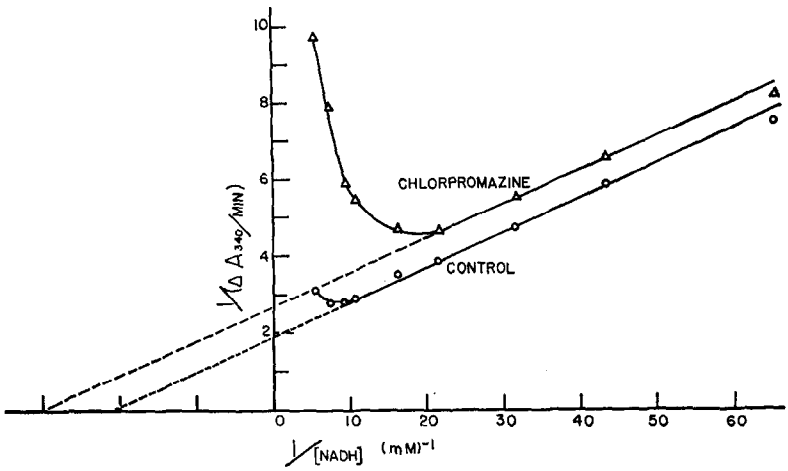

FIG. 4. Influence of chlorpromazine on the reductive amination of α-ketoglutarate at varying NADH concentrations. Double reciprocal plot of velocity versus NADH concentration. The medium was the same as that for Fig. 3, except that NADH was varied; the enzyme concentration was the same. Chlorpromazine was 0·05 mM; the pH was 8·5.

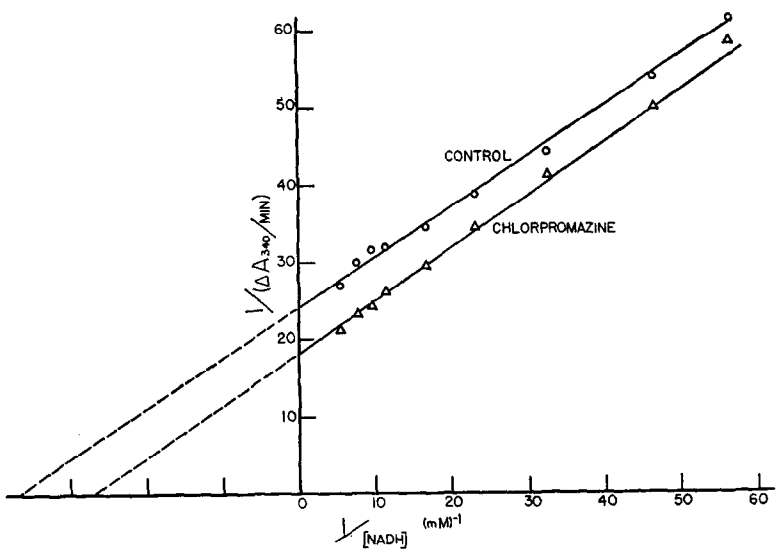

FIG. 5. Effect of chlorpromazine on the reductive amination of pyruvate at varying NADH concentrations. Double reciprocal plot of velocity versus NADH concentration. The medium was the same as that for Fig. 2, except that NADH was varied and the enzyme concentration was 0·024 mg protein/ml. Chlorpromazine was 0·05 mM; the pH was 8·5.

A comparison of the results with the two substrates suggests that chlorpromazine decreases the apparent K_m for NADH with α-ketoglutarate (considering the data obtained at the lower concentrations of NADH) and increases the apparent K_m for NADH with pyruvate. Other modifiers have also been shown to affect the binding of the coenzyme. Frieden[24] observed that, with α-ketoglutarate as the substrate, the Michaelis constant for NADPH was lowered by GTP, which inhibits this activity of

the enzyme, and was increased by ADP, which is stimulatory, and furthermore, that the NADPH binding constant was decreased by GTP and increased by ADP when measured by fluorescence quenching (no substrate added). Yielding and Holt[25] determined NADH binding constants by equilibrium dialysis and obtained similar effects with these two nucleotides. In addition, the data indicated only one binding site for NADH per protein chain with a molecular weight of 52,000. (There are about 8 chains in the monomeric form, mol. wt. 400,000, which may associate to give a polymer with mol. wt. 1·6 to 2 × 10^6.) Apparently this is the active site, and ADP and GTP did not alter the number of sites. Frieden[24] concluded that a purine nucleotide modifier is bound to a second site, but this does not mean that all of the various modifiers are bound to the same site. The possibility of another site was suggested by Yielding *et al.*[22] from evidence that binding of diethylstilbesterol was not influenced by ADP yet was affected by leucine, which is a modifier that stimulates the glutamate dehydrogenase activity.[26] This may apply to chlorpromazine, and furthermore, the changes observed with the four amines (Fig. 1) are not necessarily due to the same mechanism of interaction with the protein; for example, laurylamine might act in a different manner.

The apparent K_m for α-ketoglutarate was not affected by chlorpromazine (Fig. 6; noncompetitive inhibition).

In accord with the results with α-ketoglutarate, chlorpromazine also inhibited the oxidation of glutamate by the enzyme (Fig. 7). However, at low concentrations the amine had a greater effect with α-ketoglutarate (Fig. 1), but not simply because the enzyme concentration was half. In another experiment, 0·04 mM chlorpromazine caused a 45 per cent inhibition of activity with α-ketoglutarate (0·1 mM NADH) and only 10 per cent with glutamate and the same level of enzyme. The difference may have

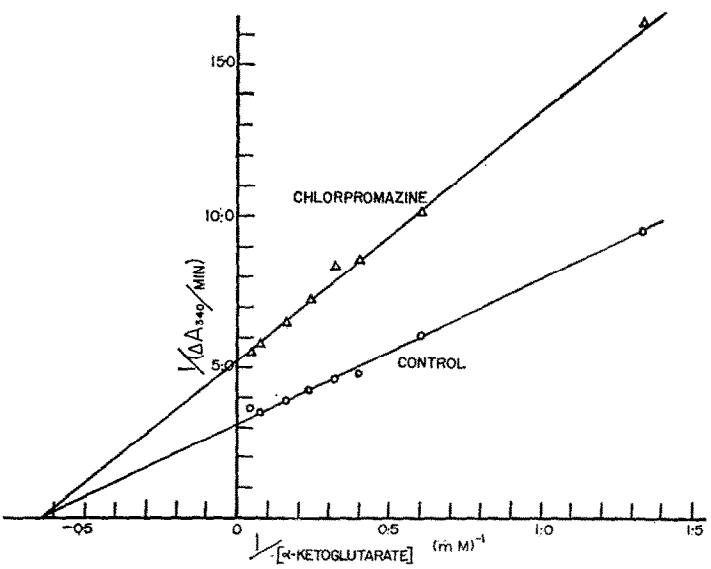

FIG. 6. Influence of chlorpromazine on the reductive amination of α-ketoglutarate. Double reciprocal plot of velocity versus α-ketoglutarate concentration. The medium was the same as that for Fig. 4, except that α-ketoglutarate was varied. NADH was 0·1 mM; chlorpromazine was 0·05 mM; the pH was 8·5.

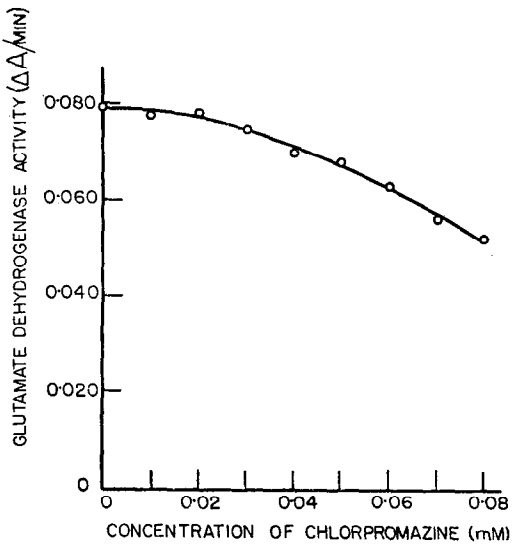

FIG. 7. Effect of chlorpromazine on glutamate dehydrogenase activity. The medium contained 50 mM NaCl, 25 mM tris-HCl buffer, 0·1 mM EDTA, 60 mM monosodium glutamate, 0·2 mM NAD, and the enzyme concentration was 0·0037 mg protein/ml. Chlorpromazine was 0·05 mM; the pH was 8·4.

been due to the presence of NADH, which does enhance the effects of other modifiers.[25]

Levy and Burbridge[5] have attributed an inhibition of uridine diphosphate glucose dehydrogenase by chlorpromazine to the free radical form of the amine. In experiments reported here, solutions of the amine were prepared just prior to use, protected from sunlight when necessary, and on standing tended to lose effectiveness. Although not tested directly, it is unlikely that the results were due to the free radical.

Glutamic dehydrogenase is a mitochondrial enzyme. It is well established that chlorpromazine *in vitro* uncouples oxidative phosphorylation and may also inhibit mitochondrial respiration.[27] With glutamate as the substrate, a decrease in oxygen uptake could result from an inhibition of electron transport as well as an inhibition of glutamic dehydrogenase. Therefore, the effects of chlorpromazine on respiration with glutamate and α-ketoglutarate were compared using rat liver mitochondria (Fig. 8). ADP was added to give maximal respiration. (The low level employed did not *per se* affect the glutamic dehydrogenase.) As the concentration of the amine was increased, levels that stimulated respiration with α-ketoglutarate were inhibitory with glutamate; at 0·2 mM the amine caused an inhibition with both substrates, but the effect was much greater with glutamate where, even with a higher control rate, oxygen uptake was less than with α-ketoglutarate. The amine certainly appeared to inhibit the glutamate dehydrogenase activity of the mitochondria. Although a difference in an effect on mitochondrial membrane permeability to the two substrates has not been ruled out, the data are entirely in accord with the results obtained on the purified enzyme. The experiments also demonstrate that, in studying the effects of any compound on mitochondrial respiration, when an inhibition is observed with glutamate as substrate, an alteration of glutamic dehydrogenase as well as electron transport should be considered.

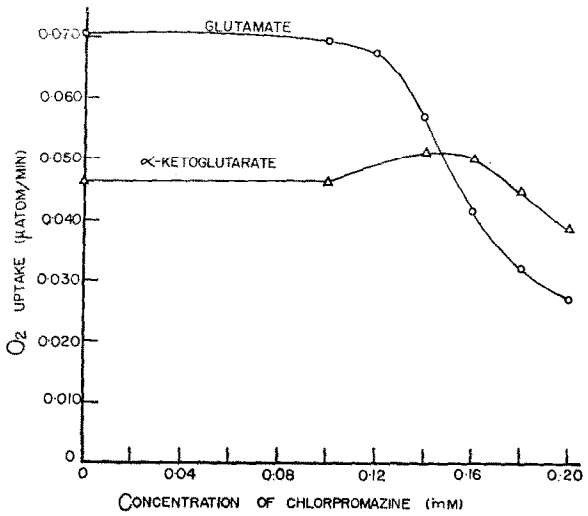

FIG. 8. Comparison of the effect of chlorpromazine on mitochondrial respiration with glutamate and α-ketoglutarate. The medium contained in 1·4 ml: 87 μmoles KCl, 10 μmoles potassium phosphate, 6·7 μmoles $MgCl_2$, 33 μmoles tris-HCl buffer, 15 μmoles substrate (monosodium glutamate or α-ketoglutarate neutralized with KOH), 0·260 μmole ADP and rat liver mitochondria (1·4 mg protein). The pH was 7·4 and the temperature 23°.

At the levels required, the amine also caused some uncoupling of oxidative phosphorylation. With 0·14 mM chlorpromazine, the ADP:O ratio with glutamate was about 60 per cent of the control ratio. Despite innumerable studies on the influence of the amine on mitochondria *in vitro*, there is still some question regarding the importance of this effect *in vivo*, which obviously would apply also to an alteration of this mitochondrial enzyme. Platt and Cockrill[28] measured the glutamic dehydrogenase activity of liver after oral administration of chlorpromazine to rats and found no significant change. However, liver does metabolize the amine and other tissues might be more sensitive. Also, the effect of the amine on glutamic dehydrogenase could be enhanced under different conditions. The activity of the enzyme within the tissues could be regulated by the purine nucleotides as discussed by Frieden.[29] A second modifier might have an effect *per se*, but, in addition, it could alter the response to compounds such as the nucleotides. Probably the most important effect would be on the glutamate dehydrogenase activity, since the alanine dehydrogenase activity is considerably less (even with a higher level of enzyme, the activity was less than one-tenth; see Fig. 1).

Klingenberg[30] has proposed that the major physiological function of glutamic dehydrogenase in liver is the incorporation of ammonia. This may be important in brain;[31] ammonia nitrogen is incorporated into the α-amino as well as the amido nitrogen of glutamine.[32] Although chlorpromazine might conceivably alter the levels of glutamate or ammonia, it should be emphasized that mitochondrial metabolism of glutamate is complex. Not only are there alternate pathways, but the reaction catalyzed by glutamic dehydrogenase is affected by other reactions.[30] The amine might have other effects on the mitochondria to alter the levels of intermediates in the tricarboxylic acid cycle or the reduction of NAD and NADP, which could influence the enzyme as well as the other pathways. The effects of chlorpromazine on glutamic dehydrogenase of brain are under investigation.

Note added in proof—Since this work was submitted for publication, Fahien and Shemisa[33] have reported that chlorpromazine inhibits glutamate dehydrogenase, mainly by increasing inhibition with NADH, and also suggested that it is bound to an allosteric site.

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