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# Effects of Chlorpromazine on Glutamate Dehydrogenase

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SUMMARY

Certain drugs which affect behavior, such as chlorpromazine, desipramine, imipramine, and amitriptyline, are inhibitors of glutamate dehydrogenase (EC 1.4.1.3). Of this group, chlorpromazine was found to be the most potent inhibitor. Chlorpromazine inhibits reactions with DPNH more than TPNH, mainly by increasing substrate inhibition with DPNH. Chlorpromazine has no effect on the reverse reaction with DPN and glutamate. This drug is not a competitive inhibitor of the coenzymes or substrates of the glutamate dehydrogenase reaction. The effect of chlorpromazine is almost completely abolished by ADP and GTP, but not ATP. These results suggest that chlorpromazine is bound to an allosteric site on the enzyme. Like GTP, chlorpromazine in the presence of DPNH produces dissociation of the enzyme.

Chlorpromazine does not inhibit glutamine synthetase (EC 6.3.1.2) or the mitochondrial glutamate-oxalacetate transaminase (EC 2.6.1.1). On the basis of these results it seems likely that the inhibition of glutamate dehydrogenase by chlorpromazine could increase brain levels of glutamine in the presence of high levels of ATP and DPNH. When the levels of ADP and DPN are high chlorpromazine has little effect on glutamate dehydrogenase. Since the levels of glutamate dehydrogenase are high in brain, liver, and kidney mitochondria, and since many compounds of diverse structures are bound to glutamate dehydrogenase, this enzyme could possibly play a role in binding and consequently concentrating some drugs in the mitochondria.

The kinetic properties of bovine heart, brain, and adrenal medulla glutamate dehydrogenase were found to be identical with those of the liver enzyme. These plus other experiments indicate that this enzyme is similar in these bovine organs. Similarly, the kinetic properties of bovine brain mitochondrial glutamate-oxalacetate transaminase are identical with those of the liver enzyme.

## INTRODUCTION

It is known that many ligands, such as ADP, GTP, and steroids, are allosteric modifiers of glutamate dehydrogenase [L-glutamate:NAD(P) oxidoreductase, EC 1.4.1.3] (1, 2). Since the level of this enzyme is high in brain (3, 4), and glutamate apparently plays an important role in the chemistry of the central nervous system (5–7), the effects of some drugs which are known to affect behavior were studied as modifiers of glutamate dehydrogenase.

It seems likely that the physiological function of glutamate dehydrogenase could be different in liver, which has an active carbamyl phosphate synthetase (8, 9), from its function in organs which lack the latter

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enzyme, such as brain. Therefore glutamate dehydrogenase might possess different properties in brain and liver. Previous studies have shown that the allosteric properties of this enzyme are the same in many bovine organs (3). However, it is known that the allosteric properties of glutamate dehydrogenase isolated from livers of various species of animals can be almost identical while the reaction at the active site (Michaelis constants of coenzymes and substrates) is quite different (9–11). Therefore, the kinetic and electrophoretic properties of glutamate dehydrogenase prepared from various bovine organs were compared.

## MATERIALS AND METHODS

*Enzymes and reagents.* Bovine liver glutamate dehydrogenase and mitochondrial glutamate-oxalacetate transaminase were prepared by methods described previously (12–14). The brain (gray matter) enzymes were prepared by similar procedures, except that the mitochondrial fraction was prepared in 0.4 M sucrose–0.02 M Tris-chloride–0.1 mM EDTA, pH 7.4 (15). The best yields of brain mitochondrial glutamate-oxalacetate transaminase and glutamate dehydrogenase were obtained by homogenizing the thawed mitochondria in 0.02 M Tris-chloride, pH 7.5 (12). These two enzyme preparations had specific activities 10- and 2-fold lower, respectively, than the crystalline liver enzymes (12, 13).

Similar methods were rather unsatisfactory for the preparation of bovine heart and adrenal medulla glutamate dehydrogenase, and therefore preparations which were 16-fold lower in specific activity than the crystalline liver enzymes were used. With these preparations corrections for DPNH oxidase activity (reaction in the absence of $\alpha$-ketoglutarate and ammonium ions in the dialyzed enzyme preparations) were necessary.

Sheep brain glutamine synthetase was obtained from P-L Biochemicals, Inc. Other enzymes and substrates were obtained from Sigma Chemical Company. Drugs were generously supplied by Geigy Chemical Corporation (desipramine and imipramine), Hoffmann-La Roche (chlordiazepoxide), Smith Kline & French (chlorpromazine), and Merck (amitriptyline). These drugs were sufficiently soluble in water to be used in these experiments, and were used at concentrations sufficiently low that they did not alter the pH of enzyme assay mixtures. Solutions of chlorpromazine were protected from exposure to light.

*Initial velocity measurements.* Enzyme assays were carried out in 0.025 M sodium arsenate with 0.1 mM EDTA, pH 7.8, at 25°. The enzyme was dialyzed against this buffer before use in assays. Reactions were followed spectrophotometrically by means of a Gilford model 2000 recorder and a Beckman DU monochromator. Standard assays were performed periodically during the course of kinetic experiments, and corrections were made for loss of enzyme activity (10–13). Since many of the drugs used in these experiments absorb light at 340 m$\mu$ (the wavelength of maximal absorption of DPNH), control experiments were performed in the absence of enzyme and in the presence of enzyme but absence of DPNH. These drugs were found to be stable during the few minutes required for initial velocity measurements. Kinetic experiments were performed at least three times, and experimental points represent average values. These experiments were essentially completely reproducible.

*Protein determination.* The concentration of glutamate dehydrogenase was determined by measuring the absorbance at 280 m$\mu$ (16).

*Electrophoresis.* Electrophoresis on cellulose polyacetate strips with a Gelman electrophoresis chamber was performed according to previously described methods (17).

*Ultracentrifugation.* Sedimentation experiments were performed in a Spinco model E analytical centrifuge at 20°.

## RESULTS

*Kinetic and electrophoretic properties of the enzyme in various organs.* The apparent Michaelis constants for TPNH, DPNH, DPN, glutamate, $\alpha$-ketoglutarate, and ammonium ions were measured with bovine adrenal medulla, brain, and heart glutamate dehydrogenase. The values of these constants were found to be the same as those previously reported for the bovine liver enzyme (13, 18). Even the complex kinetic re-

action of DPN (substrate activation) (13) and the ratio of reactivity of DPNH to TPNH were identical with all enzymes. The electrophoretic mobility was also found to be identical for the brain and liver enzymes.

Kinetic results with brain mitochondrial glutamate-oxalacetate transaminase were also the same as those previously reported for the liver enzyme (13, 18).

*Effects of drugs.* Figure 1 shows a plot of velocity of glutamate dehydrogenase with respect to the concentration of various drugs. These results can be evaluated with the general equation for the effects of modifiers on glutamate dehydrogenase (Eq. 1) (2).

$$v = \frac{V_1 + V_2 (M)/K_3}{1+[K_1/(S)][1+(M)/K_2]+(M)/K_3} \quad (1)$$

where $v$ is the initial velocity; $V_1$ and $V_2$ are, respectively, the velocities in the presence of saturating concentrations of coenzyme and in the absence and presence of saturating concentrations of the drug; $K_1$ is the Michaelis constant of coenzyme ($S$) in the absence of the drug; and $K_2$ and $K_3$ are, respectively, the apparent dissociation constants of the drug ($M$) in the absence and presence of saturating concentrations of coenzyme.[2] When the concentration of coenzyme is saturating (as is essentially the case in the experiments shown in Fig. 1), Eq. 1 can be simplified to Eq. 2.

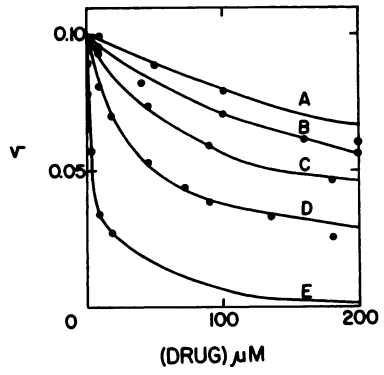


FIG. 1. *Plot of velocity of glutamate dehydrogenase with respect to concentration of amitriptyline (A), desipramine (B), imipramine (C), chlorpromazine (D), and diethylstilbestrol (E)*

The lines have been calculated with the use of Eq. 2 and the values of the constants shown in Table 1. The points represent experimental values. The concentrations of substrates used were: DPNH, 0.1 mM; α-ketoglutarate, 2.0 mM; and ammonium chloride, 50 mM. The experiments were performed in 0.025 M sodium arsenate–0.1 mM EDTA, pH 7.8, at 25°.

TABLE 1

*Values of modifier constants of drugs on glutamate dehydrogenase*

These determinations are based upon the results shown in Fig. 1 plus additional experiments with concentrations of the drugs as high as 0.5 mM. The concentration of substrates in these experiments were: α-ketoglutarate, 2.0 mM; DPNH, 0.1 mM; and ammonium chloride, 50 mM. Experiments were performed in 0.025 M sodium arsenate—0.1 mM EDTA, pH 7.8. $V_1$ and $V_2$ are velocities in the presence of saturating concentrations of coenzyme and in the absence ($V_1$) or presence ($V_2$) of saturating concentrations of drugs.

| Drug | $K_3$ | $V_1:V_2$ |
|---|---|---|
| | μM | |
| Diethylstilbestrol | 6 | 50 |
| Chlorpromazine | 32 | 6 |
| Imipramine | 80 | 4 |
| Desipramine | 200 | 8 |
| Amitriptyline | 400 | 20 |

$$(V_1 - v) = \frac{V_1 - V_2}{1 + K_3/(M)} \quad (2)$$

Therefore, from double-reciprocal plots of the change in velocity produced by the drug ($V_1 - v$) with respect to the concentration of drug added ($M$), the values of $K_3$ and $V_2$ can be determined. A summary of these determinations is shown in Table 1. Not only is Eq. 2 useful for estimating the values of these constants, but linear double-reciprocal plots suggest that there is no interaction between modifier-binding sites or more than one type of binding site; that is, the effect of the drug can be described by simple Michaelis-Menten type binding. This was found to be the case with the drugs listed in Table 1. For comparison, results with diethylstilbestrol, a drug known to inhibit glutamate dehydrogenase (1), have been added to Fig. 1.

[2] The term $K_3$ will be referred to as an apparent dissociation constant. It is known that this might not be the true value of the dissociation constant of chlorpromazine.

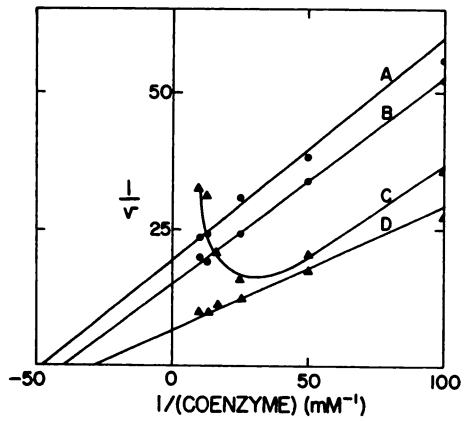


FIG. 2. *Double-reciprocal plot of velocity with respect to concentration of either TPNH (●) or DPNH (▲) in the absence (curves B and D) and presence (curves A and C) of 180 μM chlorpromazine*

Other experimental conditions were: 2.0 mM α-ketoglutarate and 50 mM ammonium chloride. Remaining experimental conditions are given in the legend to Fig. 1.

The value of $K_3$ for diethylstilbestrol is quite low; however, concentrations of 17β-estradiol [one of the most tightly bound steroids (19)] as high as 70 μM must be added to give 50% inhibition under the conditions of these assays. Ouabain, strophanthidin, phenobarbital, γ-aminobutyric acid, morphine, caffeine, quinine, and chlordiazepoxide had either no or only a slight (1.1-fold) inhibitory effect on glutamate dehydrogenase when added in concentrations as high as 0.1 mM.

Since chlorpromazine was found to be the most potent inhibitor in this group of drugs, which are known to affect behavior, additional experiments were performed with it.[3] Chlorpromazine in concentrations as high as 0.1 mM had no effect on mitochondrial glutamate-oxalacetate transaminase, glutamine synthetase, or malate or lactate dehydrogenase. In all these experiments the absorption spectrum of the chlorpromazine used was that of chlorpromazine itself and not the free radical (20). Additional irradiation of chlorpromazine (20) did not alter the inhibition of glutamate dehydrogenase, suggesting that formation of the free radical was not necessary. Also, prior incubation of chlorpromazine with the enzyme did not alter the amount of inhibition produced. Chlorpromazine is a better inhibitor of reactions with DPNH than those with TPNH, and inhibits mainly by increasing the amount of substrate inhibition by DPNH (Fig. 2). Similar results have been described pre-

[3] The results shown in Figs. 1–8 were obtained with the purer, more readily available liver enzyme. However, both the liver and brain enzymes behave identically with respect to inhibition by chlorpromazine.

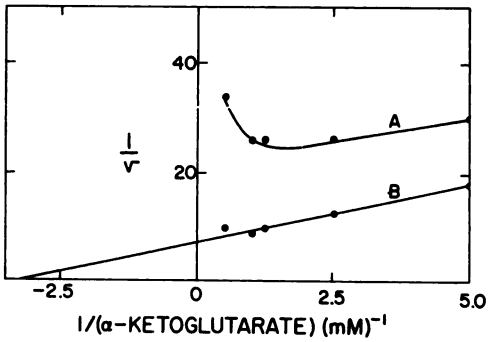


FIG. 3. *Double-reciprocal plot of velocity with respect to concentration of α-ketoglutarate in the absence (curve B) and presence (curve A) of 180 μM chlorpromazine*

Other experimental conditions were: 0.1 mM DPNH and 50 mM ammonium chloride. Remaining experimental conditions are given in the legend to Fig. 1.

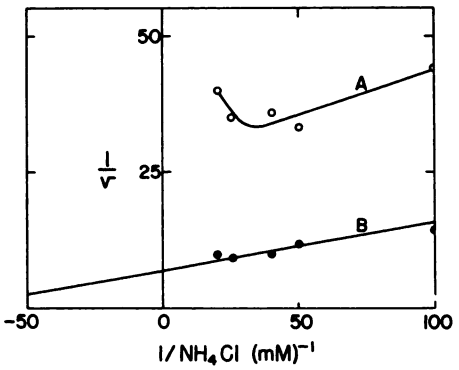


FIG. 4. *Double-reciprocal plot of velocity with respect to concentration of ammonium chloride in the absence (curve B) and presence (curve A) of 180 μM chlorpromazine*

Additional experimental conditions were: 0.1 mM DPNH and 2.0 mM α-ketoglutarate. Remaining experimental conditions are given in the legend to Fig. 1.

viously with the purine nucleotide GTP (2), which, like chlorpromazine, increases the amount of substrate inhibition by DPNH. Figures 3 and 4 show the results obtained when the concentrations of $\alpha$-ketoglutarate and ammonium ions are varied. Again, in the presence of chlorpromazine there is a greater degree of substrate inhibition. While chlorpromazine inhibits the rate of oxidation of DPNH it has no effect on the reverse reaction with DPN and glutamate. In these latter experiments either DPN was varied from 0.05 to 1 mM and glutamate was maintained constant at 10 mM, or DPN was maintained constant at 1 mM and glutamate was varied between 1 and 10 mM.

The results shown in Figs. 2–4 demonstrate that chlorpromazine is not a competitive inhibitor of the coenzymes or substrates of the glutamate dehydrogenase reaction. As shown in Figs. 5 and 6, the effect of chlorpromazine, like that of diethylstilbestrol, is decreased in the presence of either ADP or GTP, but ATP has very little effect. Like GTP, another inhibitor of glutamate dehydrogenase activity, chlorpromazine, has less effect on glutamate dehydrogenase activity in the presence of high concentrations of enzyme (21) (Fig. 7). Unlike GTP, however, chlorpromazine does not potentiate alanine dehydrogenase activity (1) (Fig. 7). While chlorpromazine has very little effect on enzyme activity when the concentration of

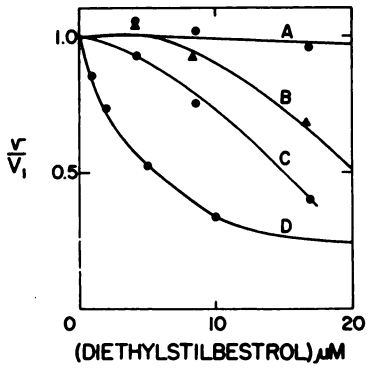


FIG. 5. *Plot of ratio of velocity in the presence to that in the absence of diethylstilbestrol with respect to concentration of diethylstilbestrol in the absence (curve D) and presence (curves A–C) of various concentrations of ADP*

The concentrations of ADP used were 0.1 mM (*A*), 0.08 mM (*B*), and 0.04 mM (*C*). Remaining experimental conditions and concentrations of substrates and DPNH are given in the legend to Fig. 1.

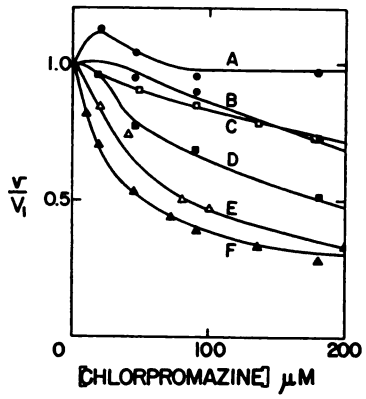


FIG. 6. *Plot of ratio of velocity in the presence to that in the absence of chlorpromazine with respect to concentration of chlorpromazine in the absence (curve F) and presence of 0.1 mM GTP (curve C), 0.1 mM ATP (curve E), or various concentrations of ADP*

The concentrations of ADP were 0.1 mM (curve *A*), 0.04 mM (curve *B*), or 0.02 mM (curve *D*).

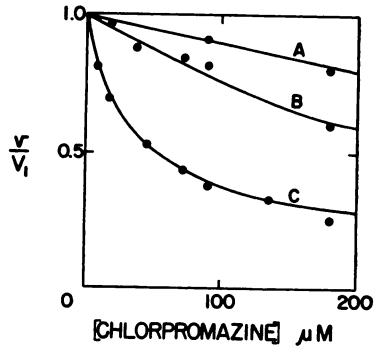


FIG. 7. *Plot of ratio of velocity in the presence to that in the absence of chlorpromazine with respect to concentration of chlorpromazine in the presence of various concentrations of enzyme*

The concentration of enzyme used in the experiments was 0.4 mg/ml (*A*), 0.02 mg/ml (*B*), or 0.4 μg/ml (*C*). In all experiments, 0.1 mM DPNH was used as coenzyme. Additional experimental conditions were: 0.9 mM pyruvate and 50 mM ammonium chloride (*A*), 0.1 mM $\alpha$-ketoglutarate and 0.1 mM ammonium chloride (*B*), and 2.0 mM $\alpha$-ketoglutarate and 50 mM ammonium chloride (*C*). Remaining experimental conditions are given in the legend to Fig. 1.

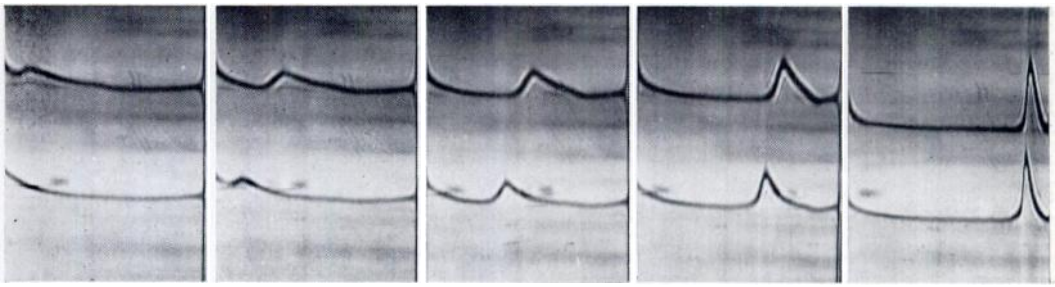

FIG. 8. *Ultracentrifugal patterns of 2.0 mg/ml solutions of bovine liver glutamate dehydrogenase in the absence (lower pattern) and presence (upper pattern) of 0.1 mM chlorpromazine*

Sedimentation is from right to left, at a velocity of 59,780 rpm; photographs were taken at 8-min intervals. These experiments were performed in 0.18 mM DPNH–0.025 mM sodium arsenate–0.1 mM EDTA, pH 7.8, at 20°.

enzyme is high, it is obviously bound under these conditions, since, in the presence of DPNH, chlorpromazine produces dissociation of the enzyme (Fig. 8).[4]

## DISCUSSION

No differences were detected among heart, brain, liver, and adrenal medulla glutamate dehydrogenase. These, plus other experiments (3), indicate that this enzyme is similar in these bovine organs, and that only its level varies to meet the physiological requirements of the organ.

Chlorpromazine and isosteres of phenothiazine, such as imipramine, desipramine, and amitriptyline, are inhibitors of glutamate dehydrogenase. On the basis of inhibition constants (Table 1) the order of specificity is chlorpromazine > imipramine > desipramine > amitriptyline. Therefore, under these experimental conditions and over this limited range of drug concentrations (10–100 μM), chlorpromazine would be the most potent inhibitor of glutamate dehydrogenase activity. Over a 10-fold higher range of drug concentration, amitriptyline would be the most potent inhibitor. Since chlorpromazine has the lowest dissociation constant, additional experiments were performed with this drug.

The formation of a free radical with chlorpromazine is apparently not required for binding. This drug had no effect on other enzymes such as glutamine synthetase, glutamate-oxalacetate transaminase, or malate or lactate dehydrogenase. Chlorpromazine inhibits mainly by increasing the amount of substrate inhibition by DPNH (Fig. 2). It has less effect on the reaction with TPNH, and no effect on the reverse reaction with DPN and glutamate. It is not a competitive inhibitor of any of the substrates of the glutamate dehydrogenase reaction. Therefore these results suggest that these drugs are bound to an allosteric site on the enzyme. Purine nucleotides decrease but do not completely abolish inhibition by chlorpromazine.

If chlorpromazine is bound to the same site as purine nucleotides, then, in the presence of 0.1 mM DPNH and 0.1 mM GTP, the addition of 200 μM chlorpromazine should activate the glutamate dehydrogenase reaction about 1.3-fold.[5] As shown in

[4] In the presence of arsenate or phosphate buffer, DPNH does not produce dissociation of the enzyme unless it is present in rather high concentrations. This can be seen in Fig. 8, where the $s_{20,w}$ in the absence of chlorpromazine and the presence of 0.18 mM DPNH is about 28. This is about the maximal sedimentation coefficient of fully associated glutamate dehydrogenase (2, 21). Under the same experimental conditions (buffer, temperature, and protein concentration) and in the absence of DPNH, the sedimentation coefficient is about 21. In the presence of chlorpromazine and DPNH the sedimentation coefficient is about 20 (Fig. 8). The effect of DPNH as a substrate inhibitor and on the molecular weight of the enzyme is complicated and is dependent upon the concentration of anions and enzyme (2, 21; L. A. Fahien, unpublished observations).

[5] These estimates are based on previously published data obtained under identical experimental conditions (18).

Fig. 6, chlorpromazine actually inhibits about 1.3-fold under these conditions. Similarly, in the presence of 0.1 mM ADP, the addition of 200 μM chlorpromazine should inhibit about 1.7-fold. As shown in Fig. 6, low concentrations of chlorpromazine slightly activate and concentrations as high as 200 μM have essentially no effect in the presence of 0.1 mM ADP. In the presence of 0.1 mM ATP the addition of 200 μM chlorpromazine should have essentially no effect. As shown in Fig. 6, the addition of 200 μM chlorpromazine in the presence of 0.1 mM ATP inhibits about 2.5-fold. Therefore, on the basis of these calculations, it would seem that chlorpromazine is not bound to the same site as purine nucleotides. Chlorpromazine enhances inhibition by GTP, has very little effect on activation by ADP, and inhibits the activating effect of ATP. It is known that ATP and GTP can increase and ADP decrease the amount of substrate inhibition by DPNH (2). ATP is an activator only when the concentration of DPNH is sufficiently low not to cause substrate inhibition (less than 0.2 mM DPNH under these conditions in the absence of chlorpromazine). In the presence of higher concentrations of DPNH, ATP is an inhibitor as a result of increasing substrate inhibition by DPNH. Therefore chlorpromazine enhances inhibition by purine nucleotides such as ATP and GTP, which inhibit at least in part by increasing the substrate inhibition by DPNH. Chlorpromazine has little effect on activation by ADP, and ADP decreases substrate inhibition by DPNH (2). Therefore these results are consistent with the concept that chlorpromazine increases substrate inhibition by DPNH (Fig. 2) and also enhances the ability of ATP and GTP to increase substrate inhibition by DPNH. In other words, if substrate inhibition by DPNH is the result of its binding to an inactive, inhibitory site [as has been suggested previously (2)], then chlorpromazine enhances binding of DPNH at this site and operates synergistically with ATP and GTP, also enhancing the binding of DPNH to the inhibitory site. Chlorpromazine has little effect in the presence of ADP, which decreases the affinity of DPNH at this inhibitory site. It is not unequivocally known if there are identical or overlapping sites for ADP and GTP (22, 23).

Chlorpromazine has less effect in assays which require a high concentration of enzyme, even though the drug is bound under these conditions, since it produces dissociation of the enzyme.[4] This is found with other allosteric inhibitors of glutamate dehydrogenase activity, such as GTP, owing in part to decreased binding of GTP to the enzyme when polymers of the enzyme are present (21). It is also due to the fact that at high concentrations of enzyme there is no substrate inhibition by DPNH even in the presence of GTP.[6] When the concentration of enzyme is low, GTP potentiates substrate inhibition by DPNH (2, 3). Chlorpromazine inhibits mainly as a result of increasing substrate inhibition by DPNH. Therefore, as with GTP, one reason there is less inhibition at high concentrations of enzyme is the absence of substrate inhibition by DPNH. While the effects of chlorpromazine and GTP are quite similar, GTP activates and chlorpromazine slightly inhibits the reaction with pyruvate. Also, GTP inhibits and chlorpromazine has no effect on the reverse reaction with DPN and glutamate.

The complexity of the glutamate dehydrogenase reaction makes it difficult at this time to develop a mechanism which completely describes the action of chlorpromazine on this enzyme. Nevertheless it is interesting to speculate that binding of chlorpromazine to glutamate dehydrogenase could in some way be related to the pharmacological properties of the drug. In these respects results obtained with chlorpromazine are different from those obtained with diethylstilbestrol and sex hormones. For example, the level of glutamate dehydrogenase is high in brain and low in target organs for sex hormones (4). The inhibition constant ($K_3$) of chlorpromazine is about 10 times lower than estimates of levels of this drug in brain after a pharmacological dose (24). While diethylstilbestrol is a potent inhibitor, naturally occurring steroids are much less potent (19). Furthermore, it is known that chlorpromazine can enter mitochondria and has a greater effect on brain than on liver mitochondria (25).

Glutamate, either directly or as a precursor of γ-aminobutyric acid, apparently

[6] L. A. Fahien, unpublished observations.

plays an important role in the central nervous system (5–7). Glutamate dehydrogenase and glutamate-oxalacetate transaminase are part of the pathway for the biosynthesis of γ-aminobutyric acid (5). The inhibition by chlorpromazine of glutamate production by glutamate dehydrogenase, but not by the transaminase, may well have physiological consequences, because most inhibition occurs when the concentration of DPNH is high. Under these conditions the levels of ATP in the cell are also high (26). ATP is not bound tightly to glutamate dehydrogenase (2) and has little effect on inhibition by chlorpromazine. Consequently, in the presence of high concentrations of ATP and DPNH, chlorpromazine would inhibit incorporation of ammonia into glutamate via glutamate dehydrogenase. For this reason, and because of the high levels of ATP, incorporation of ammonia into glutamine by glutamine synthetase might be favored. Chlorpromazine has no effect on this enzyme. If the levels of glutamate are low, this substrate of the glutamine synthetase reaction could be produced from α-ketoglutarate by the transaminase. The transaminase is probably the preferred pathway of glutamate synthesis (18), and is not inhibited by chlorpromazine. It is known that chlorpromazine increases the levels of glutamine in the brain (28). If the ADP:ATP and DPN:DPNH ratios are high, chlorpromazine would have little effect on glutamate dehydrogenase.

Many physiologically important compounds of various structures are bound rather tightly to allosteric sites of glutamate dehydrogenase. The level of this enzyme is high in brain, liver, and kidney mitochondria (3, 4). Therefore it seems possible that this enzyme could play a role in binding and consequently concentrating such drugs as chlorpromazine in the mitochondria of these organs. The amount of drug bound to glutamate dehydrogenase would be under rather sensitive control and would depend upon the concentration of enzyme and level of purine nucleotides in the mitochondria.

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