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THE INTERACTION OF CHLORPROMAZINE AND BUTYROPHENONES WITH GLUTAMATE DEHYDROGENASE

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Abstract—Experiments on the binding of radioactive chlorpromazine to glutamate dehydrogenase, at pH 7.0 and 25° in 100 mM KCl, reveal the presence of two sites for the drug with an apparent dissociation constant, $K_{D\text{app}}$, of 66 μM. In the presence of the substrates α-ketoglutarate and NADH, the affinity of one of the sites for chlorpromazine remains essentially unchanged, whereas that of the other is much lowered ($K_{D\text{app}} \sim 200$ μM). Apparently only the low affinity site of the enzyme is expressed kinetically as $K_{D\text{app}}$ for the inhibition of enzyme catalysis (in the direction of glutamate formation) is 136 μM. Each of the two sites in the hexameric enzyme molecule seems to be located in a domain formed by three polypeptide chains. K^+ and Rb^+ are competitive inhibitors of NH_4^+, the third substrate. In the presence of α-ketoglutarate and NADH, with K^+ replacing NH_4^+, the two chemically identical domains become conformationally different. Evidence is brought together, from the literature and the present work, to show that the pharmacological effects of phenothiazines and butyrophenones are due primarily to the blocking of dopamine receptors and, marginally at best, to the inhibition of glutamate dehydrogenase.

Phenothiazine and butyrophenone drugs are thought to exert their neuroleptic effects by blocking dopamine receptors in the brain [1]. These drugs were found by Fahien and Shemisa [2, 3] also to inhibit glutamate dehydrogenase (EC 1.4.1.2,L-glutamate: NAD^+ oxidoreductase) non-competitively in the direction of synthesis, that is, in the formation of glutamate:

$$\alpha KG + NH_4^+ + NAD(P)H \rightleftharpoons \text{L-glutamate} + NAD(P) + H_2O$$

They discovered that the inhibitory effect of these drugs is especially pronounced in the presence of NADH and GTP, and is almost absent in the presence of ADP and NAD^+. The functional enzyme is a hexamer of six identical polypeptide chains. Each chain of the bovine enzyme has been reported to contain 500 [4] or 501 or even 502 amino acids [5].

Fahien and Shemisa, using a variety of biophysical techniques [2, 3], showed that chlorpromazine also induces structural changes in the GDH‡ molecule and suggested that at least some of the pharmacological effects of chlorpromazine and haloperidol may be due to their interaction with GDH. In support of this they brought essentially the following evidence from the literature: (a) the level of glutamate dehydrogenase in the brain is high [6, 7]; (b) subsequent to the injection of pharmacological doses of chlorpromazine into laboratory animals, the concentration of the drug in the brain [8] exceeds by one order of magnitude the apparent dissociation constant of the complex of the drug with GDH, as obtained from kinetic measurements with the pure enzyme [3]; (c) mitochondria, which contain GDH, strongly adsorb phenothiazines; brain mitochondria adsorb chlorpromazine more strongly than liver mitochondria [9]; (d) subsequent to the injection of chlorpromazine to rats, the concentration of L-glutamine in the brain increases [10, 11].

It is plausible that the increased level of glutamine indicates inhibition by the drug of GDH-catalyzed glutamate formation [2]. Glutamate, however, is also formed from αKG by the action of glutamic: oxaloacetic transaminase (EC 2.6.1.1,L-aspartate:2-oxoglutarate aminotransferase). When GDH is inhibited, αKG becomes more available for the action of the transaminase. From glutamate and NH_4^+, which also becomes more available, glutamine is formed by the action of glutamine synthetase (EC. 6.3.1.2,L-glutamate:ammonia ligase). Neither the transaminase nor glutamine synthetase is affected by chlorpromazine [2]. It should be noted, furthermore, that GDH inhibition by the drug is strong when the level of NADH is high, and that under these conditions the cell has a high level of ATP [12], which is necessary for glutamine synthesis. As to glutamine itself, it is non-toxic; it is thought to be the product of ammonia detoxification [13] and a reservoir for glutamate in the brain [14].

The inhibition of glutamate dehydrogenase by phenothiazines and butyrophenones in the direction of synthesis only [2] probably results from the binding of these drugs to the hexameric enzyme in a specific conformation. Kinetic and spectroscopic experiments suggest that these neuroleptic drugs bind to specific sites [2, 3].

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‡ Abbreviations: $K_{D\text{app}}$, apparent dissociation constant; αKG, α-ketoglutarate; CP, chlorpromazine-HCl; [^{3}H]-CP, tritiated chlorpromazine–HCl; GDH, glutamate dehydrogenase.

Table 1. Molar extinction coefficient of some neuroleptic drugs

Compound	Medium	Wavelength (nm)	ε_M ($M^{-1} \cdot cm^{-1}$)
Chlorpromazine	0.1 N HCl	254	31,687
Spiroperidol	0.1 N HCl	248	16,347
Haloperidol	MeOH	245	12,781

In this study we report experiments on the binding of [^{3}H]-CP to GDH. We compare results with those obtained from the inhibition of the enzyme and discuss the pharmacological significance of these findings.

MATERIALS AND METHODS

Materials. Beef liver GDH was obtained from Boehringer, Mannheim, West Germany, either as a crystalline suspension in $(NH_4)_2SO_4$ or else dissolved in glycerol. We chose to work with the purified beef liver enzyme. It has been shown previously that the liver and the brain enzyme behave in identical fashion [2, 3]. Chlorpromazine–HCl was obtained from Specia, Paris, France. Haloperidol was obtained from Abic, Ramat Gan, Israel, and spiroperidol* from Dr. Daphne Atlas of our laboratory. [^{3}H]chlorpromazine (34.2 Ci/mmole) was obtained from the Dimona Nuclear Research Center, Israel. All other chemicals and biochemicals used in this study were of the highest purity.

GDH. Enzyme concentration was determined spectrophotometrically using $A^{0.1\%}_{280nm} = 0.97$ [15]. The molar concentration of the enzyme was calculated using 330,000 as the molecular weight [16]. GDH activity was measured by following the reduction of αKG by NADH ($\varepsilon_{340nm} = 6220\ M^{-1}\ cm^{-1}$) with a Gilford 2000 recording spectrophotometer. Experiments were at first conducted in 25 mM sodium arsenate 0.1 M EDTA, pH 7.8, at 25°, as described by Fahien and Shemisa [2]. As chlorpromazine is more soluble at pH 7.0 than at pH 7.8, later experiments were conducted at the lower pH, in the absence or presence of KCl (100 mM; see section "Binding of [^{3}H]-CP to GDH"). Substrate concentrations were: 50 mM NH_4Cl, 0.1 mM NADH, and 2 mM αKG, which was added last. The time course of the assay was linear up to 0.2 optical density units in a cuvette of 10 mm optical path. One enzyme unit is defined as that amount which will oxidize 1 μmole of NADH per minute at these concentrations and pH 7.0. Specific activities of the GDH preparations ranged between 8 to 17 units per mg of enzyme.

Concentration of drugs. Stock solutions of chlorpromazine and of spiroperidol were prepared in 0.002 N HCl. Haloperidol was dissolved in methanol. Concentrations were about 10 mM or less, as required. From these solutions appropriate dilutions, to be used in the experiments, were made in buffer. Concentrations of these solutions were measured spectrophotometrically, following further dilution of aliquots. The solvents used and molar extinction coefficients are given in Table 1.

Dialysis membranes. Visking dialysis tubing was boiled three times in 0.2 M EDTA, pH 7.4, for 5 min, washed with water, then allowed to stand for 24 hr in 50% acetic acid at room temperature, washed extensively with water, and stored at 4° in water or in sodium arsenate buffer.

Binding of [^{3}H]-CP to GDH. The binding of [^{3}H]-CP to GDH was determined by measuring the rate of dialysis of the drug from the enzyme–[^{3}H]-CP equilibrium mixture, using the method of Colowick and Womack [17] and the identical cells and technique described by Henis and Levitzki [18]. A full titration curve was usually obtained within 90 to 100 min, during which time and longer enzyme activity remained unchanged. The steady state of [^{3}H]-CP outflow from the upper to the lower chamber of the dialysis cell was reached in 6 to 8 min. No change occurred in outflow as a result of the increase in volume in the upper chamber. It was found that [^{3}H]-CP also binds to the dialysis membrane and that, in the presence of 100 mM KCl, this binding, though not abolished, is significantly reduced. Therefore each GDH binding experiment was run in the presence of 100 mM KCl, together with a control in the absence of GDH, in which binding of drug to membrane was measured. This latter value was subtracted from that obtained in the presence of GDH to give the specific binding of [^{3}H]-CP to enzyme. Unless otherwise specified, all binding measurements were made in 100 mM KCl, 25 mM sodium arsenate, 0.1 mM EDTA buffer, pH 7.0, at 25°.

The decrease in CP binding to membrane was not due specifically to KCl. At comparable concentrations a similar effect was obtained with lysine–HCl (data not shown).

RESULTS

Inhibition of GDH by neuroleptic drugs. The apparent binding constants of chlorpromazine, haloperidol and spiroperidol to GDH were determined from the inhibition of the GDH catalyzed reaction, as previously described [2, 3]. The inhibition curves for all three drugs were found to fit well the equation:

$$V_1 - v = \frac{V_1 - V_2}{1 + \frac{K_{Dapp}}{[D]}} \qquad (1)$$

where V_1 is the reaction velocity in the absence of drugs, V_2 the reaction velocity in the presence of saturating concentrations of drugs, v the measured velocity in the presence of drug concentration $[D]$, and K_{Dapp}, the apparent enzyme–drug dissociation constant. The plots of $(V_1 - v)^{-1}$ against $[D]^{-1}$ were

* Spiperone, see Merck Index, Ninth Edition, 1976; p. 1131.

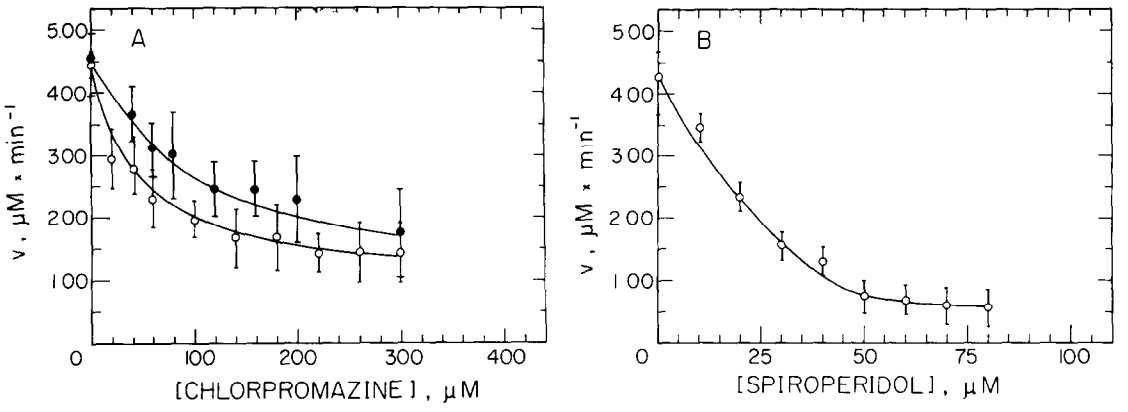

Fig. 1. The inhibition of GDH by chlorpromazine and spiroperidol. The assay mixture included: 2 mM αKG, 50 mM NH_4Cl, 0.1 M NADH, 0.116 μg GDH/ml (0.4 nM) in 25 mM sodium arsenate buffer 0.1 M EDTA, at 25°. (A) Chlorpromazine: (○——○), in the absence of KCl, pH 7.8; (●——●), in the presence of 100 mM KCl, pH 7.0. (B) Spiroperidol, in the absence of KCl, pH 7.0. Bars indicate 95% confidence limits. Curves were obtained by calculation from equation 1 (see text).

found to be linear and to yield values of K_{Dapp} using a linear regression program. Typical inhibition curves of GDH are shown in Fig. 1 and the corresponding K_{Dapp} values are included in Table 2. K_{Dapp} for chlorpromazine, which we determined under the conditions of Fahien and Shemisa [2] (Fig. 1(A), empty circles, and Table 2, top line), was similar to theirs.

Inhibition of GDH by K^+ *and* Rb^+. As binding experiments were performed in the presence of 100 mM KCl (see next section), the kinetics of enzyme inhibition were studied in the presence of this salt as well as in its absence (Fig. 1 and Table 2). We next wished to find out whether K^+ (atomic radius 1.33 Å) and Rb^+ (atomic radius 1.48 Å) are able to replace NH_4^+ (atomic radius also 1.48 Å) as GDH ligands. If this were so, binding experiments in the absence of NH_4^+ but in the presence of one of these ions would simulate conditions of the enzyme-catalyzed reaction, while occurrence of the reaction itself would be prevented. We found that K^+ and Rb^+ inhibit GDH by competing with NH_4^+. K_m for NH_4^+ and inhibition constants for K^+ and Rb^+ are given in Table 3.

Binding of [^{3}H]-*CP to GDH*. The binding of [^{3}H]-CP to GDH alone in the presence of 100 mM KCl reveals the presence of two binding sites per hexamer of equal affinity (K_{Dapp} = 66 μM; Fig. 2 and Table 2). In the presence of NADH, αKG and KCl, the affinity towards the drug of one site remains essentially unchanged, whereas that of the other site is much lowered (K_{Dapp} about 200 μM; Fig. 3 and Table 2). This lower affinity constant is an estimate, as it is impossible to obtain data at higher chlorpromazine concentrations because of the limited solubility of the drug and its relatively high binding to the dialysis membrane. These results indicate that in the presence of the substrates αKG and NADH, with K^+ replacing NH_4^+, the two initially equivalent sites become non-equivalent.

Table 2. Apparent dissociation constants for the complexes of chlorpromazine and butyrophenones with GDH

			Apparent dissociation constant (μM)			
				Enzyme kinetics		
Drug	Experimental conditions	pH	Binding experiments	This study	Other workers	References
Chlorpromazine	No KCl	7.8	ND†	45 ± 4*	32	[2]
Chlorpromazine	100 mM KCl	7.0	66 ± 4	136 ± 8*	ND	—
Chlorpromazine	100 mM KCl, 2 mM αKG, 0.1 mM NADH	7.0	61 ± 4 200 ± 30	136 ± 8*‡	ND	—
Spiroperidol	No KCl	7.0	ND	28 ± 2*	ND	—
Haloperidol	No KCl	7.0	ND	14 ± 1	4	[3]

* Average of three independent determinations and its standard error. GDH activity was measured as described in Materials and Methods, except for the presence or absence of KCl, indicated in this table.
† ND, not determined.
‡ Identical experiment to the one above.

Table 3. Apparent affinity constants for the interaction of NH_4^+, K^+ and Rb^+ with glutamate dehydrogenase*

Cation	Apparent affinity constant (mM)
NH^+	19.5 ± 1.0 (K_m)†
K^+	5.0 ± 0.4 (K_i)
Rb^+	10.0 ± 0.7 (K_i)

* GDH activity measured at pH 7.0, as described in Materials and Methods. Both K^+ and Rb^+ ions were found to be purely competitive inhibitors of NH_4^+.

† This parameter is 3.2 mM in 0.01 M Tris–acetate buffer, pH 8.0 and 25°, with NAD(P)H replacing NADH [15].

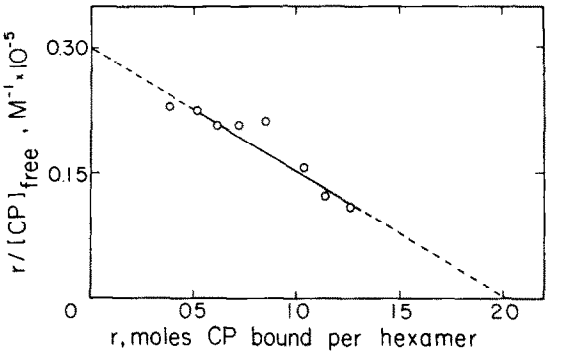

Fig. 3. Scatchard plot for the binding of chlorpromazine to GDH in the presence of 100 mM KCl, 2 mM αKG and 0.1 M NADH. Conditions as described in Fig. 2.

DISCUSSION

Direct binding studies of [^{3}H]-CP to GDH, at K^+ concentrations sufficient to saturate the NH_4^+ binding site, reveal the presence of two binding sites (Figs 2 and 3). In the absence of substrates, these two sites seem to be equivalent, with K_{Dapp} = 66 μM. In parallel studies conducted in the presence of αKG and NADH, the two sites become non-equivalent, with one site exhibiting essentially an unchanged affinity and the second a lowered affinity to the drug (61 μM and 200 μM respectively, Fig. 3 and Table 2). In kinetic experiments, in the presence of all three substrates and KCl, the apparent chlorpromazine dissociation constant is close to that of the low affinity site (136 μM; Fig. 1(A) and Table 2). It appears that under these conditions the occupancy of only the low affinity site is expressed kinetically.

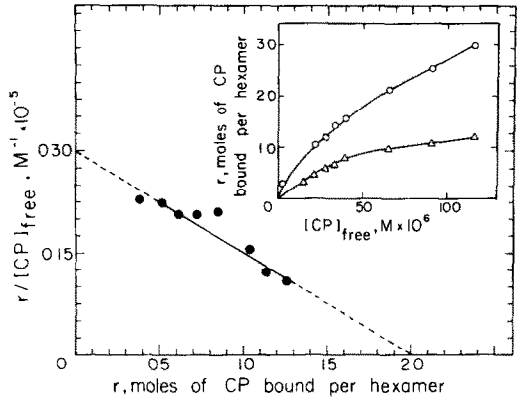

Fig. 2. Scatchard plot for the binding of chlorpromazine to GDH in the presence of 100 mM KCl and the absence of substrates, determined by the method of Colowick and Womack [13]. Initial concentrations: GDH in the upper chamber, 3.32 mg/ml, 10.1 μM; [^{3}H]-CP (342 mCi/mmole), 6.1 μM; concentrated CP solutions of same radioactivity/ml, 0.248 mM and 1.94 mM were added in small portions. Buffer flow rate through bottom chamber 1.0 ml/min; 1.0 ml fractions were collected for counting. Inset: Binding isotherms: ○, GDH plus membrane; △, GDH only. For further details, see Materials and Methods.

GDH possesses both a two-fold and a three-fold axis of symmetry ([16] and references therein). Since the stoichiometry of chlorpromazine binding is two per hexamer, it is tempting to speculate that there exists a single drug site within the domain of each of the two trimers that constitute the enzyme. This is in contrast to twelve binding sites for NADH or NAD(P)H and six for GTP [20, 21]. As the six polypeptide chains are identical, there presumably exist two NAD(P)H sites and one GTP site per chain, whereas chlorpromazine binds to a domain formed by three such chains. It seems that in the presence of αKG, NADH and K^+, the two domains, though chemically identical, are conformationally different.

Data relating to phenothiazine and butyrophenone drugs, when available for all three effects—clinical potency, GDH inhibition and the inhibition of haloperidol binding to the dopamine receptor [1]—are given in Table 4. It may be seen that the correlation of haloperidol binding inhibition with clinical potency (correlation coefficient, r, +0.972) is much better than that of GDH inhibition (r, +0.582). Moreover, our data for GDH inhibition by haloperidol and spiroperidol are in inverse relationship to their clinical potency. Over and above this, affinities for the dopamine receptor (inhibition of haloperidol binding) are greater by about three orders of magnitude than those for GDH.

One hour after intravenous injection to dogs, the concentration of chlorpromazine in the brain was 80–89 mg/kg [8], which is roughly equivalent to 250 μM, somewhat higher than the apparent dissociation constant of the low affinity site of GDH for the drug in the presence of KCl, but three to six orders of magnitude greater than K_i, the constant for the inhibition of haloperidol binding to the dopamine receptor. The specific binding of phenothiazines and butyrophenones, both to dopamine receptors in the brain and to GDH, is indeed intriguing; yet, the evidence is overwhelming that the pharmacological effects of these drugs are due primarily to the blocking of dopamine receptors and, marginally at best, to GDH inhibition.

Table 4. Comparison of the clinical potency of some phenothiazine and butyrophenone drugs with their ability to inhibit GDH and haloperidol binding to the dopamine receptor

Drug	Average clinical daily dose [1] (μmoles/kg)	K_{Dapp} GDH inhibition (μM)	Inhibition of haloperidol binding, K_i [1]* (nM)
Spiroperidol	0.058	28‡	0.25
Haloperidol	0.152	14‡, 4†	1.50
Triflupromazine	4.59	80†	2.10
Chlorpromazine	12.0	32†	10.30
Promazine	33.3	80†	71.50
		$r = 0.582$ († series)	$r = 0.972$

* pH 7.1 and 37°.
† pH 7.8 and 25° [2, 3].
‡ pH 7.0 and 25° (present work).

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REFERENCES

1. R. Creese, D. R. Burt and S. H. Snyder, *Science* **192**, 481 (1976).
2. L. A. Fahien and O. Shemisa, *Molec. Pharmac.* **6**, 156 (1970).
3. O. Shemisa and L. A. Fahien, *Molec. Pharmac.* **7**, 8 (1971).
4. K. Moon, D. Piszkiewicz and E. L. Smith, *Proc. natn. Acad. Sci. U.S.A.* **69**, 1380 (1972).
5. V. Witzemann, R. Koberstein, H. Sund, I. Rasched, H. Jornvall and K. Noack, *Eur. J. Biochem.* **43**, 319 (1974).
6. E. Schmidt, F. Schmidt, M. D. Horn and U. Gerlach, in *Methods in Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 658. Academic Press, New York (1963).
7. E. Schmidt, in *Methods in Biochemical Analysis* (Ed. H. U. Bergmeyer), Vol. 2, p. 650. Academic Press, New York (1974).
8. N. P. Salzman and B. B. Brodie, *J. Pharmac. exp. Ther.* **118**, 46 (1956).
9. M. Berger, *J. Neurochem.* **2**, 30 (1957).
10. R. S. De Ropp and E. H. Snedeker, *Proc. Soc. exp. Biol. Med.* **106**, 696 (1961).
11. N. Okumura, S. Otsuki and H. Nasu, *J. Biochem. (Tokyo)* **46**, 247 (1959).
12. B. Chance and G. R. Williams, *Adv. Enzym.* **17**, 65 (1956).
13. C. H. Letendre, K. Nagaiah and G. Guroff, in *Biochemistry of Brain* (Ed. S. Kumar) p. 353. Pergamon Press, Oxford (1980).
14. P. L. McGeer, J. C. Eccles and E. G. McGeer, in *Molecular Neurobiology of the Mammalian Brain*, p. 191. Plenum Press, New York (1978).
15. J. A. Olson and C. B. Anfinsen, *J. biol. Chem.* **197**, 67 (1952).
16. H. Eisenberg, R. Josephs and E. Reisler, *Adv. Protein Chem.* **30**, 101 (1976).
17. S. P. Colowick and F. C. Womack, *J. biol. Chem.* **244**, 774 (1969).
18. Y. I. Henis and A. Levitzki, *Proc. natn. Acad. Sci. U.S.A.* **77**, 5055 (1980).
19. C. Frieden, *J. biol. Chem.* **234**, 2891 (1959).
20. R. Koberstein and H. Sund, *Eur. J. Biochem.* **36**, 545 (1973).
21. J. Krause, M. Bügner and H. Sund, *Eur. J. Biochem.* **41**, 593 (1974).