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Drug-Protein Interactions: Evaluation of the Binding of Antipsychotic Drugs to Glutamate Dehydrogenase by Quantitative Affinity Chromatography

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SUMMARY

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The interactions of psychoactive drugs with bovine glutamate dehydrogenase were evaluated by quantitative affinity chromatography on Perphenazine-Sepharose. An affinity matrix containing a relatively low density of immobilized ligand was used to achieve competitive elution of zones of the enzyme with buffers containing soluble phenothiazines and butyrophenones. These competitive elution data indicate that all of the drugs tested bind at the same protein site. The variation of elution volume with soluble drug concentration allowed the calculation of apparent dissociation constants for the binding of these substances. Especially among the phenothiazines, the relative magnitudes of the dissociation constants for the various drugs are similar both to the relative inhibitory effects by these substances on dehydrogenase catalysis and to their relative pharmacological potencies. A close but nondirect interrelationship between drug, NADH, and GTP binding to glutamate dehydrogenase was observed by chromatographic elutions with various combinations of these substances in the eluting buffers.

INTRODUCTION

The interaction of drugs with proteins plays a central role in determining both pharmacokinetic properties and pharmacological activities (1-4). It has been observed previously that glutamate dehydrogenase (GDH[1]) is strongly inhibited by chlorpromazine and its isosteres (5-7) and by butyrophenones (7, 8). A strong correlation has been found to exist between inhibition of the enzyme by these substances and their pharmacological potencies as antipsychotic drugs (7, 8). To be sure, enzyme inhibition was recognized as only an indirect measure of drug binding per se (8). Nonetheless, the above data, when considered with the role of glutamate-α-ketoglutarate interconversion in the brain (9), have suggested that GDH could play some role in the *in vivo* mechanism of action of these

[1] The abbreviations used are: GDH, bovine glutamate dehydrogenase; EDAC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride.

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drugs. In this regard, chlorpromazine and haloperidol administration were noted to have some influence on the amounts of both glutamic acid and γ-aminobutyric acid in the brain (10, 11). Although other mechanisms for pharmacological activity also have been proposed for these drugs (3, 12, 13), the reported correlation between inhibition of GDH and pharmacological effects remains a potentially important aspect of the action of these compounds.

In view of the reported effects of antipsychotic drugs on GDH, we undertook the direct characterization of the presumed binding processes by quantitative affinity chromatography. This latter method has been employed previously to measure binding of active site ligands for such proteins as staphylococcal nuclease (14) and bovine pancreatic ribonuclease (15). In contrast to previous work, wherein only an indirect measure of drug binding could be obtained (8), the present study has allowed direct quantitative comparisons to be made of enzyme-binding properties of the various antipsychotic drugs. The relationships between the enzyme-binding sites of these drugs and those of natural ligands of glutamate dehydrogenase also were evaluated.

MATERIALS AND METHODS

All biochemical reagents used were the high purity products of Boehringer-Mannheim (Germany). Gifts included chlorpromazine, from Farmitalia (Milan, Italy); di-N-demethyl-chlorpromazine, from Dr. A. Manian (National Institute of Mental Health, Rockville, Maryland USA); triflupromazine, from Smith-Kline Corporation (USA); perphenazine, from Essex (Milan, Italy); and haloperidol and trifluperidol, from Dr. P. Janssen (Janssen Pharmaceutica, Beerse, Belgium). Sepharose 4B and epoxy-activated Sepharose 6B were Pharmacia products. Aminoethyl Bio-Gel P and EDAC were from Bio-Rad. Bovine glutamate dehydrogenase was obtained as an ammonium sulfate suspension from Boehringer-Mannheim.

Preparation of affinity matrices (see Fig. 1). Three methods were employed for coupling a phenothiazine structure to an insoluble matrix, with method A (below) yielding the product of most use in the present study. Direct exposure to light was avoided in all reactions to minimize free radical formation. Immobilized ligand concentrations achieved are reported as M, defined here as moles per liter packed gel bed volume.

A. Coupling of perphenazine to epoxy-activated Sepharose 6B. Perphenazine (200 mg) was dissolved in 5 ml of dioxane and added to 15 ml of settled epoxy-activated Sepharose 6B (15 μeq/ml) suspended in 15 ml of 0.1 N NaOH. After overnight stirring at 40°, the resin was washed sequentially with 50% dioxane, water, 0.1 M borate buffer (pH 9), and 0.5 N NaCl. In order to mask any unreacted epoxy groups on the resin, 2-mercaptoethanol (200 mg) was added and the mixture incubated in 0.1 N NaOH as above for 4 hrs. After further washings (as above), the resin was equilibrated with the standard potassium phosphate eluting buffer. In this matrix preparation, the amount of matrix-bound drug was difficult to determine due to the stability of the ether linkage of perphenazine to the resin. The composition of Cl and N found by elemental analysis of the affinity resin, as well as a determination of the unreacted drug in the washings after drug-resin coupling, both indicated that the Sepharose-bound drug was below 10^{-4} M. A quantitative value for matrix-bound ligand was obtained only indirectly, by correlating the GDH elution behavior for this drug-Sepharose with that for the matrix from method C (see below). This procedure gave a bound drug concentration of 2×10^{-5} M for the matrix of method A, henceforth referred to as Perphenazine-Sepharose; this matrix was used for all the quantitative elution studies described in this work, except as noted otherwise.

B. Coupling of di-N-demethyl-chlorpromazine to cyanogen bromide-activated Sepharose. Settled Sepharose 4B (50 ml) was activated according to Cuatrecasas (16) and subsequently reacted, in 0.1 M borate buffer at pH 9.5 and at 25% in dimethylformamide, with 70 mg of di-N-demethyl-chlorpromazine. After a 24 hr reaction, gel was washed and then equilibrated with standard potassium phosphate eluting

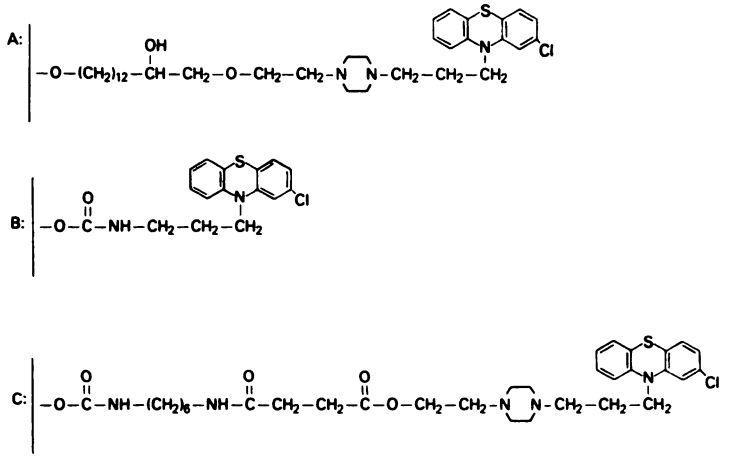

FIG. 1. *Structures of Sepharose-psychoactive drug matrices*

buffer (see below). The total concentration of drug bound to the matrix, calculated from the amount of unbound drug remaining in the washings after the coupling reaction, was 2–3 × 10^{-3} M.

C. Coupling of succinylated perphenazine to aminohexamethyl-Sepharose using soluble carbodiimide. Perphenazine (300 mg) was converted to ω-0-succinylated perphenazine with succinic anhydride (2.5 molar excess of latter) and the derivative reacted, in the presence of EDAC (600 mg), with 50 ml of settled aminohexamethyl-Sepharose 4B (prepared from Sepharose 4B and hexamethylendiamine (16)). The matrix-bound perphenazine, evaluated after its release from washed resin product by mild hydrolysis of the ester linkage between succinic acid and perphenazine (in 0.1 N NaOH at room temperature), was 5 × 10^{-5} M. This low yield of bound drug was verified by an evaluation of unreacted perphenazine in the washings after coupling.

Affinity chromatography. Chromatographic elutions were performed in the dark with a column of 0.9 × 15 cm bed volume at 5°; 1 ml fractions were collected at a flow rate of 6 ml/hr. Potassium phosphate buffer 0.02 M, pH 8, containing 0.1 mM EDTA and 2-mercaptoethanol, was used as eluting buffer unless otherwise indicated. Bovine GDH samples (0.2 mg) in a total volume of 0.4 ml of buffer with or without the appropriate concentration of competing soluble ligand were applied to the column pre-equilibrated with the buffer containing corresponding concentrations of soluble ligand. The elution position of enzyme was determined by assays of fractions for enzymatic activity, in phosphate buffer using NADH as substrate, by the assay method already reported (17).

Treatment of chromatographic data. The variation of enzyme elution volume with soluble ligand concentration, as described in affinity elution experiments, was plotted according to the equation,

$$\frac{1}{V - V_o} = \frac{K_{\overline{LM}}}{(V_o - V_m)[\overline{LM}]} + \frac{K_{\overline{LM}}[L]}{K_L(V_o - V_m)[\overline{LM}]} \quad (1)$$

derived previously for the staphylococcal nuclease case (14). Here, V = protein elution volume from affinity column (the peak fraction); V_o = volume at which protein elutes in the absence of interaction (determined in a column with thioethyloxy groups replacing perphenazine); V_m = void volume (determined by Blue Dextran 2000 elution); $[L]$ = concentration of soluble ligand; $[\overline{LM}]$ = concentration of immobilized ligand (determined as reported in the text and given in units of M (moles per liter packed gel bed volume)); $K_{\overline{LM}}$ = dissociation constant for the interaction of protein with immobilized ligand; K_L = dissociation constant for the soluble ligand-protein interaction. K_L values were calculated as the

ratio of ordinate intercept to slope of $1/(V - V_o)$ vs $[L]$ plots. $K_{\overline{LM}}$, where evaluated, was calculated from the ordinate intercept of $1/(V - V_o)$ vs $[L]$ plots using the experimentally determined values of V_o, V_m, and $[\overline{LM}]$.

RESULTS

Elution behavior of GDH on the different affinity matrices. The most useful affinity column for the present quantitative studies was that with perphenazine linked to Sepharose through ether linkage at the ω-hydroxy groups (Perphenazine-Sepharose from method A). This affinity resin was very stable and could be used over a long period of time with reproducible results. As discussed below, GDH was retarded by this matrix-bound ligand in an apparently specific manner, such that soluble perphenazine in the eluting buffer could compete with the bound ligand to decrease the enzyme elution volume.

Similar retardation of GDH and competitive elution with soluble perphenazine were also observed with the affinity resin prepared by the EDAC procedure (method C). In this case, however, the elution had to be carried out in the presence of 0.4 M KCl to avoid nonspecific adsorption probably due to ionic interactions of the enzyme with the potentially charged isourea linkage group of this matrix (18). This nonspecific adsorption is implicit in the observation of some retardation of GDH by a resin prepared as in method C but with ethoxy groups replacing perphenazine. A further disadvantage of method C is its short life, due to the low stability of the ester linkage between succinic acid and perphenazine. After repeated experiments, the resin lost bound perphenazine, a loss paralleled by a decrease in binding capacity for GDH.

The resin prepared by method B, although effective in binding GDH, retained the enzyme so tenaciously that it was not possible to remove the enzyme in an active form. The GDH could not be removed at all with chlorpromazine in the eluting buffer nor by extremes of pH, high ionic strength, or addition of other ligands or organic solvents (ethanol or dioxane) to the buffer.

Although the matrices prepared by methods B and C are potentially useful for some affinity chromatographic applications, such as purification of drug-binding proteins, these matrices were not employed further for competitive elution studies.

Dependence of the elution of GDH from Perphenazine-Sepharose on the perphenazine concentration in the eluting buffer. In the absence of any ligand in the eluting buffer, GDH was retarded significantly by the Perphenazine-Sepharose column (Fig. 2). When elution was carried out in the presence of increasing concentrations of soluble perphenazine, the elution volume of GDH decreased progressively. A linear correlation was found between $1/(V - V_o)$ and the concentration of free ligand in the eluting buffer (Fig. 1 inset). This behavior indicates competition of free and matrix-bound ligand for the same site in the enzyme molecule.

The binding of GDH to the Perphenazine-Sepharose column was found to be pH-dependent. For instance, at pH 7.2 and in absence of free ligand, the elution peak of GDH is centered at fraction 25 (from 36 at pH 8, as shown in Fig. 2, solid squares). This behavior parallels the effect of pH on the inhibition of GDH activity by perphenazine (Fig. 3), which shows decreased inhibition on going from pH 8 to 7.2.

Dependence of elution of GDH on the presence of other phenothiazine or butyrophenone drugs in the eluting buffer. The data in Figure 4 denote the chromatographic elution behavior of GDH on Perphenazine-Sepharose in buffers containing various phenothiazine drugs (perphenazine, chlorpromazine, triflupromazine, di-N-demethyl-chlorpromazine) or butyrophenone drugs (haloperidol, trifluperidol). The results indicate that in all cases a competition occurs between free ligand and matrix-bound perphenazine. This competition is very strong with some drugs (haloperidol, perphenazine) and weaker with others (trifluperidol), indicating differential affinity for the enzyme. The specificity of the competitive elution effect is supported by the finding that simple aromatic compounds, such as phenol or aniline (0.1 mM in the eluting buffer), have no influence on the

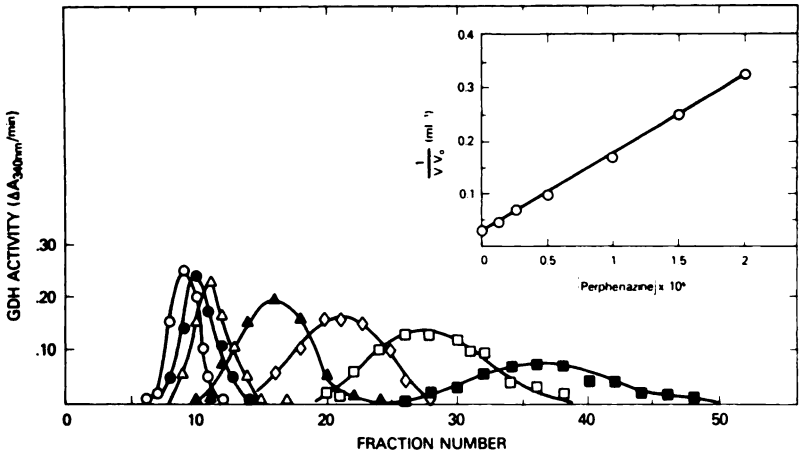

FIG. 2. *Affinity chromatographic elution profiles of glutamate dehydrogenase on Perphenazine-Sepharose in the presence of varying concentrations of soluble perphenazine in the standard elution buffer*

Samples of GDH were applied to the column equilibrated with 20 μM (○); 1.5 × 10^{-5} M (●); 1 × 10^{-5} M (△); 0.5 × 10^{-5} M (▲); 0.25 × 10^{-5} M (◇); 0.125 × 10^{-5} M (□) perphenazine or without perphenazine (■). Inset: plot of $1/(V - V_o)$ vs total soluble perphenazine concentration.

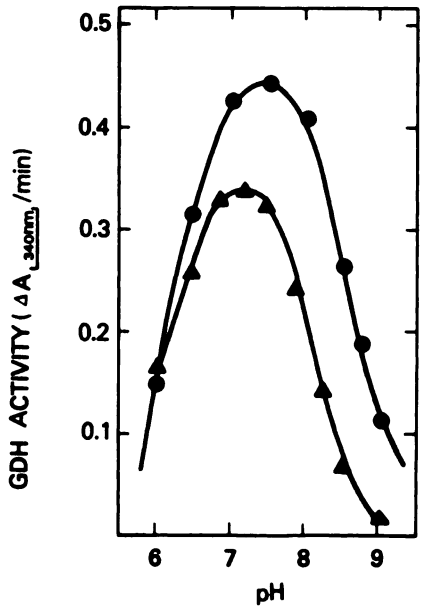

FIG. 3. *Effect of perphenazine on the reductive amination of α-ketoglutarate at varying pH*

The medium contained 0.1 M NH_4Cl, 20 mM potassium phosphate buffer, 0.1 mM EDTA, 25 mM α-ketoglutarate, 0.1 mM NADH, 2.5 μg/ml GDH and either no other additives (●) or 0.05 mM perphenazine (▲).

extent of GDH retardation.

As indicated in the inset of Fig. 4, the variation of $1/(V - V_o)$ vs drug concentration was evaluated for elutions with free ligand concentrations in the range of 10^{-6} to 2 × 10^{-5} M. The linearity of the plots suggests that all of the drugs compete at the same site in the enzyme molecule at which the matrix-bound perphenazine interacts.

Calculation of apparent dissociation constants for drugs to GDH. The $1/(V - V_o)$ vs $[L]$ plots of Figures 2 and 4 allow the calculation of dissociation constants, K_L, according to Equation 1 (see METHODS). These calculated chromatographic constants are given in Table 1. Also given, for comparison, are the apparent binding parameters calculated for the same drugs based on inhibition of GDH activity [7, 8].

Influence of other GDH ligands on the binding of the enzyme to Perphenazine-Sepharose. ADP, an activator of GDH, and GTP, one of its strongest inhibitors, were added in separate experiments to the eluting buffer, both at concentrations of 10 and 100 μM. No appreciable difference was observed in the elution peak of the enzyme as compared with that obtained in the absence of ligand, suggesting that competitive binding of these ligands and the immobilized perphenazine does not occur. On the other hand, when NADH is added to the eluting buffer, a reduction of the elution volume of GDH is observed, although it is not concentration-dependent: the peak was centered

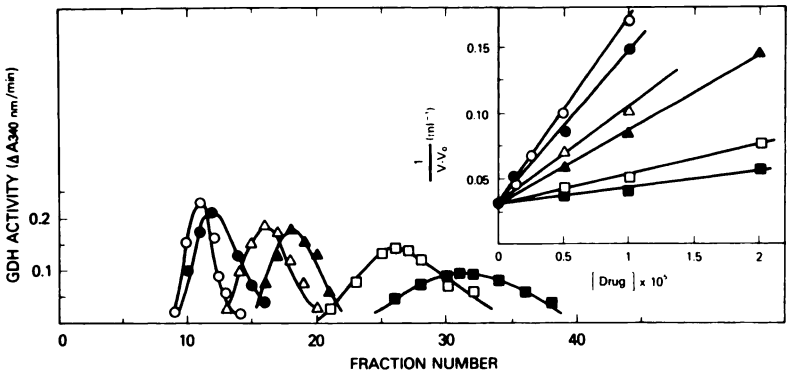

FIG. 4. *Affinity chromatographic elution profiles of glutamate dehydrogenase on Perphenazine-Sepharose equilibrated with the standard elution buffer containing 10^{-4} M of various drugs*

Perphenazine (○), haloperidol (●), chlorpromazine (△), triflupromazine (▲), di-N-demethylchlorpromazine (□), trifluperidol (■). Inset: plots of $1/(V - V_o)$ vs drug concentrations. The values corresponding to 10^{-4} M drug were obtained from the experiment reported in the main plot; the other values were from experiments from which the elution profiles are omitted from the main plot for simplicity. The experimental point at 0 M drug (same as given in Fig. 2) is included in the inset plot.

at fraction 26 (in an elution analogous to that shown in Fig. 2) both at 10 and 100 μM NADH. When NADH (100 μm) and GTP (10 μM) are present together in the eluting buffer, a stronger influence on protein elution occurs, with the elution peak decreasing to fraction 13.

DISCUSSION

In this paper we have evaluated, by quantitative affinity chromatography, the interactions of psychoactive drugs proposed previously to interact at a drug binding site (allosteric with respect to substrate) on glutamate dehydrogenase. The elution behavior of GDH on Perphenazine-Sepharose, as reported in Figure 2, demonstrates that GDH binds directly to the matrix-bound ligand and that a competition exists between this bound ligand and free ligand in eluting buffers The linearity of the $1/(V - V_o)$ vs free ligand concentration (Fig. 2 inset) indicates that the binding system conforms formally to Equation 1. Since this equation is derived for a general one-site competition interaction between matrix-bound and soluble ligand (14), the results suggest that under the conditions used here, immobilized drug binds statistically at only one site per GDH molecule at any given time. In view of the assumed multiple binding of the psychoactive drugs to the multimetric GDH (7), it is likely that the binding of only one molecule of immobilized drug per molecule of enzyme occurs due to a high degree of spacing between matrix-bound ligand molecules in the lightly substituted Perphenazine-Sepharose. The latter condition would sterically restrict the occurrence of multipoint matrix attachment of GDH. It should be noted that, where a high concentration of matrix-bound ligand occurs (in the matrix product

TABLE 1

Values of dissociation constants of psychoactive drugs to GDH as obtained by competitive elution affinity chromatography on Perphenazine-Sepharose

For comparison, kinetically-derived constants from enzyme inhibition studies also are listed.

Drug	Chromatography K_L[a]	Kinetic K_3[b]
	(M × 10^6)	(M × 10^5)
Perphenazine	2	2
Haloperidol	2.5	0.4,1.2
Chlorpromazine	4	3.2
Triflupromazine	5.8	8
Di-N-demethyl-chlorpromazine	15	20
Trifluperidol	22	14

[a] Obtained in this study, at pH 8.0.

[b] Obtained by inhibition of enzyme activity at pH 7.8 (7, 8). The parameter K_3 has been defined as the apparent dissociation constant for drug at the drug-binding site in the presence of saturating concentrations of substrate and coenzyme.

of method B), GDH binding to matrix is so tight that even competitive elution becomes difficult. The tight binding in this case is due presumably to multipoint GDH-matrix interaction. Such behavior is analogous to that observed in the polyvalent binding case of divalent immunoglobulin A to phosphorylcholine-Sepharose[2].

In view of the competitive elution behavior portrayed in Figure 2, equation 1 can be used to calculate directly the apparent dissociation constant, K_L, of the free perphenazine-glutamate dehydrogenase interaction. The dissociation constant determined in this way, $K_L = 2 \times 10^{-6}$ M, is of the same order as that for bound ligand toward GDH, $K_{\overline{LM}}$, which was calculated to be in the range of 6×10^{-6} M. However, this latter value must be considered a rough estimate only, due to the uncertainty in the determined value of $[\overline{LM}]$ for Perphenazine-Sepharose (see MATERIALS AND METHODS). The value of K_L, calculated by the ratio of intercept to slope, is independent of $[\overline{LM}]$ and thus does not suffer from this uncertainty.

As indicated by Figure 4, other GDH-inhibitory drugs with phenothiazine as well as butyrophenone structure also compete with matrix-bound perphenazine to bind the protein. This behavior strongly supports the hypothesis that all of these drugs interact with protein at the same binding site. Calculation of their dissociation constants, from plots of $1/(V - V_o)$ vs $[L]$, yields values (Table 1) which are in general proportional to their GDH inhibitory efficacies as determined from kinetic inhibition analysis. The difference of roughly one order of magnitude in the absolute values of the two sets of data of Table 1 may result from the fact that while the data obtained by competitive elution are measured under conditions wherein only protein and drug interact, the kinetically-derived K_3 values were obtained by enzymatic assay with both substrate and coenzyme present in saturating concentration (see also below). The correspondence of the relative magnitudes of the drug dissociation constants obtained by the two methods supports the contention (7) that the site of primary drug interaction on the enzyme is also responsible for enzyme inhibition. This conclusion is indicated further by the pH dependence of the two phenomena: at lower pH values, where perphenazine is a weak inhibitor (Fig. 3), binding of GDH to Perphenazine-Sepharose also is diminished. Of interest, the values of dissociation constants for antipsychotic drug binding to GDH obtained by the chromatographic method are of the same order of magnitude as the amounts of these drugs in brain during therapy (19, 20). The correspondence of enzyme inhibitory and binding efficacies, together with the previously noted (7, 8) correlation of inhibitory and pharmacological potencies, are consistent with the idea that the drug-GDH interaction has a pharmacological role.

The quantitative results with Perphenazine-Sepharose provide some insights into the relationship between the drug binding site and the binding sites for natural ligands of the enzyme. ADP and GTP, known respectively to activate and inhibit GDH activity by binding to allosteric sites (21), do not modify the elution volume of GDH when added to the eluting buffer. This result, indicating no competition with the matrix-bound perphenazine, suggests that the drug binding site is different from that for either nucleotide. (This conclusion for GTP must be considered tentative, since the binding of this molecule is low in phosphate buffers (22) such as that used in the column experiments.) NADH, which binds both to the active site and to a regulatory site on the enzyme (23), decreases the binding of GDH to the affinity column when added to the eluting buffer. It appears that in this case no direct competition with the matrix-bound perphenazine occurs, since varying the concentration of NADH in the eluting buffer produces the same incomplete decrease in the elution volume of GDH. This behavior may be interpreted to indicate that NADH modifies the enzyme structure, with this alteration resulting in a perturbation of the drug binding site. This effect of NADH would explain the lower drug affinities observed by kinetic analyses in the presence of this nucleotide ligand (Table 1). When GTP and NADH are present

[2] Eilat, D., and Chaiken, I. M., (1979) *Biochemistry*, in press.

together in the eluting buffer, the GDH elution volume is decreased further than by NADH alone. This result, which connotes an interdependence between the drug, NADH and GTP binding sites, is consistent with enzymatic studies which indicate that NADH increases the inhibitory action of GTP (23) and also with more recent preparative ultracentrifuge experiments which show positive cooperativity between the two ligands (24).

The results of the present study demonstrate that, with a suitable affinity matrix, the quantitative affinity chromatographic approach can be used to conveniently and directly evaluate drug-enzyme interaction. These studies depend only on the ability to assay eluted protein (here by enzymatic activity assay) and thus do not require the radioisotopic ligands often needed for other direct methods such as equilibrium dialysis and gel filtration. The chromatographic method should also be useful to obtain information on the interrelationships between the drug binding sites of GDH and the sites of binding of natural ligands.

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