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INHIBITION OF OX BRAIN GLUTAMATE DEHYDROGENASE BY PERPHENAZINE

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Abstract—Factors affecting the inhibition of ox brain glutamate dehydrogenase (GDH) by the antipsychotic drug perphenazine have been studied. Inhibition was found to be of mixed type with respect to 2-oxoglutarate and competitive towards NADH. However the data indicate that perphenazine binds to a site distinct from the catalytic site to which NADH binds. Perphenazine also enhanced the high-substrate inhibition by these two substrates. Inhibition by perphenazine was not affected by the allosteric effector GTP but it was enhanced by increasing pH, in the range of 6.3 to 7.6, and diminished by increasing ionic strength. Low concentrations of perphenazine relieved the inhibition of GDH by phosphatidylserine and cardiolipin. However, at higher concentrations phosphatidylserine did not interfere with the inhibition by perphenazine whereas cardiolipin relieved it. The possible significance of these interactions in terms of the behaviour of this antipsychotic drug *in vivo* are discussed.

Phenothiazines and butyrophenones have been shown to be inhibitors of glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating) EC1.4.1.3, GDH) [1–4]. Since a number of other drugs do not inhibit the enzyme and the relative inhibitory potencies of these antipsychotic drugs parallel their pharmacological potencies, it has been claimed that this inhibition of GDH may, in some way, be involved in the antipsychotic effects of the drugs [1]. However such an effect may be unlikely since a number of compounds which are not antipsychotics also inhibit the enzyme (see Ref. 4). Nevertheless this effect cannot be ignored in terms of the overall effects of these drugs.

Many preparations of glutamate dehydrogenase have been found to have suffered limited proteolytic cleavage, which results in the removal of the four aminoacids at the N-terminus, during purification [5] and this has been shown to alter the response of the enzyme to a number of activators and inhibitors [6–10]. We have recently compared the sensitivities of proteolysed and unproteolysed preparations of the enzyme to some antipsychotic drugs and shown the latter preparations to be more sensitive to inhibition [4]. The activity of glutamate dehydrogenase has been reported to depend in a complex way on a number of factors, including the NADH concentration (see Ref. 10), the assay conditions [11] and the presence of phospholipids [12–16]. In the present paper we report the results of a detailed study on the effects of these factors on the inhibition of ox brain GDH by perphenazine.

MATERIALS AND METHODS

Enzyme preparation. Ox brain GDH was purified to apparent homogeneity and shown not to have suffered significant proteolytic cleavage by the procedure previously described [5]. Enzyme concentration was determined from the absorbance at 280 mm [17] or the method of Bradford [18].

Enzyme assay. Enzyme activities were determined spectrophotometrically by monitoring the oxidation of NADH at 340 nm. Enzyme preparations were added to the assay mixtures to give concentrations in the range 0.05 to 0.4 μg/mL.

Unless otherwise stated assays were carried out at 30° in a medium containing, in a total volume of 2.5 mL, 50 mM phosphate, pH 7.4, 100 mM NH_4Cl, 80 μM NADH, the enzyme sample and 5 mM 2-oxoglutarate, which was added to start the reaction.

The effects of substrates on the inhibition by perphenazine were investigated by varying the concentrations of 2-oxoglutarate in the range 0.5 to 10 mM or the concentration of NADH in the range 10 to 160 μM. Perphenazine was dissolved in a small volume of 0.05 M HCl and brought to pH 7.0 with the appropriate buffer. Perphenazine solutions were prepared freshly each day, kept on ice and protected from light.

Suspensions of phospholipid were prepared as previously described [19].

Analysis of the effects of perphenazine. Where possible, data were plotted as $1/(V_1 - v)$ vs $1/[M]$, as described by Shemisa and Fahien [1], where V_1 is the velocity in the absence of effector, v is the velocity in the presence of the effector and $[M]$ is the concentration of the effector.

These plots were evaluated with the following equation, taken from Shemisa and Fahien [1], which is appropriate for both full and partial inhibitors:

$$1/(V_1 - v) = 1/(V_1 - V_2) + K_3/[(V_1 - V_2)[M]]$$

where V_2 is the velocity in the presence of saturating concentrations of effector, and K_3 is the concentration of effector necessary to obtain $V = (V_1 + V_2)/2$. Data were analysed by fitting the

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$(V_1 - v)$ vs $[M]$ curves by non-linear regression in order to obtain K_3 and $V_1 - V_2$.

Hyperbolic data were analysed by fitting the velocity–concentration curves by non-linear regression in order to obtain slope and intercept values of the double-reciprocal plots. Secondary plots of slope or intercept values vs the perphenazine concentration which gave them were analysed by weighted linear regression to obtain the values for their slopes and intercepts.

RESULTS

The inhibition of ox brain GDH by perphenazine was found to follow a mixed-type pattern with respect of 2-oxoglutarate, with K_i and K_i' values of 610 and 455 μM respectively (Fig. 1) and a competitive pattern with respect to NADH, with a K_i value of 170 μM (Fig. 2). Furthermore, perphenazine was found to potentiate the inhibitory effects of high concentrations of 2-oxoglutarate or NADH (Fig. 3).

Inhibition by perphenazine was found to take place in the presence of GTP (Fig. 4). Values for $(V_1 - V_2)$ were not significantly different from V_1 values, thus indicating that inhibition by saturating concentrations of perphenazine was complete in the absence or in the presence of GTP. Secondary plots showed that K_3 values for perphenazine were not affected by the presence of GTP.

The effects of perphenazine on ox brain GDH were studied in the presence of sonicates of phospholipids, under assay conditions modified as follows:

As anionic phospholipid sonicates were found to inhibit GDH strongly at 25° [19], this temperature was used instead of 30° and as increasing ionic strength has been reported to decrease inhibition of GDH by anionic phospholipids [14, 19] NH_4Cl concentration in the assay medium was 25 mM instead of 100 mM.

Figure 5 shows that the presence of phosphatidylcholine, which did not inhibit GDH activity, did not affect the inhibition of ox brain GDH by perphenazine. In contrast, perphenazine at low concentrations was found to relieve the inhibition by phosphatidylserine, whereas at higher concentrations inhibition by perphenazine took place independently of the presence of phosphatidylserine (Fig. 5B). Similarly, perphenazine at low concentrations was found to relieve the inhibition by cardiolipin, however higher concentrations of perphenazine were able to inhibit GDH in the presence of cardiolipin, although less potently than in the absence of cardiolipin (Fig. 5C).

As shown in Fig. 6A increasing concentrations of NaCl, sodium Hepes or KCl abolished the inhibition of ox brain GDH by 50 μM perphenazine, whereas increasing concentrations of LiCl, up to an ionic strength of 0.6 M, had little effect on the per-

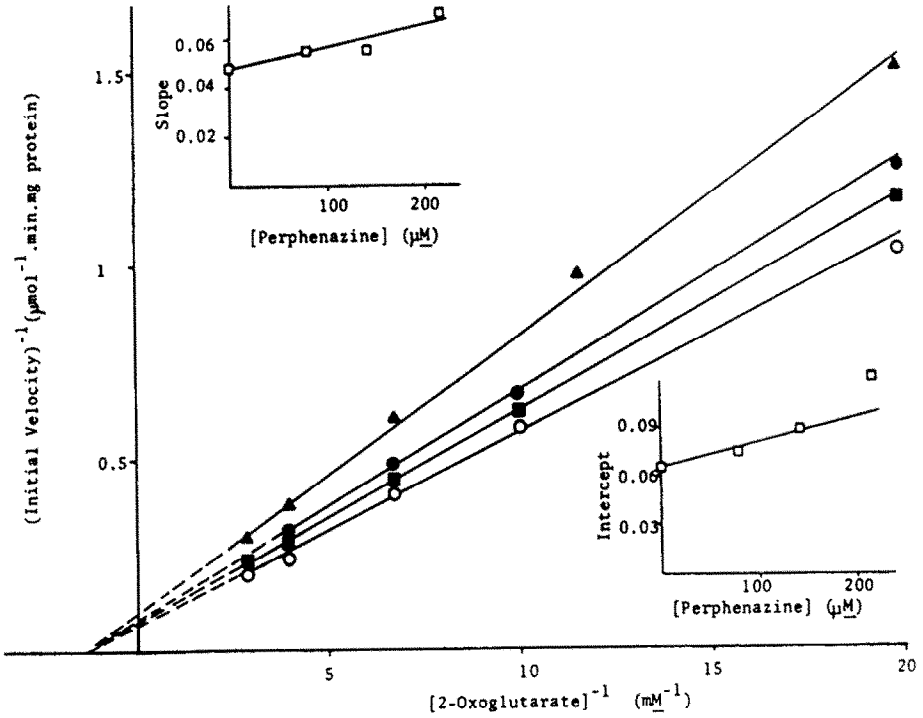

Fig. 1. Inhibition of ox brain GDH by perphenazine with respect to 2-oxoglutarate concentration. The concentration of 2-oxoglutarate was varied while the concentration of perphenazine was held constant at the following values: 0 (○); 80 μM (■); 144 μM (●) and 220 μM (▲). The results are shown as a double-reciprocal plot. Values of slope (μmol^{-1} · min · mg protein mM) and intercept (μmol^{-1} · min · mg protein) were plotted against the perphenazine concentration which gave them, as shown in the insets. The drawn lines were obtained by weighted linear regression analysis.

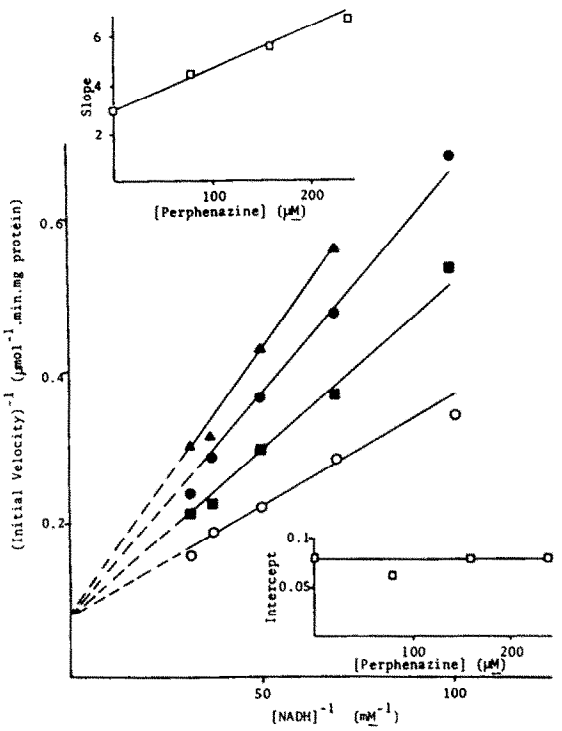

Fig. 2. Inhibition of ox brain GDH by perphenazine with respect to NADH concentration. The concentration of NADH was varied while the concentration of perphenazine was held constant at the following values: 0 (○); 81 μM (■); 162 μM (●) and 243 μM (▲). The results are shown as a double-reciprocal plot. Values of slope (μmol^{-1} · min · mg protein mM) and intercept (μmol^{-1} · min · mg protein) were plotted against the perphenazine concentration which gave them, as shown in the insets. The drawn lines were obtained by weighted linear regression analysis.

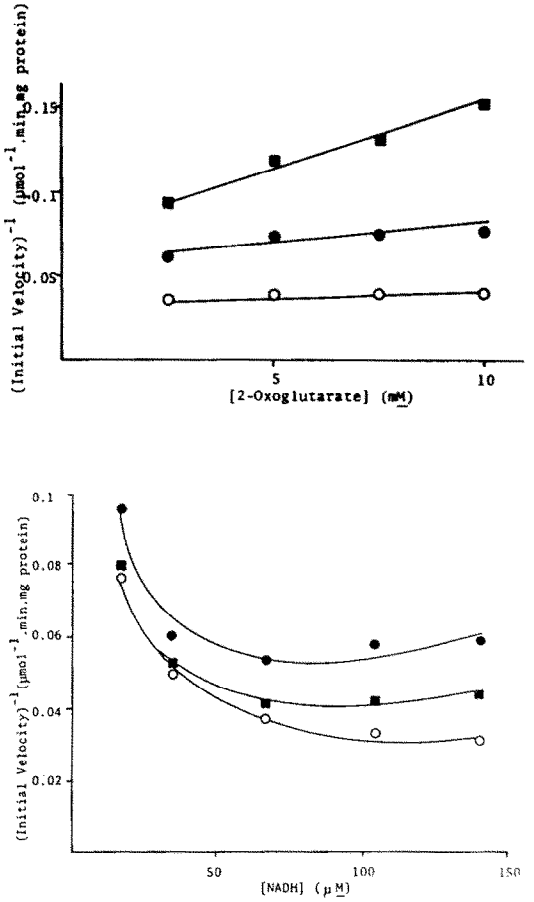

Fig. 3. Effects of perphenazine on substrate inhibition of ox brain GDH. (A) The concentration of 2-oxoglutarate was varied in the range 2.5 to 10 mM, while the concentration of perphenazine was held constant at the following values. 0 (○); 25 μM (●); 50 μM (■). (B) The NADH concentration was varied in the range 20 to 150 μM while the concentration of perphenazine was held constant at the following values: 0 (○); 80 μM (■) and 160 μM (●). The results are shown as Dixon [23] plots.

phenazine inhibition. NaCl and sodium Hepes relieved inhibition by perphenazine with a similar efficiency, but higher concentrations of KCl were required to obtain a similar effect. In contrast, increasing concentrations of Na_2SO_4 or Na^+, $K^+H_2PO_4^-$, HPO_4^{2-} decreased but did not abolish the inhibition of GDH activity by 50 μM perphenazine (Fig. 6B).

As inhibition of ox brain GDH by perphenazine was found to vary only slightly in the presence of phosphate or sulphate at an ionic strength higher than 0.2 M, buffers containing 100 mM Na_2SO_4 and 20 mM KH_2PO_4 adjusted to the desired pH between 6.3 and 7.6 were used to study the effects of pH. The ionic strength of these buffers was calculated to be 0.33 M (±0.01, range). Figure 7 shows that the inhibition of GDH by 50 μM perphenazine increased as the pH increased. GDH activity in the absence of perphenazine increased as the pH was varied from 6.3 to 7.6. The enzyme activity in the presence of perphenazine increased to a similar extent at pH values below 6.5. At higher pH values, the enzyme activity in the presence of perphenazine reached a plateau, whereas activity in the absence of effector continued to increase.

DISCUSSION

High substrate inhibition by NADH appears to be due to a non-catalytic binding site on each subunit with an affinity for this coenzyme lower than that of the catalytic site [20, 21]. The patterns of inhibition of ox brain GDH by perphenazine with respect to NADH and 2-oxoglutarate suggested that perphenazine may interact with the NADH catalytic site. The enhancement of inhibition by NADH might thus result from this interaction. However, inhibition by perphenazine was obtained under conditions where the perphenazine concentration was half the K_i value and the NADH concentrations was 5–8 K_m. As perphenazine could inhibit GDH under conditions where it could hardly bind to the NADH catalytic site, the binding site for perphenazine appeared to be distinct from the NADH catalytic site. This is consistent with the quantitative affinity chromatography experiments of Veronese *et al.* [2]

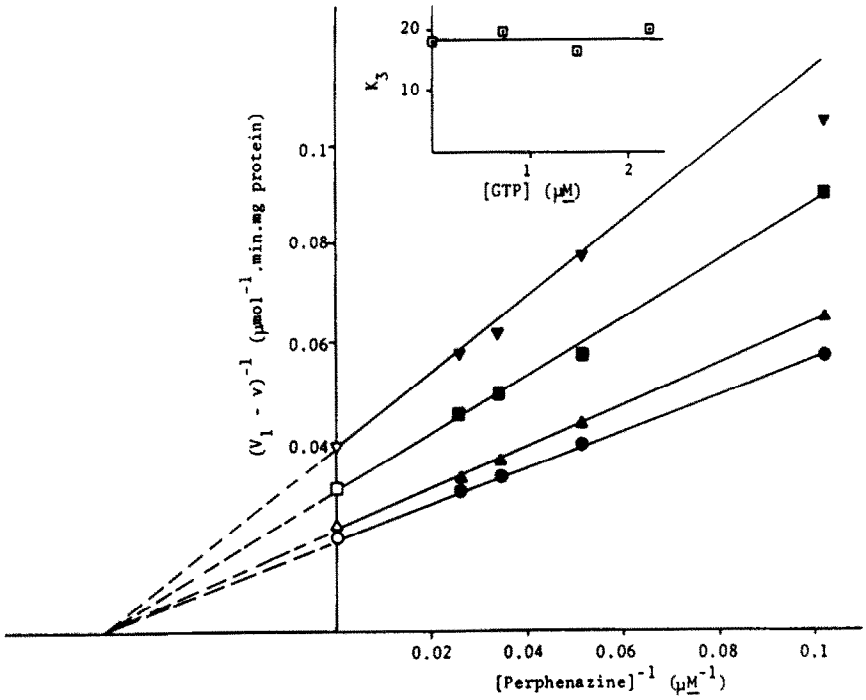

Fig. 4. Inhibition of ox brain GDH by perphenazine in the presence of GTP. The perphenazine concentration was varied while the GTP concentration was held constant at the following values: 0 (●); 0.75 μM (▲); 1.5 μM (■) and 2.25 μM (▼). Data were plotted as described by Shemisa and Fahien [1]. The open symbols on the y-axis represent $1/V_1$, K_3 (μM) values were determined by non-linear regression and were plotted against the GTP concentration which gave them, as shown in the inset.

who found that NADH decreased the binding of ox liver GDH to a perphenazine affinity column, although not by direct competition with the matrix-bound perphenazine.

Heterotropic positive interactions have been found between the inhibitory effects of 2-oxoglutarate and NADH on ox brain GDH [9]. The enhancement by perphenazine of the inhibition by one of these substrates may therefore enhance the inhibition by the other substrate. Alternatively, perphenazine may affect directly the inhibitory effects of both substrates.

Since GTP is known also to enhance NADH substrate inhibition [22], the possibility that perphenazine could bind at a GTP binding site was investigated. Inhibition of ox brain GDH by perphenazine was found not to be affected by the presence of GTP, thus suggesting that the GTP binding site and the perphenazine binding site were distinct. A similar result has been reported by Shemisa and Fahien [1]. The two sites, however, cannot be kinetically independent, since GTP and perphenazine are related by their effect on NADH substrate inhibition. As the determined values of K_3 were only apparent constants, negative interactions between GTP and perphenazine might have been concealed by the positive effects of enhanced NADH inhibition on perphenazine inhibition.

Anionic phospholipids were found to affect the inhibition of ox brain GDH by perphenazine. The presence of phosphatidylcholine, which did not inhibit GDH, in accordance with previous reports [14, 19], did not interfere with perphenazine inhibition. This suggested that phospholipid micelles did not decrease the availability of perphenazine by binding it. However, unlike phosphatidylcholine, phosphatidylserine and cardiolipin have a net negative charge at pH 7.4. Electrostatic attraction between the protonated amine group of perphenazine and the polar headgroups of the anionic phospholipids could occur and result in the absorption of perphenazine molecules at the surface of the anionic phospholipid micelles. However, as the buffer used in the present study had a high ionic strength ($I = 0.11$ M) electrostatic interactions were assumed to be very weak.

Perphenazine at low concentrations relieved the inhibition caused by either phosphatidylserine or cardiolipin. This would be expected if perphenazine was a less potent inhibitor and displaced anionic phospholipids from the enzyme. This would require the inhibition by perphenazine to be partial in nature. However, the analysis of inhibition curves indicated that this was not the case, in accordance with previous results [4]. Furthermore, if the observed activation of GDH by perphenazine resulted from the complete displacement of phospholipids, the presence of phospholipids should not interfere with the inhibition obtained at higher concentrations of perphenazine.

After the activation phase observed at low concentrations of perphenazine, the presence of phosphatidylserine did not seem to interfere with

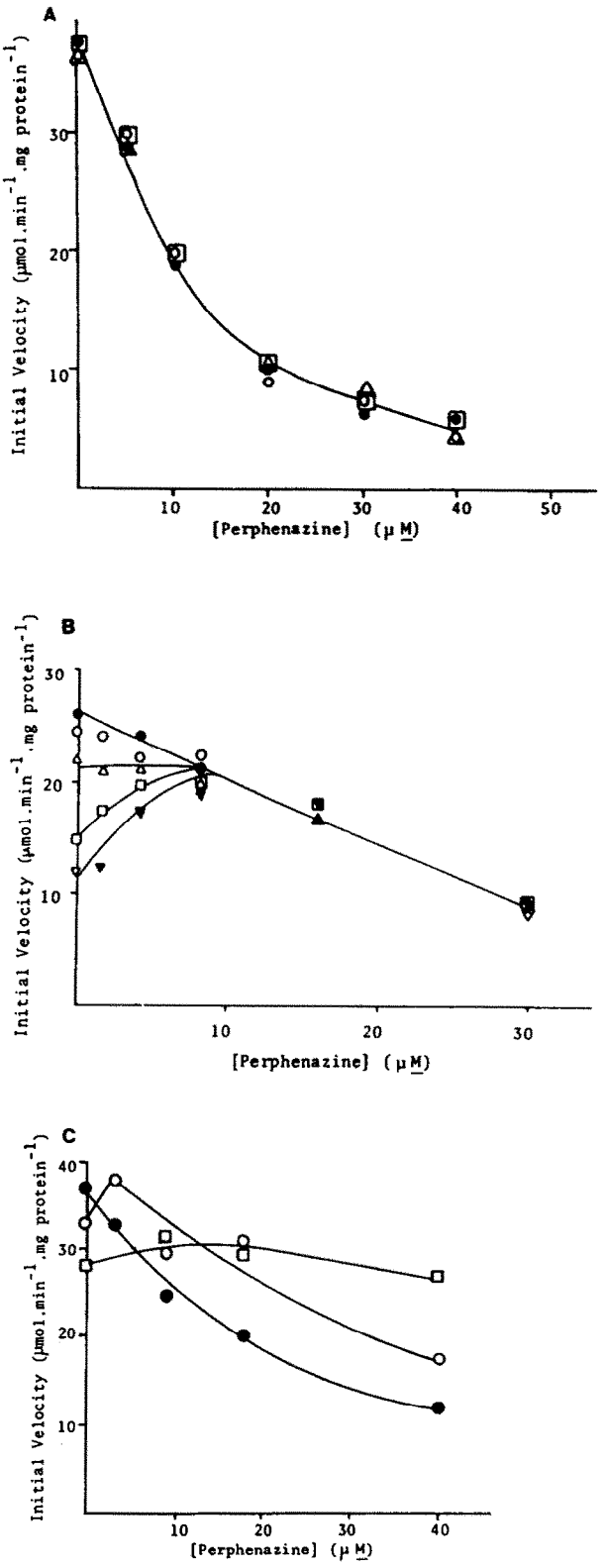

Fig. 5. Effects of perphenazine on ox brain GDH in the presence of phospholipids. The perphenazine concentration was varied while the concentration of (A) L-α-dimyristoylphosphatidylcholine (Sigma) was held constant at the following values: 0 (●); 13.3 μM (○); 26.5 μM (△) and 53.1 μM (□). (B) phosphatidylserine from ox brain (Sigma) was held constant at the following values: 0 (●); 1.8 μg/mL (○); 3.6 μg/mL (△); 9 μg/mL (□) and 18 μg/mL (▼). (C) cardiolipin from ox heart (Sigma) was held constant at the following values: 0 (●); 10 μg/mL (○) and 20 μg/mL (□).

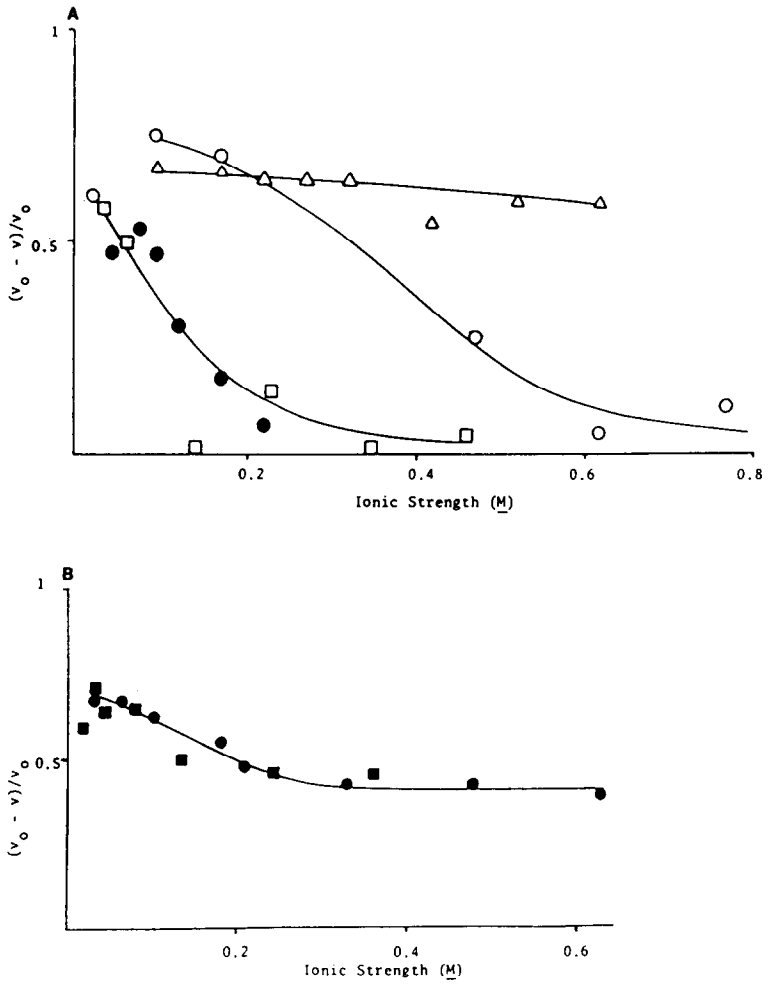

Fig. 6. Effects of salts on the inhibition of ox brain GDH by perphenazine. Ox brain GDH was assayed in the presence of increasing concentrations of the following salts: (A) LiCl (△); sodium Hepes (●); KCl (○) and NaCl (□) or (B) sodium/potassium phosphate (■) and Na_2SO_4 (●). v_0 and v are, respectively, the velocity in the absence of effector and the velocity in the presence of 50 μM perphenazine. Data were plotted as the fractional inhibition, $(v_0 - v)/v_0$, vs the ionic strength.

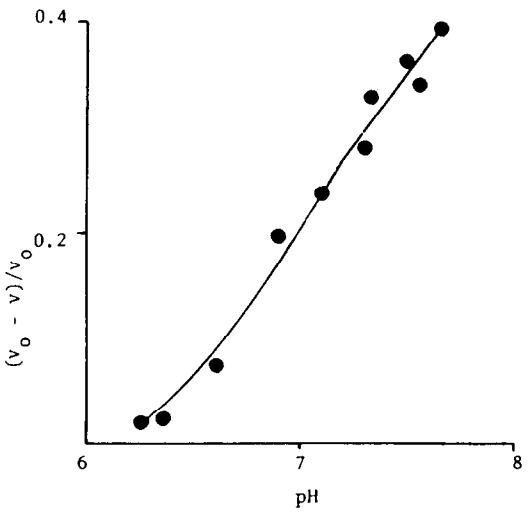

Fig. 7. Effects of pH on the inhibition of ox brain GDH by perphenazine. Ox brain GDH was assayed over a pH range from 6.3 to 7.6. v_0 and v are the velocities in the absence and presence of 50 μM perphenazine, respectively. Data are presented as the fractional inhibition, $(v_0 - v)/v_0$, vs the measured pH. The buffer contained 100 mM Na_2SO_4 and 20 mM KH_2PO_4 adjusted to the required pH with 5 M NaOH.

perphenazine inhibition, but the presence of cardiolipin decreased the extent of inhibition by perphenazine. It appeared, therefore, that perphenazine did not completely displace phospholipids from their binding sites. In other words, perphenazine and anionic phospholipids were able to bind simultaneously to GDH and modify the effects of each other on the enzyme. It is likely that phospholipid binding to GDH is complex and involves more than one site per protomer [14]. The present results might thus be interpreted in terms of multiple binding sites for perphenazine and phospholipids with mutually competitive effects occurring at only some of them.

The physiological significance of the interaction of antipsychotic drugs with mammalian GDH must depend on the concentrations of drug and the regulation of the enzyme in the mitochondrial matrix. However, it is not known whether antipsychotic drugs are concentrated in the mitochondria or not and the interactions of GDH with its mitochondrial environment are not fully understood.

The present study indicates that increasing pH, inhibitory NADH and 2-oxyglutarate concentrations would enhance inhibition by antipsychotic drugs, whereas high ion concentrations and the presence of anionic phospholipids could diminish this inhibition. Furthermore, the effects on the inhibition of GDH by phospholipids raises the possibility that the true effect of antipsychotic drugs in the mitochondrial environment might be activation, rather than inhibition, of GDH.

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