Br. J. Pharmac. (1969), 37, 459-467.

The influence of psychotropic drugs on aldolase, mitochondrial malic dehydrogenase and $Mg^{++}Na^{+}K^{+}$ adenosine triphosphatase

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1. The experiment investigated the effects of psychotropic drugs on enzymes *in vitro*.

2. Chlorpromazine inhibits mitochondrial malate dehydrogenase in concentrations of $1{\cdot}4 \times 10^{-4}$M and above. No effect was observed below this concentration.

3. A sharp inflection in the $\frac{1}{V}$/drug plot was also seen with chlorpromazine ($1{\cdot}4 \times 10^{-4}$M), trifluoperazine (1×10^{-5}M), prochlorperazine (1×10^{-4}M), and amylobarbitone (8×10^{-3}M) acting on rat brain (Na^{+}-K^{+}-) Mg^{++}-ATPase and with chlorpromazine (10^{-4}M) and imipramine (3×10^{-4}M) acting on aldolase.

Many drugs have been shown to be capable of altering the physical properties of cell membranes. For example Spirtes & Guth (1963) observed inhibition of mitochondrial swelling in the presence of chlorpromazine; anti-inflammatory drugs decrease the release of acid hydrolases from lysosomes (Tanaka & Tizuka, 1968) and tranquillizers and anti-histamines increase the resistance of erythrocytes to osmotic disruption (Seeman & Weinstein, 1966). By the use of spin labels, Hubbell, McConnell & Metcalfe (1969) have provided evidence from electron spin resonance studies that anaesthetics in low concentrations alter the crystalline structure of membrane lipids and disturb protein-lipid binding. The biological function of phospholipids is not known, but the work of Racker and his colleagues indicates that binding of membrane proteins to specific phospholipids (as occurs in intact membranes) alters their properties (Racker & Bruni, 1968). Thus if central nervous system depressant drugs alter the physical state of membrane lipids, this could produce a concomitant change in the activity of membrane bound enzyme proteins. The following experiment is an investigation of the effects of some psychotropic drugs on enzymes which occur bound to membranes to widely different extents. Two of these were purified proteins (mitochondrial malic dehydrogenase and muscle aldolase) and the third was a crude preparation of brain ($Mg^{++}Na^{+}K^{+}$ adenosine triphosphatase [ATPase]).

Methods

Malate dehydrogenase

A purified enzyme from hog heart mitochondria was used (Koch Light Laboratories Ltd.). The activity of the stock solution was 855 Racker u./mg protein and contained 8·5 mg protein/ml. The assay for this enzyme employed an incubation mixture of 3 ml. containing 0·15 μ-moles oxaloacetate (C. F. Boehringer & Soehne); 1·42 μ-moles NADH (C. F. Boehringer & Soehne); Sørensen's sodium potassium phosphate buffer, pH 7·5, 0·25 m-moles; chlorpromazine solution (May & Baker Ltd.) 0·3 ml. (or an equal volume of water); enzyme, 0·02 ml. of a 0·1% (v/v) solution of the malate dehydrogenase stock solution. The optical density of this mixture was measured at 366 mμ every minute for 5 min after the addition of the enzyme. The reaction was carried out at 21° C. The average change in optical density per minute was taken to be proportional to the velocity of the dehydrogenase-catalysed reaction.

ATPase

Adult male SAS/ICI albino mice weighing 30–40 g were used. Homogenates (4% w/v) were made of brain tissues in an ice cold solution consisting of potassium chloride 0·1 M; sodium bicarbonate 0·04 M; sodium carbonate 0·01 M; potassium cyanide 0·001 M. The incubation mixture for ATPase assay was maintained at 37° C. One ml. of the mixture contained 60 μ-moles Tris HCl buffer pH 7·0; 4 μ-moles ATP (C. F. Boehringer & Soehne); 10 μ-moles $MgCl_2$ (A.R. grade,

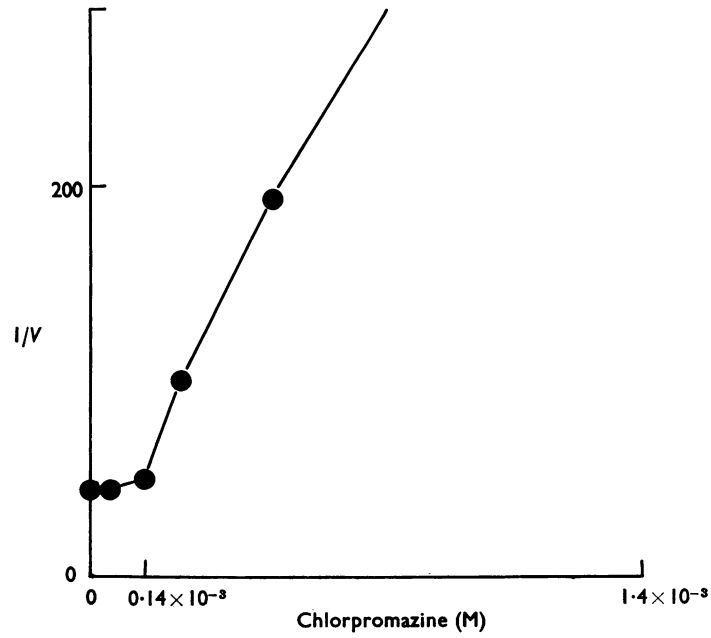

FIG. 1. Influence of chlorpromazine concentration on $\frac{1}{\text{velocity}}\left(\frac{1}{V}\right)$ of mitochondrial malic dehydrogenase. With a chlorpromazine concentration of $1{\cdot}4 \times 10^{-3}$M, no enzyme activity was detected.

Hopkin & Williams Ltd.); 0·1 ml. of brain homogenate. The reaction was started by the addition of homogenate and after incubation for 5 min was stopped by adding 1 ml. 10% perchloric acid or methanol. The amount of inorganic phosphate liberated was measured using a molybdate/ferrous sulphate method. When the concentration of ATP was varied in the enzyme kinetic studies the concentration of $MgCl_2$ was altered so that the $MgCl_2$/ATP ratio remained at 2·5. The effects of separately incorporating chlorpromazine, trifluoperazine (Smith, Kline & French Laboratories Ltd.), prochlorperazine (May & Baker Ltd.) and amylobarbitone (Eli Lilly & Co. Ltd.) into the incubation mixture were observed.

Aldolase

The assay of aldolase was a modification of the method of Jagannathan, Singh & Damodaran (1956). A commercially purified preparation of rabbit muscle aldolase was used (C. F. Boehringer & Soehne), 10 mg/ml. The reaction was carried out at 24° C and 2·5 ml. of the basic incubation mixture contained 0·25 μ-moles fructose 1,6 diphosphate (C. F. Boehringer & Soehne); 4·9 μ-moles hydrazine (B.D.H. Ltd.); 0·1 ml. of 0·01% enzyme suspension. The rate of change of optical

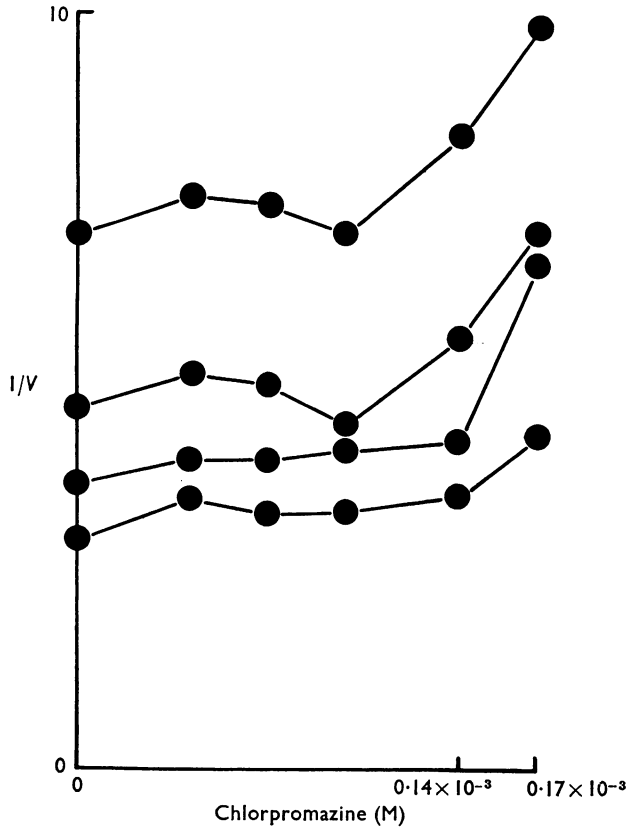

FIG. 2. Influence of chlorpromazine concentration on $\frac{1}{\text{velocity}}\left(\frac{1}{V}\right)$ of ATPase action. From top to bottom the curves represent ATP concentrations of $0{\cdot}25\times10^{-3}$, $0{\cdot}5\times10^{-3}$, $1{\cdot}0\times10^{-3}$ and $2{\cdot}0\times10^{-3}$M.

density at 240 mμ was taken to be proportional to the velocity of the aldolase catalysed reaction. Chlorpromazine and imipramine were incorporated into the incubation mixture in the concentrations indicated in the results.

Results

Chlorpromazine was found to inhibit malate dehydrogenase. Figure 1 shows the relationship between $\frac{1}{V}$ and drug concentration. The plot was biphasic with a sharp inflection at about $1{\cdot}4 \times 10^{-4}$M chlorpromazine, above which marked inhibition occurred and at $1{\cdot}4 \times 10^{-3}$M chlorpromazine there was complete inhibition of the enzyme. Below $1{\cdot}4 \times 10^{-4}$M chlorpromazine, no effect on enzyme activity was observed.

When the effect of chlorpromazine was investigated on brain ATPase a qualitatively similar result to that found with malate dehydrogenase was observed. No inhibition could be detected up to about 1×10^{-4}M, at which point inhibition began abruptly (Fig. 2). The precise point of inflection seemed to vary with the ATP concentration used, being higher at high ATP concentrations (Fig. 2).

Trifluoperazine inhibited ATPase at concentrations of 1×10^{-5}M and above. As with all the inhibitions produced in this experiment $\frac{1}{V} / \frac{1}{\text{substrate}}$ plots were linear and the inhibition was of mixed type (Fig. 3). In the plot of $\frac{1}{V}$ trifluoperazine (Fig. 4) it is possible that an inflection occurred close to the ordinate, but it was not possible to determine unambiguously whether or not the graph was biphasic. Similar results were obtained with prochlorperazine (Fig. 4).

Although this type of effect was seen with amylobarbitone (Figs 5 and 6), much higher concentrations of drug were necessary to produce enzyme inhibition. Up to $4{\cdot}0 \times 10^{-3}$M amylobarbitone produced no change in ATPase; at 8×10^{-3}M and above, inhibition occurred.

The response of aldolase to increasing amounts of chlorpromazine was not linear. No effect was observed up to $7{\cdot}5 \times 10^{-5}$M and inhibition occurred with 10^{-4}M and higher concentrations of the drug (Fig. 7). A similar response was seen with imipramine, but enzyme inhibition only occurred above 3×10^{-4}M drug (Fig. 8). With each of the enzymes the addition of sufficient amounts of any of the drugs to produce enzyme inhibition resulted in linear $\frac{1}{V} / \frac{1}{S}$ plots. In each instance the inhibition was of the mixed type (for example, Figs. 3 and 5). The estimated pseudo first order inhibitor rate constants (K_I) for the drugs are shown in Table 1.

TABLE 1. *Pseudo first order kinetic constants*

Enzyme	Drug	K_I
Malate dehydrogenase	Chlorpromazine	$2{\cdot}9 \times 10^{-4}$
ATPase	Chlorpromazine	$5{\cdot}6 \times 10^{-4}$
	Trifluoperazine	$4{\cdot}4 \times 10^{-4}$
	Prochlorperazine	$3{\cdot}1 \times 10^{-4}$
	Amylobarbitone	$5{\cdot}7 \times 10^{-3}$
Aldolase	Chlorpromazine	$1{\cdot}2 \times 10^{-4}$
	Imipramine	$5{\cdot}6 \times 10^{-4}$

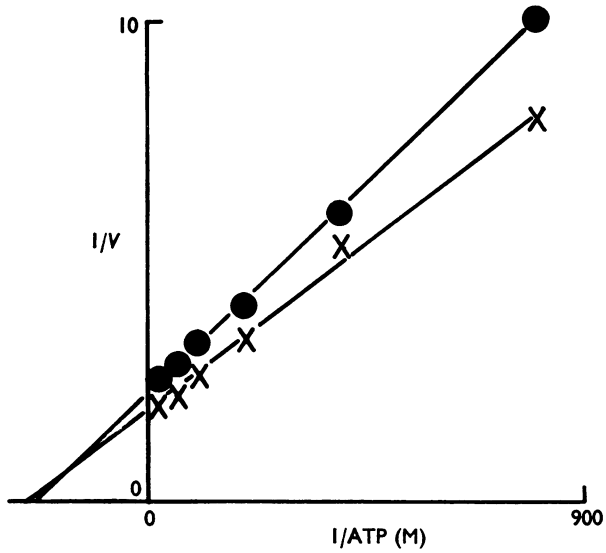

FIG. 3. Lineweaver and Burk plots of inhibition of ATPase by 10^{-5}M trifluoperazine (●); control (×).

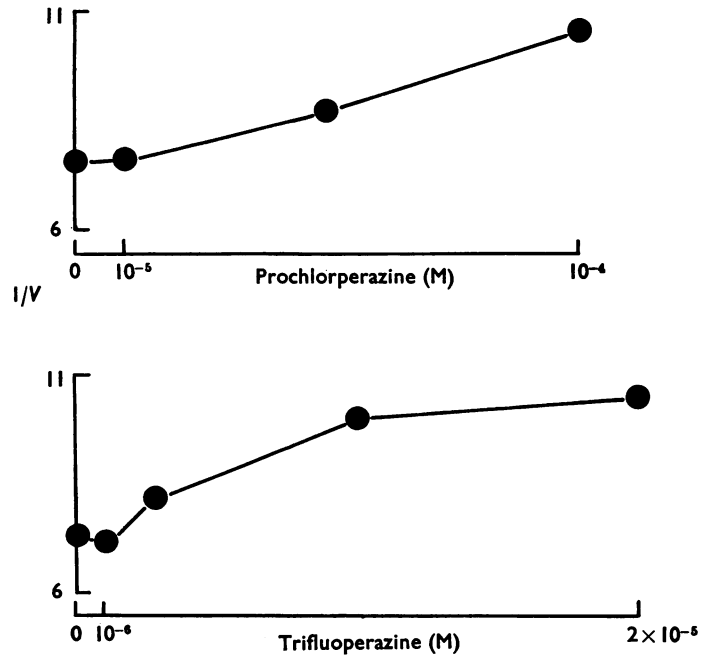

FIG. 4. Effect of prochlorperazine and trifluoperazine on $\frac{1}{\text{velocity}}\left(\frac{1}{V}\right)$ of ATPase.

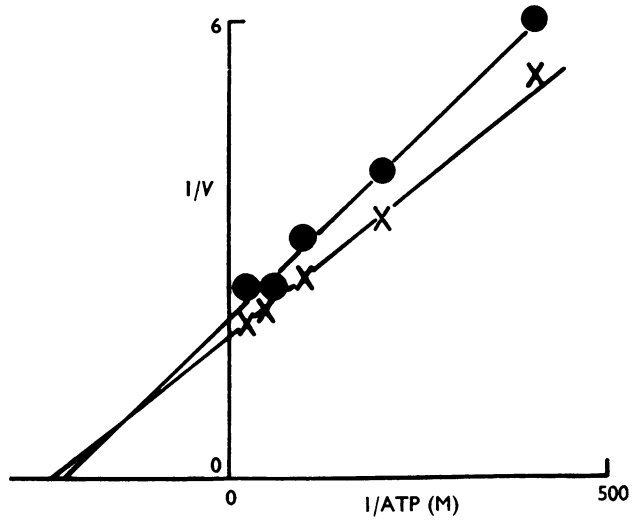

FIG. 5. Lineweaver and Burk plots of inhibition of ATPase by $1{\cdot}2 \times 10^{-2}$M amylobarbitone (●); control (×).

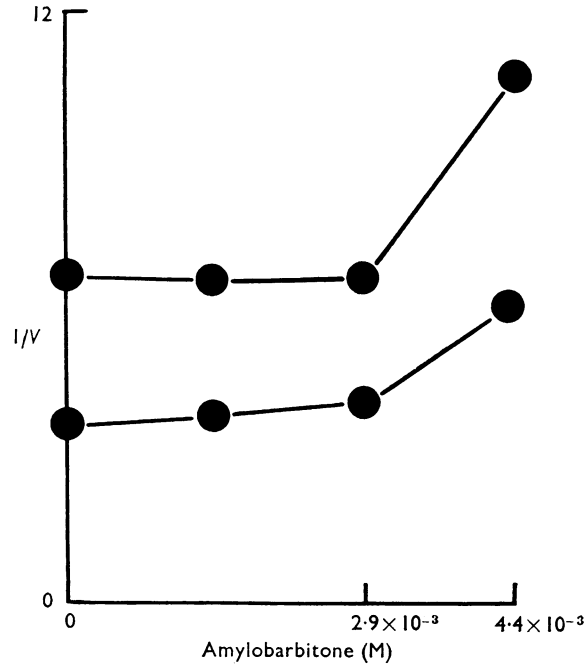

FIG. 6. Influence of amylobarbitone on $\frac{1}{\text{velocity}}\left(\frac{1}{V}\right)$ of ATPase action. The upper and lower curves are of 5 and 20 mM ATP concentrations respectively.

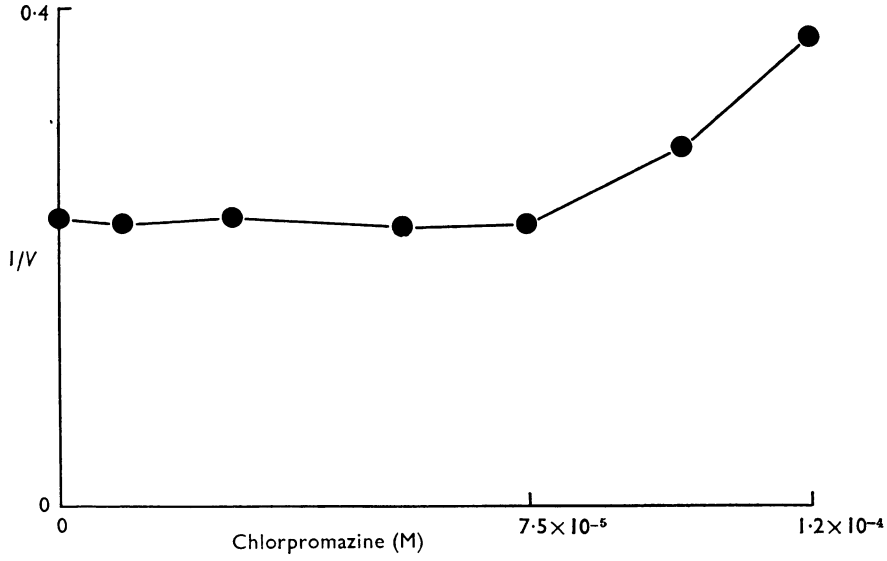

FIG. 7. Effect of chlorpromazine on $\frac{1}{\text{velocity}}\left(\frac{1}{V}\right)$ of aldolase action.

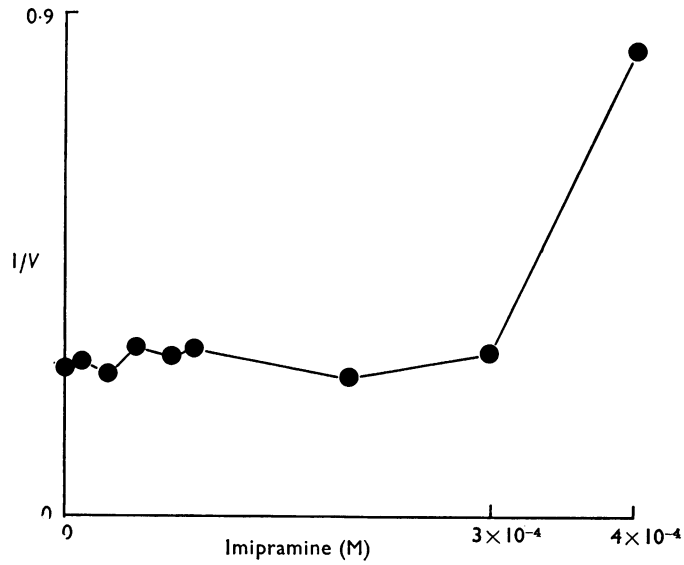

FIG. 8. Influence of imipramine on $\frac{1}{\text{velocity}}$ of aldolase action.

Discussion

In these experiments the low solubility of the drugs limited the range of concentrations tested and restricted the studies with malate dehydrogenase to the effects of chlorpromazine. Nevertheless chlorpromazine, trifluoperazine, prochlorperazine, amylobarbitone and imipramine were all capable of inhibiting the enzymes with which they could be investigated. The relationship $\frac{1}{V} / \frac{1}{\text{substrate}}$ was linear and indicated the inhibition to be of the mixed type. However, the relationship $\frac{1}{V}$/drug was not a simple one, and inhibition occurred only above a critical concentration when normal Michaelis-Menten kinetics could be applied. The inflection in the $\frac{1}{V}$/drug plot indicates that the inhibition process involves more than the simple binding of the drug to the enzyme. It is notable that all these enzymes are composed of sub-units. A possible explanation then is that the curves represent the initial portions of sigmoidal curves in which binding of the first molecule of drug induces a conformational change in the enzyme, facilitating the binding of subsequent drug molecules. Inhibition occurs only as a consequence of both binding the drug and a change in conformation.

An alternative explanation, which would not involve conformational change, is that it is not until the drug concentration is high enough to induce the binding of two or more drug molecules that inhibition occurs. Binding might involve more than one site on the protein.

The sharp change in the nature of the response of these enzymes to drugs is reminiscent of the rapid change from membrane-stabilizing to membrane-labilizing effects of tranquillizers on raising the drug concentration (Seeman & Weinstein, 1966). Seeman & Weinstein found the turning points for red cell membrane with chlorpromazine, prochlorperazine and trifluoperazine to be approximately 10^{-4}M, 5×10^{-5}M and 10^{-5}M respectively. These were the minimum concentrations in this experiment which could produce inhibition of enzyme activity. Thus it is possible that at these concentrations a single effect is produced in membrane proteins which results in both diminished osmotic resistance and inhibition of enzymic function. Our experiments indicate that this threshold phenomenon is not peculiar to tightly membrane bound enzymes, but also occurs with aldolase, which is believed to be loosely bound.

An alteration in the function of membrane proteins in response to psychotropic drugs is of interest in the light of the work of Hubbell *et al.* (1969) on the ability of anaesthetics to alter the physical state of membrane lipids. These workers also showed a dose dependent qualitative effect and above a critical concentration of anaesthetic, protein binding sites were exposed. This suggests that although drugs may affect membrane proteins the primary site of drug-tissue interaction may not be protein but membrane lipid or the region of lipid-protein binding. Recent evidence suggests that such a dissociation of lipid and protein could decrease the influence of membrane lipids on the physical state of the membrane proteins. Wallach & Zahler (1966) have investigated the optical rotary dispersion of plasma membrane preparations and shown that conformational changes of membrane proteins are induced by removal of membrane lipid. Zahler (1968) has also shown the ABO blood group activity of erythrocyte membranes is exhibited by both the separated lipid and protein components, but on re-combination the activity is greater than that of the sum

of the two separately. Similarly, Cerletti (1966) has shown that soluble, lipid depleted succinate dehydrogenase combines with phospholipids, thereby restoring its properties to what they originally were in the membrane bound form. These data provide an indication that the structure of a protein embedded in a membrane may be distorted by modification of its lipid environment. It is clear that direct interaction of a drug with a membrane bound protein and the secondary effect of a protein modified by distortion of its environment could lead to the same observed experimental consequences. Thus, although chlorpromazine in this experiment was shown to yield a similar inhibition constant when acting on the three enzymes, the mechanism of its action on aldolase (which is free of phospholipid in its purified form) may be different from its action on the tightly membrane bound enzymes.

This work was supported by a grant from the United Cerebral Palsy Research Foundation, New York.

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(*Received May* 21, 1969)