THE EFFECTS OF PROMAZINES ON MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE REACTIONS*

H. LÖW

Wenner-Gren Institute, University of Stockholm, Stockholm (Sweden)

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SUMMARY

The effects of promazine, chlorpromazine and acetylpromazine on rat-liver mitochondrial oxidative phosphorylation, phosphate–adenosine triphosphate exchange and adenosine triphosphatase reactions have been studied.

The drugs depress glutamate-supported respiration, without lowering the phosphate-oxygen ratio. They inhibit phosphate–adenosine triphosphate exchange more potently than glutamate-supported respiration.

The 2,4-dinitrophenol-induced adenosine triphosphatase catalyzed by intact mitochondria is stimulated by low, and and inhibited by high concentrations of promazines. The concentrations needed for maximum stimulation (about 50 %) are 0.01 m*M* promazine, 0.005 m*M* chlorpromazine, and 0.02 m*M* acetylpromazine. The inhibitory effect of the promazines is potentiated by amytal and partially neutralized by added flavin mono- or dinucleotide.

The Mg^{++}-activated adenosine triphosphatase of mitochondria treated with 0.1 % deoxycholate is inhibited by promazines, the concentrations needed for half inhibition being the same as those giving maximum stimulation of the dinitrophenol-induced adenosine triphosphatase of the intact mitochondria. The inhibition of the Mg^{++}-activated adenosine triphosphatase is partially relieved by dithionite.

On the basis of the analogy between the effects of promazines, described herein, and those of atebrin, reported previously, it is concluded that promazines interfere with the mitochondrial diaphorase flavin. The present findings are discussed in relation to the hypothesis that the mitochondrial diaphorase flavin participates as a high-energy phosphate carrier in phosphorylation coupled to electron transport.

INTRODUCTION

Lately much attention has been paid in the literature to the so-called tranquilizers, drugs which are widely used in psychotherapy. Chemically, they represent a rather heterogeneous collection of compounds. One group of phenothiazine derivatives, the

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; DNP, 2,4-dinitrophenol; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane.

* An account of this work has been given at the Meeting of the Swedish Biochemical Society, March, 1958[1], and at the Fourth International Congress of Biochemistry, Vienna, September, 1958[2].

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promazines, and in particular chlorpromazine, has gained a special interest. Chlorpromazine has been demonstrated to have an action on mitochondrial respiration and also on oxidative phosphorylation[3-5].

YAGI *et al.*[6] have recently shown that chlorpromazine inhibits the D-amino acid oxidase and that the inhibition is due to a competition with FAD, the prosthetic group of this enzyme.

Previous work at our laboratory led to the conclusion that the mitochondrial ATPases are parts of the enzyme system involved in the oxidative phosphorylation[7] and that the DNP-induced ATPase of intact mitochondria mainly originates from the phosphorylation occurring in the diaphorase region of the respiratory chain[8]. In a subsequent study[9] it was shown that the flavin antagonist, atebrin, exerts a marked effect on the DNP-induced ATPase reaction catalyzed by intact mitochondria, as well as on the Mg^{++}-activated ATPase catalyzed by structurally disorganized mitochondria. It was concluded that both of these ATPases involve the participation of the diaphorase flavoprotein, its prosthetic group probably serving as an intermediate carrier of high-energy phosphate[2,10,11].

In the present paper the effects of promazine, chlorpromazine and acetylpromazine on the mitochondrial ATPases are described. It is demonstrated that these effects are very similar to those previously found with atebrin. The data support the conclusion that promazines compete with the mitochondrial diaphorase flavin, and inhibit, in particular, the transfer of high-energy phosphate between phosphorylflavin and ATP.

EXPERIMENTAL

Rat-liver mitochondria, twice washed, were prepared as described previously[12].

For tests of oxidative phosphorylation and P_i–ATP exchange, mitochondria derived from 200 mg (wet weight) of liver, and suspended in 1 ml 0.25 *M* sucrose, were added to the reaction vessels. The final volume of the reaction mixture was 2 ml. The tests were performed as described previously[8].

For ATPase tests the mitochondrial pellets were suspended in sucrose to give 1 ml of a 0.25 *M* sucrose suspension containing mitochondria from 50 mg liver. In the case of deoxycholate treatment, the suspension was diluted with a deoxycholate–sucrose mixture (pH 7.5) to give a 0.25 *M* sucrose suspension containing 0.1 % deoxycholate. The deoxycholate was allowed to act for 2 min at room temperature. 1-ml aliquots of the sucrose suspension were added to the incubation tubes containing 10 μmoles Tris buffer (pH 7.5), 10 μmoles ATP (pH 7.5), 150 μmoles sucrose and, when used, 8 μmoles Mg^{++} or 0.2 μmole DNP. The final volume was 2.0 ml, and the final concentration of deoxycholate in the ATPase test was half of that used in preincubation. The tubes were incubated for 20 min at 30°, and the reaction was stopped with 1.0 ml 1.5 *M* $HClO_4$. Inorganic phosphate was determined according to the MARTIN AND DOTY[13] method as described by LINDBERG AND ERNSTER[14].

RESULTS

Fig. 1 illustrates the effects of promazine on oxidative phosphorylation supported by glutamate, and on the P_i–ATP exchange reaction as measured in the absence of added

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substrate. Promazine inhibited respiration and the accompanying phosphate uptake virtually to the same extent, thus causing no decrease of the P/O ratio. It also inhibited the P_i–ATP exchange reaction, and this inhibition was stronger than that of glutamate oxidation. The inhibition of the glutamate oxidation was not altered when 0.1 m*M* DNP was added to uncouple phosphorylation. Up to 0.3 m*M*, promazine did not inhibit the oxidation of succinate or the accompanying phosphorylation; at 0.5 m*M* promazine, the succinate oxidation and the accompanying phosphorylation were slightly depressed. Chlorpromazine acted in the same way, but it was about twice as

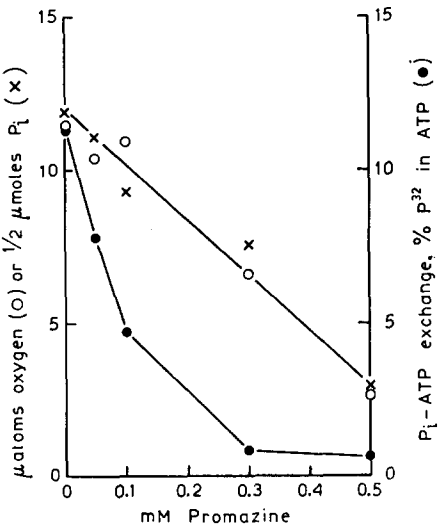

Fig. 1. Effect of promazine on the oxidative phosphorylation supported by glutamate, and the P_i–ATP exchange reaction in rat-liver mitochondria. Conditions for oxidative phosphorylation: Each Warburg vessel contained mitochondria from 200 mg (wet weight) rat-liver, 20 μmoles substrate, 30 μmoles $^{32}P_i$ (pH 7.5), 8 μmoles Mg^{++}, 3 μmoles ATP, 50 μmoles Tris (pH 7.5), 125 μmoles sucrose, and hexokinase in excess (together with 60 μmoles glucose). The final vol. was 2.0 ml, gas phase air, temperature 30° and time of incubation was 20 min. Conditions for P_i–ATP exchange: mitochondria from 200 mg liver, 30 μmoles ATP (pH 7.5), 20 μmoles P_i (pH 7.5), 8 μmoles Mg^{++}, 100 μmoles Tris (pH 7.5) and 125 μmoles sucrose. Final vol. was 2.0 ml. Temperature 22°. The % value was obtained by substracting the per cent phosphate exchanged after 3-min incubation from the value obtained after 13-min incubation

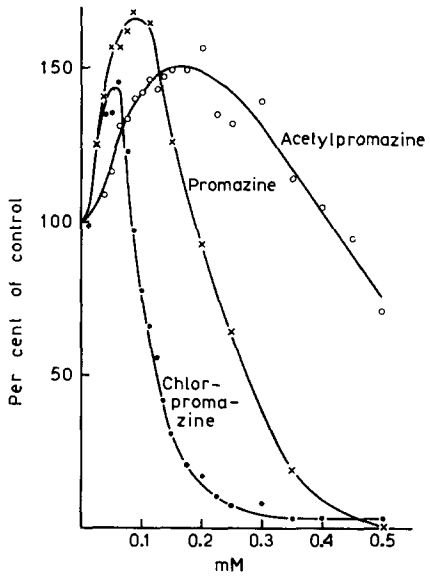

Fig. 2. Effects of promazine, chlorpromazine and acetylpromazine on the DNP- induced ATPase. Each tube contained mitochondria from 50 mg wet weight liver, 10 μmoles ATP (pH 7.5), 10 μmoles Tris (pH 7.5) and 0.1 μmole DNP in 2.0 ml 0.25 *M* sucrose, and was incubated for 20 min at 30°.

potent as promazine (*cf.* also Figs. 2 and 6). These results are essentially in accordance with those recently reported by DAWKINS *et al.*[15], but they are at some variance with earlier findings of BERGER *et al.*[5,16]. Possible reasons for this discrepancy will be discussed later.

Fig. 2 demonstrates the effects of promazine, chlorpromazine and acetylpromazine on the DNP-induced ATPase of intact mitochondria. All three drugs stimulated the DNP-induced ATPase at lower concentrations, and inhibited it at higher concentrations. This type of effect was previously[9] found to be characteristic

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of flavin antagonists. The differences seen in the degree of stimulation with the three promazine derivatives are not significant; in all three cases the degree of maximum stimulation varied between 25 % and 75 %. The concentration, on the other hand, at which maximum stimulation occurs, proved to be remarkably consistent, 0.05 m*M* with chlorpromazine, 0.1 m*M* with promazine and 0.2 m*M* with acetylpromazine.

Hydroxyzine, another drug with tranquilizing action, had a similar effect to that of the promazines, giving a maximum stimulation at 0.25 m*M* (Fig. 3). Azacyclonol, which belongs to the same group of tranquilizers as hydroxyzine, gave a maximum (about 30 %) stimulation of the DNP-induced ATPase at 1 m*M*. The well-known tranquilizer, reserpine, could not be tested because of its poor solubility at the pH of the test system.

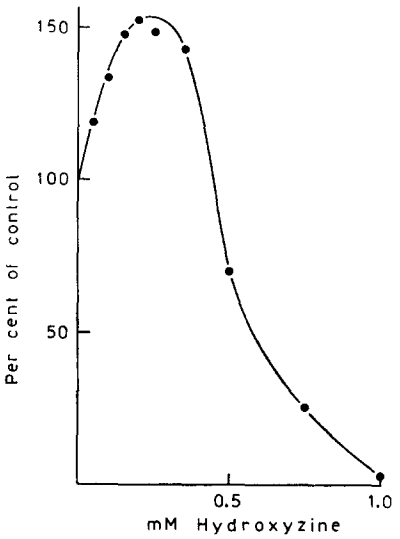

Fig. 3. Effect of hydroxyzine on the DNP-induced ATPase. Experimental conditions as in Fig. 2.

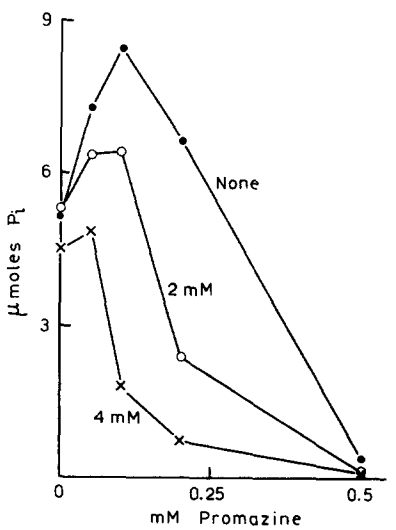

Fig. 4. Effect of promazine on the DNP-induced ATPase in the presence of amytal. Experimental conditions as in Fig. 2. Amytal was added in the concentrations indicated in the figure.

It was shown previously[9] that the inhibitory effect of atebrin on the DNP-induced ATPase can be greatly potentiated with amytal. Fig. 4 shows that if amytal is added together with promazine the inhibitory effect on the DNP-induced ATPase is markedly potentiated, and that, at sufficiently high concentrations of amytal, the stimulation caused by promazine is almost nullified. This is remarkable since promazines are known to potentiate the action of barbiturates also *in vivo*[17].

As shown in Fig. 5, the effect of promazine on the DNP-induced ATPase can be counteracted by the addition of FMN or FAD. This, too, is in conformity with previous findings with atebrin[9], although in the present case the restoration was less efficient.

Promazine and its derivatives, like atebrin, were found to be potent inhibitors of the Mg^{++}-activated ATPase of mitochondria structurally disorganized by treating with 0.1 % deoxycholate (Fig. 6). As in the case of the DNP-induced ATPase of intact mitochondria, chlorpromazine proved also here to be the most potent of the three

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compounds, followed by promazine and acetylpromazine. In all three cases, 50 % inhibition of the Mg^{++}-activated ATPase occurred at about the same concentrations as gave maximum stimulation of the DNP-induced ATPase (*cf.* Fig. 2). As can be seen in Fig. 6, the promazine inhibition of the Mg^{++}-activated ATPase does not go to completion with increasing concentrations of the inhibitors, but levels off at about 80–90 % of inhibition. The reason for this phenomenon is not yet understood.

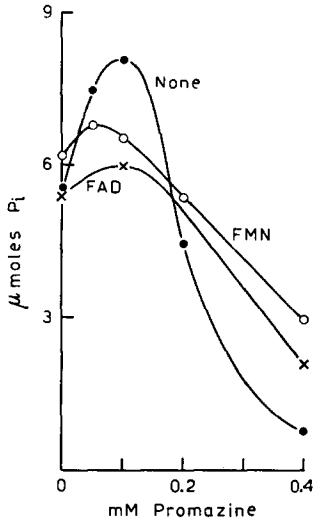

Fig. 5. Effect of promazine on the DNP-induced ATPase in the presence of FAD and FMN. Experimental conditions as in Fig. 2. Addition where indicated: FAD, 5 mM; FMN, 3.5 mM.

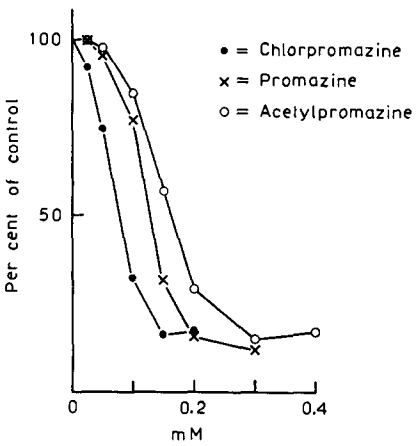

Fig. 6. Effects of promazine, chlorpromazine and acetylpromazine on the Mg^{++}-activated ATPase. For experimental conditions see experimental section. Time of incubation, 20 min; temperature, 30°.

It was previously found that addition of dithionite and cyanide to mitochondria treated with 0.1 % deoxycholate stimulates the Mg^{++}-activated ATPase. Addition of these substances, or of dithionite alone, were also found to prevent the inhibition of the Mg^{++}-activated ATPase by atebrin. In the case of promazine, as shown in Fig. 7, dithionite alone showed no significant effect in relieving the inhibition of the Mg^{++}-activated ATPase, while dithionite + cyanide gave a slight effect.

DISCUSSION

Previous studies of the action of atebrin[9] on the mitochondrial ATPase reactions led to the conclusion that these reactions involve the participation of the diaphorase flavoprotein of the respiratory chain. The following reaction schemes were proposed (a) for the DNP-induced ATPase reaction catalyzed by intact mitochondria:

$$ATP + fpH_2 \rightleftharpoons ADP + fpH \sim P \quad (1)$$

$$fpH \sim P + DPN^+ + OH^- \longrightarrow fp + DPNH + P_i \quad (2)$$

$$fp + DPNH + H^+ \longrightarrow fpH_2 + DPN \quad (3)$$

$$\text{Net: } ATP + H_2O \longrightarrow ADP + P_i,$$

and (b) for the Mg^{++}-activated ATPase reaction catalyzed by structurally disorganized

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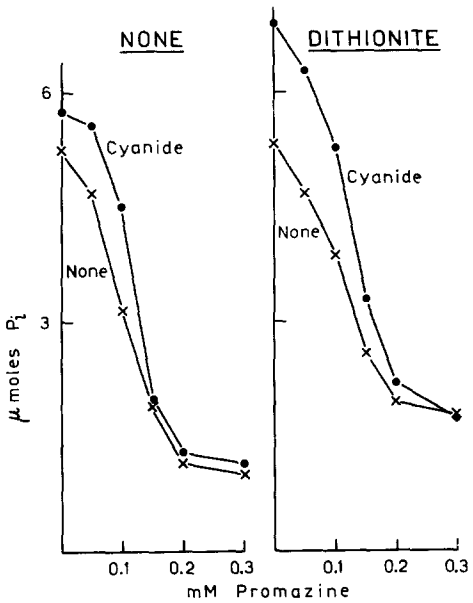

Fig. 7. Effect of promazine on the Mg^{++}-activated ATPase upon addition of dithionite. For experimental conditions see experimental section. Where indicated in the figure the concentration of cyanide was 1 mM and the added amount of dithionite was 2 μmoles per tube. Time of incubation, 20 min, temperature, 30°.

mitochondria (treated with 0.1 % deoxycholate):

$$ATP + fpH_2 \rightleftharpoons ADP + fpH \sim P \quad (4)$$

$$fpH \sim P + H_2O \longrightarrow fpH_2 + P_i \quad (5)$$

$$\text{Net: } ATP + H_2O \longrightarrow ADP + P_i$$

(fp stands for the diaphorase flavoprotein).

On theoretical grounds it was suggested[11], furthermore, that (*a*) the phosphoryl group in $fpH \sim P$ is linked to the 2-CO^--group of the isoalloxazine ring of the reduced flavin, and (*b*) that the reaction between ATP and fpH_2 according to reaction (1) may be coupled to a simultaneous reduction of fp.

The present data show that promazine and certain promazine derivatives have an action on the two ATPase reactions which is essentially identical with that of atebrin. From this it appears logical to conclude that promazines are flavin antagonists in the same sense as is atebrin. This is also borne out by work of YAGI *et al.*[6], who have shown that chlorpromazine is a competitive inhibitor of D-amino acid oxidase.

Among the derivatives tested in the present work chlorpromazine proved to be strongest as a flavin antagonist, followed by promazine itself and by acetylpromazine. It is notable that both chlorpromazine and promazine are stronger inhibitors of the reactions here studied than is atebrin. For example, to reach half inhibition of the Mg^{++}-activated ATPase, $5 \cdot 10^{-6}$ M chlorpromazine or $1.2 \cdot 10^{-5}$ M promazine was needed, compared with $5 \cdot 10^{-4}$ M atebrin[9]; the same concentrations were required to obtain a maximum stimulation of the DNP-induced ATPase. This may explain the findings that the action of promazine was more difficult to counteract by added flavins

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(in the case of DNP-induced ATPase) or by added dithionite (in the case of the Mg^{++}-activated ATPase) than was the action of atebrin[9].

Of the abundant literature published in recent years about the action of promazines on electron transport and oxidative phosphorylation[3–5, 15, 16, 18–23] only the work of BERGER *et al.*[5, 16] seems to be relevant to discussion in the present connection, since it is the only one done on liver mitochondria. The present findings differ from those of BERGER *et al.*[5, 16] in that in their system 0.2 m*M* chlorpromazine caused no inhibition of glutamate oxidation. Consequently, the concomitant decrease of the phosphate uptake gave the impression of being due to an uncoupling effect rather than, as in the present case, to a respiratory inhibition. A possibly essential difference between the test system of BERGER and co-workers[5, 16] and the present one consists in the fact that cytochrome *c* was added to the former, but not to the latter. BERGER *et al.* concluded that chlorpromazine specifically uncouples the phosphorylation connected with the oxidation of reduced cytochrome *c*. In view of the experimental data underlying this conclusion, and of recent evidence by HELPER *et al.*[23] that promazines depress the cytochrome *c* oxidase activity of liver homogenates, it appears conceivable that the addition of cytochrome *c* in BERGER *et al.* 's system might have established a by-path of the phosphorylative cytochrome *c* oxidase site, thus permitting full respiration, but with a lowered P/O ratio.

The present data seem to support the conclusion that, besides their possible action on the cytochrome *c* oxidase system, promazines also interfere, and to an even greater extent, with the functions of the mitochondrial diaphorase flavin, and particularly with its function of probable phosphate carrier in the phosphorylation connected with the oxidation of mitochondrial DPNH by diaphorase. This conclusion is also supported by the finding (*cf.* Fig. 1) that the P_i–ATP exchange reaction is considerably more sensitive to promazines than is respiration. The P_i–ATP exchange reaction has previously been shown to concern mainly the phosphorylation occurring in the diaphorase region of the respiratory chain[8].

Referring to preliminary experiments on the effects of chlorpromazine on mitochondrial respiration and P_i–ATP exchange, DAWKINS *et al.*[15] very recently arrived at the conclusion that chlorpromazine acts primarily on the mitochondrial diaphorase system. They proposed a reaction scheme, which is in agreement with the one previously postulated by LÖW *et al.*[8, 10], and further substantiated in the present paper, in that it implicates reduced flavin as a carrier of high-energy phosphate. It is difficult to agree, however, with that part of DAWKINS and coworkers'[15] scheme according to which DNP interferes with the interaction of the phosphorylated flavin with ADP. Such a possibility is strongly contraindicated both by the known inhibitory effect of DNP on the mitochondrial $[^{18}O]H_3PO_4$–H_2O exchange reaction[24], and by the marked influence of promazines on the DNP-induced ATPase, documented in the present paper.

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INCORPORATION AND ACTIVATION OF AMINO ACIDS BY DISRUPTED PROTOPLASTS OF *ESCHERICHIA COLI*

B. NISMAN*

Department of Bacteriology, University of Illinois, Urbana, Ill. (U.S.A.)

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SUMMARY

Osmotically lysed protoplasts of *E. coli* have been examined for synthetic activity. They have been found capable of activating and incorporating a large variety of amino acids. Activation has been observed with virtually every amino acid tested. The L-amino acid is the preferred substrate, the presence of the D-isomer being inhibitory.

Optimal incorporating activity requires the presence of: (1) a complete and balanced mixture of amino acids; (2) Mn^{++} at a level of $8 \cdot 10^{-3} M$; (3) ATP; (4) a mixture of the four ribonucleoside diphosphates.

* Permanent address: Institut Pasteur, Garches (S & O), France.

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