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THE EFFECT OF PHENOTHIAZINE ON THE METABOLISM OF LIVER MITOCHONDRIA

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Abstract—Phenothiazine at a concentration of 3·3 mM inhibited the oxidation by liver mitochondria of the Krebs' cycle substrates, citrate, α-oxoglutarate and L-malate, the fatty acid, octanoate, and the amino acid, L-glutamate, all of which reactions require NAD coenzymes.* NAD-dependent systems of mitochondria were inhibited by quite low concentrations of phenothiazine; for example, 10 μM produced appreciable inhibition of L-glutamate oxidation. The oxidation of succinate, which does not require NAD coenzymes, was not inhibited by 3·3 mM phenothiazine.

Phenothiazine also inhibited oxidative phosphorylation by liver mitochondria, regardless of whether an NAD-dependent substrate or succinate was being oxidized.

Inhibition of the oxidation of NAD-dependent substrates by phenothiazine was largely prevented by including NAD, GSH and nicotinamide in the respiratory reaction mixture, and it was also slightly prevented by EDTA. The inhibition was shown to be due to the loss of NAD coenzymes from mitochondria during incubation with phenothiazine.

Approximately 10 μM phenothiazone or its leuco equivalent was formed when 3·3 mM phenothiazine was incubated for 40 min with respiring mitochondria.

INTRODUCTION

PHENOTHIAZINE is widely used as an anthelmintic in sheep and cattle, usually without obvious toxic effects to the host animals. However, mortalities have been reported following anthelmintic doses in both sheep and cattle in New Zealand,[1] and its use in severely undernourished sheep may prove fatal (Franklin, unpublished data). Recent work[2] has shown that the administration of phenothiazine to sheep leads to a disturbance of liver mitochondrial metabolism, characterized by some loss of the capacity to perform the vital function of oxidative phosphorylation. In undernourished animals this loss may prove fatal.

Most work on the metabolic effects of phenothiazines has been done with derivatives, which have quite different physico-chemical and pharmacological properties from the parent compound. Phenothiazine derivatives, especially the promazines, have been shown to inhibit mitochondrial oxidations and oxidative phosphorylation.[3–8] Low[9] concluded from studying the effects of promazines on mitochondrial ATP-ase reactions that they compete with mitochondrial diaphorase flavin. This

* Abbreviations used in this paper: AMP, adenosine-5-monophosphate; ATP, adenosine triphosphate; NAD, nicotinamide-adenine dinucleotide; NADH, reduced NAD; GSH, reduced glutathione; EDTA, ethylenediaminetetra-acetic acid.

conclusion is in agreement with that of Dawkins *et al.*[7] that chlorpromazine acts primarily on the mitochondrial diaphorase system. Similarly, Yagi *et al.*[10] found that chlorpromazine inhibits D-amino acid oxidase due to competition with flavinadeninedinucleotide, the prosthetic group of the enzyme.

Although Collier and Allenby[11] reported that phenothiazine inhibited succinoxidase activity of liver mitochondria, and Abood and Romanchek[4] found that oxidative phosphorylation and ATP-ase of brain mitochondria were inhibited by the drug, no more detailed study has been made. The present study was designed to determine the effects of phenothiazine on liver metabolism under *in vitro* conditions.

MATERIALS AND METHODS

Enzyme preparations

Wistar rats were stunned and killed by decapitation. The liver was quickly removed and immersed in ice-cold 0·25 M sucrose. Livers of 2 rats were combined for each enzyme preparation. Homogenates in 0·25 M sucrose were prepared in glass homogenizers and were fractionated by differential centrifuging as described by Schneider.[12] Mitochondria were re-suspended in 0·25 M sucrose unless otherwise indicated.

Hexokinase was prepared from yeast by a modification[13] of the method of Berger *et al.*[14] The fraction precipitating between 25% and 40% by volume of ethanol was dissolved in 1% glucose and stored frozen. The optimal amount to be added of this solution was determined by assay for the appropriate system.

Reagents

Cytochrome *c*, AMP, ATP, nicotinamide, GSH, NAD, NADH, glucose, citrate, L-glutamate, succinate, L-malate, octanoate, α-oxoglutarate, EDTA, and inorganic reagents were obtained commercially. Succinic acid and octanoic acid were purified as previously described.[15] L-Ascorbic acid and α-oxoglutaric acid were dissolved and neutralized with NaOH just before use. AMP and other substrates were neutralized and stored frozen as sodium salts for 2 weeks.

Phenothiazine was a gift from I.C.I.A.N.Z. Pty. Ltd.

Methods

Oxygen uptake and carbon dioxide evolution were measured with Warburg manometers. Spectrophotometric readings were taken with a Beckman model DU or an Optica model CF4 instrument. Fluorometric readings were taken with a Farrand, model A photoelectric Fluorometer.

The standard system for respiratory experiments was: AMP 1 mM; $MgSO_4$ 6·7 mM; KCl 25 mM; NaK phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; substrate 10 mM; enzyme in 0·5 ml 0·25 M sucrose; water to 3 ml final volume; 0·1 ml 20% KOH in centre well to absorb CO_2; gas phase, air; temperature 38°; equilibration period 10 min.

The standard system for oxidative phosphorylation was the respiratory system plus glucose 33 mM, NaF 10 mM and hexokinase 0·01 ml. Inorganic phosphorus was measured by the method of Lowry and Lopez,[16] and the zero time control technique was used to determine phosphorus esterification.

Succinic dehydrogenase was assayed by the method of Slater.[17]

Cytochrome oxidase activity was measured by the method of Schneider and Potter,[18] but with 1 mM EDTA in lieu of $AlCl_3$.

Hexokinase assays were carried out either by using Nelson's estimation[19] to follow glucose disappearance,[20] or by a manometric method.[21]

Nitrogen was determined by a micro-Kjeldahl method.[22]

Phenothiazine was dissolved in ethanol and, unless otherwise indicated, it was added in 0·1 ml quantities to Warburg flasks after the addition of the enzyme suspensions and mixed by shaking to ensure thorough dispersion.

RESULTS

All experiments were done in duplicate, and repeated at least once unless otherwise stated.

Effect on mitochondrial oxidation systems

Table 1 shows that 3·3 mM phenothiazine inhibited the oxidation by liver mitochondria of the Kreb's cycle substrates, citrate, α-oxoglutarate and L-malate, of the fatty acid, octanoate, and of the amino acid, L-glutamate, all of which reactions require NAD coenzymes, but did not inhibit the oxidation of succinate. In experiments not shown in Table 1, appreciable inhibition of L-glutamate oxidation was also produced by 10 μM phenothiazine. The control figures stated in Table 1 are for flasks containing 0·1 ml ethanol, the solvent used to dissolve the phenothiazine.

TABLE 1. EFFECT OF PHENOTHIAZINE ON OXIDATIONS BY LIVER MITOCHONDRIA

Additions	No. of experiments	Oxygen uptake (μl/30 min)	Inhibition (%)
L-Malate 10mM	5	108	—
L-Malate 10 mM + phenothiazine 3·3 mM	5	53	51
Citrate 10 mM	3	92	—
Citrate 10 mM + phenothiazine 3·3 mM	3	56	29
α-Oxoglutarate 10 mM	2	132	—
α-Oxoglutarate 10 mM + phenothiazine 3·3 mM	2	83	37
L-Glutamate 10 mM	5	145	—
L-Glutamate 10 mM + phenothiazine 3·3 mM	5	86	31
Octanoate 1·67 mM + L-malate 0·67 mM	1	146	—
Octanoate 1·67 mM + L-malate 0·67 mM + phenothiazine 3·3 mM	1	108	26
Succinate 10 mM	4	144	—
Succinate 10mM + phenothiazine 3·3 mM	4	135	Within normal range

System: as in Methods. Mitochondria equivalent to 50 mg fresh liver for succinate, and 100 mg fresh liver for other substrates were added in 0·5 ml 0·25 M sucrose. Phenothiazine was added in 0·1 ml ethanol. All flasks contained 0·1 ml ethanol. The experiments were done in duplicate and the results given are the means of all experiments.

Effect of cofactors on oxidative inhibitions

Inhibition by phenothiazine of mitochondrial enzyme systems dependent upon NAD coenzymes to accept hydrogen atoms, and not of the succinoxidase system which does not require NAD, suggested the loss or inactivation of mitochondrial NAD.

Table 2 shows that addition of NAD and GSH in nicotinamide solution afforded considerable, though not complete, protection against the inhibitory effect of 3·3 mM phenothiazine on L-malate and L-glutamate oxidations by rat liver mitochondria.

Effect of EDTA on oxidative inhibitions

The stabilizing effect of the chelating agent, ethylenediaminetetraacetic acid, on mitochondrial structure and function is well established.[23–27] Lester and Hatefi[28]

TABLE 2. EFFECT OF COFACTORS AND EDTA ON MITOCHONDRIAL OXIDATIONS

Additions	No. of experiments	Oxygen uptake (μl/30 min) Control	Phenothiazine 3·3 mM	Inhibition %
L-Malate 10 mM	3	109	56	49
L-Malate 10 mM + cofactors	3	119	104	13
L-Malate 10 mM + EDTA 1 mM	2	102	81	21
L-Glutamate 10 mM	3	140	86	39
L-Glutamate 10 mM + cofactors	3	152	129	15
L-Glutamate 10 mM + EDTA 1mM	1	135	98	27

System: as in Table 1. Cofactor supplement: NAD 0·5 mM; GSH 0·67 mM; nicotinamide 40 mM. All flasks contained 0·1 ml ethanol added with or at the same time as phenothiazine. The results given are the means of all relevant experiments.

have shown that EDTA prevents the loss of bound NAD from mitochondria. It was decided therefore to investigate the possible effect of EDTA on the inhibition of mitochondrial oxidations by phenothiazine.

The data presented in Table 2 show that EDTA was less effective than the cofactors in preventing phenothiazine inhibition of L-malate or L-glutamate oxidations.

Effect of phenothiazine on mitochondrial NAD

Effect on E_{260}. Siekevitz and Potter[29, 30] have shown that much of the absorption of light at 260 mμ by acid extracts of tissue is due to the oxidized forms of NAD coenzymes. Therefore, in order to obtain an approximate measure of the effect of phenothiazine on mitochondrial NAD, the following procedure was followed. Mitochondria were incubated for 40 min in the respiratory medium containing L-glutamate with and without phenothiazine. They were then re-isolated and extracted with 5% trichloroacetic acid (TCA) as previously described.[31] The optical extinction coefficient at 260 mμ (E_{260}) of the TCA extract was then determined against a blank of 5% TCA.

Table 3 shows that although the oxidation of L-glutamate was inhibited by all four concentrations of phenothiazine used, the effect on the E_{260} value of the mitochondria was not uniform. The two higher concentrations of phenothiazine, 3·3 mM and 1 mM, increased the E_{260}, while the two lower concentrations, 0·1 mM and 10 μM, decreased

it. The rise in E_{260} value was thought to be due possibly to the uptake of phenothiazine or some metabolite of phenothiazine by the mitochondria during incubation. Suspension of phenothiazine in 5% TCA caused a rise in the E_{260} value of the TCA which was apparent after centrifuging off the precipitated phenothiazine.

Further experiments showed that the increased E_{260} values of mitochondria

TABLE 3. EFFECT OF PHENOTHIAZINE ON E_{260} OF ACID EXTRACTS OF MITOCHONDRIA

Additions	Oxygen uptake (μl/30 min)	E_{260}
Ethanol 0·1 ml	116	0·191
Phenothiazine 3·3 mM	75	0·355
Phenothiazine 1 mM	74	0·298
Phenothiazine 100 μM	71	0·146
Phenothiazine 10 μM	86	0·140

System: as in Table 1 with 10 mM L-glutamate as substrate. Mitochondria equivalent to 100 mg fresh liver/flask were added in 0·5 ml 0·25 M sucrose. Phenothiazine was added in 0·1 ml ethanol. Mitochondria were re-isolated after 40 min incubation and extracted with 5% TCA. The results shown are the means of two experiments, each done in duplicate.

incubated with high concentrations of phenothiazine, diminished with longer incubation. This is illustrated in Table 4 where it can be seen that the E_{260} values for TCA extracts of mitochondria incubated with either 3·3 mM or 0·33 mM phenothiazine decreased much more than did the E_{260} values of control mitochondria during the period 40 to 70 min after the commencement of incubation. The decrease is particularly evident for 0·33 mM phenothiazine, being probably partly off-set with 3·3 mM phenothiazine by the continued uptake of phenothiazine by the mitochondria.

TABLE 4. EFFECT OF INCUBATION TIME ON E_{260} OF ACID EXTRACTS OF MITOCHONDRIA

Additions	E_{260}	
	40 min incubation	70 min incubation
Ethanol 0·1 ml	0·144	0·136
Phenothiazine 3·3 mM	0·384	0·317
Phenothiazine 0·33 mM	0·216	0·102

System: as in Table 3. The results are the means of two experiments, each done in duplicate.

Effect on the reduction of NAD. One possible explanation for elevated E_{260} values of mitochondria was that reduction of NAD was inhibited by the higher concentrations of phenothiazine, thus producing a higher ratio of oxidized to reduced NAD. However, this explanation seemed unlikely when it was found that 3·3 mM phenothiazine did not influence the rate or quantity of NADH formed when alcohol

dehydrogenase was used to reduce NAD according to the method described by Kornberg.[32]

Effect on the total true NAD content of mitochondria. The question of whether NAD was lost from mitochondria during incubation with phenothiazine was finally decided by a fluorometric technique.[33]

Mitochondria from rat liver were incubated in the respiratory reaction mixture at 38° for 40 min with 0·01 M L-glutamate as substrate. The incubations were carried out in 100 ml Erlenmeyer flasks shaken in a constant temperature water bath. Reaction volumes were increased to 30 ml, ten-fold that of Warburg experiments, in order to allow the addition of mitochondria equivalent to 500 mg fresh liver in 2·5 ml 0·25 M sucrose per flask. Control experiments were carried out concomitantly in Warburg flasks with 3 ml reaction mixtures, to check whether the inhibitory effect of phenothiazine on oxidative metabolism was present. After incubation, the contents of the Erlenmeyer flasks were transferred quantitatively to centrifuge tubes by washing out with a modification[34] containing 25 mM nicotinamide of the medium used by Chappell and Perry.[35] Mitochondria were re-isolated by centrifuging at 0° in a Spinco model L refrigerated centrifuge at 20,000 *g* for 10 min. The mitochondria were then re-suspended in the modified medium and re-isolated from it by centrifuging. The mitochondrial pellet was then suspended in 2 ml of the medium and divided into two 1 ml aliquots. One aliquot was assayed for total oxidized and one for total reduced NAD coenzymes.[33]

The results in Table 5 show that incubation with 3·3 mM phenothiazine considerably reduced both the total oxidized and reduced NAD content of mitochondria.

TABLE 5. EFFECT OF PHENOTHIAZINE ON TOTAL NAD CONTENT OF MITOCHONDRIA

Additions	Oxidized NAD + NADP (μg/g liver)	Reduced NAD + NADP (μg/g liver)	Total NAD coenzymes (μg/g liver)
None	66·9	57·0	123·9
Ethanol 0·1 ml	62·7	53·1	115·8
Phenothiazine 3·3 mM	39·1	23·9	63·0
Phenothiazine 33 μM	39·7	49·7	89·4

System: respiratory reaction mixture as in Methods with 10 mM L-glutamate as substrate. Mitochondria equivalent to 1 g fresh rat liver were added in 5·0 ml 0·25 M sucrose containing 25 mM nicotinamide. The total volume was adjusted to 30 ml with water and the flasks were shaken for 40 min at 38° in a constant temperature water bath. Mitochondria were re-isolated and assayed for NAD coenzymes by fluorometry as described in the text. The results are the means of two experiments, each done in duplicate and are expressed as μG NAD in mitochondria from 1 g fresh liver.

Incubation with a lower concentration of phenothiazine, 33 μM, also severely depleted mitochondria of oxidized NAD coenzymes, but had little effect on the concentration of reduced NAD coenzymes, thus indicating a smaller total drain of NAD from the mitochondria.

Effect of phenothiazine on oxidative phosphorylation

Phenothiazine reduced the efficiency of oxidative phosphorylation accompanying substrate oxidations by liver mitochondria, regardless of whether the oxidations required NAD coenzymes. P/O ratios for L-malate, L-glutamate and succinate oxidations were all depressed by 3·3 mM phenothiazine.

Table 6 shows the effect of 3·3 mM phenothiazine on the P/O ratio during L-glutamate oxidation and succinate oxidation. It can be seen that phosphorus esterification accompanying succinate oxidation was considerably reduced by phenothiazine, although the rate of oxidation of succinate was not inhibited.

TABLE 6. EFFECT OF PHENOTHIAZINE ON OXIDATIVE PHOSPHORYLATION

Additions	No. of experiments	Oxygen uptake in 30 min (μ atoms)	Phosphorous esterified in 30 min (μ atoms)	P/O (μ atoms P esterified/μ atom O taken up)
L-glutamate 10 mM	5	14·5	33·3	2·3
L-glutamate 10 mM + phenothiazine 3·3 mM	5	11·3	18·8	1·7
Succinate 10 mM	3	10·9	17·3	1·6
Succinate 10 mM + phenothiazine 3·3 mM	3	10·9	9·5	0·9

System: as in Methods. Mitochondria equivalent to 100 mg fresh liver for L-glutamate oxidation, and 50 mg fresh liver for succinate oxidation were added in 0·5 ml 0·25 M sucrose. All flasks contained 0·1 ml ethanol.

Addition of 3·3 mM phenothiazine to the sucrose solution in which liver was homogenized for the release and subsequent isolation of mitochondria, had no effect on the efficiency of oxidative phosphorylation.

Effect of phenothiazine on specific enzyme systems

Cytochrome oxidase. Phenothiazine at 3·3 mM had no effect on the cytochrome oxidase activity of liver homogenates.

Succinic dehydrogenase. A concentration of 3·3 mM phenothiazine had no influence on the activity of succinic dehydrogenase in liver homogenate preparations.

Hexokinase. Hexokinase is used in the reaction mixture for measuring oxidative phosphorylation to catalyse the transfer of the terminal phosphate of ATP to glucose, to form glucose-6-phosphate, thus ensuring regeneration of the phosphate acceptor ADP, and preventing the release of inorganic phosphorus back into the reaction mixture as a consequence of hydrolysis of ATP. Inhibition of hexokinase activity by phenothiazine could have accounted for its apparent effect on oxidative phosphorylation.

Phenothiazine at 3·3 mM had no effect on rat brain hexokinase activity when measured either by glucose disappearance or by the manometric technique.

Similarly, phenothiazine at this concentration had no effect on the activity of yeast hexokinase measured manometrically.

The precipitation of phenothiazine when added to aqueous solutions, precluded the use of spectrophotometric techniques for measuring other enzymic activities.

Formation of phenothiazone

A pink colour was observed to develop when TCA was added to incubated flasks containing mitochondria and phenothiazine. This was found to be due to the formation of phenothiazone, an oxidation product of phenothiazine. The optical absorption maximum for phenothiazone in 5% TCA was found to be at 540 mμ. A standard curve was prepared for phenothiazone in 5% TCA at 540 mμ. Reference to this curve showed that supernatants collected when Warburg flask contents were precipitated with 5% TCA after incubation of respiring mitochondria and 3·3 mM phenothiazine for 40 min contained approximately 10 μM phenothiazone.

DISCUSSION

The results of this study of the effects *in vitro* of phenothiazine on mitochondrial metabolism are in agreement with those of Koch,[2] who found that the administration of phenothiazine to sheep lowered the efficiency of oxidative phosphorylation by liver mitochondria. Incubation of phenothiazine and liver mitochondria from normal rats was found, in the present study, to depress the efficiency of oxidative phosphorylation accompanying substrate oxidations, regardless of whether such oxidations required NAD coenzymes, as in the case of L-glutamate and L-malate oxidations, or not, as with succinate oxidation. Similar inhibitions of oxidative phosphorylation by phenothiazine during pyruvate and L-malate oxidations were reported by Abood and Romanchek.[4] Inhibitions or uncouplings of oxidative phosphorylation by phenothiazine derivatives have been found by Berger *et al.*,[6] Abood and Romanchek,[4] Dawkins *et al.*[7] and Judah *et al.*[8]

In addition to its effect on oxidative phosphorylation, phenothiazine has been found to inhibit substrate oxidations by mitochondrial dehydrogenases, which require NAD coenzymes to accept hydrogen atoms. Such inhibitions could be partly prevented by the addition of NAD and GSH, and slightly by the addition of EDTA, which stabilizes mitochondria against the loss of bound NAD.[28] These results suggested that phenothiazine inhibited mitochondrial oxidation systems by causing the loss of NAD coenzymes from mitochondria. Fluorometric analyses of the NAD content of mitochondria incubated with phenothiazine confirmed this suggestion.

Contrary to the findings of Collier and Allenby,[11] who reported that 0·14 mM phenothiazine inhibited 50% of the succinoxidase activity of rat liver mitochondria, the present study found that the same, and higher concentrations of phenothiazine had no effect on the oxidation of succinate by rat liver mitochondria, although considerably lowering the efficiency of the accompanying oxidative phosphorylation. However, in agreement with these authors, and with Abood and Romanchek,[4] phenothiazine was found to have no effect on cytochrome oxidase activity.

Bryhad[36] showed that phenothiazine derivatives inhibited the hexokinase activity of rat brain preparations when incubated for more than 30–40 minutes. Consequently, it was possible that the effect of phenothiazine on oxidative phosphorylation might be an experimental artefact due to inhibition of hexokinase. However, phenothiazine was found to have no effect on either rat brain or yeast hexokinase under the conditions of the present study.

TCA extraction of mitochondria after incubation with phenothiazine under oxidizing conditions revealed the presence of a pink, water-soluble substance, which was found to be phenothiazone. As the formation of this substance or its colourless,

leuco form, could conceivably influence the results of *in vitro* studies, and possibly be a factor in the metabolic effects of phenothiazine if produced under *in vivo* conditions, its influence on mitochondrial metabolism was investigated and is to be reported subsequently.

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