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THE EFFECT OF PHENOTHIAZONE ON THE METABOLISM OF LIVER MITOCHONDRIA

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Abstract—Phenothiazone at a concentration of 10 μM stimulated oxygen uptake during the oxidation by liver mitochondria of L-glutamate, pyruvate, α-oxoglutarate and L-malate. This was due to the provision of a pathway of electron transfer from NADH* to cytochrome *c* or oxygen, additional to the respiratory chain of electron carriers. Higher concentrations of phenothiazone inhibited NAD-dependent oxidations. Such inhibitions were relieved by additional NAD, or by treatment of mitochondria with EDTA. The mechanism of inhibition is considered to involve the formation of a complex between phenothiazone and NADH oxidase.

Succinoxidase of mitochondria was inhibited by concentrations of phenothiazone from 10 μM upwards. The data suggest that inhibition of succinoxidase by low concentrations of phenothiazone is mediated through NAD inhibition of succinoxidase, or through a similar mechanism. At a higher concentration, 3·3 mM, phenothiazone inhibited cytochrome oxidase, thus explaining portion of the succinoxidase inhibition by this concentration.

Phenothiazone apparently stimulated succinic dehydrogenase activity when it was isolated from the terminal respiratory chain by HCN and assayed by coupling succinate oxidation to the reduction of methylene blue. However, the apparent stimulation was due to phenothiazone itself acting as an auto-oxidizable electron donor additional to methylene blue.

Phenothiazone at and above 10 μM lowered the efficiency of oxidative phosphorylation by mitochondria for both NAD-dependent substrate succinate oxidation.

ATP-ase activity (Mg^{++}-activated) and the rate of swelling of mitochondria was unaffected by phenothiazone.

The possible relationship of phenothiazone and phenothiazine in producing their effect on metabolism is discussed. It appears likely that phenothiazone or a precursor plays a role in the overall effects displayed by phenothiazine poisoning both *in vivo* and *in vitro*.

INTRODUCTION

Studies with phenothiazine[1] showed that a pink colour developed upon the addition of trichloroacetic acid to a reaction mixture in which respiring mitochondria and phenothiazine had been incubated together at 38°. This colour proved to be due to the accumulation of phenothiazone or its leuco form, oxidation products of phenothiazine. Incubation of mitochondria with 3·3 mM phenothiazine for 30 min produced

* 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; TCA trichloroacetic acid.

an accumulation equivalent to about 10 μM phenothiazone. Since such a concentration could be formed *in vitro*, it might well participate in the metabolic sequelae of phenothiazine administration both *in vivo* and in *in vitro* systems.

Most studies on phenothiazine derivatives have not included phenothiazone. However, Collier and Allen[2] reported that it inhibited the succinoxidase activity of a beef heart preparation, and later, Allenby and Collier[3] that it inhibited hexokinase activity of rat brain.

MATERIALS AND METHODS

Enzyme preparations, reagents and methods were as previously reported.[1] In addition, the reagents, amytal and antimycin A, were obtained commercially.

Phenothiazone, melting point 161°, was kindly prepared by Mr. K. J. Farrington, M.Sc. It was added in aqueous solution or in aqueous suspension (for high concentrations).

NADH-cytochrome *c* reductase was assayed by the method of Mahler *et al.*[4]

ATP-ase activity was measured in the following system:
ATP, 5 mM; tris buffer, pH 7·5, 5 mM; sucrose 75 mM; rat liver mitochondria, thrice-washed with 0·25 M sucrose, equivalent to 50 mg fresh liver added in 1·0 ml 0·25 M sucrose; water to 2 ml final volume. The reaction mixture was incubated for 10 min at 30° before adding 2 ml 10% TCA.

Mitochondrial swelling was measured by following optical density changes at 900 mμ in the system: sucrose 0·25 M; tris buffer, pH 7·5 5 mM; substrate, where present, 1 mM; mitochondria equivalent to 50 mg fresh liver in 0·1 ml 0·25 M sucrose; water to 3·4 ml final volume; temperature 22°. The experiments were conducted both with and without 10 mM orthophosphate.

RESULTS

Effect on substrate oxidations by liver mitochondria

In contrast to the inhibitory effect of phenothiazine on NAD-dependent enzyme systems, a low concentration of phenothiazone, 10 μM, was found to stimulate oxygen uptake during the oxidation of α-oxoglutarate or L-glutamate, and to have no effect on the oxidation of pyruvate, L-malate or citrate (Table 1).

A higher concentration of phenothiazone, 0·1 mM, stimulated oxygen uptake during the first 10 min interval after equilibration for L-glutamate or pyruvate oxidation, and then became progressively inhibitory. A further increase in phenothiazone concentration to 3·3 mM inhibited pyruvate, citrate and L-glutamate oxidations from the time of equilibration.

Table 1 also shows that unlike phenothiazine, phenothiazone inhibited the oxidation of succinate. No significant stimulation or inhibition of succinate oxidation was observed during the first 10 min after equilibration, but subsequently phenothiazone progressively inhibited oxygen uptake to reach a peak of 40–50 per cent in the third 10 min interval. This degree of inhibition was then maintained.

As the greatest stimulation of α-oxoglutarate and L-glutamate oxidations occurred during the first 10 min of manometric measurements, it was conceivable that this effect of phenothiazone might occur with other substrate oxidations during the 10 min equilibration period and disappear by the time readings were made. In order to investigate this possibility, experiments were made in which phenothiazone was

tipped from side-arm compartments into the main flask contents after the initial manometer readings had been made.

The results in Table 2 show that when added in this way, 10 μM phenothiazone produced an initial stimulation of pyruvate and L-malate oxidations, as well as L-glutamate oxidation. The stimulation accompanying L-glutamate oxidation was

TABLE 1. EFFECT OF PHENOTHIAZONE ON OXIDATIVE SYSTEMS OF MITOCHONDRIA

Substrate	Phenothiazone (mM)	O_2 uptake (μl/30 min)	Change in O_2 uptake (%)	
			Inhibition	Stimulation
L-Malate	—	102	—	—
L-Malate	0·01	101	—	—
Citrate	—	111	—	—
Citrate	0·01	104	—	—
Citrate	3·3	53	49	—
Pyruvate	—	71	—	—
Pyruvate	0·01	74	—	—
Pyruvate	0·1	58	18	—
Pyruvate	3·3	20	72	—
α-Oxoglutarate	—	139	—	—
α-Oxoglutarate	0·01	172	—	24
L-Glutamate	—	120	—	—
L-Glutamate	0·01	200	—	67
L-Glutamate	0·1	111	7	—
L-Glutamate	3·3	65	46	—
Succinate	—	164	—	—
Succinate	0·01	118	28	—
Succinate	3·3	120	27	—

System: 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; mitochondria equivalent to 50 mg fresh liver for succinate, and 100 mg fresh liver for other substrates added in 0·5 ml 0·25 M sucrose; water to 3 ml final volume, 0·1 ml 20% KOH was placed in centre well to absorb CO_2; gas phase, air; temperature, 38°; equilibration period, 10 min. Results for pyruvate oxidation have been corrected for oxygen uptake due to 0·67 mM L-malate included to supply oxaloacetate. Results are the means of all experiments, which were done in duplicate and repeated at least once.

more prolonged than with other substrates. Citrate oxidation was inhibited slightly from the outset of the experiment, whether phenothiazone was present during the equilibration period or added after. There was no stimulation of succinate oxidation immediately after 10 μM phenothiazone was added from the side-arm compartments, and inhibition of the oxidation of this substrate became apparent in the second 10 min interval after such addition.

Incubation of 3·3 mM phenothiazone in the respiratory mixture at 38°, alone or with substrate, did not lead to any oxygen uptake in the absence of an enzyme preparation.

Phenothiazone at 10 μM stimulated endogenous oxygen uptake by mitochondria in the absence of added substrate, but the degree of stimulation was too slight to account for more than a small fraction of the increase in oxygen uptake in the presence of the relevant substrates.

TABLE 2. IMMEDIATE EFFECT OF PHENOTHIAZONE ON OXIDATIVE SYSTEMS

	Interval O_2 uptake (μl)															
	Pyruvate				Citrate			L-Glutamate			L-Malate			Succinate		
		Phenothiazone				Phenothiazone			Phenothiazone			Phenothiazone			Phenothiazone	
Time (Min)	Control	Main compartment 10 μM	Tipped from side-arm 10 μM	3·3 mM	Control	Main compartment 10 μM	Tipped from side-arm 10 μM	Control	Main compartment 10 μM	Tipped from side-arm 10 μM	Control	Main compartment 10 μM	Tipped from side-arm 10 μM	Control	Main compartment 10 μM	Tipped from side-arm 10 μM
10	28	32	38	23	31	29	29	35	42	40	35	34	44	64	60	61
20	26	21	24	11	31	28	26	31	40	43	34	33	32	59	42	55
30	27	13	10	4	31	26	26	32	38	40	33	31	32	60	39	45
Total	81	66	72	38	93	83	81	98	120	123	102	98	108	183	141	161

System: As for Table 1. Phenothiazone was added from side-arm compartments after 10 min equilibration, or to the main compartment before incubation.

Effect on oxidative phosphorylation

Like phenothiazine, phenothiazone was found to lower the efficiency of oxidative phosphorylation catalysed by liver mitochondria.

Table 3 shows that the P/O ratio was decreased by 33 per cent for L-glutamate oxidation and by 24 per cent for succinate oxidation by 10 μM phenothiazone. In the case of L-glutamate oxidation, the lowered P/O ratio was largely due to increased oxygen utilization, but there was also a slight decrease in phosphorus esterification. Phosphorus esterification was decreased still further with succinate oxidation to give a lower P/O ratio than normal, despite decreased oxygen uptake.

TABLE 3. EFFECT OF PHENOTHIAZONE ON OXIDATIVE PHOSPHORYLATION

Additions	P/O (μ atoms P esterified/ μ atom O used)	Reduction P/O %
Glutamate 10 mM	2·8	—
Glutamate 10 mM + phenothiazone 10 μM	1·9	33
Glutamate 10 mM + phenothiazone 3·3 mM	0·7	75
Succinate 10 mM	1·7	—
Succinate 10 mM + phenothiazone 10 μM	1·3	24

System: As for Table 1 plus glucose 33 mM, NaF 10 mM and 0·01 ml yeast hexokinase solution. Results are the means of all experiments.

Phenothiazone at 3·3 mM severely inhibited phosphorus esterification accompanying L-glutamate oxidation, depressing the P/O ratio by 75 per cent even though oxygen uptake was considerably reduced.

Addition of 10 μM phenothiazone to the sucrose solution in which liver was homogenised, depressed the P/O ratio below normal for both L-glutamate and succinate oxidations by mitochondria isolated from such homogenates.

The inclusion of supplements of NAD, GSH and nicotinamide did not reverse the effect of phenothiazone on oxidative phosphorylation.

Effect on specific enzymes

Cytochrome oxidase. Phenothiazone was found, unlike phenothiazine, to inhibit the activity of cytochrome oxidase.

Table 4 shows that 3·3 mM phenothiazone inhibited cytochrome oxidase activity of rat liver homogenate. Lower concentrations of phenothiazone, 0·1 mM and 10 μM, had no effect on cytochrome oxidase.

ATP-ase. Phenothiazone at 10 μM concentration, which was found to inhibit succinate oxidation and oxidative phosphorylation, had no effect on Mg^{++}-activated ATP-ase of fresh liver mitochondria.

Succinic dehydrogenase. Although 10 μM phenothiazone was found to inhibit succinoxidase activity, it had no effect on the activity of succinic dehydrogenase when isolated from electron transport by the terminal respiratory chain by the inhibitor, HCN, and assayed by coupling succinate oxidation to reduction of the

auto-oxidizable dye, methylene blue (Table 5). However, a higher concentration of phenothiazone, 3·3 mM, stimulated oxygen uptake considerably. There was no oxygen uptake in this system in the presence of 3·3 mM phenothiazone, but in the absence of succinate.

This anomaly was explained by experiments in which methylene blue was not added. Table 5 shows that phenothiazone itself acts as an auto-oxidizable electron transfer agent. The addition of 3·3 mM phenothiazone was as effective as 1 mM methylene blue in mediating succinate oxidation by succinic dehydrogenase.

TABLE 4. EFFECT OF PHENOTHIAZONE ON CYTOCHROME OXIDASE

Additions	Oxygen uptake (μl/30 min)
None	246
Phenothiazone 3·3 mM	143
Phenothiazone 0·1 mM	255
Phenothiazone 10 μM	261

System: as described by Schneider and Potter,[14] with the substitution of 1 mM EDTA for $AlCl_3$. Homogenate equivalent to 15 mg fresh liver in 0·3 ml distilled water was added per flask. Results are the means of 2 experiments.

TABLE 5. EFFECT OF PHENOTHIAZONE ON SUCCINIC DEHYDROGENASE

Additions	Oxygen uptake (μl/30 min)
Methylene blue 1 mM	32
Methylene blue 1mM + phenothiazone 10 μM	34
Methylene blue 1 mM + phenothiazone 3·3 mM	51
None	4
Phenothiazone 10 μM	9
Phenothiazone 3·3 mM	38

System: NaK phosphate buffer, pH 7·2, 100 mM; KCN (neutralized) 10 mM; Na succinate 10 mM; enzyme, 0·3 ml liver homogenate in 0·25 M sucrose containing 100 mg fresh liver added from side-arm compartments after 10 min equilibration; water to 3·0 ml final volume; gas phase, air; temperature, 38°. Results are means of 2 experiments done in duplicate.

NAD-L-malic dehydrogenase. A spectrophotometric method[5] was used to measure L-malic dehydrogenase activity by following the rate and amount of NADH formed. Phenothiazone was found to have no influence on the L-malic dehydrogenase activity of liver mitochondria suspended in 0·25 M sucrose, indicating that it did not alter mitochondrial permeability to the entry of either NAD or L-malate. Nor did the drug have any effect on the L-malic dehydrogenase activity of mitochondria whose permeability barriers had been abolished by suspension in water. This indicates that

phenothiazone does not directly inhibit L-malic. dehydrogenase, in agreement with the observation that phenothiazone did not inhibit L-malate oxidation by mitochondria suspended in 0·25 M sucrose under the conditions of manometric respiratory experiments.

NADH-cytochrome c *reductase.* NADH-cytochrome *c* reductase activity of liver mitochondria was assayed by following the reduction of cytochrome *c* during the reaction by the increase in optical density at 550 mμ.[4]

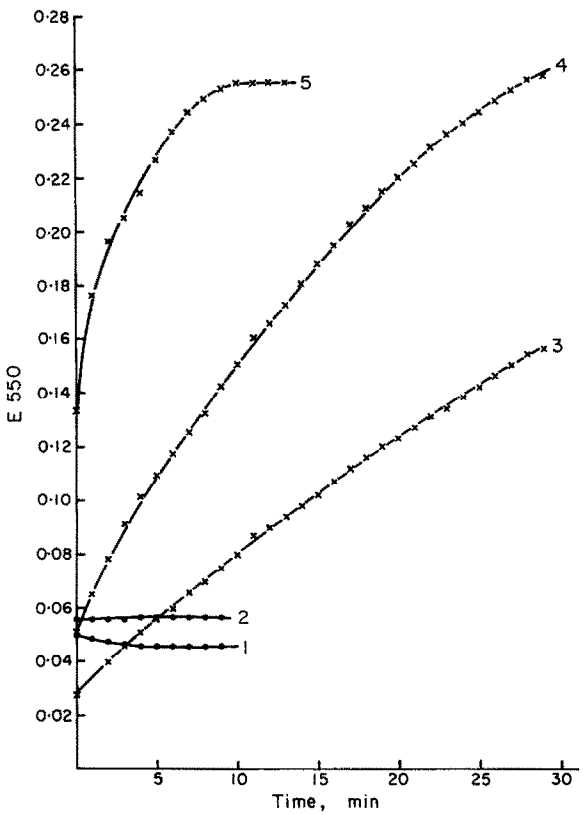

FIG. 1. Effect of Phenothiazone on NADH-cytochrome *c* Reductase

System: As in Methods. Homogenate equivalent to 2·5 mg fresh rat liver added in 0·05 ml distilled water at zero time.
1 = system plus 10 μM phenothiazone but without cytochrome *c*.
2 = system plus 10 μM phenothiazone but without cytochrome *c* and NADH.
3 = complete system.
4 = complete system plus 10 μM phenothiazone
5 = complete system plus 0·1 mM phenothiazone

Figure 1 shows that both 0·1 mM and 10 μM phenothiazone stimulated the rate of reduction of cytochrome *c* in this system. Stimulation by phenothiazone persisted, despite the addition of 2 mM amytal to inhibit NADH oxidation,[6] or of 0·67 mM antimycin A to inhibit electron transport at the cytochrome *b* level.[7]

This effect of phenothiazone was not due to stimulation of amytal-insensitive

extra-mitochondria NADH-cytochrome *c* reductase, as it was still present with mitochondria thrice-washed in 0·25 M sucrose to remove traces of extra-mitochondrial enzymes.[6]

Mechanism of phenothiazone effects on enzyme systems

Effect of cofactors on NAD-dependent oxidations. Table 6 shows that the addition of NAD and GSH in nicotanimide solution had no effect on phenothiazone inhibition of citrate oxidation.

TABLE 6. EFFECT OF COFACTORS ON NAD-DEPENDENT OXIDATIONS

Additions	Phenothiazone (mM)	O_2 uptake (μl/30 min)	Change in O_2 uptake (%)	
			Inhibition	Stimulation
Citrate 10 mM	—	102	—	—
Citrate 10 mM	3·3	53	48	—
Citrate 10 mM + cofactors	—	150	—	—
Citrate 10 mM + cofactors	3·3	76	49	—
L-Glutamate 10 mM	—	99	—	—
L-Glutamate 10 mM	0·01	135	—	36
L-Glutamate 10 mM	3·3	49	51	—
L-Glutamate 10 mM + cofactors	—	113	—	—
L-Glutamate 10 mM + cofactors	0·01	120	—	6
L-Glutamate 10 mM + cofactors	3·3	93	18	—
α-Oxoglutarate 10 mM	—	112	—	—
α-Oxoglutarate 10 mM	0·01	147	—	31
α-Oxoglutarate 10 mM + cofactors	—	118	—	—
α-Oxoglutarate 10 mM + cofactors	0·01	144	—	22

System: As for Table 1. Cofactor supplement: NAD 0·5 mM, GSH 0·67 mM in nicotinamide 40 mM. Results are the means of all experiments.

However, the cofactors decreased considerably the degree of inhibition of L-glutamate oxidation due to 3·3 mM phenothiazone. Surprisingly, the degree of stimulation of oxygen uptake during L-glutamate oxidation in the presence of 10 μM phenothiazone was also reduced (from 36 to 6 per cent) by the addition of cofactors (Table 6).

Table 6 also shows that the addition of cofactors reduced to a lesser degree the stimulation of oxygen uptake due to the presence of 10 μM phenothiazone during α-oxoglutarate oxidation.

Effect of EDTA on NAD-dependent oxidations. Inclusion of 1 mM EDTA in the reaction mixture for measuring L-glutamate oxidation did not alter significantly either the stimulation of oxygen uptake due to 10 μM phenothiazone, or the inhibition due to 3·3 mM phenothiazone (Table 7).

However, when the system was incubated with EDTA prior to the addition of phenothiazone from the side-arm compartments, complete protection against inhibition due to 3·3 mM phenothiazone was obtained. In fact, as shown in Table 7, this procedure resulted in 16 per cent stimulation of oxygen uptake in contrast to the

19 per cent inhibition by 3·3 mM phenothiazone which occurred in the absence of EDTA. It will be noted in Table 7 that the degree of inhibition in 30 min after adding 3·3 mM phenothiazone from side-arm compartments (19 per cent) was not nearly so great as when the phenothiazone was added before equilabration of the reaction mixture (48 per cent).

TABLE 7. EFFECT OF ETDA ON NAD-DEPENDENT OXIDATIONS

Additions	Phenothiazone (mM)	O_2 uptake (μl/30 min)	Change in O_2 uptake (%)	
			Inhibition	Stimulation
L-Glutamate 10 mM	—	143	—	—
L-Glutamate 10 mM	0·01	234	—	64
L-Glutamate 10 mM	3·3	75	48	—
L-Glutamate 10 mM + 1 mM EDTA	—	153	—	—
L-Glutamate 10 mM + 1 mM EDTA	0·01	239	—	56
L-Glutamate 10 mM + 1 mM EDTA	3·3	95	38	—
*L-Glutamate 10 mM	—	102	—	—
*L-Glutamate 10 mM	0·01	142	—	39
*L-Glutamate 10 mM	3·3	83	19	—
*L-Glutamate 10 mM + 1 mM EDTA	—	109	—	—
*L-Glutamate 10 mM + 1 mM EDTA	0·01	157	—	31
*L-Glutamate 10 mM + 1 mM EDTA	3·3	126	—	16

System: As for Table 1. In experiments marked *, phenothiazone or the same volume of water was added from side-arm compartments after the equilibration period. Results are the means of repeated experiments.

Effect of amytal on stimulation of oxygen uptake. As the oxidation of L-glutamate was found to be stimulated by 10 μM phenothiazone and L-glutamate oxidation is mediated through NAD, it was of interest to determine the effect of 1·8 mM amytal which inhibits the oxidation of NADH.

In the experiments shown in Table 8, 10 μM phenothiazone increased oxygen uptake during L-glutamate oxidation by 50 per cent over 30 min. The addition of amytal almost blocked the oxidation of L-glutamate unless phenothiazone was also added, when a rate of oxidation almost as high as the initial control value was achieved.

Effect of washing mitochondria on stimulation of oxygen uptake. Insensitivity of the phenothiazone-induced stimulation of L-glutamate oxidation to amytal, suggested that phenothiazone could be stimulating the activity of contaminating extra-mitochondrial NADH-cytochrome *c* reductase, which is known to be insensitive to amytal.[6] This possibility was investigated by washing mitochondria three times in 0·25 M sucrose before use.

As shown in Table 8, washed mitochondria still were stimulated by 10 μM phenothiazone in the presence or absence of 1·8 mM amytal.

Effect of phenothiazone on succinoxidase. Although it was found that phenothiazone stimulated the activity of succinic dehydrogenase by acting as an additional auto-oxidizable electron transfer agent for the enzyme, the drug was found to inhibit the activity of the intact succinoxidase system of mitochondria.

TABLE 8. EFFECT OF AMYTAL AND OF WASHING MITOCHONDRIA ON L-GLUTAMATE OXIDATION

Additions	Unwashed mitochondria		Washed mitochondria	
	Oxygen uptake (μl/30 min)	Stimulation of oxygen uptake %	Oxygen uptake (μl/30 min)	Stimulation of oxygen uptake %
L-Glutamate 10 mM	96	—	72	—
L-Glutamate 10 mM + phenothiazone 10 μM	144	50	128	78
L-Glutamate 10 mM + Na amytal 1·8 mM	16	—	12	—
L-Glutamate 10 mM + Na amytal 1·8 mM + phenothiazone 10 μM	88	450	76	530

System: As for Table 1. Mitochondria for the 2nd experiment were washed by re-suspension in and re-isolation from 0·25 M sucrose three times.

TABLE 9. EFFECT OF PHENOTHIAZONE ON THE SUCCINOXIDASE SYSTEM

Additions	Oxygen uptake (μl/30 min)		Inhibition of O_2 uptake by phenothiazone %
	Control	Phenothiazone 10 μM	
None	166	127	24
NAD 0·5 mM	136	125	8
ATP 1 mM	176	138	22
EDTA 1 mM	185	168	11
Amytal 1·8 mM	168	108	36
Antimycin A 6·7 mM	0	52	—
Mitochondria in water	141	142	—
Mitochondria in water + NAD 0·5 mM	52	48	—
Mitochondria in water + amytal 1·8 mM	170	129	24

System: As for Table 1. All flasks contained 10 mM succinate. Enzyme, rat liver mitochondria isolated from 0·25 M sucrose homogenate and re-suspended in 0·25 M sucrose unless otherwise indicated. NAD was added in nicotinamide solution to give a final concentration of 40 mM nicotinamide. Amytal was dissolved in a minimal volume of 50% ethanol and diluted to the required volume with water. Antimycin A was added in 0·05 ml 50% isopropanol. Results are the means of at least 2 experiments in duplicate.

Table 9 shows that although the addition of 1 mM ATP, which is known to influence succinoxidase activity, had no effect on phenothiazone inhibition, 0·5 mM NAD apparently reduced the degree of inhibition considerably. However, as 0·5 mM NAD itself inhibited succinate oxidation by mitochondria suspended in 0·25 M sucrose to about the same degree as did phenothiazone, the fall in percentage inhibition by

phenothiazone in the presence of NAD may simply mean that one mechanism of inhibition precluded the expression of the other. This was indicated also by the fact that 0·5 mM NAD produced the same degree of inhibition of succinoxidase in mitochondria suspended in water, and thus offering no permeability barrier to the entry of NAD, whether or not 10 μM phenothiazone was present.

The addition of 1 mM EDTA considerably reduced the inhibition of the succinoxidase system by 10 μM phenothiazone.

Addition of 1·8 mM amytal to inhibit NADH oxidation had no effect on succinate oxidation by mitochondria suspended in 0·25 M sucrose, but increased the oxidation rate of mitochondria in water (Table 9). Amytal increased the inhibition due to 10 μM phenothiazone of succinoxidase activity of sucrose-suspended mitochondria. Table 9 also shows that suspension of mitochondria in water for an hour before adding them to Warburg flasks eliminated the susceptibility of succinoxidase to inhibition by 10 μM phenothiazone. However, the addition of 1·8 mM amytal to such "water-cracked" mitochondria restored their susceptibility to inhibition by phenothiazone.

Inhibition of electron transport at the cytochrome *b* level by 6·7 mM antimycin A completely abolished succinate oxidation unless phenothiazone was added. Table 9 shows that 10 μM phenothiazone permitted 52 μl O_2 uptake in 30 min in the presence of 6·7 mM antimycin A.

Effect on mitochondrial swelling. Phenothiazone at 10 μM or 20 μM concentration did not affect the rate of swelling of mitochondrial, thrice-washed in 0·25 M sucrose as measured by following optical density changes at 900 mμ, in the absence of substrate or in the presence of 1 mM L-malate or 1 mM succinate. Phenothiazone at these concentrations was also without effect when the measurements of swelling were carried out in the presence of 10 mM orthophosphate.

DISCUSSION

Phenothiazone at the low concentration of 10 μM increased oxygen uptake in the early stages of oxidation by mitochondria of L-glutamate, α-oxoglutarate, L-malate and pyruvate, all of which require NAD as a coenzyme. As 10 μM phenothiazone permitted oxidation of L-glutamate in the presence of the inhibitor of NADH oxidation, amytal,[6] it was apparent that phenothiazone provided another pathway of electron transfer from NADH or the respective dehydrogenases to oxygen. Confirmation of this mechanism of phenothiazone stimulation of oxygen uptake was provided by the stimulatory effect of phenothiazone on NADH-cytochrome *c* reductase of mitochondria. A greater rate of reduction of cytochrome *c* was observed in the presence of 10 μM phenothiazone, despite addition of amytal to inhibit NADH oxidation, antimycin A to inhibit electron transport at cytochrome *b*, or when mitochondria were thrice-washed to remove traces of extra-mitochondrial, amytal-insensitive NADH- cytochrome *c* reductase.

Stimulation of the oxidation of the NAD-dependent substrate, L-glutamate, by 10 μM phenothiazone was largely eliminated by 0·5 mM NAD, 0·67 mM GSH and 40 mM nicotinamide. However, this supplement of cofactors itself lifted the oxygen uptake considerably and thus, by providing a near-maximal rate of oxidation in the system used, could *ipso facto* obscure the stimulatory effect of phenothiazone.

Higher concentrations of phenothiazone, 0·1 mM and 3·3 mM, progressively inhibited NAD-dependent substrate oxidations. In the case of L-glutamate oxidation, this inhibition was largely prevented by the addition of a cofactor supplement of 0·5 mM NAD, 0·67 mM GSH and 40 mM nicotinamide. However, this cofactor supplement had no effect on phenothiazone inhibition of citrate oxidation. Complete protection against phenothiazone inhibition of L-glutamate oxidation was provided by incubation of mitochondria with 1 mM EDTA prior to the addition of 3·3 mM phenothiazone. In fact, this treatment converted the effect of 3·3 mM phenothiazone from an inhibition of 19 per cent to a stimulation of 16 per cent, presumably due to the maintenance of structural integrity and NAD status of the mitochondria by the stabilizing action of EDTA.

Chlorpromazine has been shown to be a strong electron donor capable of forming a charge transfer complex with flavin mononucleotide.[8] Similarly, Orloff and Fitts[9] found that phenothiazine and its derivatives are good electron donors. The evidence compiled in the present study indicates that phenothiazone influences mitochondrial metabolism by forming a complex with the flavoprotein enzyme, NADH oxidase, or with this enzyme and its substrate, NADH. Further, the data suggest that this complex does not dissociate readily, and inhibits the initiation of electron transfer along the respiratory electron carrier chain. However, with low concentrations of phenothiazone, which stimulate oxygen uptake, the complex is either not formed or is capable of oxidation by a means other than the coupled reduction of cytochrome *b*. In any case, it is clear that low concentrations of phenothiazone provide an electron by-pass mechanism from NADH to cytochrome *c* or oxygen. The apparent protective effect of added extra-mitochondrial NAD against 3·3 mM phenothiazone inhibition of L-glutamate oxidation may be due to facilitation of this electron by-pass pathway. EDTA probably protects both by maintaining the NAD status of mitochondria at a high concentration, and by preserving the semipermeable membrane barriers and structural integrity of mitochondria, thus preventing the ready access of phenothiazone to the sites of inhibition.

Phenothiazone was also found to inhibit the succinoxidase and cytochrome oxidase systems of liver mitochondria. Inhibition of succinoxidase activity by 3·3 mM phenothiazone is probably partly due to inhibition of cytochrome oxidase. However, 10 μM phenothiazone inhibits succinoxidase strongly, but does not affect cytochrome oxidase. Inhibition of succinoxidase by 10 μM phenothiazone was found not to be due to inhibition of succinic dehydrogenase activity, which was, in fact, stimulated by phenothiazone acting as an electron-transferring auto-oxidizable agent like methylene blue. Methylene blue is, of course, a phenothiazine derivative.

Suspension of mitochondria in water to eliminate permeability barriers abolished the inhibitory effect of 10 μM phenothiazone on succinoxidase. However, the addition of amytal to block NADH oxidation, although not affecting succinoxidase activity, restored to "water-cracked" mitochondria the capacity to exhibit succinoxidase inhibition by 10 μM phenothiazone. It was found, too, that the addition of both 0·5 mM NAD and 10 μM phenothiazone to mitochondria suspended in either 0·25 M sucrose or water, resulted in an inhibition of succinoxidase activity of the same order as that due to NAD alone. It thus appears that inhibition of succinoxidase by low concentrations of phenothiazone is associated with the NAD status of mitochondria, and is mediated through the mechanism of NAD inhibition of succinoxidase.

Like phenothiazine[1] and other phenothiazine derivatives[10–13] phenothiazone lowers the efficiency of oxidative phosphorylation by liver mitochnodria for both NAD-dependent and succinate oxidations. At low concentrations of phenothiazone, the lower P/O ratio accompanying NAD-dependent oxidations is partly from increased oxygen uptake, due to the electron-shunting action of phenothiazone, and partly from decreased phosphorus esterification. For succinate oxidation at all concentrations of phenothiazone tested, and at higher concentrations of phenothiazone for other oxidations, lower P/O ratios result from inhibition of phosphorus esterification.

The production of the water-soluble product, phenothiazone, *in vivo* or *in vitro* would augment the disruptive effect of phenothiazine on liver mitochondrial metabolism, particularly with respect to lowering the efficiency of oxidative phosphorylation.

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REFERENCES

1. C. H. GALLAGHER, J. H. KOCH and D. M. MANN, *Biochem. Pharmacol.*, **14**, 789 (1965).
2. H. B. COLLIER and D. E. ALLEN, *Canad. J. Res.* **20**, 284 (1952).
3. G. M. ALLENBY and H. B. COLLIER, *Canad. J. Med. Sci.* **30**, 549 (1952).
4. H. R. MAHLER, N. K. SANKAR, C. P. VERNON and R. A. ALBERTY, in *Methods in Enzymology*, vol. 2, p. 688, Eds. S. P. COLOWICK and N. O. KAPLAN (1952).
5. G. S. CHRISTIE and J. D. JUDAH, *Proc. Roy. Soc. B.* **142**, 241 (1954).
6. L. ERNSTER, O. GALLING, H. LÖW and O. LINDBERG, *Exp. Cell. Res. Supple.* **3**, 124 (1955).
7. B. CHANCE and G. R. WILLIAMS, *Nature, Lond.* **169**, 215 (1952).
8. G. KARREMAN, I. ISENBERG and A. SZENT-GYORGYI, *Science* **130**, 1191 (1959).
9. M. K. ORLOFF and D. D. FITTS, *Biochim. biophys. Acta* **47**, 597 (1961).
10. L. G. ABOOD and L. ROMANCHEK, *Ann. N.Y. Acad. Sci.* **66**, 812 (1957).
11. L. BERGER, H. STRECKER and H. WAELSCH, *Ann. N.Y. Acad. Sci.* **66**, 806 (1957).
12. M. J. R. DAWKINS, J. D. JUDAH and K. R. REES, *Biochem. Pharmacol.* **2**, 112 (1959).
13. J. D. JUDAH, K. R. REES and M. J. R. DAWKINS, *Nature, Lond.* **183**, 821 (1959).
14. W. C. SCHNEIDER and V. R. POTTER, in *Manometric Techniques and Tissue Metabolism*, 3rd ed., p. 75, Eds. Umbreit *et al.* (1947).