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# THE EFFECTS OF PHENAZINIUM AND PHENOTHIAZINE UPON MITOCHONDRIAL METABOLISM*

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**Abstract**—The effect of the antitumor agent "phenazinium" (1,3-diamino-5-methyl phenazinium chloride) upon mitochondrial oxidations and oxidative phosphorylation has been investigated. Mitochondrial suspensions were prepared from mammalian liver and kidney. Phenazinium produced a significant degree of inhibition of mitochondrial oxidations with several substrates, and, in concentrations of 1 mM or above, induced partial uncoupling of oxidative phosphorylation. These effects are compared to those produced by phenothiazine, and differences are discussed. The compound has been shown to produce severe ultrastructural changes in renal tubule cells *in vivo*, and the significance of these changes is related to the observed toxicity of the drug.

PHENAZINE derivatives have long been known to produce striking biological effects.[1] They are also known to occur in nature, especially as the bacterial pigments pyocyanine, iodinine, the aeruginosins, and the chlorophaphins. Because of the influence of some phenazines on bacterial metabolism,[2–4] cell metabolism,[5] and oxidative phosphorylation,[6, 7] they have been examined periodically as potential antibacterial drugs[8–10] and as antitumor agents.[11–13] One such compound, 1,3-diamino-5-methyl phenazinium chloride, "phenazinium," is undergoing clinical evaluation as a potential antitumor drug, and a previous paper[5] has described its influence upon glycolysis and the pentose pathway in human red cells.

The widespread use of phenothiazines in both human medicine as tranquilizers, and in domestic animals for anthelminthic purposes, is well known. Because of the basic similarity of the structure of phenazines and phenothiazine, it was decided to study the comparative effects of representative compounds of both classes upon certain essential areas of cell metabolism. This communication represents an extension of the previous studies of the biochemical action of "phenazinium"[5] to mitochondrial metabolism, in which its behavior is contrasted with that of phenothiazine.

## MATERIALS AND METHODS

Mitochondrial suspensions were prepared from the liver and kidneys of golden hamsters and of Wistar rats. After the animal was killed by decapitation, the tissue was removed and immersed in isotonic sucrose at 0°. Homogenization was then carried out in a Potter–Elvehjem homogenizer, and mitochondria were isolated by differential

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centrifugation according to Schneider and Potter.[14] Mitochondria equivalent to 100 mg tissue were used in each Warburg flask.

Oxygen consumption was measured in the standard Warburg apparatus with the following reaction mixture (final molarities): substrate, 10 mM; $KH_2PO_4$, 13 mM; ATP, 2·0 mM; KCl, 25 mM; cytochrome c, 10 $\mu$M; mitochondrial suspension, in 0·5 ml of 0·25 M sucrose; $MgSO_4$, 0·5 mM; where substrate was α-oxoglutarate or citrate, K-malonate, 10 mM was added; water added to 3 ml final volume. $CO_2$ was absorbed by 0·1 ml 30% KOH in the center well. In the oxidative phosphorylation measurements, 0·2 ml of 0·25 M glucose, with sufficient hexokinase to give optimum rate of phosphorus transfer as determined by experiment, was added from the side arm after 10-min incubation, and potassium fluoride to 10 mM was incorporated in the mixture. At this point, "zero time" control flasks were taken off, and deproteinized with 0·2 ml 10% trichloroacetic acid. Inorganic phosphorus was determined on these and the P/O ratio determined from this figure and the inorganic phosphate remaining after the period of incubation.

Inorganic phosphate was determined by a modification of the method of Lowry and Lopez.[15] Cytochrome oxidase and succinic dehydrogenase activities were measured by the technique of Schneider and Potter.[14] Hexokinase (Calbiochem) was assayed by the technique of Allenby and Collier.[16]

Electron microscopic studies on dog kidney mitochondria were conducted with a Siemen's Elmiskop I instrument at 80 kV. Fresh renal tissue was fixed in 2·5% glutaraldehyde, post-fixed in 1% osmic acid, and dehydrated with acetone. The material was then embedded in Epon 812 and sectioned on a Porter-Blum MT-1 microtome, post-stained with 1% uranyl acetate and Reynold's lead-citrate,[17] then examined and suitable micrographs taken.

Biochemical reagents—ATP, $NADH_2$, NAD, cytochrome c, succinate, L-malate, and α-oxoglutarate were obtained from Calbiochem, Los Angeles, Calif. Phenazinium (1,3-diamino-5-methyl phenazinium chloride monohydrate) was supplied by the Clinical Branch, Collaborative Research, National Cancer Institute, U.S. Public Health Service, through the Southwest Cancer Chemotherapy Study Group. Phenothiazine was obtained from Eastman Organic Chemicals, Inc., recrystallized, and dissolved in ethanol for use.

## RESULTS

### *Oxidative reactions*

Table 1 illustrates the effect of phenazinium and phenothiazine on the oxidation of certain substrates by mitochondria. No significant differences were observed between the behavior of liver and renal cell mitochondria from either the rat or the hamster. With concentrations of 1 mM and above, however, some degree of inhibition occurred, being most noticeable with oxoglutarate and least with succinate.

### *Oxidative phosphorylation*

The results are shown in Table 2. Inhibition of oxidative phosphorylation by 3 mM phenothiazine was observed with all substrate systems tested, regardless of the observed effects upon oxygen consumption shown in Table 1. With phenazinium, the effect on oxidative phosphorylation was somewhat variable, and significant uncoupling occurred only with concentrations greater than 1 mM. There was little effect when

TABLE 1. THE EFFECT OF PHENAZINIUM AND PHENOTHIAZINE ON SEVERAL MITOCHONDRIAL OXIDATIONS

Rat liver mitochondria equivalent to 100 mg tissues were used.

| Substrate | Additions | Conc. (mM) | $O_2$ Consumption ($\mu$l/30 min) | % Stimulation (+) or inhibition (−) |
|---|---|---|---|---|
| α-Oxoglutarate | ... | ... | 125 | ... |
| | Phenazinium | 0·1 | 142 | +13·6 |
| | Phenazinium | 1 | 109 | −12·7 |
| | Phenazinium | 3 | 71 | −43·2 |
| | Phenothiazine | 1 | 114 | −8·8 |
| | Phenothiazine | 3 | 72 | −42·4 |
| L-Malate | ... | ... | 101 | ... |
| | Phenazinium | 0·1 | 117 | +14·8 |
| | Phenazinium | 1 | 104 | * |
| | Phenazinium | 3 | 78 | −22·8 |
| | Phenothiazine | 3 | 57 | −43·6 |
| Succinate | ... | ... | 133 | ... |
| | Phenazinium | 0·1 | 148 | +11·1 |
| | Phenazinium | 1 | 135 | * |
| | Phenazinium | 3 | 119 | −10·5 |
| | Phenothiazine | 3 | 128 | * |
| Citrate | ... | ... | 87 | ... |
| | Phenazinium | 1 | 84 | * |
| | Phenazinium | 3 | 72 | −17·2 |
| | Phenothiazine | 1 | 80 | −8·1 |
| | Phenothiazine | 3 | 62 | −28·7 |

* Insignificant change.

TABLE 2. THE EFFECT OF PHENAZINIUM AND PHENOTHIAZINE ON OXIDATIVE PHOSPHORYLATION WITH DIFFERENT SUBSTRATES

Rat liver mitochondria equivalent to 100 mg tissues were used.

| Substrate | Additions | Conc. (mM) | $O_2$ Consumed ($\mu$atoms/30 min) | P Consumed ($\mu$atoms/30 min) | P/O Ratio |
|---|---|---|---|---|---|
| α-Oxoglutarate | ... | ... | 10·1 | 34·3 | 3·4 |
| | Phenazinium | 0·1 | 10·5 | 34·1 | 3·3 |
| | Phenazinium | 3 | 6·7 | 20·1 | 3·0 |
| | Phenothiazine | 3 | 6·7 | 14·1 | 2·1 |
| Citrate | ... | ... | 7·0 | 18·2 | 2·6 |
| | Phenazinium | 3 | 6·4 | 13·4 | 2·1 |
| | Phenothiazine | 3 | 5·3 | 10·8 | 2·04 |
| L-Malate | ... | ... | 8·1 | 18·6 | 2·3 |
| | Phenazinium | 0·1 | 8·5 | 18·5 | 2·2 |
| | Phenazinium | 3 | 6·4 | 13·4 | 2·1 |
| | Phenothiazine | 3 | 5·3 | 10·8 | 2·04 |
| Succinate | .. | ... | 10·7 | 20·3 | 1·9 |
| | Phenazinium | 0·1 | 11·2 | 19·0 | 1·7 |
| | Phenazinium | 3 | 9·6 | 17·4 | 1·81 |
| | Phenothiazine | 3 | 10·3 | 10·3 | 1·0 |

succinate was used as substrate. The net effect was less than that due to phenothiazine. Minor decreases in the P/O ratio observed with lower concentrations of phenazinium (i.e. 0·1 mM) were probably due to the augmentation of oxygen consumption observed with L-malate, α-oxoglutarate, and succinate, though in the case of citrate this did not appear to be so.

*Effects upon specific enzymes*

No effects on the activity of succinate dehydrogenase, cytochrome oxidase, or hexokinase were observed with either phenazinium or phenothiazine in concentrations up to 3 mM.

*Structural effects of phenazinium upon tissues and cells*

Male dogs of approximately 15 kg weight were given intravenous doses of phenazinium of 7·5 mg/kg daily for 10 days. On this dosage the animals developed overt signs of toxicity, manifested by anorexia, drowsiness, and general weakness, with some signs of incoordination. The animals were sacrificed on Day 11 and the tissues examined histologically for changes indicative of toxicity. Overt signs of cell damage were confined to the kidney, which exhibited early changes of tubular necrosis. Electron microcsopic studies conducted on the kidneys showed maximal effects upon the cells of the proximal convoluted tubules. In these cells, considerable ultrastructural damage was detected, swelling and distortion, loss of cristae, and rupture of the mitochondrial membrane. These changes are clearly seen in Fig. 1, in which the appearance of the ultrastructure of a renal tubule cell from an animal treated as described with phenazinium is contrasted with that from a normal animal (Fig. 2). It should be stressed that the changes were observed only after phenazinium treatment. This is in keeping with the occurrence of a gradual rise in serum nonprotein nitrogen seen in the treated dogs, and with nitrogen retention observed in a patient receiving 5 mg/kg per day for 4 days. In the latter case, a renal biopsy revealed a marked degree of tubular necrosis, which gradually recovered after withdrawal of the drug. Renal function had previously been normal.

## DISCUSSION

It is apparent that, under appropriate conditions, phenazinium can exert an inhibitory influence upon both mitochondrial oxidations and oxidative phosphorylation. However, these effects are not marked unless the mitochondria are exposed to high concentrations of the drug. Indeed, in lower concentrations, an augmentation of oxygen consumption may be observed with some substrates, a phenomenon that may be explained by the capacity of many phenazine derivatives to act as efficient hydrogen acceptors. Higher concentrations appear to inhibit oxygen consumption, though this was not observed at concentrations less than 1 mM. The same remarks apply to oxidative phosphorylation. Minor changes in P/O ratio observed at lower concentrations of phenazinium may be explicable in terms of an increase in oxygen consumption rather than a fall in phosphorylation. At higher concentrations, however, some variable decrease in phosphorylation did occur.

Severe structural changes occurring in renal tubule cells, involving both mitochondria and endoplasmic reticulum, were observed. It appears that phenazinium is here acting as a general cellular poison, and the gross ultrastructural disturbances are

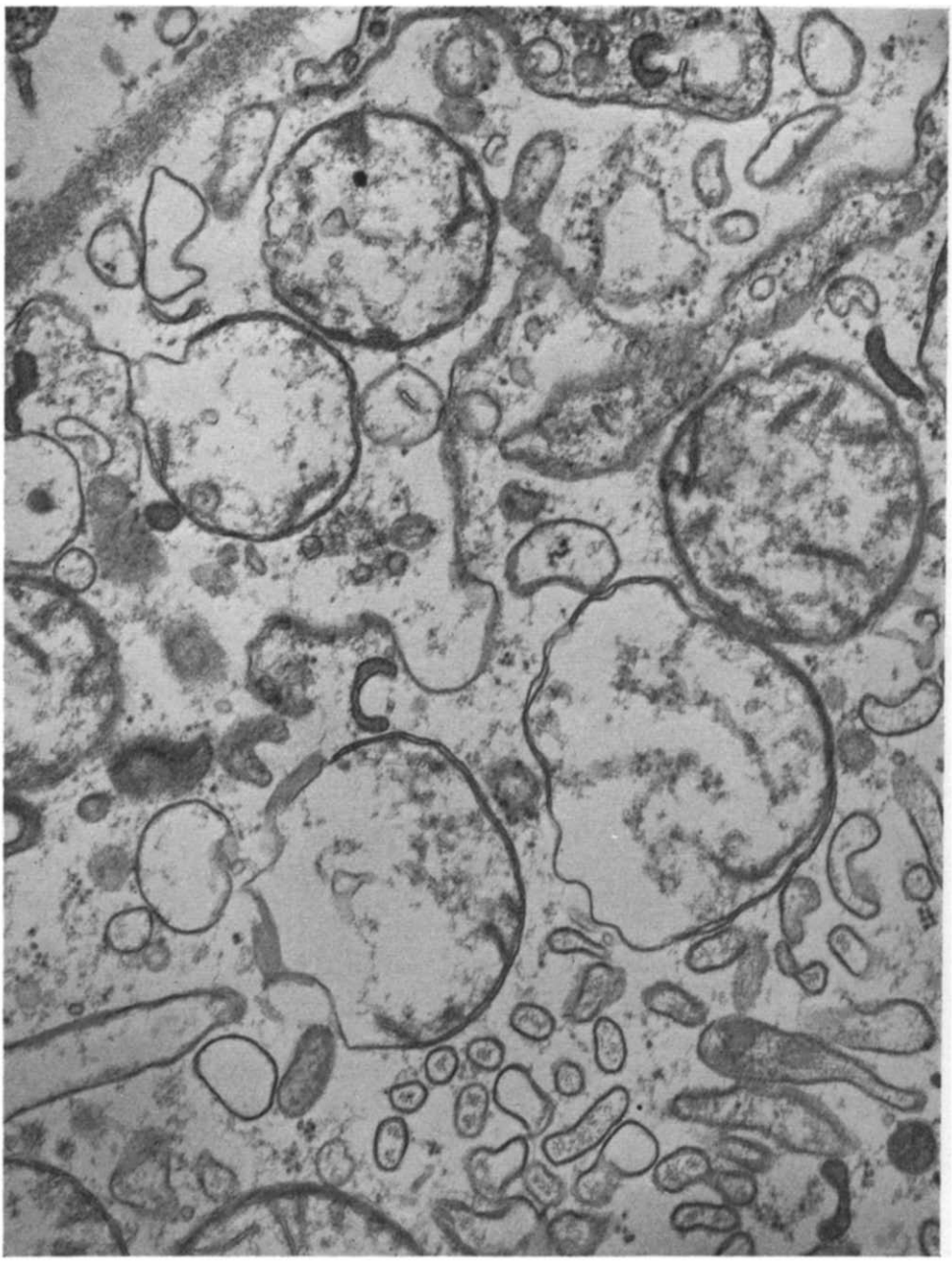

FIG. 1. The effect of prolonged high dosage of phenazinium on renal tubule cell of dog. After 10 days of phenazinium, mitochondria exhibit swelling, distortion, loss of cristae, and disruption of membrane; the endoplasmis reticulum is swollen and exhibits vesiculation and loss of continuity ($\times$ 56,000).

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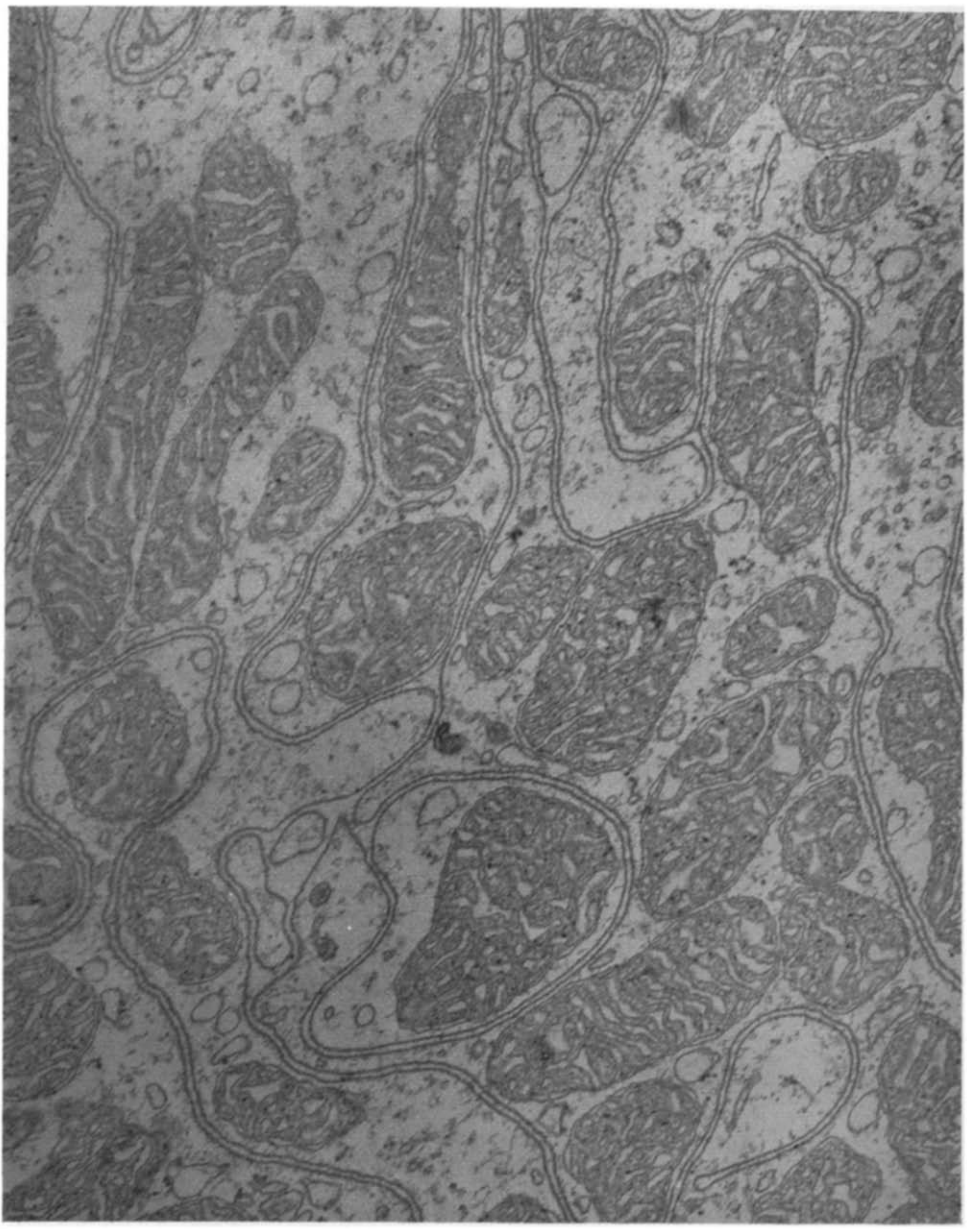

FIG. 2. Normal appearance of renal tubule cell ultrastructure of dog ($\times$56,000).

in keeping with the observed nephrotoxicity described. The drug is eliminated chiefly by renal excretion, and very high concentrations occur in urine. Preliminary experimental data from excretion studies obtained in this laboratory indicate that the renal excretion is at least in part by tubular secretion,[18] and high levels of the drug may thus occur intracellularly. This may account for the absence of overt cellular damage in other situations, as distribution studies indicate that high concentrations of the compound do not occur in other tissues.[18]

The inclusion of phenothiazine in some of the studies *in vitro* was introduced for comparative purposes. This compound, the parent substance of a host of therapeutic agents, bears a close structural resemblance to the phenazine nucleus, and some metabolic studies have been conducted with it.[18–21] Our findings are in substantial agreement with those of other authors. The majority of mitochondrial oxidations, with the notable exception of succinate, were inhibited by phenothiazine, and marked uncoupling of oxidative phosphorylation was observed at concentrations of 3 mM with all systems tested.

Neither phenazinium nor phenothiazine appeared to influence cytochrome oxidase activity. Nor could the effects of either substance upon oxidative phosphorylation be explained by inhibition of hexokinase, which was used in the reaction mixture to catalyze the phosphorylation of glucose by ATP, thus permitting regeneration of the phosphate acceptor ADP.

It would seem that the effects of both substances are complex, and require further experimental elucidation.

## REFERENCES

1. G. A. Swan and D. G. I. Felton, *Phenazines*, p. 193. Interscience, New York (1959).
2. I. C. Wells, *J. biol. Chem.* **196**, 331 (1952).
3. R. Schoental, *Br. J. exp. Path.* **22**, 137 (1941).
4. L. Birkofer and A. Birkofer, *Klin. Wschr.* **26**, 528 (1948).
5. H. B. Anstall, B. List-Young, J. M. Trujillo and W. O. Russell, *Biochem. Pharmac.* **15**, 998 (1966).
6. E. M. Case and H. McIlwain, *Biochem. J.* **48**, 1 (1951).
7. J. D. Judah and H. G. Williams-Ashman, *Biochem. J.* **48**, 33 (1951).
8. V. C. Barry and M. L. Conalty, *Am. Rev. Tuberc. pulm. Dis.* **78**, 62 (1958).
9. S. G. Brown and L. M. Hogezeil, *Lepr. Rev.* **33**, 185 (1962).
10. V. C. Barry and M. L. Conalty, *Lepr. Rev.* **36**, 3 (1965).
11. E. A. Friedheim, *Biochem. J.* **28**, 178 (1934).
12. L. R. Duvall, *Cancer Chemother. Rep.* **23**, 61 (1962).
13. M. G. Kelly, N. H. Smith and J. Leiter, *J. natn Cancer Inst.* **20**, 1113 (1958).
14. W. C. Schneider and V. R. Potter, *Manometric Techniques* (Eds. Umbreit *et al.*), p. 183 Burgess, Minneapolis (1964).
15. O. H. Lowry and J. A. Lopez, *J. biol. Chem.* **162**, 421 (1946).
16. G. M. Allenby and H. B. Collier, *Can. J. med. Sci.* **14**, 789 (1965).
17. E. S. Reynolds, *J. Cell Biol.* **17**, 208 (1963).
18. H. B. Anstall, B. List-Young and J. M. Trujillo. Unpublished data.
19. L. Abood and L. Romanchik, *Ann. N.Y. Acad. Sci.* **66**, 812 (1957).
20. G. H. Gallagher, J. H. Koch and D. M. Mann, *Biochem. Pharmac.* **14**, 789 (1965a).
21. G. H. Gallagher, *Biochem. Pharmac.* **14**, 799 (1965b).