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THE AUTOXIDATION OF A THIOL AGENT, METHYL PHENYLAZOFORMATE, INDUCED BY RAT TISSUE EXTRACTS

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Abstract—The thiol agent, methyl phenylazoformate is moderately stable in rat blood but rapidly oxidises in liver homogenates due chiefly to the action of the microsomal and mitochondrial fractions. The reaction involves enzymic hydrolysis of the ester followed by catalysis of the autoxidation of phenyldiazene, the intermediate product. This autoxidation, studied directly by generating the diazene *in situ* with chymotrypsin, occurs most readily with erythrocytes but microsomes, mitochondria and solutions of haemoglobin are also effective. Methyl phenylazoformate is found to be a partial uncoupling agent for oxidative phosphorylation in mitochondria and its action is discussed in the light of its rapid degradation by this organelle.

Substituted diazenes have been extensively used as thiol reagents [1–6] particularly methyl phenylazoformate, $C_6H_5N{=}N{-}COOCH_3$('Azoester') and diazene dicarboxylic acid—bis (dimethylamide), $(CH_3)_2NCON{=}NCON(CH_3)_2$ ('Diamide') which are both commercially available. Azoester is said to produce free radicals [7] and this has been regarded as of importance after reaction with thiol is complete. However, in the course of studies on mitochondrial thiols, it was found that azoester is rapidly and probably quantitatively autoxidised in the presence of a number of rat tissue preparations.

This paper is concerned with elucidating the mechanism for the oxidation.

METHODS

All reactions were carried out in a medium consisting of mannitol (0.25 M) containing 3-(*N*-morpholino)propane sulphonate (10^{-2} M; pH 7.4) and ethylene glycol-bis (β-aminoethyl ether)*N*,*N*′-tetra-acetic acid (10^{-4} M). The subcellular fractions from rat liver were prepared in the above medium as described by Schneider and Hogeboom [8]. Protein was assayed by a Biuret method [9] and spectrophotometry was carried out using a Unicam SP800 recording spectrophotometer. Dialyses were performed in Visking (8/32) tubing against two changes of medium at 0° for 72 hr. Oxygen uptake was measured using a Clarke oxygen electrode coupled to a Goetz 20 mV recorder. The chamber (capacity, 4 ml) was jacketed and the temperature maintained at 30° by circulating water from a thermostat. A magnetic stirrer was used to maintain constant mixing. Azoester and Diamide were both obtained from Calbiochem, San Diego, Calif. Azoester was standardised by adding it in ethanol to GSH (~5 μmole) in phosphate buffer (0.05 M; pH 7.4) under nitrogen and measuring the loss in thiol with DTNB [10] after 1 hr at 0°.

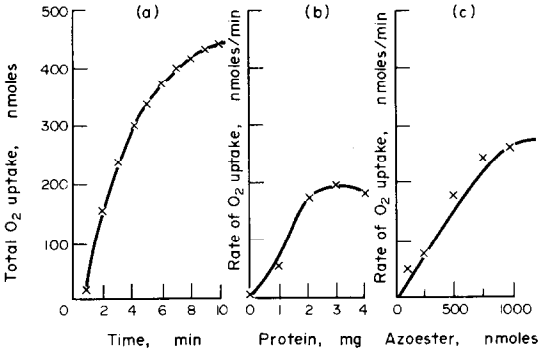

Fig. 1. Autoxidation of azoester by liver mitochondria. A mitochondrial suspension (containing 2 mg protein for (a) and (c)) was diluted in the electrode chamber to 3 ml. With stirring at 30°, azoester in ethanol (500 nmole for (a) and (b)) was added and O_2 immediately measured.

RESULTS

As shown in Fig. 1 oxygen is consumed when azoester is added to a dilute mitochondrial suspension in a reaction which is unaffected by cyanide or other respiratory inhibitors (Fig. 1). The initial rate of uptake increases with the amount of mitochondria (Fig. 1B) or azoester (Fig. 1C) in the mixture to a maximum but the total consumption of oxygen (Fig. 1A) varies linearly with the azoester concentration and when the reaction has ceased (about 15 min) 0.87 + 0.11 mole oxygen is found to be taken up per mole of azoester added. Other notable features of the reaction observed are that (i) neither the stoichiometry nor the rate of this reaction is affected by adding catalase (100 units) to the system (ii) reduction of azoester by preincubation with GSH before adding to the mitochondrial suspension abolishes subsequent oxygen uptake (iii) lysis of mitochondria with 0.1% deoxycholate does not affect the reaction rate significantly (iv) no oxygen consumption is obtained on adding diamide (up to 1 μmole) in place of azoester.

Other tissue fractions also catalyse the autoxidation of azoester (Table 1). Rat liver microsomes show the highest specific activity whereas there is very little activity present in the cytoplasmic fraction. Addition

Table 1. Catalysis of the autoxidation of azoester by preparations from rat tissues*

Tissue	Fraction	Initial rates (nmole/min) O_2 uptake	Azoester loss
—	Medium	0	4
Liver	Mitochondria	40	39
	Microsomes	210	164
	Cytoplasm	11	8
Kidney	Mitochondria	34	26
	Supernatant	29	47
Blood	Erythrocytes	0	4
	Plasma	5	15

* The fraction containing 1 mg protein was added to the medium (3 ml) containing azoester (500 nmoles) at 30° either in a spectrophotometer cuvette or in the chamber of an oxygen electrode.

of azoester to rat erythrocytes of plasma also gives little uptake of oxygen.

Azoester is known to hydrolyse slowly in neutral aqueous solution (half life, 20 min) [4]. The first formed acid is reported to decarboxylate spontaneously (estimated half life at pH 7, 0.5 sec) [11] and this allows the overall reaction to be followed from the resulting loss in extinction at 305 nm. As shown in Table 1, initial rates of ester hydrolysis, measured in this way with tissue preparations correlate well with the rates of oxygen uptake suggesting that the first reaction is rate limiting the second. However, separate catalysis is required for the oxidation because chymotrypsin which is known to catalyse a rapid hydrolysis of azoester [12] gives only a very slow initial rate of oxygen uptake (Fig. 2).

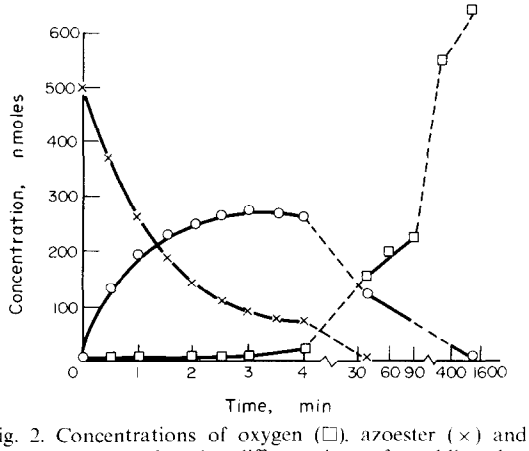

Fig. 2. Concentrations of oxygen (□), azoester (×) and phenyldiazene (○) found at different times after adding chymotrypsin to azoester. Chymotrypsin (100 μg) was added to azoester (500 nmoles) in the medium (3 ml) at 30°. Azoester losses were computed from the fall in extinction at 505 nm (E_m, 1.11×10^4) after substracting that remaining after 26 hr (6 per cent of the original). Phenyldiazine concentrations were obtained from the extinction changes at 265 nm by using the published value (11) of E_m, 0.74×10^4 and on the assumptions that no diazene remains after 26 hr, that the initial extinction, E^i, is due solely to azoester and the final extinction, E^f, to undetermined products. At time t, the fraction of the experimentally determined extinction at 265 nm, E^t, which is due to phenyldiazene is therefore given by $E^t - [(\text{Azoester}^t)/(\text{Azoester}^i)](E^i - E^f)$.

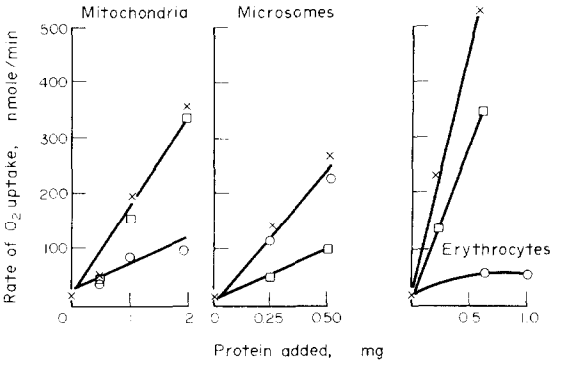

Fig. 3. Catalysis of phenyldiazene autoxidation by some tissue fractions. Chymotrypsin (100 μg) was added to azoester (500 nmol) in the medium (3 ml) in an oxygen electrode chamber. After 2 min at 30°, the tissue fraction, untreated (×), dialysed (□) or heated for 3 min at 100° (○), was added and the oxygen uptake then determined.

Phenyl diazene, the decarboxylated product of azoester hydrolysis, shows an extinction peak at 265 nm [11] and incubation of azoester with chymotrypsin in fact gives an immediate increase in the extinction at this wavelength which is then followed by a slow fall over several hours coincident with the slow uptake of oxygen (Fig. 2). Calculation of minimum phenyldiazene concentrations from the data in Fig. 2 shows that, though this compound is reported to be instantly autoxidised in acetonitrile solution [11], it is relatively stable in neutral aqueous solution. The oxygen uptake in the presence of tissue extracts is therefore due to catalysis of phenyldiazene oxidation and the reaction can thus be directly studied by generating this substance from azoester with chymotrypsin before adding the tissue extract. Under these conditions (Fig. 3) the highest specific activity is now given by the erythrocyte fraction while plasma shows only a little catalysis of the autoxidation. Among the liver subcellular fractions, microsomes are more active than mitochondria and the cytoplasm shows little activity. The effect of heating or dialysing these active fractions has also been studied. Heating has little effect on the microsomes but considerably reduces activity in the mitochondrial and erythrocyte fractions. In contrast these fractions are less affected by dialysis than the microsome fraction (Fig. 3).

The effect of erythrocytes on phenyldiazene oxidation suggests that haemoglobin may catalyse the

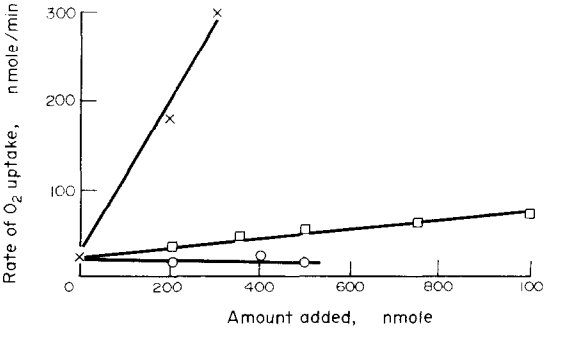

Fig. 4. Catalysis of phenyldiazene oxidation by haemoglobin (×), cytochrome c (○) or $FeSO_4$ (□). Azoester was preincubated with chymotrypsin as described in Fig. 3.

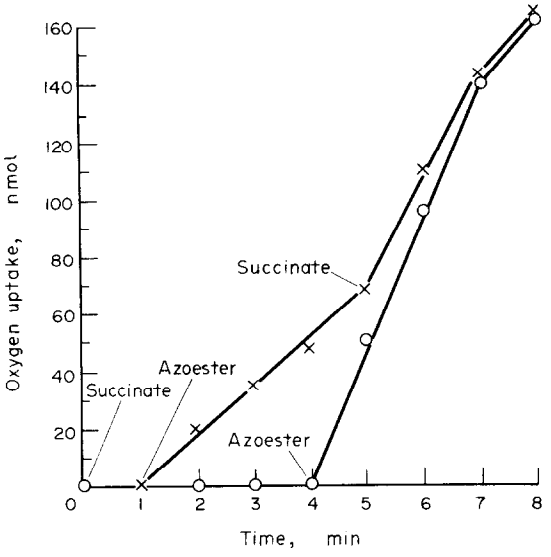

Fig. 5. Effect of azoester (100 nmole) on oxygen uptake by mitochondria (7 mg protein) in the presence of succinate (100 μmole). To a coupled suspension of mitochondria in the medium (final vol, 2 ml) was added as indicated either succinate followed by azoester (○) or azoester followed by succinate (×).

oxidation. Haemoglobin in fact has a much greater molar effect than Fe^{2+} (which can itself react with diazene) [13] while cytochrome *c* and Cu^{2+} are not active at all (Fig. 4). However, the haemoglobin catalysis can only account for a part of the erythrocyte activity and the nature of the remaining catalysis remains to be determined.

The catalysed oxidation of phenyldiazene does not appear to result in gross loss of function of subcellular particles. This is shown in the case of mitochondria by observing that when oxygen uptake from azoester has fallen off, oxidative phosphorylation still occurs on adding substrate, ADP and phosphate. Partial uncoupling has however been established by using lower azoester concentrations as illustrated in the case of succinate (Fig. 5). Such uncoupling has also been obtained with the other Krebs acids as evidenced by an increment of oxygen uptake on adding azoester to the substrate which is sensitive to the respiratory inhibitors.

DISCUSSION

The results show that the oxidation of azoester by tissue fractions occurs by the mechanism shown in Fig. 6. Two separate catalytic capacities are seen to be required. Thus there is little oxidation of azoester in the presence of either chymotrypsin (esterase activity only) or erythrocytes (oxidase activity only) but rapid oxidation when they are present together.

Catalysis of the hydrolysis of azoester by microsomes is in accordance with the localisation of most of the intracellular carboxylic acid esterases in this fraction [14]. However this explanation fails to account for the hydrolysis observed by mitochondria. Because of the hydrolysis by chymotrypsin it is possible that in this case the proteolytic enzymes known to be present in mitochondria [15] are involved in the catalysis.

The oxidation of phenyldiazene, allowing for slight losses by amidation reactions [16], approaches a value of one molecule of oxygen per mole of reagent suggesting that superoxide is first formed and subsequently oxidises other initial reaction products. Catalysis of the reaction probably occurs by a different mechanism in each tissue preparation. Thus the effects of heat and dialysis suggest that in microsomes it is due to small molecules whereas in mitochondria and erythrocytes it is associated primarily with macromolecules.

Erythrocytes may mediate an enzyme catalysed oxidation of phenyldiazene by oxyhaemoglobin since cytochrome *c* is inactive and since purified haemoglobin, which is known to form a complex with phenyldiazene [17], is much less active than erythrocytes containing roughly equivalent amounts. Several other drugs undergo oxidation in the presence of erythrocytes but the mechanism is likewise uncertain [18]. Because of the low esterase activity of plasma and erythrocytes, a substantial amount of an administered dose of azoester would be expected to reach the tissues intact for hydrolysis and oxidation. It therefore seems possible that the substance could be a useful agent for experimentally inducing a short term hypoxia.

The rapid loss of azoester by mitochondria is of particular significance in view of its demonstrated effect even at small concentrations in permanently uncoupling oxidation from phosphorylation. The implication is that the effect cannot be exerted directly. Azoester is known as a potent thioldepleting agent and this is the most likely basis for its action particularly as diamide [6] and other SH reagents [19] have similar effects. It follows that any SH groups oxidised by azoester would have to be reactive enough to succumb to the momentary exposure to the reagent which is all they could receive.

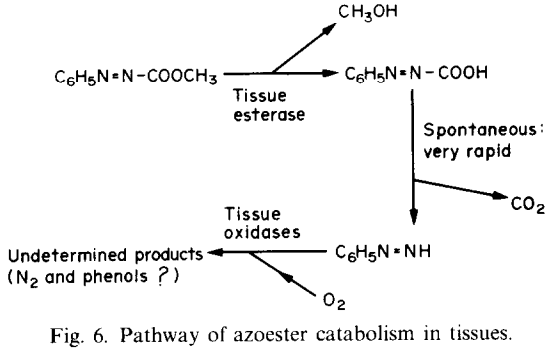

Fig. 6. Pathway of azoester catabolism in tissues.

REFERENCES

1. P. Van Caneghem, *Biochem. Pharmac.* **21**, 2417 (1972).
2. N. S. Kosower, Y. Marikovsky, B. Wertheim and D. Danon, *J. Lab. clin. Med.* **78**, 533 (1971).
3. N. S. Kosower, K. Song and E. M. Kosower, *Biochim. biophys. Acta* **192**, 1 and 23 (1969).
4. E. M. Kosower, *Nature, Lond.* **224**, 117 (1969).
5. E. N. Metz, S. P. Balcerzak and A. L. Sagone, *Blood* **44**, 691 (1974).
6. D. Siliprandi, G. Scutari, F. Zoccarato and N. Siliprandi, *FEBS. Lett.* **42**, 197 (1974).
7. P. C. Huang and E. M. Kosower, *J. Am. chem. Soc.* **90**, 2367 (1968).

8. W. C. Schneider and G. E. Hogeboom, *J. biol. Chem.* **223,** 147 (1956).
9. E. F. Jacobs, M. Jacobs, D. R. Sanadi and L. B. Bradley, *J. biol. Chem.* **223,** 147 (1956).
10. G. L. Ellman, *Archs Biochem. Biophys.* **82,** 70 (1959).
11. P. C. Huang and E. M. Kosower, *J. Am. chem. Soc.* **90,** 2362 (1968).
12. B. M. Goldschmidt. Unpublished work cited by P. C. Huang and E. M. Kosower, *J. Am. chem. Soc.* **90,** 2354 (1968).
13. J. Nicholson and S. G. Cohen, *J. Am. chem. Soc.* **88,** 2247 (1966).
14. K. Krisch, in *The Enzymes* (Ed. P. Boyer) Vol. 5, p. 43. Academic Press, New York (1971).
15. K. G. M. Alberti and W. Bartley, *Biochem. J.* **95,** 641 (1965).
16. N. S. Kosower, K. Song, E. M. Kosower and W. Correa, *Biochim. biophys. Acta* **192,** 8 (1969).
17. P. C. Huang and E. M. Kosower, *Biochim. biophys. Acta* **165,** 483 (1968).
18. G. Cohen and P. Hochstein, *Biochemistry, N.Y.* **3,** 895 (1963).
19. D. C. Gautheron, *Biochimie*, **55,** 727 (1973).