REVERSAL OF AZIDE INHIBITION BY UNCOUPLERS*

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Azide is a known inhibitor of electron transfer in non-phosphorylating submitochondrial particles with a site of inhibition at cytochrome oxidase (Keilin, 1936; Minnaert, 1961; Yonetani, 1965). In phosphorylating preparations azide also uncouples oxidative phosphorylation (Loomis and Lipmann, 1949; Slater, 1955) and inhibits some of the reactions associated with oxidative phosphorylation (Wadkins and Lehninger, 1958; Wadkins, 1961). This report on the azide inhibition of intact mitochondria demonstrates a direct relationship between the azide effects on oxidative phosphorylation and its inhibition of electron transfer.

METHODS AND MATERIALS

Rat liver mitochondria were prepared by the method of Chance and Hollunger (1961) and suspended in an assay medium containing 0.22 M mannitol, 0.05 M sucrose, 15 mM tris and 10 mM phosphate (MST-P_i). Any modifications in the medium and the pH of the medium are given in the Figure Legends. Reduced cytochromes and oxygen concentration were measured simultaneously in a double beam spectrophotometer-oxygen cathode combination by the method of Chance (Chance and Baltscheffsky, 1958).

RESULTS AND DISCUSSION

The inhibition of succinate oxidation by azide at pH 6.7 is illustrated in Figures 1A and B. The state 3 respiration is markedly inhibited with the half maximal effect occurring at about 60 μM azide. The state 4 respiration is much less sensitive and remains constant until the azide concentration exceeds 300 μM and then it is inhibited to the same level as the state 3 respiration. This "inhibition of state 4" appears to be an expression of the "uncoupling" of oxidative phosphorylation by high azide concentrations (Loomis and Lipmann, 1949; Slater, 1955).

This interpretation is supported by the data shown in Figure 1B since the State 4 inhibition is accompanied by a transition of the steady state reduc-

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tion of cytochrome c from state 4 to state 3 levels. Plots similar to Figure 1B may be made for cytochrome a (605-575 mμ or 605-630 mμ). In a typical

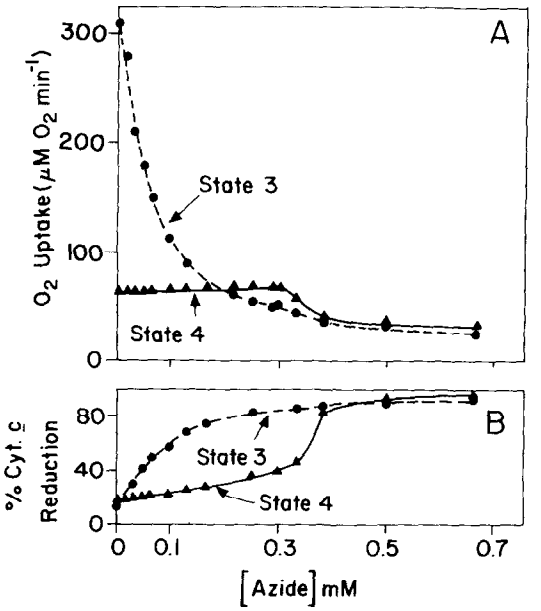

Fig. 1A,B. The azide inhibition of rat liver mitochondria. The mitochondrial suspension contained 2.2 mg protein/ml in a pH 6.9 MST-P_i medium containing 200 μM EDTA and 70 μM Mg^{++}. Three μM rotenone was added prior to the substrate which was 2 mM glutamate and 6.7 mM succinate. The oxygen uptake and cytochrome c reduction (550-540 mμ) were measured simultaneously. States 4 and 3 refer to before and after the addition of 600 μM ADP, respectively.

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experiment at pH 6.9, the azide concentrations for half maximal effect were 40 μM for inhibition of respiration (state 3), 40 μM for cytochrome a reduction and 70 μM for cytochrome c reduction. These azide concentrations for half maximal effect are pH dependent and increase about 5-fold from pH 6.6 to pH 7.6. The absolute values at a single pH were found to vary from mitochondrial preparation to mitochondrial preparation, but the relative values were quite constant for a single preparation.

The reversed control of respiration by ADP observed in Figure 1A for azide concentrations between 200 and 320 μM is characteristic of the succinate oxidation in these mitochondria. This reversed control, considered in conjunction with the reports of Wadkins and Lehninger on the effect of azide on oxidative phosphorylation, suggested that the azide inhibition might be influenced by uncouplers of oxidative phosphorylation.

An oxygen electrode tracing of such an experiment is shown in Figure 2. In this experiment the azide concentration was selected to give a slight

inhibition on addition of adenosine diphosphate (ADP). When the uncoupler FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) was added after the ADP, the respiration rate increased 4.4-fold to one-half the control rate in the absence of azide.

This release of azide inhibition by uncouplers is presented as Dixon plots (the reciprocal of the respiration rate against inhibitor concentration) in Figure 3. The reciprocal of the respiration rate is a linear function of the azide concentration at all of the uncoupler concentrations tested, but the slope of the line decreases with increasing uncoupler concentration. The azide concentration required for half maximal inhibition changed from 110 μM in the presence of ADP + Pi to 820 μM when 0.67 μM FCCP was added.

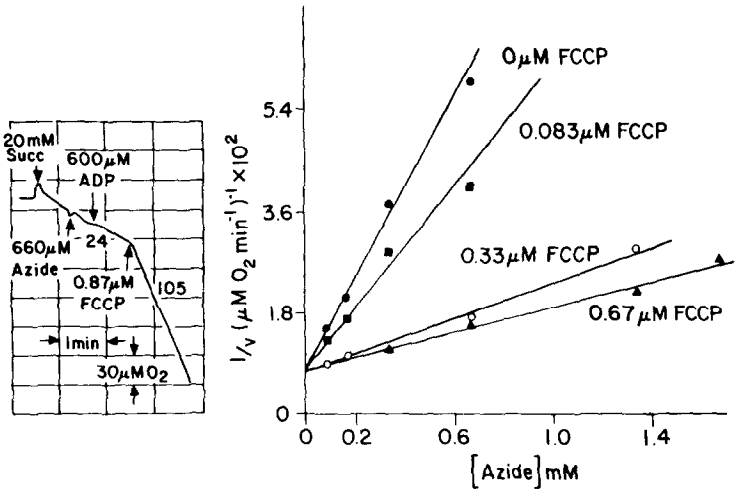

Fig. 2. The effect of uncoupler on the respiration of azide-inhibited mitochondria. Rat liver mitochondria were suspended in a pH 7.3 MST-P_i medium to a final protein concentration of 1.95 mg ml^{-1}. The order of addition was medium, mitochondria, 4 μM rotenone, then 20 mM succinate. The rate of oxygen uptake is expressed as μM O_2 min^{-1}.

Fig. 3. The release of azide inhibition by uncouplers. Rat liver mitochondria were suspended in a pH 7.3 MST-P_i medium to a final protein concentration of 2.4 mg ml^{-1}. Three μM rotenone was added before the substrate which was 16 mM succinate. In all cases the ADP concentration was 600 μM.

This release of azide inhibition is a general property of uncouplers as shown in Table I. The azide concentrations for half maximal effect were determined from Dixon plots in the presence and absence of uncoupler. The results are normalized and presented as the ratio of the azide concentration for half maximal effect in the presence of uncoupler to that in the absence of uncoupler. No attempt has been made in the data of Table I to obtain maximum possible ratios, but only to demonstrate that all of the uncouplers tested

have some influence on the azide inhibition. In each case, the uncoupler was effective in the concentration range used to uncouple oxidative phosphorylation. Three classes of uncouplers are represented: FCCP is a substituted phenylhydrazone of carbonyl cyanide, TTFB is a substituted benzimidazole, and the rest are substituted phenols.

The application of the crossover theorem to the state 4 to 3 transition by Chance (Chance and Williams, 1955) indicated a point of interaction of the electron transfer and energy conservation reactions between cytochromes c and a. It was, therefore, of considerable interest to determine the site of azide inhibition of respiration.

TABLE I

Reversal of Inhibition by Various Uncouplers

The assay medium was pH 7.3 MST-P_i. The column on the right is the ratio of the azide concentration required for half-maximal inhibition of respiration in the presence of uncoupler to that in its absence.

Uncoupler	Uncoupler Concentration μM	[Azide] + uncoupler / [Azide]
FCCP‡	0.43	4.7
"	1.7	8.9
TTFB*	1.9	4.6
"	4.9	7.0
Dicoumarol	10.8	3.3
"	21.6	3.9
Pentachlorophenol	6.6	1.4
Dinitrophenol	52.0	1.3
"	208.0	3.3

‡'carbonyl cyanide p-trifluoromethoxy-phenylhydrazone

*4,5,6,7 tetrachloro-2-trifluoromethylbenzimidazole

The liquid nitrogen temperature spectrum of the cytochromes reduced in state 3 in the presence of azide (Figure 4) was obtained by the technique of Chance and Spencer (1959). This is reduced minus oxidized difference spectrum with the reference cuvette containing mitochondria in state 2 (low substrate, high ADP). The site of azide inhibition is clearly marked by the strong reduction of cytochrome a as being between cytochromes a and a_3. The cytochrome

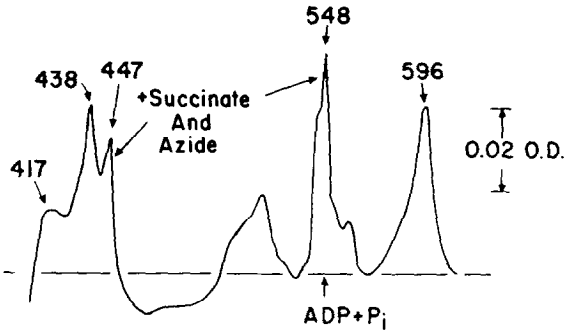

Fig. 4. A reduced minus oxidized spectrum of azide inhibited mitochondria. The mitochondria were suspended in a pH 7.2 MST-P_i medium to a final protein concentration of 6.8 mg ml^{-1}. Endogenous respiration was inhibited with 10 μM rotenone. The reference cuvette contained aerobic mitochondria with 2 mM ADP, and the measure cuvette contained mitochondria with 10 mM succinate, 2 mM ADP, and 800 μM azide. The samples were frozen by the trapped steady state technique of Chance and Spencer (1959) and the spectrum measured as liquid nitrogen temperature. The light path was 2 mm and the half-bandwidth was 1.6 mμ.

a alpha maximum is observed at 596 mμ and the Soret maximum as a double maximum at 447 and 438 mμ. It is interesting to note that the cytochrome a alpha maximum seen in Figure 4 is shifted about 5 mμ to a shorter wavelength than is observed in fully reduced mitochondria. Cytochrome a_3 is highly oxidized since its reduction would be marked by the appearance of a Soret maximum at 444 mμ. Additional data leading to this identification of the spectral forms of cytochrome oxidase will be presented elsewhere (Wilson and Chance).

The reversal of the azide inhibition by uncouplers is good evidence that the azide is inhibiting respiration by stabilizing an intermediate common to both the electron transfer and energy conservation reactions. Inasmuch as the site of azide inhibition is between cytochromes a and a_3 and not between cytochromes c and a, this interpretation would require either that an additional phosphorylation site exists between cytochromes a and a_3 or that the mechanism of coupling between the energy conservation reactions and electron transfer is more complex than was assumed in the derivation of the crossover theorem (Chance and Williams, 1955; Holmes, 1959). Although the possibility of ADP/O ratios greater than 2 for succinate oxidation have been suggested (Lynn and Brown, 1965; Smith and Hansen, 1964), the available evidence favors the existence of additional complexities in the mechanism. The existence of interaction sites both between cytochromes c and a and cytochromes a and a_3 indicate a key role for cytochrome a in the energy conservation reactions.

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