Alteration of Mitochondrial Function by *Bungarus fasciatus* Venom*

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ABSTRACT: Respiration and phosphorylation were studied following treatment of rat liver mitochondria and whole homogenates with krait (*Bungarus fasciatus*) venom. One effect was the uncoupling of energy transfer and inhibition of maximal respiration with excess substrate and adenosine 5′-diphosphate. The uncoupling activity was associated with oxidation of reduced diphosphopyridine nucleotide, while other carriers were little affected. Following treatment of mitochondria with about 5–10 μg of venom per mg of protein, adenosine 5′-diphosphate inhibited respiration (reverse acceptor control). As adenosine 5′-diphosphate inhibited respiration, cytochrome *b* and flavoprotein became reduced while pyridine nucleotide became oxidized. This reverse acceptor control was produced only by freshly reconstituted or freshly collected venom.

Snake venoms are powerful inhibitors of mitochondrial respiration (Ghosh and Chatterjee, 1948; Edwards and Ball, 1954; Ambe and Crane, 1959). The respiratory inhibition is associated with mitochondrial swelling (Petrushka *et al.*, 1959; Taub and Elliott, 1964) and release of respiratory carriers (King and Howard, 1960, 1962; Ringler *et al.*, 1960). It has been suggested that phosphorylation intermediates are among the first mitochondrial components to be attacked by venoms, and that inhibition of respiratory activity occurs as the venom subsequently attacks other components of the respiratory chain (Petrushka *et al.*, 1959).

We have found that freshly reconstituted banded krait (*Bungarus fasciatus*) venom contains at least two activities, one that causes uncoupling of energy transfer and another that induces reverse acceptor control. The uncoupling activity (I) can be produced by phospholipase A and is more stable than the activity-inducing reverse acceptor control (II). As shown below, both the inhibition of respiration and the uncoupling produced by venom action affect the oxidation states of the respiratory carriers.

Materials and Method

Venom activity was tested on whole-tissue homogenates or mitochondria prepared from rat liver. Rat liver mitochondria were prepared in 225 mM mannitol, 75 mM sucrose, and 0.01 mM EDTA, *p*H 7.1 (Chance and Hagihara, 1962). The reaction medium contained 45 mM mannitol, 15 mM sucrose, 4 mM KCl, 2 mM $MgCl_2$, 2 mM potassium phosphate buffer, and 0.002 mM EDTA, *p*H 7.4.

Homogenates were prepared in the mannitol-sucrose-EDTA medium as described by Ziegler *et al.* (1962). Specimens of *Bungarus fasciatus* were imported from southern Thailand. Venom was lyophilized immediately after it was collected (in our laboratory) by milking into a precooled wine glass, and then stored in a desiccator at −20°. The venom was reconstituted just before use, in deionized distilled water, to a concentration of 10 mg/ml. Commercial krait venom (Pure Toxin) was purchased as a lyophilized powder from the Miami Serpentarium. The commercial venom was stored and reconstituted in the same manner as the locally lyophilized venom. Fatty acid–poor bovine serum albumin was obtained from Pentex Corp., Kalamazoo, Mich.

Determination of respiratory activity was made in a sealed oxygen electrode respirometer (Strickland, *et al.*, 1961). Simultaneous recording of respiration and the oxidation state of the respiratory carriers was made with a dual-wavelength spectrophotometer (Aminco Chance), equipped with an oscillating platinum cathode (GME) in an open vessel. A silver wire anode was connected to polarizing and recording circuits previously described (Strickland, *et al.*, 1961). The change in absorbance due to DPNH oxidation was measured at 340–374 mμ, to cytochrome *b* oxidation at 430–410 mμ, and to cytochrome *c* oxidation at 550–540 mμ.

* From the Departments of Biochemistry and Biology, State University of New York at Buffalo, Buffalo, N.Y. *Received October 9, 1964.* The work reported here has been supported by grants from the following sources: U.S. Public Health Service RG-6241 (Division of General Medical Sciences) to W. B. E.: AC-00151 (Division of Accident Prevention, Bureau of State Services) to C. G.; a Public Health Service Research Career Program Award (5-K3-GM-15,514) to W. B. E.; and a Public Health Service Predoctoral Fellowship G-19119 (Division of General Medical Sciences) to A. T. (sponsored by C. G.): L. V-C. is a predoctoral trainee under training grant GM 761 (Division of General Medical Sciences), U.S. Public Health Service.

Preliminary reports of this work were presented at the 146th National Meeting of the American Chemical Society, Denver, Colo., January 1964, and the 48th annual meeting of the Federation of American Societies for Experimental Biology, April 1964.

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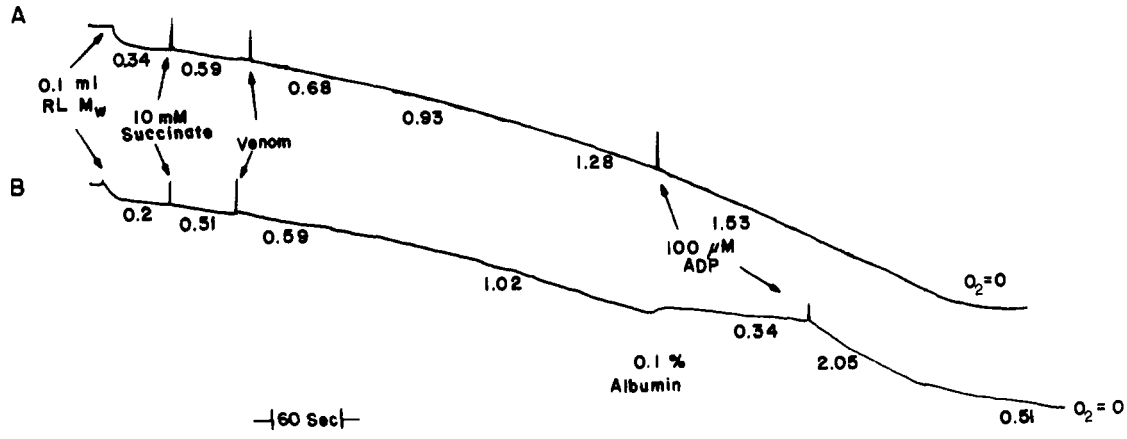

FIGURE 1: Uncoupling of homogenate respiration by venom (A) and reversal of uncoupling by albumin (B). Venom was added at a concentration of 10 μg/mg of protein. The respiratory rate is shown in μM O_2/sec under the electrode trace. Protein concentration was 1.6 mg/ml in both (A) and (B) (3.5 ml).

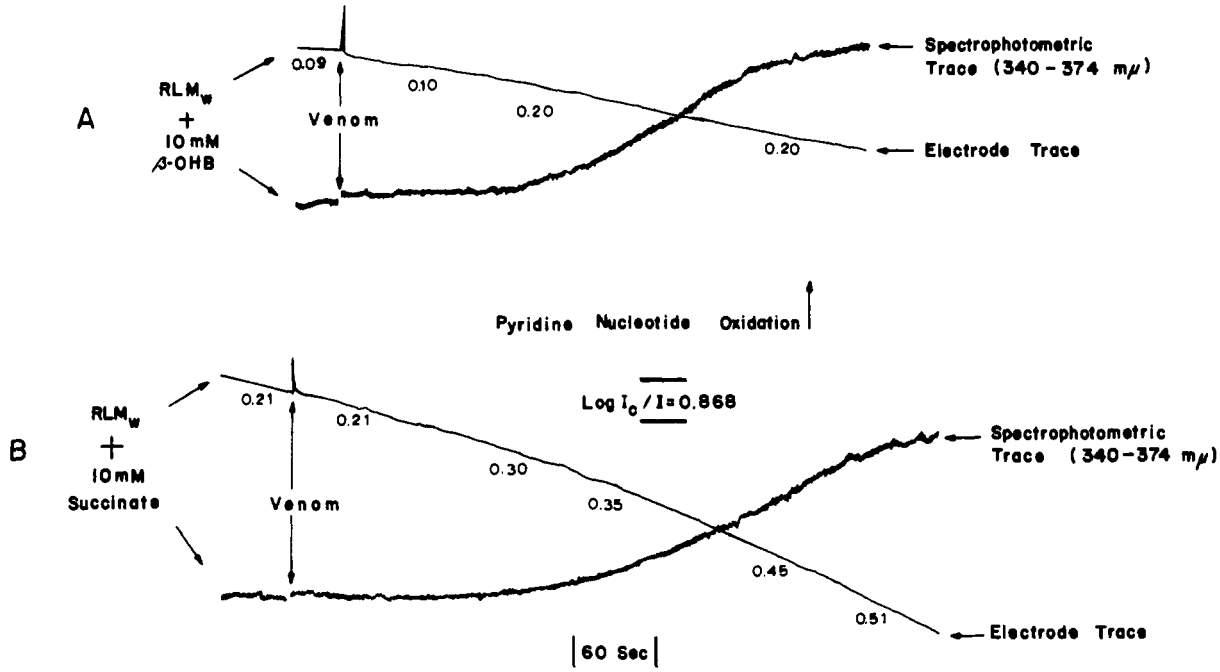

FIGURE 2: Effect of venom treatment on respiration and pyridine nucleotide oxidation state. Similar results were obtained with β-hydroxybutyrate (A) and succinate (B). Reaction medium was phosphate-buffered sucrose-mannitol-EDTA, *p*H 7.3. Respiration is shown in μM O_2/sec under the electrode trace. Venom concentration was 4.5 μg/mg of mitochondrial protein. Total mitochondrial protein was 1.8 mg/ml. Temperature was 20° (3.2 ml total volume).

Oxidation and reduction of flavoprotein was followed at 465–510 mμ.

Results

When mitochondria or whole homogenates were treated with freshly reconstituted venom, two effects were observed: an uncoupling effect (I) and a reverse acceptor control (II). The uncoupling activity persisted after the venom was subjected to the classical procedure for phospholipase A preparation (Hayaishi, 1955). The second activity was extremely labile and was accompanied by activity I. We have not found a satisfactory method to separate and/or preserve the second activity.

Activity I uncoupled oxidative phosphorylation in mitochondria as shown in Table I. Identical activity was demonstrated with whole homogenates. The uncoupling effect of the venom treatment was different from that observed with an uncoupling agent such as

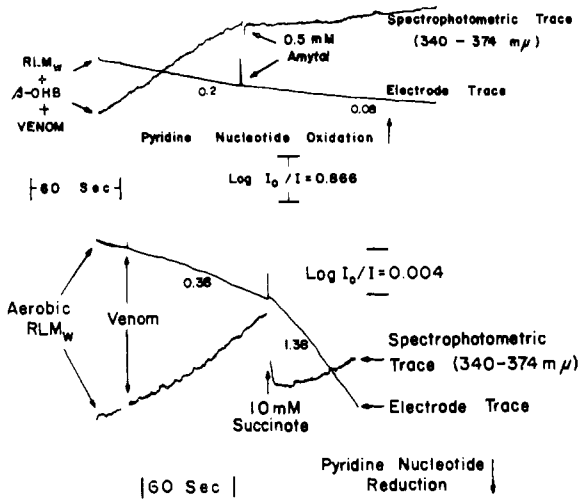

FIGURE 3: Effect of Amytal (A, upper) and succinate (B, lower) on respiration and pyridine nucleotide oxidation in venom-treated mitochondria. In (A), 40 μg of venom was added to 4.8 mg of mitochondrial protein. In (B), 20.0 μg of venom was added to 2.36 mg of mitochondrial protein. Reaction medium and other experimental conditions were the same as in Figure 2.

dinitrophenol, which allows respiration on substrate alone to proceed as rapidly as respiration in the presence of substrate and phosphate acceptor (Lardy and Wellman, 1952; Chappell, 1961a; Chance *et al.*, 1963). Rather, the effect more closely resembled the uncoupling observed in aged mitochondria by Chance and Hagihara (1962).

Treatment with venom having only activity I uncoupled both types of preparations (Figure 1). Addition of ADP did not alter the rate of increase of respiration. The addition of 0.1% serum albumin (fatty acid poor) reversed the uncoupling effect as indicated by the controlled succinate oxidation. The subsequent addition of ADP stimulated respiration about 6-fold giving an ADP/O of 1.5. Freshly reconstituted venom, as well as boiled venom containing phospholipase A activity, was equally effective in uncoupling phosphorylation.

Respiration in the presence of substrate and no phosphate acceptor was stimulated by venom, while maximal respiration with substrate and ADP was inhibited (Table I). As the venom concentration was increased, the rate of respiration on substrate alone was correspondingly increased. However, at a concentration of 1 μg venom per mg of mitochondrial protein, venom did not affect respiration in presence of succinate or β-

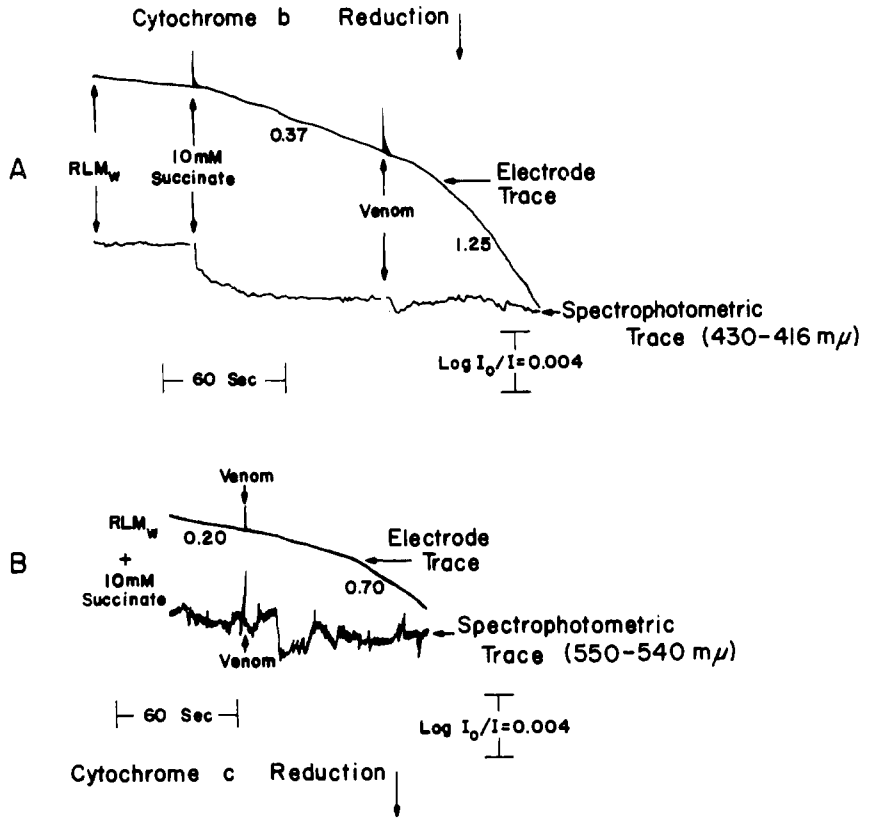

FIGURE 4: Effect of venom-induced uncoupling on respiration and oxidation states of cytochrome *b* (A) and cytochrome *c* (B). Reaction medium and experimental conditions were the same as in Figure 2. Venom concentration was about 5 μg/mg of mitochondridal protein. Mitochondrial protein concentration was 1.5 mg/ml.

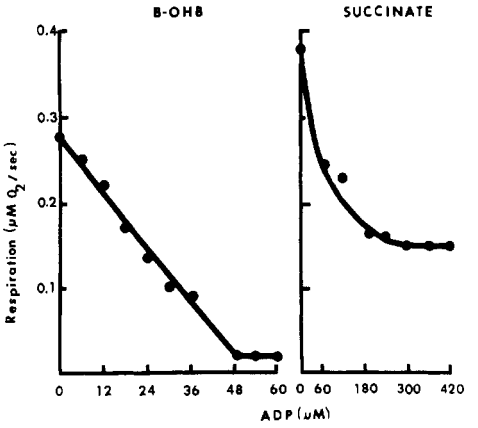

FIGURE 5: Reverse acceptor control following treatment with freshly reconstituted venom. Venom (100 μg) was used to treat 10 mg of mitochondrial protein. Other conditions were the same as in Figure 2.

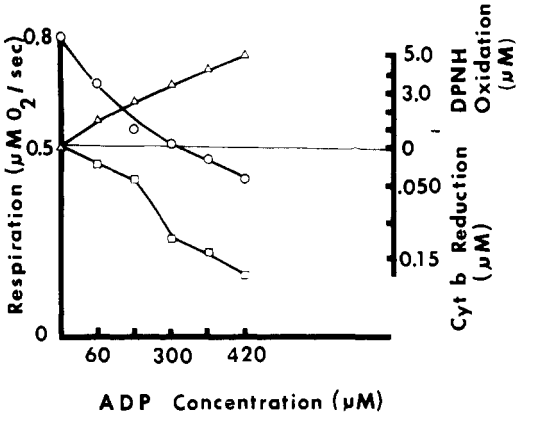

FIGURE 6: Effect of reverse acceptor control on respiration (○—○), pyridine nucleotide oxidation (△—△), and cytochrome *b* reduction (□—□) as a function of ADP concentration. Pyridine nucleotide oxidation is shown as the increase in μM of DPN^+ at 340–374 mμ. Cytochrome *b* reduction is shown as the appearance of reduced cytochrome *b* at 430–416 mμ.

TABLE 1: Effect of Venom-uncoupling Activity on Respiration and ADP/O Ratio.[a]

Substrate	Venom Treatment (μg/mg protein)	Respiration (mμmoles O_2/sec/mg N) Substrate	+ADP	ADP/O
Succinate	0.0	0.85	6.3	1.8
	1.0	0.83	6.35	1.7
	2.0	0.95	4.05	1.4
	3.0	1.21	2.91	1.1
β-Hydroxybutyrate	0.0	0.29	2.46	2.9
	1.0	0.31	2.0	2.5
	2.0	0.46	1.73	2.1
	3.0	0.52	1.67	1.7

[a] Rat liver mitochondria were suspended in phosphate-buffered sucrose-mannitol-EDTA reaction medium, *p*H 7.3. Venom treatment was carried out for 2 minutes at 0° before measurements were determined polarographically at 25°.

hydroxybutyrate or respiration in the presence of succinate and ADP. The maximal rate of succinate oxidation with excess ADP present was increasingly inhibited by greater amounts of venom.

Maximal β-hydroxybutyrate oxidation in the presence of excess ADP was inhibited by all levels of venom that were employed.

The addition of venom to mitochondria caused stimulation of respiration and oxidation of DPNH (Figure 2). The oxidation of pyridine nucleotides eventually reached a new steady state. As is evident from Figure 2, similar results were obtained with both succinate and β-hydroxybutyrate. After 5 minutes the concentration of oxidized pyridine nucleotides increased by 3 mM (of a total of 40 mM pyridine nucleotide). Respiration showed a sharp increase after 1 minute, reaching a new steady rate after 3 minutes.

Following uncoupling by venom, respiration was inhibited by Amytal, but the pyridine nucleotides continued to become oxidized (Figure 3A). In a control experiment, addition of Amytal to mitochondria respiring on β-hydroxybutyrate caused reduction of DPN^+ as well as inhibition of respiration. In contrast with the failure of Amytal to cause pyridine nucleotide reduction following venom treatment, oxidized pyridine nucleotide was reducible by succinate after venom treatment (Figure 3B). No change in the oxidation states of cytochromes *b* and *c* could be detected even after 5 minutes of venom treatment (Figure 4).

Following incubation with 5–10 mg of freshly reconstituted venom (as defined earlier) per mg mitochondrial protein, ADP inhibited respiration. At saturating concentrations, ADP inhibited β-hydroxybutyrate respiration by 90% (Figure 5) while succinate respiration was inhibited by only 70%. Half-maximal inhibition of mitochondrial β-hydroxybutyrate respiration was obtained with 25 μM ADP, while half-maximal inhibition of succinate respiration was obtained with 60–150 μM ADP. Succinate oxidation in whole homogenate was 80% inhibited by ADP following the venom action.

Treatment with triglyceride (olive oil, safflower oil) did not have any effect on respiration. However, when the triglycerides were added in the presence of freshly lyophylized whole venom, a respiratory inhibition of 40–60% was consistently observed. This inhibition was not reversed by ADP or DNP. Neither could DNP release the inhibition caused by ADP, but ADP could inhibit respiration after treatment with venom, even in the presence of DNP.

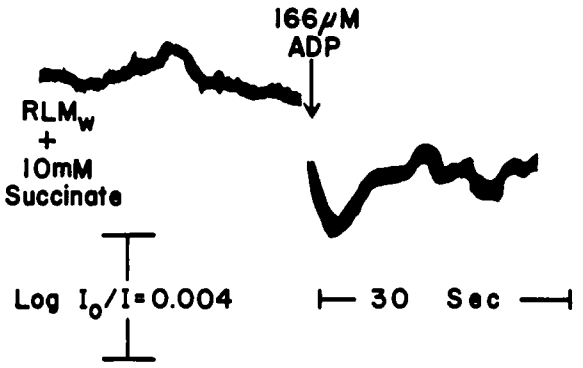

FIGURE 7: Flavoprotein reduction following the addition of ADP to mitochondria pretreated with 10 mM succinate and 10 μg venom/mg protein. Treatment was carried out for 2 minutes prior to ADP addition. Mitochondrial protein, 4.6 mg in 3.0 ml.

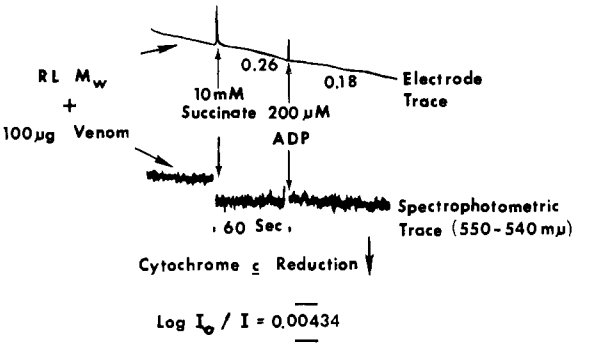

FIGURE 8: Effect of reverse acceptor control on cytochrome *c*. Venom concentration was 10 μg/mg protein. Conditions were the same as in Figure 2.

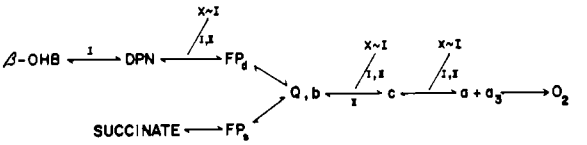

FIGURE 9: Schematic diagram of the respiratory chain showing probable sites of activities I and II. Activity I is responsible for uncoupling of the phosphorylative reactions (S~I) and activity II blocks electron transport at the cytochrome *c* level. Activity II may also have an effect at some of the phosphorylative steps.

The data presented in Figure 6 show that ADP inhibition of respiration caused reduction of cytochrome *b*, while the strong uncoupling activity permitted oxidation of DPNH to continue, an effect similar to that found in the presence of Amytal and venom (cf. Figure 3). Flavoprotein entered a more reduced state as respiration was inhibited by ADP (Figure 7). Inhibition of respiration by ADP in the presence of venom did not cause reduction of cytochrome *c* (Figure 8).

Our commercial specimen of *B. fasciatus* venom did not produce either uncoupling or reverse acceptor activity in homogenates or mitochondria. Likewise, locally lyophilized venom that had been stored at −20° for more than 1 week lost activity II, while that allowed to warm to room temperature lost both activities.

Discussion

The preceding data indicate that freshly reconstituted venom from *B. fasciatus* venom has at least two different effects on respiration and energy transfer in broken-cell preparations. One activity (I) uncouples energy transfer and the other (II) induces ADP to inhibit respiration. This last effect has been termed "reverse acceptor control" (Lehninger and Gregg, 1963; Gregg and Lehninger, 1963).

The uncoupling effect is due to the activity of phospholipase A. That this is so is indicated by the reversal of uncoupling by albumin and by the similarity of behavior between venom having only activity I and phospholipase A prepared from the same venom. Similar conclusions have been reported by Petrushka *et al.* (1959) as to the factor responsible for activity I in *Naja naja* venom.

The spectrophotometric observations show that the uncoupling activity promotes the oxidation of DPNH. Moreover, Amytal in the presence of β-hydroxybutyrate caused no reduction of the pyridine nucleotide. DPNH oxidation is typical of the uncoupled system (Chance *et al.*, 1963; Chance and Hagihara, 1962) and, in these experiments, suggests that transfer of reducing equivalents from β-hydroxybutyrate to DPN^+ is inhibited. The fact that succinate caused reduction of DPN^+ in this inhibited system lends support to the hypothesis (Chance and Hollunger, 1961; Chance and Hagihara, 1962) that the succinate-reducible DPN^+ compartment is independent of that reducible by pyridine nucleotide–linked substrates.

Reverse acceptor control causes reduction of cytochrome *b* and flavoprotein, identifying a site of inhibition between cytochrome *b* and cytochrome *c*. Pyridine nucleotide, on the other hand, continues to become oxidized, probably because of activity I. Activities I and II seem consistent with the scheme shown in Figure 9, where activity I is responsible for both uncoupling and the highly oxidized state of DPN^+, while activity II blocks electron transfer following addition of ADP. Activity I is due to phospholipase A in the presence of which free fatty acids are liberated from phospholipids (Edwards and Ball, 1954; Fleischer *et al.*, 1964). Phospholipids are necessary for the transfer of reducing equivalents from DPNH to cytochrome *c* and CoQ (Fleischer *et al.*, 1964) and, since free fatty acids are efficient uncouplers of phosphorylation (Pressman and Lardy, 1955; Lehninger and Rommert, 1959; Hulsmann *et al.*, 1960), the same acids could also account for the observed decrease in the ADP/O.

Activity II is due, mainly, to an esteraselike activity. This follows from the data on the effect of triglycerides

on respiration. Addition of triglycerides to the preparation in the absence of venom has no effect, but in the presence of venom a pronounced respiratory inhibition is observed that responds to neither ADP nor albumin. Reverse acceptor control, due to the addition of ADP in the presence of substrate and venom, is not released by addition of triglyceride. From this and other experiments (Leila Vázquez-Colón, unpublished), it appears that activity II is a powerful esterase which liberates large amounts of free fatty acids. The respiratory inhibition caused by triglycerides and ADP was not released by DNP, while ADP still inhibited respiration after treatment with venom and DNP (up to 2×10^{-4} M). This would indicate that the site of action of venom + ADP is closer to the respiratory chain than the site of action of DNP.

Solubilization of DPNH dehydrogenase (Ambe and Crane, 1959; King and Howard, 1960; Ringler *et al.*, 1960) through breakdown of phospholipids could possibly account for the inhibition of pyridine nucleotide–linked respiration. However, the experiments reported here show that mild treatment with phospholipase probably does not liberate appreciable amounts of DPNH dehydrogenase since DPNH was readily oxidized following venom treatment. Moreover, intact mitochondria were used in the present experiments while the work dealing with solubilization of dehydrogenase employed mitochondrial particles or heart particles. Since intact mitochondria are presumably less sensitive to phospholipase activity solubilization would not be expected under the conditions of these experiments, and DPNH dehydrogenase liberation is eliminated as a primary site of venom activity. Further support for this conclusion is drawn from the fact that, under similar conditions, no appreciable amount of respiratory pigments was found in the suspending medium (Taub and Elliott, 1964).

The exact mechanism of ADP inhibition of respiration is not known. The DPNH dehydrogenase or DPN^+ reductase seem to be intimately involved. In our experiments, as well as those of Lehninger and Gregg (1963) and Chance and Hollunger (1961), reverse acceptor control was found associated with high levels of DPN^+.

It seems unlikely that reverse acceptor control is mediated by oxalacetate accumulation and depletion of endogenous substrate (Chappell, 1961b; Chance and Hagihara, 1962) because, in our experiments, inhibition of respiration by ADP was accomplished before the endogenous substrate pool could be exhausted. Moreover, there was no initial stimulation of respiration by ADP, and the inhibition was observed with a substrate other than succinate. It seems more likely that ADP, possibly acting in conjunction with free fatty acid, acts directly to produce the inhibition. Considering the phosphorylation scheme proposed by Chance, by Lehninger, and by Slater:

Electron Transport

$$X + I \longleftrightarrow X\sim I \quad (1)$$

$$X\sim I + P_i \longleftrightarrow X\sim P + I \quad (2)$$

$$X\sim P + ADP \longleftrightarrow X + ATP \quad (3)$$

Where DNP is effective at the level of X~I, ADP inhibits respiration in the presence of DNP. Hence, inhibition must occur at equation (1) or at the level of electron transport. Another possibility is a competitive interaction with DNP and/or phosphate for a site in equation (2).

Acknowledgments

We would like to thank Mrs. Judy Wolf and Miss Charlyn Rhodes for skillful technical assistance.

References

Ambe, K. S., and Crane, F. L. (1959), *Science 129*, 98.
Chance, B., and Hagihara, B. (1962), *J. Biol. Chem. 237*, 3540.
Chance, B., and Hollunger, G. (1961), *J. Biol. Chem. 236*, 1534.
Chance, B., Williams, G. R., and Hollunger, G. (1963), *J. Biol. Chem. 238*, 439.
Chappell, J. B. (1961a), *Federation Proc. 20*, 50.
Chappell, J. B. (1961b), *Biol. Struct. Function, Proc. IUB/IUBS Intern. Symp. 1st, Stockholm, 1960*, 71.
Edwards, S. W., and Ball, E. G. (1954), *J. Biol. Chem. 209*, 619.
Fleischer, S., Casu, A., and Fleischer, B. (1964), *Federation Proc. 23*, 486.
Ghosh, B. N., and Chatterjee, A. K. (1948), *J. Indian Chem. Soc. 25*, 359.
Gregg, C. T., and Lehninger, A. L. (1963), *Biochim. Biophys. Acta 78*, 27.
Hayaishi, O. (1955), *Methods Enzymol. 1*, 660.
Hulsmann, W. C., Elliott, W. B., and Slater, E. C. (1960), *Biochim. Biophys. Acta 39*, 267.
King, T. E., and Howard, R. L. (1960), *Biochim. Biophys. Acta 37*, 357.
King, T. E., and Howard, R. L. (1962), *J. Biol. Chem. 237*, 1686.
Lardy, H. A., and Wellman, H. (1952), *J. Biol. Chem. 195*, 215.
Lehninger, A. L., and Gregg, C. T. (1963), *Biochim. Biophys. Acta 78*, 12.
Lehninger, A. L., and Rommert, L. M. (1959), *J. Biol. Chem. 234*, 2459.
Petrushka, E., Quastel, J. H., and Scholefield, P. G. (1959), *Can. J. Biochem. Physiol. 37*, 989.
Pressman, B. C., and Lardy, H. A. (1955), *Biochim. Biophys. Acta 18*, 482.
Ringler, R. L., Minakami, S., and Singer, T. P. (1960), *Biochem. Biophys. Res. Commun. 3*, 417.
Strickland, E. H., Ziegler, F. D., and Anthony, A. (1961), *Nature 191*, 969.
Taub, A., and Elliott, W. B. (1964), *Toxicon 2*, 87.
Ziegler, F. D., Strickland, E. H., and Anthony, A. (1962), *U.S. Army Med. Res. Lab., Fort Knox, Ky. Rept. 549.*