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INHIBITION OF OXIDATIVE PHOSPHORYLATION AND DINITROPHENOL STIMULATED ATPase BY BATHOPHENANTHROLINE

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1. Introduction

Bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) a chelating agent which is highly specific for ferrous ions, has been shown to inhibit both oxidative phosphorylation and dinitrophenol stimulated ATPase in rat liver mitochondria. The pattern of inhibition is similar to that produced by oligomycin. The inhibition of the ATPase activity caused by bathophenanthroline was reversed by adding ferrous ions; other metal ions tested were inactive.

Non haem iron proteins have been firmly established as functional components of both succinate and $NADH_2$ dehydrogenases [1,2,3]. Rieske et al. [4] have isolated another non haem iron protein believed to function as an electron carrier between cytochromes *b* and *c*.

The role of non haem iron proteins in the mitochondrion may be more complex that that of simple electron carriers. Light et al. [5] have grown yeast (*Torulopsis utilis*) on iron deficient media and have correlated the presence of a non haem iron protein in the $NADH_2$ dehydrogenase found in mitochondria with the efficient conservation of energy at the first coupling site. In the absence of the non haem iron component electron transport continued and the first site of ATP synthesis was by-passed. Vallin and Low [6,7] have also suggested that non haem iron proteins play a key role in energy conservation at the coupling sites I and II. In the electron transport system extracted from *Mycobacterium-phlei* Kurup and Brodie [8] have shown that orthophenanthroline can act as an uncoupling agent; phosphorylation could be recoupled by adding ferrous ions. All of these observations have been interpreted as showing that non haem iron proteins appear to act as electron transport components operating at the point of interaction between the electron transport chain and ATP synthesis. However, Kurup and Brodie also suggested, as an alternative explanation, that a non haem iron protein was directly involved in the ATP synthesising system. More convincing experimental evidence for the direct involvement of non haem iron proteins in the phosphorylation reactions was obtained by Butow and Racker [9] when they observed orthophenanthroline imposed respiratory control in phosphorylating submitochondrial particles and that in the presence of dithionite orthophenanthroline caused the inhibition of the $^{32}P_i$-ATP exchange reaction.

2. Materials and methods

Rat liver mitochondria were obtained from adult Sprague Dawley rats using the method described by Weinbach [10]. Sodium bathophenanthroline sulphonate (water soluble) and orthophenanthroline were purchased from Sigma Chemical Company. Bathophenanthroline (insoluble in water) was obtained from Hopkins and Williams.

Oxygen uptake was measured in a Gilson 'Oxygraph' oxygen electrode. The reaction medium (volume 1.0 ml) contained 100 μmoles KCl, 10 μmoles $MgCl_2$, 25 μmoles sucrose, 1 μmole EDTA, 15 μmoles KH_2PO_4, 100 μmoles succinate and mitochondria containing 1 mg of protein, at pH 6.8 and 25°C. Respiration was stimulated when required, by adding either 2 μmoles of ADP or 20 nmoles of DNP.

Dinitrophenol stimulated ATPase was measured by

incubating mitochondria containing 0.5 mg of protein in 1.0 ml of medium containing 100 μmoles KCl, 25 μmoles sucrose, 1 μmole EDTA, 2 μmoles ATP and 15 nmoles of DNP for 5 min. The inorganic phosphate release was measured colorimetrically using the method of Ernster et al. [11].

The amount of mitochondrial protein was estimated by carrying out a Kjedhal digestion followed by nesslerization.

3. Results

When water soluble bathophenanthroline suphonate was added to fresh mitochondria which were oxidising succinate in the presence of ADP there was a marked and immediate inhibition of the rate of oxygen uptake. This was followed 90 sec later by a second distinct phase of inhibition. When the mitochondria were in the controlled (state 4) or uncoupled state (plus DNP) the first phase of inhibition caused by bathophenanthroline was absent and only the second phase of inhibition occurred.

In fig. 1 the average rate of respiration was measured between 20 and 80 sec after adding the inhibitor (a measure of phase I inhibition) and has been plotted as a function of the inhibitor concentration. Immediate inhibition of oxygen uptake by bathophenanthroline sulphonate occurred only if the mitochondria were actively synthesising ATP. Orthophenanthroline behaved similarly; although free bathophenanthroline and αα-dipyridyl appeared to cause similar inhibitions the two phases of inhibition were not always distinct. An oxygen electrode trace, fig. 2, shows that the first phase of inhibition of respiration caused by bathophenanthroline sulphonate can be partially reversed by adding DNP. The second phase of inhibition became apparent towards the end of the trace and was thought to result from an inhibition of electron transport, probably due to interaction between the chelator and the

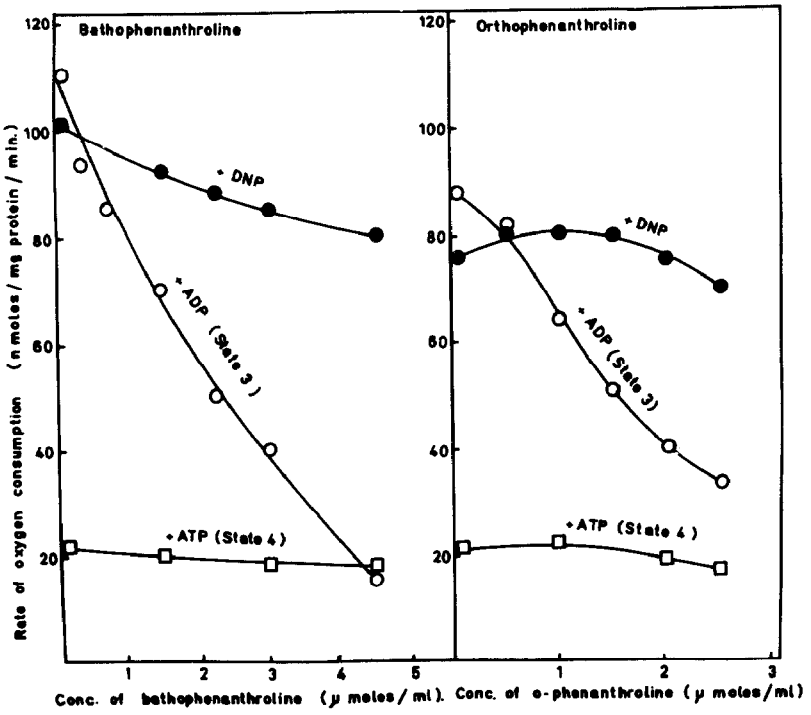

Fig. 1. The inhibition of oxygen uptake by bathophenanthroline sulphonate or orthophenanthroline. See text for experimental details. Respiration in state 4 was acheived by adding 0.25 μmoles of ADP and allowing it to be converted to ATP before adding the inhibitor.

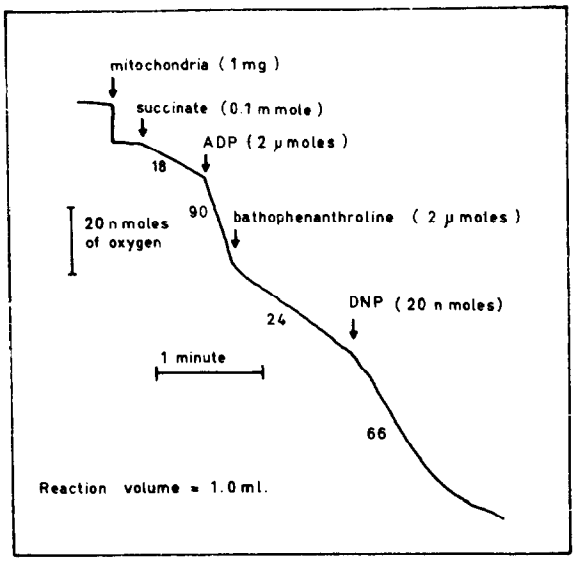

Fig. 2. An oxygen electrode trace showing the reversal of bathophenanthroline inhibition by dinitrophenol. The figures on the line respresent the rates of oxygen uptake in nmoles of oxygen consumed/min/mg protein. The lowering of the rate towards the end of the trace is due to the appearence of the second phase of inhibition.

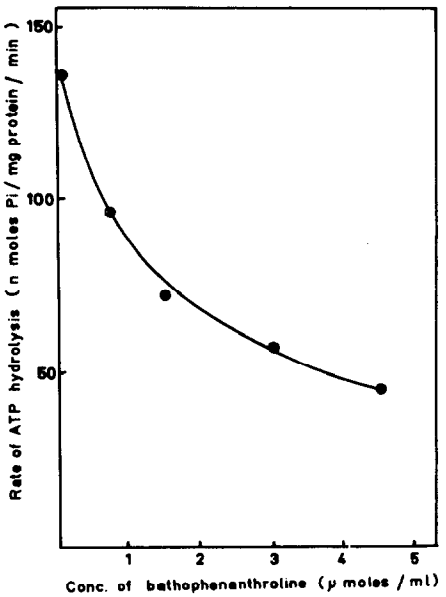

Fig. 3. The inhibition of dinitrophenol stimulated ATPase by bathophenanthroline sulphonate. The activity of the ATPase in the absence of DNP was 17.8 nmoles P_i produced/min/mg protein.

non haem iron in the succinic acid dehydrogenase. It was not possible to carry out similar experiments using NAD linked substrates as it was found that 0.2 mM bathophenanthroline sulphonate (one tenth of that used with succinate) immediately resulted in a rapid inhibition of oxygen uptake even in the presence of dinitrophenol, a result which suggests a rapid interaction with the non haem iron associated with the $NADH_2$ dehydrogenase. Preliminary experiments, using the oxygen electrode, with ascorbate + tetramethyl phenylenediamine as electron donor system were complicated by the low level of respiratory control commonly observed. Manometric experiments showed that 2 mM bathophenanthroline lowered the P/O ratio from 0.9 to 0.2 indicating an interaction of bathophenanthroline with the ATP synthesis occurring at coupling site III.

Bathophenanthroline sulphonate also inhibited the DNP stimulated ATPase as shown in fig. 3 which suggests that its point of action is located in the system synthesising ATP rather than in the electron transport chain. Therefore bathophenanthroline appears to inhibit oxidative phosphorylation in a manner similar to oligomycin.

Attempts to correlate the ability of bathophenanthroline to chelate ferrous ions with its ability to inhibit oxidative phosphorylation were made by studying the interaction between bathophenanthroline sulphonate and divalent metal ions in the ATPase assay. The results of early experiments indicated a positive interaction but were complicated because the high concentration of metal ions involved interfered with the ATPase assay. Subsequent experiments showed that free bathophenanthroline inhibited the ATPase activity at much lower concentrations than the sulphonate; in these experiments lower concentrations of metal ions which did not interfere with the assay had a positive effect. Both $FeSO_4$ and $CoSO_4$ largely removed the inhibition of ATPase activity, caused by 300 μM bathophenanthroline, if they were mixed with the chelator before the mitochondria, whereas only $FeSO_4$ reversed the inhibition if added four minutes after the mitochondria had been mixed with the inhibitor; these results are summarized in fig. 4. Other metal ions, in-

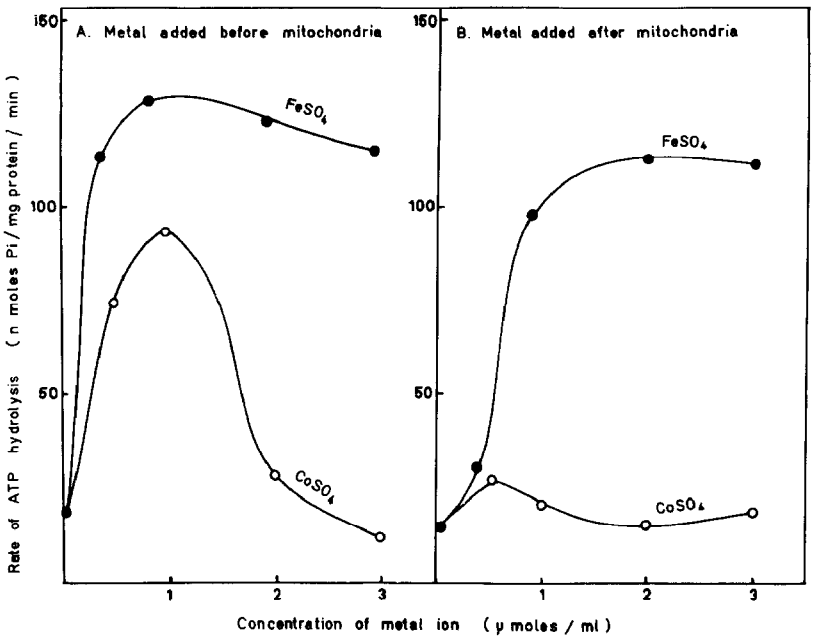

Fig. 4. The influence of divalent metal ions on the inhibition of the dinitrophenol induced ATPase casued by 0.3 μmoles/ml of bathophenanthroline. In graph B the ions were added four minutes after the mitochondria and the rate of ATP hydrolysis measured over the next four minutes.

cluding copper, zinc, nickel and manganese were tested and found to have no effect in either system.

The observation that ferrous or cobaltous ions prevent the inhibition if added before the mitochondria does suggest a connection between the ability of bathophenanthroline to chelate divalent metal ions and to inhibit oxidative phosphorylation. It does not however exclude the possibility that bathophenanthroline inhibited ATP synthesis by binding to an enzyme by a method other than chelation, and that ferrous and cobaltous ions prevented the inhibition by forming a chelated complex which was no longer able to bind with the enzyme. The reversal of inhibition by $FeSO_4$, when added to the reaction mixture after the mitochondria and the bathophenanthroline had been mixed, could be explained by suggesting that when ferrous ions chelated with bathophenanthroline the inhibitor was removed from the enzyme. However the failure of cobaltous ions to reverse the inhibition under these conditions suggests that such a mechanism is unlikely. The data is more consistent with the suggestion that bathophenanthroline caused an inhibition of oxidative phosphorylation by interacting with a non haem iron protein which is not a member of the electron transport chain. The observation of Butow and Racker [9] that orthophenanthroline imposed respiratory control in sub-mitochondrial particles and similar observations by Lee and Ernster [12] that oligomycin can also impose respiratory control in the same system strengthen the argument that these chelators interact in oxidative phosphorylation in a manner similar to oligomycin.

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