INHIBITION OF ENERGY TRANSFER AT THE PYRIDINE NUCLEOTIDE-FLAVIN SITE

Britton Chance, Gunnar Hollunger[1], and Bunji Hagihara[2]
Johnson Research Foundation
University of Pennsylvania
Philadelphia, Pennsylvania

Received May 14, 1962

Based upon studies of the Keilin and Hartree heart muscle preparation, the site of action of Amytal has been identified as lying between flavoprotein and cytochrome b (Estabrook, 1957). Nevertheless, studies of isolated mitochondria (Chance, 1956) or intact cells (Chance and Hess, 1959) suggest a site between pyridine nucleotide and flavoprotein. The similarity in the spectroscopic effects of Amytal and guanidine called our attention to the possibility that Amytal was combining an inhibition of energy transfer with an inhibition of electron transfer (Chance and Hollunger, 1961a). Such a hypothesis has the advantage of explaining both the spectroscopic response to Amytal inhibition in phosphorylating mitochondria, and the inhibition of energy-linked pyridine nucleotide reduction(Chance and Hollunger, 1960). However, the lack of evidence for a reversal of the Amytal effect by means of uncoupling agents (which was so clearly demonstrated in the case of guanidine) had for some time prevented an active consideration of this hypothesis (Chance and Hollunger, 1961a, Hollunger, 1955). A year ago, a slight reactivation of Amytal inhibited respiration was obtained (Chance and Hollunger, 1961a); more recently a higher degree of respiratory activation has been obtained

---

1. Present address: Department of Pharmacology, University of Umea, Umea, Sweden
2. Present address: Department of Biochemistry, University of Osaka Medical School, Osaka, Japan

This research has been supported in part by a grant from the National Science Foundation.

and specific effects of uncouplers on Amytal induced DPN[3] reduction are now reported.

This paper describes three types of experiments which demonstrate that Amytal combines an inhibition of electron transfer with an inhibition of energy transfer reactions in tightly-coupled mitochondria. Similar effects are observed with methylene glycol and progesterone. Both these substances have Amytal-like effects on non-phosphorylating preparations (Chance and Hollunger, 1961a, Yielding et. al. 1960). These data call attention to the need for a re-examination of the properties of a number of inhibitors to determine whether they, too, possess these two types of inhibitory effects.

Preparation and Methods

The preparations and experimental methods follow those discussed previously (Chance and Williams, 1955; Chance and Hollunger, 1961b). A detailed description of the pigeon heart mitochondrial preparation is given elsewhere (Chance and Hagihara, 1961). Details of the reaction media and other data are given in the figure legends.

Results

Partial Reversal of Amytal Inhibition by Dicumarol

Fig. 1 illustrates a considerable difference in the ADP and DIC reactivation of Amytal-blocked respiration in rat liver mitochondria. Two experiments are illustrated by the records (A and B). In these two records, oxygen concentration is measured polarographically. In record A, the aerobic suspension of rat liver mitochondria is supplemented with malate and glutamate and the respiratory rate is slow (State 4). 2.7 μM dicumarol causes an activation of respiration to 4.6 μM oxygen/sec. Addition of 325 μM Amytal causes a slowing of respiration to 3.1 μM O_2/sec. In experiment B, instead of dicumarol, 670 μM ADP is added and

3. The following abbreviations are also used: DPN: diphosphopyridine nucleotide; DIC: dicumarol; DIB: 4, hydroxy-3,5-diiodo benzoic acid butyl ester; DBP: 2,4 dibromophenol; DNP: 2,4 dinitrophenol.

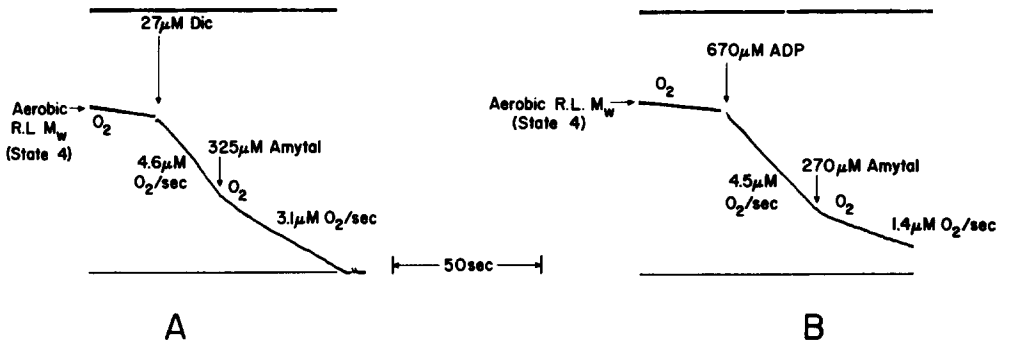

Fig. 1. A comparison of respiratory inhibition obtained on addition of low concentrations of Amytal to mitochondria treated with DIC (A) or ADP and phosphate (B). Rat liver mitochondria (1 μM cytochrome c) suspended in 0.25 M sucrose, 0.01 M KCl, 0.005 M Mg++, 0.005 M phosphate, 0.4 mM ethylene diamine tetraacetic acid, 0.01 M "tris" - HCl, pH 7.2. The mitochondria are supplemented with 4 mM malate and 4 mM glutamate as substrate, and are initially in State 4. Oxygen is recorded with the vibrating platinum micro-electrode. Respiratory rates are in μMoles O_2/liter second. 26°. (Expt. 244-6).

a respiratory rate of 4.5 μM oxygen/sec is obtained. Addition of 270 μM Amytal causes the respiration to slow down to 1.4 oxygen/sec as opposed to 3.1 μM oxygen/sec in experiment A. It would appear from this record that Amytal is able to cause more than twice as much inhibition of respiration in ADP-treated mitochondria as in dicumarol-treated mitochondria.

Reversal of DPN Reduction

The most characteristic response to Amytal inhibition of phosphorylating mitochondria is the increased reduction of pyridine nucleotide (2). It has now been found that even at high concentrations of Amytal, this increased reduction is reversed by uncoupling agents. The mitochondria are supplemented with malate and glutamate in order to reduce the DPN associated with these dehydrogenases. 2 mM malonate is added to prevent succinate-linked pyridine nucleotide reduction. Addition of uncoupling agents causes a high degree of oxidation of this pyridine nucleotide, as indicated by the "0 Amytal" trace of Fig. 2. If the experiment is repeated in the presence of 2 mM Amytal, it is seen that very little more DIB is required to cause half-maximal oxidation of the mitochondrial pyridine nucleotide than in the absence of Amytal.

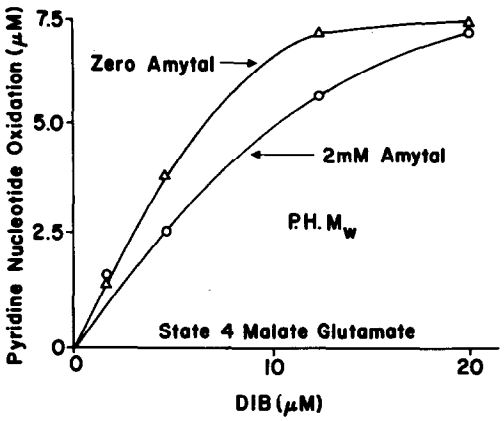

Fig. 2. Illustrating the effectiveness of an uncoupling agent in reversing the inhibition of DPNH oxidation by 2 mM Amytal. 2 mg/ml pigeon heart mitochondria, supplemented with 4 mM malate, 4 mM glutamate, 2 mM malonate, and suspended in 0.22 M mannitol, 0.05 M sucrose, 0.02 M "tris", pH 7.4, 26°. The trace at zero Amytal indicates the increment of pyridine nucleotide oxidation (measured fluorometrically) caused by successive additions of uncoupling agent (DIB). The curve labelled 2 mM Amytal shows the effect of the uncoupling agent in activating pyridine nucleotide oxidation in spite of the presence of this concentration of Amytal. (Expt. 466 A-3,4).

Similar experiments with the rat liver mitochondria using DIB or DBP as uncoupling agents also show oxidation of reduced pyridine nucleotide by uncoupling agents under conditions where ADP and phosphate are ineffective. However, the extent of oxidation in the presence of Amytal is about half that in the absence of it. A similar reactivation of the oxidation of reduced pyridine nucleotide can be obtained in mitochondria inhibited by methylene glycol or progesterone.

Reversal of Amytal Inhibition by Hexetidine

The substance, hexetidine, briefly described by Lardy and his co-workers (1958) has been found to reactivate Amytal-inhibited oxidation of glutamate plus succinate in pigeon heart mitochondria. The experiment illustrated by Fig. 3 is a polarographic recording of this phenomenon. Aerobic pigeon heart mitochondria containing 4 μM cytochrome c are supplemented with 0.4 mM glutamate, 2 mM succinate, and 4 mM phosphate. 1.9 mM Amytal has also been added. The State 4 respiratory rate of

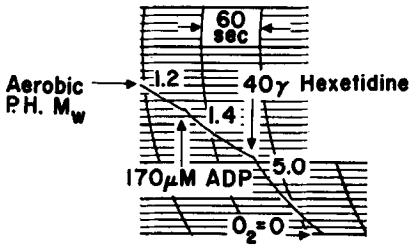

Fig. 3. The effect of hexetidine on the respiratory activity of an Amytal-inhibited pigeon heart mitochondria suspension. 4 μM cytochrome c, mannitol-sucrose-"tris" medium as described in Fig. 2. Mitochondria supplemented with 2 mM succinate, 0.4 mM glutamate, 4 mM phosphate, and inhibited with 1.9 mM Amytal. 26°. (Expt. 103-73).

1.2 μM O_2 per/sec is accelerated to 1.4 μM O_2 per/sec by the addition of 170 μM ADP. This small increase of respiration caused by adding ADP is indicative of a high degree of inhibition of electron transfer by Amytal (independent experiments have shown that the succinate as well as DPNH oxidation is Amytal-sensitive in pigeon heart mitochondria). Addition of 40λ hexetidine accelerates the respiration approximately fourfold, indicating a substantial reversal of the Amytal inhibition. The mechanism of action of hexetidine is not known in detail but it serves excellently the need for a reagent which demonstrates a sizeable reactivation of Amytal-inhibited respiration.

Discussion

Experiments outlined here describe appropriate conditions for observation of partial reactivation of Amytal-inhibited respiration by dicumarol and hexetidine, and nearly complete reversal of the increased reduction of pyridine nucleotide by an uncoupling agent (DIB). These results confirm that Amytal inhibits energy transfer (4). The fact that only partial reactivation of electron transfer is obtained in the presence of uncoupling agents indicates that Amytal inhibits electron transfer as well.

A reaction mechanism by which Amytal may block energy transfer at the DPNH-flavin site involves a specific combination of Amytal with

the high energy intermediate characteristic of this site.

$$X\sim I_d + \text{Amytal} \rightleftharpoons X\sim I_d \cdot \text{Amytal} \qquad (1)$$

Interaction of the compound with ADP and phosphate to release X, and thereby to activate respiration is inhibited, although activation by uncoupling agents is apparently permitted:

$$X\sim I_d \cdot \text{Amytal} \overset{\text{DNP}}{\rightleftharpoons} X + I + \text{Amytal} \qquad (2)$$

In addition to this effect upon energy transfer reactions, Amytal inhibits electron transfer in non-phosphorylating systems between flavin and cytochrome b (1). Thus, Amytal acts at a single site of energy transfer and a single site of electron transfer. Other data show that progesterone and methylene glycol share this property.

References

Chance, B., in "Enzymes: Units of Biological Structure and Function", edit. by Gaebler, O. H., 447 (Academic Press, New York, 1956).

Chance, B., and Hagihara, B., in "Proceedings of the Vth International Congress of Biochemistry", Moscow, 1961, (Pergamon Press, Oxford, in the press).

Chance, B., and Hess, B., J. Biol. Chem., 234, 2404 (1959).

Chance, B., and Hollunger, G., Fed. Proc., 20, 50 (1961a).

Chance, B., and Hollunger, G., J. Biol. Chem., 236, 1534 (1961b).

Chance, B., and Hollunger, G., Nature, 185, 666 (1960).

Chance, B., and Williams, G. R., J. Biol. Chem., 217, 383 (1955).

Estabrook, R. W., J. Biol. Chem., 227, 1093 (1957).

Hollunger, G., Acta Pharmacol. Toxicol., 11, Suppl. 1 (1955).

Lardy, H. A., Johnson, D., and McMurray, W. C., Arch. Biochem. Biophys., 78, 587 (1958).

Yielding, K. L., Tompkins, G. M., Munday, J. S., and Cowley, I. J., J. Biol. Chem., 235, 3114 (1960).