AN INHIBITION BY AMYTAL OF SUCCINATE OXIDATION IN TIGHTLY COUPLED MITOCHONDRIA.

Alison M. Pumphrey and Eric R. Redfearn

Department of Biochemistry, University of Liverpool, Liverpool 3, Great Britain.

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Amytal has been widely used to inhibit $NADH_2$ dehydrogenase, and is generally regarded as a specific tool for this purpose. During attempts to prepare intact mitochondria from abattoir material, it was noticed that succinate oxidation was inhibited by amytal in tightly coupled but not in aged or damaged preparations. This observation was studied further, using rat liver mitochondria, and preliminary findings are presented here.

Materials and Methods. Rat liver mitochondria were isolated in 0.25 M sucrose, after the method of Schneider and Hogeboom (1950). Except where otherwise stated, oxygen consumption was followed polarographically. Amytal (sodium 5-ethyl 5-isoamyl barbiturate) was supplied by Eli Lilly & Co., Basingstoke. Hexokinase (Type III) was obtained from Sigma Chemical Co.

Results. The effects of increasing concentrations of amytal on the oxygen uptake of a fresh liver mitochondrial preparation are shown in Figure 1. Oxidation of NAD-dependent substrates was completely inhibited by amytal (about 1 mM). Oxygen uptake in the presence of succinate was 30-35% inhibited by 1 mM amytal; further addition of amytal caused a progressive fall in the rate of oxygen uptake until, at 5 mM, 80% or more of the original activity was lost.

A similar but less intense inhibition could be demonstrated manometrically, using hexokinase (0.05 mg/mg mitochondrial protein) and glucose instead of ADP as the phosphate acceptor system. Succinate

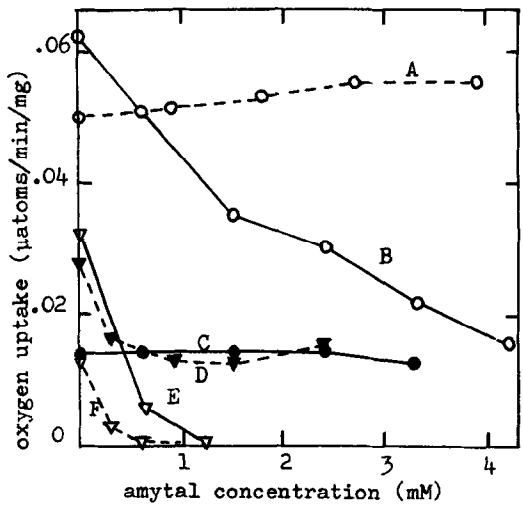

Fig. 1. The effect of amytal concentration on oxygen uptake by liver mitochondria in the presence of various substrates. The reaction mixture (4 ml) contained sucrose (0.12 M); phosphate pH 7.4 (13mM); KCl (26mM); $MgCl_2$ (13mM); mitochondria (3.6 mg protein/ml; —— fresh; --- frozen 2 hr. at -25°) Other additions: curves A,B,C, succinate (2mM); curve D, $NADH_2$ (0.6mM); curves E, F, glutamate (5mM); ADP was added in B and E. Oxygen uptake measured polarographically at 19.5°.

oxidation rates measured manometrically tended to be higher than when the polarographic method was used. (Differences of this sort have been noticed previously; see, for example, Bellamy and Bartley, 1960.) Since the increase in activity produced by manometric assay conditions was about the same at all levels of amytal tested, the percentage inhibition by amytal was lower than that found polarographically. 50-65% inhibition was reached at 6mM amytal, the highest concentration used in most manometric experiments. Though accurate measurement of initial rates in the manometers was not possible, there was some indication of a lag period during the first few minutes of the assay, more noticeable with the higher concentrations of amytal. The higher activities found manometrically were not reproduced when mitochondria were preincubated 5 minutes with gentle shaking in the usual assay medium (see Fig. 1), plus succinate and ADP (in excess, or with hexokinase and glucose), before assaying in the polarograph cell.

If the mitochondria were disrupted by freezing and thawing, succinate oxidation was no longer inhibited by amytal (Fig. 1). Oxidation of NAD-dependent substrates, on the other hand, was still completely blocked by amytal after freezing. (If the system was supplemented with NAD, or if added $NADH_2$ was used instead of an NAD-dependent substrate, some

activity was still apparent at 1 mM amytal. This residual activity was unaffected by raising the amytal concentration.)

The freshly prepared mitochondria showed a low oxygen uptake in the usual isotonic medium, containing sucrose, phosphate buffer, $MgCl_2$, in the presence of substrate and absence of ADP. The respiratory control ratio, i.e. the rate of oxygen uptake in presence of ADP divided by the rate in absence of ADP, was between 3 and 8 with succinate as substrate, in manometric as well as polarographic assays. High activities could be obtained in the absence of ADP under the following conditions:- (a) on addition of DNP (1.2×10^{-5}M), either to the usual reaction medium, or in the absence of phosphate to a Tris-HCl buffered medium; (b) on addition of EDTA (2.5 mM) and omission of $MgCl_2$ from a phosphate buffered medium; (c) on addition of $CaCl_2$ (0.38 mM) to a phosphate buffered medium; (d) on addition of deoxycholate (0.03%) to phosphate or Tris-HCl buffered media. Succinate oxidation under conditions (a) and (b) was inhibited by amytal; inhibition followed the same course as curve B in Figure 1. Succinate oxidation under conditions (c) and (d) was not significantly affected by amytal. Addition of $CaCl_2$ or deoxycholate to the polarograph cell restored the original activity to amytal-inhibited mitochondria, when succinate was used as substrate but not when NAD-dependent substrates were used.

A less detailed examination of some other barbiturates has shown that seconal and pentobarbital (5-allyl,5-(1-methyl butyl) barbiturate and and 5-ethyl,5-(1-methyl butyl) barbiturate respectively) inhibit succinate oxidation in a similar manner and at about the same concentrations as amytal. Barbitone (5-diethyl barbiturate) also inhibited succinate oxidation, but about twice the concentration was required to give the same degree of inhibition as amytal.

Discussion. Amytal inhibited the succinate stimulated oxygen uptake of tightly coupled liver mitochondria. There is close agreement between this finding and some results described previously by Hatefi, Jurtshuk

and Haavik (1961). These authors discovered a partial inhibition of oxygen uptake in the presence of succinate, by 3 mM amytal. They suggested it was due to the effect of amytal on the oxidation of malate and other NAD-dependent substrates produced *via* fumarate from succinate, rather than to an inhibition of succinic oxidase itself. The present experiments show that (in liver mitochondria at least) inhibition of oxygen uptake with succinate added as substrate differs from inhibition of oxidation of other substrates in that: (a) it needs more amytal; (b) it can be prevented or reversed by treatments which swell or disrupt the mitochondrion. These differences make it probable that the inhibitory effect of amytal on oxygen uptake in the presence of succinate is largely due to a block of the oxidation of succinate itself.

Susceptibility of succinate oxidation to amytal was retained in the presence of DNP (10^{-5}M) or EDTA (2.5 mM). These substances, although they allow oxidation to proceed without coupled phosphorylation, do not appear to cause swelling of the mitochondria; on the contrary, they are said to have a protective action in certain circumstances against swelling and ageing (Hunter and Ford, 1955; Weinbach, 1956; Tapley, 1956; Packer, 1961). Susceptibility to amytal was lost if the mitochondria were frozen, or if deoxycholate or Ca^{++} were added. These treatments are known to cause swelling and other destructive changes in mitochondrial structure, in addition to loss of respiratory control (Tapley, 1956; Siekevitz *et al.*, 1958; Chance, 1959;), though they do not necessarily destroy the whole of the phosphorylative machinery (Pritvera *et al.*, 1958). The loss of succinic oxidase susceptibility to amytal during the transition from more-or-less intact to damaged mitochondria is open to several interpretations:- (1) amytal (5 mM) might block part of the high energy bond transfer sequence, in a manner similar to guanidine (Hollunger, 1955) and oligomycin (Lardy, Johnson and McMurray, 1958; Estabrook, 1961), though possibly at a different site since amytal inhibition does not appear to respond to respond to DNP; (2) an electron-carrier essential to the

coupled system might be bypassed on damage of the mitochondrion, or else so altered that it lost its sensitivity to amytal; (3) amytal might affect the permeability of the intact mitochondrial membrane to succinate. In connection with possibility (1) above, the inhibitition of P_i-ATP exchange by amytal (Löw et al., 1958) is of interest.

Until more is known about the actions of amytal on tightly coupled mitochondria, it seems desirable to interpret with caution experiments in which amytal has been used.

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