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INHIBITION OF SUCCINATE OXIDATION BY BARBITURATES IN TIGHTLY COUPLED MITOCHONDRIA

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SUMMARY

1. Polarographic assays showed 1 mM amytal to inhibit oxidation of NAD-linked substrates almost completely, and succinate oxidation in presence of phosphate and ADP by about 30%, but had little effect on the low rates of oxidation found in absence of ADP. Amytal did not affect the stimulation of tetramethyl-*p*-phenylenediamine oxidation by ADP.

2. In manometric assays, amytal inhibited succinate oxidation to a less extent than in the polarographic method, but it stimulated oxygen uptake in absence of phosphate-acceptor system under these conditions. Phosphorylation was inhibited to a greater extent than oxidation.

3. Amytal inhibition of succinate oxidation was not prevented by 2,4-dinitrophenol or EDTA, although these substances release respiratory control by ADP. It could be prevented (or reversed) by disruption of the mitochondria and by addition of Ca^{2+}. Study of the light-scattering properties of the mitochondria indicated that prevention of amytal inhibition by Ca^{2+} was associated with a rapid swelling, greater than the reversible swelling shrinkage caused by substrates and ADP, but less extensive than the effects of other disruptive treatments.

4. These results are discussed in relation to other work which has suggested interference by barbiturates in the energy-transfer sequence of oxidative phosphorylation.

INTRODUCTION

Barbiturates have long been known to depress respiration. QUASTEL *et al.*[1,2] showed that various dialkyl barbiturates inhibited the oxidation of glucose, lactate and pyruvate by tissue slices, while the oxidation of succinate was slightly stimulated. The site of inhibition has since been more precisely located. CHANCE[3] deduced from spectrophotometric evidence that amytal (5-ethyl-5-isoamyl barbituric acid) blocks the reduction of flavoprotein by $NADH_2$ in mitochondria. ESTABROOK[4] also used

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; DIB, 4-hydroxy-3,5-diiodobenzoic acid butyl ester.

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spectrophotometric methods to show a different action in a non-phosphorylating $NADH_2$–cytochrome *c* reductase preparation from heart-muscle: amytal inhibited the reduction of cytochromes *b* and c_1 and the oxidation, not reduction, of the flavoprotein.

Amytal has generally been regarded as a specific agent for blocking electron transport in the region of the NAD-linked flavoprotein. In this capacity it has been widely employed, usually in concentrations in the range 1–3 mM. There have, however, been indications that barbiturates can influence mitochondrial reactions other than $NADH_2$-dehydrogenation. BRODY AND BAIN[5] compared the effects of a number of barbiturates and thiobarbiturates on oxygen and phosphate uptakes of brain mitochondria. While all the drugs tested inhibited both phosphorylation and oxidation of NAD-linked substrates, they all, at sub-optimal concentrations, tended to inhibit phosphorylation to a greater extent than oxidation. This preferential inhibition of phosphate uptake was more marked with the thio- than with the oxybarbiturates. The thiobarbiturates also inhibited the phosphorylation coupled to the oxidation of succinate, and BRODY AND BAIN suggested that they had an effect qualitatively similar to 2,4-dinitrophenol. SIEKEVITZ *et al.*[6] found that increasing the concentration of amytal over 0.5 mM gave a progressive inhibition of 2,4-dinitrophenol-stimulated ATPase of liver mitochondria, until at 4 mM roughly half the maximum activity had been lost. At the same time, the higher concentrations of amytal were reported to give rise to a small ATPase activity in the absence of 2,4-dinitrophenol. LÖW *et al.*[7] found that amytal inhibited ATP–P_i exchange of liver mitochondria, about 20% at 1 mM, 70% at 4 mM, and 80% at 8 mM. HATEFI *et al.*[8] showed that amytal (1.5 mM for ox-heart mitochondria, 3 mM for rat-liver mitochondria) abolished virtually all the stimulation by ADP of oxygen uptake in the presence of succinate. The observations of these last three groups were plausibly interpreted as consequences of the action of amytal on the NAD-linked flavoprotein.

A different type of action was postulated by CHANCE AND HOLLUNGER[9]. They stated that inhibition of $NADH_2$ oxidation in tightly coupled mitochondria by low concentrations of amytal was slightly relieved by uncoupling agents, and they suggested that amytal interacted with a "site-specific intermediate such as $X \sim I_d$". An inhibition of succinate oxidation by 1.5 mM amytal was also mentioned, and it was therefore suggested that amytal affected two phosphorylation sites. In a communication which appeared while the present paper was in preparation CHANCE, HOLLUNGER AND HAGIHARA[10] gave a further account of the effect of amytal on $NADH_2$ oxidation in tightly coupled mitochondria. They described in particular its effects on NAD, flavoprotein and cytochrome *b* steady-state reduction levels, and their reversal by the uncoupling agent DIB: these results were in accord with the earlier observed differences in site of action of amytal in phosphorylating and non-phosphorylating preparations[3,4]. The partial inhibition by 1.9 mM amytal of succinate oxidation in glutamate-treated pigeon-heart mitochondria in the presence of ADP was again also recorded, and was shown to be reversed by the uncoupling agent hexetidine.

During attempts to isolate tightly coupled mitochondria from abattoir material in this laboratory, the inhibition of succinate oxidation described by HATEFI *et al.*[8] and mentioned by CHANCE AND HOLLUNGER[9] was essentially confirmed. Some previously unreported features of the inhibition were noticed, however. These proved

more difficult to explain in terms of the known site of action of amytal than were the results of HATEFI *et al.*[8]. In view of the frequent use of amytal in experiments on mitochondrial systems, the problem appeared to merit more detailed study: before using amytal to inhibit selectively the NAD-flavoprotein section, it was of practical importance to determine its effect on other portions of the coupled electron-transfer chain under a variety of experimental conditions. Studies of the action of amytal and other barbiturates on oxidase activities of rat-liver mitochondria were therefore carried out.

MATERIALS AND METHODS

Mitochondria were isolated from rat liver by differential centrifugation in 0.25 M sucrose, after the method of SCHNEIDER AND HOGEBOOM[11].

Oxygen uptake was measured at room temperature, polarographically and manometrically. Polarographic measurements were made with platinum (surface area 0.16 mm^2) and silver electrodes in an open cell rotating at 60 rev./min. The silver electrode was separated from the reaction medium by a KCl–agar gel bridge. A potential of —0.6 V was applied to the platinum electrode, and the output from the electrodes was dropped across a 500 kΩ variable resistance used as a sensitivity control and then connected to a D.C. amplifier (W. G. Pye and Co. Ltd., Nanoammeter, Cat.Nos. 11342, 11352). The output of the amplifier was connected to a 1 mA, 1500 Ω recorder (Record Electrical Co. Ltd., Altrincham, Cheshire). Oxygen concentrations were calculated from figures given for Ringer's solution[12]. In calculating oxygen uptake rates, correction was made for diffusion of atmospheric oxygen into the reaction medium. The rate of diffusion was determined empirically by measuring increase of oxygen in a dilute KCl solution, made anaerobic by boiling and cooling under nitrogen, and incubated in the polarograph cell under the same conditions of temperature, rotation, etc., as were used for oxidase assays. The rate of diffusion (proportional to the difference between the actual and the saturating concentrations of oxygen in solution) was 0.022 μatom/ml/min at 18° when the solution was completely anaerobic. Manometric measurements were made in the conventional Warburg apparatus, with mitochondria in the side-arm during the 5-min equilibration period. Uptake of inorganic phosphate during manometric assays was determined after the method of MARTIN AND DOTY[13,14].

Sodium amytal (sodium 5-ethyl-5-isoamyl barbiturate) and sodium seconal (sodium 5-allyl-5[1-methylbutyl] barbiturate) were kindly supplied by Eli Lilly and Co. Ltd., Basingstoke. Solutions were freshly prepared at the start of each experiment. Since the mono-sodium salts are strongly alkaline, their addition to the reaction medium caused a rise in pH. The initial pH of reaction medium with mitochondria was about 7.2, and pH changes were checked with a pH meter. Where necessary to prevent the pH rising above 7.4, sufficient 0.15 N HCl was added to neutralize each addition of barbiturates. Contrary to an earlier report[15] seconal was found to be effective in rather lower concentrations than amytal (compare Fig. 4 with Figs. 1 and 3). This somewhat reduced complications due to pH changes. Therefore, since seconal appeared to give, qualitatively, the same results as amytal, it has been used in some of the experiments reported here.

RESULTS

Polarographic measurements of amytal inhibition

When successive additions of amytal were made to tightly coupled liver mitochondria, the rate of oxygen uptake in the presence of succinate fell progressively after oxidation of other substrates had been entirely suppressed. At 1 mM amytal, NAD-dependent substrate oxidation was negligible while oxygen uptake in the presence of succinate, phosphate and ADP was 20–30 % inhibited. Further increase in amytal concentration decreased oxygen uptake until, between 4 and 5 mM, about 80 % of the original activity was lost. The low rate of oxygen uptake which occurred in the presence of succinate and absence of ADP was not much affected by amytal. One experiment showing these results is given in Fig. 1.

Manometric demonstration of inhibition of succinate oxidation by barbiturates

One inference of the polarographic measurements was that amytal was genuinely blocking the oxidation of succinate to fumarate, and not merely (*cf.* ref. 8) the further oxidation of fumarate *via* malate. The use of amytal as a specific inhibitor of the $NADH_2$ dehydrogenase flavoprotein was, however, too well established for this inference to be immediately accepted. It seemed necessary to confirm the inhibition by other assay methods. Manometric studies were, therefore, carried out and the results of one such experiment are shown in Fig. 2.

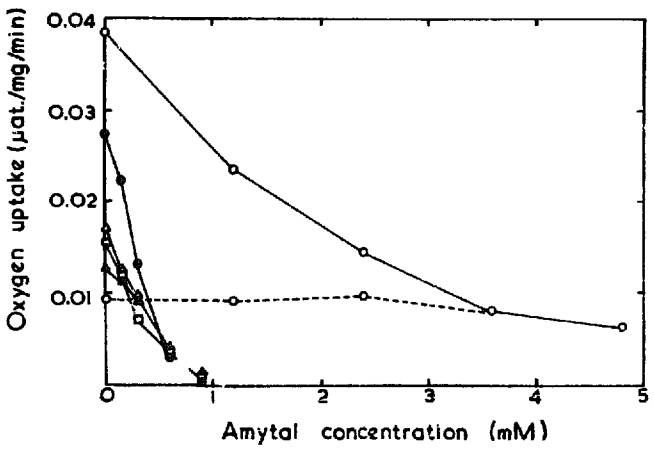

Fig. 1. Effect of amytal concentration on oxygen uptake of rat-liver mitochondria incubated with various substrates. The reaction mixture (total volume 4 ml) contained: sucrose, 56 mM; Na_2HPO_4–KH_2PO_4 buffer (pH 7.4), 50 mM; $MgCl_2$, 16 mM; mitochondrial protein, 4.7 mg protein/ml Substrates added were: O—O, succinate with ADP (0.75 mM); O---O, succinate without ADP; △—△, glutamate; □—□, β-hydroxybutyrate; ●—●, pyruvate plus malate (all approx. 2 mM); ▲—▲, glutamate plus β-hydroxybutyrate plus pyruvate plus malate (each approx. 1 mM). Oxygen uptake measured polarographically at 16.5°.

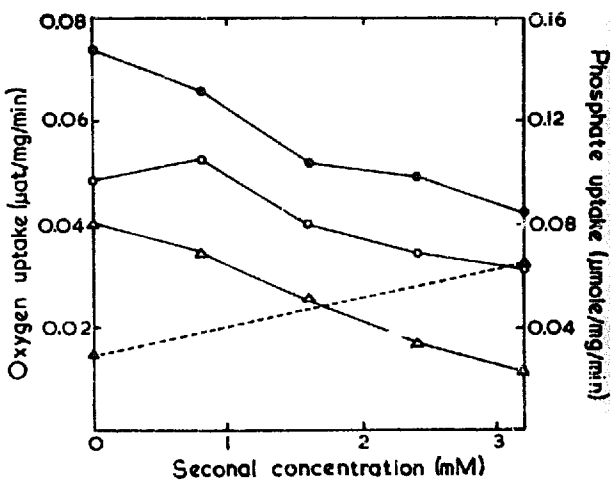

Fig. 2. Effect of seconal concentration on succinate oxidation and coupled phosphorylation, measured manometrically. The reaction mixture (total volume, 1 ml) contained: sucrose, 47–70 mM; Na_2HPO_4–KH_2PO_4 buffer (pH 7.4), 44 mM; $MgCl_2$, 13 mM; rat-liver mitochondria, 5.9 mg protein/ml; succinate, 15 mM; ADP, 0.47 mM; glucose, 37 mM, and hexokinase (Sigma Type III), 0.65 mg/ml were also added, except where indicated. ●—●, maximum rate of oxygen uptake; O—O, mean rate of oxygen uptake; △—△, mean rate of P_i uptake; ▲---▲, rate of oxygen uptake when glucose and hexokinase were omitted. Reaction carried out at 19.7°.

In most manometric assays, there appeared to be a slight lag during the first 4 or 5 min after tipping, particularly at the higher concentrations of the barbiturates. A lag was not noticeable in the polarographic assays. Comparison of oxygen uptake rates in the absence of inhibitor obtained by the two methods showed that, although there was a certain amount of scatter, the average value of succinate oxidase measured manometrically was higher than that measured polarographically at the same temperature (compare the maximum activity in Fig. 2 with Fig. 3). It was not

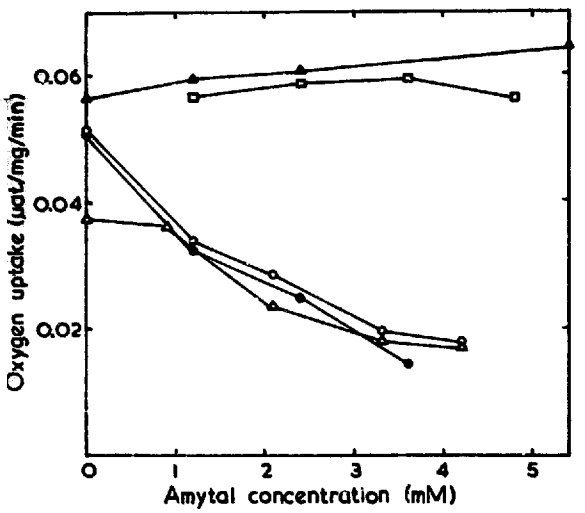

Fig. 3. Effect of various uncoupling treatments on the susceptibility of succinate oxidation to amytal. The reaction mixture (total volume 4 ml) contained: sucrose, 112 mM; Na_2HPO_4–NaH_2PO_4 buffer (pH 7.4), 13 mM; KCl, 26 mM; $MgCl_2$, 12 mM; rat-liver mitochondria, 2.2 mg protein/ml; succinate, 2 mM. Other additions or conditions were: ▲—▲, $CaCl_2$ (0.2 mM, KCl substituted for $MgCl_2$ in this assay); □—□, mitochondria frozen overnight at −25°; O—O, ADP (0.6 mM); ●—●, 2,4-dinitrophenol (12 µM); Δ—Δ, EDTA (3.8 mM, KCl substituted for $MgCl_2$ in this assay). Oxygen uptake measured polarographically at 20°.

possible to measure accurately the initial rate in manometric assays but it seems likely that this was equal to the rate in the short-term polarographic assay, and that somewhat higher rates developed after a few minutes of shaking the mitochondria in the reaction medium. No definite reason is apparent for the occurrence of a lag in the absence of inhibitor, but the more pronounced lag found in the presence of the higher concentrations of barbiturates can be explained in terms of a delayed uncoupling effect (see below). Although none of the reactants (succinate, phosphate, glucose) was added in limiting amounts, there was also a tendency for the rate of oxygen uptake to begin to fall off after about 20–30 min. For these reasons the maximum rate of uptake (measured over the 5–15 min period after tipping) is given in Fig. 2 to provide a better comparison with the polarographic data shown in Fig. 1; and the mean rate of oxygen uptake over the whole period of assay is given for comparison with the inorganic phosphate uptake. (The falling-off was unfortunately unusually marked in the control (no seconal) flask in Fig. 2: hence the atypically low mean oxygen and phosphate uptakes.)

A substantial inhibition of oxygen uptake was observed in manometric assays, but it was not as great as that routinely obtained in the polarographic method. It was found, however, that amytal or seconal stimulated the low rate of succinate oxidation observed when hexokinase and glucose were omitted from the manometer flasks (Fig. 2). This effect was not noticeable in polarographic assays, possibly because prolonged shaking with the barbiturate was needed to produce the decline in respiratory control. If this partial uncoupling is taken into account, it can be seen (Fig. 2) that high concentrations of the barbiturate abolished stimulation of oxygen uptake by the phosphate-acceptor system in manometric as well as polarographic assays.

The inhibition of phosphate uptake was rather greater than the inhibition of (manometric) oxygen uptake: thus in Fig. 2, the maximum rate of oxygen uptake was inhibited 43%, the mean oxygen uptake 36%, and the mean phosphate uptake 72% at 3.2 mM seconal. Since this concentration of seconal would have suppressed all the stimulation of succinate oxidation by ADP in polarographic assays, phosphate uptake by the manometric method might have been expected to approach zero. However, seconal and amytal do not prevent the stimulation of ascorbate–TMPD oxidation by ADP in polarographic assays (see below). It is, therefore, possible that in manometric experiments phosphorylation at the cytochrome oxidase level is still able to proceed, while phosphorylation at the cytochrome *b* level is inhibited and electron-transfer in this region is partially uncoupled. The residual phosphate uptake found in manometric experiments at high levels of barbiturate could thus be due largely to the cytochrome oxidase site.

Results similar to those in Fig. 2 were obtained with amytal. For example, in an experiment performed under similar conditions to Fig. 2 (except that both phosphate buffer and mitochondrial protein were added in about half the concentration) maximum oxygen uptake was inhibited 56%, mean oxygen uptake 53%, mean phosphate uptake 78%, and oxygen uptake in the absence of hexokinase and glucose was stimulated by 75%, by 6.0 mM amytal. The control (no amytal) P/O and respiratory-control ratios in this experiment were 1.9 and 3.3 respectively.

These results afford a partial explanation of why amytal inhibition of succinate oxidation has previously escaped much attention. The inhibition, which in any case only occurs in intact tightly coupled mitochondria (see Fig. 3) at higher concentrations than are needed to block $NADH_2$ oxidation (see Fig. 1), is more noticeable in the short-term polarographic assay than in the conventional manometric assay.

Uncoupling treatments affecting sensitivity to amytal

The requirement of the liver mitochondria for a high-energy phosphate-acceptor system for maximum succinate oxidase activity can be removed by various "uncoupling" treatments. Thus 2,4-dinitrophenol is a widely used uncoupling agent which gives high activities in the absence of added phosphate as well as of phosphate acceptor[16,17] and stimulates ATPase activity in fresh mitochondria[18]; ageing or treatment with detergents such as deoxycholate also stimulates ATPase[6]; Ca^{2+} or EDTA remove respiratory control[3]. Some conditions which release respiratory control by ADP were found to be without effect on the susceptibility of succinate oxidation to amytal: these included addition of 2,4-dinitrophenol (approx. $1 \cdot 10^{-5}$ M) and omission of Mg^{2+} with the addition of EDTA (approx. 4 mM) to the reaction medium. Other uncoupling treatments were able to prevent amytal inhibition of succinate oxidation: these included (a) freezing and thawing the mitochondrial suspension, (b) suspension of mitochondria in water instead of isotonic sucrose, (c) addition of deoxycholate (about 0.04% final concentration) to the reaction medium, (d) addition of $CaCl_2$ (about 0.6 mM) to the reaction medium. These latter treatments did not prevent the inhibition of NAD-dependent oxidases by amytal. Some examples of the effect of amytal on succinate oxidation under various uncoupling conditions are shown in Fig. 3.

Possibly because of variations in the rate of freezing and length of time kept frozen, the activity after freezing and thawing tended to vary from one preparation

to another: it was sometimes a little less than the maximum (plus ADP) activity of the fresh preparation, sometimes considerably more. The activation of succinate oxidation in the absence of ADP by deoxycholate could also be rather variable. It was affected by the amount of mitochondrial protein as well as by the concentration of deoxycholate in the medium. While low deoxycholate concentrations failed to give complete uncoupling, too high a deoxycholate/protein ratio destroyed electron-transfer as well as phosphorylation activity. Deoxycholate was effective in reversing, as well as preventing, the amytal inhibition of succinate oxidation. The activation of succinate oxidation by 2,4-dinitrophenol and its inhibition by amytal was, as expected, unaffected by the substitution of Tris–HCl buffer for phosphate in the reaction medium. Unlike 2,4-dinitrophenol, Ca^{2+} only released respiratory control in the presence of phosphate. Suboptimal concentrations of $CaCl_2$ gave only a temporary stimulation of oxygen uptake (as reported by CHANCE[19]) and the concentration of Ca^{2+} needed for irreversible release of respiratory control appeared to be proportional to mitochondrial protein concentration (very approximately 0.15 μmole Ca^{2+}/mg protein in the sucrose–phosphate–$MgCl_2$ (13 mM) medium; the effect of altering Mg^{2+} concentration, etc., was not measured). Reactivation of the amytal-inhibited succinate oxidation by $CaCl_2$ could be obtained, but only with Ca^{2+} concentrations about 5–10 times higher than those required before addition of amytal, and only after a lag of 0.5–2 min. Phosphate in the reaction medium was also found to be necessary for stimulation of succinate oxidation (in absence of ADP) by omitting Mg^{2+} and adding EDTA.

Failure of di-alkyl barbiturates to inhibit oxidation of tetramethyl-p-phenylenediamine

Respiratory control of the cytochrome oxidase portion of the electron-transfer chain has recently been demonstrated[20] using ascorbate in substrate quantities with TMPD in trace amounts. This system was studied to see if a response to amytal could be detected.

Ascorbate alone gave very low rates of oxygen uptake, unaffected by ADP. When both ascorbate and TMPD were added, a relatively high rate of oxygen uptake was found in the absence of ADP. This was several-fold higher than succinate oxidation rates obtained under the same conditions and roughly equal to succinate oxidation in presence of ADP; it was little affected by omission of phosphate from the reaction medium, with substitution of Tris–HCl buffer. ADP (with phosphate) stimulated the ascorbate–TMPD oxidation by 30–80 %. ADP/O ratios were 0.8–1.0. 2,4-Dinitrophenol gave an equal or slightly greater stimulation than ADP in presence or absence of phosphate, and $CaCl_2$ gave a similar stimulation in the presence of phosphate. Antimycin A had no measurable effect on ascorbate–TMPD oxidation.

Dialkyl barbiturates had little effect on ascorbate–TMPD oxidation, in concentrations that strongly inhibited succinate oxidation. Fig. 4 shows the results of an experiment with seconal; similar results were obtained with amytal.

Effect of uncoupling treatments on the light-scattering properties of mitochondria

Freezing and thawing, suspension in water instead of isotonic sucrose, and mixture with detergents such as deoxycholate, are all known to break up mitochondrial structure. Ca^{2+} ions induce swelling of mitochondria[21]. 2,4-Dinitrophenol and EDTA, on the other hand, have been reported to have a protective action against swelling under

certain circumstances[21,22]. In order to determine whether there was a correlation between the structural integrity of the mitochondria and the susceptibility of succinate oxidation to amytal, measurements of absorbancy at 520 mμ were carried out. The reaction media used for these experiments were similar to those used for assay of oxygen uptake, but the concentrations of mitochondrial protein were necessarily much lower, 0.1–0.05 of those suitable for polarographic or manometric measurements.

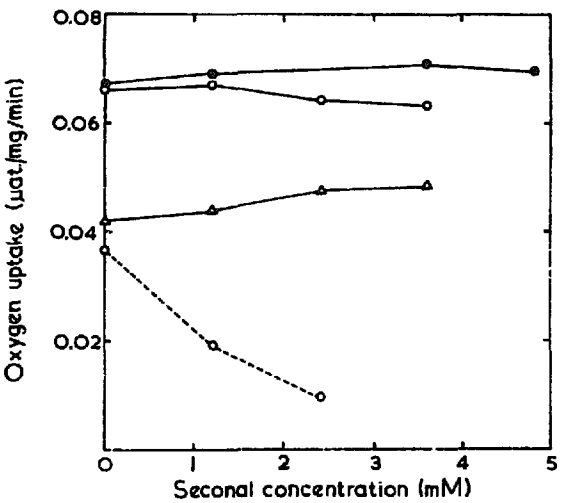

Fig. 4. Failure of seconal to inhibit ascorbate–TMPD oxidation. The reaction mixture (total volume 4 ml) contained: sucrose, 86 mM; phosphate buffer, 42 mM; $MgCl_2$, 13 mM; rat-liver mitochondria, 1.65 mg protein/ml for ascorbate–TMPD assay and 2.5 mg/ml for succinate assay. Substrates: —, ascorbate (3.8 mM) and TMPD (0.3 mM); – – – –, succinate (2 mM). Other additions were: O—O, O---O, ADP (0.25 mM); ●—●, 2,4-dinitrophenol (15 μM); △—△, no addition. Oxygen uptake measured polarographically at 17°.

Freezing and thawing, suspension in water, and addition of deoxycholate all gave a marked fall in the turbidity of the rat-liver mitochondrial suspension, resulting in a drop of 75 % or more in the absorbancy at 520 mμ. 2,4-Dinitrophenol ($2 \cdot 10^{-5}$ M) had no effect, but under appropriate conditions $CaCl_2$ rapidly reduced the turbidity of the mitochondrial suspension by 15–25 %: this was much less than other fragmenting treatments, but several-fold greater than the reversible (*cf.* Phase 1 swelling of PACKER[22]) swelling and shrinkage observed on addition of substrates and ADP. The initial rapid fall in the absorbancy at 520 mμ caused by Ca^{2+} lasted 1–4 min, and was followed by a slower decline which appeared to continue indefinitely. Since polarographic assays took only a few minutes to perform, it is presumed that only the first

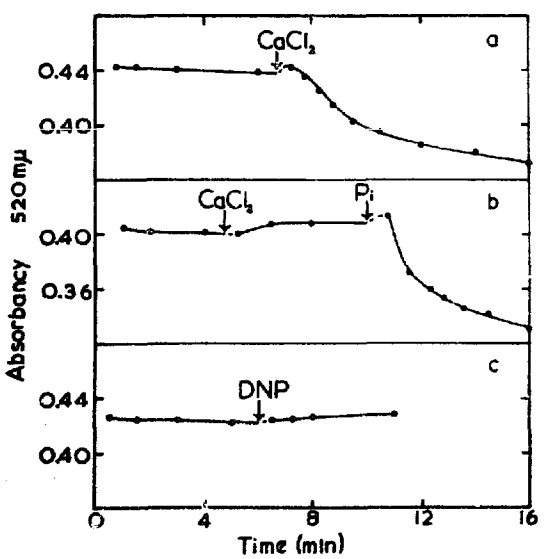

Fig. 5. Effect of $CaCl_2$ and 2,4-dinitrophenol on the 520-mμ absorption reading of rat-liver-mitochondrial suspension. The reaction medium (total volume 3 ml) in (a) and (c) contained: sucrose, 165 mM; Na_2HPO_4–KH_2PO_4 buffer (pH 7.4), 16 mM; $MgCl_2$, 15 mM; rat-liver mitochondria, 0.17 mg protein/ml, was added at zero time. In (b) Tris–HCl buffer (pH 7.4), 23 mM, was substituted for phosphate. Other additions (where indicated): $CaCl_2$, 333 μM; K_2HPO_4–KH_2PO_4 buffer (pH 7.4), 6.7 mM; 2,4-dinitrophenol, 20 μM. Correction has been made for dilution by the additions, which did not in any case exceed 1% of the total volume. Readings of 520-mμ absorption were made in a Unicam SP 600 spectrophotometer against a water blank, at approx. 20°.

rapid changes in turbidity are relevant to observed changes in enzyme activity. The minimum Ca^{2+} concentrations required for swelling were lower than those preventing amytal inhibition of succinate oxidation. This was perhaps to have been expected from the different protein concentrations used in the two measurements, and the dependence of the uncoupling concentration of Ca^{2+} on the concentration of mitochondrial protein in polarographic assays. The loss of turbidity caused by Ca^{2+} required phosphate in the reaction medium, as did prevention of amytal inhibition of succinate oxidation.

The effect of $CaCl_2$ on the absorbancy at 520 mμ and the lack of effect of 2,4-dinitrophenol, are shown in Fig. 5. Unless $CaCl_2$ had previously been added in amounts just insufficient to cause a decrease in turbidity, a lag was generally observed between stirring in excess of $CaCl_2$ and the resultant fall in absorbancy (Fig. 5). An increase of 2–4% in 520-mμ reading (more distinct than the slight rise seen in Fig. 5) was frequently associated with the lag period. This increase was presumably due to an effect of Ca^{2+} on the mitochondria, because no precipitate or increase in absorbancy was detected when $CaCl_2$ was added to the reaction medium alone. Both the duration of the lag and the appearance of an increase prior to the decrease in absorption were rather variable, however, depending on the concentration of Ca^{2+} and the presence or absence of ADP, substrates, inhibitors, etc., in the reaction mixture.

The relationship between the structural state of mitochondria and their light scattering properties is not precisely understood. Consequently, these results are not amenable to a very exact interpretation. It nevertheless seems probable that the irreversible release of respiratory control and prevention of amytal inhibition by excess Ca^{2+} in the presence of phosphate are both associated with a change in mitochondrial structure.

DISCUSSION

The oxygen uptake of tightly coupled liver mitochondria incubated with succinate, phosphate and ADP can be substantially inhibited by amytal or seconal. Two features make it impossible to attribute the whole of this inhibition to the effect of the barbiturates on the $NADH_2$-dehydrogenase flavoprotein: (a) Inhibition of $NADH_2$ and NAD-dependent substrate oxidations is complete at 1 mM amytal; succinate oxidation is 30% (or less) inhibited at this point, but increasing the amytal concentration to 4 mM suppresses 75–80% of the original activity (*i.e.* virtually all the portion stimulated by ADP or 2,4-dinitrophenol). (b) The amytal inhibition of succinate oxidation can be relieved by various treatments, freezing and thawing the mitochondria, suspending them in water instead of an isotonic medium, adding $CaCl_2$ or deoxycholate. These treatments did not prevent the inhibition of oxidation of NAD-linked substrates.

If inhibition of succinate oxidation is not due to a block of electron-transfer in the $NADH_2$-dehydrogenase region, then other modes of action must be considered. Amytal inhibition of tightly coupled succinate oxidation can be relieved by some uncoupling treatments but not by others. One possible explanation of this fact is that high concentrations of amytal may block phosphorylation coupled to succinate oxidation. The phosphorylation step affected would be at the cytochrome *b*, not the cytochrome oxidase (EC 1.9.3.1) level, since ascorbate–TMPD oxidation is not appreciably inhibited by amytal. There are many precedents for compounds producing

this type of inhibition of coupled electron-transfer, *e.g.* oligomycin[23], guanidine[24], biguanides[25,26], atractylate[27,28]. Inhibition of succinate oxidation by amytal, unlike inhibitions of electron transfer by these other agents, is not prevented by 2,4-dinitrophenol. This does not invalidate the above hypothesis, since amytal could block the energy-transfer sequence at or before the point attacked by 2,4-dinitrophenol, but it makes more careful consideration necessary.

A common feature of the treatments here found to prevent or reverse amytal inhibition of succinate oxidation is that they all show signs of damaging mitochondrial structure. The physical treatments, freezing and suspension in a hypotonic medium, can be presumed to exert their effects by purely mechanical means, and deoxycholate treatment can almost certainly be placed in the same category. Studies of the structural integrity of mitochondria, as measured by their 520-mμ light-scattering, indicate that the rapid swelling promoted by Ca^{2+} is less extensive than the damage caused by the other fragmenting procedures. This, together with its requirement for phosphate, distinguishes the Ca^{2+}-induced uncoupling from other treatments preventing amytal inhibition and prompts further speculation. It seems unlikely that Ca^{2+} prevents amytal inhibition of electron-transfer simply by directly catalysing the breakdown of a high-energy intermediate, formed in the energy-transfer sequence before the postulated amytal block. Phosphate is required for a rapid uncoupling of respiration by Ca^{2+}, and hence if Ca^{2+} acts on a high-energy intermediate, it presumably needs to be a phosphorylated one. This does not fit in with other results which place the hypothetical amytal block of energy-transfer at or before the entry of phosphate; 2,4-dinitrophenol uncouples electron-transfer in the absence of added phosphate, but it does not prevent amytal inhibition. A more convincing interpretation of the action of Ca^{2+} is that uncoupling is produced indirectly by alterations accompanying (or caused by) the swelling. The requirement for phosphate might on this basis mean that a phosphorylated intermediate in the energy-transfer sequence plays a part in the rapid swelling induced by Ca^{2+}, perhaps as a swelling agent, or as an acceptable source of energy for transport or binding of Ca^{2+} (*cf.* ref. 29). The existence of a lag between the addition of Ca^{2+} and P_i and the onset of swelling is of interest in connection with the biphasic effects of Ca^{2+} on oxygen uptake rates described by CHANCE[19] (*cf.* also ref. 29). A strict comparison of the sequence of events in these two measurements could be misleading, however, because of the difference in protein concentrations needed for absorbancy at 520 mμ and oxygen-uptake studies.

Of the uncoupling treatments studied here, those preventing amytal inhibition differ from the rest in that they are accompanied by alterations in the structural state of the mitochondria: in an enquiry into the site of action of amytal on succinate oxidation, this observation complicates the issue. It is still probable that the uncoupling treatments which release amytal inhibition do so by interrupting the energy-transfer sequence at a site closer to the electron-transfer chain than 2,4-dinitrophenol. But it is also conceivable that the uncoupling action is coincidental. Amytal could inhibit mitochondrial components other than those involved in energy-transfer. Alterations other than uncoupling could result from a breakdown of mitochondrial structure and be responsible for loss of susceptibility of succinate oxidation to amytal. However, besides the result obtained here with oxidase activities, the other effects of high concentrations of amytal, on 2,4-dinitrophenol-stimulated ATPase and ATP–P_i

exchange[6,7] must be considered. Although there is no *a priori* reason why the progressive inhibition of coupled succinate oxidation, of ATPase and of ATP–P_i exchange by 1–4 mM amytal should be due to a single action at a locus common to all three activities, the balance of probability seems in favour of this idea. The locus indicated would be a component in the energy-transfer sequence coupled to the cytochrome *b* region of the electron-transfer chain, with which both 2,4-dinitrophenol and P_i can react. No data on the effect of hexetidine on mitochondrial swelling are at present available. If this uncoupling agent proves, like 2,4-dinitrophenol, to have no swelling action, then its release of succinate oxidation from amytal inhibition[10] is of obvious interest. Hexetidine has been shown to lower the P/O of succinate oxidase and to give rise to ATPase activity[23]. This ATPase was lower than that produced by 2,4-dinitrophenol, and hexetidine added to the 2,4-dinitrophenol-stimulated ATPase assay decreased activity. LARDY *et al.* therefore suggested that hexetidine might have a stronger affinity for the same site as 2,4-dinitrophenol but be less effective in liberating P_i. Although 2,4-dinitrophenol differs from amytal in attacking (apparently unselectively) all three sites of oxidative phosphorylation, a similar argument could, of course, apply to interference by hexetidine with the amytal inhibition of coupled succinate oxidation.

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