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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION *IN VITRO* BY THE NEUROTOXIC FRAGRANCE COMPOUND ACETYL ETHYL TETRAMETHYL TETRALIN AND ITS PUTATIVE METABOLITE

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Abstract—When acetyl ethyl tetramethyl tetralin (AETT), at 10–50 μg/ml, was added to rat liver mitochondria respiring with succinate or with glutamate plus malate as substrate, the rate of mitochondrial respiration increased significantly after an initial lag period of 2–3 min. AETT also stimulated respiration in the presence of oligomycin, and at higher concentrations of AETT a phase of strongly inhibited respiration followed an initial stimulatory phase. These observations suggest that AETT uncouples oxidative phosphorylation. A diketo derivative (DK) of tetramethyl tetralin also appears to be an uncoupling agent, according to those criteria, and both compounds uncoupled mitochondria from brain as well as liver. DK and many other uncoupling agents, such as hexachlorophene (HCP), produced an immediate burst of respiration, whereas the brief lag after addition of AETT was similar to that seen after addition of triethyltin (TET).

When acetyl ethyl tetramethyl tetralin (AETT) (Fig. 1), a fragrant compound previously used in cosmetics, is fed to experimental animals, the animals become weak and ataxic, their organs and their exposed skin take on a blue coloration, and pathological changes occur in their central and peripheral nervous systems [1]. The pathological changes include ceroid inclusions in neurons and bubbling and vacuolation of the myelin sheath. The latter demyelinative changes resemble the disruption of myelin membranes observed previously with triethyltin (TET) [2], hexachlorophene (HCP) [3] and several halogenated salicylanilides [4, 5], compounds or classes of compounds to which the myelin sheath is, *in vivo*, strikingly vulnerable [2, 3] and which also uncouple mitochondrial oxidative phosphorylation [6–10]. We, therefore, have investigated changes in the respiration rates of mitochondria *in vitro* after addition of AETT or its putative metabolite, the diketo compound (DK; diacetyl tetramethyl tetralin) [11] (Fig. 1).

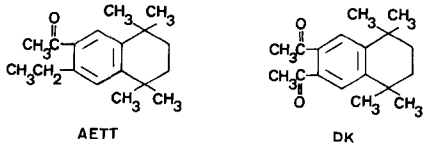

Fig. 1. Chemical formulas of AETT and DK [1, 11].

METHODS

Mitochondria were prepared from the livers of adult female Sprague–Dawley rats [12], and a crude fraction of brain mitochondria (no density gradient step) was prepared from the brains of 7-week-old female New Zealand rabbits [13]. The liver mitochondria were used in most of the experiments reported here because of their greater degree of respiratory control after isolation than is observed with brain mitochondria. Oligomycin, adenosine-5′-diphosphate (ADP), Trizma base, HCP and substrates were purchased from the Sigma Chemical Co. (St. Louis, MO); the substrates were titrated to pH 7.4 before use. TET sulfate was a gift from Dr. Kinuko Suzuki. AETT and DK were obtained from Dr. Peter Spencer, who received the AETT from Givaudan (Clifton, NJ) and the DK from Avon Products (New York, NY). All other reagents were analytical grade. Respiration measurements [14] were performed in the Gilson oxygen polarograph, and protein concentrations were determined by the method of Lowry *et al.* [15].

RESULTS

After addition of AETT to rat liver mitochondria respiring slowly (65 ngA O/min) with substrate and no phosphate acceptor, an initial lag period of 2–3 min occurred, followed by the burst of rapid respiration (178 ngA O/min) indicative of loss of respiratory control (Fig. 2, left panel, trace A). In the presence of oligomycin, mitochondria lose their capacity to respond to ADP with an increased rate of respiration but retain their ability to respire rapidly in the presence of uncouplers of oxidative phosphorylation. Trace B (Fig. 2, left panel) shows that, after phosphorylation of an aliquot of ADP (ADP:O = 1.3), addition of oligomycin blocked further mitochondrial response to ADP, whereas AETT was still capable of eliciting rapid respiration, behavior consistent with the hypothesis that AETT is an uncoupling agent. The quantities of AETT in the

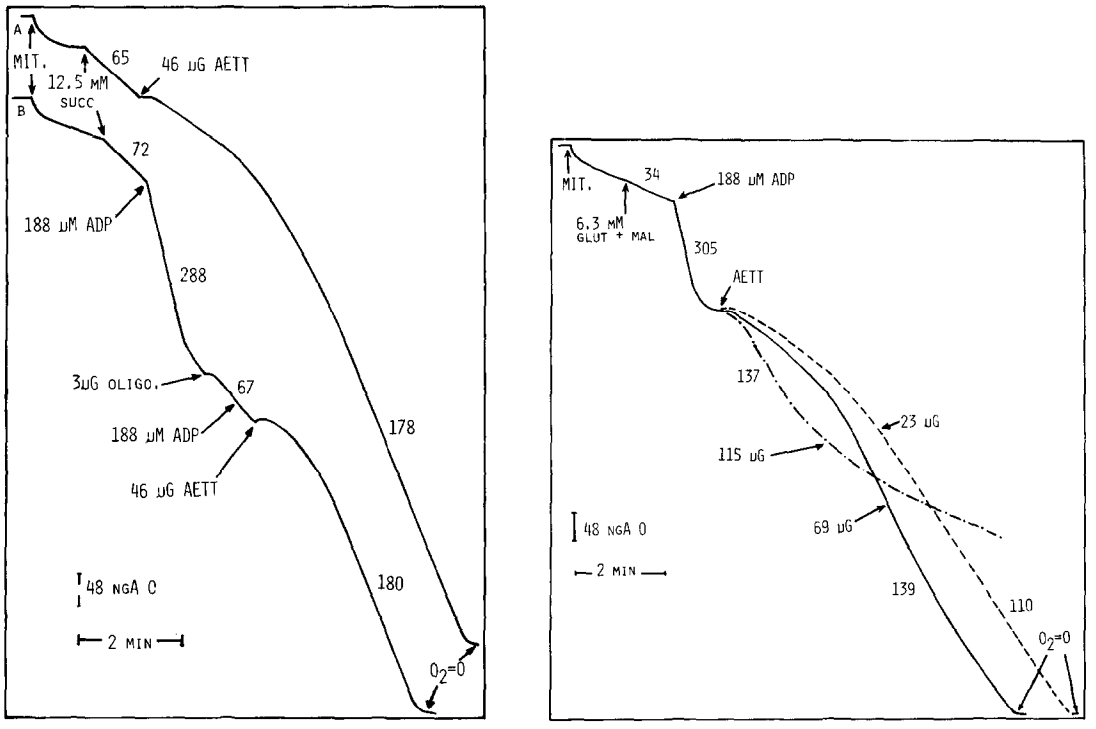

Fig. 2. Effect of AETT on the respiration of liver mitochondria. Respiration medium contained 0.25 M sucrose, 19 mM Tris–Cl, pH 7.5, 25 mM KCl, 13 mM NaCl and 19 mM Na phosphate buffer, pH 7.3. Mitochondria (2.2 mg protein) were added to 1.6 ml of medium in the electrode chamber, and subsequent additions were made as shown. The numbers next to the traces are rates of respiration in ngA O per min, and AETT was added as 3–5 µl of stock solutions in ethanol. In the right panel the dashed line is respiration after addition of 23 µg AETT, the solid line, after 69 µg AETT, and the line with dashes and dots, after 115 µg AETT.

reaction mixture are expressed in micrograms rather than in molar concentrations because the compound was added as a small quantity of a solution in ethanol and precipitated visibly in the aqueous medium. Therefore, the concentrations in solution are unknown. The insolubility of the compound may also be the reason that the AETT-stimulated respiration rate was always lower than the rate in the presence of ADP (unpublished data, and Fig. 2, left panel, trace B), whereas most uncoupling agents can stimulate respiration to a rate higher than that in the presence of ADP.

Figure 2, right panel, shows mitochondrial respiration stimulated by AETT in the presence of the NAD-linked substrate pair glutamate plus malate, after phosphorylation of an aliquot of ADP (ADP:O = 2.0). The rate of respiration was higher with 69 µg AETT than with 23 µg, but 115 µg AETT ultimately produced the inhibited rate of respiration characteristic of the mitochondrial response to excessive concentrations of uncoupling agents. The lag period prior to stimulated respiration occurred after addition of 23, 69 or 115 µg AETT, but was somewhat less pronounced with the largest amount of AETT than with lower quantities.

The putative metabolite of AETT, DK, which is a diketo derivative of tetramethyl tetralin, produced an immediate burst of rapid respiration after its addition to mitochondria in the presence of glutamate plus malate (Fig. 3, trace A). It elicited rapid respiration with succinate as substrate, as well, in either the absence (data not shown) or presence (Fig. 3, trace B) of oligomycin. Because DK was also water-insoluble and was less soluble in ethanol than was AETT, a relatively large volume of ethanol was added to the reaction mixture along with the quantity of DK (465 µg) required for maximal stimulation of respiration with succinate as substrate. The varied patterns of perturbation of the traces for oxygen utilization immediately after addition of DK (Fig. 3, traces B and C) resulted from the response of the electrode to ethanol (see Fig. 4, trace B). When respiration was supported by the NAD-linked substrates glutamate plus malate, 465 µg DK produced the inhibited rate of respiration (Fig. 3, trace C) characteristic of the response to excessive quantities of an uncoupling agent. The particular quantity of DK required to inhibit respiration depended on the identity and concentration of the substrate.

Figure 4 shows the response of liver mitochondria to TET and HCP, as well as to a large aliquot (15 µl) of ethanol, and is included for the purpose of comparison. TET produces primary demyelination *in vivo* [2] and uncouples and inhibits oxidative phosphorylation *in vitro* [6, 7]. On addition of TET to respiring mitochondria, a lag phase occurred before respiration was stimulated maximally (Fig. 4, trace A), a pattern similar to that obtained with AETT

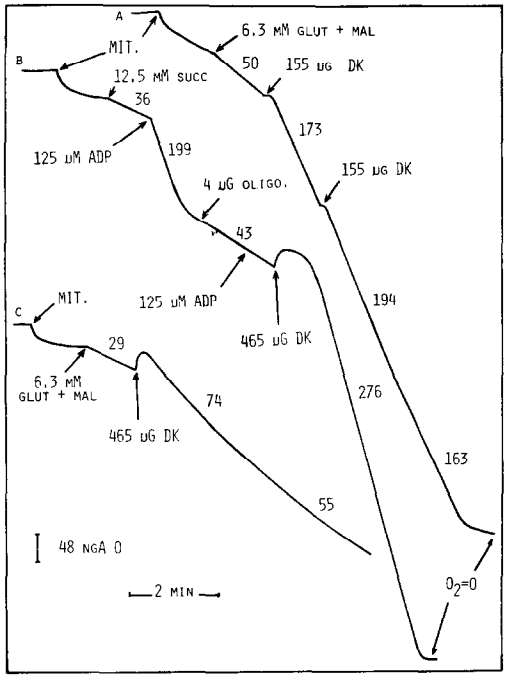

Fig. 3. Effect of DK on the respiration of liver mitochondria. Mitochondria were added to 1.6 ml of medium (same as in Fig. 2) in the electrode chamber, and subsequent additions were made as shown. The numbers next to the traces are rates of respiration in ngA O per min, and DK was added as 5–15 μl of a 112.5 mM solution in ethanol; 2.6, 2.1 and 2.7 mg mitochondrial protein were used in traces A, B and C respectively.

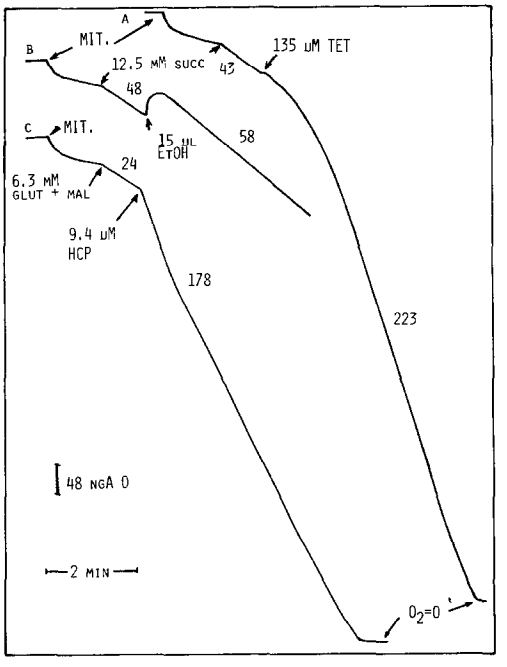

Fig. 4. Effect of ethanol alone and two known uncoupling agents on the respiration of liver mitochondria. Mitochondria (2.0 mg protein in traces A and B and 1.4 mg protein in trace C) were added to 1.6 ml medium (same as Fig. 2, left panel) in the electrode chamber, and subsequent additions were made as shown. The numbers next to the traces are rates of respiration in ngA O per min. HCP was added as 1.5 μl of a 10 mM solution in ethanol, and TET was added as an aqueous solution of the sulfate salt.

(Fig. 2). That the sulfate salt of TET, in aqueous solution, gave this pattern suggests that the small volumes (3–5 μl) of ethanol added as solvent for AETT were not required for the appearance of the initial lag. In Fig. 4, trace C shows the immediate rapid respiration produced by a typical uncoupling agent, HCP [8, 9], which also causes demyelination [3]. This trace resembles that for DK (Fig. 3, trace A).

The responses of brain mitochondria to AETT and DK are shown in Fig. 5. Phosphorylation of an aliquot of ADP demonstrated that the mitochondria were coupled (ADP:O = 1.3), and a control trace with no subsequent additions is also shown here (line with dots and dashes). AETT (dashed line) produced a rate of respiration higher than that in the control trace, after a transient lag, and, with DK (solid line) a maximum rate of respiration was reached immediately following the ethanol artifact, a pattern similar to that obtained with liver mitochondria and DK (Fig. 3, trace B). Brain mitochondria utilizing glutamate plus malate as substrates (ADP:O = 1.7) showed less stimulation of respiration by AETT and DK than did brain mitochondria utilizing succinate as substrate. With glutamate plus malate, respiration was stimulated 29 and 44 per cent by 46 μg AETT and 93 μg DK, respectively, over the rate of O_2 uptake with the substrates alone, and respiration was inhibited by additions of larger quantities of those chemicals.

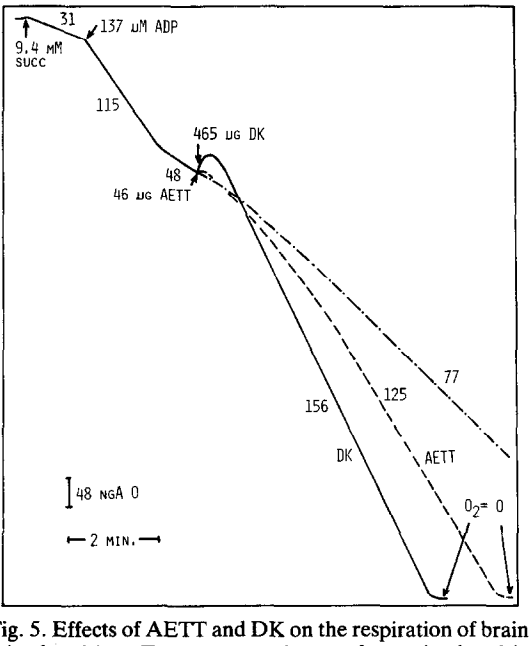

Fig. 5. Effects of AETT and DK on the respiration of brain mitochondria. Traces are shown for mitochondria (1.0 mg protein) in 1.6 ml of the respiration medium described by Clark and Niklas [13], with 20 mM KCl. The numbers next to the traces are rates of respiration in ngA O per min. The line with dots and dashes is a control trace for respiration without AETT or DK, the dashed trace is for respiration after addition of 46 μg AETT in 2 μl ethanol, and the solid line represents respiration after addition of 465 μg DK in 15 μl ethanol.

DISCUSSION

The polarographic traces shown here demonstrate that AETT and DK both affect mitochondrial respiration in a manner consistent with the conclusion that they uncouple oxidative phosphorylation. Both compounds, in appropriate quantities, release respiratory control in either the absence or the presence of oligomycin, and at higher concentrations both compounds give only a transient phase of stimulated oxygen uptake followed by an inhibited rate of respiration. Because AETT and DK both precipitated copiously on addition to the aqueous medium, a range of effective concentrations cannot be specified. It was possible, however, to demonstrate a dependence of the respiration rates on the quantities used for each compound.

Many aromatic uncoupling agents possess phenol (or thiophenol) groups which dissociate to phenolate ions and are believed to carry protons across the mitochondrial membranes, thus collapsing the proton gradient required for oxidative phosphorylation [16]. Under appropriate conditions they may carry monovalent cations as well [17]. An example of a similar effect is the increase in H^+ transport across phospholipid membranes induced by phenolic uncoupling agents [18]. Hypothetically, AETT and DK could carry protons if a keto group in each (Fig. 1) tautomerized to the enol, and the enol, in turn, dissociated. Even an enol form as weakly dissociated as that of acetophenone, for example, with a pK of 19 [19], might carry protons, since lysis of red blood cells by bis phenols appears to be independent of the strength of dissociation of those compounds [20]. Similarly, TET is believed to collapse the proton gradient by exchanging chloride ions for hydroxyl ions across the mitochondrial membrane, and organotin compounds have, indeed, been shown to exchange halide ions across erythrocyte and mitochondrial membranes and liposomes [21] and to increase the uptake of Cl^- into the brain slices [22].

The polarographic traces shown here suggest a possible distinction between two classes of uncoupling agents. This distinction is that some uncoupling agents, such as DK, HCP and most of the ones in common use, produce an immediate burst of respiration, whereas others, such as AETT and TET, produce a stimulated rate of respiration only after a 2–3 min lag time. Since AETT, which, hypothetically, dissociates to an anion, which could carry H^+ or cations, and TET, which promotes the exchange of OH^- and Cl^-, apparently belong to the same class, the distinction is not based on the charge of the ion being carried across the mitochondrial membranes. Both AETT and TET may equilibrate more slowly across mitochondrial membranes than do the other uncouplers; alternatively, AETT and TET may differ from most other uncoupling agents with respect to some unknown property.

The finding that AETT and its putative metabolite uncouple oxidative phosphorylation adds to a list of compounds which produce primary demyelination *in vivo* and which are also uncoupling agents. The characteristic pathology for this group is the inclusion of vacuoles filled with salt and water between the myelin lamellae (e.g. Ref. 2). The correlation between demyelination *in vivo* and uncoupling *in vitro* could arise either from a lesion in myelin upkeep *in vivo*, resulting from an energy deficit, or from a disruptive increase in the flux of ions and water across mitochondrial and myelin membranes brought about by common mechanisms. The results of a study where addition of ATP prevented the edema caused by infusion of DNP into the rabbit carotid artery [23] suggests a third possibility, which is a deficiency in the ATP required as substrate for the Na^+, K^+-ATPase in myelin [24]. An energy deficit might be expected to produce damage to the oligodendroglia, the cells which maintain myelin; however, ultrastructural studies have suggested that those cells remain intact during this type of toxic demyelination (e.g. Ref. 25). Furthermore, remyelination of peripheral nerves during intoxication with AETT [1, 11] suggests that Schwann cell function is largely intact. Therefore, the more likely mechanisms for demyelination are the ones in which the compounds disrupt the myelin membranes directly, or in which ATP is not available to support normal ion transport.

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