# ACETYLENEDICARBOXYLIC ACID DIMETHYLESTER (ADDM)

## A new inhibitor of oxidative phosphorylation in beef heart mitochondria

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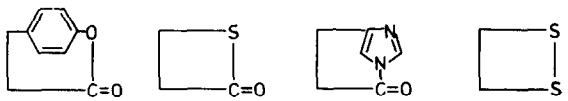
Received 10 October 1979

### 1. Introduction

To elucidate the chemistry of the ATP synthase reaction in mitochondria we maintain the working hypothesis that functional groups of the synthase may be involved in the phosphate activation, because a process which consists of the reaction between ADP, phosphate and proton(s) only, appears to us unprobable for chemical reasons [1]. We tried to associate the action of the two original protons of the chemiosmotic hypothesis [2] with a sidechain protein chemistry:




(1) (2) (3) (4)

and proposed, among other strained cyclic functions (such as, tyrosyllactones (1), thiolactones (2) and carbonylimidazoles (3)) a cyclic disulfide (4), cleavable by phosphate in a hydrophobic environment [1].

We attempted to trap the products – a thiol and a sulfenyl group ($RS^+$). The latter was proposed to react with phosphate affording a sulfenic-phosphoric acid anhydride ($RSOPO_3H_2$) [3].

*Abbreviations:* ADDM, acetylenedicarboxylic acid dimethylester (2-butynedioic acid-dimethylester); DNP, 2,4-dinitrophenol; SF 6874, 3,5-di-tert.-butyl-4-hydroxy-benzylidenmalonitrile; DCCD, dicyclohexylcarbodiimide; TMPD, tetramethyl-p-phenylene diamine

*Part of the dissertation work

Lipophilic maleimides, substituted by both an aromatic ring and alkyl chains [4] were used for trapping the thiol group, and *N*-alkyl-thioureas [5], 6-alkyl-2-thiouraciles [5], 1-alkyl-2-thioimidazoles [6] and 1-alkyl-2-thiobenzimidazoles [6] for the sulfenyl group. If the alkyl chainlength was $\geqslant 9$ carbon atoms long, the common action of all these substances on beef heart mitochondria was:

(1) Coupled respiration was inhibited, if glutamate + malate were the substrates and this inhibition reaction could not be uncoupled;
(2) When succinate or ascorbate + TMPD were the substrates, state 4 respiration was stimulated to state 3 [7–9].

If a covalent bond appeared to be formed by the corresponding maleimide [10] or if the basicity of the corresponding imidazole derivative was increased by modification of the molecule [9], the inhibitory concentration decreased significantly. The latter effect was confirmed and extended to site III by action of lipophilic alkylamines [11], which gave evidence for the inhibition of proton translocation linked to the electron transport.

A second group used in organic chemistry to trap sulfenyl groups formed during pyrolysis is the unsaturated function in esters of fumaric, maleic, acrylic, propiolic and acetylenedicarboxylic acid or in other compound such as norbornadiene and isobutyl vinylether (reviewed in [12]). Acetylenedicarboxylic acid dimethylester (ADDM):

$$CH_3OOC{-}C{\equiv}C{-}COOCH_3$$

only inhibited oxidative phosphorylation, while the dinonylester reacted like the first group of sulfenyl trapping reagents described above.

## 2. Materials and methods

Isolation of beef heart mitochondria and measurement of respiration was performed as in [10]. Rat liver mitochondria were obtained by the method in [13].

$\Delta\psi$ and $\Delta$pH determinations of beef heart mitochondria after treatment with various concentrations of acetylenedicarboxylic acid dimethylester were carried out by the $^{86}$RbCl method [14] and the [$^{14}$C] acetate method [15] as in [11].

The method in [16] was used to determine whether acetylene dicarboxylic acid dimethylester reacted with the adenine nucleotide translocase. For experimental detail, see legend of fig.4.

Acetylenedicarboxylic acid dimethylester was a product of Fluka (Buch) and distilled twice. The diethyl-, dipropyl- and dinonyl-esters were synthesized according to [17]. [$^{14}$C]Acetate, $^{3}H_2O$ and $^{86}$RbCl were obtained from New England Nuclear, [$^{3}$H]sucrose and [$^{14}$C]sucrose from the Radiochemical Centre (Amersham). All other reagents were obtained from Boehringer (Mannheim).

## 3. Results

### 3.1. *The action of acetylenedicarboxylic dimethylester (ADDM) on state 3 and state 4 respiration of beef heart mitochondria*

The effect of ADDM on coupled respiration is shown in fig.1, with glutamate + malate (expt. A), succinate in the presence of rotenone (expt. B) and

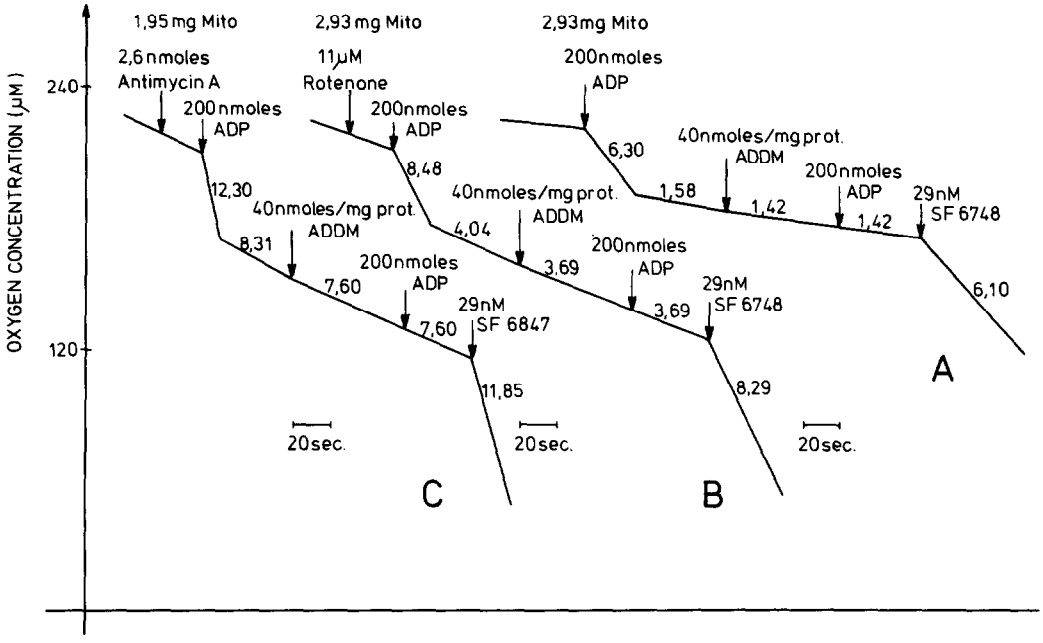



Fig.1. Effect of acetylenedicarboxylic acid dimethylester (ADDM) on the coupled respiration of beef heart mitochondria. The lines represent the output from an oxygen electrode. The numbers on the lines are respiration rates ($\mu$mol oxygen . mg protein$^{-1}$ . h$^{-1}$) at 25°C. Expt. A: Beef heart mitochondria (2.93 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 mM sucrose containing 2.5 mM glutamate, 2.5 mM D, L-malate, 5 mM malonate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl (pH 7.3). Expt. B: Beef heart mitochondria (2.93 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 mM sucrose containing 10 mM succinate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl (pH 7.3). Expt. C: Beef heart mitochondria (1.95 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 M sucrose containing 5 mM ascorbate, 0.25 mM TMPD, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl (pH 7.3).

ascorbate + TMPD (expt. C) as the substrates. About the same concentration of 40 nmol ADDM/mg protein prevented coupled respiration after 1 min incubation time with each of the substrates used, including β-hydroxybutyrate. This inhibition could be released by various uncouplers, e.g., SF 6748 and DNP, but not by 5 mg bovine serum albumin/mg mitochondrial protein. The inhibition of coupled respiration was accompanied by some inhibition of state 4 respiration. This latter inhibition increased with increasing incubation time (1–10 min), whereby the concentration of ADDM for the inhibition of the state 3–state 4 transition decreased, but could be uncoupled completely. The electron transport of state 4 mitochondria was scarcely affected by higher amounts of ADDM, except when glutamate/malate were the substrates as shown in fig.2. Here ~50% of state 4 respiration was inhibited by 300 nmol ADDM/mg protein.

### 3.2. *The action of ADDM on the membrane potential Δψ and the proton concentration gradient ΔpH of steady state mitochondria*

Neither the initial stimulation of state 4 to state 3 respiration (fig.2), if succinate was the substrate, nor the apparent partial electron transport inhibition (fig.2), if glutamate + malate were the substrates, affected the components of the protonmotive force, i.e., the membrane potential Δψ and the proton concentration gradient ΔpH, as shown in fig.3. Furthermore, this was also true for up to 70 nmol ADDM/mg protein.

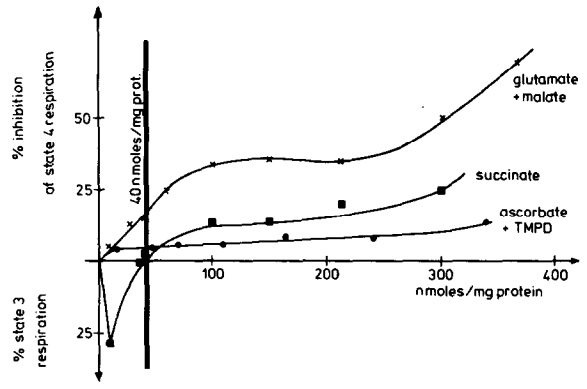



Fig.2. The action of ADDM on state 4 respiration. The conditions were the same as in fig.1.

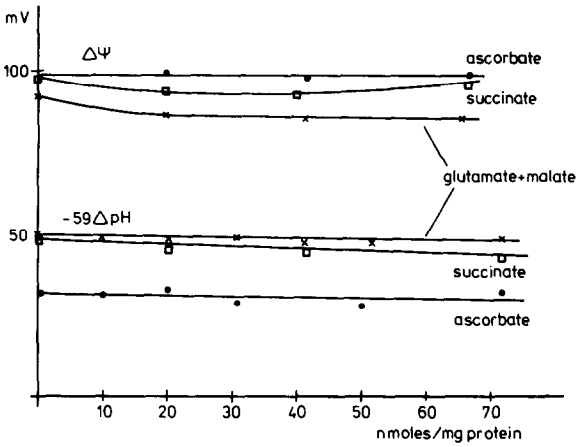



Fig.3. The action of increasing amounts of ADDM on the proton concentration gradient (ΔpH) and the membrane potential (Δψ) in steady state beef heart mitochondria. The matrix volume was determined with $^3H_2O$ and [$^{14}$C]sucrose according to [13]; the medium contained in 2.11 ml 0.2 M sucrose, 10 mM Tris–HCl., (pH 7.2), 2.7 mg protein and 1 mM of the respective substrate(s). ΔpH was determined by measuring the distribution of [$^{14}$C]acetate according to [12]; [$^3$H]sucrose was added to each sample to follow the changes of the extramatrix volume; the medium contained in 2.16 ml 0.2 M sucrose, 10 mM Tris–HCl, 25 mM Tris–acetate (pH 7.2), 2.7 mg protein and 1 mM of the respective substrate(s). Δψ was determined by a similar procedure using $^{86}$RbCl and [$^3$H]sucrose [12] (Mark III liquid-scintillation spectrometer: autoisotope program no. 9); the medium contained in 2.18 ml 0.2 M sucrose, 10 mM Tris–HCl, (pH 7.2), 50 μM RbCl, 20 nM valinomycin, 2.7 mg protein and 1 mM of the respective substrate(s) (K-salts). Each sample was incubated for 2 min with the respective substrate(s), the reaction was started with the addition of ADDM and terminated after 2 min by centrifugation.

### 3.3. *The action of ADDM on an arsenate-stimulated respiration enhanced by ADP*

To exclude that ADDM had reacted with the adenine nucleotide translocase [18], ADDM was tested in the system of [16]. Here atractylate, which is an inhibitor of the translocase [19], was reported to prevent the enhancement of arsenate-stimulated respiration by ADP, whereas oligomycin abolished both the arsenate and ADP stimulation. Although we did not obtain complete inhibition with high concentrations of either actractylate for the ADP-stimulation or of oligomycin for the arsenate stimulation, the extent of both of the inhibition reactions allowed the comparison of the action of ADDM with those of

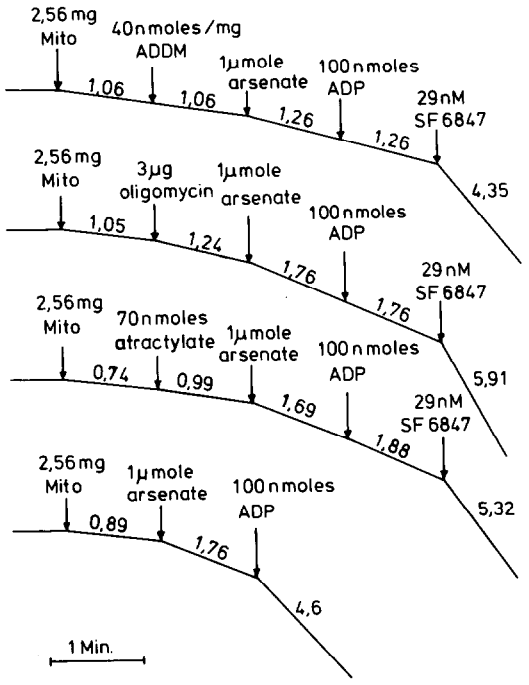



Fig.4. Effects of ADDM on arsenate-stimulated succinate oxidation in beef heart mitochondria compared to atractylate and oligomycin. The conditions were essentially those in [16]. The lines represent the output from an oxygen electrode. The numbers on the lines are respiration rates (µmol oxygen . mg protein$^{-1}$ . h$^{-1}$) at 25°C. In each case the medium contained 5 mM succinate, 0.17 µM rotenone, 80 mM KCl and 20 mM Tris–HCl (pH 7.2). The total volume was 2.4 ml. Each addition is indicated over the corresponding arrow.

atractylate and oligomycin. Thus the inhibitory concentration of ADDM (fig.1) clearly reacted like oligomycin, if succinate was the substrate (fig.4).

## 4. Discussion

Acetylenedicarboxylic acid dimethylester (ADDM), a highly reactive, unsaturated compound, inhibited coupled respiration in beef heart as well as in rat liver mitochondria, if glutamate + malate, succinate, or ascorbate + TMPD were the substrates. Each of the inhibition reactions could be uncoupled (fig.1). If the incubation time was increased from 1–10 min. the inhibitory concentration decreased from 40–15 nmol ADDM/mg protein, when glutamate + malate were the substrates. This effect was accompanied by an increased inhibition of state 4 respiration (from 15–30%), which could also be uncoupled. This partial inhibition of state 4 respiration, which could be uncoupled, resembled very much the action of oligomycin on state 4 respiration [20].

The electron transport, as shown by the action on state 4 respiration, was scarcely affected even by as high concentrations of ADDM as 300 nmol/mg protein, with the exception of a 50% inhibition if glutamate + malate were substrates (fig.2).

In steady state mitochondria, the proton concentration gradient $\Delta pH$ and the membrane potential $\Delta\psi$ were altered neither by the partial inhibitions of state 4 respiration nor by the initial stimulation of the succinate dependent state 4 respiration (fig.2). Both, $\Delta pH$ and $\Delta\psi$ remained unchanged up to double the inhibitory concentration of ADDM (fig.3). This stability of the protonmotive force was expected for a real inhibitor of the ATP synthase system. It could be shown (fig.4) that ADDM reacted like oligomycin on an arsenate-stimulated respiration, which could be enhanced by ADP, and not like atractylate. Thus it is unprobable that ADDM interacted with the adenine nucleotide translocase. However, we could not distinguish as precisely between the effects of oligomycin and atractylate as reported [16], because oligomycin failed to completely prevent the arsenate-stimulated respiration nor atractylate the ADP-enhanced one. Therefore we had to compare the extent of the inhibition.

It is known from the experience of organic chemistry [21], that the highly unsaturated and reactive ADDM not only could react with sulfenyl groups ($RS^+ X^-$), i.e., our starting hypothesis, but also with thiol-, amino-, aliphatic- and aromatic hydroxyl-groups. Since no reaction with carboxyl groups has been reported, ADDM may be assumed to react differently from DCCD. Whether the ADDM is bound covalently to $F_1$ or $F_0$, will be reported very shortly; experiments with the radioactive compound are in progress.

### Acknowledgements

The authors thank Mr B. Küchler and Mr H. Gaugler for their helpful technical assistance and especially Mr B. Küchler for the excellent drawings.

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