Inhibition of adenosine triphosphatase and respiration of rat-liver mitochondria by dinitrophenols

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INHIBITION OF ADENOSINE TRIPHOSPHATASE AND RESPIRATION OF RAT-LIVER MITOCHONDRIA BY DINITROPHENOLS*

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SUMMARY

1. The stimulating and inhibitory effect of nitrophenols on various mitochondrial systems are compared. Both respiration and ATPase are stimulated by low dinitrophenol concentrations followed by inhibition at higher concentrations. The concentration of dinitrophenol inducing maximal activity (c_{opt}) differs for ATPase, pyruvate oxidation, β-hydroxybutyrate oxidation and succinate oxidation.

2. It is concluded that dinitrophenols have a specific inhibitory action on the uncoupled respiration and on the ATPase of intact rat-liver mitochondria. This inhibition can be located between NADH and flavoprotein and between flavoprotein and cytochrome *c*. Although the third phosphorylating step is as easily uncoupled as the other two steps, no inhibition of this step by uncoupling agents occurs.

3. The inhibition by alkyldinitrophenols can be reversed by lowering the partition coefficient of the inhibitor between the mitochondrial lipid and the medium.

4. From the fact that a difference exists between c_{opt} for ATPase and for NAD-linked respiration it can be concluded that dinitrophenols act by hydrolysing a high-energy intermediate rather than by allowing respiration to proceed without the formation of a high-energy intermediate.

INTRODUCTION

Although there is still some variation of symbols, there exists nowadays no fundamental difference between the various explanations, in terms of a chemical mechanism, of the coupling of oxidation–reduction in the respiratory chain with the synthesis of ATP. It is generally accepted that during the oxidation–reduction reaction a high-energy compound (*e.g.* A $\sim$ I) is formed between one of the respiratory-chain components (A) and an unknown mediator (I). This compound reacts with P_i in an oligomycin-sensitive reaction to give a high-energy phosphate compound (*e.g.* I $\sim$ P), thereby freeing the member of the respiratory chain. Since, however, oligomycin does not react with either A $\sim$ I or P_i, we have to regard this reaction as being catalysed by an enzyme and oligomycin as an inhibitor of that enzyme. Finally the

* This article reports experiments that were part of the M.D. thesis of the author published in Dutch in April 1962 (see ref. 1).

high-energy phosphate compound reacts with ADP to form ATP (for an extensive discussion see refs. 2–4). These conclusions are summarized in the reaction scheme:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2$$

$$A \sim I + P_i \overset{(E)}{\rightleftharpoons} A + I \sim P$$

$$I \sim P + ADP \rightleftharpoons I + ATP$$

Simple as this may seem there are still many points to be investigated, *e.g.* the nature of the first high-energy compound which is thought by CHANCE AND WILLIAMS[5] to be $AH_2 \sim I$, by ERNSTER[3] to be $BH_2 \sim I$, by LEHNINGER[6] to be $A \sim I$, while SLATER[2] leaves the choice between $A \sim I$ and $BH_2 \sim I$ open. The mechanism of inhibition of the second reaction by oligomycin is also unsolved.

No agreement has been reached as to the mechanism of uncoupling of oxidative phosphorylation by phenols. The two alternative mechanisms proposed by LARDY AND ELVEHJEM[7] still remain open, although the original formulations have had to be modified in the light of newer knowledge. The two alternatives are:

(a) An uncoupling phenol (Φ) allows oxidation to proceed without phosphorylation[8–10]. The reaction mechanism of uncoupled oxidation is then given by

$$AH_2 + B \overset{(\Phi)}{\rightarrow} A + BH_2$$

and the mechanism of nitrophenol-induced ATPase by

$$ATP + I \rightleftharpoons ADP + I \sim P$$

$$I \sim P + A \rightleftharpoons A \sim I + P_i$$

$$A \sim I + BH_2 \rightleftharpoons AH_2 + B + I$$

$$AH_2 + B \overset{(\Phi)}{\rightarrow} A + BH_2$$

$$\textit{Sum} \qquad ATP \overset{(\Phi)}{\rightarrow} ADP + P_i$$

(b) An uncoupling nitrophenol reacts with the first high-energy compound to form a labile, but not energy-rich, nitrophenol compound[11,12]. The reaction mechanism of uncoupled oxidation is in that case:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2$$

$$A \sim I + \Phi \rightarrow A + \Phi - I$$

$$\Phi - I \rightleftharpoons \Phi + I$$

$$\textit{Sum} \qquad AH_2 + B \overset{(\Phi)}{\rightarrow} A + BH_2$$

Respiratory rates

These were measured polarographically[16] in the apparatus previously described or in Warburg differential manometers. The total volume of the manometer flasks was about 5 ml; the constants were calculated according to VAN DORP AND SLATER[17]. The reaction media are given in the legends to the figures.

In all experiments freshly prepared rat-liver mitochondria were used unless otherwise stated. The KEILIN AND HARTREE heart-muscle preparation[18] was prepared according to SLATER[19].

Oxidative phosphorylation

Oxidative phosphorylation was measured by the method described by SLATER[20] and SLATER AND HOLTON[21] in the following reaction medium: 15 mM KCl, 2 mM EDTA, 20 mM glucose, 30 mM inorganic phosphate, 25 mM Tris–HCl, 0.1 mM ATP, 5 mM $MgCl_2$, 50 mM sucrose, 100–150 units[22] hexokinase.

RESULTS

The effects of different concentrations of 4-isooctyl-2,6-dinitrophenol on various mitochondrial systems are illustrated in Fig. 1. Increasing the concentration between 10^{-7} and 10^{-6} M isooctyldinitrophenol first uncouples phosphorylation associated

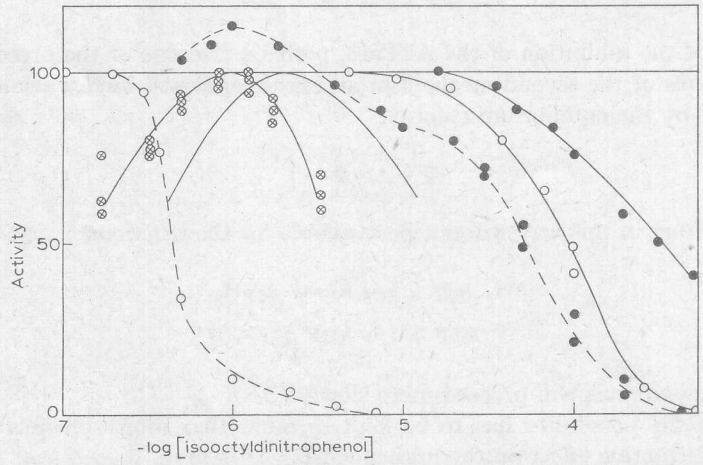

Fig. 1. The action of 4-isooctyl-2,6-dinitrophenol on different mitochondrial enzyme systems. The activity is expressed as a percentage of the uninhibited or maximally stimulated activity at pH 7.0. ○- - - -○, P:O ratio with succinate as a substrate (100% = activity without isooctyldinitrophenol); ⊗- - - -⊗, respiration with succinate as a substrate in a medium without ADP and P_i, measured as indicated in Table I (100% = maximally stimulated respiratory activity); ———, ATPase activity, data taken from ref. 14 (100% = maximally stimulated activity); ●- - - -●, respiration with succinate as a substrate in a medium with ADP and P_i. To the medium indicated in Table I were added: 30 mM P_i, 0.1 mM ATP, 20 mM glucose, about 150 units hexokinase. The O_2 uptake was measured manometrically (100% = respiratory activity without isooctyldinitrophenol). ○—○, the succinate oxidase activity of a KEILIN AND HARTREE heart-muscle preparation in 40 mM P_i, 30 mM succinate, 0.3 mM EDTA (100% = uninhibited activity); ●—●, the ATPase activity of a KEILIN AND HARTREE heart-muscle preparation (100% = uninhibited activity).

with succinate oxidation and stimulates the oxidation of succinate in the absence of P_i and ADP. Slightly higher concentrations stimulate the ATPase. The concentration of dinitrophenol giving an optimal rate of oxidation of succinate ($(c_{aq})_{opt}$) is less than that required for an optimal ATPase activity. In both cases, activity–concentration curves with quite a sharp optimum were obtained, with no plateau. This was also the case at other pH's and with other substrates.

Table I summarizes the values of $(c_{aq})_{opt}$ for 2,6-dinitrophenol and 4-isooctyl-

TABLE I

THE VALUES OF $-\log (c_{aq})_{opt}$ UNDER DIFFERENT CONDITIONS

The velocity of O_2 uptake was measured polarographically with increasing concentrations of the dinitrophenol added from a microburette. The initial volume was 3 ml and up to 0.5 ml of the dinitrophenol solution was added. The initial concentrations were 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl buffer, 5 mM $MgCl_2$, 50 mM sucrose and one of the following substrates: 20 mM pyruvate + 20 mM malate, 20 mM β-hydroxybutyrate, 60 mM succinate + 1.2 mM Amytal. 1–3 mg/ml mitochondrial protein. The values given are $-\log_{10}(c_{aq})_{opt}$ where c is the molar concentration of the nitrophenol.

		pH 5	6	7	8	9
ATPase	2,6-Dinitrophenol	4.77	4.22	3.68	3.12	—
	4-Isooctyl-2,6-dinitrophenol	6.08	5.89	5.69	5.41	—
Respiration with succinate	2,6-Dinitrophenol	6.55	5.57	4.57	3.56	—
	4-Isooctyl-2,6-dinitrophenol	—	6.80	5.90	5.00	4.05
Respiration with β-hydroxybutyrate	2,6-Dinitrophenol	5.93	5.42	4.90	4.40	3.88
	4-Isooctyl-2,6-dinitrophenol	6.92	6.39	5.87	5.30	4.74
Respiration with pyruvate + malate	2,6-Dinitrophenol	5.92	5.43	4.93	4.42	3.90
	4-Isooctyl-2,6-dinitrophenol	—	5.09	5.80	5.80	5.80

2,6-dinitrophenol at pH values between 5 and 9 for ATPase and respiration with succinate, β-hydroxybutyrate and pyruvate. The effects of pH will be discussed in greater detail in the following paper[23].

Isooctyldinitrophenol, in high concentration, inhibited the oxidation of succinate by the KEILIN AND HARTREE heart-muscle preparation (Fig. 1). In this case, also, the degree of inhibition by a given concentration of dinitrophenol depended upon the lipid solubility. The succinate and NADH dehydrogenase activities, with methylene blue as acceptor, were not affected. The cytochrome *c* oxidase activity, measured with ascorbate + cytochrome *c*, of intact mitochondria was not affected by concentrations of the dinitrophenols which strongly inhibited the oxidation of succinate, pyruvate (+ malate), glutamate, or β-hydroxybutyrate. Much higher concentrations (more than 0.5 mM isooctyldinitrophenol at pH 7.0) stimulated the cytochrome *c* oxidase activity.

It appears, then, that the site or sites of inhibition of respiration by dinitrophenols lie between NADH and cytochrome *c* in the respiratory chain. That the flavoprotein–cytochrome *c* span is involved is indicated by the sensitivity of succinate

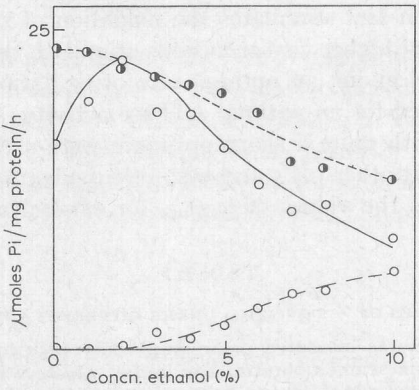

Fig. 2. Effect of ethanol on dinitrophenol-induced ATPase. pH 7.0. ○—○, 7.5 μM 4-isooctyl-2,6-dinitrophenol; ◑- - - -◑, 0.2 mM 2,6-dinitrophenol; ○- - - -○, no phenol added. The reaction was started by adding 0.05 ml of a mitochondrial suspension to the medium containing ethanol.

oxidation. The lower values of $(c_{aq})_{opt}$ for the oxidation of NAD-linked substrates than for succinate oxidation at higher pH's (Table I), despite the greater rate of oxidation of succinate, suggest that a site between NADH and flavoprotein is also inhibited, since in the concentration range between $(c_{aq})_{opt}$ for succinate and for NAD-linked substrates an increase in dinitrophenol concentration will inhibit the oxidation of the latter substrates and stimulate the oxidation of succinate.

According to the hypothesis proposed previously[14], the effectiveness of a dinitrophenol depends upon the partition coefficient, Q, between the mitochondrial

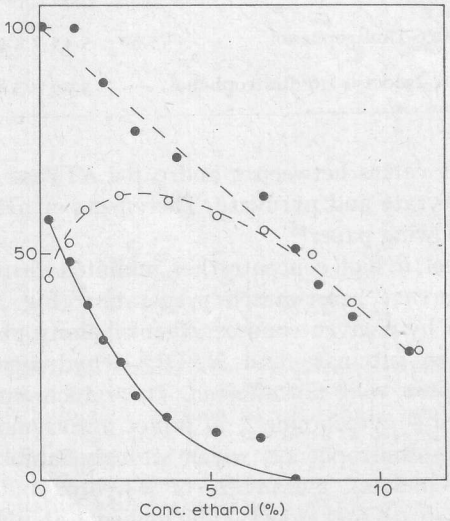

Fig. 3. Reversal by ethanol of the dinitrophenol-induced inhibition of succinate respiration. Medium as in Table I. pH 7.0. ●- - - -●, respiratory rate in absence of dinitrophenol; ○- - - -○, respiratory rate in the presence of 50 μM isooctyldinitrophenol. The rates are expressed as a percentage of the rates in the absence of ethanol or dinitrophenol. ●—●, percentage inhibition by isooctyldinitrophenol. Increasing amounts of ethanol were added to the reaction mixture already containing the dinitrophenol and mitochondria.

lipid and the aqueous medium. The addition of ethanol to the medium decreases the value of Q. The effect of increasing ethanol concentration on the ATPase induced by 2,6-dinitrophenol and the 4-isooctyl derivative is shown in Fig. 2. The concentration of the isooctyl compound used (7.5 μM) was greater than $(c_{aq})_{opt}$ at pH 7.0 (*cf.* Table I). Increasing the ethanol concentration up to about 2% caused a stimulation of the ATPase activity to the level reached with an optimum concentration of 2,6-dinitrophenol, while a further increase in ethanol concentration caused a further decline in the ATPase activity. The interpretation of these results is that increasing the ethanol concentration in the aqueous phase resulted in a decrease in the concentration of the alkyl-substituted phenol in the mitochondrial lipid, with the result that an initially post-optimal concentration became optimal and at higher ethanol concentrations sub-optimal. Fig. 3 shows that the inhibition of succinate oxidation by isooctyldinitrophenol can be reversed by the subsequent addition of ethanol.

DISCUSSION

The following evidence has been brought forward in favour of a specific inhibitory action of dinitrophenols on uncoupled respiration and on the ATPase.

(1) Concentrations which strongly inhibited the operation of the complete respiratory chain or of the ATPase had no effect on the dehydrogenase or cytochrome oxidase activities.

(2) Much higher concentrations were required to inhibit the succinate oxidase of the KEILIN AND HARTREE heart-muscle preparation.

(3) Under all conditions tested a sharp optimum in the activity–dinitrophenol concentration curve was found without any plateau.

(4) The inhibition of succinate oxidation by isooctyldinitrophenol was reversed by the addition of ethanol.

Reasons have already been given for believing that there are two sites of inhibitory action of dinitrophenols on the respiratory chain—one between NADH and flavoprotein and the other between flavoprotein and cytochrome *c*. It is generally believed that these are also sites of phosphorylation. The third phosphorylation site, between cytochrome *c* and O_2, is not inhibited, although this site is as readily uncoupled as the others, as is illustrated by the curve relating P:O ratio to dinitrophenol concentration in Fig. 1*.

The relative values of $(c_{aq})_{opt}$ given in Table I favour one of the two theories of the mechanism of action of uncoupling phenols discussed in the INTRODUCTION. According to the theory favoured by ERNSTER and his colleagues, the reaction

$$AH_2 + B \xrightarrow{\Phi} A + BH_2$$

is involved in both the dinitrophenol-induced ATPase and the uncoupled respiration. According to these authors, only the NADH–flavoprotein step makes a substantial contribution to the dinitrophenol-induced ATPase, and we could indeed confirm that this step makes the major contribution, although that of the flavoprotein–cytochrome *c* step is not negligible[25]. Since, according to this view, the reaction

* In repeated experiments we have been unable to find a plateau in this curve at about 50%, as reported by DRYSDALE AND COHN[24].

$$NADH + fp + H^+ \rightarrow NAD^+ + fpH_2$$

is promoted by dinitrophenols, it is presumably also the site of the inhibitory action by higher concentrations of dinitrophenols. In this case is it difficult to see why $(c_{aq})_{opt}$ for 2,6-dinitrophenol for the ATPase at pH's 5–7 should be more than 10 times greater than the $(c_{aq})_{opt}$ for β-hydroxybutyrate or pyruvate (+ malate) oxidation.

On the other hand, according to the mechanism of uncoupling favoured by this laboratory, there is no reason why the binding of I by Φ should have the same effect on the uncoupled oxidation and the ATPase, since the dinitrophenol-induced ATPase does not include an oxidation reaction.

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and the reaction mechanism of nitrophenol-induced ATPase:

$$ATP + I \rightleftharpoons ADP + I \sim P$$
$$I \sim P + A \rightleftharpoons P_i + A \sim I$$
$$A \sim I + \Phi \rightarrow A + \Phi - I$$
$$\Phi - I \rightleftharpoons \Phi + I$$

Sum $$ATP \xrightarrow{(\Phi)} ADP + P_i$$

The finding that uncoupling nitrophenols in higher concentrations inhibit the ATPase and the uncoupled respiration has been reported earlier[11,13,14]. At pH's around 7 relatively high concentrations of 2,4-dinitrophenol are necessary to obtain this effect; this may account for the fact that the inhibition has not been very much studied. By using alkyl-substituted dinitrophenols[14,15] this difficulty can be overcome, as these compounds have the same action on mitochondria as unsubstituted dinitrophenols, but at much lower concentrations.

These inhibitions can be explained in terms of both theories of uncoupling. In the first theory we have to assume that by some specific or non-specific action an excess of Φ inhibits the reaction:

$$AH_2 + B \xrightarrow{(\Phi)} A + BH_2$$

or, in case of the inhibition of the ATPase, perhaps also one of the preceding reactions. In terms of the second mechanism an excess of Φ will bind a relatively large amount of I by the equilibrium reaction

$$\Phi + I \rightleftharpoons \Phi - I$$

and the I bound in this way will not be available for the reactions:

$$AH_2 + B + I \rightarrow A \sim I + BH_2$$

or

$$ATP + I \rightarrow ADP + I \sim P$$

and so these reactions will proceed more slowly[11].

Thirdly the possibility has to be kept in mind that dinitrophenols inhibit by a general destructive effect on the mitochondrial structure.

It is, then, important to know whether the inhibitory effects of dinitrophenols are specific or non-specific. Experiments designed to give an answer to this question are described in the present paper.

METHODS

ATPase activity

This was measured as described by MYERS AND SLATER[13]. The reaction mixture contained 50 mM Tris–HCl buffer, 75 mM KCl, 0.5 mM EDTA, 2.0 mM ATP, 0.1 M sucrose, and about 0.13 mg/ml mitochondrial protein in a volume of 1.5 ml.