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INHIBITION BY FORMALDEHYDE OF ENERGY TRANSFER AND RELATED PROCESSES IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. Exogenous formaldehyde inhibits phosphorylating oxidation of succinate in coupled mitochondria in the presence of ADP. The inhibition of the oxidation can be released by dinitrophenol. Formaldehyde also prevents the stimulation of succinate respiration by arsenate.

2. Formaldehyde has little or no effect on respiration in the absence of ADP. Neither does it greatly affect the respiration of mitochondria which have been uncoupled by osmotic disruption or pre-treatment with dinitrophenol.

3. Formaldehyde interferes with the endergonic, anaerobic reduction of DPN^+ by succinate in phosphorylating particles of rat-liver mitochondria. Moreover, the ATP-induced contraction of intact mitochondria can be inhibited by formaldehyde or by oligomycin under the same conditions.

INTRODUCTION

Formaldehyde, one of the products of oxidative demethylation in liver mitochondria[1,2], can be oxidized to formate[3] with a P/O ratio approaching 2 (ref. 3). In addition to its role as a substrate, formaldehyde also has been observed to interfere with the respiratory activity of intact mitochondria[2]. Recent studies to be reported in this paper have shown that at low concentrations of formaldehyde the interference is largely confined to phosphorylating respiration. In this and other respects formaldehyde possesses inhibitory properties which closely parallel those of oligomycin.

Initial work had demonstrated that maximal rates of ADP-stimulated respiration could not be realized unless phosphate-acceptor and formaldehyde were added at about the same time. Delay in the addition of acceptor caused a decrease in the rate of stimulated respiration and when the delay was sufficiently long, there was no significant respiratory response to ADP. These observations suggested that exposure of the mitochondria to formaldehyde caused a time-related inhibition of the phosphorylating mechanism.

METHODS AND MATERIALS

Preparation of mitochondria

Adult male rats of the Sprague–Dawley or Holtzman strain are stunned and decapitated. The livers are quickly excised and placed in cold 0.25 M sucrose. All subsequent operations are performed with 0.25 M sucrose at 0–4°.

The livers are diced under sucrose and the fragments (0.5 g or less) are washed several times by decantation. The tissue is then homogenized for 1.5 min in sucrose, 1.0 ml per g liver, in a loose fitting Potter–Elvehjem apparatus. The homogenate is diluted with sucrose to 10.0 ml per g liver and strained through cheese-cloth.

Cells, nuclei, and debris are removed by centrifugation at 400 × *g* for 3.5 min in an International PR-1 centrifuge (conical head No. 822). The supernatant is carefully decanted until the free-flowing layer of the red pellet appears at the lip of the tube. The supernatant is centrifuged at 2200 × *g* for 20 min in the same head.

The crude mitochondria are transferred to No. 30 Spinco tubes. Sucrose is added, 3 ml per g liver, and a uniform suspension is obtained by repeatedly expressing the preparation from a 10-ml measuring pipette. The suspension is centrifuged at 14000 × *g* for 10–15 min (Spinco Model L, No. 30 rotor), and the supernatant is discarded together with free-flowing material. The upper, loosely packed layer of the pellet is removed by gentle swirling with several small portions of sucrose.

The tightly-packed layer, consisting chiefly of 'heavy mitochondria' (ref. 4), is stirred with a blunt glass rod, diluted to 3 ml per g liver with sucrose, resuspended by pipette, and centrifuged as before. The supernatant is discarded and any loose layer remaining with the final pellet is removed by swirling with sucrose.

The yield is 20–30 mg of mitochondrial protein per g of liver, wet wt.

Assays and reagents

Protein was determined by the method of OYAMA AND EAGLE[5].

Respiration was measured with a model KM oxygraph, Gilson Medical Electronics, Middleton, Wisconsin.

ADP, ATP and DPN^+ were obtained from the Sigma Chemical Company, and oligomycin from the Wisconsin Alumni Research Foundation. All of the other chemicals were of the highest commercial grade.

RESULTS AND DISCUSSION

Inhibition of the phosphorylating respiration of succinate by formaldehyde

Previous studies[3] had demonstrated that formaldehyde is oxidized rapidly at 37°. Prior to the experiments described in Table I, however, it was established that the indicated amounts of formaldehyde did not result in significant O_2-uptake at 30°, either before or after addition of ADP.

The data of Table I show that the inhibitory action of formaldehyde on succinate oxidation is confined to that fraction of respiration which is ADP-dependent. The controlled respiration, in the absence of ADP, is not affected significantly. In these experiments the mitochondria exhibited a respiratory control ratio greater than 4, but other preparations whose ratios varied from 2 to 6 were inhibited by formaldehyde to a comparable extent. The degree of inhibition increases with the form-

TABLE I

INHIBITION OF PHOSPHORYLATING RESPIRATION BY FORMALDEHYDE

Rat-liver mitochondria, freshly isolated and resuspended in 0.25 M sucrose (protein, 25 mg/ml), were assayed polarographically at 30°. Respiration was measured in a system containing: glycyl glycine (pH 7.4, 50 μmoles), $MgCl_2$ (7 μmoles), and EDTA (0.7 μmole), all in 0.35 ml; KH_2PO_4 (pH 7.4, 5 μmoles, 0.05 ml), sucrose (75–300 μmoles, 1.5 ml), mitochondria (0.5–0.7 mg protein, 0.02 ml), and, when indicated: formaldehyde (1–3 μmoles, 0.02–0.06 ml), semicarbazide (10 μmoles, 0.05 ml). Following a 2-min period for equilibration and the action of inhibitors, succinate (10 μmoles, 0.1 ml) was added. When respiration had proceeded for 2 min, ADP (0.45–0.90 μmole, 0.05 ml) was introduced with minimal admixture of air. Final volumes were 2.1–2.2 ml.

Formaldehyde (μmoles)	*Order of other additions*	*Respiration (mμ atoms O/min)*	*Respiratory control ratio*
None	(1) Succinate	51.3	4.1
	(2) ADP	210.9	
1	(1) Succinate	58.9	2.9
	(2) ADP	171.0	
2	(1) Succinate	58.9	1.1
	(2) ADP	64.6	
3	(1) Succinate	51.3	1.0
	(2) ADP	51.3	
2 + semicarbazide*	(1) Succinate	62.7	4.5
	(2) ADP	286.9	

* 10 μmoles semicarbazide were added 15 sec after formaldehyde.

aldehyde concentration. It was found also that inhibition by a given quantity of formaldehyde increases with time. In the experiments described here, at least 15 sec were required for the penetration or reactions of the formaldehyde. These results suggest that the sites which are sensitive to formaldehyde are located within the mitochondrion rather than on its outer surface.

Formate, a product of the oxidation of formaldehyde, is not an inhibitor. Thus, when formate was substituted for formaldehyde in the foregoing experiments, there was no interference with the phosphorylating respiration of succinate.

Insensitivity of uncoupled respiration to formaldehyde

The experiments summarized in Table II compare the effects of formaldehyde on coupled and uncoupled respiration. The mitochondria were uncoupled by treatment with dinitrophenol or by osmotic lysis and were shown to exhibit a rapid rate of succinate oxidation in the absence of phosphate-acceptor. This uncoupled respiration was not affected significantly by formaldehyde in concentrations which were sufficient to inhibit coupled respiration completely in the intact mitochondria. These results provided additional evidence that formaldehyde inhibits phosphorylation rather than electron transport.

The dinitrophenol-release of succinate respiration in the presence of formaldehyde

If formaldehyde inhibits coupled respiration by blocking a step in the phosphorylating sequence, it should be possible to relieve the inhibition with dinitro-

TABLE II

INABILITY OF FORMALDEHYDE TO INHIBIT UNCOUPLED RESPIRATION

Portions of the mitochondria were incubated for 2 min at 30° with dinitrophenol (2 μmoles/mg protein), or were exposed to water for 15 min at 0°. 2 μmoles of formaldehyde were used where indicated. For further experimental details, see Table I.

Mitochondrial Preparation	*Additions*	*Respiration (mμ atoms O/min)*	
		−Formaldehyde	*+Formaldehyde*
Intact	(1) Succinate	53.2	60.8
	(2) ADP	210.9	70.3
Uncoupled by dinitrophenol	Succinate	163.4	169.1
Uncoupled by osmotic lysis	Succinate	171.0	150.1

phenol[6]. This was found to be the case as demonstrated by the data in Fig. 1. When dinitrophenol was added after the formaldehyde inhibition had been established (Curve a), there was an immediate release of the respiration. In the experiment described by Curve b, the simultaneous addition of ADP and succinate was made before inhibition by formaldehyde had become complete. Under these conditions the initial rate of phosphorylating oxidation was only slightly inhibited. However, when the inhibition by formaldehyde became maximal, the rate of succinate oxidation was

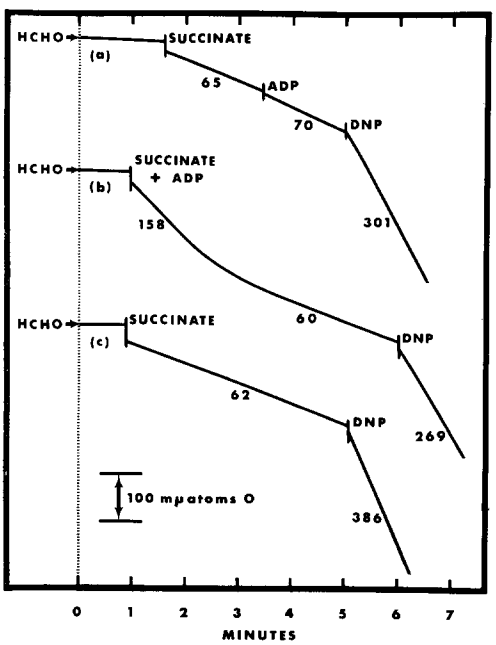

Fig. 1. Dinitrophenol-release of succinate respiration in presence of formaldehyde. Numbers below curves denote respiration in mμatoms O/min per mg protein. 2 μmoles of formaldehyde (HCHO) were present at the beginning of each incubation. Succinate (10 μmoles, 0.1 ml), ADP (0.45 μmole, 0.05 ml), and dinitrophenol (DNP) (2 μmoles, 0.02 ml) were added at the points indicated. Other experimental details are given in Table I.

returned to the lower level characteristic of controlled respiration. When added to this system, dinitrophenol again released the respiration as in Expt. a. From the data summarized by Curve c (Fig. 1), it can be seen that the controlled oxidation of succinate in presence of formaldehyde was linear throughout the period in which inhibition had occurred in Expts. a and b. Further, the controlled oxidation was essentially constant in all three cases.

Evidently, formaldehyde does not depress coupled respiration below the point of respiratory control, nor is the degree of control altered. Dinitrophenol, on the other hand, releases respiratory control whether or not there has been an inhibition of phosphorylating respiration by formaldehyde. It may be concluded, therefore, that these agents affect two different steps in oxidative phosphorylation and that the dinitrophenol-sensitive site lies between the respiratory chain and the step which is inhibited by formaldehyde.

Formaldehyde inhibition of the release of succinate respiration by arsenate

The uncoupling of respiration by arsenate is presumed to result from its substitution for phosphate in a step which otherwise leads to a stable phosphorylated intermediate[6]. In the experiments of Table III, arsenate was found to promote rapid, uncoupled respiration of succinate in the absence of added phosphate. When 2.5 μmoles of formaldehyde were present, however, most of the uncoupled respiration (84 %) was inhibited. These results suggest that formaldehyde interferes with the substitution of arsenate for phosphate and, by analogy, inhibits a reaction in the region of initial phosphorylation.

TABLE III

INHIBITION BY FORMALDEHYDE OF THE ARSENATE-RELEASE OF CONTROLLED RESPIRATION

Experimental conditions were the same as given in Table I, except that phosphate and ADP were omitted. 2.5 μmoles of sodium arsenate (0.05 ml) were added where indicated.

Formaldehyde (μmoles)	*Order of other additions*	*Respiration ($m\mu$ atoms O/min)*
None	(1) Succinate	57.0
	(2) Arsenate	201.4
2.5	(1) Succinate	53.2
	(2) Arsenate	76.0

With regard to possible mechanisms of inhibition of phosphorylation-respiration by formaldehyde, the question must be considered that formaldehyde might affect the permeability of the mitochondria to ADP. The experiments with arsenate provide evidence against such a mechanism, since the inhibition of arsenate uncoupling by formaldehyde does not involve exogenous ADP. It may be added that formaldehyde, at the level employed in these studies, apparently does not affect the morphology of intact mitochondria, insofar as no photometric changes were detected at 520 mμ.

The inhibition of ATP-dependent reduction of exogenous DPN$^+$ by formaldehyde and oligomycin

DPN$^+$ can be reduced anaerobically by succinate or sarcosine in the presence

of submitochondrial particles if energy is supplied in the form of ATP. It has been proposed that the energy pathway from external ATP to the site of DPN^+ reduction involves a reversal of the coupled reactions in oxidative phosphorylation[7–13]. The ability of formaldehyde to inhibit this energy-transfer process was tested in a system employing phosphorylating submitochondrial particles[14,15]. This preparation gave a P/DPNH ratio of 1.0, oxidized succinate, but did not catalyze any measurable reduction of DPN^+ by formaldehyde.

As shown in Fig. 2, the particles catalyzed an extensive ATP-dependent reduction of exogenous DPN^+ by succinate when the system was treated with cyanide. As expected, the reduction was inhibited immediately and completely by excess oligomycin. Formaldehyde also inhibited the reduction, but more slowly. It should be noted that when formaldehyde was present, the rate of DPNH accumulation reached a plateau in 3.5–4 min. This is the period of time required for inhibition of phosphorylating respiration in the previous experiments (see Fig. 1b).

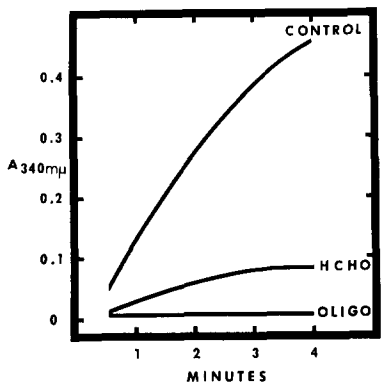

Fig. 2. Inhibition by formaldehyde or oligomycin of the ATP-dependent reduction of DPN^+ in phosphorylating sub-mitochondrial particles. Phosphorylating particles were prepared from rat-liver mitochondria[14,15]. The reduction of added DPN^+ was measured at 340 mμ in a Beckman Model DB spectrophotometer. Both cells initially contained: glycyl glycine (150 μmoles, pH 7.5), $MgCl_2$ (10 μmoles), and EDTA (2 μmoles), all in 0.5 ml; KCN (4 μmoles, 0.1 ml), DPN^+ (3 μmoles, 0.1 ml), succinate (20 μmoles, 0.2 ml), sub-mitochondrial particles (1.4 mg protein, 0.1 ml), and, when indicated: formaldehyde (HCHO) (5 μmoles, 0.1 ml) or oligomycin (OLIGO) (16 μg, 0.02 ml ethanol). After verification of equal absorbances, ATP (6 μmoles, 0.2 ml) was added to the sample cell and balanced by water in the reference. Final volumes, including appropriate amounts of water, were 3.0 ml.

The inhibition of ATP-induced contraction of mitochondria by formaldehyde and oligomycin

Mitochondrial swelling can be caused by a wide variety of reagents, and under appropriate conditions is reversible by the addition of ATP and Mg^{2+}. The ATP-induced contraction is probably energized through the reversal of at least a part of the phosphorylation sequence[16]. Evidence for such a reversal can be obtained by showing that contraction does not occur in the presence of oligomycin. The data in Table IV demonstrate that formaldehyde, like oligomycin, can prevent the ATP-induced contraction of mitochondria which have previously completed a spontaneous swelling cycle in the absence of added phosphate.

TABLE IV

INHIBITION OF ATP-INDUCED CONTRACTION OF INTACT MITOCHONDRIA BY FORMALDEHYDE OR OLIGOMYCIN

Intact mitochondria were suspended in a Tris–KCl medium without phosphate at 21°. Oligomycin or formaldehyde, where indicated, were present in the initial suspension. Changes in absorbance were followed in a Klett photometer with a No. 52 filter. ATP and $MgCl_2$ were added when minimum density had been reached (8 min, with or without inhibitor). Each Klett tube contained: Tris (74 μmoles, pH 7.5, 0.5 ml), KCl (463 μmoles, 3.2 ml), $MgCl_2$ (20 μmoles, 0.02 ml), ATP (24 μmoles, 0.2 ml), mitochondria (1.4 mg protein, 0.05 ml), and, when indicated: oligomycin (16 μg, 0.02 ml) or formaldehyde (10 μmoles, 0.1 ml). By addition of water, the volumes during the swelling phase were always 3.85 ml. Final volume in each case was 4.07 ml.

Inhibitors	*Increase of A_{520} per 2 min in presence of ATP*
None	0.176
16 μg oligomycin	0.012
10 μmoles formaldehyde	0.048

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