Biochemical Pharmacology, Vol. 31, No. 11, pp. 2025–2029, 1982.
Printed in Great Britain.

0006-2952/82/112025-05 $03.00/0
Pergamon Press Ltd.

INTERACTION OF AROMATIC ALDEHYDES WITH ISOLATED RAT LIVER MITOCHONDRIA*

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(*Received* 5 *September* 1981; *accepted* 20 *November* 1981)

Abstract—It has been suggested that aromatic aldehydes may reduce cytochrome *c* [Wolf *et al. Fedn Proc.* **39** (3), 1013 (1980)]. Therefore, interaction of the aromatic aldehydes, *p*-anisaldehyde, benzaldehyde, *p*-tolualdehyde, *p*-carboxybenzaldehyde, *p*-chlorobenzaldehyde and *p*-nitrobenzaldehyde, with rat liver mitochondria was examined *in vitro*. Although both pyruvate/malate- and succinate-mediated respiration, as well as that mediated by other citric acid cycle intermediates, were inhibited by the aromatic aldehydes (0.5 to 1.0 mM), cytochrome *c* oxidase was not inhibited by aromatic aldehydes (1.0 to 20 mM). There was a marked inhibition of succinic dehydrogenase and both ADP- and DNP-stimulated respiration by benzaldehyde (2 to 20 mM). Since both pyruvate/malate- and succinate-mediated respiration were inhibited by the aromatic aldehydes without inhibition of cytochrome *c* oxidase, several sites of inhibition, possibly both at the site of transport of substrates and the active enzymes, may exist. Benzaldehyde, 300 μM, inhibited pyruvate/malate-mediated state 3 respiration by 50% which suggests that no additional functional group or metabolism to another species is required for these inhibitory effects.

Aromatic aldehydes are used in many commercial products and are employed widely within the chemical industry. The toxicological properties of this group of compounds, however, have not been investigated extensively. Many substances, including toluenes, xylenes and benzylalcohols, are converted enzymatically to their aldehyde derivatives as part of the excretory process. For example, *p*-xylene is metabolized and excreted predominantly as *p*-toluic acid. This acid is formed after the *p*-xylene is first converted to *p*-methylbenzylalcohol by a cytochrome P-450-catalyzed reaction and then to *p*-methylbenzaldehyde and *p*-methylbenzoic acid by NAD-dependent alcohol and aldehyde dehydrogenases, respectively [1]. Some toxic effects of aromatic aldehydes on pulmonary cytochrome P-450 dependent monooxygenase systems have been reported [2]. In addition, it has been suggested that cytochrome *c* may interact with aromatic aldehydes to generate free radicals [3].

In the current study, the interaction of a series of aromatic aldehydes with mitochondrial respiration was examined *in vitro*. The possible sites of inhibition were evaluated with polarographic and enzymic techniques. The toxicological implications of these observations are reviewed.

MATERIALS AND METHODS

Animals. Adult male, specific pathogen-free rats (Charles River Breeding Laboratories, Wilmington, MA, CD strain) were used in these experiments.

Chemicals. L-Ascorbic acid, ethyleneglycol-bis (β-amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), phenazine methylsulfate (PMS), 2,4-dinitrophenol (DNP) and bovine serum albumin (BSA) were obtained from the Sigma Chemical Co., St. Louis, MO. *N,N,N',N'*-Tetramethyl-*p*-phenylene diamine HCl (TMPD) was obtained from ICN-K & K Laboratories, Inc., Plainview, NY. Aromatic aldehydes, benzaldehyde, *p*-anisaldehyde, *p*-tolualdehyde, *p*-chlorobenzaldehyde, *p*-nitrobenzaldehyde and *p*-carboxybenzaldehyde, were obtained from the Aldrich Chemical Co., Milwaukee, WI. All other substrates and cofactors were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN.

Mitochondrial preparation. The liver was removed, weighed, and homogenized [5% (w/v) suspension] with a Potter–Elvehjem tissue grinder in a medium that contained 5 mM Tris–HCl, 0.3 M sucrose and 0.5 mM EGTA, pH 7.5. The mitochondria were isolated and washed in isotonic Tris–KCl [20 mM Tris and 125 mM KCl (pH 7.4)] as described previously [4]. The final mitochondrial pellet was suspended in isotonic Tris–KCl and contained 30–40 mg protein/ml buffer.

Assay methods. Under the conditions chosen, the reaction rates were linear with time and protein concentration. All assays were performed in duplicate on each mitochondrial preparation.

Succinic dehydrogenase (SDH, EC 1.3.99.1) was

* A preliminary report was given at the annual meeting of the Federation of American Societies of Experimental Biology, San Diego, CA, April 1980 [Wolf *et al., Fedn Proc.* **39** (3), 1013 (1980)].

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assayed spectrophotometrically by measuring the rate of reduction of cytochrome *c* at 550 nm in the following reaction mixture, V_t (total volume) = 3 ml: 0.1 M potassium phosphate buffer, pH 7.6, 1 mM EDTA, 0.1% BSA, 0.05% cytochrome *c*, 0.00025% PMS, and 1 mM KCN, before and after the addition of 6 mM succinate. Under these conditions, each 2 μmoles of reduced cytochrome *c* indicates the oxidation of 1 μmole of succinate to form fumarate [5].

Cytochrome *c* oxidase (EC 1.9.3.1) was assayed polarographically by measuring the rate of oxygen consumption in the following reaction mixture, V_t = 2 ml: 75 mM potassium phosphate buffer, pH 7.2, 0.03 mM cytochrome *c*, 3.75 mM sodium ascorbate, and 0.3 mM TMPD. The rate of oxygen consumption was calculated based on the solubility of oxygen in water at 27° [6].

Mg^{2+}-stimulated adenosine 5′-triphosphatase (Mg^{2+}-ATPase, EC 3.6.1.3) was assayed by measuring the rate of oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm in the following reaction mixture, V_t = 3 ml: 30 mM Tris–HCl buffer, pH 7.4, 3 mM $MgCl_2$, 10 mM KCl, 0.5 mM NADH, 3 mM sodium ATP, 7.5 mM phosphoenol pyruvate, 10 units pyruvate kinase and 2 units lactate dehydrogenase. The rate of DNP-stimulated Mg^{2+}-ATPase was determined in the presence of 70 μM DNP. Under these conditions, each 1 μmole of oxidized NADH indicates the hydrolysis of 1 μmole of ATP [7].

Other methods. Respiration was measured polarographically with a Yellow Springs model YS 5331 Clark oxygen electrode (see Table 1 for details of the incubation conditions) [8]. Respiratory control ratios (RCR) were assessed by comparing the state 3 (ADP present) rate with the state 4b (ADP depleted) rate following complete utilization of the added ADP [9]. The ADP:O values were calculated from the amount of ADP added and the traces of oxygen uptake recorded [9, 10].

Protein was determined with the phenol reagent [11] and with BSA as the standard.

RESULTS

Effects of aromatic aldehydes on pyruvate/malate-mediated mitochondrial respiration. The effects of substituted, aromatic aldehydes on the pyruvate/malate- and the succinate-mediated respiration of mitochondria isolated from rat liver were examined polarographically. The initial experiments, employing a final concentration of 0.5 mM aromatic aldehyde, revealed inhibition of pyruvate/malate-mediated respiration as compared to control and solvent (1% ethanol) values (Table 1). Each of the aromatic aldehydes inhibited pyruvate/malate-mediated state 3 respiration to some extent, e.g., 65.9 to 26.9 ng-atom O/min-mg as compared to the solvent value of 80.4 ng-atom O/min-mg. The inhibition of state 3 respiration by *p*-tolualdehyde and *p*-anisaldehyde is reflected in the decrease in respiratory control. Four of the aromatic aldehydes, benzaldehyde, *p*-chlorobenzaldehyde, *p*-nitrobenzaldehyde and *p*-carboxybenzaldehyde, inhibited state 3 respiration and also prevented the return of respiration to state 4, i.e. state 4b is similar to state 3. Both benzaldehyde and *p*-nitrobenzaldehyde stimulated state 4a respiration as compared with the solvent value.

A representative oxygen consumption experiment is presented to illustrate the effects of the solvent (1% ethanol) and 1 mM benzaldehyde on pyruvate/malate-mediated respiration (Fig. 1a). The tracings indicate that ethanol produced only a small alteration in oxygen consumption. The presence of 1 mM benzaldehyde inhibited state 3 respiration and decreased respiratory control, i.e. no return to state 4. At 1 mM benzaldehyde, no ADP stimulation of pyruvate/malate-mediated respiration was observed and, conversely, if 1 mM benzaldehyde was added after ADP stimulation of state 4 respiration, marked

Table 1. Comparison of the inhibition of rat liver pyruvate/malate-mediated respiration by aromatic aldehydes

Experiment	Oxygen consumption* (ng-atom O/min-mg mitochondrial protein)			ADP:O†	RCR†
	State 4a	State 3	State 4b		
Control	11.0	89.7	27.6	2.53	3.2
Ethanol, 1%	19.7	80.4	26.3	2.25	3.1
p-Tolualdehyde, 0.5 mM	19.7	65.9	31.6	1.93	2.1
p-Anisaldehyde, 0.5 mM	17.1	62.0	26.3	1.88	2.4
Benzaldehyde, 0.5 mM	23.7	50.1	50.1		
p-Chlorobenzaldehyde, 0.5 mM	15.8	38.2	38.2		
p-Nitrobenzaldehyde, 0.5 mM	25.0	31.6	31.6		
p-Carboxybenzaldehyde, 0.5 mM	15.8	26.9	26.9		

* Mitochondria (3.0 mg protein) from rat liver were suspended in 2.0 ml of a medium (pH 7.4, 27°) that contained 125 mM KCl, 20 mM Tris–HCl, 2 mM K_3PO_4 and 1 mM $MgCl_2$. Aromatic aldehydes dissolved in ethanol were introduced into the medium and were followed 1 min later by the addition of 2 mM pyruvate and 1 mM malate, 0.05 mM ADP and then 0.2 mM DNP at certain intervals. Oxygen consumption was measured polarographically.

† Both ADP:O and respiratory control ratios (RCR = state 3/state 4b) were calculated from the oxygen consumption traces as described in Materials and Methods.

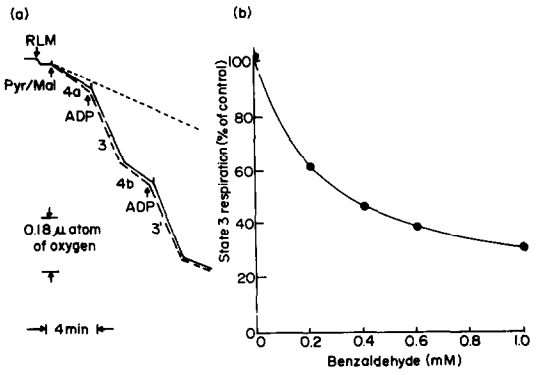

Fig. 1. Inhibition of pyruvate/malate-mediated respiration with benzaldehyde. (a) Oxygen consumption tracings. Key: (———) control, (———) 1% ethanol, and (-----) 1 mM benzaldehyde. (b) Concentration dependence of the benzaldehyde inhibition of state 3 respiration. Experimental conditions were the same as for Table 1.

inhibition occurred (data not shown). The addition of DNP (0.1 mM) was also without effect on pyruvate/malate-stimulated respiration in the presence of 1.0 mM benzaldehyde. The gradual inhibition of state 3 respiration with increasing concentrations of benzaldehyde is shown in Fig. 1b. Fifty percent inhibition occurred at 0.3 mM benzaldehyde.

Effects of aromatic aldehydes on succinate-mediated mitochondrial respiration. The inhibition of succinate-mediated respiration by aromatic aldehydes was less than that observed with pyruvate/malate-mediated respiration (Table 2). Some respiratory control remained with four of the aromatic aldehydes at a final concentration of 1 mM. Only *p*-chlorobenzaldehyde and *p*-nitrobenzaldehyde prevented the return to state 4 respiration.

A typical oxygen consumption experiment with succinate-mediated respiration is shown in Fig 2a. At 7.5 mM benzaldehyde state 3 respiration was inhibited and no respiratory control was observed (Fig. 2b). In contrast to the inhibition of the pyruvate/malate-mediated state 3 respiration, benzaldehyde at 1 mM inhibited succinate-mediated respiration 10% (Figs. 1b and 2b). The concentration-dependent inhibition of ADP- and DNP-stimulated respiration is illustrated further in Fig. 3. In each case, the ADP or DNP stimulation occurred prior to the addition of benzaldehyde.

Inhibition of mitochondrial enzymes. The possible sites of action of the aromatic aldehydes were examined further by monitoring their effects on several mitochondrial enzymes. Since benzaldehyde inhibited both pyruvate/malate- and succinate-mediated respiration in the polarographic studies, isolated mitochondria were assayed for cytochrome *c* oxidase activity (see Materials and Methods) in the absence and presence of various concentrations of benzaldehyde (0–25 mM). Within this range of benzaldehyde concentrations, there was no marked inhibition of cytochrome *c* oxidase (data not given). In contrast, the concentration dependence of the inhibition of succinic dehydrogenase by benzaldehyde, as shown in Fig. 4, indicated 50% inhibition at 11 mM benzaldehyde. At this time, the *in vitro* effects of the aromatic aldehydes on pyruvate dehydrogenase activity have not been determined.

Since it is well known that mitochondrial, latent ATPase activity is stimulated by uncouplers [12], the effects of the aromatic aldehydes, which stimulated respiration, on mitochondrial ATPase activity were measured. While DNP (70 μM) stimulated ATPase activity about 3-fold, there were varying degrees of stimulation of activity by the aromatic aldehydes. The most effective stimulator of ATPase activity was *p*-nitrobenzaldehyde, i.e. approximately 2-fold stimulation at 1 mM *p*-nitrobenzaldehyde.

DISCUSSION

Preliminary anaerobic studies revealed a gradual reduction of cytochrome *c* in the presence of each of the aromatic aldehydes [3]. Although confirmatory experiments to establish the involvement of a free radical intermediate were not possible, these results were of concern because of the central role of cytochrome *c* in mitochondria respiration. If there were a free radical intermediate formed from the aldehyde, the concomitant reduction of the cyto-

Table 2. Comparison of the inhibition of rat liver succinate-mediated respiration by aromatic aldehydes*

Experiment	Oxygen consumption (ng-atom O/min-mg mitochondrial protein) State 4a	State 3	State 4b	ADP:O	RCR
Control	15.8	130.5	19.7	1.51	6.6
Ethanol, 1%	17.2	121.7	23.7	1.56	5.2
Benzaldehyde, 1 mM	21.4	102.5	29.4	1.40	3.5
p-Carboxybenzaldehyde, 1 mM	24.5	98.9	33.7	1.31	2.9
p-Anisaldehyde, 1 mM	14.6	83.1	18.5	1.35	4.5
p-Tolualdehyde, 1 mM	16.6	77.9	26.5	1.2	2.9
p-Chlorobenzaldehyde, 1 mM	26.1	76.3	76.3		
p-Nitrobenzaldehyde, 1 mM	20.5	47.4	47.4		

* Experimental conditions were the same as for Table 1 except for the presence of 1.2 mM succinate instead of pyruvate/malate and that the aromatic aldehydes were present at a final concentration of 1 mM.

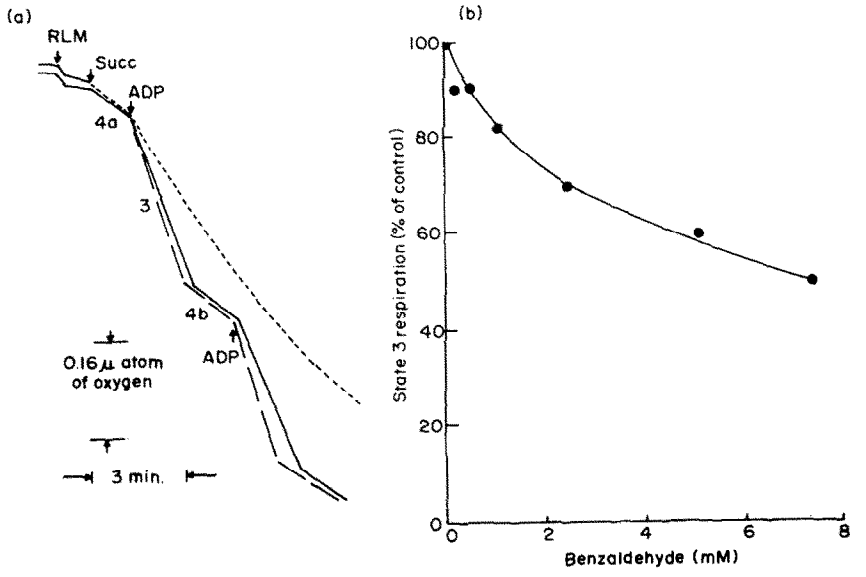

Fig. 2. Inhibition of succinate-mediated respiration with benzaldehyde. (a) Oxygen consumption tracings. Key: (———) control, (———) 1% ethanol, and (– – – –) 1 mM benzaldehyde. (b) Concentration dependence of the benzaldehyde inhibition of state 3 respiration. Experimental conditions were the same as for Table 1.

chrome *c* Fe^{3+} might saturate the electron transport chain with electrons and, thus, prevent the flow of electrons from other donors. This possibility was supported by the inhibition for both pyruvate/malate- and succinate-mediated respiration by the aldehydes; however, no marked inhibition of cytochrome *c* oxidase was observed.

The present NAD-dependent aldehyde dehydrogenase in liver may utilize the aromatic aldehydes as substrates and catalyze the conversion to the corresponding acids; however, this enzyme is located predominantly in the cytosol [13–15] and no NAD was added to the mitochondrial preparations. In addition, it is known that acetaldehyde NAD-dependent dehydrogenase present in mitochondria has a low affinity for aromatic aldehydes [16]. The effects observed may then be attributed to the various structures of the aromatic aldehydes and not to the various structures of the corresponding acids.

Since both pyruvate/malate- and succinate-mediated respiration were inhibited by the aromatic aldehydes without inhibition of cytochrome *c* oxidase, there may be several possible inhibitions, i.e. at the site of transport of substrates and the active enzymes. The effects of aromatic aldehydes on mitochondrial anion transport and pyruvate dehydrogenase were not investigated at this time. Respiration mediated by other citric acid cycle intermediates, i.e. citrate, α-ketoglutarate and L-malate, was also

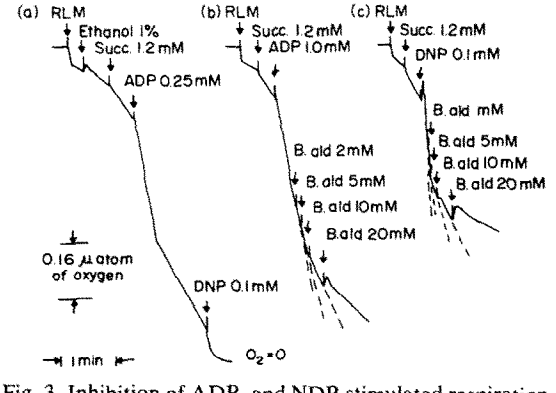

Fig. 3. Inhibition of ADP- and NDP-stimulated respiration of isolated rat liver mitochondria by benzaldehyde. Experimental conditions were the same as for previous polarographic studies except that the concentration of mitochondrial protein was 1.02 mg/ml and that benzaldehyde was added after the respiration rate had been stimulated.

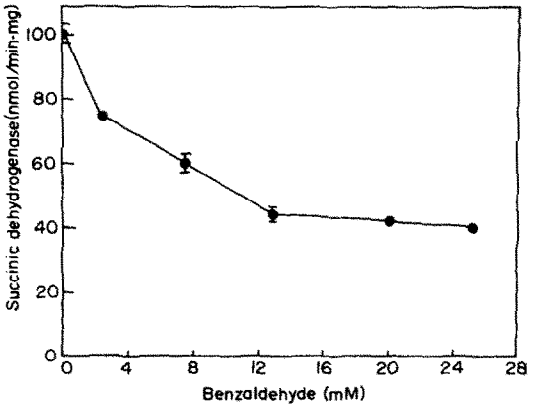

Fig. 4. Concentration dependence of the inhibition of mitochondrial succinic dehydrogenase by benzaldehyde. The experimental conditions are described in Materials and Methods. The concentration of mitochondrial protein in the assay was 0.033 to 0.038 mg/ml. Values are means ± S.E.M. for four separate mitochondrial preparations. Where not shown, the standard error is smaller than the symbol used.

inhibited by the aldehydes which indicates the likelihood of a common site of inhibition. There was a marked inhibition of succinic dehydrogenase by benzaldehyde. In addition, there was inhibition of both ADP- and DNP-stimulated respiration by benzaldehyde.

The choice of benzaldehyde for many of the experiments was based not on maximal inhibition of respiration, but rather on its relatively simple structure, i.e. no other functional groups are present. Since these effects were seen with benzaldehyde, the presence of an electron-withdrawing group is not necessary.

In summary, we have demonstrated that these aromatic aldehydes inhibited mitochondrial respiration mediated either by pyruvate/malate or succinate. Succinate dehydrogenase activity was also inhibited by benzaldehyde. Although cytochrome *c* oxidase was not inhibited by benzaldehyde, both ADP- and DNP-stimulated respiration were. Monoamine oxidase activity present in the mitochondria may generate localized concentrations of aromatic aldehydes from the corresponding amines. These effects suggest that aromatic aldehydes, such as benzaldehyde, interact with important electron transport systems independent of the presence of other functional groups and metabolism to another species.

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