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IN VITRO EFFECTS OF SUBSTITUTED TOLUENES ON MITOCHONDRIA ISOLATED FROM RAT LIVER

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Abstract—3,5-Dinitro-4-chloro-α,α,α-trifluorotoluene (DNCTT) is an intermediate in the synthesis of the herbicide trifluralin and is structurally similar to the known uncouplers of oxidative phosphorylation 2,4-dinitrophenol (DNP) and pentachlorophenol. Therefore, investigations were initiated to examine the *in vitro* effects of DNCTT on the respiration of isolated rat liver mitochondria. DNCTT (1 mM) completely inhibited mitochondrial respiration with succinate (1.2 mM) as substrate and in the presence of adenosine 5'-diphosphate (0.1 mM). Although DNP (0.2 mM) uncoupled state 4 respiration in the absence of DNCTT, DNP (0.2 mM) did not uncouple DNCTT inhibited respiration. In addition, DNCTT (2.5 mM) inhibited significantly the activity of the mitochondrial enzyme succinic dehydrogenase, but had no effect on the activity of cytochrome *c* oxidase and Mg-stimulated adenosine 5'-triphosphatase. The inhibitory effects of DNCTT were greater than those of eight other substituted toluenes that were examined. From the results of these studies, it appears that the effects of DNCTT on respiratory activity are a consequence of the inhibition of the mitochondrial enzyme succinate dehydrogenase.

3,5 - Dinitro - 4 - chloro - α,α,α - trifluorotoluene (DNCTT) is an intermediate in the synthesis of the widely used herbicide trifluralin (commonly known as Treflan). Trifluralin (3,5-dinitro-4-*N,N*-dipropyl-α,α,α-trifluorotoluene) has a low acute toxicity in mammals (acute LD_{50} oral 5.0 g/kg, mice [1]). However, it increases the incidence of hepatocellular carcinomas in female mice, while not effecting a similar increase in male mice or male and female rats [2]. When trifluralin was examined in a variety of microbial test systems, no mutagenic activity was detected [3].

Recently, environmental contamination by α,α,α-trifluorotoluene derivatives was observed in an area of northeastern Italy [4]. Some of the well water was found to be tinted yellow and smelled unpleasant. The levels of 4-chloro-α,α,α-trifluorotoluene (CTT), 3-nitro-4-chloro-α,α,α-trifluorotoluene (NCTT) and DNCTT detected in the water from the region ranged from 0.50 to 90, 0.6 to 450 and 0.7 to 56 μg/l, respectively [5]. Initial examination of these three derivatives indicated no mutagenic activity in microbial tests with and without *in vivo* and *in vitro* activation [6]. Results from the DNA repair test with human epithelial cells were positive for CTT and NCTT, but DNCTT showed no genotoxic activity [7]. Little additional information is currently available concerning the chronic toxicity of these compounds.

DNCTT is structurally similar to the known uncouplers of oxidative phosphorylation 2,4-dinitrophenol and pentachlorophenol. Therefore, investigations were initiated to examine the *in vitro* effects of DNCTT and its analogues on the mitochondrial respiration of isolated rat liver mitochondria.

MATERIALS AND METHODS

Animals. Adult male, specific pathogen-free rats (Charles River, CD strain) were used in these experiments. The animals were fed *ad lib.* on Wayne Sterilizable Lab-Blox and weighed from 200 to 250 g. The animals were decapitated at 9:00 a.m.

Chemicals. L-Ascorbic acid, ethyleneglycol-bis (β-amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), cytochrome *c* (Type III), (ethylenedinitrilo)tetra-acetic acid (EDTA), 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. Substituted, α-halogenated toluenes 3,5-dinitro-4-chloro-α,α,α-trifluorotoluene (DNCTT), 3,4-dichloro-α,α,α-trifluorotoluene (DCTT), 3-amino-α,α,α-trifluorotoluene (NH_2TT), 3-nitro-α,α,α-trifluorotoluene (NTT) and 3-nitro-4-chlorotrifluorotoluene (NCTT) were provided by Professor B. Paccagnella, Institute of Hygiene, Verona, Italy. All other substrates and cofactors were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Mitochondrial preparation. The liver tissue was quickly removed, weighed, and homogenized as a 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 [8]. The final mitochondrial pellet was resuspended in isotonic Tris–KCl and contained 30–40 mg/ml protein.

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

Succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed spectrophotometrically by measuring the rate of reduction of cytochrome *c* at 550 nm in the following reaction mixture, V_t = 3 ml: 0.1 M potassium phosphate buffer, pH 7.6, 1 mM EDTA, 0.1% BSA, 0.05% cytochrome *c*, 0.00025% phenazine methylsulfate and 6 mM succinate. Under these conditions, each 2 μmoles of reduced cytochrome *c* indicates the oxidation of 1 μmole of succinate to form fumarate [9].

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° [10].

Mg-stimulated adenosine 5′-triphosphatase (Mg-ATPase, EC 3.6.1.3) was assayed by measuring colorimetrically the liberation of inorganic phosphate [11] in the following reaction mixture, V_t = 3.0 ml: 0.03 M Tris–HCl buffer, pH 7.1, 5 mM ATP, 7.5 mM $MgCl_2$, and, when added, 0.1 mM DNP. Under these conditions the liberation of 1 μmole of inorganic phosphate indicates the hydrolysis of 1 μmole of ATP [12].

Other methods. Respiration was measured polarographically with a Yellow Springs model YS5331 Clark oxygen electrode (see Fig. 1 for details of the incubation conditions [13]). Respiratory control ratios were assessed by comparing the state 3 rate with the state 4b rate following complete utilization of the added ADP [14]. The ADP:O values were calculated from the amount of ADP added and the traces of oxygen uptake recorded [14, 15].

Protein was determined with the phenol reagent [16] and with bovine serum albumin as the standard.

RESULTS

Effects of substituted toluenes on mitochondrial respiration. The effects of ring-substituted, α,α,α-trifluorotoluenes on the respiration of mitochondria isolated from rat liver were examined polarographically. The initial experiments utilizing final concentrations of 0.25–1.0 mM substituted toluenes revealed both stimulation and inhibition of respiration. A representative oxygen consumption experiment is presented to illustrate the effects of the solvent (1% ethanol), 1 mM DNCTT and 1 mM NCTT on mitochondrial respiration (Fig. 1). Tracing 1a indicates that ethanol produced only a small alteration in oxygen consumption. The presence of 1.0 mM DNCTT completely inhibited respiration, while 1.0 mM NCTT markedly altered respiratory control, i.e. state 4b is similar to state 3.

The effects of five substituted toluenes on mitochondrial respiration are compared to control and solvent (1% ethanol) values in Table 1. A remarkable decrease in respiratory control was observed in the presence of ring-substituted, α,α,α-trifluorotoluenes such as NCTT, NH_2TT and DCTT, at a final concentration of 0.25 mM. DNP-stimulated respiration was also inhibited. The decrease in respiratory control caused by these substituted toluenes resulted from an inhibition of state 3 respiration and/or an acceleration of state 4 respiration. In contrast, NTT

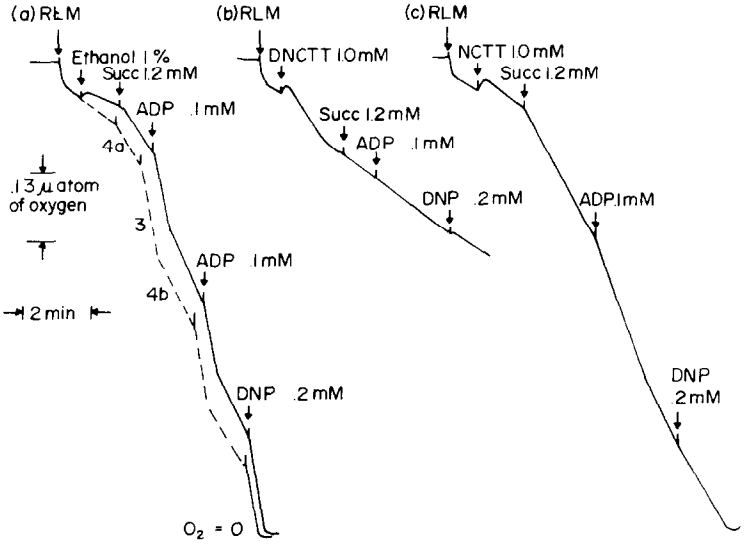

Fig. 1. Effects of ethanol, DNCTT, and NCTT on the respiratory activity of isolated rat liver mitochondria. Mitochondria (4.4 mg) from rat liver were suspended in 2.0 ml of a medium (pH 7.4, 27°) that contained 125 mM KCl, 20 mM Tris–HCl, 2 mM K_3PO_4 and 1 mM $MgCl_2$. Substituted, α-halogenated toluenes dissolved in ethanol were introduced into the medium and were followed 1 min later by the addition of 1.2 mM sodium succinate as substrate, 0.1 mM ADP, and then 0.2 mM DNP, at certain intervals. Oxygen consumption was measured polarographically. Key: (a) – – – control, 1% ethanol; (b) 1.0 mM DNCTT; and (c) 1.0 mM NCTT.

Table 1. Comparison of the inhibition of rat liver mitochondrial respiration by substituted, α-halogenated toluenes

Experiment	Oxygen consumption* (natom O/min·mg mitochondrial protein) State 4a	State 3	State 4b	DNP	RCR†	ADP:O†
Control	31.1	120.1	31.1	124.2	3.9	1.9
Ethanol, 1%	22.8	132.5	39.3	192.8	3.4	1.5
NCTT, 0.25 mM	37.3	58.0	31.1	2.1	1.9	2.0
NTT, 0.25 mM	89.0	285.5	190.8	264.9	1.9	1.5
NH_2TT, 0.25 mM	39.3	105.6	93.2	4.2	1.3	2.0
DCTT, 0.25 mM	24.9	95.2	49.7	72.5	1.9	1.7
DNCTT, 0.25 mM	8.3	8.3	8.3	8.3	1.0	

* Mitochondria (3.0 mg protein) were incubated with 0.25 mM substituted toluene dissolved in ethanol for at least 1 min before adding 1.2 mM sodium succinate, 0.05 mM ADP, and then 0.2 mM DNP. Other conditions were the same as for Fig. 1.

† 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.

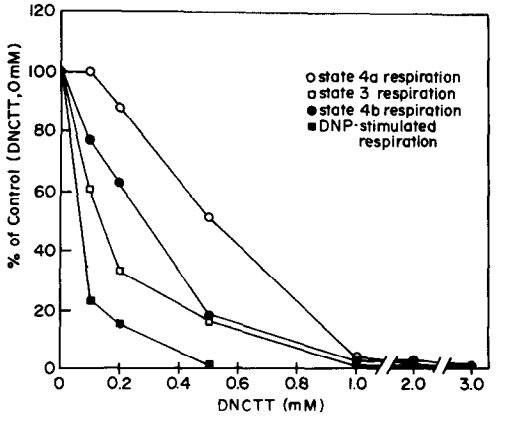

Fig. 2. Concentration dependence of the DNCTT inhibition of respiratory activity of isolated rat liver mitochondria. Experimental conditions were the same as for Fig. 1. Key: (○–○) state 4a respiration (succinate present); (□–□) state 3 respiration (ADP present); (●–●) state 4b respiration (ADP expended); and (■–■) DNP-stimulated respiration.

increased state 4a, 3 and 4b as well as DNP-stimulated respiration. Of the substituted toluenes examined, DNCTT produced the most complete inhibition of respiration. The rate of oxygen consumption in the presence of DNCTT (0.25 or 1.0 mM) was essentially the same as that of the background rate and was not stimulated by either ADP or DNP. In addition, it was observed that at final concentrations of 1 mM CTT, 2-fluoro-benzyl chloride (FBC), 2-chloro-6-fluorotoluene (CFT) and 2-chloro-6-fluorobenzaldehyde (CFB) had no effect on respiratory activity (data not shown).

The concentration dependence of the inhibition of DNCTT on mitochondrial respiration and ADP- and DNP-stimulated respiration is shown in Fig. 2. DNP-stimulated respiration was the most sensitive to DNCTT i.e., 82 per cent inhibition at 0.1 mM DNCTT. Succinate-mediated respiration, state 4a, was the least sensitive to DNCTT, i.e. only 50 per cent inhibition at 0.5 mM DNCTT. This concentration-dependent inhibition of ADP- and DNP-stimulated respiration is illustrated further in Fig. 3. In

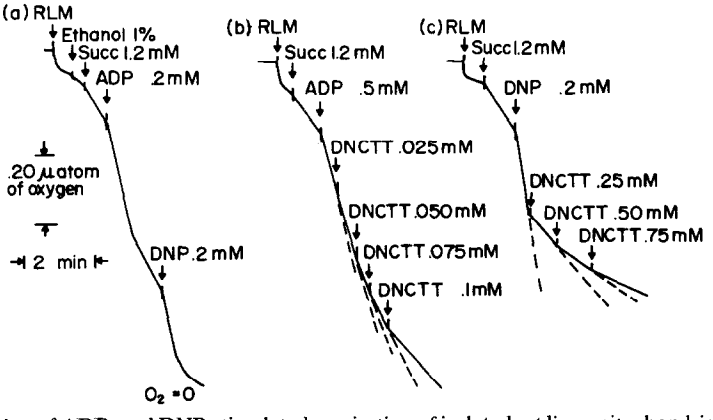

Fig. 3. Inhibition of ADP- and DNP-stimulated respiration of isolated rat liver mitochondria by DNCTT. Experimental conditions were the same as for Fig. 1 except for the concentration of mitochondrial protein (1.5 mg/ml) and that DNCTT was added after the respiration rate had been stimulated.

Table 2. Inhibition of succinic dehydrogenase and cytochrome *c* oxidase activities of rat liver mitochondria by DNCTT

Enzyme	Control (Ethanol, 2.2%)	DNCTT (2.5 mM)	Inhibition (%)
Succinic dehydrogenase	10.6 ± 1.86*	0.51 ± 0.24	94.4 ± 2.6†
Cytochrome *c* oxidase	0.143 ± 0.022	0.119 ± 0.019	10.1 ± 4.9†

* Measurements are given in μmoles/(min·mg) of mitochondrial protein and are expressed as the mean ± S.E.M. for the determinations on four separate preparations of rat liver mitochondria. Aliquots of mitochondria were incubated with 2.5 mM DNCTT for 2 min before the addition of the final assay reagent. Other conditions were as described in Materials and Methods.

† Comparison of activity of control and DNCTT-inhibited preparations by a paired *t*-test indicated a statistically significant difference for SDH only ($P < 0.05$).

contrast to the previous experiments, the ADP or DNP stimulation occurred prior to the additions of DNCTT in the tracings in Fig. 3.

Inhibition of mitochondrial enzymes. The possible sites of action of DNCTT were examined further by monitoring the effects of DNCTT on several mitochondrial enzymes. Since DNCTT inhibited succinate-mediated respiration in the polarographic studies, isolated mitochondria were assayed for succinic dehydrogenase activity in the absence and presence of 2.5 mM DNCTT (Table 2). Whereas 2.5 mM DNCTT markedly inhibited mitochondrial succinic dehydrogenase activity, only a slight effect was seen on cytochrome *c* oxidase activity. The concentration dependence of the inhibition of succinic dehydrogenase by DNCTT is illustrated in Fig. 4. This concentration–inhibition curve is similar to that obtained for the DNCTT inhibition of mitochondrial respiration (Fig. 2).

It is well known that mitochondrial, latent ATPase activity is stimulated by uncouplers and that this stimulated ATPase activity is inhibited by certain energy transfer inhibitors such as oligomycin or respiratory inhibitors [17]. Neither NCTT nor DNCTT (final concentrations of 0.01 to 1.0 mM), however, had any effect on mitochondrial ATPase activity when assayed as described in Materials and Methods (data not shown).

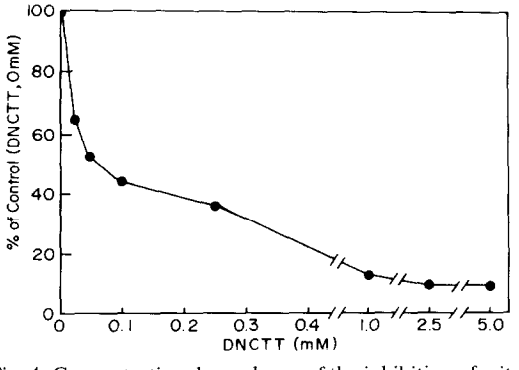

Fig. 4. Concentration dependence of the inhibition of mitochondrial succinic dehydrogenase by DNCTT. The experimental conditions are described in Materials and Methods. The concentration of mitochondrial protein in the assay was 0.50 mg/ml.

DISCUSSION

Since the recent episode of environmental contamination by the intermediates in the synthesis of the herbicide trifluralin, there has been increasing concern about the biological effects of the substituted, α-halogenated toluenes. Of special interest is the compound DNCTT since it has been detected in well water and is structurally similar to known uncouplers of oxidative phosphorylation. Although chronic toxicity studies are not available, the results of the current *in vitro* investigation indicate possible harmful effects.

A screen for an effect on the respiratory activity of isolated mitochondria reveals four levels of response to the substituted toluenes (final concentrations, 0.25–1 mM). These are: (1) no effect on respiratory activity, e.g. CTT, FBC, CFT and CFB; (2) stimulation of respiratory activity, e.g. NTT; (3) moderate inhibition of respiratory activity, e.g. NCTT, NH_2TT and DCTT; and (4) complete inhibition of respiratory activity, e.g. DNCTT. No simple structure inhibitory relationship is obvious from these results. The presence of the three ring-substituents (3,5-dinitro-4-chloro-), however, conveys marked inhibitory properties.

Detailed study of the effects of DNCTT indicates that this compound readily inhibits both state 4a and state 3 respiration in the presence of succinate and ADP, respectively, and when ADP is expended (Fig. 1 and Table 1). In addition, both ADP- and DNP-stimulated respiration is inhibited by DNCTT (Fig. 3). These data suggest that the site of action of DNCTT is before the energy transfer system. The lack of effect of DNCTT on Mg-ATPase or DNP-stimulated Mg-ATPase activities supports this suggestion.

In addition, DNCTT markedly inhibits succinic dehydrogenase, which is the initial enzyme involved in the utilization of succinate as a substrate for mitochondrial oxidation. The concentration-dependent inhibition curve obtained for SDH (Fig. 4) is strikingly similar to that obtained for the DNCTT inhibition of respiratory activity in state 3, with ADP present, (Fig. 2). As a consequence of these investigations, it appears that the effect of DNCTT on respiratory activity is a result of the inhibition of the mitochondrial enzyme SDH.

These investigations do not reveal a similarity between the effects of DNP and DNCTT on respi-

ration. It has been demonstrated, however, that some of the substituted toluenes are marked inhibitors of mitochondrial respiratory activity, especially DNCTT. In view of the marked effects of DNCTT on mitochondrial activity, further work on the precise mechanism of action and chronic toxicity of DNCTT is warranted.

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