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Dibutylchloromethyltin Chloride, a Potent Inhibitor of Electron Transport in Plant Mitochondria

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Abstract

Dibutylchloromethyl tin chloride (DBCT) inhibits coupled and uncoupled respiration of mitochondria from potato tubers, cauliflower florets and etiolated mung bean hypocotyls with succinate and L-malate but not with external NADH or TMPD/ascorbate as substrates. Using potato and cauliflower mitochondria, DBCT at 200 pmole/mg of protein gives complete inhibition only in KCl-based media and at pH 6.8. DBCT has no effect on the internal pH of mung bean mitochondria, but does cause a decrease in the membrane potential. Electron transport through the alternative oxidase is not inhibited, neither is the ATP-synthase system. DBCT appears to interact with the functionally-distinct pool of ubiquinone associated with the oxidation of succinate and L-malate.

Introduction

Trialkyltin compounds are potent inhibitors of oxidative phosphorylation [1–3] and inhibit dinitrophenol-stimulated ATPase activity [4–5] in a variety of energy-conserving membranes. The effects of such compounds on mitochondrial functions have been shown to be complex. Several modes of action

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have been ascribed to them which are to some extent dependent on the nature of the incubation medium in which the mitochondria are suspended. Thus in a nonionic suspension medium triethyltin binds to an inhibitory site on the mitochondrial membrane where it manifests a mode of action similar to that of oligomycin [6–9]. However, when mitochondria are suspended in KCl-based media, there is a considerable inhibition of dinitrophenol-stimulated oxidation of succinate or NAD^+-linked substrates, but not of ascorbate linked to TMPD[1] [10] or β-hydroxybutyrate [11, 12]. Since Cl^- ions are required to observe inhibition of uncoupled respiration, Selwyn et al. [13] postulated that the primary action of triethyltin was to facilitate a Cl^-/OH^- exchange across the mitochondrial membrane. Coleman and Palmer [10] found that triethyltin was more effective at catalyzing the Cl^-/OH^- exchange at more acid pH values. They suggested that this would result in a decrease in the matrix substrate content thus accounting for the greater sensitivity of uncoupled respiration to triethyltin in a KCl-based reaction medium. Recent observations [11, 14], however, suggest that the decrease in substrate content is not sufficient to account for the characteristics of inhibition observed in halide media. Indeed, Dawson and Selwyn [15] suggest that the observed inhibition may be due to changes in intramitochondrial pH values, due to the operation of a trialkyltin-mediated anion/OH^- exchange. This lowered intramitochondrial pH is thought to have a direct effect on the activity of the electron-transport chain rather than an effect on substrate accumulation.

Recently, a further trialkyltin derivative, DBCT, has been described [16–18], which appears to behave as a specific covalent inhibitor of mitochondrial ATPase and oxidative phosphorylation when the mitochondria are suspended in a sucrose-based medium. Furthermore, it has been suggested that this inhibitor binds specifically to a component of the ATP-synthase complex that has the properties of an intermediate of oxidative phosphorylation [17] (see refs. 19 and 20 for contrary interpretations). In an attempt to understand the complex actions of trialkyltin derivatives on mitochondrial functions, we have examined the effects of DBCT on various functions catalyzed by mitochondria isolated from potato tubers, cauliflower florets, or etiolated mung bean hypocotyls. The experimental data presented in this report strongly suggest that DBCT is a very potent inhibitor of the electron-transport chain. It is probable that DBCT inhibits the reoxidation of a portion of the ubiquinone pool.

A preliminary account of this work has been presented [21].

[1]Abbreviations: TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; DBCT, dibutylchloromethyltin chloride; MOPS, 3-(*N*-morpholino) propanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SHAM, salicylhydroxamic acid; DCPIP, 2, 6-dichlorophenol indophenol.

Materials and Methods

Preparation of Mitochondria

Etiolated mung beans (*Phaseolus aureus*) were grown for 5–6 days in a dark room maintained at 28°C. Potato tubers (*Solarum tuberosum*) and cauliflower (*Brassica oleracea*) were purchased locally. Mitochondria were prepared as described by Bonner [22]. Submitochondrial particles were prepared by the method of Rupp and Moore [23].

The mitochondria and submitochondrial particles were suspended in a solution containing 0.3 M mannitol, 1 mM EDTA, 20 mM MOPS, pH 7.4, and 0.1% bovine serum albumin.

Measurement of Respiration Rates

Mitochondrial respiration was measured polarographically in a closed vessel at 25°C in a reaction medium containing either 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate or 150 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate, final volume 2 ml. The pH was adjusted to either 6.8 or 7.2. The sequence of additions and the concentration of additional reagents are as indicated in the legends to Tables I and II.

DBCT, antimycin A (Boehringer), oligomycin (Sigma), FCCP (Boehringer), rotenone (Sigma), and SHAM (Aldrich) were dissolved in absolute ethanol. All other reagents were dissolved in distilled water and adjusted to pH 7.2.

Assay of Protein

Protein was determined by the method of Lowry et al. [24] using crystalline bovine serum albumin as standard.

Synthesis of DBCT

DBCT was synthesised from dibutyltin dichloride and diazomethane following the method described by Cain [18]. DBCT was purified by short-path distillation and collected with a bath temperature of 110–135°C at 0.1–0.2 mm of Hg (bp = 102–106°C/0.38 mm of Hg in ref. 18); *m/e* (M^+ not detected), 283 (10%, $Bu_2Sn^+CH_2Cl$), 269 (100%, Bu_2Sn^+Cl), 213 (22%, $BuSn^+HCl$), 177 (29%, $BuSn^+$), 155 (33%, $SnCl^+$), 57 (91%, Bu^+); ^{1}H-NMR (360 MHz, $CDCl_3$), δ 0.95 (6H, t, J 7Hz, $\underline{CH_3}(CH_2)_3$–), 1.40 (4H, m, $CH_3\underline{CH_2}(CH_2)_2$–), 1.50 (4H, m, $CH_3(CH_2)_2\underline{CH_2}$–Sn), 1.71 (4H, m, $CH_3\underline{CH_2}\underline{CH_2}$–), 3.36 ppm (2H, s + small doublet, J (^{1}H–C–^{119}Sn) 19 Hz, $Cl\underline{CH_2}$–Sn); ^{13}C –NMR (25.0 MHz, $CDCl_3$), δ 13 (q, J 125 Hz, $\underline{C}H_3(CH_2)_3$–), 17 (t, J 160 Hz, $CH_3\underline{C}H_2(CH_2)_2$–), 26.5 (triplet of triplets, J 125

and 4.3 Hz, CH_3 $(CH_2)_2\underline{C}H_2$–Sn), 27.5 (triplet of quartets, J 119 and 5.4 Hz, $CH_3CH_2\underline{C}H_2CH_2$–), 29 ppm (t, J 148 Hz, $Cl\underline{C}H_2$–Sn).

Results

Effects of DBCT on the Respiration of Plant Mitochondria

Potato Mitochondria. The effects of DBCT on the oxidation of various substrates by tightly coupled potato mitochondria were examined at pH 6.8 in a medium containing 150 mM KCl as the major osmotic support.

It can be seen from the data presented in Table I and Fig. 1 that DBCT is a potent inhibitor of the oxygen consumption associated with the oxidation of both succinate and L-malate in the presence and the absence of 1 μM FCCP (see Fig. 1). The uncoupler-stimulated oxidation of L-malate is maximally inhibited by 160 pmole of DBCT per milligram mitochondrial protein. The ADP-stimulated respiration is equally sensitive. In marked contrast, DBCT does not affect the oxidation of either ascorbate + TMPD or exogenous NADH via the external NADH-dehydrogenase system [25] either alone or with ADP, or uncoupling agent.

The results suggest that in a KCl-based reaction medium at pH 6.8, DBCT is acting as an inhibitor of electron transport in potato mitochondria. The site of action of DBCT lies on the substrate side of the point of entry of electrons from the external NADH-dehydrogenase into the electron-transport chain.

DBCT does not appear to be acting at a site in the ATP-synthetase system [16, 17] since (1) FCCP does not relieve the DBCT-induced inhibition of succinate and L-malate oxidation, and (2) respiratory control is shown with the oxidation of added NADH in the presence of concentrations of DBCT

Table I. Effect of DBCT on Substrate Oxidation by Potato Mitochondria[a]

Substrate	Respiration rate (nmole · min^{-1} · mg^{-1} protein)			% Inhibition of FCCP stimulated rate
	−FCCP	+FCCP	+DBCT	
NADH	47	108	99	8
Succinate	51	93	17	82
L-Malate	27	90	17	81
Ascorbate + TMPD	105	156	156	0
L-Malate + succinate	53	89	24	73

[a] 1.5 mg of mitochondrial protein were incubated in a medium containing 150 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8. The final concentrations of additional reagents were: 1 mM NADH, 10 mM succinate, 10 mM L-malate and 1 mM ascorbate with 0.1 mM TMPD. The final volume was 2 ml. Respiration was uncoupled by the addition of 1 μM FCCP; 0.2 nmole/mg DBCT was added after a linear rate was achieved.

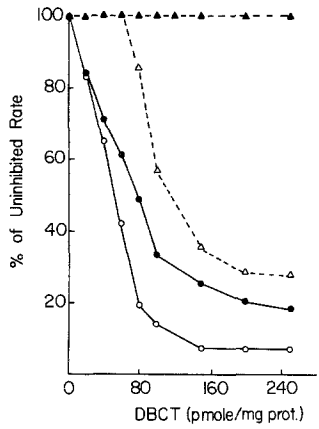

Fig. 1. Inhibition by DBCT of L-malate oxidation in mung bean and potato mitochondria. Oxygen uptake rates measured in a medium containing 150 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate, pH 6.8, and approximately 1.2 mg of mitochondrial protein, final volume 2 ml. 10 mM L-malate was used as substrate, and respiration was uncoupled by the addition of 1 μM FCCP. Rates are expressed as a percentage of the initial rate. Mung bean mitochondria: state 4 rate (▲); uncoupled rate (●). Potato mitochondria: state 4 rate (△); uncoupled rate (○).

which inhibit the oxidations of succinate and L-malate. FCCP can still uncouple the oxidation of external NADH after DBCT treatment and so DBCT does not affect the uncoupling action of FCCP.

The inhibitory effects of DBCT are not seen when (1) the pH of the medium is raised to 7.2, (2) either 150 mM KNO_3, 100 mM K_2SO_4, or 150 mM potassium isethionate is the main osmotic support of the reaction medium at pH 6.8, and (3) either 0.3 M mannitol or sucrose is the main constitutent of the reaction medium at pH 6.8, i.e., conditions analogous to those used to demonstrate DBCT inhibition of the ATP-synthase systems [16]. Observations (1) and (2) could imply that the Cl^-/OH^- antiporter system [13] is involved in the inhibitory mechanism.

The inhibition by DBCT of transport systems associated with the uptake of succinate or L-malate into the mitochondria could explain the differential effects of DBCT on the oxidations of succinate and L-malate on the one hand and the lack of effect on the oxidations of exogenous NADH and ascorbate/TMPD on the other.

From the data presented in Table I it can be seen that the rates of uncoupler-stimulated respiration of L-malate and succinate are very similar and are equally as sensitive to DBCT. The oxidation rates are, however, less than the maximum rate of oxygen consumption observed when NADH is the substrate. These observations could be due either to a coincidental similarity of the capacities of the succinate and L-malate dehydrogenase systems or the presence of a common rate-limiting step in the oxidation pathways which is not involved in the oxidation of external NADH. Since the rate of oxygen consumption with a combination of succinate and L-malate as substrates is, however, not greater than that noted with either substrate alone, it is probable that there is a common rate-limiting step in their oxidation pathways. Ubiquinone is common to the electron transport pathways and could there-

fore be a prime candidate for the rate-limiting step. However, since ubiquinone is involved in the oxidation of external NADH [25], this would imply that ubiquinone, in these mitochondria, is not a homogenous pool but is compartmentalized functionally. It is possible that DBCT is interfering with the oxidation/reduction of a compartment of the ubiquinone pool.

Cauliflower Mitochondria. Similar experiments to those described above with potato mitochondria were performed using mitochondria isolated from cauliflower florets. The results were very similar.

It was also observed that DBCT inhibited the oxygen consumption of cauliflower mitochondria when the osmotic support was 150 mM KI.

Mung Bean Mitochondria. In contrast to potato and cauliflower mitochondria, mung bean mitochondria possess a cyanide and antimycin A insensitive alternative oxidase [26], the branch point of which is currently considered to be at the level of ubiquinone [27–29]. It was therefore of importance to determine if DBCT also inhibited electron transport via the alternative pathway. This information would also give some indication as to the effect of DBCT on substrate transport. As observed with potato mitochondria, it can be seen from Fig. 1 that DBCT is a potent inhibitor of mung bean mitochondrial respiration. This is best illustrated by the results presented in Fig. 2, where DBCT was added to mung bean mitochondria oxidizing either succinate (trace A) or L-malate (traces B and C) in a 150 mM KCl-based reaction medium, pH 6.8.

As observed with potato and cauliflower mitochondria, 267 pmole DBCT per milligram protein inhibits the ADP-stimulated rate of succinate respiration (see trace A). In contrast to the observations made with potato mitochondria, DBCT did not completely inhibit oxygen consumption. The oxidation of added NADH is not affected by DBCT. Respiratory control is exhibited in the presence of DBCT (see Fig. 2, trace A). The subsequent addition of FCCP generates a rapid uncoupled rate of oxygen consumption. In the presence of antimycin A (Fig. 2, trace B) to inhibit electron transport through the cytochrome b–c_1 complex there is still a significant respiration rate, which is unaffected by the addition of DBCT. This oxygen consumption is, however, sensitive to 1 mM SHAM, indicating that this residual DBCT-insensitive consumption of oxygen is via the alternative pathway [30]. In trace C it can be seen that addition of ADP to DBCT-treated mitochondria oxidizing L-malate causes a state 4 to state 3 transition. The ADP/O ratio is 0.8 in contrast to the value of 2.4 before the addition of DBCT. This respiratory control and phosphorylation in the presence of DBCT is presumably due to electron transport through site 1 via the alternative oxidase. Thus, in the presence of DBCT, electron transport and phosphorylation can be demonstrated at site 1 (Fig. 2, trace C) and sites 2 and 3 (Fig. 2, trace A).

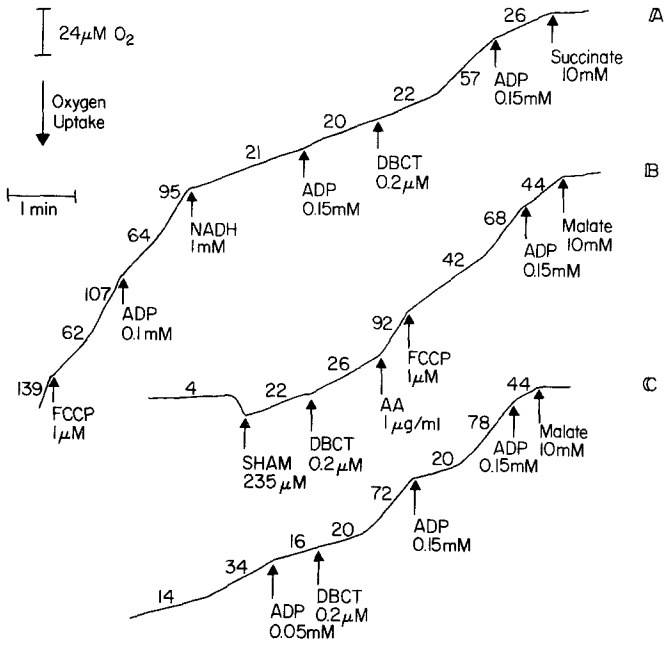

Fig. 2. Inhibition by DBCT of mung bean mitochondrial respiration. Oxygen uptake was measured in the reaction medium described in the legend to Fig. 1, with either 10 mM L-malate or 10 mM succinate as substrate. The numbers on the traces refer to rates of oxygen uptake, nmole · min^{-1} · mg^{-1} protein.

These results confirm that the site of action of DBCT does not lie in the ATP synthase system when mitochondria are suspended in a KCl-based medium.

As indicated earlier, the effects of DBCT on the respiration of potato and cauliflower mitochondria could be explained by an inhibition of the uptake of L-malate and succinate into the mitochondrial matrix. The results presented in Fig. 2 make this possible mechanism unlikely, at least in mung bean mitochondria. Thus the state 4 respiration rates associated with the oxidation of succinate and L-malate via the alternative oxidase are not affected by 270 pmole of DBCT per milligram protein (Fig. 2, traces B and C). Also the state 3 respiration rate for the oxidation of L-malate measured in the presence of DBCT (Fig. 2, trace C) is 34 nmole oxygen · min^{-1} · mg^{-1} protein. This rate is sufficiently close to the state 3 respiration rate for L-malate oxidation via the cyanide-sensitive cytochrome oxidase, 68–78 nmole of oxygen·min^{-1}·mg^{-1}protein (see Fig. 2, traces B and C), indicating that the transport of L-malate into the mitochondria is not seriously affected by DBCT.

It can also be concluded from these results that the site of action of DBCT must lie after the branch point of the alternative oxidase from the main electron transport system and prior to the introduction of electrons from the external NADH dehydrogenase.

Lack of Effect of DBCT on the Respiration Rates of Submitochondrial Particles Isolated from Mung Bean and Potato Mitochondria

DBCT had no effect on the oxidation of either succinate or NADH by submitochondrial particles prepared from mung bean and potato mitochondria in either KCl-or mannitol-based reaction media at pH 6.8. This result could be explained either by assuming a functional scrambling of the quinone pools such that electrons from "internally" produced NADH and succinate can be oxidized by the main, antimycin A sensitive electron transport chain in the presence of DBCT or that the location of the site of action of DBCT is near the cytoplasmic side of the membrane and inaccessible from the matrix side.

Effects of DBCT on the Internal pH of Mung Bean Mitochondria

As shown earlier, the inhibition by DBCT of respiration associated with the oxidation of both succinate and L-malate is dependent upon the anions present in the reaction medium. Dawson and Selwyn [15] have suggested that the operation of an anion/OH^- exchange reaction mediated by trialklytins can lead directly to an acidification of the matrix space of the mitochondria thus inhibiting electron transport. Hence a direct measurement of the effect of DBCT on the pH of the interior of the mung bean mitochondria was made.

In this experiment mitochondria were incubated with aerated substrate solution. A sample was taken after 2 min, then DBCT was added. After another 2 min a further sample was taken. The internal pH before and after the addition of DBCT was determined.

The results are presented in Table II. It can be seen that within the time scale of the experiment DBCT has no significant effect on the internal pH of mung bean mitochondria suspended in a KCl-based reaction medium with either succinate or L-malate as substrates at external pH values of 6.9 or 7.2. It should be emphasized that at pH 7.2 DBCT is ineffective as an inhibitor of electron transport.

Thus, under these experimental conditions it is clear that the inhibition of the oxidation of succinate and L-malate is not caused by an internal acidification of the matrix space. This mechanism of inhibition is also refuted by the observations that DBCT had no effects on (1) the coupled oxidation of

Table II. Effects of DBCT on the Internal pH of Mung Bean Mitochondria[a]

pH	Substrate	Additions	ΔpH ($pH_o - pH_i$)
7.2	Succinate	None	−0.51 ± 0.03
		DBCT	−0.52 ± 0.05
6.9	Succinate	None	−0.61 ± 0.04
		DBCT	−0.61 ± 0.07
6.9	L-Malate	None	−0.64 ± 0.01
		DBCT	−0.46 ± 0.07

[a]Mung bean mitochondria, 2.0 mg of protein, were suspended in a medium containing 150 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate, 20 mM HEPES (the pH had been previously adjusted to either 6.9 or 7.2), 10 μCi ^3H_2O, and 0.3 μCi[1-^{14}C]acetate followed by either succinate or L-malate (final concentrations 10 mM). The volume was 2 ml. This reaction mixture was incubated at 23°C for 2 min in a stream of oxygen. Two 0.45-ml aliquots were taken and centrifuged through a layer of silicone oil into a 14% (v/v) solution of perchloric acid. DBCT, 1.2 nmole, was added to the remaining reaction mixture. After 2 min 2 × 0.45 ml aliquots were taken and centrifuged through a layer of silicone oil, as before. The ^3H- and ^{14}C-distributions were measured in the acid extract and the supernatant fluid. Osmotic spaces were determined in a parallel run using ^3H_2O and [^{14}C]sucrose [31]. The internal pH was calculated as described by Rottenberg [32]. The results are quoted as the mean of ($pH_{outside} - pH_{inside}$) = ΔpH ± standard deviation (four determinations).

external NADH (Fig. 2, trace A) and (2) the coupled oxidation of L-malate via the SHAM-sensitive alternative oxidase system (Fig. 2, trace C).

Effects of DBCT on the Membrane Potential of Mung Bean Mitochondria

The total proton-motive force across mitochondrial membranes generated by electron transport is the resultant of the membrane potential and pH differential across the membrane [33]. It would be expected that an inhibition of electron transport would result in a decreased proton-motive force across the membrane. The experiments described in the previous paragraph showed that DBCT has no significant effect on △pH. Hence experiments were performed to investigate the effects of DBCT on the membrane potential of mung bean mitochondria. The presence of K^+ in the reaction medium where DBCT exhibited its inhibitory effects made quantitative measurements of the membrane potential using ion distribution techniques difficult. Hence, the spectral changes shown by safranine, an extrinsic probe of membrane potential, were used as a qualitative indicator of mitochondrial energization [34, 35].

Mung bean mitochondria were suspended in an aerated potassium chloride based reaction medium which contained safranine and was buffered at either pH 6.8 or 7.2. The changes in absorption of safranine induced by the addition of various reagents were recorded and are presented in Fig. 3, traces A and B.

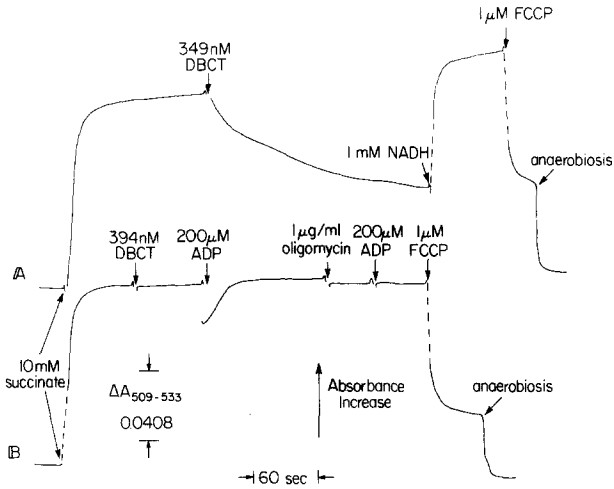

Fig. 3. Effects of DBCT on the membrane potential of mung bean mitochondria at pH 6.8 and pH 7.2. Mung bean mitochondria, 1.7 mg of protein, were added to 2.5 ml of a solution containing either 150 mM KCl, 5 mM $MgCl_2$, 10 mM orthophosphate, and 16 μM safranine, final pH 6.8 (trace A) or final pH 7.2 (trace B). Other reagents were added to the final concentrations indicated. The absorbance difference between 509 and 533 nm was measured using a Johnson Research Foundation double beam spectrophotometer.

At pH 7.2 (Fig. 3, trace B), the addition of succinate induces an energization of the membrane which is not affected by the addition of 240 pmole of DBCT per milligram protein. The subsequent addition of ADP causes a transient decrease in the potential which returns to its initial value upon subsequent transition to state 4. Oligomycin has no effect on the membrane potential but abolishes the variation previously induced by ADP. The results are in agreement with those obtained using the Rb^+ distribution technique [36]. FCCP rapidly collapses the membrane potential, which returns to its original value on anaerobiosis. In control experiments in the absence of DBCT, ADP was shown to cause cyclical changes similar to that seen in trace B. Thus, at pH 7.2 where DBCT has no effect on electron transport the membrane potential is also unaffected. However, at pH 6.8 (Fig. 3, trace A) the addition of DBCT to aerobic succinate-energized mitochondria causes a relatively slow collapse of the membrane potential. This change does not represent nonspecific damage to the membrane since the addition of NADH causes an immediate restoration of the membrane potential, which is rapidly diminished by the addition of FCCP and completely abolished by the onset of anaerobiosis. Thus, the inhibition of

electron transport by DBCT collapses the membrane potential of mung bean mitochondria.

Discussion

The experimental data presented here show that under defined conditions DBCT inhibits both ADP– and uncoupler-stimulated electron transport in plant mitochondria. Approximately 200 pmole of DBCT per milligram protein are sufficient to give complete inhibition.

A specific site of action for DBCT is envisaged because of (1) the low titer of DBCT required to inhibit electron transport (see Fig. 1); (2) the DBCT-treated mitochondria maintained energy-linked functions such as respiratory control and ATP synthesis (see Fig. 2), maintenance of membrane potential (see Fig. 3), and pH (Table II).

From the experimental results presented here it would seem probable that DBCT is acting at a site which is common to the systems which transport and oxidize L-malate and succinate. This site lies on the substrate side of the point of entry of electrons from the external NADH-dehydrogenase system to the cytochrome b–c_1 complex. The complex effects of trialkyltin salts on mitochondrial metabolism have been ascribed to one or a combination of the following mechanisms (see [12] for summary): (1) interaction with a component of the ATP-synthase system leading to an inhibition of coupled electron transport which can be relieved by uncoupling agents; (2) the physical disruption of mitochondria brought about by large scale swelling in KCl-based media; (3) the operation of an anion/OH^- exchange mechanism facilitated by trialkyltin salts. This could lead to the acidification of the matrix space of the mitochondrion which in turn is envisaged as (1) inhibiting the general metabolic activity of the mitochondrion and/or (2) leading to depletion of intramitochondrial substrates via a cascade of anion-exchange mechanisms involving the orthophosphate/OH^- exchange and the dicarboxylate/orthophosphate anion exchange.

An oligomycin-like mode of action for DBCT had already been described for mitochondria when suspended in sucrose-based media [16]. We have been able to repeat these results with rat liver mitochondria. However, this mode of action does not account for the results presented here, using plant mitochondria suspended in a KCl-based reaction medium. Thus under defined conditions respiration coupled to ATP-synthesis at sites 2 and 3 can be demonstrated in DBCT-treated plant mitochondria oxidizing NADH via the external NADH-dehydrogenase (see Fig. 2, trace A) and also at site 1 when the mitochondria are oxidizing L-malate via the SHAM-sensitive

alternative oxidase (see Fig. 2, trace C). It is significant that when plant mitochondria are oxidizing either L-malate or succinate in mannitol-based reaction media DBCT has no effect at titers up to 2 nmole · mg^{-1} protein.

Physical disruption due to DBCT-induced swelling would be expected to lead to a dissipation of the pH gradient, membrane potential, and coupled respiration. This does not happen, and energy conservation can proceed effectively in the presence of DBCT.

The addition of DBCT to mung bean mitochondria does not lead to an acidification of the matrix space. This is illustrated by the results presented in Table II. The trialkyltin depletion of substrates from the mitochondrial matrix and the induced inhibition of substrate uptake have been proposed as the basic mechanism for trialkyltin inhibition of respiration in KCl-based reaction media at pH 6.8 [9, 10, 40]. It is a seductive hypothesis, in that it involves the experimentally established anion/OH^- exchange mechanism facilitated by trialkyltin salts. It could also account for the lack of inhibitory effects of trialkyltins on SMP respiration and the inability of uncoupling agents to relieve the inhibition of respiration in coupled mitochondria.

We consider it unlikely that the effects of DBCT reported in this paper are due to an effect on the uptake of L-malate and succinate for the following reasons. It is generally accepted that the uptake of dicarboxylate substrates is driven by the pH differential across the inner mitochondrial membrane [41]. The results in Table II show clearly that the presence of DBCT has no effect on $\triangle$pH under conditions where electron transport is inhibited. Also it can be seen from the data presented in Fig. 2 that mung been mitochondria can maintain high rates of respiration via the alternative oxidase in the presence of DBCT. This indicates that both succinate and L-malate can enter the mitochondrial matrix in the presence of inhibitory titers of DBCT. This observation is in accord with findings of Skilleter [11] that after triethyltin salt treatment the substrate contents of the rat-liver mitochondrial matrix were still sufficient to saturate the dehydrogenases, e.g. rat liver mitochondria suspended in a reaction medium containing 5 mM L-malate and treated with 1 nmole of triethyltin per milligram protein have an internal concentration of 26.7 mM L-malate. (Table I and ref. 11 of [2]). Both Manger [9] and Harris et al. [40] used very low external substrate concentrations when depletion was demonstrated. A substrate concentration of 10 mM was used in the experiments reported here. Manger showed that as the external substrate concentration increased, so did the internal substrate concentration. Close inspection of the results of Manger [9] show that they do not conflict with the more extensive data of Skilleter [11]. Thus there is little data in the literature to support the hypothesis that trialkyltin-induced depletion of matrix-substrate levels is the mechanism underlying inhibition of respiration.

Furthermore experiments with intact mung bean mitochondria have

shown that the malate-DCPIP reductase activity is insensitive to concentrations of DBCT which inhibit coupled respiration via the cyanide-sensitive oxidase system (A.L. Moore, unpublished results). The rates of DCPIP reduction were the same as those for oxygen consumption with L-malate as substrate. Hence DBCT does not affect the uptake of L-malate.

Thus the only possible site of action of DBCT consistent with the data presented here and in the literature appears to be in the electron-transport chain.

The dependence of trialkyltin inhibition of respiration upon the nature of the anionic composition of the reaction medium has uniformly led to the assumption that the well-characterized trialkyltin-dependent anion/OH^- exchange mechanism is involved in the uncoupler-resistant inhibition of electron transport [42]. However, Rose and Aldridge [8] have shown that increasing concentrations of chloride, bromide, thiocyanate, and iodide ions will increase the concentration of triethyltin in the chloroform phase of a water–chloroform system while nitrate and isethionate have no such action. Thus it is possible that the requirement for specific anions to demonstrate the inhibitory effects described here simply reflects the requirements for lipid solubility of the tin complex in order to enter the mitochondrial membrane and reach the specific site of action in the electron-transport chain. The optimal pH for inhibition below neutrality may also reflect the function of a lipophilic tin complex.

It was concluded from the data presented in Table I that DBCT probably acts at a site which is common to the electron-transport chains responsible for the oxidation of succinate and L-malate. This is positioned after the branch point of the alternative oxidase from the main electron-transport chain and prior to the introduction of electrons from the external NADH dehydrogenase. An enzyme involved in the oxidation/reduction of ubiquinone associated with the internal NADH/succinate ubiquinone reductases would be a prime candidate for the site of action of DBCT. Such a site of action would require one to hypothesize some sort of functional compartmentalization of the ubiquinone pool.

The low titers of DBCT required to give total inhibition compared to the total ubiquinone content of mitochondria imply that only a specific portion of the ubiquinone pool can be affected. There are observations in the literature to support this idea. The external NADH-dehydrogenase, which is located on the external face of the inner mitochondrial membrane, also donates electrons to the ubiquinone region [25]. There is also evidence that in some mitochondria [37, 38] exogenous NADH is not readily oxidized by the antimycin A-insensitive/SHAM-sensitive oxidase. Since this alternative oxidase also accepts electrons at the ubiquinone level [37–39] it is difficult to rationalize the observations reported here on the basis of a homogeneous pool of

ubiquinone which mediates the flow of electrons between the L-malate-, succinate-, and external NADH-dehydrogenases and the cytochrome b–c_1 complex as suggested by Kroger and Klingenberg [39].

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