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INHIBITION OF EXOGENOUS NADH OXIDATION IN PLANT MITOCHONDRIA BY CHLOROTETRACYCLINE IN THE PRESENCE OF CALCIUM IONS

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Chlorotetracycline inhibits the uncoupled oxidation of exogenous NADH by Jerusalem artichoke (*Helianthus tuberosus* L.) mitochondria extensively (over 80%) and rapidly (inhibition complete in 10 s) in the presence of added Ca^{2+}. Half-maximal inhibition is observed at 15 μM chlorotetracycline in the presence of 2 mM Ca^{2+}. The oxidation of succinate is only affected marginally by chlorotetracycline plus Ca^{2+}. The inhibition of NADH oxidation and the fluorescence of CTC are well correlated. Mn^{2+} is the only other cation which shows an (increased) inhibition in the presence of chlorotetracycline. The inhibition by Ca^{2+} and chlorotetracycline disappears at acid pH, and the pH optimum in their presence is 6.4. The inhibition caused by other lipid-soluble Ca^{2+}-chelators is not reversible or is enhanced by the addition of excess Ca^{2+}. In contrast, inhibition caused by relatively water-soluble chelators is completely reversed by added Ca^{2+}. It is suggested that a neutral 1:2 complex is formed between Ca^{2+} and chlorotetracycline which can substitute for Ca^{2+} bound at sites in the lipophilic phase of the inner mitochondrial membrane, which are essential for the activity of the external NADH dehydrogenase.

Introduction

The oxidation of external NADH by plant mitochondria is dependent on Ca^{2+} bound to the membranes (See Ref. 1 and the Introduction of Ref. 2 for brief reviews). The removal of the Ca^{2+} by chelators causes a nearly complete inhibition of the oxidation. If the chelators are added during NADH oxidation (instead of prior to the addition of NADH) the inhibition takes several minutes to become complete [3]. We have suggested that the presence of substrate causes the NADH dehydrogenase to change conformation, thereby 'locking Ca^{2+} into place' and making it less accessible to chelators [3].

We have shown that chlorotetracycline binds to divalent cations in the mitochondrial membranes, but there was little or no effect on NADH oxidation [2]. Those experiments were performed only in the presence of cations which do not interact with chlorotetracycline. However, it has been reported that when Ca^{2+} is added to the reaction medium together with chlorotetracycline an inhibition is observed [4]. This effect was investigated in more detail.

It is shown that in the presence of Ca^{2+}, but not of Mg^{2+} or a range of other cations, chlorotetracycline inhibits NADH and not succinate oxida-

Abbreviations: (DM)Br_2, *N,N,N,N',N',N'*-hexamethyldecane-1,10-diamine; DPTA, diethylenetriaminepentacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*N,N',N'*-triacetic acid; Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; Tes, 2-((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-amino)ethanesulphonic acid.

tion with high affinity. The properties of this inhibition with respect to pH-dependence and kinetics are different from those observed in the presence of EGTA. Several other Ca^{2+}-chelators also exhibits a tendency towards increased inhibition of NADH oxidation in the presence of added Ca^{2+}. The results are discussed in relation to the reaction mechanism of the external Ca^{2+}-dependent NADH dehydrogenase.

Materials and Methods

Mitochondrial isolation and respiration

Tubers of Jerusalem artichoke (*Helianthus tuberosus* L.) were obtained from the University of London Botanical Supply Unit or from the local market. The mitochondria were isolated as described previously [5]. The uncoupled ($2 \cdot 10^{-7}$ M FCCP always present) oxidation of NADH (1 mM) was measured in an oxygen electrode at 25°C in a total volume of 1.0 ml the composition of which is given in the legends to the figures and tables.

Fluorescence

The fluorescence of chlorotetracycline was measured with a Perkin-Elmer MPF 3 fluorescence spectrophotometer. Unless spectra were recorded, the excitation wavelength was 380 nm (slitwidth, 10 nm) and the emission was monitored at 520 nm (slitwidth, 6 nm). The pen was always adjusted to show 80 chart units at sensitivity 3.0 in the presence of 2 mM $CaCl_2$ and 20 μM chlorotetracycline in 90% (v/v) methanol. This was done to allow comparison of the level of fluorescence between different preparations of mitochondria.

Protein determination

Mitochondrial protein was determined by the method of Lowry et al. [6] after solubilising the mitochondria in 0.5% (w/v) deoxycholate. Bovine serum albumin (Sigma A-8022) was used as the standard.

Chemicals

Chlorotetracycline was from Sigma (London) Chemical Co., Poole, Dorset, U.K., NADH from Boehringer, Corporation (London) Ltd., Lewes, Sussex, U.K., Mes, HEDTA, calcichrome, fluorescein complexone, solochrome and succinate from BDH Chemicals, Poole, Dorset, U.K., di-(*o*-hydroxyphenylimino)ethane, DPTA, 2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid, Tes and Mops from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. All other chemicals were of analytical grade and were obtained from either BDH or Sigma.

Results

Concentration dependencies

In agreement with previous results [4], chlorotetracycline inhibited NADH oxidation by Jerusalem artichoke mitochondria strongly in the presence of Ca^{2+}. Half-maximal inhibition was observed at 15 μM chlorotetracycline in the presence of 2 mM Ca^{2+} (Fig. 1). Chlorotetracycline and Mg^{2+} were much less efficient at causing an inhibition — at 50 μM chlorotetracycline the inhibition with 2 mM Mg^{2+} was only 5% whereas it was

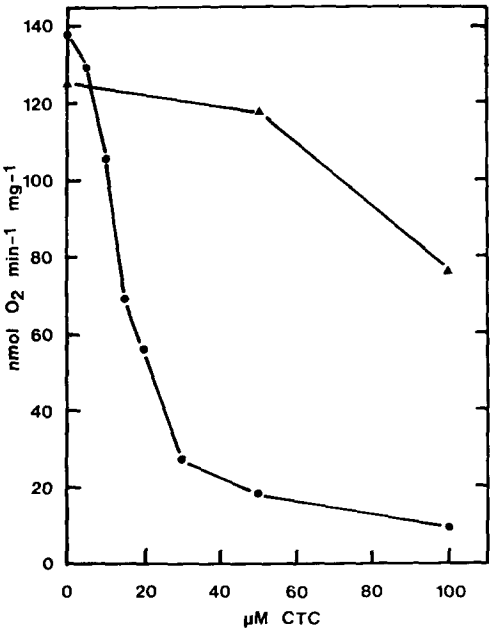

Fig. 1. The effect of the chlorotetracycline (CTC) concentration on NADH oxidation by Jerusalem artichoke mitochondria in the presence of Ca^{2+} and Mg^{2+}. The medium comprised 0.3 M sucrose, 2 mM Mops (pH 7.2), $2 \cdot 10^{-7}$ M FCCP, chlorotetracycline as indicated and 2 mM $MgCl_2$ (▲) or 2 mM $CaCl_2$ (●). Mitochondria at 0.57 mg protein/ml were present in all assays. The reaction was started with 1 mM NADH.

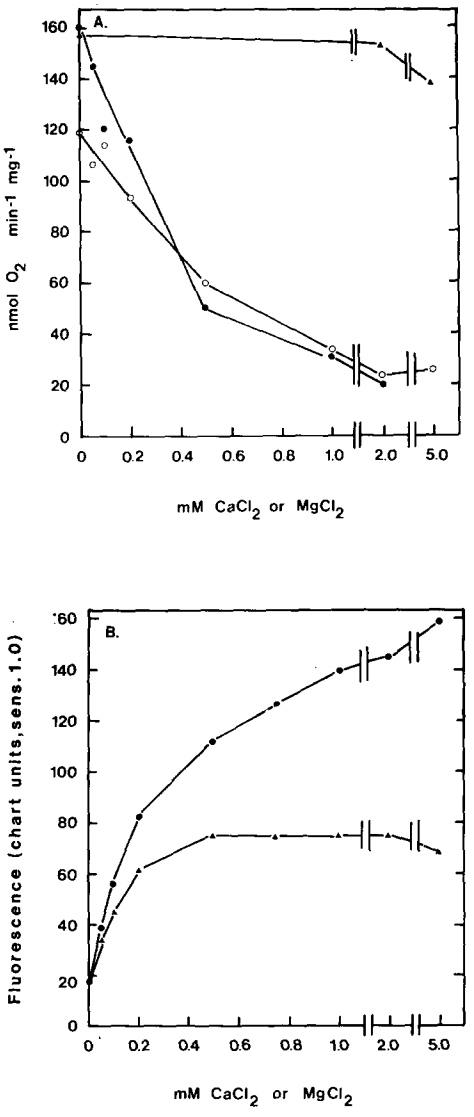

Fig. 2. The effect of the concentration of divalent cations on NADH oxidation (A) and chlorotetracycline fluorescence (B) in the presence of Jerusalem artichoke mitochondria. The medium comprised 0.3 M sucrose, 2 mM Mops (pH 7.2), 50 μM chlorotetracycline and different concentrations of (A) $MgCl_2$ (▲), $CaCl_2$ (○), $CaCl_2$ plus 2 mM $(DM)Br_2$; (B) $MgCl_2$ (▲), $CaCl_2$ (●). Mitochondria at 0.67 mg protein·ml^{-1} were present in all assays.

85% with Ca^{2+}. In another paper [2], we have shown that 50 μM chlorotetracycline causes less that 10% inhibition in a low-cation medium or in the presence of 100 mM KCl or 2 mM $(DM)Br_2$. Therefore, chlorotetracycline at 50 μM was used in the subsequent experiments.

The fluorescence of chlorotetracycline increased with increasing concentrations of chlorotetracycline in the presence of mitochondria both in the presence and in the absence of added Ca^{2+} (results not shown). Half-maximal stimulation was found at 13 μM chlorotetracycline in the presence of Ca^{2+} (16 μM in its absence), which is in very good agreement with the data on inhibition of NADH oxidation (Fig. 1). The enhancement of fluorescence by Ca^{2+} and chlorotetracycline over that in mitochondria incubated with only chlorotetracycline reached a factor 8.2 at 30 μM chlorotetracycline and decreased slightly at higher concentrations. The fluorescence with 30 μM chlorotetracycline and 2 mM Ca^{2+} was 80% complete within 30 s (results not shown).

In the presence of 2 mM $CaCl_2$ half of the 50 μM chlorotetracycline was pelleted with the mitochondria whereas no reduction in the chlorotetracycline concentration was apparent when Ca^{2+} was omitted and the mitochondria were removed by centrifugation (results not shown). Thus, it appears as if uptake of chlorotetracycline by the mitochondria and fluorescence are correlated.

Fig. 2 shows the results of experiments in which the chlorotetracycline concentration was kept constant and the concentration of Ca^{2+} or Mg^{2+} was varied. When Ca^{2+} was the only cation added, half-maximal inhibition of NADH oxidation was observed at 0.38 mM Ca^{2+} (Fig. 2A). However, this result is a mixture of two effects: (a) the inhibition of NADH oxidation by Ca^{2+}–chlorotetracycline and (b) stimulation of NADH oxidation by the increased cation concentration which is maximal around 0.5 mM [7]. To avoid the interference of (b), 2 mM $(DM)Br_2$ was added to another series of assays to create high cation conditions even at low concentrations of Ca^{2+}. We known that $(DM)Br_2$ does not inhibit NADH oxidation in combination with chlorotetracycline [2]. In the presence of $(DM)Br_2$ the half-maximal inhibition of NADH oxidation was 0.22 mM Ca^{2+} (Fig. 2A). At 50 μM chlorotetracycline, even 5 mM Mg^{2+} had only a marginal effect on NADH oxidation (Fig. 2A).

The fluorescence of chlorotetracycline was again

monitored in separate experiments (Fig. 2B). Half-maximal fluorescence (fluorescence at 5 mM Ca^{2+} taken to be maximal) was observed at 0.19 mM Ca^{2+} (Fig. 2B), in good agreement with the results on NADH oxidation (Fig. 2A). The maximum fluorescence observed with added Mg^{2+} was only half that observed with Ca^{2+} but the 'affinity' was higher — half-maximal fluorescence was at 70 μM (Fig. 2B). The addition of Mg^{2+} did not affect the position of the fluorescence peaks significantly. The excitation maximum was at 394–395 nm and the emission maximum at 518 nm (see Fig. 3 in Ref. 2 for a spectrum recorded in the absence of added divalent cations). The presence of only 50 μM Ca^{2+} shifted the maxima to 397 and 523 nm, respectively, but Ca^{2+} up to 2 mM did not shift it further (results not shown).

Specificity for the cation

Above, and in Ref. 2, it was shown that 50 μM chlorotetracycline alone or in combination with K^+, $(DM)^{2+}$ or Mg^{2+} did not affect NADH oxidation significantly. We also tested the effect of Na^+, Sr^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Cd^{2+}, La^{3+} and Tris(ethylenediamine)Co^{3+} on NADH oxidation — alone and in combination with chlorotetracycline – and on chlorotetracycline fluorescence (results not shown). Chlorotetracycline caused a significant inhibition of NADH oxidation only with Ca^{2+} (85% at 2 mM) and Mn^{2+} (51% at 2 mM).

Ca^{2+}, Mg^{2+}, Sr^{2+}, Zn^{2+} and La^{3+} all caused a significant increase in chlorotetracycline fluorescence over the control in an aqueous medium in the presence of mitochondria. When Ca^{2+} was added subsequently, the fluorescence was, in most cases, only slightly reduced compared to that with Ca^{2+} alone; only 0.5 mM Mn^{2+} caused a strong quenching and it may be significant that it was only Mn^{2+} which caused an inhibition of NADH oxidation in the presence of chlorotetracycline (results not shown).

Effect of pH

NADH oxidation had a pH optimum at pH 6.9–7.2 (Fig. 3A), in good agreement with previous results [8]. Removal of bound cations with EDTA caused a complete inhibition at neutral pH. At acid pH, the effect of EDTA disappeared, and below pH 6.0 the rate was completely unaffected by EDTA. The pH optimum in the presence of EDTA was 6.0 (Fig. 3A). It is interesting that the oxidation of NADH in the presence of EDTA is almost identical to that of NADPH oxidation

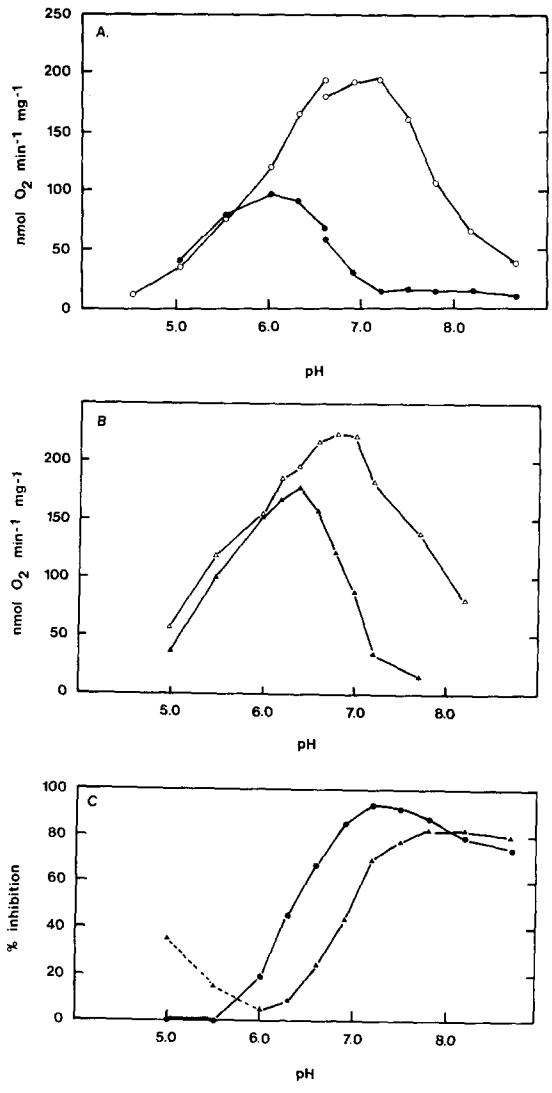

Fig. 3. The effect of pH on NADH oxidation by Jerusalem artichoke mitochondria in the presence and absence of EDTA and chlorotetracycline. All measurements were performed in a medium of 0.3 M sucrose and 0.1 M Mes (pH 4.5–6.7 in A and 5.0–7.0 in B) or 0.1 M Tes (pH 6.7–8.7 in A and pH 7.1–8.3 in B). Mitochondria at 0.64 (A) and 0.35 mg·ml^{-1} (B) were used. A, Control (○) and control plus 1 mM EDTA (●); B, control plus 2 mM $CaCl_2$ (△) and control plus 2 mM $CaCl_2$ and 50 μM chlorotetracycline (▲); C, The percentage inhibition by EDTA (●) and chlorotetracycline (▲) calculated from parts A and B, respectively.

measured in the absence of chelators [8].

When 2 mM $CaCl_2$ was present instead of the 2 mM $(DM)Br_2$ in the controls, the pH optimum of NADH oxidation was slightly displaced to pH 6.8–7.0 (Fig. 3B). The addition of chlorotetracycline in the presence of Ca^{2+} caused an almost complete inhibition at pH 7.2, but, as with EDTA, the inhibition disappeared at acid pH. The optimum in the presence of chlorotetracycline and Ca^{2+} was at pH 6.3–6.4. The degree of inhibition of NADH oxidation as a function of pH is compared for chlorotetracycline and EDTA in Fig. 3C. The curves are very similar shape, but displaced by about 0.5 pH unit — half-maximal effect is observed at pH 6.4 with EDTA and at pH 6.9–7.0 with chlorotetracycline plus Ca^{2+} (Fig. 3C).

Sequence of addition

In the experiments presented above, the inhibitors, chlorotetracycline and EDTA, were always added before the substrate. We have previously reported that when EDTA is added after NADH, at the maximal rate of oxidation, the inhibition takes several minutes to be fully effective [3]. Similar experiments were performed with chlorotetracycline and the results compared with results obtained with EDTA on the same preparation of mitochondria (Fig. 4). Although the effect of EDTA was faster than reported previously, the difference between chlorotetracycline and EDTA was very marked. The inhibition by chlorotetracycline (in the presence of Ca^{2+} Fig. 4C) was almost instantaneous and was complete within 10 s. In contrast, EDTA took at least 1 min (2 min under low-cation conditions, Fig. 4A and B) to reach the full effect.

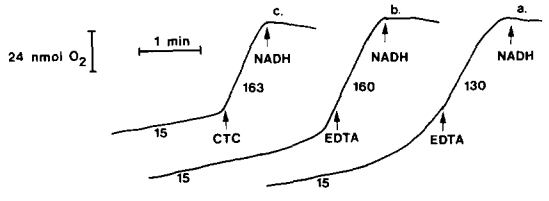

Fig. 4. Addition of chlorotetracycline (CTC) and EDTA during NADH oxidation by Jerusalem artichoke mitochondria. The medium comprised 0.3 M sucrose, 2 mM Mops (pH 7.2) plus 2 mM $(DM)Br_2$ (b) or 2 mM $CaCl_2$ (c). Chlorotetracycline (50 μM) and EDTA (1 mM) were added in (c) and in (a) and (b), respectively. Mitochondria at 0.50 mg protein·ml^{-1} were used.

Specificity of chlorotetracycline for NADH oxidation

The oxidation of succinate shares the electron transport pathway with NADH oxidation from ubiquinone to oxygen. From Table I it is clear that chlorotetracycline plus Ca^{2+} affected succinate oxidation only marginally while completely inhibiting NADH oxidation. Thus, chlorotetracycline appears to be specific for a reaction between NADH and ubiquinone, presumably that of the NADH dehydrogenase. La^{3+}, which is also known to inhibit NADH oxidation in Jerusalem artichoke mitochondria [7,9], was also a relatively specific inhibitor of the oxidation of NADH (Table I), although La^{3+} appears to bind to one of the major membrane components, which might be expected to affect several steps in the electron-transport chain [9].

The effect of other Ca^{2+}-chelators

In contrast to chlorotetracycline, chelators such as EDTA, EGTA and citrate (a) inhibit NADH oxidation by plant mitochondria in the absence of added Ca^{2+} and (b) the addition of Ca^{2+} reverses this inhibition (see Introduction for references). In Table II a range of Ca^{2+} chelators are compared

TABLE I

SPECIFICITY OF CHLOROTETRACYCLINE AND La^{3+} FOR NADH OXIDATION BY JERUSALEM ARTICHOKE MITOCHONDRIA

The oxidation of NADH was measured in 0.3 M sucrose, 0.1 M Mops, pH 7.2, as described in Materials and Methods. The mitochondria used for succinate oxidation were first activated at 25°C for 5 min in the above medium plus 0.2 mM ATP, 0.5 mM potassium phosphate and 20 μM $MgCl_2$; then, 2 mM $(DM)Br_2$ and $2 \cdot 10^{-7}$ M FCCP were added, and 20 mM succinate to start the reaction. $LaCl_3$ (200 μM), chlorotetracycline (50 μM) and $CaCl_2$ were all added to the mitochondria (0.59 mg/ml) before NADH.

Additions	NADH	Succinate
	nmol·min^{-1}·mg^{-1}	
None	136	113
200 μM $LaCl_3$	63	88
2 mM $CaCl_2$	131	114
2 mM $CaCl_2$ + 50 μM CTC	19	87
	Percentage	
Inhibition by $LaCl_3$	54	22
Inhibition by chlorotetracycline	85	24

TABLE II

THE EFFECT OF Ca^{2+} ON THE INHIBITION OF NADH OXIDATION BY JERUSALEM ARTICHOKE MITOCHONDRIA CAUSED BY VARIOUS Ca^{2+}-CHELATORS

NADH oxidation was measured as described in Materials and Methods in the presence of the chelators plus or minus Ca^{2+} as indicated. The control rate (0.26 mg protein·ml^{-1}) was 182 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$. None of the chelators affected the oxidation of malate or succinate (di-(*o*-hydroxyphenylimino)ethane and fluorescein complexone not tried).

Chelator		Absence of calcium (inhibition of NADH oxidation, %)	Presence of calcium		Effect of calcium on chelator inhibition
Name	Concentration (mM)		Concentration of calcium (mM)	Inhibition of NADH oxidation (%)	
EGTA	1	92	2	0	reversal
EDTA	2	90	3	0	reversal
DPTA	2	85	3	0	reversal
HEDTA	4	80	5	0	reversal
citrate	4	74	5	0	reversal
solochrome	1	94	3	65	partial reversal
Di-(*o*-hydroxyphenylimino)-ethane	4	43	7	43	no effect
fluorescein	2.5	55	4.5	70	enhancement
2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid	4	65	6	74	enhancement
calcichrome	2	54	4	65	enhancement

with respect to these two properties. All the chelators inhibited NADH oxidation in the absence of added Ca^{2+}. The addition of excess Ca^{2+} reversed the inhibition caused by EGTA, EDTA, DPTA, HEDTA, citrate and solochrome (the latter only partly) but had no effect on the inhibition caused by di-(*o*-hydroxyphenylimino)ethane and actually enhanced slightly the inhibition caused by fluorescein complexone, 2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid and calcichrome (Table II).

Discussion

Chlorotetracycline inhibits a large variety of processes, but, although its effect has often been linked with its metal-binding ability, its antibiotic activity at low concentrations does not appear to depend on metal chelation [10,11]. The concentration of chlorotetracycline required to inhibit processes of bacterial oxidation and respiration were in many cases significantly higher [10] than the 50 μM observed to be optimal in the present study (Fig. 1). Caswell and Hutchison [12] found no uncoupling or inhibitory effect of 20 μM chlorotetracycline on mitochondrial respiration. The substrate was not specified. Colaizzi et al. [13] reported that chlorotetracycline and certain analogues inhibited the activity of NADH-cytochrome *c* oxidoreductase from hog heart. The inhibition was competitive with respect to cytochrome *c*, but the K_i was in the 0.5–2.0 mM range and the inhibition was probably achieved through a mechanism quite different from that reported here. Nohl and Hegner [14] found that chlorotetracycline inhibited coupled succinate oxidation strongly (50%) in rat liver mitochondria with a half-maximal concentration of about 25 nmol $\cdot mg^{-1}$ protein in the presence of 2 mM EDTA and no added cations.

In the case of uncoupled exogenous NADH oxidation by Jerusalem artichoke mitochondria, chlorotetracycline was without effect in the absence of Ca^{2+} [2]. In the presence of Ca^{2+}, chlorotetracycline inhibited with high affinity (Fig. 1) and the fluorescence was well correlated with this inhibition (results not shown). That increased fluoresence was caused by chlorotetracycline in the

mitochondria was confirmed by the observation that half the chlorotetracycline was pelleted with the mitochondria in the presence of Ca^{2+}. Increased binding of chlorotetracycline to membranes upon addition of Ca^{2+} has also been observed by Deev et al. [15], Schaffer and Olson [16] and Carvalho [17]. Schaffer and Olson [16] found that half-maximal fluorescence in the presence of rat brain synaptosomes was reached at 0.3 mM Ca^{2+} in the presence of 100 μM chlorotetracycline and at 40 μM chlorotetracycline in the presence of 0.5 mM $CaCl_2$. These results are similar to those presented in Figs. 1 and 2.

The ability of a cation to form a fluorescent complex with chlorotetracycline is not correlated with its effect on NADH oxidation in the presence of chlorotetracycline. Mg^{2+} and Sr^{2+} both form fluorescent complexes with chlorotetracycline (this study and Ref. 12) but have no inhibitory effect on NADH oxidation in the presence of chlorotetracycline (this study). Mn^{2+}, which quenches chlorotetracycline fluorescence (this study and Ref. 12) is the only cation tested, besides Ca^{2+}, which forms an inhibitory complex with chlorotetracycline (this study). Caswell and Hutchison [18] observed differences in the spectral properties of the Mg^{2+} and Ca^{2+} complexes and it would be interesting to know how these cation-chlorotetracycline complexes differ in structure. Such information would provide insights into the location, shape and properties of the Ca^{2+}-binding site of the external NADH dehydrogenase in plant mitochondria (see below).

Exogenous NADH oxidation in plant mitochondria depends on Ca^{2+} bound to the inner membrane and removal of this by chelators causes a strong inhibition [1,3,4,19–22]. The ability of a given cation to reverse this inhibition depends on the stability constant of its chelator complex, e.g., Mg^{2+} can reverse EDTA- but not EGTA-inhibition [3,19,20]. Inhibition by citrate, EDTA or EGTA was always reversible by excess Ca^{2+} and chlorotetracycline thus shows a novel pattern since its inhibition was enhanced by Ca^{2+} rather than reversed (Figs. 1 and 2). Several other Ca^{2+}-chelators showed similar behaviour in that their inhibition of NADH oxidation was not affected by excess Ca^{2+} or was slightly enhanced (Table II). It is probably significant that these chelators all are relatively insoluble in water and soluble in more apolar media.

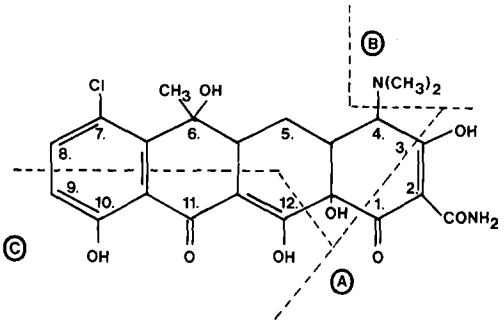

Fig. 5. Structure of chlorotetracycline and ionisable groups (A, B and C) according to the data of Laskin [10] and Asleton and Frank [24].

Chlorotetracycline has four ionizable groups, with pK values of 3.3–4.4, 6.9–8.7, 9.3–9.8 and 10.7. [23–26]. The large variability in the values for each pK is mainly due to the use of media of different polarity [24] and the presence and absence of divalent cations [25]. Asleton and Frank [24] assigned the deprotonations at 4.4, 8.1 and 9.8 in a mixed solvent system to the A, C and B groups (Fig. 5), respectively. Dobretsov et al. [26] found that the C-12 hydroxyl group (section C, Fig. 5) dissociates at pH 7.0. The presence of Ca^{2+} or Mg^{2+}, which probably bind at the C-11, C-12 (section C), C-2 and C-3 [18], causes a shift in the pK at 7.7 to 7.0 [25]. Thus, it seems to be the C group which is relevant to the present investigation.

Ca^{2+}-binding peptides contain liganding sites consisting of six oxygen atoms arising from glutamic or aspartic acid carboxyl groups and backbone peptide carbonyl groups coordinated to Ca^{2+} in approximate octahedral geometry [27]. It appears possible that C groups from two chlorotetracycline molecules could provide a similar binding site for Ca^{2+}. Such a 2:1 chlorotetracycline-Ca^{2+} complex would be neutral above pH 7, and it could be this neutral complex which is causing the inhibition of NADH oxidation (Fig. 3C).

The inhibition of NADH oxidation by EDTA shows a pH dependence with a inflection point at pH 6.4 (Fig. 3C), which is in excellent agreement with one of the pK values of EDTA [28,29]. The charge of EDTA is -3 at pH 7.0 and a 1:1

complex with Ca^{2+} is, therefore, not neutral. It should also be kept in mind that EGTA, which has no pK in the pH range 3.0–8.5 [28,29] and whose 1 : 1 complex with Ca^{2+} is neutral throughout this range, also loses its ability to inhibit NADH oxidation by plant mitochondria at pH below 7.0 [4]. Again, we must conclude that chlorotetracycline acts via a mechanism different from that of the water-soluble chelators.

Before discussing the possible mechanism by which chlorotetracycline inhibits NADH oxidation, one point merits mention in connection with the pH dependencies shown in Fig. 3. The pH optimum of NADH oxidation in the presence of Ca^{2+} (6.8–7.0) was about 0.2 pH units below that of the control (pH 6.9–7.2), where only non-binding cations (K^+, $(DM)^{2+}$) were present (Fig. 3 and Ref. 8). This might be explained by binding of Ca^{2+} to the membrane, which reduces its net negative charge. This would cause a decrease in the concentration of cations such as H^+ in the vicinity of the membrane and, therefore increase the local pH. A lower bulk pH (which is what we measure) would now be required to reach the same low local pH as in the absence of Ca^{2+}, and a lowering of the apparent pH optimum would be the result.

The inhibition of NADH oxidation by chlorotetracycline in the presence of Ca^{2+} is rapid (Fig. 4) and it is caused by the neutral 2 : 1 Ca^{2+}-chlorotetracycline complex (Fig. 3C). Although appreciable amounts of chlorotetracycline (and Ca^{2+}) are sequestered in the mitochondria under these conditions (50% of 50 μM chlorotetracycline at 0.5 $mg \cdot ml^{-1}$ gives 50 $nmol \cdot mg^{-1}$), the effect on succinate oxidation is marginal (Table I). The same is true for La^{3+}, another potent inhibitor of NADH oxidation (Table I). Therefore, the chlorotetracycline-Ca^{2+} complex appears to act on a step in electron transport unique to NADH oxidation. One possibility is that the neutral 2 : 1 chlorotetracycline-Ca^{2+} complex by entering the apolar parts of the membrane can gain access to the Ca^{2+}-binding site of the NADH dehydrogenase. There it might substitute for the bound Ca^{2+}, thereby rendering the dehydrogenase inactive. Further studies with chlorotetracycline should make it possible to learn much more about the role of divalent cations in the functioning of membranes of plant mitochondria and other plant membrane systems.

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