Characterization of Chlortetracycline (Aureomycin) as a Calcium Ionophore†

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ABSTRACT: The antibiotic chlortetracycline (aureomycin) is shown to be a potent and specific calcium ionophore. The molecule extracts calcium from an aqueous environment into a bulk organic phase in a pH-dependent manner and with a stoichiometry indicative of a 1:1 complex. The antibiotic has little affinity for other alkaline earth ions or alkali metal ions in this system. The ionophore transports calcium from one aqueous phase to another across an organic solvent barrier and induces a flux of calcium into multilamellar vesicles. The potential use of the compound in examining the role of calcium in the modulation of cellular function is discussed.

Ionophores are organic moieties capable of forming lipid-soluble complexes with metal cations. They may transport these ions across hydrophobic barriers, including artificial and biological membranes, and have been extensively employed in the investigation of a variety of biochemical phenomena (Fenton, 1977; Pressman, 1968a,b, 1973, 1976).

According to the geometry and nature of the coordination site, ionophores may exhibit differing degrees of cation specificity. In view of the central role of calcium as a second messenger in cellular activation (Berridge, 1975; Rasmussen, 1970), considerable interest has focused on ionophores that preferentially transport this cation. The antibiotics A23187 (Liu & Hermann, 1978; Pfeiffer & Lardy, 1976; Pfeiffer et al., 1974; Puskin & Gunter, 1975; Reed & Lardy, 1972), ionomycin (Liu & Hermann, 1978), and to a lesser extent X-537A (Pressman, 1973) exhibit such a selectivity, and A23187 in particular has been widely used in the study of calcium-dependent processes including stimulus-secretion and stimulus-contraction coupling (Pressman, 1973, 1976). However, such investigations have been largely confined to the use of this agent, and the availability of other calcium-specific complexones would be of great value. It has long been appreciated that chlortetracycline (CTC,[1] aureomycin) is able to form stable chelates with a number of divalent metal cations (Albert, 1953; Caswell & Hutchison, 1971a,b), and the antibiotic has been used as a fluorescent probe to examine the association of calcium with cell membranes and to monitor movements of the cation in intact cells and isolated organelles [for references, see Chandler & Williams (1978), Gains (1980), Luthra & Olson (1978), Schaffer & Olson (1976), and Täljedal (1978)]. More recently, it has been demonstrated that CTC may itself induce calcium fluxes in rat liver mitochondria and rat brain synaptosomes (Luthra & Olson, 1978; Schaffer & Olson, 1976), and it has been suggested that the compound may thus have ionophoretic properties. We wish here to demonstrate definitively that CTC is indeed a potent and highly specific calcium ionophore.

Experimental Procedures

Materials. The ionophores A23187 and CTC (free base) were generous gifts from the Lilly Research Centre (Windlesham) and Lederle Laboratories (Gosport), respectively. $^{45}CaCl_2$ was purchased from Amersham International. Oxytetracycline, phosphatidylcholine, dicetyl phosphate, cholesterol, and arsenazo III were purchased in the highest available states of purity from the Sigma London Chemical Co.

Partition of CTC and Bulk Extraction of Calcium into an Organic Phase. The partition of CTC between organic and aqueous phases was examined by shaking (24 h, 20 °C) a solution of the antibiotic (100 μM) in a mixture of butan-1-ol and toluene (3:7 v/v) with an equal volume of aqueous buffer in the presence and absence of calcium ions (100 μM). The buffer solutions (25 mM) used were acetic acid/tetramethylammonium hydroxide, pH 4–5.9, Hepes/tetramethylammonium hydroxide, pH 6.5–8.5, Tris-HCl, pH 7.3–9.3, and dimethylglycine/tetramethylammonium hydroxide, pH 8.9–10.8. The solutions were then centrifuged (15 min, 1500*g*, 20 °C) to separate the layers and aliquots carefully withdrawn. The absorbances of the solutions were measured at predetermined maxima.

The ability of CTC to extract calcium into the organic solvent was determined in a similar manner. Solutions of the antibiotic (1 mM) in butanol/toluene (2 mL) were shaken with varying concentrations (0.1–10 mM) of calcium in aqueous buffer (2 mL) as before. An aliquot (1 mL) of the organic phase was recovered and evaporated to dryness. The residue was redissolved in HCl (0.1 M, 1 mL) containing lanthanum oxide (0.3%) and the calcium content determined by atomic absorption in a Perkin-Elmer 103 spectrophotometer. In some cases, parallel experiments with the ionophore A23187 were carried out. For determination of the relative binding affinity of CTC for various metal ions, calcium and other cations were added in equimolar amounts (1 mM) to the aqueous phase, and the amount of calcium extracted by CTC (1 mM) was determined as before.

Measurement of Calcium Transport. The ability of CTC to transfer calcium from one aqueous layer to another across a butanol/toluene barrier was determined by using the recently described H tube (White & Pearce, 1982). Aqueous buffer (Hepes/tetramethylammonium hydroxide, 20 mM, pH 8.5, 1.25 mL) containing $^{40}CaCl_2$ (1 mM) was added to each limb of the tube, followed by $^{45}CaCl_2$ (3 μCi, 10–14 mCi/mg of Ca) to the right-hand limb only. The organic solvent (butanol/toluene, 3:7, 2 mL) containing the ionophore was carefully

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[1] Abbreviations: CTC, chlortetracycline; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; arsenazo III, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthalenebis(azo)]bis(benzenearsonic acid); Triton X-100, (octylphenoxy)poly(ethoxy)ethanol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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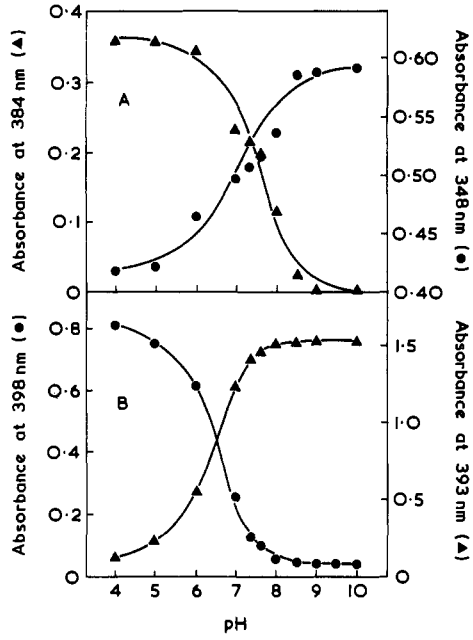

FIGURE 1: Distribution of CTC between organic (▲) and aqueous (●) phases as a function of pH in the absence (A) and presence (B) of calcium. CTC (100 μM) was dissolved in butanol/toluene (3:7, 2 mL) and shaken (24 h, 20 °C) with aqueous buffer (2 mL) containing calcium (100 μM) as appropriate. The concentration of CTC was determined by measurement of absorbance at predetermined maxima.

layered over the aqueous phases and stirred magnetically. Samples (10 μL) of the aqueous layers in both limbs were withdrawn through the organic phase at the times indicated. Aliquots of the organic solvent were similarly taken, and all samples were assayed for ^{45}Ca by scintillation counting (White & Pearce, 1982).

Preparation and Use of Multilamellar Vesicles Containing Arsenazo III. Liposomes containing the metallochromic dye arsenazo III have recently been shown to provide a sensitive method for detecting ionophoretic activity (Weissmann et al., 1980) Transport of calcium into such vesicles is manifest by a marked shift in the absorption spectrum of the entrapped dye. In the present work, such vesicles were prepared essentially by the method of Weissmann et al. (1976). Solutions in chloroform of phosphatidylcholine, dicetyl phosphate, and cholesterol (7:2:1 w/w) were reduced to dryness on a rotary evaporator. Liposomes (32 μmol of total lipid/mL) were then allowed to form by shaking them (1 h, 20 °C) in an aqueous solution (Hepes/tetramethylammonium hydroxide, 5 mM, pH 7.4) containing arsenazo III (3 mM). The liposomes were recovered by centrifugation (20 min, 10000*g*, 4 °C) and washed (3×) in buffer to remove extravesicular arsenazo III. The vesicles were finally resuspended in buffer (32 μmol of lipid in 2 mL) and the absorption spectra recorded in a Perkin-Elmer 1800 spectrophotometer. Changes in the spectrum were monitored following disruption of the vesicles with the detergent Triton X-100 or after addition of CTC.

Results

Partition of CTC and Bulk Extraction of Calcium. The partition of CTC between organic and aqueous phases was strongly dependent upon pH and the presence of calcium ions (Figure 1). In the absence of calcium, the majority of the compound was distributed in the organic layer at low values of pH and progressively partitioned into the aqueous environment as the pH was increased (Figure 1A). The reverse behavior was observed in the presence of calcium. Under these

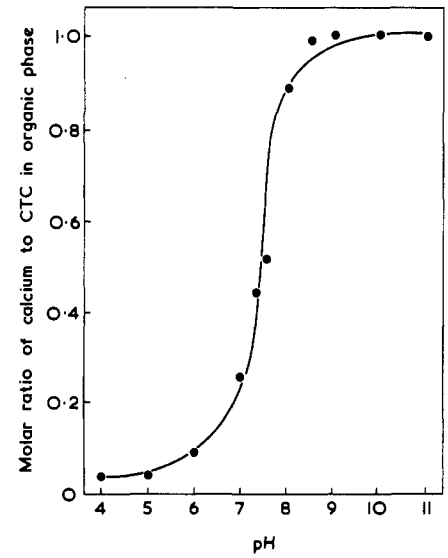

FIGURE 2: Bulk extraction of calcium by CTC as a function of pH. Organic solutions of CTC (1 mM) were shaken with aqueous solutions of calcium (1 mM) as described in Figure 1. The amount of cation extracted into the organic phase was determined by atomic absorption spectroscopy.

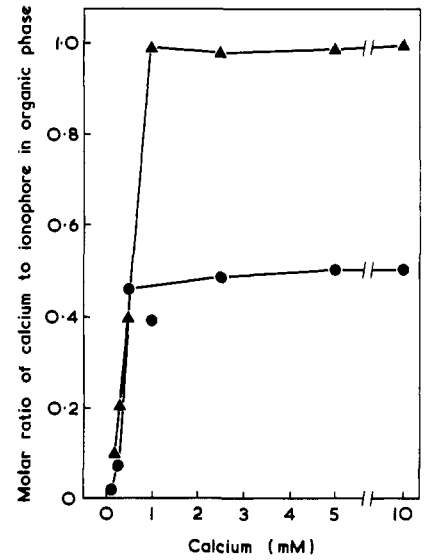

FIGURE 3: Bulk extraction of calcium by CTC (▲) and ionophore A23187 (●) as a function of metal ion concentration. The concentrations of ionophores were 1 mM, and the pH was 8.5 (Hepes/tetramethylammonium hydroxide, 25 mM). For details, see Figure 2.

conditions, the solubility of CTC in the organic solvent increased with pH so that at values above pH 8 virtually all of the antibiotic was located in this medium (Figure 1B). CTC extracted calcium ions into the organic phase in parallel fashion to reach a maximum value at pH 8–9, indicative of a 1:1 complex between the cation and the ionophore (Figure 2). This stoichiometry was confirmed by determining the molar ratio of calcium to CTC as a function of the concentration of the cation (Figure 3). As the latter was increased, CTC extracted calcium from the aqueous phase to reach a limiting value corresponding to a 1:1 complex. In contrast, but in keeping with previous reports (Liu & Hermann, 1978; Pfeiffer et al., 1974; Reed & Lardy, 1972), the ionophore A23187 formed a 2:1 complex with calcium (Figure 3).

Cation Selectivity of CTC. The ability of various mono-, di-, and trivalent ions to compete with calcium in the two-phase extraction system is shown in Table I. CTC bound calcium

Table I: Displacement of Calcium from CTC by Other Metal Ions[a]

competing cation	calcium displaced (%)	competing cation	calcium displaced (%)
Na^+	1.7 ± 0.3	Dy^{3+}	85.0 ± 3.3
K^+	1.1 ± 0.5	Eu^{3+}	72.2 ± 9.8
Ba^{2+}	7.3 ± 5.0	La^{3+}	68.4 ± 4.2
Cd^{2+}	14.7 ± 6.0	Sm^{3+}	56.6 ± 13.3
Mg^{2+}	7.6 ± 3.4	Pr^{3+}	54.5 ± 12.2
Sr^{2+}	5.0 ± 2.3	Lu^{3+}	69.0 ± 7.5

[a] Equal amounts (1 mM) of calcium and the noted cations were dissolved in aqueous buffer (2 mL, pH 7.4) and shaken (24 h, 20 °C) with CTC (1 mM) in butanol/toluene (3:7, 2 mL). The amount of calcium extracted into the organic layer was determined by atomic absorption spectroscopy and expressed relative to the amount extracted in the absence of any competing ion. Values are means ± SEM for three experiments.

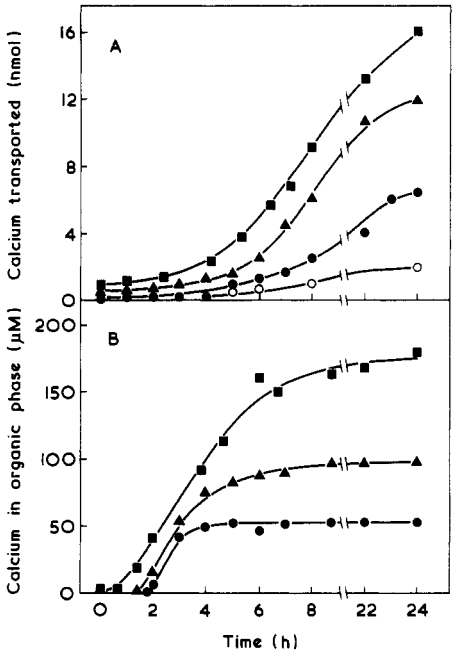

FIGURE 4: Time course of calcium transport by CTC in the H tube. The aqueous phase (Hepes/tetramethylammonium hydroxide, 20 mM, pH 8.5) in both arms of the tube contained $^{40}CaCl_2$ (1 mM). $^{45}CaCl_2$ (3 μCi) was added to the right-hand limb, and CTC was dissolved in the organic layer (butanol/toluene, 3:7) to give concentrations of 200 μM (■), 100 μM (▲), 50 μM (●), and 0 (○). Transport of calcium to the aqueous phase in the left-hand limb (A) and extraction of the cation into the organic layer (B) were measured as described in the text.

with a high degree of selectivity and showed little affinity for other alkaline earth or alkali metal ions. However, trivalent ions of the lanthanide series effectively displaced calcium to varying degrees.

Transport of Calcium in the H Tube. CTC produced an efficient transport of calcium across the butanol/toluene barrier in the H tube (Figure 4). The effect increased progressively with time and with the concentration of the ionophore. The concentration of calcium in the organic layer reached a steady-state value that was stoichiometrically equivalent to the amount of ionophore present, again indicative of the formation of a 1:1 complex. Under the same conditions, oxytetracycline (200 μM) produced no significant transport of calcium (data not shown).

Transport of Calcium in Multilamellar Vesicles. Liposomes containing arsenazo III gave a simple absorption spectrum with a maximum at ca. 550 nm, characteristic of the unconjugated dye (Figure 5). In the absence of any

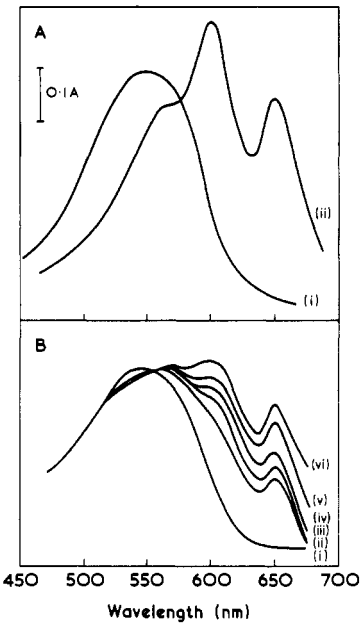

FIGURE 5: Absorption spectra of liposomes containing arsenazo III. (A) (i) Intact liposomes and (ii) liposomes disrupted with Triton X-100 (2%) in the presence of calcium (4 mM). (B) Liposomes treated with CTC (50 μM) and calcium (4 mM) for 0 min (i), 20 min (ii), 30 min (iii), 40 min (iv), 60 min (v), and 90 min (vi). The reference cell contained buffer with or without CTC as appropriate. The vertical bar corresponds to 0.1 absorbance units.

perturbation, this spectrum was stable over the time course of the experiment. However, disruption of the liposomes with Triton X-100 (2%) in the presence of calcium (4 mM) produced a marked spectral shift with the appearance of new absorption bands at 600 and 650 nm, diagnostic for the formation of the calcium–arsenazo complex. Addition of CTC (50 μM) in the absence of added calcium did not alter the signal from intact liposomes, but in the presence of the cation (4 mM) the ionophore produced a progressive spectral change indicative of calcium transport into the vesicles. Addition of excess EGTA did not significantly reverse this spectral shift, confirming that it was not due to induced dye leakage and the formation of extravesicular calcium–arsenazo complexes (Weissmann et al., 1980).

Discussion

The present study clearly establishes that CTC is a selective and efficient calcium ionophore. The molecule binds calcium with a high degree of specificity and shows comparably little affinity for other alkaline earth or alkali metal ions. CTC also complexes with lanthanide ions, which are known calcium antagonists in a number of systems (Pearce & White, 1981; Weiss, 1974). However, this effect is not likely to be of inconvenience since these ions do not occur naturally in biological materials. Of the ionophores so far tested, only ionomycin (Liu & Hermann, 1978) and A23187 (Liu & Hermann, 1978; Pfeiffer & Lardy, 1976; Pfeiffer et al., 1974; Puskin & Gunter, 1975; Reed & Lardy, 1972) show a comparable selectivity to CTC. However, these agents also exhibit a marked affinity for magnesium ions. The antibiotic X-537A transports calcium but additionally binds monovalent ions and organic amines (Pressman, 1973).

The detailed ionophoretic properties of CTC show some features in common with those of ionomycin and A23187. The partition properties of CTC are complex, but the antibiotic progressively solvates calcium into an organic environment as the pH is increased. Maximum binding occurs at about pH

8, similar to that of A23187 (Liu & Hermann, 1978; Reed & Lardy, 1972) and significantly nearer physiological values than that for ionomycin (pH 9–10; Liu & Hermann, 1978). Like the latter ionophore, CTC forms a 1:1 complex with calcium ions in contrast with the 2:1 complex formed by A23187 (Liu & Hermann, 1978; Pfeiffer et al., 1974; Reed & Lardy, 1972). CTC mediates an efficient transport of calcium ions in the H tube, but the rate is less (ca. 15%) than that previously found for A23187 under the same conditions (White & Pearce, 1982). The compound also induces calcium fluxes in multilamellar vesicles, although the compound was again less active than A23187 (Weissmann et al., 1980) in this system. Multilamellar vesicles provide a sensitive index for ionophoretic activity (Weissmann et al., 1980) and constitute a lipophilic environment more closely approximating to that of natural biological membranes. The activity of CTC under these conditions and its unique ability to discriminate between calcium and the other biologically important divalent cation magnesium suggest that it may provide a particularly useful tool in examining the role of calcium in cellular function. We have recently shown (Pearce et al., 1983) that the molecule induces calcium-dependent exocytosis and histamine secretion from mast cells, and further investigations in this direction are in progress.

Finally, the present results should be considered in terms of the common use of CTC as a fluorescent probe to monitor calcium movements during cellular activation. In such studies it is essential that the probe itself does not induce a redistribution of calcium. In view of the ionophoretic properties of CTC, this possibility clearly exists, and studies using this agent should then be conducted with considerable caution.

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