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The Migration of Divalent Cations in Mitochondria Visualized by a Fluorescent Chelate Probe

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Summary. The use of the fluorescent chelate probe, chlorotetracycline, in mitochondria is described. The probe shows a high fluorescence in the presence of mitochondria which may be ascribed to binding of the probe to membrane-associated Ca^{++} and Mg^{++}. The fluorescence excitation and emission spectra are diagnostic of binding of the probe to Ca^{++} in coupled mitochondria and Mg^{++} in uncoupled mitochondria. The fluorescence polarization spectra are diagnostic of the cations having a moderately high mobility in the membrane environment. The effects of exogenous EDTA and of endogenous Mn^{++} indicate that the probe is primarily visualizing actively accumulated Ca^{++} on the inner surface of the inner membrane. By employing the Ca^{++} transport inhibitor, Tb^{+++}, the fluorescence changes associated with metabolic alterations are shown to arise partly from cation transport and partly through alterations in the binding properties of the inner surface of the membrane. Chlorotetracycline is a probe for divalent cations associated with the membrane and is of general utility in the study of cation migrations in cellular and subcellular systems.

In attempts to gain further insight into the mechanistic process of energy conservation in mitochondria, fluorescent membrane probes have been employed (Azzi, 1969; Azzi, Chance, Radda & Lee, 1969; Gitler, Rubalcava & Caswell, 1969; Brocklehurst, Freedman, Hancock & Radda, 1970; Datta & Penefsky, 1970). The data provided from interaction of cations with fluorescence probes in membranes strongly suggest electrostatic attraction of the charged probe to the membrane surface (Rubalcava, de Munoz & Gitler, 1969; Vanderkooi & Martonosi, 1969; Gomperts, Lantelme & Stock, 1970). In mitochondria and submitochondrial particles the alterations of fluorescence intensity of probes initiated by altering the mitochondrial metabolism are opposite for anionic compared with cationic probes implying an electrical nature to the membrane change (Azzi, 1969; Gitler *et al.*, 1969; Caswell & Gitler, 1970).

Recently, Caswell and Hutchison (1971*a*, *b*, *c*) have described an entirely new variety of fluorescent membrane probe; this is the fluorescent chelate. It was demonstrated that chlorotetracycline binds to Ca^{++} and Mg^{++} with enhanced fluorescence and that the fluorescence of the cation chelate is itself a function of the polarity of the environment. The authors also demonstrated that in non-aqueous media the conformation of the Ca^{++} chelate differed from that of the Mg^{++} chelate and that this was reflected in different excitation and emission spectra for the two complexes. This enables a distinction to be drawn between Ca^{++} and Mg^{++} bound to membranes.

In this paper the probe, chlorotetracycline, is employed in a detailed examination of the role of divalent cations in mitochondrial metabolic processes. The probe is utilized in exploring the processes occurring on the inner side of the membrane of the mitochondria.

Materials and Methods

Rat liver mitochondria were prepared in the usual manner with EDTA in the homogenizing medium to extract divalent cations from the bathing solution. Chlorotetracycline was obtained from Nutritional Biochemicals Corp. and fresh solutions were prepared daily in the hydrochloride form, since the antibiotic is more stable in acid solutions.

Fluorescence was measured using a Hitachi-Perkin Elmer spectrofluorimeter as described previously (Caswell & Hutchison, 1971*a*, *b*). Fluorescence excitation and emission spectra have not been corrected for variations of exciting light intensity or photomultiplier sensitivity as a function of wavelength. Fluorescence polarization spectra were determined with polarizer attachment for the fluorimeter. z polarization was employed as well as x and y polarization in order to correct for partial polarization of the exciting light and for variable sensitivity of the photomultiplier to x and y polarized light. The correction was applied as described by Azumi and McGlynn (1962).

The distribution of chlorotetracycline between mitochondria and the external medium was determined by centrifuging the mitochondria through a layer of silicone oil. The supernatant was transferred to the fluorimeter cuvette and antibiotic was determined fluorimetrically in a medium containing 90% methanol and 2 mM $MgCl_2$.

Results

Physical Properties of Fluorescent Probe

The physical properties of chlorotetracycline chelation and fluorescence have been described in detail elsewhere (Caswell & Hutchison, 1971*a*, *b*). Of particular significance are the alteration of fluorescent quantum yield with polarity of the probe chelate environment and the spectral distinction between the Ca^{++} and Mg^{++} complexes.

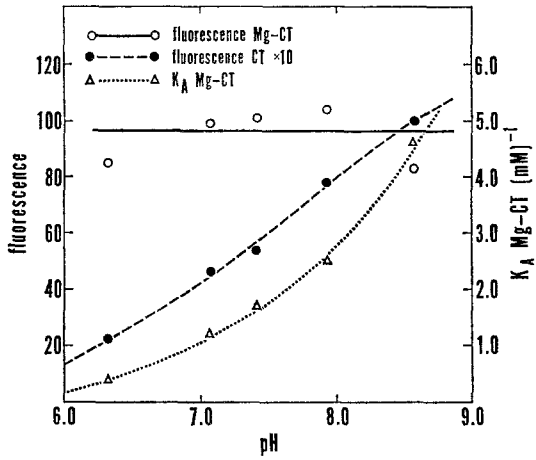

Fig. 1. Fluorescence parameters of Mg-chlorotetracycline (CT) chelate as a function of pH. The medium consists of 32 mM Na barbital; 117 mM NaCl; acetic acid to make appropriate pH; 10 μM chlorotetracycline. The fluorescence of CT is monitored directly at an excitation wavelength of 380 nm and emission of 520 nm. The fluorescence of Mg-CT and the K_A are obtained by titrating $MgCl_2$ into the incubation medium and plotting the reciprocal of the fluorescence against the reciprocal of $MgCl_2$. The intercept gives the fluorescence of Mg-CT and the slope gives the K_A

The effect of pH on the chlorotetracycline fluorescence is important in interpretation of the probe characteristics in biological membranes. The charge of the free probe and of the probe chelate are also significant factors in interpretation. Fig. 1 shows the fluorescence at excitation wavelength of 380 and emission of 520 nm of the free chlorotetracycline and the chlorotetracycline-Mg chelate as a function of pH. The affinity of the probe for the cation at the different values of pH is also shown. It is observed that the fluorescence of the native chlorotetracycline varies with the pH. This is to be expected in view of the fact that chlorotetracycline has three or possibly four dissociable groups, one of which is reported to have a pK of 7.4 (Stephens, Conover, Pasternack, Hochstein, Moreland, Regna, Pilgrim, Brunings & Woodward, 1954). On the other hand, there is virtually no variation in the fluorescence of the chelate over a pH range of 6.0 to 8.5. Within the range of external and endogenous pH expected in mitochondria, the divalent cation chelate with chlorotetracycline has a quantum yield which is independent of pH.

According to the data of other workers, chlorotetracycline has a partial negative charge at pH 7.4. The variation of affinity of the probe for Mg^{++} as a function of pH is determined by the following equation:

$$CT + Mg^{++} \rightleftharpoons CTMg^{(2-n)} + nH^+ \qquad K_D = k[H^+]^n$$

where CT is chlorotetracycline, K_D is the dissociation constant and n is the stoichiometry of H^+ reaction with CT.

CT has a net charge between 0 and -1 over the range of pH presented in Fig. 1. Since n will vary as a function of the extent of dissociation of CT, the value of log K_D will not be a linear function of pH. However, if $0 < n < 1$, then CTMg has a single positive charge. If $1 < n < 2$, then CTMg has no positive charge. The data are indicative of the chelate having a single positive charge over the pH range investigated and the constancy of the fluorescence of the chelate implies that there is no pK of the complex in the region of neutrality.

The significance of the data described above is that, if the membrane state is altered by any means, resulting in a change in fluorescence quantum yield of the bound probe, this change cannot be attributed to a change of pH of the membrane environment. On the other hand, a change in affinity of the probe for a cation may be attributed to a change in pH unless specifically excluded by other experiments. For this reason the mitochondria were well buffered in the subsequent experiments. Also in ion transport experiments, acetate was normally included in the medium to serve as a permeant anion and reduce any cation/H^+ exchange and so minimize any pH change inside the mitochondria associated with ion uptake.

Fluorescent Properties of Probe in Mitochondria

In a previous paper (Caswell & Hutchison, 1971*a*) it was demonstrated that a large fluorescence enhancement occurred when mitochondria were added to a medium containing chlorotetracycline but no divalent cation. However, divalent cations are present in the mitochondrial suspension and this fluorescence increase was attributed to binding of chlorotetracycline to divalent cations attached to the mitochondrial membrane surface. A reduction in fluorescence intensity was observed on addition of respiratory inhibitors or uncoupling agents.

Atomic absorption determinations on a suspension of mitochondria isolated for these experiments showed that Ca^{++} and Mg^{++} were both present in the suspension. The fluorescence spectroscopy shown in Fig. 2 was designed to elucidate whether the fluorescence of the antibiotic in the presence of mitochondria was associated with binding to Ca^{++} or to Mg^{++}. These fluorescence excitation and emission spectra are presented in Fig. 2*A*. A striking difference in the spectra is observed between coupled and uncoupled mitochondria. By comparison, Fig. 2*B* exhibits the spectra of chlorotetracycline when chelated either to Ca^{++} or to Mg^{++} in a metha-

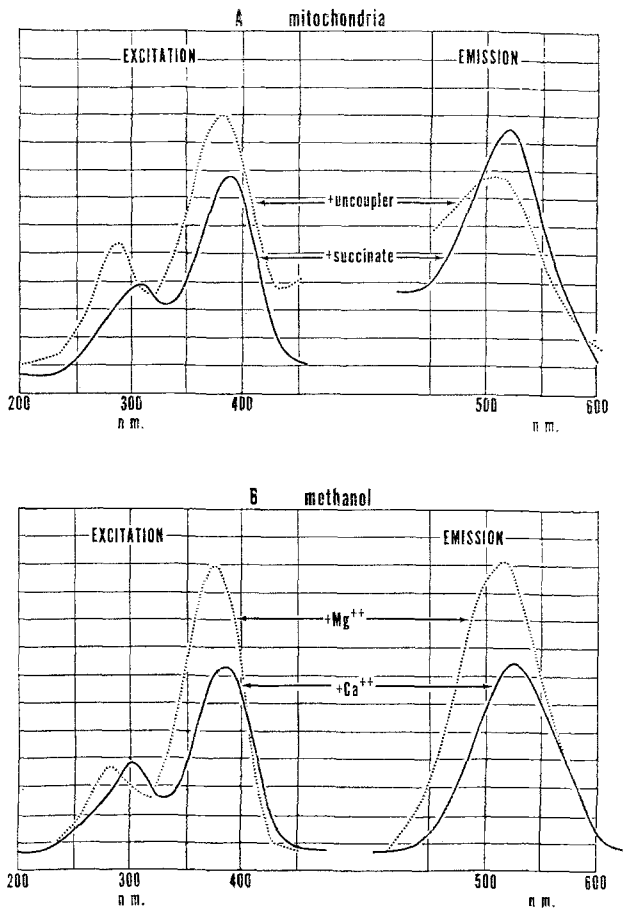

Fig. 2. Comparison of fluorescence spectra of chlorotetracycline in mitochondria and the Mg^{++} and Ca^{++} chlorotetracycline chelates in methanol. (*A*) Incubation medium consists of 250 mM sucrose; 12 mM Tris Cl; 10 μM chlorotetracycline; mitochondria 0.2 mg protein/ml and, where indicated: 12 mM Tris succinate or 1 μM *p*-trifluoromethoxy (carbonylcyanide) phenyldrazone (FCCP). pH is 7.4 and temperature 22 °C. The gain of the fluorometer amplifier is 3 times greater for the spectra in the presence of FCCP. The emission wavelength is 520 nm for the excitation spectra and the excitation wavelength is 380 nm for the emission spectra. The slit widths are 12 nm for excitation and emission. (*B*) Incubation medium consists of 90% methanol; 1 mM Tris Cl; 10 μM chlorotetracycline and 1 mM divalent cation chloride. pH is 7.4 and temperature 22 °C. Excitation and emission spectra are determined as in Fig. 2*A*

nolic solution. The comparison of Fig. 2*A* with 2*B* provides a direct and simple explanation of the variation of fluorescence spectra with state of energization of the mitochondria. Thus it is proposed on the basis of this comparison that chlorotetracycline binds primarily to Ca^{++} in the membrane in coupled mitochondria and to Mg^{++} in uncoupled mitochondria. In

coupled mitochondria the excitation peaks are located at 310 and 390 nm and in uncoupled mitochondria the peaks are at 285 and 375 nm. The values for the Ca^{++} chelate obtained from Fig. 2*B* are 305 and 390 nm and the Mg^{++} chelate gives values of 280 and 375 nm. Ca^{++} and Mg^{++} are the only divalent cations present in free form in mitochondria and atomic absorption analysis shows their presence in these mitochondria. Thus the excitation and emission spectra of chlorotetracycline in coupled mitochondria in Fig. 2*A* are identical to the spectra of Ca-chlorotetracycline in Fig. 2*B* while the uncoupled mitochondria have spectra corresponding to Mg-chlorotetracycline.

Localization of Probe in Mitochondria

Two criteria are employed to determine the localization of the probe in the mitochondria. (1) EDTA is added to the mitochondrial medium to eliminate Ca^{++} and Mg^{++} from the external phase and hence presumably to reduce the concentration of divalent cation bound to the external side of the inner membrane. (2) A paramagnetic divalent cation is added which can be transported across the mitochondrial membrane. Paramagnetic species, when they bind to tetracyclines, cause quenching of fluorescence instead of enhancement. Thus the paramagnetic divalent cation will, after accumulation, compete for any chlorotetracycline bound to the inner side of the membrane.

Fig. 3 shows the influence of EDTA on the fluorescence of chlorotetracycline bound to mitochondria. The addition of mitochondria to the incubation medium is associated with a slow increase of fluorescence caused by binding of Ca^{++} and chlorotetracycline to the membrane. The source of this Ca^{++} is the same as in Fig. 2 – namely, Ca^{++} introduced from the mitochondrial suspension. Further treatment with exogenous Ca^{++} causes an enhancement of fluorescence. Since the mitochondria are coupled, the Ca^{++} is actively accumulated and it is the accumulated Ca^{++}, which is responsible for the increased signal. Acetate has been included in the incubation medium since, in the presence of this anion, there is little exchange of Ca^{++} for H^{+} and hence any artifact of the fluorescence signal caused by alkalinization of the internal medium is minimized.

If EDTA is added after the initial appearance of fluorescence in the presence of mitochondria, then no diminution of signal is observed such as might be expected if the chlorotetracycline were bound to Ca^{++} or Mg^{++} in the external medium or on the external surface of the membrane (Fig. 3*B*). On the other hand, the succeeding addition of Ca^{++} no longer

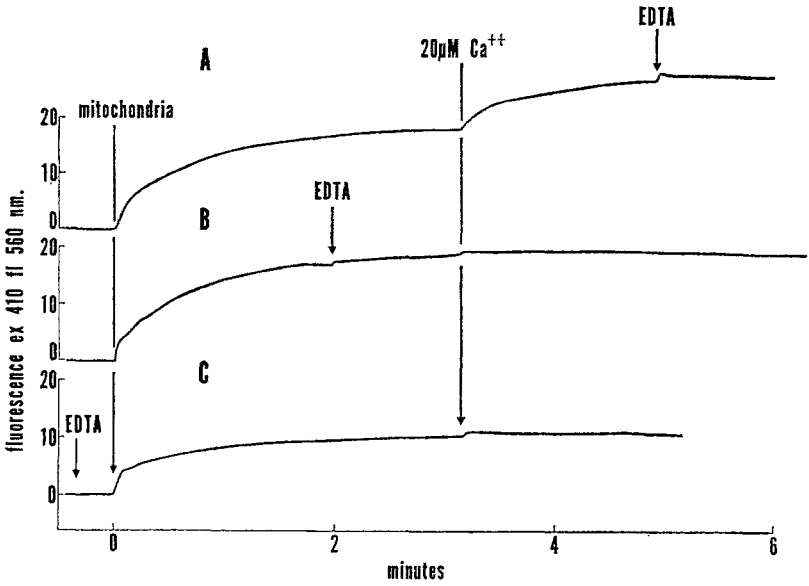

Fig. 3. Effects of EDTA on fluorescence of chlorotetracycline in mitochondria. Incubation medium consists of 250 mM sucrose; 10 mM Tris Cl; 25 mM Tris acetate; 7.5 mM Tris glutamate; 7.5 mM Tris malate; 2.5 mM KCl; 10 μM chlorotetracycline. Additions to the medium are: mitochondria, 0.6 mg protein/ml; Tris EDTA, 1 mM and $CaCl_2$, 20 μM. Final pH is 7.4 and temperature 22 °C. The light scattering artifact on addition of mitochondria is 3 units of fluorescence

is able to induce enhanced fluorescence. Thus the EDTA is able to chelate exogenous Ca^{++} and prevent transport. Any Ca^{++} in the external medium or attached to the membrane surface, but in equilibrium with the external medium, may be expected to be chelated by EDTA. It is therefore concluded that the fluorescent probe is binding to Ca^{++} accumulated within the mitochondria. Fig. 3*C* shows that, if EDTA is present in the original incubation medium, then the fluorescence associated with the mitochondria is reduced. Thus it appears that the source of part of the Ca^{++} which induces the fluorescence is the cation present exogenously in the mitochondrial suspension. The EDTA chelates this Ca^{++} and so prevents accumulation. The other source of Ca^{++} is present endogenously and is not influenced by EDTA.

The elucidation of the localization of the probe signal in the membrane is aided by the employment of paramagnetic cations to compete with the diamagnetic species, Ca^{++} and Mg^{++}. Mn^{++} is a paramagnetic cation which is transported actively at a low rate (Chappell, Cohn & Greville, 1963). When Mn^{++} binds to chlorotetracycline it causes fluorescence quenching associated with enhanced intersystem crossing from the excited

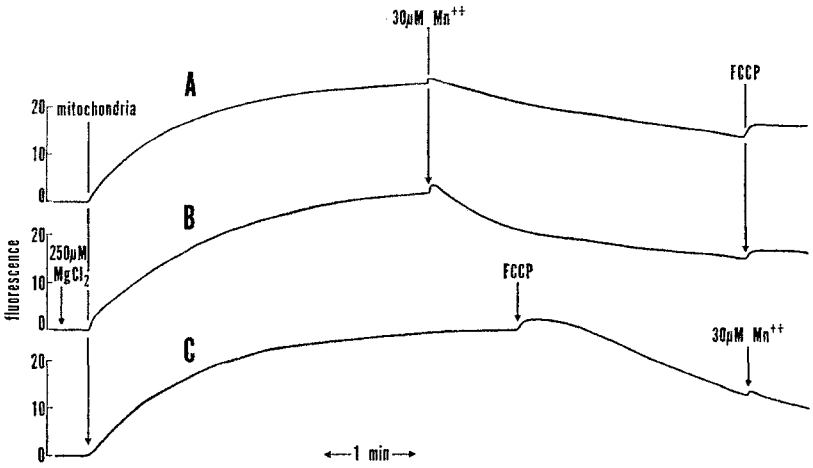

Fig. 4. Effects of Mn^{++} transport on fluorescence of chlorotetracycline in mitochondria. Incubation medium consists of 250 mM sucrose; 10 mM Tris Cl; 25 mM Tris acetate; 7.5 mM Tris succinate; 2.5 mM KCl; 10 μM chlorotetracycline. Additions to the medium, where indicated are: $MgCl_2$, 250 μM; $MnCl_2$, 30 μM; FCCP, 1 μM. Final pH is 7.4 and temperature 22 °C. Excitation is at 410 nm and emission at 560 nm

singlet state to the triplet state induced by the paramagnetism. This ion is utilized in Fig. 4 to determine the site of probe binding.

On addition of Mn^{++} to the mitochondrial suspension, there is a slow diminution of the fluorescence such as may be attributed to Mn^{++} accumulation followed by binding competitively against Ca^{++} with the chlorotetracycline on the inside of the mitochondria. This effect is independent of the presence of Mg^{++} in the medium, since Mg^{++} is not accumulated at an appreciable rate. If the mitochondria are uncoupled, then Mn^{++} has no effect on the fluorescence (Fig. 4*C*). This may be attributed to the lack of Mn^{++} accumulation when the energy source is depleted, so that Mn^{++} is unable to compete for endogenous cation binding sites.

The data presented in Figs. 3 and 4 show that the fluorescence increase of chlorotetracycline on energization of the mitochondria is associated with actively accumulated Ca^{++}. The experimental findings may distinguish two pools of divalent cation. One pool is chelated by exogenous EDTA and consists of Ca^{++} or Mg^{++} in the external aqueous phase and any Ca^{++} in equilibrium with this which is bound to the external side of the mitochondrial membrane. The second pool is not chelated by EDTA, but is influenced by actively accumulated Mn^{++}. This pool presumably consists of Ca^{++} or Mg^{++} in the matrix and divalent cation bound to the membrane after accumulation. This second part is responsible for the high

fluorescence of chlorotetracycline in energized mitochondria. However, it is not possible to determine from fluorescence measurements the spatial position of the divalent cation in the membrane. Nevertheless, it may be expected that this membrane bound cation is in closer proximity to the internal surface than the external surface.

Physical Properties of Cation Environment

The foregoing data have established that the chlorotetracycline is binding to Ca^{++} and Mg^{++} located within the mitochondria. The fluorescence is highly responsive to addition of cations which are actively accumulated and it may be considered that the fluorescence signal is a function of the endogenous divalent cation concentration. If Ca^{++} is accumulated when phosphate is present in the medium instead of acetate, then little fluorescence enhancement is observed. Addition of phosphate to a mitochondrial suspension causes a slow reduction in fluorescence. This is in accord with the view that phosphate is transported into the mitochondria and then binds endogenous Ca^{++} and so reduces its concentration in the free form and hence reduces the amount of Ca^{++} which binds to the membrane.

The question as to whether the chlorotetracycline fluorescence is a measure of internal Ca^{++} in the matrix or of endogenous Ca^{++} bound to the membrane may be readily resolved. The fluorescence in coupled mitochondria associated with the probe is far in excess of that to be observed on binding to Ca^{++} or Mg^{++} in aqueous solution. Moreover, the positions of the excitation and emission peaks of chlorotetracycline in mitochondria do not correspond with those of Ca^{++} or Mg^{++} in free aqueous solution but do correspond with those of Ca^{++} in an apolar environment. Thus, the fluorescence may be attributed to Ca^{++} in the lipophilic environment of the membrane.

It appeared that endogenous adenine nucleotides might play a role in the fluorescence signals of chlorotetracycline, since ATP and ADP are able to chelate Ca^{++}. Accordingly, the energy transfer inhibitor, oligomycin, was employed to test this possibility. This inhibitor prevents the respiration dependent synthesis of ATP from ADP. Thus, if oligomycin and ADP are added to the mitochondria, then the mitochondrial respiration remains in the energized state 4, but the adenine nucleotide transport system will cause ATP to be replaced by ADP in the matrix. However, the combination of oligomycin and ADP had no effect on the fluorescence. Divalent cations bind to ATP with higher affinity than ADP and so it might have been expected that the replacement of ATP by ADP would increase the fluores-

cence signal. However, in the experiment described above this did not occur, and it thus appears that differential binding of Ca^{++} or Mg^{++} to ATP or ADP plays no role in the chlorotetracycline signal.

Further information regarding the nature of the divalent cation binding site in the membrane may be obtained by physical study of fluorescence. Thus, fluorescence energy transfer, fluorescence polarization and fluorescence spectroscopy may all be employed as techniques in examination of the physical state of the fluorophore.

Fluorescence Energy Transfer

Brocklehurst *et al.* (1970) have described fluorescence energy transfer between the tryptophan moieties of the membrane protein and the probes, 1-anilinonaphthalene-8-sulfonate (ANS) and 2(N-methylanilino)naphthalene-6-sulfonate. They were able to conclude that the average distance between the ANS and the tryptophan moieties is less than 30 Å.

The fluorescent excitation spectrum of chlorotetracycline bound to mitochondria does not include any component which may be attributed to energy transfer from the tryptophan protein fluorescence. No quenching of the protein fluorescence signal other than may be attributed to the inner filter effect is observed after treatment of mitochondria with tetracycline. We are thus unable to observe energy transfer despite the spectral overlap and high extinction coefficient of chlorotetracycline and conclude that a considerable distance separates chlorotetracycline in the membrane from aromatic protein residues.

Fluorescence Polarization

Depolarization of fluorescence is associated with rotation of the fluorophore in the period between excitation and emission of light and may be employed to estimate the rotational mobility of the probe.

Fig. 5 shows the fluorescence polarization spectrum of chlorotetracycline bound to mitochondria in comparison with that of Ca-chlorotetracycline immobilized in glycerol and Ca-chlorotetracycline freely diffusible in methanol. The region of high polarization covers the spectrum from 360 to 420 nm. At short wavelengths the polarization is low probably as a result of intramolecular energy transfer. The comparison of the polarization of the membrane bound probe (0.18) to the probe dissolved in glycerol (0.39) and methanol (0.12) illustrates that there exists a measure of rotational

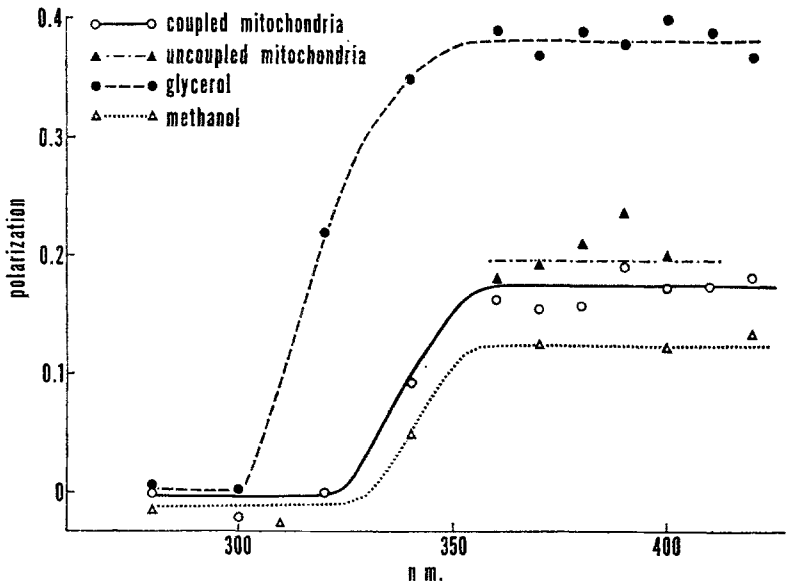

Fig. 5. Polarization fluorescence spectra of chlorotetracycline in mitochondria. Polarization measurements were made with the polarizer attachment to the spectrofluorimeter. Fluorescence signals were obtained with vertically polarized exciting light and vertically and horizontally polarized emitted light and then with horizontally polarized exciting light and vertically and horizontally polarized emitted light. The polarization, p, was determined from the equation of Azumi and McGlynn (1962). Stray light effects are minimized by incorporating a 430 nm cut-off filter in the emission path. Any stray light signal associated with mitochondrial scattering is determined in the absence of chlorotetracycline and subtracted from the chlorotetracycline fluorescence signal. The incubation medium for the mitochondria consists of 250 mM sucrose; 10 mM Tris Cl; 2.5 mM KCl; 10 μM chlorotetracycline; 12 mM Tris succinate for coupled mitochondria or 1 μM FCCP for uncoupled mitochondria. Final pH is 7.4 and the temperature 22 °C. The mitochondrial concentration is 0.4 mg protein/ml. The glycerol medium is 95% glycerol, 1 mM Tris Cl; pH 7.4. The methanol medium is 95% methanol; 1 mM Tris Cl, pH 7.4

mobility of the probe complex, but that the mobility is nevertheless impaired compared with that of freely diffusible chlorotetracycline. However, the rotational depolarization will also depend on the excited state lifetime. The quantum yield of chlorotetracycline in the membrane is higher by a factor of about 5 than that in methanol and this is indicative of a longer fluorescence lifetime of the probe in the membrane. Thus the restriction of rotational motion of the Ca^{++} chelate in the mitochondria is slightly greater than is indicated by the value of p in Fig. 5. This is further evidence of the binding of the probe in a membrane environment. Moreover, the polarization of the probe is higher than in methanol for uncoupled as well

as coupled mitochondria indicating that in uncoupled mitochondria, the fluorescent signal also has a membrane origin. The possibility that the level of polarization observed could be a reflection of concentration depolarization or scattering of the emitted light is precluded by the observation that the polarization of the fluorescence is independent of the mitochondrial concentration.

If Ca^{++} is liganded to groups in the membrane, then it may be expected that its reactivity with chlorotetracycline will be low, since the activity of free Ca^{++} in the membrane region will be low. However, if Ca^{++} is attracted to the membrane by electrostatic forces caused by charged groups at the membrane surface, then the activity of free Ca^{++} on the membrane surface will be high and so a high affinity of the probe for Ca^{++} may be expected. This is the situation observed here. It is, nevertheless, possible that the probe could bind Ca^{++} which is already liganded to membrane moieties. This would give a product which had a low mobility, since Hubbell and McConnell (1969) have demonstrated the immobility of the polar residues of the lipid matrix. Thus chelation of chlorotetracycline to Ca^{++} which is itself coordinated to phospholipids or proteins should give a product of low mobility. The observation that the mobility of the Ca-chlorotetracycline chelate is high is presumptive evidence either that the bulk of the Ca^{++} in the membrane environment is not coordinated or that any coordination is sufficiently weak that the ion may be displaced from its membrane complex by chlorotetracycline. Thus, the data of Fig. 5 indicates that the mobility is appreciable and that the Ca^{++} is electrostatically attached to the membrane surface and hence is able to move freely within the restrictions imposed by electrostatic forces.

Origin of Fluorescent Changes Accompanying Metabolic Changes of Mitochondria

The data presented above have demonstrated that Ca^{++} accumulation is a prerequisite for the appearance of the intense fluorescent signal from chlorotetracycline in mitochondria. It was established in a previous communication (Caswell & Hutchison, 1971*a*) that chlorotetracycline fluoresces with high quantum yield only when it is chelated to cations.

The question arises as to whether the fluorescence changes accompanying mitochondrial metabolic changes can be attributed entirely to an increased Ca^{++} binding which results directly from Ca^{++} accumulation or whether the fluorescence changes are reflecting in part an alteration in the capacity of the membrane for binding Ca^{++} or chlorotetracycline. This question

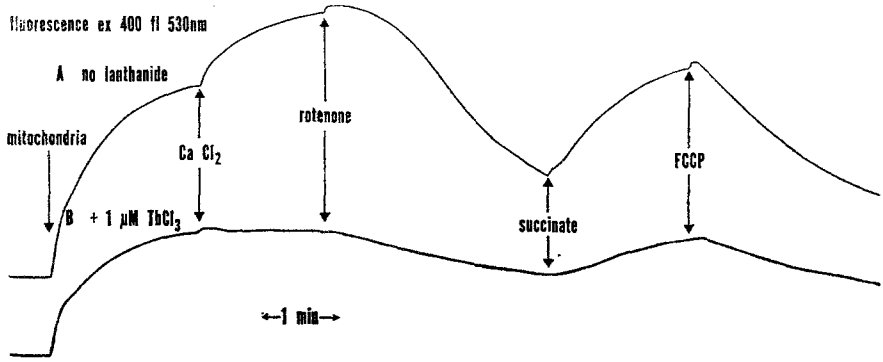

Fig. 6. Effect of inhibition of Ca^{++} transport on chlorotetracycline fluorescence in mitochondria. Incubation medium consists of 250 mM sucrose; 10 mM Tris Cl; 25 mM Tris acetate; 7.5 mM Tris glutamate; 7.5 mM Tris malate; 2.5 mM KCl; 10 μM chlorotetracycline. Additions to the medium are: mitochondria, 0.4 mg protein/ml; $TbCl_3$ 1 μM; $CaCl_2$, 10 μM; rotenone, 5 μM; Tris succinate, 10 mM; FCCP, 1 μM. Final pH is 7.4 and temperature 22 °C. Wavelength of excitation is 400 nm and emission is 530 nm

was resolved by employing lanthanide inhibitors of Ca^{++} transport (Mela, 1968). Chlorotetracycline binds to lanthanides with a high affinity. However the fluorescence caused by chelation of the lanthanide series depends on the magnetic property of the element, being low for paramagnetic and high for diamagnetic lanthanides. Accordingly, the presence of a lanthanide may be observed or masked depending on the magnetic properties of the element.

In Fig. 6, Tb^{3+} is employed as the inhibitor, since the chelate of this element has a low fluorescence and hence no interference with the Ca^{++} tetracycline signal is to be expected. The Tb^{3+} is present at a concentration sufficient to inhibit Ca^{++} transport, but insufficient to bind a significant proportion of the antibiotic. The figure shows that if Tb^{3+} is present in the incubation medium, then the fluorescence induced by mitochondria is low. Presumably the Tb^{3+} inhibits uptake of that portion of the Ca^{++} which was present exogenously in the mitochondrial suspension. Further addition of Ca^{++} causes no change in fluorescence indicating that 1 μM Tb^{3+} has inhibited Ca^{++} transport. The effect of Tb^{3+} in eliminating the fluorescence increase normally caused by Ca^{++} addition is further evidence of the necessity for the association of Ca^{++} accumulation with fluorescence increases. Successive additions of respiratory inhibitors, substrates and uncoupling agents cause alterations in fluorescence of much lower magnitude than those seen in the absence of Tb^{3+}. It has been demonstrated by Lehninger and Carafoli (1971) that lanthanides inhibit

uptake of Ca^{++}, but not outflow. Thus the small diminution in fluorescence, when rotenone is added, may be attributed to incomplete inhibition by Tb^{3+} of Ca^{++} release. The small fluorescence enhancement caused by succinate, which may not be attributed to Ca^{++} uptake, must be explained either as an increased Ca^{++} or chlorotetracycline binding to the membrane or as an increased quantum yield of the chelate in the membrane. It is therefore concluded that the Ca^{++} chlorotetracycline fluorescence signal reflects a composite effect of the Ca^{++} concentration endogenously in the mitochondria and a variable binding property of Ca^{++} to the inner side of the mitochondrial membrane; though in normal conditions the energy linked accumulation of Ca^{++} primarily determines chlorotetracycline fluorescence.

The fluorescence changes of chlorotetracycline associated with metabolic transitions have been attributed here to changes in Ca^{++} accumulation and release. It may be predicted on the basis of this conclusion that chlorotetracycline will be passively absorbed on the mitochondria in response to Ca^{++} accumulation and binding. Thus, as Ca^{++} accumulates in the membrane and chelates with chlorotetracycline, so free antibiotic will move into the membrane to replace that which has chelated to the divalent cation. Such a process should be distinguished from the variable binding of other charged fluorescent probes in that the chlorotetracycline movements are expected to be a passive response to Ca^{++} movements. This has already been seen in the fluorescence response to reagents which affect Ca^{++} transport or compete for membrane binding sites. A more direct confirmation of this prediction is shown in Fig. 7. Here the accumulation of chlorotetracycline by mitochondria has been observed directly. Fig. 7*A* shows the characteristic fluorescence signals in the presence and absence of the lanthanide inhibitor of Ca^{++} transport. Fig. 7*B* shows data concerning chlorotetracycline accumulation by mitochondria under identical metabolic conditions. In energized mitochondria there is a perfect correspondence between antibiotic accumulation and the fluorescence signal which continues when an uncoupling agent causes both the diminution of fluorescence and release of chlorotetracycline. If Tb^{3+} is present, this same correspondence holds. However, both accumulation and fluorescence are lower than in the control. Tb^{3+} is a specific inhibitor of Ca^{++} transport and cannot be expected to influence chlorotetracycline accumulation directly. Thus the data of Fig. 7 are evidence that chlorotetracycline accumulation occurs passively in response to Ca^{++} transport.

The binding parameters of chlorotetracycline to mitochondria are shown in Fig. 8. The fluorescence enhancement of chlorotetracycline on

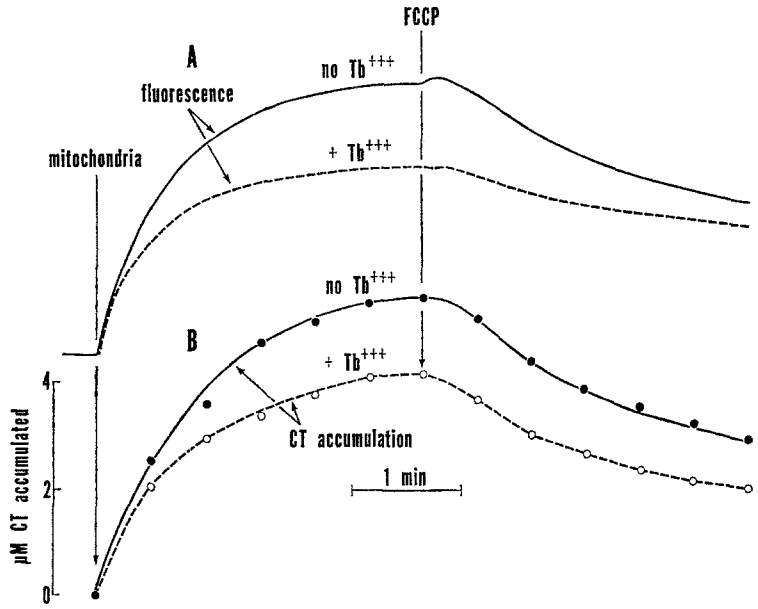

Fig. 7. Passive accumulation of chlorotetracycline by mitochondria. Incubation medium consists of 250 mM sucrose; 10 mM Tris Cl; 25 mM Tris acetate; 7.5 mM Tris glutamate; 7.5 mM Tris malate; 2.5 mM KCl; 10 μM chlorotetracycline. Additions to the medium are mitochondria, 0.6 mg protein/ml; $TbCl_3$, 1 μM; FCCP, 1 μM. Final pH is 7.4 and temperature 22 °C. In (*A*) the wavelength of excitation is 380 nm and emission is 530 nm. For (*B*), 100 μliters aliquots of the incubation system are transferred at the time indicated to a small centrifuge tube and pipetted to form the top layer of a sandwich containing 50 μliters of 13% perchloric acid lower layer and a middle layer of silicone oil. The mitochondria are rapidly centrifuged through the silicone oil so that the top layer is the supernatant. The chlorotetracycline in the supernatant is analyzed fluorimetrically in a medium containing 90% methanol; 2 mM $MgCl_2$ and 5 mM Tris Cl, pH 7.4. The fluorescence reading is standardized in comparison with known quantities of chlorotetracycline both in the presence and in the absence of 1 μM $TbCl_3$

binding to coupled mitochondria is obtained by varying the membrane concentration. The fluorescence at infinite membrane concentration is that of fully bound probe and is estimated from a double reciprocal plot. (Fig. 8*A*). The enhancement of fluorescence of chlorotetracycline over that of free probe is ca. 160 for coupled mitochondria and 110 for uncoupled mitochondria. This fluorescence is materially greater than that of Ca-chlorotetracycline in methanol. The dynamic polarity of the probe and Ca^{++} binding environment is therefore low, implying probable deep penetration of the cation in the Stern layer of the membrane. The affinity of the probe for the mitochondria is also high. The K_D for coupled mito-

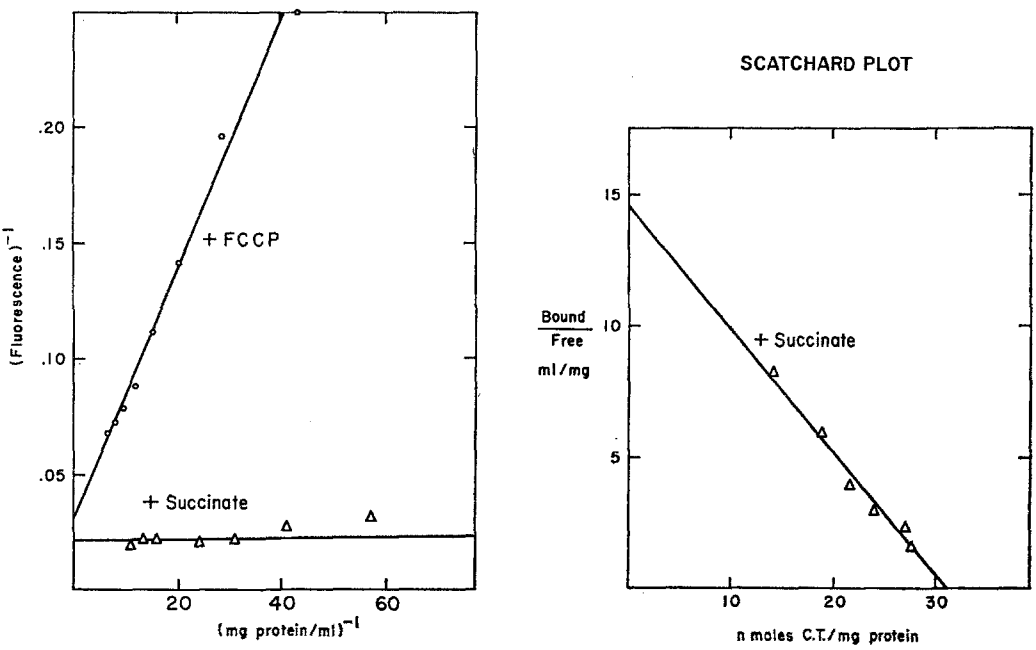

Fig. 8. Probe and membrane titrations to determine fluorescence and binding parameters. Incubation medium consists of 250 mM sucrose; 10 mM Tris Cl and either 7.5 mM Tris succinate or 2.5 μM FCCP. Final pH is 7.4 and temperature 22 °C. For the membrane titration (Fig. 7*A*) the concentration of chlorotetracycline is 5 μM; for the CT titration the mitochondrial concentration is 0.2 mg protein/ml. Excitation wavelength is 380 nm and emission is 530 nm

chondria is 2.1 μM determined from the Scatchard plot (Fig. 8*B*); this may reflect the high endogenous Ca^{++} concentration. Attempts to determine the fluorescence and binding parameters for uncoupled mitochondria are complicated by the fact that the fluorescence signal is a composite of the Mg^{++} and Ca^{++} complex. Accurate estimates have therefore not been obtained of the affinity of the chlorotetracycline in uncoupled mitochondria. Moreover, the differences between the parameters of coupled and uncoupled mitochondria would not be easy to interpret.

Discussion

This paper has attempted to fulfill two purposes. The divalent cation binding and active transport properties of mitochondria have been analyzed in detail employing fluorescent probes. However, the physical and biological

techniques employed have been of a general nature, such as might be appropriate for study of a wide range of membrane and metabolic processes. Ca^{++} plays a crucial controlling role in many cellular and subcellular transport systems. A technique for visualizing membrane cation binding could be valuable in many studies. Moreover, fluorescence techniques are exceedingly versatile by reason of the high sensitivity and many physical fluorescence parameters which may be utilized in biological studies. A few of the physical and physiological analytical methods have been illustrated in this communication.

The employment of fluorescent chelate probes as a means of visualizing membrane bound divalent cations requires that the following criteria be fulfilled: (1) The fluorescence of the uncomplexed probe should be low and that of the membrane bound cation chelate should be high. The data with mitochondria reveal that chlorotetracycline exhibits these properties admirably. (2) The probe should be able to penetrate biological membranes. The histological studies of DuBuy and Showacre (1961) combined with the present data that chlorotetracycline is binding to endogenous Ca^{++} demonstrate that this criterion is also fulfilled. (3) The chelating affinity of the probe for the cation should be fairly high but the complex should not be too strong. This latter requirement is rather critical since, if the complex is too stable, then it may migrate to regions of the membrane which would not normally be associated with the cation. If, on the other hand, the complex is fairly loose, then, as soon as the chelate migrates from the region of high cation concentration, the complex will dissociate. The affinities of cations for chlorotetracycline determined by Caswell and Hutchison (1971*a*) show that the chelating affinity is not excessive and it may be presumed that the fluorescence signal is proceeding from a region of high Ca^{++} or Mg^{++} concentration. (4) It is beneficial that the fluorescence of the chelate should be distinct for different cations. The data of Caswell and Hutchison (1971*b*) have demonstrated characteristic fluorescence spectra for Ca^{++} and Mg^{++} chelates.

The data have established that the fluorescence of chlorotetracycline may be attributed to Ca^{++} accumulated within the mitochondria when coupled respiration is occurring and to Ca^{++} and Mg^{++} in uncoupled mitochondria. The binding of divalent cations to the inner side of the mitochondria is monitored by this fluorescence technique. This is the first fluorescent reagent that has been demonstrated to probe the inner side of the membrane. The physical environment of the bound Ca^{++} is one of low dynamic polarity, partially restricted rotational mobility and considerable separation from aromatic protein residues. The data suggests that much of the membrane

bound Ca^{++} is not coordinated to membrane ligands. The accumulation of Ca^{++} and subsequent binding to the membrane surface may be directly related to energy-linked Ca^{++} transport. The Mg^{++} signal is more difficult to interpret. Mg^{++} is actively transported only at a slow rate (Johnson & Pressman, 1969). It appears from these experiments that Mg^{++} is present endogenously in normal mitochondrial preparations and remains within the organelle during mitochondrial metabolic changes.

The distinctive feature of the fluorescent chelate probe technique which separates it from other probing methods is the necessity of the probe to bind to divalent cations before the characteristic fluorescence signal may be elicited. Thus, the probe is reporting the location of divalent cations in membranes. Ca^{++} is known to play an important regulating role in many membrane systems and hence its location and environment in the membrane may give valuable information concerning physiological and metabolic processes. The superficial resemblance between chlorotetracycline fluorescence and the fluorescence of membrane probes such as ethidium bromide and ANS is not indicative of any similarity of properties of these reagents. Thus, the fluorescence changes of ethidium and ANS which accompany metabolic changes may be attributed to direct electrical alteration of the membrane surface which causes altered binding and fluorescence of the charged probes. However, the fluorescence changes of chlorotetracycline have here been demonstrated to correlate primarily with active Ca^{++} accumulation by mitochondria followed by binding of the cation to the membrane. This physiological process is not clearly related to the ill-defined membrane transition associated with energization or de-energization of the mitochondria.

This disposition of ions after they have been actively accumulated in subcellular vesicles has been largely a matter of surmise. The fluorescent chelate probe describes the properties and environment of divalent cations within vesicles. The data presented in this communication has demonstrated that Ca^{++} shows high affinity for the membrane after accumulation and the membrane bound Ca^{++} has a high activity. It has been proposed that the Ca^{++} is attracted to the membrane by electrostatic forces at the membrane surface caused by polar head groups. Cations may be accumulated at the Stern layer of all vesicular preparations through attraction to negative charges or dipoles of the membrane lipids. This is presumably the force operative in Ca^{++} binding in mitochondria. The implication of the high affinity of Ca^{++} for the membrane and the low polarity of the Ca^{++} environment is that the inner mitochondrial membrane surface has strong

negative potential with respect to the matrix. It is further to be expected that Ca^{++} accumulation and the subsequent membrane binding will reduce this negative zeta potential.

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