VISUALIZATION OF MEMBRANE BOUND CATIONS BY A FLUORESCENT TECHNIQUE

A. H. Caswell and J. D. Hutchison

Departments of Pharmacology and Microbiology

University of Miami, Miami, Florida 33152

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SUMMARY: Chlorotetracycline binds to divalent diamagnetic cations in aqueous solution with enhanced fluorescence. The fluorescence of the chelate is polarity dependent, being higher in nonaqueous solvents. If biological membranes or detergent micelles which bind cations are suspended in an aqueous phase, the fluorescence of the chlorotetracycline chelate shows enhanced fluorescence over the aqueous complex indicating migration of the divalent cation and chlorotetracycline into the nonaqueous membrane region. The fluorescence of chlorotetracycline bound to mitochondria varies as a function of the respiratory state of the mitochondria.

The application of fluorescent techniques in the study of membrane structure and function has been advanced by the introduction of probes of two basic varieties. Ionic or electrostatic probes are charged molecules, attracted to opposite charges on the membrane surface, and eliciting a concomitant alteration of fluorescence associated with movement from the aqueous to membrane environment. Such reagents are exemplified by 1-anilinonaphthalene-8-sulfonate (1,2,3,4), pyrene-3-sulfonate (5) ethidium bromide (6,7) and atabrine (8). A second class is formed by covalent binding of reagents to specific membrane groups to form fluorescent adducts (9,10).

In this paper is described a third class of membrane fluorescent agent: the fluorescent chelate. This is a reagent which binds to ions with a concomitant alteration of fluorescence spectrum and which shows a propensity for interaction with cations attached to the membrane surface. This type of reagent is exemplified by the antibiotic, chlorotetracycline.

Materials and Methods

Biological preparations were as follows: Erythrocyte membranes were prepared from whole human blood by the method of Dodge et al. (11) involving

hemolysis in a medium containing 12 mM Tris Cl; pH 7.4, 0.1 mM Tris EDTA, washing and resuspension of the erythrocyte membranes in 250 mM sucrose,10 mM Tris Cl pH 7.4. Rabbit skeletal muscle microsomes were prepared by the technique of Martonosi et al. (12), EDTA being present in the homogenizing medium. Mitochondria were prepared by the method of Schneider (13). Chlorotetracycline was purchased from Nutritional Biochemicals Corporation and fresh aqueous solutions were prepared daily.

Fluorescence was measured with a Hitachi-Perkin-Elmer MPF-2A spectrofluorometer. Fluorescence spectra are uncorrected scans. Titrations were effected normally using excitation at 380 nm and emission at 520 nm with a Wratten 55 filter in the emission path. The slit width was set at 12 nm for both monochromators.

Results and Discussion

Tetracycline antibiotics are known to bind divalent cations and histochemical studies have shown that organisms stained with tetracycline show fluorescence located in mitochondria, microsomes, bone tissue and tumors (14,15). Accordingly, the chelating and membrane binding properties of tetracyclines were investigated. In membrane studies chlorotetracycline proved superior to tetracycline, methacycline and minocycline and accordingly the interactions of chlorotetracycline are described here.

In Table I is shown the fluorescence enhancement at 380 nm excitation and 520 nm emission of chlorotetracycline and the dissociation constant on binding of the antibiotic to divalent cations. Chlorotetracycline binds Mg^{++}, Ca^{++}, Sr^{++}, and Zn^{++} giving enhanced fluorescence. On the other hand, paramagnetic species, Mn^{++} and Co^{++}, cause quenching of the fluorescence, presumably associated with a higher rate of intersystem crossing (cf Becker and Kasha (16)). The fluorescence intensity of the chelated antibiotic is a strong function of the polarity of the medium. The table shows that in 50% methanol the fluorescence of Mg-chlorotetracycline is increased by a factor of 2.9 over the fluorescence in water and the affinity of Mg^{++} is stronger by

TABLE I

Binding Constants of Chlorotetracycline to Divalent Cations

Cation	Medium	K_D μM	fluorescence enhancement over free aqueous reagent
Mg^{++}	aqueous	267	23
Mg^{++}	50% methanol	114	66
Zn^{++}	aqueous	42	10.5
Ca^{++}	aqueous	440	7.5
Sr^{++}	aqueous	65	10.7
Mg^{++}	Na dodecyl sulfate	110	101
Mg^{++}	erythrocyte membrane	370	109
Mg^{++}	microsome membrane	400	207

Media contain 12 mM Tris Cl pH 7.4; 10 μM chlorotetracycline. The chloride salt of the cation is titrated into the cuvette and the enhancement determined by a double reciprocal plot. The K_D is determined from a plot of log bound/free chlorotetracycline against log free cation. In all cases a 1:1 complex is formed. For the membrane estimates, the K_D and enhancement of Mg-chlorotetracycline are determined at a variety of membrane concentrations and the required parameters are obtained by extrapolation to infinite membrane concentration in a double reciprocal plot.

a factor of 2.3. Thus strong binding and high fluorescence are associated with nonaqueous media. Solutions of 10μM chlorotetracycline and 150mM $MgCl_2$ in different alcohols show enhancements of 17 in methanol, 36 in ethanol, 43 in n-propanol and 46 in n-butanol over the same reagents in water. The fluorescence of the unchelated antibiotic is also polarity dependent, but much less so than the metal chelate.

That chlorotetracycline binds to metal ions attached to the Stern layer of a variety of membranes is demonstrated by the fact that addition of membranes that bind cations causes a further enhancement of fluorescence of Mg^{++} chlorotetracycline. Thus sodium dodecyl sulfate, which is known to form spherical Hartley micelles and to bind cations, enhances the fluorescence as shown in Table I and, from titrations of Mg^{++} against fluorescence, it is

possible to determine apparent dissociation constants and enhancements which indicate that Mg-chlorotetracycline moves towards the nonaqueous boundary of the detergent micelle. These values represent minimum estimates, since saturation of the fluorescence titration curve occurs through two processes - binding of Mg^{++} to the membrane and binding of chlorotetracycline to Mg^{++}. The enhancement of fluorescence of chlorotetracycline caused by binding to Mg^{++} in the presence of dodecyl sulfate is 101 compared with 23 for aqueous Mg-chlorotetracycline. If a neutral detergent is used such as Triton X-100 then the Mg^{++} binding and fluorescence are those of the aqueous solution in accord with the fact that neutral detergents do not bind cations. The enhancement of fluorescence induced by membranes in the presence of divalent cations is seen also for biological membranes such as erythrocyte ghosts and muscle microsomes. Table I shows an apparent, or minimal enhancement of 109 for erythrocytes and 270 for microsomes of Mg-chlorotetracycline compared

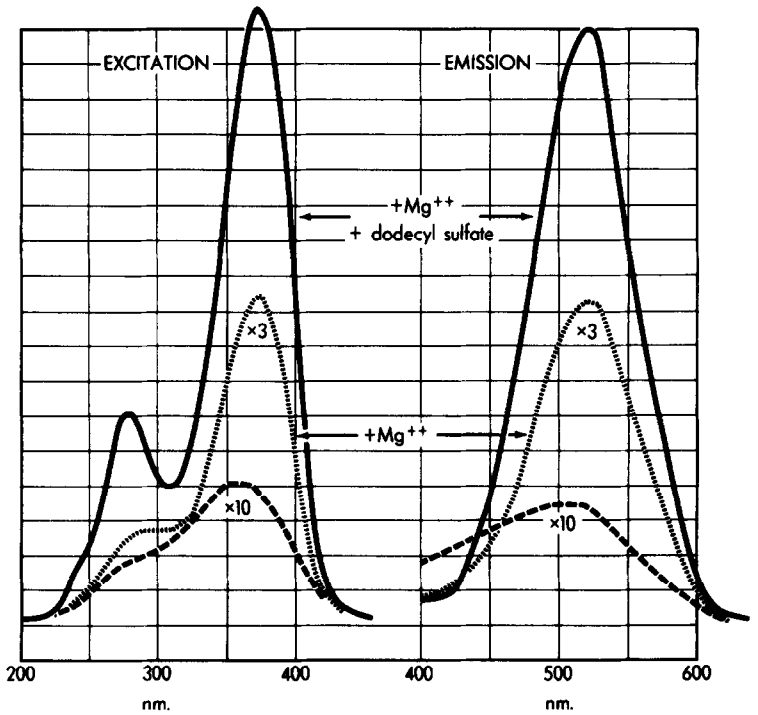

Fig. 1. Excitation and emission spectra of chlorotetracycline. Concentrations of reagents are: 10 mM Tris Cl, pH 7.4; 10 μM. chlorotetracycline and, where indicated, 250 mM $MgCl_2$; 10 mM Na dodecylsulfate.

with 23 for aqueous solution. That this is indeed a binding phenomenon is confirmed by the decrease in fluorescence of Mg-chlorotetracycline bound to membranes when 100 mM NaCl is added. The Na^{+} competes with divalent cation for the binding site and so reduces the amount of membrane bound divalent ion.

Figure 1 shows the excitation and emission spectra of chlorotetracycline, Mg-chlorotetracycline and Mg-chlorotetracycline bound to sodium dodecyl sulfate. The formation of the Mg^{++} chelate and binding to the micelle surface both affect the fluorescence yield without having marked effects either on the excitation or on the emission spectra.

While chlorotetracycline fluorescence shows little or no enhancement on binding to erythrocyte or microsome membranes in the absence of divalent cation, a different picture emerges in the case of mitochondria. Figure 2 shows that mitochondria cause an extensive enhancement of fluorescence. The enhancement builds up over a period of over 1 minute and is presumably associated with binding of chlorotetracycline to a cation bound with high affinity to the mitochondria. It is well known that repeated washing of mitochondria does not eliminate all Ca^{++} or Mg^{++}. Addition of rotenone to inhibit respiration causes a slow decrease in fluorescence while succinate restores the high fluorescence level. Subsequent addition of either uncoupling agent or antimycin causes a decrease in fluorescence. If 500μM Mg^{++} is included in the mitochondrial medium, (Fig. 2B) the fluorescence in the presence of mitochondria is actually lower than that observed if Mg^{++} is omitted; also the rate of fluorescence changes on addition of metabolites or inhibitors is slower.

The nature of the fluorescence change of chlorotetracycline in mitochondria bears a marked resemblance to that observed with ethidium (6). However, the situation may be very different, since the chlorotetracycline is fluorescing at the site of divalent cation binding. Moreover, the native antibiotic is able to penetrate membranes readily and the fluorescence observed may be sensing divalent cations bound to the inner surface of the inner

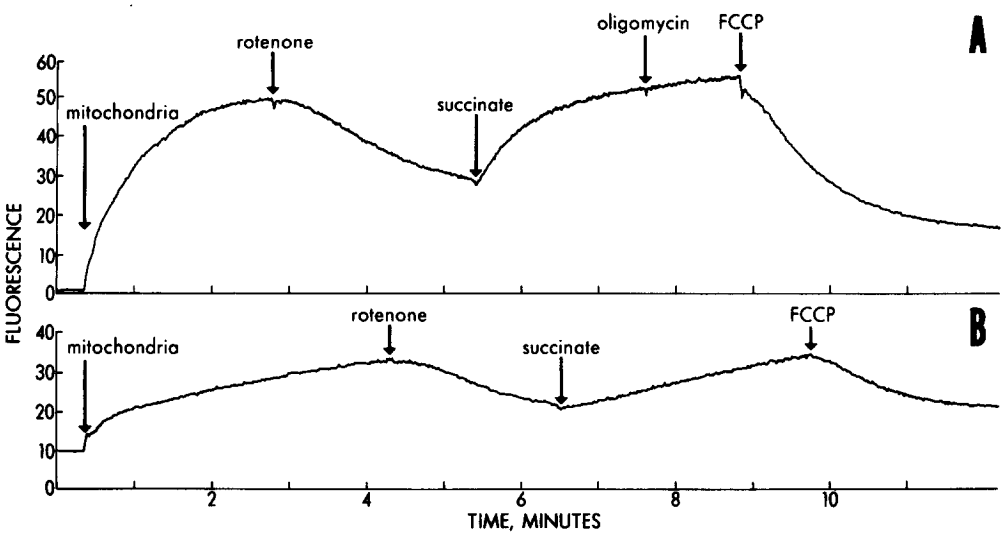

Fig. 2. Fluorescence of chlorotetracycline bound to mitochondria. The medium contains 250 mM sucrose; 10 mM Tris Cl; 7.5 mM, Tris glutamate; 7.5 mM Tris malate; 10 µM chlorotetracycline. pH is 7.4. In Fig. 2B 500µM $MgCl_2$ is also present. Additions to the medium are: mitochondria 0.6 mg. protein/ml; rotenone 13 µM; Tris succinate 7.5 mM; oligomycin 7 µg/ml; carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) 2.5 µM. Excitation is at 380 nm; emission is at 540nm. A Wratten 55 filter is in the emission path. The artifact caused by mitochondrial scattering is 3 fluorescence units. No NADH signal is detectable in the trace.

membrane. 500µM EDTA does not dislodge the fluorescence signal of chlorotetracycline bound to mitochondria. The slow onset of the fluorescence change on alteration of mitochondrial metabolism indicates that the fluorescence is not responding directly to the energy state of the energy conservation process. However, it may be reflecting a migration of ions within the membrane in response to changes in metabolism and may have implications in ion transport and control of metabolism. Chlorotetracycline, at a concentration of 20 µM, neither uncouples nor inhibits mitochondrial respiration.

The evaluation of the approach of employing fluorescent chelates in probing membrane cation binding and ion transport will require extensive experimentation with chlorotetracycline and similar reagents. However, increasing emphasis is being placed on divalent cations as control agents of membrane permeability and metabolism and it is expected that direct

visualization of cation binding will offer a means of examining ionic processes as they occur in the membrane.

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