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CHLOROTETRACYCLINE AS AN INDICATOR OF THE INTERACTION OF CALCIUM WITH BRAIN MEMBRANE FRACTIONS

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Abstract—The fluorescence of chlorotetracycline (CTC) in the presence of synaptosomes isolated from sheep brain is selectively increased by Ca^{2+} under conditions in which Mg^{2+}, Na^+, K^+, Li^+ or choline have only a small effect. The monovalent cations release bound Ca^{2+} from synaptosomes, and this effect is reflected by a decrease in the CTC fluorescence. Under optimal conditions there is a near parallelism between Ca^{2+} and CTC binding to the synaptosomes membranes, and Li^+ is the monovalent cation tested which interferes the most with the binding of both substances. These results obtained in a predominantly sucrose medium become less distinct when media simulating physiological composition are utilized, which limits the usefulness of the method. Brain mitochondria and myelin also bind Ca^{2+} and CTC. The ratio of the fluorescence signal (or CTC bound) to Ca^{2+} bound is highest of all for mitochondrial membranes, and the apparent fluorescence quantum yield of CTC is also the highest in these membranes, which suggests that the Ca^{2+} in these membranes is localized in a more apolar region than is the case for synaptosomes and myelin.

CHLOROTETRACYCLINE (CTC) has been utilized recently as a probe to follow Ca^{2+} movements in biological membranes (CASWELL & HUTCHISON, 1971; CASWELL, 1972; SCHUSTER & OLSON, 1973; LUTHRA & OLSON, 1976; HALLET *et al.*, 1972; CASWELL & WARREN, 1972; SCHAFFER & OLSON, 1976; CARVALHO & CARVALHO, 1976*a*, *b*; CARVALHO & CARVALHO, 1977). The principle of utilization of CTC to detect bound Ca^{2+} to biological membranes resides in the fact that CTC preferentially forms a complex with Ca^{2+} bound to the membrane phase. There is an increase in the fluorescence signal (CASWELL & HUTCHISON, 1971) of CTC when it binds to the membrane (CARVALHO & CARVALHO, 1976*a*, *b*; CARVALHO & CARVALHO, 1977; SCHUSTER & OLSON, 1973; SCHAFFER & OLSON, 1976).

The role of Ca^{2+} in regulating neural activity and the release of neurotransmitters is widely recognized (BLAUSTEIN, 1974; BAKER, 1972; RAHAMIMOFF *et al.*, 1975), and recently several reports have appeared on the effect of K^+, Li^+ and Na^+ on the fluxes and membrane binding of Ca^{2+} in synaptosomes (BLAUSTEIN & OBORN, 1975; BLAUSTEIN & ECTOR, 1976; BLAUSTEIN, 1974; ICHIDA *et al.*, 1976) along with other reports on the interactions of Ca^{2+} with various brain membrane fractions (LAZAREWICZ *et al.*, 1974; SWANSON *et al.*, 1974; HEMMINKI, 1974; KRISHNAN & BALARAM, 1976; KAMINO *et al.*, 1974; KAMINO *et al.*, 1975*a*, *b*; KAMINO, 1976).

We have recently utilized the CTC fluorescence technique for monitoring qualitatively Ca^{2+} interaction with membranes of sarcoplasmic reticulum (CARVALHO & CARVALHO, 1976*a*, *b*; 1977) with good results. This report contains the results of experiments in which the applicability of the CTC fluorescence technique to monitor the interaction of Ca^{2+} and other cations with synaptosomes, myelin and mitochondria isolated from sheep brain was explored. The results show that under optimal conditions the technique is relatively selective for Ca^{2+} over Mg^{2+} and that the release of Ca^{2+} bound to synaptosomes induced by Na^+, K^+ and Li^+ is reflected by a decrease in CTC fluorescence.

Abbreviation used: CTC, chlorotetracycline.

METHODS AND MATERIALS

Preparation of subcellular fractions from brain tissue. Sheep brain cortex was fractionated by differential and density gradient centrifugation in sucrose solutions according to the method described by WHITTAKER *et al.* (1964). The three fractions collected from the gradients (myelin, synaptosomes and mitochondria) were washed once with 310 mM-sucrose containing 20 mM-Tris–Cl at pH 7.4, and were resuspended in the same medium at a protein concentration of 20 mg/ml. The protein concentration was determined by the biuret method described by LAYNE (1957).

Incubation of synaptosomes and other brain fractions with chlorotetracycline in various media. Synaptosomes were incubated at room temperature in various media as described in the legends of figures, and usually contained 310 mM-sucrose and/or monovalent cations, buffered with 20 mM-Tris–HCl at pH 7.4. Chlorotetracycline (CTC) was added at a concentration of 25 μM, and synaptosomes were kept at 0.4 mg of protein/ml. When present, $CaCl_2$ was

added at 1 mM final concentration. The fluorescence changes of CTC due to Ca^{2+} were measured in 2.5 ml samples containing 1.0 mg of protein with a Perkin–Elmer Model MPF-3 spectrofluorometer with the excitation and emission wavelengths at 400 nm and 520 nm, respectively. In preliminary experiments it was shown that concentrations of CTC below 30 μM do not affect the binding of Ca^{2+} by the membranes, as was also shown by SCHAFFER & OLSON (1976).

The binding of Ca^{2+} and CTC to the membranes was measured under the same experimental conditions used in the fluorimetric measurements. The samples (5 ml containing 2 mg of protein) were either filtered through Millipore filters, or were centrifuged at 13,000 *g* for 20 min. In the experiments using filtration, the filters with the protein were washed twice with 2.0 ml of 310 mM-sucrose buffered with 20 mM-Tris–HCl pH 7.4, and then were eluted with 2 ml of 4% TCA, 0.5% La^{3+} and 4 mM-CsCl. The eluate was analysed for Ca^{2+} and Mg^{2+} by atomic absorption spectrophotometry. In the cases in which the membrane suspensions were centrifuged, the supernatants were kept for CTC analysis and the pellets were rinsed twice and re-suspended in 1.0 ml of de-mineralised water. These protein suspensions were treated with 4% TCA containing 1% La^{3+} and 4 mM-CsCl, and after short centrifugation in a bench centrifuge the supernatants were analysed for Ca^{2+} and Mg^{2+} by atomic absorption spectrophotometry, as described previously by CARVALHO & LEO (1967). The bound CTC was calculated by difference between the total CTC added to the incubation medium and the free CTC remaining in the supernatant after 13,000 *g* centrifugation, and was measured by the method described by SCHUSTER & OLSON (1973).

Incubation of synaptosomes in complex physiological media. Some experiments were performed by transferring the synaptosomal membranes to a complex physiological medium containing 20 mM-Tris, 10 mM-glucose, 1.2 mM-NaH_2PO_4, 1.3 mM-$MgCl_2$, 7 mM-maleic acid to a final pH of 7.4, 137 mM of either NaCl, LiCl, KCl or choline chloride, 25 μM-CTC and 0.4 mg of protein/ml. When added, $CaCl_2$ was at a concentration of 1.0 mM. Fluorescence measurements, Ca^{2+} and CTC binding to synaptosomes were performed in these experiments as described in the previous section.

Reagents. Chlorotetracycline was purchased from Sigma Chemical Company and solutions at pH 7.4 were prepared freshly every day. All the other reagents were of analytical grade.

RESULTS

Changes in the fluorescence of chlorotetracycline during Ca^{2+} and Mg^{2+} interaction with the synaptosomal membranes

The results shown in Fig. 1 demonstrate that the interaction of Ca^{2+} or Mg^{2+} with synaptosomes causes an increase in the fluorescence of chlorotetracycline. Figure 1 also shows that CTC fluorescence nearly parallels the binding of Ca^{2+} or Mg^{2+} by the membranes. After a rapid initial increase in cation binding and fluorescence during the first 15 s, a slower further increase occurs in the case of Ca^{2+}, but not of Mg^{2+}. At 5 min after adding the divalent cations, the Ca^{2+} and Mg^{2+} binding are 35 nmol of Ca^{2+}/mg of protein and 30 nmol of Mg^{2+}/mg of protein, but the fluorescence change induced by Ca^{2+} is about 6-fold higher than that induced by Mg^{2+} (Fig. 1).

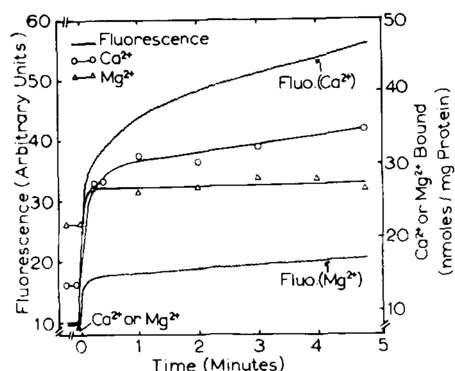

FIG. 1. Chlorotetracycline-associated fluorescence changes during Ca^{2+} or Mg^{2+} interaction with the membranes of sheep brain synaptosomes. The incubation medium contained 310 mM-sucrose, 20 mM-Tris-HCl at pH 7.4, 25 μM-chlorotetracycline, 0.4 mg protein per ml, and the reaction was started by adding 1 mM-$CaCl_2$ or $MgCl_2$. Ca^{2+} and Mg^{2+} binding to the membranes was measured by Millipore filtration as described in Methods.

Effect of various cations on the fluorescence of chlorotetracycline in synaptosomes

In order to determine the relative effect of various monovalent and divalent cations on the CTC fluorescence in synaptosomes, titrations of CTC in synaptosomal suspensions were performed with increasing cation concentrations. Figure 2 depicts the enhancement of fluorescence due to the addition of membranes to the various media containing 25 μM-CTC. The fluorescence of the probe in the suspending

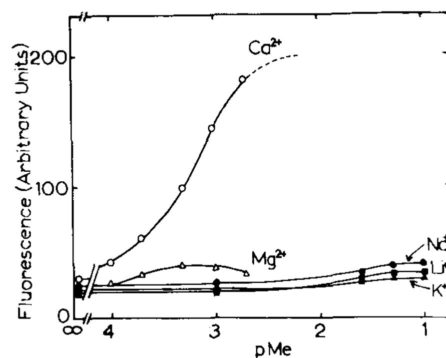

FIG. 2. Effect of monovalent and divalent cations on the fluorescence of chlorotetracycline in synaptosomes. Reaction media contained 310 mM-sucrose, 20 mM-Tris-Cl pH 7.4, 25 μM-chlorotetracycline, 0.4 mg synaptosomal protein per ml and various concentrations of cations as indicated in the abscissa as pMe (-log molarity). Fluorescence intensities were corrected for the fluorescence of chlorotetracycline observed in the absence of the synaptosomes.

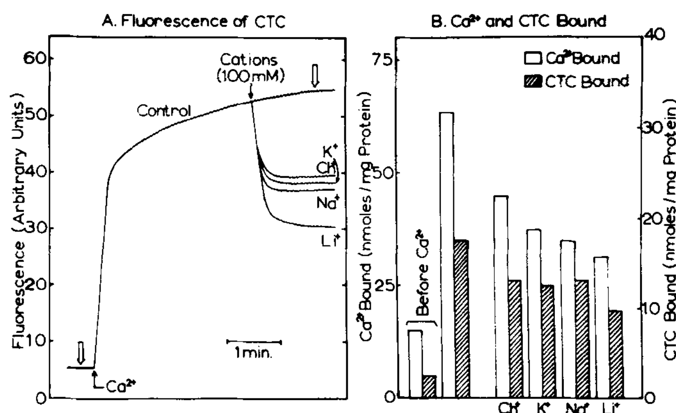

FIG. 3. Effect of the monovalent cations, Na^+, K^+, Li^+ or choline (Ch^+) on the interaction of Ca^{2+} with synaptosomes, as measured by the effect on the fluorescence (Part A) and on the binding of CTC and Ca^{2+} to the membranes (Part B). Synaptosomes (0.4 mg of protein/ml) were incubated in 310 mM-sucrose plus 20 mM-Tris–Cl pH 7.4. Ca^{2+} or monovalent cations were added as indicated in the traces. Ca^{2+} and CTC binding were measured after centrifugation as described in Methods.

media without protein added, together with membrane turbidity, were subtracted. Monovalent cations (Na^+, K^+, or Li^+) do not significantly alter the fluorescence of CTC even at concentrations as high as 100 mM. Divalent cations, Ca^{2+} and Mg^{2+}, have different effects on the fluorescence, Ca^{2+} being the more efficient one in increasing the fluorescence. Mg^{2+} increases the fluorescence of the basic medium containing CTC, in the absence of synaptosomes, much more than does Ca^{2+} (results not shown). Thus, CTC is a much more sensitive probe for detecting the interaction of synaptosomes with Ca^{2+} than with Mg^{2+}. The large background fluorescence of CTC in media containing Mg^{2+} in the absence of membranes should be taken in consideration when CTC is to be used to follow Ca^{2+} interactions with synaptosomal membranes in media containing $MgCl_2$ (BLAUSTEIN & ECTOR, 1976).

Although monovalent cations do not alter the CTC fluorescence, they are effective in significantly reducing the fluorescence increase due to the interaction of 1 mM-Ca^{2+} with the membranes. Figure 3 shows this effect for Na^+, Li^+, K^+ and choline (100 mM) when added after Ca^{2+} interaction with the membranes. Either of these monovalent cations decreases the fluorescence signal, but Li^+ is the most efficient in this respect (Fig. 3A). The effects of the cations on the CTC and Ca^{2+} binding (Fig. 3B) are approximately parallel to the effect on the CTC fluorescence. It is observed that Li^+ is the cation which releases the most Ca^{2+} from the membranes; it decreases the level from about 65 nmol to about 35 nmol/mg of protein. In the case of Li^+ the binding of CTC is also maximally decreased from 17.5 to 10 nmol of CTC/mg of protein. The higher Ca^{2+} binding observed in this experiment, in comparison with the results in Fig. 1, is due to the fact that the centrifugation method, without washing of the pellet, was utilized, whereas in the experiments reported in Fig. 1 the Ca^{2+} binding was obtained by the filtration technique with washing of the filters. Therefore, the values for Ca^{2+} binding reported in Fig. 3 are maximal values.

Figure 4 represents the effect of Ca^{2+} on the fluorescence of CTC in synaptosomes previously incubated in a complex physiological medium containing $MgCl_2$, among other substances, and either Na^+, K^+, Li^+ or choline (137 mM). The objective in this experiment was to study the interaction of Ca^{2+} with synaptosomes in a physiological medium using this fluorimetric method. Such media are usually utilized in physiological studies in which synaptosomes are used as model systems (BLAUSTEIN & ECTOR, 1976). It is observed in Fig. 4A that the fluorescence increase due to Ca^{2+} addition to the synaptosomes suspended in this complex medium is much lower than the increase observed when the membranes are incubated in a sucrose medium in the absence of cations. It is also observed that the basal fluorescence before Ca^{2+} addition is very high in comparison with that observed in sucrose medium (Fig. 3A). This must be related with the high Mg^{2+} concentration in the incubation medium. The results showing Ca^{2+} and CTC binding are represented in Fig. 4B. It is observed that the total Ca^{2+} in the membranes increased from about 7 to 10 to 25 to 30 nmol per mg of protein in all the media upon Ca^{2+} addition. Although significant differences appear to exist in the Ca^{2+} binding, it is difficult to quantify these differences by the fluorescence changes which, nevertheless, give qualitative information (Fig. 4A). The higher fluorescence level after Ca^{2+} addition for the media containing K^+ or Na^+ corresponded to a higher CTC binding to the membranes (Fig. 4A). Upon Ca^{2+} addition the binding of Mg^{2+} is only slightly decreased, so that the predominant effect on the fluorescence is caused

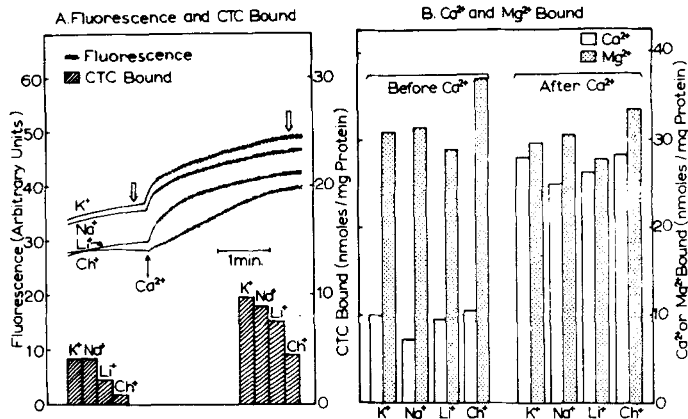

FIG. 4. Response of the fluorescence of chlorotetracycline and of Ca^{2+}, Mg^{2+} and CTC binding to Ca^{2+} (1 mM) addition when synaptosomes are incubated in complex physiological media. Synaptosomes were incubated at a concentration of 0.4 mg of protein/ml in media containing 20 mM-Tris, 10 mM-glucose, 1.2 mM-NaH_2PO_4, 1.3 mM-$MgCl_2$, 7 mM-maleic acid to a final pH of 7.4, 137 mM of either NaCl, LiCl, KCl or choline chloride and 25 μM-chlorotetracycline. The sensitivity of the fluorimeter, after Ca^{2+} addition (~~~), was set at a value three times higher than before Ca^{2+} addition (——). In part A the traces show the fluorescence of CTC developed in these media and the bars represent the binding of CTC in the various media measured at the points indicated by the arrows before and after Ca^{2+} additions. In part B, Ca^{2+} and Mg^{2+} bound to the membranes at the points indicated by the arrows before and after Ca^{2+} addition to the medium, respectively, is shown.

by Ca^{2+} binding which increases significantly (Fig. 4B).

In complex physiological medium the ratio of increase in CTC fluorescence to increased Ca^{2+} binding is much lower than observed when the experiments are performed in the non-ionic medium of sucrose.

Interaction of chlorotetracycline and Ca^{2+} *with various brain fractions*

In this section we present results of experiments in which we examined the interaction of Ca^{2+} with myelin, synaptosomes and mitochondria isolated from the same brain tissue, by following the changes in the CTC-associated fluorescence in these membrane systems. In all the systems studied the addition of 1 mM-$CaCl_2$ to a membrane suspension in sucrose promotes a rapid fluorescence increase followed by a slower increase until a maximal fluorescence is attained a few minutes after Ca^{2+} addition (Fig. 5). However, the total fluorescence increase is much

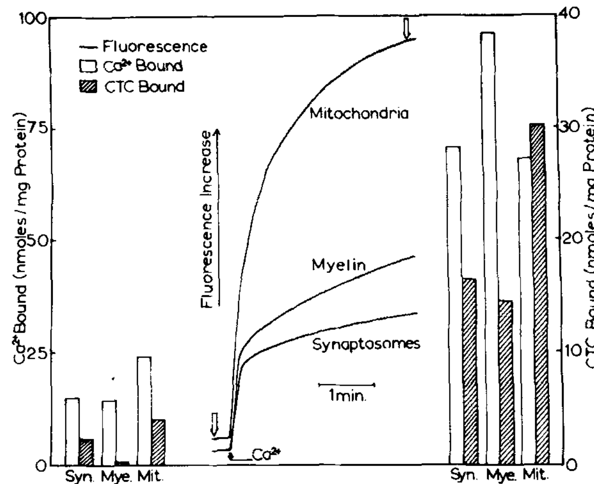

FIG. 5. Effect of Ca^{2+} on the fluorescence of chlorotetracycline in various brain fractions. The fractions (0.4 mg of protein/ml) were incubated in 310 mM-sucrose, 20 mM-Tris–Cl pH 7.4 and 25 μM-chlorotetracycline. At the center of the figure is shown the change in chlorotetracycline fluorescence upon addition of 1 mM-$CaCl_2$. The bars represent Ca^{2+} and chlorotetracycline binding to the membranes at the arrows before Ca^{2+} addition and at maximal Ca^{2+} induced fluorescence, respectively.

higher in the case of mitochondria than it is in the other two cases for the same membrane protein concentrations.

The results depicted in Fig. 5 also show the levels of Ca^{2+} and CTC binding before Ca^{2+} addition (bars at the left of Fig. 5) and 5 min. after Ca^{2+} interaction with the membranes (bars at the right of Fig. 5). The most striking difference between the three fractions is the higher CTC binding attained 5 min after Ca^{2+} addition in the case of mitochondria. This was about 30 nmol CTC bound/mg of mitochondrial protein, as compared with 17 and 14 nmol/mg of protein in synaptosomes and myelin, respectively. The ratio of CTC bound to Ca^{2+} bound is also higher in mitochondria than in the other two fractions (Fig. 5).

In order to further elucidate the difference between the fluorescence of CTC in these three membrane fractions, the possible differences in the quantum yield of CTC fluorescence in these membranes were investigated. In these experiments the CTC concentration was maintained constant (25 μM) and the amount of membrane protein was varied. Figure 6 is a double-reciprocal plot of fluorescence vs membrane protein concentration which shows rectilinearity for low protein concentrations, but reaches a limiting value for higher protein concentrations. Extrapolating the lines obtained to the y-axis, the intercept gives the maximal fluorescence (F_{max}) of CTC in the membranes, which is an indication of the quantum yield of the probe in the membranes. Comparison of the values for F_{max} obtained for the three membrane systems tested (F_{max} = 35, 60 and 100 respectively, for myelin, synaptosomes and mitochondria) reveals that the quantum yield of CTC is higher for the mitochondrial membranes than it is for the other two membrane systems. The results suggest that the higher fluorescence observed in Fig. 5 for mitochondrial membranes is

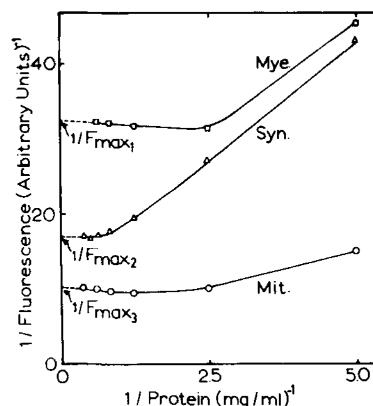

FIG. 6. Alteration in fluorescence intensity of chlorotetracycline in myelin (Mye.), synaptosomes (Syn.) and brain mitochondria (Mit.) as a function of protein concentration. Fluorescence was measured in a medium (2.5 ml) containing 310 mM-sucrose, 20 mM-Tris–Cl pH 7.4, 1 mM-$CaCl_2$, 25 μM-chlorotetracycline and protein concentrations between 0 and 2.4 mg/ml. Excitation and emission were 400 and 520 nm, respectively. Fluorescence values are corrected for turbidity due to protein addition determined in the absence of chlorotetracycline. The calculated F_{max} values are: F_{max_1} = 35, F_{max_2} = 60 and F_{max_3} = 100 fluorescence units as determined from the intercepts at the ordinates. The scale at the ordinates is multiplied by 10^3.

due to a higher probe binding per mg of protein and also to an increase in quantum yield of the probe when it reacts with these membranes (Fig. 6). Figure 6 shows that the quantum yield of CTC is lower in the myelin membranes than in the synaptosomes, but the fluorescence observed for protein concentrations of the order of those utilized in Fig. 5 (1 mg) is only slightly higher for the myelin than for the synaptosomes. This difference is accentuated at higher protein

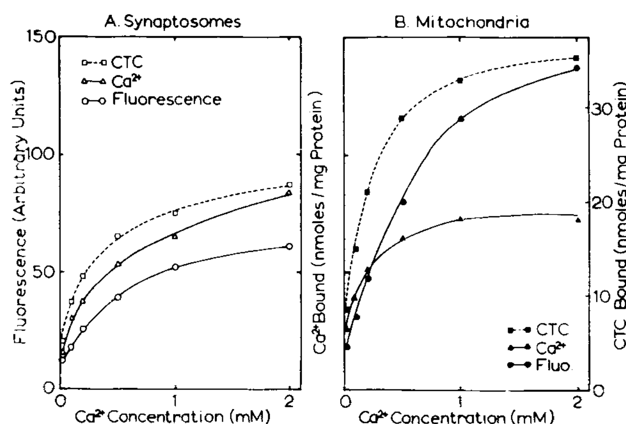

FIG. 7. Effect of increasing Ca^{2+} concentrations on the fluorescence of chlorotetracycline and Ca^{2+} binding to synaptosomes and brain mitochondria. The membranes (0.4 mg of protein/ml) were incubated as described in the legend of Fig. 5, except that the Ca^{2+} concentration was varied from 0 to 2 mM. Ca^{2+} binding was measured after 5 minutes of incubation with Ca^{2+}, and Ca^{2+} analysis was carried out on the centrifuged pellet by the procedure described in Methods. The scale for CTC binding in *A* is the same as that specified in *B*. The scale for Ca^{2+} binding in *B* is that specified in *A*.

concentrations because the CTC fluorescence reaches a maximal value in the presence of myelin at protein concentrations which are insufficient to produce maximal fluorescence in the presence of synaptosomes (Fig. 6).

The results in Fig. 7 show the effect of increasing $CaCl_2$ concentrations (0–2 mM) on the fluorescence and on the Ca^{2+} and CTC binding to the membranes of synaptosomes (Part A) or mitochondria (Part B). The effect of Ca^{2+} on the parameters studied attains near saturation for both synaptosomes and mitochondria at a concentration of 2 mM-$CaCl_2$, and it is confirmed that for all the concentrations studied the fluorescence of CTC is higher in the case of mitochondria than in the case of synaptosomes. The higher fluorescence observed in mitochondrial membranes correlates well with a higher CTC binding (Fig. 7), although about the same amount of Ca^{2+} is bound to both membrane systems. The affinity of membrane bound Ca^{2+} for CTC is probably higher in the case of mitochondria than it is in the case of synaptosomes, which probably reflects different environments for the Ca^{2+} bound in the two membrane systems (CASWELL & HUTCHISON, 1971*a*, *b*; CASWELL, 1972).

DISCUSSION

The results presented show that the interaction of Ca^{2+} with synaptosomes and other brain fractions can be followed utilizing CTC fluorescence. The fluorescence intensity of the probe increases upon Ca^{2+} addition to the suspending medium containing synaptosomes, and there is a parallel increase in Ca^{2+} and CTC binding by the synaptosomes (Figs. 1–3). Similar results were already described for these (SCHAFFER & OLSON, 1976) and other membrane systems (CASWELL & WARREN, 1972; LAU *et al.*, 1974; CASWELL, 1972; SCHUSTER & OLSON, 1973; SCHUSTER & OLSON, 1974; CASWELL & HUTCHISON, 1971; CARVALHO & CARVALHO, 1977). The method is relatively specific for Ca^{2+} over Mg^{2+}. Thus, the binding of Ca^{2+} produces a much higher fluorescence signal than the binding of a corresponding amount of Mg^{2+} (Figs. 1 and 2). Apparently, the affinity constant for the interaction between CTC and divalent cations is selectively increased for Ca^{2+} by an apolar medium such as found in the membrane phase (CASWELL & HUTCHISON, 1971; CASWELL, 1972; HALLETT *et al.*, 1972).

Another observation in Fig. 1 is that, upon Ca^{2+} addition to the synaptosomes, and after the initial phase of rapid increase in fluorescence, there is a gradual increase in the fluorescence which is accompanied by a gradual increase in Ca^{2+} binding to the membranes. This increase in Ca^{2+} binding with time is probably due to entry of Ca^{2+} to more internal binding sites which is then followed by CTC binding. This phenomenon is not observed with Mg^{2+}.

As shown in Fig. 2, monovalent cations (Na^+, K^+ and Li^+) do not directly affect the fluorescence of CTC in the synaptosomes, but they significantly decrease the fluorescence induced by Ca^{2+}, as was reported recently for the case of Na^+ (SCHAFFER & OLSON, 1976). This effect is due to the loss of Ca^{2+} and CTC from the membranes induced by the monovalent cations, which is most prominent for the case of Li^+ (Fig. 3B).

The results presented in Fig. 4 show the fluorescent signal of CTC obtained when Ca^{2+} is added to a synaptosomal suspension in complex physiological media similar to that utilized by BLAUSTEIN & ECTOR (1976). Such media contain, in addition to Mg^{2+} and other ions, either K^+, Na^+, Li^+ or choline. When CTC is added to these media, the initial fluorescence is already relatively high and there is a small fluorescence response to addition of Ca^{2+}, even though the Ca^{2+} binding increases significantly (Fig. 4). Apparently, the various cations in the suspending medium inhibit or quench the CTC fluorescence normally induced by Ca^{2+} binding to synaptosomes. These observations indicate that the utilization of CTC fluorescence to follow Ca^{2+} interaction with synaptosomes is of limited application for studies when physiological conditions are simulated. The results presented in Fig. 5 show that myelin and mitochondria also take up CTC upon Ca^{2+} addition. As in the case of synaptosomes the uptake of Ca^{2+} and CTC is due to the passive interaction of Ca^{2+} and CTC with the membranes, since no energy source is present. Thus, we are not following the energy dependent uptake of Ca^{2+} by mitochondria as was reported for liver mitochondria by LUTHRA & OLSON (1976). Nevertheless, it is of interest that the passive interaction of Ca^{2+} with brain mitochondria promotes a higher CTC binding than does the interaction of Ca^{2+} with either of other two fractions, although about the same amount of Ca^{2+} is bound to synaptosomes and mitochondria. This higher affinity of membrane bound Ca^{2+} for CTC probably reflects that the Ca^{2+}–CTC complex, which forms in the mitochondrial membranes, must be sensing a different environment. This is probably more apolar than in the case of the other two membrane systems, myelin and synaptosomes. In these systems bound Ca^{2+} may be located more superficially, where the affinity for CTC in this relatively polar environment would be diminished (CASWELL & HUTCHISON, 1971).

The results presented show that CTC fluorescence is useful in following Ca^{2+} interactions with three membrane systems isolated from sheep brain in a simple non-physiological medium of isotonic sucrose. In this medium the effects of monovalent cations on Ca^{2+} binding are readily followed by monitoring CTC fluorescence changes associated with the complex formed between Ca^{2+} bound and CTC in the membrane phase. These effects of monovalent cations on Ca^{2+} transfer between the medium and the membrane phase should be distinguished from those reported by other workers (BLAUSTEIN & ECTOR, 1976; BLAUSTEIN & OBORN, 1975; BLAUSTEIN & WIESMAN, 1970; ICHIDA *et al.*, 1976) and which

apparently reflect transmembrane fluxes of Ca^{2+}. The fluorescence technique is somewhat more limited in its application to follow movements of Ca^{2+} in membranes suspended in complex physiological media which produce initial high CTC fluorescence.

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