CIRCULAR DICHROISM OF BIOLOGICAL MEMBRANES– BRAIN MICROSOMES†

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The circular dichroism and absorption spectra of particulate and solubilized brain microsomes are reported. The corrected molar ellipticity for the membrane suspension was $-1.1 \times 10^{+4}$, between the values of axonal and oxyntic cell membranes and those for sarcotubular vesicles. No conformational change was detected which correlated unambiguously with the active cation transport function of these microsomes. Divalent cations, however, elicited a significant change in the membranes' ultra structure. Correction of suspension data for the NaI microsomes with and without sonication gave virtually identical values providing optimism for the corrections used and validating the difference in corrected values for ths suspension with and without magnesium. This indicates that, in addition to causing a change in the state of aggregation, 2 mM magnesium ion may reasonably be considered to effect a change in protein conformation.

INTRODUCTION

The characteristic circular dichroism spectra of membranes are red shifted and dampened in comparison to those for soluble α–helical proteins. That these anomalies are due to the particulate nature of the system and, indeed, are distortions has been well documented (1). With appropriate corrections, however, membrane CD spectra can be compared with respect to type and in some instances, with respect to function. For example, the corrected 224 nm molar ellipticity values of membranes form a continuum with the maximum $[\Theta]$ value being -2×10^4 for red blood cells and the minimum being -0.9×10^4 for axonal membranes. Intermediate are mitochondria $[\Theta]$ of -1.7×10^4), plasma membranes $[\Theta]$ of -1.7×10^4), and sarcotubular vesicles ($[\Theta]$ of -1.2×10^4). In addition, corrected ellipticity values may be used to compare functionally different states of membrane systems. Our recent study of oxyntic cell vesicles is a case in point.

The gastric membranes contain a HCO_3^- stimulated ATPase recently implicated in

†Number V in the series "Circular Dichroism of Biological Membranes."

*Trainee of TIAM05286 NIH Grant.

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Journal of Supramolecular Structure

the mechanism of acid secretion (2). Analysis of their corrected CD spectra indicated that they underwent a conformational change which correlated with function, in this case, active transport (3). These results encouraged us to pursue further the study of particulate systems in different functional states.

Brain microsomes were chosen for this study because they are known to contain a $Na^+ - K^+$–ATPase (4–8) maximally stimulated by sodium and potassium, and inhibited by ouabain and N-ethyl maleimide (NEM). The latter is thought to prevent the interconversion of two phosphorylated reaction intermediates (9–11). We chose this membrane system in the hope that magnesium, ATP, the cations, or inhibitors would induce a transport specific conformational change demonstrable in the corrected CD spectra.

MATERIAL AND METHODS

Membranes enriched in the $Na^+ - K^+$ – ATPase were prepared by NaI treatment (12) and Triton X–100 solubilization (13) of the 100,000 × g microsomal fraction from pressure homogenized pig brain as adapted from Skou (14). Hydrolytic activity was measured at pH 7.4 by previously described methods (15). Inorganic phosphate was assayed with the Fiske–Subbarow procedure (16), protein with the Lowry technique (17). The total ATPase activity of the NaI microsomes when measured at 37°C varied from 14.1 to 16.9 μmoles Pi mg^{-1} hr^{-1} with 82 to 97% inhibition by ouabain. The Triton solubilized ATPase activity was 5.1 μmoles Pi mg^{-1} hr^{-1}. Prior to use, the NaI microsomes, which were stored in 0.33 M sucrose, 0.2 M Tris-HCl, pH 7.4, 5 mM EDTA, were twice centrifuged at 15,000 × g for 30 minutes, each time being resuspended and homogenized in 10 mM Tris–HCl, pH 7.4, in order to wash out the sucrose. The membrane suspension was sonicated on ice with a Branson Sonifier at 5 D.C. amp for 6 minutes at 10-second intervals. Vesicle diameter did not approach the limits whereby radius of curvature would alter protein conformation. Radius of curvature becomes a significant consideration when particle diameter becomes substantially less than 0.1 μ (S. Chan, this conference).

All chemicals were reagent grade and deionized glass double distilled water was used throughout. Reagent concentrations are listed with the figures. All solutions were made with 10 mM Tris–HCl. The ATP (Sigma) solution was brought to pH 7.4 by NaOH titration. All salts were the chloride salt. Spectra were run immediately after addition of ATP and other ions where appropriate. Calculations of activity at ambient temperature (25°C) indicate that 78% ± 3%, 75% ± 3%, and 23% ± 6% of the total ATP was not hydrolyzed in the Mg state, the Mg, Na, K, ouabain state, and the Mg, Na, K state, respectively, at the end of the scan, i.e., at 190 nm. ATP hydrolysis would be minimal in the time required to reach 224 nm.

CD spectra were recorded at ambient temperature on a Cary 60 spectropolarimeter adapted with a model 6001 attachement for circular dichroism and a dynode voltage recording unit for simultaneous absorbance measurements. Cylindrical cells of 0.2 mm and 0.1 mm pathlength were used for the insoluble fractions. For the Triton solubilized material the pathlength was 1.0 mm; for the SDS solubilized membrane, 0.2 mm and 0.1 mm. The final protein concentration was between 1.0 mg/ml and 1.5 mg/ml for the NaI microsomes, the same for the SDS–NaI micro-

somes, and 0.32 mg/ml for the Triton preparation. The time constant was alternately set at 3 and 10 seconds, with the scanning speed between 2 and 15 nm/min. The range was 100 and 40 mdegrees. The CD spectra obtained from the membranous system were corrected according to the method of Urry (1).

RESULTS

The CD spectra of nonmolecularly dispersed suspensions are dampened and red shifted in their negative 224 nm extremum. This depression and wavelength maximum shift are due to absorption flattening of Duysens (1, 8) and light scattering. Therefore, before membrane spectra obtained under a variety of conditions can be compared, corrections must be applied to the data to eliminate these distortions, which may themselves vary under different experimental conditions. Accordingly, the following curves were corrected using the pseudo-reference state approach (1, 19). Corrected spectra were obtained using the approximate equation

$$[\Theta]^{corr} = \frac{[\Theta]^{obs}}{Q_A^2 Q_\sigma}$$

where $[\Theta]^{corr}$ is the molar ellipticity conservatively corrected; $[\theta]^{obs}$ is the experimentally determined molar ellipticity. Q_A is the Duysens flattening quotient (18) calculated from simultaneously recorded absorbances. The Factor Q_A^2 corrects for differential absorption flattening. Q_σ is a light scattering quotient, an absorption obscuring correction factor. The particle shape was taken as vesicular and values of Q_σ were calculated from longer wavelengths to about 206 nm and the 206 nm value was used at shorter wavelengths. This again results in minimal corrections for the positive band. Equation 1 neglects differential light scattering.

Figure 1, curve b, is the observed spectrum of the NaI microsomes containing the $Na^+ - K^+$ –ATPase. The negative peak and shoulder are at 224 nm and 210 nm, the corssover at 200 nm, and the positive maximum at 192 nm. Corrected values are shown in Figs. 2 and 4. Addition of 2 mM ATP was without effect on the experimental and calculated spectra. However, equimolar Mg–ATP (curve a) caused a 22% dampening of the observed values at 224 nm relative to that obtained in their absence. The crossover was red shifted to 202 nm. Two mM $MgCl_2$ alone had the same effect as seen with Mg–ATP. The change detected, therefore, was not attributable to the substrate, Mg–ATP, but to Mg^{2+} alone.

When activated and nonactivated states of the enzyme were compared, no difference was found in both the experimental and calculated curves (Fig. 2). The presence of Mg–ATP, Na^+ and K^+ in concentrations optimal for hydrolytic activity, altered the spectrum (curves a vs b, curves a′ vs b′), but addition of ouabain at 0.1 mM caused no further change. The same effect is seen even in the absence of ATP, i.e., with Mg^{2+}, Na^+, K^+ again in concentrations appropriate for stimulation of hydrolysis. Mg–ATP–Na and Mg–ATP–K had the same effect on the membrane; in this case the final K^+ and Na^+ concentrations were both adjusted to 120 mM to keep the tionic strength constant. The percent dampening was 24% for the uncorrected curves and 15% for the corrected. Ionic strength per se was without

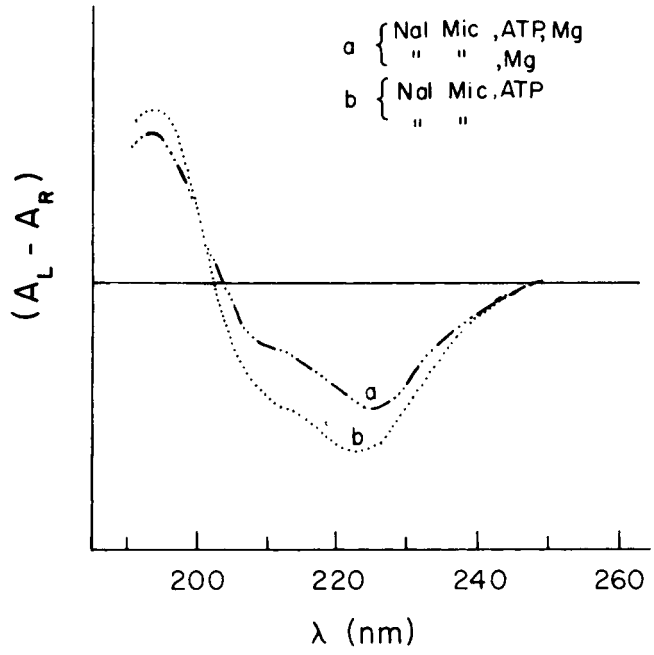

Fig. 1. CD spectra of NaI microsomes, difference absorbance ($A_L - A_R$) plotted as a function of wavelength in nanometers. Final protein concentration was 1.46 mg/ml, final ATP concentration was 2 mM, as was the $MgCl_2$ concentration.

effect in the insoluble and soluble systems. The flattening detected, therefore, was not correlated with the stimulation and inhibition characteristics of transport ATPase.

The CD spectrum of the particulate fraction in the presence of Mg–ATP, Na^+, K^+, ouabain was compared with that with Mg–ATP, Na^+, and N–ethyl maleimide. Since NEM prevents the interconversion of EP_1 and EP_2, EP_1 would accumulate in its presence. On the other hand, ouabain would enrich the system in EP_2. No difference was found which could be correlated with the relative levels of these phosphoproteins, and the two spectra approximated curves a and a′ in Fig. 2.

The only significant difference in conformation or state of aggregation of the membrane vesicles was between the fragments alone and in association with Mg^{2+}. Similar results have been reported for rough and smooth endoplasmic reticulum (20). This observation was pursued further. Ca^{2+} was even more effective in dampening the observed spectra than Mg^{2+} (Fig. 3). Two mM Mg^{2+} flattened the 224 nm negative peak 21% when compared to the microsomes alone, while 2 mM Ca^{2+} produced a 32% decrement. Increased concentrations accentuated this dampening.

To test the significance of the difference in the corrected values in Fig. 2, the membrane suspension was sonicated and the corrected values of the sonicated and unsonicated ellipticities computed (Figs. 4 and 5). It was assumed that sonic disruption altered not the secondary structure of the membrane protein, but only particle size, i.e., the extent of distortion introduced by the light scattering membranous particles. Sonication of the NaI micronsomes enhanced their spectrum at 224 nm by 35% (curve b vs curve c), but their corrected 224 nm values were virtually identical

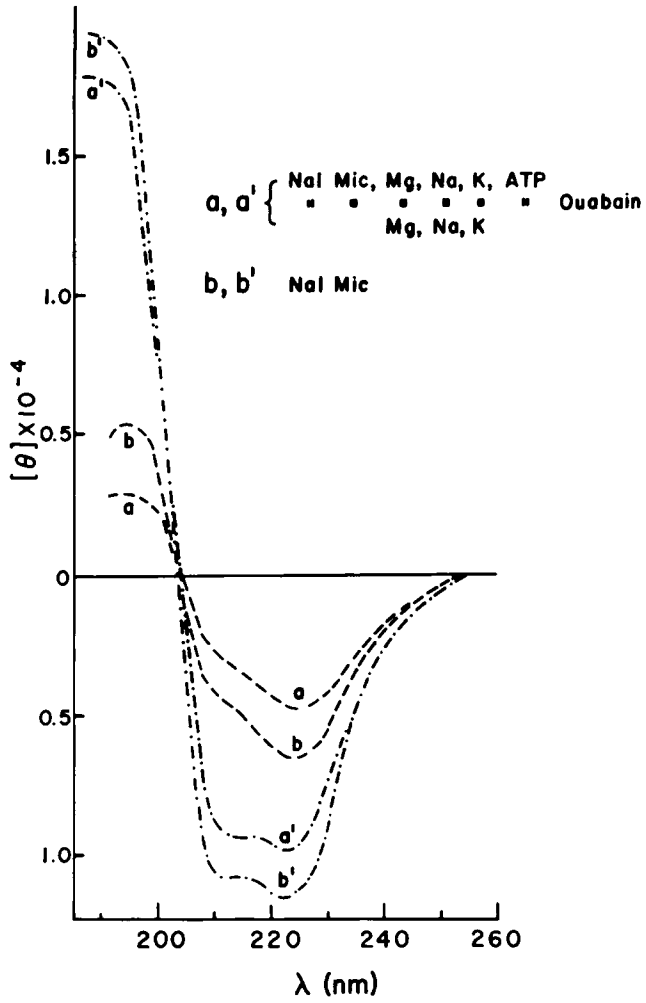

Fig. 2. CD spectra of microsomal $Na^+ - K^+$ -ATPase. Final concentrations were as follows: protein 1.46 mg/ml, 2 mM $MgCl_2$, 2 mM ATP, 100 mM NaCl, 20 mM KCl, and 0.1 mM ouabain. The corrected spectra are indicated by the primed letters.

(points b′ and c′). On the other hand, magnesium dampened both the experimental and calculated spectra points (a and a′).

In order to eliminate the distoritions of the particulate preparation, the NaI microsomes were solubilized with Triton X–100 (a soluble state in which enzymatic activity is maintained) and their CD spectra determined in the activated and nonactivated states. The results (Fig. 6) were not of the same pattern as those of the membrane fragments. Magnesium had no dampening effect. In light of these findings, it is significant that Triton X–100 did not solubilize all of the NaI microsomal material.

Recently Schwartz (21) reported a difference in the CD spectra of Lubrol solubilized $Na^+ - K^+$ -ATPase with and without ouabain over a background of $MgCl_2$ and inorganic phosphate. The apparent discrepancy between his and our data

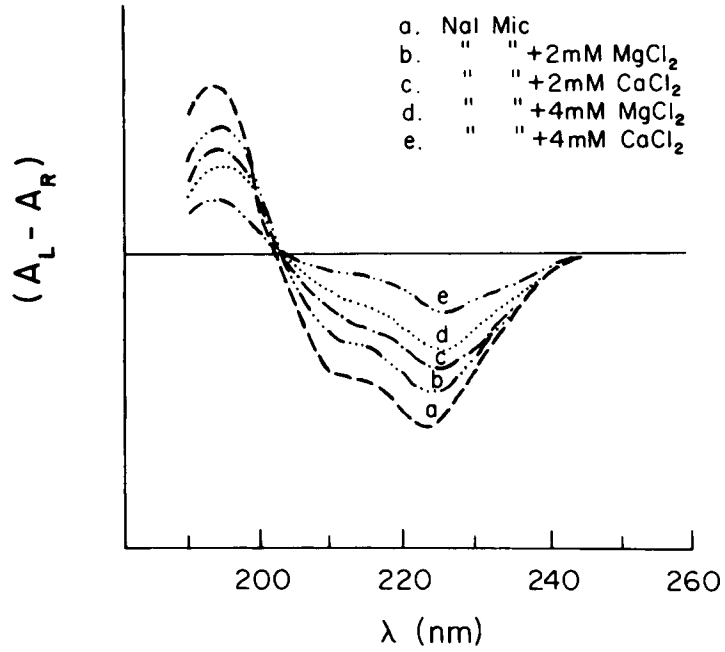

Fig. 3. CD spectra of divalent cation titration of NaI microsomes. Concentrations were the same as those described in Fig. 1.

may be due to the higher specific activity of his calf brain preparations or due to the fact that he was studying irreversible ouabain binding (22).

DISCUSSION

The purpose of this investigation was to extend the technique of CD spectroscopy to another membrane system which can be studied in functionally different states and which can be sonicated to change particle size with but a negligible amount of solubilization.

The corrected molar ellipticity value at 224 nm was -1.1×10^4, in line with the axonal membranes and with oxyntic cell and sarcotubular vesicles. One of the important findings of this investigation was the experimental support it gave to the corrected molar ellipticity values. That the corrections applied were reasonable can be seen by comparison of the corrected values in Fig. 4. The corrected ellipticity of a membrane should, within limits, be independent of the particle size even when the uncorrected spectra differ in magnitude by as much as 50%. This was found to be the case (Fig. 4). Sonication of the NaI microsomal suspension (the CD of which is given in curve b of Fig. 4) results in a 40% increase in magnitude of ellipticity at 224 nm (curve c of Fig. 4). The supernatant was examined after sonication and found to have negligible solubilized protein. Application of Eq. (1) to both curves b and c at 224 nm results in the corrected points b′ and c′. The similarity of the corrected values (points b′ and c′ of Fig. 4) for the unsonicated and sonicated samples gives reason for cautious optimism about the significance of the calculations indicating a difference between the corrected curves a′ and b′ in Fig. 2.

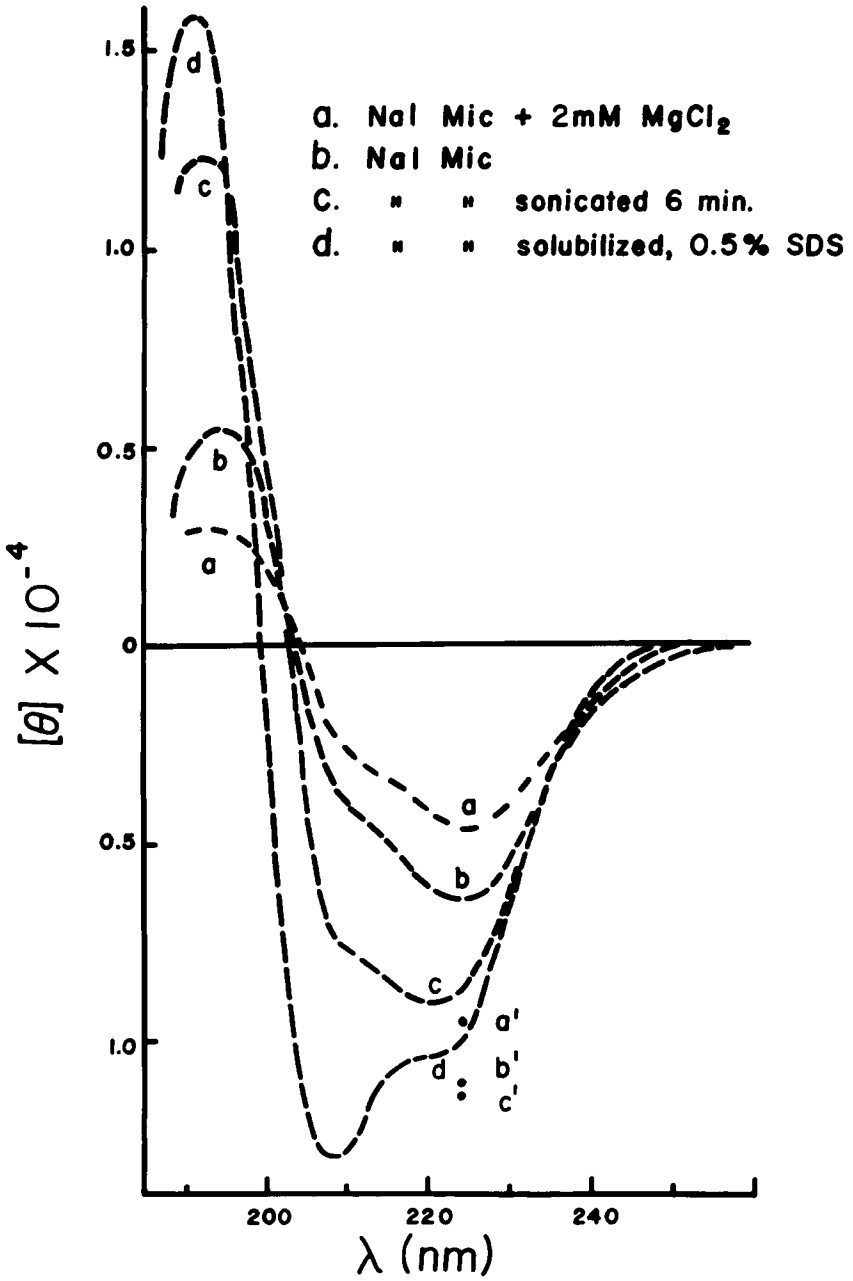

Fig. 4. CD spectra of microsomal $Na^+ - K^+$ -ATPase. Curve a was obtained upon addition of 2 mM $MgCl_2$ to the membrane suspension (curve b). Curve c was obtained after the membrane preparation was sonicated on ice for 6 minutes. Sonication reduced particle size but significantly did not solubilize the membrane, as judged by CD measurements on the supernatant of the sonicated ultracentrifuged preparation. The calculated values at 224 nm are indicated by the primes.

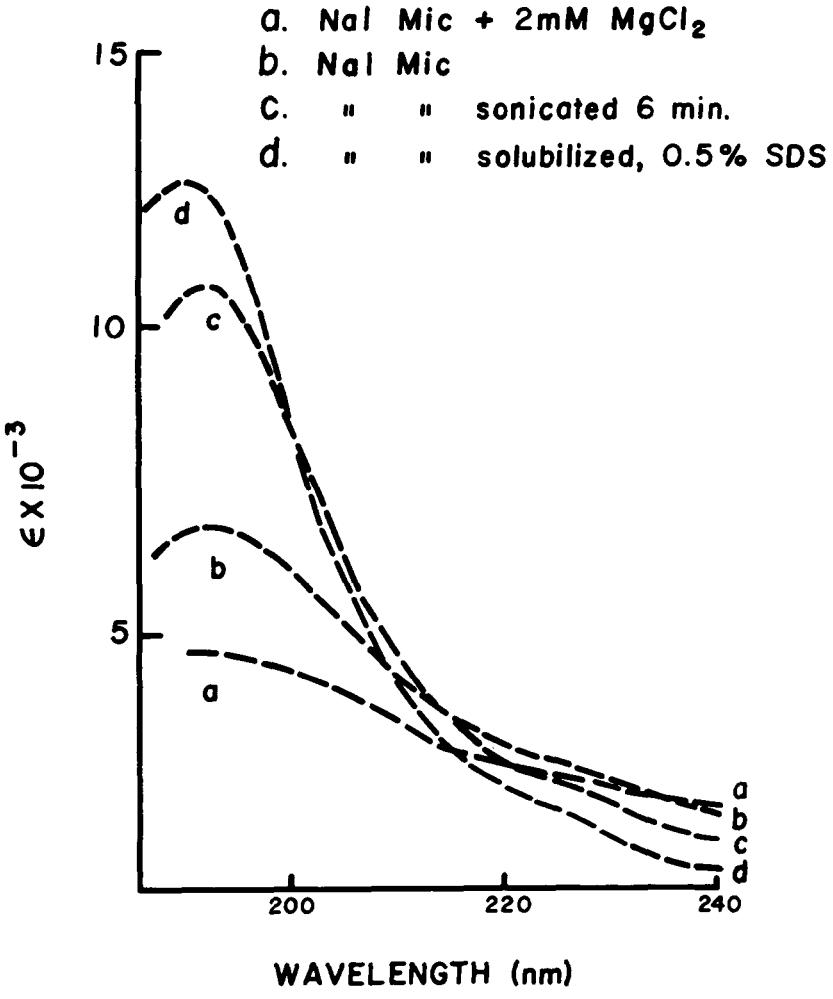

Fig. 5. Extinction coefficient of NaI microsomes as a function of wavelength. These data were obtained simultaneously with those in Fig. 4. The same legend applies.

Unlike the oxyntic cell spectra, the brain microsomes CD spectra of the functionally different activated and inactivated states were indistinguishable in both the particulate and solubilized preparations (Figs. 1, 2, and 6). The same pattern was true of the corrected spectra. The only difference was between the membrane alone and the membrane Mg^{2+} or Ca^{2+} complex. For the insoluble ATPase the magnitude of the difference between these two conditions and the red shifted crossover of the complex indicate that Mg^{2+} and Ca^{2+} caused a change in the state of aggregation of the membrane vesicles, and may also have effected a conformational change within the protein portion. This could be interpreted in terms of maintenance of membrane conformation since magnesium and calcium are vital constituents of membranes. However, any attempt to correlate this change with transport function

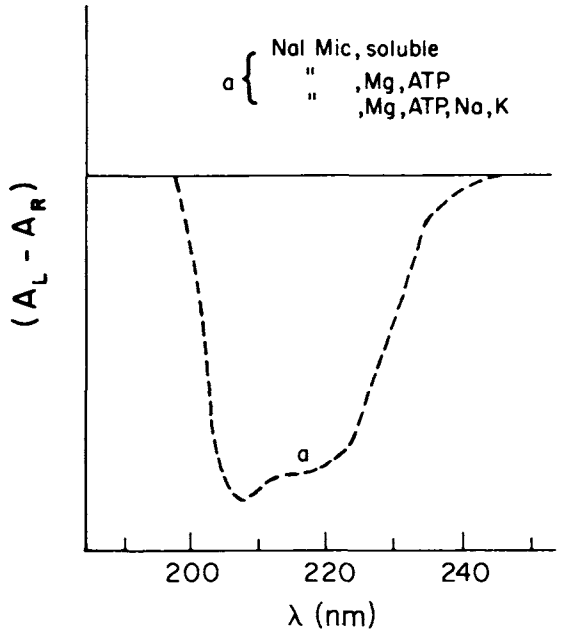

Fig. 6. CD spectra of Triton X–100 solubilized NaI microsomes. Final concentrations were the same as in Fig. 2, except for the protein, which was 0.32 mg/ml.

would be difficult, especially since the solubilized material, shown to contain active $Na^+ - K^+$ –ATPase, was insensitive to magnesium.

These results suggest that the conformational changes involved in cation translocation, if indeed there are any, are of a subtle nature involving only localized sites or less than 10% of the protein. Gross changes in major membrane polypeptide and protein conformations do not occur.

ACKNOWLEDGMENTS

This work was supported by NIH Grants AM08541 and AM09260, NSF Grants GB31075 and GB31665 and aided by a grant from the AMA Education and Research Foundation, and by financial support from the Mental Health Board of Alabama.

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