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LABELLING OF SARCOPLASMIC RETICULUM MEMBRANES WITH 1-DIMETHYLAMINONAPHTHALENE-5-SULFONYL CHLORIDE

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SUMMARY

1. Sarcoplasmic reticulum membranes were labelled with 1-dimethylaminonaphthalene-5-sulfonyl chloride (DnsCl). Analyses of the dansylated membranes demonstrated that the most of the dye was associated with ATPase (ATP phosphohydrolase, EC 3.6.1.3) and phosphatidylethanolamine in the membranes.

2. Dansylation of the membranes could be performed without significant decrease in the ATPase activity.

3. Partial differentiation of fluorescence of Dns-phosphatidylethanolamine from that of Dns-ATPase could be achieved by changing excitation wavelength; Dns-ATPase emitted in the shorter wavelength region, while Dns-phosphatidylethanolamine emmitted in the longer wavelength region.

4. Fluorescence polarization of the dye bound to the membranes indicated that both the ATPase and phosphatidylethanolamine were strongly immobilized in the membranes, while the ratio of freely rotating dye to the "frozen" dye bound to the ATPase was larger than that bound to the phosphatide.

INTRODUCTION

1-Dimethylaminonaphthalene-5-sulfonyl chloride (DnsCl), a well-known fluorescent probe which is able to bind covalently to amino acid residues of protein, has been reported to be adopted to label biological membranes [1–3]. In the membranes prepared from electric organ of *Electrophorus electrics*, the dye mainly associates with protein [1]. This is also true in erythrocyte membranes [3].

It is well known that sarcoplasmic reticulum membranes contain Ca^{2+}-activated ATPase which performs active Ca^{2+} transport into the vesicles [4–6], and that the ATPase is embedded in the membranes [7–9] as their major component. The enzyme molecule was suggested to rotate in the membranes during active Ca^{2+} transport [10].

Abbreviation: DnsCl, 1-dimethylaminonaphthalene-5-sulfonyl chloride.

To investigate the mode of existence of the ATPase in the membranes in relation to its catalytic activity, we intended to follow the fluorescence of the dye bound to the membranes prepared from rabbit skeletal muscle. The present paper deals with basic results obtained in this direction.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were of reagent grade. Sarcoplasmic reticulum membranes were prepared from rabbit skeletal muscle according to the method of Martonosi [4].

DnsCl was conjugated to the membranes according to the method described by Kasai et al. [1] with slight modifications as follows: 20 μl 25 mM DnsCl in acetone were added to 1 ml of a suspension of the membranes (5 mg in protein) in 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4). After the mixture was incubated at 22 °C for 10 min, it was cooled in an ice-bath. DnsCl in aqueous solution rapidly converts into DnsOH which is fluorescent. The dansylated membranes were freed from DnsOH by two successive filtrations on a G-25 Sephadex column equilibrated with 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4), at 4 °C, followed by centrifugation at 38 000 $\times g$ for 50 min. The labelled membranes thus prepared were practically free from DnsOH, as checked by polyacrylamide electrophoresis containing 1 % sodium dodecyl sulfate (see Results). The amount of the dye bound to the membranes was determined by absorbance at 340 nm ($\varepsilon = 4.3 \cdot 10^3$) in the presence of 1 % sodium dodecyl sulfate. The protein concentration was determined as described by Lowry et al. [11].

The ATPase activity of the membranes was calculated from the pH change of the medium in the range of 7.3–7.4, assuming that the ratio of released H^+ to hydrolyzed ATP was 0.851 in the pH range near 7.4 [12]. Solution pH was measured in a Hitachi-Horiba pH meter type F-5. The medium contained 0.16 mg protein of the dansylated membranes, 0.3 M sucrose, 2 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM potassium oxalate and 2 mM ATP. The reaction was started by the addition of 100 μM $CaCl_2$. The total volume was 5.0 ml and temperature 20 °C. For the calibration 100 μM KOH was used.

The separation of dansylated components of the membranes was performed by polyacrylamide gel electrophoresis containing 1 % sodium dodecyl sulfate. For the gel electrophoresis, the method of Fairbanks et al. [13] was adopted, but the treatment of the samples with dithiothreitol was not performed, since in this case the treatment gave no effect (see Results). The dansylated membranes (150 μg protein) were dissolved in 100 μl of the solution containing 2 % sodium dodecyl sulfate, 10 mM Tris · HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 5 % sucrose, and loaded on 5.6 % polyacrylamide gels (diameter, 7 mm). Electrophoresis was carried out with the voltage gradient at about 7–8 V/cm and the current at 8 mA/tube, using 40 mM Tris/20 mM acetate/2 mM EDTA buffer (pH 7.4) containing 1 % sodium dodecyl sulfate.

The gel was cut into slices of 5 mm thickness and the phosphorus content of each slice was determined by the method of Bartlett [14].

Fluorescence of the dye in the gel was detected by a mercury lamp with maximum wavelength at 366 nm. Membrane peptides were stained with Coomassie blue. A portion of the gel having the fluorescence was cut out and homogenated in 3 ml 2 mM Tris · HCl (pH 7.4) containing 0.3 M sucrose and 1 % sodium dodecyl

sulfate. After having been left overnight at 4 °C, the homogenate was centrifuged at 2000 × *g* for 5 min and the supernatant was subjected to fluorescence measurement. For a blank, non-dansylated membranes were treated in the same way.

Corrected fluorescence spectra were measured at 20 °C with Shimadzu corrected recording spectrofluorophotometer RF-502. For emission spectra, excitation was made at 290, 320 and 370 nm. Bandwidths were 10 nm for excitation and 3 nm for emission. For excitation spectra, emission was monitored at 510 and 550 nm. Bandwidths were 3 nm for excitation and 7.5 nm for emission. Fluorescence polarization measurements were performed with the above instrument equipped with Polacoat polarized filters as described elsewhere [15]. Bandwidths were 15 nm for excitation and 7.5 nm for emission. Special care was taken for correcting the scattered light of each sample; the reading of the intensity of non-dansylated membranes having the absorbance at 550 nm identical with that of the sample was subtracted. Temperature was regulated with a thermostated circulating water bath.

Total lipids were extracted from the sample as described by Folch et al. [16]. Separation of the lipids was performed by thin-layer chromatography, using chloroform/methanol/water (70 : 30 : 5, v/v) as solvent system. The lipids on the thin-layer plate were detected by exposing to iodine vapour, and the bound dye was detected fluorometrically by using a mercury lamp (λ_{max}, 366 nm).

Liposomes were prepared from total lipids extracted from the dansylated or non-dansylated membranes by treatment of ultrasonication of 26 KHz at 6 A for 15 min with Kokusai Electric ultrasonifier type UT 20F. The fluorescence spectra of the liposomes were measured as described above. Liposomes prepared from non-dansylated membranes were used as blank. The amount of the dye bound to phospholipid was determined photometrically with liposomes as described with native membranes. Phosphorus content in the phospholipid was determined as above.

RESULTS

Dansylation of the membranes and its effect on the ATPase activity

DnsCl was conjugated to the membranes and the amount of the bound dye was calculated from the absorbance at 340 nm to be approx. 20 nmol/mg protein. Dansylation of the membranes resulted in only a slight change in the ATPase activity (Fig. 1).

Excitation and emission spectra of the dansylated membranes

Corrected emission and excitation spectra of the dansylated membranes are shown in Figs. 2 and 3. It is noted that the emission maxima are different with different excitation wavelengths (Fig. 2). The maxima were found at 518, 523 and 529 nm, with excitation at 290, 320 and 370 nm, respectively; the emission maximum shifts towards the longer wavelength with the shift of the excitation wavelength towards the longer. In addition to the peak ascribed to the bound dye itself near 335 nm, the peak at 283 nm and the shoulder at 290 nm were observed in the excitation spectra of the dansylated membranes with emission at either 510 nm or 550 nm (Fig. 3, II and III), which are identical with the excitation spectrum of tryptophan residue of the membrane protein (Fig. 3, I). These results show the presence of energy transfer from tryptophan residue to the bound dye. The excitation maximum of the bound dye itself

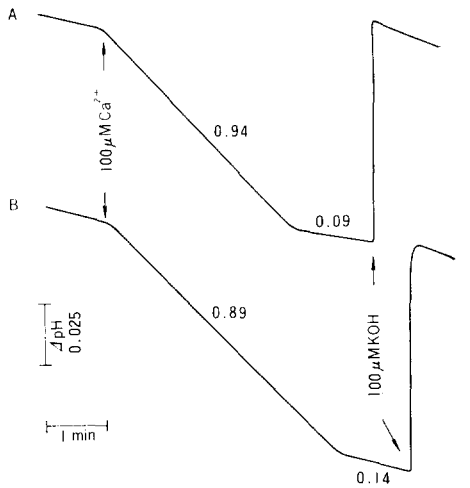


Fig. 1. ATPase activity of sarcoplasmic reticulum membranes. The ATPase activity of the membranes was measured as described in Methods. The medium contained 0.16 mg protein of the membranes, 0.3 M sucrose, 2 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM potassium oxalate and 2 mM ATP. The reaction was initiated by the addition of 100 μM $CaCl_2$. The total volume was 5.0 ml and temperature was 20 °C. For calibration, 100 μM of KOH was used. From changes in pH of the medium the ATPase activity of the membranes was calculated and expressed as μmol P_i liberated/mg protein per min in the figure. A, control membranes; B, the dansylated membranes.

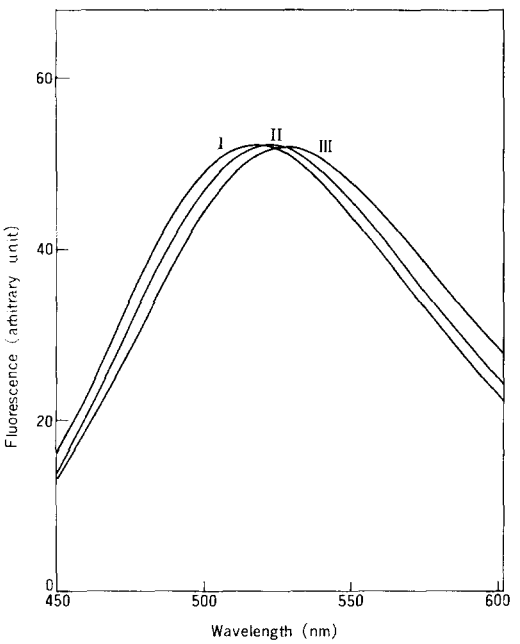
also shifted upon changing the monitored wavelengths: from 333 to 336 nm upon changing the monitored wavelengths from 510 to 550 nm, respectively.

Separation of dansylated components of the membranes by sodium dodecyl sulfate gel electrophoresis

Dansylated components were separated by using gel electrophoresis in the presence of 1 % sodium dodecyl sulfate. Under the experimental conditions described in Methods, membrane protein and phospholipid were obviously separated (see Fig. 4, A and B). The treatment with dithiothreitol showed no significant change in the peptide pattern of the membranes. However, dansylation changed their peptide pattern, causing the appearance of aggregated components (apparent molecular weight > 300 000).

Fluorometrically, two major bands were observable (band I and III) except for the aggregated components. Band I corresponds to the ATPase peptide, and band III to phospholipid. Calsequestrin, another major peptide component (band II), and other minor peptides were scarcely dansylated. DnsOH, produced in the aqueous solution, moved as a fluorescent band between bands II and III. Accordingly, the contamination of DnsCl in the sample was considered to be negligible as judged by fluorescence (Fig. 4, D).

Bands I and III were extracted from the gel and the emission and excitation spectra were measured in the presence of 1 % sodium dodecyl sulfate (Figs. 5, 6). The emission maximum of the dye bound to the ATPase was found at 523 nm and that of



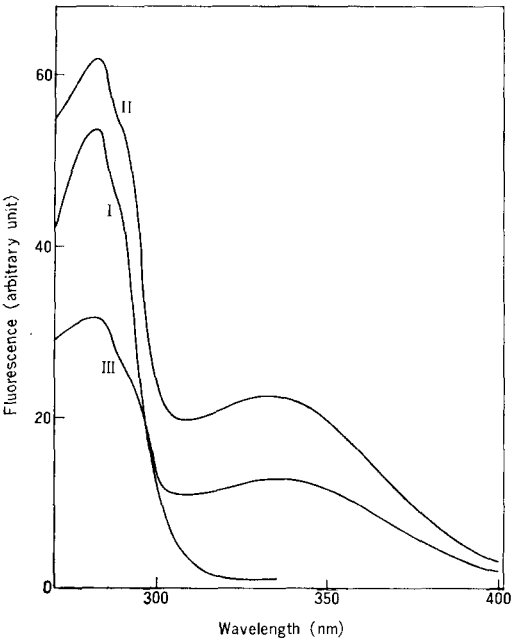


Fig. 2. Corrected emission spectra of the dye bound to the membranes. Measurements were performed in a medium containing 0.6 mg protein of the dansylated membranes, 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4). The total volume was 3.0 ml and temperature was 20 °C. Excitation was made at 290 nm (I), 320 nm (II) and 370 nm (III). Spectra were normalized to the same maximal intensity.

Fig. 3. Corrected excitation spectra of the dye bound to the membranes. Measurements were performed in a medium containing 0.24 mg protein of the dansylated membranes, 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4). The total volume was 3.0 ml and temperature was 20 °C. Emission wavelengths were fixed at 345 nm (I), 510 nm (II) and 550 nm (III).

the dye bound to phospholipid at 548 nm, and both emission maxima did not change with the two different excitation wavelengths (320 and 370 nm).

The excitation spectra of the dye bound to the ATPase in the presence of 1 % sodium dodecyl sulfate still had the peak at 283 nm, suggesting the presence of energy transfer from tryptophan to the dye, although the apparent efficiency seemed much lower than that of native membranes. However, the excitation spectra of the dye bound to phospholipid provided no evidence for the energy transfer. The excitation maxima of fluorescence of the dye bound to the ATPase and phospholipid were 332 and 337 nm, respectively. Independence of the excitation maximum upon emission wavelengths monitored (510 and 550 nm) suggests that only a single emission center exists in both systems.

Effect of addition of sodium dodecyl sulfate on fluorescence spectra of the dansylated membranes

Changes in emission maximum and the fluorescence intensity of the dansylated membranes in the presence of various concentrations of sodium dodecyl sulfate are summarized in Table I. Upon excitation at either 320 nm or 370 nm, the emission

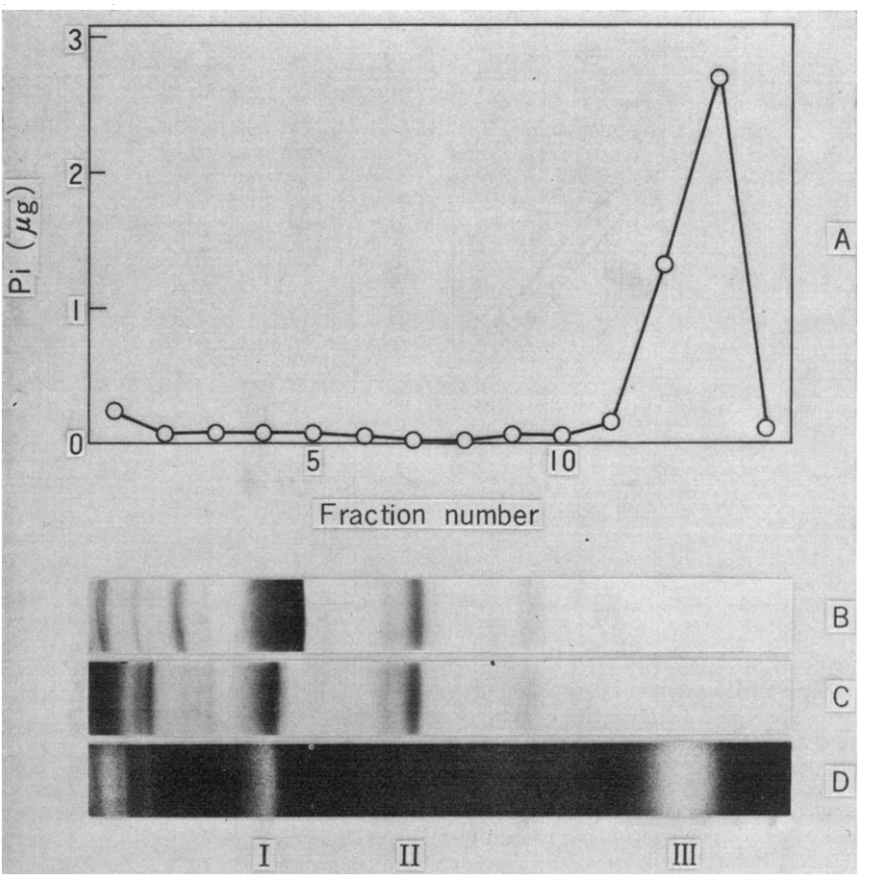


Fig. 4. Electrophoretic separation of membrane components in polyacrylamide gel containing 1 % sodium dodecyl sulfate. Two major peptides, the ATPase and calsequestrin, were numbered I and II, respectively, and number III corresponds to phospholipid. A, P_i content of slices of 5 mm thickness of the gel; B, non-dansylated membrane peptides stained with Coomassie blue; C, dansylated membrane peptides stained with Coomassie blue; D, dansylated membrane components detected by a mercury lamp (λ_{max}, 366 nm).

maximum shifted towards the longer wavelength with increasing concentration of sodium dodecyl sulfate. However, the extent of red shift upon excitation at the longer wavelength was much larger than that at the shorter wavelength. The fluorescence intensities decreased with increasing concentration of sodium dodecyl sulfate.

Fluorometric analyses of lipid fraction

Total lipids were extracted from the dansylated membranes or from band III of the polyacrylamide gel and separated by thin layer chromatography. As shown in Fig. 7, the dye was mostly associated with phosphatidylethanolamine, and R_f value of the dansylated phosphatide was slightly higher than that of the native phosphatide. When extracted from the gel, the pattern of the total lipids on the thin layer plate was scarcely modified.

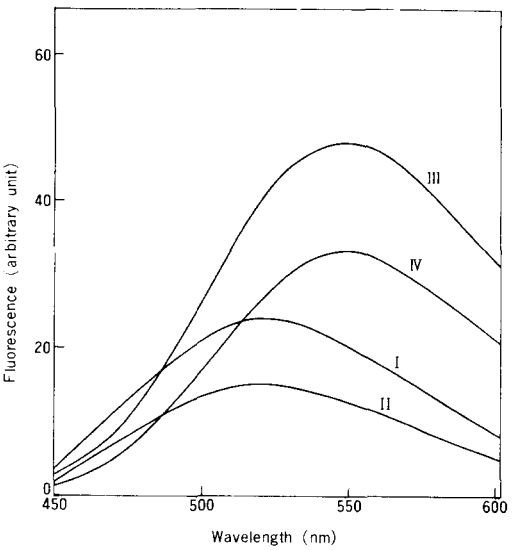


Fig. 5. Corrected emission spectra of the dye bound to the ATPase and phospholipid extracted from the polyacrylamide gel. I and II, the dye bound to the ATPase with excitation at 320 nm (I) and 370 nm (II), respectively; III and IV, the dye bound to phospholipid with excitation at 320 nm (III) and 370 nm (IV), respectively.

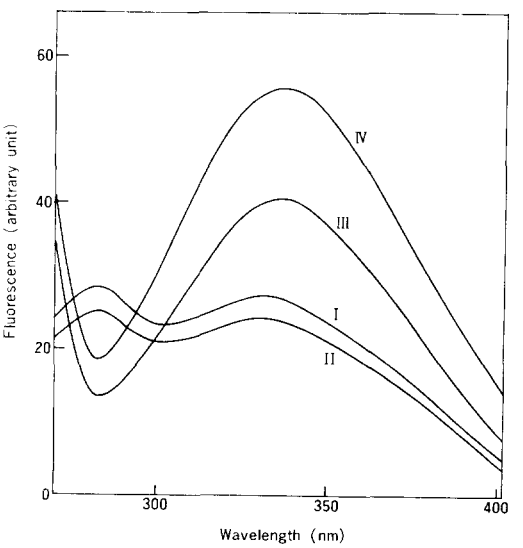


Fig. 6. Corrected excitation spectra of the dye bound to the ATPase and phospholipid extracted from the polyacrylamide gel. I and II, the dye bound to the ATPase with emission at 510 nm (I) and 550 nm (II), respectively; III and IV, the dye bound to phospholipid with emission at 510 nm (III) and 550 nm (IV), respectively.

TABLE I

EMISSION MAXIMUM AND RELATIVE FLUORESCENCE INTENSITY OF Dns-SARCOPLASMIC RETICULUM MEMBRANES IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF SODIUM DODECYL SULFATE

Corrected fluorescence spectra were measured as described in Methods. The medium contained 0.2 mg protein of the dansylated membranes, 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4). The total volume was 3.0 ml and temperature was 20 °C. Various concentrations of sodium dodecyl sulfate were attained by adding small amount of the concentrated solution in the medium and the correction of increased volume was done for estimating relative fluorescence intensities.

Sodium dodecyl sulfate	Excitation wavelength (nm)	Emission maximum (nm)	Relative fluorescence intensity
None	320	523	100
	370	529	100
0.1 %	320	525	77
	370	534	75
0.2 %	320	531	60
	370	542	60
0.4 %	320	533	54
	370	545	53
1.0 %	320	534	48
	370	548	45

Fluorescence maxima of liposomes prepared from the dansylated membranes are summarized in Table II. In liposomes the bound dye emitted in the longer wavelength region and the emission maxima upon excitation at the shorter and longer wavelengths were identical even when the liposomes were dissolved with 1 % sodium dodecyl sulfate. The decrease in fluorescence intensity was also observed as in the case of the dansylated membranes (Table I).

The amount of the dye associated with phospholipid estimated for liposomes prepared from the dansylated membranes was approx. 14 nmol/mg phospholipid, on the assumption that there exists 25 μg phospholipid per 1 μg P_i.

Polarization measurements of the fluorescence of the dansylated membranes

In order to differentiate the dye bound to the ATPase from that bound to phosphatidylethanolamine, polarization degree (P) of the fluorescence of the dye was measured at shorter and longer wavelength regions; for the shorter wavelength region, the dye bound to the membranes was excited at 320 nm and it emitted at 510 nm ($P_{320\rightarrow 510}$), and for the longer one, excited at 370 nm and emitted at 550 nm ($P_{370\rightarrow 550}$). It is reasonably assumed that the P value of the dye bound to the ATPase corresponds to the value of $P_{320\rightarrow 510}$, and the P value of the dye bound to phosphatidylethanolamine to the value of $P_{370\rightarrow 550}$. As shown in Fig. 8, the values of $1/P$ were plotted against T/η, assuming that the viscosity (η) of the solvent is identical with that of pure sucrose solution. In the dansylated membranes, the values of $P_{320\rightarrow 510}$ were significantly smaller than those of $P_{370\rightarrow 550}$ under isothermic conditions. When the temperature (T) was changed from 5 to 41 °C at the fixed sucrose concentration of 0.3 M, $1/P$ increased (Fig. 8, I and III). However, when η was

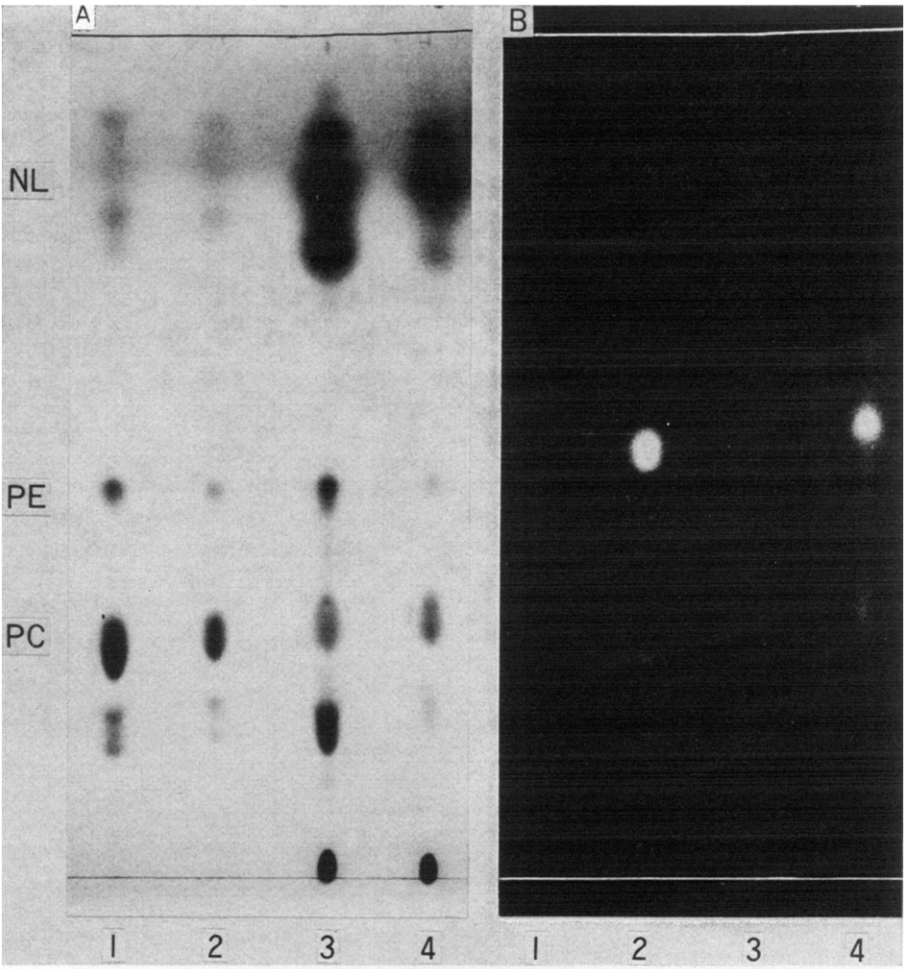


Fig. 7. Separation by thin layer chromatography of lipids extracted from the dansylated membranes or band III of the polyacrylamide gel. A, Chromatographic profile of total lipids detected by exposing to iodine vapour. Total lipids extracted from non-dansylated membranes (1), from the dansylated membranes (2), from band III of the gel to which non-dansylated membranes were applied (3), and from band III of the gel to which the dansylated membranes were applied (4). NL, neutral lipids; PE, phosphatidylethanolamine; and PC, phosphatidylcholine. B, fluorescence of the dye of the same plate detected by using a mercury lamp.

changed by adding solid sucrose at 20 °C, no significant variation of $1/P$ occurred at high values of T/η (Fig. 8, II and IV), while at low values of T/η, the downward curvature of $1/P$ appeared in both curves of II and IV. On the other hand, in liposomes prepared from the dansylated membranes including only the dye bound to phosphatidylethanolamine, the values of $P_{320\rightarrow510}$ and $P_{370\rightarrow550}$ were identical (0.163) at the conditions described in Table II.

TABLE II

EMISSION MAXIMUM AND RELATIVE FLUORESCENCE INTENSITY OF LIPOSOMES OF TOTAL LIPIDS EXTRACTED FROM THE DANSYLATED MEMBRANES

Corrected fluorescence spectra were measured as described in Methods. The medium contained liposomes prepared from the dansylated membranes equivalent to 0.79 mg phospholipids, 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4). The total volume was 3.0 ml and temperature was 20 °C. Small amount of concentrated sodium dodecyl sulfate solution was added to attain 1 % concentration and the correction of the volume was made for estimating relative fluorescence intensities.

Sodium dodecyl sulfate	Excitation wavelength (nm)	Emission maximum (nm)	Relative fluorescence intensity
None	320	537	100
	370	537	100
1 %	320	548	63
	370	548	66

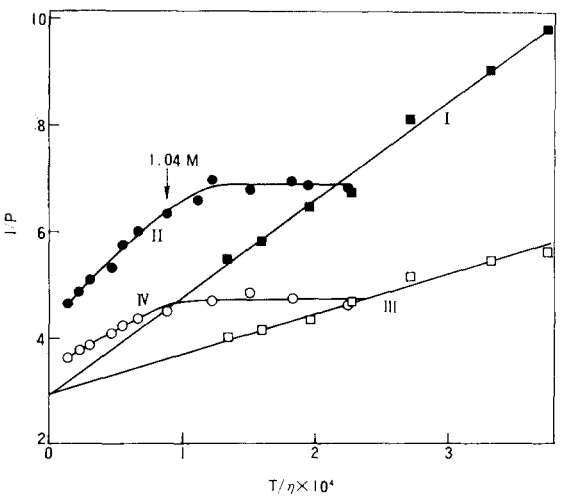


Fig. 8. Effects of temperature and viscosity of the medium on the fluorescence polarization of the dye bound to the membranes. The medium contained 0.6 mg protein of the dansylated membranes, 0.3 M sucrose and 2 mM Tris · HCl (pH 7.4). The total volume was 3.0 ml. Temperature of the medium was regulated with a thermostated circulating water bath and viscosity of the medium was changed by adding solid sucrose to the medium. The degree of the fluorescence polarization of the bound dye in the shorter wavelength region was measured with excitation at 320 nm and emission at 510 nm. I, temperature of the medium was changed from 5 to 41 °C at the fixed concentration of 0.3 M sucrose. II, the sucrose concentration was changed from 0.3 to 1.9 M at 20 °C. The degree of the fluorescence polarization of the bound dye in the longer wavelength region was also measured with excitation at 370 nm and emission at 550 nm, at different temperatures (III) and sucrose concentrations (IV).

DISCUSSION

The present results clearly demonstrate that DnsCl reacts with both the ATPase and phosphatidylethanolamine of sarcoplasmic reticulum membranes (Figs. 4, 7), as in the case of erythrocyte membranes [3]. The present observation that calsequestrin was scarcely dansylated might indicate that calsequestrin exists on the interior surface of the membranes as suggested by Stewart and MacLennan [17] or that the protein exists on the exterior surface of the membranes as suggested by Thorley-Lawson and Green [18] but has a weak reactivity to the dye.

Assuming that the amount of phospholipid is half of that of protein in intact membranes and that the ATPase also amounts to half of the total protein in sarcoplasmic reticulum membranes, it can be deduced that nearly one mol of DnsCl binds to one mol of the ATPase. Although one mol of the ATPase molecule contains a number of amino acid residues which can react with DnsCl [8], one to one binding of DnsCl with the ATPase may suggest the presence of a specific binding site of the ATPase molecule for the dye. In the same way, 1 mg of phospholipid was equivalent to about 1.25 μmol of phospholipid (mean molecular weight, 800), and approx. 4 % of phosphatidylethanolamine was conjugated with the dye, taking into account the fact that approx. 30 % of phospholipid was phosphatidylethanolamine in sarcoplasmic reticulum membranes of the mammalian preparation [19].

Considering that the ATPase in sarcoplasmic reticulum membranes was dansylated without significant loss of enzyme activity, our labelling method seems to be rather gentle (Fig. 1). However, higher molecular weight components appeared in the gel electrophoresis, which indicates the occurrence of aggregation of dansylated peptides. The same phenomenon was observed with dansylated erythrocyte membranes [3].

It is considered that the fluorescence having the emission maximum at 518 nm in the dansylated membranes (Fig. 2, I) could be ascribed to the dye bound to the ATPase, since the fluorescence is attributable to the energy transfer from tryptophan to the dye bound in the neighborhood. Accordingly, the fluorescence in the relatively longer wavelength region could be due to the dye bound to phosphatidylethanolamine. This consideration is supported by the results that the emission maximum of liposomes prepared from the dansylated membranes exists at the relatively longer wavelength (537 nm). It can be further verified by the experimental data using sodium dodecyl sulfate. In the presence of 1 % sodium dodecyl sulfate, the emission maximum of the dansylated membranes upon excitation at 370 nm was found at 548 nm which was identical with that of the dye bound to phospholipid extracted from the polyacrylamide gel (Fig. 5, III and IV), and the emission maximum of the dansylated membranes upon excitation at 320 nm was at 534 nm (Table I) which was close to that of the dye bound to the ATPase (Fig. 5, I and II). Therefore, the dye bound to the ATPase and that bound to phosphatidylethanolamine could be partially differentiated fluorometrically by changing the excitation wavelength; fluorescence excited at 320 nm demonstrates mainly that of the dye bound to the ATPase and fluorescence excited at 370 nm reveals mostly that of the dye bound to phosphatidylethanolamine.

It is noted that the fluorescence polarization values of the dye bound to the membranes at the shorter ($P_{320\rightarrow510}$) and longer ($P_{370\rightarrow550}$) wavelength regions were remarkably different under isothermic condition (Fig. 8, I and III). These

results cannot be explained by the difference of the excitation wavelength, since the values of $P_{320\rightarrow510}$ and $P_{370\rightarrow550}$ of liposomes prepared from the dansylated membranes were the same. Therefore, it can be considered that the environment of the dye bound to the ATPase is different from that bound to phosphatidylethanolamine. It is also noted that $1/P$ values were independent of T/η at high values upon changing the concentration of sucrose (Fig. 8, II and IV), indicating that the above two components are strongly immobilized in the membranes. The same conclusion has been reported for proteins in biological membranes [1, 2]. The downward curvature of $1/P$ at low values of T/η observed upon changing the concentration of sucrose is similar to the case of Dns-protein conjugates and pyrenbutyric-protein conjugates reported by several researchers [20–24]. They proposed a model containing both a freely rotating fluorescent dye molecule and a "frozen" one, in which a temperature-dependent distribution between the two states exists. According to this model, the fraction of residues in "frozen" state (f)* is expressed as a function of the apparent limiting polarization ($P_{0_{app}}$) and the true one ($P_{0_{true}}$) as follows:

$$(1/P_{0_{app}} - \tfrac{1}{3}) = 1/f(1/P_{0_{true}} - \tfrac{1}{3})$$

In this case, $1/P_{0_{app}}$ is referred to the intercept of the asymptote of $1/P$ upon changing the concentration of sucrose at high values of T/η (Fig. 8, II and IV) and $1/P_{0_{true}}$ can be estimated from that of the asymptote of $1/P$ observed upon changing temperature (Fig. 8, I and III). As the values of $P_{0_{true}}$ in the shorter and longer wavelength regions were found to be identical, the difference in the values of $P_{0_{app}}$ in two measurements ($P_{320\rightarrow510}$ and $P_{370\rightarrow550}$) suggests that the fraction of freely rotating fluorophore bound to the ATPase is larger than that bound to phosphatidylethanolamine.

The other notable facts demonstrated in the fluorescence polarization study are the sucrose accessibility to the dye bound to the membranes and the relatively low value of $P_{320\rightarrow510}$ (0.146 at 20 °C), in contrast to the polarization degree of the dye bound to the excitable membranes (0.24 at 22 °C) [1] where most of the dye was bound to the protein. These facts again indicate that considerable amount of the dye bound to the ATPase is in the freely rotating state. In this connection, the electron microscopic observation of sarcoplasmic reticulum membranes should be considered, viz., the presence of surface particles which were supposed to be the hydrophilic extension of the ATPase [17]. The present results may be reasonably understood by the assumption that some of the dye bound to the ATPase is associated with the surface particles.

On the other hand, most of the dye bound to phosphatidylethanolamine seems to be in the "frozen" state. Therefore, the naphthalene core of the dye molecule might be buried in the hydrophobic region of lipid bilayer of the membrane, since the naphthalene core of the dye can be adjacent to the acyl side chain of the phosphatide according to a space-filling model.

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* In the literatures [23, 24], f in the equation has been referred to fraction of a dye rotating freely, but it should be corrected to be that of a dye in "frozen" state, as described here, according to Wahl and Weber [20].

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