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The Mechanism of Voltage-Sensitive Dye Responses on Sarcoplasmic Reticulum

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Summary. The mechanism of voltage-sensitive dye responses was analyzed on sarcoplasmic reticulum vesicles to assess the changes in membrane potential related to Ca^{2+} transport. The absorbance and fluorescence responses of 3,3′-diethyl-2,2′-thiadicarbocyanine, 3,3′-dimethyl-2,2′-indodicarbocyanine and oxonol VI during ATP-dependent Ca^{2+} transport are influenced by the effect of accumulated Ca^{2+} upon the surface potential of the vesicle membrane. These observations place definite limitations on the use of these probes as indicators of ion-diffusion potential in processes which involve large fluctuations in free Ca^{2+} concentrations. Nile Blue A appeared to produce the cleanest optical signal to negative transmembrane potential, with least direct interference from Ca^{2+}, encouraging the use of Nile Blue A for measurement of the membrane potential of sarcoplasmic reticulum *in vivo* and *in vitro*. 1,3-dibutylbarbituric acid (5)-1-(*p*-sulfophenyl)-3 methyl, 5-pyrazolone pentamethinoxonol (WW 781) gave no optical response during ATP-induced Ca^{2+} transport and responded primarily to changes in surface potential on the same side of the membrane where the dye was applied. Binding of these probes to the membrane plays a major role in the optical response to potential, and changes in surface potential influence the optical response by regulating the amount of membrane-bound dye. The observations are consistent with the electrogenic nature of ATP-dependent Ca^{2+} transport and indicate the generation of about 10 mV inside-positive membrane potential during the initial phase of Ca^{2+} translocation. The potential generated during Ca^{2+} transport is rapidly dissipated by passive ion fluxes across the membrane.

Key Words: sarcoplasmic reticulum, Ca^{2+} transport, membrane potential, potential-sensitive dyes, surface potential, excitation-contraction coupling

The contraction and relaxation of skeletal muscle is accompanied by large fluxes of calcium across the sarcoplasmic reticulum membrane. Without compensating ion fluxes the active transport of calcium into the sarcoplasmic reticulum should generate an inside-positive membrane potential. A potential change of opposite sign is expected to occur during Ca^{2+} release. These potential changes could have major physiological importance in the regulation of excitation-contraction coupling.

Analysis of the ionic composition of the sarcoplasmic reticulum and surrounding cytoplasm by electron X-ray microprobe in resting and fatigued muscles did not reveal significant Cl^- and K^+ gradients across the sarcoplasmic reticulum membrane (Somlyo, Shuman & Somlyo, 1977; Somlyo et al., 1978). Although the data do not rule out transient changes of membrane potential, it appears unlikely that a sustained resting potential is generated in the sarcoplasmic reticulum by calcium uptake during relaxation *in vivo*.

The apparent absence of Cl^- and K^+ gradients across the sarcoplasmic reticulum in resting muscle is consistent with the high permeability of the membrane to anions and cations (Duggan & Martonosi, 1970; Jilka, Martonosi & Tillack, 1975; Kasai & Miyamoto, 1976*a*, *b*; Meissner & McKinley, 1976). The estimated ion fluxes through anion and cation channels in the sarcoplasmic reticulum are sufficiently fast to neutralize potential changes which may take place during Ca^{2+} uptake or release. Therefore investigation of the electrical changes connected with Ca^{2+} fluxes requires conditions which minimize compensating ion fluxes.

A generation of inside-positive membrane potential[1] during Ca^{2+} uptake into reconstituted ATPase-phosphatidylcholine vesicles was observed by Zimniak and Racker (1978), using 8-anilino-1-naphthalene sulfonic acid as fluorescent probe. In reconstitut-

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[1] Inside-positive or -negative membrane potential in sarcoplasmic reticulum is denoted as positive or negative potential, respectively.

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ed vesicles containing only the purified Ca^{2+} transport ATPase the anion and cation permeability was sufficiently small to observe sustained membrane potentials.

Indirect evidence for electrogenic transport of calcium into sarcoplasmic reticulum was later obtained by Meissner (1979) and Beeler (1980*a, b*) who found that the absorbance signal and fluorescence of carbocyanine dyes caused by artifically imposed inside-negative membrane potential, was sharply diminished during ATP-induced calcium transport, with stimulation of the rate of Ca^{2+} uptake. As yet there is no direct evidence that Ca^{2+} transport by sarcoplasmic reticulum generates inside-positive membrane potential.

The reliability of the conclusions concerning the electrical changes connected with Ca^{2+} transport is largely dependent upon the interpretation of the mechanism by which "voltage-sensitive" probe molecules respond to membrane potential.

Voltage-sensitive optical probes fall into two general classes:

1. Oxacarbocyanines, oxadicarbocyanines, thiacarbocyanines, thiadicarbocyanines, indodicarbocyanines, and oxonol dyes contain a delocalized positive or negative charge, which makes them relatively permeant to lipid bilayers (Sims, Waggoner, Wang & Hoffman, 1974; Waggoner, 1976, 1979; Cohen & Salzberg, 1978; Freedman & Laris, 1981). These dyes redistribute across and within the membrane under the influence of transmembrane potential, and fluorescence or absorbance changes result when an increase in the internal dye concentration causes the binding of the dyes to the membrane and other cell constitutents with the formation of dye-dimers and larger aggregates.

The positive cyanine dyes, 3,3′-diethyloxadicarbocyanine (Beeler, Russell & Martonosi, 1979; Russell, Beeler & Martonosi, 1979*a*), 3,3′-dipentyloxadicarbocyanine (Madeira, 1978), 3,3′-dipropylthiadicarbocyanine (Ueno & Sekine, 1978; Dupont, 1979), and 3,3′-diethylthiadicarbocyanine (Beeler, 1980*a, b*), respond with optical changes during Ca^{2+} uptake by sarcoplasmic reticulum vesicles.

2. The second class of voltage-sensitive dyes (e.g., 8-anilino-1-naphthalene sulfonic acid, merocyanine 540, 1,3-dibutylbarbituric acid-(5)-1-(*p*-sulfophenyl)-3 methyl, 5-pyrazoline pentamethinoxonol (WW 781), contain localized charges and penetrate only slowly through bilayers. Their response to membrane potential is complex, but involves primarily the redistribution of bound dye within the membrane (Conti, 1975; Cohen & Salzberg, 1978; Freedman & Laris, 1981).

Among the negative dyes, 8-anilino-1-naphthalene sulfonic acid (Vanderkooi & Martonosi, 1969, 1971*a, b*; Haynes & Chiu, 1978; Ueno & Sekine, 1978; Zimniak & Racker, 1978), merocyanine 540 (Salama & Scarpa, 1978; Russell, Beeler & Martonosi, 1979*b*) and oxonols (Akerman & Wolff, 1979; Russell et al., 1979*b*) were reported to produce optical changes during Ca^{2+} transport by sarcoplasmic reticulum.

Absorbance and fluorescence changes of Nile Blue A (Bezanilla & Horowicz 1975; Oetliker, Baylor & Chandler, 1975; Vergara, Bezanilla & Salzberg, 1978; Baylor, Chandler & Marshall, 1981), indodicarbocyanine (Oetliker et al., 1975; Baylor et al., 1981), merocyanine 540 (Landowne, 1974; Nakajima, Gilai & Dingeman, 1976; Salama & Morad 1976; Vergara & Bezanilla, 1976) and WW 781 (Vergara & Bezanilla, 1979; Baylor et al., 1981) have been correlated with action potential, tension development and Ca^{2+} fluxes during excitation in living muscle fiber.

Under suitable conditions most of these dyes respond to transmembrane potential with absorbance or fluorescence changes as defined by the Nernst equation. However, the probes are also sensitive to changes in surface potential caused by the binding of cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, La^{3+}) and anions (Cl^-, ATP^{3-}) to the membrane (Vanderkooi & Martonosi, 1969, 1971*a, b*; Russell et al., 1979*b*; Krasne, 1980*a, b*). As the intravesicular Ca^{2+} concentration during active Ca^{2+} transport reaches 20–30 mM, causing large changes in the surface potential due to "screening" and binding of Ca^{2+} to membrane sites, the effect of Ca^{2+} upon the probe response requires serious consideration.

The purpose of this report is to analyze the properties of 3,3′-diethyl-2,2′-thiadicarbocyanine (di-S-C_2(5)), 3,3′-dimethyl, 2,2′-indodicarbocyanine (di-I-C_1(5)); Nile Blue A, oxonol VI and 1,3-dibutylbarbituric acid (5)-1-(*p*-sulfophenyl)-3 methyl, 5-pyrazoline pentamethinoxonol (WW 781) as potentiometric probes on sarcoplasmic reticulum. The relative contribution of transmembrane potential and Ca^{2+} to the optical response of the dye is evaluated during Ca^{2+} transport. The results are consistent with an electrogenic Ca^{2+} pump, but much of the optical response of the various probes during Ca^{2+} translocation is due to the effect of accumulated Ca^{2+} upon the surface potential of the vesicle membrane. The observations place strict limitations upon the use of these probes as indicators of ion diffusion potential in processes which involve large fluctuations in free Ca^{2+} concentration.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle essentially as described earlier (Nakamura, Jilka, Boland & Martonosi, 1976), except that the homogenization medium contained 0.3 M sucrose and 10 mM imidazole (pH 6.6). The microsome fraction collected at 36,000 × *g* was washed first with 0.2 M sucrose, 10 mM imidazole (pH 6.6), and then twice with a solution contain-

ing 10 mM imidazole (pH 6.6), 10 mM Mg-maleate and either 0.15 M K-glutamate, 0.15 M choline-Cl or 0.3 M glycine. Protein concentration was determined by either the Lowry (Lowry, et al., 1951) or the biuret (Gornall, Bardawill & David, 1949) method.

Calcium accumulation by sarcoplasmic reticulum vesicles was monitored by Millipore filtration using ^{45}Ca as a tracer (Martonosi & Feretos, 1964) or spectrophotometrically using the calcium indicator, arsenazo III (DiPolo et al., 1976). ATPase activity was measured by assaying the inorganic orthophosphate liberation according to Fiske and Subbarow (1925).

For absorption measurements an Aminco DW-2 spectrophotometer was used. Fluorescence excitation and emission spectra were measured on a Varian SF-300 spectrofluorometer.

3,3′ Diethyl-2-2′-thiadicarbocyanine (di-S-C_2(5)) was obtained from Eastman Kodak (Rochester, N.Y.). 3,3′ Dimethyl-2-2′-indodicarbocyanine (di-I-C_1(5)) and Nile Blue A were kindly supplied by Dr. W.K. Chandler, Department of Physiology, Yale University. WW 781 was a gift from Dr. J.C. Freedman, Department of Physiology, S.U.N.Y. Upstate Medical Center, N.Y. Oxonol VI was synthesized by Molecular Probes Inc. (Plano, Tex.). The stock solutions of all dyes were made in ethanol at concentrations of 0.5–1 mg/ml and stored in the dark at −20 °C no longer than 3 weeks. Valinomycin, glutamic acid, gramicidin, glycin and adenosine 5′-triphosphate were supplied by Sigma Chemical Co. (St. Louis, Mo.). $^{45}CaCl$ was purchased from ICN Chemical (Irvine, Cal.). A23187 was kindly provided by Dr. Robert Hamill of Eli Lilly and Co. (Indianapolis, Ind.). Arsenazo III, obtained from Aldrich Chemical Co. (Milwaukee, Wis.), was purified before use according to Kendrick (1976).

Results

A complete description of the relationship between membrane potential and the Ca^{2+} fluxes across sarcoplasmic reticulum requires the measurement of changes in surface potential as well as the transmembrane potential during ATP-dependent Ca^{2+} uptake, and either passive Ca^{2+} release or Ca^{2+} release through reversal of the Ca^{2+} pump.

The structures of the various dyes used in these studies are given in Fig. 1. 3,3′-diethyl-2,2′-thiadicarbocyanine (*I*), 3,3′-dimethyl, 2,2′-indodicarbocyanine (*II*) and Nile Blue A (*III*) are positively charged and are freely permeant across biological membranes. The optical response of 3,3′-diethyl 2,2′-thiadicarbocyanine to membrane potential involves primarily the accumulation of the dye in vesicles with negative potential, followed by aggregation and binding of the dye to the membrane (Sims et al., 1974; Waggoner, 1976; Cohen & Salzberg, 1978). In the case of 3,3′-dimethyl 2,2′-indodicarbocyanine, aggregation of the dye is less significant and the optical response to potential is largely attributable to the interaction of the dye with the membrane. The mechanism of Nile Blue A response has not been systematically investigated. Oxonol VI (*IV*) has one delocalized negative charge. It is freely permeant across the membrane and accumulates in vesicles with inside-positive membrane potential; the optical signal arises from changes in membrane-dye interactions. WW 781 (*V*) contains one loclized and one delocalized negative charge. Its permeability across the membrane is limited and the dye response to potential reflects redistribution of the dye within the membrane or at the membrane-water interphase.

The differences between the mechanisms by which various dyes respond to potential facilitates the assessment of surface potential contribution to the overall response.

(I) 3,3′-Diethyl-2,2′-Thiadicarbocyanine (Di-S-C_2(5))

(II) 3,3′-Dimethyl-2,2′- Indodicarbocyanine (Di-I-C_1(5))

(III) Nile Blue A

(IV) Oxonol VI

(V) 1,3, Dibutyl barbituric acid (5)-1-(P-sulfophenyl)-3methyl, 5pyrazolone pentamethin oxonol (WW781)

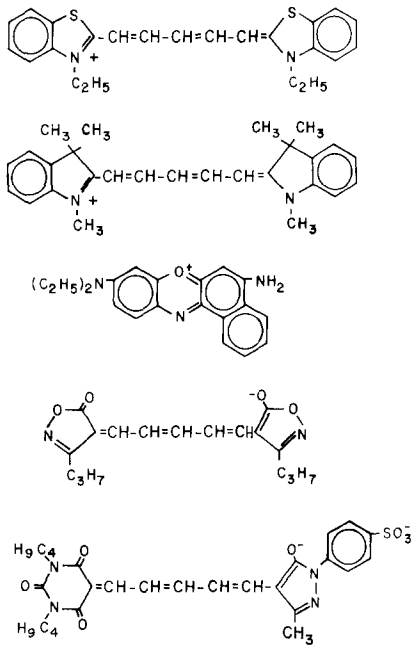


Fig. 1. Structures of the potentiometric probes. For details *see* text

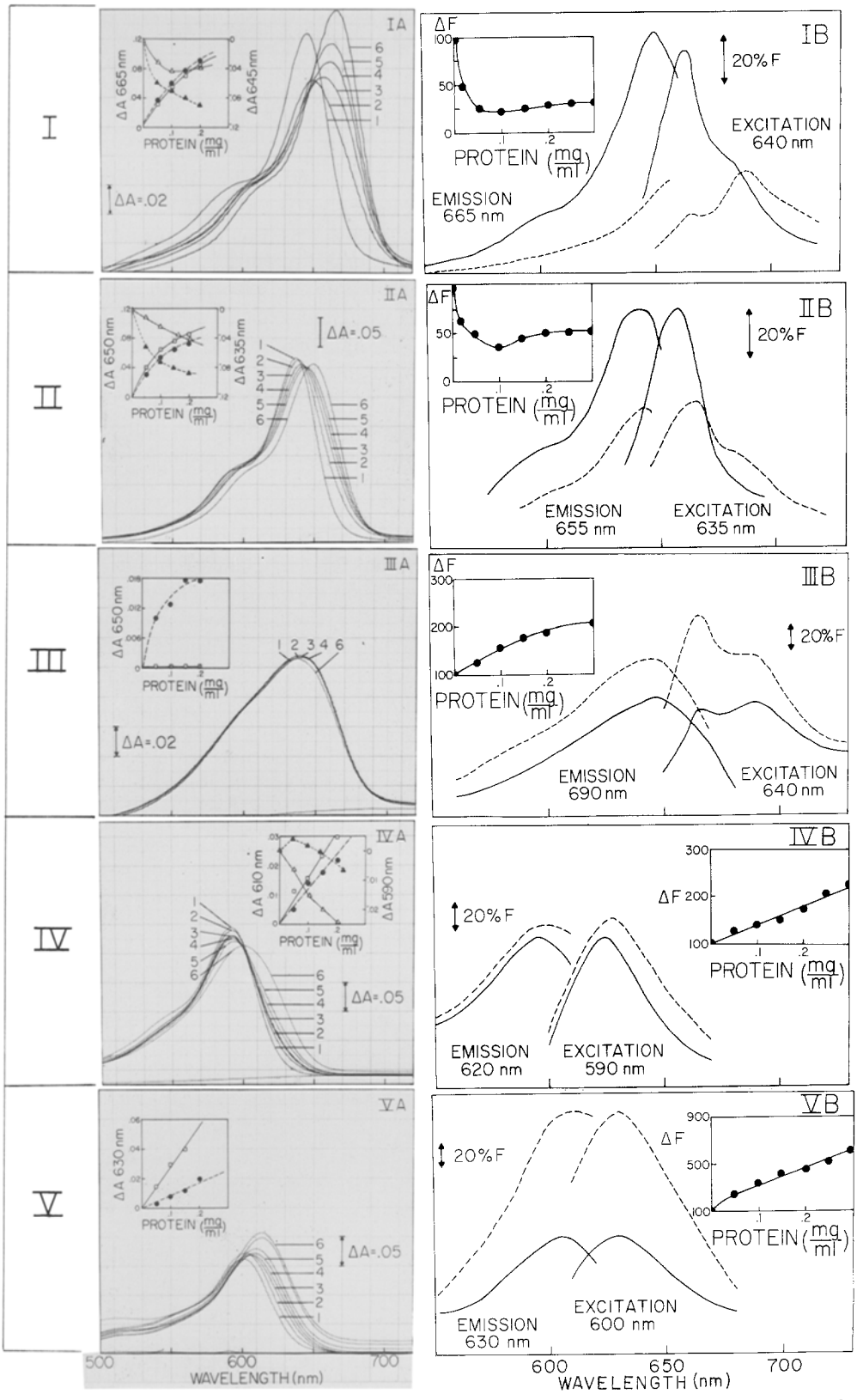


Fig. 2. Effect of sarcoplasmic reticulum vesicles on the absorbance (*A*) and fluorescence (*B*) spectra of di-S-C_2(5) (*I*), di-I-C_1(5) (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*). (*A*) The absorbance spectra of the dyes (1 μg/ml) were measured in a medium containing 0.15 M K-glutamate and 10 mM imidazole (pH 6.6) (*1*). Sarcoplasmic reticulum vesicles were added to both the sample and reference cells to a final protein concentration of 50 (*2*), 100 (*3*), 150 (*4*), 200 (*5*) and 550 (*6*) μg/ml. *Insert:* Change in absorbance as a function of the microsome concentration measured in 0.15 M K-glutamate (△, □) or 0.3 M sucrose (●, ▲) and 10 mM imidazole (pH 6.6). *Left ordinate:* ●, □; *right ordinate:* △, ▲. (*B*) The corrected excitation and emission spectra of the dyes (1 μg/ml) were measured in 0.3 M sucrose, 10.0 mM imidazole (pH 6.6) in the absence (——) and presence (– – –) of sarcoplasmic reticulum vesicles (100 μg protein/ml). *Insert:* Change in the dye fluorescence intensity as a function of microsome concentration. The excitation and emission wavelengths are given in the Figure

As the absorption and fluorescence spectra of the various dyes depend in a complex manner upon the dye concentration, the dye:membrane ratio and the ion composition of the assay system, meaningful interpretation of the optical response to membrane potential requires information on all these effects.

Effect of Sarcoplasmic Reticulum Vesicles on the Absorbance and Fluorescence of the Various Probes

Binding of di-S-$C_2(5)$, di-I-$C_1(5)$, oxonol VI and WW 781 to sarcoplasmic reticulum vesicles causes a shift in the absorption maximum to longer wavelengths (Fig. 2*A*). With increase in membrane concentration the absorbance of dyes *I, II* and *IV* increases at wavelengths of 665, 650 and 610 nm, respectively; the change is similar in media containing 0.3 M sucrose or 0.15 M K-glutamate (Fig. 2*A*, inserts). At wavelengths of 645 and 635 nm the absorbance of dyes *I* and *II* decreases with increasing membrane concentration and the absorbance change is greater in media containing 0.3 M sucrose than in 0.15 M K-glutamate. The opposite relationship was obtained with the negative dye oxonol VI, at 590 nm.

With WW 781 (*V*) the increase in ΔA_{630} is greater in K-glutamate than in 0.3 M sucrose medium.[2] Only slight change in the absorbance of Nile Blue A (*III*) was observed at 650 nm in 0.3 M sucrose and essentially no change in 0.15 M K-glutamate at membrane protein concentrations ranging from 0 to 0.2 mg/ml.

The large absorbance differences between media containing 0.15 M K-glutamate and 0.3 M sucrose suggest the influence of surface potential upon the dye binding to the membrane. Screening of negative surface charges by K^+ ions decreased the interaction of positive dyes (*I, II* and *III*) and increased the interaction of negative dyes (*IV* and *V*) with the membrane. These observations are consistent with the results of equilibrium dye-binding studies carried out in media of 0.3 M sucrose or 0.15 M K-glutamate, at various pH's (Table 1). The binding of di-I-$C_1(5)$, a positive dye, is less, while that of oxonol VI, a negative dye, is greater in media containing 0.15 M K-glutamate as compared with 0.3 M sucrose. Similarly a decrease in negative surface charge density by lowering the pH of the medium from pH 7.0 to 5.0 promoted the binding of oxonol VI and reduced the binding of di-I-$C_1(5)$ to the membrane, as expected for ionic interactions between charged groups on the membrane and the charged dye molecules.

Table 1. Effect of K-glutamate and pH on the binding of di-S-$C_2(5)$ (*I*), di-I-$C_1(5)$ (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*) to sarcoplasmic reticulum vesicles[a]

Dye	Medium	pH	Percent dye bound
I	K	6.6	62.9
	S	6.6	80.2
II	K	6.6	18.9
	S	6.6	31.2
III	K	6.6	12.8
	S	6.6	33.5
IV	K	6.6	11.5
	S	6.6	2.5
V	K	6.6	32.1
	S	6.6	5.3
II	K	5.0	13.9
	K	6.0	18.0
	K	7.0	19.9
IV	K	5.0	23.4
	K	6.0	16.6
	K	7.0	13.5

[a] The media contained 10 mM imidazole at the indicated pH, 50 µg microsomal protein per ml, 0.5 µg/ml dye and either 0.15 M K-glutamate (medium K) or 0.3 M sucrose (medium S). Incubation time: 1–2 hr; temperature: 22 °C. The absorbance of the solutions was measured before and after the removal of microsomal proteins by centrifugation at 145,000 × *g* for 30 min, at the following wavelengths: 650 nm (*I*), 636 nm (*II*), 640 nm (*III*), 592 nm (*IV*) and 606 nm (*V*). The absorbance of the dye was corrected for light scattering by the vesicles. The percentage dye bound to microsome was calculated from the difference of the absorbance of the solutions before and after centrifugation.

Di-S-$C_2(5)$ (*I*) and di-I-$C_1(5)$ (*II*) respond with a decrease in fluorescence intensity upon excitation at 640 and 635 nm, respectively, in the presence of 0.1–0.2 mg sarcoplasmic reticulum protein per ml (Fig. 2*B*, insert). The decrease in fluorescence intensity is caused by a shift in the excitation and emission maxima to longer wavelengths upon binding of dyes *I* and *II* to the membrane, without major change in the quantum yield of fluorescence (Fig. 2*B*). The intensity of fluoresence of Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*) increases with membrane concentration, with little change in the emission or excitation maxima.

The absorbance and fluorescence changes of di-S-$C_2(5)$, di-I-$C_1(5)$ and Nile Blue A show a tendency for saturation above 0.1 mg protein per ml, while the changes observed with oxonol VI (*IV*) and WW 781 (*V*) are proportional to protein concentration up to 0.3 mg/ml (Fig. 2*B*, insert).

[2] WW 781 (1 µg/ml) in media containing 0.15 M K-glutamate 10 mM imidazole, 1 mM Mg-maleate, 50 µM Ca-maleate, and 100 µg microsomal protein per ml gives a stable absorption maximum at 600 nm. Upon addition of A23187 (1 µg/ml) a rapid decrease in absorbance occurs; $A_{1/2}$ is reached in about 5 min at 15 °C. ATP (100 µM) inhibited the absorbance change. The absorbance change is not observed if microsomes are omitted, if Ca^{2+} is removed with EGTA, if A23187 is replaced by ionomycin (a Ca ionophore), or if K-glutamate is replaced by sucrose, glycine or KCl. The absorbance change is not light dependent. Its mechanism is unknown.

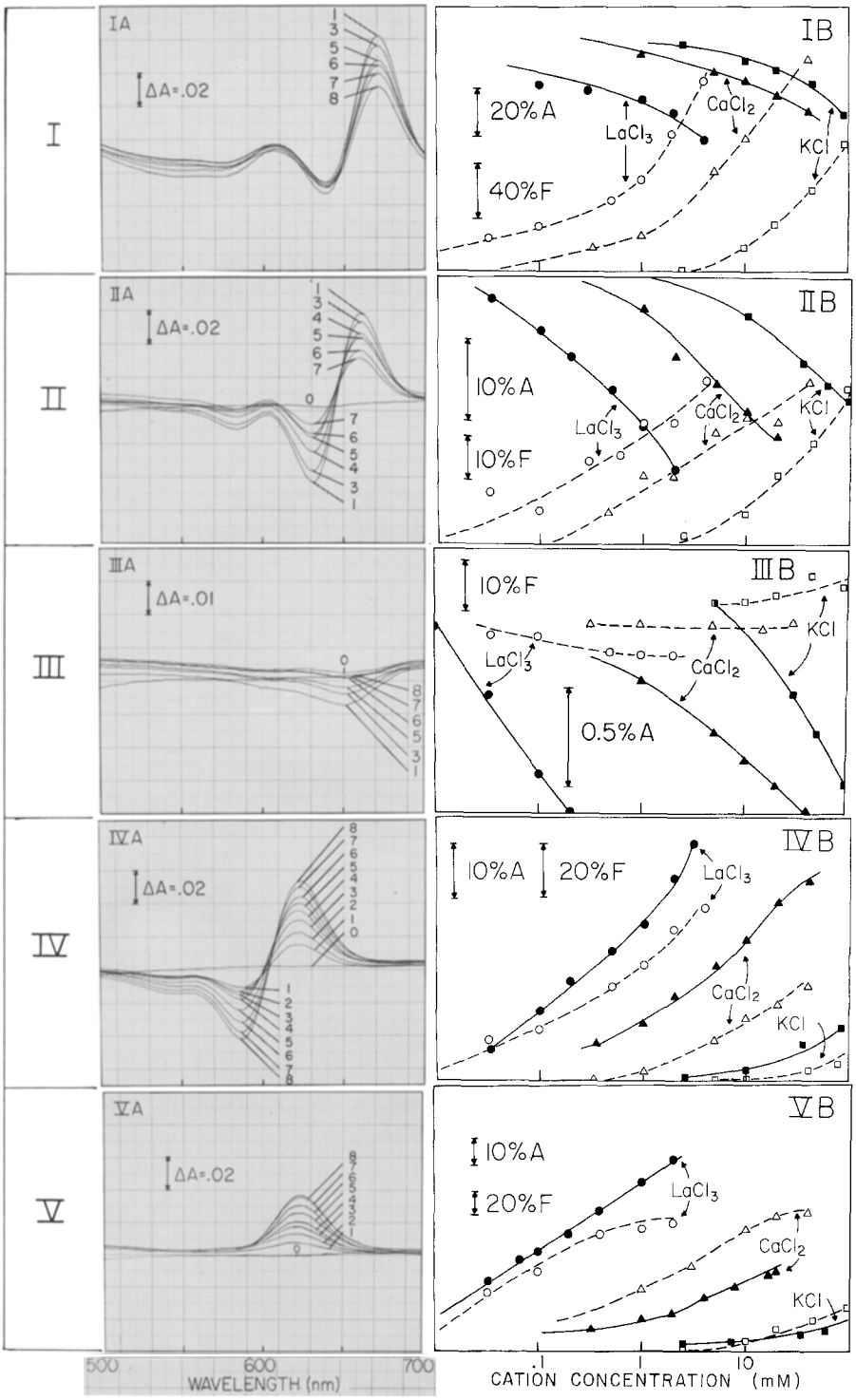


Fig. 3. Effect of cations on the absorbance and fluorescence of di-S-C_2(5) (*I*), di-I-C_1(5) (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*) in the presence of sarcoplasmic reticulum vesicles. (*A*) Effect of Ca^{3+} on the difference absorbance spectrum of sarcoplasmic reticulum vesicles. The baseline (*O*) was recored in the presence of 0.3 M sucrose, 10 mM imidazole (pH 6.6) and 1 μg/ml dye in both the sample and reference cells. Sarcoplasmic reticulum vesicles (100 μg protein/ml) were added to the sample cell (*1*). $CaCl_2$ was then added to both sample and reference cells to a final concentration of 0.33 (*2*), 1 (*3*), 2 (*4*), 5 (*5*), 10 (*6*), 20 (*7*), and 40 (*8*) mM. (*B*) Changes in the absorbance (●, ▲, ■) and fluorescence (○, △, □) of microsomes stained with the dyes upon the addition of $LaCl_3$ (●, ○), $CaCl_2$ (▲, △) and KCl (■, □). The medium contained 0.3 M sucrose, 10 mM imidazole (pH 6.6) 1 μg/ml dye and 100 μg protein/ml. For the absorbance measurements, sarcoplasmic reticulum vesicles were added to the sample cell; the salts were added to both sample and reference cells. No significant change in the dye fluorescence was observed upon the addition of salts in the absence of sarcoplasmic reticulum vesicles. *IB*: *λabs* 670–640 nm; *λex* 640 nm; *λem* 665 nm. *IIB*: *λabs* 660–640 nm; *λex* 635 nm; *λem* 655 nm. *IIIB*: *λabs* 690–650 nm; *λex* 640 nm; *λem* 690 nm. *IVB*: *λabs* 624–588 nm; *λex* 580 nm; *λem* 640 nm. *VB*: *λabs* 628–592 nm; *λex* 590 nm; *λem* 640 nm

Effect of K^+, Ca^{2+} and La^{3+} Upon the Absorbance and Fluorescence of Probes I–V

The difference absorption spectra, caused by the binding of various dyes to sarcoplasmic reticulum (Fig. 3*A*, line 1), are used as reference in evaluating the effect of cations upon the optical properties of the dyes.

With the positive dyes di-S-C_2(5) (*I*), di-I-C_1(5) (*II*) and Nile Blue A (*III*), addition of 0.3–4 mM Ca^{2+} decreases the difference absorbance measured in the presence of 0.1 mg sarcoplasmic reticulum protein per ml, without significant change in the wavelength of the absorption maxima and minima (Fig. 3*A*). With dyes *I* and *II* the decrease in difference absorbance with increasing Ca^{2+} concentration is accompanied by an increase in fluorescence intensity (Fig. 3*B*). Qualitatively similar changes were observed upon addition of K^+ or La^{3+}, to systems containing microsomes and dyes *I, II* or *III*.

As calcium reversed the absorbance change of di-S-C_2(5) and di-I-C_1(5) caused by the addition of sarcoplasmic reticulum vesicles (Fig. 3), the effects of Ca^{2+} upon the difference absorbance spectrum of the positively charged dyes probably reflects a decrease in dye binding to the membrane and an increase in free dye concentration. The relationship between K^+, Ca^{2+} and La^{3+} concentration and the dye binding reflected by the absorbance change may be explained by screening of surface charges and by a direct effect of cation binding upon the surface charge density.

The effect of K^+, Ca^{2+} and La^{3+} upon the absorbance and fluorescence of Nile Blue A in the presence of sarcoplasmic reticulum is relatively small.

The negative dyes (oxonol VI and WW 781) respond with an increase in difference absorbance and fluorescence upon addition of K^+, Ca^{2+} or La^{3+} (Fig. 3). In this case the change in difference absorbance caused by cations is in the same direction as that obtained upon binding of the dyes to sarcoplasmic reticulum (Fig. 2). This suggests that cations promote the binding of negative dyes to the membrane presumably by screening of negative surface charges and by increasing the positive surface charge density. The small absorbance changes in the 500–550 nm wavelength range with dyes *I, II, III*, and *V* may be due to light scattering and are of questionable significance.

Effect of Ca^{2+} Upon the Binding of Dyes I–V to Sarcoplasmic Reticulum

At dye/protein ratios similar those used in Fig. 3 approximately one-half of di-S-C_2(5) and WW 781, and close to 20% of di-I-C_1(5) Nile Blue A and oxonol VI are bound to sarcoplasmic reticulum vesicles (Table 2). Calcium (37–40 mM) decreased the binding of positive dyes (*I–III*) and increased the binding of negative dyes (*IV–V*) to sarcoplasmic reticulum, in agreement with the suggestion that surface charge density plays a significant role in the binding of the dyes to the membrane.

Table 2. Effect of Ca^{2+} on the binding of di-S-C_2(5) (*I*), di-I-C_1(5) (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*) to sarcoplasmic reticulum vesicles

Dye (λ, nm)	[Dye] (μg/ml)	Additions (mM)	% Dye bound		Δ % Bound due to Ca^{2+}	
			2–4 hr	1 day	2–4 hr	1 day
I (650)	0.5	84 glycine	59.7	54.5	−15.9	−21.5
		37 $CaCl_2$	43.8	33.0		
	5.0	80 glycine	66.6	62.3	− 8.6	−11.9
		40 $CaCl_2$	58.0	50.4		
II (636)	1.5	84 glycine	21.2	18.0	−10.5	− 9.2
		37 $CaCl_2$	10.7	8.8		
III (640)	0.5	84 glycine	18.4	8.1	− 5.4	− 4.1
		37 $CaCl_2$	13.0	4.2		
	5.0	80 glycine	20.2	13.1	−11.0	− 6.9
		40 $CaCl_2$	9.2	6.2		
IV (592)	0.5	84 glycine	17.0	11.8	+ 4.1	+ 8.3
		37 $CaCl_2$	21.1	20.1		
	5.0	80 glycine	10.5	9.9	+ 4.4	+ 2.6
		40 $CaCl_2$	14.9	12.5		
V (606)	0.5	84 glycine	45.5	43.4	+19.9	+24.4
		37 $CaCl_2$	65.4	67.8		
	2.6	80 glycine	20.2	19.6	+15.5	+21.1
		40 $CaCl_2$	35.7	40.7		

[a] The media contained 0.15 M K-glutamate, 10.0 mM imidazole (pH 6.6), 1.0 mM Mg-maleate, 50 μg/ml microsomal protein and dye, glycine, or $CaCl_2$ at the concentrations indicated in the Table. The percentage of dye bound to the vesicles after various incubation times was determined by centrifuge transport as described in Table 1, using the wavelengths indicated in the first column for absorbance measurements.

Effect of the Sarcoplasmic Reticulum Membrane Potential on Dye Absorbance and Fluorescence

The membrane potential of sarcoplasmic reticulum vesicles affects the distribution of permeant dyes between the intra- and extravesicular medium. The resulting increase or decrease in the internal dye concentration alters the amount of dye bound to the inner membrane surface. This influence of membrane potential on dye-membrane interaction is reflected in the difference spectra of di-I-C_1(5) and oxonol VI shown in Fig. 4 (*II* and *IV*).

An inside-positive membrane potential was generated by a 60-fold dilution of choline-Cl-equilibrated sarcoplasmic reticulum vesicles into K-glutamate medi-

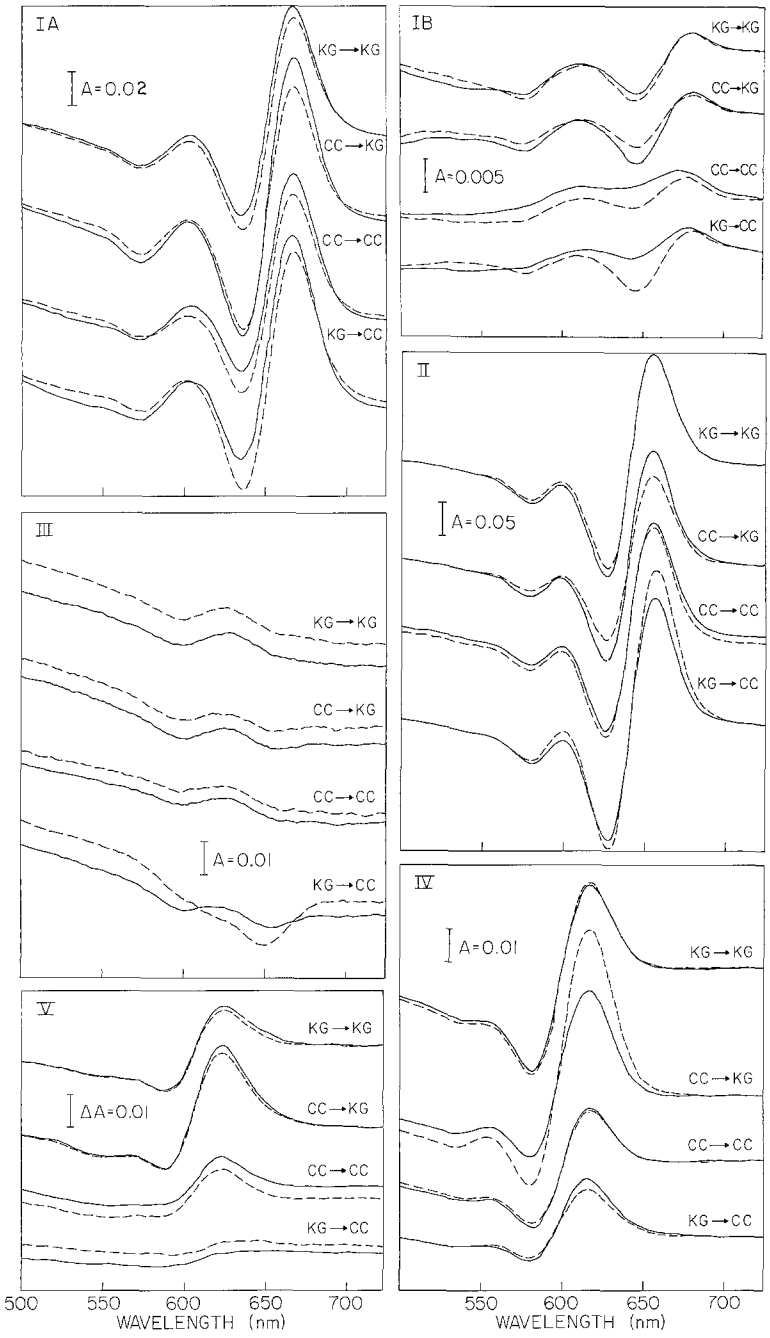


Fig. 4. Effect of membrane potential across the sarcoplasmic reticulum vesicles on the difference spectra of di-S-$C_2(5)$ (*IA, IB*) di-I-$C_1(5)$ (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*). Microsomes (6 mg protein per ml in *IA-V* and 60 μg/ml in *IB*) equilibrated in solutions containing 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, and either 0.15 M choline-Cl (CC) or 0.15 M K-glutamate (KG) were diluted 60-fold into a medium containing 0.15 M choline-Cl or 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate and 1 μg/ml dye. The absorbance spectra were recorded immediately (20 nm/sec) (---) or approximately 5 min later (——) after the spectrum had stabilized. Temp. 15 °C. Under these conditions the preimposed potential is dissipated in about 5 min. In *IB* the conditions were similar to those in *IA* except that the final microsomal protein concentration was 1 μg/ml and the concentration of di-S-$C_2(5)$ was 5 μg/ml

um. Membrane potential of the opposite polarity was formed by dilution of K-glutamate vesicles into choline-Cl medium. The amplitude of the difference spectrum of di-I-$C_1(5)$ produced by sarcoplasmic reticulum vesicles with positive membrane potential, as indicated by the difference between solid and dashed lines in Fig. 4*II* CC → KG, is smaller than that obtained at zero membrane potential (Fig. 4*II* KG → KG or CC → CC), suggesting exclusion of the dye from the vesicles. Inside-negative membrane potential (Fig. 4*II* KG → CC) increased the difference spectrum of di-I-$C_1(5)$ compared with vesicles of zero potential as would be expected if the dye is accumulated within the vesicles.

In contrast, inside-positive membrane potential (Fig. 4*IV* CC → KG) caused a large increase in the difference spectrum of oxonol VI, a negative dye, while a small decrease in difference absorbance accompanied inside-negative potential (Fig. 4*IV* KG → CC).

The response of di-S-$C_2(5)$ to changes in membrane potential is similar to di-I-$C_1(5)$ (Fig. 4, *IA*

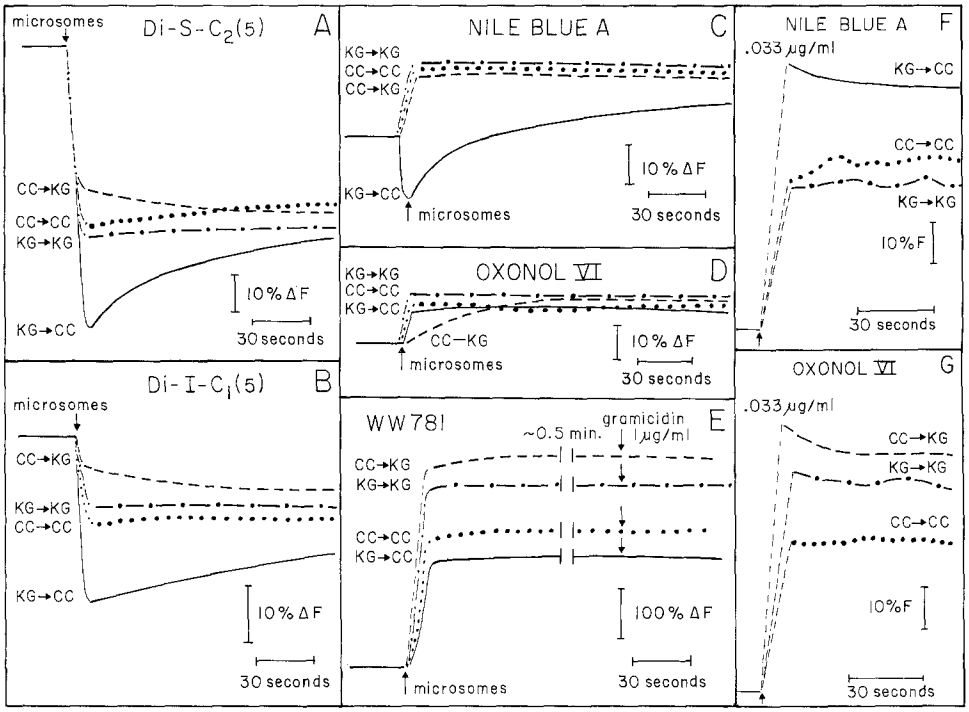


Fig. 5. Effect of membrane potential on the fluorescence intensity of di-S-C_2(5) (*A*), di-I-C_1(5) (*B*), Nile Blue A (*C, F*), oxonol VI (*D, G*) and WW 781 (*E*). (*A–E*) Sarcoplasmic reticulum vesicles (32 mg protein/ml), equilibrated in 10 mM imidazole (pH 6.6) 1 mM Mg-maleate and either 0.15 M choline-Cl (CC) or 0.15 M K-glutamate (KG), were diluted 750-fold into a medium containing 0.15 M choline-Cl or 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, and 1 μg/ml dye. Final protein concentration: 42 μg/ml. The fluorescence intensity before the microsome addition is defined as 100%. Dilution of choline-Cl equilibrated microsomes into K-glutamate medium (CC- - - - - - - →KG) generated an inside-positive membrane potential, while dilution of K-glutamate equilibrated vesicles into choline-Cl (KG——→CC), forms a negative membrane potential. Dilutions from CC→CC (· · ·) or from KG→KG (–·–·–) serve as controls. Temp=15 °C. In the experiments *F* and *G* microsomes (46 mg protein/ml) were diluted 1500-fold into media of identical composition as described above, except that the dye concentration was 0.033 μg/ml. Final protein concentration was 30 μg/ml. Excitation wavelengths: 640 nm (*A, B, C, F*), 590 nm (*D, G*), and 600 nm (*E*). Emission wavelengths: 665 nm (*A*), 660 nm (*B*), 670 nm (*C, F*) and 630 nm (*D, E, G*)

and *IB*). The magnitude of the response is dependent on the dye-to-protein ratio. At 100 μg/ml protein and 1 μg/ml di-S-C_2(5) (Fig. 4 *IA*) the percentage absorbance change caused by inside-negative potential is relatively minor, compared with the changes observed with 1 μg/ml protein and 5 μg/ml di-S-C_2(5) (Fig. 4 *IB*).

It is likely that at the lower dye-to-protein ratio (Fig. 4 *IA*), a greater portion of the dye is bound to the membrane already at zero potential, decreasing the amount of free dye which can be accumulated by the vesicles with inside-negative membrane potential. At high di-S-C_2(5): protein ratio a weak absorption band appears around 550 nm under the influence of negative potential, which may indicate the formation of dye-dimers.

Nile Blue A responds to negative membrane potential with a decrease in absorbance at 640 nm. It is not known whether this absorbance change is due to accumulation of Nile Blue A by sarcoplasmic reticulum vesicles or to reorientation of the dye within the membrane (Fig. 4, *III*). No Nile Blue A absorbance change was observed with positive membrane potential (Fig. 4, *III*).

Under our experimental conditions, WW 781 did not respond to either inside-positive or -negative membrane potential (Fig. 4, *V*). Major absorbance changes are not expected since WW 781 is a nonpermeable dye and redistribution of the dye across the membrane would not occur.

With the exception of di-S-C_2(5) there is no clear indication of the formation of dye-dimers and larger dye-aggregates with any of the dyes tested under the conditions of these experiments.

The fluorescence of di-S-C_2(5) and di-I-C_1(5) in the presence of sarcoplasmic reticulum vesicles is also dependent on the membrane potential (Fig. 5, *A* and *B*). An increase in fluorescence is observed upon generation of inside-positive membrane potential, compared to vesicles with zero potential. Inside-negative potential decreased the fluorescence intensity. These results are consistent with the exclusion of the dye

from the vesicles by positive potential, and dye accumulation in vesicles with negative potential.

The fluorescence response of Nile Blue A and oxonol VI to membrane potential was dependent upon dye concentration. At low concentrations of Nile Blue A (0.033 μg/ml) negative membrane potential (KG→CC) increased the fluorescence intensity, compared with vesicles of zero potential (Fig. 5*F*). At higher Nile Blue A concentrations (≃0.31–1 μg/ml) negative potential caused a decrease in fluorescence intensity (Fig. 5*C*). Both responses can be explained by accumulation of Nile Blue A in the vesicles under the influence of negative potential with subsequent binding of the dye to the membrane. At low dye concentrations binding of the dye to the membrane increases the fluorescence intensity as shown also in Fig. 2. At high dye concentration the amount of bound dye on the membrane reaches levels where self-quenching decreases the fluorescence intensity.

Similarly, the increase in the fluorescence of oxonol VI with inside-positive membrane potential (CC→KG) at low dye concentrations results from binding of the dye to the membrane (Fig. 5*G*), while the decrease in fluorescence under similar conditions at high oxonol VI-to-protein ratios is due to self-quenching (Fig. 5*D*).

As experiments in living muscle fibers are generally performed at high protein:Nile Blue A ratios, positive fluorescence signals are expected to reflect negative potential (Bezanilla & Horowicz, 1975).

There was no significant effect of positive potential on the fluorescence of Nile Blue A, and of negative potential on the fluorescence of oxonol VI at protein/dye ratios of 42.

Binding of WW 781 to sarcoplasmic reticulum vesicles led to a large increase in fluorescence (Figs. 2 and 5*E*). There was no measurable change in the fluorescence caused by negative or positive membrane potentials, but small changes would have been hidden by the much larger fluorescence change taking place upon dye binding to the membrane. The high steady fluorescence level in the K-glutamate as compared with the choline-Cl dilution medium may be related to the large difference absorbance at 625 nm in K-glutamate as compared with choline-Cl media (Fig. 4*V*). The absorbance difference suggests that the amount of WW 781 bound to the membrane is promoted by K^+ in accordance with the data in Table 1.

Effect of Probes I–V Upon the ATPase Activity and Ca^{2+} Transport

Meaningful analysis of potential changes related to Ca^{2+} fluxes requires that at relevant concentrations (1–10 μg/ml) the optical probes would not influence significantly the ATPase activity and Ca^{2+} transport. As shown in Table 3 the rate of Ca^{2+}-dependent ATP hydrolysis and ATP-dependent Ca^{2+} uptake in the presence of 5 mM oxalate was not influenced by any of the five dyes at a concentration of 1 μg/ml. At 10 μg/ml dye concentration moderate inhibition of Ca^{2+} transport was obtained with di-S-C_2(5), oxonol VI and WW 781. The rate of Ca^{2+} transport in the absence of oxalate was slightly inhibited by di-S-C_2(5) and di-I-C_1(5) and activated by Nile Blue A and WW 781 but these effects are not dependent on dye concentration between 1 and 10 μg/ml (Fig. 6).

Table 3. Effect of di-S-C_2(5), di-I-C_1(5), Nile Blue A, oxonol VI and WW 781 on the rate of calcium uptake and the calcium-dependent ATPase activity of sarcoplasmic reticulum vesicles[a]

Additions	Rate of Ca^{2+} uptake μmole Ca/mg protein/min)	Ca^{2+}-dependent ATP hydrolysis (μmole/Pi/mg/min)
Control (no dye)	1.22	0.95
Di-S-C_2(5)		
1 μg/ml	1.22	0.85
10 μg/ml	0.88	1.13
Di-I-C_1(5)		
1 μg/ml	1.18	0.95
10 μg/ml	–	1.13
Nile Blue A		
1 μg/ml	1.22	1.09
10 μg/ml	1.22	0.93
Oxonol VI		
1 μg/ml	1.22	0.88
10 μg/ml	0.88	0.70
WW 781		
1 μg/ml	1.44	0.70
10 μg/ml	0.94	0.81

[a] The rate of oxalate-supported calcium uptake was measured in a medium of 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 5 mM Mg-maleate, 5 mM K-oxalate, 1 or 10 μg/ml dye, 100 μM arsenazo III, 1 mM ATP and 100 μg protein/ml sarcoplasmic reticulum vesicles. The absorbance of the calcium-arsenazo III complex was monitored in the dual-wavelength mode at 660 nm using 685 nm as a reference wavelength. ATPase activity was measured in a medium containing 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 5 mM Mg-maleate, 0.5 mM EGTA, with or without 0.45 mM Ca^{2+}-maleate. The reaction was started by the addition of 0.45 mM ATP and stopped 4 min later with 2% TCA. Inorganic phosphate was measured by the method of Fiske and Subbarow (1925). The difference between activities measured in the presence and absence of Ca^{2+} is denoted as Ca^{2+}-dependent ATPase activity. Temperature: 25 °C

Effect of ATP-Induced Ca^{2+} Uptake Upon the Difference Spectra of the Various Dyes

ATP-induced accumulation of Ca^{2+} decreases the difference absorbance of di-S-C_2(5) and di-I-C_1(5) caused

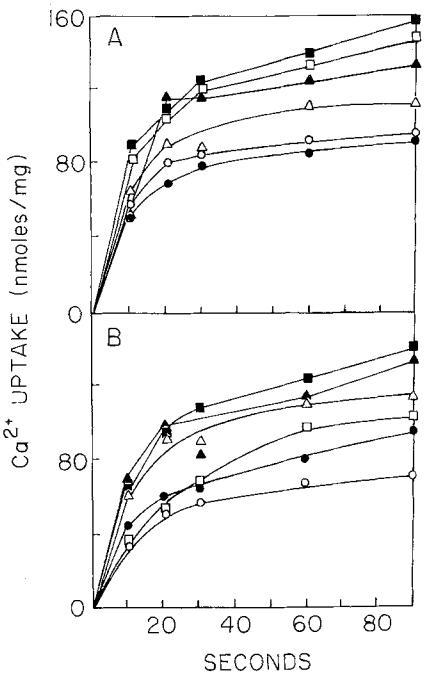


Fig. 6. Effect of di-S-$C_2(5)$, di-I-$C_1(5)$, Nile Blue A, oxonol VI and WW 781 on calcium uptake by sarcoplasmic reticulum vesicles. Calcium uptake was measured in a medium containing 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 80 μM ^{45}Ca-maleate (10 μCi/ml), and 100 μg microsomal protein/ml. Di-S-$C_2(5)$ (●), di-I-$C_1(5)$ (○), Nile Blue A (■), oxonol VI (□) or WW 781 (▲) were added to the uptake medium at final concentrations of 1 μg/ml (*A*) or 10 μg/ml (*B*) 1 min before calcium uptake was initiated. with 1 mM ATP. A control without dye was also included (△). Aliquots (50 μl) were removed at 1, 20, 30, 60 and 90 sec after the addition of ATP and diluted into 3 ml of 0.15 M K-glutamate, 10 mM imidazole, 1 mM Mg-maleate, 1 mM ATP and 5.0 mM EGTA. The samples were rapidly passed through Millipore filters. The amount of calcium uptake was calculated from the radioactivity retained in the filter. Temp. = 15 °C

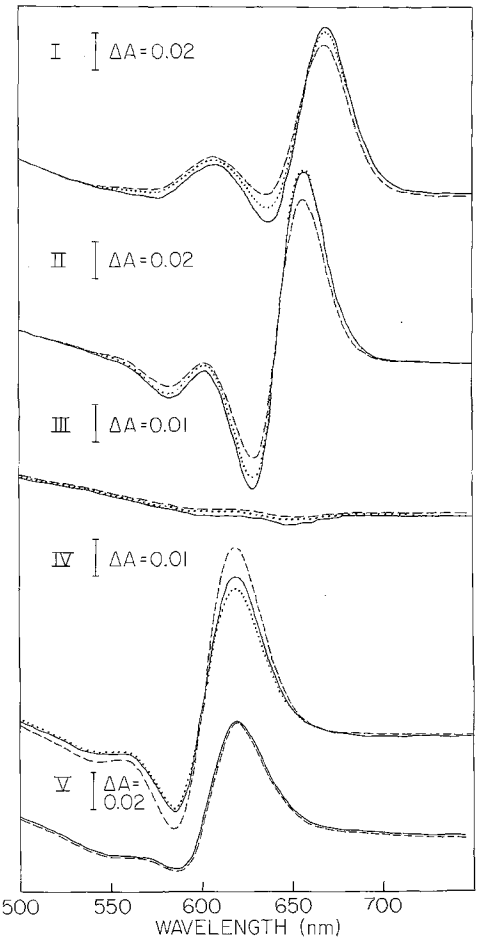
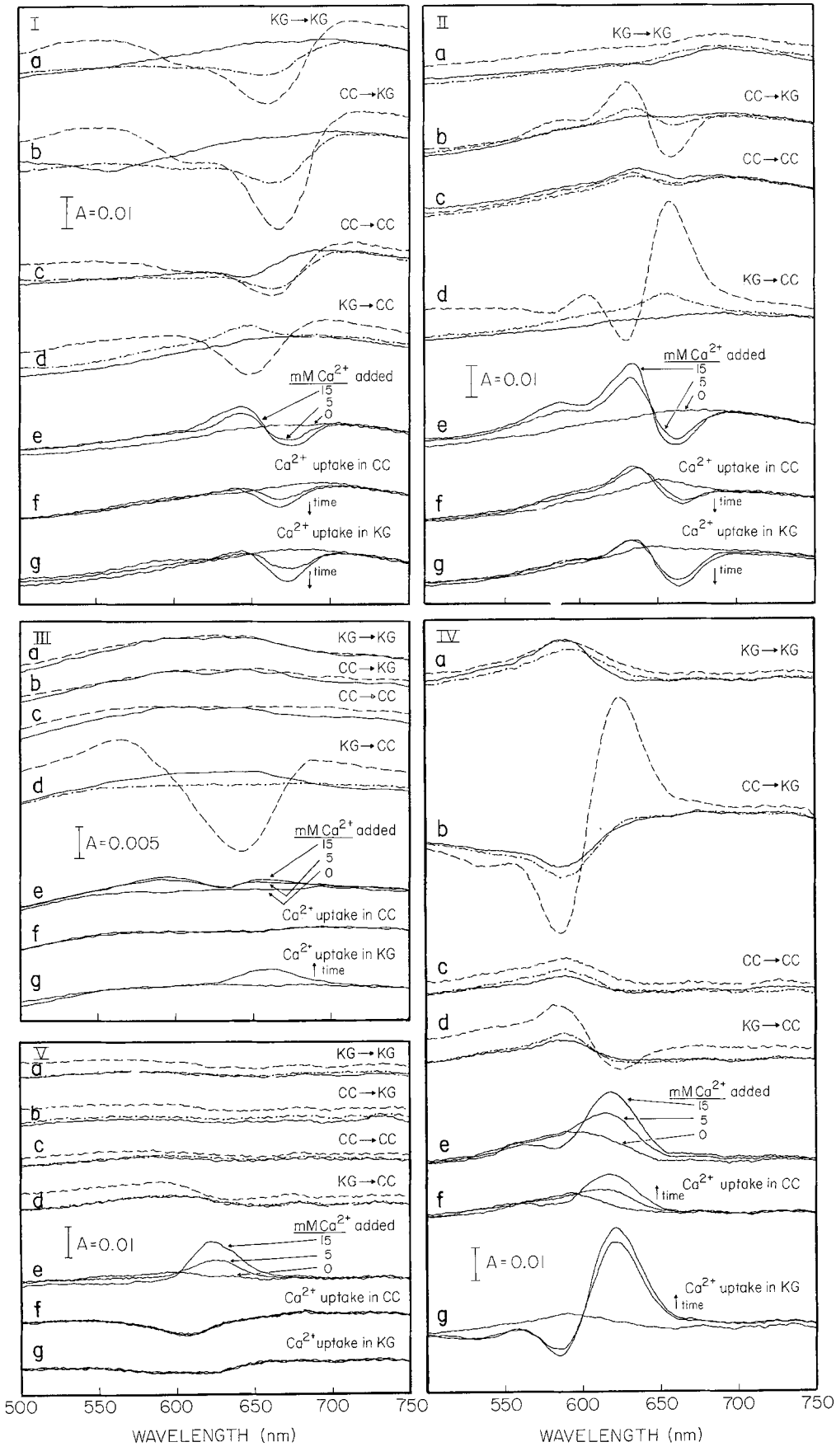


Fig. 7. Effect of ATP-induced Ca^{2+} uptake by sarcoplasmic reticulum vesicles on the difference spectra of di-S-$C_2(5)$ (*I*), di-I-$C_1(5)$ (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*) caused by microsomes. The difference absorbance spectra were measured at 15 °C in a medium containing 0.15 M K-glutamate, 10 mM imidazole, 1 mM Mg-maleate, 50 μM Ca^{2+}-maleate and 1 μg/ml dye in both the sample and reference cells. Sarcoplasmic reticulum vesicles were added to the sample cell to a protein concentration of 100 μg/ml (——). Ca^{2+} uptake was initiated by the addition of 0.1 mM ATP to both sample and reference cells and the difference spectrum was recorded 45 sec later (– – – –). The calcium ionophore A23187 (1 μg/ml) was added to the sample cell and the spectra were recorded 45 sec later (· · · ·)

by dye binding to sarcoplasmic reticulum (Fig. 7, *I* and *II*). The absorbance change produced by Ca^{2+} accumulation persists as long as Ca^{2+} is retained by the microsomes. Release of Ca^{2+} from the vesicles with A23187 (1 μg/ml) restores the absorbance at the absorption maximum close to the starting level (Fig. 7, *I* and *II*).

With oxonol VI as indicator, ATP-induced Ca^{2+} uptake increases the difference absorbance; this change is also completely reversed by A23187 (Fig. 7, *IV*). With positive (*I*, *II*) as well as negative dyes (*IV*) the absorbance change caused by active Ca^{2+} uptake is similar in sign and magnitude to that obtained upon addition of 20–30 mM Ca^{2+} to sarcoplasmic reticulum vesicles (Fig. 3). Since in actively loaded vesicles the estimated intravesicular Ca^{2+} concentration is of the order of 20–40 mM, these spectral changes are consistent either with an effect of increased intravesicular Ca^{2+} concentration upon the dye response or with the generation of inside-positive membrane potential due to electrogenic Ca^{2+} transport.

Only slight changes were observed in the absorbance of Nile Blue A and WW 781 during ATP-induced Ca^{2+} transport (Fig. 7, *III* and *V*). Externally added WW 781 was also insensitive to changes in intravesicular Ca^{2+} concentration produced by passive loading of the vesicles with Ca^{2+} and dilution into EGTA-containing media (not shown). Since WW 781 responded with absorbance change to externally added Ca^{2+} (Fig. 3), it appears that WW 781, an impermeable dye, is sensitive primarily to changes in surface potential on the surface of the microsomes where the dye is present.


I
II
III
IV
V
a
b
c
d
e
f
g
KG→KG
CC→KG
CC→CC
KG→CC
A=0.01
A=0.005
mM Ca2+ added
15
5
0
Ca2+ uptake in CC
Ca2+ uptake in KG
time
500
550
600
650
700
750
WAVELENGTH (nm)

Comparison of the Absorbance Changes of Dyes I–V Caused by Transmembrane Potential, Externally Added Ca^{2+}, and ATP-Dependent Active Ca^{2+} Transport

The influence of membrane diffusion potential upon the absorbance of the probes was studied by dilution of vesicles pre-equilibrated in 0.15 M choline-chloride (CC), or 0.15 M K-glutamate (KG) media into isoosmotic K-glutamate or choline-chloride solutions. Transfer of vesicles pre-equilibrated in choline chloride (CC) into K-glutamate (KG) generates inside-positive potential, while transfer from K-glutamate into choline-chloride generates an inside-negative potential. Transfer of vesicles into solutions of identical composition is not expected to change the membrane potential and serves as control.

The positively charged dyes di-S-C_2(5) and di-I-C_1(5) respond with characteristic changes in absorbance to inside-positive (Fig. 8 *Ib* and *IIb*) and inside-negative (Fig. 8 *Id* and *IId*) membrane potential. In the case of di-I-C_1(5) the absorbance change caused by inside-positive potential of about 100 mV (Fig. 8, *IIb*) is similar in magnitude and shape to the effect of 5–15 mM Ca^{2+} added to the outside medium (Fig. 8, *IIe*) or to the changes induced by ATP-dependent Ca^{2+} transport in choline-chloride (Fig. 8, *IIf*) or K-glutamate media (Fig. 8, *IIg*). The optical response to inside-positive potential or Ca^{2+} is best explained by a decrease in the concentration of bound dye within the vesicles (Table 2). The optical response of di-I-C_1(5) to inside-negative potential (Fig. 8, *IId*) is the mirror-image of that obtained with inside-positive potential (Fig. 8, *IIb*) or Ca^{2+} (Fig. 8, *IIe, f, g*), and probably reflects the accumulation of the dye within the vesicles, with an increase in the amount of the dye bound to the membrane.

With di-S-C_2(5) the wavelength dependence of the response to inside-positive potential (Fig. 8, *Ib*) is again similar to the changes caused by externally added Ca^{2+} (Fig. 8, *Ie*) or by ATP-mediated Ca^{2+} transport (Fig. 8, *If, g*) suggesting a common mechanism, i.e., the exclusion of the dye from the vesicles with a decrease in the concentration of bound dye. An absorption minimum characterizes the response of di-S-C_2(5) to inside-negative potential (Fig. 8, *Id*). At the relatively low dye concentration used in these experiments (1 μg/ml), much of the dye is bound to the membrane even in the absence of potential (Table 2); the optical change presumably reflects reorientation of already bound dye under the influence of membrane potential.

Nile Blue A shows a unique sensitivity to inside-negative membrane potential (Fig. 8, *IIId*). Inside-positive potential (Fig. 8, *IIIb*) or ATP-induced Ca^{2+} uptake (Fig. 8, *IIIf, g*) produced little or no change in the absorbance. Only a minor absorbance change was observed upon addition of 5–15 mM calcium (Fig. 8, *IIIe*). Therefore, under these experimental conditions Nile Blue A serves as a relatively selective probe for measurement of inside-negative membrane potential.

Oxonol VI, a permeable negative dye, gave a large change in absorbance to inside-positive potential (Fig. 8, *IVb*). The difference absorbance spectrum caused by externally added Ca^{2+} (Fig. 8, *IVe*) or ATP-induced Ca^{2+} uptake (Fig. 8, *IVf, g*) is similar to that observed with inside-positive potential (Fig. 8, *IVb*). In the light of the data in Fig. 2 *IV* and in Tables 1 and 2 the shape of the difference spectra suggest that inside-positive potential (Fig. 8, *IVb*) or the presence of Ca^{2+} within (Fig. 8, *IVf, g*) or outside the vesicles (Fig. 8, *IVe*) promotes the accumulation and binding of oxonol VI in the vesicles. Inside-negative membrane potential gave a smaller signal of opposite sign (Fig. 8, *IVd*) which is consistent with an increase in free dye concentration.

WW 781 is a strongly negative dye with limited permeability to biological membranes. It responds with an increase in absorbance at 625 nm to 5–15 mM Ca^{2+} added to the external medium (Fig. 8, *Ve*). Little or no absorbance change occurred during ATP-

Fig. 8. (facing page) Comparison of the effect of membrane potential, Ca^{2+}, and ATP-mediated Ca^{2+} uptake on the absorbance spectra of di-S-C_2(5) (*I*), di-I-C_1(5) (*II*), Nile Blue A (*III*), oxonol VI (*IV*) and WW 781 (*V*). (*a–d*) Difference spectra due to membrane potential. The baseline (——) was recorded using a medium containing 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 1 μg/ml dye and either 0.15 M K-glutamate (*a* and *b*, KG) or 0.15 M choline-Cl (*c* and *d*, CC) in both the sample and reference cells. Sarcoplasmic reticulum vesicles (100 μg protein/ml) were then added to the reference cell and allowed to equilibrate for 10 min. Sarcoplasmic reticulum vesicles (6.0 mg/ml protein) equilibrated in 10 mM imidazole (pH 6.6), 1 mM Mg-maleate and either 0.15 M K-glutamate (*a* and *d*, KG) or 0.15 M choline Cl (*b* and *c*, CC) were diluted 60-fold into the sample cell medium. The spectra were recorded at a speed of 20 nm/sec immediately following microsome addition (– – –) and 5 min later (–·–·–), after the signal had stabilized. Kinetic studies indicate that within 5 min preimposed transmembrane potential is nearly completely dissipated. (*e*) Difference spectra due to Ca^{2+}. The sample and reference cells contained 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 100 μg/ml microsomal protein and 1 μg/ml dye. $CaCl_2$ was added to the sample cell to a final concentration indicated in the Figure. (*f–g*) Difference spectra due to ATP-mediated calcium transport. The sample and reference cell contained 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 0.5 mM EGTA, 0.45 mM Ca-maleate, 1 μg/ml dye and 100 μg/ml microsomal protein and either 0.15 M choline-Cl (*f*, CC) or 0.15 M K-glutamate (*g*, KG). ATP (0.15 mM) was added to the sample cell and the difference spectrum was recorded at a speed of 20 nm/sec immediately after ATP addition and 2 min later

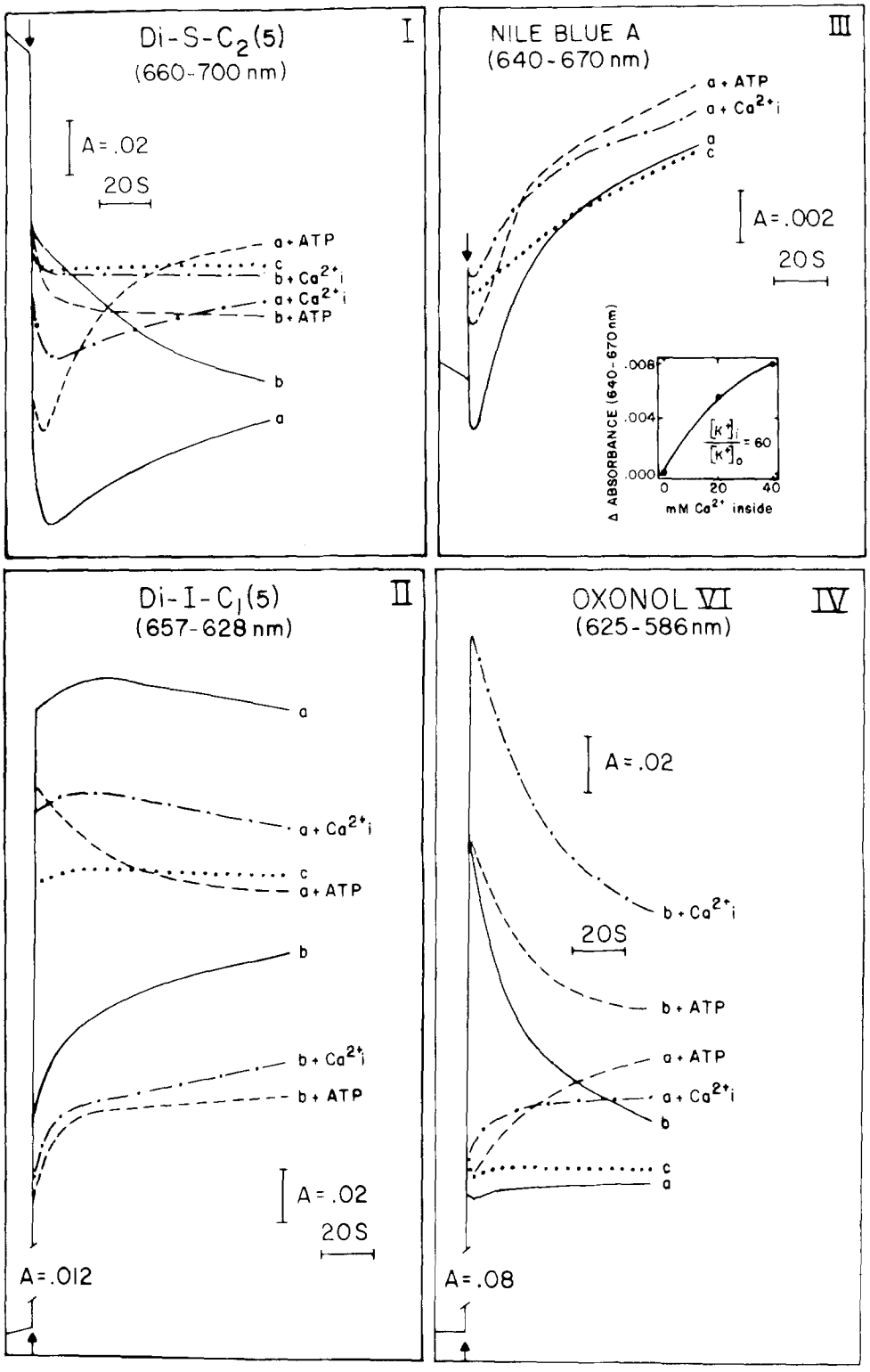


Fig. 9. Effect of membrane potential across sarcoplasmic reticulum vesicles on the absorbance of di-S-$C_2(5)$ (*I*), di-I-$C_1(5)$ (*II*), Nile Blue A (*III*), and oxonol VI (*IV*). Microsomes (6.0 mg protein/ml) equilibrated in media containing 10 mM imidazole (pH 6.6) 1 mM Mg-maleate and either 0.15 M K-glutamate and 40 mM glycine (*a*), 0.15 MK-glutamate and 20 mM Ca-maleate ($a+Ca^{2+}i$), 0.15 M choline-Cl and 40 mM glycine (*b*), 0.15 M choline-Cl and 20 mM Ca-maleate ($b+Ca^{2+}i$), or 0.34 M glycine (*c*) were diluted 60-fold (↑) into a medium containing 0.34 M glycine, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 1 μg/ml dye and 0.5 mM EGTA if Ca^{2+} -loaded microsomes were added. In some experiments 0.15 mM ATP and 0.1 mM Ca-maleate were also present in the dilution medium ($a+$ATP, $b+$ATP). The absorbance was measured in the dual wavelength mode at the indicated wavelength pairs. Temp.: 15 °C. *Insert:* Microsomes (6.0 mg/ml) equilibrated in 0.15 M K-glutamate, 10 mM imidazole (pH 6.6) and various concentrations of Ca-maleate as indicated on the abscissa and glycine to maintain a constant ionic strength, were diluted 60-fold into an isotonic medium containing 0.38 M glycine, 10 mM imidazole (pH 6.6) and 1.5 mM EGTA. The ΔA indicates the difference absorbance of Ca^{2+}-containing minus Ca^{2+}-free samples measured immediately after dilution, with extrapolation to zero time

induced accumulation of Ca^{2+} within the vesicles (Fig. 8, *Vf, g*) or with either positive or negative membrane diffusion potential of close to 100 mV (Fig. 8, *Vb, d*). We presume that the binding of external WW 781 to the membrane is promoted by the presence of 5–15 mM Ca^{2+} in the bathing medium (Table 2) through its effect upon the surface potential at the outer membrane surface. Intravesicular Ca^{2+} at similar concentrations has no effect presumably because WW 781 did not penetrate into the vesicles. Therefore, under these conditions WW781 appears to be a selective probe for the surface potential of microsomes. Incorporation of the probe within the vesicles may permit measurement of surface potential changes on the inside surface.

Effect of Intravesicular Ca^{2+} Concentration and ATP-Induced Ca^{2+} Transport Upon the Response of Dyes I–IV to Artificially Imposed Membrane Potential

The data in Figs. 3, 4 and 8 suggest that potentiometric probes respond to changes both in surface potential and in diffusion potential. As the optical response of the probes during ATP-induced Ca^{2+} transport is likely to include contributions from changes in surface potential caused by the binding of accumulated Ca^{2+} to the membrane, and from changes in transmembrane potential, if the Ca^{2+} transport is electrogenic, the assessment of the relative magnitude of these contributions is of importance. The experiments given in Fig. 9 were designed to this end.

Inside-negative or inside-positive potentials of close to 100 mV were generated by dilution of microsomes equilibrated in 0.15 M K-glutamate or 0.15 M choline-Cl into isotonic media containing 0.34 M glycine. Microsomes equilibrated with glycine and diluted into glycine medium served as control (Fig. 9*c*). The positive dyes (di-S-C_2(5) (*I*) and Nile Blue A (*III*) respond to inside-negative potential with a decrease in absorbance which returns slowly to control levels as the potential is dissipated (Fig. 9, *Ia, IIIa*). The absorbance response to inside-negative potential is rapidly abolished when ATP-induced Ca^{2+} transport is initiated at the time of dilution (Fig. 9, *I* and *IIIa*+ATP). The final absorbance level after the ATP-dependent Ca^{2+} accumulation is completed (60–90 sec) is similar to that obtained with vesicles passively loaded with 20 mM intravesicular Ca^{2+} (Fig. 9, *Ia*+Ca_i *IIIa*+Ca_i). These observations indicate that intravesicular Ca^{2+} decreases the response of Nile Blue A and di-S-C_2(5) to negative potential. The dependence of this effect on intravesicular Ca^{2+} concentration is given in Fig. 9 *III* insert for Nile Blue A, at 100 mV negative potential. At zero potential Ca^{2+} had no effect on the absorbance of Nile Blue A (Fig. 8, *IIIe*).

Only slight absorbance change was observed with inside-positive as compared with zero potential using di-S-C_2(5) (Fig. 9, *Ib*), while Nile Blue A gave no response (not shown), in accord with the data in Fig. 8 (*IIIb*).

di-I-C_1(5) responds to inside-negative potential with an increase in absorbance at 657–628 nm (Fig. 9, *IIa*), which is also diminished by 20 mM intravesicular Ca^{2+} (Fig. 9, *IIa*+Ca_i) or ATP-induced Ca^{2+} transport (Fig. 9, *IIa*+ATP). Inside-positive potential decreased the absorbance of di-I-C_1(5) compared with control (Fig. 9, *IIb*); the absorbance is further reduced in vesicles passively (Fig. 9, *IIb*+Ca_i) or actively loaded with Ca^{2+} (Fig. 9, *IIb*+ATP).

The negative oxonol VI responds to inside-positive potential with a large transient increase in absorbance that is markedly enhanced by Ca^{2+} (Fig. 9, *IVb*+Ca_i). ATP-induced Ca^{2+} transport decreased the rate of spontaneous decay with a time course that resembles the time course of Ca^{2+} accumulation (Fig. 9*IVb*+ATP). The weak response of oxonol VI to inside-negative potential (Fig. 9, *IVa*) is enhanced by passive (Fig. 9, *IVa*+Ca_i) or active loading (Fig. 9, *IVa*+ATP) of the vesicles with Ca^{2+}.

In summary, these observations indicate that high intravesicular Ca^{2+} achieved either by active transport or passive loading of the vesicles greatly diminished the absorbance change of di-S-C_2(5), di-I-C_1(5), and Nile Blue A caused by artificially imposed inside-negative potential. The absorbance change due to positive membrane potential was increased by intravesicular Ca^{2+} in the case of oxonol VI and decreased with di-I-C_1(5).

The time course of the decay of potential signal was not appreciably different for any of the dyes between Ca^{2+}-free and Ca^{2+}-loaded microsomes, suggesting that the effect of Ca^{2+} is not due to changes in the permeability of the membrane.

Relationship Between Membrane Potential and the Absorbance Change of di-I-C_1(5); Effect of Intravesicular Ca^{2+} on the Absorbance Change

Dilution of vesicles equilibrated in 0.15 M K-glutamate into isotonic media of lower K-glutamate concentration generates inside-negative membrane potential. The maximum potential at $[K_i^+]/[K_o^+]$ gradients of 60, 15, and 3.75 is −100, −66, and −33 mV, respectively, as calculated from the Nernst equation (Fig. 10, left of ordinate).

Inside-positive potential was generated by dilution of vesicles equilibrated in 0.15 M choline-Cl into isotonic media of varying choline-Cl concentration. The estimated maxiumum potential corresponding to $[Cl_i^-]/[Cl_o^-]$ ratios of 3.75, 15 and 60, respectively,

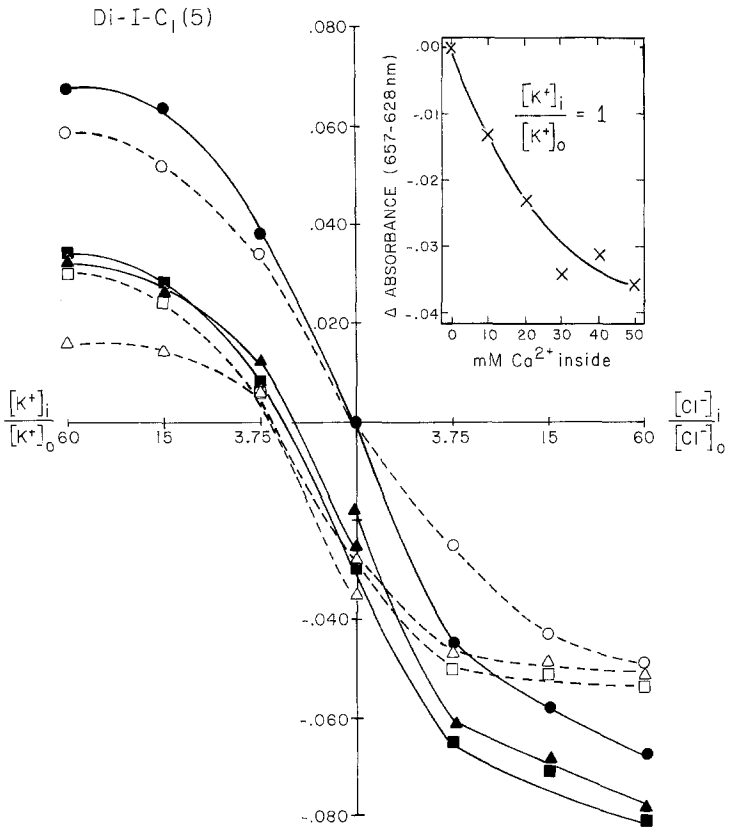


Fig. 10. Relationship between the membrane potential of sarcoplasmic reticulum vesicles and the absorbance of di-I-C_1(5). *Left of ordinate:* To generate inside-negative membrane potentials, sarcoplasmic reticulum vesicles (6 mg protein/ml) equilibrated in 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 40 mM glycine, 1 mM Mg-maleate were diluted 60-fold into isotonic media containing K-glutamate and glycine at varying ratios, 10 mM imidazole, 1 mM Mg-maleate and 1 μg/ml di-I-C_1(5) (○, ●). *Right of ordinate:* To generate inside-positive membrane potentials, sarcoplasmic reticulum vesicles, (6 mg protein/ml) equilibrated in 0.15 M choline-Cl, 40 mM glycine, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate were diluted 60-fold into isotonic media containing choline-Cl and glycine at varying ratios, 10 mM imidazole, 1 mM Mg-maleate and 1 μg/ml di-I-C_1(5) (○, ●). The absorbance change was measured in the dual wavelength mode using the wavelength pair 657–628 nm. The data are presented as the difference in the absorbance change in the presence of a K^+ or Cl^- gradient and that obtained by a control dilution in which the sarcoplasmic reticulum vesicles were equilibrated in a medium similar to the dilution medium. To test the effect of ATP-mediated Ca^{2+} uptake upon the optical response in a parallel experiment, 0.15 mM ATP and 100 μM Ca-maleate were added to the dilution medium before the addition of microsomes (△, ▲). The effect of intravesicular Ca^{2+} upon the optical response was tested by equilibration of sarcoplasmic reticulum vesicles with 20 mM Ca-maleate before dilution. In these experiments 0.33 mM Tris-EGTA was included in the dilution medium to keep the extravesicular [Ca^{2+}] below 10^{-7} M (□, ■). The absorbance change was obtained either by extrapolation to the time of dilution (●, ▲, ■) or 15 sec after the dilution (○, △, □). *Insert:* Relationship between the internal Ca^{2+} concentration of sarcoplasmic reticulum vesicles and the absorbance change of di-I-C_1(5), measured in the dual-wavelength mode using the wavelength pair 657–628 nm (extrapolated to the time of dilution). The experiment was carried out essentially as described above. Sarcoplasmic reticulum vesicles were equilibrated in a medium containing 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1.0 mM Mg-maleate and various concentrations of Ca-maleate, as indicated in the Figure, and glycine to maintain a constant ionic strength. The isotonic dilution medium contained 0.15 M K-glutamate, 0.10 M glycine, 10 mM imidazole (pH 6.6), 1.0 mM Mg-maleate, 1.5 mM EGTA and 1 μg/ml di-I-C_1 (5)

is 33, 66 and 100 mV inside-positive (Fig. 10, right of ordinate).

Under these conditions the voltage-response curve of 1 μg/ml di-I-C_1(5) measured immediately after dilution is nearly symmetrical. Inside-negative potential of 100 mV increases, while inside-positive potential of 100 mV decreases the absorbance by 0.067 absorbance units compared with vesicles of zero potential. The absorbance change slightly decreases 15 sec after dilution, as the potential is slowly dissipated.

The absorbance change levels off at inside-negative potentials of 66–100 mV due to decrease in the free dye concentration of the medium, and at inside-positive potentials of 66–100 mV due to decrease in intravesicular dye concentration. The absorbance increase with inside-negative potential is consistent with the accumulation of the positive dye within the vesicles, with increase in the concentration of the dye bound to the membrane. By analogy the decrease in absorbance with inside-positive potential reflects extrusion of the dye from the vesicles and a decrease in the concentration of bound dye.

Using vesicles pre-equilibrated with 20 mM Ca^{2+}, the voltage-response curve of di-I-C_1(5) shifts to the

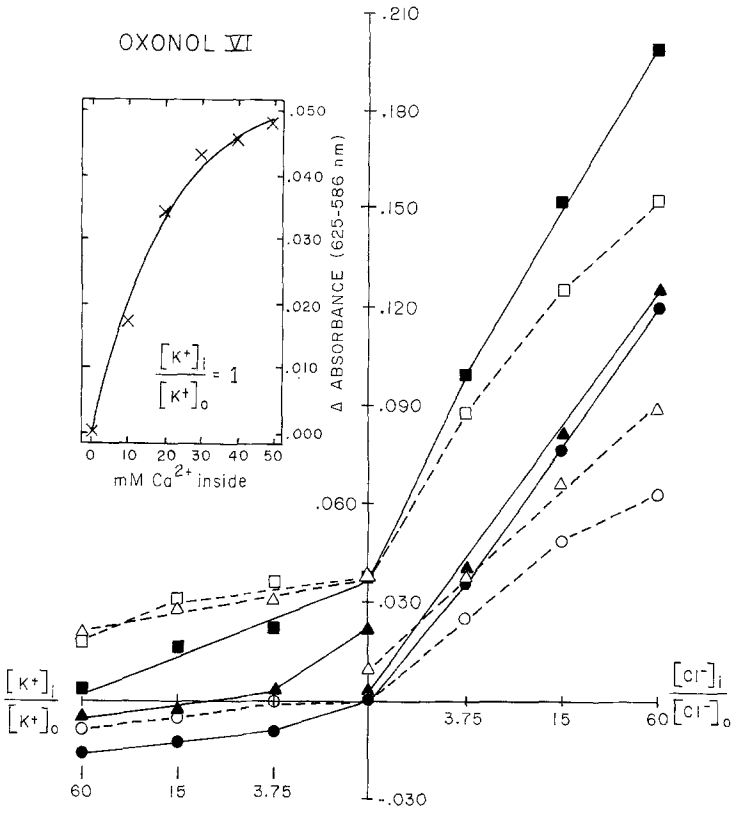


Fig. 11. Relationship between the membrane potential of sarcoplasmic reticulum vesicles and absorbance of oxonol VI. The conditions were identical to those described in the legend to Fig. 10 except that 1 μg/ml oxonol VI was present in the dilution medium instead of di-I-$C_1(5)$. The absorbance change was measured in the dual beam mode using the wavelength pair 625–586 nm. For other details *see* text and legend to Fig. 10

left compared with Ca^{2+}-free microsomes at the same membrane potential; the absorbance response to inside-negative potential decreases ($\Delta A = 0.034$ at -100 mV) and to inside-positive potential increases ($-\Delta A = 0.08$ at $+100$ mV). The relationship between the intravesicular Ca^{2+} concentration and the absorbance of di-I-$C_1(5)$ in the absence of K^+ or Cl^- gradients is given in Fig. 10 insert. An intravesicular Ca^{2+} concentration of 20 mM causes a decrease in absorbance by 0.025–0.03. Due to the presence of EGTA in the dilution medium the contribution of extravesicular Ca^{2+} to the absorbance change is negligible. We assume that Ca^{2+} exerts this effect by decreasing the negative surface potential on the interior surface of the membrane, thus reducing the amount of bound di-I-$C_2(5)$ in accordance with data in Table 2. The change in absorbance caused by 20 mM intravesicular Ca^{2+} corresponds to about +30–40 mV estimated potential difference based on the data in Fig. 10.

ATP-induced Ca^{2+} uptake by sarcoplasmic reticulum vesicles caused a similar shift in the voltage-response curve of di-I-$C_1(5)$, after extrapolation to the time of dilution, as does 20 mM intravesicular Ca^{2+}. A significant further depression of the absorbance response was observed both at inside-negative and at inside-positive potentials after a 15-sec incubation. During this time the vesicles accumulate 0.08–0.12 μmole Ca/mg protein (Fig. 6). Assuming an intravesicular volume of 3–5 μl/mg protein (Duggan & Martonosi, 1970) Ca^{2+} transport under these conditions would yield intravesicular Ca^{2+} concentrations of 20–30 mM. Therefore the change in the absorbance response of di-I-$C_1(5)$ during ATP-incuced Ca^{2+} transport is attributable largely to the accumulation of intravesicular Ca^{2+}.

Relationship Between Membrane Potential and the Absorbance Change of Oxonol VI

The voltage-response relationship of the negative oxonol VI dye was established by the approach described in Fig. 10. The voltage response curve is asymmetric, with sharp decrease of the magnitude of absorbance change at inside-negative potentials (Fig. 11, left of ordinate), presumably due to depletion of intravesicular dye.

The absorbance response to inside-positive potential, extrapolated to the time of dilution, follows the Nernst relationship between estimated potentials of 0 and 100 mV. Intravesicular Ca^{2+} (20 mM) increases the absorbance at 625–586 nm and the relationship between intravesicular [Ca^{2+}] and absorbance change at $[K_i]/[K_o] = 1$ indicates a tendency for saturation above 20 mM Ca^{2+} concentration (Fig. 11 insert). Ca^{2+} probably promotes the binding of negative oxonol VI to membrane by decreasing the negative surface potential of sarcoplasmic reticulum. The effect

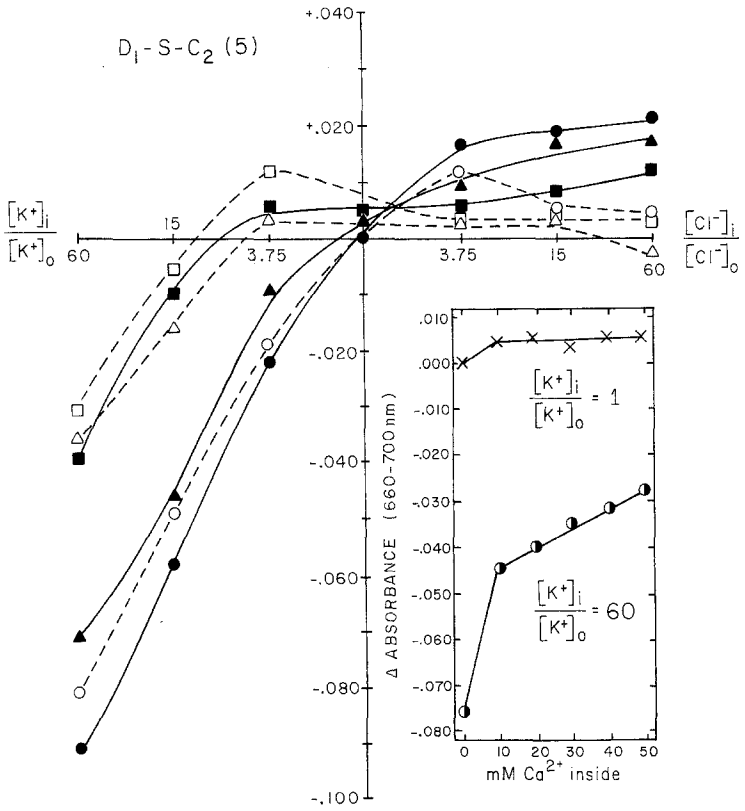


Fig. 12. Relationship between the membrane potential of sarcoplasmic reticulum vesicles and the absorbance of di-S-C_2(5). The experimental conditions were similar to those described in the legend to Fig. 10 except that 5 μg/ml di-S-C_2(5) was present in the dilution medium instead of di-I-C_1(5). *Left of ordinate:* To generate inside-negative membrane potentials, sarcoplasmic reticulum vesicles (3 mg protein/ml) equilibrated in 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate and 40 mM glycine were diluted 60-fold into an isotonic medium containing K-glutamate and glycine at varying ratios, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, and 5 μg/ml di-S-C_2(5). *Right of ordinate:* To generate inside-positive membrane potentials, sarcoplasmic reticulum vesicles (3 mg protein/ml) equilibrated in 0.15 M choline-Cl, 10 mM imidazole (pH 6.6), 1 mM Mg^{2+}-maleate and 40 mM glycine were diluted 60-fold into isotonic medium containing choline-Cl and glycine at varying ratios, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, and 5 μg/ml di-S-C_2(5). The absorbance change was measured in the dual beam mode using the wavelength pair 660–700 nm. *Insert:* The relationship between the internal Ca^{2+} concentrations of microsomes and the absorbance change of di-S-C_2(5) at zero potential (×–×) and at ≃100 mV negative potential (◑–◑). For other details *see* text and legend to Fig. 10

of ATP-induced Ca^{2+} uptake on the dye response is less pronounced than the effect of 20 mM intravesicular Ca^{2+}.

Relationship Between the Membrane Potential of Sarcoplasmic Reticulum Vesicles and the Absorbance of di-S-C_2(5)

The voltage-response curve of di-S-C_2(5) is markedly asymmetric. At inside-negative potentials of 33–100 mV the absorbance change at 660–700 nm follows the Nernst relationship, but at inside-positive potentials the sensitivity of the dye is sharply reduced (Fig. 12). In the absence of transmembrane potential ($[K_i]/[K_o]=1$) passive loading of vesicles with 10–50 mM Ca^{2+} had essentially no effect on the absorbance at 660–700 nm, while the absorbance change caused by negative potential ($[K_i]/[K_o]=60$) is markedly diminished by 10–50 mM intravesicular Ca^{2+}, compared with Ca^{2+}-free vesicles (Fig. 12 insert). The effect of ATP-induced Ca^{2+} uptake after 15 sec of incubation is similar to that observed with vesicles passively loaded with 20 mM Ca^{2+}. Due to the effect of intravesicular Ca^{2+} on the absorbance response of di-S-C_2(5), the voltage response curves of Ca^{2+}-free and Ca^{2+}-loaded microsomes cross over in the vicinity of zero potential.

The decreased sensitivity of di-S-C_2(5) to potential in the presence of ≃20 mM Ca^{2+} may largely explain the abolition of dye response to preimposed inside-negative potential during Ca^{2+} transport (Fig. 9).

In these experiments 50 μg/ml microsomal protein and 5 μg/ml di-S-C_2(5) were used. One may argue that under these conditions most of the dye is bound to microsomes and therefore dye redistribution under the influence of potential is limited. For this reason

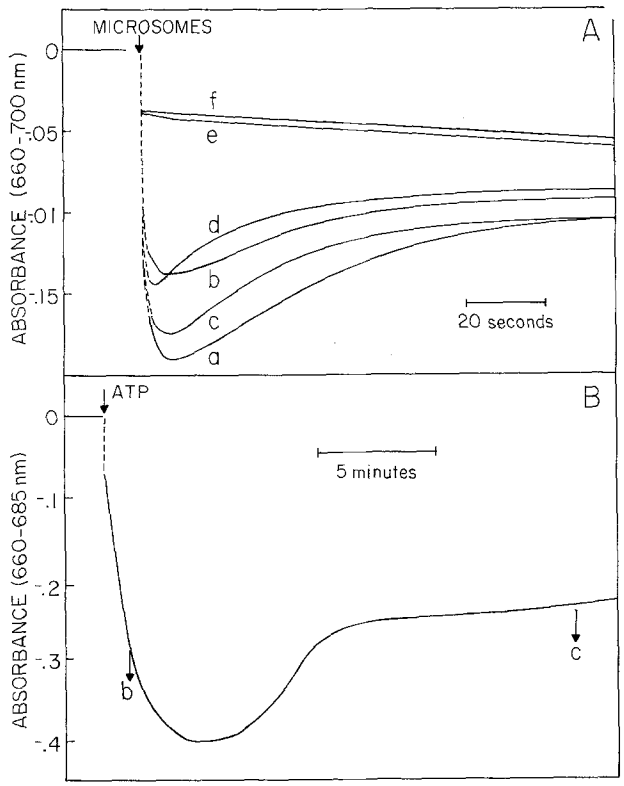


Fig. 13. Effect of Ca^{2+} -loading on the potential response of sarcoplasmic reticulum vesicles measured with di-S-$C_2(5)$. (*A*) –(*a*) Sarcoplasmic reticulum vesicles (1 mg protein per ml) equilibrated in 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate and 150 μM Ca-maleate were diluted 100-fold into 0.3 M glycine, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 2.5 mM EGTA, 1 μM valinomycin and 5 μg/ml di-S-$C_2(5)$. Temp. = 15 °C. (*b–c*) Same as "*a*" except ATP (1.0 mM) was added to the sarcoplasmic reticulum vesicles 1 (*b*), and 20 (*c*) min before the dilution. (*d*) Same as *a*, except that the dilution medium contained 100 μM Ca-maleate and 0.44 mM ATP but no EGTA. Temp. = 15 °C. (*e*) Same as *a*, except sarcoplasmic reticulum vesicles were incubated in 0.3 M glycine instead of 0.15 M K-glutamate. (*f*) Same as *d*, except sarcoplasmic reticulum vesicles were incubated in 0.3 M glycine instead of 0.15 M K-glutamate. (*B*) Rate of calcium uptake by sarcoplasmic reticulum vesicles. Calcium uptake was initiated by the addition of ATP (1.0 mM) to medium containing 0.15 M K-glutamate, 10.0 mM imidazole (pH 6.6), 1.0 mM Mg-maleate, 150 μM calcium-maleate, 50 μM arsenazo III, and 1 mg microsomal protein per ml. Temp. = 25 °C. The absorbance was measured in the dual wavelength mode at the indicated wavelength pairs. *b* indicates time of dilution of microsomes in Fig. 13*A*, *b*. *c* indicates time of dilution of microsomes in Fig. 13*A*, *c*

the effect of membrane potential upon the optical response of di-S-$C_2(5)$ was reinvestigated at a protein/dye weight ratio of 2 (Fig. 13). Even under these conditions loading of the vesicles actively (Fig. 13*Ab*, *c*, *d*) or passively (not shown) with close to 20 mM Ca^{2+} greatly diminished the optical response to negative potential. As the effect of ATP-induced Ca^{2+} uptake upon the dye response persisted long after net Ca^{2+} uptake ceased (Fig. 13*B*), the simplest explanation of these observations is that intravesicular Ca^{2+} inhibits the redistribution response of di-S-$C_2(5)$ to potential. Ca^{2+} (40 mM) had no effect upon the absorption spectrum of di-S-$C_2(5)$ in the absence of microsomes at dye concentrations ranging from 0.5–50 μg/ml.

At the earliest time that could be measured (2–3 sec after dilution), there was little difference in the optical response between vesicles preloaded for 1 min with Ca^{2+} (Fig. 13*b*) and vesicles in which Ca^{2+} uptake was initiated at the time of dilution (Fig. 13*d*), but the rate of return of the signal to zero potential was slightly faster in vesicles which actively pump calcium (Fig. 13*d*). This difference may indicate the generation of some positive potential during Ca^{2+} transport. ATP-induced Ca^{2+} uptake in absence of negative potential had no influence on the absorbance at 660–700 nm (Fig. 13*e*, *f*).

Absorbance Response of di-I-C_1 (5) and Oxonol VI to Calcium Uptake by Sarcoplasmic Reticulum Vesicles. Separation of the Contribution of Surface Potential and Ion Diffusion Potential

The experiments described in Figs. 9–12 suggest that much of the optical response of di-S-$C_2(5)$, di-I-$C_1(5)$, Nile Blue A and oxonol VI during ATP-induced Ca^{2+} transport by sarcoplasmic reticulum is attributable to the accumulated calcium. If inside-positive membrane potential is generated during ATP-induced Ca^{2+} transport, this effect is submerged in the response of the probes to Ca^{2+}.

In order to separate the contribution of transmembrane potential generated by electrogenic Ca^{2+} translocation from the overall probe response the effect of valinomycin upon the absorbance of di-I-$C_1(5)$ and oxonol VI was analyzed during ATP-mediated Ca^{2+} transport in the presence of K^+ (Fig. 14). Valinomycin (5 μM) promotes K^+ fluxes across the membrane without interfering with active Ca^{2+} transport (Fig. 14*C*). During ATP-mediated Ca^{2+} transport in K-glutamate medium in the presence of valinomycin di-I-$C_1(5)$ (Fig. 14*A*) and oxonol VI (Fig. 14*B*) are expected to respond only to changes in surface potential caused by the accumulated Ca^{2+}, since valinomycin prevents the generation of ion diffusion potential. In the absence of valinomycin the absorbance changes of the two dyes during Ca^{2+} transport reflect changes in surface as well as ion diffusion potentials. Therefore, the difference between the traces obtained in absence and presence of valinomycin is assumed to represent the diffusion potential component of the probe response (.....).

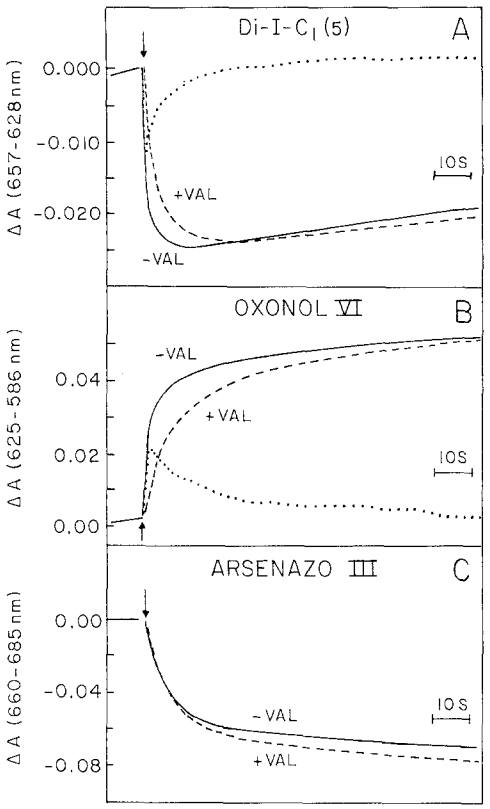


Fig. 14. Absorbance response of di-I-C_1(5) and oxonol VI to calcium uptake by sarcoplasmic reticulum vesicles. (*A* and *B*) The medium contained 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 60 μM Ca-maleate, 50 μM EGTA, and either 1 μg/ml di-I-C_1(5) (*A*) or oxonol VI (*B*) and 100 μg/ml microsomal protein. Calcium uptake was initiated by the addition of 0.5 mM ATP (↓). The difference between the optical response in the absence of valinomycin (——) and in the presence of 5 μM valinomycin (– – –) is shown by the dotted line (· · · ·). The absorbance was measured in the dual wavelength mode at the indicated wavelength pair. (*C*) Calcium uptake. Di-I-C_1(5) or oxonol VI were replaced with 100 μM arsenazo III and the uptake of calcium was monitored in the dual wavelength mode at 660–685 nm in the absence (——) or presence (– – –) of valinomycin. Temp. = 15 °C

With both di-I-C_1(5) and oxonol VI the diffusion potential contribution appeared as a transient spike which dissipated within 30 sec, while Ca^{2+} accumulation continued. The maximum absorbance change attributable to diffusion potential was 0.012 for di-I-C_1(5) and 0.018 for oxonol VI, which correspond to inside-positive membrane potentials of 10 and 13 mV, respectively, based on the data of Figs. 10 and 11. According to McKinley and Meissner (1978) close to 2/3 of the sarcoplasmic reticulum vesicles (Type I) contain K channels of sufficiently high K conductance that they are not expected to maintain significant transmembrane potential even in the absence of valinomycin. Therefore, the effects of valinomycin shown in Fig. 14 presumably refer to Type II vesicles which do not contain K^+ channels.

Effect of Ca^{2+} Uptake upon the Fluorescence Response of Dyes I–IV to Preimposed Negative and Positive Potentials

Dilution of sarcoplasmic reticulum vesicles equilibrated in 0.15 M K-glutamate medium into 0.15 M choline-chloride is expected to generate an inside-negative potential of close to 100 mV. Di-S-C_2(5) and di-I-C_1(5) respond to this potential with a rapid decrease in fluorescence intensity (Fig. 15*A*, *B*) which slowly returns to control levels as the potential is dissipated (Fig. 15*A*, *B*, bottom lines). Addition of ATP (↓) after dilution to initiate active Ca^{2+} transport accelerated the return of the signal toward zero potential (Fig. 15*A*, *B*, bottom and middle lines), while ATP added before dilution of the vesicles diminished the optical signal (Fig. 15*A*, *B*, top lines). Nile Blue A gave a similar response (Fig. 15*C*). These observations are consistent with the data in Fig. 9.

Dilution of vesicles from 0.15 M choline-Cl into 0.15 M K-glutamate medium generates inside positive potential of close to 100 mV. Oxonol VI responds to this potential with a decrease in fluorescence intensity, compared with control vesicles of zero potential. There is a slow increase in fluorescence as the potential is dissipated (Fig. 15*D*). Addition of ATP either before or after dilution had no effect on the rate of return of fluorescence to control levels, suggesting that Ca^{2+} transport into inside-positive vesicles did not significantly alter the membrane potential. These experiments establish a consistency between conclusions based on absorbance and fluorescence measurements.

Influence of Membrane Potential Upon the Rate of Ca^{2+} Transport

The experiments described in Figs. 13 and 14 suggest the generation of a positive membrane potential during ATP-dependent Ca^{2+} transport. In turn, under conditions similar to those described in Fig. 15, inside-negative membrane potential increased, while inside-positive potential decreased the rate of Ca^{2+} uptake by sarcoplasmic reticulum vesicles (Fig. 16), compared with control samples in which no potential was generated upon dilution or in which the potential was allowed to decay before initiation of Ca^{2+} transport by addition of ATP. The increased rate of Ca^{2+} uptake in K^+-glutamate as compared with choline-Cl medium is due to the activation of Ca^{2+}-ATPase by K^+.

Discussion

These studies indicate that dyes which are used to monitor transmembrane potentials are also sensitive

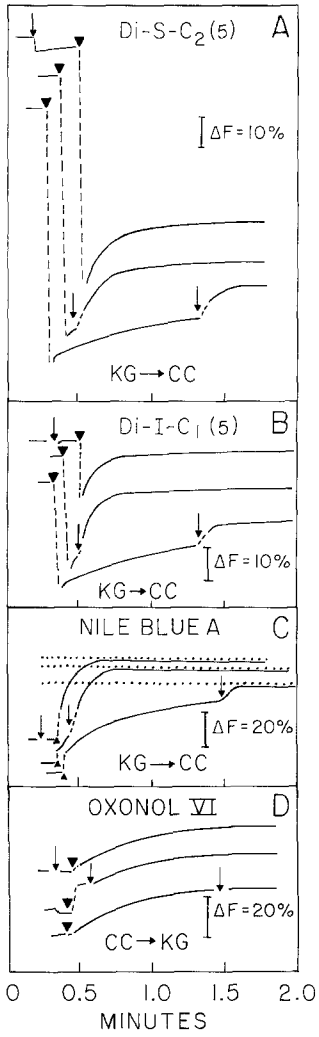


Fig. 15. Effect of ATP-mediated calcium uptake by sarcoplasmic reticulum vesicles on the fluorescence response of di-S-$C_2(5)$ (*A*), di-I-$C_1(5)$ (*B*), Nile Blue A (*C*) and oxonol VI (*D*) to the membrane potential generated by K^+ or Cl^- gradients. Inside-negative membrane potential (*A–C*) was generated by 300-fold dilution of sarcoplasmic reticulum vesicles (33 mg protein/ml), equilibrated in 0.15 M K-glutamate (KG), 10 mM imidazole, pH 6.6, and 1 mM Mg-maleate, into 0.15 M choline-Cl (CC), 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 50 μM Mg-maleate, 50 μM Ca-maleate and 1 μg/ml dye (*A–C*). Inside-positive membrane potential was generated (*D*) by diluting sarcoplasmic reticulum vesicles (33 mg protein/ml) equilibrated in 0.15 M choline-Cl, 10 mM imidazole (pH 6.6) and 1 mM Mg-maleate 300-fold into a medium containing 0.15 M K-glutamate, 10 mM imidazole (pH 6.6), 1 mM Mg-maleate, 50 μM Ca-maleate and 1 μg/ml oxonol VI. ATP (0.1 mM) was added either before dilution, or 10 sec (middle line) or about 1 min (bottom line) after dilution of the microsomes. ATP addition (↓). Microsome addition (▼). Excitation wavelength: 640 nm (*A, B, C*) or 590 nm (*D*). Emission wavelength: 665 nm (*A*), 660 nm (*B*), 670 nm (*C*), and 630 nm (*D*). Dotted lines (· · · · · ·) represent zero potential control traces. The fluorescence intensity before dilution of the vesicles is defined as 100%

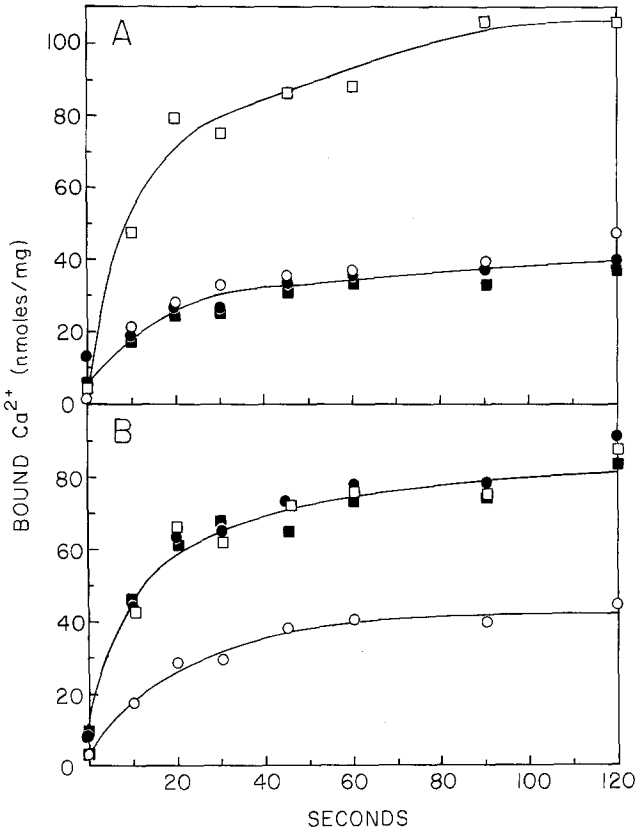


Fig. 16. Effect of membrane potential on calcium transport by sarcoplasmic reticulum vesicles. (*A*) Sarcoplasmic reticulum vesicles (32 mg protein per ml) equilibrated in 0.15 M choline-Cl (○, ●) or 0.15 M K-glutamate (□, ■), 10 mM imidazole (pH 6.6) and 1 mM Mg-maleate were diluted 100-fold into 0.15 M choline-Cl. 10 mM imidazole (pH 6.6), 1 mM Mg-maleate and 120 μM ^{45}Ca-maleate (1 μCi/ml) at 15 °C. Ca^{2+} uptake was initiated with 1.0 mM ATP at the time of dilution (○, □) or 1 hr later (●, ■) after the microsomes had equilibrated with the dilution medium. Aliquots (200 μl) were removed at various times after initiation of Ca^{2+} uptake and passed through Millipore filters. The filters were washed with dilution medium containing 5 mM EGTA in place of ^{45}Ca maleate. (*B*) Similar to the above experiment, but the dilution medium contained 0.15 M K-glutamate instead of choline-Cl

to surface potential. Changes in surface charge density and the screening of surface charges will result in absorbance or fluorescence signals with spectral characteristics that are in most cases indistinguishable from signals evoked by changes in transmembrane potential. The problem of distinction between the effects of surface and transmembrane potentials is particularly difficult in ion translocating systems, such as the sarcoplasmic reticulum, where major changes in surface charge density, caused by the binding of accumulated calcium, occur together with changes in transmembrane potential.

The simultaneous use of dyes with localized or delocalized positive or negative charges permitted us to compare their responses under identical experimental conditions and arrive at a consistent interpretation of the mechanisms responsible for the absorbance and fluorescence response to membrane potential.

The partitioning of permeant dyes across the sarcoplasmic reticulum membrane is governed by the membrane potential, and under suitable conditions the relationship between the absorbance or fluorescence of the dyes and the transmembrane potential follows the Nernst relationship. Dyes with delocalized negative charges (oxonol VI) accumulate in compartments with positive potential, while dyes with delocalized positive charges (di-S-C_2(5) and di-I-C_1(5)), accumulate in compartments with negative potential (Sims et al., 1974; Waggoner, 1976). The increase in dye concentration under the influence of potential promotes the binding of the dyes to the membrane, with changes in absorbance or fluorescence as the ratio of bound/free dye increases. Aggregation of di-S-C_2(5) contributes to the optical response at high dye/protein ratios, as indicated by the appearance of the characteristic absorption band of dimers and polymers in the spectrum (Fig. 4 *IB*; Sims et al., 1974); this effect is probably minor in the case of di-I-C_1(5) and oxonol VI.

Dyes with localized negative charges (WW 781) do not redistribute significantly across the membrane (Baylor et al., 1981; Freedman & Laris, 1981) and much of the dye response may arise from reorientation of dye molecules within the membrane under the influence of potential. The binding of penetrating dyes to the membrane is influenced by changes in surface potential on either side; with the nonpenetrating dye, WW 781, the effect is restricted to the side of the membrane where the dye is bound.

Cations influence surface potential through "screening" of surface charges and by changing surface charge density due to interaction with the membrane (McLaughlin, 1977). This is reflected in the effects of K^+, Ca^{2+}, La^{3+} and H^+ upon the binding of the dyes to the membrane (Tables 1 and 2). Such effect is clearly observed during ATP-dependent transport of Ca^{2+}, which increases the intravesicular Ca^{2+} concentration to 20–30 mM. Furthermore, the usual experimental conditions for generation of membrane diffusion potentials involve establishment of large ion gradients which necessarily influence surface potential.[3] It appears that the effect of Ca^{2+} accounts for much of the optical response of di-S-C_2(5), di-I-C_1(5) and oxonol VI during ATP-dependent Ca^{2+} transport. Nile Blue A and WW 781 were relatively insensitive to changes in intravesicular Ca^{2+} in the absence of transmembrane potential.[4]

The effect of Ca^{2+} upon the optical response of the probes is established by the following criteria:

1. Passive loading of sarcoplasmic reticulum vesicles with 10–50 mM Ca^{2+} altered the absorbance and fluorescence of di-S-C_2(5), di-I-C_1(5) and oxonol VI in a manner similar to that observed during active Ca^{2+} transport. With di-I-C_1(5) and oxonol VI, the difference absorbance spectrum caused by Ca^{2+} was similar to that produced by inside-positive potential. The absorbance of externally added WW 781 was influenced only by changes in medium Ca^{2+} concentration; neither passive nor active loading of the vesicles with Ca^{2+} caused detectable change in the absorbance or fluorescence of external WW 781.
2. The optical changes during Ca^{2+} transport are roughly proportional to the amount of accumulated Ca^{2+} and persist as long as the Ca^{2+} is retained by the vesicles. In contrast, the response of the dyes to artificially imposed potential is transient due to the high permeability of the membrane for small ions.
3. The presence of valinomycin during Ca^{2+} transport produced only a small transient change in the response of di-I-C_1(5) and oxonol VI to Ca^{2+} transport under essentially voltage-clamped conditions. The observations are consistent with the generation of about 10 mV inside-positive potential during the first 5–10 sec of Ca^{2+} accumulation. The valinomycin-induced change rapidly diminished, while the absorbance change due to Ca^{2+} uptake increased.
4. Inclusion of 5 mM oxalate into the Ca^{2+} uptake medium reduced the absorbance change of di-S-C_2(5), di-I-C_1(5) and oxonol VI during Ca^{2+} uptake, while it had no effect upon the magnitude of optical response to artificially imposed membrane potential in the absence of calcium. These observations suggest that oxalate influenced the dye response by lowering the intravesicular Ca^{2+} concentration due to precipitation of calcium oxalate.
5. The optical responses caused by passive or active loading of the vesicles with calcium were reversed by the calcium ionophore, A23187, which caused the release of accumulated Ca^{2+}.

Calcium increases the binding of negative and decreases the binding of positive dyes to the membrane. These changes in dye binding are sufficient to explain the absorbance and fluorescence changes in passively Ca^{2+}-loaded sarcoplasmic reticulum vesicles and account for much of the optical change during ATP-dependent active Ca^{2+} transport. Qualitatively similar effects were observed with monovalent and trivalent cations. The effectiveness of cations increases with valency, but the concentration dependence of the effect does not permit clear distinction between contributions due to screening of surface charges and

[3] Establishment of K^+ gradient could also influence Ca^{2+} transport by causing pH changes within the vesicles through K^+–H^+ exchange. Furthermore, K^+ has a direct effect on the kinetics of Ca^{2+} translocation.

[4] The optical signal of Nile Blue A due to negative transmembrane potential is diminished by 20 mM intravesicular Ca^{2+}.

the binding of cations to the membrane. Due to its effect on dye binding to the membrane, calcium increases the absorbance response of di-I-$C_1(5)$ and oxonol VI to artificially imposed positive potential, and decreases the response of di-S-$C_2(5)$, di-I-$C_1(5)$, Nile Blue A, and oxonol VI to inside-negative potential. This is reflected in a shift of the voltage-response curves of di-I-$C_1(5)$ and oxonol VI in the presence of 20 mM Ca^{2+} (Figs. 10 and 11). With di-S-$C_2(5)$, the effect of Ca^{2+} on the voltage response curve is more complex and causes attenuation of probe response by Ca^{2+} at both negative and positive potentials (Fig. 12). There is no indication that Ca^{2+} affects significantly the rate of re-equilibration of the dyes across the membrane. Ca^{2+} (10–50 mM) had no influence upon the absorbance of di-S-$C_2(5)$, di-I-$C_1(5)$, Nile Blue A and oxonol VI in the absence of membranes and on the absorbance of di-S-$C_2(5)$ in the presence of egg phosphatidylcholine-cholesterol vesicles.

The rate of ATP-dependent active Ca^{2+} transport is increased by preimposed inside-negative potential (Fig. 16) and decreased by positive potential. The absorbance and fluorescence changes of di-S-$C_2(5)$, di-I-$C_1(5)$, Nile Blue A and oxonol VI caused by a preimposed negative potential are rapidly attenuated during ATP-dependent Ca^{2+} transport (Figs. 9, 13, 15). These observations together with the data in Fig. 14 suggest that Ca^{2+} transport is electrogenic, but the magnitude of the inside-positive potential generated is uncertain. Although Ca^{2+} transport abolished within 30 sec optical signals caused by a preimposed negative potential of 100 mV, much of this effect is apparently due to the influence of intravesicular Ca^{2+} upon the dye response to potential. Under the conditions of Fig. 14, the transmembrane potential generated during Ca^{2+} transport is estimated to reach about 10 mV. These experiments reveal only potential generated in Type II vesicles which do not contain K^+ channels (McKinley & Meissner, 1978). Potentials generated in Type I vesicles during Ca^{2+} transport are rapidly neutralized by compensating ion fluxes and would not be detectable under the conditions of Fig. 14. Technical difficulties prevented the application of rapid kinetic methods which could resolve early changes in transmembrane potential related to the fast initial phase of Ca^{2+} transport.

As the response of various optical probes to membrane potential is heavily influenced by changes in surface potential, clear interpretation of the probe response is difficult in systems where major fluctuations of ion concentrations occur during physiological activity. The problem becomes even more complex in excitable tissues where potential changes occur in surface as well as intracellular membranes, with distinct time courses, accompanied by changes in ion concentration. Furthermore, part of the dye introduced into cells is concentrated within the mitochondria, and fluorescence changes may accompany changes in mitochondrial function.

In frog semitendinosus muscles stained with Nile Blue A the fluorescence intensity increases in response to single stimuli (Bezanilla & Horowicz, 1975; Vergara et al., 1978). The change in fluorescence intensity starts early in the falling phase of the action potential and the threshold for the fluorescence response is indistinguishable from the contractile threshold. The time course and magnitude of the fluorescence signal suggest that it is related to changes associated with the sarcoplasmic reticulum (Bezanilla & Horowicz, 1975; Vergara et al., 1978). Essentially similar observations were made using indodicarbocyanine as potential probe (Oetliker et al., 1975). The nonpermeable dyes, merocyanine (Vergara & Bezanilla, 1976) and WW 781 (Vergara & Bezanilla, 1979) added externally to intact muscle fibers respond primarily to changes in the surface membranes and T-tubules.

The intrinsic birefringence change and the responses of cyanines, Nile Blue A and WW 781 during muscle activation follow significantly different time courses and the optical changes contain several components of distinct polarization (Baylor et al., 1981). Therefore, interpretation of these changes in terms of a known underlying mechanism, such as the Ca^{2+} release from sarcoplasmic reticulum, is uncertain. Furthermore, as shown in this report, signals previously attributed to inside-negative potential generated in sarcoplasmic reticulum during Ca^{2+} release may reflect in part a direct effect of Ca^{2+}. Since the effect of Ca^{2+} on the dye response is usually more pronounced at large protein/dye ratios, it is expected to be prevalent in experiments with living muscle.

Of the five dyes tested *in vitro*, Nile Blue A appeared to produce the cleanest signal to inside-negative potentials, with least interference from Ca^{2+}. Therefore, the Nile Blue A fluorescence signals obtained during muscle activation may represent the best evidence to date for the generation of inside-negative potential in sarcoplasmic reticulum during activating Ca^{2+} release (Bezanilla & Horowicz, 1975; Vergara et al., 1978). As binding of Nile Blue A to sarcoplasmic reticulum vesicles was accompanied by an increase in fluorescence intensity, the results in living muscle suggest that activation promotes the entry of Nile Blue A into the sarcoplasmic reticulum with subsequent binding to the membrane. Conformational changes in contractile proteins may also influence the dye response if Nile Blue A like dihexyloxacarbocyanine (Scordilis, Tedeschi & Edwards, 1975) is bound to myofibrils.

If the Nile Blue A signal during muscle activation indicates a negative potential in sarcoplasmic reticulum, this potential change during a single twitch lasts only for about 50 msec (Bezanilla & Horowicz, 1975). The Nile Blue A signal coexists during part of its course with the Ca^{2+} transient measured by calcium indicators (Kovacs, Rios & Schneider, 1979), raising the possibility that the membrane potential of sarcoplasmic reticulum regulates the rate of Ca^{2+} transport. As shown in Fig. 16, the rate of Ca^{2+} uptake by isolated sarcoplasmic reticulum vesicles is enhanced by negative potential and in turn the potential signal is attenuated during Ca^{2+} uptake (Figs. 9 and 15). It is plausible to assume that the negative potential generated in living muscle during activation facilitates the subsequent reabsorption of calcium, which, in turn, accelerates the disappearance of negative potential. This possibility may be tested in several ways:

1. Inhibition of Ca^{2+} uptake by injection of EGTA into living muscle fibers should prolong the Nile Blue A signal, since the dissipation of negative potential under these conditions would depend only upon the passive ion fluxes across the sarcoplasmic reticulum. EGTA should abolish the Nile Blue A signal if it arises from interaction of Ca^{2+} with myofibrils or the Ca^{2+}-ATPase on the outer surface of sarcoplasmic reticulum, or if EGTA interferes with the Ca^{2+} release.
2. The rate of Ca^{2+} reabsorption into sarcoplasmic reticulum should be faster after a depolarizing pulse of short duration while the negative potential still exists, compared with depolarizing pulses lasting for several seconds, which may allow the dissipation of negative potential. A negative potential in sarcoplasmic reticulum during activation could arise from electrogenic Ca^{2+} release or from ionic current flow between the T-tubules and the sarcoplasmic reticulum (Mathias, Levis & Eisenberg, 1980).

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