

The Location of the Calcium Precipitating Protein in the Sarcoplasmic Membrane

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Fluram, a fast reacting reagent for primary amino groups does not label proteins enclosed in reconstituted sarcoplasmic vesicles (SR vesicles) when applied at low reagent/protein ratios. Hence, Fluram does not penetrate through SR membranes under these conditions. Likewise, the membranes of erythrocytes prevent the reagent from reacting with hemoglobin. However, at high reagent/protein ratios the membranes of the SR vesicles disintegrate. In contrast, liposomal membranes prepared from SR lipids are not affected even at a high degree of labeling. Disintegration of SR membranes starts to occur when the phosphatidylethanolamine fraction is completely substituted by Fluram and the transport protein is labeled with 3–4 mol of Fluram per 100 000 d. When closed SR vesicles are labeled at low Fluram/protein ratios, the calcium precipitating protein is four times more intensely labeled than the transport protein. The labeling of the calcium transport protein in closed vesicles excludes its location in the intravesicular space. In SR vesicles solubilized with deoxycholate the relative degree of labeling of the calcium precipitating protein remains unchanged while the transport ATPase is more intensely labeled at the expense of the labeling of the amino lipids. The relative degree of labeling of the protein components depends not only on the number of labable groups but also on the rates with which these groups react with Fluram. Therefore, the experimental data do not give quantitative information concerning the distribution of the protein components in the SR membranes.

Introduction

In living skeletal muscle the total concentration of calcium present inside the sarcoplasmic reticulum (SR) has been estimated to range from 6 to 20 mM (Winegrad¹). The ATP dependent accumulation of calcium performed by isolated vesicular SR fragments yields similarly high internal total calcium concentrations at the cessation of net calcium uptake (*cf.* Hasselbach²). Skeletal muscle requires such a relatively large store of calcium for continuous activation.

However, since an ionized calcium concentration exceeding 0.5 mM at the internal surface of the SR membranes severely counteracts calcium translocation, the SR membranes can only remove calcium from the contractile system at a sufficient rate if most of the calcium in the sarcoplasmic reticulum is chelated (Makinose and Hasselbach³, Weber⁴). Depending on the amount and the affinity of the chelating agent the concentration of ionized calcium can be considerably lower than the total calcium concentration. The phosphate residues of the internal membrane phospholipids have been considered

as calcium binding sites (Carvalho⁵). More recently, much attention has been given to calcium binding proteins as sites for calcium sequestration^{6–8}. Small quantities of these proteins have been isolated from purified SR membrane preparations and characterized by their calcium binding ability^{6–8}. The suspected function of the calcium sequestering proteins requires them to be located in the intrareticular space. The following observations have been used as arguments supporting this location.

a. In contrast to the calcium transport protein the main calcium binding protein is not cleaved when intact vesicles are treated with trypsin^{9–11}. b. Diazoaminophenyl dextran (40 000 mol.weight) applied to intact vesicles cross-links only the transport ATPase without affecting the calcium binding protein⁹. c. When SR vesicles are opened by treating them with cholate, the electron dense material observed in the internal space of some vesicular fractions disappears together with the calcium binding component¹².

On the other hand, arguments against an intravesicular location have been put forward by Thorley-Lawson and Green¹³. The authors consider the

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Abbreviations: Fluram, 4-phenylspiro[furan-2(3H),1'phthalan]-3,3'-dione; DOC, deoxycholate; SDS, sodium dodecyl sulfate.

fact that alkaline solutions containing EDTA¹⁴ can extract the calcium binding protein as incompatible with its intravesicular location. They supported this notion by labeling the calcium binding protein with iodide produced extravesicularly from potassium iodide, H₂O₂ and lactoperoxydase. None of the presented arguments can be considered to be unambiguous. In this report the use of Fluram¹⁵ as a membrane label is described. The reagent reacts extremely fast with free amino groups of proteins and lipids. Although hydrolytic cleavage also proceeds rapidly, it can be neglected as long as the protein concentration is chosen appropriately. Thus the reagent practically reacts quantitatively with all available amino groups.

In the following, experiments are described showing that Fluram fulfills the requirement of an external label for the location of the major calcium binding protein, the calcium precipitating protein, in the SR membranes.

Materials and Methods

The SR vesicles were isolated and purified according to the procedure described by de Meis and Hasselbach¹⁶. The SR lipids were extracted from the SR suspension (20–30 mg/ml) with 10 volumes of ethanol and ether (2:1). The suspension was stirred for 20 min at room temperature and the precipitated protein was removed by centrifugation. After evaporation of the organic solvents the lipids were redissolved in chloroform and methanol (2:1) and subsequently dried under vacuum. Lipids not used immediately after preparation were kept at –18 °C. Erythrocytes from freshly drawn blood were washed 3–4 times by centrifugation at room temperature with 4 volumes of 0.15 M NaCl containing 1 mM EDTA, pH 7.2.

Liposomes and SR vesicles containing protein

SR lipids were solubilized with DOC (0.5–1.0 mg DOC per mg of lipid) in 0.1 M KCl, 0.05 M K-phosphate, pH 7.2. 100 mg ovalbumin were added to 5 ml of the lipid-DOC solution containing 30 mg of lipids. The clear solution was dialyzed for 3 days (4–6 °C) against 0.3 M sucrose and 0.2 M KCl, 0.05 M K-phosphate, pH 7.2.

The liposomes were separated from excess ovalbumin by centrifugation in a swinging bucket rotor Spinco SW 25 at 25 000 rpm for 3 hours through 0.5 M sucrose containing 0.2 M KCl. For the determination of protein enclosed by the lipids

the protein was lightly labeled with Fluram for easier detection. Liposomes enclosed approximately 25% w/w ovalbumin/lipid.

For the preparation of SR vesicles containing ovalbumin SR vesicles were dissolved in a solution containing DOC (0.5–1.0 mg/ml of vesicular protein), 0.05 M K₂HPO₄ and 0.1 M KCl mixed with ovalbumin and further treated as described for the preparation of the ovalbumin containing liposomes. After separation from excess protein the liposomes and the reconstituted vesicles were resuspended in 0.5 M sucrose. The reconstituted SR vesicles enclosed 75%, 80% and 120% w/w ovalbumin/SR protein in three experiments.

Labeling of membrane proteins and lipids with Fluram

The membrane preparations (2 mg/ml) were suspended in 0.1 M KCl, 0.05 M borate buffer, pH 8.3. 0.5 M sucrose was added to liposomes and reconstituted vesicles. The suspension was vigorously shaken and Fluram dissolved in acetone was rapidly added. The volume of acetone never exceeds 2% of the volume of the suspension. Usually the volume of the suspension was 5 ml. The volume was increased to 10 ml when larger quantities of protein were labeled as for the preparation of calcium precipitating protein or for the labeling of erythrocytes. Unless otherwise stated, Fluram labeling was performed at room temperature (20–22 °C).

Separation of protein and lipids

a. Native vesicles

The protein was precipitated and the lipids extracted by the addition of 5 volumes of ethanol and ether 2:1. To assure complete protein precipitation when small quantities of protein were labeled, unlabeled protein was added for coprecipitation. After vigorous stirring for 20–30 min the protein was spun down and the lipid containing organic phase was saved for fluorescence determination. The protein was washed with 70% acetone and subsequently dissolved in a solution containing 0.05 sodium borate and 1% SDS.

b. Liposomes and reconstituted vesicles

The labeled liposomes were diluted with 0.1 M NaCl, 0.5 M sucrose and three aliquots were taken. One aliquot remained unchanged, the two others were treated with DOC 1.0–2.0 mg/mg of protein to release the enclosed ovalbumin. After centrifugation for 1 hour at 50 000 rpm the fluorescence in the supernatant fluid and in the pellet dissolved in 0.05 M sodium borate, 1% SDS were measured.

c. Erythrocytes

After labeling, the erythrocytes were spun down and the supernatant containing extracellular protein was saved. Subsequently, the erythrocytes were hemolyzed by addition of water. The fluorescence was determined in the first supernatant, in the hemolysate, and in the precipitated membrane ghosts after solubilization in sodium borate 0.05 M, 1% SDS. Hemoglobin exhibits a small intrinsic fluorescence which was determined in an unlabeled sample. This fluorescence was not diminished by prolonged dialysis.

The isolation of calcium precipitating protein from Fluram labeled SR vesicles

200–300 mg protein of SR vesicles labeled with 0.1 μ mol Fluram per mg was treated with 30–45 mg DOC in 0.4 M KCl, pH 7.0 at 0 °C. The suspension was diluted with 0.4 M KCl to give a final volume of 100 ml. Centrifugation at 50 000 rpm in a Spinco 50 Ti rotor precipitated the calcium transport protein. The precipitate was resuspended in 0.1 M NaCl. In most cases this procedure yielded a preparation which exhibits only one band in SDS gel electrophoresis. The clear supernatant was dialyzed for 12 hours against 0.1 M KCl to reduce its DOC content. Addition of 10 mM CaCl_2 to the dialyzing fluid precipitated the calcium precipitating protein together with transport protein not removed during high speed centrifugation. The precipitate was spun down and dissolved in 1 M NaCl containing 10 mM K-phosphate, pH 7.2. The undissolved material was removed by centrifugation. The protein was precipitated by a second dialysis against 0.1 M KCl, 10 mM CaCl_2 . The precipitate was dissolved in 0.1 M NaCl + 10 mM EDTA, pH 7.0. The yield was variable but usually 10–15 mg of calcium precipitating protein were obtained from 200–300 mg vesicular protein. After Fluram labeling, aliquots for the determination of the fluorescence of the total membrane protein and the membrane lipids were taken as described before. Fluorescence of the labeled calcium binding protein was determined after solubilization in 0.05 M borate and 1% SDS.

The measurement of fluorescence

Fluorescence intensity was measured at a wavelength of 390 nm for excitation and of 480 nm for emission in aliquots of the protein and lipid solutions diluted with 0.05 M sodium borate and 1% SDS. The spectrofluorimeter (Hitachi) Perkin Elmer MPF 2A was adjusted to the same sensitivity by using the solid fluorescing standard. Fluram

standards were prepared by labeling excess protein with different quantities of Fluram. As long as the protein concentration exceeds 2 mg per ml and the concentration of Fluram ranges between 0.04–0.2 mM, Fluram reacts quantitatively with the SR vesicles as demonstrated in Fig. 1. The half lifetime of Fluram in the reaction media at pH 8.3 is 5 sec at 20 °C and 30 sec at 0 °C.

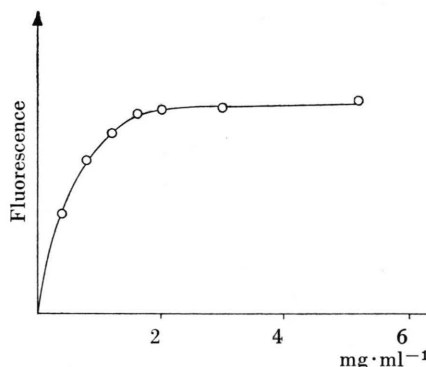


Fig. 1. Titration of Fluram with SR vesicles. Abscissa: protein concentration in the labeling assay; Ordinate: fluorescence intensity in arbitrary units. Fluram concentration 0.2 mM, $T=20$ °C. Other conditions as described in Methods. Note that at protein concentrations exceeding 2 mg/ml the fluorescence remains constant indicating that Fluram reacts quantitatively with the vesicular components.

Gel electrophoresis

Gels containing 7% polyacrylamide, 0.2% $\text{N,N}'$ -methylenebisacrylamide were polymerized with sodium persulfate and $\text{N,N,N}',\text{N}'$ -tetramethylethylenediamine. 0.1 M Tris-bicine, pH 8.2, 0.1% SDS was used as solution in the gel and in the electrode chambers for protein separation. The protein samples were dialyzed against 10 mM tris-bicine, pH 8.2, 0.1% SDS before application to the separating tubes 0.5 cm $\times$ 7 cm. The gels were fixed in 20% sulfosalicylic acid, stained with 0.25% Coomassie brilliant blue, dissolved in methanol, acetic acid and water 5:1:5 and destained in 10% acetic acid containing 2% methanol. To record the fluorescence photographically, unstained gels were illuminated with UV light 366 nm. The camera was equipped with a barrier filter (Schott, Mainz). Scanning of the destained gels were performed with a disc attachment for a spectrophotometer PMQ II at a wavelength of 550 nm and yielded the following distribution 75%, 8.2%, 3.3%, 12.5% for the calcium transport ATPase, the calcium precipitating protein⁶, a calcium binding protein^{21,22} and unknown poorly separated components, respectively. The figures should be taken with some reserve since, due to a number of difficulties inherent in

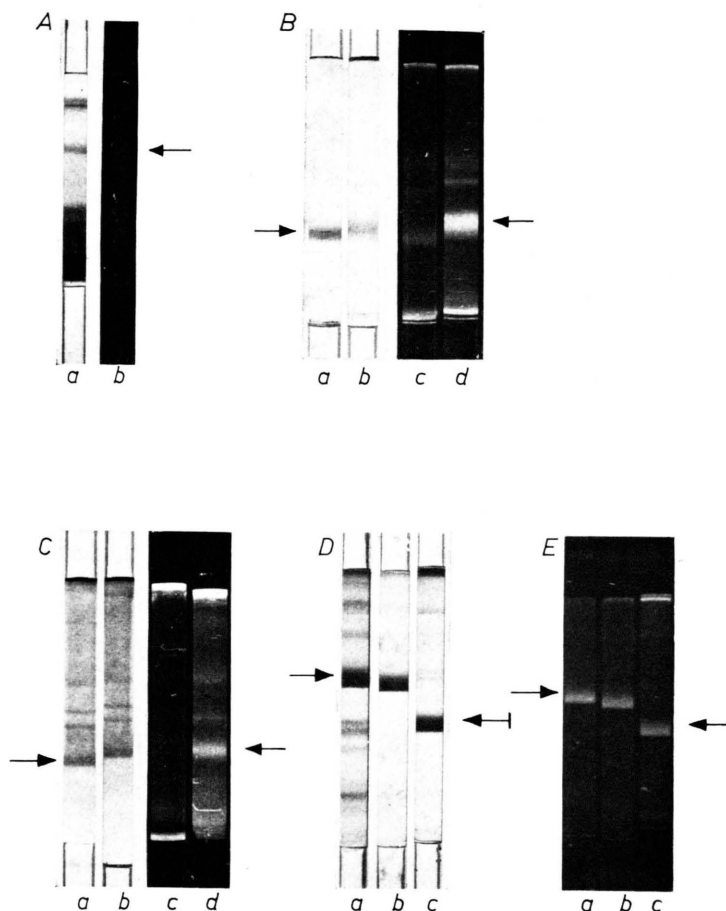


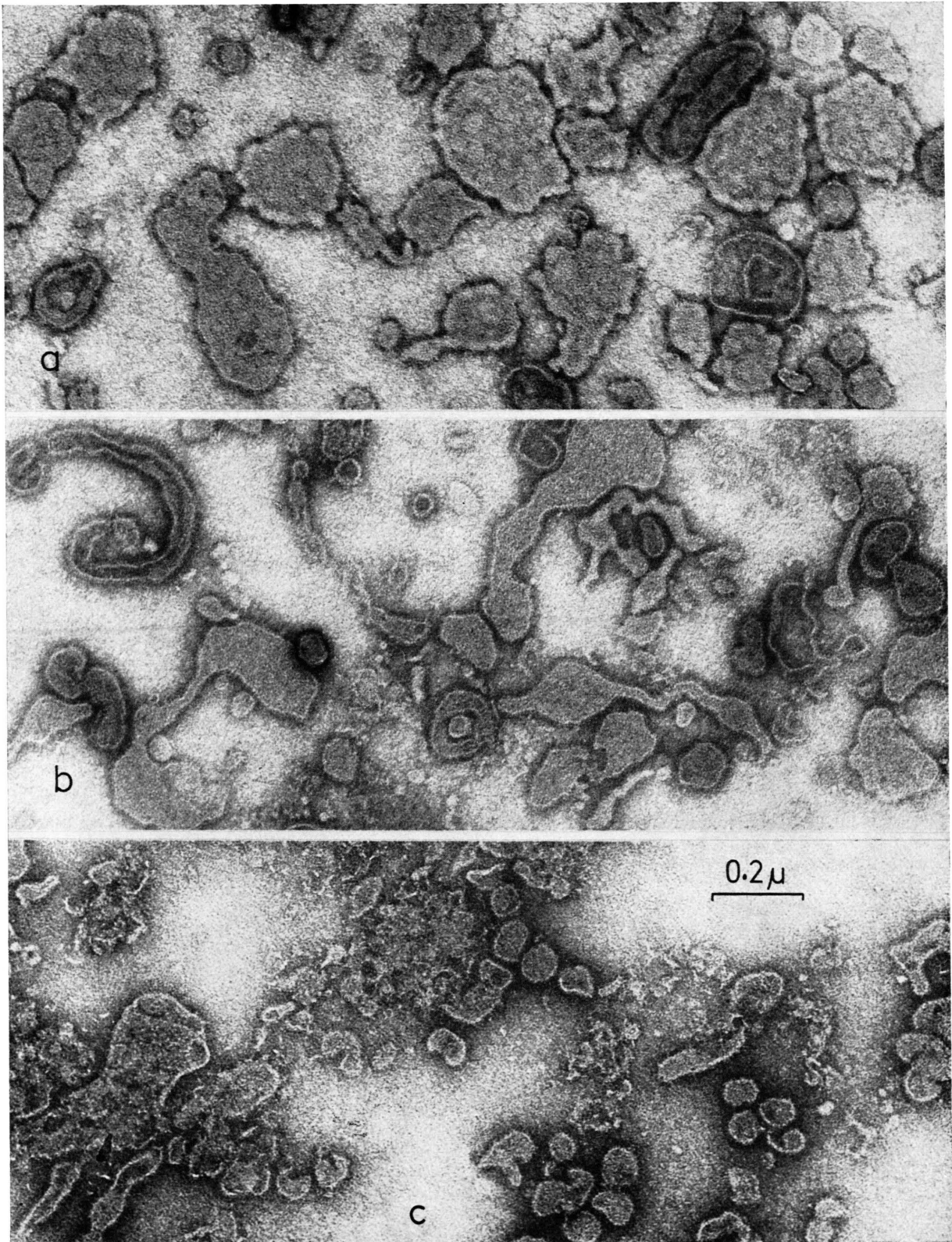
Fig. 2. Gel electropherograms of preparations labeled with Fluram.

A. Erythrocyte membranes. Aa, protein pattern, Coomassie brilliant blue staining. Ab, only one component, either the glycoprotein and/or component a, is faintly fluorescent ($\leftarrow$).

B. Liposomes from SR lipids with enclosed ovalbumin ($\leftarrow$). Ba, protein pattern (Coomassie brilliant blue staining), Bb, protein pattern, Bc, fluorescence pattern of the liposomal preparation obtained after removing nonenclosed ovalbumin by centrifugation through a sucrose layer, and Bd, fluorescence pattern of liposomes lysed with 1% SDS before labeling with Fluram. The minor bands moving more slowly than ovalbumin are contaminations present in the ovalbumin preparations. Liposomes prepared from 10 mg of phospholipids were treated with 10 μ mol Fluram. The lipid layer nearly completely protects ovalbumin from being labeled by Fluram.

C. Reconstructed SR vesicles with enclosed ovalbumin ($\leftarrow$). Protein pattern before (a) Coomassie brilliant blue staining and (b) after lysis; fluorescence pattern, (c) Fluram labeling of closed vesicles, (d) labeling after lysis. Note that the transport protein after reconstruction and labeling with Fluram does not enter the gel.

D. Protein pattern and E. fluorescence pattern after labeling of native vesicles, of isolated transport and of calcium precipitating protein. Da, Ea, native vesicles, Db, Eb, transport protein ($\leftarrow$), Dc, Ec, calcium precipitating protein (%).



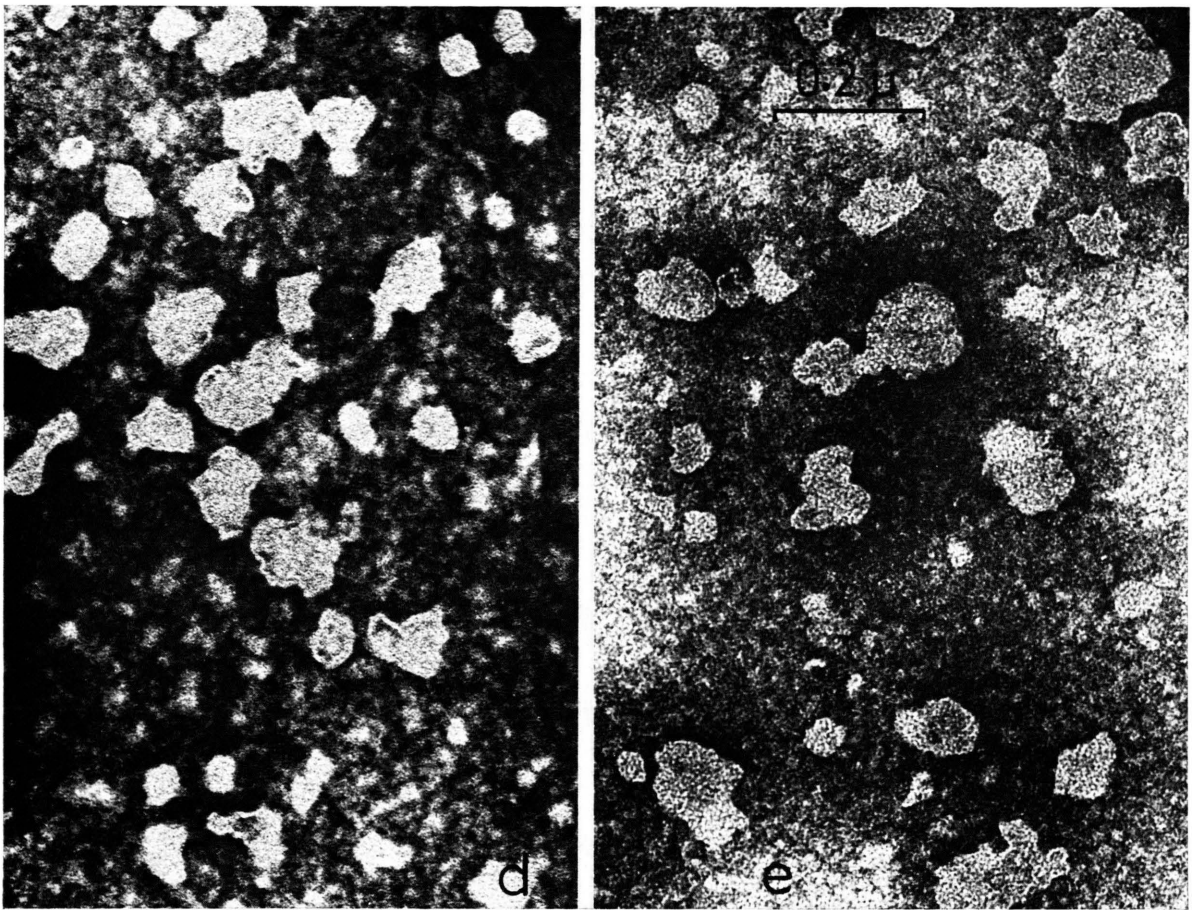


Fig. 3 d and e.

Fig. 3 a—e. Electron micrographs of SR vesicles of rabbit skeletal muscle treated with various amounts of Fluram. Negative staining with 1% potassium phosphotungstate $\times 100\,000$. (Bar in the figure indicates $0.2\,\mu\text{m}$). a. SR vesicles treated with $0.06\,\mu\text{mol}$ Fluram/mg of protein. Closed vesicles of various shape and size displaying a fine surface granularity can be observed. b. after treatment with $0.14\,\mu\text{mol}$ Fluram/mg of protein, vesicles more heterogeneous in shape and size than in a. are seen. Broken down vesicular fragments are also evident. c. sample with added $0.30\,\mu\text{mol}$ Fluram/mg of protein. Small vesicular structures mixed with open membranes and amorphous debris are predominant. d, e, liposome preparations negatively stained with 1% potassium phosphotungstate. No differences in size and shape of liposomes between a control sample d. and one treated with $0.5\,\mu\text{mol}$ Fluram/lipid (e) can be observed.

both membrane proteins, a rigorous calibration for quantitative gel electrophoresis using Coomassie brilliant blue for staining proves difficult. However, the figure is in good agreement with that found by scanning unstained gels at a wavelength of 280 nm.

Electron microscopy

For electron microscopy, samples of control and Fluram treated SR vesicles and liposomes were negatively stained on collodion and carbon coated grids according to standard procedures and observed in the electron microscope within a few minutes after air drying. A 1% potassium phosphotungstate aqueous solution adjusted to pH 7.0 with KOH was used. A Siemens Elmiskop 101 electron microscope equipped with a specimen cooling device was used with a double condenser illumination, 300 μm Pt and 30 μm Pt condensers and 30 μm Pt objective aperture, accelerating voltage of 80 kV and an emission current of 20 μA . Pictures were taken at magnification⁵ ranging from $\times 20\,000$ to 50 000.

Chemicals

All general chemicals were reagent grade and were obtained from Serva, Heidelberg, and Merck, Darmstadt. Ovalbumin 90% pure was purchased from Serva, Heidelberg. Fluram was obtained from Hoffmann-La Roche, Grenzach, Germany BRD.

Results

The use of Fluram for the analysis of membrane topology requires an unambiguous proof that it does not penetrate the SR membranes. Penetration can be recognized only when markers which can be labeled by the reagent if it penetrates are present in the space enclosed by the membrane. In erythrocytes, hemoglobin ideally fulfills the function of an internal marker. When hemolyzed erythrocytes are treated with Fluram, it reacts practically exclusively with hemoglobin. If, however, intact erythrocytes are treated with the same amount of

Table I. Fluram labeling and membrane enclosed proteins. The results are expressed as percentage of total fluorescence intensity F_0 of labeled erythrocytes and SR vesicles, respectively. Erythrocytes and SR preparations were labeled and lysed as described in Methods. The relative fluorescence intensities of the lysates and of the precipitated membranes are given. The erythrocytes were washed before lysis. The small fluorescence observed in the lysate accounts quantitatively for the unspecific fluorescence found in unlabeled controls (*). The small increase of the fluorescence in the supernatants produced by the lysis of the preparations indicates that the SR membranes largely protect the enclosed protein from Fluram labeling. The decrease of the fluorescence/protein ratio $F \cdot Pr_a / F_0 \cdot Pr$ ($Pr_0 = 1$ = protein concentration present in the supernatants after lysis) indicates the release of protein which was not or only weakly labeled. The lysis causes a small loss of the labeled material which is precipitated by centrifugation. The experiments in which ovalbumin or serum albumin were labeled before they were enclosed during reconstitution of the SR vesicles show that most of the enclosed material is released by DOC lysis. If, therefore, enclosed unlabeled proteins were labeled, a great loss of the fluorescence of the precipitate of the reconstituted vesicles should have occurred during lysis. The fluorescence in the supernatant of nonlysed preparations is due to either an incomplete removal of nonenclosed protein or its spontaneous release from the liposomes or reconstituted vesicles during the labeling procedure.

Preparation and labeling conditions	Supernatant		$\frac{F}{F_0} \cdot \frac{Pr_0}{Pr}$	Precipitate	
	unlysed [%]	lysed [%]		unlysed [%]	lysed [%]
Human erythrocytes					
600 mg total protein					
20 μmol Fluram	—	12	—	—	62
10 μmol Fluram	—	11	—	—	80
(*)	—	11	—	—	—
SR vesicles reconstituted in the presence of ovalbumin 100 $\text{mg} \cdot \text{ml}^{-1}$					
μmol Fluram mg prot.^{-1}					
0.05	18	—	3.0	81	—
	—	23	1.0	—	67
0.1	17	—	—	80	—
	—	16	—	—	75
0.1	19	—	2.1	78	—
	—	23	1.0	—	79
Ovalbumin labeled with Fluram	6	—	0.5	—	—
enclosed during reconstitution of SR vesicles	—	48	1.0	94	38
Serum albumin labeled with Fluram	21	—	1.33	79	—
enclosed during reconstitution of SR vesicles	—	75	1.0	—	17

Fluram, no Fluram specific fluorescence is found after hemolysis in the membrane free hemoglobin solution (Table I). The total specific fluorescence is present in the membrane ghosts separated from the hemoglobin solution by centrifugation. In the gel electropherogram of the labeled membranes only a faintly fluorescing band can be detected. Its position corresponds to substances with a molecular weight of approximately 100 000 d and may be either the glycoprotein or the component A protein (Fig. 2 A *)¹⁷.

Since the vesicular fragments of the SR membrane lack a substance whose internal location is certainly known, incorporation of ovalbumin as an internal marker has been attempted. As described in Methods, vesicular lipids as well as the dissolved lipoprotein complex enclosed considerable amounts of ovalbumin which had been lightly labeled with Fluram for easier detection. As illustrated by Fig. 2 B the lipid envelope nearly completely prevents Fluram from reacting with the enclosed ovalbumin. Fluram was applied in sufficient quantity for labeling lipid and ovalbumin as shown in gel Bd which was obtained from a preparation labeled by Fluram after the liposomes had been destroyed by SDS. Essentially the same results were obtained with reconstituted SR vesicles containing ovalbumin (Fig. 2 C). When the reconstituted vesicles were labeled and subsequently precipitated by centrifugation, approximately 15% of the total labeled material was found in the supernatant. Either some ovalbumin remained attached to the external surface of the vesicles or some vesicles were destroyed during separation from the ovalbumin solution or during the labeling procedure. Addition of DOC (1.0–2.0 mg/mg) does not release additional fluorescing material into the supernatant while the enclosed protein is liberated. This was controlled by measuring protein fluorescence (290–330 nm) or the fluorescence of enclosed Fluram labeled ovalbumin (Table I). These results demonstrate that under the applied conditions Fluram neither penetrates the liposomes nor the reconstituted SR membranes. Therefore, we assumed that the SR membrane does not allow Fluram to enter the intravesicular space.

When native vesicles are reacted with Fluram, the aminophospholipids, mainly phosphatidylethanolamine and the calcium transport ATPase, the

calcium precipitating protein and minor protein components are labeled. To obtain a discernable fluorescence of the different proteins in gel electrophoresis the vesicles must be labeled with more than 0.15 μmol Fluram/mg of protein. At this degree of labeling the vesicular membranes are destroyed so that the internal surface of the vesicles become accessible to Fluram. This membrane destruction starts to occur at an application of 0.14–0.17 μmol Fluram per mg of vesicular protein (Figs 3 a, b, c **). The destruction of the SR vesicles by Fluram is obviously produced by the reaction of Fluram with the protein component after the Fluram concentration exceeds a critical level because, in contrast to the SR vesicles, liposomes prepared from SR lipids are not visibly affected at a similar extent of labeling (Figs 3 d, e). As shown by Fig. 4 Fluram reacts preferentially with the lipid component and without any sign of discontinuity

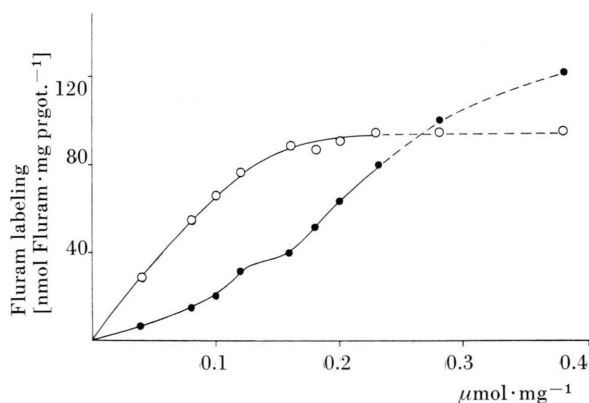


Fig. 4. Titration of native vesicles with Fluram. Abscissa: added quantities of Fluram, $\mu\text{mol}/\text{mg}$ of protein. Ordinate: μmol Fluram reacted with aminophospholipids and protein. The labeling conditions are described in Methods. This experiment was performed at 0 °C. The labeling assay contained 10 mg of protein in a volume of 5 ml, pH 8.3. ○—○, labeled lipids; ●—●, labeled protein; ○---○, corrected for labeled protein soluble in ethanol ether. Note the plateau which lipid labeling reaches when 0.1 μmol Fluram/mg of protein have reacted. Since Fluram cannot penetrate the vesicular membrane, the labeled aminophospholipids must be located at the external surface of the vesicles. The quantity of 0.1 μmol of vesicular protein corresponds to all aminophospholipids present in the vesicular membrane. A mean value of 0.12 ± 0.02 $\mu\text{mol}/\text{mg}$ of vesicular protein was obtained in 25 titrations. Note further that the protein labeling rapidly increases after the phospholipids are fully labeled. At higher degrees of labeling the protein becomes partially soluble in ethanol ether. The soluble protein was separated from the lipids by thin layer chromatography.

* Figs 2 A—E see Table on page 602 a.

** Figs 3 a—e see Tables on page 602 b and c.

in the titration curve until the label reaches an amount of $0.16 \mu\text{mol}$ Fluram/mg of protein. This observation combined with the impermeability of the SR membranes for the reagent and the stability of the liposomal structure strongly suggests a highly asymmetric arrangement of the SR membrane lipids with the aminophospholipids facing the external membrane surface. When the lipids are labeled with approximately $0.12 \mu\text{mol}$ Fluram per mg of vesicular protein, Fluram reacts solely with the protein. As with other labels the membrane protein undergoes a drastic structural change when it was reacted with approximately $0.04 \mu\text{mol}$ per mg of protein^{18,19}. Consequently, the dye can only be used as a reliable surface label when applied at ratios below $0.1 \mu\text{mol}$ per mg of protein. The results of four experiments in which the fresh vesicles were labeled with $0.05 - 0.1 \mu\text{mol}$ per mg of protein and subsequently separated in their components (transport ATPase, lipid and calcium precipitating protein) are presented in Table II. The fluorescence of the protein of the transport ATPase and the vesicular lipids are measured with reference to the fluorescence of the native vesicles per mg of protein, F_0 . Two figures are given for the fluorescence of the calcium precipitating protein. First, the fluorescence is given for one mg of purified protein $F_{sp} \cdot 100/F_0$ and secondly, from this value the fluorescence of the calcium precipitating protein in the membrane is calculated using the distribution of the sarcoplasmic proteins in our preparations. These pre-

parations contain 8% calcium precipitating protein. The specific labeling of the calcium precipitating protein is considerably higher than that of the transport ATPase and it does not change when the vesicular structure is destroyed by DOC.

Discussion

The results demonstrate that Fluram can be used for labeling external membrane sites. As long as it is applied in amounts lower than that of the available amino groups in the membrane, it reacts nearly quantitatively with membrane proteins and amino-lipids. Added in excess, it is hydrolytically cleaved in a few seconds to yield a non fluorescing component. In contrast to most other reagents it is possible to determine the degree of labeling in advance and thus avoid excessive labeling of the sarcoplasmic membrane. The structure collapses when the degree of protein labeling reaches $0.04 \mu\text{mol}/\text{mg}$ of protein. The SR membrane becomes permeable and the interior space of the vesicles can be reached by the label. This is very likely also the case with various other labels. Furthermore, some labels may even penetrate the membrane directly as it has been put forward by Tsai *et al.*²⁰ who demonstrate that iodine could easily penetrate the membranes of the erythrocytes when iodide oxydation was performed by the addition of hydrogen peroxide. The fact that the calcium precipitating protein is labeled by Fluram even under the most gentle conditions

Table II. Fluram labeling of protein and lipid components of native and DOC treated SR vesicles. Native vesicles and vesicles (300 mg of protein) made leaky by the addition of DOC (0.5 mg/mg of protein) were labeled with $0.05 - 0.1 \mu\text{mol}$ Fluram/mg of protein as described in Methods. Subsequently, the transport protein, the membrane lipids and the calcium precipitating protein were isolated from aliquots. The fluorescence intensity is expressed as percentage of the fluorescence intensity of native vesicles related to their protein content $F \cdot 100/F_0$. The fluorescence intensity of the calcium precipitating protein is given for one mg of isolated protein itself (F_{sp}) as well as the partial fluorescence intensity of the calcium precipitating protein within the native vesicles. Both values are related to F_0 . Note that in the presence of DOC the degree of Fluram labeling of the preparations is somewhat reduced and that DOC causes a reduction of lipid labeling with a corresponding increase of protein labeling. Labeling of the calcium precipitating protein is not affected by DOC. Its labeling obviously does not depend on the physical state of the vesicles and/or the presence of DOC.

Native vesicles		Calcium transport protein		Vesicular lipids		Calcium precipitating protein		Calcium precipitating protein	
closed $F \cdot 100/F_0$	lysed $F \cdot 100/F_0$	closed $F \cdot 100/F_0$	lysed $F \cdot 100/F_0$	closed $F \cdot 100/F_0$	lysed $F \cdot 100/F_0$	closed $F_{sp} \cdot 100/F_0$	lysed $F_{sp} \cdot 100/F_0$	closed $F \cdot 100/F_0$	lysed $F \cdot 100/F_0$
100	88	22	32	72	52	94	80	7.4	6.3
100	93	16	24	67	59	69	95	5.5	7.6
100	89	21	31	54	49	58	56	4.6	4.5
100	103	28	42	—	—	52	48	4.2	3.8
Average values	93.2	21.7	32.2	64	53	68	69	5.4	5.5

strongly supports its external location in the SR membrane. This arrangement seems to be inconsistent with the findings that trypsin cannot hydrolyze the calcium binding protein as long as it is embedded in the membrane and that externally applied diazoaminophenyl dextran crosslinks the ATPase whereas the calcium precipitating protein is not affected and can be separated from the cross-linked material by gel electrophoresis⁹.

This ambiguity would be resolved if the calcium binding protein is located at sites in the surface of the membrane not accessible for molecules with a diameter larger than 30–40 Å. This would imply the presence of some kind of crypts in the membrane in which the calcium precipitating protein is hidden.

At present, the possibility that part of the calcium precipitating protein may span the membrane cannot be excluded. Even if the calcium binding domains of the calcium precipitating protein were to be located internally, the contribution which the protein could give to calcium storage must be considered to be relatively unimportant. It would be saturated only at a free calcium concentration of 10 mM, and at a free calcium concentration of 1 mM only 30% of its sites would be occupied²¹. At an ionized calcium concentration of 1 mM less than 30% of the stored calcium would be bound to the calcium precipitating protein. This is in agreement with Meissner's observation that fractions of SR vesicles rich in precipitating proteins do not store significantly more calcium than fractions containing only little of them¹². The other calcium binding proteins which are not precipitated by calcium contribute even less to calcium storage because they occur only in small amounts in the SR membranes and their constants for calcium binding do not allow chelation of appreciable amounts of calcium²².

Another aspect of these labeling experiments is the fact that the degree of labeling of the calcium precipitating protein does not change when the vesicular structure is destroyed by DOC. This is in contrast to the increased labeling of the transport ATPase and the reduced labeling of the lipid fraction. The labeling of the different constituents with Fluram occurs in proportion to the number of the labile groups present in the vesicular constituents and also to the rates at which these groups react with the label. As a further difficulty the possibility must be taken into account that the labeling reaction is influenced by the presence of the detergent. The

invariance of the labeling of the calcium precipitating protein on the one hand and the variance observed in the labeling of the protein of the transport ATPase and of the lipids can be reconciled by the following consideration. The vesicles are labeled with Fluram according to the distribution of the reactive amino groups in the membrane protein a_0 , in the lipid constituent b_0 and in the calcium precipitating protein c_0 . The different rates of labeling are taken into account by introducing r , s and t as relative rate factors. Hence, the relative degree of labeling for the calcium precipitating protein is

given by the expression $\frac{t \cdot c_0}{r \cdot a_0 + s \cdot b_0 + t \cdot c_0}$. DOC or

other disruptive treatments make internal binding sites a_i , b_i and c_i available for labeling. It is assumed that the internal sites represent constant fractions of the external sites $a_i = \alpha a_0$, $b_i = \beta b_0$, $c_i = \gamma c_0$. The relative rates with which Fluram reacts in the presence of the detergent are taken into account by introducing r' , s' and t' . For lysed vesicles the relative degree of labeling of the calcium precipitating proteins is given by the expression

$$\frac{t' c_0 (1 + \gamma)}{r' a_0 (1 + \alpha) + s' b_0 (1 + \beta) + t' c_0 (1 + \gamma)}.$$

For the calcium precipitating protein, the transport protein and the membrane lipids in closed and lysed vesicles the following three relationships are obtained:

$$\begin{aligned} & \frac{t' c_0}{r a_0 + s b_0 + t c_0} \\ &= \frac{t' c_0 (1 + \gamma)}{r' a_0 (1 + \alpha) + s' b_0 (1 + \beta) + t' c_0 (1 + \gamma)}, \end{aligned} \quad (1)$$

$$\begin{aligned} & \frac{u r a_0}{r a_0 + s b_0 + t c_0} \\ &= \frac{r' a_0 (1 + \alpha)}{r' a_0 (1 + \alpha) + s' b_0 (1 + \beta) + t' c_0 (1 + \gamma)}, \end{aligned} \quad (2)$$

$$\begin{aligned} & \frac{v s b_0}{r a_0 + s b_0 + t c_0} \\ &= \frac{s' b_0 (1 + \beta)}{r' a_0 (1 + \alpha) + s' b_0 (1 + \beta) + t' c_0 (1 + \gamma)}. \end{aligned} \quad (3)$$

u and v are the labeling ratios of lysed and closed vesicles as they are observed in the experiment for the membrane proteins and the membrane lipids respectively. From Eqns (1), (2), and (3) the relations

$$u = \frac{t r' (1 + \alpha)}{t' r (1 + \gamma)}$$

are obtained:

$$v = \frac{t s' (1 + \beta)}{t' s (1 + \gamma)}$$

These relations demonstrate the dependence of the labeling ratios on the rates of labeling and on the distribution of the respective components. If the latter two relationships are introduced in Eqns (1), (2) or (3) the following identity is obtained

$$r a_0 + s b_0 = u r a_0 + v s b_0$$

asserting that at a constant degree of labeling of the calcium precipitating protein in closed and lysed vesicles the total fluorescence of transport protein plus membrane lipids coincides in both preparations

although the labeling ratios may change. The values for a_0 , b_0 , c_0 and the coefficients α , β and γ which determine the distribution of the membrane components cannot be obtained without further information. Although the preferential labeling of the aminophospholipids by Fluram strongly suggests a value of zero for β and a value of 0.10–0.12 $\mu\text{mol/mg}$ of vesicular protein for b_0 , there are still too many unknown parameters remaining for an estimate of a_0 , c_0 , α and γ .

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