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# STUDIES ON THE HETEROGENEITY OF SARCOPLASMIC RETICULUM VESICLES

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## Summary

Isolated sarcoplasmic reticulum vesicles from rabbit white muscle were separated into a light (15—20% of total microsomes) and a heavy (80—85%) fraction by density gradient centrifugation.

The ultrastructure, chemical composition, enzymic activities and localization of membrane components in the vesicles of both fractions were investigated.

From the following results it was concluded that both fractions are derived from the membranes of the sarcoplasmic reticulum system of the muscle:

(i) The protein pattern of both fractions is essentially the same, except for different ratios of acidic, $Ca^{2+}$-binding proteins.

(ii) The 105000 dalton protein of the light fraction cross-reacts immunologically with the $Ca^{2+}$-dependent ATPase of the heavy fraction.

(iii) $Ca^{2+}$-dependent ATPase, although of different specific activity, is found in both fractions. After rendering the vesicles leaky, specific activities in both fractions reach the same value.

The light fraction was found to consist of "inside-out" vesicles by the following criteria:

(i) No $Ca^{2+}$ accumulation can be measured and the $Ca^{2+}$-dependent ATPase activity is low and variable.

(ii) The rate of trypsin digestion is lower and, compared to the heavy microsomes, a different ratio of degradation products is obtained.

(iii) The sarcoplasmic reticulum membrane has a highly asymmetrical lipid distribution. This distribution of aminophospholipids is opposite to that in vesicles of heavy fraction.

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Abbreviations: DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EDTA, ethyleneglycol-bis(β-aminoethylether)-*N,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; TNBS, 2,4,6-trinitrobenzenesulfonate.

The light sarcoplasmic reticulum fraction has a higher phospholipid to protein ratio than the heavy one. This is consistent with the possibility that the two fractions derive from different parts of the sarcoplasmic reticulum system.

## Introduction

The sarcoplasmic reticulum membrane is an especially suitable system for study of the molecular organization of biological membranes because of its structural and functional simplicity [1].

Fragments of sarcoplasmic reticulum, obtained as a result of homogenization of muscle, reseal to form vesicles, which during differential centrifugation appear in the microsomal fraction. It has been established long ago [2] that the fraction of heavy muscle microsomes, sedimenting between 8000 and 30000 × *g*, is able to accumulate calcium. This was the reason why this fraction has been commonly used for studies.

It has been shown that the vesicles of heavy sarcoplasmic reticulum fraction display an asymmetry in the transverse plane. This concerns ultrastructure [3—6] and localization of proteins [7—11] and phospholipids [12,13].

The light microsomal fraction from muscle, sedimenting between 30000 and 100000 × *g*, has previously been shown not to accumulate calcium [2,14] and hence it has not been a subject of interest for authors studying the role of sarcoplasmic reticulum system in muscle contraction. Our recent studies [15] have indicated that the light fraction also originates from the sarcoplasmic reticulum membrane. These studies [15], as well as those of Chevallier et al. [16], have suggested that "inside-out" vesicles of sarcoplasmic reticulum membrane might be present in the light microsomal fraction.

Recently in several laboratories the total microsomal fraction, sedimenting between 8000 and 100000 × *g*, has been fractionated by means of sucrose density gradient centrifugation [15,17—22]. However, because of divergent results concerning ultrastructure [15,17,19,21,22], chemical composition [15, 18—20,22] and the activity of $Ca^{2+}$ transport [15,17—22] of the vesicles present in the light microsomal fraction, the origin of these vesicles has still remained unclear.

The aim of the present work was to characterize the vesicles of the light muscle microsomal fraction in detail, and to compare the orientation and localization of proteins and phospholipids in the membrane of vesicles of both light and heavy fraction, in order to determine whether the vesicles in the light fraction are indeed "inside-out" oriented.

## Materials and Methods

### *Preparation of sarcoplasmic reticulum fractions*

Sarcoplasmic reticulum vesicles were obtained essentially as described previously [15]. The trimmed fast skeletal muscles from rabbit were minced and homogenized with a Polytron PT-20 (3 × 15 s at 4.5 speed) with 5 vols. of 20 mM imidazole, pH 7.2/100 mM KCl (called throughout this paper imidazole/KCl buffer). The homogenate was centrifuged at 4500 × *g* for 20 min, and

the supernatant was centrifuged at 8000 × *g* for 30 min. Both pellets were discarded.

*Heavy sarcoplasmic reticulum fraction.* The 8000 × *g* supernatant was centrifuged at 30000 × *g* for 60 min. The pellet was suspended in imidazole/KCl buffer, layered on a continuous sucrose density gradient ranging from 8.5 to 68% sucrose and centrifuged at 130000 × *g* for 120 min. Usually two main layers were obtained. The minor layer located at 25—30% sucrose consisted of light microsomes, present as a contamination in the 30000 × *g* pellet. The main fraction, forming the layer at 30—37% sucrose, was collected, diluted with imidazole/KCl buffer and centrifuged at 100000 × *g* for 60 min. The pellet, suspended in imidazole/KCl buffer, was used as the heavy sarcoplasmic reticulum fraction.

*Light sarcoplasmic reticulum fraction.* The supernatant obtained after centrifugation at 30000 × *g* (see above) was centrifuged subsequently at 100000 × *g* for 60 min and the pellet was suspended in imidazole/KCl buffer. This crude, light sarcoplasmic reticulum fraction revealed the activity of phosphorylase *b* (approx. 7.0 μmol $P_i$/mg protein per min) and contained glycogen (approx. 0.45 mg/mg protein), indicating the presence of glycogen-protein particles [15]. For further purification, the fraction was washed with imidazole/KCl buffer by centrifugation at 100000 × *g* for 60 min. This procedure, leading to the removal of almost all glycogen-protein particles, was repeated three times. This partially purified light sarcoplasmic reticulum fraction had only traces of phosphorylase *b* activity and of glycogen (Table I). It was layered on a continuous sucrose density gradient ranging from 8.5 to 68% surcose and centrifuged at 130000 × *g* for 120 min. The main fraction found at 25—30% sucrose was collected, suspended in imidazole/KCl buffer and used as the light sarcoplasmic reticulum fraction.

The light fraction accounted for 15—20% and the heavy one for 80—85% of total microsomes sedimenting between 8000 and 100000 × *g* for 1 h.

Contamination with other cell organelles was monitored using glucose-6-phosphatase and 5′-nucleotidase [23] activities for endoplasmic reticulum and sarcolemma membranes, respectively, and phosphorylase *b* activity [24] and glycogen content [25] for glycogen-protein particles. Both purified sarcoplasmic reticulum fractions had very low activities of 5′-nucleotidase and glucose-6-phosphatase (Table I).

TABLE I

ACTIVITY OF MARKER ENZYMES IN THE LIGHT AND HEAVY SARCOPLASMIC RETICULUM FRACTIONS

Limit values are given. Number of determinations in parentheses.

| Sarcoplasmic reticulum fraction | (nmol $P_i$/mg protein per min) | | | Glycogen (μg/mg protein) |
|---|---|---|---|---|
| | Glucose-6-phosphatase | 5′-Nucleotidase | Phosphorylase | |
| Light | 1.5—2.5 (5) | 0.7—1.2 (5) | 150—200 (10) | 45—50 (10) |
| Heavy | 0.5—1.0 (5) | 0.5—1.0 (5) | 0.00 (10) | 0.00 (10) |

*Determination of calcium accumulation and ATPase activities*

Total ATPase activity was determined in a mixture of 100 mM KCl/20 mM imidazole, pH 7.2/5 mM $MgCl_2$/5 mM ATP/5 mM potassium oxalate/0.1 mM EGTA/0.5 mM $CaCl_2$/100 $\mu$g microsomal protein in a total volume of 2 ml. For determination of $Mg^{2+}$-dependent ATPase activity, the medium contained 1 mM EGTA and $CaCl_2$ was not added. The reaction was started by addition of ATP. After 5 min incubation at 25°C the reaction was stopped by addition of 1 ml 4% SDS. The liberated inorganic phosphate was determined according to Fiske and Subba Row [26]. The activity of $Ca^{2+}$-dependent ATPase was calculated as the difference between total ATPase activity and $Mg^{2+}$-dependent ATPase activity. Calcium accumulation was measured in the same mixture as for determination of total ATPase activity except that $^{45}CaCl_2$ was added. After incubation the mixture was filtered through a Millipore filter (HA, 0.45 $\mu$M) according to a procedure described previously [27]. Radioactivity of the filtrates was counted in a Chicago Nuclear Corp. gas-flow counter with a Micromil window.

*Detection of the ATPase with antiserum*

100 $\mu$g microsomal protein from light fraction was solubilized in a mixture of 0.5% Triton X-100/0.2% SDS/0.5% deoxycholate/50 mM borate buffer, pH 8.3 and centrifuged at 100000 × *g* for 60 min. Proteins present in the supernatant were labeled with a fluorescent dye, fluorescamine. For that purpose a solution of fluorescamine in acetone was rapidly added to the supernatant to a final concentration of 0.15 $\mu$mol/mg protein. After 10 min incubation in ice the solution was treated with 0.25 ml of rabbit anti-rat ATPase serum. The specific immunoprecipitates, which had been formed after 72 h incubation in ice, were recovered by centrifugation at 20000 × *g* for 30 min, and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were examined for fluorescence in ultraviolet light. The heavy sarcoplasmic reticulum fraction was used as a control.

*Extraction of sarcoplasmic reticulum vesicles with EDTA*

Sarcoplasmic reticulum vesicles (5 mg protein/ml) in imidazole/KCl buffer were extracted for 30 min with 1 mM EDTA at pH 8.0 and 4°C with constant stirring. Subsequently, the suspension was centrifuged at 100000 × *g* for 60 min.

*Digestion of sarcoplasmic reticulum vesicles with trypsin*

Digestion of sarcoplasmic reticulum vesicles (5 mg protein/ml) with trypsin was carried out at 25°C in a solution containing imidazole/KCl buffer and 5 mM $CaCl_2$, with a ratio of trypsin to sarcoplasmic reticulum protein of 1 : 100 (w/w). Digestion was stopped by the addition of soya-bean trypsin inhibitor (4 mg/mg trypsin) in imidazole/KCl buffer. The suspension was centrifuged at 100000 × *g* for 60 min and the pellets were used for further analysis. In some experiments, the reaction was stopped by boiling the digested samples in the presence of 1.3% SDS, 0.33% 2-mercaptoethanol and phosphate buffer, pH 7.0.

*Digestion of sarcoplasmic reticulum vesicles with phospholipases*

*Phospholipase C.* Sarcoplasmic reticulum vesicles (about 10 mg protein), suspended in imidazole/KCl buffer in a final volume of 2 ml were incubated at 20°C for 10 min with 0.5 units of phospholipase C from *Bacillus cereus* in the presence of 4 mM $CaCl_2$. The reaction was terminated by addition of a chloroform/methanol mixture (1 : 2, v/v) and lipids were extracted according to Bligh and Dyer [28].

*Phospholipase $A_2$.* The incubation medium was the same as described for phospholipase C except that it contained 0.25 M sucrose and 20 mg bovine serum albumin per ml. Phospholipase $A_2$ from pig pancreas was added at a ratio of 0.5 units per 10 mg of sarcoplasmic reticulum protein. After 10 min incubation at 20°C, the reaction was stopped by addition of 10 mM EGTA and rapid cooling. The suspension was layered on 0.5 M sucrose and centrifuged at $30000 \times g$ for 30 min. The pellet was washed three times with imidazole/KCl buffer containing 1% bovine serum albumin. Serum albumin was removed by washing with cold imidazole/KCl buffer. The pellet was suspended in the same buffer and lipids were extracted according to Bligh and Dyer [28].

*Labeling of sarcoplasmic reticulum vesicles with 2,4,6-trinitrobenzenesulfonate (TNBS)*

Light and heavy sarcoplasmic reticulum fractions (20 mg protein) were incubated for 60 min at 25°C in a medium containing imidazole/KCl buffer and 0.25 M sucrose and various concentrations of TNBS. After incubation, the suspensions were centrifuged at $50000 \times g$ for 60 min and pallets were suspended in imidazole/KCl buffer. Lipids were extracted according to Bligh and Dyer [28].

*SDS-polyacrylamide gel electrophoresis*

Electrophoresis was carried out on 7.5% polyacrylamide slab-gels according to Weber and Osborn [29]. Proteins were stained with Coomassie Brilliant Blue R-250.

*Chemical analysis*

Total lipids were extracted according to the procedure of Folch at el. [30] or Bligh and Dyer [28] and separated by two-dimensional silica gel thin-layer chromatography as described by Rouser et al. [31]. Spots containing lipids were detected by iodine vapour, scraped into tubes and digested with perchloric acid. Silica gel was removed by centrifugation and inorganic phosphate was determined in the supernatants according to Chen et al. [32].

Protein and glycogen content were determined according to Lowry et al. [33] and Krisman [25], respectively, total cholesterol according to Searcy and Bergquist [34], and inorganic phosphate according to Fiske and Subba Row [26].

*Chemicals*

ATP, EGTA, trypsin and soya-bean trypsin inhibitor were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. $^{45}CaCl_2$ was obtained from the Institute of Nuclear Research, Swierk, Poland. TNBS was obtained from ICN

Pharmaceuticals, Cleveland, Ohio, U.S.A., and fluorescamine from Serva Chemical Company. Other reagents were of analytical grade. Organic solvents were distilled before use. Phospholipase C from B. cereus was a gift of Dr R.F.W. Zwaal and phospholipase $A_2$ from pig pancreas was a gift of Dr A.J. Slatboom from the University of Utrecht. Anti-ATPase serum was donated by Dr D.H. MacLennan, University of Toronto, Canada.

## Results

### *ATPase activities and calcium accumulation*

Both sarcoplasmic reticulum fractions showed a $Ca^{2+}$-dependent ATPase activity; however, this activity was much lower in the light than in the heavy fraction and varied from one preparation to another (Table II). Rendering the vesicles leaky to $Ca^{2+}$ by an EDTA treatment abolished this variability. Moreover, after such a treatment the $Ca^{2+}$-dependent ATPase activity reached almost the same value in both light and heavy microsomes (Table II). Unlike the $Ca^{2+}$-dependent ATPase activity, the $Mg^{2+}$-dependent ATPase activity was slightly higher in the light than in the heavy fraction and EDTA treatment had no effect on that activity in both fractions (Table II).

In contrast to the heavy fraction, practically no $Ca^{2+}$ accumulation by the light fraction was observed (Table II).

### *Protein and lipid composition*

As revealed by SDS polyacrylamide gel electrophoresis, the protein pattern of both fractions was virtually the same. However, some differences in the relative proportions of individual bands were observed. The 105000 dalton $Ca^{2+}$-dependent ATPase accounted for about 70% of the total protein in the heavy sarcoplasmic reticulum fraction and for 75–80% in the light one. The identity of the 105000 dalton band in the light fraction with the ATPase protein present in the heavy fraction was proved by immunoprecipitation (Fig. 1, gels $L_1$ and $H_1$).

Additional protein bands present in both sarcoplasmic reticulum fractions

TABLE II

ATPase ACTIVITY AND CALCIUM ACCUMULATION OF THE LIGHT AND HEAVY SARCOPLASMIC RETICULUM FRACTIONS

$Ca^{2+}$-dependent ATPase activity was calculated as the difference between total and $Mg^{2+}$-dependent ATPase activity. Limit values are given, with the number of determination in parentheses.

| Sarcoplasmic reticulum fractions | ($\mu$mol $P_i$/mg protein per min) | | $Ca^{2+}$ accumulation ($\mu$mol $Ca^{2+}$/mg protein per 5 min) |
|---|---|---|---|
| | $Mg^{2+}$-dependent ATPase | $Ca^{2+}$-dependent ATPase | |
| Light | | | |
| Original | 0.25—0.30 (10) | 0.20—0.60 (10) | 0.00—0.02 (10) |
| EDTA treated | 0.24—0.30 (5) | 0.80—1.10 (5) | — |
| Heavy | | | |
| Original | 0.14—0.16 (20) | 0.90—1.00 (20) | 3.00—4.00 (10) |
| EDTA treated | 0.15—0.17 (5) | 1.10—1.20 (5) | — |

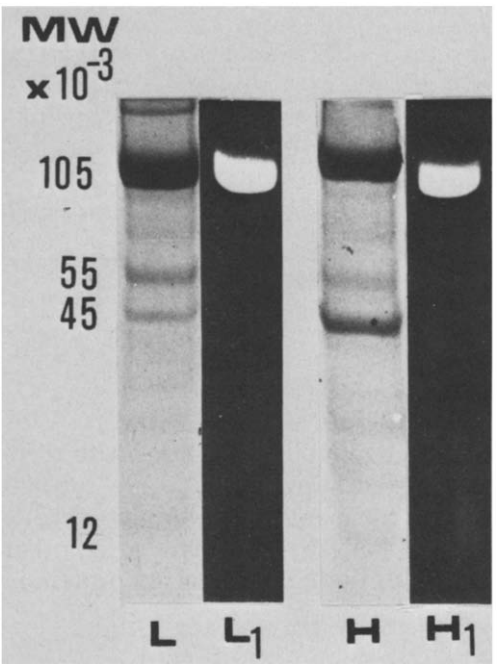


Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins of the light and heavy sarcoplasmic reticulum fractions. L and H, protein pattern of intact light and heavy sarcoplasmic reticulum fraction, respectively; $L_1$ and $H_1$, immunoprecipitates formed during the reaction between solubilized light or heavy sarcoplasmic reticulum fraction (fluorescamine-labeled) and anti-ATPase serum; unstained gels were illuminated with an ultraviolet lamp. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in 7.5% slab-gels. For details see Materials and Methods. MW, molecular weight.

corresponded to the peripheral proteins: calsequestrin ($M_r$ = 45000) and high affinity $Ca^{2+}$ binding protein ($M_r$ = 55000). The heavy fraction contained more calsequestrin than high affinity $Ca^{2+}$ binding protein. Treatment of the vesicles with EDTA led to removal of about 20% of total proteins form both fractions. This released material was subjected to SDS polyacrylamide gel electrophoresis. From proportions of peak areas of corresponding protein bands on gel densitograms it was estimated that calsequestrin accounts for about 15—17% of the total protein of the heavy fraction versus 3—5% of the high affinity $Ca^{2+}$ binding protein. In the light fraction this ratio was inverted, 4—6% of calsequestrin versus 10—12% of high affinity $Ca^{2+}$ binding protein.

The proteolipid was present in the vesicles of both sarcoplasmic reticulum fractions in similar amounts.

The ratio of phospholipids to protein was almost one and a half times higher in the light than in the heavy sarcoplasmic reticulum fraction, although the phospholipid pattern and cholesterol content were very similar in both fractions (Table III). As shown in Table III, phosphatidylcholine was the major phospholipid in the vesicles of both fractions. Phosphatidylethanolamine was the second major phospholipid, followed by phosphatidylinositol, phosphatidylserine and sphingomyelin.

TABLE III

LIPID CONTENT AND COMPOSITION OF THE LIGHT AND HEAVY SARCOPLASMIC RETICULUM FRACTIONS

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin. Limit values are given. Data of 10 experiments.

| Sarcoplasmic reticulum fractions | Phospholipid/protein (mg/mg) | (% of total phospholipid) | | | | | Cholesterol (μg/mg protein) |
|---|---|---|---|---|---|---|---|
| | | PC | PE | PS | PI | Sph | |
| Light | 0.9—1.1 | 70—73 | 12—13 | 3.4—3.7 | 7.6—7.7 | 4.0—4.2 | 10—20 |
| Heavy | 0.7—0.75 | 74—76 | 13—14 | 3.5—3.6 | 7.5—7.8 | 3.0—4.2 | 15—25 |

*Proteolytic degradation of sarcoplasmic reticulum proteins*

Digestion of the vesicles of both sarcoplasmic reticulum fractions with trypsin in the presence of 5 mM $CaCl_2$ resulted in the degradation of the 105000 dalton ATPase and in the formation of four fragments of molecular weights 55000, 45000, 30000 and 20000 (Fig. 2, gel H). In agreement with other authors [8,35], the first degradation products of the 105000 dalton protein in

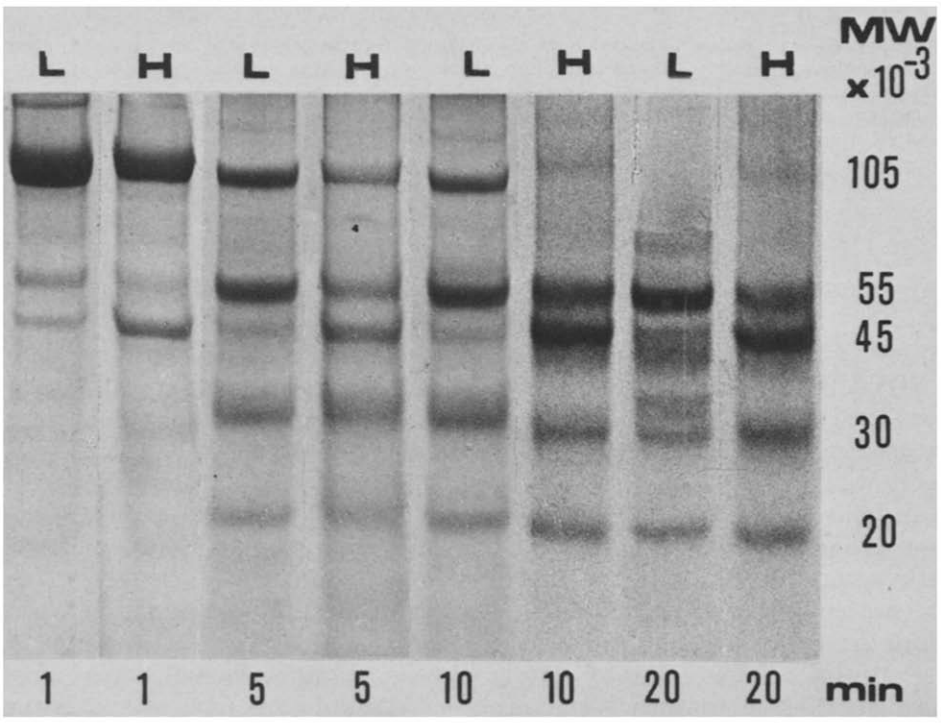


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the proteins of the light (L) and heavy (H) sarcoplasmic reticulum fractions digested with trypsin. Digestion with trypsin was carried out in the presence of 5 mM $CaCl_2$ for the time indicated in the figure. Trypsin to sarcoplasmic reticulum protein ratio was 1 : 100. Digestion was stopped by addition of soya-bean trypsin inhibitor, the vesicles were centrifuged for 60 min at 100000 × *g* and the pellets were used for electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on 7.5% slab-gels.

the heavy fraction had molecular weights of about 55000 and 45000 (Fig. 2, gel H). The latter fragment was relatively resistant to trypsin, whereas the 55000 dalton fragment was cleaved to 30000 and 20000 dalton fragments (Fig. 2, gel H). The 105000 dalton protein present in the vesicles of the light sarcoplasmic reticulum fraction was more resistant to trypsin than the same protein in the vesicles of the heavy fraction (Fig. 2, gel L). Although the products of degradation had virtually the same molecular weight, an essential difference between both sarcoplasmic reticulum fractions was observed. During digestion of the light sarcoplasmic reticulum fraction the 55000 dalton fragment was degraded much slower than the corresponding fragment in the heavy fraction, while the 45000 dalton fragment was split faster than the analogous fragment in the heavy fraction (Fig. 2, gel L).

The treatment with EDTA makes the sarcoplasmic reticulum vesicles permeable to macromolecules [36]. As a result of the treatment of vesicles of both

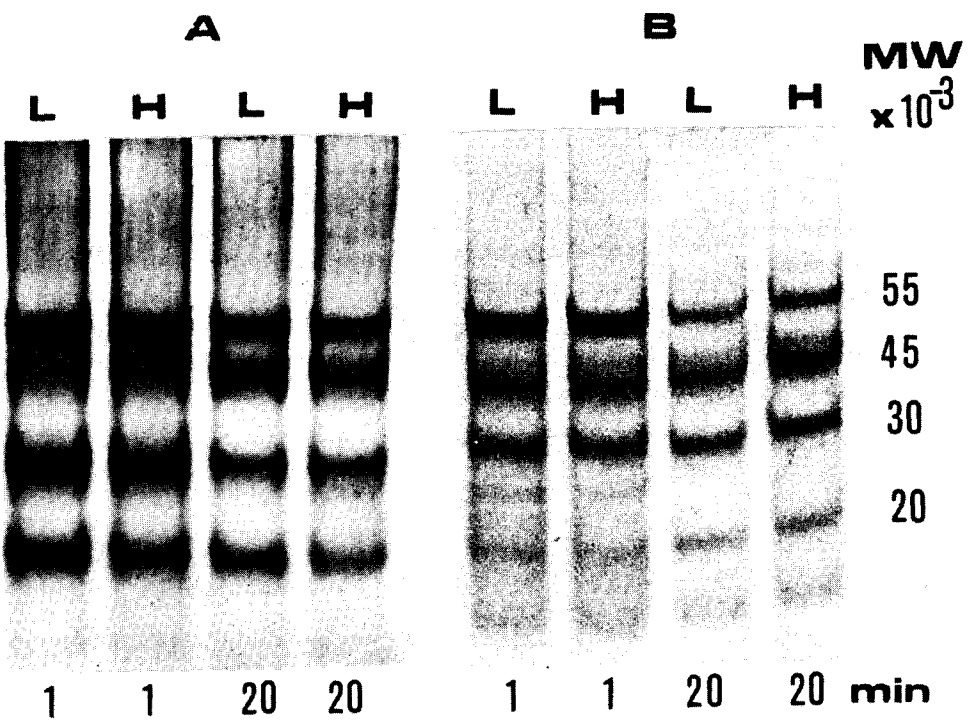


Fig. 3. Trypsin digestion of vesicles of the light (L) and heavy (H) sarcoplasmic reticulum fraction treated with EDTA or with EDTA and Triton X-100. A, vesicles of sarcoplasmic reticulum fractions were extracted with 1 mM EDTA at pH 8.0 and centrifuged for 60 min at 100000 $\times g$. Pellets, suspended in imidazole/KCl buffer, were digested with trypsin in the presence of 5 mM $CaCl_2$ under the same conditions as described in the legend to Fig. 2. The reaction was stopped by addition of soya-bean trypsin inhibitor. The vesicles were centrifuged and the protein pattern of pellets was analyzed. B, vesicles of both sarcoplasmic reticulum fractions treated with EDTA and then additionally extracted with Triton X-100 (2 mg/mg protein) were centrifuged at 100000 $\times g$ for 60 min. Triton X-100 extracts were digested with trypsin in the presence of 5 mM $CaCl_2$. The reaction was stopped by heating the digested samples in the presence of 1.2% sodium dodecylsulfate, 0.33% 2-mercaptoethanol in phosphate buffer, pH 7.0. The conditions of gel electrophoresis were as described in the legend to Fig. 1.

sarcoplasmic reticulum fractions with EDTA, the 105000 dalton ATPase became much more susceptible to trypsin and the difference in the digestion rate between both fractions disappeared. In some experiments the pellets obtained from the vesicles treated with EDTA were extracted with Triton X-100 in order to solubilize the membrane. This latter treatment did not produce any additional changes in the rate of splitting in both sarcoplasmic reticulum fractions and in the pattern of the obtained peptides (Fig. 3, gels A, and B).

*Digestion of sarcoplasmic reticulum vesicles with phospholipases*

After 10 min incubation of intact vesicles of both sarcoplasmic reticulum fractions with phospholipase C, about 40—45% of phospholipids were hydrolyzed. Most of the diglycerides, which were formed during digestion, remained bound to the membrane. Under these conditions the activity of $Ca^{2+}$-dependent ATPase was virtually unchanged. During this limited digestion, the individual phospholipids were hydrolyzed to a very different extent, as shown in Table IV. Prolongation of the time of digestion with phospholipase C led to almost complete hydrolysis of all phospholipids (about 90% of the total phospholipid), concomitantly with the loss of $Ca^{2+}$-dependent ATPase activity.

The treatment of both sarcoplasmic reticulum fractions with phospholipase $A_2$ in the presence of serum albumin led to degradation of about 40% of phospholipids after a short time of incubation. Again, different classes of phospholipids show different susceptibilities to hydrolysis (Table IV). After a prolonged exposure to the enzyme, an almost complete hydrolysis of all phospholipids took place. Most of the lysocompounds formed during digestion with phospholipase $A_2$ were released from the membrane and were bound by serum albumin.

After treatment of the vesicles of both fractions with EDTA the difference in the digestion pattern between both sarcoplasmic reticulum fractions disappeared and either phospholipase led to almost complete degradation of all phospholipids in a very short time.

TABLE IV

DIGESTION OF THE VESICLES OF LIGHT AND HEAVY SARCOPLASMIC RETICULUM FRACTIONS WITH PHOSPHOLIPASES C AND $A_2$

The vesicles of both sarcoplasmic reticulum fractions were digested with phospholipase C from *B. cereus* or with phospholipase $A_2$ from pig pancreas at 20°C for 10 min in the presence of 4 mM $CaCl_2$. For details see Materials and Methods. For abbreviations see Table III. Limit values of six experiments are given.

| Sarcoplasmic reticulum fractions | Phospholipase | (% hydrolysis) | | | | |
|---|---|---|---|---|---|---|
| | | PC | PE | PS | PI | Sph |
| Light | C | 43—45 | 15—18 | 8—12 | — | 10—12 |
| | $A_2$ | 40—45 | 15—20 | 8—10 | 90—95 | — |
| Heavy | C | 45—50 | 64—80 | 80—90 | — | 3—5 |
| | $A_2$ | 42—48 | 70—80 | 90—92 | 5—10 | — |

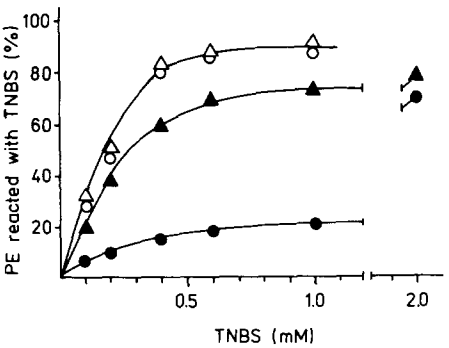


**Fig. 4. Labeling of phosphatidylethanolamine (PE) by TNBS. Intact or EDTA-treated vesicles of both fractions (see Materials and Methods) were incubated in the presence of TNBS at the concentrations indicated in the figure. After 2 h incubation samples were centrifuged for 30 min at 100000 × *g*. Pellets were washed with imidazole/KCl buffer and lipids were extracted according to Bligh and Dyer [28] and separated by thin-layer chromatography. The reaction of phosphatidylethanolamine with TNBS was measured by following the loss of phosphatidylethanolamine. In both cases inorganic phosphate was determined in the scraped spots by the method of Chen et al. [32]. Intact vesicles of light (●) and heavy (▲) fraction, and EDTA-treated vesicles of light (○) and heavy (△) fraction.**

*Labeling of the sarcoplasmic reticulum vesicles with TNBS*

Fig. 4 shows the amount of phosphatidylethanolamine labeled with TNBS in intact or in EDTA-treated vesicles of both sarcoplasmic reticulum fractions. About 70% of this phospholipid present in the heavy, but only 20% in the light sarcoplasmic reticulum fraction reacted with TNBS at a concentrations lower than 1 mM.

After solubilization of the vesicles with deoxycholate or their treatment with EDTA, about 85—90% of phosphatidylethanolamine was labeled in both sarcoplasmic reticulum fractions. The percentage of phosphatidylserine (not shown) which reacted with TNBS in the heavy and light sarcoplasmic reticulum fractions were very similar to that of phosphatidylethanolamine.

## Discussion

We have recently reported [15] that vesicles of the light microsomal fraction from muscle are not able to accumulate calcium despite the fact that they still reveal a $Ca^{2+}$-dependent ATPase activity and that their protein pattern is very similar to that of the heavy fraction, which is active in $Ca^{2+}$ uptake. Contrary to our observations, some authors reported previously that the light microsomal fraction does accumulate calcium [19—22]. Interestingly, some of them have found [20,22] that the light fraction has a low $Ca^{2+}$-dependent, but a relatively high $Mg^{2+}$-dependent and $Ca^{2+}$-independent ATPase activity.

The protein composition of the light microsomal fraction is also a controversial matter. According to Meissner [19] the vesicles present in this fraction contain almost exclusively the 105000 dalton ATPase; according to other investigators, in addition to this protein also a 55000 dalton one is present [20, 21]. On the other hand, Heilman et al. [22] have recently found that vesicles of the light fraction contain only low amounts of a protein corresponding to the ATPase and, instead, they contain several proteins of molecular weights

lower than that of the ATPase. Different isolation procedures might account for this variability.

In spite of different results, most authors have indicated on the basis of ultrastructure, chemical composition and activity of $Ca^{2+}$ transport that the vesicles of the light microsomal fraction are derived from sarcoplasmic reticulum [15,19—22]. The following results of the present work fully support this view: (i) the protein pattern of the light fraction is essentially the same as that of the heavy one, except for the higher amount of high affinity $Ca^{2+}$ binding protein than calsequestrin in the former, (ii) the immunoprecipitation test with anti-ATPase serum indicates that the 105000 dalton protein in the light fraction represents the sarcoplasmic reticulum ATPase, (iii) both fractions contain the same number of SH groups per molecule of ATPase reacting with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (15 SH equivalents reacting directly with DTNB and about 25 reacting after solubilization with SDS; Korczak, B., unpublished results).

If both the light and the heavy fraction originate from sarcoplasmic reticulum, the question arises why two kinds of vesicles are formed after fragmentation and resealing. One possibility is that they originate from different regions of the native membrane; this hypothesis will be discussed later. The second possibility, which of course does not exclude the first one, is the formation of inverted and right side-out vesicles. The sarcoplasmic reticulum membrane, like several other biological membranes [37], displays an asymmetry in the transverse plane. This has been shown by ultrastructural analysis of this membrane using both negative staining and freeze-etching [3,6,7], by antibody precipitation [7], limited proteolysis of membrane protein [8,35], fluorescence probes [9,13], labeling with $^{125}I$ [10], localization of free SH groups [5], and localization of aminophospholipids [12,13]. Because of this asymmetry, inverted vesicles are expected to behave differently from normal ones.

Some features of the two sarcoplasmic reticulum fractions obtained previously by us seemed to indicate that the vesicles of the light fraction are indeed "inside-out" oriented [15]. Chevallier et al. [16], using the freeze-etch technique, have also recently found that most of the vesicles of light sarcoplasmic reticulum fraction display an inverted structural asymmetry in comparison to the vesicles present in the heavy fraction. Further support to the view that the vesicles in the light fraction are inverted comes from several morphological and biochemical observations. The morphological observations (Sarzała, M.G., Michalak, M and Gulik-Krzywicki, T., unpublished results) are as follows: (i) no 4 nm particles are seen on the surface of the vesicles of the light fraction as revealed by negative staining technique, (ii) vesicles fixed with tannic acid appear asymmetrical in thin sections with an opposite sidedness in vesicles from both fractions, (iii) most of the vesicles of the light fraction display the inverted type of fracture plane. The results of biochemical studies concern the topology of the ATPase protein, localization of its SH groups [38], localization of peripheral proteins [39] and distribution of phospholipids in the membrane of both sarcoplasmic reticulum fractions.

Differences between both sarcoplasmic reticulum fractions in the rate of digestion of the ATPase molecule with trypsin and in the tendency of accumulation of different fragments of this protein are in favour of the opposite orien-

tation of ATPase molecules in both fractions. The difference in the peptide pattern obtained during digestion between the vesicles of both fractions disappears after treatment with EDTA, when vesicles become permeable to macromolecules [36] and presumably to trypsin.

In contrast to the vesicles of the heavy fraction, there is no reaction between the vesicles of the light fraction and thiolated Sepharose 4B, a reagent nonpenetrating through vesicular membrane [38]. In view of the observations [5] that SH groups are localized on the outer surface of the vesicles of heavy sarcoplasmic reticulum fraction, the lack of SH groups interacting with a nonpenetrating reagent in the light fraction is further evidence for the inverted orientation of the vesicles of this fraction. If the impermeability of the membrane to ATP is taken into account, the inverted vesicles should have no ATPase activity. The experimental finding that such an activity, although variable, can be observed in the light fraction, could be explained by some degree of leakiness of all or some vesicles to ATP and $Ca^{2+}$; the extent to which the vesicles are imperfectly resealed could vary in different preparations. An alternative explanation would be a variable content of right-side-out vesicles in the light fraction. We do not consider it very likely, however, because of the lack of $Ca^{2+}$ accumulation even in preparations with a relatively high ATPase activity.

Pronounced differences between the vesicles of both sarcoplasmic reticulum fractions have been also found in the content of individual phospholipids in both leaflets of the membrane bilayer, as revealed by the digestion with phospholipase and labeling with TNBS. The results of this paper show that phosphatidylethanolamine and phosphatidylserine are digested with phospholipase C or $A_2$ to 70% and 80%, respectively, in the heavy fraction, but only to about 20% in the light fraction. This may indicate that these phospholipids are located mainly in the outer leaflet of the membrane bilayer of heavy sarcoplasmic reticulum vesicles and in the inner leaflet in the light vesicles. Similar localization of aminophospholipids has been found with TNBS labeling. It should be noted that our results concerning distribution of phosphatidylethanolamine in the heavy sarcoplasmic reticulum fraction are in agreement with those found by Vale [12] and Hidalgo and Ikemoto [13].

The differences in the digestion of individual phospholipids with phospholipases and their labeling with TNBS between intact vesicles of both fractions disappeared when the vesicles were made leaky by EDTA treatment. Again, the simplest explanation of these results is that the light vesicles are inverted.

The simple assumption that the light and heavy vesicles differ only by the sidedness of their membrane, cannot explain all our findings, especially the different lipid to protein ration in the two fractions. However, in addition to the transverse asymmetry of sarcoplasmic reticulum membrane, its lateral asymmetry has been also recently described, mainly on the basis of morphological analysis [21,40]. The particle distribution in freeze-fracture preparation of the whole muscle seems to be not homogenous in various regions of the sarcoplasmic reticulum system. The density of particles is higher in the longitudinal elements than in terminal cisternae [21,40]. These observations led Scales et al. [21] to the suggestion that the sarcoplasmic reticulum vesicles with low and high particle density, originate from terminal cisternae and longitudinal elements, respectively.

The high phospholipid content found in this work and the low number of particles observed by Scales et al. [21] and us (Sarzała, M.G. and Gulik-Krzywicki, T., unpublished results), seem to suggest that the vesicles of the light fraction derive from terminal cisternae. The possibility cannot be excluded that these membrane fragments which have a high phospholipid to protein ratio and, therefore, different physicochemical properties under the conditions used by us for homogenization, have a tendency to reseal in an opposite way and, consequently, to become "inside-out" oriented.

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