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ROLE OF CHOLESTEROL IN THE Ca^{2+} UPTAKE AND ATPase ACTIVITY OF FRAGMENTED SARCOPLASMIC RETICULUM

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SUMMARY

With the use of continuous sucrose density gradient a highly purified preparation of vesicles of fragmented sarcoplasmic reticulum was obtained and its lipid content was determined.

Short mild treatment of fresh vesicles with aqueous diethyl ether or aqueous heptane removes only a part of the cholesterol. Aqueous heptane treatment does not lead, contrary to that of aqueous diethyl ether, to the loss of the ability of vesicles to actively accumulate Ca^{2+}. Exhaustive extraction of lyophylized vesicles with dry diethyl ether or dry heptane enables removal of all cholesterol and most of the other neutral lipids without any changes in the Ca^{2+} uptake ability. Thus, the results show that the loss of specific properties of fragmented sarcoplasmic reticulum as a result of treatment with aqueous diethyl ether is due to the specific action of this solvent but not a result of the removal of some part of cholesterol from the membranes. Contrary to phospholipids, cholesterol and other neutral lipids seem to be bound "loosely" to the membranes of sarcoplasmic reticulum; can be easily extracted with apolar solvents in the absence of water and do not play any direct role in the specific function of this biomembrane system.

Cholesterol can be bound to the sarcoplasmic reticulum vesicles in large amounts.

INTRODUCTION

It is already well established that vesicles of fragmented sarcoplasmic reticulum exhibit the specific capacity to accumulate Ca^{2+} coupled with the splitting of ATP. (For review see ref 1.)

Several authors[2-4] have recently observed that a short, mild treatment of the vesicles with aqueous diethyl ether causes an uncoupling of Ca^{2+} accumulation from the ATPase activity; in spite of a loss of Ca^{2+} uptake capacity, Ca^{2+}-dependent ATPase is even increased. Fiehn and Hasselbach[3] interpreted this phenomenon as being due to the leakiness of vesicular membranes as a result of treatment with diethyl ether.

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The authors found that only a small part of cholesterol esters was extracted under these conditions, and assumed that the appearence of leaks in the membranes was due to the removal of this lipid constituent.

It seemed, however, difficult to understand how a removal of a minute amounts of cholesterol esters could be sufficient to explain a loss of ability of reticular membranes to accumulate Ca^{2+} on the basis of leakiness. Hence, in this work an attempt was made to examine this problem in detail with the use, under various conditions, of other solvents besides diethyl ether and to correlate the removal of particular lipids with the changes in the specific function of this muscle subcellular fraction. These studies seemed to be also appropriate in view of the important role of cholesterol in biological membranes postulated currently[5]. Part of the results was presented on the 7th FEBS Meeting in Varna[6].

MATERIAL AND METHODS

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared from rabbit leg and back skeletal muscle as previously described[7]. Muscle mince was homogenized in 5 vol. of 0.1 M KCl and 20 mM histidine buffer (pH 7.2); fraction sedimenting between 5000 and 30000 $\times$ *g* (crude microsomal fraction) was collected, suspended in 0.25 M sucrose and purified in a continuous sucrose density gradient essentially according to Sreter[8]. Usually 5 ml of microsomal suspension (15–20 mg protein/ml) was layered on top of 50 ml of sucrose density gradient system prepared from equal volumes of 0.25 and 2 M neutralized solutions of sucrose, containing 1 mM EDTA. After centrifugation for 60–90 min at 2500 $\times$ *g* in MSE Refrigerator centrifuge two layers were obtained, the upper one not penetrating into the gradient, the lower located usually 6–8 cm below. Both fractions were collected and centrifuged at 90000 $\times$ *g* for 1 h. Pellets were suspended in 0.1 M KCl and 20 mM histidine buffer (pH 7.2).

Chemical determinations

ATPase activity was measured in 2 ml reaction mixture containing 0.1 M KCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM histidine (pH 7.2), 2.5 mM potassium oxalate and vesicles of sarcoplasmic reticulum (0.025–0.050 mg protein/ml). Reaction was started by addition of 2.5 mM ATP and after 5 min incubation at 25 °C was stopped with 2 ml 10% trichloroacetic acid. The activity of Ca^{2+}-dependent ATPase was obtained by subtracting the basal ATPase measured in the presence of 1 mM ethylene glycol bis-(β-aminoethyl ether) tetraacetate instead of $CaCl_2$.

Ca^{2+} uptake was measured in the same reaction mixture as for determination of the ATPase activity with the exception that $^{45}CaCl_2$ was used, instead of $^{40}CaCl_2$. After incubation for 5 min at room temperature $^{45}Ca^{2+}$ bound to the vesicles was removed by filtration through Milipore filters (HA 0.45 μm). Samples of the filtrate were counted in Chicago Nuclear Corp. gas-flow counter with Micromil window.

Total lipids were extracted using Folch *et al.*[10] or Blight and Dyer[11] procedures. Lipids were analyzed by thin-layer chromatography in a system containing *n*-hexane–diethyl ether–formic acid (6:4:0.16, by vol.). The content of total sterols was determined according to Searcy *et al.*[12]. Lipid phosphorus was determined according to

Bartlett[13]. Phospholipid content was calculated by multiplying the amount of phosphorus by 25.

Homogeneous suspensions of cholesterol were prepared by 2 min sonication with cooling in 0.1 M KCl and 20 mM histidine buffer (pH 7.2) using MSE, London sonicator.

Protein was determined by biuret method[14] using bovine plasma albumin as a standard.

Electron microscopy

Samples of pellets were fixed in 6 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) postfixed with 1 % buffered OsO_4, dehydrated with increasing concentrations of ethyl alcohol and acetone and finally embeded in Epon. Sections were cut in a Reichert ultramicrotome, stained with uranyl acetate and poststained with lead citrate.

For negative staining samples of suspension of sarcoplasmic reticulum vesicles were placed on Formvar-coated grids and stained with 2 % ammonium molybdate (pH 7.2) as previously described[15]. All specimens were examined in a JEM 7A electron microscope.

Materials

ATP and cholesterol were purchased from E. Merck, Darmstadt, Germany, EGTA from Sigma Chemical Co., U.S.A., $^{45}CaCl_2$ was obtained from the Institute of Nuclear Research, Poland and [4-^{14}C]cholesterol from the Radiochemical Centre, Amersham, England. All organic solvents were distilled before use.

RESULTS

Purification of the fragmented sarcoplasmic reticulum

The heavy microsome fraction of skeletal muscle obtained by differential centrifugation yielded in the continuous sucrose density gradient two fractions. The lighter one contained homogenous population of sarcoplasmic reticulum vesicles, freed of mitochondrial and miofibrillar contaminations, whereas lower layer from the sucrose density gradient contained myofibrils, fragments of mitochondria and usually only some vesicles originating from sarcoplasmic reticulum (Fig. 1). Negatively stained vesicles were more or less spherical with the surface covered with small, about 40 Å particles, as previously described by other workers[16,17] (Fig. 2). In the presence of $MgCl_2$, ATP, oxalate and $CaCl_2$ the average Ca^{2+} uptake was 3.60 μmoles Ca^{2+} per mg protein. In the electron microscope an appearance of nodular deposits of calcium oxalate was easily visible inside vesicles. When the suspension of sarcoplasmic reticulum vesicles after loading with calcium oxalate was fractionated on sucrose density gradient, as described by Graeser *et al.*[18], the bottom fraction consisted entirely of loaded vesicles (Fig. 3).

Vesicles of fragmented sarcoplasmic reticulum purified as described in Material and Methods were found to contain about 60 mg of lipids per 100 mg of protein. About 75 % of lipids consisted of phospholipids, two third of which was lecithin. In the neutral lipid fraction predominanted triglycerides. Besides smaller amount of diglycerides, cholesterol was present in the amount of about 4.4 mg per 100 mg of

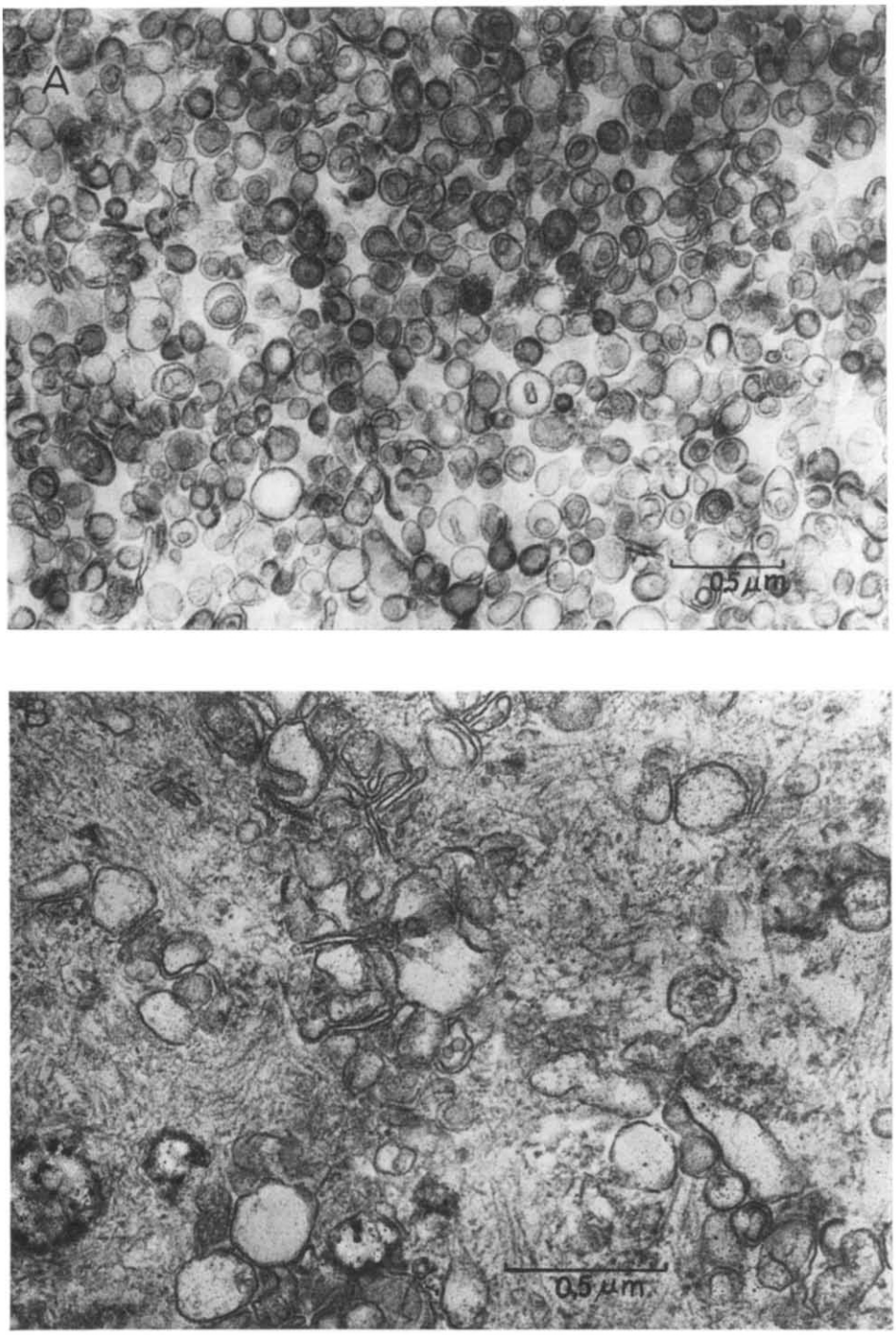

Fig. 1. Electron micrograph of thin sections of (A) upper and (B) lower fraction from continuous sucrose density gradient.

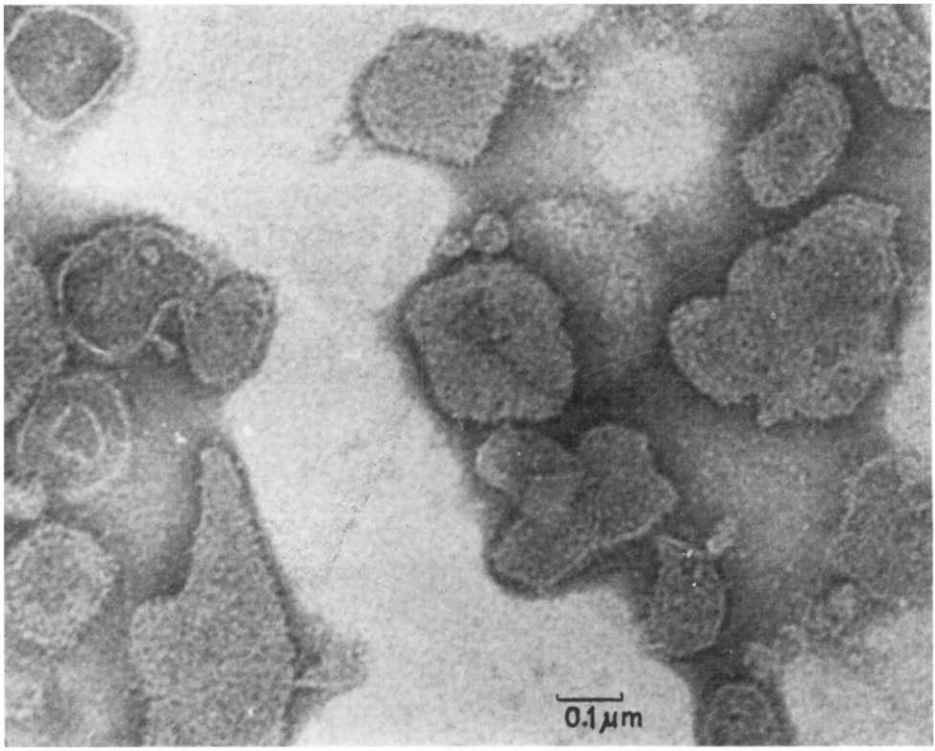

Fig. 2. Purified vesicles of the sarcoplasmic reticulum negatively stained with 2% phosphomolybdate.

protein, from which only about 10% was esterified. The amount of lipids found in the vesicular membranes in the present work is somewhat higher than that previously obtained by us[19] and by other workers. This is most probably due to the fact that vesicles of much higher purity were used at present.

Treatment of vesicles with aqueous solvents

In agreement with Fiehn and Hasselbach[3] we found that a mild treatment of vesicles with 6–8% diethyl ether caused a rapid decrease of the ability to accumulate calcium (Fig. 4). Simultaneously, an increase of Ca^{2+}-dependent ATPase took place. With much higher amounts of diethyl ether the activity of Ca^{2+} dependent ATPase also considerably decreased. However, when instead of diethyl ether heptane was used, its effect was completely different. No uncoupling of the ATPase activity from Ca^{2+} uptake was observed and, instead, an increase of both of these activities occurred. Even at the ratio of heptane to water equal one to one, Ca^{2+} uptake was still equal to that of the control. In spite of the pronounced difference between the effect of these two solvents on the properties of vesicular membranes the amounts and patterns of lipids extracted did not differ substantially (Fig. 4). In both cases cholesterol, especially in the esterified form, was the predominantly extracted lipid at low solvent to water ratio. The amount of total cholesterol being extracted increased slightly with the increasing proportion of solvent in the extracting mixture, reaching a value

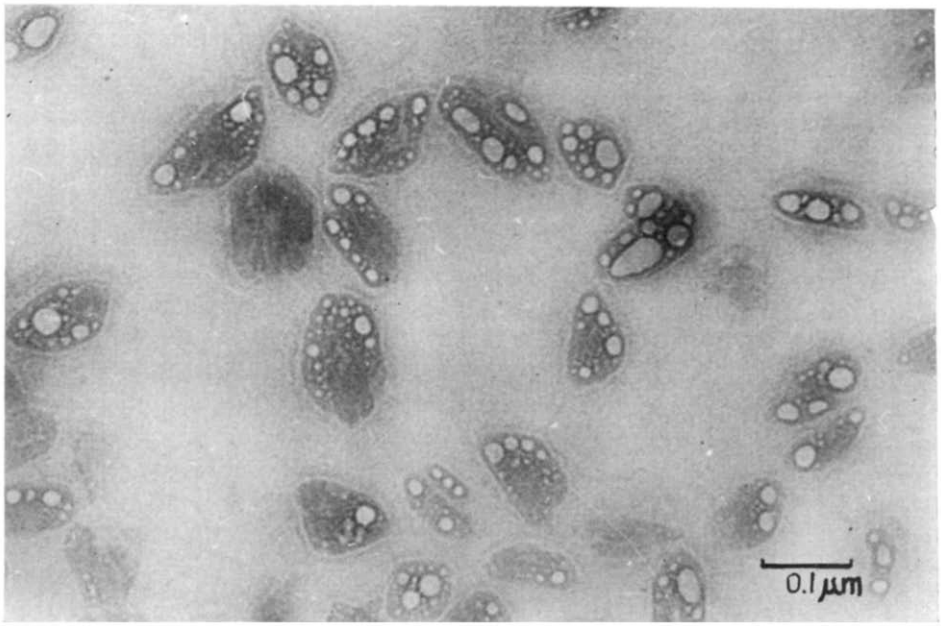

Fig. 3. Purified vesicles of the sarcoplasmic reticulum loaded with calcium oxalate. Incubation mixture contained: vesicles (1 mg protein/ml), 0.1 M KCl, 20 mM Tris–maleate (pH 6.5), 5.0 mM $MgCl_2$, 5.0 mM potassium oxalate, 5 mM ATP, 1 mM EGTA and 0.5 mM $CaCl_2$ added dropwise at every 30 s during stirring. After incubation for 5 min at 20 °C the suspension was centrifuged for 30 min at 100000 × *g*. Pellet was suspended in 0.5 ml 0.25 M sucrose and layered on the sucrose density gradient, according to ref. 18 and centrifuged in a Spinco SW-39L swing-out rotor for 1 h at 39000 rev./min. Fraction sedimented at the bottom of the tube was collected and negatively stained with 2% ammonium molybdate.

of about 25–30 % of total cholesterol at the highest solvent to water ratio examined. Concomitantly with cholesterol some triglycerides, diglycerides and fatty acids, but only traces of phospholipids were extracted.

Repeating extraction with water-solvent mixture lead during each extraction to the removal of a new portion of cholesterol and traces of phospholipids. The highest amounts of phospholipids were removed when an excess of diethyl ether in the presence of water was used. Even in this case however, the quantity extracted accounted only for a few percent of the total amount of phospholipids present in the vesicles. All the results showed that the changes in the ATPase activity and Ca^{2+} uptake depended on the kind of solvent used, but not on the amount of cholesterol removed.

Treatment of lyophylized vesicles with dry solvents

When the treatment with the solvents was performed on dried vesicles the obtained pattern was entirely different. For this purpose lyophylized vesicles were used. Control determinations showed that freeze–drying of sarcoplasmic reticulum fragments did not change activity of Ca^{2+}-dependent ATPase or Ca^{2+} storage ability.

During a single, 1 h extraction of dried vesicles with diethyl ether about 75 % of the total cholesterol, including all cholesterol esters, was removed (Table I). Dry

heptane extracted under the same conditions somewhat less, about 50 % of cholesterol. Other neutral lipids were at the same time also removed but in the amounts somewhat smaller than these of cholesterol (about 40 and 20 % of the total amount with diethyl ether and heptane, respectively). In both cases only few percent of phospholipids

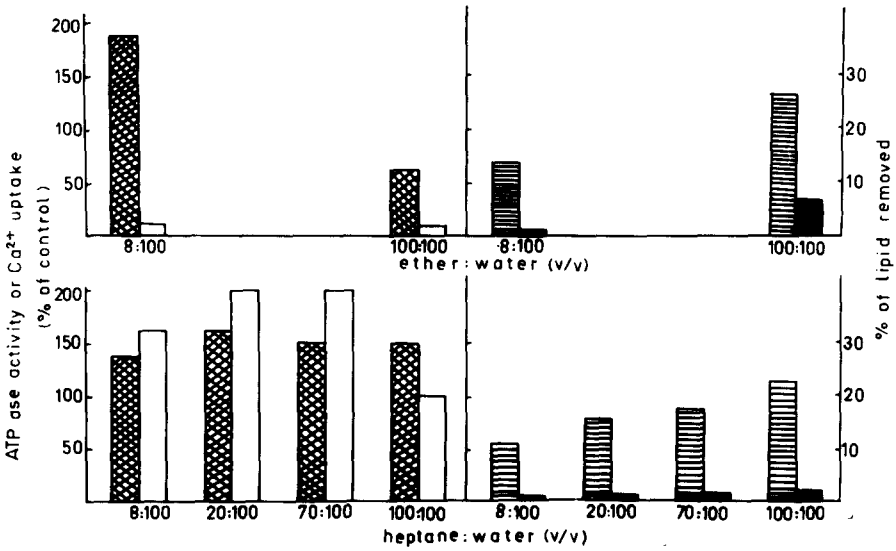

Fig. 4. Effect of single treatment of sarcoplasmic vesicles with solvent–water mixtures on the ATPase activity, Ca^{2+} uptake and removal of cholesterol and phospholipids. Vesicles suspensions (1–5 mg protein/ml) in 0.1 M KCl and 20 mM histidine (pH 7.2) were incubated with various amounts of organic solvents for 20 min at room temperature in closed tubes with gently shaking. After incubation samples were centrifuged for 1 h in Spinco ultracentrifuge at 100000 × *g*. Pellets were resuspended in KCl–histidine medium and taken for the ATPase activity and Ca^{2+} uptake measurements. Aliquots of supernatants were extracted 3 times with an excess of required solvent. Extracts were collected and evaporated. Lipids were dissolved in chloroform and taken for the cholesterol and phospholipid phosphorus determination. Checkered bars, ATPase activity; empty bars, Ca^{2+} uptake; dashed bars, total cholesterol; full bars, phospholipids.

TABLE I

REMOVAL OF LIPIDS FROM THE LYOPHYLIZED SARCOPLASMIC RETICULUM VESICLES

20 mg of lyophylized vesicles were extracted with 10 ml of dry solvent at 4 °C for 1 or 20 h. Extracts were collected and evaporated. The total amount of lipids extracted was determined by weighting. Cholesterol and phospholipids were determined as described in Material and Methods.

Treatment	*Lipids removed (mg/100 mg protein)*			
	Total	*Phospholipids*	*Neutral*	
			Cholesterol	*Others*
Diethyl ether				
1 h	11.2	2.6	3.2	5.4
20 h	12.0	3.2	3.6	5.2
Heptane				
1 h	7.2	2.4	2.0	2.8
20 h	9.0	3.2	2.5	3.3

were released. The increase of the time of extraction to 20 h did not increase substantially the amount of lipids extracted (Table I). As a result of two to three reextractions with ether the remaining 25 % of cholesterol was removed. A similar amount was released during reextraction with heptane. Since the third extraction with the

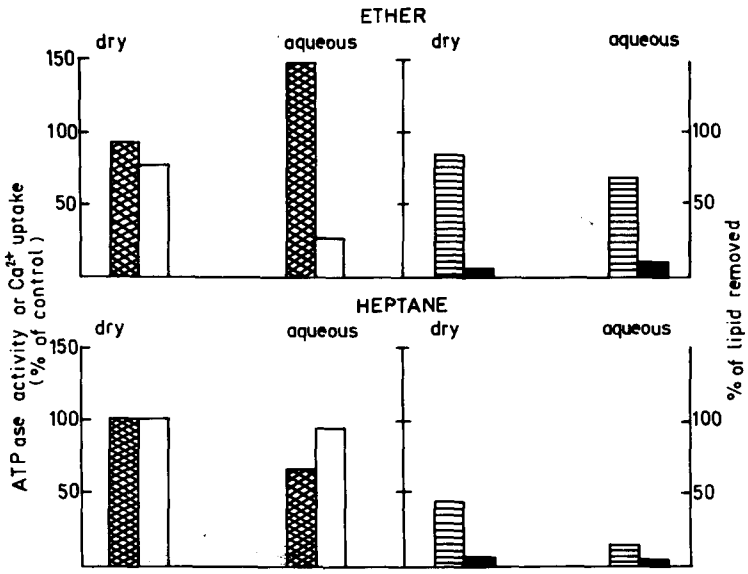

Fig. 5. Extraction of lyophylized vesicles with organic solvents. A. Extraction with dry solvents. 20 mg of lyophylized vesicles were extracted twice for 1 h with 20 ml of required solvent. Extracts were collected for lipid determinations. Residue, after careful evaporation off the solvent, was suspended in KCl–histidine medium and taken for ATPase activity and Ca^{2+} uptake measurements. B. Extraction with aqueous solvents. 20 mg of lyophilized vesicles were suspended in 4 ml KCl–histidine medium, gently shaken for 20 min with 1 ml of diethyl ether or heptane and then centrifuged in Spinco ultracentrifuge. After resuspension of the pellets in KCl–histidine medium, ATPase activity and Ca^{2+} uptake were measured. From the supernatants lipids were extracted. For details see legend to Fig. 4. Checkered bars, ATPase activity; empty bars, Ca^{2+} uptake; dashed bars, total cholesterol; full bars, phospholipids.

latter solvent did not remove any lipid material it seemed that about one third of cholesterol remained not extracted from the vesicles with dry heptane. Some part of glycerides and practically all phospholipids were not removed from lyophylized vesicles by either of the solvent used.

Fig. 5 compares the removal of lipids and the changes in the specific properties as a result of extraction of freeze-dried sarcoplasmic reticulum vesicles. One can see that in this case not only heptane but also diethyl ether, in spite of the removal of most of cholesterol, caused only a slight decrease of both Ca^{2+} storage ability and activity of Ca^{2+}-dependent ATPase. On the other hand, when water was added to the system, in agreement with the results discussed above, a substantial difference between the effect of diethyl ether and that of heptane was observed. The treatment with heptane–water mixtures did not, but that with diethyl ether–water mixtures did drastically change properties of the vesicles. All these results showed that the decline of Ca^{2+} accumulation ability was not directly related to the removal of cholesterol or its esters.

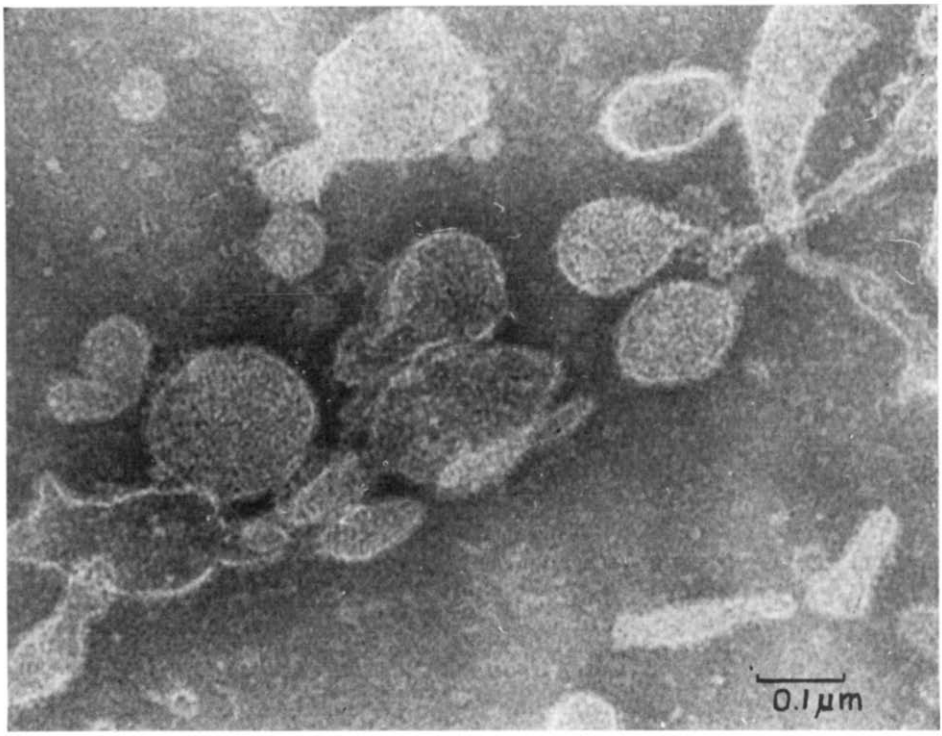

Fig. 6. Lyophylized vesicles of sarcoplasmic reticulum extracted with dry diethyl ether and negatively stained with 2% ammonium molybdate. Magnification: 114000 ×.

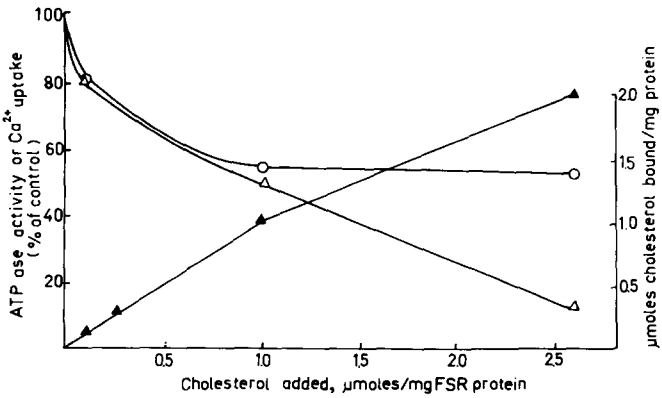

Fig. 7. Binding of cholesterol to sarcoplasmic vesicles and its effect on the ATPase activity and Ca^{2+} uptake. Samples of vesicles (1 mg protein/ml) were incubated with ultrasonically dispersed [^{14}C]cholesterol for 30 min. After incubation samples were centrifuged at 105000 × *g* for 30 min. Pellets were resuspended in the standard KCl–histidine medium. In one portion lipids were extracted and the radioactivity of bound cholesterol was measured. In the other portion ATPase activity and Ca^{2+} uptake were measured. ▲, binding of cholesterol; ○, ATPase activity; △, Ca^{2+} uptake. FSR protein = fragmented sarcoplasmic reticulum protein.

When the residue of lyophilized vesicles after extraction with dry diethyl ether or heptane was reextracted with the mixtures of either one of these solvent with the absolute ethanol the remaining neutral lipids and a large part of phospholipids were released. Concomitantly, activity of Ca^{2+}-dependent ATPase and Ca^{2+} uptake were completely abolished.

Negatively stained preparations of sarcoplasmic reticulum vesicles showed after treatment with aqueous diethyl ether, in agreement with Graeser *et al.*[4], an appearance of transparent patches. The latter were not observed after extraction of fresh vesicles with heptane–water mixture. No visible changes in size or shape were also found as a result of exhaustive extraction of lyophylized vesicles with dry diethyl ether or heptane (Fig. 6). In all these cases small 40-Å particles covering outer surface of native vesicles were still present.

Binding of cholesterol by the vesicles

Sarcoplasmic reticulum vesicles easily bound considerably amounts of cholesterol added in the form of sonicated aqueous suspension. Fig. 7 shows that cholesterol added in the amounts up to about 2.5 μmoles per mg protein, *i.e.* several times in excess over the amount of cholesterol originally present, was almost entirely bound by the vesicles. The extent of binding was the same whether fresh, native vesicles, vesicles devoid of cholesterol by the extraction with dry diethyl ether after lyophylization, or vesicles delipidated according to Folch *et al.*[10] were used. Concomitantly a parallel decrease of both, Ca^{2+}-dependent ATPase and Ca^{2+} uptake, took place, reaching about 50 % inhibition when about 1 μmole of cholesterol was bound per 1 mg of vesicular protein. Higher amounts of bound cholesterol caused further decrease of Ca^{2+} uptake ability without change in the ATPase activity. Cholesterol-treated vesicles had a tendency to aggregation. In the negatively stained vesicles treated with cholesterol the surface seemed to be rather smooth and 40-Å particles were less prominent. It is difficult to decide at present whether these phenomena were connected with some lytic effect of cholesterol bound to the vesicles or a result of much more difficult staining of vesicles covered with a layer of bound cholesterol.

DISCUSSION

Method of purification of sarcoplasmic reticulum vesicles, used first by Sreter[8] and employed in this work, seems to be the simplest and fastest method yielding fragmented sarcoplasmic reticulum of a high purity. It has an advantage over the methods in which discontinuous sucrose gradient was used[20] since it does not require an ultracentrifuge with a swing-out rotor but only a low-speed centrifuge with cooling. It is also certainly better than a treatment with 0.6 M KCl (ref. 21) which removes only myofibrillar impurities but not contaminations with mitochondria. Besides, one cannot exclude that high KCl concentrations remove some material from the vesicular membranes.

The present results indicate that from the point of view of extractability one can differentiate between particular classes of lipids in the membranes of sarcoplasmic reticulum. Neutral lipids seem to be "loosely" bound and hence can be relatively easily removed with nonpolar solvents. They seem not to play any essential role in the specific function of this subcellular structures, that is in the active transport of

Ca^{2+}. Even among neutral lipids it is also possible to distinguish two fractions of different extractability. About one third of cholesterol is not removed from freeze-dried vesicles with dry heptane; part of other neutral lipids is not extracted with either of the solvent used. On the other hand, phospholipids are bound to the vesicular membranes much more "strongly" and this class of lipids is virtually not removed by nonpolar solvents, and only with the solvents of higher polarity. In agreement with previous studies in which phospholipases for decomposition of vesicular phospholipids[9,22,23] were used, the present results employing extraction with various solvents show that whenever phospholipids are removed from the membranes the loss of specific function of sarcoplasmic reticulum vesicles occurs.

Similar studies on the effect of various solvents performed previously on the erythrocyte ghosts by Parpart and Ballentine[24], as well as Roelofsen and co-workers[25,26] also enabled a distinction between various classes of lipids classified from the point of view of their extractability and, consequently, of the kind of binding to protein. Thus, diethyl ether removed from the lyophylized red cell ghosts all sterols and about 20 % of phospholipids which were not essential for the activity of ($Na^+ + K^+ + Mg^{2+}$)-activated ATPase[26].

Lipids of red cell ghosts contain up to about 40 % of cholesterol and 55 % of phospholipids[27], hence, their pattern considerably differs from that of lipids of fragmented sarcoplasmic reticulum. In spite of this some analogy between both kinds of membranes can be drawn. In both cases cholesterol is only "loosely" bound and can be removed with the use of solvents of very low polarity, whereas phospholipids are in general "tightly" bound. Thus, the results of this work fit well to these recently proposed structures of biomembranes[27,28], according to which they are built from a protein lattice penetrated by lipid cylinders. Phospholipids are supposed to be bound through their hydrocarbon chains with the apolar regions of protein segments and through the heads with the polar groups of proteins, whereas cholesterol and possibly other neutral lipids are supposed to be associated chiefly with the apolar sites of the strongly bound phospholipids, and consequently are only "loosely" bound.

The present observations show that the effect of small amounts of diethyl-ether, only sufficient to saturate aqueous phase, which causes drastic changes in the properties of sarcoplasmic reticulum cannot be directly related to a specific removal of particular lipid and especially cannot be due to the removal of minute amounts of cholesterol esters, as it was suggested by Fiehn and Hasselbach[3]. On the one hand the presence of water during extraction with heptane does not abolish Ca^{2+} uptake and, on the other hand, ether also does not alter properties of sarcoplastic reticulum in the absence of water, in spite of the removal of all cholesterol. Alterations in the ultrastructure of vesicles are parallel to the biochemical changes. All present results indicate that leaks appearing in sarcotubular vesicles as a result of a treatment with aqueous diethyl ether are due to a very specific effect of this mixture causing some conformational changes in the structure of the membrane. Similar phenomenon was observed by Roelofsen and co-workers who found that the treatment of lyophylized red cell ghosts with wet diethyl ether inhibited the activity of ($Na^+ + K^+ + Mg^{2+}$)-activated ATPase, contrary to dry diethyl ether, being without effect.

One may speculate, consistently with the suggestions made by Roelofsen *et al.*[29], that at critical concentration of diethyl ether essential bounds between

phospholipids and protein are weakened, altered or even broken. It is possible that after treatment with aqueous diethyl ether phospholipids do not occupy anymore their initial binding sites on the membrane proteins. This view seems to be supported by our unpublished observations showing that the digestion of phospholipids bound to sarcoplasmic reticulum membranes with phospholipase C, proceeding very slowly with native vesicles, is considerably increased when traces of diethyl ether are added to the incubation medium.

Sarcotubular vesicles can bind large amounts of cholesterol. Our experiments[6] with a polyene antibiotic-filipin, used as a fluorescence probe is order to characterize polarity of the environment of bound cholesterol seem to indicate that added cholesterol is bound to the outer surface of sarcoplasmic reticulum membranes. This view is consistent with the results of this work showing that added cholesterol gradually inhibits both, Ca^{2+}-dependent ATPase and Ca^{2+} uptake, as well as with the morphological changes in the ultrastructure of vesicles trated with cholesterol. All present results show that the effect of cholesterol is different from those of oleate[7,15] or lysolecithin[9,23,30].

The effect of various sterols on the properties of sarcoplasmic reticulum fragments was studied previously by Martonosi[31] who found that, contrary to some other sterols, cholesterol up to the concentrations used in the present work, did not affect Ca^{2+} transport and ATPase activity. At the moment no resonable explanation for the discrepancy between these and the present results can be found. Perhaps the state of dispersion of cholesterol was different in both works and this led to different extent of binding of lipid to the vesicles. This is, however, difficult to reconcile since the latter was not measured by Martonosi.

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