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EFFECTS OF DIETHYL ETHER AND THYMOL ON THE ULTRASTRUCTURAL AND BIOCHEMICAL PROPERTIES OF PURIFIED SARCOPLASMIC RETICULUM FRAGMENTS FROM SKELETAL MUSCLE

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SUMMARY

1. Studies were conducted to determine if the biochemical alterations of sarcoplasmic reticulum fragments produced by diethyl ether or thymol treatment were related to the membrane ultrastructure.

2. Concentrations of diethyl ether or thymol that abolished Ca^{2+}-accumulating ability but did not reduce the Ca^{2+}-activated ATPase activity caused the formation of transparent patches on the surface of negatively stained vesicles.

3. Higher concentrations of diethyl ether or thymol caused a decrease in the Ca^{2+}-activated ATPase activity, an apparent loss of the 40-Å subunits and an increased irregularity in the vesicle surface structure. The vesicle shapes observed in sectioned preparations were also altered.

INTRODUCTION

The morphology of isolated sarcoplasmic reticulum fragments has been studied by several workers in attempts to establish the structural requirements for Ca^{2+} accumulation *in vitro*. Observations of negatively stained preparations have revealed many tadpole-shaped structures with their outer surface covered with 40-Å subunits[1-4]. Trypsin treatment, which abolished Ca^{2+}-accumulating ability and ATPase activity, caused swelling of the globular portion of the "tadpoles" and loss of the 40-Å subunits[1,4]. Ether and thymol treatment of sarcoplasmic reticulum fragments abolished the Ca^{2+}-accumulating ability and relaxing activity of these preparations, respectively[5-8]. However, little information has been obtained concerning the structural modifications produced by these latter treatments.

The present study was conducted to determine if the diethyl ether- and thymol-induced alterations of the biochemical properties of sarcoplasmic reticulum fragments were correlated with the ultrastructure of the membranes. The results suggest a definite relationship between the changes in Ca^{2+}-accumulating ability and in ATPase activity and the appearance of the membrane surface structure.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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METHODS

Preparation and purification of sarcoplasmic reticulum fragments

Sarcoplasmic reticulum fragments were prepared from the longissimus dorsi of pigs and rabbits essentially according to the procedure of MARTONOSI AND FERETOS[9]. These preparations were extracted for 1 h with 0.6 M KCl–5 mM histidine (pH 7.2) to solubilize contaminating actomyosin, and the sarcoplasmic reticulum fragments were reprecipitated by centrifugation[3]. The precipitate was resuspended in 0.1 M KCl–5 mM histidine and further purified on a sucrose density gradient[10]. Only the upper layer (which contained membrane fragments with the highest specific activity for Ca^{2+} accumulation) was used in these studies.

Diethyl ether and thymol treatment

Diethyl ether treatment of purified sarcoplasmic reticulum fragments was conducted using the procedure of INESI *et al.*[11] with minor modifications. Preparations were incubated for 10 min at 25° with 0–20 % (v/v) diethyl ether in 0.1 M KCl–5 mM histidine (pH 7.2). The suspensions were then cooled rapidly in ice, the excess ether was removed by aspiration and the sarcoplasmic reticulum fragments were precipitated by centrifugation at 30000 × *g* for 60 min. The precipitates were resuspended in KCl–histidine. A similar treatment with 0–4 mM thymol in 0.1 M KCl–5 mM histidine (pH 7.2) was employed.

Ca^{2+} uptake

Ca^{2+}-accumulating ability was determined using $^{45}Ca^{2+}$, with the reaction conducted at 25°. The sarcoplasmic reticulum fragments were removed from the medium (see legend of Fig. 1) after 15 min by means of Millipore filters[9]. The radioactivity of the filtrates and of appropriate standards was determined by liquid scintillation counting.

ATPase activity

Incubations (see legend of Fig. 1) were conducted at 25°, with aliquots withdrawn and added to 2 vol. of 7.5 % (w/v) ice-cold trichloroacetic acid at 1, 2, 3 and 5 min after the initiation of the reaction. The precipitated proteins were removed by centrifugation, and the inorganic phosphate was determined by the method of FISKE AND SUBBAROW[12].

Electron microscopy

Samples for embedding were fixed in 3 % biological grade glutaraldehyde and were post-fixed in 1 % osmic acid[13]. The samples were then soaked overnight in a 1:1 mixture of 95 % ethanol and 5 % aqueous uranyl acetate. They were subsequently dehydrated in ethanol and propylene oxide and were embedded in an Epon–Araldite resin mixture[14].

Suspensions of sarcoplasmic reticulum fragments were placed on parlodion and on carbon-coated grids and were negatively stained with 1 % potassium phosphotungstate (pH 7.0)[15]. Bovine serum albumin (0.01 %) was often added to facilitate the spreading of the stain. The grids were observed and photographed at 80 kV accelerating voltage in a Siemens Elmskop I electron microscope which was equipped with a liquid-N_2 cooling device.

Other methods

Protein concentrations were determined by use of the biuret procedure[16]. ATP was treated with Dowex 50-X8 in the H^+ form to remove contaminating Ca^{2+} (ref. 17). Distilled water which had been passed through a mixed-bed ion-exchange resin was used for all solutions.

RESULTS

The effects of diethyl ether treatment on Ca^{2+} uptake and ATPase activities of purified pig sarcoplasmic reticulum fragments are shown in Fig. 1. Similar results were obtained with rabbit muscle preparations. Ca^{2+}-accumulating ability was depressed nearly 50 % with the 5 % diethyl ether treatment and was practically abolished at the 10 % diethyl ether level. In spite of this loss in activity, the Ca^{2+}-activated ATPase (or "extra ATPase" of HASSELBACH AND MAKINOSE) was markedly increased by 5 and 10 % diethyl ether treatment. The ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)-ATPase (or "basic ATPase" of HASSELBACH AND MAKINOSE[18]) was not affected by diethyl ether. These results are in agreement with those of INESI *et al.*[11]. However, 20 % diethyl ether caused a reduction of the Ca^{2+}-activated ATPase compared to 10 % diethyl ether-treated preparations, while previous results indicated that the ATPase activity remained high at both concentrations (INESI *et al.*[11]). The fact that oxalate was used in the present system and not in the previous studies might explain this disagreement.

Although thymol has been shown to inactivate the relaxing factor[5,8], its action on the Ca^{2+}-accumulating ability and ATPase activity has not been previously examined in detail. Treatment with 2 mM thymol nearly abolished Ca^{2+} uptake (Fig. 2). The Ca^{2+}-activated ATPase activity was increased, but not to the extent that occurred with the diethyl ether treatment. The Ca^{2+}-activated ATPase was markedly inhibited by 4 mM thymol. The ATPase activity in the presence of EGTA

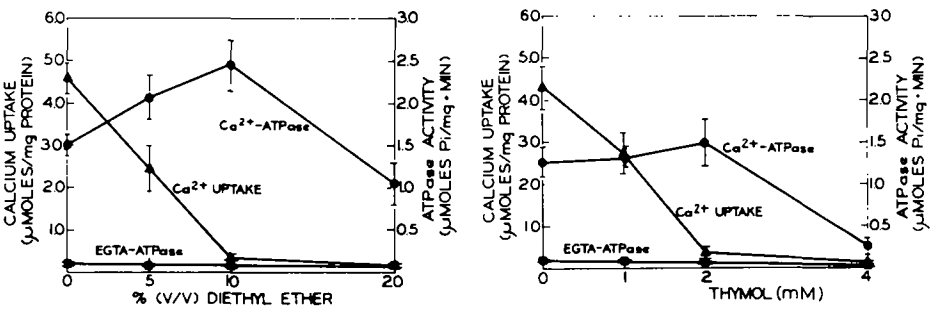

Fig. 1. Effects of diethyl ether treatment on sarcoplasmic reticulum Ca^{2+} accumulation and ATPase activity. Ca^{2+} uptake medium: 0.1 M KCl, 5 mM histidine (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, 5 mM $K_2C_2O_4$ and 0.2 mM $CaCl_2$ containing $^{45}Ca^{2+}$. Ca^{2+}-activated ATPase medium: same as for Ca^{2+} uptake, except the $CaCl_2$ was not radioactive. EGTA–ATPase medium: 0.1 M KCl, 5 mM histidine (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, 5 mM $K_2C_2O_4$, and 1 mM EGTA. Protein concentrations: Ca^{2+} uptake, 0.03 mg/ml; Ca^{2+}-activated ATPase, 0.03 mg/ml; EGTA–ATPase, 0.20 mg/ml. Each point represents the mean of 5 determinations ± S.E.

Fig. 2. Effect of thymol treatment on sarcoplasmic reticulum, Ca^{2+} accumulation and ATPase activity. Assay media and protein concentrations were identical to those used in Fig. 1. Each point represents the mean of 5 determinations ± S.E.

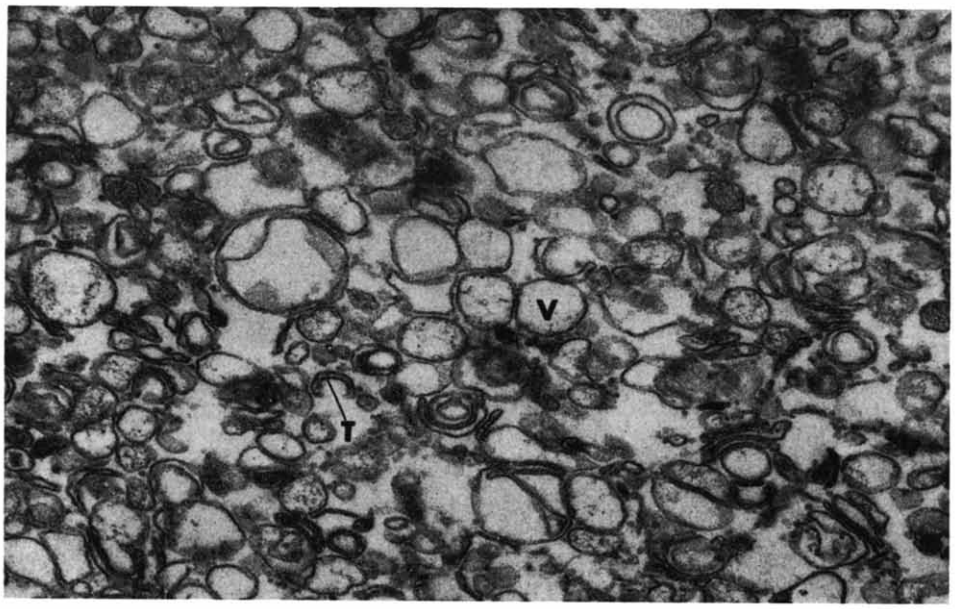

Fig. 3. Purified sarcoplasmic reticulum fragments (sectioned through middle of pellet). Most of the membranes appeared as vesicles (V) and tubules (T). × 40000.

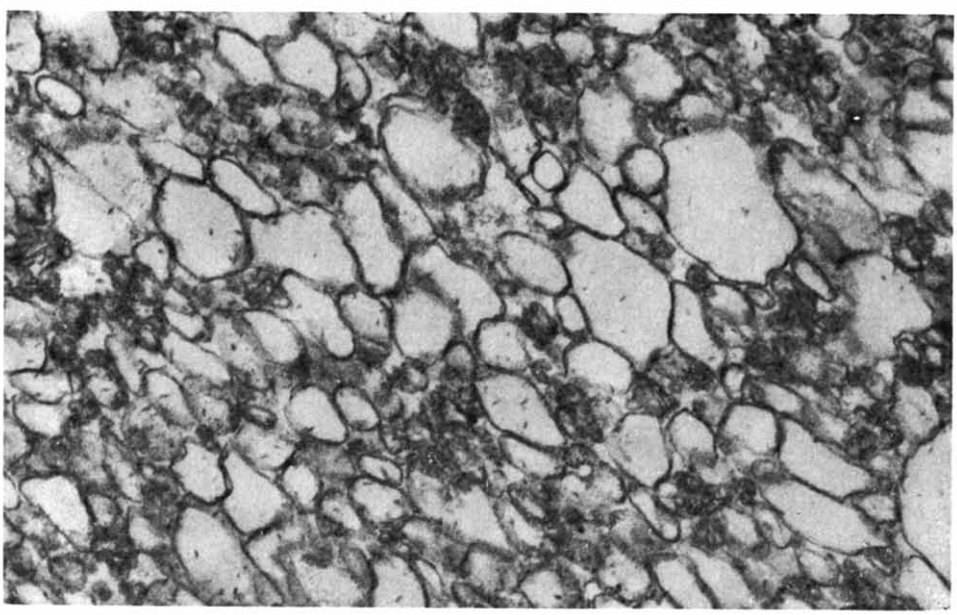

Fig. 4. Purified sarcoplasmic reticulum fragments which had been treated with 20% diethyl ether (sectioned through middle of pellet). The vesicles were closely aggregated, and the membranes were thinner than in control preparations. × 40000.

declined with increasing thymol concentrations, in contrast with that of diethyl ether-treated preparations. In spite of the minor differences noted, 10% diethyl ether and 2 mM thymol both appeared to uncouple the ATPase activity from the Ca^{2+} transport mechanism.

Electron micrographs of control, 20% diethyl ether-treated and 4 mM thymol-treated sarcoplasmic reticulum fragments are shown in Figs. 3, 4 and 5. The control preparations had many single- and double-contoured circular vesicles (V) which were interspersed with numerous tubular (T) fragments. The appearance and composition of this purified preparation was very similar to unpurified fractions which have been observed by others[5,19,20].

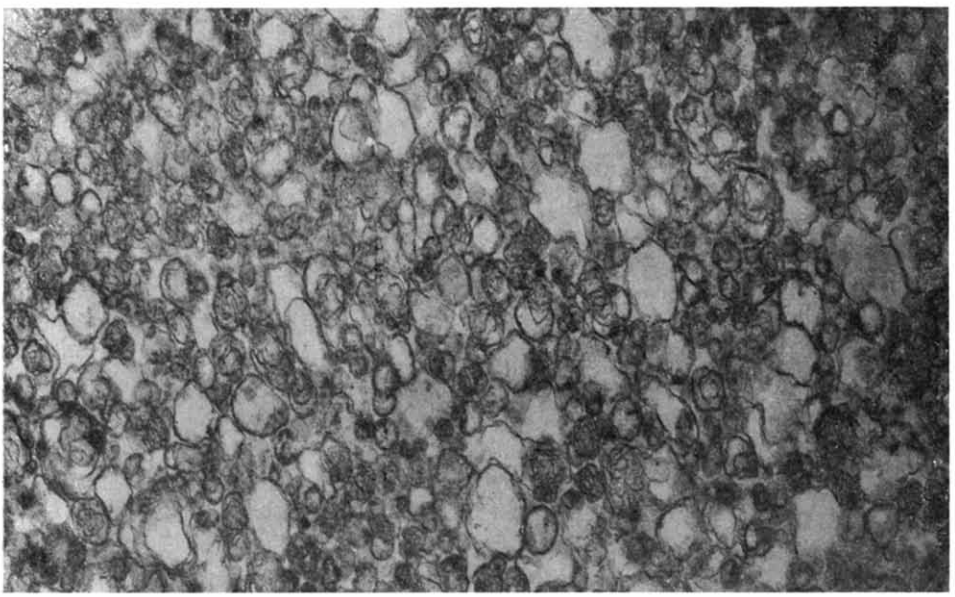

Fig. 5. Purified sarcoplasmic reticulum fragments which had been treated with 4 mM thymol (sectioned through middle of pellet). The interiors of some of these vesicles contained thread-like processes and granular material. × 40000.

The diethyl ether-treated preparations (Fig. 4) contained mainly swollen, irregularly shaped vesicles, with practically no tubules. The vesicles were closely aggregated, with the walls of adjoining vesicles appearing to be fused. The membranes were continuous but thinner than in control preparations. These observations are in agreement with results of previous work[11].

The appearance of the thymol-treated membranes differed from both the control and the diethyl ether-treated preparations (Fig. 5). Part of the membranes were swollen and empty looking like those that had been treated with diethyl ether. Others, however, had interiors which appeared to contain thread-like processes and granular material. The origin of this apparent internal structure could not be explained.

The appearance of negatively stained control sarcoplasmic reticulum fragments is shown in Figs. 6 and 7. The vesicles were mainly circular in shape, with some pos-

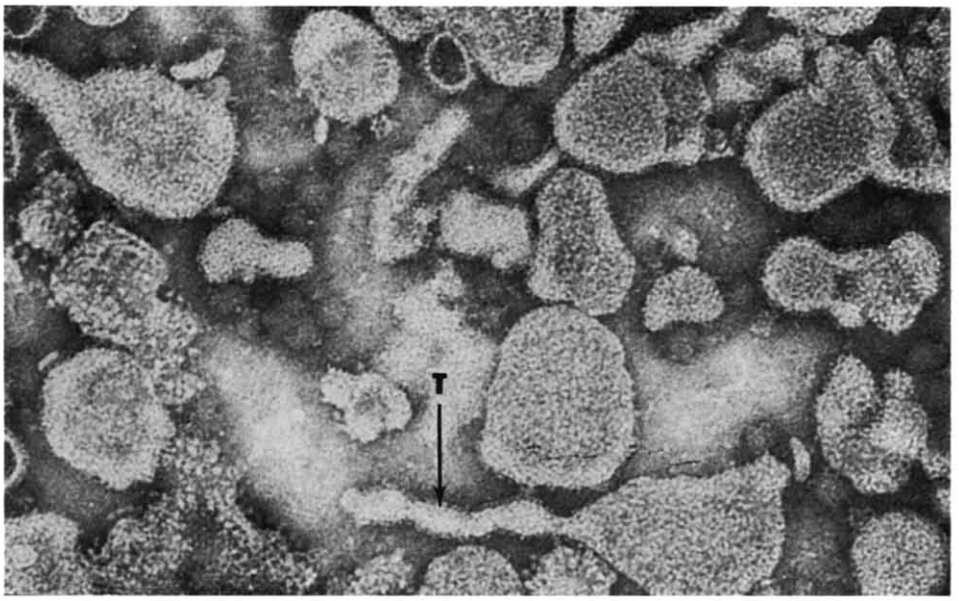

Fig. 6. Purified sarcoplasmic reticulum fragments (negatively stained). The vesicles were generally circular in shape, with some possessing tails (T). The surface of the membranes was usually granular in appearance. × 120000.

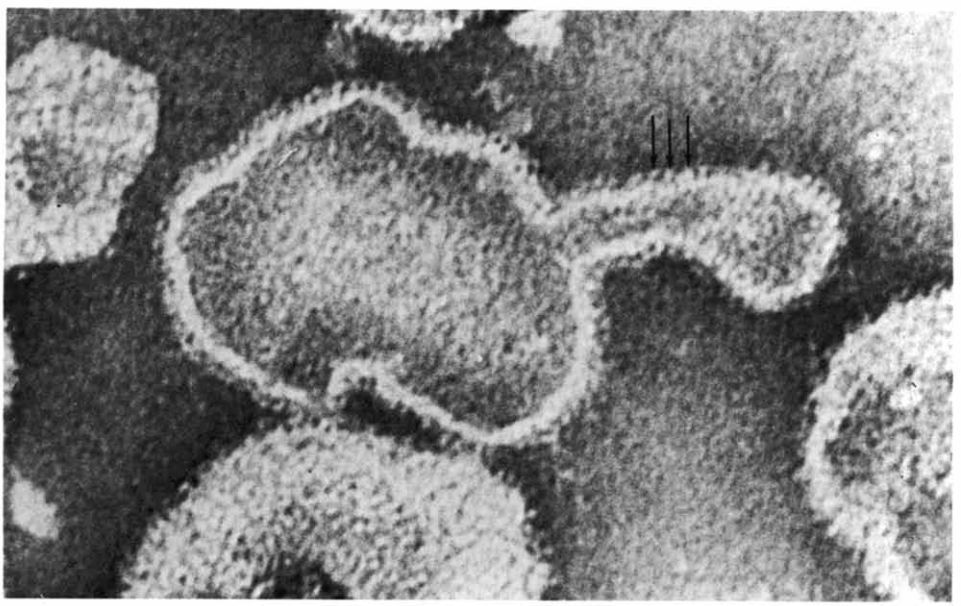

Fig. 7. Purified sarcoplasmic reticulum fragments (negatively stained). The edge of the vesicles was lined with spherical subunits (arrows) which were 35–40 Å in diameter. × 300000.

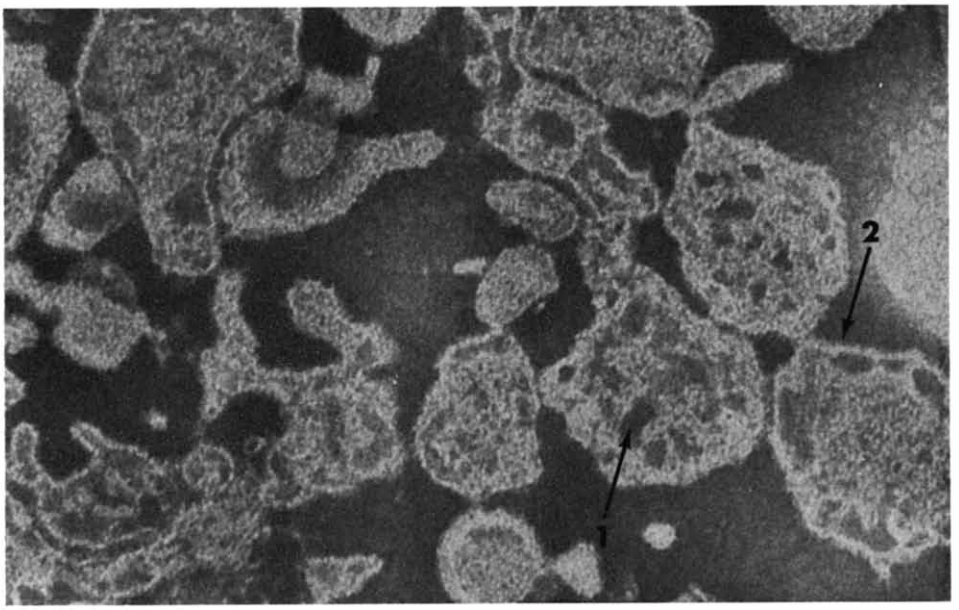

Fig. 8. Purified sarcoplasmic reticulum fragments which had been treated with 10% diethyl ether (negatively stained). The surface of the membranes was no longer completely granular but had patches that were transparent (Arrow 1). The subunits (Arrow 2) along the edge of the membrane were less prominent. × 120000.

sessing tails (T) of various lengths. Neither pig nor rabbit sarcoplasmic reticulum fragments had the preponderance of tadpole-shaped membranes which have been found previously with mouse-muscle preparations[1,4]. Most of the membranes had a granular surface structure when viewed face on. The edge of the membrane was lined with spherical subunits which were approx. 35–40 Å in diameter (arrows, Fig. 7).

Fig. 8 shows the structure of negatively stained sarcoplasmic reticulum fragments which have been treated with 10% diethyl ether. The surface of the membranes was no longer completely granular but had patches (Arrow 1) that appeared to be transparent. The subunits along the edge of the membrane were less prominent, but they appeared to form a continuous fringe around the membrane (see Arrow 2). Thus the transparency of the surface areas probably reflected an increased membrane permeability to the stain rather than a loss of the subunits. The observation that no protein or phospholipid was released as the result of diethyl ether treatment[11] also suggested that the changes noted were more likely due to conformational changes in the membrane rather than an actual extraction of material. However, the removal of neutral lipid, sterols or some other component cannot be excluded as a possibility.

Treatment with 20% diethyl ether usually resulted in a loss of subunits and promoted an aggregation of the vesicles.

The effect of 2 mM thymol treatment on the vesicular structure is shown in Fig. 9. The appearance of the vesicles was very similar to that of 10% diethyl ether-treated preparations. The membranes had irregular transparent patches that interrupted the granular surface structure. Also, the subunit fringe was less apparent.

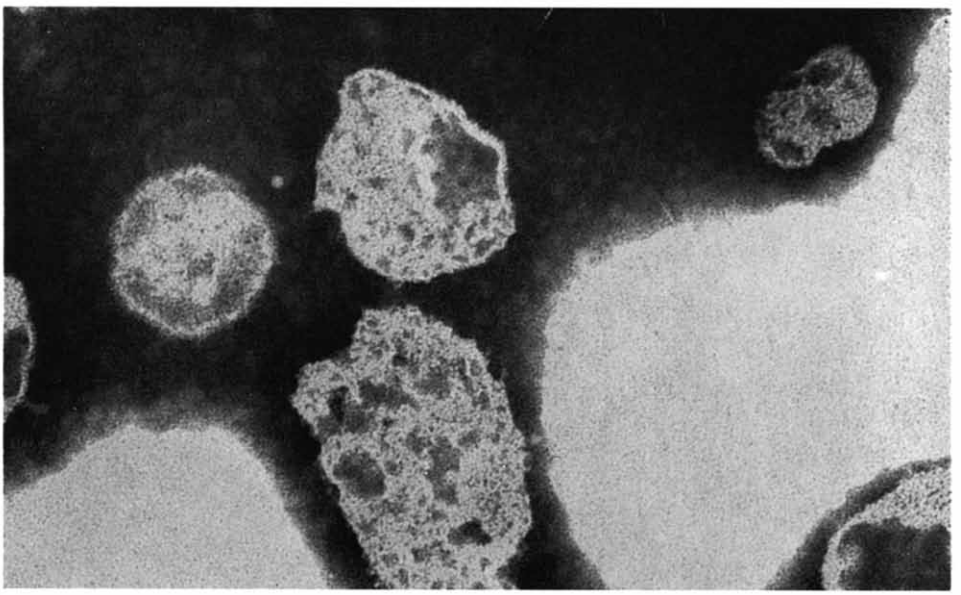

Fig. 9. Purified sarcoplasmic reticulum fragments which had been treated with 2 mM thymol (negatively stained). The granular surface structure was interrupted by transparent patches, similar to that found with 10% diethyl ether treatment. × 120000.

The 4 mM thymol-treated sarcoplasmic reticulum fragments rarely had visible subunits and also tended to aggregate.

The loss in Ca^{2+}-accumulating ability caused by diethyl ether or thymol treatment without loss of the transport ATPase suggests that the membrane permeability was altered without changing the Ca^{2+} pump mechanism. However, preliminary experiments with ferritin incubation revealed that very few granules reached the vesicle interiors.

DISCUSSION

The presence of the 40-Å subunits in membrane preparations treated with 10% diethyl ether or 2 mM thymol suggested that their structural integrity was not the primary requirement for Ca^{2+}-accumulating ability. The apparent loss of subunits in 20% diethyl ether- and 4 mM thymol-treated sarcoplasmic reticulum fragments might explain, however, the decreased Ca^{2+}-activated ATPase activity found in these preparations. The possibility that diethyl ether and thymol might cause the formation of membrane holes could explain the uncoupling of Ca^{2+} uptake and the Ca^{2+}-activated ATPase; however, preliminary experiments with ferritin suggested that if any holes existed, they would be smaller than 110 Å.

It should be realized that the images produced by negative staining merely reflected the permeability and penetration of the stain in relation to the membranes examined. Thus the structures observed may not be a true representation of the actual membrane architecture in solution. However, it can be concluded that diethyl ether and thymol changed the membrane structure to such an extent that the membrane–stain interactions were altered.

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