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PROPERTIES OF PARTICLES PREPARED FROM SARCOPLASMIC RETICULUM BY DEOXYCHOLATE

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SUMMARY

Treatment of sarcoplasmic reticulum from rabbit skeletal muscle with deoxycholate, and subsequent removal of the detergent by Sephadex chromatography, yielded non-aggregated particles of 100–200 Å in diameter. The particles represented 90% of the membrane proteins and phospholipids, had an ATPase activity as high as the untreated membrane and retained only traces of deoxycholate. Sucrose and other polyalcohols stabilized the ATPase activity of the particles.

INTRODUCTION

Sarcoplasmic reticulum vesicles were recently shown to dissolve in deoxycholate[1]. Subsequent dilution resulted in spontaneous reaggregation and vesicle formation with concomitant reactivation of the ATPase and calcium pump[1]. The present report shows that stable non-aggregated particles with high ATPase activity and retaining only a trace of deoxycholate can be prepared from sarcoplasmic reticulum membranes. These particles, which represent more than 90% of the membrane components and are essentially free of deoxycholate, are needed to study the conditions for membrane assembly, a study which is technically difficult in partially dissolved and spontaneously reaggregated preparations.

MATERIALS AND METHODS

Preparation of sarcoplasmic reticulum membranes

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle by differential centrifugation in 0.3 M sucrose, and purified from glycolytic enzymes and contractile proteins by extraction with 0.6 M KCl (ref. 2). The isolation procedure was as follows. The animals were killed by decapitation, and the back and hind leg muscles were quickly removed. All subsequent steps were carried out in the cold. The minced tissue in 100 g portions was homogenized with 400 ml of 0.3 M sucrose containing 1 μg diphenyl-*p*-phenylene diamine per ml. Homogenization was carried out in a Waring blendor operating at top speed for a total of 2 min. The homogenate was centrifuged at 2500 $\times$ *g* for 15 min, and the supernatant was centrifuged at 10000 $\times$ *g* for 40 min to remove mitochondrial particles. The sarcoplasmic-reticulum fraction was obtained by centrifugation of the supernatant at 38000 $\times$ *g* for 60 min. The

sediment was resuspended in 0.6 M KCl at a concentration of 1 mg per ml and centrifuged at 38 000 × *g* for another 60 min. The KCl-extracted sarcoplasmic reticulum was transferred to a low-ionic-strength medium by resuspension and centrifugation in 0.3 M sucrose, and was stored at −20°. As estimated from succinate-dehydrogenase determination the microsomal membrane proteins contained less than 1% proteins originating from mitochondria.

ATPase activity determined at 30° was 2–3 μmoles ATP hydrolyzed per mg protein; and calcium storing capacity measured in the presence of oxalate was 2–2.5 μmoles Ca^{2+} per mg protein. The sarcoplasmic reticulum fraction isolated in sucrose contained 10–20% muscle contractile proteins, various glycolytic enzymes, AMP deaminase (EC 3.5.4.6) and high activity of adenylate kinase (EC 2.7.4.3) (0.3–0.5 μmole of ATP formed from ADP per min/mg protein at 30°). Washing of sarcoplasmic-reticulum fraction thus obtained with 0.6 M KCl solution eliminated most of the enzyme contaminants except for AMP deaminase which tends to be retained in the membrane fraction.

Reagents

Carboxyl ^{14}C-labeled deoxycholic acid was obtained from Nuclear Research Chemicals and was of more than 99% radiopurity. Unlabeled deoxycholic acid was recrystallized three times from ethanol. Phosphoenol pyruvic acid was synthesized according to the method described by CLARK AND KIRBY[3]. It was recrystallized at least once as the monocyclohexylamine salt and was converted to the potassium salt before use. All other reagents were of analytical grade.

Analytical methods

Protein was measured with the Folin-phenol reagent[4] using crystalline serum albumin as standard. Lipids were extracted from membranes by the method of FOLCH *et al.*[5], and the phosphorus in the phospholipid fraction was determined according to AMES[6]. ATPase activity was determined by measuring the amount of P_i liberated from ATP with phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) as ATP-regenerating system. The reaction mixture in a final volume of 0.5 ml contained imidazole–HCl buffer (pH 7.0) 5 mM; ATP, 4.5 mM; $MgCl_2$, 5 mM; KCl. 100 mM; $CaCl_2$, 0.1 mM; phosphoenolpyruvate, 4 mM; pyruvate kinase, 30 μg/ml, The reaction was started by the addition of enzyme, and was terminated by the addition of an equal volume of 10% trichloroacetic acid. An enzyme unit is defined as the amount of enzyme required to liberate 1 μmole of P_i per min at 30°. Succinate dehydrogenase (EC 2.3.99.1) was measured by the method of GREEN *et al.*[7]. Adenylate-kinase activity was determined as the amount of ATP formed from ADP by coupling with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and following the amount of NADPH formed at 340 mμ. AMP deaminase (EC 3.5.4.6) was determined according to NIKIFORUK AND COLOWICK[8].

Radioactivity was measured in a scintillation counter, using the scintillation solution described by BRAY[9].

Negative staining was accomplished by diluting the samples with 2% phosphotungstic acid adjusted to pH 6.8. A drop of the diluted sample was placed on a grid covered with a thin film of formvar reinforced with evaporated carbon. After 20 sec the drop was blotted off with filter paper and the grid was allowed to air-dry. The specimens were examined in a RCA EMU-3G electron microscope operated at 100 kV.

RESULTS

Experiments to solubilize the sarcoplasmic reticulum membranes by freezing and thawing, sonication or washing with EDTA were unsuccessful. Anionic detergents, on a weight basis, were found to be more effective in solubilizing sarcoplasmic reticulum membranes than were the cationic or nonionic detergents. The relationship between the concentration of membrane material and the degree of solubilization obtained at various detergent concentrations is shown in Fig. 1. Solubilization was

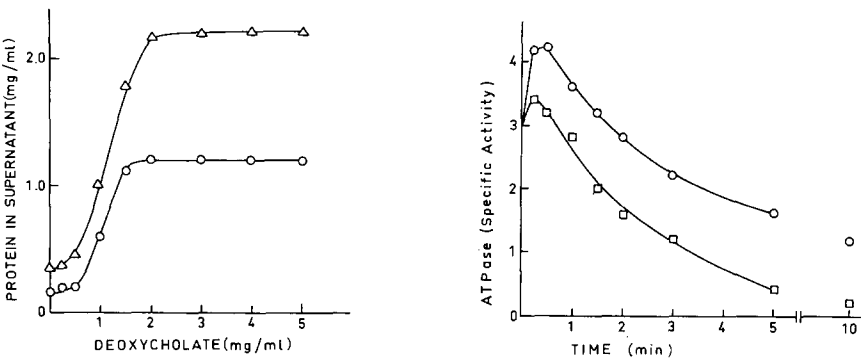


Fig. 1. Membrane solubilization at various concentrations of detergent. Membrane suspensions in 0.5 M sucrose containing the indicated final protein concentration were incubated for 30 min at 30° with various amounts of deoxycholate. The amount of solubilized protein was determined in aliquots of the supernatant solution after centrifugation for 60 min at 100000 × *g*. △—△, protein, 2.4 mg/ml; ○—○, protein, 1.2 mg/ml.

Fig. 2. ATPase inactivation by deoxycholate. Deoxycholate was added to membrane suspensions to give a membrane protein to detergent ratio of 1:2 (w/w). Aliquots of the extraction mixture at 0 and 30° respectively were taken at various times after the addition of deoxycholate and diluted 1:50 in the ATPase assay solution. ATPase activity after dilution was linear for at least 15 min. ○—○, 0°; □—□, 30°.

dependent on the ratio of detergent to membrane protein and not on the absolute detergent concentration. The time course of sarcoplasmic reticulum ATPase inactivation by deoxycholate is described in Fig. 2. Immediately after the addition of detergent to the membranes there was an increase in ATPase activity followed by a fast decay of activity. The half-life time of ATPase activity was about 2 min at 30° and 8 min at 0°. Fractionation of deoxycholate-extracted membranes on a Sephadex column is the quickest procedure for removal of detergent[10,11]. However, under these conditions the ATPase activity was extremely unstable even when the concentration of deoxycholate in solution was reduced to 1/500 of its original value. In preliminary studies it was found that high concentrations of polyalcohols such as ethylene glycol, glycerol or sucrose stabilized the ATPase activity. Fractionation of deoxycholate-extracted membranes in sucrose media is described in Fig. 3. Although the bulk of the deoxycholate was retained on the column a small amount came off with the fraction containing the protein and phospholipid. That such a chromatographic pattern could not be the result of impurities in the deoxycholate preparation is evident from the symmetrical peak of deoxycholate when chromatographed without membranes (Fig. 4b).

Recoveries of phospholipid, protein and ATPase activity are listed in Table I. A 100-fold purification from deoxycholate with essentially quantitative recovery of phospholipid and protein was obtained. When a purified fraction was rechromatographed on a Sephadex column only a further 2-fold purification from deoxycholate was achieved in the second run. Fig. 4 shows that when the deoxycholate-treated membranes were kept at 0° for 1 h and then chromatographed on Sephadex, the same amount of deoxycholate was obtained in the membrane fraction but there was a complete loss of ATPase activity.

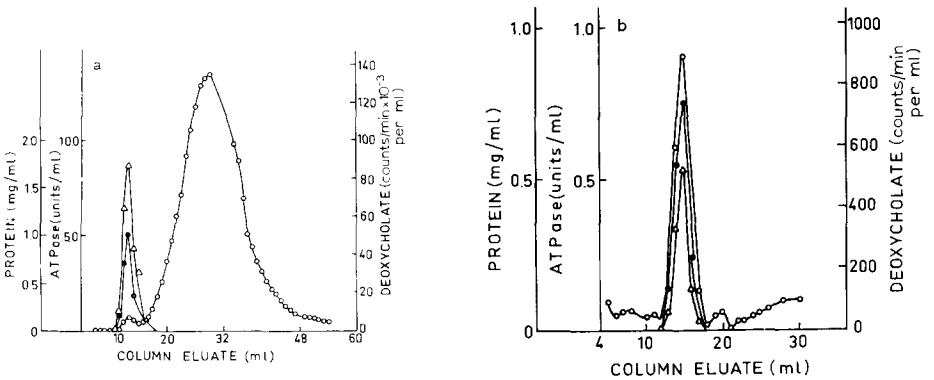


Fig. 3. (a) Chromatography of deoxycholate-treated membranes on a Sephadex G-50 column. (b) Rechromatography of an aliquot of the membrane peak fractions on Sephadex G-50. For experimental details see Table I. △—△, protein; ○—○, radioactivity; ●—●, ATPase.

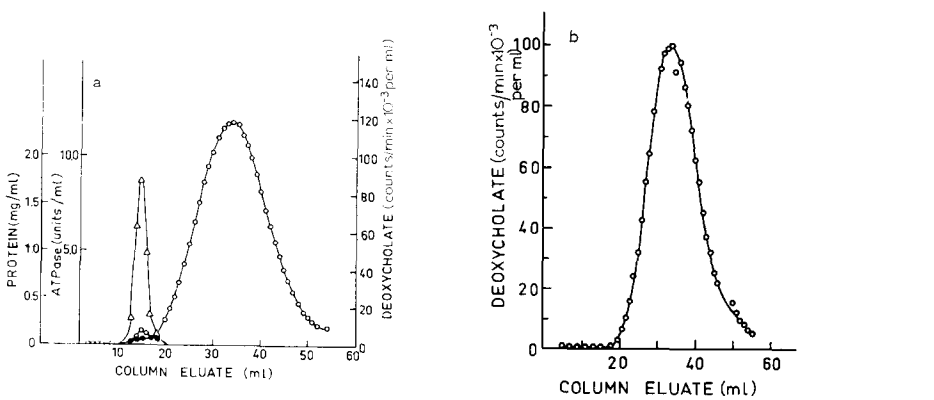


Fig. 4. Chromatography of deoxycholate-treated membranes on Sephadex G-50. (a) Same conditions as in Table I but incubated for 1 h at 0° in the presence of deoxycholate before chromatography. (b) Chromatography of carboxyl ^{14}C-labeled deoxycholate without membranes. △—△, protein; ○—○, radioactivity; ●—●, ATPase.

The effect of sucrose on stabilization of ATPase activity is described in Fig. 5. Sucrose exerted its effect not only when the detergent was present but also after it had been removed by Sephadex chromatography.

Fig. 6 is an electron micrograph of negatively stained sarcoplasmic reticulum membranes from rabbit skeletal muscle. The fragmented sarcoplasmic reticulum

TABLE I

THE SEPARATION OF SOLUBILIZED MEMBRANES FROM DEOXYCHOLATE ON SEPHADEX G-50

A suspension of membranes, in 0.5 M sucrose and 5 mM imidazole–acetate buffer (pH 7.8) containing 10 μM EDTA, was extracted with a solution of potassium deoxycholate to give a protein to deoxycholate ratio of 1:1. 1 ml of extract was immediately placed on a Sephadex G-50 column (1 cm × 40 cm) and eluted with the sucrose–buffer–EDTA solution. Fractions of 1 ml were collected and aliquots analyzed for protein lipid phosphorus, ATPase activity and radioactivity.

	Protein		*Lipid-P*		*ATPase*		*Deoxycholate*		*Purification****
	mg	*Recovery* (%)	*μmole/mg protein*	*Recovery* (%)	*Specific activity*	*Recovery* (%)	*Counts/min* $\times 10^{-3}$	*Counts/min* $\times 10^{-3}$ *per mg protein*	
Original sample	5.8		0.51		2.2*		2356	403	
Column eluates fractions 11–15	5.3	92	0.49	96	3.1	140	22	4	100
Fractions 16–50	0	0	0	0	0	0	2200		
Rechromatographed fractions**	—	90	0.48	94	2.5	116	4	1.6	250

* Specific activity of the untreated membranes.
** 1 ml of the membrane peak fractions was rechromatographed under the same conditions.
*** Purification is the ratio of deoxycholate per mg protein of the dissolved membranes to that of a purified preparation.

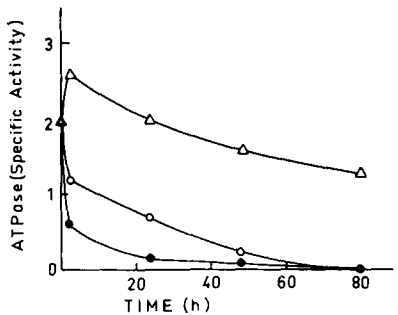
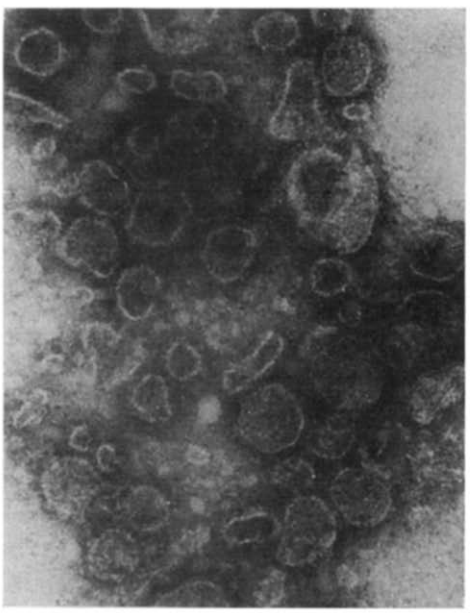


Fig. 5. The influence of sucrose on ATPase inactivation by deoxycholate. (1) △—△, chromatographed in the presence of 0.5 M sucrose (see Table I); (2) ●—●, chromatographed under the same conditions as (1), but in a medium not containing sucrose; (3) ○—○, chromatographed as (1) and then rechromatographed in the absence of sucrose.

fraction is composed of membrane-bound vesicles with a diameter between 600 and 1600 Å and a membrane thickness of about 60–70 Å. Although originating from what is described as smooth endoplasmic reticulum, negatively stained preparations of fragmented sarcoplasmic reticulum show that the surface of the vesicles is covered by projections of 50 Å diameter. Such projections have been described by MARTONOSI[13] in sarcoplasmic reticulum and by PREZBINDOWSKI *et al.*[14] in a negatively stained preparation of smooth endoplasmic reticulum from rat liver.



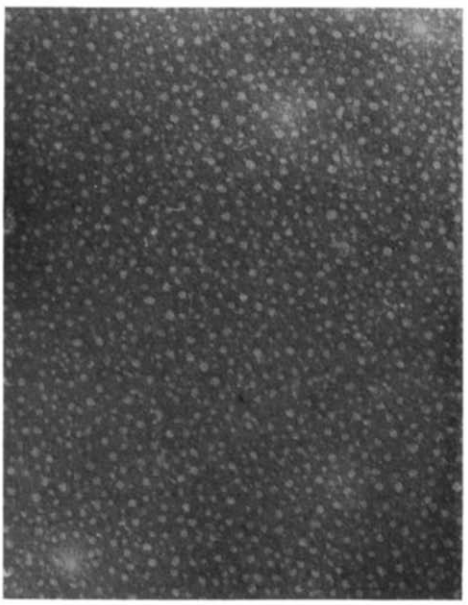


Fig. 6. Sarcoplasmic reticulum vesicles negatively stained with phosphotungstic acid. This preparation consists of empty vesicles ranging in size from 600 to 1600 Å. In many places the surface of the vesicle membrane is lined with 50-Å particles. × 67000.

Fig. 7. Particles prepared from sarcoplasmic reticulum by deoxycholate negatively stained with phosphotungstic acid. The only visible structures in this preparation are particles ranging in size between 100 and 200 Å diameter. × 67000.

Fig. 7 is an electron micrograph of the particles prepared from sarcoplasmic reticulum by solubilization with deoxycholate and subsequent removal of deoxycholate by chromatography on a Sephadex column. The particles were of 100–200 Å diameter with irregular borders. There were no visible clumps or aggregates. However, at the present state of resolution it is difficult to distinguish whether the particles themselves contained any substructure.

At the low ionic strength employed for solubilization and purification the particle preparation showed little tendency to aggregate although it possessed high ATPase activity. The membrane particles were not precipitated by centrifugation for 60 min at 100000 × *g*, and 90% of the material passed through a Millipore filter with a 0.22 μ pore size, whereas the original microsomes were precipitated after 60 min at 38000 × *g* and were retained on a Millipore filter with 0.45 μ pore size. When divalent cations such as Ca^{2+} or Mg^{2+} were added to the membrane particles

turbidity developed; this also occurred with intact sarcoplasmic reticulum membranes which tend to aggregate in the presence of Mg^{2+} or Ca^{2+} (ref. 11). However, reaggregation of the membrane particles with either Mg^{2+} or Ca^{2+} did not restore active Ca^{2+} uptake.

DISCUSSION

In order to study the functions of a complex membrane system, it is desirable to obtain a soluble preparation, which is much more easily handled than the insoluble membranes. Detergents are the agents most widely used to achieve such a task, and many of them yield preparations with interesting properties. However, it is of equal importance to remove the detergent from the solubilized membranes as it affects the new preparation, the new properties being those of a secondary complex between detergent and various membrane components.

It has been suggested that deoxycholate is a suitable detergent for disaggregation of the membranes of the sarcoplasmic reticulum[1,10]. In the study reported here, when deoxycholate was employed in a concentration which ensures complete disaggregation, it was necessary to include a high concentration of sucrose in order to obtain ATPase activity similar to that of the untreated membranes. Small amounts of deoxycholate were retained in the membrane fractions even after repeated Sephadex chromatography, probably owing to the formation of a complex which is not readily dissociated.

The term solubilization which is often used in membrane studies, is only an operational criterion and does not imply that a molecular solution or even a homogeneous suspension of building blocks is obtained. It implies, however, that a drastic reduction in size has taken place. The electron micrographs made before and after treatment of the sarcoplasmic reticulum with deoxycholate (Figs. 6 and 7) indeed show that the vesicles had been transformed into particles of 100–200 Å in diameter. As the untreated vesicles have a membrane with a thickness of 60–70 Å, the particles as they are cannot be considered as membrane building blocks unless it is assumed that disaggregation also resulted in unpacking and expansion of the lipoprotein complex. The particles with their irregular outlines might be the result of the unfolding of what was previously a tightly-packed lipoprotein membrane, or they might be the result of a random disaggregation of a continuous membrane. Whatever the origin of these particles, their small size and fully preserved catalytic activity make them a useful tool in studies of membrane structure and function.

It was reported recently that treatment of membranes of the sarcoplasmic reticulum with detergents, at a concentration which causes only partial solubilization, results in a preparation which upon dilution or removal of detergent on a Sephadex column spontaneously reaggregates to form vesicles with an active calcium pump[1]. The preparation reported here differs from the above-mentioned preparation in the following respects. Although it represents more than 90% of the membrane components, and has an ATPase activity as high as the original membrane, it does not tend to aggregate spontaneously upon removal of detergent. Surprisingly, the spontaneous aggregation reported by MARTONOSI[1] is not affected by Mg^{2+} or Ca^{2+}, whereas the particles described in the present work readily aggregate when divalent cations are added. Preliminary aggregation experiments have not yet enabled us to

reconstitute an active calcium pump. Further investigation should show whether spontaneous reaggregation to form an active calcium pump[1] is dependent upon the presence of residual membrane structures (not completely disaggregated by deoxycholate), or whether the particles alone are sufficient for the reconstitution, provided that exact conditions are met.

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