Effect of Lipid Modification on Fusion of Sarcoplasmic Reticulum Vesicles

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Freeze fracture technique ascertains that sarcoplasmic reticulum vesicles fuse during anisodiametric dehydration which leads to the formation of sheetlike structure of a mean extension of 5000 Å. Modification of the membrane lipids by phospholipase A_2 digestion or the incorporation of deoxycholate facilitates the coalescence of the vesicles, while it is completely prevented by lipid removal. Membrane fusion during anisodiametric dehydration is considered as resulting from the close contact of highly curved edges from which the membrane proteins have been excluded.

Introduction

The sarcoplasmic reticulum is a muscular membrane structure with a very high degree of morphological and functional specialization. Its main protein component is an ATP-driven calcium pump which is an essential part of the system involved in the regulation of muscle's contractile activity by calcium release and removal (*c. f.* ref. [1]). Rapid removal of considerable quantities of calcium ions which are required for the activation of the contractile system is guaranteed by the large surface of the sarcoplasmic membranes. They form a complex network of tubules and cisternae around the myofibrils. Calcium ions are not only translocated across the tubular membranes but also moved inside the tubules longitudinally to the cisternae [2]. The latter are characterized by special contacts with the plasma membrane and its tubular invagination [3]. During excitation calcium is most likely set free from the cisternal elements of the reticular membranes. Apart from the specialized physiological function of the membranes, they are marked by a high degree of morphological stability. The complex arrangement of the membranes is preserved, although they are permanently deformed during muscular activity. Only extensive muscle shortening exceeding the physiological limits presumably causes changes in the structure of the sarcoplasmic reticulum which affect the excitability of the muscle [4, 5]. On the other hand, the membranes respond very sensitively by swelling to small changes of the ionic environment of the muscle [6]. Swelling finally results in the formation of membrane vesicles. Similar structural changes occur during the procedure of isolation of the membranes leading to the formation of vesicular fragments. They are tightly sealed and characterized by a low permeability for calcium ions [7–9]. The ability of the sarcoplasmic tubules to form closed vesicles is a most remarkable property of their membranes. These morphological changes require a high mobility of all membrane constituents to which presumably the high degree of the unsaturation of the membrane lipids essentially contributes [10, 11].

In connection with the attempt to prepare highly ordered membrane structures suitable for X-ray analysis, our interest was focussed on the deformability of the sarcoplasmic membranes and led us to study the structural change which occurs when flat pellets of isolated vesicles were unisometrically dried [12, 13]. We have shown that the shape of the isolated vesicles is astonishingly stable. Even, when during sedimentation high gravitational forces are applied for a long period of time, the vesicular cross sections remain nearly circular. Only, when more than 50% of the water content of the vesicular pellet is removed, the vesicles start to collapse and disclike structures appear in which adjacent vesicles seem to merge [12]. In this paper the occurrence of membrane fusion will be substantiated by applying freeze fracturing technique and by a morphometric analysis. The same approach will be applied to sarcoplasmic membranes whose lipid and protein constituents have been modified.

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Materials and Methods

Sarcoplasmic vesicles were isolated from rabbit skeletal muscle by the method of Hasselbach and Makinose [14]. The deoxycholate treatment of the vesicles was performed at a deoxycholate/protein ratio of 0.1 to 0.2 in a solution containing 0.3 M KCl, 0.1 M potassium phosphate, pH 7.0. The protein suspension contained usually 10 mg protein/ml. After centrifugation for one hour at 45 000 rpm in a Ti 50 rotor (Spinco) the protein pellet was resuspended in 0.1 M KCl and dialyzed against 0.1 M KCl, 0.01 M potassium phosphate, pH 7.0 for 24 hours.

The membrane phospholipids were hydrolyzed by phospholipase A_2 (EC 3.1.1.4.). 100 mg vesicular protein were treated for 2 hours at room temperature (20 °C) with 1 mg phospholipase A_2 at pH 7.0 in the presence of 1 mM $CaCl_2$. To deplete the membrane protein of the hydrolysis products, 100 mg of phospholipase A_2 digested vesicles were treated with 1 g of acetone washed bovine serum albumin in 100 ml of 0.1 M KCl.

Flat vesicular pellets were prepared as described previously by centrifugation in polyallomer tubes of sarcoplasmic vesicles suspended in solutions containing 0.05 M KCl, 0.02 M histidine pH 7.0 and 7% glycerol [12]. In a number of experiments the solutions contained either 1 mM EGTA or 1 mM $CaCl_2$. The polyallomer tubes, 12×1.2 cm, were equipped with inserts having a flat surface with an elevated rim. The vesicles were sedimented at 4 °C for one hour at 35 000 rpm. To guarantee fast and homogeneous drying of the pellet the total amount of the sarcoplasmic protein per pellet was kept between 5 and 7 mg. For the preparation of thin sections the pellet material was treated as described by Stromer and Hasselbach [12]. For freeze fracturing studies samples of the various pellets were transferred to a golden specimen holder and frozen in liquid Freon-22 cooled with liquid nitrogen. Specimens were fractured at -100 °C in a Balzers BAF 301 device (Balzers AG, Balzers, Liechtenstein) and shadowed with platinum-carbon and carbon. The replicas were cleaned in a sodium hypochlorite solution, rinsed in distilled water and picked up on uncoated grids. A Siemens Elmiskop 101 electron microscope equipped with an anticontamination device and operating at 80 kV was used.

For measuring the length of uninterrupted membrane profiles they were traced on transparent paper, and the total length of 20 to 30 profiles obtained from one plate were summed by a line integrator as used for measuring distances on maps. With the same instrument the mean diameter of elliptically deformed vesicles was determined by summing up long and short diameters of approximately 100 vesicles of the particular preparations. The birefringence analysis was performed as previously described [12] with preparations oriented on a cover slip perpendicular to the direction of the flat membrane pellet.

Results

When flat pellets of sarcoplasmic membranes are freeze-fractured perpendicular to the plane of the preparations which have lost approximately 75% of their water content by anisotropic drying in the presence of glycerol, continuous membrane profiles appear which extend over more than 5000 to 8000 Å (Fig. 1 a). A comparison with nondehydrated preparations shows that the large profiles must result from the coalescence of several vesicles (Fig. 1 b). Fusion is preceeded by flattening of the spherical vesicles and close apposition of their external membrane leaflet as previously shown in sectioned preparations [12, 13]. In freeze-fractured preparations a more or less regular grainy structure between the membrane leaflets becomes apparent. These structures represent most likely interdigitating membrane particles from opposing membranes. When the dehydrated preparation is fractured in the plane of the pellet, large membrane areas of low particle density are continuous with areas densely covered with particles (Fig. 1 c). Their diameter of 80 Å is identical with that in nondehydrated preparations. The fracture faces of low particle density apparently originate from large continuous membrane areas. In contrast, the particulate fracture faces appear quite irregular. They are presumably composed of only partially fused membrane areas. Although these pictures unambiguously prove the occurrence of membrane fusion, it is rather difficult to estimate in pictures of freeze-fractured preparations how many confluent vesicles are typically involved in forming an uninterrupted sheet. Therefore, in four sectioned preparations (*c. f.* ref. [12]) the length of 20 – 30 uninterrupted membrane profiles was measured as described in Methods, and a mean length of 3900 ± 300 Å was found. This distance is 3.7 times larger than the length which the profiles

b
c
a

of unfused vesicles would attain on flattening (comp. Table I). Hence, approximately 6 to 10 vesicles must have been fused during dehydration to form these extended structures. The extent of fusion, *i. e.* the fraction of vesicles which has undergone fusion irrespective of the size of the fusion product, is even more difficult to quantify. Figures between 10% minimal and 50% maximal may be given as a cautious estimate. Fusion was not observed in preparations the protein constituent of which has been labelled and cross-linked with mercuri-phenylazo-ferritin while the formation of flattened closely apposed membrane areas is not affected [12, 15]. When such preparations are freeze-fractured, ferritin particles are not visible indicating that even after decoration and cross-linking of the membrane proteins the fracture plane passes through the hydrophobic interior of the bilayer (unpublished observations).

Table I. Dimensions of different sarcoplasmic reticulum vesicles.

Preparations	Diameter of sarcoplasmic vesicle preparations measured in		
	sectioned material [nm]	negatively stained material [nm]	freeze fractured material [nm]
Native vesicles	82.0 *	115.0 *	
	78.0 ± 3.0	113.0 ± 6.0	120.0 ± 9.0
Vesicles treated with phospholipase A_2	37.0 ± 3.5	62.0 ± 2.5	78.0 ± 5.0
Vesicles treated with phospholipase A_2 and albumine	—	61.0 ± 3.0	79.0 ± 5.0
Vesicles treated with DOC 0.1 mg/mg protein	53.0 ± 1.5	—	—

200–300 vesicular diameters were measured in groups of 50 with a distance integrator. The standard error refers to the value of the respective groups.

* Values from Hasselbach and Elfvin [15].

The incorporation of small amounts of amphiphiles into the isolated membrane fragments must be considered to be a relatively mild modification of the interaction of the membrane components. The application of deoxycholate at low amphiphile protein ratios (~ 0.1) does not remove lipids nor protein from the membrane nor does it affect the calcium-activated ATPase [16, 17]. However, deoxycholate incorporation leads to a loss of the ability of the sarcoplasmic vesicles to accumulate calcium. Structurally, deoxycholate causes a significant reduction of the mean size of the vesicles from 800 to 600 Å (Table I, Fig. 2 a). When the water content of the pellets prepared from deoxycholate treated membranes is reduced, structures of very high density are formed. Not only the extravesicular space as observed for native vesicles but also the intravesicular space disappears nearly completely. The high density of the material makes it difficult to demonstrate the presence of extended membranes or any other structure in sectioned material. However, when the preparations are fractured, continuous membrane profiles appear which extend over several thousand Å (Fig. 3 a). They are regularly coated with typical sarcoplasmic particles. Fracturing in the plane of the flattened membrane pellets reveals large membrane areas (Fig. 3 b).

When the vesicular preparations are treated with deoxycholate at higher deoxycholate protein ratios (0.2) and dialyzed overnight to remove most of the amphiphile, large and very flexible membrane structures appear already after relatively small water losses and on further drying densely packed lamellar structures are formed (Fig. 2 b). Apart from the addition of amphiphiles phospholipase A_2 digestion has successfully been applied to produce

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Fig. 1. Electron micrographs of three replicas of freeze fractured pellets of sarcoplasmic reticulum illustrating the effect of dehydration on the vesicular membranes.

a) Perpendicular fracture through a flat strongly dehydrated pellet. Lamellar structures with a characteristic periodicity separated by more or less regular grainy structures can be recognized.

b) Loose pellet as a control. Only vesicular structures with 80 Å intramembraneous particles or with smooth face are represented.

c) Same sample as in a) fractured parallel to the plane of the pellet. Large membrane areas almost devoid of particulate structures and areas densely covered with 80 Å particles can be observed.

The appearance of none of the preparations changes when the glycerol containing solution in which dehydration was performed contained either 1 mM EGTA or 1 mM $CaCl_2$. The mean weight loss of the dehydrated preparations a) and c) amounts to 70–75%. Bars in the figures indicate 2000 Å.

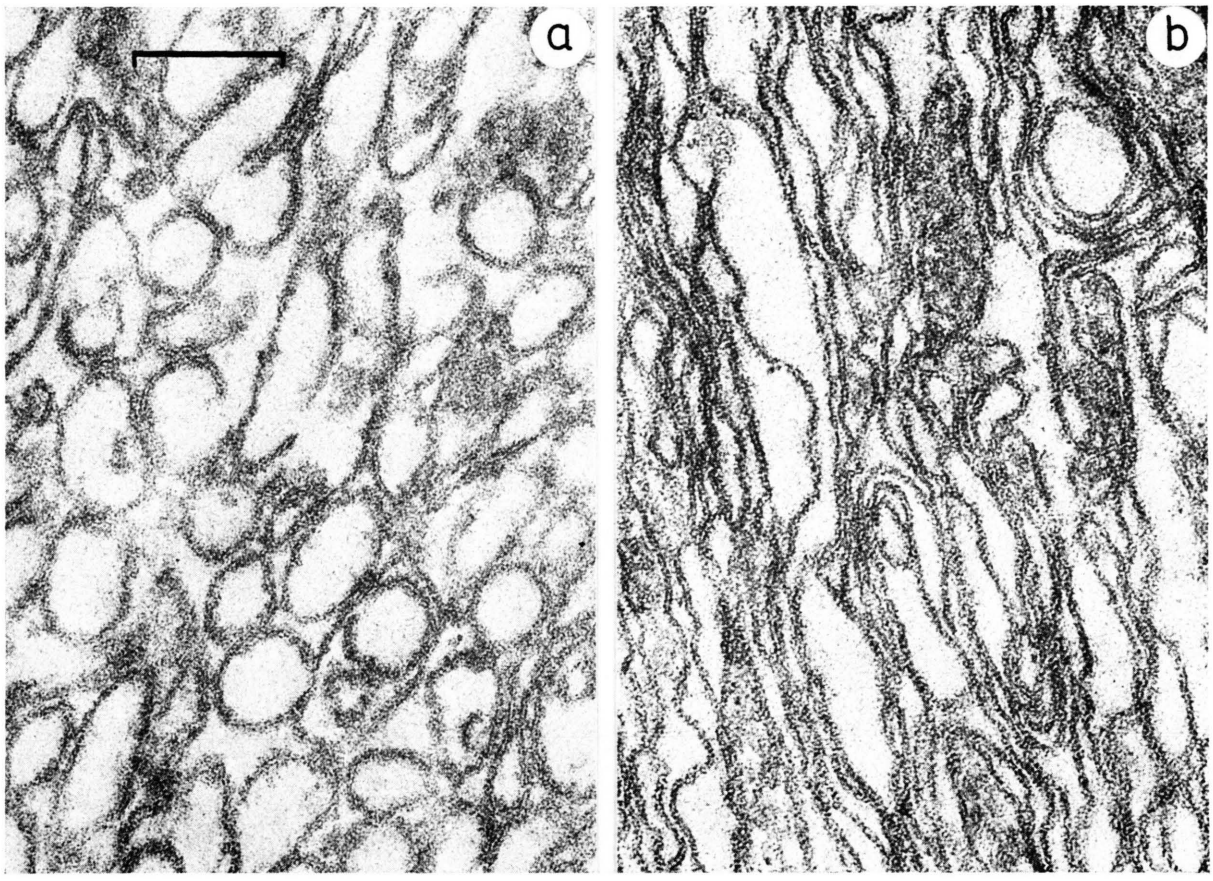

Fig. 2. Thin sections through two different pellets of sarcoplasmic membranes treated with deoxycholate.

a) Pellet of a preparation treated with 0.1 mg deoxycholate/mg protein and dehydrated for 85 min. Weight loss 34%. Individual vesicular structures with a mean diameter of 600 Å and a well outlined unit membrane can be observed.

b) Pellet obtained from a sample treated with 0.2 mg deoxycholate/mg protein for 1 h, dialysed overnight to remove the amphiphile. Medium degree of dehydration weight loss 54%. Large lamellar structures are present throughout the figure. — Uranyl acetate and lead citrate stain.

Bar: 2000 Å.

functionally modified membrane preparations. These preparations, like deoxycholate treated preparations, are unable to store calcium while their calcium-dependent ATPase and other calcium-dependent interactions with ATP or other phosphate compounds are preserved [10]. The replacement of the diacylglycerophospholipids by mixtures of fatty acids and lysocompounds of lecithin and cephalin results in a considerable size reduction of the vesicles [18]. Their mean diameter is diminished to approximately 400 Å (Table I, Fig. 4 a). Since neither lipids nor substantial quantities of sarcoplasmic proteins are removed by this procedure, the number of smaller vesicles must have increased by division of the larger ones. In freeze-fractured preparations mostly small circular profiles are present which are filled with coarse particles (Fig. 4 b). With a few exceptions the characteristic smooth or particulate fracture faces characteristic for native vesicles are absent. Drying in aqueous glycerol gives rise to densely packed preparations in which freeze-fracturing reveals continuous profiles (Fig. 4 c). The role of membrane lipids for the observed transformation of vesicular to sheetlike structures induced by dehydration becomes apparent when water is removed from lipid deprived preparations. Such preparations are obtained by treating phospholipase A_2 digested vesicles with albumin. Albumin

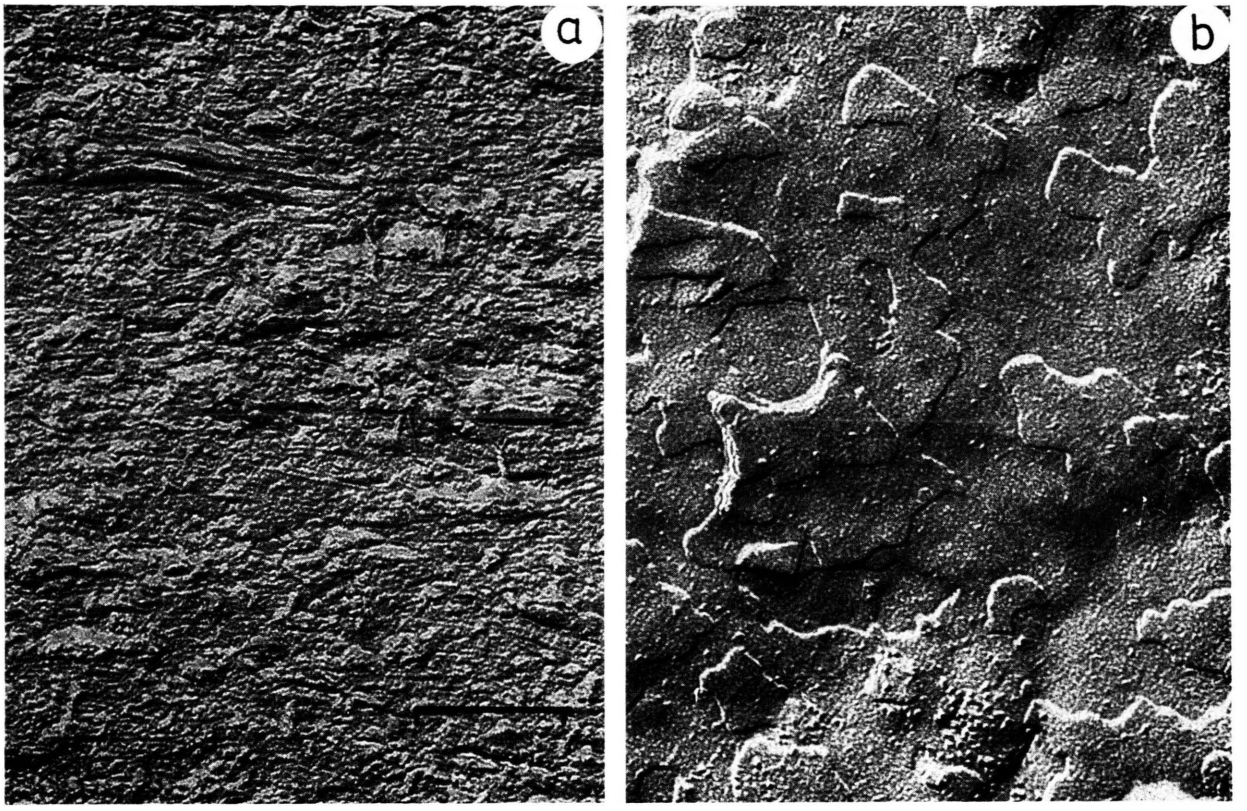

Fig. 3. Replicas of a strongly dehydrated flattened pellet of sarcoplasmic membranes treated with deoxycholate 0.2 mg/mg protein as described in Materials and Methods and freeze fractured perpendicularly (a) and parallel (b) to the plane of the pellet. While in **a** continuous membrane profiles coated with typical particles can be observed, large membrane areas are easily recognizable in **b**. (Compare also **a** with Fig. 1 a and **b** with Fig. 1 c.) Bar: 2000 Å.

extracts the fatty acids completely and approximately 60% of the lyso compounds [10]. Although the lipid bilayer must have been completely destroyed by this procedure, the vesicular shape of the sarcoplasmic vesicles is retained. The lipid deprived vesicles neither collapse nor fuse when they are dried in aqueous glycerol (Fig. 4 d).

On excessive drying the vesicular profiles are somewhat deformed. It is difficult to determine quantitatively the degree of flattening. Yet, even this small degree of deformation is sufficient to give rise to an intrinsic birefringence of the preparation the direction of which is positive, perpendicular to the long axes of the vesicles or the plane of the pellet. Since the lipid bilayer does not exist anymore, the measured birefringence must result from an anisotropic arrangement of highly ordered presumably helical domains in the transport ATPase molecule (Fig. 5).

Discussion

The controlled dehydration of flat pellets of sarcoplasmic vesicles imbibed with aqueous glycerol induces structural changes in the membrane which reflect membrane properties like elasticity and fluidity. A sequence of structural changes induced by dehydration of pelleted vesicles could be demonstrated by electron microscopic studies of sectional material [12, 13]. Yet, its analysis is rendered difficult by the considerable increase in density of the preparation during drying, together with bending and distortion of the membranes. These difficulties could be overcome by applying freeze-fracturing technique. The formation of extended membrane profiles arising from the fusion of a great number of vesicles could unequivocally be demonstrated. As previously shown, in sectioned material the spherical shape of the vesicles is affected only after

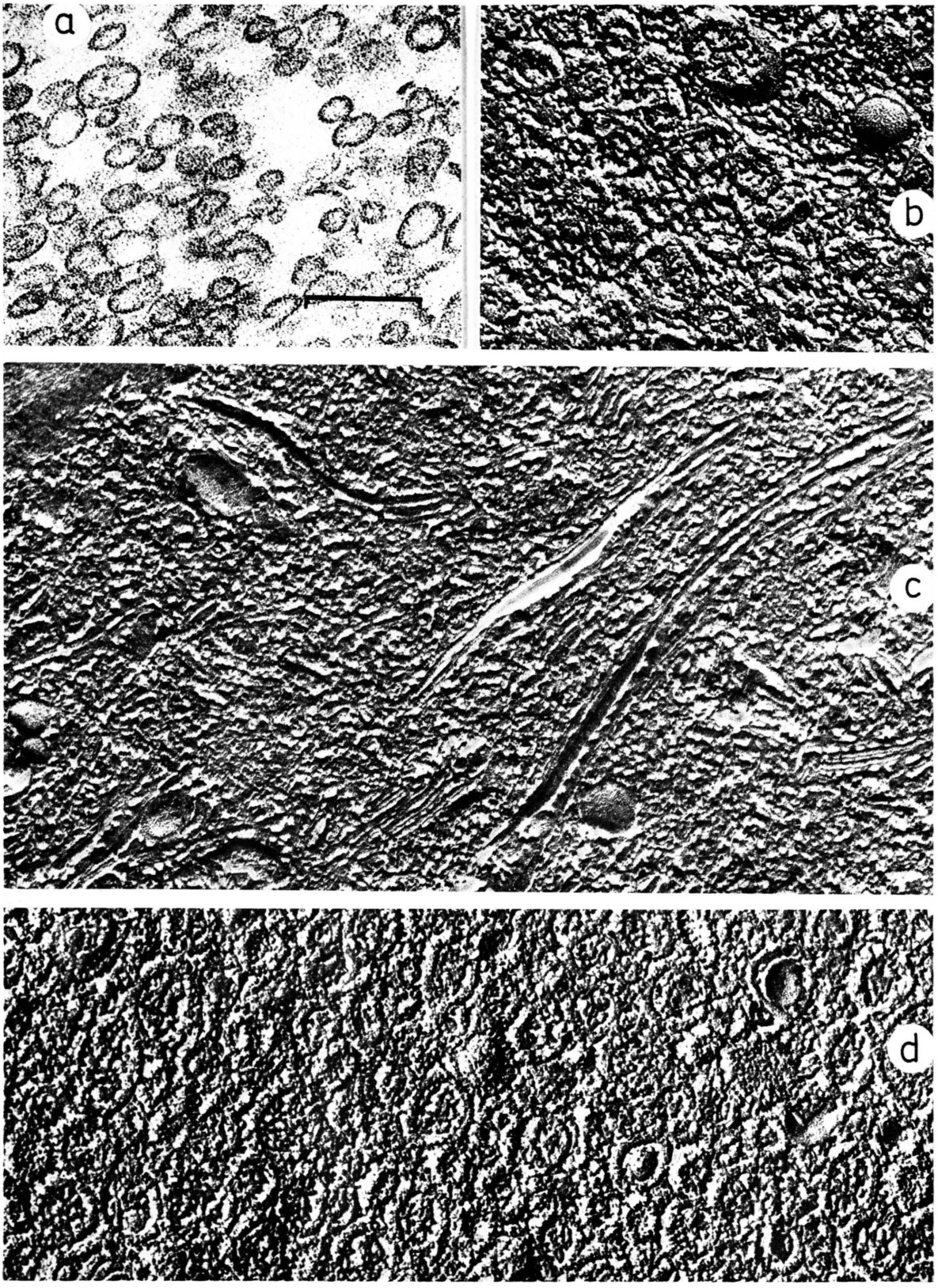
a
b
c
d

Fig. 4. Electron micrographs of phospholipase A_2 digested sarcoplasmic membranes.

a) Thin vestopal section through a sample fixed only with glutaraldehyde. Small vesicular structures with a mean diameter of 400 Å and a prominent asymmetry of the membrane are recognized. — Uranyl acetate and lead citrate stain (*c. f.* ref. [26]).

b) Replica of a freeze fractured loose pellet. Small circular profiles filled with coarse particles are predominant.

c) Replica of a flat densely packed pellet of the sample as in **b** which has been dried. Continuous profiles of the sheetlike structure can be observed. Weight loss ~70%.

d) Replica of the same sample as in **c** dehydrated after removal of splitting products of phospholipase digestion with albumin. Only circular profiles with coarse particles are represented. Weight loss ~70%.

Bar: 2000 Å.

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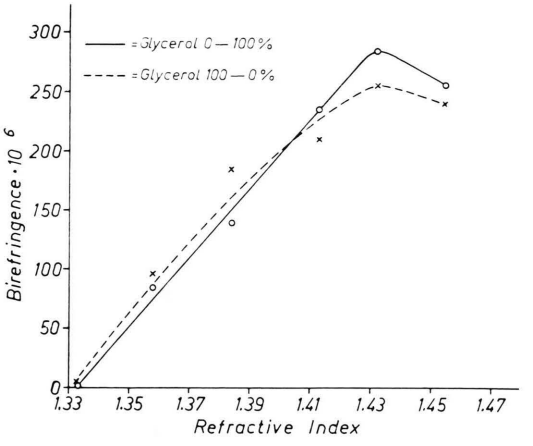

Fig. 5. Dependence on the refractive index of the imbibing medium of birefringence of slices of dehydrated pellets of lipid depleted sarcoplasmic vesicles. The refractive index was adjusted by the addition of glycerol to the medium. The birefringence is the difference between the refractory indices μ_{II} and μ_I determined when the electric field component of the polarized light is parallel or perpendicular to the normale of the membrane pellet. To exclude irreversible change in the preparation birefringence was measured with increasing as well as with decreasing concentrations of glycerol.

the water has been removed from the extra vesicular space. At this stage of drying the membranes of the vesicles are in close apposition and there is no indication that the membranes of adjacent vesicles may fuse to form a common intravesicular space. This behaviour of the vesicles is neither affected by the removal nor by the addition of calcium ions. Evidently, calcium ions, although bound in considerable quantity by the membranes, do not induce segregation of the membrane components, a process which has been postulated to be a prerequisite for membrane fusion [19, 20]. Fusion of the sarcoplasmic membranes was also observed after dehydration had led to water losses from the intravesicular space leading to the formation of disc-shaped vesicles. Fusion occurs at their highly curved edges. As to the changes of the membrane structure occurring at these regions, recent observations on liposomes may have some bearing. It has been shown that areas of different curvatures in bilayers are formed under conditions where different lipid components segregate and form isolated domains [21]. If different domains in a bilayer adopt different curvatures, a change in the curvatures produced by mechanical forces as they are exerted during dehydration on the vesicular pellets, must reciprocally induce a segregation of membrane components [22, 23]. Those components which can adopt the smallest curvature will be enriched at the curved edges of the vesicles. It is, therefore, conceivable that at the edges of the vesicles the small phospholipid molecules will be accumulated while the protein constituents will be shifted to the flat areas. The highly curved edges are structures which must be considered to be very unstable as compared to a flat bilayer. Close contact of the curved edges of the vesicles in the concentrated pellet will allow the system to reduce this instability by membrane fusion. A similar mechanism for membrane fusion has first been proposed by Lucy [24]. Fusion apparently takes place more easily in preparations whose lipid bilayer has been modified. The diminished stability of modified vesicles causes a considerable reduction of their curvature and the vesicles collapse already at the initial state of water removal. Deamer [25] reported similar structural changes of sarcoplasmic vesicles which were treated with lysolecithin. The importance of the lipids as the deformable matrix in the membrane is conversely stressed by the rigidity of the lipid deprived preparations. The residual lipids of approximately 0.1 μmol/mg protein consisting mainly of lysolecithin and lysocephalin are apparently insufficient to allow a deformation of the proteinaceous shell of the vesicles.

The mobility of the membrane components against each other is not only an essential requirement for the initial shape changes during controlled dehydration leading to a close apposition of the membranes but also for the segregation of lipids and proteins occurring at the edges of the membrane discs. Crosslinking of the proteins in the outer membrane leaflet does not interfere with the formation of discs, however it prevents the formation of elongated membrane sheets from the discs [12]. This observation supports the view that fusion is initiated by the formation of protein free membrane areas the coalescence of which is promoted by their small radii of curvature. The stability of the membranes *in vivo* and of the isolated vesicles is most likely guaranteed by the fact that the forces exerted on the isolated membranes by thermal motion or in the living muscle by mechanical deformation do not provide enough energy to separate fusible lipid domains from the lipoprotein matrix of the membranes.

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