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SARCOPLASMIC RETICULUM
V. THE STRUCTURE OF SARCOPLASMIC RETICULUM MEMBRANES

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SUMMARY

Electron microscope analysis of skeletal muscle microsomes was carried out by negative staining with potassium phosphotungstate. The size and shape of microsomes are influenced by species differences, the conditions of preparation and storage. The outer surface of the microsomal membrane is covered by subunits of 40 Å diameter, with center to center separation of 90–120 Å.

Chemical modification of microsomal membrane by treatment with phospholipase C, digitonin, or trypsin produced characteristic changes in the surface structure, shape or size of microsomes, parallel with the loss of Ca^{2+} transport function. The surface structure of microsomes does not indicate temperature-dependent structural changes of the kind observed on liver cell surface membranes, and the Arrhenius plot of ATPase activity is linear.

INTRODUCTION

The heavy microsome fraction of skeletal muscle consists of vesicles and tubules bounded by a phospholipid–protein membrane of 70–80 Å thickness[1]. It is generally believed that these elements are largely derived from the sarcoplasmic reticulum, although small admixtures of T-system, sarcolemma and mitochondrial fragments are likely to be present.

The microsome particles contain the KIELLEY–MEYERHOF[2,3] ATPase, and a powerful Ca^{2+}-transport apparatus[4–7], which forms an important link between the excitation and contraction in skeletal muscle.

The ATPase activity and Ca^{2+}-transport processes are uniquely dependent on the structural integrity of the membrane. Both ATPase activity and Ca^{2+} transport are inhibited by treatment of microsomes with phospholipase C, parallel with the hydrolysis of lecithin into phosphorylcholine and diglycerides. The process is accompanied by the formation of diglyceride droplets in association with the microsome membrane. The phospholipase C-treated membrane retains its unit membrane structure and is able to combine with finely dispersed synthetic phospholipids resulting in complete restoration of its ATPase and Ca^{2+} transport activities[1,8–11].

These observations support the idea that in the structural stability of biological membranes, non-phospholipid materials (proteins, sterols) play an important, although rarely emphasized role.

In the present report a detailed morphological description of rabbit and mouse microsome preparations is presented together with some correlative biochemical data on the ATPase activity and Ca^{2+} transport.

EXPERIMENTAL PROCEDURES

Skeletal muscle microsomes were prepared from predominantly white leg muscles of mice, rabbit, rat and frog, according to the procedure earlier reported by us[8,9]. This method includes washing of crude microsome suspension with 0.6 M KCl in order to remove adhering actomyosin contamination.

Electron microscope studies were carried out on a Philips EM 200 electron microscope operating at 60 kV accelerating voltage. A double condensor system and, in some cases, cooling stage were used.

Specimens were mounted on formvar- or parlodion-coated grids and negatively stained with 0.5 % potassium phosphotungstate (pH 7.0) or 0.5 % uranyl acetate (pH 5.0). Preservation of surface detail was considerably better with potassium phosphotungstate.

ATPase assay. Incubation was generally carried out in a solution of 0.1 M KCl, 10 mM histidine, 5 mM $MgCl_2$ and 5 mM ATP at 25° as described earlier[8,9]. Total volume, 2 ml. Protein concentration, 0.05–0.1 mg/ml. Incubation was stopped after 1–10 min with 0.5 ml 10 % trichloroacetic acid and 1–2-ml aliquots were used for assay of inorganic phosphate, by the technique of FISKE AND SUBBAROW[12].

Ca^{2+} uptake was measured by Millipore filtration technique[8]. Incubation medium contained 0.1 M KCl, 5–10 mM histidine (pH 7.3), 5 mM oxalate, 5 mM $MgCl_2$, 5 mM ATP and 10^{-4} M $^{45}CaCl_2$. Protein concentration, 0.02–0.1 mg/ml. Total volume, 2 ml. Incubation time, 5–15 min. Reaction was stopped by filtration through Millipore filter. Radioactivity of filtrate was determined by Packard liquid scintillation counter according to LOFTFIELD AND EIGNER[13].

Cholesterol determination. Cholesterol was extracted with a ethanol–acetone (1:1, v/v) solution. After precipitation with digitonin the cholesterol digitonide was washed by repeated centrifugation as described by SPERRY AND WEBB[14]. The digitonin content of the precipitate was determined using the anthrone method of VAHOUNY *et al.*[15].

Materials. Distilled ion-exchanged water was used throughout. The source of the special reagents was as follows: cholesterol (Calbiochem.), digitonin (Mann Research Laboratories), saponin (Fisher Scientific Co.), ATP (Pabst Laboratories, Inc.). Trypsin (EC 3.4.4.4) and subtilisin (EC 3.4.4.16) were purchased from Sigma Chemical Co. and crystalline bacterial protease Novo from Novo Industries A/S.

RESULTS

Morphology of microsomes

Microsome preparations, obtained by differential centrifugation from rabbit or mouse skeletal muscle, consist of membrane-bound vesicles whose shape, size and fine structure are strongly influenced by experimental conditions.

In fresh preparations, isolated from mouse leg muscles, tadpole-shaped particles predominate, which consist of a spherical head (diameter 800–1300 Å) and a tail of

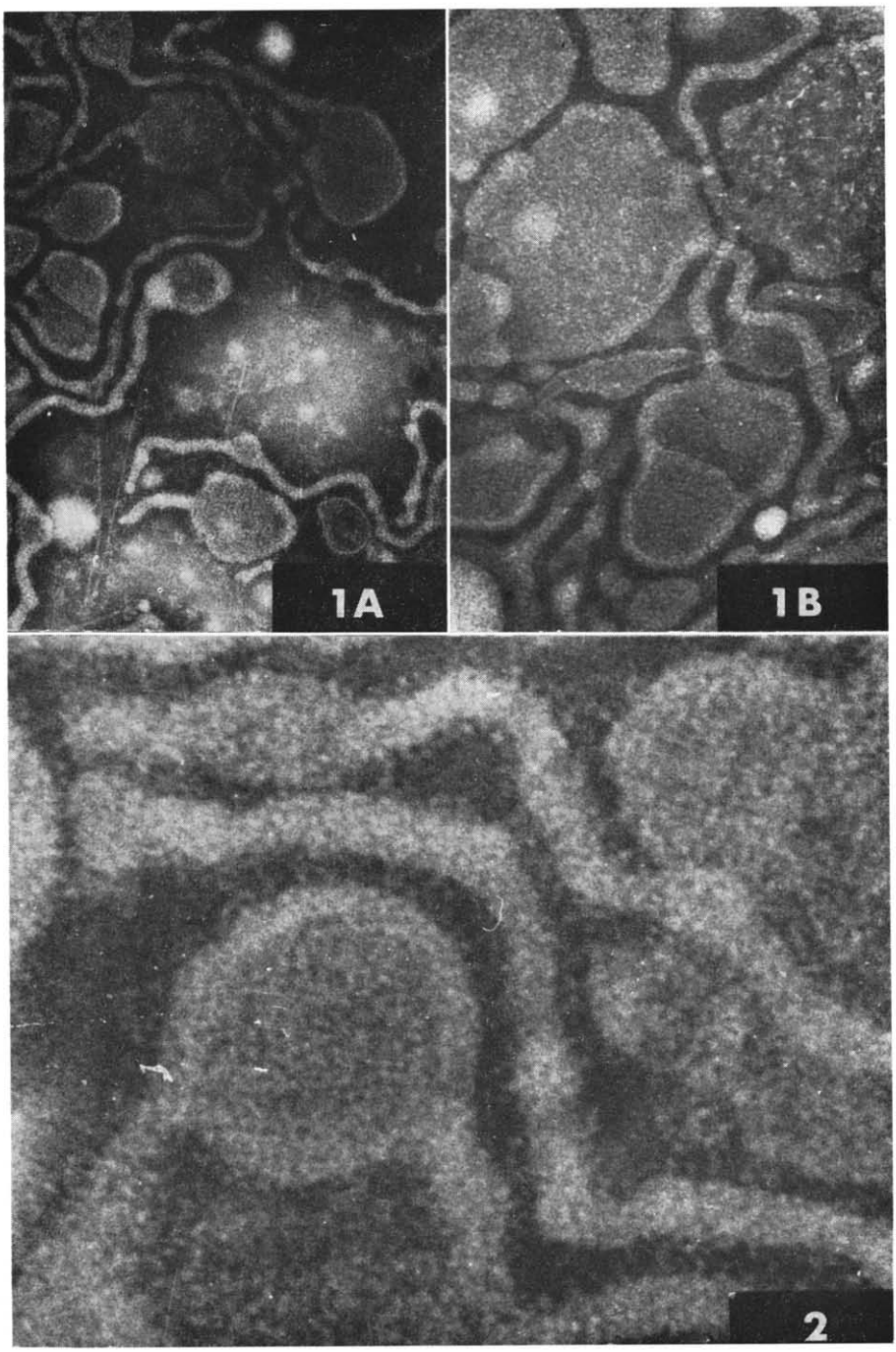

Fig. 1. Mouse microsomes. Mouse microsome preparation was isolated from leg muscles of 40–50-day-old homozygous normal mice of the 129/J subline obtained from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Me. Preparation was completed within 3 h after the animals were killed, and following negative staining with 0.5% potassium phosphotungstate, the specimens were immediately viewed. Magnification: A, × 108000; B, × 176000.

Fig. 2. Mouse microsomes. For details see legend to Fig. 1. Magnification: × 520000.

variable length (Figs. 1A, 1B and 2). The diameter of the tail is about 130–310 Å, not including the spherical particles attached to the surface. On the basis of tail length, the microsomes appear to fall into three classes. In a large portion of the particles, the length of the tail is about 0.7 μ (0.6–0.8 μ). The next class is characterized by a tail length of about 0.2–0.3 μ. A relatively small number of particles have no or very small tubular projections.

A number of particles with long tail projections display a characteristic differentiation of the head portion, which appears to be divided into a section situated in the area where the tail originates, and a dense spherical compartment which is barely penetrated by the stain and is located distant from the origin of the tail (Figs. 1A, 1B, 2). This type of differentiation is evident on less than 10 % of the particles.

Rabbit microsome preparations also contain tadpole-shaped particles, but their occurrence is less frequent, the length of the tail is variable and the tubular projections are usually thicker than those observed in mouse microsome preparations.

The surface of the microsomes is densely covered with spherical particles of approx. 40 Å diameter (Figs. 1A, 1B, 2). By virtue of their small size, the microsomal particles can be clearly distinguished from the elementary particles of the mitochondria, especially in pictures which contain small amounts of fragmented mitochondria, permitting direct comparison of the two types of elements.

Membrane thickness in negatively stained microsome preparations can be measured only after sufficient permeability increase takes place to permit the penetration of phosphotungstate into the particles. The average thickness of the membrane of a large number of microsomes, disregarding the row of particles, fell between 60 and 80 Å. The inner surface of microsomal membrane is devoid of particles.

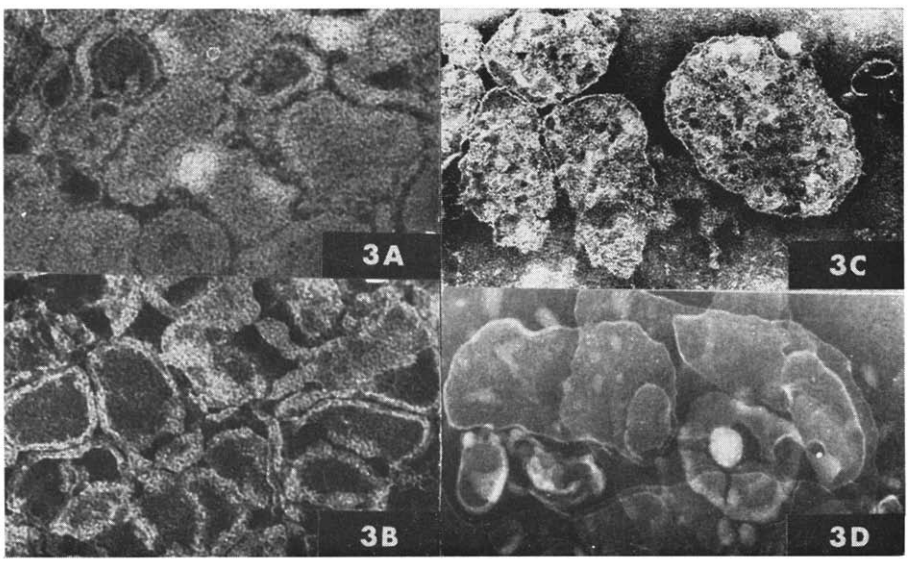

Fig. 3. Effect of trypsin on microsomes. Trypsin treatment was carried out in a medium of 0.1 M KCl, 10 mM histidine, 0.5 mM $CaCl_2$ and 0.5 mg trypsin per ml. Concentration of microsomal proteins, 5.6–10.5 mg/ml. The series of pictures was compiled from different experiments. Control microsomes (Fig. 3A; × 90000) were incubated at room temperature for 2 h without trypsin. Duration of trypsin treatment was 10 min for B (× 69000), 1 h for C (× 64000) and 2 h for D (× 80000), at room temperature.

During storage for a few days in ice, an increasing fraction of microsomes becomes permeable to negative stain. Vesicles of spherical or elongated shape become predominant over the tadpole-shaped particles, implying a change in microsomal shape with aging. These changes are not accompanied by the loss of subunit particles from the surface.

The effect of trypsin on the structure of microsomal membrane

The structure of microsomal membrane undergoes significant changes as a result of trypsin treatment (Figs. 3A, B, C, D). After even a short digestion, the number of tadpole-shaped particles decreases, the membrane permeability to potassium phosphotungstate increases and the subunit particles become less prominent (Fig. 3B).

After prolonged trypsin treatment, the microsomal membrane undergoes fragmentation with the frequent appearance of small microsomes of 300–400 Å diameter. Ultimately, smooth-surfaced, translucent membrane ghosts are frequently obtained (Figs. 3C, 3D).

The effect of proteolytic enzymes on the ATPase activity of rabbit skeletal muscle microsomes is illustrated in Fig. 4. Trypsin, Novo proteinase and subtilisin produce a transient activation of ATPase activity, which coincides with the previously reported inhibition of Ca^{2+} transport[16] and with the rapid solubilization of 20–30 % of the microsomal proteins. Prolonged incubation of microsomes with proteolytic

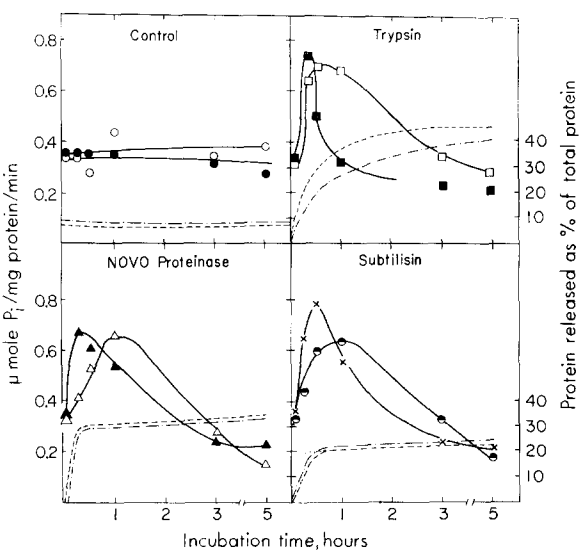

Fig. 4. Effect of proteolytic enzymes on microsomes. Microsomes (5.4 mg protein per ml) were incubated with trypsin, Novo proteinase or subtilisin (0.2 mg/ml each) for times indicated on the abscissa in a medium containing 20 mM Tris (pH 8.0) and, when indicated, 5 mM ATP. Aliquots were taken at intervals of protein remaining in the supernatant following centrifugation at 18000 × *g* for 1 h at 2°. ATPase activity was measured at room temperature in a medium containing 10 mM histidine (pH 7.3), 5 mM $MgCl_2$, 5 mM ATP and 0.2 mg microsomal protein per ml in a total volume of 2 ml. The amount of protein released into the supernatant by proteolytic enzymes is expressed as % of total microsomal protein. Open symbols represent experiments carried out in the presence of 5 mM ATP. ——, ATPase activity; – – –, protein liberation without ATP; –·–·–, protein liberation with ATP.

enzymes results in inhibition of ATPase activity, accompanied by a slow release of microsomal proteins, which results in the morphological changes represented in Figs. 3C and 3D.

The effect of digitonin on microsomes

Electron microscope analysis of digitonin-treated microsomes revealed the presence of rigid tubules with an apparently structureless wall (Fig. 5), which are similar to those observed by DOURMASCHKIN, DOUGHERTY AND HARRIS[17] in digitonin-treated liver cell membrane preparations, and considered by them to be modified cell membranes. The rigid tubules lie frequently adjacent to apparently intact microsomes with the characteristic subunits attached to them.

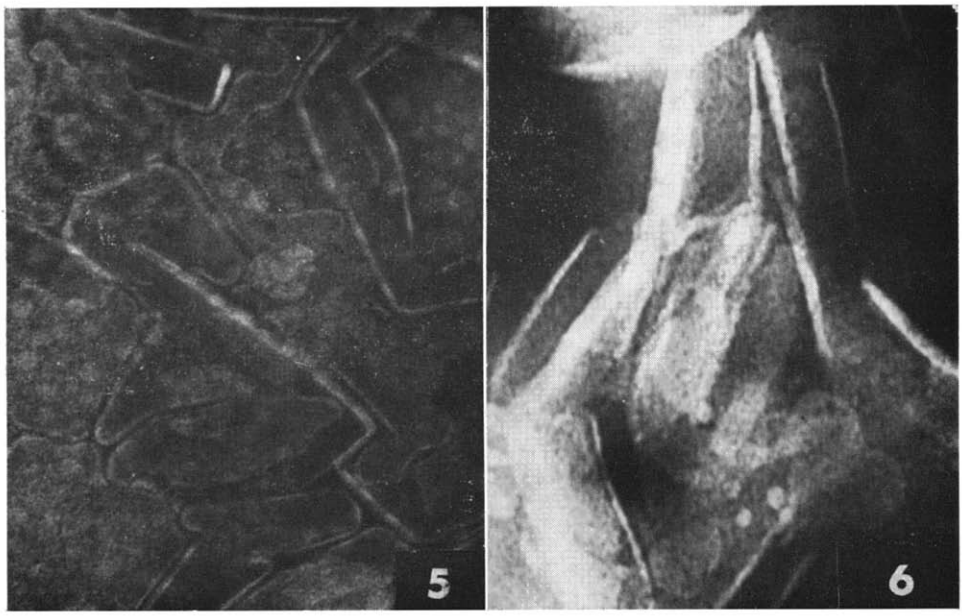

Fig. 5. Effect of digitonin on microsomes. Rabbit skeletal muscle microsomes were treated with 0.25 % digitonin for 2 h at room temperature. The microsomes were centrifuged at 3000 rev./min for 10 min. The supernatant was negatively stained with 0.5 % potassium phosphotungstate. Magnification: × 100000.

Fig. 6. Electron microscope appearance of cholesterol–digitonin complexes. 0.64 ml of a 1 mg/ml cholesterol solution in acetone–ethanol (1:1, v/v) was slowly diluted with H_2O to 5 ml and 1 ml of 2 % digitonin was added with stirring. After a 1-h incubation in ice, the opalescent suspension was negatively stained with 0.5 % potassium phosphotungstate. Magnification: × 188000.

Particles indistinguishable from those found in digitonin-treated microsome preparations, form occasionally in mixtures of pure cholesterol and digitonin (Fig. 6), although the predominant form of the cholesterol–digitonin complex is that of a spherical micelle.

Skeletal muscle microsomes contain about 0.02–0.025 mg total cholesterol per mg protein, about 90 % of which is present as free cholesterol (Table I). Digitonin treatment, followed by repeated washing, leads only to a small decrease in the free cholesterol content as expected from the low solubility of cholesterol digitonide.

TABLE I

CHOLESTEROL CONTENT OF SKELETAL MUSCLE MICROSOMES

n = number of determinations. Cholesterol content is expressed as mg cholesterol per mg protein. Microsomes (protein concentration 5.1–7.0 mg/ml) were treated with 0.3 % digitonin in a medium buffered with $5 \cdot 10^{-2}$ M histidine for 24–48 h in ice. The microsomes were diluted to a protein concentration of 0.6–0.8 mg/ml with H_2O and were centrifuged at 28000 × g for 45 min. The sediment was resuspended in original volume of H_2O and centrifugation repeated. Cholesterol determinations on control and digitonin-treated microsomes were carried out as described under EXPERIMENTAL PROCEDURES.

Free cholesterol						*Total cholesterol*					
Control microsomes			*Digitonin-treated microsomes*			*Control microsomes*			*Digitonin-treated microsomes*		
Cholesterol content	*n*	*S.E.*	*Cholesterol content*	*n*	*S.E.*	*Cholesterol content*	*n*	*S.E.*	*Cholesterol content*	*n*	*S.E.*
0.0153	22	± 0.0016	0.0145	11	± 0.0010	0.0194	12	± 0.0014	0.020	2	—

Treatment of microsomes with digitonin inhibits Ca^{2+} uptake, while the ATPase activity is slightly activated (Fig. 7). The inhibition of the Ca^{2+} uptake and the activation of ATPase activity are not reversed by addition of cholesterol in a wide range of concentrations.

Effect of phospholipase C on microsomes

Treatment of microsomes with phospholipase C inhibits the ATPase activity[3] and Ca^{2+} transport[10,11,18]. In spite of these profound biochemical changes, the surface structure of the microsomal membrane remains similar to that of control microsomes. Part of the surface of phospholipase C-treated microsomes is covered with a diglyceride

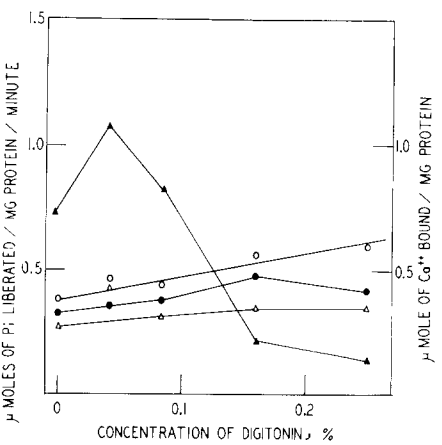

Fig. 7. Effect of digitonin on the ATPase and Ca^{2+} uptake of microsomes. Freshly prepared microsomes were incubated with digitonin (0–0.25 %) at room temperature in the presence of 5 mM histidine buffer (pH 7.3), for 2 h. Protein concentration, 2.5 mg/ml. At the end of incubation, the solution was centrifuged at 3000 rev./min for 10 min and the ATPase activity and Ca^{2+} uptake measured as described under EXPERIMENTAL PROCEDURES. Protein concentration during ATPase assay: ○—○, 0.062 mg/ml; ●—●, 0.125 mg/ml; △—△, 0.25 mg/ml. Protein concentration in Ca^{2+} uptake measurements: ▲—▲, 0.022 mg/ml.

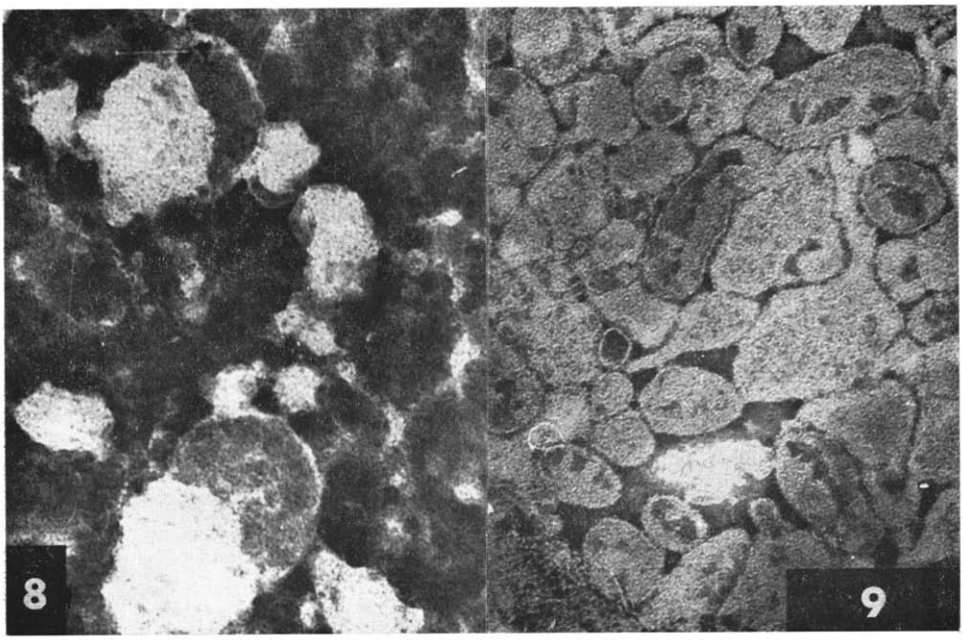

Fig. 8. Effect of phospholipase C on microsomes. Phospholipase C treatment was carried out as described earlier[18], using 0.5 mg phospholipase C per ml and 5.7 mg microsomal protein per ml. Incubation at room temperature for 1 h. Magnification: × 104 500.

Fig. 9. Effect of temperature on the surface structure of rabbit skeletal muscle microsomes. Microsomes were exposed to room temperature for 2 h in a solution of 0.1 M KCl, 10 mM histidine, prior to staining with 0.5 % potassium phosphotungstate. Magnification: × 76 000.

droplet, which appears as a transparent spherical contour on negative staining (Fig. 8).

Temperature effect on microsomes

BENEDETTI AND EMMELOT[19] reported a marked change in the surface structure of liver cell membrane depending on whether the staining was carried out at 2° or 37° temperature.

Although the subunit particles became less prominent after incubation of microsomes for 2 h at 25° or 37°, no definite mosaic pattern appeared (Fig. 9). The Arrhenius plot of the temperature dependence of microsomal ATPase did not indicate any sharp transition in the range of 0–40° (Fig. 10) which could be correlated with the transformation of the membrane from particulate into mosaic structure.

Effect of saponin on microsomes

Adsorption of saponin to erythrocyte and liver cell membranes causes the appearance of a hexagonal array of pits surrounded by rings[17,20,21].

Treatment of microsomes with saponin (1–2 %) for 5–6 h at 0° causes only small and variable inhibition of the Ca^{2+} uptake with no change in the ATPase activity. Although the subunit structure of the microsomal surface became less prominent after

saponin treatment, the characteristic mosaic structure described for other types of membranes was not observed.

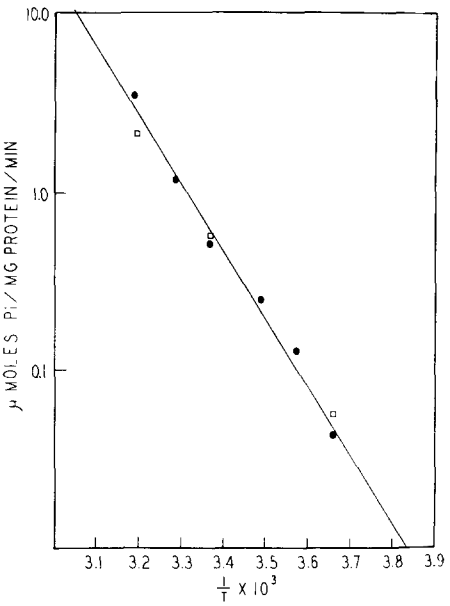

Fig. 10. Effect of temperature on the ATPase activity of skeletal muscle microsomes. ATPase activity was measured in a medium containing 10 mM histidine (pH 7.3), 5 mM $MgCl_2$, 5 mM ATP, and microsomes at a protein concentration of 0.05 mg/ml. Incubation time was 30 min at 0° and 8°, 10 min at 15°, and 5 min at 25°, 32°, and 38° (●—●). In some experiments 10^{-4} M $CaCl_2$ was added to the above incubation medium (□—□).

DISCUSSION

As represented by DAVSON AND DANIELLI[22], biological membranes consist of a bimolecular leaflet of phospholipids coated on both sides with proteins. While the basic content of this model is supported by a large body of evidence[23], important structural features were recently recognized in a variety of biological membranes which were not predictable on the basis of the original unit membrane theory. Such differentiation of biological membranes is shown by the presence of elementary particles on the membranes of mitochondria[24,25], liver cells[19], and Rous sarcoma virus[17]. Patterns consisting of a hexagonal array of subunits were observed on the synaptic disc of the goldfish Mauthner cell[26].

The presence of distinct particles of about 40 A diameter on the surface of sarcoplasmic reticulum membranes is reported in the present paper. The particles are clearly observed on microsomes negatively stained within a few hours after preparation. Prolonged aging, treatment with trypsin, saponin, or exposure to 25–40° temperature for a few hours, results in a moderate reorganization of the surface structure which makes the subunits less prominent.

Transformation from particulate to a mosaic-type surface structure was re-

ported to occur at elevated temperatures[19] and following saponin treatment[17] on different surface membranes. The temperature-dependent transformation of liver cell membranes is assumed to accompany the change of the lamellar phospholipid framework into a micellar one at higher temperatures.

Surprisingly, the temperature dependence of the microsomal ATPase did not indicate any sharp change in the range of 0–40° which could be correlated with some structural change of the membrane, although the ATPase activity of microsomes is known to be dependent upon phospholipids. Furthermore, the surface structure of skeletal muscle microsomes did not assume the characteristic mosaic pattern shown by cell surface membranes, after prolonged exposure to temperatures of 25–37° or following saponin treatment. Even phospholipase C-treated microsomes which lost 60–70 % of their phospholipid content displayed a surface structure rather similar to control microsomes. These observations tend to emphasize the structural role of non-phospholipid constituents in membranes of sarcoplasmic reticulum.

The changes in the surface appearance of microsomes subjected to prolonged trypsin treatment might be explained by the susceptibility of the surface particles to tryptic hydrolysis.

The physiological role of the observed subunits is unknown. Their identification with the ATPase enzyme implicated in the Ca^{2+} transport was attempted using the histochemical procedure of TICE AND BARRNETT[27], without conclusive results.

DOURMASCHKIN, DOUGHERTY AND HARRIS[17] concluded that the rigid tubular structures which appear in preparations of liver cell membranes after treatment with digitonin represent modified cell membranes. Under similar experimental conditions, identical structures are formed in digitonin-treated skeletal muscle microsomes, and in water suspensions of cholesterol and digitonin. These latter observations suggest that the structures probably represent cholesterol digitonides rather than modified microsomal membranes. An explanation must be sought for the fact that while the rigid tubules form abundantly when microsomes are treated with digitonin, in cholesterol-digitonin suspensions small micellar droplets prodominate. The loss of Ca^{2+} transport in digitonin-treated microsomes suggests that cholesterol is in some way involved in the Ca^{2+} accumulation process.

It is generally argued that, during the isolation of skeletal muscle microsomes, the sarcoplasmic reticulum undergoes fragmentation.

The frequent occurrence of tadpole-shaped particles in fresh microsome preparations isolated from rat, mouse and rabbit muscles might indicate that large segments of the sarcoplasmic reticulum membranes remain intact during the preparation procedure. It must be emphasized, however, that the shape, as well as the surface structure of microsomes, is probably influenced by the preparation procedure, making all conclusions regarding membrane morphology entirely tentative.

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REFERENCES

1 J. B. FINEAN AND A. MARTONOSI, *Biochim. Biophys. Acta*, 98 (1965) 547.
2 W. W. KIELLEY AND O. MEYERHOF, *J. Biol. Chem.*, 176 (1948) 591.
3 W. W. KIELLEY AND O. MEYERHOF, *J. Biol. Chem.*, 183 (1950) 391.
4 S. EBASHI, *J. Biochem. Tokyo*, 50 (1961) 236.
5 S. EBASHI AND F. LIPMANN, *J. Cell Biol.*, 14 (1962) 389.
6 W. HASSELBACH AND M. MAKINOSE, *Biochem. Z.*, 333 (1961) 518.
7 W. HASSELBACH AND M. MAKINOSE, *Biochem. Biophys. Res. Commun.*, 7 (1962) 132.
8 A. MARTONOSI AND R. FERETOS, *J. Biol. Chem.*, 239 (1964) 648.
9 A. MARTONOSI AND R. FERETOS, *J. Biol. Chem.*, 239 (1964) 659.
10 A. MARTONOSI, *Biochem. Biophys. Res. Commun.*, 13 (1963) 273.
11 A. MARTONOSI, *Federation Proc.*, 23, Suppl. 5 (1964) 913.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 R. B. LOFTFIELD AND A. A. EIGNER, *Biochem. Biophys. Res. Commun.*, 3 (1960) 72.
14 W. M. SPERRY AND M. WEBB, *J. Biol. Chem.*, 187 (1950) 97.
15 G. V. VAHOUNY, R. M. MAYER, I. H. ROE AND C. R. TREADWELL, *Arch. Biochem. Biophys.*, 86 (1960) 210.
16 A. MARTONOSI, *J. Biol. Chem.*, 243 (1968) 71.
17 R. R. DOURMASCHKIN, R. M. DOUGHERTY AND R. J. C. HARRIS, *Nature*, 194 (1962) 1116.
18 A. MARTONOSI, J. DONLEY AND R. A. HALPIN, *J. Biol. Chem.*, 243 (1968) 61.
19 E. L. BENEDETTI AND P. EMMELOT, *J. Cell Biol.*, 26 (1965) 299.
20 A. D. BANGHAM AND R. W. HORNE, *Nature*, 196 (1962) 952.
21 A. M. GLAUERT, J. T. DINGLE AND J. A. LUCY, *Nature*, 196 (1962) 953.
22 H. DAVSON AND J. F. DANIELLI, *The Permeability of Natural Membranes*, Cambridge University Press, Cambridge, 2nd ed., 1952.
23 J. B. FINEAN, *Progr. Biophys. Mol. Biol.*, 16 (1966) 143.
24 H. FERNÁNDEZ-MORÁN, T. ODA, P. V. BLAIR AND D. E. GREEN, *J. Cell Biol.*, 22 (1964) 63.
25 D. F. PARSONS, *Science*, 140 (1963) 985.
26 J. D. ROBERTSON, *J. Cell Biol.*, 19 (1963) 201.
27 L. W. TICE AND R. J. BARRNETT, *J. Cell Biol.*, 15 (1962) 401.