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PROTEASE DIGESTION OF MEMBRANES
ULTRASTRUCTURAL AND BIOCHEMICAL EFFECTS

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SUMMARY

(1) Nagarse, a bacterial protease, was permitted to react with sarcoplasmic reticulum, submitochondrial and plasma membranes. Gel electrophoresis indicated that all polypeptides were labile to the enzyme, and therefore must be at least partially exposed at membrane surfaces. However, hydrolysis did not proceed to completion, and in each membrane 30–50 % of the original protein mass remained after extensive digestion. Gel patterns showed that remaining polypeptide fragments were in the range of 10000 molecular weight.

(2) Amino acid analysis of the original protein and membrane-bound digestion product was performed. Only minor changes were observed following digestion, suggesting that the peptide fragments remaining with the membrane did not have specialized amino acid compositions.

(3) Freeze-fracture analysis of Nagarse-treated sarcoplasmic and plasma membranes showed that particulate structures were present, although particle density and asymmetry of distribution between fracture faces were decreased. In submitochondrial membranes, digested membranes were indistinguishable from the original membranes in particle density and distribution. We conclude that high molecular weight polypeptides are not required for the production particulate structures in freeze-fracture images of membranes.

INTRODUCTION

Freeze-fracture electron microscopy has been extensively used to investigate biological membrane structure (for reviews, see refs. 1–3). Freeze-fracture images of different membranous systems have varying appearances. For instance, lamellar phase lipid and myelin membranes appear smooth [1, 4], whereas more complex

Abbreviation: TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

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membranes (chloroplast, mitochondrial, plasma membrane) appear as smooth sheets interrupted by particulate structures [5–7]. When particles are present, there is often an asymmetric distribution of particles between the two fracture faces [8–10].

The interpretation of the appearance of various membrane fracture faces is a current problem in membrane biology. If it is accepted that the freeze-fracture method normally parts membranes along hydrophobic planes [11–13] then the particles may represent membrane proteins extending into or through a bilayer of lipid. In several systems, including sarcoplasmic reticulum [14] rod outer segments [15] and lipid monolayers and bilayers penetrated by hydrophobic apoprotein [16, 17] particles have been correlated with the presence of proteins. However, the precise manner in which particles are produced by the freeze-fracture method is still obscure.

One approach to this problem is to perturb the membrane protein in some controlled fashion and observe the effects on the freeze-fracture image. For instance, Engstrom [18] digested erythrocyte membranes with pronase, a proteolytic enzyme, and found considerably fewer particles in the digested membranes. This result suggested that the particles were correlated with membrane protein. Tillack et al. [19] treated erythrocyte membranes with lithium diiodosalicylate, which tends to dissociate lipid-protein complexes, and also found that particle density decreased in fracture faces.

In the present study, we have treated several membranes (sarcoplasmic reticulum, submitochondrial membranes and erythrocyte membranes) with Nagarse, a bacterial protease of broad specificity. The digestion process was followed over a period of time by measuring membrane protein and enzymatic activity, and by correlating gel electrophoresis patterns with freeze-fracture images. We addressed the following questions.

(1) Are there differences in the mode of digestion of the various membrane polypeptides by Nagarse?

(2) Does digestion release a random assortment of amino acids, or are there specific amino acids or polypeptides which remain associated with the membrane?

(3) Does digestion affect the size, density or distribution of the particles observed by the freeze-fracture method?

MATERIALS AND METHODS

Preparation of lobster muscle microsomes

Sarcoplasmic reticulum was isolated from 4–6 freshly dissected lobster abdominal muscles [14]. The whole muscles were homogenized for 30 s in 4 vols. of cold 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (TES) buffer (pH 7.0) in a cooled Waring blender. All operations were performed at 0–4° C. The homogenate was centrifuged at $5000 \times g$ for 5 min and the supernatant was filtered through cheese cloth. The pellets were resuspended by homogenization with half the original buffer volume and centrifuged again at $5000 \times g$. Both supernatants were combined and centrifuged for 15 min at $12000 \times g$, and the pellets were discarded. The supernatant was centrifuged at $40000 \times g$ for 40 min, and the microsomal pellets were combined and resuspended in 0.6 M KCl/10 mM TES buffer (pH 7.0) to remove extraenous protein [20]. After 60 min incubation at 0 °C the suspension was centrifuged at $40000 \times g$ for 40 min. The pellets were resuspended in 10 mM

TES buffer and centrifuged at 40 000 × *g* for 40 min, and the final pellets were combined and resuspended in 0.25 M sucrose/10 mM TES (pH 7.0) to a protein concentration of approximately 10 mg/ml. Aliquots of the sarcoplasmic reticulum preparation were frozen in liquid N_2 and stored at −80 °C. Biochemical parameters of stored sarcoplasmic reticulum preparations (calcium uptake, ATPase activity) were similar to those of fresh preparations.

Preparation of mitochondria and submitochondrial membranes

Rat liver mitochondria were prepared according to the method of Stancliff et al. [21]. Submitochondrial membranes were prepared according to the method of Gregg [22] using three 5 min sonications in ice. After sonication, larger fragments were removed by centrifugation at 25 000 × *g* for 20 min. The supernatant was centrifuged at 100 000 × *g* for 60 min and the pellets were resuspended in 0.25 M sucrose 1 mM Tris · HCl (pH 7.4)/0.1 mM EDTA to approx. 20–30 mg protein/ml. Aliquots were stored by freezing as described for sarcoplasmic reticulum.

Preparation of human erythrocyte plasma membranes

Erythrocytes were obtained by venipuncture with a heparinized syringe (10 units/ml blood) and washed 3 times in phosphate-buffered 0.85 % saline. Plasma membranes were prepared according to Dodge et al. [23] and used the same day.

Nagarse digestion of membranes

Samples of sarcoplasmic reticulum and submitochondrial membranes were suspended in 1 ml of 10 mM TES (pH 7.0) at a concentration of 5 mg/ml, with varying concentrations of Nagarse, and stirred magnetically at 25 °C. Plasma membranes were suspended in 2 ml of the same buffer system to a concentration of 1.5 mg/ml, together with indicated quantities of Nagarse. After varying intervals, aliquots were removed for gel electrophoresis and enzyme assays. In order to obtain pellets for electron microscopy, the remaining sample was centrifuged through a subphase of 0.25 M sucrose/10 mM TES (pH 7.0) at 100 000 × *g* for 60 min. The supernatant, containing most of the Nagarse, was removed by aspiration and the surface of each pellet was carefully rinsed with cold 10 mM TES buffer.

Enzyme assays

ATPase activity of sarcoplasmic reticulum and submitochondrial membranes was measured by the pH change accompanying ATP hydrolysis [14, 24] at pH 7.5. Succinate oxidase activity was measured according to King [25] with a Clark oxygen electrode. Details may be found in the figure and table legends. Protein was estimated according to lowry et al. [26] using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Gel electrophoresis was carried out according to the procedure of Melnick et al. [27] and molecular weights were standardized with bovine serum albumin, bovine liver catalase, pepsin, trypsin, lysozyme and horse heart cytochrome *c* (Sigma Inc., St. Louis, Mo.). In order to be certain that Nagarse was inhibited by the sodium dodecyl sulfate/mercaptoethanol cocktail, membranes and Nagarse were mixed directly in the cocktail at 80 °C. As discussed in the Results section, no hydrolysis

products were observed, and we assume that changes in the gel patterns reflect hydrolysis only during the incubation period at 25 °C.

Freeze-fracture electron microscopy

Pellets of control and treated membranes were mixed with sufficient glycerol to give a final concentration of 20–30 % glycerol. The pellet and glycerol were stirred on ice for 10 min, and samples were freeze-fractured according to Fisher and Branton [28]. The results described for freeze-fracture analysis were obtained from combined data of three different experiments on each membrane system.

Amino acid analysis

Protein samples were hydrolysed under vacuum for 24 h in 6 M HCl at 110 °C, and amino acid analyses were performed with a Beckman model 121 analyzer.

Reagents

Nagarse (EC 3.4.21.14), a crystalline bacterial protease ($80 \cdot 10^4$ punits/g), was a product of Nagase Ltd., Osaka, Japan, and was a kind gift of Dr. I. Nishigaki. ATP and oligomycin were purchased from Sigma Inc., St. Louis, Missouri.

RESULTS

Loss of membrane protein and enzyme activity during digestion

All membrane protein components were readily attacked by Nagarse, with solubilization of a major fraction of the original membrane protein (Fig. 1). However, hydrolysis did not proceed to completion and an equilibrium was reached after approx. 20 min, in which 30–50 % of the original protein mass remained with centrifugal pellets of the digested membranes.

In preliminary studies the ATPase activity of sarcoplasmic reticulum was

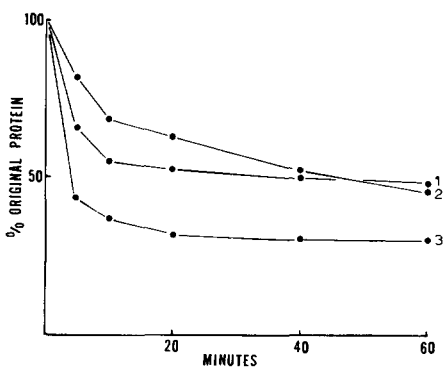


Fig. 1. Loss of protein from membranes during digestion with Nagarse. Membrane protein (3–5 mg) was incubated with Nagarse (Nagarse : protein = 1 : 10) in 1 ml of 10 mM TES buffer (pH 7.0). After varying times, samples were centrifuged through 0.25 M sucrose 10 mM TES (pH 7.0) at 100 000 × *g* for 60 min in order to remove Nagarse and stop hydrolysis. Pellets were taken up in a small volume of buffer for protein determinations and the amount of protein was compared with that in pellets which were not treated with Nagarse. (1) Sarcoplasmic reticulum; (2) plasma membranes; (3) submitochondrial membranes.

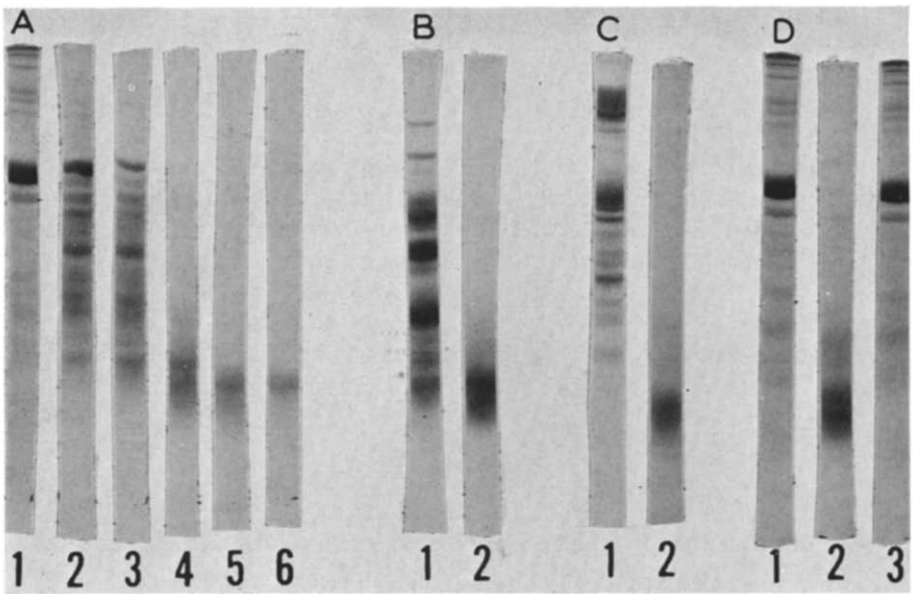


Fig. 2. Effect of Nagarse digestion on gel patterns of membrane proteins. (A) Sarcoplasmic reticulum. Gels 1–6 are control and ratios of 10^4, 10^3, 10^2, 10 and 2 mg protein/mg Nagarse, respectively. Conditions are as in Fig. 1, with a 60 min digestion period. (B, C) Submitochondrial membranes and plasma membranes, respectively, were digested as described above for sarcoplasmic reticulum, but only the control and 2 : 1 protein : Nagarse ratios are shown. All gels correspond to 60 μg protein. Similarly treated membranes were used for the freeze-fracture analyses described in Figs. 3, 4, 5 and 6. (D) Sarcoplasmic reticulum. This represents a test of the sodium dodecyl sulfate/mercaptoethanol inhibition of Nagarse digestion. Gel 1 = control. Gel 2 shows that hydrolysis occurred in sodium dodecyl sulfate /mercaptoethanol at room temperature. In gel 3, the mixture was heated to 80 °C. and digestion was completely inhibited under these conditions.

measured during enzymatic digestion. At a Nagarse: membrane protein ratio of 1: 10, the ATPase activity was totally inhibited after 5 min digestion. Even at ratios of 1: 100, inhibition was complete after 40 min digestion. From such experiments it was apparent that varying the Nagarse: protein ratio permitted control over digestion rates of specific proteins bound to membranes. Therefore, varyious ratios of Nagarse: protein by weight were used to digest membrane protein over a period of 60 min, and resulting hydrolysis products were compared by gel electrophoresis. At ratios which inhibited the ATPase activity of sarcoplasmic reticulum, the ATPase band in gels was also diminished or absent (Fig. 2A). Ratios of Nagarse: protein greater than 1: 100 attacked all of the original membrane polypeptides, and the hydrolysis products formed a diffuse band in the region of 10 000 daltons. Lower ratios of Nagarse: protein produced new bands with molecular weights of 66 000, 47 000 and 29 500, suggesting that the major ATPase polypeptide was not attacked randomly by the enzyme.

Digestion of submitochondrial membranes with Nagarse

In contrast to sarcoplasmic reticulum, submitochondrial membranes are relatively complex, with numerous protein components related to oxidative phos-

TABLE I

EFFECT OF NAGARSE ON ATPase AND SUCCINATE OXIDASE ACTIVITY OF SUBMITOCHONDRIAL MEMBRANES

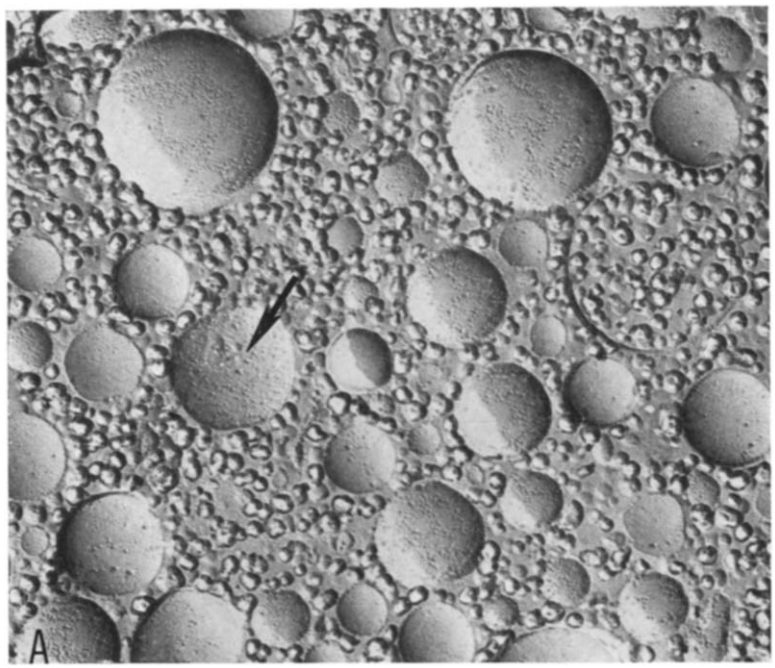
Submitochondrial membrane preparations (5 mg protein) were incubated 60 min with Nagarse, as described in Fig. 1. For ATP hydrolysis measurements, the reaction mixture contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 1 mM Tris · HCl (pH 7.5), 5 mM ATP and 50 μg of submitochondrial membrane protein $\pm$oligomycin, 20 μg/mg protein. Succinate oxidase activity was measured with a Clark oxygen electrode in a reaction mixture containing 0.1 M potassium phosphate (pH 7.8), 38 mM succinate, 28 μM cytochrome *c* and 0.24 mg/ml submitochondrial membrane protein in a total volume of 5 ml.

Protein : Nagarse ratio	ATPase activity (μmol · mg^{-1} · min^{-1})		Succinate oxidase (natoms 0 · mg^{-1} · min^{-1})
	−Oligomycin	+Oligomycin	
Control	1.73	0.25	367
10^4	1.25	0.12	304
10^3	1.05	0	258
10^2	0.15	0	92
10^1	0	0	0
2 : 1	0	0	0

phorylation. We reasoned that perhaps one or more of these components might prove to be resistant to enzymatic hydrolysis, as suggested by Tillack et al. [19] for certain proteins of the erythrocyte membrane. Therefore the activity of two enzymes, ATPase and succinate oxidase, and the gel patterns were compared during Nagarse digestion (Table I and Fig. 2B). The ATPase and succinate oxidase activity were inhibited by Nagarse digestion at higher ratios. The ATPase activity was somewhat more rapidly attacked, probably because of its localization on membrane outer surfaces. In contrast to submitochondrial membranes from beef heart [29] succinate oxidase activity of liver mitochondria was also attacked by Nagarse. Fig. 2B shows the results of gel electrophoresis. Submitochondrial membrane polypeptides were digested to lower molecular weight components in the 10000 range, similar to the results with sarcoplasmic reticulum.

Digestion of erythrocyte plasma membranes

Erythrocyte plasma membranes were relatively resistant to enzymatic hydrolysis, since Nagarse treatment at a ratio of 1: 10 for 60 min did not completely digest higher molecular weight polypeptides (not shown). However, treatment at ratios of 1: 2 for 60 min (Fig. 2C) produced the result observed with sarcoplasmic reticulum and submitochondrial membranes, that is, disappearance of all original polypeptide bands and replacement by a diffuse 10000 dalton band. Since hydrolysis of phospholipids could potentially affect the interpretation of our results, erythrocyte membranes were also used to test the Nagarse enzyme for phospholipase activity. Chloroform/methanol extracts of the membranes were prepared at 0 time and after 60 min of digestion at 1 : 2 ratios of Nagarse: protein. Analysis of the extracts by thin-layer chromatography showed no detectable differences in the lipid composition, and we concluded that lipase activity was not a significant factor.



In order to be certain that digestion was not occurring in the sodium dodecyl sulfate/mercaptoethanol mixture prior to loading the gels, sarcoplasmic reticulum membrane protein and Nagarse were mixed in the cocktail in various conditions. As shown in Fig. 2D, Nagarse was not inhibited by sodium dodecyl sulfate/mercaptoethanol at room temperature. However, heating the mixture for 2 min at 80 °C completely inactivated the enzyme. The latter conditions were used for all experiments described in this paper.

Freeze-fracture images of Nagarse-digested membranes

It was clear from the above results that Nagarse treatment had a dramatic effect on the protein components of three different membranes. After 60 min digestion at Nagarse: protein ratios of 1: 2 or 1: 10, about half the original protein remained, enzyme activities were inhibited and only diffuse low molecular weight bands appeared on gels. The freeze-fracture images of the treated membranes might be expected to be considerably altered, but we were surprised to find remarkably few changes in the ultrastructural appearance of fracture faces.

Fig. 3 shows control and treated sarcoplasmic reticulum membranes, which underwent the most marked alterations during digestion. Apparent fusion of sarcoplasmic reticulum vesicles occurred during digestion, and the average area per vesicle increased 4-fold. Surfaces remained particulate, with approximately half the original particle density remaining (combined count from concave and convex fracture faces).

Fig. 4 shows submitochondrial membranes. Despite the fact that gel electrophoresis patterns indicated that polypeptides larger than 10 000 daltons were absent,

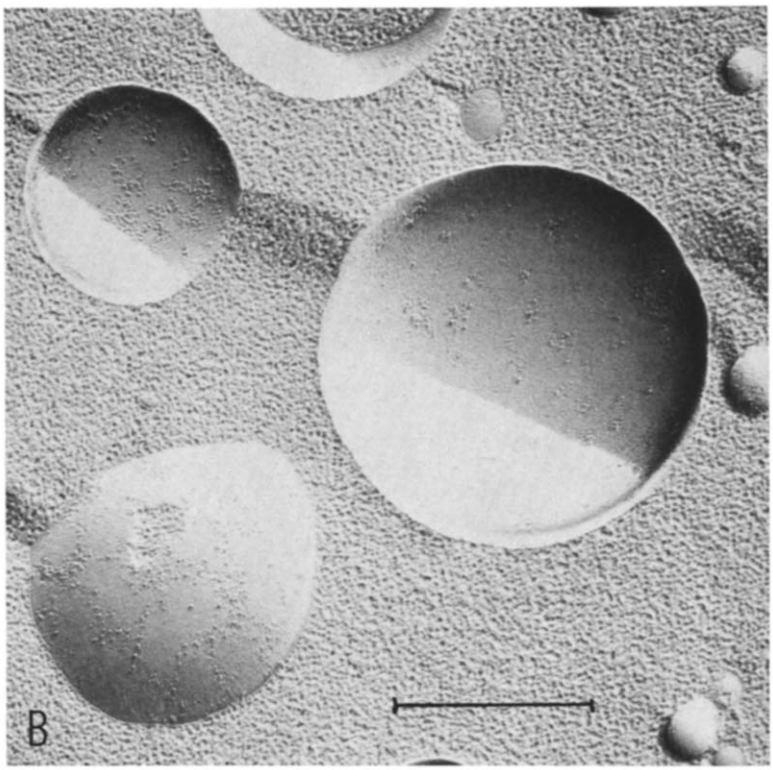
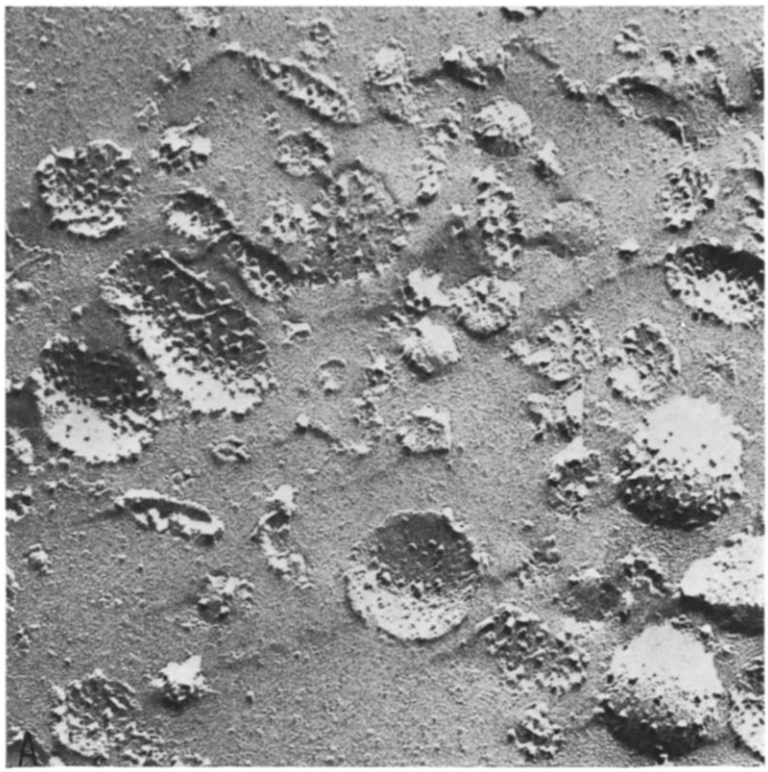


Fig. 3. Effect of Nagarse digestion on sarcoplasmic reticulum preparations. (A) Microsomes of control preparations have particles on concave fracture faces, and convex faces are relatively smooth. The arrow shows one exception, which may represent a contaminating membrane. (B) Microsomes treated with Nagarse (Nagarse : protein ratio = 1 : 2) still exhibit particles, although at lower densities. Particles are equally distributed on concave and convex fracture faces. Apparently fusion of vesicles has occurred: the average diameter has increased 2-fold and the average vesicle area 4-fold. Bar shows 0.5 μM.

the freeze-fracture images of digested membranes were indistinguishable from those of the controls. Fig. 5 shows erythrocyte ghost membranes. Again particles remained on both fracture faces, although reduced in number on the A face. The B face appeared to be unaltered by digestion. In all three membranes no obvious change in particle diameter was found. Since Nagarse digestion caused fusion of sarcoplasmic reticulum vesicles (but not submitochondrial membrane vesicles) we also checked erythrocyte membranes for fusion, using phase contrast microscopy. Nagarse digestion had no apparent effect on the size or shape of the ghosts, and we concluded that digestion caused neither fusion nor breakdown of erythrocyte plasma membranes.

Fig. 6 summarizes results obtained with the freeze-fracture method. With the exception of submitochondrial membranes, Nagarse digestion reduced the total number of particles observed per unit membrane area by about half. In all three membranes the original asymmetric distribution of particles was diminished, and particles became more randomly distributed between concave and convex fracture faces. This effect was most clearly demonstrated in sarcoplasmic reticulum membranes.



Amino acid analysis of digested membranes

About half the original protein mass remained with membranes following Nagarse digestion. It was of interest to determine whether the amino acids of remaining polypeptide fragments could represent hydrophobic species protected from hydrolysis by virtue of being embedded in the membrane lipid phase. One might also expect that hydrophilic species located on membrane surfaces would be diminished, since they would be more readily attacked by the enzyme. Therefore, amino acid analysis was carried out on the original membranes and on digested membranes, and these results are shown in Table II. In general, no dramatic alterations of amino acid composition were apparent. The content of strongly hydrophobic amino acids was moderately increased in plasma membranes and submitochondrial membranes. However, only phenylalanine increased in sarcoplasmic reticulum, while other hydrophobic amino acids decreased. Sarcoplasmic reticulum also varied in that anionic amino acid content (aspartic and glutamic combined) increased with digestion, while the total anionic amino acid content of the other membrane species decreased. In all three membranes the content of lysine and arginine decreased after Nagarse treatment. No general trends could be detected among the other possible groupings of amino acids.

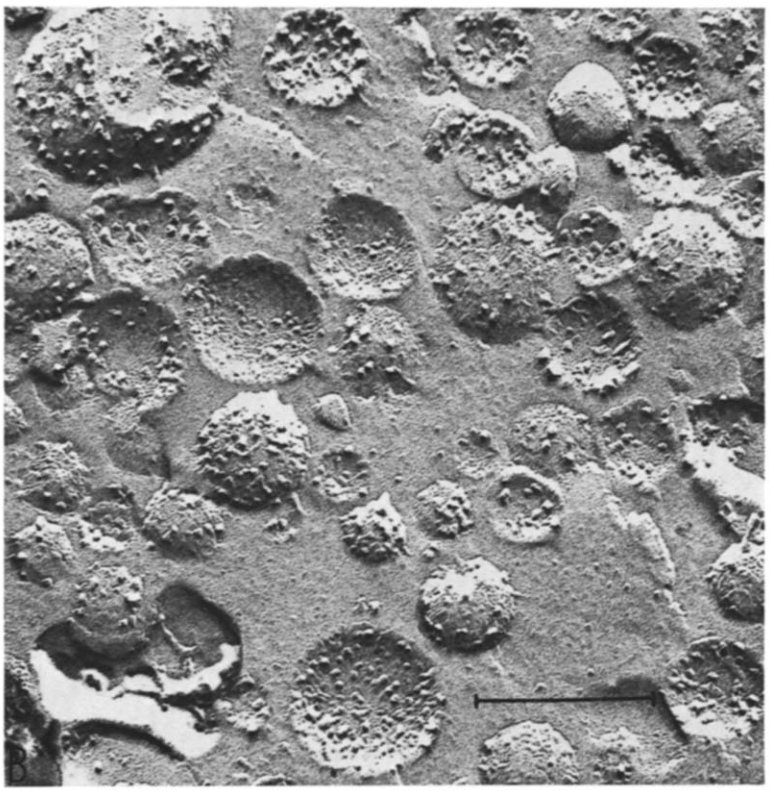


Fig. 4. Effect of Nagarse digestion on submitochondrial membranes. (A) Controls. (B) Nagarse-treated membranes, conditions as in Fig. 3. Superficially there is little effect of Nagarse digestion on the appearance of fracture faces. Bar shows 0.1 μm.

DISCUSSION

The results reported here show that Nagarse can readily digest protein of three different membrane species and produce lower molecular weight polypeptides. An important result is that none of the original polypeptides in any of the three membranes was resistant to hydrolysis. This suggests that every polypeptide must be at least partially exposed at membrane surfaces. About half the original protein mass as measured by the Lowry method remained bound to the membrane and was resistant to further hydrolysis. The digestion product is presumably correlated with diffuse, low molecular weight bands which appear on polyacrylamide gels, and may represent fragments of polypeptides which are protected from enzyme action by their position within the lipid bilayer.

There was some question concerning the accuracy of the Lowry method in measurements of polypeptide remaining with membranes following digestion. However, the amino acid analyses were performed on measured aliquots of control and digested membranes whose protein content had been determined by the Lowry method. The total amino acid content of the samples therefore provided a second determination of the amount of polypeptide remaining with the digested membranes.

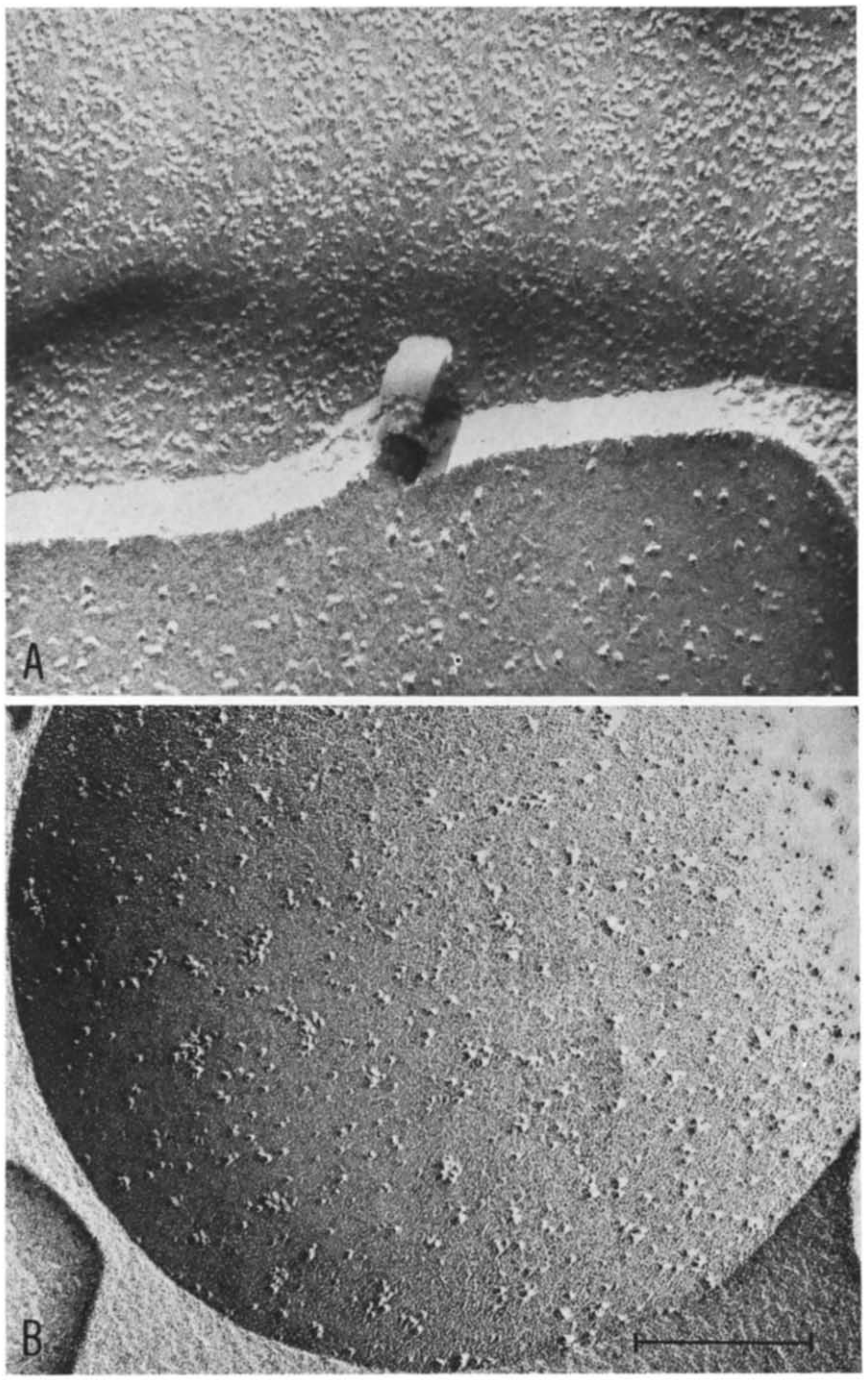


Fig. 5. Erythrocyte plasma membranes. (A) Control shows both A and B fracture faces, with particles primarily on the A face. (B) After Nagarse digestion the A face still contained particles, but fewer in number. B faces (not shown) also exhibit particles, but the particle density was unchanged. Bar shows 0.1 μm.

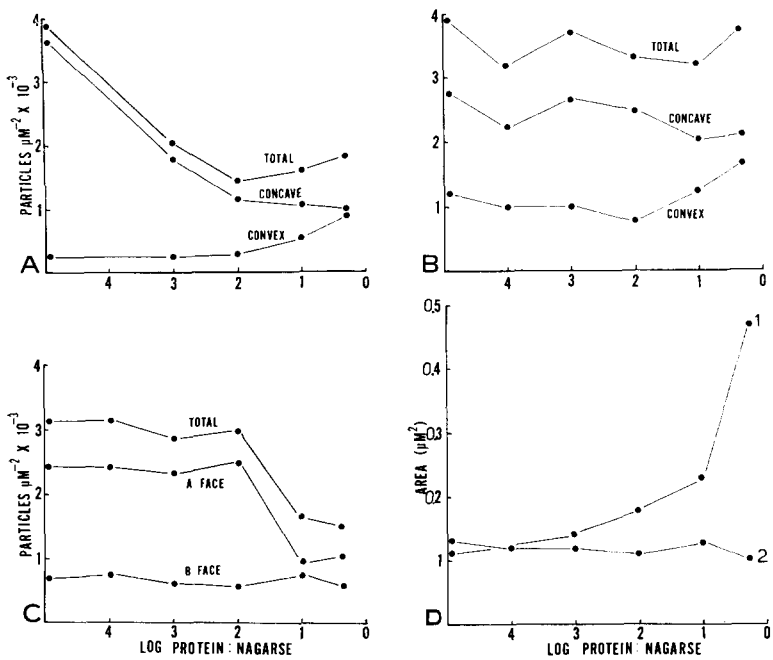


Fig. 6. Summary of particle count and distribution in sarcoplasmic reticulum, submitochondrial membranes and erythrocyte plasma membranes during varying degrees of Nagarse digestion. Abscissae in all figures show log of protein : Nagarse ratios by weight, which was varied from 10^4 : 1 to 2 : 1. Controls were treated identically, but no enzyme was present. (A) Sarcoplasmic reticulum. With increasing Nagarse concentration, particle density decreased on concave faces and increased on convex fracture faces. The total number of particles per unit area (combined count of both faces) decreased by about half. (B) Submitochondrial membranes. Nagarse digestion had little effect on particle count or distribution, although there was a trend toward more symmetric distribution between concave and convex faces. (C) Erythrocyte plasma membranes. Only at highNagarse concentrations did significant loss of particles from the A face occur. The count on the B face was unaffected. (D) Comparison of average vesicle area in sarcoplasmic reticulum (1) and submitochondrial membranes (2) during Nagarse digestion. Nagarse produced little effect on submitochondrial membranes, but at higher Nagarse : protein ratios sarcoplasmic reticulum membranes apparently fused to produce much larger vesicles. Each data point was produced by measuring particle density or vesicle diameter for all the vesicles in a single electron microscopic field. Typically this would include 10 (plasma membrane) to 100 (sarcoplasmic reticulum, submitochondrial membrane) measurements. Results from three experiments were averaged.

Since the Lowry method and the amino acid analyses gave similar values for polypeptide content, we concluded that the Lowry method reliably estimated the protein mass remaining after digestion.

Particulate structures could be observed on fracture faces of the membrane preparations throughout the digestion period (Figs. 3–5). Sarcoplasmic reticulum and plasma membranes (but not submitochondrial membranes) often showed considerable aggregation of particles, as demonstrated in previous studies of pronase- and trypsin-treated membranes [18, 30, 31]. In all three membrane systems, we observed asymmetric distribution of particles between the two fracture faces of untreated controls. Nagarse treatment tended to randomise the distribution of

TABLE II

EFFECT OF NAGARSE DIGESTION ON AMINO ACID CONTENT OF MEMBRANE PROTEINS

Membranes were digested for 1 h at 25 °C at a Nagarse : protein ratio of 1 : 10, then centrifuged through sucrose to remove enzyme. Compositions are given as mol percent. Tryptophan and cysteine were not determined.

	Sarcoplasmic reticulum		Submitochondrial membranes		Plasma membranes	
	Control	Digested	Control	Digested	Control	Digested
Phe	1.7	6.0	4.1	5.0	3.7	4.9
Tyr	2.4	2.2	3.1	3.2	1.9	2.2
Leu	9.1	4.9	9.7	10.4	10.9	10.6
Ile	4.2	2.9	3.1	4.1	2.5	2.9
Met	2.3	2.1	2.4	.4	1.1	1.9
Val	5.4	3.9	4.4	5.2	3.7	4.4
Ala	9.1	9.4	10.5	9.7	8.9	8.4
Asp	13.4	17.7	9.9	10.4	11.0	9.9
Glu	12.5	12.5	11.3	10.0	14.9	11.8
Lys	6.4	4.6	6.6	6.4	5.2	4.6
Arg	4.3	2.6	4.9	4.7	4.9	4.1
Ser	7.9	9.4	7.7	7.6	10.3	12.3
Thr	5.9	4.6	5.4	5.7	5.5	5.2
Gly	8.1	8.1	9.5	9.2	7.6	8.5
Pro	5.6	7.3	5.2	5.6	5.3	6.2
His	1.6	1.7	2.0	2.4	2.5	1.9

particles (Fig. 6). Previous studies have shown that the asymmetric distribution of particles was also lost in reconstituted membranes formed from solubilized sarcoplasmic reticulum protein and lipid [10] as well as in trypsin treated sarcoplasmic reticulum [30, 31]. In the present study, it is possible that loss of asymmetry reflects movement of the polypeptide fragments from their original position in the bilayer to more random locations. This result and those of previous investigators suggest that the typically asymmetric distribution of particles in freeze-fracture images does reflect a specific placement of polypeptides in the lipid phase, and is not necessarily an artifact of the freeze-fracture method.

The most striking finding was that particulate structures could still be observed in the digested membranes by freeze-fracture electron microscopy, even though the molecular weight of the remaining peptides was in the neighborhood of 10 000. It should be noted that this cannot be considered a precise value, because of the inherent limitations of sodium dodecyl sulfate gel electrophoresis in molecular weight ranges below 13 000. However, it is clear from the gel patterns that the original membrane proteins are attacked, and that the resulting fragments produce diffuse bands below those of cytochrome *c* (M_r 12384) in calibration gels. We conclude that high molecular weight polypeptides are not required for production of visible particulate structures in freeze-fracture images. This leaves the problem of understanding how the remaining peptides, which individually would be too small to be resolved by

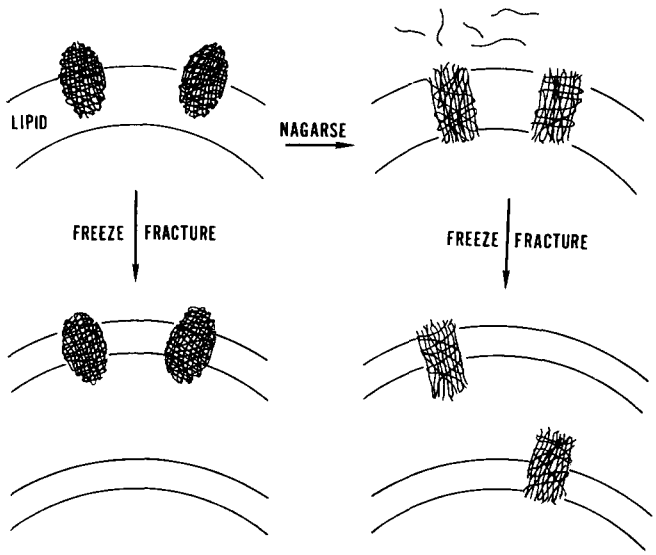


Fig. 7. Possible mechanism which would conserve particles in fracture faces following Nagarse digestion. See text for details.

the freeze-fracture method, may produce visible particles in the resulting images. Fig. 7 shows a working hypothesis for the manner in which Nagarse could attack membrane polypeptides but still conserve membrane particles. For convenience, we will use the ATPase of sarcoplasmic recticulum as an example.

In the diagram, the coiled polypeptide has been placed within the lipid phase, such that a major portion of the coil resides in the exterior half of the lipid bilayer. Thus, a fracture plane produced during freeze-fracture would leave particles primarily on the concave face. This placement of polypeptide, originally predicted from freeze-fracture observations [9, 10, 14] is in agreement with recent X-ray diffraction results of Marquart et al. [32], which locate the protein of sarcoplasmic reticulum in the outer half of the membrane.

A polypeptide of 100 000 molecular weight in an extended conformation would be approximately 4000 Å in length and would require considerable coiling to fit within the thickness of a membrane. To produce polypeptide fragments in the range of 10 000 molecular weight, a protease might attack the chain at 10 sites. Hydrophilic fragments produced near the surface of the membrane would be lost to the aqueous phase, leaving 10 000 dalton fragments (400 Å length) in the lipid phase, which would be protected from further hydrolysis. Residual coiling of the 400 Å polypeptides would presumably stabilize their continuing presence at the original site. Thus, a fracture plane would still show a particulate discontinuity at the site of the original polypeptide. Longer periods of digestion, for instance the extended incubations used by Engstrom [18], could permit further hydrolysis and dispersal of the fragments away from the original site, finally producing a smooth membrane fracture face. In the shorter digestion period of the present study, the total particle density in sarcoplasmic reticulum and plasma membranes decreased only by about half, and there was no decrease in submitochondrial membranes.

The present results are in accord with those of Segrest et al. [33] who found that freeze-fracture particles could be produced in egg lecithin preparations by the membrane-penetrating peptide of the human erythrocyte MN-glycoprotein. These investigators suggested that particles were produced when multimers of the peptide (M_r 3 700) were formed within the plane of the lipid bilayer. The multimer molecular weight was estimated to be approx. 45 000–85 000, and would be composed of 10–20 peptides. We presume that the particles demonstrated in Nagarse-treated membranes are analogous to the multimers described above. Segrest et al. also found that particle density depended on concentration of the peptide fragment in the membrane. Particles became discernable at molar lipid: protein ratios of 120 : 1, which they termed the "critical multimer concentration." Similar calculations may be carried out with the Nagarse-treated membranes. Given that about half the protein mass remains following digestion, and an average molecular weight of 10 000 for the peptide fragments, the lipid: protein ratios in Nagarse-treated membranes are in the range of 25 : 1. This clearly exceeds the critical multimer concentration determined by Segrest et al., and it is possible that similar forces help stabilize the polypeptide aggregates of Nagarse-treated membranes.

These findings can be compared with the results of Packer et al. [7] who used freeze-fracture analysis to study mitochondrial membranes of wild and petite mutant yeast. The petite mutant lacks many of the enzyme activities of oxidative phosphorylation, and in a sense is a genetic analog of the protease-digested membranes described here. It was found that the petite mutant and wild yeast mitochondrial particles were indistinguishable in size, density and distribution. Thus, both the yeast studies and the present investigation suggest that freeze-fracture analysis of membrane particles is not necessarily sensitive to fairly marked alterations of component proteins.

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