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STRUCTURAL AND FUNCTIONAL MODIFICATIONS INDUCED IN MUSCLE MICROSOMES BY TRYPSIN

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SUMMARY

1. The effects of increasing concentrations of the proteolytic enzyme, trypsin, upon the membranes of the microsomal fraction from rat muscle have been investigated. At low trypsin concentrations, changes in X-ray diffraction characteristics and in the appearance of condensed systems in electron micrographs indicate modification of lipoprotein structure.

2. These changes are closely associated with a loss in ability to accumulate calcium, and are interpreted as being related to a change in lipoprotein structure which produces physiologically leaky vesicles. In this range of trypsin concentrations, morphological breakages in the walls of many of the vesicles are observed and may be related to the changes in lipoprotein structure.

3. Higher concentrations of trypsin produce further modification of the membrane morphology and loss of ATPase activity.

INTRODUCTION

Several characteristics of the microsomal fraction obtainable from skeletal muscle render the system quite favourable for assessing the potential of specific methods for inducing structural modifications of membrane systems. A high yield of microsomes is obtained and they possess several readily assayable biochemical activities, some of which may be related to the structural integrity of the system[1–5]. Moreover, the fraction consists of a relatively homogeneous population of smooth surfaced vesicles with essentially similar morphologies that give well defined X-ray diffraction data when in condensed form. Tryptic digestion, which has been used extensively for modifications of membrane preparations and has recently been applied to muscle microsomes[5,7], was used in this study to achieve structural modification of microsomes isolated from rat skeletal muscle.

MATERIALS AND METHODS

Preparation of muscle microsomes

Microsomes were prepared from the leg muscles of young adult rats of both sexes according to the method of MARTONOSI AND FERETOS[8]. At the KCl extraction

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stage a modification was introduced in that the microsomes, at a protein concentration of approx. 5 mg/ml, were treated with a final concentration of 1.1 M KCl, 0.005 M histidine (pH 7.3) for 3 h. This treatment extracted only a little additional protein and did not affect the biochemical activities (see also ref. 5), but it was found to lead to slightly sharper diffraction patterns. The suspension was then centrifuged at 100 000 $\times g_{av}$ for 40 min. The resultant pellet was suspended in buffered water (sufficient $NaHCO_3$ to bring the pH to 7.4) to bring the final concentration to approx. 0.1 M with respect to KCl. Storage overnight usually followed at this stage, and caused no appreciable impairment of metabolic activities or diffraction characteristics (see also ref. 8). The following morning the suspension was centrifuged at 100 000 $\times g_{av}$ for 20 min and the resultant pellet suspended in 10 vol. of buffered water and recentrifuged. The pellet was redispersed in buffered water and the volume adjusted to give a final protein concentration of 5 mg/ml (biuret determination).

This preparation is considered to consist principally of fragmented sarcoplasmic reticulum[1], but may also contain a small proportion of fragmented mitochondria as demonstrated by recoveries in the fraction of approx. 10 % of the succinate dehydrogenase activity of the original homogenate.

Trypsin treatment

Trypsin treatment was carried out by incubating 1 vol. of microsomal suspension at 5 mg protein/ml with 0.1 vol. of 0.05 M phosphate buffer (pH 7.6), containing the appropriate amount (0–1000 μg) of trypsin. The reaction was terminated after 20 min at 37° by cooling the tube in ice and adding 0.1 vol. of 0.05 M phosphate buffer (pH 7.6), containing soya bean trypsin inhibitor equivalent to 1.2 times the amount of trypsin present in the incubation mixture.

This mixture was sampled immediately for ATPase determination. For Ca^{2+} uptake measurements the mixture was first diluted by the addition of 3.8 vol. of cold buffered water (giving a total volume of 5 ml). For diffraction and microscopy the mixture was centrifuged at 100 000 $\times g_{av}$ for 20 min (the supernatant at this stage was sampled for estimation of protein content), and the pellet suspended in 40 vol. buffered water and recentrifuged. The resulting pellet was then taken for X-ray diffraction analysis or electron microscopy, or both. Control experiments were carried out using microsomes subjected to the same procedures but omitting the trypsin.

Protein determination

Protein determination was carried out by a biuret method[9]. Values expressed for the supernatants from trypsin digestions are those obtained after subtraction of the appropriate amount of trypsin and trypsin inhibitor. Although trypsin might be expected to cause some fall in the protein yield due to hydrolysis of peptide bonds, the observed fall in total peptide bond content is quite small. For example at 28 % protein liberation the total biuret colour had decreased only to 94 %.

ATPase determination and Ca^{2+} uptake measurement

ATPase (Mg^{2+} activated) activity was determined in aliquots of the trypsin digestion mixture at 37° according to the procedure of MARTONOSI AND FERETOS[8]. Ca^{2+} uptake was determined on the diluted tryptic digestion mixture using ^{45}Ca and filtration by Millipore discs, according to the method of MARTONOSI AND FERETOS[8].

X-ray diffraction

Samples taken from washed pellets were allowed to dry slowly at 95 % relative humidity in an atmosphere of nitrogen. During this time the membranes became close-packed and oriented, and low angle diffractions were then recorded from the dried material with both slit-focussed and point-focussed X-ray beams. Wide angle diffraction patterns were also recorded.

Electron microscopy

Samples of the washed pellets were fixed in 6.5 % glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4), post-fixed with 1% osmium tetroxide (pH 7.4) and embedded in araldite. Specimens at intermediate and final stages of drying were prepared for electron microscopy by fixation in 1% osmium tetroxide and embedding in araldite. Thin sections were post-stained with uranyl acetate (25 % in methanol) or lead citrate[10] and sometimes with both successively. Negatively stained specimens were prepared from a suspension of microsomes in 2% phosphotungstate (pH 7.4).

Materials

Trypsin from beef pancreas (type III, 2× crystallized), and trypsin inhibitor from soya bean (type IS, 2× crystallized) were obtained from Sigma Chemical Company, London. Trypsin solutions were made up from the dry powder at frequent intervals as concentrated stock solutions in 0.05 M phosphate buffer (pH 7.6), and kept at —20° to minimize breakdown. Immediately before use, these stock solutions were thawed and the desired dilutions then prepared using 0.05 M phosphate buffer (pH 7.6). In spite of these precautions, slight deteriorations with time in the effectiveness of the trypsin solutions were noted. Conclusions have therefore been drawn mainly from series of simultaneous experiments.

RESULTS

Protein and peptide liberation resulting from tryptic action

The relationship between concentration of trypsin in the incubation medium and amount of protein and peptide released is shown in Fig. 1.

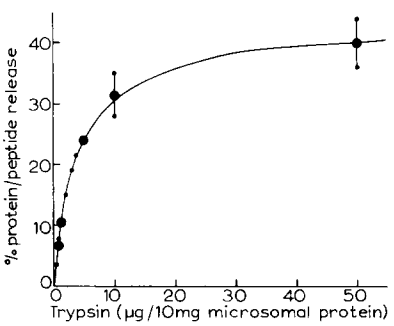


Fig. 1. Effect of trypsin concentration upon release of protein and peptides. Trypsin treatment was carried out as in the section on MATERIALS AND METHODS. Following the addition of trypsin inhibitor the mixture was centrifuged at 100000 × g_{av} for 20 min and aliquots of the supernatant then taken for protein estimation. •, results from a single experiment; ●, means of three experiments.

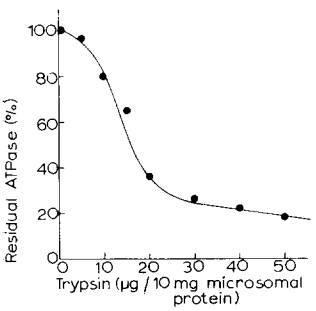
Tryptic action did not release phospholipid from the microsomes. Although it was not possible to perform phospholipid analysis directly on the supernatant, analysis of washed pellets of trypsin treated material demonstrated a fall in protein: phospholipid ratio which exactly paralleled the percentage of original protein liberated into the supernatant.

Effect of trypsin treatment on ATPase activity

The effects of increasing concentrations of trypsin in the incubation medium on the activity of Mg^{2+} stimulated ATPase are shown in Fig. 2.

Effects of trypsin treatment on calcium uptake activity

The rapid and dramatic loss of calcium uptake activity at low concentrations of trypsin is illustrated in Fig. 3.



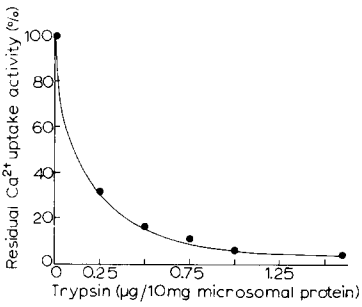


Fig. 2. Effect of trypsin concentration upon ATPase activity. Trypsin treatment was carried out as in the section on MATERIALS AND METHODS, and after the addition of trypsin inhibitor, the mixture was sampled directly for ATPase determination. Specific activity of the untreated microsomes was 3.22 μmoles P_i released per mg protein per min at 37°.

Fig. 3. Effect of trypsin concentration upon calcium uptake activity. Trypsin treatment was carried out as in the section on MATERIALS AND METHODS, and, after addition of trypsin inhibitor and water (3.8 × volume of microsomes treated) the mixture was sampled for Ca^{2+} uptake activity. The untreated microsomes took up 1.71 μmoles Ca^{2+} per mg protein in 15 min at 22°.

Since calcium uptake was measured by a filtration method there are two alternative explanations for the loss in calcium uptake activity.

(1) Clumps of vesicles which previously did not pass the filter may be disaggregated so that the calcium containing material would then pass the filter[5], or (2) alternatively the vesicles may become leaky to calcium as a result of the tryptic action. At the levels of trypsin used in the present experiments (Fig. 3) the latter alternative is favoured since the magnitude of the decrease in calcium uptake is considerably greater than the fall in turbidity of the trypsin-treated suspension (Table I). Parallel experiments in which ultracentrifugation replaced filtration as the method of separating the microsomal material showed that calcium was not removed from the medium to a significantly greater extent. This would suggest that both filtration and centrifugation were separating a population of essentially leaky vesicles (Table I). Furthermore, at levels of trypsin sufficient to cause the fall in calcium uptake activity, microsomal protein (measured as $A_{280\ m\mu}$) was retained by the filter to the extent of approx. 95% (Table II). Although disaggregation and passage through the filter was

detected following higher levels of tryptic action (Table II) it was obviously of minor importance at low levels.

Electron microscopy

In electron micrographs of thin sections, this preparation of muscle microsomes from rat skeletal muscle normally featured a population of predominantly spherical smooth surfaced vesicles (Fig. 4a). After staining with uranyl acetate the membranes showed the customary trilamellar structure.

Treatment with trypsin produced a progressively increasing number of apparently disrupted vesicles and also a progressive modification to the form of flattened sacs and individual strands (Fig. 4b). The flattened sacs showed clearly the preservation of a trilamellar type membrane (Lower right) and although this was more difficult to demonstrate for the individual strands it could be seen to persist inmany instances (Lower left). Disruption of a proportion of the vesicles was apparent soon

TABLE I

CALCIUM UPTAKE MEASUREMENT BY FILTRATION AND BY CENTRIFUGATION METHODS FOLLOWING TRYPSIN TREATMENT. EFFECT OF TRYPSIN CONCENTRATION UPON THE TURBIDITY OF THE MICROSOMAL SUSPENSION

Trypsin treatment was carried out as in the section MATERIALS AND METHODS. After the addition of trypsin inhibitor and water (3.8 × volume of the microsomes treated) samples were removed for calcium uptake determination and for turbidity measurement at 520 mμ. At the termination of the calcium uptake incubation, one series of reaction mixtures was filtered, (Millipore type HA 0.45 μ) and a second series centrifuged (125000 × g_{av} 20 min). The filtrates or supernatants were then sampled for ^{45}Ca estimation.

Trypsin concentration μg/10 mg microsomal protein	*Loss in Ca^{2+} uptake activity (%) (filtration experiment)*	*Loss in Ca^{2+} uptake activity (%) (centrifugation experiment)*	$\frac{A_{520\,m\mu}}{initial\ A_{520\,m\mu}}$ *in %*
0	0	0	0
0.2	81	73	11
0.4	87	95	24

TABLE II

PASSAGE OF MICROSOMAL MATERIAL THROUGH MILLIPORE FILTERS FOLLOWING TRYPSIN TREATMENT

Trypsin treatment was carried out as in the section MATERIALS AND METHODS. After the addition of trypsin inhibitor and water (3.8 × volume of the microsomes treated), 0.2 ml of the mixture was diluted with 3.6 ml water *plus* 0.2 ml 0.05 M phosphate (pH 7.6). One sample was read directly and a second sample filtered through Millipore filter (type HA 0.45 μ) before reading.

Trypsin concentration μg/10 mg microsomal protein	$\frac{A_{280\,m\mu}\ after\ filtration}{A_{280\,m\mu}\ before\ filtration}$ *in %*
0	0
1	4.5
10	25
100	90

after the stage at which calcium uptake properties were lost (Fig. 3) but the changes to collapsed vesicles and to strand forms occurred mainly during the stages at which ATPase activity was lost (see Fig. 2). The tendency seemed to be to convert most of

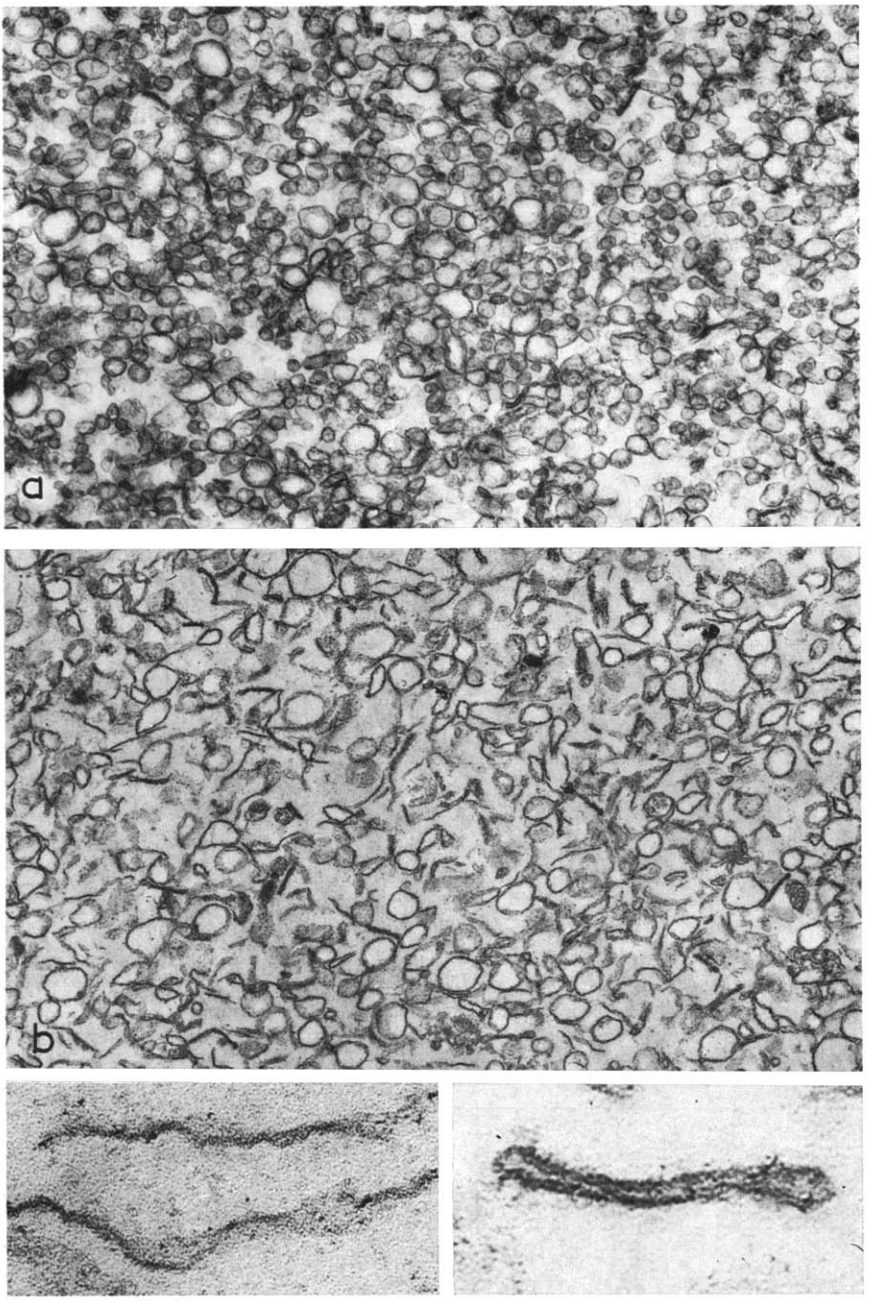


Fig. 4. Electron micrographs of muscle microsome preparations. a, Standard preparation, × 20000; b. microsomes modified by trypsin treatment, × 20000. Insets. Examples of strand form (left) and collapsed vesicle (right) in trypsin modified preparation, × 160000.

the membrane material to the strand form and in one preparation which showed a loss of about 50 % protein (as peptide) after prolonged trypsin treatment, few vesicles could be seen. However, complete loss of ATPase activity (Fig. 2) could be produced without approaching this extreme of morphological change. Fig. 7 correlates these with other changes.

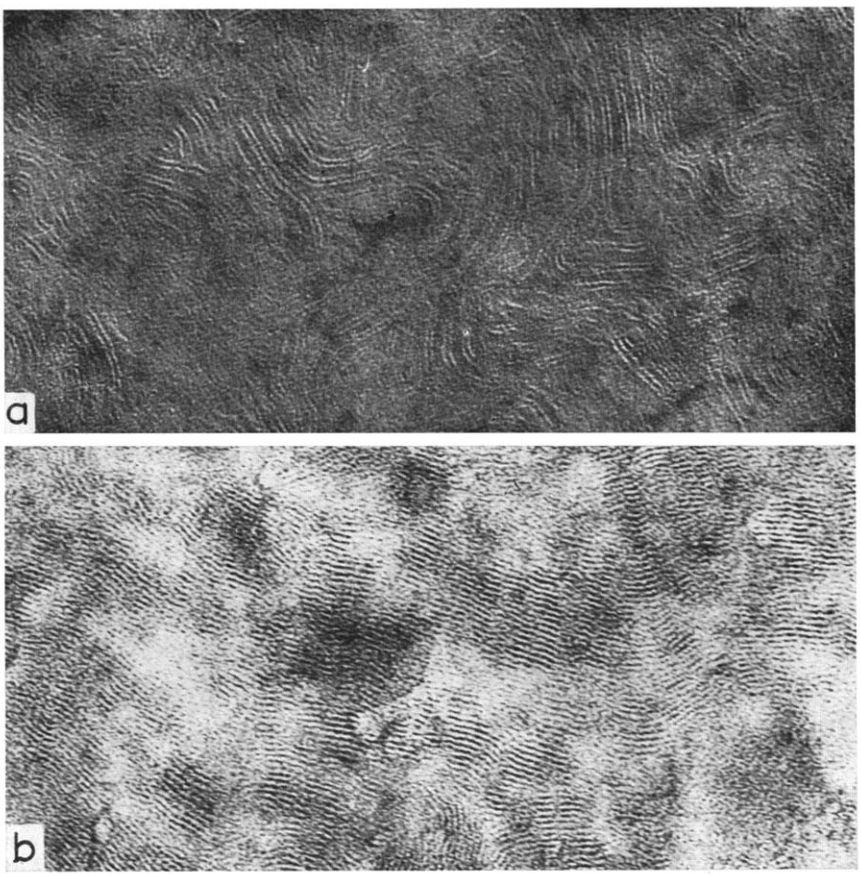


Fig. 5. Electron micrographs showing layered structures which predominate in condensed preparations of microsomes. a, Standard preparation, × 160000; b, Trypsin modified microsomes, × 160000. The major layering shown in (a) features a repeat which includes two thicknesses of membranes and measures about 150 Å overall: the fine layering in (b) gives a repeat layer measuring about 50 Å.

Electron micrographs of sections through condensed preparations of untreated microsomes featured areas of regular layering in which the repeating layer appeared to involve two trilamellar units and to measure about 150 Å overall (Fig. 5a). The micrographs were such as to suggest that when each vesicle collapsed as water was removed the inner dense lines eventually fused to form a single dense line whilst the outer dense lines of adjacent vesicles remained separated by a very narrow band of low density. Comparable preparations of trypsin treated material showed areas of regular layering which were markedly different from those of the starting material.

This modified layering featured a regular alternation of dense and light bands with a periodicity of about 50 Å (Fig. 5b). In the very early stages of trypsin treatment preparations showed both types of layering (which might be referred to as normal and fine layering) and also suggestions of some intermediate forms, such as one which featured alternations of pairs of closely spaced dense lines with rather wider bands of low density. However, such condensed preparations were very difficult to prepare for electron microscopy and the observations did not lend themselves to quantitative assessment.

X-ray diffraction

X-ray diffraction patterns, both low angle and wide angle, have been obtained from preparations of untreated muscle microsomes at various levels of hydration[6], but for the purpose of comparison of untreated and trypsin digested microsomes, only the nitrogen-dried preparations have been considered.

Although the drying process itself is known to modify the molecular organization of membranes, the diffraction patterns can nevertheless be used in a comparative way to detect changes initiated by the trypsin treatment.

The low angle pattern of air-dried microsomes normally featured four bands which could be related as simple orders of a lamellar repeat of about 160 Å, together with two sharper and unrelated bands at 59 Å and 38 Å (Fig. 6). The intensity of the 59 Å band was very variable and it could be eliminated completely by heating the dried preparation at 40 to 60° for a few minutes. The effect of tryptic digestion on the intact microsomes was to eliminate the four lamellar diffractions from the pattern of the dried preparation, leaving only the two sharp bands at 59 Å and 38 Å (Fig. 6). Again, the 59 Å band could be eliminated by heating. A significant decrease in the intensities of the lamellar diffractions was already apparent at the stage where

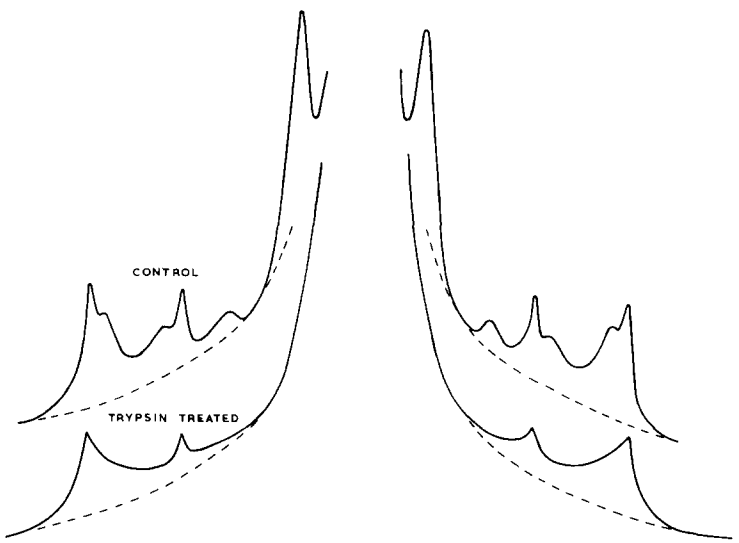


Fig. 6. A comparison of microdensitometer traces of low angle X-ray diffraction patterns obtained from dried preparations of muscle microsomes. The control shows the first four orders of diffraction from a lamellar periodicity of about 160 Å together with two independent reflections at 59 Å and 38 Å. The trypsin treated preparation shows only the 59 Å and 38 Å reflections.

tryptic digestion had destroyed the ability of the microsomes to take up calcium, but complete elimination of the bands was achieved only at slightly higher trypsin concentrations. These changes are summarized in diagrammatic form in Fig. 7.

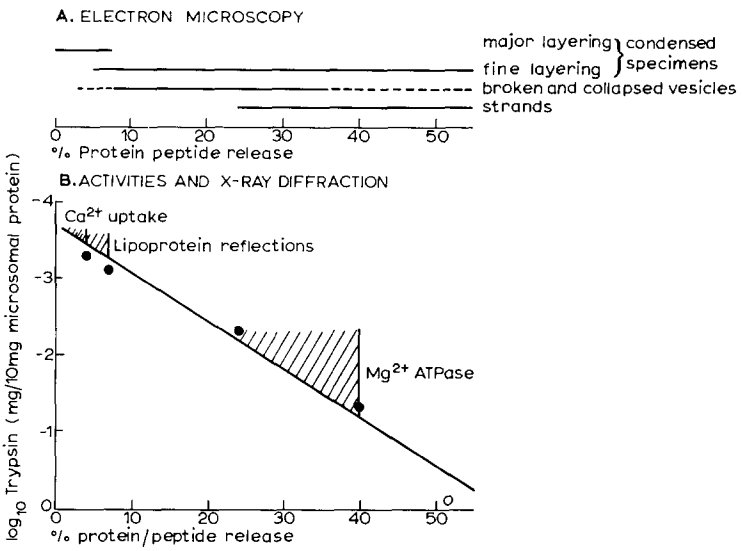


Fig. 7. Diagrammatic representation of the effects of trypsin on the structure and activities of muscle microsomes. A, the lines indicate the approximate range in which the various features are observed. ———, a major feature; – – – –, a minor feature. B, the relationship between protein/peptide release and trypsin concentration is given by the line down through the experimentally observed points. Superimposed upon this line are hatched regions (the first two overlapping) denoting the range over which the feature is observed to disappear. In regions to the left of cross hatching the feature is shown to maximum extent, to the right of cross hatching the feature has completely disappeared.

The wide angle diffraction pattern of muscle microsomes dried for a few (3–4) h featured a diffuse ring centred on a Bragg spacing of about 4.5 Å and another relatively broad peak at about 10 Å. In time (usually overnight) a sharper line at 4.2 Å became superimposed on the broad 4.5 Å ring. The main effect of tryptic digestion was to cause this sharper 4.2 Å reflection to appear more quickly.

DISCUSSION

The experimental data has provided evidence for changes in biochemical activities and for modifications of membrane structure induced by the action of the proteolytic enzyme, trypsin. From an examination of the sequence and magnitude of these changes there is evidence of a degree of correlation between them. These changes are most readily followed by reference to the diagrammatic representation in Fig. 7.

Considering first the relatively rapid loss in the ability of the microsomes to accumulate calcium, this is associated with the loss of only a small amount of protein from the system, although significant changes in the retained protein may also have occurred by this time. At this early stage there is little appreciable change in the structural data. However, changes in the X-ray diffraction patterns and in the electron microscopy do become apparent very soon afterwards, and these may still be relevant to the loss of activity. Although the decrease in the intensities of the lipoprotein

reflections do not become pronounced until a slightly later point than the onset of the Ca^{2+} uptake loss, it is possible that modification of lipoprotein structure begins earlier than can be detected by the diffraction method and that the ability of the microsomes to accumulate calcium is sensitive to slight modifications or to limited and localized breakdown in membrane structure.

When the diffraction changes have become appreciable it is also apparent from electron micrographs that many of the membrane vesicles are now broken open and some have collapsed to give flattened sacs. Again, it may be that it is only when the vesicle disruptions have become relatively gross that they become prominent in electron micrographs, but that smaller lesions are introduced much earlier and render the vesicles leaky to calcium before they can be detected by other means. Certainly a breaking open of the vesicles as is eventually observed in electron micrographs and which in turn results from modifications of lipoprotein structure such as those subsequently detected by X-ray diffraction, would provide a simple explanation of the very rapid loss of ability to accumulate calcium before there is any appreciable decrease in ATPase activity.

Leakiness of trypsin-treated vesicles has been suggested by INESI AND ASAI[7] to explain the effect on Ca^{2+} uptake in muscle microsomes. The levels of trypsin used by these workers were, however, considerably greater than those used in the present experiments. When a full report of their work is published, it may be possible to relate their findings on the effects on surface detail (together with other effects seen by MARTONOSI[5]) with the physical modifications observed here at lower trypsin concentrations.

Although trypsin treatment eventually leads to a complete disappearance of the lipoprotein bands from the X-ray diffraction pattern of the condensed membrane system, and electron micrographs then show a lamellar system of a type that might be attributed to a purely lipid phase, there is nevertheless more than 50% of the original protein or peptide still remaining in the membrane system, and the modified microsomes, even at the "stranded" stage, continue to show a trilamellar feature. Moreover, a purely lipid material in aqueous dispersion would not be expected to exist in the form of extended strands such as are featured in the later stages of tryptic digestion. Consequently it would seem likely that the residual strands are still lipoprotein or lipopeptide in nature when in the dispersed state although dehydration to the condensed state may lead to a final breakdown of lipid-protein or lipid–peptide association so that the ordered lamellae seen in electron micrographs may eventually involve only lipid material. The electron micrographs of intermediate stages of trypsin treatment are not yet sufficiently clear-cut and reproducible to permit reliable interpretation, but they may eventually provide important clues concerning the location of the ATPase protein which is inactivated or removed during the conversion of the spherical or ellipsoidal shaped vesicles to flattened or stranded forms during the main phase of tryptic digestion.

NOTE ADDED IN PROOF (Received January 9th, 1969)

The work of INESI AND ASAI referred to above has now been published in full[11]. With respect to the initial action of trypsin the work reported above is in general agreement with the findings and interpretations of these authors. Further work is,

however, required before any correlation between the effects on surface detail seen by these authors and by MARTONOSI[5] can be related to the physical modifications demonstrated by X-ray diffraction and the appearance of thin sections of condensed material.

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