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Changes of Lysosomes in Autolysis

Histochemical, Biochemical and Electron Microscopic Studies in the Early Stage of Autolysis

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Introduction

A distinct class of cytoplasmic particles containing several acidic hydrolases was isolated by deDuve et al[1]. and called "Lysosomes". They suggested further that the organelles might play an important role in the process of degeneration or death of tissue cells by releasing their enzymes. The "lysosome" concept has introduced so-called "suicide bag" hypothesis in the mechanism of cellular death. The hypothesis has implied the release of the enzymes from particles as a trigger for initiation of cell necrosis. If the lysosome alter in the early stage of autolysis and release the enzymes into surrounding cytoplasms, the autolytic change may be recognizable morphologically, especially by electron microscopy, as the alterations of the lysosomal membranes and degradations of the cytoplasmic components in the vicinity of the altered lysosomes due to the hydrolytic enzyme actions. And at the same time, the enzyme release resulting from the destruction of the lysosoma lmembrane can be measured biochemically using the acid phosphatase activity as a marker enzyme of lysosome. Though there have been several reports[2~7] concerning with the alterations of lysosome and its release of enzymes during necrosis *in vivo* and *in vitro*, it is not yet established whether or not the lysosomal change, either morphologically or biochemically, acts as an initiating factor at the early stage of autolysis. The author examined the lysosomal changes morphologically and biochemically to elucidate the influences of the lysosomal enzymes released in the process of autolysis on the cytoplasmic components, and also the mechanism by which cytoplasmic degradation takes place *in relation to* the lysosomal changes.

Materials and Methods

Male Wistar albino rats weighing approximately 200 g were used. Being maintained on the standard laboratory diet for at least 5 days, they were fasted overnight prior to sacrifice. After the sacrifice by decapitation, the liver was removed aseptically and sliced about 5 mm in thickness. Each slice was placed in the sterile moist chamber and incubated at 37°C. The moist chamber was simply constructed with a Petri dish, a steel mesh and a moist filter paper placed at the bottom of the dish. This apparatus (moist chamber) was sterilized in an autoclave before use. The incubation time was as follows: 0, 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 hrs. for the light microscopic examinations, and 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8 hrs. for the biochemical and electron microscopic experiments. For the histochemical examinations, the incubated slice was

fixed in the 10% neutral buffered formalin containing 1% of calcium chloride[8,9]. After 24-hr fixation, the slice was cut in half of the thickness, and the one half was sectioned 5–10 μ thick by the freezing microtome for the staining of acid phosphatase (AcPase) and of fat by Sudan III, the other was embeded in paraffin as usual manner for the staining of hematoxylin and eosin, methylgreen-pyronin and PAS. The histochemical demonstration of AcPase activity was carried out by Gomori's method[10] with slight modification. A mixture of 100 ml of 0.12 g lead nitrate in 0.05 M acetate buffer pH 5.0 and 10 ml of 0.3 g disodium glycerophosphate (β-, Sigma Co.) in distilled water was prewarmed at 37°C and filtered before use. Sections were then rinsed tenderly in distilled water and dipped in a 1–2% ammonium sulphate solution for about 10 seconds, rinsed in distilled water again and mounted on slide glasses, with or without nuclear counterstaining. As mounting medium, freshly prepared glycerin-gelatin was used.

Preparation of homogenate and subcellar fractions: Whole homogenates, lysosome containing fractions and supernatants were made from the incubated liver tissues at each time point in 1) 0.25 M sucrose and 2) 0.25 M sucrose buffered with 0.01 M tris-maleate solution at pH 7.2, using Potter-Elvejem[11] homogenizer tube with a Teflon pestle. The whole homogenate was prepared with 2 g of liver tissues in 18 mℓ of homogenizing medium in chilled environment.

The lysosomal fraction was prepared by sedimenting the postmitochondrial fraction at 22,000×g for 10 minutes, washed twice and was resuspended in 0.25 M sucrose or in buffered 0.25 M sucrose[12].

The supernatant fluid was obtained from the homogenate by centrifuging at 100,000 ×g for minutes in Spinco type centrifuge (Type 40 P Hitachi preparative Ultracentrifuge) with rotor No. 40[12].

To examine the degree of release of the AcPase from the lysosome, the AcPase activity was measured on both homogenate and supernatant fluid at each time point by the method described below. To compare the degree of release of the AcPase *in vitro* with that *in vivo*, homogenate and lysosomal fraction were incubated simultaneously in a water bath at 37°C. At each time of incubation, the aliquots of them were assayed for the AcPase activity. The AcPase activity was determined by the measurement of finally produced inorganic phosphorus in the homogenates, lysosomal fractions and supernatants by the method of Fiske and Subbarow[13].

For the determination of the total activity, Triton X-100 was added to the incubating medium in the final concentration of 0.1% (V/V)[14], and for the free activity the incubating medium without addition of Triton X-100 was used. The activity of cathepsin was also measured in one series by the method of Gianetto and DeDuve[15].

In order to examine the influence of the various concentration of sucrose and Trition X-100 on the lysosomal membrane, AcPase activity was measured on the aliquots of lysosomal fractions prepared from liver tissues incubated in the medium containing Triton X-100 or sucrose in various concentration for 0, 30 minutes and 1 hr. The range of the concentration of Triton X-100 was from 0.00001% to 0.1%, and that of sucrose from 0.025 M to 0.25 M.

To test the influence of ATP on the lysosomal membrane, the lysosomal fractions

were incubated with and without the addition of ATP in the concentration of 10^{-3} M (Na- salt, Sigma) and were centrifuged at 22,000×g for 15 minutes at each time. The sediments were served for the measurement of both total and free activity.

For the electron microscopic study, the incubated liver tissues were fixed in 2% osmium tetroxide solution buffered with veronal acetate adjusted at pH 7.4[16] for 2 hrs., dehydrated in a series of graded alcohol and embedded in Epon 812 by the method of Luft[17]. The thin sections were cut with the Porter-Blum microtome and stained in the uranyl acetate or lead citrate solution by the method of Raynolds[18] and observed on Hitachi HU-llA and HS-7 electron microscope. Some of the tissue blocks were prepared with saturated uranyl acetate solution dissolved in 70% alcohol and examined mainly for the ultrastructural studies of the lysosomal membranes.

Results

Histochemistry: Lysosomes which were demonstrated by the AcPase activity as deposits of lead sulfide reaction product were seen as discrete granules along bile canaliculis of hepatic cell plates (Fig. 1, 2). Granules were ranged from 0.5 to 1.0 μ in diameter and observed smaller in size and lower in density in the hepatic cells in the centrolobular area than those in other areas as noted by Novikoff and Essner[19]. There were no morphological changes of the granules at 30-minute, 1-hr and 2-hr incubation. The firstly

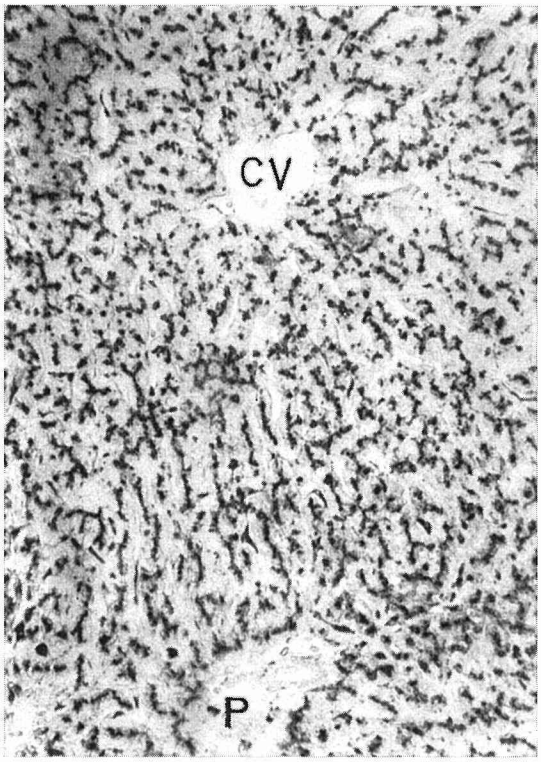

Fig. 1

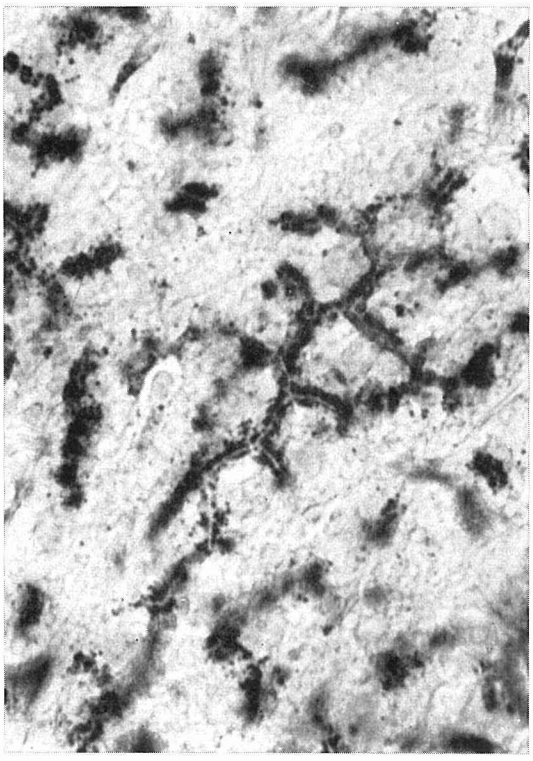

Fig. 2

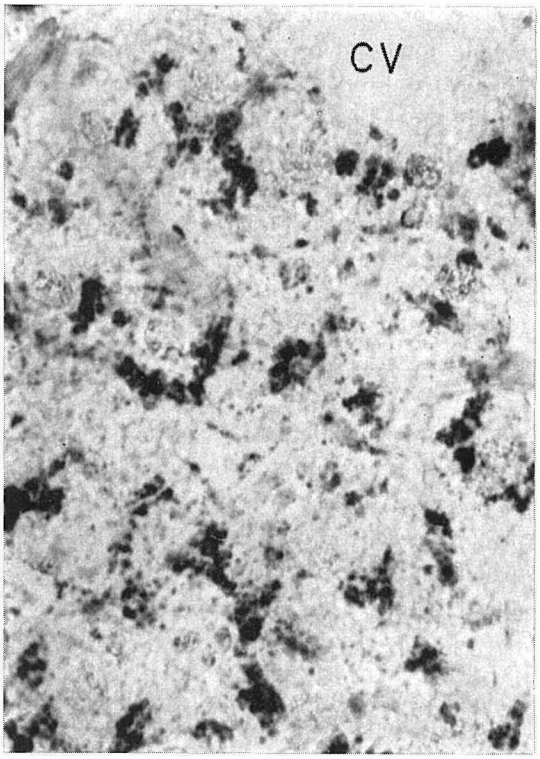

Fig. 3

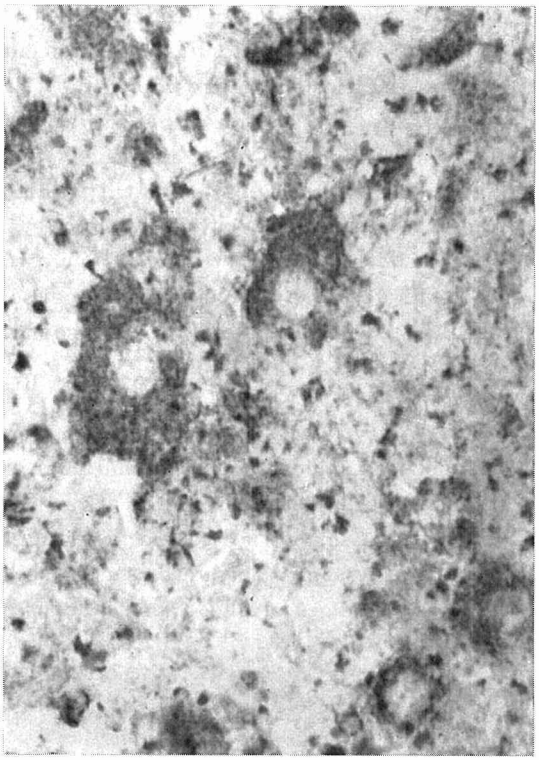

Fig. 4

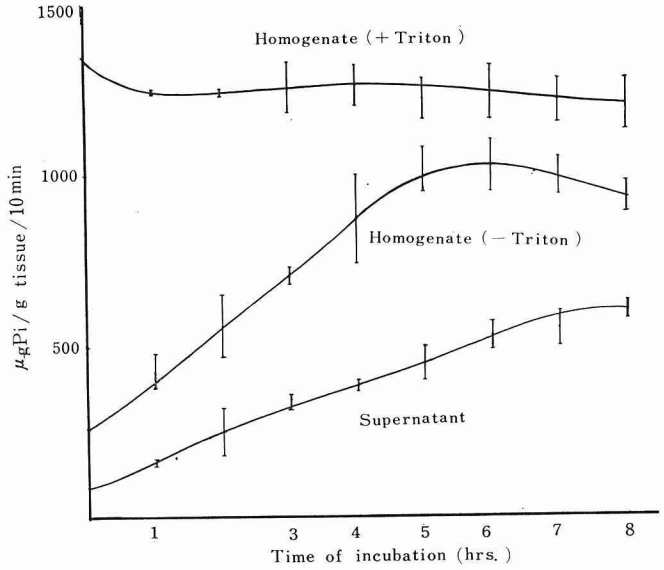

Fig. 5 AcPase activities of both homogenate and supernatant.

discernible morphological change of the granules was found at 3-hr. incubation by light microscopy. The granules in hepatic cells around the central vein showed poorly defined contour with decreased density (Fig. 3). The enzymatic activity was not confined to the granules but seemed to disperse into cytoplasm. In process of incubation, these changes became distinct and gradually extended to peripheral area of the lobule. At 7-and 8-hr, incubation no positively stained granules were observed in the hepatic cells in the central and mid-zonal areas while slight and dispersed stained granules were found in peripheral areas (Fig. 4).

Bichemistry: To examine the degree of the release of the lysosomal enzymes, the total AcPase activity of homogenate was compared with the free activity at each time of incubation. The activity of the supernatant at each time point was also compared with the total activity. Since the activities of the AcPase in the supernatant both with and without Triton X-100 showed almost the same value, the experiment without Triton X-100 was preferred in the examination. As shown in Fig. 5, the AcPase activity of Triton-X-100 free homogenates at each time point increased almost lineally by 6 hrs., and decreased slightly thereafter. The activity in supernatant increased also lineally but showed approximately the half value of that in Triton X-100 free homogenate at each time point. The activity in Triton-added homognate showed no change for 6 hrs. and decreased thereafter. The activity of cathepsin at each time point showed almost similar curve to that of AcPase activity (the figure is not demonstrated in this paper). Homogenates prepared in 0.25 M sucrose alone, showed only 10% increase in the activity at 3-hr incubation, whereas in lysosomal fraction prepared in 0.25 M sucrose almost 50% increase at 3-hr incubation. On the other hand, the activity of homogenate prepared in

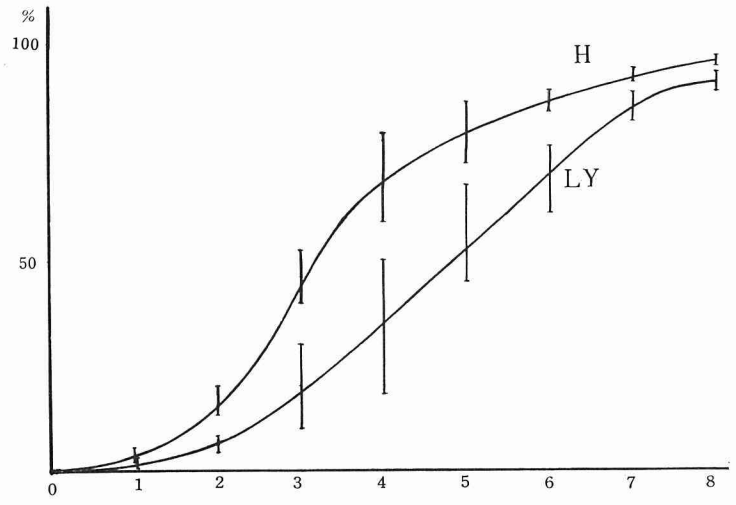

Fig. 6 AcPase activities of homogenate and lysosomal fractions prepared by tris-maleate buffered 0.25 M sucrose.
H—homogenate, Ly—lysosomal fraction.
Expressed as percent to total activities.

tris-maleate-buffered 0.25 M sucrose showed more than 40% increase at 3-hr incubation, whereas the activity of lysosomal fraction showed 20% increase (Fig. 6).

To examine the fragility of the lysosomal membrane, the free activity in various concentrations of Triton X-100 and sucrose were measured on lysosomal fractions incubated for 0, 30 minutes and 1 hr. It is known that total activity can be assayed without any inhibitory effect on the enzyme by treatment with Triton X-100. In the solutions containing the detergent, lysosome showed no release up to 0.001% of Triton X-100 and slight release in 0.01% of Triton X-100 at 0 time of incubation. The lysosomal fraction incubated for 30 minutes showed a slightly increased activity up to 0.001%, and in 0.01% solution showed more than twice of activity compared with that 0 of time of incubation.

To examine the effect of ATP on the lysosomal membrane, the AcPse activity was measured and compared on the lysomal fractions incubated with and without the addition of ATP in the concentration of 10^{-3}M. The total activity of the AcPase of both ATP-added lysosomal fraction and fraction without ATP showed no change until 6-hr incubation. The free activity of ATP-added lysosomal fractions showed no increase until 6-hr. incubation and the activity showed plateau as shown in Fig. 7, the lysosomal fraction without addition of ATP showed gradual increase in the activity; 14% increase at 3-hr incubation, 52% at 4.5-hr. incubation and 86% at 6-hr incubation.

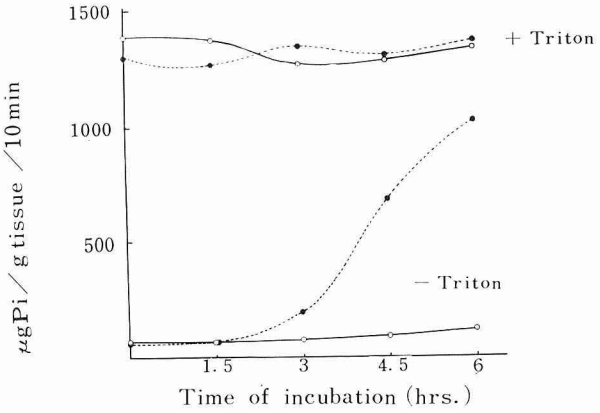

Fig. 7 AcPase activity of the lysosomal fraction with and without addition of ATP.
○——○ with ATP, ●·····● without ATP.

Electron microscopy:

30 minutes: The most conspicuous change at 30-minute incubation was seen on the cell membrane of the sinusoidal border of hepatic cell. Microvilli, normally seen in this site, were almost completely disappeared and instead of them many blebs and protrusions were observed (Fig. 8). The bulk of the blebs appered as a clear area devoid of any cytoplasmic organelles. Occasionally, apparent ruptures of cell membranes were encountered. Swelling and bleb formation of the microvilli of the bile canaliculi can be seen in some instances, but not conspicuous. The lateral cell membrane showed little change. Lysosomes, round-or oval-shaped, 0.5-1.0 μ in diameter and clearly defined by the single

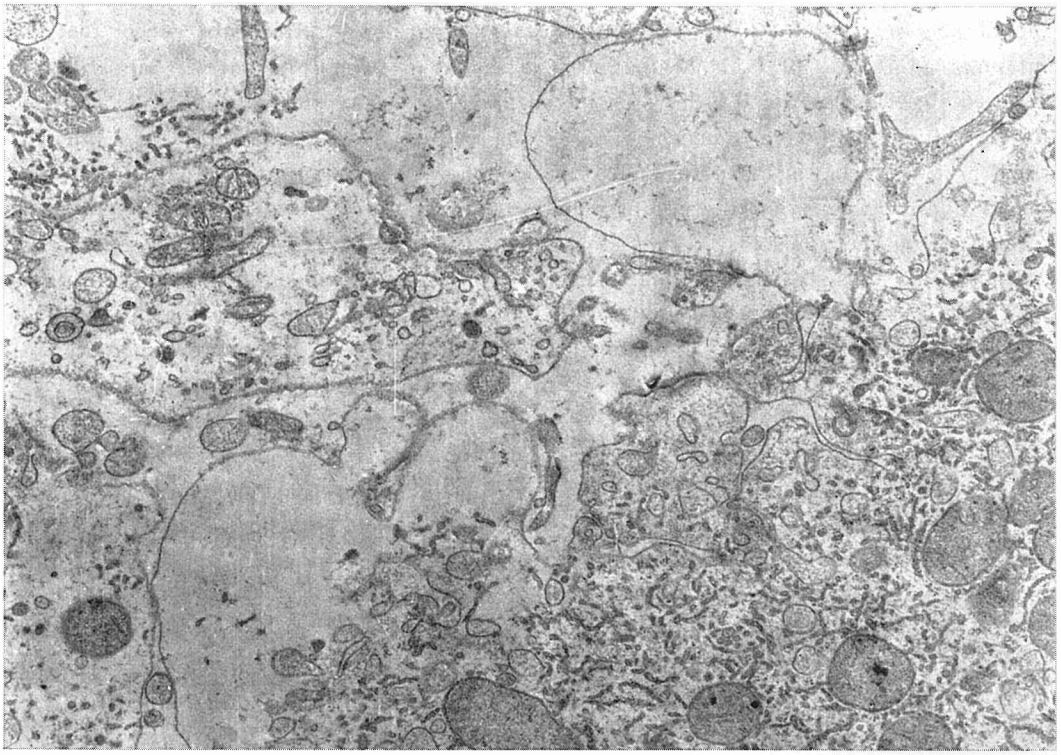

Fig. 8

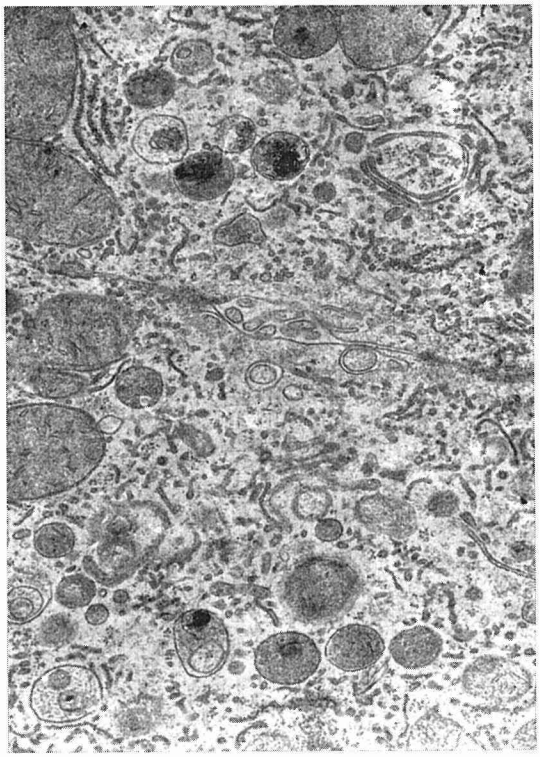

Fig. 9

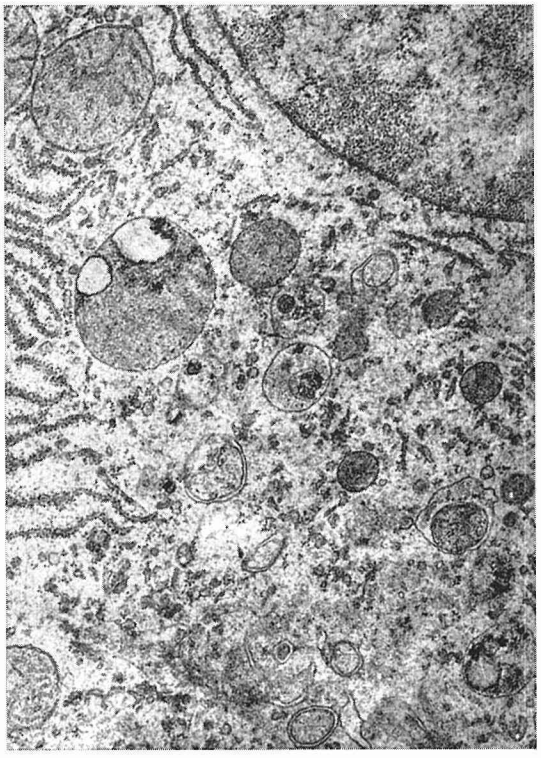

Fig. 10

membrane in control tissues, did not show any alterations at this time except that the granular aggregates of their content were observed in several instances. Mitochondria showed slight swelling and a decrease in the matrical density and occasionally a decrease in the number or loss of matrical granules was noted. However the structural changes such as rupture of the membrane, deformation of cristae were not apparent.

1 hour: Changes of the cell membrane of the sinusoidal border; Bleb formation or protrusion of the sinusoidal border of the hepatic cell were still noted. On almost all parts of the sinusoidal border, the microvilli completely disappeared. The interruptions of the sinusoidal border were often seen. Morphological alterations of the microvilli of the bile canaliculi seen at 30 minutes became more marked. The villous pattern was completely disappeared and bleb formation or protrusion of bile canalicular membrane became conspicuous. But the density of blebs or protrusions was the same as that of cytoplasmic matrix of other portion. Lysosomal changes such as increase in size, swelling, decrease in density and rupture of the limiting membrane seen at 30-minute incubation were more conspicuously observed (Fig. 9). Mitochondrial changes found at 30-minute incubation, such as swelling, separation of outer membrane from inner membrane were more pronounced. Moreover, changes of mitochondrial contours such as attenuation, elongation, binary fission, scalloped-shaped and punching-out contour were conspicuous, and rupture of the outer membrane could be seen. At the site where the outer membrane

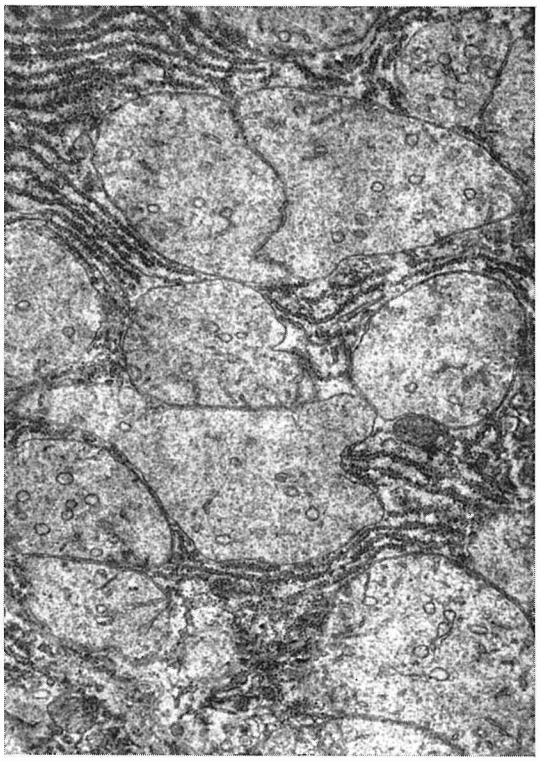

Fig. 11

Fig. 12

showed discontinuity, tongue- like extrusions of the inner membrane were noted. The bizarre configuration of Golgi-complex were seen at this time point. On the ER, detachment of the ribosomes from the ER were pronunced and the smooth ER showed vesicular profiles.

2–4 hrs.: Mitochndrial changes mentioned above were more marked and the most prominent changes in these stages. Dense amorphous inclusions near the inner membrane, swelling of the cristae, complete loss of the intramitochondrial granules, protrusion of the inner membrane at the site where the outer membrane ruptured were seen. Density of the matrix was also decreased. Lumen of the bile canaliculi were observed full-filled by the bleb formation of the microvilli. Lysosomes, in general, showed a decrease in density, some of which appeared to be empty. Some lysosomes, especially larger ones, contained the membranous structures and granular aggregations in their content (Fig. 10).

5–6 hrs.: Mitochondria were highly swollen Some of them lost their outher membrane and had the dilated tubular profiles of the mitochondrial cristae. Among the sowllen mitochondria, other cytoplasmic organelles such as vesiculated smooth ER, empty and occasionally granule contained lysosomes near the bile canaliculis could be seen. Partially, the laminated rough surfaced ER had their ribosomes (Fig. 11). The intracellular space were dilated markedly.

Discussion

Chronologically, in this experiment, the first discernible change was on the membranous structure, that is, sinusoidal border of the hepatic cell showed bleb formation or extrusion of the plasma membranes and disappearance of microvilli at 30-minute incubation. These alterations became more prominent at 1-hr incubation. The bile canaliculus also showed disappearance of their microvilli and extrusion of the membrane into the bile canalicular lumen. These findings agree well with the reports of Trump et al[20]. on the mouse liver incubated in a moist chamber, and also of Bassi et al[21]. on the ischemic rat liver. These alterations in plasma membrane were considered to be due to the probable increase in permeability of the membrane itself. Thus, influx of fluid may occur resulting in the bleb formation.

As shown in Fig. 9, disruption of the lysosomal membrane was found to be more prominent as the incubation time proceeded. It is suggested that the fragility of the membrane were continued to increase, like that of the plasma memebrane above mentioned. A cosiderably low activity of AcPase in the isolated lysosomes after the autolytic treatment as compared with the enzyme activity in the control, both determined in the presence of Triton X-100, is assumed to be attributed to denaturation of the enzyme induced by autolytic mechanisms. The same phenomenon was also observed on lysosomes isolated from the incubated tissue slices. The pH of the lysosomal fraction and the homogenate incubated *in vitro* at 37°C in 25 M sucrose was lowered to 5.0, while that of homogenate was 6.0 at the end of the incubation. Under these conditions AcPase in the lysosomal fraction may be released far more and earlier than that in the homogenate. At 3-hr incubation, more than 50% and 10% of the enzyme were released from lysosomal fraction and homogenate. These findings might be in accordance with the results obtained

by Sawant et al[22]., who reported that in acidic environments the lysosomes were more labile and the accesibility of the enzyme to its substrate was also enhanced as compared with those in neutral medium. Lysosomes may be most stable in neutral environment as suggested by Sawant et al. In contrast to the above mentioned finding, relase of the enzyme from lysosomes was found to be more pronounced in homogenate than in the isolated fraction, when tris-maleate buffered sucrose (pH 7.2) was used (Fig. 6). At the end of 8 hrs. of incucation, the pH of the buffered medium was estimated and almost no changes were noted. This result indicates that under these conditions, namely, at the same temperauture, at the same pH and in the same osmotic pressure, lysosomes in homogenate are more labile than those in the isolated state. A mechanisms is presumed to be present in homogenate that makes lysosomal membranes labile and thereby enhances the release of the enzymes, though further investigations are now in progess.

In addition to this mechanism, lowering of the pH of the incubation media was also accounted for the decreased stability of the membranes. It is well known that intracellular energy generation is required for maintenance of the membrane structures. In the autolyzed rat liver, it was reported that a significant impairment of oxidative phosphorylation and loss of respiratory co-factors occur, thus results in a reduction of ATP generation[23]. Furthermore, a certain ATP-level was reported to be essential for the protection of oxidative phosphorylation against autolytic state on the one hand, and for the maintenance of permeability barrier of cellular membranous strutres on the other hand. Similarly to autolyzed liver, the liver tissues of rats following in situ anoxia were reported to show a striking decrease in oxidative and phosphorylative metabolic activities[24] resulting in an increase in permeability of the membrane structures. In these aspects, it is appropriately expected that ATP exhibits a protective effect on lysosomes against the increase in permeability of these membranes and further the enhanced rupture. As is shown in Fig. 7, the lysosomal fraction which was prepared in tris-maleate buffered 0.25 M sucrose solution and added with ATP showed almost no increase in the release of AcPase during 6 hrs. of incubation, whereas approximately 80% of the total enzyme activity was released without the addition of ATP. In other words, ATP should be emphasized as the important factor responsible for the maintenance of cytoplasmic membranous structures in general.

In autolyzed liver tissues, certain biochemical changes were reported to be mediated through release of lysosomal enzymes, though definite ultrastructural changes were not found in cytop asmic areas adjacent to injured lysosomes in the present experiments. This finding is in accordance with those reported by some investigators[25~28].

Summary

1. Changes of lysosomes of rat hepatic cells in the process of autolysis were investigated histochemically, biochemibally and electron microscopically on the aseptically incubated liver tissues.

2. Lysosomes identified by histochemical examinations of AcPase activity were seen as discrete granules along the bile canalicli of the hepatic cell plates. After 3 hrs. of the incubation, the granules in the hepatic cells surrounding the central vein showed

poorly defined contour with decreased density, and the enzymatic activity was not confined to the granules but appeared to disperse into the cytoplasm. With the time of incubation, these changes became distinct and gradually extended to peripheral area of the lobules.

3. Biochemically the AcPase activity in the supernatant fraction and also the activity available in the homogenate showed the gradual increase in the process of incubation. The addition of ATP to the isolated lysosomes protected the membrane against the release of the enzymes concerned.

4. Electron microscopically, among the various morphological changes of the cytoplasmic organelles observed, characteristic changes of the cytoplasmic membranes on the sinusoidal border and those of bile canaliculi and certain alterations of mitochondria were conspicuously noted prior to the ultrastructural alterations of lysosomes.

It is concluded that the changes in cytoplasmic membranes of the hepatic cells in the early stages of autolysis precede the release of lysosomal hydrolases, whereas the ATP-level is lowered, the releasing of lysosomal enzymes is accelerated and leads to the lytic changes of the cytoplasm in the later stages.

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From Fig. 1 to Fig. 4, light micrographs show the AcPase activity demonstrated histochemically by Gomori's method using β-glycerophosphate as stubstrate.

Fig. 1. The acid phosphatase activity of rat liver tissue of control group. The positive stained granules arealmost evenly distributed throughout the heptic lobule. CV— central vein, P— portal area. 100×.

Fig. 2. The high magnification of a part showed in Fig. 1. The pericanalicular arrangement of the granules is noticed. 400×.

Fig. 3. Liver tissue incubated for 3 hrs. at 37°C. The granules which showed the AcPase activity in control tissue are poorly defined with decreased density. CV—central vein. 400×.

Fig. 4. Liver tissue incubated for 8 hrs. at 37°C. The positive stain was no more observed except in the hepatic cells in peripheral area with slight and dispersed activity. 400×.

From Fig. 8 to Fig. 12, electron micrographs of liver tissues incubated various time intervals at 37°C. Stained by uranyl acetate.

Fig. 8. 30 min. incubated liver tissue. Hepatic cell membrane facing to the space of Disse showing the bleb formation and disappearance of microvilli. 11,500×.

Fig. 9. Pericanalicular part of 1 hr. incubated liver tissue. Rupture of the limiting membrane are seen in several lysosomes.
Mitochondria are swollen and separation of the outer membrane from the inner membrane are seen in some mitochondria. 13,000×.

Fig. 10. 2 hrs. incubated liver tissue showing membranous structures and granular aggregations in some lysosomes. A large lysosome have vacuoles and lost the clear zone along the limiting membrane. At the upper right, a part of nucleus showing aggregation of chromatin can be seen. At the underpart of the picture, a bile canaliculus which has intensely swollen microvilli can be seen. 14,000×.

Fig. 11. 5 hrs. incubated liver tissue. A part of hepatic cell cytoplasm which is occpied by swollen mitochondria. 20,000×.

Fig. 12. 5 hrs. incubated liver tissue. A part of hepatic cell nucleus showing perinuclear condensation of chromatin. 10,5000×.