ELECTRON MICROSCOPY OF LYSOSOME-RICH FRACTIONS FROM RAT THYMUS ISOLATED BY DENSITY-GRADIENT CENTRIFUGATION BEFORE AND AFTER WHOLE-BODY X-IRRADIATION

Y. E. RAHMAN, M.D.

From the Division of Biological and Medical Research, Argonne National Laboratory, Illinois

ABSTRACT

Fractions from rat thymuses were isolated by sucrose density-gradient centrifugation, before and after 1000 r whole-body x-irradiation, and examined by electron microscopy. Cytochrome oxidase and acid phosphatase activities of these fractions were tested as well. Electron-opaque bodies with diameters ranging from 0.10 to 0.35 μ, with a mean of 0.25 μ, were found in fractions having high acid phosphatase activity, while the fractions rich in cytochrome oxidase consisted mostly of mitochondria. After irradiation, there was an increased ratio of dense bodies to mitochondria. These particles are considered to be lysosomes similar to those identified in other rat tissues. Their relationship to the mitochondria is discussed.

On the basis of biochemical data and centrifugation experiments on rat liver, the existence of a morphologically distinct group of particles between mitochondria and microsomes has been deduced by de Duve and co-workers. These authors suggested the name "lysosome" and defined it as follows (4): "(1) dimensions corresponding, in 0.25 M sucrose and on the assumption of a spherical shape, to a mean diameter of 0.4 μ and an average density of 1.15, both properties showing a fairly wide dispersion around these mean values; (2) an enzymic equipment lacking several key enzymes of oxidative metabolism, but comprising a number of easily soluble hydrolases (hence the name lysosomes) having further in common an acid pH optimum; (3) a surrounding membrane of lipoprotein nature, which effectively prevents the enzymes from escaping from, as well as their respective substrates from penetrating into the particles; (4) the simultaneous release of all internal enzymes in soluble and fully active form following injuries to the membrane as caused by various treatments."

Attempts were made by Novikoff, Beaufay, and de Duve (16), using the electron microscope, to identify morphologically the lysosomes isolated from rat liver by differential centrifugation. In every fraction showing high acid phosphatase activity, they found, besides varying amounts of mitochondria and microsomes, particles which contained many dense granules and often possessed an internal cavity and an external single-layered membrane. These particles had a mean diameter of 0.37 μ and could be identified with the dense peribiliary bodies described by Rouiller (19) and by Palade and Siekevitz (17). Such a distinct morphological entity might be identified in tissues other than liver, as suggested by Straus (20) and Farquhar and Palade (9) in the kidney, and by Cohn and Hirsch (2) in rabbit polymorphonuclear leucocytes.

Besides the indirect evidence mentioned above,

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a direct relationship between these dense bodies and acid phosphatase is provided by histochemical studies by Holt in rat liver and kidney (12, 13), by Essner and Novikoff (7, 8) in rat and human liver, by DeMan *et al.* (6) in mouse liver, and by Barka, Schaffner, and Popper (1) in the reticuloendothelial cells of liver, spleen, thymus, and lymph nodes of rat.

We have studied the changes in activities of two of the lysosomal enzymes, acid phosphatase and

This paper reports the results of the electron microscopic study of the lysosome particles isolated from rat thymus by density-gradient centrifugation.

METHODS

Density-Gradient Centrifugation Procedure

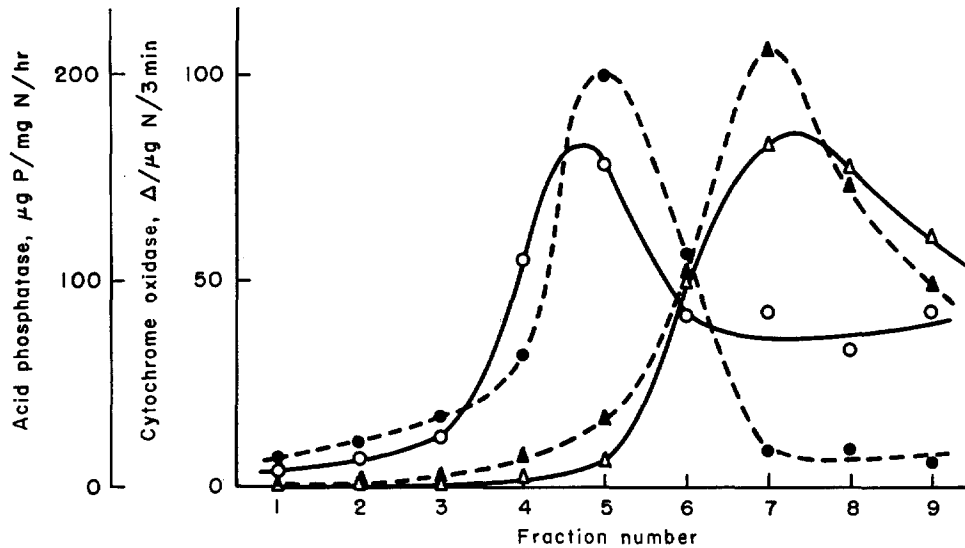Female Holtzmann rats, 45 to 50 days of age, weighing between 130 and 150 gm, were killed by

FIGURE 1

Acid phosphatase and cytochrome oxidase distribution pattern in fractions separated by density-gradient centrifugation of rat thymuses. Acid phosphatase from non-irradiated thymus ○—○, from irradiated thymus ●—●. Cytochrome oxidase from non-irradiated thymus △—△, from irradiated thymus ▲—▲.

β-glucuronidase, in the rat thymus after whole-body x-irradiation (18), and found that, 24 hours after a single dose of radiation, the specific activities of both acid phosphatase and β-glucuronidase increased up to 130 per cent and 150 per cent of the control value, respectively, after a dose of 1000r and up to 280 per cent and 260 per cent after a dose of 200 r. This increase, of course, might be due solely to the selective nitrogen loss of the lymphoid tissue (a selective loss of lymphocytes in which lysosomes might not be found). However, if it were a true reflection of a "relative increase" of lysosomes, the increased specific activities would suggest that the use of irradiated thymus might provide a better chance of isolating the lysosome particles.

decapitation; the irradiated rats were killed 24 hours after receiving a single dose of 1000 r. The thymuses were removed and chilled at once in 0.25 M sucrose at 0°C. Thymuses of four to six rats were used, which provided sufficient tissues for six to eight gradient tubes. The thymus glands were homogenized, with a glass homogenizer fitted in a plastic test-tube, in 9 ml of sucrose solution for each gram of tissue. One ml of the thymus homogenate was layered on top of a gradient tube. Each gradient tube was prepared by layering successively 2 ml of 30, 25, 20, 15, and 10 per cent sucrose into a 12 ml plastic centrifuge tube (21, 22, 23). The tubes were stored in a cold room at about 4°C for 16 to 20 hours before use. Centrifugations were performed in the SCR swinging rotor of the Lourdes centrifuge operated at 10,000 RPM (14,900 g) for 30 minutes,
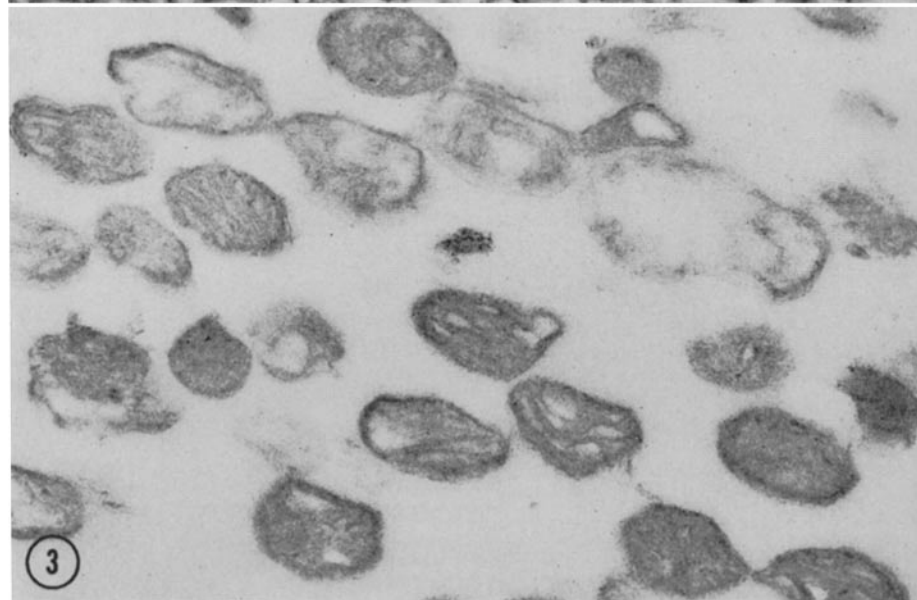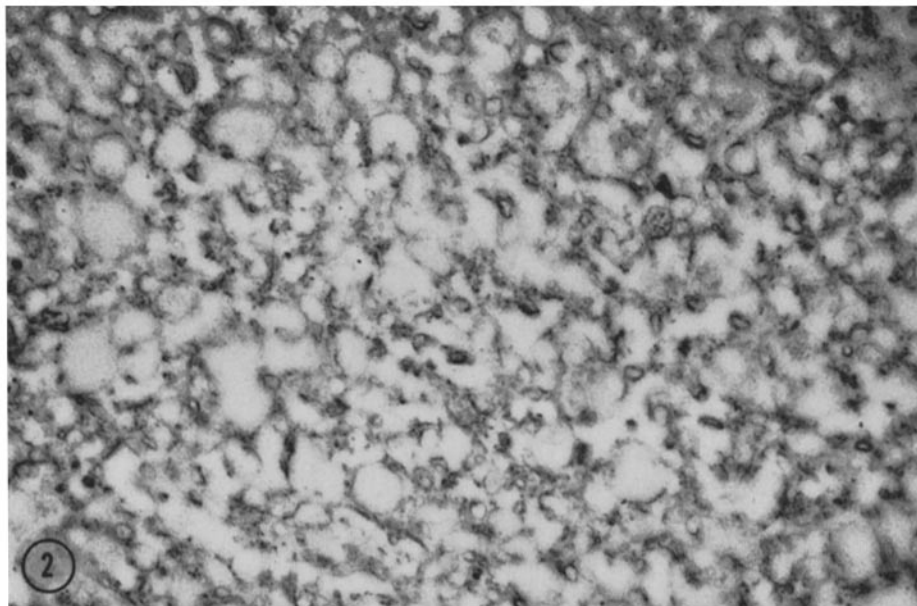
FIGURE 2

Fraction 3 of normal rat thymus, consisting mainly of fragments of microsomes. × 20,000.

FIGURE 3

Fraction 6 of normal rat thymus. Mitochondria with their characteristic cristae can be seen. A few are swollen and damaged. These mitochondria have a mean diameter of 0.30 μ, the range being from 0.20 to 0.40 μ. × 52,500.

and nine successive 1 ml fractions each were carefully isolated with a syringe and a specially bent needle, proceeding from the top to the bottom of the tubes. The last 2 ml, which contained a small amount of cell debris in clumps, was pooled and considered as the bottom layer.

Enzymes Assays

Cytochrome oxidase, used as tracer enzyme for the presence of mitochondria in the fractions, was assayed by the spectrophotometric method of Cooperstein and Lazarow (3). Acid phosphatase, used as tracer enzyme for the presence of lysosomes, was tested following the method described by Gianetto and de Duve (10). These assays were carried out on all ten fractions as well as the original homogenate.

Electron Microscopy

Of the ten fractions obtained from each gradient tube, the first two and the last two fractions were discarded, since the top two fractions contained mostly soluble proteins and the last two contained cell debris. Because of the minute quantity of tissue in each fraction, fractions of the same level from eight different gradient tubes were pooled to provide sufficient material for electron microscopic observation. These fractions were then centrifuged at 30,000g for 30 minutes and the pellets obtained were fixed for 1 hour in 1 per cent osmium tetroxide buffered with Veronal to pH 7.4; the material was dehydrated and then embedded in prepolymerized methacrylate (three parts of n-butyl- and two parts of ethyl-) with 2 per cent Luperco CDB as initiator. The whole procedure before embedding was performed at 0°C. Then the methacrylate was polymerized at 60°C for 16 to 20 hours. Thin sections, usually 50 to 100 mμ, were cut on a Porter-Blum microtome and observed in a Siemens Elmiskop I electron microscope operated at 80 kv.

X-Irradiation Procedure

Besides the non-irradiated control animals, a set of four to six animals was used for each experiment. They were given a single dose of 1000 r at an average dose rate of 190 r per minute, delivered by a 250 kv machine with 0.5 mm Cu and 3.0 mm Bakelite filters; the target distance was 29 cm and the rats were rotated during exposure.

RESULTS

Enzyme Activities

As can be seen from Fig. 1, the peak specific activity of acid phosphatase was in fraction 5, and that of cytochrome oxidase was in fraction 7. Neither of the enzyme distribution patterns, to judge by their peak specific activities, was significantly altered after x-irradiation. In fractions 6 to 9, the specific activity of the acid phosphatase was much higher for the control thymuses than for the irradiated thymuses (Fig. 1). This was probably due to the higher viscosity of the DNA contained in the control thymuses, which was sedimented in small clumps to the bottom of the gradient tubes and hence pulled down some of the lysosomes.

Electron Microscopic Observations of Isolated Fractions

Fractions 3 (Fig. 2) and 4 consisted mostly of fragments of microsomes, although a few "dense bodies" were occasionally seen, particularly in fraction 4.

Fraction 6 (Fig. 3) consisted of a small amount of tissue, mostly mitochondria.

Fraction 7 from both irradiated and non-irradiated rats (Fig. 4) consisted almost exclusively of mitochondria. Many of these were damaged, probably because they were put into an unphysiological medium, such as sucrose, and had gone through a rather long procedure of isolation.

Fraction 5 isolated from irradiated rat thymuses (Fig. 6), which showed high acid phosphatase activity, contained, besides a fair amount of easily recognized mitochondria, particles of the sort found by Novikoff, Beaufay and de Duve (16) in the acid phosphatase–rich fractions isolated from rat liver and called by them "dense bodies." The dense particles found in thymus had diameters ranging from 0.10 to 0.35 μ, with a mean of 0.25 μ. They were bounded by a single-layered membrane, and dense granules could be seen withni them. Some of these particles (Fig. 5 and Fig. 6)

FIGURE 4

Fraction 7 of normal rat thymus. It consists almost exclusively of mitochondria. One electron-opaque body (arrow) can be seen in the left lower corner of the micrograph. The mitochondria seen here have a mean diameter of 0.30 μ, the range being from 0.20 to 0.40 μ. $\times$ 70,000.
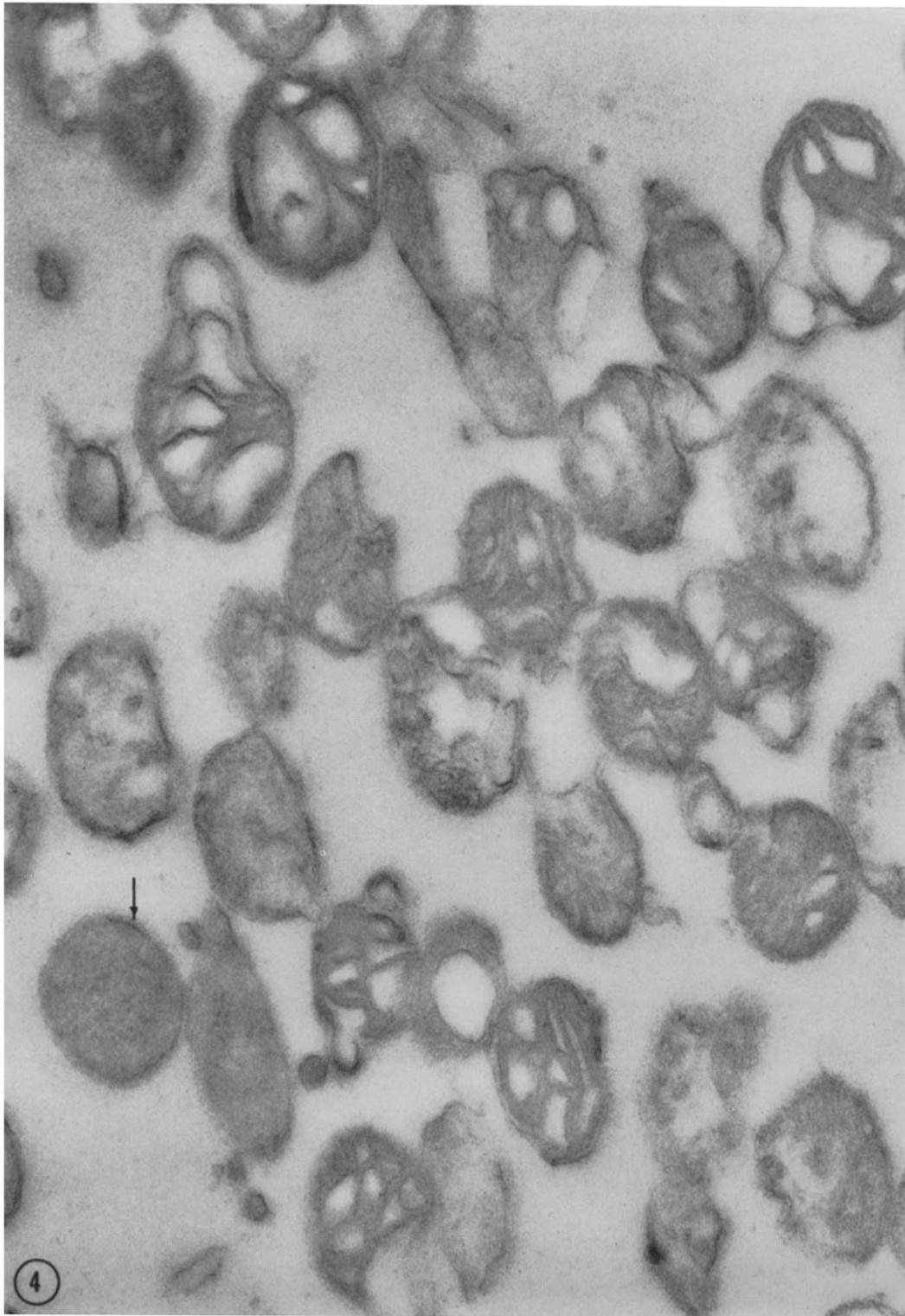
showed an internal cavity like that found in dense bodies from rat liver (13, 16).

Fraction 5 isolated from thymuses of non-irradiated rats contained larger numbers of mitochondria, among which, as compared to fraction 5 obtained from the thymuses of irradiated rats, far fewer typical dense particles were seen (Fig. 5). The dense granules within these particles (in non-irradiated as well as irradiated thymuses) were less in quantity than in the particles isolated from rat liver.

DISCUSSION

It has been deduced from experimental data that lysosomes in the thymus are associated with no more than 3 to 5 per cent of its total nitrogen; the task of isolating such a minute amount of these organelles is really a tedious one. Another difficulty is that the diameter of these particles varies within a fairly wide range, so that with the present centrifugation method it is almost impossible to obtain a clear cut separation of lysosomes from other particles. The result of this study seems to confirm the assumption that there is a better separation of lysosomes from the thymus after whole-body x-irradiation, apparently because of a selective retention of cells rich in lysosome particles.

It has been reported by Kallman and Kohn (14), and recently confirmed by Hofman, Stanković and Allegretti (11), that there are two independent cell populations in the thymus, one radiosensitive and the other relatively radioresistant. From the results we obtained on the study of acid phosphatase and β-glucuronidase activity of rat thymus after whole-body x-irradiation (18), it seems reasonable to assume that the lysosomes are located more abundantly (perhaps exclusively) in the cytoplasm of the radioresistant group of cells in the thymus. Whether this specific distribution of lysosomes has a well defined physiological meaning, we have no way of knowing from the present study.

From the study by Hofman et al. (11) as well as our study on acid phosphatase and β-glucuronidase activity of rat thymus (18) after a lower dose of whole-body x-irradiation, it seems possible that we might have a still better chance to purify the lysosomal fractions by irradiating the rats at a dose in the range of 150 to 300 r, since the relative decrease in radiosensitive cells seems to be more pronounced at this low x-ray dose level.

Meijer and Willighagen (15) reported recently that they found an increased activity of acid phosphatase and β-glucuronidase in the liver and spleen of mice after intraperitoneal administration of various macromolecular substances. If these increases in enzyme activity were directly related to the number of lysosomes in the tissue, one could well expect that, after a suitable treatment (i.e. x-ray or different chemicals) which leads to a selective retention (or possibly de novo formation) of the lysosome particles in different tissues, it would be possible to obtain a better purification of this group of organelles and thus provide morphological identification as well as direct evidence of the relationship between these particles and the group of so-called "lysosomal" enzymes.

This study shows beyond doubt that the lysosomes reported to occur in rat liver and kidney are also present in the rat thymus, since the dense particles in the thymus are similar to those seen in rat liver. The mean diameter of thymus lysosomes seems to be slightly smaller than that of liver lysosomes; however, it may be pointed out that the mean diameter of thymus mitochondria is also smaller than that of rat liver mitochondria (21).

The dense granules within the particles isolated from the thymus are much fewer than the dense granules in the particles isolated from the liver.

FIGURE 5

Fraction 5 of normal rat thymus. Among the mitochondria a few electron-opaque bodies can be seen (arrows). × 48,000.

FIGURE 6

Fraction 5 of rat thymus, 24 hours after 1000 r whole-body x-irradiation. Mainly electron-opaque bodies (arrows) which have a mean diameter of 0.25 μ, the range being from 0.10 to 0.35 μ. An internal cavity can be seen in a few of these particles. × 28,000.
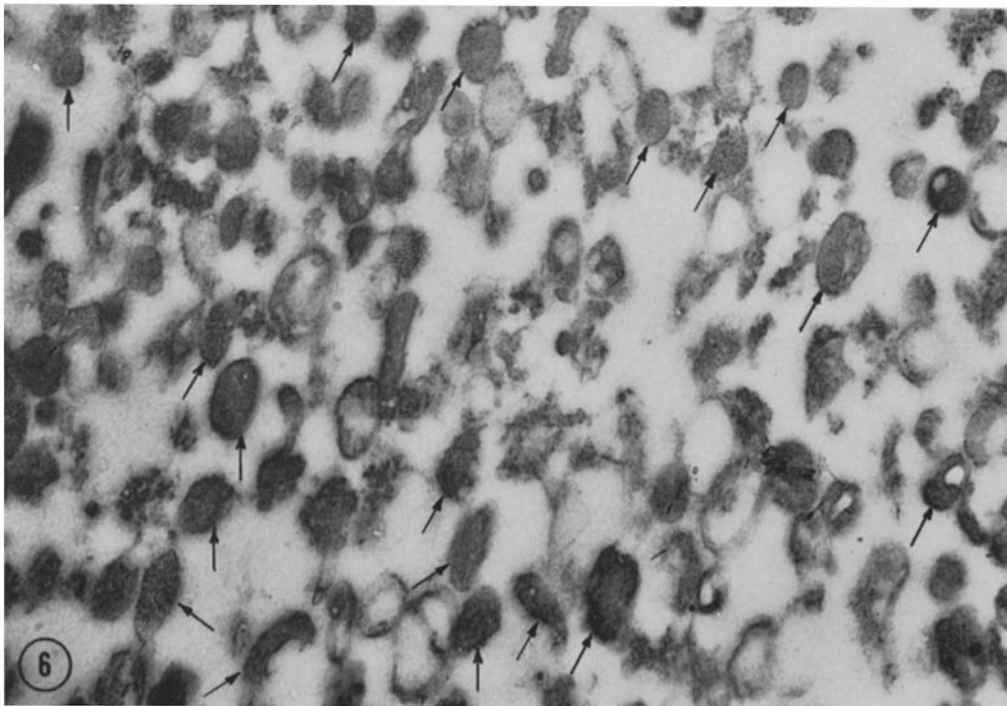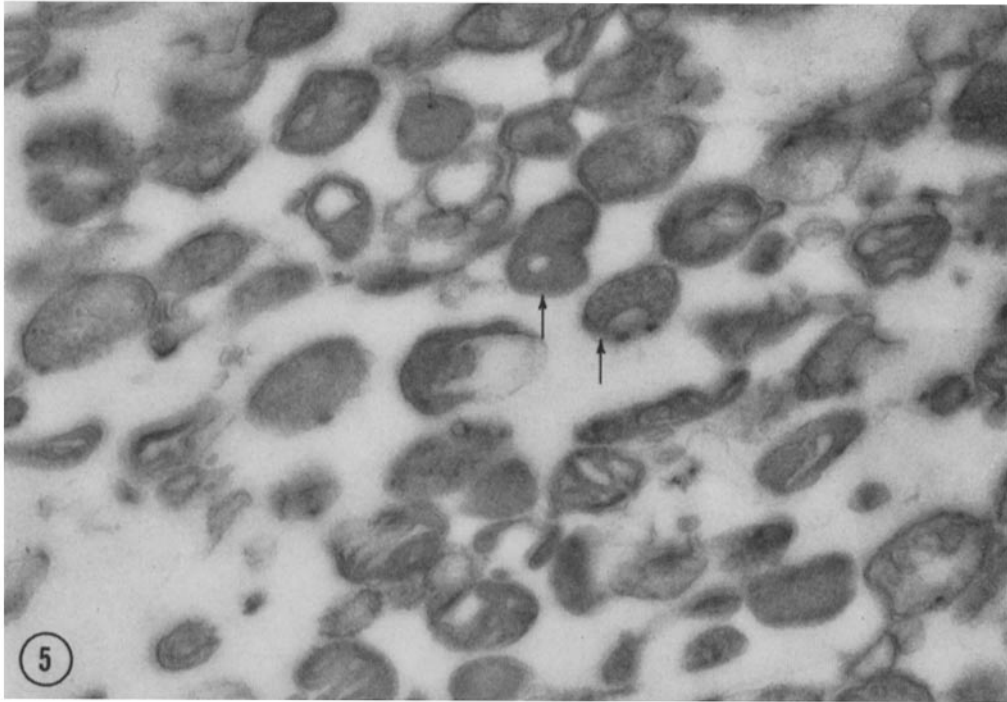
Since it has been shown by de Duve and coworkers (5) that these granules could be ferritin-iron micelles, the difference found between thymus and liver in respect to the quantity of these dense granules can be easily explained in view of the fact that the liver is one of the principal organs in which iron metabolism takes place.

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