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Intracellular Modulation in Cellular Immunity

3. Macrophage Lysosome Formation and Intracellular Interaction with Phagosome in Murine Leprosy[1, 2, 3]

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Macrophages play an important role in tissue defense against pathogens in chronic granulomatous infections (5). Studies of the morphologic changes of macrophage lysosomes and the macrophage lysosomal enzyme responses in murine leprosy (*M. lepraemurium* infection) suggest that macrophage lysosomal response is an integral part of acquired cellular immunity (8). This role is primarily suggested by the acceleration of cellular lysosomal enzymes response associated with suppression of infection in murine leprosy (7). The prolonged association and interaction of macrophage and pathogen in murine leprosy provide a useful model for studying intracellular enzyme modulation, especially in view of the relative absence of associated hypersensitivity as evidenced by lack of lesion caseation.

Evidence of lysosomal enzyme synthesis in stimulated macrophages has been found by electron microscopic radioautographic tracings. The technic allows visualization of the dynamic relationship between lysosomes and phagosomes of immunized macrophages during active stages of interaction with the infecting pathogen.

MATERIALS AND METHODS

Labeling of macrophages. Four C_3H mice were injected subcutaneously with complete Freund adjuvant *M. lepraemurium* antigen four weeks prior to their infection with 10^7 *M. lepraemurium* intraperitoneally. Five days following the intraperitoneal infection the mice were injected intraperitoneally with H^3-L-leucine having a specific activity of 1.53 millicurie per milligram (200 millicuries per millimole), diluted in physiologic saline to 100 microcuries per milliliter. This was administered at a dose of 50 microcuries per animal. A chaser containing cold L-leucine of 2.5 mgm. in 0.5 ml. was given at five minutes. The peritoneal cell collection technic was that previously reported (7). The cell suspensions were centrifuged at 1,000 rpm for 10 minutes and then fixed in 1 per cent osmium tetroxide in 0.1 M phosphate buffer for one hour at 4°C. The resulting pellets were washed with cold 70 per cent alcohol, and processed in routine Epon embedding for electron microscopy.

Radioautography. Sections about 0.08 micron thick were cut with a Porter-Blum microtome and placed on Formvar coated grids. Procedures for making uniformly thin films of Ilford L-4 Nuclear Research Emulsion and applying it on the sections were based on the method of Caro and Van Tubergen (2). After refrigerated incubation for two months in a tightly sealed box containing desiccant (Drierite), the grids were developed in Microdol X and fixed with Kodak rapid fixer and then stained for eight minutes in a 50 per cent alcoholic solution of saturated uranyl acetate followed by five minutes in Reynold's lead (6).

Counting of grains. Multiple grids with specimen sections from each time period were examined and photographed with a RCA-EMU 3c electron microscope. All large macrophages (mononuclear phagocytes) that contained phagocytized bacilli in phagocytic vacuoles were counted. Background grains were so low as to be

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virtually absent in the field not containing sectioned material. The occasional extracellular grains accounted for less than five per cent of the total grain tally. Grains that were found with a large portion over the organelles (i.e., lysosomes, phagosomes and Golgi complexes, etc.) were included as labels of the organelles but, if more than half of the grain was outside the organelle, then it was regarded as label of cytoplasmic matrix. Grains in the cytoplasmic matrix and those over the endoplasmic reticulum, mitochondria and Golgi complex were grouped together as distinct from the combined grains over phagocytic vacuoles (phagosomes) and over lysosomes. Nuclear grains were not included in the analysis.

RESULTS

Five different grids with 34 macrophages were obtained for the five minute tracing, six different grids with 41 macrophages for the 30 minute tracing, six different grids with 32 macrophages for the 60 minute tracing, and four different grids with 35 macrophages for the 120 minute tracing. The macrophages had an average of nine grains per cell, the range being four to 15 grains. Those cells in which the plane of section included Golgi complex were analyzed separately. The results of all counts are presented in Tables 1 and 2.

At five minute tracing time, the grains were randomly distributed in the cytoplasm (Fig. 1, 2) being predominately in the cytoplasmic matrix and endoplasmic reticulum. After 30 minutes, the grain counts over or around the Golgi complexes increased (Table 2). In some cells of the 30 minute tracing, as many as 50 per cent of the grain counts were over the Golgi complex (Fig. 3). Many grains were over the prominent Golgi vesicles and some labels were already present in the adjacent newly formed lysosomes. These newly formed lysosomes were small and usually had in their centers a homogeneous dense matrix that was separated from the membrane-shell by a clear space (Fig. 4). They were larger when located further away from the Golgi complex. At 60 minutes, more labels were found over the newly synthesized lysosomes and over the phagocytic vacuoles (Table 1). At 120 minutes there were significant increases in the concentration of the grains over the lysosomes and the phagocytic vacuoles as compared with the five minute tracing (Table 1), (Figs. 5, 6).

In summary:

1. An increasing number of labels appeared in the lysosomes and phagosomes as the tracing time increased. At 120 minutes more than one-fourth of the cytoplasmic grains were either on the newly formed dense granules or already inside the phagocytic vacuoles (phagosomes).

2. Although the number of cells that had been cut through the Golgi complex zone (near the indentation of the kidney shaped nucleus) was small, the results indicated peak counts at 30 minutes and lowest counts at 120 minutes (Table 2). This difference in grain counts is statistically significant.

TABLE 1. *Distribution of grains over the cytoplasmic structures in peritoneal macrophages after 5 min., 30 min., 60 min. and 120 min. exposure to tritiated-L-leucine.*

Cytoplasmic structures	Per cent distribution			
	5 min.	30 min.	60 min.	120 min.
Golgi complexes Cytoplasmic matrix	95	82	76	71
Mitochondria Phagocytic vacuoles Dense bodies	5	18	24	29
Total cells	34	41	32	35
Total grains	284	410	351	374

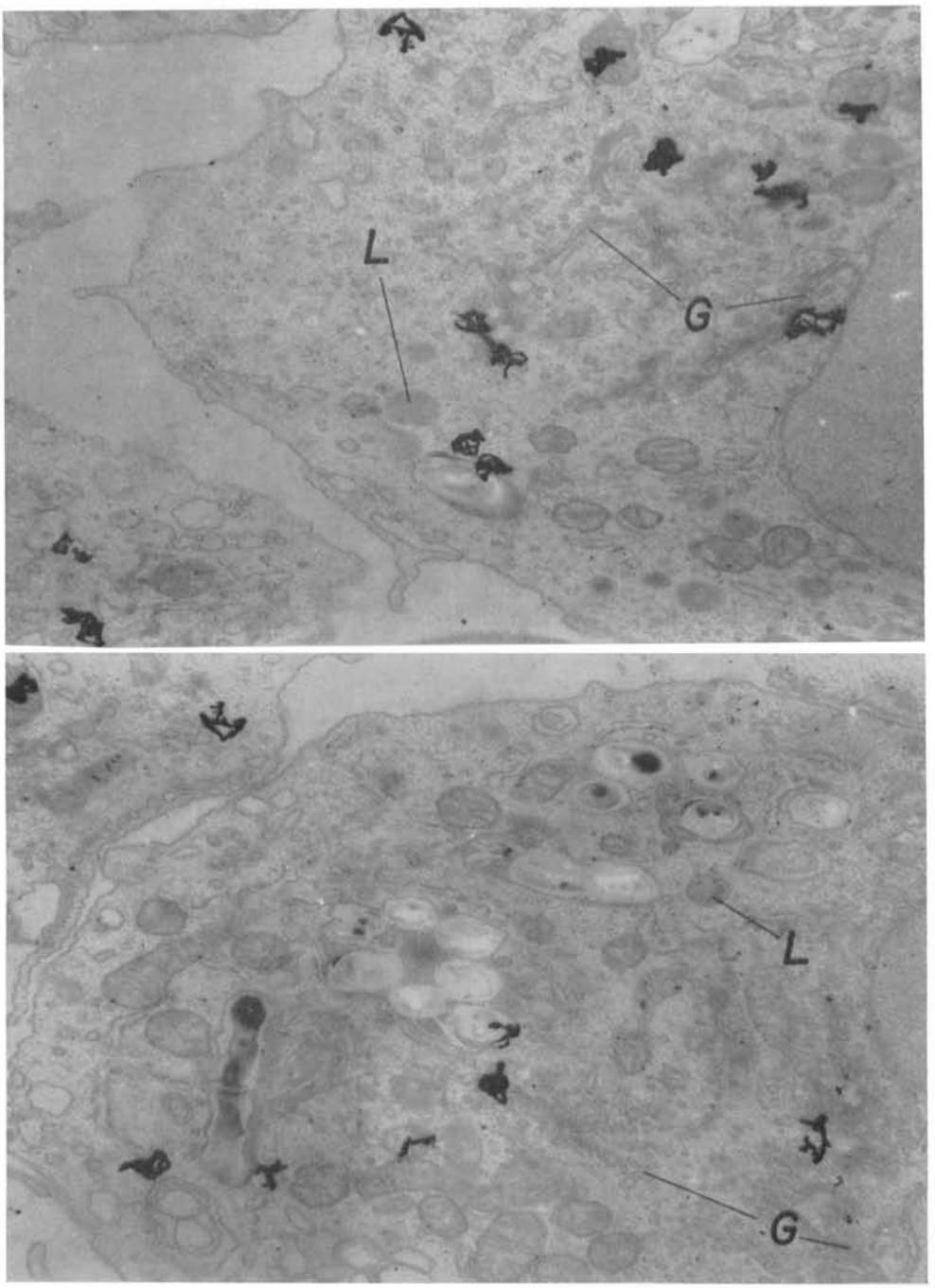


FIG. 1 and FIG. 2. Thin section electron micrographs of a peritoneal macrophage at 5 minute tracing with H^3-L-leucine. Note the hyperplastic Golgi complexes (**G**) surrounded by small newly formed lysosomes (**L**). At this time point, the grains are randomly distributed. One grain in Figure 1 and 2 grains in Figure 2 are found over the Golgi complexes. Magnification: 25,000×.

TABLE 2. *Per cent of label over Golgi complex in peritoneal macrophages after 5 min., 30 min., 60 min. and 120 min. exposure to tritiated-L-leucine.*

	Distribution			
	5 min.	30 min.	60 min.	120 min.
No. of cells	11	8	8	5
Total number of grains	108	90	94	58
Per cent of grains on Golgi complex	21	28	25	12

3. The number of grains over the nuclei was less than 15 per cent and was fairly constant throughout the time period studied. These grains were not included in the analysis.

4. The appearances of the macrophages and their cellular organelles represented that of the stimulated macrophages. The Golgi complexes in all the counted cells were hyperplastic and surrounded by small vesicles and small, probably nascent, lysosomes.

DISCUSSION

These results suggest that the newly synthesized protein is initially present in the cytoplasmic matrix and later transferred to the Golgi complex. The formation of lysosomes by the Golgi complex as indicated by this later shifting of grain counts is in agreement with the pattern of formation of zymogen granules in pancreatic acinar cells (1), and the formation of neutrophile granules in myelocytes (4).

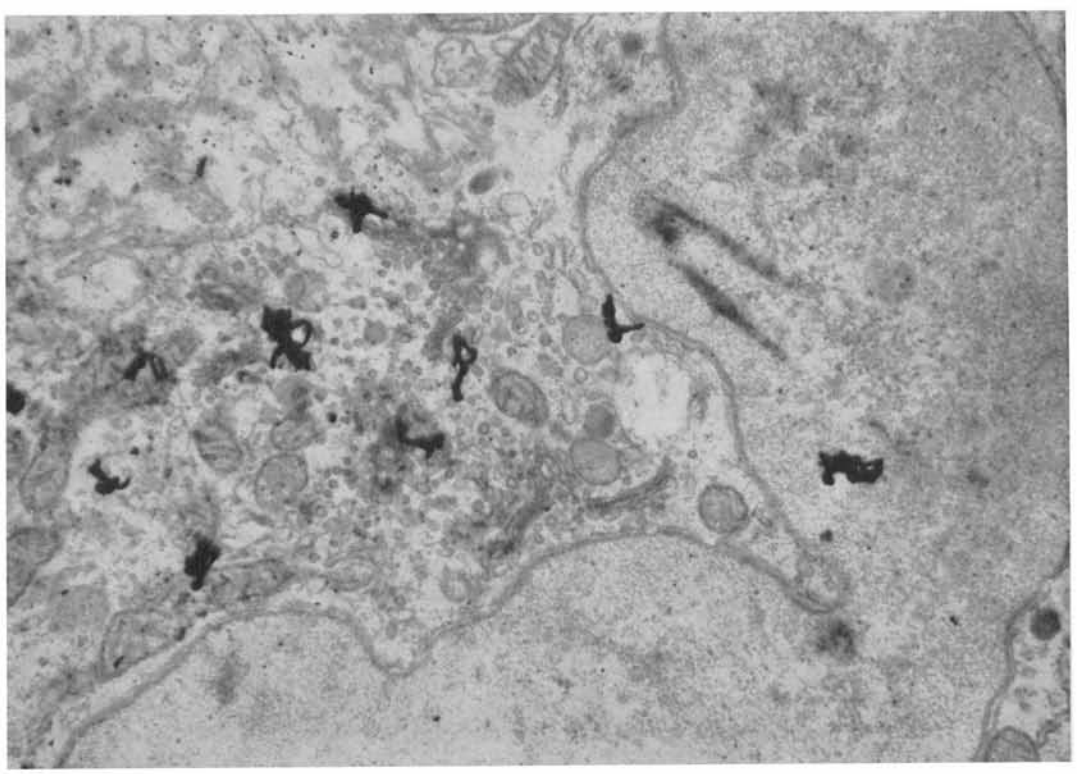


FIG. 3. Thirty minutes H^3-L-leucine tracing. Grains are concentrated over the area of Golgi complex. Magnification: 25,000×.

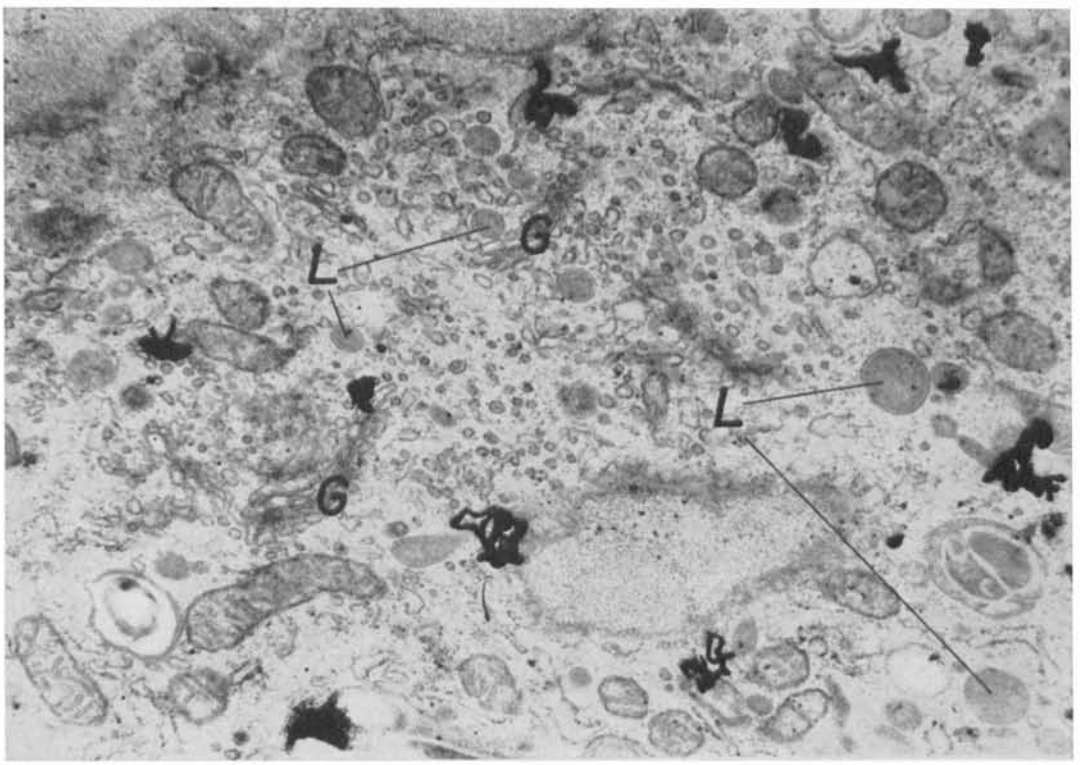
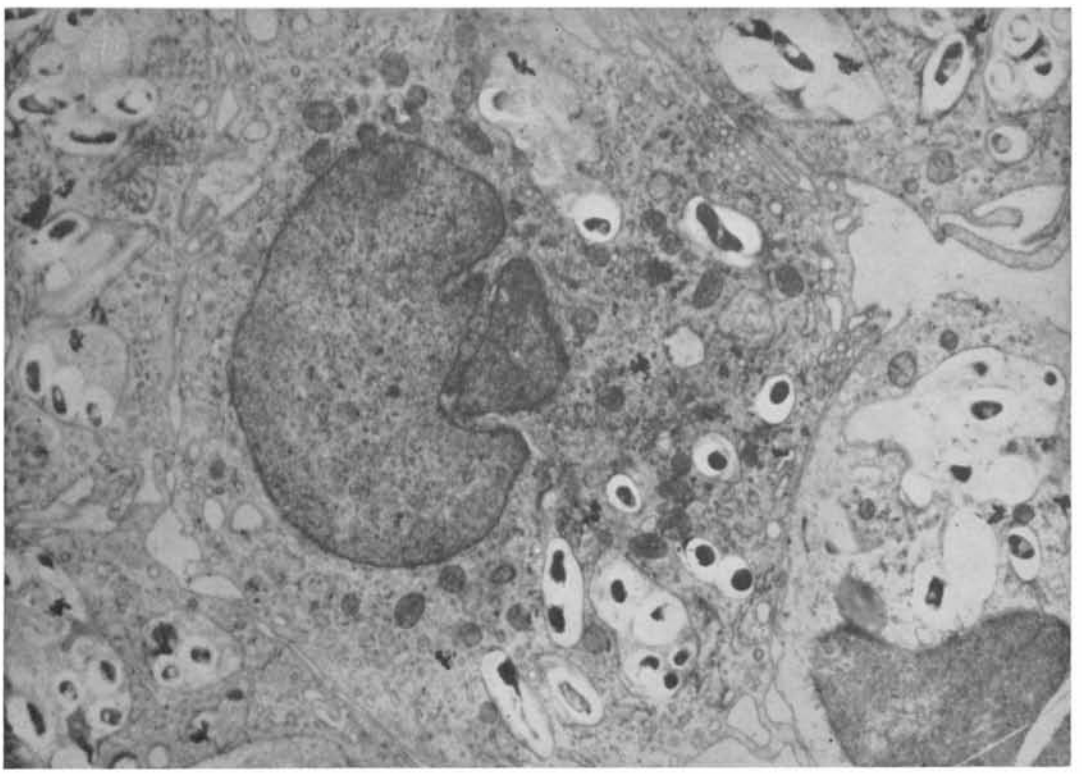


FIG. 4. Thirty minutes H^3-L-leucine tracing. Golgi complex (G) is hyperplastic and there are adjacent small lysosomes (L). Magnification: 25,000×.

In vitro studies of lysosome formation in cultured macrophages by Cohn *et al* [3] led to the same conclusion. The difference between Cohn's observation and those here reported is the time at which the peak labeling appeared in the Golgi complex. The peak Golgi complex labeling time in these experiments was at 30 minutes, while in Cohn's observations, it was at 60 minutes. It is possible that the *in vivo* conditions in the present experiments favor cell metabolism, and secondly that macrophages in these experiments were stimulated by prior immunization as well as the intracellular presence of bacilli. This difference, if significant, might be an indication that the rate of protein synthesis in the stimulated macrophages is increased. Since in this particular experiment the animals were all immunized with *M. lepraemurium* antigen in Freund's adjuvant and this was observed to have enhanced the host resistance, it is probable that the increased rate of lysosome production is associated with enhanced cellular immunity.

SUMMARY

An electron microscopic, radioautographic study of stimulated protein synthesis of peritoneal macrophages revealed that isotope labeled amino-acid was incorporated into protein which has the lysosome enzyme distribution pattern and follows the same path of lysosome transportation leading to phagocytic vacuoles. The H^3-L-leucine activity was initially present in the cytoplasmic matrix and endoplasmic reticulum. At 30 minute tracing time the highest activity appeared at Golgi complexes. At 120 minute tracing time, significant increase of grain count was detected either over the newly formed lysosomes or already inside the phagocytic vacuoles (phagosomes).



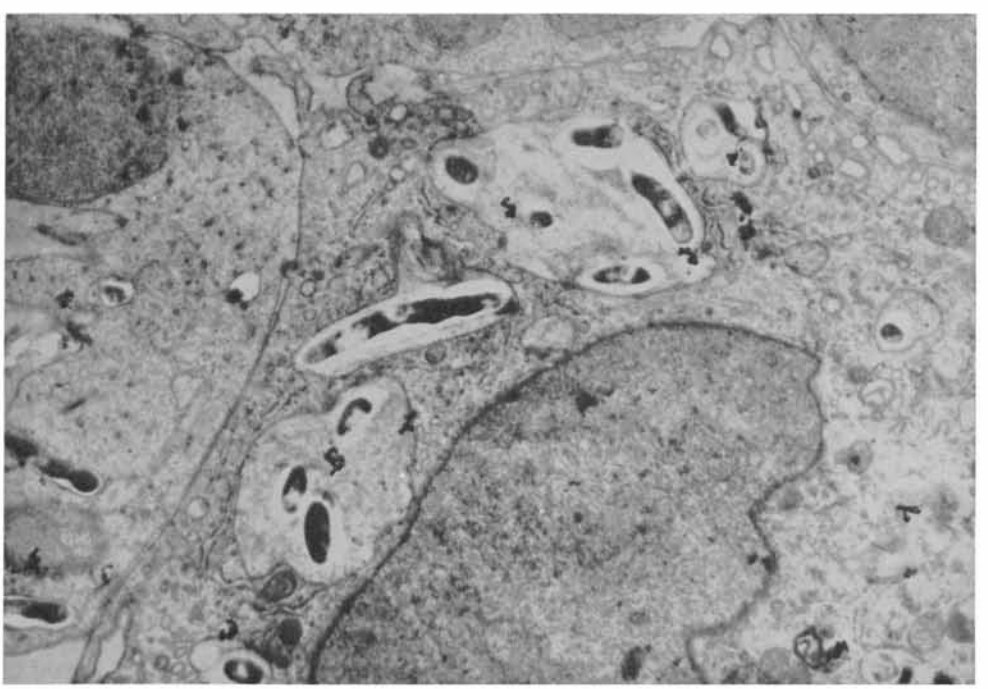


FIG. 5 and FIG. 6. One hundred and twenty minutes H^3-L-leucine tracing. There are significantly more grains incorporated into the phagocytic vacuoles (phagolysosomes). Magnification: 12,000× and 18,000×.

RESUMEN

Un estudio electron-microscópica y radioautográfico de síntesis de proteina estimulado en macrófagos peritoneales reveló que aminoácido rotulado con un isótopo fuera incorporado dentro de la proteina teniendo el modelo de distribución de lisosoma-enzima, y sigue el curso de transporte conduciendo a vacuolas fagocíticas. La actividad de H^3L-leucina fue presente al principio en la matriz citoplásmica y reticulum endoplásmico. En trazadores a 30 minutos la actividad mas alta apareció en complejos de Golgi. A 120 minutos un aumento significante de "grain count" fue detactado o sobre las lisosomas nuevamente formadas o ya dentro de las vacuolas fagocíticas (phagosomas).

RÉSUMÉ

Une étude au microscope électronique, de même que des investigations radio-autographiques de la stimulation de la synthèse des protéines dans les macrophages péritonéaux, ont révélé que l'amino-acide marqué par isotope était incorporé dans la protéine qui possédait le profil de distribution enzymatique correspondant aux lysosomes, et suivait le même chemin de transfert lysosomique qui mène aux vacuoles phagocytaires. L'activité en H^3-L-leucine était, au début, présente dans la matrice cytoplasmique et dans le réticulum endoplasmique. Au bout de 30 minutes de'enregistrement du tracé d'incorporation, l'activité la plus forte s'est manifestée au niveau des complexes de Golgi. Au bout de 120 minutes d'enregistrement du tracé, une augentation significative du nombre de grains a été détectée soit dans les lysosomes nouvellement formés, soit dans ceux qui etaient déjà dans les vacuoles phagocytaires (phagosomes).

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