By 20 days after injection, most of the labeled cells in the lymph nodules occurred in the peripheral parts of the nodules. Some vacuolated cells were now unequivocally labeled, but few lymphocytes were labeled any longer. Most of the labeled cells, both in the lymph nodules and in the circulating blood, were leucocytes and vacuolated cells. At 60 days after the administration of tritiated thymidine, most of the blood cells in the lymph nodules were unlabeled (Figure 2). However a few scattered labeled leucocytes and vacuolated cells occurred in the connective tissue below the atrial epithelium and in the blood channels.

Discussion. The blood cells of *Styela clava* constitute a renewing[8] cell system with a renewal time on the order of several weeks. As suggested by other authors[1,2], blood cell proliferation occurs both in the lymph nodules and in the blood channels. The possible blood cell transformations are shown in Figure 3. Although both lymphocytes and leucocytes proliferate, the lymphocyte is probably the more primitive blood cell type. It is ultrastructurally the most undifferentiated blood cell type[9] and may also be capable of differentiating into germinal cells[10] and somatic cells other than blood cells[11]. Presumably the lymphocytes differentiate into leucocytes. However, since the leucocytes divide and also probably represent several separate cell types, the leucocytes themselves might be composed of stem, dividing transit, or non-dividing transit[12] components. Since the differentiated vacuolated cells do not divide, they must be differentiating from a precursor cell type; whether this precursor is a lymphocyte or a leucocyte could not be determined in the present investigation. Based upon morphological criteria, however, vacuolated cells have been reported to differentiate from intermediate cell types and not from lymphocytes in other ascidians[7,13].

Blood cells are renewed in insects[14] and mammals[15] but not in echinoderms[16] where blood cells have characteristics of expanding[8] cell populations. In mammals, proliferating cells are most concentrated in the bone marrow, lymph nodes, and spleen, but, like in *Styela*, they also occur in the circulating blood and in the connective tissue. In *Styela*, other elements of the vascular system (the heart and connective tissue lining the blood channels) comprise expanding cell populations[17].

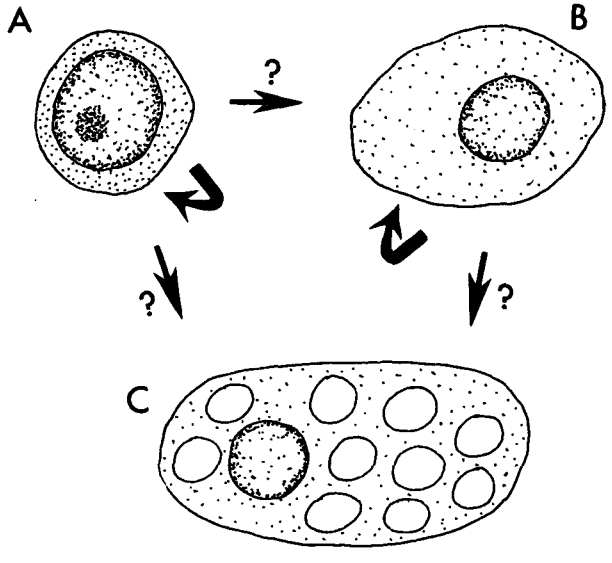

Fig. 3. Transformation of blood cell types in *Styela clava*. A, lymphocyte; B, leucocyte; C, vacuolated cell.

Summary. The blood cells of *Styela clava* were shown by autoradiography with tritiated thymidine to be renewed after several weeks. Proliferating lymphocytes and leucocytes occurred in the lymph nodules and blood channels of the body. Vacuolated cells did not proliferate but differentiated from a precursor cell type.

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Aggregations of Dense Granules in Mitochondria of Active Pulmonary Lymphatic Endothelial Cells

In a previous study[1] we investigated the rôle of the peribronchovascular lymphatics in the clearance of intratracheally instillated ferritin and carbon particles. Both tracers reached the lymphatic lumen mainly via the open intercellular junctions. Ferritin particles were moreover absorbed by the endothelial cells and accumulated, probably to be digested[2], in secondary lysosomes.

The purpose of the present study was to investigate if these activities of the lymphatic endothelial cells are associated with morphological changes of their mitochondria, as it is well known that more active mitochondria display an altered fine structure[3].

We now demonstrate aggregations of small, dense, and more or less rounded granules (300 à 800 Å) occurring in mitochondria of pulmonary lymphatic endothelial cells, which had endocytosed ferritin, were fixed in a mixture of glutaraldehyde and osmium tetroxide, and stained with uranyl acetate and lead citrate. Although the precise nature of these granules, which have not been reported before to the best of our knowledge, remains unexplained, it is suggested that these aggregations are related to an in-

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crease of the endothelial cell metabolism which was stimulated by the endocytosis and digestion of the ferritin particles.

Material and methods. In 16 neonatal rabbits 0.05 cm^3 ferritin (NBC Cleveland, Ohio; 2 × crystallin and cadmium free) or 0.05 cm^3 India ink (Günther und Wagner, Hanover, C 11/1431) was intratracheally instillated. The animals, as well as the 3 control animals, were sacrified by an i.p. injection of an overdose of nembutal (30′, 4, 24, 28 h and 5 days after instillation of ferritin; 30′, 6, 8, 28 h and 5 days after instillation of India ink).

In all animals, lung tissues were fixed by 2 methods: 1. either the usual fixation with glutaraldehyde 2.5% (at 4 °C, 2 h) buffered with phosphate at pH 7.2, followed by postosmification in 1% OSO_4 (phosphate buffered, pH 7.2); 2. or a fixation for 1 h in a solution of 1% OSO_4 and 1.25% glutaraldehyde cacodylate buffered at pH 7.0 (4°C), followed by postosmification in 2% OSO_4 cacodylate buffered at pH 7.0 for 3 h at 4 °C (Trump fixation)[4]. After fixation, all tissue blocs were embedded in epon and cut into thick (1 μm) and thin sections. The peribronchovascular lymphatics were studied with the electron microscope (Philips 300 A) on ultrathin sections which were either stained with uranyl acetate and lead citrate or not stained at all.

Observations. Control animals. According to the plane of sectioning, the mitochondria of the pulmonary lymphatics appear as rounded to elongated (length 1.6 μm), sometimes even more irregular organelles revealing their wellknown basic pattern[5]. Their matrix is finely granulated (granule diameter 60 à 95 Å) and may contain some larger electron dense granules (diameter 160 à 300 Å), probably calcium and phosphate[5].

Intratracheal instillation of ferritin. At low magnification and after Trumps' fixation and staining, electron dense, round to oval dots (diameter 300 à 800 Å) are seen in the matrix of the mitochondria of the pulmonary endothelial cells which have absorbed ferritin (Figure 1). These dense spots often occur at the mitochondrial periphery, never in the intracristal or the peripheral (i.e. between the inner and the outer membrane) space. They are often near the inner mitochondrial or the cristal membrane (Figures 1, 2 and 4). At higher magnification they appear constituted by small more or less rounded and electron dense granules with a diameter ranging from 45 to 150 Å (Figure 2 and 4). The granules do not reveal a particular disposition and their number per aggregation varies (Figure 2). Though it is difficult to ascertain the exact number of granular aggregations per mitochondrion, it appears largest in the lymphatic endothelial cells which contain most ferritin. These endothelial cells contain also the most numerous mitochondria with dense granules (Figure 1).

Similar aggregations of dense granules were not found in mitochondria of lymphatic endothelial cells, either after classical fixation with glutaraldehyde and postosmification, or on non-stained sections (Figure 3).

Intratracheal instillation of carbon. In this group of animals, aggregations of dense mitochondrial granules were never seen. Still, there is transport of carbon from the lung alveoli to the lymphatic lumina, but no endocytosis of these particles by the lymphatic endothelial cells[1].

Discussion. Clusters of small dense particles were reported in the matrix of isolated mitochondria and in mitochondria of intact cells from different tissues[6] which had been incubated with strontium, barium or calcium. They are supposed to result from a transport and intramitochondrial accumulation of Sr^{++}, Ba^{++} or Ca^{++}, as mitochondria are capable of accumulating[7] large amounts of divalent cations by an energy-dependent process[8]. Analogous intramitochondrial granules were also described in various diseases, and on morphological grounds were considered to represent calcium accumulations[9,10].

Though the morphology as well as the localization of all these aggregations closely mimick the hereby reported dense granular aggregations, they are not identical. Indeed, the earlier described aggregations are not only electron opaque after fixation and staining with heavy metals, but also after fixation with aldehydes and without staining[9], indicating an intrinsic electron opacity. The hereby reported mitochondrial granules, on the contrary, are not seen either after a glutaraldehyde fixation (with postosmification), or on non-stained sections. They are only detected on stained sections and after fixation with a mixture of glutaraldehyde and osmium tetroxide; thus they do not possess a perceptible intrinsic electron opacity and are not visible with all fixation methods. Therefore, we do not suppose that they correspond to accumulations of Sr^{++}, Ba^{++} or Ca^{++}, though this is suggested by their localization and their morphology. The mitochondria in the lymphatic walls do not, moreover, reveal any structural desorganization as usually seen after uptake of large amounts of Ca^{++}[11].

Though in our study the dense and granular mitochondrial aggregations are only seen after an endocytosis of ferritin, one may accept that the aggregations are not made of ferritin molecules, or of their digestion products. Ferritin particles[12] have indeed a distinct intrinsic electron opacity and a rather uniform and round structure (diameter ± 110 Å). The intramitochondrial aggregations are, moreover, seen in the early stages after intratracheal instillation, when the amount of ferritin absorbed in the endothelial cells is still small and when there is not yet an accumulation of ferritin in secondary lysosomes.

After an intratracheal instillation of carbon particles which reach the lymphatic lumen via the intercellular junctions and are not absorbed by the lymphatic lining cells[1], no aggregations of dense granules were seen in the lymphatic mitochondria. This apparently indicates that the aggregations are most probably related to (endocytotic) activities of the lymphatic endothelial cells. These activities, which include e.g. the formations of vesicles

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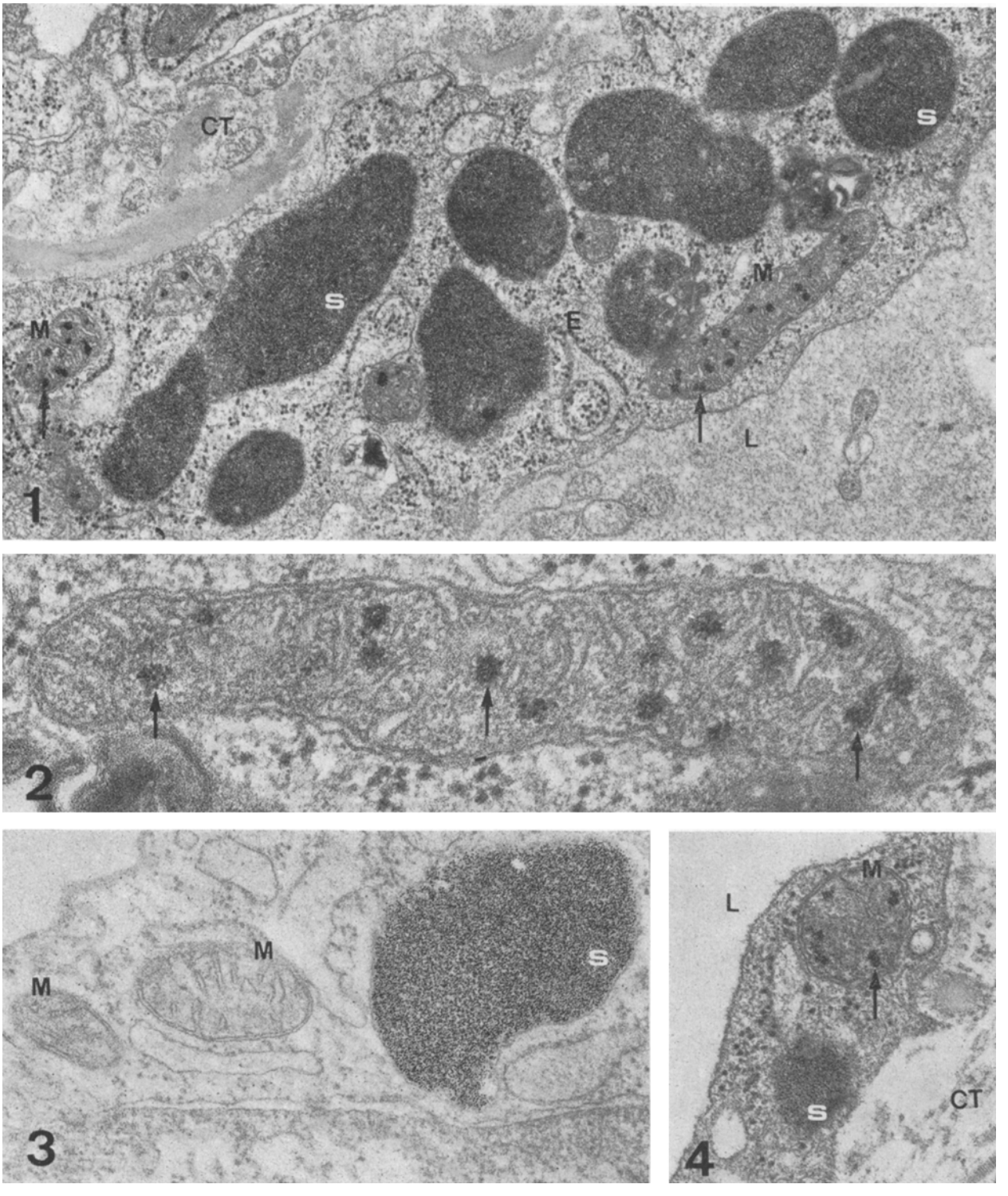

Fig. 1. Detail of a pulmonary lymphatic endothelial cell (E) 28 h after intratracheal instillation of ferritin (Trump fixation, uranyl acetate and lead citrate staining); several secondary lysosomes (S) are filled with ferritin. The matrix of the mitochondria (M) contains electron dense round to oval spots (arrows); CT, connective tissue; L, lymphatic lumen; ×31,350.

Fig. 2. Higher magnification of a mitochondrion from Figure 1. The dense intramitochondrial spots (arrows) are composed of small dense granules, that do not reveal any particular disposition; ×99,750.

Fig. 3. Detail of a pulmonary lymphatic endothelial cell 24 h after intratracheal instillation of ferritin (Trump fixation, but no staining). Although many ferritin particles are piled up in a secondary lysosome (S), the mitochondria (M) do not reveal any aggregations of dense granules; ×51,300.

Fig. 4. Detail of a pulmonary lymphatic endothelial cell (E) 24 h after intratracheal instillation of ferritin (Trump fixation, uranyl acetate and lead citrate staining). Dense spots (arrow) constituted by small granules occur at the periphery of the mitochondrion (M); CT, connective tissue; L, lymphatic lumen; S, secondary lysosome with ferritin; ×51,300.

and of parts of the cell membrane[13], require a larger energy expenditure, which could in turn induce morphological changes such as the appearance of granular aggregations in the energy producing mitochondria. This is also suggested by the large number of mitochondria containing aggregations as well as of aggregations per mitochondrion in the later stages after instillation when huge amounts of ferritin are absorbed and accumulated in the endothelial cells.

The observation reported here had, to the best of our knowledge, not been described before. Similar aggregations were not seen in our studies in mitochondria, either of alveolar macrophages or of alveolar epithelial cells, though these cells exhibit pronounced endocytotic activities[1]. This could indicate that the aggregations are specific for certain cell types (such as the lymphatic endothelial cells) under certain physiological conditions (i.e. high increase of the cell metabolism).

The precise nature of the granular aggregations in the mitochondria of lymphatic endothelial cells remains obscure. From this morphological study we can only conclude that they represent an accumulation of substances (ions, enzymes or other organic material) which play (or have played) a rôle in the increased respiratory activity of the mitochondria in response to an increased cell metabolism. The clustered appearance of the granules, which are often closely related to the inner or cristal membrane, may be explained by the occurrence of binding-places (possible on the membranes themselves) for these substances.

Résumé. Nous avons étudié les mitochondries dans les cellules endothéliales de lymphatiques pulmonaires chez des lapins nouveau-nés après instillation intratrachéale de ferritine ou de charbon. Les mitochondries des cellules qui ont endocyté de la ferritine et qui ont été fixées au glutaraldéhyde et au tétroxide d'osmium contiennent après coloration à l'acétate d'uranyle et au citrate de plomb des aggrégations de granules plus ou moins rondes (diamètre de 300 à 800 Å) qui d'après nous, n'ont pas encore été décrites auparavant. Quoique la nature précise de ces granules reste inconnue, il est possible qu'elles soient en relation avec une augmentation du métabolisme des cellules endothéliales, stimulées par l'endocytose et la digestion des particules de ferritine.

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Electron Cytochemical Demonstration of -SH Groups in the Synaptic Vesicles of Photoreceptor Cells with the Mixture of Zinc Iodide-Osmium Tetroxide

The mixture of zinc iodide-osmium tetroxide (ZIO) introduced by AKERT and SANDRI[1] in electron microscopy, stains totally different kinds of synaptic vesicles[1–5] and other subcellular components.

ZIO stains membranous structures similarly to conventional fixatives (glutaraldehyde, osmium tetroxide) in electron microscopy. However, electron opaque deposits, connected or not with membranes, may be seen in some structures. These electron opaque deposits characterize a positive ZIO reaction.

In the retina, MAILLET[6] observed with light microscope that ZIO stains the outer and inner plexiform layers. Electron microscopic studies made in our laboratory revealed a positive reaction in the synaptic vesicles of the outer[7,8] and inner[9] plexiform layers and in the rod outer segments in different species. Under certain conditions, the vacuolar system of photoreceptor cells was also reactive[10].

Synaptic vesicles do not show the same reactivity in both plexiform layers. When the fixation is made at 4 °C, only the vesicles of the outer plexiform layer react[7,8]. Their reactivity is variable and negative vesicles are frequently observed. On increasing the temperature (20 °C or more), a greater number of positive vesicles are observed, and the reaction is also seen in the synaptic vesicles of the inner plexiform layer[9].

The significance of ZIO reaction in synaptic vesicles has not yet been established. Synaptic vesicles with different transmitters are ZIO positive. Experiments made in vitro showed that many substances react with ZIO giving a black precipitate. Among them amino acids with –SH groups were amongst the most reactive. This fact, and other considerations[8], led us to think that –SH groups could be responsible of ZIO reaction. To test this hypothesis the effect of –SH reagents on ZIO reaction was studied in the synaptic vesicles of the photoreceptor cells of the rat.

Retinas from adult Wistar rats were quickly dissected after decapitation and incubated in toto at room temperature according to one of the following schedules: 1. in phosphate buffer 0.1 *M*, pH 6.9 for 30 min; 2. in 5 m*M* dithioerythritol (DTE) in the same buffer for 30 min; 3. in 0.1 *M* N-ethyl-maleimide (NEM) in the same buffer for 30 min; 4. incubation for 30 min in medium 2, washing for 5 min in the buffer, incubation for 30 min in medium 3.

After incubation the retinas were washed for 5 min in the phosphate buffer and a few seconds in distilled water to eliminate phosphates and fixed in ZIO at 4 °C for 2 h. After 10 min of fixation the retinas were teased in small blocks in a drop of ZIO. Tissue blocks were prepared for electron microscopy after fixation. Dehydration was done in ethanol and embedding in Epon 812. Ultrathin sections were stained with lead citrate and studied in a Siemens Elmiskop 1 electron microscope. ZIO was prepared as previously described[11].

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