MEMBRANE RECYCLING AND ENDOCYTOSIS IN *PARAMECIUM* CONFIRMED BY HORSE-RADISH PEROXIDASE PULSE-CHASE STUDIES

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SUMMARY

*Paramecium caudatum* cells were pulse-chased in horseradish peroxidase (HRP) using pulses of 30 s to follow endosome (endocytotic vesicle) formation from defaecating digestive vacuoles and 3-min pulses to follow the movement and fate of these vesicles in the cell. Endosomes formed during the 30 s in HRP are heavily labelled with HRP reaction product and are mostly flattened. Some align along microtubules that point toward the cytopharynx. Diskoidal vesicles at the cytopharynx are unlabelled in unchased cells. Cells exposed to a 3-min HRP pulse contain varied amounts of HRP-labelled diskoidal vesicles at the cytopharynx but no labelled vesicles near their closed cytoprocts. Labelled vesicles are also found aligned along the cytopharyngeal microtubular ribbons. Diskoidal vesicles are no longer labelled after a 47-min chase. Use of pulse-chase HRP cytochemistry supports the hypothesis that the membrane of digestive vacuoles retrieved at the cytoproct is moved along microtubular ribbons directly to the cytopharynx where the membrane enters the diskoidal vesicle pool. This membrane appears to enter neither the Golgi nor lysosomal systems in its passage. HRP reaction product is also found in vesicles near the parasomal sacs, in some secondary lysosomes and in small spherical vesicles that are probably trichocyst membrane fragments. Possibly some membrane from parasomal sacs or condensing digestive vacuoles may also enter the diskoidal vesicle pool but apparently not membrane from trichocysts.

INTRODUCTION

Previous electron-microscopic studies led us to believe that the membrane of digestive vacuoles in *Paramecium caudatum* is recycled and used again to form at least part of the membrane of new digestive vacuoles. This belief was based on a timed study of membrane retrieval at the cytoproct (cell anus) of *Paramecium* (Allen & Wolf, 1974) and on the observation that diskoidal vesicles which had previously been observed to accumulate at the cytopharynx (Jurand & Selman, 1969; Pitelka, 1969) were seen to fuse with the cytopharyngeal membrane, thus providing new membrane for the growing nascent food vacuole (Allen, 1974). In addition, some 40 ribbons of microtubules arise at the left side of the cytopharynx and fan out into the endoplasm, where some pass toward the cytoproct (Allen, 1974). Diskoidal vesicles become firmly attached to these ribbons (Allen, 1975) and are apparently guided by them toward the cytopharynx. The motive force for this movement is not known. In our initial studies...
it was postulated that the tubular endosomes (endocytotic vesicles) retrieved at the cytoproct become transformed into diskoidal vesicles which are then recycled as new digestive vacuole membrane.

The weakest link in this recycling model was the lack of direct evidence for the transformation of the endosomes, retrieved at the cytoproct, into diskoidal vesicles prior to or at the time of their being shuttled back to the cytopharynx. Other possibilities, that the diskoidal vesicles originate from condensing digestive vacuoles, from endocytosis at parasomal sacs (indentations of the plasma membrane next to basal bodies) or from the Golgi apparatus, could not be ruled out. The fate of the endosomes derived from the defaecated vacuoles could also have been different from that postulated. In higher animal cells endosomes generally pass either to the lysosomal system, where their contents are digested (Silverstein, Steinman & Cohn, 1977), or they pass to the Golgi apparatus (Farquhar, 1978) for processing before their membrane again enters the transitional vesicle membrane pool. Entering this pool may be an intermediate step in the return of this membrane once again to the plasma membrane.

Our present study was designed to investigate the hypothesized connexion between the endosomes at the cytoproct and the diskoidal vesicles at the cytopharynx. Using the cytochemical tracer, horseradish peroxidase (HRP), we show that the endosomes at the cytoproct contain HRP reaction product and that with pulse-chase studies the reaction product can be found in the diskoidal vesicles aligned along the cytopharyngeal microtubular ribbons and finally at the cytopharynx. We believe this confirms our hypothesis that membrane of old digestive vacuoles is directly reutilized to form at least some of the membrane of new digestive vacuoles.

**MATERIALS AND METHODS**

*Experimental procedure*

*Paramecium caudatum* was cultured axenically on medium described in Fok & Allen (1979). Cells used in this study were in mid-log phase, about 4-days post-inoculation. In the 3-min pulse experiments cells were placed in a medium containing polystyrene latex spheres (PLS) 0.9μm in diameter (Dow Chem. Co., St. Louis, MO.) This marker medium was prepared by adding one drop of PLS to 1.5 ml of axenic culture fluid and sonicating to disperse the PLS. 0.5 ml of culture fluid containing 0.0125 g HRP was then added to the above to make a total of 2 ml. This marker medium was placed on a depression slide and about 1 ml of cell suspension was added to it. At the end of 2.5 min the cells were collected in a micropipette and at exactly 3 min a drop of these cells was placed in fixative (0 min chase), while the remainder were chased in fresh axenic culture medium without PLS for 4, 27 and 47 min.

In the experiments designed to follow the uptake of HRP at the cytoproct, cells were first incubated for 30 min in axenic culture medium containing PLS, 0.9μm in diameter, so that most, if not all, of the digestive vacuoles would contain PLS which make the vacuoles visible under the dissecting microscope. Toward the end of this 30 min incubation individual cells were transferred to a depression slide and about 1 ml of cell suspension was added to it. At the end of 2.5 min the cells were collected in a micropipette and at exactly 3 min a drop of these cells was placed in fixative (0 min chase), while the remainder were chased in fresh axenic culture medium without PLS for 4, 27 and 47 min.

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Glutaraldehyde-fixed cells were incubated in a solution containing H2O2 and 3, 3'-diaminobenzidine (DAB, Sigma Chem.
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Co., St. Louis, MO.) according to the procedure of Graham & Karnovsky (1966). Control cells were handled identically except that the cells were not incubated in the marker medium.

Preparation for electron microscopy

Cells were fixed for 15 or 30 min in 1 or 2 % glutaraldehyde buffered in 0-05 or 0-1 M cacodylate, pH 7-2. The cells were then washed in the same buffer and incubated in DAB medium. Following incubation, the cells were postfixed in 1 % OsO₄ buffered in 0-05 M cacodylate, pH 7-2, for 30 min at room temperature. The cells were then washed in distilled water and preincubated with either 1 % aqueous uranyl acetate (UA) or 0-5 % UA in 50 % ethanol. Dehydration was carried out only in a graded series of ethanol since propylene oxide tends to dissolve the PLS. Cells were embedded in Epon 812.

Individual cells were serially sectioned in the longitudinal direction to ensure that all sites of endocytosis could be detected and the presumptive movements of the various endosomes could be followed. Sections were collected on Formvar-coated 1-hole grids. The sections were observed unstained or after post-staining with 0-5 % uranyl acetate in ethanol and with 0-2 % lead citrate. A Hitachi HU-11A electron microscope operated at 75 kV was used.

RESULTS

Our study consisted of two sets of experiments. In the first set a short (30 s) pulse of HRP was given to individual Paramecium cells after they were observed under the dissecting microscope to be ready to excrete a digestive vacuole. The second set of experiments consisted of placing cells in HRP for 3 min after which the cells were either fixed or transferred by pipette to fresh medium for various chases. The formation and disposition of HRP-containing vesicles within the cell could be followed by using these pulse-chase studies of individual cells.

HRP uptake at the cytoproct

Defaecation in Paramecium caudatum is rapid and the cytoproct is closed within 30–60 s of opening (Allen & Wolf, 1974). During this short period all of the digestive vacuole membrane is retrieved by endocytosis; this process is probably mediated by the layer of microfilaments located on the cytoplasmic surface of the membrane of the defaecated vacuoles (Allen & Wolf, 1974). In the present study cells which defaecated during or just prior to the 30-s exposure to HRP and which were fixed without further chase contain a large mass of endosomes just inside the closed cytoproct (Fig. 1). These endosomes are labelled with reaction product following the incubation of the cells in DAB. These endosomes may be tubular, cup-shaped or diskoidal but the one characteristic which they have in common is that there is very little luminal space in all but a few of them. The outside width of the flattened endosomes or tubular diameter is usually around 60 nm and the lumen is filled with electron-opaque HRP reaction product.

Filamentous material is found interspersed between the endosomes. Microtubules are seen to pass through the mass of vesicles and filaments (Fig. 1). These microtubules, for the most part, arise from the basal bodies adjoining the cytoproct as well as from the top of the cytoproct ridge (Allen & Wolf, 1974). However, some of the microtubules may have their origin at the cytopharynx.

The microtubules are usually organized in bundles or ribbons and invariably angle
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away from the cytoproct in an anterior direction aligned along the cytopharynx-to-cytoproct axis. Even after only 30 s of HRP exposure, followed by immediate fixation, some HRP-containing endosomes are already associated with microtubules in a linear fashion near the cytoproct end of the cytopharynx-to-cytoproct axis (Fig. 2). These vesicles, although flattened, have a rather irregular shape rather than the uniformly regular diskoidal shape exhibited by the pool of vesicles at the cytopharynx.

When the cytopharynx of the 30-s exposed cell was observed no diskoidal vesicles with HRP reaction product could be found. Nor did scanning the rest of the cell reveal HRP-labelled diskoidal vesicles originating from other sites. Two cells were sectioned which had been exposed to HRP for 30 s and then chased for 90 s before fixation. Apparently the cytoproct had not opened in either cell during the critical 30-s period since neither cell had HRP-containing endosomes in the vicinity of the cytoproct or the cytopharynx (Fig. 3). However, these cells do contain HRP reaction product in some lysosome-like bodies (Fig. 4) and in a few small vesicles lying near the parasomal sacs both at the cells’ surfaces and next to their buccal cavities (similar to those in Fig. 7).

One cell fixed 3 min following the 30-s period in HRP had apparently not defaecated either, since there are no HRP-containing vesicles at the cytoproct and only a very few HRP-containing vesicles at the cytopharynx (Fig. 5). However, the fact that a few diskoidal vesicles contain reaction product suggests that a small percentage of the labelled diskoidal vesicles may have an origin other than the cytoproct. A few lysosomes are also labelled in this cell.

**HRP uptake and distribution following 3-min exposure**

0-min chase. All cells which had been exposed to HRP for 3 min but not chased contain many labelled diskoidal vesicles. In one cell fixed immediately after the 3-min HRP exposure, a large accumulation of HRP-containing vesicles along with a large HRP-containing remnant of the digestive vacuole are found just under the closed cytoproct (Fig. 6; serial sections confirmed the cytoproct was closed). Some labelled diskoidal vesicles are found along microtubular ribbons and at the cytopharynx. HRP is also found in vesicles near parasomal sacs (Fig. 7) and within digestive vacuoles which had been formed during the 3-min incubation in HRP (Fig. 8). These vacuoles also contain latex spheres which were used as additional markers, since the activity of HRP in digestive vacuoles seems to be short-lived. Not all vacuoles laden with latex spheres contain HRP reaction product, indicating that the activity of the HRP is quickly lost. Very young digestive vacuoles which contain latex marker show some

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Fig. 1. HRP-labelled vesicles next to the cytoproct (c) of *P. caudatum* fixed after being exposed to HRP for 30 s, 0 chase. The flattened and tubular vesicles are remnants of the defaecated digestive vacuole membrane. Microtubules (arrows) pass through the vesicle mass. × 35 000.

Fig. 2. HRP-labelled vesicles of irregular shape aligned along microtubules in the cytoproct-to-cytopharynx axis in the same cell as in Fig. 1. × 35 000.

Fig. 3. Diskoidal vesicles at the cytopharynx unlabelled with HRP reaction product. Cell exposed to HRP 30 s and chased 90 s. × 25 000.
Fig. 4. Some secondary lysosomes contain HRP label in cells exposed to HRP 30 s and chased 90 s before fixation. They lie next to a digestive vacuole. × 22000.

Fig. 5. Cell exposed to HRP for 30 s and chased for 3 min contained only a few labelled diskoidal vesicles (arrows) at the cytopharynx and none at the cytoproct (not shown). cc, cytopharyngeal cord. × 35000.

Fig. 6. HRP-labelled vesicles and a remnant of a digestive vacuole next to the cytoproct (c) in a cell exposed to HRP 3 min with no chase. Serial sections showed the cytoproct was closed which may have resulted prematurely before all the membrane was endocytosed. × 16000.

Fig. 7. HRP-labelled vesicles also lie next to a row of parasomal sacs (ps). Cell exposed to HRP for 3 min with 0 min chase. × 40000.

Fig. 8. Digestive vacuoles formed during a 3-min exposure to HRP and latex spheres (l) usually contain HRP label and latex. Evaginations arising from these vacuoles are also labelled. × 30000.
large invaginations of their membranes into the cytosol (Fig. 8). These invaginations also contained HRP.

4 min chase. Cells fixed after a 4-min chase contain no labelled vesicles near the cytoproct but have a large number of labelled diskoidal vesicles at the cytopharynx and along the cytopharyngeal ribbons, particularly in the vicinity of the cytopharynx (Fig. 9). These labelled diskoidal vesicles are dispersed randomly amongst unlabelled ones. Reaction product is also found in vesicles near parasomal sacs and in a small number of lysosomes.

In addition, cells of this time period have a very large number of HRP-labelled small spherical vesicles (as in Fig. 12) found in a layer at a level usually occupied by the proximal ends of trichocysts, except that in these cells the trichocysts had been discharged. These are presumably the remnants of trichocyst membranes which are also known to be retrieved following trichocyst discharge (Hausmann & Allen, 1976). In these cells the trichocysts apparently were discharged during the 3-min exposure to HRP and, since the fusion site at the plasma membrane is very rapidly sealed off following discharge, HRP was trapped in the retrieved vesicles.

18–27 min chase. Cells chased for a longer period following HRP exposure are all alike in that they have no labelled vesicles near the cytoproct but all have a varied number of diskoidal vesicles containing HRP reaction product at the cytopharynx (Fig. 10). The cytopharyngeal ribbons are typically associated with labelled vesicles, particularly near the cytopharynx (Fig. 11).

During this time HRP reaction product is also found in vesicles near parasomal sacs, in the presumed trichocyst membrane vesicles (Fig. 12) and in some secondary lysosomes (Fig. 13) that contain paracrystalline material (Fok & Allen, 1979). However, none of these types of vesicles accumulates predominantly at the cytopharynx, as far as we can tell.

Latex-containing digestive vacuoles at these chase times which were formed during the 3-min exposure to HRP, never contain reaction product.

47 min chase. By 47 and 50 min the HRP-labelled diskoidal vesicles have disappeared from the cells. HRP reaction product can still be found in clumps of presumed trichocyst membrane but not in other endosomes or in diskoidal vesicles at the cytopharynx (Fig. 14). Cells at all times may have an electron-opaque reaction product in the cristae of mitochondria (Fig. 9) and in peroxisomes due to the presence of endogenous peroxidatic enzymes in these organelles.

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Fig. 9. A mixed population of HRP-labelled and unlabelled diskoidal vesicles at the cytopharynx in a cell exposed to HRP for 3 min and chased 4 min. A nearby mitochondrion contains endogenous peroxidatic activity in its cristae. × 25,000.

Fig. 10. HRP-labelled diskoidal vesicles at the site of digestive vacuole membrane growth, the left cytopharyngeal lip. 3 min HRP exposure followed by 18 min chase. × 16,000.

Fig. 11. Both HRP-labelled and unlabelled diskoidal vesicles are aligned along the cytopharyngeal ribbons. 3 min HRP exposure, 18 min chase. × 30,000.
Fig. 12. Clumps of small HRP-labelled vesicles are associated with a filamentous cytosol. The vesicles are presumably the remnants of trichocyst membrane. They lie close to dictyosomes of the Golgi apparatus (brackets). 3 min HRP exposure, 27 min chase. × 50,000.

Fig. 13. HRP-labelled lysosome from cell exposed to HRP 3 min and chased 27 min. The lysosome contains an HRP-labelled paracrystalline array and a luminal membrane coat showing periodic globules. × 50,000.

Fig. 14. Diskoidal vesicles at the cytopharynx of cells exposed to HRP for 3 min and chased 47 min are completely unlabelled. × 25,000.
Control cells

Cells incubated in DAB which have not been placed in HRP-containing axenic medium sometimes contained reaction product in the cristae of mitochondria and in peroxisomes but not in the lysosomal system, parasomal sacs or presumptive trichocyst vesicles. This reaction product in control cells is due to endogenous peroxidatic enzymes.

Discussion

Direct recycling of digestive vacuole membrane

The fate of endosome membranes which have been retrieved from the plasma membrane varies, depending on the cell type. Some endosome membrane seems to be recycled. The membrane may be recycled (a) by first fusing with lysosomes, (b) after fusing with the Golgi cisternae or Golgi vacuoles before becoming part of an exocytic vacuole, or (c) directly. In the last case the endosomes become exosomes without first becoming part of another organelle system such as the Golgi apparatus or lysosomal membrane system. Endosomes are known to fuse with lysosomes in many cell systems (Holtzman et al. 1973; Steinman & Cohn, 1972; Steinman, Silver & Cohn, 1974) and it has been proposed that parts of the membrane originating from the endosomes may be pinched off either before or following fusion with lysosomes and shuttled back to the plasma membrane (Dean, 1977; Silverstein et al. 1977). Fusion of endosomes with the Golgi apparatus has also been documented (Pelletier, 1973; Gonatas et al. 1975; Farquhar, Skutelsky & Hopkins, 1975; Herzog & Farquhar, 1977; Farquhar, 1978). Presumably this membrane is then differentiated and used in packaging proteins for export. The idea of direct recycling of membrane, where an endosome is taken into the cell at one point and moved through the cell to another point where it again becomes part of the surface membrane by exocytosis, has had little experimental or morphological evidence to support it. However, this latter method seems to be occurring in Paramecium (Allen, 1974) and may also occur in other ciliates such as Tetrahymena (Nilsson, 1976; Allen & Wolf, 1979), although the membranes of these cells have not been studied as extensively as Paramecium (Allen, 1978).

The present study confirms the fact that the membrane of defaecated digestive vacuoles is retrieved at the cytoproct by endocytosis. Cells that have expelled a digestive vacuole during a 30-s exposure to HRP contain a mass of labelled endosomes under the closed cytoproct. These vesicles are not all diskoidal in shape but they all contain a minimum of luminal space; they are tubular or flattened and their lumens are full of reaction product. They apparently contain only a glycanalyx which lines the luminal surface of the membrane. A few of these labelled vesicles are already aligned along microtubules in the cytopharynx-to-cytoproct axis but none of the diskoidal vesicles at the cytopharynx is labelled. Cells that have been incubated 3 min in HRP followed by a chase of 27 min or less all have labelled diskoidal vesicles at the cytopharynx and along the cytopharyngeal ribbons. The presence of labelled vesicles at
the cytoproct seems to depend on how recently defaecation has occurred. Complete removal of vesicles from the cytoproct zone seems to be quite rapid, occurring in less than 3 min. Cells that had been chased following the 3-min exposure to HRP never have HRP-labelled vesicles close to the cytoproct.

This study thus confirms our previously proposed model (Allen, 1974) that the membrane vesicles retrieved at the cytoproct are transformed into a flattened disk shape and are almost immediately linked to microtubular ribbons. In a short time, there is a transfer of these labelled vesicles along the microtubular ribbons from the cytoproct to the cytopharynx. This type of membrane reutilization can be termed direct recycling since there is no evidence that retrieved membrane passes first to the lysosomal or Golgi systems on its way back to the cytopharynx. The endosomes seem to be reutilized without extensive modification. However, modification at the molecular level is presumably possible. These diskoidal vesicles form a membrane pool from which new digestive vacuole membrane is derived. However, after freeze-fracturing the membranes of the diskoidal vesicles as well as the nascent vacuoles have a very different appearance from that of the membranes around actively digesting vacuoles (Allen, 1978). Membrane modification seems to be possible at several stages as the vacuoles age. This modification seems to involve membrane removal and insertion by a series of fusion events occurring at the vacuole membrane. In the end the membrane retrieved from the old vacuole must be returned to its original freeze-fracture appearance.

Accounting for other HRP-labelled vesicles

HRP seems to be taken into Paramecium caudatum by at least 3 routes separate from the cytoproct. The first of these is at the parasomal sacs, the shallow indentations of the plasma membrane associated with basal bodies in both the somatic and oral regions of the cell. Parasomal sacs and pellicular pores have been shown to be sites of endocytosis in other ciliates (Noirot-Timothée, 1968; Rudzinska, 1977) and is now confirmed in Paramecium. Parasomal sacs and pellicular pores may also be sites of exocytosis as well (Franke, Eckert & Krien, 1971; Suchard, 1978; Furuya & Allen, 1979).

A second route of HRP uptake is through digestive vacuole formation since very young digestive vacuoles also contain HRP reaction product. Newly formed digestive vacuoles are seen to develop very quickly some characteristic invaginations which would be spherical except that one or more fingerlike indentations penetrate from the wall into the lumen of these invaginations. These invaginations contain reaction product. A few tubular invaginations also arising from these young vacuoles contain HRP reaction product as well. The larger invaginations may be one source of the labelled lysosomes we observed. In addition lysosomes may acquire their HRP by the fusion of unlabelled lysosomes with HRP-containing endosomes of another origin, such as the endosomes from the parasomal sacs. We will deal with the fusion events in digestive vacuole membranes in a subsequent paper.

A third route for HRP uptake other than at the cytoproct appears to occur during trichocyst discharge. This discharge is extremely rapid (Hausman & Allen, 1976). However, the opening of the fusion sites at the cells surface is apparently long enough
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for HRP to get into the empty sac which results from discharge. Further vesiculation of these empty sacs gives rise to a large number of small spherical vesicles which are enmeshed by filamentous material and which accumulate in a zone between the ectoplasm and endoplasm of these cells. Cells which have discharged most of their trichocysts have a much larger number of these HRP-containing vesicles than do cells which still contain most of their trichocysts. Clumps of these vesicles frequently lie close to dictyosomes of the Golgi apparatus which are found in abundance at this exoplasmic-endoplasmic interface. However, no HRP reaction product was seen in the Golgi cisternae so that fusion of these vesicles with some part of the Golgi apparatus has not been demonstrated. Clumps of HRP-labelled vesicles were still present 50 min after exposure of the cell to HRP. Diskoidal vesicles in the same cell were completely unlabelled. This may indicate that the trichocyst membranes do not become part of this pool. Further study will be required to follow the ultimate fate of trichocyst membranes.

Origin of diskoidal vesicles

Do all diskoidal vesicles come from endosomes of egested vacuoles? Our study suggests that, although a large number of diskoidal vesicles have their origin at the cytoproct, not all come from this site. In cells which have not egested a food vacuole during the short 30-s exposure to HRP, as judged by the absence of large numbers of HRP-labelled vesicles in the cytoproct-to-cytopharynx axis, a few labelled diskoidal vesicles can still be found at the cytopharynx following a 3-min chase. These diskoidal vesicles may arise either as a result of endocytosis occurring at the parasomal sacs, since these endosomes frequently appear flattened, or as a result of the removal of membrane from condensing digestive vacuoles, a process which occurs within the first few minutes after vacuole formation (Alien, 1978). It would appear that only the first endosomes removed from a condensing vacuole will contain active HRP, since the HRP is quickly inactivated in these vacuoles. Tubular evaginations of very young vacuoles have been observed to contain HRP. Like the endosomes at the cytoproct these vesicles may be capable of shape transformation and recycling to the cytopharynx. This would account for the few labelled diskoidal vesicles found at the cytopharynx in cells chased briefly but which apparently have not defaecated a vacuole in the presence of HRP. Membranes of young digestive vacuoles are identical in freeze-fracture appearance to diskoidal vesicles (Alien, 1978). The structural similarity of these 2 membrane pools may facilitate membrane fusion and exchange. On the other hand, the apparent dissimilarity of parasomal sac membrane from the diskoidal vesicle membrane, based on freeze-fracture views of the plasma membrane, with which parasomal sacs are continuous, as well as of fractured parasomal sacs, raises the possibility that they may not usually find their way to the cytopharynx to enter the digestive vacuole membrane system. However, on the basis of the present results, the transformation of these endosomes into diskoidal vesicles cannot be ruled out.

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