THE MECHANISM OF MITOCHONDRIAL EXTRUSION FROM PHENYLHYDRAZINE-INDUCED RETICULOCYTES IN THE CIRCULATING BLOOD

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ABSTRACT

The mechanism of mitochondrial extrusion from reticulocytes was studied in whole blood from dogs made anemic by treatment with phenylhydrazine hydrochloride. The initial stage of preparation for mitochondrial extrusion was attraction of vesicles to mitochondria. There was subsequent encirclement of the organelle and other bodies, such as ferritin, by coalesced vesicles forming double membrane-limited vacuoles. Large vacuoles were formed from the union of single vacuoles, and they were usually situated near the periphery of the cell. Fusion of the outer membrane of vacuoles with the plasmalemma of the reticulocyte provided a route for exposure and release of mitochondria and other material to an extracellular location. An extracellular mitochondrion, therefore, was confined by its original double membrane, and a third membrane was derived from the internal boundary of vacuoles.

In the process of erythropoiesis, the mammalian erythroblast extrudes its nucleus to become a reticulocyte. Reticulocytes have been reported to be characterized by the presence of ribosomes, ferritin, mitochondria, occasional remnants of Golgi apparatus, and vesicles of rhopheocytosis when observed by the electron microscope (1). Most of these vesicles are of the "coated" or "fuzzy" type (2). Reticulocytes in the peripheral blood are believed to require only a few days to develop into mature erythrocytes, which are devoid of mitochondria and nuclei (3). An explanation of the sequential process of extrusion of organelles from reticulocytes has not been adequately described. The present paper is an effort to describe this mechanism in whole blood from dogs made anemic by treatment with phenylhydrazine.

MATERIALS AND METHODS

Three dogs became anemic as a result of injections with phenylhydrazine hydrochloride. The optimum dosage level for the production of anemia was found to be three subcutaneous injections, administered every other day, at a level of 16 mg/kg of body weight. Blood from such anemic dogs was fixed in 3% glutaraldehyde, postfixed in 1% OsO_4, and embedded in Araldite. Thin sections on grids were stained with uranyl acetate and lead citrate (4) prior to their being examined with a Philips EM200 electron microscope.

RESULTS

In low magnification micrographs (Fig. 1), reticulocytes were seen to contain cytoplasmic vesicles, abundant mitochondria, moderate numbers of free ribosomes, aggregates of ferritin, and micropinocytotic vesicles and invaginations on the plasmalemma. Many of these vesicles and invaginations on the cell membrane were of the fuzzy or coated type. Although both mono- and polyribosomes were present in reticulocytes, it seemed that monoribosomes predominated over polyribosomes. There apparently was a decrease in the number of these cellular particles in the

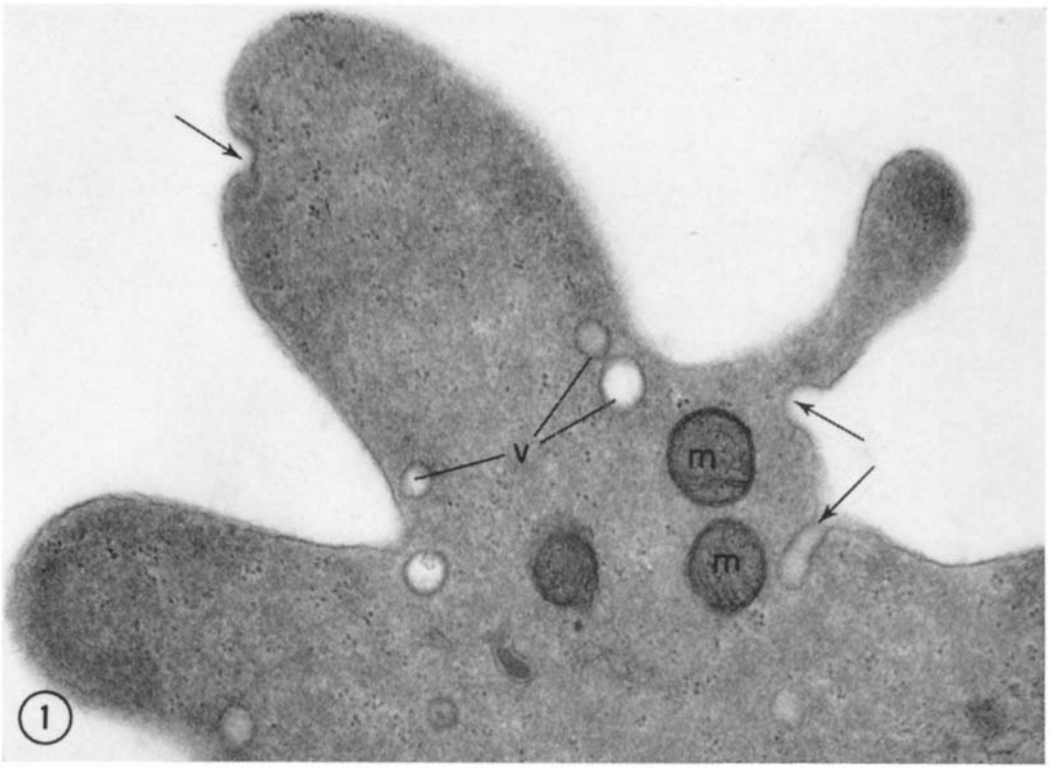
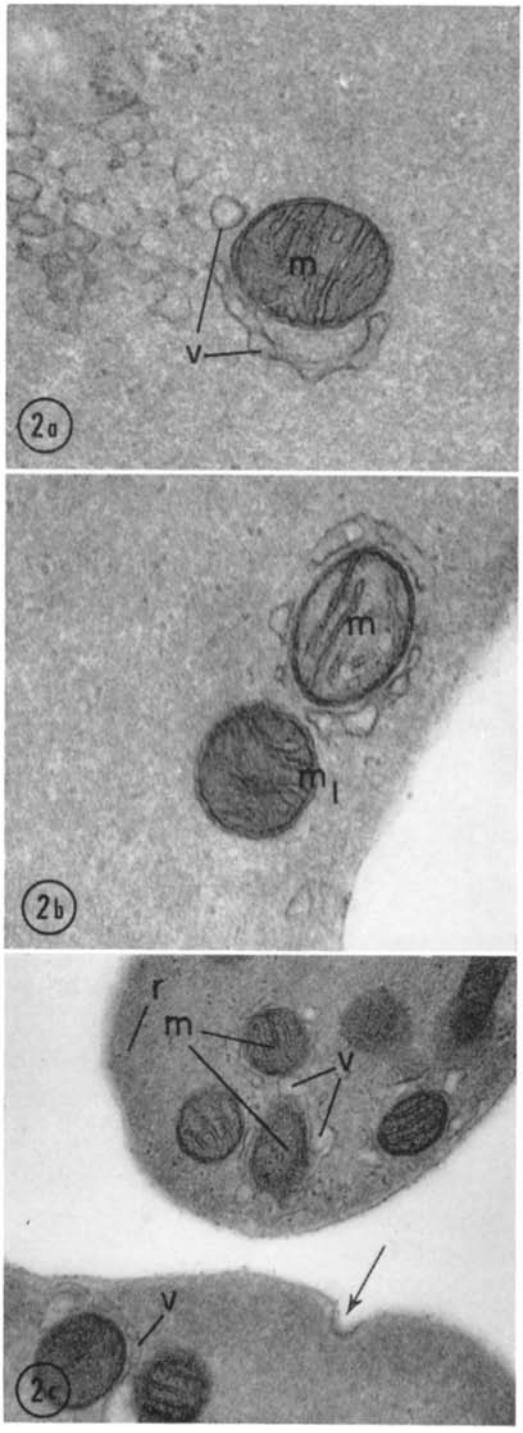


FIGURE 1 Canine reticulocyte. There are mitochondria (*m*) and vesicles (*v*) in the cytoplasm as well as coated invaginations (arrows) on the plasmalemma. × 20,000.

progression of cell maturity from the reticulocyte to the erythrocyte stage.

In micrographs of reticulocytes, mitochondria generally were observed in clusters of two to four, or occasionally more. These organelles were limited by a double membrane, and lamellar folds of cristae mitochondriales completely transversed the body of the organelle. Usually cytoplasmic vesicles were seen in close proximity to mitochondria (Fig. 2). Such vesicles were of various sizes and shapes, from round to oval to elongated. Usually chained together, vesicles encircled varying portions of the periphery of mitochondria (Fig. 2). By such a process, a series of vesicles seemed ultimately to completely surround the organelles. There was a definite impression that large vesicles had formed from the coalescence of smaller ones (Fig. 2). After mitochondria had become completely surrounded by a series of abutted vesicles, there was a generalized compression of vesicles along with fusion of membranes of adjacent ones at the polar regions of each. As a result of this process, mitochondria were confined by inner and outer vesicular membranes which were superimposed over the double limiting membranes of the mitochondria. The inner vesicular membrane often, but not consistently, enveloped the entrapped mitochondria rather intimately, although a layer of hemoglobin sometimes was interposed between the outer mitochondrial and inner vesicular membranes. Free vesicles in the vicinity of the encircled mitochondria often fused with the outer vesicular membrane, and then ballooned the external vesicular membrane outward (Fig. 3). This often resulted in a prominent separation of the internal and external vesicular membranes. Usually a single mitochondrion was subjected to encirclement by vesicles, but occasionally up to three or more neighboring mitochondria appeared to be entrapped by a series of irregularly arranged and abutted vesicles (Fig. 4). Encirclement of more



than one organelle by a common external vesicular membrane also occurred as a result of fusion of the limiting boundaries which surrounded previously encircled mitochondria. When one or more bodies had become limited by a distinct and complete outer vesicular membrane, the resulting structure was designated as a mature vacuole or an inclusion (Fig. 5). Mitochondria within vacuoles often appeared to have undergone various stages of degeneration. This conclusion seemed warranted because the organelles were more electron opaque than typical mitochondria, and they did not contain well-defined cristae. Mitochondria often contained various amounts of ferritin (Fig. 6).

Vacuoles contained structures other than mitochondria. Masses of hemoglobin, unlimited by membranes, and ribosome-like granules also accumulated in vacuoles (Fig. 7). In addition, membrane-bounded aggregates of ferritin were observed in vacuoles (Fig. 8).

Heavily populated mature inclusions containing one or more mitochondria and other bodies generally were observed close to the plasmalemma of the reticulocyte (Fig. 9). This peripheral location apparently was the site from which materials were released from reticulocytes. Just prior to extrusion, it seemed that the outer vesicular membrane of a vacuole united with the cell membrane of the reticulocyte (Fig. 10). This process of membrane fusion apparently provided a mechanism for exposure and release of mitochondria and other bodies within vacuoles to an extracellular position, without causing disruption of the plasmalemma of the involved reticulocyte. Thus, when extracellular mitochondria were observed in pockets of the cell membrane of the reticulocyte, they were confined by the original double mem-

FIGURE 2 Vesicles are present in proximity to mitochondria in a reticulocyte. *a*, coalesced and single vesicles (*v*) partially surround a mitochondrion (*m*). × 35,000. *b*, one mitochondrion (*m*) is almost completely encircled by vesicles. Another mitochondrion (m_1) is unassociated with vesicles. × 35,000. *c*, the lower reticulocyte has a fuzzy invagination of the plasmalemma (arrow). Both mitochondria in the cell are partially encircled by vesicles (*v*). In the upper reticulocyte, two mitochondria (*m*) in a nest of organelles are apparently being entrapped by a series of vesicles (*v*). Free ribosomes (*r*) are also evident. × 20,000.

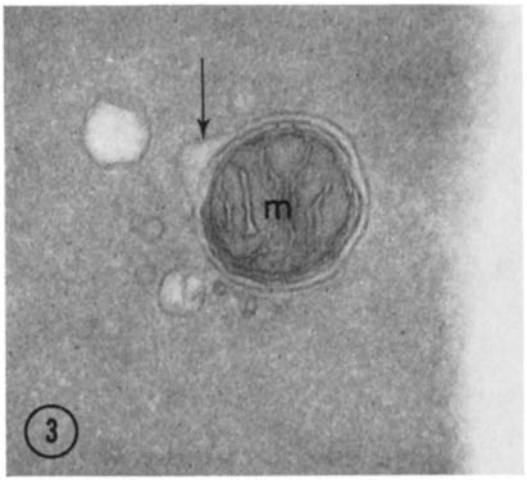


FIGURE 3 A mitochondrion (*m*) is almost encircled by a series of coalesced vesicles; another vesicle (arrow) has fused with the outer vesicular membrane to balloon it outward. × 35,000.

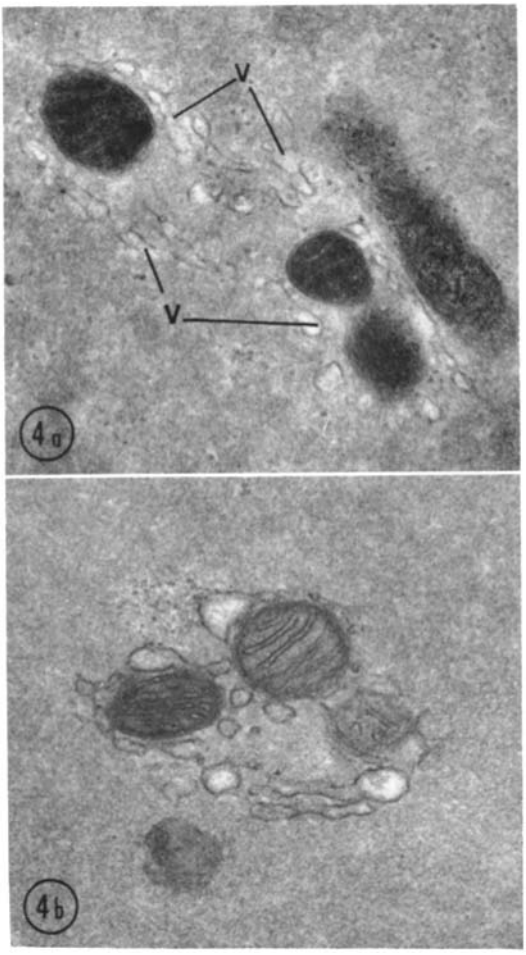


FIGURE 4 *a*, a series of vesicles (*v*) appear to be surrounding three mitochondria. × 20,000. *b*, vesicles of various sizes and shapes have almost encompassed three mitochondria. × 20,000.

brane and a third membrane derived from the internal boundary of coalesced vesicles which originally surrounded them (Fig. 11). In the same way, small portions of hemoglobin and ferritin granules became extracellular.

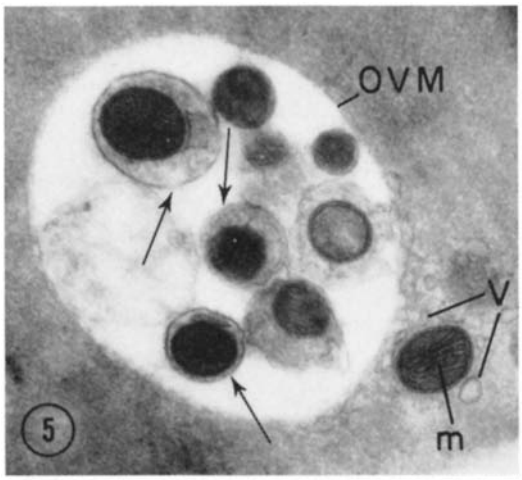
DISCUSSION

The fine structure of reticulocytes has been described (1, 5–8), but a sequential mechanism for the loss of mitochondria from reticulocytes in the process of maturation to erythrocytes has not been explained.

Vacuoles have been described in human reticulocytes, some of which were induced by splenectomy (9); it was suggested that these inclusions were probably instrumental in the disposal of materials from the cells. The inclusions were membrane-enclosed and contained mitochondria, ribosomes, smooth membranes, hemoglobin, and ferritin. The vacuoles generally were located close to the cell membrane of the reticulocyte. Since the reaction product for acid phosphatase activity occasionally was demonstrated in such vacuoles, they were designated as cytolysosomes or autophagic vacuoles.

With few exceptions, vacuoles in the reticulocytes of dogs, as described in this paper, and humans (9) seem to be very similar except that ferritin-laden mitochondria were seen in vacuoles of canine reticulocytes but not in the human cell. The presence of ferritin particles in the dog reticulocyte mitochondria is understandable because phenylhydrazine causes intravascular hemolysis.

An explanation for the development of vacuoles in reticulocytes has not been set forth (9). In the work reported herein, a possible mechanism is advanced for the formation of vacuoles in reticulocytes. This process involves the encirclement of organelles by the fusion of membranes of many cytoplasmic vesicles. Apparently, such cytoplasmic vesicles are derived from pinocytotic vesicles on the plasmalemma of reticulocytes



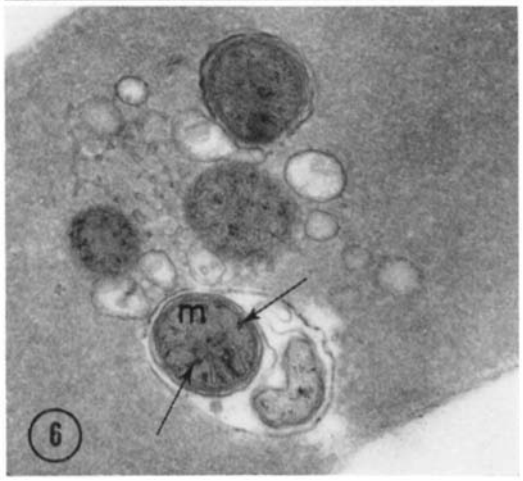
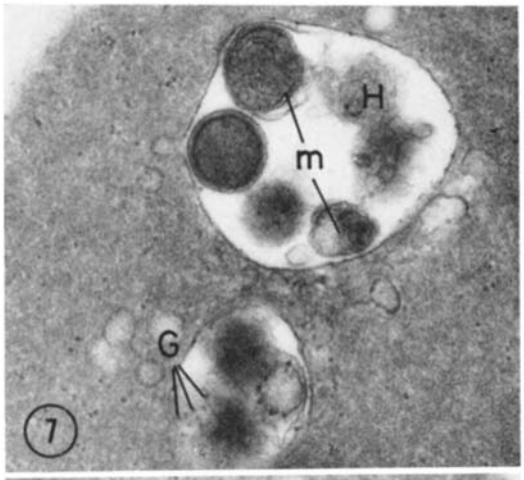


FIGURE 5 A mature vacuole is limited by an uninterrupted outer vesicular membrane (*OVM*). The inner vesicular membranes (arrows) surround each organelle. Probably another mitochondrion (*m*), after being encircled, will be added to the vacuole by fusion of vesicular membranes (*v*). × 15,000.

FIGURE 6 A mitochondrion (*m*) in a vacuole contains electron-opaque granules (arrows), probably ferritin. × 20,000.

(Fig. 12). Attention has been directed to the rich vesicular network in reticulocytes (8), and it has been postulated (8, 10) that such a vesicular network may result from the coalescence of many small vesicles.

It is suggested from the appearance of micrographs in this paper that vacuoles, or inclusions, are utilized by reticulocytes as an innoxious method by which mitochondria and various other cellular products are discarded by the cell in the final stage of red cell maturation. Release of the contents of vacuoles to an extracellular location involves fusion of the external membranes of vacuoles with the plasmalemma of the involved reticulocyte. As a result, extracellular mitochondria are limited by a trilaminar membrane, the two inner ones being an integral component of mitochondria, and the external one being derived from the in-



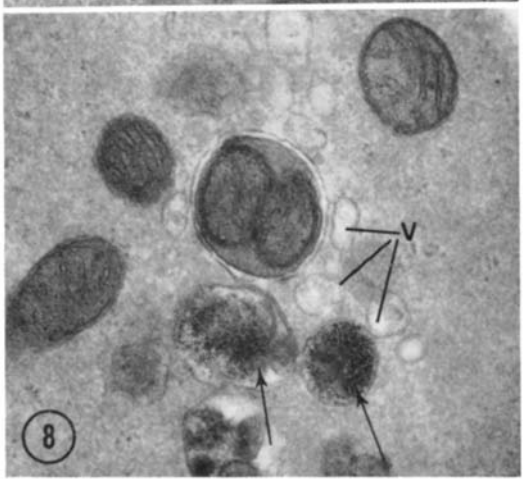
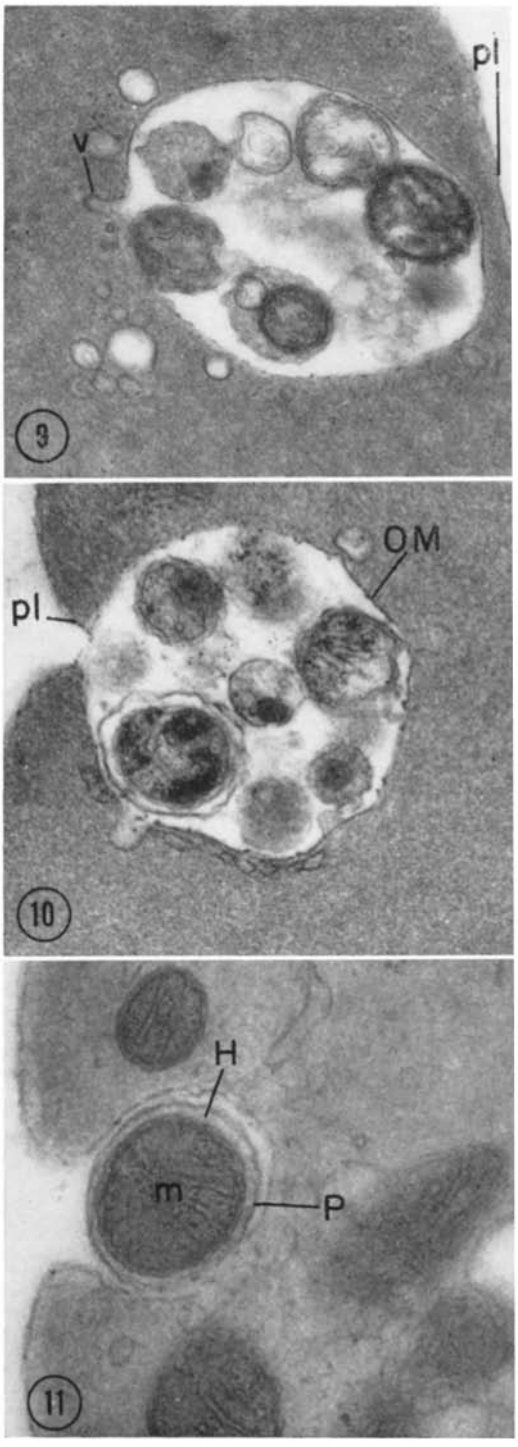


FIGURE 7 Material that appears to be nonmembrane-limited hemoglobin (*H*) is present in a vacuole, along with mitochondria some of which (*m*) appear to be degenerated. Ribosome-like granules (*G*) are present in the lower vacuole. × 20,000.

FIGURE 8 Two vacuoles contain ferritin (arrows). Notice the large, coalesced vesicles (*v*) in the area. × 20,000.



ternal membrane of the vacuole. Whether this same mechanism of extrusion occurs with mitochondria produced physiologically is not determined in the present work.

The reticulocyte diminishes in size as it matures into an erythrocyte (6). The role of pinocytotic vesicles derived from the plasmalemma in the formation of membranes surrounding vacuoles could account for diminution of cell size in maturation of erythropoietic cells. There was partial compensation for the loss of plasmalemma which resulted from the formation of pinocytotic vesicles because the outer limiting membranes of vacuoles are returned to the plasmalemma of erythropoietic cells when the components of vacuoles assume an extracellular location.

Rough-surfaced endoplasmic reticulum has been described in mouse reticulocytes (8), and remnants of Golgi apparatus have been noted in mouse reticulocytes (1). Neither of these organelles was observed in canine reticulocytes.

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FIGURE 9 A complex inclusion is located close to the plasmalemma (*pl*) of the reticulocyte. A vesicle (*v*) has fused with the outer limiting membrane of the inclusion. × 20,000.

FIGURE 10 The outer membrane (*OM*) of an inclusion has fused with the plasmalemma (*pl*) of the reticulocyte. The contents of the inclusion are free to enter the extracellular medium. × 20,000.

FIGURE 11 A free mitochondrion (*m*) in a pocket of the plasmalemma of a reticulocyte is surrounded by its double membrane, a layer of hemoglobin (*H*), and a third membrane (*P*) derived from the internal vesicular membrane. × 35,000.

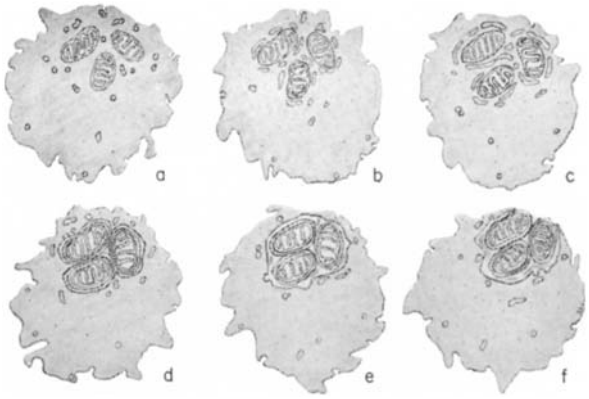

FIGURE 12 Schematic drawing indicating the possible mechanism of mitochondrial extrusion from reticulocytes. *a*, attraction of vesicles to mitochondria; *b* and *c*, partial encirclement of mitochondria by vesicles; *d*, complete encirclement of three mitochondria, so that each is surrounded by internal and external vesicular membranes; *e*, fusion of the limiting boundaries surrounding previously encircled mitochondria, with the formation of an inclusion containing three mitochondria; *f*, extracellular mitochondria are confined by their original double membrane and a third membrane derived from the internal vesicular membrane.

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