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ULTRASTRUCTURAL ASPECTS OF MATURE *CYPRINUS CARPIO* ERYTHROCYTES. *

ADOLPHO BRUNNER JR., JOSÉ RAFAEL R. COIRO, CLARA Y. MITSUTANI, MARIA ANGÉLICA S. CARVALHO DOS SANTOS and HÉRCULES MENEZES
Laboratory of Electron Microscopy, Instituto Butantan

ABSTRACT: Mature *Cyprinus carpio* erythrocytes show a phenomenon of Feulgen positive vesicle formation, begining from a mitochondrion juxtaposed at the nuclear membrane. While condensed chromatin enters the mitochondrion, modifications occur in the lamellar inner structure of the organelle, giving rise to a vesicle which detaches from the nucleus and displaces itself through the cytoplasm. Some aspects suggest that the vesicle content is expelled from erythrocytes. It seems that this is a general event for other mature nucleated vertebrate erythrocytes. Other vesicles of unknown origin, some originating from degenerated mitochondria, and others from the nuclear membranes, were found.

A dense amorph material, never found in erythrocytes of other vertebrates, was frequently present in the nucleoplasm, and in the cytoplasm associated to the smooth endoplasmic reticulum. Marginal bands, thinner than those found in erythrocytes of other groups, were observed.

UNITERM: Ultrastructure of *Cyprinus carpio* erythrocytes.

INTRODUCTION

Information is scarce on the cytology of mature fish erythrocytes, especially at the ultrastructural level. Concerning the mature erythrocytes of other vertebrates, except mammals, there are only a few interesting references with regard to the ultrastructure of mature chicken [6,9], bothropic species [14], amphibians [5,6,10,19,21] and toadfish [10] erythrocytes.

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Address: Caixa postal 65 - S. Paulo - Brazil

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The structural components of mature erythrocytes differ from group to group, depending on the physiological behaviour of the respective specimens. However, such components as the marginal band [6, 9, 10, 14, 19, 21], and vesicles carrying chromatinic material [2,5,6,14], are common occurrences in all mature nucleated erythrocytes.

This paper reports on the ultrastructure of mature *Cyprinus carpio* erythrocytes in comparison with findings in other nucleated erythrocytes.

MATERIAL AND METHODS

Blood samples were obtained by cardiac puncture from adult specimens free of hemoparasites, without the use of anticoagulants, or using 10% v/v of 2% EDTA solution adjusted to pH 7.3 — 7.4 by the addition of a 4% $NaHCO_3$ solution, resulting in a final concentration of 0.15% anticoagulant.

Rosenfeld staining

This staining according to Rosenfeld [17], has been employed for the evaluation of the general hematological aspects, and to assure the absence of parasitism.

Supravital staining

One drop of blood was added to 10 drops of a 0.65% NaCl solution, containing 0.1% brilliant cresyl blue, to evaluate the proportion of erythrocytes with basophilic reticulum.

Feugen reaction

In an attempt to verify whether the erythrocyte vesicle content could be in part DNA, thin blood smears were submitted to the reaction according to Lison [12]. Control smears were previously treated by 0.5% DNase in O.1M phosphate buffer (pH7.2), for 50 min at 37°C, and by the buffer only, under the same conditions.

Electron microscopy

Thin blood smears were submitted to hemolysis after drying for 4-6 h under environmental conditions, in a 0.80% NaCl solution containing 2.5% v/v concentrated formalin [3], or a 2.5% v/v aqueous glutaraldehyde solution [8], followed by phosphotungstic acid staining, and several washings in distilled water [3]. Some hemolysed smears were then covered by a thin palladium film at a 15.°-20.° shadow-casting angle.

For thin sectioning, fixation of the blood was performed as follows [1]: to 15 drops of blood, 15 drops of 2% glutaraldehyde in 0.20M Millonig's buffer [15] were added, drop by drop, each of which followed by slow agitation; after 30 min, the suspension was diluted with 2-3 volumes of 1% glutaraldehyde in the buffer, and fixed for 2 h. Erythrocytes were washed three times in the buffer, and fixed for 20 min in 1% osmium tetroxide in the same buffer. After staining in an 1% aqueous uranyl acetate solution for 30 min, erythrocytes were dehydrated in the alcohol series, and embedded in Polylite 8001-P [7]. Thin sectioning was done in MT-1 and MT-2 Porter-Blum microtomes; the sections were stained by lead citrate [16].

All preparations were examined in the ÜM 100b and Elmiskop I electron microscopes, at 60 Kv, from 2,500 to 20,000 magnification.

RESULTS

Blood smears stained according to the Rosenfeld technique showed 2.5% to 3.0% of immature erythrocytes, as well as absence of parasites in the peripheral blood. Almost the same proportion of immature erythrocytes was found in blood supravitally stained with brilliant cresyl blue, in which these red cells are characterized by a variable amount of basophilic reticulum. A high number of mature erythrocytes presented from one to four basophilic granules individually disposed at several cytoplasmic sites. Positive results for the Feulgen reaction, were hardly visible in the cytoplasm. A good visualization of positive results was possible in the vicinity of the nuclei. However, they could be mistaken for nuclear protrusions. Erythrocytes of blood smears submitted to partial drying, and then hemolysed, show an ellyptical stroma containing a central nucleus, granular and rod-shaped forms with variations in diameter and length of about 0.40 μ or 0.25 — 0.60 μ x 1.20 — 1.50 μ, respectively; in general, the granules are more electron-dense than the rod-shaped forms (Figs. 1, 2). Thin section of mature carp erythrocytes, commonly contain different kinds of vesicles, mitochondria in various degenerative stages, and moderately dense amorph masses, more or less compact and agglomerated, concomitantly present in the cytoplasm, associated to the endoplasmic reticulum, and in the nucleoplasm (Figs. 3, 4, 8, 9); an amorph mass can be seen in the nuclear membrane pore, between cytoplasm and nucleoplasm (Fig. 5).

Mitochondria and erythrocyte nuclei are frequently connected by an apposition of the external mitochondrial and nuclear membranes (Fig. 4), or through the passage of dense chromatinic material into the organelles (Figs. 5, 6). Such connections occurs always at nuclear region where chromatinic material is juxtaposed to the nucleolema, at sites of variable distances from the nuclear pores, through which hemoglobinized cytoplasm enters the nucleoplasm (Figs. 3, 4, 8, 9, 11). Convoluted chromatinic threads, invade mitochondria still presenting their chracteristic structure (Figs. 5, 6), or organelles which already had suffered a modification (Fig. 8). Mitochondria that had received chromatin, modify and detach themselves from the nucleus giving rise to free vesicles (Fig. 9) containing dense, fibrous, as well as granulated material.

Free vesicles containing myelin-like figures are constantly present in erythrocytes. They seem to originate from the external nuclear membrane, as suggested in figures 10 and 11; some vesicles presenting myelin-like figures originate from degenerated mitochondria which had not received chromatinic material (Fig. 7). Vesicles apparently of other types, contain mainly small vesicles or besides these small round elements, a fine fibrous material (Fig. 3a); several vesicles contain only a few membrane traces or nothing at all (Figs. 3,9). Vesicles carrying granular, fibrous, dense, amorphous materials, and myelin-like figures or traces of membrane, were found approaching the invaginated plasmic membrane (Figs. 12, 13). These vesicles may be simple, or may agglomerate, fusing among themselves.

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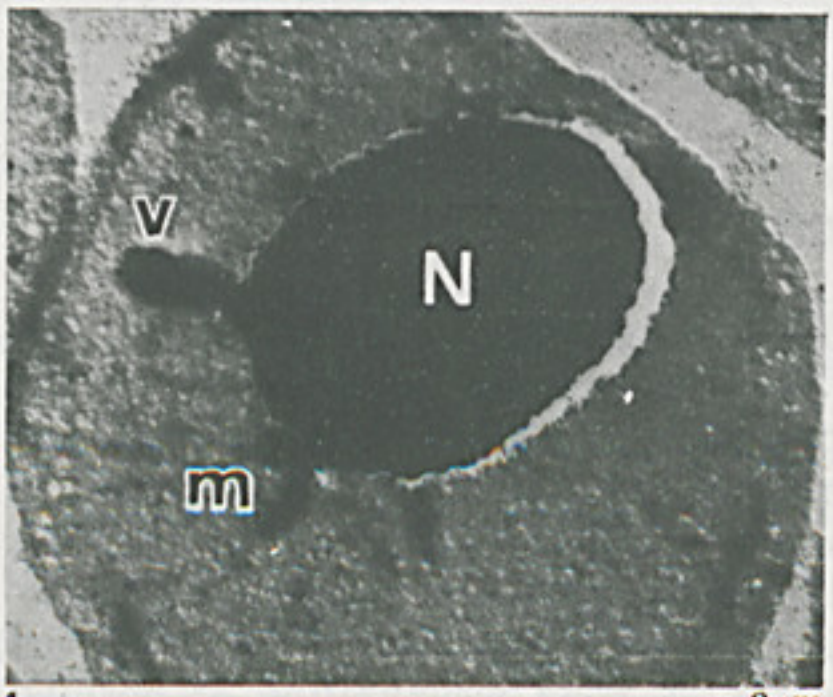
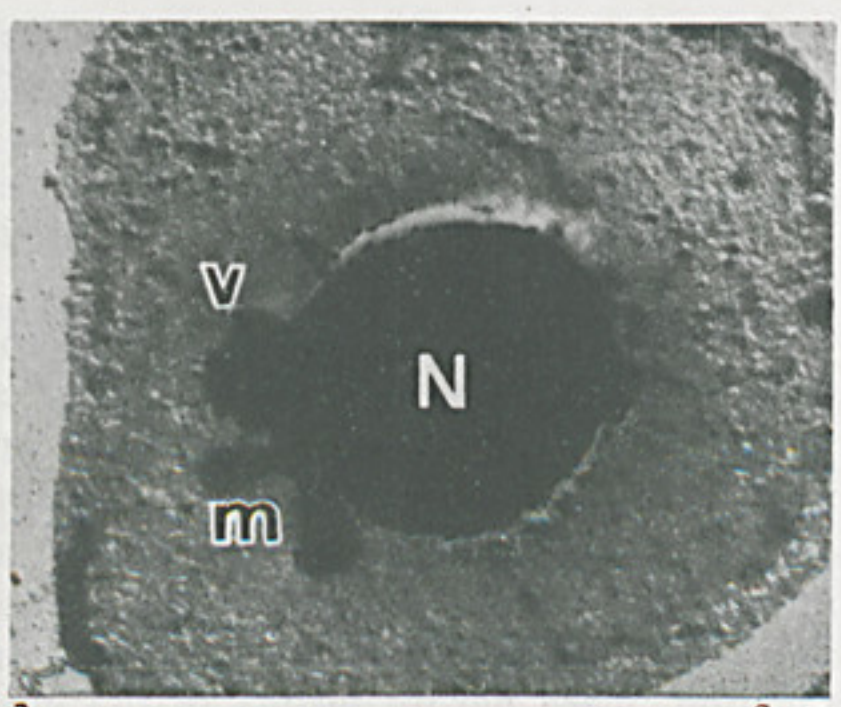


1 2μm



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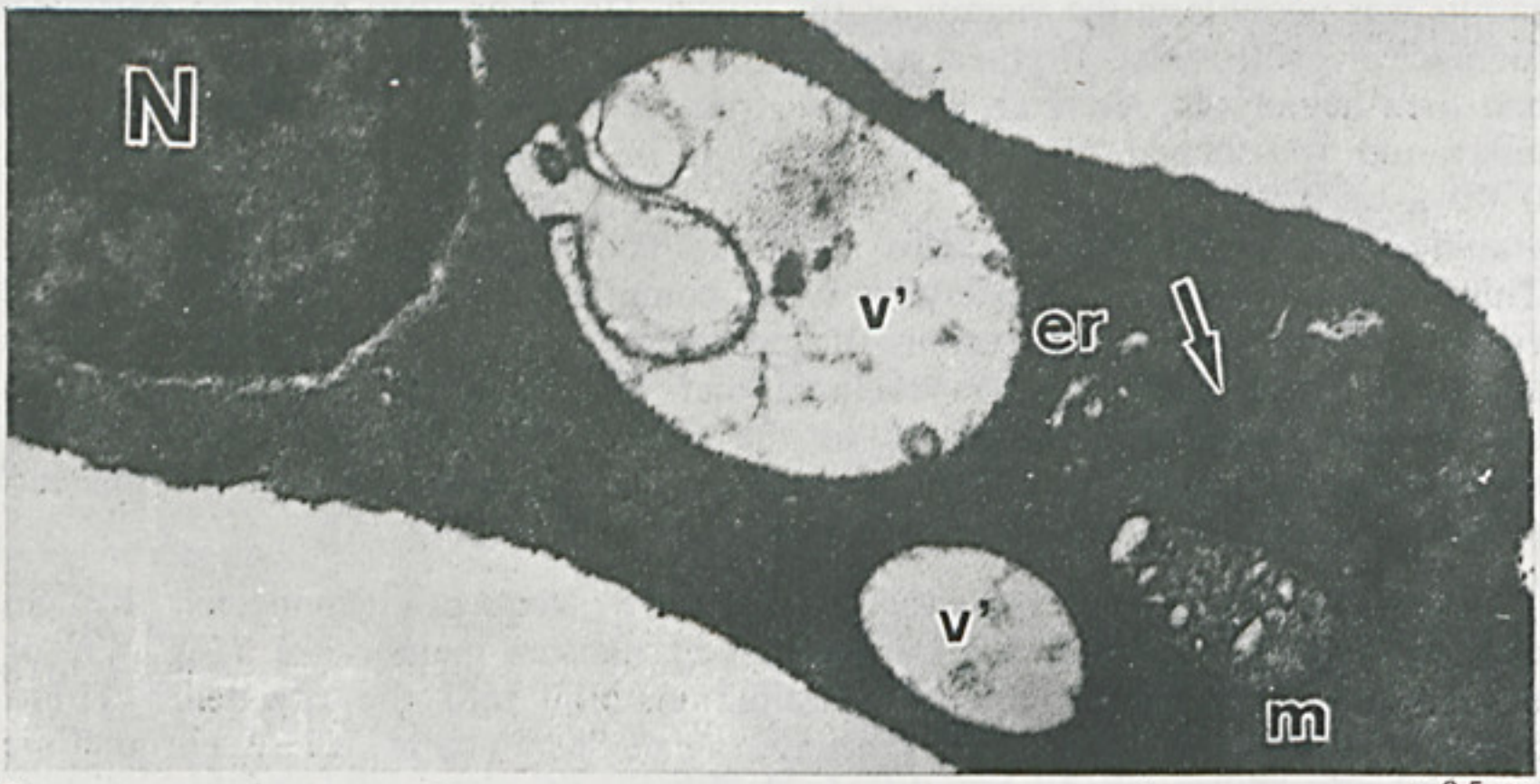
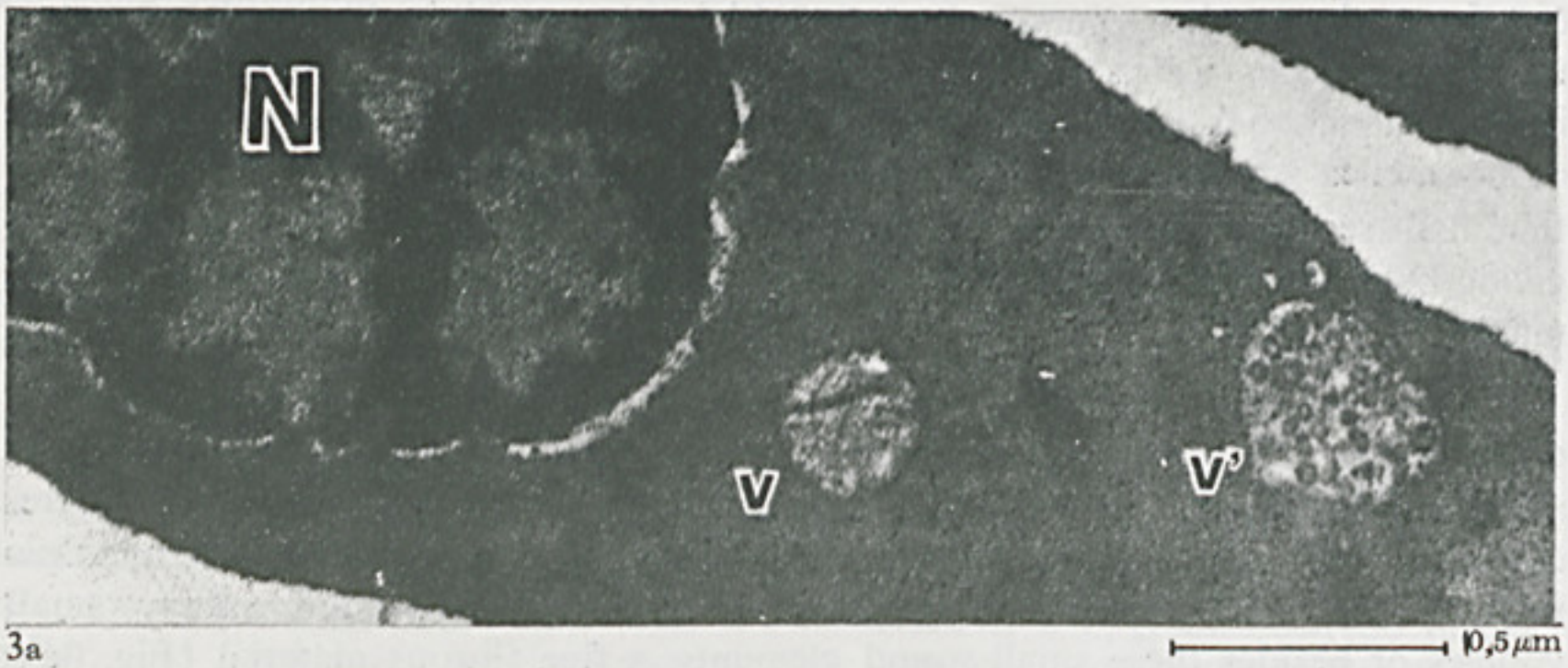


3 0,5μm



3a 0,5μm

MATURE CARP ERYTHROCYTES

Figs. 1 and 2 - Cells from hemolysed blood smears. (N) nuclei; (m) mitochondria; (V) large vesicles.

Figs. 3 and 3a - Thin section of the same cell. (N) nucleus; (m) degenerated mitochondrion: (er) endoplasmic reticulum; (arrow) amorph material; (V) vesicle of mitochondrial origin; (V') vesicles of unknown origin.

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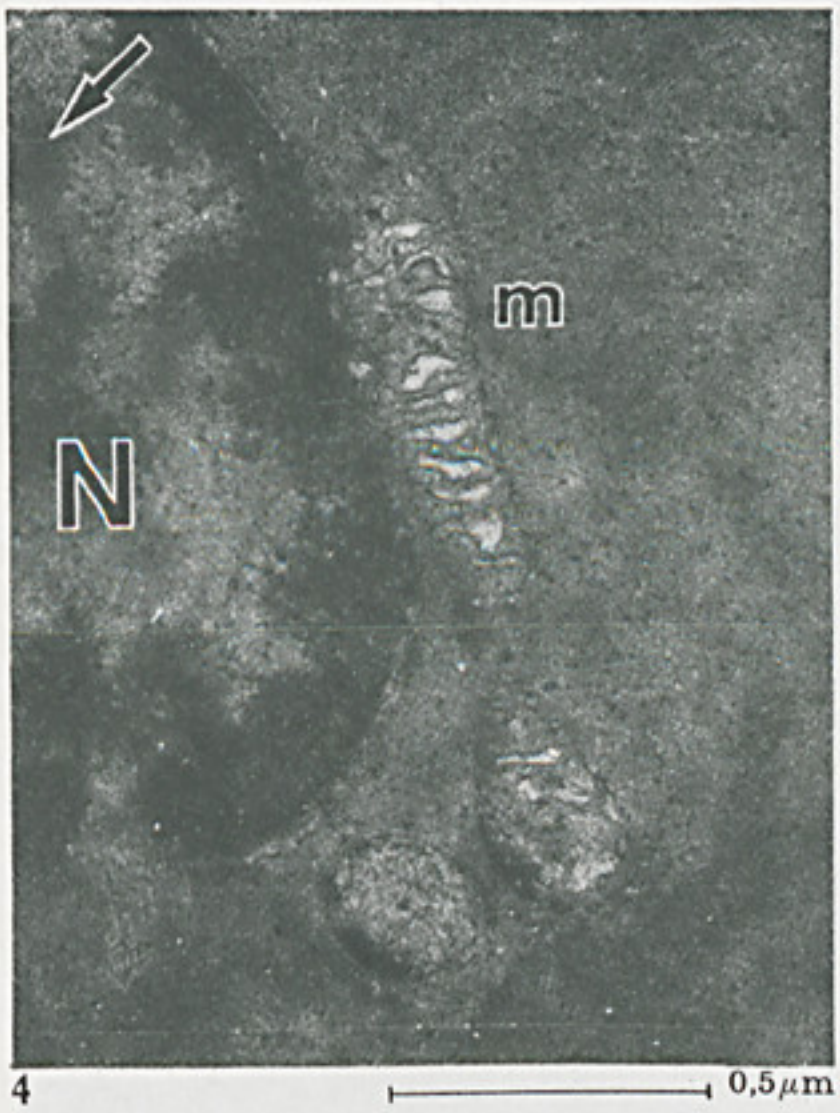

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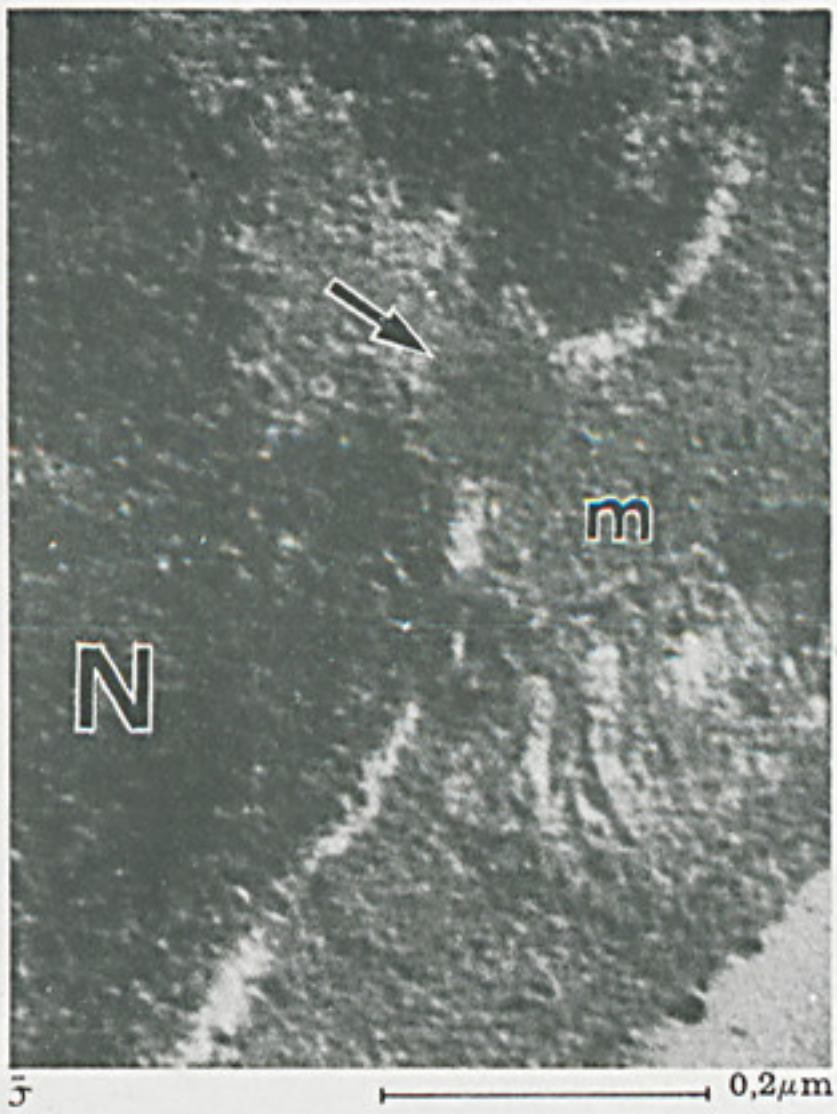

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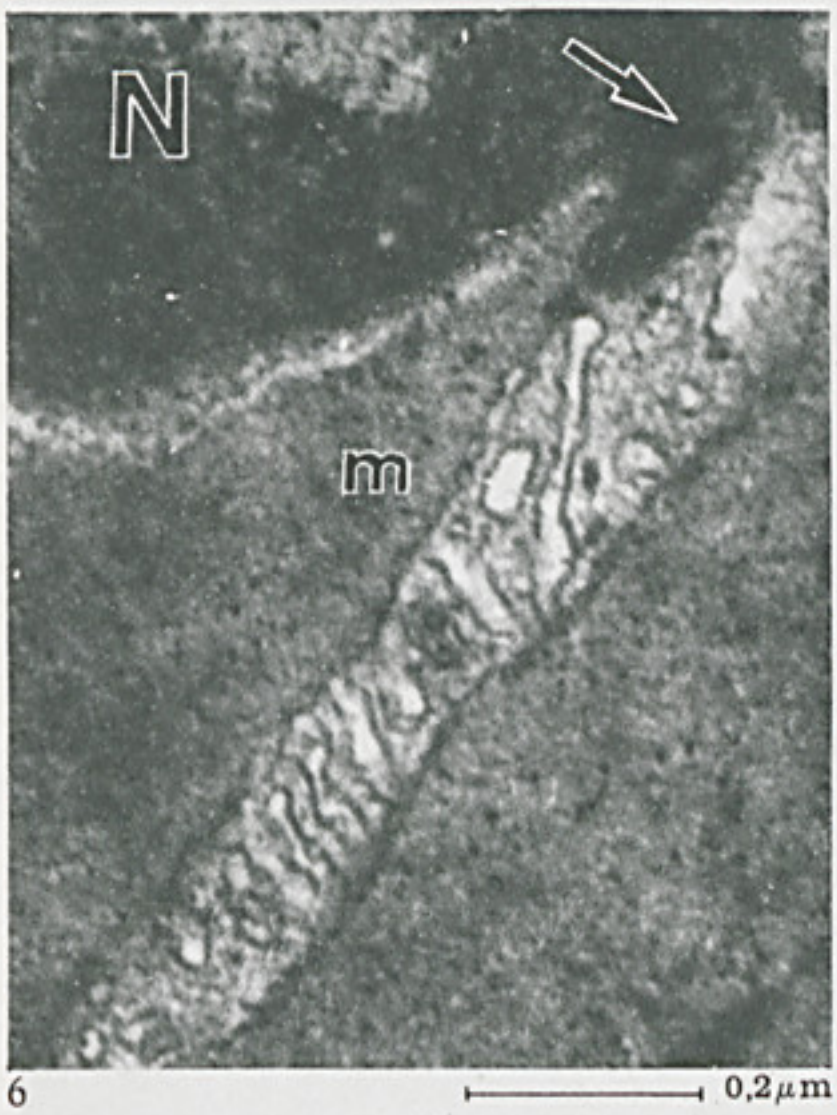

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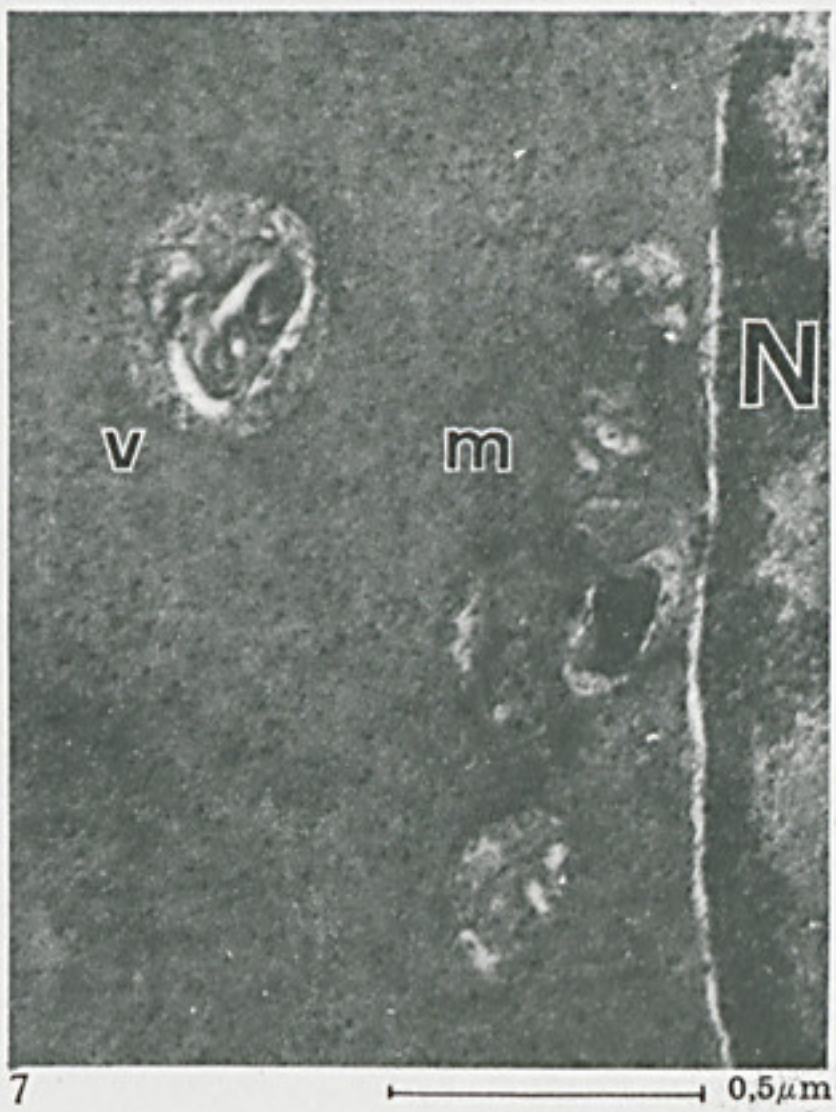

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THIN SECTIONS OF MATURE CARP ERYTHROCYTES

Fig. 4 - (N) nucleus; (m) mitochondrion juxtaposed at the nuclear membrane; (arrow) amorph material.

Fig. 5 - (N) nucleus; (m) mitochondrion receiving chromatinic material; (arrow) amorph material in the nuclear membrane pore.

Fig. 6 - (N) nucleus; (arrow) passage of condensed chromatin into a partially modified mitochondrion (m).

Fig. 7 - (N) nucleus; (m) partially modified mitochondrion containing dense material; (V) vesicle, possibly of mitochondrial origin, containing myelin figures.

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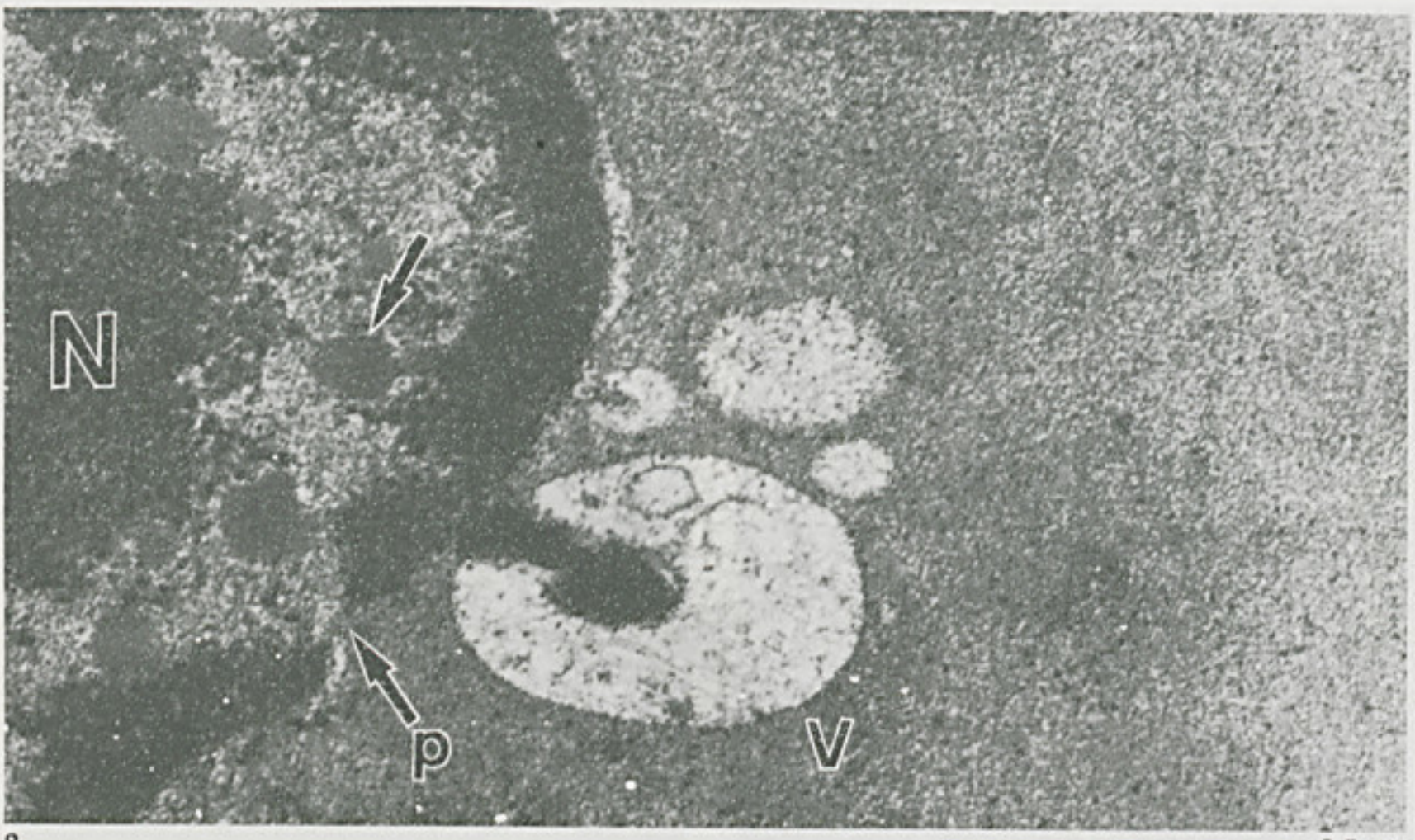
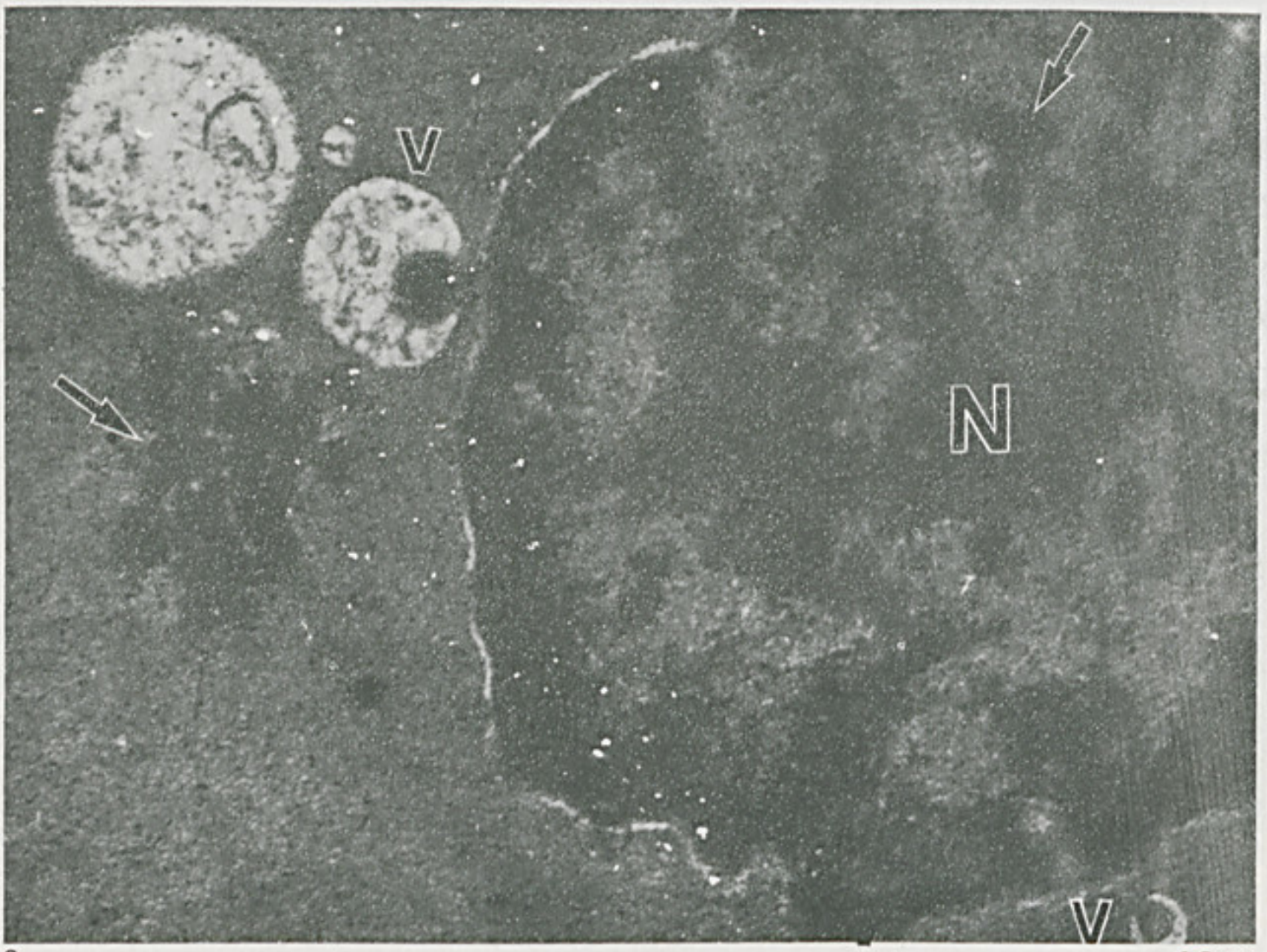


8 0,5μm



9 0,5μm

THIN SECTIONS OF MATURE CARP ERYTHROCYTES

Fig. 8 - (N) nucleus; (arrow) amorph mass; (p) - nuclear membrane pore; (V) a recently constituted vesicle; nearly detached from the nucleus, showing condensed chromatinic material.

Fig. 9 - (N) nucleus; (arrows) amorph masses in cytoplasm and nucleoplasm; (V) vesicle containing dense material, recently detached from the nucleus.

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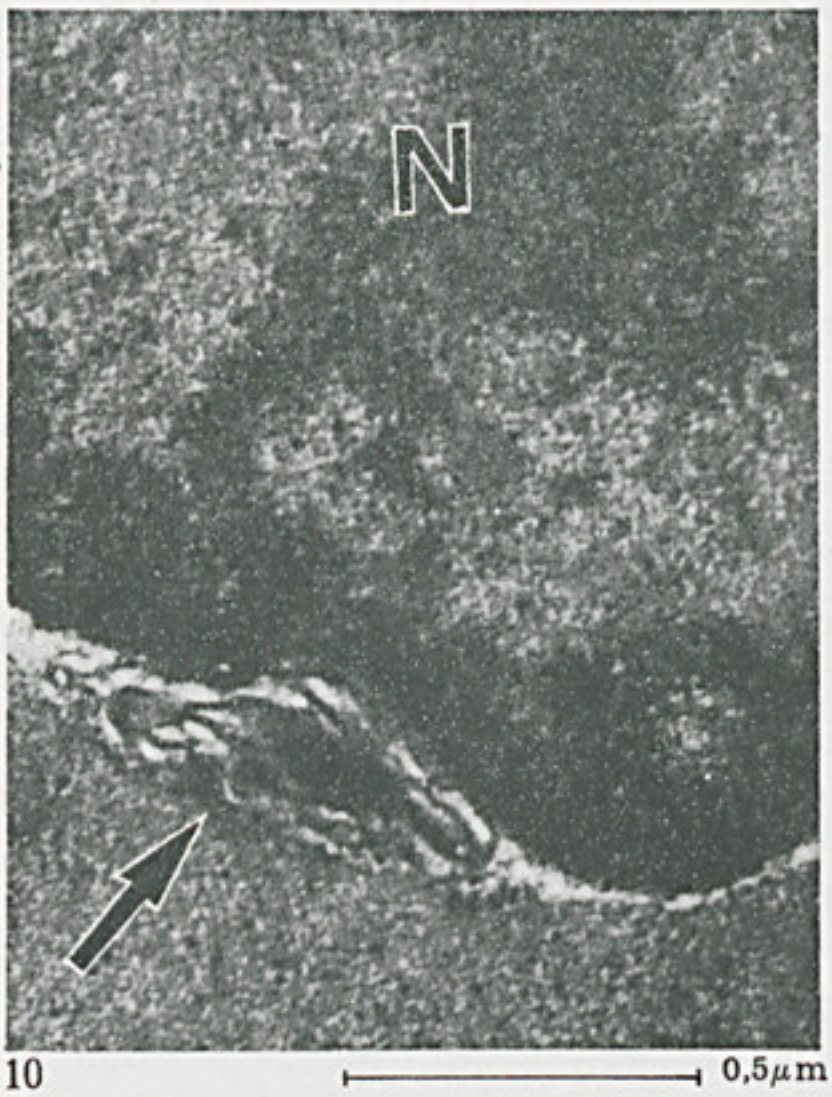
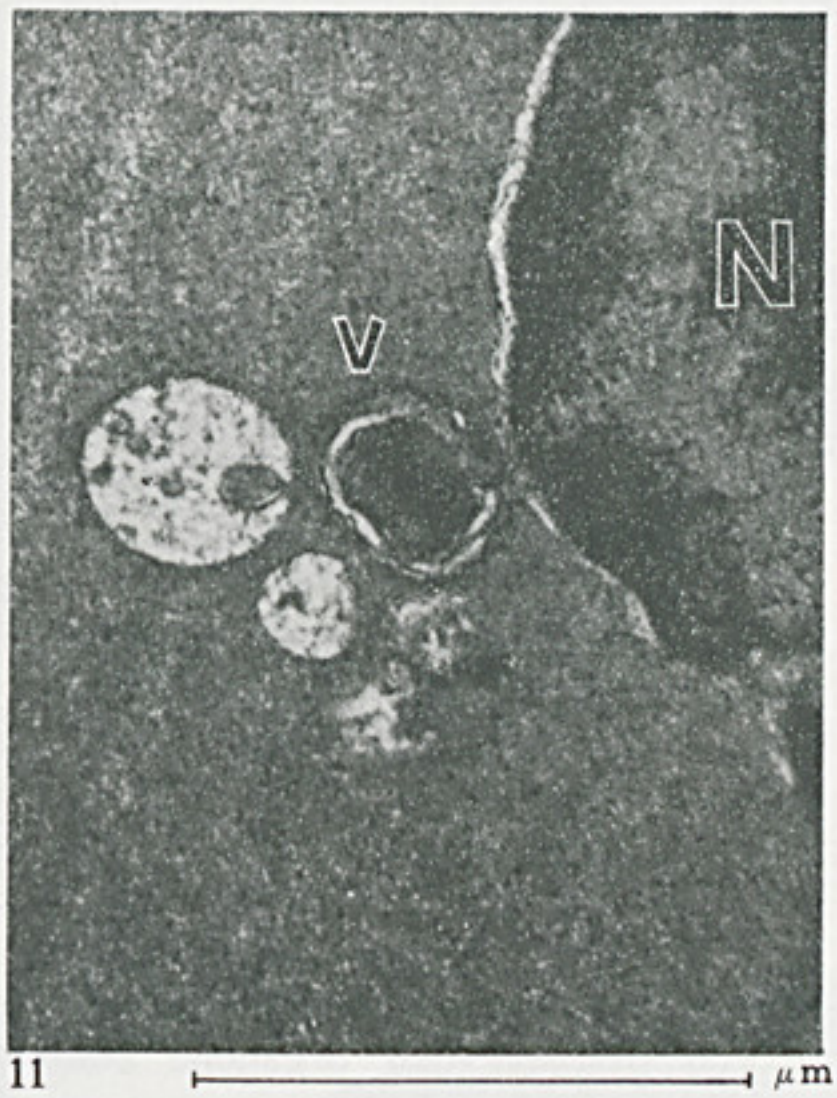
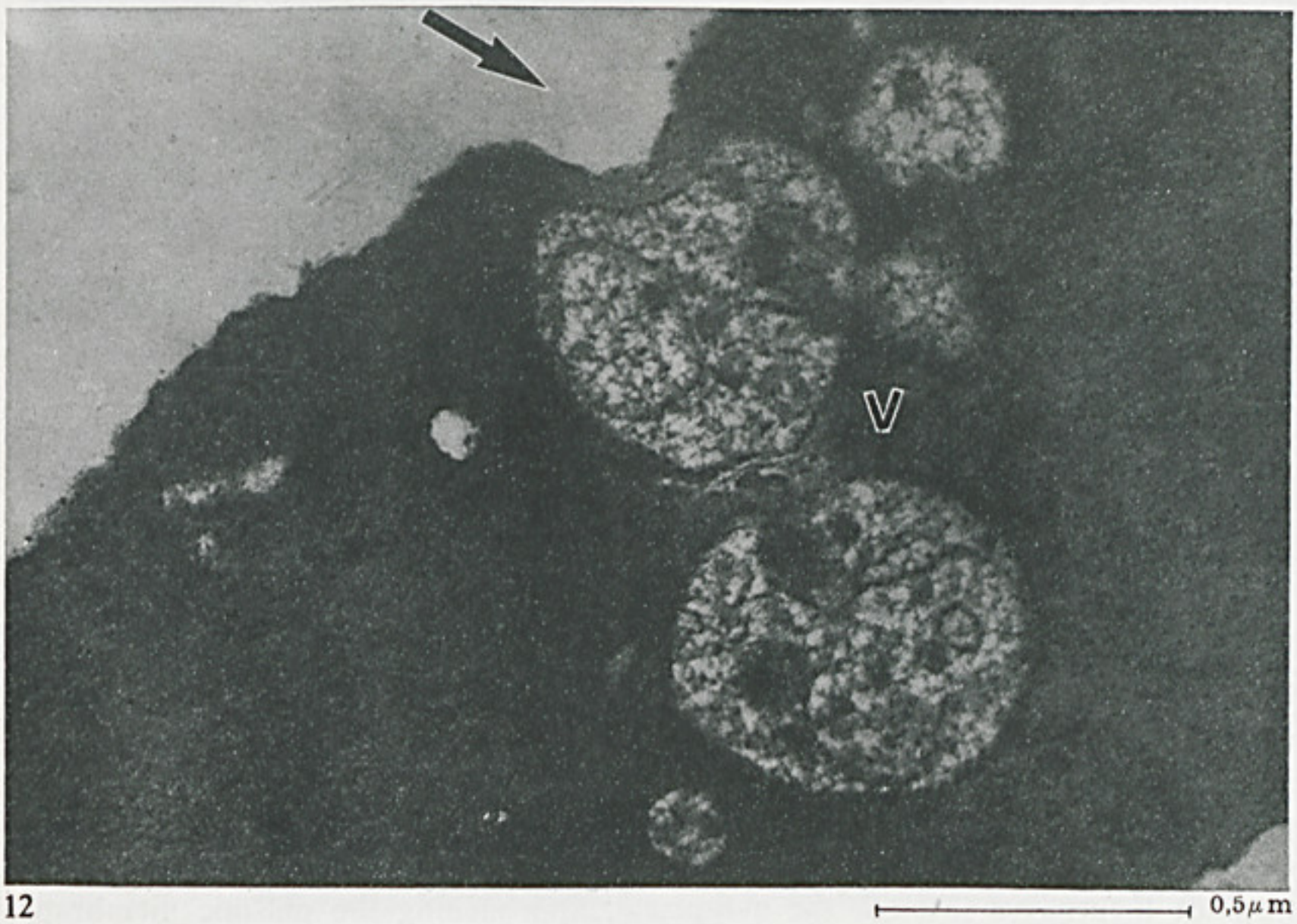


10 0,5μm



11 μm



12 0,5μm

THIN SECTIONS OF MATURE CARP ERYTHROCYTES

Fig. 10 - (N) nucleus showing invagination. A convoluted membrane within the increased perinuclear space can be observed (arrow).

Fig. 11 - (N) nucleus; (V) vesicle conataining a myelin-like figure, almost detached from the nucleus.

Fig. 12 - (V) vesicles fusing among themselves, approaching the plasmic membrane at an invaginated region (arrow).

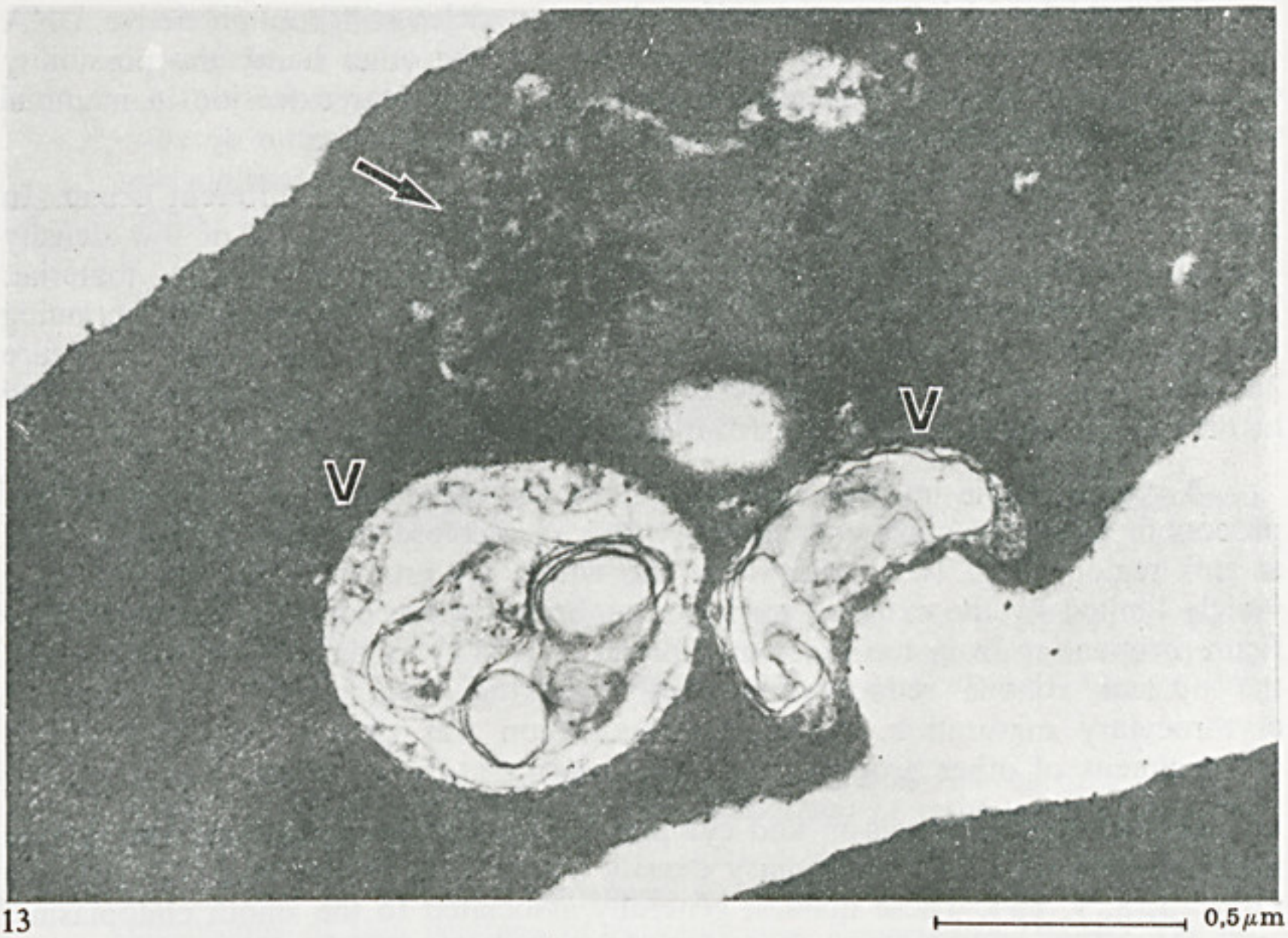
Several sections of mature erythrocytes show relatively thin marginal bands ranging from 140 to 250 mμ in diameter. They are frequently detected in longitudinal sections (Fig. 14), and occasionally in cross sections (Fig. 15), where fifteen to eighteen microtubules of 18 to 21 mμ in diameter can be seen.

DISCUSSION

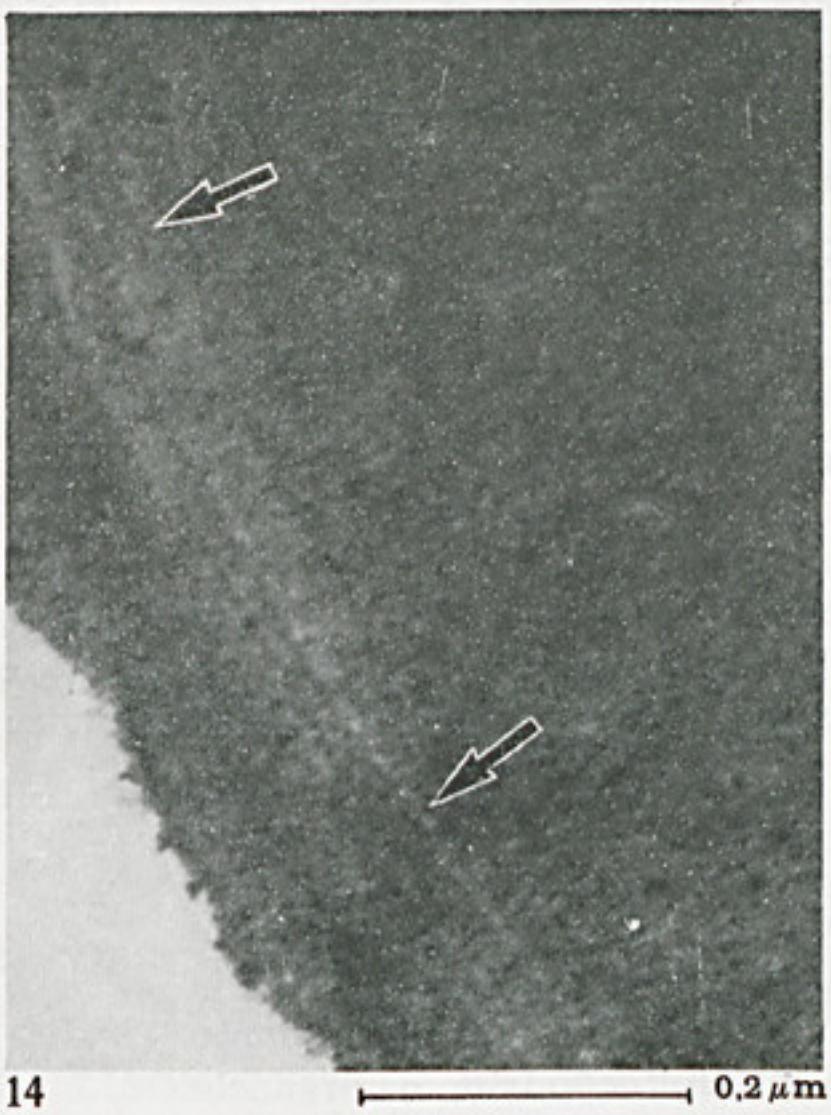
The absence of parasitism in the peripheral blood of *Cyprinus carpio* specimens, as confirmed through the Rosenfeld staining [17], allows secure interpretations on hemolysed, as well as on sectioned erythrocytes. In the former instance (Figs. 1, 2), the rod-shaped forms, generally disposed around the nucleus, can be taken as mitochondria, according to earlier demonstrations on immature mammalian erythrocytes [4,20]. This interpretation is also supported by thin sectioned erythrocytes, where mitochondria are observed in the same dispositions (Figs. 4, 6, 7, 11). The granular forms, more electron-dense than the rod-shaped ones (Fig. 2) present themselves in the same disposition, and possibly correspond to the vesicles carrying nuclear material (Figs. 8, 9). Their higher density may be due to the condensed chromatinic content. Hemolysis of red blood cells in partially dried smears, permits a global visualization of their organelle constitution, as well as on the disposition of such elements.

The outstanding phenomenon is the formation of vesicles carrying chromatinic material. They originate from mitochondria, an event which seems to be general for mature chicken [6], bothrops snake [14], frog [5] and carp [2] erythrocytes. Feulgen reaction, done on erythrocytes from specimens of all those vertebrates, was always positive· although these results were not conclusive. However, mature frog erythrocytes showed labelled vesicles when ^{3}H-thymidine was given to animals, previously turned anemic through phenylhydrazine in order to activate erythropoiesis, and thus enhancing the incorporation of this DNA basis [13]. Obviously chromatin labelling persists until the last erythrocytic maturation stage where the phenomenon of vesicle formation takes place. This occurrence was never detected in immature forms through Feulgen reaction or electron microscopy.

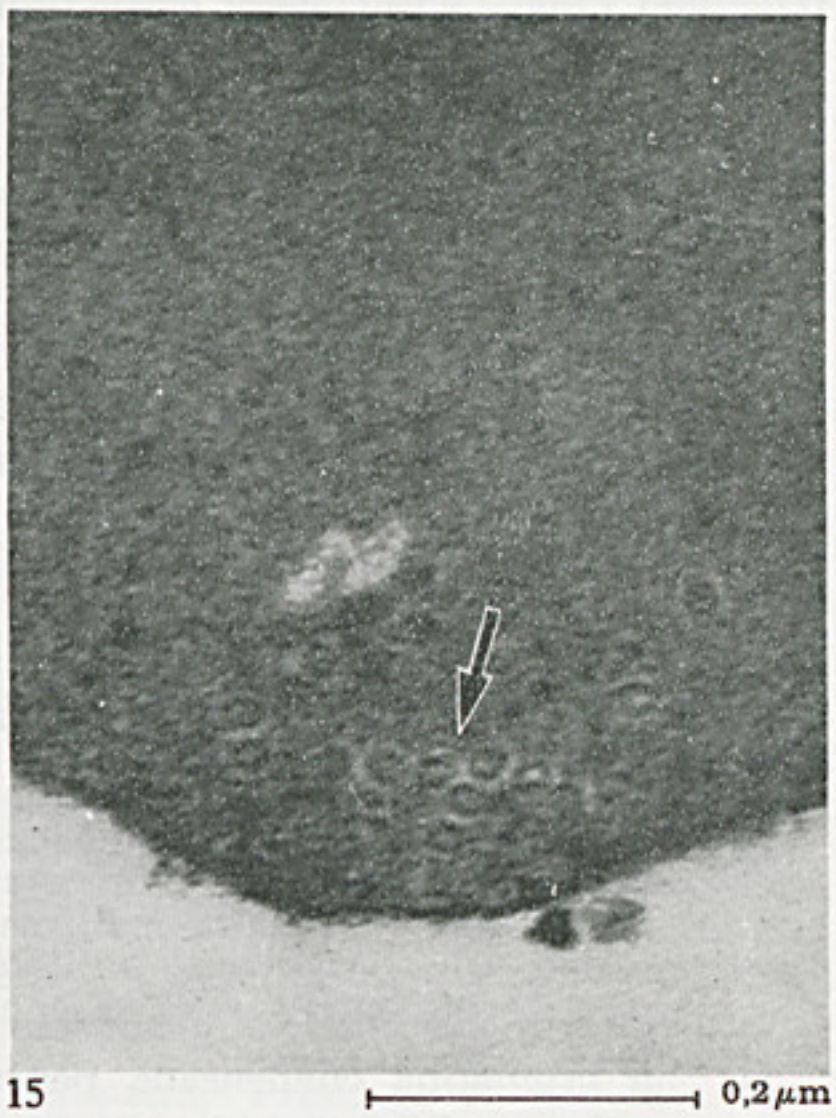
Formation of vesicles begins by mitochondria juxtaposition on the nuclear membrane at regions where chromatin is disposed (Fig. 4); hence, this occurs at points more or less distant from the nuclear pores through which hemoglobinized cytoplasm enters the nucleoplasm (Figs. 3, 4, 8, 11). While condensed chromatin penetrates into the mitochondrion, a disorganization of its inner lamellar structure gradually occurs (Fig. 6). After cessation of chromatin penetration, mitochondria may still present vestiges of their structure (Fig. 7) or lose it completely, as shows in figures 8 and 9. These modified organelles displace themselves through the cytoplasm, approaching the plasmic membrane. At this region the membrane invaginates (Figs. 12, 13), suggesting a possible occurrence of a phenomenon of exocytosis, like that which may occurs in final maturation stages of mammalian reticulocytes [11,18]. When inner mitochondrial membranes disappear, organelle diameters increase gradually from about 0.14 (Fig. 4) to 0.40 μ (Fig. 8); vesicles in the proximity of the plasmic membrane reach diameters ranging from 0.60 (Fig. 12.) to 0.70 μ (Fig. 13).



13 — 0,5 μm



14 — 0,2 μm



15 — 0,2 μm

THIN SECTIONS OF MATURE CARP ERYTHROCYTES

Fig. 13 - (V) vesicles near the plasmic membrane, one of which already contacting the invaginated plasmic membrane; (arrow) amorph mass partially surrounded by the smooth endoplasmic reticulum.

Fig. 14 - Longitudinally sectioned microtubules, constituting the marginal band (arrows).

Fig. 15 - Transversally sectioned microtubules (arrow).

It would be of interest to verify whether vesicles still contain active DNA or only products from the DNA degradation. On the other hand, the possibility of a correlation between this phenomenon and the nuclear extrusion in mammal orthochromatic erythroblasts, shoud be considered.

Several vesicles commonly found in erythrocytes are of different origin. In figures 3 and 3a it is difficult to ascertain the origin of vesicles of low density and of the one containing small vesicles among granular and fibrous material. The degenerated mitochondrion of figure 3, distant from the nucleus, continuing the degenerative process, could originate the moderately dense vesicle of figure 3a. Mitochondria may also degenerate (Fig. 7) through modifications occurring in their inner lamellar structure, from which myelin figures rise.

A region of the nucleus may invaginate, accompanied by a convolution-like process of the inner nuclear membrane, and an increase of the perinuclear space at this region (Fig. 10). Afterwards the whole set protrudes, giving rise to a vesicle limited by the external nuclear membrane whose content is a myelin-like figure originating from the inner membrane (Fig. 11). This may be related with the nuclear volume reduction possibly occurring even in the last stage of erythrocytary maturation. No such phenomenon was detected in erythrocytes of specimens of other groups.

Erythrocyte nucleoplasm and cytoplasm frequently contain some amorphous material presenting an intermediary density between chromatin and both plasmas (Figs. 3, 8, 9, 13). These masses, generally associated to the smoth endoplasmic reticulum (Figs. 3,13), were never found in erythrocytes of other groups. Their nature and origin are unknown, but the presence of such a mass in the nuclear membrane pore (Fig. 5), and its position sometimes distant from the nucleus (Figs. 3, 13) may, although remotely, suggest a nuclear origin.

The marginal bands of carp erythrocytes are thin, compared to that found in erythrocytes of other groups [6,9,10,14,19,21], specially as regards the diameter, about 700mμ of the chelonian erythrocyte band [8]. Correspondingly the number of microtubules is also lower, although the possibility that degenerative bands of old erythrocytes had been observed, can not be excluded.

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RESUMO: Eritrócitos maturos de *Cyprinus carpio* mostram um fenômeno de formação de vesícula Feulgen positiva, originária de um mitocôndrio justaposto à membrana nuclear. Enquanto a cromatina condensada penetra no mitocôndrio, o organelo sofre modificações na estrutura lamelar, dando origem a uma vesícula que se destaca do núcleo e se desloca através do citoplasma. Alguns aspectos sugerem que o conteúdo da vesícula é eliminado do eritrócito. Este mecanismo parece ser geral para os eritrócitos maturos nucleados de outros vertebrados. Outras vesículas de origem desconhecida, algumas originadas de mito-

côndrios degenerados e outras da membrana nuclear, foram observadas.

Um material denso e amorfo, nunca encontrado em eritrócitos de outros vertebrados, estava frequentemente presente no nucleoplasma e no citoplasma, associado ao retículo endoplasmático liso. Bandas marginais, de menor diâmetro que as de eritrócitos de outros grupos, foram observadas.

UNITERMO: Ultra-estrutura de eritrócitos de *Cyprinus carpio*.

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