Mem. Inst. Butantan
35: 1-39, 1971.

ERYTHROCYTARY MATURATION IN RODENTS. *

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SUMMARY: Erythrocytary series of peripheral rabbit and hamster blood have been studied by methods of hemolysis and thin sections at the ultrastructural level.

The presence of filamentous or rod-like mitochondria, that disintegrate to circular membranous forms, has been demonstrated when hemolysis was done in suspension in distilled water.

After nuclear extrusion, occurring generally in the orthochromatic erythroblast, concomitantly with active contraction and expansions, an increase of mitochondria occurs. This is coincident with the higher intensity of hemoglobin synthesis, as verified by other authors.

Besides the organelles, as mitochondria and polyribosomes, which participate in hemoglobin synthesis, there is the Golgi Complex, probably acting in the reticulocyte water and salts balance during maturation, as well as vesicles and a smooth endoplasmic reticulum.

The final maturation of the erythrocyte is characterized by the increase of hemoglobin concentration followed by a progressive disappearance of all the cytoplasmic structures and the rise of the characteristic discoidal biconcave form, constituted by only an ordinate paracrystalline structure at the molecular level.

UNITERMS: Erythrocytary Maturation.

INTRODUCTION

An accurate study of mammalian erythrocytes shows that the cell during the maturation process, undergoes successive structural and biochemical transformations which confer a functional specificity to it. Theses modifications arise in a cell at the beginning of differentiation, and therefore, without any definitive morphologic and physiologic characteristics. In this respect, erythropoiesis is a very illustrative phenomenon, well known nowadays. Every maturation phase was followed up by the classical staining and cytochemical techniques for optical microscopy. Proerythroblast, the first phase of differentiation, is followed by the basophilic, polychromatophilic and acidophilic erythroblast; the latter gives rise to the non nucleated cell, namely reticulocyte, that develops to the mature erythrocyte.

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In animals considered as normal, the transitional phases follow the synchronism law of nucleocytoplasmic evolution, occuring a parallelism between cytoplasmic and nuclear transformations during the maturation of the blood cells; this synchronism did not occur under pathological conditions (Bessis, 1961). In this sequence of evolution, mitosis takes place, followed or not by cytoplasmic division (Berman, 1947; Wolff and Hofe, 1951), and loss of the nucleolus in the basophilic eròthroblast; further, a gradual decrease of basophilia occurs, and hemoglobin synthesis takes place in the polychromatophilic phase; the acidophilic erythroblast shows a marked decrease of nucleoplasmic relationship at the picnotic stage of the nucleus, and a reduction in number of mitochondria that arrange themselves generally near the nucleus. At this phase the nucleus vanishes through cariolysis, carioexis or extrusion, the latter being the more frequent phenomenon (Astaldi et al., 1950; Leonardi, 1951).

Right after this phase, the reticulocyte develops, showing a more intense hemoglobin synthesis in accordance with comparative microspectrophotometric determinations for mature erythrocytes, reticulocytes, erythroblasts and purified hemoglobin, corresponding to the extinction at 4060 Å wave length (Seno, 1953). The immature erythrocyte, or reticulocyte is characterized by granules and filaments at vital or supravital staining with brilliant cresyl blue, Janus green B or other dyes. The nature of the reticulum thus formed has been studied exhaustively by Cesaris-Demel (1907). The granules were denominated A substance, and the filaments, B substance; these structures are known as "Substantia granulo-filamentosa". It has been discussed also whether or not this structure could be an artifact, resulting from the precipitation of the dyes employed. The majority of authors consider "Substantia granulo-filamentosa" as a real artifact on the basis of their experimental results.

Dustin (1947) and Theorell (1950) came to the conclusion that the mammalian reticulocytes contain a diffuse basophilic substance, resulting in a granular agglomeration in the presence of basic dyes. They demonstrated that basophilia is due to the presence of ribonucleoproteins, since this structure is not evidenced after treatment of the reticulocytes with ribonuclease; Burt et al. (1951), and Thoma (1959) confirm these observations. Kosenow (1952), when observing young erythrocytes stained in increasing acridin-orange H-concentrations by fluorescence microscopy, demonstrated an increase of granules and filaments. He concludes, as others do, that this fact is due to the presence of diffuse nucleic acid. Bessis (1954) holds the hipothesis of an artifact due to dye and ribonucleoprotein precipitation, and does not admit the preexistence of the so-called "Substantia granulo-filamentosa".

However, the preexistence of a structure in non stained reticulocytes has been observed through dark field microscopy by Simmel (1926) and Seyfarth (1927), as well as by phase contrast microscopy after hemolysis (Lüdin, 1949), or in intact reticulocytes (Sano, 1965). The nature of this structure and its relationship to the "Substantia granulo-filamentosa" was enlightened by electron microscopy with various techniques (Brunner et al., 1956; Braunsteiner et al., 1956; Vallejo-Freire and Brunner, 1958; Brunner, 1962).

Structures that constitute the Golgi complex, or vesicles and dictyosomes, were observed in normal and lead poisoned guinea-pigs as well as in rabbit embryo erythrocytes. Concentric lamellar structures of unknown function, described for

several kinds of cells, were also observed in reticulocytes (Brunner and Vallejo-Freire, 1964).

Basophilia and all the structures of the reticulocytary phase disappear gradually, giving rise to a mature erythrocyte with the characteristic discoidal and biconcave form that contains only a structure at the molecular level.

In the present work, morphologic aspects of some evolutive phases at the ultrastructural level are analysed, occurring during the maturation of the erythroblast up to the definitive red blood cell. With reference to the above considerations, various questions arise, mainly related to physiology. The following facts are quoted: a — "Substantia granulo-filamentosa" is of essential mitochondrial nature; b — the different aspects of mitochondria, when reticulocytes are hemolysed by different techniques; c) — the quantitative increase of mitochondrial structures after nuclear extrusion; d — in consequence of the former phenomenon, the mechanism that leads to the quantitative increase of mitochondria, as well as the degenerating process of these organelles; e — the components of the Golgi complex in the young erythrocytes; f — the possible existence of a molecular arrangement in the normal mature erythrocyte.

MATERIAL AND METHODS

Guinea-pigs (*Cavia porcellus*) weighing 350-400 g, newborn rabbits (*Oryctolagus cuniculus*), rabbit and hamster embryos (*Mesocricetus auratus*), 16-22 and 10 days old, respectively, were used.

Bleeding anemia in guinea-pigs.

To increase the number of reticulocytes in blood, 5 to 6 ml of blood were withdrawn daily by cardiac punction during 5 days; 2-3 days after the last bleeding, blood was collected for examination. The Giemsa stained smears show reticulocytes recognized by their diffuse basophilia, with a percentage varying between 10 and 15%.

Lead poisoning anemia in guinea-pigs.

One ml of an 1% aqueous lead acetate solution was injected subcutaneously during 3 to 9 days. Blood was withdrawn by cardiac punction after 3-7 days. Reticulocytosis varied from 6 to 50%, and the proportion of erythroblasts was about 6%.

Blood samples with reticulocytosis and erythroblastosis.

Blood samples of newborn rabbits were obtained by cardiac puncture; from hamster and rabbit embryos, by sectioning the umbilical cord. Embryonic blood consists merely of megaloblasts, megalocytes, erythroblasts and reticulocytes; the percentage of reticulocytes is about 20% in newborn rabbits; erythroblasts are rarely found in the peripheral blood.

Hemolysis.

Technique A — Thin blood smears were prepared on collodion-coated histological slides and allowed to dry at room temperature for 5-10 hs, and hemolysis

was performed in 0.8% NaCl solution containing 2.5% formalin. The stromas were then stained for 5-10 min 1% aqueous phosphotungstic acid solution, pH about 3.5, washed in distilled water and dried at room temperature. The films, carrying the stromas, were transferred to metal grids for examination (Brunner and Vallejo-Freire, 1956).

Technique B — One ml of blood was washed once in 0.85% NaCl solution and hemolysed in 20 ml distilled water.

Technique C — Washed blood was hemolysed in 20 ml of 0.2% NaCl solution containing 2.5% formalin.

In the latter two instances, 2 ml of a 2% O_sO_4 aqueous solution were added to the samples after hemolysis. After 5 min the ghosts, or stromas, were centrifuged at 60 g for hemoglobin elimination, and washed three times in distilled water; the sediment was suspended in 4 or 5 ml of distilled water and then dropped over collodion-coated metal grids. After 50 or 60 min the excess of water was absorbed with filter paper, and the preparations air dried at room temperature (Brunner and Vallejo-Freire, 1956).

Replica

To investigate the existence of structures on the erythroblast nucleus, blood smears were fixed with O_sO_4 vapor for 1 h, and then covered with a 1% collodion amyl acetate solution, and tilted at an angle of about 50° to the horizontal, for 30 min. After drying, the replicas were detached from the smears and transferred to metal grids.

Treatment with ribonuclease

Smears hemolysed according to technique A, without staining, were kept in a M/7 veronal-acetate buffer, pH 6.8, containing 0.1% ribonuclease (General Biochemical Inc) at 37°C for 1 h; controls were treated under the same conditions. All smears were washed in distilled water and stained for 5 to 10 min in an 1% aqueous phosphotungstic acid solution. After washing and drying the films with the adhering stromas or ghosts were transferred to metal grids.

Most of all these preparations were submitted to the shadow-casting process, with chromium or palladium.

Retraction of the inner erythrocyte material by osmic acid.

Peripheral blood erythrocytes of normal guinea-pigs were fixed for 18 to 20 h in NaCl solutions of concentrations varying between 0.60 and 0.40%, containing 10% formalin. Part of each suspension was centrifuged at 60 g and the sediments suspended or not in 1% aqueous phosphotungstic acid solution for 15 min; the remaining part of each suspension was centrifuged and the sediments suspended in 1% aqueous osmic acid solution for 15 min.

The erythrocytes treated with phosphotungstic or osmic acid were, after dehydration, embedded in methyl and butyl-methacrylates, and ultrathin sections prepared for examination.

Supravital staining with Janus green B.

Reticulocytes of guinea-pings with bleeding anemia, were stained with Janus green B, adding 2 or 3 blood drops to 3 or 4 ml of 0.85% NaCl in a series of dye dilutions from $1x10^{-4}$ to $5x10^{-6}$. After 5 min, and when the staining at the $1x10^{-4}$ dilution is microscopically visualized, the erythrocyte suspensions were centrifuged at 60 g, and the sediments suspended in an 1% osmic acid solution in veronal-acetate buffer of 7.4 pH. For fixation of the dye, and to increase the contrast at the electron microscope, an equal volume of an aqueous solution, containing 3.0% $HgCl_2$ and 5.6% Kl was added to the suspension after 5 min, according to the technique of Seno et al. (1958). After washing in distilled water, the erythrocytes were dehydrated in alcoholic series; the passages should be as short as possible because the Janus green B-dye fixative complex is soluble in alcohol.

Fixation end embedding techniques for ultrathin sectioning.

A few blood drops of rabbit and hamster embryos were added to an 1% $KMnO_4$ solution in veronal-acetate buffer, pH 7.4. After 5 min the suspension was centrifuged at 60 g for 10 min, the supernatant discarded, and the sediment suspended in an 1% O_sO_4 solution in "Subtosan" or veronal-acetate buffer for 10 min. Blood of normal and lead acetate treated guinea-pigs was fixed only in an 1% O_sO_4 solution in veronal-acetate buffer, pH 7.4, for 15-20 min. Erythrocytes embedded in a 3:7 methyl and butyl methacrylate mixture, containing 1% benzoil peroxide, were dehydrated in alcoholic series; embeddings prepared in Epon 812+, and in Crystic-araldite++ were firstly dehydrated in acetone series, or when dehydrated in alcoholic series, they were finished in 100% acetone. The time allowed for erythrocytes in each dehydrant medium was about 15 min, and passages from one medium to another were made by successive centrifugations up to the final embeddings.

Sections were cut in a Porter-Blum microtome with glass knives, and harvested on collodion or carbon-coated metal grids. To increase the contrast of the sections of Epon or Crystic-araldite inclusions, lead citrate staining was employed, according to the procedure of Reynolds (1963).

\+ Epon mixture (Luft, J., 1961), modified

Epon 812	.....	5.3 ml
DDSA	.....	2.0 ml
NMA	.....	3.8 ml
DMP-30	.....	2% $^{v}/_{v}$

++ Crystic-araldite mixture (Weigl, D.R. and Kisielius, J.J., 1967)

Crystic 196	.....	7.5 ml
Araldite	.....	2.5 ml
Dibutyl phtalate	.....	1% $^{v}/_{v}$
Benzoil peroxide	.....	0.05 ml

Comparative study of the number and area of mitochondria in immature erythrocytes.

The criterion adopted in the computation of sectioned cells or from hemolysed smears, to determine the total mitochondrial area per early reticulocytes, and erythroblasts, is based on the following facts: 1 — The reticulocyte loses gradually the mitochondria as it develops into the mature erythrocyte; 2 — in an immature erythrocyte population exist, therefore, besides erythroblasts, reticulocytes of any maturation phase, from that containing the highest number of mitochondria to the end phase of evolution. In each preparation, only cells showing the highest number of mitochondria have been considered, to verify whether the early reticulocytes actually contain a higher number of those organelles than the erythroblasts. The values obtained for mitochondrial areas in thin sections of nucleated and non nucleated erythrocytes in relation to the respective frequencies of the erythrocytes, considered in this work, were graphically represented.

Determination of the mitochondrial areas in sections or after hemolysis of the smears.

The areas are estimated from the weight of the mitochondria, cut out from the photographic paper used for the copies or enlargements of the negatives, with relation to the medium paper weight per area unit. The total mitocondrial area per erythrocyte was converted to μ^2.

The preparations were examined and photographed with the Siemens ÜM 100 b and Elmiskop I electron microscopes of the Instituto Butantan. The magnifications were from x 1200 to x 20000; an acceleration potencial of 60 KV was applied.

RESULTS

There are no morphological differences between erythroblasts, reticulocytes, and erythrocytes of guinea-pigs, rabbits and hamsters, except for those guinea-pigs with lead acetate intoxication, whose immature erythrocytes present mitochondria much larger than the ones of normal animals.

Blood of hamster and rabbit embryos obtained from the umbilical cord, and examined in Giemsa-stained smears, contains besides erythroblasts, 10 to 15% of megaloblasts; no mitosis occurs in the blood of 18 to 19-days old rabbit embryos; in the embryos of 16 days, only two mitoses could be observed.

In peripheric blood of lead poisoned guinea-pigs with an aproximately 50% reticulocytosis, and a 5-6% erythroblastosis, only a few basophilic erythroblasts were found.

Megaloblasts are easily distinguished from erythroblasts by their much larger diameter, and by the small nucleus, generally located at the center of the cells; reticulocytes are readily distinguished by the electron dense reticulum at different stages, and by the absence of a nucleus (Figs. 1 and 2).

Erythroblasts

Hemolysed smears prepared in accordance with technique A, show erythroblasts with a fine membrane, containing large electron dense filaments and gra-

nules. The nucleus, larger than that of megaloblasts, are of irregular periphery (Fig. 3), sometimes located at an excentrical position, probably during the extrusion phase.

Erythroblasts, when hemolysed in suspension in distilled water without partial drying, according to technique B, present granules and circular forms within the folded membrane. Under those conditions of hemolysis, no filaments nor rods have been observed. Their irregular nucleus may be found at the center or at the periphery of the stroma (Fig. 4). The same structures are also found in the subsequent evolutive reticulocytary phase, where a more detailed analysis has been done.

Ultrathin cell sections undergo less drastic treatment, and are integrally analysed without any hemolytic procedure. Megaloblasts contain a more electron dense cytoplasm as compared to erythroblasts, with several mitochondria, vesicles and endoplasmic reticulum. The nucleus is small and heterogeneous (Fig. 5).

Only one mitotic figure was observed in hundreds of preparations of ultrathin rabbit and hamster embryo sections. Fig. 6 shows a possibly basophilic erythroblast in mitosis, corresponding to an intermediary phase between prophase and metaphase; the double nuclear membrane shows large gaps at several regions with a mitochondrion at one of the extremities; the other organelles dispose themselves close to the membranes. Chromosomes are less electron dense than cytoplasm, and apparently individualized. The cell shows an irregular configuration, probably due to protoplasmic movements, ocurring during mitosis.

The erythroblast in Fig. 7 is constituted by an electron dense cytoplasm, compared to other types of cells, because at this stage it contains already synthesized hemoglobin. Some sections show mitochondria of about 0.3 μ in diameter, disposed near the agranular endoplasmic reticulum. This structure is sometimes associated with the Golgi complex. In other cytoplasmic regions there is a fine, dense granulation, identifyed as ferritin, enclosed in a membrane; these granulations may agglomerate to form masses of about 0.03 to 0.1 μ, situated between double membranes of mitochondrial nature. The ferritin granules may also be aligned, forming parallels as if corresponding to the mitochondrial lamellar structure (Fig. 8). The nucleus (Fig. 7), generally less electron dense, presents however, regions similar in density to the cytoplasm; it is limited by a double membrane that shows small pores.

At the acidophilic maturation phase that precedes that of nuclear extrusion, the cell shows an irregular configuration, and a homogeneously dense excentric nucleus limited by a double membrane, generally without pores. Cytoplasmic contractions and expansions are observed at a more advanced evolutive phase, giving rise to several lobes, one of which contains the nucleus. At this stage of development the proximity of mitochondria is commonly noted near the nuclear membrane (Fig. 9).

Reticulocyte

Well-marked cytoplasmic contraction and expansion movements persist still in the earlier reticulocyte after the extrusion of the erythroblast nucleus (Fig. 10). This reticulocyte from lead poisoned guinea-pigs, shows mitochondria with dia-

meters of about 0.90 μ, corresponding on the average to three times the diameter of mitochondria in normal animal reticulocytes; the internal membrane, as well as the lamellas, are disrupted.

This anucleated, basophilic, and therefore immature erythrocyte presents the same structures already described for erythroblasts, when hemolysed according to the three techniques. Threre are differences, however, as to quantity, of large filaments or circular forms, according to technique A or B, respectively. The recently constituted reticulocytes present large filaments and rods in a higher number and length (technique A) than the erythroblasts; also the number of circular forms is higher in less evolved reticulocytes, in conformity to technique B for hemolysis in suspension in distilled water, as will be seen later.

The large filaments may attain 10 μ in length, or more, and show diameters ranging from 0.20 to 0.30μ; generally, they are continuous to an irregular region of the same density. Their extremely variable dispositions present a radial, convoluted or undefined configuration (Fig. 11). There are diffusively distributed granules, ranging from 0.10 to 0.15 μ in diameter, which may appear in a large number, exceeding sometimes one hundred; these granules disintegrate through treatment with ribonuclease under the above exposed conditions. In erythrocytes slightly shadowcasted, thin filaments are clearly visible whose extremities maintain contact with the border of the stroma, and apparently with the large filaments too (Fig. 12).

Reticulocytes of lead poisoned animals show the same structures; the large filaments, however, have diameters of about 0.75 μ (Fig. 13), corresponding, on the average, to three times that observed in normal animals.

When reticulocytes are supravitally stained with Janus green B at dilutions from $1x10^{-4}$ to $5x10^{-6}$ in physiologic saline, and hemolysed in partially dried smears, according to technique A, they show large and irregular filamentous structures of accentuated electron density; thin filaments and granules are not visible at low dilutions (Fig. 14). At higher dilution of Janus green B, these immature erythrocytes show clearly all the structures (Fig. 15). Through ultrathin sections, reticulocytes stained with dye at dilutions of $1x10^{-4}$, show well contrasted mitochondria due to mercuric chloride and potassium iodide, that form a dense complex with the dye within these organelles, close to the internal membranous structures, thus reducing the intertrabecular space; this complex is also near the agglomeration of granules from 100 to 150 Å, and the enlarged endoplasmic reticulum (Fig. 16); the controls show reticulocytes with the same structural aspects as unstained cells, when submitted to the same treatment.

Reticulocytes from blood hemolysed in suspension in distilled water (technique B) contain circular forms from 0.3 to 1.0 μ as well as granules from 0.15 to 0.20 μ (Fig. 17). In these preparations, no large nor thin filaments were found. When reticulocytes have been hemolysed in hypotonic NaCl and formalin (technique C), they show forms similar to the circular ones, but not individualized; granules are also present in these red cells (Fig. 18). Ultrathin sections of these erythrocytes show that mitochondria became volumous, presenting sections of about 0.08 μ, as in Fig. 19; this initially filamentous mitochondrion shows several constrictions, due to osmotic changes.

The ultrathin sections of intact blood present reticulocytes of less electron density than the mature erythrocyte. This fact is due to the lower hemoglobin concentration in immature cells. A varying number of mitochondria is found in transverse, oblique or longitudinal sections of rods and filamentous forms; sections of greater diameters measure up to 0.35 μ in normal material. Transverse and longitudinal sections from the agranular endoplasmic reticulum were observed. The diameter of these canaliculated systems are of about 0.07 μ (Fig. 20); this reticulum is continuous to the plasmic membrane as in other kinds of cells, existing however, temporarily in reticulocytes (Fig. 21); frequently a relation of proximity to the mitochondria is observed (Figs. 7 and 20). Some cells show membranous structures, constituting the dictyosomes and vesicles of the Golgi complex as in Fig. 22; the distance between the membranes is of approximately 0.02 μ.

Quantitative considerations on erythroblast and reticulocyte structures.

Through hemolysis by technique A, structures of erythroblasts and reticulocytes may be observed integrally, thus providing a more suitable way for comparative studies on the quantity of these structural elements within these cells. In cach preparation, only cells with a higher number of large filaments were taken into account, considering that they diminish gradually during the reticulocyte maturation phase. By using technique A for hemolysis of partially dried smears, we observed that the quantity of large filaments with respect to number and extension, is higher in reticulocytes than in erythroblasts. These structures of erythroblasts with excentric nucleus may present areas of about 8.5 μ^2 (Fig. 23); in erythroblasts with the nucleus already in the extrusion phase, the area of filaments and rods decreases considerably (Fig. 24), and a value of 3.6 μ^2 was found. The newly constituted reticulocytes show an increase of large filaments, attaining twice the above values, taking into account only the areas of erythroblasts still containing an excentric nucleus; the anucleated erythrocytes of Figs. 25, 26, 27, and 28 show areas of 8.6, 12.7, 16.7, and 17.2 μ^2, respectively. These cells are still of an irregular shape due to protoplasmic movements that occur during the extrusion of the nucleus at the late evolutive erythroblastic phase.

The immature erythrocytes, hemolysed in suspension in distilled water, according to technique B, show also a higher number of circular forms in the reticulocytes when compared with erythroblasts (Figs. 29 and 30).

By the ultrathin sectioning method a confrontation was performed based on statistical values, whereas the presence of a higher or a smaller number of mitochondria depends on the level of the sections as well as on the evolutive erythrocytary stage, since mature red blood cells are destitute of any structure. Hence, in each preparation only erythroblasts and reticulocytes with a higher number of mitochondria were considered.

Determinations were done in 138 erythroblasts and 191 reticulocytes, and the percentual frequency of cells were listed in the Table; the mitochondrial area per cell has been converted into μ^2 with interval of 0.1 μ^2 and represented in the histogram of Fig 31.

Erithroblasts — 138		Reticulocytes — 191		
E B	%	Mitoch. área/cell (μ^2)	R C	%
14	10.1	0.0 —— 0.1	1	0.5
34	24.6	0.1 —— 0.2	4	2.1
32	23.9	0.2 —— 0.3	3	1.6
20	14.5	0.3 —— 0.4	13	6.7
18	13.0	0.4 —— 0.5	18	9.4
7	5.1	0.5 —— 0.6	19	10.1
5	3.6	0.6 —— 0.7	15	7.9
4	2.9	0.7 —— 0.8	17	8.9
4	2.9	0.8 —— 0.9	21	11.0
		0.9 —— 1.0	23	12.1
		1.0 —— 1.1	10	5.2
		1.1 —— 1.2	9	4.7
		1.2 —— 1.3	8	4.2
		1.3 —— 1.4	5	2.6
		1.4 —— 1.5	6	3.2
		1.5 —— 1.6	7	3.6
		1.6 —— 1.7	3	1.6
		1.7 —— 1.8	1	0.5
		1.8 —— 1.9	1	0.5
		1.9 —— 2.0	1	0.5
		2.0 —— 2.1	2	1.0
		2.1 —— 2.2	2	1.0
		2.2 —— 2.3	0	—
		2.3 —— 2.4	0	—
		2.4 —— 2.5	0	—
		2.5 —— 2.6	0	—
		2.6 —— 2.7	1	0.5
		2.7 —— 2.8	0	—
		2.8 —— 2.9	1	0.5

TABLE

Genesis and disappearance of mitochondria.

These aspects were only observed in a few reticulocytes. Several sections show mitochondrial structures apparently in formation (Figs. 32 and 33). A possible canalicular system, sectioned longitudinally, shows two parallel and convoluted membranes (Fig. 34), and it seems that the inner membrane of the whole gives rise to a double lamella by invagination; the external one may constitute the limiting membrane of a newly formed mitochondrion. A relationship between canalicules and partially formed mitochondria is well suggestive in Figs. 35 and 36. More complex inner structures of mitochondria, resulting from bifurcations or bilateral expansions of the double lamellas, were also observed.

Several aspects of mitochondrial structures, suggesting a gradual breakdown, were also found in some reticulocytes (Figs. 37 and 38). Mitochondria lose the external limiting membrane, then the internal one; finally, only double lamellas, or traces of them, are seen.

Erythrocyte.

This final stage of the evolutive erythrocytary process in characterized by its structural simplicity at the cytological level. Through the techniques A, B, or C, for osmotic hemolysis, only the remnants of the folded plasmic membrane, namely stroma, can be observed.

When red cells are fixed integrally by formalin in hypotonic saline, and embedded as above described for ultrathin sectioning, their transversal sections show biconcave forms of enlarged diameters due to osmotic changes, provoked under these conditions; these sections show electron dense granules attached one to another by thin, more or less long filaments. As a whole these elements give an aspect of an irregular meshwork with the larger and more agglomerated granules concentrated at the periphery (Figs. 39 and 40).

If erythrocytes fixed by formalin in hypotonic saline are suspended in an aqueous ormic acid solution before dehydration, they present biconcave forms, but now compact, and therefore with a high electron density and normal dimensions, as if fixed by formalin or osmic acid in an isotonic medium. This is due to a retraction of the inner components of the erythrocytes; the membrane, or stroma, remains expanded and partially detached from the erythrocytary content. Three dimensionally this membrane constitutes large sacs, enclosing the retracted material (Figs. 41 and 42).

In some erythrocytes, partially hemolysed by disruption of the membrane during manipulation previous to treatment with osmic acid, no such phenomenon has been observed.

DISCUSSION

In this study, cytologically less heterogeneous material has been used, closely related to other investigations on the isolation of a possible intrinsic maturation factor in cells of the erythrocytary series. Peripheral blood of anemic animals or

embroyonic blood is a more adequate material than bone marrow or erythropoietic liver. Early embryonic blood is also favorable since leuco — and thrombocytopoiesis does not occur at this uterine growth stage. Lead poisoned animals present a much longer maturation period in the erythrocytary series, according to Seno et al. (1953), thus allowing the detection of all structures from active phases to disappearance.

Structures observed after hemolysis.

The blood of embryos hemolysed in partially dried smears (tecnique A), allows an observation of at least three elements, relatively preserved as a whole, common to megaloblasts, erythroblasts and reticulocytes: granules that correspond to the polyribosomes, large filaments or rods, and thin filaments well defined in Figs. 2, 12, 13, and 15, constituting the agranular endoplasmic reticulum, better examined in ultrathin sections. The large filaments are identified as mitochondria in accordance with the following facts: 1 — they are stainable with ferric hematoxylin and acid fucsin for mitochondrial evidence by the methods of Regaud and Altmann, respectively; 2 — they decrease quantitatively as the reticulocyte develops to a mature erythrocyte, the same ocuring with mitochondria; 3 — in erythroblasts as well as in reticulocytes of lead poisoned animals, they increase in diameter, on the average, about three times that of normal animals, comparing Fig. 13 with others; the same enlargement is observed in mitochondria as seen in Fig. 10 in comparison with Fig. 20 of normal animal reticulocyte (Vallejo-Freire and Brunner. 1958); 4 — they are morphologically comparable with the mitochondria in fibroblasts from tissue culture medium examined as a whole (Porter, 1953); 5 — they increase in size when in contact with a hypotonic medium (Brunner and Vallejo-Freire, 1956); the same occurs with mitochondria as observed by Lewis and Lewis (1915), Zollinger (1948), and Vallejo-Freire and Brunner (1958).

Partial drying of the smears preserves relatively the large filaments or mitochondria without coagulation of the hemoglobin, now existent in the polychromatiphilic erythroblasts, thus allowing hemolysis (technique A); when hemolysis is performed in suspension in distilled water (tecnique B), the mitochondria increase in size up to disintegration, resulting circular forms (Figs. 4, 17, 29, and 30) as observed by Bernhard et al. (1949). Intermediary forms may be obtained when hemolysis is performed according to technique C, in a less hypotonic medium containing formalin (Fig. 18). These structures can be observed in sections, as in the reticulocyte shown in Fig. 19, whose inicially filamentous mitochondrion increased in size, consequently giving rise to sectors limited through constrictions. The disruption of these membranous septa gives origin to circular forms constituted by the limiting mitochondrial membrane, traces of the inner membrane and double lamellas.

Reticulocyte granules, ranging from 0.10 to 0.15 μ, clearly visible in Figs. 11, 12, 17, and 18, did not change morphologically when hemolysed by any one of the three techniques, A, B, or C. Each granule is morphologically similar to the polyribosomes described by Warner et al. (1962) as tetra, penta, and hexamerous forms, from lysed reticulocytes, constituting the site of globin synthesis. These granules disintegrate partially when treated with ribonuclease; remnants

possibly represent the proteic part of the ribosomes. In reticulocytes stained with Janus green B, these granulations precipitate with the dye, as will be shown later.

Mitochondria and "Substantia granulo-filamentosa"

Our observations on reticulocytes treated with Janus green B agree with those of Kosenow (1952) which refer to the concentrations of the dye used. In reticulocytes treated with a $1x10^{-4}$ dilution, the structures are detected with some difficulty at optical microscopy. Figure 14 shows a reticulocyte supravitally stained with this dilution, and hemolysed according to technique A, where only a single, long and pronounced electron dense structure can be observed. In these preparations the large filaments or mitochondria, the polyribosomic granules and the thin filaments or endoplasmic reticulum, agglomerate with the dye. Reticulocytes treated with higher dilutions, e.g., $5x10^{-6}$, show clearly all those structures, independent one of each other (Fig. 15). Ultrathin sections of the stained reticulocytes show mitochondria of increased contrast after $HgCl_2$ and KI fixation. The membranes, mainly the double lamellas are thicker than in controls, reducing therefore the intertrabecular space (Fig. 16). Surrounding these mitochondria, sections of the enlarged endoplasmic reticulum and agglomerated particles were found, identified as ribosomes by their density and dimension.

Lazarow and Cooperstein (1953) suggest that the cytochomoxidase system takes part in the staining of mitochondria with Janus green B; this enzymatic system is probably responsible for the specificity of this staining. Rubinstein et al. (1956) determined cytochromoxidase in the mitochondrial fraction of rabbit reticulocytes.

Mitochondria are the predominant structural elements in immature erythrocytes; they may therefore be regarded as the essential component of the "Substantia granulo-filamentosa" in those cells stained with Janus green B, considering that the dye fixes itself within the mitochondria, and that the resultant ribonucleoprotein precipitate surrounds these organelles. Thus, a pre-existent structure is indirectly evidenced at the optical microscope. The concept of "Substantia granulo-filamentosa" is restricted merely to the mitochondria of unstained reticulocytes, only hemolysed after partial drying of the smears.

Observations on some evolutive phases.

The embryonic, megaloblastic, or primordial erythroblastic series are distinguishable from the definitive or secondary erythroblastic series through their cells of larger dimension, contain hemoglobin in higher concentration, even at the nucleated phases (Fig. 5). In comparison with the definitive erythroblasts there is an evolutive cytoplasmic anticipation in relation to the nucleus.

According to Jolly (1923) the definitive erythroblasts arise in 14-day rabbit embryos. In spite of a low mitosis frequency, and the reduced examination field at the electron microscope, a possible basophilic erythroblast has been detected, showing a mitotic figure at an intermediary phase between prophase and metaphase. In this latter stage, the nuclear membrane disappears, and the individualized chromosomes arrange themselves at the equatorial region of the cell. Fig. 6 shows

a nuclear membrane with long interruptions, and at one point, one mitochondrion arrange itself close to one of the extremities of this membrane. The well defined chromosomes, as a morphological entity, are less electron dense than cytoplasm, and apparently will dispose themselves at the equatorial region of the erythroblast. These dislocations are accompanied by cytoplasmic movements which confer to the cell an irregular configuration.

The erythroblast in Fig. 7, more frequently observed in the preparations, may be considered as polychromatophilic due to its relative dense cytoplasm, containing now synthesized hemoglobin. Some of the mitochondria arrange themselves close to the endoplasmic reticulum whose function probably consists in conducting material between those organelles and blood plasma. Fig. 21 shows a reticulocyte whose plasmic membrane is continuous with the endoplasmic reticulum, constituting a pore of about 180 Å. These pores exist only temporarily and disappear during maturation.

In some hypochromic anemias, morphological aspects of hemoglobin biosynthesis is easier to be detected because of the slowness or interruption of the process in which iron, originating from the reticular cells, participates in the formation of the heme prostetic group. Using this material, Bessis and Breton-Gorius (1957) detected part of the iron cycle through electron microscopy, from its incorporation into the erythroblast, as ferritin, its allocation in mitochondria at the intertrabecular space, to finally, its dispersion in the cytoplasm in form of fine grains, by disruption of the mitochondrial membranes. Rimington (1957) attained from a suspension of liver cell mitochondria, some stages of hemoglobin biosynthesis. In normal, immature red cells, these enzymatic reactions are rapid; it is, therefore, difficult to detect some of these aspects at the electron microscope. Fig. 8 shows a fine ferritin granulation disposed between membranes of mitochondrial nature. The mitochondria decrease quantitatively up to the acidophilic stage of erythroblasts, as observed by Sorensen (1960) and confirmed by our observations. However, these organelles increase in number in the less evolutive phase of reticulocytes, as shown by comparative examinations.

The nucleus of erythroblasts (Fig. 7) is heterogenously electron dense, presenting regions of the same density as the cytoplasm. This material is often continuous to the cytoplasm through pores of the double nuclear membrane, a fact also verified by Grasso et al. (1962), and Skutelsky and Danon (1967) in mouse erythroblasts. Simpson and Kling (1967) reported the same fact for dog erythroblasts, and suggested the presence of hemoglobin in the nucleus. The same observation was also described in amphibian erythroblasts, where the nuclear hemoglobin arranges itself in a paracrystalline form, an arrangement not suggested for cytoplasmic hemoglobin (Fawcet and Witebsky, 1964). Through cytochemical reactions and absorption determinations at a wave length of 4047 Å in human and rat erythrocytes, Carvalho (1953) suggested the presence of the heme group in the nucleus. Davies came to the same conclusion through measurements with the same wave length and by electron microscopy of bird and amphibian erythroblasts.

A few hamster erythroblasts showed a less electron dense material extruded from the nucleus into the cytoplasm. According to Simpson and Kling (1967) this Feulgen positive material could be the result of a karyolytic process, giving rise

to Jolly bodies. Summarizing, there exist in the nucleocytoplasmic relationship of erythroblasts, intranuclear regions of the same electron density as in cytoplasm, which absorb wave lengths of 4047 Å, corresponding, therefore, to the heme group, and sometimes less electron dense cytoplasmic regions, deriving from the nucleus and containing desoxiribonucleic acid.

At the beginning of the extrusion process the erythroblast shows active contraction and expansion movements, and the nucleus disposes itself excentrically up to its location within a cytoplasmic lobe (Fig. 9). These movements have been studied through microcinematography by Comandon and Jolly (1923), and by Bessis and Bricka (1952). Observations of this phenomenon are relatively frequent in blood smears, stained by usual methods, of embryos, or of animals with erythroblastosis (Albrecht, 1951; Brunner, 1968). The here observed agglomeration of some mitochondria in close proximity to the nuclear membrane, is not to be seen in the preceding phases. These aspects were also observed by Sorenson (1960), Grasso et al. (1962), Skutelsby and Danon (1967), Orlic et al. (1965). Simpson and Kling (1967) suggest that these mitochondria provide the energy necessary for extrusion; they also may be related to the constitution of a new membrane which is required at the site of the nuclear extrusion, according to Skutelsky and Danon (1967). The nuclear membrane pores disappear, and the nucleus becomes homogeneously electron dense. However, some authors, as Skutelsky and Danon 1967) described a heterogeneous nucleus, apparently liberated.

The nuclear extrusion process lasts for at least ten minutes, from inicial contractions to definite elimination, under examination conditions (Bessis and Bricka, 1952).

With exception of our findings and the ones of Zamboni (1965), the supposedly liberated nucleus seems to be surrounded by a thin cytoplasmic layer, containing one or more mitochondria (Grasso et al., 1962; Orlic et al., 1965; Simpson and Kling, 1967; Skutelsky and Danon, 1967, and Weiss, 1965). Details correlated with this phenomenon might be elucidated by serial sectioning of those erythroblasts or by an examination of a suspension of nuclei now liberated, and without cells. Depending on the level of sectioning, the nucleus may appear as already liberated; it must be considered that the erythroblasts present at this stage a well pronounced irregular configuration. It is quite possible that the few nuclei devoid of any cytoplasmic layer, observed by us and by Zamboni (1965) are the result of an artefact. Further investigations of numerous and totally isolated nuclei, may supply more detailed information.

The active movements still persist in the reticulocytes, as shown in Fig. 10. After this new evolutive stage the cell resumes its normal configuration which may be spherical or ovid, as shown in Fig. 20. At these evolutive stages the mitochondria increase in number and length, and assume considerably long, filamentous forms. At no time, erythroblasts show long structures similar to those observed during the reticulocytary phase.

By hemolysis method, technique A, and through comparative analysis, it can be verified that the number of mitochondria is higher in reticulocytes than in erythroblasts (Figs. 23-28). In this way however, mitochondria, disposed under or over the nucleus, are not detected, an error partially eliminated through the

replica method that shows that rarely erythroblast mitochondria are found over the nucleus. On the other hand, the majority of erythroblasts, used for such comparisons, presented a nucleus practically eliminated, a condition under which all organelles may be observed.

Tecnique B for hemolysis in suspension in distilled water, has also been used to compare the number of circular forms, resulting from the disintegration of mitochondria of those nucleated and anucleated cells. The results are the same (Figs. 29, 30).

The less developed reticulocytes (Figs. 28 and 30) present accentuated high amount of filamentous or circular forms, compared to the more evoluted and more frequent cells. This aggrees with the statistical values obtained with the ultrathin sectioning method. Reticulocytes, presenting mitochondria during the formation and growth process, are less frequent too, as will be shown below. Both observations suggest that the increase as well as the decrease of mitochondria is a relatively rapid process within the anucleated cells, thus reducing the probability of a frequent detection of these aspects.

Through ultrathin sections of erythroblasts and reticulocytes, values on mitochondrial areas per cell, were obtained and distributed, according to the histogram in Fig 31. This histogram shows a different distribution of classes among the two kinds of evolutive cells, which is in aggreement with the observations aforementioned, about those cells examined by the hemolysis method. The comparative histogram, concerning mitochondrial area, is more significant, although comparisons with respect to the number of these organelles per cell show also differences. A mitochondrion may be sectioned at variables planes, depending on its disposition within the cell, resulting either a diminute or a very large area.

Figure 31 shows high erythroblast frequencies, corresponding to small mitochondrial areas of about 0.30 μ^2; the frequencies decrease at about 0.90 μ^2, the highest value obtained. The inverse occurs in reticulocytes, up to 0.95 μ^2, although the larger areas of about 2.85 μ^2 correspond to low frequencies.

The increase of mitochondrial structures in reticulocytes evidently results from the formation of new organelles, or the growth of the pre-existent ones; it is quite possible that both phenomena are involved (Figs. 32-36). There are no data on the origin of mitochondria from canalicules as suggested for reticulocytes. Hoffman and Grigg (1958) reported the formation of mitochondria in onion root tip cells, rat thymus, and mouse lymph nodules, frequently ocurring during mitosis, what accounts for their similarity. The relationship between nuclei and mitochondria in those cells is the same; it has been verified that the external nuclear membrane is continuous to the mitochondrial membrane, suggesting that mitochondria are originating from it. The same relationship has been observed by Brandt and Pappas (1959) in the ameba *Pelomyxa carolinensis,* frequently in nuclear evagination. In other cases, the increase of mitochondrial number is due to the division of pre-existent organelles, as in rabbit oocytes (Blanchette. 1961). An important fact is Steinert's observation (1961) on the rhytydia phase of *Tripanosoma mega* from "in vitro" cultures, where the mitochondrion is derived from the Feulgen positive kinetoplast. After the completion of its development,

the mitochondrion is liberated, individualizing itself. This could be related to the findings of Chèvremont (1963) in fiblroblasts, whose mitochondria contain DNA, detected by Feulgen reaction. Further studies have shown that an incorporation of ^{3}H-thymidine occurs in those cells. These not yet generalized facts could explain the cytogenetical phenomena related to mitochondria.

The mechanism of hemoglobin biosynthesis in reticulocytes is not yet clear, due to the lability of the messenger RNA, only synthesized in the presence of DNA, according to the hypothesis on nucleocytoplasmic genetic relations of Jacob and Monod (1961). No DNA has been yet detected in reticulocytes. Some authors, however, as Nathans et al. (1962) suppose, that in such case, the messenger RNA would be well stable and synthesized at the nucleated phase. They noted that the incorporation of aminoacids is higher than the degradation of RNA, as determined by liberated ^{32}P measurements. Burny (1962) concluded that there exists RNA synthesis in reticulocytes, because ^{32}P was incorporated and distributed to the messenger and transfer RNA. On the other hand, Seno et al. (1963) verified that an incorporation of labeled uridine did not occur in these erythrocytes, concluding therefore, that any kind of RNA may be synthesized. Pinheiro et al. (1963) reported identical results, demonstrating that also labeled citydine and adenin are not incorporated at this erythrocytary phase.

The increase of mitochondrial structures at the reticulocytary phase coincides with higher capacity for hemoglobin synthesis at this evolutive phase, according to the microspectrophotometric determinations of Seno (1958). This aggress also with the equal or higher degree of ^{59}Fe incorporation by those erythrocytes in relation to erythroblasts, as observed by autoradiographic determinations in human bone marrow cultures (Lajtha and Suit, 1955; Suit et al., 1957). In rabbit bone marrow, the degree of ^{59}Fe incorporation is the same in both evolutive phases, but it increases highly in reticulocytes of the peripheral blood (Suit et al., 1957). Evidently these three facts are correlated with hemoglobin biosynthesis, considering that mitochondria take an important part in the biochemistry of these immature erythrocytes.

Reticulocytes of normal animals, intoxicated guinea-pigs, and embryos, show a membrane system, consisting of vesicles and dictyosomes (Fig. 22). This structural set composes the Golgi complex, as in other kinds of cells. The observation that the region which involves the contractile vacuoles of some protozoans, reduces the osmic acid, as occurs in the vertebrate Golgi complex, leads Nassonov (1924) to suggest the existence of a homology between these two structures. This hypothesis has been confirmed by Gatenby et al. (1955) through electron microscopic examination of protozoans and spongeans. This apparent homology involves the Golgi complex, as a control system for the osmotic equilibrium in cells, although other functions were atributed to this structure. It is possible that in reticulocytes this structure is involved in the loss of sodium, potassium, calcium, and phosphorus, whose concentrations are higher than the ones in mature red blood cells, according to Kay (1930), Rappaport et al. (1944), and Kruszynski (1955). Consequently, the elimination of water which is 5% higher in reticulocytes (Gaffney, 1957; Lowenstein, 1959) occurs too.

Reticulated, concentric or convoluted lamellar structures were described in basophilic myelocytes (Pease, 1956), epithelial cells from convoluted proximal mouse kidney tube (Clark, 1957), L-60 cells (Dales and Siminovitch, 1961),

and in epithelial cells from salamander branch (Schulz and de Paola, 1961). The function of these structures is unknown, just as the one rarely observed in reticulocytes from lead poisoned animals (Brunner and Vallejo-Freire, 1964). Similar structures were also found in smooth human megacolon fibers, and less frequently in the normal colon (Kiss, 1968). Jones (1966) reported an intramitochondrial formed lammellar structure, in early erythroblasts from fetal rats, arising from degenerated mitochondria, and followed by its extrusion from the cell, suggesting that this might be a normal secretory lipoprotein process.

All these structures, above described, disappear gradually as occurs with mitochondria. The external membrane is the first to disappear, followed by the internal one, and finally the double lamellas, clearly to be seen in Figs. 37 and 38. These less frequent reticulocytes from lead poisoned guinea-pigs does not present any mitochondria of increased volume nor disrupted lamellas, morphologically characterizing those immature erythrocytes (Fig. 10). The reticulocytes in Figs. 37 and 38 present a cytoplasm of low electron density due to its low hemoglobin content, and its mitochondria present themselves in degeneration, their function having ceased by now. According to Rouiller (1960) the degenerated mitochondrial forms vary in aspect, showing a disrupted or a single integral membrane. In the malignant melanoma of the ciliary body, similar aspects were found (Toledo, in press). Jones (1966) showed degenerated mitochondria of early erythroblasts, with the same forms as shown in reticulocytes.

The final stage of the evolutive erythrocytary process is characterized by distinctly shaped red blood cells. The obtained results confirm indirectly the existence of a structure at the molecular level. Its hemoglobin content (Bessis, 1961) attains about 33 per cent, e.g., in human blood cells.

Several authors suspect that there might exist a certain order in the interior of red blood cells. Dervichian et al. (1947), through X-ray diffraction of horse erythrocytes, came to the conclusion that there is a paracrystalline arrangement of hemoglobin molecules. Perutz (1948) suggests that the inner components of erythrocytes do not arrange themselves at random, in view of the molecular weight of hemoglobin and the red cell volume. Hitherto, it has also been shown that hemoglobin can crystallize in the red cells of some animals under certain conditions (Ponder, 1945). Pauling et al. (1949), in aggreement with the conclusions of other investigators, ascribe falciform anemias to a molecular intraerythrocytary disease. However, it seems that physicochemical surface phenomena, due to several factors in the blood plasma, are responsible for the shape of erythrocytes, as shown by Furchgott and Ponder (1940), Ponder (1948) and Bessis and Bricka (1950). Spherical or sea urchin forms were restored to their discoidal form by treatment of washed red cells, with normal plasma, albumin, glucose, and other substances; this was even observed in ghosts or stromas, void of hemoglobin. Besides these facts it is also known that the peripheral region of erythrocytes is more dense than the more internal one (Bessis and Bricka, 1950), where hemoglobin exists as a solution of high concentration. The periphery is constituted of lipoproteins and glucosides with only a small amount of hemoglobin (Ponder, 1948). Thus, the ultra-structure cannot be regarded merely as a sac, containing hemoglobin, or as a spongework in which hemoglobin is contained. The experimental results support the hypothesis that the structure seems to be something between these two alternatives (Bessis, 1961).

Guinea-pig erythrocytes, fixed by formalin in hypotonic saline, present an increased volume, maintaining however its discoidal-biconcave form (Figs. 39 and 40). These red cells show a peripheral electron dense region, limited by a very thin film, and a less dense internal region. The sections show an irregular meshwork, constituted by granules, larger and more agglomerated at the periphery than the ones from the central region; very thin filaments, long or short, link the granules to each other. When those erythrocytes are suspended in an aqueous osmic acid solution, after "fixation" with formalin, they resume their normal dimensions through retraction of their content, which becomes compact and highly electron dense, their membrane remaining stretched (Figs. 41 and 42). This aggrees with the observations of Dervichian et al. (1947), that the membrane might be independent of the inner erythrocytary structure. If erythrocytes were partially hemolysed through disrupture of the membrane, this retraction, provoked by osmic acid, did not occur, what might be explained by the partial loss of their content.

These observations aggree also with the aforemenitioned experimental results, suggesting that, at least in part, besides external factors, also an internal molecular arrangement would be responsible for the shape of red blood cells.

CONCLUSIONS

1. Megaloblasts, erythroblasts, and reticulocytes hemolized in partially dried blood smears, and integrally examined, present large filaments and rods identified as mitochondria, and thin filaments, corresponding to the endoplasmic reticulum and polyribosomic granules, diffusively distributed.

2. When hemolysis is performed in suspension in distilled water, without partial drying, the filamentous mitochondria disintegrate, giving rise to circular forms. Intermediary structures between the filamentous and circular forms are observed when hemolysis is interrupted.

3. Erythroblasts and reticulocytes, supravitally stained with Janus green B show an agglomeration of endoplasmic reticulum, and a dye-polyribosomic precipitate, surrounding the mitochondria. As a whole, this constitutes the so-called "Substantia granulo-filamentosa". This concept is restricted to the filamentous or rodlike mitochondria of those unstained cells, only hemolysed in partially dried smears.

4. The nucleus of erythroblasts shows equal electron dense regions as cytoplasm, continuous with the latter through the pores of the nuclear membrane. This fact and the specific absorption determinations at wave length of 4047 Å (Carvalho, 1953; Davies, 1961), or 4060 Å (Seno, 1958), suggest the presence of hemoglobin or of the heme-group within the nucleus.

5. The nucleus of erythroblasts is eliminated by extrusion, a process not yet completely elucidated. Aspects which may suggest a karyolysis or karyorrexis process, were rarely observed.

6. The number of mitochondria in the less evoluted reticulocytary stage is higher than in the nucleated phase. This coincides with the higher isotopic iron

uptake (Laitha and Suit, 1955; Suit et al., 1957), and with the higher activity of hemoglobin biosynthesis in reticulocytes in relation to erythroblasts (Seno, 1958).

7. The increase in mitochondrial number in early reticulocytes is due to the formation of new organelles, arising from canalicules. This increase might also result through growth of the already existent mitochondria.

8. Erythroblasts and reticulocytes show an endoplasmic reticulum close to the mitochondria, and continuous to the plasmic membrane, constituting pores at the cell surface. The function of this canalicular system is supposed to conduct material between the inner structures, mainly the mitochondria, and blood plasma.

9. The Golgi complex observed in reticulocytes is identical with that of other kinds of cells. It is quite possible that its function is related to the salt-water equilibrium between cells and blood plasma.

10. All these structures disappear gradually as the concentration of hemoglobin increases. Mitochondria disappear through sucessive loss of the external membrane, followed by the internal one, and finally by the double lamellas.

11. The mature erythrocyte has probably an internal structure at the molecular level, independent of the stroma or the limiting membrane. This molecular arrangement would be, in part, responsible for the discoidal and biconcave form of the erythrocyte.

RESUMO: A série eritrocitária do sangue periférico de embriões de coelho e hamster foi submetida a estudos ao nível ultraestrutural pelos métodos de hemólise, segundo três técnicas, e através de cortes ultrafinos. Pelo método de hemólise em esfregaço foi constatada a presença de mitocôndrios filamentosos ou em bastonete, que se desintegram em formas membranosas circulares quando a hemólise se processa em suspensão em água destilada.

Após a extrusão nuclear, geralmente ocorrente no eritroblasto ortocromático, acompanhada de movimentos citoplasmáticos ativos de contrações e expansões, foi constatado um aumento da quantidade de mitocôndrios, fato coincidente com a maior intensidade de síntese de hemoglobina, segundo verificações de outros autores.

Além dos organelos participantes na síntese hemoglobínica, como mitocôndrios e poliribosomos, foi observada ainda a presença do complexo de Golgi, provàvelmente com função no equilíbrio da água e sais no reticulócito, no decorrer da maturação. Foi constatada a presença de vesículas e de retículo endoplasmático liso.

A maturação final do eritrócito caracteriza-se pelo aumento crescente na concentração em hemoglobina com o desaparecimento gradual de tôdas as estruturas citoplasmáticas e a aquisição da forma discoidal bicôncava característica, constituida apenas por uma estrutura ordenada, paracristalina, ao nível molecular.

UNITERMOS: Maturação eritrocitária.

ACKNOWLEDGEMENTS.

The author is indebted to Prof. Dr. Paulo Sawaya, Director of the Departamento de Fisiologia Geral e Animal at the Universidade de São Paulo, for his helpful orientation during the course of this work.

To the sucessive Directors of the Instituto Butantan, Dr. Aristides Vallejo-Freire, Prof. Dr. Lucio Penna de Carvalho Lima, and Dr. Jandyra Planet do Amaral for material support, and for facilities and conditions provided during the realization of this work.

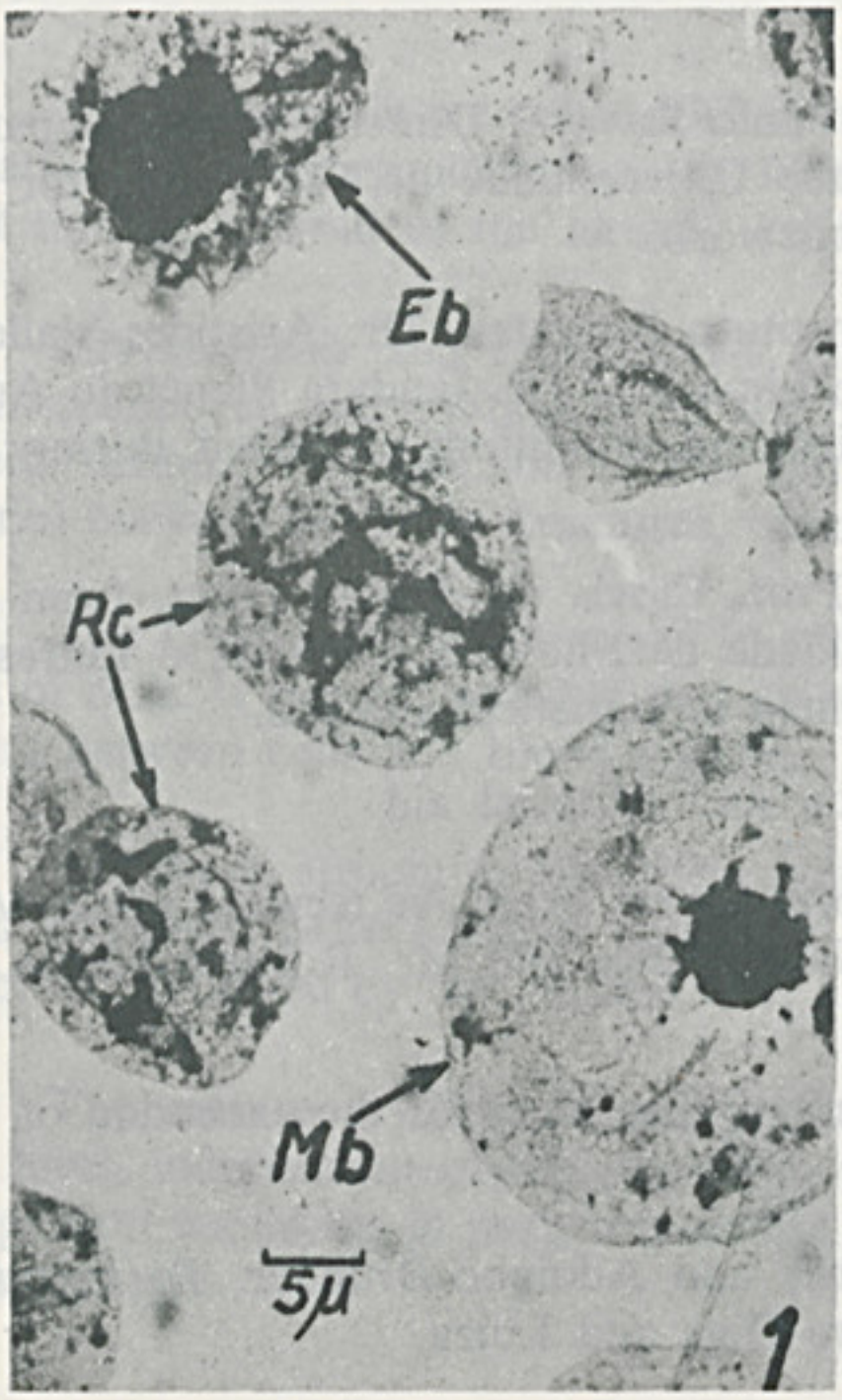
To Prof. Ary J. Dias Mendes and Prof. Clóvis de Araújo Peres, from the Departamento de Estatística of the Faculdade de Filosofia, Ciências e Letras at the Universidade de São Paulo, for their help in statistical analysis.

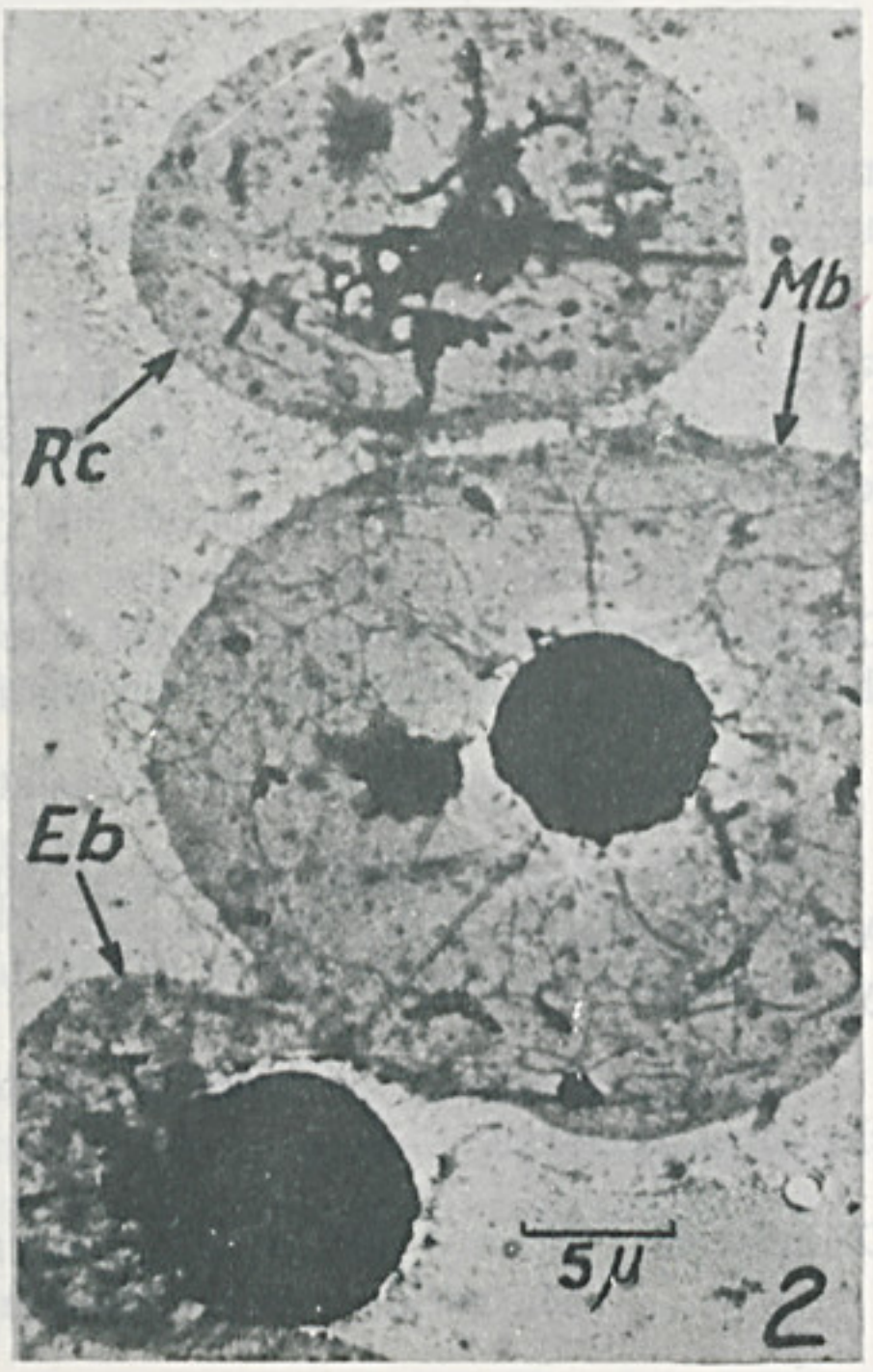
To Mrs. Sibylle Heller for translation and editorial aid.

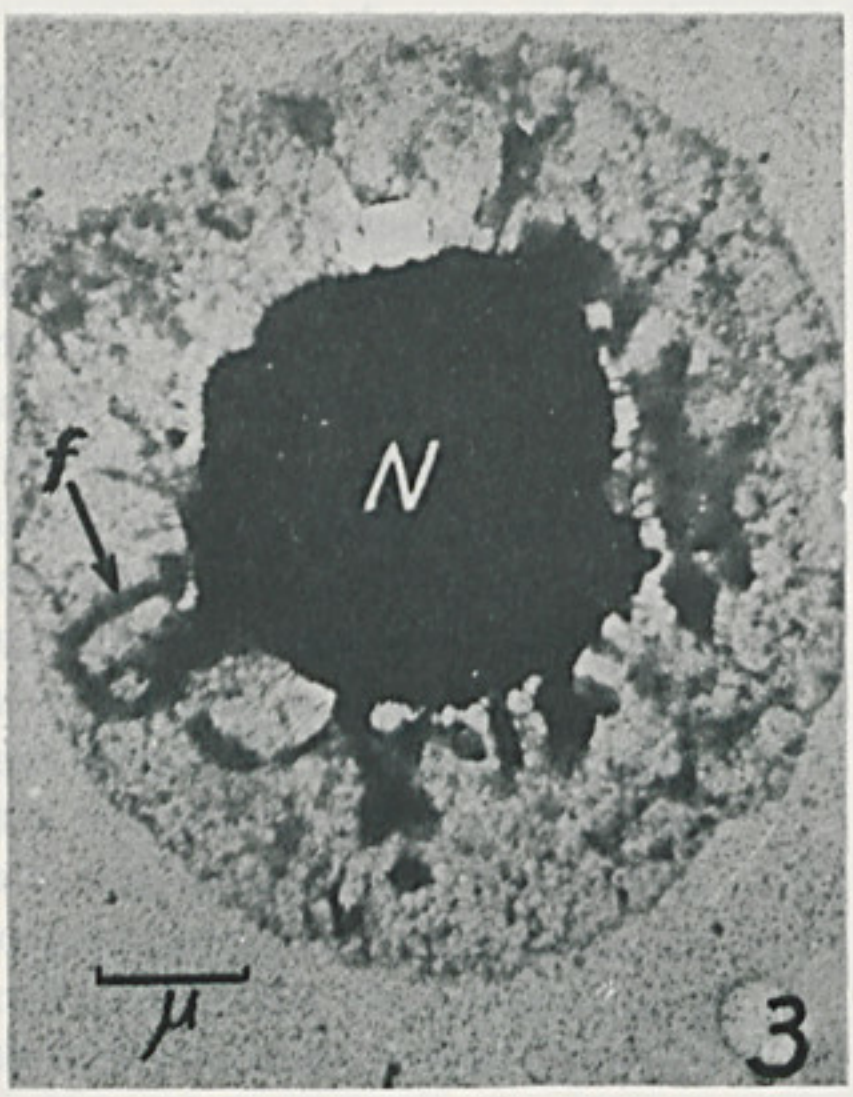
To Mr. José Antônio de Toledo Bilotta for his extensive tecnical work; Miss Alice Henrique Pedreira, Mr. Eduardo Navas Neto, and Mr. Roberto Navas for typewriting.

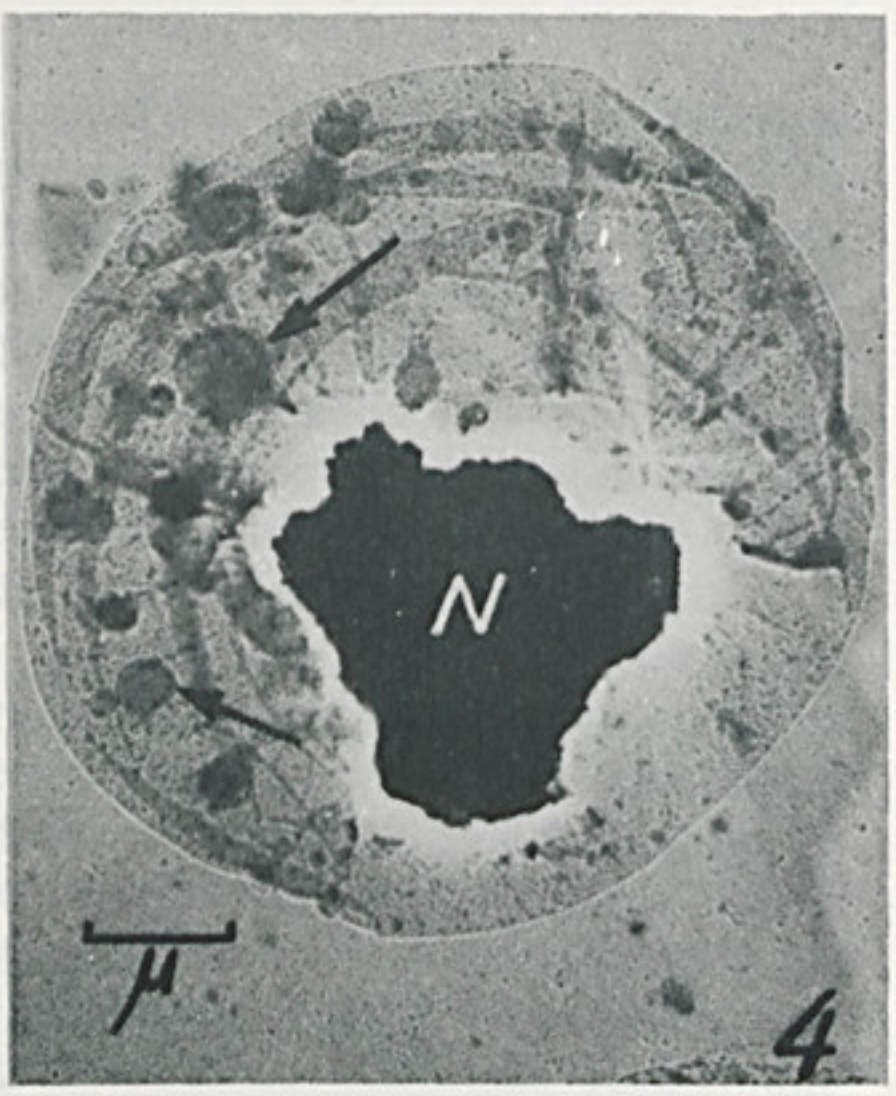
To Mr. Taufic Aued, and Mr. Antônio Seixas Neto for the execution of the electromicrographs.

Dedication: To my parents, Olga Brunner and Adolpho Brunner. To my wife, Margarida and my daugthers Olga and Luiza.







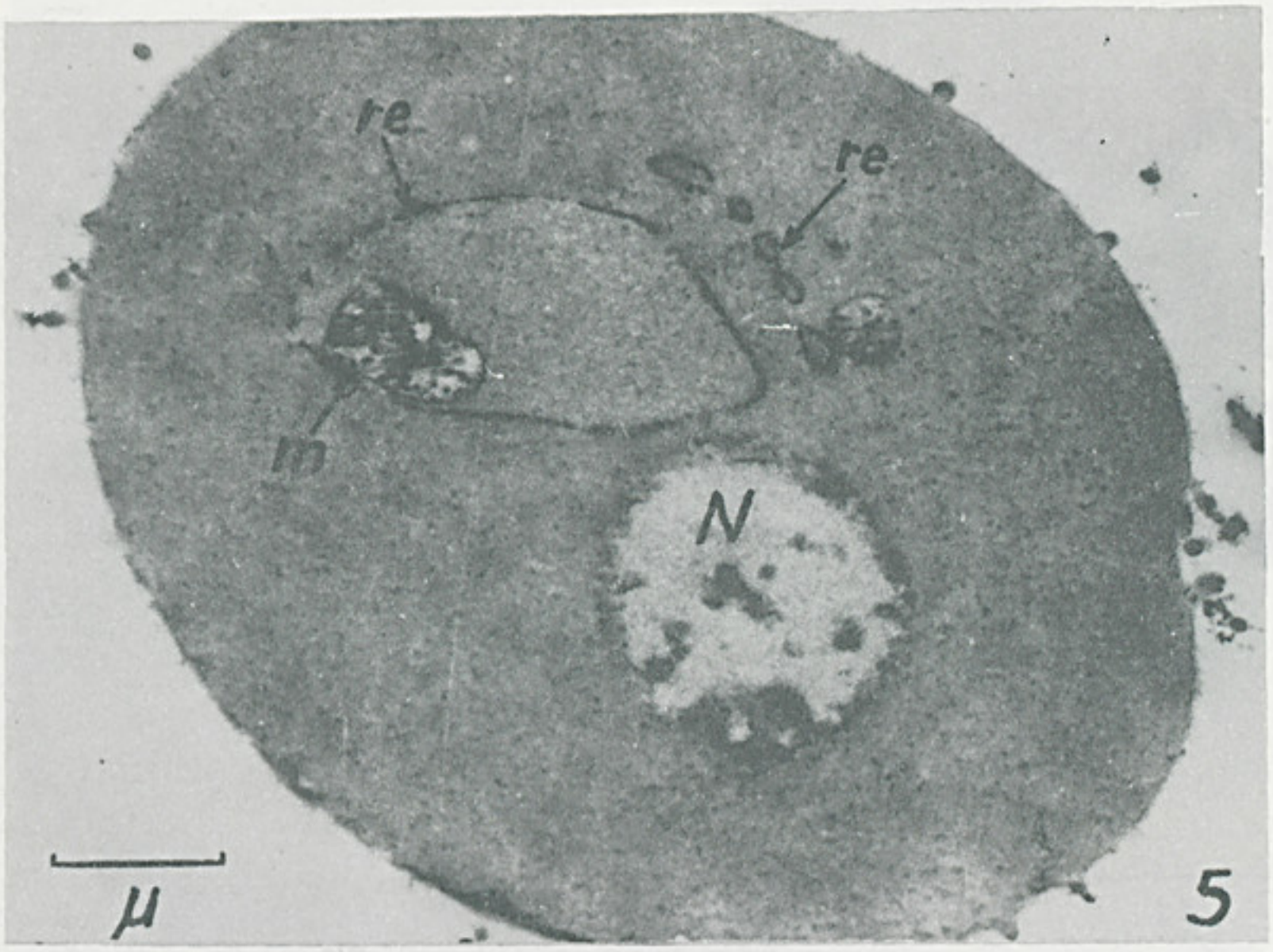


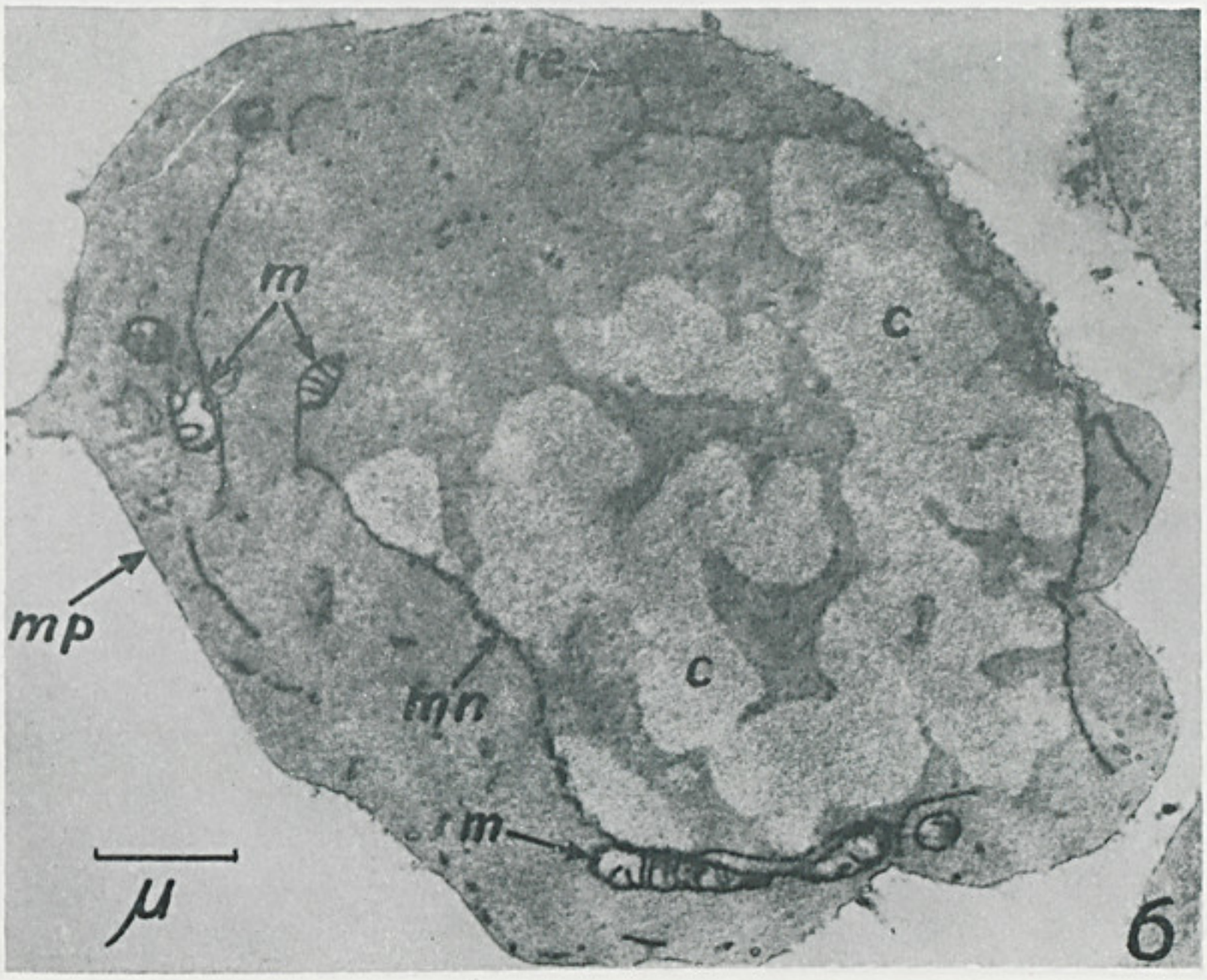
Hemolysed embryo blood.

Figs. 1 and 2. — *Mb* — megaloblasts; *Eb* — erythroblasts; *Rc* — reticulocytes. Technique A.

Fig. 3 — Erythroblast. *N* — nucleus; *f* — large filaments. Technique A.

Fig. 4 — Erythroblast. *N* — nucleus; *arrows* — circular forms. Technique B.

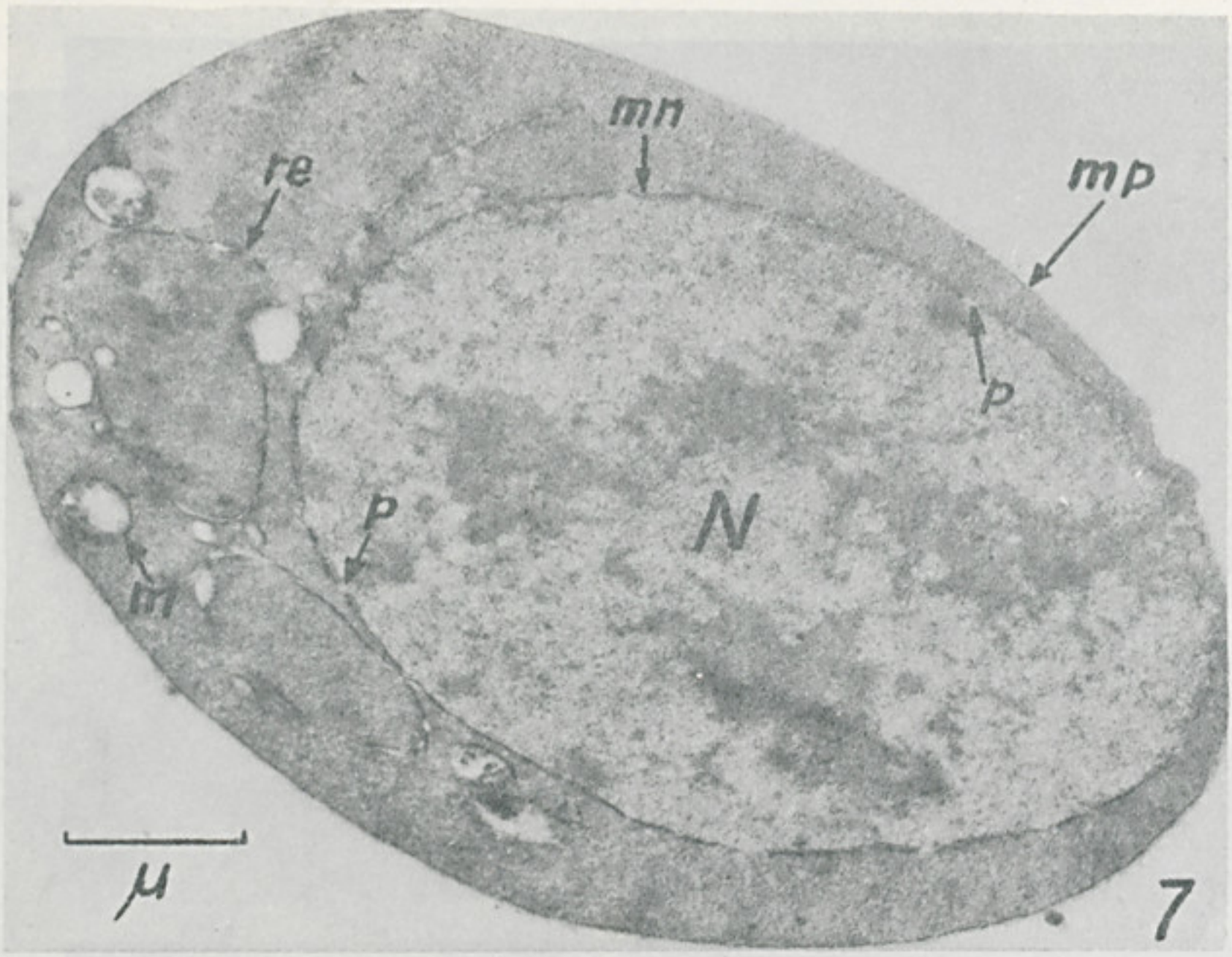


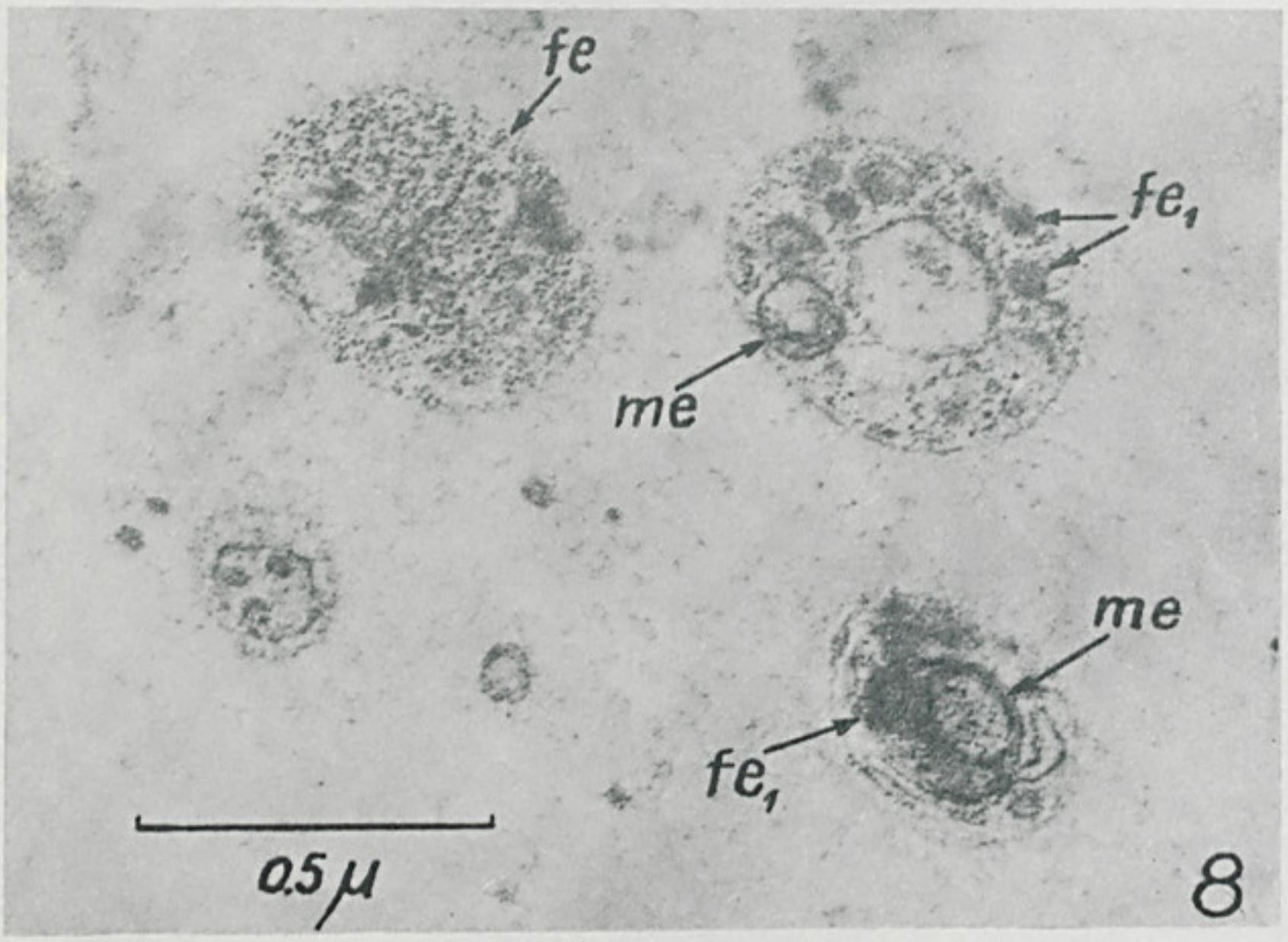


Embryo blood. Thin sections stained with lead acetate. Epon 812.

Fig. 5 — Megaloblast. *N* — nucleus; *m* — mitochondria; *re* — endoplasmic reticulum:

Fig. 6 — Mitotic erythroblasts, *c* — chromosomes; *mn* — nuclear membrane; *m* — mitochondria; *re* — endoplasmic reticulum; *mp* — plasmic membrane.

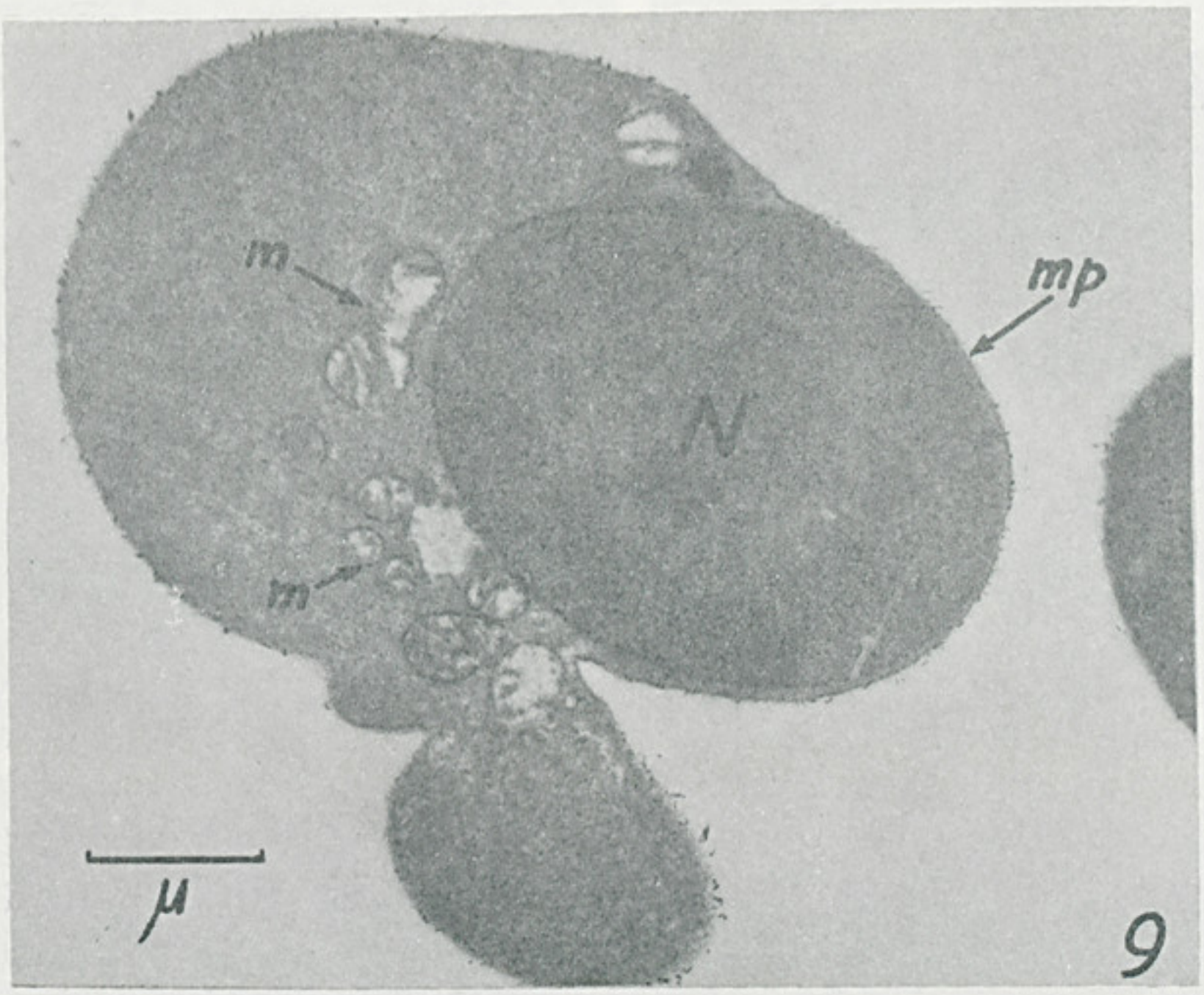


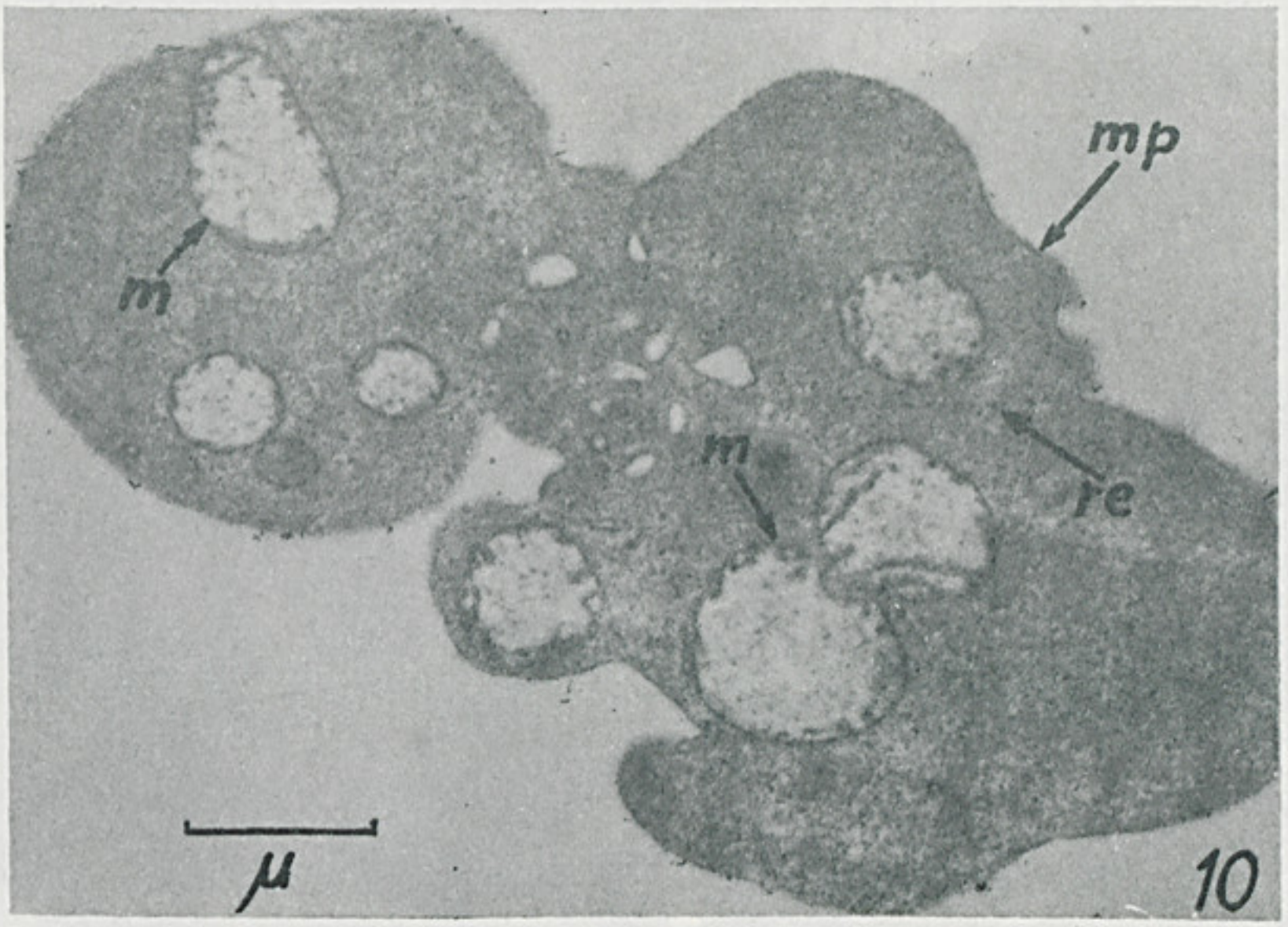


Embryo blood. Thin sections stained with lead acetate.

Fig. 7 — Polychromatophilic erythroblast. *N* — nucleus; *p* — pores; *mn* — nuclear membrane; *m* — mitochondria; *re* — endoplasmic reticulum; *mp* — plasmic membrane. Epon 812.

Fig. 8 — Erythroblast. *fe* — ferritin grains; *me* — mitochondrial membrane; fe_1 — electron dense agglomerated grains. Crystic-araldite.

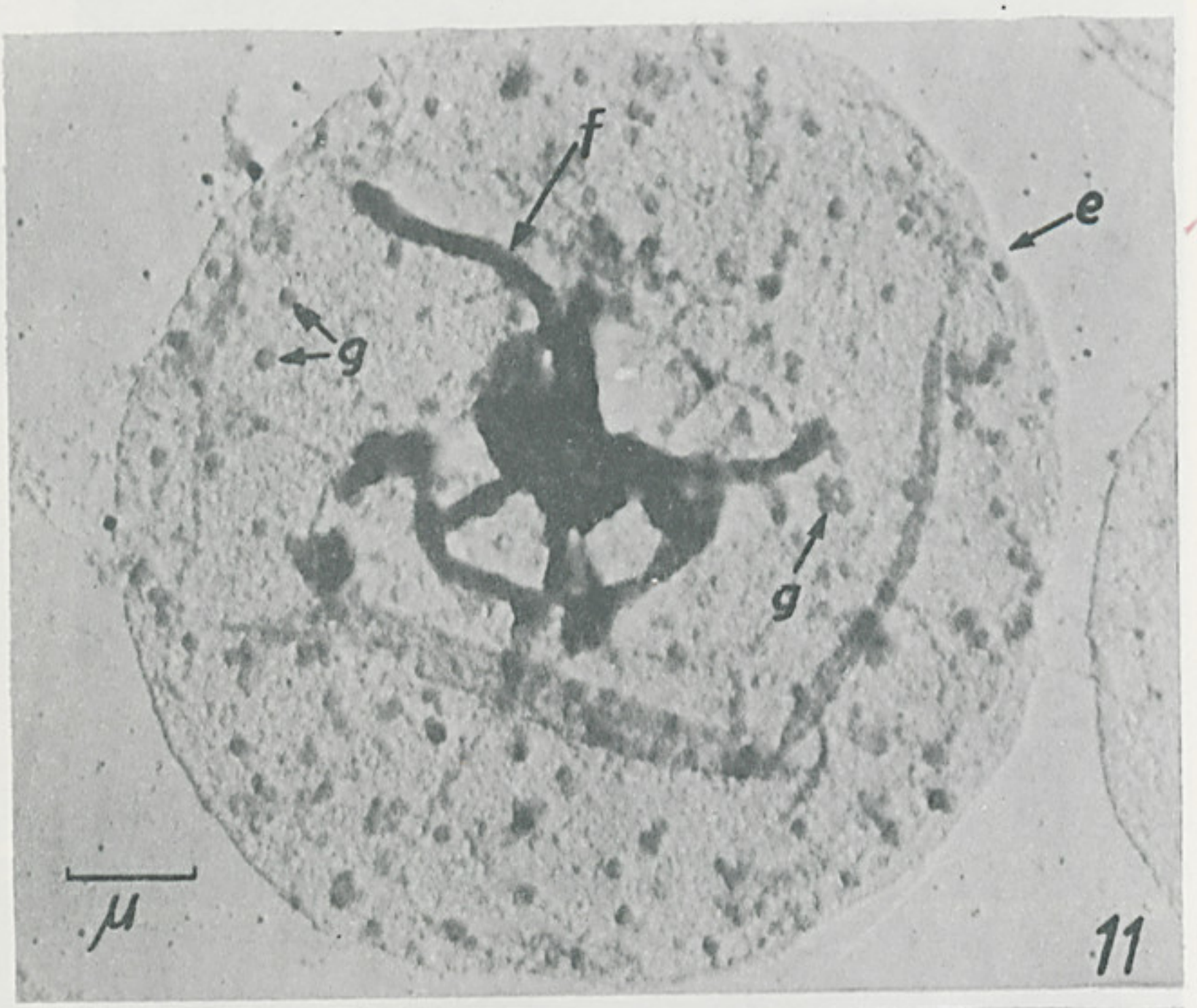


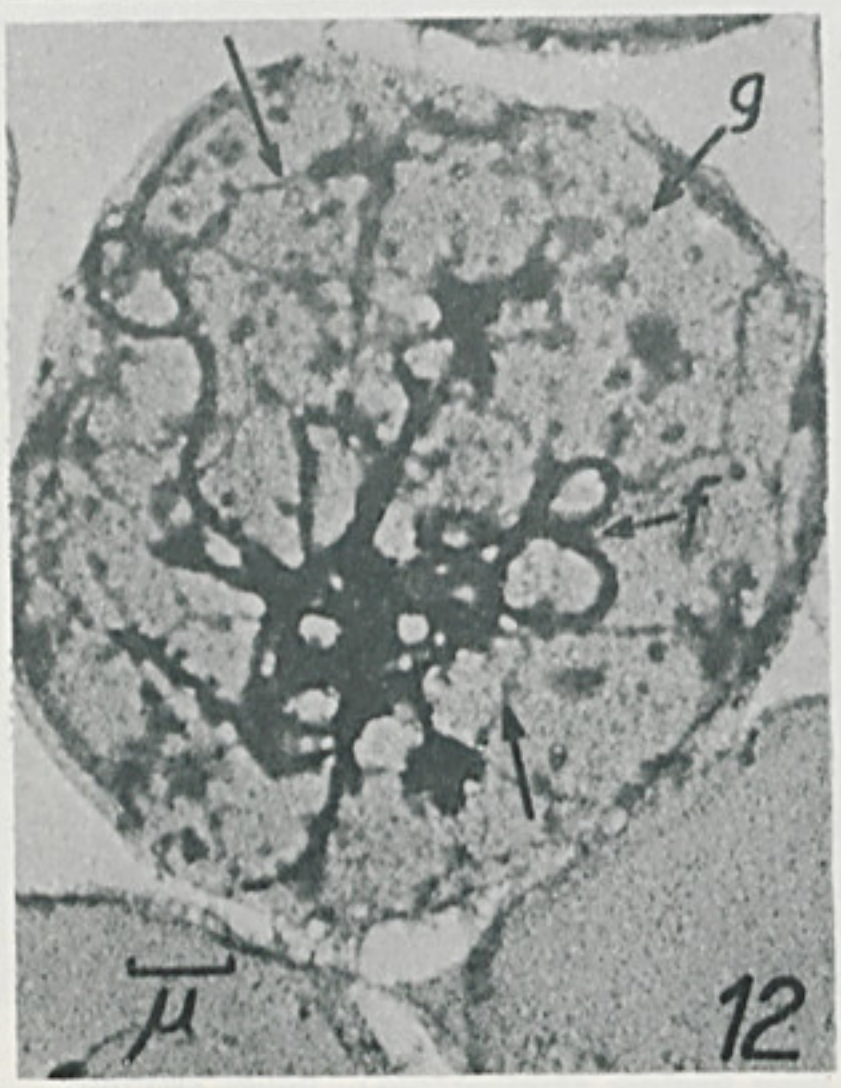


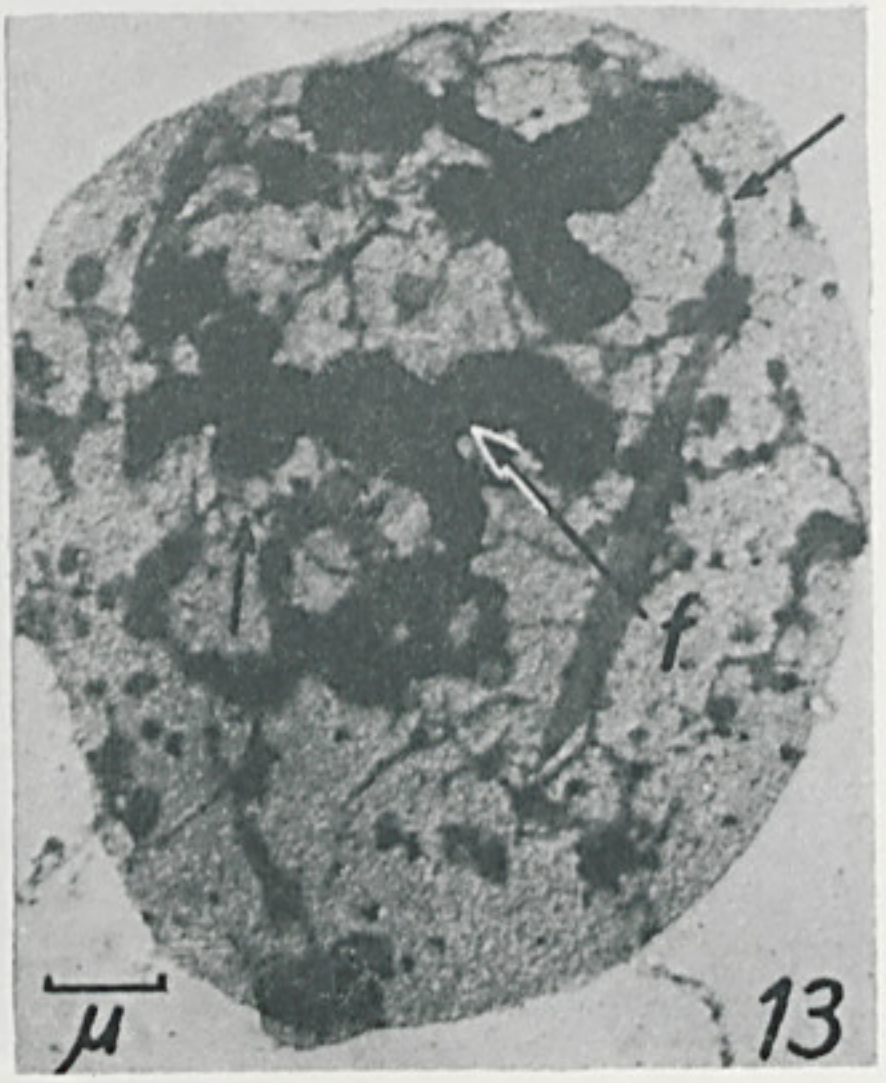
Blood of lead poisoned guinea-pigs. Methyl-butyl methacrylate.

Fig. 9 — Acidophilic erythroblast. *N* — nucleus; *m* — mitochondria; *mp* — plasmic membrane.

Fig. 10 — Early reticulocyte. *m* — mitochondria; *re* — endoplasmic reticulum; *mp* — plasmic membrane.





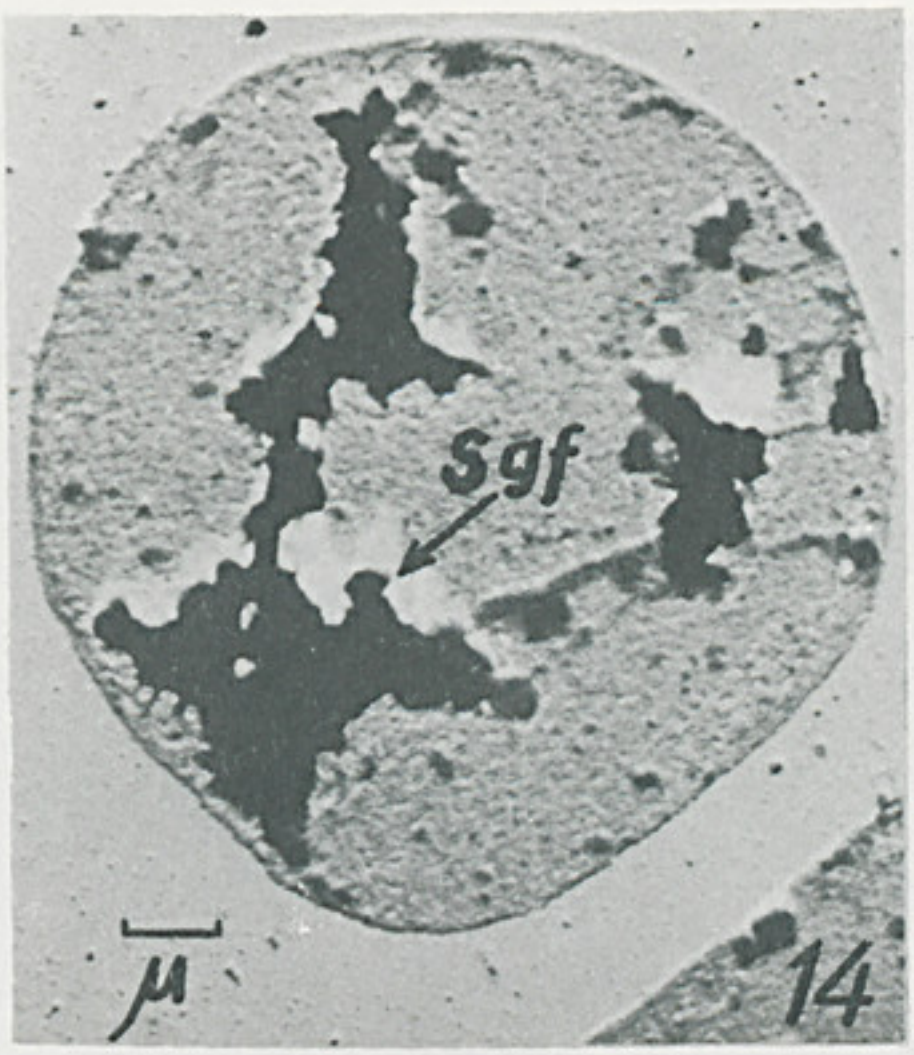


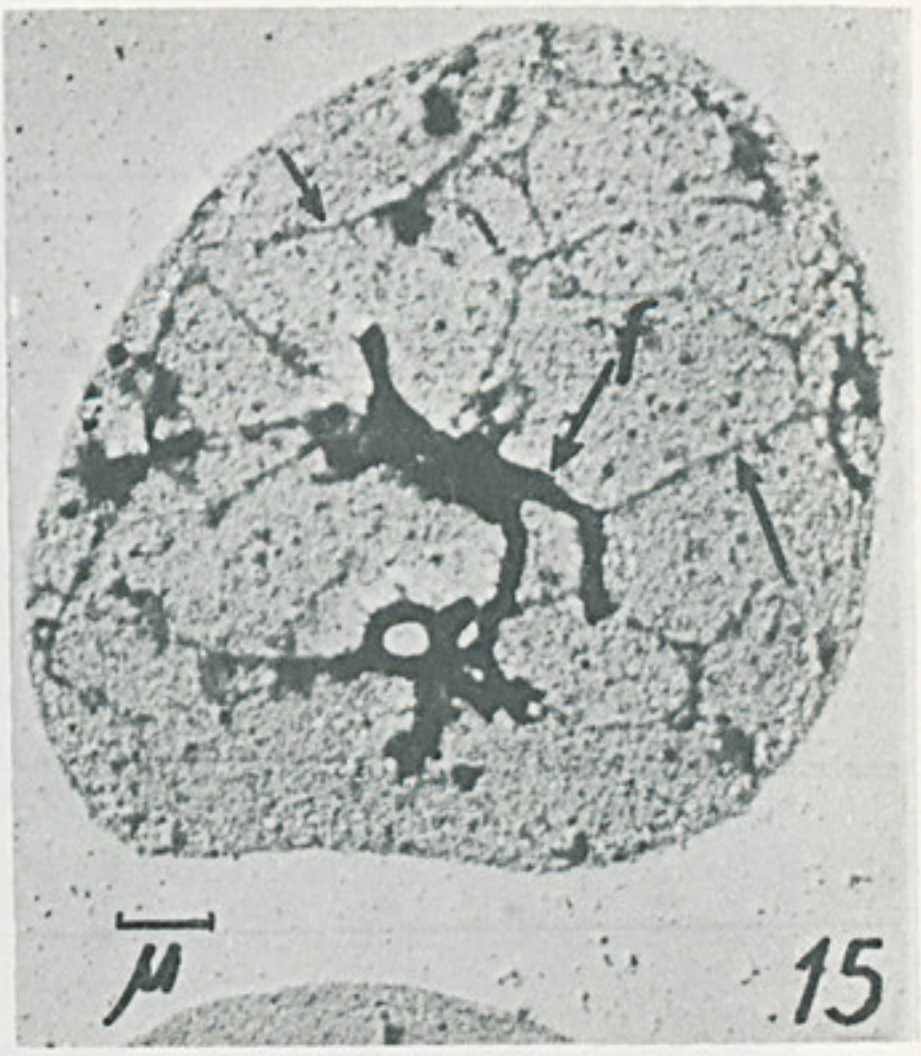
Hemolysed reticulocytes of guinea-pig blood (Technique A).

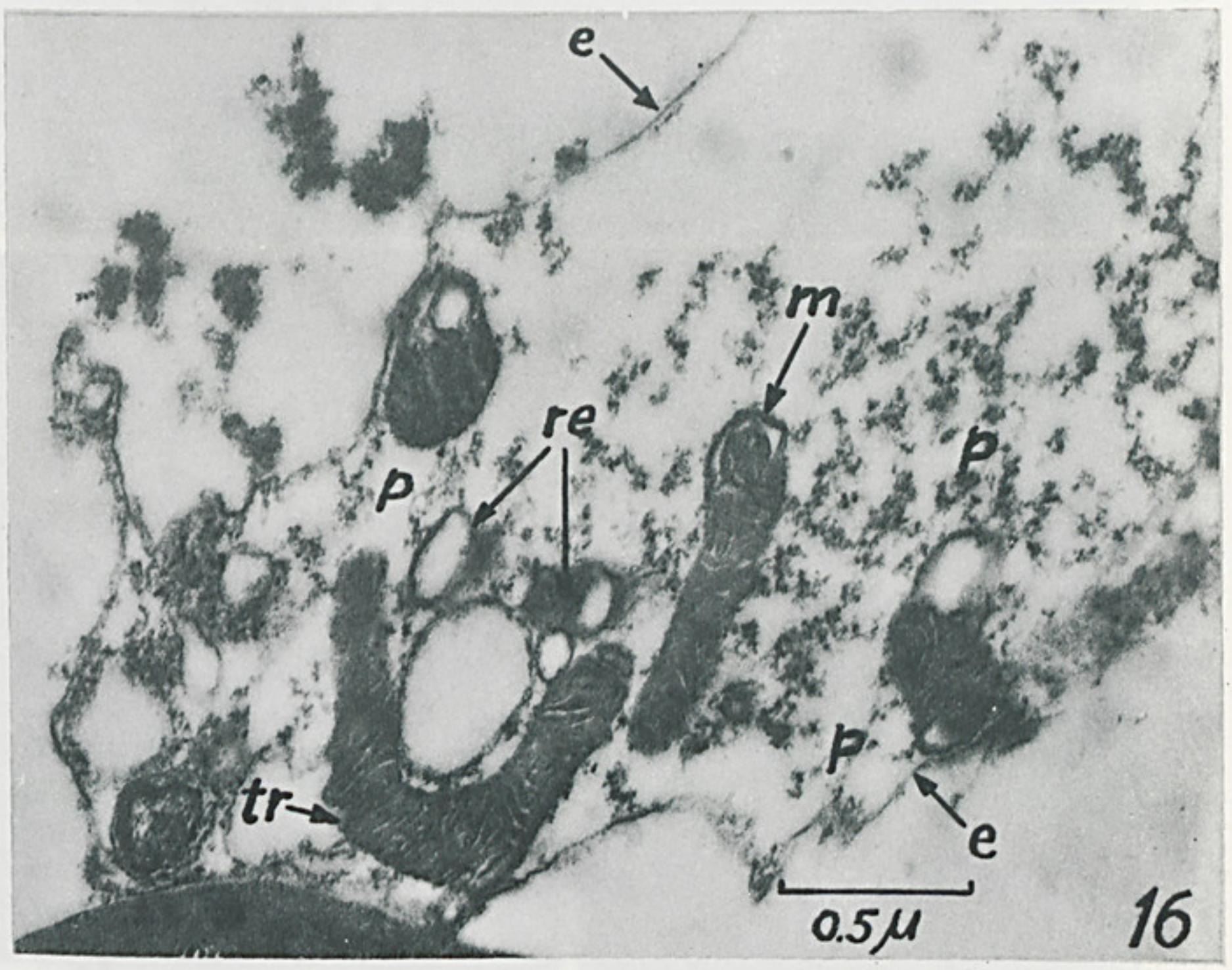
Fig. 11 — Bleeding anemia. *f* — large filaments; *g* — polyribosomes; *e* — stroma.

Fig. 12 — Bleeding anemia. *f* — large filaments; *g* — polyribosomes; *arrows* — thin filaments.

Fig. 13 — Lead poisoning anemia. *f* — large filaments; *g* — polyribosomes; *arrows* — thin filaments.





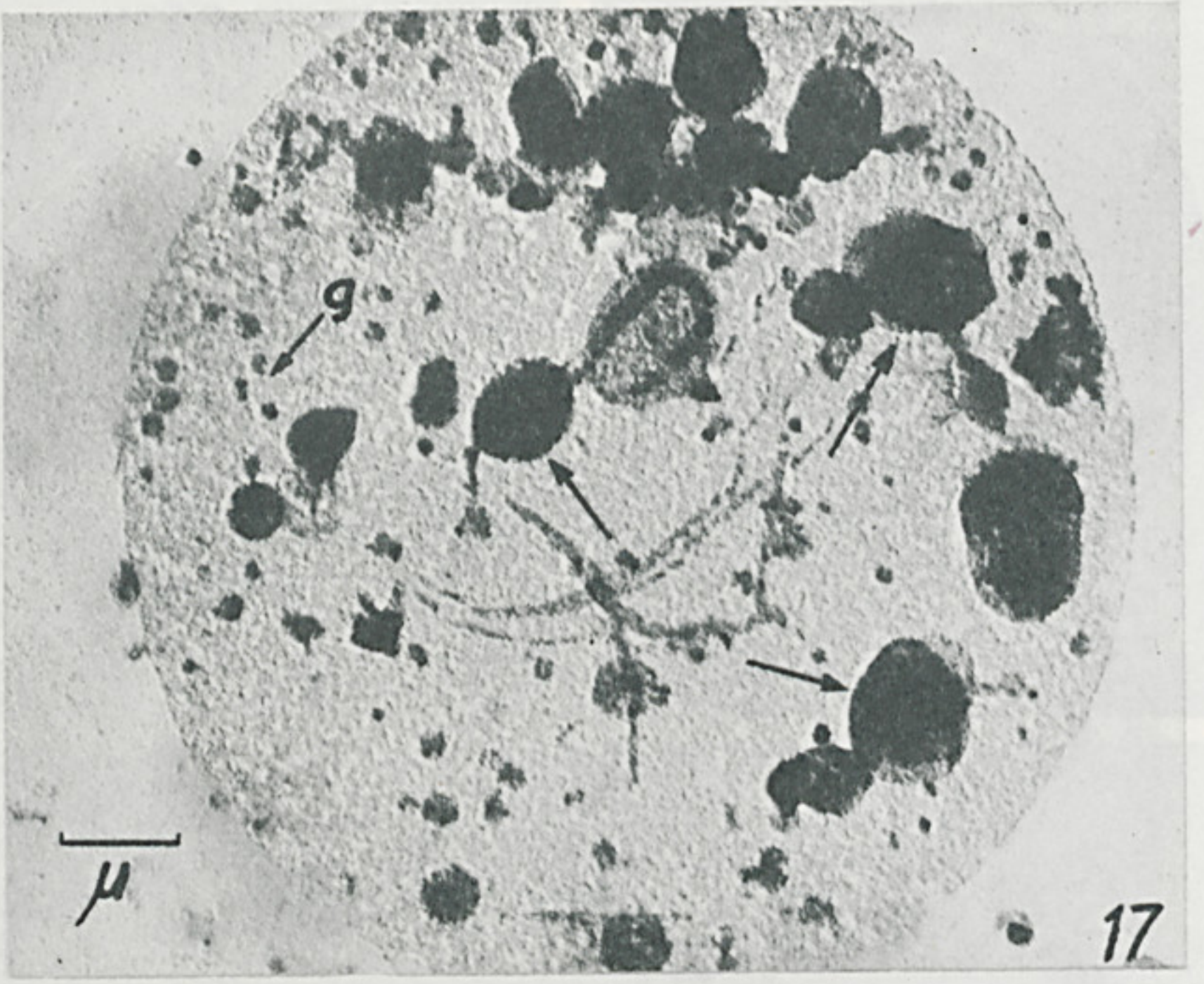


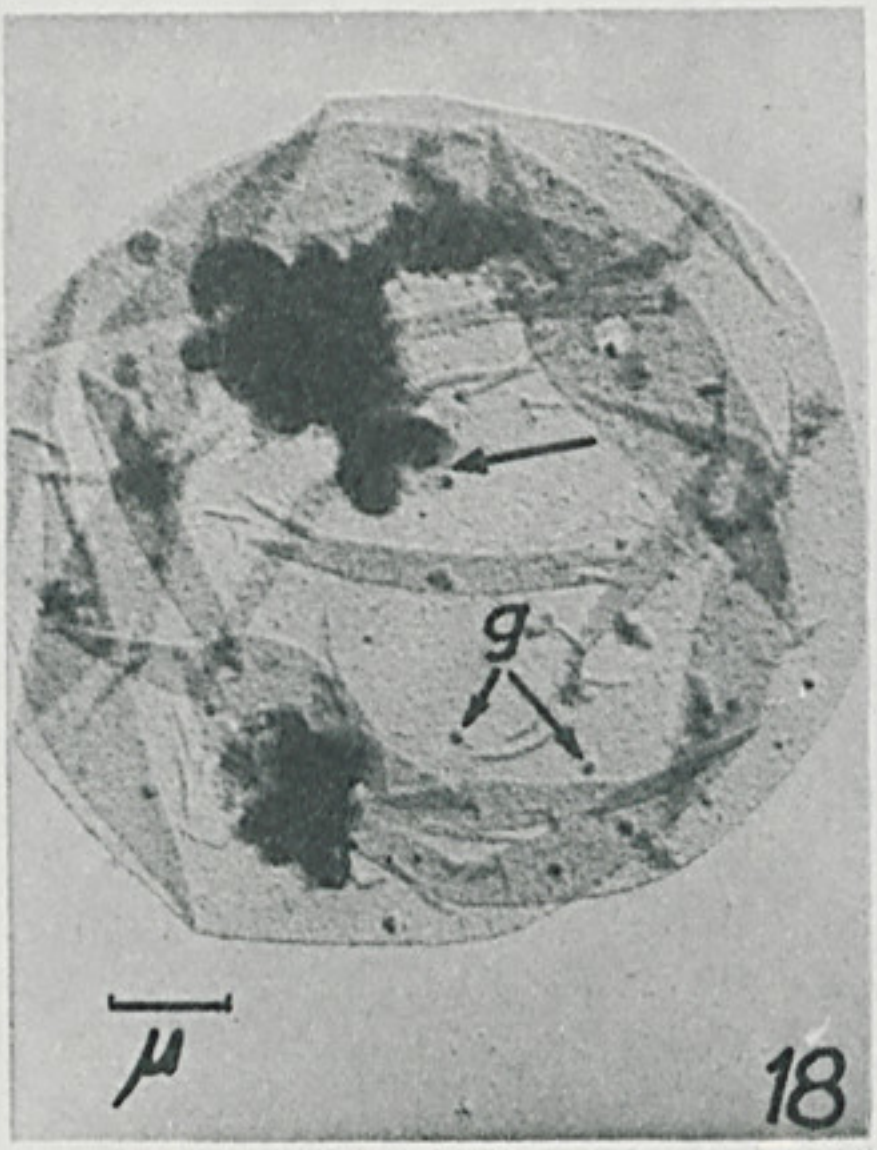
Bleeding anemia. Reticulocytes supravitally stained with Janus green B.

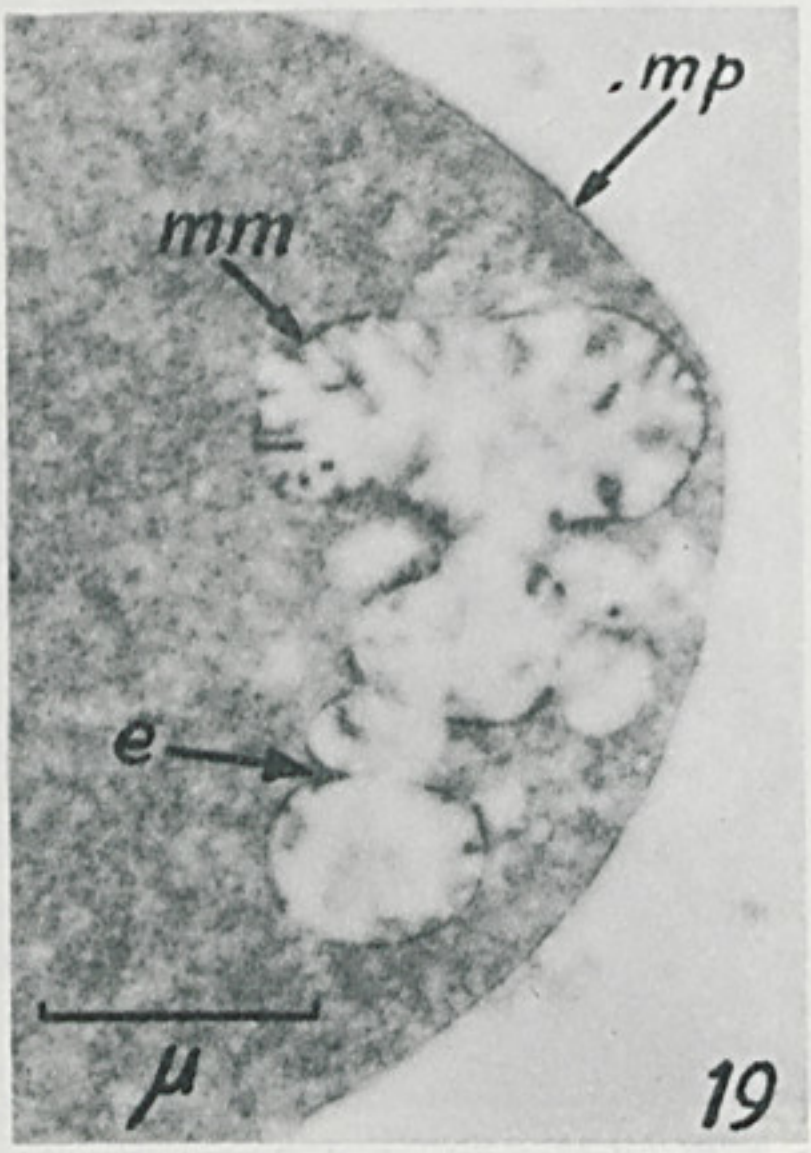
Fig. 14 — Dye at $1x10^{-4}$. *Sgf* — "Substantia granulo-filamentosa". Technique A.

Fig. 15 — Dye at $5x10^{-6}$. *f* — large filaments; *arrows* — thin filaments. Technique A.

Fig. 16 — Thin section. Dye at 1x10 . *m* — mitochondria; *tr* — double lamellas; *re* — endoplasmic reticulum; *p* — polyribosomes; *e* — stroma. Methyl-butyl methacrylates.





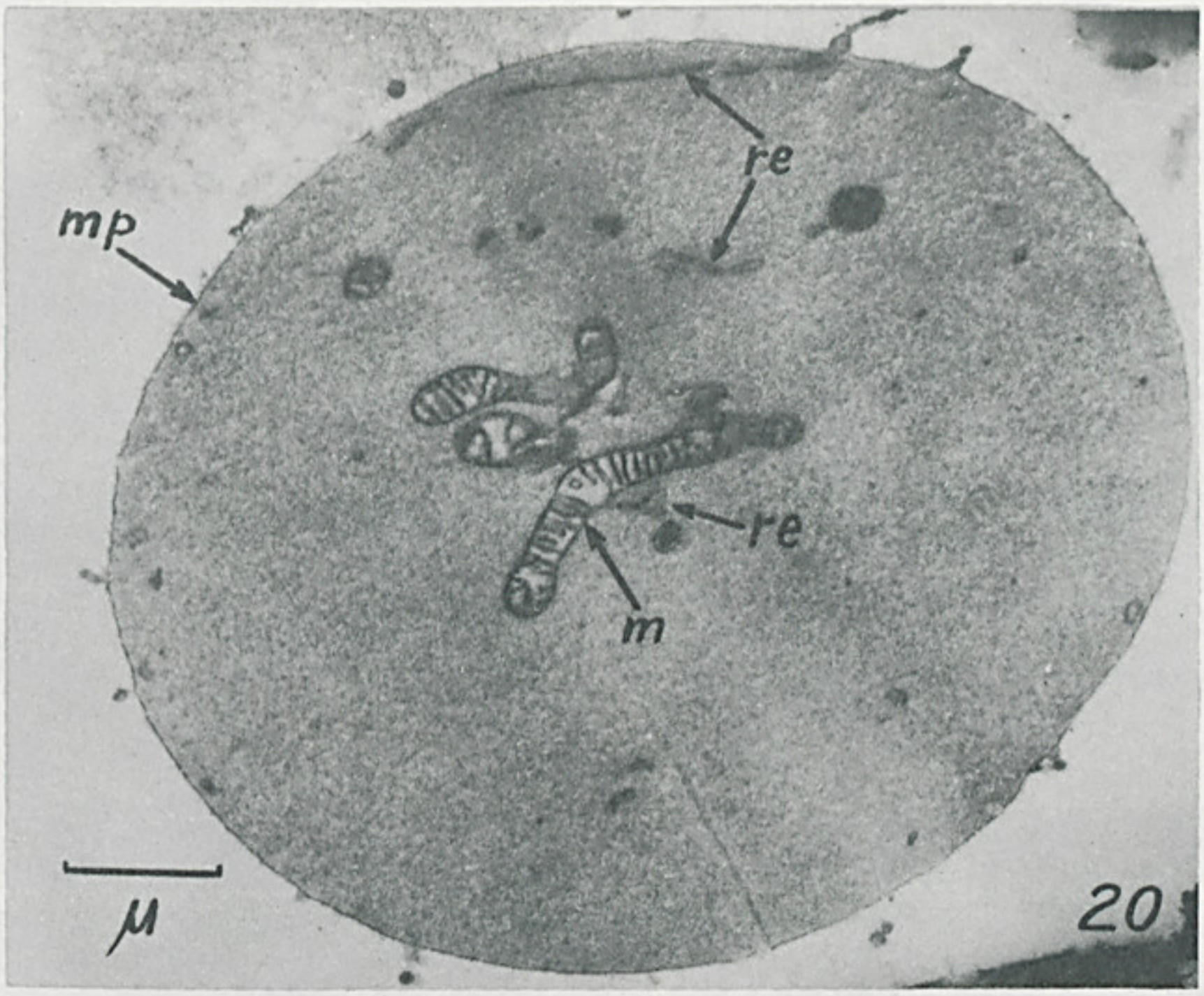


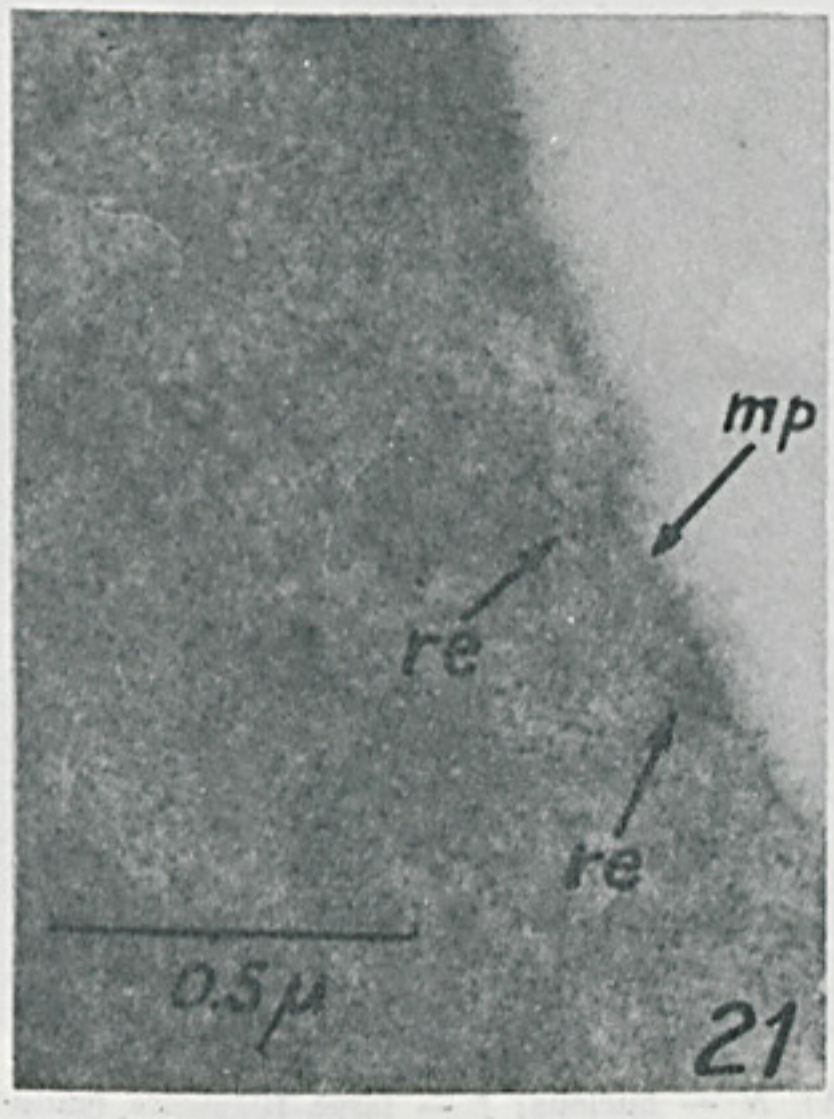
Bleeding anemia. Reticulocytes.

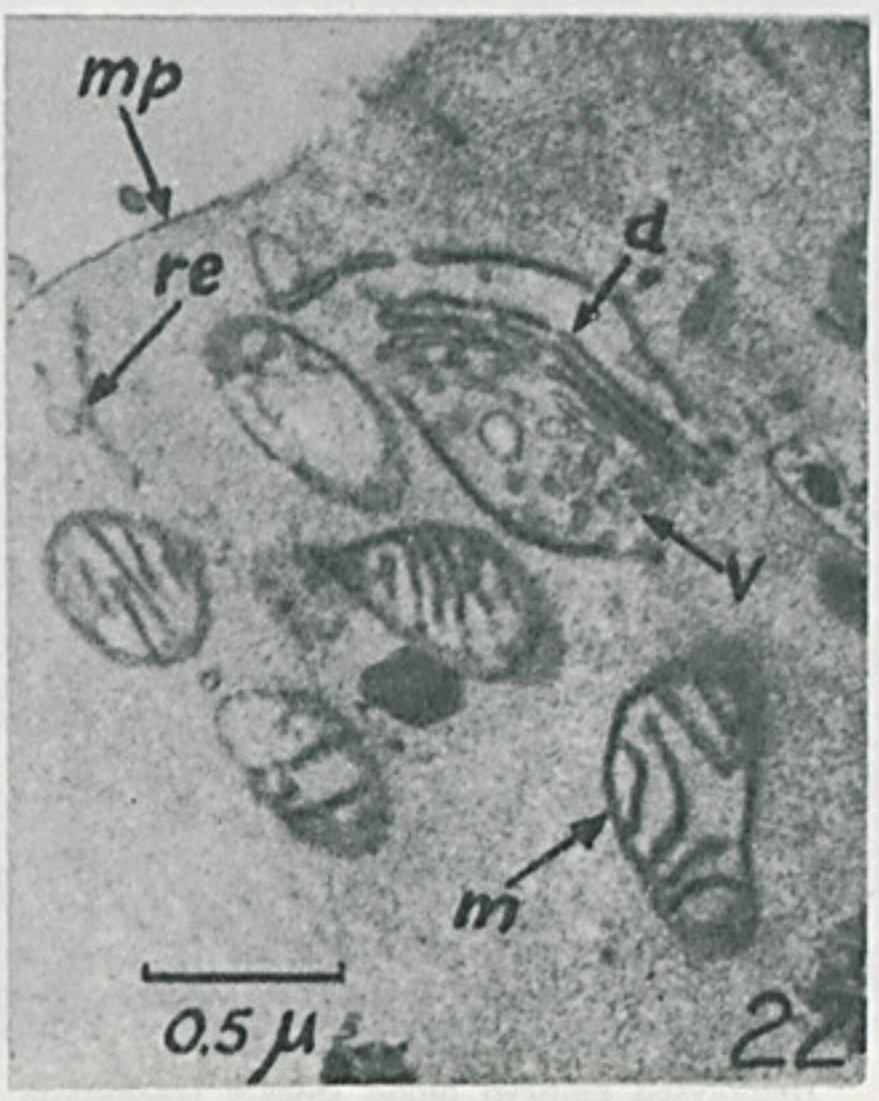
Fig. 17 — Hemolysis (technique B). *Arrows* — circular forms; *g* — polyribosomes.

Fig 18 — Hemolysis (technique C). *Arrows* — not individualized circular forms; *g* — polyribosomes.

Fig. 19 — Thin section. *e* — constrictions; *mm* — mitochondrial membranes; *mp* — plasmic membrane. Methyl-butyl methacrylates.





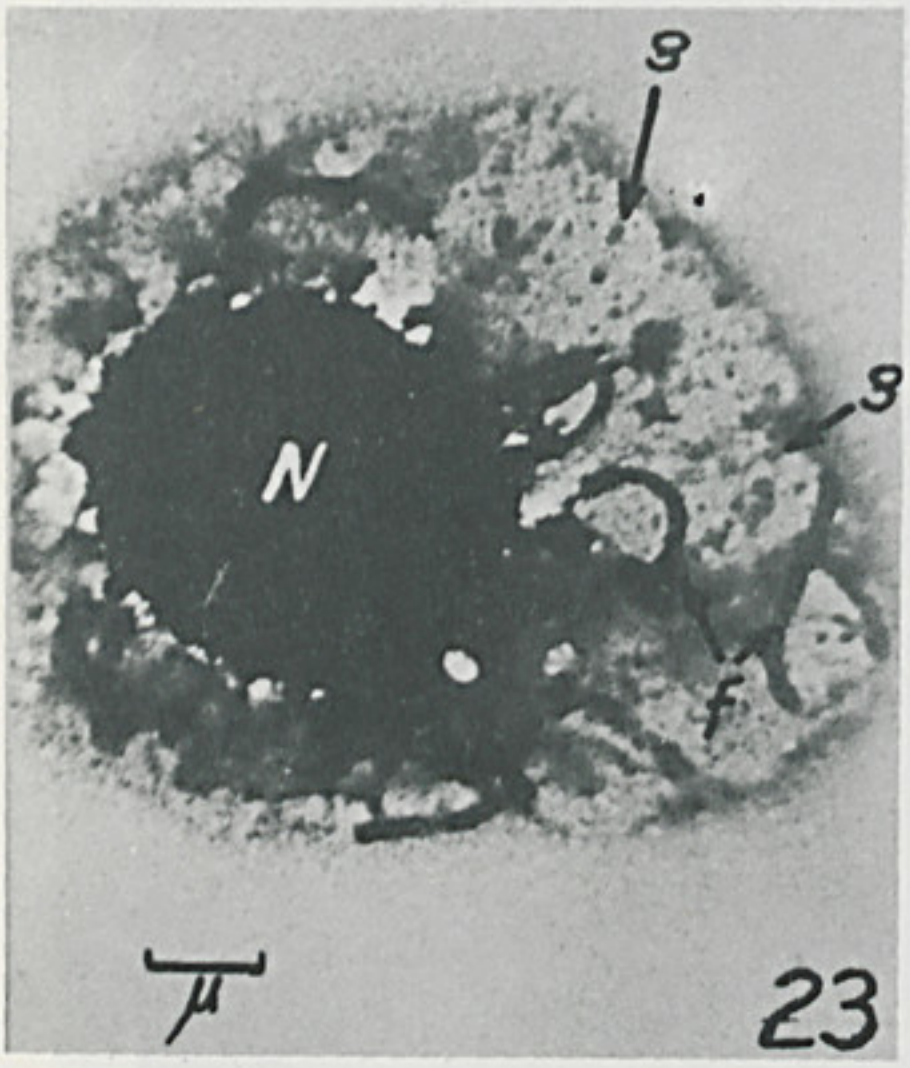


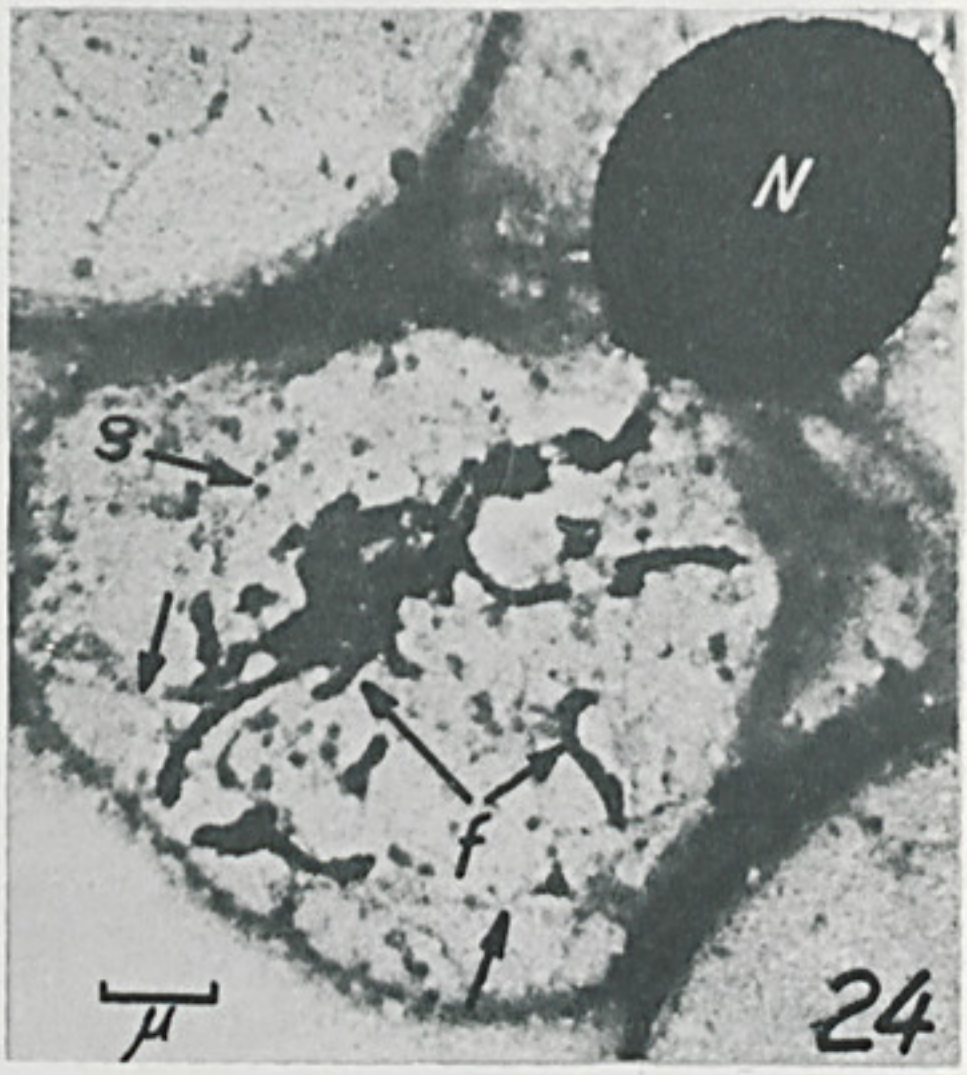
Thin sections of reticulocytes. *mp* — plasmic membrane; *re* — endoplasmic reticulum; *m* — mitochondria.

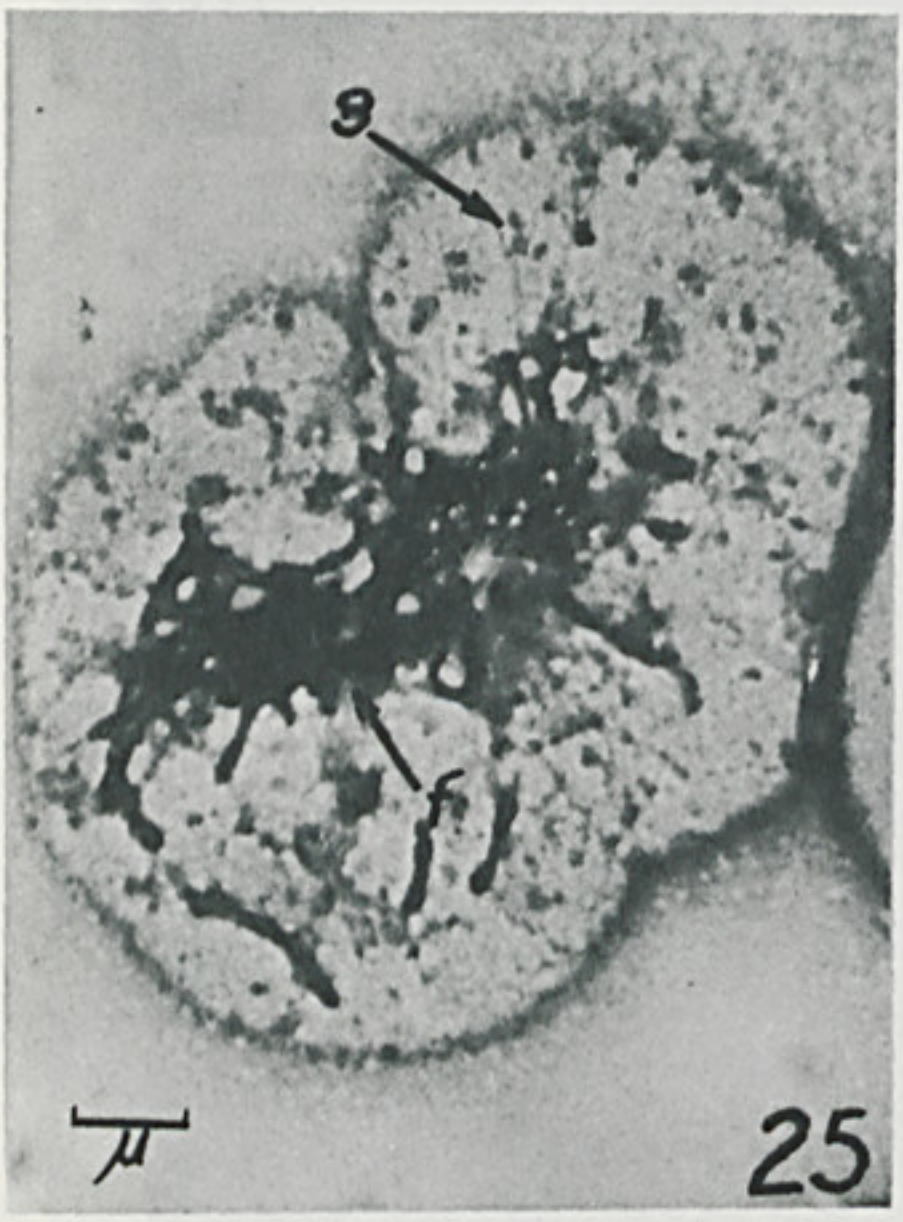
Fig. 20 — Embryo blood. Epon 812; lead acetate.

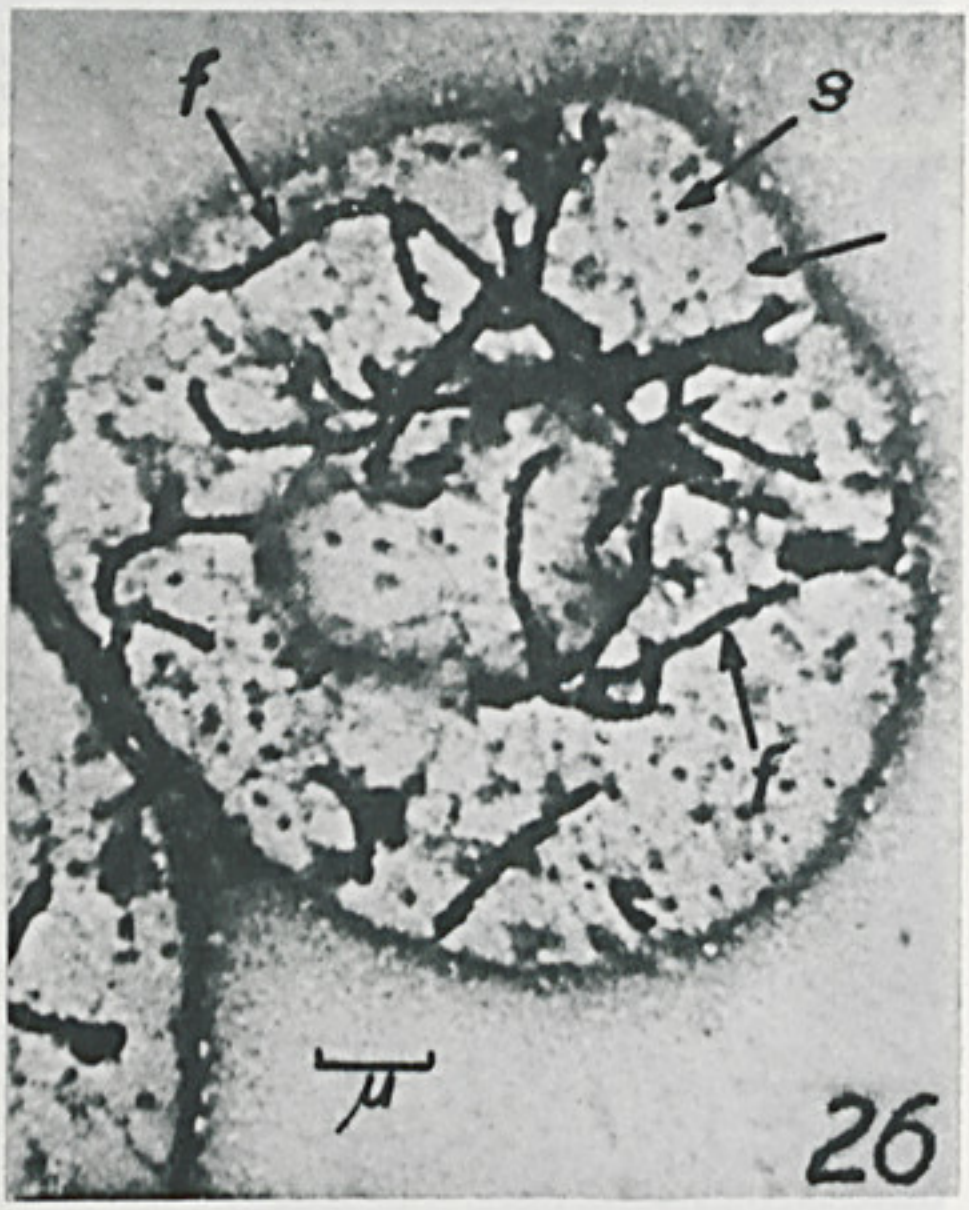
Fig. 21 — Lead poisoned guinea-pig blood. Methyl-butyl methacrylates.

Fig. 22 — Embryo blood. *d* — dictyosome; *v* — vesicles. Epon 812; lead acetate.









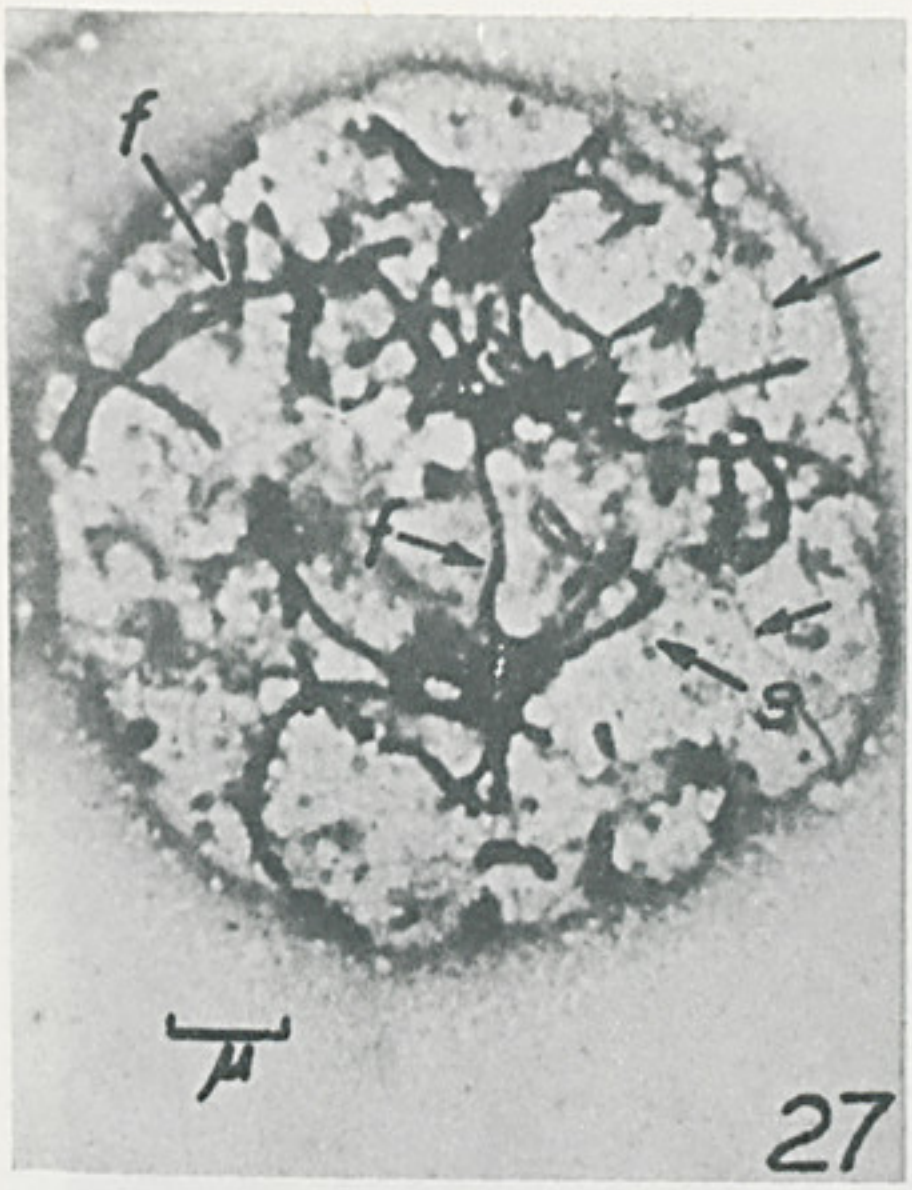
Hemolysed embryo blood smears (technique A). *N* — nucleus; *f* — large filaments (mitochondria); *arrows* — thin filaments (endoplasmic reticulum); *g* — polyribosomic granules.

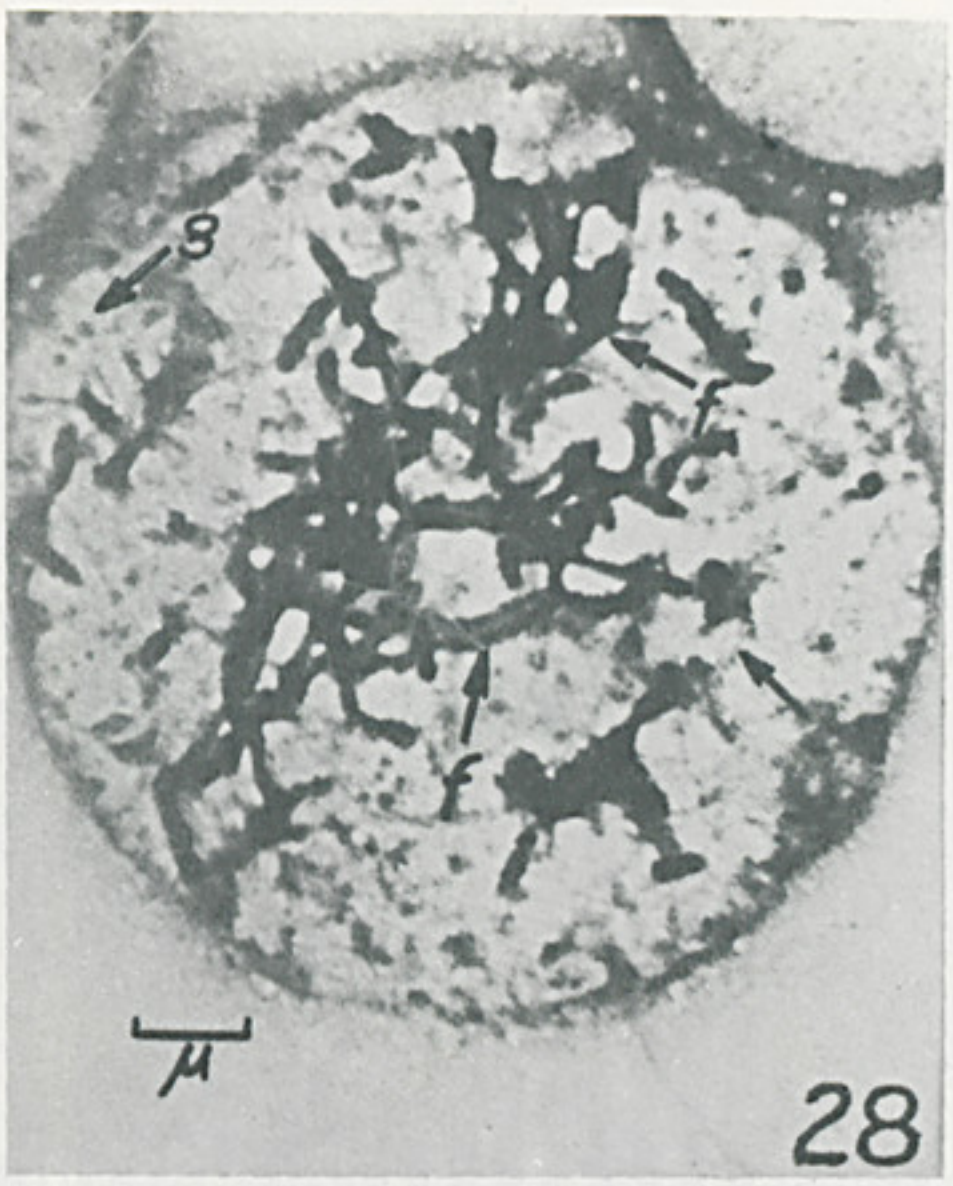
Fig. 23 — Erythroblast. Mitochondrial area (f) — 8.5 μ^2.

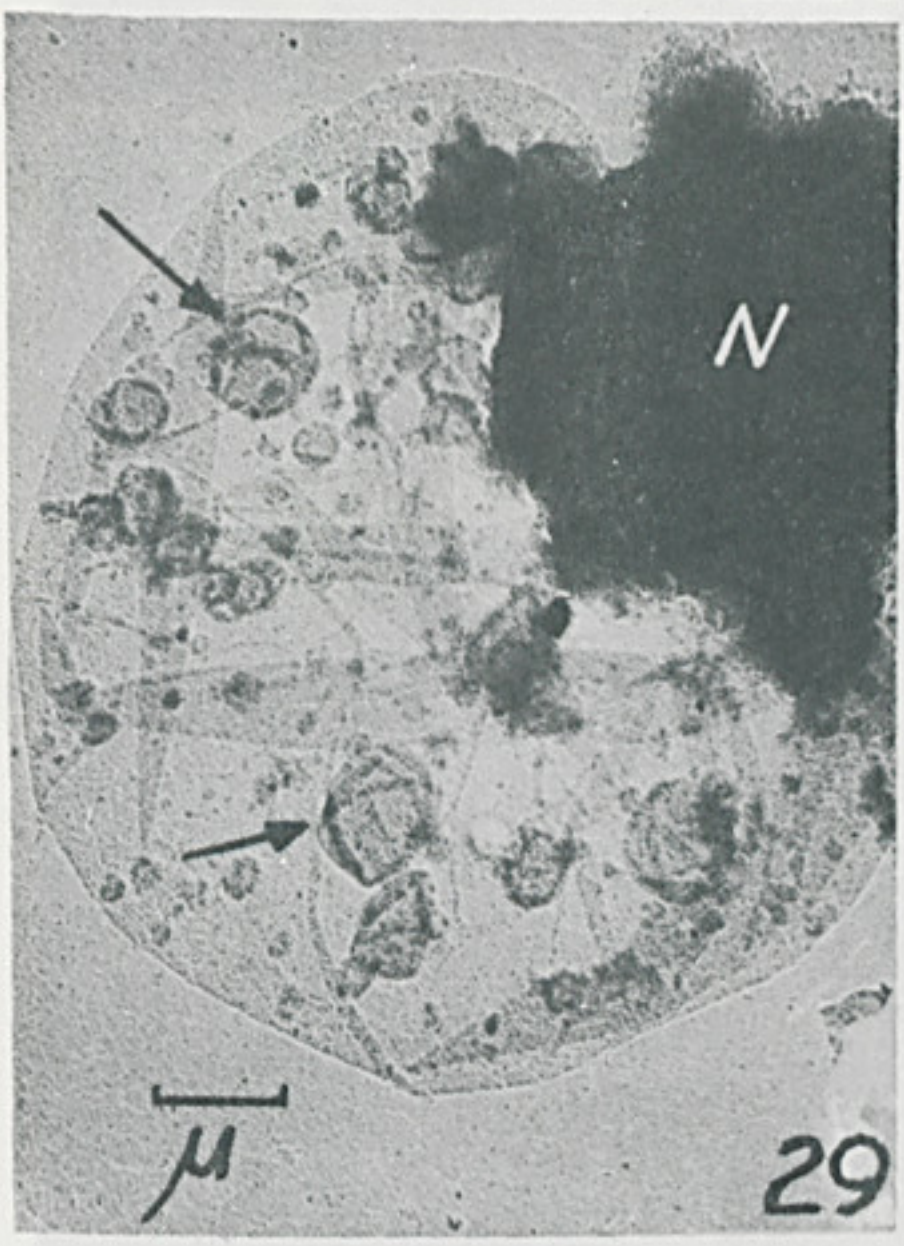
Fig. 24 — Erythroblast. *f* — 3.6 μ^2.

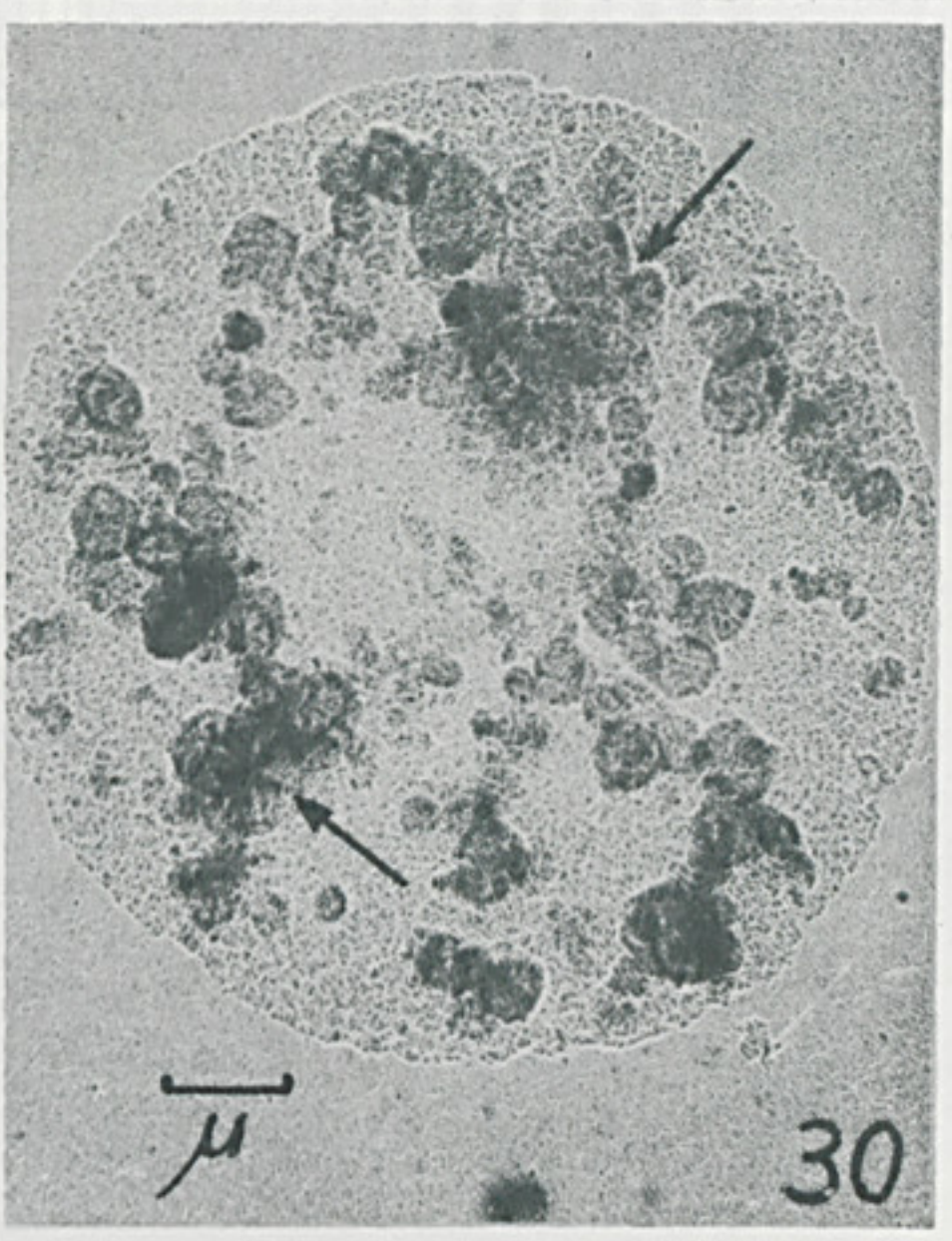
Fig. 25 — Reticulocyte. *f* — 8.6 μ^2.

Fig. 26 — Reticulocyte. *f* — 12.7 μ^2.









Hemolysed embryo blood smears (technique A). *Arrows* — thin filaments (endoplasmic reticulum); *g* — polyribosomic granules; *f* — large filaments (mitochondria).

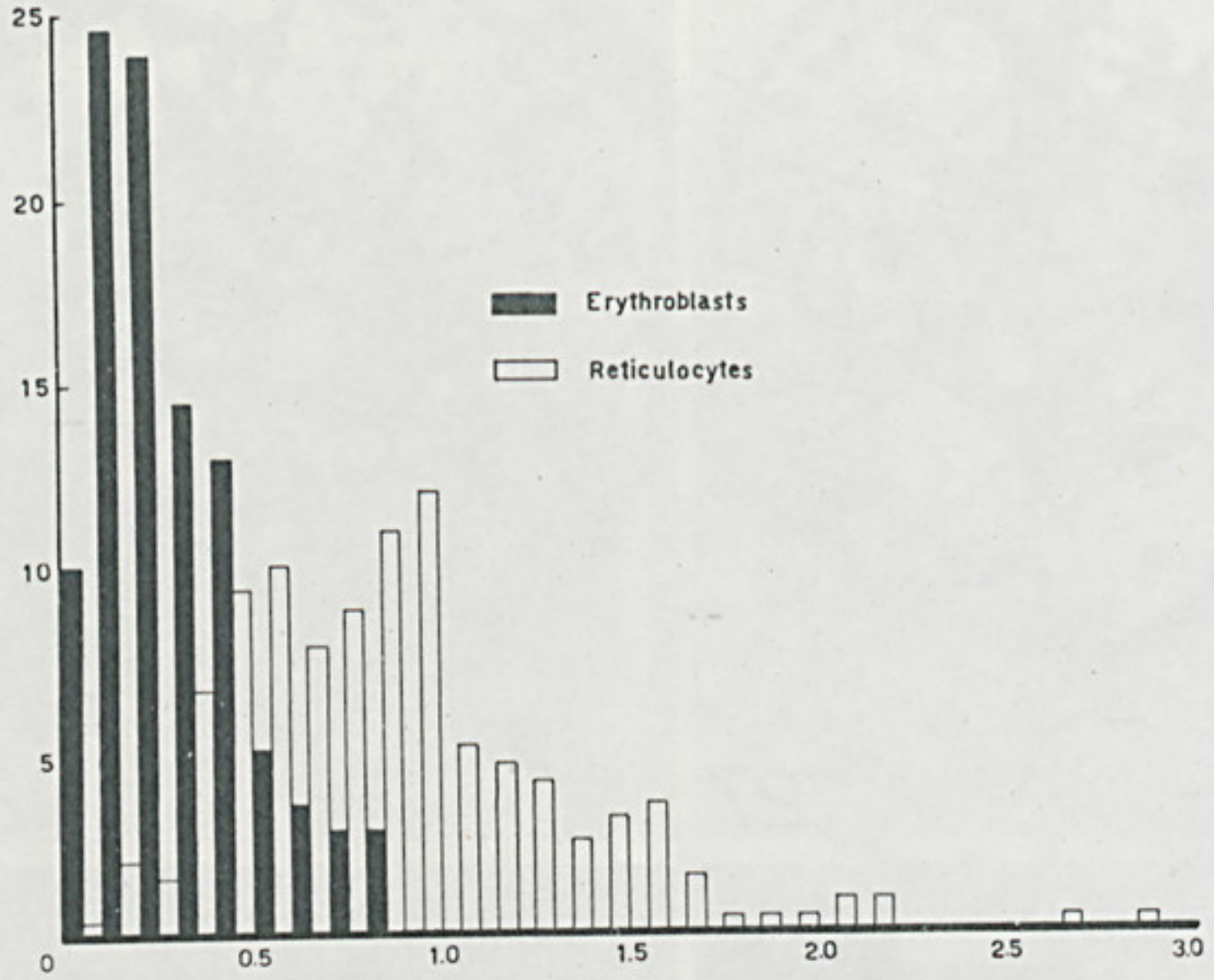
Fig. 27 — Reticulocyte. *f* — 16.7 μ^2.

Fig. 28 — Reticulocyte. *f* — 17.2 μ^2.

Hemolysed embryo blood (technique B). *Arrows* — circular forms (disintegrated mitochondria).

Fig. 29 — Erythroblasts. *N* — nucleus.

Fig. 30 — Reticulocyte.



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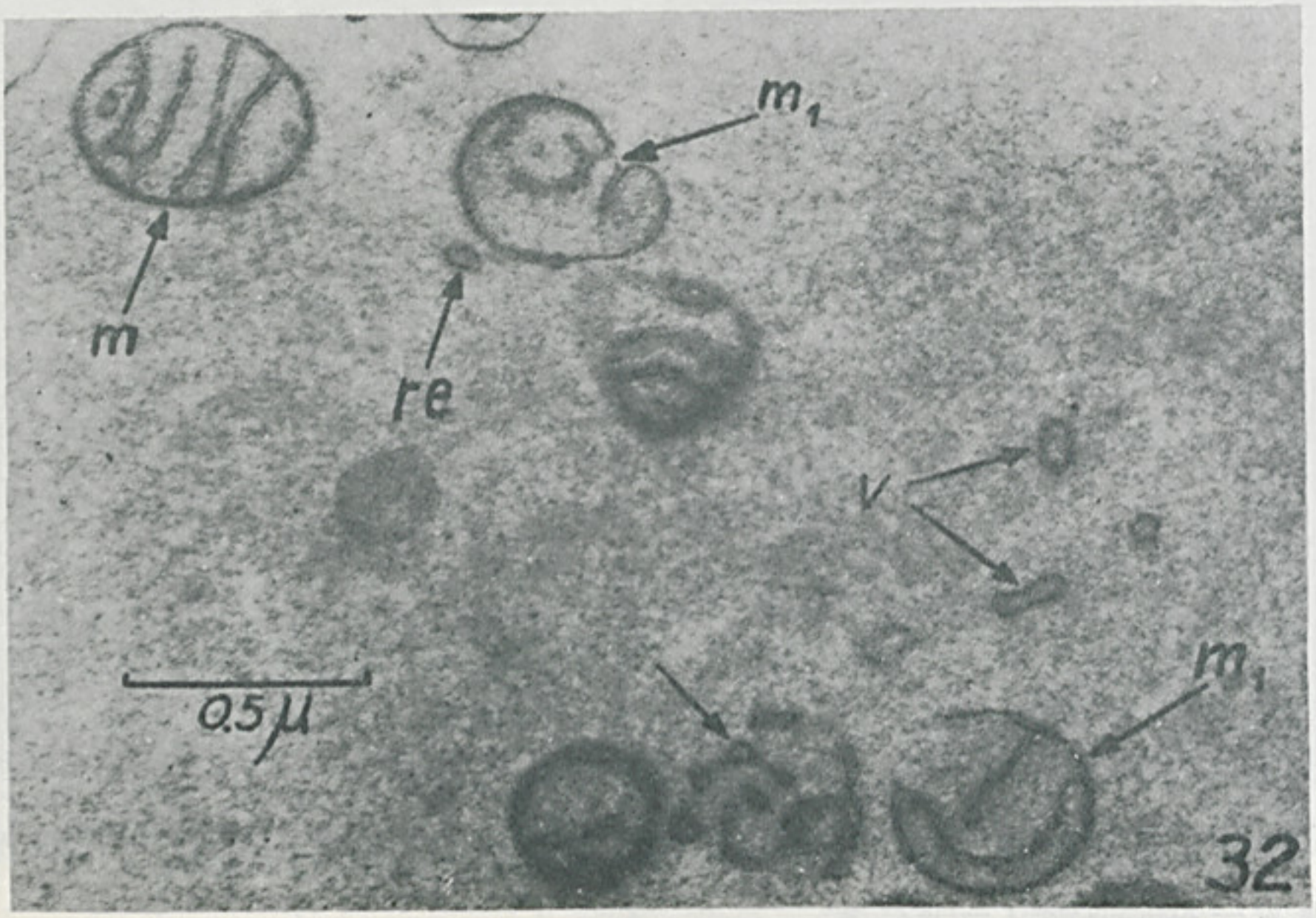
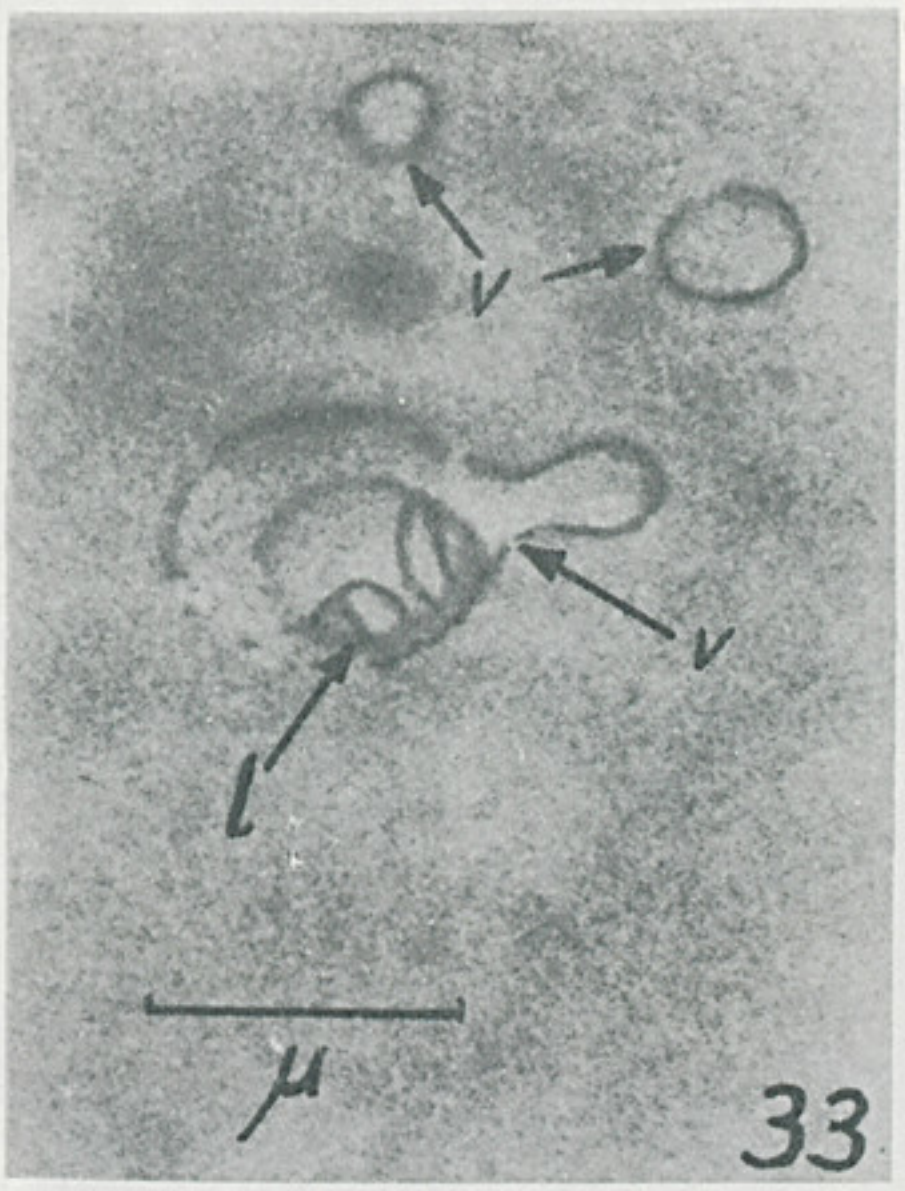


32

Fig 31 — Frequency distribution histogram presenting mitochondrial areas (μ^2)/ cell — abscissa — of sectioned erythroblasts and reticulocytes with intervals of 0,1 μ^2. Ordinate: % of cells.

Fig. 32 — Mitochondriogenesis in a reticulocyte. m_1 — mitochondria formation; *m* — mitochondrion; *re* — endoplasmic reticulum; *V* — vesicles; *arrows* — canalicules. Epon 812; lead acetate.



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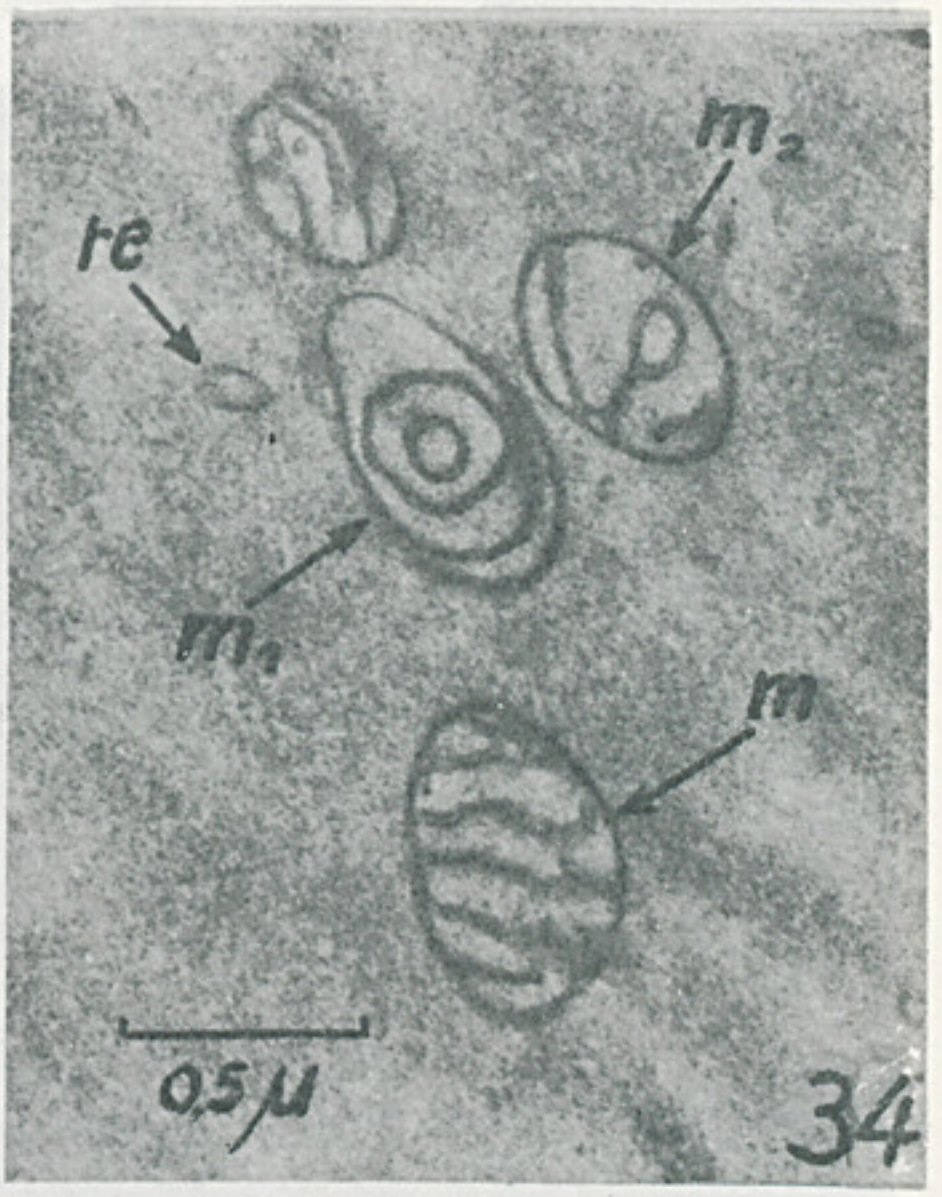

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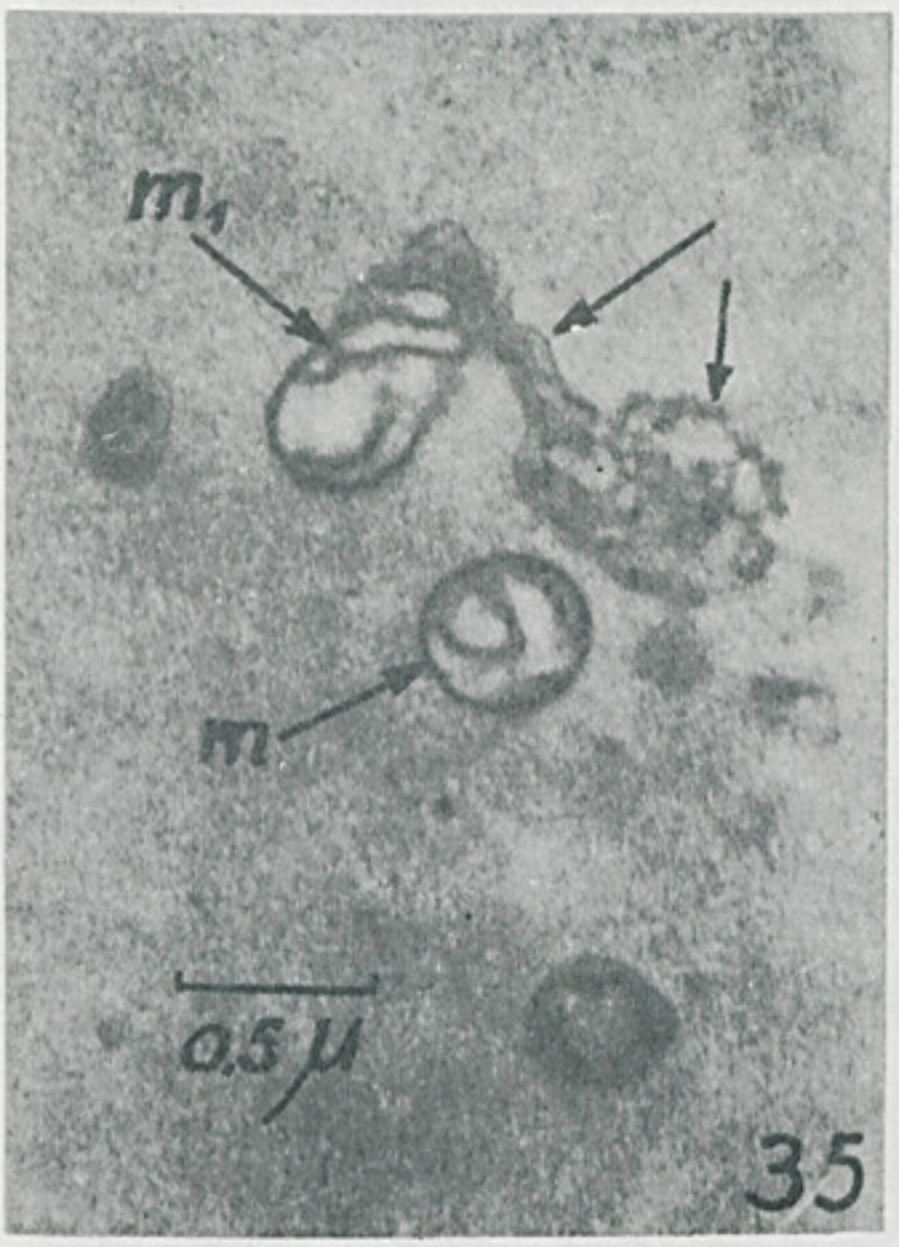

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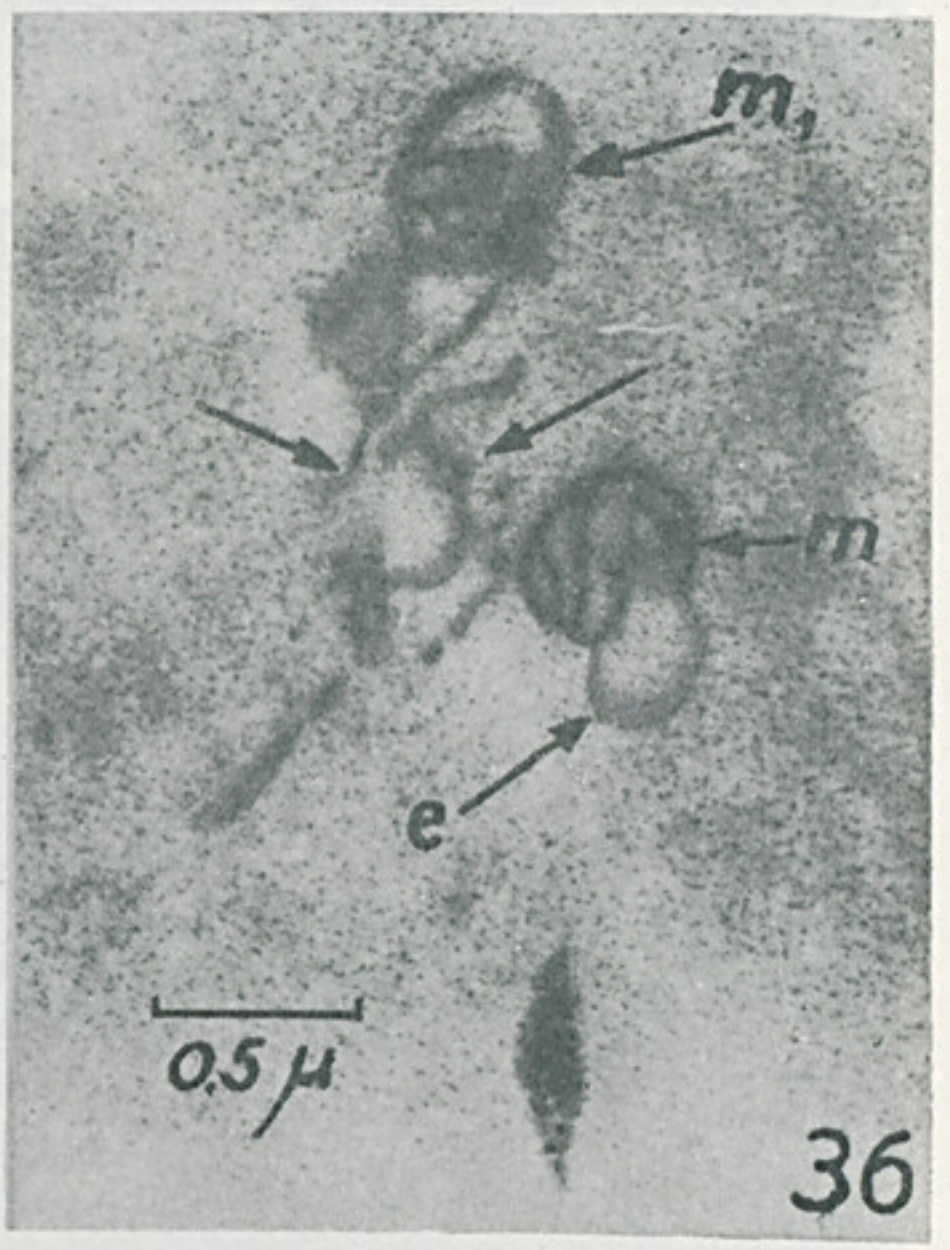

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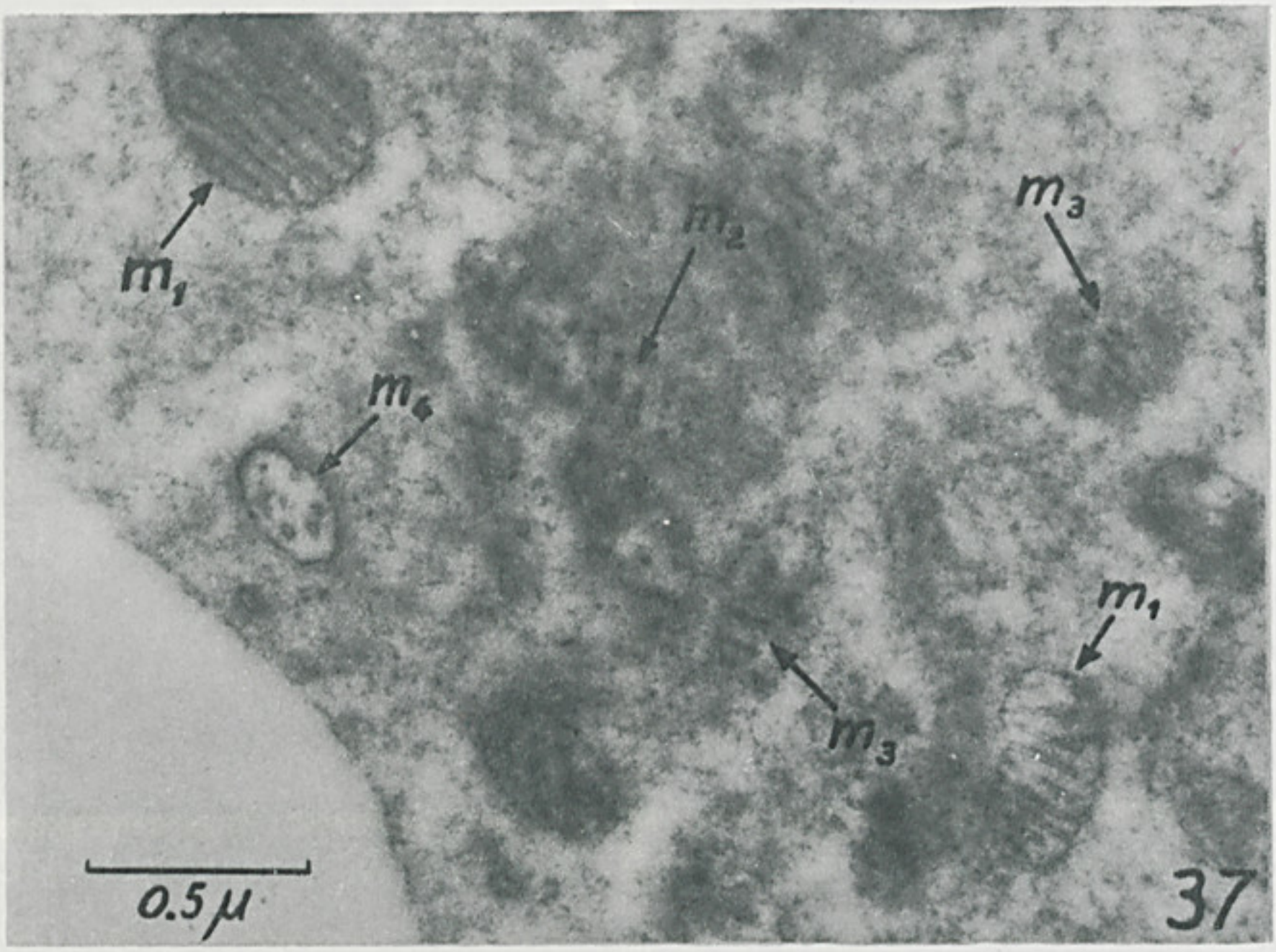
Mitochondriogenesis in reticulocytes. Epon 812; lead acetate.

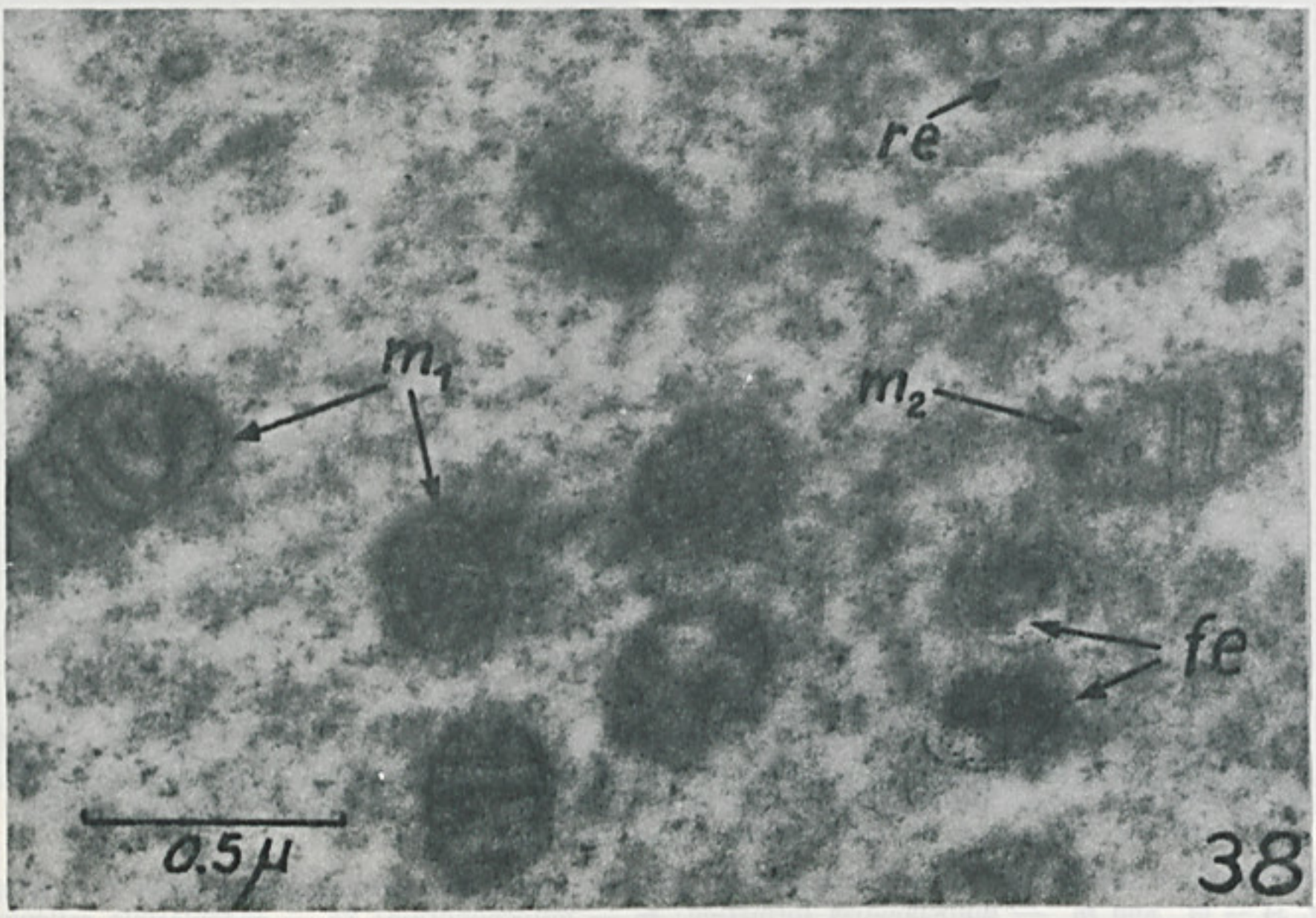
Fig. 33 — 1 — double lamellas; *V* — vesicles.

Fig. 34 — m_1 and m_2 — mitochondria formation; *m* — mitochondrion; *re* — endoplasmic reticulum.

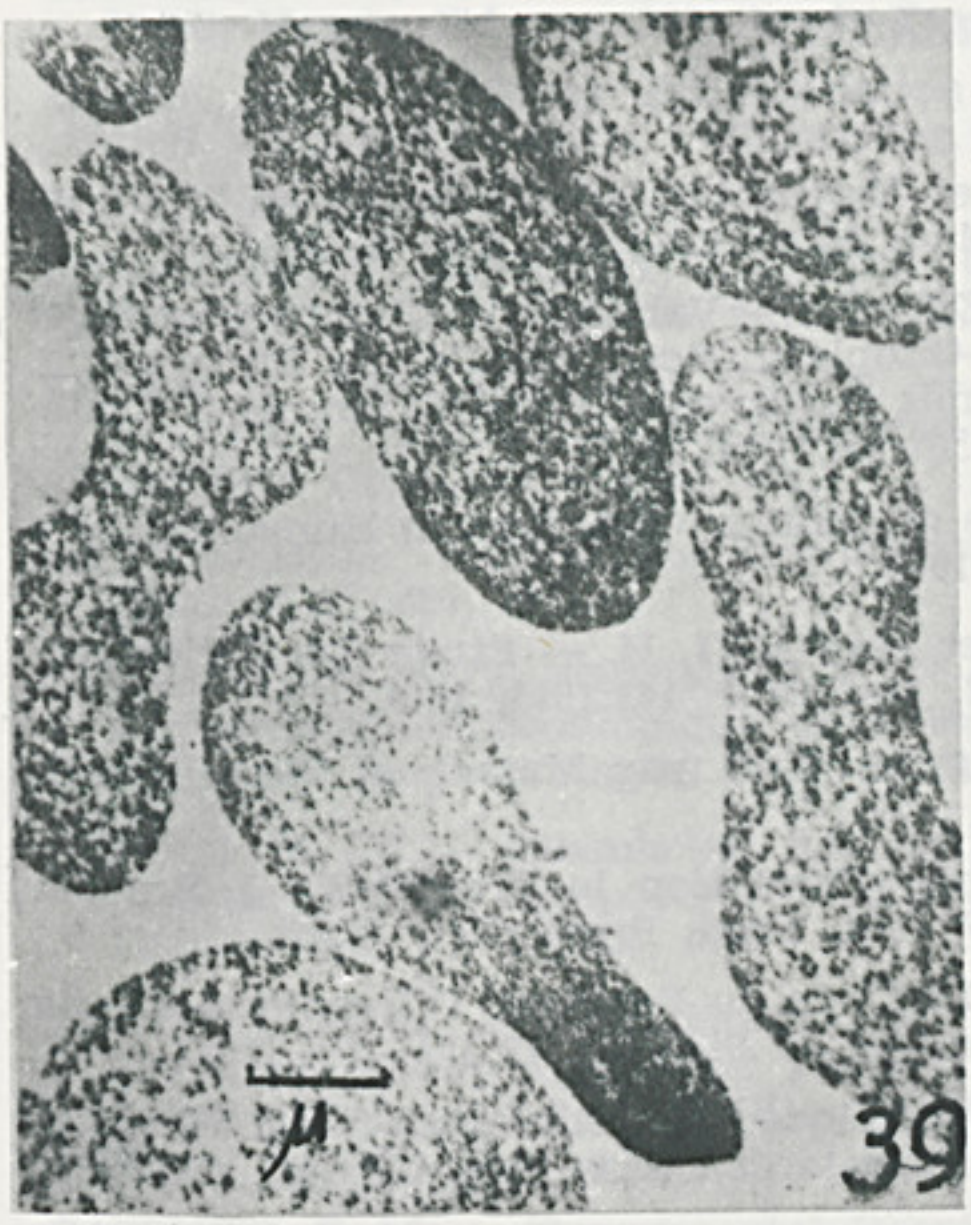
Fig. 35 — m_1 — mitochondrion formation; *arrows* — canalicules; *m* — mitochondrion.

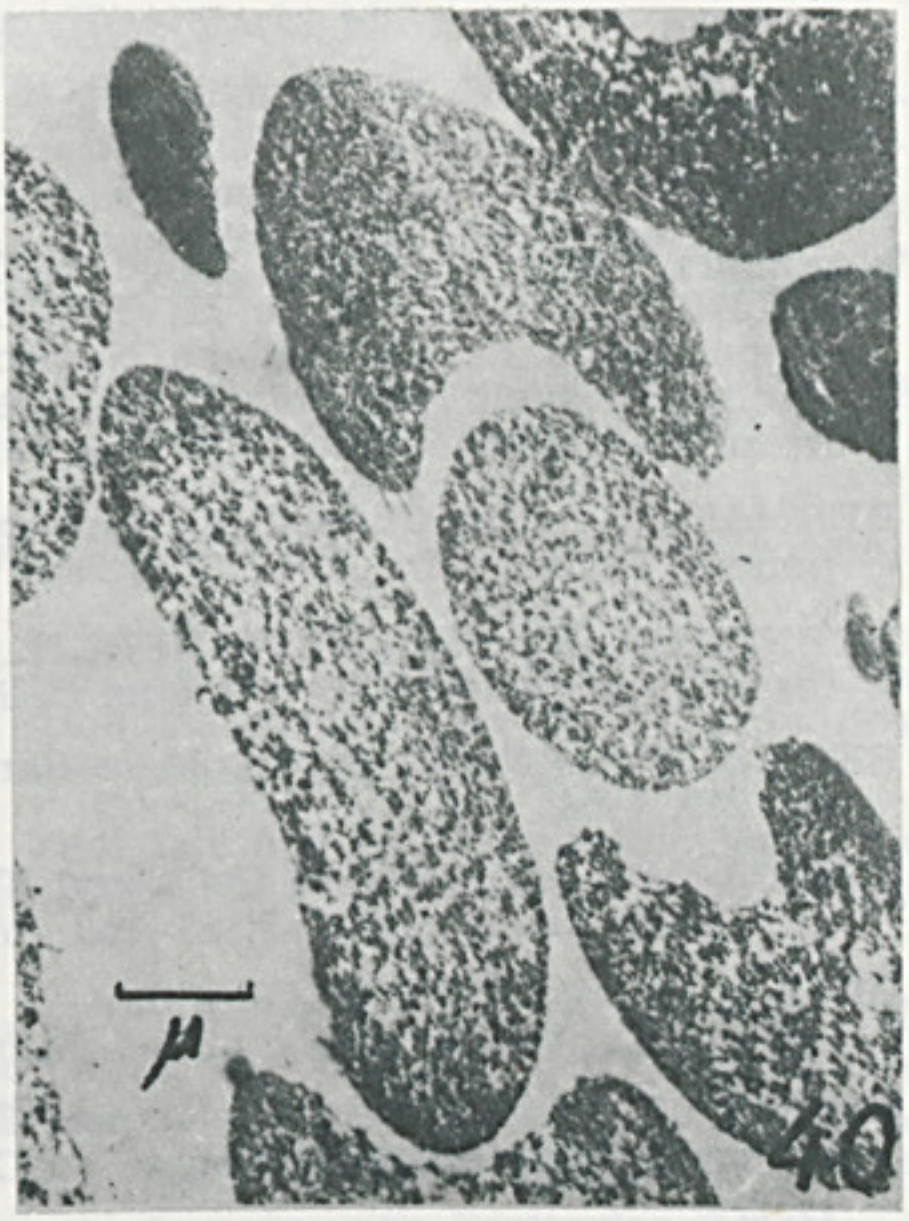
Fig. 36 — m_1 — mitochondrion formation; *arrows* — canalicules; *m* — mitochondrion with a membranous expansion (e).

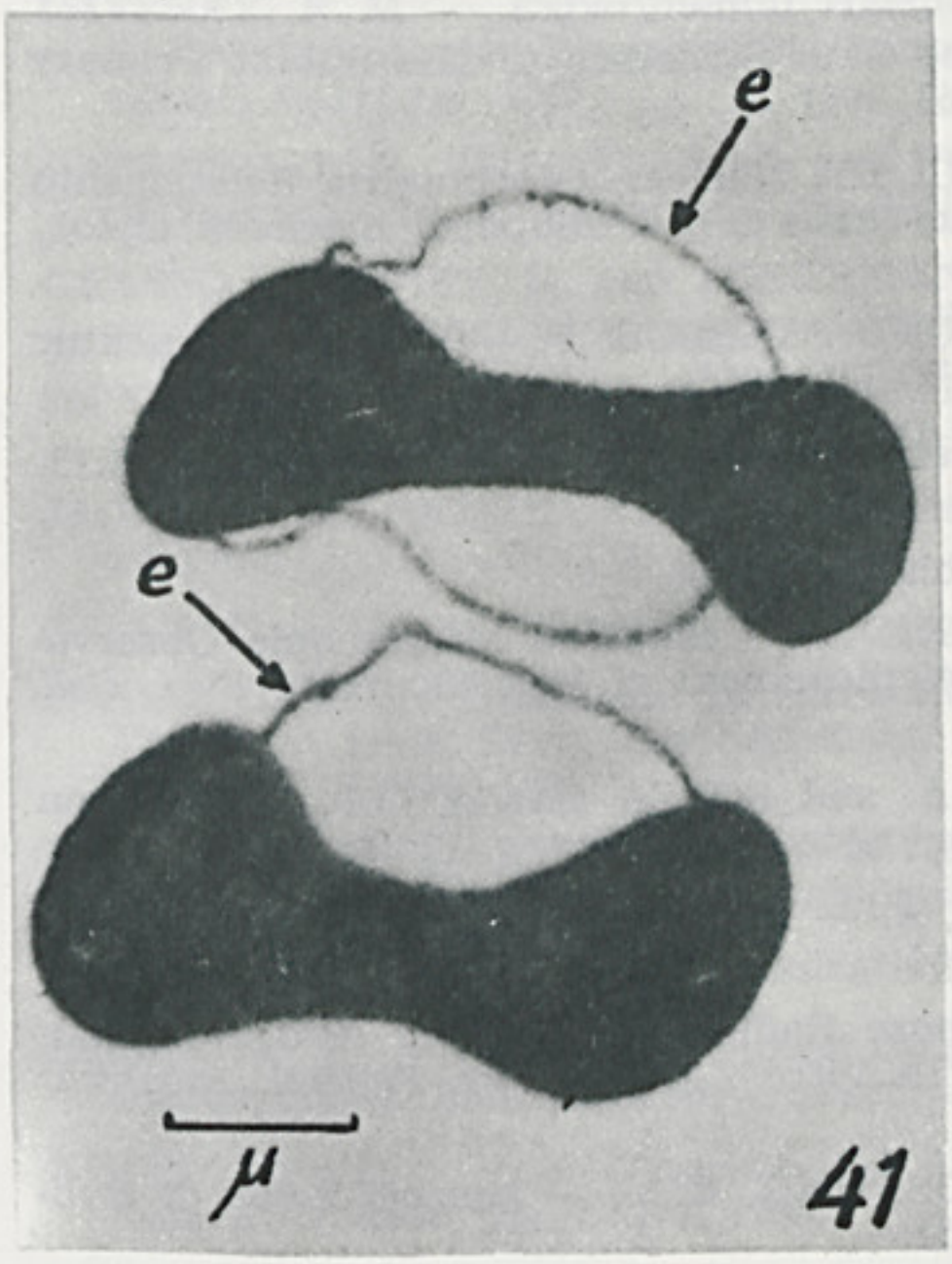


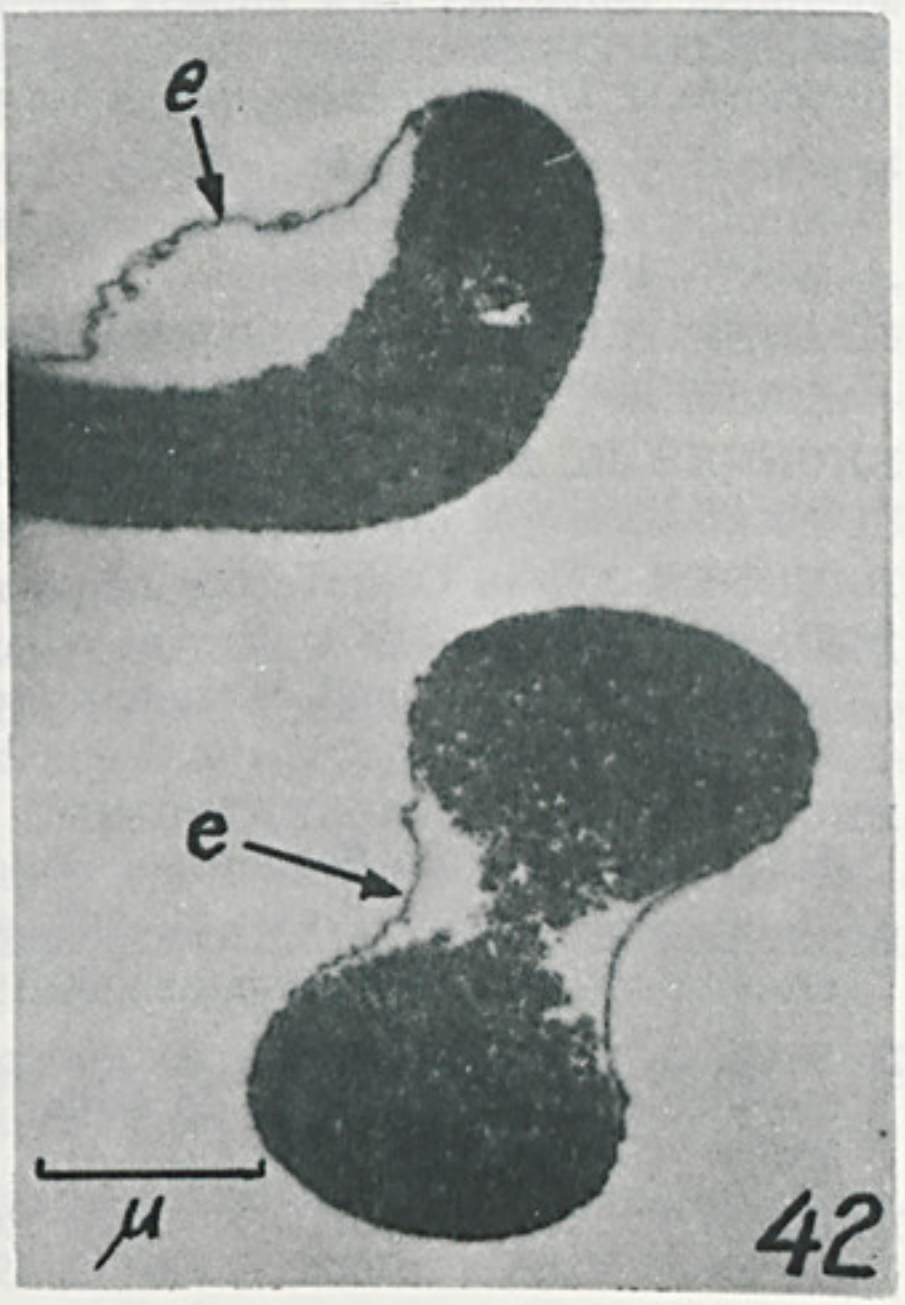


Figs. 37 and 38 — Reticulocytes of lead poisoned guinea-pigs, presenting degenerative mitochondrial forms (m_1, m_2, m_3); m_4 — mitochondrion with digitiform membrane system; *fe* — ferritin granules; *re* — endoplasmic reticulum. Methyl-butyl methacrylates.









Mature erythrocytes of normal guinea-pigs, fixed with formalin in hypotonic NaCl solution. Embedding in methyl-butyl methacrylates.

Figs. 39 and 40 — Erythrocytes stained by phosphotungstic acid.

Fig. 41 and 42 — Erythrocytes treated by aqueous osmic acid solution after "fixation" with formalin in a hypotonic medium; *e* — envelope or membrane.

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Recebido para publicação em 10/8/71