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COMPARATIVE STUDY ON THE ULTRASTRUCTURE OF THE ELEMENTS OF THE AVIAN AND MAMMALIAN ERYTHROCYTIC SERIES. CORRELATION WITH HEMOGLOBIN BIOSYNTHESIS. *

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ABSTRACT: Ultrastructural studies of the erythron of birds and mammals were made in smears of stromata and in ultrathin sections of erythrocytes from the peripheral blood of normal and anemic animals.

Polyacrylamide gel electrophoresis and spectrophotometry were also used to detect intrahemosomal hemoglobin and the presence of heme group in the supernatants of fraction lysates. Chromatin extrusion in mature avian erythrocytes was correlated to nuclear extrusion in mammal orthochromatic erythroblasts. Furthermore, the nature of "Substantia Granulo-Filamentosa" (Sgf) was studied in birds and mammals, as well the degree of cell maturation as reaveled by polysome countings per μ^2. The marginal band has been investigated in ultrathin sections and smears of hemolysed blood.

Results may be summarized as follows:

1. In smears of hemolysed blood, filaments of larger diameter representing Sgf may be identified as mitochondria, whereas those of smaller diameter are interpreted as hemosomes.

2. In bleeding anemias, Sgf is almost exclusively made out of mitochondria.

3. The genesis of hemosomes is the same in birds and mammals and is related to hemoglobin biosynthesis.

4. No connection exists between intranuclear hemoglobin and synthesis blockade. This is related to chromatinic extrusion concomitant to polysomic dissociation.

5. In bleeding anemias avian and mammalian responses of the hemopoietic tissue differ in reaction time.

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UNITERMS: Ultrastructure of avian and mammalian erythrocytes. Hemoblobin biosynthesis.

INTRODUCTION

Through microspectrophotometry it has been demonstrated that during erythrocyte maturation basophilia decreases as hemoglobin concentration increases (Thorell [93], 1950). In basophilic erythroblasts a decrease of ribonucleoprotein is noted, as well as the disappearance of the nucleolus. The Golgi complex, other structures, and organelles (Orlic et al. [69], 1965; Simpson & Kling [91], 1967) have been observed.

The polychromatophilic erythroblast is characterized by a decrease in the number of mitochondria; hemoglobin synthesis is initiated, conferring a higher cytophasmic density in relation to the basophilic erythroblast.

In orthochromatic erythroblasts the membrane pores tend to disappear, and chromatin undergoes condensation. Still in this phase the nucleus is extruded, and less frequently suffers karyolysis or karyorrhexis according to the observations of Astaldi et al. [1] (1950) and Leonardi [59] (1951). After the extrusion, the reticulocyte phase follows, showing an intense hemoglobin synthesis in the peripheric blood (Seno [87], 1958; Fantoni et al. [41], 1968; Brunner [14], 1968), which results in the appearance of the mature erythrocyte.

The first observations of microtubules in lower vertebrate erythrocytes were made by Ranvier [80] (1875), Dehler [35] (1895) and Nicholas [67] (1896). Meves [65] (1903) observed these structures in avian erytrocytes by supravital staining, and suggested that the microtubules, composing the marginal band, confer to them their chracteristic biconvex shape. Weidenreich [97] (1905) considered the band as an artifact due to the staining technique. Fawcett [42] (1959) however, was able to ascertain by electron microscopy the actual existence of the marginal band in erythrocytes of the toadfish (*Opsanus tau*). Fawcett & Witebsky [43] (1964) described the band as formed by 25 parallel tubules of about 200 Å each, and state that this band is responsible for the elliptical shape of lower vertebrate erythrocytes. They proposed the term "endoplasmic ring" for this structure of both erythrocytes and thrombocytes of the toadfish. Grasso [46] (1966) supposed that the function of the band is closely related to the shape and elasticity of mammalian erythrocytic cells.

Endocytosis is the generic term given to any type of incorporation of substances into the cell (Bessis [6], 1972). Lewis [60] (1931) observed the incorporation of droplets, 1-5 μ in diameter, calling this mechanism pinocytosis. Endocytosis of dense substances such as ferritin has been analysed by Bessis & Breton-Gorius [7] (1956) and Bessis [5] (1958). Parks & Chiquoine [70] (1957) observed this mechanism for silver compounds.

Ribonucleoproteins are constituted of about 50% RNA, and 50% proteins (Dintzis et al. [38], 1958). Warner et al. [96] (1962), found in reticulocytes that ribonucleoproteins can present themselves from monosomic to polysomic forms. The arrangement most frequently found is the pentamerous one whose monosomes are united by a thin filamentous m-RNA bridge. Mathias et al. [63] (1964) demonstrated that this bridge of ribonucleic nature is sensitive to RNase. Glowacki & Millette [45] (1965) believed that in the course of maturation a

total disaggregation of pentamerous polysomal forms directly to monomerous forms would occur, contrary to Marks et al. [62] (1963) and Rifkind et al. [83] (1964) who affirm that this disaggregation is gradual, i.e., pentamerous, tetramerous, trimerous, dimerous, and momomerous. Burka & Marks [24] (1967), maintain that the last RNA to disappear is the t-RNA, although no more globin synthesis occurs in the already nearly mature reticulocyte. The polysomic activity during the protein synthesis is higher in the polychromatophil and the orthochromatic erythroblasts (Rifkind et al. [83], 1964), as well as in the less mature reticulocytes (Glowacky & Millette [45], 1965). Based on the theory of Jacob & Monod [51] (1961) as to the relationship between nucleus and cytoplasm it is possible to assume that the m-RNA and t-RNA are synthesised still in the erythroblastic phase, since reticulocytes do not contain any more DNA. There is evidence that biosynthesis of the r-RNA precursors occurs within the nucleolus (Brown & Gurdon [12], 1964; Penman et al. [72], 1966; Edström & Daneholt [40], 1967; Izawa & Kawashima [50], 1968). Seno et al. [88] (1963) and Pinheiro et al. [75] (1963) demonstrated the absence of DNA synthesis both in reticulocytes and the different RNA types.

Brunner & Vallejo-Freire [22] (1964) found convoluted forms in reticulocytes, similar to myelin figures, which can be observed in a great variety of cells, such as mature erythrocytes about to lyse (Policard et al. [76], 1957), branchial epithelium of salamander (Schultz & De Paola [86], 1958), cultured cells of guinea-pig testicles infected with viruses and rickettsiae (Brunner, 1961 — personal communication), mitochondria of leucocytes within the epithelium of patients with American tegumentary leishmaniosis (Coiro et al., 1973 — umpublished observations), and mitochondria of pancreatic cells of normal adult rats (Coiro & Souza Dias, 1973 — umpublished observations).

Since the work of Cesaris-Demel [26] (1907) up to present, the pre-existence of organelles in reticulocytes has been discussed although the work of Simmel [90] (1926), Seyfart [89] (1927), Sano [85] (1955) and Brecher [11] (1958), allowed cleary the observation of the "Substantia granulo-filamentosa" ("Sgf"). Even so, other authors considered the "Sgf" as an artifact resulting through the applied staining technique, due to ribonucleoprotein precipitation caused by supravital dyes such as brilliant cresyl blue and Janus green B (Dustin [39], 1947; Thorell [93], 1950; Burt et al. [25], 1951; Bessis [4], 1954; Thoma [92], 1959).

Bernhard et al. [2] (1949) observed under the electron microscope reticulocytes after osmotic hemolysis. This procedure allowed the observation of circular membranous intrareticulocytary forms whose nature also was much discussed by various authors as Braunsteiner & Bernhard [9] (1950), Bessis [3] (1950), Peters & Wigand [74] (1950), Wolpers [100] (1956), Brunner & Vallejo-Freire [21] (1956), Jung [54] (1959) and Hug et al. [49] (1959). Brunner & Vallejo-Freire [21] (1956) however, found filamentous forms, granules, and fine filaments in hemolysed reticulocytes after partial drying. At this same period of time, Braunsteiner et al. [10] (1956), and Brunner et al. [23] (1956) confirmed the presence of mitochondria in those cells. Later, Brunner [13] (1962) clearly demonstrated the mitochondrial and ribosomic nature of the "Sgf" in association with the dye. Granules and fine filaments were interpreted as ribosomes and smooth endoplasmic reticulum (ER) respectively.

Reimann [81] (1942) working with chicken proerythrocytes concluded that

the hemoglobin biosynthesis is not related to the nucleus but to the "Sgf". Jensen et al. [52] (1953) verified that hemoglobin biosynthesis is related to the amount of "Sgf". Brunner & Mombrum [19] (1972) demonstrated in mammalian reticulocytes the presence of organelles, which, on account of their ultrastructural similarity with mitochondria, were denominated mitochondrion-like organelles (MLO). Brunner et al. [20] (1972) suggested that the final hemoglobin biosynthesis occurs in the MLO or hemosomes.

The purpose of the present work is to analyse comparatively the ultrastructural aspects os the avian and mammalian erythron, and to elucidate several points in relation to morphology and physiology. Accordingly, we planned a study on the following: a) origin and nature of the cytoplasmic vesicles present in avian erythrocytes; b) ultrastructural identity of avian and mammalian "Substantia granulo-filamentosa"; c) genesis of the mitochondrion-like organeles; d) hemoglobin presence in MLO from birds; e) estimation of the cell maturity degree in the avian erythron.

MATERIAL AND METHODS

Material

Avian blood

Adult chickens (*Gallus gallus*), 2.5 — 3.0 kg. Blood was harvested from the marginal wing vein or by cardiac puncture.

Embryos, 14-18 days old, and newborn chicks. Blood was harvested by cardiac puncture.

Adult chickens with anemia induced by bleedings, withdrawing daily 30-40 ml of blood during 2-3 days. One to two days after the last cardiac puncture, blood was harvested.

Adult chickens with anemia induced by phenylhydrazine in an 1% aqueous solution in a 1 ml/kg ratio, by subcutaneous injections per 4 days. Five days after the last dosis the blood was harvested.

Mammalian blood

Adult guinea-pigs (*Cavia porcellus*), weighing 250-300 g, with phenylhydrazine-induced anemia injected in an 1% aqueous solution in a 1 ml/kg ratio, during 3 successive days. Three days after the last dosis, blood was withdrawn.

Adult guinea-pigs (*Cavia porcellus*) 250-300 g each, with anemia induced by successive bleedings by cardiac puncture, withdrawing 5 ml during 5 successive days. Three days after the last puncture, blood was harvested.

Rabbit embryos (*Oryctolagus cuniculus*), 16-20 days old. Blood was harvested after sectioning the umbilical cord.

Human blood

Blood from a patient with acquired hemolytic anemia was harvested by venous puncture.

Methods

Rosenfeld [84] staining (1947, modified)

The modification consisted in the dilution of the dye in a 1:4 ratio on the slide, with degasified distilled water, leaving the diluted dye in contact with the smear for 2 h.

Supravital staining with brilliant cresyl blue

One gram dye was dissolved in 1 liter of a 0.85% (1:1000) saline solution. Cells were stained in a 1:9 ratio of blood drops and dye respectively.

Feulgen reaction (Lison [61], 1953, modified)

The original indications of Feulgen-Rosembeck (1924) were followed except for the fixation of the smears, which was carried out in methanol for 3 min.

Hemolysis. Technique A — According to Brunner & Vallejo-Freire [21] (1956). Technique B — Followed the procedure of technique A, but with a modified fixing process introduced for mature erythrocytes. Instead of formol, 25% glutaraldehyde in 90 ml NaCl at 0.80% in an amount of 10 ml was used mainly in order to improve the degree of preservation of stroma structures, as for instance the marginal band, and organelles (Coiro et al., 1973 — unpublished observations). Technique C — The same as A, however, modified as to the partial drying. The drying lasted for 5 min at 40°C in order to obtain circular forms (Coiro & Brunner, 1972 — unpublished observations). Technique D — Hemolysis in suspension to obtain circular forms in mammalian blood (Brunner & Vallejo-Freire [21], 1956).

Fixation

Blood was harvested into an anticoagulant consisting of 0.75 ml of 2% EDTA, and 0.25 ml of 4% sodium bicarbonate in a 1 ml per 10 ml ratio.

Double fixation

Fixation in a glutaraldehyde gradient followed by osmium tetroxide in Millonig buffer (pH 7.3) (Brunner & Coiro [16], 1973).

Direct fixation in potassium permanganate followed by osmium tetroxide in veronal acetate buffer (pH 7.3).

Simple fixation

Direct fixation in 1% osmium tetroxide in Millonig buffer (pH 7.3) for 20 min.

Fixation in hypotonic medium for phosphotungstic acid (PTA) staining (Brunner & Mombrum [19], 1972).

For blood fixed in glutaraldehyde and osmium tetroxide, the staining was done in an aqueous 1% uranyl acetate solution (Kellenberger et al. [56], 1958) for 30-40 min.

Dehydration was carried out in the alcohol series at 30%, 50%, 70%,

95%, 100% with pure acetone in a 1:1 ratio, and finally in pure acetone, for 10 min each.

Pre-imbibition — Imbibition — Embedding

Pre-imbibition was carried out in a 1:1 Polylite 8001 (Coiro et al.[33]; Coiro & Brunner[28], 1972) and pure acetone mixture for 1-2 h.

Polylite 8001 mixtures

A —	Polylite 8001	10 ml	(Coiro et al.[33], 1972)
	Benzoyl peroxide	0,2 g	
B —	Polylite 8001	9 ml	(Coiro & Brunner[29], 1973)
	Dibutylphtalate	1 ml	
	Benzoyl peroxide	0,2 g	
C —	Polylite 8001	7 ml	(Coiro[27], 1972)
	Benzoyl peroxide	0,2 g	
	Polylite T 200	3,0 ml	

Imbibition of all material was carried out with Polylite 8001 or Polylite 8001-P (Polylite with a plastifyer) for 24 h in intermittent resuspension. Embedding was carried out in gelatin capsules, n.º 0 or n.º 00. Into all capsules, 5 drops of blood imbibed in polyester were placed, and made up with the polyester in use. The cells were then centrifuged at 550Xg for 30 min. Polymerization was achieved in an incubator at a constant temperature of 58ºC for 72-96 h.

Ultramicrotomy — final staining — electron microscopy

MT-1, and MT-2 (Porter Blum) ultramicrotomes were used with crystal or diamond knives. Ultrathin sections were stained by lead citrate (Reynolds[82], 1963), and washed in degasified distilled water. Electron microscopes Siemens ÜM 100b, Elmiskop I, and Zeiss EM-9S2 were used for the examinations. Accelerating potencies varied from 60 to 100 Kv.

Fractionation, organelle isolation, and hemoglobin determination in avian blood

Blood was harvested by cardiac puncture from 60 embryos, 16 days old. Immature erythrocytes were fractionated, and the mitochondrion-like organelles (MLO) were isolated, washed and lysed according to the following procedure: a) addition of 4 ml blood to 8 ml of a solution prepared according to Glowacki & Millette[45] (1965); b) centrifugation of the cell suspension at 200Xg (900 rpm R=21 cm) for 10 min; c) resuspension of the sediment in a 0.32 M sucrose solution, 10 times its volume, according to Weinbach[98] (1961); d) homogenization in a Potter-Elvehjem tube, in the cold at about 1,000 rpm with 10 movements; e) centrifugation of the homogenate at 1,350Xg (R=21 cm) for 10 min; f) centrifugation in the cold of the supernatant at 14,830Xg (15,000 rpm — Spinco L, rotor 40) for 10 min; g) three washings by resuspension of the pellet with 0.32M sucrose, and controlling of the fraction's purity degree by electron microscopy; h) harvesting the last washing supernatant to be used as

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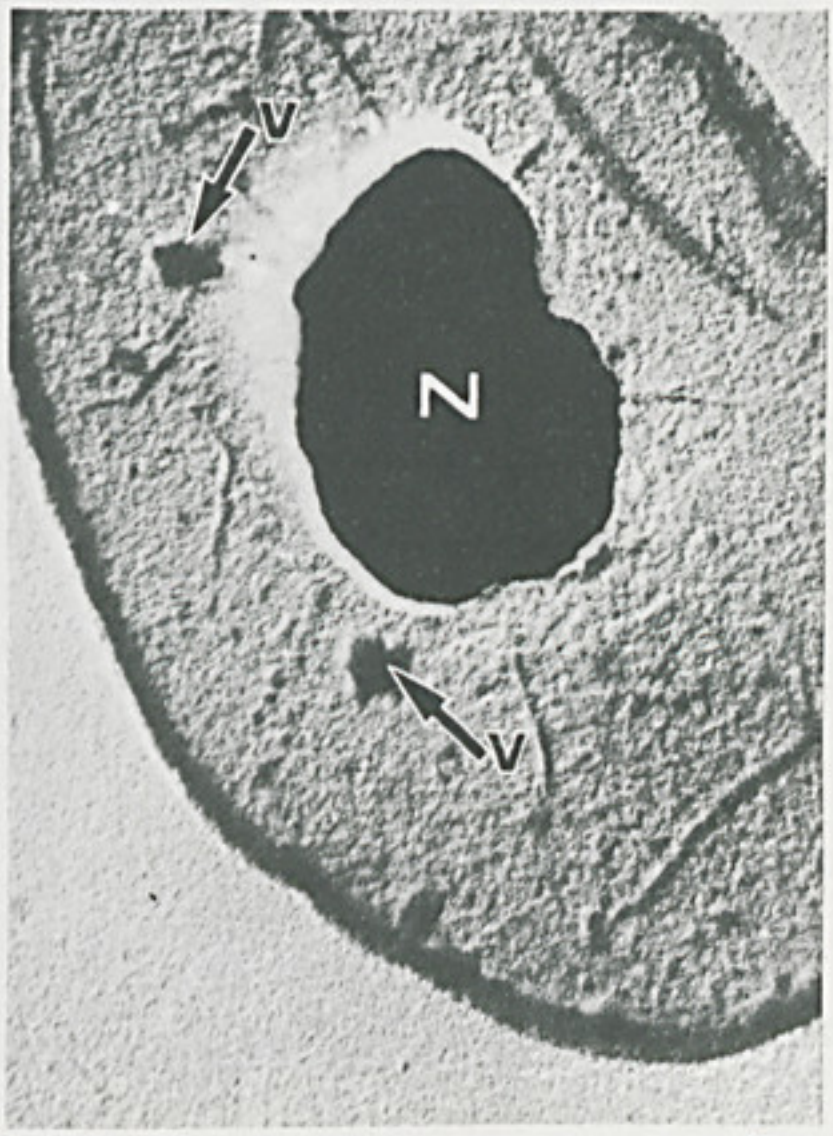

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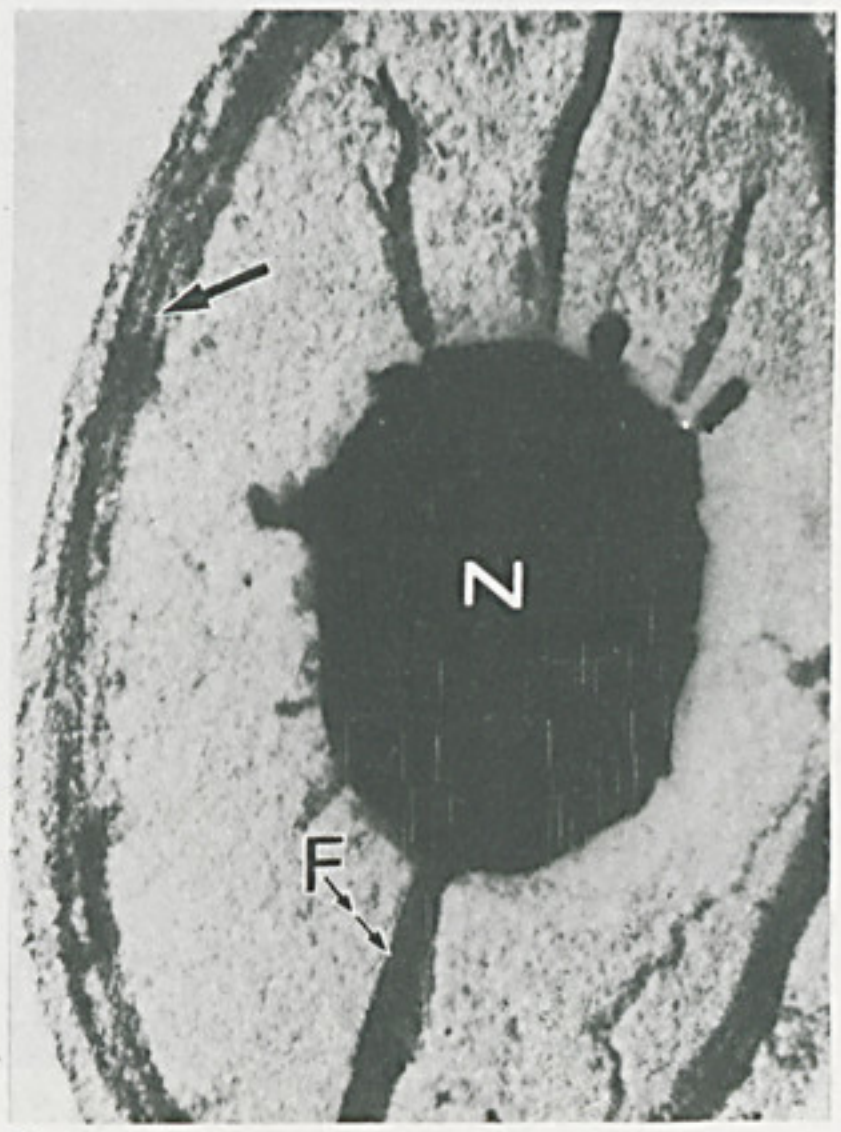

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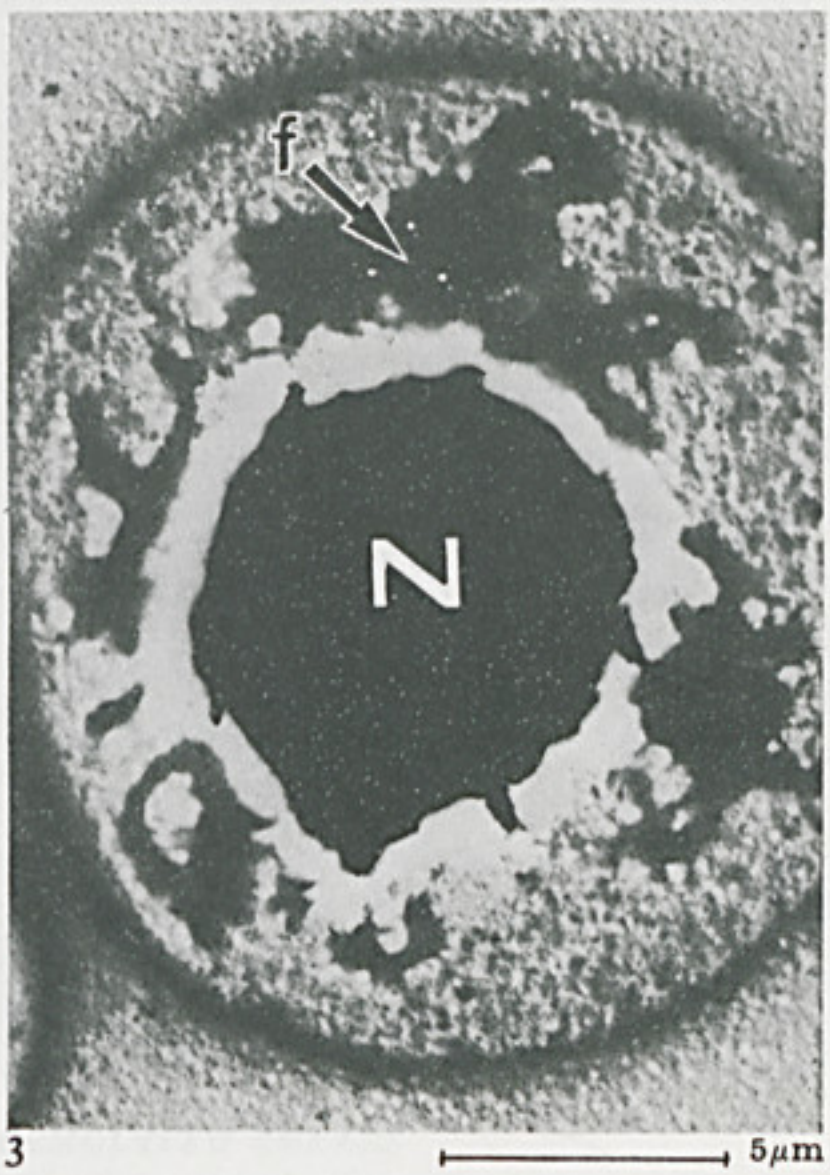

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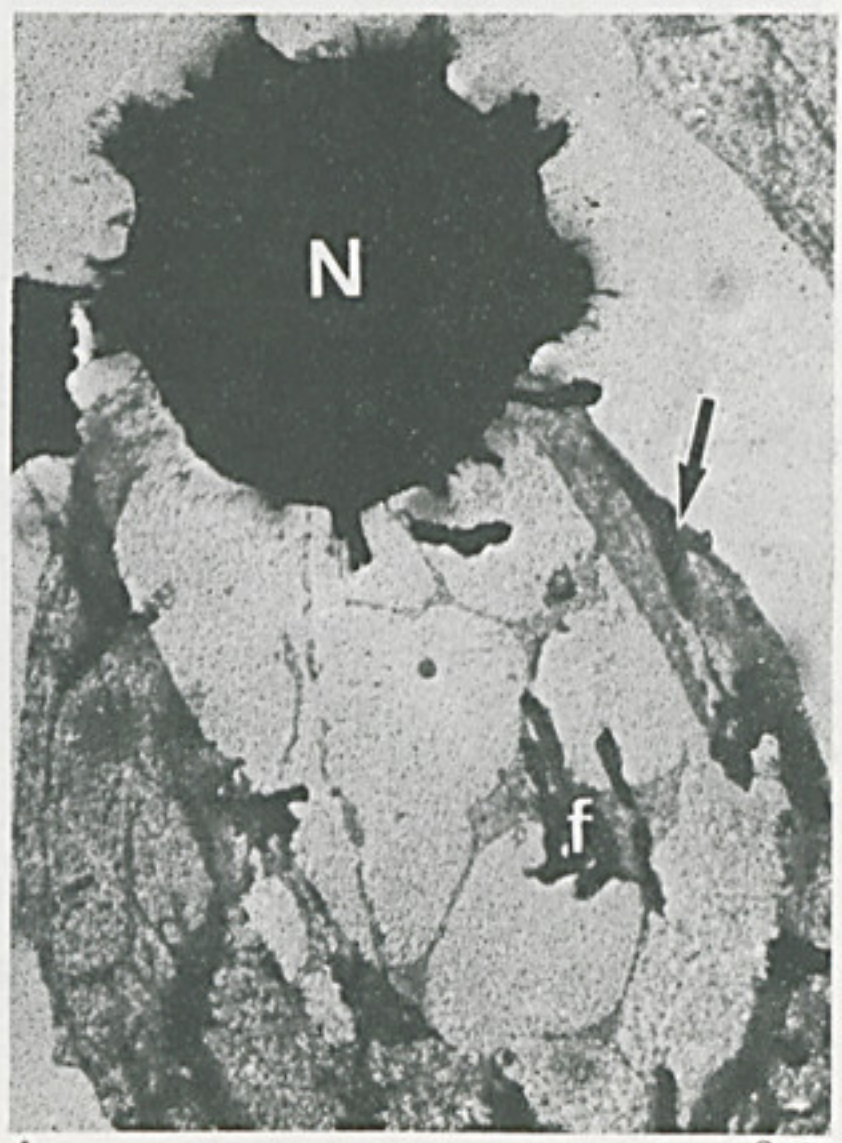


4 2μm

HEMOLYSED SMEARS

Figs. 1 and 2 - Mature avian erythrocytes: 1 - (N) nucleus; (V) vesicles. 2 - (N) nucleus; (F) fold; (arrow) microtubule bundle.

Fig. 3 - Immature avian erythrocytes: (N) nucleus; (f) filaments.

Fig. 4 - Orthochromatic mammal erythroblast: (N) nucleus in extrusion; (f) filaments (Sgf); (arrow) stroma.

control; i) osmotic lysis by resuspending the pellet in 3 ml degasified distilled water; j) centrifugation of the suspension at 20,000Xg for 10 min, and concentration in a vacuum chamber of the supernatants and control; k) determination of hemoglobin by electrophoresis.

Fractionation, organelle isolation and hemoglobin determination in mammalian blood

For mammalian blood, several modifications of the procedure were adapted. In item "f", 10,000Xg instead of 14,830Xg was used, and in item "g", 5 instead of 3 washings were carried out.

Electrophoresis in disc polyacrylamide gel, and spectrophotometry of the concentrated supernatants

The method of Dietz & Lubrano [37] (1967), was used for electrophoresis, and spectrophotometry was performed in accordance with the method of Kampen & Zijlstra [55] (1964).

Estimation of the degree of erythrocyte maturation (Brunner & Coiro, 1973 — unpublished observations)

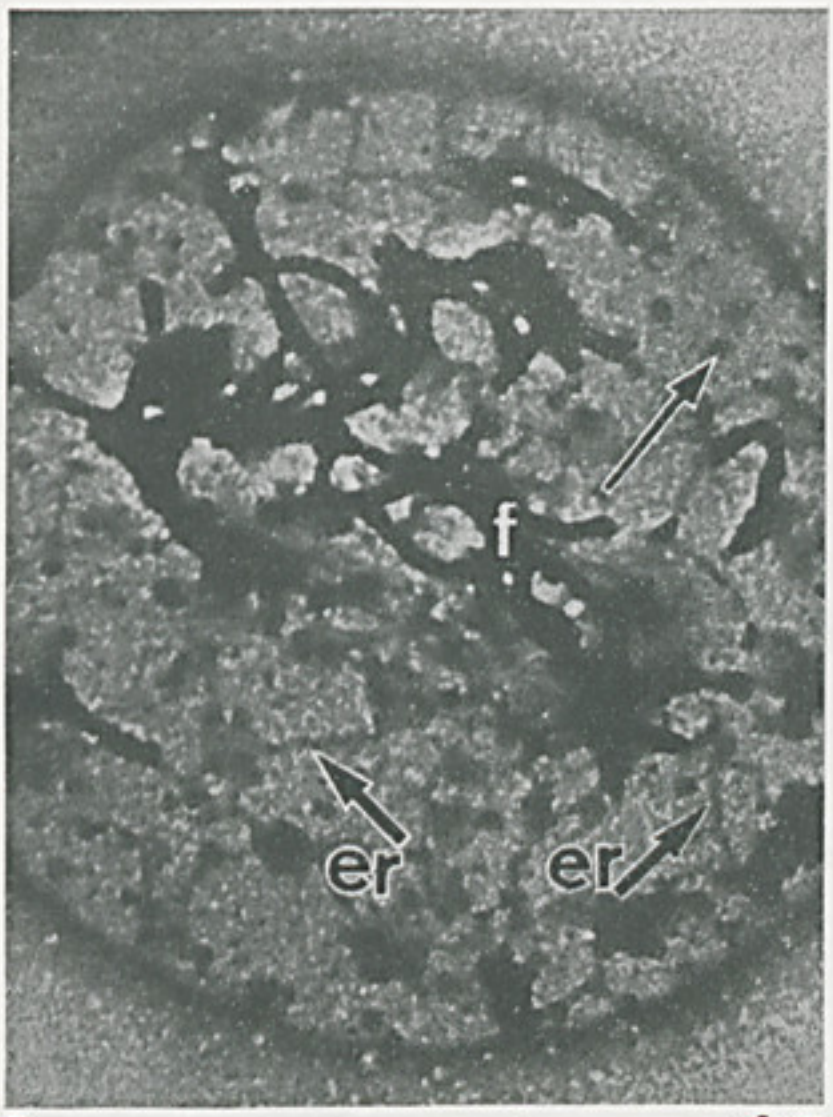
In mammals, the orthochromatic phase is easily recognized by the irregular cytoplasm configuration as well as by the eccentric disposition of the nucleus. Therefore, this phase has been selected as the point of reference for the polysome countings.

In birds, in the orthochromatic phase no evidence of irregular cytoplasmic configuration or perceptible nuclear eccentricity can be found. Therefore, an identification of the various maturation phases is very difficult. Basophilia of a cell, in a higher or lesser degree, depends on the amount of polysomes. Knowing the frequency of the polysomes, and their variations in the orthochromatic mammalian erythroblast ($x \pm y$), the determination of the polysome frequencies in immature avian cells allows a close estimate of their identity.

A value higher than $x + y$ would allow the identification of an avian erythroblast at least as polychromatophilic; a value between $x \pm y$, as orthochromatic, and less than $x - y$, as proerythrocyte, corresponding to the reticulocyte of mammals.

To attain these results, the following procedure was used:

1) Estimation of the negative magnification;
2) weighing of the photographic paper in order to estimate a determined area per gram;
3) weighing of the cytoplasm free of organelles, nucleus, and other structures for subsequent estimate of the cytoplasmic area;
4) conversion of the cytoplasmic area to μ^2;
5) counting of the polysome number in the whole cytoplasmic area, and estimate of the polysome number per μ^2.



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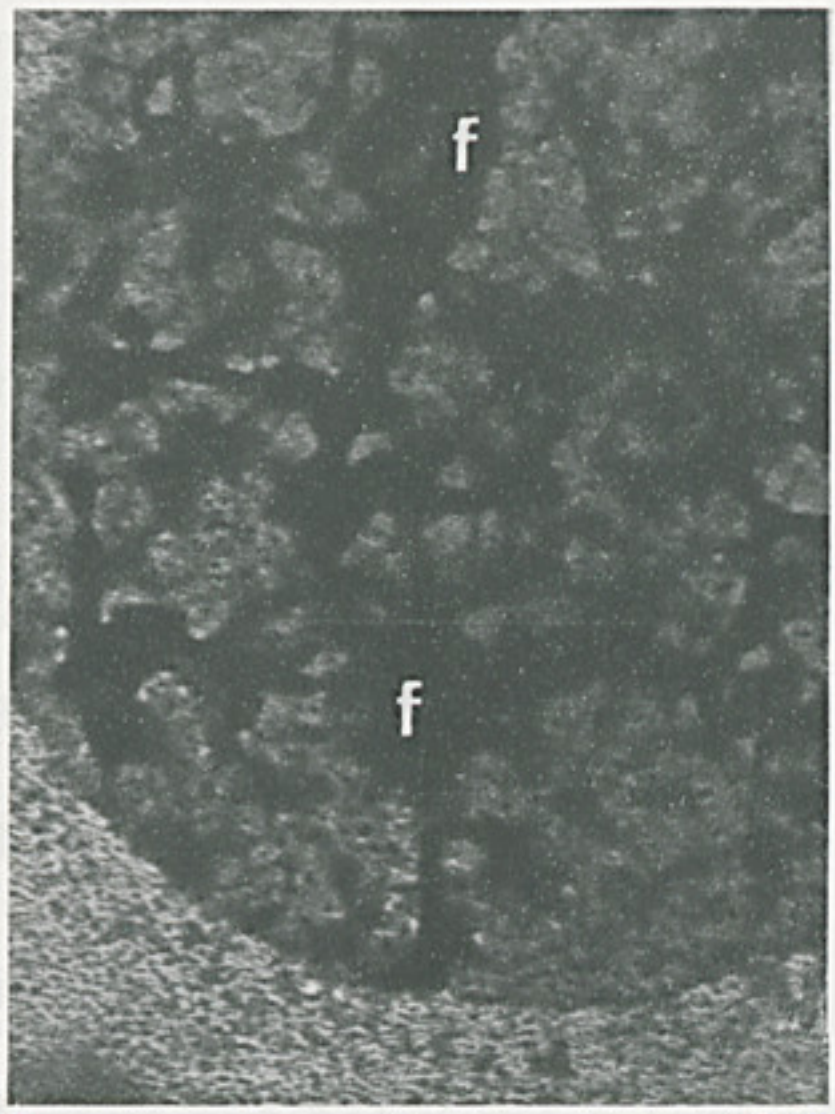
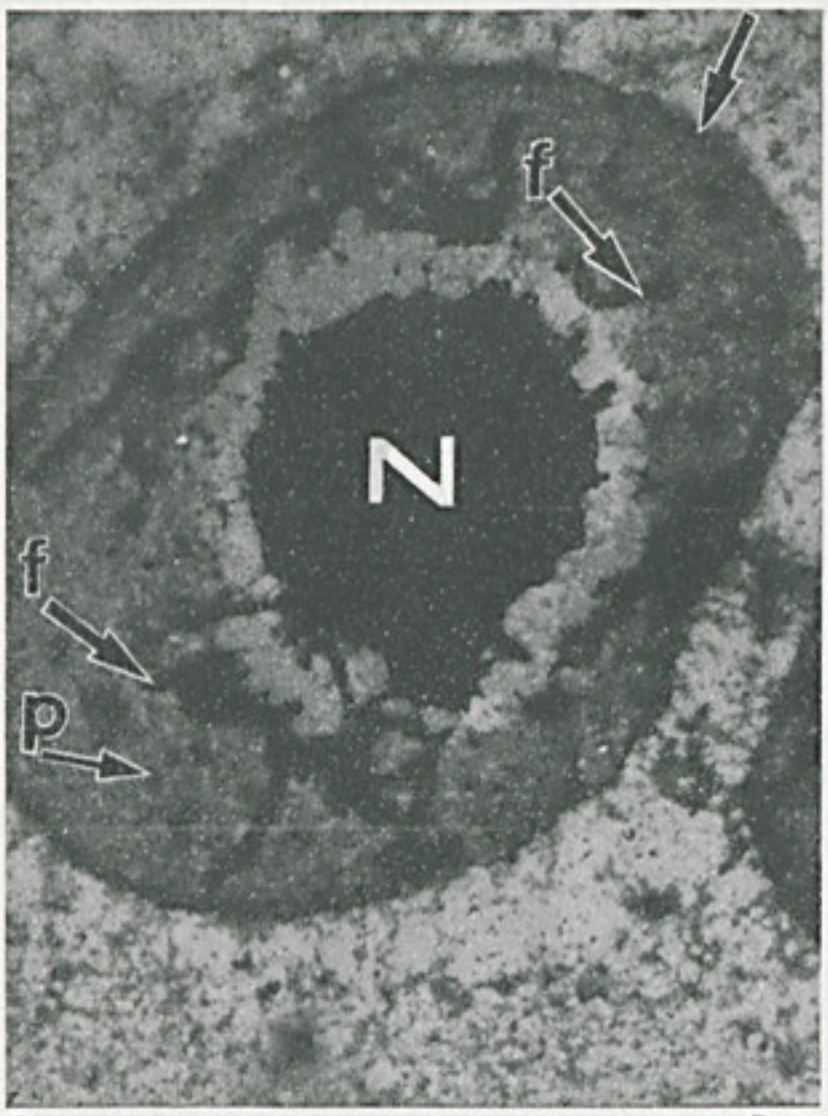


6 5μm



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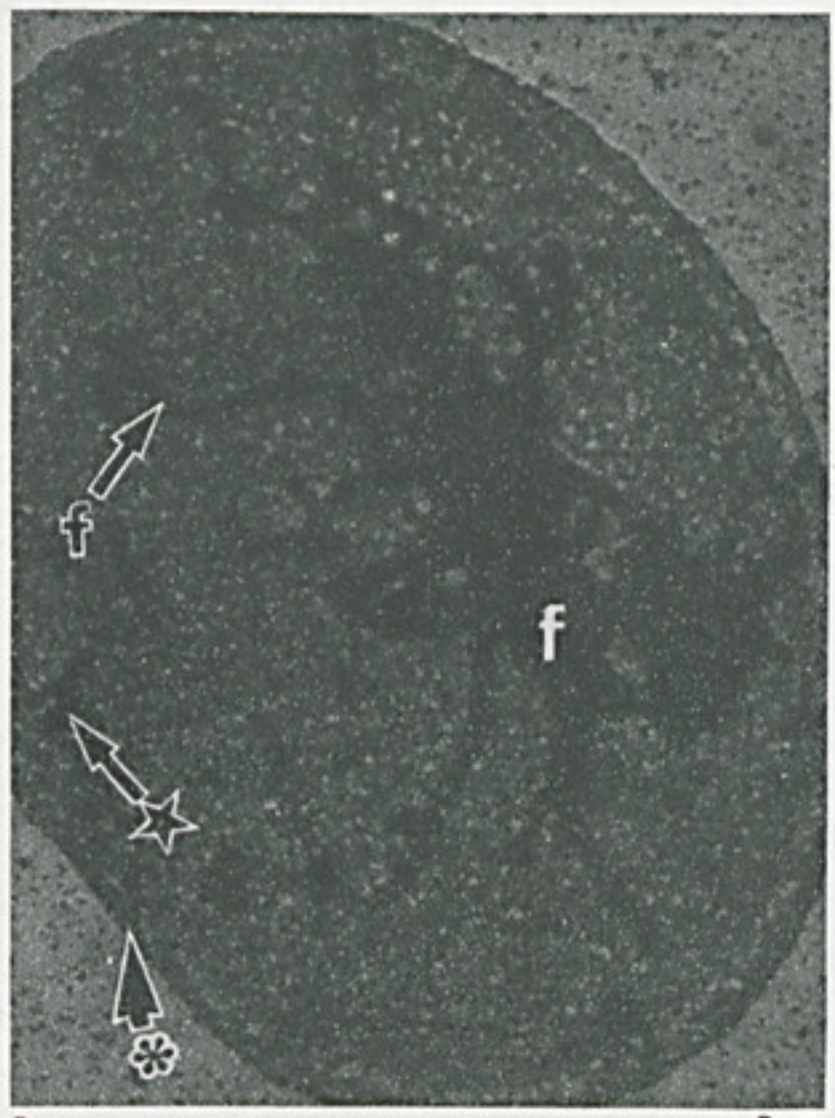

8 2μm

HEMOLYSED SMEARS

Figs. 5 and 6 - Mammal embryo reticulocytes (Prohematia): 5 - (er) endopalsmic reticulum (arrow) polysome; (f) filaments. 6 - (f) a great number of filaments

Fig. 7 - Immature cell of avian embryo: (N) nucleus; (f) filaments; (p) polysome; (arrow) stroma.

Fig. 8 - Reticulocyte (Prohematia) from phenylhydrazine intoxicated adult mammal: (f) filaments; (asterisk) stroma; (star) segmented filament.

Calculation of the polysome number per μ^2 can be made according to the following formula:

$$Np/\mu^2 = \frac{Pp.\ A^2\ .\ Np}{Pc\ .\ Ap}$$

Np/μ^2 = number of polysomes per μ^2;

Pp = weight of the known area of the photographic paper;

A^2 = increase of the photographic magnification, squared;

Np = polysome number in the cytoplasm free of other structures;

Pc = weight of the cytoplasm free of nucleus, organelles, and other structures;

Ap = known area of the photographic paper.

RESULTS

Rosenfeld [84] staining (1947 — modified)

In order to estimate the percentage of the different immature forms, this staining was used for the normal blood of chickens, embryos, as well as for the blood of animals with anemia induced by successive bleedings or phenylhydrazine intoxication.

Blood \ Immature forms	Basophilic erythroblast	Polychromatophil erythroblast	Orthochromatophil erythroblast
Embryos - 15 days	0,8%	11%	7%
Bleedings	1,4%	14%	6%
Phenylhydrazine	1,4%	18%	9%

Brilliant cresyl staining

Through this staining blood samples of normal animals and others with anemia of different types were examined. The immature forms are characterized by the presence of the "Substantia granulo-filamentosa".

Aves	Embryos	Phenylhydrazine	Bleedings	Normal adult
	100%	30%	30%	0,5%
Mammals	Embryos	Phenylhydrazine	Bleedings	Human with acquired hemolytic anemia
	100%	30%	30%	30%

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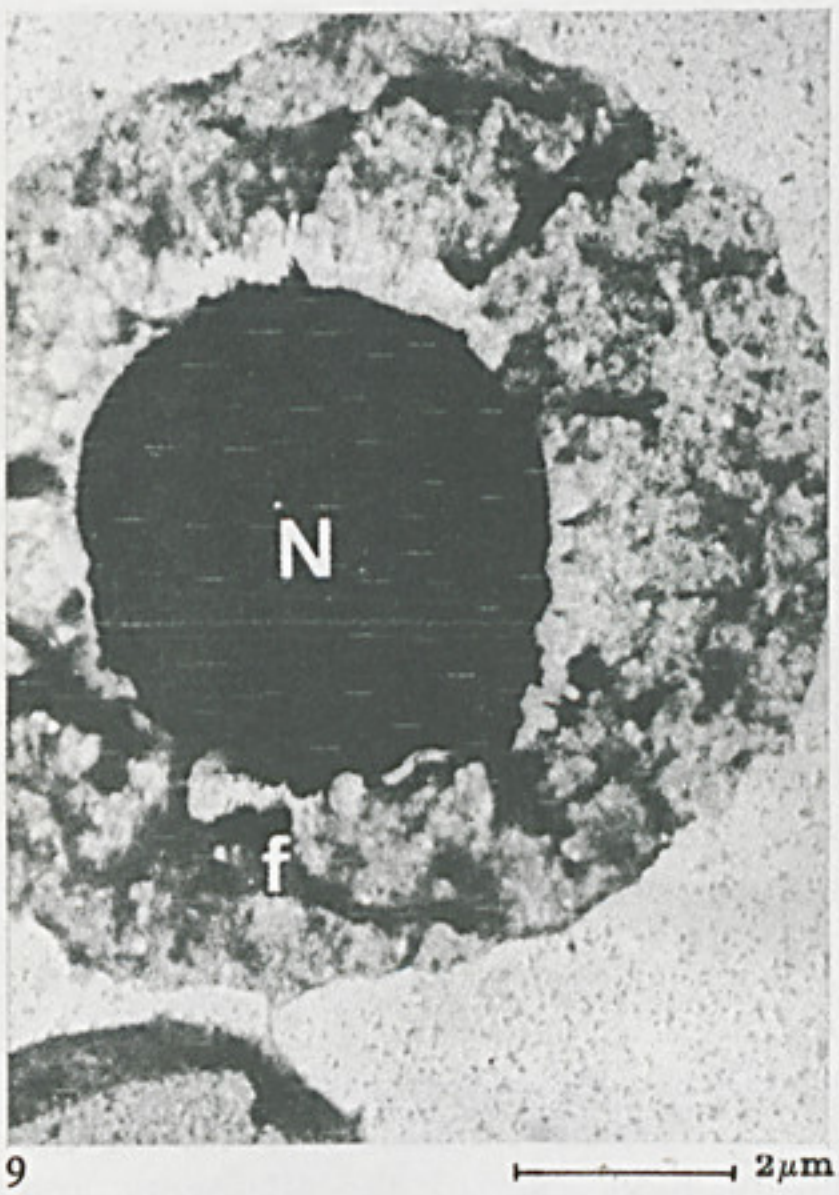
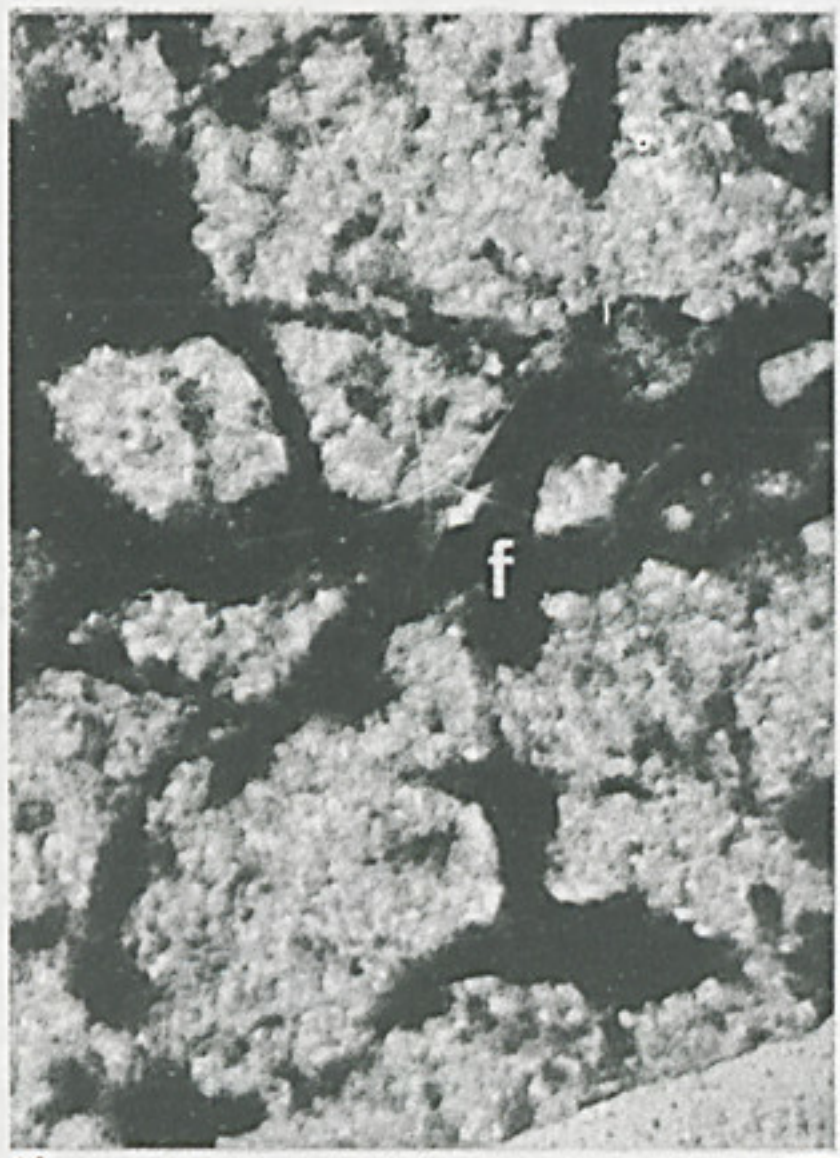


9 2μm



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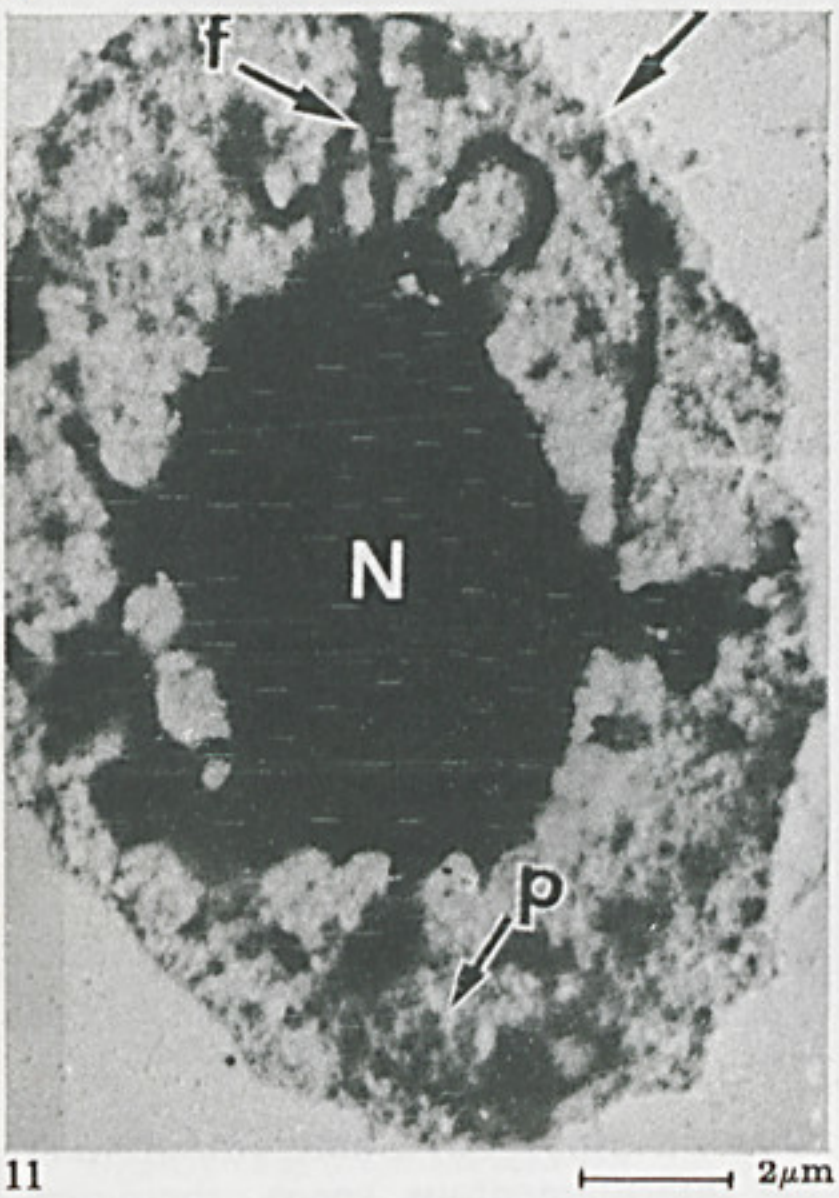
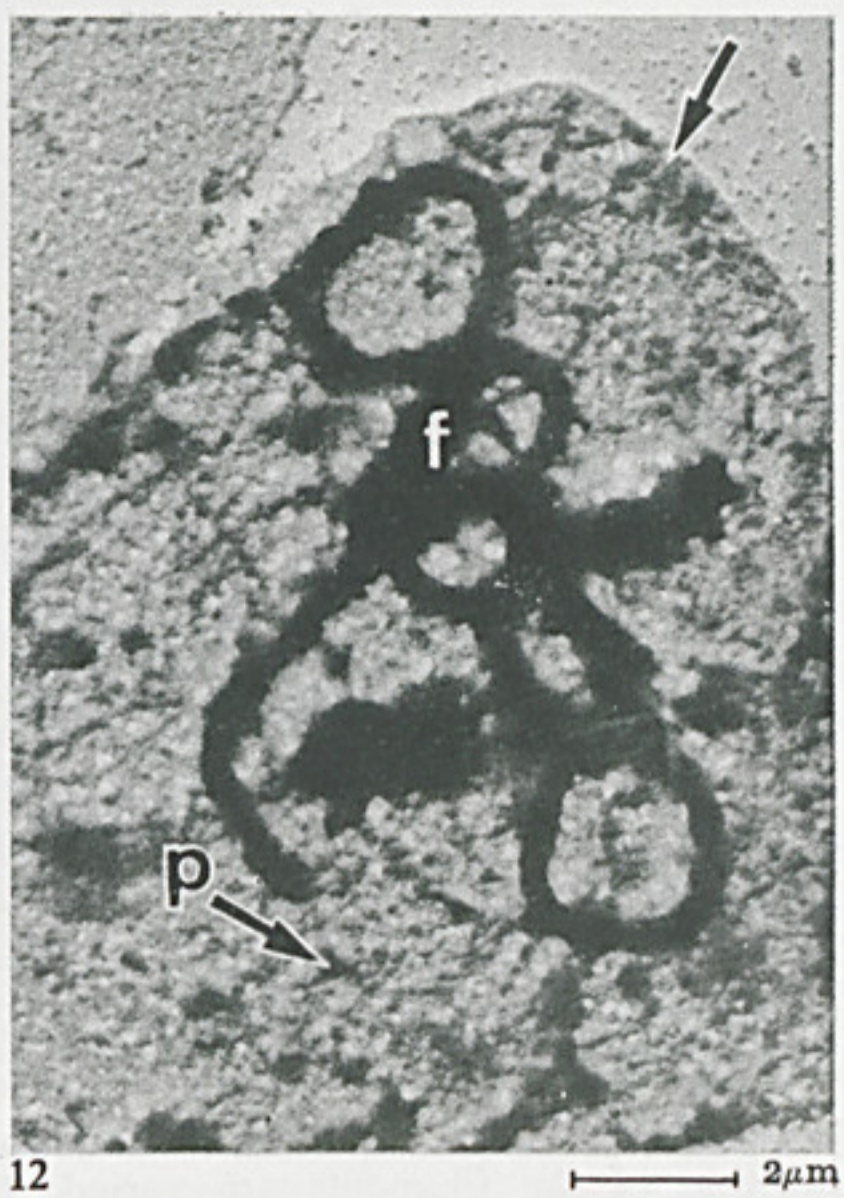


11 2μm



12 2μm

HEMOLYSED SMEARS

Figs. 9 and 10 - Erythroblast and prohematia (reticulocyte) of a patient with acquired hemolytic anemia: 9 - (N) nucleus; (f) filaments. 10 - (f) filaments.

Figs. 11 and 12 - Immature erythrocyte and prohematia (reticulocyte) from bled adult chicken and mammals: 11 - (N) nucleus; (f) filaments; (p) polysome; (arrow) stroma. 12 - (f) filaments; (p) polysome; (arrow) stroma.

Feulgen reaction

The presence of Feulgen positive cytoplasmic vesicles, close to or distant from the nucleus has been verified. In DNase treated control smears, the cytoplasm did not present any Feulgen positive structures.

Hemolysis

Adult animal blood

In birds, most of the stromas are elliptical and with a dense central nucleus. Bodies with dimensions between 0.42 x 0.64 μ and 0.88 x 1.2 μ, apparently with the same nuclear density, can be observed at variable sites either distant from the nucleus or very close. The number of these bodies varies from 1 to 3 (Fig. 1). At the periphery of many stromas, filamentous formations can be seen of about 300 Å in diameter, parallel among themselves and to the periphery (Fig. 2). In mammals, the stromas are circular and with scarce dense granules.

Embryo blood

In the avian and mammalian stromas, dense filaments of variable configuration and disposition are observed; the mean diameter is 0.19 μ. Among the filaments, granules are seen with less density than that of the filaments and with a mean diameter of 0.20 μ. Dense filaments in mammals reach a mean diameter of 0.15 μ. Often it is possible to observe extremely thin filaments with a diameter of about 660 Å, whose extremities seem to be in contact with the stroma's periphery, and the dense filaments. In the anucleated mammalian stromas, dense filaments are less numerous and present a diameter of about 0.16 μ (Figs. 3 and 5).

Blood from adult birds and mammals with anemia induced by phenylhydrazine and human blood from patients with acquired hemolytic anemia

Avian stromas present dense central nuclei; around them, dense filaments in a much variable disposition and configuration are seen. Among the filaments there are granules of less density than that of the nucleus. Many filaments become fragmented, giving rise to segments of indefinite contour. In the preserved filaments the diameter is of abount 0.19 μ In mammals, the filaments achieved a mean diameter of 0.17 μ, and within the interfilamentous space, granules with a mean diameter of 0.15 μ are seen (Figs. 7 to 10).

Blood from birds and mammals with anemia induced by successive bleedings

In birds, stromas present themselves circular or elliptical with dense filaments, however, in a smaller number than in embryos. Configuration and position of the filaments are varied. In the interfilamentous space, there are scarce granules of little density, and with diameters of about 0.15 μ, with dense filaments achieving up to 0.20 μ in diameter. Mammalian filaments, however, may reach up to 0.30 μ in diameter, disposition and configuration varying from one stroma to another. Between the filaments, granulation of little density and diameters of about 0.15 μ can be detected (Figs. 11 and 12).

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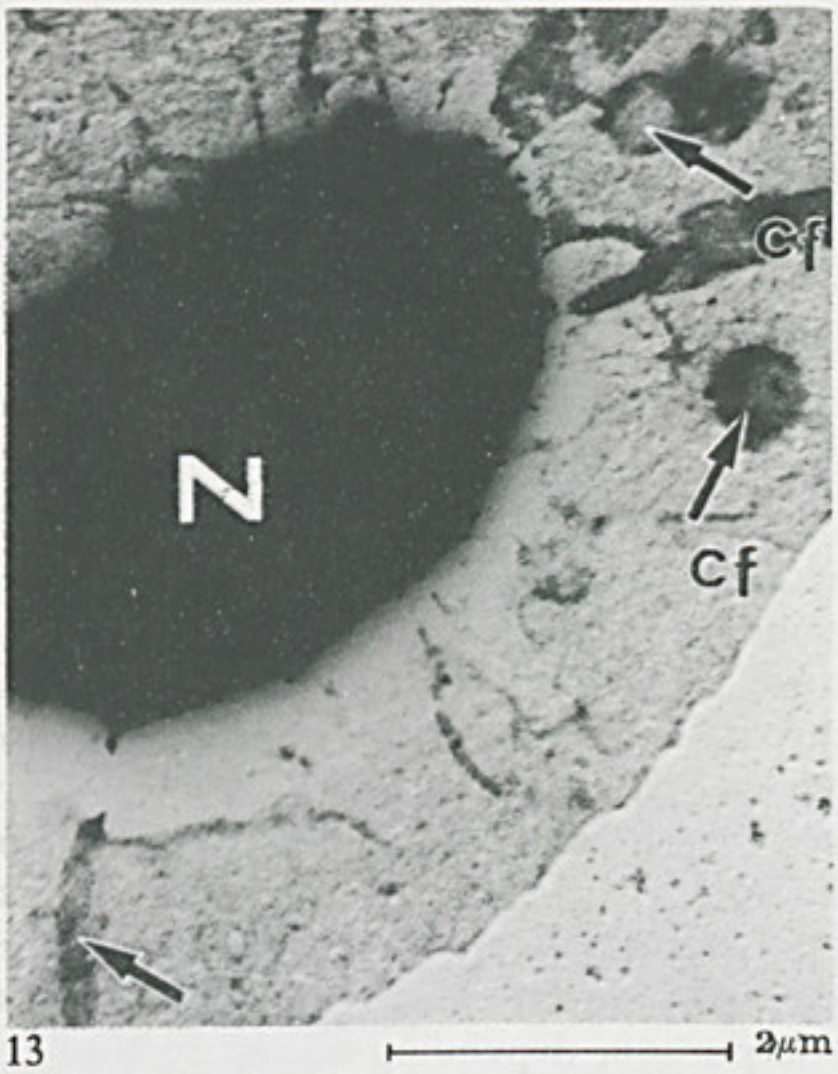
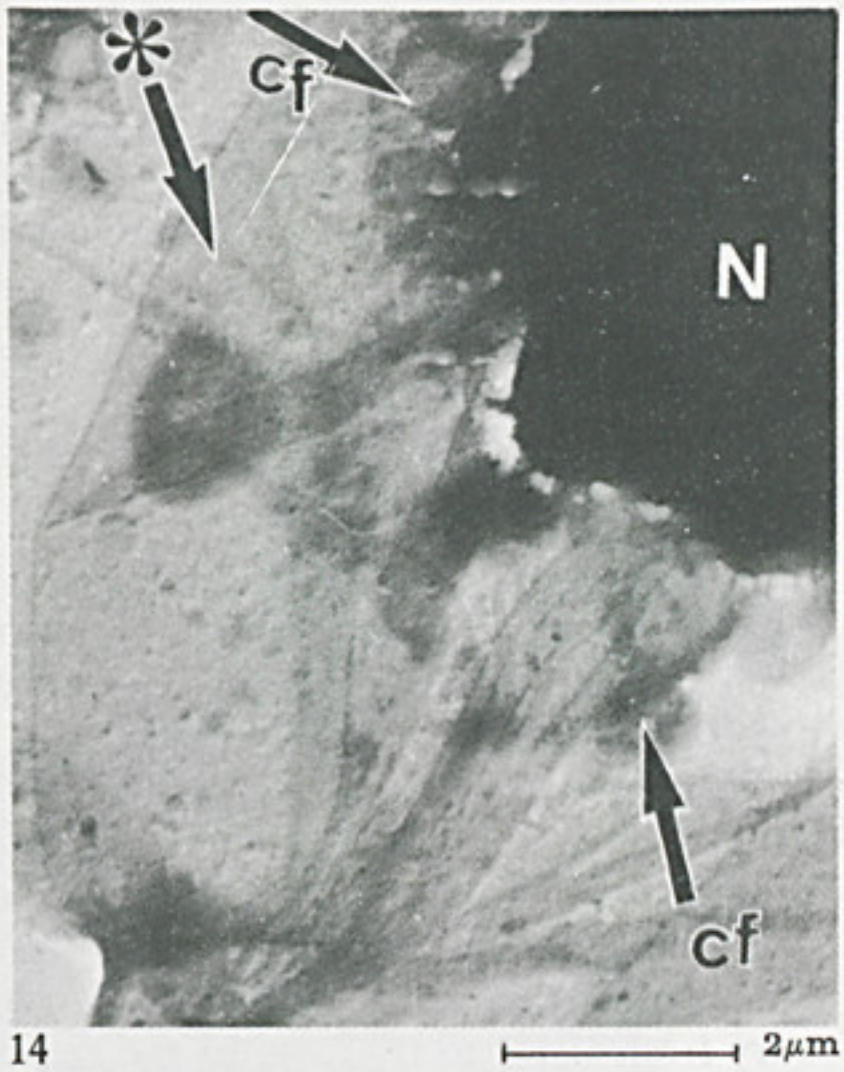


13 2μm



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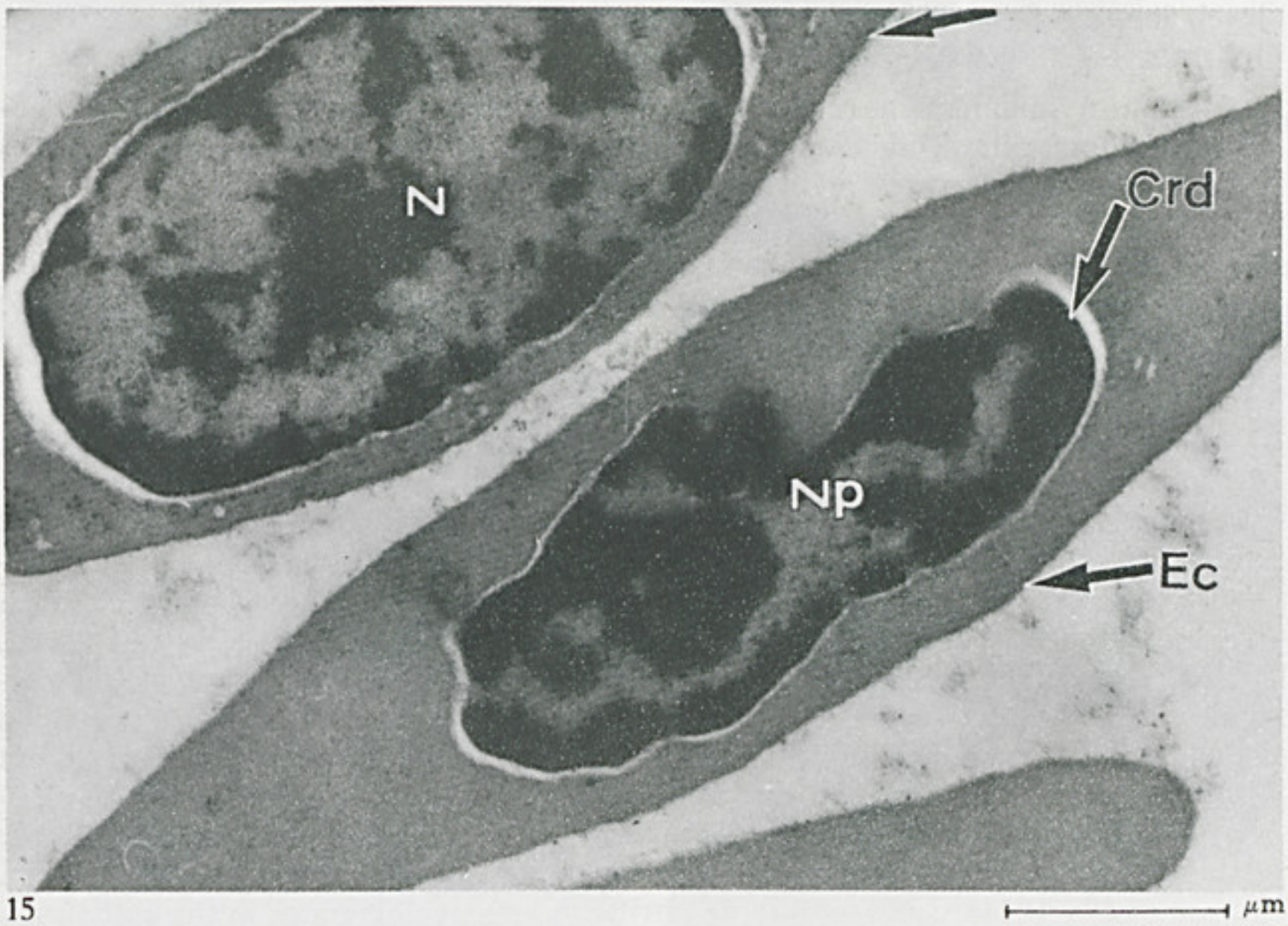

15 μm

HEMOLYSIS IN SUSPENSION

Figs. 13 and 14 - Immature chicken embryos erythrocyte and mammal embryos erythroblast: 13 - (N) nucleus; (cf) circular forms; (arrow) fold. 14 - (N) nucleus; (cf) circular forms; (asterisk) stroma.

ULTRATHIN SECTIONS

Fig. 15 - Mature avian erythrocyte: (Ec) mature erythrocyte; (Np) picnotic nucleus; (crd) dense chromatin; (N) nucleus (chromatin is not condensed); (arrow) immature erythrocyte.

Circular forms

Immature cell submitted to hemolysis in suspension give rise to distinctly circular bodies, with diameters of about 1.57 μ. In birds, the same circular forms are obtained through hemolysis in smears after a very rapid drying (Figs. 13 and 14).

Ultrathin sections

Blood of adult birds and mammals

In birds, erythrocytes present a pycnotic nucleus. Through the nuclear membrane pores, penetration of hemoglobinized cytoplasm can be observed, conferring to the caryoplasm a density close to that of the cytoplasm. Vesicles are seen, in which, when justaposed, chromatin passing from the nucleus to their interior is clearly visible. These vesicles show formations of a fibrous or membranous aspect. In the cytoplasm, less dense lamellar formations are also present, suggesting degenerating mitochondria, as well as lamellar formations with dense particles in the interlamellar space. Occasionally it is possible to obserse pinocytosis, Golgi complex, and smooth endoplasmic reticulum, whose diameters vary from 0.02 to 0.07 μ. In the peripheric portion, depending on the sectioning level, long parallel microtubules are detected with a diameter of about 270 A. In transversal sections, microtubules show an interior void of density.

Mature mammalian erythrocytes show a dense cytoplasm containing hemoglobin molecules with diameters ranging from 55-60 Å. There are no traces of any type of structures or organelles (Figs. 15 to 18).

Blood of avian and mammalian embryos, adult birds and mammals with phenylhydrazine-induced anemia, and blood of humans with acquired hemolytic anemia

In the nuclei of immature avian erythrocytes, chromatin is not compacted. Through the membrane pores, the passage can be observed of hemoglobinized cytoplasm to the caryoplasm, that becomes nearly as dense as the cytoplasm. In the latter, typical mitochondria are observed with variable form, position, and dimension, and with a mean diameter of 0.20 μ. Frequently, longitudinally lamellated bodies are found, and less frequently, bodies with oblique or even without any lamellae. In the interlamellar space, dense particles are seen, ranging between 80-100 Å diameter, identical to those of the hemoglobin molecules of the cytoplasm. Polysomic granulations can be observed in high numbers, as compared to monosomic forms, whose diameters are of about 150 Å. Many cells show an intense pinocytotic activity. Near the site of pinocytosis, vesicles containing dense particles and a diameter of about 100 Å, are detected, which, by their aspects, dimension, and density suggest to be ferritin. In some cells it is possible to observe ferritin as free agglomerates in the cytoplasm, denominated hemosiderin. At other regions this ferruginous mass becomes amorph, and is enveloped by a smooth membrane similar to that of the smooth endoplasmic reticulum. Sometimes, this material is seen involvel in a smooth membrane, with rarefied internal points, presenting a honeycomb-like aspect. Microtubules, composing the marginal band, are detected, in disposition, form, and aspect identical to the microtubules of mature erythrocytes, including their average diameters of 270 Å.

COIRO, J.R.R. Comparative study on the ultrastructure of the elements of the avian and mammalian erythrocytic series. Correlation with hemoglobin biosynthesis.
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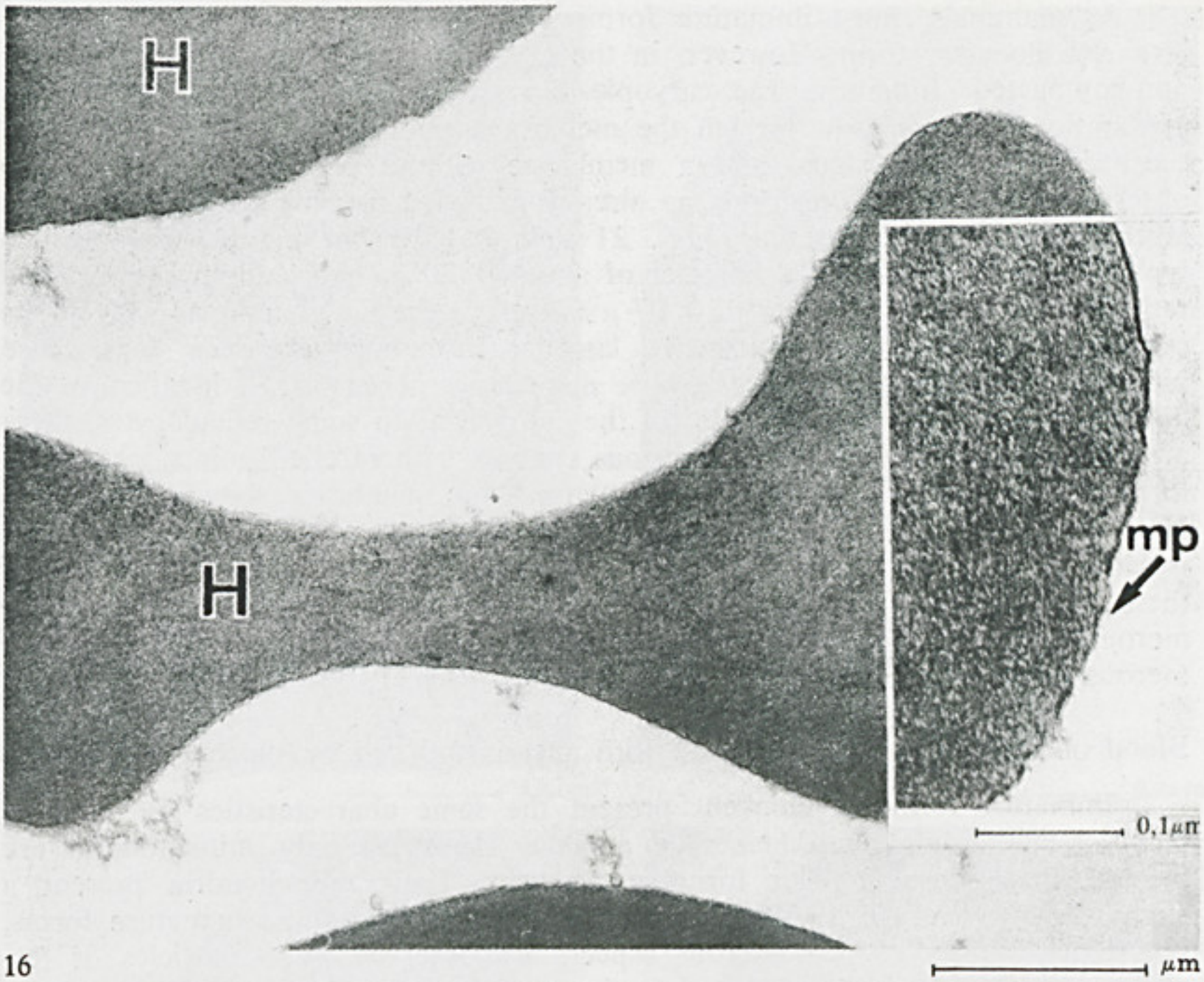

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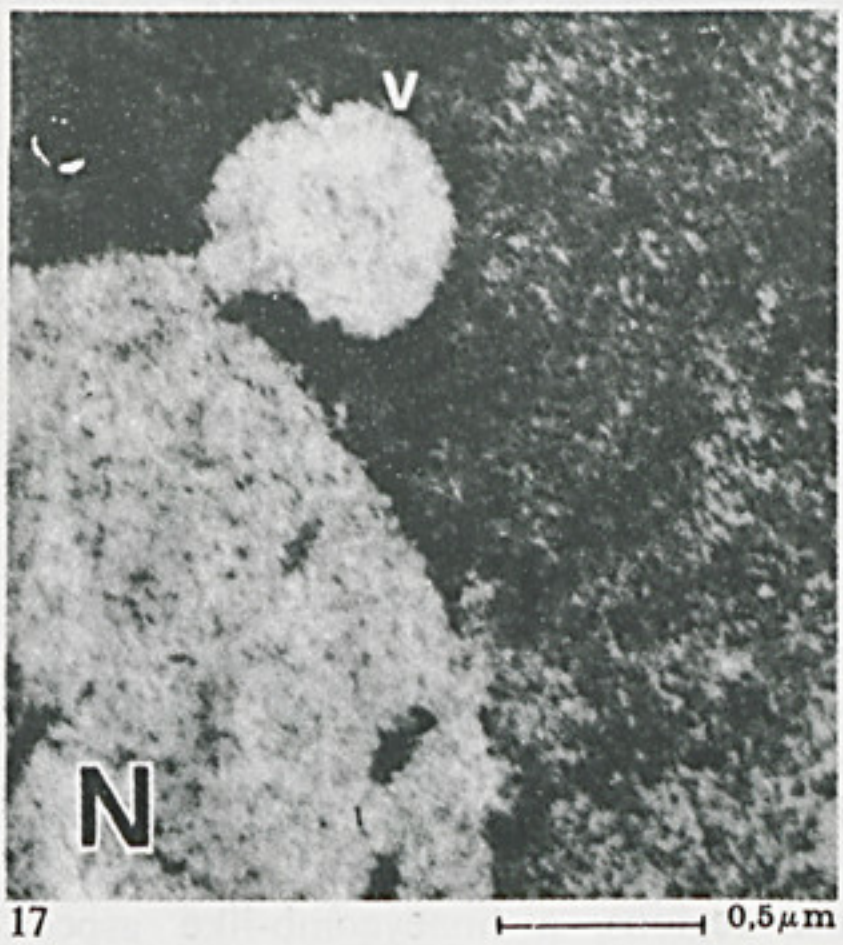

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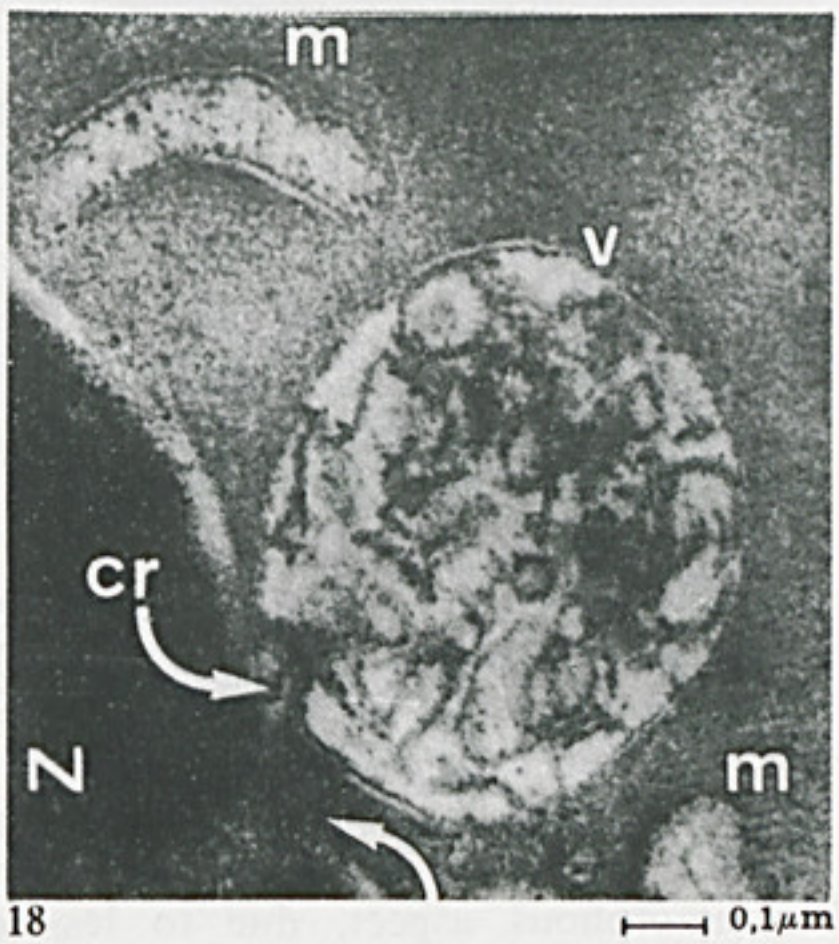

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ULTRATHIN SECTIONS

Fig. 16 - Normal human "hematia" (mature erythrocyte): (mp) plasmic membrane (inset shows details of the membrane unit); (H) hematia.

Figs. 17 and 18 - Mature avian erythrocytes: 17 - (N) nucleus; (V) vesicle originated from a mitochondrion (permanganate fixation). 18 - (N) nucleus; (cr) chromatin; (m) mitochrondria; (V) vesicle originated from a mitochondrion; (arrow) show hemoglobin penetration into the nucleus. (Gluraldehyde gradient fixation).

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In mammals, most immature forms present themselves anucleated, i.e., in a reticulocytary form. However, in the erythroblastic forms, the nuclei show non-compacted chromatin, and caryoplasm with a high density caused by the penetration of cytoplasm through the membrane pores. A few cells present an eccentric pycnotic nucleus, and a membrane without pores. It is possible to observe in these immature forms an already extruded nucleus covered by a fine layer of cytoplasmic material (Figs. 21 and 22). In the cytoplasm there are typical mitochondria with a diameter of about 0.20 μ, and smooth endoplasmic reticum with a mean diameter of 0.07 μ as well as the Golgi comples with all its constituents. Besides these elements, lamellar formations are seen with dense interlamellarly disposed particles whose diameter is about 60 Å, identical to the diameters of hemoglobin molecules of the cytoplasm. In some reticulocytes, there are dense masses bound by membranous systems with rarefied points, conferring to them a honeycomb-like aspect. The cytoplasmic membrane shows pinocytosis. Near the pinocytotic region in the cytoplasm there are agglomarates whose isolated particles are of the same density and diameter as ferritin. Diameters of these agglomerates vary from 0.03 to 0.08 μ. Polysomic granulations of pentamerous form are observed, distributed in the cytoplasm, and less often, monomerous forms near the polysomic ones (Figs. 24 to 31, 33, 37 to 40).

Blood of adult birds and mammals with anemia induced by successive bleedings

Immature forms of chickens present the same characteristics as those of embryos and adults with hemolytic anemia. However, only mitochondria are present, and dense lamellar forms are absent. Their mitochondria present a slightly higher average diameter, i.e., about 0.25 μ. In these immature forms, polysomes, mostly of pentamerous aspect, are observed. Also particles of ferruginous material can be detected in form of hemosiderin or as free granules in the cytoplasm. The nucleus of the erythroblastic forms show besides loose chromatin, the penetration of cytoplasmic material through the membrane pores, the caryoplasm thus acquiring approximately the same density as the cytoplasm (Figs. 34 to 36).

Aspects of the organelle genesis in immature erythrocytes of the peripheric avian and mammalian blood

The beginning of the phenomenon is characterized by the condensation of the plasmic membrane at determined sites, followed by the formation of an invagination, i.e., pinocytosis. Next to this site, pinocytotic vesicles with a dense and thick membrane are observed. In continuation, several pinocytotic vesicles blend together, originating a larger one with abundant dense particles attached to the membrane. This vesicle gradually loses its restraining membrane, and the inner particles become free, originating agglomerates of ferruginous material in the cytoplasm. This ferruginous material is then encircled by a smooth membrane similar to the smooth endoplasmic reticulum and acquires an amorphous aspect, due to less dense regions, a honeycomb-like aspect. In many mammalian cells an association has been observed between mitochondria connected by a dilatation of low density, and the honeycomb-like bodies or bodies already with an outline of lamellar formation, with dense interlamellar particles. These lamellated bodies in cells at the end of maturation, present ruptures in their membranes, and the dense inner particles spread to the surrouding cytoplasm.

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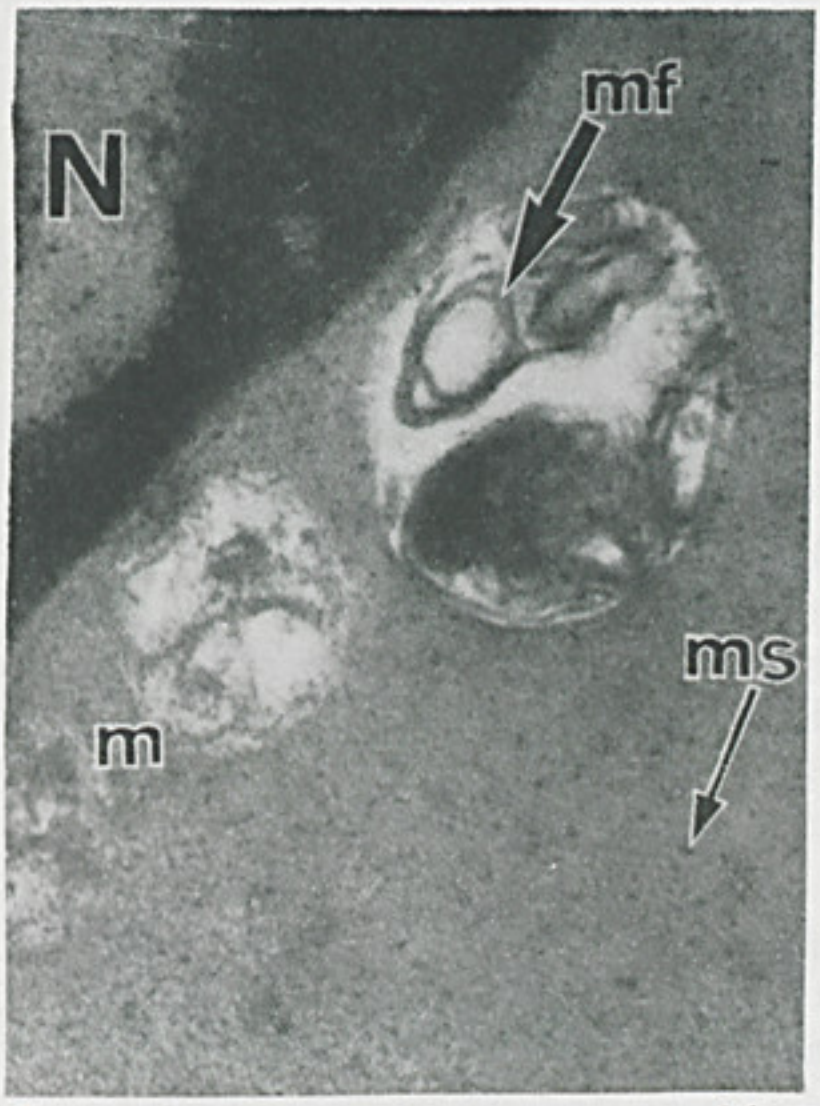
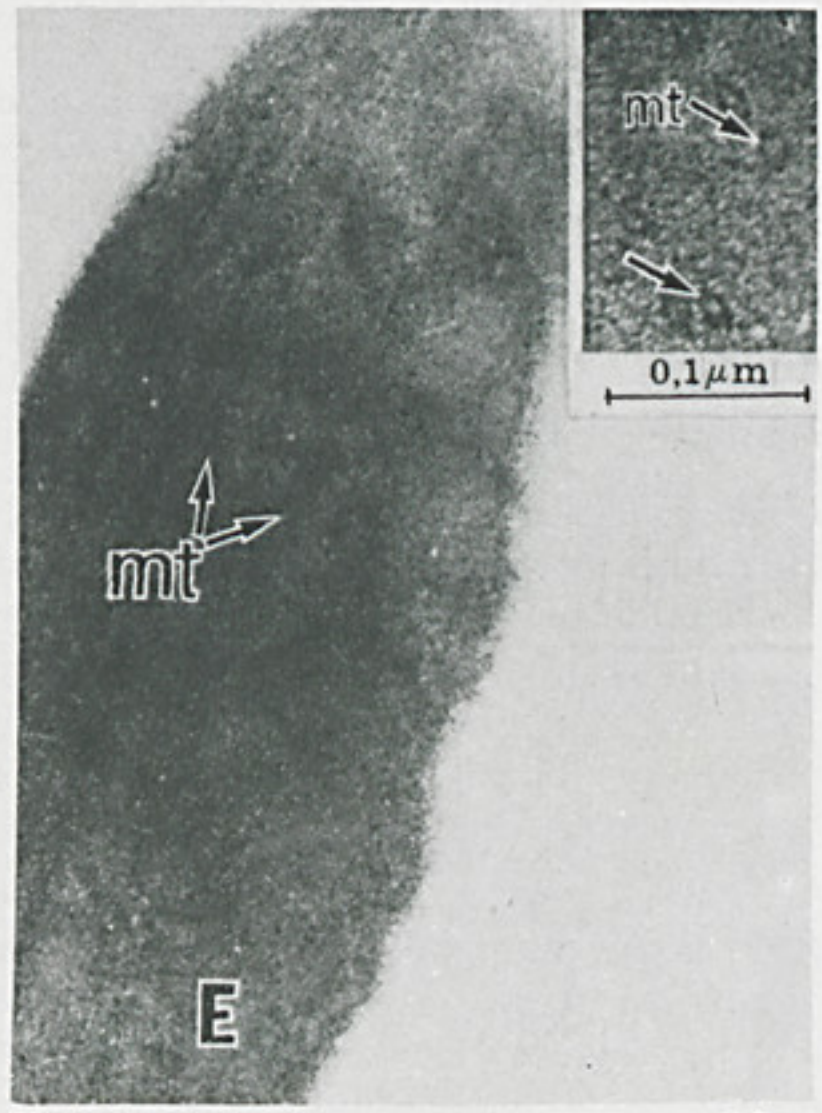


19 0,2μm



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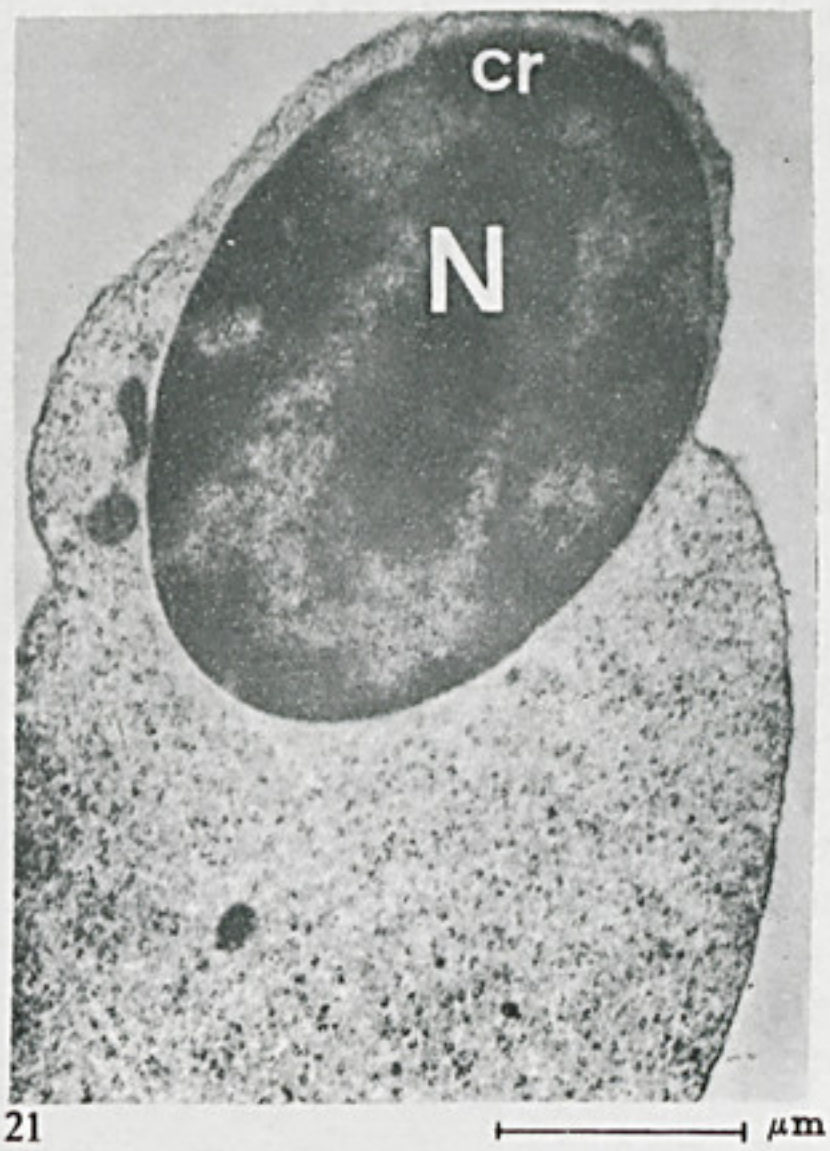

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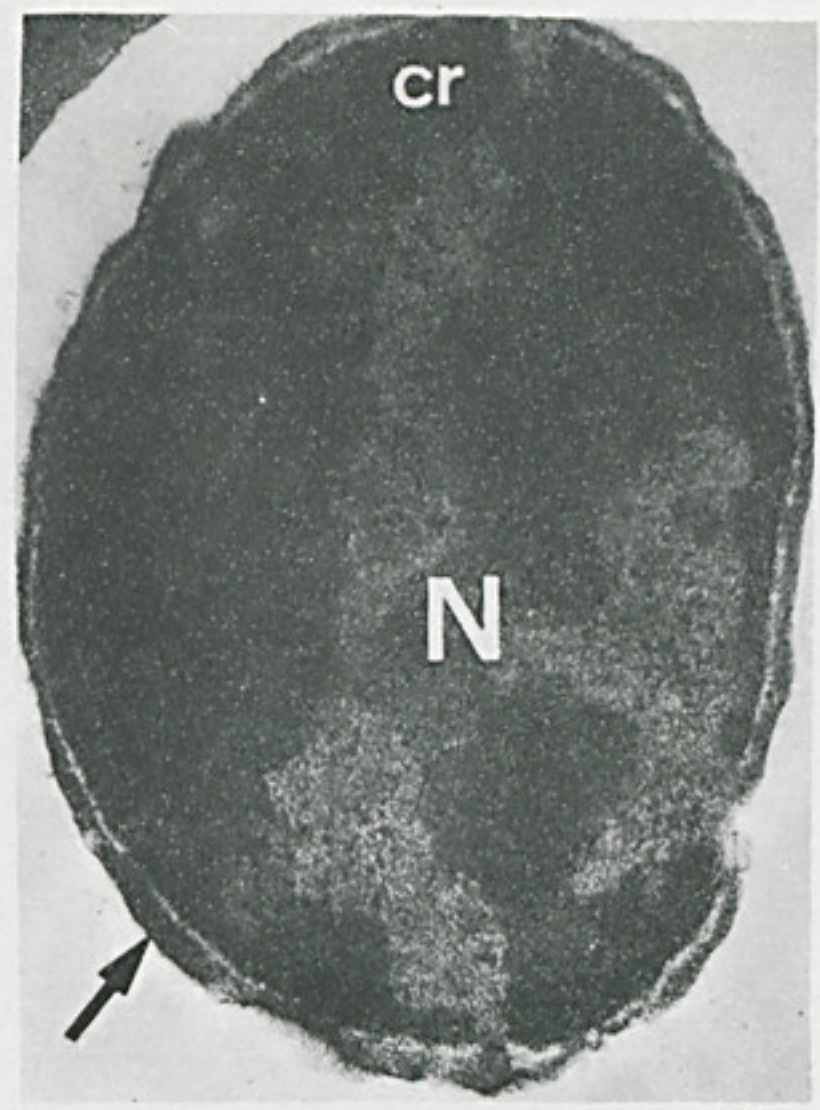

22 0,5μm

ULTRATHIN SECTIONS

Fig. 19 - Proerythrocyte II: (N) nucleus; (m) aberrant mitochondria; (mf) myelin figure; (ms) monosomic forms.

Fig. 20 - Mature avian erythrocyte: (mt) microtubules in longitudinal section; (E) mature erythrocytes. (inset shows transversal sections).

Fig. 21 - Typic mammals orthochromatic erythroblast: (N) nucleus (cr) compacted chromatin.

Fig. 22 - Orthochromatic mammal erythroblast: (N) free nucleus; (arrow) cytoplasm.

Estimate of the erythrocyte maturation degree

In orthochromatic mammalian erythroblasts the polysome number varies from 58-65 per μ^2. In immature avian cells, the countings revealed 80, 65, 40, and 16 polysomes per μ^2.

Fractionation, organelle isolation, and hemoglobin determination. Blood f avian embryos (*Gallus gallus*), and blood of adult chickens (*Gallus gallus*) with anemias induced by phenylhydrazine, and successive bleedings

Cytoplasmic hemoglobin of the blood of these animals, when stained with benzidine, reveals two components of about the same concentration in polyacrylamide gel. The concentrated supernatants of the lysate revealed hemoglobin of identical patterns, in healthy embryos as well as in those with phenylhydrazine induced anemia (Figs. 46 and 48). For animals with anemia induced by successive bleedings, no traces of hemoglobin were found in the concentrated supernatants of the last washings, the same as for the controls.

Blood of mammalian embryos (*Oryctolagus cuniculus*), adult mammals with phenilhydrazine induced anemia (*O. cuniculus* and *Cavia porcellus*), with acquired hemolytic anemia (humans), and with anemia induced by successive bleedings (*C. porcellus*)

Cytoplasmic hemoglobin of the blood of these animals, when stained with benzidine, reveals one single component in polyacrylamide gel. Concentrated supernatants of the lysate reveal hemoglobin of the same pattern, in embryos and in animals with anemia induced by phenylhydrazine or with acquired hemolytic anemia (Figs. 47 and 49). On the other hand, the animals with anemia due to successive bleedings did not reveal any trace of hemoglobin, the same as the controls, represented by the concentrated supernatants of the last washings.

Macroscopic aspects, and control of purity degree of the pellets by electron microscopy

In embryonic blood of chickens and mammals, as well as in adult avian and mammalian blood with phenylhydrazine induced anemia and in the blood of humans with acquired hemolytic anemia, it is observed that integral pellets possess a reddish-pink central region, that becomes light brown through the washings. After osmotic lysis, the pellets begin to show a fine granular membrane with an undefined color. In animals with anemia induced by successive bleedings, the pellets show a yellowish central region, even after the washings. After osmotic lysis, the sediments acquire the aspect of a fine, nearly imperceptible membrane. Examined by the electron microscope, the integral pellets are constituted by sac-like formations, in general ellyptical, filled with dense material. On the other hand, the lysed pellets show a less dense interior. Furthermore the lysed pellets acquire a greater volume (Figs. 42 to 45).

Spectrophotometry

In birds and mammals, spectrophotometric determinations in the concentrated supernatants of lysates and in hemoglobin were carried out at 5.400 Å.

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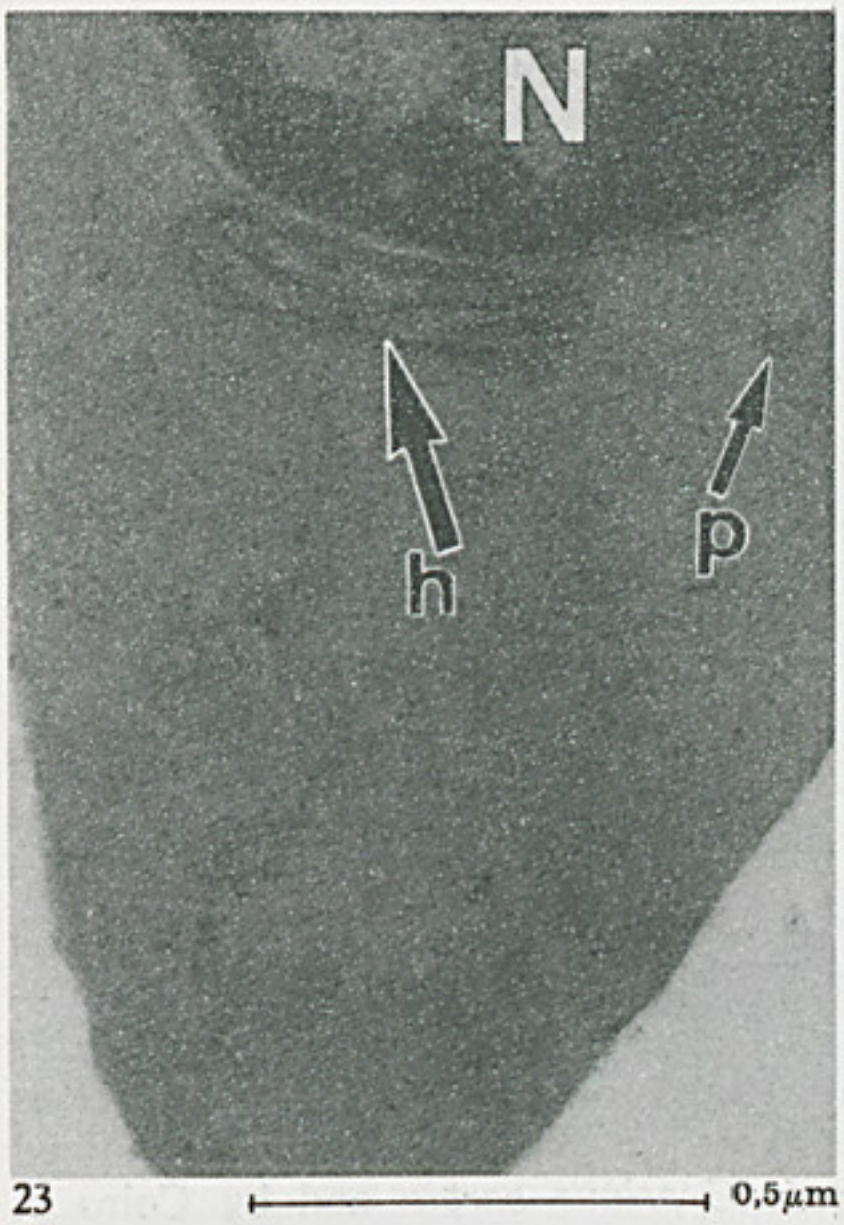
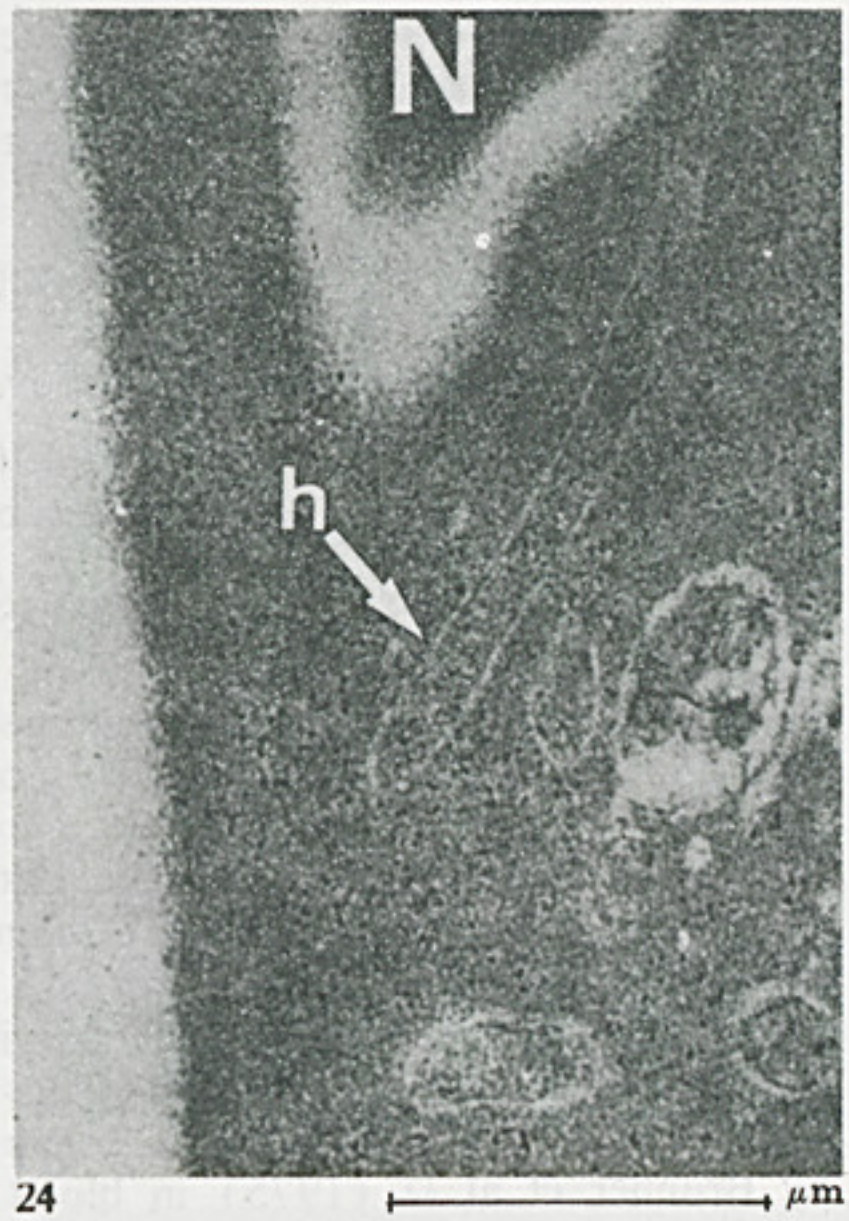


23 0,5μm



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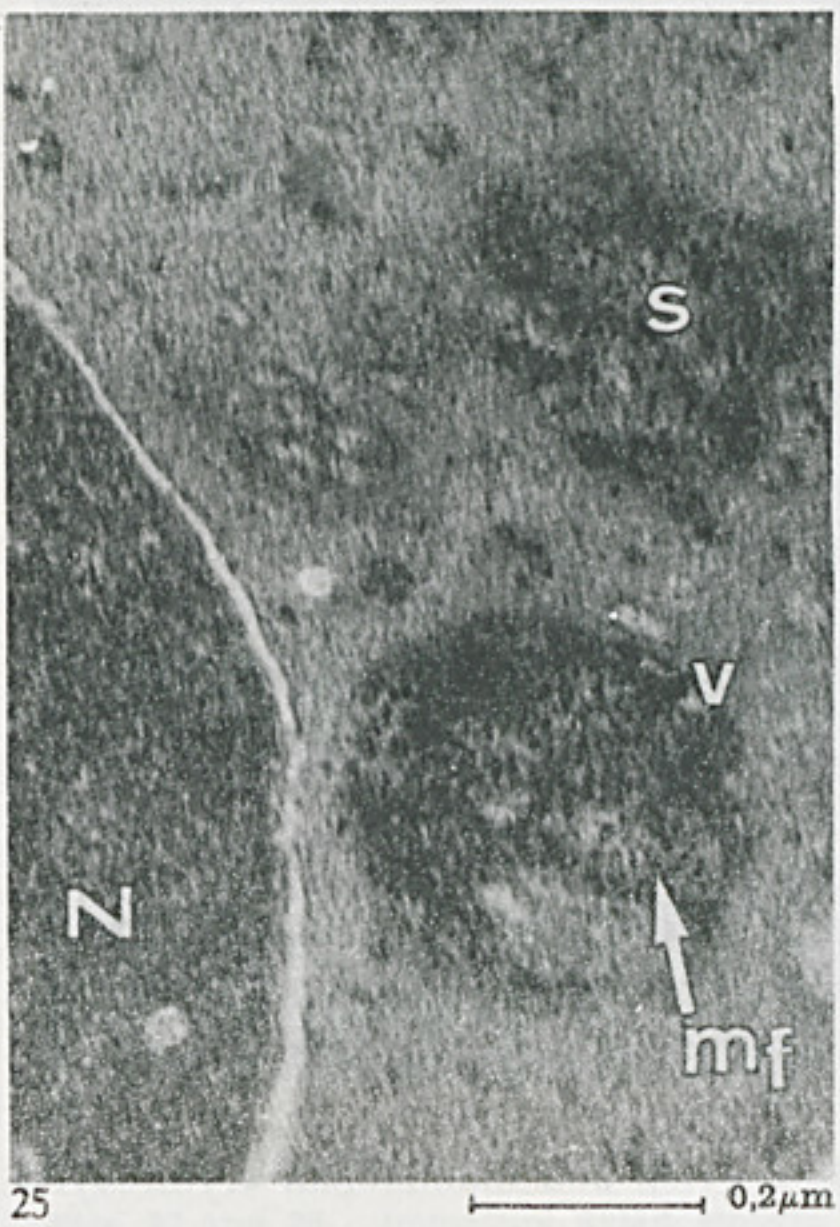
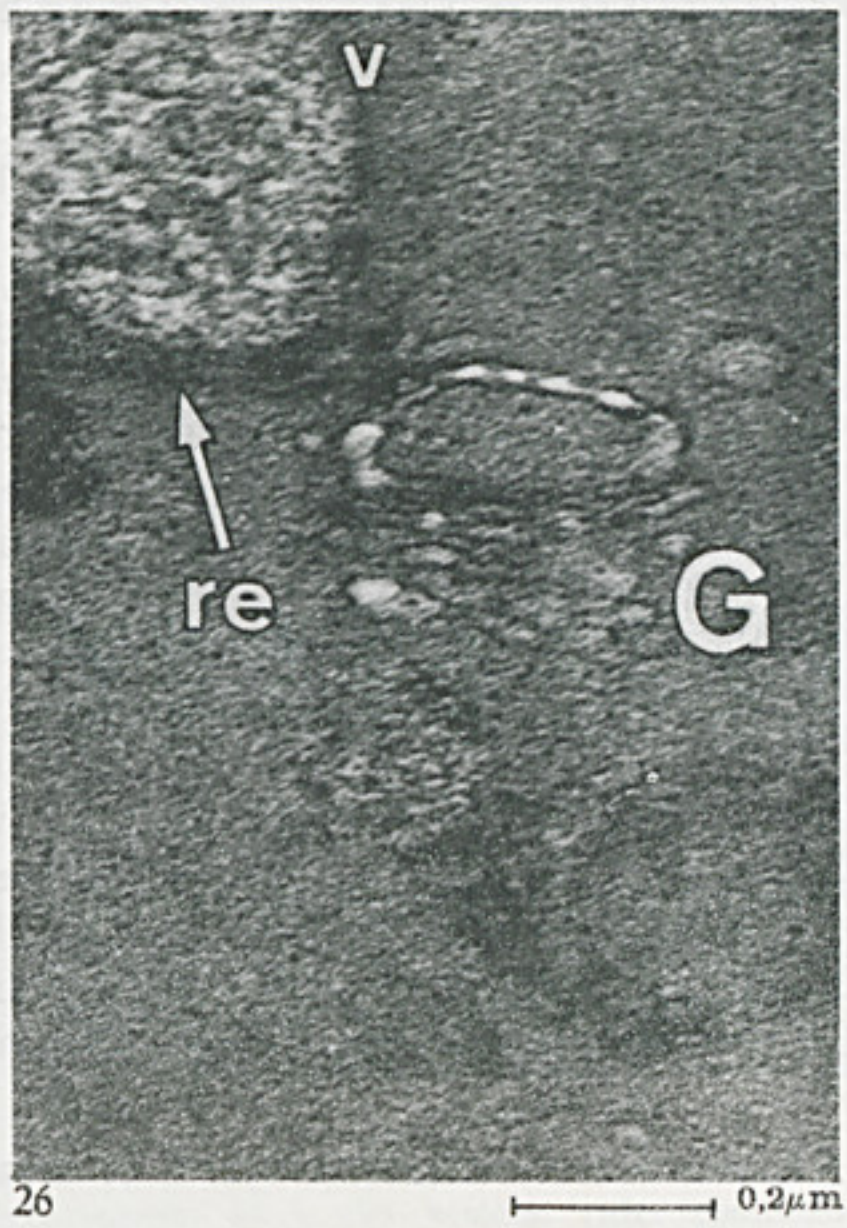


25 0,2μm



26 0,2μm

ULTRATHIN SECTIONS

Figs. 23 to 26 - Immature avian embryos erythrocytes: 23 - (N) nucleus; (p) polysome; (h) hemosome with longitudinal lamellae. 24 - (N) nucleus; (h) hemosome filled with hemoglobin molecules. (Osmium tetroxide fixation). 25 - (N) nucleus; (mf) ferritin molecules; (s) hemosyderine. 26 - (G) Golgi complex; (re) endoplasmic reticulum; (v) vesicles.

Absorbance was observed in the lysate supernatants, suggesting the presence of the hemo group. In some control supernatants (last washings) some absorbance was observed, but much less than that of the corresponding lysate supernatant.

DISCUSSION

Mature erythrocytes — Feulgen positive vesicles

O'Brien [68] (1960), Davies [34] (1961), Wilt [99] (1962) and Grasso et al. [47] (1962) found hemoglobin in erythroblast nuclei. Tooze & Davies [94] (1963), suggested that hemoglobin could act as histone, causing DNA condensation. According to Moore & Brown [66] (1968), the presence of large amounts of intranuclear hemoglobin could act by a negative retroalimentation mechanism, and interrupt heme and globin synthesis. Based on our findings related to the Feulgen positive vesicles, as well as on the observations in hemolysed smears and ultrathin sections, we present another hypothesis. Ultrathin sections show continuity (Fig. 17) or close connection between vesicles and nucleus (Fig. 18). In the cases of close connections, passage of condensed or filamentous chromatin into the vesicles is observed (Fig. 18). In chickens (Coiro, 1972 — unpublished observations), as well as in *Gallus, Bufo* and *Liophis* (Menezes et al. [64], 1972), Feulgen positive vesicles apparently originating from the nucleus are observed. The mitochondrial origin of those vesicles was clearly determined by Brunner et al. [17] (1975) in blood from *Cyprinus carpio* and by Coiro et al. [32] (1974) in blood from *Gallus, Bufo, Bothrops* and *Cyprinus*. Menezes et al. (1974 — unpublished observations), and Coiro et al. [32] (1974) observed, after labelling *Bufo ictericus* erythrocytes with ^{3}H-tymidine, that those vesicles of mitochondrial origin, filled with chromatin, show DNA or the product of its degradation. Such findings suggest that the blocking of DNA activity may also be related to chromatin reduction, and not only to a negative retroalimentation mechanism, because, as already know, hemoglobin is synthesized in mammalian erythroblastic forms, in avian erythroblasts as well as in proerythrocytic forms, even when their nuclei had received a large amount of hemoglobin through the pores of the membrane. Actually, we observed that at the moment when the vesicles appear, filled with chromatinic material, erythrocytes may still contain monosomes, indicating desaggregation of polysomes and cessation of globin synthesis, and therefore hemoglobin synthesis (Fig. 19). Comparing the phenomenon of nuclear extrusion in orthochromatic mammalian erythroblasts (Figs. 4, 21 and 22), much more frequent than caryolysis or caryorhexis according to Astaldi et al. [1] (1950), with the partial extrusion of chromatinic material in avian erythrocytes, it may be suggested that these phenomena possibly could be correlated, in spite of the differences as to the periods of hemoglobin synthesis. While reticulocytes work intensely at synthesis, the loss of chromatinic material in avian erythrocytes is concomitant with hemoglobin synthesis cessation.

Considerations on the structures of immature avian and mammalian erythrocytes

Bernhard et al. [2] (1949) observed circular forms in hemolysed cells in suspension. Origin and nature of these circular forms were much discussed by Braunsteiner & Bernhard [9] (1950), Peters & Wigand [74] (1950), Wolpers [100] (1956), Jung[54] (1959) and Hug et al.[49] (1959). Through hemolysis in immature

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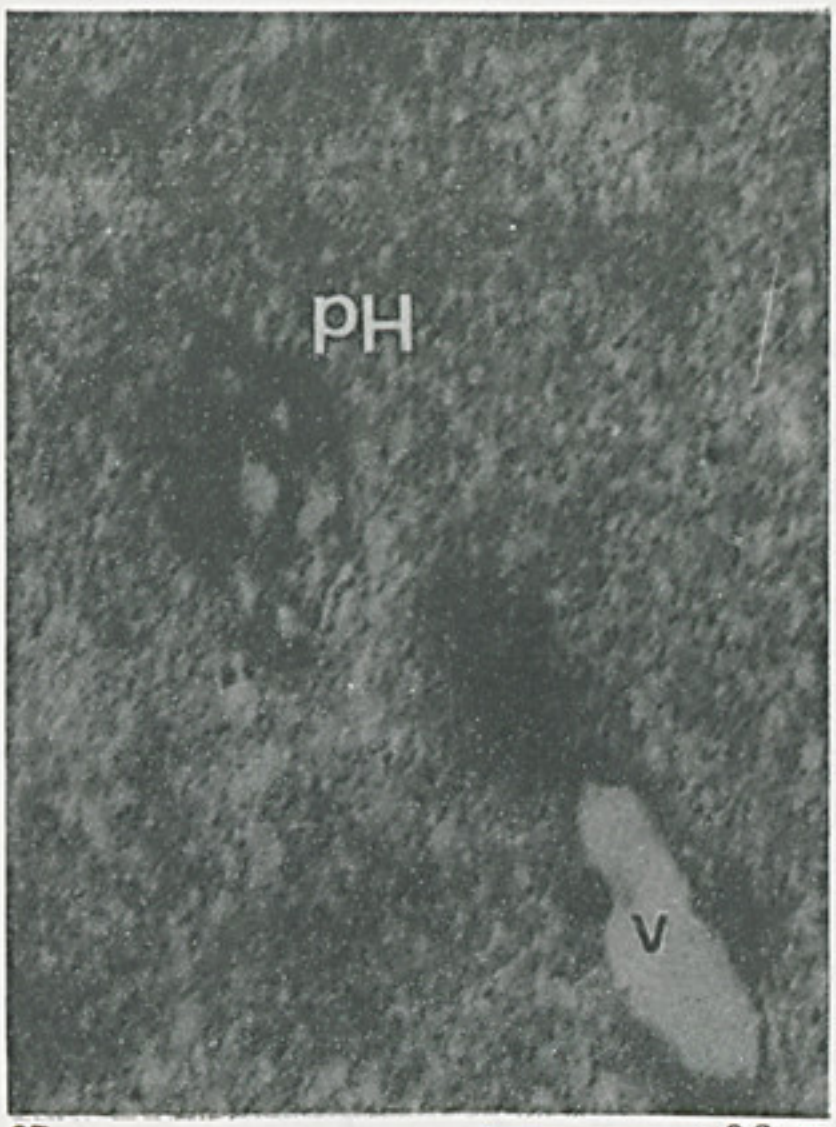

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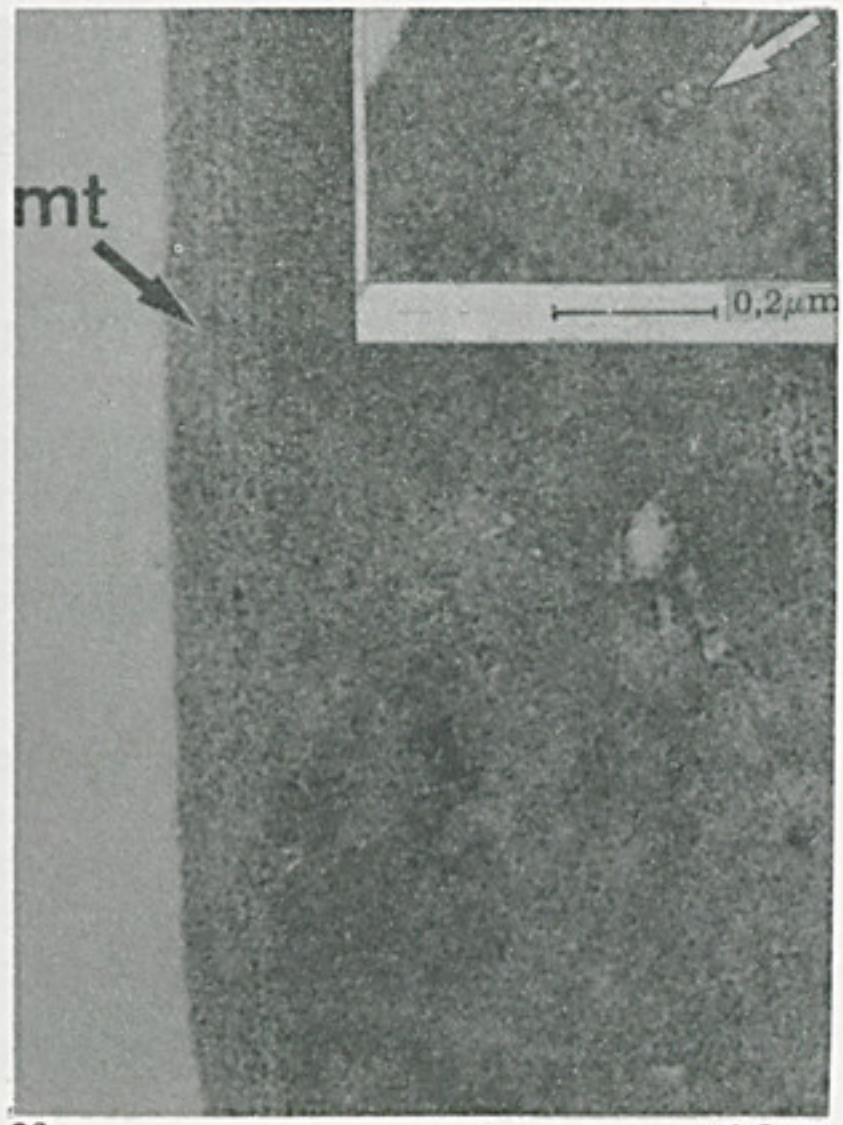

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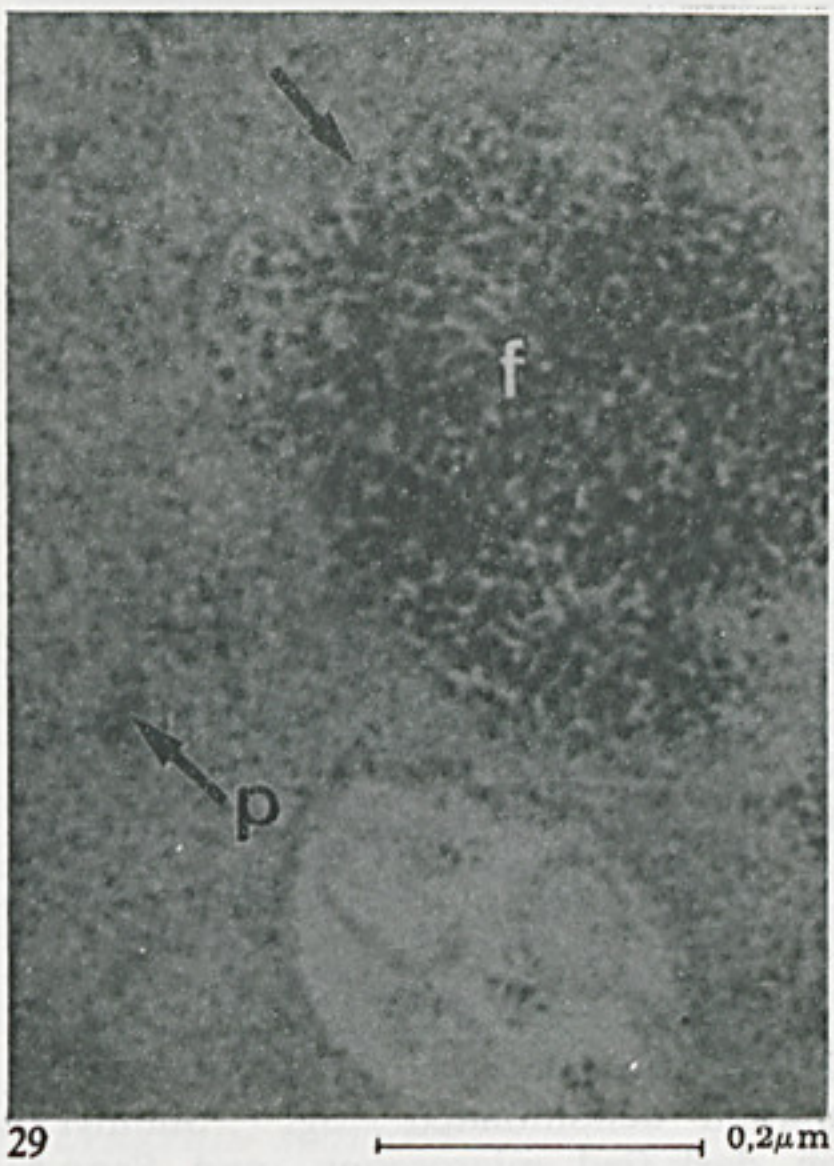

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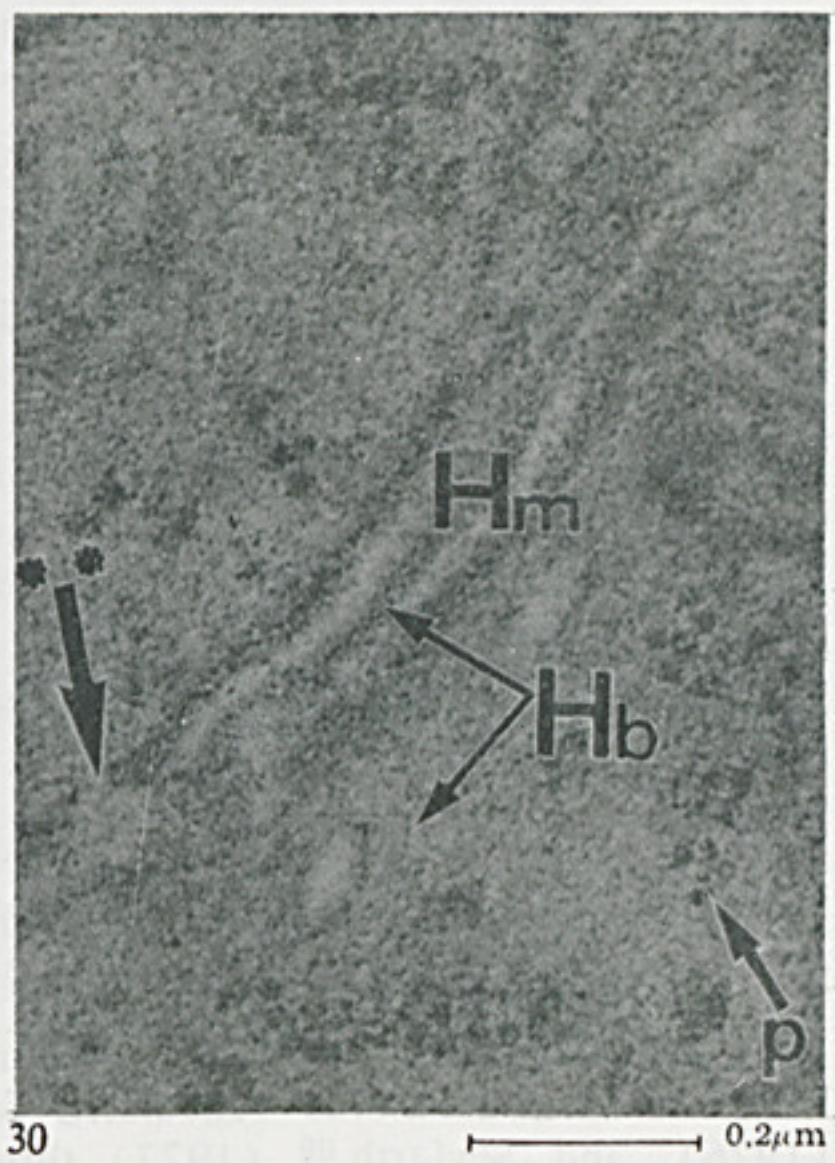

30

ULTRATHIN SECTIONS

Figs. 27 and 28 - Immature avian embryo erythrocytes: 27 - (V) vesicle; (pH) prohemosome. 28 - (mt) longitudinally sectioned microtubules. Inset shows transversal section. (Osmium tetroxide fixation).

Fig. 29 and 30 - Mammal embryo reticulocytes (prohematia): 29 - (f) ferritin; (p) polysome; (arrow) smooth membrane. 30 - (p) polysomes; (Hm) mature hemosome; (Hb) hemoglobin molecules; (double asterisk) linking-body.

cell smears, Brunner & Vallejo-Freire [21] (1956), obtained dense filamentous structures with a diameter of about 0.20 μ, and extremely fine filaments with a mean diameter of 600 Å, as well as granules of 0.25 μ in diameter (Fig. 5). Such granules were interpreted as polysomes, since they desintegrate when submitted to RNase, traces of proteic material remaining at the site (Brunner [14], 1968). As to the filaments, they were interpreted as smooth endoplasmic reticulum similar to the interpretation of Porter [79] (1953), when he observed very fine filaments of 500-700 Å in diameter, in integral mesothelial rabbit cells. The dense filaments about 0.20 μ in diameter were considered to be mitochondria. This interpretation is justified by the fact, that these dense filaments have an affinity with ferric hematoxilin (Régaud) and acid fuchsin (Altmann), also used for the identification of the forementioned organelles. Brunner et al. [23] (1956) and Braunsteiner et al. [10] (1956), found filamentous mitochondria in mammal reticulocytes. It has also been observed that these filaments are reduced in numbers when immature cells evolve to maturity, the same as observed with mitochondria. It is important to point out that in mammalian erythroblasts and reticulocytes with lead intoxication, the dense filaments in hemolysed smears increase three fold in diameter on the average, and that this fact occurs also in mitochondria (Vallejo-Freire & Brunner [95], 1958).

Porter [79] (1953), in integral endothelial rabbit cells, observed dense filaments with diameters of about 0.20 μ morphologically interpreted as mitochondria. Such result coincides with the ultrastructural measurements and aspects of the dense avian and mammalian immature erythrocyte filaments that we have observed (Figs. 5 and 6).

Brunner & Vallejo-Freire [21] (1956) and Vallejo-Freire & Brunner [95] (1958) verified that in immature mammalian cells submitted to hemolysis in suspension, without interruption by a fixative, the filamentous mitochondria become desintegrated, giving rise to circular forms (Fig. 14). However, these authors obtained intermediate forms when hemolysis was performed in a less hypotonic medium, containing formol. In ultrathin sections they also observed that filamentous mitochondria increase in volume at determined sites, separated by constrictions. However, if an interruption had not occurred, the intermediate forms would have given rise to circular forms.

In birds, due to the intense agglutination during hemolysis in suspension, circular forms were obtained through hemolysis in smears, with a modification, i.e., rapid drying at a temperature of 40°C. The circular forms thus obtained presented a diameter of about 0.68 μ (Fig. 13).

Relationship between "Substantia granulo-filamentosa" (Sgf), mitochondria, and other structures

The immature avian and mammalian forms, stained by brilliant cresyl blue, show a dark-blue reticulated formation denominated "Sgf". Simmel [90] (1926), and Seyfarth [89] (1927) through dark-fied microscopy, and Sano [85] (1955) through phase contrast microscopy, observed intrareticulocytary structures. Since these structures only were visualized with definition in stained reticulocytes, many investigators interpreted the reticulum as an artifact. On the other hand, reticulocytes treated with a supravital dye and subsequently treated with Giemsa did not show diffuse basophily, thus leading to the supposition that both dyes evidence one and the same structure. Dustin [38] (1947) showed

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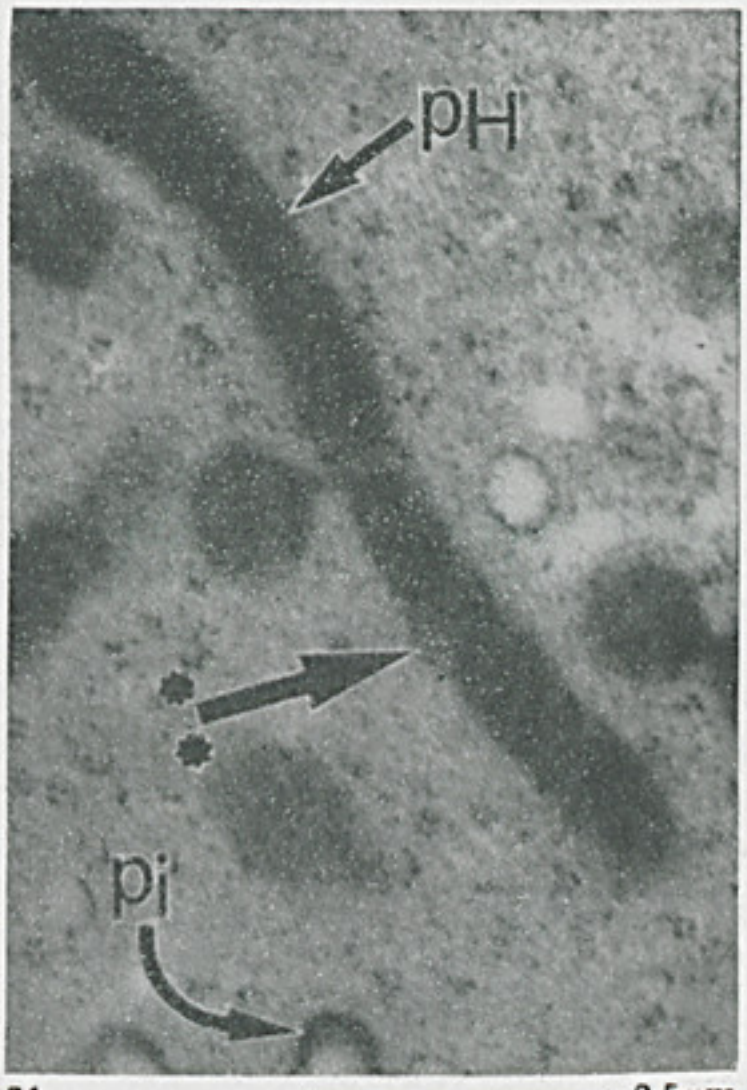
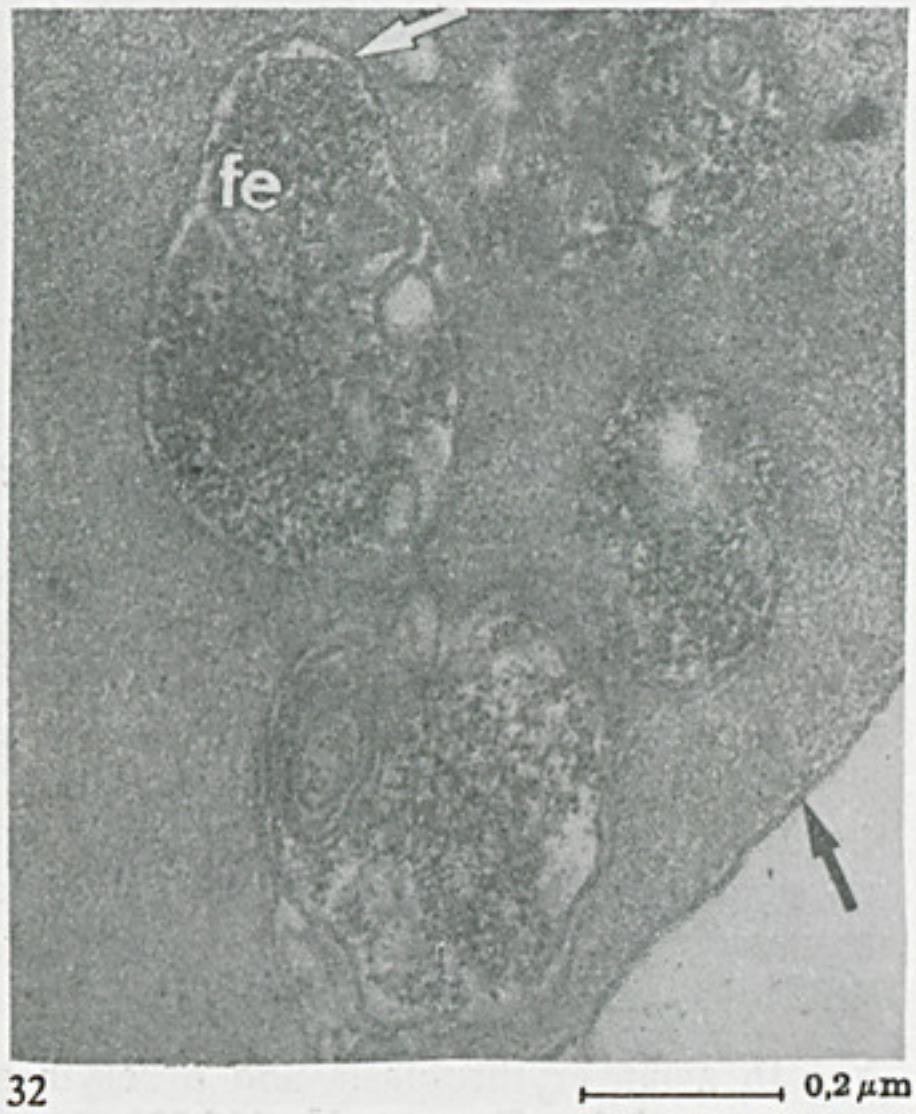
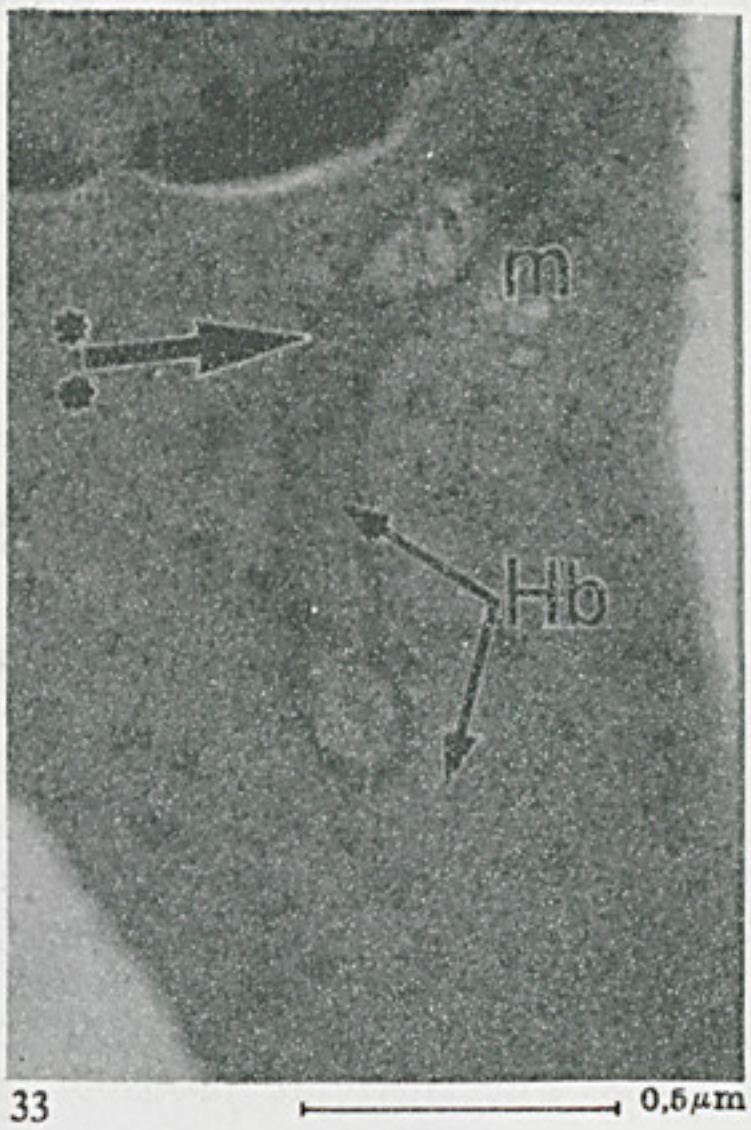


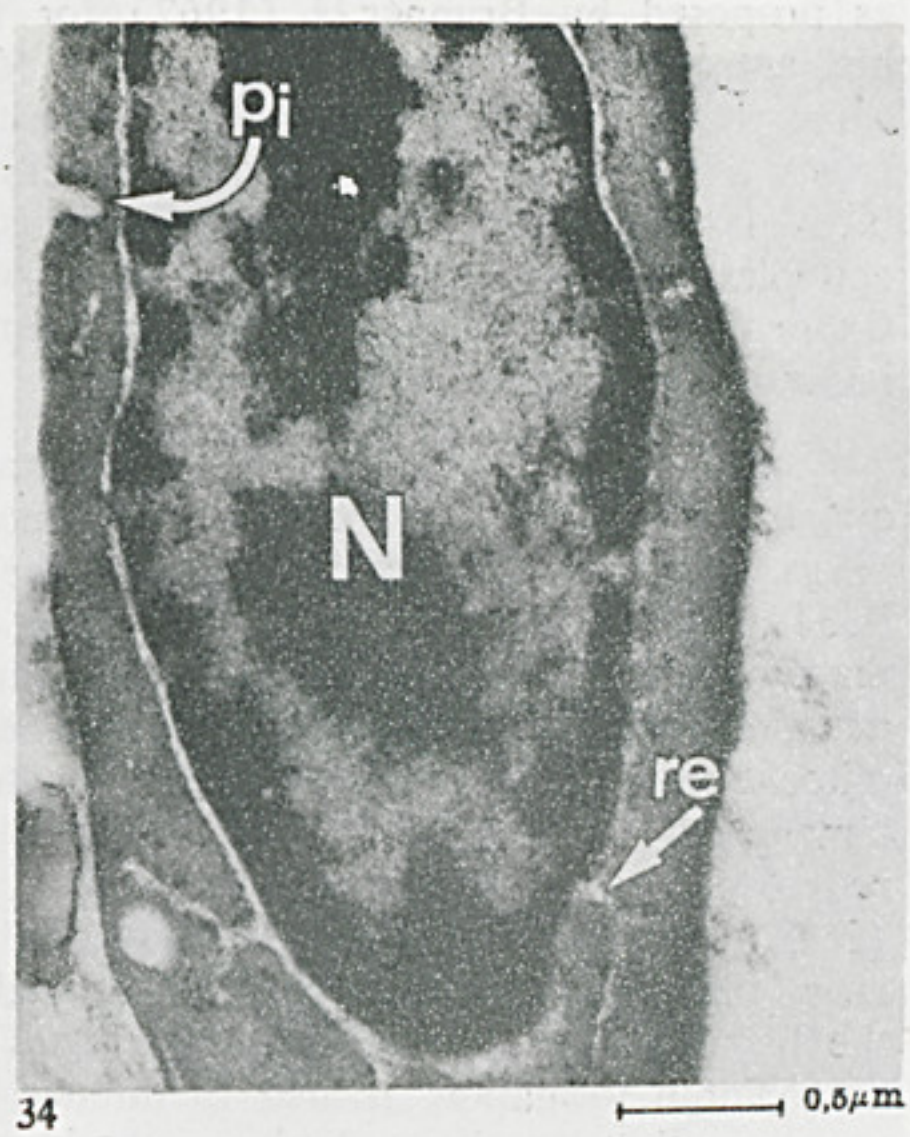
31 0,5 μm



32 0,2 μm



33 0,5 μm



34 0,5 μm

ULTRATHIN SECTIONS

Figs. 31 and 32 - Reticulocytes or "prohematia" of mammal embryos, and a patient with acquired hemolytic anemia: 31 - (pH) prohemosome; (double asterisk) linking-body; (pi) pinocytosis (Fixation by formalin in hypotonic medium). 32 - (fe) ferritin; (arrows) membrane unit.

Figs. 33 and 34 - Immature avian erythrocytes of embryos and adults with bleedings anemia: 33 - (m) mitochondrion; (Hb) hemoglobin molecules; (double asterisk) linking-body. 34 - (pi) pinocytosis; (re) endoplasmic reticulum. Immature cells obtained by successive bleedings, present a developed endoplasmic reticulum.

that basophily was given by the cytoplasmic ribonucleic acid, since there was no basophily in reticulocytes previously treated by RNase. When reticulocytes evolve to erythrocytes a RNA decrease occurs (Burt et al. [25], 1951; Thoma [92], 1959) and at the same time hemoglobin concentration increases up to 30% (Bessis [6], 1972).

Kosenow [57] (1952) and Brunner [13] (1962) also concluded the same as to the concentration of the acrydine-orange and Janus green B dyes. With a dilution higher than 1 x 10^{-4} and hemolysis in smears it was possible to observe larger filaments, polysomal granules, and thin filaments corresponding to the smooth endoplasmic reticulum. Brunner [13] (1962) observed Janus green B stained reticulocytes in a 5 x 10^{-5} dilution in hemolysed smears, with none of the structures presenting aglomerations. The same author, in ultrathin sections of reticulocytes previously treated with Janus green B, observed that the mitochondria displayed dye in their interior, smooth endoplasmic reticulum, and ribosome particles next to the dye. Since the mitochondrion is the predominant structural element in the reticulocyte, it is possible to consider it to be the main component os the "Sgf". It is necessary to point out that the concept os "Sgf" in non-stained reticulocytes is restricted only to mitochondria (Brunner [13], 1962).

In avian erythroblasts and proerythrocytes we used the same concentration as proposed by Brunner [13] (1962) for the mammalian reticulocytes. When brilliant cresyl blue was used in a 1 x 10^{-3} concentration, the reticulocyte became well evidenced. Due to the simillarity of immature avian and mammalian forms, i.e., evidencing the same structures, we may infer that the "Sgf" in birds is also constituted by filamentous mitochondria agglomerates, smooth endoplasmic reticulum and ribosomes with dye, and furthermore affirm that these mitochondria are elements fundamental for the "Sgf" formation in immature avian cells (Fig. 3).

With hemolysis in immature cell smears of humans with acquired hemolytic anemia, filaments were observed with diameters varying between 0.14 - 0.30 μ (Figs. 9 and 10). In rabbit-embryos, the filaments show variations of 0.11 - 0.31 μ (Fig. 5).However, it has been observed that filaments with greater diameter (0.30 μ) correspond to the filaments of reticulocytes of guinea-pigs anemic through bleedings (Fig. 12). These filaments of a larger diameter are mitochondria of a proportional size in ultrathin sections. It was verified that filaments of animals with bleeding anemia show a larger diameter when compared to filaments of embryos, or nimals with phenylhydrazine induced anemia (Figs. 6 and 8). This fact leads to the conclusion that the obtention of only large filamentous mitochondria is related to the type of induced anemia. In the bleeding type, we remove erytrocytes and other components important for hemoglobin formation, related to thinner filaments. The latter are probably organelles related to hemoglobin synthesis.

Brunner [14] (1968), in a comparative study on the number and extension of "Sgf" in orthochromatic erythroblast and reticulocytes verified that the area was about 7.5 μ^2 in erythroblasts, whereas in mature reticulocytes it reached about 17.2 μ^2, i.e., a 100% increase of the filamentous structures (Figs. 6 and 4). Reimann [81] (1942) and Jensen et al. [52] (1953) in birds, and Brunner [24] (1968) in mammals, indicated the dependence between the higher intensity of hemoglobin synthesis and the amount of "Sgf". Brunner & Mombrum [19] (1972)

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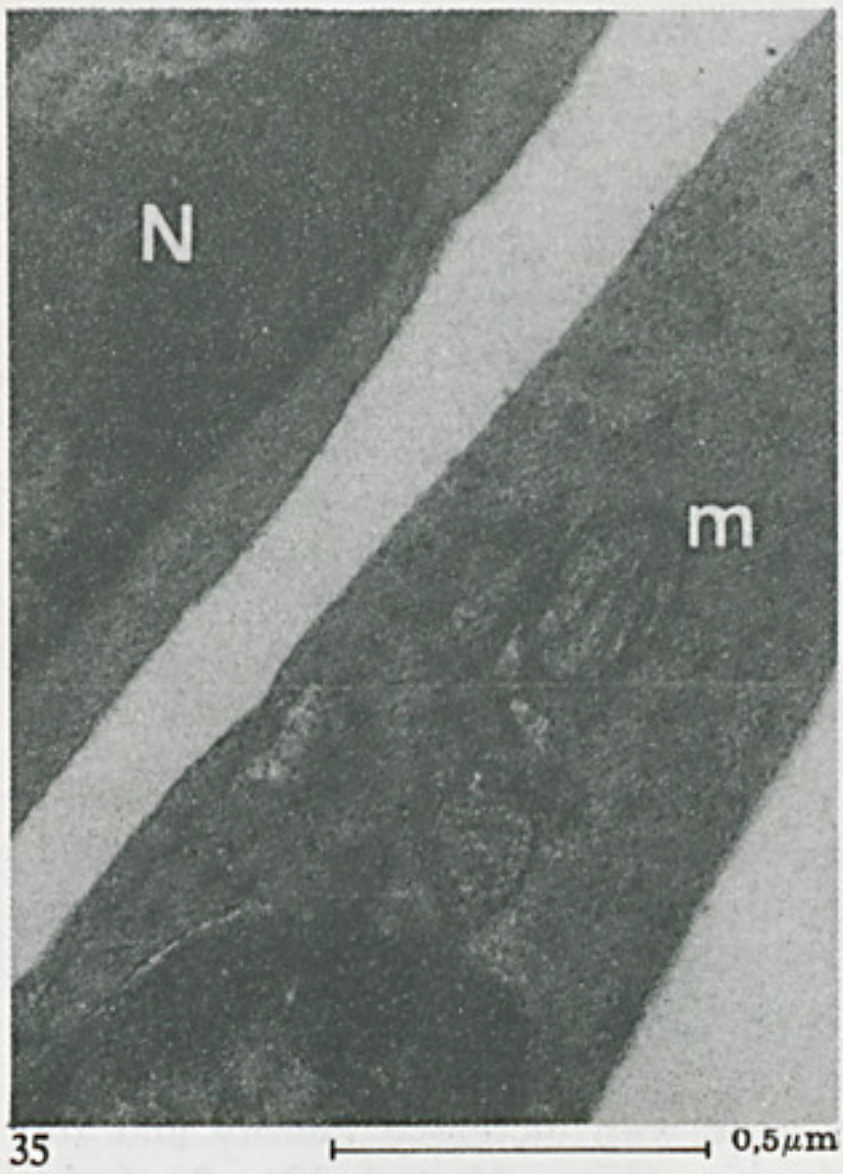
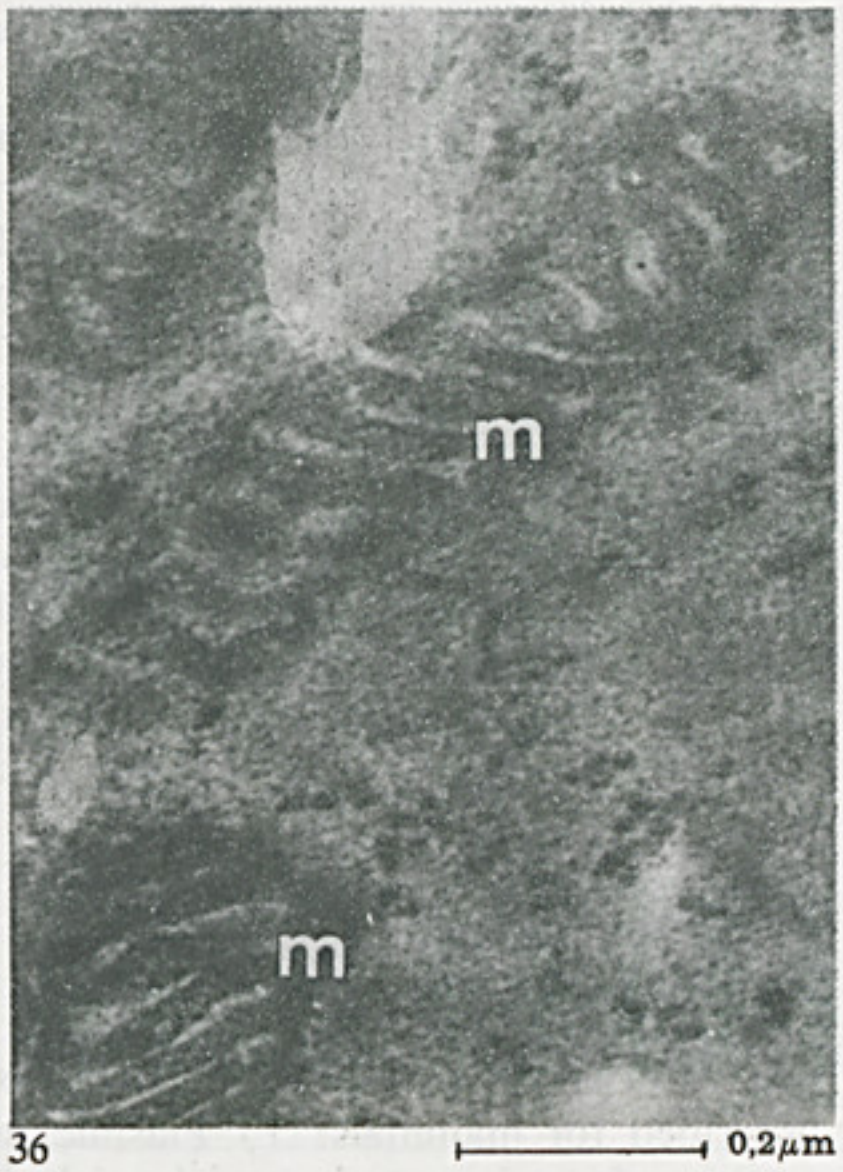
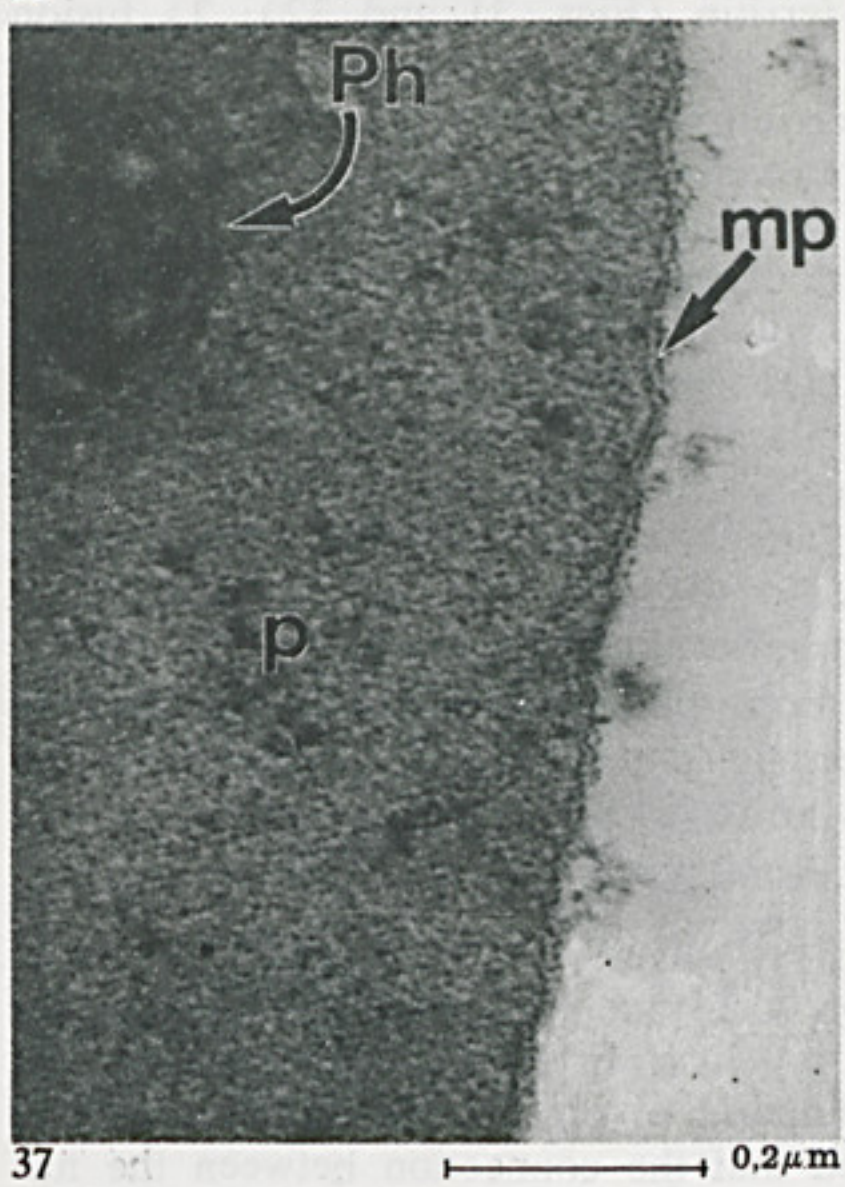


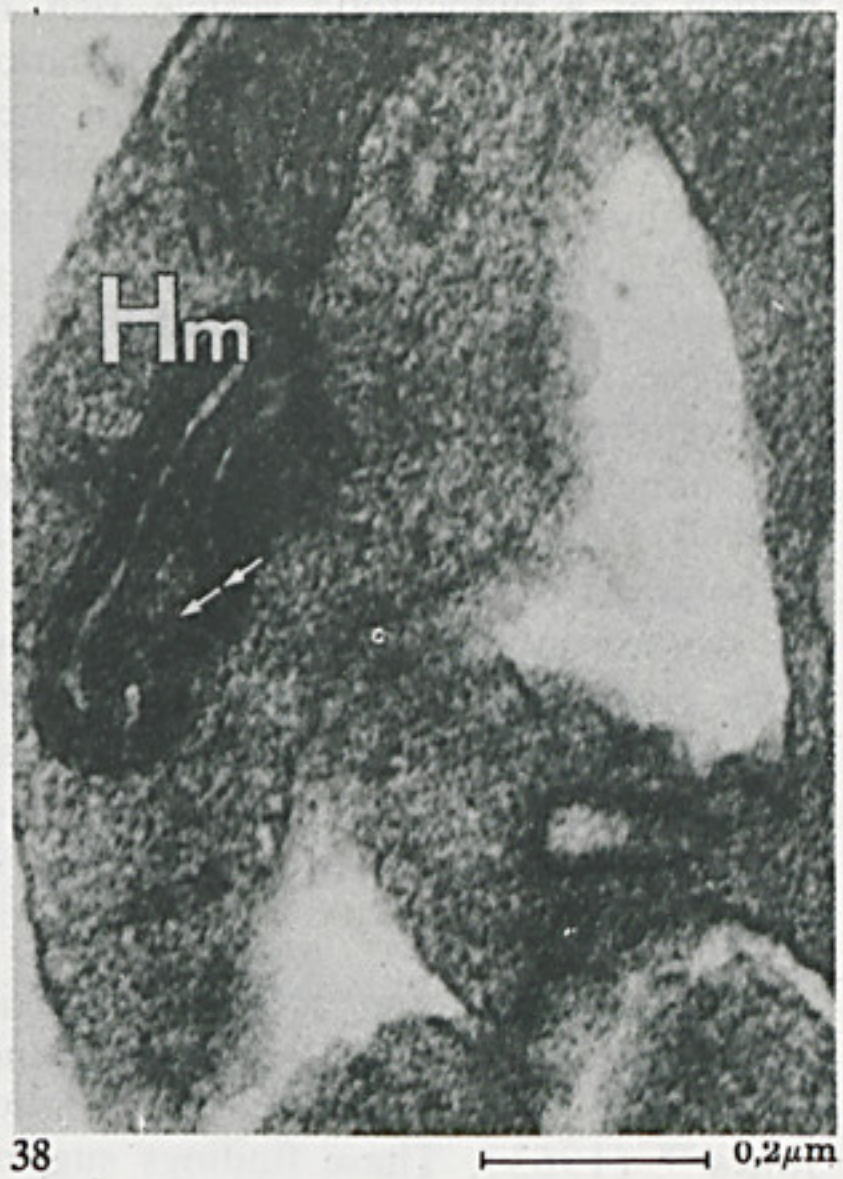
35 0,5μm



36 0,2μm



37 0,2μm



38 0,2μm

ULTRATHIN SECTIONS

Fig. 35 and 36 - Immature avian erythrocyte and mammal reticulocyte (prohematia) from sucessive bleedings. 35 - (N) nucleus; (m) typical mitochondrion. 36 - (m) mitochondria.

Figs. 37 and 38 - Immature avian erythrocyte (phenilhydrazine intoxication) and human prohematia (reticulocyte) from acquired hemolytic anemia. 37 - (p) polysome; (Ph) prohemosome; (mp) plasmic membrane showing clearly the three components. 38 - (Hm) mature hemosome; (arrow) hemoglobin molecules.

investigated the mechanism of the filament increase in rabbit-embryo reticulocytes, and demonstrated the existence of an organelle probably formed "de novo", which they denominated mitochondrion-like organelle (MLO). Brunner et al. [20] (1972), suggested that the final hemoglobin biosynthesis occurs in the MLO, called hemosomes (Fig. 29). The filaments of immature cells of animals anemic through bleedings are in fact mitochondria. This statement is supported by the findings related to the dimensions found in hemolysed smears, ultrathin sections, and electrophoresis of the lysed fraction supernatant, that showed absence of hemoglobin synthesis (Brunner et al. [18], 1973; Coiro et al. [31], 1973), in mammals and birds, respectively. However, in mammalian embryo reticulocytes (Brunner[14], 1968) and in avian proerythrocytes (Coiro et al.[31], 1973) as well in immature forms from individuals with hemolytic anemia, the filaments ("Sgf") correspond, in their majority, to hemosomes (Figs. 3 and 8). Evidently, the "Sgf" filament increase in reticulocytes is related to the number of hemosomes and not to the number of mitochondria.

Hemosome genesis in birds

Brunner & Mombrum [19] (1972) described the genesis of mitochondrion-like organelles (MLO). Brunner et al. [20] (1972), proposed the term hemosome for these MLO. In birds (Fig. 41), hemosome genesis follows the same stages described for mammals: 1) Plasmic membrane with increased density and formation of a pinocytotic vesicle rich in ferritin (Figs. 31 and 32); 2) fusion of the pinocytic vesicles and formation of a larger vesicle; 3) disappearance of the vesicle's walls and release of the ferritin molecules in the cytoplasm (Fig. 25); 4) at the time when the ferritin molecules become amorph they are encircled by a smooth membrane similar to the smooth endoplasmic reticulum (Figs. 25, 26 and 29); 5) the inner part of the smooth membrane folds into the element in formation parallel to the joining of both free ends. In this way the molecules of ferruginous material jointly with the globin synthesised in the polysome remain separated from the cytoplasm. These transformations give rise to a honeycomb-like body; this set was called prohemosome (Figs. 27, 31 and 39); 6) the prohemosome may appear attached to a mitochondrion through an enlarged element we called "linking body" (Figs. 30, 31 and 33 — arrows); 7) the assemblage of prohemosome and mitochondrion joined by the linking body gives rise to the hemosome. This hemosome already shows longitudinal lamellae, but still with one of the transversally lamellated ends derived from the same mitochondrion (Figs. 30 and 31 — arrows); 8) mature hemosome, whose walls disintegrate, allowing scattering of hemoglobin into the cytoplasm (Fig. 40).

The enzyme that acts upon the iron for heme formation was detected in mitochondria of rat hepatocytes, and in duck erythrocytes by Labbe & Hubbard [58] (1961), as well as in mitochondria of pig hepatocytes by Porra & Jones [78] (1963). These findings suggested that the connection between the mitochondrion and a prohemosome is related to the iron for heme formation within the prohemosome, and moreover interferes in the growth of this prohemosome furthering structural protein synthesis. Synthesized globin in polysome (Warner et al. [96], 1962) is taken off the cytoplasm together with ferruginous particles and polysomes, as observed by Brunner et al. (unpublished observations), in HeLa cells induced to hemoglobin synthesis. Finally, energy necessary to com-

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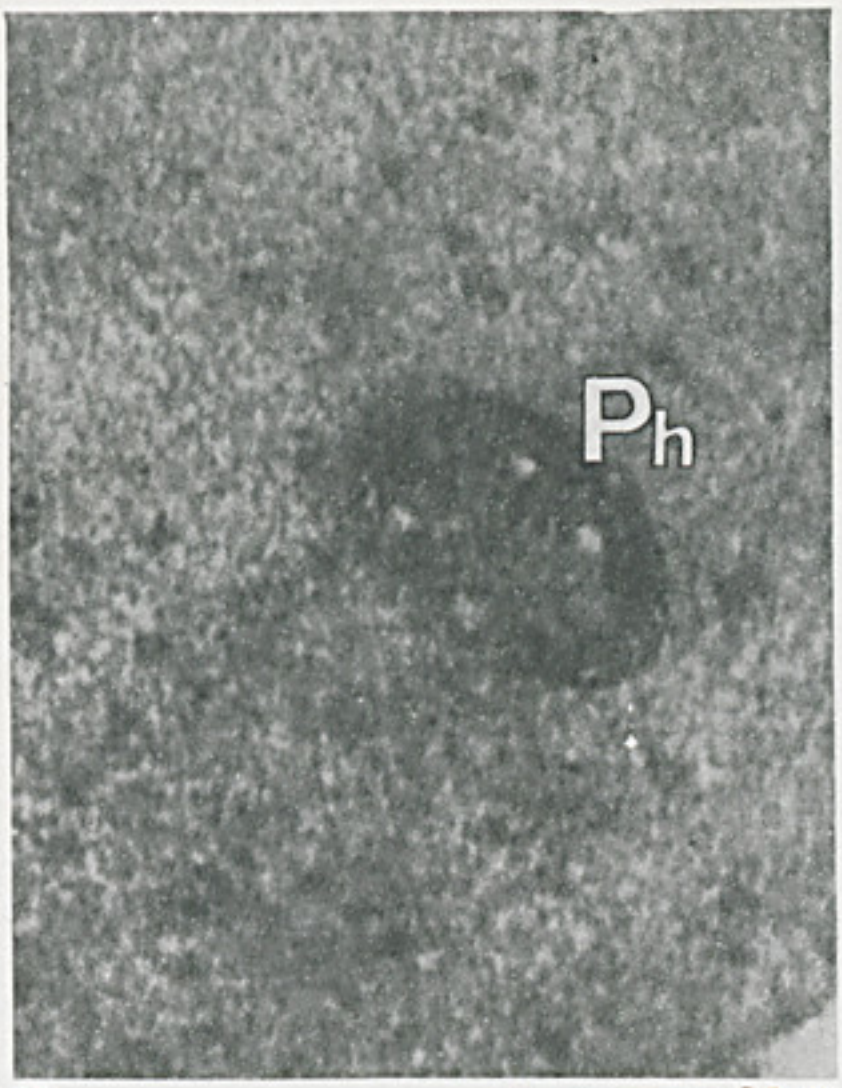

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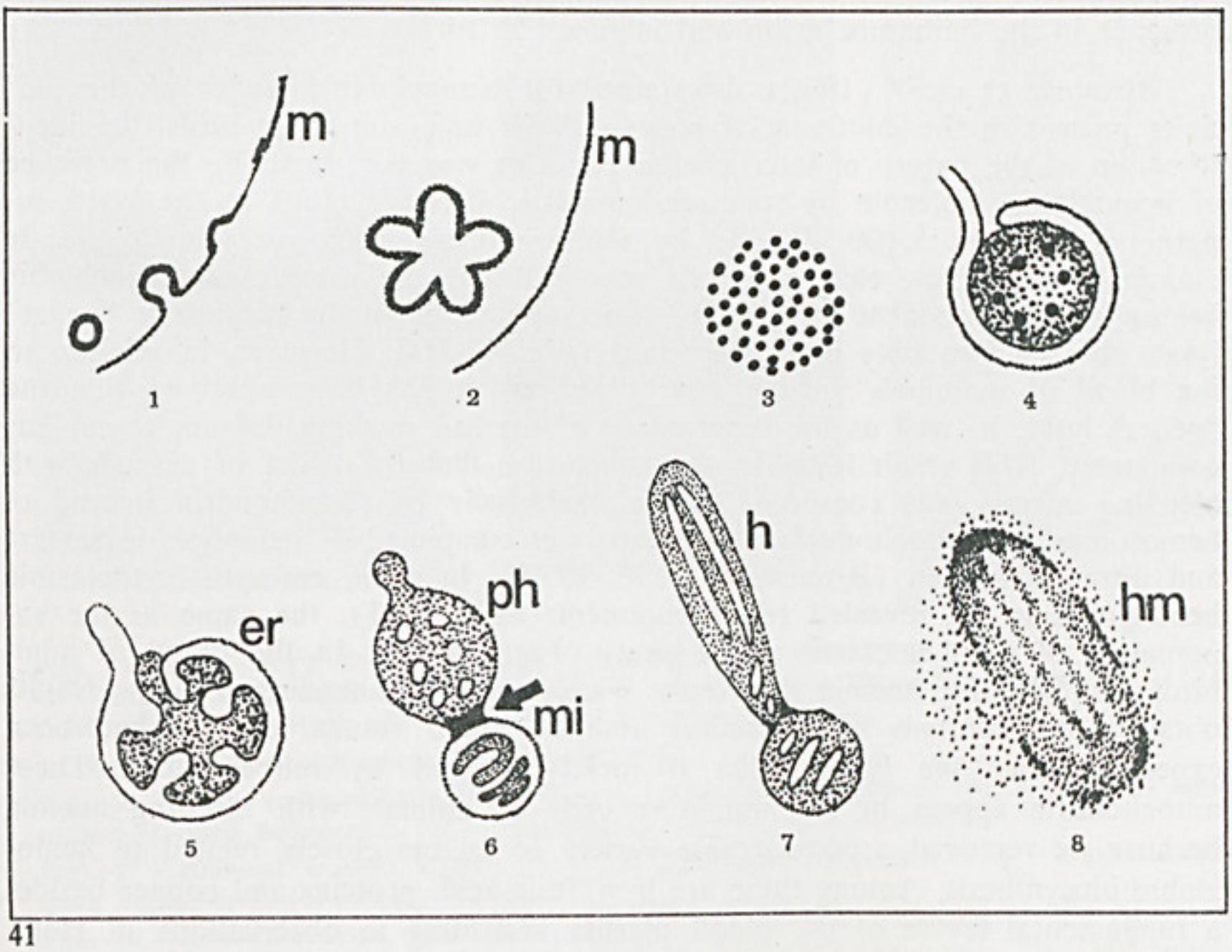

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ULTRATHIN SECTIONS

Figs. 39 and 40 - Human prohematia (reticulocytes) from acquired hemolytic anemia: 39 - (Ph) Prohemosome (see the honeycomb-like aspect). 40 - (Hm) mature hemosome.

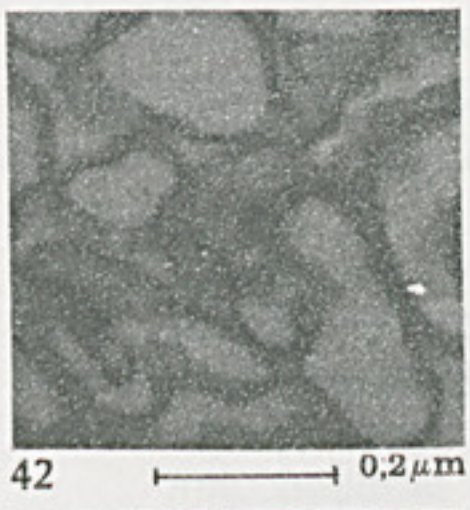
Fig. 41 - Schema of hemosome genesis.

bine heme and globin, also wold be provided by the mitochondrion associated to the prohemosome.

Hammel et al. [48] (1963) described hemoglobin synthesis in nuclear fractions of avian erythrocytes. However, the nuclear fraction had been contamined by cytoplasmic particles. It is probable that these particles were hemosomes, the elements responsible for the final hemoglobin biosynthesis, contributing, in this case, with erroneous results. In general, hemosomes have longitudinal lamellae (Fig. 30). Perhaps this lamellar disposition is due to the pressure exerted by the hemoglobin molecules on the circular lamellae; these pressures would flatten the circular lamellae of the prohemosome, dislocating them alongside the greater axis of the hemosome in formation. The mature hemosome is characterized by abundant hemoglobin molecules within the interlamellar space (Fig. 40). It is possible that the hemoglobin release into the cytoplasm depends on a gradient where the hemosome would distribute the molecules in less hemoglobinized regions of the cytoplasm, a displacement of the hemosome thus occuring from the more concentrated region to the less concentrated region. This may well be so, because immature avian or mammalian cells, hemolysed in smears, were never found with the same type of filamentous hemomose distribution (Figs. 3, 5, 6, 7 and 9).

Relationship between hemoglobin confirmation by electrophoresis and hemosome presence in the immature avian and mammalian forms

Brunner et al. [20] (1972) determined the hemoglobinic nature of the particles present in the interlamellar spaces of the hemosomes. In birds, the identification of the nature of interlamellar particles was confirmed by the presence of hemoglobin molecule by spectrophotometric determinations in the lysed supernatant read at 5.400 Å, and by electrophoresis in polyacrylamide gel. In mammalian embryos, electrophoresis revealed that the cytoplasmic hemoglobin has only one component (Fig. 47-I). The supernatant of the hemosome "pellet" lysate showed also only one component (Fig. 47-III). However, in relation to the blood of mammals anemic after bleedings, it has been observed that the "pellet" lysate as well as the supernatant of the 3rd washing did not reveal any component. This result leads to the conclusion that the pellet of animals with bleeding anemia was composed nearly exclusively by mitochondria instead of hemosomes and mitochondria; this results was completed by hemolysis in smears and ultrathin section (Brunner et al. [18], 1973). In avian embryos, cytoplasmic hemoglobin in gel revealed two components (Fig. 46-I), the same as the supernatant of the hemosome pellet lysate (Fig. 46-III). In the blood of adult birds with bleeding anemia the results were negative, coinciding with the results obtained in mammals with bleeding anemia. These results in birds had been expected, since the lysed pellet is mostly formed by mitochondria. These mitochondria appear in the immature cells of animals with bleeding anemia because we removed a considerable variety of factors closely related to hemoglobin biosynthesis. Among these are iron, folic acid, proteins and copper besides a fundamental factor of the blood plasma according to observations in HeLa induced to hemoglobin synthesis, by Brunner et al., 1975 (unpublished observations). This statement was corroborated by the positive results obtained by electrophoresis, when daily withdrawn blood was homogenized and returned to the same bled animals by intraperitoneal route. In parallel, in hemolysed



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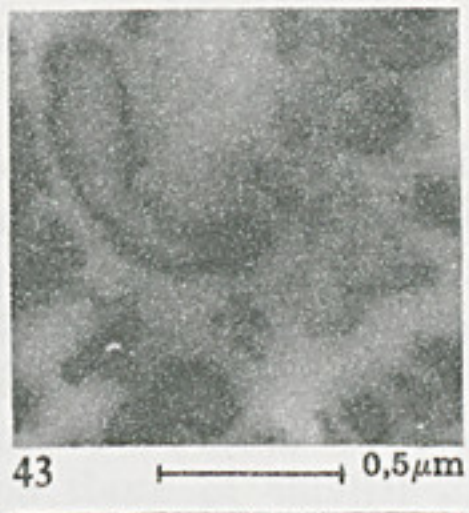

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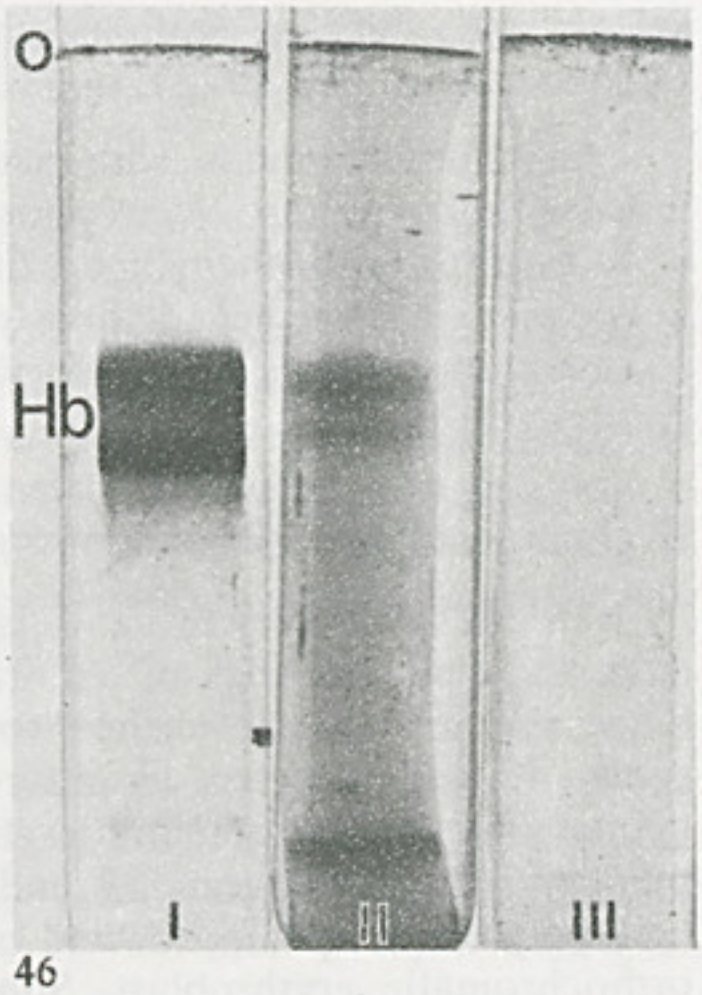

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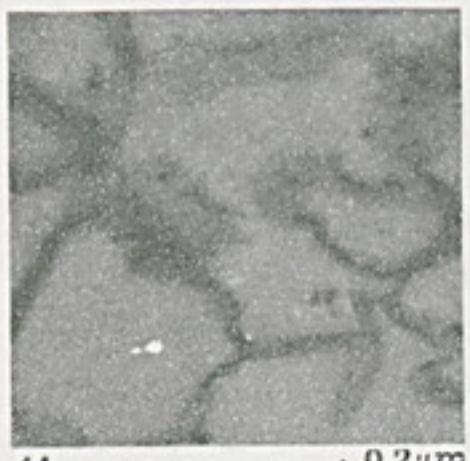

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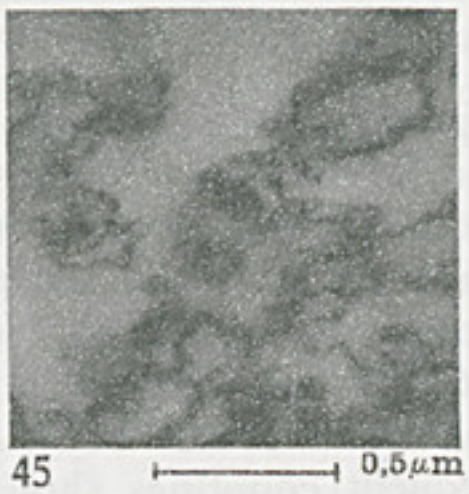

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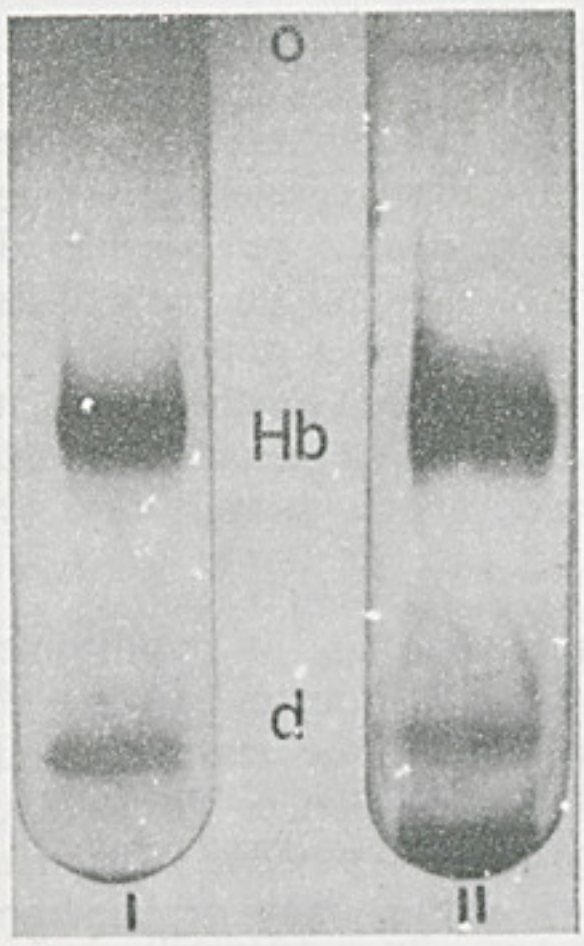

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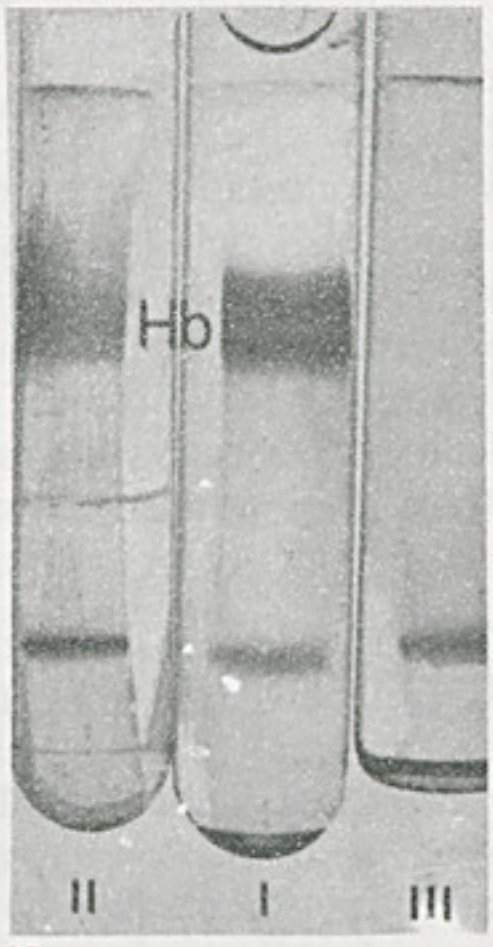

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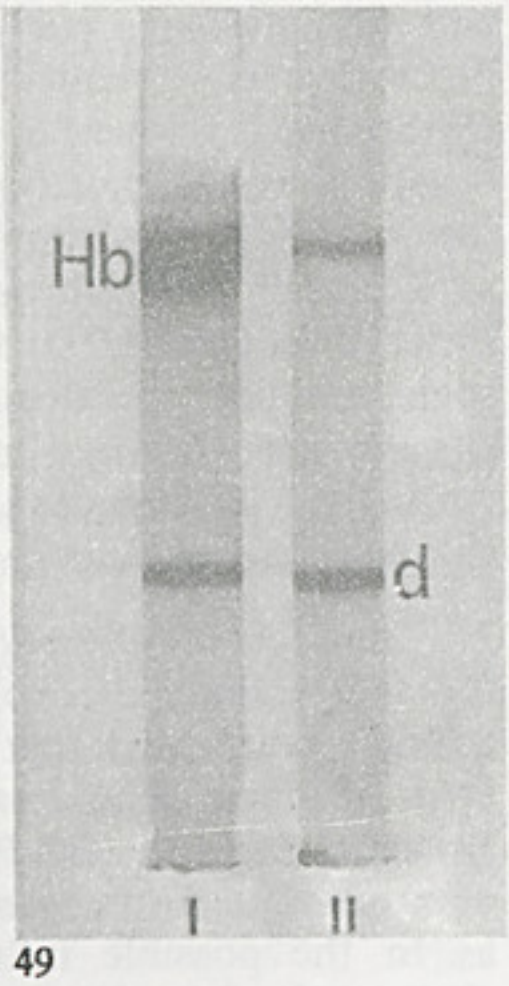

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Figs. 42 to 45 - *ULTRATHIN SECTIONS OF HEMOSOME PELLETS:*
42 - Integral avian hemosome pellet. 43 - Integral mammal hemosome pellet. 44 - Lysed avian hemosome pellet. 45 - Lysed mammal hemosome pellet.

ELECTROPHORESIS IN POLYACRYLAMIDE GEL

Figs. 46 - Avian embryos blood: I - (Hb) cytoplasmic hemoglobin; (o) origin. II - supernatant of lysed hemosomes. III - last washing supernatant (control).

Figs. 47 - Mammal embryo blood: I - (Hb) cytoplasmic hemoglobin. II - supernatant of lysed hemosomes; (o) origin; (d) dye.

Figs. 48 - Immature avian erythrocytes from phenilhydrazine intoxication: I - (Hb) cytoplasmic hemoglobin; (d) dye. II - (Hb) supernatant of lysed hemosomes. III - last washing supernatant (control).

Figs. 49 - Human immature erythrocytes (prohematia and erythroblasts) from acquired hemolytic anemia: I - (Hb) cytoplasmic hemoglobin. II - supernatant of lysed hemosomes; (d) dye.

smears and ultrathin sections, the presence of typical hemosomes was verified (Brunner et al. [18], 1973).

In adult mammals with acquired hemolytic (Fig. 49) or phenylhydrazine induced anemia, the electrophoretic results are the same as in embryos (Fig. 47). In birds with phenylhydrazine induced anemia, the presence of components in polyacrylamide gel demonstrates that the hemoglobin synthesis in these recuperating animals is cytologically the same as the hemoglobin synthesis in embryos (Fig. 47).

Evaluation of the maturation degree

In the avian erythron, it is possible to determine, through morphological data, the characteristics of an orthochromatic erythroblast and a proerythrocyte. Since the mammalian orthochromatic erythroblast is morphologically recognizable, it was selected as reference for the polysome countings. In several mammalian orthochromatic erythroblasts the counts pointed out a polysome number varying between 58 and 61 per μ^2. In birds, one of the cells showed 65 polysomes per μ^2, and therefore could be classified as a probable orthochromatic erythroblast. Subsequent countings showed variations of 8, 16, and 49 polysomes per μ^2. The values between 8 and 16 demonstrated that the proerythrocytes nearly reached the point of definitive transformation into erythrocytes. However, it is possible to find slightly basophile cells where basophily is evidenced only by monosomic forms from the desintegrated polysome forms (Fig. 19). In this case, although basophilic, they cannot be considered as a proper proerythrocyte. Therefore, it is necessary to denominate these cells Proerythrocytes II. To the proerythrocytes, although presenting a reduced polysome number, the denomination Proerythrocyte I is proposed. Furthermore, it is suggested that the reticulocyte forms of mammals should be called more correctly "prohematia", since the anucleated reticulocyte no longer can be characterized as a cell.

Fawcett & Witebsky [43] (1964), stated that the marginal band is responsible for the maintenance of the ellipsoidal form of avian erythrocytes. Grasso [46] (1966) reaffirmed that the marginal band is responsible for the elasticity and form of mammalian erythrocytes. Dervichian et al. [36] (1947), Ponder [77] (1948), Perutz [73] (1948) and Pauling et al. [71] (1949), suggested the possible existence of a molecular arrangement in the "hematia", however, without any explanation as to the possible influence of this arrangement on the discoidal bi-concave shape of the mature mammalian forms. Furchgott & Ponder [44] (1940), Ponder [77] (1948) and Bessis & Bricka [8] (1950), affirmed besides an inner molecular arrangement, the shape could be confered by a physical-chemical phenomenon related to the surface of the "hematia". Brunner [13] (1962) investigating "hematias" maintained in a hypotonic medium, and treated with osmium tetroxide, came to the conclusion that the biconcave form could be essentially conditioned by an inner structural arrangement. The statements of this author are supported by the fact that no marginal band, forming microtubules, were found in mammalian erythrocytes throughout the course of this study, except the microtubule remnants of the achromatic fuse in still immature forms (Grasso [46], 1966; Jones [53], 1969; Brunner [15], 1972). Actually, if a marginal band existed in erythroblasts, mammal "prohematias" and "hematias" could easily show it in ultrathin sections, e.g., figures 20 and 28, or in hemolysed

smears (Coiro et al.[30],1973) (Fig. 2), the latter being very efficient for the identification of marginal band forming microtubules.

CONCLUSIONS

1 — Avian erythroblasts and proerythrocytes, as well as mammalian erythroblasts and reticulocytes show, in hemolysed smears after partial drying, dense and filamentous structures. Those of larger diameter are mitochondria, and those of smaller diameter, hemosomes. Among these filaments, extremely thin thread-like structures appear, corresponding to the smooth endoplasmic reticulum. Besides these structures there are scattered polysomic granules in the stroma.

2 — In the mammalian immature forms submitted to hemolysis in suspension without prior drying, mitochondria and filamentous hemosomes undergo disintegrations which originate the circular forms. Mitochondria and hemosomes of the immature avian forms also give rise to the same circular forms, however, only when hemolysed in smears after a rapid drying at 40°C.

3 — Avian erythroblasts and proerythrocytes, when supravitally stained, show the "Substantia granulo-filamentosa" consisting of mitochondria and hemosomes besides the dye and the precipitated polysomic granules. In the immature forms obtained by bleeding of adult chickens, the "Substantia granulo-filamentosa" consists of only mitochondria besides the dye and the precipitated polysomic granules, showing therefore an identical behaviour as the immature mammalian forms.

4 — Hemosomes, those organelles responsible for hemoglobin biosynthesis, originate from the smooth membranes that agglomerate ferruginous material together with globin. From this phase onwards, transformations occur that originate a prohemosome associated to a mitochondrion, a phase that gives rise to a mature hemosome. The phenomenon of avian hemosome formation is similar to that of mammals.

5 — Spectrophotometric determinations in the lysed fraction supernatant, and the cytoplasmic hemoglobin were performed at 5.400 Å, demonstrating the presence of the heme group. By electrophoresis, hemoglobin was detected in the supernatant of the lysed hemosomic fractions of avian and mammalian embryos as well as in adult animals with hemolytic anemia, except in those animals with anemia induced by successive bleedings in which no hemoglobin was found through electrophoresis of the lysed fraction supernatant.

6 — The presence of hemoglobin in mammalian erythroblast nuclei, and avian erythroblasts, proerythrocytes and erythrocytes is not related with a blocking of synthesis; it is a consequence of the pressures of molecules that penetrate through the nuclear pores.

7 — In mammalian erythroblasts the total loss of chromatinic material occurs by extrusion, karyolysis or karyorrhexis, the level of hemoglobin synthesis continuing and even increasing in reticulocytes. In chickens, the blocking of hemoglobin synthesis seems to be related to the extrusion of a small

chromatin portion into the vesicles, parallel to the disaggregation from polysomic forms to monosomic forms.

8 — In chickens, vesicles which receive the chromatinic material originate from mitochondria fixed at the nuclear membrane and gradually lose their ultrastructural features. This phenomenon is concomitant to the cessation of hemoglobin synthesis.

9 — In successive-bleeding anemias of chickens and mammals, the results differ as to the period of time of the reaction in the erythropoietic tissue. In chickens, the interval between the beginning of the bleedings and the observation of the maximal reaction, confirmed by means of a proerythrocytosis in the peripheral blood, is of about 24 h, compared with the maximal reaction in mammals where the interval can be up to 72 h.

10 — As to the cytological aspect, the mechanism of hemoglobin synthesis is identical in adults with hemolytic anemia in recuperation phase, as well as in embryos. This similarity occurs in both chickens and mammals.

11 — Determination of the degree of the mammalian erythroblast maturation by the counting method of polysomes per area unit, taking as point of reference the orthochromatic phase, characterized by the eccentric nucleus, allows an evaluation of the maturation degree of avian erythron phases, where no characteristic morphological evidence is found as a point of reference for identification.

12 — The less immature avian forms, when stained according to Rosenfeld, may show some basophilia due to the presence of monomere forms originating from dissociated polysomes. Such forms must not be regarded as immature, considering the cessation of globin synthesis. For the cell in this maturation phase the term Proerythrocyte II is proposed. For the cells still containing polysomic forms, the term Proerythrocyte I is suggested.

13 — In chickens, in immature as well as in mature forms, microtubules were found to constitute the marginal band, as observed in hemolysed smears and ultrathin sections. In mammals, no microtubules were detected, except their remnants, constituents of the achromatic fuse.

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RESUMO: Estudos ultra-estruturais de eritron de aves e mamíferos foram realizados no sangue periférico de embriões e de animais adultos normais ou anemizados, mediante o emprego de diferentes métodos, como a hemólise em esfregaço e cortes ultrafinos. Foram utilizados também a eletroforese em gel de poliacrilamida e a espectrofotometria, com a finalidade de detectar hemoglobina intrahemossomal, bem como a presença do grupo hemo nos sobrenadantes de lisados de diferentes frações. Estudou-se ainda a extrusão cromatínica nos eritrócitos maturos de aves, correlacionando-a com a extrusão do núcleo de eritroblastos ortocromáticos de mamíferos e comparou-se a gênese dos hemossomos em mamíferos e em aves. Também foi estudada a identidade da "Substantia granulo-filamentosa" (Sgf) de aves e mamíferos, bem como a avaliação do grau de maturação de células do erítron de aves, através de contagens de polissomos por μ^2. Por fim, verificou-se a existência de banda marginal, através de cortes ultrafinos e de esfregaços de sangue hemolisado.

Os resultados mostram que:

1. Nos esfregaços de sangue hemolisado ("Sgf") em aves, os filamentos de maior diâmetro são mitocôndrios e os de menor diâmetro são hemossomos.

2. Nas anemias por sangrias sucessivas, a "Sgf" é constituida quase que exclusivamente por mitocôndrios.

3. Os hemossomos das aves têm gênese idêntica à dos mamíferos e participam da biossíntese de hemoglobina.

4. A presença de hemoglobina intranuclear, não está relacionada ao bloqueio da síntese, o qual se relaciona com a extrusão cromatínica concomitante a uma desagregação polissômica.

5. Nas anemias por perda de sangue, as respostas do tecido hematopoiético em aves e mamíferos diferem no tempo de reação.

UNITERMOS: Ultra-estrutura do erítron em aves e mamíferos. Biossíntese de hemoglobina.

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