Mem. Inst. Butantan
37: 335-344, 1973

HEMOSOME AND HEMOGLOBIN BIOSYNTHESIS IN EMBRYOS AND IN REGRESSIVE ANEMIAS

A. BRUNNER JR.
J. R. R. COIRO
M. L. SCHWANTES
A. R. SCHWANTES

Laboratório de Microscopia Eletrônica, Instituto Butantan,

SUMMARY — Hemosomes are organelles. Which increase in number at the reticulocytary stage in the peripheral blood of embryo-rabbits, and of adult humans and guinea-pigs in hemolytic regressive anemias. Through fractionation of immature erythrocytes, isolation and lysis of hemosomes, and electrophoresis of the lysate supernatants, hemoglobin bands were obtained. This does not occur in bleeding anemia, in which reticulocytes contain mostly mitochondria.

These facts suggest that the final hemoglobin biosynthesis may occur in the hemosomes. A hypothesis of an association between mitochondrion and pre-hemosomal structures is discussed.

UNITERMS — Hemosome and hemoglobin biosynthesis.

INTRODUCTION

As was stated in the peripheral blood of rabbit-embryos, organelles increase in number at the reticulocytary stage (1).They were termed mitochondrion-like organelles (MLO) on account of their structural similarity to mitochondria, and because neither DNA nor aspects suggesting any division have been detected up to the present. This increase is due to the newly formed MLO which arise from the interrelation of smooth membranes with ferruginous micelles (2), incorporated by immature erythrocytes through pinocytosis (9). This results in the appearance of a pro-MLO followed by a definitive MLO, generally constituted by longitudinal lamellae (2). Size measurements of particles within the interlamellar space of the organelles and of particles dispersed in the hemoglobinized cytoplasm of immature embryo erytrocytes, as well as electrophoresis of the supernatant of the lysed fraction, showed that hemoglobin molecules are present within these organelles, for which the term hemosome has been proposed (3).

This paper shows that hemoglobin biosynthesis in reticulocytes of adult humans and of guinea-pigs, both with hemolytic regressive anemia, depends on

This research has been supported by the Conselho Nacional de Pesquisas, Fundação de Amparo à Pesquisa do Estado de São Paulo and Fundo Especial de Despesas do Instituto Butantan.

Address: C.P. 65,/05504, São Paulo, Brasil.

the hemosomes, the same as occurs in immature erythrocytes of newborn and embryonic rabbits. The behavior of hemosomes during the maturation of reticulocytes, as well as a possible association between pre-hemosomal structures and mitochondrion, are discussed.

MATERIALS AND METHODS

1. *Blood from newborn and embryo-rabbits.*

Blood samples with about 20% reticulocytes were obtained from newborn-rabbits by cardiac puncture. Other samples were collected, sectioning the umbilical cord of 18-day-old rabbit-embryos, and were found to contain, besides definitive erytroroblasts and reticulocytes, a few primitive erythroblasts and erythrocytes.

2. *Bleeding anemias in guinea-pigs.*

Five to 6 ml of blood were withdrawn by daily cardiac puncture from 4 animals weighing 400 g, during 5 days; 2-3 days after the last bleeding, the blood samples contained 10-15% reticulocytes.

3. *Saponin-induced hemolytic anemia in guinea-pigs.*

Four animls, weighing 350 g, received daily subcutaneous 0.1 ml injections of 1% saponin in an isotonic sodium chloride solution, for 3 days; 4-5 days after the last injection, the blood samples contained 15 to 20% reticulocytes.

4. *Human blood.*

Two samples of venous blood from a patient with acquired hemolytic anemia presented a reticulocytosis of about 30%, and less than 1% erythroblasts.

Blood samples obtained by cardiac puncture were collected in a 2.5% v/v of an 1.0% EDTA and 1.0% sodium bicarbonate solutions, in equal volumes. All samples were supravitally stained with 0.1% brilliant cresyl blue in isotonic sodium chloride, in order to determine the reticulocyte percentage. To increase this concentration, the blood samples were submitted to centrifugation at 60Xg for 5 min; the supernatant contained about twice the inicial percentage of reticulocytes.

Hemolysis of blood smears.

Thin blood smears were prepared on collodion-coated histological slides and allowed to dry at room temperature for 18-20 h; hemolysis was performed in a 0,8% sodium chloride solution containing 2.5% formalin. The smears were then stained for 7 min. in an 1% aqueous phosphotungstic acid solution, washed in distilled water and dried at room temperature. The films were transferred

to copper grids and submitted to the shadow-casting process with palladium (4).

Fixation and embedding for thin sectioning.

1. All blood samples were fixed in 2% glutaraldehyde in Millonig's phosphate buffer (pH 7.3) (11) for 1 h, followed by fixation in 1% osmium tetroxide in the same buffer. After staining in an 1% aqueous uranyl acetate solution for 30 min., the blood was dehydrated and embeddel.

2. Other blood samples obtained from rabbit-embryos were fixed for 18-20 h in 10% formalin in 0.50% sodium chloride solution. After washing in 0.50% saline solution, staining was done in an 1% aqueous phosphotungstic acid solution for 1 h. Dehydration was initiated in 70% alcohol, containing 1% phosphotungstic acid, for about 10 min. at 4°C; then the staining continued for 2-3 h at room temperature in the same alcohol; dehydration finished in the alcohol series without staining.

Araldite (10) and Polylite (5) were used as embedding media. Sections were obtained in a Porter-Blum MT-1 microtome and stained with lead citrate (12).

All preparations were examined in UM 100b and Elmiskop I electron microscopes with magnifications of X 1,300 to X 40,000, at 60 and 80 Kv.

Demonstration of hemoglobin within the organelles.

Immature red blood cells were fractionated, the organelles isolated and lysed according to the following procedure: 1. Blood is poured on 8-12 ml of a 0.153M NaCl, 0.005M KCl, 0.005M $MgCl_2$, 1 x 10—^{4}M EDTA and 0.04M phosphate buffer (pH 7.2). 2. Centrifugation of the cell suspension for 10 min at 200Xg, discarding of the supernatant, and resuspension of the sedimented cells (cell volumes: 0.6 — 2.0 ml) in 10-fold their volume of a 0.32M sucrose and 0.03 phosphate buffer. 3. Homogeneization in a Potter-Elvehjem tube at 1,000 rpm for 3 min at 4°C. Centrifugation of the homogenate at 1,350Xg, for 10 min. (sometimes steps 3 and 4 were repeated, resuspending the 1,350Xg sediment). 5. Centrifugation of the 1,350Xg supernatants, at 4°C, for 10 min. at 10,000Xg. 6. Resuspension of the organelle sediments in 0.32M buffered sucrose, and 5-fold washing of the fraction by sucessive resuspension and centrifugation at 10,000Xg for 10 min. The sediments were lysed by resuspension in 3-4 ml distilled water. 7. After the lysis was copleted, the suspensions were centrifuged at 19,000Xg for 20 min. The supernatants were used for electrophoresis, and the sediments were fixed and embedded for electron microscopic examination.

The supernatants of the lysed organelles and of the last washing medium were liophylized in a vacuum chamber, and later hydrated for electrophoresis. Diluted hemoglobins, obtained from the supernatants of 10,000Xg centrifugation (step 5), were used for comparisons. All samples were run on disc electrophoresis in polyacrylamide gel as described by Dietz and Lubrano (7). A

current of 2.5mA applied per tube for 40 min. at 5°C, and the hemoglobin bands were identified by benzidine reagent.

RESULTS

Morphological observations. Hemosomes are largely found in less mature reticulocytes and generally present two or three longitudinal lamellae. Their interlamellas space is filled with agglomerated hemoglobin molecules, which confers to the organelles a higher density than that of the hemoglobinized cytoplasm. They present diameters varying from 0.12 to 0.15μ (Figs. 1a and 1b) which increase to 0.20μ or more when the hemosomes present a higher number of hemoglobin molecules. The presence of these hemosomes is then only recognized by remains of the interlamellar space, as seen in Fig. 1c. The dense honeycomb-like body, or prohemosome, is continuous to a mitochondrion, as can be observed in Fig. 2a. In other instances, the region of continuity is slightly enlarged, and at the opposite extremity of the pro-hemosome a typical hemosome arises (Fig. 2b). Often hemoglobin molecules are also observed in the organelle extremity constituted by oblique or transverse lamellae (Fig. 2c). In figures 2a and 2b the organelles are highly electron dense due to phosphotungstic acid staining. These immature embryo erythrocytes, when examined in hemolysed smears, present filaments of different lenghts, whose diameters range from 0.11 to 0.21μ; erythroblast contain a lower number of filaments than reticulocytes (Figs. 3a and 3b).

Thin sections of reticulocytes of guinea-pigs with bleeding anemia show a high number of characteristic mitochondria measuring 0.25 to 0.30μ in diameter (Fig. 4a); pro-hemosome or hemosomes are rarely found. In hemolysed smears, filaments with 0.24 to 0.30μ in diameter or more were observed (Fig. 4b). Reticulocytes of saponin-induced hemolytic anemia in guinea-pigs, as well as reticulocytes, and a few erythroblasts, of the peripheral blood of a human with acquired hemolytic anemia, contain besides mitochondria, typical hemosomes (Fig. 1b) such as the ones found in immature embryo erythrocytes (Fig. 1a). Hemolysed blood smears show reticulocytes containing filaments whose diameters range from 0.13 to 0.33μ.

Organelle isolation and lysis. Electrophoresis. The pellets of 10,000Xg centrifugations, isolated from reticulocytes of newborn rabbits, embryo-rabbits, guinea-pigs with saponin-induced hemolytic anemia and a human with acquired hemolytic anemia, presented a rose-pink to reddish colour. The final pellets, after five washings with sucrose solution (Fig. 5a), turned to a brownish colour. After the osmotic lysis with distilled water (Fig. 5b) the sediments were clearly visible, although sprayed on the wall of the centrifuge tubes. The cell volumes obtained by centrifugation of the blood at 200Xg, after the concentration of reticulocytes, ranged from 0.60 to 0.85 ml. The pellets of the 10.000Xg centrifugation, isolated from reticulocytes of guinea-pigs with bleeding anemia presented a pale-yellow colour, even starting from a cell volume of 2.0 ml. After the osmotic lysis, the fine sprayed sediment was hardly visible.

Disc electrophoresis in polyacrilamide gel of the concentrated last washing supernatants of all hemosomal fractions showed no visible band. No visible hemoglobin band was obtained from the supernatant of the mitochondrial fraction lysate of reticulocytes from bled guinea-pings. The supernatant of the hemosome lysate, corresponding to reticulocyte organelles from saponin-induced hemolytic anemia, showed two distinct hemoglobin bands, and the supernatant of hemosome lysates corresponding to newborn-rabbits, rabbit- embryos and human immature erythrocytes, presented only one sharp hemoglobin band (Figs. 6a and 6b).

DISCUSSION

The increase of filaments in reticulocytes, after extrusion of the nucleus by the orthochromatic erythroblast (1), as observed in hemolysed smears, (Figs. 3a and 3b) is due to "the rising of benzidine positive organelles (2), later termed hemosomes (3), and not a result from organelles division, as is the case of mitochondria. This increase may be correlated with the increase of the hemoglobin synthesis rate observed in anucleated immature erythrocytes (8, 13) and can be found also in the human peripheral blood.

Initially, pinocytotic vesicles, carrying ferruginous material, fuse among themselves and ferritin granules appear as simple clusters, later surrounded by a double membrane; the ferruginous clusters turn into dense bodies by the gradual disappearence of the ferritin granules. The dense bodies transform themselves in honeycomb-like bodies, or pro-hemosomes, giving rise to the definitive hemosomes (2). They contain hemoglobin molecules within the generally longitudinal interlamellar space, as was demonstrated through fractionation of reticulocytes, organelle isolation (Fig. 5a), lysis (Fig. 5b), and electrophoresis of the lysate supernatant (Fig. 6a). When hemosomes are filled to a high degree with hemoglobin molecules, or are "mature", the hemoglobin spreads out to the cytoplasm in consequence of the disruption of internal and external membranes of the organelle (Fig. 1c). The extremely variable disposition of hemosomes, found in hemolysed erythroblasts and reticulocytes, are possible due to their dislocation in the cytoplasm of the living cells, thus explaining the uniformity of hemoglobin distribution. Comparable results were achieved from erythroblasts and pro-erythrocytes of 16-day-old chick-embryos blood, concerning the presence of hemosomes, and obtainment of hemoglobin bands througs electrophoresis of the supernatant of their lysate (6).

It is difficult to explain the way by which the hemosomes grow, since neither DNA nor RNA have yet been detected in these organelles, and how the energy for final hemoglobin synthesis is supplied. Hypothetically, an association between mitochondria and pro-hemosomes might provide the necessary conditions for hemosome growth and their possible biosynthesis function, as suggest Figs. 2a and 2b. Subsequently, mitochondria may behave as hemosomes (Fig. 2c).

First, to verify if in reticulocytes of adult animals with regressive anemia hemoglobin biosynthesis is conditioned by the presence of hemosomes, guinea-pigs were submitted to successive bleedings. But, reticulocytes present only typical mitochondria, and rare hemosomes (Fig. 4a); no visible hemoglobin band was obtained through electrophoresis of the supernatant of the lysed mitochondrial fraction. Anemia induced under these conditions, causes insufficiency of iron, protein as well as other factors, and in consequence, recuperation proceeds slowly. Through hemolysis of partially dried smears (4) a marked difference in diameters is evident between hemosomal (Fgs. 3a and 3b) and mitochondrial (Fig. 4b) filaments of reticulocytes from rabbit-embryos and guinea-pigs with bleeding anemia, respectively. The former vary from 0.11 to 0.21μ; mitochondria diameters vary from 0.24 to 0.30μ. Correspondingly, this marked difference is also observed in sections (Figs. 1a and 4a).

In hemolytic anemias, as the one induced in guinea-pigs by daily injections of a saponin solution, no iron, protein and other material necessary for the biosynthesis of hemoglobin are withdrawn. Sections of reticulocytes show typical hemosomes, besides mitochondria, and hemolysed reticulocytes contain filaments ranging from 0.15 to 0.37μ in diameter. Electrophoresis from the supernatant of the lysed hemosomal fraction showed two distinct hemoglobin bands. Similar results were obtained with the blood from the patient with acquired hemolytic anemia, whose reticulocytes show also typical hemosomes (Fig. 1b); filaments with 0.11 to 0.33μ in diameter were found in hemolysed reticulocytes, and one sharp hemoglobin band was obtained by electrophoresis of the supernatant of the lysed hemosomal fraction (Fig. 6b).

The above results suggest that the final hemoglobin biosynthesis may take place within the hemosomes. Positive benzidine reaction (2) demonstrated the presence of heme in the course of their formation. Globin, synthesized in polysomes (14, 15), could be already present during the growth of hemosomes, combining to heme within the organelle (2). This does not occur when elements necessary for hemoglobin biosynthesis are insufficient. Consequently, reticulocytes contain only mitochondria, and there scarcely ever occurs hemosome genesis, ,as happens in bleeding anemia. In reticulocytes of adults with hemolytic regressive anemias the cytological modifications observed during maturation are the same as in immature embryo erythrocytes.

RESUMO — Hemossomos são organelos que aumentam em número na fase reticulocitária no sangue periférico de embriões de coelho, e do homem e cobaios adultos nas anemias hemolíticas regressivas. Através do fracionamento de eritrócitos imaturos, isolamento e lise dos hemossomos, eletrofo-rese dos sobrenadantes dos lisados da fração, foram obtidas bandas de hemoglobina. Na anemia provocada por sangrias, em que os reticulócitos não contêm praticamente hemossomos, mas apenas mitocôndrios, nem mesmo traços de bandas de hemoglobina são obtidos.

Estes fatos sugerem que a biossíntese final da hemoglobina pode ocorrer nos hemossomos. Uma hipótese sobre a possível associação entre estruturas pre-hemossômicas e o mitocôndrio é discutida.

UNITERMOS — Hemossomo e biossíntese de hemoglobina.

ACKNOWLEDGEMENTS

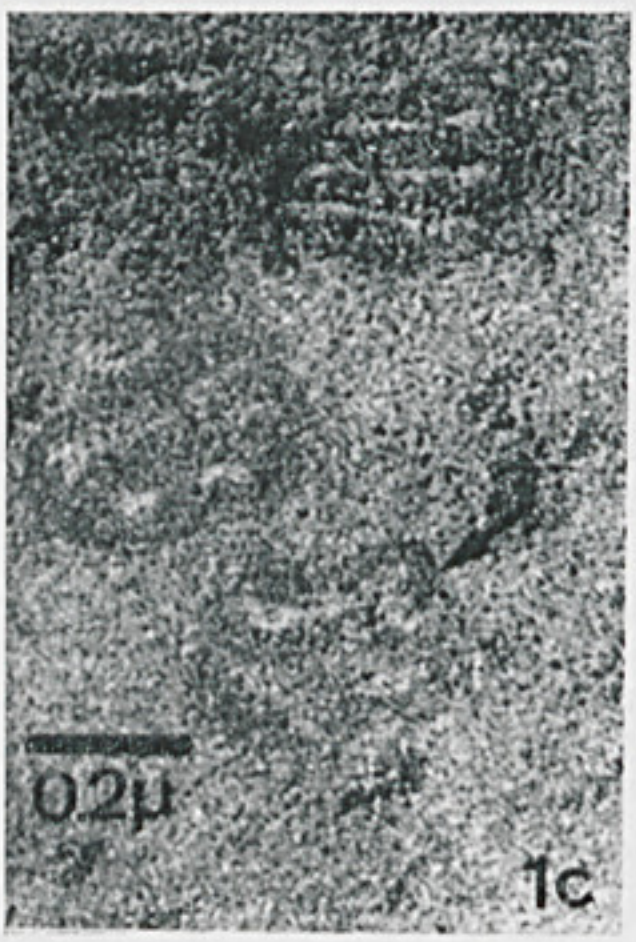
The authors wish to thank Mrs. Vera Mondin Weisz, Mr. Alipio Silva Gonzales and Mr. Heitor Costa for their excellent technical assistance. And Mrs. Sibylle Heller for her editorial aid and translation.

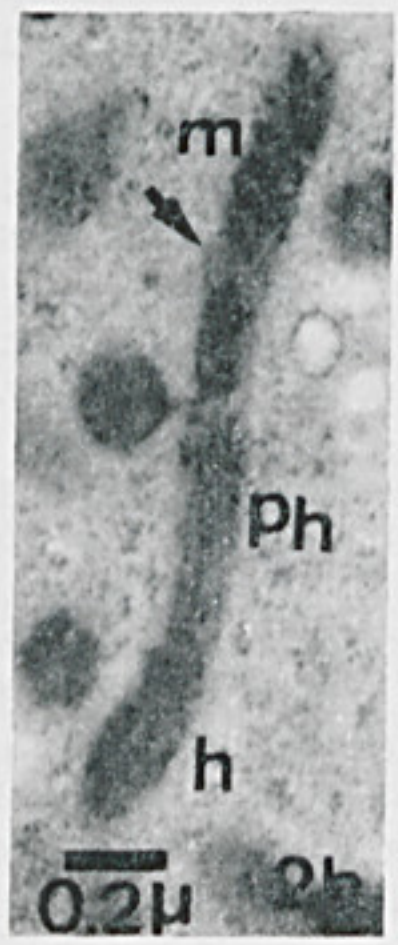
The human blood samples were obtained through courtesy of Dr. Gastão Rosenfeld, Director of the Laboratório de Hematologia Clínica, São Paulo, Brasil.

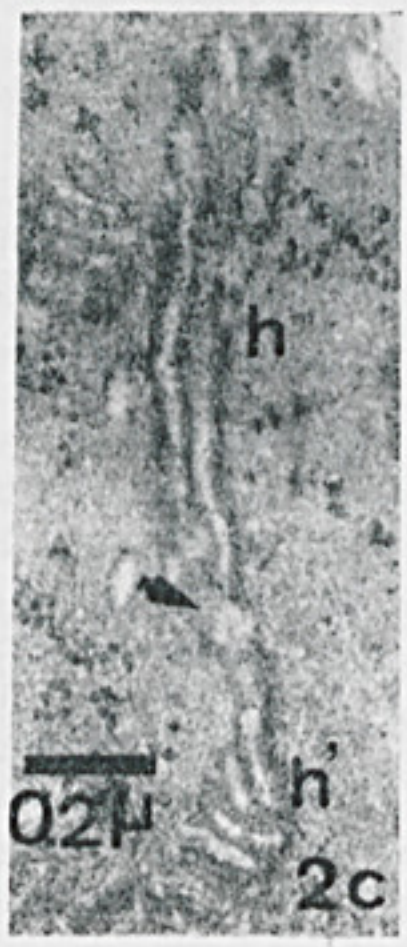
REFERENCES

1. BRUNNER JR., A. — Increase of mitochondria at the reticulocytary stage. *Ciência e Cultura,* **24**: 468-476 (1972).
2. BRUNNER JR., A. & MOMBRUB, I. C. — Genesis of mitochondrionlike organelles in reticulocytes of rabbit-embryo blood. *Ciência e Cultura,* **24**: 448-451 (1972).
3. BRUNNER JR., A., SCHWANTES, A. R., SCHWANTES, M. L. & BEÇAK, W. — Hemoglobin in immature erythrocyte mitochondrion-like organelles. *Experientia,* **28**: 569-571 (1972).
4. BRUNNER JR., A. & VALLEJO-FREIRE, A. — Electron microscopic observations on granules and filaments (reticulosomes) of reticulocytes. *Exp. Cell Res.,* **10**(1): 55-62 (1956).
5. COIRO, J. R. R. & BRUNNER JR., A. — Polylite 8001: A new embedding me dium for electron microscopy. *Rev. Microsc. Electr.,* **1**(1): 12 (1972).
6. COIRO, J. R. R., BRUNNER JR., A., SCHWANTES, M. L. & SCHWANTES. A. R. — Hemoglobin in mitochondrion-like organelles of immature chick-embryo erythrocytes. Mem. Inst. Butantan, **37**: 327-334, 1973.
7. DIETZ, A. A. & LUBRANO, T. — Separation and quantitation of lactic dehydrogenase isoenzymes by disc electrophoresis, *Anal. Biochem.,* **20**: 246-257 (1967).
8. FANTONI, A., DE LA CHAPELLE, A., RIFKIND, R. A. & MARKS, P. A. — Erythroid cell development in fetal mice: Synthetic capacity for different proteins. *J. Mol. Biol.,* **33**(1): 79-91 (1968).
9. JONES, O. P. — Selective binding sites for the transfer of ferritin into erythroblasts. I. Preliminary report. *J. Nat. Cancer Inst.,* **35**: 139-151 (1965).
10. LUFT, J. — Improvements in epoxy resin embedding methods. *J. Biophy. Biochem. Cytol.,* **9**: 409-414 (1961).
11. MILLONIG, G. — Advantages of a phosphate buffer of OsO_4 solutions in fixations. (Abstract). *J. Appl. Phys.,* **32**(8): 1.637 (1961).
12. REYNOLDS, E. S. — The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.,* **17**: 208-212 (1963).
13. SENO, S. The structure and the function of reticulocyte. *Acta Haem. Jap.,* **21**: 171-181 (1958).
14. WARNER, J. R., RICH, A. & HALL, C. E. — Electron microscope studies of ribosomal clusters synthesizing hemoglobin. *Science,* **138**: 1.399-1.403 (1962).
15. WARNER, J. R., KNOFF, P. M. & RICH, A. — A multiple ribosomal structure in protein synthesis. *Prof. Nat. Acad. Sci.,* **49**: 122-129 (1963).

Recebido para publicação em 26.X.1974
Aceito para publicação em 26.XI.1974







Thin sections of adult human (Fig. 1b) and rabbit-embryo reticulocytes (Figs. 1a, c, 2a, b and c). Glutaraldehyde/OsO_4 fixation: Figs. 1a, b, c and 2c; formalin fixation and phosphotungstic acid staining: Figs. 2a and b.

Fig. 1a — Several hemosomes (h) slightly more electrondense than the cytoplasm are seen. Two of them are longitudinally sectioned. Polyribosomes (arrows) are diffusely distributed.

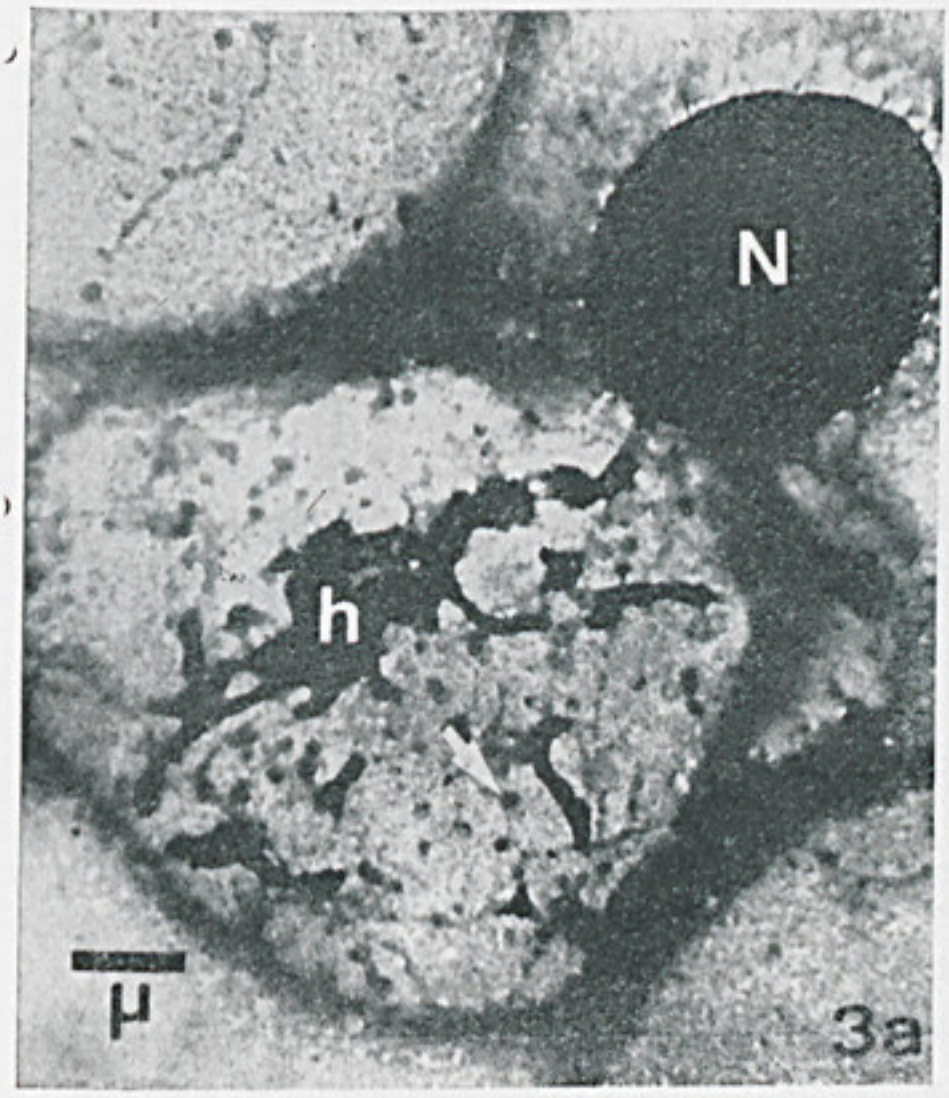
Fig. 1b — A typical hemosome (h), constituted by longitudinally disposed double lamellae and limited by an external membrane.

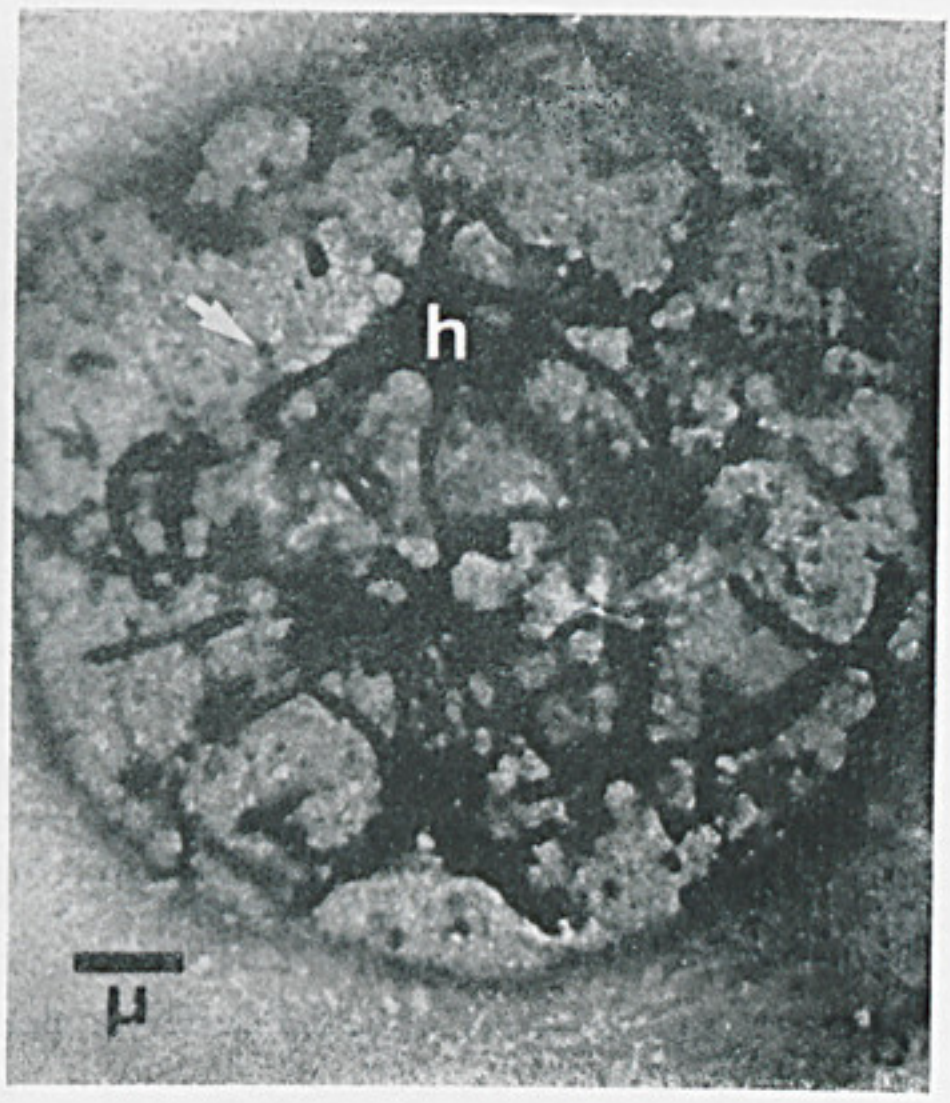
Fig. 1c — Three "mature" hemosomes completely filled with hemoglobin molecules, which confounds them with the hemoglobinized cytoplasm. From one hemosome, hemoglobin molecules spread out to the cytoplasm (arrow).

Fig. 2a — A honeycomb-like organelle or pro-hemosome (ph) is continuous to a mitochondrion (m), suggesting a possible association between these organelles.

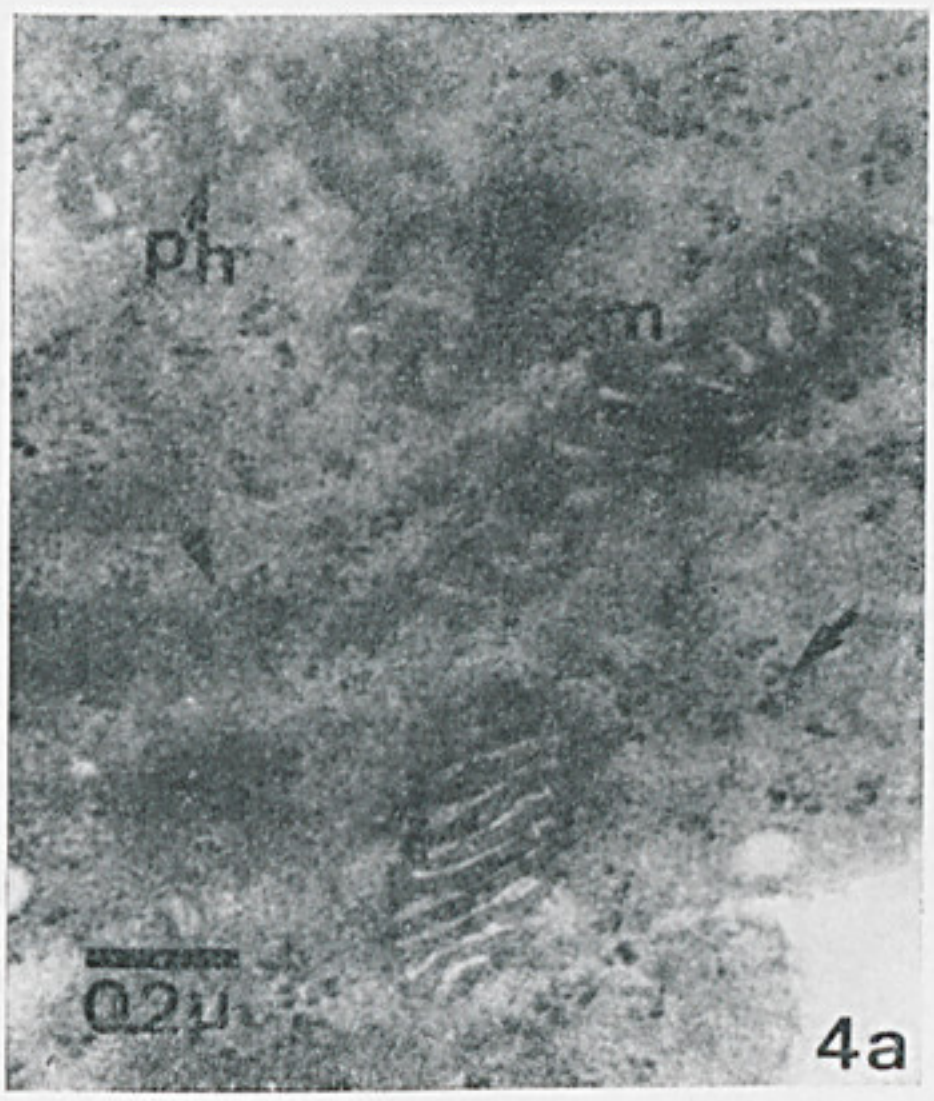
Fig. 2b — Continuous with the extremities of a long pro-hemosome (ph), a hemosome (h) and a mitochondrion (m) are seen. The region of connection between *ph* and *m* is slightly enlarged (arrow).

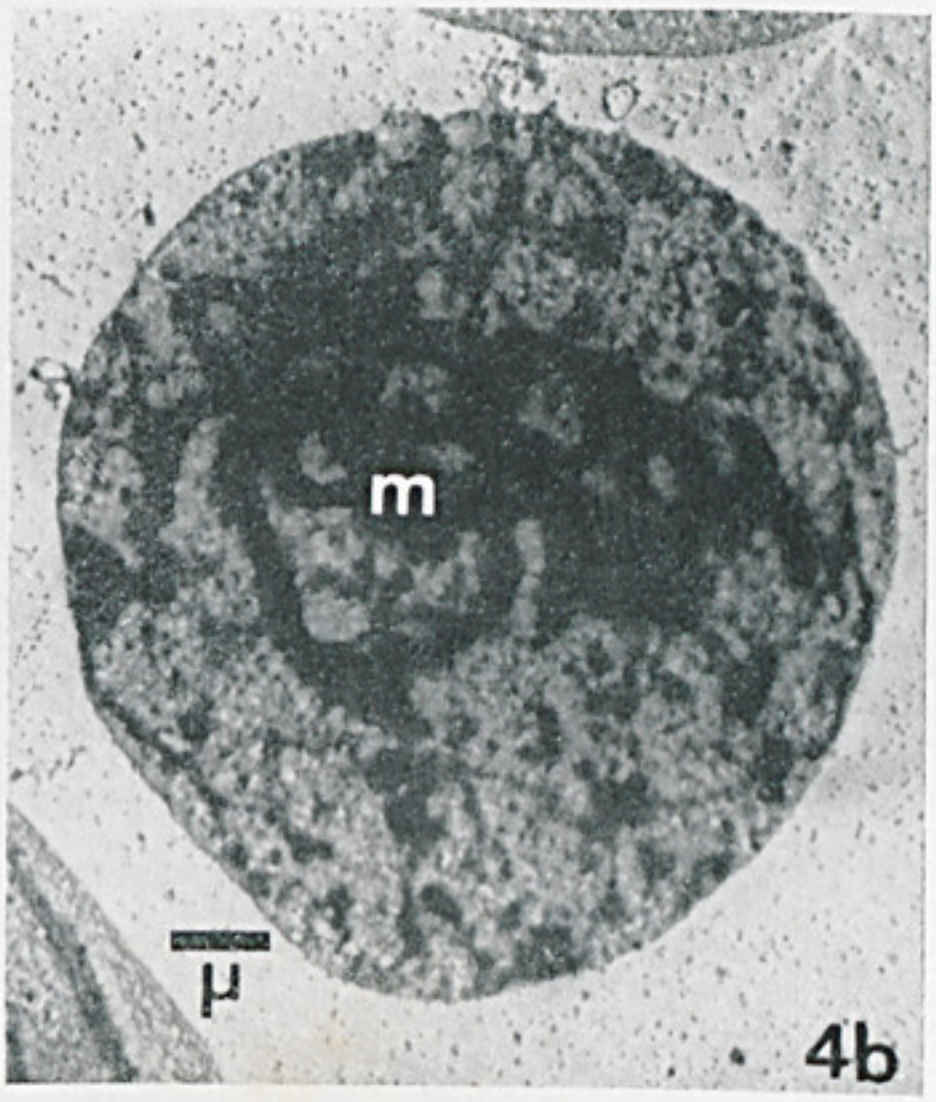
Fig. 2c — The long hemosome (h) is continuous to a hemosome constituted by transverse and oblique lamellae (h') which could have been a mitochondrion. A dilatation between *h* and *h'* can be observed (arrow).





Figs. 3a and 3b — Hemolysed orthochromatic rabbit — embryo erythroblast, extruding its nucleus (N) and reticulocyte, respectively. Filamentous and rod-shaped hemosomes (h), and polyribosomes (arrow) are observed.

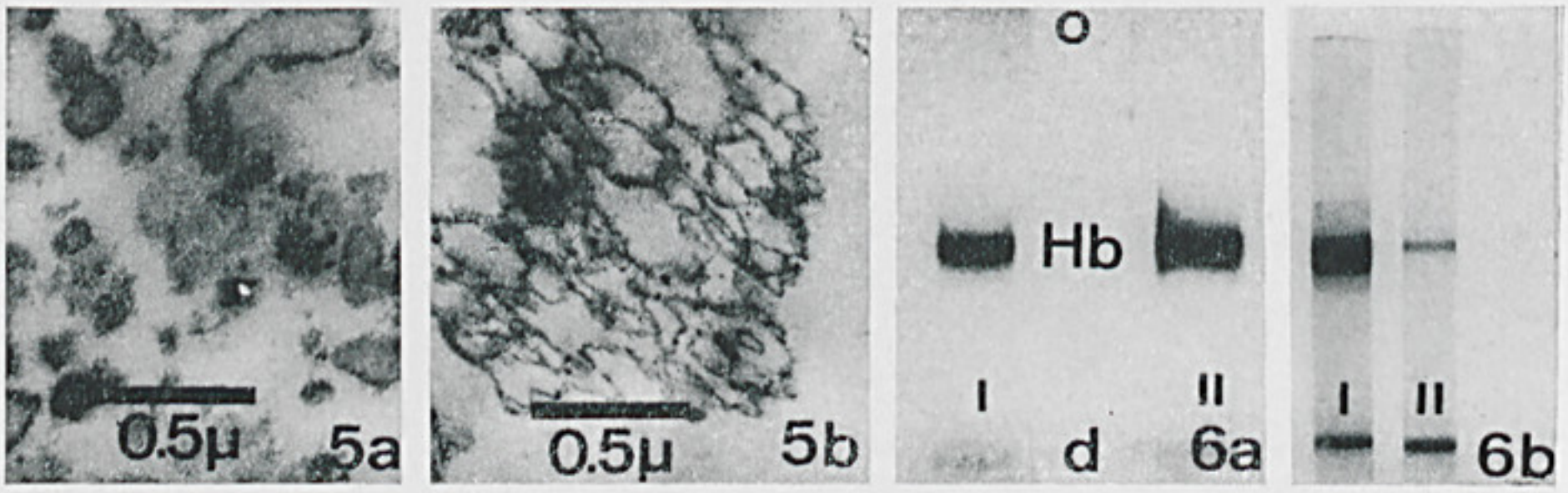




Reticulocytes of guinea-pigs with bleeding anemia.

Fig. 4a — Thin section showing mitochondria (m) of higher diameters than hemosomes, and polyribosomes (arrows). Pro-hemosomes (ph) and hemosomes are rarely found.

Fig. 4b — Hemolysed reticulocyte presenting filamentous and rod-shaped mitochondria (m) of higher diameters than filamentous hemosomes of Figs. 3a and 3b.



Sections of hemosomes obtained from reticulocyte fractionation.

Fig. 5 — After five washings; lamellae were not preserved.

Fig. 5b — After osmotic lysis for electrophoresis of the supernatant.

Electrophoresis patterns of the hemoglobin from the supernatants of 10,000Xg centrifugation (I) and from hemosome content (II); *o* — origin; *Hb* — hemoglobin; *d* — dye.

Fig. 6a — From rabbit-embryo reticulocytes.

Fig. 6b — From reticulocytes of humans with acquired hemolytic anemia.

ÍNDICE DE AUTORES

ÍNDICE DE ASSUNTOS