ULTRASTRUCTURAL STUDIES OF RED BLOOD CELLS FROM THYROXIN-TREATED *RANA CATESBEIANA* TADPOLES

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During thyroxin-induced metamorphosis of *Rana catesbeiana* tadpoles, hemoglobin synthesis in the circulating red blood cells initially declines to very low levels. This period is followed by the synthesis of a new hemoglobin which is characteristic of the adult bullfrog. Radioautographic studies on smears of whole blood cells have indicated that the production of adult hemoglobin is associated with the appearance of a new cell type which differs from mature tadpole blood cells in size and shape (Moss and Ingram, 1965; 1968 *a* and *b*; DeWitt, 1968). Recently it has been shown that the thyroxin-induced switch in hemoglobin synthesis is also accompanied by striking changes in the synthesis of erythrocyte ribosomal RNA. These include an initial breakdown of ribosomal RNA followed by an increase in RNA synthesis at the time of appearance of frog hemoglobin (McMahon and DeWitt, 1968).

It was expected that biochemical changes occurring during the switch in hemoglobin synthesis could be correlated with changes in cell morphology. Ultrastructural studies were, therefore, initiated to examine the cell types present in the peripheral blood following the administration of thyroxin to bullfrog tadpoles. These results clearly demonstrate the existence of two distinct red blood cell types which appear to differ significantly in their synthetic activities.

MATERIALS AND METHODS

Rana catesbeiana tadpoles without hind legs were purchased from the Lemberger Co., Oshkosh, Wis., and kept at 20°C in tap water containing 2.5×10^{-8} M L-thyroxin. After various periods of thyroxin treatment, tadpoles with hind leg/tail ratios greater than 0.25 were anesthetized by chilling in ice water and carefully dissected open. Aortae and other large vessels were clamped and immediately bathed and injected with 5% glutaraldehyde in 4.5×10^{-4} M $CaCl_2$, 0.065 M sodium phosphate buffer, pH 7.3. Very short lengths of artery containing clumped blood cells were removed and fixed for an additional 3 hr at 4°C in fresh glutaraldehyde solution. The tissue was washed overnight in cold $CaCl_2$-sodium phosphate buffer containing 1% sucrose. Postfixation was done in 1% osmium tetroxide in the same buffer without sucrose. Tissue was then rapidly dehydrated in a graded series of cold ethanols, immersed in polypropylene oxide, and embedded in Epon 812. Silver sections (approximately 500–600 A in thickness) were cut on the LKB Ultrotome III (LKB Instruments, Inc., Rockville, Md.) with glass or diamond knives, picked up on uncoated grids, and stained in uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Half-micron sections for light microscopy were stained on glass slides with toluidine blue–pyronin by the method of Bennett and Radimska (1966). Micrographs were made in the Philips EM 300 electron microscope.

RESULTS

Mature Tadpole Erythrocyte of Control and Thyroxin-Treated Animals

Light microscopy of stained sections shows a large, centrally located nucleus within a homogeneous cytoplasm. All sections are consistent with a regular, flattened ellipsoidal shape (Fig. 1 *a*). Electron microscopy demonstrates ultrastructural features similar, although not identical, to those observed in mature erythrocytes of *Triturus cristatus* (Tooze and Davies, 1967), and which indicate that the mature tadpole red blood cell retains some capacities for synthetic and other metabolic activity (Fig. 2 *a*).

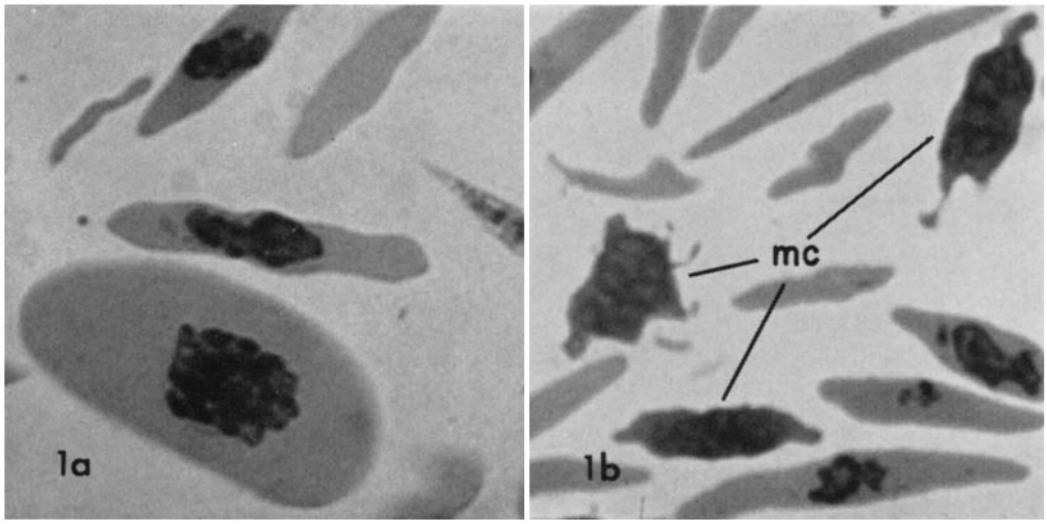


FIGURE 1 Light micrographs of sectioned erythrocytes stained with toluidine blue–pyronin. (*a*) Control tadpole; (*b*) thyroxin-treated tadpole. Microcytic cell (*mc*). × 2150.

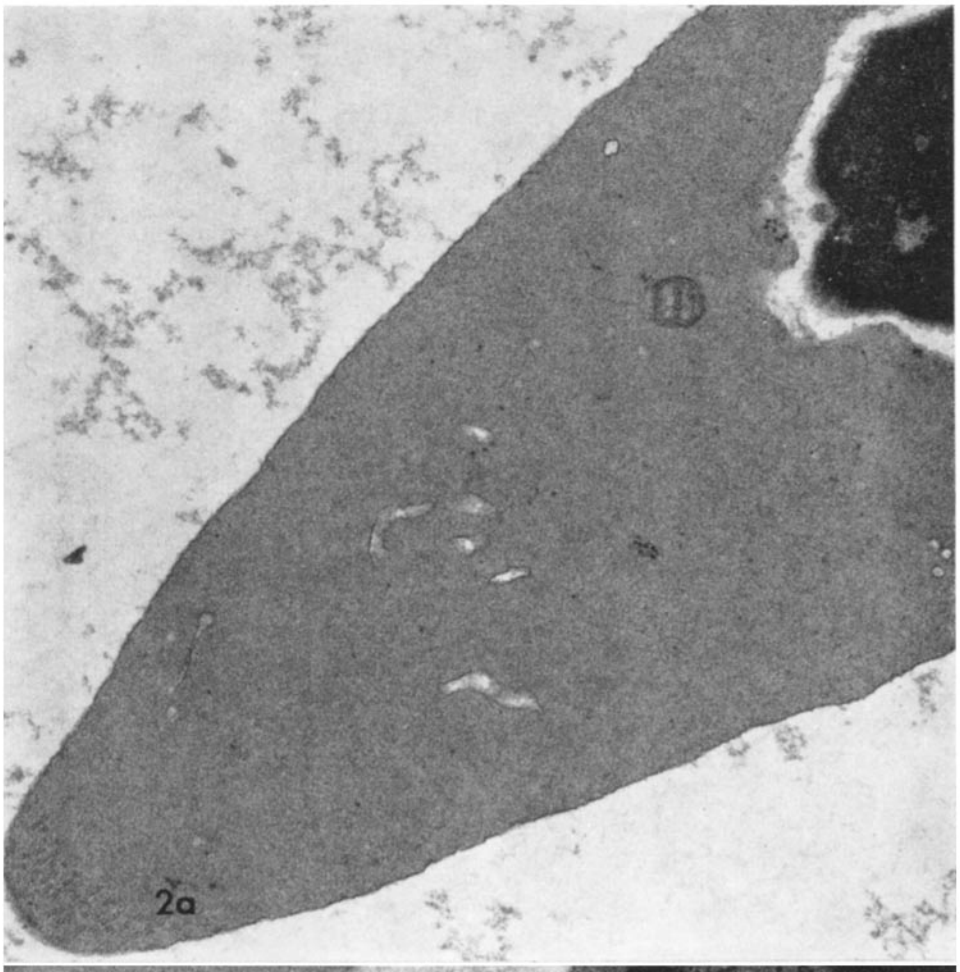
Polyribosomes, though relatively few, are invariably present in the cytoplasm. However, the concentration of polyribosomes and single ribosomes appears to vary somewhat from cell to cell as noted by Tooze and Davies (1967) in mature newt erythrocytes. Intact mitochondria are found, as well as myelin figures and membranous extrusions from the cell surface, which suggest degeneration and expulsion of mitochondria (Simpson and Kling, 1968; Sekhon and Beams, 1969). Remnants of a Golgi apparatus are present and an associated centriole pair has been demonstrated in several cells. The cytoplasm of most cells contains some scattered ferritin, but in no case has this material been found concentrated in membrane-bounded vacuoles. The concentration of ferritin granules attached to the plasma membrane varies considerably. Micropinocytotic vacuoles are occasionally found and appear to remain confined near the cell surface. A marginal band (Fawcett and Witebsky, 1964; Gall, 1966) is always present (Fig. 2 *a*). The nucleus is characterized by very large masses of highly condensed chromatin. At least one nucleolus is present; it is of the fibrillar-granular type (Hay, 1968) with no nucleolonema discernible in any case.

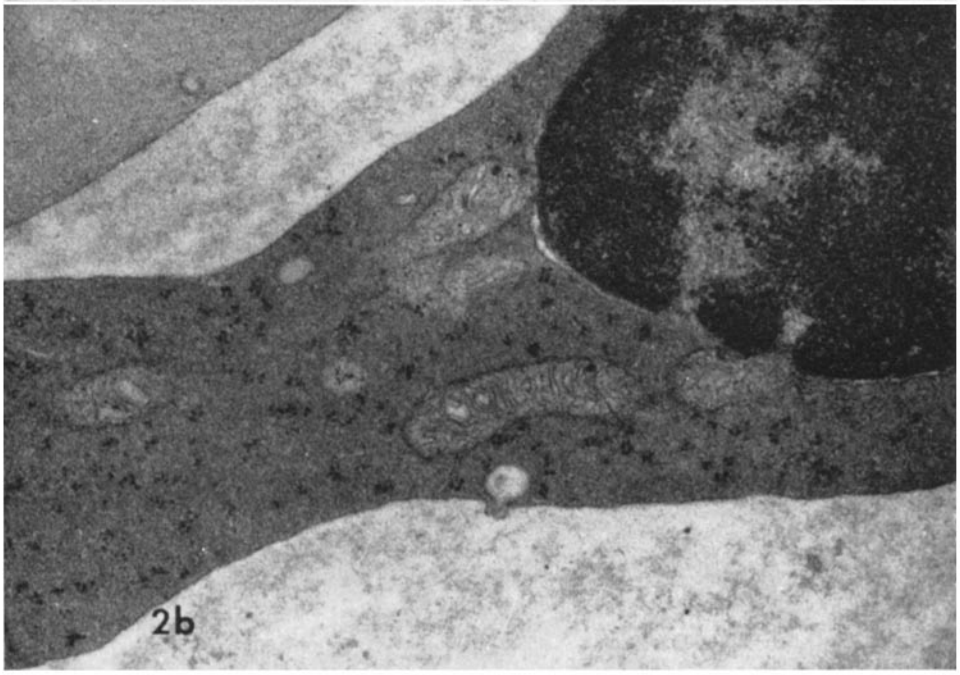
Microcytic Erythroid Cell of Thyroxin-Treated Tadpoles

This cell type can be unequivocally identified by light microscopy of half-micron sections of peripheral blood. Cell outlines are more irregular and crenulated than are those of mature tadpole erythrocytes, and several large cytoplasmic projections are often apparent. The nuclear-cytoplasmic volume ratio is greater than in tadpole erythrocytes, and the cytoplasm stains more intensely with toluidine blue–pyronin dye (Fig. 1 *b*).

Details of fine structure also identify the microcytic cell as a distinct erythroid cell type (Fig. 2 *b*). Ribosomes are extremely numerous, and the vast majority of them are present in polyribosomal

FIGURE 2 (*a*) Mature tadpole erythrocyte showing few cytoplasmic organelles, single ribosomes, or polyribosomes. Microtubules are apparent at the periphery of the cell. (*b*) Microcytic erythroid cell induced by thyroxin treatment of tadpoles. Mitochondria, pinocytotic vesicles, and numerous polyribosomal structures are apparent. The cytoplasm is considerably more electron-opaque than in the mature erythrocyte. Fig. 2 *a*, × 32,000; Fig. 2 *b*, × 32,000.


2a


2b

structures. A small fraction of ribosome clusters are closely associated with the outer layer of the nuclear envelope. Elements of an endoplasmic reticulum are infrequently seen and mostly present as smooth profiles, although very occasional small clusters of ribosomes are clearly attached.

Micropinocytotic vacuoles with a characteristic coated membrane are often seen at the surface of the microcytic cells (Fig. 3). In addition, the internal cytoplasm of these cells contains a localized system of vacuoles, possessing a coated membrane structure which appears very similar to that of the micropinocytotic vacuoles. Their diameters or widths are large compared to those of the endoplasmic reticulum or Golgi apparatus found in this cell type. On this criterion and that of membrane structure, they are quite distinct from these latter cytoplasmic membrane systems. The interior of these vacuoles is very electron lucent compared to the surrounding cytoplasmic matrix, and there is no evidence of ferritin particles within their coated membranes. Similar vacuoles are very infrequent in mature tadpole erythrocytes and never clustered into an apparent system of vacuoles. A marginal band is also found in microcytic cells, although there may be a smaller number of microtubules in it than in mature erythrocytes.

Individual ferritin granules, approximately 50–55 A in diameter, are found scattered in the cytoplasm. Unlike the case in many mature tadpole erythrocytes, very little or no ferritin appears bound to, or concentrated at, the plasma membrane. However, microcytic cells always contain in the internal cytoplasm at least one large membrane-bounded vacuole with an extremely dense concentration of ferritin particles. The arrangement of these particles fails to show the paracrystalline array characteristic of some siderosomes in erythroid cells of other species (Bessis, 1961).

Mitochondria are common and generally show obliquely transverse cristae. Configurations suggesting mitochondrial degeneration are found, and appear similar to the cytolysomes described by Tooze and Davies (1965). Mitochondrial extrusion figures appearing identical to those reported by Sekhon and Beams (1969) have also been observed. Elements of a Golgi apparatus are present, and occasional sections demonstrate an associated pair of centrioles.

Microcytic cell nuclei are highly heterochromatized, with extended regions of condensed chromatin bordering the nuclear membrane. Heterochromatization in this cell type, however, appears to be considerably less extensive than in the mature erythrocyte. Also, greater areas of electron-lucent material extend from nuclear pores well into the nuclear interior; these regions contain many electron-opaque granules that are distinct from the heterochromatin. A nucleolus

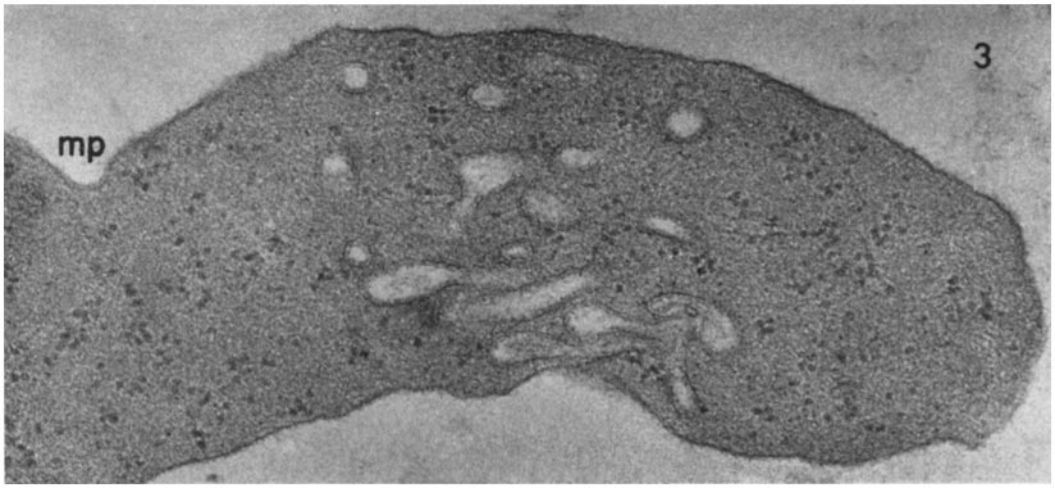


FIGURE 3 Vacuoles, present in the microcytic cell, possessing coated-membrane structure similar to that of the micropinocytotic invagination (*mp*). × 52,000.

of the fibrillar-granular type has also been demonstated.

The two erythroid cell types differ noticeably in the electron opacity of the cytoplasmic matrix. This is especially evident in sections from thyroxin-treated tadpoles that include microcytic and mature erythroid cells in the same field.

DISCUSSION

Only one erythroid cell type, representing the mature tadpole erythrocyte, is found in the peripheral blood of *Rana catesbeiana* tadpoles. Structurally, it is identical to the cell type that constitutes the majority of erythroid cells in thyroxin-treated tadpoles. The existence of occasional free polyribosomes in addition to ribosome clusters on the nuclear membrane and endoplasmic reticulum indicates that these mature erythrocytes retain a very limited capacity for protein synthesis. The presence of a fibrillar-granular nucleolus, mitochondria, and pinocytotic activity suggests continuing synthesis of ribosomes (Hay, 1968) as well as other metabolic functions. Peripheral blood cells of *Rana catesbeiana* tadpoles have been shown to synthesize only hemoglobin characteristic of the larval form (Moss and Ingram, 1965; 1968 *a* and *b*). From the ultrastructural data reported here, it is reasonable to conclude that the equivalent cell type in thyroxin-treated tadpoles contains only this form of hemoglobin, and may continue to synthesize larval hemoglobin at very low levels.

Thyroxin treatment is followed by the appearance of a new erythroid cell type in peripheral blood of tadpoles, very probably identical to the microcytic cell described by DeWitt (1968), which synthesizes adult hemoglobin. It also appears similar to the "crenulated" cell which is present during metamorphosis in other *Rana* species (Holleyfield, 1966).

Details of fine structure indicate that the microcytic cell is capable of high synthetic activity and is a relatively immature erythroid cell. The abundance of polysomes is a most striking feature, and is consistent with the requirements of a cell type that is rapidly producing the new form of hemoglobin. Abundance of cytoplasmic inclusions, increased pinocytotic activity, and lesser heterochromatization are further suggestive of relative immaturity compared to both mature tadpole and adult frog erythrocytes (Vankin, Brandt, and DeWitt, unpublished). Ultrastructural studies on erythropoiesis in the newt spleen (Tooze and Davies, 1967) have indicated that maturation of the newt erythrocyte is also accompanied by a reduction in the number of single ribosomes and polyribosomes, a condensation of the nuclear chromatin, and a reduction in the number of cytoplasmic organelles.

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