Histol Histopathol (1997) 12: 147-170

Histology and Histopathology

Invited Review

The phylogenetic odyssey of the erythrocyte. IV. The amphibians

C.A. Glomski, J. Tamburlin, R. Hard and M. Chainani

State University of New York at Buffalo, Department of Anatomy and Cell Biology, School of Medicine, Buffalo, New York, USA

Summary. Amphibians manifest permanently nucleated, oval, flattened, biconvex erythrocytes. These cells demonstrate a cytoskeleton which is responsible for their morphogenetic conversion from a sphere to an ellipse and imparts to their cellular mass reversibility of traumatic deformation. The class Amphibia has the largest of all erythrocytes attaining volumes greater than 10,000 femtoliters in the *Amphiuma*. The large dimensions reflect evolutionary processes, genomic size, ploidy and the relative size of other somatic cells. Conversely, the erythrocyte count and hemoglobin concentration of these species are low. Occasional denucleated red cells can be seen in the peripheral blood but may attain levels of 90-95% of the total circulating population in certain members of the tribe Bolitoglossini (e.g. *Batrachoseps attenuatus*). These erythroplastids retain the marginal band thus remaining different from mammalian erythrocytes. Embryologically, erythropoiesis initiates in the yolk sac and then progresses to the kidney, liver, and possibly spleen. The yolk sac cohort is transitory and is successively replaced by the larval and definitive populations of erythrocytes. Red cell production (along with thrombocytopoiesis) in adult urodeles is conducted intravascularly in the spleen. In anurans this organ is usually the major site although the liver also serves as a secondary locus for this activity. Medullary (bone marrow) erythropoiesis makes its phylogenetic debut in anurans and typically occurs during heightened hemopoiesis following metamorphosis or hibernation. Maturation of the erythrocyte in the circulation is commonplace (especially in urodeles) while proliferation at this site is inducible by splenectomy and/or hemolysins. Erythrocyte-related values demonstrate variable differences associated with age, weight, season, gender, and environment.

Key words: Erythrocyte, Erythropoiesis, Hemopoiesis, Hemoglobin, Amphibian, Bone marrow

Offprint requests to: Professor Chester A. Glomski, State University of New York at Buffalo, Department of Anatomy and Cell Biology, School of Medicine, Sherman Hall 309, Buffalo, New York 14214, USA

Introduction

Hemoglobin is an unique, ancient respiratory metallo-pigment whose specialized functions are demonstrably enhanced by its micro-environmentalization in a passive-flowing, circulating cell as opposed to free physical solution in the plasma as seen at the invertebrate level (Glomski and Tamburlin, 1989). The degree of its polymerization, association with interactive enzyme systems, and the structure of its globin chains confer upon the compound a spectrum of qualities of oxygen binding/release that can be viewed as tailored for certain stages of life (e.g. embryonic vs. adult) and specific organisms. The emergence of the earliest erythrocytes in certain invertebrates and the phenomenon of erythropoiesis in the lowest vertebrates, the primitive (cartilaginous) and modern (bony) fish have been appraised in subsequent reports of this series (Glomski and Tamburlin, 1990; Glomski et al., 1992). The piscine erythrocyte along with its mode of production can be considered prototypes of this cell and its genesis throughout all other submammalian vertebrates. The establishment of transient and definitive populations of erythrocytes, generation of red cells in a certain group of organs, and the elaboration of erythrocytes with reasonably characteristic dimensions, hemoglobin concentrations, and other cytologic features in cohorts such as families and species of the chondroichthyes and osteichthyes is paralleled at higher phylogenetic levels. Many factors that have an impact upon erythrocellular qualities and values have been identified. The purpose of the present report is to consider erythropoiesis at the next phylogenetic level, i.e. among the members of the class Amphibia. Erythropoietic development in amphibians readily lends itself to the experimental identification and delineation of the precepts that this process observes. Furthermore, it offers an insight into and is somewhat predictive of this activity in higher animals including man.

The Amphibians were the first vertebrates to abandon evolutionarily an exclusively aquatic environment and adopt respiratory mechanisms that permit a terrestro-aquatic existence. Consequently larval

gills are likely to be replaced by lungs in adults and cutaneus respiration is also adopted. Indeed, in some cases lungs are acquired even though gills are permanently retained. Amphibians' erythrocytes express the cytologic characteristics that these cells present in fish. However, they also manifest changes presumably reflective of their hosts' new habitat and activities. Their structural dimensions, number in circulation and other aspects of cellular expression are particularly varied, often novel. As in fish, the earliest erythropoietic activity occurs in the yolk sac followed by internal organs as the kidney, spleen, or liver. Some amphibians further incorporate a new erythropoietic locus, the bone marrow, which has been subsequently universally adopted by the higher vertebrates.

Taxonomic organization

The amphibians are so-named because most members lay their eggs in water which develop into aquatic larvae and subsequently undergo metamorphosis into terrestrial adults. Thus, they live in both aquatic and terrestrial environments. All surviving amphibian species are included in three orders, the Caecilia, Urodela (Caudata or tailed amphibians) and Anura (tailless amphibians). The simplest are the caecilians which are wormlike animals without limbs, limbgirdles or a significant tail. Their body is segmented by circular folds, the skin is smooth and devoid of scales. Caecilians are tropical, fossorial creatures that burrow and live in forest soil and river banks. The sirens (members of the Urodela) conversely, though elongated and somewhat wormlike, have a pectoral girdle with tiny front legs but no hind limbs. They have external gills and a distinct tail. Sirens are exclusively aquatic, have a limited distribution in the USA, and only a few species are extant. Salamanders have both fore- and hindlegs, a distinct neck between the head and long body, and a permanent tail. Salamander larvae in all but rare instances are aquatic, developing in permanent or temporary, and even subterranean waters. Typically, but not necessarily, metamorphosis involves loss of gills and closure of gill slits. The larval stage may be short as a few days or as long as several years. Some species never metamorphose and permanently retain larval characteristics as do *Necturus maculosus* the mud puppy, *Ambystoma mexicanum* the axolotl, and *Cryptobranchus alleganiensis* the hellbender. Such primitive, aquatic urodelans have particularly large erythrocytes. Frogs comprise the majority of the anurans and make up the largest and most widely recognized group of amphibians. They are anatomically characterized by their short tailless body, fore- and hind-limbs, the latter of which are particularly elongated. Most frogs have a free-swimming larval stage termed a tadpole or polliwog which after a growth period undergoes metamorphosis. Toads are similar in structure to frogs, the overt difference is the presence of prominent glands within the skin which give it a «warty» appearance.

Some salamanders replace their lost gills with lungs while others (Plethodontidae) do not. Both types of urodeles nevertheless, along with the (lunged) anurans, utilize cutaneous respiration which is accomplished by extensive capillary beds within the epidermis. Among newts 75% of respiratory exchange is cutaneous while the lungs supply the rest. In lungless salamanders the skin is responsible for an even greater proportion with the remainder being effected by the buccal mucous membranes. Cutaneous respiration accounts for approximately 30% of the oxygen absorption in the aquatic African clawed toad *Xenopus laevis* and 50% in *Rana esculenta*, the edible frog (Foxon, 1964). Cutaneous oxygen consumption in the Andean frog *Telmatobius marmoratus* exceeds its pulmonary uptake by 40-60% (Ruiz et al., 1983). Awareness of this method of oxygenation aids in understanding how some amphibians can repeatedly switch from gaseous to aquatic environments (or vice versa) without difficulty, as well as how hibernation under various conditions becomes tolerable. The efficacy of cutaneous respiration is implied by the observation that some anurans and urodeles completely devoid of erythrocytes and plasma hemoglobin can still maintain their existence (DeGraaf, 1957; Ewer, 1959; Grasso and Sheppard, 1968).

The erythrocytes of an amphibian were first observed by Leeuwenhoek (1683, 1722) when he examined the blood of a frog with his microscope. He noted and illustrated their oval configuration (Fig. 1). The credit of recognizing the shape of the red blood cells throughout the Amphibia, their inordinately huge dimensions in some urodeles and their otherwise large size in the rest of this class, however, belongs to George Gulliver. This investigator, the author of hematology's counterpart of «Gulliver's Travels.» (i.e. his «explorations» in comparative erythrology), described and compared erythrocytes throughout the classes and orders of vertebrates. His 1862 and 1875 reports presented woodcuts than dynamically document the morphology of the amphibians' erythrocytes in context with those of other vertebrates (Fig. 2).

Urodelan erythropoiesis

The permanently larval *Proteus anguineus* (an olm with a native habitat limited to underground caverns in the Balkan peninsula) is a representative primitive

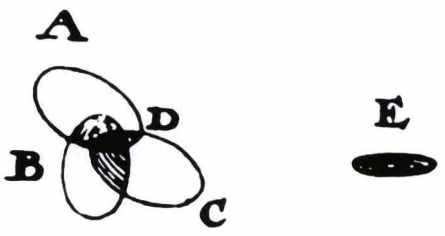

Fig. 1. Anton Van Leeuwenhoek's drawings of frog erythrocytes. Opera Omnia, Seu Arcana Naturae, p 54. Leiden, 1722.

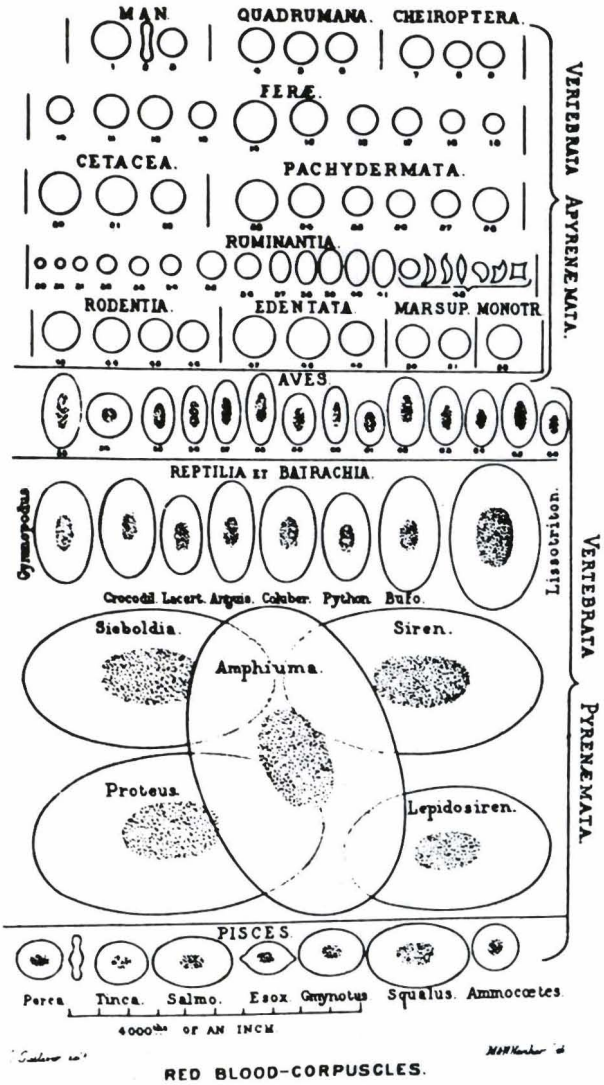

Fig. 2. The classic woodcut of Gulliver (1875) illustrating the sizes and configurations of vertebrate erythrocytes. The huge size of the erythrocytes in the permanently larval, tailed amphibians is illustrated (e.g. *Amphiuma* (*tridactylum*) the Congo eel, *Siren* (*lacertina*) the greater siren, and *Proteus* (*anguineus*) the olm). The *Lepidosiren*, the South American lungfish, though not an amphibian, is noteworthy because it is a representative of the Dipnoi which also manifest similarly large red cells. Note the intermediate dimensions of the erythrocytes in *Bufo* (*bufo*), the common Eurasian toad. *Esox* (lucius), the northern pike, and *Squalus* (*acanthias*), the dogfish shark are included in pisces red cells. Man's erythrocytes are illustrated in the first three drawings (upper left). The first is a surface view, the second is viewed «on edge» while the third figure is a red cell after extraction of its hemoglobin. The terms pyrenaemata and apyrenaemata designate species whose red cells respectively have or do not have nuclei. Erythrocytes are drawn to scale. Each small division in the scale at the base of the drawing represents four thousandths of an inch; resultant magnification in the present photograph is x600. George Gulliver Esq, F.R.S., began his professional career in the Birtish armed forces in 1827. He was appointed assistant surgeon to the royal regiment of horse guards (Blues) circa 1830, Fellow of the Royal Society in 1838, and Hunterian professor of comparative anatomy and physiology to the Royal College of Surgeons in 1861. He regularly authored papers on the comparative morphology of erythrocytes for a period of more than 30 years with his last paper appearing in 1875.

salamander whose erythropoietic status has been historically documented (Jordan, 1932a, Fig. 3). Gulliver's repertoire includes its red cell. Its erythropoiesis, as in most if not all urodeles, is limited to the spleen (Barrett, 1936). The erythroid progenitor, according to Jordan, occurs in two morphologic phases, the hemocytoblast and the lymphocyte. It arises in the spleen, enters the venous sinuses and begins erythroid differentiation. The major extent of maturation plus some mitotic activity is thereupon conducted in the blood. This pattern of erythropoiesis parallels that expressed by some primitive fish e.g. the cyclostomes, in

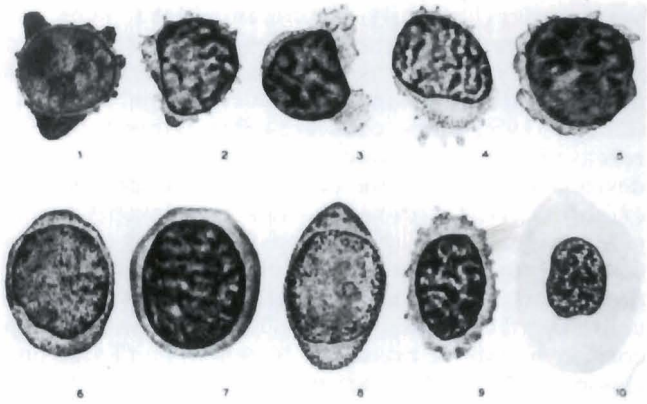

Fig. 3. Development of the erythrocytes in the urodele *Proteus anguineus*. Upper row, first cell: large hemocytoblast followed by three lymphocytes (lymphoid hemoblasts of Jordan). The last cell (5) is a proerythroblast with a nuclear pattern that is characteristic of the large lymphocyte. Lower row: (6) a proerythroblast judged as slightly more mature because of its oval shape even though it does have a hemocytoblastic nucleus. The following two cells (7, 8) are erythroblasts manifesting maturation beyond the proerythroblast stage. The last two cells are a further developed erythroblast (9) and a mature erythrocyte. Blood smears, Wright's stain. x 700. Jordan, 1932a.

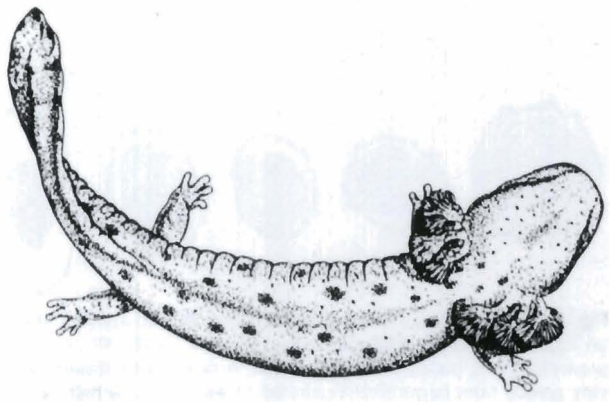

Fig. 4. *Necturus maculosus*, mudpuppy or waterdog. An entirely aquatic, neotenous amphibian with huge erythrocytes. It has 3 pairs of feathery, plumelike gills that are colored maroon due to its circulating red cells. This animal's use in biologic studies is incidently reflected in the observation that it was first found in the Connecticut River in Massachusetts circa 1931 after excess laboratory specimens had been released from Amherst College (Warfel, 1936). C.H. Pope, 1944.

which red cell maturation also occurs in the circulation. The closely related mudpuppy *Necturus maculosus* also leads a solely aquatic existence but has a much wider geographic distribution (much of eastern USA). This species has feathery, red to maroon gills, imparted by its erythrocytes. It is a common specimen in vertebrate anatomy courses and its red cells are often examined because of their magnitude (50x30 μm, Fig. 4, Table 1). Erythropoiesis in the *Necturus* is restricted to the spleen although seasonally or following hemorrhage considerable erythrocellular differentiation may occur in the general circulation (Dawson, 1931, 1932).

Among higher urodeles, specifically the true salamanders (family Salamandridae) such as *Notophthalmus (Triturus) viridescens* the red-spotted newt, and *Cynops pyrrhogaster* the Japanese fire-bellied newt, the spleen is similarly the erythropoietic locus (Ohuye, 1932). The cells are often if not typically released into the blood early and complete their development while in the circulation. The spleens of *T. cristatus*, *N. viridescens*, and presumably other salamanders have a subcapsular outer zone of (erythropoietic) red pulp containing erythroid progenitors, venous sinuses, reticular fibers, reticular cells, and fixed macrophages. The (inner)white pulp consists mainly of masses of lymphocytes (Tooze and Davies, 1968). Other potential sites such as liver or kidneys are not enlisted for red cell generation in adult urodeles. (Among primitive fish intestinal intramural splenic-type tissue or an independent spleen is the typical erythropoietic organ, in modern teleosts such as *Carassius auratus* the goldfish, conversely, the kidney with or without splenic participation assumes this function.) Salamanders demonstrating a largely aquatic existence (i.e. «newts») as *N. viridescens* have the same erythropoietic site(s) as purely terrestrial salamanders, e.g. the woodland, red-backed salamander *Plethodon cinereus* (Jordan, 1932b, 1938). Granulopoiesis in urodeles is conducted in the capsule of the liver and sometimes, especially in the permanently larval species, in the mesonephros (Barrett, 1936). The complete segregation of erythropoiesis and granulopoieisis is characteristically urodelan and does not occur in any other group of vertebrates.

Jordan and Speidel (1930), and Ohuye (1932) studied Wright's stained blood films from splenectomized salamanders; such animals transfer their erythropoiesis to the peripheral blood. Consequently all developmental stages of the erythrocyte are identifiable under ideal conditions (Fig. 5). Jordan and Speidel envisioned erythropoiesis as initiating from a lymphocyte-like cell, the hemocytoblast. It evolves into a committed erythroid precursor, the proerythroblast. The latter is a round cell whose round nucleus occupies almost the entire cell; the cytoplasm is a narrow circumferential band. With maturation, the nucleus contracts, the chromatin coarsens, while the cytoplasm reflecting the accumulation of hemoglobin assumes shades of yellowish brown, orange, or a final reddish yellow. In the last stages of differentiation both the nucleus and cytoplasm become increasingly oblong resulting in an oval cell with a relatively large amount of cytoplasm housing a condensed, central,

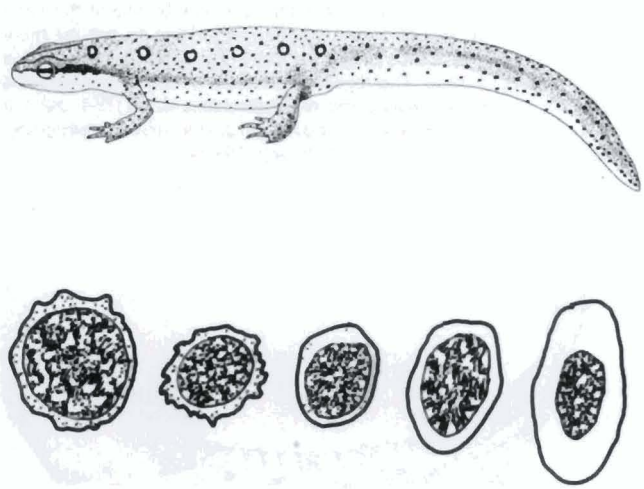

Fig. 5. *Notopthalmus* (*Triturus*) *viridescens*, the red-spotted newt with an illustration of its erythroid differentiation. First cell, left: a large proerythroblast (megaloblast of Jordan, a cell whose dimensions can vary greatly from larger than illustrated to less than one half its present diameter). Interpretations of Grasso's observations (1973a,b) suggest the large «megaloblastic» proerythroblast represents a cell in late interphase or already withdrawn from the cell cycle (G_0 state) while smaller cells at this level of differentiation probably are recently formed daughter cells. The second cell is a smaller proerythroblast while the third and fourth cells are differentiating erythroblasts. The last cell is a mature erythrocyte. Wright-stained blood film 1 year post-splenectomy; modified from Jordan and Speidel, 1930, De Graaf and Rudis, 1983. Amphibians and Reptiles of New England, Univ. MA Press.

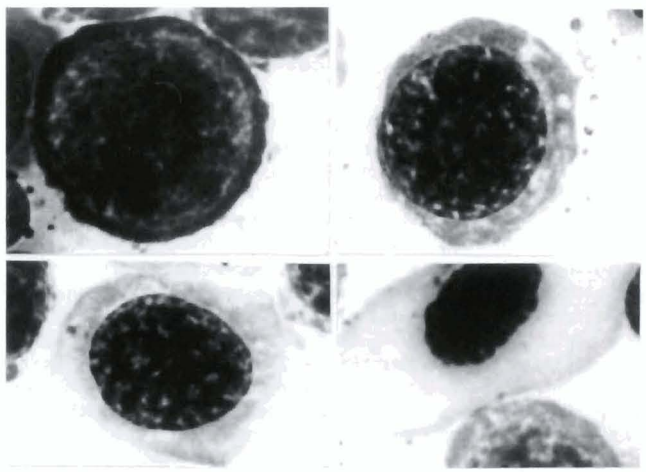

Fig. 6. Erythroid differentiation in the crested newt *Triturus cristatus*. Upper left: a probable hemocytoblast, the nucleus stained purple and the cytoplasm stained blue in the original preparation. Upper right: basophilic erythroblast, the nucleus stained blue at this stage and it demonstrated the checkerboard chromatin pattern that is a hallmark of developing erythroid cells in essentially all species. Lower left: polychromatophilic erythroblast. Lower right: mature erythrocyte. Air dried, MGG-stained films from the spleen. x 1,200. Tooze and Davies, 1967, with permission of J. Cell. Sci.

correspondingly oval nucleus.

More recently, erythropoiesis in the newt (*N. viridescens* and *T. cristatus*) have been reexamined at both bright field and ultramicroscopic levels (Tooze and Davies, 1967; Grasso et al., 1966, 1968, 1973a,b). Air-dried, May-Grünwald-Giemsa stained smears (imprints) of spleens were used by Tooze and Davies to characterize the erythropoiesis of the crested newt (Fig. 6). The hemocytoblast was identified as a «lymphoid» cell (i.e. a round cell with a round nucleus), larger than the usual lymphocyte, having minimal cytoplasm and little or no azure granulation. It manifests a definitely purple nucleus that is fairly homogeneous or somewhat particulate, and has a basophilic (blue) cytoplasm. The investigators considered their candidate progenitor comparable to the hemocytoblast of Jordan. This cell differentiates into the proerythroblast, a cell with a blue-staining nucleus and increased cytoplasmic basophilia. It is the most immature, recognizable cell committed to the erythroid line. The subsequent maturational stages are classified as the basophilic erythroblast (characterized by a smaller nucleus, increasingly defined blocks of chromatin, and deep blue cytoplasm), and the poly-chromatophilic erythroblast (recognizable by its further reduced, sometimes ovoid nucleus, and gray cytoplasm). The latter tinctorial quality is the result of the presence in common of eosinophilic hemoglobin and basophilic RNA. The mature erythrocyte is the next (and last) step in development. Under transmission electron microscopy (TEM) the youngest erythroid precursor in *T. cristatus* that Tooze and Davies could identify with certainty was the counterpart of the basophilic erythroblast of light microscopy (Fig. 7). Under TEM it has a large nucleus with dense packets of chromatin along its periphery, a few that are randomly scattered, and many smaller dispersed aggregates. Large nucleoli are present. The interchromatin areas, historically termed the nuclear sap, are not amorphous at the ultrastructural level but contain numerous osmiophilic, partly RNAase sensitive granules. The nuclear envelope has a double membrane organization and exhibits numerous nuclear pores. The cell's defining cytoplasmic feature is the high density of ribosomes which are packed so tightly that individual ribosomes are indistinguishable. It is responsible for the intense cytoplasmic basophilia under light microscopy. Other features include numerous mitochondria, rare smooth and rough endoplasmic reticulum, centrioles, and Golgi apparatus. The plasma membrane often exhibits pinocytotic projections and invaginations, as seen in developing erythroid cells in other non-mammalian (e.g. ichthyic) and mammalian species. Polychromatophilic erythroblasts are recognized ultrastructurally on the basis of larger masses of heterochromatin and a cytoplasm comprised essentially of homogeneous electron-dense material photometrically identifiable as hemoglobin. Well defined nucleoli persist, as do interchromatin granules, while the nuclear sap has an increased density due to the presence of infiltrated hemoglobin. Dispersed ribosomes and polyribosomes are now identifiable, due at least in part, to an increase in cytoplasmic volume. Lysosomes are identifiable in basophilic and polychromatophilic erythroblasts. They assist in the formation of autophagic vacuoles which degrade effete organelles and promote their exocytosis as also observed in the newt *Pleurodeles waltl* (Sean et al., 1980) and other species. Nearly mature and mature red cells demonstrate diminished numbers or absence of ribosomes, occasional mitochondria, and increased hemoglobinization of the cytoplasm. The presence of hemoglobin in the nucleus of the mature erythrocyte and its continuity with cytoplasmic hemoglobin via the nuclear pores is a pattern common to nucleated red cells. It has been demonstrated per analytical microscopy in the leopard frog *Rana pipiens* as well as the chicken *Gallus domesticus* (Davies, 1961).

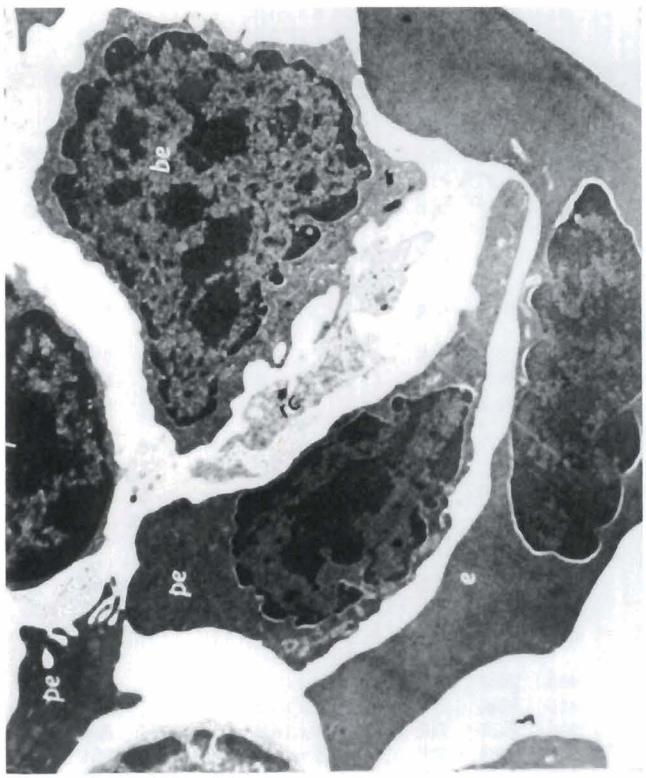

Fig. 7. Developing erythrocytes within a sinus in the spleen of the crested newt *Triturus cristatus*. The somewhat pyramidal shaped cell in the upper left field is a basophilic erythroblast (be). Note the minimal clumping of chromatin and the narrow rim of cytoplasm. The adjacent, lower cell in the middle of the field is a polychromatophilic erythroblast (pe). The large flattened cell along the right margin is a mature erythrocyte (e); its homogeneous electron-dense cytoplasm is a result of its hemoglobin content. The partly included spherical cell in the left margin is a lymphocyte. TEM x 4,000. Tooze and Davies, 1967, with permission of J. Cell. Sci.

Erythrocytic cytoskeleton

Amphibian erythrocytes possess a unique cytoskeleton that is similarly expressed throughout all other

Table 1. Erythrocyte counts and related values in representative amphibians.

	RBC	Hct	Hb	L x W	MCV	MCH	MCHC
Order urodela (Caudata)							
Family Cryptobranchidae, giant salamanders							
Cryptobranchus alleganiensis alleganiensis[16], hellbender	73	43.3	10.1	49.8 x 26.8	5973	1388	23
Cryptobranchus alleganiensis bishopi[16], Ozark hellbender	93	40.1	8.3	43.5 x 24.6	4323	897	21
Family Proteidae, olms							
Necturus maculosus[1], mud puppy or waterdog	20	21.4	4.6	52.8 x 28.2	10070	2160	22
Necturus maculosus[14,18]	-	19	4.5	56.4 x 38.1	-	-	-
Necturus maculosus[8]	28	-	-	40.5 x 28.2	-	-	-
Proteus anguineus[21]	-	-	-	60 x 35	-	-	-
Family Amphiumidae, Congo eels							
Amphiuma means[1], two-toed amphiuma	30	40	9.4	62.5 x 36.3	13857	3287	24
Amphiuma tridactylum[14, 18, 36], three-toed Congo eel	-	23	5.7	65.3 x 36.6	-	-	-
Family Ambystomatidae, mole or North American salamanders							
Ambystoma mexicanum[12], axolotl	-	27.7	7.5	-	-	-	27
Ambystoma maculatum[17], spotted salamander	143	-	-	-	-	-	-
Ambystoma maculatum[8]	53	-	-	37.9 x 23.9	-	-	-
Ambystoma tigrinum[8], tiger salamander	81	-	-	34.5 x 19.0	-	-	-
Ambystoma tigrinum melanosticum[38], Northwestern tiger or blotched tiger salamander	166 (?)	40	9.4	-	2414	567	24
Dicamptodon ensatus[11], Pacific giant salamander	49	24.2	4.4	51.4 x 29.3	4938	880	16
Family Salamandridae, true salamanders, Newt family							
Notophthalmus (Triturus) viridescens[13, 22, 23], red-spotted newt	89	25	4.8	37.8 x 23.8	-	-	19
Triturus cristatus carniflex[24], alpine crested newt	121	33.9	10.8	30.7 x 18.8	2820	892	32
Taricha granulosa[11], rough-skinned newt, West American salamander	110	36.7	9.5	40.4 x 25.9	3336	837	13
Taricha granulosa[19, 20], sea level	95	-	7.5	36 x 20	-	789	-
Taricha granulosa[33] (field 15 °C, spring)	84	-	8.9	-	-	1010	-
Taricha granulosa[33] (laboratory 15 °C, spring)	76	-	7.1	-	-	980	-
Pleurodeles waltl[42], Iberian or Spanish newt	172	39.1	10.5	-	2290	618	27
Family Plethodontidae, lungless salamanders							
Pseudotriton ruber schencki[2]	124	25.7	7.7	-	2073	621	30
Pseudotriton montanus montanus[2], eastern mud salamander	115	24.6	7.2	-	2139	626	29
Plethodon glutinosus[2, 8], slimy salamander	105	23.1	6.8	33.4 x 20.2	2200	648	29
Plethodon cinereus[8], redback salamander	91	-	-	30.7 x 16.3	-	-	-
Desmognathus quadramaculatus[2, 8], black-bellied salamander	152	23.6	6.2	32 x 16.7	1552	408	26
Desmognathus ochrophaeus carolinensis[2, 8], Allegheny Mountain salamander, mountain dusky salamander	145	23.4	7.4	30.7 x 12.7	1613	510	32
Desmognathus monticola[2, 8], seal salamander	133	22.5	7.0	29.4 x 14.8	1691	526	31
Gyrinophilus porphyriticus[2, 8], spring (purple) salamander	100	23.3	6.5	-	2330	650	28
Order Anura (Salientia)							
Family Pipidae							
Xenopus laevis[27], African clawed toad	-	-	-	19.7 x 13.6	1280	-	-
Xenopus laevis[35, 39]	897	43.3	16.4	17.5 x 10.5	483	183	38
Family Ranidae, true frogs							
Rana catesbeiana[1], American bullfrog	440	29.3	7.8	24.8 x 15.4	671	179	27
Rana catesbeiana[41]	312	27.1	6.5	24.6 x 16.5	874	208	24
Rana catesbeiana[28, 14]	329	23.5	5.7	26.1 x 13.5	-	-	-
Rana esculenta[9], edible frog							
prewinter male	480	21.6	8.8	19.8 x 13.7	459	184	41
prewinter female	420	23.6	7.1	20.2 x 13.9	554	169	31
winter male	320	21.8	7.2	25.7 x 14.2	707	246	33
winter female	250	19.8	5.8	24.9 x 14.0	840	251	30
Rana ridibunda[37] male, lake frog (summer)	515	-	9.8	-	-	190	-
Rana ridibunda[37] (winter hibernation)	539	-	11.0	-	-	204	-
Rana tigerina[3, 29] male Indian bullfrog (monsoon season)	1850	34.4	12.5	22.4 x 13.5	186	68	36
Rana tigerina[3, 29] female (monsoon season)	1670	36.4	11.3	22.4 x 13.5	217	68	31
Rana tigerina[34] male 90 grams	750	20.2	12.8	16.9 x 10.5	269	171	63
Rana tigerina[34] female 90 grams	700	22.5	12.4	16.3 x 10.9	321	177	55
Rana cyanophlyctis[3, 29] male Indian skipper frog (monsoon season)	1610	32	7.7	16.4 x 10.0	219	54	24
Rana cyanophlyctis[3, 29] female (monsoon season)	2060	33	7.8	16.4 x 10.0	184	44	24
Rana cyanophlyctis[3] male (winter)	1190	24	7.7	-	204	67	32
Rana cyanophlyctis[3] female (winter)	1320	20	9.6	-	169	82	48
Rana breviceps[3] male (monsoon season)	1250	27.4	9.5	-	219	76	35

Rana breviceps[3] female (monsoon season)	1470	26.9	8.3	-	182	56	31
Rana pipiens[4], leopard frog	319	24.7	6.8	23.8 x 16.2	773	212	27
Rana pipiens[41]	341	22	6.6	21.6 x 13.8	653	192	29
Rana temporaria[23, 26], European common brown or grass frog	408	-	-	20.0 x 13	-	-	-
Family Bufonidae, true toads							
Bufo cognatus[5], great plains toad	520	25.8	10.6	-	497	204	41
Bufo melanostictus[15, 29] male, common Indian or black spined toad	690	32.1	11.0	18.4 x 10.2	465	159	34
Bufo melanostictus[30, 40] male wt. 50 g	1220	20.0	13.6	16.6 x 14.2	168	112	69
Bufo melanostictus[30, 40] female wt 50 g	850	14.4	10.6	17.7 x 14.2	169	125	74
Bufo melanostictus[31] male wt. 50 g	730	38	15.0	-	516	204	39
Bufo melanostictus[31] female wt. 64 g	511	24.9	9.3	-	487	183	38
Bufo spinulosus[6], low altitude	707	38.5	10.4	18.5 x 13.2	544	148	27
Bufo spinulosus[6], high altitude	768	34.6	9.8	16.8 x 12.5	451	129	28
Bufo americanus[17], Eastern American toad	658	-	-	-	-	-	-
Bufo viridis[37] male, green toad (summer)	523	-	10.4	-	-	199	-
Bufo viridis[37] male, (winter hibernation)	587	-	10.9	-	-	186	-
Family Leptodactylidae, neotropical frogs							
Telmatobius marmoratus[7]	795	35.5	11.2	17.6 x 13.0	448	144	31
Telmatobius halli[7]	892	34.3	9.8	16.1 x 11.5	390	119	29
Telmatobius pefauri[7]	560	19.3	7.6	15.4 x 11.5	338	115	32
Telmatobius peruvianus[7]	602	26.9	9.5	17.4 x 12.8	446	158	35
Telmatobius culeus[32], Lake Titicaca frog	729	27.9	8.1	-	394	110	28
Family Hylidae, tree frogs							
Hyla versicolor[17, 25], gray tree frog	615	-	-	21.7 x 14.6	-	-	-
Hyla chrysoscelis[25]	-	-	-	18.7 x 13.1	-	-	-
Hyla arborea[27]	-	-	-	15.1 x 10.6	1390	-	-
Order Caecilia (Apoda, Gymniophona)							
Family Typhlonectidae							
Typhlonectes compressicaudus[10]	-	37.6	11.3	50 x 12.5	-	-	-
Family Caeciliidae							
Boulengerula (Afrocaecilia)taitanus[11]	680	40	10.3	22.1 x 15.6	588	151	26

RBC: erythrocytes thousand/mm^3 or µl; Hct: hematocrit; Hb: g hemoglobin/deciliter; L x W: length x width (µm); MCV: mean cellular volume (µm^3 or femtoliters, fl); MCH: mean cellular hemoglobin (pg); MCHC: mean cellular hemoglobin concentration (wt/vol%, g Hb/100 ml rbc). [1]: Wintrobe, 1933; [2]: Reynolds et al., 1973;[3]: Samantaray, 1984, 1985; [4]: Rouf, 1969; [5]: Paulson et al., 1987; [6]: Ruiz et al., 1989; [7]: Ruiz et al., 1983; [8] Vernberg, 1955a; [9]: Sinha, 1983; [10]: Toews et al., 1978; [11]: Wood et al., 1975; [12]: Gahlenbeck et al., 1970; [13]: Pitkin, 1983; [14]: Lenfant et al., 1967; [15]: Biswas et al., 1985; [16]: Jerrett et al., 1973; [17]: Hutchinson et al., 1965; [18]: Hartman et al., 1964; [19]: Friedmann, 1971; [20]: Friedmann, 1970; [21] Jordan, 1932a; [22]: Grasso and Shephard, 1968; [23]: Jordan, 1938; [24]: Frangioni et al., 1987; [25]: Matson, 1990; [26]: Andrew, 1965; [27]: Goniakowska, 1970 (dimensions per measurements in suspension, volume calculated as elliptic cylinder not per Wintrobe extrapulation); [28]: Kuramoto, 1981; [29]: Pai et al., 1988; [30]: Banerjee, 1988; [31]: Choubey et al., 1986; [32]: Hutchinson et al., 1976; [33]: Friedmann, 1974; [34]: Mishra et al., 1983; [35]: Hadji-Azimi et al., 1987; [36]: Gulliver, 1875; [37]: Zhukova et al., 1979; [38]: Roofe, 1961; [39]: Merkle, 1989; [40]: Banerjee, 1983; [41]: Kaloustian et al., 1982; [42]: Deparis et al., 1975. Some erythrocyte indices have been calculated from reported data per Wintrobe formulas.

classes of submammalians. It made its initial appearance in some invertebrates' erythrocytes (e.g. the arcid clam *Noetia ponderosa*) and has persisted in the fish through the avians. The erythrocytic cytoskeleton consists of three major components, a marginal band (MB), the membrane skeleton (formerly termed the cell surface-associated cytoskeleton), and intermediate filaments (IF) of the vimentin class (Cohen, 1978, 1982, 1991; Cohen et al., 1982; Fig. 8). The MB is a bundle of continuous subplasmalemmal microtubules that encircle the cell like a belt in the plane parallel to the flat surface of the cell. The membrane skeleton (MS), in turn, is an interwoven network of nonmammalian spectrin, actin, ankyrin, and band proteins 3 and 4.1 which completely encloses and is in close contact with the marginal band. It lines the inner surface of the entire plasmalemma consequently enveloping the nucleus. The IFs are thought to connect the nucleus to the surrounding MS with some fibers bypassing the nucleus to link the opposing inner cell surfaces.

The marginal band begins to appear early in erythroblastic development and persists throughout the cell's life span (Grasso, 1973b). It is the ultrastructural cause for the development of single and double pointed erythrocytes which can consistently be seen in populations of developing amphibian red cells (Twersky et al., 1995). These cells reflect the presence of incompletely developed, pointed marginal bands in intermediate stage, immature erythroid cells. The thickness of the marginal band i.e. the number of microtubules in cross section parallel the size of the cell. A linear correlation between the length of a red cell and the logarithm of the number of microtubules per MB has been demonstrated by Goniakowska-Witalinska and Witalinski (1974, 1976). *Rana pipiens* has a marginal band composed of 24 microtubules in its ~22 µm long red cells, the alpine newt *Triturus alpestris* with 38 x 22 µm rbc exhibits 90 microtubules in its MB, while the larger cells of *Salamandra salamandra* the largest, most widely recognized salamander in Europe (42 x 27 µm)

have 200. The giant cells of the Congo eel *Amphiuma tridactylum* (65 x 36 μm) have hundreds of microtubules in their >1.0 μm thick MB (Cohen, 1978).

The cytoskeleton is responsible for several critical aspects of erythrocellular development and function. The MB acts as an internal frame supporting the cell surface from within. It has the properties of flexibility, resistance to bending, and capacity to return to equilibrium shape. It thus helps sustain a red cell's geometry and also enables it to return to original shape following deformation due to collisions etc. while in circulation. The membrane skeleton imparts the quality of elasticity to the erythrocytic membrane. This fibrous reticulum is normally under tension and applies forces asymmetrically across the MB. It is responsible for the erythroblast's maturational conversion from a sphere to an ellipse and for its flattened form, a streamline configuration that enhances cell flow in plasma. Even the human erythrocyte, a biconcave disc under static conditions, is reversibly converted to a (rheologically improved) ellipse under conditions of large vessel flow. Furthermore in the human erythrocellular defect, hereditary elliptocytosis, normoblasts in the bone marrow are geometrically normal (i.e. spherical). However, when their derived erythrocytes are released into the circulation they assume an ellipsoidal form as a result of appropriate conformation to large vessel flow but are unable to return to the normal discoidal shape when the forces are rescinded because of an inherent defect in the cell membrane. This is illustrative of a probable principle that the ellipsoid is the design of choice for erythrocytic transportive flow.

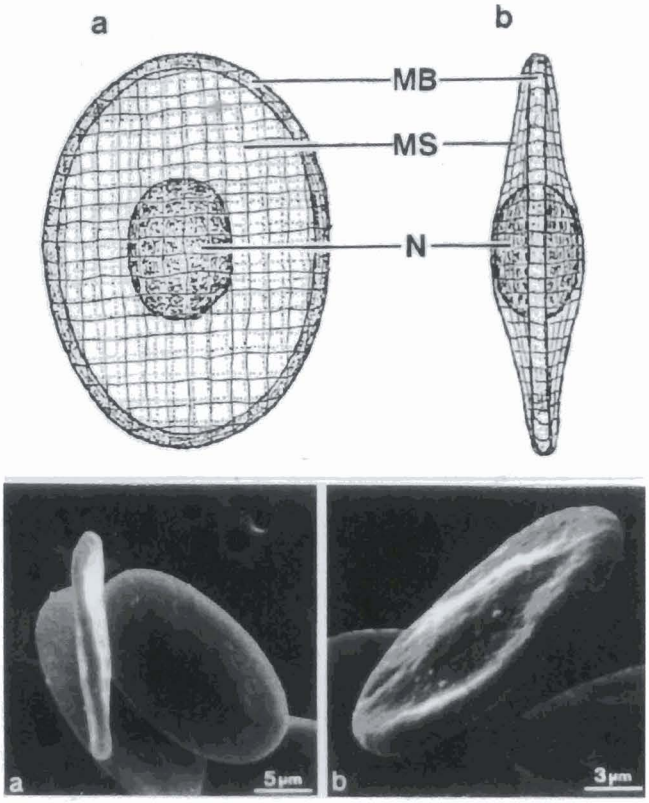

Fig. 8. Upper: Diagramatic model of the nucleated erythrocyte cytoskeleton. The face (a) and edge views (b) illustrate the marginal band (MB) and the membrane skeleton (MS). The membrane skeleton is in close contact with and completely encloses the marginal band. It holds the MB under asymmetrical tension causing the erythrocyte to assume an ellipsoidal configuration. The presence of a prominent nucleus (N) results in a central bulge and the biconvex profile typical of amphibian red cells. The intermediate filaments (the third component of the cytoskeleton) which are believed to connect the inner surface of the plasmalemma to the nucleus or to the opposing inner cell surface are not included in the drawings. Joseph-Silverstein and Cohen, J. Cell Biol, 1984, vol. 98, p 2124 by copyright permission of the Rockefeller University Press. Lower: Scanning electron micrographs, edge (a) and oblique views (b) of erythrocytes from the frog *Rana pipiens*. The nuclear bulge, biconvex configuration and oval discoid form of the cell are identifiable. Cohen W.D., Int. Rev. Cytol. vol. 130, 1991 with permission of Academic Press.

The amphibian cytoskeleton is present in the numerous denucleated erythrocytes of the California slender salamander *Batrachoseps attenuatus*, *B. major* (and predictably, some other members of the tribe Bolitoglossini) (Cohen, 1982; Villolobos et al., 1988). These lungless salamanders are exceptional in that they commonly have up to 95% anucleate red cells in circulation, with both nucleated and denucleated cohorts demonstrating the MB and membrane skeleton. The denucleated cells arise perhaps by amitotic division of the nucleated erythrocytes, as initially proposed by Emmel (1924) and subsequently supported by Cohen (1982). The presence of this ultrastructural organization forms the basis of the tenet that these unusual anucleate amphibian red cells are not the equivalent of mammalian denucleated erythrocytes because the latter (with the possible exception of the Camelidae) do not manifest a marginal band at any time during adult erythromorphogenesis.

Erythropoiesis post-splenectomy

The identification of the precursor of the proerythroblast has been addressed in the newt *T. cristatus* by exploitation of the cited fact that when splenectomized, its erythropoiesis shifts to the circulating blood. Further, when such newts are administered acetylphenylhydrazine they sustain a transient loss of all circulating erythrocytes (Grasso, 1973a,b). At 11-14 days post-administration of the hemolysin numerous primitive cells appear in the blood and comprise 50-60% of the cell population (the remainder are granulocytes, thrombocytes, lymphocytes, but not erythrocytes). Their diameter in stained smears is 30-45 μm, their nuclei are round/oval, large, leptochromatic (thin chromatin strands) and possess nucleoli. The cytoplasm is fragile and exhibits minimal to moderate basophilia. Under TEM they have exceedingly open, delicate nuclei and initially the cytoplasm is devoid of organelles (Fig. 9). With time the complement of ribosomes increases and an amorphous cytoplasmic material also appears

suggesting a synthesis of hemoglobin. Metabolic studies imply that these primitive cells collectively termed erythroid precursor cells (EPC), proliferate in the blood and are engaged in RNA, heme and protein synthesis (Grasso, 1973a,b). By 15-18 days they are replaced, having differentiated into basophilic and polychromatophilic erythroblasts (Fig. 9). Studies indicate that the synthesis of ribosomal (r)RNA and messenger (m)RNA occurs during the interval from the primitive erythroid precursor to the early polychromatophilic erythroblast, at which point production ceases. The ultimate erythroid precursor (source of the EPC) likely a cell with lymphocytic morphology, remains to be definitively identified.

Splenectomy with or without acetylphenylhydrazine does not induce hepatic or renal erythropoiesis in the salamander (Ohuye, 1932; Grasso, 1973b) while hepatectomy does not result in splenic granulopoiesis (Jordan, 1938). The sharp demarcation between a urodele's erythropoietic and granulopoietic tissues is thus maintained even under severe hemopoietic stress.

Exposure of unoperated warty newts *Triturus cristatus carniflex* (collected in Florentine Italy) to

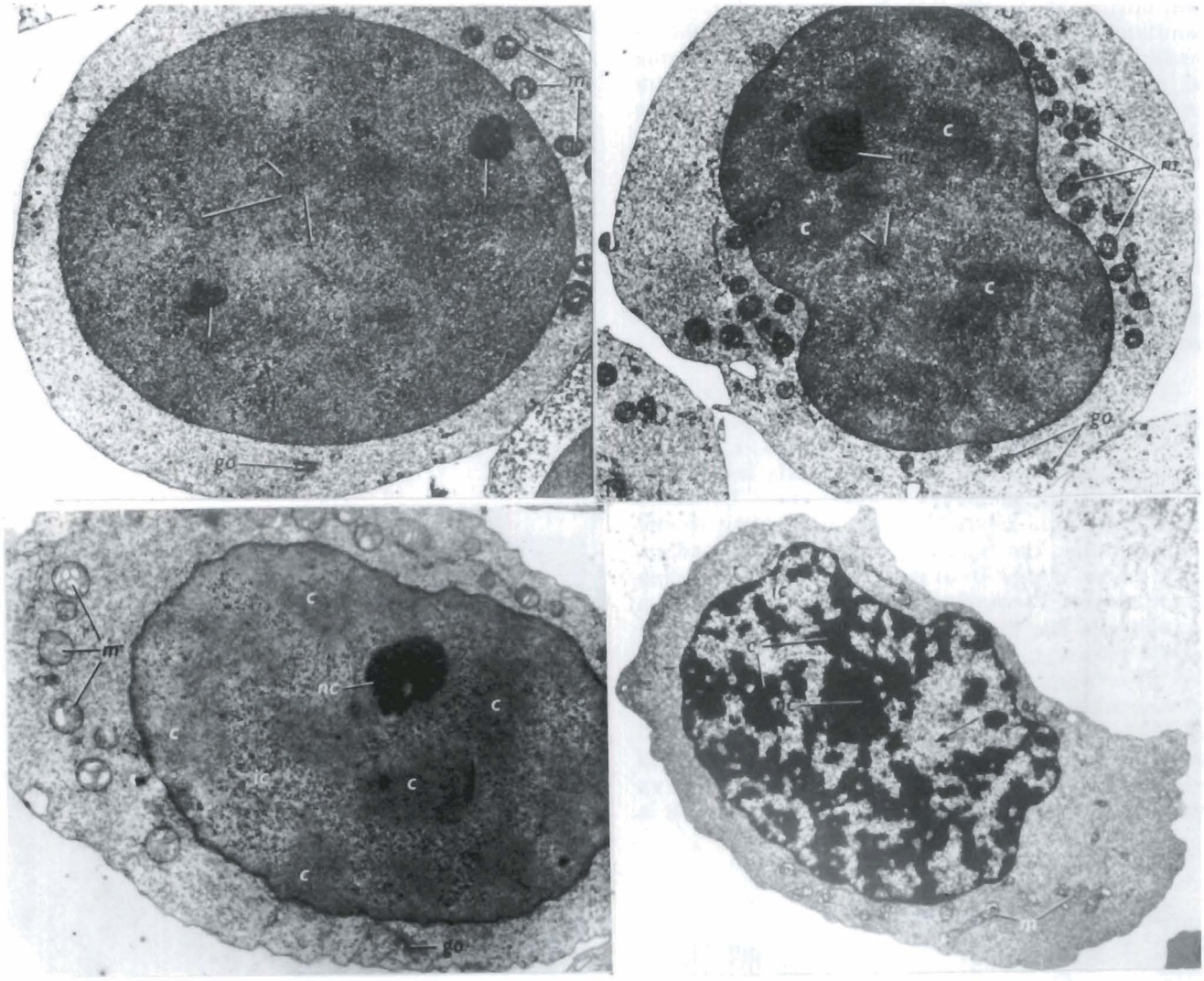

Fig. 9. Erythroid cells in the circulation of splenectomized crested newts *Triturus cristatus* after administration of acetylphenylhydrazine. Upper left: an erythroid precursor cell (EPC). This precursor of the proerythroblast has a large prominent nucleus with very delicate granular chromatin. RNAase-sensitive interchromatin granules (poorly resolved) are indicated by 3 bars in the center of the nucleus; two prominent nucleoli are also indicated. Several mitochondria are identifiable. TEM. x 6,500. Upper right: Another EPC. This cell has blocks of chromatin (c) that are more distinct than those in the prior cell. A prominent nucleolus and an area containing poorly resolved interchromatin granules are indicated by bars. An aggregation of mitochondria (m) is present. The cytoplasm contains flocculent material of low density. TEM. x 5,300. Lower left: Basophilic erythroblast. Chromatin blocks (c), interchromatin areas (ic) containing a prominent nuclear granular component, and a large electron-dense nucleolus (nc) are readily apparent. The cytoplasm contains a small Golgi complex (go) and ribosomes. The production and accumulation of ribosomes is a major morphologic indicator of a basophilic erythroblast. TEM. x 6,800. Lower right: early polychromatophilic erythroblast. The clumps of chromatin (c) are much more condensed and electron-dense than in the previous less differentiated cells. The cytoplasm is moderately dense, believed to be due to the accumulation of hemoglobin. TEM. x 3,500. Grasso, 1973a,b; with permission of J. Cell Sci.

acetylphenylhydrazine and a 5 month follow-up reveals that erythropoiesis is discontinuous and rhythmic in this and presumably other salamanders (Frangioni and Borgioli, 1987a,b). The drug synchronized the erythropoiesis in this cohort of newts and revealed that the length of each proliferative cycle is about 4-5 weeks and that the life span of the red cells is 50-60 days. Mitotic indices of splenic erythroid cells documented periods of intense proliferation followed by reciprocal intervals of quiescence. These episodes correlated with the periodic entry of erythroblasts into the blood. Certain Bowman's renal corpuscular cells became hypertrophied and granulated. The latter also enlarge in response to elevated levels of lactic acid (a concomitant of anemia and hypoxia) and are a proposed site of erythropoietin synthesis. A particular point illustrated by this experiment is that normal oscillatory erythropoietic activity is technically difficult to demonstrate in a random population of unorchestrated animals. Drug-induced synchronization unmasks this rhythmicity. Urodelan erythropoiesis apparently differs from that of mammals among whom the process is continuous.

Medullary lymphogranulopoiesis and erythro-thrombocytopoiesis

Of evolutionary significance is the observation that although medullary (bone marrow) erythropoiesis does not occur in urodeles, (and forerunners, i.e. fish), lymphogranulopoiesis is phylogenetically initiated at this site in some members of this group. Urodeles manifesting this characteristic all belong to the Plethodontidae, the family of lungless salamanders, among whom at least 10 of its 20 odd genera including *Demognathus*, *Plethodon* and *Hydromantes* have a granulocytic and lymphoid but not erythropoietic marrow (Barrett, 1936, 1947; Curtis et al., 1979). Well developed, active bone marrow is found in *Desmognathus fuscus* (Fig. 10), *Plethodon glutinosus* and *Plethodon cinereus*, the dusky, slimy and red-backed salamanders respectively. This activity is identifiable in the larval forms including the permanent larva *Typhlomolge rathbuni* the Texas blind salamander. It is usually demonstrable in all bones large enough to have a medullary cavity e.g. pelvic girdle, vertebrae, skull and long bones. Myeloid bone marrow (erythropoietic and granulopoietic), however, makes its debut in the Anura.

Erythropoiesis in urodeles is accompanied by thrombocytopoiesis. In a survey of representatives from all urodelan families except the Hynobiidae (Asian land salamanders), the spleen was found to be the sole erythropoietic and thrombocytopoietic organ (Barrett, 1936). Both processes are conducted intravascularly in the same areas of the spleen, and following splenectomy, generation of both cell lines is transferred to the blood. Erythropoiesis and megakaryopoiesis (the mammalian equivalent of thrombocytopoiesis) have demonstrable linkages e.g., both erythrocytic and megakaryocytic *in vitro* cultures are stimulated by erythropoietin while mammalian thrombopoietin similarly promotes cellular proliferation of these cell lines. Further, in mammals the two cells may share the same immediate progenitor. If a unique relationship between erythro- and thrombocyto-poiesis does exist, it appears that it first became histologically evident in the amphibians.

Caecilian erythropoiesis

Caecilian erythrocytes conform to the morphology presented by the other amphibians. Erythroblasts, immature and occasional mitotic erythroid cells are found in the circulation. *Typhlonectes compressicaudus*

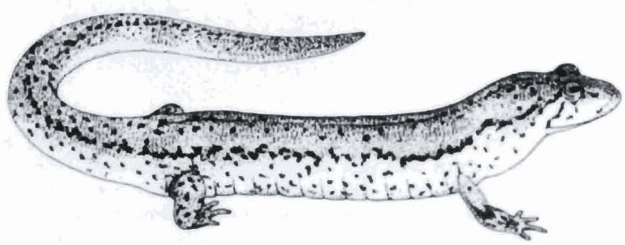

Fig. 10. *Desmognathus fuscus*, northern dusky salamander. A typical lungless salamander (Plethodontidae) among whom intramedullary hemopoiesis is first initiated. The marrow, however, is lympho-granulopoietic and not erythropoietic. DeGraaf R. and Rudis D., 1983, Amphibians and Reptiles of New England, Univ. MA Press.

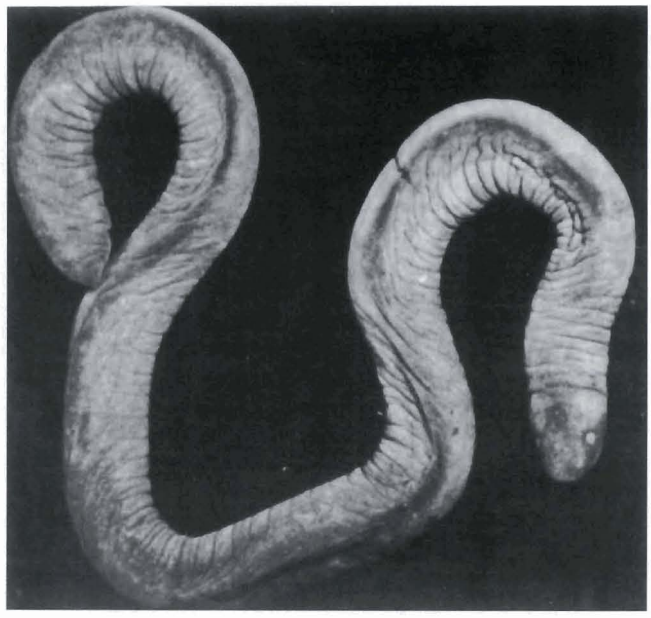

Fig. 11. *Typhlonectes compressicaudus*. An aquatic, air-breathing, fresh water caecilian; length of specimen: 328 mm. This is one of the few caecilians whose erythrocytic profile including red blood cell indices have been established. The wormlike, limbless, segmented organization of this animal is readily observed. Taylor E, 1968, The Caecilians of the World, Univ. Kansas Press.

an Amazonian, aquatic caecilian has huge erythrocytes that often demonstrate small clusters of azure cytoplasmic granules (presumed lysosomes) (Toews and MacIntyre, 1978; Boschini Filho, 1979, cited by Turner, 1988) (Fig. 11). Their dimensions (50 x 12.5 μm) approach that of primitive, aquatic urodelan rbc. The red cells of the terrestrial *Ichthyophis kohtaoensis* (native to Koh Tao Island, gulf of Thailand) are not as large, ~30 x 12 μm, while those of *Boulengerula (Afrocaecilia) taitanus*, also a terrestrial species (indigenous to the Teita Hills of Kenya, East Africa) are smaller, 22.1 x 15.6 μm (Wood et al., 1975; Zapata et al., 1982). Caecilian erythropoiesis has the same organ distribution as in the urodeles. In *I. kohtaoensis* it is restricted to the spleen, the liver is granulopoietic, and the marrow is inactive (Zapata et al., 1982). The blood volume of *Typhlonectes compressicaudus* is noteworthy in that it represents 25% of its body weight. This is in contradistinction to the usual much lower values e.g. *Rana pipiens* (9%) and man (7%).

Anuran erythropoiesis

Erythroid generation in anurans initiates, as in other amphibians, from the hemocytoblast or its presumed variant, a pluripotential lymphocyte. The maturation of erythroid precursors in the common European toad *Bufo (vulgaris) bufo* and African aquatic toad *Xenopus laevis* have been illustrated by Jordan (1938) and Hadji-Azimi et al. (1987) respectively (Fig. 12). Erythroblasts are identifiable in anuran circulating blood e.g., the leopard frog *R. pipiens*, the Egyptian *R. mascareniensis* as well as the *Bufo (vulgaris) bufo*, *B. viridis* and *B. regularis*. The progressive increase in cell size and change in shape (round to oval) of the circulating developing erythroid cells have been documented in the latter two species (Khalil and Elfeky, 1986a). Erythroplastids are a common incidental finding in anurans. The spleen is the chief erythropoietic organ of most anurans and in contrast to the urodeles, this organ is also likely to serve as a locus for granulopoiesis (Jordan, 1938). Erythrocytic development (along with thrombocytopoiesis) occurs within the splenic sinuses while granulopoiesis is extravascular. The liver (subcapsular region) frequently also selves as a site for erythropoiesis (and granulopoiesis) (Schermer, 1967; Turner, 1988). In addition, it is among the anurans that erythrocellular production first appears in bone marrow albeit on a variable basis i.e. typically following metamorphosis and hibernation. Thus in *Rana esculenta*, *R. pipiens*, and the tree frog *Hyla arborea* erythropoiesis is conducted in the spleen, liver (to a lesser extent) and, during the metamorphic and spring awakening periods, in the bone marrow (Foxon, 1964) (Fig. 13). The bone marrow and kidneys may also become erythropoietic after splenectomy. Rarely, the marrow is proposed as the major, possibly discontinuous erythropoietic locus (e.g., *R. temporaria* the European brown or grass frog, *R. catesbeiana* the

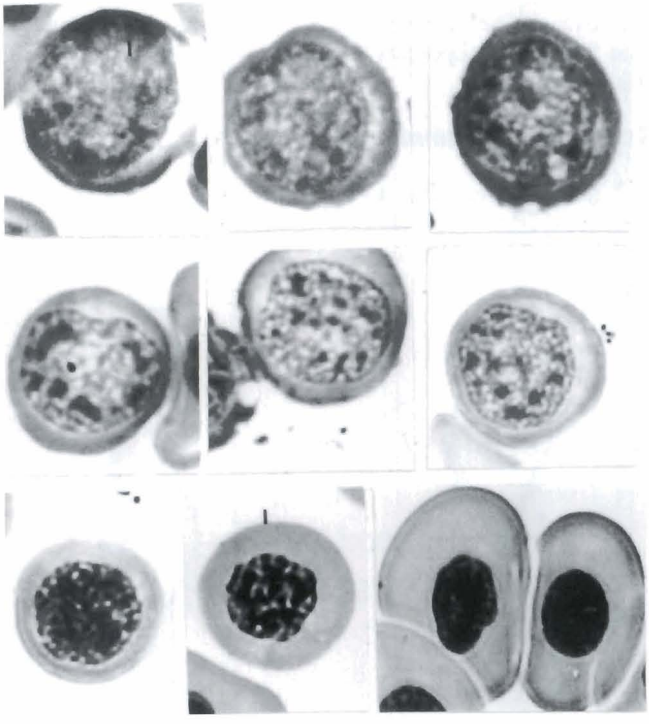

Fig. 12. Development of erythrocytes in *Xenopus laevis*. Top row: first two cells, proerythroblasts; third cell, erythroblast I. Second row: first cell, erythroblast I; second and third cells, erythroblast II. Third row: young erythrocyte I, young erythrocyte II, and mature erythrocytes respectively. From imprints of liver (subcapsular zone) except mature erythrocytes which were observed in blood smears. May-Grunewald-Giemsa stain. x 1,650. Reprinted with permission Dev. Comp. Immunol. 11, 1987. Hadji-Azimi et al., Atlas of adult *Xenopus laevis laevis* hematology, Pergamon Press.

Fig. 13. *Rana pipiens*, leopard frog. One of the more highly hematologically-investigated anurans. Erythropoietic bone marrow is first phylogenetically observed in anurans such as this species. Noteworthy is the fact that this occurs only during periods of heightened erythropoietic activity during spring or following metamorphosis. Otherwise the spleen, as in urodeles, is the primary center for red cell production. DeGraaf R. and Rudis D., 1983, Amphibians and Reptiles of New England, Univ. MA Press.

bullfrog) (Foxon, 1964; Maniatis and Ingram, 1971). The spleen and bone marrow are erythropoietic in *Bufo bufo*, the Egyptian green toad *B. viridis*, and *B. regularis* the panther toad, the most common and widespread toad in Africa (Andrew, 1965; Khalil and Elfeky, 1986b). Conversely, the bone marrow in *Xenopus laevis* does not assume this function (Thomas and Maclean, 1974; Hadji-Azimi et al., 1987). Histologic examination of its femoral marrow (young and old toads of both sexes) reveals juxtaendosteal, paravenous granulopoiesis but no erythropoiesis (Tanaka, 1976).

The administration of phenylhydrazine to *X. laevis* results in transitory anemia and temporary loss of all erythrocytes. As in the newt, the recovery is a circulatory phenomenon. Light and ultramicroscopy of the spleen and liver reveal phagocytosis of the damaged rbc. These organs also release a single burst of basophilic erythroblasts which multiply and mature in circulation to restore the original population (Thomas and MacLean, 1974, 1975; Chegini et al., 1979). The bone marrow remains erythropoietically uninvolved.

Erythropoiesis in bone marrow

The histology of erythropoietic (red) bone marrow in *R. pipiens* was described by Jordan (1919) while its ultrastructure during an erythropoietically inactive but granulopoietic period has been detailed by Campbell (1970). For a baseline comparison, the hemopoietically inactive marrow of the Japanese newt *Cynops (Diemictylus) pyrrhogaster* is grossly yellow, crowded with adipose cells and without prominent vascularization or a central vein (Ohuye, 1932; Tanaka, 1976). Following splenectomy, this newt's marrow develops an increased blood supply but still does not initiate red cell production. Erythropoietic bone marrow, alternatively, demonstrates proliferation of erythroid and leukocytic cells, along with a persistence of fat cells (Jordan, 1919). An artery enters the femoral medullary cavity at the mid shaft and divides into two vessels that run in opposite directions along the axis of the bone. These yield branches that terminate as a venous sinusoidal network that drains in *R. catesbeiana* into a central thin walled venous channel as well as into veins paralleling the endosteum (Tanaka, 1976). Under TEM the sinuses are seen to be lined by single flattened cells with an elongate nucleus; cytoplasmic organelles such as rough ER and free ribosomes are present. These cells are non-phagocytic, devoid of a basal lamina, joined to one another by tight junctions and may exhibit pores containing traversing granulocytes. Developing erythrocytes (and thrombocytes) are seen only within the sinuses while leukocytes arise and differentiate in intersinusoidal spaces. Additionally, as noted in the lymphogranulopoietic marrow of *Plethodon glutinosus* the slimy salamander, pinocytotic vesicles are abundant in the sinusoidal endothelium and gaps are identifiable between some adjacent cells (Curtis et al., 1979).

The phylogenetic onset of medullary erythropoiesis in the frog is a hematologic landmark. Foxon (1964) suggests that marrow erythropoiesis is a functional expression and not an evolutionary mechanism. Other views propose that the marrow was «selected» because bone offers protective shielding from ionizing radiation for the radiosensitive hemopoietic stem cells (Cooper et al., 1980; Zapata et al., 1995). Organisms (fish) inhabiting an aquatic medium would not require this osseous insulation since they benefit from the shielding effects of water. The absence of hemopoietic marrow in anuran (aquatic) larvae is cited as consistent with this hypothesis. Indirect support is likewise derived from the contemporary depletion of stratospheric ozone which has led to increased tropospheric ultraviolet-B radiation and resultant lethal effects upon embryos of wild, UV-B sensitive species such as *Bufo boreas* the western toad and *Rana cascadae* the cascades frog (Blaustein et al., 1994).

The emergence of a lympho-hemopoietic bone marrow is a late phylogenetic acquisition. The importance of its stromal cells (fibroblastic cells, adventitial cells, endothelium and other cells) in establishing the microenvironment required for hemopoiesis has been cited by Zapata et al. (1995). In lower forms that are marrow-less or have an inactive marrow the stromal components of certain organs (e.g. kidney) are believed to elaborate a microenvironment that fosters hemopoiesis (and mimics that of active bone marrow).

Embryonic and larval erythropoiesis

The first red cells to appear in amphibians arise in the blood island, a V-shaped, midline plate of splanchnic mesoderm (ventral blood island mesoderm, ventral

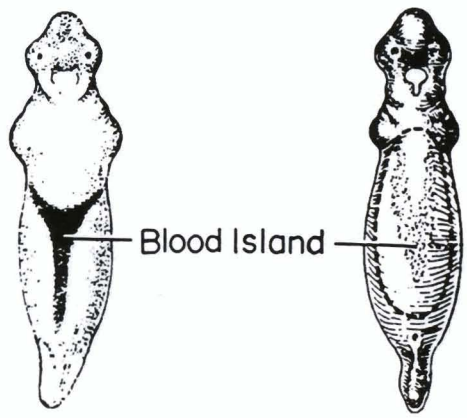

Fig. 14. Amphibian embryos demonstrating the blood island. Left: *Ambystoma* (*Amblystoma*) *mexicanum*, axolotl, Harrison stage 31. The embryo was subjected to the benzidine reaction thus localizing the blood island, Slonimski, 1931. Right: *Ambystoma punctatum* , (*Amblystoma maculatum*) Harrison stage 32, 28 somites. The stippled area represents the blood island; the dotted line indicates that area surgically excised by Goss, 1928. The V shape of this structure is identifiable in both embryos; its outline can frequently be seen with the naked eye.

aspect of lateral plate mesoderm) located ventral to the yolk mass extending from the blastopore to either side of the liver primordium (Figs. 14-16). The blood island is recognizable *in situ* before heartbeat by the benzidine reaction for hemoglobin (Slonimski, 1931). The erythroid progenitors are committed prior to their morphologic differentiation and can be cultured following explanation from the gastrula.

The first analyses of the amphibian blood island were performed by Federici (1926) and Goss (1928) who extirpated blood islands from *Rana temporaria* and *Ambystoma maculatum* embryos before the development of the circulation. The larvae which developed following this procedure had fewer or an absence of erythrocytes in circulation, thus demonstrating that blood islands are the earliest source of erythrocytes. Other species verifying this origin include *Hyla regilla*, *Rana japonica*, *R. pipiens* and *B. bufo* (Fernwald, 1943; Hollyfield, 1966; Turpen et al., 1979, 1981). These first generation red cells are impermanent and do not persist throughout larval life. Their early disappearance has been cited in *Ambystoma jeffersonianum* the Jefferson salamander and *R. pipiens* (Cameron, 1941; Turpen et al., 1979, 1981). The half life of the latter ranid's red cells is ~40 days; they disappear from circulation by larval age 70 days.

The second site of erythropoiesis is the kidneys as seen in *R. esculenta*, *R. temporaria*, *R. catesbeiana*, *R. pipiens* and *R. japonica* larvae (Hollyfield, 1966). The question of the source of the precursors of these blood cells was addressed by Hollyfield who reciprocally exchanged blood islands between 2n and 3n *R. pipiens* embryos before erythrocytic development and demonstrated that rbc circulating in older, mid-larval amphibians arise from cells other than those in the blood island. A similar reciprocal exchange of pro- or mesonephric anlagen revealed that graft ploidy red cells comprised the blood of mid-larval tadpoles. That is, they originated from the transplanted non-blood island tissue.

These observations have led to the formulation of the concept that the erythrocytes originating in the amphibian blood island are not only the first red cells to develop but that they also undergo programmed cell death (Zon, 1995). Yolk-associated erythrocytes first appear in fish and are produced by all higher species. The persistence of this ontogenetic recapitulation is underscored by the fact that human primitive generation, yolk sac rbc have a marginal band (signifying their equivalence to submammalian red cells), even though the definitive series does not include this organelle in its development (van Deurs and Behnke, 1973). Blood island rbc exhibit morphology, kinetics and hemoglobins other than the adult's cells. Recently, it has been surprisingly discovered that a minor cohort of red cells in the blood of mature *X. laevis* have blood island cell characteristics (Bechtold et al., 1992). These studies were performed on transplantation-derived chimeras and employed analysis of the DNA content of circulating cells. The possibility of an erythropoietic model whereby a second (non-primitive) population of cells migrates from the yolk sac to contribute to the definitive erythroid pool has also been proposed (Zon, 1995).

A study of cytogenetically labeled, grafted mesoderm in *R. pipiens* (grafted 67 and 72 hr, Shumway embryonic stages 15-16) and analyzed at 15-41 days (Taylor and Kollros larval stages III-IX) has implicated lateral plate (dorsal anterior) mesoderm in the region of the presumptive mesonephros as the definitive source of hemopoietic stem cells which colonize the blood-forming organs (Shumway, 1940; Taylor and Kollros, 1946; Turpen et al., 1981). Donor, dorsal anterior mesoderm-derived hemopoietic cells were identifiable in the pronephros, mesonephros, spleen, and blood. The

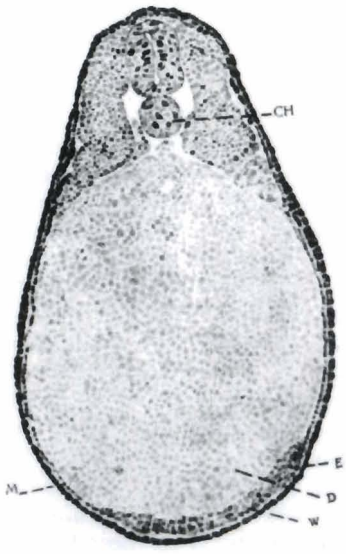

Fig. 15. A transverse section of *Ambystoma mexicanum*, axolotl embryo, Harrison stage 32 illustrating the yolk mass (yolk-filled endoderm cells) (D), mesoderm (M), blood island (W), surface ectoderm (E) and notochord (CH). Slonimski, 1931.

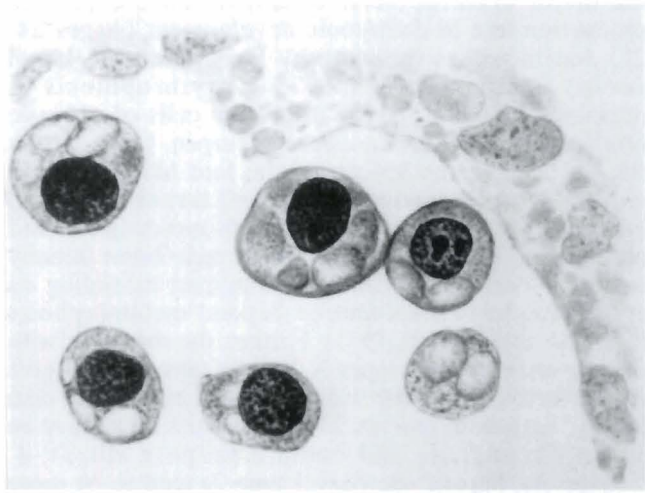

Fig. 16. Yolk sac generation erythroblasts in a section of *Ambystoma mexicanum*, axolotl embryo demonstrating a positive benzidine reaction in both the cytoplasm and nuclei. The cells continue to demonstrate yolk vacuoles. Slonimski, 1931.

hemopoietic precursor cells are initially located posterior to the pronephros (stage 14, 62 hr) and migrate interstitially (stages 15-17) to a more anterior location (Turpen and Knudson, 1982). A suggestion of hematopoietic cell differentiation is observed at embryonic stage 19 (120 hr) when a group of cells appear to arise from the dorsal hypomere clustered around the developing aorta. (The hypomere is the most ventral subdivision of the mesoderm, it gives rise to the splanchnopleure and somatopleure). By stage 20 such cells can be seen both outside and inside the dorsal aorta and within the pronephric sinus. Theoretically, they are available for vascular distribution. Cultures of *Xenopus laevis* lateral plate mesoderm with or without concomitant presence of dorsal lateral plate mesoderm reveal that not only is the former tissue (blood island) the sole source of this species' early erythrocytes but also that its activity is regulated by factors produced by dorsal mesoderm (Kau and Turpen, 1983; Turpen and Smith, 1985). In addition as in other anurans, the latter is also the source of hemopoietic stem cells which populate its mesonephros. This organ, in turn, is the reservoir of erythropoietic (and other hemic) progenitors of definitive hemopoiesis.

Cytocentrifugal harvesting of cells associated with the pronephroi and aortae of stage 20 leopard frog embryos has permitted the identification of candidate pluripotential hemopoietic stem cells (Turpen and Knudson, 1982). They are large, 35-40 μm in diameter, have an eccentric, leptochromatic nucleus and a prominent nucleolus. The cytoplasm contains pigment inclusions and numerous yolk granules imparting to it a foamy, voluminous, macrophage-like appearance.

The pronephros becomes hemopoietically active in *R. pipiens* at Shumway stage ~25 and is predominantly granulopoietic; at Taylor-Kollros larval stages I-VI <10% of its hemopoietic cells are erythroid. The liver, the major erythropoietic organ, initiates erythroid production late in embryonic development (stages 24-25) and increases this activity throughout the larval period (Turpen et al., 1979). Erythropoiesis is intrasinusoidal and derived from stem cells of extrinsic origin (dorsal anterior mesoderm) (Turpen, 1980). A few rbc develop in the spleen of the late tadpole. In *R. catesbeiana* (American bullfrog) larval erythroid production varies inversely between mesonephric and hepatic loci. The kidney is the major contributor initially but later the liver becomes predominant extending its erythroamplificative function beyond metamorphosis (Maniatis and Ingram, 1971). Further, the rbc differ with the site of origin. At stages X-XII the mesonephroi give rise to larval type 1 rbc which are oval and longer than hepatic rbc (mean 26.9 vs. 23.5 μm). The former have an eccentric nucleus and contain tadpole Hb Td-4. Conversely, hepatic rbc (larval type 2) tend to be more round, are centrally nucleated and present hemoglobins Td-1, -2, -3 (Broyles, 1981; Broyles et al., 1981a,b). Erythropoiesis in *R. temporaria* exhibits a parallel ontogenetic sequence (Foxon, 1964). In early tadpoles the kidney houses all hemopoiesis while in older larvae the liver assumes erythro-thrombocytic production.

Larval erythrocytes whose morphology differs form definitive rbc are also seen in *R. pipiens*, *X. laevis*, and *Bufo melanostictus*. In some instances, their nuclei are round, the chromatin is less clumped, the cytoplasm may have increased basophilia, and the cells incorporate labeled metabolites - indicators of synthetic activity. The production of different hemoglobins during embryonic/larval and adult periods has been retained throughout phylogeny from fish through man. The axolotl a neotenous urodele, switches from larval to adult hemoglobin even without metamorphosis (Ducibella, 1974). The hematologic data deemed demonstrative of an ancestral relationship between the coelacanth *Latimeria chalumnae* and the tetrapods rests on the molecular similarities of this ancient fish's and *R. catesbeiana* larval hemoglobins (Gorr et al., 1991, 1993). Of potential evolutionary relevance is the fact that erythropoietin, the major regulator of erythropoiesis in man, is synthesized in the kidney and to a lesser extent in the liver, the major sites of erythropoiesis in the larval frog.

During metamorphic climax hepatic erythropoiesis in *R. catesbeiana* switches from larval to adult Hb production (Okazaki et al., 1982), while its renal erythroid production presumably declines and ceases. The liver retains its primacy as the erythrosynthetic locus in froglets. Larval to adult hemoglobin «switching» in an amphibian was first described in *R. catesbeiana*; it develops four larval and four adult hemoglobins (Turner, 1988). Induction of anemia in adult *R. catesbeiana*, *R. pipiens* and *X. laevis* can result in partial reversion to larval hemoglobin synthesis.

In review, the amphibians generate three different erythrocytic populations. The first is the blood island generation which is comparable to the human yolk sac-derived cohort. The second is the larval renal-hepatic group with «tadpole» hemoglobin(s) (akin to the human fetal hemoglobin), and finally the definitive erythrocyte with adult hemoglobin(s).

Denucleated erythrocytes

Occasional denucleated erythrocytes are observed in the peripheral blood of amphibians. The erythroplastids have been noted in species as *Proteus anguineus*, *Plethodon cinereus*, *Cynops pyrrhogaster*, *R. pipiens*, *Notophthalmus viridescens*, *Taricha granulosa* the rough-skinned newt, and *Desmognathus fuscus* (Emmel, 1924; Ohuye, 1932; Jordan, 1938; Friedmann, 1970). The incidence rarely exceeds 1-3% of the circulating rbc. The most unusual expression of this characteristic is obtained in a few Bolitoglossini, a tribe of lungless salamanders. Among these select species 80-95% of the circulating erythrocytes may be anucleate. Originally *Batrachoseps attenuatus*, *B. pacificus*, and *B. major* were the sole amphibians recognized to have almost exclusively denucleated erythrocytes in their blood

(Eisen, 1897; Emmel, 1924; Duran-Jorda, 1951; Cohen, 1982) (Fig. 17). Other bolitoglossine genera with some members presenting this hemic picture have since been identified (*Oedipina*, *Nototriton*, *Thorius*, and *Bolitoglossa*) (Villolobos et al., 1988). The denucleated cells are elliptical due to the persistence of the cytoskeleton, sometimes round, varied in size, and at times pointed at one end (because of a damaged marginal band) (Cohen, 1982). The variability in size of the erythroplastids may reflect nuclear extrusion that is less efficient in amphibians than it is in mammals. These notable lungless salamanders have in common a diminutive physique being either proportionately miniaturized (e.g., the South American *Bolitoglossa rufescens*) or extremely attenuated with small limbs (e.g., *B. attenuatus* and *Oedipina uniformis*) (Fig. 18). Not all salamanders presenting these physical features, however, have numerous denucleated rbc, e.g., *Batrachoseps campi* which has a standard blood picture.

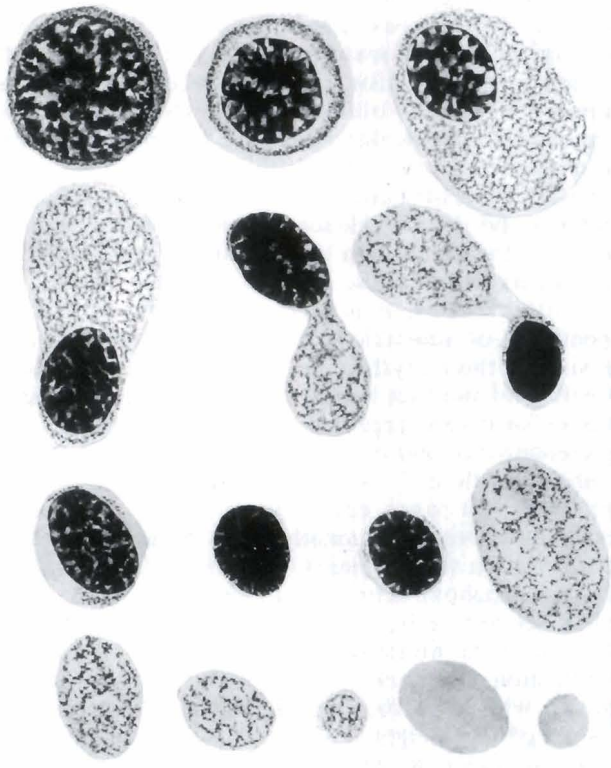

Fig. 17. Development of erythroplastids (denucleated erythrocytes) in the peripheral blood of *Batrachoseps attenuatus* the slender salamander as interpreted by Emmel (1924). The series begins with a young erythroblast and an older, still circular erythroblast. The next four cells are erythrocytes revealing eccentric nuclei, some of which also demonstrate «cytoplasmic segmentation» of Emmel. The third row illustrates three erythroid nuclei with or without a small rim of cytoplasm, and one erythroplastid. The remaining structures (bottom row) are erythroplastids of varying dimensions and shapes. The cells are stained with brilliant cresyl blue and counterstained with Wright's stain. The cytoplasmic precipitate documents the presence of ribonucleoprotein. The last two erythroplastids are older forms devoid of this material.

B. attenuatus larvae like their mature counterparts and unlike the usual larvae have predominantly anucleate rbc in their blood (Emmel, 1921, 1924). It has yet to be established whether other erythroplastid-rich species have comparable rbc during their larval period. It is recognized that the nucleus, because of its own oxygen requirements diminishes the complement of an erythrocyte's tissue-available oxygen. Thus oxygen transport by erythroplastids offers advantages in respiratory economy that are more than a reduction in cell size and expulsion of an inactive, space occupying mass. The extrusion of the erythroid nucleus, seen occasionally in fish and more commonly in amphibians (as well as some oceanic light fish as *Maurolicus mülleri* which have only anucleate rbc) can be viewed as a portent of erythrocellular changes to appear at higher evolutionary levels.

Amphibian erythrocytes: their dimensions

The amphibians possess, as demonstrated by Gulliver (1873, 1875; Fig. 2), Alder and Huber (1923), Wintrobe's indices (1933), and other workers, the largest red cells found in vertebrates. The very largest are produced by primitive, aquatic, permanently larval urodeles, notably *Amphiuma means* whose red cells are visible to the naked eye (Gulliver, 1875), have dimensions of 63 x 36 μm, a volume of ~14,000 fl (femtoliters or μm^3), and are about 150 times as large as man's rbc. Other inordinately large rbc are seen in the urodelans *Proteus anguineus* (60 x 35 μm) and *Siren lacertina* (56 x 32 μm) (Fig. 2). Erythrocytes with slightly lesser diameters and a 5,000-10,000 fl volume include those generated by *Necturus maculosus* the mud puppy, *Cryptobranchus alleganiensis* the hellbender and *Dicamptodon ensatus* the Pacific giant salamander (Table 1). The latter is an atypical member of this group because it resides in both aquatic and terrestrial environments. *T. compressicaudus* an Amazonian caecilian has red cells this size. The lateral profile of such erythrocytes is also impressive. The Congo eel's rbc have a thickness of 5 μm along the flange and a 16

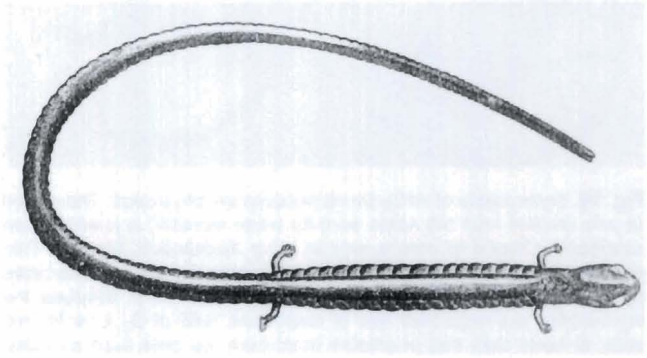

Fig. 18. *Oedipina uniformis*. This species demonstrates the body structure that is typical of the lungless salamanders that manifest large numbers of denucleated erythrocytes in the circulation, i.e. a markedly attenuated body with miniaturized limbs.

μm height in the nuclear region; the comparable values for the hellbender's cells are 2 μm and 7 μm. Goniakowska (1970) reports that the red cells in *Ambystoma mexicanum* the neotenous axolotl (~38 x 28 μm, 7100 fl, Fig. 19) and *S. salamandra* the European fire salamander (~42 x 27 μm, 7800 fl) have central thicknesses of 8.4 and 8.9 μm respectively. The amphibians with somewhat smaller erythrocytes are generally the more modern urodeles, i.e. the mole, true, and lungless salamanders (Ambystomatidae, Salamandridae, Plethodontidae). Their rbc are ~30-40 μm long, ~15-25 μm wide and usually have cellular volumes of 1500-3000 fl. Typical species are *Ambystoma tigrinum* the tiger salamander, *Taricha granulosa* the rough-skinned newt, and *Desmognathus quadra-maculatus* the black-bellied salamander (Table 1).

Anurans have the smallest cells among the amphibians which nevertheless surpass in dimensions those from avians and mammals. They generally have red cells with long and short diameters of 15-25 μm and 12-15 μm. Their mean cellular volumes exhibit a broad range, some attain levels exceeding 1500 fl (*X. laevis, R. temporaria* and *B. bufo*; Goniakowska, 1970) while most others are 300-700 fl (e.g. *R. catesbeiana* and *Bufo cognatus* the great plains toad) (Table 1). Direct measurement of rbc in suspension from *X. laevis*, *Hyla arborea*, R. *esculenta*, *R. temporaria* and *B. bufo* reveals a central thickness of ~6 μm for these cells (Goniakowska, 1970).

It is generally promulgated that primitive species have larger erythrocytes. As a group, however, fish have smaller red cells than amphibians. While cartilaginous fish can be expected to manifest larger erythrocytes than the modern teleosts, the former class' cells are still smaller than those generated by primitive and many modern urodeles (Glomski et al., 1992). For example *Squalus acanthias* the dogfish shark, a well known chondroichthian, and the teleost *Esox lucius* the northern pike produce rbc with corpuscular volumes of 900 and 170 fl respectively (Fig. 2). The «small» red cells of anurans are also likely to be bigger than those of modern fish. Thus although the evolutionary significance is not fully understood, amphibians have larger erythrocytes than fish and the dimensions of these hemic cells progressively diminish thereafter among the higher classes. This relationship is effectively visualized in Gulliver's woodcut (Fig. 2).

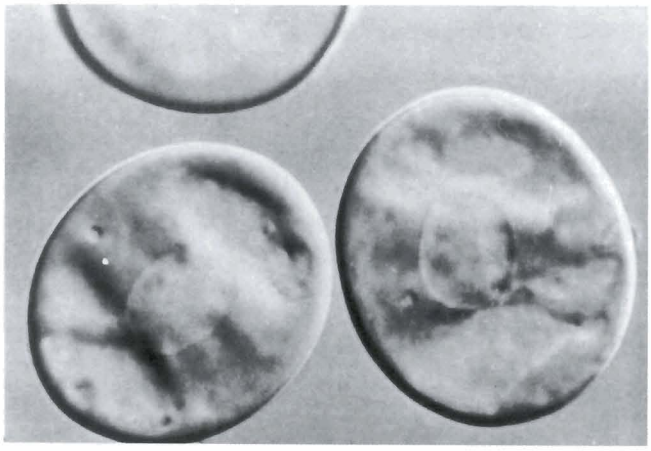

Fig. 19. Erythrocytes of *Ambystoma mexicanum*, the axolotl. The axolotl (a term derived from the Aztec word for water monster) is a well known salamander found in nature only in Lake Xochimilco, Mexico. This species is a neotenous salamander with large erythrocytes approximately 38 x 28 x 8 µm, cellular volume 7100 fl. Because the erythrocytes are large their rate of respiration, 0.65 µl O_2 x 10^{-3}/hr/mg cells, is lower than that of smaller amphibian red cells such as those from *Rana temporaria* 1861 fl, 1.6 µl O_2x10^{-3}/hr/mg cells and *Hyla arborea*, 1390 fl, 2.3 µl O_2 x 10^{-3}/hr/mg cells (Goniakowska, 1970). Differential interference contrast microscopy, x 1,800. Photomicrograph courtesy of Dr. F. de La Tour Du Pin; Bessis, M., The Life cycle of the Erythrocyte, Sandoz, 1966.

In evolution the early vertebrates probably invaded brackish water first, and later, fresh waters (Szarski, 1968). As a result, their body cells including erythrocytes increased in size, partly in response to osmotic differences while cellular metabolism concomitantly diminished because of reduced cell surface to mass ratios. The enhanced cell size is proposed to have evolutionarily persisted (and even increased) in species that retained a low metabolic rate and/or sustained environmentally-related fluctuations in osmotic concentrations of their body fluids (e.g. amphibia and dipnoi). Hence the amphibians mainifest large erythrocytes on the basis of recognized evolutionary possibilities.

Smith (1925) demonstrated that the basal energy expenditure of amphibians is inversely proportional to the size of their erythrocytes. This relationship is a reflection of the fact that the amphibians particularly urodeles have very large somatic cells, that red cells are representative of and possess the same amount of DNA as other somatic cells as well as being of corresponding size, and that large cells exhibit lower levels of metabolic activity than smaller cells because of lower surface area to volume ratios (and related factors). As a result the metabolic activity of an amphibian is low, as is that of its red cells, a condition which is inversely reflected in the dimensions of these cells. In Smith's investigation *Amphiuma means* and *Necturus maculosus* (species with huge rbc) had the lowest production of CO_2 per g/body weight while the concurrently evaluated *R. catesbeiana*, *R. pipiens*, and *Bufo americanus* demonstrated progressively higher CO_2 production rates and correspondingly smaller erythrocytes. The quantitation of oxygen consumption by isolated urodelan and anuran red cells (e.g. *S. salamandra* < *A. mexicanum* < *R. temporaria* < *B. bufo* < *H. arborea*) verifies the inverse proportionality of red cell size and oxygen utilization (Goniakowska, 1970, Fig. 19). With evolutionary progression individual red cell volume tends to decrease with the smallest rbc being found in high energy-consuming, homeothermic species.

An illustration of the tenet that a relationship in size

usually exists among an animal's various cells (Szarski, 1968) is seen in the dimensions of the leukocytes that accompany the erythrocytes in an amphibians's blood. Circulating rbc in *Cryptobranchus a. alleganiensis* or its variant *C. a. bishopi* are representative of primitive urodeles, ~45 μm long; their neutrophils are equally ponderous, 35 μm in diameter (Jerrett and Mays, 1973). Man with his «mammalian size» rbc has granulocytes one half to one third as large.

The size of an amphibian's erythrocytes is also correlated with its genome i.e. the quantity of its nuclear DNA (Olmo, 1973; Szarski, 1976; Olmo and Morescalchi, 1978). Indeed, Kuramoto (1981) has proposed that a rbc's surface area might be usable as a simple indicator of genomic size. In his study of Asian urodeles and anurans a significant positive concordance was demonstrable between the magnitude of the nucleus and the dimensions of the erythrocyte, as well as the size of either the nucleus or cell and its genomic quantity. The amount of DNA per erythrocyte predictably increases with the degree of evolutionary advancement of a species but also with the degree of preservation of its gene sequences. The amphibians have retained a large genomic constituency of repeated DNA sequences («genetically void» DNA), accounting in some respects for the large amount of DNA in some amphibians' red cells and their resultant huge size. A high degree of variability in the amount of nuclear DNA is also a characteristic of amphibians and distinguishes them from most other tetrapods (Mazin, 1980). While the teleostean and avian red cells have 1-7 pg DNA per nucleus, various caecilians have 7-28 pg, Plethodontidae (lungless salamanders) exhibit 20-72 pg and Ambystomatidae (mole salamanders) express 44-105 pg DNA in their nuclei. *Amphiuma* and *Necturus* have the remarkably high 190 pg DNA/nucleus. Anurans have smaller erythrocytes than the urodeles and tend to maintain an appropriate 5-15 pg DNA in their nuclei.

A relationship between the genome and the size of the red cell is also evident in hybrids. *Ambystoma jeffersonianum* frequently crosses in nature with *Ambystoma laterale* the blue-spotted salamander generating triploid offspring termed *A. temblayi* and *A. platineum* the silvery salamander (Uzzell, 1964; Freytag, 1974; Fig. 20). The mean surface area of erythrocytes from these four species, respectively, are 720, 710, 1040 and 1090 μm^2 (Uzzell, 1964). Another hybrid complex resulting from cross fertilization of *A. laterale* and *A. texanum* the smallmouth salamander yields diploids, triploids, and tetraploids with erythrocytes of increasing dimensions (723, 921 and 1127 μm^2 surface area) (Licht and Bogart, 1987). Comparable findings are obtained in anurans. *Rana esculenta* a hybrid of Europe's *Rana lessonae* the little waterfrog or pool frog and *R. ridibunda* the marsh frog, occurs in diploid and triploid forms with red cells having mean lengths of 25 and 31 μm (Blommers-Schlösser, 1990). The look-alike gray tree frogs (diploid) *Hyla chrysoscelis* and (tetraploid) *Hyla versicolor* are separable by vocal calls and also by red cell dimensions which are specific enough to yield a 93% accuracy in wild, Ohio species identification (Matson, 1990).

Polyploid Iberian newts *Pleurodeles waltl* reveal a homeostatic maintenance of their normal quantitative erythrocellular relationships (Deparis et al., 1975). Thus, the increased red cell volumes associated with 3n and 4n genomes are accompanied not only with a proportionately reduced erythrocyte count but also with a normal range Hct, Hb and MCHC. This accommodation loses some precision in pentaploids.

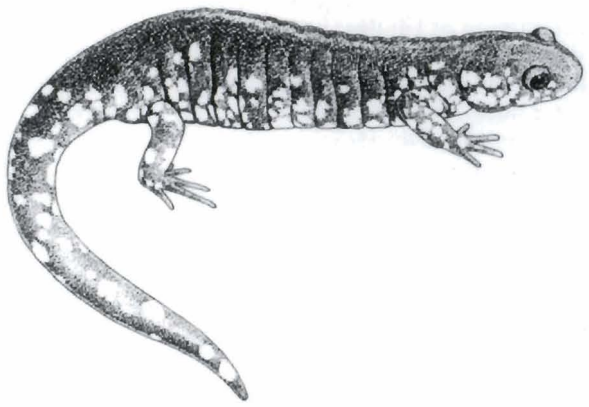

Fig. 20. *Ambystoma laterale*, blue-spotted salamander. This species crosses with *Ambystoma jeffersonianum* the Jefferson salamander, as well as with *Ambystoma texanum* the smallmouth salamander, to yield polyploid offspring, whose erythrocytes have dimensions greater than those of diploid specimens. The phenomenon of polyploidy and the resultant generation of erythrocytes with enhanced cell volumes, is a common phenomenon in both anurans and urodeles. De Graaf R. and Rudis D., 1983, Amphibians and Reptiles of New England, Univ. MA Press.

Erythrocyte counts

Erythrocyte counts in the amphibians are generally the lowest for all vertebrates. It is also an accepted precept that red cell counts in amphibians and other species tend to be inversely correlated with the volume (size) of the erythrocytes (Wintrobe, 1933). That is, the numbers of erythrocytes per unit volume of blood increase as animal groups manifest smaller red cells. While the commonly accorded ranges for erythrocyte counts in fish and mammals are 500,000-2,500,000 and 5.0-12.0 million cells/mm^3 of blood respectively, the range for urodeles, in contrast, is ~25,000-200,000 rbc/mm^3 and that for frogs and toads is ~500,000-1,5000,000 rbc/mm^3 (Table 1). Amphibian red cell counts demonstrate marked intra- and interspecific variation, perhaps a reflection of the less precise regulatory feedback mechanisms of amphibians compared with those of homeothermic vertebrates (Hutchison and Szarski, 1965). The extreme lowest red cell counts are expressed by the Proteidae (e.g. *Necturus*) and the Amphiumidae (e.g. *Amphiuma*) which have only about 30,000 cells in a cubic millimeter

(microliter) of blood. A modest 70,000-90,000 rbc/μl is obtained in *Cryptobranchus alleganiensis* (Table 1). In analyses which were conducted either «during the breeding season» (Kuramoto, 1981) or during June/July (Hutchison and Szarski, 1965), the counts for salamanders belonging to the families Hynobiidae (Asian land salamanders), Salamandridae and Ambystomatidae ranged from 100,000-200,000 cells/mm^3 (Table 1). The broad range of counts obtained from a given species, even within the constraints of a single investigation is illustrated by differences of 100,000 cells/mm^3 between the high and low red cell counts observed in *T. granulosa* (mean 111,000 mm^3) and in *Ambystoma maculatum* the spotted salamander (mean 143,000/mm^3) (Hutchison and Szarski, 1965, Fig. 21). Low counts were also derived for the latter urodele (53,000/mm^3) in another experiment (Vernberg, 1955a). The Plethodontidae, in the preponderance of reported data, also have mean erythrocyte counts of approximately 100,000-200,000/mm^3, e.g. *Plethodon glutinosus* the slimy salamander and *Gyrinophilus porphyriticus* the purple salamander each with 100,000 rbc/mm^3 (Vernberg, 1955a; Reynolds and Pickard, 1973; Davic and Gallati, 1979; Table 1). The extended ranges in plethodontid rbc counts also led Reynolds and Pickard to conclude that a marked intrapopulation variation to be the norm. In a comparison of the profiles of *Plethodon cinereus* and *Plethodon glutinosus* the redback and slimy salamanders (Vernberg, 1955b), the latter urodele exhibited a lower erythrocyte count (56,000 vs. 91,000 cells/mm^3), correspondingly larger red cells, a lower level of activity, and a lower consumption of oxygen per unit body weight. This is consistent with the concept that larger rbc are likely to be found in subjects having lower metabolic rates (as well as lower numbers of red cells/mm^3 blood).

The erythrocyte counts of anurans are almost universally much higher than those of urodeles. The lowest levels of circulating erythrocytes are in the order of 200,000 cells/mm^3 (e.g. *Bombina orientalis* 184,000 cells/mm^3) while most commonly the counts are considerably higher but less than one million/mm^3 (e.g. *R. pipiens* ~320,000 and *Bufo americanus* the eastern American toad 660,000) (Table 1). The Indian bullfrog *Rana tigerina* and *R. cyanophylctis* the skipper frog have red cell counts in excess of one million/mm^3 (as well as small rbc, 200 fl) (Table 1).

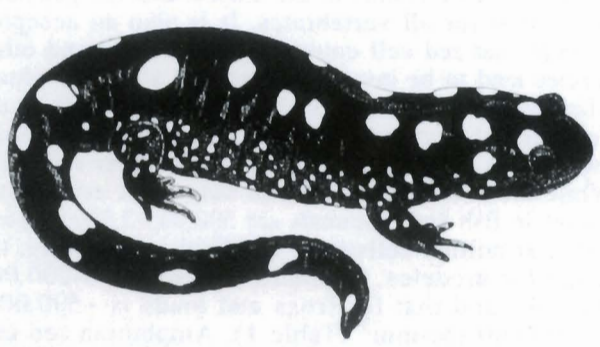

Fig. 21. *Ambystoma maculatum*, spotted salamander. A recognized characteristic of amphibians' erythrocyte counts is their marked inter- and intraspecies variation. This mole salamander (salamanders that spend the greater part of the year burrowed in the earth) exhibits this erythrocellular profile. In the derivation of its mean rbc count yielding ~150,000 cells/mm^3 blood, differences of 100,000 cells between the high and low counts have been noted. DeGraaf R. and Rudis D., 1983, Amphibians and Reptiles of New England, Univ. MA Press.

Hemoglobin, hematocrit, red cell indices

Hemoglobin levels in most amphibians have a range of 5-10 g/deciliter (dl, 100 ml) of blood, the general limits expressed by most fish but significantly lower than 12-18 g/dl, the arbitrary mammalian hallmark (Table 1). True toads (Bufonidae) tend to exhibit higher concentrations than the salamanders, 10 g/dl is not unusual, as seen in *Bufo cognatus* the great plains toad (Table 1). Spadefoot toads, e.g. *Scaphiopus couchi* Couch's spadefoot, have among the highest Hb levels attained by amphibians, typically ~12 g/dl, determined in field specimens, at different times of the year (Seymour, 1973).

Hematocrit values (packed red cell volume, ml rbc/100 ml blood) parallel the hemoglobin concentration of an animal's blood. Amphibian hematocrits are typically in the spectrum of 20-35% (i.e. the rbc comprise this percentage of the blood volume) (Table 1). In addition it is recognized that amphibian (and other submammalian) hematocrit values do not represent the oxygen-carrying potential that is equivalent to comparable mammalian hematocrits because amphibians' red cells are nucleated and a mammal's cells are not. Because the erythrocytic nucleus is a hemoglobin-poor, space-occupying mass within the cell, the amphibian Hct is consequently an indicator of a lower concentration of Hb than it would be if it were composed of denucleated erythrocytes. Extrapolation of Wintrobe's data (1933) leads to the suggestion that the aggregate nuclear mass represents ~15-20% of the hematocrit.

The erythrocyte indices offer an insightful standard for the comparison of the sizes and hemoglobin content of red cells among taxonomic groups and individuals (Wintrobe, 1933) (Table 1). The mean cellular volume (MCV) has proved to be the most useful of the three indices and provides an accurate quantitation of cell size as it is in the blood (as opposed to dry films). The mean cellular hemoglobin (MCH) denotes the amount of hemoglobin in an average circulating erythrocyte. The MCH of urodelans ranges from ~500-3000 pg Hb/cell while the anurans, due to their smaller cells, present a MCH spectrum of approximately 75-250 pg. In comparison, the MCH of modern bony fish is <100 pg and that of man is 30 pg. The mean cellular hemoglobin concentration (MCHC) indicates what proportion of an average erythrocyte can be considered devoted to hemoglobin (wt/vol, g Hb/100 ml rbc) (Table 1). The MCHC is usually 25-34% in the urodeles and anurans.

In some instances however, MCHC's of 40% or greater are derived (Table 1). Such values merit reconsideration because it has been generally accepted that the maximum attainable cellular concentration of Hb is ~36%. Whether these data are biased because of technical considerations or some factors peculiar to amphibian blood remains to be established.

Erythrocytic variation among amphibians

Erythrocyte based values in amphibians present variable intraspecific differences associated with age, season, environment, and sex. The erythrocyte count, hematocrit and hemoglobin levels have been found to increase as a function of body weight (males and females) in the terrestrial, Indian black spined toad *Bufo melanostictus* (Choubey et al., 1986). For example, during the growth period from 25 to 50 g in males the rbc count increased from 490,000 to 730,000/mm^3, Hb increased from 11.8 to 15 g/dl, and the Hct rose to 38% from 26.8%. All specimens were caught during April and analyzed one week after capture. A parallel observation regarding Hb and body weight for both sexes of this species caught during winter has also been recorded (Mishra and Mittra, 1988). Conversely, in another investigation of this anuran (with month(s) of capture unspecified) the rbc counts and Hb of similar weight males exhibited a negative correlation with body weight increase (rbc count fell from 1550 to 1220 x $10^3/mm^3$ and the Hb decreased to 13.6 g/dl from an initial 15.2 g/dl) (Banerjee, 1988). The Hct, however, increased as in the prior study. In *R. tigerina* the red cell count, Hb, and Hct increased with growth from 50-125 g in the males and 50-170 g in the females followed by a recession (except the Hct in females) to an intermediate range (Mishra and Banerjee, 1983). Adult Guatemalan highland toads *Bufo bocourti* express a significantly higher hemoglobin level (mean 10.6 g/dl) than their juveniles (Stuart, 1951). *Bufo boreas* the North American western toad has a Hb level that is positively correlated with body weight (Stiverson and Packard, 1974). However, the concentration of Hb does not increase proportionately with increments in weight, and in fact, this ratio has an inverse expression. In an investigation of three species of northern appalachian *Desmognathus*, although the acknowledged variation of the rbc counts was present, each species nevertheless revealed a positive correlation between the erythrocyte number and body size (Davic and Gallati, 1979). The erythrocyte count and Hb concentration are lower in larval anurans than in adults. The MCH is also greater in mature *R. catesbeiana* and *R. pipiens* than in their larval cells.

In a study of Javanese *Bufo melanostictus*, Church (1961) observed that its growth is accompanied by an increase in the size of the red cell and other somatic cells, i.e. auxetic growth. In a planimetric analysis of camera lucida drawings from tadpoles through adults the mean erythrocytic surface area increased with the size of the toad. The largest rbc were twice the size of the cells in the smallest tadpoles. A generally positive correspondence of the weight of this toad with its MCV (both sexes) has also been noted for a cohort native to India (Banerjee, 1983, 1988) but not by Choubey et al. (1986). A correlation of weight (or age) and erythrocyte size was noted in a survey of wild *Rana lessonae*, *R. ridibunda*, and *R. esculenta* (2n and 3n) (Blommers-Schlösser, 1990). The mean sizes of erythrocytes of juveniles were consistently smaller than the rbc from the same genotypic adults. Berger (1988) indicates that smaller European water frogs (e.g. *R. esculenta*) exhibit smaller erythrocytes.

In a year-long series of bimonthly analyses a significant seasonal difference in the Hct was encountered in wild, adult, aquatic red-spotted newts (*N. viridescens*) during July vs. March (28.1% and 21.6%) (Pitkin, 1983). Erythrocyte counts and Hb concentrations in wild *Taricha granulosa* monitored for 17 months exhibited a cyclic pattern with highest values in winter (prior to spring breeding) and lowest in late summer (Friedmann, 1974). The subjects were predominantly males which are said to be physically exhausted after breeding and which recoup their deficits during summer. This cyclic pattern, though subdued, was also identifiable in laboratory-housed animals maintained at a constant 15 °C, with little breeding activity and without migration. In contrast, the same wild species collected in May and acclimated for four weeks at 4 °C or 20 °C (normal winter and summer temperatures) generates a significantly higher Hct and Hb in the newts maintained at the elevated temperature (Wood, 1991). Erythrocytes from *Triturus carniflex* newts (collected in northern Italy) demonstrate decreased cytoplasmic peroxidase and increased localized acid phosphatase activity during hibernation as opposed to the active period (June). Barni et al. (1993) suggest these may be indicators of the advanced maturation of the circulating rbc during a period of diminished hemopoiesis.

The erythroid profile of female (males not analyzed) *R. pipiens* varies during the year (Kaplan and Crouse, 1956, Fig. 13). The rbc count and Hb maintain intermediate levels during the winter, begin to decrease in March, and reach their lowest values in April (rbc) and July (Hb). Then a roughly parallel increase is initiated which peaks in September/October followed by a recession to the winter picture. The MCV is maximal during the January-March interval, probably representative of a population mature erythrocytes which were released during prior seasonal erythropoiesis. A reduction in plasma volume apparently occurs in March/April as evidenced by an elevated Hct in the presence of diminishing Hb and rbc levels. In *Rana cyanophlyctis*, an aquatic frog that breeds throughout the year, the red cell count is greatest (males and females) during the rainy season (June-August) (Samantaray, 1985). Hematocrits exhibit a corresponding curve with the highest values in the summer (March-April) and rainy season, and the lowest

during winter. The MCV is greatest in summer (280 fl male, 270 fl female) and decreases through the subsequent rainy and winter seasons (to 204 fl male and 169 fl female) (Table 1). *Bufo viridis* the green toad and *R. ridibunda* demonstrate consistently higher red cell and Hb levels (in both sexes) during hibernation than they do in summer (Zhukova and Kubantsev, 1979, Table 1). Erythrocyte counts and Hb levels in males and females of Iraqi wild *R. esculenta* are significantly higher during the pre-winter period than the (non-hibernating) winter whereas the Hct remains stable (Sinha, 1983, Table 1). This winter picture may reflect a reduction in plasma volume plus a drop in total body rbc and Hb levels. The red cells are more elliptical and the MCV is larger in winter (males 707 fl vs. 460 fl), representative of a population of mature rbc. The circulating erythrocytes of *Rana esculenta* captured during underground hibernation (January, in Italy) are significantly different from those collected during the active period (July) (Barni et al., 1992). The erythrocytes in hibernating frogs are more morphometrically homogeneous, have decreased minor to major axis ratios for both the nucleus and cytoplasm (i.e. more oval shaped), demonstrate decreased staining with acridine orange presumably relecting a diminished concentration of ribosomes, and react less avidly with the nuclear fluochrome propidium iodide, indicating a more condensed and less active DNA. The observations are viewed as revealing the presence of a more mature population of rbc and slowed hemopoiesis. The intracellular sequestration of erythrocytes within the hepatocytes of this species during hibernation has also been noted (Barni and Bernocchi, 1991). Both cell types exhibit normal morphology. The function of this phenomenon and its net effect upon the level of circulating erythrocytes is unknown. *Xenopus laevis* with a phenylhydrazine-induced hemolytic anemia recover more rapidly if maintained at warm temperatures (18 °C vs. 12 °C), an observation consistent for a poikilotherm (Chegini et al., 1979).

The impact of geographic elevation upon erythropoiesis is well recognized. *Telmatobius culeus* the Andean Lake Titicaca frog resides at a high altitude (3800 m) (Hutchison et al., 1986) (Table 1). When it is acclimatized to a lower, oxygen-richer altitude (350 m) while still maintained at the usual ambient temperature (10 °C), the erythrocyte count diminishes significantly. Other Andean high altitude (≥ 3000 m) telmatobids such as *T. halli* exhibit greater red cell counts, Hb levels, MCHC values, and smaller red cells than concurrently compared low altitude fellow Leptodactylidae and the common Chilean toad *Bufo chilensis* (Ruiz et al., 1983; Table 1). The same investigators (Ruiz et al., 1989) further analyzed the erythropoietic profiles of adult *Bufo spinulosus* garnered at low (sea level to 2700 m) and high (3200-4500 m) elevations. The highland toads exhibited significantly smaller red cells, a significantly higher MCHC, and a tendency for a higher erythrocyte count (also smaller body size, 26 g vs. 45 g) than their lower-residing cohorts (Table 1). *Pseudacris triseriata* chorus frogs collected in the Rocky Mountains along an altitude gradient from 1500-3000 m failed to reveal a correlation of Hb concentration with the elevation of residence (Packard and Stiverson, 1976). Guatemalan *Bufo bocourti*, a species normally found at elevations >1700 m, did not enhance its Hb level upon exposure to higher elevations up to 3200 m, but did manifest a significantly higher Hb than the compared lowland toad *Bufo marinus* (Stuart, 1951). *Ambystoma macrodactylum* the long-toed salamander collected from low and high elevations and acclimatized at 800 m for 60 days demonstrated an increased Hct but not Hb, when subsequently transfered to 2400 m for two weeks (Howard and Wallace, 1980). Though unexplained, this response could be due to a release of increased numbers of immature, incompletely hemoglobinized erythrocytes.

A hypothesis that a microcytic erythrocytosis is the response of the newt to hypoxia (perhaps analogous to exposure to high altitude) is derived from the experience of Frangioni et al. (1987). They observed that phenylhydrazine-treated wild newts *T. cristatus carniflex* (from the environs of Florence, Italy) at eight weeks post exposure presented depressed Hb and Hct levels as well as a normal erythrocyte count comprised of rbc with a low MCV. However, when the presumed tissue hypoxia in similarly treated newts was eliminated by maintaining the salamanders in a hyperbaric chamber at 1.5 atmospheres, the replacement red cells were normocytic (normal MCV) even though the rbc count, Hb and Hct were at anemic levels. Within the limits of this study it appears that the response to lowered levels of oxygen in the short term is directed towards enhanced erythropoiesis and a production of smaller (more geometrically oxygen-exchange efficient) red cells. A consistent response is seen in *Bufo melanostictus* the common Indian toad exposed to a simulated altitude of 7000 m for 96 hr at 32 °C (Biswas and Boral, 1985). It presents a profile of hemoconcentration (decreased blood and plasma volumes, increased Hb, Hct, rbc count), an elevated red cell mass, and reduced MCV and MCH.

Sexual dimorphism of erythrocyte-related parameters has been noted at times, although the conditions that foster this phenomenon are poorly defined. In a year-long study of Hb and Hct levels in aquatic *N. viridescens* (Pitkin, 1983), females had significantly higher hematocrits (27.7%) than males (23.4%). Roofe (1961) found the opposite in wild *Ambystoma* tigrinum (locale: Wyoming, 2100 m). Rbc counts, Hct and Hb of *Bufo melanostictus* are greater in males than females (Choubey et al., 1986; Banerjee, 1988; Mishra and Mittra, 1988; Table 1). A significant sex-linked difference in rbc counts this time favoring females, is reported for the aquatic *Rana cyanophlyctis* during the rainy season, female 2.06 vs. male 1.61 million rbc/mm^3, while a concurrent analysis of two other ranids, *R. tigerina* and *R. breviceps* did not reveal any sex-related differences (Samantaray, 1984). In *R. tigerina* grouped by weight, the rbc count and Hct

tended to be higher in females while the reverse was the case with regard to the Hb concentration (Mishra and Banerjee, 1983). *R. temporaria* and *R. esculenta* have been cited as illustrating rbc counts and Hb values that are somewhat higher in males (Arvy, 1947; Schermer, 1967). A similar profile has been attributed to *R. esculenta* native to Iraq, i.e. higher rbc and Hb levels in males compared with weight-matched females (Sinha, 1983, Table 1). The same, statistically significant bias is observed in *R. pipiens* (Kaplan, 1951). The hematocrit is also consistently higher in males, except during the spawning season (March-April) when both sexes express equal values (Kaplan and Crouse, 1956). Adult male *R. ridibunda* collected in Krasnodar, Russia have significantly higher rbc counts (but not Hb) that females during summer and the hibernation season (Zhukova and Kubantsev, 1979).

Gender-related differences in the erythrocytic MCV have been noted in some higher vertebrates (e.g. chicken and man). The MCV in *Bufo melanostictus*, according to Choubey et al. (1986), is greater in males across the species' weight spectrum; in 60 g toads the volumes are male 534 fl and female 487 fl. In another analysis of this toad, gender bias was not detected (and the cellular volumes were markedly smaller, specimen wt. 60g, MCV 200 fl) (Banerjee, 1988). The mean cellular volume of Iraqi wild, similar weight (25-30 g) *R. esculenta* is greater in females than males in pre-winter and winter seasons (Sinha, 1983; Table 1).

In summary, the amphibian model of erythropoiesis is markedly varied and has the broadest range of expression observed in a Class of vertebrates. It is a system that in many cases optimally lends itself to the decipherment and analysis of the principles that govern erythropoiesis. It remains incompletely understood and a continued source of fascination.

Acknowledgements. The authors are indebted to Professor Loralee Kerr and Mrs. Susan Stegmeir of the Entomology, Fisheries and Wildlife Library, University of Minnesota for their support. Introduction to the subject matter was made possible by Professors John Rebuck and R. Dorothy Sundberg.

References

Alder A. and Huber E. (1923). Untersuchungen über Blutzellen und Zellbildung bei Amphibien und Reptilien. Folia Hematol. 29, 1-22.

Andrew W. (1965). Pisces and amphibia. In: Comparative hematology. Grune and Stratton. NY. pp 90-104.

Arvy L. (1947). Le dimorphisme sexuel sanguin chez *Rana temporaria* L. et *Bufo vulgaris* Laur. Comptes Rendus Séanc. Soc. Biol. 141, 457-459.

Banerjee V. (1983). Morphometry of erythrocytes of *Bufo melanostictus* with relation to season and body weight. Biol. Bull. India 5, 28-32.

Banerjee V. (1988). Erythrocyte related blood parameters in *Bufo melanostictus* with reference to sex and body weight. Environ. Ecol. Kalyani 6, 802-806.

Barni S. and Bernocchi G. (1991). Internalization of erythrocytes into liver parenchymal cells in naturally hibernating frogs (*Rana esculenta* L.). J. Exp. Zool. 258, 143-150.

Barni S., Nano R., Bertone V. and Prosperi E. (1992). Ultrastructure and cytochemistry of circulating erythrocytes during the annual cycle of *Rana esculenta* L. Comp. Haematol. Int. 2, 166-169.

Barni S., Nano R. and Vignola C. (1993). Changes of haemopoietic activity and characteristics of circulating blood cells in *Triturus carniflex* and *Triturus alpestris* during the winter period. Comp. Haematol. Int. 3, 159-163.

Barrett W.C. Jr. (1936). A comparative survey of hemopoietic loci in urodele amphibia, with especial reference to the bone marrow of the plethodontidae. Folia Haematol. 54, 165-192.

Barrett W.C. Jr. (1947). Hematopoiesis in the European plethodontid, *Hydromantes italicus* with reference to phylogeny. Anat. Rec. 98, 127-136.

Bechtold T.E., Smith P.B. and Turpen J.B. (1992). Differential stem cell contributions to thymocyte succession during development of *Xenopus laevis*. J. Immunol. 148, 2975-2982.

Berger L. (1988). Principles of studies of European water frogs. Acta Zool. Cracov. 31, 563-580.

Biswas H.M. and Boral M.C. (1985). Tissue weights and adaptation response of the toad after 96 hours of exposure to simulated high altitude - a body fluid and hematological study. Int. J. Biometeor. 29, 347-352.

Blaustein A.R., Hoffman P.D., Hokit D.G., Kiesecker J.M., Walls S.C. and Hays J.B. (1994). UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? Proc. Natl. Acad. Sci. 91, 1791-1795.

Blommers-Schlösser R.M.A. (1990). On the occurrence and identity of triploids of *Rana kl. esculenta* Linnaeus and *R. lessonae* Camerano in the Netherlands anura: ranidae. Bijdr. Dierkd. 60, 199-207.

Boschini Filho J. (1979). Elementos figurados do sangue periferico de *Typhlonectes compressicaudus*. Descricao das formas eritrocitarias aos niveis optico e electronico. Bol. Fisiol. Anim. (Sao Paulo) 3, 33-38 .

Broyles R.H. (1981). Changes in the blood during amphibian metamorphosis. In: Metamorphosis, A problem in developmental biology. 2nd ed. Gilbert L.I. and Frieden E. (eds). Plenum press. N.Y. pp 461-490.

Broyles R.H., Dorn A.R., Maples P.B., Johnson G.M., Kindell G.R. and Parkinson A.M. (1981a). Choice of hemoglobin type in erythroid cells of *Rana catesbeiana*. In: Hemoglobins in development and differentiation. Stamatoyannopoulos G. and Nienhuis A.W. (eds). A. R. Liss. NY. pp 179-191.

Broyles R.H., Johnson G.M., Maples P.B. and Kindell G.R. (1981b). Two erythropoietic environments and two larval red cell lines in bullfrog tadpoles. Dev. Biol. 81, 299-314.

Cameron J.A. (1941). Primitive blood-cell generations in *Amblystoma*. J. Morphol. 68, 231-239.

Campbell F.R. (1970). Ultrastructure of the bone marrow of the frog. Am. J. Anat. 129, 329-356.

Chegini N., Aleporou V., Bell G., Hilder V.A. and MacLean N. (1979). Production and fate of erythroid cells in anaemic *Xenopus laevis*. J. Cell Sci. 35, 403-415.

Choubey B.G., Shankar A. and Choubey B.J. (1986). Haematological investigations of Himalayan toad *Bufo melanostictus* Schneider in relation to sex and size. Biol. Bull. India 8, 106-114.

Church G. (1961). Auxetic growth in the Javanese toad, *Bufo melanostictus*. Science 133, 2012-2014.

Cohen W.D. (1978). Observations on the marginal band system of nucleated erythrocytes. J. Cell Biol. 78, 260-273.

Cohen W.D. (1982). The cytomorphic system of anucleate non-mammalian erythrocytes. Protoplasma 113, 23-32.

Cohen W.D. (1991). The cytoskeletal system of nucleated erythrocytes. Int. Rev. Cytol. 130, 37-84.

Cohen W.D., Bartlet D., Jaeger R., Langford G. and Nemhauser I. (1982). The cytoskeletal system of nucleated erythrocytes. I. Composition and function of major elements. J. Cell Biol. 93, 828-838.

Cooper E.L., Klempau A.E., Ramirez J.A. and Zapata A.G. (1980). Source of stem cells in evolution: In: Development and differentiation of vertebrate lymphocytes. Horton J.D. (ed). Elsevier. NY. pp 3-14.

Curtis S.K., Cowden R.R. and Nagel J.W. (1979). Ultrastructure of the bone marrow of the salamander *Plethodon glutinosus* caudata: plethodontidae. J. Morphol. 159, 151-184.

Davic R.D. and Gallati W.W. (1979). Erythrocyte number in three species of northern Appalachian *Desmognathus* amphibia, urodela, plethodontidae. J. Herpetol. 13, 354-356.

Davies H.G. (1961). Structure in nucleated erythrocytes. J. Biophys. Biochem. Cytol. 9, 671-687.

Dawson A.B. (1931). Hemopoiesis in *Necturus*. Anat. Rec. Am. Soc. Zool. 51, 72-73.

Dawson A.B. (1932). Hemopoietic loci in *Necturus maculosus*. Anat. Rec. 52, 367-380.

DeGraaf A.R. (1957). A note on the oxygen requirements of *Xenopus laevis*. J. Exp. Biol. 34, 173-176.

Deparis P., Beetschen J.-C. and Jaylet A. (1975). Red blood cells and hemoglobin concentration in normal diploid and several types of polyploid salamanders. Comp. Biochem. Physiol. 50A, 263-266.

Ducibella T. (1974). The occurrence of biochemical metamorphic events without anatomical metamorphosis in the axolotl. Dev. Biol. 38, 175-186.

Duran-Jorda F. (1951). Haemopoiesis as seen in *Batrochoseps attenuatus*. Acta Med. Scand. 140, 183-192.

Eisen G. (1897). Plasmocytes; the survival of the centrosomes and archoplasm of the nucleated erythrocytes, as free and independent elements in the blood of *Batrochoseps attenuatus* esch. Proc. Calif. Acad. Sci. series 3, Zool. 1, 3-72.

Emmel V.E. (1921). Hematological and respiratory conditions in the larval stages of the lungless amphibians. Anat. Rec. 21, 56.

Emmel V.E. (1924). Studies on the non-nucleated elements of the blood. Am. J. Anat. 33, 347-405.

Ewer D.W. (1959). A toad *Xenopus laevis* without haemoglobin. Nature 183, 271.

Feder M.E. (1976). Lunglessness, body size, and metabolic rate in salamanders. Physiol. Zool. 49, 398-406.

Federici E. (1926). Recherches experimentales sur les potentialitiés de l'ilot sanguin chez l'embryon de *Rana fusca*. Arch. Biol. 36, 465-487.

Fernald R.L. (1943). The origin and development of the blood island of *Hyla regilla*. Univ. CA. Publ. Zool. 51, 129-147.

Foxon G.E.H. (1964). Blood and respiration. In: Physiology of the amphibia. Vol. I. J.A. Moore ed. Academic Press. London. pp 151-209.

Frangioni G., Borgioli G. and Martini R. (1987). Microcythemia from anemic hypoxia and normal erythropoiesis in the newt. J. Exp. Zool. 244, 183-186.

Frangioni G. and Borgioli G. (1987a). Intermittent erythropoiesis in anemic newts. J. Exp. Zool. 241, 207-215.

Frangioni G. and Borgioli G. (1987b). Periodic changes in the organs involved in the erythropoiesis of anemic newts. J. Exp. Zool. 243, 409-416.

Freytag G.E., Grzimek B., Kuhn O. and Thenius E. eds (1974). Amphibians. In: Grzimek's Animal Life Encyclopedia. Vol. 5. Grzimek B. ed-in-chief. Van Nostrand Reinhold. NY. pp 273-456.

Friedmann G.B. (1970). Differential white blood cell counts for the urodele *Taricha granulosa*. Can. J. Zool. 48, 271-274.

Friedmann G.B. (1971). Altitudinal variations in the red blood cell count and haemoglobin content of urodele blood. Can. J. Zool. 49, 565-568.

Friedmann G.B. (1974). The annual cycle of red blood cell count and haemoglobin level in the urodele *Taricha granulosa* on southern Vancouver Island. Can. J. Zool. 52, 487-494.

Gahlenbeck H. and Bartels H. (1970). Blood gas transport properties in gill and lung forms of the axolotl *Ambystoma mexicanum*. Respir. Physiol. 9, 175-182.

Glomski C.A. and Tamburlin J. (1989). The phylogenetic odyssey of the erythrocyte. I. Hemoglobin: the universal respiratory pigment. Histol. Histopathol. 4, 509-514.

Glomski C.A. and Tamburlin J. (1990). The phylogenetic odyssey of the erythrocyte. II. The early or invertebrate prototypes. Histol. Histopathol. 5, 513-525.

Glomski C.A., Tamburlin J. and Chainani M. (1992). The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience. Histol. Histopathol. 7, 501-528.

Goniakowska L. (1970). The respiration of erythrocytes of some amphibians *in vitro*. Bull. Acad. Polon. Sci., Ser. Sci. Biol. 18, 793-797.

Goniakowska-Witalinska L. (1974). Respiration resistence to hypotonic solutions and ultrastructure of erythrocytes of *Salamandra salamandra*. Bull. Acad. Polon. Sci., Ser. Sci. Biol. 22, 59-66.

Goniakowska-Witalinska L. and Witalinski W. (1976). Evidence for a correlation between the number of marginal band microtubules and the size of vertebrate erythrocytes. J. Cell Sci. 22, 397-401.

Gorr T. and Kleinschmidt T. (1993). Evolutionary relationships of the coelecanth. Am. Sci. 81, 72-82.

Gorr T., Kleinschmidt T. and Fricke H. (1991). Close tetrapod relationships of the coelacanth *Latimeria* indicated by haemoglobin sequences. Nature 351, 394-397.

Goss C.M. (1928). Experimental removal of the blood island of *Amblystoma punctatum* embryos. J. Exp. Zool. 52, 45-63.

Grasso J.A. (1973a). Erythropoiesis in the newt, *Triturus cristatus* Laur. I. Identification of the «erythroid precursor cell» J. Cell Sci. 12, 463-489.

Grasso J.A. (1973b). Erythropoiesis in the newt, *Triturus cristaus* Laur. II. Characteristics of the erythropoietic process. J. Cell Sci. 12, 491-523.

Grasso J.A. and Shephard D.C. (1968). Experimental production of totally anaemic newts. Nature 218, 1274-1276.

Grasso J.A. and Woodward J.W. (1966). The relationship between RNA synthesis and hemoglobin synthesis in amphibian erythropoiesis. J. Cell Biol. 31, 279-294.

Gulliver G. (1862). On the red corpuscles of the blood of vertebrata, and on the zoological import of the nucleus, with plans of their structure, form, and size on a universal scale, in many of the different orders. Proc. Zool. Soc. London 91-103.

Gulliver G. (1873). Measurements of the red blood corpuscles of Batrachians. Proc. Zool. Soc. London. 162-165.

Gulliver G. (1875). Observations on the sizes and shapes of the red corpuscles of the blood of vertebrates, with drawings of them to a uniform scale, and extended and revised tables of measurements.

Proc. Zool. Soc. London 474-495.

Hadji-Azimi I., Coosemans V. and Canicatti C. (1987). Atlas of adult *Xenopus laevis laevis* hematology. Dev. Comp. Immunol. 11, 807-874.

Hartman F.A. and Lessler M.A. (1964). Erythrocyte measurements in fishes, amphibia, and reptiles. Biol. Bull. 126, 83-88.

Heusser H.R. (1974). Amphibians. In: Grzimek's animal life encyclopedia. Vol. 5. Chapters 4-6. Grzimek B. ed-in-chief. Van Nostrand Reinhold. NY. pp 357-456.

Hollyfield J.G. (1966). The origin of erythroblasts in *Rana pipiens* tadpoles. Dev. Biol. 14, 461-479.

Howard J.H. and Wallace R.L. (1980). Effects of altitude on selected blood parameters in populations of the salamander *Ambystoma macrodactylum*. Comp. Biochem. Physiol. 65A, 243-245.

Hutchison V.H. and Szarski H. (1965). Number of erythrocytes in some amphibians and reptiles. Copeia (3), 373-375.

Hutchison V.H., Haines H.B. and Engbretson G. (1976). Aquatic life at high altitude: respiratory adaptations in the Lake Titicaca frog, *Telmatobius culeus*. Respir. Physiol. 27, 115-129.

Jerrett D.P. and Mays C.E. (1973). Comparative histology of the hellbender, *Cryptobranchus alleganiensis* in Missouri. Copeia (2), 331-337.

Jordan H.E. (1919). The histology of the blood and the red bone-marrow of the leopard frog, *Rana pipiens*. Am. J. Anat. 25, 437-480.

Jordan H.E. (1932a). The histology of the blood and the blood-forming tissues of the urodele, *Proteus anguineus*. Am. J. Anat. 51, 215-251.

Jordan H.E. (1932b). The blood and blood-forming organs of the salamander *Plethodon cinereus*. Anat. Rec. 54 Supp. 45-46.

Jordan H.E. (1938). Comparative hematology. In: Handbook of hematology. Vol. 2. Downey H. (ed). Hafner Publishing Co. Inc., NY. pp 700-862.

Jordan H.E. and Speidel C.C. (1930). The hemocytopoietic effect of splenectomy in the salamander *Triturus viridescens*. Am. J. Anat. 46, 55-90.

Kaloustian K.V. and Dulac R.W. (1982). Relationships between red blood cell indices and the effects of thryroxine in three species of amphibians. Comp. Biochem. Physiol. 73A, 427-430.

Kaplan H.M. and Crouse G.T. (1956). Blood changes underlying the seasonal resistance of frogs to disease. Copeia (1), 52-54.

Kau C.-L. and Turpen J.B. (1983). Dual contribution of embryonic ventral blood island and dorsal lateral plate mesoderm during ontogeny of hemopoietic cells in *Xenopus laevis*. J. Immunol. 131, 2262-2266.

Khalil R.M. and Elfeky N.K. (1986a). Observations on the blood cells of *Bufo viridis* Laurenti, *Bufo regularis* Reuses and *Rana mascareniensis* Dumerila and Bibron anuran amphibian. J. Coll. Sci., King Saud Univ. 17, 67-85.

Khalil R.M. and Elfeky N.K. (1986b). The blood forming tissues and organs of *Bufo viridis* Laurenti, *Bufo regularis* Reuses and *Rana mascareniensis* Dumerila and Bibron anuran amphibia. J. Coll. Sci., King. Saud. Univ. 17, 87-98.

Kuramoto M. (1981). Relationships between number, size and shape of red blood cells in amphibians. Comp. Biochem. Physiol. 69A, 771-775.

Lenfant C. and Johansen K. (1967). Respiratory adaptations in selected amphibians. Respir. Physiol. 2, 247-260.

Leeuwenhoek A.V. (1683). An abstract of a letter from mr. Anthony Leeuwenhoeck of Delft about generation by an animalcule of the male seed. Animals in the seed of a frog. Some other observables in the parts of a frog. Digestion, and the motion of the blood in a feavor. Phil. Trans. Royal Soc. London 13, 347-355.

Leeuwenhoek A.V. (1722). Opera omnia, seu arcana naturae, ope exactissimorum microscopiorum, detecta experimentis variis comprobata, epistolis ad varios illustres viros. Leyden. pp 49-64.

Licht L.E. and Bogart J.P. (1987). Comparative size of epidermal cell nuclei from shed skin of diploid, triploid and tetraploid salamanders genus *Ambystoma*. Copeia (2), 284-290.

Maniatis G.M. and Ingram V.M. (1971). Erythropoiesis during amphibian metamorphosis. I. Site of maturation of erythrocytes in *Rana catesbeiana*. J. Cell Biol. 49, 372-379.

Matson T.O. (1990). Erythrocyte size as a taxonomic character in the identification of Ohio *Hyla chrysoscelis* and *H.* versicolor. Herpetologica 46, 457-462.

Mazin A.L. (1980). Amounts of nuclear DNA in anurans of the USSR. Experientia 36, 190-191.

Merkle S. (1989). Sexual differences as adaptation to the different gender roles in the frog *Xenopus laevis* Daudin. J. Comp. Physiol. B 159, 473-480.

Mishra V. and Banerjee V. (1983). Haematology of *Rana tigrina* erythrocytes and related parameters with relation to body weight. Ann. Zool. 20, 25-32.

Mishra D.R. and Mittra A. (1988). S-V length and haemoglobin content in blood of the tropical anuran *Bufo melanostictus* Bloch and Schneider, a correlation study. Indian J. Comp. Anim. Physiol. 6, 129-133.

Morris M.A. (1984). Comparison of erythrocyte sizes in *Ambystoma texanum*, *A. laterale* and *A. jeffersonianum*. J. Herpetol. 18, 197-198.

Ohuye T. (1932). Hemocytopoietic effect of splenectomy in the newt, *Diemyctylus pyrrhogaster*. Sci. Rep. Tohoku Imp. Univ. ser. 47, 49-64.

Okazaki T., Ishihara H. and Shukuya R. (1982). Changes in the density of circulating erythrocytes of the bullfrog tadpole, *Rana catesbeiana*, in relation to the transition of hemolgobin during metamorphosis. Comp. Biochem. Physiol. 73B, 309-312.

Olmo E. (1973). Quantitative variations in the nuclear DNA and phylogenesis of the amphibia. Caryologia 26, 43-68.

Olmo E. and Morescalchi A. (1978). Genome and cell sizes in frogs: a comparison with salamanders. Experientia 34, 44-46.

Packard G.C. and Stiverson R.K. (1976). Blood hemoglobin concentration in chorus frogs *Pseudacris triseriata*: relationship to body size and altitude. Am. Midl. Nat. 96, 482-487.

Pai R.M. and Shanbhag S.V. (1988). On erythrocytes of certain Indian amphibians. Comp. Physiol. Ecol. 13, 103-110.

Paulson B.K. and Hutchison V.H. (1987). Blood changes in *Bufo cognatus* following acute heat stress. Comp. Biochem. Physiol. 87A, 461-466.

Pitkin R.B. (1983). Annual cycle of body size and blood parameters of the aquatic adult red-spotted newt, *Notophthalmus viridescens*. J. Exp. Zool. 226, 373-377.

Reynolds A.E. and Pickard B.L. (1973). Normal blood values in some plethodontid salamanders. Herpetologica 29, 184-188.

Roofe P.G. (1961). Blood constituents of *Amblystoma tigrinum*. Anat. Rec. 140, 337-340.

Rouf M.A. (1969). Hematology of the lepoard frog, *Rana pipiens*. Copeia (4) 682-687.

Ruiz G., Rosenmann M. and Veloso A. (1983). Respiratory and hematological adaptations to high altitude in *Telmatobius* frogs from the Chilean Andes. Comp. Biochem. Physiol. 76A, 109-113.

Ruiz G., Rosenmann M. and Veloso A. (1989). Altitudinal distribution

and blood values in the toad, *Bufo spinulosus* Wiegmann. Comp. Biochem. Physiol. 94A, 643-646.

Samantaray K. (1984). A comparative study of hematology of three species of *Rana*. J. Adv. Zool. 5, 42-47.

Samantaray K. (1985). Studies on hematology of Indian skipper frog, *Rana cyanophlyctis* Schneider. Comp. Physiol. Ecol. 10, 71-74.

Schermer S. (1967). The frog. In: The blood morphology of laboratory animals. 3rd ed. F.A. Davies. Philadelphia. pp 171-187.

Sean K.E., Lassalle B. et Boilly B. (1980). Ultrastructure de l'érythrocyte du triton *Pleurodeles waltlii* Michah. Can. J. Zool. 58, 1193-1199.

Seymour R.S. (1973). Gas exchange in spadefoot toads beneath the ground. Copeia (3), 452-460.

Shumway W. (1940). Stages in the normal development of *Rana pipiens*. I. External form. Anat. Rec. 78, 139-147.

Sinha R.C. (1983). Haematological studies on the prewintering and wintering frog, *Rana esculenta*. Comp. Biochem. Physiol. 74A, 311-314.

Slonimski P. (1931). Recherches expérimentales sur la genèse du sang chez les amphibiens. Arch. Biol. 42, 415-477.

Smith H.M. (1925). Cell size and metabolic activity in amphibia. Biol. Bull. 48, 347-378.

Stiverson R.K. and Packard G.C. (1974). The relation of blood hemoglobin concentration to body size in the toad *Bufo boreas*. Comp. Biochem. Physiol. 49A, 673-676.

Stuart L.C. (1951). The distributional implications of temperature tolerances and hemoglobin values in the toads *Bufo marinus* Linnaeus and *Bufo bocourti* Brocchi. Copeia (3), 220-229.

Szarski H. (1968). Evolution of cell size in lower vertebrates. In: Problems of lower vertebrate phylogeny. Orvig T. (ed). Interscience Publishers. NY. pp 445-453.

Szarski H. (1976). Cell size and nuclear DNA content in vertebrates. Int. Rev. Cytol. 44, 93-111.

Szarski H. and Czopek G. (1966). Erythrocyte diameter in some amphibians and reptiles. Bull. Acad. Pol. Sci. Ser. Sci. Biol. 14, 433-437.

Tanaka Y. (1976). Architecture of the marrow vasculature in three amphibian species and its significance in hematopoietic development. Am. J. Anat. 145, 485-498.

Taylor E.H. (1968). The caecilians of the world. A taxonomic review. Univ. Kansas Press. Lawrence. pp 1-848.

Taylor A.C. and Kollros J.J. (1946). Stages in the normal development of *Rana pipiens* larvae. Anat. Rec. 94, 7-24.

Thomas N. and Maclean N. (1974). The blood as an erythropoietic organ in anaemic *Xenopus*. Experientia 30, 1083-1085.

Thomas N. and Maclean N. (1975). The erythroid cells of anaemic *Xenopus laevis*. I. Studies on cellular morphology and protein and nucleic acid synthesis during differentiation. J. Cell Sci. 19, 509-520.

Toews D. and MacIntyre D. (1978). Respiration and circulation in an apodan amphibian. Can. J. Zool. 56, 998-1004.

Tooze J. and Davies H.G. (1965). Cytolysosomes in amphibian erythrocytes. J. Cell Biol. 24, 146-150.

Tooze J. and Davies H.G. (1967). Light- and electron-microscope studies on the spleen of the newt *Triturus cristatus*: the fine structure of erythropoietic cells. J. Cell Sci. 2, 617-640.

Tooze J. and Davies H.G. (1968). Light and electron microscopic observations on the spleen and the splenic leukocytes of the newt *Triturus cristatus*. Am. J. Anat. 123, 521-526.

Turner R.J. (1988). Amphibians. In: Vertebrate blood cells. Rowley A.F. and Ratcliffe N.A. (eds). Cambridge University Press. Cambridge. pp 129-209.

Turpen J.B. (1980). Early embryogenesis of hematopoietic cells in *Rana pipiens*. In: Development and differentiation of vertebrate lymphocytes. Horton J.D. (ed). Elsevier. NY. pp 15-24.

Turpen J.B. and Knudson C.M. (1982). Ontogeny of hematopoietic cells in *Rana pipiens*: precursor cell migration during embryogenesis. Dev. Biol. 89, 138-151.

Turpen J.B. and Smith P.B. (1985). Dorsal lateral plate mesoderm influences proliferation and differentiation of hemopoietic stem cells derived from ventral lateral plate mesoderm during early development of *Xenopus laevis* embryos. J. Leukocyte Biol. 38, 415-427.

Turpen J.B., Turpen C.J. and Flajnik M. (1979). Experimental analysis of hematopoietic cell development in the liver of larval *Rana pipiens*. Dev. Biol. 69, 466-479.

Turpen J.B., Knudson C.M. and Hoefen P.S. (1981). The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. Dev. Biol. 85, 99-112.

Twersky L.H., Bartley A.D., Rayos N. and Cohen W.D. (1995). Immature erythroid cells with novel morphology and cytoskeletal organization in adult *Xenopus*. Protoplasma 185, 37-49.

Uzzell T.M. Jr. (1964). Relations of the diploid and triploid species of the *Ambystoma jeffersonianum* complex amphibia, caudata. Copeia (2) 257-300.

van Deurs B. and Behnke O. (1973). The microtubule marginal band of mammalian red cells. Z. Anat. Entwickl. Gesch. 143, 43-47.

Vernberg F.J. (1955a). Hematological studies on salamanders in relation to their ecology. Herpetologica 11, 129-133.

Vernberg F.J. (1955b). Correlation of physiological and behavior indexes of activity in the study of *Plethodon cinereus* Green and *Plethodon glutinosus* Green. Am. Midl. Nat. 54, 382-393.

Villolobos M., León P., Sessions S.K. and Kezer J. (1988). Enucleated erythrocytes in plethodontid salamanders. Herpetologica 44, 243-250.

Warfel H.E. (1936). Notes on the occurence of *Necturus maculosus* Rafinesque in Massachusetts. Copeia (4), 237.

Welsch U. and Starck M. (1986). Morphological observations on blood cells and blood cell forming tissues of *Gymnophiona*. Mem. Soc. Zool. Fr. 43, 107-115.

Wintrobe M.M. (1933). Variations in the size and hemoglobin content of erythrocytes in the blood of various vertebrates. Folia Haematol. 51, 32-49.

Wood S.C. (1991). Temperature acclimation of respiratory function in the salamander *Taricha granulosa*. Respir. Physiol. 85, 371-381.

Wood S.C., Weber R.E., Maloiy G.M.O. and Johansen K. (1975). Oxygen uptake and blood respiratory properties of the caecilian *Boulengerula taitanus*. Respir. Physiol. 24, 355-363.

Zapata A., Gomariz R.P., Garrido E. and Cooper E.L. (1982). Lymphoid organs and blood cells of the caecilian *Ichthyophis kohtaoensis*. Acta Zool. Stockh. 63, 11-16.

Zapata A., Torroba M., Vicente A., Varas A., Sacedón R. and Jiménez E. (1995). The relevance of cell microenvironments for the appearance of lympho-haemopoietic tissues in primitive vertebrates. Histol. Histopathol. 10, 761-778.

Zhukova T.I. and Kubantsev B.S. (1979). Changes in the blood composition of salientians during hibernation. Soviet. J. Ecol. 9, 379-382.

Zon L.I. (1995). Developmental biology of hematopoiesis. Blood 86, 2876-2891.