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**Cellular morphogenesis and the cytoskeleton in amphibian
erythrocytes**

Ginsburg, Mary Fuday, Ph.D.

City University of New York, 1992

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CELLULAR MORPHOGENESIS AND THE CYTOSKELETON
IN AMPHIBIAN ERYTHROCYTES

by

Mary Fuday Ginsburg

A dissertation submitted to the Graduate Faculty
in Biology in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
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Abstract

CELLULAR MORPHOGENESIS AND THE CYTOSKELETON
IN AMPHIBIAN ERYTHROCYTES

by

Mary Fuday Ginsburg

Adviser: PROFESSOR WILLIAM D. COHEN

"Two things are required from every specialized treatise:
it should clarify its subject and, in the second place,
but actually more important,
it should tell us how and by what methods we can attain it
and make it ours."

(Pseudo-Longinus, prob 1st century, On the Sublime)

The nucleated erythrocytes of non-mammalian vertebrates provide an excellent model system for studying cytoskeletal function during cellular morphogenesis. Previous work by several laboratories has suggested that the marginal band is important in establishing cell shape as such cells undergo transition from mitotic erythroblast to flattened ellipse; however, much of this work has been done by manipulation of mature cells of various animals. The research presented herein attempted to extend such findings by an analysis of marginal band development and its possible correlation with cell morphogenesis during maturation of larval axolotl erythrocytes, with emphasis on the population in the spleen,

which is not connected to the general circulation at early developmental stages in this organism. Approaches included tabulation of cell shapes as a function of larval age and a variety of morphological studies of erythrocytic cells at different stages of development, using such techniques as scanning electron microscopy of whole cells, transmission electron microscopy and immunofluorescence of cytoskeletons, and cell culture.

The results were consistent with the hypothesis that the marginal band plays a role in determination of cell shape during development and were used as the basis for a preliminary model for the morphogenesis of these erythrocytes. More importantly, however, the work extended the excellent work of Duprat and Flavim (1976) in demonstrating the value of the axolotl spleen as a developmental system deserving of study. It is hoped that this dissertation may serve as a foundation for further inquiry, even as did theirs.

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Introduction

Although much is known about the structure and function of the cytoskeleton, there are many gaps in the information about its development, particularly as related to cell morphogenesis. The research presented in this thesis approaches the development of one component of the cytoskeleton, the marginal band of non-mammalian red blood cells, and its role in cell shape formation, using amphibian erythropoiesis as an accessible system for study.

The Marginal Band: A Unique Cytoskeletal Element

The nature of the cytoskeleton has been one of the oldest and most intriguing problems of modern cell biology. As the name implies, this intricate array of microtubules, intermediate filaments, and microfilaments, together with various associated proteins, forms a supporting structure for the maintenance of cell shape. In addition, various cytoskeletal structures and their components play a dynamic role in such vital aspects of cell physiology as axoplasmic transport and cell division. Developmentally, the cytoskeleton assumes varied geometry and composition at different stages of cell division and morphogenesis, and study of its differentiation can contribute much to an understanding of cell development in general.

Probably because they are the largest of the cytoskeletal components, microtubules were the earliest ones to be noticed. These are especially conspicuous when aggregated, as they are in the marginal band, which was

first documented by Ranvier, who observed a peripheral fibrous bundle in nucleated erythrocytes in 1875 (Dustin, 1984). However, Meves (1911) states that Dehler was the first, in 1895, to recognize that it was a marginal ring, rather than a thick membrane surrounding the corpuscle. Meves continued the seminal work on marginal bands, using the red cells of the fire salamander, Salamandra salamandra. Recognizing the marginal band as a cytoskeletal element although the term was unknown, he attributed the red blood corpuscle's ability to resume its original shape upon cessation of external forces to the "elasticity inherent in the marginal ring, which allows it to return to its natural state." [Dustin (1984), however, incorrectly credits Jolly with being the first to link the marginal bundle with erythrocyte shape.] Meves recognized that the ring was "composed of many ultrafine filaments running in parallel or, what is just as possible, of a single, uninterrupted filament which is wound into a skein along the edge of the blood disk" (Meves, 1911, I.2., p. 2, of translation), thus stating concisely a scientific question which is still unanswered eighty years later. As a matter of historical interest, it may be noted that axonal neurotubules and the microtubules of the spindle were noticed and documented near the end of the nineteenth century, a few years later than Ranvier's initial observations on the marginal band (mentioned by Dustin, 1984).

After such a prescient beginning, further progress in understanding microtubules in general and marginal bands in particular awaited the development of biological electron microscopy and of modern biochemical techniques. Beginning with Fawcett's electron microscopic observations on the marginal band in 1959 and his suggestion of its involvement in cell shape maintenance, and continuing to the present, many varied aspects of the cytoskeleton have been the subject of intensive research. Meves's ring is now known to consist of microtubules and associated proteins arranged circumferentially around the elliptical cell in the plane of flattening. The term "microtubule" did not itself enter the scientific vocabulary until Slautterback (1963) coined the term in a 1963 Journal of Cell Biology paper. It is most appropriate that the nucleated erythrocyte, which shares only with the thrombocyte and platelet the possession of a marginal band as a unique configuration of the cytoskeleton, remains one of the primary systems for the study of this important aspect of cellular structure and function.

Among the laboratories engaged in studies of the marginal band, Cohen's is one which in previous years has investigated it in a variety of systems, ranging from the camel (Cohen and Terwilliger, 1979) to invertebrates (Cohen, Nemhauser, and Cohen, 1983; Nemhauser, Ornberg, and Cohen, 1980). One emphasis of this work has been the evaluation of the marginal band function in cell shape determination. To that end, development of a dependable method for lysis of

the erythrocytes, to enable viewing of the bands under moderate magnification with phase microscopy, has made such cells accessible to a variety of experiments (Cohen, 1978). Such work resolves the problem of explaining the elliptical shape of non-mammalian erythrocytes by theories such as analogy to the contour of a mercury drop in contact with a cylindrical surface (Davison, 1959)! Goniakowska-Witalinska and Witalinska (1976) found a positive correlation between red cell size and marginal band thickness/microtubule number. This has been supported and extended by results in other laboratories (Cohen, 1978). In addition to its assumed morphogenetic role, the marginal band serves an ongoing function in preserving mature erythrocyte shape against forces of deformation (Joseph-Silverstein and Cohen, 1984; Joseph-Silverstein and Cohen, 1985), much as Meves (1911) originally proposed.

The morphogenetic role of the marginal band has been studied in chicken erythrocytes by Barrett's laboratory (Barrett and Scheinberg, 1972; Barrett and Dawson, 1974). Murphy (Murphy, Grasser, and Wallis, 1986) and Solomon (Kim, et al., 1987) have examined the formation of the marginal band in chicken erythrocytes and have found microtubules emanating from a microtubule-organizing center at the early stages of band development. They have not, however, attempted a correlation between the changes in the microtubule arrangement and the evolving cell shape, nor do they refer to the observation of pointed cells as a possible

intermediate stage. Such a study of the development of the marginal band and the concomitant morphogenesis from spherical erythroblast to elliptical erythrocyte is of interest in terms of both cytoskeletal function and red blood cell differentiation, and is the major focus of this dissertation.

Erythrocyte development

It is most convenient for cell and developmental biologists that the marginal band occurs in erythrocytes, which have long served as a model system for the study of differentiation. Unlike the majority of eukaryotic cells, they undergo constant elimination and renewal, and can be obtained with relative ease. Thus, unless a study focuses on embryonic aspects, the biologist may not require access to fetal tissue, and may in fact be able to use blood samples without killing the animal. Vertebrate blood cells begin differentiation early in development and continue to proliferate throughout the life of the organism in a delicately controlled system of self-renewal. The sequence of erythroid differentiation has long been defined, and seems consistent from species to species. Hemoglobin, a unique and plentiful cell component, changes with embryonic development and may be used as a marker as well as a subject of study. The presence of hemoglobin also can aid in cell identification. Many researchers, again going back at least to Ranvier (1875), have used this system for their developmental studies.

A variety of animal systems have been used for the study of blood cell differentiation, with choices often being made on the basis of convenience of a given animal. When fetal hematopoiesis is studied, it is important to consider the site(s) of such development. Blood cell formation in various fish occurs in scattered sites, including the spleen, kidney, and peripheral blood (Rowley et al., 1988). Phylogenetically, the bone marrow first functions in hematopoiesis in amphibians, but only seasonally or transitionally, and only in the Anurans (Siegel, 1970). In reptiles, considered a transitional group, bone marrow functions in hematopoiesis throughout life. Turner (1988) states that in all three amphibian orders the spleen is considered a major site of red cell production and destruction. According to Siegel the spleen is the sole erythropoietic site in normal post-embryonic Urodeles throughout life, although Dawson (1930) reported erythroid regeneration in the circulation. (See Discussion.) In adult Anurans the spleen functions as the sole or major site except immediately after metamorphosis and hibernation, when the bone marrow becomes active. In Anuran larvae, however, the mesonephroi and the liver are the main sites of erythropoiesis.

The axolotl as a system for study

The work on which this thesis is based has endeavored to examine in parallel the appearance and development of the erythrocyte marginal band and the morphogenesis of the cell

itself in the neotenuous urodele Ambystoma mexicanum (the axolotl).

Amphibians in general are convenient for developmental studies because their embryos are large and easily staged, and because much literature is available about them. The last attribute applies with a vengeance to the axolotl, named after the Aztec god of games and grotesque beings, for since 1855 not a year has passed without at least one paper about it (Smith, 1989)! Although neotenuous, this urodele can be induced to metamorphose in thyroxine (Fox, 1983). It is convenient to acquire the embryos from the NSF-sponsored Axolotl Colony at Indiana University, and these embryos can be viewed through the clear jelly surrounding their eggs and easily staged (Schreckenbergs and Jacobson, 1975; Bordzilovskaya and Dettlaff, 1979). The young embryos may be stored in a state of arrested development at 1°C for up to a month, after which they return to a normal sequence upon restoration to 18°C (Ginsburg, Twersky, and Cohen, 1987). Unlike chicken blood cells, which have been used extensively in other laboratories, the erythrocytes of this species are large (20-25 μm long axis) and the marginal band relatively thick; thus both are easily viewed by phase contrast microscopy.

The primary, or primitive generation, red blood cells of prehatching amphibian embryos derive from the large ventral blood island, from which erythroblasts round up and are released to the circulation, where they continue to

proliferate and complete differentiation (Fox, 1984; Goss, 1928). According to Goss, after the erythrocytes from the blood island are discharged into the circulation (about Harrison stage 37), the blood cells proliferate and develop throughout the the vascular system. (His studies used Ambystoma punctatum.)

Although blood formed in the large ventral blood island (Cameron, 1941) undergoes development in the circulation of the embryo and early larva, presumably with a morphological sequence similar to that found later, the emphasis in this dissertation is on erythropoiesis in the larval spleen because this is the more convenient system for study. As pointed out by Duprat and Flavin (1976), using Pleurodeles waltlii, red blood cells develop there in an apparently homogeneous population. The spleen during this time is a closed, red sac isolated from the general circulation from the second or third day post-hatching until the tenth to twelfth day post-hatching.

Because the earliest erythropoiesis is that of cells derived from the ventral blood island, this thesis work included an examination of the prehatching embryonic cells in an attempt to correlate their shape and ultrastructure with those of the larval splenic cells. In addition to various other morphological analyses, the cells were studied in terms of the number of yolk platelets present at various stages. This is in keeping with the finding of Cameron (1941) that the primitive erythrocytes of Amblystoma

jeffersonianum change developmentally in size, shape, and yolk platelet number, in a manner so specific that the generations may be specified by determining these factors.

For purposes of structural comparison, some additional studies have been performed on the erythrocytes of the neotenus adult.

Experimental approach

Both whole and lysed cells have been examined using a variety of morphological techniques. Phase contrast microscopic evaluation of whole cell shape as a function of larval age and/or splenic size has been used to follow statistically a progression of cell morphology from sphere to ellipse. Shape analyses have been enhanced by the use of scanning electron microscopy of isolated cells, and correlations with marginal band configuration facilitated by both standard and high voltage transmission electron microscopy.

To substantiate the identification of the varied cell shapes observed in the spleen as normally occurring stages of development, it is of interest to know whether they may be found in the circulating blood as well. To evaluate this, the morphologies of the erythroid cells in the larval circulation have been studied by direct observation of the flowing blood and by scanning electron microscopy of circulating cells fixed both after isolation and in situ.

Interestingly, observations of axolotl microcirculation show the deformability seen in other nucleated erythrocytes

by Bloch (1962), Gaehtgens, Will, and Schmidt (1981a, 1981b), and Koyama et al. (1982). Scanning electron microscopy of the circulating axolotl erythrocytes fixed in situ seems to add to the data on deformability. Such observations on circulating cells in the gills of the living larvae have excited a keen interest in the hydrodynamics of nucleated blood flow, but a variety of circumstances have precluded more thorough analyses.

Phase microscopic observations of lysed cells at the various stages of cellular morphogenesis, made by Dr. Cohen with the student's help, showed pairs of dots in points of several cytoskeletons, indicating the possible presence of centrioles. This suggested the need for further appraisal of marginal band conformation, and led to extensive electron microscopic studies.

Of course, the most definitive observation of erythroid morphogenesis would be the viewing of the same cell throughout its differentiation. To this end, various methods of culturing the splenic erythroid cells have been attempted, using the methods of Duprat and Flavin (1976) as a starting point. This has enabled some preliminary observations of fields of differentiating cells, with promising but incomplete results to date.

Summary of major objectives and technical approaches

The approaches mentioned above were designed to fulfill the following objectives:

A. The overall goal was the determination of the progression of marginal band formation and the concomitant sequence of erythroid cell morphogenesis from spherical erythroblast to elliptical erythrocyte, and the elucidation of any correlation between the two.

B. In pursuit of this multi-faceted objective, it was deemed important to determine the three-dimensional shape of the cells at various times, and to learn the stage at which flattening actually takes place. The scanning electron microscopy contributed toward this goal. At the light microscope level of shape determination, additional experiments were performed on slowly rotating fixed cells in viscous Polybed 812, and living cells in methyl cellulose (Carolina Biological).

C. In the study of the forming marginal band, the goal of determining centriole presence and microtubule arrangement was aided by transmission electron microscopy, including high voltage, on cytoskeleton whole mounts. This aim was also furthered by immunofluorescence microscopy with anti-tubulin as the first antibody, to confirm the presence of tubulin and define the location and arrangement of the microtubules in the developing marginal band at the various stages of cellular morphogenesis.

Because microtubules have been shown to disassemble at low temperatures, cells were exposed to such temperatures and then rewarmed to determine whether the marginal band

could be disrupted by such exposure, and, if so, whether this would affect cell shape.

D. Culturing of splenic erythroid cells was attempted in the hope of clarifying the exact sequence of the sphere to ellipse progression, the role and time of appearance of any intermediate forms, and the time of flattening of the cells.

E. In order to verify the general applicability of the results on larval axolotl splenic cells to other amphibian erythroid cells, the work was expanded and clarified, as appropriate, by study of circulating larval cells, adult erythrocytes, and Xenopus tadpole cells. Initially, research had centered on primitive axolotl red cells, in part because of Cameron's (1941) stating that the number of yolk platelets could be used as an indication of the stage of erythroid differentiation, but preliminary studies and a literature search suggested that the study of splenic cells might be more fruitful. However, results obtained with the primitive cells have been used to confirm and extend those with the splenic cells.

F. A broader practical objective of this study has been the development of a convenient experimental system for examination of cytoskeletal development and cellular morphogenesis.

Materials and Methods

Thus the study of microtubule formation and cell morphogenesis during axolotl erythropoiesis utilized the following techniques: determination and employment of optimal media and mechanisms of animal storage, growth, staging, and manipulation; evaluation of cell shapes by application of a variety of visualization and evaluation procedures to both circulating and isolated cells; study of microtubule appearance by preparation plus cytological and ultrastructural analysis of cell cytoskeletons; investigation of the effect of cold exposure on splenic erythrocytes; and attempts to culture immature cells in the hope of observing shape development.

The Media

All animals were generally maintained in the water in which they arrived or in commercial spring water (usually Poland) purchased from a grocery. (A discussion of the advantages of using a dilute salt solution and of water quality in general may be found in Armstrong, Duhon, and Malacinski (1989).)

Salt Solutions

A variety of media and salt solutions were tested to determine the optimal one(s) for preserving the erythroid cell morphology. (See results section for details.) The salt solutions used included Holtfreter's and Steinberg's:

Holtfreter's Solution: (Brothers, 1977)

<u>Solution A</u>		<u>Solution B</u>	
NaCl	3.5 g	NaHCO ₃	0.2 g
KCl	0.05 g	H ₂ O	500 ml
CaCl ₂	0.1 g		
H ₂ O	500 ml		

Autoclave separately if sterility is desired. Mix 1:1 for use. Adjust pH to 7.4 with NaOH. (Some workers prefer 7.7).

Steinberg's Solution: (Brothers, 1977)

NaCl	3.4 g
KCl	0.05 g
Ca(NO ₃) ₂ · 4H ₂ O	0.08 g
MgSO ₄ · 7H ₂ O	0.205 g
Tris Buffer (Base)	0.56 g
H ₂ O	To 1 liter total
1.0 N HCl	To pH 7.4, about 4 ml.

Sterilize by Millipore filtration if needed.

Amphibian Ringer's (used for adult axolotl blood):

KCl	0.14 g
NaCl	6.50 g
CaCl ₂	0.12 g
NaHCO ₃	0.2 g
NaH ₂ PO ₄ · H ₂ O	0.012 g
H ₂ O	To 1 liter total

It was determined that, although blood released from the circulation would clot, no anti-clotting factor was necessary to prevent splenic erythroid cells from doing so. When required, 20-40 U/ml heparin was added, the larger amount being used for adults.

These tests led to the use of Wolf and Quimby Amphibian Medium (Gibco #350-1835) when the cells were to be used for a fairly lengthy counting, and to Holtfreter's, usually containing 0.1% glutaraldehyde, when the cells were to be fixed. In general, when the experiment was to involve cell lysis, the spleen was opened directly into the lysing medium.

Lysing medium

Cell lysis was carried out with Cohen's (1978) detergent-containing microtubule-stabilizing medium (lysing medium, LyM) consisting of 100 mM PIPES (piperazine-N-N'-bis (2-ethane sulfonic acid)), 1 mM $MgCl_2$, 5 mM EGTA (ethyleneglycol-bis-(b-aminoethyl ether) N,N'-tetraacetic acid), 10 mM TAME (p-tosyl arginine methyl ester HCl), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), freshly added, and either 0.6% Brij or 0.4% Triton X-100 at pH 6.8. The stabilizing solution with no detergent, used for rinsing cytoskeletons, is referred to as PEM, after the component initials.

The organisms

Axolotl embryos

As mentioned in the introduction, axolotl embryos may be obtained from the Axolotl Colony at Indiana University (c/o Dr. George M. Malacinski, Department of Biology, Indiana University, Bloomington, Indiana 47405). The embryos can be staged easily because their jelly coats are transparent. The embryos, usually received at an early gastrula stage, may be stored at 1°C for up to a month, with consequent interruption of growth. They resume normal development upon restoration to 18°C. (See Results--Observations on embryo viability, below.) After about four to five weeks at low temperature, the viability of the embryos begins to decrease. About a month prior to desired use, they are transferred to 100mm Petri dishes or finger

bowls and kept in spring water at 18°C, where development proceeds normally. (According to Bordzilovskaya et al. (1989), successful embryo development has occurred over the temperature range 10°C–28°C, but most laboratories use 18°C–20°C for axolotls of all ages. Little is known about the natural history of the axolotl in the wild.)

Maturation and Staging

The embryos proceed from gastrula to hatching in 20 to 24 days at 18°C. Photographs and sketches by Schreckenberg and Jacobson (1975) and Bordzilovskaya and Dettlaff (1979) were used in staging the embryos, except during the early work on primitive erythrocytes, prior to finding these articles. In the latter case, careful descriptions of the following embryonic features were recorded: embryo length, degree of gill and dorsal fin development, and eye maturation. These notations were later utilized retroactively to arrive at the stages. Both sets of investigators based their series on the work of Harrison (1969 republication) with Ambystoma maculatum, but Schreckenberg and Jacobson did their work at 18°C, whereas Bordzilovskaya and Dettlaff used 20°C. An updated, thorough discussion of axolotl staging, not available during the bulk of the experimental work, may be found in Bordzilovskaya et al., 1989. A particularly useful aspect of their paper is the provision of a method for prediction of stage progression at different temperatures in the 16° to 25°C range. They also give a table comparing their staging with

those of Schreckenberg and Jacobson and of Harrison. A brief synopsis of the staging, as used in this work, is given in Table 1.

Table One
SUMMARY OF STAGING AS USED IN THIS WORK

<u>BD Stage</u>	<u>SJ Stage</u>	<u>Time</u>	<u>L(mm)</u>	<u>Features</u>
36	36-37	130 (5 1/2)	7.1	Short sprouts of gills. Straight body axis. Elongated fin. Eye bulge visible.
37	38	177 (7)	7.5	Gills longer; point ventroposteriorly.
38	39	178 (7)	7.9	Gills sprout filaments. Eyes pigmented. Fins extensive.
39		220 (9)	9.0	1st gill: 2 pairs of filament sprouts; 2nd and 3rd: 3 prs. each. Eyes and skin more pigmented.
40		240 (10)	9.3	Gills longer, with more filaments: 4 pr. on 1st; 6-7 on 2nd and 3rd.
41	40	265 (11)	10.0	Longer gills with more, longer filaments. Increased pigmentation. Larva turns onto belly, then hatches.
42		296 (12)	10.5	Gills extend beyond forelimb buds. Mouth outlined.
43		342 (14)	11.3	Mouth broken through.

(Time is in hours from first cleavage. Approximate equivalent number of days is given in parentheses below each hour indication.)

(BD Staging was done at 20°C, and SJ Staging at 18°C.)

See Bordzilovskaya et al. (1989) for stages prior to 36; no source has been found for staging after stage 43. In this thesis times subsequent to Stage 40 are given as "days post-hatching."

Thus one sees that according to the staging described by Schreckenbergr and Jacobson (at 18°C), hatching occurs during stage 40; in the description by Bordzilovskaya and Dettlaff (at 20°C), it occurs in stage 41. By the latter scheme, stage 43 begins on the third day post-hatching, and it seems probable that all of our splenic studies are carried out during or after that stage.

Where there was a conflict between Bordzilovskaya and Dettlaff and Schreckenbergr and Jacobson, the staging by the former was used. With embryos, physical features and length were used, and the time of development was ignored. After hatching, however, the age was given as days post-hatching, and no attempt was made to determine stage.

Removal of intact larval spleen

By about day 2 or day 3 post-hatching, each larva develops a spleen which is visible to the naked eye as a tiny red dot underneath the skin on the left side slightly ventral and caudal to the gill. From then until it joins the circulation (about day 12) the spleen exists as a closed, bright red sac, which may usually be pinched off intact with a pair of watchmaker's forceps. Thus the developing erythroid population may be studied in isolation, without "contamination" from blood cells of other origin, such as the blood islands.

Usually, spleens are usable from about day 4 post-hatching, when they are about 0.3-0.4mm in length, until day 10 or 11, when they may exceed 1.0mm in length. In a

majority of axolotls the spleen begins visibly to connect to the general circulation about day 12 post-hatching.

When ready for use, the axolotl larvae are anesthetized with tricaine methanesulfonate (MS-222, or Finquel), 1:5000, and viewed with a dissecting microscope. Their spleens may generally be seen and evaluated through the skin, so that only those animals with suitable spleens are used. (The others recover nicely when removed from the anesthetic.)

When the experiments involve unhatched embryos, they are released from their jelly coats by gentle pulling with two pairs of watchmakers' forceps and placed into 1:10,000 Finquel. It should be noted that hatched larvae require a higher Finquel concentration than do the embryos, usually at least 1:5000 for the former, and 1:10,000 for the latter.

The procedures developed for removal of the intact larval spleen involve the careful removal of the skin and fascia with watchmaker's forceps to expose the gastrointestinal tract. A pair of forceps with hand-sharpened points is then used to grasp the base of the spleen where it is attached to the stomach. This sac-like structure usually remains intact until it is opened into the appropriate solution by gentle tearing with two pairs of forceps. Whole erythropoietic cells, which are already heavy with hemoglobin, settle fairly quickly in any of the normally used solutions; if placed into lysing medium, settling of the lysed cells takes longer.

Studies with circulating cells

For studies with circulating cells, blood was released from either the gill insertion or the heart, using a simple severing of the gills or body with a Teflon-coated razor blade, or a pinching-off of the gills with forceps. The blood was then allowed to pump onto a slide into Amphibian Ringers or Holtfreter's medium (sometimes with 0.1% glutaraldehyde). Blood of unhatched embryos flowed freely out of body when gills were cut; after hatching, blood tended to clot when gills were cut. This may be due to developmental appearance of clotting factors or to greater sensitivity to Finquel-induced bradycardia.

Adult axolotls

To determine differences as well as similarities between larval and adult axolotl erythroid cells, an adult was requested from the Axolotl Colony and was used within a few days of arrival. (Adults provided by the Colony are those which have outlived their breeding usefulness.) After partial anesthesia with 1:1000 Finquel, it was killed by decapitation. Circulating blood was collected in heparinized (20 U/ml) Amphibian Ringer's, and was washed in additional Ringer's with three centrifugations and resuspensions. Aliquots were placed on polylysine-coated cover slips and formvar-covered grids and prepared for immunofluorescence and scanning electron microscopy. (See below for details.) Pieces of spleen, liver, and kidney (all possible hemopoietic organs) were put into 2.0%

glutaraldehyde in 0.1M Sorenson's phosphate buffer, pH 7.4; in addition, some pieces of each organ were partially lysed in triton-based lysing medium before fixing in 2.0% glutaraldehyde in PEM. Fixed tissues were washed in buffer, post-fixed in 1% OsO_4 in the same buffer, washed, dehydrated, infiltrated, and embedded in Polybed 812 (Polysciences).

After it was noted that the adult erythrocytes in Amphibian Ringer's were predominantly discoidal, rather than elliptical, when viewed by phase microscopy, a smear of undiluted blood was examined. No difference in cell shape was seen (But see Discussion).

Xenopus laevis

In order to compare erythroid cell shape in the axolotl with that of another amphibian, tadpoles of Xenopus laevis were studied briefly. Tadpoles staged within a narrow interval by the supplier were obtained from Nasco (Fort Atkinson, Wisconsin) and were kept in spring water at 18°C until they were at stages judged from the literature to be useful for looking at erythropoietic cells. Closer estimates of stages were made with the help of the Normal Table of Xenopus laevis (Daudin) by Nieuwkoop and Faber (1967). Criteria employed were those of length and external features, as given in the plates and tables, with particular attention to gill appearance, number of intestinal revolutions, xanthophore location and appearance, and degree of hindlimb development.

For phase contrast microscopic observations of both intact erythroid cells and cytoskeletons, blood was obtained from circulation (either from the gills or by cardiac puncture), liver, and/or spleen.

Direct observations of circulating blood

Anesthetized axolotls resting in a pool of anesthetic in a 35mm petri dish were observed using the Zeiss Model IM35 inverted phase microscope. Lightly pigmented areas of the gill or ventral surface were selected for circulation studies. In properly chosen areas the blood flow could be seen clearly. Successful photography used a (Phase 1) high focal-length 32x lens and Kodak T-Max3200 film, with the automatic exposure mechanism of the camera set for ASA 3200, and the light source transformer set for maximum illumination ("12"). Clear micrographs were obtained only from capillaries where the flow velocity was relatively slow. Such capillaries were also used for calculation of cell velocity, with the added criterion of maximal observable length within the viewing field. Timing was with a stop watch. Length was measured by switching the view to the video monitor and making measurements on the enlarged image.

In order to compare observed cell deformations with those in the literature, which often stated the circulation velocity, it was thought that it would be useful to know the cell velocity in the axolotl larvae. Several attempts were made to evaluate the more typical, faster flow by recording

flow on the Panasonic AG-6050 VCR at a speed setting of 2 hours (per standard VHS tape), then playing it back at the slower setting of 12 hours or 24 hours. This unfortunately proved futile because of the instability of the images during such playback (a characteristic of the machine).

Cell Counting

Erythrocytes were recognizable in phase microscopy by their reddish-orange hue. As appropriate, they were measured, their shapes were evaluated, and any yolk platelets present in prehatching cells were counted.

For counts of erythropoietic cells of different shapes, cells were deposited into either Holtfreter's solution or Wolf and Quimby medium, with or without 0.1% glutaraldehyde. Promptly counted cells without fixative had apparent shapes indistinguishable from those with fixative. Counting was generally done with cells in a hanging drop to prevent the cell flattening over time which could occur on a glass slide; controls, in which a hanging drop was inverted after counts were taken, showed no difference between this method and use of an immediately-viewed hanging drop. With care, movement of the microscope stage was possible without rearrangement of the cells, and the use of cell counters facilitated nearly continuous viewing of a drop of cells without losing track of which had or had not been evaluated. The microscope used was a Zeiss phase microscope with a long-focal-length lens, either 16x or 32X.

During counting at the microscope, the cell counts were entered directly into a Tandy TRS-80 Model 100 Portable Computer (Radio Shack, Fort Worth, Texas) and later uploaded to an AST Premium/386C desktop computer (AST Research, Inc., Irvine, California). The data were sorted and analyzed with Paradox and Quattro Pro (both by Borland International, Scotts Valley, California); statistical assessments were performed using Minitab Data Analysis Software and Handbook (Minitab, Inc., State College, Pennsylvania). [Other software without which the thesis could not have been completed were Microsoft Windows and Microsoft Word (Microsoft Corporation, Redmond, Washington), Mace Utilities (Paul Mace Software, Ashland, Oregon), and Norton Utilities (Symantec, Cupertino, California)].

Cytoskeleton Preparation

Cytoskeleton preparation was with lysing medium, LyM, and either Brij or Triton X-100, at pH 6.8. To avoid twisting of the marginal band, fixative was generally omitted for the phase contrast light microscopic studies. The erythrocytes of this species are large (20-25 μm long axis) and the marginal band relatively thick; thus both are easily viewed in phase contrast. Cells with yolk platelets retained them after lysis.

Whole Mounts

Cytoskeletons were prepared as whole mounts for viewing in the transmission or high voltage electron microscope as follows: Grids were coated with 0.35% (silver film), 0.5%,

or 0.8% (gold film) formvar in ethylene dichloride. After drying, the formvar side of the grid was coated with polylysine (Sigma P-1399, MW 260K; 1%) by exposure for 20 min., followed by four washes with water. Grids were placed onto the grid support of Fullam's EFFA grid centrifuging tube, and the cylinder screwed into place. In most cases, the support was covered with a small piece of Parafilm prior to adding the grid; this seemed to help prevent formvar breakage with wide mesh grids. Four drops of the lysing medium were added to keep the grid moist.

One or more spleens were opened into 0.5 ml of Brij-based lysing medium in a well of a Boerner-Thomas Micro Test Slide; glycerol was added to 5% final concentration. The lysing red blood cells were transferred to a centrifuge tube, and the well in which the spleens were opened was washed with the lysing medium. Centrifugation was at the top speed of the clinical centrifuge for 5 min. This resulted in adhesion of the cytoskeletons to the coated grids. Each grid was removed from the centrifuge tube with self-closing anti-capillary action watchmaker's forceps, where it remained for processing. (In this procedure, and in every method involving cells or cytoskeletons on grids, the grids were held in forceps which were placed on moistened filter paper in 100 mm Petri dishes; the dishes were covered between manipulations and fluid changes. This was done to avoid drying of the samples.) Each of the following fluid changes involved wicking the fluid off with

bibulous paper and replacing it by a single drop added to the grid: PEM rinse twice, 5 min. fixation with 0.05% glutaraldehyde and 1% formaldehyde in PEM; water rinse twice; 30 sec. uranyl acetate, 0.1% in water. After air drying, each grid was screened with the phase contrast microscope to determine appropriateness for TEM.

Viewing of grids was in the Hitachi HS-8 at 50 kV or in the Hitachi H600 at 75 or 100 kV, the latter being particularly useful in observing detail with thicker films. Various methodological aspects which should be noted here include the use of thicker-than-usual formvar, the centrifugation of the cytoskeletons, and the use of a very brief exposure to dilute uranyl acetate in the negative staining. These modifications were found empirically to yield the most satisfactory results for the following reasons. The use of the conventional formvar, from an 0.25% solution, gave a film whose thinness under the weight of numerous cytoskeletons led to frequent breakage in the electron beam; on the other hand, the thick film achieved by use of 0.8% concentration was too dense for viewing of detail in most cases. Ultimately, a solution of 0.35% or 0.5% was found to be the most effective. Centrifugation resulted in a greater number of cytoskeletons adhering to the grid and a lower degree of marginal band twisting than did natural settling. Use of brief staining with very dilute uranyl acetate was essential to avoid obscuring detail with excess metal deposit.

High voltage electron microscopy

In hopes of seeing the cytoskeleton with some depth of focus, and of clarifying the presence, orientation, and connections of centrioles, whole mounts of splenic erythrocytes, prepared as above, were taken to the NIH High Voltage Electron Microscope Facility (Biotechnology Resource, Center for Laboratories and Research, New York State Department of Health) in Albany. They were examined at 1 million volts.

Many of the interesting cellular structures were viewed at different tilts of the microscope stage, with the degree of tilting chosen to optimize stereo viewing. The graphs of optimum tilt angles by Hudson and Makin (1970) were used for this. The paired prints prepared from the high voltage negatives were mounted according to the stereo viewing guidelines of Dr. Min J. Song at the Albany facility (personal communication).

Scanning electron microscopy

Isolated cells

With the goal of looking at the cell shape and surfaces of isolated erythroid cells from various sources, they were prepared for scanning electron microscopy. Grids coated with 0.35% formvar were treated with 1% polylysine, usually for 20 min. A drop of 0.1% glutaraldehyde in Holtfreter's medium was placed on a grid, and a spleen opened into the drop, so that the erythropoietic cells were released. Alternatively, circulating pre-hatching or larval cells were

released into the drop by cutting the axolotl open at heart level with a razor blade, and allowing the circulating cells to flow out. They were permitted to settle for 20-30 min., after which the fluid was drawn off and replaced with 2.5% glutaraldehyde in Holtfreter's. Any phase photography for correlative purposes was done during this period. Fixation was continued for 15 min., after which the grid was washed and dehydrated. Critical point drying was with the Tousimis SAMDRI Model PVT-3B. Grids were coated with gold-palladium in the Hummer sputter coater for 2-5 min., giving an approximate thickness of 200-400 Å, and examined in the Hitachi H600 TEM/SEM (called the H6001A when used for SEM), generally using a 25 kV beam acceleration.

Erythrocytes in situ

In the special case of scanning electron microscopy of circulating erythrocytes in situ, gills of anesthetized axolotls were pinched off with forceps and placed in 2.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After 60 min., the gills were washed, then dehydrated in graded series of ethanol; critical point drying was in the Tousimis SAMDRI. (Unmounted larval gills are, of course, very small, and after fixation they are fragile. It was found that the best way to critical-point dry them was to place them, at the 50% or 70% ethanol step, in a small square of lens paper, after which the paper was quickly but securely folded and put into the Tousimis specimen basket. Dehydration and drying were continued with this package. After drying, the

paper was opened and the beige gill pieces could be lifted gently off the white paper.) The gills were affixed to grids using double-backed tape, and carefully opened under the dissecting microscope. They were coated for 4 min. in the Hummer sputter coater and viewed in the Hitachi H6001A.

Transmission electron microscopy

In order to look at the fine structure of the various erythroid cells and cytoskeletons, they were prepared for transmission electron microscopy. For circulating pre-hatching erythrocytes, circulating larval cells, and adult cells, gill embeddings were prepared: gills were pinched off and placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. In some cases the gills were subjected to partial lysis prior to fixation or simultaneously with light fixation in 0.1-0.2% glutaraldehyde in LyM (subsequently referred to as "lyse-fix"). Fixation with 2.5% glutaraldehyde followed. In all cases, post-fixation in 1% OsO_4 in the same buffer was followed by washing, dehydration in a graded ethanol series, infiltration, and embedding in Poly/Bed 812 mixture (Polysciences, #-08792). Thin sections, cut on the Porter-Blum microtome MT II, were viewed in the Hitachi HS-8 or H600. For splenic cells whole spleens were embedded, or released cells were allowed to settle onto a polylysine-coated Permanox slide which had been scored with a gel cutter. The steps from fixation through embedding were then carried out on the slide, frequently with a second slide placed on top of the Polybed

during hardening. When this worked well, the hardened Polybed containing the cells would simply lift off the slide, and the cells could be viewed in the thin embedment with the phase microscope, enabling selection of cells to section. The scoring assisted in localization. The same procedure was used for splenic cytoskeletons, with lysis taking place on the slide, in the hope that the selectivity so gained would enable the specific sectioning of pointed cytoskeletons with possible dots/centrioles.

The electron microscopy was performed with either the Hitachi HS-8 at 50 kV or the Hitachi H600 at 75 kV.

Immunofluorescence

Immunofluorescence studies were deemed necessary in order to confirm the tubulin composition of the marginal bands and to attempt to clarify its structure in immature cells. For these observations, spleens were opened into Triton-based lysing medium on ethanol-washed, polylysine-coated glass cover slips. Cytoskeletons were allowed to adhere for 10 min., rinsed with TAME-containing PEM, and fixed for 20 min. in 4% paraformaldehyde in PEM. After rinsing with PEM, subsequent washes between incubations were with PBS, pH 7.4. Blocking by incubation with 1% BSA for 30 min. at 37°C generally preceded a 30-min. (37°C) incubation with sheep anti-tubulin (Southern Biotechnology Associates, Inc. #7000-01). Final incubation was with fluorescein-conjugated rabbit F(ab')₂ anti-sheep IgG (affinity purified; Southern Biotech., #6010-02) for 30 min. After washing,

cover slips were mounted using 1:1 glycerol:PBS, and viewed in a Zeiss phase contrast microscope with epi-fluorescence illumination. (The filter arrangement, suited for fluorescein, consisted of a blue-interference exciter, wavelength 455-490; an FT 510 chromatic beam splitter; and a green interference barrier filter, wavelength 520-560.)

Effect of low temperature exposure on splenic erythrocytes

In order to determine the effect, if any, of cold exposure and rewarming on splenic red blood cell shape, pooled erythrocytes, from several larvae from the same clutch of eggs, were exposed to low temperature. Larvae used were five days post-hatching, having hatched within a few hours of each other, and were selected for relative uniformity of spleen size, about 0.6mm, and appearance. Six spleens were opened into five drops of Wolf and Quimby medium, and the cells mixed well. Small drops were placed on cover slips which were then inverted over Boerner-Thomas Micro Test Slides and sealed with a small amount of vaseline. One set of such hanging drops was placed in a sealed plastic bag in an ice slurry, and the other was left at room temperature. Cell shapes in both sets were evaluated and counted under phase microscopy both before and after two hours of such exposure.

Cold-exposed cells were re-counted one hour after being returned to room temperature. Controls were also checked for absence of shape changes.

In a second type of experiment, cells were counted in hanging drops suspended over wells as above. They were then refrigerated at 1°C for one hour or for two days, after which the cells were re-examined. Controls were treated the same but were kept at room temperature. Although the glassware used for the two-day control had been autoclaved, and the medium was sterile, the technique was not strictly aseptic, and the two-day controls became infected.

Injection of dye into spleen

In an effort to confirm that the larval spleen is a closed sac, not connected to the general blood flow, blue food coloring was injected into the spleen and observed for entry into the circulation. A hand-pulled glass pipet was filled and emptied by a micropipette mouth tube. Unfortunately, various efforts to make such injections through the animal's skin were unsuccessful because of its toughness, and therefore it was necessary to remove enough to expose the spleen. These manipulations were performed using a dissecting microscope, and the circulation was observed with both it and the Zeiss microscope. The axolotl was kept moist during this time with Finquel solution. Observations were limited to 15-30 min. because of the occurrence of some non-splenic bleeding. Although useful data were obtained by visual inspection, photographic recording of the results was thwarted by insufficient contrast of the blue dye against the axolotl skin pigments and organ color.

Erythropoietic cultures

In diverse attempts to determine the exact sequence of erythroid morphogenesis by culturing the larval splenic cells, a variety of media, cell distribution methods, and dishes were assayed. Both Wolf and Quimby Amphibian Medium (Gibco #350-1835) and Leibovitz L-15 medium , 50% or 100%, (Gibco #320-1415), with fetal calf serum (Gibco #200-6140) at concentrations of 0%, 10%, or 15%, were tested. Hy-Clone iron-supplemented bovine calf serum (#A-2151-D) was also tried at 10% and 15% concentrations. In all cases media contained 100 U/ml penicillin and 100 µg/ml streptomycin. Fungizone at 250 µg/ml was also tried but not used consistently.

Prior to dissection, larvae were washed successively in 1% sodium hypochlorite (Chlorox), 70% ethanol in Holtfreter's, and three separate dishes of sterile antibiotic-containing Holtfreter's, after which the spleens were removed using sterile technique. An attempt was made to improve sterility by washing stage 39 or 40 axolotl eggs as above and dejelling the embryos, placing them into 25% sterile Holtfreter's medium containing 50 U/ml penicillin and 50 µg/ml streptomycin. They were then allowed to develop until spleens were the desired size, and the larvae were treated as above. This was the method of choice after it was found to improve the percentage of uncontaminated cultures (See Neff, 1984).

Several approaches to cell apportionment were tried: pooling of cells from several spleens, with aliquots ranging from one to about a hundred cells placed into 35 mm Petri dishes, 16-well plates, or 96-well microwell plates; cells released from a single spleen opened into one of the above; spleens, one per dish, placed into 35 mm Petri dishes (which had been scratched to aid in keeping and locating cells in place), opened, and left with erythroid cells spilling out around each spleen. The last technique was the method which achieved the most success in terms of both apparent cell division and erythroid differentiation. The Bionique Chamber/Dish (Bionique Laboratories, Bloomingdale Road, Saranac Lake, NY 12983) was also tried; this had the advantage of being sealable to help preserve sterility and humidity, but the disadvantage of having glass, which was of course resistant to identifying scratches. Another approach used Costar Transwell dishes, which have 6.5 mm wells with a 3.0- μ m pore membrane between an upper and a lower compartment (Costar #3411). A few isolated erythroid cells were placed on the lower surface, and either a piece of liver or a whole but opened spleen was placed above the membrane, in the hope that erythropoietic factors from the organ would diffuse to the isolated cells and promote their differentiation while permitting viewing of only a few cells. This approach showed some promise.

Each culture was examined immediately, using the Zeiss inverted microscope model IM35, sometimes photographed using

the Contax model 137 camera, and placed at 18°C in a cubical dessicator (with distilled water instead of dessicant) to preserve humidity and aid sterility. In most cases counts of various cell shapes were made at this point, and other pertinent information, such as apparent cell health and any membrane distortion, were noted. After a minimum of an hour the cultures were examined again and the most promising one (in terms of plentitude of healthy but immature erythroid cells) was selected for video photography with a Dage-MTI 70 series video camera model 2640C9 attached to the microscope, a Conrac Model 2640 Monochrome Monitor, and the Panasonic AG-6050 VCR, using a recording speed of 48 or 72 hours (per six-hour VHS tape). (Note that during videotaping these cells were exposed to continuous light from the microscope, and to resultant temperatures equal to or above room temperature, despite the constant use of the air conditioner.) The next day, or the day after that, this culture as well as those left in the 18°C-incubator were examined and sometimes photographed.

Results

The research procedures described above have generated a body of informative data, much of which is micrographic in nature. A perlustration of the results has contributed to the goal of a further understanding of the development of non-mammalian erythroid cell shape and of the possible role of the marginal band in that process. An integrative construction has led to a model for the correlation of cellular morphogenesis and marginal band formation.

Such an holistic approach must await the discussion, however, and the results are presented in more or less chronological order as obtained. The data fall into the broad categories of embryo viability, media effects, frequency and micrographic features of various embryonic blood cells, statistical assessment of larval erythroid shapes, cytological and ultrastructural studies of those larval cells and their cytoskeletons, observations on circulating cells, temperature manipulations of isolated cells, attempts at culturing, and auxillary studies of adult axolotl and larval Xenopus erythrocytes.

However, it is felt necessary to insert a note on terminology of the erythroid cytoskeleton: Lazarides and Woods (1989) define the cytoskeleton of nonmammalian erythroid cells as consisting of three interconnected sections: the marginal band, a system of intermediate filaments, and a "plasma-membrane associated skeletal network" (p. 428), which they call the "membrane-skeleton".

Mammalian cells, they state, lose all components except the last. This thesis work has emphasized the marginal band and its role in erythroid morphogenesis, but as will be seen in the Discussion, all three cytoskeletal components seem important in the formation of the cellular shape, and, in the case of nucleated blood cells, in the maintenance of that shape during flow. Cohen et al. (1982a) define "trans-marginal band material (TBM)": "In terms of cellular location, the TBM is quite likely the nonmammalian counterpart of the 'membrane cytoskeleton' that underlies the mammalian erythrocyte membrane bilayer." This is further clarified in Bartelt et al., (1982), where, like Lazarides, the authors refer to the nonmammalian erythroid cytoskeleton as composed of "a marginal band of microtubules, a spectrin-containing network of trans-marginal band material (TBM), and intermediate filaments attached to the nucleus." (p. 278) In Nemhauser et al., 1983, however, "TBM" is abandoned without explanation and the term "SAC" is introduced for "surface-associated cytoskeleton". Thus SAC=membrane skeleton (MS). In this thesis, the two terms are used interchangeably, with some preference being given to the term "membrane skeleton" because it seems to be more commonly applied in today's literature. TBM, which, according to Dr. Cohen (personal communication), includes all non-marginal band cytoskeletal material visible in negatively stained whole mounts, is used only in citing references which use that term.

Observations on embryo viability

In order to facilitate optimal use of the Ambystoma mexicanum embryos received from the Axolotl Colony, their viability under cold storage conditions was assessed. Detailed records of axolotl development showed that the embryos, which usually arrived at the gastrula stage, may be stored for nearly four weeks at 1°C without significant insult to their ability to develop. Upon arrival, the embryos were transferred, in the water in which they arrived, to a large (600-800ml) beaker, which then was covered lightly with a petri dish and placed in a refrigerator kept at 1°C. After being transferred to 18°C (in Petri dishes or finger bowls), they resumed development. When put at 18°C immediately upon arrival, with no cold storage, an average of 94% of the embryos developed normally to hatching (See Table 2). After one to three weeks' cold storage, this average did not change significantly (90% in weeks 1 and 2; 89% in week 3). The first significant decline in fraction of embryos developing normally was seen in those refrigerated for 27 days; the average for days 27 to 35 was 59%. This decreased still further with longer storage; the average for those stored for more than 6 weeks was 37%. A slight (1-2 days) lengthening of the average time to hatching was also seen in those stored longer than four weeks. This is shown in tabular form in Table 2 below:

Table Two			
Days at 1°C	# embryos	Percent normal	Percent deformed
0	98	93.8	6.1
1	23	91.3	8.6
2	20	100	0
3	12	100	0
4	12	66.7	33.3
5	9	100	0
6	6	100	0
7	21	81.0	19.0
8	70	95.7	4.2
10	13	100	0
12	13	30.8	69.2
13	24	100	0
15	8	87.5	12.5
22	36	94.4	5.5
23	14	78.6	21.4
25	12	66.7	33.3
26	21	100	0
27	53	54.7	45.2
30	60	68.3	31.7
33	5	0	100
36	18	27.8	72.2
42	82	23.2	76.8
46	3	33.3	66.7
53	34	52.9	47.1
60	43	60.5	39.5
63	9	0	100

Summary by time period			
Days at 1°C	# embryos	Percent normal	Percent deformed
0	98	94	6
1-13	223	90	10
15-26	91	89	11
27-33	118	59	41
36-63	189	37	63

Deformities included (not all in the same embryo): failure to progress at all beyond stage 25-27; enlargement of head without comparable growth of body; curtailment of gill and eye development about stage 36-37; failure of body to straighten as it lengthened. In such cases the embryos rarely progressed to hatching. A minority of embryos hatched but with such abnormalities as a curved or twisted

spine, small or missing gills, undeveloped eyes. No internal organs or blood cells were examined in any of the deformed embryos or larvae. No attempt was made to correlate type or degree of deformity with days of refrigeration.

Unfortunately, although the data as presented in Table Two seem to present a fairly reliable guide to safe length of embryo storage at 1°C, a statistical analysis shows that the gathering of data was not as well planned as it should have been. In order to perform statistical analyses, the original data, recorded as number of normal embryos per total in a Petri dish, were used, with a separate entry for each dish. (It should be pointed out that the percentages in Table Two were calculated using numbers pooled from the Petri dishes of each time group. This was a grave error, from a statistical point of view, for which there is no excuse.) The results of a simplified data analysis are on the next page:

[Days stored = days at 1°C; No. PDs = number of Petri dishes whose embryos were evaluated for normal development]

Days stored	No. PDs	Mean percent normals	Std. Dev. mean	95% Conf. interval for the mean
0	7	0.94	0.05	0.80-1.05
1	2	0.96	0.06	0.70-1.10
2	2	1.00	0.00	0.80-1.20
3	1	1.00	0.00	0.60-1.30
4	1	0.67	0.00	0.30-1.10
5	1	1.00	0.00	0.60-1.30
6	1	1.00	0.00	0.60-1.30
7	2	0.80	0.15	0.55-1.05
8	5	0.96	0.41	0.80-1.10
10	1	1.00	0.00	0.60-1.30
12	1	0.31	0.00	-0.50-0.65
13	2	1.00	0.00	0.80-1.20
15	1	0.87	0.00	0.55-1.15
22	3	0.94	0.05	0.80-1.10
23	1	0.79	0.00	0.60-1.30
25	1	0.67	0.00	0.30-1.10
26	2	1.00	0.00	0.80-1.20
27	4	0.56	0.39	0.40-0.75
30	5	0.70	0.27	0.55-0.80
33	1	0.00	0.00	-0.35-0.35
36	2	0.27	0.04	0.05-0.50
42	6	0.23	0.19	0.10-0.40
46	1	0.33	0.00	0.00-0.70
53	3	0.53	0.09	0.40-0.80
60	3	0.60	0.06	0.40-0.85
63	1	0.00	0.00	-0.35-0.35

The data, as they stand, are so scattered that an application of statistics seems almost meaningless. Many of the standard deviations are zero only because there was only one sample! Nevertheless, this treatment does illustrate the dangers of poor planning of experiments; although a few data were gathered at each time point because the initial goal was to determine how long the embryos might be stored safely, more convincing numbers would have been recorded by using fewer time points with more dishes of embryos at each. The one useful conclusion obtained here is that there does indeed seem to be a sharp decrease in healthy embryo survival after about one month's cold storage.

Because our studies used axolotl larvae only until about twenty days post-hatching, it is not known whether cold storage has any effect upon later development.

Effects of Different Media on Cell Shape

Because literature indicated that a variety of physiological solutions formulated for amphibians were used in diverse applications, several different formulations were evaluated in terms of their effect on axolotl erythrocyte morphology. Observations were made with the phase microscope, first on cells in hanging drops of the test solution, followed as appropriate with examination with the same drops lying as puddles on the cover slip. Control observations were made with the cells in 0.1-0.5% glutaraldehyde in Holtfreter's, in which ellipses were well-formed. Some distinctions between the reactions of circulating and splenic cells were found, and subsequent experimental procedures were modified accordingly.

After several minutes in 100% Holtfreter's solution, splenic cells (from 0.5-0.8mm spleens of 6-8-day-post-hatching larvae) developed crinkly membranes, and a few lysed; membranes of circulating cells crinkled fairly rapidly. Therefore, various dilutions of Holtfreter's were tested. In 10% solution the splenic cells rounded somewhat while retaining their basic shapes, and circulating cells tended to lyse. In 40% Holtfreter's, cells seemed to maintain shapes well, although some membrane crinkling and

lysis occurred after 45 minutes. There was, however, not much further change after 2 hours. In this case, spheres showed the most crinkling. In 50% Holtfreter's there was mild, very slow (10 minutes) lysis of ellipses; discs looked fine at 15 minutes, at which time there was some lysis of pointed ends of single-pointed cells. At 45 minutes, there was increased lysis of ellipses and some crinkling of spheres' membranes. However, the distribution of cell types seemed to remain the same. At 2 hours, cells looked the same as at 45 minutes. With circulating blood, there was slow crinkling of ellipse membranes, but discs seemed unaffected. Use of 60% Holtfreter's with splenic cells resulted in moderate cell lysis, with ellipses appearing especially vulnerable and spheres least so. Most cells, however, looked fine for up to 2 hours, and shapes were still easily interpretable. Circulating erythrocytes seemed to have a greater tendency to show membrane crinkling than splenic cells. In 70% solution, there was fairly extensive cell lysis.

In Amphibian Ringers, splenic cells retained their normal shape over several minutes, with only a slight crinkling of the membrane afterwards, but circulating cells had a very high incidence of shape distortion.

In Steinberg's, circulating ellipses seemed to have good shape retention but to generally have somewhat granular cytoplasm.

Using circulating blood from 4-week larvae, it was found that PBS, pH 7.4, resulted in partial lysis of a few ellipses, although most ellipses seemed unaffected. In this case, there were more spheres and discs than expected for that age, but it was not determined whether this was an artifact of the medium, or a peculiarity of that particular larva.

The most consistent shape maintenance seemed to be in full strength or somewhat diluted (to no less than 80% strength) Wolf and Quimby medium. This was thus the solution of choice for most unfixed long-term exposures.

Discs and slightly elliptical cells seemed most susceptible to non-optimal osmotic effects. In general, spheres seemed to withstand osmotic insult better than ellipses.

Studies on Embryonic Erythroid Cells (Stages 36-40)

Both the embryonic axolotl circulation and the larval spleen have a population of erythroid cells which includes a spectrum of cell shapes from spherical to elliptical. Because of Cameron's finding (1941) of a correlation between number of yolk platelets in a primitive erythroid cell and that cell's shape and stage of differentiation, these aspects were investigated and extended by cytoskeletal and ultrastructural studies. There was a reasonable but not perfect correlation between developmental stage (of erythrocytes) and number of yolk platelets: although most spheres had at least four yolk platelets, an occasional one

lacked them, and, contrary to the general trend, a few ellipses had as many as eight.

Unfortunately, however, the student must apologize for the fact that insufficient data were gathered to do more than generalize with a qualitative statement that there were a decrease in number of yolk platelets and a lessening in size variability with increasing age. Although the work on splenic cells supplanted that on the primitive ones, it would have been wiser to have continued the collection of counts of yolk platelets by larval stage and erythroid shape just long enough to be able to give some quantitative data. The erythroid cells of the primitive line did show a much greater variability in shape and size than did the splenic cells. In axolotls from stage 37 to 39, cells ranged in size from 15-33 μm (long axis) and nuclei from 7-13 μm ; yolk platelets varied from less than 2 μm to more than 8 μm but were usually less than 6 μm . Later in development (stage 40 and larval), erythrocytes had a more uniform size of 20-26 μm .

In the mixture of spheres, discs, ellipses, and partial ellipses from stage 38 embryos, diameters and long axes ranged from 15 to 33 μm ; yolk platelets varied in number from none to seventeen per cell, although most cells had eight or fewer. It was at this stage that the first marginal bands were found, in elliptical or disc-shaped cells, including some with eight yolk platelets. However,

considerable variability was seen from animal to animal, and within a given animal.

In most spawns studied, the primitive erythrocytes of Stage 39 had a somewhat narrower range of cell diameter, about 20-30 μm ; nuclear diameter was about 12 μm or larger. Yolk platelets per cell varied from none to nineteen, with fewer (than in Stage 38) having more than eight; cells containing 16 yolk platelets were all spheres or discs, and ellipses were found containing as many as eight. Lysis revealed fewer than 5% (all ellipses) with MBs.

In some groups hatching together, gill circulation at stage 39 or 40, as viewed in living animals with 10X phase, seemed to have more than 90% ellipsoid erythrocytes. Circulating erythrocytes liberated from these stage 39 animals were seen under phase to be partially elliptical but when lysed most lacked visible MBs; most also had very large nuclei, 22-26 μm diameter, i.e., not much smaller than the cells themselves.

Results with stage 40 and newly hatched larvae were also variable. Some had mostly elliptical cells nearly all of which had MBs; others had predominantly spherical cell populations with almost no MBs visible upon lysis.

Scrutiny of circulating cells indicated a lower general deformability than that seen in larval circulation (see below). Careful observation indicated that this was related to a kind of steric hindrance from the existence of the yolk platelets. Cells whose nubby appearance indicated the

presence of several of these often slowed to a near halt as they squeezed like knockwursts through narrowed capillaries. In addition, as noted above, many cells were larger than those seen at later (larval) stages, and this also interfered with smooth rapid flow.

Observations were also made on dividing cells in hanging drops: at first these appeared cylindrical, and could be made to roll in the Ringer's; as the furrow deepened to the point where the daughter cells appeared separated but still adjacent, both seemed to flatten. This impression was enhanced by their ability to flip in a discoid manner when flushed with fresh liquid.

The prehatching cells frequently had a nubby surface, probably due to the yolk platelets. Such cell features may be seen by phase microscopy (Figures 1 and 2) and in scanning electron micrograph Figure 3. Figure 1, a photograph of part of the gill of a freshly killed Stage 39 embryo, shows a wide blood vessel crowded with cells of varied shape and size. Cells a and b have a knobbed appearance, probably due to the yolk platelets. Figure 2, of isolated erythrocytes from a Stage 39 embryo, shows a rough-surfaced cell. Figure 3, a scanning electron micrograph, shows the extent of the rough, nubbed surface. In many early embryos, particularly those of Stages 38 and 39, smooth ellipses were conspicuous by their scarcity, both in observations with the phase microscope and on the grids

prepared for scanning. One of the very few mature-appearing ellipses which were found may be seen in Figure 4.

Lysing the cells enabled clearer viewing of the yolk platelets, which may be seen in Figure 5. Yolk platelets were found associated with their cells only in lysed cells with marginal bands; the (frequently numerous) bare nuclei remaining after lysis of immature erythroblasts lacked yolk platelets, and many small refractile objects, interpreted as platelets, were found scattered in the preparation. In cells with marginal bands such maneuvers as tapping on the slide or the pressure of the high-power lens on the cover slip showed that the yolk platelets move within the confines of the membrane skeleton. The variability of yolk platelet number was also observed more clearly in lysed than in whole cells.

An additional interesting feature seen in lysed cells (viewed in phase) of the primitive erythroid line was the presence of pairs of dots, interpreted as probable centrioles, in discs and ellipses. An example of each may be seen in Figures 5b and 5c. Such pairs of dots were seen frequently (in contrast to the case with larval splenic cells), and whenever found were seen to lie near the marginal band.

For electron microscopy primitive erythrocytes in gills of stage 39 and 40 embryos were prepared by the lyse-fix technique (Cf. Materials and Methods, TEM). This contributed to ease of viewing internal cell structures

without interference by hemoglobin. A variety of cells was seen, but it proved impossible to define all stages of erythroid development in the sections used. In most instances where microtubules were found, they were already bundled into a band, often visible at both ends. This was the case in cells with rounded profiles as well as in cells apparently more elongated (not shown).

Finding yolk platelets in the electron micrographs was problematic because of the thinness of the sections; perhaps (serial) sectioning of a complete cell would enable viewing of these structures. However, interesting details of the erythroblast fine structure are observed in Figure 6, in which distinct microtubules are bundled into a band whose component microtubules are quite clear. The Golgi apparatus is prominent as are the dense chromatin clumps. This figure has a centriole near the marginal band microtubules, and the microtubules comprising the centriole itself are quite distinct. In the cross-section of the marginal band are seen a few faint lines bridging the distance between individual microtubules. There are gaps in the cell membranes caused by the lysis, which enhances the visibility of the organelles by liberating the electron-dense hemoglobin. Some of these gaps are indicated by small arrows. Some breaches in the nuclear membrane have also resulted. Figure 7, which shows microtubules in a longitudinal section of the marginal band, also has a

mitochondrion with severely dilated cristae. Clumps of ferritin may be seen in the cytoplasm of these cells.

The number of microtubules was determined in cross-sections of several stage 39 erythroblasts: 110, 114, 108, and 111 in four different cells which were judged to be polychromatophilic using criteria set forth by Orlic, Gordon, and Rhodin (1965) and Tooze and Davies (1967) [See Discussion]. A single cell with a more homogeneous nucleoplasm and a paucity of organelles, thus tentatively identified as an orthochromatophilic erythroblast, had only 90 microtubules.

The mitochondria, ribosomes, endoplasmic reticulum, and Golgi complex contribute to defining this and similar cells as immature.

Thus, although the work on the embryonic axolotl erythrocytes was abandoned in favor of the larval splenic cells, these preliminary results indicate a general correlation between number of yolk platelets and stage of development, and an apparently early formation of the marginal band with probable involvement of centrioles.

Erythroid development in the larval spleen

During the early larval period, in which the spleen is visible as a red spot and is removable intact from the animal beginning with the third or fourth day post-hatching, the erythroblasts seem to undergo a sequence of morphogenesis from sphere to pointed teardrop, to flattened pointed cell, to flattened disc, to mature ellipse.

Statistically, there is an age-dependent decrease of spherical erythroblasts and a concomitant increase in ellipses. This sequence has been followed as a function of larval age by phase microscopy with details furnished by electron microscopy. Both whole and lysed cells have been examined.

Cell Shape Evaluation

Initial evaluation of the varied morphology of splenic erythroid cells utilized viewing by phase microscopy, with the cells either in hanging drops or in puddles on the slide. This approach demonstrated the expected variety of shapes, which were defined in a logical manner. Rounded and refractile cells which could be made to roll, ball-like, in a hanging drop, were identified as spheres, or S. Single-pointed teardrop-shaped cells and single-pointed cells which appeared more flattened were counted as singly-pointed, or SP. Double-pointed cells, usually rare, were designated as DP, double-pointed, whether they were flat or rounded. [Late in the course of the project it was realized that the rounded pointed cells (which had some refractility and seemed rotund or football-like in hanging drops) might be distinct stages, separate from the flattened ones (which were non-refractile and possessed a nuclear bulge, a circumferential edge, and thinned, grey-orange cytoplasm), but circumstances did not permit a reconstruction of the data. (See Discussion.)] Flattened, non-refractile cells which were circular or nearly so were named discs, or D.

Discs were easily distinguished from spheres because of their non-refractility, nuclear bulge, and fairly obvious circumferential edge; they also tended to have a larger diameter than the spheres. More elongated cells were called ellipses, E. Although axes were not specifically measured during shape characterizations, retrospective measurements from photographs indicate that cells enumerated as ellipses had an eccentricity (major axis/minor axis) of 1.5-3.2, with the majority about 1.9. Discs had a lower eccentricity, generally 1.0-1.2. Representative cells may be seen in the field of cells in Figure 8, in which the living cells are spilling out of a spleen in a culture (in 50% Leibovitz L-15, 10% fetal calf serum).

The spectrum of cell forms was analyzed in terms of variations between splenic and circulating erythrocytes, alterations in morphology as a function of larval age and splenic size, and distribution of possible intermediate structures such as pointed cells.

Splenic-Circulating Differences

A few otherwise apparently normal larvae were unusual in having spleens still isolated from the general circulation later than 12-13 days post-hatching. Both splenic and circulating erythroid cells were counted using several such larvae at (post-hatching) ages from 13 to 48 days. As may be seen in Table Three, larvae in which both splenic and circulating cells were counted showed variable differences in distribution of morphology between the two compartments.

As expected in a pool of younger cells, the splenic constituents generally show a weighting toward spheres. It should also be noted that with the exception of Larva #6, the percentage of pointed cells in the spleen was considerably higher than in the corresponding circulatory pool:

Table Three								
Circulatory vs. Splenic Cell Shapes in Older Larvae								
Lar No.	Cell Source	Tot ctd	%E	%D	%S	%SP	%DP	Tot %Ptd
1	Circ.	107	68.6	26.7	2.9	1.9	0.0	1.9
	Spleen	334	57.2	19.8	7.5	12.0	3.0	15.0
2	Circ.	249	79.5	19.3	0.8	0.4	0.0	0.4
	Spleen	179	31.3	48.6	12.3	5.6	2.2	7.8
3	Circ.	296	67.9	30.4	0.7	0.7	0.3	1.0
	Spleen	295	37.3	45.8	5.1	10.5	1.0	11.5
4	Circ.	223	84.7	4.5	9.4	1.4	0.0	1.4
	Spleen	255	51.0	18.8	22.4	6.7	0.8	7.5
5	Circ.	243	78.2	8.2	8.2	3.7	0.8	4.5
	Spleen	248	38.7	29.6	30.6	7.7	1.2	8.9
6	Circ.	269	61.4	9.7	23.8	4.1	0.7	4.8
	Spleen	275	27.7	27.7	39.3	3.7	0.7	4.4

Larval ages (days post-hatching) were:

No. 1:	18
No. 2:	13
No. 3:	15
No. 4:	17
No. 5:	48
No. 6:	26

Unfortunately, the student was unable to analyze the data in this table statistically, for which deficiency apologies are presented. Because each group of figures was taken from an individual larva, selected for an unusual characteristic, it seems difficult to make adequate comparisons and analyses.

Similar trends were seen in younger larvae (where the spleen's isolation from the general circulation was expected), but the differences between circulating and splenic blood in terms of cell shapes was not as pronounced as in older animals. In both the circulating and splenic compartments, however, there was a general, albeit irregular, decrease in percentages of spheres and increase in proportion of ellipses with increasing larval age. This is illustrated in the two following graphs (Text Figures 1 and 2, p. 58). (Note: In the graphs, as well as elsewhere, "larval age" refers to days post-hatching.)

Because the numbers of larvae of each age were small, the graphs give only an indication of general trends, rather than concrete evidence of inexorable shape progression. Nevertheless, the application of nonparametric statistics indicates some significance in the figures:

Kruskal-Wallis analysis of the changes in number of spheres in the circulation shows differences significant at the 5% level ($H = 14.37$). An examination of the sign confidence intervals indicates that the greatest differences lie between ages 6 and 9, with well-separated confidence intervals at a high confidence.* (Minitab Data Analysis Software and Handbook were used for statistical analyses.)

*Because nonparametric methods are based on ranking of medians, rather than on means and standard deviations, the degree of confidence is strongly dependent on number of observations, and it is rare to derive an exact confidence.

AGE	N	MEDIAN	ACHIEVED CONFIDENCE	CONFIDENCE INTERVAL
4	3	30.40	75%	25.50-35.29
5	3	31.38	75%	24.73-35.34
6	6	33.18	78%	26.79-40.00
			97%	15.58-40.53
7	4	31.54	88%	18.89-44.21
9	5	10.38	94%	9.24-21.78
12	2	35.80	50%	33.30-38.30
17	2	9.42	50%	8.03-10.81

When applied to the values at these ages, the Mann-Whitney Confidence Interval and Test indicates a significant difference at 1.37%. The point estimate for the difference in their medians is 18.88; the confidence interval is 96.4% for the difference 5.1-30.2.

A similar analysis applied to the spheres of the spleen shows the existence of differences significant at the 5% level by Kruskal-Wallis.

AGE	N	MEDIAN	ACHIEVED CONFIDENCE	CONFIDENCE INTERVAL
4	3	52.67	75%	47.62-54.74
5	3	31.21	75%	24.64-36.89
6	6	34.97	78%	30.77-44.64
			97%	23.31-54.03
7	4	35.77	88%	25.00-46.54
9	5	22.04	94%	14.96-36.69
12	2	19.42	50%	17.42-21.42
17	2	22.35	50%	20.15-24.55

In this case, the Mann-Whitney Confidence Interval and Test shows the difference between age 4 and age 9 medians to be significant at a p value of 3.7%, with a point estimate of 29.75, and a 96.3% confidence interval of 10.9-39.8.

Among the ellipses in the circulation, Kruskal-Wallis analysis shows existence of significant differences at the p 5.0% level.

AGE	N	MEDIAN	ACHIEVED CONFIDENCE	CONFIDENCE INTERVAL
4	3	25.90	75%	17.65-34.23
5	3	23.94	75%	21.55-25.27
6	6	19.44	78%	13.68-25.77
			97%	11.89-43.61
7	4	23.45	87%	15.79-31.11
9	5	47.32	94%	29.21-58.49
12	2	39.60	50%	36.29-42.91
17	2	84.75	50%	81.18-88.33

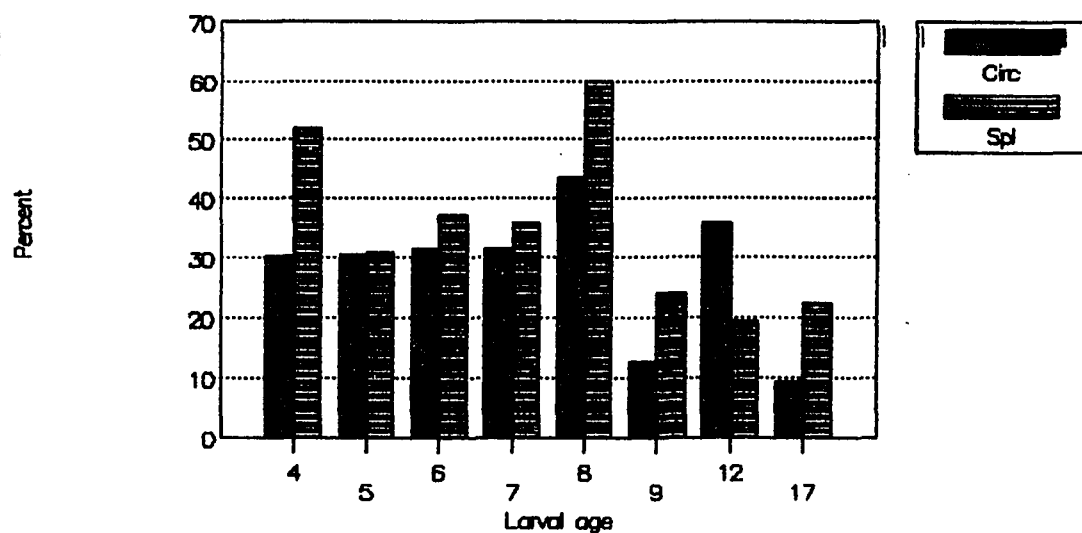
Use of these sign confidence intervals for medians led to the choice of ages 6 and 9 for application of the Mann-Whitney Confidence Interval and Test; this shows that the difference between the medians is significant at $p = 1.37\%$. The point estimate for the difference is 26.70, and the 96.4% confidence interval for the difference is 3.7-39.5.

For ellipses in the spleen, the Kruskal-Wallis test indicates differences significant at $p = 1.0\%$ ($H = 17.09$). Again, the sign confidence intervals were used to select data pairs for the Mann-Whitney Confidence Interval and Test.

AGE	N	MEDIAN	ACHIEVED CONFIDENCE	CONFIDENCE INTERVAL
4	3	13.35	75%	10.94-19.05
5	3	21.21	75%	15.94-22.33
6	6	10.79	78%	8.92-18.35
			97%	7.25-36.82
7	4	14.23	88%	10.60-17.86
9	5	32.24	94%	24.03-39.81
12	2	46.69	50%	43.86-49.53
17	2	50.98	50%	46.99-54.98

The difference between ages 6 and 9 is not significant at $p = 5.0\%$ (but is significant at 5.52%). The difference between ages 7 and 9 is significant at 2.0% (point estimate 18.31; 96.3% confidence interval 6.9-28.4), and that between ages 4 and 9 is significant at 3.7% (point estimate 17.63; 96.3% confidence interval 5.0-28.9).

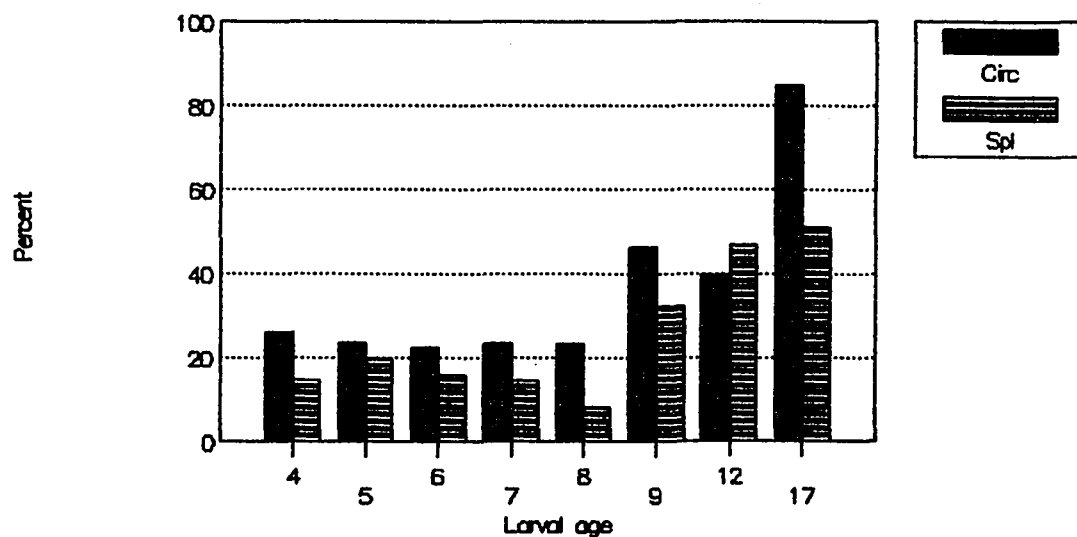
Change in Percent Spheres by Age In Circulating and Splenic Blood



Text Figure 1

Text Figure 2

Change in Percent Ellipses by Age In Circulating and Splenic Blood



In ten larvae over seventeen days post-hatching, in which the spleen was visibly connected to the circulation, average circulating cell percentages were: 77.7% ellipses, 16.8% discs, 2.5% spheres, 2.4% single-pointed, and 0.4% double-pointed. (Because of the spleen's connection to the circulation, no attempt was made to tabulate its contents.)

This manifestation of the separation of the two erythroid populations prior to the connection of the spleen to the circulation is given further credence by the fact that blue food coloring injected into the spleen remains there as the circulating blood continues to course through the vessels. Unfortunately, it proved impossible to photograph this phenomenon due to a lack of sufficient contrast between the dye-injected spleen and the surrounding tissue, including the dark gastrointestinal tract and the epidermal pigmentation. However, observation of the spleen and its surroundings during approximately 15 minutes after injection of the spleen showed no leakage of the dye from the spleen. The observations were brief because of the necessity for using opened larvae. (See Methods.) This experiment was performed four times, with 9- to 10-day-old larvae which had large (approx. 0.9-1.1 mm) spleens apparently isolated from the circulation.

In addition, the spleen's development in the living larva could be watched in lightly pigmented animals. Several such larvae were followed over several days, usually beginning with day 4 post-hatching. Usually, on day 10 or

11 a small finger could be seen to protrude from the spleen; this was followed by a second projection within the next twenty-four to thirty-six hours. These lengthened over the ensuing two to three days until they joined the main circulation. This progression is roughly diagrammed in Text Figure 3. Of course, the shape of the living spleen is not a perfect oval, nor do the presumptive vessels emerge from exactly opposite sides of the organ. As in the cases of erythroid cell maturation and other aspects of development, there are individual variations in the timing and manner of connection of the spleen to the general circulation.

Text Figure 3

DEVELOPMENT OF SPLENIC CONNECTIONS TO THE CIRCULATION



Four to five days after larval hatching, spheroid spleen elongates, usually becoming ovoid.



Spleen continues to elongate over several days.



Small protrusion appears from one, or both, ends, about day 10-11.



Protrusion lengthens, lying against stomach.



Further extension occurs; by day 11-12, this is seen at both ends.



Both splenic extensions lengthen and narrow, and begin to look very much like blood vessels.



Final connection to the circulation usually occurs about day 12-13 post-hatching.

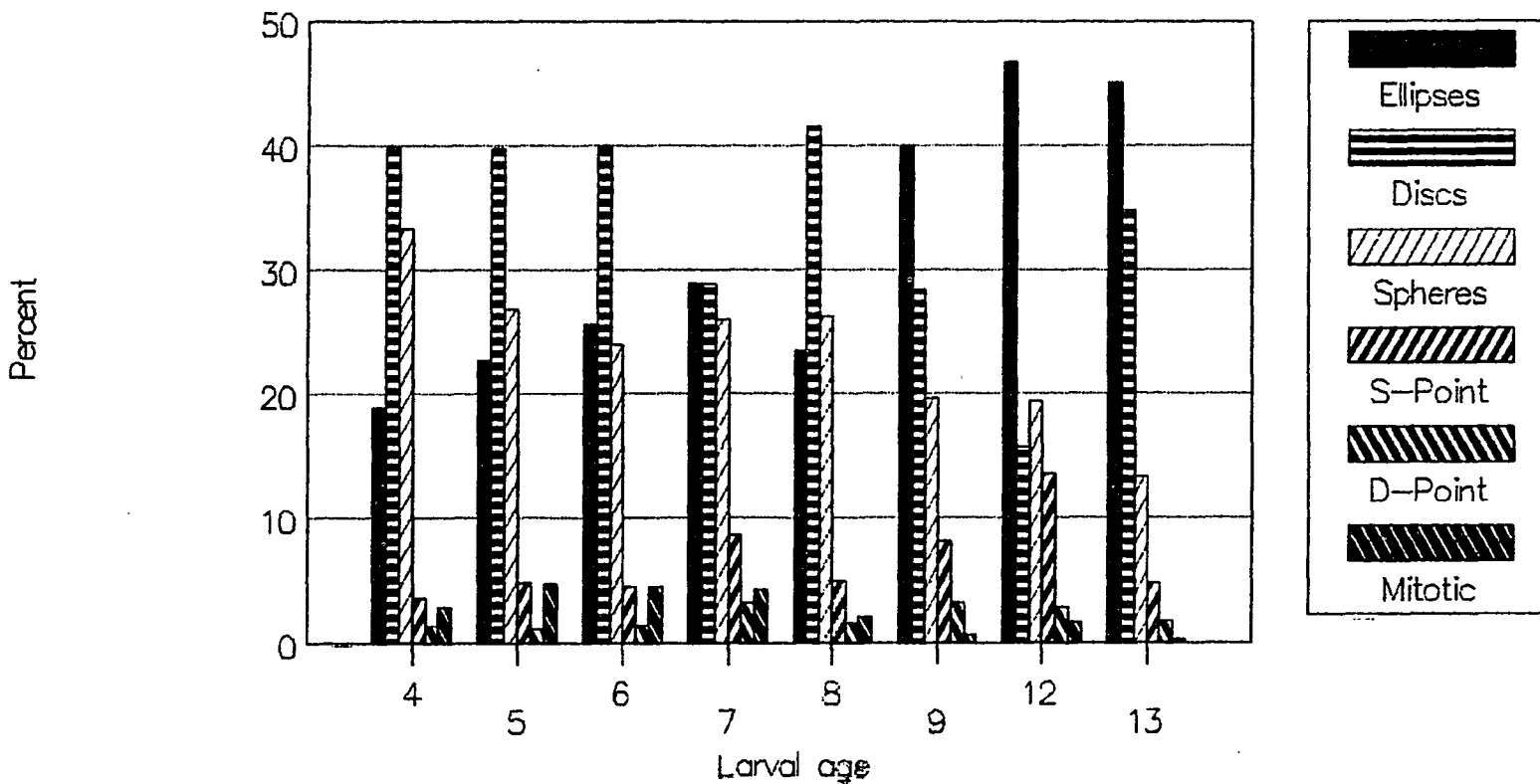
Distribution of cell shapes by larval age

Because the spleen is a closed system during early larval development, emphasis was placed on evaluation of the variation of cell shapes during early development, from post-hatching age of three days through day twelve or thirteen, when the spleen usually connects with the circulation. With the hope of determining a developmental progression of erythrocyte morphology, counts of cell shapes from 105 spleens of larvae ranging in age from four to thirteen days were performed. (Spleens of three-day-old larvae proved too small to obtain.) Such spleens ranged in size from 0.3 to 1.3 mm length but varied greatly in plumpness. [In fact, some of the longer (1.1 mm) spleens were extremely slender and probably contained a lower volume than considerably shorter, but much plumper, organs.] There was only a rough correlation between larval age and splenic length, with two four-day larvae having 0.6mm spleens, and one nine-day larva possessing one of only 0.3mm. The correlation might have been somewhat better had it seemed feasible to estimate splenic volumes. Counts of total cell contents were performed on small spleens with few cells, and a minimum of 200 cells were counted when spleens contained a sufficient number to do so.

Text Figure 4 shows the cell shape distribution as a function of larval age. In general, the percentage of ellipses increases with increasing age and size, while that of spheres decreases.

Averages of Percentages of Various Splenic Cell Shapes

Text Figure 4



The proportion of discs shows no regular variation, but rather seems somewhat stable as a function of larval age. A significant number of mitotic cells is seen only at young ages and in relatively small spleens.

It therefore seems senseless to perform statistical analyses on the percentages of discs, and the occurrence of pointed cells in apparent waves also seems to preclude them from fruitful analysis. Therefore, statistical procedures are limited to spheres and ellipses.

A Kruskal-Wallis test for the data on spheres in the spleen indicates a significant difference at the 0.5% level ($H = 25.16$). A one-way ANOVA indicates a significance at the 1% level ($F = 4.24$). Both sign confidence intervals (seen below) and t-intervals indicate the usefulness of comparison of ages 4 and 7 with ages 9, 12, and 13.

AGE	N	MEDIAN	MEAN	STDEV	CONF	CONF INTER
4	10	35.27	33.02	6.55	89%	32.98-36.89
					98%	23.13-38.06
5	8	23.93	26.93	12.25	90%	20.21-26.09
					97%	19.76-31.21
6	11	24.64	24.03	5.18	93%	19.73-29.18
					99%	17.21-29.62
7	19	25.00	25.97	8.55	94%	22.76-31.88
					98%	22.75-32.10
8	4	26.46	26.28	9.33	88%	17.09-35.14
9	21	19.87	19.63	6.92	92%	17.29-22.73
					97%	16.62-23.33
12	17	20.19	19.48	6.93	86%	17.37-21.93
					95%	13.10-24.13
13	5	12.46	16.33	9.54	94%	7.194-29.35

Application of the Mann-Whitney Confidence Interval and Test shows significant differences between age 4 and age 9 ($p = 0.04\%$), age 12 ($p = 0.06\%$), and age 13 ($p = 1.2\%$), and between

age 7 and age 9 ($p = 4.7\%$), but not between age 7 and ages 12 and 13 ($p = 8.2\%$ and 11.3% , respectively).

The same analyses applied to the ellipses show significant overall differences by Kruskal-Wallis, and indicate the value of comparisons of similar ages.

AGE	N	MEDIAN	MEAN	STDEV	CONF	CONF INTER
4	10	19.80	19.51	4.70	89%	17.90-22.35
					98%	17.08-24.23
5	18	21.77	22.69	11.09	90%	15.97-24.47
					97%	15.94-24.83
6	11	22.66	25.65	7.70	93%	20.49-33.10
					99%	18.95-36.82
7	19	27.71	28.93	8.88	94%	23.48-34.91
					98%	22.41-38.10
8	4	23.90	23.50	9.80	87%	11.15-35.04
9	21	40.29	40.07	8.15	92%	38.29-42.23
					97%	36.87-42.32
12	17	45.29	46.69	5.71	86%	42.91-50.40
					95%	42.73-50.65
13	5	38.43	45.09	14.11	94%	31.53-66.57

Application of the Mann-Whitney Confidence Interval and Test indicates significant differences between age 4 and ages 9, 12, and 13, as well as between age 7 and ages 9, 12, and 13.

As shown in Table Four, the occasional appearance of large numbers of pointed cells seems to lack a strict correlation with age. Rather, at all larval ages, there are individual axolotls with a relatively high percentage of such cells.

Table Four
Individual Larvae with Large Proportions of Pointed Cells

<u>Larva No.</u>	<u>Age</u>	<u>%SP</u>	<u>%DP</u>
1	4	12.5	0.0
2	4	6.1	9.1
3	5	11.8	1.7
4	5	16.7	2.4
5	6	15.8	0.7
6	6	11.9	4.4
7	7	14.6	0.0
8	7	14.8	11.8
9	8	12.0	3.4
10	9	27.9	13.9
11	9	10.6	4.2
12	12	13.6	2.9
13	19	12.0	3.0

Once again, the student lacks the expertise to statistically analyze this data.

Microscopic Verification of Cell Shapes

Although phase microscopy was adequate for a numerical evaluation of the occurrence of various larval cell shapes, it was necessary to use additional micrographic methods to examine the details of both whole and lysed cell structure. Figure 9 shows scanning and phase micrographs of intact cells of the varied shapes found. Micrographs of the corresponding cytoskeletons, immunofluorescent (lysed) cells, and, when available, whole mounts (lysed) viewed with transmission electron microscopy are included as well. As seen here, the various shapes seem to be authentic forms, consistently appearing regardless of the method of preparation. Each type of micrograph shows unique features of the cells: the surface topography in the scanning electron micrographs, the general overall morphology (as used in cell counts) in the phase pictures, and various

characteristics of the marginal band in the lysed cells. The size variations are also of interest: all cells except those in the TEM column are at the same magnification (see bars on lower right). [An elaboration on this will be presented in the Discussion.]

Scanning microscopy of splenic erythroid cells

The scanning micrographs of the isolated splenic erythroid cells show the same variety of shapes recorded above. In addition to confirming general shape, they permit some assessment of the surface features of the cells. An overview of many cell types lying on formvar may be seen in Figure 10; although there are several single-pointed cells, there is no obvious double-pointed one in this micrograph. Several spheres are present. A scanning view of cells in situ in an opened larval spleen may be seen in Figure 11 (Done by Dr. Laura Twersky, to whom thanks are due, and previously published in Ginsburg, Twersky, and Cohen, 1989).

Such micrographs also present various significant features of the cells, including the folds and ridges in the membranes of immature cells, of possible significance in circulation (See Discussion), nuclear bulges, and variety of points. Figure 12 shows the larval erythroid diversity of both general morphologies and surface details. In 12b a sphere with "excess" membrane may be studied. The spheres in 12a also have a somewhat shaggy surface; in contrast, some spheres, not shown, lack extraneous membrane but possess an interesting pattern of surface pits. As

expected, the spheres have smaller dimensions than more mature cells: the range seen in this set of SEMs is from 7.2-9.6 μm diameter. The same dimensions apply to each sphere of the pairs apparently completing mitosis in Figure 12a.

A useful aspect of the scanning microscopes is their ability to tilt the specimen. This is particularly helpful in confirming points of cells. In Figure 12d the nuclear hump and spheroid character of the cell may be seen, whereas viewing of only 12c, at a tilt of 50° from 12d, gives a rather flattened impression. This humped cell is particularly interesting because it seems to have acquired/be acquiring points at either end prior to any flattening. Although other views are not shown here, tilting of the grid holding the double-pointed cell of Figure 12e confirmed that each end was sharply pointed. Other views of double-pointed cells may be seen in Figures 12f and h, illustrating variations in degree of flattening and of point elongation. The single-pointed cell of 12i was also examined at severe tilts of the microscope stage, verifying that the point is neither an artifact of preparation nor an aberration of imaging. The limitations of this technique, however, may be seen in Figure 12b, in which the structure adjacent to the sphere was interpreted to be an ellipse which had become misshapen during preparation. Tilted views (not photographed), as well as the measured dimensions, indicated that it was in fact an

ellipse, but it turned out to be an artifact. A disk may be seen in Figure 12g, and an ellipse in Figure 12j.

Circulating cells: Varied shapes

In order to confirm the varied cell shapes as actually occurring outside as well as within the spleen, circulating erythrocytes were observed in the microcirculation of gills or unpigmented ventral areas of anesthetized axolotls. In narrow vessels, up to about 30 μm wide, and in wider vessels with rapid flow, the blood cells tend to align with their long axes parallel to the flow. In wide capillaries the cells tend to align randomly, often with several cells piled together in rouleaux-like fashion, with their long axes perpendicular or angled to the flow. (It should be noted that in many of the larger vessels the circulation is so rapid that individuals cannot be discriminated.) Rapidly moving cells are seen to undergo deformations as they enter smaller capillaries, course around bends, or encounter other cells or the capillary walls. The cell's bulge moves toward the leading end, and the following end folds along the long axis, parallel to the direction of flow. These cells resemble fish swimming stiffly along the vessel. In relatively slow flow, this fold is usually gentle and untwisted, as though the MB curved slightly inward from end to end. A twisting of the folded tail is seen (1) in rapid flow; (2) in extreme capillary narrowing; (3) when cells round corners. Such twisting or folding of the following edge causes an elliptical cell to appear pointed. An

apparently pointed cell frequently can be observed to resume its elliptical shape when it enters an uncongested wide area. It is therefore impossible to identify as singly-pointed any cell whose point is at the trailing end. An occasional cell observed with a solitary point at its leading end can be designated as single-pointed. Since the trailing point of an apparently double-pointed cell cannot be identified as such with any certainty, it is impossible to designate any circulating cell as double-pointed. In addition to ellipses and pointed cells, a rare sphere or disc can be observed. A sphere tends to move freely through the circulation, rolling like a ball. Discs, however, tend to become stuck in narrow vessels, indicating that they lack the deformability of ellipses. (Such an obstruction, or a simple narrowing of the vessel, could be observed to cause a temporary traffic jam among the following cells.) In only one instance has deformation been noted which was not along the lengthwise axis; in this case, an ellipse rounding a sharp bend folded parallel to its short axis with about one third of its length curved gently around the bend.

Some of the varied circulating shapes may be seen in Figure 13. (In each micrograph the direction of flow is indicated by the arrow.) Cells with following points which appear to be folds or twists (indicated by T) may be seen in (a), (b), (e), and (f); cells in (c) and (f) and the forward one in (e) may each have a leading point (P) as well. The presence of a forward point probably indicates the

possession of at least a single "true" point. There is a sphere in (a). The double-pointed cell in (d), with a fairly flattened appearance, may in fact be double-pointed.

Circulating erythrocytes ranged in length from 21 to 33 μm ; spheres measured about 19 μm diameter.

Measured velocities ranged from 323 $\mu\text{m}/\text{sec}$ to 45.5 $\mu\text{m}/\text{sec}$. These probably represented the lower end of the complete velocity spectrum, due to difficulties in determining those in the faster range; as mentioned earlier, in fact, flow in some areas of the capillaries was so rapid that individual cells could not be distinguished.

Scanning electron microscopy was performed on circulating cells from larval gills in an effort to clarify cell morphologies. Unfortunately, it proved difficult to open the gill circulatory vessels in such a manner as to preserve the location of the erythrocytes in them. The critical-point-dried blood cells, perhaps understandably, tended to scatter around the surface of the opened gill. Comparisons of the lengths of several cells from different preparations with measurements of unfixed cells show a shrinkage of about one-third.

Scanning electron micrographs of erythrocytes isolated from the circulation also show deformability of cells and pseudo versus true points. Ellipses as seen in Figures 14 are relatively smooth, but there is probably sufficiently extra membrane to allow twisting such as seen in Figure 15. This is also true of the slightly pointed cell in the center

of Figure 14, which was in fact shown to be an ellipse by tilting of the grid (not shown). Of additional interest in this figure are the several ellipses displayed at various angles and the possibly mitotic cell. The twisted cell in Figure 17 was found by varied tilting to be a simple ellipse (Cf. Figure 16). On the other hand, this set of figures (taken with the grid tilted at +23 and -23 degrees, respectively) shows a true double-pointed cell and a single-pointed cell lying on the grid bar. Other double-pointed cells may be seen in Figure 18. The points seem convincingly real in these views.

Cytoskeletal studies

Phase Microscopy

Because a prominent goal of the research was to attempt a correlation between the development of cell shape and that of the cytoskeleton, several morphological approaches were used to evaluate cytoskeletal formation. These included phase microscopy, immunofluorescence studies, and electron microscopy of both thin sections and whole mounts.

The phase microscopic studies of lysed axolotl splenic cells showed that the marginal bands, and thus the cytoskeletons, displayed the same variety of shapes as seen among the whole cells. In addition, a new objective arose from observations made by Dr. Cohen of pointed cytoskeletons possessing a pair of dots in or near the point. Such dots, rarely found in elliptical cytoskeletons, were postulated to

be centrioles. One objective of this thesis work was to clarify the nature of the dots.

This was pursued via both serial sectioning of monolayer cytoskeleton embedments and transmission electron microscopy of cytoskeleton whole mounts. Transmission electron microscopy of cytoskeleton whole mounts seemed to promise some possibility of quicker success than use of thin sections, and these cytoskeletons were viewed with both standard and high voltage electron microscopy.

Transmission Electron Microscopy of Cytoskeletal Whole Mounts

Transmission electron microscopy of the whole mounts of the cytoskeletons gave insight into the character and disposition of the cytoskeleton, including the membrane skeleton (MS) as well as the marginal band, their possible correlation with the shapes of the intact cells, the location of the nucleus, and the presence and nature of presumed centrioles. All cytoskeletal formations postulated to lie on the developmental path from sphere to ellipse were found among these whole mounts; these included possible spheres with nascent marginal band and MS, single- and double-pointed cells (frequently with structures interpreted as centriole pairs), discs, and ellipses.

An overview of several such cytoskeletons may be seen in Figure 19. Both single-pointed cells and ellipses are somewhat twisted, but the conformation of the marginal band and the disposition of the MS are clear. The naked nuclei

may be from spheres: presumably lysis of a sphere prior to its beginning synthesis of its cytoskeleton would result in a lone nucleus. It is, of course, difficult to discern the cytoskeleton at low magnification until a reasonable proportion of it is complete. At the electron microscope level, however, careful scrutiny may permit viewing of incomplete cytoskeletons.

As an example, it is possible that Figures 20 and 21 show cytoskeletons of spheres which are beginning to develop their membrane skeletons and marginal bands. At first glance, that of Figure 21 appears to be at a slightly earlier stage than does that of Figure 20, but the obscuring of part of the former by the grid bar makes this an uncertain conclusion. Note that in both cases, the microtubules seem to be emanating from a single site, rather than growing in more than one part of the cell, but there is no clear centriole pair visible.

Inspection of lysed erythroid cells at presumably later stages of maturation shows a wide variation of cytoskeletal shapes, presence of centriole-like structures in many cells, a high degree of marginal band and MS development, and evidence of extensive filamentous connections to the nucleus. In many, though not all, pointed cytoskeletons, possible centrioles were found in or near the points. In some instances, the marginal band has frayed so that individual microtubules have been negatively stained, with resultant clear structural visibility.

Microtubules in Figures 22 and 23 are rendered with particular clarity because of the peculiar twisting and lifting of the marginal band away from the rest of the cytoskeleton. Of additional interest in Figure 22 is the end of the cytoskeleton, seen enlarged in the insert. Microtubules of varying lengths are seen to extend outward, thus forming a tapered point. The ellipse seen in part in Figure 24 also has a partially disrupted MS, which facilitates its examination because there are areas where it is present only in a single layer, rather than the more usual collapsed double layer. Less sharply delineated but of interest because of the state of the microtubules at one pointed end is the cytoskeleton of Figures 25 and 26. The enlargement of the pointed end in Figure 26 shows microtubules which appear to be fraying but may instead be joining at the end of their morphological development.

An additional goal of the whole mount technique had been to determine whether or not centrioles were actually present in some single- or double-pointed cytoskeletons. The results were suggestive but not definitive. Many instances were found of structures whose size, orientation, and occurrence in pairs were consistent with their being centrioles. Examples may be seen in Figures 27, 28, and 34-39. The marginal band of Figure 36 seems to be fairly well bundled, with an apparently well-developed MS. Figure 28, however, has more dispersed microtubules near the point. The difficulty of identifying centrioles in a cytoskeleton

at low magnification is obvious from a glance at Figure 36. The dark blobs near the point (within the dotted box) might be thought to be artifacts until seen at larger magnification (Figures 37, 39) to have the characteristic shape and size of centrioles. The possible centrioles of Figures 27, 28, and 35 all have the right-angle orientation typical of centrioles, or an arrangement close to that; those in Figure 29 seem to be oriented end-to-end, but it must be remembered that this could be due to flattening during whole mount preparation. The staining of these specimens is too dense to permit viewing of the internal structure of the putative centrioles; such visualization was attempted with the high voltage electron microscopy.

The elliptical cytoskeleton seen in Figure 31 definitely lacks centrioles and has a compact marginal band with minimal fraying.

High Voltage Electron Microscopy

We were fortunate to be able to try to extend the TEM results with microscopy at the NIH High Voltage Electron Microscope Facility in Albany. Many of the same whole mounts of splenic erythrocyte cytoskeletons used for TEM were subjected to high voltage electron microscopy in the hope that this would enable a better resolution of the possible centrioles, and facilitate an examination of the relationship between the membrane skeleton (MS) and the microtubules. This proved to be the case to some degree, particularly with stereo pairs which give a three-

dimensional representation of both centriole-like structures and membrane skeleton conformation.

The compactness of the microtubules in an elliptical cytoskeleton, seen in the marginal band of Figure 31, is seen again in Figures 32 and 33. The latter shows filamentous aspects of the MS with particular clarity. Perhaps of greater interest, however, are the pointed cytoskeletons, with their tantalizing suggestions of centriole pairs as well as microtubules. In Figure 34 such structures are barely discernable, but the enlargement in Figure 35 shows not only the possible centrioles, but also some of their internal structure and plausible relationship to the microtubules. Note that some microtubules course around the proximal centriole-like structure, whereas others appear to enter or traverse it. Nearly all seem to converge on the distal figure. Another entity, seen in relation to the rest of the cytoskeleton in Figure 36, may be examined as a stereo pair in Figure 37 to gain a three-dimensional sense of some of the internal structure and that of the microtubules. This section of the cytoskeleton is further enlarged, with slight underexposure, in Figure 39, to enliven one's imaginative visualization of microtubular structure. Another conjectural centriole pair is seen in a stereo view in Figure 38.

Immunofluorescence

Splenic erythrocyte cytoskeletons treated with anti-tubulin followed by fluorescein-conjugated second antibody

show the expected heavy binding to the marginal band. The labeled marginal bands of the variously shaped cytoskeletons allow analysis of the band compactness at different developmental stages. As seen in Figures 40 a and b, the beginnings of the marginal band in the spherical erythroblast may be seen with immunofluorescence in a way not possible with other techniques. Here the microtubules appear to emanate from a single pole and follow the curvature of the sphere, much like the longitudinal lines on a (world map) globe (Thanks to Dr. K. Lyser for this simile!).

Individual, unbundled microtubules may also be seen in Figure 40d, which shows a double-pointed cytoskeleton, and in Figures 40c and h, single-pointed cytoskeletons. Microtubules are tightly packed into a uniformly fluorescent wide band in the double-pointed cytoskeleton of Figure 40e, a single-pointed one in Figure 40k, a disc in Figure 40j, and an ellipse in Figure 40l.

Transmission Electron Microscopy of Thin Sections

The attempt to find and analyze centrioles in the cytoskeletal embedments was suspended when the whole mount venture began to give fruitful data, and time, unfortunately, did not permit resumption of that perscrutation. This was indeed unfortunate, for only such an approach would have given convincing evidence for the identification of the centriole-like structures as true centrioles. However, thin sections as viewed in the

transmission electron microscope did succeed in giving further insight into the general structure of the erythroid cells of the larval axolotl spleen. Among the features seen in thin sections of cytoskeletons are the nuclei, the microtubules, and other members of the cytoskeleton, including the membrane skeleton (MS). Lysing and embedding apparently subjected many of the cells to distortion, as exemplified in Figure 41; this cell is of particular interest because of a centriole pair, seen enlarged in b and c. The proximity of the centrioles to a set of longitudinal microtubules is not analyzable because of the cell distortion, but they are close to a membrane skeletal remnant and were most likely near the periphery in the intact cell.

Micrographs of unlysed cells, Figures 42 and 43, illustrate the value of lysis but also reveal microtubules and ferritin granules.

Effects of low temperature on splenic erythrocytes

In keeping with the postulate that if the microtubules of the marginal band could be disrupted by exposure to low temperature, cell shape should be affected, such exposure did indeed cause some morphological changes in splenic erythroid cells, particularly in the ratio of spheres to ellipses. (As mentioned in the Methods and Materials, all experiments were performed with the cells in hanging drops in Wolf and Quimby medium, with the slides sealed to prevent evaporation. It was hoped that this method would avoid any

shape changes due to contact with the glass, as well as provide any short-term nourishment the cells might need.) The following are percentages of various shapes found in the same group of pooled cells from 0.6mm spleens of five-day-old larvae, after two hours at room temperature (about 22°C) and at 0°C (in ice water bath):

	<u>Ellipses</u>	<u>Discs</u>	<u>Spheres</u>	<u>S-Pointed</u>	<u>D-Pointed</u>
Room Temp	46.9	31.8	15.1	4.2	1.9
0°C	27.2	30.0	39.3	2.8	0.7

All of the cold-exposed cells that were counted as Ellipses or Discs, except for one ellipse, had a more rotund appearance than the room temperature cells enumerated as Ellipses and Discs.

When these cold-exposed cells were watched over the next hour as they warmed nearly to room temperature, no shape changes were noted.

Using 0.5mm spleens from 5-day-old larvae, three sets of red cells were counted, refrigerated for two days at a recorded temperature of 1-2°C, and recounted. In two cases, these showed a shape conversion to 100% spheres and discs from 56.1% and 75.5%, respectively; in the third one, where the original count included 43.2% spheres and 44.5% discs, only spheres could be found at the end of the cold exposure.

A one-hour cold exposure of pooled cells from three 0.65-0.7mm spleens from six-day larvae resulted in the

following shape changes, given in percentages of cells counted:

	<u>Ellipses</u>	<u>Discs</u>	<u>Spheres</u>	<u>S-Pointed</u>	<u>D-Pointed</u>
Pre-ice	57.6	6.3	23.4	7.0	5.7
Cold	8.9	40.0	34.8	8.1	8.1

Many of the pointed ones observed after hypothermic treatment were quite spheroidal rather than flattened.

A six-hour cold exposure of cells from 0.5mm spleens of 6-day larvae resulted in conversion of 22.7% spheres to 100% spheres and slightly ellipsoid spheres.

In all cases the uncooled controls did not undergo the shape changes, although the cells left at room temperature for two days became infected, with attendant membrane disruptions.

Larval splenic cultures

The culture attempts were moderately successful in that some erythroid cell differentiation, and possibly a small amount of proliferation were found to occur. The best data, promising though limited, came from old-fashioned visual observation and still microphotography. Many experiments were videotaped, but unfortunately the videotaping did not capture many hoped-for details. Even when undocumented, however, there was an overall general impression, even in the difficult-to-analyze video tapes, that splenic erythroid

cells do undergo morphogenetic changes and some proliferation in culture.

Besides the disappointment with the video technology, the single most vexing problem in the culture attempts was a high rate of infection, which, however, is common with amphibian cultures. Not surprisingly, this was lowest (as low as 30%) with whole opened spleens in Petri dishes and highest (as high as 90%) in sets using pooled splenic cells aliquoted to microwells. Transwell dishes also had a high rate of infection. Because interest was focused on cell proliferation and differentiation, rather than on possible characteristics of long-term cultures, apparently successful cultures were not continued for more than a few days. Thus the longest period during which cells were maintained was five days.

Because attention was centered on the question of changes within any particular culture, the many culture attempts were not tabulated systematically. However, as a general summary, cultures prepared included:

Cells from single or several pooled spleens, aliquoted to microwell plates: 150-200 separate cultures, using various media. These served as a preliminary medium-screening and showed that 50% Leibovitz L-15 or 80% Wolf and Quimby, each with 10% fetal calf serum, gave the best cell survival. For unexplored reasons, use of iron-supplemented serum resulted in excessive cell lysis. The use of 15% fetal calf serum also increased lysis over that seen with

10% serum. Under all conditions tested, a minimum of about thirty cells seemed essential for cell survival, and the few indications of cell division or differentiation were seen only with groups of more than seventy cells.

Pooled or single-spleen cells aliquoted to larger wells or to 35 mm Petri dishes: 25-50 separate cultures. In these cultures, only cells which remained grouped within a few millimeters of each other survived; "strays" tended to lyse. Wolf and Quimby medium, when used, rapidly turned a deep purplish-red, indicating an increase in pH. No proliferation or differentiation was seen in these sets.

Cells from single spleens placed in bottom chamber of a Transwell dish: 8-10 with whole, opened spleen in top compartment; 6 with liver in top compartment. Liver was not satisfactory as used, because the blood and tissue spread in the compartment, causing a shadow which hindered viewing of the lower compartment. Modest results seen by examination of a group of almost fifty cells which had been placed in the lower level of a Transwell dish with a spleen above included several mitotic cells and a small increase in cell number after about 14 hours in the incubator. In another of the dishes with a spleen above, conversion of a few spheres to ellipses was noted after about thirty hours; the intervening stages were not viewable due to technical difficulties. These results seemed promising for possible further pursuit. However, the dishes used were samples from the manufacturer, and it seemed advisable to improve the

methodology before purchasing additional (fairly costly) dishes.

Whole spleens in 35 mm Petri dishes: about twenty sets. By the time this was tried, it seemed that the best medium for culturing splenic erythroid cells was 50% Leibovitz L-15 containing 10% fetal calf serum. In such cultures, the spleens flattened as additional cells were released, resulting in more cells over a larger area than initially; checking the possibility of cell proliferation would have required counting, which was unfortunately not done. Changes in cell morphology were also seen. Results from two such cultures (using still phase microphotography) are seen in Figures 44 and 45. Figure 44a (taken shortly after explantation) and b (at 24 hours), shows a probable change in predominant cell shape from spherical to elliptical in an opened spleen (in a 35mm Petri dish) after 24 hours in culture. Many SP and DP cells, as well as ellipses, may be seen in b. A similar development is seen in Figure 45a and b. The second explant shows fewer ellipses at 24 hours than does the first explant, but there are many pointed cells and a possible mitotic one (45b).

Preliminary video results indicate that changes in individual cells do occur in culture and might be analyzable under appropriate conditions. Specific developments included: (1) A sphere which became single-pointed over about an hour, undergoing an ostensible struggle as the membrane bulged, retracted, and bulged, alternately until

the point seemed stable; (2) A sphere which became single-pointed with intervening "amoeboid" movements; (3) A single-pointed sphere which flattened while remaining single-pointed over the time span of one hour, 20 minutes, but did not change further over the next two hours before it moved out of the field; (4) A single-pointed cell which seemed to become elliptical over about nine hours.

Structure of Adult Axolotl Erythrocytes

Intact Cells

Although the focus of the work was on developmental questions, larvae do become adults, and it seemed appropriate to look briefly at the erythrocytes of adult axolotls. Three adult axolotls, which were no longer useful as breeders, were kindly provided by the Axolotl Colony, and their blood was examined by phase contrast microscopy. To my astonishment, the majority (est. 85%) of the erythrocytes were discoidal, rather than elliptical (Figure 46 a-b). With the thought that perhaps the suspending medium was having some strange effect, cells were examined in heparinized and unheparinized Amphibian Ringer's, in Holtfreter's, in Sorenson's, in Sorenson's with 2.5% glutaraldehyde, and as drops of undiluted blood. No difference in cell shape was seen in these cases. This was true of all the animals studied, but one adult, white pigmented, had virtually 100% discs. To evaluate viewing conditions, fresh Rana pipiens blood, obtained through the kind assistance of Heinz Wesch, was briefly observed, both

"straight" and in heparinized Amphibian Ringer's from the same stock as that used for one of the axolotls. These erythrocytes were "their normal happily elliptical selves".

At 30-33 μm diameter, the discs were larger than larval discs or ellipses. As seen in Figure 46b, the nuclei seem somewhat smaller and less elliptical than those of larval ellipses. Figure 46c, a scanning micrograph, gives the impression that in some cases, at least, the membrane "hugs" the nucleus rather closely, rather than tapering out to the rim caused by the marginal band. The rare ellipses seen in the scanning electron microscope looked much like larval ellipses.

Cytoskeletons

The generally discoidal character of the adult erythrocyte cytoskeletons is seen in both phase and immunofluorescent preparations (Figures 46 d-f). In both d and e one gains an impression of the marginal band's being less compact at one side of the disc than the other. Two elliptical immunofluorescent bands seemed frayed at both ends but compact in the middle.

Xenopus Larval Erythroid Cells

Cell Counts

Xenopus tadpoles were used for evaluation of erythroid cell shapes in order to determine whether the same apparent progression of cell forms, and the same intermediate shapes, might apply in an anuran species. Because the literature indicates that the liver and mesonephroi are the major sites

of larval erythropoiesis in anurans, the emphasis was placed on the red cells of the liver. (It must be kept in mind, however, that the liver is not an isolated organ like the larval axolotl spleen.) A few spleens were also analyzed. Although this was not done with a full series of Xenopus stages, and the sampling was too small and the count variability too great to give truly convincing data, the results suggested that there is indeed a change in the predominant cell shape as the animal matures. The larvae of only two stages (Nieuwkoop and Faber, 1967) were examined: Stage (St. in Table 6) 49 (11 animals) and Stage 58 (start of metamorphosis) (4 animals). Table five gives the average percentages found for the different shapes, using data pooled from the different animals. No statistical analysis was attempted because of the small sample sizes; although it would have been of considerable interest to perform a lengthy series of shape evaluations, it was felt that the simple indication of a possible shape progression was sufficient for the purposes of the studies at that time.

Table Five
Xenopus Erythroid Cell Shapes

St.	Ellipses	Discs	Spheres	Single-Point	Double-Point
in liver:					
49	37%	21%	29%	10%	3%
58	57%	31%	9%	2%	0%
in the spleen:					
49	13%	16%	67%	2%	1%
58	61%	28%	9%	2%	0%
in the circulation:					
49	65%	18%	15%	2%	0%
58	67%	24%	7%	2%	0%

These data show that the general trend is a decrease of spheres and increase in ellipses with tadpole development. The averages do not, however, indicate the great variability seen among individuals. An aspect of particular interest is the range of percentages of pointed cells in the liver of the eleven animals at Stage 49: 1%-26%, SP; 0%-10%, DP. Another is the difference between the percentages seen in liver and spleen in the same animal: the spleen frequently had more spheres and fewer ellipses plus discs than did the liver. Table Six gives the data from three Stage 49 individuals to show both the difference among heart (H), spleen (S) and liver (L) and the variability among animals:

Table Six
Xenopus Erythroid Cells in Different Organs

No.	Org.	Ellipses	Discs	Spheres	Single-Point	Double-Point
1	H	80%	16%	2%	3%	0%
1	L	62	21	5	12	1
1	S	0	0	94	0	0
2	H	50	33	13	3	0
2	L	29	33	30	5	1
2	S	34	23	36	5	1
3	H	67	30	1	1	0
3	L	39	34	18	6	2
3	S	14	33	51	1	1

(The same explanation for the lack of statistical analysis applies to this table as did to Table Five.)

Intact Xenopus Cells: Phase and Scanning Microscopy

Scanning microscopy confirmed the variety of cell shapes seen with phase. Only a single-pointed cell and an ellipse are shown (Figures 49 and 50), but spheres and a

double-pointed cell were also observed. The different cell shapes as seen with phase microscopy are shown in Figure 47 a and b.

Cytoskeletons: Phase Microscopy

Of course, far fewer cytoskeletons were examined than in the axolotl. However, two single-pointed ones were found to have paired dots in the point. One of these is shown in Figure 48. Unfortunately, immunofluorescence was not performed, but it is assumed that the results would have been comparable to those found with the axolotl cells.

Figure 1. Erythroid cells within gill of a freshly killed Stage 39 embryo. Note nubby appearance. Yolk platelets are visible in cell b. Cells seem to have a relatively large nuclear-cytoplasmic ratio. Phase microscopy. Bar = 10 μm . x750.

Figure 2. Erythrocytes isolated from Stage 39 embryo. Lower cell has rough appearance, probably due to internal yolk platelets. Upper cell is out of focus, so degree of lumpiness cannot be discerned. Phase microscopy. Bar = 10 μm . x430.

Figure 3. Two erythrocytes from Stage 39 embryo. Note knobby surfaces, particularly evident in the lower one. Scanning electron microscopy. Bar = 5 μm . x2000.

Figure 4. Smoother erythrocyte from same preparation as Figure 3. Scanning electron microscopy. Bar = 5 μm . x2000.

Figure 5. Cytoskeletons from circulating cells of Stage 39 embryos. Many yolk platelets (yp) may be seen in (a). Both (b), which has no apparent yolk platelets, and (c) have paired dots (arrows) near the marginal band. Phase microscopy. Bar = 10 μm . x1130.

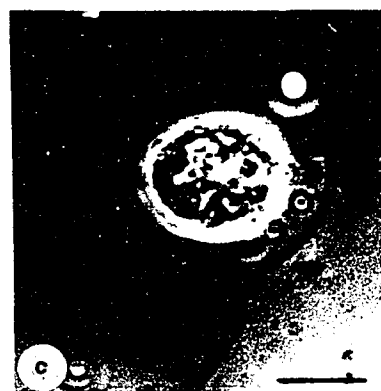
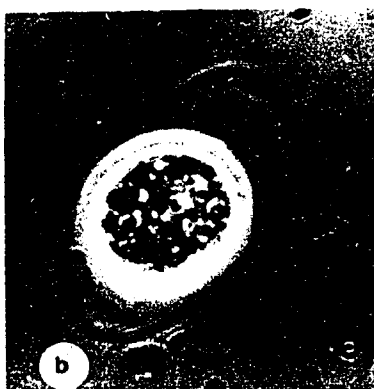
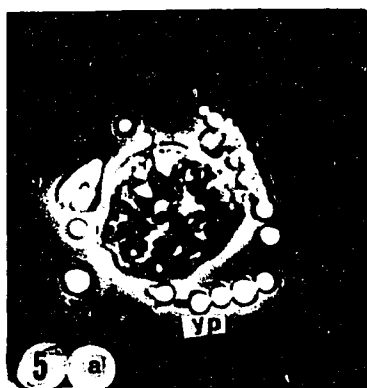
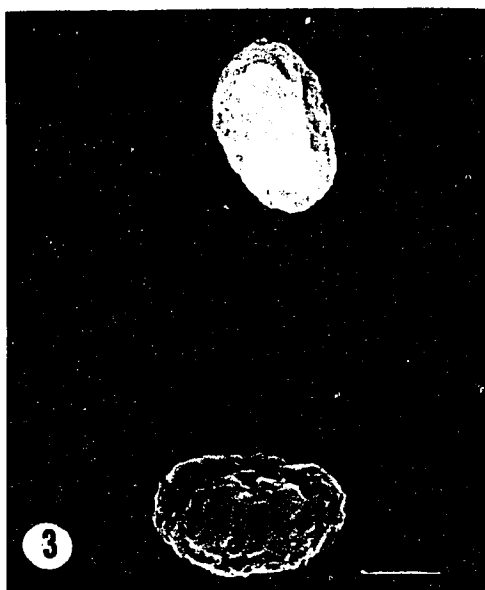


Figure 6. An enlargement of the centriole-containing end of an erythroblast, slightly underexposed to facilitate viewing of microtubules and nearby structures. In addition to the microtubules of the marginal band (M), the centriole (ce) and Golgi (G) may be seen clearly. Examination of the microtubules discloses some fine lines between some of them, possibly the bridges holding them together. Arrows indicate membrane discontinuities caused by the lysis. (r=ribosomes) Transmission electron microscopy. Bar = 0.5 μ m. X64,000.



Figure 7. A longitudinal view of microtubules in a lyse-fixed gill erythroblast. The mitochondrion (mi) is dilated. Ferritin particles (fe), both clumped and dispersed, may be seen. MT: microtubules. Transmission electron microscopy. Bar = 0.5 μ m. X 39,800.

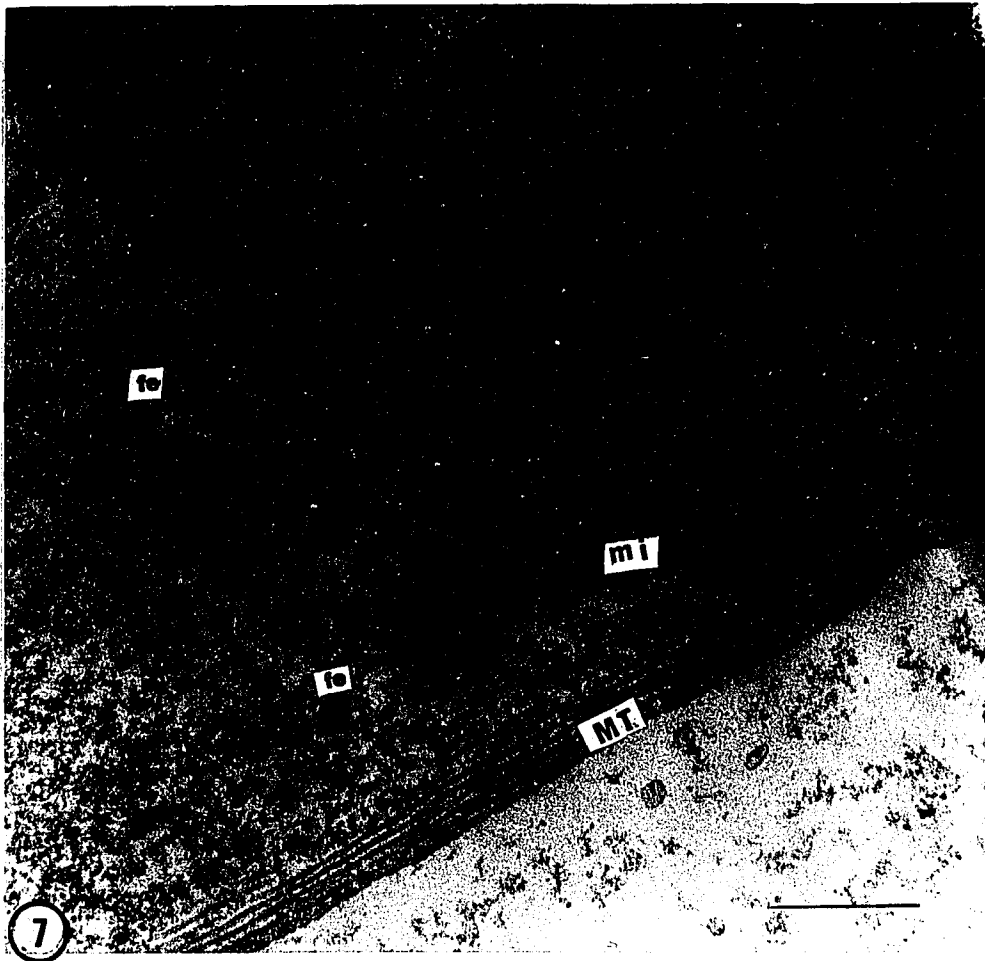


Figure 8. Erythroid cells of various morphologies spilling out of living spleen in culture (50% Leibovitz L-15 medium, 10% fetal calf serum). Spheres (S), double- and single-pointed (DP, SP), discs (D), and ellipses (E) may be seen. Note that the SP near the upper edge of the micrograph is much more refractile and spherical than the two other, more flattened, SPs. Lines in background are scratches on plastic dish. Phase microscopy. Bar = 100 μ m. X260.

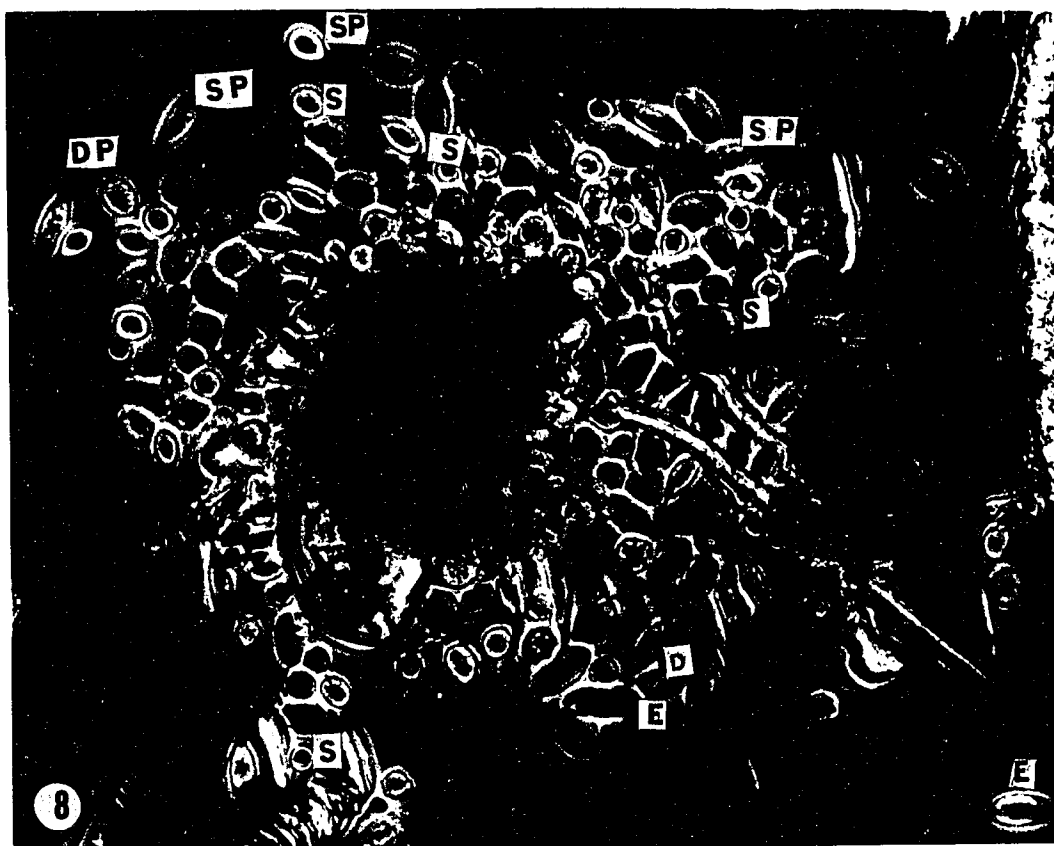


Figure 9. A micrographic table of the various erythrocyte morphologies found, arranged by method of viewing. SEM-Scanning electron microscopy; PHASE-Phase microscopy; LYSED-Phase microscopy of cytoskeletons; IM-Immunofluorescence, using anti-tubulin as the primary antibody, of lysed cells; TEM-Transmission electron microscopy of whole mounts of cytoskeletons. All cells were isolated from larval spleens by methods presented above. Bars = 10 μ m. X900 for first four columns; X1100 for TEM.

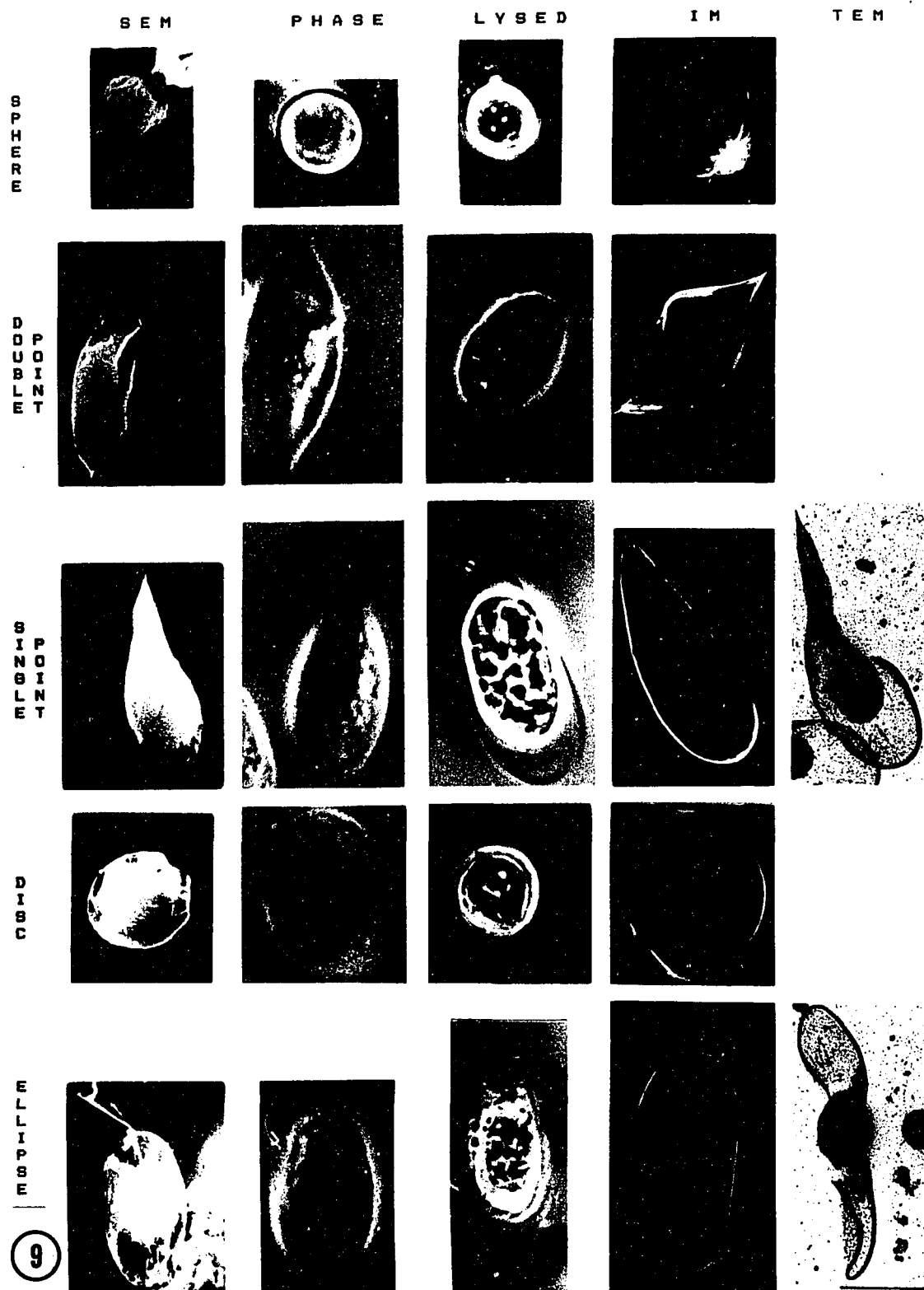


Figure 10. Splenic erythroid cells of varied morphology lying on formvar-coated grid. Readily recognizable shapes are spheres, single-pointed cells, a disc, and ellipses. Note mitotic cell near end of cytokinesis in upper left space. Scanning electron microscopy. Bar = 20 μm . X550.

Figure 11. Opened larval spleen (sp) lying on stomach (st). Cells visible in situ include spheres, single-and double-pointed, discs, and ellipses. Used with permission of Dr. Laura Twersky, to whom thanks are due (previously published in Ginsburg, Twersky, and Cohen, 1989). Scanning electron microscopy. Bar = 50 μm . X200.

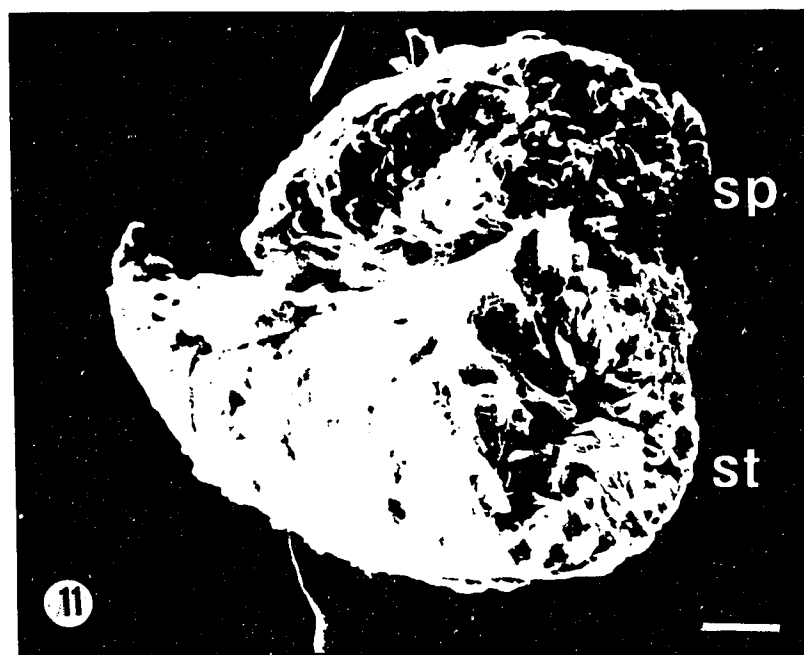
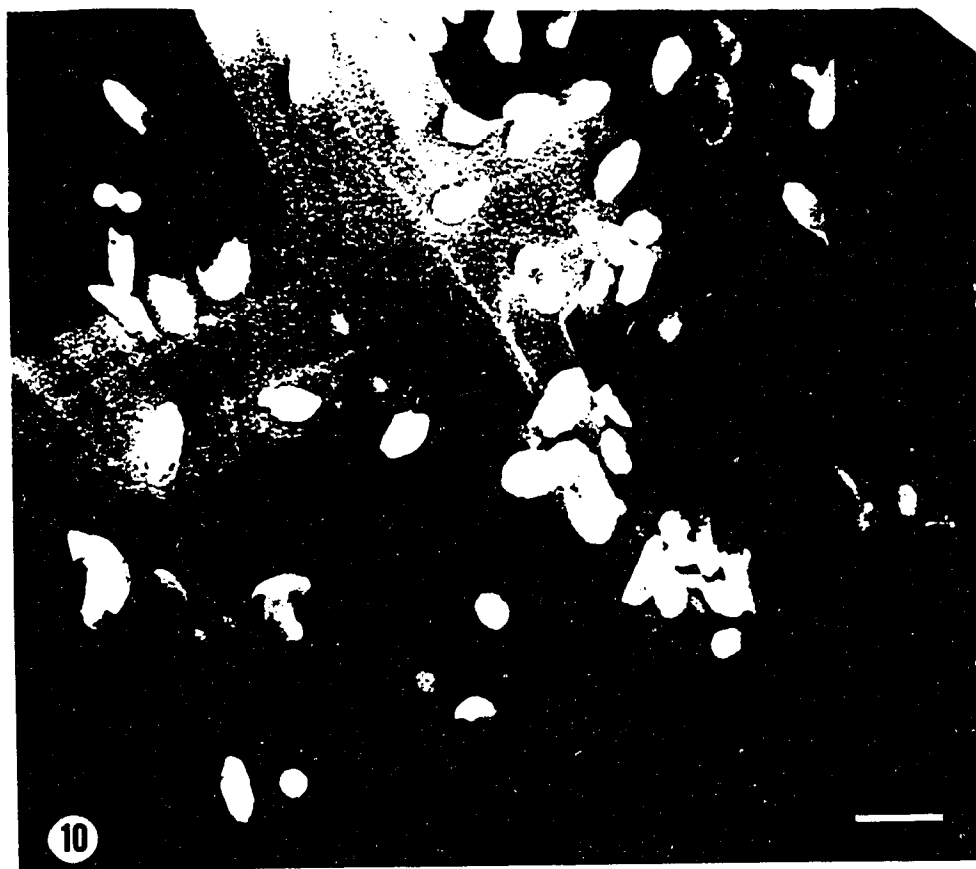


Figure 12. Various erythroid cell shapes delineated by scanning electron microscopy. Spheres may be seen in a and b. Two views of one sphere with beginning points may be seen in c and d; d emphasizes the nuclear bulge. Double-pointed cells, with varying degrees of flattening, may be seen in e, f, and h; i shows a single-pointed cell. A disc is presented in g, and an ellipse in j. (Considerable shrinkage has occurred.) Bar = 10 μ m. X3000.

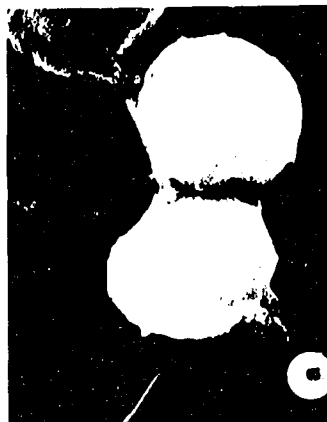
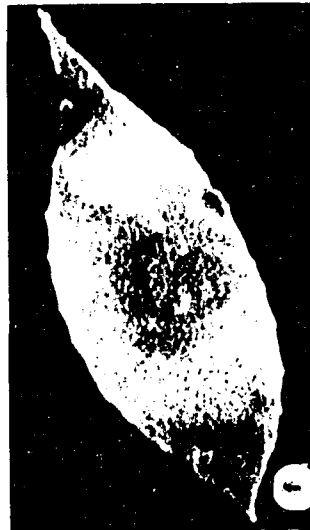
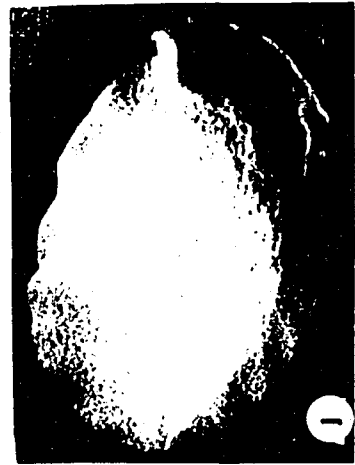


Figure 13. Phase micrographs of circulating erythrocytes in vessels near the surface or in the gills of living, anesthetized axolotl larvae. The arrows indicate the direction of blood flow. Twists of the cells believed to be induced by circulatory forces are indicated by T and possible "true" structural points by P. S in a is a possible sphere. In d, DP? indicates a possible double-pointed cell. Bar = 20 μ m. X460.



Figure 14. Several ellipses in various orientations. Possibly mitotic cell at left side. Scanning electron microscopy. Bar = 5 μm . X2000.

Figure 15. Two twisted ellipses. Scanning electron microscopy. Bar = 5 μm . X2000.

Figure 16. (From top left:) A single-pointed cell (on the grid bar; other, rounded end seen, but not included in micrograph), a double-pointed cell, and an ellipse. The grid was tilted at -23° . Scanning electron microscopy. Bar = 5 μm . X2000.

Figure 17. The same field as Figure 16, tilted at $+23^\circ$. The ellipse appears somewhat twisted when viewed from this angle, and the double-pointed cell is seen from a different perspective. Scanning electron microscopy. Bar = 5 μm . X2000.

Figure 18. Two double-pointed cells with somewhat "shaggy" membrane. Scanning electron microscopy. Bar = 5 μm . X2000.



Figure 19. An overview of whole mounted cytoskeletons from larval spleen, showing a single-pointed one (SP), ellipses (E), and bare nuclei (N). MS: membrane skeleton; MB: marginal band. Transmission electron microscopy. Bar = 10 μ m. X2000.

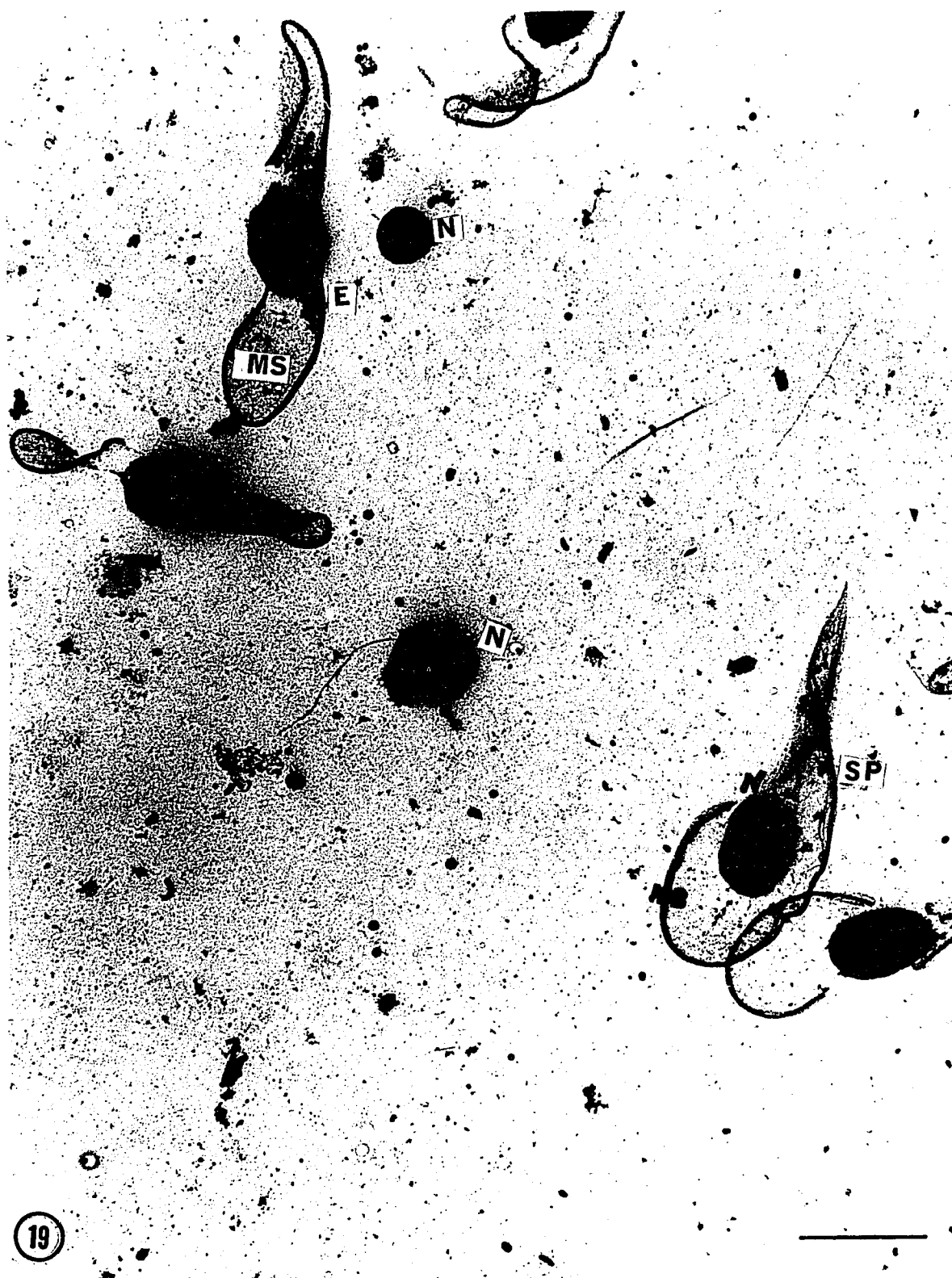


Figure 20. Whole mount of cytoskeleton, possibly of a sphere which may be beginning to develop a membrane skeleton, SAC (surface-associated cytoskeleton, a variant term), and marginal band, MB? Transmission electron microscopy. Magnification as in Figure 21.

Figure 21. Whole mount of cytoskeleton, possibly of a sphere with a beginning MS and marginal band, mt (microtubules), at an earlier stage than that seen in Figure 20. Bar = 5 μ m. X4800.

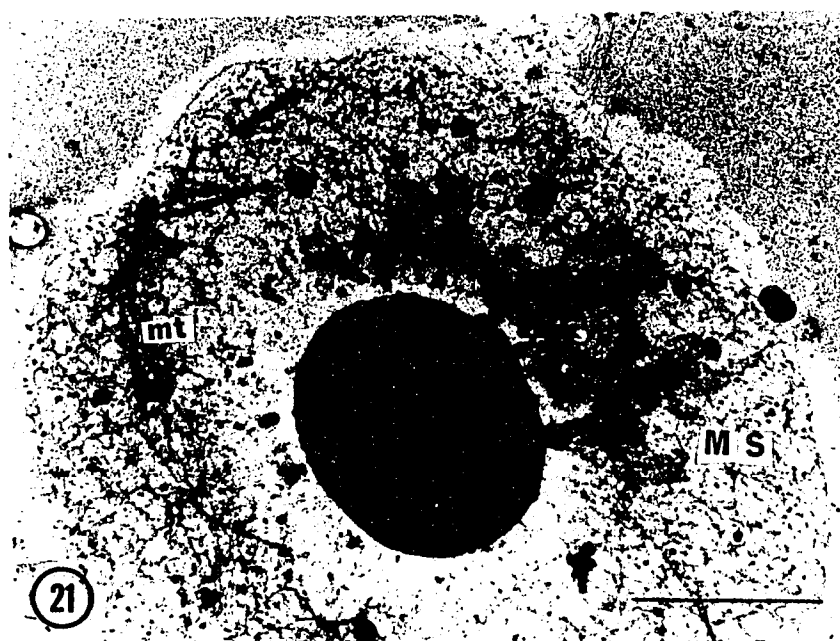


Figure 22. Whole mount of cytoskeleton of a pointed cell, showing microtubules. Transmission electron microscopy.

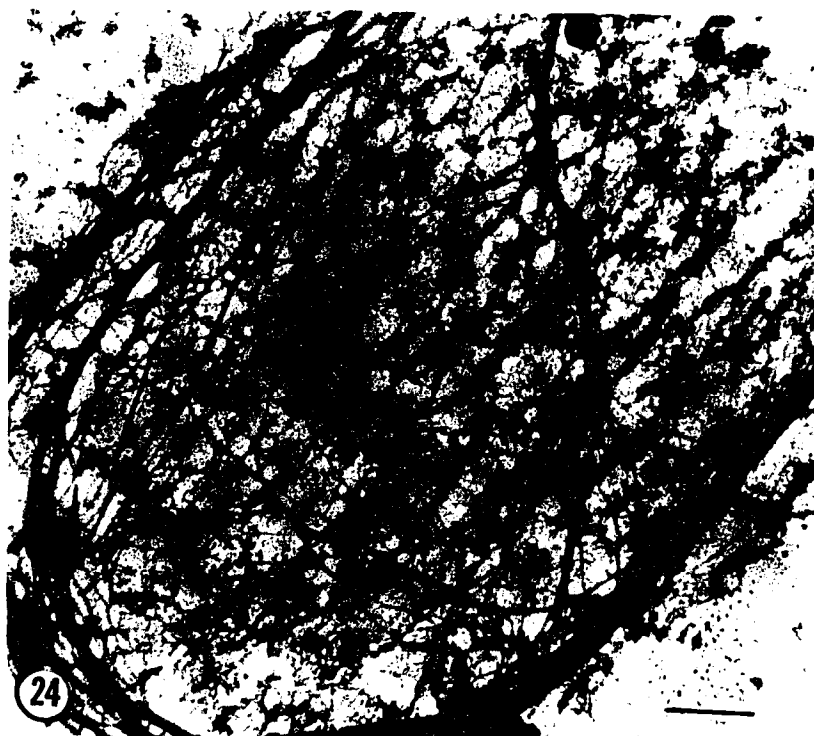
Bar = 1 μ m. X7900.

Insert i. Enlargement of the point, showing microtubules and their ends. Bar = 0.25 μ m. X20,000.

Figure 23. Enlargement of cytoskeleton of Figure 32, showing microtubules. Transmission electron microscopy.

Bar = 1 μ m. X22,600.

Figure 24. Whole mount of one end of cytoskeleton of an elliptical cell, showing microtubules. The marginal band appears somewhat frayed, which facilitates viewing of the microtubules. Transmission electron microscopy. Bar = 1 μ m. X11,800.



Figures 25 and 26. Whole mount of somewhat pointed end of otherwise elliptical cytoskeleton, at low and high magnifications, showing microtubules, mt. Microtubules appear to be in the process of curving around to form an elliptical shape. To the right in Figure 25 may be seen individual microtubules which seem to have not yet joined the main bundle. The same is true of the microtubules forming the point and those between the point and the marginal band, seen in both figures. Transmission electron microscopy. Figure 25: Bar = 2 μ m. X6400. Figure 26: Bar = 1 μ m. X19,600.



Please note that on this page an attempt has been made to present the micrographs in a sort of collage, with the shapes of the pictures complementing those of the biological structures.

Figure 27. Possible centrioles (ce) near point of cytoskeleton. The putative proximal centriole is nearly on end, but the density of the micrograph precludes viewing of any inner structure. TEM. Bar = 0.5 μ m. X33,000.

Figure 28. Possible centrioles at point with very dispersed microtubules, some of which appear unfinished (see arrows at ends). TEM. Bar = 0.5 μ m. X21,100.

Figure 29. Possible centrioles which seem aligned end-to-end, a relatively unusual orientation. Small arrows indicate possible emergence of microtubules. Larger arrows indicate position in relation to rest of cell: TE-to end; TN-to nucleus. TEM. Bar = 0.2 μ m. X30,600.

Figure 30. Double pointed cytoskeleton with partially dispersed microtubules, many along length of cell from end to nucleus. TEM. Bar = 2 μ m. X4100.

Figure 31. Cytoskeleton of ellipse with minimal fraying of marginal band microtubules (see arrows). Note bit of MS lying outside of band (due to manner of settling onto the grid). TEM. Bar = 2 μ m. X3600.



Figures 32 and 33. Whole mount of elliptical cytoskeleton viewed via high-voltage electron microscopy. Marginal band appears tightly bundled. HVEM. Figure 32: Bar = 2 μm . X3000. Figure 33: Bar = 1 μm . X9700.

Figure 34. Single-pointed cytoskeleton with marginal band, MS, and possible centrioles, seen enlarged in Figure 35. HVEM. Bar = 1 μm . X6400.

Figure 35. Enlargement of section of Figure 34, giving a sense of microtubules and their possible relation to the putative centrioles. Notice the tufts of material along the sides of the "ce" structure near the tip, as well as the faintly visible tubules within it. Many microtubules of the marginal band are clearly distinguishable. HVEM. Bar = 0.5 μm . X40,000.

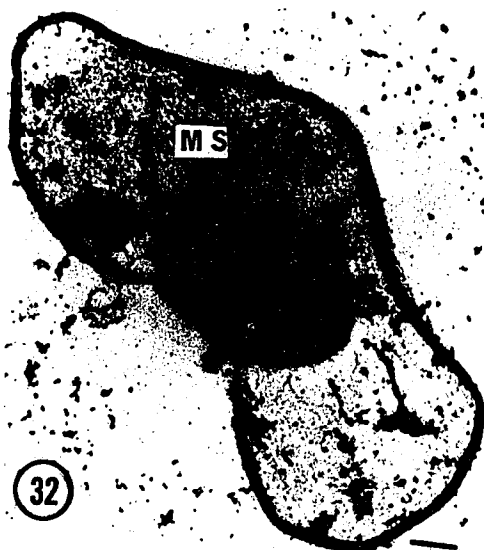


Figure 36. One end of a pointed cytoskeleton, showing difficulty of distinguishing possible centrioles, in dotted box. These are enlarged in Figure 37. Marginal band is well-developed and relatively tightly bundled. HVEM. Bar = 1 μm . X5500.

Figure 37. Enlargement of Figure 36 structures. An imaginative viewer using stereo glasses might visualize a microtubule sheaf seeming to pass over the "centriole" nearer the point, with apparently none touching it. Some internal structure of the lower "centriole" may be discerned; ends of some microtubules of the other may be seen toward the point. [View by aligning left lens of the glasses directly over the left-hand picture.] HVEM. Bar = 0.2 μm . X21,400.

Figure 38. Aspects of internal structure of the possible centriole which seem to be visible in stereo include microtubules apparently running end to end, some protruding from "pericentriolar material," somewhat tufted ends, and a middle stripe of dense material. Microtubules seem to go around/over the "centriole", a few possibly touching/entering the ends. With intent gazing (and imagination!) one can see a faint film, possibly a portion of MS, overlying these structures. HVEM. Bar = 0.5 μm . X25,800.

Figure 39. Further enlargement of Figure 37, non-stereo. Microtubules of both "centrioles" and band are visible. HVEM. Bar = 0.2 μm . X50,000.



Figure 40. Immunofluorescent splenic erythrocyte cytoskeletons.

a and b show spherical erythroblasts with beginning microtubules growing out around the sphere with apparently little or no bundling.

c displays a possibly spherical single-pointed cell.

d-g show various forms of double-pointed cells, with firm bundling of the microtubules in e-g. There is evident rounding of one end of a (probably) double-pointed cell in g.

h, i, k demonstrate different single-pointed cells. Note the "tennis-racket" appearance of the cytoskeleton in h; it appears that the single point is in the process of rounding and losing its point.

j shows a discoidal cytoskeleton.

l shows an elliptical marginal band with tightly bundled microtubules.

Bar = 5 μ m. X1700.

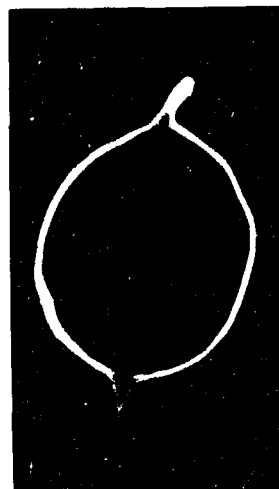


Figure 4la. Thin section of a lysed larval splenic cell, showing outline of cell membrane as well as the nucleus and such cell remnants as mitochondrial membranes. Note pair of possible centrioles (enlarged below) near upper end, as well as nearby bundle of microtubules. Transmission electron microscopy. Bar = 1 μ m. X9400.

Figure 4lb. Enlargement of the centrioles seen in Figure a. Notice also the microtubules. Transmission electron microscopy. Bar = 0.2 μ m. X35,000.

Figure 4lc. Further enlargement. Transmission electron microscopy. Bar = 0.1 μ m. X61,700.

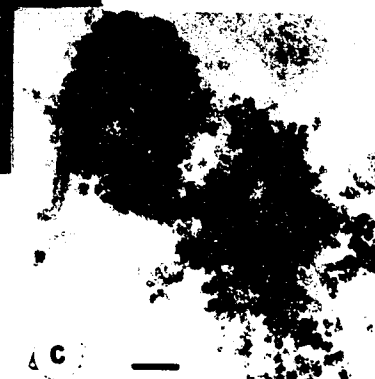
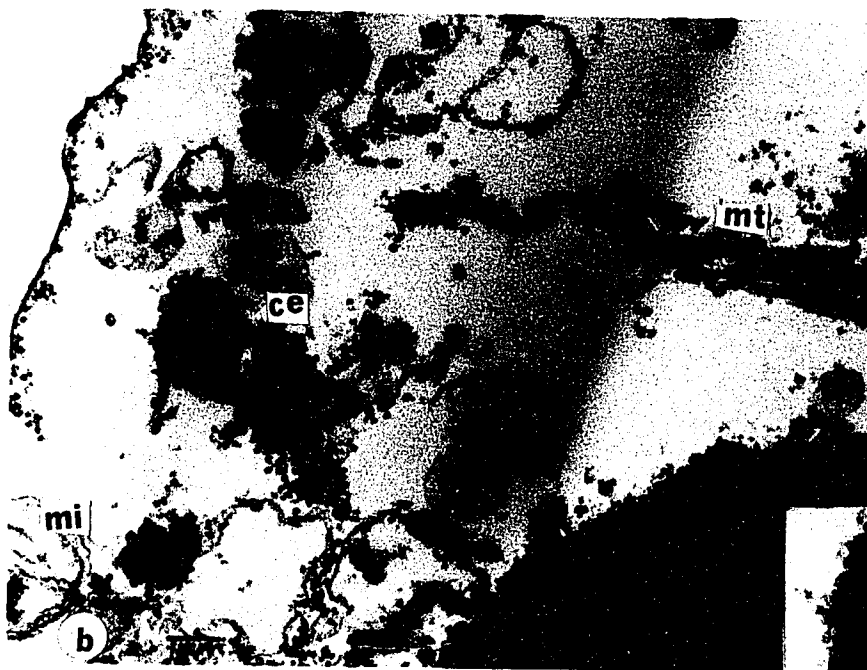
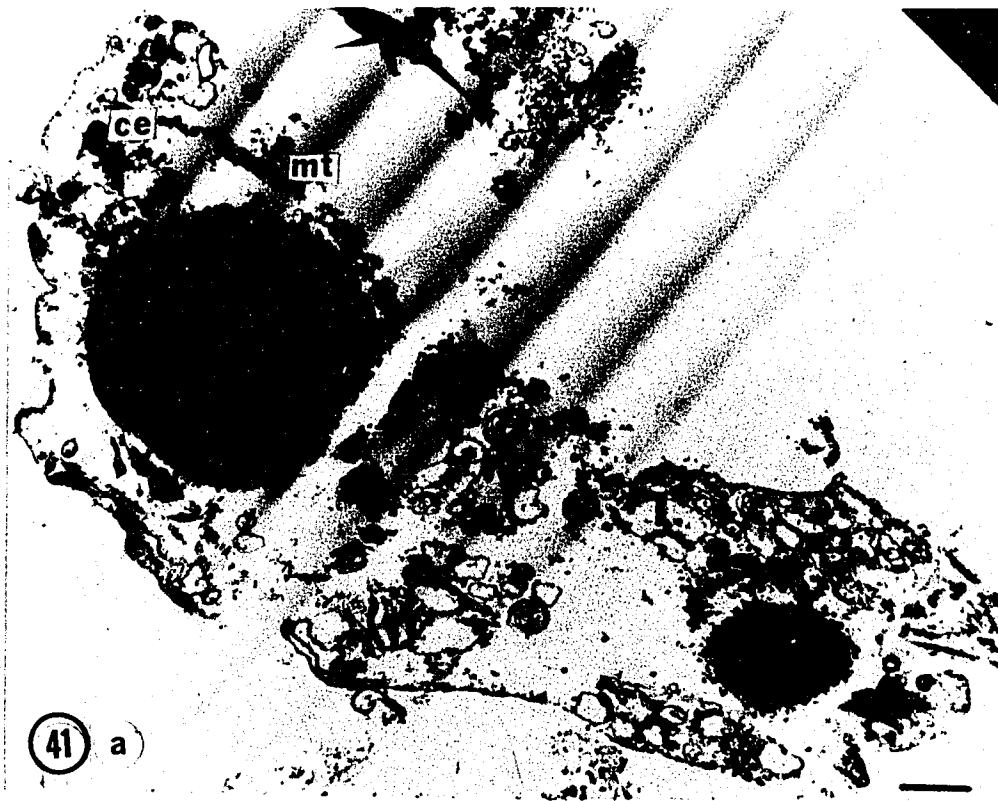


Figure 42. Unlysed larval splenic erythroid cell, showing the difficulty of distinguishing microtubules when obscured by hemoglobin. Transmission electron microscopy. Bar = 1 μm . X8000.

Figure 43 a and b. Enlargements of the two ends of above unlysed cell. At this magnification the microtubules are fairly obvious. Transmission electron microscopy. Bar = 0.1 μm . X60,000.

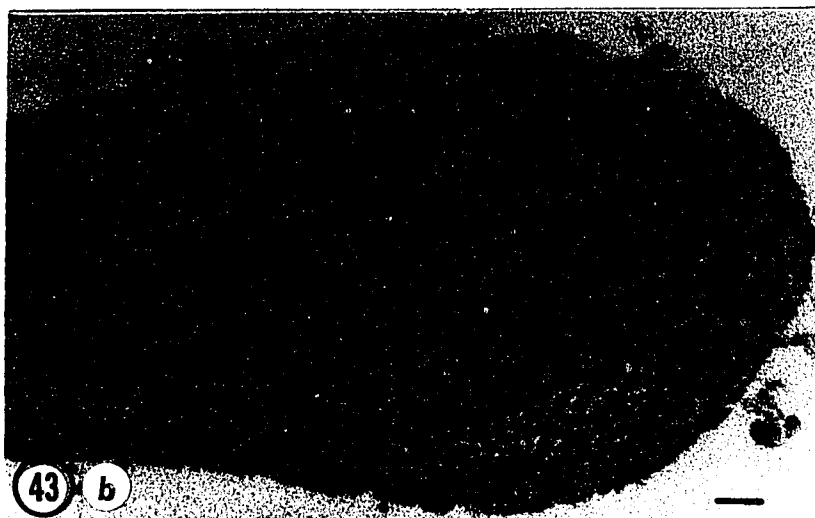
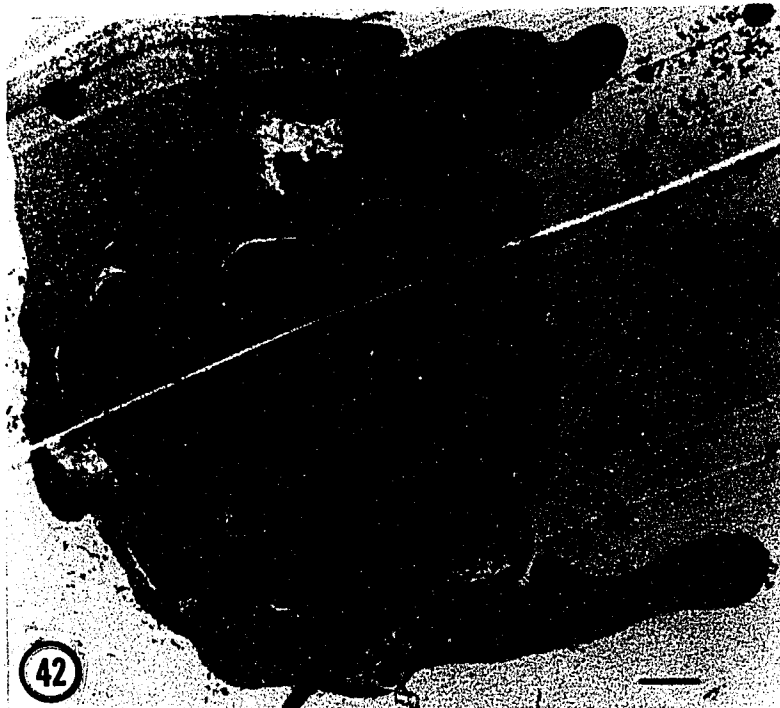


Figure 44. Cultured spleen from 6-day axolotl larva. Spleen was opened and placed into 50% Leibovitz L-15 containing 10% fetal calf serum in a 35-mm Petri dish which had been scratched on the inside bottom to aid locating of cells. The small arrows on the two micrographs indicate the same site on the dish, verified by two intersecting scratches. Phase microscopy. Bar = 100 μ m. X215.

a. Taken within 1 hour of explantation. Note the large number of refractile, spherical or somewhat spherical cells.

b. Taken 24 hours later. Note the greatly increased number of elliptical cells, and the decreased number of spheroid cells. Many pointed cells may be found. The cells have spread outward from the spleen, and there appears to have been cell proliferation as well. However, it must be remembered the greater number of cells seen here may be due simply to release of cells from the opened spleen.

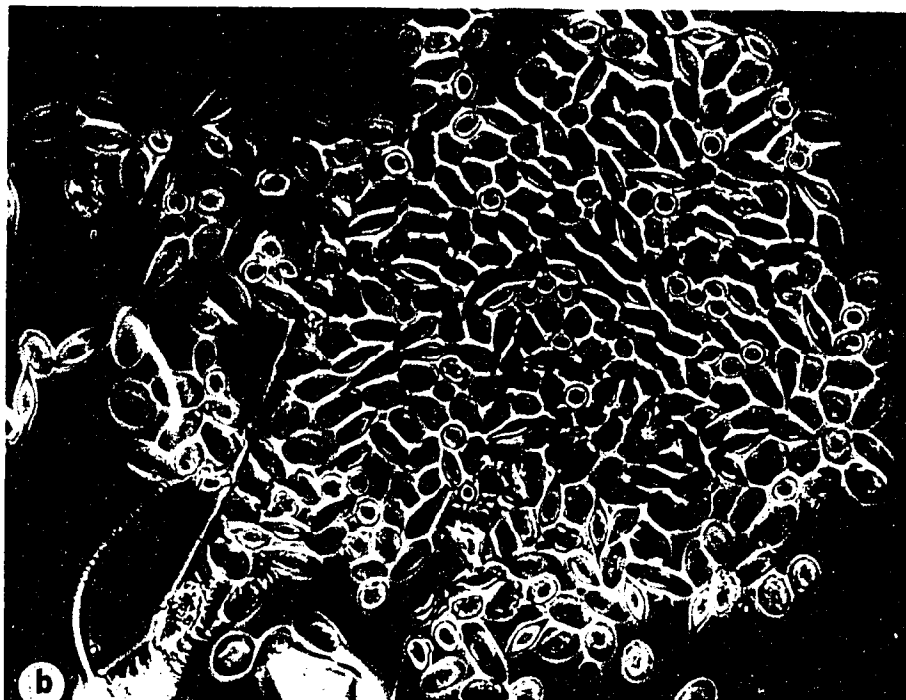
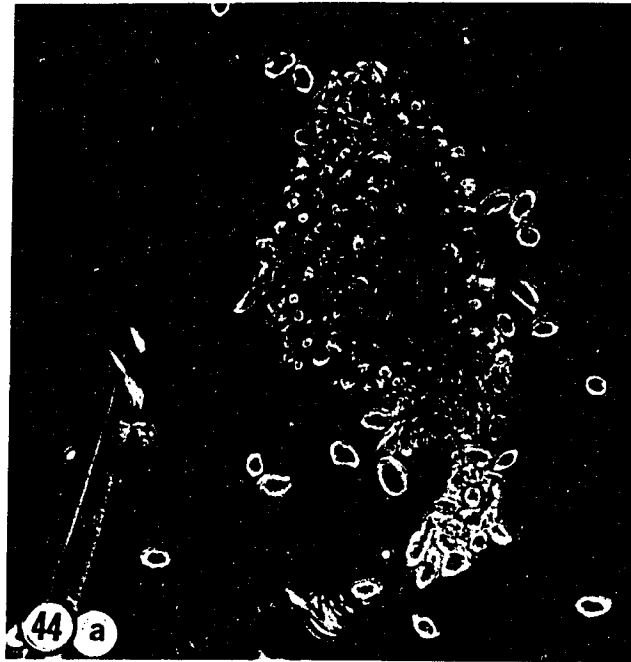


Figure 45. Another splenic culture prepared with the same techniques as that of Figure 44. Phase microscopy. Bar = 100 μ m. X215.

a. Photographed within an hour of explantation. Again, most cells are highly refractile.

b. Taken 24 hours later. Fewer cells seem to have moved from the central splenic area than in Figure 44, but there are a decrease in number of refractile cells and an increase in ellipses and pointed cells. Of particular interest is the mitotic cell on the left.

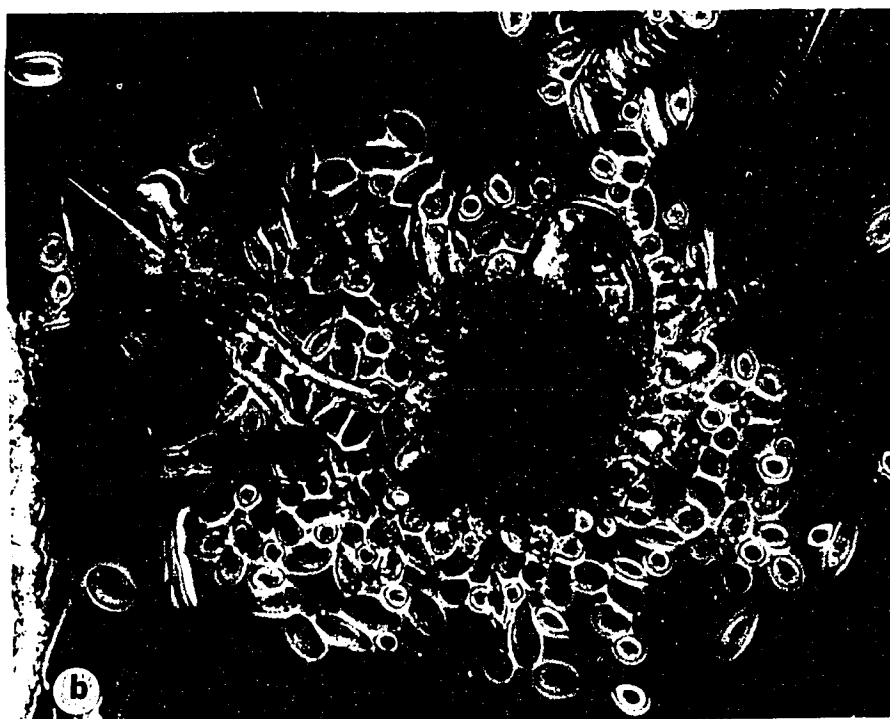
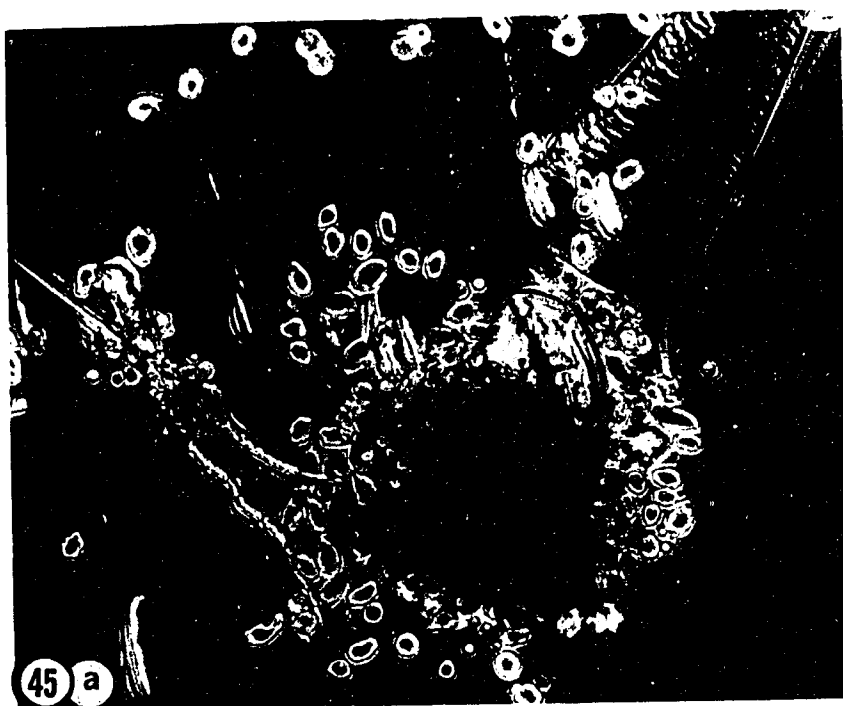


Figure 46. Adult axolotl erythrocytes.

- a. Intact cells in Amphibian's Ringer's. Phase microscopy. Bar = 50 μm . X330.
- b. Intact cells in Amphibian's Ringer's. Nuclei are easily observed. Phase microscopy. Bar = 10 μm . X800.
- c. Intact cells prepared by usual method of critical point drying. As usual, some shrinkage has occurred with maintenance of shape. Scanning electron microscopy. Bar = 5 μm . X1100.
- d. Cytoskeletons viewed by phase microscopy. Bar = 10 μm . X700.
- e. Immunofluorescent marginal bands of cytoskeletons. Bar = 10 μm . X750.
- f. Immunofluorescent round marginal band. Bar = 10 μm . X1800.

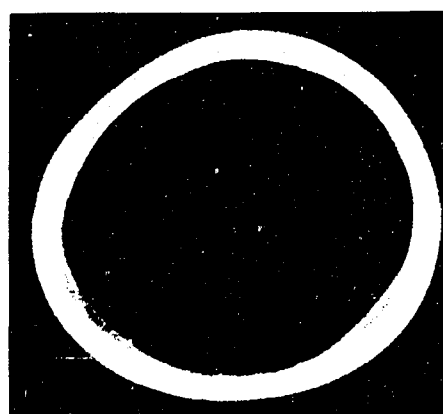
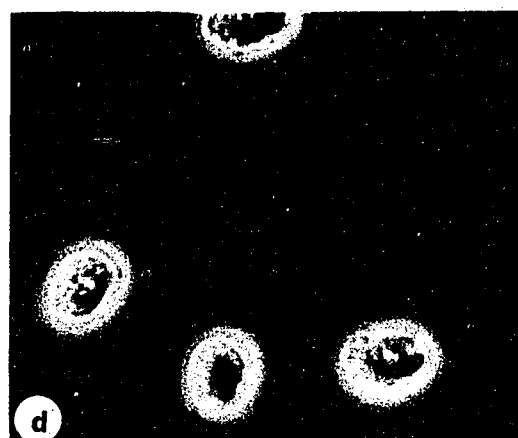
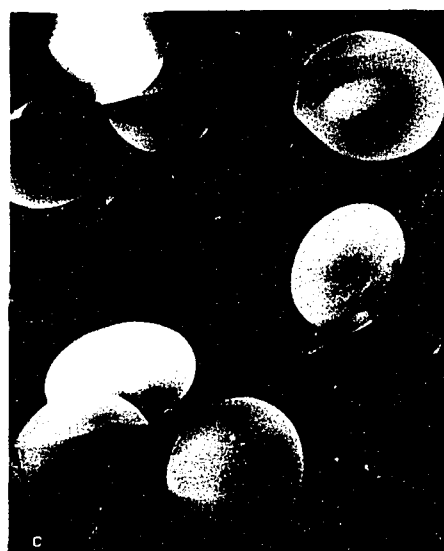
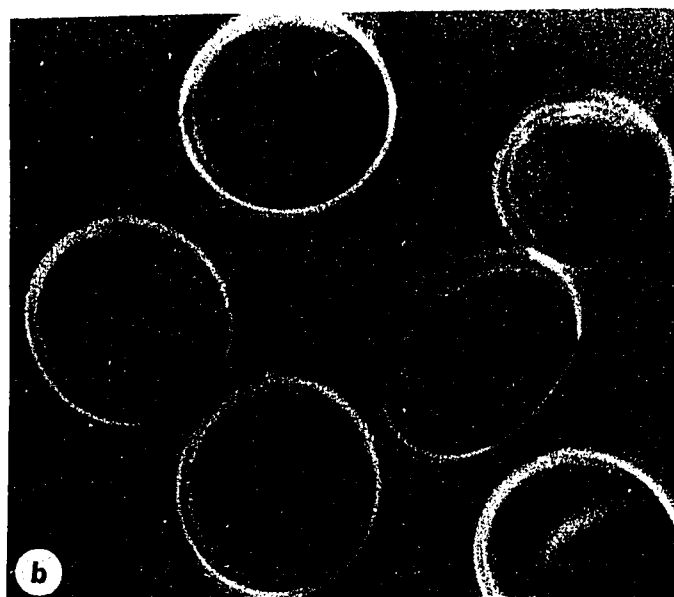
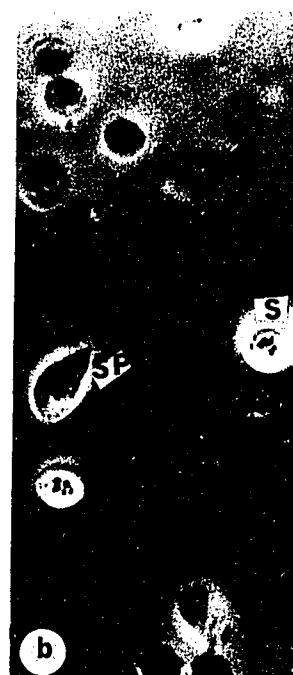
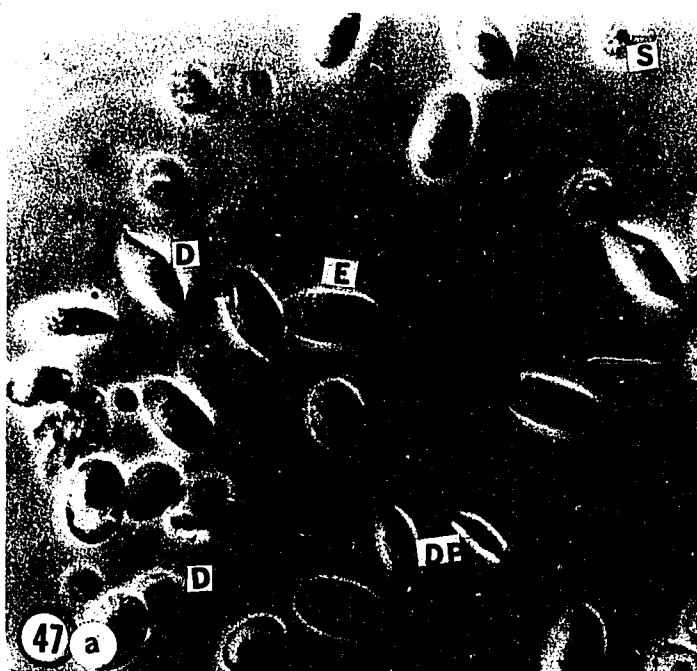


Figure 47 a and b. Xenopus tadpole (Stage 49) liver erythroid cells in hanging drop in Holtfreter's, showing variety of cell shapes. Phase microscopy. Bar = 10 μ m. X730.

Figure 48. Single-pointed erythroid cytoskeleton from liver of Stage 49 Xenopus, showing marginal band and pair of dots in point. Phase microscopy. Bar = 0.5 μ m. X2400.

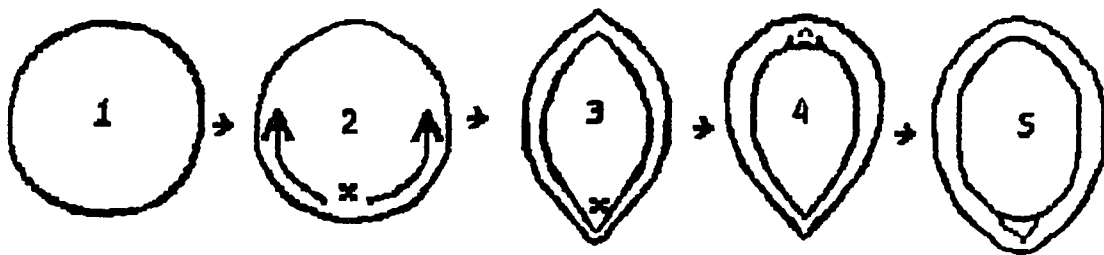
Figure 49. Single-pointed intact Xenopus erythroid cell (liver, Stage 49). Scanning electron microscopy. Bar = 2 μ m. X5000.

Figure 50. Intact Xenopus elliptical erythroid cell (liver, Stage 49). Scanning electron microscopy. Bar = 2 μ m. X5000.



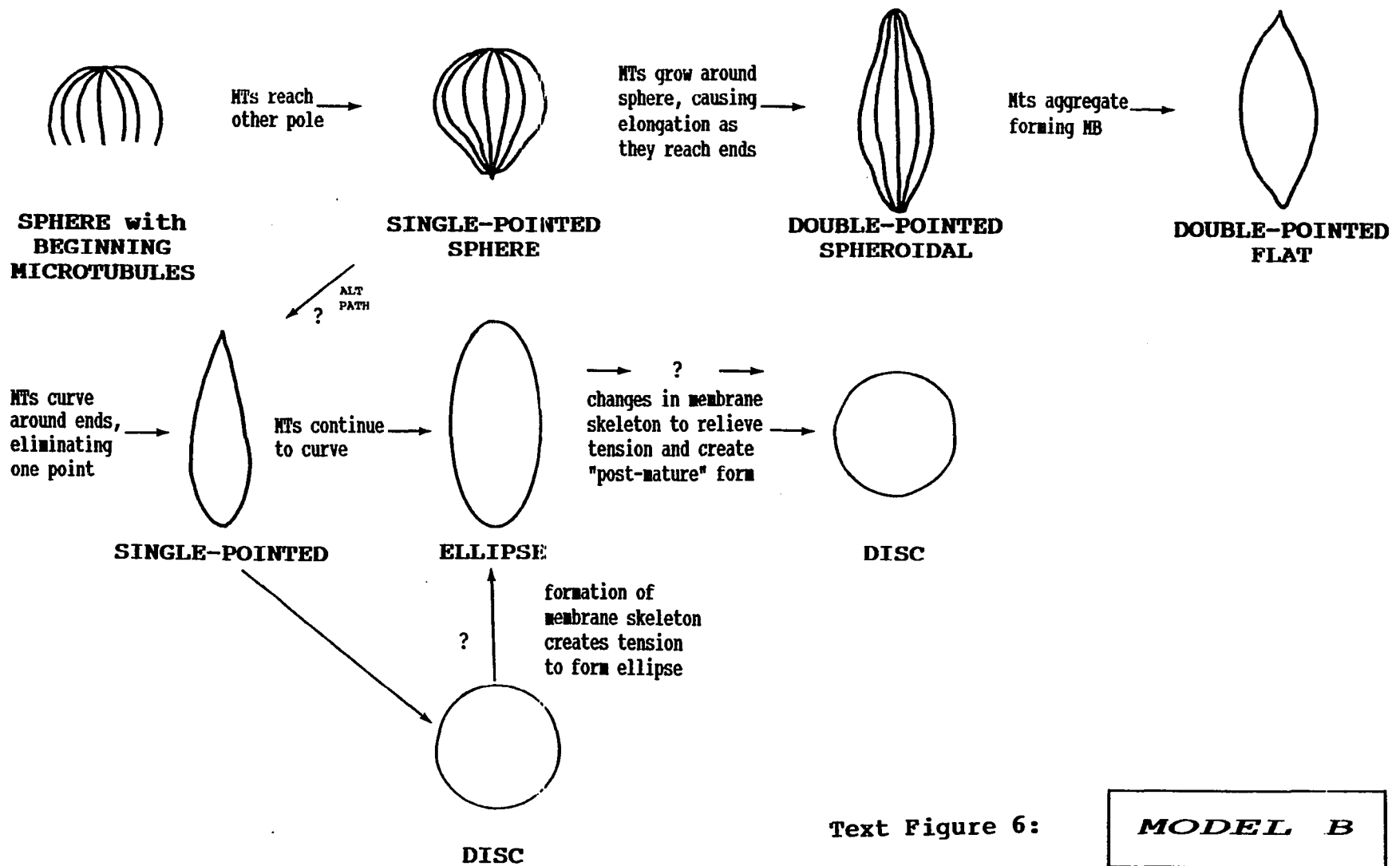
Discussion

Through the various morphological and statistical techniques described above, possible sequences of erythroid morphogenesis and the role played by the marginal band in the larval axolotl spleen cells may be postulated. One hypothetical sequence is that posited by Nemhauser and Cohen, in which two bundles of microtubules begin to form in the spherical erythroblast, curving as they grow distally and producing a double-pointed cell; when the distal point is closed by curvature of the microtubules, a single-pointed cell results; the completion of the marginal band by closure at the proximal point results in an ellipse (Nemhauser, Joseph-Silverstein, and Cohen, 1983; Ginsburg, Twersky, and Cohen, 1989). This model, from Nemhauser, Joseph-Silverstein, and Cohen, 1983, and Cohen and Ginsburg, 1986, is shown below:



Text Figure 5: Model A (Cohen-Nemhauser Model)

However, additional data presented in this thesis suggest that the situation may be more complex:



In each instance in Model B, postulated centrioles would be positioned at the top, but their presence in the model, or in the discussion thereof, should not be interpreted as a claim for any proof of their existence.

(a) Microtubules begin to form from a single locus underneath the plasma membrane of the spherical erythroblast (which at this point has a "shaggy" membrane). The membrane cytoskeleton is not yet formed, and the microtubules grow from a (postulated) microtubule-organizing center (MTOC), perhaps a centriole pair, in an apparently unguided manner, resulting in a "bird's-cage" appearance. During this time the membrane skeleton (MS), or surface-associated cytoskeleton, begins to form.

(b) When the microtubules reach the area of the sphere distal to the MTOC, they push against the membrane, forming a point on the surface.

(c) The resulting tension also creates a point at the "MTOC-end" so that a double-pointed sphere results. The microtubules continue to grow and push against the plasma membrane, until excess membrane has "smoothed out", causing elongation of the cell into a double-pointed, spheroidal ellipse with a relatively smooth surface.

(d) Perhaps in response to molecular signals from the MS, the microtubules begin to bundle at opposite "sides" of the ellipse, resulting in flattening of the cell. This flattening may be due to tension from the newly aggregated marginal band, to strain exerted by the membrane skeletal

components, or (most likely) to a combination of the two. Once flattened, the cell has a prominent bulge due to the nucleus.

(e) Under an unknown type of guidance from the rest of the cytoskeleton, microtubules continue growth, but now with a defined placement as they curve around the points next to the newly formed marginal band. The protruding tubules which caused the points also curve to lie against the others, resulting in the loss of one point (that distal to the MTOC). A single-pointed cell results.

However, inasmuch as both spheroidal and flattened single-pointed cells are seen, it is proposed that developmental constraints are sufficiently lax to permit some microtubules to curve around prior to bundling. It is possible, in fact, that vagaries of timing of the formation of other cytoskeletal components may result in a certain percentage of cells bypassing the double-pointed stage altogether, with packing of the tubules taking place after they have curved around to head back toward the MTOC (See ALT PATH between single-pointed sphere and single-pointed cell).

(f) The microtubules at the sole pointed end curve around until the point is gone.

A variety of evidence (see page 186ff) indicates that the MS is largely responsible for the tension forcing the marginal band into an elliptical, rather than a more natural circular, shape. It seems likely that most cells go

directly from single-pointed ellipse to ellipse, with a smaller proportion becoming discs prior to forming ellipses. It is proposed that the fineness of the coordination between microtubule growth and the formation of the rest of the cytoskeleton determines which path a given cell takes.

An alternative, or additional, possibility, is that the disc is a post-mature form, assumed by the cell when for some reason the MS breaks down.

Evidence for this model derives mainly from the work with the larval splenic erythroid cells. Therefore, although the studies of embryonic primitive blood cells preceded those of the larval cells, the results with the latter system will be discussed first, in terms of the model(s) whenever appropriate, as well as with reference to the work of other investigators and to possible further experiments. (Throughout the following pages, the term "model" will refer to Model B.)

The Erythropoietic Sequence

Documentation for the model consists of inferences from the differential cell shape counts by age, observations of cells in culture, and deductions from various morphological studies. It had been hoped that the correlation of marginal band development with the evolution of cellular morphology would be a straightforward task. Of course, this was not the case, and difficulties began with the supposedly simple task of determining the erythropoietic sequence. The

optimal approach would have been to perform histological blood smears, with stains, at the outset, in order to clarify the sequence of erythroid cell development. The failure to do this has resulted in a dependence upon the literature to relate the experimental findings to the classical stages of erythropoiesis.

Fortunately, Grasso and Woodard (1967) have found, with rabbit and Triturus, that "the erythropoietic process within two widely different vertebrates occurs in an essentially similar manner." Classically, this has been described in histological terms, but in recent years several laboratories have looked at erythropoiesis at the ultrastructural level (Fish: Sekhon and Maxwell, 1970; Amphibian: Tooze and Davies, 1967, and Grasso, 1973 a, b; Avian: Edmonds, 1966, and Small and Davies, 1972; Mammalian: Kovach et al., 1967, and Orlic, Gordon, and Rhodin, 1965). Because the light microscopic definitions have depended to a large degree upon staining characteristics, correlations between the two level of structural analysis have not always been straightforward. However, general characteristics of the erythropoietic process involve a decrease in cell size, a change in external cell morphology from spherical to flattened, a decrease in the nuclear/cytoplasmic ratio, a condensation or elimination of nuclear material, and an accumulation of hemoglobin and loss of cytoplasmic organelles. Grasso and Woodard (1967) give the sequence as: hemoblast--> proerythroblast--> basophilic erythroblast--> early, mid-,

then late polychromatic erythroblast--> reticulocyte--> mature erythrocyte. As described by them, by Charlemagne (using the axolotl) (1972), and by Tooze and Davies (with Triturus cristatus) (1967), cells at these stages have the following characteristics: Stem cells are lymphocyte-like, large, with abundant granular cytoplasm. These appear as reticular cells in groups of 6-8; individual cells detach from the group and become hemoblasts (also called hemocyto blasts), which are very large, 55-75 μm , having cytoplasm with irregular granules. In the proerythroblast, the nucleus/cytoplasmic (volume) ratio has increased to 0.6-0.7, and the chromatin has lost its reticular appearance while increasing in density. There are numerous ribosomes and mitochondria. The basophilic erythroblast is smaller, about 30-35 μm in the axolotl, with a nuclear/cytoplasmic ratio of 0.5, according to Charlemagne, who describes the cell at this stage as perfectly spherical. (Note, however, that in the absence of scanning microscopy, it is uncertain how this was determined.) Tooze and Davies think that this may be the earliest stage recognizable as erythroblastic in the electron microscope, due in part to the abundance of ribosomes. The polychromatic erythroblast has a smaller, denser nucleus, which has about one-third the volume of the cytoplasm. The eosinophilic hemoglobin together with the basophilic ribosomes give the cytoplasm its polychromatic appearance. Reticulocytes show a further decrease in nuclear size and increase in density; ribosome density

continues to decrease, but, according to Charlemagne, cytoplasmic basophilia never completely disappears (in contrast to the case in mammalian cells). The mature axolotl erythrocyte has been found by Charlemagne to be 30-35 μm long in young animals and 31-33 μm in adults, and he gives the nuclear/cytoplasmic ratio as 0.2-0.25. The nucleus of the mature erythrocyte is condensed and transcriptionally inactive but, in chickens, at least, may be reactivated experimentally (Dieterlen-Lievre 1988).

When Grasso (1973a, b) used splenectomized, phenylhydrazine-treated (therefore anemic) adult newts, he found that basophilic erythroblasts appeared in the circulation about day 14 after treatment; these then took about eight to eleven days to proceed to the polychromatic erythroblast stage, and an additional ten to fifteen days to complete maturation into fully mature erythrocytes. (It is the orthochromatophilic erythroblast, described by Orlic as the mammalian stage after polychromatophilic erythroblasts, which becomes enucleated in mammals to form the reticulocyte.)

The timetable of development found by Grasso and Woodard (1967) does not fit at all well with the much briefer one apparently operative in axolotl embryos and larvae. At least part of this discrepancy may be due to the fact that the waves of erythropoiesis seen in their treated newts were stimulated by toxic treatment, although erythroid precursor cells are also found in blood of these animals in

normal erythropoietic activity during spring breeding. Also, growth in adults, when it occurs, may be expected to be slower than in rapidly developing immature forms. It is significant that when Grasso (1973b) produced anemia in newts with acetylphenylhydrazine, but left their spleens intact, there were two overlapping erythropoietic waves at eight and eleven days: the first was splenic with many basophilic erythroblasts, whereas the second occurred in the blood and consisted first of the appearance of erythroid precursor cells. There are also, as pointed out by Slack (1989), profound species differences in amphibian embryology, and the axolotl may have a quite different hematopoietic timetable than does the newt.

Cell Morphology: Structure of Larval Splenic Erythroid

Cells

Lacking in most of the literature, as well as in this thesis, is a clear correlation of cell shape with the cytology and the fine structure, in both the primitive and the definitive erythroid lines. Although various correlative microscopic studies were failures, much of the research presented here did cast some light on morphological aspects of axolotl erythrocytogenesis. It was the investigation of the larval splenic cells which constituted the bulk of the work and contributed most heavily to the model for cell morphogenesis with marginal band formation.

Integral to the basic premises of the thesis was the observation by Duprat and Flavim (1977) that the spleen is a

closed erythropoietic system for several days post-hatching. Several pieces of evidence tended to confirm their work. These included the differences in cell shape distribution between the circulating and splenic compartments, the failure of dye injected into the spleen to join the general circulation, and the visual observation of the process of the spleen's connection to the circulation. Thus it seemed valid to use the erythropoiesis of the larval spleen while still isolated from the general circulation as a model for this type of cell differentiation.

As seen in Table Three and in Graphs One and Two, a striking difference in percentage of any given cell shape often exists between the two erythroid compartments. In both the spleen and the circulation, however, the percentage of spheres decreased and that of ellipses increased as the larvae matured. This is logical in view of the proposed maturation process from spheres to ellipses; presumably there is a very early point in development at which all putative red blood cells are spherical erythroblasts. However, because the blood cell population is a continually self-renewing one, it is not surprising to find immature cells at all stages of the animal's life.

Although the shape progression was not smooth in any of the data sets, and the average numbers in some of the age groups did not "fit" the trend, the general tendency was shown to be statistically significant by nonparametric procedures. The nonparametric approach was used because (1)

the apparently random occurrence of large numbers of pointed cells at most ages distorts the population distribution from the normal; (2) the outlier values for percentages of spheres and ellipses, caused again by unusual numbers of pointed (or mitotic) cells, distort the median-based (nonparametric) results less than mean-based (parametric) figures.

The application of statistics does, however, raise pertinent questions regarding the experimental strategy. Two vital aspects of all endeavors which seek to extrapolate a theory about a population from a sampling are the normality of the population and the randomness of the sampling. Although of course the distribution of cell shape by age would not be expected to be a normal one, it is quite possible that the distribution of cell shapes for a given larval age from animal to animal would be, provided adequate controls were exercised and sufficiently large numbers studied. Thus studies should involve the evaluation of large numbers of splenic populations from comparable animals, care being taken to use as many from the same spawn for a given set of counts or experiments as possible. (Some spawns did seem to display aberrant morphological development, but unfortunately no systematic attempt was made to control for spawn number.) In addition, much larger numbers of spleens, probably at fewer age points, should have been analyzed. For example, from each of several spawns of 100 embryos each, counts could have been made from

20 animals each at larval ages four, seven, nine, and twelve days. This would be possible if the cells could be fixed and held for counting and would give a much better sense of the true statistical validity of the results. A variety of controls should be instituted to assure random selection of animals from a given spawn: one factor that might have skewed the results is the fact that the slowest swimmers in a given Petri dish were taken for analysis, so that the oldest larvae tended to be the most vigorous. Thus it seems in retrospect that the experiments were poorly planned for the type of quantitative analysis desired.

Cell Proliferation and Differentiation

Although the percentages of immature erythroid shapes (non-ellipses) were larger in the axolotl spleen than in the circulation, varying numbers of mitotic, spherical, pointed, and discoid erythroblasts were found consistently in the circulation at all stages of larval development. The presence of such cells in the circulation may be attributed to their release from erythropoietic organ(s) prior to complete maturation or to continuation of cell proliferation and development in the circulation (quite possibly the case with the axolotl larvae).

This is in keeping with the finding of other researchers, who have determined that cell proliferation and differentiation can take place in the circulation, despite the fact that for many years most literature has proclaimed the spleen as the sole erythropoietic site in urodeles. The

existence of erythroid cell maturation and even division in the circulation (in addition to erythropoietic organs) has been documented in several species (Avian: Dieterlen-Lievre (1988); Amphibian: Dawson (1930), Jordan and Speidel (1930a), Frangioni and Borgioli (1987b); Fish: Sekhon and Maxwell (1970), Pica and Della Corte (1987)). As far back as 1930 Dawson reported mitosis in basophilic cells in the circulation of Triturus viridescens and other urodeles and found all transitional morphological forms in the circulation of thirteen of the fourteen species examined. Interestingly, he included a pointed cell in his sequence: "Ordinarily during maturation, the cell, which is at first spherical, changes to a spindle form and finally becomes a flattened oval. At the same time the nucleus usually becomes small and dense, and eventually acquires the oval form to conform with the changed outline of the cell itself." Pica et al. (1986) found that erythroblasts pass into the circulating blood of the lizard and continue to differentiate there. The same laboratory (Pica and Della Corte, 1987) ascertained that "a variable number of basophilic and acidophilic erythroblasts" of the Elasmobranch Torpedo marmorata (Risso) leave the spleen to continue maturation in the blood stream. In the dogfish Scyliorhynchus stellaris L., according to D'Ippolito et al. (1985), "the presence in the circulating blood of basophilic erythroblasts, some of them in mitosis and of acidophilic erythroblasts, confirms that the complete maturation of

erythrocytes occurs in circulating blood as is already known in all the Vertebrates with nucleated red cells." However, the occurrence of "many mitotically dividing young erythrocytes" found by Sekhon and Maxwell (1970) in the "Pacific hagfish," Eptatretus stoutii, and by Jordan and Speidel (1930) in Myxine glutinosa has seemed to be an exception to the general findings in other species. Mammalian yolk-sac-derived erythroid cells proliferate and differentiate in the fetal circulation (Rifkind et al., 1974).

The work of several laboratories with anemic and/or splenectomized animals has given further evidence for non-splenic sources functioning in addition to the spleen in erythroid cell production. Jordan and Speidel (1930a) found evidence that the general circulation had taken over the erythroid functions of the spleen in splenectomized Triturus viridescens, including "extensive multiplication...of the hemoblasts, proerythroblasts, and erythroblasts." Grasso (1973 a,b) found red cell production in the circulation of splenectomized, chemically anemic newts. Chegini et al. (1979) found that in phenylhydrazine-caused anemia, Xenopus spleen and liver released basophilic erythroblasts into the circulation, where they proliferated and differentiated; however, they found no erythroblasts in the circulation of nonanemic Xenopus. Dawson (1930) found that "differentiation of the immature elements of the blood is apparently favored by a sluggish circulation such as

occurs in the venous sinusiods." Frangioni and Borgioli (1988) found significant Triturus red cell production in areas of the heart cavity, as well as in the circulation, after splenectomy accompanied by acetylphenylhydrazine anemia. They raise the interesting point that the clustering of erythropoietic tissue in the heart is surprising in view of the probable mechanical stress caused by the heart's pulsation.

In view of the findings by others as well as in this research, it seems possible to postulate that perhaps cells form in the erythropoietic organ, eg., the spleen, and proliferate and differentiate there to varying degrees; normally a certain fraction escape to continue development in the circulation but under some conditions, such as seasonal or exertional demands, this fraction increases to allow a greater number to reach maturity in the circulation. It also seems likely that, at least in the case of the axolotl, the erythroid cells of the primitive line continue to proliferate and mature in the circulation until the spleen and its erythroid population have developed sufficiently to meet the growing animal's needs. At that point, presumably, the spleen completes connection to the circulation and begins releasing red blood cells. It would be of interest to look more closely at the circulating cells during the period of closed splenic development to determine their source, perhaps via surface antibody or hemoglobin identification. A more thorough study of those axolotls

whose spleens fail to join the circulation might also be productive.

Shape Development as Indicated by Cell Counts

The apparently random occurrence of individual larvae with relatively large percentages of pointed cells in the splenic population (see Table Four) might be interpreted as a result of erythropoietic waves, in which a group of stem cells are released from the mitotic cycle and are induced to begin differentiation together. This hypothesis is supported by the findings of Dieterlen-Lievre (1988) in quail embryos, where primitive erythroblasts are released from the yolk sac simultaneously and mature in the circulation in synchrony, and by the work of Frangioni and Borgioli (1987a, b) with the newt. The latter investigators noticed that individual adult newts (Triturus cristatus carnifex (Laurenti)) showed high erythropoietic activity, whereas others had none. This variation was independent of the seasonal fluctuations they have documented. It is also relevant that they found a "discontinuous, rhythmic erythropoiesis" in anemic Triturus, which they believe to be an exaggeration of normally occurring oscillations. This, they comment (Frangioni and Borgioli, 1987a, b), may explain, "not only the often marked differences between the counts made on untreated newts and the mean seasonal values but also the discordances in the literature on the trend of the same seasonal cycle."

The differential cell counts indicated, in addition, that the morphological progression begins with spherical erythroblasts and ends with mature elliptical erythrocytes. The sequence of the intervening steps as outlined in the model is based upon a combination of morphological evidence and logical deduction. However, it must be kept in mind that, in the absence of a successful erythropoietic culture, there are no data which unambiguously support the proposed intermediate stages.

Shape Development as Elucidated by Morphological Studies

Spherical Erythroid Cells

According to Rowley (1988), basophilic erythroblasts in agnathans and elasmobranchs are spherical with a central nucleus. Pica et al. (1986) found basophilic erythroblasts of lizard (Podarcis s. usicula Raf.) to be round, occasionally mitotic, but acidophilic ones to be ellipsoidal. The description of the basophilic cell as having but a thin rim of cytoplasm around the nucleus correlates well with phase studies here in which the nucleus of a sphere after lysis appears nearly as large as does the whole unlysed sphere.

Although in one sense the scanning micrographs of spheres presented here do little but support the validity of the shape assignments, they also show details of surface morphology, such as membrane folds or shagginess (See Figure 12b). The excess membrane might be developmentally important, possibly as a reservoir for extensibility as the

cell shape changes, and might therefore be an area for investigation.

In terms of the proposed model for erythropoiesis presented here, the initial stage (a) of a sphere with beginning microtubules is presumably a basophilic erythroblast. In an electron micrograph of a basophilic erythroblast of Triturus cristatus Tooze and Davies (1967) found scattered microtubules, much as one would expect to see in a sphere just beginning a marginal band, such as the one with immunofluorescent microtubules seen in Figures 40a, b. Here the technique of indirect immunofluorescence allows visualization of microtubules which are not yet bundled into a marginal band and are therefore too thin to see by other microscopic techniques. These figures show microtubules growing out from a single locus on the circumference of a sphere. Their peripheral deployment was observed by focusing up and down with the microscope. They are more or less symmetrically arrayed in b, but somewhat less so in a, perhaps because of the apparent earlier state of development of the microtubules in a, or because of variation in the timing of microtubule bundling. This distribution indicates that the initial tubule growth proceeds without any guidance into the final (bundled) arrangement.

This is in partial agreement with Murphy's findings (Murphy, Grasser, and Wallis, 1986) in developing chicken erythroblasts, that "microtubules associated with the centrosome extended outwards forming a loose wreath that

occupied the space between the nuclear membrane and the cell surface." His immunofluorescence micrographs, however, show less "globally uniform" microtubule growth than seen here in Figures 40 a and b; rather, the arrangement seems like that of an open-bottomed bird's nest, indicating the possibility of some guidance of the microtubules into an equatorial grouping. Murphy identifies these "wreath cells" as polychromatophilic erythroblasts. Of particular interest in Murphy's work is the apparently exclusive presence, in the developing and mature marginal band, of an erythrocyte-specific beta tubulin which is not present in erythroblasts prior to the beginning of hemoglobin synthesis and marginal band initiation.

However, Tooze and Davies (1967) also found a bundle of longitudinal microtubules in a basophilic erythroblast in thin section; this bundle seems to contain at least eight fairly closely packed microtubules. The micrograph does not show clearly how near these are to the periphery, but the authors state that microtubule bundles are "usually lying near the periphery of the cell." In their study of yolk sac erythroid cells in the early chick embryo, Small and Davies (1972) found basophilic erythroblasts to have small bundles of microtubules at the cell perimeter, and, rarely, "almost fully formed marginal bands." Murphy, on the other hand, found that consolidation of the microtubules into an organized bundle occurred after the completion of fully formed circles. In primitive chick embryo erythroblasts

Solomon's group (Kim et al., 1987) found formation of microtubule bundles which were not confined to the periphery but instead coursed throughout the cytoplasm. Within a day most of these had coalesced into a thick peripheral band. Perhaps, then, there is temporal variation in the formation of lateral association which result in the bundling of the microtubules, sometimes beginning at an early stage in the spherical erythroblast, before the tubules have even reached the opposite end, and sometimes occurring only after the completion of all microtubule circles.

The possible sphere shown in the whole mount of Figure 20 has a fairly large proportion of bundled microtubules, with only a few scattered and apparently growing out in "bird's cage" fashion. This would be in accord with SEM Figures 12c and 12d which show incipient points at both ends of the sphere along with a possible "rim" which might be indicative of bundled microtubules. However, these micrographs present no evidence against the possibility of many single microtubules traversing the bulge from tip to tip (as seen in b of model). The presence of these two points, at opposite ends, in a cell which is still basically spheroid indicates that in at least some of the cases the microtubules exert point-forming pressure against both ends, so that the double-pointed stage may be hypothesized to precede the single-pointed stage (unless, as postulated, the cell sometimes goes directly to the single-pointed elliptical form). Figure 21, on the other hand, shows more

diffuse microtubule growth, such that if it continued in the same directions the result might be a cage similar to that seen in immunofluorescent Figure 40b. (It must be remembered, however, that even if present, the postulated cage-like growth of the microtubules around the sphere may not be evident in the whole mount micrographs, due to flattening during preparation, which involves centrifugation of the cytoskeletons onto the grid. What is needed to confirm or negate the hypothesis of dispersed microtubule growth is a method to look at an unflattened cytoskeleton, perhaps via an in situ lysis, fixation, and embedment at a stage when many spherical erythroblasts are expected, followed by painstaking serial sectioning and three-dimensional reconstruction, or via confocal microscopy.)

Perhaps the most probable scenario is a loosely controlled outgrowth of microtubules such that a certain proportion remain diffuse until they reach the end distal to the MTOC, there beginning bundling and/or curvature back toward their origin, whereas others begin aggregation while still quite short and grow in concert with their neighbors. This possibility may be seen in Figure 40, where the microtubules of b seem to be growing out symmetrically and those of a appear to have both preliminary bundling and a preferred directionality. The work of Euteneuer, Ris, and Borisy (1985) on marginal-band microtubule polarity seems to support this hypothesis. Looking at erythrocyte marginal bands in Rana catesbiana, Necturus maculosus, and

Sternotherus odoratus, they found a mixture of polarity patterns of microtubules within a species along with a consistency of polarity pattern along the entire length of any given erythrocyte. Assuming, as is common, "that all microtubules initiated by a centrosome have the same polarity," they point out that "an evenly mixed polarity pattern may indicate that microtubules arose uniformly about the centrosome and were consolidated into two bundles running in opposite directions" (presumably after each microtubule had completed at least one circle). Any bias in direction of outgrowth would give an uneven mixture of polarities.

Such variations could be made possible by rather slight discrepancies in the timing of the synthesis of such factors as microtubule-associated proteins (MAPs) and MS proteins. Whether or not the outgrowth from the presumed microtubule organizing center is random awaits analysis at the molecular level.

Pointed Cells: Double and Single, Spheroid and Flat

Tooze and Davies (1967) describe cells of the next stage, the polychromatic erythroblasts, as cells where "the amount of cytoplasm relative to the nucleus has increased due, no doubt, to both an increase in haemoglobin content and nuclear shrinkage." Their micrographs of such erythroblasts show elongated cells, including one which apparently has one rounded end, with the other end slightly pointed. (One must, of course, remember the pitfalls of

extrapolating three-dimensional shape from a few thin sections.) They also state that in this stage "Bundles of gently curved microtubules lie around the periphery of the cell. There seems to be an increase in the frequency of tubules." They found that mature erythrocytes have smaller nuclei than at earlier stages, and that "microtubules... present in the mature cell...form a marginal band like that described by Fawcett." Thus it seems that basophilic erythroblasts are rounded, with either no marginal band or one which is not yet completed. Probably the pointed cells seen in phase and scanning microscopy and represented by the intermediate, double- and single-pointed, structures of the model are polychromatophilic (or polychromatic) erythroblasts. Few of the authors who present classical hematological studies mention the presence of pointed cells. Jordan and Speidel (1930) illustrate spindle cells from both Myxine glutinosa and Lampetra wilderi but describe them as a separate cell type, rather than as a stage in erythroid differentiation. It is of interest, however, that they mention a curving of the pointed processes, "especially in the early stages of elongation"; these may be compared with the curved points of the double-pointed cell seen in the SEM, Figure 12h. Sugiyama (1926) describes "a few spindle-shaped red cells in the blood of chicks, not only in embryonic life but also after hatching....sometimes with one end pointed and the other rounded, sometimes with both ends pointed." He found these both in embryos and in chicks.

His drawings look very much like the pointed cells found among the developing erythrocytes of the axolotl. (He also cites descriptions of spindle-shaped cells found in the late 19th Century by M. Lowit and P. Knoll in poikilothermic adults, Rana larvae, and trout embryos.)

Murphy (Murphy, Grasser, and Wallis, 1986) does not mention the presence of pointed cells in his work on chick bone marrow erythropoiesis, but several may be noted in one of his figures which shows both phase and immunofluorescent views.

The existence of pointed cells as an intermediate form is indicated in the phase microscopic studies evaluating cell shape by larval age and in the meager culture work. Their presence is confirmed by such scanning electron micrographs as Figures 12e, f, and h which show both semi-cylindrical and flattened double-pointed cells. Although only a somewhat rounded single-pointed cell is shown here (12i), flattened single-pointed cells are seen frequently as well. That such pointed cells also exist in the circulating population is also documented both by phase microscopic observations (Figure 13) and, more convincingly, by scanning electron microscopy (Cf., eg., Figure 16-18). The detection of both rotund and flattened pointed cells, double- and single-pointed, leads to a model with alternate pathways.

The hypothesis that the variety of pointed cells may be due to differences in the timing of bundling of the microtubules is supported by the immunofluorescence results.

In Figure 40d may be seen a marginal band whose microtubules are not (yet?) all tightly grouped together. This cytoskeleton is obviously double-pointed. Unfortunately, an obviously spherical pointed cell was not found among the immunofluorescent samples. The only single-pointed cytoskeleton seen with such splaying is that in Figure 40h, whose apparent partial bundling indicates that the microtubules may be in the process of aggregating. The partial rounding at the pointed end of the same cytoskeleton indicates that the microtubules which extend out to the point are curving around to lead to the eventual loss of the point. This is also seen in Figure c, whose shape may indicate that a disc is being formed (or a sphere being reshaped). These results imply that most bundling of the microtubules may occur while the cell is at a double-pointed stage, but more data are needed. Pointed cells with unseparated microtubules, such as those in Figures e and f, were a more common occurrence. It would be of value to determine the polarity of the microtubules in such cytoskeletons as those in d and e; in accord with the work of Euteneuer, Ris, and Borisy (1985), the polarity should be the same in all microtubules if, as it appears, they have not yet begun to curve around to grow back to the centrosomal pole. Such marginal bands as those in f, g, h, and i, on the other hand, should logically have microtubules of mixed polarity. It seems unlikely that the axolotl splenic erythrocytes would have many bands with the uniform

polarity found by Euteneuer, Ris, and Borisy (1985) in Rana and Sternotherus, because this would seem to require a single bundle of microtubules growing in one direction and curving around more or less in concert. No trace of such band morphology was seen in this work. The relative scarcity of pointed cells presumably indicates that once microtubules begin to grow (in the sphere), the remainder of development into an ellipse may happen quickly. Perhaps only a good culture system can give a definitive answer to the timing question.

Electron microscopy of whole mounts generally confirmed observations from immunofluorescence, showing that the microtubules are only weakly bundled, if at all, in spheres (Figure 20 and 21), and may or may not show tight bundling in pointed cells. Unbundled microtubules in pointed cells may be seen in Figures 22, 26, 28, and 30.

Unanswered is the question of what causes the microtubules to bundle, as well as when they do so, in a perfect hoop with the two half-bands stretched to opposite sides of the ellipse. The participation of the microtubule-associated protein MAP2 seems likely; a MAP2-like factor isolated from Bufo marinus erythrocytes by Centonze and Sloboda (1986) was found by them to induce microtubule aggregation in vitro. It is tempting to postulate that the newly formed MS gives some kind of guidance in the aggregation and location of the band. Perhaps the tracts postulated to guide the growth of the reformation of the

marginal band after dissolution by cold or drug exposure (Cf. Centonze, Ruben, and Sloboda, 1986) may develop at this point in cell differentiation as the microtubules move to their final alignment just underneath the MS.

It would be of particular value to study the formation of the band, using immunofluorescence, in a defined population of developing cells, such as one synchronized by acetylphenylhydrazine (See below). In their elegant reassembly experiments, Nemhauser, Joseph-Silverstein, and Cohen (1983) were able to show pointed cells which had only partial marginal bands emanating from the centrioles at the points. Unfortunately, in the experiments with the axolotl cells, all splenic pointed cytoskeletons had marginal bands which were already nearly or fully completed. Incomplete microtubules in forming marginal bands were seen only in spherical cells. Of course, in the case of the axolotl, the cytoskeleton seen was the newly forming one, rather than a reassembling one. Results found in band reformation, pertinent and useful as they are, do not necessarily represent the full details of the original band development. Also, one might consider that in the rewarming experiments samples could be selected for having undergone marginal band reassembly for only a short time, i.e., 2 and 5 min., whereas the stage of development reached by any given axolotl splenic cell was a matter of chance. Systematic analysis of cytoskeletons from maturing cells at progressive developmental times is necessary for a complete elucidation

of the microtubule growth and association. Once a system for doing so was elaborated, additional information could be derived by the use of immunological labeling with gold as a second antibody and viewing the labeled uncompleted microtubules with the electron microscope. (Experimentally more difficult, a further path of investigation might attempt labeling of living erythroblast, for example, with fluorescently or radioactively labeled or biotinylated tubulin, to study the dynamics of marginal band growth and bundling.)

Centriole Presence in Pointed Cells

The model hypothesizes the presence of centrioles at the presumed origin of microtubule growth. Phase microscopy of pointed cytoskeletons showed pairs of dots in the points which were tentatively identified as centrioles by their size, density, and pairing (Cohen and Nemhauser, 1980; Nemhauser, Joseph-Silverstein, and Cohen, 1983).

Neither conventional nor high voltage electron microscopy of whole mounts verified the presence of centrioles, although many of the structures seen within pointed cytoskeletons seemed to the student to be strong candidates. If, in fact, they were centrioles, it might be possible to modify the approach used in order to clarify the relations/connections with the rest of the cytoskeleton, particularly the marginal band. As seen in Figures 27-28, 35, and 37-39, structures seen meet several criteria of centrioles: right-angle orientation, cylindrical shape, and

size (about 0.2 μm diameter and 0.35-0.50 μm long) (Vorobjev and Nadezhdina, 1987; Cohen and Nemhauser, 1980).

(Centrioles, which may contain other important structures in addition to the centriolar cylinder, consisting of nine triplets of MTs, are relatively constant in form and size, about 0.2 μm diameter and 0.2-0.5 μm long. [Vorobjev and Nadezhdina, 1987])

In addition, although not optimal in terms of showing relationships to the marginal band, electron micrographs of thin sections show centrioles in some instances (Figures 41 a-c, as well as Figure 6 of primitive cells). Sections from lysed spleen embedments were often difficult to interpret without serial sectioning because of cytoskeleton distortion. More attention should have been given to sectioning and electron microscopy of isolated erythropoietic cells embedded in monolayers, for those might have given clearer results, especially since that method enabled the viewing of the cells under phase prior to sectioning. It could have allowed optimal correlation of external shape with internal structures. An alternative method, contemplated but not attempted, might have been to remove the formvar containing whole mounts from their grids, after examination by electron microscopy, and to carefully embed them directly into epon. Thus, with prior knowledge of the exact location of possible centrioles, serial sectioning followed by electron microscopy might have been singularly

fruitful in confirming (or disproving) the structures seen in the whole mounts as actual centrioles.

It had been hoped that it might be possible to obtain some anti-centrosome serum, which would have been most useful in determining the centriole presence, particularly in spheres, whose lack of a developed cytoskeleton made them elusive in whole mounts and in thin sections. If sufficient anti-centrosome antibody were available, a double labeling experiment together with anti-tubulin and appropriate second antibodies might be useful.

The Centriole as a possible microtubule organizer

Various studies have indicated that the centrioles and the centrosomes (structures surrounding the centrioles) operate as microtubule-organizing centers, or MTOCs.

"Electron microscopy has shown that microtubules entering the cell center may be attached there to electron-dense structures" (Vorobjev and Nadezhdina, 1987, p. 227).

Nemhauser, Joseph-Silverstein, and Cohen (1983) found that the pericentriolar material of centriole pairs in blood clam erythrocytes seems involved in the microtubule nucleation of marginal band reassembly during rewarming after use of low temperature to disassemble the marginal band. Mature erythrocytes of this species retained a pair of centrioles associated with the marginal band, and at the earliest stages of rewarming (2') all microtubules present were short and focused on the centriolar pole, which was pointed.

Formation of thin continuous bands continued to emanate from

the pole, which remained pointed. By fifteen minutes, cytoskeletons had continuous marginal bands, but with fewer interconnections and connections to SAC than in controls; bridges between microtubules were seen at two hours. In uncooled preparations, cells which were pointed had their centrioles at the point. The microtubules of the marginal band were not continuous with those of the centriolar cylinders but seemed to insert into the pericentriolar material, or to pass between the centrioles and the SAC. The results thus support the postulate of centrioles in these species as MTOCs. Apparently both centrioles are active, "since MTs emanate from each of them throughout reassembly....

Nucleation of MTs for MB construction in this cell type is...presumed to be a function of pericentriolar material, as in various other microtubule systems, with the centrioles serving to organize and/or anchor...material." (p 986)

Many authors have described pericentriolar satellites radiating out along the length of the centriole. These are homogeneously staining dense masses composed of a head which is a microtubule-nucleating center, and a foot, which fixes the head to the centriolar cylinder. Vorobjev and Nadezhdina (1987) state that Vorobjev and Chentsov (1977 in Tsitologiya 19) made counts of pericentriolar satellites in serial cross sections of centrioles in axolotl hemopoietic tissue and found a variable number of satellites (from two to thirteen, usually four to seven); in this tissue the

satellites were arrayed along the full length of the centriolar cylinder.

Such observations emphasize that serial sectioning of cytoskeletons such as that seen in Figure 35 should have been pursued. In this figure, microtubules may be imagined to insert at different sites along the length of the centriole-like structure, and it would have been of great interest to have determined whether this were the case, and if so, whether the insertions were actually into the pericentriolar material.

According to Vorobjev and Nadezhdina (1987), the number of microtubules polymerized on an MTOC in vitro is proportional to the tubulin concentration; it also varies with MTOC type. In vitro, preferential growth of microtubules occurs from an organizing center when there is enough tubulin for elongation from the positive end but too little for spontaneous polymerization. It is postulated that cell conditions permit microtubule growth only on primers of microtubule-nucleating centers, each giving rise to one microtubule. It is thought that local ionic conditions in the organizing center region favor microtubule assembly. Vorobjev and Nadezhdina (1987) summarize the data on in vitro microtubule polymerization activity on organizing centers and conclude that an MTOC is capable of inducing assembly of microtubules under conditions excluding their spontaneous polymerization, and that the MTOC must

contain microtubule growth primers, whose biochemical composition is not clear.

It is also of interest that mature red blood cells which lack centrioles, such as those of the dogfish and the chicken, nevertheless reassemble their marginal bands upon rewarming (Cohen, et al. 1982a; Miller and Solomon, 1984). This indicates that the centriole is not always essential for the reformation, at least, of microtubules. Both Brinkley (1985) and Vorobjev and Nadezhdina (1987) present the possibility that a MTOC may act in part by containing an assembly-promoting environment which has a lowered threshold for microtubule assembly. It is possible that during marginal reformation, the environment is more conducive to assembly, perhaps through such factors as tracks in the membrane skeleton or MAPs already present on or near nascent microtubules. The postulate of a role for tracks or some other peripheral determinant is supported by Solomon's work with chicken erythrocytes: Miller and Solomon (1984) found that during *in vivo* marginal band reassembly the number of microtubule profiles in a cell seldom exceeded two before completion of a complete circumference by one; Swan and Solomon (1984) used an *in vitro* system to show that purified tubulin would form a marginal band in the presence of extracted cytoskeleton. Brinkley (1985) refers to regulation by "dispersed templates in the matrix or ground substance of the cytoplasm" (p. 160), but work such as

Solomon's indicates that microtubule regrowth guides are more likely in or on the membrane skeleton.

The lack of a role for a MTOC in the mature erythrocyte is supported by the general absence of this structure among the overwhelming majority of such cells studied. The presence of possible centrioles (paired dots) very near the marginal band in many primitive elliptical erythrocytes from axolotl embryos remains an intriguing puzzle.

Mature Erythrocytes: Ellipses and the Puzzling Discs

The trend toward an increase in proportion of ellipses with increasing larval age, the relative abundance of ellipses in the larval circulation, and the body of literature on non-mammalian erythrocytes all contributed to the inclusion of an ellipse as the final stage of morphogenesis in the model. Such cells were seen to be elliptical by all morphological approaches (See Figures 10, 12, 13, 14, 19, 31, and 401). As expected, all ellipses and discs found were flattened, with an equatorial rim and a nuclear bulge (See Figures 12g and j and 14). They also generally had bands with tightly congregated microtubules (See Figures 40 j and l).

[It should be noted here, as a cautionary interjection, that micrographs must be interpreted circumspectly: one structure in Figure 12 b was confidently identified as a folded or bent ellipse, based upon elliptical appearance during various tilts of the H6001A stage, and upon axes of approximately $26\text{ }\mu\text{m}$ x $11\text{ }\mu\text{m}$ (after correction for about 30%

shrinkage), but this was later proven not to be the case. Similarly, most cell identifications, unless confirmed by a second methodology, should be treated as probable, rather than definitive. (In the particular case of Figure 12b, computerized image analysis of photographs at different tilts might have helped.) However, it is hoped that the care taken to ensure consistency of cell identification conditions has decreased the probability of massive errors.]

The inclusion of a step in the model whereby the flattened single-pointed cell transforms directly to an ellipse is based in part on the fact that most pointed cells were elongated rather than rounded. At no time, with any method, was a pointed disc seen; rather, it seemed that as soon as the marginal band had lengthened the cell began to assume an elliptical contour. (Of course, in the absence of a visible MS, one might argue that the mere tension of the unflexed microtubules pushing against the membrane resulted in elongation of the cell.)

Thus arose the hypothesis that in this system the disc is not an obligatory intermediate form but rather a variant; if the MS (See later in Discussion) tends to shape the ellipse, and if this is present early, then an ellipse should be the preferred shape. That the MS helps determine ellipticity may be seen from the ellipse-to-circle transformations found by Cohen (1978) in erythrocytes of Notophthalmus viridescens during pepsin digestion of semilysed (sic) cells; the marginal band circularized before

release of nucleus, and the isolated band circularized as well. Cohen hypothesized "that the TBM is normally under tension, applying such forces asymmetrically across the MB. The otherwise more circular MB (and the cell) would thus be converted to an ellipse, with TBM tension accounting for cell flatness as well. Both MB and TBM acting together as a system would be required to produce the flattened elliptical morphology of nucleated erythrocytes....The proposed combination of MB strain and TBM tension results in a system with flexibility and deformability, as well as the potential for returning rapidly to normal equilibrium morphology upon removal of deforming forces...." (p.269-270) The "decision" of any given cell whether or not to achieve ellipticity via an intermediate disc may depend upon subtle variations in the timing of the membrane skeleton's formation and the affixing of intermediate filaments onto it. The elliptical shape would seem to indicate a taut cytoskeleton, the disc a more relaxed one. If the completion of those binding domains contributing to a firm tension across the cell precedes, or is concurrent with, the completion of the marginal band, the erythrocyte might be expected to proceed directly to an elliptical shape; if, however, the final attachments develop after the completion of the marginal band, an intermediate disc should result.

The hypothesis that the disc is an "optional" intermediate in erythroid morphogenesis is at odds with the work in avian cells, where the ellipse always seems to be

preceded by a flattened circular disc. Small and Davies (1972) state clearly, "The change to the polychromatophilic stage is accompanied by the appearance of an ordered band of microtubules, the marginal band, which is associated, as with the primitive generation, with a change in shape of the cell from a roughly spherical to a lenticular form, the cell at this time being circular in plan view." Barrett and Scheinberg (1974) also found that flattening of cultured spheroidal erythroid cells preceded their becoming "slightly elliptical" by 2 3/4 hours. Evidence for the role of microtubules of the marginal band in determining this flattening was described by Barrett and Dawson (1974). Dieterlen-Lievre's work (1988) also supports the concept of the disc as a general intermediate form, rather than as the alternate pathway indicated in the model. The role of the marginal band in such morphogenesis is mentioned in the Dieterlen-Lievre article (p. 285): "Young [avian] erythroid cells in the early stages of postmitotic maturation display a marginal band of microtubules that seem to play a critical role in the acquisition of the flat discoidal shape."

These same researchers have found that in avian erythroid development the microtubules of the marginal band decrease in numbers as the cell matures. Although this thesis research included no systematic counting of microtubule number in the marginal bands of young versus mature erythroid cells, the general impression was that the number did not decrease as it does in avian red cells. This

agreed with Small and Davies (1972) who found that the number of microtubules in Triturus polychromatic erythroblasts was similar to that in mature erythrocytes. They suggest that this may indicate that the marginal band plays a greater role in erythrocyte shape maintenance in the amphibian than in the chicken. In evaluating this, it might be useful to consider the different physiologies of the two classes, including blood osmolality and circulatory rates. Amphibians are poikilothermic and remain in a moist environment, whereas birds are homeothermic, have a very high metabolic rate, fly, and breathe dry air. This might result in differences in physiological adaptations, so that the amphibian cells require the firmer structure provided by a greater number of microtubules. There might also be some differences in the composition of the membrane skeleton. It would be interesting to compare microtubule numbers during erythroid development in reptiles and fish.

The decrease in microtubule number found in the avian marginal band implies some sort of "microtubule-trimming" control which may explain the lack of microtubule overlap found by Euteneuer, Ris, and Borisy (1985) in mature erythrocytes. Such micrographs as Figures 40 c, f, and g and Figure 26 show an "excess" microtubule length, which, to avoid overlap, must either continue around the cell once more to the origin or be trimmed to abut or join opposing microtubules at that site in the band. (The question of

"loose ends" versus completed rings in the marginal band is an interesting problem in its own right.)

It is assumed that a cell, once flattened due to bundling of the microtubules in an equatorial position, would not return to a more rotund shape. This does not preclude the possibility, however, that changes in the MS to relax the tension across the band might cause a change from an ellipse to a disc.

However, a problem regarding acceptance of the ellipse as the final stage of morphogenesis arose when circulating erythrocytes from three adult axolotls were examined.

Adult Axolotl Erythrocytes

The few adult axolotls whose erythrocytes were examined possessed a majority of discoid rather than ellipsoid cells. This was puzzling, in view of the literature and of personal observations of cells of adult Rana pipiens, Triturus viridescens, and Amphiuma tridactylum, most of whose cells were elliptical. In the erythropoietic model presented here, the adult discs have been accommodated by a conjectural step labeled "changes in MS to relieve tension and create 'post-mature' form." However, in the absence of analysis of erythropoiesis in adult axolotls, the possibility that discs are formed directly, with no intervening elliptical stage, must be kept in mind. (In the course of the work with the adults, samples of probable erythropoietic tissue, the liver, spleen, and kidney, were fixed and embedded.

Unfortunately, time did not permit sectioning and microscopy of these.)

Ducibella (1974) has studied the changes in axolotl red cells with normal development and with thyroxine-induced metamorphosis. He found that larval, but not adult, red cell shape was affected by the isolation medium: they were normally elliptical in 67% Liebovitz but crinkled and spherical in ACD (citric acid-trisodium citrate-dextrose). Thus he used this difference as a determinant of cell type. (Note, however, that ACD used was at 2°C, whereas Liebovitz was at 20°C, so that temperature may play a predominant role.) His photographs of larval (younger than 100 days) versus neotenus adult (300 days and older) erythrocytes indicate the presence of a substantial proportion of disc-shaped cells in the adult, with virtually all larval cells being elliptical; however, he shows many fewer discs than found in this work. The current work is in accord with his in finding that adult erythrocytes are longer than the larval, about 28-32 μm compared to about 22-26 μm . The size difference found by Ducibella was, however, larger. (Note, however, Charlemagne's finding (1972) that mature erythrocytes are 30-35 μm in young animals and 31-33 μm in adults, i.e., not significantly different. Apparently, though not stated or presented photographically, these cells were all or mostly ellipses.) Of particular interest was the correlation found by Ducibella between the appearance of

adult versus larval hemoglobin and the cells' morphology in ACD.

Similarly, Dorn and Broyles (1982) have found morphological differences, as well as differences in hemoglobin type and in cell density, among the erythrocytes of Rana tadpoles and adults. In their photographs, the tadpole (Stage XXI) cells appear as oblate ellipsoids, like short loaves of French bread, whereas the mature adult (Stage XXIV) erythrocytes are flattened ellipsoids, with the nucleus bulging prominently at the center while the cytoplasm is flattened out to the marginal band. The immature adult erythroid cells (Stage XXII) are discoid in shape, but with the same prominence of nuclear bulge and flattening of the cytoplasm ("fried-egg-like") as seen later. The nuclei of the mature adults appear elliptical, whereas those of the immature adult erythrocytes are more rounded. The latter cells were found to increase in number as metamorphosis proceeded. They matured in shape and increased in density after the tadpole erythrocytes disappeared.

In this thesis the axolotl adult red cells seem more like the immature adult cells pictured by Dorn and Broyles. It is not known whether this is due to the fact that the axolotl is neotenuous, or to some other aspect of axolotl physiology. Neither is the reason for the cell shape discrepancy between this work and that of Ducibella (1974) apparent. The adults used in this work were probably older

than those used by Ducibella, having participated in several breeding seasons. Also, the rigors of shipment from Indiana might have had an effect upon the animals' physiology, and using them fairly quickly after their arrival may have introduced an aberration. Ducibella's paper implied that his animals lived in his laboratory until use. Also, various amphibians have been shown [Frangioni and Borgioli (1988)] to have seasonal differences in erythropoiesis, and it might be of value to examine this aspect.

The strict developmental compartmentalization of the Rana hemoglobins, found by Broyles and coworkers, seems significant. It would be of interest to expand Ducibella's work by determining and comparing the hemoglobin contents of axolotl erythrocytes at various stage of development from prehatching through metamorphosed adult.

In view of the shape differences, to which the MS may contribute, comparison of axolotl larval versus adult cytoskeleton compositions by PAGE electrophoresis might illuminate this problem. Such a project would, of course, be difficult because of the small quantities of larval sample readily available. The student had hoped to utilize frozen, pooled larval cytoskeletons in an attempt at such electrophoretic analysis, but never did so.

Thus the brief examination of circulating adult erythrocytes actually complicated the model, necessitating the inclusion of a discoidal "post-mature" form. It would be of interest to induce metamorphosis in adult axolotls

with thyroxine to determine whether such a physiological change resulted in a prevalence of elliptical mature cells, as is found in other amphibian species. In the same vein, some of the experiments done for the thesis might be repeated in Ambystoma tigrinum, "the axolotl's close relative which undergoes anatomical metamorphosis during normal development" (Ducibella, 1974). Additional information might be found by a long-term indagation of erythropoiesis in adult axolotls with chemically induced anemia (See below, Suggestions for Further Research). Another possible approach might be the culturing of cells from adult axolotls, possibly after induction of anemia, in the hope that the timing of the disc appearance could be clarified.

Correlation of Marginal Band Formation with Morphogenesis

Thus the correlation of the formation of the mature ellipse with the rounding of the marginal band and with classical erythropoietic staging still requires clarification, as does the closely related question of when erythroid flattening actually occurs. Cameron observed erythrocyte flattening to begin about stage 38, and found two groups of the blood cells, spheres with 16 yolk plates and flattened ellipses with 8 per cell. He interpreted his observations as indicating an even distribution of yolk platelets with each mitotic divisions, including at least three such divisions of elliptical cells. As mentioned above, a greater body of evidence indicates that spheres are

generally (always?) the form which undergoes mitosis. According to Dieterlen-Lievre (1988), avian "red cell precursors are spheroidal, becoming flattened after the final mitotic division." After that, they become circular disks, acquiring the elliptical form during further maturation.

Because much of the model depended upon the identification of cell shapes by phase microscopy, an experiment to attempt to correlate three-dimensional shape with cell cytology was begun but not completed. This involved viewing of cells from the same pool in both phase and scanning, as well as an attempt to view them in slow movement in a viscous solution. Cells from ten plump spleens of six-day larvae were combined in a few drops of 80% Wolf and Quimby medium. After mixing, cells were deposited on formvar- and polylysine- coated hexagonal 100-mesh grids. The plan had been to examine and photograph these grids under phase, and then to prepare them for scanning electron microscopy. Preliminary tests had shown that, although some cells were lost in the fixation and critical point drying, they did not move, so that the arrangement of cells found on the hexagonal grid pattern could be accurately correlated with cells viewed in the SEM. An additional sample was to have been taken from the pooled cells for viewing, both fixed and unfixed, in methyl cellulose solution, whose viscosity would enable slow rotation of the cells. It is the (admittedly highly

personal) opinion of this student that this line of inquiry might have provided a useful addition to the research.

Another approach to the study of cell shape lies in the physiological or chemical manipulation of the cytoskeleton or other aspects of cellular metabolism or development. One method essayed was that of exposing splenic cells to low temperatures.

Effects of Cold Exposure on Erythroid Cell Shape

Because cold exposure usually leads to microtubule disassembly, axolotl erythroid cells were incubated at low temperature in the hope of confirming the relationships between cell shape and microtubule presence. Results from such exposure of isolated splenic erythroid cells are indicative of an effect on the shape-stabilizing cytoskeleton. A six-hour exposure at 0° - 1°C led to an apparent conversion of cells of mixed morphologies from 23% to 100% spheres, and a striking though less dramatic partial conversion of ellipses to spheres ensued after a one- to two-hour exposure.

The erythrocyte marginal bands of many species are known to be cold-labile (Cohen et al., 1982; Cohen, Langford, and Sloboda, 1982), and it is reasonable to postulate that the shape change seen in the axolotl larval erythroid cells is due to cold-induced band disassembly. However, the marginal bands of amphibian erythrocytes have been reported to be cold-stable (Behnke, O., 1970), and this has been found to be true in the case of adult Rana pipiens.

The lability may be different, nevertheless, for larval cells, which have been found to differ from adult cells in osmotic stability and deformability (Hollyfield, 1966).

Many reports indicate that rewarming of cold-labile microtubules results in their reassembly (Cohen et al., 1982; Dustin, 1984; Miller and Solomon, 1984).

Unfortunately, one-hour rewarming of cooled axolotl splenic cells did not lead to restoration of elliptical shapes. This time, however, may have been insufficient for thorough warming and reassembly.

Future work involving the effect of low temperature might include the following:

- (1) Watching the shape changes at closer intervals during cooling.

- (2) Longer rewarming to determine whether this would lead to restoration of original shapes.

- (3) Lysis of aliquots of cells at each stage of isolation, cooling, and rewarming in order to ascertain presence and state of marginal bands and, possibly, centrioles. This should enable correlation of cell shape at these stages with cytoskeleton state.

- (4) If (2) and (3) showed a marginal band disassembly and reassembly correlated with changes in cell morphologies, a repeat rewarming in the presence of the microtubule assembly inhibitor colchicine (or colcemid).

(5) Determination of whether the presence of taxol inhibits band disassembly and/or shape change in the same conditions.

(6) Repetition of the above experiments with intact spleens, or even with splenic cultures, rather than isolated cells.

(7) Because it would be of interest to determine whether cold lability of cell morphology decreases as cells fully mature, repetition of cooling/rewarming experiments at different stages of axolotl development, eg., in older larvae when spleens appear ready to join circulation, or in adults. Along with a comparison of results with circulating versus splenic blood, these might demonstrate whether there is a time during development at which shape is fixed. (See below for discussion.)

(8) Of course, electron microscopy and immunofluorescence, as well as phase microscopy, of the cytoskeletons in the above experiments would aid in interpretation.

Cohen et al. (1982) found that the marginal band of dogfish erythrocytes was cold-labile, but that band disassembly did not affect cell morphology. Cells used were from the circulation of adult fish, so that they might be assumed to be fully mature. The trans-marginal-band network remained during cooling, although in cytoskeletons it collapsed around the nuclei. It reverted to its original state, however, when the marginal band reassembled (assuming completely normal microscopic appearance) upon rewarming.

Conversely, Cohen (1978, 1982) found that dissolution of the TBM in both nucleated (Notophthalmus viridescens) and anucleate (Batrachoseps) erythrocytes led to circularization of the marginal band.

In their work with avian erythroid cells, Barrett and Scheinberg (1972) and Barrett and Dawson (1974) found that mature flattened cells (maturity judged by cytological criteria) did not revert to spheres upon cooling, as immature ones did. Barrett and Dawson (1974) also found that the microtubules of the more mature cells displayed less cold lability, with a majority taking longer to disassemble and a few failing to disassemble at all. Similarly, Kim et al. (1987) found an increased resistance to microtubule-depolymerizing drugs and an enhanced precision of band formation after cold depolymerization in mature erythrocytes.

Such results indicate that the final morphology of the erythrocytes is dependent on the totality of cytoskeletal interactions, including not only the marginal band but also the filaments of the MS and a variety of accessory proteins.

Other Aspects of the Erythroid Cytoskeleton

The complexity of the erythroid cytoskeleton has been shown by many investigators in both anucleate mammalian and nucleated nonmammalian red blood cells. At maturity the mammalian cells have only the spectrin-actin skeletal network associated with the plasma membrane, whereas the

nonmammalian cells contain a much more complex cyoskeleton with more structural components (Lazarides and Woods, 1989).

Both types of cells possess a MS, a filamentous network "closely applied to the cytoplasmic surface of the membrane" (Steck, 1989, p. 210). Briefly, this is composed of long filaments of spectrin crosslinked by short filaments of actin into a hexagonal or pentagonal lattice. Protein 4.1 reinforces the spectrin-actin association and also helps link the array to the membrane via the integral membrane protein glycophorin. The attachment of the skeleton to the membrane is further mediated by ankyrin's interaction with a subunit of spectrin and with band 3, the membrane anion transporter. (Lazarides and Woods, 1989; Steck, 1989)

In addition to the MS, nucleated nonmammalian erythrocytes possess a marginal band and a network of intermediate filaments (composed of vimentin) between the nucleus and the cell surface (Lazarides, 1987).

Among the more elegant work showing this cytoskeletal intricacy is that of Centonze, Ruben, and Sloboda (1985, 1986) in which they examined platinum-carbon replicas of Bufo marinus erythrocyte cyoskeletons and identified various protein components using immunogold labeling and immunofluorescence. Electron micrographs of the replicas show filaments and cross bridges as well as microtubules and when viewed in stereo give a clear conception of their structural interrelationships. The MS's character as a lattice enveloping the remaining cell contents is apparent,

as is the close association of the marginal band with the MS. Using immunological techniques the authors found the MS to contain alpha spectrin and actin. The cross-bridging filaments linking the microtubules within the band were identified antigenically as MAP 2. These cross-bridges are believed to be a major factor in the stabilization of the marginal band. A network of 9-11 nm filaments extending between the nucleus and the cell periphery was seen in some preparations and was determined to contain both vimentin and synemin. These filaments are thought to anchor the nucleus, keeping it in position within the cytoskeleton. In addition, there seem to be connections between the marginal band and the MS, protein composition unknown, which result in the band's appearing in some micrographs to be suspended in the MS. This, the authors postulate, "would explain the ability of the microtubules in some erythrocytes to be depolymerized by cold or drug treatment and then repolymerize in the same position when the destabilizing conditions are reversed" (Centonze, Ruben, and Sloboda., 1986, p. 386).

The development of the MS was merely a peripheral aspect of this thesis, the emphasis being on the formation of the marginal band. However, any study of cellular morphogenesis must perpend all aspects of the cytoskeleton, including the MS. In the current work, much of the filamentous material of the MS appears to be positioned by the time that the microtubules have begun to grow (See, eg., Figure 21). Unfortunately, spheres with even earlier

beginnings of marginal bands were not easily identifiable. A useful approach might be the use of double-label immunofluorescence (for example, anti-tubulin with fluorescein-labeled second antibody and anti-actin or vimentin with rhodamine-labeled second antibody) with systematically isolated cytoskeletons from spleens of young larvae. Another technique might involve the injections (into spleens or into cultured cells) of labeled precursors in order to follow synthesis of the various cytoskeletal components. Any attempt to clarify precisely the timing of the formation of the various cytoskeletal elements must investigate very early stages.

One indication of this in axolotl cells was the apparently early restriction of nuclear movement. It was noted that, similarly to the results found by Cohen (1978) with adult Rana and Amphiuma, the nucleus of the lysed axolotl erythroid cell, of both the primitive and definitive lines, was resistant to displacement. Tapping or pressing the coverslip, though causing movement of yolk platelets, if present, did not cause significant movement of the nucleus. This was true at all stages where there was a marginal band visible at the light microscope level (thus the band was completed), and was interpreted to indicate that the development of the cytoskeletal filaments from the nucleus to the membrane (Goldman et al., 1985) occurs at an early stage. An additional possibility is Cohen's hypothesis that

"...TBM pressure against nuclei would account for their positional stability...and for the nuclear bulge seen in all such cells viewed edgewise." (Cohen, 1978, p. 270)

Support for the postulate of early MS formation was found in the assumption of an elliptical shape by the developing erythroid cell while still pointed and in the visible presence of an extensive MS in all whole mounts possessing a completed marginal band of any shape (See Figures 19-26, 30-34, 36).

This, then, is one example of the interactions of the various domains of the nonmammalian erythroid cytoskeleton in contributing to cell shape formation and maintenance. Another aspect is the mechanism by which the microtubules of the marginal band position themselves appropriately in an equatorial band.

The question of tracks guiding microtubule growth has been raised by several investigators, including Centonze, Ruben, and Sloboda. (1986). Developmentally, however, it seems likely that such tracks are established by the marginal band and any proteins linking it to the MS after the microtubules have grown around the cell and bundled into a fairly tight ribbon. Evidence for this includes immunofluorescent micrographs (Cf. Figure 40 a, b) showing that during initial growth in the spherical erythroblast the tubules of the band splay out more or less symmetrically from a central point (putative centriole). In view of the apparent difference in shape maintenance between mature and

larval cells after low temperature exposure, experimental suggestion #7 above could be of particular significance. Presumably only after a given stage of cytoskeletal maturation would regrowth of the marginal band microtubules be guided into a cohesive bundle. Lazarides (1987) states, "The determinants initiating MB formation during development do not appear to be the same as those specifying the site of MB reassembly in vitro after tubulin depolymerization in mature cells." (p. 353)

It would be of especial interest to determine when the rest of the cytoskeleton, such as MAPs and the intermediate filaments, mature into place. It is probable that synthesis and assembly precede binding into a firm structure resistant to alteration; variations in cold lability of shape at different ages could reflect this. As reviewed by Lazarides and Woods (1989), ankyrin and protein 4.1 are the last constituents of the MS to be synthesized and assembled, and it is possible that their binding is the final step giving stability to the red cells. Murphy, Grasser, and Wallis (1986) propose microtubule association and attachment to the cell membrane as the final step in marginal band formation.

Nemhauser, Joseph-Silverstein, and Cohen (1983) postulate: "It appears possible, therefore, that cell 'maturation' entails modification of the SAC material such that it no longer requires the MB for shape maintenance under the experimental conditions....If the SAC were more

elastic or fluid in immature cells, then it might be deformed and shaped by the growing MTs within.

"We visualize the sequence of events during erythropoiesis as follows: the shape of the SAC and the cell coincide, with the cell initially being roughly spherical... Cells would begin to flatten at the 'initiation stage' as MTs made contact with the SAC and applied pressure against it. Further growth of MTs to the 'pointed stage' could elongate the SAC along the axis from the centrioles to the opposite side of the cell. Concurrently the SAC would constrict in the same plane in a direction perpendicular to that axis, resulting in a flattened teardrop-shaped cell. Subsequently, by passing peripherally to the points at which the centrioles are located, the MTs could close the ellipse and attenuate cell pointedness." (p 989)

Note, however, that this thesis postulates that the microtubules, growing without tension imposed by the SAC, first form points in a rotund, or spheriodal cell, causing elongation by the pressure of the microtubules at either end pushing out the excess membrane of the sphere. Only then, it is hypothesized, do the constraints imposed by the simultaneously (but possibly independently) developing MS result in flattening of the pointed cell. This hypothesis is supported by the presence of both rotund and flattened pointed cells and by two observations of living pointed cells (one single-pointed and one double-pointed) apparently undergoing a sort of contorted struggle during which

flattening occurred. It seems likely that the variations in the developmental sequence which are proposed in the model may be due to permutations in the chronology of the marginal band maturation vis-a-vis that of the MS.

As discussed by Lazarides (1987) and Lazarides and Woods (1989), assembly of the erythroid cytoskeleton is controlled both spatially and temporally. In their review, Lazarides and Woods (1989) state that in mammalian and avian erythropoiesis the expression and assembly of ankyrin, which mediates attachment of the spectrin-actin array to the cytoplasmic face of the plasma membrane, and protein 4.1, which reinforces the spectrin-actin interaction and contributes to membrane binding, occur late in development. They postulate that this "may ensure that full mechanical stability of the MS and the red blood cell as a whole occur as one of the last steps in erythroid morphogenesis well after the cells have become postmitotic." (p. 441) Steck's (1989) speculations about the genesis of (mammalian) red cell shape seem to agree: he suggests that the MS is "elastic in the short term," but "plastic in the long term," so that its assembly on the plasma membrane imprints the skeleton with the cell contour. During development in the nonmammalian erythrocyte, such a characteristic of the MS would result in its sharing with the marginal band the responsibility for shape genesis and maintenance.

Such questions of the timing and interactions of various developmental events contributing to cellular

morphogenesis are not limited to erythroid cells, although such cells furnish a convenient system for study. As maintained by Oliver and Berlin (1982), it is not possible to select one cytoskeletal component over another as the primary cell shape regulator, whether during development or during such dynamic events as chemotaxis or phagocytosis. Also, although not discussed here, the plasma membrane plays a role in its own shape determination, through its molecular asymmetry and through its interactions with the cytoskeleton. Studies of membrane fluidity indicate less membrane protein movement at regions of increased cytoskeleton-membrane interactions and more at sites of decreased cytoskeletal density (Oliver and Berlin, 1982).

Presumably, such cytoskeletal interactions with the membrane in the mature erythrocyte enable the cell at rest to maintain its shape even in the absence of the marginal band. There is evidence, however, that the marginal band contributes to shape maintenance during circulation (See below, section on circulation).

In the current work the cytoskeletal components other than the marginal band are seen only in the whole mounts (both TEM and HVEM) and in some thin sections (TEM). Clarification of the relationships among the various cytoskeletal elements might be most effectively accomplished via high voltage electron microscopy of serial semi-thick sections, followed by computerized reconstruction of the cytoskeletal structure. Since the only immunofluorescence

data was acquired with anti-tubulin, no conclusions may be drawn about the composition of the filaments seen in the electron micrographs. One obvious difficulty is the absence of any clear indications of developmental stage of a given thin section. Correlative microscopy similar to that done by Centonze, Ruben, and Sloboda. (1985) utilizing immunogold labeling could be very useful in defining the protein composition of the cytoskeletal components at various stages of development. Anemia-induced developmental synchronization (See below) might make such an approach especially fruitful, particularly in view of the pattern of spectrin subunit switching seen in early erythroid development (Lazarides and Woods, 1989). It would also be gratifying if an axolotl (or any non-mammalian species) strain with a mutation in the cytoskeleton, similar, perhaps to the spherocytic trait in mice, could be found (or developed) for study.

Culture of Erythroid Cells

Preliminary results achieved with the cultures of larval splenic cells indicate that the technique is a promising one for future study of erythroid differentiation.

Duprat and Flavin (1976) have described successful cultures of differentiating erythroblasts from the spleens of Pleurodeles waltlii, using techniques which served as a basis for those described here. They found Wolf and Quimby medium, which is buffered with NaHCO_3 , to give the best results, probably because they used a 2.5% CO_2 atmosphere

for incubation. In the absence of such a controlled atmosphere, Leibovitz L-15 medium, which is formulated for use in free gas exchange with the air, seems preferable. The use of media which are dilute in comparison with those used for mammalian and avian cultures is in keeping with the fact that amphibians have more dilute body fluids than most other vertebrates (Hronowski and Armstrong, 1977). For long-term culturing of axolotl epithelial cells, Neff (1984) used a medium consisting of 55 parts L-15, 25 parts H₂O, and 20 parts fetal bovine serum, a somewhat richer medium than that used here, and one which might be tried in any subsequent work. Work reported by Carver and Meints (1977) found that a more dilute culture medium, MC33, 225 mOsm (about 10% less than that of undiluted Wolf and Quimby), allowed frog erythroid cell activity over a nine-day period, longer than achieved by other workers, who often stopped cultures due to cell lysis. They also found that bovine and normal frog serum "appear to depress activities related to the erythropoietic process when compared to media without protein." (This article, found after the cessation of the culture work, would be worth close attention by anyone hoping to culture amphibian erythroid cells.)

The culturing of poikilotherm tissues is in general a more difficult task than that of homeotherms, in part due to a greater risk of contamination because these animals frequently harbor systemic microorganisms or parasites (Wolf, 1979). Amphibian embryos do not develop within a

sterile environment, such as an egg or a uterus, but within a jelly coat which itself can become a breeding ground for infectious agents. Axolotl cells have been found even more resistant to established culturing methods than those of other amphibians (Frost, Neff, and Malacinski, 1989). The method of sterilely removing the larvae from their gels prior to hatching, and allowing further development in sterile, antibiotic-supplemented 25% Holtfreter's, aids in maintaining sterility.

The preliminary results described here, as well as some one-to-three hour observations of the cultures with the microscope alone, indicate that the times required for shape changes in culture are of the nature of several hours. The shape statistics given above do not contradict this. It is of interest that the work of Hagopian, Lippke, and Ingram (1972) with erythropoietic cultures from chicken embryos showed slowed cycle times after the first day in culture.

It is not known what, if any, effect the elevated temperatures of the microscope stage during prolonged videotaping may have had on cell growth. A convenient temperature probe was not available, but measurements with standard thermometers placed near the culture dishes were as high as 28°C. Frost, Neff, and Malacinski (1989) maintain their cultures at 20°-23°C, and Wolf (1979) recommends "a temperature slightly above that preferred by the intact animal" (p. 470), or 22-25°C for amphibian cells. He also indicates 38°C as the maximum for amphibian cell culture.

It is thus possible that the elevated temperatures on the microscope stage had no deleterious effect, and even that the 18°C used in the incubator was below optimum.

One inconvenient aspect of the cultures was the tendency of the cells to move out of the field of view, or out of the plane of focus. Scratches in the bottom of the Petri dish seemed to restrict them somewhat, and cells also seemed inclined to stay relatively near the spleen once released. Of course, the use of very few erythroid cells, perhaps in a Transwell dish as suggested, might solve this problem; the use of a fibrin clot or agar(ose) might also be beneficial, regardless of the number of cells used.

Despite such difficulties, however, the results here agree with those of Duprat and Flavin (1976) in that erythroid cells seemed to differentiate, changing from spherical to elliptical shapes, and to proliferate modestly. The culture work does seem to support the following stages of erythroid morphogenesis: sphere-->single-pointed sphere -->single-pointed flattened cell--->--->ellipse. It was disappointing that the culture work did not define the other aspects of the morphogenetic sequence, including the developmental position of the disc. However, the failure to clarify the sequence of shape changes by observing complete erythroid differentiation seems to be a technical one.

Two possible methods seem most appropriate to try to achieve the goal of looking at individual cells as they differentiate: (1) A sparse or single-cell culture, in

which the researcher may follow the development with a microscope alone; (2) An opened spleen which is photographed at frequent intervals or, possibly, videotaped with an improved technology. A third possibility, really a variation of (2), is the use of a whole spleen in a plasma or agarose clot to immobilize the splenic cells; also any factors from the spleen might act on individual cells through the agar. When Metcalf and Bradley (1970) cultured hematopoietic avian cells in agar, they found that important factors included fetal calf serum batch, agar used, cell density, and the presence of feeder cells which seemed to elaborate a factor diffusible through agar. Although Duprat and Flavim (1976) found that the presence of spleen fragments was not essential for culture maintenance, Jones and Elsdale (1963) have reported a minimum of 30 cells as necessary for axolotl culture survival. In the experiments done here, the use of whole spleens certainly gave better results, but it is not known whether this is due to cell-cell interaction or to some diffusible factor. If the latter is the case, a few cells might be induced to differentiate in a Transwell dish, with a spleen above the membrane. Although mammalian erythropoietin has been found to be ineffective on nucleated erythroid cells (Goldwasser, 1966), there is some evidence for an erythropoietic factor found in adult amphibians, and this might be isolated, and used for larval cells. In culturing Rana spleen, Carver and Meints (1977) found an erythropoiesis-promoting effect of

serum or plasma from adult Rana which had been stimulated to erythropoietic activity through phenylhydrazine- or phlebotomy-induced anemia. It is noteworthy, however, that Duprat and Flavin (1976), Hagopian, Lippke, and Ingram (1972), and Wainwright and Wainwright (1975) all found nucleated erythroid cell development in standard media with no added erythropoietic factor. On the other hand, if the presence of a certain number of cells is essential, the use of the whole but opened spleen may be the best method. Using dispersed chicken blastodiscs as the source for erythroid cell differentiation, Wainwright and Wainwright (1975) found that there was a specific inoculum size (1 blastodisc/ml medium) which was optimal for development of erythroid cells; either an increase or decrease impaired erythropoiesis. Broyles et al. (1981b) found that in cocultures of Rana tadpole liver with kidney, the liver blood cells became enriched in kidney-specific hemoglobins. This effect required physical contact between the two organs, which Broyles postulates to indicate a factor from the kidney which may be unable to diffuse far.

Because erythroid cells do not form monolayers in vivo, it might be worthwhile to try culturing in suspension, although this of course would preclude continual monitoring. Hanging drop cultures, which have been found to be effective for culturing axolotl heart tissue (Hill and Lemanski, 1979), were tested once but became infected. Further trials

could be useful as an additional technique to try to encourage cell differentiation.

A routinely successful culture system would eventually permit various pharmacological manipulations, such as the use of colchicine, to clarify aspects of morphogenesis. Furthermore, comparison of splenic erythroid cultures with ventral blood island cultures, such as those achieved by Smith and Turpen (1985) using Rana pipiens, would constitute a more distant but desirable goal.

(Two excellent articles, found after these preliminary experiments were done, are "Primary and Long-Term Culture of Axolotl Cells" (Frost, Neff, and Malacinski, 1989) and "Surgical Manipulation of Embryos" (Asashima, Malacinski, and Smith, 1989).

Circulating Erythrocytes

The original goal in looking at the axolotl larval circulation was simply to confirm the validity of cell shapes seen in the spleen. However, observations of the cells as they flowed gracefully through the capillaries aroused an interest in the topic of their deformability and the contributions of the various cytoskeletal elements to it.

Cell Deformability and the Cytoskeleton

The flow of blood through the tiny vessels of the axolotl, like all fluid flow, is subject to the laws of fluid dynamics. Much methodology for studying various

aspects of blood flow is based upon use of drawn blood in glass capillaries. This has given rise to the concept that the flow is laminar and adheres strictly to equations describing such flow, such as Poiseuille's equation, which expresses flow as being directly proportional to the effective pressure gradient along the vessel and to the fourth power of the vessel radius, and inversely proportional to vessel length and the fluid's coefficient of viscosity. Such equations, however, assume that fluid flow is laminar, moving as infinitely thin layers past each other. The shear stress which tends to impede this flow is directly proportional to the shear rate, or the relative velocity between two adjacent layers. Viscosity is the ratio of shear stress to shear rate.

In vivo studies, such as those of Bloch (1962), have shown that flow in the living organism is much more complex. In the case of blood, total viscosity is determined not only by viscosity of the plasma but also by such cell contributions as hematocrit, cell aggregation and cell deformability. It is increased as the proportion of cells increases. Mammalian erythrocytes also tend to aggregate into rouleaux, resembling a stack of coins; this increases the viscosity further, for aggregated cells can form "particles" that are stiffer and less subject to deformation and more subject to axial migration. A tendency for such piling up of erythrocytes within the vessels was noted in the axolotl circulation; this aggregation seems to increase

after a lengthy period under the microscope, when the anesthesia, inactivity, and lessened hydration probably contribute to traumatic sludging. Other influences on viscosity per se include temperature, which can affect plasma viscosity, cell shape, cell wall elasticity, and internal cell viscosity. The viscous resistance encountered during capillary flow is also influenced by the ability of the cells to deform and align for smooth flow.

In order to perform their primary function of oxygen delivery, the erythrocytes of all organisms must be able to traverse capillaries smaller than themselves. This requires their undergoing deformation without suffering a permanent loss of shape.

The ability of the erythrocyte to change its shape in response to a deforming force is called its deformability. As discussed by Chien (1987), this deformability can vary in type and degree, involving a change in cell curvature, surface area expansion/contraction, or twisting. The cell's deformability is influenced by its intracellular fluid viscosity, its geometry, and its membrane's surface viscosity, bending stiffness, and shear elastic modulus. (The shear elastic modulus, a measure of stiffness, is defined as the ratio of the unit shear stress, due to lateral sliding of layers against each other, to the unit strain, or displacement relative to the original position.) The direction and magnitude of the stress (force per unit area) determine the mode and extent of deformation.

In the human, erythrocyte rigidity is related to the cell contents as well as to the cell membrane, which can move around the cell contents, "much as a tank tread would move around the wheels" (Charm and Kurland, 1974). Shear deformation ratio is a function of the ratio between the viscosity of the external fluid and that of the cell contents, and the deformability of the red cells is a major cause of the decrease in blood viscosity at high shear rates. Charm and Kurland (1974) describe this: "Cells deform into prolate ellipsoids, and the membrane rotates around the cell contents at shear stresses of 5 dynes/cm² and above. Movement of the cell membrane around the cell contents is the mechanism by which red cells adapt themselves to flow. Hemoglobin is subjected to shear, the cell assumes the properties of a fluid drop, and whole blood behaves as an emulsion rather than as a suspension."

Schmid-Schonbein and Gaehtgens (1980) found that nucleated red blood cells do not show the membrane tank-treading seen in mammalian cells. The absence of organelles in the latter contribute to a highly fluid cytoplasm, which contributes to a continuous viscous deformability. The internal structures of the nucleated cells would be expected to hold the cytoplasm in place to a much greater degree, and thus limit the extensive shape changes seen in non-nucleated erythrocytes. In fact, Waugh and Evans (1976) state that in nucleated erythrocytes, "The plasma membrane is firmly attached to two sides of the centrally located nucleus with

the cytoplasmic fluid forming an annulus around the nucleus." This secure positioning is no doubt due to the vimentin-containing intermediate filaments identified by Centonze, Ruben, and Sloboda (1985) (See above).

The deformability of the red blood cell influences the ease with which it can flow through small vessels. When the blood flows through very small capillaries, the blood cells are distorted, so that they can pass through. Occasionally, however, an erythrocyte occludes the vessel, and flow in that area ceases. When this happens, flow in some nearby small branches may run backwards until the obstructing cell is forced through. Even without obstruction, the flow in the smallest vessels of the gill and ventral trunk varies in rate and direction. Thus it is similar to mammalian capillary flow which "is intermittent and often reverses direction" (Friedman, 1976).

Observations on the flow of axolotl erythrocytes through the gill vessels reveals a greater degree of twisting and deformation than expected for cells whose shape is maintained by a marginal band. During flow, many cells appear to have single points streaming in fish-tail fashion behind them. In several cases the "pointed" end may be observed to have a partial twist about halfway between the end and the site where the nucleus probably stops (Figures 13 c and f, and 15). This twisting leads to a pointed profile, similar to a swimming fish. In addition to this twisting, the cells show a degree of folding ability, so

that in going around a curve, the ellipse can bend. As the cells are watched, however, they are seen to resume elliptical shape when the velocity decreases or the curves straighten. Thus it is essential that circulating cells be thoroughly studied before assigning a pointed shape to them. The few cells which "lead" with a point (Figures 13 c-e), with or without a trailing point, could probably be identified as singly-pointed, but an apparently doubly-pointed cell could possess either one or two points. In fact, the difficulty in assigning shapes is emphasized by Bloch's (1962) finding that undistorted forms existed for only a few milliseconds in arterioles. With his high speed cinematography, he was the first to see that considerable deformation could occur in vessels larger in diameter than the cells.

Spheres are more easily distinguished, for they look unmistakably like balls. The few discs observed seem to have less tendency to deform than ellipses, but the number has been too small to be conclusive. The discs do seem to have a greater propensity to get stuck.

In a comparison of capillary flow (in glass capillaries) of duck and human erythrocytes, Gaehtgens, Will, and Schmidt (1981a) found that the nucleated cells had a lower deformability. The mode of deformation seen by him was similar in the two species, and in accord with the observations of this study: a radial folding of the cell with displacement of the intracellular contents into the

leading end. Even when "relatively minor" changes were seen in the nucleated cells, eg., in large capillaries, the nature of the change involved "displacement of intracellular fluid associated with occasional 'kinking' of one of the tapered ends of the ellipsoid." As might be expected with an inferior ability to deform, the avian cells showed a lessened stability of flow orientation. (Note that Gaehtgens' description of mammalian erythrocyte flow orientation indicates a higher degree of alignment with the tube axis than that found by Bloch in the living rat.) Gaehtgens presented viscometric data to show that lower deformability of the avian cells resulted in their decreased ability (relative to mammalian cells) to yield to the external shear forces resulting from flow. He also showed (Gaehtgens 1981b) that relative apparent viscosity of a nucleated erythrocyte suspension is much more dependent upon hematocrit and capillary diameter than is that of a non-nucleated cell suspension. In the former, the Fahraeus effect, or the dynamic decrease in the circulation hematocrit as a result of a velocity difference between the cells and the plasma, plays a prominent role in determining viscous resistance to flow. The Fahraeus effect in such cases is dependent upon hematocrit changes, with the result that a low capillary hematocrit would be advantageous for flow in the animals having nucleated erythrocytes. As discussed by Gaehtgens, the presence of the nucleus (restricting the ability of the cell to act like a resilient

teardrop as well as geometrically impeding the inward folding of the membrane) and of the marginal band (which probably tends to spring back to its elliptical shape from a twisted one) may play a role in limiting the nucleated cell's deformability. He also discusses various possible mechanisms to compensate for the flow restrictions resulting from this lowered deformability. These include a lowered capillary hematocrit or a greater number of capillaries per unit tissue volume (which has in fact been found for brain tissue of birds versus mammals); it is also of interest that the mean corpuscular hemoglobin concentration was found to be nearly twice as high for ducks as for humans.

The current study indicated a better, albeit unquantitated, alignment of the flowing cells with the stream direction than that found by Gaehtgens. Possible reasons for this could be an effect of the surface of the vessel endothelium, a consequence of the pulsatile character of the flow in the living organism, and a result of the branching of the capillary bed.

Scanning electron micrographs show the existence of all shapes in the circulation. Figures 15 through 17 also demonstrate how the angle of viewing of an ellipse may contribute to misinterpretation of a "point."

Many of the scanning electron micrographs (Cf. Figures 15, 17, 18) show cells with folds of membrane, some so much as to have a "shaggy" appearance. It may be possible to postulate that, whereas mammalian RBC's deformability is due

in large part to excess surface area ($140 \mu\text{m}^2$, compared to the $97 \mu\text{m}^2$ required for a sphere of its volume), the deformability of the non-mammalian RBC is due partly to excess membrane as seen in such SEMs, but also to the ability of the MB to twist and return to its original shape. Although not calculated here, presumably there is sufficient slack in the membrane and MS to permit the folding and twisting. Gaehtgens, Will, and Schmidt (1981a) did compare the surface to volume ratio of human (non-nucleated) and duck (nucleated) erythrocytes and found that the cells had the same relationship, 1.52, although the avian cells were much larger in volume.

The spheres, which lack complete MBs, are seen to have a greater shagginess (excess membrane) than the other shapes. A sphere, by virtue of its shape, is less deformable than discs and ellipses. As pointed out by Burton (1966), a sphere with no excess membrane would be at risk of damage in small capillaries because it would be unable to deform without stretching, and probably damaging, the membrane.

Membrane deformability seems to be determined to a large degree by the cytoskeleton underlying the lipid bilayer and associated with it through protein-protein and protein-lipid interactions (See above). Most studies of this have dealt with mammalian cells, where the spectrin-actin-protein 4.1 associations appear to play an important role in membrane stability, as well as in the development of

the erythroid biconcave discoid shape (Mohandas, Chasis, and Shohet, 1983; Lazarides and Woods, 1989). After studies in which they cross-linked spectrin, Schmid-Schonbein and Gaehtgens (1980) concluded that spectrin was essential for maintaining the excess membrane material upon which deformability depends. The erythrocyte's important ability to return to its original shape after deformation is dependent upon membrane stability, defined by Chasis and Shohet (1987) as "the maximum extent of deformation that a membrane can undergo and still completely recover its original shape." They propose a folding and unfolding of spectrin, coupled with rearrangement of other skeletal proteins, as a primary factor enabling reversible deformation of the erythrocyte membrane. Waugh and Evans, studying the viscoelasticity of the membrane itself, found that the erythrocytic membranes of the classes Amphibia, Aves, and Osteichthyes were five to ten times more resistant to deformation than those of Mammalia. Those of Reptilia were two to three times more resistant than those of the other non-Mammalia. Mammalian cells, possessing a large excess membrane in the cavity of the biconcave disc, can undergo a linear increase of up to 230% without a change in surface area (Mohandas, Chasis and Shohet, 1983). In non-mammalian erythrocytes, lacking this, flexibility of the MB is also thought to play a large role in reversible deformability.

Joseph-Silverstein and Cohen (1984a) found that dogfish erythrocytes subjected to mechanical stress underwent deformation more readily when marginal bands were absent. Similar results were obtained with blood clam erythrocytes (Joseph-Silverstein and Cohen 1984b). They hypothesize that "the marginal band functions to resist deformation up to some threshold value, above which the cell becomes deformed, and subsequently promotes rapid recovery of normal cell shape." In agreement with this, observations on circulating axolotl erythrocytes indicate that the marginal band deforms beneath the cell membrane in response to hydrodynamic forces, and effects a prompt restoration of shape upon cessation or lessening of those forces.

Waugh and Erwin (1989) measured the deformation of isolated marginal bands as a function of applied force. They found that the band has a bending rigidity three orders of magnitude greater than that of the cell membrane, and a high resistance to increases in length. These characteristics would stabilize the cell against indentations at the rim and limit the cell's potential degree of lengthening. The latter attribute agrees well with Bloch's findings that avian cells lengthen to a much smaller degree than do human cells under similar conditions of capillary flow. Interestingly, bands isolated under conditions which increased their stiffness tended to twist when stretched, which seemed somewhat reminiscent of the twisting seen in circulating cells (Waugh and Erwin, 1989).

Criticism of experiments

The inability to observe and quantitate blood flow in any but the regions of slowest flow was a considerable disadvantage, particularly since the frequency and degree of cellular deformation might be expected to increase at higher rates of flow. Also, measurement of cell velocity proved difficult, for often the flow rate was too great to be measured. The necessity for choosing capillaries where flow was lethargic enough to permit stop-watch timing from one end of the viewed length to the other limited the choice to an unrepresentative population: most flow was considerably faster, and most capillaries soon twisted out of view. Velocities found by Bloch in frog arterioles of 25-35 μm diameter ranged from 2.6-19.2 mm/sec (considerably faster than measured here in axolotl capillaries). Bloch also found in the microvascular systems of living frogs, mice, rats, and rabbits that when the capillary diameter was larger than the red blood cell, the flow was often too rapid to distinguish the orientation.

However, his success in studying microvascular hemodynamics using high speed cinerecording is encouraging. Perhaps a videorecorder with greater image stability and playback speed flexibility than the Panasonic AG-6050 VCR would facilitate such a study. The principle of determining velocity by recording at one speed with playback at a slower one is valid but needs more sophisticated equipment for its execution.

In the absence of a large number of scanning micrographs showing erythrocytes in their vessels, there is a question regarding the correspondence between the shapes of isolated cells and their true shape in vivo. Aspects mitigating against ready acceptance of the SEMs are the finding of twists in splenic red blood cells and the known effect of glutaraldehyde in causing twists in cytoskeletons. It might be expected that splenic cells, presumably subject to no hydrodynamic force, would not show twisting until after release into the circulation. On the other hand, the observation of many instance of twisting in the living circulation point to its having an important role. Also, in view of the large number of untwisted ellipses seen in the SEMs, it is difficult to attribute the twisting of a minority of cells merely to an artifact of glutaraldehyde fixation. Perhaps the twisting seen in both splenic whole cells and in cytoskeletons may be interpreted as a demonstration of the readiness of the marginal band to twist when circumstances demand it. (In an appraisal of 19 micrographs of twisted cells, including splenic, circulating, and lysed, the mean of the ratio (distance from end to twist)\(total length) was 0.25 with a range of 0.16-0.34, SD 0.06.)

One felicitous outcome of this work is that the axolotl gill and ventrally lying trunk blood vessels appear to constitute a possible system for study of amphibian

microvascular rheology in the living organism without surgery.

Further avenues of study utilizing this system might include: quantitation of cell deformability with high speed cinematography equipment; viscosity measurements if a microviscometer is available; (farfetched:) recording of flow before and during microinjection of lysing medium to try to "catch" marginal band twisting "in the act."

Possible Generality of the Model

Some further support for the model may be extracted from the work with the blood of the pre-hatching axolotl embryos. Although only partially investigated, cells of the primitive erythroid line may undergo a sequence of morphogenesis similar to that in the definitive cells, and it is reasonable to postulate a phylogenetic continuum of erythroid development from invertebrates to mammals.

Phylogenetic aspects of erythrocyte occurrence and development

As might be expected where such important functions as oxygen delivery and immunity are concerned, blood cell structure and biochemistry are highly conserved throughout much of the animal kingdom.

Invertebrates

Some invertebrates have erythrocyte-like cells which resemble nucleated non-mammalian erythrocytes in having a

biconvex shape, amorphous cytoplasm with sparse organelles, respiratory pigment distributed more or less uniformly in both nucleus and cytoplasm, and a marginal band of microtubules (Ratcliffe and Millar, 1988). Erythrocytes containing hemoglobin or hemerythrin have been found in such diverse invertebrates as Anadara transversa and Noetia ponderosa (blood clams, Ph. Mollusca), Phascolopsis gouldii (peanut worm, Ph. Sipuncula), Travisia japonica (a polychaete, Ph. Annelida), and Cucumaria miniata (sea cucumber, Ph. Echinodermata) (Cohen and Nemhauser, (1985). The annelids Glycera gigantea and Goniada maculata have erythrocytes which attain a diameter of 100 μ m (Andrew, 1965). Unfortunately, little is known about the development of invertebrate blood cells, although it has been found that many annelids have segmentally arranged hemopoietic organs (Andrew, 1965).

In addition to being present in these erythrocytes, marginal bands have been found in arthropod clotting cells (Nemhauser et al, 1980; Cohen and Nemhauser, 1985). As pointed out in these papers, the occurrence of marginal bands in the amebocytes of Limulus polyphemus, which can be traced back to the Cambrian period about 500 million years ago, indicates that this organelle system may indeed be ancient.

Vertebrates

Jordan and Speidel (1930b) state that the hagfish Myxine glutinosa, a cyclostome, is the lowest vertebrate

with specialized hemocytopoietic tissue, a spleen which "occurs in scattered and diffuse condition in the intestinal wall." Interestingly, it is also the lowest chordate with hemoglobinated red cells, although, as seen above, cells with hemoglobin may be found in some invertebrates. (According to Andrew (1965), the blood cells of the lower, non-vertebrate chordates, *Amphioxus* and *Balanoglossus*, seem to correspond to the "finely granular amebocytes" or "lymphocytes" of invertebrates.)

Throughout the vertebrate phylogenetic tree, the process of hemopoiesis is amazingly consistent, with the same basic structural sequence being followed, although the sites vary from species to species. This is true of both erythroid and immune-system cells.

Hematopoietic stem cell origin and migration

In all vertebrates, even in placental mammals, which have almost no yolk, the earliest blood vessels and cells arise in the yolk sac (eg., in birds and mammals) or the yolk-laden splanchnopleure of the midgut (in amphibians) (Kau and Turpen, 1983; Hopper and Hart, 1985). The early mesenchyme cells become free cells known as hemocytoblasts, the stem cells for a variety of blood cells including erythroblasts. In the early embryo these cells are aggregated to form a blood island on the ventral surface of the yolk sac; thus this extra-embryonic source of primary blood cells is referred to as the blood island of the yolk sac. It has been a matter of interest, and sometimes of

controversy, to developmental biologists to ascertain the embryonic origin of the blood cells at various stages of development, and to determine whether the primitive erythrocytes derive from a different embryonic compartment from the source of later (definitive) blood cells.

One widely-held hypothesis, questioned by Turpen, Turpen, and Flajnik (1979), is that "pluripotential hematopoietic stem cells, as well as primary erythroid cell lines, differentiate de novo from the mesoderm of the embryonic yolk sac; these yolk sac-derived stem cells become blood-borne and colonize various intraembryonic hematopoietic sites." If, in fact, the axolotl spleen is populated by blood-borne hematopoietic stem cells, previous (Duprat and Flavin, 1976) and current evidence that it is a closed system until nearly two weeks post-hatching is hard to explain. The concept of a single source of stem cells, by which the spleen and other hematopoietic organs must be colonized, is supported by the early work of Goss (1929) with Amblystoma (sic) punctatum embryos in which he found that removal of the primitive blood island at an early stage (stages 26 to 34) resulted in development of larvae with blood cells totally lacking or greatly reduced. Neither the spleen nor the liver, when they developed, assumed erythropoietic functions; he found that the spleens of such treated animals were small, with as great a reduction of hemoglobin-containing elements as was found in other organs. "No instances were found where erythrocytes seemed to be

forming from the endothelium or from any other type of cell, except the circulating erythroblasts."

Cameron (1941), on the other hand, found that in untreated Ablystoma (sic) jeffersonianum, distinctive new erythrocytes appeared about 24 hours post-hatching (stage 41). Observing this "fourth class of plump, deeply staining, red cell with no yolk plates," he concluded that they were a new type of post-hatching erythrocyte "from the usual sources," but from other hematopoietic centers than the blood islands. This result shows some discrepancy from the current work, where the spleen as the post-hatching erythropoietic source does not appear until about three days after hatching.

The postulate of two separate stem cell compartments is supported by recent work by Kau and Turpen (1983), using reciprocal transplants of ventral blood island mesoderm and dorsal lateral plate mesoderm between cytogenetically distinct embryos (at Nieuwkoop and Faber stages 14-19). This has shown that in Xenopus the ventral blood island mesoderm gives rise to stem cells that colonize the blood and thymus, entering the (early) circulation before or at stage 40 and remaining through stage 49. Later stem cells arise from the dorsal lateral plate mesoderm and colonize the thymus and mesonephros (but not the blood); cells derived from these do not appear in the circulation until stage 49 or after. Similar experiments with Rana pipiens show that blood island derivatives predominate in early

circulation, but that the hematopoietic cells of liver seem to be derived from an extrinsic cell source (Turpen, et al., 1979). Turpen's hypothesis of two stem cell compartments, separated in space and time, is in accord with Cameron's findings. The work of Deparis and Jaylet (1984) with the newt Pleurodeles waltlii gives further support, for they have found that in the anterior trunk region, the ventromedial lateral plate mesoderm gives rise to blood stem cells which last the lifetime of the animal, whereas in the middle and posterior trunk regions the mesoderm produces the blood island which generates a transitory erythrocyte population, which they term "primary blood cells." They conclude that there is "either a real [Their use of "real" is undefined.] regional differentiation of the mesoderm, or a regional differentiation due to an inducing effect of an already regionalized endoderm."

The evidence is thus strong that in a variety of amphibians, including the axolotl, the early circulating blood cells, seen prior to hatching and soon thereafter, are of ventral blood island origin. Most current evidence indicates that the later blood derives from an intra-embryonic source, perhaps via migration of erythropoietic organ precursors and/or stem cells. However, both the immediate and the ontogenic source of red cells between hatching and the joining of the spleen to the circulation remains problematic. Occasional cells with one or two yolk platelets are found in the circulation after hatching, but

these are infrequent. Probably the blood islands have furnished those in the circulation, as well as many of the circulating cells which lack yolk platelets, having received none during division or having used theirs. Do new cells arise during this time from division and development of circulating cells, or do the blood-island-derived cells colonize another organ and proliferate there? The occasional appearance of structures resembling yolk platelets in the erythroid cells of the young spleen seems to argue for the latter possibility. However, there is ample evidence in the literature for erythroid proliferation in the circulation of many species: Grasso (1973a) states that in the absence of a spleen, the blood is the normal erythropoietic locus for adult Triturus; Sekhon and Maxwell (1970) describe extensive cell proliferation and differentiation in the blood stream of the hagfish Eptatretus stoutii. Dawson (1930) reported a lack of mitotic figures in the blood of adult Amblystoma (sic), although he found mitoses in the blood of a large variety of other urodeles. In the current work, however, observations of circulating cells in apparent mitosis, as well as at immature developmental stages, support the possibility that at least until the spleen has matured and joined the circulation, the blood supply derives from circulatory proliferation of either yolk-sac-derived cells or stem cells populating the vascular system from another source.

It seems possible that hemopoietic tissue migration takes place during the early morphogenetic cell movement of gastrulation, thus forming or populating the presumptive spleen, and that subsequently the erythropoietic cells form from the wall of the spleen. Charlemagne (1972) states categorically that the primitive erythrocytes from the blood islands are replaced at or around (Fr. des) hatching by red cells generated in the spleen. Romanoff (1959) states that the first visible anlage of the avian spleen is a condensation of mesenchymal cells in the dorsal mesentery, and that other mesenchymal cells begin differentiation there as erythroblasts or lymphoid hemocytoblasts. In addition, the quail-chick marker technique has shown that avian stem cells in bone marrow are of extrinsic origin (Dieterlen-Lievre, 1988).

In summary, then, the important question of the embryonic source of the definitive red blood cells is still unresolved. However, there is a substantial body of work to support the theory that the definitive erythrocytes derive from a separate source from that of the primitive cells, in which case the evidence that the spleen is a closed cell-generating organ, from the time of its organogenesis until it joins the general circulation, is not refuted.

Potential Methods for Further Study

This problem, as well as the path of migration of cells from blood islands or other areas of the mesoderm, might be clarified by use of tracer dye or radioactive isotope,

immunological lineage, further transplantation studies, or classical dye injection experiments. According to Dieterlen-Lievre (1988), "An array of developmental antigens has been detected on the surface of erythrocytes or erythroblasts at different stages of development" in chickens. The same is probably true of amphibians, and these could aid in answering a variety of developmental questions.

The developmental site also seems involved in the choice of hemoglobin type, as seen in the work of Broyles and coworkers (Broyles et al., 1981a). They have found that in Rana the differentiation microenvironment seems to be involved in the type of hemoglobin made by the erythrocyte. Conveniently, in Rana tadpoles the microenvironments are in different organs: the kidneys, which give rise to oblong erythrocytes with acentric nuclei, and the liver, which generates elliptical cells with central nuclei. The former cells are enriched in hemoglobin Td-4, the most slowly migrating hemoglobin, whereas the latter erythrocytes contain hemoglobins Td-1, 2, and 3. Broyles et al. (1981b) found, in organ cocultures of liver with kidney, that liver-generated erythrocytes shifted their hemoglobin pattern toward that of kidney red cells. They also found a different cellular architecture in the erythroid foci of the two organs. They hypothesize that the determination of Hb type is made, in part, "at the level of the hemopoietic stem cell (or at least, at the level of a precursor cell that is

not identifiable either structurally or functionally as an erythroid cell)." This existence of biochemically distinguishable fetal and adult hemoglobins (which seems to be true of most organisms) might provide an avenue for further study of the primitive versus definitive erythrocytes of the axolotl. It should be noted, however, that not all species present the neatly segregated hemoglobin synthesis found in Rana; Rifkind et al. (1974) state that there is no evidence relating the switch from fetal to adult hemoglobin in humans to either cell lineage or a change in erythropoietic locus.

Structure of Prehatching Axolotl Erythroid Cells

Although probably deriving from different stem cell origins, prehatching erythroid cells seem to undergo a developmental sequence very similar to that of the larval cells. Despite being less thorough than the work with the larval splenic cells, the study of the prehatching embryonic cells tends to confirm the the former, particularly in terms of the occurrence of different cell shapes and of centrioles near the marginal band.

The blood island, which is the first erythropoietic site, extends along the mid-ventral surface of the embryo. It has been manipulated by various researchers (Goss, 1929; Kau and Turpen, 1983) in their efforts to determine its role. In the current work, however, it was appropriate and easier to use the circulating cells, usually from the gills,

for morphological study. In retrospect, this is viewed as a flaw in the experimental design, for a comparison of circulating and unreleased erythroid cells might have clarified such aspects as the correlation of yolk platelet number with age/stage, the time of appearance of the marginal band, and the degree to which development continues in the circulation after release.

The primary feature which distinguishes erythrocytes from prehatching axolotls from later cells is the presence of yolk platelets. These influence the surface of the cells as viewed in the SEM and remain as a prominent detail even after cell lysis. It must be remembered, however, that although the presence of yolk platelets is at least indicative of blood island origin, their absence does not necessarily indicate a different origin.

The sizes of the cells seen in the SEM are smaller than expected, leading to concern about the possibility of excessive shrinkage in the preparation. However, the ellipse of Figure 4 is 20 μm in length, which compares well with other SEMs. Therefore, unless the more primitive cells are in some manner subject to more shrinkage than normal, their sizes as seen in Figure 3 (12 μm and 13 μm) may be reasonable. On the other hand, phase figures 1 and 2 show cells of 23 μm (1a) to 31 μm (Figure 2). (Broyles et al. (1981b) found that immature erythrocytes in Rana were significantly smaller than more mature ones. Grasso (1973b)

explains a small cell in his scanning micrographs as a probable "recently formed daughter cell.")

These prehatching erythroid cells show considerable variation, in developmental stage, size, nuclear size, number of yolk platelets, and marginal band presence. This would be consistent with the hypothesis of a blood island stem cell population from which cells are released, some continuing to divide and others beginning differentiation into mature erythrocytes. It is also consistent with Benbassat's (1970) finding of extensive pleomorphism in tadpole erythrocytes, which contrasts with the uniformity of shape and size of RBCs of adult frogs. Dawson (1930) also commented on irregularity of cell size during erythroid maturation, which he attributed to unequal growth rates and the high degree of mitotic activity.

In Figure 5 b and c, a pair of dots may be seen near the marginal band in each cytoskeleton. This finding was fairly common with the primitive elliptical cells, in contrast to the case with the splenic erythroid cytoskeletons, in which paired dots were confined almost exclusively to pointed structures. [Among the many splenic cytoskeletons observed there was only one case with such dots near an elliptical (non-pointed) marginal band.] No whole mounts of primitive erythroid cytoskeletons were prepared, so it is not possible to state with certainty that these dots are centrioles, but their size and density are consistent with this hypothesis. The possibility that the

cells of the primitive erythroid line keep centrioles later in development than do those of the definitive line would be, of course, an interesting question for further study.

It is also noteworthy that preliminary work done with Dr. Cohen found pointed cytoskeletons among the lysed erythrocytes of Stage 39 axolotls. In at least one of these, a pair of dots within the point was noted, and hypothesized to be centrioles, in analogy with findings in lysed red cells of axolotl larval spleen (Ginsburg, Twersky, and Cohen 1989), and with Dr. Cohen's previous work on the skate (Cohen, 1986) and the blood clam Noetia ponderosa (Nemhauser, Joseph-Silverstein, and Cohen, 1983). As in those cases, the pointed primitive cells were postulated to be stages of erythroid cell maturation in a sequence from sphere to ellipse. Although this work on the primitive cells was not pursued using cell counts and whole mount preparation, these findings tend to confirm the validity of the models in terms of the occurrence of pointed cells as an intermediate stage in erythroid morphogenesis.

The stages of erythroblast development as seen in TEMs were identified using the work of Orlic, Gordon, and Rhodin (1965) with mouse and Grasso and Woodard (1967) and Tooze and Davies (1967) with Triturus. With one probable exception, only basophilic and polychromatic erythroblasts were identified here, mainly by nuclear appearance. It should be emphasized that the standard classification of erythroblasts is based upon light microscopy, including

staining characteristics, and in retrospect it seems that some classical hematology would have been a useful addition to this work. Staging using electron micrographs is not as straightforward as might be desired. For example, the intact Golgi of Figure 6 indicates a basophilic erythroblast, but the highly clumped chromatin suggests a polychromatophilic one. In some cells viewed by electron microscopy but not shown here, there seemed to be a discrepancy in developmental stage as judged by nuclear structure; for example, in some cases the presence of a nucleolus implied an earlier stage than the basophilic or polychromatophilic erythroblast indicated by chromatin condensation (Orlic, 1970). Dawson (1930) found, during springtime blood regeneration in a variety of adult urodeles that "differentiation of the nucleus and cytoplasm is not always closely correlated, as one or the other may lag considerably in its maturation, giving rise to more or less atypical transition forms." It is also of interest that Orlic found many mitochondria to be swollen at the proerythroblast stage, whereas in this series of micrographs swollen mitochondria are (still?) seen in the polychromatophilic erythroblasts. Grasso, finding such swollen mitochondria, along with dilated Golgi, after the basophilic erythroblast stage, suggested the possibility of organelle degeneration accompanied by autolysis as a normal aspect of the developmental process. Rowley (1988) describes loss of most erythrocyte organelles by lysosomal

digestion during maturation. The remnants appear as "myelin whorls" which are either expelled or stored. However, in the current work the swelling of the mitochondria could also be due to the partial cell lysis. This lysis accounted for the breaks in the cell and nuclear membranes and for the elimination of hemoglobin from the cells.

Although membrane-enclosed ferritin (siderosomes) has been found in some erythroblasts (Orlic, 1970), only free particles were seen in the thin sections prepared in this work. It is thought that ferritin within the cell cytoplasm is used for heme synthesis, although iron-binding transferrin may be the main source (Orlic, 1970).

In all cases seen here the microtubules appeared to have already assembled into a marginal band. However, the rounded cell seen in Figure 6 may in fact represent a sphere with microtubules associating only at the "poles", perhaps just prior to the formation of a point. If this were the case, a bird-cage-like microtubule arrangement might be expected around the circumference, similar to that seen in the model. Serial sectioning would have clarified this and should have been done. In general, of course, it would be of interest to apply the same variety of techniques used with the larval cells to these and earlier stages. (It is in any case difficult to find uncompleted marginal bands prior to microtubule bundling.) It seems, however, that the marginal band forms early in the erythropoietic scheme.

This has also been seen in the hagfish (Sekhon and Maxwell, 1970).

The observation of ellipses without apparent MBs was a surprise. One possible reason for this is observer inexperience. Possibly some MBs were "tucked " under nuclei and not noticed. It seems unlikely that MBs were in fact absent from true ellipses.

Few yolk platelets were found by electron microscopy, which could be due to the vagaries of sectioning. In the phase microscopic observations there was only rough agreement between the yolk platelet counts found by Cameron in Ambystoma jeffersonianum (1941) and the counts done here. The numbers of cells counted by him greatly exceeded those examined here, which, of course, gave him better statistics. However, at (Harrison) stage 38, he found that nearly all primary red cells had eight yolk platelets, whereas in these axolotls only 28% was found to do so at Stage 38, 28% having more and 44% having less. The best agreement was at stage 40, at which he found nearly all cells to be elliptical and to have two yolk platelets. Several factors could account for the discrepancies, including current investigator inexperience both in staging axolotls and in counting yolk platelets. Most of this work used retrospective staging, the articles by Schreckenber and Jacobson (1975) and Bordzilovskaya and Dettlaff (1979) not yet having been found. An additional discrepancy was that the much larger-than-normal erythrocytes found by him in Ambystoma

jeffersonianum were not found here in Ambystoma mexicanum. Cameron found erythrocyte flattening to begin about stage 38 (12 days after fertilization), at which time the blood cells fell into two groups, spheres with 16 yolk plates per cell and flattened ellipses ("spindle or lens-shaped") with 8 per cell. [It would be of interest to know whether the "spindle-shaped" cells seen by Cameron were in fact the double-pointed cells seen in this work. One of his photographs indicates that this may be the case.] At stage 39 (14 days), "most of the cells are flat ovoid disks and the most numerous class has four yolk plates each," with about one-third having eight plates. He found that at hatching (stage 40, day 15) most red cells had two yolk plates each. All cells seen by him on day 16 (stage 41, post-hatching) were "shaped like the characteristic amphibian erythrocyte. Numerous cells with two yolk plates each and a few with one yolk plate represented the expected mitotic generations. The relatively small number with one yolk plate and the large number of pale, irregular, 'empty' cells indicated that at or soon after the division giving rise to one-plate cells the yolk plates were exhausted or disintegrated." (Cameron, 1941, p. 234)

Cameron's work indicates that elliptical erythrocytes of the primitive line may undergo mitosis, dividing their yolk platelet supply as they do so. His is not the only evidence against the almost intuitive supposition that erythroid cells should divide while at a spherical blast

stage. Jordan and Speidel (1930a) averred, "Any of the stages may exhibit division by mitosis." Dawson (1931) saw mitosis of elliptical amphibian erythrocytes, with division in a plane parallel to the MTs of the MB. Grasso (1973b) reported finding mitosis in late reticulocytes in newts. Goss (1929), however, stated that new erythrocytes in Ambystoma punctatum formed only from circulating erythroblasts, and the work of several more contemporary laboratories indicates that it is the hemoblast or erythroblast which undergoes mitosis (See also Cell Morphology...: Cell Proliferation and Differentiation, above). The disposition or movement of the marginal band is difficult to visualize if, in fact, some flattened cells do undergo mitosis.

Xenopus Erythroid Cells

The brief experiments with Xenopus indicate that anuran erythroid cells undergo shape changes similar to those seen in the urodele Ambystoma mexicanum. Although differences were seen among axolotl individuals, with animals of the same stage showing considerable variability in cell shape distribution, particularly of pointed cells, the inconsistencies seemed more pronounced in Xenopus. It is possible that this is due, at least in part, to the greater developmental age at which Xenopus was used, compared to the axolotl, which was studied just prior to and during early definitive erythropoiesis. Presumably, when

animals are younger and thus closer to the beginning of erythropoiesis, the waves of erythroid development are extended in time so that they appear more distinct than at a later stage. Also, animals of the same stage are closer to each other developmentally when younger than when older. This would accord with the findings of Frangioni and Borgioli (1987), about the occurrence of newt erythropoiesis in cycles of waves. These authors describe this coexistence of overlapping waves of erythropoiesis as an explanation for many of the contradictions found in the literature about amphibian erythropoiesis and conclude, "it is highly probable that research on newt erythropoietin (sic.) using traditional methods will always give contradictory results."

The counts made from the heart (circulation), liver, and spleen of the same animal imply that, contrary to some literature (Turner, 1988) which gives the kidney as the main erythropoietic source in Xenopus, red cell differentiation may take place also in the liver and spleen. Of course, this work did not approach the question of the site of Xenopus erythroid cell proliferation; however, the presence of a large number of spheres in the spleen suggests that some cell division might occur there. Also of significance is the indication that erythroid differentiation is not synchronized between the two organs, although the effects of dilution of immature liver cells by more mature circulating cells cannot be ruled out.

The Model

Thus the evidence obtained in the course of this thesis work gives some support for the model, but much more work is needed. It has not been established unambiguously that each sphere becomes an ellipse, or that all ellipses were once spheres. It has not been proven that the pointed cells are true developmental stages, and not some aberrant forms. Such proof requires either a totally successful culture system, or some type of tagging of immature cells, such as autoradiography. However, although the model as presented here may not be correct in all aspects (or even most), it can serve as a framework for further experimentation. A "good model" has been described (Hirschfeld, 1970) as "one that, with a minimum of components, each of which is physically or physiologically meaningful and measurable, will describe the known behavior of a system to an acceptable degree of precision....The omission of unnecessary components is almost as important as the inclusion of necessary ones." By this definition, the model as presented and supported here seems to be adequate, for each component (or stage or figure) is consistent with the data and should be testable by further experimentation. It is not, however, a model which excludes other possibilities.

Once this or another model is confirmed, it may be extended and refined by study of such aspects of non-mammalian erythropoiesis as: kinetics of the process of morphogenesis; kinetics of microtubule elongation and

bundling; molecular biology of controlling mechanisms for marginal band formation and interaction with the rest of the cytoskeleton; comparison, at both the molecular and cellular and levels, of initial band formation with its reformation; triggers for cell division versus differentiation.

These, however, are beyond the aims of this thesis, whose goals might be furthered by application of some of the following suggestions.

Suggestions for Further Research

Erythroid Development in Anemic Ambystoma

An alternative approach to the study of erythrocyte development is the production of anemia, which has been used to good effect by Broyles, Chegini *et al.* (1979), Frangioni and Borgioli (1987, 1988), Grasso (1973a, b), Maclean, Maniatis, Carver and Meints (1977), and others. It might be of interest, therefore, to induce anemia in adult axolotls and compare the results with larval.

From the literature, the method of inducing anemia seems to be quite simple: merely adding to the animals' water acetylphenylhydrazine, which selectively destroys red blood cells by damaging their redox system (Frangioni and Borgioli, 1987). This method is more reliable (and probably kinder) than such alternative methods as bleeding or starvation. For several months after such treatment, erythropoietic activity (at least in newts) occurs in fairly synchronized waves, whose timing seems consistent for a

given species (Grasso, 1973a, b; Frangioni and Borgioli, 1987).

This would also have the advantage that, if the anemia successfully resulted in a surge of erythropoietic activity, a large number of erythroid cells might become available for study. Such an induction of a degree of synchrony could be useful in studying larval erythropoiesis as well; it would be most advantageous if, for example, a wave with a large percentage of pointed cells were found during recovery from acetylphenylhydrazine anemia. A particularly desirable application of such synchrony would be the development of a timetable of development, so that cells could be arrested at planned stages, such as the (possible) one when only a few microtubules begin to emanate from centrioles. (It might even be possible to select a population for culturing which was enriched in a given phase of development).

The availability of a large number of synchronized developing cells would of course open the door to many types of experiments difficult or impossible to do with the tiny amounts obtained from normally growing larvae. The method of Dorn and Broyles (1982) could be used to purify cells at different stages of development so that gel techniques could be used to determine cytoskeletal components.

It is of interest that recent experiments in Cohen's laboratory (Twersky, Moogan, and Cohen, 1990), using adult Xenopus laevis made anemic with phenylhydrazine, have shown that spherical, discoidal, and pointed cells appear in the

regenerative erythroid population. The percentage of spheres declined before that of the points, which is in accord with the model presented here. However, the percentage of discs declined prior to that of the pointed cells, which seems strange. It seems plausible, however, that in some cases the MS might develop sufficiently early to cause tension which results in flattening of a sphere as the microtubules begin to grow. If this does occur, the tubules might form a marginal band from the outset, in which case pointed cells would be expected to be flat, rather than rotund. This emphasizes the possible importance, discussed earlier, of a variability in the developmental timetable, and reintroduces the question of the degree of such variability, whether interspecies or intra-individual.

High Voltage Electron Microscopy of Serial Sections

Serial semi-thick (0.25-0.75 μm) or thick (1-2 μm) sectioning of some of the monolayer cytoskeletal embeddings which enabled visualization and selection of pointed cytoskeletons had been planned and begun. It had been hoped that high voltage electron microscopy of these sections would enable analysis of the relationships of the microtubules to any centrioles present and to other components of the cytoskeleton, such as surface associated membrane skeleton. Another experimental goal involved correlative immunofluorescence and electron microscopy of these sections (Rieder and Bowser, 1985).

An effort to clarify the relationship of the external, three-dimensional shape of the developing erythroid cells with their cytology and ultrastructure might include the following approaches: (1) Correlation of phase micrographs with scanning micrographs of the same cells, as outlined in the previous paragraph; (2) Histological or immunofluorescent staining of cells prior to embedding, or embedding of cells in an "alternative carrier" (such as polyethylene glycol) which allows such staining as well as thin or semi-thin sectioning, so that the same or alternate sections could be subjected to both light and electron microscopy; (3) Further pursuit of the scanning-transmission correlative microscopy begun by Teresa Moogan, a student in the Graduate Fine Structure course, which would have done much to illuminate this problem. (4) Determination of haptens responsible for classical staining reactions and use of antibodies to them for immunoelectron microscopy following light microscopic studies; (5) Use of radioactively or immunologically labeled hematological stains, followed by appropriate ultrastructural techniques.

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