

BLOOD FORMATION IN THE AFRICAN LUNGFISH, UNDER NORMAL CONDITIONS AND UNDER CONDITIONS OF PROLONGED ESTIVATION AND RECOVERY¹

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FOUR PLATES (ONE HUNDRED FIGURES)

AUTHORS' ABSTRACT

The blood cells of the African lungfish, *Protopterus ethiopicus*, are very large and resemble those of urodeles. Leucocytes are especially plentiful and rich in variety, including eosinophils, special eosinophils, 'meta-eosinophils' with atypical granules, monocytes, thrombocytes, lymphocytes, and basophils.

The chief hemocytopoietic organs are the spleen, intestine, and kidneys. The lungfish spleen, embedded in the wall of the stomach, represents an intermediate phylogenetic stage between the disperse intra-enteral type of the hagfish and the compact extra-enteral type of other vertebrates.

Erythrocytes are formed in the spleen pulp, granulocytes in the granulocytopoietic organ of the intestine and in the capsules of kidneys, gonads, and spleen. Thrombocytes and monocytes are differentiated in the spleen and general circulation. Basophils arise in the spleen and intestine. Lymphoid cells of all types arise in the spleen. Evidence is presented bearing upon the hemocytopoietic capacity of the various types. Cells with 'Russell bodies' also occur in the spleen.

In lungfishes subjected to long periods of dry estivation, erythrocytopoiesis practically ceases. Granulocytes, however, appear to play an important role, possibly in fat metabolism. The large variety of meta-eosinophils, a unique feature of the lungfish, appears to be associated with the habit of estivation.

Recovery from estivation may show numerous amitoses of erythrocytes in the general circulation. Other cells which divide in this manner are young thrombocytes, granulocytes, monocytes, and lymphoid hemoblasts.

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INTRODUCTION

The lungfishes constitute an especially interesting group from the hemocytopoietic standpoint. There has, however, been no recent or detailed work on their blood and blood-forming organs. These animals, being native to Africa, South America, and Australia, are not readily available for study and experiment.

Preliminary observations revealed that both erythrocytes and leucocytes are of exceptionally large size, being comparable in this respect to the blood cells of urodeles. Parker

('92) in his monograph on the African lungfish remarks that this animal is probably richer in leucocytes than any other vertebrate. Not only are the leucocytes relatively numerous, but they exhibit an extraordinarily wide variety of types. Another interesting characteristic of the lungfish is its habit of existing in a torpid condition for months in moist or even dry earth after the evaporation of the water in which it is living. That such estivation should have a profound effect on the hemocytopoietic activities seemed most probable.

Through the courtesy of Prof. H. W. Smith, of New York University, material of the African lungfish, *Protopterus ethiopicus*, was obtained. This included normal young and adult animals, as well as animals subjected by Doctor Smith to various degrees and lengths of estivation.

MATERIAL

Material from the animals listed below was available for this study.

No. 1. Young lungfish, 14 cm. in length, about two years old, collected in Africa during the latter part of July, 1928, placed in a small can of slightly moist earth for two months (torpid condition), then in water for five days (active condition), and killed.

No. 2. Adult lungfish, about 60 cm. in length, possibly six or more years old, taken from the lake in Africa and killed immediately, July 28th.

No. 3. Several small lungfish fry, 2.5 cm. in length, about two months old, collected in July and killed immediately.

No. 8. Lungfish collected about middle of July, no food given, kept alive in dry estivation from November, 1928, to October, 1929, killed without being returned to water.

No. 13. Lungfish collected in July, kept alive in dry estivation from November 1, 1928, for 410 days, placed in water for thirteen days, infection appearing. Died during night and tissues fixed next morning. During the last two days of life, treatment with potassium permanganate was resorted to in an attempt to overcome the infection.

No. 21. Lungfish collected in July, kept alive in dry estivation from November 1, 1928, for 427 days; replaced in water for ten days and killed, as it seemed about to die after the appearance of superficial infection. An injection of thyroxin (0.5 mg.) twenty-one days before death resulted in 100 per cent rise in metabolic rate. Original weight, 182 grams; dry estivation weight, 133 grams; postestivation weight after water absorption, 152 grams.

No. 29. Lungfish collected in July, subjected to dry estivation from November 1, 1928, to January 21, 1929, being kept at 90°F. for three weeks and 103° F. for four days; placed in water January 21, 1929, died on fourth day, an infection being present.

Nos. 51 to 70. Twenty lungfishes fixed in 10 per cent formalin, varying in size from 15 cm. to 35 cm., thought to be from two to four years old.

No. 33. Lungfish collected in July, 1928, kept in water until January 10, 1930, being fed regularly; killed by cold in transportation between Detroit and New York, tissues, however, showing little post-mortem change.

No. 34. Lungfish with history like that of no. 33.

Blood smears were available for study from animals nos. 1, 8, 13, 21, 33, 34. These were stained according to the Wright technic. The tissues were fixed in the Zenker-formol mixture of Helly and stained with eosin-azure mixture of G-iemsa, or with eosin-azure following dilute Delafield hematoxylin after the method of Maximow.

The regions which are of chief interest for the study of hemocytopoietic activity are the following:

1. The general circulation for the variety of types of blood cells.
2. The spleen for the processes of lymphocytopoiesis, erythrocytopoiesis, granulocytopoiesis, erythrocytaphagic and granulocytophagic activity, and for its place in the general scheme of spleen phylogeny.
3. The spiral-valve region of the intestine for its granulocytopoietic organ.
4. The kidney and gonad capsules for granulocytopoiesis.

OBSERVATIONS

The general circulation

Erythrocytes. Mature, young, and senile erythrocytes are present in the general circulation (figs. 1 to 6). The resemblance to conditions in the salamander is striking. The ancestral cell is the lymphoid hemoblast (hemocytoblast). An early stage in erythrocytopoiesis is represented by the proerythroblast (fig. 1). The nucleus in this stage is often somewhat condensed, granular, and tachychromatic, the nuclear membrane sharply defined, and the cytoplasm deeply stained,

with sometimes a non-homogeneous appearance. Irregular outline of the border of the cell may also characterize this stage. While the pro-erythroblast figured (fig. 1) is of large size, many cells at this stage are much smaller and should not be confused with cells of the lymphoid hemoblast group. The pro-erythroblast nucleus always presents a dark blue color as compared with the purple or light blue of the ancestral lymphoid hemoblast. Similar conditions have already been described in detail for salamanders (Jordan and Speidel, '30). Numerous transitional forms may be found between the pro-erythroblast stage and the adult erythrocytes (figs. 2 to 4). Progressive increase in hemoglobin gives the characteristic staining reactions of the cytoplasm. The nucleus loses its somewhat granulated appearance and large deeply stained chromatin blocks are formed, with a tendency to thread formation. In the final stages contraction of the nuclear membrane may leave these projecting somewhat at the border. The shape, both of nucleus and cytoplasm, changes from spherical to oval.

Mitoses of erythroblasts are fairly common in the blood smears of normal animals (nos. 33, 34). They are not to be seen in the estivating specimens. In the splenic sinuses of animal no. 2, freshly taken from the lake in Africa, numerous mitoses of hemoblasts and of all stages in erythrocyte differentiation are present (fig. 37). Blood smears of this specimen were not, however, available.

Amitosis of erythrocytes. In an animal (no. 1) which was placed in water for five days after two months of estivation, amitoses of erythrocytes are numerous (fig. 16). Though some of these cells appear to be not entirely mature, others seem to have their full complement of hemoglobin.

In a few cells, definite cell plates between the daughter cells can be recognized—an observation which we believe precludes the possibility of the amitotic appearance being the result of artifacts. Very early stages in the process can be recognized under high magnification (figs. 93, 94). The first indication is a slight thinning of the nucleus near the center and the

appearance of a fairly definite 'nuclear plate' (figs. 93, 94). A slight indentation at the edge of the nucleus is also usually present (fig. 93). The cytoplasm also shows elongation and central constriction (figs. 94 to 97). As the nuclear halves draw apart, fine lines of chromatin usually still connect the two halves (figs. 94 to 97 and 16). Although in a few cases a sharp cell plate can be noted (fig. 16), the more usual condition shows the central region reduced to a fine thread-like connection (fig. 97).

A random count under high magnification of 10,000 erythrocytes in animal no. 1 showed fourteen indubitable cases of amitosis and thirteen other probable cases (early stages). In some low-power fields three or four examples were to be seen. That the amitotic impulse does not always result in nuclear division is suggested by the cell of figure 98. We are inclined to interpret this as a case of cytoplasmic segmentation—a process which is common to many urodeles, but which is quite rare in lungfish. It is quite obvious that the daughter halves in amitosis are practically always of distinctly different size, the discrepancy often being considerable. Amitoses of erythrocytes occur in some of the lungfishes other than no. 1, but they are much less numerous.

Senile erythrocytes. The most usual type of senile erythrocyte presents a pale shadowy cytoplasmic envelope and swollen, lightly staining, purple nucleus. At a later stage the cytoplasm disappears entirely and the nucleus becomes progressively less definite.

Another type of senile cell has the 'soap-bubble' type of nucleus (fig. 5). In this type the nucleus is vacuolated and the nuclear membrane distended in an irregular manner, taking on a lobulated appearance. As degeneration proceeds the nucleus progressively stains more green and less blue. Many degenerating erythrocytes of this type are present in our estivating animals. We have noted this type of degeneration in salamanders (Jordan and Speidel, '30) and it has recently been described in some detail in infected *Triturus* (Nigrelli, '29).

A third type of erythrocyte degeneration occurs occasionally, a type which may be termed 'intracellular nuclear fragmentation' (fig. 6). The chromatin material of the nucleus is scattered throughout the cell. Both cytoplasm and the chromatin masses often show degenerative vacuolation. In some erythrocytes a densely pycnotic nucleus occurs with or without vacuoles.

Erythroplastids. Erythroplastids are exceedingly scarce, though there is evidence of occasional cytoplasmic segmentation (fig. 98).

Leucocytes. Leucocytes are especially numerous in proportion to erythrocytes in the lungfish blood smears. Many types occur, including lymphoid hemoblasts (large lymphocytes), small lymphocytes, thrombocytes, eosinophilic granulocytes, special eosinophilic granulocytes, meta-eosinophilic granulocytes, monocytes, macrophages, basophilic granulocytes, and lymphoid cells with acidophilic inclusions.

Lymphoid hemoblasts (large lymphocytes). The lymphoid hemoblasts are large lymphocyte-like cells (figs. 7, 11, 17). The nucleus contains a moderate number of fair-sized chromatin blocks and varies in staining reaction from blue to purple. Plasmosomes, though conspicuous in fixed material, do not show in the smears. The light blue cytoplasm is homogeneous, or slightly granulated, with weakly basophilic (bluish) granules. Pseudopods may be present. That some of these cells undergo degeneration is clear. Transitional forms with lightly staining nucleus and cytoplasm, followed by typical degenerating stages, suggest that many of these cells never give rise to anything other than a lymphoid cell. Other transition stages, however, seem to point to this type of cell as the progenitor of the large thrombocyte and monocyte as well as erythrocyte.

Small lymphocytes. Small lymphocytes occur in moderate numbers. They present a number of chromatin blocks in the nucleus which stain relatively deeply. The cytoplasm is usually somewhat granular. In many cases the nuclei are practically naked, with the thin cytoplasmic rim reduced to a

minimum. The nucleus may be slightly grooved or lobulated. A few basophilic or azurophilic granules may be present. Often lymphocytes with little or no cytoplasm so closely resemble thrombocytes which have also lost their cytoplasm that a distinction cannot be definitely made. Of special interest is the occasional occurrence of large eosinophilic inclusions in small or medium-sized lymphocytes (fig. 22).

Thrombocytes. The thrombocyte is characterized by a small condensed nucleus and very little cytoplasm, which usually presents some reddish or reddish-violet granulation (fig. 14). The nucleus is oval or spherical, and stains deeply. Grooving of the nucleus may be observed in many cases. The delicate pale-staining outer rim of cytoplasm is not always conspicuous, but may be seen in thrombocytes that have clumped (fig. 14).

Stages in the development of thrombocytes occur in the general circulation. The youngest thromboblats resemble the medium-sized or small lymphoid hemoblats (figs. 12, 13). The nucleus is slightly more dense. The cytoplasm is characterized by mixed granules, both bluish and reddish ones being conspicuous. At a later stage the bluish granules disappear and are replaced by reddish ones. The outer portion of the cytoplasm becomes rather delicate and often shows processes. The granules may be present all around the nucleus, but there is a tendency for segregation at one or both poles. In later stages the nucleus becomes still more dense and the cytoplasm more scanty.

Mitosis and amitosis. One interesting example was seen of a thromboblatt in mitosis in the circulation. The cell was in early prophase, and the cytoplasmic granules were mixed, both blue and red ones being visible. We have previously recorded two cases of thromboblatt mitosis in the circulating blood of splenectomized salamanders (Jordan and Speidel, '30).

Another observation showed that thromboblats may divide by amitosis (fig. 15). Several other cases of probable amitosis of young thrombocytes were seen. These observations

were made on the blood of a young lungfish (no. 1) placed in water for five days after being in slightly moist earth for two months. This is the same animal which displayed such a large number of erythrocytes dividing by amitosis.

Monocytes and macrophages. Typical monocytes are present in fairly large numbers (figs. 8, 9). The nucleus may be reniform as figured, or it may be spherical, elongate, lobulated, or double. It presents a purplish tinge in Wright's stain, or in some preparations a light blue color. The chromatin blocks often have a 'smeared' appearance. The cytoplasm may be characterized by a mixed weak reddish and bluish granulation. An archoplasmic area may be easily seen. There is a distinct tendency for the reddish granules to be aggregated about this area (fig. 8). However, the reddish granulation may be entirely absent. The contour of the cell may be smooth or it may show irregular processes. Great size variation has been noted. In general, the monocyte is of a size comparable to the large lymphoid hemoblast and often it is somewhat larger. However, a few small cells have been seen possessing distinct monocyte characteristics.

Monoblast stages have been seen which are transitional between the hemoblast (fig. 7) and the monocyte (fig. 8). A young monocyte has a spherical nucleus and often a mixed blue and red granulation. Some cells of this type approach in appearance the thromboblats, so that it is not always easy to distinguish between the two.

The cytoplasm of the monocyte may show vacuoles as well as granules and present a 'stringy' appearance (fig. 9), similar to conditions in the salamander. Cells of this type may become quite active in phagocytosis. One of these is illustrated (fig. 10). Among the ingested materials of these monocytic macrophages may be recognized much eosinophil debris.

Several examples quite suggestive of multiplication of monocytes by amitosis have been seen. Both monocytes and macrophages are quite numerous in animals no. 21, 13, and 8. Whether this is a result of estivation, or of infection, or both is not certain.

Eosinophilic granulocytes. Large eosinophils with coarse granules are conspicuous (fig. 18). The nucleus is often approximately spherical or slightly indented, but it may also be lobular, or even occur in two or three separate parts. An archoplasmic area devoid of granules is visible. The cytoplasmic ground-substance stains a pale bluish gray. Variations in size are marked. Plastids of these cells may be seen occasionally. Rarely, eosinophils with pigment granules occur in the general circulation. Similar pigmented eosinophils have been seen in the circulating blood of the salamander.

Coarse-granule eosinophils in all stages of senility and degeneration may be found. These are particularly in evidence in animals subjected to long periods of estivation. There is often coalescence of the granules into large masses which then may lose their eosinophily. Cells sometimes appear to break into several parts. Another type of degeneration is characterized by a progressive lightening of staining reaction, both of granules and nucleus. In some cases pigment granules are produced as a late stage of the degenerative process.

Special eosinophilic granulocytes. Eosinophils with fine granules are numerous (fig. 19). The nucleus is ovoid, sometimes reniform, lobular, ring-like, or double. An archoplasmic area may, or may not, be visible. The cytoplasmic ground-substance stains a pale bluish gray.

Degenerative forms of this type of cell are frequently met with, especially in animals subjected to estivation. Degeneration usually causes the cell to stain with a pale watery appearance, the nucleus and granules losing their distinctness (fig. 20).

Basophilic granulocytes. Basophils with metachromatic granules (lilac or violet) occur abundantly (fig. 21). The nucleus is usually approximately spherical. It may, however, be bibbed or grooved. The granules may be coarse or fine. The cells often present a somewhat degenerate appearance, with nuclear structures indistinct. Some cells seem to show

liquefaction and disappearance of the granules. Young stages of basophils may also be seen which contain a single row of metachromatic granules and a young-looking nucleus. In these young cells may sometimes be seen the weakly bluish granules characteristic of the lymphoid hemoblast, which is considered the progenitor.

The hemocytopoietic organs

Spleen. The spleen of the lungfish is the chief organ for erythrocytopoiesis. It is in close relation to the stomach, being embedded in the wall and covered by peritoneum and muscle (figs. 47, 48). The pancreas is immediately beside the spleen and has a similar relation to the stomach (fig. 48).

In the young lungfish (fig. 48) the spleen presents a central core of lymphoid cells, surrounded by a region of pulp cords and sinuses, and a thin peripheral capsular region. Connective-tissue strands extend at intervals between the outer capsule and the central lymphoid mass, thus dividing the pulp into lobular areas. From the central lymphoid core lobular projections of lymphoid cells extend into the pulp areas. The spleen of the older lungfish (60 cm. in length) resembles that of the younger animal except that with the increase in size there has also gone increase in number of lobules of splenic pulp, as well as increase in the lobulation of the central lymphoid mass. Some of the lobules of lymphoid tissue appear to have become separated from the central mass. A study of each of these regions in the spleen discloses characteristic functions.

In the central lymphoid mass lymphocytopoiesis is actively carried on. In the splenic pulp erythrocytopoiesis takes place. Senile granulocytes and erythrocytes are disposed of by macrophages, reticular cells, and endothelial cells in this region. A certain amount of extravascular granulocytopoiesis and intravascular thrombocytopoiesis may also be seen. In the capsular region granulocyte production and destruction may both be active. The latter process is marked by extensive pigmented areas of degeneration.

The central lymphoid mass. Lymphocytopoiesis. In the central lymphoid mass there are present lymphocytes of large, medium, and small size, the last type predominating. Reticular cells form the framework, and there are small arteries at intervals, but no veins. The lymphocytes migrate peripherally into the splenic pulp.

The larger and medium-sized lymphocytes usually present the typical vesicular or 'sieve' type of nucleus, the chromatin being in the form of a few large angular blocks and fine granules scattered through the linin. One or more plasmosomes may be present, staining red with the eosin-azure combination (figs. 52, 56). This type appears to be the usual hemoblast, the progenitor of erythrocytes, monocytes, and granulocytes. It is identical with the hemoblast type found in the granulocytopoietic organ of the intestine (fig. 23), which in that locus gives rise to granulocytes. In some of the large and medium-sized lymphocytes the large chromatin blocks may be numerous and radially arranged (figs. 55, 59), somewhat resembling, therefore, the small lymphocyte type. The small lymphocytes (fig. 63) usually possess a moderately condensed nucleus with large angular chromatin blocks arranged in more or less radial fashion (wheel arrangement). Plasmosomes are usually not present, though in some cases they may be seen. They are not as conspicuous as in the larger lymphocytes. A few small-sized lymphocytes with nuclei of the vesicular type may be found (fig. 60), as well as intermediate types (figs. 61, 62).

There are, therefore, lymphoid cell types (figs. 52 to 63) which may be classified as follows: 1) Large lymphocytes with *a*) amblychromatic nucleus (fig. 52), *b*) pachychromatic nucleus (fig. 55) with radial arrangement of chromatin blocks ('cartwheel' type), *c*) intermediate nuclei between *a* and *b* types (figs. 53, 54). 2) Medium-sized lymphocytes with *a*, *b*, and *c* types of nucleus (figs. 56 to 59). 3) Small lymphocytes with *a*, *b*, and *c* types of nucleus (figs. 60 to 63). Types 1*c* and 3*a* are least numerous. The large number of transitional lymphocytes with respect to size, arrangement of

chromatin, amount of chromatin, and occurrence of plasmosome suggest that all of the lymphoid cell varieties are genetically related. Mitoses of medium-sized and large lymphocytes are common. Small lymphocytes, however, seem not to multiply in this fashion. On the contrary, the large number of deeply lobulated and even binucleated forms indicates the probability that some multiplication of small lymphocytes by amitosis occurs (figs. 87 to 89).

The smaller lymphocytes with coarse chromatin granules in the nucleus are especially conspicuous near the periphery of the lymphoid core. They appear to arise from the less differentiated lymphoid cells nearer the center, and to migrate into the splenic pulp. Some undergo degeneration (figs. 78 to 80) and some remain as lymphocytes in the general circulation. This, however, accounts for only a portion of them. Many, after reaching the venous sinuses of the spleen, function as the progenitors of thrombocytes.

The reticular cells of the lymphoid core present nuclei much like those of the large lymphocytes (figs. 81, 82). The chromatin is sparse and in the form of fine granules except for a few larger blocks. A plasmosome is usually distinguishable. The boundary of the irregular cytoplasm cannot ordinarily be traced exactly, as it extends out in interlacing processes. In some of the cells a faint cytoplasmic granulation may be made out.

The close resemblance of the nucleus of a reticular cell to the vesicular sieve-like nucleus of many of the lymphocytes (fig. 83) makes it seem probable that some reticular cells by rounding up may become transformed into lymphoid hemoblasts. Many reticular cells contain granules (fig. 84). This condition is especially noticeable in regions from which most of the lymphoid cell content is gone ('exhausted' areas) and in reticular cells of the splenic pulp. In many cases these granules appear to be derived from senile granulocytes and ingested by reticular cells. However, in other cases the granules appear to be a product of the reticular cells.

The splenic pulp region. Erythrocytopoiesis. The most striking feature of the spleen pulp is the large number of proerythroblasts and erythroblasts. These cells are conspicuous in the venous sinuses of this region and in the meshes of the pulp cords. The youngest stages of development of these cells plainly indicate the usual mother cell. It is the lymphoid hemoblast of large or medium size with vesicular, sieve-like nucleus. Stages in the differentiation of three kinds of lymphoid hemoblasts into erythroblasts are shown (figs. 64 to 75); in one row a medium-sized vesicular hemoblast with the amblychromatic nucleus persisting well into the erythroblast stage (figs. 64 to 67); in the next row a typical 'intermediate' type of hemoblast with pro-erythroblast and erythroblast stages following (figs. 68 to 71); and in the next row, a hemoblast with lobulated nucleus followed by pro-erythroblasts and erythroblast also with lobulated nucleus (figs. 72 to 75). The pro-erythroblast stage may be recognized by a slight change in the staining reaction of the cytoplasm from the gray-blue of the hemoblast to a slightly more purple tinge. The nucleus becomes less sieve-like; i.e., the fine chromatin granules on the *linin* are no longer so obvious. The blocks of chromatin become less angular and in some cases slightly swollen, in others slightly elongated. These chromatin blocks stain less deeply blue and less sharply. The plasmosome persists, but is often slightly less conspicuous.

The erythroblast is characterized by pronounced hemoglobin formation resulting in a definite pinkish-staining reaction. The nucleus may show some contraction, but the early erythroblast nucleus may be as large as that of the *hemocyto*-blast ancestor. The sieve appearance of the earlier stages is usually practically gone, especially in the later erythroblasts. The chromatin blocks are still less angular. They are more numerous and in many cases show a tendency to elongate and coalesce with adjacent *granules* into threads. Plasmosomes are still visible, though often not very obvious (figs. 36, 38). In some cells there is exhibited a tendency on the part of the cytoplasm to become finely granulated. The later erythro-

blasts exhibit a more condensed nucleus, which stains more deeply, and a larger amount of cytoplasm which becomes more eosinophilic (fig. 38). The further differentiation of erythroblasts into erythrocytes consists in the growth and elongation of the cytoplasmic mass accompanied by elaboration of more hemoglobin. This latter process results in more deeply red-staining cytoplasm. The nucleus becomes smaller, more concentrated, and more deeply staining. The individual chromatin blocks become less distinct. Intravascular erythrocytopoiesis only was observed.

An interesting feature of the erythrocytopoietic process in the lungfish is that pro-erythroblasts and erythroblasts may show nearly as much, and similar, variation in morphology as their hemoblast progenitors. This affords a useful key for determining which kinds of lymphoid cells may act as progenitors of erythrocytes. The evidence indicates that all kinds may function as ancestors, with the probable exception of the smaller lymphocytes with coarse-granule nuclear chromatin (fig. 63). This last type of cell may pass into the circulation as a small lymphocyte; it may transform in the splenic sinuses into a thrombocyte (figs. 90 to 92) ; or it may degenerate in the spleen without ever reaching the circulation (figs. 78 to 80). But pro-erythroblasts and erythroblasts with similar coarse-granule chromatin nuclei are not in evidence. Thus, we consider the cells of figures 52 to 58, 60, and 61 capable of erythrocytogenesis, but not those of figures 62, 63, and 59.

Thrombocytopoiesis. Thromboblats and thrombocytes are quite numerous in the region of the splenic pulp. It is obvious that active production of this type of cell takes place in this locus. One variety of mature thrombocyte is approximately spherical or oval (fig. 91). The cytoplasm is relatively scant and may or may not present a faint granulation in eosin-azure sections. In blood smears the outer part of the cytoplasm is delicate and often presents irregular processes ; in sections, however, the outer edge is generally smooth and regular. The nucleus is greatly condensed, the chromatin

being in the form of large granules rather closely massed together, so that in many cases some fusion of granules has occurred. Grooving of the nucleus may or may not be visible. It seems typical for a few of the chromatin granules to take the azure stain very deeply, giving a slightly pycnotic appearance.

Another variety of thrombocyte is elongated or spindle-shaped (fig. 92). In such cells the chromatin is drawn out in the direction of the long axis of the cell. There are almost always one or more distinct surface grooves along the nucleus. Acidophilic granulation may be conspicuous at both poles, at one pole, or extending entirely about the nucleus.

The spherical type of thrombocyte predominates. The younger thromboblats closely resemble the smaller lymphocytes of the periphery of the lymphoid mass. The inference is that the small (or medium-sized) lymphocytes with coarse-granule chromatin function largely as mother cells for thrombocytes. Differentiation takes place only intravascularly, at least as regards the acidophilic granulation of the cytoplasm. The transitional types of small and medium-sized lymphocytes may also become transformed into thrombocytes. However, no cases have been noted which suggest a similar transformation of the large lymphocytes with vesicular nucleus.

Monocytopoiesis. While monocytes are probably formed in the general circulation, as has already been noted, they may also arise in the spleen. They may be derived from lymphoid hemoblasts or from reticular cells. Many become phagocytic, disposing particularly of large numbers of granulocytes and senile erythrocytes. One of these cells is figured (fig. 45) which contains ingested material, but which still presents the young hemoblast nucleus of vesicular type with conspicuous red-staining plasmosome. Similar phagocytic activity is exhibited by monocytes or macrophages in the liver and in the granulocytopoietic capsules of the kidneys (fig. 50) and gonads, and in the wall of the gut.

Granulocytopoiesis. Eosinophils with coarse granules. Eosinophils are formed in great numbers in the wall of the

intestine, in the spiral-valve region. A special granulocytopoietic organ is located at this level (fig. 49). As in the case of the spleen, this organ lies in the submucosa internal to the muscle layers. Anteriorly, this organ reaches the spleen. Posteriorly, it extends practically to the end of the intestine, with some variation in its degree of development. Other granulocytopoietic regions are the capsules of spleen, kidneys, and gonads.

The usual ancestral cell for the coarse-granule eosinophil is the large lymphoid hemoblast with vesicular nucleus (fig. 23). This cell corresponds to the large lymphoid hemoblast with amblychromatic nucleus which has been described in the spleen. In the early stages of development the young eosinophil nucleus is much like that of the hemoblast (fig. 31), and the cytoplasm may contain mixed bluish and reddish granules (figs. 32, 33). Mitoses are frequent (fig. 34). In the later stages the nucleus becomes larger, sometimes indented, lobulated or even double. The granules become larger and the archoplasmic area becomes more distinct (fig. 35). The cytoplasmic ground-substance stains a light grayish blue. The plasmosome may persist quite late in development.

In the spleen also, particularly in the capsular region, young eosinophils may be seen developing. In some cases a hemoblast ancestry is indicated, but in other cases the cells appear to differentiate directly from reticular cells. It is probably true also that in the intestinal wall, kidney, and gonad capsular regions, coarse-granule eosinophils sometimes take origin directly from reticular cells.

Special eosinophils. The special eosinophil is characterized by fine eosinophilic granules and by a nucleus of the polymorphous type (fig. 30). This cell is regarded as the homologue of the neutrophil of higher vertebrates. Special eosinophils may be seen arising in the granulocytopoietic organ of the gut wall. These cells develop from the lymphoid hemoblast with vesicular nucleus. The red granules may or may not be preceded by blue ones (fig. 24). Active multiplication by mitosis of granuloblasts occurs (fig. 25). The nucleus under-

goes some lobulation (fig. 28), often suffering loss of nuclear material in the process. An archoplasmic area sometimes becomes visible in the early stages (fig. 27). Nuclear condensation also is quite characteristic (fig. 28).

In the later stages there is an increase in nuclear lobulation in many but not all of the cells. The proportion of cytoplasm to nucleus increases (fig. 30). In a few cases cells were observed containing coarse granules as well as fine ones (fig. 29).

In the spleen this type of cell is also found in early stages of differentiation. The ancestral cell in this locus is the hemoblast, or it may be a lobulated lymphocyte. It is also probable that the reticular cell may function directly as the mother cell for the special eosinophil in the intestinal wall organ and in the capsules of kidneys and gonads.

Meta-eosinophils. In all of the granulocytopoietic areas there are cells containing granules which exhibit other varieties of staining reactions, such as pink, purple, green, yellow, colorless, and mixed (figs. 39 to 43). Cells of this sort are termed 'meta-eosinophils.' Many of these cells are young and appear in no way degenerate forms. In fact, a number have been seen in amitosis (fig. 39). They appear to be of the same general nature as the coarse-granule eosinophils. The green granulocytes are found only in close proximity to the peritoneum. That some of these cells represent senile or abortive eosinophils seems certain. Faint pink, yellow, and colorless granules occur in cells that are in all probability senile eosinophils. A continuous series of transition stages showing senility in these cells can be traced. Meta-eosinophils are also present in the general circulation.

The occurrence of coarse granulocytes with mixed red and blue granules (fig. 40) is of special interest to us, as these cells are obviously homologous to those previously noted by us in the turtle thymus and spleen (Jordan and Speidel, '28).

Basophils. Young basophils have been seen in the spleen and in the general circulation. They appear to come from the small or medium-sized lymphoid hemoblasts. In a young

basophil the nucleus is spherical or slightly lobulated (fig. 44) and a single row of basophilic granules may be present. An archoplasmic area is not usually visible at this stage. Further development consists in growth of the cytoplasm, formation of more metachromatic granules, and indentation or lobulation of the nucleus. The nucleus becomes hazy and indistinct. While direct origin from the hemoblast may be traced, the general characteristics of basophils suggest degeneration or abortive development.

In the circulation young basophils may sometimes present a bluish granulation as well as the definitive metachromatic granulation. The granules may be fine, coarse, or of intermediate sizes, varying in much the same manner as in eosinophils. Late stages in basophil development or senile stages may be characterized by partial vacuolation, as if a result of liquefaction of the granules. In some regions, as in the intestinal wall, the connective-tissue-cell origin of basophils is conspicuous.

Disposal of granulocyte debris. Correlated with the large number of granulocytes in lungfish blood and certain tissues, there occurs conspicuous granulocyte degeneration. Both eosinophils and special eosinophils fragment in large numbers, the debris being ingested by macrophages (figs. 10, 45). There are progressive changes in the staining reactions of the granules. Various shades of pink, yellow, and brown may characterize the granules, formerly eosinophilic. Some may be quite colorless. These changes may occur inside macrophages or they may occur without macrophage influence. In late stages of change pigment is produced. This may vary from light tan or brown to deep black. Large masses of pigmented material formed from granulocyte debris constitute one of the most prominent features of the spleen. A similar condition is found in the wall of the intestine, in the kidney (fig. 50) and gonad capsules, in the liver, and to a less extent in other loci. Elimination of some of this material appears to take place by way of the intestinal lumen, some also by way of the kidney (figs. 99, 100) and liver cells.

The reticular cells and endothelial cells of the venous sinuses of the spleen seem also to play a part, at least at times, in the disposal of granulocyte debris. Granules which stain a light pink, or which are practically colorless, may be present in the cells of the reticular framework of the spleen and in the endothelial cells of the sinuses (figs. 84 to 86). In view of the extensive granulocyte degeneration, the presumption is that these granules, partly at least, represent former eosinophil granules which have been ingested.

Disposal of erythrocyte debris. Senile erythrocytes are found in the general circulation and in the venous sinuses of spleen, liver, and other organs. They are especially prominent in the spleen pulp. All conceivable stages of degeneration are to be seen of whole erythrocytes, of naked erythrocyte nuclei, and of cytoplasmic fragments. The staining reaction varies from red, green, or yellow to various shades of brown. Some fragments are almost colorless. The degenerating nuclei vary from deep blue, or black, to deep green.

The fragments may be free or they may be in macrophages. Some macrophages seem to specialize on the ingestion of cytoplasmic fragments, some on nuclear material ; a few show both kinds ; and many show other types of debris, such as granulocyte debris. Pigment formation seems to occur to some extent in the later stages of degeneration. Both reticular cells and the endothelial cells of the venous sinuses contain granules which to some extent may represent erythrocyte debris.

Observations upon other organs

The kidneys. The granulocytopoietic capsule of the kidney contains a variety of granulocytes. Some activity in granulocytopoiesis is apparent, a number of young eosinophils and of young special eosinophils being present.

More conspicuous, however, are the indications of degenerative change. Senile granulocytes are numerous, containing granules of various colors : green, yellow, and pink. Some are colorless. Deeply pigmented material represents a late

stage. Macrophages with ingested material display a tendency to congregate. An occasional mass of lymphoid cells occurs (fig. 50).

Suprarenal cells were not seen in relation to the kidney. Typical chromaffin cells are, however, conspicuous in the heart—a condition which has been described for the lungfish.

The gonads (ovaries). Conditions in the connective-tissue capsule of the ovaries are very similar to those of the kidney, although both granulocytopoiesis and the degenerative processes are less pronounced.

The liver. The liver of the lungfish is not an important organ from the standpoint of blood-cell formation. In the venous sinuses occur some of the younger stages of erythrocytes and thrombocytes, but these are not numerous. An occasional locus of either granulocyte or erythrocyte degeneration may be seen. The liver cells may show pigmented granules, which probably represent the products of erythrocyte degeneration about to be excreted as bile pigment.

The heart. Little of interest was found in the heart from the standpoint of hemocytopoiesis. Unlike the salamander, there occurs no marked number of immature red cells, nor is there any indication of rounding up of reticulo-endothelial cells, as seen by Wituschinski ('28) in the axolotl heart. One interesting feature is the presence of a number of chromaffin cells. These occur in small nests or groups. Cartilage is also quite prominent in the wall of the heart in certain regions.

The stomach. The stomach lining shows migrating granulocytes and macrophages. Near the peritoneum are metacosphils with purple granules. Basophils are also numerous.

Bursa entiana. The bursa entiana is located just below the stomach. The internal wall or lining is raised into a number of deeply pigmented folds. The pigmented appearance is caused by numerous cells containing dark pigment granules which appear to be in process of migrating through the lining. Many of these are true pigment cells, but others are macrophages loaded with ingested debris. The lower portion of the

spleen and the upper portion of the granulocytopoietic organ of the intestine may both be seen at this level.

The pancreas. The pancreas is unimportant from the standpoint of hemocytopoiesis. It is in close relation to the spleen and is embedded in the stomach wall in a similar fashion. Its capsule shows some granulocytopoiesis, as does that of the spleen. There may also be seen areas of granulocyte degeneration. Near the peritoneal edge are the characteristic purple granulocytes with coarse granules. The deeply pigmented appearance of the pancreas is caused by numerous pigment cells (chromatophores) scattered throughout the organ.

The lungs. The lungs are rather simple sac-like organs, in general appearance much like those of the salamander. They are not important as regards blood-cell formation or destruction. Granulocytes are conspicuous in the pulmonary capillaries and also in the connective tissue underlying the epithelium.

Vertebral column. Sections through the vertebrae failed to reveal any locus of hemocytopoietic activity. Notochord and cartilage appeared as the only constituents, no trace of marrow being seen.

Muscle. Sections through muscles associated with the vertebral column revealed nothing of interest for this study. Neither in normal nor in estivating animals was anything apparent indicative of blood-cell activity.

The granulocytes of kidney and gonad capsules are in close proximity to perirenal fat, and may be important agents in its transportation. In some preparations the granulocytopoietic area is seen to be continuous with the fat area.

Estivation

Animals subjected to long estivation periods showed marked changes in the blood and hemocytopoietic organs. The best specimen for observation of the blood changes proved to be no. 8, an animal in dry estivation for one year.

As compared with normal animals, the blood contains a large number of degenerating granulocytes (fig. 20). These include both eosinophils and special eosinophils. Early stages in this process are represented by cells in which the granules are somewhat less distinct in outline and the nucleus less sharply defined, with a tendency to stain less deeply. In later stages the cell features are still less distinct. Many cells present a pale-staining watery appearance (fig. 20). The granules in the latest stages become practically colorless, and the nucleus becomes pale blue and almost homogeneous. In a few cases pigment formation accompanies the degenerative process.

Another conspicuous change in the animals subjected to estivation was the absence in the blood of the monocyte. With return of the animals to water, monocytes reappeared in large numbers. This may, however, have been correlated with infection, which attacked animals nos. 21 and 13. There is no trace of mitosis or amitosis of blood cells in the estivating animals. On the other hand, many erythrocytes are to be seen in various stages of senility and degeneration.

In the hemocytopoietic organs changes from the normal condition are also conspicuous. In the spleen and kidney there occurs a great amount of eosinophil destruction. Large masses of degenerating cells with pigment are being handled by macrophages. In the kidney the tubule cells show many deeply pigmented granules passing through them (fig. 99), the inference being that these cells slowly excrete the products of eosinophil degeneration. To a less degree there appears to be similar activity in the liver.

The granulocytes are much more conspicuous in the spleen and intestinal granulocytopoietic organ. In the spleen almost the entire area outside of the central lymphoid mass is packed with eosinophils. The spleen pulp appears to be practically without its normal erythrocytopoietic activity. The cords of lymphoid cells surrounding the spleen pulp in the normal animal had been almost entirely replaced by granulocytes. The intestinal granulocytopoietic organ also appears to be a solid

mass of granulocytes. Although the granulocytes are everywhere conspicuous, there is little or no indication of active formation of new granulocytes. Such activity is at a low ebb.

Mixed-granule eosinophils or meta-eosinophils, i.e., cells containing both basophilic and eosinophilic granules, were noted particularly in the estivating animal no. 8 (fig. 40). Whether or not they are produced as a result of the estivation is not known. It seems to us that these cells constitute merely one more variety of meta-eosinophil.

Animal no. 21, which was replaced in water after a long period of estivation and in addition given an injection of thyroxin, shows active blood and hemocytopoietic organs. In the general circulation occur erythrocytes in all stages of development from hemoblasts (figs. 1 to 4). Senile stages are also quite numerous. The unusual types represented in figures 5 and 6 are present. The intracellular nuclear-degeneration type of erythrocyte was so common in animals nos. 29 and 21 that random sample counts were made. Of 1000 erythrocytes in the general circulation, 235 showed this kind of degeneration in no. 29 and forty-five showed it in no. 21.

Eosinophils and special eosinophils in all stages of degeneration are conspicuous. Monocytes and macrophages are numerous and quite active in the ingestion of debris, especially the remnants of eosinophils and special eosinophils. In animal no. 21 amitoses of hemoblasts (fig. 17) and of monocytes were seen. This latter type of cell is present in all stages of development, the ancestral cell being the hemoblast. The reddish granulation when present is always conspicuous about the archoplasmic area. In some cells the granules are distributed in circular bands near the periphery. Great size variations occur.

The macrophages (figs. 9, 10) are obviously later differentiation forms of monocytes, usually with ingested material in the cytoplasm. These cells ordinarily lack the reddish granulation and archoplasmic area. The cytoplasm appears moderately delicate, presenting a faint granulation, vacuoles, and processes.

In the spleen amitoses of eosinophils and of meta-eosinophils were observed (fig. 39). In certain areas these are quite numerous, distinct cell plates being sometimes visible, as in the cell figured. A large number of eosinophils and meta-eosinophils possess nuclei of great interest. Each nucleus presents several long lobular extensions from a central region, as if under the influence of a strong 'amitotic urge' which is being expressed in a somewhat abnormal manner. Mitoses of eosinophils and special eosinophils are also numerous.

The lymphoid cords of the spleen have an exhausted appearance, as if these areas had been largely drained of their cellular content. This is the appearance also of the splenic pulp and capsular zones.

Stages in the differentiation of erythrocytes, thrombocytes, macrophages, and special eosinophils were seen. Mitoses of hemoblasts occur and lobulation and amitoses of the smaller lymphocytes. In addition to the reticular-cell mode of origin of hemoblasts, animal no. 21 also exhibited their origin from endothelial cells of venous sinuses. These cells undergo typical stages of rounding up and separating from the sinus wall. Sometimes several separate in a group.

The cells of the kidney tubule show vacuoles and granules (fig. 100) that contrast markedly with the condition in animal no. 8, which was not placed in water after the long estivation period. The general active appearance of the kidney of no. 21 suggests that much of the pigmented eosinophil debris that accumulates in estivation is excreted.

*Comparison of hemocytopoietic organs of lungfish fry,
young lungfish, and adult*

In the fry two months old there is as yet no marked definition of central lymphoid core, spleen pulp, and spleen capsule (fig. 47). The spleen shows little or no differentiative activity. Proliferation of hemoblasts dominates the entire organ. Erythrocytes and thrombocytes, therefore, are formed especially in the general circulation and sinuses of the liver.

The kidneys afford the chief locus for granulocytopoiesis, the special eosinophils being formed here in large numbers. Eosinophils and meta-eosinophils with coarse granules are not present here, nor are they to be seen in the general circulation. These types appear only later in development. Basophils, monocytes, and macrophages were not seen. Yolk granules persisting from the *egg* are in evidence in the cells of liver and *notochord*, and in association with the posterior part of the alimentary tube.

In the young lungfish two years old the spleen pulp is an active locus for erythrocyte differentiation. Eosinophils with coarse granules are conspicuous, as are also the special eosinophils with fine granules. Monocytes, macrophages, thrombocytes, and basophils are in evidence. Degenerative activity is apparent.

The kidney differs from that of the fry in exhibiting coarse-granule eosinophils as well as cells containing granules of purple, pink, yellow, and other shades. Special eosinophils are also numerous. Degeneration of granulocytes is quite conspicuous, especially in the capsular region, associated with the presence of macrophages and the formation of pigment.

A granulocytopoietic organ is well developed in the intestine, active in the production of eosinophils and special eosinophils.

The largest lungfish in our collection, 60 cm. long and estimated to be six or more years old, shows some advance in hemocytopoietic differentiation over the younger fishes. The spleen is much larger, its regions are more definitely marked out, the central lymphoid core shows lobulation, with some lobules detached from the main mass. The presence of exhausted areas usually at the boundary zone between lymphoid core and spleen pulp is noticeable (fig. 51). The cells, themselves, appear more mature. The special eosinophils show a greater proportion of cytoplasm to nucleus (fig. 30), and the nucleus is more lobulated or polymorphous.

The eosinophils likewise show similar differences, though perhaps not quite so marked. Vacuolation may be exhibited

by both eosinophils and meta-eosinophils. There is more active erythrocyte differentiation in the spleen pulp. Russell-body cells (fig. 46) are more in evidence.

DISCUSSION

The foregoing observations on the hemocytopoietic organs and blood of the lungfish reveal a number of interesting features which are worthy of special discussion. The lungfish is particularly notable for the transitional position of the spleen from the phylogenetic viewpoint ; the general resemblance of its blood to that of urodeles ; the variety of lymphoid cell types in the spleen of the adult ; the number and variety of granulocytes, including meta-eosinophils ; the decided effects on the blood and hemocytopoietic organs of prolonged estivation and recovery.

Hemocytopoietic loci and the phylogeny of the spleen

In vertebrate evolution the spleen is represented in cyclostomes by scattered cords of hemocytopoietic tissue in the wall of the alimentary tube. Each cord has a central venous sinus region where erythrocytopoiesis takes place and a peripheral region where granulocytopoiesis occurs. This is the condition in the hagfish *Myxine* (Jordan and Speidel, '30). In another cyclostome, the lamprey, the hemocytopoietic tissue is somewhat more localized, and is aggregated into a typhlosole-like mass which projects into the dorsal wall of the intestine. The centrally located arteries are ensheathed by lymphoid tissues. Surrounding this is a region of pulp cords and sinuses, which may completely encircle the intestine. Granulocytes are also produced in this region.

In the lungfish a further advance is to be noted. The spleen is here a very definite organ, but it is nevertheless embedded in the wall of the stomach, being covered externally by the muscle of the gastro-intestinal wall. The rather sharp distinction between central lymphoid mass, intermediate splenic pulp, and peripheral cords has already been pointed out. While the spleen is to some extent a granulocytopoietic organ,

this function has been largely assumed by other regions, such as the granulocytopoietic organ of the intestine and the capsule of the kidneys and gonads.

A still further advance in spleen evolution is represented by the conditions in the other vertebrates in which the spleen becomes entirely separate from the alimentary tube wall. Thus the condition of the lungfish, with the spleen as a definite compact organ, but still embedded in the stomach wall, may be considered as a transitional stage between the scattered hemocytopoietic tissue of the cyclostomes in the gut wall and the compact spleen of other vertebrates entirely separated from the alimentary tube.

The lungfish with its primitive salamander-like lungs foreshadows the amphibian types. It is interesting to note that the blood of the lungfish bears marked resemblances to that of the salamander (compare Jordan and Speidel, '30). Although blood cells of most fishes are of small size, those of the lungfish are enormous, being of a size comparable to those of urodeles. Other resemblances that may be mentioned are the similarities in the erythrocytopoietic process, the various immature cells being much alike at similar stages; the occurrence in the general circulation of immature cells of the erythrocyte series; the similar degenerative types of erythrocytes; the similarities of the monocytes, basophils, eosinophils, and thrombocytes.

Erythrocyte amitosis in the lungfish also seems entirely similar to that described for *Necturus* (Charipper and Dawson, '28). Among the differences in the two forms may be mentioned the great variety of meta-eosinophils in the lungfish and the difference in locus of granulocytopoiesis; in the lungfish the alimentary tube wall, kidney, and gonad capsules, in the salamander the capsule of the liver.

The lymphocyte types and their hemocytopoietic capacity

The various types of lymphoid cells have already been described. These are of such variety and of such size in the lungfish that a study of their hemocytopoietic capacity is

much more readily made than in most animals. Special attention has therefore been paid to the early pro-erythroblasts and erythroblasts to obtain information as to their probable origin. It seems entirely clear that the usual erythrocyte mother cell is the large or medium-sized hemoblast with typical vesicular sieve-like nucleus containing one or more acidophilic plasmosomes. Both pro-erythroblasts and erythroblasts with nuclei of this type occur in large numbers. The plasmosomes often may be seen in late erythroblast stages. The nucleus of pro-erythroblast and erythroblast may sometimes be slightly lobulated, as may that of the hemoblast mother cell.

Lymphocytes which correspond to the small lymphocyte of higher vertebrates do not appear to give rise to erythrocytes. These cells have the pachychromatic type of nucleus with coarse chromatin blocks, cart-wheel arrangement, and very little cytoplasm. Pro-erythroblasts and erythroblasts never present nuclei of this sort. The fate of some of these cells is to undergo progressive degeneration (figs. 76 to 80). Others circulate in the blood stream as small lymphocytes. But a large number migrate into the splenic sinuses and there undergo transformation into thrombocytes. Transition stages are numerous between the vesicular-nucleus type of hemoblast and the coarse-granule nucleus types. The real problem is to what extent these transition stages may retain their original erythrocytopoietic capacity. Judging from the character of many of the earliest pro-erythroblast nuclei, it seems probable that these transitional forms may also give rise to erythrocytes.

The evidence is somewhat similar for the origin of granulocytes and monocytes. In each case the usual ancestral cell is the vesicular-nucleus type of hemoblast. However, the transitional lymphoid cell types can by no means be eliminated as potential granulocytes, monocytes, and thrombocytes.

An interesting feature of the lymphoid mass of the lungfish spleen is that it presents small hemoblasts with vesicular nucleus (i.e., small-sized large lymphocytes') and large lym-

phoid cells of the small lymphocyte type (i.e., 'large-sized small lymphocytes'). Small-sized hemoblasts like those of figures 60 and 61 were noted by Danchakoff ('16) in her experimental work on chick allantoic grafts. Such small hemoblasts were interpreted by her as resulting from intense mitotic activity on the part of larger hemoblasts.

In a recent study of the spleen in a number of fishes, including elasmobranchs, teleosts, and a single specimen of lungfish, *Calamoichthys*,² Yoffey ('29) concludes that the small round lymphoid cell is the progenitor of erythrocytes. An examination of his figures showing erythrocytopoiesis, however, does not seem to bear out this idea. Unfortunately, Yoffey makes no mention of thrombocytes in the spleen—cells which are sometimes confused with small lymphoid cells. His figure 4, plate 3, represents a cell, which he interprets as a transition form between a small lymphoid cell and an erythroblast. This cell to us, however, appears to be a fairly typical young thrombocyte. Characteristic features are the acidophilic cytoplasm faintly granulated, and the 'grooved' nucleus with chromatin drawn out somewhat into threads. We have seen many thrombocytes of this type in the spleen, both in lungfish (fig. 91) and in salamander. Furthermore, the cell of his figure 5, an early erythroblast, presents a nucleus which to us indicates a probable derivation from the large lymphoid cell with vesicular type of nucleus. Yoffey's series of figures begins with a cell containing coarse chromatin granules (fig. 1), which develops into a cell with finer granules and more vesicular nucleus (fig. 5), which then leads to a coarse-granule type (figs. 6, 7). The early part of this series seems questionable.

Seasonal and regional activity of the spleen

In animals which undergo periods of hibernation and reawakening, such as the frog, salamander, turtle, and horned toad, we have already pointed out that there occur cyclical or

² While *Calamoichthys* is sometimes classified with the lungfishes, it is perhaps more usually classified with the ganoid fishes.

seasonal hemocytopoietic changes. This holds true also for the lungfish, though in this case the torpid state is induced by lack of water rather than by cold.

Not only is there evidence of seasonal variations, but there is also evidence of regional activity in the spleen, followed sometimes by exhaustion and regeneration. In animal no. 2 the spleen is very active. Many small areas are visible in the spleen pulp zone, or in the boundary zone between central lymphoid mass and spleen pulp, from which practically all cells have been drawn with the exception of the reticulo-endothelial framework. Two of these areas may be seen in the low-power photograph (fig. 51), though the condition is much more striking with higher magnification.

In animal no. 21 the entire spleen appears relatively exhausted, as if very recently drained of its cellular content. This is somewhat like the splenic exhaustion induced in frog tadpoles by thyroid treatment, and in frogs by experimental anemia (Jordan and Speidel, '23 and '24). In some areas there is evidence of regenerative process, such as we have described for the horned toad (Jordan and Speidel, '29).

Significance of erythrocyte amitosis

The amitotic activity exhibited by the erythrocytes of lungfish no. 1 is so striking that it must be of significance. This animal, five days in water after two months in a small can of earth, is in an early stage of recovery from the torpid condition. Increased metabolic rate and respiratory rate place an added burden on the erythrocytes as oxygen-transporting agents. Multiplication of erythrocytes, therefore, would seem to be a natural physiological adjustment accompanying the change from torpid to active state. Not a single example of erythrocyte division by mitosis, however, was seen in the blood of this animal; only the amitotic type was in evidence. Contrasting sharply with this is the condition in normal well-fed lungfishes, nos. 33 and 34, in which erythrocyte mitoses were common and amitoses not to be seen.

Observations on salamanders are of interest in this connection. Mitosis of erythrocytes is the rule, both in normal animals and in splenectomized animals. However, Dawson ('28) has observed that erythrocyte amitosis occurs in *Necturus* blood plasma which has been allowed to stand for several days. Cases have also been found in normal *Necturus* blood.

These observations strongly suggest that amitosis represents a rapid response to a sudden change in environmental conditions. It is of the nature of a sudden emergency reaction. Its duration is short-lived. Mitosis represents the response to more gradual change ; it represents the method of normal replenishment, and of replenishment over a long period of time, as in year-old splenectomized salamanders. In general, also, amitosis appears in relatively mature cells ; mitosis, in less mature cells—a fact which fits in with the idea just stated. In sudden emergencies fully differentiated cells are in demand. In more gradual crises the younger less differentiated cells, which are easily susceptible to mitosis, are adequate to multiply, then differentiate in time to care for the deficiency created by the changed conditions.

Another interesting feature of animal no. 1, that should be mentioned in this connection, is that mitoses of young leucocytes are very common in the granulocytopoietic organ of the intestine. This contrasts sharply with the occurrence in this animal of erythrocyte amitosis only. The deficiency of leucocytes created by the return of the animal to water is not so critical as the deficiency of erythrocytes. Thus, the leucocyte deficiency is cared for by the normal granulocytopoietic regions with somewhat heightened mitotic activity.

In animal no. 21 a more critical leucocyte condition is presented than in animal no. 1. The long estivation period of 427 days, the thyroxin injection, and the infection after return to water are all contributing factors. Monocyte and granulocyte amitoses are here regarded as emergency reactions elicited by these factors. Granulocyte mitoses are also exhibited by this animal.

The granulocytes; senility and relation to pigment formation

In many ways the granulocytes in the lungfish are of exceptional interest. They are by all odds the most plentiful and of the greatest variety we have seen in any vertebrate. They arise from connective-tissue cells in many localities, and may also arise from hemoblasts. Furthermore, they degenerate in great numbers, accompanied by pigment formation and ingestion by macrophages. They also appear to play an important role during the estivation periods.

Senility in an eosinophil of the coarse-granule type may be exhibited in a variety of ways. In some cells the granules become progressively less eosinophilic, staining various shades of light red or pink, or orange or yellow, and finally become almost colorless. The nucleus at the same time takes the stain less and less. Or the granules may be replaced by (possibly transformed into) pigment granules. A few cells containing both eosinophil granules and pigment granules have been observed both in the lungfish and in the salamander. In general, however, pigment granules are formed after phagocytosis by macrophages. Or, the granules may become atypically basophilic. A number of eosinophils have been seen in the granulocytopoietic organs which contain granules showing various degrees of basophily. This color reaction does not appear to be quite a normal basophilic color. The occasional presence also in these cells of colorless granules suggests that they are about to degenerate. Similar cells have been noted in the thymus of the turtle (Jordan and Speidel, '28) and also in the spleen.

Still another way in which granulocytes may become senile is by method of cytoplasmic segmentation or nuclear extrusion. Examples of large cytoplasmic fragments have been noted in the circulation; also a few nuclei somewhat degenerate with small cytoplasmic fragments still attached which show their eosinophil-granule nature. Free red granules from eosinophils are commonly to be seen in the spleen and other regions.

Senility of the special eosinophils is also easily followed. There is here, too, a tendency for the granules to become colorless in old worn-out cells. Pigment formation was not seen in these except after ingestion by macrophages. Whether the plentiful occurrence of degenerating granulocytes in the estivating lungfish means that these cells have a specific relation to this process is not clear.

The ingestion of degenerating eosinophils by monocytes or macrophages is very conspicuous in spleen, kidney, intestine, and also may occur in the capsule of the gonad, in the liver, lungs, gills, and elsewhere. In the fish subjected to a year of estivation it is even more evident. The nuclear and cytoplasmic debris is broken down in the macrophage and transformed into pigment, at first a golden brown or yellow, then later a deep brown or black. The nuclear material may be immediately transformed into the deeper shades of pigment. In the kidney and liver, excretion of pigment, presumably partly eosinophil pigment, takes place. In the spleen there is also ingestion of free eosinophil granules by the cells of the reticulo-endothelial system.

Granulocytes and fat metabolism

That the granulocytes in the lungfish may be of special importance in relation to fat metabolism seems probable. Unfortunately, living specimens were not available for tests on this question. However, the Nile-blue-sulphate treatment shows in salamanders and in frog tadpoles that the eosinophils are loaded with neutral fat or fatty acid in the form of granules. These granules appear to be the same as the eosinophilic granules. Ready intracellular transportation of fat is thus assured.

The various colors of eosinophil and meta-eosinophil granules in the lungfish, as brought out by the eosin-azure stain, may depend largely on the state of the fatty material; i.e., whether it is in the form of neutral fat, or saturated or unsaturated fatty acid. An excellent comparison of the staining reactions of yolk granules is afforded by our sections of lung-

fish fry. In a two-months-old specimen there is a great deal of yolk material present. This is particularly prominent in sections near the caudal part of the body, but may also be noted in liver and notochord. The yolk granules stain various shades of pink, red, green, purple, yellow, and some may even be colorless. These shades of staining reaction are somewhat comparable to those of the granules of the granulocytes, especially the meta-eosinophils. It is probable that the granules of these granulocytes contain chemical compounds much like those of the yolk granules.

We are thus inclined to interpret the rich variety of granulocytes as being related to the estivation habit, the granules being partly composed of fat or fatty materials and affording an intracellular mechanism for fat transportation.

An interesting point concerning granulocytes is that neither the eosinophil nor the meta-eosinophil is present in the two-months-old specimen. All eosinophils appear to be of the special eosinophil variety containing only the fine granules. Apparently the time factor is of importance. That is, the large granules are formed by the accumulative process from smaller ones. Coalescence has often been seen. In the fry not enough time has elapsed for the coarse-granule formation. In the older specimens subjected to estivation coarse granulocytes of all varieties are numerous.

Another hypothesis suggests itself in this connection. In the fry there is still a plentiful amount of granular yolk material from the egg. Correlated with its disappearance is the appearance of the eosinophils and meta-eosinophils. These cells in the adult may play a role similar to that played by the yolk granules in the fry; i.e., to furnish a supply of partly formed material (fats, fatty acids, and other compounds) available for the metabolic needs of the body. In other vertebrates, likewise, the eosinophils make a relatively late appearance; e.g., in the chick Sugiyama ('26) states that they appear only in the last few days of incubation.

Monocytes and macrophages

The lungfishes subjected to dry estivation followed by return to water have proved to be excellent for the study of monocytes. All stages in development of these cells are found in the circulation. The two kinds of monocytes with respect to presence or absence of reddish granulation of the cytoplasm occur. Also, numerous definite transition stages indicate the further development of the monocyte into a full-fledged macrophage. Clark and Clark ('30) have recently reported such transition by direct observation on living frog tadpoles.

The significance of the large number of monocytes and macrophages in the circulation of our estivation specimens is not entirely clear. It may be merely a reaction to superficial infection which was obvious in some animals. Or it may represent a reaction elicited by the large number of degenerating granulocytes, both of the coarse eosinophilic and special varieties. This latter idea is probably correct, as no infection was observed in some animals. However, since our estivating specimens are few in number, any conclusion is necessarily uncertain.

Thrombocytes

The observation that the small lymphocyte in the lungfish is the usual mother cell for the thrombocyte contrasts with the condition in the salamander, in which the usual mother cell is the large hemoblast. This condition is strikingly reflected in the splenic lymphoid cell content in the two animals: the small lymphocyte predominating in the lungfish, the large lymphocyte in the salamander. Fundamentally, this merely means that in the lungfish, nuclear differentiation (i.e., development of coarse-granule chromatin and lobulated or grooved nucleus) may precede the appearance of the acidophilic granules in the cytoplasm. In the salamander the acidophilic granulation may appear before the nucleus differentiates or changes from the primitive hemoblast type. Another point of interest is that the small spherical type of

thrombocyte predominates in the lungfish, whereas the large-sized elongated type is quite numerous in the salamander. This difference may be correlated with the type of progenitor in each case.

Probably not enough emphasis has been placed on the two types of thrombocytes in vertebrates. As has been indicated, the lungfish presents a small spherical or oval type of cell and an elongated or spindle type. Similar types have been observed in the frog, in salamander and several other *urodeles*, and in cyclostomes. We have not been certain as to the relative age of the two types. In general, the small spherical condensed type seems a bit more highly differentiated. But it does not necessarily pass through the elongated stage in its development. The clumping tendency is exhibited to a greater degree by the small condensed type. This type also shows most clearly the delicate outer zone of cytoplasm which fuses readily in clumping.

The condition in the hagfish is of great interest in this connection. We have pointed out that in this primitive form a rather unique spindle-shaped cell is present in large numbers, besides a typical thrombocyte of the spherical condensed kind (Jordan and Speidel, '30). The former type displays relatively little clumping tendency, and it is quite doubtful whether it should be classified as a thrombocyte. However, it must greatly aid the clotting process by its mere structure with elongated processes. This type greatly outnumbers the clumping type of spherical thrombocyte. In the lungfish and salamander the two kinds are equally conspicuous. In frogs the clumping type is more numerous, and in higher vertebrates it is still more so. Thrombocyte evolution thus appears to present a progressive numerical decrease in the elongate type of 'morphological thrombocyte' which aids clotting by its structure ; and progressive increase in the spherical condensed type of 'physiological thrombocyte,' which aids clotting by the release of substances which take part in fibrin formation.

Double origin of pigment cells

Observations on the lungfish emphasize once more the possible double origin of pigment cells. That many of the pigment cells are formed directly from their mesenchymal ancestors is clear, and some derive their pigment directly from the egg. The degeneration of eosinophils gives another possible origin of pigment cells. In fact, some cells have been seen which contain eosinophil granules and pigment granules with no particular evidence of degenerative change.

Much of the pigment in spleen, kidney capsule, gonad capsule, alimentary tube, and other loci is the result of granulocyte degeneration. Macrophages may or may not assist in the production of this pigment.

This process of eosinophil degeneration into pigment which is so pronounced in lungfish is now recognized as also being present in many other forms we have studied, such as frog, turtle, and salamander. It is probable that the pigmented debris of this sort so prominent in hemocytopoietic areas has often been wrongly interpreted as products of erythrocyte degeneration.

SUMMARY

The blood cells of the African lungfish, *Protopterus ethiopicus*, are very large and in many respects resemble those of urodeles. Leucocytes are especially plentiful and rich in variety, including eosinophils, special eosinophils, 'meta-eosinophils,' monocytes, thrombocytes, lymphocytes, and basophils.

The chief hemocytopoietic organs are the spleen, kidneys, and intestine. Erythrocytes are formed in the splenic pulp, the usual mother cell being the lymphoid hemoblast with vesicular, sieve-like nucleus. Eosinophilic and special eosinophilic granulocytes are formed in the granulocytopoietic organ of the spiral-valve region of the intestine, to a less extent in other loci, such as the capsule of kidneys, gonads, and spleen. Meta-eosinophils with granules showing atypical staining reactions are also formed in the granulocyto-

poietic areas. These are thought to be related to fat metabolism, which becomes of special importance in the lungfish in relation to estivation.

Lymphocytes of all types arise in the spleen. Intravascularly in the splenic sinuses, thrombocytes are differentiated from the smaller lymphocytes. Monocytes are differentiated in the spleen and general circulation. Basophils are formed in the spleen and intestinal wall. 'Russell-body' cells are conspicuous in the spleen.

Macrophages and cells of the reticulo-endothelial system are especially active in the ingestion and disposal of senile granulocytes, particularly in the spleen, kidneys, and intestine. Elimination of this *débris* largely in the form of pigment appears to be by way of the kidneys, intestine, and liver.

In fishes subjected to long periods of dry estivation, the granulocytes become more prominent throughout the tissues and in the circulation. Many undergo degeneration. Erythrocytopoiesis practically ceases. Senile erythrocytes often present the appearance of intracellular nuclear degeneration.

Fishes replaced in water after estivation may exhibit general recovery of the hemocytopoietic tissues. Erythrocyte amitoses may become very numerous. Amitosis has also been observed in the case of thrombocytes, granulocytes, monocytes, and lymphoid hemoblasts.

The spleen of the lungfish, embedded in the wall of the stomach internal to the muscular layers, represents an intermediate phylogenetic stage between the disperse intra-enteral type of the hagfish and the aggregate extra-enteral type of other vertebrates.

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PLATE 1'

EXPLANATION OF FIGURES

Figures 1 to 22 represent cells from blood smears (Wright's stain); 23 to 46 represent cells from sections (azure II-eosin stain). The cells in figures 1 to 7, 9 to 11, 17, 20, 39, and 45 are from lungfish no. 21; 8, 12 to 16, 18, 19, 21 to 29, 31 to 35, 40 to 42, and 44 are from lungfish no. 1; 30, 36 to 38, and 46 are from lungfish no. 2; 43 is from lungfish no. 8. While all cells have been drawn to the same scale, some differences in size are accounted for by differences in the degree of spreading of cells in the smear preparations. X 750.

1 Pro-erythroblast. An early stage in the erythrocyte series. It may be distinguished from the hemoblast by the characteristic deep blue nucleus with granulated appearance. The cytoplasm is usually non-homogeneous, often faintly granular. The nucleus of the lymphoid hemoblast is more purple or light blue (compare figs. 7, 11, 17).

2 Transition stage between pro-erythroblast and erythroblast. Condensation of the nucleus is apparent.

3 Erythroblast with obvious hemoglobin production. Further contraction of nucleus.

4 Mature erythrocyte.

5 Erythrocyte showing 'intracellular nuclear degeneration.' The nucleus presents a typical inflated appearance.

6 Erythrocyte showing nuclear fragmentation. The granules represent chromosome remains, degeneration having taken place during mitosis.

7 Lymphoid hemoblast. The nucleus presents a purplish tinge.

8 Monoocyte with typical archoplasmic area near nuclear indentation and reddish granulation throughout the cytoplasm, but most conspicuous about the archoplasmic area.

9 Monocyte (or macrophage) showing no reddish granulation or archoplasmic area. Cytoplasmic vacuoles and processes are conspicuous. This is a more highly differentiated cell than that of figure 8.

10 Macrophage with ingested material, some of it representing eosinophil debris.

11 Lymphoid hemoblast. The nucleus stains a light blue.

12 Thromboblast with mixed reddish and bluish granules in the cytoplasm. 'Grooving' of the nucleus is slightly indicated in two places.

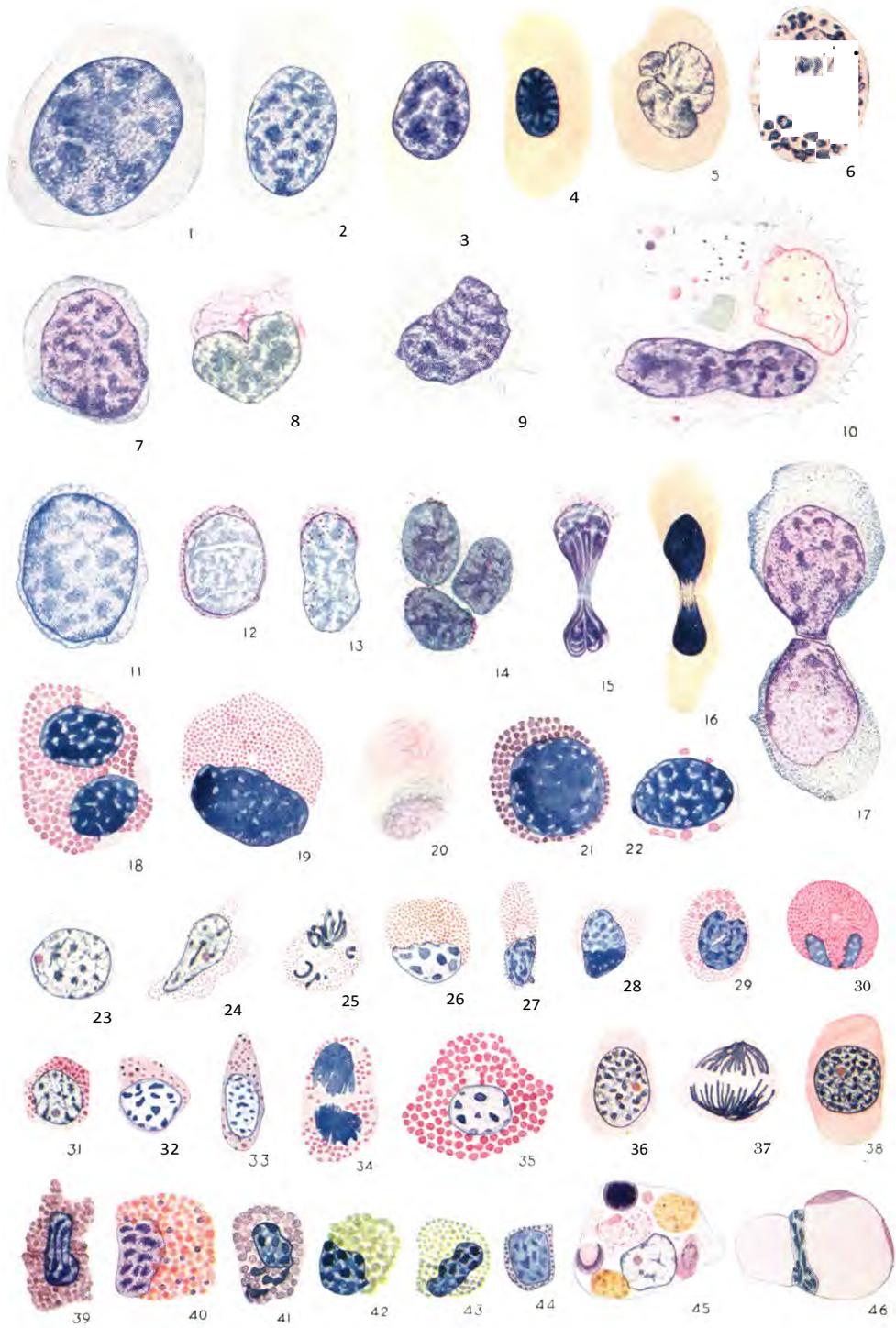
13 Elongated thromboblast.

14 Mature thromboocytes, showing the characteristic clumping reaction with fusion of the delicate pale blue peripheral cytoplasm. The reddish granulation is apparent in one cell at both poles, in another at only one pole, and in the third it is absent entirely.

15 Thromboblast in amitosis. The lower half of the nucleus shows characteristic surface grooves, and the cytoplasm exhibits the thromboblast type of mixed blue and red granulation.

Many of the figures of plates 1 and 4 were drawn by Alice Clark Mullen.

- 16 Erythrocyte in amitosis. The cell-plate region indicates definite separation between the daughter cells.
- 17 Lymphoid hemoblast in amitosis. This cell may also be interpreted as an early transitional stage from a lymphoid hemoblast toward a young monocyte.
- 18 Eosinophil.
- 19 Special eosinophil.
- 20 Senile special eosinophil.
- 21 Basophil with coarse granules.
- 22 Small lymphoid cell with acidophilic inclusions.
- 23 Hemoblast with vesicular sieve-like type of nucleus, containing acidophilic plasmosome (taken from granulocytopoietic organ of the intestine).
- 24 Young special eosinophil (granulocytopoietic organ of intestine).
- 25 Young special eosinophil in mitosis (granulocytopoietic organ of intestine).
- 26 to 28 Young special eosinophils from spleen. Condensation of nucleus, with sometimes some extrusion of nuclear material, occurs.
- 29 Special eosinophil containing some coarse as well as fine granules. The nucleus is of the ring type.
- 30 Mature special eosinophil from large lungfish, showing small nucleocytoplasmic ratio. An arehoplasmic area is visible.
- 31 Young eosinophil with typical vesicular nucleus, showing acidophilic plasmosome (granulocytopoietic organ of intestine).
- 32 and 33 Young eosinophils containing mixed blue and red granules (splenic capsule).
- 34 Young eosinophil in mitosis.
- 35 Young eosinophil with full-sized granules. An arehoplasmic area is visible.
- 36 Erythroblast (splenic sinus).
- 37 Erythroblast in mitosis (splenic sinus).
- 38 Later erythroblast with acidophilic plasmosome still persisting.
- 39 Meta-eosinophil in amitosis (spleen).
- 40 Meta-eosinophil with mixed red and blue granules (granulocytopoietic organ of estivating lungfish).
- 41 Meta-eosinophil with coarse purple granules. Nuclear extrusion is visible (splenic capsule).
- 42 Meta-eosinophil with coarse green granules (splenic capsule).
- 43 Meta-eosinophil with fine green granules (splenic capsule).
- 44 Young basophil (spleen).
- 45 Macrophage with ingested materials. The nucleus is of the hemoblast type with acidophilic plasmosome and vesicular appearance.
- 46 'Russell-body' cell (spleen).





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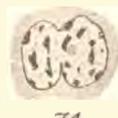
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PLATE 3

EXPLANATION OF FIGURES

All of the cells in this plate were taken from the spleen of animal no. 2, and drawn to the same magnification, X 1000. Figures 52 to 63 show the variations in types of lymphoid cells. Figures 64 to 75 are given to show how erythroblasts may vary in somewhat the same manner. Figures 76 to 80 indicate a common fate of the small lymphocytes with the coarse-granule type of nucleus.

52 Large hemoblast with vesicular sieve-like nucleus (**amblychromatic** nucleus). **Acidophilic** plasmosome.

53 and 54 Transitional forms between cells of figures 52 and 55.

55 Large hemoblast with small lymphocyte-like type of nucleus, the chromatin granules being large and numerous (**pachychromatic** nucleus) and showing radial arrangement. **Acidophilic** plasmosome.

56 Medium-sized hemoblast with vesicular (**amblychromatic**) nucleus. **Acidophilic** plasmosome.

57 and 58 Transitional medium-sized hemoblasts between the cells of figures 56 and 59. **Acidophilic** plasmosome.

59 Medium-sized hemoblast with small lymphocyte type of nucleus (**pachychromatic** nucleus). **Acidophilic** plasmosome.

60 Small-sized hemoblast with vesicular type of nucleus (**amblychromatic** nucleus). **Acidophilic** plasmosome.

61 and 62 Transitional forms between cells of figures 60 and 63.

63 Small-sized lymphocyte (**pachychromatic** nucleus) with coarse chromatin granules radially arranged ('cart-wheel' type).

64 Vesicular lymphoid hemoblast.

65 Vesicular hemoblast in early prophase of mitosis.

66 Pro-erythroblast showing typical vesicular-type nucleus.

67 Erythroblast showing typical vesicular-type nucleus. This cell should be compared with the erythroblasts of figures 71 and 75.

68 and 69 Hemoblasts of transitional type.

70 Early erythroblast with nucleus somewhat similar to those of figures 68 and 69.

71 Later erythroblast.

72 Hemoblast with grooved or lobulated nucleus.

73 Pro-erythroblast with grooved or lobulated nucleus.

74 Young erythroblast with grooved or lobulated nucleus.

75 Erythroblast with grooved nucleus. The **acidophilic** plasmosome is still present.

76 Typical small lymphocyte with coarse chromatin granules radially arranged.

77 Lobulated small lymphocyte. A small **acidophilic** plasmosome is present.

78 Small lymphocyte showing early degeneration of the nucleus.

79 Degenerating small lymphocyte.

80 Late stage of degeneration of small lymphocyte.

PLATE 4

EXPLANATION OF FIGURES

Figures 81 to 92 represent cells from the spleen of lungfish no. 2; 93 to 98 are from blood smears of the general circulation of lungfish no. 1; 99 and 100 represent kidney tubules from animals nos. 8 and 21. X 1000.

81 Reticular cell from the reticular syncytium of the spleen. The vesicular sieve-like nucleus with plasmosome bears a marked resemblance to that of the hemoblast.

82 Reticular cell with fibers showing in the cytoplasm.

83 Hemoblast.

84 Reticular cell containing acidophilic granules in the cytoplasm.

85 Reticulo-endothelial cell containing acidophilic granules in the cytoplasm.

86 Endothelial cell lining a vein sinus. Most of these cells show the acidophilic granules in the cytoplasm.

87 to 89 Stages suggesting amitosis of small lymphocytes in the spleen.

90 Lymphocyte with nucleus of the pachychromatic type, for comparison with the thrombocytes of figures 91 and 92.

91 Thrombocyte of the spherical type. This type of cell is easily confused with lymphocytes in sections. The cytoplasm, however, stains less deeply and in well-differentiated material faint acidophilic granulation may be present. Grooving of the nucleus may often be seen.

92 Thrombocyte of the elongated spindle type. This cell, if seen in transverse section, might be mistaken for a small lymphocyte.

93 Early stage of amitosis in an erythrocyte. The nucleus shows elongation and a transverse groove near the middle. This middle area appears less dense. The cytoplasm shows no elongation or constriction.

94 Early stage of erythrocyte amitosis, slightly later than that of figure 93. The nuclear halves show definite separation, leaving a central light transverse area. The cytoplasm shows constriction near the middle.

95 Erythrocyte amitosis; later stage than that of figure 94.

96 and 97 Successively later stages in erythrocyte amitosis. Compare also with figure 16.

98 Erythrocyte showing the 'amitotic urge,' the nucleus not, however, being involved. This results in cytoplasmic segmentation.

99 Kidney tubule from animal no. 8 after dry estivation for one year. Three large masses of pigment granules are shown just outside the tubule. This is largely debris of granulocytes. Deeply pigmented granules are also in process of excretion by the kidney tubule cells. Compare with figure 100.

100 Kidney tubule from animal no. 21, showing conditions after 427 days of dry estivation followed by replacement in water for ten days. The kidney tubule cells are vacuolated and in places show light tan pigment granules. The whole appearance of this kidney suggests active excretion. Most of the deeply pigmented debris, such as shown in figure 99, has apparently been eliminated.

