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A PHYLOGENETICAL SURVEY OF HEMOCYTOPOIETIC TISSUES IN SUBMAMMALIAN VERTEBRATES,

WITH SPECIAL REFERENCE TO THE DIFFERENTIATION OF THE LYMPHOCYTE AND LYMPHOID TISSUE

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INTRODUCTION

Comparative survey of blood cells and blood-forming tissues in lower vertebrates has attracted much interest of many investigators. Innumerable work in this field of hematology have been reviewed by *Maximow* (1927), *Jordan* (1938), and *Amano* (1948). In a survey of the literature we realize that most of previous investigators devoted their effects chiefly to cytological observations of blood cells, little attention being paid to the histological study of blood-forming tissues. The monophyletic theory of *Maximow* and *Jordan* on the origin of blood cells, especially that of the latter researcher, that the lymphocytes are identical with the primitive blood cells and may differentiate under proper stimulation into any other type of blood cells, either erythrocytes or thrombocytes, granulocytes or monocytes, is chiefly based on the cytological observations of blood cells from the phylogenetical viewpoint. This view appears to be rather widely accepted by other investigators who dealt with blood cells of lower vertebrates (*Yoffey*, 1928–9; *Dawson*, 1932; *Duthie*, 1939; *Cotton*, 1951; and many others).

However, the monophyletic concept concerning the interrelationship of blood cells seems to contradict with the following observations. It must first be noticed that, according to this theory, the hemocytoblast or larger lymphocyte is the first blood cell to develop. Nevertheless, in the course not only of ontogenesis but also of phylogenesis the differentiation of the genuine lymphocyte and lymphoid tissue is considerably delayed as compared with the differentiation of myeloid cells and myeloid tissues. *Amano* (1949) demonstrated in his ontogenetical studies of the early development of blood cells in chickens by supravital technique that the first blood cells, which develop at about 7-somite stage and were called hemocytoblasts or large lymphocytes by *Maximow* and *Jordan*, are not true

lymphocytes but belong to the erythroid series of cells (megaloblasts or primitive erythroblasts), because most of them are tinged with yellowish color, a sign of the presence of a small amount of hemoglobin. He further showed that true lymphocytes appear in the blood at 2 weeks of incubation, coincident with the appearance of lymphoid tissue in the embryo.

Our observations of blood-forming tissues in lower vertebrates disclosed that the first blood-forming tissue to develop is the granulocytopoietic center in the spiral valve of intestine of cyclostomes, the lymphocytopoietic tissue develops for the first time in the spleen and intestine of elasmobranchs, and the erythrocytopoietic tissue appears to differentiate in the spleen as late as at the level of urodeles. It was further revealed that at the level of urodeles the three blood-forming tissues, namely the granulocytopoietic, the lymphocytopoietic and the erythrocytopoietic make their appearance in different areas, quite widely separated from one another. These observations indicate that in lower vertebrates erythropoiesis, granulopoiesis and lymphopoiesis take place quite independently without any mutual interrelationship.

The above-mentioned sequence of differentiation or segregation of blood-forming tissues at successive evolutional levels seems to be of particular importance as casting light upon the problem of interrelationship of blood cells. The descriptions by the previous investigators, who stood on the monophyletic viewpoint as regards the origin of blood cells, cannot be regarded as reliable, since they confused genuine lymphocytes with the ancestral cells of other series and often failed to distinguish lymphocytopoietic from other blood-forming tissues. Some authors such as *Drezewina* (1905) and *Downey* (1909) referred to all sorts of blood-forming tissues as lymphiod. Keeping these facts in mind, the author intended in the present research to re-investigate the evolutional history of blood cells and blood-forming tissues, with special emphasis on the differentiation of the lymphocyte and lymphoid tissue.

The work was carried out in the laboratory of Professor Bunsuke Osogoc and constitutes a part of his extensive study of the lymphocyte and lymphoid tissue. I wish to express my thanks to him for constant guidance in the course of the research.

MATERIALS AND METHODS

As material the following different classes of vertebrates were used. The majority of the animals were collected and fixed for study during the period from April to August. Some material, e.g., frogs and newts, were collected at different seasons in order to follow seasonal variations in blood formation.

I. Cyclostomes

1. Entosphenus japonicus (5)
2. Lampetra planeri (10)
3. Ammocoetes branchialis (the larval stage of Lampetra) (30)

II. Fishes

A. Elasmobranchs

1. Mustelus manazo (5)
2. Dasyatis akajei (sting-ray) (5)

B. Teleosts

1. Cyprinus carpio (carp) (5)
2. Carassius auratus (crusian carp) (5)
3. Goldfish (5)
4. Mugil cephalus (gray mullet) (5)
5. Seriala quinqueradiate (yellow-tail) (3)
6. Scomber japonicus (mackerel) (5)
7. Lateolabrax japonicus (perch) (3)
8. Sebastodes güntherii (3)
9. Chasmichthys dolichognatus (goby) (5)
10. Spheriodes pardalis (swellfish) (5)

III. Amphibians

A. Urodeles

1. Megalobatrachus japonicus (giant salamander) (5)
2. Triturus pyrrhogaster (newt) (20)
3. Hynobius nigrescens (3)
4. Hynobius lichenatus (3)
5. Hynobius tokyoensis (5)
6. Hynobius dunni (3)

B. Anurans

1. Bufo vulgaris japonicus (toad) (10)
2. Rana nigromaculata (frog) (10)
3. Rana japonica Guenther (5)
4. Rana catesbina Show (bullfrog) (5)

IV. Reptiles

1. Elaphe quadrovirgata (stripted snake) (5)
2. Eumeces latisculata (lizard) (5)
3. Clemmys japonica (tortoise) (5)
4. Amyda japonica (soft-shelled turtle) (3)
5. Alligator sinensis (2)

V. Birds

1. Domestic fowl (White Leghorn) (100)
2. Anas phatyrhynchas domestica (duck) (10)

3. Coturnix coturnix japonica (quail) (10)
4. Columba liva domestica (pigeon) (10)
5. Passer montanus saturatus (sparrow) (15)
6. Milvus lineatus lineatus (kite) (3)
7. Corvus coronoides japonicus (crow) (2)
8. Sallus aquaticus indicus (water-rail) (1)

The blood samples were taken from the heart, tail or from the subcutaneous vein, and smear preparations were made. After the blood examination, each animal was sacrificed by exanguination, and stamp preparations and sections were made from various organs, chiefly those which were suspected to contain blood-forming loci. The tissues were fixed in 10% formol or Zenker-formol and embedded in paraffin or celloidin. The blood smears as well as the stamp preparations of organs were stained with *Giemsa* or *May-Giemsa*, and the tissue sections with *Mayer*'s acid hemalum and eosin or azur II.

I. CYCLOSTOMES

Blood cells.

In the blood of cyclostomes the following five types of blood cells are to be readily distinguished: erythrocytes, special granulocytes (neutrophil), eosinophiles, thrombocytes, and lymphoid cells. The following descriptions are chiefly based on observations of blood of the larval lamprey, Ammocoetes branchialis.

The erythrocyte, much larger than that of higher vertebrates (about 30μ in length and 20μ in width), assumes the shape of an ellipsoidal disk and contains an eccentric oval dense nucleus. Special granulocytes are much smaller cells having a bean-shaped or variously polylobulated nucleus.

The cytoplasmic granules of these cells are fine and appear to be staining either purple or reddish (amphophil). Eosinophiles are seldom found in blood smears, but they occur more numerously in sections of the blood-forming tissue. The granules are much coarser than those of the speccial granulocytes.

In the blood of cyclostomes thombocytes appear in relatively large numbers. In most instances the nucleus of the thrombocyte assumes the shape of a spindle cell, but it may take spherical or ellipsoidal form. These cells exhibit a marked tendency to clump in groups. The chromatin granules are fairly coarse and usually elongated in the direction of the long axis of the nucleus. The cytoplasm is very scanty, being confined to both poles of the nucleus, and contains fine reddish granules which are clustered at one or both poles of the nucleus. The above-mentioned features may be considered as characteristic of the thrombocyte since these are also found in thrombocytes of higher vertebrates.

In the blood of cyclostomes, lymphoid cells possessing a nucleus composed of

dense aggregation of chromatin with scanty basophilic cytoplasm, which correspond to the mammalian small lymphocytes, are not discernible, but those having a large spheroidal nucleus with delicate chromatin net work and surrounded by fairly abundant basophhilic cytoplasma, that is, the cells which are identical with the lymphoid hemoblasts of *Jordan*, are often met with. Since, however, at the evolutional level of cyclotomes lymphocytopoietic tissue is not yet differentiated, as will be mentioned later, these cells can hardly be regarded as genuine lymphocytes. It seems more reasonable to consider them to be ancestral cells of other blood corpuscles, especially of erythroid series.

Blood-forming tissue.

In the ammocoetes stage of Lampetra the spiral valve of the intestine is almost completely filled with hemopoietic tissue, which represents the chief site of blood-cell formation (Figs. 1–3). In the adult stage, however, the spiral valve becomes shrunk to a large extent, or is substituted entirely by adipose tissue, and its hemopoietic tissue has practically disappeared (Figs. 4–5).

The blood-forming tissue in the spiral valve of larval Lampetra is composed of agranular and granular cells densely packed in the meshes of reticular stroma (Fig. 6). A longitudinal artery runs through the axis of this tissue. This artery gives off numerous radial capillaries which connect with capilliform venous sinusoids. These sinusoids are enveloped by blood-forming tissue. The fundamental structure of the spiral valve thus closely resembles that of mammlian spleen. For this reason *Jordan* (1930) and *Osogoe* (1954) claim that the spiral valve of the larval lamprey constitutes an initial step in the evolution of spleen.

The chief cellular elements of this tissue are, as noted above, agranular and granular cells, the latter far predominating over the former. The agranular cells are identical with the so-called lymphoid cells. Among them larger and smaller forms can be distinguished. The larger forms possess a round vesicular nucleus with basophilic cytoplasm and may be regarded as immature forms. These forms appear preferentially in the central zones of the spiral valve near the longitudinal artery, either singly or in clusters. The smaller forms are cells having a round pachychromatic nucleus with very scanty cytoplasm like mammalian lymphocytes. The latter forms occur predominantly in the peripheral parts of the spiral valve, especially in the proximity of the venous sinusoids. These cells often appear inside or outside of the sinusoids and are arranged in a line along the endothelial wall (Fig. 7). In other portions they are rather diffusely scattered and do not show any tendency to form dense aggregations, a tendency characteristic of genuine lymphocytes of higher vertebrates. Therefore, the smaller lymphoid cells can hardly be regarded as true lymphocytes. It seems likely that these cells belong to the thrombcytic series, probably thromboblasts, since no other sources of supply of blood thrombocytes are to be seen elsewhere. No inter-

mediate forms between the larger or smaller celles and the erythrocytes were recognized. Ancestral cells of erythrocytes comparable to the erythroblasts of higher vertebrates were not seen in the hemocytopoietic tissue of the spiral valve.

The granular cells constitute the major elements of the blood-forming tissue of the spiral valve. The overwhelming majority of these cells are developing special granulocytes possessing fine amphophil granules in their cytoplasm (Fig.8). Eosinophiles also appear in relatively large numbers, most of which being scattered singly. Basophil granulocytes or mast cells were not met with in this tissue. The immature granulocytes are quite similar to the myelocytes of higher vertebrates. Successive stages of maturation of these cells comparable to the promyelocytes, metamyelocytes and mature granulocytes with variously polylobulated nuclei were to be seen. Mitosis of these cells were numerously found. It seems evident therefore that the blood-forming tissue of the spiral valve chiefly concerns with granulocytopoiesis. In short, it can be said that in the cyclostomes only granulocytopoitic tissue is differentiated.

II. FISHES

A. Elasmobranchs

Blood cells.

In the blood of elasmobranchs the following seven types of blood cells are to be discriminated: erythrocytes, special granulocytes, eosinophiles, basophiles, thrombocytes, and lymphoid cells. The morphologic features of these cells are the same for both Mustelus and the sting-ray. The result of differential counts of blood cells in the sting-ray is given in Table I.

TABLE I.

Differential count of blood cells in blood smears of the sting-ray.

Erythroblasts	7.1%
Thromboblasts	3.5
Lymphoid cells	51.4
Pseudoeosinophiles	34.7
Eosinophiles	3.0
Basophiles	0.3

In the blood of the sting-ray erythroblasts appear in relatively large numbers (see Table I). The cytoplasm of these cells is either basophilic or polychromatic. The basophilic erythroblasts are characterized by the coarse nuclear structure, often assuming a wheel-spoke arrangement, and by the "thick" looking appearance of the basphilic cytoplasm. The red cell assumes the shape of an ellipsoidal disc, having a round or oval dense nucleus in the center. The erythroblasts and

red cells are often in mitosis. The special granulocytes contain fine pseudo-eosinophilic granules in their cytoplasm. The eosinophiles and basophiles contain coarse granules and are similar both in appearance and in stainability to those of mammals.

Thrombocytes also appear in fairly large numbers in the blood of the sting-ray. The nucleus of these cells does not assume the spindle shape but is elongated to a more or lesser degree, and the coarse chromatin granules are extended in the direction of the long axis of the nucleus. The cytoplasm is very scanty and usually contains extremely fine reddish granules which are clustered at one or both poles of the nucleus. These cells exhibit a marked tendency to clump in groups. The features noted above are characteristic of the thrombocyte throughout the entire submammalian vertebrates.

Lymphoid cells appear numerously in the blood of the sting-ray. The nucleus of these cells is usually spherical and generally characterized by the coarse nuclear structure with a sharply outlined, thick nuclear membrane. The cytoplasma is slightly basophilic, and very scanty in the smaller forms but may be abundant in the larger forms. The large majority of these cells contain no cytoplasmic granules, but the remainder possess either fine or coarse reddish granules or sometimes both (Fig. 9). The proportions of three types of lymphoid cells in the blood of sting-ray are shown in Table II.

TABLE II.

Differential count of lymphoid cells in blood smears of the sting-ray.

Type I, without granules	36.6%
Type II, with fine granules	9.4
Type III, with globular granules	4.1
Type IV, with both granules	1.3

The lymphoid cells without cytoplasmic granules may be regarded as true lymphocytes, because in the sting-ray and Mustelus the lymphocytopoietic tissue is clearly differentiated in the spleen as well as in the mucous membrane of the intestine, as will be described later. The lymphoid cells with fine or coarse cytoplasmic granules are thought to be of the thrombocytic series, probably thromboblasts, since the cytoplasmic granules are similar in stainability to those of the thrombocyte.

Granulocytopoietic foci.

In both Mustelus and the sting-ray, chief granulocytopoietic tissues are found in the interstitial spaces of the ovaries and testes as well as in the *Leydig*'s organ of the esophagus. The gonads of elasmobranchs contain a very extensive myeloid tissue composed essentially of developing granulocytes densely packed in

the meshes of the reticular stroma. The *Leydig*'s organ consists of ventral and dorsal longitudinal folds of the mucous membrane of the esophagus which are almost completely filled with granulocytopoietic tissue similar to that of the gonads (Fig. 10). In these myeloid tissues numerous capilliform sinusoids are embedded.

The predominating cellular element of the myeloid tissues is the pseudo-eosinophil or special leucocyte at various stage of development. There occur also eosinophiles with coarse granules in relatively large numbers. Numerous mitosis of granulocytes, especially half-mature forms, are usually seen. A few large lymphoid cells possessing vesicular nucleus and basophilic cytoplasm without granules appear scattered among granular cells. These cells are considered to be ancestral cells of granulocytes (myeloblasts), since intermediate forms between these cells and immature granulocytes are seen.

Although the hemopoietic tissues in the gonads and in *Leydig*'s organs consist essentially of developing granulocytes, yet they also contain small lymphoid cells, thrombocytes and erythroblasts in lesser numbers. Small lymphoid cells occur usually aggregated as either small or relatively large dense clusters in some portions of the myeloid tissue, being fairly well demarcated from the surrounding tissue. Thrombocytes and erythroblasts appear scattered singly within the sinusoids in much smaller numbers.

The spleen of elasmobranchs is claimed by *Jordan* (1924) to be essentially a mass of myeloid tissue. However, our observations gave evidence against Jordan's view. It was demonstrated that the spleen of Mustelus and the sting-ray is almost entirely devoid of myeloid tissue but consists predominantly of lymphoid tissue, as will be mentioned below.

Lymphocytopoietic loci.

Extensive lymphoid tissue is present in the spleen around arterioles and constitutes white splenic pulp. This tissue consists almost exclusively of densely aggregated small lymphoid cells and is fairly distinctly separated from the red pulp where erythrocytes and myeloid leucocytes occur numeroulsy (Fig. 11). The white splenic pulp of elasmobranchs has thus a fundamental structure essentially similar to that of mammalian spleen. A comparative survey of this tissue revealed that it constitutes one of the most important structural elements of the spleen throughout the entire vertebrate series except for cyclostomes (*Osogoe*, 1954). From the phylogenetical viewpoint, therefore, the white splenic pulp of elasmobranchs should be regarded as homologous to the so-called "periarterial lymphoid sheaths" of the spleen of higher vertebrates and seems to represent one of the chief lymphocytopoietic centers.

Cytological study of the cellular constituents of the spleen of the sting-ray in stamp preparations disclosed evidence which supports this view. As shown

in Tables III and IV, lymphoid cells account for nearly 90% of all cells, and the overwhelming majority of lymphoid cells (about 76%) contain no cytoplasmic granules and correspond to the lymphoid cells which occur most numerously in the blood.

TABLE III

Differential count of blood cells in stamp preparations of spleen of the sting-ray.

Erythroblasts	2.3%
Thrombocytes	0.8
Lymphoid cells	88.9
Pseudoeosinophiles	6.4
Eosinophiles	0.6
Basophiles	0.3
Monocytes	0.7

TABLE IV.

Differential count of lymphoid cells in stamp preparations of spleen of the sting-ray.

Type I, without granules	76.1%
Type II, with fine granules	8.0
Type III, with globular granules	3.9
Type VI, with both granules	0.9

Dense collections of small lymphoid cells similar in appearance to the white pulp of spleen, though much smaller in size, were also found in the subepithelial layer of the mucous membrane of intestine (Fig. 12). In this connection it is of interest to note that many lymphoid cells occur in the intestinal epithelium. As will be mentioned later, lymphoid cells occur more or less numerously in the intestinal epithelium together with lymphoid cell collections in the subepithelial layer, not only in the submammalian vertebrate series except for cyclostomes but also in mammals. Accordingly, the occurrence of lymphoid cells in the intestinal epithelium may be regarded from the phylogenetical viewpoit as an evidence for the presence of genuine lymphocytes.

As noted above, there is sufficient evidence to indicate that the lymphoid tissues of the spleen and intestine of the elasmobranchs are homologous to those of mammals and therefore should be considered to be composed of genuine lymphocytes. For this reason we regard the non-granulated lymphoid cells, which were found most numerously in the blood, as true lymphocytes. It is worthy of special emphasis that the lymphocyte and lymphoid tissue in a narrow sense first appear at the evolutional level of elasmobranchs.

Erythrocytopoietic and thrombocytopoietic tissues.

Neither erythrocytopoietic nor thrombocytopoietic loci in the form of defi-

nite blood-forming tissue could be demonstrated anywhere. The formation of erythrocytes and thrombocytes appears to take place in the general circulation.

B. Teleosts

Blood cells.

Nucleated erythrocytes, special granulocytes (amphophil or pseudoeosinophil polylobulated leucocytes), eosinophiles, thrombocytes and lymphoid cells are to be seen in the blood of the teleost fishes. Basophiles are not constantly demonstrable in smears. Most of thrombocytes appear in the forms of spindle cells and are easily distinguishable from the lymphoid cells. The majority of lymphoid cells are similar in appearance to the mammalin lymphocytes.

As in the blood of elasmobranchs, immature cells, the erythroblasts in particular, were expected to occur in the blood of teleost fishes, but their occurrence was not clearly verified in smears.

Granulocytopoietic loci.

In all species examined except for Mugil cephalus (gray mullet), an extensive granulocytopoietic tissue was found in the intertubular portion of the kidney (mesonephros) (Fig. 13). This tissue consists almost exclusively of developing granulocytes densely packed in the meshes of the reticular stroma. In it there are many capilliform sinusoids embedded. The reticular cells are actively phagocytic as revealed by the injection of trypan blue. Only a few small lymphoid cells are found either intermingled among myeloid tissue or in the capilliform sinusoids.

Cotton (1951) claims in his study on hemopoisis in certain teleost fishes that the chief sites of blood formation are in the kidney and spleen. He states that in the roach only the kidney shows activity while in the perch activity is limited to the spleen, and that in the trout spleen and kidney are both active. However, in our material, even in the gray mullet whose kidney showed only slight activity of granulopoiesis, active participation of spleen in granulopoiesis could not be verified. The testes and ovaries showed no granulopoietic activety.

Finally it should be added that the bone cavity of the teleosts is very limited and contains no hematopoietically active marrow.

Lymphocytopoietic loci.

In the teleost fishes lymphoid tissues are found in the spleen around arterioles and in the subepithelial layer of the intestine. These lymphoid tissues are however not so extensive and consist of less densely aggregated lymphoid cells, as compared with the corresponding tissues of elasmobranchs. In some of the species examined (e.g., Scomber japonicus, Sebastodes güntherii and Spheriodes perdalis) the lymphoid cells in the spleen are diffusely scattered throughout the reticular

stroma while in others they are aggregated into small or large masses around arterioles. In the latter case, the lymphoid masses around the splenic arterioles correspond to the so-called "periarterial lymphoid sheaths" of mammalian spleen. Likewise, the lymphoid tissues of mucous membrane of intestine may be considered to be composed for the most part of genuine lymphocytes, because many of lymphoid cells are migrating into the intestinal epithelium. Since the occurrence of lymphoid cells in the intestinal epithelium is common not only in submammalian vertebrates but also in mammals, this phenomenon may be regarded as characteristic of genuine lymphocytes.

Besides lymphoid cells there occur numerous eosinophiles intermingled with lymphoid cells in the mucous membrane of intestine. Since a few immature forms are mixed among mature forms, this area seems to be the seat of proliferation of eosinophiles. Occasionally these cells also appear in the intestinal epithelium.

III. Amphibians

A. Urodeles

Blood cells.

Nucleated erythrocytes, special granulocytes (amphophil polylobulated leucocytes), eosinophiles, basophiles, thrombocytes, monocytes, and lymphoid cells are to be distinguished in the blood of urodeles. The small lymphocytes in smears are similar in appearance to the mammalian lymphocytes. The blood of the newts (Triturus pyrrhogaster) shows a very high percentage of basophiles (19–45%). (See Table V.) Immature cells such as baso- and polychromatophil erythroblasts and myelocytes also appear more or less numerously. The erythroblasts in the peripheral blood are often in mitosis. The proportions of these blood cells in the newts at different seasons are given in Table V.

Table V

Differential counts of blood cells in blood smears of the newt at different seasons (average 3–5 animals each).

Cell Type \ Season	Winter	Spring	Summer
Erythroblasts	0.97%	36.19%	7.46%
Red cells in mitosis	0	0.30	0.18
Neutrophil myelocytes	0.12	0	0.35
Neutrophil metamyelocytes	1.22	0	3.20
Neutophil leucocytes	6.20	4.57	12.96
Eosinophil leucocytes	0.25	0.43	0.18
Basophil leucocytes	45.13	23.32	19.54
Lymphocytes	43.19	33.47	51.51
Moncytes	0.85	0.28	2.13
Pigment cells	0	0	0.18
Unclassified cells	2.07	1.44	2.31

It is of especial interest that the blood picture of the newts undergoes a considerable seasonal variation, and that in spring erythroblasts occur very numerously (about 36%) while in winter they are scarce (below 1%). The fact indicates that in spring erythroblasts are liberated from the erythropoietic loci into the general circulation to a large extent and erythropoiesis is taking place in the blood.

Granulocytopoietic loci.

In urodeles the kidney participates neither in granulopoiesis nor in other blood formation. The chief sites of granulocytopoiesis in urodeles are found in the liver. In Triturus pyrrhogaster and in all four species of Hynobius examined, such foci are limited to the sub-serous layer, but in the giant salamander (Megalobatrachus japonicus) the interlobular, periportal spaces also participate in granulopoiesis to a large extent (Fig. 14).

The granulocytopoietic loci in the liver of urodeles consist essentially of developing granulocytes embedded in the reticular stroma, being intermingled with a few scattered small lymphoid cells. Neither erythrocytopoiesis nor thrombocytopoiesis occur in these areas (Fig. 15).

In all species examined the bone marrow consisted almost exclusively of adipose tissue and showed no hematopoietic activity (Fig. 16). The blood forming bone marrow has been reported to exist only in the Plethodontidae, the terrestrial urodeles (*Schaëfer*, 1935 and *Barrett*, 1936).

Lymphocytopoietic loci.

In urodeles lymphoid tissues are found, as in the teleost fishes, in the spleen around arterioles and in the subepithelial layer of mucous membrane of intestine. In the spleen of Hynobius nigrescens the lymphoid cells are densely aggregated around arterioles and constitute the so-called "periarterial lymphoid sheaths". However, in the spleen of other species examined they appear more evenly distributed throughout the reticular stroma, and accordingly the periarterial lymphoid sheaths appear less well-defined. In the subepithelial layer of the mucous membrane of intestine the lymphoid cells are more diffusely scattered and never aggregated into difinite masses. In the intestinal epithelium lymphoid cells are also found but in much smaller numbers than in teleost fishes.

The above-noted findings in urodeles are essentially similar to those in teleost fishes, although lymphoid tissues in urodeles are less well defined.

In the mucous membrane of intestine of urodeles eosinophiles also appear but in a much smaller number as compared with those in teleost fishes.

Erythrocytopoietic and thrombocytopoietic loci.

In urodeles erythropoiesis takes place in the spleen. Erythroblasts occur numerously in the the spleen of the newts. In the splenic pulp of the newts,

baso-, poly- and orthochromatophil erythoblasts appear either scattered singly or aggregated into small clusters. The number of these cells however undergoes a marked seasonal variation, being most numerous and densely aggregated in winter (Fig. 17) while in spring and summer becoming scarce. When contrasted with the seasonal variations in the percentage of erythroblasts in the blood (see Table V), the erythropoietic activity of spleen seems to vary in inverse proportion to that of the blood. It is conceivable, therefore, that in spring and summer erythroblasts are shifted from the spleen to the general circulation to a large extent.

Barrett (1936) reports that erythropoietic loci are definitely present in the heart of urodeles, especially the areas between the muscle trabeculae of the auricles and ventricle. Examination of our meterial, however, disclosed no evidence which enable us to verify the observations of *Barrett*, except that in the heart of the newts, which were collected in winter, there occurred a few small clusters of erythroblasts between the muscle traveculae of the ventricle.

In our material, thrombocytopoietic loci comparable to the erythrocytopoietic foci mentioned above occurred neither in the spleen nor in other organs. A few thromboblast-like cells were found in the spleen, but they do not aggregate into definite masses as do the erythroblasts.

B. Anurans

Blood cells.

In the blood of anurans there occur the same types of blood cells as observed in the blood of urodeles. A few immature cells, the erythroblasts in particular, also occur in the peripheral blood. The small lymphoid cells are quite similar in appearance to the mammalian small lymphocytes.

Granulocytopoietic loci.

At the evolutional level of anurans the hemopoietic bone marrow is definitely differentiated and granulocytopoiesis is limited almost exclusively to this area together with erythropoiesis. It is worthy of notice, however, that the bone marrow of anurans functions as the chief site of blood formation only for a short post-hibernation period. During the period from April to August, the free marrow cells are depleted to a large extent. During this period, the femoral bone marrow of Bufo vulgaris assumes the appearance of fatty marrow. In the femoral bone marrow of Rana nigromaculata, Rana japonica and Rana catesbina, there remains only a thin layer of granulocytopoietic tissue in the subendosteal region, while the central portion of the marrow consists almost exclusively of fatty tissue, with scattered small foci of erythroblasts that occur along the venous sinuses (Fig. 18).

During the period from April to August, the kidney of Bufo vulgaris and Rana catesbina contained rather extensive granulocytopoietic foci in the intertubular tissue, in conjunction with small erythropoietic foci. The kidney of Rana nigromaculata and Rana japonica, on the other hand, showed no hematopoietic activity. Thus granulocytopoiesis in the kidney of anurans is inconstant and appears to represent a compensatory mechanism for the depleted bone marrow.

Lymphocytopoietic loci.

In general, lymphoid tissue is better developed in anurans than in urodeles. In the spleen small lymphoid cells often aggregate into definite masses surrounding an artery (central artery). These lymphoid masses quite simulate the periarterial lymphoid nodules of mammals, but germinal centers never appear in these masses (Fig. 19). In some individuals, however, lymphoid masses were quite absent. In such instances lymphoid cells are evenly distributed throughout the reticular stroma. The cause of such individual variation is not known, but in view of the fact that lymphoid tissue rapidly disappears after inanition or malnutrition, the variation may be attributable to some nutritional factors.

As in urodeles, lymphoid cell collections also occur in the subepithelial layer of mucous membrane of intestine of anurans, but nodule-like masses never appear in this area. Like the periarterial lymphoid masses of spleen, these structures are subject to a considerable individual variation, probably due to some nutritional factors. In the intestinal epithelium lymphoid cells appear in the same manner as in urodeles.

In addition, lymphoid cell collections were found in the interlobular spaces of the liver of some anurans, especially Bufo vulgaris, Rana nigromaculata and Rana catesbina. These are small nodular masses composed almost exclusively of densely packed, small lymphoid cells (Fig. 20). From the phylogenetical viewpoint, they may be considered to correspond to the granulocytopoietic foci that appear in the interlobular spaces of the liver of the giant salamander, Megalobatrachus japonicus (see Fig. 14.).

Erythrocytopoietic and thrombocytopoietic loci.

In anurans, erythrocytopoietic loci occur almost exclusively in the bone marrow in conjunction with the granulocytopoietic foci, and preferentially in the central portion of the marrow along the venous sinuses. In the kidney of Bufo vulgaris and Rana catesbina, small erythrocytopoietic foci were also found scattered in the intertubular tissue, together with granulocytopoietic tissue. In other species no such foci were found in the kidney. As stated already, the blood-formation in the kidney of some anurans appears to be compensatory to the marrow function, since the bone marrow was depleted to a large extent during the period from April to August, during which we collected the material.

The formation of thrombocytes appears to take place in the bone marrow, but thrombocytopoietic loci comparable to the erythropoietic foci could be demonstrated with certainty neither in the bone marrow nor in other organs.

IV. Reptiles

The hemopoietic bone marrow is well developed in reptiles. In adult stage both granulocytopoiesis and erythrocytopoiesis (and presumably thrombocytopoiesis as well) are confined to the bone marrow. Both the mode of blood formation in the bone marrow and the types of blood cells in the circulation of reptiles are essentially similar to those of mammals, except that in the former the circulating red cells are nucleated and giant ancestral cells of thrombocytes, the megakaryocytes, are absent in the bone marrow. Only lymphocytopoietic tissue occurs for the most part outside of the bone marrow.

Jordan (1927) reports an active participation of spleen in the production of granulocytes, erythrocytes and thrombocytes in a lizard, Phrynosoma solare. Examination of our material, however, revealed no evidence to indicate production of myeloid cells in the spleen. In the spleen of the lizard, tortoise and soft-shelled turtle that we have examined, lymphoid nodule is well developed surrounding an artery (central artery) (Fig. 21). Moreover, similar lymphoid masses are also present around the capillary sheaths in the spleen of the tortoise and soft-shelled turtle (Fig. 22). In the spleen of these animals capillary sheaths are very well developed, so that lymphoid masses surrounding these sheaths occur more numerously than those around the central arteries, as described previously by *Osogoe* (1954). Both lymphoid masses constitute the white splenic pulp, often becoming confluent. In both masses germinal centers never appear, however.

It is a characteristic feature of the reptiles that lymphoid masses occur numerously in the liver and, though less numerously, also in the bone marrow. In the liver of the tortoise and soft-shelled turtle, lymphoid tissue appears in the interlobular periportal spaces either as diffuse cell infiltrations or as nodule-like formations (Fig. 23). Germinal centers never appear in these lymphoid masses. In the bone marrow of the toroise, soft-shelled turtle and alligator, lymphoid tissue appears as diffuse cell infiltration without formation of nodule-like masses (Fig. 24). These findings seem to be of great sgnificance from the phylogenetical viewpoint since the liver and bone marrow of birds contain abundant lymphod masses, as will be metioned in the next section.

In the subepithelial layer of mucous membrane of instestine of reptiles lymphoid cells are also diffusely collected as in that of amphibians and other lower vertebrates. In some portions these cells aggregate densely into nodular masses simulating the solitary follicles of mammalian small intestine. In the intestinal epithelium lymphoid cells occur more numerously in reptiles than in amphibans.

V. BIRDS

At the evolutional level of birds, the hempoietic bone marrow reaches its height of development and its histological picture approaches that of mammlian bone marrow. Both the mode of blood formation in the bone marrow and the types of blood cells in the circulation of birds are similar in essentials to those of mammals. Nevertheless, the marrow of birds still differ from that of mammals in some respects. It must first be noticed that in birds denucleated red cells do not appear in the circulating blood and megakaryocytes are quite absent in the bone marrow. In addition, there is a striking difference in that in birds abundant lymphoid tissue normally occur in the bone marrow while the marrow of mammals is usually devoid of such tissue.

The amount of lymphoid tissue in the marrow of birds varies considerably among different species. The lymphoid masses of marrow appear either as sharply circumscribed, nodule-like masses, or in the form of diffuse cell infiltrations which are irregular in shape and not sharply separated from the surrounding myeloid tissue (Fig. 25). Usually, germinal centers do no appear in these masses.

Abundant lymphoid tissue also occurs in the liver of birds as in that of reptiles. The sites of occurrence of lymphoid masses are in the interlobular periportal spaces, where these masses appear either as diffuse cell infiltrations or as nodule-like masses, but without formation of germinal centers.

The spleen of birds consists for the most part of lymphoid tissue. It contains innumerable well developed, periarterial lymphoid masses. In addition, there occur lymphoid masses which surround the capillary sheaths. It is worthy of notice that the latter masses appear more numerously than the former. The red pulp is very narrow in cotrast with the well developed white pulp which is composed of lymphoid tissue. In this area neither erythropoiesis nor granulopoiesis takes place.

In the subepithelial layer of mucous membrane of intestine there occur diffuse lymphoid cell collections simulating those of other vertebrates. But lymphoid cells seldom appear in the intestinal epithelium.

VI. VARIATION IN THE CHIEF SITES OF BLOOD FORMATION AT DIFFERENT LEVELS OF EVOLUTION.

As briefly stated in the introdction, variation in the chief sites of erythropoiesis, granulopoiesis and lymphopoiesis in lower vertebrates is of special interest as thrawing light on the problem of interrelationship of blood cells.

As described in the preceding sections, the first blood-forming tissue to develop is the granulocytopoietic center in the spiral valve of intestine of the cyclostomes. At the level of cyclostomes, however, neither erythrocytopoietic nor lymphcytopoietic tissue is differentiated. At the succeeding level, represented by

the elasmobranchs, both granulocytopoietic and lymphocytopoietic tissues become segregated, but they are situated quite separately in different organs, the former being in the ovaries or testes while the latter in the white pulp of spleen and in the subepithelial layer of intestinal mucosa. In the teleost fishes, the chief sites of granulocytopoiesis are in the intertubular spaces of kidneys, while the chief lymphocytopoietic loci are located in the spleen and intestine as in the elasmobranchs. At the successively higher level, represented by the urodeles, the three hemopoietic tissues—erythropoietic, granulopoietic and erythropoietic—become differentiated altogether for the first time. At this level the three hemopoietic tissues are still clearly separated from one another. Namely, the erythropoietic tissue occurs in the red splenic pulp; the granulopoitic tissue in the subcapsular layer of the liver (and in the giant salamander in the interlobular spaces as well); and the lymphopoietic tissue in the white pulp of spleen and in the intestinal mucosa. In the more advanced stages, represented by the anurans, reptiles, birds and mammals, the hemopoietic bone marrow becomes differentiated, so that erythropoiesis and granulopoiesis (and probably thrombocytopoiesis as well) are confined to this area. However, the hemopoietic bone marrow does not function through all seasons till the levels of reptiles. In the anurans, the bone marrow exhibits its proper functions only for a short, post-hibernation period.

As outlined above, the chief sites of hemopoiesis, granulopoiesis in particular, varies at different levels of evolution. In this connection the rôle of spleen in hemopoiesis is of especial interest. It is known that the spiral valve of intestine of the cyclostomes is homologous to the spleen of higher vertebrates (*Jordan*, 1930 and *Osogoe*, 1954). Since the spiral valve contains hemopoietic tissue consisting for the most part of developing granulocytes, it can be stated that at the level of cyclostomes the spleen is the chief site of granulopoiesis. In the elasmobranchs the condition becomes sharply altered. Namely, the spleen is almost completely devoid of myeloid tissue but composed mainly of lymphoid tissue. At this level the chief sites of granulopoiesis are located in the ovaries and testes. In the teleosts the corresponding sites are in the kidney and in the urodules in the liver. In the successively higher levels, represented by the reptiles, birds and mammals, the bone marrow becomes differentiated and function as the the chief site of granulopoiesis in conjunction with erythropoiesis and thrombopoiesis. Thus, the chief site of granulopoiesis are located outside of the spleen throughout the entire vertebrate series higher than the cyclostomes, and the spleen contains no myleoid tissue in most of these animals. Likewise, the spleen cannot be regarded as an important site of erythropoiesis, since no definite erythropoietic foci appear in the spleen of any animals examined except that of some urodeles, the newts in particular. It must be noticed, moreover, that even in the newts the erythropoietic activity of spleen is greatly reduced in summer.

The above-mentioned view is in sharp contrast to the opinion of *Jordan*

and others, who claim that the spleen plays an important rôle not only in granulopoiesis but also in erythropoiesis. As briefly stated in the introduction, most of the previous investigators who dealt with blood cells and blood-forming tissues of lower vertebrates stood on the monophyletic viewpoint as regards the origin of blood cells. They believe that lymphoid cells or lymphocytes are identical with the primitive blood cells and may differentiate into any other type of blood cells. Our observations have demonstrated, however, that at early evolutional levels, represented by the elasmobranchs, teleosts and urodeles, the chief sites of either granulopoiesis or erythropoiesis are located in the places quite different from the chief sites of lymphocytopoiesis. The fact implies that granulocytopoiesis, erythrocytopoiesis and lymphocytopoiesis take place quite independently without any mutual interrelationship, and thus offers a strong evidence aginst the monphyletic view above referred to.

Finally, some mention should be made of the importance of the general circulation as the site of erythropoiesis. The present research has demonstrated that, at the evolutional stages lower than the anurans, no definite foci of erythropoiesis appear anywhere except for the general circulation. It is worthy of notice that in spring the blood of the newts shows a very high percentage of erythroblasts (about 36%) which are often in mitosis. (See Table V.) In the blood of the sting-ray erythroblasts also appear in a relatively high percentage (about 7%). (See Table 1.) Though no blood count was made in other animals, it can be stated in general that the blood of lower vertebrates contain numerous erythroblasts and other immature cells. The fact has already been noticed by *Jordan* (1930), who claims that one aspect of the evolution of blood cells in vertebrates is the progressive elimination of the undifferentiated blood cells from the general circulation and its replacement by the more highly differentiated ones.

VII. DIFFERENTIATION OF THE LYMPHOCYTE AND LYMPHOID TISSUE.

In this section a brief discussion will be made of the question, "at whas level of evolution do the lymphocyte and lymphoid tissue first come into appearance?" In this connection the evolutional history of the lymphoid tissue of spleen — the so-called periarterial lymphoid sheaths — should be noticed.

In the cyclostomes, the spleen is the chief site of granulopoiesis, and true lymphoid tissue is not yet differentiated anywhere. At the next higher evolutional level, represented by the elasmobranchs, the ovaries and testes take over the granulocytopoietic function of spleen, and the spleen becomes chiefly devoted to lymphocytopoisis. Thus in the spleen of elasmobranchs the periarterial lymphoid masses are very well developed. It should be emphasized here that these

lymphoid masses have a fundamental structure essentially similar to that of mammalian spleen, and that the corresponding masses constitute one of the chief structural elements of spleen through all classes of the vertebrates series except for the cyclostomes, although the degree of their development varies to a large extent in different species of animals (cf. *Osogoe*, 1954). Accordingly, there seems to be no doubt that the periarterial lymphoid masses of elasmobranchs may be regarded as homologous to those of mammals and hence as true lymphoid tissue. Similar lymphoid tissue is also differentiated in the intestinal mucosa of elasmobranchs and other higher vertebrates.

It may be concluded, therefore, that true lymphoid tissue first comes into appearnce as early as at the evolutiona level of elasmobranchs. Since lymphocytes are produced in the lymphoid tissue and delivered to the general circulation, the above discussion naturally leads to the conclusion that genuine lymphocytes occur in the circulating blood at all levels higher than the elasmobranchs. This was confirmed by our cytological observations of blood cells of elasmobranchs to some extent. However, final certainty on this point must await further investigation, because in lower vertebrates the morphological features characteristic of the lymphocyte are obscure, so that distinction between lymphocytes and other blood cells, the thrombolasts in particular, is often difficult.

In association with this problem the occurrence of lymphoid cells in the intestinal epithelium deserves special attention. It was demonstrated that lymphoid cells occur more or less numerously in the intestinal epithelium, together with lymphoid cell collections in the subepithelial layer, throughout the submammalian vertebrate series higher than the cyclostomes. Since this phenomenon is very common in the intestine of mammals, it may be regarded as an evidence for the presence of genuine lymphocytes in submammalian vertebrates. On the basis of these observations the conclusion may also be drawn that genuine lymphocytes first appear at the evolutional level of elasmobranchs.

Finally, the relationship between the appearance of genuine lymphocytes and the segregation of the lymph vessel system must be considered from the phylogenetical viewpoint. It is known that in the elasmobranchs the lymph vessel system is not yet differentiated. According to *Glaser* (1933) and *Kihara* (1940), this system becomes differentiated for the first time at the level of teleost fishes. Taking these facts in mind, *Amano* (1948) believes that genuine lymphocytes never appear until the differentiation of the lymph vessel system takes place. This view does not agree with our observations. The present research gave evidence that genuine lymphocytes may appear as early as at the evolutional level of elasmobranchs in which the lymph vessel system is not yet differentiated.

SUMMARY

A phylogenetical survey of hemocytopoietic tissues was made of the submammalian vertebrate series from cyclostomes up to birds, with special emphasis on the differentiation of the lymphocyte and lymphoid tissue. The chief results obtained are as follows:

1. At the level of cyclostomes, only granulocytopoietic tissue is differentiated in the spiral valve of intestine which corresponds to spleen of higher vertebrates. Neither erythrocytopoietic nor lymphocytopoietic tissue is found anywhere at this level.

2. At the succeeding level, represented by the elasmobranchs, both granulocytopoietic and lymphocytopoietic tissues become segregated, but they are situated quite separately in different organs, the former being in the ovaries and testes while the latter in the white pulp of spleen and in the subepithelial layer of intestinal mucosa.

3. In the teleost fishes, the kidney becomes the most important organ of graulocytopoiesis, while the chief lymphocytopoietic centers are located in the spleen and in the intestinal mucosa as in the elasmobranchs. No erythropoietic locus is found in any organ at this level.

4. At the still higher level, represented by the urodeles, the three hemopoietic tissues — erythropoietic, granulopoietic and lymphopoietic — become differentiated altogether for the first time, but they are still clearly separated each other. Namely, the erythropoietic tissue occurs in the red splenic pulp; the granulopoietic tissue in the subcapsular layer of the liver (and in the giant salamander in the interlobular spaces as well); and the lymphopoietic tissue in the white pulp of spleen as well as in the intestinal mucosa. Hemopoietic bone marrow is not yet differentiated at this level.

5. In the most advanced stages, represented by the anurans, reptiles and birds, the hemopoietic bone marrow is differentiated and functions as the chief erythropoietic and granulopoietic organ; while the chief lymphocytopoietic centers are the same as in the urodeles, fishes and elasmobranchs.

6. The spleen is an important seat of lymphocytopoiesis throughout the vertebrate series, with the exception of cyclostomes in which this organ (the spiral valve of intestine) represents the chief granulopoietic center. Only in urodeles does the spleen participate in erythropoiesis. In other animals it contains no myeloid tissue.

7. Lymphocytopoietic tissue comes into appearance for the first time in the spleen and in the intestinal mucosa at the level of elasmobranchs. Other evidences were also presented that genuine lymphocytes first appear at this level.

8. In lower vertebrates, the general ciculation contains abundant immature red cells and seems to play an important rôle in erythropoiesis.

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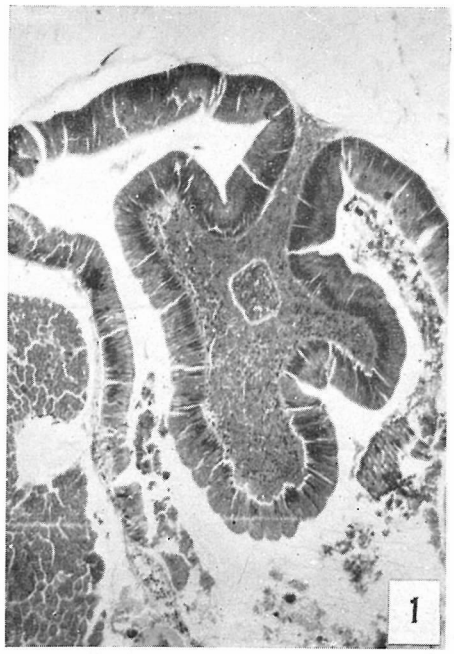
EXPLANATION OF PLATE I

1. Transverse section through the spiral valve of the intestine of Ammocoetes branchialis (larval stage of Lampetra planeri), at the height of the liver.

2. Transverse section through the spiral valve of the intestine of Ammocoetes branchialis, at the height between the liver and kidney.

3. Transverse section through the spiral valve of the intestine of Ammocoetes branchialis, at the height of the kidney.

4. Transverse section through the intestine of Lampetra planeri (adult stage of Ammocoetes branchialis), at the height of the testis. The intestine and its spiral valve become shrunk to a large extent. Hemopoietic tissue is completely absent in the spiral valve. Clumps of spermatozoa are seen upper to the left of the intestine.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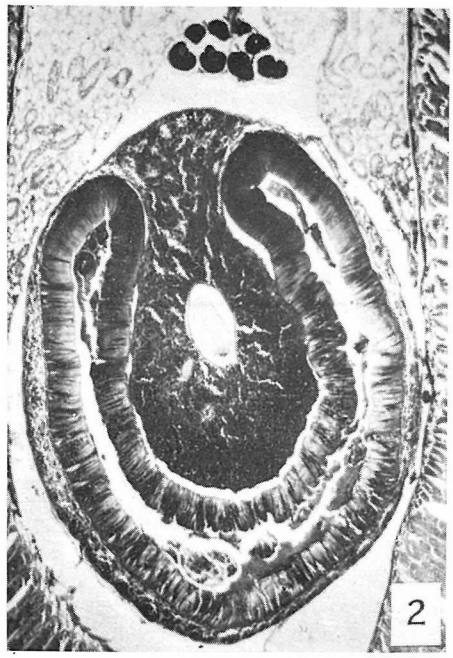
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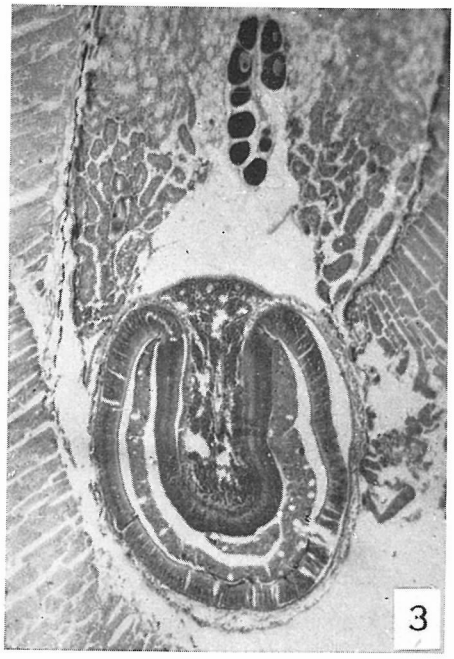
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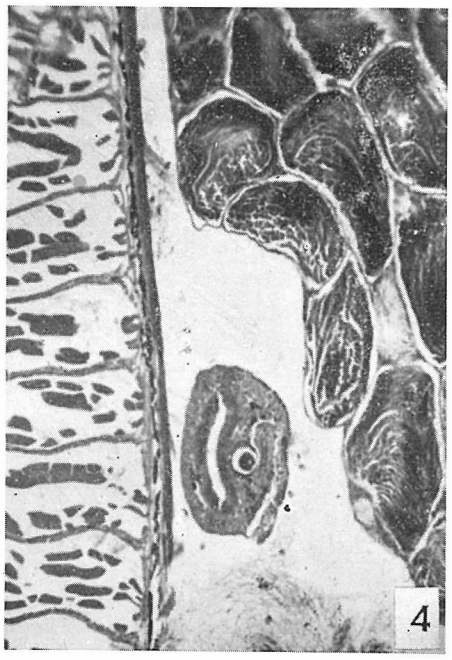
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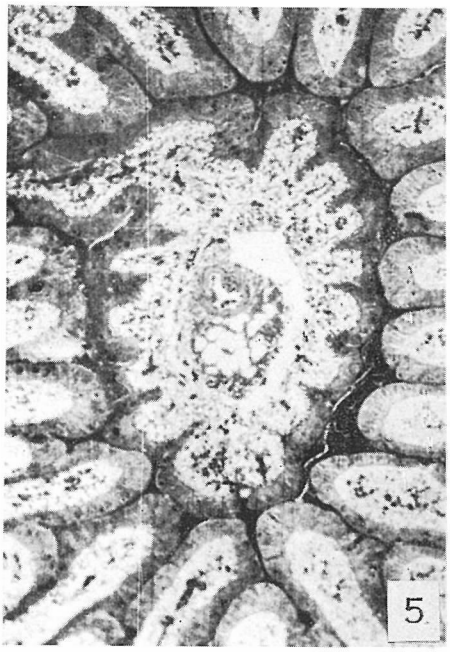
EXPLANATION OF PLATE II

5. Transverse section through the spiral valve of the intestine of Entosphenus japonicus (adult stage). The spiral valve is devoid of hemopoietic tissue. A clump of fatt cells are seen in the central portion of the spiral valve.

6. Net work of reticular fibers in the spiral valve of the intestine of Ammocoetes branchialis. Stained by Gömöri's method.

7. Capilliform sinusoides in the hemopoietic tissue of the spiral valve of the intestine of Ammocoetes branchialis. Notice numerous small lymphoid cells (presumably thromboblasts) are arranged in a line along the sinusoidal walls.

8. Imprint preparation from the spiral valve of Ammocoetes branchialis. Notice the majority of cells are of the granulocytic series possessing variously polylobulated nuclei.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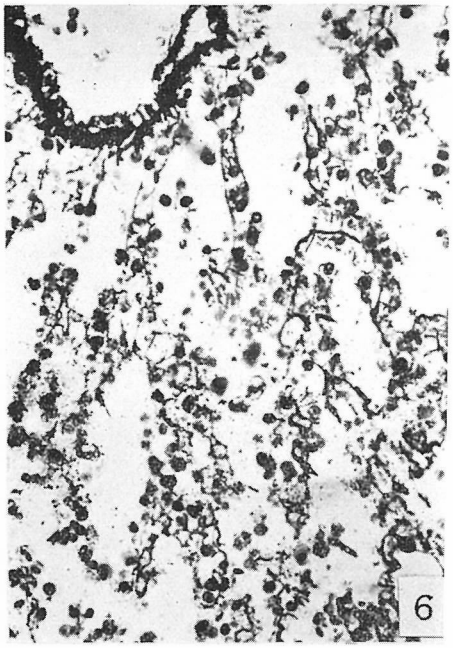
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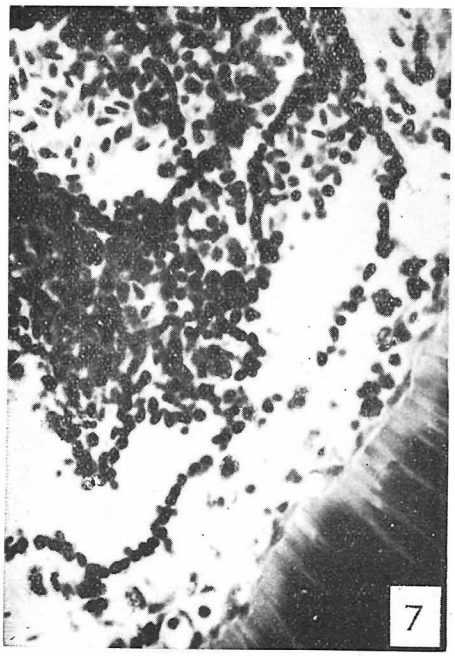
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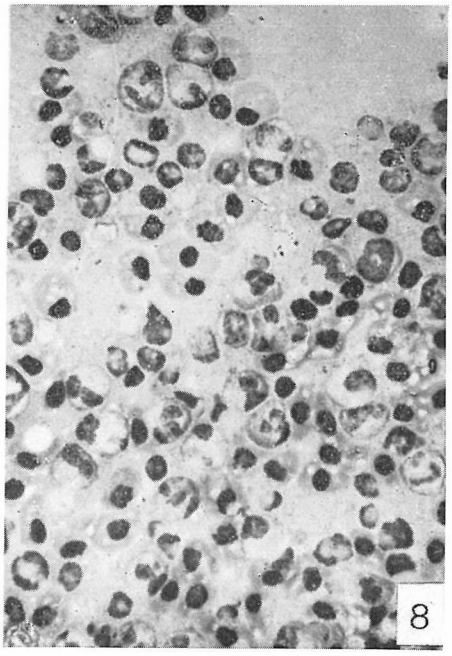
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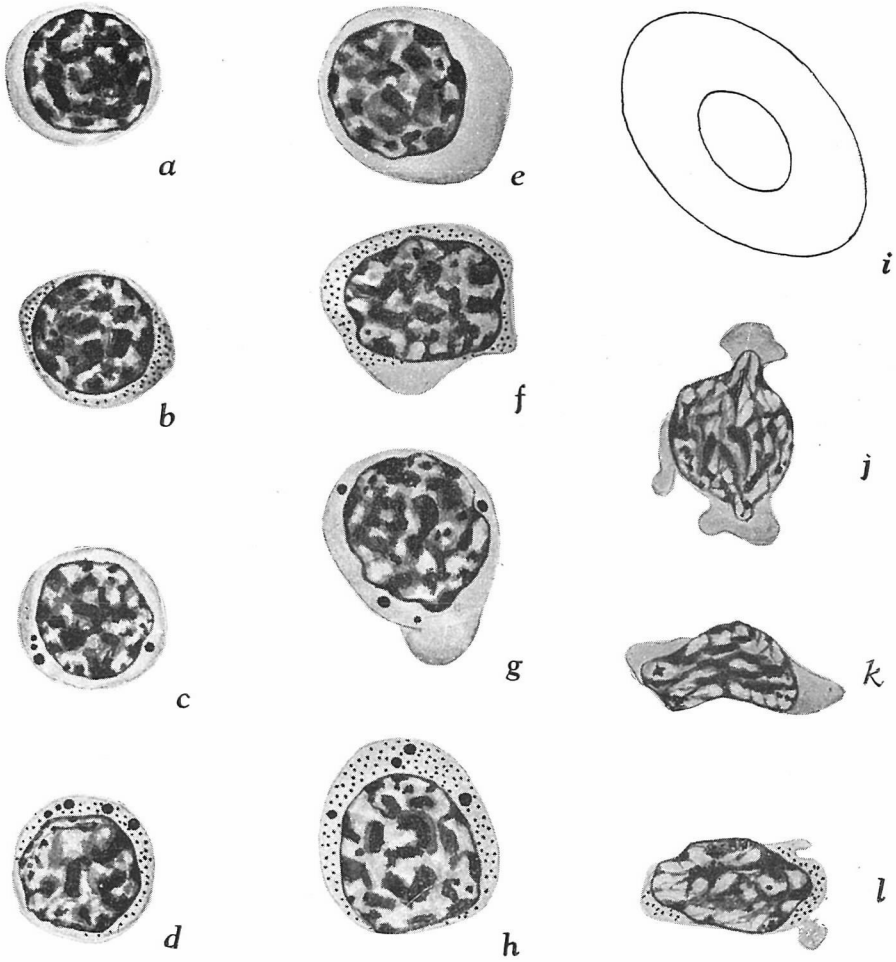
EXPLANATION OF PLATE III

9. Various types of lymphoid cells and thrombocytes of the sting-ray.
   a–d : lymphoid cells of smaller size
   e–h : lymphoid cells of larger size
   i : erythrocyte
   j–l : thrombocytes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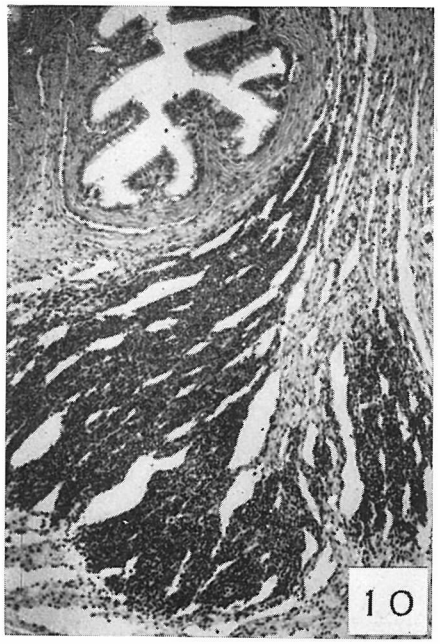
EXPLANATION OF PLATE IV

10. Leydig's organ in the mucous membrane of the esophagus of the sting-ray. The dark-staining tissue is composed chiefly of developing granulocytes.

11. Periarterial lymphoid sheath in the spleen of the sting-ray.

12. Nodule-like aggregations of lymphoid cells in the subepithelial layer of the intestinal mucosa of the sting-ray.

13. Hemopoietic foci in the intertubular spaces of the kidney of the crusian carp (Carassius auratus).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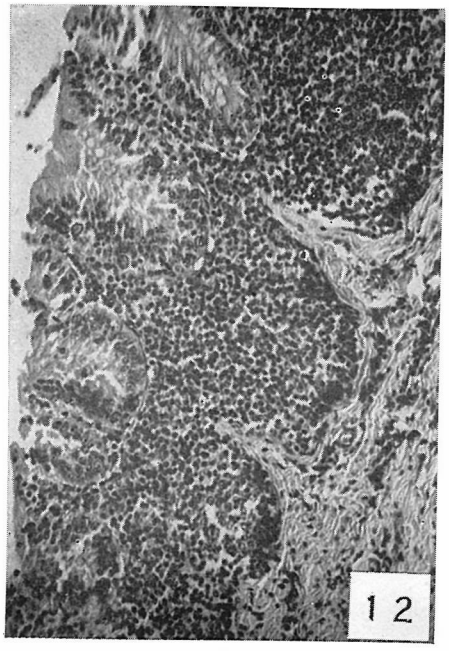
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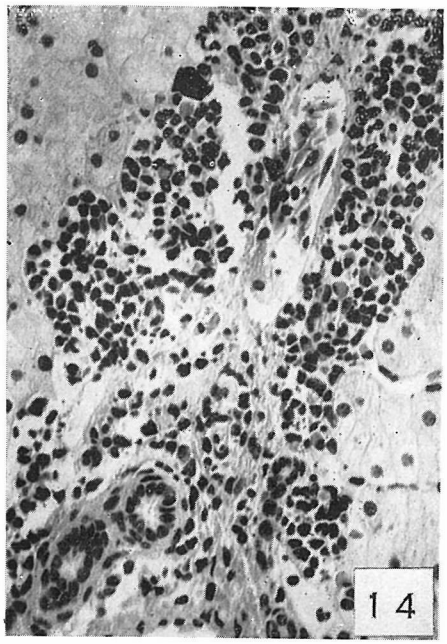
EXPLANATION OF PLATE V

14. Hemopoietic tissue in the interlobular space of the liver of the giant salamander (Megalobatracus japonicus).

15. Higher magnification of a portion of the hemopoietic tissue in the interlobular space of the liver of the giant salamander. Notice the majority of cells are of the granulocytic series.

16. Bone marrow of the newt (Triturus pyrrhogaster). No hemopoietic lous is seen in the marrow.

17. Spleen of the newt (Triturus pyrrhogaster) in spring. Numerous dark-staining cells represent erythroblasts.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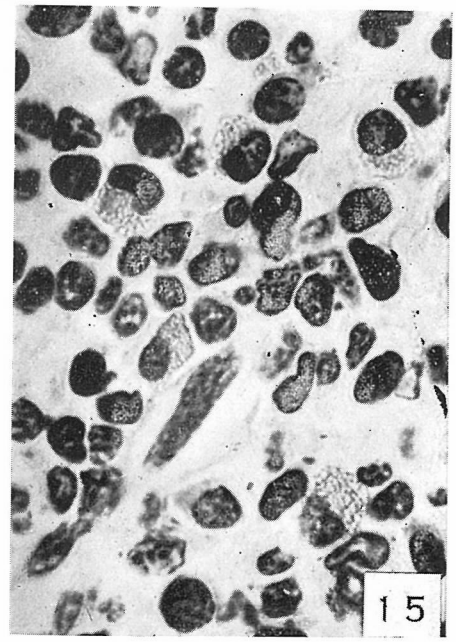
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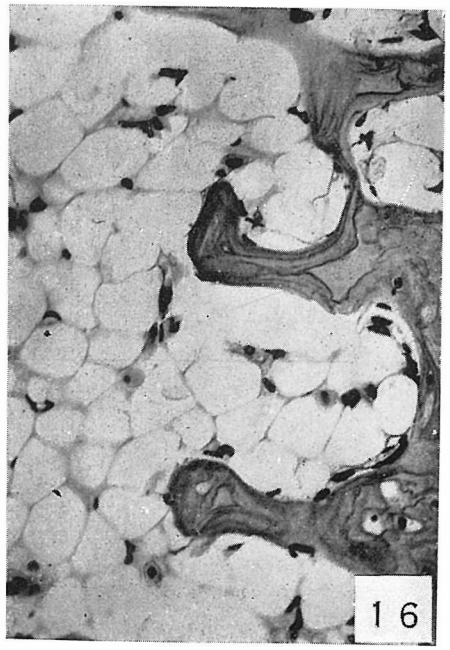
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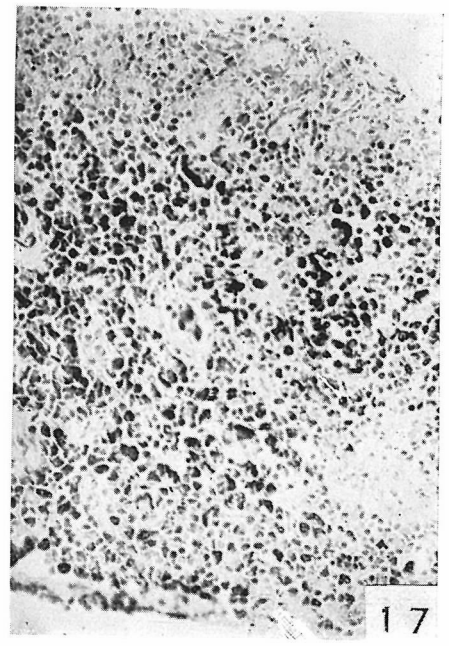
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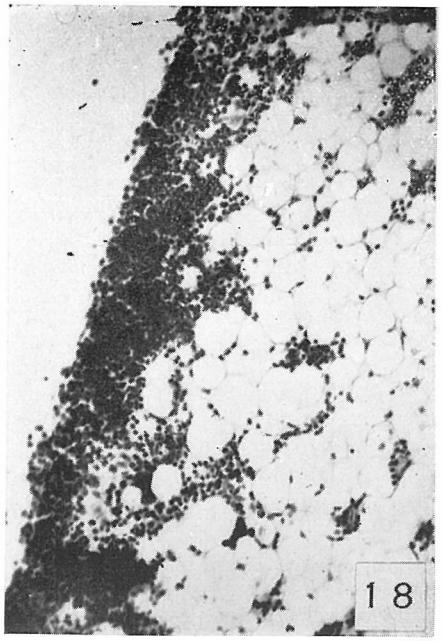
EXPLANATION OF PLATE VI

18. Femoral bone marrow of the bullfrog. In spring and summer, the marrow is depleted to a large extent and there remains only a thin layer of hemopoietic tissue in the subendosteal region.

19. Periarterial lymphoid sheath in the spleen of the bullfrog, which is composed of densely aggregated lymphoid cells.

20. Lymphoid cell collection in the liver of the bullfrog adjacnt to the central vein.

21. Periarterial lymphoid sheath in the spleen of the tortoise (Clemmys japonica).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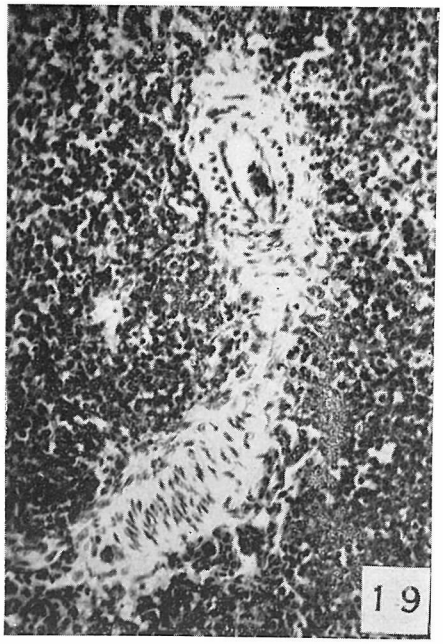
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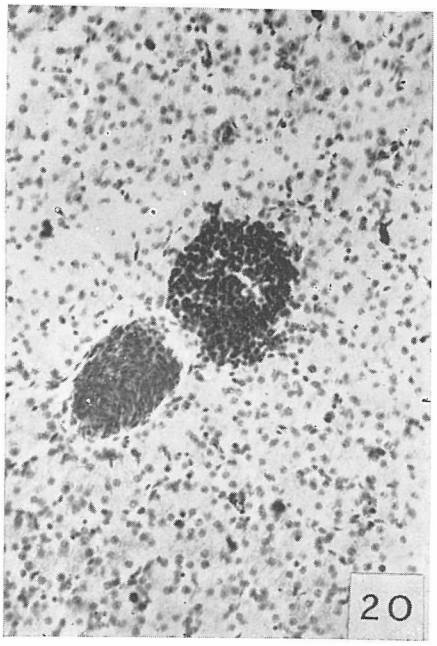
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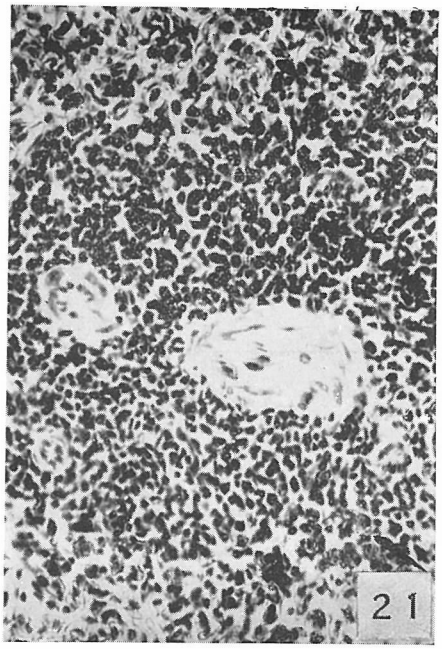
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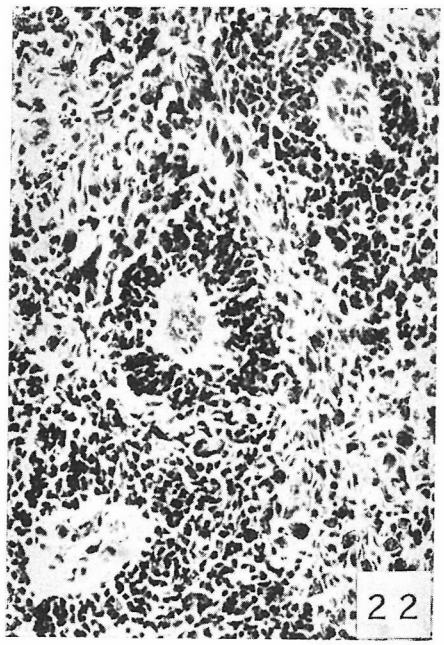
EXPLANATIOON OF PLATE VII

22. Lymphoid masses around the capillary sheaths (Schweigger-Seidel's sheathed capillary).
23. Lymphoid mass in the interlobular space of the liver of the tortoise (Clemmys japonica).
24. Diffuse lymphoid cell aggregation in the bone marrow of the soft-shelled turtle (Amyda japonica).
25. Nodule-like lymphoid aggregation in the bone marrow of the domestic fowl (White Leghorn).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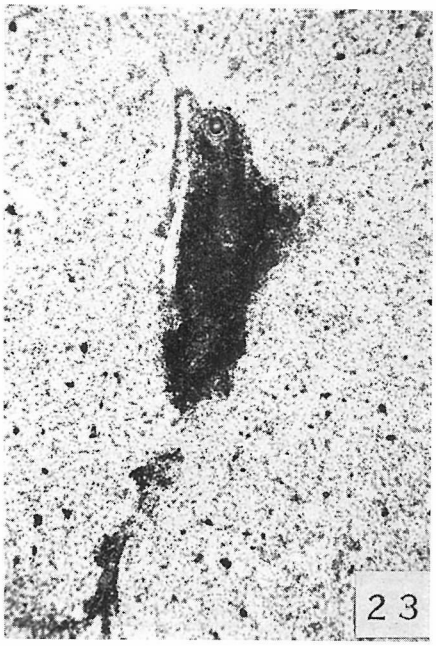
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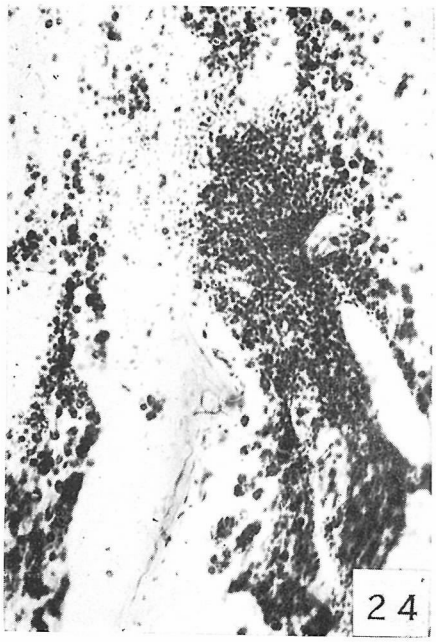
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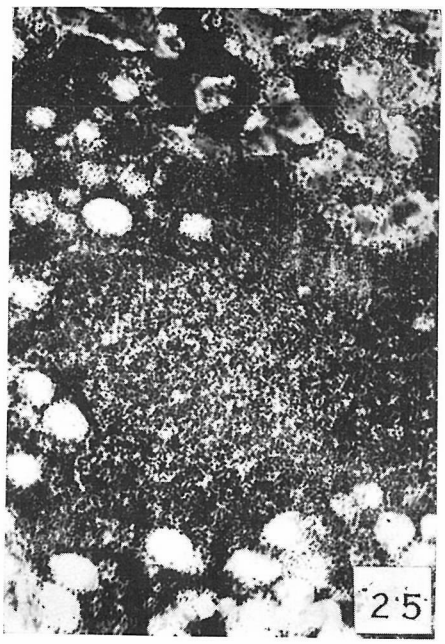
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