GRANULAR LEUCOCYTES IN THE CHICK SPLEEN AND YOLK SAC

LIGHT AND ELECTRON MICROSCOPIC STUDIES ON THE DEVELOPMENT OF GRANULAR LEUCOCYTES IN THE CHICK SPLEEN AND YOLK SAC

by

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SCOPE AND CONTENTS:

Imprints and tissue sections of chick yolk sac and spleen from 4 days incubation to 2 days post hatching were studied with the light microscope after staining with May-Grünwald Giemsa stain. A sequence for the development of heterophils was determined from imprints of the spleen, and some stages in eosinophil and basophil development were described. The cells observed in the spleen were compared with those from the yolk sac. The time of appearance and location of granulocytes in both organs is described. Preliminary electron microscopic observations were made on the granulocytes found in the 14 day embryonic spleen, and three types of granules were described. The morphology of yolk sac blood vessels mid way through incubation is discussed.

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INTRODUCTION

The development of the microscope by the middle of the seventeenth century revealed many structures invisible to the naked eye. Blood was a substance examined early and in 1658 Jan Swammerdam saw red blood corpuscles in frog blood. Antony Lewenhoeck (1674) accurately described the red blood cell as "globules" which were circular in the mammal and oval in birds and fishes, and observed that the red colour resided in the cells, not the fluid. William Hewson (1770-1773) recognized the lymphocyte in blood as well as its origin from lymphoid tissue; he also showed that the erythrocytes were not globules but biconcave discs. In 1841, William Addison described the granulocyte and identified pus cells with granulocytes, thus showing something of the function of leucocytes. The first reliable description of eosinophils and neutrophils was given by Wharton Jones in 1846 when he distinguished the finely granular neutrophils from the more coarsely granular eosinophils. Granulocytes were not considered as morphological entities until Max Schultze (1865), in a study of blood on a warm stage, was able to differentiate finely granular leucocytes with two or more nuclear masses from the coarsely granular cells, lymphoid elements and mononuclear cells.

Blood cell studies were revolutionized by the work of Ehrlich during the latter half of the nineteenth century. Ehrlich had become interested in differential staining of cells and tissues using the newly produced aniline dyes produced by the German chemical industry. Using the triacid stain, eosin-indulin-glycerine, on blood films and tissues, Ehrlich, on the basis of colour reaction, was able to distinguish the various blood cells, particularly those of the granulocyte series. With the use of aniline dyes and their differential staining of cell components, the various blood cells could be readily distinguished. Many authors observed cells which were interpreted as early developmental stages and various developmental sequences were postulated.

During the late nineteenth century and first quarter of the twentieth, many studies of blood and blood forming organs were reported; most of these studies were concerned with mammals and in particular with humans, with relatively few on the non-mammalian vertebrates. This emphasis on mammals has persisted to date.

Comparatively few morphological studies on blood cells were made after 1925 (around which time many key references are to be found), until the introduction of suitable preparatory methods for biological materials allowing the examination of biological tissue on the electron microscope. While ultrastructural studies have provided much information allowing the interpretation of various functions for blood cells, many

problems are still unanswered. Such problems include the development of blood cells especially in non-mammalian animals, the origin of the stem or stem cells, the position of the ubiquitous lymphocyte, the functions of the various blood cells and their fates. The present study attempts to follow and compare the development of granulocytes (particularly heterophils) in the spleen and yolk sac of the avian embryo.

Sequence of Development of Haemopoietic Organs in the Bird.

During embryonic growth, organs develop according to an overall integrated pattern. It is for this reason that a brief introductory chapter on the sequence of development of haemopoietic organs in the bird is included to indicate that while the yolk sac and spleen are the organs of primary consideration in this thesis, they are not mutually exclusive nor are they the only organs involved in haemopoiesis. References for this chapter are to be found mainly in Romanoff (1960) and Lillie (1952).

Apart from the first few days of development, the avian embryo receives nutrients and carries out respiratory exchange via its circulation. Yolk is the primary source of nutrient and a well developed vitelline circulation is found early in life. At 24 hours during the normal development of the chick embryo there is a marked difference in the

appearance of the proximal and distal parts of the area opaca (Fig. 1). The area into which mesoderm has grown is termed the area opaca vasculosa and at this stage has a dense mottled appearance due to the aggregation of mesodermal cells into blood islands from which are formed blood vessels and blood cells. At this time the first traces of a reddish colouration may be seen here. Between 24 and 33 hours of incubation when the embryo has approximately 8 pairs of somites, the area vasculosa has increased rapidly in size and resembles a network due to anastomosing of the blood islands which form a plexus of blood vessels on the surface of the yolk. Differentiation of the blood islands begins at the periphery of the area vasculosa and extends towards the body of the embryo; by 33-35 hours this vascular plexus has formed an afferent vessel and makes contact with the omphalomesenteric veins originating within the embryo. During the period of 33-38 hours, the heart develops and along with it blood vessels begin to form within the body of the embryo. At about 40 hours, omphalomesenteric arteries develop; the proximal portion from branches of the dorsal aorta and the distal portion from the extraembryonic vascular area. Circulation begins at about 40 hours with the opening up of all these vessels. During intra-and extraembryonic blood vessel formation, blood cells have been forming at the same time in the yolk sac.

Haemopoiesis of erythrocytes first occurs in the blood islands of the yolk sac at about 2 days, with granular leucocytes arising extravascularly in the vicinity of yolk sac vessels on the third and fourth days of development. Blood forming activity in the yolk sac increases to reach a maximum between 10 and 15 days of incubation, after which it falls off and ceases between the eighteenth and twentieth day. The decrease in yolk sac haemopoiesis coincides with increased embryonic haemopoiesis, which occurs as early as the 8-9 somite eg. in the dorsal aorta (Romanoff, 1960). Clumps of blood cells may form in areas of lesser importance including the endothelium of the heart and aortic vessels, loose mesenchymal tissue dorsal to the somites and in the head.

The liver in birds is much less important as a blood forming organ than in mammals. There are two embryonic periods of marked blood formation in the loose perivascular reticulum in the liver of the chick, erythropoiesis from the seventh to the ninth day, granulopoiesis beginning at the eleventh day, reaching a maximum at 14-15 days and ceasing by hatching. The chick spleen is moderately erythropoietic between days 8 and 10 although many haemocytoblasts may be found in the loose mesenchymal syncytium. Granulopoiesis in the spleen begins at about the eleventh day with the differentiation of haemocytoblasts to eosinophilic granulocytes with a maximum intensity of activity at days 16-17.

In the bone marrow haematopoiesis begins on the eighth or ninth day of incubation with definitive haemopoiesis starting on the tenth or twelfth day with the differentiation of haemocytoblasts from extravascular cells. Some blast cells enter the vessels where they give rise to red blood cells whilst other remain in an extravascular position to produce eosinophilic leucocytes. Granulocytes are found in the tissue which will form the thymus during the eighth day of incubation; haemocytoblasts gather on the surface and by the tenth or eleventh day of incubation have begun to differentiate into granulocytes. There is only very slight erythropoiesis in the thymus. Both erythrocytes and granulocytes are found in the Bursa of Fabricius; here haematopoiesis is at a maximum during the last days of incubation and ceasing a few days after hatching.

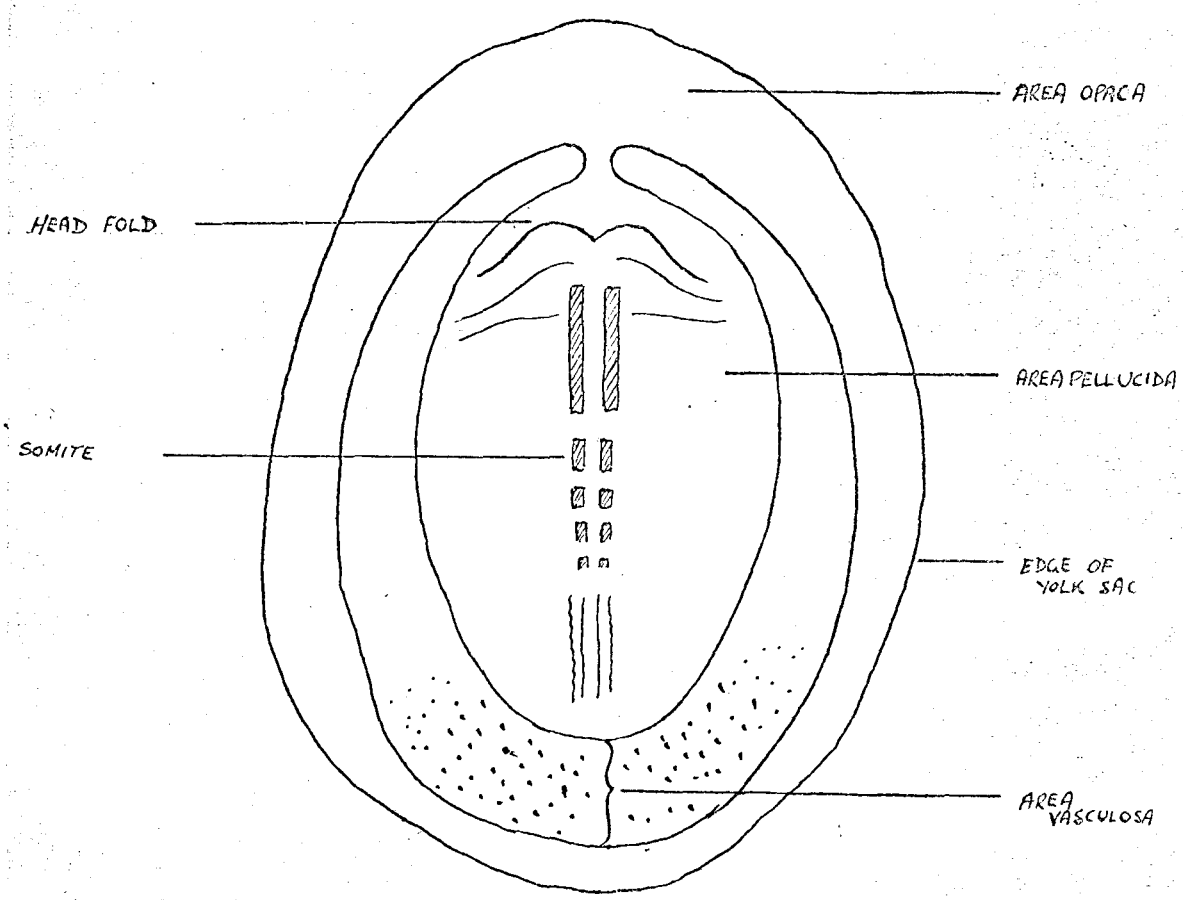


Fig. 1. 26-29 hour embryo, approximately 4 somite stage, showing the main feature of the blastoderm and the initial area of blood cell and vessel formation.

TERMINOLOGY

Before proceeding further, I shall now list the terms which I shall use for the various blood cells described in the thesis. A major problem in haematology has been the profusion of names (terms) used by different authors for the same cells or the use of the same name for different cells. I shall synonomize, wherever possible, the terms used by former authors.

In the present work, the earliest recognisable cell of the blood cell series is termed a stem cell. This cell is characterized as a large round cell with basophilic cytoplasm and round large pale staining nucleus. In the past this cell was called blast cell, haemocytoblast, angioblast or blood island cell by Sabin (1920) and Murray (1932) who also used the term haemoangioblast, lymphocytoblast or haemocytoblast or primitive blood cell by Dantschakoff (1916), undifferentiated stem cell or lymphocyte by Maximow (1909) and lymphoidocyte by Pappenheim (1898).

All cells which follow the blast cell are considered as progressive stages in development towards mature cells. Mature basophils are readily recognized by their round blue-purple granules. Mature heterophils are cells which possess rod-shaped orange specific granules. Cells with round specific granules slightly more red in colour than those seen in heterophils are eosinophils.

The greatest confusion has arisen over the heterophils and eosinophils. Synonyms for heterophils include pseudoeosinophils (Sabin 1921, Sugiyama 1926), eosinophilic granulocyte (Forkner 1929), neutrophil (Sandreuter 1951). Eosinophils have been termed pseudoeosinophilic granulocyte (Forkner 1929) and acidophilic amorphous granular leucocyte (Sandreuter 1951).

Lucas and Jameroz (1961) in their "Atlas of Avian Haematology" used the terms heterophil and eosinophil as I have done. I have also adopted the names for various stages in the development of granulocytes as used by Lucas and Jameroz, i.e. promyelocyte, mesomyelocyte, metamyelocyte, and mature granulocyte. The characteristics of these cells are given in the section "Observations and Results".

THEORIES OF BLOOD CELL DEVELOPMENT

Cunningham, Sabin and Doan (1925) posed the problem of blood cells and their origin in a series of questions, which are still pertinent today.

1. "Do the red blood cells and white blood cells come from a specific common stem cell differentiated from mesenchyme in embryonic life?

2. Are polymorphonuclear leucocytes and cells of the lymphoid series derived from a common stem cell?

3. What is the position of the lymphocytes, fully differentiated or capable of differentiation into other cell types?

4. Is it possible to trace free cells of the blood and tissues under normal or pathological conditions to any fixed cell in the haemopoietic organs or other tissue?

5. What is the relationship of the large mononuclear cells in the circulation to granulocytes, lymphocytes and phagocytic mononuclears?"

Theories on the origin and relationship of blood cells fall into two broad classes, the monophyletic and polyphyletic theories. The monophyletic theory postulates the origin of all blood cells, red and white, from a single stem cell. The polyphyletic theory suggests that each blood cell line arises from distinct separate stem cells, i.e., there exists a variety of stem cells each giving rise to a single blood line.

Throughout the years each theory has had its adherents; early monophyletists included Pappenheim, Maximow, Danstchakoff, and Jordan, while the polyphyletists included Schridde, Naegeli, Stackard, Sabin.

The polyphyletists agree with the concept of a single stem cell for both red and white cells only in that both can be traced back to an origin in the mesenchyme.

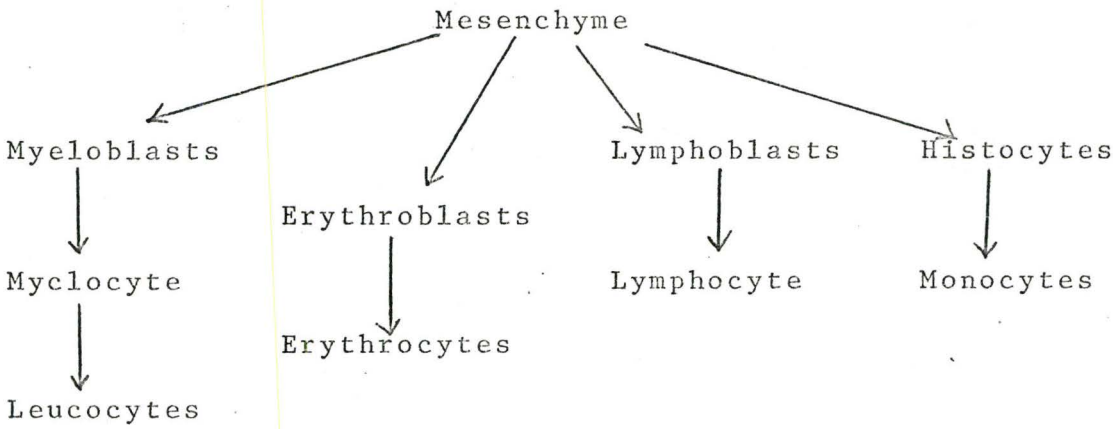
Schridde (1923), from studies of human embryos derived all blood cells directly from endothelial cells. In the early embryo primary erythroblasts, formed intravascularly from endothelium, proliferated to become the primary generation. White cells appearing late in embryonic life were developed from large basophilic cells extravascularly derived from endothelium of the liver; this cell he termed the myeloblast and considered it the parent of other granulocytes. At the same time a smaller basophilic cell, also produced extravascularly, was designated by Schridde as a basophilic erythroblast which gave rise to the definitive series. Later, in spleen and bone marrow, erythroblasts were produced extravascularly, in clumps budding from endothelial tubes and mature erythrocytes emptied into the circulation. Naegeli (1931) considered that the three blood cell types were specific and that the only undifferentiated cell was that of the embryonic mesenchyme; some of these undifferentiated cells remained in the adult near blood vessels, and were available for further differentiation when required. During the development of

erythrocytes from mesenchyme, Naegeli suggested that two cell types were produced, megaloblasts and normoblasts; the normoblast differentiated in the adult to normal red cells. The granulocytic series was derived from mesenchymal cells passing through the stage of myeloblast. Similarly lymphocytes too originated from mesenchyme; the first generation was comprised of small lymphocytes (from mesenchyme) and gave rise to large lymphocytes of the lymph gland which in turn produced the small lymphocytes of the circulation.

Sabin (1920) working on the development of blood in the area vasculosa in living chick embryos gave the basis for a theory of independent origin of red and white cells directly, without an intermediate stem cell, from different types of fixed-tissue cells. Endothelial cells were seen to divide and to form the blood islands and red blood cells. At a later stage in embryonic development, single cells in the extravascular mesenchyme differentiated into pseudoeosinophilic leucocytes (heterophils) but Sabin was unable to determine from which specific cell they were derived although she believed them to arise directly from mesenchyme. By reducing the bone marrow of pigeons through starvation, Doan, Cunningham and Sabin (1924) demonstrated that red blood cells were formed by proliferation of the endothelium in the bone marrow; after starvation only reticular and endothelial cells plus some fat cells remained; on stimulation by feeding young erythrocytes were produced intravascularly while the

reticular cells became the source of extravascular granulocytes.

The most widely accepted of the polyphyletic theories was that of Schilling (1928) based on the observation of dry smears.



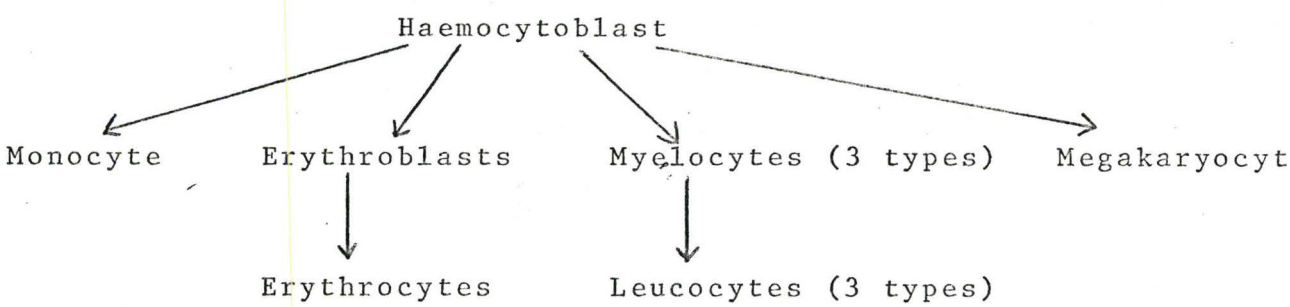
This scheme of Schilling illustrates the concept of polyphyletic theory.

In the monophyletic theory, it is assumed that a single mother cell arises in embryonic life from mesenchyme and throughout embryonic and adult life retains a definite character so that depending on the environment of the cell it can give rise to both red and white cells. Pappenheim (1898) reported that he was able to follow the development in adult mammals of red cells from a mononuclear cell with a strongly basophilic cytoplasm. This cell he called a lymphoidocyte and considered it developed from the primitive mesenchyme at the same time as the endothelium of blood vessels. The lymphoidocyte was then the first free cell stage of both red and white cells with the erythrocyte developing

intravascularly and leucocytes extravascularly from it. Danstchakoff (1907-1916) came to similar conclusions from studies on the development of blood cells in the chick embryo, using azure eosin stains. Islands of cells developed from mesenchyme in the area vasculosa. A thin endothelium originating from island cells surrounded some of these islands to form a network of vessels outside of which remained some cellular clumps. She termed these island cells "primitive blood cells" as soon as the individual cells separated within the clumps and noted that they had the appearance of lymphocytes. Danstchakoff named both the groups of free cells, i.e., those located intravascularly and those extravascularly, lymphocytes; the extravascular cells became granulocytes while those within the vessels either retained their lymphocytic appearance and proliferated or else differentiated into red blood cells. Thus all blood cells were derived from mesenchyme; later she called this mesenchymal stem cell a haemocytoblast.

Red cell development was described by Maximow (1907-1909) in the rabbit embryo as developing intravascularly. Further he reported that cells proliferated from the lining of embryonic vessels; these cells he concluded were free undifferentiated stem cells and not specific precursors of erythrocytes. The undifferentiated stem cell, termed a lymphocyte, he believed could give rise to all other blood cells and at the same time could also give rise to strains of undifferentiated cells throughout life. In bone marrow

of adults, he found red cell formation to take place extra-vascularly and so concluded that in the embryo the red cells produced are temporary and replaced by cells formed later in the bone marrow of the adult. In the mongoose embryo, Jordan (1917) derived a primitive stem cell from endothelium and mesenchyme in connective tissues which could produce under the proper conditions, erythrocytes, granulocytes and lymphocytes. He supported the concept that since mesenchyme is the origin of blood cells, endothelium and mesothelium (both of which are from mesenchyme) must also retain their haemopoietic potentials. Ferrata (1918) found a cell similar to the primitive cell of Maximow which he believed was derived from mesenchyme and gave rise exclusively to primitive erythroblasts. From these were derived a generation of red cells. Later in embryonic development the same cell gave a polyvalent basophilic cell, similar to Pappenheim's lymphoidocyte, which he believed could produce all the varieties of blood cells. Basically the monophyletic theory can be represented as follows.



Position of the Lymphocyte

The identity of the common stem cell of the monophyletists has led to controversy over the position of the lymphocyte in the formation of blood cells. The similarity of a cell in the bone marrow to the large lymphocyte and the similarity in morphology of the lymphocyte to basophilic mononuclear cells of embryonic and adult life, lead to the conclusion that the lymphocyte retained primitive characteristics and was able to give rise to all other cells of the circulation. Dominici (1920) supported this concept with evidence of the formation of myelocytes in the germinal centres of the lymph nodes of rabbit, suggesting that these myelocytes developed from lymphocytes. Furthermore, under severe conditions of bleeding, lymphocytes could transform to macrophages, transitional cells and polymorphonuclear leucocytes without any intermediate stages of myelocyte formation. Downey and Weidenreich (1912) concluded that in the lymph glands and spleen, reticular cells produced large lymphocytes of the germinal centres, which divided to produce small lymphocytes. They also found macrophages being formed by rounding up of reticular cells, and indicated a continuous series between macrophages and lymphocytic series. Granular leucocytes were derived in the lymph gland from small and medium sized lymphocytes. Maximow (1923) reported from *in vitro* cultures of lymphoid tissue from mammalian bone marrow that pseudoeosinophilic and eosinophilic myelocytes appeared

amongst growing fibroblasts and reticular cells of newly formed tissue. This he interpreted as evidence that leucoblasts were formed from large lymphocytes but also in part from small lymphocytes which were able to increase in size giving rise to myelocytes.

Other authors believed that the lymphocyte is a highly specialized or specific cell which is incapable of metaplastic activity. Schridde (1923) suggested that lymphocytes developed from lymph-vascular endothelium during embryonic life and later collected to form lymph nodes which produced only small lymphocytes. Large lymphoblasts were derived from earlier formed lymphocytes and located in the germinal centers of lymph glands; adult lymphocytes were produced by mitosis of the lymphoblasts. He maintained that there were granules specific in type and arrangement for the lymphocyte group and concluded that the lymphocyte was a fully differentiated cell incapable of producing granulocytes. Downey (1924), from studies of normal and leukemic human and mammalian organs concluded that the myeloblast was the real stem cell of the myeloid cells of the marrow and that lymphocytes of lymph nodes and spleen were formed by mitosis of their own cell type from the reticulum. However, he believed that the lymphocytes were capable of further differentiation since those located in other areas, e.g. intestinal mucosa and thymus, might differentiate into granular leucocytes. In myelogenous and lymphatic leukemias he stated that the myeloblast might

serve as the stem cell for both lymphocytes and myeloid cells. Thus he regarded the lymphocyte as partially differentiated but still with some myeloid potentialities that were revealed only under abnormal conditions.

BLOOD CELL DEVELOPMENT IN THE BIRD

The marked differences in the appearance of the red and white blood cells lead early authors to the conclusion that these cells must have different origins. Due to the relatively short duration of the yolk sac and that white cells had not been found in the vessels of young embryos, investigators concluded that erythrocytes developed exclusively in the blood islands and that leucocytes differentiated later within the body of the embryo.

Dantschakoff (1908) defined the problem of blood cell differentiation in the form of five questions:

1. "What is the genetic relationship between leucocytes and erythrocytes in the growing organism and embryo?

2. How do the different types of leucocytes originate?

3. Is there a common stem cell?

4. Where and how do the first erythrocytes originate in the embryo?

5. Where and how do the leucocytes originate later?"

In answer to some of these questions, Dantschakoff (1908), from studies on the chick embryo concluded the first vessels appeared in the area vasculosa; later this venous capillary network became the blood forming organ. Blood cells developed in this area in two locations; haemoglobin-containing cells developed intravascularly while extravascularly there developed granulocytes. She stressed a

common origin of both cell types and that the common stem cell, the haemocytoblast, remained throughout life. In the initial stage of development of the area vasculosa, Dantschakoff described blood islands being formed by local growth of the mesoblast which had no connection with the endoblast, and from these mesoblast cells arose both blood cells and endothelium. "Primitive blood cells" formed a syncytium which then separated to give simultaneously two stem cells. Primitive erythroblasts, transitory cells producing primitive erythrocytes, were replaced later by what Dantschakoff described as a new and better form of red blood cell, termed by others the definitive erythrocyte. The second stem cell was the large lymphocyte, a cell which retained its position as a "primitive blood cell" and acted as a common stem cell for all other blood cells. Extravascular derivatives of the "primitive blood cell" produced acidophilic myelocytes, granulated cells which matured and formed polymorphonuclear leucocytes possessing acidophilic rod shaped granules in the cytoplasm.

Sabin (1920) studied living chick blastoderm which had developed to the 2-3 somite (approximately 24 hours) stage and was able to trace the origin and formation of blood vessels. She observed the differentiation from mesoderm of a new type of cell which she termed the angioblast. These cells first arose in the embryonic membranes and continued to differentiate in the walls of the yolk sac during the third and fourth day of incubation. Sabin distinguished primitive mesoderm and

blood islands; the former gave rise to coelom and the vascular system while the blood islands were described as a group of cells that could produce haemoglobin and develop into haemoblasts. Angioblasts then differentiated from mesoderm, their cytoplasm became more basophilic and granular and they tended to form syncytial clumps. From these clumps, endothelium and blood islands (erythroblasts) differentiated and by liquefaction at the center produced blood plasma; the endothelium also gave rise to erythroblasts. Sabin did not describe the development of white cells as she stated that in the two day incubated chick there are no cells present distinguishable as leucocytes or their precursors. However, she posed two questions: 1) whether all white cells developed outside the vessel from mesenchyme which had not undergone angioblast differentiation, and 2) whether endothelium produced true lymphocytes?

Murray (1932) used a culture technique whereby fragments of primitive streak containing presumptive haemopoietic cells were removed from chick embryos and incubated in a medium of embryo extract and plasma, fixed and stained with Giemsa. The nomenclature used by Murray for the early stages of *in vitro* development of blood basically agrees with that of Sabin (1920) in that he distinguished between angioblasts and blood islands. Angioblasts were those cells derived from mesenchyme which produced endothelial vessels and blood islands; however, Murray preferred the term haemangioblast to angioblast as he suggested that endothelium and blood developed from the

mass of cells. Murray's results dealt entirely with the development of red blood cells, endothelium and "wandering" amoeboid cells with little mention being made regarding the appearance of granular leucocytes. Haematopoiesis was found to occur in any region of the posterior three-quarters of the blastoderm and cultures of this region all produced large numbers of haemoglobin-containing cells. Endothelium developed particularly in the area opaca and area pellucida by the flattening of the peripheral cells of the haem-angioblasts; he does not disregard the excavation process of Sabin (1920). The histogenesis of the red blood cell agreed with that of Dantschakoff. In addition he described a stem cell, similar to the large lymphocyte of Dantschakoff (1916), which was the same size as an erythroblast and contained a large nucleus surrounded by a rim of basophilic cytoplasm with some eosinophilic granules, considered by Murray to be artefacts. Murray described some cells which he suggested were young eosinophils but no indication is given to the location of these cells.

As a sequence to her work on the development of red blood cells and vessels in the living blastoderm, Sabin (1921) studied the development and differentiation of the three types of white cells. Pieces of the area pellucida consisting of the three germ layers, ectoderm, two layers of mesoderm, and endoderm were cultured in a hanging drop. A new type of cell differentiated from the mesoderm in which the cytoplasm

became densely basophilic and azurophilic; these cells divided and stayed together to form a syncytial mass. These masses then joined together to form a network of plexuses and vessels which were described as being formed by the liquefaction of the central part of these cell masses. A progressive differentiation of the angioblasts at the 2 somite stage (approximately 24 hours incubation) was described starting at the periphery of the area vasculosa and extending towards the embryo. Erythroblasts were found in the vessels of the outer margin of the area opaca at the 7-11 somite stage (approximately 30-40 hours incubation). Red blood cells began to differentiate at two days of incubation and on the third day endothelial cells gave rise to monocytes and clasmatocytes of connective tissue . Also, on the third day granulocytes were reported to develop from mesoderm and wander into the blood vessels. Differentiating lymphocytes were not seen in the walls of the yolk sac but were reported to appear in the circulation on the fourth day, becoming abundant at the fifth and sixth days. By the third day there had appeared a finely granulated cell analogous to the neutrophilic myelocyte of mammals which Sabin called a pseudoeosinophile. The cell appeared close to a vessel and at first could not be distinguished from angioblasts. However, unlike angioblasts, they tended to separate after division to give clumps of four or more cells with dense azurophilic cytoplasm. The nucleus became eccentric while the center of the cell became occupied by a centrosphere

made more obvious by the presence of pink staining granules arranged in a crescent shape around it. These cells then moved towards a vessel. Sabin described the migration of one through the wall halfway between two endothelial nuclei; the wall bent inwards until finally the cell entered the lumen. The remaining cells of the clump lined up behind this first cell and also entered the lumen. Sabin suggested that the two stem cells, the angioblasts producing red blood cells and histocytes, and the granuloblasts, have a common ancestor in a mesenchymal cell, not a differentiated stem cell or haematoblast. Thus, the cells of the blood were not so markedly cut off from those of the connective tissue as to have a separate stem cell. However, Sabin could not demonstrate a common stem cell.

Doan, Sabin and Cunningham (1925) analyzed the bone marrow of pigeons. The birds were starved in order to produce a much simpler system in the bone marrow in which only three cell types were present, endothelial, reticular and fat cells. On resumption of feeding, normal structure of red bone marrow was quickly regained, during which time cell proliferation could be followed. They concluded that there was a direct relationship between endothelial cells of the marrow vascular bed and the first generation of red blood cells, that these cells developed intravascularly throught life and gained access to the general circulation by venous sinuses. Leucocytes arose from fixed connective tissue cells of the marrow

parenchyma and their maturation and life history to the stage of leucocyte was entirely extravascular in the intervascular spaces of the marrow. The most mature forms of leucocytes were found nearest the endothelium i.e. growth and maturation proceeded towards the vessel.

Sugiyama (1926) reported that in the 3½ to 4 day chick embryo the mesenchyme around the vessels separated from the plexuses with the cells losing their cytoplasmic processes to round up and form free extravascular myeloblasts. The cytoplasm of these cells contained a few round refractile neutral red granules; during maturation the numbers of granules increased and became arranged in rosettes, the large numbers of mitochondria initially found throughout the cytoplasm decreased and were seen finally only at the periphery. The nucleus changed shape from round to semi-spherical to kidney shaped and then to horseshoe shaped in the mature cell. Typical myelocytes were found in the area pellucida on day 4 or 5 and later the granules became fusiform (elongate); the author reported their passage through the endothelial wall into the vessel on day 5 but stated that they were seldom seen in the embryonic circulation. From day 7 on there was an increase in absolute numbers of the granulocytes. Sugiyama called the granulocytes which were the first to appear in embryonic blood, pseudoeosinophils. True eosinophils with round eosinophilic granules in the mature forms were found generally during the last days of incubation although exceptionally as early as day 7; they appeared to be

mainly produced in the spleen. Sugiyama expressed difficulty in determining true eosinophils from immature pseudoeosinophils. He noted that basophils appeared on day 14.

Forkner (1929) employing supravital staining techniques with neutral red studied blood and bone marrow cells from adult domestic fowl to obtain information on motility, phagocytic power, mitochondrial content, fragility and life history of these cells. He described a mature granular leucocyte, a polymorphonuclear eosinophil (actually a heterophil), with a lobulate nucleus and chromatin existing as a reticular network; rod shaped specific granules irregular in size and shape, stained red with eosin in fixed preparations and golden yellow with neutral red in supravital films. Mitochondria were rarely seen but when present appeared as delicate filaments or dots. The "polymorphonuclear eosinophils" were actively motile and Forkner suggested that they might be comparable functionally to polymorphonuclear neutrophils of human blood. A second type of cell, polymorphonuclear with pseudo-eosinophilic granules (eosinophils), possessed round granules which differed from the "polymorphonuclear eosinophils (heterophil) by taking very little colour with neutral red. The nucleus was bilobed and mitochondria were commonly found. The cell was rarel as active and motile as the "polymorphonuclear eosinophil" (heterophil). Basophils had a single irregular or round nucleus possibly lobulate in which the chromatin was diffuse; the cell was filled with red specific granules and only

slightly motile. He grouped myelocytes into three classes according to the numbers of granules in the cytoplasm: A, the earliest type and the stage after the myeloblast contained 1-10 specific granules; B contained a greater number of granules than A but not the full quota as C which however still retained the mononuclear character. The granules of the myelocytes were nearly always round or globular in contrast to the elongate ones of the mature cells. The young specific granules of myelocytes stained more intensely with neutral red. Mitochondria were readily seen but became less conspicuous with the accumulation of granules. "Pseudoeosinophilic myelocytes" and polymorphonuclear pseudoeosinophils were only occasionally seen in the bone marrow and were identical with those in the circulation; but Forkner does not give a description of the immature forms of the polymorphonuclear pseudoeosinophils (eosinophils). Also Forkner gave some average figures for the numbers of cells found in the blood of adult chickens.

Total Leucocytes/cubic mm	24,586
"Eosinophils with rods" (heterophils)	34.72%
"Pseudoeosinophils" (Eosinophils)	1.76%
Basophils	4.21%

Downey (1938) pointed out that there was a considerable degree of confusion concerning the terminology of avian granulocytes, due partly to the fact that the finely granular polymorphonuclear granulocytes of mammals were replaced by cells with elongate granules (heterophils) with a similar

staining property to true eosinophils, also the initial granules of both heterophils and eosinophils were spherical and of approximately equal size. Thus, in birds distinction may be impossible in sections of bone marrow except perhaps in the pigeon where the granules of true eosinophils retain their rounded shape. Downey adopted the terminology of Keyes (1929) by calling the group of special granulocytes with elongate granules, heterophils. Downey (1938) described the mature forms of granulocytes in the pigeon; heterophils had a conspicuous bilobed nucleus (polymorphous) with rod shaped sometimes slightly beaded granules which stained bright red with Wright's stain. Some cells, appearing smaller with less conspicuous nuclei and stouter, longer granules staining more purplish were thought to represent older stages of development. In the bone marrow, heterophil granules originally spheroidal developed into rods and except in early and late stages were all oxyphilic. Eosinophils were described as having a bilobed nucleus located towards the periphery of the cell and granules usually coarser, spherical and of uniform size. Basophils occuring in smaller numbers had the appearance of degenerating cells; Downey suggests that they might even be degenerated eosinophils.

A good description of the morphology of developing granulocytes in adult chicken and starlings was given by Sandreuter (1951). She studied blood smears, smears and sections from liver, spleen and bone marrow stained with

May-Grünwald Giemsa stain and a peroxidase reaction. Neutrophils (heterophils) were described as crystal granulated leucocytes and the stages in their development divided into stages. Promyelocyte I cells contained progranules, fine subspherical granules, azurophilic and few in number, which were found along the periphery of the cell and over the nucleus. The round nucleus usually contained two nucleoli which appeared as dark coloured bodies in a diffuse chromatin. In cells of the stage promyelocyte II, the azurophilic granules appeared much coarser and some were described as being coloured only at their periphery. The nucleus often appeared crescent shaped. The cytoplasm was lighter in colour and the azurophilic granules had multiplied and enlarged. Definitive acidophilic granules were seen at this stage and found in the indentation of the nucleus; consequently azurophilic granules tended to be found at the periphery of the cell. In myelocytes, the originally round acidophilic granules became "oat-shaped" inclusions. The nucleus became kidney shaped and surrounded by many varied coarsely shaped eosinophilic granules while azurophilic granules were found singly along the periphery of the cell. By the metamyelocyte stage, the nucleus had become more deeply indented and contained dense chromatin. There was a continuation of the development of the specific eosinophilic granules; Sandreuter makes no mention of the fate of the azurophilic granules. The other granulocyte cell line, termed by Sandreuter acidophilic amorphous granular leucocyte

(eosinophils), was similarly divided into several developmental stages. In the promyelocyte stages, the nucleus was round and eccentrically situated in the cell, nucleoli were difficult to distinguish, the cytoplasm appeared homogeneous and strongly basophilic and there were no light spheres present in contrast with the neutrophils (heterophils). At Promyelocyte I stage, the first specific orange granules were small bodies situated in the broadest part of the cell and were sharply contrasted forms with slight refractility similar to that of the definitive granules. In Promelocyte II stage, the number of granules had increased. At the myelocyte stage, the granules, varying in size and colour, had enlarged and filled all the basophilic cytoplasm. The nucleus of the metamelocyte was more or less indented and the rounded and highly refractile eosinophilic granules had become more uniform in size and colour. She gave little information on basophils. Sandreuter gave the following diameters for the various blood cell types in the normal adult chicken: lymphocytes 5-10μ, plasma cells 10-13μ, basophil no measurment, neutrophils 10-12μ, and eosinophils 10-12μ.

Sandreuter considered that the crystalloid granulated avian leucocyte (heterphil) corresponded to the mammalian neutrophil and the amorphous granulated avian leucocyte (eosinophil) to the mammalian eosinophil.

Lucas and Jameroz (1961) in their "Atlas of Avian Haematology" described the morphology of both red and white

blood cells, stained with Wright's and May-Grünwald Giemsa stains, at various stages of their development.

These authors did not express their ideas on the origins of the blood cells but quote briefly some of the authors already discussed in this literature review. Since in this present work the terminology and some of the criteria for differentiating granulocytes has followed that of Lucas and Jameroz, a detailed table (Table I) of their descriptions of granulocyte stages of development appears in the section on "Observations and Results." Briefly their sequence for heterophil development is as follows: the first cell identified was a large basophilc cell the granuloblast; this was followed by a metagranuloblast which possessed similar characteristics although the nucleus was less distinct. Magenta and specific granules first appeared at the promyelocyte stage; definitive rods were present by the mesomyelocyte stage, half the number of definitive rods were present in the metamyelocyte and the mature heterophil contained the full complement of orange refractile rods. During maturation, the nucleus changed shape from being round or ovoid to lobed, and the chromatin material became condensed into clumps.

Lemez (1964), in a comprehensive review of blood cells of chick embryos, gave the time of appearance and numbers of the various stages of development of erythrocytes and leucocytes. The primitive erythrocytes (haemocytoblasts) appeared on the second day and were found in smears up to days 3 and 4. Soon

after day 2 the parent cell of the thrombocyte series appeared and by day 4 haemocytoblasts of the definitive erythroid series were present; from day 5 until hatching only haemocytoblasts were found in smears. Also on day 4, cells closely resembling haemocytoblasts of the red blood cell series appeared extravascularly giving rise to the first neutrophil (heterophil) leucocytes. In the adult hen, the percentages (these are Lemez's figures; there are considerable variations, depending on the author, see pg. 27) in the blood of various leucocytes present are given as:

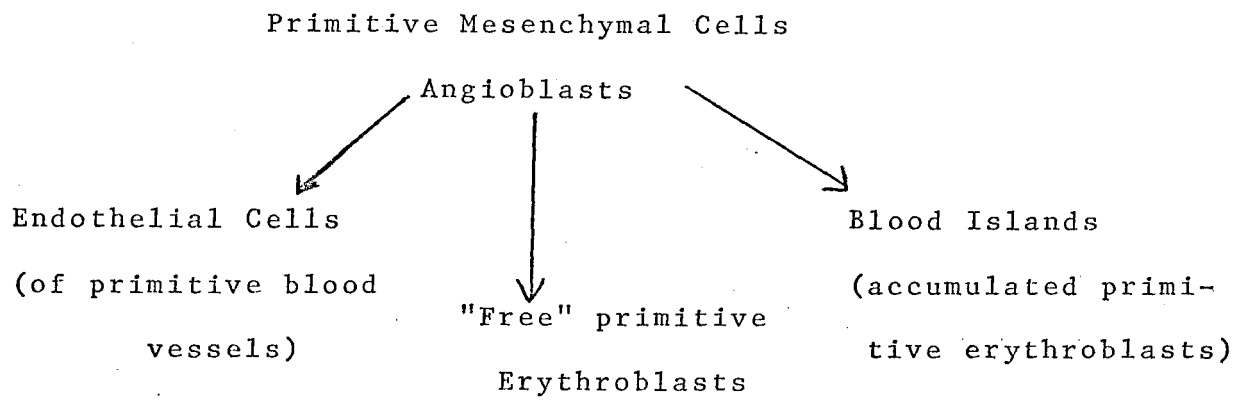
Neutrophils	27.5%
Eosinophils	3.7%
Basophils	2.8%
Lymphocytes	60.4%
Monocytes	5.5%
(Plasma Cells	0.5% not found in the embryo)

In the embryo these cells appear in the circulation successively in the same order as above. Neutrophils appeared about day 4 or 5, eosinophils, basophils and lymphocytes were always present in smears from the fourteenth day onwards, although eosinophils are seen occasionally from 11 days and basophils and lymphocytes from 12 days.

Houser, Ackerman and Knouff (1961) using phase contrast microscopy, studied the posterior part of the chick yolk sac near the junction of the areas pellucida and opaca (splanchnopleura nearest coverslip) which had been cultured for 60 hours and described the formation of the first erythropoietic areas and

and blood vessel formation. Although these authors do not discuss specifically the formation of leucocytes it is of interest to mention the description of this phenomenon in light of the same work performed by earlier authors (Sabin, 1920; Dantschakoff, 1908). Vasculogenesis began in the area pellucida with the condensation of mesenchymal cells into clusters or strands first in the mid-portion, then extending anteriorly and posteriorly. These cords which develop from mesenchyme were termed angioblasts and showed some degree of morphological and cyto-differentiation, i.e., mesenchyme cells destined to become primitive vessels, possessed cytoplasm which became more refractile, cytoplasmic borders which became more indistinct and processes between cells which became shorter and thicker. These last two points presumably lead to the concept of earlier authors of a syncytium. In addition, round and rod shaped mitochondria became more prominent and the nucleus less refractile with a prominent nucleolus. The angioblastic cords developed progressively wider and thicker by further differentiation of primitive mesenchymal cells, the cords anastomosing to form a network in the mid portion of the area pellucida; this network was completed by 42-44 hours. The authors stated that vessels could be formed in two ways; more commonly small slit-like spaces appeared between the cells of the central angioblasts, then joined up and formed a hollow tube. Within the tube there were few cells, mainly degenerating angioblasts and some red blood cell precursors; blood plasma

accumulated as extracellular fluid from degeneration and lysis of angioblasts. In the second method of blood vessel formation, cords became hollow without change in internal structure; at the periphery, the cells became more elongate and took on the characteristics of endothelium, i.e., spindle shaped cells with more homogenous cytoplasm, smaller nuclei and less prominent nucleoli. A narrow space developed between the central mass and the endothelium; this central mass was termed the blood island. By 48 hours, the area pellucida had a functional vascular system, with new vessels continuing to form from primitive mesenchyme; after 50-55 hours blood vessels had lost their single layered appearance as mesenchymal cells arranged themselves parallel to the endothelial wall. With the change to this multilayered structure, little active erythropoietic activity were seen in the area pellucida. During the second day, primitive erythroblasts came from three sources, 1) angioblasts remaining within the vessel cavity which had not degenerated 2) blood islands and 3) directly from endothelial walls by mitosis provided they had their attachments before commencing to divide. Blood islands in the area pellucida lay adjacent to the endothelial wall with only one surface touching, and it was suggested by Houser *et al* that the blood islands may actually arise from the endothelial cells. The results of these workers may be summarized in the following way:



No mention was made in this paper on the development of granulocytes.

In summary, light microscopic studies have shown that confusion exists over the identification of heterophils and eosinophils. The origin and development of granulocytes in the yolk sac is still not known, although it is agreed that they arise extravascularly. The identity and relationship of the stem cell is not solved except in so far as authors agree that blood cells originate from mesenchyme. Most work has been concentrated on the development of red blood cells and blood vessels.

Electron microscopic studies on the developing yolk sac are few and most information on the development of granular leucocytes comes from observations on the bone marrow. What little work that has been done on the yolk sac involved the formation of blood vessels and development of the red blood cell up to the seventh day of incubation. There appears to be little information on the relationship of mesoderm to endoderm, structure of endoderm or the structure and relationship

of cells (angioblasts?) outside the vessel and in "cords" suspending the vessels from the ectoderm in which granulocytes are found.

A short report on the ultrastructural relationships between mesoderm and endoderm in the area vasculosa of the chick blastoderm during blood island formation was given by Mato, Aikawa and Kishi (1964). At the 3 somite stage mesodermal cells have no contact with endodermal cells. By the 4 somite stage some close contacts were seen between projections of endodermal cells to the mesoderm. Later during this stage of development there appeared to be a membrane connection between the two cell types. At the 5 somite stage, electron dense material was present in the space between endodermal and mesodermal cells but no explanation was given for the appearance or presence of this material and from the few micrographs presented in the paper, the material appeared to me as either some form of debris or a rather blotchy basement membrane. Filamentous structures were present along the apical cell membrane of the endoderm, and the free surface occasionally showed microvilli. In later stages of 7 somites, mesodermal cells near the interstice became flattened but the cytoplasm of these cells was no different from that of cells near the center of the blood islands. A change in the cytoplasm of these flattened cells occurs at stage 8 somites as they develop into endothelium; mitochondria and endoplasmic reticulum were more abundant than in blood cells. A small part of the

endothelial cells were in contact with endodermal cells but those cells destined to be blood cells had no contact with the endoderm.

Edmonds (1966) studied a series of chick blastoderms incubated for 24 hours to 7 days and described the ultrastructural changes during the development of erythrocytes. By removing pieces of yolk sac and orienting these in such a way as to be able to cut sections through the entire thickness of the sac, he was able to identify cells not only by their structure but also by position. Angioblasts, cells in and from splanchonic mesoderm not surrounded by endothelium, were described as large irregular oval cells with a large nucleocytoplasmic ratio; the nucleus possessed a prominent nuclear membrane and pores, and contained a large irregular nucleolus and diffuse chromatin. Free ribosomal granules in the cytoplasm were often found in a rosette pattern. Endoplasmic reticulum was sparse, mitochondria were rod shaped and frequently seen in close relation to Golgi complex. The Golgi itself consisted of flattened cisternae with dilated ends and free vesicles. Cells of the angioblastic cords did not form a syncytium as previous authors believed, but were closely packed cells with two types of association, protoplasmic interdigitating processes and attachment areas similar to desmosomes. The lumen and intravascular blood islands were formed by the enlargement of intercellular spaces; peripheral cells flattened to become the endothelium; central cells remained as the first blood

islands. No cell degeneration was reported during this process, contrary to previous reports (Sabin 1920, Houser et al 1961). Cells remaining in the blood islands had a fine structure similar to that of the angioblasts. Two types of cells were described by Edmonds as arising from the islands, one a large immature cell retaining the morphology of island cells, numerous mitochondria, free ribosomes and a prominent Golgi, similar to the multipotential cells found in the haemopoietic tissue (Pease, 1956); and secondly, round or oval cells which had begun their maturation to primitive erythrocytes, i.e., showing a decrease in nucleocytoplasmic ratio, a less distinct nucleolus, less abundant endoplasmic reticulum, a decreased Golgi complex, mitochondria decreased in size and were less frequently associated with Golgi. With maturation these changes became more conspicuous, with denser material, probably haemoglobin, filling the cytoplasm.

Pinocytosis increased through the first two-thirds of incubation after which it decreased. By day 5, the most prominent cell was the mature primitive erythrocyte, a large electron dense cell, with few organelles. At this time, a large increase in the number of stem cells was seen which Edmond suggested developed into the definitive red blood cell series. Maturation of the definitive series is similar in many respects to that of the primitive series.

Stem cells and older generation granulocytes were reported by Edmonds adjacent to the endothelial lining in an

extravascular position. Early granulocytes were described as having a variable shaped nucleus with prominent nucleolus, numerou free ribosomes and scattered mitochondria, a small amount of rough endoplasmic reticulum and a prominent Golgi with many vesicles (Bainton and Farquhar 1967). Edmonds 1966, identified immature granulocytes by their time of appearance, between 72-96 hours, the basophilia of the cytoplasm and the similarity in morphology of the cell compared to previous light microscopy description, i.e., eccentric nucleus and cytoplasm containing sperical granules. Stem cells and maturing granulocytes were described as being extravascular and abutting the endothelial wall. The precursor of the granulocyte had a variable shaped nucleus with prominent nucleoli, many free ribosomes in the cytoplasm and scattered mitochondria and only a small amount of rough surfaced endoplasmic reticulum. The Golgi complex was well formed, consisting of flattened lamellae surrounded by numerous small vesicles, situated near the indentation of the nucleus. Only early stages in granulocyte formation were seen and Edmond's reported that a progression could be seen as regarding the development of the granules; they first appeared as small clear vesicles, these coalesced to form larger partially filled ones which eventually produced large spherical membrane bound granules containing electron dense material. He stated the similarity between the intravascular and extravascular stem cells suggested a possible multipotential stem cell with the differentiation pathway depending on the environment i.e. a monophyletic

origin.

Campbell (1967) studied the bone marrow of 3-4 week old chicken and adult pigeons using both light and electron microscopy. He described erythropoiesis within the sinuses and granulocytopoiesis in the extravascular spaces. With light microscopy, cells within the sinuses appeared similar to the reticular cells of bone marrow except they were elongated. Extravascularly large non-granular cells identical with intravascular haemocytoblasts were identifiable as the stem cells of the granulocyte series. The nucleus of the promyelocyte was similar to that of the haemocytoblast but characteristically the cytoplasm contained a few small round granules found in areas where there was loss of basophilia. Myelocytes contained numerous round granules, the majority of which were large; the nucleus was round or oval with dense chromatin and small nucleolus, and the cytoplasm had lost its basophilia. With Azur II stain, Campbell was not able to distinguish heterophils from eosinophils at the light microscope level. However, with the elctron microscopy he was able to resolve this problem. It is worthwhile to describe in some detail the fine structure of the stages in the development of granulocytes found by Campbell, and also of the erythroid series, particularly the earlier stages where confusion may occur. Haemocytoblasts possessed large nuclei, the chromatin of which was loosely scattered but with some densation into clumps along the nuclear membrane, nuclear

pores appeared to communicate between these masses i.e. less dense area, opposite a pore. The cytoplasm contained large mitochondria, free ribosomes often arranged as polyribosomes, isolated endoplasmic reticulum and small Golgi apparatus. Coated vesicles were seen in the cell membrane close to the sinus wall. The cell was flattened along the sinus wall with the space between the two being regular and filled with an electron dense material perhaps a cement substance. In the basophilic erythroblast, the nuclear chromatin was dense, nucleolus smaller and mitochondria fewer and smaller. Ribosomes were scattered singly or in small groups; endoplasmic reticulum and Golgi were reduced but coated vesicles were still present. Electron dense material probably haemoglobin, first appeared in the cytoplasm of the polychromatic erythroblasts; it was diffuse and scattered through the cytoplasm but absent round the ribosomes which had decreased in numbers. Nuclear chromatin had become coarse and condensed, and an electron dense material, possibly haemoglobin, filled the interchromatin spaces with a continuity of nuclear and cytoplasmic haemoglobin at nuclear pores. Coated vesicles were still present. There were few mitochondria and endoplasmic reticulum and Golgi comples were absent. In the mature erythrocytes, the nucleus was oval with dense chromatin nuclear haemoglobin present and continuous with that of the cytoplasm; a few mitochondria and scattered ribosomes and a few coated vesicles were found.

Extravascularly the stem cell (haemocytoblast) possessed a large nucleus the chromatin of which was scattered with some condensation along the nuclear envelope; a large nucleolus was present. The cytoplasm contained free ribosomes either singly or in clumps, a few cisternae of the endoplasmic reticulum and large mitochondria. Golgi apparatus was seen in some sections as well as coated vesicles at the cell membrane. Campbell was not able to distinguish promyelocytes from myelocytes. However, he distinguished two types of myelocytes - one containing two populations of granules differing in size and shape and identified as heterophil myelocytes, the other containing only round granules of a fairly uniform size identified as eosinophil myelocytes. Heterophil myelocytes had abundant rough surfaced endoplasmic reticulum, an elarged Golgi apparatus, dense nuclear chromatin and a dense smaller nucleolus. Granules in the cytoplasm were of two types. One type arising near the cell surface and described as an expanded vesicle packed with fibrous material which became with maturity, oval and contained fibrillar material; their origin was unknown. The other type, which was more abundant, arose from dense conglomerations of globular material loosely packed within vesicles; more mature ones had a compact core on which globular material appeared to be condensing; the mature granule had a less dense matrix than the immature. Since the globular material of these granules was seen within the Golgi complex and in small

membrane bodies associated with the Golgi, it was suggested that the material was formed (packaged) here, becoming pinched off into vesicles which migrated through the cytoplasm to accumulate in the immature granules. The nucleus of the heterophil metamyelocyte was elongate and the chromatin clumped along the nuclear membrane. Golgi complex was smaller and there was a decrease in amount of rough surfaced endoplasmic reticulum. Pinocytotic vesicles were common near the cell periphery. At this stage, the majority of granules were spindle shaped with a dense homogeneous matrix; all the granules appeared the same in that the fibrillar type had either disappeared or become indistinguishable from the globular type. The round granules of the eosinophil myelocytes were more homogeneous in size, shape and content, as the cell matured; the granule contents became denser. Immature granules were shown to contain small membrane bound vesicles, similar to vesicles occurring near the Golgi and within material was present similar to that of the immature granules; this was interpreted as evidence for a Golgi origin of the eosinophil granule. Granules from eosinophilic metamyelocytes and mature cells resembled mature granules of the myelocyte. Mature heterophil myelocytes were shown to pass through the sinus wall into the lumen; also closely associated with the wall were haemocytoblasts. This wall was seen as a continuous layer only broken by temporary pores for the migrating granulocytes. In Campbell's study, no structural differences

were seen between the stem cells either intravascularly (proerythroblasts) nor extravascularly (myeloblasts) located. Haemocytoblasts were observed in the same sections, eliminating any changes due to fixation.

Since very few studies have been made on the ultrastructure of developing avian granulocytes, mention will now be made of certain important studies of granule development in the mammal.

The earliest ultrastructural study of mammalian granulocytes was made by Pease (1956) on the bone marrow of guinea pigs. He found a range of intermediate cells in the development of neutrophils, eosinophils and erythrocytes from a common myeloblast. Neutrophil granules were described as being of two main types, a homogeneous granule with low electron density, found predominatly during later development, while in the earlier stages of differentiation, a larger more osmophilic granule within a vacuole was more common. Myeloblasts and reticular cells were distinguished by their large and well defined nucleolus (these features disappear from the other cell lines as they specialize) and large "watery" mitochondria which condensed with maturity of the cell. In the promyelocyte stage of neutrophils and eosinophils, endoplasmic reticulum developed and later decreased.

In a very comprehensive paper, Bainton and Farquhar (1966) reported on the origin, nature and distribution of granules in polymorphonuclear leucocytes from the bone marrow

of normal rabbits. The material was prefixed in glutaraldehyde, followed by post fixation in OsO_4. On the basis of size, density, time and mode of formation, two types of granules were described, azurophilic and specific, with both being formed by the Golgi complex. However, they originated from different faces of the complex and appeared at different times of cell maturation. The larger and more dense azurophilic granules were formed during the promyelocyte stage from the concave (proximal) surface of the Golgi by budding of large numbers of small vacuoles with a dense core which then aggregated to form a secreting granule before condensing. Specific granules, with a finely granular content, were pinched off as vesicles from the convex (distal) face during myelocyte stages. In the mature polymorphonuclear leucocyte, 80-90% of the granules were specific. These authors suggested that the change in the ratio of azurophilic to specific granules during the myelocyte stage had three possible causes: a) reduction in azurophil granules by repeated mitosis b) lack of new azurophilic granule formation after the promyelocyte stage and c) continuation of specific granule production.

Fedorko and Hirsch (1966) followed the formation of granules in the heterophilic myelocytes of rabbit bone marrow, using tritiated lysine and electron microscopic autoradiographic method. Granules of mature polymorphic leucocytes were considered primary lysosomes. These authors

were able to show that after thirty minutes the total label (37%) in the cytoplasm rose over the Golgi complex and after 3-4 hours fell (to 11%), coincident was an increase (to 32%) recorded over the granules. The time for incorporation and flow of the label was the same for myelocytes *in vivo* and *in vitro*. Fedorko and Hirsch suggested that these results demonstrated a function of the Golgi complex in incorporating (packaging) certain basic amino acids into cytoplasmic granules. No transition zone was demonstrated between the rough surfaced endoplasmic reticulum and Golgi. The label over the Golgi complex was often found associated with vacuolar structures containing dense material, termed nascent granules. No polarity of the Golgi complex was reported but the results did indicate the function of at least a part of the Golgi in granule formation.

A similar study of the development of the neutrophil from bone marrow of cats has been made by Ackerman (1968), and agrees essentially with the work of Fedorko and Hirsch (1966).

In summary, electron microscopic studies on avian granulocytes are few. Edmonds (1966) has shown that at 7 days cells containing dense membrane bound granules can be found. These granules appeared to be formed by the condensation of small vesicles containing electron dense material and are the precursors of the mature spindle shaped heterophil granules. Campbell (1967) studing the bone marrow of adult

chickens with both light and electron microscopy, was able to distinguish heterophils and eosinophils only with the electron microscope. Heterophil myelocytes contained two populations of granules, one arising near the cell surface and containing fibrillar material, (the actual origin of this granule type was unknown); the second more abundant type arose by condensation of vesicles containing globular material and were associated with the Golgi comples. Campbell followed the disappearance of the fibrillar granules and the maturation of the globular ones to spindle shpaed granules of the mature heterophil; no mention is given as to the fate of the heterophil fibrillar granule. Eosinophils contained only round granules of a fairly uniform size, the granule became denser as the cell matured.

In mammals more electron microscopic studies have been made. Bainton and Farquhar (1966) reported two populations of granules in rabbit polymorphonuclear leucocytes and suggested that they were produced from opposite faces of the Golgi complex. Fedorko and Hirsch (1966) did not see this polarity but their autoradiographic studies demonstrated the involvement of the Golgi apparatus in granule production.

MATERIALS AND METHODS

Eggs from a white Leghorn variety* were incubated on their sides in a Thelco incubator at 38 C. Humidity of the incubator was maintained by water in the bottom of the incubator. New (1966) suggests a relative humidity of 60% for optimum hatchability. However, in the present study while no humidity measurements were made it was assumed that the humidity was at least 60% and, from graphs presented by New, hatchability was not greatly affected by humidities higher than 60%. The eggs were rotated approximately through 45° twice each day.

During early studies it was found that embryos developed normally until about day 17 to hatching; after this time all embryos died. Dissection of these embryos revealed a certain amount of haemorrhage; the skin was blotchy with patches of blood clots, lungs contained blood, heart had stopped at systole (atria expanded with a blood clot) and there were blood clots in the peritoneal cavity. It was thought that death might have been caused by poor ventilation in the incubator. Circulation of the air in the incubator was improved by installing a small aquarian pump, which pumped air through the water at the bottom of the

* Supplied by Standard Biological Company, Toronto

incubator. Although the air flow was small, it was sufficient to cause air turnover preventing dead spaces in the incubator and at the same time keeping the humidity high. Later, with this improved circulation of air, viable embryos were obtained to hatching; some chicks were allowed to hatch.

Specimens of embryos and yolk sacs from two days incubation to hatching were prepared for light and electron microscopic studies. The greatest number of specimens were taken during the middle period of development i.e. 12 days to 14 days, as preliminary studies showed a great increase in granulocyte production in the yolk sac and within the embryo at this time. Eggs were removed from the incubator, opened at the blunt end using coarse forceps; as much of the albumen as possible was removed and the yolk decanted into chick Ringer (Appendix I) solution. In the case of young embryos, the yolk sac plus embryo was removed by cutting around the vitelline membrane outside of the area vasculosa and the membranes peeled off. With older embryos this was more difficult and the whole egg was immersed in Ringer's solution before the shell was carefully removed, without tearing membranes or blood vessels, leaving the yolk floating. In both techniques, the dissected yolk sacs were then gently agitated to remove excess yolk material and transferred to petri dishes containing Ringer solution. At this point the embryos were described according to their stage of development (Hamburger and Hamilton, 1951).

Preparation of Cells for Light Microscopic Studies

Light microscopic studies were carried out on cells prepared by the technique of touch imprinting, smearing and tissue sectioning. Cells were obtained from spleen, yolk sac, yolk sac vessels, bone marrow and blood from embryos of approximately 2 days incubation to approximately 2 days post hatching.

Touch Imprints and Smears

Prior to imprinting and smearing of cells, glass slides were thoroughly cleaned in 70% alcohol and polished with clean muslin. The spleen, which lies dorsal and lateral to the stomach in the bird, was removed using fine forceps and excess moisture removed by touching it to the surface of clean filter paper. When the spleen was removed from embryos older than 14 days, a surface of the organ was cut with scissors or a sharp scalpel and the cut surface gently touched to the slide; however, due to the fragile nature of spleens from embryos younger than 14 days, it was impossible to cut a surface without disrupting the whole organ. In these, the spleen was touched to the slide surface without cutting and splenic cells adhered adequately. Great difficulty was was encountered in attempting to obtain the spleen from embryos earlier than 9 days incubation, due to location of the organ and its extremely fragile nature.

Vessels dissected from the yolk sac were imprinted; however, after staining and studying these preparations, few

cells other than red blood cells from the lumen of the vessel were found. Consequently, smears of yolk sac vessels were made to obtain granulocytes from the yolk sac. Blood vessels either by themselves or the vessel plus the thick fold of tissue in which the vessel was suspended were carefully dissected out under chick Ringer solution using fine scissors. With the aid of a dissecting microscope, the folds were seen to be most prominent from 9 days on but decreased in thickness during the latter third of embryonic life. The thin vessels, both at the periphery and from areas of the yolk sac close to the embryo, were removed. A piece of vessel touch-dried on filter paper and placed on a cleaned slide was carefully teased and smeared using a fine camel hair brush.

Bone marrow smears from embryos of 12 days incubation and older were prepared. The developing bone, usually the tibia, was removed from the embryo and the surrounding tissue cleared away. The bone was then cut and the marrow gently squeezed out and smeared on a slide with a paint brush.

An attempt was made to sample blood from the heart and vitelline circulation from all embryos; this, however, was particularly difficult from the early stages of development, i.e. before 10 days, mainly because of the small volume of blood present and the difficulty of placing a capilllary tube in the vessel or heart. Fine capillary tubes were drawn and used to puncture the heart of older embryos. The

drop of blood obtained was then placed on a cleaned slide and a smear prepared by pulling out the drop with another slide. Blood was obtained from vitelline vessels by removing a vessel, quickly placing it on a slide and then gently forcing out the blood from in the vessel.

In all cases imprints and smears were air dried or fixed over iodine vapour for approximately 5 minutes or until the cells appeared brown. The slides were then stained with May-Grünwald Giemsa staining procedure (Jacobson and Webb, 1952, Appendix I).

Tissue Sections

Tissue blocks were removed from the spleen, fixed 4 to 6 hours in Zenker's fixative, dehydrated, cleared in Terpineol and embedded in Tissuemat (MP 58°C). Sections were cut at 5 thickness, mounted and stained with the May-Grünwald Giemsa technique of Strumia (1935-36) modified by the author (Appendix I).

Tissue blocks from the yolk sac were obtained as follows. The whole yolk sac was spread on a filter paper and placed in a petri dish containing Zenker's fixative for 4-6 hours (Appendix I). Then the fixative was replaced with 70% alcohol after which blood vessels or blood islands (depending on the age of the embryo) were dissected from three separate areas: close to the body of the embryo, anterior periphery and posterior periphery of the yolk sac. These tissues were treated similarly to the blocks of spleen.

The remaining yolk sac tissue was stored in 70% alcohol in sealed plastic envelopes. Early in this study several different fixatives, including Bouin's, 10% Formalin, Calcium Formol (Appendix I) were tried in an attempt to obtain a better staining reaction with May-Grünwald Giemsa stain; however, Zenker's fixative gave the best results and was used in all later studies.

Histochemical Methods

Observations on cells stained by the May-Grünwald Giemsa method indicated that granulocytes containing basophilic granules (i.e. basophils) could be readily distinguished from granulocytes containing eosinophilic granules, i.e. eosinophils and heterophils, subtle differences could be distinguished in the colour of their granules but this was much less evident in the early developmental stages of the cells. To distinguish more readily between the developmental lines of heterophils and eosinophils and to obtain additional information about the nature of the specific granules, several histochemical techniques were employed on cells prepared by sectioning, imprinting and smearing. Since mammalian eosinophils contain a peroxidase, several peroxidase techniques were used on the avian tissue. These included a modified Undritz Peroxidase reaction (Undritz, 1952) on smears, imprints and sections, Graham's Naphthol Pyronin (Graham, 1916) and Copper Peroxidase reaction (Sato and Yoshimatsu 1925) on smears and imprints. Further, since granules of mammalian

neutrophils are considered lysosomal, an attempt was made to demonstrate acid phosphatase in the bird granulocytes by the lead nitrate method of Gomori (Comori, 1950) on smears and imprints. Smears, imprints and sections were stained to determine the presence of Periodic Acid Schiff positive material using an aqueous method (MacManus 1948, Ackerman 1960) and an alcoholic method (MacManus and Murray, 1960). The details of these histochemical methods are given in Appendix II.

Preparation of Cells for Electron Microscopic Studies

Tissues for electron microscopic studies were selected from the following areas in the yolk sac and embryo: yolk sac vessels adjacent and peripheral to the body of the embryo and the spleen, from embryos at two days incubation to hatching but with the majority of specimens removed around 12 days. Embryos and their yolk sacs were harvested and staged similarly to that described in the previous section on light microscopy.

Vessels of the yolk sac were either dissected out individually, removing only the vessel or the vessel plus the thicker fold of tissue in which the vessel was suspended. In the case of some of the "thin walled vessels", the vessel with the surrounding area vasculosa was removed. In the initial studies, small tissue blocks were removed under fresh Ringer solution and then transferred to a drop of Palade osmium tetroxide fixative (Palade, 1952; Appendix III) where the tissue was further cut into small blocks about 1 mm square

using a clean razor blade. They were then immediately transferred to clean glass stoppered bottles containing fixative and processed through the steps indicated in Appendix III. Finally the tissue was transferred using a tooth pick to a capsule filled with Epon embeddant into which a small label had been inserted; the tissue was allowed to sink to the bottom and orientated as necessary. These capsules were heat polymerised first at 37° C for 16 hours and then at 60° C for 24 hours and finally allowed to cure at room temperature for 3-4 days before sectioning.

Several modifications were applied to the basic technique. In order to give better cytoplasmic fixation, to minimize the occurrence of artefacts and to compare the two methods of fixation, a second fixation method was used. A 3.5% gluteraldehyde solution was made up in both chick Ringer and Sorenson's phosphate buffer (Appendix III). Whole yolk sacs plus embryos were transferred directly to the gluteraldehyde fixative and fixed for half an hour. During this time dissections of the vessels were carried out and under these conditions, a much cleaner dissection could be made. Small tissue blocks were transferred directly to Palade buffered osmium fixative and carried through as described previously. Gluteraldehyde, made up in Sorenson's phosphate buffer, was used throught the later work after initially observing that the pH of the solution made up in Ringer was very low and that material fixed in this solution

suffered a great deal of visible shrinkage. The pH of the Sorenson's buffer was 7.2 - 7.4 which was closer to that of the final osmium fixative.

Blocks, sectioned from material prepared by the initial technique and those in which the tissue had been prefixed in gluteraldehyde but embedded in epon of the original 7.3 ratio, gave sections which although thin (grey or silver) showed a considerable amount of secondary chatter. In spite of varying the knife angle, cutting speed or depression of the moving arm of the microtome this problem remained, suggesting the embeddant was too soft, possessing some elasticity causing vibrations as the block passed over the knife. In an attempt to overcome this problem a harder epon mixture was used (Appendix III). However, the blocks produced from this mixture proved to be too hard and had a tendency to chip rather than section no matter what combination of the variables on the ultramicrotome was used. In addition the tissue appeared to be "squeezed". This suggested poor impregnation and to overcome this, the glass bottles, containing the tissue and the various concentrations of Epon in the last three stages of the procedure, were attached to an inclined turntable and agitated to aid impregnation. Sectioning did not improve and the orinal epon mixture (7.3) was used again.

Glass knives were prepared using an LKB knife maker (Type 7801B). A small reservoir of masking tape was attached to the back of the knife and sealed with paraffin wax,

and the knife clamped in the knife carriage of an LKB Ultramicrotome (Type 4801A). Sections were cut as thin as possible, to obtain grey sections (500-600A), and floated onto the water surface in the reservoir where they were flattened using chloroform vapour and finally picked up on an uncoated copper grid (E. Fulham. Type 300 mesh).

Contrast in the sections was improved by the use of heavy metal staining technique. Originally, single lead citrate stain (Reynolds, 1963) for membranes was used (Appendix III) but was later replaced by a method using uranyl acetate and lead citrate (Appendix III) which gave good contrast both for membranes and cytoplasm.

Sections were examined at low power with a Zeiss (EM9) electron microscope and at higher powers with an RCA (EMU3H) electron microscope.

Details of type of film and paper used in preparation of photographs may be found in Appendix IV.

OBSERVATIONS AND RESULTS

Light Microscopic Results

Spleen

1. Description of Granulocytic Cells

Imprints of spleen from normal chick embryos of 8, 9, 10, 11, 12, 17, and 18 days of incubation and of 2 day old chicks were examined. Cells from chick embryos of 12 days incubation were studied in detail because from preliminary observations of the spleen and yolk sac very active granulopoiesis was present at this time. All imprints were stained with the May-Grünwald Giemsa combination of Pappenheim blood stain; areas of study were chosen where the cells were evenly spaced to form a monolayer.

At 12 days a large number of cell types of the granulocytic, erythroid and lymphoid series were seen. By considering the nature of the cytoplasm, i.e. the degree of basophilia as shown by the intensity of blue staining, the presence, shape and location and different staining of granules and vacuoles, the pattern of chromatin present in the nucleus, construction of a series of developmental stages in the maturation of heterophils, eosinophils and basophils was attempted. The stages have been divided into divisions similar to those of Lucas and Jameroz (1961), i.e. stem cell,

promyelocyte, mesomyelocyte, metamyelocyte and mature granulocyte. Table I summarizes their descriptions of these stages in the heterophil, eosinophil, and basophil series. Because the cells are seen in all stages of development it is sometimes difficult to place a particular cell in a well definable compartment, i.e. to determine whether a cell is in late mesomyelocyte or early metamyelocyte stage.

(i) Cells of the heterophil Series (Figs. 2-18)

Measurements of cells (size indices) are shown in Table II.

Promyelocyte (Figs. 2, 3, 5, 7, 10, 12, 13)

The first cell recognisable as a granulocyte was the promyelocyte. These were large round cells (average size index 227.4) with a high nucleocytoplasmic ratio. The cytoplasm stained blue (i.e. basophilic) and contained a few irregular clear vacuoles. Some vacuoles containing faintly orange staining material were interpreted as procursors of later granules. Small dense magenta coloured refractile granules were present, tending to be located mainly over the nucleus. The nucleus, round and situated slightly off the centre of the cell, contained diffuse chromatin material staining a pale mauve colour. The terms early, mid and late promyelocyte have been used in order to attempt to differentiate promyelocytes of different ages. Early promyelocytes possessed basophilic cytoplasm with clear vacuoles, very faint orange granules and magenta granules situated over the

TABLE I

Description of heterophil, eosinophil and basophil developmental sequences (Summarized from Lucas and Jameroz, 1961)

STAGE	HETEROPHIL	EOSINOPHIL	BASOPHIL
Granuloblast	Large round cell, basophilic, chromatin delicate reticulum.	same	same
Metagranuloblast	No granules. Nucleus eccentric, chromatin aggregated.	Nucleus eccentric but more defined. Vacuoles uniform. Chromatin clumps larger. Precursor substance for specific granule present.	Vacuolization of cytoplasm. Scattered magenta granules less tendency to form rings. Nucleus eccentric, coarse chromatin.
Promyelocyte	Nuclear boundary indistinct. Magenta granules and rings, orange spheres in vacuolated cytoplasm.		
Mesomyelocyte	Definitive rods from orange spheres, half of total number of definitive rods. Some nuclear condensations.	Definitive granules present. Nucleus more definite chromatin more condensed.	Smaller cell, nucleus tends to central, less than half of total number of granules. Granules water soluble.
Metamyelocyte	Smaller cell. Cytoplasm with more than half of total number of definitive rods. Nucleus bean shaped. Chromatin condensation.	More than half of total number of granules. Nucleus round indented or constricted Chromatin clumped irregularly.	Contains half of total number of basophilic granules. Nucleus near centre dense chromatin network.
Mature	Full complement of rods, nucleus lobed. Chromatin condensed into clumps.	Full complement of round granules. Nucleus lobed, chromatin clumped close together.	Full number of basophilic granules. Nucleus single body.

TABLE II

Size indices of developmental cell stages of spleen heterophils of 12 day chick embryos

DEVELOPMENTAL STAGE	NUMBER	LONG AXIS (l.a.) (μ)	SHORT AXIS (l.a.) (μ)	SIZE INDEX (l.a. X s.a.)	AVERAGE
Promyelocyte	1	14.0	14.0	196	
	2	16.0	14.8	236	
	3	14.8	14.0	206	
	4	16.0	16.0	256	
	5	16.0	15.2	243	
					227.4
Mesomyelocyte	1	12.8	14.0	179	
	2	13.6	14.0	190	
	3	12.8	16.0	204	
	4	14.0	14.0	196	
	5	16.0	15.6	240	
					201.8
Metamyelocyte	1	10.4	11.2	121	
	2	11.6	12.8	148	
	3	16.8	14.8	249	
	4	10.8	10.4	112	
	5	14.0	14.0	196	
					165.2
Mature	1	10.0	10.8	108	
	2	12.0	10.4	124	
	3	10.0	10.4	104	
	4	12.0	12.8	154	
	5	13.2	12.0	158	
					129.6

nucleus (Figs.2, 3). The nucleus was round and usually central. Mid-promyelocytes have characteristics mid-way between early and late stages. A later stage of promyelocytes (Figs.10, 13) was seen which was similar except for the more eccentrically located nucleus, larger and more numerous magenta coloured granules over the nucleus and more numerous pale orange granules in the cytoplasm.

Mesomyelocytes (Figs. 2-8, 10-12, 15, 18)

Mesomyelocytes were classified primarily on the presence of orange and magenta granules and the persistence of clear vacuoles in the cytoplasm. They were smaller cells than the promyelocytes (average size index 201.8). Since the terms mesomyelocyte also encompasses a progression of morphological stages, again the terms early, mid and late have been used.

Early mesomyelocytes (Figs.2, 6a, b, 7a) are similar to the late promyelocytes except that the nucleus tended to be more eccentrically located in the cell and the orange cytoplasmic granules stained more densely. Late mesomyelocytes (Figs. 3-5, 8, 11) possessed many large round orange refractile granules which filled the cytoplasm and a few persisting magenta granules. The nucleus was eccentrically situated in the cytoplasm and there may be some clumping of the mauve staining chromatin. Mid-mesomyelocytes fall into a category between early and late; these will sometimes be referred to as mesomyelocytes (Fig. 4).

Early, mid and late mesomyelocytes fell into groups based on the amount of basophilia of the cytoplasm, and were classified as type 1 or type 2. Type 1 mesomyelocytes (Figs. 2, 3, 5) exhibited much less basophilia in the cytoplasm than type 2 mesomyelocytes (Figs. 6a, b, 7a).

Metamyelocytes (Figs. 2, 3, 6, 7, 8, 9, 13, 16)

The metamyelocyte was a smaller round cell (average size index 165.2) with the nucleus comprising about one third the cell area. The cytoplasm was less basophilic than that of the mesomyelocyte and stained a rather pale blue but was still usually visible. There was no difference in degree of cytoplasmic basophilia in the metamyelocyte stages. The predominant large spherical orange granules were much more distinctly delineated and more refractile; one or two magenta granules may persist in ring form, (Figs. 6a, 7b, 8).

The nucleus was round and eccentrically placed in the cell; its staining properties varied so that two types could be distinguished. In some cells, type 1, the nuclear chromatin was pale mauve staining and rather indistinct (Figs. 2, 7a, 8) although patches of condensed chromatin could be seen at the periphery of the nucleus. The other type, type 2, possessed an eccentric nucleus containing mauve staining coarser chromatin (Figs. 3, 6a). The cytoplasm was blue and contained many orange granules as well as a few magenta granules.

Again, for convenience, early, mid and late metamyelocytes are distinguished on the basis of the shape of the orange granules and the presence of magenta granules. In the early metamyelocyte the cytoplasm is packed with round refractile orange granules (Fig. 6a) and a few magenta granules over the nucleus; some may be in ring form (Figs. 7a, b). In the late metamyelocyte, there is a predominance of orange refractile granules; a single or very few magenta granules may still be found over the nucleus (Fig. 8). Some of the definitive granules have become elongated in the late metamyelocyte (Figs. 9, 13) and the magenta granules have disappeared.

Mature Heterophil (Figs. 5, 7a, b, 9, 13, 17)

This is the end cell of this developmental line and is the smallest stage (size index 129.6). The most characteristic feature of this stage is the presence of elongate spindle shaped refractile granules staining orange with May-Grünwald Giemsa stain (Figs. 5, 7a, 9). The nuclear densely clumped chromatin (Fig. 7b) stained mauve to pale blue. Usually the nucleus was bilobed (Fig. 5) although a few cells possessed three or more lobes (Fig. 7b). The cytoplasm stained very pale blue or not at all.

In summary the changes which have taken place during the maturation of the heterophil are as follows: There is a decrease in cell size (size index of 227.4 dropping to 129.6). The nucleus becomes smaller, more eccentrically

located and finally lobed; the chromatin becomes coarser. The cytoplasm loses its basophilia and eventually stains a very pale blue or not at all. During the development there is a change in the population of granules present in the cell from early stages where most prominent granules were denser magenta in colour and large and small granules could be found throughout the cytoplasm but mainly over the nucleus. Concurrently with these magenta granules, there were also present faintly staining orange granules in the indentation of the nucleus. The orange granules increased in size and number while magenta ones decreased, some becoming very large and reddish in colour. Finally only spindle shaped orange refractile granules were present in the mature heterophil.

Cells have been seen (Figs.7, 10, 14, 18) in which the nuclei display an unusual pattern of chromatin material. In these cells the nucleus is round or ovoid and situated slightly off cell centre, taking up at least one third of the cell area. The chromatin instead of being difuse or irregularly clumped is present as very discrete bundles making up a network and staining a reddish purple colour. This type of nucleus appears to exist in cells which would be classified as heterophil promyelocytes, mesomyelocytes and stem cells on the basis of the characteristics of their cytoplasm and are found in close proximity to cells of this type. Close examination of the chromatin showed that it was in bundles and not in a continuous dense strand, and this

would suggest that these cells are not in prophase of mitosis but that the pattern is possibly due to densely staining heterochromatin.

(ii) Cells of the esoinophil series (Figs. 13-16)

Imprints of spleens of 12 day embryos possessed very few cells which could be termed eosinophils. The most mature eosinophils recognised are described and then the less mature ones. No size indices are given as not enough cells were seen for measurements.

Metamyelocyte or Mature eosinophil (Figs. 13, 15, 16)

This cell was round; the cytoplasm stained a very dense basophilic blue (Figs. 13, 14) and contained only round orange refractile granules which stained somewhat denser than those of heterophils. The nucleus was slightly eccentric, not lobed and contained coarse chromatin which stained deep purple-mauve (Fig. 13). None of the granules in the cytoplasm were very large in comparison to the large round orange granules of meso- and metamyelocytes of the heterophil.

Mesomyelocyte (Fig. 14)

In this early stage, the nucleus was oval and occupied about half the cell area; more cytoplasm was visible than in the later stage and stained heavily basophilic. Round refractile orange granules of a uniform size were present in the cytoplasm; no magenta granules were seen. The nucleus was eccentric, non-lobed and consisted of dense

chromatin material staining a red-mauve colour. This cell was similar to cell 1, Fig. 15 except that there was more cytoplasm present and there were some clear vacuoles.

(iii) Cells of the basophil series (Figs. 17, 18)

Few basophils were seen in the spleen at 12 days; a developmental sequence could not be constructed.

Immature basophil (Fig. 17)

This was a medium sized cell in which the cytoplasm stained a very pale blue. Only dense purple granules were seen which were situated mainly round the periphery of the cell, although a few could be found over the nucleus. The nucleus was large and its diffuse chromatin stained pale purple.

Mature basophil (Fig. 18)

The mature basophil was a round cell smaller than the mature heterophil; the cytoplasm where visible stained pale blue. The nucleus was not lobed, eccentric and stained pale blue, often being indistinguishable from the cytoplasm, the chromatin being diffuse. Round dense purple granules the most noticeable feature of this cell, packed the cell but in some cases were not found over the nucleus. These granules were quite distinct from those of any other cell line, being much more purple in colour and of a more or less uniform size, whilst the magenta granules of the immature heterophils existed in various sizes.

2. Description of Stem (Blast) Cells (Figs. 2, 6a, b, 7b, 10,13)

Three types of stem cell have been identified from imprints of the 12 day spleen; the differences are primarily of colour of the nucleus and its chromatin pattern.

The first type (Figs. 6a, b, 10) is a large cell, the cytoplasm was very basophilic staining a deep blue; there were some clear spaces in the cytoplasm. The nucleus was large, round or ovoid and the chromatin material was coarse but not in clumps and stained red-purple. A blue staining nucleolus may be visible. This cell was similar to the blast cell of the erythrocytes as described by Lucas and Jameroz (1961). In the present work, cells very similar to this stem cell can be seen in which there are small dense purple-magenta granules in the cytoplasm and over the nucleus as well as faint orange granules in the cytoplasm. For this reason it is suggested that the stem cell described above may give rise to granulocytes.

A second stem cell (Figs.6a, b) is again a large cell. Its cytoplasm is blue and the nucleus round or ovoid. In contrast to the first type of stem cell, the nucleus stained very pale blue and its chromatin was in the form of extremely fine strands. A slightly denser blue nucleolus is seen. In addition to this cell one can see similar cells in which small dense granules had developed and further, cells with the same type of nucleus but with orange and magenta

granules in the cytoplasm.

A third type of stem cell (Fig. 11) was similar to the type one stem cells; i.e. a large cell with a round or ovoid nucleus filling about two thirds of the cell. However, it differs from type 1 in that the cytoplasm was less basophilic and the nucleus stained a pale red-purple colour and had chromatin as a fine network. Very early granulocytes with the above characteristics have been seen with faint orange granules and small magenta granules in the cytoplasm and over the nucleus.

Tissue Sections of Spleen (Figs. 19-24)

Paraffin sections of spleen stained with modified May-Grünwald Giemsa were studied in order to determine the location of granulocytes in that organ, and their developmental sequences. It was found impossible to differentiate heterophils from eosinophils, and basophils were not seen although these three cell lines could be seen in imprints from 12 day old spleens. At 12 days granulocytes with orange staining granules could be found throughout the spleen tissue (Figs. 20-22), lying in cords of splenic cells from the edge to the center of the organ. Granulocytes, in close association with splenic cells (Figs. 20-22) were found deep in the tissue of the spleen distant from the spleen sinuses, bordering them occasionally within the sinus and along large vessels entering the spleen (Fig. 22). There were no specific concentrations of granulocytes in any area of the spleen.

Although the staining properties of cells in paraffin sections were not the same as those seen in imprints and smears (for example, orange granules were less refractile and the orange colour was less brilliant, tending to be more reddish), it was possible to determine some different stages in the development of these cells. In Fig. 20, four cells are interpreted as representing different stages in the development of heterophils. A heterophil promyelocyte (Fig. 20, cell 1), is a large cell with a large pale staining nucleus, taking up about half the area of the cell, containing faintly clumped chromatin material and a large nucleolus. Basophilic cytoplasm and faint orange granules to one side in the slight indentation of the nucleus were seen; two large denser granules were present. A heterophil mesomyelocyte (Fig. 20, cell 2) is a smaller cell, with a pale staining eccentric nucleus, larger clumps of chromatin substance and prominent nucleolus, slightly basophilic cytoplasm containing some clear vacuoles and orange staining granules with some denser staining (redder) granules near the nucleus. A heterophil late meso-or early metamyelocyte (Fig. 20, cell 3) possesses a small eccentric nucleus and prominent orange refractile granules, some having a dense rim. Mature heterophils can be seen (Fig. 20, cell 4; Fig. 22, cell 2) containing elongate orange refractile granules; the nuclei in these cells are not easily seen. Similar forms were present throughout the spleen.

Studies of sections of 10 day embryonic spleens showed that all the above stages could be found (Figs. 23, 24) although there were many fewer granulocytes than in the 12 day spleen. Generally they were found near the periphery.

Yolk Sac

Tissue Sections

(i) Blood vessels, granulocytes, endodermal cells as seen in the yolk sac of 12 day embryos (Figs. 25-35)

Examination of paraffin sections of the yolk sac of 12 day chick embryos which had been fixed in Zenkers fixative and stained with modified May-Grünwald Giemsa stain, showed the presence of two types of blood vessels. I have termed these "thick walled" (Fig. 25) and "thin walled" vessels (Fig. 26). The differences between the vessels is due to the presence or absence of clumps of cells lying outside the single layer of endothelial cells forming the vessel wall, producing grossly "thick" and "thin" vessels. The extra-vascular areas or clumps could be divided into three parts consisting of a pale center, a denser periphery and an area between the clumps (Figs. 27-29). The centrally located cells are developing red blood cells (Figs. 28-29); their cytoplasm is pale or colourless. In more advanced clumps the cytoplasm of these cells has a faint red colour (Fig. 29). Their nuclei are round to oval and contain condensed masses of chromatin; the nucleus stains a bluish green colour.

Surrounding these paler areas are cells in which the cytoplasm stains much more basophilic (blue); the nuclei of these cells are round and contain a prominent nucleolus. Small condensations of chromatin material may be present. These groups or clumps of cells are thought to be developing "auxilary" vessels (my term) forming outside the main central vessel.

The more basophilic cells are possibly haemocytoblasts or blood island cells similar to those present in the initial stages of blood vessel formation in the yolk sac. Although it is rather difficult to see a direct transition between the cells of the periphery and those of the center, there is a gradual change in the density of the staining properties of the cytoplasm of cells from the edge to the central region; the cells become much less basophilic. These basophilc peripheral cells may be the stem cells for the central erythrocytes.

In all the sections studied at this time of development, no connections were seen between the lumens of the central vessel and the auxillary vessels. Examination of the 12 day yolk sac under the dissecting microscope does show that certain vessels i.e. those which hang down into the yolk material and which tend to have a thickened appearance, were suspended by a cord of material; this cord under high power was composed of cells similar to the so-called clumps around the vessel. Around the vessels and clumps were endodermal cells. With the dissecting microscope, this main suspended

vessel was seen to have attached to it many smaller vessels which appeared to form loops starting and finishing on the central vessel. These too were covered by endoderm and in some, red blood cells could be seen. This arrangment of loops gave a convoluted appearance to the vessels, and was much more prevalent towards the periphery of the yolk sac where the vessels divide. Other types of blood vessels were seen with the dissecting microscope, which were much thinner walled (in the context used above) and lay close to the ectoderm; in tissue sections these vessels (Fig. 26) had no clumps of cells surrounding them. These vessels contained more blood cells than the others; such vessels were tentatively identified as veins. Small spheres of yolk were seen with the naked eye around all the vessels.

The third area around the thick vessels is located outside the main vessel and between the individual clumps of cells. Large numbers of granulocytes were found in this area (Figs. 28, 30, 31). These cells were round to ovoid, the cytoplasm was not usually visible being packed with orange refractile granules, most of which were round although occasionally elongated. No magenta granules were seen but a few ring shaped orange coloured granules were observed (Fig. 31). The bilobed nucleus stained blue, and contained coarsely clumped chromatin material; nuclei with single and triple lobes were also seen. Stages in the development of granulocytes in sections could not be determined. It was not

possible to distinguish heterophils from eosinophils nor were any basophils seen. In sections both single eccentric indented nuclei (Fig. 31) and bi- and trilobed nuclei were seen to possess clumped chromatin. However, care must be taken when comparing the nature of chromatin material in sections and imprints as the methods of fixation are quite different. Those cells with cresent shaped nuclei are considered immature and are regarded as mesomyelocytes or metamyelocytes; and although the cell line cannot be identified with any accuracy, they are believed to be heterophils as no eosinophils were seen in smears of yolk sac vessels and very few in spleens of this age. Some granulocytes were seen where the granules had a denser rim and the granules stained a reddish orange colour; such granules may correspond to the magenta ringed ones of imprints and smears from yolk sac vessels and spleen.

A fourth type of cell was observed in this extravascular area (Fig. 27). This was a large cell, round or oval in shape, with strongly basophilic cytoplasm. The nucleus was large with small clumps of chromatin and a prominent elongated nucleolus. This cell has been termed a blast (or stem) cell by the author.

Within the lumen of the thick vessels are found different stages in the development of erythrocytes as well as cells which have the same morphology as the basophilic cells on the periphery of the auxillary vessels. A well

developed endothelium, consisting of elongate cells with spindle shaped nuclei, is present and in certain areas (Fig. 32) there are round intravascular cells with sparse cytoplasm and round pale nuclei resembling those of the blast cells in the clump. Some of these cells appeared to be attached to and were possibly being budded from the endothelium.

Granulocytes were not confined solely to extravascular areas of the thick vessels but could be found in large groups around smaller vessels near the ectodermal suface of the yolk sac. Figure 33 shows such a vessel developing under the ectoderm; it has a similar structure to those already described. The granulocytes appear to be at the same stage of development as those around the large vessels. Some granules are in ring form and are slightly more densely coloured at the rim. A few elongated loose granules were seen in this section, perhaps the result of a damaged mature heterophil. Granulocytes with orange refractile granules were also seen deep in endoderm (Fig. 34) often as a long thin chain of cells stretching between two blood vessels. Close examination of such granulocytes often showed them to be outside the endothelium of a small vessel; no other cells apart from endodermal cells were present in these areas. Single granulocytes were sometimes found quite isolated in the endoderm, again with no other cell type around. In some vessels granulocytes approached the wall; these may possibly enter the lumen of the vessel (Fig. 35).

Large and small stem (blast) cells were seen around all the "thicker vessels". Large "thin walled" vessels were found in the same sections as thick ones. Endodermal cells with eccentric nuclei,often difficult to distinguish, and large vacuoles enclosing yolk remnants abut all the vessels.

(ii) 4 day embryo (Figs. 36, 37)

Granulocytes (Figs. 36, 37) were found extravascularly, close to thin walled vessels in the yolk sac of embryos incubated for 4 days. This was the earliest time that such cells were seen. Three different cells were seen: 1) a large cell with a basophilic blue cytoplasm containing some eosinophilic round orange refractile granules, and slightly eccentric round nucleus with a prominent nucleolus, 2) a smaller cell with an ovoid nucleus containing clumps of chromatin and with orange granules found all over the cell, 3) and a smaller cell which possessed a blue staining nucleus and many specific round granules. Because of the difference in cell sizes and the numbers of granules, the first cell described above is considered as the more immature, possibly a promyelocyte, and the last two as late meso-or early metamyelocytes.

Many small vessels were seen lying close to the ventral surface of the ectoderm as well as larger vessels, surrounded by a stalk of endodermal cells, protruding into the yolk. Endodermal cells were large and the small amount of non-vacuolated cytoplasm present stained acidophilic. The cells contained many vacuoles. These were large and small

often with yolk material in which case they stained pink with modified May-Grünwald Giemsa stain; otherwise they appeared empty. Two types of yolk granules were seen, those with a uniform granular appearance (Fig. 36 YG1) and others where the center of the yolk granule appeared much paler (Fig. 36 YG2). The nuclei of the endodermal cells were located in the cell towards the small vessels lying under the ectoderm; the large vacuolar bulk of the cell lies in the yolk.

Apart from endodermal cells and their nuclei, no other cells were recognizable generally. Cells similar to the small stem cell present outside vessels at 12 days were seen within the 4 day vessel but rarely extravascularly. These are round basophilc cells with a round pale staining nucleus and a prominent nucleolus. These cells may be precursors of the red cell series.

(iii) 5 day embryo (Figs. 38-42)

At five days incubation the yolk sac had a well defined circulatory system and sections showed that although there were no "thick walled" vessels as described in the 12 day yolk sac, some vessels had begun to sink away from the surface ectoderm coming to lie deeper in the yolk material (Fig. 38). No vessels were seen with large numbers of cells outside the endothelium. Occasionally one or two stem cells could be found (Fig. 38) in such a position. Besides the larger vessels containing well developed red blood cells, many smaller vessels were seen near the surface of the yolk

sac lying just under the ectoderm; these vessels were packed with early stages in the development of red blood cells and basophilic stem cells (Figs. 38, 39). In several cases these stem cells (Figs. 39, 40) appeared to be migrating out of the small vessels into the larger thin walled vessels (Fig. 38).

By 9 days incubation the thick walled vessels were well established and the yolk sac had much the same appearance as that at 12 days.

In an attempt to determine the origin of the extra-vascular clumps of cells, an examination of the yolk sac at both 12 days and 5 days was carried out. The peripheral area of the yolk sac of 12 days had the general appearance of earlier 5 day yolk sac sections; changes were seen in the endodermal cells as areas closer to the embryo were studied. Endodermal cells were packed with large and small granules of yolk and possessed readily visible blue staining nuclei found at various levels of the cell, while those cells towards the embryo were larger more vacuolated cells with fewer nuclei visible and were in a position towards the ectoderm or blood vessel.

Both in the periphery of the 12 day and in all areas of the 5 day yolk sac, small blood vessels lined with an endothelium and packed with stem cells (Figs. 39, 40, 41) were found; stem cells either completely filled the vessel or lined the endothelium. Often three vessels existed together, a

a central vessel with a thick endothelial lining, with two vessels on either side, in which the endothelium was drawn-out and much thinner. Some of these vessels had clumps of stem cells within and not existing as free clumps. Often in such vessels (Fig. 42) with well defined endothelial wall and containing red blood cells, and situated under the ectoderm, numerous granulocytes were located on the endodermal side of the vessel. Finally, the whole was surrounded by a larger developing vessel which contained several stem cells giving the impression that the granulocytes are in the lumen of the second vessel as no separating endothelial wall can be seen.

Figure 41 shows a small vessel with some stem cells outside. In this developing area of the yolk sac, vessels were surrounded by stellate shaped mesenchymal cells, but no transitional stages from mesenchyme to blood cells or vessel cells were seen. Granulocytes were always seen outside the developing vessels (Figs. 39, 42).

Granulocytes were present extravascularly in the 5 day yolk sac (Fig 29) either singly or two, never as cords or clumps around the vessel. Granulocytes were seen only individually (Fig. 29) or in twos, never as the cords or clusters around vessels as is seen at 12 days.

Granulocytes from smears (Figs. 43-51)

Smears of yolk sac vessels and the thickened tissue around the vessels were prepared from embryos of 9 to 12 days

incubation. Although many of the cells were damaged, some intact cells were found and compared to cell stages found in imprints of spleen. Blood cells were similar to those seen in the spleen. At 10 days (Figs. 43-46, 48, 49), all four developmental stages of heterophils were present as well as mature cells. No cells definitely recognisable as eosinophils were observed and basophils were rarely seen (Fig. 47). There were no differences found in the cell types seen in 9, 10, 11 or 12 day smears.

Two types of blast cells were seen (Figs. 43, 50, 51); both had basophilic cytoplasm, large nucleo-cytoplasmic ratio. However, the nuclei differed. One contained a large pale purple blue nucleus with diffuse chromatin, in the other the chromatin was coarser and stained more intensly purple. These colour differences were not easily demonstrated with colour photomicrography. The line to which these blast (stem cells) belong is not known.

Electron Microscope Results

Spleen

1. Cells from 14 day embryos (Figs. 52-59)

With the electron microscope, granulocytes from the spleens of 14 day old embryos were readily identified by the presence of granules of varying degrees of density and structure (Figs. 51-59). Three types of granules have been identified in the same cell (Fig. 55). The granule types are described

as follows: 1) A large round dense granule surrounded by a single membrane which may be closely applied to the substance of the granule or in some cases (Fig. 55) leaves a"perigranular" space. At low magnification the content of the granule (Fig. 55) appeared uniformly dense although at higher magnifications it was seen to be composed of fine granular material in a homogeneous matrix (Fig. 56). In the electron micrographs presented, the majority of this type of granule appeared to be round, both large or small. Elongate granules of similar structure were also seen (Fig. 54). 2) The second type of granule had a similar fine granular structure (Figs. 55-57) but the granule itself was not uniformly dense. There were larger or smaller patches of clear areas. These clear spaces could be few in number or take up a large part of the granule so that it appeared blotchy (Figs. 56, 57) or produce a granule in which the electron dense material was concentrated in areas around the periphery or as a core in the center (Fig. 57). There appeared to be a progression in the development of this type of internal structure, from being dense with few clear areas to having a very loose structure. 3) The third granule type was much less distinct and less often seen (Figs. 55, 57); it tended to be elongate and about the size of the smaller dense granules. The content of this appeared to be made up of a fibrillar material aligned along the long axis of the granule. An indistinct membrane either surrounded it or was broken or absent.

A fourth possible type of granules (Fig 56) was a membrane bound loose condensation of electron dense material not unlike that of granule 2 but much less dense; it may also be found in a more dense form (Fig. 55), surrounded by a membrane less tightly applied to the internal material.

With the limited number of micrographs available it was not possible to form a developmental sequence of the granulocytes. However, from the type of granules found in the cells, it was thought that some stages could be identified. Light microscopic studies indicated that three types of granules could be identified on the basis of their staining properties: dark staining magenta granules both large and small with some larger ones having a ring-like appearance; round orange granules varying in the intensity of colour becoming more intense and refractile in older cells i.e. meso-and metamyelocytes; and elongate granules of the mature heterophil. From the electron microscope results it was not possible to identify eosinophils or basophils, and considering the morphology of the granules and nucleus, the cells in the micrographs were identified as heterophils. The large round granules of type 1 may correspond to the orange staining granules seen in imprints and sections stained with May-Grünwald Giemsa. Those granules in which the matrix appeared to be breaking up leaving a core or a ring of electron dense material are thought to correspond to the magenta rings seen in late promyelocytes and mesomyelocytes of heterophils (Figs. 52, 53).

Elongate granules similar to granule type 1 were seen in some cells (Fig. 54), and were possibly derived from the round granules. Little can be said about type 3 granule; few are seen and may be confused with degenerating mitochondria, although in Fig. 55 the two present at the periphery of the Golgi complex do not resemble mitochondria. The fourth type of granule appeared to be a vacuole with electron dense material present and may possibly be a developing granule.

Although no sequences of cell development have been seen two stages were identified. Figure 1 shows a meso-myelocyte and is identified tentatively as a heterophil. In this cell the nucleus was situated eccentrically, chromatin material appeared evenly distributed with slight condensation around the periphery, nucleolar material was faintly visible. Granules present were types 1, 2, and 3; they were all round. A Golgi complex was prominent (Fig. 55), consisting of flattened lamellae with very small vesicles associated with it at the edge and on the face towards the nucleus. These vesicles were small, some containing a diffuse dense material, and others appearing to have a denser periphery and relatively clear central area. The vesicles appeared to exist singly with no indication of their associating into groups or clusters. There is, however, one vacuole (Fig. 55) situated towards the nucleus in which there is a dense core with an internal structure of small round vesicles, around the core is less dense material; the whole is surrounded by a membrane. The

cell contains few mitochondria which tend to be located around the Golgi complex. There are many ribosomal granules in the cytoplasm both as single particles or in clusters; swollen rough surfaced endoplasmic reticulum containing some diffuse material is also seen. The cell has the appearance of active synthesis. Similar types of cells are seen in Figs. 58, 59 ; in both these cells there is more swollen endoplasmic reticulum and although no nucleus can be seen, it is believed that these two cells represent mesomyelocyte stage.

Figure 54 shows a heterophil metamyelocyte; there are several spindle shaped granules while round ones are still present. The nucleus is lobed, small vesicles are present and may be covered with ribosomal granules or have a coating and a large amount of swollen rough surfaced endoplasmic reticulum is still present. The cells bordering this granulocyte possess several small coated pinocytotic vesicles. Such vesicles are not definitely seen in this granulocyte (Fig. 54) although two can be seen forming in the granulocyte of Fig. 59. Also present is a myelin figure, its significance and origin is not known.

Other cells are seen (Fig. 52) but it is not possible to identify with certainty which of these cells represents stem cells (of granulocytes or erythrocytes) and which are parenchyma. By a consideration of size, cell 1 and possibly cell 2 may be stem cells; cell 1 in particular is larger, has

an ovoid nucleus with a prominent nucleolus.

Yolk Sac (Figs. 60-62)

Material obtained from the yolk sac proved very difficult to section. Fig. 60 shows a cell, thought to be an early developmental stage of a blood cell surrounded by endodermal cells. The nucleus is round with diffuse even chromatin and a nucleolus. The cytoplasm is filled with large vacuoles many of which are clear though some contain particles of dense material. Some vacuoles appear to have a distinct membrane while others lack a surrounding membrane. Close examination of those with an internal structure suggest that they may be expanded mitochondria, the internal structure being remains of cristae (it was for this reason the author attempted to change the fixation and embedding procedure). Membranes of the endoplasmic reticulum can be seen, and many ribosomal particles between the vacuoles. There is close association of this "blood cell" with the adjacent endodermal cells; at one point the cells interdigitate and a desmosome can be seen (Fig. 60).

Some of the structure of blood vessels and endodermal cells were seen. Fig. 61 shows part of the wall of a blood vessel; it consists of a thin but well defined endothelium. The endothelial cells rather than abutting end to end are arranged in an interdigitating fashion so that cells overlap each other with long processes. At the lumen side, coated pinocytotic vesicles are readily seen. Other vesicles are present

in the cytoplasm and may have dense granules on their membranes. The pinocytotic vesicles occurred less frequently on the endodermal side (Fig. 62). The nucleus of the endothelial cell is elongate and the nuclear membrane swollen in places. Spaces between endothelial cells and between endothelium and endoderm were filled with some form of dense material, a basement membrane was associated with the endodermal cell but there were also numerous particles (possibly ribosomal or lead precipitate from over-staining). Nucleated red blood cells were seen in the lumen of the vessels, the perinuclear space of these cells was swollen; this was believed to be a fixation artefact. Endodermal cells appear to be only loosely attached to the endothelium by small cell processes (podia); coated pinocytotic vesicles are seen on this surface (Fig. 62).

Endodermal cells seen with the light microscope appeared as large vacuolated cells. Nuclei was difficult to locate and tended to be situated towards a blood vessel, and were surrounded by a small amount of cytoplasm with light microscopy. The vacuolar nature of the cells varied according to where the section was made, vacuoles being apparently empty in "older areas", i.e. towards the embryo, and filled with yolk granules at the periphery of the sac. This general picture was confirmed with the electron microscope. The cells appeared large and filled with a great many vacuoles (Figs. 60, 62), some reaching an extremely large size and almost fill the cell. Many of the membrane bound vacuoles

were clear, although some contained dense particles (these may be a lead precipitate). Yolk granules were present (Figs. 61, 62) and were either densely granular with clear areas or consisted of a less dense periphery and denser core. There were also compound vacuoles which contained yolk granules and other membrane structures (Fig. 60). Myelin figures, very commonly found in endodermal cells (Fig. 62), appeared to grade in size. Some appeared small and loosely packed while extremely large ones could be found.

Histochemical Results

Histochemical Results (Fig. 63)

Smears, imprints and sections were stained for peroxidase, PAS positive material and the presence of acid phosphatase. None of these staining techniques provided a method for clearly identifying eosinophils from heterophils.

Undritz II Peroxidase Method for Imprints and Sections

Imprints of spleen and sections of yolk sac from embryos of 10-18 days were studied and in no case was a positive reaction, as indicated by the presence of a greenish yellow colour obtained in any of the granulocytes, mature or immature. The cytoplasm of red blood cells did give a greenish yellow colour indicating peroxidase activity. Granulocytes stained similarly, in this reaction, to those treated with the ordinary May-Grünwald Giemsa combination except that the colours were less brilliant, perhaps due to the

weaker staining solutions employed. Although of no advantage for observations on imprints, the May-Grünwald Giemsa stain plus peroxidase, stained tissue sections better than ordinary May-Grünwald Giemsa technique for sections.

Periodic Acid Schiff Reaction

No clear distinction was obtained between eosinophils and heterophils although PAS positive granules in many leucocytes were seen in 18 day spleen imprints and tissue sections. Cells in tissue sections, gave a much weaker reaction so that although granulocytes could be seen the granules appeared as clear spaces both in aqueous and alcoholic PAS techniques. Yolk granules gave a strongly positive reaction and some positive material was also found associated with blood vessels. Imprints of 18 day spleens stained with alcoholic PAS contained granulocytes with PAS positive round granules (Fig. 68) which considering the size of the cell, shape and position of the nucleus were interpreted as meso-myelocytes and metamyelocytes. These cells contained many clear vacuoles as well as granules. Granules of mature heterophils did not stain. Controls of 12 and 18 day spleen imprints and yolk sac sections, digested for 20-30 minutes with diastase, showed very faint pink granules.

Copper Peroxidase for Leucocyte Peroxidase

This stain gave negative results.

Graham's α-Naphthol Pyronin

This stain gave negative results.

Gomori Acid Phosphatase

Imprints stained with this technique gave unsatisfactory results. There was much debris which may have been loose granules or lead precipitate; little information could be obtained form such slides although it was possible in some cells to see faint brown granules.

FIGURES

Symbols used for Photomicrographs

Magenta granules	m
Vacuoles	v
Round orange granules	o
Elongate (spindle) orange granules	e
Magenta ring-type granules	mr
Nucleolus	*
Cord Splenic cells	c
Spleen sinus	s
Vessel	Vs
Red blood cells	r.b.c.
Basophilic cells of auxillary vessel	BC
Endoderm	Ed.
Developing vessel	dev. vess.
Granulocytes	Gr
Ectoderm	Ect.
Yolk granule	YG
Mesodermal cells	M

Imprint 12 day Embryo Spleen stained
with May-Grünwald Giemsa stain

Fig. 2 x1520

Cell 1 Heterophil early promyelocyte. Note basophilic cytoplasm containing small dense magenta granules (arrow m) mainly over the round nucleus, faint orange granules (arrow o) high nucleo-cytoplasmic ratio, chromatin in diffuse network staining mauve.

Cell 2 Heterophil early mesomyelocyte, (type 1) Note the cell possess the same characteristics as cell 1 but has more prominent orange granules (arrow o)

Cell 3 Stem cell. Note basophilic cytoplasm with some vacuoles, high nucleo-cytoplasmic ratio, chromatin material in network staining mauve.

Cell 4 Stem cell. Similar to cell 3 above but with a more prominent nucleolus.

Cell 5 Heterophil early metamyelocyte. Cytoplasm stains pale blue where visible and is packed with large round orange refractile granules.(o) Nucleus stains mauve but is less well delineated than in mesomyelocytes. Four magenta granules are seen (arrow m)

Fig. 3 x2000

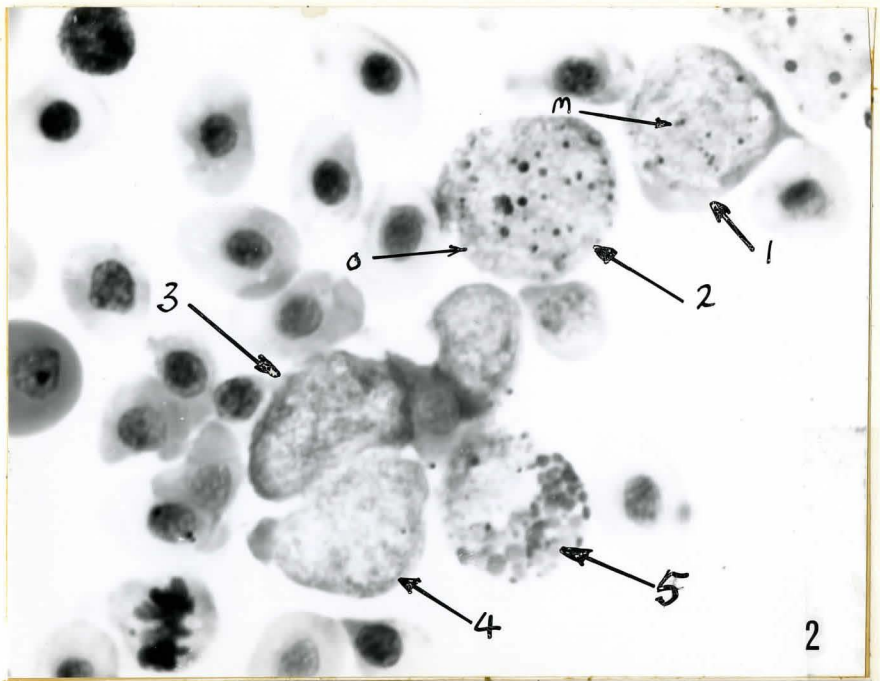
Cell 1 Heterophil early promyelocyte. Cytoplasm intensly basophilic, contains a few clear vacuoles near the periphery(v) Some faintly orange granules and small dense magenta granules are found over the nucleus. Nucleus is large, round, high nucleo-cytoplasmic ratio, chromatin in coarse network stains mauve.

Cell 2 Heterophil late mesomyelocyte, (type 1). Cytoplasm less basophilic than type 2; large clear vacuoles many large round orange refractile granules; large magenta granules are present over the nucleus. Nucleus eccentric, less distinct chromatin stains pale mauve.

Cell 3 Heterophil mid-mesomyelocyte, (type 1). Nucleus eccentric and more distinct and coarse chromatin stains mauve, cytoplasm less basophilic than type 2.

Cell 4 Heterophil mesomyelocyte, (type 1). Contains both orange granules and few magenta granules. Cytoplasm is only slightly basophilc. Nucleus stains pale blue and is not easily distinguished from cytoplasm.

Cell 5 Heterophil early metamyelocyte, cytoplasm is pale blue and packed with orange granules most of which are round although some may be elongate. Nucleus is elongate and slightly bean shaped, slightly clumped chromatin stains pale mauve.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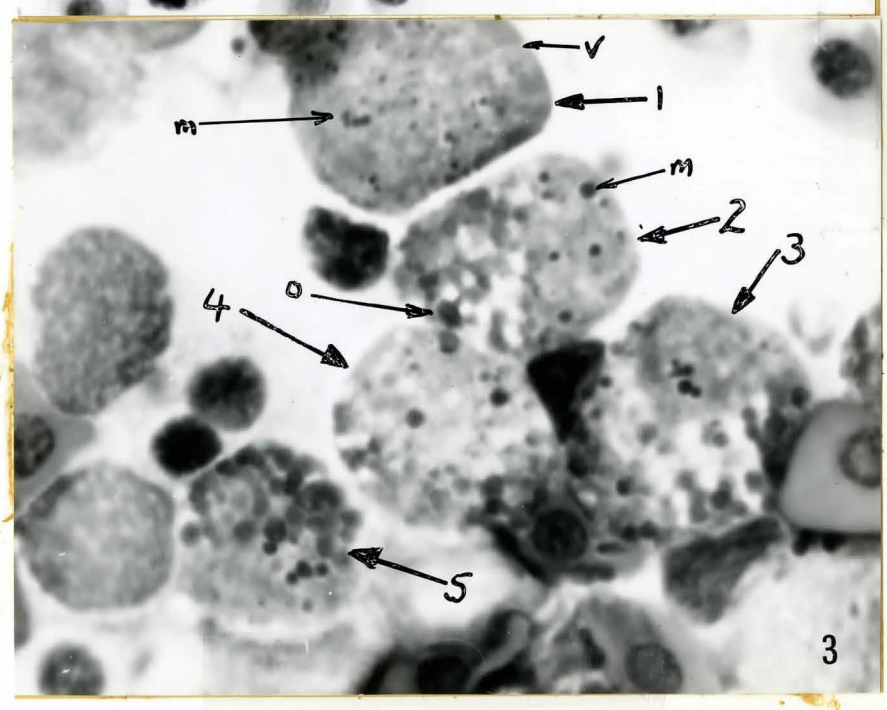
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Imprints from 12 day Embryo Spleen Stained with May-Grünwald Giemsa stain

Fig. 4 x1700

Cell 1 Heterophil early mesomyelocyte (type 2) strongly basophilic, cytoplasm with large round orange granules and magenta granules showing a range in size present over the nucleus. Nucleus is mauve coloured and indented.

Cell 2 Heterophil, mesomyelocyte (type 1). Cytoplasm is less basophilic than that seen in cell 1, bright orange granules, magenta granules. Nucleus has slightly coarser chromatin stains mauve, not indented.

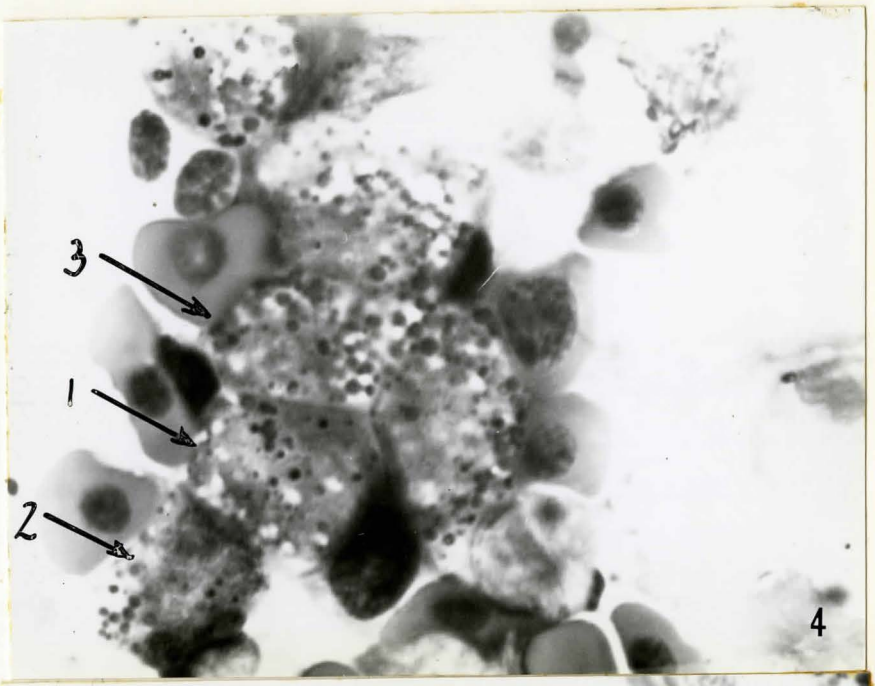
Cell 3 Heterophil, late mesomyelocyte (type 1); (or early metamyelocyte) cytoplasm pale blue, few magenta granules, many large orange refractile granules. Nucleus eccentric with slightly clumped chromatin staining pale mauve.

Fig. 5 x2000

Cell 1 Heterophil, promyelocyte, Basophilc cytoplasm small round faint orange granules, small magenta granules over nucleus. High nucleo-cytoplasmic ratio, nucleus eccentric diffuse chromatin stains mauve.

Cell 2 Heterophil late mesomyclocyte (type 1) Cytoplasm pale blue, clear vacuole and round orange refractile granules filling most of cytoplasm, few magenta granule rings over the nucleus. Nucleus eccentric, chromatin forming some clumps stains mauve. Decrease in nucleo-cytoplasmic ratio.

Cell 3 Heterophil, mature. Elongate orange refractile granules, bilobed nucleus condensed chromatin stains pale mauve.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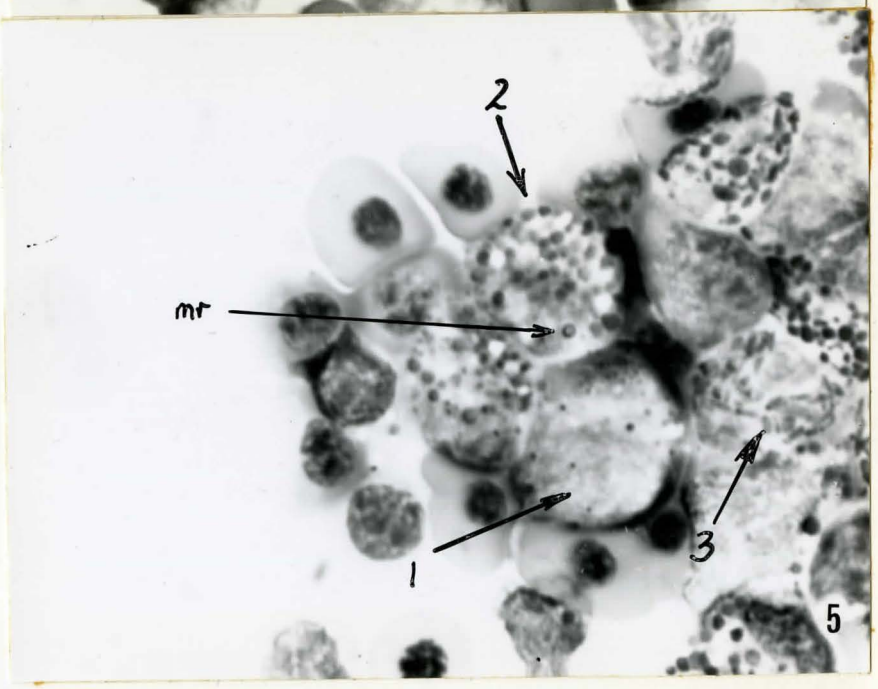
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Imprint from 12 day Embryo Spleen stained with May-Grünwald Giemsa stain

Fig. 6a x1560

Cell 1 Heterophil early mesomyelocyte (type 2). Note orange and magenta granules round nucleus, purple-mauve staining, chromatin in dense uniform pattern. The cytoplasm is much more basophilic than type 1 cells.

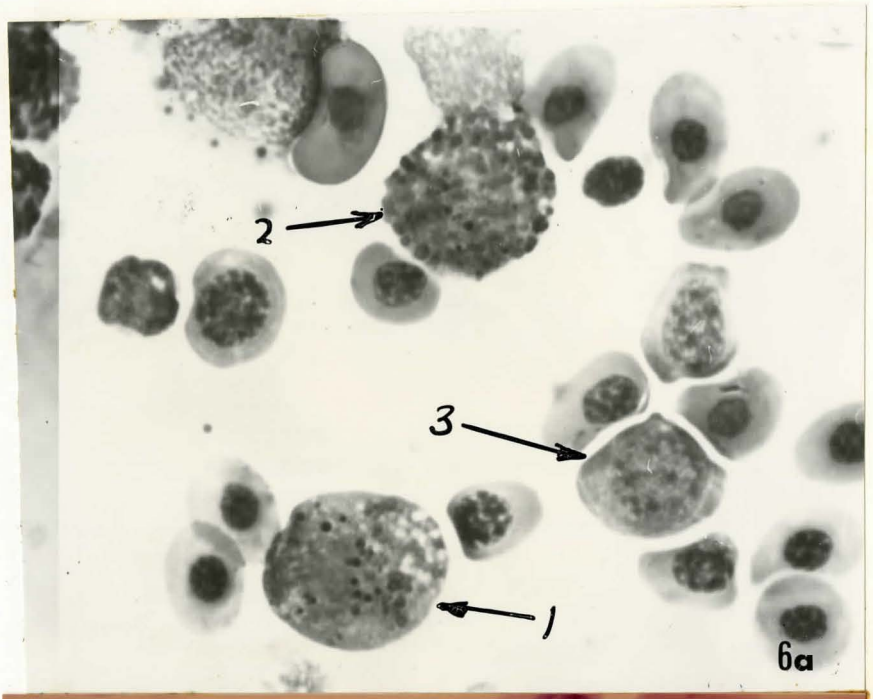
Cell 2 Heterophil early metamyelocyte. Note many orange refractile granules, few magenta granules over the nucleus, cytoplasm slightly basophilic, nucleus eccentric, coarse mauve staining chromatin.

Cell 3 Stem cell. Small, basophilic cytoplasm with some vacuoles. Nucleus stains purplish mauve, chromatin coarse. Possibly of erythroid series although has the features of the heterophil promyelocyte.

Fig. 6b x1900

Cell 1 Heterophil early mesomyelocyte (type 2). This is the same cell as the cell 1 in figure 6b.

Cell 2 Same as cell 3 in figure 6a.



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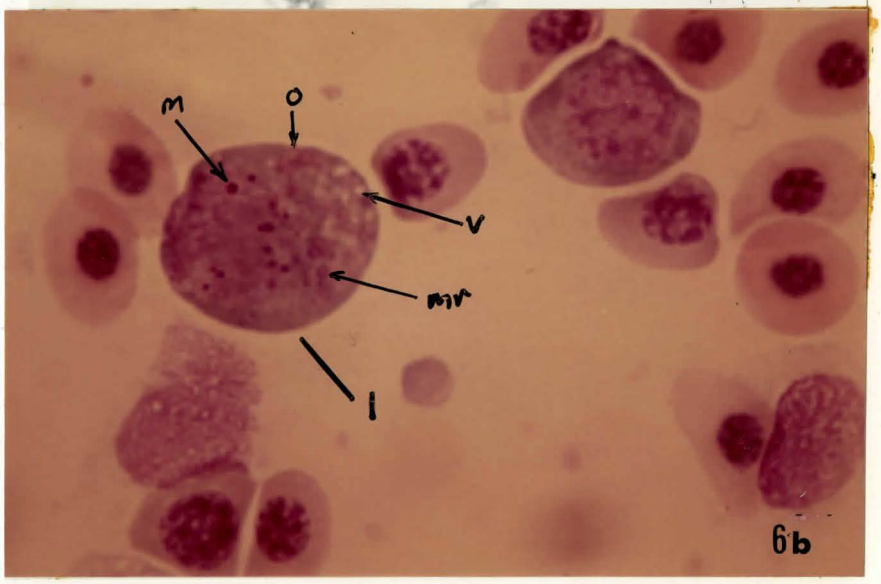


6b

Imprint of 12 day Embryo Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 7a x1560

Cell 1a Heterophil, mature. Nucleus is purple blue, bilobed with clumps of chromatin material. All the granules are elongate spindle shaped. The cytoplasm is not visible.

Cell 1b Mature heterophil.

Cell 2 Heterophil, early mesomyelocyte (type 2). Basophilic cytoplasm, both orange and magenta granules. Nucleus stains pale mauve, chromatin diffuse

Cell 3 Heterophil, early metamyelocyte (or eosinophil mesomyelocyte). Little cytoplasm, contains many large round orange refractile granules, two red magenta granules over the nucleus. Nucleus stains pale mauve.

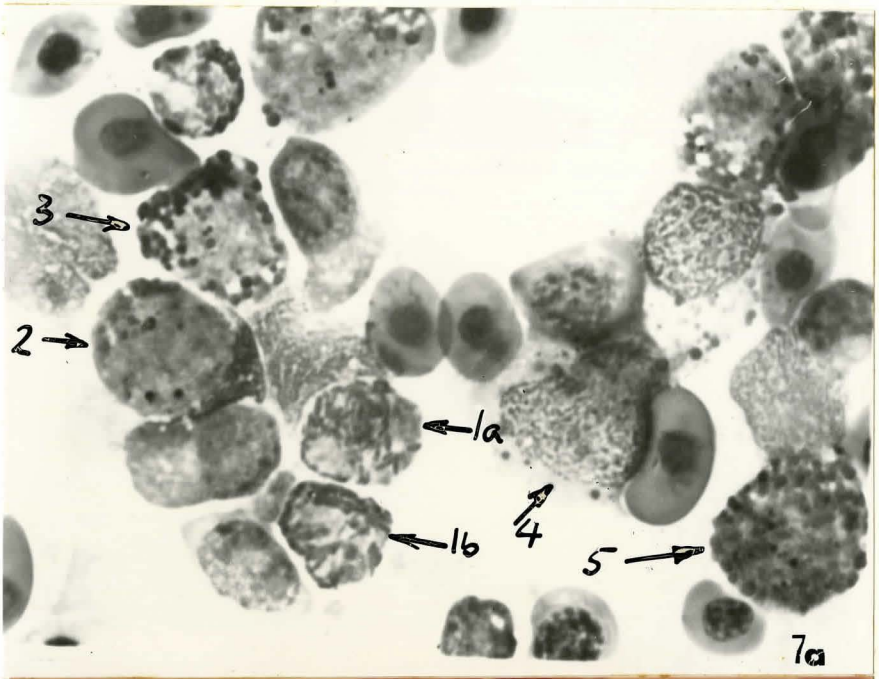
Cell 4 Heterophil, promyelocyte. Note chromatin condensed into small clumps.

Cell 5 Heterophil, early meta-myelocyte (same as cell 2, Fig. 6a).

Fig. 7b x 1900

Cells 1a, 2, 3 and 4 as in Fig. 7a

Note two small blast cells (arrow), basophilic cytoplasm with some vacuoles, red-mauve nucleus with some chromatin condensation.



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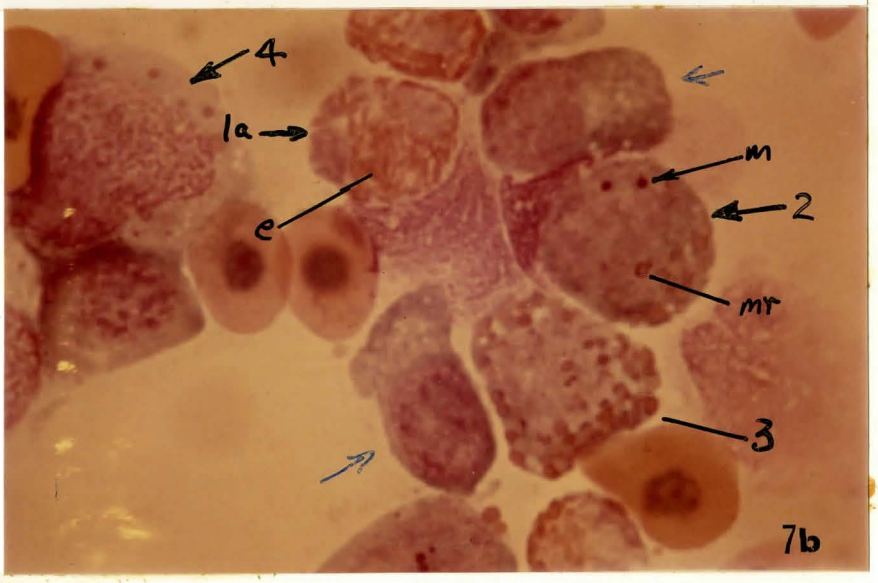

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Imprint from 12 day Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 8 x1540

Cell 1 Heterophil mesomyelocyte (type 1). Cytoplasm is basophilic; both magenta and orange granules are present. Nucleus stain pale mauve colour, chromatin in fine network. Nucleus not deliniated from cytoplasm.

Cell 2 Heterophil, late mesomyelocyte (type 1). Contains more round orange granules and fewer magenta granules than in cell 1. The cell is slightly smaller.

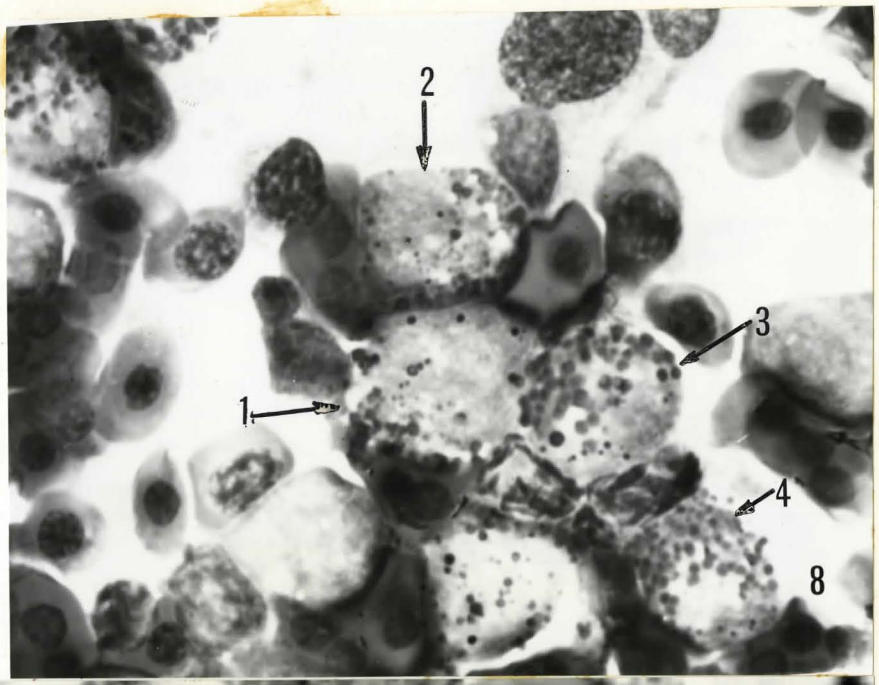
Cell 3 Heterophil, early metamyelocyte. Cytoplasm very pale blue, predominance of large round orange granules although magenta granules are still present. Nucleus eccentric indistinct chromatin clumped were seen

Cell 4 Heterophil, late metamyelocyte. Pale blue cytoplasm filled with large orange granules, few magenta granules over the nucleus. Nucleus indistinct but at one edge contains coarse mauve staining chromatin.

Fig. 9 x1870

Cell 1 Heterophil, late metamyelocyte. Pale blue cytoplasm; granules refractile orange-most are spindle shaped but some remain round, bilobed nucleus contains coarse clumps of mauve staining chromatin.

Cell 2 Heterophil mature. Colourless cytoplasm. All granules are orange, refractile and spindle shaped. Nucleus is lobed; clumps of chromatin stains mauve. This cell is smaller than cell 1.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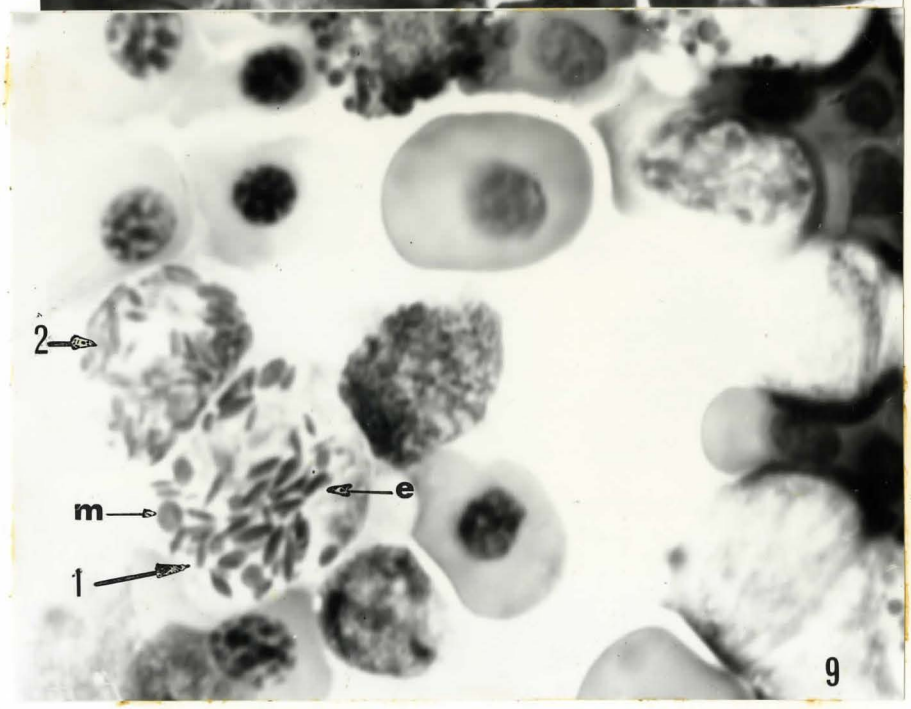
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Imprints from 12 day Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 10 x1900

Cell 1 Heterophil, late promyelocyte. Slightly basophilic cytoplasm, contains clear vacuoles, faint orange granules and magenta granules over the nucleus. Nucleus eccentric diffuse, chromatin staining pruple-red.

Cell 2 Medium sized blast cell. Basophilic cytoplasm.

Cell 3 Large blast cell. Basophilc cytoplasm, large nucleus network of slightly coarse chromatin, nucleolus (*)

Cell 4 Heterophil mesomyelocyte (type 1). Slightly basophilc cytoplasm orange and magenta granules. Nucleus, ovoid and contains dense clumps of chromatin staining red-purple.

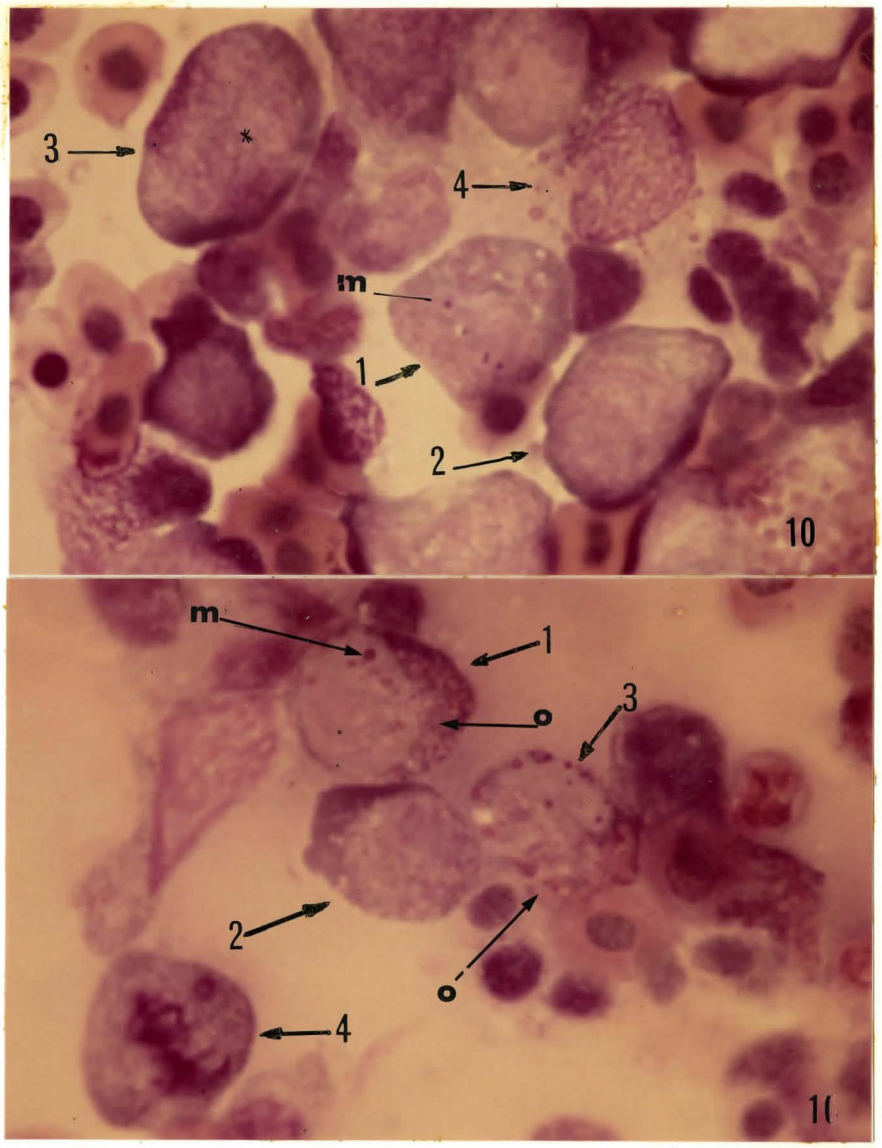
Fig. 11 x1900

Cell 1 Heterophil, early mesomyelocyte (type 1) Cytoplasm basophilic contains many round orange granules, magenta granules present over the nucleus. Nucleus eccentric, diffuse chromatin material staining blue purple.

Cell 2 Medium sized blast cell (type 3). Basophilic cytoplasm with some clear vacuoles. Nucleus eccentric pale mauve colour with blue nucleolus. Chromatin evenly distributed.

Cell 3 Heterophil, late mesomyelocyte (type 2). Basophilic cytoplasm contains many round orange granules, magenta granules mainly over the nucleus. Nucleus eccentric and indented stains blue purple; chromatin diffuse.

Cell 4 Dividing Blast Cell.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Imprint from 12 day Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 12 x1900

Cell 1 Heterophil early mesomyelocyte (type 1). Slightly basophilc cytoplasm with many clear vacuoles, and round orange granules. Nucleus is eccentric contains diffuse chromatin and stains mauve, a nucleolus (*) can be seen. No magenta granules are present suggesting this cell may possibly be an eosinophil.

Cell 2 Heterophil promyelocyte. Basophilic cytoplasm. Clear vacuoles some with very faint orange granules, no magenta granules. This cell may possibly be an eosinophil.

Cell 3 Stem (Blast) cell. Thin rim of basophilic cytoplasm large pale mauve nucleus.

Cell 4 Stem (Blast) cell, more basophilic cytoplasm than cell 3. Nucleus paler than cell 3

Cell 5 Basophil, mature. Purple granules pale nucleus staining mauve, cytoplasm has not stained. (This cell may be in a terminal developmental stage i.e. have functioned.)

Fig. 13 x1900

Cell 1 Heterophil metamyelocyte. Note round and elongate orange granules, little visible cytoplasm. Nucleus is horse-shoe shaped stains purple mauve, with coarse chromatin.

Cell 2 Heterophil mature. Note elongate orange granules, lobed nucleus with clumps of chromatin

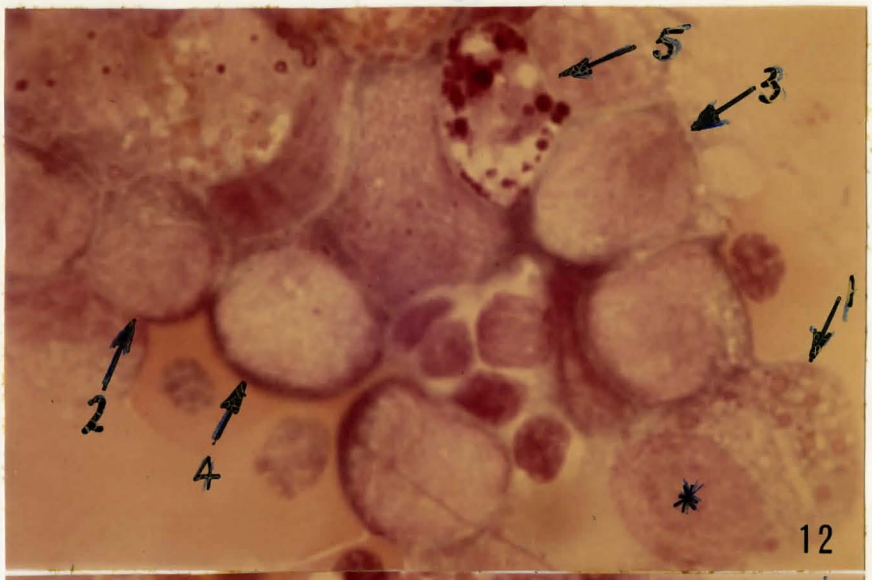
Cell 3 Heterophil, mature

Cell 4 Eosinophil, early. Note coarser chromatin of nucleus staining purple large round orange granules. This cell may be damaged.

Cell 5 Eosinophil, only part of the cell is illustrated. The nucleus stains more intensly red/purple than heterophils, and only round orange granules (arrow) are present.

Cell 6 Heterophil, late promyelocyte (type 2). Note basophilic cytoplasm.

Cell 7 Blast cell. Less basophilic cytoplasm than type 1, possibly type 2 stem cell.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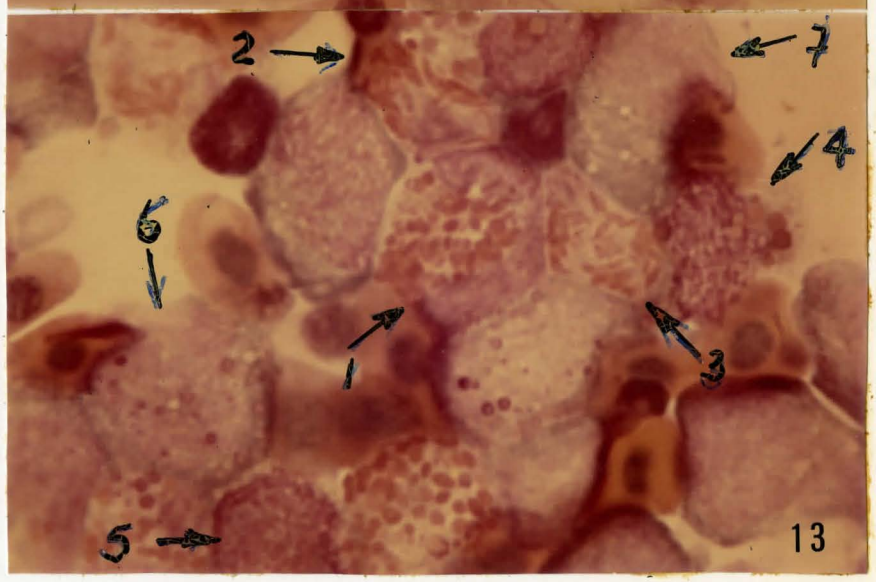
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Imprint from 12 day Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 14 x 1700

Cell 1 Eosinophil, mesomyelocyte. Cytoplasm basophilic containing small round granules some pale staining, others more dense and refractile. The nucleus is round and eccentric; coarse chromatin is evenly distributed and stains red-purple i.e. more intense than that of the heterophil.

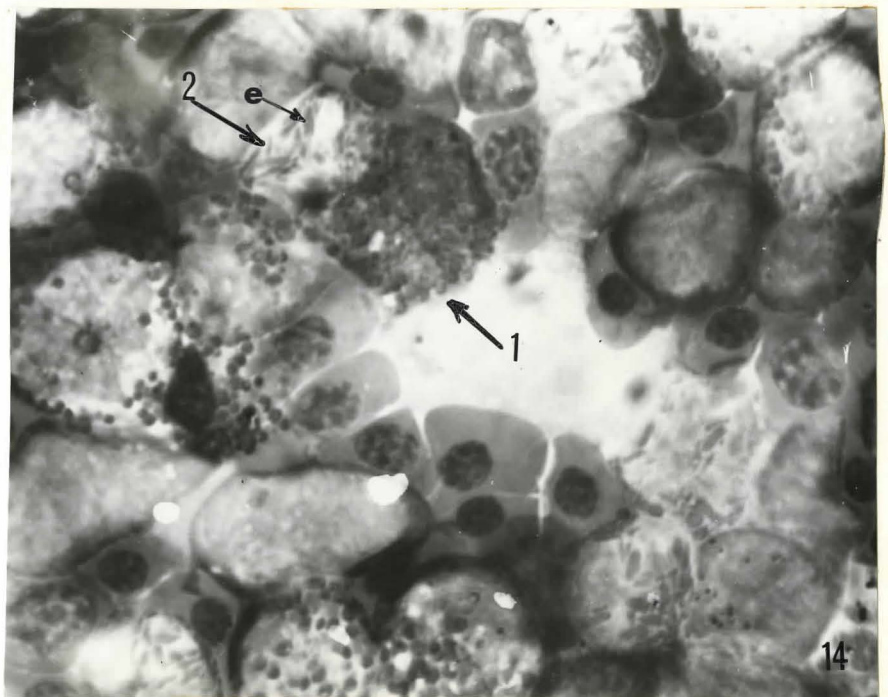
Cell 2 Heterophil mature. Note elongate rod shaped granules.

Fig. 15 x2000

Cell 1 Eosinophil mature (or Eosinophil, metamyelocyte). Cytoplasm where visible is very basophilic. Granules are round, orange and refractile and stain slightly denser than those of the heterophil. The nucleus is slightly eccentric stains red-purple, chromatin is coarse.

Cell 2 Heterophil, late mesomyelocyte. Cytoplasm very pale blue, contains large round orange granules and one magenta granule. Nucleus eccentric, indented, chromatin diffuse and stains pale mauve.

Cell 3 Heterophil mesomyelocyte. Cytoplasm pale blue, contains orange and magenta granules. Nucleus, eccentric diffuse chromatin staining more densly than that of cell 2.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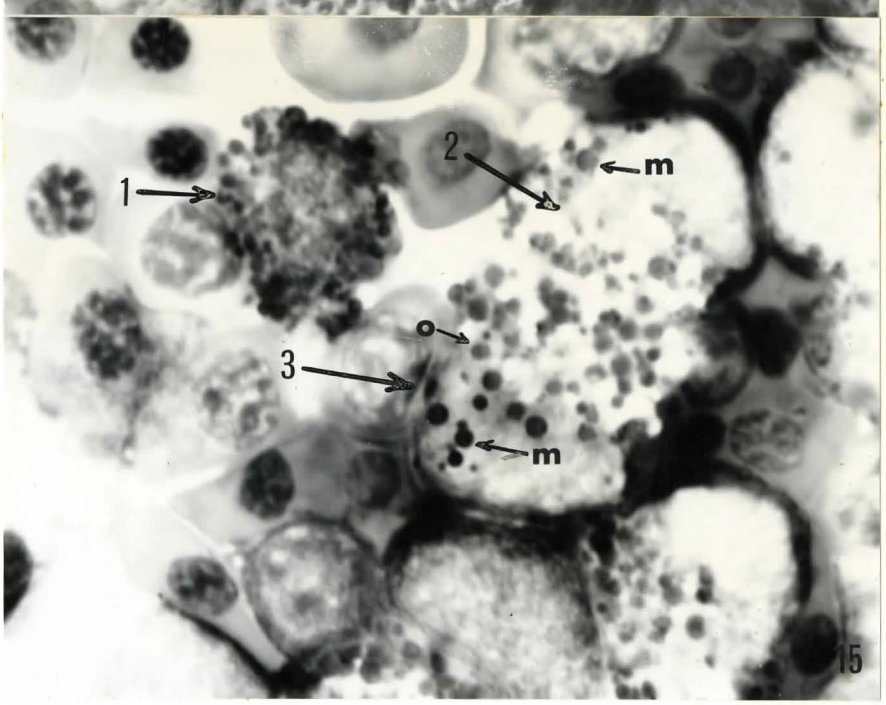
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Imprint from 12 day Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 16 x1660

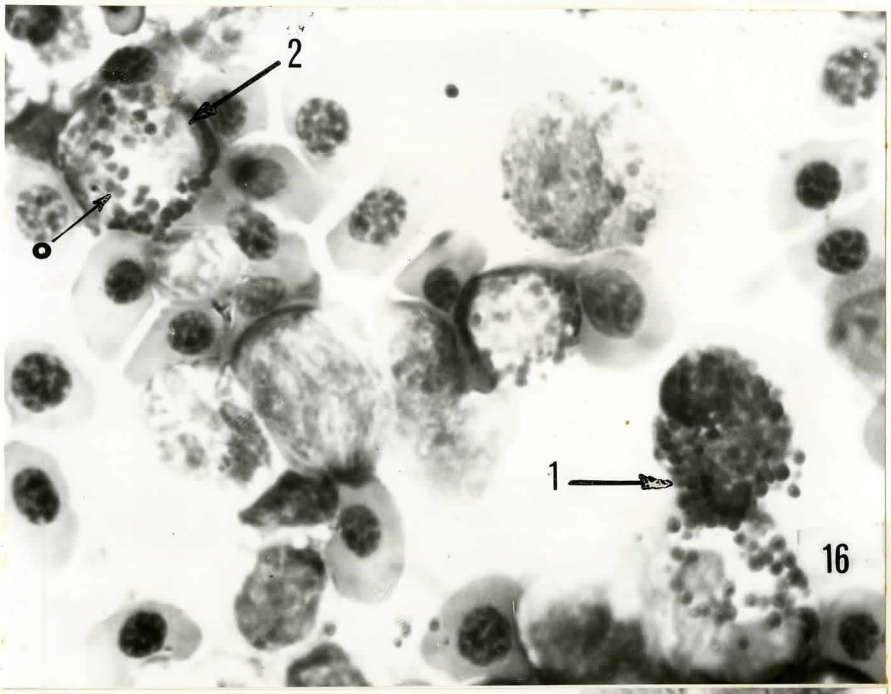
Cell 1 Eosinophil, mature. Basophilic cytoplasm containing round refractile orange granules. Nucleus eccentric coarse chromatin stains intensly red-purple.

Cell 2 Heterophil, early metamyelocyte. Orange granules, some round and some beginning to elongate.

Fig. 17 x1580

Cell 1 Basophil immature; stage not known. Cytoplasm stains very pale blue. Dark purple granules situated mainly around the cell periphery. Nucleus large, chromatin diffuse and stains pale purple blue.

Cell 2 Heterophil mature. Rod shaped orange granules and bilobed nucleus.



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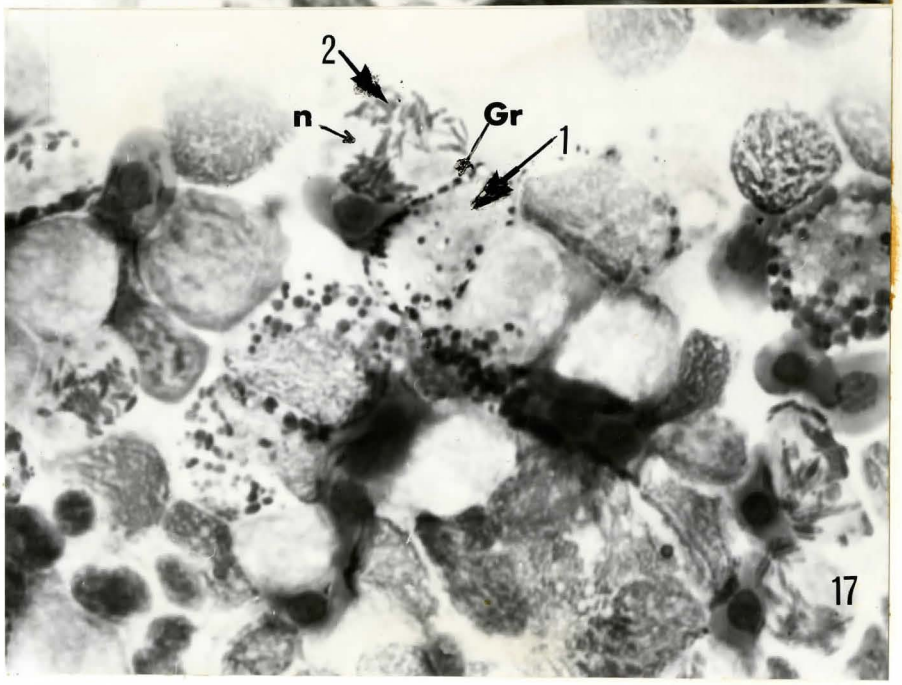


17

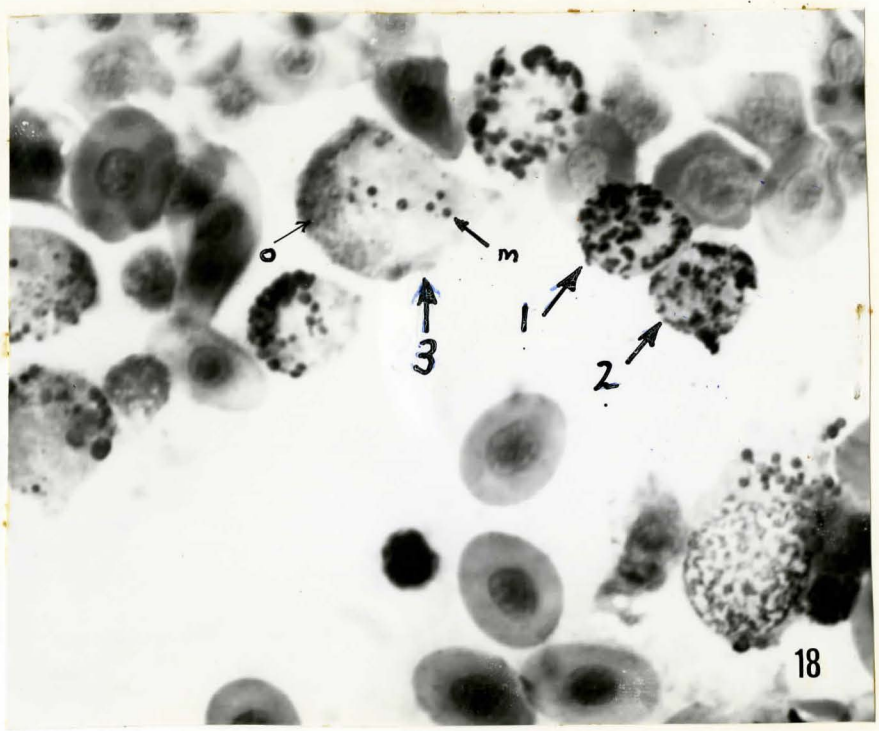
Imprint from 12 day Spleen Stained
with May-Grünwald Giemsa Stain

Fig. 18 x1670

Cell 1 Basophil mature. Barely visible cytoplasm, stains pale blue. Round nucleus stains pale blue, chromatin is diffuse. Dark purple granules pack the cell.

Cell 2 Basophil, mature

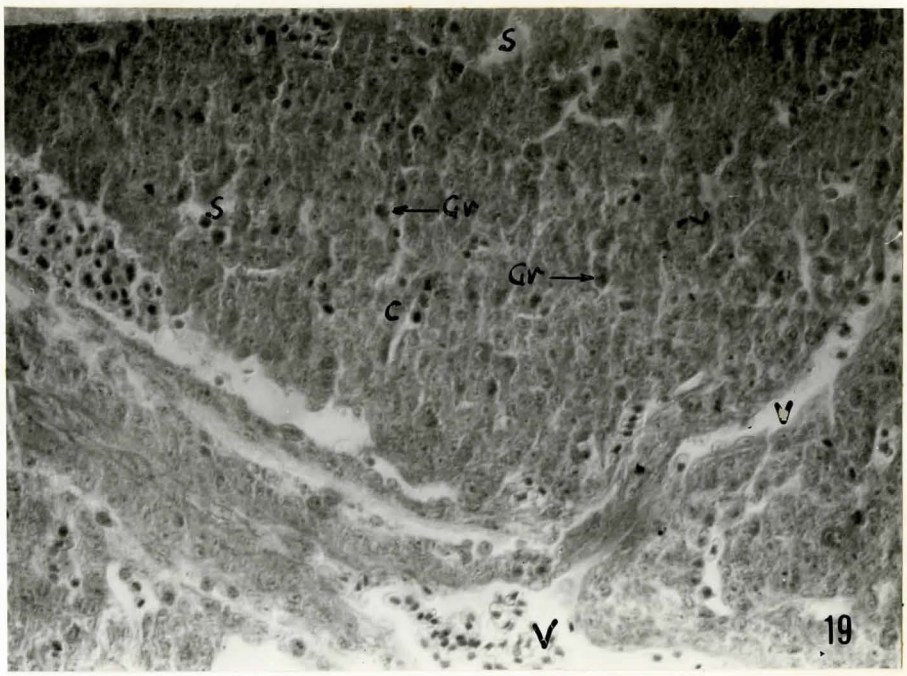
Cell 3 Heterophil, mesomyelocyte (type 2) Basophilic cytoplasm, orange and magenta granules.


o
m
1
2
3
18

Sections of 12 day Embryo Spleen Sectioned at 5μ and stained with May-Grünwald Giemsa stain for paraffin sections

Fig. 19 : x400

Low power view of spleen. Note the cords (c) of splenic cells and sinuses (s).
Granulocytes and blast cells are seen within the splenic tissue (arrows).


S
S
Gv
Gv
C
V
V
19

Sections of 12 day Embryo Spleen Sectioned at 5μ and stained with May-Grünwald Giemsa stain for paraffin sections

Fig. 20a x1500

Cell 1 Heterophil, promyelocyte. Note, basophilic cytoplasm containing faint orange granules in the area of the indentation of the nucleus (larger dense granules were also seen but are not illustrated in the photomicrographs). The nucleus is large and ovoid, stains pale blue; prominent nucleolus and the chromatin is in small clumps.

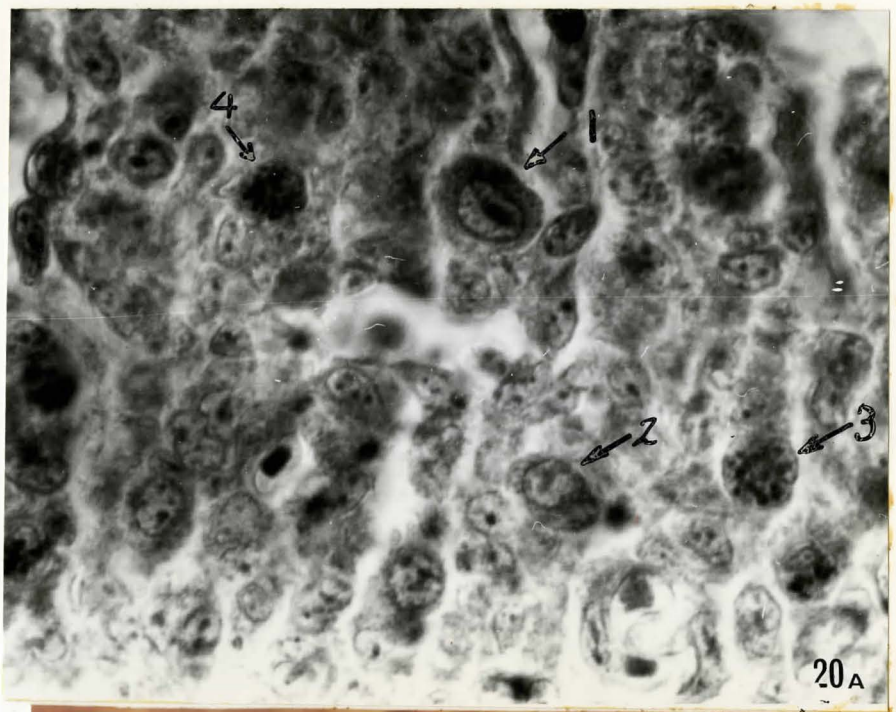
Cell 2 Heterophil mesomyelocyte. Smaller cell, than the promyelocyte is slightly basophilic and contains some clear vacuoles, more prominent orange granules situated in the broadest part of the cytoplasm. The nucleus is small eccentric and indented, stains very pale blue and has a prominent nucleolus.

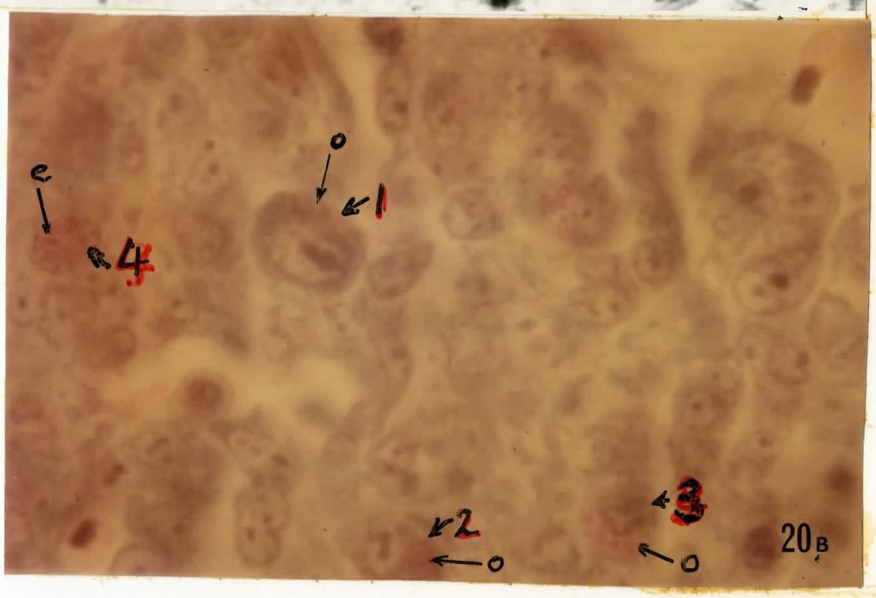
Cell 3 Heterophil metamyelocyte. Cytoplasm contains many round orange refractile granules; the nucleus is eccentric.

Cell 4 Heterophil, mature. Elongate orange, refractile granules. Nucleus stains blue and is bilobed.

Fig. 20b x1500

Colour photomicrograph of 20 a
Cells 1, 2, 3 and 4 are the same as those in Fig. 20a.


4
1
2
3
20A


0
1
4
2
0
3
0
20B

Sections of 12 day Embryo Spleen Sectioned at 5μ and stained with May-Grünwald Giemsa stain for paraffin sections

Fig. 21 x1500

Granulocytes (arrows) situated extravascularly to a large vessel (v) entering the spleen.

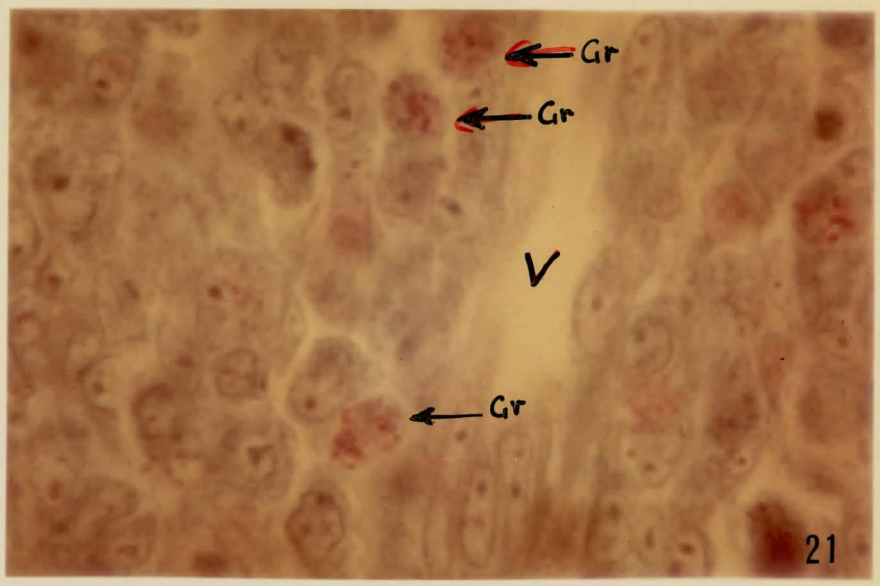
Fig. 22 x1500

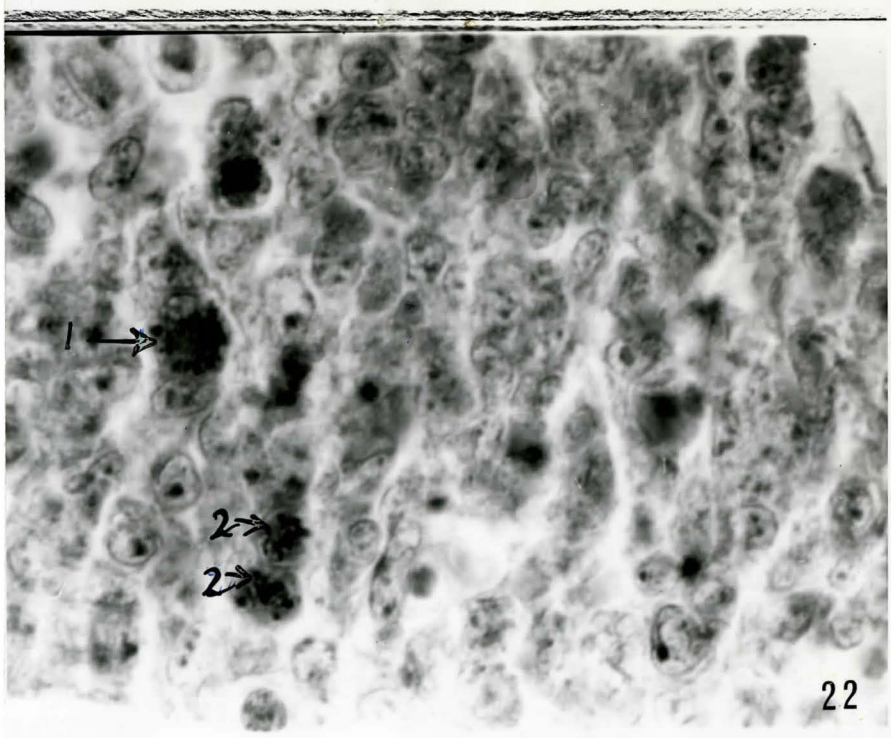
Granulocytes developing in cords of spleen cells, near the periphery of the organ.

Cell 1 Heterophil, metamyelocyte. (or late mesomyelocyte), cytoplasm is packed with round orange granules; some granules have a ring form.

Cell 2 Heterophil, mature. Note elongate granules which stain orange.

#7.9





Sections of 10 day Embryo Spleen Sectioned at 5μ and stained with May-Grünwald Giemsa stain for paraffin sections

Fig. 23 x1500

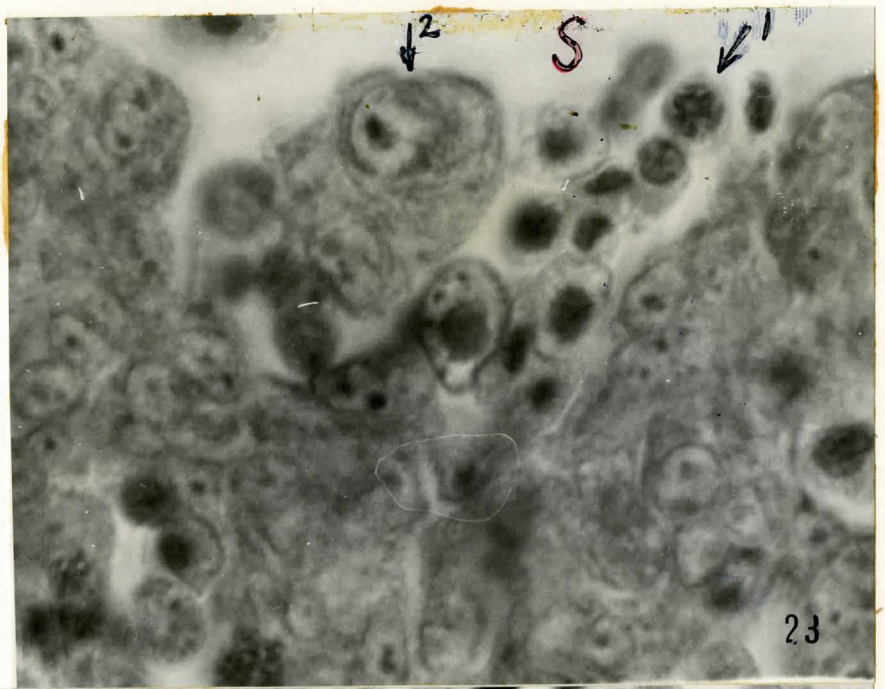
Cell 1 Granulocyte (heterophil) late mesomyelocyte or early metamyelocyte, in the lumen of a sinus (s)

Cell 2 Heterophil, promyelocyte. Large cell with basophilic cytoplasm containing very faint orange granules. Nucleus stains pale is round and contains a prominent nucleolus.

Fig. 24 x1500

Cell 1 Heterophil mature. Note elongate granules which stain orange.

Cell 2 Heterophil metamyelocyte. Cytoplasm contains round and elongate granules which stain orange. A large red blood cell (rbc) is seen in the sinus (s).



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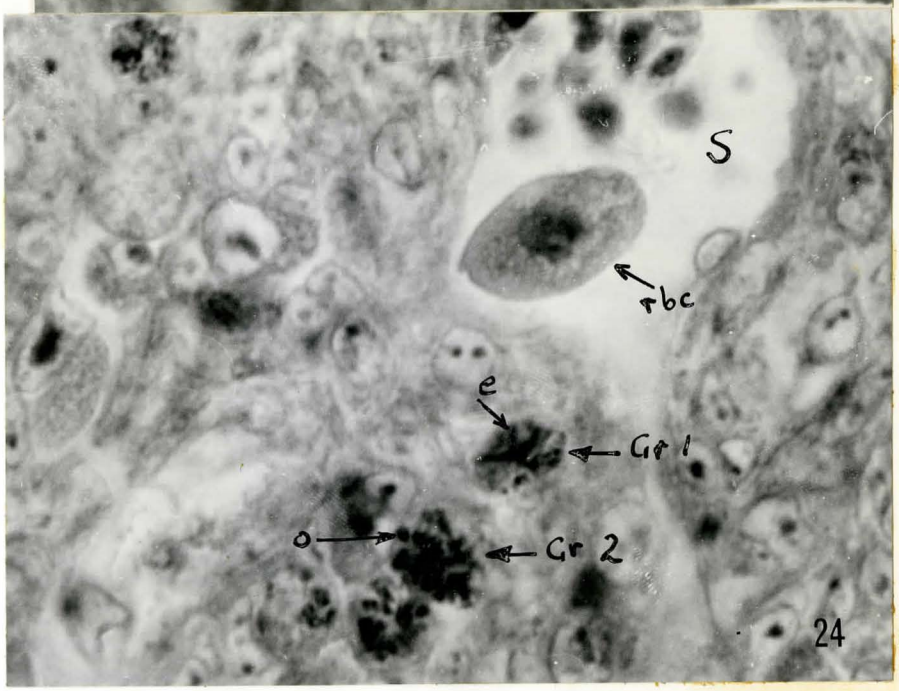


24

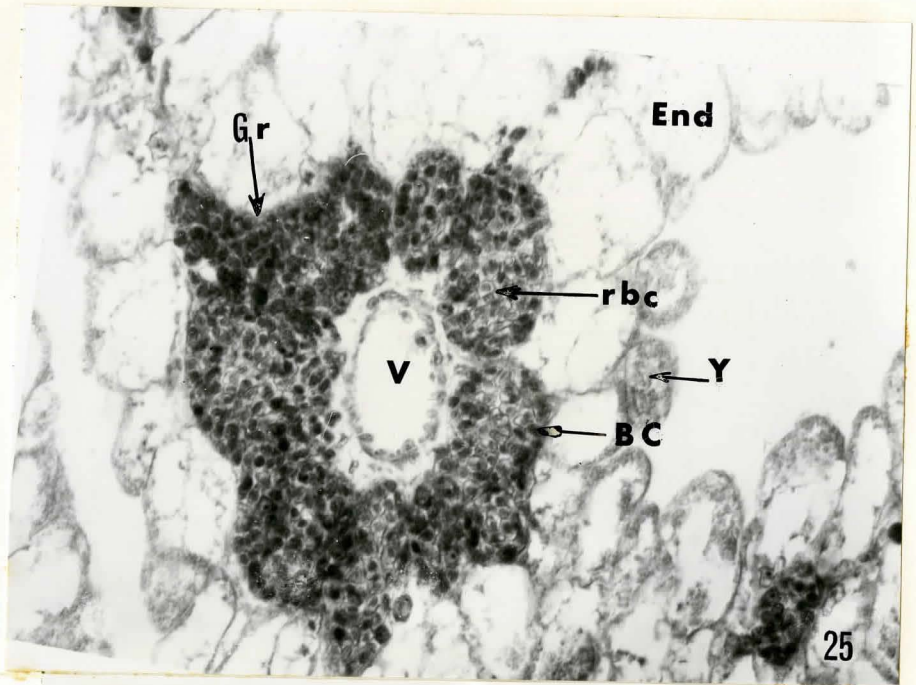
Sections of Yolk Sac Vessels from 12 day Embryos fixed in Zenker's fixative sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

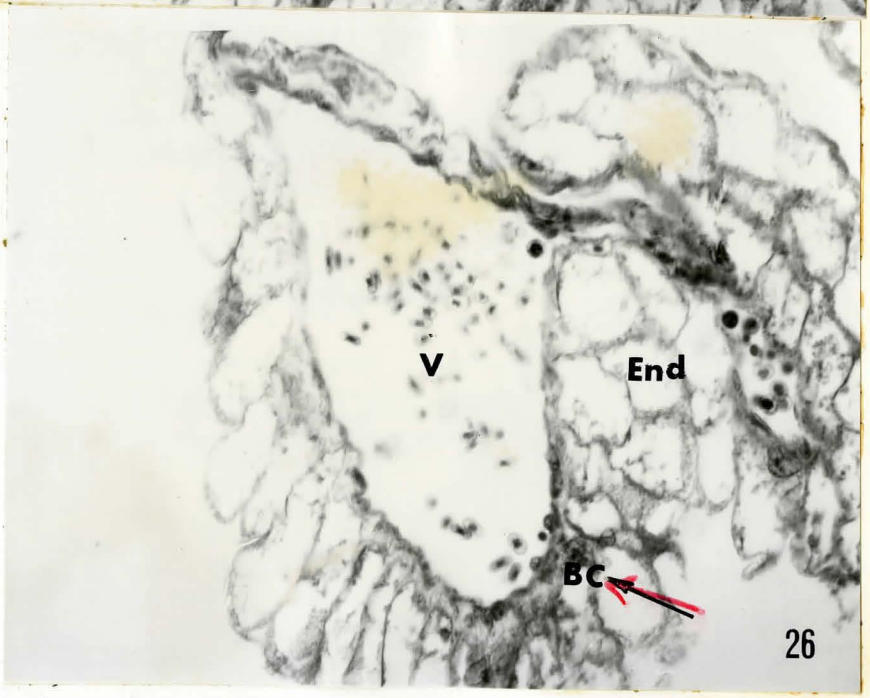
Fig. 25 x400

"Thick walled" vessel (v). Note the clumps of cells surrounding the main vessel, clumps or auxillary vessels consist of basophilic cells at the periphery (arrow b) and developing red blood cells at the centre. Granulocytes (arrowed) are situated between the clumps.

Fig. 26 x370

"Thin walled" vessel. Note the absence of auxillary vessels, and few basophilic cells (arrows) lying outside the vessel. The surrounding endoderm (e) is composed of large vacuolated cells with most of the cytoplasm lying towards the vessel.


Gr
End
rbc
V
Y
BC
25


V
End
BC
26

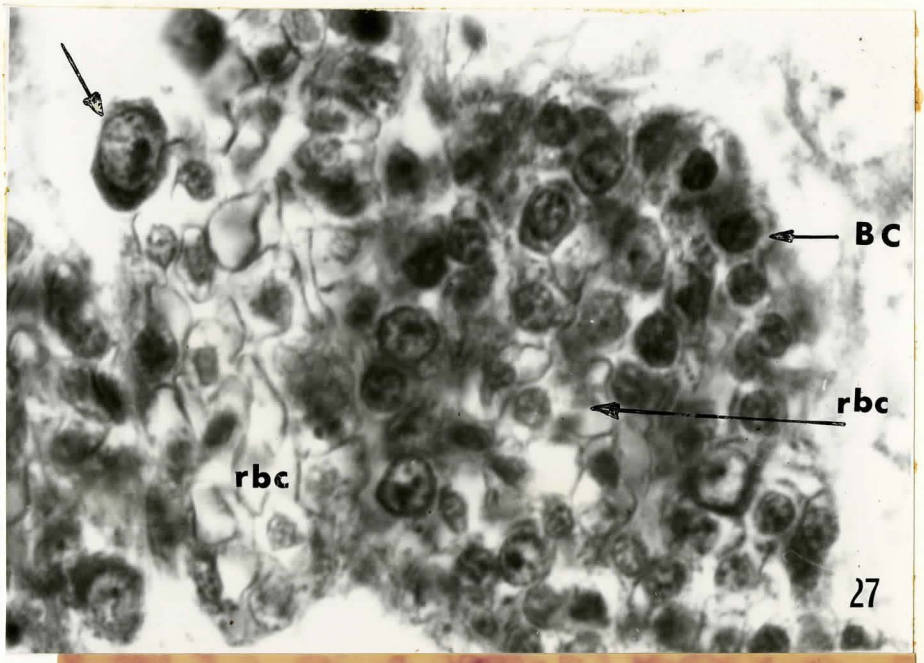
Sections of Yolk Sac Vessels from 12 day Embryos fixed in Zenker's fixative sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

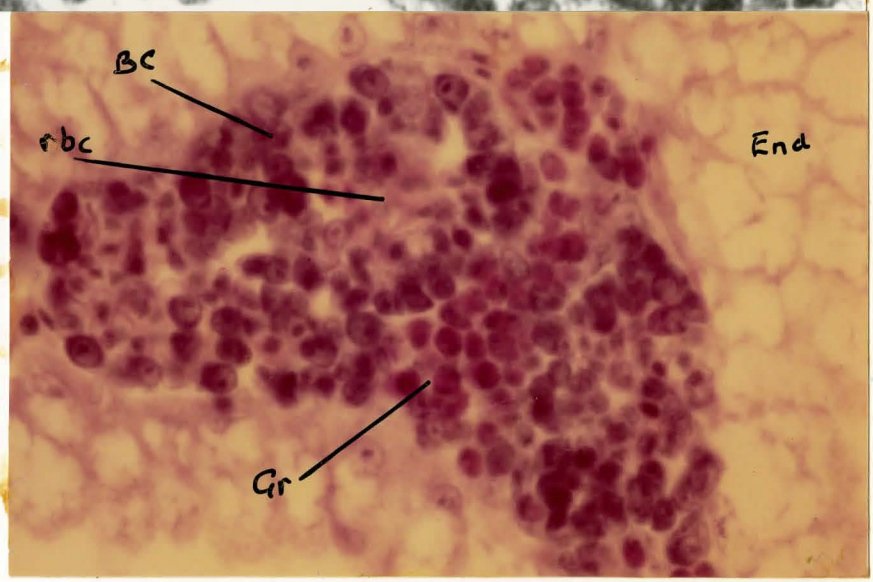
Fig. 27 x1500

Developing auxillary vessel, with the more basophilic blast cells lying at the periphery; the paler area to the centre is developing red blood cells. There are no cells which appear to be flattening to form an endothelium. Note the large blast cell (arrow)

Fig. 28 x800

Developing vessel within the ectoderm. Note the cord of granulocytes, orange-red granules and blue single or lobed nuclei.


BC
rbc
rbc
27


BC
rbc
End
Gr

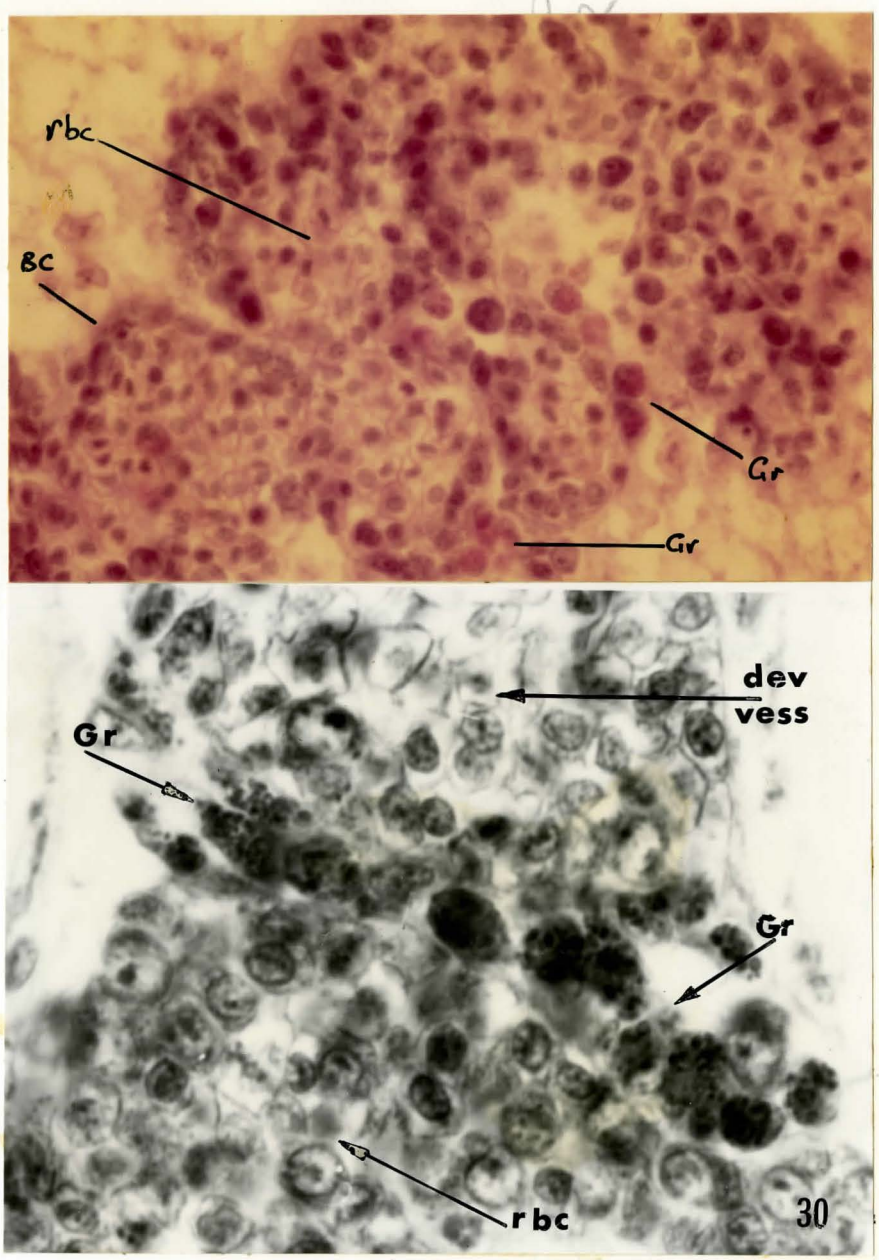
Sections of Yolk Sac Vessels from 12 day Embryos fixed in Zenker's fixative sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

Fig. 29 x800

Group of developing vessels in which the central area is occupied by red blood cells. A few granulocytes are seen extravascularly.

Fig. 30 x1600

Cord of granulocytes between the basophilic blast cells of the periphery of a developing vessel (arrowed)


rbc
BC
Gr
Gr
dev
vess
Gr
Gr
rbc
30

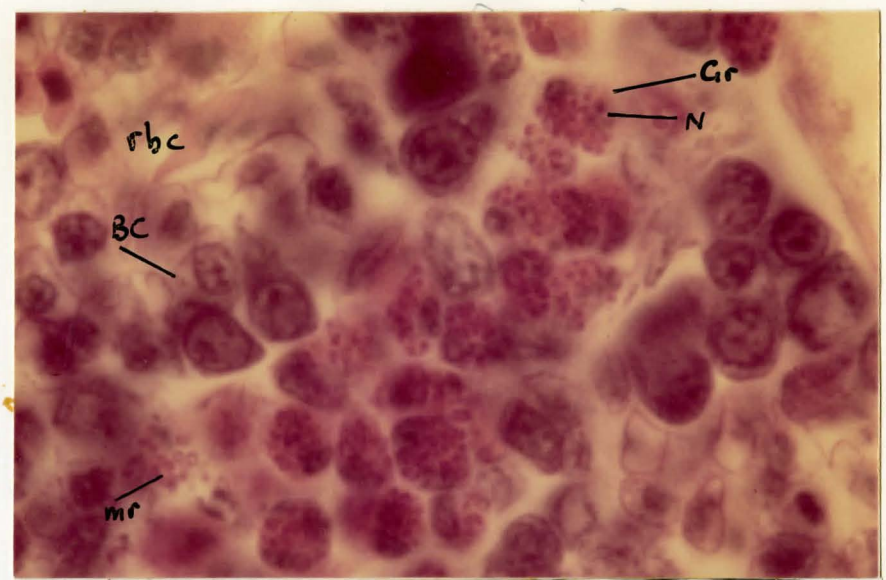
Sections of Yolk Sac Vessels from 12 day Embryos fixed in Zenker's fixative sectioned at 5 and stained with May-Grünwald Giemsa stain for tissue sections

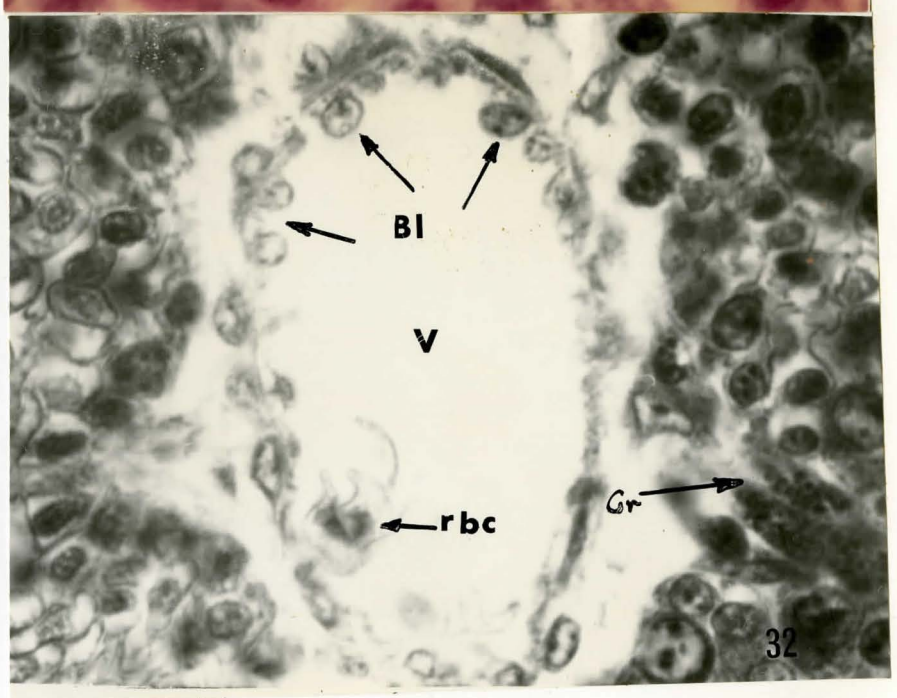
Fig. 31 x1400

Granulocytes between two auxillary vessels. Granules appear reddish orange most are round, a few are in ring form, (arrow n). The nuclei of these cells stain blue, most are eccentrically placed and indented; there are also lobed forms.

Fig. 32. x1500

Endothelium of a thick walled vessel, blast cells similar to those found in the auxillary vessels appear to be budding from the endothelium (arrows). The space between the endothelium and the surrounding cells may be a fixation artefact. Cord of granulocytes is seen in the right had corner (arrow).





32

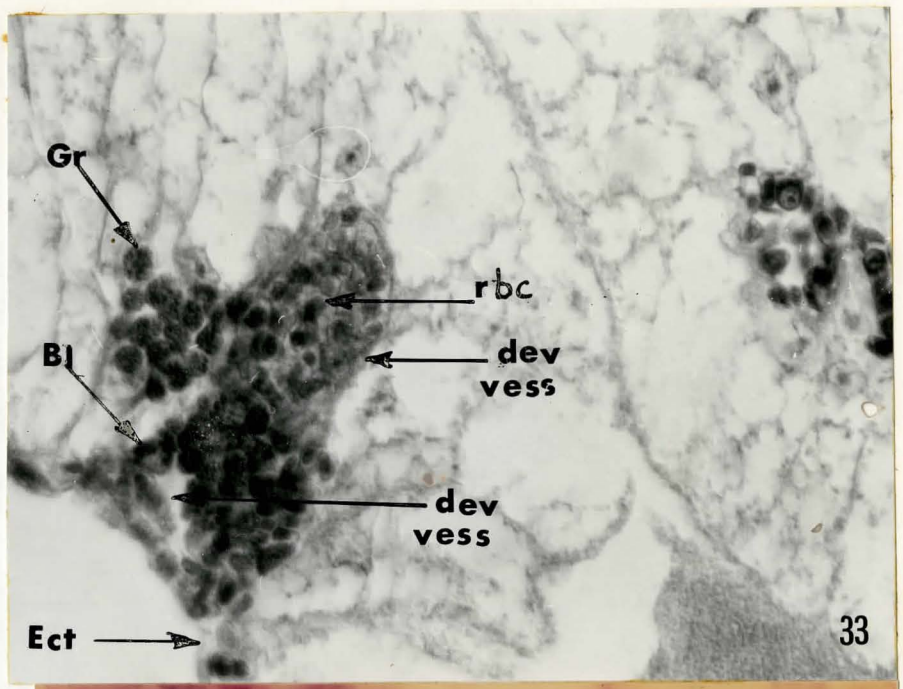
Sections of Yolk Sac Vessels from 12 day Embryos fixed in Zenker's fixative sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

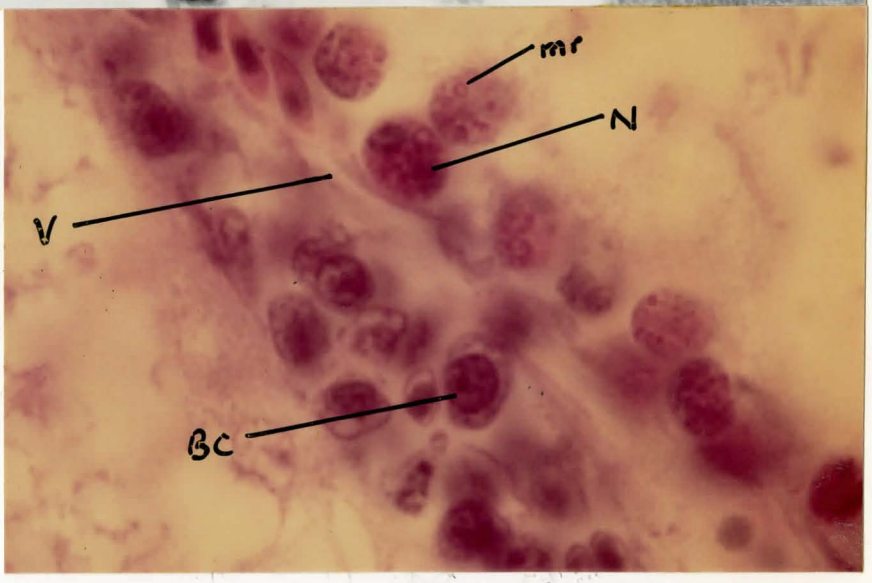
Fig. 33 x720

Two small vessels developing (dev. vess) immediately under the ectoderm. Granulocytes (Gr) are seen in the endoderm outside these vessels. Ectoderm (Ect.). Developing red blood cells (Dev. RBC) Blast cell (BC).

Fig. 34 x1400

Granulocytes deep in the ectoderm, and extravascular to a small vessel which often appear to connect larger vessels. Note the ring shaped granules and indented and bilobed nuclei of the granulocyte.

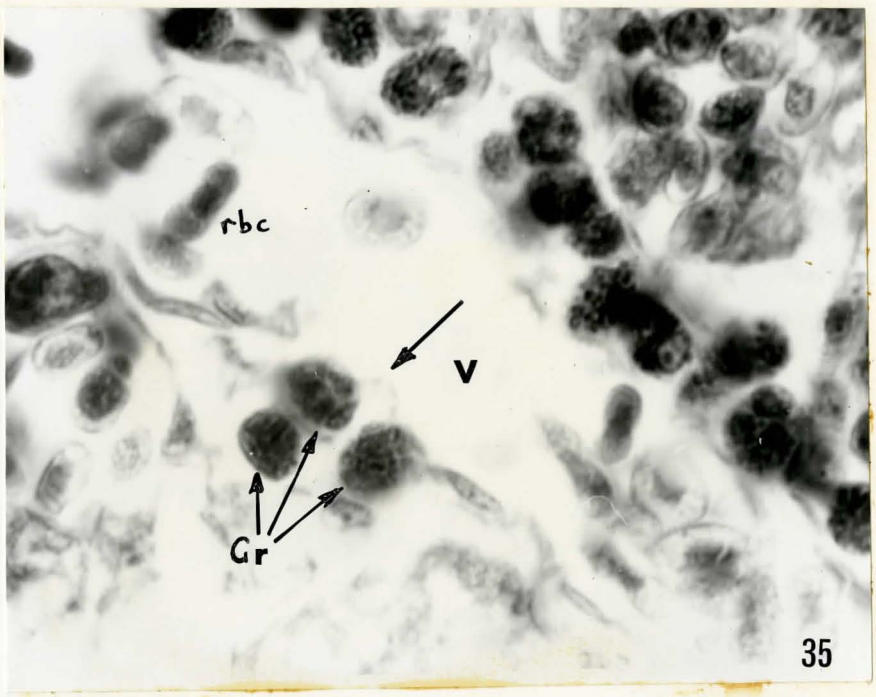

Gr
rbc
Bl
dev
vess
dev
vess
Ect
33


mr
N
V
Bc

Sections of Yolk Sac Vessels from 12 day Embryos fixed in Zenker's fixative sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

Fig. 35 x1500

A group of three granulocytes close to the endothelium of a vessel (these may possibly be entering the vessel lumen) (arrow).


rbc
V
Gr
35

Sections from 4 day old Yolk Sac sectioned at 5μ
and stained with May-Grünwald Giemsa
stain for tissue sections

Fig. 36 x1330

Three granulocytes lying extravascularly to a small vessel under the ectoderm. The endothelium of the vessel, and ectoderm are clearly seen.

Cell 1 Promyelocyte heterophil (or early mesomyelocyte). Large cell with basophilic cytoplasm, round nucleus situated to one side of the cell, with a prominent nucleolus. A few pale orange granules are present in the cytoplasm.

Cell 2 Heterophil mesomyelocyte. Nucleus is round; cytoplasm contains many round orange granules.

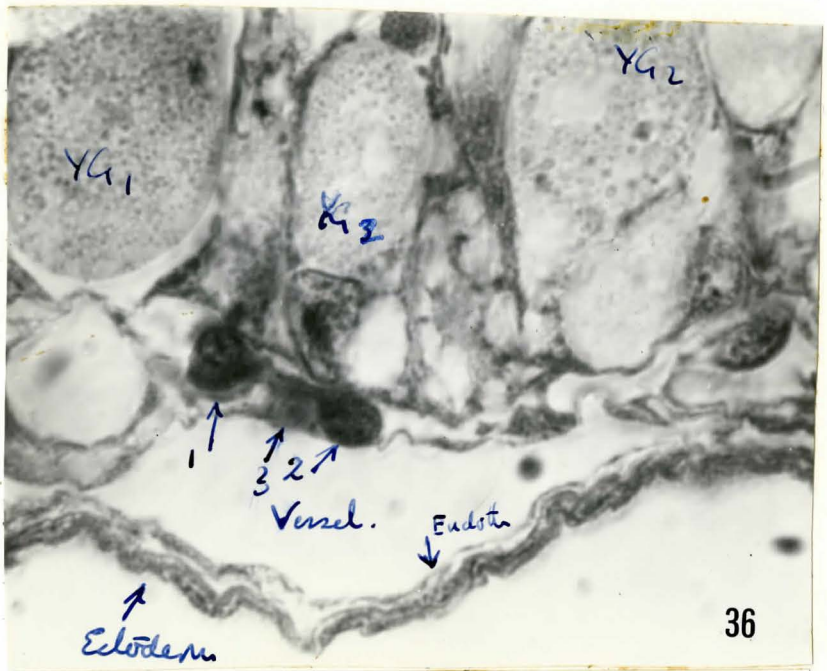
Cell 3 Heterophil mesomyelocyte (or metamyelocyte). Nucleus is more elongate.

Two types of the yolk granules (YG) are seen within the endodermal cells; YG1, has a uniform granular appearance, YG2, is granular but with a clear centre.

Fig. 37 x1330

Cell 1 Heterophil mesomyelocyte. Round orange granules in the cytoplasm.

Cell 2 Stem cell. Round cell with basophilic cytoplasm. The nucleus is round and occupies 75% of the cell, and contains a prominent nucleolus. Chromatin may be in small clumps.



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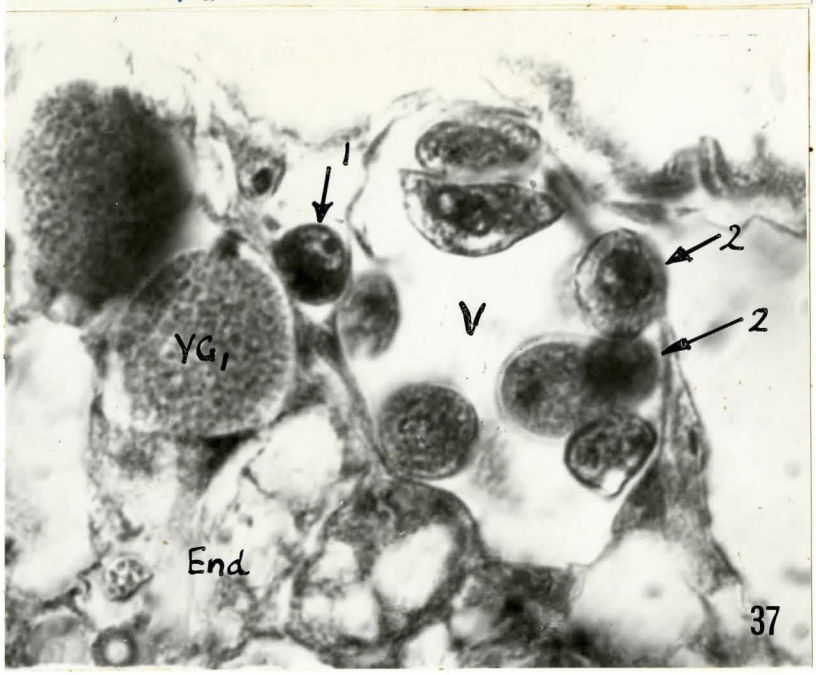


37

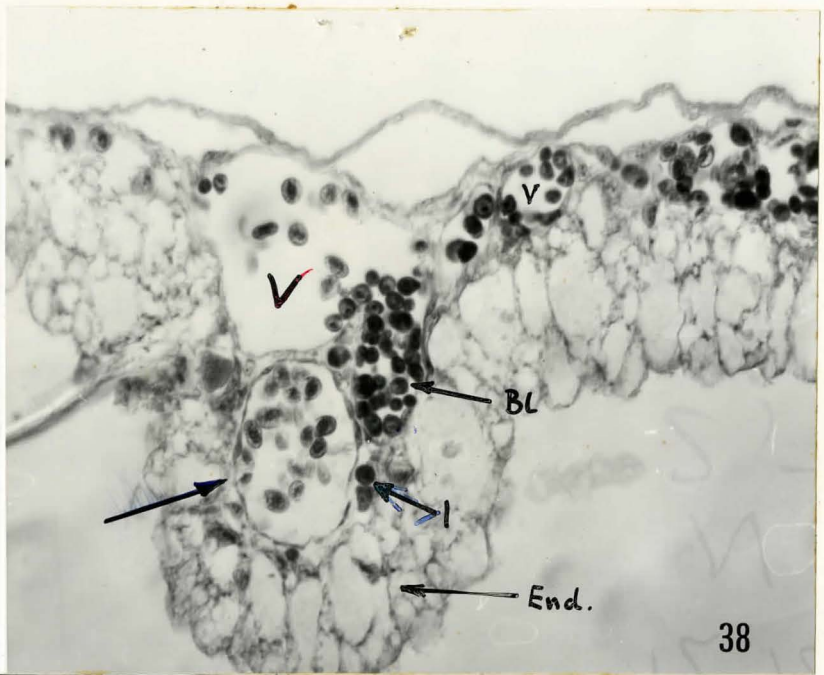
Sections from 5 day of Yolk Sacs sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

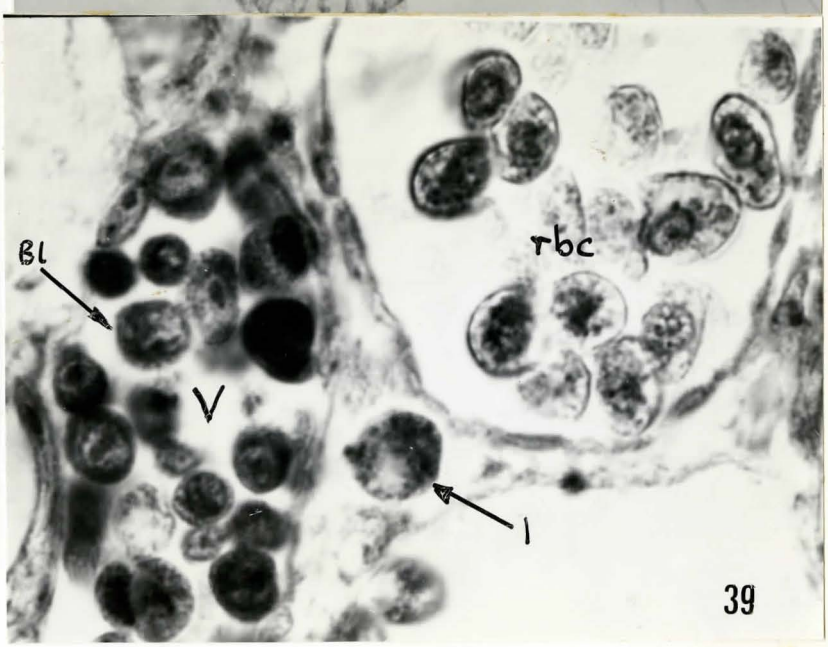
Fig. 38 x1000

Large (arrowed) and small vessels are seen. The large vessel deep in the yolk material has a well developed endothelium. A single stem (blast) cell (cell 1) lies outside. Next to this vessel is a smaller vessel packed with blast cells which appear to be passing into a large vessel (V) situated under the ectoderm. Many small vessels are seen, containing blast cells, under the ectoderm.

Fig. 39 x1400

Cell 1 Late mesomyelocyte heterophil (or early metamyelocyte) situated extravascularly between two blood vessels. The cytoplasm is basophilic and contains many round orange granules; nucleus is eccentric and stains pale blue; the vessel contains many round basophilic blast cells.


V
V
Bl
l
End.
38


Bl
rbc
V
l
39

Section from 5 day old Yolk Sac sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

Fig. 40 x1500

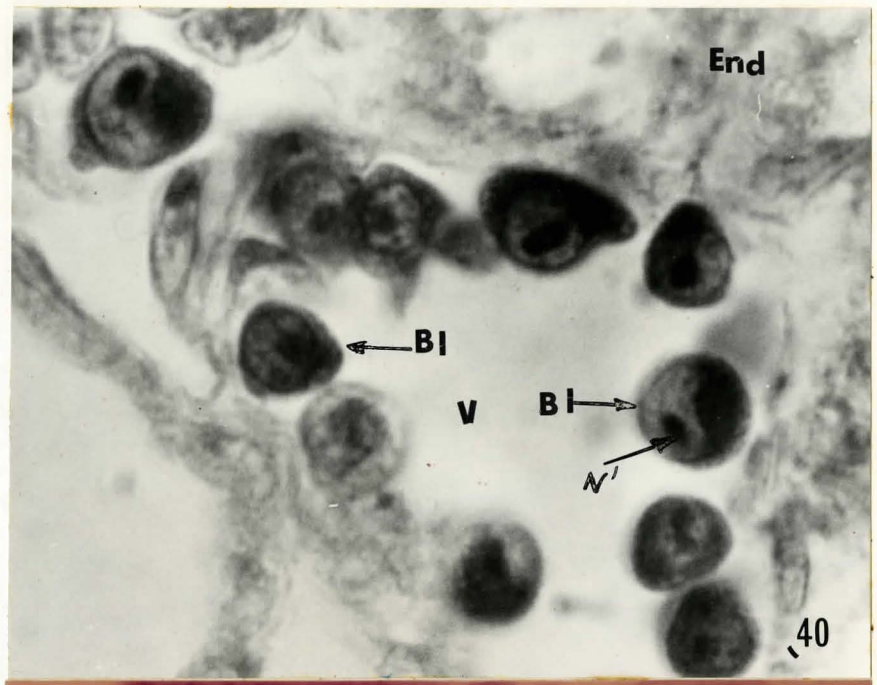
Small vessel containing basophilic blast cells the nuclei of which may be indented, stain pale blue and contain a prominent nucleolus. These are believed to be the stem cells of the erythrocyte series.

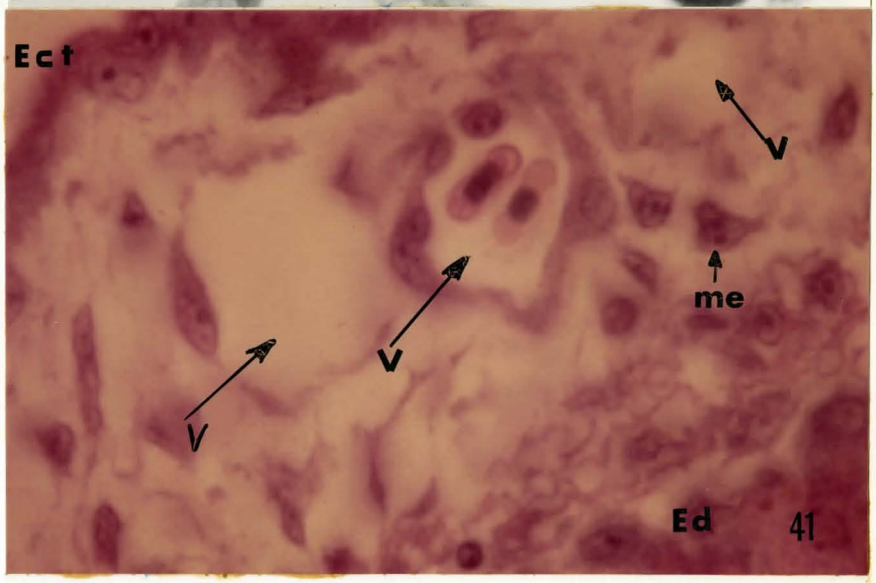
Section from the periphery of 12 day Yolk Sac sectioned at 5μ and stained with May-Grünwald Giemsa stain for tissue sections

Fig. 41 x1200

3 small blood vessels (arrows) are illustrated. The centre one contains two red blood cells and a thick endothelium. This vessel is flanked by two with rather a thin endothelium.

Note the stellate mesodermal cells (Me), and the endodermal (Ed) cell packed with yolk material.

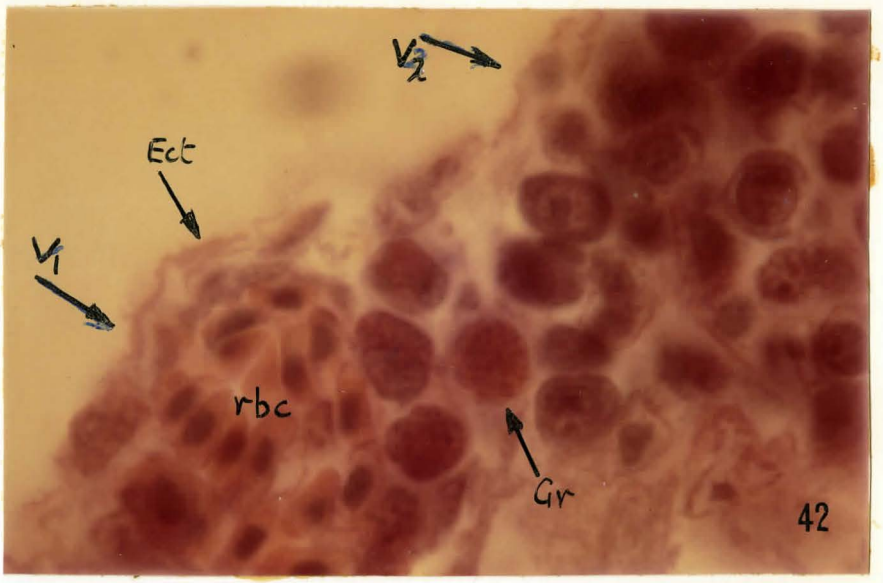

End
Bl
V
Bl
N'
40


Ect
V
me
V
V
Ed
41

Section of 12 day Yolk Sac sectioned at 5μ stained with May-Grünwald Giemsa stain for tissue sections

Fig. 42 x1200

Blood vessels lying under the ectoderm at the periphery of the yolk sac. Vessel 1, is well developed and contains red blood cells. Granulocyte; these lie extravascular to this vessel as well as to a developing vessel 2.


V2
Ect
V1
rbc
Gr
42

Smears made from Yolk Sac Vessels of 9, 10, 11 and 12 day old Embryos; smears were either air dried or fixed over Iodine Vapour and Stained with May-Grünwald Giemsa Stain

Fig. 43 x1580

10 day Yolk Sac Vessel

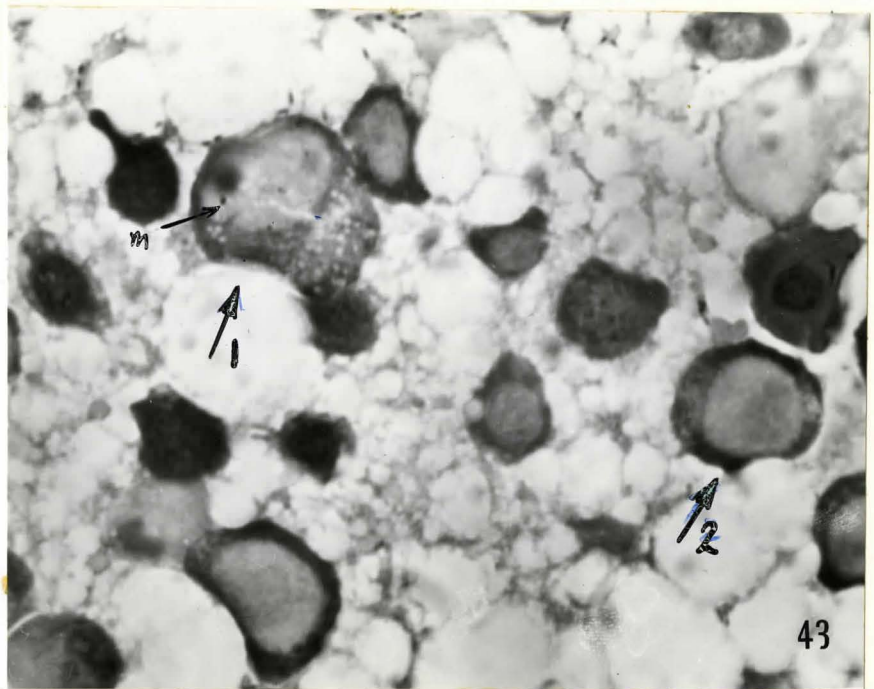
Cell 1 Heterophil late promyelocyte, from 10 day yolk sac vessel; the cytoplasm is basophilic and contains clear vacuoles, magenta granules and faint orange granules. The nucleus, eccentrically placed in the cell possesses chromatin diffuse and stains pale blue.

Cell 2 Basophilic blast cell of erythroid series

Fig. 44 x1580

10 day Yolk Sac Vessel

Cell 1 Heterophil, mesomyelocyte. Basophilic cytoplasm containing more prominent orange granules, magenta granules some in the ring form still present. The nucleus is eccentric, chromatin material diffuse and stains pale blue.



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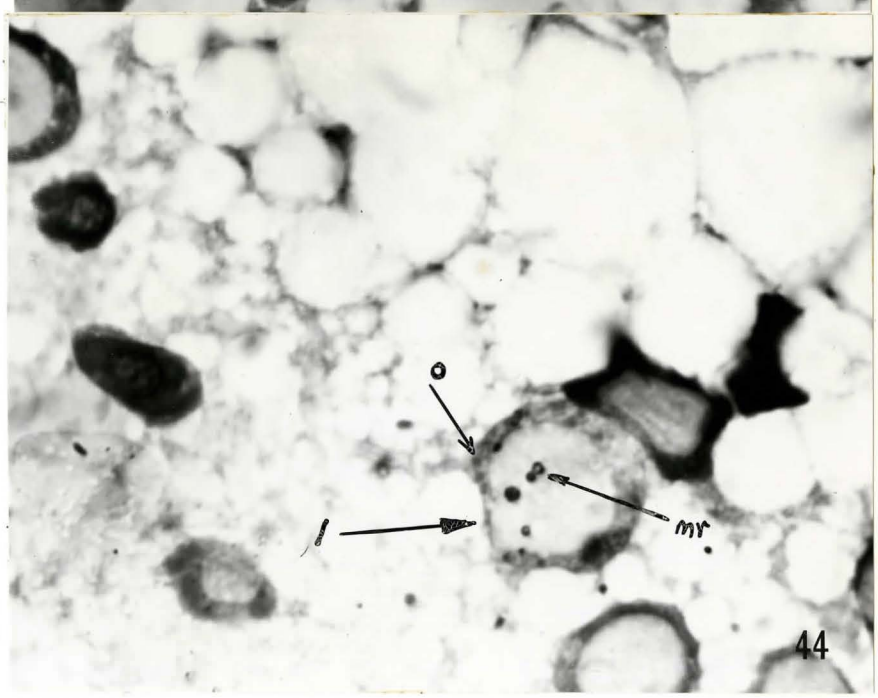


44

Fig 45 x1580

Smear from 10 day old Yolk Sac Vessel

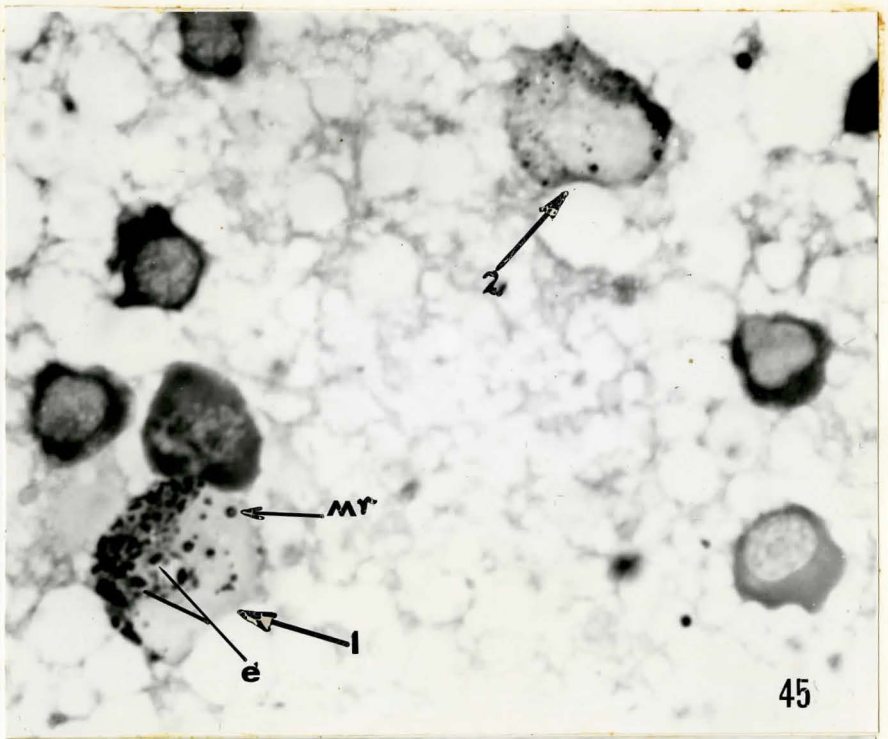
Cell 1 Heterophil metamyelocyte; cytoplasm is packed with round and elongate orange granules. There are some ring-like magenta granules over the nucleus which is indented and stains pale blue.

Cell 2 Heterophil mesomyelocyte

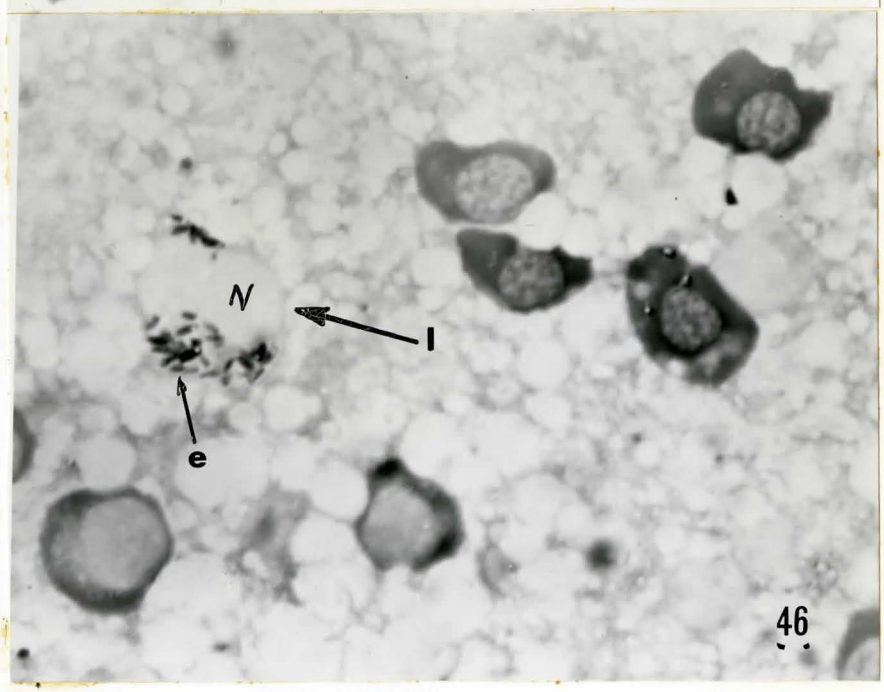
Fig. 46 x1700

Smear from 10 day old Yolk Sac Vessel

Cell 1 Heterophil, mature. Very pale blue cytoplasm containing elongate spindle shaped refractile granules. The nucleus is bilobed and stains pale blue.



45



46

Smear from 11 day old Yolk Sac Vessel stained with May-Grünwald Giemsa stain

Fig. 47 x1500

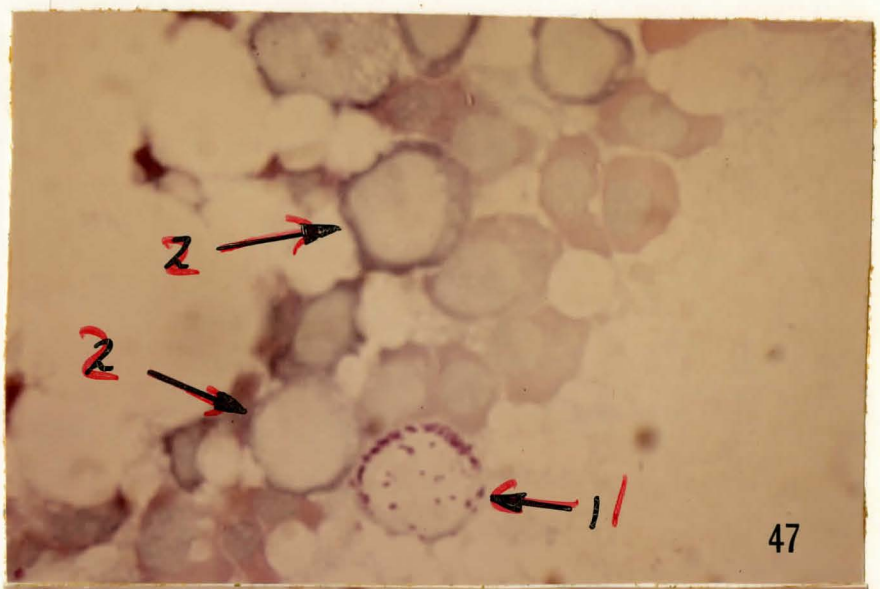
Cell 1 Basophil, immature. Purple granules are present at the periphery of the thin rim of cytoplasm which stains only slightly basophilic. There are a few purple granules over the pale blue staining nucleus. Chromatin of the nucleus is very diffuse.

Cell 2 Blast cell with thin rim of slightly basophilic cytoplasm; the nucleus stains pale blue and has diffuse chromtin.

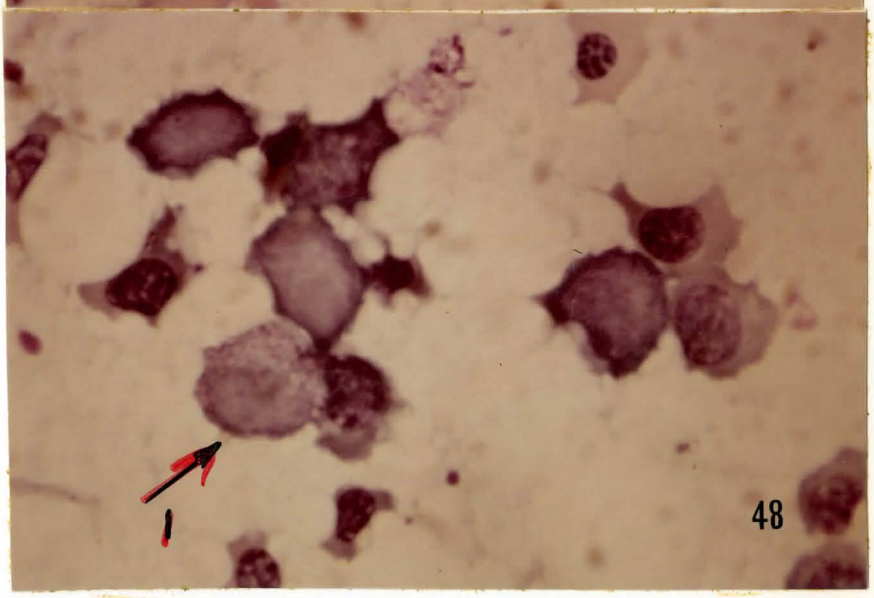
Smear from 9 day Yolk Sac Vessel stained with May-Grünwald Giemsa stain

Fig. 48 x1500

Cell 1 Mesomyelocyte (Heterophil, late mesomyelocyte or eosinophil mesomyelocyte). The cytoplasm is basophilic and contains many orange granules and some clear vacuoles; no magenta granules are seen. The purple and the chromatin material is diffuse. The nucleus appears indented.



47



48

Vessel stained with May-Grünwald Giemsa stain

Fig. 49 x1500

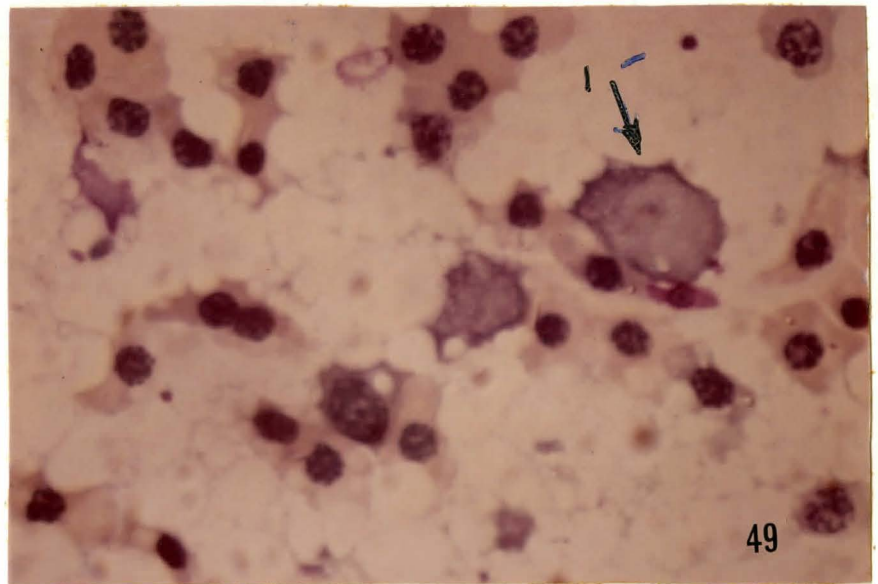
Cell 1 Heterophil, promyelocyte. Note the basophilic cytoplasm containing faint orange granules; small magenta granules are present in the cytoplasm and at the edge of the nucleus (not well illustrated in the photomicrograph). The nucleus is large and pale blue staining, with diffuse chromatin.

Stem (blast) Cells

Fig. 50 x1500

Cell 1 Type 1; large round cell, basophilic cytoplasm, large round to ovoid nucleus with diffuse chromatin which stains pale purple blue.

Cell 2 Type 2; smaller cell, basophilic cytoplasm round to ovoid nucleus with coarser condensed chromatin which stains more densely purple.



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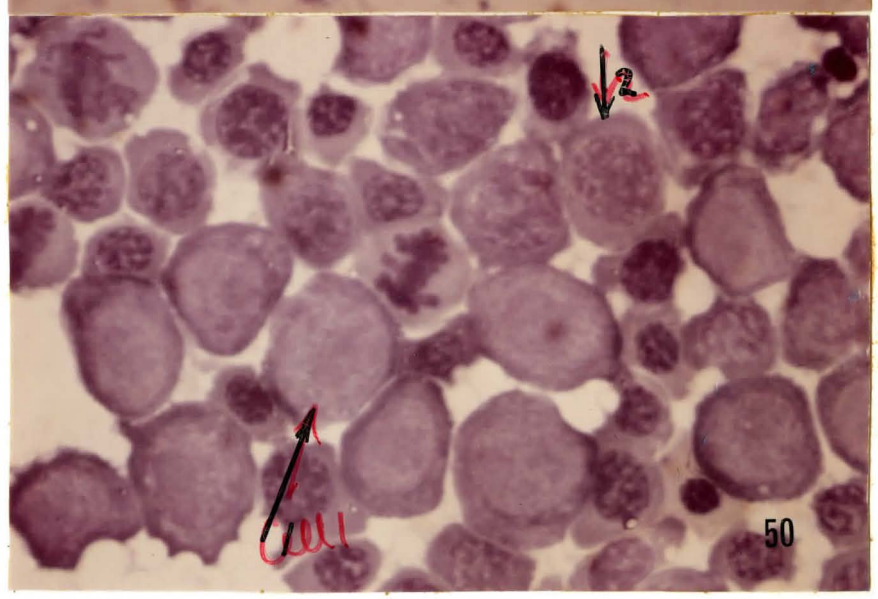


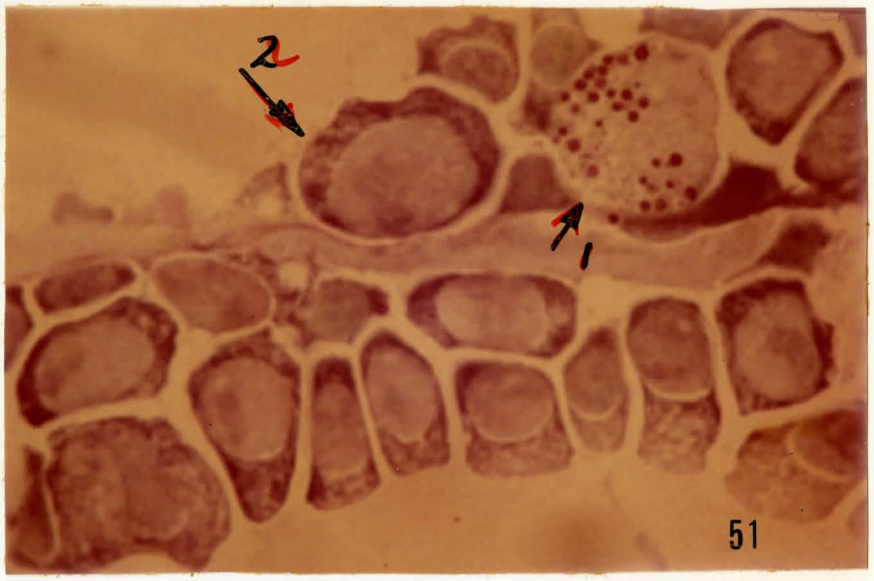
50

Smear from 12 day Yolk Sac Vessel

Fig. 51 x1500

Cell 1 Heterophil late mesomyelocyte. Cytoplasm slightly basophilic contains clear vacuoles, orange granules and magenta rings and granules. The nucleus is eccentric, indented chromatin is diffuse and stains pale blue purple.

Cell 2 Stem (blast) cell. Basophilic cytoplasm ovoid nucleus stains blue-purple, diffuse chromatin. Some vacuoles in the cytoplasm. (May be a granuloblast) Type 2.


2
A1
51

Symbols used for Electron Micrographs

Granules	G_1, G_2, G_3, G_4
Golgi complex	G
Endoplasmic reticulum	er
Nucleus	N
Mitochondria	M
Desmosome	des.
Pinocytotic vesicle	PV
Vacuoles	V
Myelin figure	My
Nucleolus	N
Compound Vacuole	CV
Endodermal cell	End.
Red blood cell	r.b.c.

Explanation of Figures

Electron Micrographs of 14 day old Chick Embryo Spleen prefixed in Gluteraldehyde postfixed in 2% Osmium, sectioned and stained with Lead Citrate

Fig. 52

Cell 1 Heterophil, mesomyelocyte. 3 granule types are present, dense round, floccular and fibrillar. Golgi complex can be seen, some endoplasmic reticulum; nucleus is eccentric little evidence of chromatin clumping

Cell 2 (Heterophil, mesomyelocyte possibly) possesses two granule types, dense round and floccular.

Cell 3 This cell is larger than surrounding splenic cells and may represent a blast cell.

Total Magnification 9000X

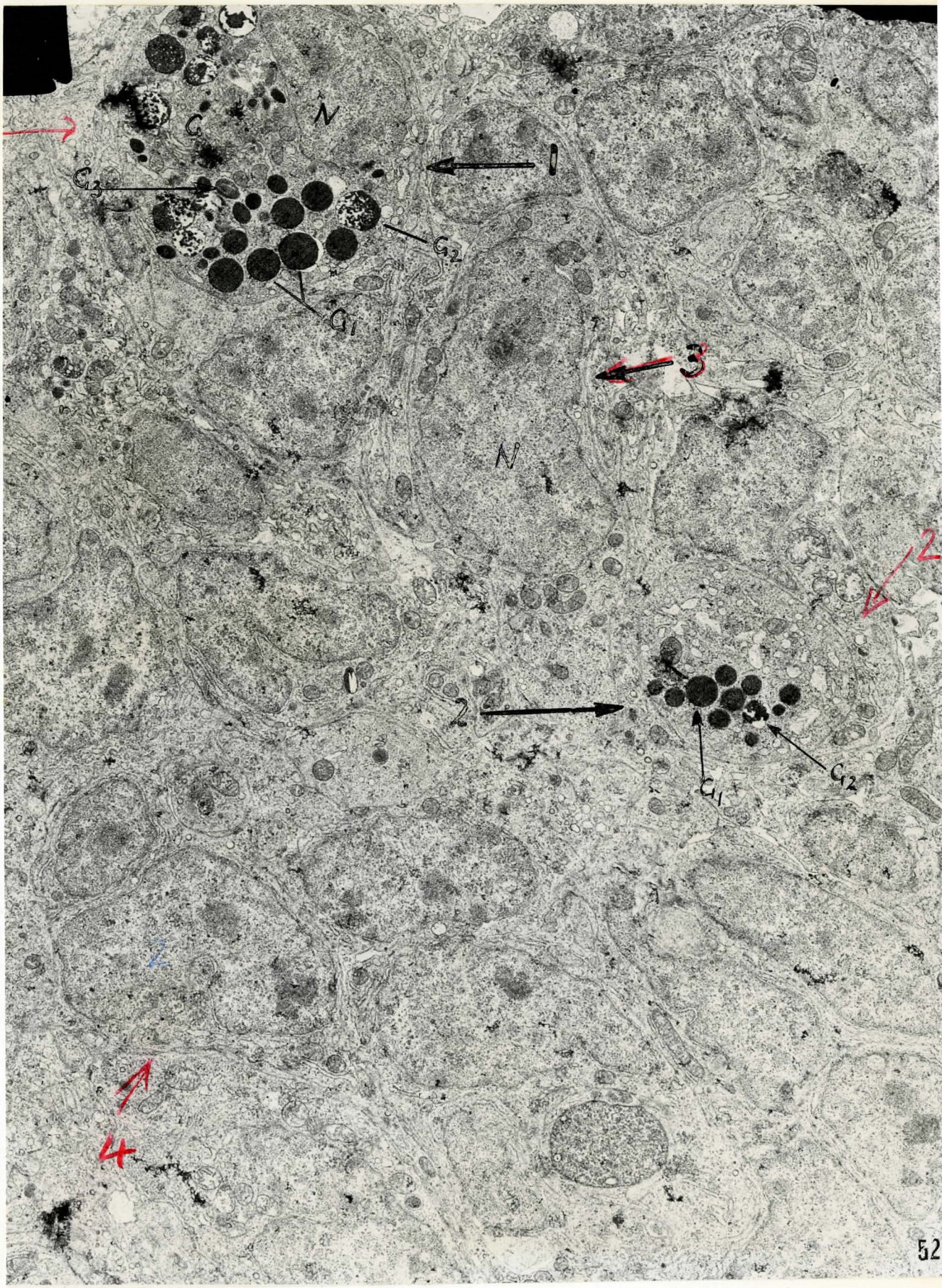

G
N
1
G_3
G_2
G_1
3
N
2
1
2
G_1
G_2
2
1
4

Fig. 53

Cell 1, 2, 3 Heterophil, mesomyelocytes, two types of granule present, dense (G1) and floccular (G2) (Broken)

Cell 4 Heterophil, early metamyelocyte. Two types of granule present, some of the dense (G1) ones have become elongate, a lobe of the nucleus can be seen. (N)

Total Magnification 9000X

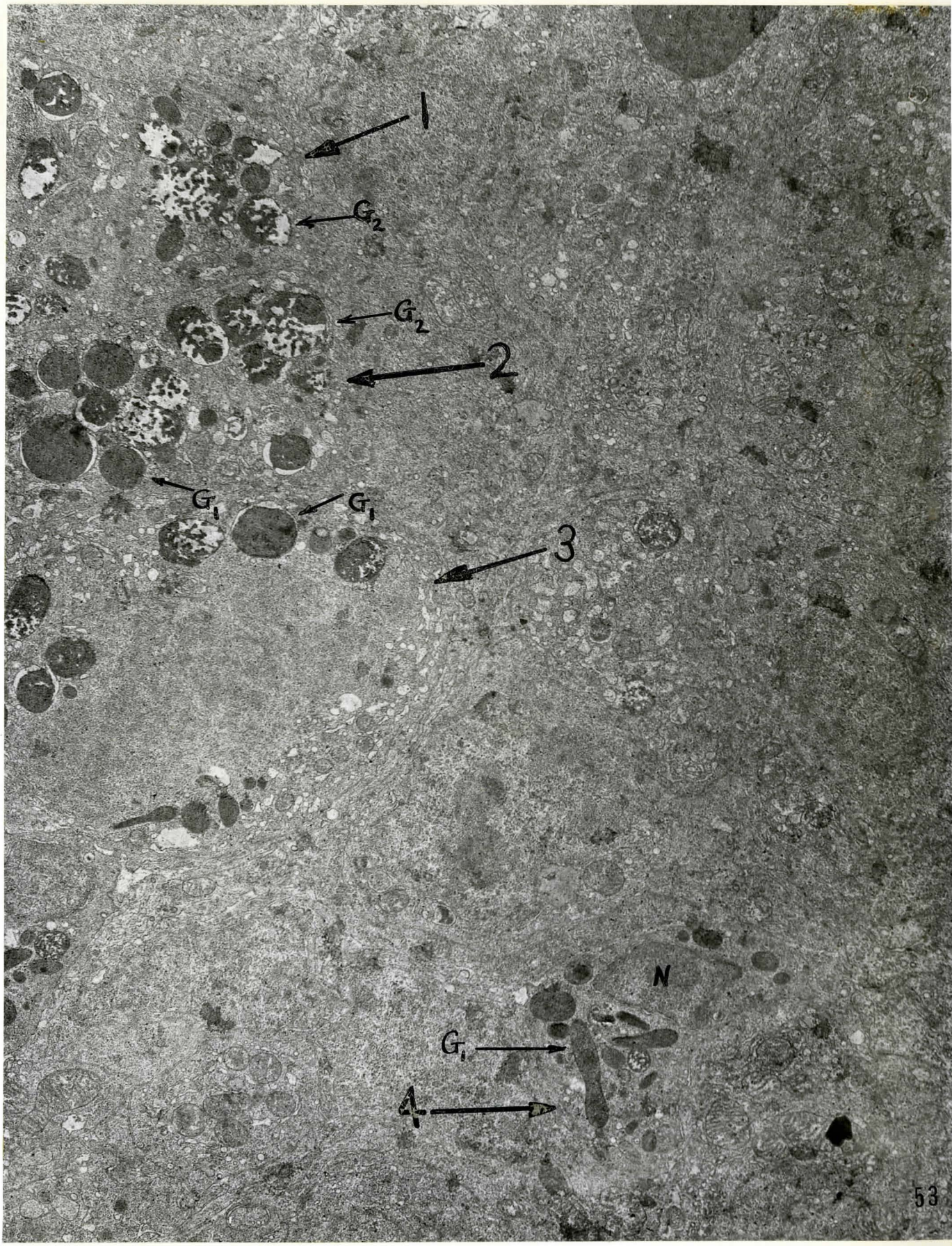

1
G2
G2
2
G1
G1
3
N
G1
A1

Fig. 54

Heterophil, metamyelocyte. Three types of granule present (G 1,2,3). Some dense granules have become spindle shaped indicating metamyelocyte stage. The nucleus appears as three lobes (N). There is some evidence of pinocytotic activity at the cell surface (arrows). Various clear round vacuoles are present.

Total Magnification 30,000X

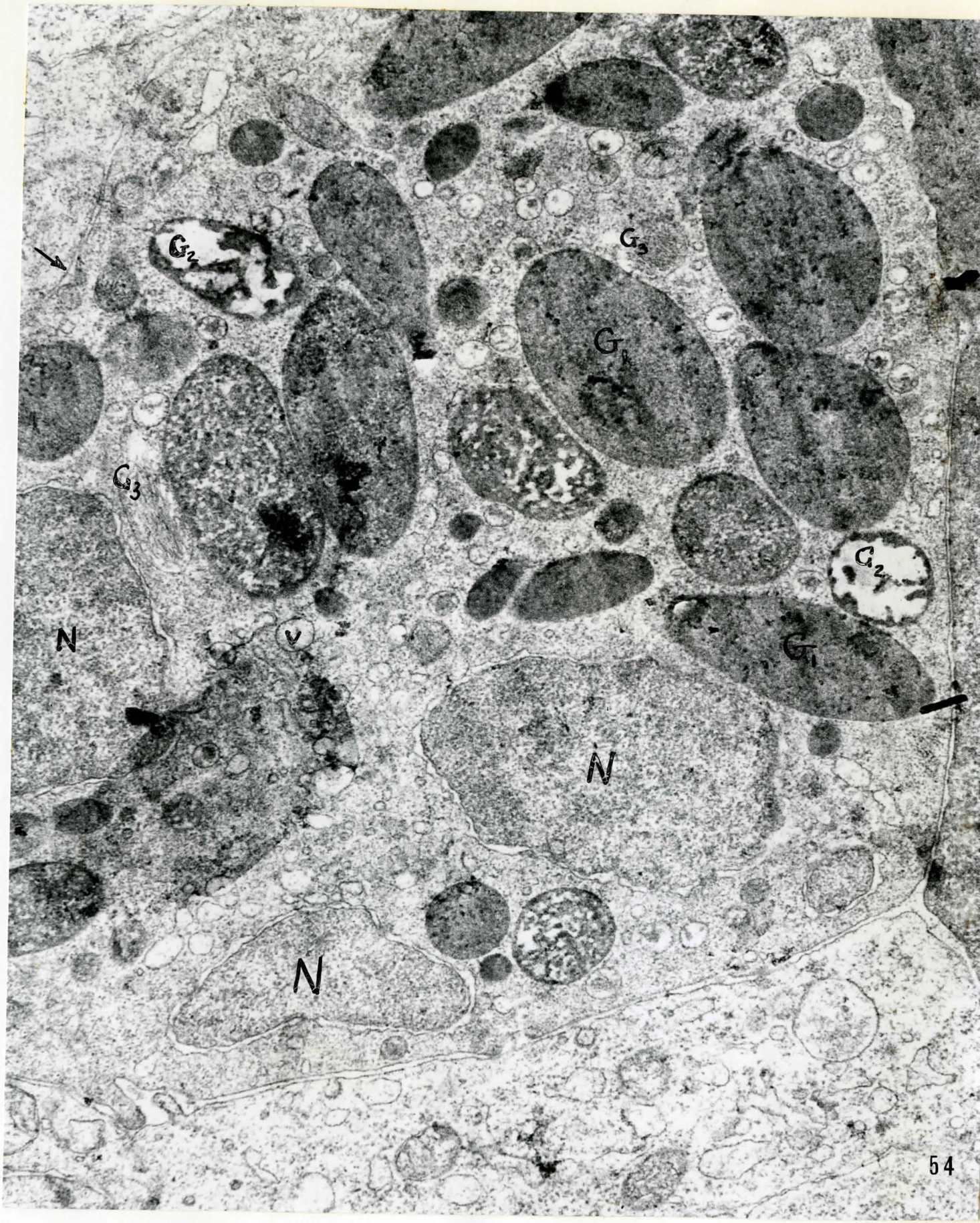

G2
G3
G1
G3
G2
G1
N
v
N
N

Fig. 55

Higher magnification of granules and Golgi (G) area from mesomyelocyte of Fig. 52. Three types of granules are shown, dense floccular (or broken) fibrillar. A small dense vacuole (arrowed) can be seen to be made up of small vesicles. Many small dense vesicles are associated with the Golgi complex.

Total Magnification 30,000X

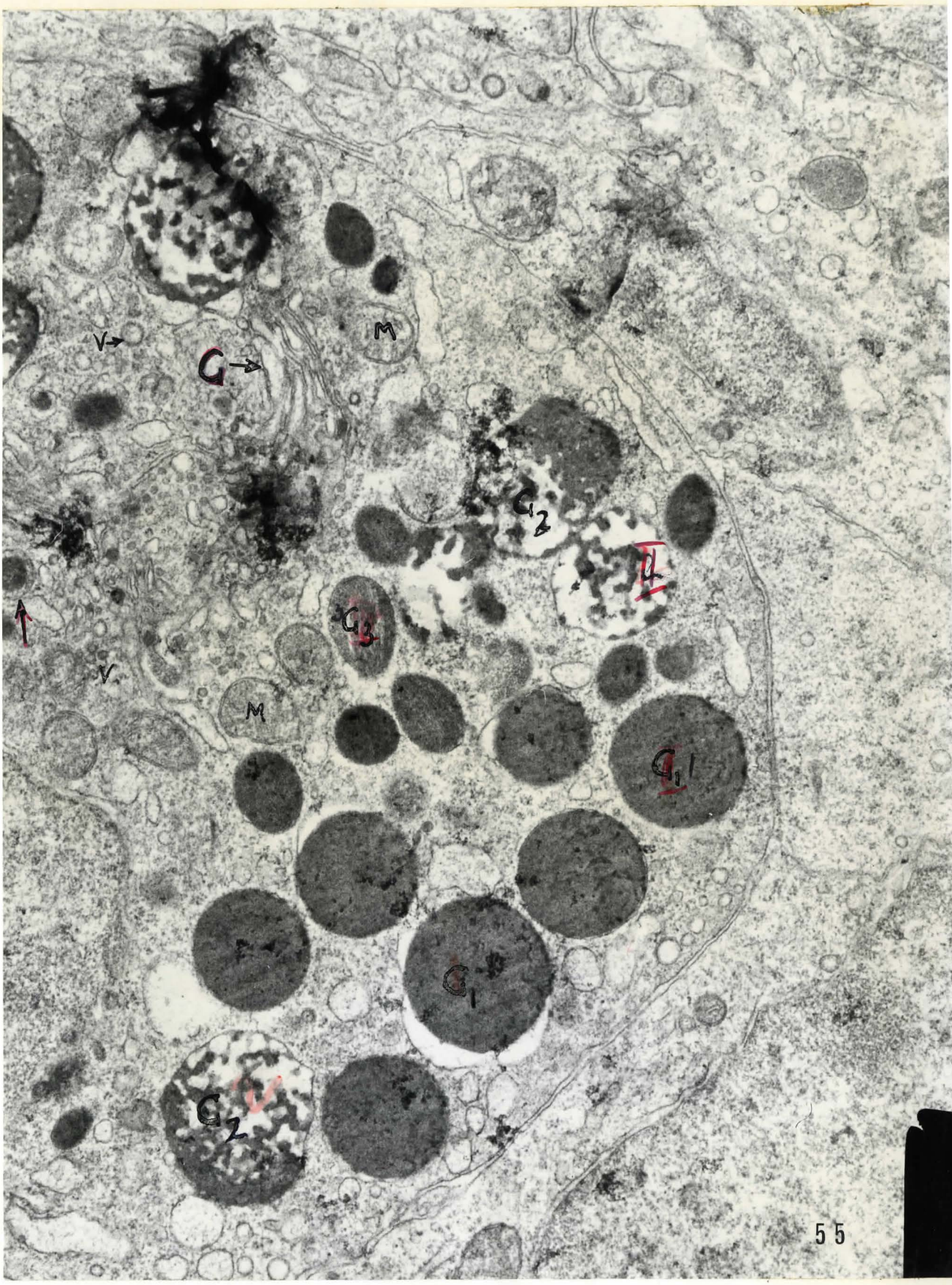

V
G
M
G_2
II
G_3
A
V
M
G_1
G_1
G_2

Fig. 56

Higher magnification of the three types of granule 1)dense 2) floccular (broken) 3) fibrillar. Granule 1 and 2 have a similar homogeneous granular appearance whilst G3 appears to be fibrous. 4 may be a condensing type 1 granule. (Some lead precipitate present).

Total Magnification 90,000X

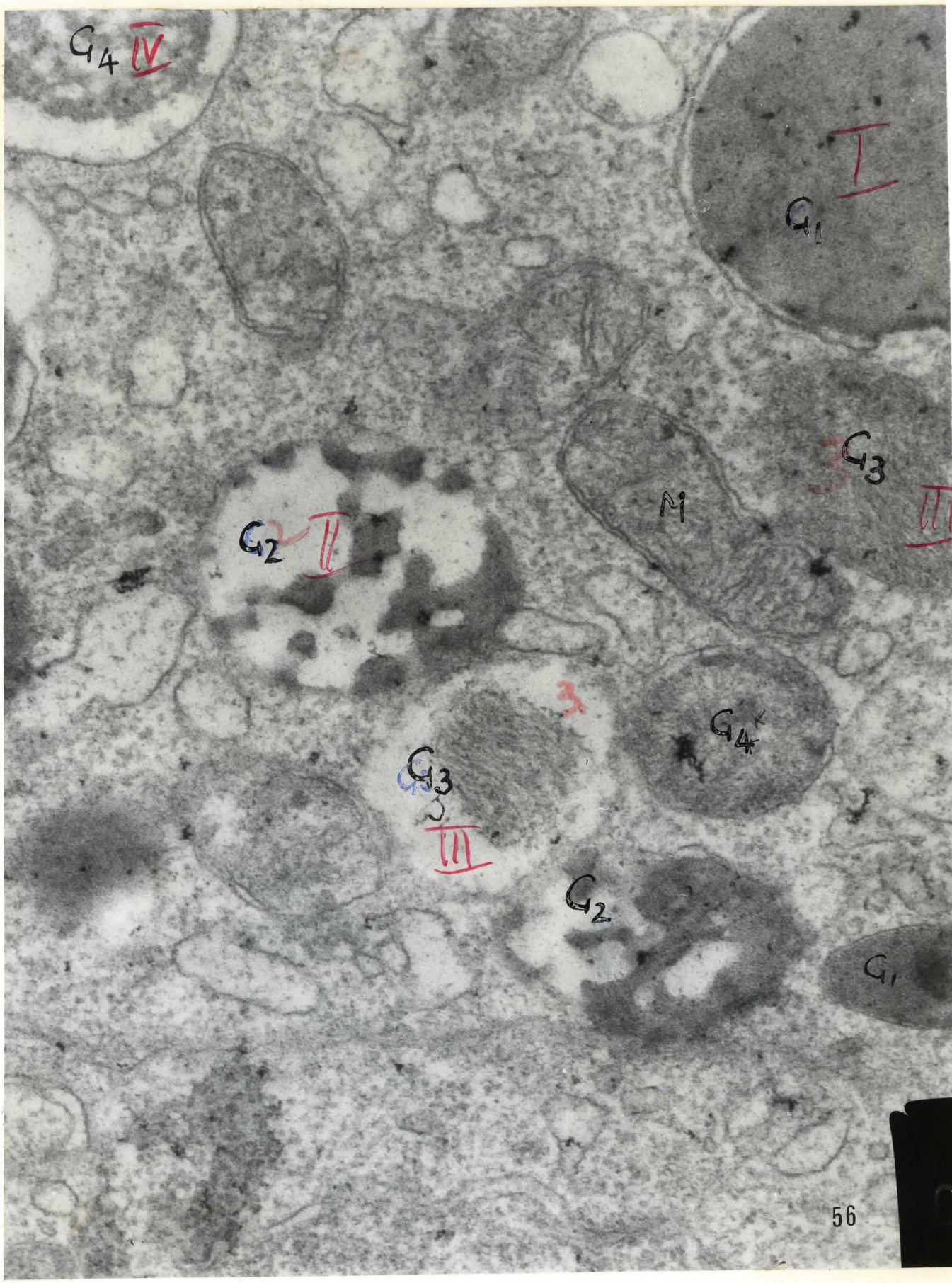

G4 IV
I
G1
G3
M
II
G2
3
G4
G3
III
G2
G1

Fig. 57

Higher magnification of granules type 2 and 3. These are present in a heterophil, part of the nucleus (N) is shown. Type 2 granule has a distinct membrane in places. This may be broken (arrowed) or lost. *

Total Magnification 90,000X

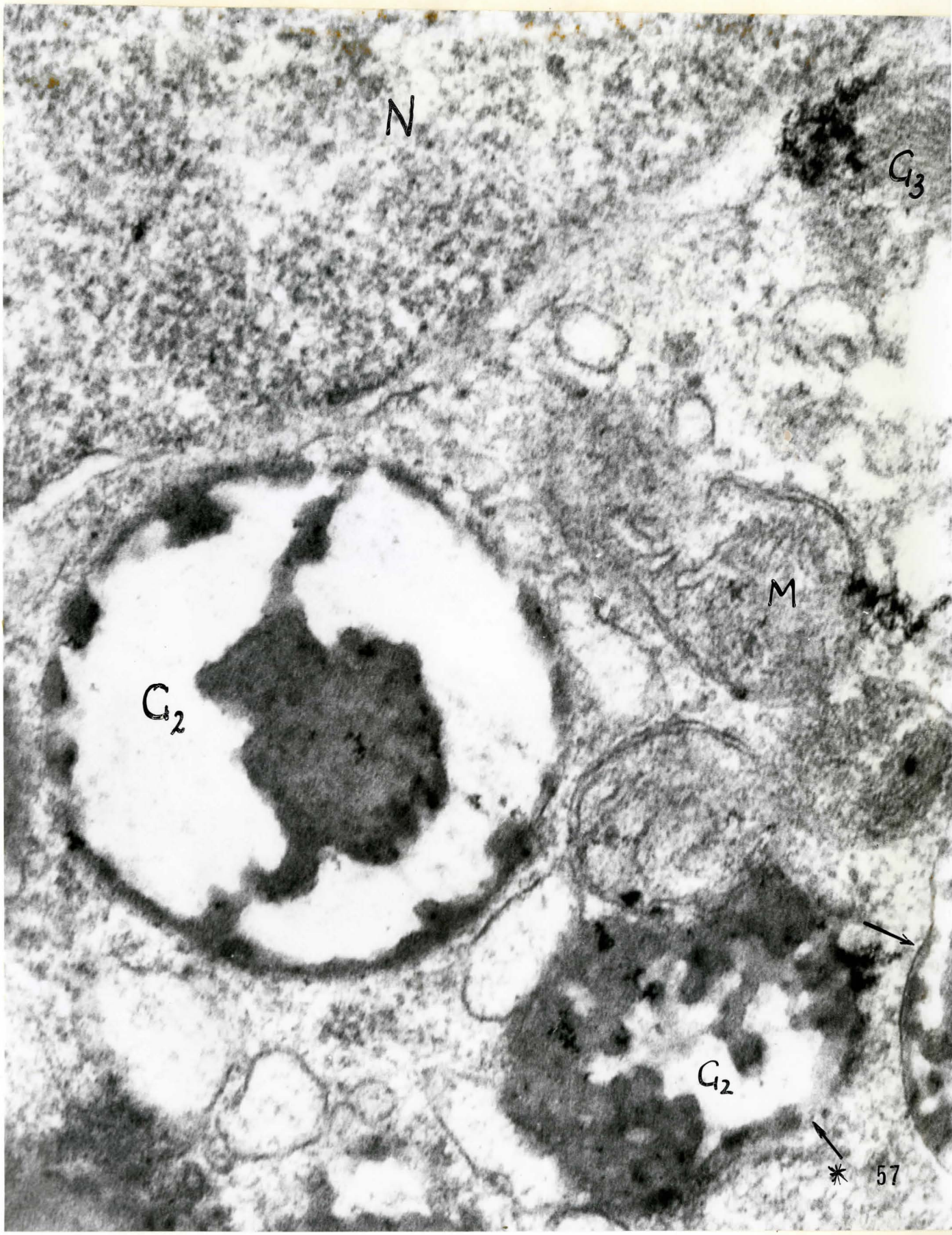

N
G3
M
G2
G2
57

Fig. 58

Heterophil, mesomyelocyte, three granule types; swollen endoplasmic, reticulum and vacuoles.

Total Magnification 30,000X

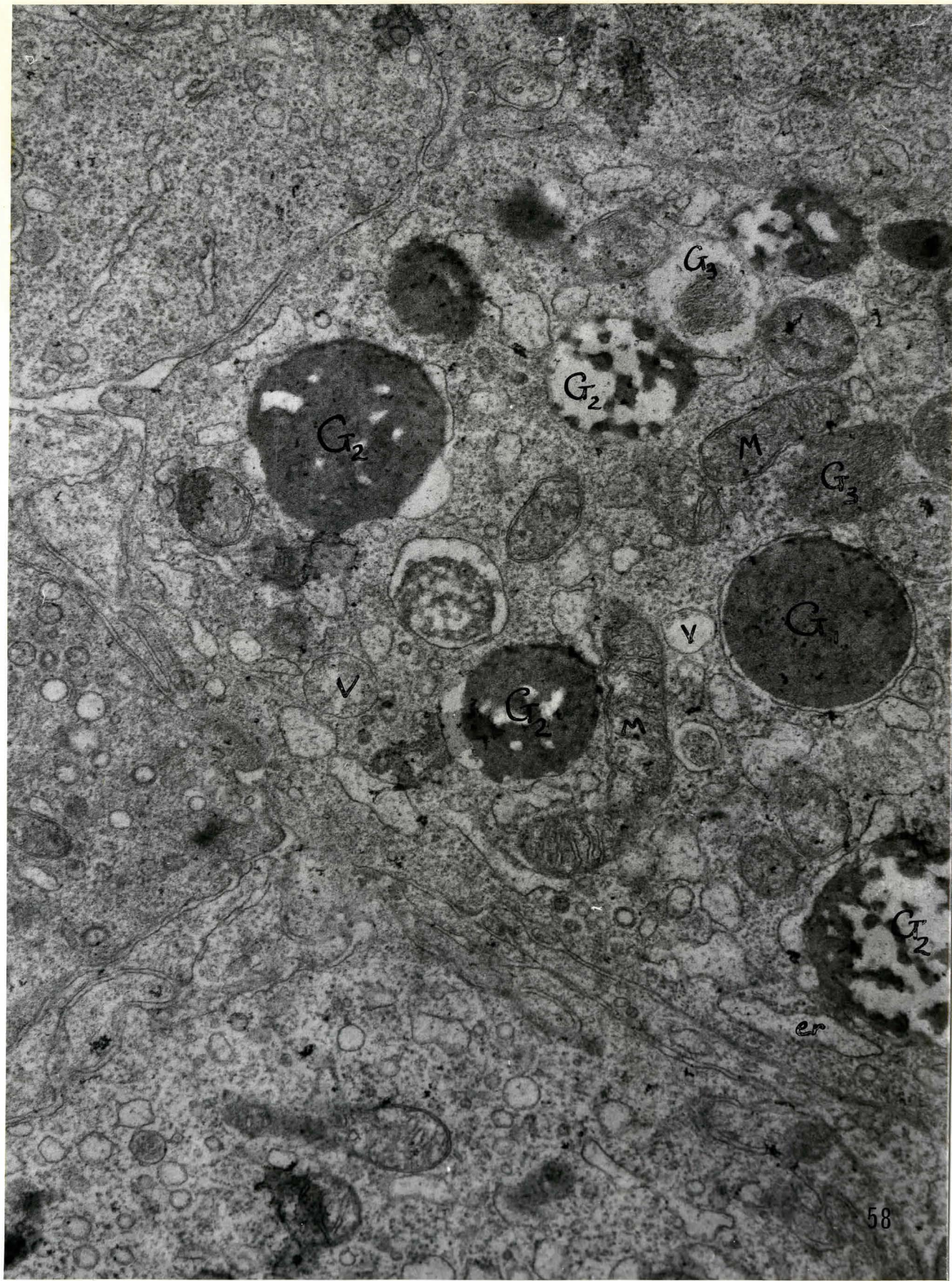
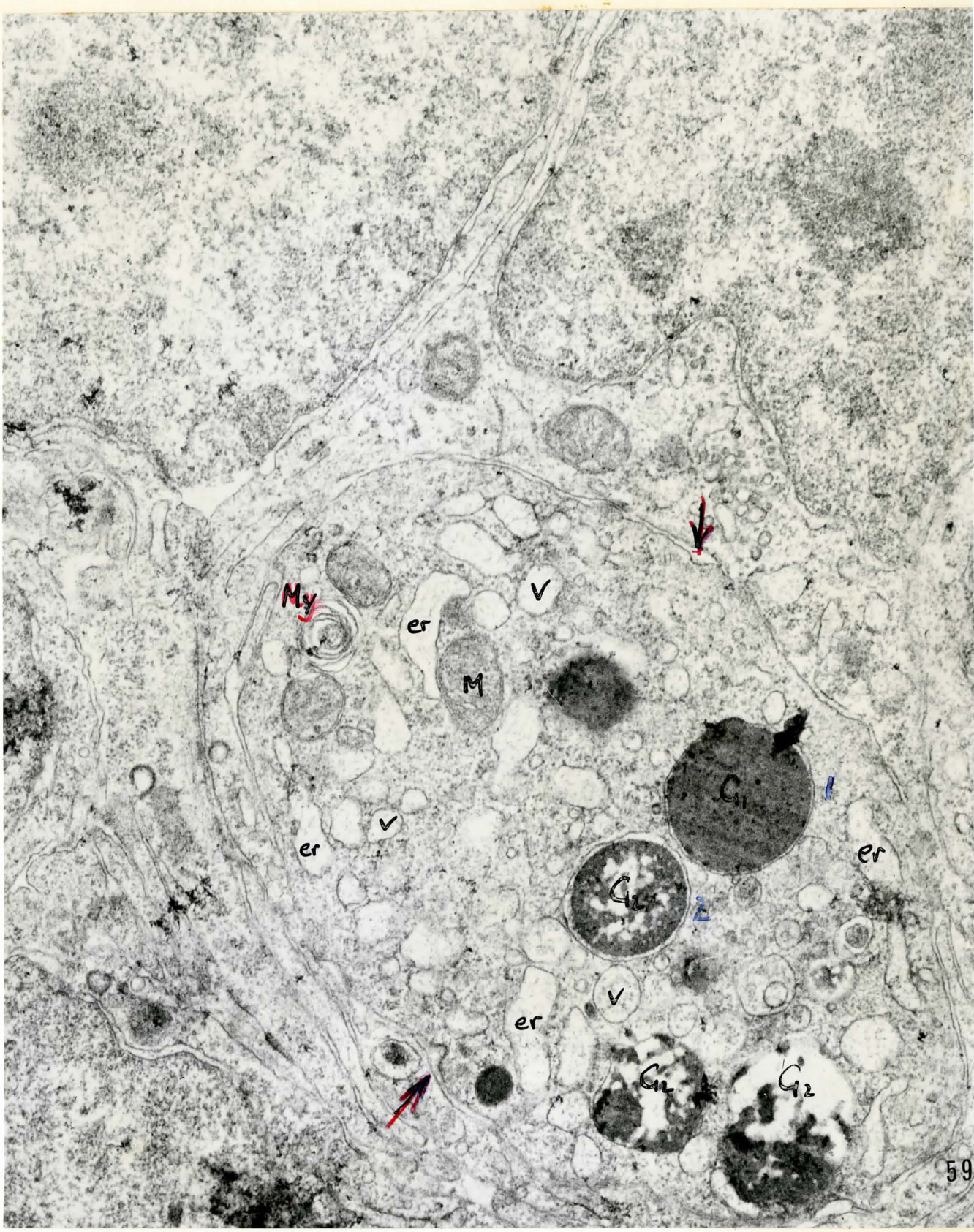

G3
G2
G2
M
G3
G2
V
G1
V
G2
M
G2
er

Fig. 59

Heterophil, mesomyelocyte, showing many vacuoles and swollen endoplasmic reticulum. Nucleus is not seen in this section. Myelin figure is seen in the cytoplasm (My). Coated pinocytotic vesicles can be seen forming (arrowed). Granules of type 1 and 2 are indicated.

Total Magnification 30,000X


My
er
V
M
V
er
G1
1
er
G2
2
V
er
G2
G2

Yolk Sacs from 3 and 14 day Embryos fixed in 2% Osmium sectioned and stained with Lead Citrate

Fig. 60

Early stage of development of blood cell, round cell with round central nucleus (N) prominent nucleolus (N'); cytoplasm contains many vacuoles and disrupted mitochondria (fixation artefact). The cell may possibly be a haemoangioblast. It is in close association with the large vacuolated endodermal cell by means of small processes and a desmosome (arrow)
Endodermal cell (Ed) contains many clear vacuoles both large and small, Myelin figure (My), and yolk granules YG. Compound vacuole (CV).

Total Magnification 16,000X

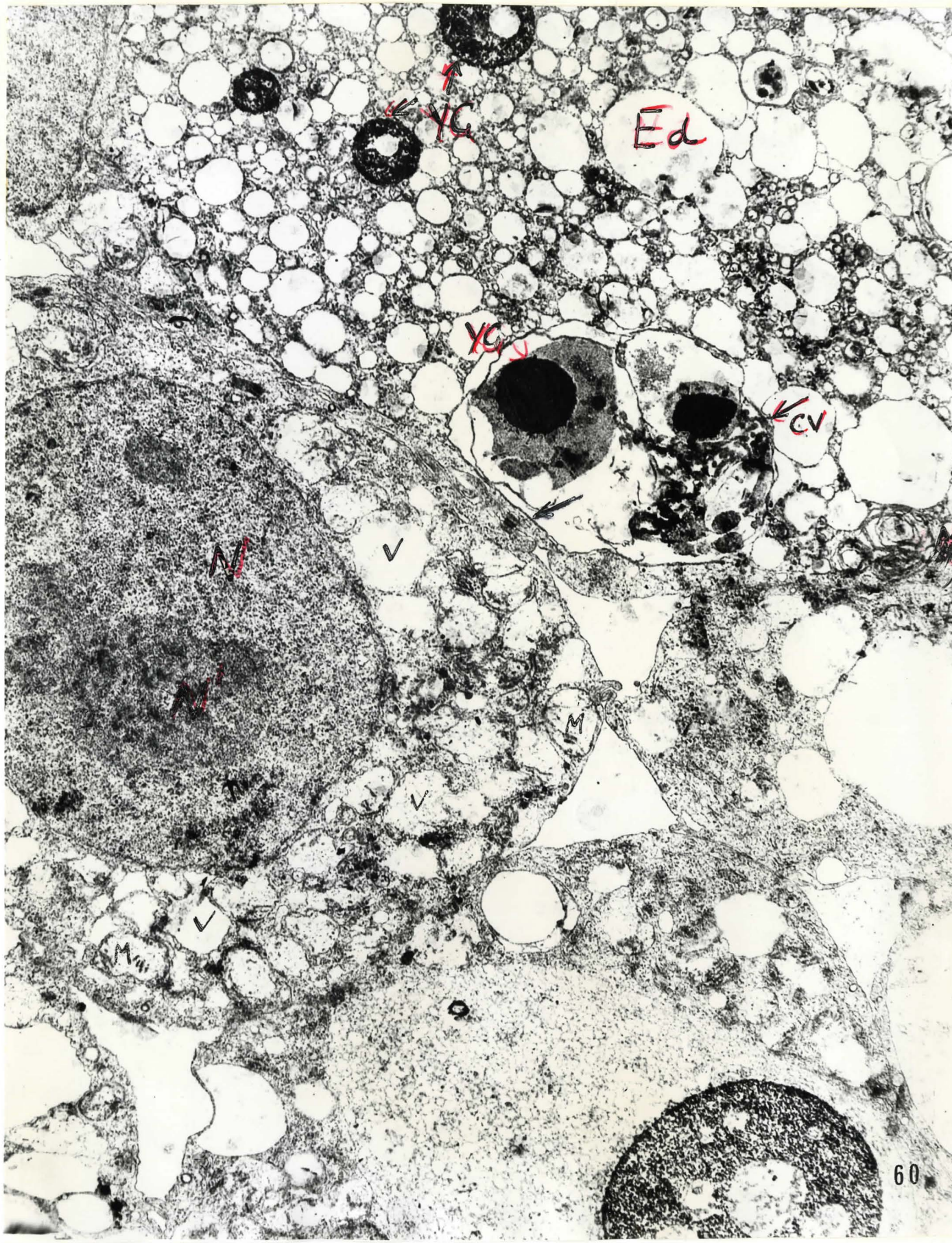

YG
YG
Ed
CV
M
M
V
V
V
M
M
60

Fig. 61

Endothelium of yolk sac blood vessel. Endothelial cells elongate and overlap each other; several coated vesicles are seen to be forming on the lumen side of these cells. The lumen contains nucleated red blood cells with an expanded perinuclear space (fixation artefact)

Total Magnification 23,000X

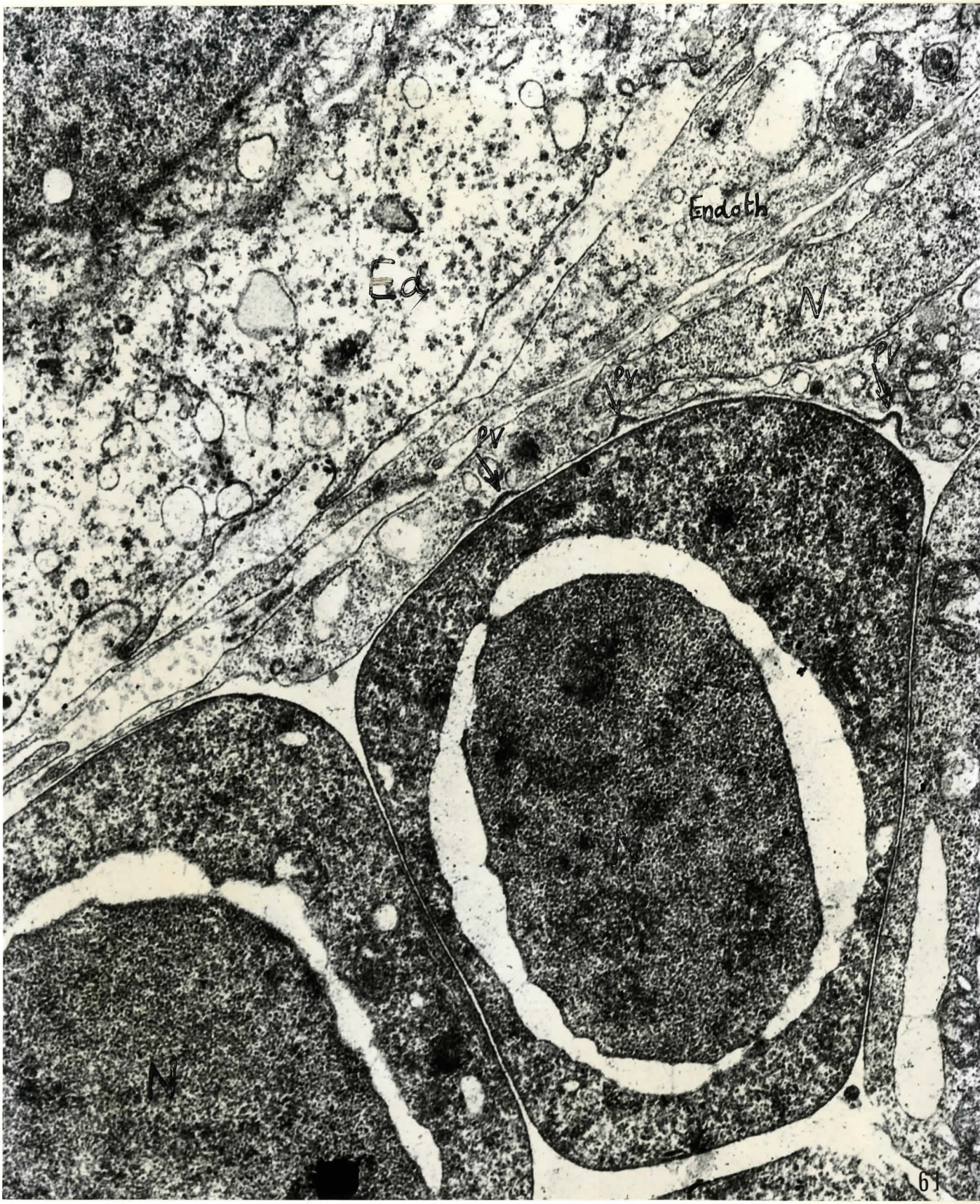
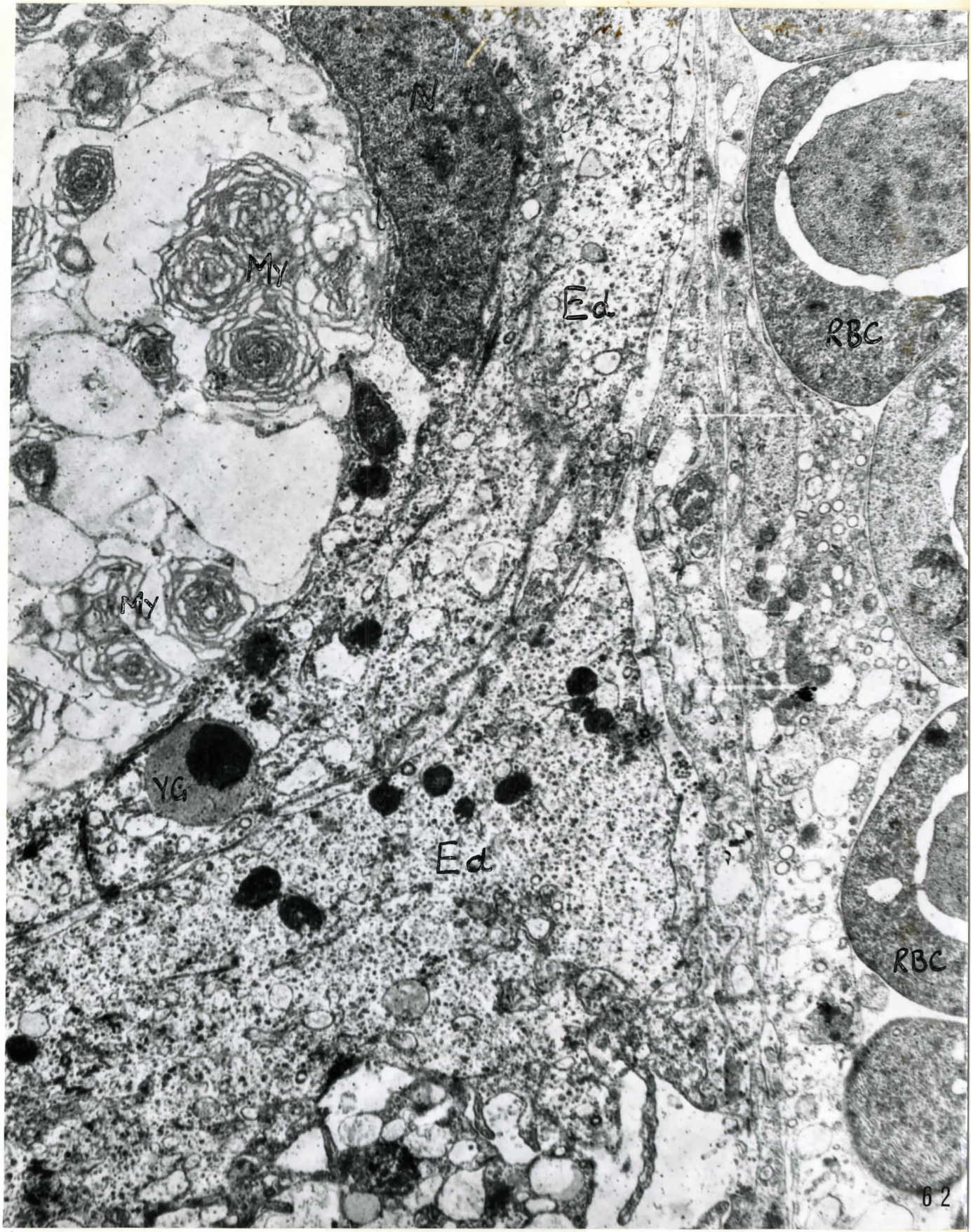

Endoth
Ed
N
PV
PV
PV
N
N

Fig. 62

Endothelium of yolk sac vessel, adjacent to it is a large endodermal cell (Ed) the nucleus is small and situated towards the vessel. Cytoplasm is sparse; much of the cell is taken up by a large vacuole containing many myelin figures (My). Yolk granules (YG) are present.

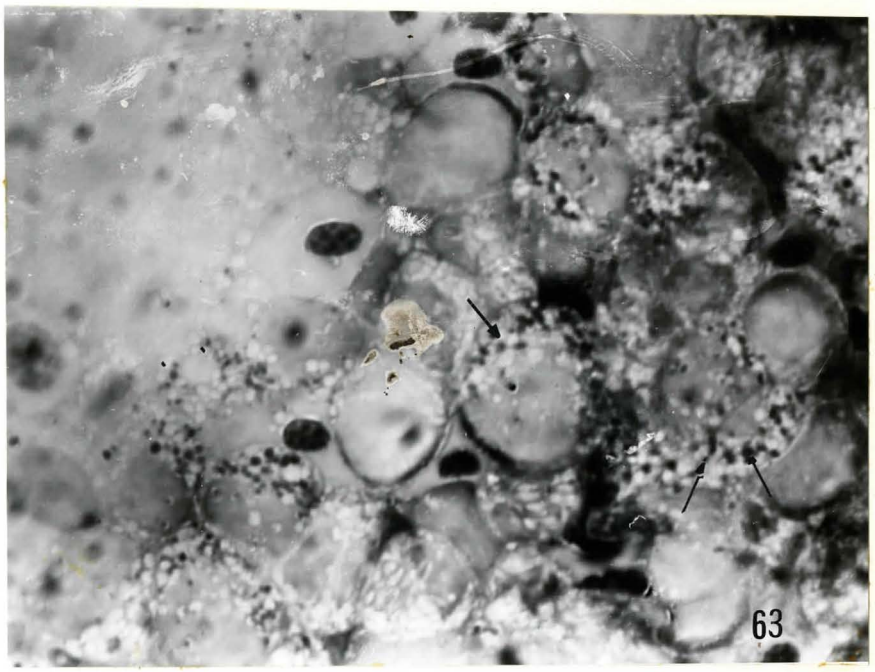
Total Magnification 11,500X


N
My
Ed
RBC
My
YG
Ed
RBC
62

Imprint of 18 day Embryo Spleen digested with Diastase stained with Alcoholic P.A.S. and counter stained with Harris' Haematoxylin

Fig. 63 x1580

P.A.S. positive granules (arrows) are seen in cells which are indentified as mesomyelocytes


63

DISCUSSION

Time of Appearance of granulocytes in spleen and yolk sac

Granulocytes, mostly promyelocytes and early mesomyelocyte were seen in the yolk sac sections at 4 days always extravascularly either singly or in twos. I could not confirm Sabin's (1921) findings that they were usually in groups of four. By 9 days of incubation cords of granulocytes were present outside the main yolk sac vessels and between the clumps of cells which formed the auxillary vessels. At 12 days, granulocytes existed in great numbers as cords and clumps of cells outside the well developed yolk sac vessels and also as strings of cells in the endoderm between vessels. Fewer observations were made after 12 days incubation but it did appear that in the last days of incubation, i.e. from about day 18 to 21, the numbers of granulocytes present in the yolk sac had decreased. It would then appear that the most active period of granulopoiesis in the yolk sac was around day 12, and with the decrease in size and thickness of the yolk sac towards the end of incubation, granulopoiesis also decreased. Sabin (1921) reported granulocytes in area pelucida cultures at 3 days. Sugiyama (1926) while expressing difficulty in identifying heterophils and eosinophils did find granulocytes in the area pellucida of the chick between

three and a half and four days although granulocytes were seldom seen in the circulation at this time. Lemez (1964) in his review of chicken blood states that granulocytes appear in the embryonic circuation at about 4 to 5 days but in no great numbers until after day 11. The present work confirms the time of appearance of granulocytes in the yolk sac of previous authors.

Before day 10, the chick spleen was very fragile and difficult to remove without damaging; however, some imprints were obtained at 8 and 9 days in which early stages of heterophils could be found. At 10 days, heterophil granulocytes in all stages of development could be found, although promyelocytes and mesomyelocytes predominated. By 12 days, the spleen showed a tremendous increase in the numbers of granulocytes present; although heterophils were the main type present, a few eosinophils and basophils were identified.

Comparing the time of appearance and the numbers of granulocytes (although no counts were made) in the spleen and yolk sac large numbers of granulocytes were present at 12 days. Whereas in the yolk sac, the numbers showed a steady increase to that time, in the spleen there was a rapid increase between day 11 and 12. In both tissues, mitotic cells were found only in small numbers and very rarely seen in the granulocytes themselves. This raises the question of how does the increase in granulocyte number come about, particularly in the spleen where the increase is dramatic. Possible

explanations for the low number of mitotic figures include: 1) there may be a small limited number of cells capable of mitotic division; these are actively dividing to produce granulocytic stem cells which develop to maturity with little or no division, so that at any one time only a few cells are in mitosis or 2) perhaps the spleen and yolk sac are populated by imigration of stem cells from other areas of the embryo; however, in the chick at 12 days the yolk sac and spleen are the major haemopoitic organs.

There is a sequence in the importance of yolk sac and spleen as granulopoietic organs. Granulocytes first appear in the yolk sac with a peak in numbers around 12 days after which they decrease. At day 12, there is a great increase in numbers found in the spleen but this large number is maintained to and after hatching while those in the yolk sac decrease. According to Sandreuter (1951), the granulopoietic capacity of the spleen decreases after hatching when it is replaced by an increase in granulopoiesis in the bone marrow a short time before hatching. The bone marrow then becomes the major organ of granulopoiesis during post embryonic life. In the present work large numbers of granulocytes were observed in the spleen at hatching and at two days post hatching.

A question arises concerning the stimulus involved in the initiation of granulopoiesis in each organ, first in the yolk sac then spleen and finally in the bone marrow. It is not until the latter third of development that there is any

bone marrow present so that we would not expect to find granulocytes there before that time. The yolk sac is well developed and functions early in development so again one might expect to see granulocytes there early in embryonic development. Thus the time of appearance of blood cells is dependent on the development of the functional haemopoietic organ. Whether the granulopoietic stimulus is general, i.e. stimulates each haemopoietic organ similarly or is specific, i.e. a separate stimulus for each haemopoietic organ, is unknown.

Location of granulocytes in spleen and yolk sac

The present light microscopic results from paraffin sections of yolk sac agree with the findings of previous authors with regard to the location of granulocytes (Dantschakoff 1916, Sabin 1920, 1921, Sugiyama 1926). Developmental stages of granulocytes were recognised and always found extravascular to all blood vessels, the main well developed vessels as well as to the developing auxillary vessels and clumps of blast cells surrounding vessels. In sections of spleen, granulocyte developmental stages were observed; these were found throughout the spleens of 12 day old embryo, in the cords of splenic cells at the centre and periphery of the organ, adjacent to the sinuses and occasionally within the sinus.

Development of granulocytes

In the present study of avian granulocytes eosinophils were not easily indentified and few basophils were present at the time of development which was most studied; consequently,

the early stages of these two granulocytes were difficult to characterise and the discussion will concentrate mainly on the development of heterophils.

In paraffin sections of yolk sac at 12 days, granulocytes were seen which could be defined as mature or immature by the shape of the nucleus and presence of refractile orange granules. Immature cells possessed ovoid nuclei which with development became indented and finally lobed; this agrees with the observations of Sugiyama (1926). However, Sugiyama states that the number of red refractile granules increased andformedrosettes; while the present author agrees with the increase in granule number they were not distributed to form rosettes. The granules, first round and faintly orange showed increases in intensity of colour and refractility and finally became rod shaped. The developmental stages of heterophils found in sections of the spleen were found to be identical to those of the yolk sac. Eosinophils and basophils were not identified in sections of these tissues although they were identified in smears and imprints of both organs. Magenta granules so obvious in heterophils seen in imprints and smears were not observed in sectioned material. Although ring-like granules were seen, they stained orange in sections while in imprints they were always magenta coloured. This suggests to the author the action of May-Grünwald Giemsa stain is different in tissue sections and smears, perhaps due to the different fixation techniques. No mention is made by the

earlier authors (Sabin 1921, Sugiyama 1926) of the magenta granules.

While paraffin sections of yolk sac and spleen taken at 12 days did reveal the presence and location of granulocytes, imprints and smears of these tissues allowed a much better understanding of the developmental stages. Identical stages of development of heterophils were found in both yolk sac and spleen. The promyelocyte, was a large cell with basophilic cytoplasm containing a round to slightly indented nucleus, with diffuse chromatin, occupying about one-half the area of the cell. In sections, faint orange coloured granules were seen. However, in whole cells from imprints, two types of granules were identified; dense large and small magenta coloured granules were present in the cytoplasm and over the nucleus and faint orange granules in the indentation of the nucleus. Although very early promyelocytesdid vary in size and staining reactions of the nucleus and cytoplasm (see discussion of stem cell), the middle and late promyelocytes appeared to be identical.

Mesomyelocytes were smaller cells than the promyelocytes. They possessed cytoplasm less basophilic, a nucleus more eccentrically located in the cell and a more prominent nucleolus than the promyelocyte. Cells from sections showed orange round granules and clear vacuoles in the cytoplasm. Cells from imprints and smears showed the presence of clear vacuoles and that both the magenta and orange coloured granules had increased

in number; the orange granules had increased in size and in their intensity of stain reaction. Two types of cells with the above characteristics were seen in imprints and smears: those in which the cytoplasm stained intenselybasophilic and others with less basophilic cytoplasm; these two cell types will be discussed later.

Metamyelocytes from smears and imprints had fewer magenta granules; those which were seen were often ring shaped. The orange granules were more round in shape and more refractile and in the later stages some elongate granules in addition were found both in sections and imprints. The mature heterophil of sections and imprints was recognisable due to its elongated granules and lobed nucleus with coarse basophilic chromatin. Changes in shape of the nucleus were most pronounced at the metamyelocyte stage where it had become greatly indented; it was not definitely lobed until the mature stage.

In summary, during the development of the heterophil the following changes occur: general decrease in cell size, corresponding decrease in size of the nucleus, nuclear morphology changes from round to indented to lobed, chromatin changes from diffuse to coarse, change of definitive granule morphology from small round to large round and finally elongate, initial increase in numbers of magenta granules followed by decrease and finally extinction, change in morphology of magenta granules from small to large to ring shaped.

Only two stages in the developmental sequence of eosinophils were recognised; the immature form possessed a round to oval eccentric nucleus, dense chromatin staining red-mauve and the cytoplasm was basophilic and contained round refractile orange granules of uniform size. In the more mature eosinophil the slightly eccentric nucleus was round, containing coarse chromatin which stained a dense purple mauve colour, the cytoplasm contained only round refractile orange granules staining more densely than those of the heterophils. The most characteristic difference between these cells and those of the heterophil series was the coarseness and more densely staining nucleus. These cell descriptions are similar to those of Lucas and Jameroz (1961) except that the present author places less emphasis on the degree of basophilia of the eosinophil and believes that their granules stained more densely orange than those of the heterophil but not orange-red as suggested by Lucas and Jameroz.

Again only two stages in basophil development were seen. In the immature form the nucleus was round with diffuse chromatin which stained pale purple, dense purple granules were present in the cytoplasm which was only slightly basophilic. The mature basophil was a smaller cell with the same characteristics but the cytoplasm was packed with dense purple granuls. These descriptions agree with those of previous authors (Sandreüter, 1951).

Most of the early literature describes only mature forms of the three types of granulocyte and the descriptions of Forkner (1929) and Downey (1938) agree basically with the present work, although their staining techniques were different. However, the present author disagrees with Downey's interpretation of basophils which he states have the appearance of degenerating cells; while they are present in smaller numbers than the heterophils, as seen in the imprints studied they appear as normal healthy cells.

The first detailed description of the developmental stages of granulocytes in adult birds (chicken and starling) was given by Sandreuter (1951). In early stages of heterophils she wrote of two types of granules, orange and azurophilic. In the present work as well as that of Sandreuter, the stain used was May-Grünwald Giemsa. The present author prefers to call the denser granules magenta but they are believed to be the same as the azurophilic, although the choice of adjective may be a personal interpretation of colour. I agree with Sandreuter that these are the first dense granules to appear in the cell and that their numeral increase is followed by a decrease with their eventual disappearance from the cell as the heterophil matures; it appears to me that they are the same granule. Sandreuter described the earliest granules, progranules, as small azurophilic granules near the periphery of the cell and over the nucleus. These were found in the present work but also faint orange granules were seen near

the nucleus in the earliest granulocyte. I believe that the magenta granules and definitive orange granules may arise at the same time. According to Sandreuter the orange granules do not appear until her second developmental stage, promyelocyte 2 when the azurophilic granules appeared coarser and more densely staining at their periphery (corresponding to my magenta-ring type granule). The differences between the stage of appearance of orange granules in Sandreuter's and the present work may reside in a difference of terminology, i.e. her promyelocyte may correspond to my early mesomyelocyte; however, I found that magenta-rings were a feature of heterophils later in development, in late meso-or early metamyelocytes. The morphology of the nuclei of the different stages during development as described by Sandreuter, i.e. ovoid to kidney shaped to deeply indented and with the chromatin pattern changing from diffuse to condensed, was the same as I found except I did not find that nucleoli were as prominent in the early stages as she did. Sandreuter found in her promyelocyte 2 stage, (probably identical to my mesomyelocyte which corresponds to the mesomyelocyte of Lucas and Jameroz 1961), that magenta granules were present only over the nucleus. I found magenta granules over the nucleus and scattered throughout the cytoplasm.

In her summary Sandreuter stated that heterophils correspond in origin to neutrophils of mammals. This may be true for adult bone marrow but in other tissue e.g. embryonic

spleen and yolk sac this may not be true; for example, the avian yolk sac in early stages of evelopment is an extremely active haemopoietic organ while in mammals it is small and rudimentary.

Lucas and Jameroz (1961) in their "Atlas of Avian Haematology" describe very exact stages in the development of heterophils. However, examination of smears and imprints from spleen indicates very many more cell morphologies than they have described. These cell morphologies must be fitted into the developmental cell sequence. Development of a cell is a continuous process and as such the division of the sequence into stages is always arbitrary.

In the present work although the naming of the stages of heterophil development has been adopted from Lucas and Jameroz (1961), the author is not in full agreement with their criteria for all the divisions, nor do Lucas and Jameroz seem to differentiate sufficiently the early stages of heterophils and eosinophils, in my opinion. I agree with the description of heterophil granuloblasts and progranulocytes, but not with the mesomyelocyte stage. According to my definition at this stage magenta granules are still present as dense bodies or rings and the specific orange granules are round, agreeing with Sandreuter's myelocyte stage. Lucas and Jameroz suggest that the ring granules are more a feature of the promyelocyte. Also at this stage I found two types of mesomyelocyte in monolayers of cells stained with

May-Grünwald Giemsa: type 2 with very basophilic cytoplasm, and type 1 less so; both cells contained magenta and orange granu

Sandreuter (1951) and Lucas and Jameroz described the heterophilic mesomyelocyte as possessing less basophilic cytoplasm (corresponding to my type 1 mesomyelocyte) than the eosinophilic mesomyelocyte; a further difference was that the eosinophil contained no magenta granules. On the basis of cytoplasmic basophilia, i.e. criterion of Lucas and Jameroz, my type 2 mesomyelocytes would be considered eosinophilic mesomyelocytes and my type 1 mesomyelocyte, the true heterophilic mesomyelocyte. However, neither Sandreuter (1951) nor Lucas and Jameroz (1961) found magenta granules in eosinophils. Since I found magenta granules in both type 1 and 2 mesomyelocytes, and on the basis of absence or presence of magenta granules, then these cells must belong to the heterophil series. However, as there is little information on eosinophils in the present work, care has been taken about a definite conclusion on these two cells. The possibilities are that the developing eosinophil may possess magenta granules (extremely unlikely), or that one of these cells, the more basophilic type 2, has more ribosomal material and so stains more intensely basophilic. This suggests that perhaps it is more actively synthesising protein. I question the reliability of degrees of basophilia as a means of distinguishing two cell lines as used by Sandreuter, and Lucas and Jameroz. Both cells (heterophils and eosinophils) produce granules and are thus actively

synthesising cells and so it would be expected that both have many ribosomes, with subsequent cytoplasmic basophilia. Identification would reside more with the granules, which unfortunately are both acidophilc, and nuclear morphology. From my observations magenta granules are still present at the mesomyelocyte stage. Lucas and Jameroz regard them as a feature of their promyelocyte and that mesomyelocytes already contain some definitive rods, (not illustrated in their atlas). Rods were not seen in the present work until much later. Again some of these discrepancies may be related to different definitions of the stages of development. However, I believe that the cells which I saw were both heterophil mesomyelocytes. The differences in degrees of basophilia of the mesomyelocytes may be due to the possibility of more than one developmental type for the heterophil, this will be discussed under stem cells. I suggest that the two types of heterophil mesomyelocytes may be different phases of development; i.e. type 2 cell with greater cytoplasmic basophilia may represent a phase of greater synthetic activity than type 1 cell.

Metamyelocytes contain large orange granules, the refractility of which has increased throughout development; a few of the granules may become spindle shaped and ring forms of the magenta granules are occasionally present. The nucleus was much more indented with some chromatin condensation. Lucas and Jameroz (1961) make no mention of the remaining

magenta rings. Their definition of metamyelocytes resides in the presence of half the number of definitive rods and condensation of chromatin material, (again this is not well illustrated). While a few cells were seen in the present work with spindle as well as round granules, cells other than mature heterophils were not seen which contained only a few spindle granules. Sandreuter (1951) for this stage observed the condensation of the chromatin and increasing indentation of the nucleus while definitive granules became progressively more elongate in metamyelocytes. Neither Sandreuter nor Lucas and Jameroz commented on the fate of the magenta granule, although they were present to Sandreuter's myelocyte stage and to the promyelocyte of Lucas and Jameroz. I believe that the magenta granules exist through to at least the mesomyelocyte and a few may remain in ring form to the metamyelocyte, after which they disappear from the cell. Possible fates of these magenta granules include: transformation of the magenta granule to the definitive granule; destruction or extrusion from the cell. These three suggestions will be discussed further in the section on electron microscopy.

The morphology of the mature heterophil as presented here agrees with the findings of previous authors (Sabin 1921, Forkner 1929, Sandreuter 1951, Lucas and Jameroz 1961). It is a cell characterised by elongate spindle shaped granules and a lobed nucleus containing clumped chromatin.

Stem Cells in Spleen

In any study of blood cell development, the identification of the stem (blast) cell presents many problems. For instance, in figures 329 and 330, presented by Lucas and Jameroz (1961) of the 12½ day embryo spleen it is difficult to differentiate erythroblasts, metagranuloblasts (presumably heterophil) and granuloblasts; all appear to have the same degree of cytoplasmic basophilia, nuclear pattern and staining reactions although the granuloblast is smaller.

In the present work three stem (blast) cells of varying morphology and staining reaction were seen. The first, type 1, was a large basophilic cell with a round nucleus containing chromatin which stained red-purple and appeared similar to the erythroblast of Lucas and Jameroz. The second stem cell type 2, which is not reported by Lucas and Jameroz was less basophilic and the nuclear chromatin which existed as fine strands stained very pale blue. The third variety type 3, similar to the first but stained less intensely and had a finer chromatin pattern. Of these three types of stem cells, type 2 was most readily distinguished from types 1 and 3; types 1 and 3 were more similar to each other.

I believe it is very difficult to distinguish as did Lucas and Jameroz (1961) blast cells which eventually give rise to erythrocytes and granulocytes. By the criterion of Lucas and Jameroz type 1 stem cell of my work should be considered as a cell from which erythrocytes develop.

However, I found some cells indentical to the type 1 blast cells except for the presence of a few magenta and faint orange granules. These early promyelocytes, from morphological evidence, appear to have arisen from type 1 stem cell.

Originally, I considered that my stem cell type 2 might be a precursor of basophils because its cytoplasmic and nuclear staining properties were the same as those seen in early basophils. However, early promyelocytes, identical to the type 2 stem cells except for the presence of magenta and orange granules were observed. This suggests that heterophils could arise from this variety of stem cell.

Similarly, early heterophil promyelocytes were observed with nuclear and cytoplasmic characteristics identical to my type 3 stem cell, again with the addition of magenta and orange granules. Thus, I found early heterophil progranulocytes which, on morphological evidence, could have arisen from each of the three types of stem cells reported here.

The position of these stem cells remains uncertain. Since heterophils appear to arise from each stem cell, then there appears to be no one specific stem cell for heterophil granulocytes. Of course, again, the three types may represent different stages in the cell history of a single stem cell, each capable of producing magenta and orange granules. Furthermore, the similarity in morphology of the erythroblast of Lucas and Jameroz to my types 1 and 2 stem cells might be evidence for a common stem cell for erythrocytes and granulocytes.

The implication of the lymphocyte as a stem cell for red blood cells and granulocytes has been favoured by the presence of large numbers of lymphocytes in bone marrow and other haemopoietic organs of many animals (guinea pig, rabbit, man, bird). Only indirect evidence supports the contention that the lymphocyte is a stem cell and this is by no means universally accepted; those who believe it, generally do not claim that the lymphocyte is the only stem cell.

Elves (1967) raises the important question whether the lymphocytes of the marrow, spleen, lymph nodes, circulation, etc. are identical in structure and function. There is a reluctance of the polyphyletic school to accept the small lymphocyte as a multipotential stem cell and polyphyletists argue that the small cells in the bone marrow which resemble lymphocytes are not really lymphocytes. Morphologically there is little difference in lymphocytes from the various haemopoietic organs; some may be smaller, the life span may vary. Recently Bimes (1962) has found a non-specific esterase in small lymphocytes of bone marrow which is not found in those of the blood. Bloom (1937) reported the differentiation of small lymphocytes to eosinophilic myelocytes and granulocytes in cultures of lymph duct cells of Ascaris-immunised rabbits. Downey (1948) from studies of rabbit thymus concluded that granulocytes developed from the small lymphocyte. McCulloch and Till (1965) have also demonstrated potential

differences in morphologically similar lymphocytes. In mouse bone marrow, they reported colony forming units of cells morphologically indistinguishable from the small lymphocyte which could produce clones of myeloid, erythroid or megakaryocytic cells in the spleens of lethally irradiated mice. These cells were found in the peripheral blood but not in the thymus nor thoracic duct lymph. They suggest that the colony forming cells are limited stem cells in the sense that they are not capable of contributing to the immunological lymphocyte series but differentiate to granulocytes, erythrocytes, and megakaryocytes. Yoffey (1960) from mammalian bone marrow studies implicates the small lymphocyte as the multipotential stem cell. The small lymphocyte he believes is a comparatively inactive form which sooner or later becomes capable of reactivation to a new cycle of growth and development.

In the present work, I could not distinguish with certainty lymphocytes from cells I believe to be blast cells, in smears and imprints of spleen and yolk sac. Lucas and Jameroz (1961) do not give descriptions of lymphocytes which could be identified separately from the stem cells. In tissue sections, I saw large stem cells with basophilic cytoplasm, ovoid nucleus and prominent nucleolus which were very similar to the totipotential cell in the chick thymus described and illustrated by Moore and Owen (1967).

Development of granules

The main finding arising out of the present electron microscopic studies was the presence of three types of granule, the large round dense type 1, the broken or flocculant type 2 and fibrillar type 3. As to the origin of type 1 and 2 granules, one can speculate that they are derived from the Golgi complex; many small vesicles containing dense material were seen in this region although they did not seem to be condensing to form granules or precursors of granules. The granules in the cells (heterophil mesomyelocytes and early metamyelocytes) were large and presumably of a "maturer nature." Both granules, type 1 and 2, appeared to have a similar internal composition, but whether they are related cannot be stated at present. However, due to their size and the nature of the material found within both granules, I believe that they are different stages in the development of the same granule. A sequence of events may be postulated from the compact type 1 granule to the broken type 2; the reverse sequence has not been ignored, but due to the appearance of broken membranes, a dense rim of material at the periphery in granule 2 and the presence of small irregular spaces in type 1, I feel that the process is one of breakdown from granule 1 to granule 2.

Comparing granules as seen with the electron microscope with those from the light microscope, I suggest that type 2 granule could give the ring-like magenta granule seen in

heterophil pro-, meso-, and metamyelocytes when stained with May-Grünwald Giemsa stain and observed with light microscopy. Heterophil pro-, meso-, and metamyelocytes, seen with the light microscope also have large round orange granules. On morphological characteristics and numbers present, type 1 granules seen in the electron micrographs may correspond to these orange granules. Also elongate type 1 granules have been seen in electron micrographs of early metamyelocytes (or late mesomyelocytes), again suggesting that they are indeed the definitive orange granules as seen in light microscopy. This raises the question of the position of type 2 granule, for apart for its less compact and broken nature it appears similar to type 1 in electron microscopic observations. However if this granule represents the ring magenta granule of light microscopy then it possess a chemical composition different from type 1. But the possibility does exist that type 1 and type 2 granules are different stages in the development of a single variety of granule with type 1 giving rise to type 2. I suggest that only some of the type 1 granules are destined to become definitive granules and that others are broken down in some way within the cell. Histochemically I was not able to differentiate the magenta and orange granules; the difference in staining reaction seen in light microscopy may be due to changes in the chemical nature of those which will or are in the process of breaking down. Granules were not seen to be extruded from the cell.

However, the question of these two granule types remains uncertain, for from light microscopic studies small dense magenta coloured granules of early heterophils exist at the same time as developing orange granules.

Edmonds (1966) although studying mainly erythropoiesis in the early chick yolk sac, did find a maturing granulocyte (illustrated in his figure 11) with granules and clear vacuoles. His granules appeared to be similar to the two types described in the present work; Edmonds makes no comment on the origin or the significance of these granules. Campbell (1967) in his electron microscopic study of chicken and pigeon bone marrow illustrates what he refers to as "globular" and "fibrillar" granules in heterophil leucocytes and states that they are both present at the metamyelocyte stage. His "globular" granule has the same morphology as Edmond's dense granule and granules 1 and 2 of the present work. Campbell states that this granule arises from the Golgi complex as membranous vesicles containing globular material, which condense to form a granule. The "fibrillar" granule described by Campbell has been seen in the present work, and is termed type 3, in very small numbers. Campbell suggests that they are formed near the periphery of the cell (although this could be debated after examination of his micrographs, plates 8, 9, 10) and do not appear to have a Golgi origin. This granule, he says, disappears or becomes indistinguishable from the "globular" types by the metamyelocyte stage. He makes no mention of

magenta or azurophilic granules in his light microscopic observations. Very few fibrillar (type 3) granules have been seen in the present work and little comment can be made although from their size and number it seems unlikely that they are definitive granules. Campbell makes no comment on the fate of those globular granules which appear in his micrographs to be disintegrating and although he describes two types of granules in the heterophil myelocyte it does not explain the differences seen with the light microscope between magenta and orange granules, and the disappearance of the magenta ones. Although he states that fibrillar granules do not appear after the metamyelocyte stage and suggests these granule to be the same as Sandreuter's azurophilic granules, their numbers and shape shown in Campbell's micrographs as well as those seen in the present work, do not justify their identification as magenta or azurophilic granules. Campbell differentiates heterophils and eosinophils by the fact that eosinophils have only one population of granules which arise from the Golgi complex. However, he shows only one micrograph of an eosinophil in which the granules although less dense could be classified as heterophil "globular" type. No cells were seen in the present electron microscopic study which could be identified as eosinophils or basophils.

Structure of Yolk Sac Vessels

The present studies concentrate on that period of development of the yolk sac around 12 days and were primarily

involved in following the formation of granulocytes; nevertheless some interesting observations have been made concerning blood vessels themselves. Romanoff (1960) states that folds in the yolk sac first appear on day 4 after which there is an increase in their thickness which decreases towards the end of incubation. In the present study at 12 days, these folds are very prominent and vessels are suspended in the fold by cords of basophilic cells which are similar to the angioblasts as described by other authors (Sabin 1920, Houser et al 1961).

To my knowledge there is no information in the literature on the presence and nature of the "clumps" of cells surrounding the thick walled vessels. The present observations suggest that these areas may be compared to the development of blood vessels in the early blastoderm, i.e. that these clumps are forming "auxillary vessels" presumably for increased area for yolk absorption. The outer basophilic cells found on the periphery of the clump are angioblasts and there is a progressive differentiation to erythrocytes towards the centre. The mass of cells is not a syncytium as was believed by Danstchakoff (1908) and Sabin (1920) and later disproved by Houser et al (1961) and Edmonds (1966). Cells at the centre have a reddish tint presumably due to the presence of haemoglobin; however, no endothelium has been identified and the situation appears similar to that described by Houser et al. No connections have been seen from

these auxillary vessels to the main vessels although these might be found by taking serial sections, as gross examination under the dissecting microscope did show circulating red cells in small loop vessels coming off main yolk sac vessels.

Where these basophilic angioblastic cells come from has posed a problem not solved by the present studies. They are present in large numbers, yet very few cells in mitosis are to be found. Studies of earlier yolk sacs (5 days) and of areas of 12 day yolk sacs still proliferating gave no further clues; it was hoped to find vessels in such areas with this type of cell actively proliferating outside the vessel, or to see mesenchymal cells rounding up to produce the cells as did Sugiyama (1926). The problem was further complicated by finding vessels with well defined endothelial walls but packed with basophilic angioblasts and developing red blood cells, often such a vessel could be found close by larger thin walled vessels and apparently feeding basophilic angioblasts cells into its lumen. These cells (angioblasts) then became aligned along the intravascular endothelial wall. Where the extravascular cells come from remains a mystery; possibly they are budded from the endothelium but this was not seen in the sections studied.

Other larger more basophilic cells with a large nucleus and prominent nucleolus were seen at the periphery of the clump. These may represent stem cells, possibly of the granulocytic series. They do have the appearance of the

totipotential stem cell described by Moore and Owen (1967) in the developing chick thymus.

With electron microscopy blood vessels were seen in which the endothelial cells overlapped by means of long processes so that the vessel wall was not composed of a single layer of cells, but rather formed an interdigitating lamella. This was shown by Edmonds (1966) although his micrographs are indistinct. Coated pinocytotic vesicles were a common feature of the endothelial cells being more abundant on the lumen side of the vessels; this suggests some sort of transport across from the vessels to the surrounding tissue.

Suggestions for further study

In studies on avian granulocytes both in the adult and the embryo there is a continuing problem of the identification of heterophils and eosinophils. Lucas and Jameroz (1961) state that they are able to distinguish these two cell types by the degree of basophilia of the cytoplasm morphology and staining properties of the granule and the coarseness of the chromatin material in the nucleus. Sandreuter (1951) gives similar differences, and from the present work some cells have been seen which possess these characters, although they are few in number and the author is not convinced of the reliability of these criteria of identification when faced with the multitude of cell types seen in an imprint of spleen, for example. For this reason a number of histochemical techniques were employed but were

unsuccessful for the discrimination of the two lines. The results showed that with the peroxidase type reaction no positive results were obtained, there was P.A.S. positive material in the granules of some leucocytes which was not digested with diastase, but the cells themselves could not be identified. This was similarly true for acid phosphatase reactions. From studies of mammalian granulocytes it has been shown that there are peroxidase reactions which distinguish heterophils and eosinophils and a more intensive study of these methods on the avian granulocytes may provide a method for separating these two cell lines. The problem of identification of mature cells is difficult but is further increased in developmental stages where many more cell types with more types of granules are seen. Detailed histochemical methods may give an answer allowing the cells to be placed in the correct cell line, and provide information on their chemical composition.

Basophils are less difficult to identify in the mature form, but again there is some difficulty in the very early stages. Histochemical studies might give much information on the early stages.

In heterophils two populations of granules in the same cell raise many problems of origin, fate and relationship; there has been very little electron microscopical work on the avian granulocytes and that which has been performed has not given clear answers to these problems. Many more

electron microscopic studies have been carried out on the leucocytes of mammals, and it would be of great interest to follow up some of these studies using the bird rather than mammals. For example in the rabbit Bainton and Farquhar (1966) have investigated two populations of granules in the polymorphonuclear leucocytes and have suggested a polarity in the Golgi complex, with one type of granule arising from one face of the Golgi and the other from the other face. Fedorko and Hirsh (1966) have not found this. However, the possibility of a Golgi polarity producing the two populations of granules in the avian heterophil is fascinating. Sandreuter's (1951) observation that the azurophilic granules of bird heterophils are located on the periphery of the cell (an observation which I could not confirm) and Campbell's (1967) hypothesis that his "fibrillar" granules, corresponding to Sandreuter's azurophilic granules, do not originate in the Golgi, raises the question whether some granules might not have a site of origin other than the Golgi apparatus. If so the conventional conceptofgranule formation, i.e. manufacture in the rough surfaced endoplasmic reticulum, storage and condensation in the Golgi and final release from that organelle, would need to be modified in some cases.

Use of radioactive labelling techniques in combination with electron microscopy applied to avian heterophils could provide concrete information regarding the function of the Golgi complex and the sequence of events in granule formation.

Ackerman (1968) using such techniques suggests that, in cats, both types of granules arise from Golgi. If this were to be the case in the chick then why, if the granules arise at the same time and from the same place, do they give different staining reactions with conventional blood stains.

Eosinophils are very few in number in the adult, as well as embryonic bird (Lemez 1964 and present study). If it were possible to induce an increase in their numbers (eosinophilia) by perhaps the introduction of foreign material, bacteria or parasitic infection, means of identification and resolution of the developmental sequence of the eosinophils might be determined.

Radioactive tracers might be of some use in the determination of the stem cells. However, how does one label selectively enough to be able to follow different types of stem cells in the haemopoietic organs. In addition there is the problem of cell imigration as shown by Moore and Owen (1967) where they found a migration of cells from the yolk sac to the thymus using the technique of chromosome markers in the chick embryo. This migration may also be true of the spleen, and there may be no reason to suppose that the migration is not in the other direction as well.

CONCLUSIONS

1. Imprints and tissue sections, stained with the May-Grünwald Giemsa method, of spleen and yolk sac of chick embryos aged 4 days to 2 days post hatching were examined with light microscopy. Large numbers of granulocytes in different stages of development in the spleeen were present at 12 days; this was the period of most intensive study.

2. A developmental sequence for the heterophils was determined in the spleen imprints stained with May-Grünwald Giemsa. From the promyelocyte through the mesomyelocyte and metamyelocyte to the mature heterophil the following progressive changes take place: decrease in cell size, decrease in cytoplasmic basophilia, decrease in numbers of first magenta granules concurrent with increase in the number of the definitive orange granules which are first round then become elongated, decrease in the size of the nucleus, change in nuclear position and shape from central round to eccentric lobed, and progressive clumping of the chromatin.

Granulocytes were found throughout the spleen in tissue sections, mainly in the cords of spenic cells, some could be seen in the sinuses. Before 10 days few could be found, between 10 and 12 days there was an enormous increase. Stages in the development of heterophils seen in tissue sections were less easy to determine, but agreed with those

seen in imprints except no magenta granules were observed.

3. Early and late stages in the development of eosinophils were described. The more mature eosinophils compared to heterophils possessed only round orange granules which packed the cytoplasm; the cytoplasm where seen appeared to be more basophilic. The nucleus with May-Grünwald Giemsa method stained purple red and had a coarser chromatin pattern than the heterophil. The earlier stage possessed this same coarse purple nucleus and contained only orange granules. No eosinophils were found in sections of spleen.

4. In spleen imprints, mature basophils were recognized by the presence of round purple granules which filled the cytoplasm and a pale blue nucleus. Immature basophils had a pale blue nucleus and only purple granules at the periphery of the slightly basophilic cytoplasm. No basophils were seen in sections of spleen.

5. Granulocytes were first seen in sections of yolk sac at 4 days. In all yolk sacs studied from 4 days to hatching granulocytes were always found in an extravascular location, in the early yolk sacs either singly or in twos and later often as cords of cells outside the vessels. The greatest numbers were found around 12 days.

6. Heterophils, observed in smears and sections of yolk sac, comprised most of the granulocytes of this organ. Eosinophils were not seen in smears or sections and basophils were found only rarely in smears. Comparison of the

granulocytes found in the yolk sac showed them to be the same as those of the spleen.

7. Three types of stem (blast) cells were identified in the spleen and two types in the yolk sac. All are considered as possessing the potential to give rise to heterophil granulocytes since progranulocytes were seen identical to each of the stem cells except for the addition of magenta and orange granules.

8. Electron microscopic studies revealed the presence of three types of granules in granulocytes from 14 day embryo spleens. These were: 1) round, dense membrane bound 2) round, broken or floccular, membrane bound 3) elongate fibrillar around which a membrane was not always visible. The cells in which they were seen were identified as heterophils. It is suggested that there is a progresion in the development of the granules from type 1 to type 2, and that type 2 granule may correspond to the magenta ring-type granule seen in light microscopy. Elongate type 1 granules were observed and it is thought that this granule, i.e. round type 1, is the precursor of the spindle shaped granule of the mature heterophil.

9. Two types of blood vessels were seen in the yolk sac, thin and thick walled. Those with thick walls possess a well developed endothelium and the thickness of the vessel is due to the presence of clumps of cells lying outside. These clumps are considered to represent developing auxillary

vessels. At the periphery of the clumps round basophilic stem cells (erythroblasts) possibly lost their basophilia and developed into centrally located red blood corpuscles. No connection between these auxillary vessels and the main vessel were seen. It is thought that these clumps represent blood vessel formation similar to that seen in the very early yolk sac. The origin of these clumps is not known. Granulocytes were found in cords between the auxillary vessels.

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"Selected Histochemical and Histopathalogical Methods" Pub. C. C. Thomas, Springfield, 111.

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"The Lymphocyte". Int. Rev. Cytol. 7: 236-293.

Undritz, E. 1952.
Peroxidase reaction. Sandroz "Atlas of Haematology" Sandroz Ltd. Basle, Switzerland, p. 28.

APPENDIX I

Fixatives

Zenker's Fluid

Stock Solution	Potassium dichromate	2.5 g
	Mercuric chloride	5.0 g
	Distilled water	100.0 ml

Immediately before use, 5 ml of glacial acetic acid were added to 95 ml stock Zenker.

Bouin's Fluid

Picric Acid (saturated aqueous solution)	75 ml
Formaldehyde solution (40%)	25 ml
Glacial Acetic Acid	5 ml

Calcium Formol

Formaldehyde	10 ml
Calcium Chloride anhydrous	1.0 g
Distilled water to make	100 ml

10% Formalin

Formaldehyde	100 ml
Distilled water	900 ml

Chick Ringer Solution, New (1966)

Anhydrous gm/litre of solution	
Sodium chloride	9.0 g
Potassium chloride	0.42 g
Calcium chloride	0.24 g
Distilled water	1000 ml

Dissolve salts in water

May-Grünwald Giemsa Stain for Imprints

Reference: Jacobson, W. and Webb, M 1952. Exp. Cell Res. 3: 163-183.

Fixation:

Wet imprints were fixed rapidly over Iodine vapour until they appeared deep brown (approximately 5 minutes). The slides were dried thoroughly, washed in methanol 5 minutes then stained.

Alternatively, wet imprints were air dried and then stained directly or air dried and fixed in methanol. This latter step was not found essential.

Solutions:

May-Grünwald (Jenner's) Stain

0.3 g of the stain was dissolved in 100 ml methanol at 50°C; this was allowed to stand 24 hours and was filtered each time before use.

Stock Giemsa

50 ml of glycerol were heated in an oven to 55°C, 0.3 g Giemsa stain added and allowed to stand overnight to dissolve. 50 ml methanol were added the next day.

Stock Buffer (Sorenson's Phosphate Buffer pH 6.5)

68 ml 0.1M sodium dihydrogen phosphate (15.6 g in 1000 ml distilled water)

32 ml 0.1M sodium hydrogen phosphate (14.2 g in 1000 ml distilled water)

Dilute Buffer

Stock Buffer	70 ml
Distilled water	140 ml

Dilute Giemsa (working solution)

Stock Giemsa	30 ml
Dilute Buffer	210 ml

Buffer Rinse

Stock Buffer 22.5 ml made up to 675 ml with distilled water.

Staining Procedure:

1. Methanol 5 minutes
   (This step may be omited with air dried imprints)
2. May-Grünwald solution 12 minutes
3. Dilute Giemsa solution 20 minutes
4. Buffered Rinse 3 changes of 2 minutes each
5. Blot with filter paper, dry thoroughly and mount in synthetic resin.

Results:

DNA of nucleus	reddish purple
RNA	light blue
Erythrocytes	orange cytoplasm
Heterophils	orange granules
Eosinophils	orange red granules
Basophils	deep purple granules

May-Grünwald Giemsa for Tissue Sections

Reference: Strumia, M. M. 1935-6. Lab. and Clin. Med. 21: 930-934. from Man. Hist. and Special Staining Technique. Armed Forces Inst. Path. p. 111 (Modified by I.G.M.)

Fixation: Zenker's Fluid 4-6 hours

Technique: Paraffin sections cut at 5μ.

Solutions:

Stock May-Grünwald (Jenner)

0.3 g of the stain was dissolved in 100 ml methanol at 50° C, this was allowed to stand 24 hours and was filtered each time before use.

Stock Giemsa

50 ml of glycerol were heated in an oven to 50° C, 0.3 g Giemsa stain added and allowed to stand overnight. 50 ml methanol were added the next day.

Stock Buffer (Sorensen's Phosphate Buffer pH 6.5)

68 ml 0.1M sodium dihydrogen phosphate (15.6 g in 1000 ml distilled water)

32 ml 0.1M sodium hydrogen phosphate (14.2 g in 1000 ml distilled water)

Dilute Buffer

Stock Buffer	70 ml
Distilled water	140 ml

Buffer Rinse

Stock Buffer 22.5 ml made up to 675 ml with distilled water

Working Jenner's Stain

Used as stock solution

Working Giemsa Stain

50 drops of stain in 50 1 dilute buffer

Acetic Acid Rinse for Differentiation

1 drop glacial acetic acid in 40 c.c. distilled water

Staining Procedure:

1. Deparaffinise sections through two changes of xylene, absolute alcohol, 95% alcohol to distilled water
2. Remove mercury precipitate with 1% alcoholic iodine solution if Zenker fixed 5 minutes
3. Wash in running tap water 5 minutes
4. Sodium Thiosulphate, 5% aqueous solution 5 minutes
5. Running tap water 15 minutes
6. Distilled water rinse
7. Methyl alcohol 5 minutes
8. Jenner's stain 10 minutes
9. Working Giemsa 1 hour

   (Check; the nuclei should be distinct blue against pink/rose cytoplasm)

10. Differentiate in acetic acid water 10 seconds
11. Buffer rinse 3 changes, 2 minutes each
12. 70% alcohol
13. 95% alcohol Very rapidly-less than 10 seconds
14. 100% alcohol

15. Xylene 2 changes

16. Mount in resin

Results:

Nuclei	blue
Granular leucocytes	orange-red granule

May-Grünwald Giemsa Stains

Probable Mode of Action:

Reference: Thompson, S. W. 1966. "Selected Histochemical and Histopathological Methods". Publisher: C. C. Thomas

Staining mixtures of Romanowsky-type stains i.e. Giemsa and Wright, consist of measured amounts of methylene blue and its lower metachromate homologs azure A or B, preferably azure B. A neutral dye is prepared which contains chloride ions due to excess of basic dye by precipitation with a measured quantity of eosin. The precipitate is soluble in alcohol; usually methyl alcohol is used as a solvent. Glycerin is used to increase the capacity of the alcohol to dissolve the dye.

In addition to the measured quantities of methylene blue and azure B and/or A which are present in such dye solutions at the time of preparation, additional quantities of lower homologs (e.g. Azur A,B, methyl violet) of methylene blue are formed by oxidation as the solution ages, i.e., the metachromate material in the stain increases with age. In blood smears and tissue sections, the basophilic (blue) staining of nucleoli and cytoplasm is due to the methylene blue component; this affinity for methylene blue can be abolished by hydrolysis of the tissue with exonuclease before staining. The anionic component of the nucleolus and cytoplasm which stains with the cationic methylene blue is presumed to be ribonucleic acid. Nuclei stain metachromatically red to

purple by the azure components of the stain; the reaction can be prevented by prior treatment with deoxyribonuclease and so it is presumed that DNA is one substance which has an affinity for azur A and/or B. The eosinophilic granules and erythrocytes are stained by the eosin component of the stain.

The precise colour depends on several factors, concentration of the chromotrope, interspace distance between reactive ionic groups on the surface of the chromotrope, concentration of the metachromatic dye in the staining solution, the isoelectric point of the substrate and the pH of the staining solution, e.g. one or more metachromatic dyes may be present. If the dye is present in too great a concentration it may not stain metachromatically since metachromasia is roughly inversely proportional to the concentration of the dye. So it may be possible that one of the homologs of methylene blue may be present in too great a concentration to stain metachromatically whereas the other or others may be in a sufficiently low concentration to produce metachromasia. With the variations on this and the other factor, different staining reactions can be produced.

If the tissue section is differentiated for a sufficient period in alcohol after staining with Giemsa, the metachromasia will be reversed and the nuclei will stain orthochromatically (blue).

APPENDIX II

Undritz Peroxidase Reaction, modified for smears and imprints

Reference: Undritz, E. 1952. Sandoz Atlas of Haematology. Sandoz Ltd., Basle, Switzerland. p. 28.

Fixation: Air dried

Solutions:

May-Grünwald Stain

Giemsa Stain: 3-4 mls Stock Giemsa in 100 ml carbon dioxide free distilled water

Peroxidase: Enough Benzidine to cover a knife point is dissolved in 6 ml 96% alcohol; the solution is diluted with 4 ml distilled water and 0.02 ml hydrogen peroxide (30) added. In a closed vessel, the reagent will keep for 5 days.

Giemsa Peroxidase: Add 0.1 ml peroxidase reagent to every 10 ml of water before mixing with Giemsa.

Staining Procedure:

1. May-Grünwald 5 minutes
2. Giemsa Peroxidase 15-30 minutes
3. Wash in tap water
4. Dry
5. Mount in synthetic resin solution

Results:

DNA, RNA colour similar as with ordinary MGG except colours are paler.

Eosinophils give strong positive reaction-greenish yellow. All other cells react negatively except a few basophils which give only a weak reaction. This method is suitable for eosinophils and their precursors particularly in the bone marrow. It is the best method for differentiating rabbit blood.

Copper Peroxidase Method for Leucocyte Peroxidase in Blood Smears

Reference: Sato, A. and S. H. Yoshimatsu, 1925. Am. J. Dis. Child 29: 301-312: from Thompson, S. W. "Selected Histochemical and Histopathological Methods". p. 715.

Fixation: Air dried

Solutions:

I. Copper Sulphate 0.5%

Copper sulphate	500 mg
Distilled water	100 ml

II. Benzidine Saturated

Benzidine Base	200 mg

Dissolve as much of the base as possible, filter. Add 4 drops hydrogen peroxide (3%) to filtered solution. Keep in the dark. If I and II are mixed, should get a blue colour.

III. Aqueous Safranin 1%

Safranin	1 g
Distilled water	100 ml

Staining Procedure:

1. Flood slide with solution I. After 30-60 seconds, decant as much of solution as possible.
2. Flood the slide with solution II and pour off after 2 minutes
3. Safranin (solution III) 2 minutes (in jar)
   (Carbol fuchsin (1 part in 5) may be used as a counter stain)
4. Wash in running tap water.
5. Air dry.

Results:

Peroxidase granules (myeloid leucocytes)	bluish green
Eosinophil and basophil granules	intense blue
Lymphocytes	red
Red blood cells	unstained

Peroxidase II Reaction to determine Eosinophils in Tissue Sections

Reference: Gross, R. 1962. "The Eosinophil". In: H. Braunsteinder and D. Zucker-Franklin (ed.). The Physiology and Pathology of Leucocytes. Grune and Stratton, New York and London.

Fixation: Tissues are kept in formal alcohol for 24 hours. Then fully dried and embedded in paraffin.

Solutions:

a. 10% formol alcohol

formalin 40% 10 parts

alcohol 96% 90 parts

b. May-Grünwald solution buffered at pH 7.2

May-Grünwald stock 1 part

Sorensen's phosphate buffer 8 parts

c. Giemsa-peroxidase solution buffered at pH 7.2

Sorensen's phosphate buffer diluted 15 parts

Peroxidase reagent 0.15 parts

Giemsa stock solution 0.2 parts

d. Peroxidase reagent after Undritz

A pinch of benzidine in 6 ml of 96% alcohol to which is added 4.0 ml distilled water and 0.02 ml 3% hydrogen peroxide.

e. Sorensen's buffer diluted

distilled water 9 parts

Sorensen's buffer 1 part

test pH after dilution

Solutions b and d should be prepared prior to use, particularly if H_2O_2 is concerned.

Staining Procedure:

1. Xylol
2. Graded alcohols
3. Sorensen's buffer diluted
4. May-Grünwald solution b - 20 minutes warmed to 35° C
5. Wash--tap water
6. Counter stain·Giemsa peroxidase 4 minutes
7. Rinse in buffered saline
8. Dry on filter paper
9. Briefly to 96% alcohol
10. Absolute alcohol
11. Acetone
12. Xylol
13. Mount

Periodic Acid Schiff Reaction for Glycogen and Glycoprotein

Reference; Ackerman, G. A. 1960, Cytochemistry of the Lymphocyte. In: "The Lymphocyte and Lymphocytic Tissue". p. 28-55. Ed. J. W. Rebuck, Paul B. Hocker, New York 1960.

Fixation: Fix air dried film in formalin vapour - 5 minutes

Method:

1. Fix as above
2. Rinse briefly in distilled water
3. Place films in 0.5% aqueous periodic acid - 10 minutes
4. Wash in distilled water
5. Immerse in Schiff Reagent for 20 minutes (use pipette rinsed with Sulphurous acid to transfer Schiff's reagent from stock bottle to staining dish)
6. Wash in 3 changes of sulphurous acid rinse - 2 minutes

   Prepare: 10% Aqueous (Na) metabisulphite 6 ml
   1 N HCI 6 ml
   Distilled water 110 ml
7. Wash in running tap water 5 - 10 minutes
8. Counterstain if desired with 0.5 - 1.0% Aqueous methyl green - 2 minutes
9. Wash in running tap water
10. Blot dry and mount

Results:

Intense red purple indicates glycogen

Deep pink indicates glycoprotein or mucopolysacchrides

Note: Formalin vapour fixation binds glycogen tightly to protein in the cell so that saliva or amylase digestion fails to extract the glycogen. An alcoholic fixative (eg. Abs. methanol or Abs. ethanol or Carnoy's fixative for 5 minutes) will permit the extraction of glycogen by saliva or amylase. However, some water soluble glycoprotein or mico-protein are extracted with alcoholic fixation.

Glycogen: When salivary digestion is employed the fixed films are exposed to several changes of saliva for time of 20 to 30 minutes, then proceed as for step 2. Glycogen is denatured by comparison of control preparation with those exposed to saliva. Reduction in PAS positive material in digested film indicates glycogen.

Additional References:

Mayhoe, F. G. J., D. Guagline, and R. J. Fleurans. Consecutive use of Periodic acid Schiffs Techniques in the study of Blood and Bone Mammalian cells. Brit J. Haemat 6:23. 1960.

Wachstein, M., Distribution of Histochemically Demonstrable Glycogen in Human Blood and Bone Mammal Cells. Blood 4:54 1949.

Periodic Acid - Schiff Reaction

Reference: MacManus J.F.A., (1948) Stain Technology 23:99 Armed Forces Inst. Path. modification. p. 132.

Fixation: Neutral Buffered 10% Formalin, Zenker's formic solution, Bouin's Fluid, Absolute Alcohol.

Technique: Cut paraffin sections 5 - 8 μ

Solutions:

1N HCI

HCI Acid conc. (Sp. grav. 1.19)	83.5 ml
Distilled water	916.5 ml

Schiffs Reagent

Basic Fuchsin	1 g
IN HCl	20 ml
Sodium metabisulphite	1 g
Activated Charcoal	2 g
Distilled water	200 ml

Dissolve basic fuchsin in hot distilled water. Bring to boiling point. Cool to 50° C. Filter and add 20 ml HCI. Cool and add 1 g sodium metabisulphite (or anhydrous sodium bisulphite). Allow to stand overnight in dark. Add charcoal and filter. The filtrate should be colourless. Store in dark at 0 to 4° C; should keep for several months; if becomes pinkish discard.

Test for Schiff's Leuco Fuchsin Solution:

Few drops into 10 ml 37 - 40% Formaldehyde. If

solution turns reddish purple rapidly it is good. If reaction is delayed and resultant colour is deep blue purple solution is breaking down.

0.5% Periodic Acid Solution

Periodic Acid Crystals	0.5 g
Distilled water	100 ml

0.2% Light Green Counterstain

Light green crystals	0.2 g
Distilled water	100.0 ml
Glacial Acetic Acid	0.2 ml

or Harris's Haematoxoxylin

Diastase Solution

Diastase	0.5 g
Distilled water sterile	100 ml

Store in refrigerator - good for 1 week

Staining Procedure:

1. Xylene
2. Absolute alcohol
3. 95% Alcohol
4. If Zenker-fixed, remove mercury precipitate in iodine, wash in water and decolourize in hypo solution.
5. Rinse in distilled water
6. Periodic acid solution 5 minutes
7. Rinse in distilled water
8. Schiff's Leuco Fuchsin 15 minutes
9. Running tap water - 10 minutes for pink colour to develop
10. Stain Harris's Haematoxylin 4 minutes

11. Rinse tap water
12. Differentiate in Acid Alcohol 3-10 quick dips
13. Wash in tap water
14. Dip in ammonia water to blue
15. Wash in running tap water 10 minutes
16. 95% Alcohol
17. Absolute alcohol 2 changes
18. Xylene 2 changes
19. Mount

*If PAS reaction with digestion required, cover sections with saliva for 20 minutes (3 changes). Rinse mouth before. Rinse section in running tap water for 10 minutes and wash in distilled water.

Results:

Glycogen, mucin, colloid and most basement membranes pink to red.

Nuclei - blue

PAS Reaction (Alcoholic)

Reference: MacManus, J. F. A. and R. W. Murray, 1960. Staining Methods: Histochemical and Histological, New York, Paul B. Hocker and Co.

Solutions:

Periodic acid

Periodic acid	1 g
90% Ethyl Alcohol	100 ml

Keep in dark, solution is unsatisfactory if it turns brown.

Schiff's Reagent

Basic Fuchsin CI 42500	0.5 - 1.00 g
Distilled water	85 ml
Sodium Metabisulphite	1.9 g
N HCI	15 ml

Sodium Bisulphite

Sodium metabisulphite	0.5 g
Distilled water	100 ml

Procedure:

1. Deparaffinize - 70% Alcohol (Remove $HgCl_2$ in Iodine in in 70% alcohol)
2. Alcoholic Periodic Acid 2 hours
3. Wash 90% Alcohol
4. Hydrate quickly to water
5. Treat with Schiff's Reagent 10 minutes

6. Transfer to sulphite solutions 3 changes 1.5 - 2 minutes
7. Wash in running water 5 minutes
8. Counterstain
9. Dehydrate, clear, mount

For imprint follow the steps for both aqueous and alcoholic without the preliminary deparaffinizing steps.

Lead Nitrate for Acid Phosphatase

Reference: Gomori, G. 1950, Stain Technology 25: 81-85

Staining Procedure:

1. Sections

   Cold acetone, paraffin; cold formalin frozen sections

   Cut 10-15μ

   If formalin fixed should be dried to ensure adhesion.

2. Incubate at 37 for ½-16 hours in 0.01 M sodium β - glycerophosphate in 0.05 M Acetate buffer pH 5.0 containing 0.004 M Lead Nitrate
3. Wash briefly
4. Immerse in dilute Yellow Ammonium Sulphide 1-2 minutes
5. Wash
6. Counterstain with 1% aqueous Eosin 5 minutes
7. Wash
8. Mount in glycerine jelly

Control - Incubate without Na β glycerophosphate

APPENDIX III

Technique for the Preparation of tissue Blocks for Electron Microscopy

Modified Palade Fixative

Reference: Palade C. E. (1952)

Buffer

Sodium Veronal	2.89 g
Sodium Acetate	1.15 g
Distilled water	100 ml

This buffer was stable and could be kept at 4 C for several months.

Osmium Tetroxide

A 2% solution was prepared by breaking a washed ½ g capsule of osmium tetroxide (Fisher Co.) in a clean glass stoppered bottle. 25 ml of distilled water were added and the crystals allowed to dissolve. The solution was stored at 4° C until required.

Fixative:

2% Osmium tetroxide	6.25 ml
Veronal Acetate Buffer	2.50 ml
Distilled water	1.25 ml
0.1 N HCI	2.50 ml

Hydrochloric acid was added gradually until the pH of the fixative lay between 7.3 and 7.5 The fixation solution was prepared immediately prior to use from reagents stored at 4 C and throughout its preparation care was taken to ensure that all glassware was clean and free from contamination which tended to reduce the osmium producing brown colouration

of the solution and form a precipitate.

Epoxy Resin Mixtures for the preparation of Epon

Solution A

Epon 812 Resin (Fisher)	62 ml
Dodecenyl Succinic Anhydride (Fisher)	100 ml

Solution B

Epon 812 Resin (Fisher)	100 ml
Methyl Nadic Anhydride (MNA)	89 ml

Both solutions were thoroughly mixed by mechanical and manual stirring to ensure complete mixing; these solutions were stored at 4 C in glass jars with tightly fitting lids. The embedding medium was prepared prior to use from solutions A, B and the accelerator Dimethylamino-methyl-phenol.

Solution A	7 ml
Solution B	3 ml
Dimethylamino-methyl-phenol	0.15 ml

This embedding medium was mixed thoroughly. All solutions were measured out using dry and clean glassware; the glassware when not in use was stored under absolute alcohol. The capsules used for the final embedding were either gelatin (Parke Davis Co. Size 00) or plastic Beem capsules, and were kept under dry conditions.

Procedure;

1. Fixation in 2% osmium tetroxide 1¼ hours
2. Wash in distilled water 2 changes 15 minutes
3. Dehydrate in absolute alcohol 2 changes 30 minutes each

4. Transitional solvent Propylene Oxide 2 changes 30 minutes each

5. Epon: Propylene Oxide 2:1 1 hour

6. Epon: Propylene Oxide 3:1 3-4 hours

7. Epon 1 hour

8. Embed 1 hour

Gluteraldehyde Fixation

Reference: Sabatini, D. D., Miller F. and Barnett P. J. 1964. Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope. J. Histochem. Cytochem. 12: 57-71.

Solutions:

Sorenson's Phosphate Buffer

Solution A 7.1 g Na_2HPO_4 in 500 ml distilled water

Solution B 6.8 g KH_2PO_4 in 500 ml distilled water

Working Solution

7 parts of solution A to 3 parts of solution B. This gave a 0.1 M solution with a pH between 7.2 and 7.4.

Chick Ringer Solution

Sodium Chloride	9.0 g/l
Potassium Chloride	0.42 g/l
Calcium Chloride	0.24 g/l

Fixative

14 ml of 25% gluteraldehyde were added to 86 ml of phosphate buffer or chick ringer solution, to give 3.5% gluteraldehyde solution.

Procedure:

The entire yolk sac or dissected spleen was fixed for 2 hours before transferring directly to 2% osmium tetroxide.

Epon Mixtures:

	A	:	B
Soft	2	:	1
Medium	1	:	1
Hard	3	:	7

The second embedding mixture used was Solution A:B = 1:1

Staining for Electron Microscopy

Reference: Reynolds, E. S. 1963. The Use of Lead Citrate at high pH as an Electron-opaque stain in Electron Microscopy. J. Cell. Biol. 17: 208-212.

1. Lead citrate

   Lead Nitrate 1.33 g

   Sodium Citrate 1.76 g

   Distilled water 30 ml

The stain was prepared using clean glassware, the suspension was shaken vigorously for one minute and allowed to stand with intermittent shaking for 30 minutes. 8.0 ml of carbonate free N NaOH was added and the volume made up to 50 ml with distilled water, the pH of the solution adjusted to approximately 12. Sections were stained by floating the grids section side down in a drop of staining solution. Drops of solution were placed on a piece of cleaned dental wax in a petri dish containing some pellets of NaOH to provide a CO_2 free atmosphere. In all cases contamination of the solution was avoided and the material set up as rapidly as possible. The most satisfactory staining time was found to be 25 minutes, after which the grids were washed in 0.02 N NaOH, distilled water, and dried on filter paper in a covered petri plate.

2. Lead Citrate and Uranyl Acetate

Distilled water	10 ml
Lead Citrate	0.035 g
10 N NaOH	0.1 ml

The solution was shaken until all the lead citrate had disolved.

6% Uranyl acetate in 100% Methanol

The technique of using drops of stain on dental wax and the avoidance of contamination was the same as above.

Procedure:

1. Filter Uranyl acetate and stain at 37° C for 15 minutes
2. Rinse in 100% Methanol
3. Stain in Lead citrate about 90 seconds
4. Rinse in Distilled water
5. Dry on filter paper

APPENDIX IV

Photographic Techniques

All light photomicrographs were taken with a Leica (Type MD) camera attachment to a Leitz Ortholux microscope. Kodak Blue and Green XZ filters were used for all black and white photography. Kodak High contrast copy film was used for all black and white photomicrographs, developed in Kodak Microdol-X developer for 5 minutes, washed in tap water and fixed for at least 15 minutes in Kodak Rapid Fixer, washed in running tap water for at least half an hour. Then a few drops of Kodak Photo Flo-200 were added to water; the film removed and dried.

Prints were made using Agfa Bovira single weight paper, developed in Dektol, washed in tap water and fixed in Kodak Rapid Fixer diluted for use with papers.

Several types of colour films were used for colour photomicrography, the most successful was High Speed Ektachrome EH-135, although some prints are included using Kodachrome II Professional film Type A.

For the electron microscopic work with the Zeiss microscope, Scientia 23D56 film was used, and for the R.C.A. microscope Kodak (2x10") Plates. Both films were developed using Kodak 19 Developer, washed and fixed with Kodak Rapid fixer for film.