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The Technique and Diagnostic Value of Ehrlich's Method of Staining the White Blood-Corpuscles.

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The tide of active and exhaustive investigation which so long expended its force upon the elucidation of the problem of the genesis of the red blood-cell has now turned to that of the much-neglected white blood-corpuscle, which latter seems to have been well-nigh submerged and lost sight of in the first onward rush. This reawakened interest we owe largely to the uninterrupted study of inflammatory processes, but more especially of late to our widening knowledge of the part played by the phagocyte in its warfare against bacterial invasion, its determined onslaught or cowardly retreat. To the discovery of leucæmia by Virchow,* to Cohnheim's † teaching of the emigration of the white cell in pathological processes, and to Max Schultze's ‡ obser-

* Virchow's *Archiv*, Bd. v, p. 43.
† *Ibid.*, Bd. xl, p. 1.
‡ *Archiv f. mikrosk. Anat.*, Bd. i, p. 1.

vation of the morphological dissimilarity of the white cells in circulating blood, we owe our earliest important advances in this direction. But the great strides of recent years were rendered possible by the discovery by Ehrlich * of the distinctive reactions of white blood-cells with aniline colors, and that by means of these the white cells could be divided into several groups with sharply defined characteristics. Yet, although the first steps of these important discoveries were published some twelve years ago, the number of Ehrlich's disciples has been so small, the literature of the subject so scanty and so confused with other subjects, that, despite its great interest, no complete attempt has been made to gather these observations together and present the subject concisely to English readers in such a form that they could easily grasp and carry out the really simple technique, or draw from their results the possible diagnostic deductions of the highest importance. These means which allowed Ehrlich to distinguish elements which were morphologically similar and again to class together cells of very different habitus were furnished by his discovery that in the protoplasm of the white blood-cells there existed both fine and coarse granules, which were characterized by their reaction to (staining by) certain colors or groups of coloring matters, and, further, that the same kind of granules were always found in the same kinds of cell elements. This distinction was based upon the fact that the aniline colors can be divided into three groups—acid, basic, and neutral (the latter resulting from a combination of the two others). For example, the eosinophile † or α granulation is stained by each and all of the acid coloring stuffs and by no others, while other granules react only to basic, or still others only

* Ehrlich. *Verhandl. d. physiol. Gesellschaft z. Berlin*, 1878–'79, No. 8, No. 20.

† *Ueber eosinophile Zellen.* Schwarze. Inaug. Diss., Berlin, 1880.

to neutral colors. These granules, thus far seven in number, Ehrlich called α, β, γ, δ, ϵ granules, etc. Of these, only three are of importance in the study of human blood—viz., the α or eosinophile granule, the γ or mast-cell granule, and the ϵ or neutrophile. The α and γ are found in man and in all animals; the ϵ only in man. Of the remaining four, two are found only in guinea-pigs and rabbits, and two in birds alone.* They may exist in great numbers in the body of the cell, and do not in any way involve the nuclei. Besides their reaction to colors, they can be distinguished from one another by their size, form, and solubility. Those found in man are more or less round, varying in size in different kinds of cells from the fine, dust-like ϵ or neutrophile, to the larger, distinct, rounded α or eosinophile. Rarely in birds crystalloid or octaedral forms are seen. Each cell contains but one kind of granule, and those in the same cell and in each one of the same kind of cells are always of the same size. The fact that these cells containing granules are produced in the marrow and sometimes in the spleen, but never in the lymphatic glands, together with the observation that certain granules are limited to certain species, is thought to argue strongly for the existence of the closest relation between the granules and the chemical function of the cell.

The technique of demonstrating these cells is easily acquired. The necessary apparatus is exceedingly simple and is comprised in the following list: A copper plate, $12 \times 4 \times \frac{1}{8}$ inch; a tripod or metal filter stand upon which the plate can be balanced securely; a Bunsen burner or alcohol lamp; a small thumb forceps; stains; filter paper; Canada balsam dissolved in xylol; cover glasses; slides; and a $\frac{1}{12}$ oil immersion lens.

* Ehrlich. *Charité-Annalen*, Bd. xii.

To secure preparations for staining the procedure is as follows: Lay out four cover glasses, previously cleaned with alcohol, upon a hard smooth surface near at hand, together with a slide having a smooth and preferably ground and rounded edge. Clean the pulp of a finger or the lobe of the ear carefully with soap and water or alcohol to remove dirt or dead epidermis, and dry it thoroughly. Puncture the part selected by the quick stab of a needle, preferably one with a ground triangular point, as such punctures are less painful and, even when slight, bleed freely. Take the slide in the fingers as one holds a pen, and, wiping away the blood, draw the edge of one end through the blood as it flows. Then, placing a finger of the other hand upon the edge of a cover glass to steady it, quickly put the end of the slide, held at an angle of 45°, fairly upon the cover next the finger and draw it rapidly across the cover, leaving a thin film of blood, which immediately dries in the air. This procedure requires a little dexterity, and its success depends upon the rapidity with which the fresh blood from the capillary is transferred to the cover, where the individual cells lie entirely separate and are instantly fixed by exposure to the air without undergoing any change of shape. This is repeated with each of the covers, the finger-tip and edge of the slide being each time wiped free from coagulating blood. These spreads when dry will keep indefinitely, but should be handled entirely with the forceps, as the thin film of moisture which forms on them from the fingers alters the hæmoglobin. Further fixation is, however, necessary before they are subjected to the solvent properties of the stain. This is accomplished by subjecting them to a temperature of 120° to 130° C. Balance the copper plate upon the tripod, and place the Bunsen burner or alcohol lamp beneath one end, so the flame a little more than touches it. In about half an hour an equilibrium will have

been established between the continuous source of heat and the radiation from the plate, so that any given point on the plate will have the same persistent temperature. Beginning at the cool end of the plate, and dropping water upon it progressively toward the flame, a point will be found where the drop is instantly converted into steam. This represents approximately 100° C., or boiling point. About the width of the cover glass nearer the flame the cover glass should be laid, face upward, at a constant temperature estimated at 120° to 130° C., and left there for two hours. They are then ready for staining, and may be laid, face downward, in a drop of the stain placed upon a slide, and may remain there for the time peculiar to each stain, after which they are freely washed in running water, dried between fine filter paper, and mounted in Canada balsam dissolved in xylol, which has been heated slightly upon the slide to drive off the excess of xylol. Balsam prepared with the usual solvent, chloroform, decolorizes the specimens and should not be used.

Staining Solutions.—Of these, two are necessary to bring out all the diagnostic factors: First, to demonstrate the eosinophile granulations, the nuclei of both white and red cells, and the hæmoglobin of the red cell:

℞ Eosin. cryst., Nigrosin, Aurantia, ... āā 10·0;
Glycerini..... ..... 100·0.

These are carefully rubbed together in a glass or porcelain vessel for fifteen minutes, and placed in a glass-stoppered bottle. Eosin stains the eosinophile granules a purple red. Nigrosin stains all the nuclei black. Aurantia stains the hæmoglobin—*i. e.*, the protoplasm of the red cells—a reddish yellow. The neutrophile granules of the polynuclear cells are not stained by this solution. To bring out

these neutrophile granules another stain must be used, as follows: *

℞ Saturated aqueous solution orange G. 125 c. c.;
Saturated solution of acid fuchsin in twenty-per-cent. alcohol..... 125 "
Add gradually, shaking continually:
Saturated aqueous solution methyl-green..... 125 "
Alcohol absolute..... 75 "

This solution must stand for several weeks until the precipitate which forms has sunk to the bottom, and the fluid for use at any time must be drawn from the center with a pipette to avoid the sediment. This stain improves with age, acts in a few minutes, and does not overstain if left much longer.

The basic methyl green and acid fuchsin combine on standing to form a neutral color which stains the neutrophile granules violet. The methyl-green stains the nuclei green. The acid fuchsin colors the eosinophile granules red, and the orange-G imparts a yellow color to the hæmoglobin.

For rapid use where time presses a concentrated eosin glycerin solution washed off with water will quickly color the eosinophile cells, but a better solution is the following: †

℞ Eosin. cryst..... 0·5;
Hæmatoxylin..... 2·0;
Alcohol absol., Aq. destill., Glycerin., ... āā 100·0;
Acid. acet. glac..... 10·0;
Alum in excess so that it lies at the bottom.

* Gollasch in *Friedländer und Eberth, Mikroscopische Technik.* Ber n, 1889.

† Gollasch. *Loc. cit.*

After standing in the light for three or more weeks this stains in a few hours. The nuclei are then dark blue, the hæmoglobin yellow, and the eosinophile granules red purple. All the above-mentioned stains are washed off in water, the cover glasses dried in the air or between fine filter paper and mounted in Canada balsam dissolved in xylol.

Normal blood, according to H. Reinecke, contains 7,100 to 7,300 white cells per cubic millimetre, or 1 white to 720 red.* These figures are subject to wide variation in health, a proportion of 1 to 500 or 1 to 1,000 falling within normal limits. During the period of digestion, one to two hours after an important meal, the proportion of white cells or leucocytes may be increased to 1 to 150 or even 1 to 100. Such an increase, which is of temporary duration and which consists of a simple augmentation of such white elements as are already present in normal blood, is called leucocytosis, a term simply descriptive of a condition and in no sense implying any pathological change.

We now come to the consideration of the different kinds of white cells found in normal circulating blood, and here 't is that Ehrlich and his disciples have laid the foundation for all subsequent progress in the differentiation of five

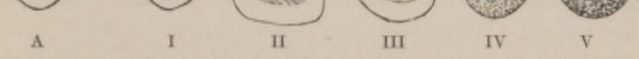

FIG. 1.—A, Red blood-cell. White blood-cells: I, Lymphocyte. II, Large uninuclear leucocyte. III, Transitional form. IV, Multinuclear, neutrophile leucocyte with ε granulations. V, Eosinophile cell with α granulations.

cardinal forms, the comprehension of which will be facilitated by reference to the accompanying schematic drawing (Fig. 1).

* *Fortschr. d. Med.*, 1889, No. 11, p. 409.

I. *Lymphocytes.*—Uninuclear small forms, most nearly approaching the red cells in size, and having a single deeply staining nucleus nearly filling the cell body. The protoplasm of the latter is represented by a faint circular rim, sometimes crescentic and visible only at one side, sometimes spindle-shaped, having the nucleus in the middle. These lymphocytes were first proved by Virchow to be derived exclusively from the lymphatic glands, whence their name. They contain no stainable granules in their protoplasm and constitute about twenty per cent. of the white cells in normal blood. Einhorn* states that within normal limits they may be increased even as high as forty-four per cent., but not much decreased. In pathological cases, however, their number may be markedly reduced, not only relatively so as to be only ten per cent. or even five per cent., but also absolutely decreased (diabetes, phosphorus poisoning, phthisis, hæmorrhagic diathesis, etc.). In lymphatic leucæmia they are both relatively and absolutely increased, and are of great diagnostic importance, fifty per cent. or more being found in such cases, or even ninety-three per cent. in one fatal case. In stained specimens the lymphocytes vary often considerably in different cases, sometimes presenting small dark, in others slightly larger and lighter nuclei with more surrounding protoplasm. Variations often occur rapidly in the same case, but the significance of the change is not known. This occasionally complicates the usually easy differentiation of forms I and II. In those instances where the cell body has a spindle shape it perhaps presents its original form in the lymph gland.

II. *Uninuclear Leucocytes.*—Larger forms of variable size up to twice the diameter of a red cell, having, when stained, a single large ovoid, pale nucleus, and a well-marked border of protoplasm. They are derived from the medulla of

* *Fortschr. d. Med.*, iii.

the long bones, and also, Ehrlich maintains, from the spleen. In normal blood they are not numerous, constituting with III only five to seven per cent. of the white cells. Increased numbers indicate the presence of cachexia. No stainable granules occur in the protoplasm in normal blood.

III. *Uninuclear Transition Form.*—This cell is derived from II and is similar in size and color to its progenitor. The cell body, however, is slightly smaller and its nucleus more or less kidney-shaped, which is the first step toward a division of the nucleus. According to the degree of the further nuclear development, it may show the first traces of neutrophile ϵ granulation in the protoplasm between the horns of the nucleus.* From this is evolved the following form, which is the last of this subseries:

IV. *Multinuclear Neutrophile Form with ϵ Granulations.* —These contain a nucleus of polymorphous figure resembling the letters S V Y Z E, which stains rather feebly, or

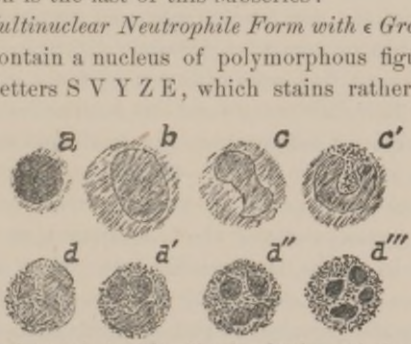

FIG. 2.—Drawn from a single slide. Showing development of uninuclear into multinuclear leucocytes. *a*, lymphocyte, size of red cell, introduced for comparison; *b*, large uninuclear leucocyte; *c*, transition form; *c'*, transition form with neutrophile granulations between the horns; *d d' d'' d'''*, multinuclear neutrophile leucocytes showing various stages in the development of the nuclei and the neutrophile granulations.

else several small darkly staining nuclei just held together by threads, or in the mature form entirely separate. They contain in their protoplasm numerous fine dust-like granules,

* Spilling. *Ueber Blutuntersuchungen bei Leukämie.* Inaug. Diss., 1880.

which stain only with stains of neutral reaction, resulting from a mixture of acid and basic colors; hence they are called by Ehrlich polynuclear neutrophile cells. They are of variable size, larger than red cells, but smaller than the large mononuclear from which they are derived. They constitute sixty-five to seventy per cent. of the white cells, are capable of emigration from the blood-vessels into the tissues, and are alone found in pus. In leucocytosis they are the only form of white cell which is increased. The neutrophile granules are not visible in unstained specimens, nor are they made apparent by any other stains than those of neutral reaction. Now, although the uninuclear leucocyte does not show neutrophilic granulations, it possesses all the other morphological and microchemical characteristics of the multinuclear cell. In leucæmic blood we find the links necessary to definitely determine their relationship, for we find cells with very sparse ε granulations, which in their habitus occupy an intermediate position. Therefore Ehrlich believes that the multinuclear is evolved from the uninuclear form. He shows that while the original large nucleus of the uninuclear cell is developing by the progressive stages above mentioned into the four or five nuclei of the multinuclear form, other changes are taking place in the cell body by which it gradually assumes the neutrophile granulation, and its protoplasm acquires an increased contractility, which gives the cell the amœboid motion necessary to pass through the walls of the vessels in inflammation. It seems probable that this transformation or development takes place after the uninuclear cell reaches the general circulation, and that the blood-forming organs send out their product as raw material to be developed in the nutritive media of the circulating plasma. If this is so, it follows, theoretically, that altered conditions of the plasma should affect the development of the cells,

This is exactly what Ehrlich shows to occur in severe cachexiæ (tuberculosis, carcinoma), where in the impoverished blood there seems to be a stoppage or prolongation of the developmental process, for in cachexiæ the uninuclear outnumber the multinuclear; in leucæmia the examples of the intermediate stages are well marked and very numerous. In health these changes presumably take place so rapidly as to disclose few traces of the metamorphosis.*

V. *Eosinophile Cells with α Granulations.*—These are the white cells which strike one at once in a fresh specimen of blood as containing rather large, yellowish, fat-like granules. They are not related to the former series having their origin in the marrow. Their granules are easily stained by all acid colors, such as eosin, whence their name. Thus stained, the granules appear of a purplish-red color, noticeably larger than those of the neutrophile cells, while their nuclei, one to three in number, stain less darkly than those of the multinuclear leucocytes If an eosinophile cell has been broken in the process of spreading the cover glass, the granules lie loose in groups and have been mistaken by some observers for micrococci. From these, however, they can be easily distinguished by their staining affinities. Eosinophile cells are variously estimated as constituting two to four per cent. and five to seven per cent. of normal blood. It is probable that the former figures are most correct. They are present in large numbers both in blood and sputum of asthmatic patients, also in prostatic secretion, and in the blood of patients with certain affections of the skin (lymphodermia, pemphigus, etc.); therefore Ehrlich's † dictum that they are formed exclusively in the marrow, and that an increase in their numbers points to chronic changes in the blood-forming organs, is now under discus-

* Ehrlich. *Loc. cit. Ztschr. f. klin. Med.*, Bd. i.

† *Ztschr. f. klin. Med.*, Bd. i.

sion. Their absolute number is often increased to a high degree in myelogenous and lieno-myelogenous leucæmia. On the other hand, the eosinophile cells are decreased in severe cachexiæ (phthisis, lupus, vitium cordis, anæmia, diabetes, carcinoma*)—that is, in those cases in which leucocytosis exists with increase of the mononuclear and polynuclear forms. Von Jaksch believed that their increase alone, without other changes, was presumptive evidence of beginning leucæmia, but they have since been found to be increased in numerous other conditions.† Von Jaksch also bases the diagnosis of his anæmia infantum pseudo-leucæmica in part upon their total absence or greatly lessened numbers. ‡

Passing now from the consideration of normal blood, there remain to be mentioned several stainable forms which, as they occur in the blood only in pathological conditions, are also of great diagnostic importance.

Mast-cells, having γ granulations, easily stainable in basic aniline colors alone (basophile), and appearing only in the blood during pathological processes (leucæmia), and then only in very small numbers.# They were formerly supposed to be derived from the lymph glands. Ehrlich opposes this view. He has found them in large numbers in a case of myelogenous leucæmia. ‖

Myelocytes.—These are large uninuclear cells differing from those found in normal blood in that they contain ϵ or neutrophile granulations. While they are normally formed and found in the bone marrow, in cases of myelogenous leucæmia alone do they escape into the circulating blood in

* Alderhoff. *Prag. med. Woch.*, 1891, No. 8.

† Müller u. Rieder. *Archiv f. klin. Med.*, Bd. 48.

‡ Ueber Diagnosis u. Therapie d. Erkrank. d. Blutes. *Med. Wander Vorträger*, 21, Berlin, 1890.

Ueber Mastzellen. Inaug. Diss., Berlin, 1880.

‖ Ehrlich. *Deut. med. Woch.*, 1883, p. 871.

considerable numbers. Their great value in diagnosis is at once apparent.*

Nucleated red cells, whose nuclei are stained by any of the agents which stain the nuclei of the white cells. They are easily distinguished from the latter cells, in that their surrounding zone of protoplasm takes the same stain as the hæmoglobin of the other red cells. Three forms are distinguished, corresponding to the three forms, or more properly speaking sizes, of non-nucleated red cells—microcytes, normocytes, megalocytes—and are called respectively microblasts, normoblasts, and megaloblasts. The microblast, found and described by Ehrlich,† is very rarely seen. Normoblasts, as the prefix implies, are nucleated red cells of the average size of the ordinary red cell. The nuclei may be uniformly dark or there may appear an irregular and heavily beaded network upon a lighter background within the nucleus. It is believed with Rindfleisch that the nucleus escapes from the red cell after it is fully formed, and, after gathering about itself hæmoglobin for another perfect cell, again escapes to repeat the cycle. Free nuclei may often be found in the stained blood of certain pathological cases, and nucleated reds are likewise seen in which the nucleus is of dumb-bell shape, in the process of division, or where it has divided into two, three, or four nuclei before escaping. The nucleated red cell may be found in all forms of advanced anæmia, whether traumatic, secondary, or primary (pernicious); also in acute metallic poisoning (As, P, etc.), and in leucæmia.

Megaloblasts or gigantoblasts are very large nucleated red cells, having a paler reticulated nucleus of much larger size than those of the last class. The surrounding zone of

* Ehrlich. *Deut. med. Woch.*, 1883, p. 871.

† Ehrlich. *Verhandl. d. Gesellschaft d. Charité Aerzte z. Berlin*, 1880.

hæmoglobin also often stains badly and has a dingier color. Ehrlich believes these to be a return to an embryonal type. It is also thought that, after the escape of the nucleus from the cell, it dies and thus ceases to continue the formative cycle. They are therefore pathognomonic of very serious disturbance in the cytogenic organs, and are found only in the late stages of leucæmia and pernicious anæmia. Still further, their presence in the blood, taken together with certain other factors, is absolutely necessary to the establishment of the diagnosis of pernicious anæmia.

Turning now to the practical results of these examinations, we are at once confronted by the question: Having discovered a certain combination of factors in the condition of the blood, what definite diagnostic conclusions can be drawn from it? It has not been within the scope or purpose of this paper to treat of the familiar methods of estimating the corpuscular richness of the blood or the percentage value of its hæmoglobin, the importance and necessity of such estimates having been long ago established and conceded. Nor is it contended that a diagnosis can always be made by these methods of staining without careful consideration of the clinical course and objective symptoms in any given case, but that used to supplement the usual methods, which too often leave us in doubt, they are invaluable in definitely confirming and often in alone determining the diagnosis. Eliminating largely, therefore, such factors as are still mooted and incorporating chiefly those which are generally accepted, we arrive at the following basis for diagnosis:

Chlorosis.—Red cells in the majority of cases nearly normal in numbers, of average size and good shape, but the individual corpuscles strikingly pale, with relative decrease in the percentage of hæmoglobin. Rouleaux well formed, leucocytosis slight if present. In rarer cases poikilocytosis

and marked reduction in the numbers of the red cells, more likely to be present if the reduction in hæmoglobin is below sixty per cent. This latter condition often considered a mixed form and called chloro-anæmia.

*Simple Anæmia, Primary.**—Decrease in the percentage of red corpuscles and hæmoglobin to approximately the same degree. Variation in size and shape of the red cells. No leucocytosis or change in white cells.

Secondary Anæmia.—Decrease in the percentage of the red corpuscles and hæmoglobin to approximately the same degree. Imperfect formations of rouleaux. Leucocytosis, hence increased numbers of polynuclear neutrophile cells, but also numbers of large uninuclear leucocytes without granulations. Often marked variation in the size and shape of the red corpuscles, microcytes predominating. Poikilocytosis or schistocytosis. Nucleated red cells of normal size (normoblasts). The intensity of these changes varies considerably with the degree of the anæmia.

Pernicious Anæmia, Primary.—Rouleaux not formed. Enormous decrease in the number of red cells, with distinct excess of the hæmoglobin percentage. Very marked variations in shape, poikilocytes or schistocytes, also in size with microcytes and megalocytes; the latter largely predominate. Large red cells with very large nuclei, megaloblasts or gigantoblasts. Never absolute leucocytosis. The excess of megalocytes and presence of megaloblasts are considered to be diagnostic.

Pseudo-leucæmia.—In the early stages no noticeable change. In the later stages well-marked signs of simple anæmia, with rather decided simple leucocytes, clearly distinguished, however, from leucæmia by the absence of eucæmic changes in the white cells.

Leucæmia in General.—The earliest sign in the blood

* Von Limbeck. *Grundriss einer klin. Pathologie d. Blutes.* Jena, 1892.

of a beginning leucæmia seems to be an increase in the eosinophile cells. It has been thought that in connection with the clinical history a probable diagnosis could be advanced upon this basis, but it must be remembered that the eosinophile cells have now been shown to be increased in many other conditions.* In advanced cases there is an excessive and persistent increase of leucocytes, the ratio of white to red varying from 1 to 20 to 1 to 1. Reduction in the red cells and correspondingly in hæmoglobin. Faulty formation and decreased resistance in the individual red cell, with poikilocytosis. Formation of Charcot's crystals in the drawn blood on standing.

Leucæmic cases may be divided into four classes, but mixed cases most commonly occur.

Lymphatic Leucæmia.—Predominance of small uninuclear lymphocytes over all other white cells. Eosinophile cells decreased. No nucleated red cells. Proportion of white to red seldom above 1 to 20, even ante mortem. Marked anæmia.

Lienal Leucæmia.—Predominance of large uninuclear leucocytes (without neutrophilic granulations). No nucleated red cells. No myelocytes. No increase of eosinophile cells. Cases of pure lienal leucæmia have been reported, but the exclusive involvement of the spleen without the involvement of other cytogenic organs has been denied.

Myelogenous Leucæmia.—Characterized by the appearance of abnormal forms—*i. e.*, myelocytes or large uninuclear leucocytes with neutrophilic granulations formed in the marrow and present in the blood. Eosinophile cells much increased, including large forms which have escaped from the marrow. In the earlier stages normoblasts; in

* Neusser. *Wiener klin. Woch.*, 1892, No. 4.

the later stages megaloblasts. In rare cases the red cells show division of their nuclei.

Lieno-myelogenous Leucæmia.—The most common of the mixed forms presenting the characteristics of both the lienal and myelogenous forms.

Upon these lines the diagnosis of diseases of the blood or blood-forming organs is becoming firmly established. The subject is a living one. Each month brings new light and reveals further possibilities. Questions once mooted are passing from debatable ground into the realm of certainty. To the scientific student these methods of staining open up a most interesting and fascinating field for investigation. To the general practitioner and careful diagnostician the following advantages are presented :

1. Earlier and more exact diagnosis.
2. More definite prognosis.
3. More intelligent and therefore more active and successful therapeutic efforts.

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