THE SUPRAVITAL METHOD IN THE STUDY OF THE CYTOLOGY OF BLOOD AND MARROW CELLS

By JOSEPH L. SCHWIND, PH.D.

SINCE the intensive application of the supravital method of studying the cells of the blood and hematopoietic organs by Sabin and her students and associates, there has been much discussion of the value of this technic. These investigators believed that purely morphologic studies of the blood had reached an impasse, and in pioneering the new, somewhat more physiologic method, they became convinced, perhaps not unnaturally, that in many ways it was superior to the study of dried stained smears. Unfortunately, the criteria used in the identification of some of the cell types have been found in the intervening years to be unreliable. Moreover, as will be shown below, the supravital method is ill adapted for the study of the erythroid series in blood dyscrasias. As a result of the first study of the developing red cells with this technic, much confusion was introduced, not only with regard to the terminology of erythropoiesis, but also in the fundamental concept of the nature of pernicious anemia. Investigators working from dried stained smears have therefore been extremely critical of the supravital method, but occasionally adverse criticism has been carried beyond the justified facts.

The object of the present paper is (1) to reconsider the value of supravital preparations as a method of studying the cells of the blood and hematopoietic tissues after considerably more experience with the method than was possessed by previous critics and to arrive at a balanced view of its usefulness and practicality; (2) to report various changes in technic which were made during the present study; (3) to correct certain erroneous concepts which occurred during the pioneer work on the method, cited above; (4) to attempt to correlate the terminology applied to the developing marrow cells when seen in supravital preparations with that applied to similar cells seen in dried stained smears; and (5) to discuss briefly the bearing of the above findings on the current theories of hematopoiesis.

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†The manuscript of this paper was in the hands of the Editors when Dr. Schwind died suddenly, of coronary thrombosis on May 20, 1948. Editorial suggestions for revisions and condensation of certain portions of the paper were carried out by his colleagues, Drs. Elbert B. Ruth and George M. Guest.
SUPRAVITAL METHOD IN CYTOLOGY OF BLOOD

This paper is the result of the experience gained by studying both supravital preparations and dried stained smears* of (1) most of the cytologically abnormal bloods which were found in the Albany Hospital during the years 1937–1945, as well as of about 400 other bloods referred by outside physicians, (2) the bone marrow obtained from 240 sternal punctures performed on patients during the same period, and (3) incidental studies of the blood and bone marrow of chick embryos, rabbits, guinea pigs, rats and pigeons. The substance of this paper is based, however, on human material.

It must be appreciated at the outset that when using the supravital method, one is working with cells which are, to a large extent, still alive and extremely fragile. All technical procedures must therefore be carried out with meticulous care. A mild slurring of technic in staining dried smears results in a slide which is somewhat less than perfect, but with the supravital method the resulting slide is useless. Technic will be discussed, therefore, in detail in the hope that novices at the method may be spared many of the difficulties which were encountered by the present writer. Neutrophil granules, many neutral red vacuoles and some mitochondria are extremely small and must be optically resolved to individual entities before certain cells can be identified, and it is of primary importance, therefore, that the best optical conditions be maintained. A binocular microscope is a necessity. Curiously, neither the proponents of the supravital method nor its severest critics have ever pointed out the importance of good microscopy in its use, though certain errors in the first papers dealing with the method, and some criticisms of it, are directly attributable to inadequate optical systems.

OPTICAL SYSTEM AND ILLUMINATION

To obtain dependable results with the supravital method, apochromatic oil immersion objectives of not less than N.A. 5.3 should be used with compensating oculars and an achromatic condenser, preferably of N.A. 1.4. The maximum amount of resolution must be obtained, and therefore the full aperture of the objective lens must be utilized by immersing the condenser.† Good vision is much more of a problem in supravital preparations than with dried stained smears, as when the slide is improperly lighted the cells appear hazy. Controlled† (Koehler) illumination should be used. Controlled illumination‡ is prefer-

* Most of the dried stained smears were made with Wright's stain; some were stained with May-Grünwald-Giemsa. Giemsa is a better stain, but relatively few physicians are familiar with the appearance of blood and marrow cells stained with it. For close study of the nucleus in a Wright's preparation, a combination of a No. 15 and a No. 45 Wratten filter on the light source, as suggested by Waterman, will greatly increase the contrast between chromatin and parachromatin.

† Unless the condenser is immersed, an oil immersion lens actually works at N.A. 1.0, regardless of its rated N.A.

‡ Directions for setting up the optical system in controlled (Koehler) illumination are given in most books on microscopy, although the method is not nearly as well known or as widely used as it should be. This type of illumination is possible only with light sources equipped with a focussing device on the lamp condenser and which have a field diaphragm between the lamp condenser and the mirror of the microscope. The microscope and lamp should be fixed in a permanent position. Brief directions for setting up the system are included here for the convenience of the reader. For oil immersion work, one proceeds as follows: using the flat side of the mirror, (1) check the centering of the substage condenser; (2) with the aid of a handmirror, focus the image of the filament of the light source on the substage iris diaphragm of the microscope (which is closed for the purpose) by means of the focussing device on the lamp condenser; (3) place the slide carrying the object to be studied on the stage of the microscope, and, having im-
able to "critical" illumination because it permits the use of microscopic slides which may vary slightly in thickness and because the condenser can be refocussed for every field if this is necessary. Only the flat side of the substage mirror should be used, and a first-surface mirror is of definite advantage. The hazy appearance of the cells in supravital preparations can further be avoided by using a total magnification not greater than 600 times the numerical aperture at which the lens is actually working.

Less light is needed for the study of supravital preparations than for the study of sections or dried stained blood smears. Nevertheless, a band filament projection bulb is the best light source. A band filament bulb, however, gives far too much light, and the excess must be removed by neutral density filters. The glare of too intense illumination should never be controlled by closing the substage diaphragm. In addition, in order to have standard conditions for color comparisons, a transparent daylight filter should be used. The daylight filter must be transparent, for otherwise the image of the filament cannot be focussed on the substage diaphragm. When the optical system is properly set up in this way, the field of the microscope is brilliantly lighted, but there is no glare. Small foreign objects, such as dust particles between the elements of the condensing lens of the lamp, are clearly visible in the field of the microscope and must be removed.

STAINING

Successful supravital preparations depend primarily upon proper staining. All of the precautions emphasized by Sabin are necessary to obtain absolutely clean slides. The slides must be of good quality, noncorrosive, free from bubbles or scratches, and not thicker than 0.12 mm., as otherwise the field diaphragm cannot be brought into focus by the condenser. They must be washed with soap and water, rinsed, soaked in bichromate cleaning solution for at least twenty-four hours and thoroughly rinsed again in tap water. This latter step should be carried out with a piece of bolting cloth tied over the outer of the tap to remove algae and other micro-organisms which are present, and which sometimes stick to the slides. They are then rinsed in distilled water to remove the salts present in tap water, and are stored in an airtight container in 95 per cent alcohol, which makes them easy to dry. Just before spreading the stain on them, they are dried with lintless cloth, such as surgical gauze. If care is taken to prevent lint or dust from getting onto the slides, flaming may be omitted. Coverslips are prepared in the same way. After being washed with soap, both slides and coverslips should be handled with slide forceps only.

Slides are covered with dye in the manner originally described by Sabin: the slide is held in a horizontal position and flooded with a proper dilution of dye, which is then drained off and the slide allowed to dry in a vertical position. This gives a thin, uniform film of dye on the greater part of the slide when the humidity of the atmosphere is low, as the alcohol in which the dye is dissolved quickly evaporates. In the summer, however, when the humidity is high, the slides must be dried in a stream of hot air (obtained most conveniently from a cheap commercial hair drier), for in a humid atmosphere the absolute alcohol will take up enough moisture to ruin the dye film before evaporating. Films dried in a stream of hot air are not as uniform as those from which the alcohol is allowed to evaporate at room temperature, but they are quite satisfactory since a slight unevenness in the distribution of dye is of no practical importance.
Vital neutral red and Pinacyanole* are used as stains. The stock solutions are prepared by dissolving 0.2 Gm. of neutral red and 0.05 Gm. of Pinacyanole in 50 cc. of absolute alcohol in separate vials. This makes a saturated solution of neutral red, and in handling the stock solution care must be taken not to agitate the container, for dye particles on the slides will interfere with the even spreading of the drop of blood. The stock solution of neutral red will keep indefinitely, but that of Pinacyanole must be renewed every six months. The solution for making supravital slides is prepared by adding 30 drops (0.38 cc.) of stock neutral red and 9 drops (0.17 cc.) of stock Pinacyanole to 5 cc. of absolute alcohol and mixing thoroughly before making films on the slides. The dye solutions are made up in absolute alcohol because it evaporates quickly from the slides. Pinacyanole, which stains the mitochondria a deep blue color, and which was introduced by Hetherington, is used in place of Janus green because it has a number of advantages over that classic mitochondrial stain. It does not interfere with the staining of the vacuolar system with neutral red, and does not fade out of the mitochondria for many hours except in very exceptional types of cells. When stained with Janus green,† the mitochondria begin to fade after a relatively short period of time. One can never be certain, therefore, that a cell containing a few mitochondria is truly showing the total number of them. Pinacyanole, moreover, stains the nuclear chromatin a purplish blue color without interfering with the motility of the cell, whereas Janus green in sufficient concentration to stain the nucleus is toxic. On the other hand, Pinacyanole is light-sensitive, so that the stock bottle and the films on prepared slides must be kept in the dark. Prepared films will not keep longer than two weeks.

Staining with neutral red alone, as was done in early work with the supravital method, is inadequate except for the more mature cells of the granulocytic series. Both neutral red and Pinacyanole are used in every film, as the number and size of the mitochondria are often of considerable help in identification. In the first reports, considerable emphasis was placed on the rosette of vacuoles in monocytes as a means of identifying that cell type. It has been shown by many writers that rosettes are not specific for any one type of cell. The present writer has also found that there is much more variation in the number, size and color of neutral red vacuoles scattered in the cytoplasm of various types of blood and marrow cells than had originally been supposed.

To make the supravital preparation, a small drop of blood, marrow fluid, tissue scraping suspended in plasma, or other material to be studied is placed in the center of a scrupulously clean coverslip, which is then inverted and very gently lowered onto the dye film on a prepared slide. The drop should be of such a size as to cause the red blood cells to spread out in a single layer. Thicker preparations cause the leukocytes to round up instead of being flattened by the coverglass, and their cytology is correspondingly more difficult to study. If air bubbles are trapped in the blood film, the cells may not spread out properly. If too little blood is placed on the coverslip or if the slip is dropped onto the slide, the weight of the coverglass will rupture the cell membrane of many of the leukocytes, and many others will not stain properly. Injured cells very frequently cannot be identified. Every supravital preparation of blood or marrow contains a varying number of neutrophils in which the neutrophilic granules do not stain properly with neutral red. Such cells, which also show no movement of the cytoplasm when studied in the warm box, have been called "nonmotile leukocytes" by Sabin, and are regarded by most investigators using the supravital method as the final stage in the life cycle of the neutrophilic granulocytes. Other authors believe that the failure of the neutrophil granules to stain is due to injury rather than aging. This interpretation may be the more correct one as these cells are found most frequently at the thinned-out periphery of the blood film in slides, and more of them occur in poorly made preparations than in good ones. Schweizer found that the variable number of such cells in preparations made from successive drops of blood from the same puncture wound could not be accounted for on the basis of random distribution, but was due to some uncontrollable factor.

Blood for supravital study must always be taken directly from the subject, and never mixed with anticoagulants as they often cause distortion of the morphology of the cells and interfere with staining. The

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* Pinacyanole was obtained from the Research Laboratory, Eastman Kodak Company, Rochester, N. Y.
† In the discussion which followed the presentation of a preliminary note on this work, Dr. Edna Tompkins pointed out that mitochondria can be studied by either method, but that comparisons sometimes lead to difficulty.
properly sized drops can be most easily obtained from a small puncture wound made with a No. 11 Bard-Parker knife, the base of which has been pushed into a cork in order to provide a handle. It is always advisable to make from four to six supravital films, as one can never be sure that any one preparation will be satisfactory. The amount of dye in the dried film on the slide must be varied according to the number of cells per unit volume which are to be studied, as Sabin pointed out. The concentration described above is suitable for white blood counts below 10,000 per cu. mm., twice that concentration is necessary for blood counts ranging from 10,000 to 40,000, and it must be tripled for higher counts. When studying marrow obtained by sternal puncture in human patients, samples should be taken directly from the puncture needle. Preparations should be made with all three concentrations of dye, as the degree of cellularity of the marrow fluid cannot be predicted in any individual. There is great variation in the cellularity of marrow obtained in normal subjects and even in the leukemias one may find all variations from the most extreme hyperplasia to practically acellular marrow. Dried smears should also be made from all punctures, not only in order to have a permanent record, but because the presence of the megaloblastic type of erythropoiesis cannot be detected with any degree of certainty in supravital preparations.

After the supravital preparations have all been made, they are sealed with a low melting point vaseline (Salvolene) to prevent the evaporation of water from the plasma and subsequent crenation of the erythrocytes. This is best done by having the solidified vaseline in a 5 cc. syringe to which is attached a short 23 gage hypodermic needle with the point removed. The vaseline can be forced out of the syringe slowly by pushing in the plunger with the palm of the hand, while running the needle along the edge of the coverslip. Preparations in which the coverslip is inadvertently moved during the sealing process should be discarded.

In the supravital method, as originally used, the microscope and lamp were enclosed within an incubator or warm box, kept at 37.5 C. The use of an illuminating system as described above would necessitate a rather elaborate construction to permit adjustments to the optical system while working. It has been found that cells can be studied just as well at room temperature, if they are first placed in an incubator at 37 C. for twenty minutes. At the end of that time, the cells of normal blood are well stained and ready for study. Longer periods of incubation may be used if necessary. Staining will take place at room temperature, but much more slowly. This makes it possible to transport supravital preparations for some distance. Blood films on supravital slides have been transported 30 miles in cool weather, and were in excellent staining condition on arrival at the laboratory. The cells in a supravital preparation are exposed to all of the unabsorbed dye present in the film, and therefore will continue to stain more and more heavily. When the slides are studied at room temperature the continued staining goes on at a slower rate, thus allowing a longer period for study. As time passes, the vacuoles grow larger, usually new ones appear in the cytoplasm, the mitochondria take on a deeper and deeper blue and finally begin to swell, the nuclei change shape and form trefoils and other bizarre patterns, and eventually most of the cell types become unrecognizable. All observations should therefore be made within an hour and a half of the time the slide is made when the room temperature is 70 F. Motility cannot be studied at room temperature, except during the warmest weather. The neutrophils round up as the slide cools off following its removal from the incubator. The monocytes also withdraw their numerous fine pseudopodia and round up, thus losing the characteristic irregular outline observed when they are motile at body temperature. It has been suggested by Casey and Rosan9 that if it is impossible to study the films at once, they be put into the icebox to delay the staining process. In the writer’s experience, however, preparations kept in the icebox are never quite as satisfactory as those studied immediately, even though great care is taken to warm them up gradually.

The use of warm stage attachments is not recommended as it is impossible to bring the condenser to focus so far above the stage of the microscope.

The intensity of staining of a given cell is controlled by a number of factors: the strength of the stain, the amount of time it is allowed to act, the number of cells per unit area on the slide, the position of the cell in the film (the drop of fluid washes some of the dye film ahead of it as it spreads), so that the center of the drop is less deeply stained than the periphery), the amount of injury and the nature of the cell itself. Minor variations in the thickness of the dye film in various parts of the slide are therefore of no importance.

A frequent criticism of the supravital method is that the colors in the cells are so faint that proper visualization is impossible. The intensity of the colors produced by the dyes used in supravital staining...
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is relatively faint, compared to the brilliance obtainable on dried smears. However, by equipping the laboratory with light-proof window shades, the apparent brilliance of the coloration can be greatly increased, and there should be no difficulty in obtaining sufficient color contrast. The origin of this criticism is most probably the optical system and the kind of light filters used by the critic. For example, with the standard type of medical student’s microscope and a frosted blue glass as a light source, one cannot resolve the mitochondria of human monocytes sufficiently well to distinguish them from the smaller neutral red vacuoles.

CYTOLOGY

The appearance of most of the cells seen during supravital examination of the blood and bone marrow has been well described by Cunningham,22 as well as in the papers already cited. Only certain topics on which the present study yielded new information or on which a change of concept is necessary will be discussed.

TABLE I. — COMPARISON OF POLYNUCLEAR COUNTS IN PERNICIOUS ANEMIA

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<th>Weighted mean</th>
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<td>II</td>
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<td>6</td>
<td>9</td>
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<td></td>
<td>Wright’s</td>
<td>13</td>
<td>24</td>
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<td>Wright’s</td>
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<td>Supravital</td>
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<tr>
<td></td>
<td>Wright’s</td>
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The Neutrophilic Series

Sabin believed that the determination of the Schilling index and Arneth counts could be done more advantageously from dried stained smears than from supravital preparations. This is undoubtedly true if Janus green is employed, for it does not stain the nucleus in nontoxic concentration. Moreover, if the microscope is kept in the warm box the constant movement of the neutrophils makes observation and counting of the nuclear lobes very difficult. However, if Pinacyanole is used, the nuclei stain a bluish purple color and are easy to see. Comparisons of “polynuclear counts”24 of the number of lobes in 100 neutrophils in the peripheral blood of 5 cases of pernicious anemia, made from supravital preparations and from dried smears, are shown in table 1. A portion of the nucleus was not counted as a lobe unless the nuclear membrane on the two sides of the constricted area was fused to form a clearly visible thread. On dried films, nuclear lobes frequently touch each other, and occasionally one finds cells in which parts of the nucleus overlap. This makes accurate counting difficult. In supravital preparations the nuclear lobes never overlap, as the nucleus remains in one plane, and they touch each other only rarely. It can be seen in the table that the weighted mean of the number of lobes
is always higher in the counts made from supravital preparations. This is also seen from differential counts made on normal bone marrow (table 2).* In this table, it will be noted that the 90 per cent central normal range of segmented neutrophils is higher in the supravital counts than in those made from dried smears stained with Wright's stain, whereas in the band neutrophils, the situation is reversed. While making the differential counts on which this table is based, 4,711 cells were counted in these two classes in the smears stained with Wright's stain, of which 1,651 were segmented neutrophils and 3,070 were band forms. On the supravital slides, 4,596 cells were counted in the two classes, of which 1,854 were segmental forms and 2,742 were bands. Counts made from supravital preparations have greater objective accuracy because the individual lobes of the nucleus can be distinguished more easily. As a matter of fact, Schilling indices made from the same dried stained smear in different laboratories are subject to great variation, owing to the difficulty in deciding whether a nucleus whose lobes are pushed close together in the drying process is a segmented form or a band.

Segmented neutrophils show no mitochondria in their cytoplasm with the concentration of dye and the time of incubation ordinarily used in the present work. However, as Cunningham and Tompkins showed, if the cells are somewhat overstained a small number of mitochondria appear in the periphery of the cytoplasm. This is also true of forms younger than the segmented neutrophils. Classification of myelocytes C, metamyelocytes, band and segmented forms of granulocytic cells must therefore be based on the number of specific granules and the shape of the nucleus. Occasionally in a supravital preparation the distinction of myelocytes C and metamyelocytes may be a little arbitrary, for as Sabin pointed out in her first papers on the method, nuclei are more elongated in the living cell than in dried smears.

Cunningham, Sabin and Doan divided the myelocytes into three groups, designated A, B, and C in the order of increasing age and differentiation. Myelocyte C has a full complement of specific granules and only a few mitochondria around the periphery of the cell which may not even stain with the usual concentration of dye. Myelocyte B has less than the full number of specific granules and many mitochondria, which are scattered at random in the cytoplasm outside of the clump of specific granules. The specific granules are grouped close together around a small clear area of cytoplasm. This clear area, which can still be seen in some segmented eosinophils, is usually called the centrosphere, centrosome or cytocentrum, but it seems more likely to the present writer that it has some relation to the Golgi apparatus of the cell.† Around the periphery of the group of specific granules there are

* Table 2 was not included with the manuscript, nor were Dr. Schwind's colleagues able to find it elsewhere among his papers. References to it, however, have been retained, should discussion of some of the data included prove of value. Ed.

† Just why this clear area was originally called the cytocentrum is not obvious. This locus is marked by a rosette of neutral red vacuoles which has a clear center in the myeloblast before specific granules are present. A similar rosette is present in the pronormoblast and in some lymphocytes and monocytes, as well as in many other types of cells. The centriole or cytocentrum (as the writer understands it) is an organoid which takes a prominent part in mitotic cell division, and it seems rather unlikely that the specific granulation would develop around it. In gland cells, the secretory granules are formed near the
a considerable number of vacuoles which vary greatly in size and range in color from an orange yellow to a deep blackish red. These vacuoles were shown in Simpson and Deming's illustrations, but were apparently not noticed by Cunningham, Sabin and Doan. Myeloblasts B are the largest of the granulocytic cells, and are of striking appearance. They are easily identified. They correspond to the promyelocytes seen in dried smears, and there is good correlation between the number of promyelocytes of the dried smears and the myelocytes B found in the supravital slides in duplicate counts made from the same marrows (table 2). These cells show more neutrophilic granules in supravital slides than they do with Romanowsky stains. This was noticed by Cunningham, Sabin and Doan, but it is very difficult to demonstrate objectively. It can be definitely proved only in abnormal bloods and marrows in which the older cells of the series are not present to complicate the comparison, such as the marrow of agranulocytosis in which there is an "arrest" in the promyelocyte stage, or in the marrow and peripheral blood of acute myeloblastic leukemia. Recently, the marrow of a case of agranulocytosis was studied, in which the oldest cells were neutrophilic promyelocytes, which when stained with Wright's stain had an acidophilic spot (the clump of neutrophil granules) about one-fourth the diameter of the cell in size. In the supravital preparations, these cells were myelocytes B, but the number of neutrophil granules was very much greater, and the cells appeared to have about half of their mature number. Similar observations have been made on 3 cases of acute "myeloblastic" leukemia in which the predominant cells were actually very early promyelocytes.

Myelocyte A was defined by Cunningham, Sabin and Doan as containing "a few, not more than 10, neutral red granules clumped together in the cytoplasm." Myelocyte A should be redefined as a myeloid cell containing a small clump of specific granules which can be definitely identified as such surrounding the cytocentrum, in which the total diameter of the clump is not greater than three times the diameter of the cytocentrum contained within it. The specific granules do not first form at the periphery of the clump, as Simpson and Deming claim, but in the center of the future clump, next to the cytocentrum. The vacuoles around the periphery of the clump of neutrophil granules disappear as the cell matures from a late myelocyte B to the next stage, myelocyte C.

The exact relations of the developing specific granules and the neutral red vacuoles which surround the cytocentrum in myelocyte A could be studied to much greater advantage in the eosinophilic line of cells than in the neutrophils. However, eosinophilic myelocytes A occur so seldom, even in diseases in which there is a pronounced eosinophilia, that not enough observations have been made to warrant a statement in this line of cells. In the neutrophilic series, the problem is complicated by the fact that the first neutrophil granules have an almost identical coloration with the vacuoles which surround them at the cytocentrum. Some of the cell...
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vacuoles, when they first appear, are just as small as the neutrophil granules. In eosinophilic myelocytes B, the specific granules next to the cytocentrum are somewhat smaller than those lying farther peripherally. If the same thing is true of neutrophilic cells, as seems likely, it explains the difficulty of visualizing the early granules, for the mature granules are so small they are almost at the lower limit of vision. It has also been found that the slides must be incubated for half an hour instead of the customary twenty minutes if all of the early neutrophilic granules are to be stained. One neutrophil granule cannot invariably be distinguished from one tiny neutral red vacuole, but when a sufficient number of them are present, they can be distinguished from most vacuoles without too much difficulty. The process of development of the neutrophil granules has been studied intensively in 2 cases of acute myeloblastic leukemia, the details of which will be reported in another paper. In these cases, differential cell counts were made with Wright's stain, supravital staining, and the peroxidase method. In the great majority of cases, a rosette of neutral red vacuoles is present in the myeloblast before any neutrophilic granules can be seen. When the latter appear, they are first found next to the cytocentrum, inside the rosette. No cell was counted as a myelocyte A unless the neutrophilic granules could definitely be distinguished from small vacuoles. The number of such cells in the counts was always considerably higher than the number of early myeloid cells having an acidophilic spot with Wright's stain (the first evidence of the formation of specific granules), showing again that the neutrophilic granules can be stained earlier with neutral red than they can on dried smears. It also shows that some cells which on Romanowsky preparations would be called myeloblasts and leukoblasts from their nuclear pattern and which, moreover, have a homogeneously basophilic cytoplasm are, as a matter of fact, promyelocytes in which the neutrophilic granules have not stained in the dried smears. Myelocytes A are therefore cells which are usually classified as myeloblasts on Romanowsky preparations. The number of peroxidase-positive cells in the blood of these 2 cases was almost entirely accounted for by cells present in the blood which are known to be peroxidase positive. Myelocytes A must therefore be peroxidase negative instead of positive as had been supposed. A preliminary note has been published of some of these observations.

Cells of the myeloid series which are too young to contain specific granules should be referred to as myeloblasts when studied with supravital staining. This term therefore includes the myeloblasts and leukoblasts seen on dried smears. Most of the supravital myeloblasts, as was shown by Simpson and Deming and by Hall contain rosettes of neutral red vacuoles, as well as other neutral red vacuoles scattered in the cytoplasm. These scattered vacuoles seem to be of two types, one staining a deep port wine red and the other an orange yellow. The deep red vacuoles are not merely a later stage in the staining process of the orange colored ones, for they are of a deep color when they first appear. Some cells contain only the rosette, others only the scattered vacuoles. Some myeloblasts contain no neutral red vacuoles at all, and never develop them even after prolonged staining. Such cells are in the minority, but are the only ones included in the definition of the myeloblast by Cunningham, Sabin and Doan. There is no characteristic arrangement
of mitochondria in myeloblasts stained with the supravital method which might serve invariably to distinguish them from the larger cells of the lymphocytic line. As a rule, myeloblasts and large lymphocytes are sufficiently different in appearance so that they can be properly identified. One case of acute leukopenic myeloblastic leukemia came under observation, however (Case 311), in which an erroneous diagnosis of lymphatic leukemia might have been made from supravital studies on the peripheral blood, had a sternal puncture not been done.

The indistinct nature of the nuclear chromatin in supravital preparations is a great handicap in attempts to study the derivation of the myeloblast. No cells were seen in the course of this study which the author would feel confident to say were "pre-myeloblasts" or "primitive cells," as described by Cunningham, Sabin and Doan. From what is known from dried smears, one would expect a primitive cell to have a leptochromatic nucleus instead of an extremely pachychromatic one. The differences in the appearance of primitive cells and lymphocytes in supravital preparations are not obvious to the present writer. This does not necessarily imply that "primitive cells" are nonexistent, as most of them were described from simplified marrows, with which he has had no experience. The appearance of reticulum cells, primitive cells and premyeloblasts in supravital preparations is in need of further study.

**Monocytes**

When studied by the technic here recommended, practically all of the monocytes are rounded up, since they withdraw their numerous fine pseudopodia as the slide cools off to room temperature. They therefore have a more definite cell wall than when studied in the warm box. In the few cases of monocytic leukemia which were studied, most of the cells kept their pseudopodia extended. The cytoplasm has a pale greenish yellow coloration, but the "ground glass" appearance usually ascribed to it has never been seen by the present writer. The mitochondria of the monocytes are slender, short rods, much smaller than those of lymphocytes, myeloblasts or myelocytes A and B. The identification of monocytes is accomplished by the peculiar color of the cytoplasm, the character of the mitochondria, and the number and color of the neutral red vacuoles contained in the cytoplasm.

Monocytes contain a large but variable number of neutral red vacuoles, which may or may not form a rosette. Human monocytes usually do not show rosettes unless the staining time has been prolonged. Those of the rabbit's blood almost always show a rosette. The neutral red vacuoles of monocytes stain a salmon brown color, and differ considerably in this respect from most of the vacuoles seen in lymphocytes, which range from a light yellow to a deep red. Too much emphasis was placed on the rosette as a means of identification of monocytes in the early papers on the use of the supravital method. Many types of cells contain rosettes of neutral red vacuoles.10

Bloom20 found that in normal rabbits, most of the monocytes of the circulating blood could be sharply separated from lymphocytes when studied with supravital staining. A complete series of transition forms between the two types of cells is present, however, in the blood of animals infected with B. monocytogenes. Transi-
tion forms were also present on dried stained smears. Bloom concluded that monocytes were developed from lymphocytes in the blood stream.

In the peripheral blood of the white rat, Bloom found that monocytes and lymphocytes could not be distinguished from one another in supravital preparations. More than half of the lymphocytes have a rosette, and those not having one contain many more neutral red vacuoles in the cytoplasm than are found in other species. Many of the lymphocytes of this species are quite large, so that there is no size difference to aid in differentiating these two types of cells. The present writer has also found that in the albino rat there is little if any difference in the size of the mitochondria of lymphocytes and monocytes, as there is in human cells, and agrees with Bloom that in the rat these cells cannot be identified with certainty with the supravital method.

During the present study there was no difficulty in identifying monocytes or in distinguishing them from lymphocytes in human material. Duplicate counts of peripheral blood from dried smears and from supravital preparations were always in agreement. In bone marrow obtained by sternal puncture, however, a slightly higher number of monocytes (table 2) was found on the supravital preparations than on the dried smears. This was most likely due to misclassifying some of the monocytes as leukoblasts on the smears. In making differential counts from marrow smears, every cell encountered must be classified, and occasionally individual cells are not as well preserved or as well stained as would be ideally desirable.

The study of the younger stages of the development of monocytes in supravital preparations is attended by difficulties similar to the study of such cells on dried stained smears. In the younger monocytic cells the nucleus is rounded in shape, the number of neutral red vacuoles is decreased, and the mitochondria are somewhat larger. There is no way, with the present technic at any rate, of distinguishing them morphologically from some large lymphocytes or from myeloblasts. Relatively undifferentiated cells of the monocytic line are identified as monoblasts only because they are in association with older, undoubtedly monocytic cells. In Romanowsky preparations, as is well known, a similar situation pertains: the younger cells do not have as many azure granules, the nucleus is round, the chromatin network finer, and the cells are indistinguishable from the myeloblasts of the bone marrow or the lymphoidocytes of the lymph nodes. As far as the objective facts, obtained from microscopic study, are concerned, supravital staining offers no more evidence of a separate stem cell for the monocyte than does the study of dried stained smears.

**Macrophages (Reticulum Cells)**

These cells were studied only as they occurred in marrow films obtained by sternal puncture from human patients, and in the peripheral blood of cases of erythroblastosis foetalis. Because of the large number of neutral red vacuoles of varying color and the phagocytized debris which they contain, these cells are the most conspicuous cells found in supravital preparations. They are much more conspicuous than in dried stained films, where the debris is not nearly as well seen and the vacuolar system not evident. More of them are found in supravital preparations:
than in dried films made of the same marrow, probably because many of them are broken up in pulling the smear. In marrow from hemolytic anemias it is common to find macrophages which have ingested considerable numbers of red blood cells, sometimes as many as seven. In dried films made from the same marrow the macrophages contain few if any ingested red cells. It is no exaggeration to say that our concepts of the nature of this cell type would be almost completely erroneous if they had been studied only from dried stained smears and sections.

The supravital method (in the present writer's opinion) offers no evidence that macrophages and monocytes are separate cell types, as was deduced from supravital studies by Sabin, Doan and Cunningham. Monocytes and active macrophages have even fewer resemblances when seen in supravital preparations than they do on dried smears, but when the proper type of material is studied, all stages intermediate in structure can be observed. This has been done in studies of the blood of severe erythroblastosis foetalis, in which there is an enormous destruction of erythrocytes. Every stage in transition from ordinary monocytes to very large macrophages can be found relatively easily in the more severe cases of this disease. Recently, in one case in which the infant died during the second postnatal day, all the transitional stages shown in Masugi's Plate XIII, figures 1-11, could be found within fifteen adjacent microscopic fields on the same slide. It might be added that all the transition forms and the active macrophages are found to contain mitochondria when Pinacyanole is used as the mitochondrial stain, although according to the literature this is not true when they are stained with Janus green.

Lymphocytes

The cytoplasm of lymphocytes, as previously described by many workers, varies in color from a faint yellow to a complete lack of any coloration. Cells which have yellowish cytoplasm in supravital or unstained fresh preparations have intensely basophilic cytoplasm in Romanowsky preparations. The mitochondria are large rods and granules, but have no characteristic arrangement. The heavy blocks of chromatin in the nucleus can usually be seen.

A normal lymphocyte was said by Simpson to have not more than eight neutral red vacuoles. Cunningham, Sabin and Doan and Cunningham and Tompkins agree that lymphocytes contain a very small number of neutral red staining bodies. This statement is true if only the lymphocytes of normal peripheral blood are studied. The large lymphocytes of infectious mononucleosis, for example, may contain from fifteen to twenty neutral red vacuoles in their rosettes. In the bone marrow of patients with agranulocytosis, large lymphocytes are occasionally found which may have more than thirty neutral red vacuoles. The present writer finds more neutral red vacuoles in the larger varieties of lymphocytes than are shown in the plates of Cunningham and Tompkins and Tompkins and Cunningham, possibly as a result of not using Janus green, which is said to antagonize staining with neutral red.

* It is also possible that Pinacyanole causes the formation of more vacuoles than does Janus green.
infections and toxemias and following drug therapy are for the most part unknown at the present time.

The lymphocytes of infectious mononucleosis have a characteristic appearance. These cells are of large or intermediate size and their cytoplasm has a faint yellow tint. The nucleus, which may appear round on dried smears, is almost always bean shaped. The mitochondria are large rods or granules, and in the larger cells sometimes appear to be bigger than normal. There is a rosette of neutral red vacuoles in the bay of the nucleus which, during the first hour of staining, is usually a light yellow color, but which subsequently in some cells turns a dark red. Gall, in his supravital studies, has pointed out that the large body which stains a scarlet red in the normal small lymphocytes of the blood is absent from the cell typical of infectious mononucleosis. Lymphocytes such as are described above do not occur (or at least occur very rarely) in human subjects past early childhood, and their presence in suspected cases of infectious mononucleosis is sometimes of considerable diagnostic help. Similar cells probably occur in typhoid fever, according to Tompkins and Cunningham, but in the present writer's experience the types of cells present in the lymphocytoses of septic conditions and in infectious lymphocytosis are of quite different appearance as they do not have the rosette and their neutral red vacuoles have a red or orange coloration. Typical cases of infectious mononucleosis are easy to diagnose from the clinical appearance of the patient, the total white blood count, the heterophil antibody test, the differential count and the appearance of the typical cells in the blood smears, but it is sometimes very difficult to distinguish atypical cases from early acute lymphatic leukemia or early Hodgkin's disease. In a number of cases, when the evidence from dried smears was not quite convincing, the use of supravital staining has made possible a more confident diagnosis.

In some cases of acute lymphatic leukemia there is also a qualitative change in the lymphocytes. The number of neutral red vacuoles is greatly increased and many of them are abnormally large for a given time of staining. Many of them stain a deep blackish red. The mitochondria are much smaller than normal and in some cells are dustlike in appearance. They have a tendency to clump together near the nucleus. The nuclei of these cells frequently take on bizarre shapes, such as trefoils and quatrefoils, within twenty minutes of the time the slide is made, while in most normal cells such changes in nuclear morphology do not take place for a considerable time after incubation. These qualitative changes in the lymphocytes will enable one to make, on rare occasions, a diagnosis of acute leukemia from supravital slides before any forms containing nucleoli are found to substantiate such a diagnosis from a dried stained smear. Unfortunately, not all cases of acute lymphatic leukemia show these changes. Of the 8 cases studied, 4 did not show them; in these the size and distribution of the mitochondria was normal. In 3 of them, the number, staining and size of the neutral red vacuoles was normal; in the fourth case there was a reduction in the number of vacuoles, while the size and staining were normal. In one case, the nuclei quickly took on bizarre shapes, but in the other 3 they remained rounded or bean shaped throughout the period of observa-
tion. Potter and Ward\textsuperscript{4} found that lymphatic leukemia of mice is not characterized by any qualitative changes in the lymphocytes, the larger younger cells having merely a greater number of mitochondria than the smaller, more mature ones. Qualitative changes in the cytology of the lymphocytes resembling those of acute lymphatic leukemia were also seen in one case of Hodgkin's disease.\textsuperscript{12} The absence of qualitative change in the morphology of the lymphocytes cannot be depended on to rule out this disease in differential diagnosis, and such cases must therefore be diagnosed from dried stained smears by the presence of lymphoblasts which contain nucleoli.

Cunningham, Sabin and Doan\textsuperscript{5} defined a lymphoblast as a cell of the lymphocytic series which contained no neutral red vacuoles. This definition must be considered as being faulty as there are occasionally small lymphocytes in the peripheral blood, which, while they are undoubtedly mature forms, do not have any bodies staining with neutral red in the cytoplasm. Scrapings of lymph nodes show many more such cells, but on dried smears they are found not to contain nucleoli, and therefore should be called lymphocytes instead of lymphoblasts. Conversely, in acute lymphatic leukemia one finds many cells having nucleoli which also contain neutral red vacuoles. The above mentioned authors judge the relative age of a lymphocyte by the number of mitochondria it contains. A detailed restudy of the development and maturation of the lymphocytic series of cells with the supravital method is greatly needed.

**Plasma Cells and Myeloma Cells**

Human plasma cells usually have a faint yellowish cytoplasm, although in some cells the cytoplasm is grey in color. They contain many orange staining neutral red vacuoles which lie near the eccentric nucleus and therefore in the geometric center of the cell. A rosette is not always present, but the vacuoles are arranged in a demilune, and are apparently the cause of the perinuclear light area seen in dried smears and in sections. Most of the mitochondria, which are large like those of lymphocytes, lie scattered around the periphery of the demilune of vacuoles, but the outer rim of cytoplasm is empty. Heavy blocks of chromatin can be seen in the nucleus.

Typical myeloma cells are of the same size and shape as plasma cells, but their appearance is much different. The faintly yellow cytoplasm is completely filled with scattered rod shaped mitochondria, and most of them contain no neutral red vacuoles. Some cells contain a small number of small, darkly staining vacuoles. Of the 3 cases studied from sternal puncture, only one showed transitional forms between myeloma cells and plasma cells. Beizer, Hall and Giffin\textsuperscript{42} also found transitional forms between these two cell types in one of ten cases.

**Lymphosarcoma Cells**

Two per cent of the cells in the peripheral blood of a case of lymphosarcoma, diagnosed from a biopsied lymph node, were abnormal. On dried smears they were 13 micra in diameter, with a scanty rim of moderately basophilic cytoplasm and a finely leptomchromatic nucleus. In supravital preparations they were usually about 16 micra in diameter, with a rim of light grey cytoplasm about 1
micron wide surrounding the nucleus. The otherwise round nucleus was usually indented slightly at one place. Most of the mitochondria were moderately coarse granules, somewhat smaller than those found in normal small lymphocytes. There were less than 20 neutral red vacuoles which varied in size from being just visible to 2 micra in diameter, and which varied in color from orange to deep red. In a few cells there were large clumps of material which stained like the mitochondria, but which were sometimes 2 micra in diameter. Such masses of mitochondrial material have not been previously reported and their significance is unknown. The lymphosarcoma cells of this case differed greatly in their appearance from those previously reported from supravital studies by Wiseman and Tompkins and Cunningham. These cells evidently vary more widely in appearance on supravital slides than has been supposed.

Erythropoiesis

A great deal of confusion has resulted from the supravital studies of Doan, Cunningham and Sabin on the origin and maturation of the red blood cells. These authors divided the nucleated red blood cells into three groups: megaloblasts, erythroblasts and normoblasts, in the order of their increasing age and differentiation. They defined a megaloblast as the first generation of red blood cells, and applied the definition to erythropoiesis in the embryo as well as in adult life. Most clinical hematologists since Ehrlich’s time have regarded megaloblasts as a separate line of red cells present in the marrow in cases of pernicious anemia, which gives rise to the large sized non-nucleated red cells characteristic of the disease, and which never occurs in normal marrow stimulated to erythropoiesis by hemorrhage. As will be shown later, the cells from the yolk sac and the earliest cells in bone marrow do not closely resemble one another in properly stained supravital preparations. The definition of Doan, Cunningham and Sabin has been uncritically accepted by many American and by some English authors. Continental investigators as well as some of the Americans and English with whom the present writer agrees, regard this view of the megaloblast as a misconception. As Scott has pointed out, this problem is not a quibble in terminology without importance or interest, for upon it depends the concept and interpretation of the pathology of pernicious and the allied anemias.

Ehrlich pointed out that the first red cells present in the embryonic circulation are identical with some of the megaloblastic cells found in the blood and bone marrow of pernicious anemia patients. These cells, which are probably best termed as “primitive erythroblasts,” bear a remarkable resemblance to pernicious anemia megaloblasts. Although they differ from the latter cells in certain chemical and physiologic ways, and there are minor cytologic differences in their maturation process, many primitive erythroblasts are indistinguishable from similar stages of erythropoiesis in the marrow of pernicious anemia.

Doan, Cunningham and Sabin, defined a megaloblast as the first generation of developing red cells. As morphologic criteria for the identification of this cell they seem to have depended mostly on the uniform, granular precipitation of neutral red in the “basophilic substance” of the cytoplasm and on the rapid fading of Janus
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green from the large mitochondria, as well as on the "faintest possible trace of hemoglobin in the cytoplasm." The phenomena of neutral red staining of developing red cells were, however, inadequately studied in their material. Such cells, stained with neutral red and Janus green, are shown in their Plate V, but it will be noticed that neutral red vacuoles are shown in only one cell. All erythroblastic cells contain neutral red vacuoles when exposed to proper concentrations of the dye and the earlier ones in mammalian marrow may contain a large number—80 or more, some of which may form a very conspicuous rosette. The failure of these authors to notice them can be explained only by the assumption that the concentration of neutral red in their marrow preparations was insufficient to stain the vacuoles. Some of their marrow preparations were made by flattening a sample, obtained by trephine, beneath the coverslip, and it is quite possible that in the presence of such a large number of cells the concentration of neutral red was inadequate. This would not explain their absence from the cells shown in their figures 44 and 45 which were obtained by puncture of the marrow cavity, and which, in the present writer’s experience, should have had the largest number of vacuoles.

The uniform precipitation of neutral red in the "basophilic substance" of the cytoplasm, which was taken as the outstanding characteristic of the megaloblast, is a quantitative staining phenomenon which is not entirely specific for developing red cells. It is true, as the above authors state, that in blood obtained by cardiac puncture from chick embryos of 40-48 hours of incubation, practically all the red blood cells are filled with precipitated neutral red when concentrations of dye such as are used for normal human blood are present on the slide. However, by decreasing the amount of neutral red, it is possible to have the neutral red vacuoles and the mitochondria of all the cells stained, without any precipitate being present. This precipitation of neutral red can also be seen in the developing erythrocytic series in human marrow, but the writer regards such portions of his supravital preparations as overstained. It is possible to stain the neutral red vacuoles and mitochondria of such cells, as well as the neutral red vacuoles, specific granules and mitochondria of all the other cells present in the marrow without causing any precipitate. Neutral red will precipitate in the cytoplasm of early red cells more quickly in a given concentration of dye than in other cells, but by increasing the concentration a similar precipitate can be caused to form in the plasma cells of human marrow, and the intermediate lymphocytes of the guinea pig’s blood. It was also observed in atypical lymphocytes present in the blood of a case of Hodgkin’s disease, where it occurred in the concentration of dye ordinarily used for peripheral blood studies. The precipitation of neutral red in the cytoplasm is therefore a rather nonspecific quantitative staining phenomenon, and, when it is recalled from the section of the present paper dealing with staining how many factors enter into the depth of staining achieved in a given cell under observation, such precipitation is obviously useless as a criterion for the identification of any one cell type.

The fading of the mitochondria when studied with Janus green, which was also considered to be a characteristic of the megaloblast, does not occur if the cells are
stained with Pinacyanole, and would in any case not be a very helpful criterion. A fading of the stain from the mitochondria, even when stained with Pinacyanole, was observed in the atypical lymphocytes of the case of Hodgkin's disease mentioned in the previous paragraph. These cells, although they were definitely lymphocytes when studied on dried smears, therefore fulfilled all of the conditions necessary to classify them as 'megaloblasts.'

According to the writer's observations, the earliest cells of normal marrow which can be definitely called developing red cells when stained supravitally are large, measuring 2.0–2.5 micra in diameter, and correspond to the pronormoblast stage of the Romanowsky technics. These cells have a thin nuclear membrane and very finely divided chromatin in which one or more nucleoli can usually be observed. The cytoplasm has a faint but very definite yellow color. Most myeloblasts have a colorless cytoplasm in supravital preparations, though a few also have a yellowish coloration. The cytoplasm usually contains a clump of dark orange red neutral red vacuoles, which are usually not larger than one-half micron in diameter, which are placed very close together, and form a very-conspicuous rosette near the nucleus. In addition there may be other neutral red vacuoles, sometimes 20–30 in number, which lie about the periphery of the rosette or are attached to the nuclear membrane all over its outer surface. Some cells do not have a rosette but merely have the neutral red vacuoles attached to the nuclear membrane. At present it is not known if these cells are more primitive than the rosette-containing pronormoblasts as they may well be, for the younger myeloblasts also have no rosettes. The mitochondria are short, coarse rods or granules, and are very numerous. They are limited in the distribution to the area immediately surrounding the nuclear membrane or sometimes merely surrounding the rosette, instead of being scattered throughout the cytoplasm as they are in the myeloblast. Neutral red does not precipitate in the cytoplasm unless the preparation is overstained.

As these cells mature to the stage called the basophilic normoblast on Romanowsky preparations, the nucleus becomes relatively smaller, the nucleoli disappear, mitochondria are less numerous and more slender in appearance, and in some cells appear to have increased in length. Practically all of the mitochondria now lie in the vicinity of the rosette, which is also smaller than in the previous stage. Most of the vacuoles which at first are attached to the nuclear membrane have now disappeared. These cells are 15–18 micra in diameter. Subsequently, during the stage called the polychromatic normoblast on dried smears, which is still smaller in diameter, the rosette breaks up, and the vacuoles may scatter in the cytoplasm. The mitochondria are further reduced in size and number and may be either clumped together or scattered. Finally, the nucleus is believed to become pyknotic and ready for extrusion or dissolution in what corresponds to the orthochromatic normoblast stage in smear preparations. In every normal blood one can find non-nucleated erythrocytes containing from one to fifteen neutral red vacuoles which may lie close together or be scattered in the cytoplasm. These cells are reticulocytes, but according to the writer's counts on bloods which had high percentages of reticulocytes when stained with brilliant cresyl blue, they represent only about 40 per cent of
the total number. Some of the non-nucleated erythrocytes which contain neutral red vacuoles show a small number of mitochondria also present. Occasionally one finds an erythrocyte with a few mitochondria, but no neutral red.

The cytoplasm of the pronormoblast when studied in supravital preparations has a faint yellow color. The intensity of this color is no greater than that seen in the cytoplasm of many other cells, such as the lymphocytes of infectious mononucleosis, plasma cells or myeloma cells, and the present writer sees no reason for supposing that any hemoglobin is present. This yellow color apparently occurs in all cells which show an intense basophilism of the cytoplasm with Romanowsky methods. As the cells mature and develop hemoglobin, the yellow color gradually becomes more intense and gradually takes on the characteristic greenish tinge of the erythrocytes which contain their full complement of hemoglobin. In the writer's opinion it is impossible to say just when the first hemoglobin appears, as there is no sharp color change. The hemoglobin develops homogeneously throughout the whole cytoplasm, and does not appear in a crescent at the edge of the cell where there is no precipitate of neutral red, as described by Doan, Cunningham and Sabin in the erythroblasts of the 3 day chick embryo. This crescent is merely the cytoplasm of the older cells in which no precipitation has occurred. It does contain hemoglobin, because, as has been already mentioned, all the cells of the chick embryo contain hemoglobin as soon as they are free in the circulation. The disappearance of the rosette, the reduction in the number of neutral red vacuoles and mitochondria and the gradual deepening of the yellow color of the cytoplasm as hemoglobin is developed in it are so variable that only three stages in erythropoiesis can be conveniently and definitely distinguished. These are (1) the earliest stage, a large cell with a rosette and additional neutral red vacuoles and many mitochondria, here called the pronormoblast and presumably being the cell called the megaloblast in adult marrow by Doan, Cunningham and Sabin; (2) the most mature nucleated red cells, with a small number of neutral red vacuoles and scanty mitochondria, which is filled with hemoglobin and in which the nucleus is becoming pyknotic, called simply the normoblast by the above authors, and corresponding to the orthochromatic normoblast of dried smears; (3) all the intermediate stages, which were called by these authors the erythroblasts, and which would include the basophilic normoblasts, polychromatophilic normoblasts and possibly some of the younger orthochromatic normoblasts of dried stained preparations. The process of hemoglobinization is harder to follow in the supravital preparations where it is manifested only by the deepening of a faint yellow color than in Romanowsky preparations where it shows itself by the change in cytoplasmic coloration from deep blue to an orange red, and does not lend itself as well to arbitrary staging. Fine nuclear detail cannot be studied on supravital preparations, even though the nucleus is stained with Pinacyanole. This also makes it more difficult to judge accurately the relative age of a given cell.

The megaloblastic line of cells such as occurs in pernicious and the allied anemias was studied carefully to see if they differed in any way from the normoblastic cells found in normal marrow. As has been previously reported, the appearance of the two lines of cells is identical in supravital slides, except that the megalob-
blastic cells are larger in corresponding stages. Occasionally one can see that the nuclear chromatin is finer than in the corresponding stage of the normoblastic line, but supravital preparations are so unsatisfactory for the study of nuclear detail that no confidence can be placed in the observation. Size is also a poor criterion, for there is no way to distinguish a megaloblastic cell which has just divided and is therefore somewhat smaller than normal from a normoblastic one which is about to divide and is therefore larger than usual. The identification of megaloblastic cells in supravital preparations was tested in a number of cases of anemia in which the diagnosis was difficult to make. Sternal marrow puncture was necessary in order to make a diagnosis, and supravital preparations made from the marrow were studied before any of the dried stained smears were examined, in order to avoid any mental bias. In several cases the writer had convinced himself that the predominant strain of cells were megaloblasts, only to find on the dried smears that only normoblasts were present. The true megaloblastic cells and the normoblastic line usually cannot be distinguished in supravital preparations, and the supravital method must be taken to be unsatisfactory for diagnostic work involving the erythroid cells. Diagnostic work in the anemias should be done only from dried, Romanowsky-stained smears.

Doan, following his concept that the megaloblast is the earliest stage in the process of erythropoiesis, regards pernicious anemia as a maturation arrest in the earliest stage of the process and has proposed that the erythropoietic substance present in liver extract be called the "erythrocyte maturing factor." There is no real reason, however, for believing that the low red cell count in addisonian pernicious anemia is due to a maturation arrest in the sense that the low white count in agranulocytosis is sometimes said to be due to a "maturation arrest," by which is meant that no cells beyond a certain stage of development are being produced in the marrow. What actually happens in agranulocytosis seems to be that the etiologic agent destroys the whole neutrophil series, and by the time the sternal puncture is done the marrow is recovering and neutrophils have regenerated as far as the stage at which the marrow is apparently "arrested." The bone marrow of agranulocytosis at the height of the disease is hypoplastic, whereas in most cases of pernicious anemia it is hyperplastic. Differential counts from dried smears of pernicious anemia marrow show that the percentage of the earliest stage in red cell production (promegaloblast) may be no greater in this disease than in marrows obtained from severe hemolytic anemias or following severe hemorrhage (pronormoblasts). This is also shown in tables of differential counts by Scott. There is no cytologic evidence that there is any maturation arrest of the abnormal megaloblastic series of cells, for all stages up to non-nucleated megalocytes are present (Jones). When studied on dried smears, there is certainly no great increase of the earliest erythroid cells found in normal marrow. The action of liver extract seems to be to allow the dysplastic megaloblastic series of cells to be replaced by the normoblastic line. In the rare "refractory" anemias of the aregenerative type, of which the author has seen 2 cases, the youngest erythroid cells greatly outnumber the more mature types, but liver extract is ineffective. It is difficult to see how anyone accepting Doan's definition of a megaloblast can distinguish the marrow of
pernicious anemia from that of ordinary hemolytic anemia, erythroblastosis fetalis or posthemorrhagic states.

Doan, Cunningham and Sabin believed that the red cell line develops from endothelium in the area vasculosa of the embryo and the intersinusoidal capillaries of the bone marrow of the adult. There is no doubt that this is true in avian and mammalian embryos and in adult birds, but their evidence, which was obtained from sectioned material, in adult mammals is somewhat less convincing. In Romanowsky smears, as is well known, it is possible to find stages intermediate in nuclear structure between myeloblasts and pronormoblasts or promegaloblasts, if the latter happen to be present. The number of these is not as great, however, as would be expected, and Schleicher has recently adduced good evidence that the erythrogenic line may come directly from reticulum without going through a myeloblast stage. In supravital preparations, cells intermediate in cytoplasmic structure can also occasionally be found. Such cells have a yellowish cytoplasm, the mitochondrial pattern may be that of the pronormoblast or may closely resemble that of the myeloblast, but the rosette of neutral red vacuoles is absent, and only a few other neutral red vacuoles are present. Such cells are intermediate in structure between the white and red cell lines, but so few of them have been studied that a definite statement as to their significance must be reserved until further studies can be made.

**Differential Counting**

It is frequently said that differential counts can not be made accurately from supravital preparations. Such statements are without foundation in fact, provided that the supravital method is properly used. In most instances there is no significant difference between counts made with this method and those made from Romanowsky preparations. In certain bloods, such as those obtained in the acute leukemias, when large numbers of unusually fragile cells are present, differential counts made from supravital preparations are more accurate, as fewer cells are damaged in making the preparation.

**Discussion**

In Downey's "Handbook of Hematology," the two articles on the supravital method are followed by Hall's article, which was intended to be an evaluation of its use. However, because of technical factors which have been discussed in the section of this paper dealing with illumination, the method was a complete failure in his hands. He was unable to differentiate monocytes, large lymphocytes, and leukoblasts, and could see no difference between some large lymphocytes and proerythroblasts (pronormoblasts). He was unable, using the supravital method, to separate the three types of acute leukemia. Accurate differential counting was difficult at the best of times, and, when the cells mentioned above occurred in the slide, impossible. Because of the prominent position given to this article in the "Handbook," and the almost completely erroneous impression of the supravital method which it gives, it is felt that his objections to it should be discussed in some detail.
JOSEPH L. SCHWIND

Hall felt very strongly that supravital staining would never replace the stained dried smear method as a routine procedure in clinical laboratories. In this viewpoint, he was quite correct, for the average clinical laboratory is unable to obtain dependable results with the most simple type of technic. The supravital method can be used as a routine method if it seems desirable to do so, though it is somewhat more cumbersome. It is used routinely in Doan's laboratory.

Hall lists eight disadvantages of supravital staining, not all of which are valid criticisms. His objections to the method, with appropriate comment by the present writer, follow:

1. Supravital films are not permanent, and must be examined as soon as they are made. They cannot be saved for reference. This is indeed a great disadvantage, but it is more of a nuisance than a fact which disqualifies supravital staining as a technic of investigation.

2. The nucleus does not stain. On dried smears, the discrimination between monocytes and leukoblasts or between lymphocytes and proerythroblasts (pronormoblasts) may depend entirely on the nuclear pattern of the cell. These cells can be differentiated from each other in supravital preparations by a competent observer by means of the arrangement, number and color of the neutral red vacuoles and the number, size and arrangement of the mitochondria without reference to the nucleus. Moreover, it will be recalled that if Pinacyanole is used, the nucleus will stain, and that counts of the number of lobes in the nucleus made from supravital preparations have more objective accuracy than those made from dried smears. More important is the fact that the failure to stain the nuclear chromatin sufficiently well for close study completely vitiates the supravital method for diagnostic work on the anemias, because it is impossible to distinguish normoblastic from megaloblastic developing red cells.

3. The failure of the cytoplasm to stain is a decided disadvantage. This is a disadvantage only in trying to stage the red cell line into basophilic, polychromatic and orthochromatic forms as is done on dried smears. The very gradual development of hemoglobin in the cytoplasm makes it impossible to find similar stages in supravital preparations, as has already been pointed out, but in other cells the lack of cytoplasmic staining does not make the slightest difference. Basophilic stippling of the erythrocytes cannot be seen in supravital preparations, it is true, but the significance of this phenomenon is by no means clear.

4. Qualitative changes in the appearance of the neutrophil granules such as toxic granulations and the larger brick red granules seen in the neutrophils of pernicious anemia are not evident in supravital preparations. Cells which show toxic granules on dried smears show an increased number of neutral red vacuoles in supravital preparations,23 and, moreover, since the advent of sulfonamide drugs and penicillin, toxic granules have lost a great deal of their prognostic significance. No difference between normal neutrophil granules and those in pernicious anemia could be seen in supravital preparations during the course of this study. The large brick red granulation seen on dried smears in this disease is not a criterion on which too much reliance can be placed in making a diagnosis. Polynuclear counts or the demonstration of megaloblastic cells by sternal puncture are far more satisfactory diagnostic criteria.

5. Nothing is known of the appearance of azure granulation on supravital preparations. This criticism is rather beside the point, since cells which contain it on dried smears can be identified by other means in supravital preparations. Moreover, azure granulation is not an unmixed blessing as is shown by the varied terminology applied to azure containing cells by hematologists working from dried stained smears.

6. Parasitic inclusions do not stain for long periods of time and hence may be overlooked. Hall quotes this from Tompkins22 with reference to malarial parasites. The writer found, however, that with the technic recommended in this paper, malaria parasites were well stained at the end of twenty minutes and while not as conspicuous as in dried smears, should certainly not be overlooked. Kurloff bodies in the lymphocytes and monocytes of the guinea pig are, however, much more striking in supravital preparations than they are on stained smears.

7. Hall believed that differential counting on supravital preparations was unsatisfactory. He points out that the cells are not as vividly stained as in Romanowsky preparations and consequently in order to over-
look as few cells as possible in supravital films the count must be made slowly and with extreme care."

By darkening the room by means of light-proof window shades, the apparent brilliance of the colors in supravital slides can be greatly increased and there is no reason for overlooking any cells at all if the preparation is a good one. A slide in which "small lymphocytes devoid of neutral red vacuoles obscured by one or two overlying erythrocytes" such as Hall mentions is improperly made, as the cells should not be overlapping. Lucia and Lucia, working on the guinea pig, claim that the tendency of cells to bunch together in fresh preparations make differential counting difficult. However, there should be no bunching of cells in properly made fresh preparations except in the rarely encountered human individuals in whom autoagglutinins or cold agglutinins are present. The dependability of any laboratory method varies with the technical skill of the person using it. The writer finds that differential counts can be made just as rapidly from supravital preparations as from dried smears. The time necessary to do a differential count is determined by the character of the cells in the smear, and not by the method used. If a sufficient number of very abnormal cells are present, differential counting becomes a very time consuming procedure, even on dried smears. Duplicate differential counts on bone marrow made with the two methods compared very favorably. Epstein and Tompkins found that counts made from supravital preparations and sections of marrow agree very well.

There are several other disadvantages to supravital staining in addition to those mentioned by Hall which greatly limit its usefulness and practicability. The first of these is the expense of adequate microscopic equipment, which precludes its use in any but research laboratories. Secondly, it is necessary to have a knowledge of optics considerably greater than is possessed by the average laboratory worker. Thirdly, proper interpretation of results with the method for clinical use requires a great amount of experience. All in all, the supravital method is far too complicated to be used for routine differential counting in hospital laboratories. For this purpose it offers no advantages over dried smears and in addition has, as was just discussed, a considerable number of grave disadvantages. Because of the rather rudimentary type of equipment which is available and the small number of hours which can be given to it, the present writer regards attempts to teach supravital staining to medical students as wasted.

Some English investigators have felt that supravital staining was of definite advantage in the separation of the types of acute leukemia. The present writer would agree with this only to a very limited degree, and feels that this method is of definite advantage only in rare cases, though the staining of the early neutrophil granules before they can be seen in Romanowsky preparations is occasionally very welcome corroborative evidence in "myeloblastic" leukemia. Its use has some advantage in acute lymphatic leukemia, but as has been pointed out, in about half of the cases there is little or no cytologic change in the leukemic cells. Some cases of acute leukemia, especially when in the leukopenic phase, cannot be satisfactorily identified from studies of the peripheral blood and recourse must be had to sternal marrow puncture.

In the writer's opinion, there is nothing in the cytology of the developing blood cells when studied with the supravital method which would favor a polyphyletic interpretation of hematopoiesis rather than a monophyletic view. Myeloblasts cannot always be distinguished from lymphocytic cells. The early stages in the development of monocytes also cannot be invariably identified. This was admitted by Cunningham, who further stated that any method which would make this
possible would usher in a new era in hematology. Studies of the peripheral blood in erythroblastosis foetalis have convinced the writer that supravital studies do not support the view that monocytes and macrophages are separate cell types. In view of the large amount of evidence, it can no longer be doubted that lymphocytes may also give rise to macrophages. Whether they go through a monocyte-like stage in the process is still an open question, and depends somewhat on how a monocyte is defined. Bloom,31 it will be recalled, found that in rabbits with B. monocytogenes infection there were many transition forms between monocytes and lymphocytes. How commonly such forms occur in other conditions is still to be determined. In supravital preparations one can also occasionally find cells which are intermediate in structure between pronormoblasts or promegaloblasts and myeloblasts, so that in this instance also there is cytologic evidence favoring a monophyletic interpretation. The extreme polyphyletic view held by Sabin, Cunningham and Doan does not of necessity stem from the use of the supravital method. It has been shown in experimental embryology that chemical differentiation precedes structural differentiation, and it may well be that there is more truth in the polyphyletic viewpoint than might be suspected on purely morphologic grounds.

In the research laboratory, the supravital method has a number of uses. Mitochondria can be studied much more easily by this method than on sections. Individual specific granules of the leukocytes stand out against the colorless background of the cytoplasm with much greater definition than in stained preparations. Small differences in degree of maturity in developing granulocytic cells can be detected much more accurately than in smears. Polynuclear counts can be made with greater accuracy in supravital preparations. Macrophages are found in greater numbers than on stained smears and a much better concept of their state of activity is obtained by supravital staining. Occasionally it permits an earlier diagnosis in acute lymphatic leukemia or infectious mononucleosis than would be possible from dried smears. In puzzling cases where it has no advantage over dried smears, it sometimes gives corroborative evidence which enables one to interpret a blood picture with greater confidence. Finally, it should not be forgotten that despite the disadvantages inherent in the method, supravital staining has brought about a tremendous increase in knowledge of the cells of the blood and hematopoietic organs.

Summary

1. Dependable results with supravital staining can be obtained only with apochromatic objectives, compensating oculars, an achromatic condenser, controlled illumination and a transparent daylight filter on the light source.
2. Proper technic is of the utmost importance in making supravital slides.
3. Pinacyanol is preferable to Janus green as a mitochondrial stain.
4. Supravital preparations should be studied in a darkened room to increase the apparent brilliance of the staining.
5. More accurate counts of the number of lobes in the nucleus of neutrophils can be made with the supravital method than from dried stained smears.
6. Myelocytes C in supravital preparations correspond to the myelocytes seen on dried smears, while myelocytes B are the promyelocytes.
7. Myelocyte A has been redefined. The neutrophil granules of these cells do not as a rule stain on dried smears, and these cells are therefore classed as myeloblasts or leukoblasts in the latter type of preparation. They are peroxidase-negative.

8. Myeloblasts may or may not contain neutral red vacuoles, and may or may not contain a rosette.

9. Supravital staining is a good technic for the identification of monocytes from human blood and marrow, and is the method of choice for the study of macrophages.

10. Supravital preparations of the peripheral blood of severe cases of erythroblastosis foetalis show all transition stages between monocytes and macrophages, and there is no reason for regarding these cells as separate types.

11. Lymphocytes contain many more neutral red vacuoles than have been previously described.

12. The lymphocyte of infectious mononucleosis has a characteristic appearance, and can be used as a means of diagnosis.

13. Qualitative changes do not always occur in the lymphocytes of acute lymphatic leukemia.

14. Myeloma cells have a characteristic appearance in supravital preparations.

15. Sarcoma cells in the peripheral blood are more variable in appearance than had been supposed.

16. The precipitation of neutral red in the cytoplasm is a quantitative staining reaction and is not specific for the earliest stages in red cell formation.

17. The appearance of the developing red cells in supravital preparations is described.

18. The megaloblastic line of developing red cells which is present in pernicious anemia cannot be distinguished in supravital preparations from the normoblastic line found in normal marrow or in marrow after hemorrhage or hemolysis.

19. The supravital method therefore cannot be used for diagnostic work involving erythropoiesis.

20. There is no evidence of any kind indicating that a maturation arrest occurs in pernicious anemia.

21. Cells intermediate between pronormoblasts and myeloblasts are occasionally seen in supravital preparations.

22. Differential counts can be made from supravital slides just as accurately as from dried stained smears, and if many fragile cells are present, more accurately.

23. The cytology of the developing blood cells as seen in supravital preparations would support a monophyletic view of hematopoiesis just as well as the polyphyletic theory.

REFERENCES


JOSEPH L. SCHWIND

19 ———: The development of leucocytes, lymphocytes and monocytes from a specific stem-cell in adult tissues. Contributions to Embryology 16: 227–76, 1925.
29 ———: The relationships between lymphocytes, monocytes and plasma cells. Folia haemat. 37: 63–69, 1928.
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THE SUPRAVITAL METHOD IN THE STUDY OF THE CYTOLOGY OF BLOOD AND MARROW CELLS

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