HISTOCHEMICAL METHODS APPLIED TO HEMATOLOGY*

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CHEMICAL cytology deals with the chemical characterization of substances in their natural locations within cells. Its principal aim is to provide a morphologic basis for the understanding of the chemical and functional activities of cells. It attempts to develop histologic staining methods which will have chemical significance and which will characterize and differentiate various kinds of cellular lipids, carbohydrates, proteins, enzymes and inorganic substances. In addition to the use of staining methods, the chemical and physical milieu of cells can be explored by other procedures and technics, for example, by means of polarized light, ultraviolet light, x-ray diffraction spectra and the electron microscope, to name a few. The present investigation involves the application of a number of methods of chemical cytology to the cells of blood and bone marrow. The results illustrate, we hope, how, by contributing to an understanding of the chemical composition and activity of blood cells, chemical histology can advance the subject of hematology.

The chemical cytology of normal blood and bone marrow has been under investigation for some time in this laboratory. Previous papers have concerned the use of various histochemical procedures for the demonstration of lipids, nucleoproteins, glycogen, acid and alkaline phosphatases, and the phenomenon of metachromasia (Wislocki and Dempsey; Wislocki, Bunting and Dempsey1). The present study is a continuation of these lines of investigation. Additional data are presented, including information obtained from a more extensive examination of human blood cells.

The previous observations were carried out mainly on deparaffinized sections of bone marrow. We have now adapted some of the histochemical procedures for use on imprint preparations of bone marrow and on smears of peripheral blood. As a result of these modifications and also from a growing familiarity with the various technics, our previous observations have been considerably extended. The results of the present study will be presented under the headings of lipids, nucleoproteins, metachromasia, phosphatases and glycogen. In addition to the two papers referred to above, a few other existing investigations in this field will be briefly cited or reviewed.

Material and Methods

The material for the present study was obtained from 2 young rhesus monkeys and a number of human subjects.† Blocks of marrow from the femurs of the 2

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† The human material was obtained from the Hematological Laboratory of The Pratt Diagnostic Hospital, Boston, Massachusetts, through the interest and courtesy of Dr. William Dameshek.
monkeys were immersed in various fixatives, and from others touch or imprint preparations made on glass slides were placed in identical fixatives.*

Human material consisted of smears of bone marrow and peripheral blood from normal subjects as well as from patients suffering from pernicious anemia, multiple myeloma, Gaucher’s disease, lymphocytic, myelocytic and monocytic leukemia, and other pathologic conditions. Peripheral blood was obtained by finger puncture, and marrow by aspiration, usually from the sternum.

For the staining of lipids with sudan black B, blocks of marrow and imprints were fixed in 10 per cent neutral formalin. Subsequently, frozen sections and imprints were immersed briefly in 70 per cent alcohol and stained in a saturated solution of sudan black B for one and seven minutes. Smears of peripheral blood and bone marrow were also fixed by immersion in 70 per cent alcohol for a few seconds, or by placing them for five seconds in a mixture of 10 per cent formalin—1 part, and 95 per cent ethyl alcohol—9 parts (Bailiff and Kimbrough†). These preparations were then stained in sudan black B for periods ranging from ten minutes to one hour. They were dipped briefly in 70 per cent alcohol and dried. They could then be counterstained, if desired, with Wright or Wright-Giemsa stain.

Nuclear basophilia was studied by means of the Feulgen reaction following fixation of pieces of bone marrow in Zenker’s fluid. For smears of marrow and blood, fixation in 95 per cent alcohol for thirty seconds was utilized and found to be very satisfactory. Light green was used as a counterstain.

For the study of cytoplasmic basophilia, imprints, smears, and blocks of tissue were fixed in Zenker’s fluid and stained with eosin and methylene blue. To determine whether cytoplasmic basophilia present in some cells might be due to the presence of ribonucleoprotein, control sections were treated with ribonuclease† before staining them. For this purpose, the sections were digested at a temperature of 60 C. for three hours in a 0.1 per cent crystalline ribonuclease solution buffered with sodium barbital to a pH of 6.75.

Another means for the characterization of cellular basophilia consists of measuring the dye-binding capacity of tissue elements photometrically for methylene blue over a range of pH. For this purpose, the tissues were fixed in Zenker’s fluid, imbedded in paraffin, sectioned at 5 micra and mounted on glass slides. After deparaffinization, the sections were stained in methylene blue according to the method described by Dempsey and Singer† and Dempsey, Wislocki and Singer.5 The intensity of staining of various substances was determined by measurements made with a photometer (see fig. 1, Dempsey, Bunting, Singer and Wislocki6). The figures obtained for light absorption were plotted against pH to form a graph relating dye-binding to the acidity of the staining solution.

Staining of metachromatic substances was carried out on smears, imprints and

* We are indebted to Mrs. Edith Herman for her assistance in the preparation of the material by the various technics employed.

† Crystalline ribonuclease was kindly provided by Dr. M. Kunitz of the Rockefeller Institute for Medical Research, Princeton, N. J.
paraffin sections of tissues fixed in 4 per cent basic lead acetate,* according to the
method of Holmgren.7 Sections cut at 4 micra were stained in a 0.5 aqueous solution
of toluidin blue, resulting in a lavender or purple coloration of the metachromatic
components of the tissue. Metachromasia observed following this procedure may
be due to the presence of mucopolysaccharides, ribonucleoproteins or substances of
unknown composition.2 That attributable to ribonucleoprotein can be identified
by exposing control sections to ribonuclease (see above).

For demonstrating the presence of glycogen, smears, imprints and blocks of
tissue were fixed in a solution of absolute alcohol, formalin and picric acid (Ross-
man’s fluid). Sections and imprints were then stained by the Bauer-Feulgen method.
Since glycogen is dissolved by saliva, control sections so exposed were prepared.

Alkaline phosphatase was demonstrated by the Gomori method,9 as modified
by Dempsey and Deane.8 Blocks of tissue, smears and imprints were fixed in chilled
80 per cent alcohol. The sections and imprints were then incubated for three and
six hours in a solution of sodium glycerophosphate at pH 9.4.

LIPIDS

Foreword. Increasing attention is being accorded sudan black B as a histologic
stain for lipids. With this dye, Sheehan11 observed briefly that the leucocytes
of human blood became variously tinged. The neutrophilic leucocytes were filled
with small, deeply stained granules, whereas the larger granules of the eosino-
philic leucocytes appeared to possess merely a surface layer of lipid. The monocytes
usually contained lipid granules, while the small and large lymphocytes were
always quite free. Myelocytes possessed many sudanophilic granules, and myelo-
blasts usually had a small number, while lymphoblasts showed no sudanophilia
at all. McManus,13 studying films of human blood and marrow stained with
sudan black B, reported briefly that the cytoplasm of the neutrophilic leucocytes
was packed with fine sudanophilic granules, whereas the lymphocytes and monocytes
were unstained. Furthermore, granules stained by sudan black B were
reported as being present in the “cells of the late myeloblast series.” Ralph14
presented an extremely brief report on human blood, examined with sudan black
B, to the effect that the granules of neutrophiles, eosinophiles and monocytes
contained phospholipids as well as lipids extractable with acetone. Lymphocytes
and thromboplastids, on the contrary, were said to contain no phospholipids.

Wislocki and Dempsey1 studied bone marrow and peripheral blood of several
young rhesus monkeys in formalin-fixed frozen sections stained with sudan black
B. They verified the fact that both neutrophilic and eosinophilic leucocytes of the
circulating blood show intense black staining of their granules as do also the
corresponding myelocytes of the bone marrow. Lymphocytes of the blood and
tissues did not contain lipid particles, but cells which were diagnosed as being

* Merck’s reagent lead subacetate (Pb(CH2COO)2·Pb(OH)2 mol. wt. 566.52) has been used. The
stock must be protected from exposure to air since combination with carbon dioxide results in an insolu-
ble compound. The 4 per cent solution should be made up freshly before using.
monocytes appeared to have a few black particles in their cytoplasm. In megakaryocytes, minute sudanophilic particles were observed, and in blood platelets similar lipoidal dots were encountered. In a subsequent study, evidence was offered suggesting that the sudanophilic material in the cytoplasm of the megakaryocytes might represent mitochondria.

Bailiff and Kimbrough examined normal and abnormal peripheral human blood stained with sudan black B, followed by May-Grunwald Giemsa stain to aid in the differentiation of cell types. They noted that the granules of the eosinophiles were sudanophilic and possessed unstained, clear centers. Basophilic leucocytes, lymphocytes and monocytes were unstained.

From the previous account it will be noted that there is general agreement regarding the behavior of the granular leucocytes, myelocytes and lymphocytes. Of the monocytes, McManus, as well as Bailiff and Kimbrough, claim that they do not stain, whereas Sheehan, Ralph, and Wislocki and Dempsey describe them as being variously tinged.

Excepting Bailiff and Kimbrough, none of the investigators specifically mentions the reaction of the basophilic leucocytes. Because these elements are relatively scarce and with the technic employed difficult to identify, Wislocki and Dempsey and Wislocki, Bunting and Dempsey turned their attention to tissue mast cells. In formalin-fixed frozen sections of several human organs, including uterine cervix and mammary gland, they observed mast cells in which a portion of the normal complement of granules was stained by sudan black. In contrast to the partial or incomplete staining of the granules of mast cells, the granules of tissue eosinophiles were completely and readily stained in similarly prepared formalin-fixed, frozen sections. Meanwhile, Montagna and Noback have reported that sudan black B reveals stained granules in the mast cells of the rat, when applied to frozen sections of tissues fixed in formal calcium-cadmium.

Observations. The present observations on sudanophilia have been made upon human blood cells from both blood stream and bone marrow. Sudanophilic granules are present in the neutrophilic leucocyte-precursors from the time when specific granules first make their appearance. The sudanophilic granules, staining a grayish brown to black color, are in evidence throughout the series and from their number and size appear to be identical with the specific neutrophilic granulations (fig. 2c). In the myelocyte stage they can be seen overlying the nucleus.

The granules of the eosinophilic leucocytes and of the corresponding myelocytes are sudanophilic. As noted by several investigators, the granules are characterized by a darker, sudanophilic periphery and a clearer, possibly totally unstained, interior (fig. 2a). This peculiarity differentiates their granules from those of the neutrophilic leucocytes.

The basophilic leucocytes in the peripheral blood of several patients with chronic myelogenous leukemia have been examined. Their granules prove to be sudanophilic (fig. 2d), varying in size and ranging in intensity of staining in individual cells from pale gray to deep black. Many of the smaller granules stain deeply, often appearing solidly black, whereas the larger granules are as a rule paler and contain a clear center. In contrast to the basophiles, the granules of
eosinophiles exhibit marked uniformity in regard to depth of staining and size. In the blood of 2 patients in which the monocytes were increased and hence readily identifiable, a variable scattering of sudanophilic cytoplasmic particles was observed (fig. 2f). These varied in size and number from a few faint dots up to a dusting of the cytoplasm with quite evident black particles. Since a counterstain tends to obscure these faint particles, monocytes are best studied without its use.

Fig. 1. Curves illustrating and comparing the dye-binding capacity of the cytoplasm of megakaryocytes, mast cell granules, Nissl substance and the hyaline matrix of cartilage. The several tissues were fixed in Zenker's fluid, sectioned, stained with methylene blue, and their dye-binding capacity determined according to a method presented in detail elsewhere (Dempsey et al.6). The cytoplasm of megakaryocytes fails to stain below pH 4.0, in this respect being identical with characteristic nucleoproteins such as Nissl substance or cell nuclei. The granules of mast cells and the matrix of hyaline cartilage, on the contrary, exhibit very much stronger acid dissociation, so that staining is not abolished even at pH 1.6 (cf. Wislocki and Dempsey1 and Dempsey et al.6). Their signatures are characteristic of sulfate-containing acid mucopolysaccharides.

In human material, sudanophilia was not observed in either megakaryocytes or platelets. In the megakaryocytes and platelets of the monkey, on the contrary, minute sudanophilic dots have been described,1 and from their shape, size and number these appear to be mitochondria.2 In the smears of human material prepared in the manner described, conditions are possibly not so favorable for the staining of these small particles.

We have not encountered lipids in lymphocytes in any of our preparations. Gaucher cells have not been described as being sudanophilic, but they are rich in cerebrosides (Thannhauser38). Because sudan black B appears to have an affinity for a greater variety of lipids than the other sudan dyes currently used in histology, we exposed smears known to contain Gaucher cells to this dye. No sudanophilic
Fig. 2. Individual cells from smears of human bone marrow, fixed in 70 per cent alcohol for a few seconds, stained with sudan black B for 10 minutes, and with the exception of d., counterstained with Wright-Giemsa stain. All cells in figures 2 and 3 were drawn with a X 10 ocular and X 50 objective.

a. An eosinophilic myelocyte containing granules of uniform size, each composed of a sudanophilic shell surrounding an unstained core.

b. Two adult erythrocytes showing a dark tinge, superimposed on their normal color, following exposure to sudan black.

c. Orthochromatic nucleated red blood cell, whose cytoplasm is tinged by sudan black, but not so deeply as adult erythrocytes.

d. Basophilic leucocyte, stained only with sudan black, exemplifying the variation in size of the granules, and their lesser number as compared with eosinophilic granules. Each granule exhibits a dark periphery and light center. The granules in occasional basophilic leucocytes are completely blackened, more especially those of smallest size.

e. Polymorphonuclear neutrophilic leucocyte, revealing sudanophilia of its specific granules.

Fig. 3 (a, b and c). Individual cells from smears of peripheral human blood and bone marrow, fixed for 30 seconds in 95 per cent alcohol, then stained by the Feulgen method which is specific for deoxyribonucleoprotein. Counterstained with light green. (d) A megakaryocyte from human bone marrow, stained by the Bauer-Feulgen technic.

a. Two adult red blood cells, one containing a Howell-Jolly body which is stained red and is Feulgen positive, and three blood platelets which are negative.

b. A polymorphonuclear leucocyte, the nucleus of which is Feulgen positive.

c. A megakaryocyte whose nucleus also is Feulgen positive. It was observed above that blood platelets are Feulgen negative, and it is noteworthy that the cytoplasm of megakaryocytes is also negative.

d. A megakaryocyte from a section of human bone marrow which was fixed in a solution of absolute alcohol, formalin and picric acid, then stained by the Bauer-Feulgen technic and counterstained with light green. Note pinkish-lavender color of cytoplasm and the scattered deeper staining granules in the neighborhood of the nucleus. Unlike the Bauer-Feulgen reaction of true glycogen encountered in the neutrophilic leucocytes, this reaction in the cytoplasm of human megakaryocytes is not prevented by exposure to saliva.
material could be demonstrated, and it must be concluded that, unlike some other phospholipids, cerebrosides have no affinity for sudan black.

We have observed a faint staining of the red blood cells by sudan black. Adult erythrocytes and the antecedent orthochromatic and polychromatic nucleated red blood cells are diffusely and faintly tinged (fig. 2b, c). The more mature the cell stage, and the longer the preparations are exposed to the dye, the darker the cells become. According to Parpart and Dziemian, the structural meshwork (stræmata) of the red blood cells is composed chiefly of lipids and proteins. Among the lipids, phospholipids predominate, while cholesterol makes up the remainder (cf. Höber). Nevertheless, Baker found tests for phospholipids inconclusive in the erythrocytes of the frog and mouse. In view of these considerations, we are not prepared to interpret the observed staining of the erythrocytes by sudan black B.

NUCLEOPROTEINS

Foreword. Two kinds of nucleic acids exist in cells, depending upon the nature of the sugar incorporated into their structure. One, containing a desoxyribose sugar, is called desoxyribonucleic acid, whereas the second contains a ribose sugar and is designated as ribonucleic acid. Nucleic acids are strongly charged compounds and have a strong affinity for basic dyes. Moreover, their basophilia is not destroyed by conjugation with proteins so that the nucleoproteins are also strongly basophilic.

Although nucleoproteins have long been known to display pronounced basophilia and therefore to be among the substances in tissues which react with basic dyes, the positive identification of them and their accurate localization in cells have only recently been accomplished. Three methods have been developed which are of importance in this regard. These are the staining method of Feulgen and Rossenbeck for desoxyribonucleoprotein, the use of ribonuclease developed by Dubos, Brachet and Kunitz, and the utilization by Caspersson of the specific ultraviolet absorption of nucleoproteins. To these should be added a recent method for measuring by photometric means the dye-binding capacity of tissue elements over a range of pH (Dempsey and Singer; Dempsey, Wislocki and Singer).

The Feulgen method consists in the freeing of the aldehyde groups of the desoxyribose sugar by acid hydrolysis followed by the Schiff test for aldehydes. The procedure is apparently specific for desoxyribonucleoprotein (cf. Stowell) and is confined entirely to the nuclei of mammalian cells or to the immediate products of their disintegration or dissolution, as will be shown below. The nucleolus is Feulgen-negative because it is composed of ribonucleoprotein.

The discovery and preparation of an enzyme capable of depolymerizing ribonucleic acid paved the way for the cytologic localization of ribonucleoproteins. Brachet described the ability of ribonuclease to abolish cytoplasmic basophilia in a variety of animal cells, including pancreatic, hepatic and intestinal epithelium, nerve cells and lymphocytes. Wislocki and Dempsey observed the disappearance of cytoplasmic basophilia from erythroblasts, myelocytes, lymphocytes and some other basophilic cells of the marrow following this procedure. More recently, Wislocki, Bunting and Dempsey have noticed a moderate basophilia of
the cytoplasm of megakaryocytes. This basophilia is abolished by treatment with ribonuclease, indicating the presence of ribonucleoprotein in these cells. In addition to various cytoplasmic structures, a number of investigators have noted the disappearance of basophilic staining in the nucleolus after treatment with ribonuclease.

Other basophilic substances, occurring variously in the cytoplasm and in ground substances, may be composed of mucopolysaccharides or of substances of unknown composition. With the photometric method referred to above, which registers the intensity of staining of selected cell constituents over a range of pH, characteristic and specific curves or "signatures" may be obtained for a variety of basophilic substances, including nucleoproteins and mucopolysaccharides.

Observations. In the present study we have continued the use of these methods in the study of blood. By the Feulgen method the cell nuclei of human marrow stand out distinctly as reddish purple objects, indicating the presence of deoxyribonucleoproteins (fig. 3). The dense chromatin of the polychromatic normoblasts stains more intensely than the finer chromatin of the younger stages of the red blood cells. The nucleoli are unstained. Imprints of bone marrow stained less well than histologic sections.

Howell-Jolly bodies and blood platelets were noteworthy. The Howell-Jolly bodies were stained by the Feulgen technic, indicating that they are nuclear material consisting of deoxyribonucleoprotein (fig. 3a). Blood platelets, on the contrary, were negative, indicating that they contain no nuclear material (fig. 3a). Voit and Kempa have described a positive reaction for platelets, when stained in thick films, but our observations do not bear out their contention. The delicacy of the reaction in the Howell-Jolly bodies suggests that the reaction would occur similarly in platelets provided that they contained nuclear material. In accord with the negative findings in platelets, it is noteworthy that no Feulgen-positive material is encountered in the cytoplasm of megakaryocytes (fig. 3c).

It should be recorded also that neither the basophilic material in the cytoplasm of reticulocytes nor the cytoplasmic stippling of erythrocytes reacts at all, indicating that neither of these structures contains deoxyribonucleoprotein.

The use of ribonuclease on human marrow cells confirms the previous observations of Wislocki and Dempsey on monkey marrow. The cytoplasmic basophilia of the red blood cells precursors, as well as of the basophilic stem cells of the leucocytes, is abolished by the use of this enzyme. This holds true of myeloblasts and promyelocytes, as well as megakaryocytes. The cytoplasmic basophilia of lymphocytes and plasma cells is likewise abolished.

These findings are of interest in view of the recent important discovery that a major function of lymphocytes is to produce globulins which participate in immune reactions (Dougherty and White; Ehrich and Harris). Consequently the globulin synthesis of the lymphocytes can now be related to the cytoplasmic nucleoproteins which are responsible for the basophilic properties of the cells (Dempsey and Wislocki).

By means of the photometric method, described above, for the characterization of basophilic staining, we have concerned ourselves with the cytoplasmic baso-
philia of megakaryocytes. The cytoplasm of this cell interested us particularly because Wislocki, Bunting and Dempsey had arrived at the conclusion, after some previous uncertainty, that it exhibits a moderate amount of basophilia attributable to ribonucleoprotein. The "signature" obtained by the photometric method confirms this conclusion (fig. 1). The curve for the cytoplasm coincides, in regard to degree of acid dissociation and extinction around pH 4.0, with Nissl substance, a nucleoprotein which fails characteristically to stain below pH 4.

For comparison, the graph contains curves for the hyaline matrix of cartilage and the granules of mast cells, both of which consist of strongly acid mucopolysaccharides, the staining of which is not abolished even at pH 1.5. Thus we have proof from the use of ribonuclease, as well as from the photometric signature, that the cytoplasm of megakaryocytes contains ribonucleoprotein.

We have not succeeded as yet in testing blood platelets with either ribonuclease or by the photometric method.

**Metachromasia**

Metachromasia is a property, which some basophilic substances possess, of changing the color of certain dyes such as toluidin blue or thionin from blue to red. The principal group of substances exhibiting metachromasia is the acid mucopolysaccharides, but nucleoproteins, after certain methods of fixation, also stain metachromatically. Many of the cells of bone marrow exhibit variably faint metachromatic reactions which are prevented by the use of ribonuclease, a result indicating that the reactions are due to ribonucleoproteins. For example, the cytoplasm of megakaryocytes, following fixation in 4 per cent basic lead acetate, especially in the bone marrow of guinea pigs, is noticeably metachromatic when stained with a 0.5 per cent aqueous solution of toluidin blue, but this staining is prevented by the use of ribonuclease (Wislocki, Bunting and Dempsey). The granules of the mast cells of the connective tissues are intensely metachromatic due to the presence of an acid mucopolysaccharide, possibly heparin as claimed by Holmgren and Wilander. This reaction is not prevented by ribonuclease (Wislocki, Bunting, and Dempsey) and the granules yield a photometric "signature" characteristic of acid mucopolysaccharides (fig. 1). The granules of basophilic leucocytes are known to exhibit metachromasia, but we have not tested them photometrically.

**Phosphatase**

**Foreword.** The amount and distribution of alkaline phosphatase in various blood cells have been investigated to some degree in a variety of animals. Wachstein has described this enzyme in normal and abnormal cells of human blood and bone marrow; according to him, it is not present in red blood cells, lymphocytes, monocytes or eosinophilic leucocytes of circulating blood, but is present in a variable number of neutrophilic leucocytes. In marrow, staining was uneven and involved neutrophilic leucocytes and occasional nucleated red cells. Megakaryocytes were as a rule negative. Wislocki and Dempsey have described various features of the distribution of alkaline phosphatase in the hemopoietic tissue and blood of
rhesus monkeys, while Deane has reported on its presence in the leucocytes of rats. The last-mentioned investigator used nucleic acid, fructose diphosphate, glucose-b-phosphate and adenylic acid as substrates in addition to glycerophosphate. Recognizable differences in blood cells, following the use of these several substrates, have not been very great.

Wislocki and Dempsey report upon the presence of alkaline phosphatase in variable quantities in the cytoplasm of polymorphonuclear neutrophilic leucocytes, metamyelocytes, myelocytes and lymphocytes. As a rule, the enzyme occurs in both cytoplasm and nucleus. No information appears to exist on the occurrence of phosphatase in basophilic leucocytes of either blood or marrow. However, the granules of tissue mast cells of the rat have been shown to contain alkaline phosphatase by Noback and Montagna and Wislocki and Dempsey. Contrariwise, in human tissue eosinophiles, Wislocki, Bunting and Dempsey encountered none of the enzyme. In keeping with an observation on human marrow (Wachstein), little or no phosphatase was observed in the cytoplasm of megakaryocytes in the bone marrow of the monkey, and insofar as platelets could be evaluated, none was demonstrable.

Only brief references exist to the occurrence of acid phosphatase in the cells of the hemopoietic tissue. Gomori reported the blood cells of all species studied as being negative. Deane refers briefly once to its occurrence in occasional polymorphonuclear leucocytes in the rat. In the granules of the mast cells of rats, both Montagna and Noback and Wislocki, Bunting and Dempsey have described acid phosphatase. In the granules of human tissue eosinophiles the latter investigators have not encountered any.

Observations. The present observations concern the demonstration of acid and alkaline phosphatases in both tissue sections and imprints of rhesus monkey's bone marrow with glycerophosphate as substrate. In the case of both enzymes there is far more phosphatase visible in the imprint preparations than in the sectioned material. The procedure for both imprints and sections was identical, except that in the case of the latter, paraffin imbedding was employed. A similar experience has been reported recently by Montagna and Noback, who found that in mast cell granules, far less acid phosphatase was present after imbedding the tissue in paraffin than when paraffin infiltration was eliminated. These authors offer the possible suggestion that the enzyme is partially denatured during infiltration in paraffin at high temperatures.

In the present material, alkaline phosphatase is readily identified in neutrophilic leucocytes, metamyelocytes and myelocytes, being most abundant in the latter. It is diffusely and variably present in both cytoplasm and nuclei. The cytoplasm of the megakaryocytes shows a diffuse faint staining. The nucleated red cells are uniformly negative in both cytoplasm and nuclei.

Imprints of monkey marrow prepared for acid phosphatase reveal an abundance of this enzyme in contrast to its almost complete absence in sectioned material. It is predominantly nuclear in location, staining the nuclei of nearly all of the cells present in the marrow. A few of the cell types can be definitely identified. Myelo-
cyes, in addition to diffuse nuclear and cytoplasmic staining, often exhibit a brownish granulation of their cytoplasm. Metamyelocytes and polymorphonuclear leucocytes are readily identified by brown staining of their nuclei. Nucleated red cells and megakaryocytes are also recognizable by differentiation of their nuclei.

**Glycogen**

*Foreword.* Neukirch,\(^1\) in 1910, observed material stained by iodine or Best’s carmine method for glycogen in human neutrophilic leucocytes of inflammatory exudates. The material was soluble in saliva and consequently he decided that it must be glycogen or some closely related carbohydrate. Similarly, Stahl, Horstmann and Hilsnitz,\(^2\) utilizing iodine vapor, observed staining of the granules of the neutrophilic metamyelocytes and leucocytes; this they attributed to the presence of glycogen without, however, testing it with saliva.

In human blood platelets Neukirch\(^3\) found central bodies tinged by iodine and stainable with Best’s carmine, but these were insoluble in saliva. Stahl, Horstmann and Hilsnitz\(^2\) also encountered iodiphilic granules in human platelets as well as a very few, seemingly identical cytoplasmic particles in megakaryocytes. However, they did not test any of these granules for their solubility in saliva.

Wislocki and Dempsey\(^1\) investigated the presence of glycogen in the blood cells of rhesus monkeys by means of the Bauer-Feulgen technic and by an ammoniacal silver nitrate method devised by Mitchell and Wislocki.\(^3\) The diagnosis of glycogen was checked by routine saliva-treated controls. Both methods were successful and positive when applied to appropriately fixed material. Glycogen was identified with regularity in bone marrow in the cytoplasm of neutrophilic leucocytes and metamyelocytes. None was seen in the other cell types including megakaryocytes. Glycogen was also found quite regularly in the rim of cytoplasm of the ordinary fat cells of the marrow. In peripheral blood a positive Bauer-Feulgen reaction occurred solely in the polymorphonuclear leucocytes. No glycogen was demonstrable in the blood platelets.

*Observations.* In the present investigation we have repeatedly observed glycogen in neutrophilic metamyelocytes and leucocytes in smears of human blood and bone marrow after staining with the Bauer-Feulgen method and using saliva controls. Glycogen is minimal in neutrophilic metamyelocytes and increases as the cells mature into leucocytes. It is not present in myeloblasts, myelocytes or the red blood cell series.

On re-examining monkey marrow, glycogen was found quite regularly in the cytoplasm of the fat cells. Moreover, in 1 young rhesus monkey, interstitial glycogen was encountered, although nothing similar was noticed in the marrows of 3 other monkeys.

In peripheral human blood, glycogen was encountered solely in the neutrophilic leucocytes. This finding agrees with those of Wagner\(^3\) who has recently shown by chemical analysis that the granular leucocytes are the only carriers of glycogen in normal human blood.

As reported above, Wislocki and Dempsey found the Bauer-Feulgen and ammoni-
acal silver methods for glycogen negative in the megakaryocytes and blood platelets of rhesus monkeys. In contrast to the monkey, the megakaryocytes of human marrow are faintly Bauer-Feulgen positive, but this material is not digested by saliva (fig. 3d). Besides a faint pink color, the cytoplasm of the megakaryocytes contains a variable amount of reddish granular material. The platelets, on the contrary, like those of the monkey, are entirely negative. This finding is consistent with the results of Wagner\textsuperscript{37} who identified pentose sugar in human blood platelets but found no glycogen.

Glycogen increases in amount in the neutrophilic leucocytes as they mature, and consequently it characterizes the differentiated functional state of this cell instead of its developmental stages. The glycogen does not appear to be localized or bound in the neutrophilic granules, for, as in other glycogen-bearing cells, it usually shifts by fixation to one side of the cell. Besides glycogen, the neutrophiles contain alkaline phosphatase, which is extremely variable in amount and maximal in quantity in the myelocytic stages. Alkaline phosphatase is encountered in association with glycogen in various tissues of the body and its presence has been related to the synthesis of glycogen which takes place through the dephosphorylation of hexose phosphate by this enzyme (cf. Dempsey and Wislocki\textsuperscript{32}). Alkaline phosphatase within the neutrophiles tends to be diffusely distributed in the cytoplasm rather than segregated in the neutrophilic granules. In mast cells, on the contrary, in which phosphatase is also abundant, the enzyme is quite definitely localized in the granules.

Confirming Wachstein,\textsuperscript{28} we have observed that polymorphonuclear neutrophiles which have entered the tissues in inflammatory areas contain increased amounts of alkaline phosphatase. It would be interesting to know whether simultaneously the glycogen content of the neutrophilic leucocytes is altered. Neukirch\textsuperscript{31} reports that the neutrophilic leucocytes in exudates contain a far greater quantity of glycogen—as revealed by iodine or Best’s carmine stain—than normal leucocytes, but this observation deserves to be re-examined and checked by modern methods.

**Summary**

In table 1 we have attempted briefly to characterize some of the principal blood cell types by means of the various histochemical methods. The data assembled in the table are derived from the present study as well as from the previous papers from this laboratory. The condensed phrases of the table should be referred back to the observations and discussions presented in the text. The spaces which are left blank do not indicate negative reactions but signify merely that critical data are still outstanding.

Besides offering new observations, the present paper summarizes some recent literature on the chemical histology of the cells of blood and bone marrow. The investigation concerns the demonstration and characterization of lipids, nucleoproteins, mucopolysaccharides, acid and alkaline phosphatases and glycogen by a variety of recently developed technics. It serves to illustrate how histochemical procedures can be utilized to explore the chemical composition and functional activities of cells.
Table 1.—Brief characterization of the principal blood cell types by histochemical reactions for lipids, nucleoproteins, phosphatases and glycogen

<table>
<thead>
<tr>
<th>Cell</th>
<th>Lipid (sudan black B)</th>
<th>Ribonucleoprot. (cytoplasmic basophilia)</th>
<th>Acid phosphatase</th>
<th>Alkaline phosphatase</th>
<th>Glycogen (Bauer-Feulgen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblast (Sabin)</td>
<td>Negative</td>
<td>Strongly positive</td>
<td>Nucleus positive</td>
<td>Positive in cytoplasm and nucleus</td>
<td>Negative</td>
</tr>
<tr>
<td>Myelocyte</td>
<td>Granules uniformly positive</td>
<td>Nucleus positive</td>
<td>Nucleus positive</td>
<td>Positive in cytoplasm and nucleus</td>
<td>Negative</td>
</tr>
<tr>
<td>Metamyelocyte</td>
<td>Granules uniformly positive</td>
<td>Nucleus positive</td>
<td>Nucleus positive</td>
<td>Positive in cytoplasm and nucleus</td>
<td>Faintly positive</td>
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<tr>
<td>Polyneut.</td>
<td>Granules uniformly positive</td>
<td>Nucleus positive</td>
<td>Nucleus positive</td>
<td>Variably positive in cytoplasm and nucleus</td>
<td>Positive</td>
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<tr>
<td>Eosin. Myelocytes and Leuco.</td>
<td>Granules uniformly positive</td>
<td>Nucleus negative</td>
<td>Nucleus negative</td>
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<td>Tissue Eosin.</td>
<td>Granules uniformly positive</td>
<td>Granules negative</td>
<td>Nucleus negative</td>
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<td>Baso. Leuco.</td>
<td>Granules variably positive</td>
<td>Cytoplasm very faintly tinged</td>
<td>Nucleus positive</td>
<td>Nucleus negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Mast Cells</td>
<td>Granules variably positive</td>
<td>Cytoplasm very faintly tinged</td>
<td>Nucleus negative</td>
<td>Granules positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Early Erythroblast</td>
<td>Negative</td>
<td>Cytoplasm very faintly tinged</td>
<td>Nucleus positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Late Erythroblast</td>
<td>Faintly tinged</td>
<td>Faintly tinged</td>
<td>Nucleus positive</td>
<td>Nucleus positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Normoblast</td>
<td>Faintly tinged</td>
<td>Negative</td>
<td>Nucleus positive</td>
<td>Nucleus positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>Minute dots (monkey)</td>
<td>Negative</td>
<td>Nucleus positive</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>Megakaryocyte</td>
<td>Minute dots (monkey)</td>
<td>Moderately positive</td>
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<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Blood platelets</td>
<td>Minute dots (monkey)</td>
<td>Strongly positive</td>
<td>Cytoplasm and nucleus variably positive</td>
<td>Negative (cit. Wachstein)</td>
<td>Negative</td>
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<tr>
<td>Lymphocytes</td>
<td>Negative</td>
<td>Strongly positive</td>
<td>Cytoplasm and nucleus variably positive</td>
<td>Negative (cit. Wachstein)</td>
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<td>Monocyte</td>
<td>Minute granules</td>
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REFERENCES

HISTOCHEMICAL METHODS APPLIED TO HEMATOLOGY

JACK J. RHEINGOLD and GEORGE B. WISLOCKI