Binding of Acid and Basic Dye at Varied pH by Blood and Bone Marrow Cells of Man

With Observations of Blood and Bone Marrow Stained with Serra’s Method for Arginine

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THE CAPACITY of cellular structures, particularly those formed of protein, to bind acid and basic dye at varied pH has provided insight into their electrochemical character. Solutions rich in protons (H+) repress the acid dissociation of tissue proteins whereas solutions poor in protons favor such dissociation. Such conditions are shown in the following formulas using an amino acid as a model.

\[
\begin{align*}
R - \text{OOOC} - C - \text{NH}_2 & \quad \underset{\text{alkaline solution}}{\rightleftharpoons} \quad R - \text{OOOC} - C - \text{NH}_3^+ \quad \underset{\text{acid solution}}{\rightleftharpoons} \\
& \quad \text{H} \\
\end{align*}
\]

In acid solutions tissues tend to acquire a net positive charge and bind acid dye. Binding of basic dye, on the other hand, is enhanced in basic solutions where tissues tend to possess a net negative charge. A measure of the acid or basic strength of a given structure is afforded by its ability to bind basic or acid dye over a range of pH. The approximate isoelectric point of such a structure is located at that pH where binding of acid and basic dye are equivalent.

In the present investigation these principles have been adapted to the study of blood and bone marrow cells of man. This paper includes a review of earlier applications of this approach to the study of blood cells, and the results of experiments intended to provide additional information on the electrochemical composition of blood cell components. Smears have been stained over a wide range of pH, reagents have been used to block selectively or destroy certain chemical groups in blood cells, and observations have been made of smears stained with Serra’s adaptation of the Sakaguchi method for arginine.

In a recent review, Singer has given an extensive discussion of the factors involved in dye binding by tissues, together with a complete bibliography.

PREVIOUS INVESTIGATIONS

Following the works of Bethe and Loeb and Pischinger, which showed the usefulness of dye solutions of varying hydrogen ion concentration in determining isoelectric points of...
BINDING OF DYES AT VARIED pH BY BLOOD CELLS

tissue structures and model tissues, a series of investigators studied the reactions of blood cells to solutions of acid and basic dyes buffered to different pH. Most of these papers, which were published in the late 1920's and early 1930's, are primarily concerned with the determination of isoelectric points of blood cell components. The technic usually included the use of air dried blood smears, alcoholic fixation, concentrated dye solutions and short staining times.

Mommsen estimated the uptake of an acid (cyanol) and a basic (toluidin blue) dye by components of red blood cells, neutrophils, eosinophils and lymphocytes of human blood. His blood smears were dried for 12 to 24 hours at 37.5 C., fixed for 4 minutes in methyl alcohol, and stained with a 2 per cent solution of toluidin blue or cyanol in aqueous buffers. Smears were also stained with Giemsa's stain. While attention was mainly given to the positions of isoelectric points, located where intensities of staining with acid and basic dye were equal, the range and relative intensity of staining were reported. Table 1, constructed from Mommsen's data, summarizes many of his observations.

Schwartz-Karsten also studied the isoelectric points of various components of blood cells and sought to relate his findings to those of Mommsen. He used only a basic dye, methylene blue. Air dried, human blood smears were fixed in ether-alcohol 30 minutes, and stained in M/500 methylene blue buffered in acetic acid-acetate and phosphate systems. He appears to have located isoelectric points at those pH at which structures began to bind the basic dye. This interpretation must involve a degree of error because any complex protein possesses a certain number of negatively charged groupings even on the acid side of the isoelectric point. This interpretation as well as differences in method could reasonably account for the small differences in the isoelectric points which they reported viz: erythrocytes, 5.9 (Schwartz-Karsten) and 6.5 (Mommsen); perinuclear lymphocytic cytoplasm 5.0 and 5.25; peripheral lymphocytic cytoplasm 4.5 and 4.5. In addition, Schwartz-Karsten presented data on platelets, whose isoelectric point he fixed at pH 5.3 to pH 5.6, and granules of monocytes, whose isoelectric points were judged to be at pH 4.5 to pH 5.0. Both he and Mommsen stated that Giemsa's stain was needed to stain the granules of neutrophilic leukocytes, although he suspected that they were stained with methylene blue beginning at pH 4.5 and 5.9. Stained with basic dye alone, the granules varied in color intensity. The staining reactions of basophilic granules was not described as such in any of the studies reviewed.

Seke believed that the pores of fixed red cell membranes were too narrow to freely admit an acid dye without previous softening by immersion in water. Thus, he found that the isoelectric point of water soaked erythrocytes was at a higher pH than that obtained by Mommsen and Schwartz-Karsten. His value for human erythrocytes was at pH 7.2. Yasazumi and Seke showed that the uptake of acid dye by erythrocytes is inhibited by fixation in formalin. By the uptake of an acid dye (ponceau) and a basic dye (toluidin blue)
over a pH range extending from pH 2.2 to pH 8.0, Yasazumi found the isoelectric points of erythrocytes fixed in alcohol to be at pH 6.5 and of those fixed in formol-alcohol (1:9) at pH 5.5. Seke reported similar results following fixation of blood smears in formalin. Zeiger summarized some of the data on alcohol and other fixatives and the effects of fixation upon isoelectric points as determined by dye uptake. The decrease of acidophilia following formalin fixation has been frequently observed by histologists and is considered to be due to coupling of free amino groups of tissues with formaldehyde resulting in a considerable decrease in the basicity of the tissues. The isoelectric point of gelatin and collagen is displaced about one pH unit to the acid side by formaldehyde tanning.

The influence of temperature on staining was studied by Ochs who used smears of human blood fixed for 4 minutes in methyl alcohol and stained at pH 4.8, 6.0, and 6.8 for 1 hour in eosin or in methylene blue in concentration of 1:2000 at temperatures of 2, 22, and 42 C. While the pH of the staining solution was the more important variable, the intensity of staining tended to increase with increasing temperature.

Data on the dye binding capacity of blood cells of vertebrates other than man are given in the works of Kindred, Pulcher and Seke. In the studies summarized above the acid and basic dyes were used separately. Tolstoouhov has contributed data on the uptake by human blood cells of eosin and methylene blue used in combination and has suggested optimum proportions and hydrogen ion concentrations in staining solutions. Considerations of factors influencing the staining of blood smears by the complex Romanowsky-type stains are beyond the scope of this paper. The reader is referred to the works of Lillie for experimental data and bibliography on these stains.

**MATERIALS AND METHODS**

Slide smears were made from normal human blood obtained from 3 individuals, as well as from blood of 2 individuals with chronic myelogenous leukemia who had received no specific treatment in the six months before the preparations were made. Coverslip smears of normal human bone marrow aspirated from the iliac crest were obtained from 2 individuals and provided through the courtesy of Dr. M. Greenberg of Boston City Hospital.

Blood smears were air dried at room temperature, approximately 23 C., for 24 to 48 hours and then fixed in absolute methyl alcohol for 24 hours at room temperature. They were then allowed to dry and stored in the refrigerator at 4 C. until staining. The period of storage varied from a few hours to three weeks. Recently prepared coverslip smears of bone marrow were fixed and stored in the same manner as the blood smears.

Preparations of blood and bone marrow treated in the way described above were stained by an acid or basic dye in 5 x 10^-4 M concentration, in 1500 cc. of aqueous staining solution, maintained at a pH within the pH range 0.05 through 12.3 at intervals of approximately 0.5 pH unit. The solutions were continuously stirred and maintained at a temperature of 25 C. by immersion of their containers in a water bath. The smears were generally stained for either 3 or 24 hours, although in a few instances they were stained for 72 hours.

The principal dyes employed were an acid dye, light green, and a basic dye, methylene blue. A given solution contained only one dye. Eosin Y, and in some cases light green, was used to evaluate the effects of nitrous acid, pyridine in acetic anhydride, pyridine in acetic acid, pyridine alone, and ethanol-ether upon the acid dye-binding power of various components of blood cells. Eosin Y and methylene blue, moreover, were combined in a single staining solution to facilitate identification of cell structures in blood smears, from pH 3.8 to 7.4. The dyes were manufactured by the National Aniline Division of the Allied Chemical and Dye Corporation and were certified for histologic use by the Biological Stain Commission.

The following buffers were used: sodium acetate—glacial acetic acid pH 3.8-5.3; monosodium phosphate—disodium phosphate 5.9-7.4; sodium veronal—hydrochloric acid 7.0-8.9; disodium phosphate—sodium hydroxide 9.0-12.3.

Buffer strength was 0.01 M. Staining baths at pH 3.0 were attained with hydrochloric acid and sodium chloride, and at pH 2.0 to as low as pH 0.05 with hydrochloric acid and water and hydrochloric acid alone.
BINDING OF DYES AT VARIED pH BY BLOOD CELLS

Following staining, the preparations were washed in distilled water, dehydrated in tertiary butyl alcohol, cleared in xylol and mounted in Permount.

The method of staining at controlled pH described above was developed in this laboratory and is described more completely in several publications from this department.15-17

Smears of normal blood from 6 individuals were acetylated in acetic anhydride in pyridine (1:1) as described by MacManus and Cason.18 Their procedure was modified, however, in the proportion of reagents, in that the period of incubation was increased to 24 and 48 hours and in the temperatures used: 45 C., 37.5 C. and room temperature. Acetylated smears were hydrolyzed in 0.1 N KOH for 20 minutes at room temperature. A longer period of hydrolysis resulted in dissolution of the smears. Smears were treated with nitrous acid in the manner reported by Friedenwald et al.,19 except that NaNO2 instead of KNO3 was used. Preparations were extracted with pyridine, and with pyridine and acetic acid, for 60 hours at 70 C. and with boiling ethanol-ether (2:1) for 1 hour.

Hemolyzed blood smears were made from a drop of distilled water mixed with a drop of blood. An alternative method consisted of breathing over all or a portion of an unfixed smear. The slides were allowed to dry in air and were fixed and stained at pH 3.0 and 8.9 in the manner described above for smears of blood and bone marrow.

Comparison of color intensities of normal blood, blood treated with nitrous acid or by acetylation, or extracted by pyridine, alone or in acid, and ethanol-ether and leukemic blood were made with a Bausch and Lomb comparison ocular used with matched microscopes.

The possibility that a given cell structure was dissolved in a staining solution at a pH where it failed to stain, was evaluated by attempting to stain the very structure which did not stain at a pH where it was known to be capable of binding dye.

Coverslip smears of normal human blood from 4 individuals and of normal human bone marrow from 5 additional individuals were fixed in formaldehyde and methyl alcohol (1:10) and stained with Serra's adaptation20 for histologic preparations of Sakaguchi's chemical method for arginine. The bone marrow smears were obtained through the courtesy of Dr. Janet Watson, Kings County Hospital, Brooklyn.

The blood and bone marrow for these studies were obtained from 16 individuals. Two persons provided blood for each of the experiments reported: otherwise blood and bone marrow for staining at varied pH, procedures for blocking or destroying amines, and the Serra test for arginine, were obtained from different individuals.

Absorption spectra of dye solutions were obtained with a DU Beckman spectrophotometer.

OBSERVATIONS

The data presented below, except where stated, were obtained from smears stained with light green or methylene blue for 24 hours. Staining for 3 hours gave much the same results, except as concerns neutrophilic and basophilic granules. Staining for 72 hours with light green at pH 5.6 was less intense than for 24 hours.

Mature erythrocytes bound light green throughout the entire range of pH studied (12.3 to 0.05), staining most intensely at pH 3.0. At pH 12.3 staining was so faint that it was scarcely recognized. Mature erythrocytes were stained with methylene blue from pH 12.3 to about pH 8.0. In bone marrow preparations where erythrocytes were several layers thick, staining with basic dye was recognized as low as pH 6.0. Staining was most intense at high pH and diminished in intensity with decrease in pH.

In hemolyzed smears, studied at pH 3.0 with acid dye and at pH 8.9 with basic dye, free hemoglobin was stained and red cell ghosts were unstained. Furthermore, intact erythrocytes displayed most intense staining peripherally and were but faintly stained or unstained in their concavities.

Treatment of smears with nitrous acid, and acetic anhydride in pyridine
at 37.5°C and room temperature, greatly reduced the intensity of staining of erythrocytes by eosin at pH 7.7. Staining was eliminated by acetylation at 45°C. Extraction of smears with pyridine, pyridine in acetic acid, or ethanol-ether had no effect upon the staining of erythrocytes. The staining capacity of acetylated erythrocytes was almost completely restored after treatment with KOH.

Staining of the cytoplasm of nucleated red blood cells depended upon their level of maturation. Basophilic and polychromatophilic erythroblasts and polychromatophilic erythrocytes were stained with methylene blue over the same pH range as the basophilic cytoplasm of lymphocytes and other white blood cells. The cytoplasm of polychromatophilic erythroblasts and normoblasts, and polychromatophilic erythrocytes, was stained with acid dye over the same range as mature erythrocytes.

Nuclei of red blood cells stained in the same way as nuclei of other blood cells and are described in a separate passage.

The staining of red blood cells of normal and leukemic blood was alike.

The cytoplasm of lymphocytes and monocytes was similar in its affinities for light green and methylene blue. Methylene blue was bound in decreasing amounts from pH 12.3 through 3.9 and light green from pH 0.05 through pH 8.9, and, often, pH 11.0. Cytoplasmic granules of lymphocytes and monocytes could not be sufficiently well identified to define their staining characteristics. The region of the centrospore was differentiated in most of the lymphocytes and monocytes stained with methylene blue above pH 7.7 and with light green below pH 6.0, being lightly stained at these pH in contrast to the more intensely stained peripheral cytoplasm. Below pH 7.7 with methylene blue, and above pH 6.0 with light green, cytoplasmic staining was so faint that the centrospheral region was not distinguished. In a few mononuclear cells the centrospheral region stained more intensely than the rest of the cytoplasm at about pH 5.5 with acid dye.

The staining features of large and small lymphocytes were alike save for their nuclei, the reactions of which are presented in a subsequent passage.

The staining of lymphocytes and monocytes of the leukemic blood was the same as that of the normal blood.

The ground substance of mature polymorphonuclear neutrophils bound light green through pH 8.9, and, occasionally, pH 10 to 11, with most intense staining at pH 3.0. The intensity of staining at pH 6.7 to 8.9 was very faint. Methylene blue was bound from pH 12.3 through 3.9 in most neutrophils. In many of them, however, staining was extinguished between pH 5.5 and 7.7. The latter group showed no differences in nuclear polymorphism and cell size from those which stained through the entire pH range from 12.3 through 3.9. The ground substance of eosinophils and basophils stained in the same manner as that of neutrophils except that almost all eosinophils and all basophils bound methylene blue through pH 3.9.

Neutrophilic granules were stained by methylene blue from pH 12.3 through pH 9.0 to 10.0 with a 3 hour staining period, but when the staining time was increased to 24 hours, the dye was bound through pH 5.3. The response of the granules to acid dye could not be evaluated because marked cytoplasmic staining masked them. Eosinophilic granules bound light green from pH 0.05 through pH 12.0, but were not stained at pH 12.3. Below pH 10.0 the granules were deeply
stained but above it they became very faint in color. Most of the granules were stained deeply about their periphery, with centers more lightly stained. Nevertheless, about 10 per cent of the granules of any given cell were stained uniformly. Eosinophilic granules could not be stained with basic dye. Basophilic granules were stained metachromatically with methylene blue through pH 1.0 through 8.0 but the granules were soluble in more acid and alkaline solutions. They could not be stained with light green, an observation made from a leukemic blood which contained approximately 20 per cent basophils whose granules were seen as negative images.

Blood platelets bound methylene blue from pH 12.3 through pH 3.9 and light green from pH 0.05 through pH 8.9. The central portion of the platelets tended to stain more intensely than the peripheral part but a hyaloplasm could seldom be differentiated. Megakaryocytes, observed in normal bone marrow and, occasionally, in blood smears of leukemic blood, displayed the same range of cytoplasmic staining as blood platelets.

The cytoplasm of immature granulocytes stained in the same manner as that of lymphocytes and monocytes with the exception that forms less mature than metamyelocytes failed to bind acid dye above pH 5.3 or pH 6.7. Eosinophilic and basophilic granules, in whatever stage of development they were found, were stained as in mature cells. Frequently eosinophilic granules were heavily surrounded by basophilic material. In leukemic blood eosinophilic myelocytes often contained several orthochromatic basophilic granules. In smears of leukemic blood stained with eosin and methylene blue several cells, in all other respects typical basophils, contained a few eosinophilic granules. Neutrophilic or indifferent myelocytes contained granules which possessed the same range of staining with methylene blue as mature neutrophils, although the granules were frequently coarser. In these myelocytes the cytoplasmic basophilic material, instead of presenting a homogeneous appearance as in blast forms and mononuclear cells, was often fragmented and difficult to differentiate from granules.

The cytoplasm of white blood cells and blood platelets responded similarly after acetylation at 37.5 C. and at room temperature, and after treatment with nitrous acid. Compared with untreated cells, cytoplasmic staining with eosin at pH 7.7 was moderately to considerably diminished. In smears acetylated at 45 C. cytoplasm had no affinity for eosin at pH 7.7. Staining was almost completely restored after hydrolysis of acetylated smears with KOH. Following acetylation at 45 C. for 48 hours eosinophilic granules failed to stain with eosin at pH 7.7. After treatment with nitrous acid or acetylation at 45 C. for 24 hours staining of the granules was considerably diminished at this pH. Less intensive acetylation had only slight effect upon their uptake of eosin. Their staining capacity was restored after immersion of acetylated smears in KOH. Extraction with pyridine or pyridine in acetic acid or ethanol-ether did not diminish the granules’ acidophilia.

The nuclei of all blood cells responded alike to acid and basic dyes. Chromatin, or basic chromatin, bound methylene blue from pH 12.3 through pH 0.05 in the case of lymphocytes; normoblasts and granulocytes. In immature blood cells or those containing a loose chromatin network, staining with basic dye could be recognized only to about pH 3.9, and in some very immature cells, only to pH
4.8 or pH 5.5. The nuclei also stained with acid dye. In most instances the acid dye stained homogeneous material, nuclear sap, while in some cases it colored fibrillar material, termed oxychromatin. In mature granulocytes and lymphocytes the areas stained with light green were reduced to narrow spaces between heavy masses of chromatin. In these cells staining with acid dye extended to pH 8.9, but in cells where acidophilic nuclear substance was more abundant staining with light green extended to pH 12.3.

**Fig. 1.—Eosinophil stained with Serra method for arginine.**

**Fig. 2.—Range of staining of blood cell components with light green and methylene blue from pH 0.05 to pH 12.3.** The range given for chromatin is that of granulocytes, and for erythrocytes' uptake of light green, that of erythrocytes observed in blood smears.

Treatment of smears with nitrous acid reduced nuclear acidophilia to a slight degree. Following the most intensive acetylation procedure nuclear affinity for eosin at pH 7.7 was moderately diminished; in some cells after acetylation nuclei bound acid dye homogeneously. Pyridine alone, pyridine in acetic acid, or ethanol-ether had no effect upon nuclear acidophilia.

The nuclei of all blood and bone marrow cells were colored a faint to moderately intense orange with the Serra procedure for arginine. The nuclear reactions were generally diffuse but occasionally the color was sharply restricted to areas within the nucleus which appeared to correspond with nuclear sap. The cytoplasm of mature granulocytes was slightly positive while that of mononuclear cells and immature blood cells was moderately or, occasionally, intensely colored.
Eosinophilic granules gave a positive reaction. They were solidly stained with little variation from granule to granule, and typically, were the deepest colored of all blood cell structures (fig. 1). Neutrophilic granules could not be recognized. Under low power, especially where the smear was folded on itself, erythrocytes appeared faintly positive but under higher powers they did not seem stained. Plasma, particularly in bone marrow preparations, was often colored a faint orange.

Discussion

In this discussion factors influencing dye uptake and measurement of dye uptake, as affect the present study, will be considered. This will be followed by a consideration of the relations of the present observations to those of previous investigators and, finally, by an attempt to correlate some of the observations with known chemical data.

Factors Influencing Dye Uptake

Predictably close approximation of the dissociation curves or isoelectric points of tissue structures by their affinity for acid and basic dyes at varied pH is difficult to attain, because binding of dye and measurement of bound dye in smears and tissue sections depend upon conditions of staining in addition to pH. Dye uptake by tissue structures is influenced by fixation, the nature of the dye and its concentration, the buffer and buffer concentration, the temperature and duration of staining, and pH. Blood and bone marrow smears were fixed in absolute methyl alcohol. Methyl alcohol possesses no marked chemical affinity for acid and basic tissue groups but changes from the electrochemical character of the native state of tissue structures must be expected to follow its use due to the physical changes of denaturation induced by fixation.

Under conditions of staining where the pH is near neutrality the dye is, in effect, completely dissociated, and differences in dye uptake by tissues at different pH reflect differences in ionization of tissue components. At about pH 2.5 a solution of light green is about 25 per cent as intensely colored as at pH 7.5 and above, a difference due, in all likelihood, to a greater degree of acid dissociation at higher pH. At low pH dye binding capacities of tissue structures may not be satisfied because of a decrease in concentration of ionized dye.

Portions of the same batch of dye dissolved in aqueous buffers of different pH may differ from one another in chemical composition as well as in pH. These differences are more profound than the reversible differences in acid dissociation referred to in the above paragraph. It is unlikely, however, that significant chemical change in methylene blue or light green occurred in the present investigation since measurable oxidation of methylene blue occurs only under more drastic conditions than used in the work reported here, and solutions of light green used in this investigation kept at pH 2.4 and 12.0 for 24 hours had identical absorption spectra when the pH was adjusted to 7.5 with concentrated HCl or NaOH.

By the nature of compounds formed with dyes and tissue structures different buffers may cause differences in dye uptake. In the present study four buffer
systems were used and variations in the amount of dye bound by tissues at the pH where buffers are changed may be due to differences in buffers as well as to changes in tissue ionizations.

Variation in temperature, duration and dye concentration in tissue staining are significant primarily as they influence maximal uptake of dye, or equilibrium staining. For example, as Singer and J. M. Weiss showed (unpublished), light green was maximally bound by fibrin in less than 24 hours, while approximately 80 per cent of equilibrium was attained in 2 to 3 hours. The dye concentration and temperature employed in the present study were the same as theirs.

However, pH is the most important of the variables in the staining solution, which influence dye uptake by tissues. The influence of factors in addition to pH presented above are, for the most part, indeterminate and limit the conclusions which may be drawn from study of tissues stained at different pH.

Measurement of Dye Uptake

Especially in homogeneous substances, dye uptake can be precisely measured by extraction of the total amount of bound dye. Less precise methods must be used for approximating dye bound by most structures in tissues, especially if the dye binding capacities of intracellular structures are sought. In the present study primary attention has been given the pH ranges over which blood cell structures were stained, and no attempt has been made to quantitatively judge the absolute or relative amounts of dye bound at a given pH or from pH to pH. Under the same conditions of staining, color intensities of similar structures in normal, acetylated and nitrous acid-treated, pyridine-extracted, pyridine in acetic acid extracted, ethanol-ether-extracted, and leukemic blood cells have been compared.

Relations of Present Observations to Those of Previous Investigators

Differences in the staining capacities of blood cells reported here from those given by Mommsen, Schwartz-Karsten and others, may reasonably be attributed to differences in technic. Blood cell structures, in the present work, tended to bind acid dye at higher pH and basic dye at lower pH than reported previously. The ground cytoplasm of white cells, for example, was stained with acid dye through pH 8.9, whereas according to Mommsen, it was unstained above pH 7.0. The increased range of staining is probably due to the long periods of staining used in the present study which allowed maximal uptake of dye. The importance of time as an element permitting maximal dye uptake is shown by the staining of neutrophilic granules in the present work which were unstained after 3 hours of staining with methylene blue at pH 5.3, but were stained at that pH after 24 hours.

Seke’s requirement that erythrocytes be immersed in water after fixation to enhance uptake of acid dye was probably met in the present study by staining smears in dilute aqueous solutions of dye for 24 hours.

Cytochemical Significance of Certain Experimental Findings

Several patterns of staining are apparent in the present experimental material. Although any attempts to ascribe chemical significance to them must be guarded
BINDING OF DYES AT VARIED pH BY BLOOD CELLS

because of limitations in technic, some of the observations do appear to warrant correlation with known chemical data.

Erythrocytes were stained with acid dye throughout the range of pH studied. The decrease in intensity of staining below pH 3.0 is probably due primarily to dye association, and possibly to extraction of some hemoglobin from the red corpuscles. Above pH 3.0 the gradual decrease in staining must be attributed to loss of charge of positively charged tissue groupings as they yield protons to the dye baths. Unfortunately the data do not permit construction of a curve whose points of inflection would indicate successively dissociating groups.

Adult human hemoglobin is rich in the basic amino acids, containing 36.3 residues of histidine, 44.0 of lysine and 12.6 of arginine per molecule. In addition, end group analysis has disclosed 5 terminal α-amino groups provided by valine. Depending upon pH, acid dye may be secured to hemoglobin by the imidazolium group of histidine, α-ammonium groups of valine, ε-ammonium groups of lysine and guanidinium groups of arginine, whose pK in free amino acids are, respectively, 6.00, 9.62, 10.53 and 12.48. In protein these pK values may be modified, tending to be lower than those of corresponding free amino acids. Below about pH 5.0 all of the above mentioned groups bind acid dye to hemoglobin. Above about pH 6 to 7 ammonium and guanidinium groups contribute to acid dye binding. (This method does not permit differentiating α- from ε-ammonium groups whose pK in protein may overlap.) Above about pH 11.0 staining with acid dye must be attributed to guanidinium groups.

Acetylation at 45 C. or treatment of smears with nitrous acid, procedures used to block or destroy primary and secondary amines and possibly guanidino groups, destroyed or greatly reduced the affinity of red cells for acid dye at pH 7.7. Extraction with pyridine under conditions more drastic than those of Baker for the extraction of phospholipids, with pyridine in acetic acid or ethanol-ether, did not affect binding of acid dye. Phospholipids are sources of unacetylizable quaternary amines and the diminished intensity of staining following the above extraction procedures make it unlikely that those compounds bound acid dye.

Although eosinophilic granules were stained with almost undiminished intensity to about pH 10.0, at pH 12.0 they were but faintly colored and at pH 12.3 unstained. After acetylation at 45 C. the granules were not stained and following treatment with nitrous acid the granules were faintly stained, with eosin at pH 7.7, suggesting that acetic anhydride in pyridine was more effective than nitrous acid under the conditions used in these experiments, in blocking amines.* Treatment of acetylated smears with KOH typically restored the affinity of eosinophilic granules for acid dye. Their strong basicity, which resists extraction by pyridine, alone or in acid or by ethanol-ether, diminution or elim-

* R. D. Lillie (Blood 7: 1042, 1952) treated blood smears with nitrous acid and evaluated the effect upon acidophilia of red blood cells and eosinophilic granules with Giemsa stain. He found that the eosinophilia of eosinophilic granules persisted following exposure to nitrous acid and that red cells were stained yellowish green instead of orange pink. In the present author's experience, the use of acid dye alone is more satisfactory for evaluating eosinophilia than Romanowsky stains or mixtures of acid and basic dyes because the acid dye binding capacity of a given cell structure may be masked by binding of basic or neutral stain from dye mixtures.
ination of their acidophilia by acetylation or treatment with nitrous acid, strongly suggest that the eosinophilic granules are rich in arginine, a suggestion confirmed by the Serra test.

Consistent binding of acid dye to about pH 9.0 and considerable reduction in acidophilia following exposure to nitrous acid or acetylation suggest that the positively charged compounds of the ground cytoplasm of white blood cells and platelets consist in large part of α- and ϵ-ammonium groups. That arginine is present is indicated by frequent or occasional staining to pH 10 to 11 and results with the Serra test.

A pattern of staining displayed by the cytoplasmic ground substance of many of the circulating white cells and all of the immature blood cells was binding of methylene blue through the pH range 12.3 to 3.9. Cells rich in ribonucleoprotein are stained with methylene blue over a similar range of pH and enzymatic and ultra-violet absorption methods have clearly demonstrated ribonucleoprotein in the cytoplasm of blood cells.

In the thinly drawn blood smears studied, red blood cells failed to bind basic dye below pH 7.9 as judged by examination of the smear. In portions of the bone marrow smears where red blood cells were several layers thick, on the other hand, staining was recognized down to pH 6.0. Thus, low concentration of basophilic material may account for the failure of the cytoplasm of certain mature neutrophils to take up basic dye below pH 5.5, 6.0 and 7.7. Conversely high concentration of nucleoprotein may account for the ability of nuclei whose chromatin is densely packed to bind basic dye through pH 0.05. Most nuclei bind methylene blue to pH 3.9, a finding typical of ribonucleoprotein.

The combination of strong affinity for basic dye and metachromasia displayed by basophilic granules, is typical of half esters of sulfuric acid and carbohydrate. A similar pattern of staining is shown by mast cell granules whose basophilia has been attributed to the sulfate esters of heparin.

Persistence of a moderate degree of acidophilia after acetylation or treatment with nitrous acid suggest that quaternary amines or other compounds unaffected by these procedures contribute to nuclear acidophilia or that these reagents do not combine with all hydrogenated amines. Extraction with pyridine, or pyridine in acetic acid, or ethanol-ether had no effect upon nuclear affinity for eosin. It is of interest that nuclei are commonly stained in the Baker test for phospholipid after extraction with pyridine. The capacity for acid dye through pH 12.3 shown by many nuclei, diminution of acidophilia following acetylation and exposure to nitrous acid, and positive reaction with the Serra test indicate the presence of arginine in the nuclei studied. Hamar, in his studies of the amino acid composition of thymus nuclei, found that 30.7 per cent of amino acid N (as total N) of thymus histone was contributed by arginine.

In a paper on the cytochemistry of eosinophilic granules of horse leucocytes, Vercauteren reported staining of the granules with Serra's method for arginine. They were but faintly stained with an alcoholic solution of eosin after extraction by boiling ethanol-ether for 1 hour. Eosinophilia of these granules was, accordingly, attributed primarily to phospholipid. Phospholipid extracted from horse leucocytes, moreover, could bind eosin.

Warren and McManus have adapted the Sakaguchi oxine test for arginine for histological preparations and reported, in a survey of tissues, that blood gave
no reaction. Their method differs from the Serra method primarily in the use of 8-hydroxyquinoline, or oxine, instead of α-naphthol.

SUMMARY

In acid solutions, tissues tend to acquire a net positive charge and bind acid dye; binding of basic dye is enhanced in basic solution where tissues tend to possess a net negative charge. A measure of the acid or basic strength of a given tissue structure is afforded by its ability to bind basic or acid dye over a range of pH.

The capacity of blood cell components to bind light green and methylene blue in the pH range 0.05 to 12.3, and their affinity for eosin after acetylation and treatment with nitrous acid, procedures used to block or destroy primary and secondary amines and guanidino groups, was studied. Observations were also made of blood smears stained with the Serra test for arginine.

Erythrocytes bound light green through pH 12.3 and methylene blue to pH 6.0. The ground substance of white blood cells and immature blood cells stained with light green through about pH 9 consistently, and often to pH 11: methylene blue was bound through approximately pH 4.0. Eosinophilic granules bound light green through pH 12.0, and did not bind methylene blue. Basophilic granules bound methylene blue in the pH range 8.0 to 1.0 but were dissolved in more acid and basic solutions: they bound no acid dye. Neutrophilic granules were stained with methylene blue through pH 5.3. Nuclei of immature blood cells and monocytes bound methylene blue to about pH 4.0; polymorphous nuclei and nuclei of lymphocytes and normoblasts were stained very faintly to pH 0.05. Many nuclei bound light green to about pH 12.0.

Nuclear and cytoplasmic acidophilia was reduced or destroyed by acetylation or treatment of smears with nitrous acid. Nuclei and cytoplasm of blood cells were stained with the Serra test. Eosinophilic granules were stained strongly.

The cytochemical significance of certain experimental findings have been discussed.

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Binding of Acid and Basic Dye at Varied pH by Blood and Bone Marrow Cells of Man: With Observations of Blood and Bone Marrow Stained with Serra's Method for Arginine

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