BENZIDINE AS A PEROXIDASE REAGENT FOR BLOOD SMEARS AND TISSUES.*

G. S. GRAHAM.

(*From the Pathological Laboratory of the Albany Medical College and the Albany Hospital.*)

The oxidizing substance present in the leucocytes of the blood and in their forbears of the bone marrow is of considerable theoretical and practical interest. The question as to whether this oxidizing substance is a true enzyme or merely an unorganized catalyst, does not here concern us. The balance of evidence seems to indicate that it is in fact an enzyme of the so-called peroxidase type and as such it will be spoken of in this paper. The presence of this body in the leucocytes of myelogenous type and its absence in the lymphocytic series is a convincing biological argument for the fundamental dissimilarity of these two cell groups. It follows that a microchemical method capable of demonstrating the presence or absence of the peroxidase may occasionally be of considerable practical value in the study of blood smears from its identification of cells whose nature is not adequately revealed through the use of the usual stains. Such a method is capable also of giving very interesting pictures when applied to tissues. Here it has a distinct value, particularly for teaching purposes, on account of the strong emphasis laid by it upon the granule-bearing cells. The student struggling in the early hours of his second year pathology with the problem of differentiating the cell types in acutely inflamed tissues comes to view this granule-bearing series with a heightened interest once he has studied sections in which they stand out in sharp contrast with all other histological elements, and it is not difficult now to convince him that they are not to be confused with members of the lymphocytic or any other series.

* Received for publication July 2, 1918.

The list of substances that may be used in vitro as reagents for the oxidizing ferments is a long one, but not all of them are adaptable to the methods of histology, where for one reason or another the list must be considerably contracted. In a previous paper[1] there was described a peroxidase method employing alpha naphthol and pyronin. It was and is believed that this method has certain advantages over previous ones. Occasion may here be taken to state that the technic originally advised for the staining of blood smears was later modified. It was found that speedier and perhaps more constant results could be obtained by exposing the fixed preparation for five minutes to a mixture of alpha naphthol solution and pyronin as advised for section staining, washing under the tap, differentiating and removing excess of alpha naphthol by thirty seconds' washing in forty per cent alcohol, again washing in water, and counterstaining with a one per cent aqueous solution of methylene blue. Owing to the disorganization of the drug and dye markets incident to the war, the method has become impracticable, since both the reagents used are now unobtainable from the dealers. In order that certain lines of inquiry employing the method might be continued it became necessary therefore to adopt some other reagent, and after trial of a number of the well-known enzymatic indicators it was found that benzidine lends itself readily to the purpose. On account of its established use in clinical pathology this reagent will be found on most laboratory shelves. Aside from the greater availability of the benzidine, the method now described has advantages over the alpha naphthol method in that it is simpler, the reaction rather more clear cut, and, finally, there can be less question as to the specific nature of the granular reaction obtained since the dye set up in the granule needs no reinforcement such as is necessary with alpha naphthol.

Technic.—Preparation of the reagent: To ten cubic centimeters of forty per cent alcohol are added a few crystals of benzidine and .02 cubic centimeter of hydrogen peroxide.

The benzidine is very sparingly soluble. It has been customary to use as much as may be taken up "on the tip of a small knife blade." The preparation of the reagent may be simplified by making up a small stock bottle of forty per cent alcohol containing two-tenths per cent of hydrogen peroxide and adding the benzidine to small portions as they are withdrawn for immediate use.

Application to blood smears. — The smear should be as fresh as possible. Fix the dried smear for a minute or two in a mixture consisting of forty per cent formaldehyde one part, ninety-five per cent alcohol nine parts. The fixative should be freshly prepared. Unfixed preparations show the granular reaction but do not take the counterstain satisfactorily. Wash off the fixative under the tap and replace with the benzidine solution. Allow a reaction time of five to ten minutes. Five minutes is usually sufficient. Wash under the tap and counterstain with Loeffler's alkaline methylene blue solution for thirty seconds.

The neutrophilic and eosinophilic granules are stained a warm brown color. Those of neutrophilic type are small, irregularly rounded, rather variable in size and where seen most distinctly tend to occur as short linear aggregations of two to several units. The eosinophilic granule is of large size, round or elliptical in shape and characteristically refractive. The brown stain appears to be held in a mantle or envelope bounding a central relatively unstained substance. The identification of the mast cell is not yet certain, but it is believed that its basophilic granule does not react in most cases although in rare instances cells that have been tentatively placed in this group have shown a few scattered granules of a dense greenish-brown color entirely unlike that found in the cells above described. These granules vary in size but are often as large as the eosinophilic type. The cytoplasm of these cells in whole or in part shows a faint purplish-red metachromatism and occasional vacuole-like spaces. As in the alpha naphthol method, the endothelial leucocyte, "large mononuclear" or "transitional" cell

often has a few vaguely defined granules giving a positive peroxidase reaction. The leucocytic nuclei are dark blue while those of the lymphocytes have a slight purplish tone. The erythrocytes are greenish yellow to greenish blue. The color is due to the counterstain. These cells show no color change under the action of the benzidine unless the reagent is allowed to act for periods of half an hour or more, when they gradually take on a brownish tone. When thus stained they lose much of their affinity for the counterstain. Platelets are blue.

Application to tissues. — The material should be fixed in formalin. Freshly cut frozen sections are placed in the benzidine solution for five minutes, washed in water, counterstained rather deeply with hematoxylin, washed in several changes of water, dehydrated with eighty per cent followed by ninety-five per cent alcohol, and cleared on the slide by the xylol method. Neutralized balsam is best for mounting although the preparations are by no means so sensitive to acidity of this medium as are alpha naphthol stained sections. The stain is permanent.

Discussion. — The literature records several methods that have been proposed for the use of benzidine as a peroxidase reagent, but none appear to have been entirely satisfactory. Fischel[2] claims priority for its use. He used an alcoholic solution containing slight amounts of hydrogen peroxide. His results with tissues are considerably at variance with the present findings, including as they do positive reactions in nucleus or cytoplasm of various parenchymal cells, and even in the cytoplasm of the lymphocytes. He complains of an abundant precipitate thrown down in the sections and of the alcohol solubility of the granular coloring matter, and was led to replace the benzidine by benzidine monosulphate of sodium. He appears, however, to have had difficulty in securing uniform results with the latter, nor was he successful in finding suitable counterstains, while his preparations faded in from three days to one week. Kreibich[3] attempted to improve the former method. He concluded that the variable

results obtained with the benzidine sodium compound were due to its alkalinity, and developed a method for stabilizing the reagent by neutralizing or acidifying it. He used an aqueous solution containing traces of acid alcohol or an aqueous solution of equal amounts of benzidine and the benzidine sodium salt to which was added a small amount of hydrogen peroxide. Fiessinger and Rudowska[4] used a two per cent solution of benzidine in absolute alcohol followed by a weak solution of hydrogen peroxide. Applied to unfixed blood smears their reagent gave a blue coloration of the leucocytic granules but it failed to evoke a reaction when applied to tissues. Loele[5] used an aqueous solution of benzidine to which he added a trace of hydrogen peroxide and a little methylene blue solution. Methylene blue was used also as a counterstain.

The method proposed in the present paper is believed to be simpler and better than any of those enumerated, whether applied to blood smears or to tissues. As concerns blood smears it must be emphasized that the specimen to be stained must be as fresh as possible if best results are to be obtained. If exposed to sunlight the enzyme of the neutrophilic granule deteriorates rapidly, and even when protected from the light it gradually loses its oxidizing property, so that after a week or ten days the granular stain loses its sharpness and non-staining areas begin to make their appearance in the cells. As time goes on the relative number of "live" granules slowly diminishes until the entire cytoplasmic body is completely free of them. Unexposed smears three or four weeks old may occasionally react fairly well, particularly if exposed to the benzidine for twenty or thirty minutes, but these findings do not invalidate the general rule. The eosinophilic granule is more resistant and may react vigorously after all trace of the neutrophilic type is lost.

It must be emphasized also that the benzidine used must be as free as possible from spontaneous oxidative changes. Old discolored preparations give a well-marked reaction both in smears and in tissues but the resulting stain is diffuse and the individual granules fail to stand out as the sharply isolated

bodies seen after the use of a proper reagent. The same heavy but diffuse reaction was obtained with a "technical" benzidine occurring as a fine purple-gray powder.

The color imparted to the granule is of some interest. In the methods above reviewed the color obtained has usually been a blue or green, although Kreibich states that, with increasing alkalinity of his reagent, shades of brown were obtained. With the present method the granule is at first greenish blue but quickly changes to brown and up to a certain limit the brown color increases in depth and intensity with lengthening exposure to the reagent. If the staining process is watched under the microscope the polymorphonuclear nucleus appears to take on a faint greenish coloration, but this is soon obscured by the deepening color of the granules. Individual cells vary in the intensity of their granular staining. This has been noted both with the alpha naphthol and with the present reagent, and there have been some indications that variations in the leucocytic reaction may accompany certain conditions of disease.

The question here raised is an old one that claimed sporadic interest in the early days of modern hematology. It is possible, however, that the use of a method such as is here proposed may open a new avenue of approach and it is hoped that the limited observations thus far made along this line may eventually be amplified in a systematic way. Fiessinger and Rudowska noted a variable leucocytic reaction to their benzidine reagent and followed up the suggestion by studying the leucocytes in a number of diseases without, however, achieving any very conclusive results.

The change of color from green or greenish blue to brown that is produced by the peroxidase action on benzidine is seen to best advantage when frozen sections of acutely inflamed tissues are placed in the benzidine solution. If the tissue holds large numbers of leucocytes the whole section will immediately take on a bright greenish-blue color, or if restricted areas are the seat of leucocytic accumulation such areas stand out prominently. The primary color is, however, very evanescent, and gives way in the course of a minute or

two to a light brown which slowly deepens in tone for a period of ten to fifteen minutes. It has been customary to remove the sections from the solution after five minutes, since the color is at this time well developed and the counterstain is more effective than after a longer exposure. If sections are watched under the lens, it is seen, as in blood smears, that the leucocytic nucleus early takes on a barely perceptible green color. Further, this slight color change may, at any rate in such sections as those of an acutely inflamed appendix, be shown also by the nucleus of the smooth muscle cells, and possibly by that of other cells. It is, however, difficult to make out such slighter and perhaps non-specific color reactions because of their transient nature. At its green stage the dye is not alcohol-resisting so that cleared preparations cannot be obtained. Attempts to watch the process by covering the section with a cover-glass and displacing the water with a benzidine solution have been unsuccessful, since in such a closed chamber the reaction fails to appear. Variations in the granular content of the individual leucocytes may be seen in sections. While some cells may contain very few, others are heavily laden. Loss of granules has been interpreted as evidence of degenerative changes in the cells. Cells giving the heaviest granular reaction frequently show also a dense brownish central mass that fails to stain with hematoxylin. It is probable that this is due to a masking of the nucleus by cytoplasmic material rather than to a reaction on the part of the nucleus itself, since the percentage of such cells increases with increased exposure to the reagent and they are always abundant in sections stained with solutions made up from old or impure benzidine. The leucocytes in blood smears subjected to prolonged reaction also show a more or less complete loss of the nuclear affinity for the counterstain and seem to take on a slight brownish color.

In tissues the seat of gangrenous inflammatory changes, the benzidine sometimes gives a diffuse brownish staining of all the stroma elements in a smaller or larger area. Thus in a prostate gland and in an ulcer of the skin and subcutaneous tissues the stroma underlying necrotic surfaces appears to

have been saturated with the reacting substance so that the latter is evidenced in all the tissue elements including the walls of blood vessels. Similarly larger or smaller sectors or strata of the appendicial wall may show a diffuse brown staining in acute suppurative appendicitis. Thus far no material has been obtained and fixed in a condition fresh enough to show whether such a diffuse staining occurs in perfectly fresh tissue and that it is not due merely to the post-mortem or post-operative diffusion of the ferment substance. But there is a suggestion here of the possibility that the enzymatic material may be distributed through the tissues as a protective substance. The same question is raised by the occurrence in acutely inflamed tissues of scattered cytoplasmic particles filled with granules giving a vigorous peroxidase reaction. They are obviously fragments of leucocytes and sometimes contain basic staining masses of nuclear material. They arise in part by the separation from intact cells of pseudopod-like projections from the cytoplasmic body and in part by more extensive break-up of the parent cell. Under the usual tissue stains a breaking up of the leucocytes is of course frequently observed. It is taken as evidence of cell death and we are accustomed to thinking of the resulting cellular fragments as dead material. The fact that such masses may still bear an active ferment substance suggests, however, that the protective function of the leucocyte may not cease even with the fragmentation of the original cell, but that the resulting particles may still be active in the work of defense against noxious agents.

While no extensive study of the various tissues as stained by benzidine has been possible, it may be stated that no evidence has been found to support the claims of widespread peroxidase reactions in the body cells advanced by some of the German writers. The reaction appears sharply limited to the myelogenous series. It is interesting, however, to note that the endothelial cells of the vessels may occasionally take up the ferment substance. This ability is particularly well developed in the endothelial cell of the hepatic sinusoids, a cell that is notoriously active in picking up foreign matter of

various types. In acute inflammatory conditions, and particularly in general peritonitis, these cells may contain considerable quantities of the peroxidase. A similar action has been noted previously[1] in acute myelogenous leukemia.

Benzidine-stained sections of the spleen are of considerable interest. Here there is found, as would be expected, a variable number of reacting cells. When few they tend to accumulate in a zone about the splenic corpuscles. In non-infectious conditions they are almost all of polymorphonuclear type, although occasional nononuclears appear. In infectious conditions these granular mononucleated forms may be greatly increased in number. Thus in a case of acute generalized peritonitis in a child the pulp cords are everywhere crowded with them almost to the total exclusion of the non-reacting forms usually present. The reacting cells vary in size, some being no larger than a large lymphocyte with only a narrow cytoplasmic rim bearing a few feebly-reacting granules, while others are definite myelocytes with large vesicular nucleus and abundant cytoplasmic substance. The nucleus is usually round but may be indented or U-shaped, and the cell body may be round, oval or polyhedral. In sections stained by the Eosin Methylene Blue method many of these granular cells may be recognized readily as myelocytes, but when comparison is made with sections from the same material stained with benzidine and hematoxylin it is apparent that many of the reacting cells must have been overlooked. The cells that particularly fail of recognition under the usual stains are the smaller and presumably less fully differentiated forms. Such cells are in fact indistinguishable excepting for their positive benzidine reaction from the type often referred to rather vaguely as "splenic cells." The indication is that under certain conditions the myeloblast and myelocyte may occur in the spleen in much greater numbers than is commonly realized and the further question arises whether such cells may not be derived from a prototype always present in numbers in the pulp cords where they differentiate slowly under normal conditions but very rapidly and widely under the influence of appropriate stimuli.

REFERENCES.

1. Graham, G. S. Journ. Medical Research, 1916, xxxv, 231–242.
2. Fischel, R. Wien. klin. Woch., 1910, xxiii, 1557.
Fischel, R. Münch. med. Woch., 1910, lvii, 1203.
3. Kreibich, C. Wien. klin. Woch., 1910, xxiii, 1443–45.
4. Fiessinger, N., and Rudowska, L. Arch. de med. exper. et de l'anat. path., 1912, xxiv, 585.
5. Loele, W. Ergeb. d. all. Path., etc., 1912, xvi, 776.

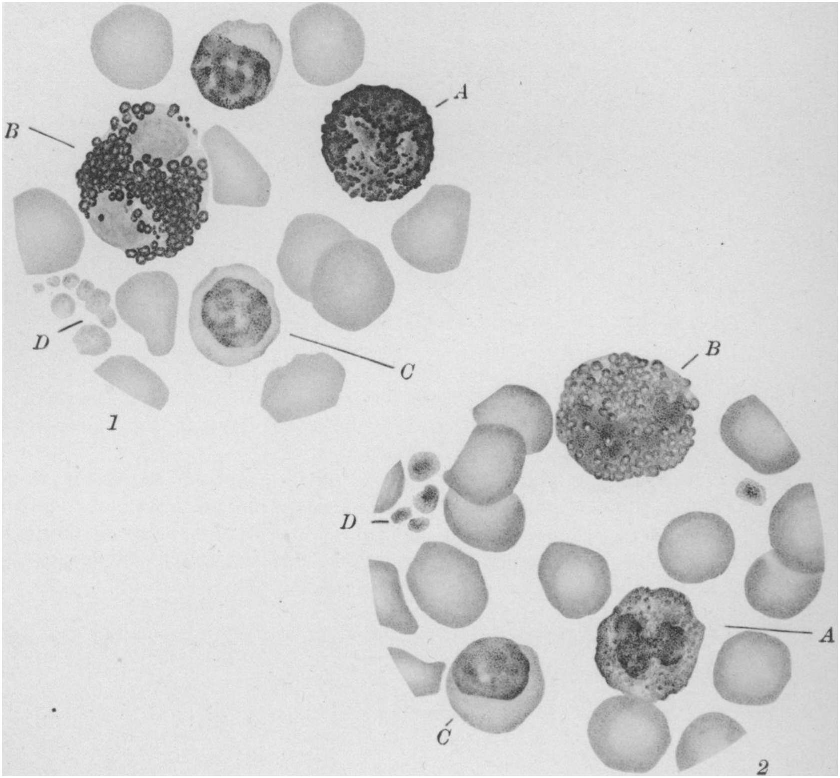
EXPLANATION OF PLATE I.

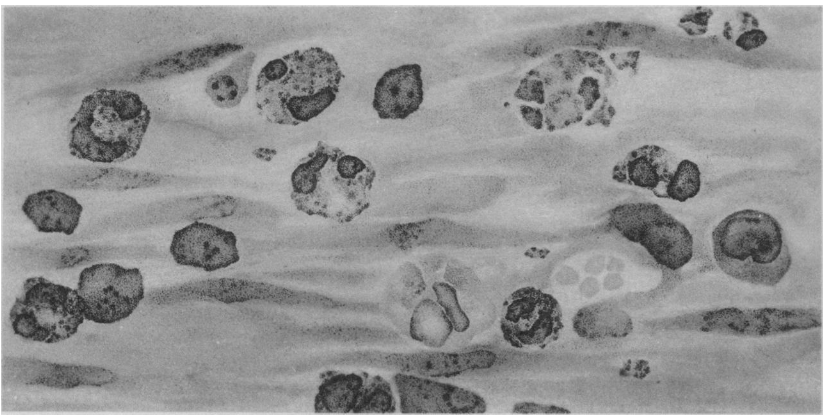
FIGURE 1. — Normal blood stained by the alpha naphthol-pyronin method. A, neutrophile; B, eosinophile; C, lymphocyte; D, platelets. Granules in A and B are stained a deep purple red.

FIGURE 2. — Normal blood stained by the benzidine method. A, neutrophile; B, eosinophile; C, lymphocyte; D, platelets. Granules are brown.

FIGURE 3. — Acute appendicitis. Frozen section; benzidine reagent followed by hematoxylin. Numerous polymorphonuclears: an endothelial leucocyte containing blood pigment and ferment substance. Note leucocyte with pseudo-pod-like formations, also the granule-containing masses believed to be fragments of neutrophiles.

[I am indebted to Miss M. R. Oliver for the colored drawings here reproduced in black and white.]





3