

## Supravital Blood Studies, Using Acridine Orange Fluorescence

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**A**LTHOUGH FLUORESCENCE microscopy had its beginnings over half a century ago, it has only recently gained widespread usage as a research tool, and practical applications have yet to be fully exploited. Blood has been one of the tissues most extensively studied by fluorochroming,<sup>1</sup> but few reports have been published in English. In addition, the previously reported saline dilution technics for supravital fluorochroming have been rather cumbersome.<sup>2,3</sup> It is the purpose of this report to describe a simplified method for supravital fluorochroming with acridine orange. Some preliminary histochemical observations based on the metachromatic property of the dye are included.

### METHODS

Microscope slides bearing a dried film of acridine orange were prepared similar to the method of Schwind<sup>4</sup> for supravital staining with neutral red and pinacyanole. Since the solubility of acridine orange is relatively low compared to that of neutral red,<sup>5</sup> 0.1 Gm. of the dye (National Aniline, Lot 14834) was added to 50 ml. of 95 per cent ethyl alcohol to make a stock saturated solution and the excess dye was allowed to settle. Five drops of the undisturbed saturated supernatant were added to 5 ml. of 95 per cent alcohol. Slides previously washed in detergent and stored in alcohol were dried with a lint-free cloth. The dilute dye solution was flooded over the surface of the horizontal slide and the excess solution was drained away. The slides were allowed to dry in the vertical position producing a uniform thin film of dye. They were then stored in a partitioned box for future use.

Films of blood, marrow, and fresh L. E. buffy coat were prepared by placing a small drop of the specimen to be examined on a clean coverslip. The coverslip was then inverted and dropped on a slide bearing the film of acridine orange. The drop, when suitably small, spread out to form a layer of one-cell thickness. The edges of the coverslip were sealed with petrolatum to prevent drying.

The specimen was then examined with a Zeiss fluorescence microscope with the Osram HBO 200 mercury vapor lamp ultraviolet source. Two BG12 exciter filters, one 4 mm., the other 3 mm. in thickness were used in combination with a GG4 barrier filter to exclude unabsorbed ultraviolet. Either an OG4 or OG5 barrier filter was also used to control the amount of transmitted blue light, since the BG12 filters allow both near ultraviolet and blue-violet excitation. Specimens were satisfactorily reproduced in color transparencies by photography using 35 mm. daylight film with an exposure index of daylight 100. Exposures ranged from 30 seconds to 3 minutes when using the 100X oil immersion objective and 12.5X compensating eyepiece.

### RESULTS

Fresh finger-stick peripheral blood was examined using dye dilutions from thirty drops of saturated solution in 5 ml. of 95 per cent alcohol up to one drop

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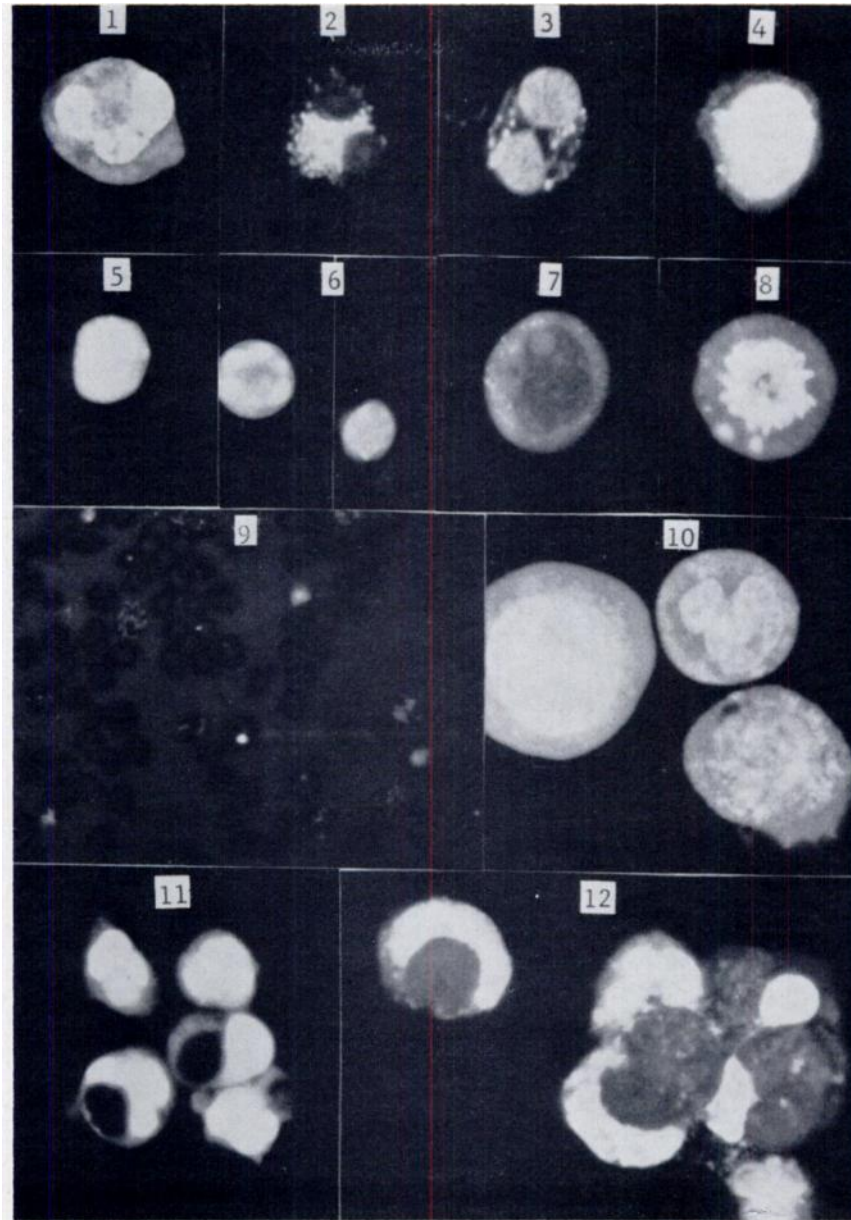
of saturated solution in 25 ml. 95 per cent alcohol. In stronger concentration, the leukocytes were stained uniformly orange and were completely nonmotile. In weaker concentrations, the leukocytes retained their motility undisturbed, but were only very weakly fluorescent. Occasional preparations containing large numbers of leukocytes also required more dye for adequate staining, so that the concentration of five drops of saturated alcoholic solution of acridine orange in 5 ml. 95 per cent alcohol was chosen as the optimal concentration for preparation of slides. At this concentration, the dye was not detectable with white light microscopy.

At optimal concentration of dye, the formed elements of peripheral blood showed distinctive metachromatic fluorescence. The neutrophilic polymorphonuclear leukocytes exhibited bright green nuclei with well demarcated chromatin network and bright orange cytoplasmic granules. After incubation several hours at room temperature, large orange cytoplasmic vacuoles appeared, similar to those previously described in supravital staining with neutral red.<sup>6</sup> Eosinophiles stained similarly, but the cytoplasmic granules were larger in size and more regular in shape. The eosinophilic granules occasionally developed a yellowish cast rather than being bright orange. The granules of basophilic leukocytes were bright yellow. Lymphocytes had bright green nuclei and usually contained a single large bright red round cytoplasmic granule located near the nuclear indentation. Occasionally more than one red cytoplasmic granule was present. The red granules of lymphocytes usually faded completely after intense U-V stimulation lasting several minutes. Monocytes were readily distinguished by their typical folded or incompletely lobulated large nuclei. In addition, the cytoplasm of monocytes was a more dense green, approaching the density of the nucleus, and usually several red cytoplasmic granules were present. Occasionally large irregular reddish-orange granules were widely distributed in the cytoplasm.

Mature erythrocytes showed no fluorescence. Reticulocytes, however, exhibited a bright orange reticulum. The Howell-Jolly bodies in erythrocytes from a postsplenectomy patient were bright yellow and were readily distinguished from the erythrocytic reticulum.

Intact platelets were not often noted in fresh finger-tip blood, but in venous blood anticoagulated with EDTA they were noted to be very pale green with pale orange granules.

Bone marrow cells fluoresced in a fashion similar to those of peripheral blood. Rarely the nucleoli of blast cells contained orange granules but more often merely exhibited an increased green density, probably due to associated nucleolar DNA. The agranular cytoplasm was pale green. The nonspecific granules of promyelocytes were orange and the nucleoli were a slightly more dense green than the finely distributed nuclear chromatin. Erythrocytic precursors could be distinguished readily when hemoglobinization had begun by dark nonfluorescent cytoplasm surrounding a dense green nucleus. More immature erythroid elements were distinguished only by the characteristic coarse network of nuclear chromatin pattern and occasionally contained a few small orange cytoplasmic granules. Megakaryocytes stained uniformly yellowish-



**Figs. 1-12.**—(1) Polymorphonuclear neutrophilic leukocyte. (2) Eosinophilic leukocyte. (3) Basophilic leukocyte. (4) Monocyte. (5) Lymphocyte. (6) Microblast (left) and lymphocyte (right). (7) Promyelocyte. (8) Mitotic cell (peripheral blood). (9) Reticulocytes; Howell-Jolly body (lower center). (10) Myeloblast; segmented neutrophile; neutrophilic myelocyte. (11) Erythrophagocytosis. (12) L. E. cells.

Illustration 9 (X500); 1-8 and 10-12 (X1250).

Illustrations 6, 7, 8 and 10 from subacute myelogenous leukemia, peripheral blood.

Illustration 1-11 OG5 barrier filter.

Illustration 12 OG4 barrier filter.

green, the nucleus being more dense than the cytoplasm. Plasma cells were also most often entirely green, but some were noted to contain a few pale orange cytoplasmic granules.

Preliminary studies of peripheral blood in leukemias indicated that blast cells could be readily distinguished by their dense green nucleoli. Microblasts appeared smaller than the usual blasts and the majority of the nuclear chromatin was aggregated into large green globular clumps just within the nuclear membrane leaving the central portion of the nucleus very pale, occasionally containing a nucleolus. The microblast could be distinguished from a lymphocyte by having neither the red cytoplasmic granules nor the diffuse uniformity of the nuclear chromatin pattern of the latter.

The L.E. cell phenomenon was also studied by the supravital acridine orange technic, using the buffy coat from clotted blood incubated two hours at room temperature. The L.E. body was found to be a very pale homogeneous green and altered nuclei were noted to range from a bright homogeneous green to almost colorless. Erythrophagocytosis in negative preparations was particularly prominent by the dark nonfluorescent appearance of the ingested erythrocyte. An occasional tart cell was noted in negative preparations, but was recognizable by the persistence of the nuclear chromatin pattern and lack of homogeneity of the inclusion.

#### DISCUSSION

As predicted by Kurnick,<sup>7</sup> the diamidines have proved to be useful histochemical fluorescent stains. In fixed tissues stained with acridine orange at pH 3.6 to 5.2, nuclear chromatin is greenish-yellow and cytoplasmic RNA crimson red.<sup>8</sup> Above pH 6, tissues stain diffuse red. The metachromatic differentiation of nucleic acids persists after fixation in absolute alcohol, alcohol-acetic acid, or Carnoy fluid, but is inhibited by formalin or Bouin fixation.<sup>9</sup> The distinctive orange fluorescence of cytoplasmic RNA in rapidly proliferating malignant cells stained with acridine orange has provided a valuable clinical tool in the cytologic diagnosis of cancer.<sup>10-12</sup>

Beers<sup>13-15</sup> and Steiner<sup>16</sup> have studied the binding of acridine orange by ribonucleic acid, deoxyribonucleic acid and polyadenylic acid. The red fluorescent complex I with RNA presumably involves both the phosphate and base of each nucleotide unit along the chain of the polymer, whereas the green complex II with DNA apparently involves only the terminal phosphate group of the polymer. The binding of the dye by the two kinds of sites of the polymer is influenced by such factors as pH, ionic strength, magnesium and calcium.<sup>11</sup>

Amino groups in the 3 or 6 position (2,8 position in the British chemical nomenclature)<sup>17</sup> on the acridine ring system have been shown to be important in the ability of the dye to stain living nuclei.<sup>18</sup>

Although blood has been extensively studied by fluorochroming,<sup>1</sup> few reports have been published in English. Metcalf<sup>19</sup> tabulated the appearance of blood elements using 14 fluorochromes in the detection of malaria. The erythrocytic reticulum has been demonstrated both in fixed smears and supravital preparations in investigations by Leonhartsberger<sup>20</sup> and Kosenow.<sup>2</sup> Kosenow<sup>3</sup> also described the appearance of the nucleated cellular elements of blood

stained supravitaly in 1:10 or 1:20 dilutions of blood in acridine orange 1:20,000 in .875 per cent saline at pH 6.0–7.0. We have used a simplified method of supravital fluorochroming with acridine orange similar to the method of Schwind<sup>4</sup> for staining with neutral red.

Of major interest is the appearance of the characteristic metachromatic differentiation of the red complex I with RNA and the green complex II with DNA in leukocytes of whole blood, which is normally alkaline at about pH 7.4. The color differentiation with supravital acridine orange is identical to that seen in fixed tissues,<sup>8</sup> fixed exfoliative cells,<sup>12</sup> and our own unpublished studies of fixed blood cells stained at controlled pH values below 4. This finding corroborates the findings of Rous<sup>21</sup> and others<sup>22</sup> in studies using litmus, bromphenol blue, and other indicators that intracellular granular acidity of living macrophages may reach pH values as low as 3. Although factors other than pH affect color differentiation, the findings with acridine orange conform rather well to those with other indicators. Fluorochromes thus may prove to be invaluable as indicators of intracellular pH, especially in view of their relatively low toxicity *in vivo*.<sup>18</sup>

By reason of decreased staining with methyl green and the resultant pyroninophilia of the L. E. body in methyl green-pyronin staining, the L. E. bodies have been considered to contain partially depolymerized DNA.<sup>7</sup> Klemperer,<sup>23</sup> however, pointed out that the pyroninophilia should not have been ascribed to the depolymerized state of the deoxyribose nucleic acid, since it might well have been due to the protein content of the L. E. body. Godman<sup>24</sup> furthermore determined that there was no significant decline of methyl green staining of DNA in L. E. bodies which could not be accounted for by protein interference, and that the DNA was not detectably "depolymerized" or altered in state in systemic lupus. It was therefore postulated that the L.E. transformation entailed influx of protein normally foreign to the nucleus, displacement of histone from combination with DNA, and association of the DNA with the new protein which was then responsible for masking the anionic groups of DNA.

In supravital studies with acridine orange, the L. E. bodies stained a very pale homogenous green and altered nuclei ranged from bright green to almost colorless. This finding would support the previous conclusion that the change in staining reaction of the L. E. body is due to blockage of binding sites for acridine orange by the interaction of L. E. factor and DNA. If the previous conclusion by Beers<sup>14</sup> is correct in that the formation of the green complex II occurs with the binding of acridine orange to the terminal phosphate group of the polymer only, then the interaction of L. E. serum with DNA must block the terminal phosphate group also. Therefore, the terminal phosphate group may be one site of combination of the L. E. factor with DNA.

#### SUMMARY AND CONCLUSIONS

A simple method for supravital fluorochroming of blood with acridine orange has been described. The supravital method is of value in distinguishing nucleated cellular elements, reticulocytes and erythrocytic inclusions. Preliminary studies of peripheral blood in leukemia indicate that it may be helpful in differentiating certain abnormal cellular elements. The chemistry of acridine

orange with respect to metachromatic color differentiation of DNA and RNA has been discussed. Based on supravital observations of the L. E. cell phenomenon, speculation has been offered as to the site of interaction of the L. E. factor with DNA.

#### SUMMARIO IN INTERLINGUA

Un technica simple pro fluorochromation supravital de sanguine con acridina orange ha essite describe. Le technica supravital es de valor in distinguer inter elementos cellular nucleate, reticulocytos e inclusiones erythrocytic. Studios preliminari de sanguine peripheric in leucemia indica que illo pote facilitar le differentiation de certe elementos cellular anormal. Le chimia de acridina orange con respecto a differentiation metachromatic de acido disoxyribonucleic e acido ribonucleic ha essite discutite. Speculationes basate super observationes supravital del phenomeno de cellulas L.E. ha essite offerite con respecto al loco de interaction del factor L.E. con acido disoxyribonucleic.

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