THE AUTOMATION OF CYTOCHEMICAL METHODS FOR AUTOMATED CYTOPHOTOMETERS

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The development of an automated differential white blood cell counter is reviewed. After the red cells have been lysed, the white cells are counted by staining and passing through an electro-optical chamber in liquid suspension, surrounded by a laminar, or sheath, stream. Staining procedures were made specific for each type of leukocyte, and separate channels were used for counting each type. Staining intensity and characteristics of the various types of blood cells are discussed. They relate to enzyme levels and the effect on differentiation and identification of the cells. Since reasons for some of the design features are not obvious, discussion of the relevant problems is included. Several applications that go beyond routine differential counting are described.

When the design and production of a differential white blood cell counter was initially proposed at Technicon Instruments Corporation, much of the basic information for its development was already available. Indeed, this was a necessary precondition for the large investment commitment required by a private-sector corporation to undertake production of such equipment.

A definite correlation had been established between the intense staining produced by basic stains in certain cell granules and the presence of heparin in the cells containing these granules (1, 28). Techniques had been demonstrated for staining and counting these "basophils" in liquid suspension in volumetric chambers by conventional microscopy (5, 29). Also, an improved understanding of the critical salt concentration phenomenon by which cations in solution compete against basic dyes for binding sites on polyanions such as heparin (21, 27, 32) contained the background by which a staining procedure could be made more specific for basophils (9), and then automated.

Similarly, it had been shown that a nonspecific esterase contained in human monocytes was specific for that cell type (26). This esterase could work on a number of substrates and was inhibited by fluoride. The original technique called for two colors of esterase reaction; one in the presence of fluoride, one without fluoride. Cells containing only the latter reaction product (no fluoride present) were classified as monocytes. Although the procedure would be cumbersome to automate, the concept of a specific enzyme as a tracer for a specific cell type was very promising for attainment of the goal of differential leukocyte classification.

The presence of the enzyme myeloperoxidase in granules of neutrophils and eosinophils had been well described (3, 12), while segregation of eosinophils by their specific tinctorial (eosinophilic) properties was already in common use (31).

Thus, the positive identification of lymphocytes by a simple contrast technique was the only missing link in differential classification of the common leukocytes of human blood. At first it was thought that counting lymphocytes by difference would be sufficient (24), but the occasional presence of abnormal cells required a further subclassification (22, 23) which is described in the legend to Figure 1.

With regard to optical cell processing, the field had matured to the point where prototypes of a rapid cell spectrophotometer had been placed in Stanford Hospital and elsewhere by IBM to demonstrate their value (10). The main drawback of this instrument was the frequent plugging of the optical flow chamber during operation. However, this problem had been solved in another field by the use of concentric laminar (or sheath) streams (6), and this solution has since been incorporated into the majority of optical flow counters.

Finally, the technology for automatic wet chemical analysis (30) seemed perfectly applicable to the development of contrast in single cells suspended in a liquid. A great variety of continuous-flow biochemical methods already
 existed, including simple cell-counting techniques (14), some of these even using hemolyzing agents by which red cells would be eliminated from the count.

In some preliminary work done with the rapid cell spectrophotometer, the requirement for refractive index matching also had been established (25).

Had all this basic information been available to one individual, he would have advised us:
1. Do not try to get the whole procedure by one technique, but count enough cells in each so that the result is reproducible and valid.
2. Find an internal reference against which to count the cells. Preferably, count all cells by a general identifier; then count specific cells by a second identifier.
3. The general techniques of histochemistry and microscopy are still important when the procedures are automated. In this case, they are: (a) cell fixation; (b) red cell hemolysis; (c) production of color contrast by carefully controlled chemical reaction and (d) control of the refractive index of the suspending liquid for optimal processing in a simple optical device.

In general, this would have been sound advice, but there were some surprises in store for us during the development.

**MYELOPEROXIDASE**

When viewed in retrospect, it seems surprising that so little attention is given to myeloperoxidase by the majority of hematologists. It is used routinely in the laboratory to identify morphologically difficult cells as belonging to the myeloid series (when the stain is positive). The research literature had good references to differences in peroxidase, and other enzyme content at various stages of cell maturation (4). However, the knowledge had not been applied to cytokinetics or to clinical problems before the availability of automation.

Hindsight tells us that there could possibly be more than one important myeloperoxidase to use in cyto-differentiation. However, the retrospectoscope had not yet been designed when the project started, and each clue had to be analyzed independently.

Three findings are judged to be more important than the rest:
1. Various simple methods of differentially staining eosinophils and neutrophils with myeloperoxidase.
2. Existence of great patient-to-patient variability of neutrophil peroxidase activity when the stain is used at low pH.
3. Possibility of developing specific and separate myeloperoxidase inhibitors for different cell types.

Ansley and Ornstein (2) first discovered that neutrophils and eosinophils have different pH optima for myeloperoxidase reactivity. On this basis, the first design of the differential cell counter had a peroxidase channel that counted stained eosinophils only; there was another channel that counted both eosinophils and neutrophils. Such an instrument was actually built and evaluated. It was shown that this instrument over-counted eosinophils at times. Table I contains these results, and shows that the over-count was frequently found in blood samples with band cells and other less mature neutrophils. It was because of these findings that the optical signature of peroxidase-stained cells was examined in more detail. To do this, we decided to build a two-dimensional oscilloscope display on which to depict the measured light absorption and light scatter from single cells. Such displays had previously been used in the RCS designed at IBM (11). The patterns obtained when an ensemble of such cells is collected in a single display had significance well beyond explaining overcounting of eosinophils. It would be used as a shorthand code to explain many of the later findings and developments.

Figure 1 depicts a two-dimensional display in which cells from a normal individual are fixed in formaldehyde and stained with 4-chloro-1-naphthol and H₂O₂ at pH 3.2 after lysing the

**Table I**

<table>
<thead>
<tr>
<th>Hematologist D Eosinophil Count Distorted</th>
<th>Elevated Bands or Metamyelocytes and Elev. Ferrooxidase Activity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.3 Method</td>
<td>Normal volunteers</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hospital routine</td>
<td>45</td>
</tr>
<tr>
<td>pH 3.2 Method</td>
<td>Normal volunteers</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Hospital routine</td>
<td>2</td>
</tr>
</tbody>
</table>
red cells with acetic acid. The absorbed light is on the X axis, while the narrow forward scatter of light is measured along the Y axis. Note the clear separation of clusters of eosinophils, neutrophils, monocytes, and lymphocytes. This is an ideal picture. If all bloods behaved this well, the instrument could have been built with this single channel. However, basophils, when present, appear in the same region as monocytes, and the staining intensity of both monocytes and neutrophils is not constant from patient to patient. These variations seem to have clinical meaning, as do the morphologic variations of cells. Examples of these will be given below.

When the new system was assembled and reevaluated, separation of eosinophils and neutrophils was well maintained, at least if thresholds were set in two dimensions. With the light-scattering threshold as an aid, a study was made of staining-intensity distribution of a large sample of normal people and of patients with reactive conditions, using constant staining conditions and optical analysis. The results indicated that normal individuals usually had a monodisperse staining which, however, varied between individuals. Samples from patients with inflammation tended to have a more complex distribution.

Thus, we could specify that the engineers devise a moving threshold to separate monocytes from neutrophils and another moving threshold that would indicate the degree of departure from a monodisperse distribution of staining intensity of neutrophils.

Figure 2 is a flow diagram for producing cells stained for myeloperoxidase activity. The diluted sample is mixed with cooled formalin buffered to pH 6.8. Cooling is necessary because proteins precipitate in the plasma if formalin is added at an uncontrolled temperature. The sample is then heated to 55°C. During this heating step, plasma and red cell catalases that compete for hydrogen peroxide are inactivated, but myeloperoxidases remain active. Next, the sample is cooled, and cold acetic acid is added to

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**Fig. 1.** Two-dimensional oscilloscope display of cells stained for myeloperoxidase activity. Straight lines represent thresholds that are set by the operator on a reference standard, except for the indicated moving thresholds, which the instrument adjusts dynamically for each specimen. Stained cells are distributed as discussed in the text. Lymphocytes and other unstained agranulocytes produce scatter signals (along the ordinate) which increase with size. They also produce small pseudoabsorption signals (along the abscissa) by the same mechanisms. Therefore large lymphocytes and most blasts appear in the upper left hand box directly in line with the major axis of the lymphocyte population.

**Fig. 2.** Diagram for myeloperoxidase staining by continuous flow. Small circles represent the peristaltic pump action. All flow is from left to right and downward. Volume of proportional flow is indicated in ml/min. As indicated, coils pass through heat transfer media under thermostatic control.
lyse the red blood cells and to bring the pH to 3.2. This is followed by the addition of 4-chloro-
1-naphthol in oxydiethanol and a separate solu-
tion of dilute hydrogen peroxide, and incuba-
tion proceeds at 55°C for 2 min.

Benzidine is not used for color development
because it would react with formaldehyde and
form a Schiff base. The 4-chloro-1-naphthol was
first used with horseradish peroxidase by Nak-
an, and appears superior to benzidine in
blood cell work because the reaction product is
largely limited to intracellular granules,
whereas with benzidine there is frequent crys-
tal formation extending beyond granules and
even beyond cells.

As the cells exit from the 55°C incubator coil,
the suspension is diluted with propylene glycol.
By this dilution, the refractive index of the
solution is brought to 1.385. The purpose of
raising the refractive index is to diminish the
amount of light scattered by individual red cell
ghosts and by platelets. It is more important to
do so in this channel than with the monocyte or
basophil channels because a stronger fixation is
used here so both platelets and stroma are bet-
ter preserved.

After all these additions, the effluent flows
into the optical device for cell-by-cell measure-
ment. During the course of optimizing this au-
tomated cytochemical reaction for electro-opti-
cal reading, several interesting phenomena
were uncovered:
1. Since the peroxidase reaction is very
rapid, it tends to go to completion in each cell
before the sample arrives at the optical view
chamber. Yet the intensity of staining varies
under easily controllable conditions such as pH
and halide ion concentration (2, 13). In particu-
lar, halide acted as a quantitative inhibitor.

Since the initial automated dilution used eth-
ylenediaminetetraacetate (EDTA) in saline, we
felt that substitution of a nonpolar solute in the
diluent might enhance the staining intensity and
provide better control. Indeed, a 5% solu-
tion of dextrose, fructose, or galactose did aug-
ment the staining intensity. Sucrose and lacto-
se were about one-half as effective. However,
this effect, which was an extraordinary en-
hancement, turned out to be independent of the
halide inhibition; i.e., the augmentation oc-
curred in the presence or absence of saline.

2. The use of Tween 20 in the prediluent had
a similar effect on staining intensity. A combi-
nation of monosaccharides and Tween 20 fur-
ther improved the stain intensity and provided
a better separation of monocytes from neutro-
phils.

However, this is a good example of the best
biologic solution not matching system require-
ments. The detergent action of Tween 20 coun-
teracted the effect of bubbles in the flowing
stream to such an extent that integrity of anal-
ysis of individual samples was no longer possi-
ble.

This problem was not solved in time to incor-
porate the optimum stain into the production
system. Subsequently, it was found that use of
a combination of detergents such as Tween 20
and Triton X100 had a stronger effect on stain-
ing than on the fluids of the system.

Since we were not assured of solving this
problem in time for production, a means of
accounting for monocytes and basophils by a
three-channel approach was devised. The other
two channels and the method of justifying the
100% are detailed below.

3. In some other experiments analyzing the
peroxidase channel of the instrument, an at-
tempt was made to control the intensity of
staining by causing a combined colored and
noncolored reaction product to precipitate lo-
ally in the cell. In previous studies, combining
acidine orange and cetylpyridinium was found
to produce a controlled degree of staining in test
objects such as ion exchange spheres (15).
Availability of such control of staining inten-
sity would have been very useful in the optical
calibrations of the instrument. This goal was
never achieved. However, the experiments led
to the discovery of specific inhibitors of eosino-
phil and neutrophil myeloperoxidase. Each one
can be inhibited completely while the other
retains full activity.

The most intriguing part of this study was
the structural similarity of the inhibitors; thus,
resorcinol inhibits eosinophil activity only,
while 4-hydroxyresorcinol only affects neutro-
phils and monocytes. Phloroglucinol, which is
5-hydroxyresorcinol, inhibits both eosinophil
and neutrophil activity, as do pyrogallol, which
is 3-hydroxycatechol, and hydroquinone, the
para di-hydroxy compound. The ortho group
tends to specify neutrophil inhibition, while the
meta compounds seem to inhibit eosinophils.
This inhibition may or may not be associated
with formation of a colorless deposit (Table II).
Also, the behavior is somewhat different when the inhibitor is preincubated with cells. Often the effect is stronger, but in several examples, such as m-chlorophenol and m-cresol, preincubation is without effect. A few compounds actually did produce colorless deposits in neutrophil or eosinophil granules, but none of these was independent of inhibitory activity. Thus, after studying some 80 compounds in this series, we abandoned our attempt at stoichiometric stain control.

The experience was of some clinical value because in an occasional patient with bizarre-appearing cells and unusual staining reaction, an inhibitor such as resorcinol can differentiate eosinophilic from neutrophilic leukemia. The example in Figure 3 illustrates two cases. On the left in each case is the control stain; on the right, resorcinol is added to 4-chloro-1-naphthol as part of the automated incubation condition. Note that in the upper example, the staining intensity did not alter, so the patient most likely is suffering from a bizarre neutrophilia. In the lower example, the bizarre cells which looked like eosinophils on the slide, and stained like eosinophils by peroxidase staining, could be inhibited with resorcinol.

MONOCYTES

The initial observations of Braunsteiner (26) on the specificity of esterase staining were confirmed in our work. It seemed, however, that the reaction had not been optimized. Two directions of development were required: first was the optimization of conditions for the enzyme; second, optimization of the reaction for the use of whole-blood suspensions in a continuous flow system. Both these objectives were achieved by close cooperation between Drs. Ornstein and Ansley at Mt. Sinai Hospital and a group at Technicon.

After many experiments, the possibility of doing a double esterase stain in a continuous flow channel was abandoned. The alternative was to optimize activity of the monocyte enzyme in preference to any other cell activity.

By performance of a pH staining series, the optimum for the system was determined to be close to 6 (present specification is 6.2). At higher pH, the substrate and diazonium compounds are both unstable, and break down. At lower pH, the enzyme activity decreases rap-

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**TABLE II**

*Examples of Specific Myeloperoxidase Inhibitors Acting on Specific Blood Cells*

<table>
<thead>
<tr>
<th>Reagent Added</th>
<th>Combined Stain&quot;</th>
<th>Preincubated&quot;</th>
<th>Colorless Deposit&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Control</td>
<td>+++ + b</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>Resorcinol</td>
<td>+ +</td>
<td>0</td>
<td>0 +</td>
</tr>
<tr>
<td>4-OH-Resorcinol</td>
<td>+++</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Dopamine</td>
<td>+++ +</td>
<td>0</td>
<td>0 +</td>
</tr>
<tr>
<td>M-Chlorophenol</td>
<td>0</td>
<td>+++</td>
<td>0 +</td>
</tr>
<tr>
<td>M-Cresol</td>
<td>+++ +</td>
<td>+</td>
<td>0 +</td>
</tr>
<tr>
<td>2,4-Dinitroso-Resorcinol</td>
<td>+</td>
<td>0</td>
<td>+++</td>
</tr>
</tbody>
</table>

" Staining reagents are as in reference 20.

b Symbols +, 0 refer to staining intensity or amount of deposit.

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![Examples of the use of myeloperoxidase inhibitors in clinical diagnosis, by means of two-dimensional pictures. See text for clinical case discussion.](image_url)

**FIG. 3.** Examples of the use of myeloperoxidase inhibitors in clinical diagnosis, by means of two-dimensional pictures. See text for clinical case discussion.
TABLE III
Esterase Staining Patterns of Monocytes, Neutrophils and Lymphocytes

<table>
<thead>
<tr>
<th>Substrate Class</th>
<th>Monocytes</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A pH 6.0</td>
<td>Diffuse cytoplasmic staining that is fluoride sensitive</td>
<td>Unstained in most persons (weak diffuse fluoride-sensitive staining in a few persons)</td>
<td>A few stained grains in some lymphocytes</td>
</tr>
<tr>
<td>B pH 7.0</td>
<td>From no to few to many stained granules</td>
<td>Many stained granules</td>
<td>A few stained grains in some lymphocytes</td>
</tr>
<tr>
<td>C pH 7.0</td>
<td>From no to few to many stained granules</td>
<td>Many stained granules</td>
<td>Unstained</td>
</tr>
</tbody>
</table>

Substrate classes: (all substrates are esters of 1-naphthol, and are listed under each class in order of speed and intensity of stain). Class A: butyrate, propionate, acetate, 4-bromobutyrate, 3-chloropropionate, gamma-aminobutyrate, caproate, epsilon-aminocaproate. Class B: 2-chloropropionate, 2-bromopropionate, 2-bromobutyrate, ethoxy acetate, chloroacetate. Class C: N-acetyl-DL-alanine, N-acetyl-L-leucine, N-acetyl-L-alanyl-L-alanyl-L-alanine, N-acetyl-L-phenylalanine. Note: basophils and eosinophils do not stain.

idly. However, Davis and Ornstein (7) had a wealth of experience with diazotized basic fuchsin at slightly acid pH. This diazonium salt couples extremely rapidly with free alpha naphthol, forming an azo dye which precipitates close to the site of enzymatic substrate cleavage without significant diffusion.

A series of substrates were used experimentally; Table III summarizes their cell specificity and degree of activity. Alpha naphthyl butyrate was finally chosen as a compromise between enzyme activity and solubility in the aqueous staining mixture containing the cell suspension.

However, storage of α naphthyl butyrate in aqueous solution causes spontaneous breakdown of this substrate. Even in the most anhydrous, but water-soluble, solvents this spontaneous deterioration was an intractable problem. In the original design, therefore, the instrument operator was expected to prepare fresh solution in oxydiethanol daily, and to discard the excess at the end of the day. Subsequently, it was found\(^2\) that the substrate was stable in a mixture of methanol and oxydiethanol for up to 6 months.

Stability of the diazonium salt of basic fuchsin, hexazonium pararosaniline, was never achieved. Since the compound is used for its high reactivity, this is not at all surprising. The problem of instability was solved by synthesizing the compound from stable precursors in the continuous flow apparatus.

\(^2\) Lee M: Technicon Instruments Corp.; Private communication.

Figure 4 is a diagram of the whole monocyte flow system, showing the mixing of diluted whole blood with a buffered formalin mixture containing eserine sulfate. This stream mixes, after a controlled time of fixation, with a stream which contains hexazonium-pararosaniline, cacodylate buffer, naphthyl butyrate and saponin as a lysing agent. The last two are stored as a single solution. Note that basic fuchsin in dilute sulfuric acid and sodium nitrite are mixed and permitted to diazotize for two minutes before the buffer is added.
After the fixed cells are mixed with the substrate-coupler-buffer solution, incubation for color reaction is allowed to proceed. The possibility of substrate utilization by plasma cholinesterase and the pseudocholinesterase of red cell stroma is minimized by the addition of eserine sulfate which inhibits these enzymes.

It is of interest that an excess of eserine sulfate does not inhibit the monocyte enzyme, whereas the potent phosphorylated inhibitors of cholinesterase used as insecticides will inactivate monocyte esterase at extremely low concentration. Vapona, for example, will inhibit monocyte esterase at $10^{-12}$ molar concentration. Presence of a vapona vaporizer in the room where this histochemical reaction is proceeding is sufficient to diminish activity. Even more strange is the fact that people exposed to the same vapors in living and working quarters have perfectly active monocyte esterase unless these cells are subsequently exposed in vitro.

Visual inspection and measurement of the effluent cells confirm that the deposition of reaction product is limited to monocytes (18). Figure 5 shows how the combination of a light-scattering and a light-absorption measurement can automatically classify monocytes separately from the remaining cells.

Figure 6 is the same blood sample stained identically, except that fluorode has been added to show correspondence of this enzyme to the one described by Braunsteiner.

However, experience has shown that staining intensity of monocytes varies considerably, and that in occasional individuals the stain is completely inactive. How the instrument handles this problem is discussed elsewhere (22).

Experience has also shown that there is an occasional individual with mild staining of a few neutrophils by this esterase. Staining of these cells is also inhibited by fluorode. The highest level of staining of these neutrophils is used as a borderline condition by which the threshold is set for counting monocytes in this esterase channel. As an extremely rare occurrence, immature granulocytes contain a fluorode-stable esterase which stains intensely and causes some confusion in counting. However, in these situations, it is more appropriate to review the slide of the patient's blood than to depend on automatically classified cells.

As a final note on monocytes, incubation time for color development is only 5 min. In most methods, a minimum of 30 min incubation is required to achieve this level of contrast in cells. When these optimum and constant conditions are used to examine hundreds of blood specimens, the original observations of Braunsteiner are seen to hold true with the few exceptions noted above. Hence it was considered that the use of the nonspecific esterase would be a satisfactory replacement for the morphologic recognition of monocytes in an automated device (20).

Problems of chemical engineering as applied to cytochemistry are well illustrated in the design of an automated channel for monocytes. Here is an example of the constraints with which we worked—and the solutions employed. It must be obvious that other solutions exist, but many of them are not compatible with the solutions to other component problems.

1. Hexazonium pararosaniline is unstable in aqueous solutions, yet is desirable as the most rapid azo coupler available at pH 6. On-line preparation of the diazonium compound was the solution adopted, as previously mentioned. However, this created a risk for the system—the accidental introduction of basic fuchsin without simultaneous introduction of sodium nitrite. Downstream, basic fuchsin would mix with formaldehyde solution and form a sticky precipitate that had a tendency to build up on the internal surface of the glass tubing of the flow system. Carefully timed serial introduction of these constituents was required. In addition, a valve to stop dye introduction during the standby mode prevented accidental elimination of nitrite. For example, the nitrite solution has a tendency to degas (probably nitrous acid gas) under even the mild pressure changes existing in the peristaltic pumping system. Because the resulting bubbles in the tubing physically replaced solution and caused changes in proportioning, a recycling scheme was developed to eliminate bubbles.

2. Actually, a variety of effects interacted to produce buildup of precipitated material on the inside of glass tubing in the monocyte channel.

(a) The remaining instability of the diazonium compound at pH 6 is the most troublesome of these effects, because it cannot be directly prevented. It was observed that this buildup was more severe when the system was operated without blood samples, but was always present. However, the possibility of inhibiting the ad-
herence to glass led to the trial of detergents as an additive. The first one tried, Tween 20, caused some inhibition of monocyte esterase. In contrast, Brij 35 at the required concentration had no effect on the enzyme activity. It was also worth remarking that use of Brij 35 during routine operation permitted more rapid cleaning of the system between runs. In spite of all these precautions, routine cleaning is required at 4-hr intervals. Naturally, the exchange of cleaning solutions for reagents is performed in an automatic cycle of valve changes. The cleaning solutions are commercial Clorox, followed by water containing Brij 35.

Fig. 5 and 6. Two-dimensional oscilloscope display of blood cells after processing in the esterase channel. In Figure 6 (right), sodium fluoride has been added to the buffer solution and illustrates total inhibition of monocyte esterase. The remaining cells to the right of the main population cluster represent eosinophils, where light loss is not due to stain, but to high angle light scatter of the refractile granules. Optical correction of this feature would require use of a much smaller field depth and, therefore, a narrower sample stream in the optical view chamber. Horizontal line is a threshold defining presence of a cell. Vertical line is the threshold separating monocytes from other cells.
(b) Instability of naphthyl butyrate in aqueous media also contributed to buildup. As the substrate breaks down to its components, butyric acid and naphthol, the latter is co-precipitated with the diazotized basic fuchsins. This is the expected capture reaction within monocytes. However, when it happens outside of cells the precipitate is free floating. As such, it is one of the causes of buildup, and may also cause false monocyte counts when larger masses of precipitate pass through the optical counting chamber.

If one uses freshly dissolved substrate in diethylene glycol (2, 2'-oxydiethanol), it is sufficiently stable for one day of operation. "Sufficiently stable," in this context, means that no visible precipitate is present in the completely mixed effluent of the manifold. If the effluent is stored for several minutes, precipitates do form, and the suspension is not usable. This degree of instability was not completely predictable. It was resolved, in part, by reducing the pH of the reaction mixture immediately after incubation was complete.

(c) Part of the buildup was protein precipitate. As it accumulates over a 4-hr period in the incubation coils, it is continuously exposed to formalin hexazonium pararosaniline solution at elevated temperature. As expected, the protein becomes more and more cross-linked. During the cleaning cycle, there is a tendency for the precipitate to be dislodged as a single long cast which collapses at constrictions in the tubing and can cause complete obstruction. It was necessary to increase the strength of the cleaning solution with sodium hydroxide and to speed up the flow during the cleaning cycle in order to overcome this problem.

**BASOPHILS**

A number of different dyes were tested for developing contrast in basophils. Neutral red, in a technique modified from Shelley's counting procedure, gave good visual contrast (29); the basophil granules stood out in brilliant red against a background where cell structure was almost invisible. This is precisely the effect desired by Shelley for the absolute count of basophils. However, it was difficult to count basophils against a reference of unstained cells to establish a percentage when the unstained cells also were barely refractile. Also, the contrast as measured electro-optically was much lower than visually, because the cells were transparent in that part of the spectrum where conventional photodiodes and photomultipliers are most efficient.

Similar problems were experienced with toluidine blue. Although good visual contrast was established between the pink basophil granules and blue nuclei of all cells, the electro-optical system did not register this contrast effectively.

Acridine orange fluorescence was tried experimentally. However, the requirement of a high-powered light source for this specific purpose would have made the instrument much more expensive.

The spectral qualities of alcin blue seemed ideal for our purpose (17). As noted in Figure 7, this dye has a broad absorption peak that extends from the visible into the near infrared. This compares well with the combined peak output of photomultiplier and light source.

Acridine blue had previously been used in histochemical differentiation of acid mucopolysaccharides, including heparin (32); however, nuclear staining and some protein background always seemed to occur when this technique was used.

The same author had also described a technique using cetylpyridinium as the precipitant for tissue acid mucopolysaccharides that were subsequently stained (32). A technique combining acridine blue staining and cetylpyridinium has similar properties to cetyl ammonium ions which had been used (5) to lyse red cells for basophil chamber counts. For chamber counts

![Fig. 7. Light-scattering spectra of a basophil and a lymphocyte after staining by the acridine blue technique. A micro-spectrophotometer was set up to simulate the optical configuration of the automated system. At 500 nm the signatures are similar, but at 750 nm, quite different.](image)
it was used in combination with aluminum chloride, but the reasons were not given. In our hands, a combination of a quaternary ammonium ion and a trivalent cation were required for lysis without causing aggregation (rafting) of the RBC membranes. The rafting would, of course, cause an optical artifact and would have the tendency to plug the constricted portion of the flow cell.

Thus, by using the proper concentration balance between alcian blue, lanthanum chloride and cetylpyridinium chloride and by controlling the pH of the reaction mixture to about 2.2, a clean suspension of leukocytes is achieved, with only the basophils stained blue. The red cell membranes fragment under these conditions and do not interfere with the light-scattering measurements. Figure 8 is a representation of basophils and unstained cells classified by two light-scattering colors. Note that for the unstained cells, the degree of light scattering by the two colors is equal, but that cells containing alcian blue scatter less at the red and infrared than at the green-blue part of the spectrum. This was also established in cell-by-cell measurement with visual location of the cells under ideal microscopic conditions (17).

Lines in Figure 8 represent thresholds. Evidence for presence of a cell is defined as an electronic pulse generated by an object passing the light beam and causing a minimum amount of light to scatter into the green-light measuring device. A basophil is defined as a cell that scatters considerably more green light than red light, as delineated by the angular threshold.

APPLICATIONS

As one of the first specifications for using cytochemical stains in continuous flow, we stated that the principles used by cytochemists must still apply. If we adhere to those principles, it must follow that the majority of methods devised for complete automation of a cytochemically oriented differential white blood cell counter also apply to semi-automated methods where only the electro-optical measurement has been mechanized. The same experience also applies to manual methods. The detail of a series of these has recently been published (20). This publication also reports on combining methods, for example, of monocyte esterase with peroxidase, or of "monocyte esterase" with one more specific for neutrophil granules. Visual examination of single cells could generally confirm the specificity of the various enzymes. Using a static cytophotometer, one could even reproduce the two-dimensional patterns of staining intensity and light-scattering intensity under conditions where the morphologic identity of each cell is visually confirmed (17-19).

There are also exceptions to these specifications. For example, there frequently are peroxidase-containing granules in monocytes. The enzyme in these granules behaves like the enzyme we associate with neutrophils, and is not like the eosinophil enzyme. Usually, this cross-contamination is small, and the cell classifications are not disturbed. Occasionally, peroxidase staining becomes so strong in the monocyte that cells are grossly misclassified in the instrument. From the clinical viewpoint these are interesting cases, because correlation with disease may be present. Two examples of such correlation are myelomonocytic leukemia, where cells at some stage of the disease develop both strong esterase and strong peroxidase activity.3 In the presence of severe chronic infection, there is occasionally an intense monocyte peroxidase reaction. The neutrophils may or may not overlap monocytes.

3 Atamer M: Grasslands Medical Center, New York; Private communication.
in staining intensity. I have been shown an example of each by Drs. Krastinova and Karmen.4

Another exception to specificity is toxic granulations. As may be expected from the azurophil nature of these granules, they behave like basophils with alcian blue. The number and degree of basophilia of the granules will determine whether or not the cells cross the threshold on the basophil channel.

To take account of some of these exceptions, the instrument was designed with an additional count, reported as the "Remainder." In situations with overlapping cell counts, the remainder is negative. With enzyme deficiencies, the remainder is positive.

Enzyme deficiencies have been noted both in monocyte esterase and in myeloperoxidase. In both there are mild and complete deficiency states, and as expected the complete deficiencies are much more rare. Genetic studies on several families are presently under way.5,6

To date I have not observed an example of eosinophil peroxidase deficiency in humans. One example of eosinophil deficiency was seen in a mongrel dog. I have suggested that the deficiency might be used as a genetic tag in developing a new strain. It may be a significant coincidence that the name of this dog is "Love."

An intriguing set of two-dimensional pictures illustrating myeloperoxidase deficiency was shown by Debauche (8). A female patient with Hodgkins disease in a quiescent stage was shown to have complete deficiency of myeloperoxidase in neutrophils, as determined on the instrument (Fig. 9a). After a full-term pregnancy, she delivered an infant who was partially deficient in myeloperoxidase (Fig. 9b). At the time of delivery (Fig. 9c), the maternal sample indicated complete deficiency except for approximately 15 cells out of 10,000. These 15 cells had staining intensity of partial peroxidase deficiency as observed in the newborn offspring. It is of interest to speculate on the transplacental dilution of maternal blood with fetal cells. If the degree of dilution is in the range of 0.15%, it would be difficult to detect by any other means.

CONCLUSION

A large and varied number of problems challenging to the histochemist were experienced during the design and construction of a commercial automated leukocyte differential counter. These were due both to the constraints of instrument design and to the nature of the biologic variables.

Beyond the encountered problems of design lay rewarding new insights into the biochemistry, pathology, and genetics of blood, as studied with a new tool. It is with hope of improving medical care that one offers these observations to the medical community.

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4 Krastinova L, Karmen A: Einstein College of Medicine, New York; Private communication.
5 Pierre R: The Mayo Clinic, Rochester, New York; Private communication.
6 Simonian Y: University of Utah, Salt Lake City, Utah; Private communication.
poration, Tarrytown, N.Y.) for their help with the peroxidase inhibitor studies; Dr. L. Ornstein (Mount Sinai School of Medicine) and Dr. Ansley for their data on naphthol ester substrates; and H. Mansberg and Dr. K. Roth (Technicon Instruments Corp.) for continuing cooperation through the course of these studies.

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