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BRIAN H. MAYALL

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What is This?
DEOXYRIBONUCLEIC ACID CYTOPHOTOMETRY OF
STAINED HUMAN LEUKOCYES

I. DIFFERENCES AMONG CELL TYPES

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The deoxyribonucleic acid (DNA) content of individual human leukocytes was estimated
cytophotometrically using visible light and spreads stained either with gallocyanin-chrome
alum following ribonuclease digestion or with the Feulgen reaction. When the cells were
measured on a scanning cytophotometer, significant differences in stain intensity were
found among slides. Significant differences also were found among the leukocyte types. In
gallocyanin-chrome alum preparations, monocytes measured 16% higher than small
lymphocytes and 13% higher than neutrophilic granulocytes. In Feulgen preparations,
monocytes measured 4% higher than small lymphocytes and 6% higher than neutrophils.
These differences among cell types were independent of donor and stain intensity. Mea-
surements of cells within types and within slides frequently showed close agreement, but it is
only in this very limited context that the data are consistent with the hypothesis of DNA
constancy. Measurements made on a two-wavelength cytophotometer showed a divergence
of only 2.1% relative to similar measurements made on the scanning cytophotometer,
which suggests that the differences observed among cells and types are unlikely to be
artifacts of the instruments. Over-all, the data indicate either that there is variability in
DNA content or that DNA is not being expressed correctly by the measured stain content.

The estimation of the nuclear deoxyribonucleic
acid (DNA) content of individual cells remains
as one of the more important and common
applications of cytophotometry, and reflects
both the success of the available methods and
the central role played by DNA in the biology
of normal and abnormal cells. Cytophotometry
provided some of the earliest evidence suggesting
DNA constancy (21), while the studies of later
investigators led to the discovery of the relation-
ship of DNA metabolism to gene reproduction
(8) and to the cell cycle (35, 38). Swift's studies
provided significant support for the hypothesis
of DNA constancy, confirming results obtained
by the chemical analysis of counted cells (4, 28).
Patau and Swift (29) and Richards, Walker and
Deeley (30) discussed DNA constancy at some
length. Stated in its simplest terms, the hypothe-
sis postulates that karyotypically identical cells
at the same stage in their cell cycle will all
contain the same amount of DNA. Funda-
mentally, the hypothesis is of the type that can
never be proven, but it is supported by available
evidence, it has found wide biologic applicability
and it is important in the interpretation of
cytophotometric data. If measurements on cells
show no significant variation beyond that which
can be ascribed to random instrument error, the
hypothesis is supported within the accuracy of
the experiment and, if significant differences are
found, such differences negate the hypothesis
only when they cannot be accounted for by (1)
changes in chromosomal complement, (2) nuclei
involved in DNA synthesis or (3) errors in
measurement including systematic biases in the
instrument and variability in staining (30).
Thus, when differences are found between species
or in pathologic conditions, they can be related
to changes in genetic constitution (1, 2, 7, 20,
31). However, when significant differences are
found between karyotypically identical cells,
it is then pertinent to ask whether these repres-
ent departures from DNA constancy, or whether
they are due to inaccuracies in the techniques of
cytophotometry; it is with this problem that the
papers in this series are concerned.

The most convincing cytophotometric evi-
dence supporting the hypothesis of DNA con-
stancy is provided by the study of James (18),

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in which he exploited the 0.94% instrument error of a modified integrating scanning densitometer to measure the Feulgen DNA stain content of rat liver cells. Analysis of the variance associated with his measurements of tetraploid cells showed that the differences in stain content among individual cells were significant in terms of the instrument error, but that the coefficient of variation attributable to the cells was only 0.56%.

There are, however, a number of situations in which appreciable differences are found in the DNA stain content of cells expected to show DNA constancy. A prime example is provided by several studies of mammalian leukocytes. These cells are thought to be nondividing diploid cells, yet cytophotometry has frequently shown major differences in their DNA stain content. This was first shown by Atkin and Richards (3). Using an integrating microdensitometer to measure Feulgen-stained human vaginal smears, they found that small lymphocytes read about 10% lower than cervical epithelial cells. Hale (10) found a 10% decrease in the Feulgen staining of both small lymphocytes and neutrophilic granulocytes relative to the less mature cells in the bone marrow.

Differences are not always seen in the DNA stain content of leukocytes. Stich, Florian and Emson (34), using the two-wavelength method, found only integer ratios among the Feulgen stain content of lymphocytes, polymorphonuclear leukocytes and mitotic figures of normal intestinal epithelial cells. Den Tonkelaar and van Duijn (36) specifically looked for differences between leukocytes and liver and kidney cells in the rat using Feulgen preparations and photographic colorimetry. They did not differentiate between the different types of leukocytes, but state that their preparations contained mainly neutrophils, with some lymphocytes and monocytes also present. Although the sensitivity of their method was such that they would have readily detected differences of 10%, they found no evidence of any differences between the leukocytes and the other cells. Garcia (12), in an extensive study, used two-wavelength cytophotometry to measure the Feulgen stain content of human leukocytes. In this study, he found no differences among the cell types, but he has subsequently found differences in studies in which he used the two-wavelength method, the two-area method and scanning cytophotometry (13, 14). He feels that his earlier results may have reflected differences in the criteria used to select cells for measurement rather than an effect peculiar to the two-wavelength method.

Hale and Cooper (17) provided evidence that the decreases in the stain content of lymphocytes could be reversed without any change in DNA content. They found that, when cultured lymphocytes underwent blastic transformation following stimulation with phytohemagglutinin, their Feulgen stain content reverted to normal levels prior to the detectable incorporation of any tritiated thymidine.

Killander and Rigler (19) also studied the blastic transformation of human leukocytes. They showed that there was a 12% increase in the Feulgen DNA content of cells exposed to phytohemagglutinin for 24 hr relative to cells exposed for only 1 hr, but they did not give values for untreated cells. In addition, they demonstrated that within the 1st hr of stimulation there was an 8-fold increase in the DNA-phosphate groups available for binding to acridine orange, thus indicating that blastic transformation is associated with marked changes in the structure and organization of nucleoprotein. However, the exact relationship between the Feulgen reaction and the changes indicated by acridine orange staining remains to be clarified. Torelli et al. (37) found no change in Feulgen DNA following blastic transformation. They suggest that their inability to demonstrate any change may be explained by differences in their staining technique or by the marginal sensitivity of their instrument.

Both the differences among leukocyte types and the changes associated with blastic transformation suggest that DNA staining may be a function of the physical state of the chromatin. Mayall (22) observed a relationship between cell flattening and stain content for human leukocytes, and Garcia (13, 14) related the stain content of both human and rat leukocytes to nuclear area. Unpublished data from the author's laboratory indicate that mouse leukocytes show differences comparable to those found with rat and human leukocytes and that the gallocyanin-chrome alum stain content of mouse small thymocytes is about 30% less than that of diploid liver or kidney cells from the same animal. An even more dramatic difference in stain content
was reported by Gledhill et al. (15), who showed that the differentiation of spermatids into spermatocytes in the bull testis is associated with a 50% decrease in Feulgen stain content without any appreciable change in the ultraviolet absorption as measured at 265 nm. Working with gallo-
cyanin-chrome alum-stained human metaphase cells at various stages of compaction, Mayall and Mendelsohn (24) and Mendelsohn et al. (25) demonstrated that an increase in total stain content of 10% occurs when the mean chromosome length increases by 40%. In general, it seems that the more compact or dense is the nuclear chromatin, the lower is the DNA stain content.

In the results presented in this paper, human leukocytes showed differences among types comparable to those reported by Atkin and Richards (3) and by Hale (16). These differences occurred independently of donor, staining procedure or measuring instrument.

MATERIALS AND METHODS

Slide preparation: Blood was obtained by venipuncture using a syringe and needle previously rinsed with heparin (1:1000). The blood was layered over cold flotation medium (2 parts 1% methylcellulose in 0.8% saline, 1 part 50% w/v sodium Hypaque (8)), and allowed to stand at room temperature while the red blood cells formed rouleaux and sedimented out. When the volume of leukocyte-rich serum was about half the original blood volume, it was decanted off and centrifuged at 800 rpm for 5 min. The pellet of leukocytes was resuspended in about one-tenth of the original volume of serum. A small drop of the resuspended cells was placed on a clean microscope slide, and was spread with another slide. The spreads were dried quickly with a hot air blower and then the slides were plunged into absolute ethanol at -70°C. The ethanol was allowed to stand at room temperature while the red blood cells were resuspended cells were washed in two changes of distilled water. A small drop of the original volume of serum. A small drop of the original volume of serum. A small drop of the original volume of serum. A small drop of the original volume of serum. A small drop of the original volume of serum.

Staining: The spreads were stained specifically for DNA using either gallocyanin-chrome alum or the Feulgen reaction.

The gallocyanin-chrome alum stain was prepared by Einarson's method (10), modified as follows:

Potassium chromium sulfate, 5 g, is dissolved in 100 ml distilled water, placed in a covered flask and heated in a boiling water bath.

Gallocyanin (National Aniline C.I. 51030), 150 mg, is shaken vigorously with 100 ml distilled water at room temperature for 2 min and filtered. The filter paper containing the insoluble residue is shredded and added to the hot chrome alum solution. The mixture is left in the water bath for 15 min, and then is removed, allowed to cool and filtered. The filtrate is kept for 2 or 3 days, is refiltered to remove any surface scum that may have formed, has its pH adjusted to 1.64 with the addition of 1 N hydrochloric acid and then is ready to use.

Before staining, the slides were hydrated using a graded alcohol series and were treated with ribonuclease solution (30 mg Worthington ribonuclease in 100 ml distilled water at 37°C for 3 hr) followed by two changes of distilled water of 5 min each. The slides were immersed in the staining solution for 3 days at room temperature, after which they were washed in two changes of distilled water, dehydrated through the alcohol series transferred to xylene, drained and allowed to dry. The slides were mounted in Cargille refractive index oil, carefully chosen to match the refractive index of the cells which usually was found to be 1.556 for these preparations.

When this method is used, the nucleoli and the cytoplasm of the leukocytes remain unstained, and the nuclei stain a clear gray-blue.

The Feulgen reaction was carried out following the protocol given by Deitch (9) modified in the following details: hydrolysis was in 5 N hydrochloric acid at room temperature for 1 hr, the Schiff reagent was prepared using National Aniline basic fuchsin (C.I. 42510) and a total of only 6 min was spent in the three wash solutions. Oil of refractive index 1.552 usually was the most appropriate for mounting.

CYTOPHOTOMETRY

Measurements were made with either a two-wavelength or a scanning cytophotometer.

The scanning instrument is the mechanical image plane scanner of CYDAC. The main features of CYDAC and its operation as a CRT flying spot scanner have been described (5, 26), while the specific features peculiar to the use of the mechanical scanner as an integrating cytophotometer are the subject of the second paper in this series. The spectral band width of CYDAC was defined by an interference filter nominally centered on 566 nm (peak at 572 nm, half-band width 17 nm), which is close to the spectral absorption peaks of both the gallocyanin-chrome alum and the Feulgen preparations. The objective was a 40X oil immersion apochromat, NA = 1.00, and the condenser aperture was set to NA = 0.3.

B. H. Mayall and M. L. Mendelsohn, manuscript in preparation.
The two-wavelength instrument is an automatic cytophotometer, DATEM, and has been described in detail elsewhere (23). This instrument was used to measure only a single gallocyanin-chrome alum preparation. The wavelengths were chosen to give the necessary absorption ratio by measuring small apparently homogeneous areas from several cells on this slide; the mean value was used, and corresponded to wavelengths of 560 nm and 450 nm. The objective was a 100X planachromat, NA = 1.25, and the condenser aperture was set to NA = 0.3.

RESULTS

CYDAC measurements: Over a period of months, slides were prepared from the blood of four apparently healthy adults and were stained with either gallocyanin-chrome alum (GCA) or with the Feulgen reaction. For each spread, the stain contents of monocytes, neutrophilic granulocytes and small lymphocytes were measured on the mechanical scanner of CYDAC; 10, 15 or 20 cells of each type were measured. The mean and the coefficient of variation were calculated for each set of measurements, and these are given in Table I.

The average stain intensity shows highly significant interslide variation. When the means of the three cell types are pooled for each slide, the interslide coefficient of variation is 11% for the 12 GCA preparations and 33% for the seven Feulgen preparations.

The variation within each slide, both among types and among cells within types, is significantly less than the variation among slides. The differences among cell types within each slide were expressed as ratios of the means of the measurements for the individual types, and the double sided t test was used to test significance. Table I tabulates both the ratios and the results of the significance tests. For the 12 GCA preparations, monocytes are significantly greater than small lymphocytes in all cases, and significantly greater than neutrophils in six cases; in addition, neutrophils are significantly greater than small lymphocytes in seven cases and significantly less in two cases. For the seven Feulgen preparations, monocytes are significantly greater than small lymphocytes in five cases, and significantly

<table>
<thead>
<tr>
<th>Stain and Donor</th>
<th>Means</th>
<th>Coefficient of Variation</th>
<th>Ratio of Means</th>
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<tbody>
<tr>
<td></td>
<td>Monos</td>
<td>Neutros</td>
<td>Lymphs</td>
</tr>
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<td>-----------------</td>
<td>-------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>GCA M</td>
<td>15.15</td>
<td>11.12</td>
<td>12.64</td>
</tr>
<tr>
<td></td>
<td>17.92</td>
<td>12.73</td>
<td>14.20</td>
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<td></td>
<td>21.44</td>
<td>16.28</td>
<td>16.78</td>
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<tr>
<td>Feulgen M</td>
<td>6.69</td>
<td>6.50</td>
<td>6.66</td>
</tr>
<tr>
<td></td>
<td>6.69</td>
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<td>10.28</td>
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<tr>
<td></td>
<td>12.05</td>
<td>11.27</td>
<td>11.21</td>
</tr>
</tbody>
</table>

* Means are expressed in absorbance units (area (square microns) times optical density at 566 nm).
* Significantly different from 1.000 at the 1% level.
* Significantly different from 1.000 at the 5% level.
greater than neutrophils in six cases; and neutrophils are significantly greater than small lymphocytes in one case and significantly less in four cases.

The average differences among cell types over the entire experiment were calculated from the means of the different sets of ratios for all of the slides stained by the same method, and the double sided t test was used to test the significance of these mean ratios. Both the mean ratios and their significances are given in Table II. On the average, monocytes in GCA preparations are 16% greater than small lymphocytes and 13% greater than neutrophils. With Feulgen preparations, the differences are somewhat less, and the monocytes are 4% greater than small lymphocytes and 6% greater than neutrophils. All four of these percentages are significantly greater than zero. However, the differences between neutrophils and small lymphocytes are not significant with either stain.

It is possible that the differences among cell types are a function of the intensity of staining. To test for such a relationship, the stain intensity was taken as the mean of the measurements for the three cell types on each slide, and the regression of the ratio between cell types on stain intensity then was analyzed for each of the three sets of ratios and for both of the stains. In none of the six cases is the regression significant at the 5% level.

The coefficients of variation tabulated in Table I estimate the variance associated with the measurements made within types and within slides. The mean and standard deviation of the coefficients are 4.5 ± 2.1% for the 36 sets of measurements from the GCA preparations, and 3.1 ± 1.8% for the 21 sets from the Feulgen preparations. The statistical significance of these average residuals cannot be evaluated because of the significant heterogeneity among the coefficients of variation. However, in one of the GCA sets and in eight of the Feulgen sets, the coefficients indicate that the total variation associated with the measurements is less than the upper limit of the estimated replication error, and in four of these Feulgen sets the coefficients actually are less than the lower limit of replication error. All of these nine cases are compatible with zero residual variation, but the latter four cases raise doubt about the validity of applying an external estimate of measuring error to the results of this experiment.

There still is the possibility that the differences found among cell types and among cells within types are peculiar to the measuring system and stem from a systematic bias present in the scanning cytophotometer used in this experiment. The following experiment, involving an instrument based on a different cytophotometric principle, explores this possibility further.

**CYDAC versus DATEM:** A population of 45 leukocytes, consisting of 15 cells of each of the three leukocyte types, was chosen from a single GCA-stained preparation. These cells were mapped photographically and their stain content was measured both with the mechanical scanner of CYDAC and with DATEM, the two-wavelength instrument. The measuring sequence was repeated on different days to give three measurements for each of the slides stained by the same method, and the double sided t test was used to test the significance of these mean ratios. Both the mean ratios and their significances are given in Table II. On the average, monocytes in GCA preparations are 16% greater than small lymphocytes and 13% greater than neutrophils. With Feulgen preparations, the differences are somewhat less, and the monocytes are 4% greater than small lymphocytes and 6% greater than neutrophils. All four of these percentages are significantly greater than zero. However, the differences between neutrophils and small lymphocytes are not significant with either stain.

### TABLE II

**Mean Ratios between Cell Types, Combined Data, CYDAC Measurements**

<table>
<thead>
<tr>
<th>Stain</th>
<th>No. of Preparations</th>
<th>Mean Ratio ± 1.S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mono-neutro</td>
</tr>
<tr>
<td>Galloycyanin-chrome alum</td>
<td>12</td>
<td>1.130 ± 0.044*</td>
</tr>
<tr>
<td>Feulgen</td>
<td>7</td>
<td>1.063 ± 0.017*</td>
</tr>
</tbody>
</table>

* Significantly different from 1.000 at the 5% level.
* Significantly different from 1.000 at the 1% level.
Each cell on each instrument. The data for each instrument were normalized by dividing every measurement by the mean for all the measurements on that instrument.

The means of the normalized replicate measurements for each cell are plotted in Figure 1, with CYDAC measurements along the abscissa and DATEM measurements along the ordinate. If there were perfect agreement between measurements with the two instruments, then all of the points would lie on the line of unit slope passing through the origin. Although there is some spread in the measurements, the values tend to lie along this line and to fall into loose clusters according to cell type. The correlation coefficient between the instruments is 0.87 and the slope of the regression through these measurements does not differ significantly from 1.0. For several of the monocytes, there is considerable divergence between the measurements on the two instruments; this is probably due to inaccuracies associated with the DATEM measurements, as these cells gave transmission values which were at the limits for the accurate solution of the two-wavelength equations by DATEM's computer.

When the normalized measurements from the two instruments are pooled, the mean of the monocytes is 15.3% greater than the mean of the small lymphocytes, and 9.2% greater than the mean of the neutrophils. In addition, the mean of the neutrophils is 5.6% greater than the mean of the small lymphocytes. All of these differences are significant at the 1% level using the double sided t test.

These relationships were analyzed further by entering the normalized measurements into an analysis of variance with the results given in Table III. There is a significant divergence between measurements on the two instruments which can be ascribed mainly to the outlying monocytes. Nevertheless, the divergence between the instruments is significantly less than the differences found among cell types and even is significantly less than the differences found among the individual cells within each type. The replication error associated with the two-wavelength measurements is 3.7%, while an error of 1.7% is associated with the scanning measurements.

**DISCUSSION**

Leukocytes from peripheral blood have many properties that make them suitable objects for DNA cytophotometry (11, 16). They are readily obtained and prepared as spreads of well flattened, isolated, intact and classifiable cells. Furthermore, they are postmitotic diploid cells that are not involved in the synthesis of DNA. These properties of leukocytes have led to their widespread use as internal cytophotometric standards. Leukocytes are very useful for es-

*The University of California computer program, BMD08V, was used for the calculations, and the design was specified as hierarchical with a mixed model (33).*
establishing the ploidy values of unknown populations, but they are not suited to use in establishing the presence or absence of small differences in DNA content because of the uncertainties that still accompany leukocyte cytophotometry.

The measurement associated with any cell is determined by three factors: the cellular DNA content, the stoichiometry of the DNA staining reaction and the accuracy with which the stain content is measured. In addition, the measurement will include an element of statistical uncertainty which is composed of the variations associated with staining and measurement and the possible variation associated with DNA content. In order to test the hypothesis of DNA constancy rigorously, it is necessary first to isolate the variation due to staining and measurement and then to estimate the variation attributable to DNA content. In practice it is not possible to isolate each factor completely, but in any case the composite residual variation associated with measurements places an upper limit on the possible variation in DNA content.

In the data presented in this paper, only a limited and partial isolation of the variables is possible. Nevertheless, the data still can be analyzed in some detail, and the results interpreted in terms of the three factors. The results of such an analysis show that:

1. Large and highly significant variation is found in stain intensity from slide to slide. This effect is seen with both GCA and Feulgen preparations, but is larger with the latter. Interslide variability could not be correlated with any observed characteristics of the slides or with intraslide variability, and it is much too large to be due to any known source of instrument error. As it is found even among slides from the same individual, it represents a major source of uncertainty that can be ascribed to neither biologic nor instrument variability. This uncertainty affects interslide measurements primarily, but one must anticipate that a poorly defined error of this magnitude is likely to influence intraslide measurements as well. Slide to slide variations have been found by others, even when great care was taken to eliminate potential sources of variation (32, 35). The simplest explanation of interslide variation is that it reflects uncontrolled changes in the stoichiometry of the staining reactions. In the present experiments, time in absolute alcohol, age of staining solutions and changes in room temperature were not controlled closely and all could have contributed to the interslide variability. However, it seems likely that the intensity of staining is influenced more by other uncontrolled factors and in particular by the degree of chromatin denaturation associated with cell flattening and spread preparation.

2. Within slides, monocytes generally measure significantly greater than small lymphocytes and neutrophilic granulocytes. With Feulgen staining, the mean differences are about 5%, and so are about half those reported by Atkin and Richards (3) and by Hale (16). GCA preparations show the same effect, but in this case the mean differences are about 15%. Furthermore, the GCA preparations show greater variability, both for cells within types and for the ratios between types. With either stain, there frequently are significant differences between small lymphocytes and neutrophils, but these differences are not consistent and cease to be significant when data from the different slides are pooled.

3. For single types of leukocytes on single slides, the measured variation among cells averages 4.5% for GCA preparations and 3.1% for Feulgen preparations. When allowance is made for the random measuring error, the residual variations average 4.2% and 2.6% respectively. The coefficient of variation associated with any given set of measurements places an upper limit on the variation of DNA content among the cells of the set. There is significant heterogeneity among the coefficients, but for some of the sets the variation of the measurements is consistent with the hypothesis of DNA constancy. However, it is only within this very limited context that constancy is demonstrated. The same measurements show significant variation among types and among slides, and it is clear either that DNA content varies or that DNA is not being expressed correctly by measured stain content.

4. Significant differences among cell types and among cells within types are found with both scanning and with two-wavelength cytophotometry. In spite of the dissimilarity of the two methods, there is close agreement between measurements made on the scanning and the two-wavelength instrument used in these experiments. When Mendelsohn and Richards (27) compared measurements made with a scanning and with a two-wavelength instrument, they found a high degree of proportionality with a coefficient of
variation between instruments of 3.4%. In the present data, the coefficient of variation between the instruments is 2.1%. This implies that, for 95% of the measurements, readings on one instrument will agree with readings on the other instrument to within 4.2%. Furthermore, the results indicate that the differences found among cells cannot be ascribed to an artifact that is peculiar to the mechanical scanner of CYDAC. It is conceivable that the nonlinearity associated with the two measuring systems could be almost identical, but it would seem more reasonable to infer that the differences observed among cells reflect a phenomenon which is independent of the measuring instrument and that these differences will be found with any cytophotometric method of adequate resolution.

These differences in the measured stain content of human leukocytes cannot be considered as exceptions to DNA constancy until it is shown conclusively that they are due neither to systematic bias associated with the cytophotometric methods nor to variation in the DNA staining reactions. The observed differences correlate closely with some of the morphologic features used to identify and classify the cells; thus the large nucleus and fine chromatin patterns characteristic of monocytes are associated with high stain values, while the clumped and dense chromatin of small lymphocytes and neutrophils is associated with low values. Differences in chromatin compaction can have both optical and chemical consequences, and so could be the source of photometric and stoichiometric variability. These effects will be examined further in subsequent papers.

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