Microspectrophotometric Determination of Desoxyribonucleic Acid in Megaloblasts of Pernicious Anemia

By Edward H. Reisner Jr., M.D. and Roy Korson, M.D.*

With the isolation of vitamin B₁₂ and its demonstration as the active anti-pernicious anemia substance of liver,²,³ attention was focused on some of its biochemical properties. It was shown that in the growth requirements of various micro-organisms, vitamin B₁₂ could be replaced by various desoxyribosides,⁴,⁵ from which evidence its role in desoxyriboside and nucleic acid synthesis could be inferred. Pteroylglutamic acid (PGA) has been shown to be replaceable by thymine in the growth of certain bacteria⁶ and Shive⁷ has shown that both B₁₂ and PGA are intimately involved in the synthesis of both amino acids and purine and pyrimidine bases. In man, the test-tube relationships of PGA and thymine have been reproduced clinically⁸ and thymidine in amounts up to 150 mg. has been shown to produce slight reticulocyte activity but has not been able to effect remission in pernicious anemia.⁹ From these studies it seemed possible that the deficiency state in pernicious anemia might lead to a decrease in the amount of desoxyribonucleic acid in the megaloblast nucleus, and that the paler staining properties of such nuclei might be attributable to a lack of that substance.

The microspectrophotometric technic originated by Caspersson¹⁰ provides a valuable aid in the quantitative estimation of cellular constituents of the magnitudes found in individual cells. Thorel¹¹,¹² has used this technic to determine the amounts of ribonucleic acid in maturing blood cells by measuring their absorption of ultraviolet light. With the aid of refinements devised by various authors¹³ it is now possible to measure the relative amounts of desoxyribonucleic acid (DNA) using the Feulgen stain,¹⁴ and its degree of polymerization with methyl green.¹⁵

The present studies were undertaken to see if there was any demonstrable difference between normal red blood cell development and that seen in pernicious anemia in terms of the nuclear DNA measured throughout the maturation process and in nuclei of comparable stages of maturity in the marrows of patients with pernicious anemia before and after treatment. Also, a study of the degree of polymerization of DNA in the cell nuclei before and after treatment was made, using methyl green as the dye. The validity of making photometric measurements on blood films has been established and a complete discussion of the details is published elsewhere in a paper by Korson.¹⁶

Methods and Materials

Aspirations of sternal marrow from normal human subjects and 9 patients with pernicious anemia, nutritional macrocytic anemia and megaloblastic anemia of infancy were obtained

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before treatment with liver extract, folic acid or vitamin B₁₂, and after treatment when
the red blood count had reached a level of over 3,000,000 or the reticulocytosis had subsided.
Smears were made on clean glass slides taking care to make them of as uniform thickness as
possible. The slides were then fixed in 95 per cent methyl alcohol, air dried, and stained with
Feulgen stain (prepared according to Stowell) after a twelve minute hydrolysis at 56 C.

A graded series of red cell nuclei 11.3, 9.5, 7.0, 5.3 and 4.5 μ in diameter was measured in a
normal (post-hemorrhagic) subject and in a patient with pernicious anemia before and
after treatment. These sizes were chosen because they represented various stages in erythro-
blast maturation, including basophilic and pro-normoblasts and megaloblasts and inter-
mediate cells down to orthochromatic normoblasts and megaloblasts, and they were all
sizes that occurred in relatively large numbers on the slides. For the remaining patients,
nuclei of 9.5 μ diameter (a size common to the treated and the untreated states) were meas-
ured through a “core” of 7.0 μ diameter. Previous study had indicated that the nuclei
were flattened and essentially cylindrical in shape. With such material it is possible to
measure a “core” through the nucleus and to use the extinction of this “core” as the ex-
tinction of the whole nucleus. The size of the “core” used in measuring each nucleus is
given in table 1. Control slides which contained unstained cells were used to estimate the
nonspecific light loss due to differences in refractive index within the absorbing material.
These blanks were so low in extinction (.004 to .000) as to be negligible.

**TABLE 1.—Diameter of Nucleus and “Core” Used for Measuring Extinction**

<table>
<thead>
<tr>
<th>Diameter of Nucleus (in microns)</th>
<th>Diameter of Core (in microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.3</td>
<td>7.0</td>
</tr>
<tr>
<td>9.5</td>
<td>7.0</td>
</tr>
<tr>
<td>7.0</td>
<td>5.3</td>
</tr>
<tr>
<td>5.3</td>
<td>4.4</td>
</tr>
<tr>
<td>4.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

The uniformity of the smears themselves is evident from the strikingly similar extinc-
tions obtained for nuclei of the same diameter on various slides.

The apparatus used was similar to that described by Pollister and Moses. The photo-
metric light source was a zirconium arc lamp, Western Union type J-100, a Farrand inter-
ference filter with maximum transmission at 550 μ being used for relative monochromatic-
ity. Transmitted light was measured by an RCA photomultiplier tube (931-A) and recorded
on a Weston microammeter.

In 6 cases, a second set of slides was stained with methyl green solution. This was made
by dissolving 150 mg. of purified methyl green (recrystallized several times from chloro-
form) in 100 cc. of the following mixture: phenol 0.5 Gm., glycerin 20 cc., 95 per cent ethyl
alcohol 2.5 cc., water 100 cc. The slides were stained for forty-five minutes at 56 C., then
rinsed in ice water. After being blotted dry, they were differentiated overnight in tertiary
butyl alcohol at room temperature, rinsed in xylol and mounted in clarite. This
is the technic of Pollister and Leuchtenberger. The methyl green slides were measured with light passed through a Farrand interference
filter with maximum transmission at 630 μ.

**RESULTS**

The results are depicted in tables 2 and 3 and figure 1. In interpreting them
it should be emphasized that if nuclei of the same diameter and thickness give
the same extinction values, the absorbing material within them is present in a
constant amount. It can be seen that there was no significant deviation in the
total amount of DNA (Feulgen stain) or the amount of polymerized DNA (methyl green stain), before or after treatment.

**Table 2.—Mean Extinction Values and Standard Error of the Mean for Feulgen and Methyl-Green Stained Erythroblast Nuclei All .5 μ in Diameter.**

*From Human Marrow Aspirations*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Feulgen</th>
<th></th>
<th>Methyl-Green</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Treatment</td>
<td>After Treatment</td>
<td>Before Treatment</td>
<td>After Treatment</td>
</tr>
<tr>
<td><strong>No. Nuclei</strong></td>
<td>Extinction</td>
<td></td>
<td>Extinction</td>
<td></td>
</tr>
<tr>
<td>1. Nutritional macrocytic anemia</td>
<td>20 0.250 ± .009</td>
<td>30 0.247 ± .007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Megaloblastic anemia of infancy</td>
<td>30 0.244 ± .007</td>
<td>5* 0.244 ± .008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Pernicious anemia</td>
<td>10* 0.232 ± .007</td>
<td>20 0.242 ± .005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Pernicious anemia</td>
<td>35 0.249 ± .005</td>
<td>27 0.238 ± .005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Pernicious anemia</td>
<td>30 0.240 ± .004</td>
<td>35 0.240 ± .005</td>
<td>20 0.136 ± .003</td>
<td>10* 0.133 ± .005</td>
</tr>
<tr>
<td>6. Pernicious anemia</td>
<td>30 0.231 ± .005</td>
<td>40 0.237 ± .006</td>
<td>11* 0.131 ± .005</td>
<td>20 0.143 ± .003</td>
</tr>
<tr>
<td>7. Pernicious anemia</td>
<td>50 0.248 ± .005</td>
<td>20 0.242 ± .006</td>
<td>5* 0.148 ± .008</td>
<td>20 0.141 ± .002</td>
</tr>
<tr>
<td>8. Pernicious anemia</td>
<td>35 0.231 ± .006</td>
<td>20 0.235 ± .007</td>
<td>12* 0.146 ± .003</td>
<td>20 0.150 ± .034</td>
</tr>
<tr>
<td>9. Pernicious anemia</td>
<td>40 0.256 ± .006</td>
<td>30 0.248 ± .005</td>
<td>15 0.140 ± .004</td>
<td>20 0.148 ± .003</td>
</tr>
<tr>
<td>10. Normal</td>
<td>30 0.231 ± .003</td>
<td></td>
<td>20 0.138 ± .003</td>
<td></td>
</tr>
</tbody>
</table>

* In these smears hypocellularity made it difficult to find larger numbers of nuclei of the desired diameter.

**Table 3.—Extinction Values of Nuclei of Various Diameters in Normal Patients and Patients with Pernicious Anemia in Relapse—Treated and Untreated**

<table>
<thead>
<tr>
<th>Diam. of Nucleus (in Microns)</th>
<th>Normal</th>
<th>Untreated Patients</th>
<th>Treated Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.3</td>
<td>10</td>
<td>0.185</td>
<td>10</td>
</tr>
<tr>
<td>9.5</td>
<td>10</td>
<td>0.232</td>
<td>20</td>
</tr>
<tr>
<td>7.0</td>
<td>10</td>
<td>0.400</td>
<td>10</td>
</tr>
<tr>
<td>5.3</td>
<td>10</td>
<td>0.508</td>
<td>15</td>
</tr>
<tr>
<td>4.5</td>
<td>10</td>
<td>0.649</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 3 shows that nuclei of the same size, whether they are from normal subjects, or from treated or untreated pernicious anemia patients, show approx-
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imately the same extinction. This suggests that an equal amount of DNA is present in each case and that the rate of change of DNA concentration with increasing maturity of the cell is also the same in each instance.

That DNA is actually lost from the nuclei during maturation is seen from the graph in figure 1. As a reference curve the extinction values that would be obtained if the DNA remained constant in the progressively shrinking nuclei were plotted against nuclear area. This is based on the fact that extinction times area gives a measure of the absorbing material present and for a constant amount of absorbing substance as area decreases, extinction increases. The three experimental curves are all lower than the reference curve by practically the same amount, indicating a similar rate of DNA loss for each one.

![Graph](image)

**Fig. 1.**—Change of extinction with cell size. The dotted line represents a hypothetical curve calculated on the basis of no change in the amount of DNA per nucleus (E × A = K). The solid line shows that DNA is actually decreased as the cell matures.

**DISCUSSION**

Thorell\(^1\) has shown that as red cells mature there is a rapid decline in the quantity of cytoplasmic ribonucleic acid (RNA) during the earlier stages of maturation (between the pronormoblast and basophilic normoblast) after which it rapidly disappears with further maturation of the cell. In contrast, in pernicious anemia in relapse there is a continued high concentration of RNA in the cytoplasm throughout the maturation process. Our results show that the nuclear DNA undergoes a steady and gradual diminution to the lower limits of accurate measurement imposed by nuclear diameter. Furthermore, there is no discernible difference in the rate of loss of DNA from the nuclei of normal blood cells and those of pernicious anemia in relapse or after treatment, or in the amounts of total or polymerized DNA.

Swift\(^1\) has shown that in polyploidy the DNA is proportional to the increase
in the number of chromosomes, e.g., diploid, tetraploid, octoploid. Since one would not expect the chromosomal make-up of a patient with pernicious anemia to change following treatment, it appears more reasonable to hypothesize that a lack of cell constituents essential for rapid DNA formation would impede the formation of extra amounts of DNA required for mitosis and bring about the condition of retarded blood formation characteristic of pernicious anemia.

The megaloblasts of pernicious anemia are characterized by a more particulate type of chromatin than are the normoblasts of corresponding maturity. Such structural characteristics are easily observed in marrow smears stained with one of the Romanovsky stains. Since the substance in the nucleus stained by this group is probably DNA, the same phenomenon should be seen with Feulgen stained preparations. The accompanying photographs (figure 2) taken under identical conditions, show a more particulate chromatin in the nuclei of an un-

![Megaloblasts](image)

**Fig. 2.**—Megaloblasts (*left*) 9.5 μ in diameter—before treatment. Normoblasts (*right*) 9.5 μ in diameter—after treatment. Both slides stained with Feulgen.

...treated patient, and a generally “lighter” appearance of these nuclei. This “lightness” is not borne out, however, by photometric measurement of hundreds of cells. It may be that the nuclei with a less particulate and more clumped structure are actually later resting phase nuclei. Perhaps this clumping precedes an increase in DNA for the ensuing mitotic division, and since the number of cell divisions is less frequent in pernicious anemia, one sees less pre-divisional aggregation. In any event, it is shown by the methyl green studies that such change in chromatin structure is not accompanied by a change in the polymerization of DNA.

**Summary**

1. In 9 patients with various types of megaloblastic anemia responding to treatment with vitamin B₁₂, folic acid or liver extract, no significant deviations from the normal amounts of total or polymerized DNA were observed in the nuclei of red blood cells in marrow smears.

2. During the maturation of megaloblasts in the bone marrow there is a gradual loss of nuclear DNA.
3. This pattern is quantitatively and qualitatively similar for normal marrow and for that of pernicious anemia in relapse and after treatment.

REFERENCES


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