A MICROSPECTROPHOTOMETRIC STUDY OF RED CELL NUCLEI DURING PYKNOSIS*

By ROY KORSON,‡ M.D.

(From the Department of Zoology, Columbia University, New York)

(Received for publication, September 19, 1950)

Most observations of the process of pyknosis, generally considered to be a reflection of nuclear degeneration, have been confined to morphological changes and little is known of the chemical alterations involved. A microspectrophotometric technique, with which quantitative measurements of cell constituents can be made, has been found to be applicable to a cytochemical study of pyknosis—in this case, the physiological pyknosis of erythropoiesis in which the primitive erythroblast nucleus shrinks, loses its delicate reticular structure, and becomes through successive stages the densely staining, clumped nucleus of the normoblast.

Leuchtenberger (1), using a similar technique, followed the nucleoprotein changes attending nuclear pyknotic degeneration in mouse sarcoma 180.

Thorell (2) studied the red cell maturation process cytochemically, but concerned himself primarily with cytoplasmic alterations.

The present study had a threefold purpose: (1) to learn whether blood smears are measurable microspectrophotometrically; (2) to compare erythropoietic pyknosis with that accompanying necrosis; (3) to determine the fate of the red cell nucleus during maturation.

Material and Methods

Human bone marrow was obtained by sternal puncture, without anticoagulant.[1] The marrow was spread in as uniform a manner as possible on ordinary glass slides. These were then air-dried, fixed in absolute methyl alcohol for 3 to 5 minutes, and dried again. The slides were stained with Unna-Pappenheim's phenol–methyl green mixture for 45 minutes at 56°C. They were then rinsed in ice water, blotted dry, and differentiated overnight in tertiary butyl alcohol at room temperature.

* This investigation was carried out during tenure of a postdoctoral Research Fellowship from the United States Public Health Service, National Cancer Institute.

‡ Present address: Department of Pathology, University of Vermont College of Medicine, Burlington.

[1] Bone marrow was kindly supplied by Dr. E. H. Reisner, Jr., Hematology Division of the New York University Post-Graduate Medical School.

Methyl green has been shown to stain highly polymerized desoxyribose nucleic acid (DNA) specifically, and to combine with this nucleic acid stoichiometrically (3–5).

Loss of methyl green stainability during erythropoiesis would indicate either a depolymerization of DNA or a loss of that material from the nucleus. These alternatives can be resolved by subjecting the methyl green-stained slide to the Feulgen reaction, which is a measure of total DNA, of high and low polymer type. The Feulgen hydrolysis removes the green color of the methyl green. If the Feulgen stainability decreases at the same rate as the methyl green, then DNA is actually being lost from the nucleus. If the Feulgen value remains constant and the methyl green stainability is decreased, depolymerization alone is occurring (1).

After measurement of methyl green colorization, the same slides were stained by means of the Feulgen reaction, following the method outlined by Stowell (6). Hydrolysis was carried out at 56°C. for 12 minutes.

Measurements of the amounts of dye combined with DNA were made with a microspectrophotometer which has been described by Leuchtenberger (1), and Pollister and Moses (7). The light source was a J-100 Western Union zirconium arc lamp. Approximately monochromatic light was obtained by means of Farrand interference filters, the wave lengths used being those of maximal absorption peak of the dyes (550 millimicra for Feulgen and 625 millimicra for methyl green). The transmitted light was amplified by A.C. power-supplied RCA photomultiplier tubes—931-A for Feulgen, and 1P22 for methyl green.

In practice the nucleus to be measured was located in the microscopic field. Its image was projected into the plane of an iris diaphragm situated above the microscope. The diaphragm was closed down to exclude everything but a concentric area through the nucleus (see Table II and discussion of Nuclear Flattening below) and the transmission of the nucleus recorded on a Weston microammeter *via* the phototubes. As a background for 100 per cent transmission, an empty area on the slide, containing no cells but covered by mounting medium and a coverslip, was used. The transmission of the specimen was converted to extinction (or optical density) from the equation $E = \log \frac{1}{T}$, where E is extinction and T is transmission. It is the extinction value which varies directly with the amount of absorbing material in the light path, and which gives a quantitative estimate of the substances measured (1, 8).

Experimental Procedure

Before measuring the graded series of nuclei selected as representative of erythropoietic classes, it was necessary (1) to establish the validity of using films rather than sections, (2) to determine the uniformity of cell thickness in such films, and (3) to determine what portion of the whole nucleus should be measured.

Reproducibility of Readings.—Ten to fifteen readings were made on the same nucleus (stained with Feulgen dye), under the same optical conditions, on successive days. The stability of the instrument was such that repeated readings of light transmission varied no more than 2 per cent.

Degree of Nuclear Flattening.—Nuclear flattening was estimated by measuring the extinction of concentric "cores" taken through the nuclei.

If the nuclei were spherical as in A, Fig. 1—and the light being measured travelled through the nucleus in the direction shown by the arrow, the light absorbed by each part of the whole

nucleus (distance a to a') would be variable, depending on which part of the nucleus the light passed through. The path of the light at the sides of the nucleus would be shorter than that near the central axis. The mean optical path is the average of these various lengths. In a smaller core (distance b to b') the distance through the nucleus which the light travels becomes more uniform, and the mean distance travelled is longer. Similarly in a still smaller core (c to c') the various paths are even more uniform and the mean path is again longer. Since absorption varies directly with thickness (*i.e.*, it is greater when the mean path is

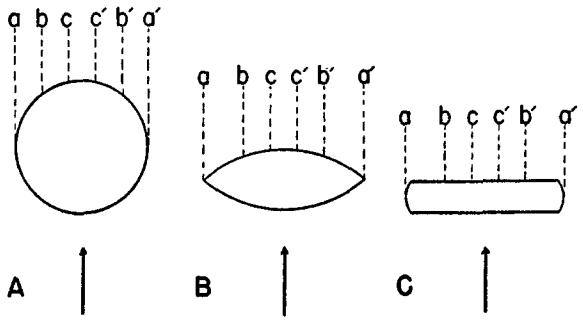

FIG. 1. Diagrammatic representation of nuclear flattening, and length of optical path through nuclei flattened to different degrees.

TABLE I

Extinction Values of Concentric Cores through Erythroblast Nuclei 11.3 Micra in Diameter

Core diameter	Extinction
μ	
8.5	0.152
8.0	0.152
7.0	0.155
6.1	0.155
5.3	0.155
3.5	0.155

greater) the extinction obtained in the last case should be higher than in the first. This phenomenon was verified on spherical nuclei (mouse liver stained with Feulgen dye).

If the nuclei were partially flattened as in B, Fig. 1, the same effect, higher extinctions as one chooses a concentric core nearer the axis, would be observed, but the difference in extinctions would not be as great.

If the nuclei were completely flattened (C, Fig. 1), the mean thickness would be virtually the same throughout, the very slight rounding of corners being undetectable. Hence whatever core was used, the extinction obtained would be the same. Table I shows a series of measurements made on erythroblasts 11.3 micra in diameter, using progressively smaller concentric cores.

The constancy of the extinction values suggests complete flattening, the nuclei being essentially short cylinders. Similar measurements made on all sizes of nuclei used in this study showed them to be flattened in the same way.

Uniformity of Nuclear Thickness.—On a given slide, extinction values were obtained for similar cells of the same diameter (Table II). The uniformity of thickness was found to be quite good, for extinctions clustered closely about a mean value. The mean varied little from slide to slide, indicating a constancy in the smears.

Distribution of Absorbing Material.—The Feulgen-positive and methyl green–stained material is distributed in the earliest cells as a finely dispersed granular substance. In the later elements it is a densely packed, uniformly dark material. While the ideal substance for photometry is a homogeneous one, and while distributional errors are difficult to estimate, they need not introduce large discrepancies. Indeed if such errors could be entirely corrected, the results reported here would be enhanced.

If one measures photometrically, material distributed in clumps, the extinction values obtained will be too low, due to the passage of light unopposed through the non-absorbing

TABLE II

Extinction Values of Erythroblast Nuclei 9.5 Micra in Diameter, on Two Slides

Slide 1		Slide 2	
Nucleus	Extinction	Nucleus	Extinction
1	0.26	1	0.24
2	0.21	2	0.24
3	0.22	3	0.24
4	0.27	4	0.25
5	0.26	5	0.24
6	0.24	6	0.24
7	0.24	7	0.25
8	0.24	8	0.27
9	0.25	9	0.28
10	0.26	10	0.26
Mean.....	0.245	Mean.....	0.251

areas. An estimate of the quantity of absorbing material will then be too low. This error is eliminated as the absorbing material becomes homogeneous.

Thus, the relative values for nuclear DNA in the early erythroblasts, in which distribution is less homogeneous, would if anything be too low. Corrected values would heighten the contrast reported under Results.

Other errors such as "non-specific" light loss due to diffraction and refraction of light by the structural arrangement of the cell can be wholly corrected for (8). This is done by measuring appropriate "blank" slides which have the same morphological features as the test material, but which lack stain. The extinction of such "blanks" can be subtracted from the total extinction, giving the absorption of the dye alone. Blank slides were made for all cell types measured and the extinction values obtained. These were so low (0.004 to 0.000) as to be negligible.

Types of Cells Measured.—The type of cell used, its size, and the diameter of core employed in each case are given in Table III. All nuclei were in the resting stage; no dividing cells were measured. The sizes of the nuclei were chosen because they represented distinct stages of pyknosis and because they occurred in relatively large numbers on the slides. The cells are classified by the nomenclature of Dameshek and Valentine (9).

The use of the core technique in which the extinction of a central portion of the nucleus equals the extinction of the whole structure is validated by the cylindrical nature of the nuclei in the smears. Cores were employed electively to avoid diffraction losses around the

TABLE III

Cell Types Measured, with Diameters of Nuclei and Cores Used

Type of cell	Diameter of nucleus	Size of core
	μ	μ
Pronormoblast	13.9	7.0
Basophilic normoblast	11.3	7.0
Polychrome normoblast	9.5	7.0
Polychrome normoblast	7.0	5.3
Orthochromatic normoblast	5.3	4.4
Orthochromatic normoblast	4.5	3.5

TABLE IV

Relative Amounts of DNA (Expressed as Extinction × Area) in Nuclei of Various Sizes during Erythropoiesis

Feulgen			
No. of nuclei measured	Diameter	Mean extinction	Extinction × area
	μ		
10	13.9	0.131	19.8
55	11.3	0.192	19.3
40	9.5	0.248	17.5
55	7.0	0.426	16.3
50	5.3	0.538	11.8
50	4.5	0.685	10.8
Methyl green			
20	11.3	0.062	6.2
20	9.5	0,083	5.9
20	7.0	0.134	5.2
15	5.3	0.177	3.9
10	4.5	0.225	3.6

edges of the nuclei and to keep the diameter of the area measured within the cathode size of the phototubes. The phototubes employed gave a linear response in current for increase in area of light measured up to a maximum circle of 8.5 micra in diameter. The largest core used was 7 micra.

RESULTS

Extinction and DNA content of pyknotic cells, as measured photometrically, are summarized in Table IV.

It follows from the equation expressing the Beer-Lambert law that the product of nuclear area times its extinction gives a measure of the total amount of absorbing material present. This value can be stated in absolute terms if the extinction coefficient of the absorbing material is known. On the other hand, relative values can be utilized if the extinction coefficient is treated as a constant (10). No attempt is made here to state absolute amounts of DNA per nucleus, but rather relative amounts in the various nuclei.

The Feulgen-stained nuclei show a progressive loss of absorbing material as the nucleus undergoes maturation and pyknosis. Similarly, the methyl green series shows a progressive loss of staining material.

Table V shows ratios of extinction with Feulgen dye to extinction with methyl green for each nuclear size.

A comparison in the Feulgen series reveals the DNA content of the smallest cells to be about one-half (55 per cent) that of the largest ones. In Leuchtenberger's series of pyknotic sarcoma cells (1), the smallest measured cell had 52 per cent of the DNA of the larger "fresh" cells.

TABLE V

Ratio of Extinction with Feulgen Dye to Extinction with Methyl Green for Nuclei of Various Sizes during Erythropoiesis

Nuclear diameter	Feulgen/methyl green ratio
μ	
11.3	3.1
9.5	3.0
7.0	3.1
5.3	3.0
4.5	3.0

DISCUSSION

The results of the foregoing experiments in which it is shown that human erythroblasts undergoing maturation show a steady decline in the total amount of DNA in the nucleus bear on two interesting questions. The first is whether the physiological pyknosis of erythropoiesis is a process similar to that of nuclear degeneration seen in pathological tissue. The constancy of the ratios indicates a parallel rate of decrease of absorption for both dyes. This can be interpreted as a loss of DNA rather than depolymerization alone. In terms of such loss of DNA there is a strikingly close agreement between the largest and smallest cells measured in bone marrow, and those in pathological material. In the sarcoma cells, studied by Leuchtenberger (1), however, an initial depolymerization of DNA was noted before the gradual loss of that material occurred. There are several possible explanations for the absence of this phe-

nomenon in the present results. For example, the basophilic erythroblasts used here are not the earliest cells in the erythropoietic series. Perhaps depolymerization has already taken place in the first cells of the present series, and it may be that the earliest erythroblasts in the marrow would show an equal amount of Feulgen- and methyl green–staining material. Again, all the films measured here were air-dried. It is conceivable that this process depolymerizes some of the DNA in all nuclei—enough at least to give the 3 to 1 Feulgen to methyl green ratio obtained in all cases.

Additional intimation of a common "pyknotic process" is found in data reported elsewhere (11) which show that in normal human beings, those with pernicious anemia in relapse, and those with treated pernicious anemia, pyknotic nuclei are quantitatively similar in respect to DNA loss.

The second question to which an answer is suggested concerns the fate of the mammalian red cell nucleus. General opinion is divided into two groups (12). One group (13, 14) maintain that the nucleus is extruded as a unit from the cells. Indeed some authors claim to have seen nuclei extruded or in the process of being extruded. The second group (15, 16), on the other hand, suggest that the nucleus undergoes dissolution in the cell and thus disappears. Davidson, for example (16), says, "From [my] observation of bone marrow and blood, the nucleus disappears by intracellular solution through the process of pyknosis, karyorrhexis, and karyolysis." And Di Robertis (17) says of nuclear senescence, "The process of pyknosis is generally accomplished by a shrinkage of the nucleus and the disappearance of its structural details (perhaps due to dissolution of the nucleic acid into the cellular sap)."

It is this latter point of view that appears to be substantiated by the data presented. There is a loss of half the DNA by the time the normoblastic stage is reached. Later normoblastic nuclei might show still further loss of DNA but smaller nuclei were not amenable to the photometric technique described. That the nucleic acid is not simply moved out to the cytoplasm but is degraded or actually lost from the cell is seen from the complete absence of all stain in the cytoplasm.

While it could be argued that the remaining normoblastic nucleus is extruded as such, there is no clear cut evidence of such an occurrence. Again, it seems important to emphasize that even if such a nuclear extrusion took place, it would not be a whole, intact nucleus that was lost, but only a small, shrunken fraction of the original structure, containing little more than half of the nucleic acid found at an earlier stage.

SUMMARY

1. A microspectrophotometric apparatus was used to measure nuclear absorption in fixed and stained human bone marrow smears.

2. The validity of the measurements was established.

3. The total and polymerized desoxyribose nucleic acid (DNA) of erythroblast nuclei were measured in relative units, and a gradual loss of DNA from the red cell nucleus during maturation was noted.

4. The loss in DNA is comparable quantitatively to that occurring during pathological pyknosis in sarcoma cells.

5. The continual loss of nuclear material during erythropoiesis supports the conception that the nucleus disappears by intracellular dissolution, not by extrusion in the intact form.

The author is indebted to Professor A. W. Pollister, Department of Zoology, Columbia University, for many helpful suggestions.

BIBLIOGRAPHY

1. Leuchtenberger, C., *Chromosoma*, 1950, **3,** 449.
2. Thorell, B., *Acta med. Scand.*, 1947, **129,** suppl. 200, 1.
3. Kurnick, N. B., *J. Gen. Physiol.*, 1950, **33,** 243.
4. Kurnick, N. B., and Mirsky, A. E., *J. Gen. Physiol.*, 1950, **33,** 265.
5. Pollister, A. W., and Leuchtenberger, C., *Proc. Nat. Acad. Sc.*, 1949, **35,** 111.
6. Stowell, R. E., *Stain Techn.*, 1945, **20,** 45.
7. Pollister, A. W., and Moses, M. J., *J. Gen. Physiol.*, 1949, **32,** 567.
8. Pollister, A. W., and Ris, H., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12,** 147.
9. Dameshek, W., and Valentine, E., *Arch. Path.*, 1937, **23,** 159.
10. Ris, H., and Mirsky, A. E., *J. Gen. Physiol.*, 1949, **33,** 125.
11. Reisner, E. H., Jr., and Korson, R., *Blood*, in press.
12. Smith, P. E., and Copenhave, W. M., *in* Bailey's Textbook of Histology, Baltimore, Williams and Wilkins Co., 11th edition, 1944, 173.
13. Maximow, A. A., and Bloom, W., A Textbook of Histology, Philadelphia, W. B. Saunders Co., 5th edition, 1948, 89.
14. Plum, E. M., *Blood*, 1947, Special Issue No. 1, 42.
15. Cooke, W. E., *Brit. Med. J.*, 1930, **1,** 433.
16. Davidson, L. S. P., *Edinburgh Med. J.*, 1930, **37,** 425.
17. Di Robertis, E. D. P., Nowinski, W. W., and Saez, F. A., General Cytology, Philadelphia, W. B. Saunders, 1st edition, 1949, 334.