CYTOCHEMICAL STUDIES OF NUCLEIC ACIDS AND PROTEINS IN ERYTHROCYTIC DEVELOPMENT*

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The process of erythrocytic production offers an excellent system for the study of the relationship between nucleic acids and proteins during differentiation. All stages of development can be obtained in a single given tissue at a given time, and hemoglobin can be used as an indication of the specialization attained. Reticulocytes have provided a relatively homogeneous system for biochemical studies on the relationship of hemoglobin synthesis to reticulocyte RNA.[1-11] While such investigations are obviously of considerable experimental value, they do not reveal the modifications which had occurred prior to the reticulocyte stage and which undoubtedly influenced the metabolic behavior of the reticulocyte. Furthermore, biochemical studies of blood cell formation are limited by the marked heterogeneity in cell populations of various hematopoietic organs and the considerable difficulties in achieving a satisfactory separation of the many cell types.

On the other hand, cytochemical studies can be advantageously employed, since the need for separation of the various cell types is eliminated and cell identification within a given sample rendered possible through direct examination. This investigation examines the sequence of changes in nucleic acid and hemoglobin amounts during the process of development by means of microphotometry and radioautography.

Materials and Methods.—Livers were obtained from 19- or 23-day-old fetal rabbits after administration of Nembutal to the mother. Slides for microphotometric measurements were prepared by touching the freshly cut surface of the liver to a glass slide. These touch preparations or imprints were immersed for 1–2 min in absolute methanol or 10% formalin (pH 7.0) buffered in Sorensen phosphate buffer and subsequently air-dried. For RNA determination, the preparations were stained with Azure B,[13] and mounted in a mounting medium whose refractive index matched that of the cells (see Table 1). For measurement of total protein, Azure B was removed in water adjusted to pH 2.0 with 0.1 *N* HCl and slides restained in 0.001% fast green (FCF) at pH 2.0.[14] The preparations were remounted in a matching refractive index medium and total protein measured in the same cells used for RNA determinations. Hemoglobin measurements were made in the Soret band (420 mμ) on imprints which were lightly stained in Azure B to facilitate recognition and identification of the various erythropoietic cells. At 420 mμ, the light Azure stain gave unmeasurably low extinctions and therefore did not interfere with hemoglobin absorption. Table 1 summarizes the techniques employed for each cellular component. The microphotometric instrumentation and details of the methods used have been previously described.[15] In calculating amounts of bound dye or hemoglobin, the formula M = EA was used where M = amount of bound dye or hemoglobin, E = extinction, and A = area as determined on camera lucida drawings with the polar planimeter. The amount of DNA (Feulgen) per nucleus was calculated by the two-wavelength method.[15, 16]

To justify the use of the formula M = EA on imprints, it was necessary to demonstrate the uniform thickness of individual cells upon the slide. This was performed by obtaining extinctions of variable-sized concentric plugs starting at the center of the cell and progressively increasing plug size until almost the entire cell was included in the field. If the cell was of uniform thickness throughout, one would expect the extinctions obtained at all plug sizes to be equal. Since the extinctions showed no significant variation, it was concluded that the cells in imprints were uniformly flattened disks (see Korson[17]).

TABLE 1

SUMMARY OF TECHNICAL PROCEDURES

Substance measured	Stain	Fixative	Wavelength in mμ	Microphotometric method	Refractive index of mounting medium (n)
1. RNA	Azure B	methanol	530	plug[15]	1.564
2. RNA	Azure B	formalin	530	plug	1.564
3. Total protein	Fast green	methanol	536	plug	1.564
4. Hemoglobin	Natural pigment	methanol	420	plug	1.560
5. Hemoglobin	Natural pigment	formalin	420	plug	1.560
6. DNA	Feulgen reaction	formalin	480; 501	two-wavelength[15, 16]	1.564

Radioautographic analysis of RNA synthesis was performed on 19-day fetal rabbits. The mother was anesthetized with intravenous Nembutal and laparotomy performed. Two hundred μC of tritiated cytidine (specific activity 1.25 C/mM) was injected into the placenta by passing a fine bore needle through the uterine wall. At the end of 6 hr, the mother was sacrificed, and the livers excised from the injected fetuses. Tissues were fixed in cold (0–5°) 10% buffered formalin (pH 7.0) or Zenker-formol for 1 hr and embedded in paraffin. After extractions to ensure specificity of incorporated label, slides were coated with stripping film (Kodak AR-10) and developed after a suitable exposure period. Grain counts were made over the nucleolus, nucleus, and cytoplasm after staining in hematoxylin-eosin-azure[18] or Azure B, and the count expressed in number of grains per square micron. The correction for background was included in the data.

Identification of the various erythropoietic cells was based on the nuclear morphology and cytoplasmic tinctorial properties. In addition, it was found that nuclear area could be arbitrarily used in cellular identification and, for this reason, values were plotted against this parameter. The sequence of erythropoietic cells was considered as follows: hemocytoblast→ proerythroblast→ basophilic erythroblast→ polychromatophilic erythroblast→ normoblast→ reticulocyte→ erythrocyte.[19]

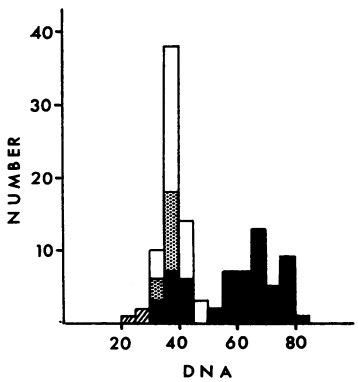

FIG. 1.—Amount of DNA per erythroblast nucleus. Open bars represent late normoblast nuclei; stippled bars represent nuclei in the process of extrusion; cross-hatched bars represent extruded nuclei; solid bars represent a mixed population of erythroblast nuclei exclusive of late normoblast nuclei. Photometric units in abscissa are calculated by the two-wavelength method.[16]

Results.—(1) *DNA:* Microphotometric measurements of DNA in a randomly selected population that included all stages in the developmental sequence result in the composite or "pooled" data depicted in Figure 1. The data show a bimodal distribution into 2C and 4C classes, as well as a number of interclass values characteristic of a proliferating system of cells. In a randomly selected population of late normoblasts, however, all fell into the 2C class, suggesting the cessation of DNA synthesis and mitotic activity at this stage. A similar distribution of DNA values was observed in nuclei which appeared to be in the process of extrusion. Only after extrusion was completed was a depression in DNA amount per nucleus indicated.

(2) *RNA:* (a) *Microphotometry:* The amount of cytoplasmic RNA per cell was initially high in the hemocytoblast or stem cell (Fig. 2). In successive stages of development, cytoplasmic RNA exhibited a constant and gradual decrease throughout the process of maturation (Fig. 2). No detectable Azure B dye was bound by mature erythrocytes; the slight absorption (E = 0.041) at 530 mμ was shown by curve analysis to be due to porphyrins. The decline in RNA amount per cell was

TABLE 2

INCORPORATION OF H^3 CYTIDINE (200 μC) PRESENTED TO NINETEEN-DAY RABBIT FETUS FOR SIX HR

Stage	Grains/μ^2* Cytoplasm	Nucleus	Nucleolus	No. cells
HCB-PrE	0.17	0.42	1.42	40
BE	0.14	0.35	...†	13
PE	0.067	0.061	0	21
Subsequent stages	0	0	0	

* After correction for background.
† Value not significant due to small size of nucleoli in cells sampled.

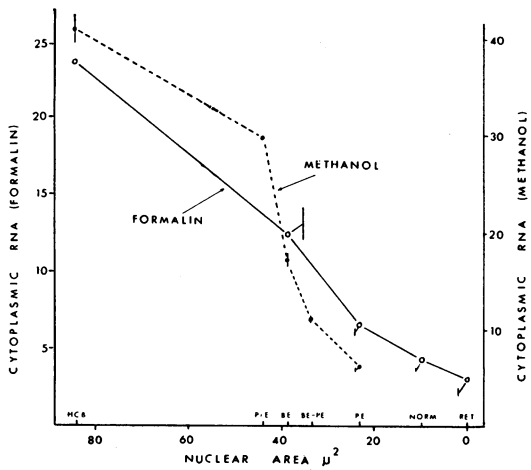

FIG. 2.—Means and standard errors of cytoplasmic RNA amounts throughout blood cell development. HCB = hemocytoblast; PrE = proerythroblast; BE = basophilic erythroblast; PE = polychromatophilic erythroblast; NORM = normoblast; RET = reticulocyte. Photometric units in ordinate are calculated from extinction X area in square microns.

accompanied by a progressive decrease in RNA concentration (following a slight increase in the basophilic erythroblast) and also by a substantial decrease in cell size measured in terms of cytoplasmic area (see below).

(b) *Radioautography:* With presentation of H^3 cytidine for 6 hr, marked incorporation into RNA was observed in the hemocytoblast, proerythroblast, and basophilic erythroblast (Table 2). All later stages exhibited no incorporation of labeled precursor. In the labeled stages, the highest activity was found in the nucleolus, followed successively by the nuclear and cytoplasmic compartments. In the polychromatophilic erythroblast, a light label was found equally distributed over the nucleus and cytoplasm. This label could result from cells that had differentiated during the 6 hr presentation time. This explanation is supported by the fact that less than 20 per cent of 122 cells were labeled and that the label did not show a nuclear concentration. Therefore, it was concluded that only the earliest stages exhibited RNA synthesis and that, if subsequent stages were capable of RNA synthesis, there was a substantial decrease in their ability to incorporate the precursor.

(3) *Protein:* Cytoplasmic total protein, measured in the same cells used for RNA determinations, decreased between the hemocytoblast and polychromatophilic erythroblast and then increased sharply to the reticulocytic stage (Fig. 3). The initial decline in cytoplasmic protein observed in the earlier phases of development accompanied a substantial decrease in cytoplasmic area. The mean area of the hemocytoblast was 59 μ^2 compared to a value of 14 μ^2 in the polychromatophilic erythroblast, while extinctions remained constant or were slightly increased during the same period. The increase observed in later phases was attributed to intensive synthesis of hemoglobin and is in agreement with microchemical and ultraviolet absorption measurements made in bone marrow erythroblasts by Thorell.[20] Soret band measurements showed the sharpest increase in total amounts of porphyrin per

cell occurring during the later developmental stages (Fig. 4). Because of the low extinctions obtained at 420 mμ prior to the polychromatophilic erythroblast, hemoglobin amounts for these stages may be overestimated by nonspecific light loss. The nuclear protein measurements showed a decline throughout the erythropoietic process which was accompanied by a decrease in nuclear area (Fig. 3). No measurements were made of nuclei which had been extruded.

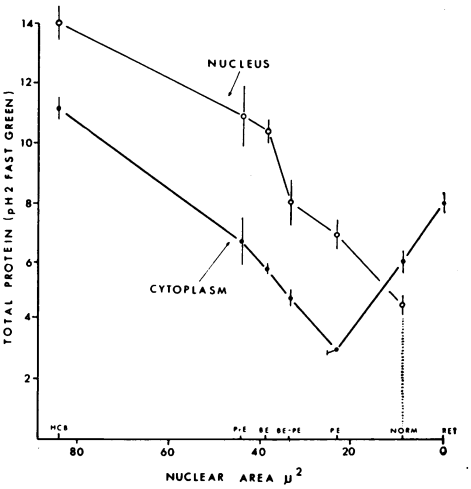

FIG. 3.—Means and standard errors of total protein amounts in the nucleus and cytoplasm at different stages of blood cell development. The curve of cytoplasmic changes represents the pooled results of two experiments. Photometric units in ordinate are calculated from extinction X area in square microns.

Discussion.—The results (Fig. 5) suggest that the metabolically stable RNA complex demonstrated in reticulocytes[11, 21–23] is established at the time of differentiation into the erythrocytic line. This interpretation is supported by several of our observations which can be considered in two categories: (1) the lack of cytidine incorporation into late erythroblast RNA, and (2) the inverse relationship between RNA and hemoglobin.

(1) *Lack of cytidine incorporation into erythroblast RNA:* The absence of H^3-cytidine incorporation into the RNA of erythroid cells subsequent to the basophilic erythroblast stage presumably reflects the lack of ribosomal RNA synthesis in erythroblasts which, in turn, results in a gradual loss of cytoplasmic RNA. Although our data do not preclude the possibility that an extractable soluble RNA fraction is in a state of turnover, this seems unlikely since biochemical results have been reported in which the rate of RNA turnover in reticulocytes has been considered too low to represent formation of a messenger RNA.[11, 21–23] Furthermore, Marks *et al.* pointed out that the low incorporation of C^{14}-guanine in whole cell preparations could be due to leukocytic contamination or to terminal addition of the label to reticulocyte soluble RNA.[11]

(2) *Inverse relationship between RNA and hemoglobin:* From Figures 3 and 4, it is evident that the most active period of hemoglobin synthesis occurs in the later stages when cytoplasmic RNA is relatively low (see also review by Errera[24]). Ackerman has reported that histochemical demonstration of protein-bound sulf-

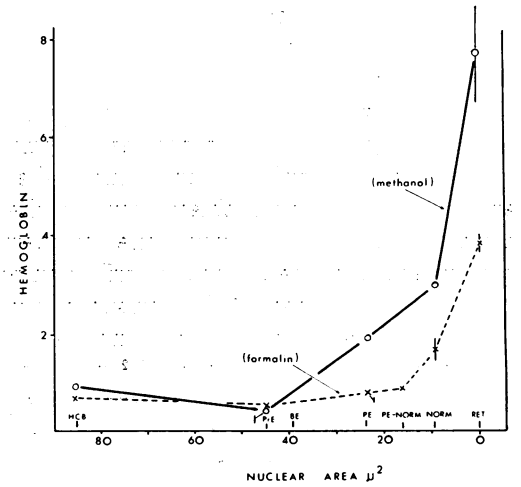

FIG. 4.—Means and standard errors of hemoglobin amounts during erythrocytic production. Photometric units in ordinate are calculated from extinction X area in square microns.

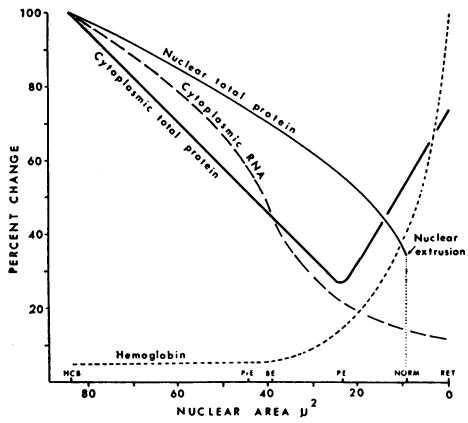

FIG. 5.—A summary of the data showing the cytochemical changes involved in blood cell production.

hydryls and histidine presumed to be associated with the globin moiety was most intense during this same period.[25] Although it can be assumed that some globin is present in earlier stages, on the basis of Ackerman's results and electron microscopic observations,[12] its presence is masked by the loss of proteins other than hemoglobin which constitute the bulk of cellular proteins during earlier periods.

The "delayed" appearance of hemoglobin (relative to RNA) is in direct contrast with many cellular systems where protein synthesis is usually accompanied or preceded by a period of intense RNA synthesis. The delay is explicable if the presence of a stable informational complex is postulated, a complex which is probably integrated with a small potentially "active" ribosomal fraction. In this way, a decrease in cytoplasmic RNA represented by the loss of an "inactive" ribosomal fraction is possible without hindering the cell's ability to synthesize hemoglobin. Indeed, electron microscopic examination of forming erythroid cells indicated the presence of both single ribosomes and ribosomal clusters.[12] Recent studies of hemoglobin synthesis in rabbit reticulocytes utilizing incorporation of labeled amino acids into ribosomes of intact cells and cell-free preparations indicated that less than 20 per cent of the total ribosomes were associated with newly-formed peptides.[11] In a similar system, Warner *et al.* found the "active" ribosomal fraction chiefly associated with a pentameric ribosomal complex which sedimented at 170S.[10] Single ribosomes with a sedimentation rate at 76S were demonstrated to be "inactive" in the incorporation of labeled amino acids.

The nuclear alterations observed during erythropoiesis are the result mainly of a decrease in total protein and probably water, but do not arise from a loss of DNA, as has been suggested.[17] It is evident from Figure 1 that the entire population of erythroid elements except the late normoblast exhibits 2C and 4C classes and several interclass values characteristic of a population undergoing DNA synthesis and mitosis. All normoblasts contain the diploid DNA level typical of a nondividing tissue. If, however, one considers the mean of all DNA values at each particular stage, the bimodal distribution would be obliterated and a gradation of mean values obtained in a fashion suggestive of a DNA loss, as reported by Korson.[17]

Several investigators have concluded that the normoblast nuclei disappear through intracellular karyolysis[17,26] rather than by extrusion.[31] However, apparent nuclear expulsion has been frequently observed by us both in the light and electron microscope. Furthermore, a 2C amount of DNA was found in extruding nuclei; lower values were found only in extruded nuclei.

It would seem, then, that the primary function of erythroblast nuclei during the greater part of the erythropoietic process is to provide a means for proliferation and that, after the basophilic erythroblast stage, the nucleus plays a minor role, if any,

in further differentiation of the system. Support for this conclusion can be drawn from several observations. Electron microscopic examination of hemocytoblasts and proerythroblasts revealed the presence of several possible pathways of nucleocytoplasmic exchange which were gradually lost in later stages.[12] In addition, it has been shown that the nucleus may be expelled in the polychromatophilic erythroblast during the normal course of development as well as under anemic stress.[27] Marrow cells *in vitro* exhibit a tendency to extrude their nuclei prior to active hemoglobin synthesis, but incorporation of heme precursors continues to occur.[28] Finally, the occurrence of hemoglobin synthesis in the nonnucleate reticulocyte is further evidence that nuclear presence is not required for synthesis of hemoglobin.[2-11] In view of these findings, one cannot assume that the presence of hemoglobin described within nuclei of later erythroblasts[12] or the mature nucleated erythrocytes of lower vertebrates[29,30] plays a major functional role in differentiation. As we have pointed out earlier from fine structure studies, its presence in nuclei appears to be a later acquisition, long after the establishment of a definite developmental pattern.

We conclude that differentiation along the erythrocytic lineage results in the establishment of a metabolically stable informational RNA complex in the earliest stages which is retained throughout the remainder of the developmental process. After the establishment of this complex, there is a loss of RNA synthesis, evident from the lack of H^3-cytidine incorporation, and a gradual decrease in cytoplasmic RNA levels. At a point when the RNA content is relatively low, intensive hemoglobin synthesis is initiated, which suggests that the stable informational complex is integrated into a small "active" ribosomal fraction. This "active" fraction must be retained while the larger "inactive" ribosomal population is deleted. After the basophilic erythroblast stage, nuclei continue to divide, thus enabling proliferation of the system, but eventually become mitotically inactive and are expelled from the cell. The role of the nucleus, then, would seem to be twofold: (1) in the earliest stages, the formation of the stable informational complex; and (2) to provide a means of cellular proliferation.

Summary.—Microphotometric measurements of various stages of red blood cell formation indicated a gradual loss of cytoplasmic RNA throughout the process. In later stages both total protein and hemoglobin revealed a concomitant increase. Autoradiographic analysis of RNA synthesis utilizing tritiated cytidine showed no incorporation after the basophilic erythroblast stage. DNA measurements revealed a bimodal distribution into 2C, 4C, and intermediate values in all stages except the late normoblast. The latter contained the 2C amount of DNA, while extruded nuclei revealed a decrease in DNA. It was concluded that: (1) a stable informational RNA complex is formed only in the earliest developmental phases and is retained throughout the process; (2) the loss of RNA in early stages is chiefly limited to an "inactive" ribosomal fraction while the "active" fraction is retained; and (3) the nuclei serve both as the origin of the stable messenger and as a means for proliferation of the system.

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*ANALYSIS OF UNLINKED SUPPRESSORS OF AN O° MUTATION IN SALMONELLA**

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The nature of the operator region of an operon in bacteria has been primarily defined in terms of the characteristics of its mutant forms. One type of mutant alteration of an operator, designated O°, results in the nonfunctioning of the associated structural genes, regardless of the conditions of growth.[1] Evidence has been presented which indicates that an operator does not act via a cytoplasmic product. Jacob and Monod have suggested that it acts as a receiver of controlling signals (i.e., the receptor of the repressor) and as the initiating point for the transcription into messenger RNA of the information carried by the structural genes of