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Abstract

Using the erythroid cells of Rana nigromaculata the hemoglobin synthesis has been studied in the relation of DNA and RNA contents. Results showed that the hemoglobin synthesis starts in the early stage of erythroblast but becomes marked just before the complete maturation. RNA contents drops markedly in the later stage of maturation. Measurement of DNA contents by Feulgen reaction suggested the termination of the mitosis just before the prematuration. From these results the author concludes that the RNA which will act as the template for the globin synthesis, develops from the early stage of erythroblast but the templation is accelerated in the terminal stage of maturation and the marked acceleration in hemoglobin synthesis in this stage.

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QUANTITATIVE STUDIES OF NUCLEIC ACIDS IN THE CELL WITH MICROSPECTROPHOTOMETER. II. NUCLEIC ACID AND HEMOGLOBIN CONTENTS IN ERYTHROID CELLS OF FROG AND HEN

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In the previous paper from the view points of optical interference and the mathematical calculation methods the author[1] scrutinized the method for microspectrophotometry on the nucleus of lymphocyte smeared and stained with Feulgen reacton, establishing an improved method, by which the contents of desoxyribonucleic acid (DNA) per cell can be estimated more accurately comparing to the usual method, but the scrutinization is also nessesary for the method of the treatment of the cell for the estimation of the intracellular substances. In this paper, the scrutinization of the pretreatment for the quantitative estimation of DNA and ribonucleic acid (RNA), has been carried out on the red cells of frog and hen. Measurement of the hemoglobin (Hb) contents per cell also has been carried out and the correlation between the contents of nucleic acids and that of hemoglobin is pursued.

MATERIALS AND METHODS

As the materials the erythroid cells from the circulating blood of the healthy adult female hen, *Gullus domesticus*, and of the normal frog, *Rana nigromacurata*, are used.

The blood from the wing vein of the hen or from the heart of the frog were smeared on the quartz slide and dried and fixed with sublimate alcohol[2], 50 per cent formalin[3,4], Carnoy's fluid[5] or acetic alcohol[6]. As the apparatus for the microspectrophotometry the microspectrophotometer (MSP) of Olympus Co. improved by SENO[7,8,9] and the author[1,9] has been employed. To settle the absorption spectra of nucleic acid and hemoglobin the absorption curves have been drawn on the aqueous solution of yeast

RNA (Merk), 1 per cent solution of 17.8μ depth, and horse crystalline hemoglobin obtained by the method of SUZUKI, 3 per cent solution of 33μ depth. The standard curves, from which the constant for the calculation of the absolute value of the contents of each substance can be derived, are drawn by the absorption intensities measured on the aqueous solutions of different concentration at 2600 Å in RNA and at 4060 Å hemoglobin respectively.

For the estimation of the contents of RNA and DNA per cell by using the ultra-violet ray of 2600 Å the following procedures of the alternated treatment and measurement have been performed;

a) The blood was smeared on a quarz slide, dried and fixed with methanol or acetic alcohol. b) On these fixed cells the estimation of the extinction of nucleic acids as total hase been carried out at 2600 Å. c) Then re-estimation of the extinction at 2600 Å after treating the cell with RNase, 0.1 per cent aqueous solution, pH 6.78, for one hour at 60°C. (RNase has been obtained from cow pancreas by the method of McDONALD). d) And finally the estimation of the extinction using the same ray after the subsequent treatment with 0.3 M trichlor-acetic acid (TCA) exposing 1 hour at 100°C. or 10 per cent perchloric acid (PCA) 30 minutes at 70°C (for the estimatin of extinction refer to the first report). For the calculation in the case of the cells oval in shape the diameters of the circles having the equal areas were used as the diameter of the cells.

The extinction by RNA can be obtained by eliminating the value given by (b) from that given by (a). The extinction of DNA can be obtained by eliminating the value given by (c) from that given by (d). For the extimation of DNA by Feulgen reaction the blood was smeared on a glass slide and dried and fixed with acetic alcohol by the method of MARRIAN[5], and then hydrolized and stained by the method of SHIBATANI[4]; modified by the author i. e. the hydrolysis for 20 minutes at 50°C. or 5 minutes at 60°C.

For the measurement of hemoglobin the method reported by SENO[7,8,9] was used. Gross estimation of the contents of DNA and RNA have been made on the other smears of the same sample by staining with methyl green-pyronin[12,13]

OBSERVATIONS AND RESULTS

On the smears of the blood of frog stained by methyl green-pyronin there appear several members of the young red cell which are rather round in shape having large round nuclei and strong pyronophilic cyto-

plasm. But the majority of red cells are matured ones, rather oval in shape with the wide cytoplasm having no affinity to pyronin. The former correspond to the basophilic erythroblast of mammals and the latter the denucleated matured red cell. Among these youngest and matured cells there appear the cells in the various stage of maturation. In the smears of hen's blood, the young cells seen in frog blood can not be seen. Some cells have a slight affinity to pyronin and the other have no affinity. The former correspond to the reticulocyte of the mammals and the latter to the matured red cell. Treatment with RNase induced the complete disppearrance of the pyronophlicity of the unchanged staining of the nucleus by methyl green.

Observations on the smeared cells under monochrome light without staining proved that the youngest cells of frog show a strong absorption of 2600 Å both in the nucleus and cytoplasm. and negative or slight absorption of 4060 Å in the cytoplasm and in the nucleus (Fig. 1). Morderately matured

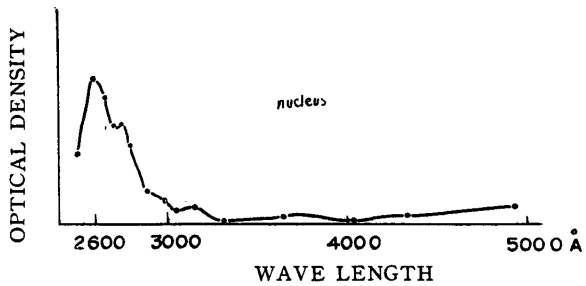

Fig. 1. Absorption spectrum of a nucleus of young red cell of frog, fixed with methanol, under the microspectrophotometer (MSP) using the high pressure-mercury lamp as a light source.

erythroblasts show the reduced absorption of 2600 Å in cytoplasm, but a marked absorption of 4060 Å. Some slight absorption of 4060 Å also appears in the interchromatin space of nucleus (Fig. 2). Matured cells of both frog and hen show the strongest absorption at 4060 Å in cytoplasm and slight absorption in the nucleus. Absorption at 2600 Å markedly decreases in the cytoplasm of this stage but still a slight absorption (Fig. 3). Treatment with RNase results in the marked reduction of the absorption at 2600 Å in the cytoplasm in the erythroblast. But still some slight absorption in cytoplasm can be recognized. Further treatment with TCA resulted in the disappearance of the absorption at 2600 Å in the nucleus and some reduction of the absorption at 4060 Å in cytoplasm. But in this case the treatment causes a marked shrinkage of

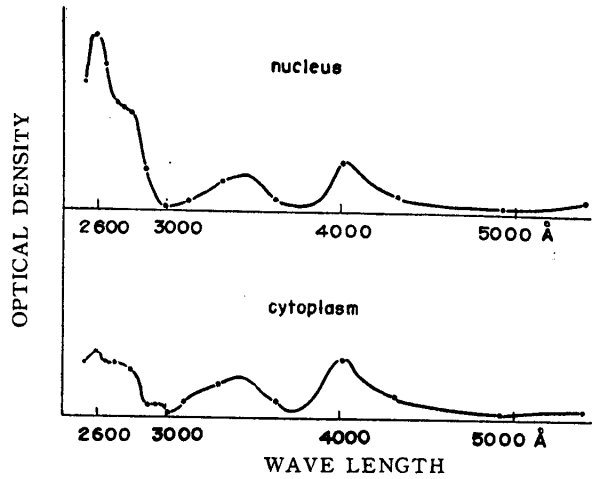

Fig. 2. Absorption spectra of a nucleus and cytoplasm of moderately matured erythroid cell of frog drawn under the MSP. The reduced absorption of 2600 Å in cytoplasm and a moderate absorption at 4060 Å can be observed. but a slight absorption of 4060 Å also appears on the nucleus.

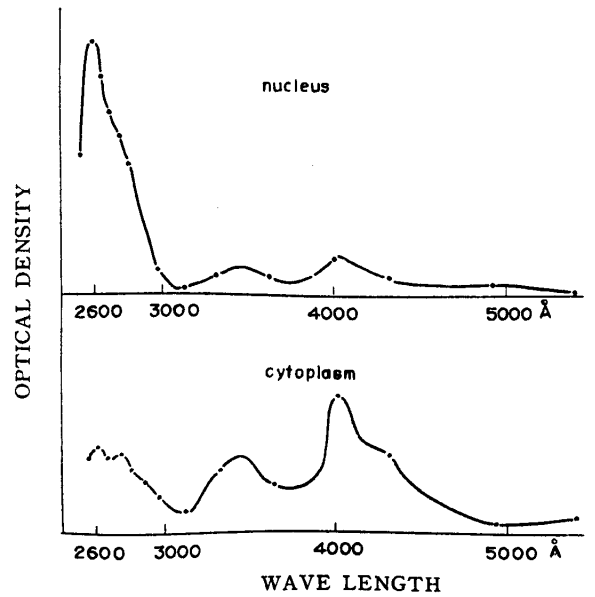

Fig. 3. Absorption spectra of the nucleus and the cytoplasm of a matured red cell of frog. A slight absorption at 2600 Å in cytoplasm probably by Hb and the increased absorption in nucleus by the picnosis of nucleus. The strongest absorption at 4060 Å in cytoplasm and slight absorption in nucleus can be observed.

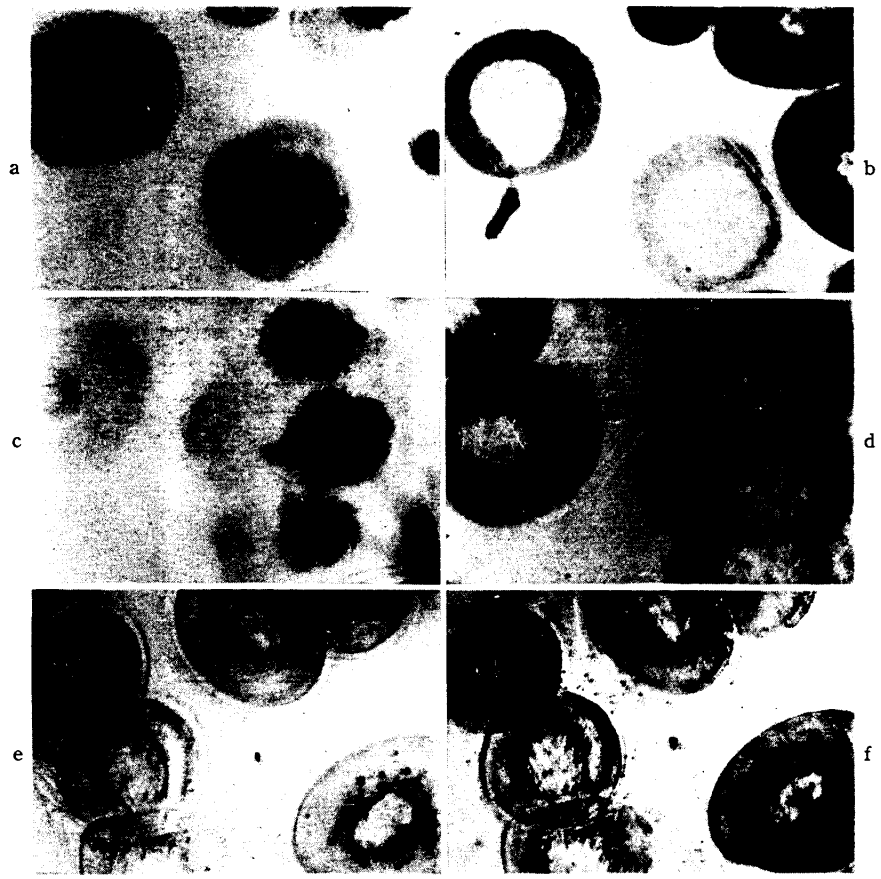

Fig. 4. a. Two young red cells of *Rana nigromaculata* in the early erythroblast stage fixed with methanol and taken at 2600 Å. Marked absorption both in the nucles and the cytoplasm. The left one is the youngest and the right one the more matured.

b. Two young cells in the early erythroblast stage of the same sample with *a*, taken at 4060 Å. Right one is the youngest having a slight absorption in the cytoplasm but almost negative in the nucleus. The left one is the more matured one, marked absorption in the cytoplasm and a slight absorption in the nucleus.

c. Three cells in the later erythroblast stage taken at 2600 Å after treatment with RNase. The middle one is the younger and other two are those in the endostadium of the later erythroblast stage, still a moderate absorption in cytoplasm probably by Hb.

d. Three cells in the later erythroblast stage of the same sample with *c*, taken at 4060 Å. These cells having a marked absorption in the cytoplasm and in the nucleus.

e. Five matured red cells taken at 2600 Å after treatment with RNase and then with TCA. A marked optical diffraction at the round of nuclear and cell membrane. The absorption of cytoplasm probably by Hb.

f. Five matured red cells of the same sample with *e*, taken at 4060 Å. A marked decrease of the absorption in cytoplasm due to the extraction of Hb by TCA treatment and the diffraction at cell and nuclear membrane can be observed.

the cell, which can be recognized by the furrow formation surrounding the cell, and may results in the disturbances in the microspectrophotmetry (Fig. 4). But by the treatment with perchloric acid the absorption at 2600 Å in the nucleus disappears without resulting in the shrinkage of the cell.

The absorption curves obtained with the yeast RNA and the horse crystalline hemoglobin showed the maximum absorption at 2600 Å in RNA and 4060 Å in hemoglobin. RNA showd no absorption at 4060 Å, but fairly a high absorption of hemoglobin at 2600Å, showing the possibility that the existence of hemoglobin in a high concentration affects the values of nucleic acid obtained from the absorption intensity at 2600 Å (Fig. 5).

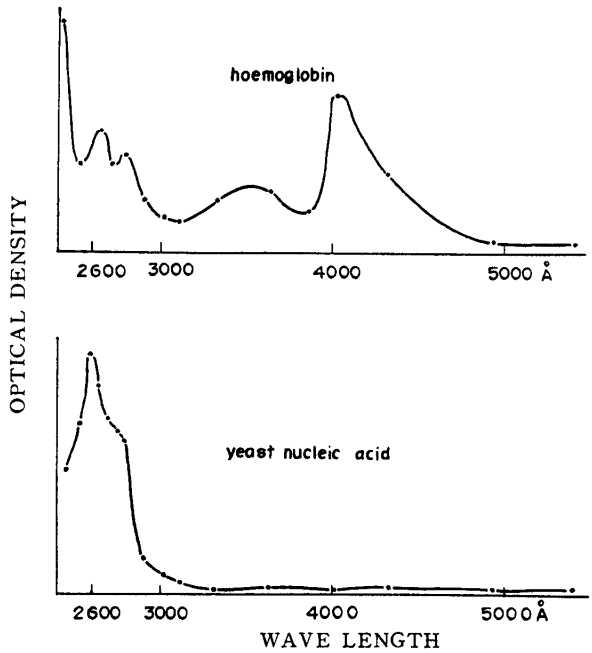

Fig. 5. Absorption spectra of Hb (a) and of RNA (b). Drawn by the MSP of Olympus Co. on an aqueous solution of 3 per cent crystalline Hb, 33 μ in depth, and on a 1 per cent aqueous solution of Yeast RNA, 17.8 μ in depth, respectively.

The standard curves drawn with the values obtained on the yeast RNA solutions of 0.1—3.0 per cent in the depth of 17.8 μ measuring at 2600 Å shows a straight line as indicated in Fig. 6, showing that absolute value of nucleic acid can be calculated from the absorption intensity at 2600 Å ; the derived constant is $K_2=3.3$.

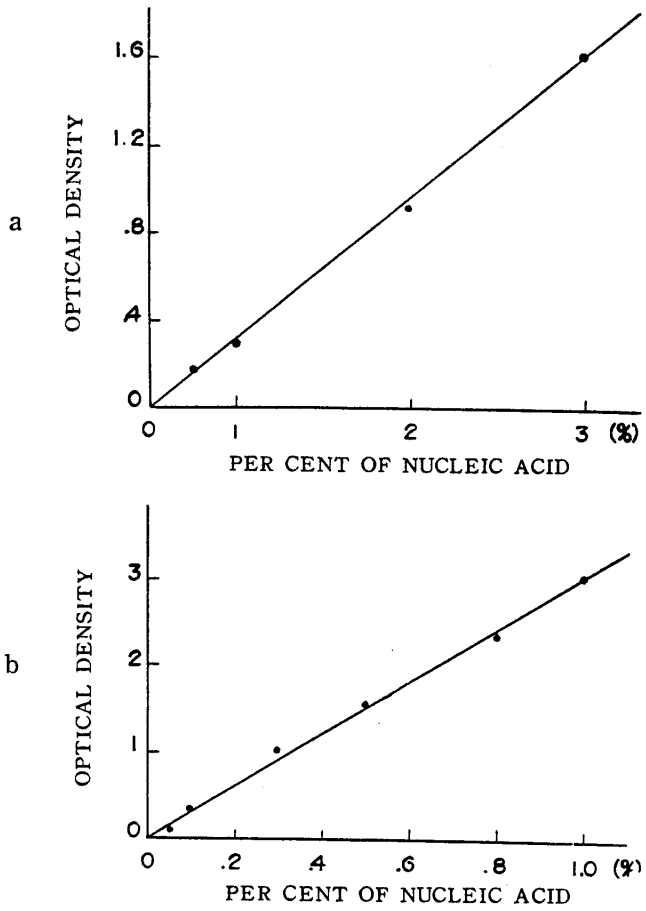

Fig. 6. The relation between the concentration and the optical density of RNA measured on the liquid films of yeast nucleic acid (Merk) solution of 0.1—3.0 per cent in the depth of 17.8μ measuring at 2600 Å by MSP. The relation is straightforward and satisfies the Lumbert-Beer's law. The derived constant is $K_2=3.3$

a; in higher concentrations, *b*; in lower concentrations

Concerning the standard curve of Hb and the constant derived from the curve, refer to the former reports by SENO[7,8,9] and the author[9].

On these fundamental observations the calculation of the contents of RNA and DNA and hemoglobin per cell have been carried out dividing the cell into the groups of each maturation stage. The calculation have been performed according to the formula $C_Q = K\pi \int |x| f(x)\,dx$, where $\int |x| f(x)dx$ shows the volume of the solid obtained by rotating the

extinction curves drawn along the cell diameter with the spotted light of 0.67 μ in diameter. For the precise method refer to the first report. The estimation has been carried out on the frog cells dividing into three groups according to their maturing grades; the young erythroblast, the moderately matured erythroblast and the completely matured cell. In each group the estimation has been made on 30 cells and the mean value has been obtained. According to the data calculated at 2600 Å the nucleic acid contents of the young erythroblast are 14.9×10^{-9} mg. in RNA and 14.1×100^{-9} mg. in DNA per cell, the moderately matured ones, 14.1×10^{-9} mg. in RNA and 16.1×10^{-9} mg. in DNA and the matured ones 9.39×10^{-9} mg. in RNA and 17.9×10^{-9} mg. in DNA. Hemoglobin contents derived from the absorption are 26.5×10^{-9} mg. in the young cell, 32.2×10^{-9} mg. in the moderately matured ones and 60.8×10^{-9} mg. in the matured cell. These are shown with the curves in Fig. 7 and 8. These data show that RNA decreases gradually with the maturation of cells and hemoglobin is synthesized most markedly in the later stage of maturation. The values of DNA showed some increase with the maturation. The values obtained by Feulgen reaction showed a fairly marked decrease in DNA contents with the proceeding of maturation of cell. Of these contradictory data it seems to suggest that the absorption of hemoglobin will be superimposed where the estimation of DNA is carried out using the ray of 2600 Å as is supposed from the observation on the absorption curves drawn on the crystalline hemoglobin, which shows some absorption at 2600 Å and yet the treatment with TCA results in the escape of Hb making impossible to reveal the

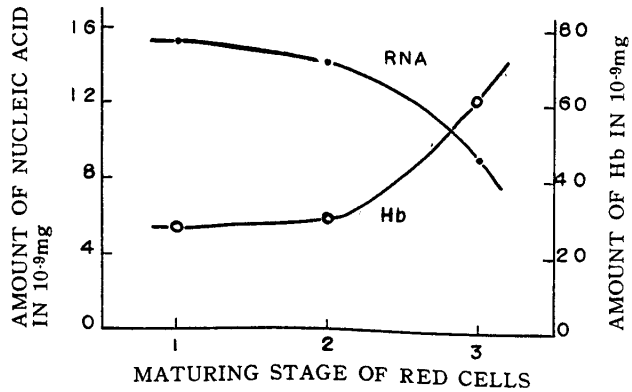

Fig. 7. The relation between RNA and Hb contents in various maturation stages of erythroid cells of frog. Hb synthesis starts at an early stage of erythroblast and becomes marked just before the complete maturation. RNA content drops markedly in the later stage of maturation.

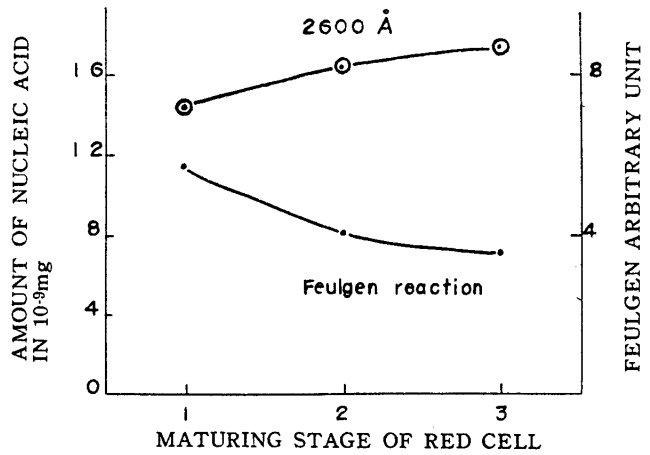

Fig. 8. The relation between the obtained value by Feulgen reaction and ultraviolet absorption in matured red cells of frog. The value from the 2600 Å absorption gradually increases along with the maturation of red cell but the value from the Feulgen reaction decreses. The former include many artefacts and the latter are reasonable ones from the activity of cell division (see text).

grade of superimposing rate of the absorption of hemoglobin to that of DNA. The contents of DNA of the matured cell of hen whose DNA contents have been known to be constant has been calculated by the same method but the materials are fixed with acetic alcohol and Hb extracted, and the final treatment for the estimation of DNA the 10 per cent perchloric acid was used, by which DNA is removed without resulting in the shrinkage of the cell. The DNA contents have been calculated as 2.04×10^{-9} mg. per cell as the mean value from 30 cells, which is slightly lower than that given by MIRSKY and RIS[14] on the nucleus of the red cell of hen. Therefore, in the case of the estimation of DNA of the red cell without removing Hb, the values become high and the estimation by Feulgen reaction will give the more reliable results. As indicated in Fig. 9 the distribution of individual DNA contents in the bone marrow cells of the blood depleted anemic rabbits are found to be wider than in the younger cells. And by the maturation of the cell the range of the distribution becomes smaller, i. e. to the diploid value in the DNA contents. The distribution of the younger cells in wider range in DNA contents will show the proceedings of the cell division and the syhthesis of DNA. Then the decreasing curve in DNA contents with the maturation of the cell will be reasonable one showing the lower rate in cell division in the more matured cells.

In the next, on the beses of these results it has been aimed to obtain the absolute value of DNA from the values obtained by Feulgen reaction

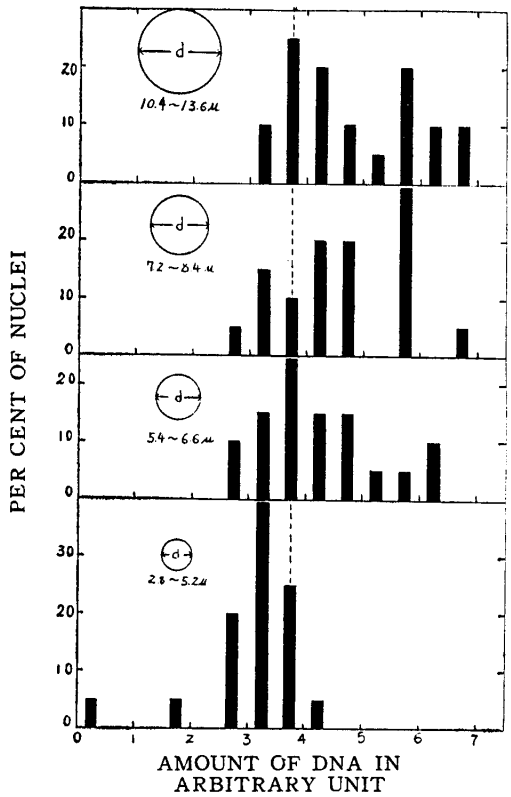

Fig. 9. Individual DNA contents in the nuclei of the bone marrow erythroblasts of the blood depleted anemic rabbits in the various developmental stages.
d : nuclear diameter. The upper are young ones and the lower are matured ones.

on the matured red cells of hen. But the results showed that the color intensity given by Feulgen reaction shows the marked differences according to the changed method by fixation as indicated in Table 1 and yet even in the case stained with the same method a fairly marked difference in the color intensity in each smear. Therefore, the arbitrary unit derived from the cells on the same slide and yet on the restricted part

Table 1. The changes in color intensity of Feulgen nuclear reaction by various fixatives on the blood cells of the same species (*Gullus domesticus*). (hydrolysis at 60°C. for 5 minutes)

Fixative	Number of cells	Cell diameter	Feulgen arbitrary unit
50% formalin	10	2.91	1.76 (± 0.07)
Sublimate alcohol	10	3.61	5.00 (± 0.17)
Carnoy's fluid	10	3.74	6.52 (± 0.10)
Acetic alcohol	10	4.13	5.36 (± 0.13)

only, where the distribution of the cell is found not to be heterogenous, can be used as the standard for the calculation.

From these results the reliable data of RNA contents can be given by measuring at 2600 Å after the treatment with RNase. Hemoglobin values measured at 4060 Å does not change before and after the treatment with RN-ase. Accurate values of DNA contents can be obtained by Feulgen reaction but only after the adequate pretreatment.

DISCUSSION

Even when the method of MSP has been done accurately, the error will be the result if the treatment of the material for spectrophotometric measurement is inadequate. The absorption at 2600 Å in wave length for the measurement of nucleic acid is dependent on the purin and pyrimidine bases, whose absorption maximums are found near 2600 Å. And as suggested by POLLISTER and RIS[15] the absolute value of nucleic acid in the individual cell can be obtained by using the absorption at 2600 Å: the value of RNA by the difference between the values obtained before and after the treatment with RNase, and that of DNA by the difference between the values obtained before and after the treatment with TCA. But the swelling or shrinkage of the cell by the treatments causes sometimes the changes in optical diffraction or refraction which may severely affect the trace absorption values. Especially, the TCA treatment causes a marked shrinkage of cells by which the phase contrast effect, diffraction and refraction are increased which become marked by illuminating with light of the short wave length. And yet by exposing to TCA hemoglobin which shows some absorption at 2600 Å, is extracted to some extent. Then the treatment with TCA for the detection of DNA amount in the case of red cell by using the ray of 2600 Å must be discarded. The increasing tendency of DNA value with the maturation of the cell will not give the true change in the contents of DNA. The DNA contents are measured at 2600 Å by using PCA, whose effect on the shrinkage of the cell is less comparing to that of TCA, *seems the to give* a reliable results.

Concerning the Feulgen nuclear reaction STOWELL[16], ELY and ROSS[17] determined the specificity of this reaction to DNA revealing the mechanism of the reaction between DNA and dye. DI STEFANO investigated the changes of the color intensity given by Feulgen reaction by changing the period of hydrolysis for DNA with the scrutinization by methyl green staining, showing that the Feulgen reaction can be used for the measurement of DNA. LESSLEY[19] showed that this reaction does not satisfy the

LUMBERT-BEER's law in the case where the contents of DNA is small but in a range of the concentration of DNA as generally found in a cell the law is satisfied. And WALKER[20] observed that the absorption intensity of Feulgen color coincides with that found at 2600 Å on DNA. SHIBATANI and NAORA[4] also suggested that the absorption intensity of Feulgen color accurately proportionates to the amount of DNA if the pretreatment is adequate, i.e. the adequate period of hydrolysis and the adequate pH for the reaction. But the author's observation shows that the color intensity are changed by changing fixatives. This will be due to the different extraction grade of labile DNA observed by MARRIAN[6]. Therfore, it is still a problem whether or not the treatment with 50 per cent formalin for the measurement of DNA as reported by NAORA could give the reliable results.

Moreover, the amount of protein, especially that of histone, which acts as to inhibit the Feulgen reaction[21] can be taken into consideration. By the author's observation the Hb also seems act as to inhibit the Feulgen reaction, because the DNA contents in maturd red cell given by Feulgen reaction give a smaller value than that found in lymphocyte. But in the cast of the cells containing Hb. the data obtained by Feulgen reaction will be more reliable than that obtained by 2600 Å

The amounts of ribonucleic acid calculated by using the ray of 2600Å will be reliable ones because RNase does not extract Hb and the absorption at 2600 Å by Hb can be eliminated by deducing the value obtained after treating with RNase. Hb values are also reliable ones, because nucleic acid does not absorb the ray at 4060 Å and the absorption curves derived from cytoplasm and nucleus show only the curve of pure nucleic acid superimposed with that of pure Hb. The absorption of cytochrome and others is negligible because the cytoplasm of leucocytes does not show any absorption at 4060 Å so long as this apparatus is used.

From these data it can be said that in the red cell of frog the Hb synthesis starts in an early erythroblastic stage but more than half of that found in the matured cell is synthesized in the prematuration stage where the contents of RNA rapidly decrease and the cell division almost stops. Therfore, the RNA as the template for globin synthesis will develop from the early erythroblastic stage and the templation completes in the later stage and the rapid synthesis of hemoglobin ensues.

CONCLUSION

Using the erythroid cells of *Rana nigromaculata* the hemoglobin synthesis has been studied in the relation of DNA and RNA contents. Results

showed that the hemoglobin synthesis starts in the early stage of erythroblast but becomes marked just before the complete maturation. RNA contents drops markedly in the later stage of maturation. Measurement of DNA contents by Feulgen reaction suggested the termination of the mitosis just before the prematuration. From these results the author concludes that the RNA which will act as the template for the globin synthesis, develops from the early stage of erythroblast but the templation is accelerated in the terminal stage of maturation and the marked acceleration in hemoglobin synthesis in this stage.

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