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Studies on the relation between heme and nucleic acid syntheses in erythroid cell. II. Nucleic acid synthesis in erythroblast of anemic rat treated with aminopterin and bromouracil*

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Abstract

With the bone marrow of anemic rats, which had received the repeated injections of phenylhydrazine once a day for three to four days, the effects of aminopterin and bromouracil on the nucleic acid metabolism of erythroblasts were observed in vivo experiment. The injection of aminopterin suppressed DNA synthesis with the lowered labeling index as observed by the incorporation of ^3H -thymidine into DNA in vitro. But the grain count per cell showed the level similar to that of anemic control. RNA synthesis was not interfered by AP injections. These results indicate that AP mainly suppresses the thymidilate kinase. Bromouracil showed no such effect even on the administration of a large dose. On the basis of the data obtained from the experiment by using AP, a discussion was made on the correlation between DNA synthesis, nuclear function and the cell specialization.

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STUDIES ON THE RELATION BETWEEN HEME AND NUCLEIC ACID SYNTHESSES IN ERYTHROID CELL

II. NUCLEIC ACID SYNTHESIS IN ERYTHROBLAST OF ANEMIC RAT TREATED WITH AMINO- PTERIN AND BROMOURACIL

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In the previous paper (1) the author reported that the synthesis of DNA is closely correlated to the erythroid cell specialization, i.e., in anemic rat the suppression of DNA synthesis enhances the hemoglobin synthesis and denucleation at early stage of specialization. This has been elucidated by the injection of aminopterin (AP) which is known as the analogue for folic acid and suppresses the purine synthesis and also the methylation of uracil. The injection of AP brings about severe inhibition of the mitosis of erythroid cell, whereas the injection of bromouracil (BU), which is also known as the inhibitor of DNA synthesis, does not show any actual effect on the mitosis of erythroblasts and their specialization. In this paper, it is demonstrated that DNA and RNA syntheses of the erythroblasts from the animals treated with AP is severely arrested but the nucleic acid metabolism is hardly affected by treating with BU.

MATERIALS AND METHODS

Adult Wistar rats weighing about 200g were used. All the animals were made anemic by the repeated injections of phenylhydrazine, daily for three to four days, by the same method as reported in the previous paper (1). On the recovery stage of phenylhydrazine anemia, i.e. two to three days after the last injection of phenylhydrazine, the animals were divided into three groups; the first group receiving the subcutaneous injection of aminopterin, the second group bromouracil and the third group no further treatment as anemic control. Dose and method of the injection of the medicaments were the same as those reported in the previous paper (1). The animals treated with aminopterin and their anemic controls were killed a few days after two injections of AP, 2mg per 100g body weight per day for two successive days, and those treated with BU and the controls were killed one day after three injections, 25mg per 100g

body weight per day for three successive days.

DNA contents per cell were measured by microspectrophotometry on the cells smeared and stained by Feulgen reaction. The fresh bone marrow tissue from femur was taken on an object glass added with one drop of rat serum and crushed gently sandwiching between two object glasses and the cells were isolated from the tissue. One drop of the bone marrow-serum mixture was smeared on the cover slide glass of $0.18 \times 25 \times 50$ mm, dried, fixed with methanol and stained with Feulgen reaction by the method of SHIBATANI for microspectrophotometry. On these cells photometry was carried out by using the microspectrophotometer of Olympus Kogaku Co., employing the two-wavelength method (2, 3, 4) at $565\text{m}\mu$ and $507\text{m}\mu$.

For the observation of nucleic acid synthesis the incorporation of ^3H -thymidine into DNA and ^3H -uridine into RNA were observed by autoradiography. The cells of the femur bone marrow were detached from the tissue crushing gently in a glass homogenizer by adding cool Hanks' solution containing a trace of heparin. The inner tube of the homogenizer was moved up and down quite slowly for two to three times. The free cells were washed two times with Hanks' solution by repeated centrifugation. The sedimented cells were resuspended in an equal volume of Hanks-serum mixture, and one ml of the cell suspension was incubated with 0.02ml of ^3H -thymidine solution ($100\mu\text{C}$ per ml) at 37°C for 30 minutes shaking gently. Immediately after incubation, the cells were washed two times with Hanks-serum mixture, and the sedimented cells were smeared, dried and fixed with methanol. These smears were mounted with liquid film emulsion, SAKURA NR M-1, exposed for 7 days at 4°C , developed and stained with Giemsa at pH 5.0.

For the observation of RNA synthesis the incorporation of ^3H -uridine was observed by the same method as in the observation of DNA synthesis incubating the cells with 0.1ml of ^3H -uridine solution ($10\mu\text{C}$ per ml) and 0.1ml of cold thymidine (1mM per ml). The specific activities of these isotopes were 5C per ml in ^3H -thymidine and 2.7C per ml in ^3H -uridine and both of them were obtained from the Radiochemical Center in England.

RESULTS

Severe anemia developed after the injections of phenylhydrazine chloride, once a day for three to four days. Macrocytosis was also induced as in previous paper, showing almost all the red cells were formed by skipping one cell division as judged from the cell diameter (20). The treatment of these anemic animals with AP injection resulted in a marked suppression of erythropoiesis in bone marrow. The findings were nearly the same as those observed in the previous experiment (1). By AP injections big erythroblasts having red-stained cytoplasm appeared in the bone marrow with the accentuated macrocytosis in the circulating blood. But BU injection did not show such effect. The mitosis of the erythroblasts was sup-

pressed by aminopterin administration but not by bromouracil as in the cases of previous observations (1).

On these animals with anemic controls the DNA contents of erythroblast per cell were estimated by microspectrophotometry on the bone marrow smears stained with Feulgen reaction. In anemic controls which were sacrificed four to five days after the last injection of phenylhydrazine the DNA level of erythroblasts showed a tendency to decrease gradually with the advance of cell specialization. The DNA level began to decrease around early basophilic stage (9—11 μ in nuclear diameter) and minimized at polychromatic stage (6—7 μ) where it reached nearly one half value, probably haploid level (Fig. 1a). The cells corresponding to orthochromatic erythroblast in nuclear diameter (5—5.5 μ) have rarely been encountered.

The erythroblasts from the animals of phenylhydrazine anemia treated with aminopterin showed a very low level of DNA contents. Twenty-four hours after the first AP injection many cells showed nearly the similar DNA level as that of anemic control (Fig. 1b), but 24 hours after the second AP injection most of the cells showed a low DNA level, about one half of that of anemic control (Fig. 1c). Thus, most of the erythroblasts at early (9—11 μ in nuclear diameter) and late basophilic stages (7—9 μ) and showed to be nearly at haploid level in DNA contents. More mature cells, the cells comparable to polychromatic cells in their cell size, were rarely encountered.

In animals treated with bromouracil the bone marrow cells retained their DNA level at nearly the same as that of anemic control even after the injection of 50 mg per 100g for two days (Fig. 1d). But after three injections, 75 mg BU per 100g for three days, many cells of 6—11 μ in nuclear diameter showed slightly low DNA levels (Fig. 1e).

The DNA synthesis of erythroblast observed by the incorporation of ^3H -thymidine into DNA revealed that the cells synthesizing DNA were reduced in number among those from AP-treated animal (Fig. 2), whereas BU treated animal showed no marked change compared to anemic controls (Fig. 3). Grain counts per cell were nearly the same in all three groups (Figs. 2, 3).

In the ^3H -uridine incorporation into RNA, the cells from the animals treated with AP showed a slight reduction in labeling index, but no appreciable change in the animals treated with BU. Grain count per cell stood always nearly at the same level in all the animals (Figs. 4, 5).

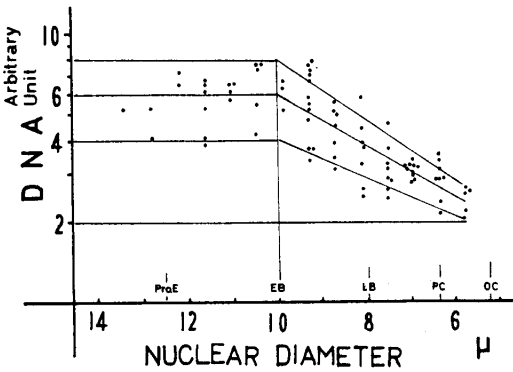


Fig. 1-a

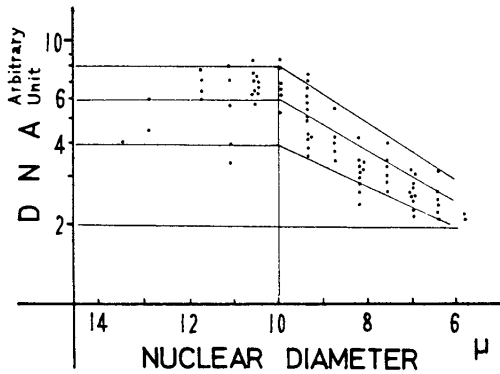


Fig. 1-b

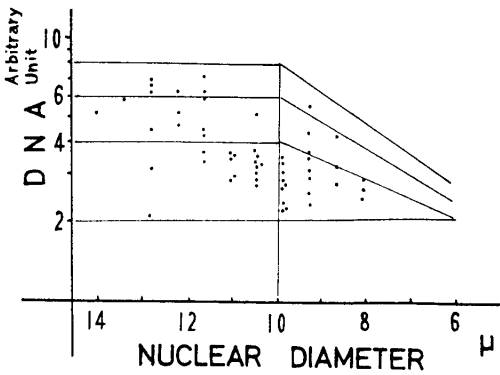


Fig. 1-c

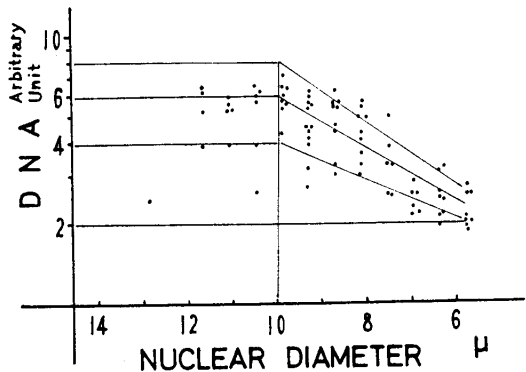


Fig. 1—d

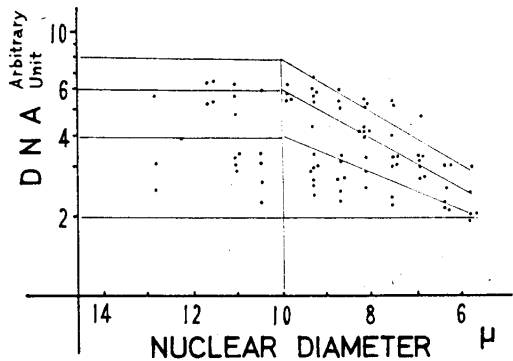


Fig. 1—e

Fig. 1 DNA contents (arbitrary unit) of erythroblast, estimated by micro-spectrophotometry, 24 hours after the last injection of the medicaments.

- Fig. 1a anemic control
- Fig. 1b After one AP injection, 2mg per 100g
- Fig. 1c After two AP injections, 2mg per 100g daily for two days
- Fig. 1d After two BU injections, 25mg per 100g daily for two days
- Fig. 1e After three BU injections, 25mg per 100g daily for three days

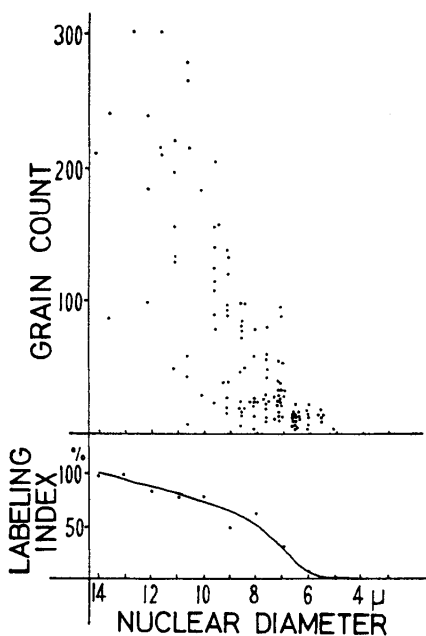


Fig. 2—a

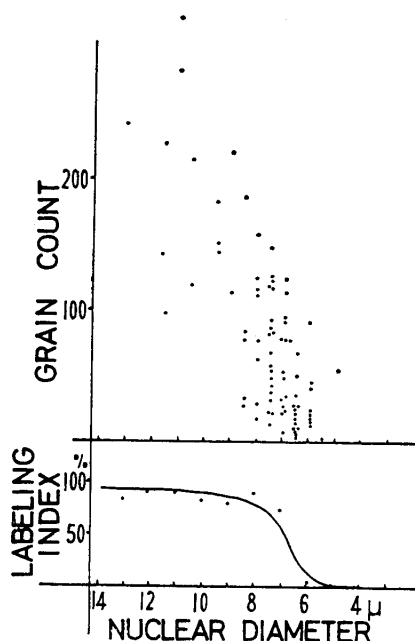


Fig. 2—b

Fig. 2 ^3H -thymidine incorporation into DNA of erythroblasts of the animal treated with single injection of aminopterin (AP), 2 mg per 100 g body weight. The cells were taken 24 hours after the AP injection and incubated for 30 minutes with ^3H -thymidine *in vitro*.

- (a) Grain count and labeling index of the erythroblasts from the anemic animal treated with aminopterin
- (b) From anemic control

DISCUSSION

The purpose of this experiment was to verify the supposition that the suppression of DNA synthesis by aminopterin (AP) will promote the cell specialization or the latter proceeds independent of the suppressed DNA synthesis which was reasonably deduced from the previous experiment, showing the suppression of mitosis by AP to result in the hemoglobin synthesis and denucleation of erythroblast at early stage of its specialization. In this experiment it was revealed that the administration of AP actually inhibited the DNA synthesis of erythroblast as understood from the lowered labeling indices by the incorporation of ^3H -thymidine, while BU administration failed to show such an effect, which did not accelerate the erythroid cell specialization.

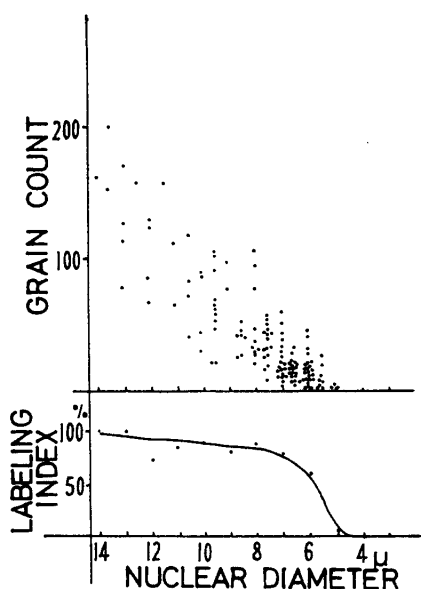


Fig. 3—a

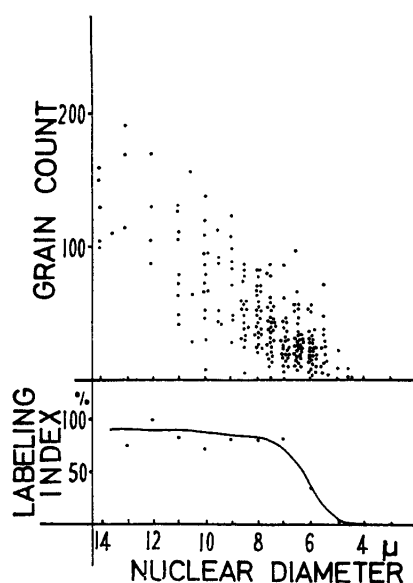


Fig. 3—b

Fig. 3 ^3H -thymidine incorporation into DNA of erythroblasts of the animals receiving three injections of bromouracil (BU) once a day for three days, 75 mg as total per 100 g body weight. The bone marrow cells were taken 24 hours after the last BU injection and incubated with ^3H -thymidine for 30 min. *in vitro*.

- (a) Grain count and labeling index of the cells from the anemic animals treated with bromouracil.
- (b) From anemic control

As discussed precisely in the previous paper AP is the folic acid antagonist and inhibits highly the activity of the thymidilate kinase or the methylation of deoxyribose uridine monophosphate (dRUMP) to deoxyribose thymidine monophosphate (dRTMP) but not the incorporation of thymidine into DNA. It may be reasonably thought that the grain count by ^3H -thymidine-incorporation into DNA should not be affected by AP administration. Actually, data showed no change in the incorporation of ^3H -thymidine into DNA in the animals treated with AP. This means that the suppression of DNA synthesis is mainly due to thymidine deficiency but not to the defects of purine ring formation. Many cells were kept at G_1 -phase without entering into S-phase probably because of deficiency of thymidine and the suppressed mitosis is the result. This view is supported by the DNA content per cell which showed a very low level in the animals receiving AP injection. After two AP injections the DNA of many erythroblasts became to hypodiploid and haploid levels.

RNA synthesis was not affected by AP as judged from the incorpora-

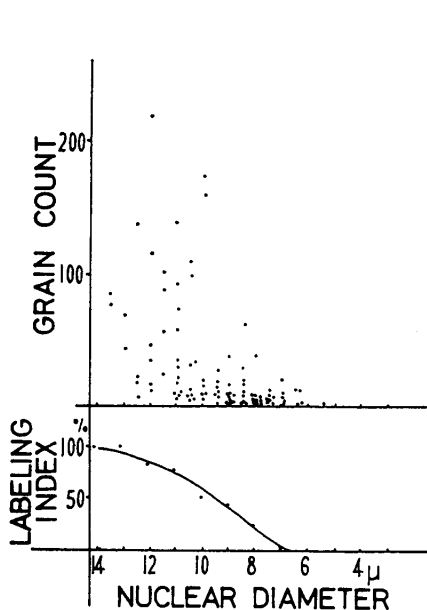


Fig. 4—a

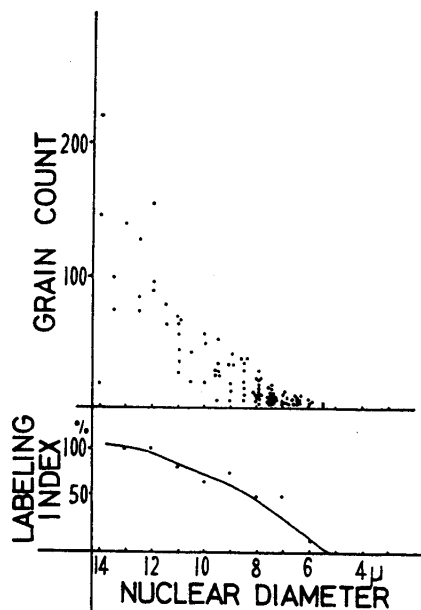


Fig. 4—b

Fig. 4 ^3H -uridine incorporation into RNA of erythroblasts. Bone marrow cells were taken 24 hours after a single aminopterin injection and incubated with ^3H -uridine for 30 min, at 37°C *in vitro*.

- (a) Grain count and labeling index of erythroblasts from AP treated animal.
- (b) From anemic control

tion of ^3H -uridine. This proves again that AP did not suppress purine formation. The protein or hemoglobin synthesis proceeded irrespective of the suppression of DNA synthesis. The cytoplasm of erythroblast in early stage stained red and rich in hemoglobin. This does not necessarily mean the accelerated hemoglobin synthesis. However, it is very probable as the maturation of cell is actually promoted by denucleation. It is still uncertain at present what is the control mechanism of the hemoglobin synthesis which will be controlled at translation level, though histone has been supposed to be the very controller by SENO (19).

By AP injection the denucleation occurred in the early specialization stage as in the rabbit receiving mass red cell transfusion (TAKEBAYASHI) (5). This may mean that the acceleration of the somatic protein synthesis results in the suppression of DNA synthesis and the accelerated denucleation, because in the denucleated cell hemoglobin synthesis is accelerated in the presence of O_2 . The control of denucleation is also a very important factor for the red cell specialization and seems to be concerned with

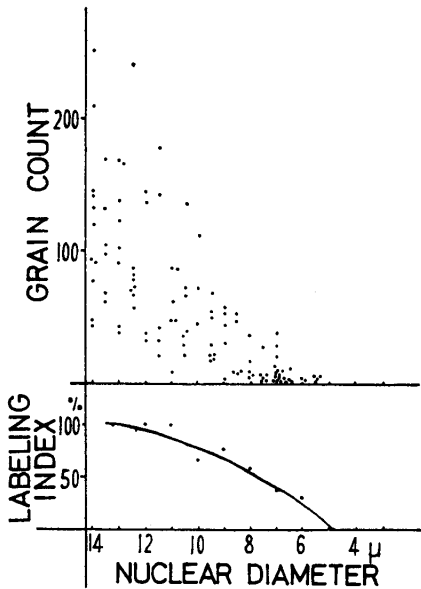


Fig. 5—a

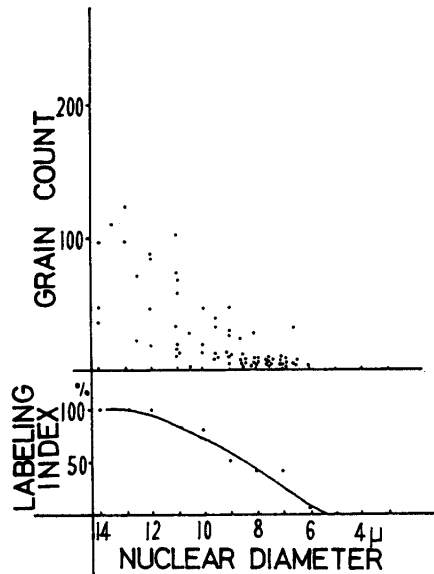


Fig. 5—b

^3H -uridine incorporation into RNA of erythroblast from the anemic animals, receiving three injections of bromouracil (BU) once a day for three days, 75 mg as total per 100g body weight. The bone marrow cells were obtained 24 hours after the last injection of bromouracil, and incubated with ^3H -uridine for 30 min. at 37°C *in vitro*.

- (a) Grain count and labeling index of the cells from the BU treated animal.
- (b) From anemic control

the lowered DNA synthesis, but what control mechanism is involved remains a problem at present.

SUMMARY

With the bone marrow of anemic rats, which had received the repeated injections of phenylhydrazine once a day for three to four days, the effects of aminopterin and bromouracil on the nucleic acid metabolism of erythroblasts were observed *in vivo* experiment. The injection of aminopterin suppressed DNA synthesis with the lowered labeling index as observed by the incorporation of ^3H -thymidine into DNA *in vitro*. But the grain count per cell showed the level similar to that of anemic control. RNA synthesis was not interfered by AP injections. These results indicate that AP mainly suppresses the thymidilate kinase. Bromouracil showed no such effect even on the administration of a large dose. On the basis of the data obtained from the experiment by using AP, a discussion was made on the correlation between DNA synthesis, nuclear function and the cell speciali-

zation.

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REFERENCES

1. SHIGEHISA, M.: Studies on the relation between heme and nucleic acid syntheses in erythroid cell. I. Effects of aminopterin and bromouracil on hemopoiesis and hemoglobin synthesis in anemic rat. *Acta Med. Okayama* **22**, 251, 1968
2. ORNSTEIN, L.: The distributional error in microspectrophotometry. *Lab. Invest.* **191**, 250, 1952
3. PATAU, K.: Absorption microphotometry of irregular shaped objects. *Chromosoma* **5**, 341, 1952
4. MENDELSON, M. L.: The two-wavelength method of microspectrophotometry. I. A microspectrophotometer and tests on model systems. II. A set of tables to facilitate the calculations. *J. Biophysic and Biochem. Cytol.* **4**, 707, 1958
5. TAKABAYASHI, J.: Effect of mass blood-transfusion on erythroid cell differentiation in anemic rabbit. I. An evolutionary change in the cell specialization process. *Acta Med. Okayama* **21**, 251, 1967
6. LOCHAYA-VIMOKESANT, S., WINCHELL, H.S., FISH, M.B. and POLLYCOVE, M.: Decreased *de novo* DNA-thymine synthesis and increased thymidine-³H incorporation into DNA by human bone marrow cultured with aminopterin. *Proc. Soc. Exp. Biol & Med.* **126**, 621, 1967
7. DIDERHOLM, H.: Inhibitory effect of aminopterin and 5-bromodeoxyuridine on the multiplication of Simian virus 40. *Acta Pathol. Scand.* **59**, 87, 1963
8. SLAVÍKOVÁ, V. and SLAVÍK, K.: The influence of some 4-amino analogue of tetrahydrofolate coenzymes on purine biosynthesis. *Biochim. Biophys. Acta* **71**, 604, 1963
9. SCHOENBACH, E.B., WEISSMAN, N., GOLDBERG, B. and FISHER, J.: Nucleic acid change in sarcoma 180 following administration of aminopterin. *Proc. Soc. Exp. Biol. & Med.* **80**, 559, 1952
10. PRUSOFF, W. H., TEPLY, L. J. and KING, C. G.: The influence of pteroylglutamic acid on nucleic acid synthesis in *Lactobacillus casei*. *J. Biol. Chem.* **176**, 1309, 1948
11. HENSON, D., PINKERTON, H.: Effect of aminopterin on the replication of the murine salivary gland virus. *Nature* **202**, 419, 1964
12. GONTCHAROFF, M. and MAZIA, D.: Developmental consequences of introduction of bromouracil into the DNA of sea urchin embryos during early division stages. *Exptl. Cell Res.* **46**, 315, 1967
13. ROWND, R.: The buoyant density of 5-bromouracil-labeled DNA. *Biochim. Biophys. Acta* **134**, 464, 1967
14. HACKETT, P. JR and HANAWALT, P.: Selectivity for thymine over 5-bromouracil by a thymine-requiring bacterium. *Biochim. Biophys. Acta* **123**, 356, 1966
15. GRASSO, J. A., WOODARD, J. W. and SWIFT, H.: Cytochemical studies of nucleic acids and proteins in erythrocytic development. *Proc. Natl. Acad. Sci.* **50**, 134, 1963
16. GRASSO, J. A. and WOODARD, J. W.: The relationship between RNA synthesis and

- hemoglobin synthesis in amphibian erythropoiesis. Cytochemical evidence. *J. Cell Biol.* **31**, 279, 1966
17. SCHERRER, K., MARCAUD, L., ZAJDELA, F., LONDON, I. M. and GROS, F.: Patterns of RNA metabolism in a differentiated cell: A rapidly labeled unstable 60S RNA with messenger properties in duck erythroblasts. *Proc. Natl. Acad. Sci.* **56**, 1571, 1966
 18. TAKEBAYASHI, J.: Effect of mass blood-transfusion on an erythroid cell differentiation in anemic rabbit: II. Denucleation in early stage of erythroid cell specialization, with special reference to RNA- and hemoglobin synthesis. *Acta Med. Okayama* **21**, 267, 1967
 19. SENO, S.: Differentiation of erythroid cell. *Acta Path. Jap.* **16**, 457, 1966
 20. SENO, S., MIYAHARA, M., ASAKURA, H., OCHI, O., MATSUOKA, K. and TOYAMA, T.: Macrocytosis resulting from early denucleation of erythroid precursors. *Blood* **24**, 582, 1964