Acta Medica Okayama

Volume 24, *Issue* 5 1970 *Article* 1

OCTOBER 1970

Studies on the mechanism of supravital staining; supravital staining of blood cell nucleus

Katsuko Yoshii*

*Okayama University,

Studies on the mechanism of supravital staining; supravital staining of blood cell nucleus*

Katsuko Yoshii

Abstract

For the purpose to reveal the relation between cell death and nuclear stainability by supravital staining with basic dyes observations have been made on the cells of bone marrow, peripheral blood and lymph node from anemic and non-anemic rabbit, rat, mouse and chicken, and thymus from young mouse. The cells were stained supravitally in blood serum, isotonic saline, calcium chloride and sucrose solutions with the dyes; brilliant cresyl blue (B. C. B), Nile blue (N. B.), neutral red (N. R.), Janus green (J.G.) and eosin (E.). The following results were obtained: 1. In the presence of blood serum all the living cell nuclei observed were not stained supravitally, except some mature erythroblasts and nucleated red cells. 2. In isotonic saline, CaCl2 and MgCl2 solutions all the erythroid cell nuclei were stained deep by B. C. B., N. B., N. R., slightly by J. G. but not by E. In stainability the younger the cell is the deeper in its nuclear staining. The nuclei of other cell strains were not stained. 3. In isotonic sucrose the nuclei of mature granulocyte were also stained by B. C. B. and N. B. but not by other dyes. The nuclei of lymphoid cells and myeloid cells appeared pale without being stained by any dyes. The nuclei of erythroblasts in sucrose solution were stained deeper with B. C. B. and N. B. than those in isotonic saline. The differences between supravital stainability of the nuclei among the cells belonging to different strain and among those of the same strain but in different maturation stage and the nuclear staining after cell death have been discussed from the possible dissociation of DNA from histone.

---

*PMID: 4107856 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **24**, 493—507 (1970)

STUDIES ON THE MECHANISM OF SUPRAVITAL STAINING ; SUPRAVITAL STAINING OF BLOOD CELL NUCLEUS

Katsuko YOSHII

Department of Pathology, Okayama University Medical School, Okayama, Japan (Director: Prof. S. Seno)

Received for publication, August 12, 1970

During the first half of this century, there appeared a number of works on the supravital staining of the cells (1-9). These works were conducted for the purpose to observe cell organellae-like mitochondria and other cytoplasmic granules (2, 8, 5, 12), or to reveal physicochemical properties of cytoplasm (3, 4, 14), and in some cases, to observe the permeability of cell membrane (1, 13-18). These papers have materially contributed to the establishment of the basic concept of cell organellae, transport, pH of cytoplasm, etc.

Since then the development of the fine technique for electronmicroscopy, e. g. preparation of thin section and osmification has made it possible to observe the fine structure of each cytoplasmic organellae, and the progress in the techniques of biological application of the variety of isotopes have opened the door to observe the transport of various substances through cell membrane.

Thus by the advance in such techniques in biological research, vital staining of the cell as a tool for the study of cell physiology and morphology lost much of its value. In recent years, works on supravital staining are rarely encountered except for some cytologists using the supravital staining of the cells by eosin and other dyes to differentiate living cells from dead ones (5, 14, 16, 19-22).

As pointed out by SENO (22), however, studies of the vital staining of the cell may be reviewed in the light of modern concept of molecular biology, because these basic dyes easily combine with acidic DNA or with RNA only by mixing them in test tube, but in living cell these basic dyes hardly stain nucleic acid even when they penetrate into the cell. The phenomenon seems to indicate the presence of such a special and labile high molecular conformation in living cytoplasm that inhibits the binding of the basic dye with nucleic acid and degrades easily by cell death.

It is generally believed that if the cell nucleus is stained by some dyes like Janus green or methylene blue, it indicates cell death. But problem

has been left without being solved decisively since the work of SUGIYAMA (21), who reported some fibroblast nuclei can be stained with a series of dyes supravitally. He also described of the works of his predecessors that the nuclei of the kidney epithelial cells, striated muscle cells, corneal cells and spermatocytes can be stained vitally with some dyes. The question is why DNA combines with basic dyes in some living cells but not in others. Among blood cells it has been also reported that the nuclei of erythroblasts are stained by some basic dyes supravitally (5, 28), though SUGIYAMA thought these should be dead cells.

For further studies of vital staining on the basis of molecular biology the author aimed at the confirmation of this phenomenon and searched for the theoretical background of supravital staining of nucleus by using the cells from bone marrow, spleen and lymph nodes of rabbit, rat and mouse, and erythroblasts and red cells of chicks, and thymus cells of young mouse. In this paper it is reported that the nucleus of the living cell is not stained by the supravital staining in the usual medium, blood serum, but by changing the medium, *e. g.* by removing serum or by reducing ionic strength in environment, erythroblasts, nucleated red cells and some granulocytes are stained supravitally.

MATERIALS AND METHODS

Adult Wistar rats weighing about 200 g, ddN mouse about 25 g, and adult rabbits about 2.5 kg of both sex were used. Of these animals, the cells from peripheral blood, bone marrow, spleen and lymph node of mesenterium were observed after being stained supravitally.

Besides these, nucleated red cells from young leghorn chick and thymus cells from young mouse of ddN strain were also used for the observation.

Of them some animals were made anemic by blood depletion to obtain the erythroid bone marrow, *i. e.* in rat and mouse the blood was drawn from retro-orbital sinus, 0.2—0.3 ml at a time and twice every other day in mice and 4 ml once in rat and the bone marrow cells were taken one week after the first blood depletion.

In rabbits, blood was drawn from ear vein, 20 ml per day for 3 consecutive days, and the bone marrow cells were observed 5 to 6 days after the first blood depletion. The chickens were used without blood drawing.

The animals were sacrificed under anesthesia by cutting carotid in rabbit, by decapitation in mouse and chickens and by puncture of the heart in rat.

After sacrifice the femurs and mesenterial lymphnodes were taken out in all animals and thymus in young mouse. Bone marrow tissues were obtained by removing the epiphysis of femur with bone scissors and drawing the tissue with small pincette. A piece of fresh bone marrow tissue was sandwiched between two

object glasses adding one drop of medium, rabbit serum, physiologic saline, isotonic $CaCl_2$, $MgCl_2$ and sucrose solutions. Then the tissue was spread to a thin layer by pressing gently. By repeating such treatment two or three times, the cells were freed in the medium.

For supravital staining, one drop of the cell suspension was taken on an object glass, and was added another drop of dye solution which was prepared with the same medium as that used for cell suspension (25). In the case using serum for the cell suspension, a drop of dye solusion in pure ethanol was taken on an object glass and dried and then one drop of cell suspension was added to the dye film (26, 27). The cell suspension and dye were mixed thoroughly and sealed with cover-slide and paraffin for microscopy. Microscopic observations were carried out at 37°C for 30 minutes after the sample preparation in general.

For the observation of lymphoid cell the mesenterial lymph node and thymus were cut into small pieces by scissors in wet, and a piece of tissue was sandwiched between two object glasses, a drop of medium added, cells were freed from the tissue and observed after being stained supravitally as in the case of bone marrow cells.

For the dyes, 4 basic and one acidic dyes were used; brilliant cresyl blue (B. C. B), Nile blue sulfate (N. B), neutral red (N. R.), Janus green (J. G.) and eosin (E.). With these dyes five solutions were prepared for each dye; pure alcohol, physiologic saline, isotonic $MgCl_2$, $CaCl_2$ and sucrose solutions. Concentrations of the dye were 0.5 % in B. C. B. and N. B., 0.005 % in N. R., 1.0 % in J. G. and 0.5 % in E. Excepting alcohol solution, the dye solutions often gave low pH, 3.6—3.7 in B. C. B. and N. B. solutions, pH 1.7 in N. R. solution, and each solution was divided into two parts, one was adjusted to pH 7.1 by using 1 N and 0.1 N NaOH, and the other was left intact.

For the observation of the changes in supravital stainability by cell death, some preparations were added wit $2\times10^{-3}M$ KCN or monojod acetate in saline solution, both of which were neutralized with 0.1 N HCl and were adjusted to $10^{-3}M$ in final concentration.

OBSERVATIONS AND RESULTS

First, the observations were made of blood cells and lymphoid cells stained by the conventional method (25), in which one drop of blood was added with a drop of basic dye saline solution, *e. g.* B. C. B. solution whose pH was not adjusted. By this method the cells were stained supravitally by basic dyes as reported by various authors (5, 6, 7, 8, 9, 11); namely, the cells of myeloid or granulocyte series gave stained granules by B. C. B., N. B. with pale unstained nucleus.

Lymphoid cells also showed some granular organellae stained by these dyes and J. G., but the nucleus remained unstained.

Most of erythroblasts also gave pale nuclei being left unstained but some of them showed stained nuclei, though these were only a few in

number. The cells having the stained nuclei were small ones at final maturation stage, otho- or polychromatic stage. The nuclei of the mature nucleated red cell of chiken were also stained deep. Reticulocytes showed the stained reticulum, though it should be an artefact formed by aggregation of ribosomes, endoplasmic reticulum and mitochondria with basic dyes (31).

Janus green stained mitochondria of all the cells, granulocytes, erythroid cells and lymphoid cells, but failed to stain the cell nuclei. Reticulum of reticulocyte was also stained by this dye though it took a fairly long period of time compared to the staining by B. C. B. or N. B.

Neutral red stained some structures in cytoplasm, probably Golgi vesicles and others (12) in the cells of myeloid and lymphoid series. The nuclei of these cells were not stained by N. R. Some mature red cell nuclei were stained light by this dye, but most of the erythroid cell nuclei were pale.

Eosin did not stain any cell granules nor nuclei. The data are summarized in Table 1.

TABLE 1. THE SUPRAVITAL STAINABILITY OF THE BONE MARROW AND LYMPHOID CELLS OF ADULT RABBIT SUSPENDED IN RABBIT SERUM AND MIXED WITH AN EQUAL VOLUME OF DYE-SOLUTION IN SALINE WHOSE pH WAS NOT ADJUSTED.

Dyes	Series of Granulocyte		Erythroid Cell		Reticulocytes	Lymphoid cells	
	N.	C.	N.	C.		N.	C.
B. C. B.	−	++	+~++*	++~+++	+	−	+
N. B.	−	++	+~++*	++~+++	+	−	+
N. R.	−	+	±	+	+	−	+
J. G.	−	+	±	+	+	−	+
E.	−	−	−	−	−	−	−

B. C. B., N. B., N. R., J. G., and E.: Refer to the text.
+++ : stained deeply, ++ : stained moderately, + : stained light, ± : labile in stainability, − : not stained, * : only a few cells at the final maturation stage were stained.
Method : See the text.
N : Nucleus, C : Cytoplasm

In the presence of KCN or monojod acetic acid, nuclei of many erythroblasts and myeloid and lymphoid cells were stained within 5 to 10 minutes after addition of the agents, especially stained deeply by B. C. B., N. B. and N. R. Eosin stained the cells diffusely. The number percent of the cells having nuclei stained with the basic dyes increased with the lapse of time in proportion to the increase in the number of E -stained cells.

Observations reveal that except the erythroid cell the staining of

nuclei with the basic dyes like B. C. B. and N. B. by the method of general use means the cell death as has been believed. And the supravital staining by E. proves to be a good method to acertain the cell death.

Subsequent observatioins were made on the cells suspended in saline instead of blood serum and stained by mixing with the dye solution in saline, whose pH was adjusted to pH 7.1. The bone marrow cells from non-anemic rat, mouse, rabbit and chicken served as materials.

In this series of experiments the erythroid cell showed a marked increase in the affinity of the nucleus to the dyes, though the myeloid and lymphoid cells did not show any appreciable difference in their stainability from these in the former experiments. Namely, the nuclei of all erythroblasts at varying maturation stages were stained supravitally in the physiologic saline and the more matured ones were stained deeper (Table 2) in contrast to the cells suspended in blood serum and mixed with dye saline solution without pH adjustment where the nuclei of the small sized erythroblasts at final maturation stage were solely stained.

TABLE 2. SUPRAVITAL STAINABILITY OF THE BONE MARROW AND LYMPH-NODE CELLS OF NORMAL RABBIT AND CHICKEN IN PHYSIOLOGIC SALINE (pH 7.1)

Dyes	Myeloid Cells		Mature Ebl. and Nucleated R. B. C.		Young Ebl.		Reticulocyte	Lymhoid Cell	
	N.	C.	N.	C.	N.	C.		N.	C.
B. C. B.	−	++	++~+++	++	+~++	+++	+	−	+
N. B.	−	++	++~+++	+	+~++	+++	+	−	+
N. R.	−	+	+	+	−	+	+	−	+
J. G.	−	+	+	±	−	+	+	−	+
E.	−	−	−	−	−	−	−	−	−

B. C. B., N. B., N. R., J. G. and E.: Refer to the text.
N. C., +++, ++, +, ±, −: Refer to Table 1.
Ebl.: Erythroblast, R. B. C.: Red blood cell

Among the dyes tested, N. B. and B. C. B. stained the erythroid cell nuclei very deeply as well as cytoplasm (Fig. 1). J. G., which did not stain any cell nucleus in the former experiment, stained the nuclei of mature erythroblasts and nucleated erythrocytes, but not the immature ones.

In this experiment E. did not stain any kind of cells. Myeloid cells and mature granulocytes hardly showed any increase in the affinity to dyes even in saline solution. Their nuclei appeared always pale irrespective of the source of the cells, though the cytoplasmic organellae and granules were stained distinctly as in those suspended in serum. When the time of microscope observations exceeded 30 to 60 minutes, then the nuclei of

some cells were also stained by the basic dyes, and nearly the same number percent of cells were also stained by E.

The incubation of these cells with KCN, $10^{-3}M$ in final concentration, resulted in the penetration of the basic dyes into the nuclei of some cells as early as after five minutes. Number percent of the granulocytes whose nuclei were stained by B. C. B. was nearly the same as that of granulocytes stained by E. After 60 minutes, the nuclei of a large number of myeloid cells from the bone marrow were stained by B. C. B. and the cell itself by E., but in both cases 20 to 30 per cent of the total cells remained unstained. These cells proved to have surived through the treatment as attested by their cytoplasmic movement. Monojod acetic acid given instead of KCN yielded nearly the same results, though the cell damage seemed to be rather severe than in KCN.

Observations on the bone marrow of anemic animals, rat, mouse and rabbit showed the erythroid cells to be distinctly different from myeloid cells and mature granulocytes in supravital stainability, when they were exposed to the dyes in saline solution, pH 7.1. The bone marrow of anemic animals was rich in young undifferentiated erythroblasts including early basophilic and proerythroblasts, and the nuclei of these immature cells were stained by B. C. B. and N. B. as in non-anemic animal. But it was shown clearly that younger the cell and nuclei the less was their dye affinity. With the advance of cell maturation the nuclei were stained deeper and those of late basophilic erythroblast were stained fairly deep and those of poly- and orthochromatic erythroblasts were stained diffusely cobalt blue. Myeloid cells and granulocytes remained unstained as in the case of normal animals (Fig. 2). The extruded nuclei, which were found in reticulum cell or macrophage, were also stained deep by B. C. B. and N. B., though the nucleus of macrophage itself remained unstained. N. R. also stained the nuclei of erythroblasts, but J. G. stained only the cells in advanced maturation stages.

In those cells from anemic bone marrow observations were also carried out by adding KCN and monojod acetic acid, $10^{-3}M$ in both cases (Figs. 3, 4). The results were nearly the same as in the former experiments showing the appearance of myeloid cells and granulocytes having the nuclei stained by N. B. and B. C. B. and the cells stained by E. The number of the stained cells increased with the lapse of time.

The cells from lymph nodes and thymus of non-anemic rat, mouse and rabbit were also observed being suspended in saline and stained supravitally by mixing with the basic dyes and E. dissolved in saline solution (pH 7.1). The experiment revealed no cell being stained by E. nor any

nucleus by N. B., B. C. B., J. G. and N. R. (Fig. 5), nor in the cells suspend in serum and exposed to the dyes in saline without pH adjustment as well. The cytoplasm was also stained slightly by the basic dyes giving only a few granular staining (Tables 1, 2). With the lapse of time the cells were stained by E. and the nuclei by the basic dyes. Cell death occurred in lymphoid cells much earlier than that in granulocyte.

On exposing to KCN or monojod acetic acid, the nuclei of lymphocytes and thymus cells became stainable supravitally by B. C. B. (Fig. 6), N. B., N. R. and their cytoplasm by E.

The experiments described above clearly indicate that the nuclei of living erythroblasts can be stained supravitally by the basic dyes, B. C. B., N. B., N. R. and J. G. and the stainability is promoted by removing serum from environment. In myeloid cells, granulocytes, lymphoid cells and thymus cells, the nuclei have never been stained supravitally in the conditions described. Except erythroblasts, stainability of the nucleus by the basic dyes is comparable to the stainability of the cell by E. irrespective of the source of the cell.

Supravital stainability of the erythroid cell nuclei was also examined in isotonic $CaCl_2$ and $MgCl_2$ by the method similar to that in the former experiment with physiologic saline, to find out the effect of cations in the media, *i. e.* in one series of experiment the cells were suspended in isotonic $CaCl_2$ and added with the dye solution in isotonic $CaCl_2$ whose pH was adjusted to pH 7.1 and in other series $MgCl_2$ was used instead of $CaCl_2$.

The observations gave nearly the same findings as in the cells stained supravitally in saline, indicating that Ca^{++}, Mg^{++} have the identical effect on the stainability of erythroid cell nuclei as Na^{+}.

The nuclei of myeloid and lymphoid cells remained pale in all cells, but were stained by the dyes when they were left for a certain period in an unfavorable environment or exposed to KCN and monojod acetic acid.

The above experiments indicate that serum seems to act as to inhibit the stainability of the nucleus of erythroblast and the ions act as to promote the stainability as long as the cell is viable. Therefore, the author tried to remove all the ions from the medium and to stain cells by the same dyes. For this purpose isotonic or 9.25 percent sucrose solutions was used as the medium for cell suspension and also for dye solution whose pH was adjusted to pH 7.1. Here all the cell strains, the cells from bone marrow of normal rat, mouse, rabbit and chicken, lymphoid cells from the lymph node of rat and the thymus cells from young mice were employed.

In the isotonic sucrose solution the nuclei of erythroid cells have been

stained distinctly by B. C. B. and N. B. just as in the physiologic saline solution. Besides these, the nuclei of granulocytes were also stained by B. C. B., N. B. and N. R. in the sucrose medium, slightly but distinctly (Table 3). The grade of stainability of granulocyte nuclei was far less

TABLE 3. SUPRAVITAL STAINABILITY OF ERYTHROID, MYELOID AND LYMPH-NODE CELLS FROM AN ANEMIC RAT IN SUCROSE SOLUTIONS.

Dyes	Myeloid cells and Granulocyte		Mature Erythroblast		Immature Erythroblast		Reticulocyte	Lymphoid Cell	
	N.	C.	N.	C.	N.	C.		N.	C.
B. C. B.	−∼+	++	++∼+++	+	+∼++	+++	+	−	+
N. B.	−∼+	++	++∼+++	+	+∼++	+++	+	−	+
N. R.	−∼+	+	+	+	−	+	+	−	+
J. G.	±	+	+	+	−	+	+	−	+
E.	−	−	−	−	−	−	−	−	−

B. C. B., N. B., N. R., J. G. and E.: Refer to the text.
N, C., +++, ++, +, ±, −: Refer to Table 1.
Method: See the text.

than that of erythroid cell nuclei but this has never occurred in any other medium used. The phenomenon was common one to all the granulocytes from different kind of animals but all the nuclei of granulocytes were not stained in an equal intensity; some granulocytes generally encountered had their nuclei not at all stained. The nuclei of lymphocytes from lymph node and thymus remained unstained as before, nor E. stained any cell.

The results indicate clearly that the nuclei of living granulocytes can be stained supravitally by the basic dyes like B. C. B. and N. B. in the medium containing no ion in which the granules in the cytoplasm of granulocyte kept its normal behavior in movement.

Further, the author observed the cells from normal animals stained supravitally by the method of SABIN (26) which is a conventional method for the supravital staining. The method has been somewhat modified by putting one drop of ethanol solution of dye instead of making dye film on object glass. The dye droplet is dried and one drop of cell suspension in serum is added, mixed, and covered with a cover slide and paraffin, so as to expose the cells to the dyes in pure blood serum. In this instance, all the cell nuclei observed were kept without being stained by any dyes used, except the mammalian erythroblasts at final maturation stage and chick erythrocytes, whose nuclei were stained only slightly by B. C. B. and N. B., though their nuclei were not stained by the other dyes, J. G. and N. R. (Table 4).

The last experiment was carried out with rabbit bone marrow cells.

TABLE 4. SUPRAVITAL STAINABILITY OF THE CELLS OF BONE MARROW, LYMPHNODE FROM AN ANEMIC RAT IN RABBIT SERUM.

Dyes	Myeloid cells and Granulocyte		Mature Erythroblast		Immature Erythroblast		Reticulocyte	Lymphoid Cell	
	N.	C.	N.	C.	N.	C.		N.	C.
B. C. B.	−	+++	−∼+	+	−	+	+	−	+
N. B.	−	+++	−∼+	+	−	+	+	−	+
N. R.	−	+	−∼+	+	−	+	+	−	+
J. G.	−	+	−	+	−	+	+	−	+
E.	−	−	−	−	−	−	−	−	−

B. C. B., N. B., N. R., I. G. and E.: Refer to the text.
N. C., +++, ++, +, −, − : Refer to Table 1.
Method : See the text.

The cells were stained supravitally by B. C. B. and N. B. in the medium of saline as just mentioned. A few minutes later the cover slide was removed, one or two drops of serum were added to the cell suspension and covered again with a cover slide. In these instances, the deep blue color of erythroblast nuclei once stained by the dyes in saline mostly faded after the addition of blood serum. A similar phenomenon was also observed with the erythrocytes of chicken. The pictures resembled those of the erythroblast stained supravitally in serum with B. C. B. or N. B. Observations indicate that these basic dyes seem to have a stronger affinity to serum protein in the environment than to DNA in living cell.

COMMENT

As just described, it is generally thought that if the cell nucleus is stained by supravital staining with basic dyes such as methylene blue, Janus green, etc. then the cell is dead (5, 14).

Present observations have proved that this is true as long as the supravital staining in the presence of blood serum is concerned. Generally the supravital staining of the blood cell was done on dye film by Sabin's method and it means the interaction of living cell and the dye in pure blood plasma. The nuclei of the cells that had not been stained supravitally by the basic dyes became stainable when they were kept *in vitro* for a certain period under an unfavorable condition or under the influence of some respiratory inhibitor-like KCN and of glycolytic inhibitor like monojod acetic acid, or by the mechanical damage to result in cell death.

The present observations have also revealed that if the blood serum, probably serum protein, is removed from the medium and the cell is suspended in isotonic saline, $CaCl_2$, $MgCl_2$, or sucrose solutions, then the

nuclei of erythroid cells in salt solution and erythroid cell and granulocyte in sucrose solution, were stained supravitally by all the dyes used except E. The fact indicates that the concept of supravital stainability of cell nucleus by some dyes to mean cell death should not be generalized but limited to the cases where the staining is made in blood plasma, serum or the media containing blood serum. The present experiment has also proved that the supravital stain by E. (19) is a very useful tool to differenitiate the living cells from dead ones.

The phenomenon that the nuclei of erythroblasts are stained with some basic dyes by supravital staining has been pointed by SUGIYAMA (21) as early as in 1924 and also by SENO *et al.* recently (28). SUGIYAMA described that the erythroblasts having the nuclei stained supravitally should be the dead cell, but in his paper (21) he has also stressed the presence of nuclear staining of living cell from the observations of his predecessors and himself, though he pointed out some works reported as vital staining of the nuclei of kidney epithelial cells or the cells of lower animal, by indigo carmine, acid fucshin, Boldeau red, Congo red, etc. would possibly be the postmortem staining.

The present observations, however, clearly indicate that the nuclei of living mammalian erythroblasts and chick nucleated red cells are stained by B. C. B., N. B., N. R. and by J. G. in the medium free of blood serum protein. After nuclear stain, they gave normal morphologic picture without swelling and E. which stained dead cells selectively, does never penetrate into these cells by supravital staining.

The inhibition of the nuclear staining by blood serum, as just mentioned, should be due to the binding of the positively charged dye with serum proteins, albumin and globulin, both of which are charged negative in the media around pH 7.0. Ca and Mg ions in the medium give the identical effect on the supravital stainability of the cell as Na ion as long as the medium is kept isotonic. But the ions themselves should be of important factor for the dye penetration, as erythroid cell nuclei were more deeply stained in isotonic sucrose solution than in the salt solution and nuclei of granulocytes which were never stained in salt solution, were stained by N. B. and B. C. B. in sucrose solution.

As the intracellular distribution of the ions and small molecules and the high molecular conformations of living cell cytoplasm and membrane will not severely be arrested on being affected by the changes in ions and other molecules in environment, as long as the medium is maintained isotonic and free of toxic substances and the cell continues to live. Consequently, the changes in the penetration of the dye into the cell and the

nucleus can readily be understood in relation to the changes in the characteristics of the dyes induced by the changes in environmental components.

The basic dyes used, B. F. B., N. B. etc. are charged slightly positive when dissolved in water, but the charge will be much reduced in the presence of ions like Na^{+}, Ca^{++} etc. in the medium and dye molecule will become more hydrophobic because of the reduced activity of the charged group. This may explain the promotion of the dye penetration in sucrose solution.

The most important finding in this observation is that there are distinct differences in the nuclear stainability by supravital staining with the dyes among the cells of different strain, and even those belonging to the same strain. As just described, in physiologic saline the erythroblast nuclei were stained deep by B. C. B. and N. B., but in the cells of other kinds no nucleus was stained supravitally by the same dyes under the same environment.

The nuclear staining of erythroid cell observed was similar in all cases irrespective of animal strains, but distinct differences were observed in stainability between immature erythroblasts and mature ones. As the nuclei of nucleated red cells of chicken were also stained supravitally the phenomenon seems not to be directly related to the peculiar fate of mammalian erythroid nuclei, the denucleation.

The simple test made on the cells stained supravitally by N. B., where the cells were smeared, dried, fixed with methanol and observed under microscope, proved that the dye stained erythroid cell nuclei did not fade through the fixation procedure, while the dye stained cytoplasmic granules of myeloid cells and granulocytes faded away completely by the same treatment.

The result suggests that the electrostatic binding occurrs between basic dye and DNA of erythroid cell nuclei. Such a binding occurred also in other cells but only after the cell death. By cell death, the loosening of the binding between DNA and protein, probably histone, may be induced, because the cell membrane will become permeable for some ions which will act to loosen the bindings between DNA and histone, and yet the nuclear membrane has pores indicating that there seems to be no inhibitory barrier for the penetration of the dye from cytoplasm into the nucleus.

If the nuclear supravital staining by the dyes is solely due to a weak binding between DNA and histone, histone of the myeloid and lymphoid cells should be different from that of erythroid cell, or differ in each cell strain, and the nature of histone should change with the advance in cell specialization so as to loosen the binding between DNA and histone.

Actually, the fact that the changes in histone occurs by cell specialization has been proved by ALFKEY and associates and BERLOWITZ (29, 30).

Thus the supravital staining of nuclei of living cells by the dye may give an information for the changes in the chemical characteristics of histone which will be closely related to the cell specialization or gene activation.

SUMMARY

For the purpose to reveal the relation between cell death and nuclear stainability by supravital staining with basic dyes observations have been made on the cells of bone marrow, peripheral blood and lymph node from anemic and non-anemic rabbit, rat, mouse and chicken, and thymus from young mouse. The cells were stained supravitally in blood serum, isotonic saline, calcium chloride and sucrose solutions with the dyes; brilliant cresyl blue (B. C. B), Nile blue (N. B.), neutral red (N. R.), Janus green (J. G.) and eosin (E.). The following results were obtained:

1. In the presence of blood serum all the living cell nuclei observed were not stained supravitally, except some mature erythroblasts and nucleated red cells.

2. In isotonic saline, $CaCl_2$ and $MgCl_2$ solutions all the erythroid cell nuclei were stained deep by B. C. B., N. B., N. R., slightly by J. G. but not by E. In stainability the younger the cell is the deeper in its nuclear staining. The nuclei of other cell strains were not stained.

3. In isotonic sucrose the nuclei of mature granulocyte were also stained by B. C. B. and N. B. but not by other dyes. The nuclei of lymphoid cells and myeloid cells appeared pale without being stained by any dyes. The nuclei of erythroblasts in sucrose solution were stained deeper with B. C. B. and N. B. than those in isotonic saline.

The differences between supravital stainability of the nuclei among the cells belonging to different strain and among those of the same strain but in different maturation stage and the nuclear staining after cell death have been discussed from the possible dissociation of DNA from histone.

ACKNOWLEDGEMENT

The author thanks Prof. S. SENO for invaluable suggestions during the experiment and painstaking revision of the manuscript, and also expresses gratitude to Dr. AWAI for kind advice and technical help.

REFERENCES

1. OVERTON, E.: *Jahrb. f. Wiss. Bot.* **34**, 669, 1900
2. MICHAELIS, L.: Arch. microscop. Anat. u. Entwicklingsmech. **55**, 558, 1900
3. KOEHRING, V.: *J. M. orph. and Physiol*, **49**, 45, 1930
4. KELLER, R.: Die Elektrozität in der Zelle, Mahrisch-Ostrau, 3, *Auflage*, 1932
5. SUGIYAMA, S.: Studies on supravital staining. **1**, 1929 (in Japanese)
6. SUGIYAMA, S., HIRANO, M. *et al.*: Studies on supravital staining **2**, 1930 (in Japanese)
7. SUGIYAMA, S., MORI, K. *et al.*: Studies of supravital staining **3**, 1931 (in Japanese)
8. KIYONO, K.: Studies on vital staining, *Nankodo Tokyo*, 1931 (in Japanese)
9. KIYONO, K., SUGIYAMA, S. and AMANO, S.: Die Lehre von vitale Färbung, *Kyoto*, 1938
10. NOVIKOFF, A. B.: *The cell* **2**, 199, 1961
11. AMANO, S.: Basis of hematology, *Maruzen, Tokyo*, 1948 (in Japanese)
12. SENO, S., HABA, K., MAKI, T. and NAKAMOTO, T.: *Acta Haem. Jap.* **21**, (No. 2 supple.) 332, 1958
13. GELLHARN, E.: Gesamtgebiet der Physiologie der Pflanzen u. *der Tiere* **16**, 92, 1929
14. YAMAHA, G.: *General cytology.* **44**, 343, 1949
15. DAVSON, H. and DANIELLI, J. F.: The permeability of natural membrane, *Publisher Cambridge*, 1952
16. RUZICKA, V.: *Zschr. f. wiss. Mikros.* **22**, 91, 1905
17. SENO, S., MATSUOKA, I., UTSUMI, K., HAYASHI, H. and AMANO, I.: *Acta Med. Okayama* **12**, 18, 1958
18. SENO, S., OHGUCHI, M. and YOSHIZAWA, K.: *Le Sang* **28**, 687, 1957
19. EATON, M. J., SCALA, A. R. and TEWELL, M.: *Cancer Res.* **19**, 945, 1959
20. SENO, S., YOKOMURA, E., VKAHORI, F., KOSHIBA, K. and NAKATSUKA, A.: *Acta Med. Okayama* **18**, 173, 1964
21. SUGIYAMA, S.: *Nippon Biseibutsu-gaku-zassi* **18**, 1775, 1924 (in Japanese)
22. SEEO, S.: *Acta Haem. Jap.* **29**, 72, 1965 (in Japanese)
23. SUGIYAMA, S.: *Nippon Biseibutsu-gaku-zassi* **18**, 1423, 1824
24. GIESE, A. C.: Cell Physiology, *Saunders*, Philadelphia, 1962, p. 144
25. WINTROBE, M. M.: Clinical hematotogy, Lea and Febiger, Philadelphia, 1964, p. 91
26. SABIN, F. R.: Johns Hopkins Hospital Bulletin, **34**, 277, 1923
27. MORI, K.: K*anazawa Ikadaigaku juzenkai Zassi* **33**, No 8, 1928 (Refer to Reference 5)
28. SENO, S., ODA, T., TSUDA, T., YOSHIZAWA, K., SAKAI, A., OKAZAKI, H. and KUBO, Y.: *Acta Med. Okayamn* **11**, 300, 1957
29. LITTAU, V. C., BURDICK, C. J., ALLFREY, V. G. and MIRSKY, A. E.: *Proc. N. A. S.* **54**, 1204, 1965
30. BERLOWITZ, L.: *Proc. N. A. S.* **54**, 476, 1965
31. SENO, S., YOSHIZAWA, K., NAKAMOTO, T. and KANDA, S.: *Folia Haem.* **2**, 269, 1958

Explanations of Figures

1. The erythroid cells from normal chicken bone marrow. They were suspended in physiologic saline and stained with brilliant cresyl blue (B. C. B.) suspended in saline (pH 7.1). The nuclei of erythroblasts (Eb) and mature nucleated red blood cell (E) are stained distinctly, while the nuclei of granulocyte (G) are not stained. The cytoplasm of basophilic erythroblasts is stained deep blue diffusely but the cytoplasm of granulocyte is stained granular. Photographed 10 minutes after the preparation.

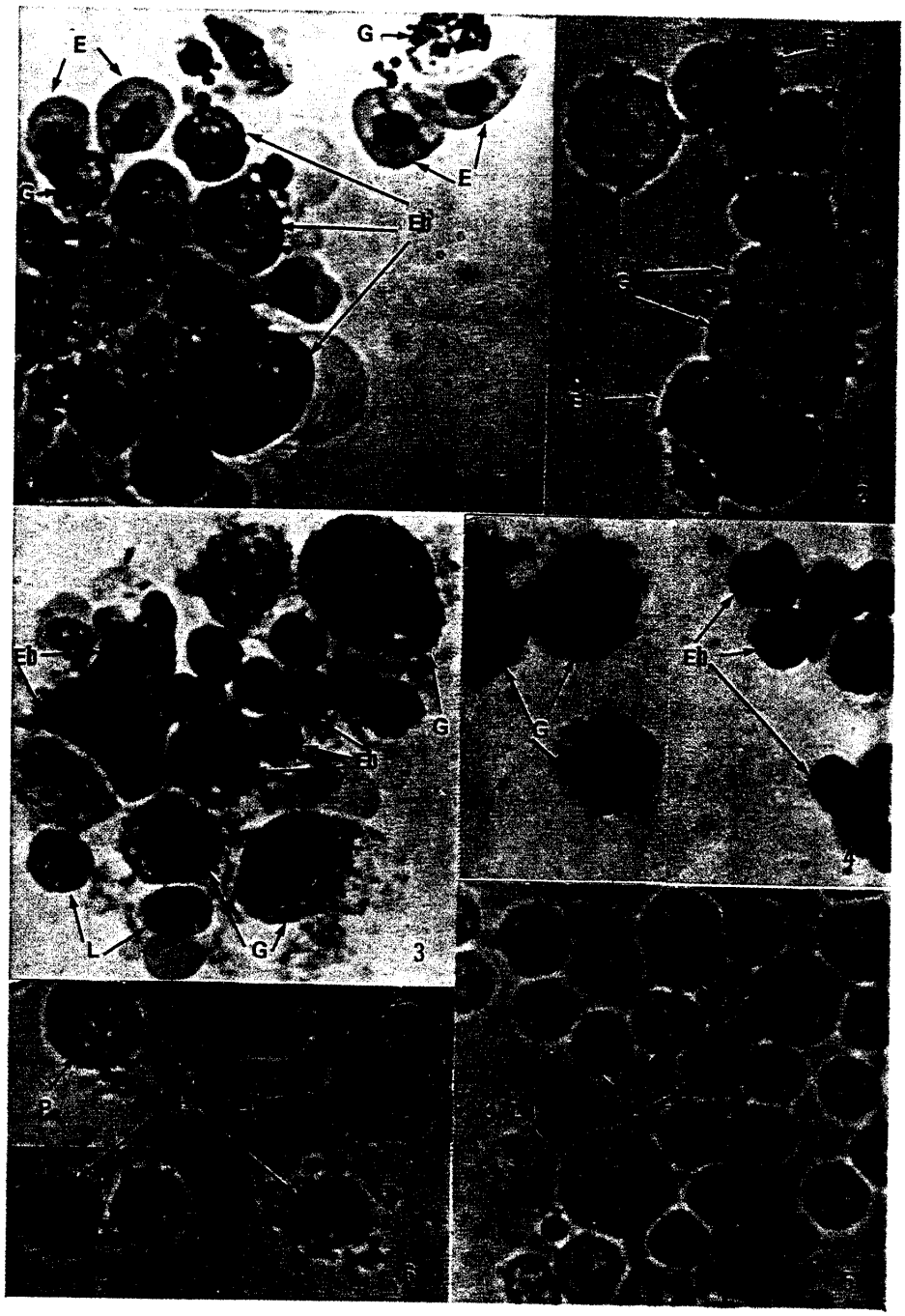
2. The cells of bone marrow from an anemic rat. The cells were suspended in physiologic saline and stained supravitally with B. C. B. by the same method as in Fig. 1. Nuclei of the erythroblast (Eb), one basophilic erythroblast appearing at the bottom of the picture and two polychromatic and one orthochromatic erythroblasts at the top, are stained distinctly. The cytoplasm of these basophilic and polychromatic erythroblasts is stained blue deeply. The three cells appearing unstained are of granulocyte or myeloid cells (G). Note the staining of some granular components in cytoplasm of myeloid cells. Photographed 5 minutes after preparation.

3. The cells of bone marrow of an anemic rat. They were suspended in physiologic saline and treated by monojod acetic acid ($10^{-3}M$) for 3 hours and then stained supravitally with B. C. B. dissolved in saline. Besides the nuclei of erythroblasts (Eb), the nuclei of myeloid cells (G) and lymphocyte (L) are stained clearly.
Method: see the text.

4. The cells of bone marrow of an anemic rat. They were suspended in physiologic saline, exposed to KCN ($10^{-3}M$) for 2 hours, and then stained supravitally with neutral red (N. R.). The nuclei of myeloid cells and granulocyte (G) as well as these of erythroblast (Eb) are stained red.
Method: See the text.

5. The cells from the mesenterial lymph-nodes of a normal mouse. The cells were suspended in physiologic saline and stained supravitally by mixing with N. R. saline solution. The nuclei of the lymphoid cells, both of small (L) and large sized ones (Lb) are not stained by the dye. The dye stains slightly a part of cytoplasm, probably some part of Golgi apparatus.

6. The lymphoid cells from the mesenteric lymph node of a mouse. They were suspended in physiologic saline solution and treated with KCN ($10^{-3}M$) for 30 minutes, and then stained supravitally with B. C. B. The most nuclei of lymphocyte (L) and lymphoblast (Lb) are stained deep, but some nuclei are stained only slightly.
P: Plasma cell


E
G
E
G
Eb
Eb
G
Eb
L
G
3
Eb
G
4