Histochemical Study on the Phosphatase Distribution in Rabbit Bone Marrow Cells

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Since the introduction of histochemical methods for the demonstration of phosphatase activity by Gomori and Takamatsu, many investigations have been made on the distribution of this enzyme in various tissues under normal and pathological conditions, including those on blood and bone marrow. This author has also attempted to study the distribution of acid and alkaline phosphatases in bone marrow cells, in the course of histochemical studies on hemopoietical tissues. In this experiment, some findings were noticed which had been not so fully described in the previous reports on similar cells. The most noteworthy finding was the negative reaction of mitotic chromosomes in both acid and alkaline ranges, which was completely opposed to the opinions of many authors, such as Danielli and Catcheside, Kurgelis, Brachet, Rabinovitch and Andreucci, and Sulkin and Gardner.

In last few years, on the other hand, specificity of the reaction pattern obtained by Gomori-Takamatsu methods has been called in question, especially, several investigators deciding the positive reaction in nucleus as nothing but an artefact. But whether chromosomes of the dividing cells are poor in phosphatase activity or utterly lack in it is still in question, as not being reported on these phenomena by any investigator. In this paper, specificity and significance of these reaction patterns are also discussed.

EXPERIMENTAL

Material and Methods

Normal rabbit bone marrow was made to imprints on slides and treated as described below. Sodium-beta-glycerophosphate was used as the substrate of incubation mixtures for both alkaline and acid phosphatases.

Alkaline phosphatase: Air-dried imprints were dipped in dilute alcohol-ether-celloidin solution for about 10 seconds without any preceding fixation.
Thus coated slides were kept on a slant for several minutes until the celloidin became half dried, and immersed in absolute alcohol for removal of remaining ether, and next in 90 and 70% alcohol to harden the celloidin. Moderate drying of coated celloidin prior to immersing into alcohol was essential to prevent the celloidin membrane from getting off during the following treatments. After washing in tap water, slides were then incubated in Gomori's revised substrate mixture\(^9\) (pH 9.0–9.4, tested with thymol blue paper) for 3–24 hours at 37°C, and visualized according to cobalt-sulfide method. Some slides were counterstained with methyl green.

Control slides were inactivated by means of fixation for at least 10 minutes with 0.5% mercuric chloride solution or Zenker-acetic, prior to incubation. Moreover, according to Danielli,\(^10\) these inactivated slides were incubated in another substrate mixture containing a small amount of 3% hydrogen peroxide solution (final volume per cent was 5–10) instead of magnesium salt, in order to examine unspecific adsorption of calcium phosphate which may occur during the long period of incubation.

Acid phosphatase: Imprints were fixed for not over half minute in chilled acetone or 10% neutralized formalin or 10% neutralized formalin-saline, and rinsed in water. Then, slides were incubated for 3–24 hours at 37°C in the substrate mixture described by Rabinovitch et al.\(^7\)^\(^11\) Ph of the acetate buffer used was 4.4–4.8 (tested with brom cresol green paper). Visualization was carried out in dilute ammonium sulfide solution.

Control slides were inactivated as similar as in alkaline phosphatase. In order to examine unspecific adsorption of lead phosphate, following test introduced by Gomori\(^12\) in his critical study on the alkaline phosphatase was applied in this case of the acid one. An inactivated slide and non-inactivated one were made up to a pair, being superimposed together face to face each other. Both opposite faces were separated by a narrow space supported with insertion of cover glass between the ends of the two slides. Such a pair was incubated for 15–24 hours in the usual substrate mixture.

Observations

**Alkaline phosphatase**

In briefly incubated slides, positive reactions were evident only in the cytoplasm of pseudoeosinophilic myelo- or metamyelocytes and polymorphonuclear granulocytes, while their nuclei remained unstained (Fig. 1). These reactions were diffused in the cytoplasmic backgrounds among the specific granules which were recognized as unstained round particles. Promyelocytes showed very slight tint in their nuclei and cytoplasm in some specimens incubated for 6 hours.

With prolongation of incubation period, being kept for more than 15 hours, juvenile cells were brought to be visible accompanying simultaneous appearance of positive reaction in almost all nuclei of all cellseries. Such a nuclear reaction was diffuse and even in its pattern, and
scarcely seemed to be located within any specific structures, such as chromatin or nucleoli. After prolonged incubation near to 24 hours, cytoplasmic reaction of pseudoeosinophilic granulocytes had become apparently slighter than that of briefly incubated ones, while the blackening of their rod-shaped or segmented nuclei had been intensified.

Large round-nuclear cells without cytoplasmic granulations were also visible as faint gray shadow-like figures in such slides. As nuclei of small erythroblasts (probably, polychromatic and orthochromatic ones) had shown occasional staining after such prolonged incubation, these round cells were supposed to include not only myeloblasts but also early erythroblasts. It is very difficult, however, to identify cytologically to what cell-series each of them belongs.

Mitotic figures of these blast-cells in division exhibited a characteristic reaction pattern as represented in Figs. 2 and 3. Their chromosomes either showed nothing of the reaction or, if any, only a minimal one forming a striking contrast to considerably intensified impregnation on the cytoplasm. It must be taken in consideration here, that the greater part of the nuclei of resting cells in the same slides show more or less certain positive reactions. These general reactions found in most of the nuclei result from the prolonged incubation kept for more than 15 hours, indispensable to visualize the mitotic figures as most of them belong to these so-called juvenile cells which were beforehand observed as large round-nuclear cells.

Eosinophilic granules showed entirely negative reaction standing out against the dark background as well as pseudoeosinophilic granules. It was always easy thanks to these distinct negative figures of the granules, to distinguish pseudoeosinophilic and eosinophilic promyelocytes and myelocytes from other cell-series. Unspecific (azurophilic) granules of promyelocytes and basophilic granules could not be identified in any of the slides.

Mature erythrocytes did not show any reaction.

Megakaryocytes represented only faint and diffuse tint in their nuclei and border zone of their cytoplasm when the slides were incubated for long period. Reaction of the platelets was not observed.

Reaction patterns of monocytes, lymphocytes, and plasma cells yet remain not clear, as they can not be distinguished respectively from each other, nor from other round-nuclear cells. At least, however, reaction of these cells was not observed in briefly incubated slides. Those of lymphocytes and lymphoblasts were also searched using the imprints obtained from mesenteric lymph node as the materials. They exhibited only a slight and diffuse reaction in their nuclei after long incubation.

Inactivated slides treated simultaneously did not show any sign of
reaction. Another control slides incubated in the substrate solution which contained hydrogen peroxide showed far different results. Namely, though occurrence of reaction was almost not to be discernible except slightest signs within 3–6 hours of incubation, but after 15 hours, all nuclei of all cell-series had been pretty blackened remaining their cytoplasm unaffected. In the very case, pattern of the nuclear stain conformed almost exactly to that of in May-Giemsa stained ones, increasing in its intensity as maturation of the nuclei in both of white and red cell-series. For instance, blackening of the pycnotic nuclei of normoblasts was most prominent as they are usually. Mitotic chromosomes were stained infallibly, though their contour was not invallably distinct. Coincident results between these controls and experimental cases were restricted to the fact that both of them were negative in the reaction of cosinophilic and pseudoeosinophilic granules. Impregnation in the cytoplasm occurred after more prolonged incubation near to 24 hours, being accompanied with simultaneous blackening of mature red corpuscles.

Acid phosphatase

In the reaction pattern of acid phosphatase, there was no great difference between each cell-type and cell-series. Generally, almost all of the nuclei were impregnated most intensely in somewhat slighter cytoplasm, except certain cytoplasmic constituents, such as eosinophilic granules. In this experiment, it has become questionable whether the nuclear pattern of acid phosphatase takes after that of conventional hematological stain as claimed by Rabinovitch and Andreucci.\(^7\) Large round nuclei containing distinctly unstained nucleoli in them (probably the nuclei of myeloblasts, proerythroblasts, etc.) took the pattern of fairly fine granular in reaction which was apparently coincided to that of chromatin. But the granular reaction was distributed more dispersedly than the network of basichromatin, and never identified was the so-called “nucleolus associated chromatin,” which is usually stained with basic dyes and Feulgen technic. Moreover, the nuclei of granulocytes and late erythroblasts showed thread-like reaction which seemed to be located in nuclear sap or oxychromatin, but at least not in basichromatin. Such a thread-like reaction of nuclei was most remarkable in the acetone-fixed specimens (Figs. 4 and 5). Other formalin-fixed ones, however, also showed very similar reaction pattern when they were incubated briefly (Figs. 6 and 7).

Mitotic nuclei represented negative reaction, as well as in alkaline phosphatase, but the outline of individual chromosomes was much better preserved (Figs. 5 and 6). Most of the cytoplasm of these dividing cells were stained more intensely than those of the resting cells. Such a characteristic staining pattern was never altered in quality with choice of the fixatives and length of the incubation period. In thick portion, however,
or in surrounding zone around solid clot of tissue in the same slides, asters of dividing nuclei showed occasional diffuse brown tint, and moreover, some of them were impregnated deep brown-black prominently standing out against light brown cytoplas.

In the case of the acid phosphatase too, the evidence of specific granules only makes it possible to make a discrimination between the so-called juveniles of granulocytic series and those of the other series. Eosinophilic granules were readily distinguishable by their intense reaction even in the slides incubated briefly (Fig. 7).

Cytoplasmic reactions of pseudoeosinophilic series were somewhat more variable. Most of the slides showed negative figures of the granules in slightly stained cytoplasm, while occasional granular reaction in cytoplasm was also recognized in some specimens. Such a granular reaction, however, was found mainly in the cytoplasm of promyelocytes and myelocytes, though that of polymorphonuclear ones in the same slides was stained more diffusely. These granules were not apparently coincided with the unspecific granules in their number and locality, when compared with control slides stained by means of May-Giemsa method, being obtained from the same animal. Basophilic granules were not identified.

Mature red corpuscles were not stained.

Coarse and intense reaction of the nuclei of megakaryocytes was observable in every case, but the pattern was somewhat different from the structures of the Feulgen stained nuclei. Their cytoplasm also showed intense granular tinting (Fig. 8), but the distribution pattern of these granules and that of azurophilic dusts (Schriddle's granulation) were not exactly identical. Reaction of platelets was not found.

As regards reaction patterns of lymphocytes, monocytes, and plasma cells, only lymphocytic series was to be distinguished from the latter two, using lymph node imprints as the materials. Positive reaction of lymphocytes and lymphoblasts was recognizable only in their nuclei, but none in their cytoplasm (Fig. 9). Pattern of their nuclear reaction was granular and somewhat aggregated, especially in the nuclei of mature cells. Monocytes and plasma cells were considered to present some reaction at least in their nuclei, probably in so similar a pattern to that of lymphocytes, as make it very difficult to differentiate each of them. Nuclei of fat cells and reticulum cells were also impregnated.

No sign of specific reaction appeared in the control slides incubated in usual manner. To the inactivated ones of the slides superimposed in pairs, on the other hand, took place certain adsorption of lead phosphate. After 15 hours of incubation, almost all cells had been stained brown-yellow, and the nuclei deeper than the cytoplasm, chromosomes being also stained with indistinct outlines, though general pattern of the
impregnation was considerably diffuse and hazy. After 24 hours, vigorous
impregnation had been observed, strictly locating within basicchromatin
of nuclei. Thus, nuclear pattern of these slides became to have strong
resemblance to that of conventional hematological stain, showing intense
impregnation of mitotic chromosomes standing out in relief against practi-
cally unstained cytoplasm (Fig. 12). Comparing Fig. 12 with Figs. 4-7,
differences between impregnation of chromatin caused by secondary
adsorption of lead salt and granular or thread-like reaction due to enzy-
matic activity are very easily recognizable.

The other slides of such pairs, the active ones, showed noteworthy
results. After 15 hours, they had represented the reaction pattern same
with that of usual active slides, showing distinct negative figures of mitotic
chromosomes. After 24 hours, difference between the pattern of the
active slide and inactive one superimposed together had become minimal.
Nuclear pattern approached to that of Giemsa stained ones, exhibiting
either prominent or slight diffuse tint of asters of mitotic chromosomes
(Figs. 10 and 11). Intensely stained asters stood out prominently against
paler or negligibly stained cytoplasm (Fig. 11), and on the other hand,
cytoplasm surrounding diffusely tinted asters showed moderate reaction
(Fig. 10). Such cytoplasmic reactions were only a difference discrimi-
nating the active slides from the inactive ones of the same pair.

Staining pattern of dividing cells in the thick portion surrounding
solid clot of tissue in the usual active slides has strong resemblance to that
in the active slides of superimposed pairs described here. It may also
be due to secondary adsorption took place in such a portion.

DISCUSSION

Histochemical demonstration of alkaline phosphatase in bone marrow
cells was made previously by Wachstein,13) Wislocki and Dempsey,14)
Rheingold and Wislocki,15) Plum,16) Takeuchi,17) and as for both acid
and alkaline ones, exhaustive studies were made by Rabinovitch et al.7111).1
Moreover, Horii et al.18) had made phylogenetical comparative study on
the both phosphatase activities in blood cells obtained from various species.
Among these many observations, there exists no great difference, and the
results presented here are also not inconsistent in their principles with
the precedings, except some points, especially concerning with acid phos-
phatase as described below.

Specific reaction in the cytoplasm of erythroblasts and plasma cells
described by Rabinovitch and Andreucci,7) and granular reaction local-
ized in the Golgi-zone in lymphocytes observed by Rabinovitch et al.7111)
and by Kazikawa19) as well, could not be found here. Nuclear pattern
in acid phosphatase in this experiment resembled rather to the preceding:
Except these little differences, the most outstanding fact in the present observations is the negative reaction of mitotic chromosomes in both acid and alkaline ranges. This finding is perfectly contradictory against almost all of the reports published before which described positive phosphatase activity in nuclei, especially against observations of some authors insisting on the positive reaction located in chromosomes of various tissue cells. For instance, Danielli and Catcheside observed alkaline phosphatase activity situates in the Feulgen positive bands of the chromosomes obtained from salivary gland of *Drosophila*, and Krugelis suggested the existence of desoxyribonucleotidase activity in the nuclei and chromosomes. Newman, Feigin, Wolf and Kabat confirmed recently the existence of activity of a certain group of alkaline phosphatase in the nuclei, using various substrates, inhibitors, and accelerators.

In last few years, the specificity of sites of the reaction obtained by Gomori-Takamatsu method have been criticized exhaustively. In these critical studies, great importance has been attached to the possibilities of the diffusion of enzyme itself or reaction product (calcium phosphate or lead phosphate) and the secondary adsorption of them by cellular constituents. Above all, nuclear staining in the alkaline phosphatase was decided as an artefact by Gomori and Novikoff. Some attempts to prevent such a technical error were also tried by Bélanger and Goetsch, Reynolds and Bunting, both reports describing the negative reaction of nuclei.

Specificities of the results about acid phosphatase distribution also could not stay free from criticism. Noback and Paff studied on the intracellular distribution of acid phosphatase activity in fibroblast, and found diffusion and shifting of positive reaction from cytoplasm into nuclei through nuclear membrane with prolongation of incubation period. Newman, Kabat and Wolf demonstrated natural affinity of tissues to lead salt which was especially vigorous between pH 5.0–7.0. Attempts to check these artefacts were also made by Goetsch *et al.* and Takeuchi and Tanoue, and nuclei remained unstained in both of their experiments. This author also observed previously the occurrence of lead salt precipitation on the smears and imprints of bone marrow when they were fixed with basic lead acetate, but it was seen to be strictly localized within basichromatin of nuclei. This is fairly agreed with the observation in this paper on unspecific impregnation occurred in the inactivated imprints with superimposing test according to Gomori.

The observations about absence or slightness of nuclear phosphatase activity, obtained histochemically as described, have many agreeing points with what has been introduced bio- and cytochemically with differential
fractionation of cellular constituents. Berthet et al.\textsuperscript{29,30}) studied recently on the intracellular distribution of acid phosphatase in rat liver with such technic, and found out that the enzyme was naturally bound to mitochondria in non-active state, and when it was released out from the bond with mitochondria under various conditions, it acquired its whole enzymatic activity, and might be adsorbed secondarily in fully active state by various structural constituents, such as mitochondria, nuclei, and others. Such a migration of acid phosphatase itself may possibly occur in the conventional Gomori technic, because, according to Berthet's experiments, the enzyme-mitochondria binding is protected under O°C.

Concerning such doubtfull existence of phosphatase activity in nuclei, negative reaction of chromosomes is not to be regarded as so incomprehensible a phenomenon. It is noticed in this report, however, especially in the case of the alkaline phosphatase, that the negative reaction of mitotic figures is observed in such slides, in which almost other nuclei are stained considerably, as the positive reaction of these actively proliferating juvenile cells has been produced only after prolonged incubation over 15 hours, just as Takeuchi\textsuperscript{17} also observed. Perhaps in these slides, diffusion and secondary adsorption of calcium phosphate have taken place already, because reaction of the cytoplasm of polymorphonuclear granulocytes seems weaker in its intensity than that of briefly incubated slides, while the reaction of nuclei is brought to be visible. Such an inversion of the site of reaction during incubation is very similar to Moe's\textsuperscript{31}) observations on the intestinal mucosa, which he has attributed to the diffusion of enzymatic product but not of enzyme itself.

There arises, therefore, an interrogation whether the diffusion of calcium phosphate from chromosomes causes such negative figures. This doubt may be resolved by taking into consideration the results of Danielli's test, which is supposed to reveal natural affinity of the cells to calcium phosphate. In this test, no difference of adsorbing affinity was found between resting nuclei and dividing ones. Moreover, appearance of adsorption in the cytoplasm occurred more later than in nuclei.

Educing from these results of Danielli's test, it is completely difficult to consider that adsorption of calcium phosphate takes place only in the cytoplasm of dividing cells, remaining their chromosomes unaffected, or that chromosomes represent less affinity to calcium phosphate than cytoplasm or resting nuclei. Accordingly, negative reaction of chromosomes indicates conceivably loss or decrease of enzymatic activity in nuclei during mitosis. Then it becomes not so unreasonable to consider that a certain portion of nuclear reaction is attributed to enzymatic activity, even though the greater part of it caused after long period of incubation may be due to unspecific adsorption of calcium phosphate.
In the case of the acid phosphatase, on the other hand, negative reaction of mitotic chromosomes is not a specific phenomenon restricted to dividing cells. Positive reaction in resting nuclei is dispersedly granular or thread-like in its pattern and is considerably located in nuclear sap or oxychromatin, but not in basichromatin. Negative reaction of chromosomes, therefore, may be regarded as an expression of natural absence of the acid phosphatase activity in chromatin.

Various staining patterns of dividing cells in active slides of the superimposed pairs are highly significant in their import. After 15 hours of incubation, they showed specific enzymatic activity alone, while another slides of the same pairs, the inactive ones, represented only slight and hazy impregnations. Reaction observed in the similar slides incubated for 24 hours was a total result of enzymatic activity and secondary adsorption. In these slides, nuclei and cytoplasm of the dividing cells showed various staining patterns, which were gradated into three orders as followings: slightly stained asters of chromosomes in moderately stained cytoplasm; moderately stained asters in moderately stained cytoplasm; and intensely stained asters in slightly or negligibly stained cytoplasm. And similar cells in the inactivated slides of the same pairs showed asters invariably stained intensely in unstained cytoplasm. Comparing with the natural affinity to lead salt in basichromatin as described previously by this author, such a gradation of impregnation described here is undoubtedly nothing but the representation of diffusing and shifting process of enzymatically produced hydrolysates. It is conclusive, therefore, that intense impregnation in acid range restricted to chromosome and chromatin described by Rabinovitch and Andreucci is an artefact, and moreover, that the greater part of the positive reaction obtained with incubation within 15 hours may be regarded as the representation of the real site of acid phosphatase activity.

Another noteworthy finding is the considerably intensified reaction in the cytoplasm of the cells during mitosis, in both acid and alkaline ranges. This increased reaction in alkaline range may be attributed to increased enzymatic activity, educing from the results of Danielli’s test, while the significance of that in acid range remain obscure, whether it is due to real increase of the enzymatic activity.

In the preceding paper, this author noticed that, on the contrary to division of general somatic cells, intensity of cytoplasmic basophilia of the blast-cells of bone marrow had never decreased during mitosis. This fact is very interesting when compared with the increased alkaline phosphomonoesterase activity in the cytoplasm of the similar cells, which may suggest accelarated hydrolysis of ribonucleic acid. Accordingly, in the case of division of these blast-cells, it seems that vigorous synthesis of
ribonucleic acid may be simultaneously proceeding, while total ribonucleic acid content in the cytoplasm is not apparently altered. Significance of the decrease of alkaline phosphatase activity in the chromosomes is not clear.

**Summary**

Intracellular distributions of acid and alkaline phosphatase activities in bone marrow imprints obtained from normal rabbit were studied histochemically according to Gomori-Takamatsu methods.

The most outstanding result obtained in this experiment was that the negative reaction of mitotic chromosomes in both acid and alkaline phosphatases, which was accompanied with simultaneous intensification of reaction in the cytoplasm. Although such reaction patterns in both phosphatases are very similar, significances are different from each other.

Negative or minimal reaction of the chromosomes in alkaline phosphatase represents loss or decrease of enzymatic activity, and increased reaction in the cytoplasm of the same cell shows increased enzymatic activity. On the other hand, negative figures of mitotic chromosomes in acid reaction do not seem to be the specific phenomena restricted to cell division and should be interpreted as the expression of the natural absence of acid phosphatase activity in chromatin of nuclei.

**References**

2) Gomori, Arch. Path., 1941, 32, 189.
7) Rabinovitch and Andreucci, Blood, 1949, 4, 580.
11) Rabinovitch, Junqueira and Mendes, Science, 1948, 107, 322.
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24) Goetsch, Reynolds and Bunting, ibid., 1952, 80, 71.
30) Berthet, Berthet, Applemans and de Duve, ibid., 1951, 50, 182.

Note at proof-reading: After this manuscript had been submitted for publication, the writer received several papers dealing with nuclear phosphatase kindly sent from Drs. Richterich, Novikoff and Goetsch. Richterich\(^33\) discussed on nuclear phosphatase with reference to many literatures, Novikoff\(^34)(35\) described negative alkaline phosphatase reaction of nuclei of intestinal mucosa and mitotic chromosomes of regenerating liver, and according to Goetsch and Reynolds,\(^36\) acid phosphatase is not detectable in cell nuclei.

Explanation of Figures

Fig. 1. Alkaline phosphatase reaction after 6 hours of incubation. Positive reaction is restricted within the cytoplasm of polymorphonuclear granulocytes and myelocytes.

Figs. 2 and 3. Negative reaction of mitotic figures in alkaline phosphatase after 24 hours of incubation. Small spot in Fig. 2 shows a late erythroblast.

Fig. 4. Acid phosphatase reaction in acetone-fixed slide incubated for 24 hours, showing dispersedly granular and thread-like reaction in nuclei. Negative reaction of nucleoli is also visible.

Fig. 5. Similarly treated slide as the above, showing distinct negative figures of mitotic chromosomes.

Figs. 6 and 7. Acid phosphatase reaction in formalin-saline-fixed slide after 6 hours of incubation. Fig. 7 represents a dividing eosinophilic myelocyte containing intensely stained granules in its cytoplasm.

Fig. 8. Acid phosphatase reaction in nuclei and cytoplasm of megakaryocyte in formalin-fixed slide incubated for 24 hours.

Fig. 9. Acid phosphatase reaction in lymph node imprint fixed with formalin-saline, and incubated for 16 hours.

Fig. 10. Reaction in an active slide of a superimposed pair incubated for 24 hours in acid substrate mixture. Moderate and diffuse tint of mitotic asters is visible.

Fig. 11. Different portion from the same slide, showing intense impregnation of chromosomes and chromatic materials.

Fig. 12. Reaction in an inactivated slide of the same pair superimposed together with the slide represented in Figs. 10 and 11. Intense and distinct impregnation is restricted to chromatic materials.