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Hikaru Asakura*

*Okayama University,

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Abstract

The mechanism of induction of anisocytosis was studied with experimental anemia of rabbits induced by blood depletion and phenylhydrazine chloride injection. The Price-Jones' curves of erythrocytes from anemic animals showed a large variety of red cell size, indicating the appearance of abnormally large sized cells. RNA contents of some reticulocytes in anemia were comparable to those of polychromatic and late basophilic erythroblasts. The result proved that in severe anemia a large number of erythroblasts are denucleated at earlier maturation stages, in most cases at polychromatic, and some at late basophilic and some at orthochromatic stages, resulting in anisocytosis.

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MECHANISM OF INDUCTION OF ANISOCYTOSIS

Hikaru ASAKURA

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

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The normal size of the matured human erythrocyte is from 6.0 to 9.0 μ and its average size is 7.0 μ in diameter[1] or from 7.0 to 10 μ and its average 8.5 μ in diameter in wet[2]. The erythrocyte larger than 9.0 μ is called macrocyte and smaller than 6.0 μ is called microcyte.[1] Commonly, anisocytosis is vaguely admitted as the abundant appearance of these macro- and microcytes in the circulating blood[1]. For the classification on the size of the erythrocyte the distribution curve of the erythrocyte, known as the Price-Jones' curve[3], is figured with respect to the cell diameter. The curve shows a specific symmetrical pattern with a peak at the value of average diameter of about 7.5 μ in normal, more than 7.5 μ in macrocytosis and less than 7.5 μ in microcytosis. In traditional way of judging the curve so obtained, however, the existence of the reticulocyte is not taken into consideration in spite of abundant appearance of reticulocytes in various anemic conditions, in which anisocytosis is frequently encountered. It is generally believed that macrocytosis in anemia is mainly due to the appearance of large sized reticulocytes, but it is uncertain that the big reticulocyte reduces its volume to a normal red cell by maturation.

SUIT *et al.*[4], LAJTHA and SUIT[5] and BORSOOK *et al.*[6] stressed that macrocytosis in panic state of anemia should be due to the early denucleation of erythroblasts. In normal rabbit the greatest population of red cells is found at those of 6.5 μ in diameter by our observation on smeared cells, but the smallest ones are about 5.0 μ and the largest ones 9.0 μ. This means that there is a big difference in cell volume between larger and smaller ones. This seems to suggest that the denucleation of red cell can occur at varying maturation stage of erythroblast even in normal state, provided that the erythroblasts reduce their volume by one half at each cell division[7], and shrinkage does not occur after denucleation[6].

In view of this the author studied the diameter of red cells and erythroblasts and their ribonucleic acid (RNA) contents in anemic rabbits. As the result it was found that in anemia a large number of cells are denucleated at polychromatic stage producing large size cells, some resulting in anisocytosis at orthochromatic stage.

MATERIALS AND METHODS

Adult male rabbits of about 2.5 kg body weight fed on soybean mash precipitants and green grass were used. They had about 5.5 million red cells per cu mm of blood and 80 to 90 % of hemoglobin contents (SAHLI). They were divided into three groups, the first for hemolytic anemia, the second for blood depletion, and the third for control.

Hemolytic anemia was induced by the subcutaneous injection of phenylhydrazine on the back of the rabbits, 1.5 cc of 2.5 % neutralized phenylhydrazine chrolide solution once a day for three to four days. Price-Jones' curve, red cell count and reticulocyte number were observed at selected intervals. On the fourth day after the last injection, when the anemic condition seemed to be the initial stage of recovery (red cell count; about 2.0 to 2.5 million per cu mm, hemoglobin contents; about 30 % and reticulocyte; about 80 to 90 %), the rabbits were sacrificed by bleeding. The circulating blood and bone marrow cells were smeared with or without supravital staining with Nile blue. They were counterstained with Giemsa for routine observation and some of them were stained with Azure B for the estimation of RNA by microspectrophotometry[8,9].

Anemia by blood loss was induced by depletion from ear vein, about 30 cc a day for seven days by sectioning auricular vein. The blood was taken directly into the test tube containing 3 cc of 3.8 % sodium citrate to prevent coagulation, and reinjected into the subcutaneous tissue of the same rabbits for the purpose of preventing iron defficiency by blood loss. On the eighth day the red cell count was 3.0 to 3.5 million per cu mm, hemoglobin contents 40 to 50 % (SAHLI) and reticulocyte 40 to 60 %. The tests on blood were carried out as in the cases of hemolytic anema.

Control animals were fed on the same food as in the experimental animals and injected with saline solution subcutaneouly, the volume corresponding to that of phenylhydrazine or depleted blood observed as in the cases of experimental animals.

All the blood samples observed were those of circulating blood from the ear vein and bone marrow cells from femoral marrow.

For the supravital staining of reticulocyte for general observation the method devised by SENO and others[10] was used.

For the measurement of red cell diameter thin smears dried rapidly by using heater and fan, fixed with methanol for one minute and stained with Giemsa for fifteen minutes were, used. The Price-Jones' curve was drawn on the cell measured in the similar condition by using Kellner type of ocular lens with micrometer ($\times 10$) from Nippon Kogaku.

The measurement of RNA was carried out on reticulocytes and erythroblasts of the bone marrow smears. The bone marrow tissue was ground by glass homogenizer adding a small amount of homologous blood serum by moving the core cylinder gently up and down two or three times. The marrow cell suspension thus obtained was stained supravitally by mixing with an equal volume of 0.1 % Nile blue saline solution for about five seconds on the thin cover glass, smeared and dried as mentioned above and fixed with formol gas for five minutes. One half side of the smear was mounted with 0.5 cc of 0.1 % solution of DNase containing 0.02 M of $MgCl_2$ and treated for 24 hours at 37 °C in moist chamber and then washed and stained with 0.025 % solution of Azure B following the method of FLAX and HIMES[8]. As for the part of smears treated with DNase, RNA contents of reticulocyte and erythroblast in various stages of maturation were estimated quantitatively with a microspectrophotometer (MSP) of Olympus Kogaku Co. For the measurement the two-wavelength method[9,11] was applied. The RNA contents were calculated from the table of MENDELSOHN[9]. The maturation stage of erythroblasts was interpreted from the nuclear diameter according to the principle described by WEICKER[7].

It was helpful to check the maturation stage of erythroblasts treated with DNase exactly as those found on the adjacent side of smear which had not been treated with DNase, whose nuclei could be stained with Giemsa. Some smears were treated with 0.02 % solution of RNase, pH 6.5, for 30 minutes at 60 °C prior to the staining with Azure B to confirm that the dye stained RNA specifically. On each smear about 100 cells at each maturing stage of erythroblasts and reticulocytes were estimated.

RESULTS

Diameter of the reticulocyte and the matured erythrocyte in circulating blood of normal and anemic rabbits: The Price-Jones' curve in normal rabbit which was obtained by observing 200 red cells in each blood sample gave the similar distribution pattern in both of matured red blood cells and reticulocytes. The peak of the reticulocyte (7.0 μ) shifted to the right comparing to that of the matured red cell (6.5 μ), suggesting some are reduced in diameter in the course of maturation from the reticulocyte to the red blood cell (Fig. 1a). In blood-depleted rabbits the patterns of the curves of both matured red cells and reticulocytes were about the same as those of control animal, respectively. In phenylhydrazine anemia the curves showed a completely different pattern from normal and blood-depleted animals, showing two peaks in both matured red blood cells and reticulocytes (Fig. 1c). In any case, with normal, blood-depleted and phenylhydrazine injected animals, the mean diameter of reticulocytes was

larger than that of matured red cells.

Therefore, it is generally said that reticulocytes are larger than matured red cells in size, indicating some shrinkage by maturation. The distribution curve of the red cells of the blood-depleted rabbits showed somewhat wider bases in Price-Jones' curve as compared with those from normal animal (Fig. 1b). Especially, in hemolytic anemia this tendency was marked and showed a peculiar pattern having two peaks as just mentioned (Fig. 1c). This pattern was recognized only in phenylhydrazine anemia, but not in blood-depleted anemia. The significant difference in blood picture of the phenylhydrazine anemia from blood-depleted anemia was that the reticulocyte percentage was markedly low in the latter (40 to 60%) comparing to the former (80 to 90%).

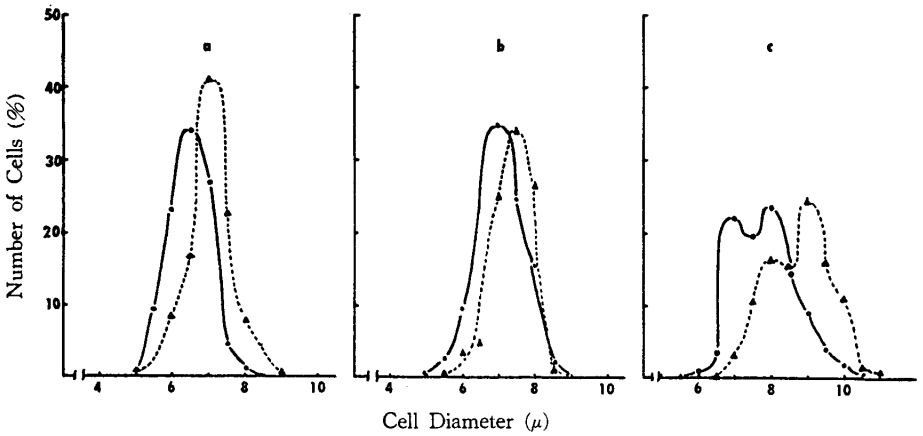

Fig. 1 Price-Jones' Curves of Matured Red Blood Cells and Reticulocytes of a Normal and Two Anemic Animals.
a; normal animal; 2.500g, ♂, R.B.C. 5.74 millions, R.C.: 2.6%. b: hemorrhagic anemia; 2.720g, ♂, R.B.C.: 3.77 miilions, R.C.: 58%. C: hemolytic anemia; 2.420g, R.B.C.; 3.24 millions, R.C.: 95%. solid lines: R.B.C. broken lines: R.C.

Cell diameter of the marrow erythroblast: The curve drawn on erythroblasts gave the exponential curves concerning both of the cell and the nuclear volumes which were calculated from the diameters. The dense distribution of the nuclear diameter in the normal was found at around 5.5 μ, 7.0 μ, and 9.0 μ, showing reduction in the nuclear volume by one half around these points (Fig. 2a). The curve of the cellular volume also shows a similar exponential pattern indicating reduction of the cellular volume by one half at each cell division (Fig. 2b). The pattern of the curves from normal and two anemic groups were almost the same (Fig. 3a, b).

Corresponding to Weicker's view about the relation of the nuclear diameter and the reduction of the cell volume by one half at each cell division, it is said

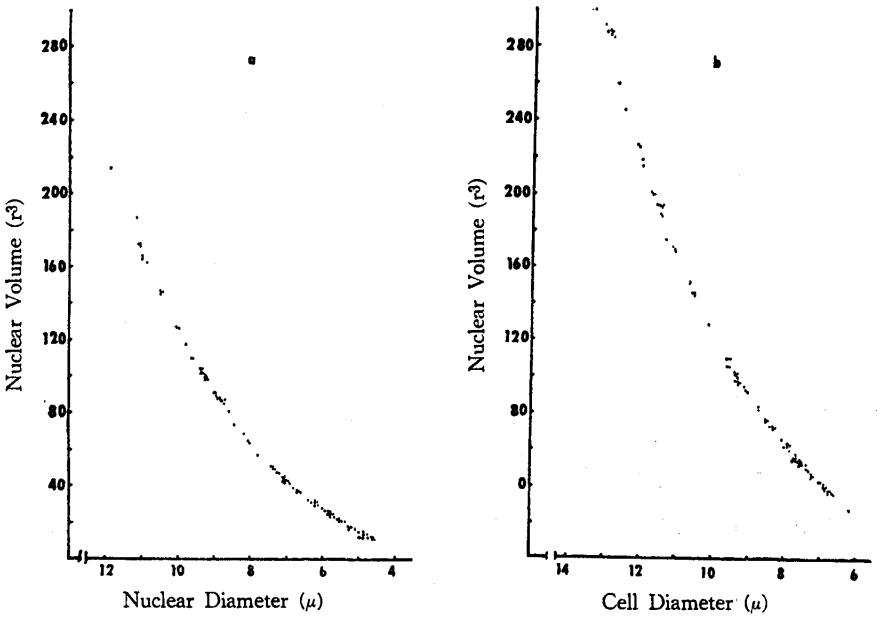

Fig. 2 Nuclear and Cell Volume of Normal Erythroblasts

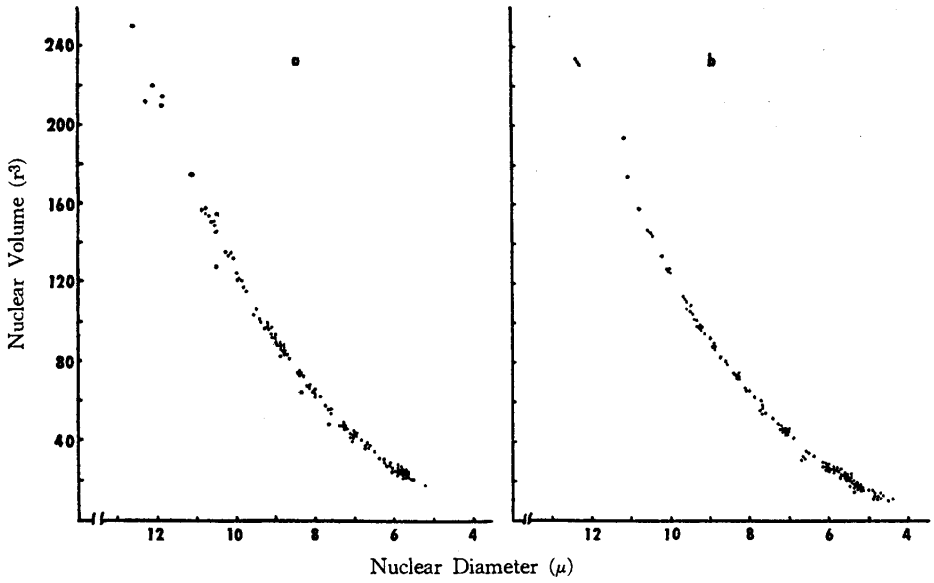

Fig. 3 Nuclear Volume of Erythroblasts in Anemic Animals
a: hemorrhagic anemia b: hemolytic anemia

that these points indicate definite stages of maturation; that is, orthochromatic, polychromatic and late basophilic stages, respectively.

In anemic animal, it was found that most of reticulocytes were larger than the cytoplasm of erythroblast in orthochromatic stage in volume. Their volumes were rather comparable to those of the cytoplasm of polychromatic erythroblasts as calculated from the diameter (Table 1).

Table 1 The Volume of Marrow Reticulocytes and Cytoplasms of Erythroblast of Normal, Blood-let and Phenylhydrazine Injected Anemia (mean from about 30 cells)

		Cell Diameter	Nuclear Diameter	Cytoplasmic Volume	Numbers Measured
Normal	RC	6.59 μ	—	38.03	30
	OEbl	7.26 μ	5.00 μ	28.36	29
	FEbl	8.45 μ	6.62 μ	50.07	26
Blood-Let	RC	6.91 μ	—	43.20	30
	OEbl	7.57 μ	5.81 μ	33.50	22
	PEbl	8.02 μ	6.27 μ	40.92	30
Phenylhydrazine	RC	7.34 μ	—	50.69	30
	OEbl	7.95 μ	5.43 μ	38.12	30
	PEbl	9.08 μ	6.90 μ	56.18	30

The cytoplasmic volume is arbitrarily defined as $Cr^3 - Nr^3$ in erythroblasts: Cr and Nr are the radius of cell and nucleus, respectively.
RC: reticulocyte, OEbl: orthochromatic erythroblast, PEbl: polychromatic erythroblast

In normal animal the cytoplasmic volume of reticulocytes was far smaller than that of polychromatic stage. It should be comparable to that of orthochromatic erythroblast, just as deduced from the RNA level of the reticulocyte in the following data. Some larger value than that was expected would be due to the fact that reticulocytes of normal animal include a large number of disc-shaped reticulocytes.

RNA contents of reticulocyte and erythroblast: The RNA contents of the reticulocytes in bone marrow of anemic animals were greater than those from normal animals. In normal animal the RNA level of reticulocytes was comparable to that of orthochromatic erythroblast, the stage just before denucleation in normal condition. But in anemic animal many reticulocytes contained abundant RNA whose level was comparable to that of polychromatic erythroblast and some to the basophilic erythroblast in phenylhydrazine anemia. This indicates that in severe anemia large reticulocytes appear whose RNA contents are much greater than those of orthochromatic erythoblasts (Table 2).

Table 2 RNA Contents of Marrow Erythroblasts and Reticulocytes

Normal			Blood-let			Phenylhydrazine		
RC	OEbl	PEbl	RC	OEbl	PEbl	RC	OEbl	PEbl
80.8	144.8	222.1	132.7	176.8	194.2	251.6	140.0	251.0
60.5	93.5	197.0	122.1	120.2	170.0	200.2	137.5	222.2
60.2	90.6	184.2	120.5	111.2	154.2	167.0	135.5	218.2
55.5	89.3	150.9	100.0	93.2	149.5	150.2	123.1	209.0
54.3	88.6	148.0	96.4	89.9	148.2	148.2	121.1	200.8
54.0	84.4	147.6	95.5	85.5	156.9	137.8	118.1	185.5
52.5	81.2	141.2	95.3	82.9	133.0	132.6	118.0	176.2
51.4	76.5	135.8	93.6	82.6	127.2	129.0	115.3	151.0
51.2	76.0	126.0	93.4	81.0	113.8	120.2	113.0	141.5
47.3	75.2	114.2	91.0	81.0	111.5	120.1	111.5	141.0
47.0	74.5	107.0	89.3	67.0	108.7	117.1	110.0	138.5
43.2	68.3	105.4	87.9	67.0	107.5	116.8	108.3	137.8
41.0	64.25	104.0	86.8	63.0	105.25	116.0	106.3	137.2
40.9	62.4	86.25	84.6	59.6	104.0	114.5	105.8	135.0
40.8	62.25	84.7	84.2	58.5	99.9	113.2	105.8	133.0
40.5	61.6	84.25	81.8	56.4	99.7	109.3	101.9	132.5
38.7	61.5	80.7	77.2	52.6	92.5	108.5	99.6	130.0
37.6	56.6	79.4	76.5	50.0	91.4	101.2	99.3	127.8
37.42	55.5	79.0	70.7	49.95	80.8	97.0	96.6	125.0
37.4	52.9	75.8	70.2	43.7	80.3	96.5	94.75	120.5
37.4	52.75	75.7	69.1	32.6	78.5	96.3	92.6	113.0
37.19	52.5	73.3	65.4	22.0	78.5	90.4	91.7	110.7
35.8	51.6	67.75	65.4		78.4	88.5	90.5	110.2
35.42	48.2	67.2	63.2		78.0	82.0	89.6	108.9
34.22	47.42	61.6	61.3		76.7	81.4	82.4	108.9
33.05	45.7	51.8	60.5		76.6	73.3	79.2	106.0
31.23	44.2		53.6		76.5	71.9	78.6	99.5
30.82	40.0		48.3		66.5	71.4	77.25	94.0
27.4	35.4		48.2		65.9	66.3	76.2	69.1
27.3			47.6		56.5	61.2	73.25	58.0

RNA content, arbitrarily unit

DISCUSSION

According to WINTROBE[12] reticulocytes are larger than fully matured red blood cells in diameter and are reduced in their size by maturation. However, it is suggested that the reticulocyte arises by skipping subdivision of the polychromatic erythroblast and matures without reduction in its size[6]. In contrast, by the author's observation on the rabbits it appears that some reduction in volume

should occur by maturation both in normal and anemic cases. However, big reticulocytes do never decrease in volume in the course of maturation to the normal size. FICHSEL[13] and FICHSEL, GELLISEN, WALTHER and WEICKER[14] described that in some stage of severe anemia of rabbit caused by phenylhydrazine, the volume of the reticulocyte becomes twice as large as the mature erythrocyte. From these observations they proposed that reticulocytes divide themselves into two red cells which mature to the normal size erythrocytes, but the reticulocyte does not divide further as revealed by the observation with isotope labeling method. The grain count of H^3-leucine incorporated into the reticulocyte is not reduced in their number by maturation[15].

In all likelihood, the materials observed by FICHSEL and associates were those containing both normal size, mature red cells formed before the phenylhydrazine injection and those newly formed big reticulocytes after the injection. An important point to be borne in mind is that the reticulocytes in phenylhydrazine anemia are twice as large as the normal red cells in volume. In phenylhydrazine anemia a marked reticulocyte crisis can be seen, 80 to 90% in recovery periods. Therefore, in the recovery stage of these cases almost all of mature red cells should be those from regenerated reticulocytes.

Under such conditions all the erythroblasts were denucleated at polychromatic stage in anemia, and the Price-Jones' curve should give one peak in each of mature red cells and reticulocytes. This shows that there exist two kinds of cells, smaller and larger ones. Comparing the curves from anemic animals with those of normal animals, the left side peak of the curves from phenylhyrazine anemia corresponds to the peak of normal animal, suggesting that smaller red cells are formed by denucleation at orthochromatic stage. Now, it is probable that in anemia, especially in severe anemia, big reticulocytes appear and they mature to large size red cells, and some others pass through the normal maturation stage producing the cells of normal size.

Now, I need the fact to establish that the big reticulocytes in anemia are actually formed by denucleation at polychromatic or more younger stages which should be found in the bone marrow of anemic animal. In anemic animal there is an actual increase of the stem cells, proerythroblasts, basophilic and polychromatic erythroblasts but rather fewer of orthochromatic erythroblasts in number, as revealed by SHIBATA[16] and SENO and collaborators[17]. Both of these observations on diameter of reticulocytes and erythroblasts in orthochromatic stage and the reduced number per cent of orthochromatic erythroblasts in anemia seem to suggest that the denucleation occurs at earlier stage of maturation of erythroblast.

The estimation of RNA contents of reticulocytes may give the conclusive evidence for this supposition. SENO and his collabolators[18] reported that new

RNA synthesis does not occur in reticulocytes in rabbit, and PINHEIRO and his co-worker[19] also stressed the same phenomenon by observing rat reticulocyte in phenylhydrazine anemia.

Consequently, RNA level of reticulocytes should be equal or lower comparing with that of the erythroblasts just before denucleation. The highest RNA level of reticulocyte will indicate the possible denucleation stage of erythroblast. As mentioned above, the RNA level of some reticulocytes of anemic animal measured by Azure B staining is just comparable to those of polychromatic erythroblast. It is clear that in severe anemia a large number of reticulocytes can be formed by the denucleation of polychromatic eryfhroblasts, skipping the terminal cell division to orthochromatic stage. This fact explains decisively the appearance of big reticulocytes in anemia with resultant macrocytosis or anisocytosis with co-existence of normal size red cells.

As for the reduction in diameter occurring at maturation of the reticulocyte, its mechanism is as yet uncertain. However, the reticulocyte just denucleated is round in shape, flexible in cell membrane and rather low in hemoglobin contents and flattened by smearing, while the mature red cell is originally disc-shaped and high in hemoglobin contents. And there is a probability that the smearing of the reticulocyte makes it flat and wide comparing with the mature red cell.

Concerning the mechanism of denucleation BORSOOK[20] states that erythropoietin will be responsible and when the stimulus of the erythropoietin is exerted on bone marrow, red cell size grows larger than normal, suggesting many reticulocytes arise directly from the younger erythroblasts, skipping the expected cell division. But it is uncertain whether or not erythropoietin is solely responsible for the denucleation of erythroblast.

STOHLMAN[21,22] also proposed that the size of the reticulocyte was nearly proportional to the cytoplasm of erythroblasts from which they were derived by skipping mitosis. SUIT *et al.*[4] likewise suggested that a portion of the reticulocyte is formed from early normoblast without intervening cell division, but they did not clearly show at what stage of maturation the denucleation occurred.

In my observation on phenylhydrazine anemia the diameter of the reticulocytes is from 6.0 to 9.0 μ, slightly dense distribution around 8.0 μ in the bone marrow. This implies that the skipping of mitosis has occurred around the stage of $K_{1/4}$ of WEICKER[7], that is the stage of polychromatic erythroblast. If confined only at 9.0 μ, the stage coincides with the late basophilic erythroblast, and if only at 6.0 μ, orthochromatic stage. Consequently, the denucleation occurs at the late basophilic stage in the earlier case, mostly at polychromatic stage, but not at the stage of the stem cells.

Thus, it can be concluded that the denucleation does occur at various stages

of maturation in anemia later than late basophilic stage, resulting in macrocytosis or anisocytosis.

SUMMARY

The mechanism of induction of anisocytosis was studied with experimental anemia of rabbits induced by blood depletion and phenylhydrazine chloride injection. The Price-Jones' curves of erythrocytes from anemic animals showed a large variety of red cell size, indicating the appearance of abnormally large sized cells. RNA contents of some reticulocytes in anemia were comparable to those of polychromatic and late basophilic erythroblasts. The result proved that in severe anemia a large number of erythroblasts are denucleated at earlier maturation stages, in most cases at polychromatic, and some at late basophilic and some at orthochromatic stages, resulting in anisocytosis.

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