Nucleic Acids of Rabbit Reticulocytes


Thorell, using the technic of ultraviolet light absorption developed by Caspersson and his associates (see Caspersson for references), showed that the concentration of nucleic acid in the cytoplasm of immature red cells was greater than that in mature cells. Using chemical methods, he also showed that the concentration of phosphorus (chiefly nucleoprotein phosphorus) remaining after successive removal of the acid-soluble phosphorus and the lipid phosphorus was greater in the red cells of rabbits that had been subjected to repeated hemorrhage. Many of these immature red cells were polychromatophilic and probably would have appeared as reticulocytes had they been stained supravitally with brilliant cresyl blue. This finding lent support to the earlier suggestion of Kay that there is a greater concentration of nucleic acid phosphorus in reticulocytes.

Other workers have made both chemical and histochemical studies on the immature cells of bone marrow and on primitive red cells from the yolk sacs of embryos, but few observations have been made on the reticulocytes of peripheral blood. The most comprehensive observations are those of Dustin who, using the ribonuclease method of Brachet showed that the basophilic substance in the cytoplasm of reticulocytes is destroyed by ribonuclease.

In his experiments on the red cells of rabbits subjected to hemorrhage, Thorell did not estimate pentose and desoxypentose nucleic acids separately. It seemed of value, therefore, to determine the concentration of pentose and desoxypentose nucleic acids in suspensions of red cells containing immature cells and to compare the results with those obtained with the histochemical methods. Reticulocytes were produced in the peripheral blood stream of rabbits either by the administration of phenylhydrazine or by successive bleedings. There was an increase in the concentration of nucleic acid in the red cells, coincident with an increase in the number of reticulocytes. This nucleic acid was of the pentose rather than the desoxypentose type. The basophilia of the polychromatophilic cells was found to disappear after treatment with the ribonuclease or after hydrolysis with normal hydrochloric acid. Desoxyribonuclease was without effect.

In addition to the increase in the concentration of pentose nucleic acid there was also an increase in the concentration of both acid soluble phosphorus and lipid phosphorus in cell suspensions containing reticulocytes. Incidentally, data are presented on the concentration of acid soluble phosphorus, lipid phosphorus and nucleic acids in red cells and polymorphonuclear leukocytes from normal rabbits. Details of the distribution of the various lipids in the red cells and in the polymorphonuclear leukocytes of the rabbit have already been described.

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Methods

Animals. The rabbits used were fully grown. No attempt was made to control the age, sex, weight or the dietary condition of the animals.

Red Cells. Blood was withdrawn from the marginal ear vein of a rabbit using heparin as an anticoagulant. After centrifuging at 3000 r.p.m. the plasma and buffy coat layer were aspirated off. The complete analysis in duplicate was done on 1.5 to 2 Gm. packed cells.

Reticulocytes. Reticulocytes were produced in one of two ways: (1) Hemorrhage. From 50 to 60 ml. blood was withdrawn from the marginal ear vein each day for four or five days. In this way a reticulocytosis of 20 to 50 per cent was produced. (2) Phenylhydrazine. A 3.3 per cent solution of phenylhydrazine hydrochloride was injected subcutaneously as described by Rapoport, Guest and Wing. The total dose for each animal was varied somewhat according to the degree of anemia that developed. A reticulocytosis of 35 to 50 per cent was produced.

Polymorphonuclear Leukocytes. Polymorphonuclear leukocytes were obtained from the peritoneal cavity of the rabbit by the method of de Haan, the details of which have already been described by Cram and Rossiter. The cells, 95 per cent of which were polymorphonuclear leukocytes, were concentrated by centrifuging, washed with isotonic saline, packed at 3000 r.p.m. in a tared centrifuge tube, allowed to drain for thirty minutes and then weighed. The complete analysis was done in quadruplicate on 1 to 1.5 Gm. packed cells. On microscopic examination the cells appeared similar to those circulating in the blood. The possibility that they may have differed, either physiologically or chemically, from normal cells cannot be excluded.

Cytologic Methods. Reticulocyte counts during the course of bleeding and phenylhydrazine experiments were made as described by Wintrobe using blood from the marginal ear vein. The smears, supravitaly stained with brilliant cresyl blue, were counterstained with Wright's stain.

For the cytologic experiments blood films were made on coverslips from a resuspension of the packed cells used for chemical determinations. The films were fixed in absolute methyl alcohol for five minutes except for those smears to be stained with May-Grünwald methylene blue-cosin. Some preparations in each experiment were stained supravitaly with brilliant cresyl blue. There was no evidence that the heparin or the centrifugation altered the cell characteristics.

Ribonuclease and desoxyribonuclease (crystalline, Worthington Biochemical Laboratory, Freehold, N. J.) were made up in normal saline in a concentration of 0.25 mg./ml. Fixed films were immersed in these solutions for one hour at 37 C. Hydrolysis with N-HCl (essentially the first step in the Feulgen procedure) was carried out at 60 C. for ten minutes.

Analytical Methods

Hemoglobin was determined by the cyan-hematin method of King and Gilchrist. A sample of crystalline hemin of known iron content was used as a standard.

Hematocrit and red cell count were determined as described by Wintrobe. Mean corpuscular volume and mean corpuscular hemoglobin were calculated as described by Wintrobe.

Phosphorus-containing compounds were determined by the method of Schneider. Phosphorus was estimated by the modification of the method of Fiske and Subbarow described by King. In some instances the pentosenucleic acid phosphorus and the desoxypentose-nucleic acid phosphorus was determined by the method of Schmidt and Thannhauser, while in others the phosphorus of each type of nucleic acid was calculated from the result obtained from the color reactions for the sugars, as described by Schneider. Pentose was determined by the orcinol method of Mejaun and desoxypentose by the diphenylamine reaction described by Dische. A specimen of yeast sodium ribonucleate was selected from a number of commercial preparations for the colorimetric standard for the ribose color reaction and a preparation of thymus sodium desoxyribonucleate was used as the standard.

* Kindly provided by Dr. G. C. Butler, Department of Biochemistry, University of Toronto.
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for the determination of desoxypentose. The results given by the two methods were in fair agreement.

TABLE 1.—Mean Concentration of Phosphorus Compounds in Rabbit Blood Cells (mg. P per 100 Gm. Fresh Tissue)

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Treatment</th>
<th>Red Cells</th>
<th>Polymorphonuclear Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid soluble P.</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>57.4</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.5</td>
<td>S.E.</td>
</tr>
<tr>
<td></td>
<td>Lipid P.</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10.2</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.3</td>
<td>S.E.</td>
</tr>
<tr>
<td></td>
<td>Pentosenucleic acid P.</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.79*</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.06</td>
<td>S.E.</td>
</tr>
<tr>
<td></td>
<td>Desoxypentosenucleic acid P.</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.57*</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.06</td>
<td>S.E.</td>
</tr>
<tr>
<td></td>
<td>Total nucleic acid P. (by addition)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Total nucleic acid P. (by P. estimation according to Schneider)</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

* By the method of Schneider.† By the method of Schmidt and Thannhauser.

TABLE 2.—Hematologic Findings of Rabbits Treated with Phenylhydrazine or Subjected to Daily Hemorrhage

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Treatment</th>
<th>Reticulocytes (%)</th>
<th>Hemoglobin (Gm. per 100 ml.)</th>
<th>R.B.C. (10⁶ cells per cu. mm.)</th>
<th>Hematocrit (%)</th>
<th>M.C.V. (cu. μ)</th>
<th>M.C.H. (γγ)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>1.6</td>
<td>13.0</td>
<td>6.83</td>
<td>42</td>
<td>62</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Phenylhydrazine (4 days)</td>
<td>48.4</td>
<td>7.4</td>
<td>2.67</td>
<td>24</td>
<td>90</td>
<td>27.7</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>1.9</td>
<td>10.3</td>
<td>6.08</td>
<td>38</td>
<td>63</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td>Phenylhydrazine (4 days)</td>
<td>49.5</td>
<td>4.7</td>
<td>1.65</td>
<td>13</td>
<td>79</td>
<td>28.5</td>
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<tr>
<td>3</td>
<td>Control</td>
<td>1.9</td>
<td>11.9</td>
<td>6.26</td>
<td>40</td>
<td>64</td>
<td>19.0</td>
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<td></td>
<td>Phenylhydrazine (5 days)</td>
<td>39.9</td>
<td>4.6</td>
<td>1.14</td>
<td>10</td>
<td>88</td>
<td>40.4</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>2.1</td>
<td>13.9</td>
<td>6.56</td>
<td>44</td>
<td>67</td>
<td>21.2</td>
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<tr>
<td></td>
<td>Phenylhydrazine (5 days)</td>
<td>50.3</td>
<td>4.3</td>
<td>1.25</td>
<td>10</td>
<td>80</td>
<td>34.3</td>
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<tr>
<td>6</td>
<td>Control</td>
<td>1.8</td>
<td>11.2</td>
<td>5.44</td>
<td>36</td>
<td>66</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>Hemorrhage (4 days)</td>
<td>33.2</td>
<td>3.3</td>
<td>2.19</td>
<td>16</td>
<td>73</td>
<td>15.2</td>
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<tr>
<td>7</td>
<td>Control</td>
<td>1.7</td>
<td>13.9</td>
<td>6.83</td>
<td>44</td>
<td>65</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>Hemorrhage (5 days)</td>
<td>44.5</td>
<td>4.8</td>
<td>1.99</td>
<td>16</td>
<td>80</td>
<td>24.3</td>
</tr>
</tbody>
</table>

Results

Normal Red Cells and Polymorphonuclear Leukocytes

Table 1 gives the mean values and the standard error of the mean for the concentration of phosphorus compounds in both normal red cells and polymorphonuclear leukocytes of the rabbit. The concentration of nucleic acid
phosphorus and lipid phosphorus was much greater in the polymorphonuclear leukocytes than in the red cells. There was a small but consistent concentration of both pentose and deoxypentose nucleic acid in the red cells. In the white cells the concentration of deoxypentose nucleic acid was more than four times that of the concentration of pentose nucleic acid. The concentration of acid soluble phosphorus was of the same order in both types of cell.

Table 3.—Phosphorus Compounds in Red Cells of Rabbits Treated with Phenylhydrazine or Subjected to Daily Hemorrhage (mg., P., per 100 Gm. Fresh Tissue)

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Treatment</th>
<th>Reticulocytes (v&lt;sub&gt;%&lt;/sub&gt;)</th>
<th>Acid soluble P.</th>
<th>Lipid P.</th>
<th>Pentose nucleic acid P.</th>
<th>Deoxypentose nucleic acid P.</th>
<th>Total nucleic acid P. (by addition)</th>
<th>Total nucleic acid P. (by estimatation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>1.6</td>
<td>58.5</td>
<td>11.0</td>
<td>0.9*</td>
<td>0.4*</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Phenylhydrazine (4 days)</td>
<td>48.4</td>
<td>84.0</td>
<td>18.6</td>
<td>17.3*</td>
<td>1.8*</td>
<td>19.1</td>
<td>18.4</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>1.9</td>
<td>68.0</td>
<td>10.4</td>
<td>0.8*</td>
<td>0.4*</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Phenylhydrazine (4 days)</td>
<td>49.5</td>
<td>81.6</td>
<td>20.3</td>
<td>17.7*</td>
<td>2.7*</td>
<td>20.4</td>
<td>20.9</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>1.9</td>
<td>56.6</td>
<td>10.4</td>
<td>0.7*</td>
<td>0.4*</td>
<td>1.1</td>
<td>1.0</td>
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<tr>
<td></td>
<td>Phenylhydrazine (5 days)</td>
<td>39.9</td>
<td>82.9</td>
<td>15.3</td>
<td>10.2*</td>
<td>1.5*</td>
<td>11.7</td>
<td>10.9</td>
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<tr>
<td>4</td>
<td>Control</td>
<td>2.1</td>
<td>74.0</td>
<td>11.9</td>
<td>1.0*</td>
<td>0.4*</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Phenylhydrazine (5 days)</td>
<td>50.3</td>
<td>83.5</td>
<td>18.2</td>
<td>20.1*</td>
<td>5.3*</td>
<td>25.4</td>
<td>24.7</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>1.6</td>
<td>52.5</td>
<td>12.1</td>
<td>0.9*</td>
<td>0.3*</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Hemorrhage (4 days)</td>
<td>24.3</td>
<td>68.5</td>
<td>12.2</td>
<td>11.6*</td>
<td>1.4*</td>
<td>13.0</td>
<td>11.3</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>1.8</td>
<td>58.5</td>
<td>9.2</td>
<td>0.8*</td>
<td>0.5*</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Hemorrhage (4 days)</td>
<td>33.2</td>
<td>89.2</td>
<td>15.6</td>
<td>13.4*†</td>
<td>1.2†</td>
<td>14.6</td>
<td>12.5</td>
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<tr>
<td>7</td>
<td>Control</td>
<td>1.7</td>
<td>64.5</td>
<td>11.0</td>
<td>1.0*</td>
<td>0.5*</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Hemorrhage (5 days)</td>
<td>44.5</td>
<td>82.9</td>
<td>18.5</td>
<td>19.3*†</td>
<td>2.9†</td>
<td>22.2</td>
<td>22.0</td>
</tr>
</tbody>
</table>

* By the method of Schneider.53
† Confirmed by the method of Schmidt and Thamnhauser.54

Reticulocytes

Table 2 gives the percentage of reticulocytes in the blood of rabbits both before and four or five days after the administration of phenylhydrazine or the beginning of daily bleedings. In each instance the treatment caused a considerable fall in the concentration of hemoglobin, the red cell count and the hematocrit. There was an increase in both the mean corpuscular volume and the mean corpuscular hemoglobin of the cells containing a high percentage of reticulocytes. However, the figures for the mean corpuscular hemoglobin must be treated with some reserve. In the animals treated with phenylhydrazine there was considerable hemolysis. The figure reported for the hemoglobin concentration represents not only the hemoglobin that was in the cells, but also much extracellular hemoglobin derived from lysed cells.
Table 3 gives the concentration of phosphorus compounds in the red cells both before and after phenylhydrazine administration or daily bleedings. In each instance there was an increase in the concentration of acid soluble phosphorus and lipid phosphorus in the cells containing the high percentage of reticulocytes. However, the greatest increase was in the concentration of nucleic acid which in every instance increased by a factor of more than 10. The increase in nucleic acid was almost entirely confined to the pentosenucleic acid fraction, the increase in concentration of deoxypentosenucleic acid being, in comparison, slight.

**Figure 1**—Effect of hemorrhage on the chemistry of the red blood cells of two rabbits. Each arrow indicates a bleeding of 50 to 60 ml. Hb., hemoglobin concentration in Gm. per 100 ml. blood. Retic., reticulocytes per cent. NA, total nucleic acid. PNA, pentosenucleic acid. DNA, deoxypentosenucleic acid. All nucleic acid figures are in mg. phosphorus per 100 Gm. packed cells.

Figure 1 shows the time course of the changes in the red cells of two animals after hemorrhage. As the concentration of hemoglobin decreased, the percentage of reticulocytes in the blood increased. Coincident with this increase in the number of reticulocytes there was an increase in the concentration of nucleic acid, almost all of which was of the pentose type. Several days after the last bleeding the concentration of hemoglobin had returned to normal values, the reticulocytosis had disappeared, and the concentration of nucleic acids had decreased to the low value of the untreated animals. This is convincing evidence that the increase in pentosenucleic acid concentration was due to a high concentration in the reticulocytes.
Cytologic Observations

In all the experiments a marked polychromasia was observed when films were stained with Giemsa, May-Grünwald or Wright’s stains. The majority of the basophilic cells were, like the reticulocytes, much larger than the normal erythrocytes. Companion preparations, supravitally stained with brilliant cresyl blue and subsequently stained with May-Grünwald methylene blue-eosin, showed that all the reticulocytes were polychromatic but that there were always a number of faintly basophil cells that did not show any reticulum. The count of basophil cells usually showed an increase of one-third over the reticulocyte count. The basophilia could be demonstrated by staining for two minutes in 0.01 per cent thionine but the borderline cells were difficult to distinguish. When stained with Pappenheim’s methyl green-pyronin all the cells were stained red by the pyronin and there was no evidence of staining with methyl green. Polychromatic cells could not be demonstrated when the films were treated with ribonuclease prior to staining. On the other hand, neither desoxyribonuclease nor saline alone altered the picture significantly on comparison with untreated controls. Hydrolysis with N-HCl also abolished the polychromasia. The preparations were stained with either May-Grünwald or with thionine. The few leukocytes in these preparations acted as an additional control.

The majority of the cytologic observations were made on the rabbits subjected to hemorrhage. Identical results were obtained in one experiment on a rabbit treated with phenylhydrazine and on blood from a child who had suffered an acute hemolytic crisis.

Discussion

The results described above show that in comparison to mature red cells the immature cells of peripheral blood are rich in nucleic acid, most of which is the pentose type. This view is supported by the histochemical studies which confirm and extend the earlier observations of Dustin.24 Thorell135–136 showed that in bone marrow the concentration of pentosenucleic acid is greater in the cytoplasm of the more immature cells. Subsequently Davidson et al.25 showed that the concentration of pentosenucleic acid per cell (i.e., per unit of desoxypentosenucleic acid) in the bone marrow of patients with pernicious and other megaloblastic anemias decreases as the bone marrow becomes more mature as the result of therapy. Our results would indicate that the greater concentration of pentosenucleic acid in immature red cells can be demonstrated not only in the bone marrow, but also in the peripheral blood.

These findings are of interest in relation to existing knowledge of the reticulum of the reticulocyte. The nature of this substance has long been the subject of debate. Excellent reviews are those of Howell,22 Hawes,21 Key,27 Davidson,19 Orten a and Nittis.18 It is now generally believed that on supravital staining many of the basophilic red cells become reticulated. It is thought that the reticulum is formed by the combination of the basophilic substance of the cytoplasm with the supravital dye, although recent observers using the technics of phase microscopy27 and electron microscopy3, 4 have suggested that organized structures are present in lysed reticulocytes before treatment with brilliant cresyl blue.
Whether these structures are cytoplasmic or nuclear in origin is of interest, for there are those who have maintained that the reticulum of the reticulocytes is derived from fragments of a disintegrating nucleus. Davidson and McCrie, although reserving an open mind on the subject, make the statement that "the microscopic evidence is in favor of the view that the reticulum is not cytoplasmic, but a product of nuclear degeneration." It is now well established that the principal nucleic acid of the nucleus is of the desoxypentose type, whereas that of the cytoplasm is of the pentose type. It would thus appear that the nucleic acid of the immature red cell is of cytoplasmic rather than of nuclear origin. The relatively small increase in desoxypentosenucleic acid that accompanies the large increase in pentosenucleic acid (fig. 1) is probably due to the presence of such Feulgen positive nuclear remnants as Cabot rings and Howell-Jolly bodies in the immature cells (Rheingold and Wislocki), or perhaps, to the presence of a few nucleated red cells.

Other workers have determined the concentration of phospholipid in both the red cells and the polymorphonuclear leukocytes of the rabbit (see Parpart and Dziemian and Burt and Rossiter, for references). Lipid has also been demonstrated histochemically in polymorphonuclear leukocytes. The finding that reticulocytes contain more lipid phosphorus than mature red cells is of interest and is being investigated further. Using either the histochemical test for phospholipid described by Baker or staining with sudan black B, Bloom and Wislocki found that immature red cells contained more lipid than the mature forms.

The finding that the concentration of acid soluble phosphorus in reticulocytes is greater than that in mature red cells confirms an earlier report of Rapoport, Guest and Wing.

Reticulocytes thus differ from red cells in having a greater concentration of acid soluble phosphorus, phospholipid and pentosenucleic acid. The association between pentosenucleic acid and synthesis of protein has been stressed by the Stockholm school (for reviews see Schultz, Caspersson, Brachet and Hyden). This association is evident in the precursors of the red cells found in the bone marrow. Here hemoglobin and other proteins are being synthesized rapidly. Claude stressed the high degree of association between pentosenucleic acid and glycolysis. Glycolysis takes place very largely in the microsomes of cells. These are cytoplasmic structures containing both pentosenucleic acid and lipid, chiefly phospholipid. In this regard it is interesting to note that reticulocytes, cells which contain more pentosenucleic acid and phospholipid than mature red cells, also both respire and glycolyse more rapidly than the mature cells. It is thus apparent that this is a further example of the association between pentosenucleic acid and cell metabolic activity. It may be, as suggested independently by Muller and Spiegelman (see also, Spiegelman and Kamen), that nucleic acids play a direct role in the energy transfer system of the cell.

**Summary**

1. The concentration of acid soluble phosphorus, lipid phosphorus and nucleic acid phosphorus was determined in the red cells and polymorphonuclear leuko-
The concentration of acid soluble phosphorus was of the same order in both types of cells, but there was more lipid phosphorus and nucleic acid phosphorus in the polymorphonuclear leukocytes than in the red cell. Over 80 per cent of the nucleic acid in white cells was of the desoxypentose type.

2. Immature cells were produced in the peripheral blood stream of rabbits either by the administration of phenylhydrazine or by successive bleedings. Either treatment caused a fall in the concentration of hemoglobin, the red cell count and the hematocrit with an increase in the mean corpuscular hemoglobin. There was also an increase in the concentration of acid soluble phosphorus and lipid phosphorus and a great increase in the concentration of nucleic acid phosphorus which was coincident with the increase in number of immature cells as judged by the reticulocyte count. Almost all of the excess nucleic acid was of the pentose type.

3. The basophilia of the immature cells disappeared after treatment with ribonuclease or after hydrolysis with normal hydrochloric acid. Desoxyribonuclease was without effect.

4. These results suggest that the basophilic substance of the immature rabbit red cell, and presumably the reticulum of the reticulocyte, is of cytoplasmic rather than nuclear origin.

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