The Maturation Rate of Reticulocytes

By MARIO BALDINI AND IVO PANNACIULLI

The red blood cells, after release from the bone marrow, continue their "maturation" in the peripheral blood. Here, the immature red cells, the reticulocytes, become mature erythrocytes. The time necessary for the reticulum substance to disappear has been defined as the "maturation time" of reticulocytes.

In the past, the maturation time of reticulocytes has been studied by in vivo and by in vitro technics. In vivo studies were performed in pernicious anemia patients during reticulocyte "crisis" \(^1\) \(^3\) \(^8\) \(^9\) or in animals treated with phenylhydrazine. \(^4\) Other experiments were done transfusing normal blood into aplastic subjects. \(^5\) \(^7\) The main limitations of all these experiments were that they could only be performed in abnormal and often unstable conditions, and that, in humans, they could not be repeated in series. Studies in vitro were done by Nizet, Seip, and others. \(^8\) \(^8\) \(^11\) \(^12\) \(^13\) \(^19\) \(^21\)

It has been demonstrated on numerous occasions that reticulocytes in vitro are transformed into mature red cells by an aerobic fermentative process. \(^3\) \(^11\) \(^13\) The in vitro requirements of the cells are not the same as those in vivo, although in vitro the environment remains substantially unmodified during the experiment, and the effect of variables can be excluded. Furthermore, experiments in vitro can be reproduced ad libitum with controlled conditions which are indispensable for a systematic comparative study. In vitro studies, therefore, present many advantages.

The first part of the present investigation was concerned with the establishment of a technic and the demonstration of its validity. The maturation time of reticulocytes was then studied in different types of anemias. With increased red cell regeneration, the number of reticulocytes in the circulating blood becomes increased, and younger forms with greater amounts of reticulum substance also appear. Obviously, the presence of younger reticulocytes causes an increase in the maturation time of these cells. The question, however, of whether in some conditions the maturation time of reticulocytes can also be increased because of a "delayed" rate of maturation, due to a metabolic defect of these cells, has never been clearly defined. This problem has been investigated in the present study, and the findings are of special interest with reference to the pathophysiology of erythropoiesis in anemias.

Method for the Study of Reticulocyte Maturation in Vitro

Method of culture.—Petrof's glass flasks of 20 ml. capacity were used as culture chambers (see figure 1). They were provided with a long neck measuring 20 cm. in length and 1

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Fig. 1.—The apparatus for the culture of reticulocytes consisted of a waterbath at 37 C., provided with an agitator which kept the culture flasks in motion during the time of the experiment.

The bulb of the flasks remained immersed in a waterbath at 37 C., while the long neck emerged above the water, preventing the concentration of the content by evaporation. The volume of the blood plus culture medium in each flask was 5 ml., occupying only one-fourth of the flask volume. Increase in hydrostatic pressure in the flasks was avoided by fitting a 20 cm. long capillary tube into the rubber stopper in the neck of the flasks. The capillary tube was long enough to prevent bacterial contamination of the culture. Glass equipment and culture medium were autoclaved before use.

In order to prevent adhesion of the reticulocytes to the glass walls of the container, the flasks were siliconized, and during the experiment they were gently agitated mechanically (30 times per minute). The agitation also prevented sedimentation of the cells with resultant variations in nutritional and respiratory conditions.

After various trials of different saline mixtures, it was found that an isotonic buffered citrate solution and glucose served best as an anticoagulant and culture medium. The mixture contained: 3.8 per cent Trisodium citrate, 1 part; phosphate buffer solution, 2 parts; glucose, 500 mg. 100 ml.

Blood was drawn by venipuncture from the patients in fasting condition and mixed with the above solution in a 7 to 3 proportion. Experiments were performed at least in duplicate.

A few drops of blood were used to determine the initial number of the reticulocytes (the "zero time" of the experiment). Every 4 hours a few drops were aseptically removed from each of the two flasks, and reticulocytes were counted. The culture time was arbitrarily limited to 12 hours. During this period of time the number of reticulocytes usually decreased to about 20 per cent of the initial value. It would have been ideal to extend the time of culture until the last surviving reticulocyte could be observed. However, after 12 hours, the maturation curve usually approached an asymptote, and results were variable.

*The buffer contained 60 volumes of 0.11 M disodium phosphate and 40 volumes of 0.11 M monosodium phosphate.
No appreciable change in the culture medium was found during the first 12 hours. The pH remained between 7.1 and 7.3, and hemolysis of erythrocytes was minimal (18 to 25 mg. per cent of free hemoglobin). Only about 20 per cent of the glucose was consumed during the incubation. Additional glucose was essential for the culture of reticulocytes. It has been shown that after incubation for 6 to 8 hours at 37 C., without addition of glucose, the blood glucose level falls to 10 per cent of the initial value. After this time, hemolysis is usually observed and the maturation of reticulocytes becomes delayed or arrested.6

**Method of observation.**—The few drops of blood removed from the flasks at each sampling time were placed in a paraffinized container and mixed with an equal volume of 1 per cent solution of Brilliant Cresyl Blue in normal saline. After 20 to 30 minutes in a moist chamber, the sample was again carefully mixed, and 5 to 6 smears were made.

Reticulocytes were counted by dark-field microscopy. By this method (Nizet’s method), even small particles of reticulum substance could be detected because they assumed a bright golden-yellow color in the dark field. Basophilic granules did not usually absorb the stain, although they sometimes showed a slightly visible brownish tint. Heinz bodies assumed a reddish-brown color. Counts were done on the central area of the smear. A minimum of two smears and a total of 5000 erythrocytes were counted for each blood sample unless reticulocytes were less than 10 per mille, in which case 8000 erythrocytes were counted.

**Number and maturation time of normal reticulocytes.**—Reticulocyte number and in vitro maturation time were studied in blood samples from 82 normal adults of varying age and sex.

The average value for the number of reticulocytes was 15.4 per mille (±6.5 S.D.). In six normal subjects the reticulocyte number was determined on six consecutive days. The variations found were between +3.6 and −4.5 per cent of the average normal value. The narrow range of the daily variations suggested that the reticulocyte count stays essentially constant in normal subjects.

The in vitro maturation time of reticulocytes from the above 82 normals was studied, and the results were averaged. The disappearance of reticulocytes during the 12 hours of culture, expressed in per cent of the values at time 0, was as follows:

<table>
<thead>
<tr>
<th>Culture time, hours</th>
<th>Reticulocyte number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>63.7</td>
</tr>
<tr>
<td>8</td>
<td>34.7</td>
</tr>
<tr>
<td>12</td>
<td>17.9</td>
</tr>
</tbody>
</table>

The graphic representation of the results showed that the disappearance of reticulocytes cultured in vitro was an exponential function (fig. 2). For sake of more accuracy the T1/2 for each experiment was obtained by mathematical analysis of the experimental results rather than by visual extrapolation on semilog paper. The integral equation applied was the following:

\[ y = y_0 e^{-kt} \]  
(1)

This equation expressed the number of reticulocytes surviving as a function of time, \( y_0 \) being the initial number of reticulocytes, \( e \) being the base of the Napierian logarithms (2.718 . . . ), and \( y \) the number of reticulocytes present at any time \( t \) of the culture period; \( k \) was the maturation constant of reticulocytes. Expressed in logarithmic form, equation (1) was as follows:

\[ \ln y = \ln y_0 - kt \]  
(2)

The half-life (T1/2) was calculated from the above equation:

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*The reticulocyte counts in this study will be all expressed in number of reticulocytes per 1000 red cells. Throughout this paper “S.D.” stands for “1 standard deviation.”
Fig. 2.—The maturation curve of normal reticulocytes in culture. By averaging the results obtained in 82 experiments with normal reticulocytes it could be shown that the maturation curve in vitro was an exponential function.

\[ T_{1/2} = \frac{\ln 1/2}{k} \]

The average half-life of normal reticulocytes obtained from 82 experiments was 4.8 hours (±1.9 S.D.).

In order to determine whether the results of the reticulocyte maturation experiments were reproducible in one single subject, blood from 6 normal individuals was cultured on 6 consecutive days. Variations of the maturation time were shown to be within a narrow range, from +14 to −12 per cent of the value observed on the first day.

**FURTHER STUDIES ON THE CULTURE METHOD**

*Effect of trisodium citrate on the staining property of reticulocytes.*—It had previously been suspected\(^6\) that trisodium citrate could inhibit the staining properties of reticulocytes and result in falsely low counts. Our observations showed that when compared with heparin, trisodium citrate did not produce any decrease in reticulocyte counts.

Samples of blood were taken from five normal subjects. One part of each sample was diluted with 3.8 per cent trisodium citrate (7 parts blood, 3 parts diluent) and the other with 1 per cent heparin (5 drops heparin solution per 10 ml. blood). Reticulocytes were stained and counted as described above. The two anticoagulants gave similar results, an average value of 15.5 per mille with trisodium citrate against 16.3 per mille with heparin.

*Effect of heparin on the culture of reticulocytes.*—Other investigators have cultured reticulocytes without culture medium, but with small amounts of heparin.\(^5\) The results obtained did not agree with ours. The effect of heparin on the reticulocyte maturation time and on the degree of hemolysis of the incubated blood was, therefore, studied in the following experiments.

Cultures of blood from 11 normal subjects were prepared with powdered heparin,\(^1\) 6 mg./10 ml. of blood, without addition of culture medium. After 12 hours the samples showed increased hemolysis: 66 mg. per cent of free hemoglobin as against 25 mg. per cent in the controls. The reticulocyte maturation time was also increased, with a \(T_{1/2}\) of 11 hours as compared to 4.8 hours for the controls.

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\(^*\)The values of reticulocyte maturation time in this study are expressed in hours and decimals of hour.

\(^{1}\)Liquaemin Sodium, Organon, Inc.
When, in 4 subsequent experiments, heparin was increased to 10 mg., 10 ml. of blood, the degree of hemolysis increased further (72 mg. per cent of free hemoglobin), although the reticulocyte maturation time did not change.

The question of whether delay of maturation and increased hemolysis were produced by the addition of heparin or by the lack of the balanced salt-glucose solution was then investigated. In a series of 16 experiments, it was observed that the addition of 2 to 6 mg. of powdered Liquemin to samples of blood diluted with salt-glucose solution in the usual manner produced only a moderate increase in hemolysis (45 mg. per cent of free hemoglobin), while no delay in the maturation time was observed.

From these results, it was concluded that heparin per se had no direct effect on the maturation time of reticulocytes, but that the absence of the salt-glucose solution caused delay of maturation and increased hemolysis.

**Effect of free hemoglobin.**—The effect of free hemoglobin in the culture medium on the maturation time of reticulocytes was studied.

Free hemoglobin was obtained by placing 0.5 ml. of blood in 2 ml. of hypotonic phosphate buffer. Once lysis of the red cells had occurred, 2 ml. of hypertonic phosphate buffer were added to yield a final solution isotonic with the plasma and of pH 7.2. This mixture (hemoglobin dissolved in phosphate buffer) was added in varying amounts to 19 flasks containing blood plus standard salt-glucose mixture so that the culture medium in the different samples contained free hemoglobin in concentrations from 90 to 230 mg. per cent. Controls were run by culturing blood from the same samples in the usual manner. A \( T_{1/2} \) of 5.4 hours was obtained in both samples and controls and was independent of the free hemoglobin added.

**Correlation between in Vitro and in Vivo Life-Span of Reticulocytes**

The turnover rate of cells in the peripheral circulation depends on two parameters, their number and their life-span. On the assumption that all the red cells come into the peripheral circulation as reticulocytes, the daily production of red cells should equal the daily production of reticulocytes. From this principle the following equation can be derived:

\[
\frac{\text{number of retics.}}{\text{life-span of retics.}} = \frac{\text{number of red cells}}{\text{life-span of red cells}}
\]

Since three parameters of this equation can be determined in vivo, the time that reticulocytes spend in the peripheral circulation after release from the bone marrow can be indirectly calculated. To the normal individual, the above equation can be applied as follows:

\[
\text{life-span of retics. (days)} = \frac{120 \times 15.4}{1000}
\]

The figure of 1.8 days, i.e., 43 hours is obtained. The life-span of reticulocytes obtained in vitro was much below this value, the average \( T_{1/2} \) of reticulocytes from 82 normal subjects being 4.8 hours. It seemed evident, therefore, that when cultured in vitro in the system described above, the reticulocytes had a shorter life-span than in vivo. The method, however, could still be applied to a comparative study of reticulocyte maturation in anemias, since it could be shown that a straight correlation existed between in vitro and in vivo maturation time of reticulocytes. This was demonstrated in the following experiments.

In a group of 11 patients in which there were variable degrees of increased
hemolysis, the life-span of the red cells was determined by the Cr\textsuperscript{51} technic, and simultaneously the life-span of reticulocytes was determined by the in vitro technic. The diagnoses in these patients were hereditary spherocytosis (2 cases), autoimmune hemolytic anemia (4 cases), chronic lymphocytic leukemia (2 cases), and 1 case each of lymphosarcoma, multiple myeloma, and myelofibrosis with myeloid metaplasia (table 1).

Not all the patients were anemic, but all of them had variable degrees of reticulocytosis, from 33 to 327 per mille. Care was taken to select only patients who had a stable hematocrit during the time of study. The red cell survival was measured with the use of the Cr\textsuperscript{51} method of Donohue et al.\textsuperscript{16} From the apparent Cr\textsuperscript{51} survival time \( (T_{1/2}) \), the mean cell life (MCL) of the red cells was calculated with the use of the method proposed by Hughes-Jones and Mollison.\textsuperscript{17} To simplify the calculations, the daily turnover rate of the red cells was expressed per 1000 red cells and was obtained by the following equation:

\[
\text{Red cell turnover/1000/24 hrs.} = \frac{1000}{ \text{Red cell life-span (days)}}
\]

For each patient, the in vitro maturation curve of the reticulocytes was determined. From the \( T_{1/2} \) value of the curve and from the number of reticulocytes per mille present in the sample at time zero, the number of reticulocytes which would have disappeared in 24 hours was obtained by extrapolation on semilog paper. The daily turnover values for the red cells were then compared with those for the reticulocytes. It was seen that not only in normals but also in patients with increased hemolysis the daily erythrocyte turnover values calculated from the in vitro life-span of the reticulocytes were always greater than their correspondent values obtained from the life-span of the red cells (table 1). This confirmed the fact that the life-span of the reticulocytes determined in vitro was always shorter than their actual life-span in vivo. However, the values obtained with both methods were in linear relationship (fig. 3). This was more evident for higher turnover values than for the lower ones, probably because with higher degrees of red cell turnover, the red cell life-span and/or the reticulocyte count and maturation time could be determined with greater accuracy. Thus, it seemed that the maturation time of reticulocytes determined in vitro closely paralleled the variations present in vivo. The method was therefore valuable for a comparative study of reticulocyte maturation in different blood disorders.

**The Reticulocyte Maturation Rate in Anemias**

The maturation time of reticulocytes kept in culture is a function of two factors: (1) the stage of development of the reticulocytes, younger reticulocytes requiring a longer period of time to mature into red cells, (2) the rate of disappearance of the reticulum substance, a function of the metabolic state of the reticulocytes.

In the following group of experiments the in vitro maturation rate of reticulocytes from patients with different types of anemia was investigated. The method of analysis of the experimental results had to be different from the one
Table 1.—Reticulocyte and Red Blood Cell Turnover in Normals and in Patients with Increased Hemolysis

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>Hematocrit (%)</th>
<th>No. per mille</th>
<th>Maturation time, hrs.</th>
<th>Turnover (per 000 cells per 24 hours)</th>
<th>Cr\textsuperscript{51}-RBC survival (T&lt;sub&gt;1/2&lt;/sub&gt;, days)</th>
<th>Mean cell life of RBC (days)</th>
<th>RBC turnover (per 000 cells per 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal subjects (average values)</td>
<td>44</td>
<td>15.4</td>
<td>4.8</td>
<td>14.9</td>
<td>28.3</td>
<td>129</td>
<td>8.3</td>
</tr>
<tr>
<td>2</td>
<td>Hereditary spherocytosis</td>
<td>25</td>
<td>327</td>
<td>11.0</td>
<td>256</td>
<td>4.2</td>
<td>6.6</td>
<td>150.0</td>
</tr>
<tr>
<td>3</td>
<td>Autoimmune hemolytic anemia</td>
<td>27</td>
<td>262</td>
<td>7.0</td>
<td>237</td>
<td>5.6</td>
<td>9.0</td>
<td>130.0</td>
</tr>
<tr>
<td>4</td>
<td>Autoimmune hemolytic anemia</td>
<td>41</td>
<td>45</td>
<td>9.3</td>
<td>44</td>
<td>14.0</td>
<td>32.0</td>
<td>31.0</td>
</tr>
<tr>
<td>5</td>
<td>Autoimmune hemolytic anemia</td>
<td>28</td>
<td>92</td>
<td>6.0</td>
<td>86</td>
<td>13.0</td>
<td>29.0</td>
<td>35.0</td>
</tr>
<tr>
<td>6</td>
<td>Autoimmune hemolytic anemia</td>
<td>35</td>
<td>40</td>
<td>6.0</td>
<td>37</td>
<td>19.0</td>
<td>46.0</td>
<td>22.0</td>
</tr>
<tr>
<td>7</td>
<td>Chronic lymphocytic leukemia</td>
<td>32</td>
<td>62</td>
<td>7.2</td>
<td>57</td>
<td>21.0</td>
<td>57.0</td>
<td>17.5</td>
</tr>
<tr>
<td>8</td>
<td>Chronic lymphocytic leukemia</td>
<td>36</td>
<td>33</td>
<td>5.3</td>
<td>31</td>
<td>22.0</td>
<td>80.0</td>
<td>12.5</td>
</tr>
<tr>
<td>9</td>
<td>Lymphosarcoma</td>
<td>45</td>
<td>63</td>
<td>8.1</td>
<td>57</td>
<td>19.6</td>
<td>48.0</td>
<td>21.0</td>
</tr>
<tr>
<td>10</td>
<td>Multiple myeloma</td>
<td>27</td>
<td>56</td>
<td>7.6</td>
<td>51</td>
<td>23.0</td>
<td>72.0</td>
<td>13.8</td>
</tr>
<tr>
<td>11</td>
<td>Myelofibrosis</td>
<td>23</td>
<td>38</td>
<td>9.5</td>
<td>32</td>
<td>21.0</td>
<td>64.0</td>
<td>15.6</td>
</tr>
</tbody>
</table>

Fig. 3.—The values of reticulocyte turnover in vitro showed a direct relationship with the in vivo red cell turnover values, determined by the Cr\textsuperscript{51}-red cell survival technic.
applied above because the anemic patients had populations of reticulocytes different from the normal, usually with increased numbers of younger forms. Therefore, the maturation time of only those reticulocytes which were of an age group comparable to those of normals was measured.

The maturation stage of reticulocytes could be distinguished morphologically with the use of the classification into five groups (0, I, II, III and IV) proposed by Heilmeyer and Westhäuser. Group 0 of this classification included red cells which still contained both nucleus and reticulum. The nucleus was usually pycnotic and sometimes was represented by only a small remnant. Group I represented the youngest forms of true reticulocytes which possessed a dense reticulum occupying the greater part of the cells. In group II the reticulum was looser and often did not occupy the whole cell. In group III threads of reticulum and isolated granules were present. Group IV included cells with only isolated granules; one or two threads of reticulum could still be present in one single area of the cell. It is universally accepted that reticulocytes pass through all these consecutive stages of maturation to become mature red cells. The progressive sequence of the changes in the differential count of reticulocytes cultured in vitro also supports this concept. During the initial period of culture the more profound decrease was noted in the most immature groups with little and, sometimes, no apparent change in the more mature ones. Later, the more mature reticulocytes also decreased in number and disappeared.

The following is a typical example:

<table>
<thead>
<tr>
<th>Culture time, hours</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.1</td>
<td>37.2</td>
<td>56.7</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>2.4</td>
<td>17.5</td>
<td>43.8</td>
<td>63.7</td>
</tr>
<tr>
<td>8</td>
<td>0.7</td>
<td>6.5</td>
<td>27.5</td>
<td>34.7</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>2.5</td>
<td>14.9</td>
<td>17.9</td>
</tr>
</tbody>
</table>

It is obvious that the life-span of reticulocytes would appear to be longer in blood samples containing younger, i.e., more immature reticulocytes than in those containing only the more mature ones. For a comparative study of the maturation of reticulocytes from different patients to be significant it was necessary to determine the maturation time of each individual reticulocyte group. In doing this, two types had to be distinguished: (1) groups which had no precursors, i.e., no groups of more immature type, and (2) groups with precursors. The maturation curve of the groups with no precursors was obtained from the experimental values by applying equation \( I \) as in the above experiments. In the case of groups with precursors the same equation could not be used, since these groups had a rate of formation superimposed upon the rate of disappearance. These two superimposed rates could only be separated by mathematical analysis.

The method of analysis selected for this study was based on the following consideration; if, for example, group II and III were taken together and considered as a single "unit," an all-inclusive number of reticulocytes was ob-
tained which decreased during the time of culture by maturation of group III into group IV. In fact, all the reticulocytes of group II had to pass through group III to reach group IV. In other words, to determine the maturation curve of a given group of reticulocytes during the time of culture, it was sufficient to consider the variations in the total reticulocyte number of a “unit” including the said group and all the precursors, since the disappearance curve of this “unit” was controlled by the maturation of the most developed group present in it.

The maturation time of each individual reticulocyte group was, then, determined graphically by plotting the maturation curve of the above “units” on semilog paper. For convenience, the Y intercept at time 0 was corrected to 100 by straight proportion. The maturation time of the most mature group of each “unit” was expressed as the amount of time for one-half the cells of this group to disappear. As an illustration, if 40 reticulocytes of group III were present at time 0, the maturation time of this group was determined by finding on the graph the amount of time for the total number of cells of the “unit” including groups I, II and III to decrease from 100 to 80.

By this method of analysis the 82 culture experiments with blood from normal subjects were first evaluated. Reticulocytes of group III and group IV were found to be present in all subjects with an average value of 5.86 (±3.2 S.D.) and 8.95 (±5.4 S.D.) per mille, respectively. A few reticulocytes of group II were present in only 50 per cent of the cases, with values varying from 0.1 to 2 per mille.

The values for the maturation time of the different reticulocyte groups showed rather wide variations, probably due to the low number of reticulocytes in normal blood. The maturation of group IV had a T1/2 of 2.9 hours (±1.1 S.D.), while group III had a T1/2 of 3.6 hours (±2.1 S.D.). The T1/2 of group II was 3.4 hours. The latter could only be estimated with wide approximation, because group II was only represented by a few reticulocytes and was found in only 50 per cent of the subjects. Since groups III and IV were present in all the normal subjects, the sum of the half-lives of these two groups was used as an index for comparison in the study of the reticulocyte maturation rate in hematologic disorders. This total value for normal reticulocytes was 6.5 hours (±2.4 S.D.).

The reticulocyte maturation rate index, i.e., the maturation time of group III and IV, was then estimated in a series of patients with different types of anemias. The study included 11 patients with hereditary spherocytosis, 6 patients with autoimmune hemolytic anemia, 4 with Cooley’s anemia, 5 with pernicious anemia in relapse, 6 with iron deficiency anemia, 4 with anemia due to acute blood loss, and 6 with anemia of chronic uremia. The findings are summarized in table 2 and figure 4. The results clearly showed that in some anemias the maturation rate index of reticulocytes was constantly and sometimes conspicuously higher than normal, indicating a delay in the disappearance rate of the reticulum substance of erythrocytes in these disorders. No relationship was found between degree of delay in the reticulocyte maturation and reticulocyte or hemoglobin level in these patients. Acceleration of the maturation of reticulocytes was not seen in any type of anemia.
The most marked abnormality was found in the patients with Cooley's anemia, in which the average maturation rate index was 27.5 hours, i.e., 4.2 times the normal value. In the 6 uremic patients in which the BUN varied from 70 to 197 mg. per cent, the maturation rate index was also consistently above the normal. The mean value obtained was 19.8, i.e., 3 times the normal. However, there was no direct correlation between BUN level and reticulocyte maturation rate in these patients. In one of them (case 37) reticulocytes were unusually increased due to a recent hemorrhage, and the maturation rate of reticulocytes was still conspicuously prolonged (16.3 hours). In the 5 patients with pernicious anemia in relapse the maturation rate index of reticulocytes was increased to variable degrees, with an average value of 12.3 hours. Following treatment with vitamin B₁₂ the reticulocyte maturation rate rapidly returned to normal in all cases (fig. 5). In the patients with hereditary sphero- cytosis the maturation rate of reticulocytes was normal in most cases and somewhat prolonged in others, with an average value of 8.3 hours. Similar results were obtained in the patients with autoimmune hemolytic anemia. In the 6 patients with iron deficiency anemia the reticulocyte maturation rate was normal in all but one case, in which it was found to be lower than normal. However, the mean value of 5.2 hours was still in the normal range. The patients with acute post-hemorrhagic anemia were studied at the time in which the highest level of reticulocyte response was reached. The maturation rate of reticulocytes was always found to be normal in these patients.
Table 2.—Hematocrit and Reticulocyte Data in Normal Subjects and in Patients with Various Types of Anemia

| No. | Hematocrit (%) | Reticulocytes (per mille) | Absolute reticulocytes (/cu.mm.) | Reticulocyte maturation rate, in vitro (T112
| Group | | | | group III + Tci
| | | | group IV; hours) |
|------|----------------|--------------------------|-------------------------------|----------------------------------|
| 82 subjects | 44 (± 5 S.D.) | 15.4 (± 6.5 S.D.) | 77,000 (± 23 × 10³ S.D.) | 6.5 (± 2.4 S.D.) |

**Hereditary Spherocytosis**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>119</td>
<td>327,250</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>200</td>
<td>548,000</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>192</td>
<td>576,000</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>394</td>
<td>1,221,000</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>339</td>
<td>813,600</td>
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<tr>
<td>6</td>
<td>29</td>
<td>390</td>
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<td>7</td>
<td>34</td>
<td>217</td>
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<td>37</td>
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**Autoimmune Hemolytic Anemia**

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**Cooley’s Anemia**

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**Pernicious Anemia**

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**Iron Deficiency Anemia**

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**Acute Bleeding Anemia**

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<td>98</td>
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Table 2.—Continued

Anemia of Uremia

| No. | Hematocrit (%) | Reticulocytes (per mille) | Absolute reticulocytes (per cu.mm.) | Reticulocyte maturation rate in vitro (T1/2, group III + T1/2, group IV, hours) | BUN (mg.%)
|-----|----------------|--------------------------|-------------------------------------|--------------------------------------------------------------------------------|-------
| 37  | 19             | 308                      | 585,100                             | 16.3                                                                             | 90    |
| 38  | 24             | 22                       | 58,265                               | 8.0                                                                              | 143   |
| 39  | 22             | 24                       | 60,500                               | 33.8                                                                             | 197   |
| 40  | 19             | 34                       | 58,276                               | 36.6                                                                             | 171   |
| 41  | 22             | 12                       | 30,000                               | 13.3                                                                             | 188   |
| 42  | 23             | 51                       | 130,000                              | 10.9                                                                             | 70    |
| Mean| 21.5           | 75                       | 153,690                              | 19.8                                                                             | 143   |

Fig. 5.—In cases of pernicious anemia the delayed reticulocyte maturation rate normalized rapidly after treatment was begun. At the peak of the reticulocyte response the reticulocyte maturation was always normal.

**DISCUSSION**

The aim of this investigation was not to establish the exact life-span of reticulocytes in the circulating blood, but rather to compare the rate of reticulocyte maturation in pathologic conditions. The method used was thought to be suitable for such study, because it gave reproducible results with values of reticulocyte turnover which paralleled those found in vivo by the Cr51-red cell survival technic.

Whether all red cells leave the bone marrow as reticulocytes or some completely mature within the bone marrow cavity, coming into the peripheral blood as mature red cells, has been a subject of frequent discussion. Studies by Istomova and Nizet in animals and by Riddle, Koller, and Seip in humans have demonstrated that there are no reserves of mature red cells in the bone marrow. However, the problem still seems to be controversial, especially with regard to pathologic states. The present study does not prove either theory. However, the fact that the values of Cr51-red cell turn-
over were in linear relationship to the in vitro values of reticulocyte turnover would favor the former hypothesis, since it seems difficult to accept that the same percentage of reticulocytes should complete their development into mature red cells within the bone marrow in normal conditions as in cases of increased blood regeneration. Reticulocytosis is usually associated with the appearance of younger reticulocytes in the circulating blood.

The life-span of reticulocytes in the circulating blood depends on the age at which they are released from the bone marrow, and on the rate of disappearance of the reticulum. The study of this rate and its derangements was the main object of the present investigation, and from the results obtained it is clear that in some anemias a definite delay in the rate of reticulocyte maturation occurs. This maturation defect of reticulocytes was found to be more pronounced in Cooley’s anemia, anemia of chronic uremia and pernicious anemia in relapse. This phenomenon most probably reflects a metabolic abnormality of the erythron in these disorders. The reticulum substance is known to be a remnant of the erythroblastic cytoplasm, since it is known to consist of RNA. Mitochondria and endoplasmic reticulum have been seen in reticulocytes. For this reason these cells are capable of heme synthesis, while mature red cells are inert. Significantly, the most marked increase in maturation rate of reticulocytes was found in Cooley’s anemia, the anemia of chronic uremia and in pernicious anemia in relapse. In the former two conditions a maturation defect of the normoblasts has been demonstrated by bone marrow culture, and in the latter Thorell has shown spectrophotometrically that there is a defect in the disappearance of RNA from the cytoplasm during cellular maturation. Other investigators have also shown a defect in reticulocyte maturation in pernicious anemia. Conversely, a normal maturation rate of reticulocytes was obtained in iron deficiency anemia, in which the disappearance of RNA in the normoblasts is known to occur in normal fashion, in spite of decreased hemoglobin synthesis. It is not yet known why occasional patients with hereditary spherocytosis or autoimmune hemolytic anemia showed a moderate delay in the maturation of reticulocytes.

The finding of a delayed maturation rate of reticulocytes in some anemias is of relevance in the study of erythrokinetics, i.e., the quantitation of red cell production and destruction in blood disorders. In erythrokinetics, the “reticulocyte erythropoietic index” is calculated from the ratio between the absolute number of circulating reticulocytes in the patient and the normal number. This calculation disregards the fact that an increased reticulocyte count in the peripheral circulation may be the combined result of three variable factors. (1) increased output of reticulated red cells, (2) increased numbers of younger reticulocytes with longer life-span, and on occasion (3) a delayed disappearance rate of the reticulum substance. This may explain why we have often found, in the study of the erythrokinetics of anemias, that the erythropoietic index derived from the reticulocyte count correlated poorly with the erythropoietic indexes calculated from the Cr\textsuperscript{51}-red cell survival and the Fe\textsuperscript{59}-red cell incorporation.

The data presented in this study indicate that the reticulocyte count in the
circulating blood, the oldest and probably still the most useful clinical sign of increased red cell production, may not be an accurate index for the quantitation of erythropoiesis.

SUMMARY

An in vitro culture technic for the study of reticulocyte maturation was described. The method gave reproducible results and proved to be of value in the comparative study of reticulocyte maturation in blood disorders. By this method it was shown that variations in the reticulocyte maturation in vitro paralleled similar variations present in vivo.

The maturation of reticulocytes from patients with different types of anemia was investigated. In some anemias the in vitro maturation of reticulocytes was prolonged, not only because younger reticulocytes were present in the blood, but also because the rate at which the reticulum substance disappeared was delayed. This was particularly evident in the anemia of chronic uremia, in Cooley’s anemia and in pernicious anemia in relapse. In only occasional cases of hereditary spherocytosis and of autoimmune hemolytic anemia was the rate of reticulocyte maturation found to be moderately delayed. In patients with iron deficiency anemia or bleeding anemia it was always normal.

From the above findings the following conclusions were derived:

1. The reticulocyte number in the circulating blood is the resultant of three variables: (a) the rate of output of new reticulocytes from the bone marrow; (b) the stage of maturation at which reticulocytes are delivered into the peripheral circulation; (c) the rate of disappearance of the reticulum substance.

2. The number of reticulocytes in the circulating blood cannot be indiscriminately used as a precise index of red cell production in erythrokinetics.

3. There is good reason to believe that a defect in the rate at which the reticulocytes mature in the circulating blood is an index of a similar defect in the process of erythroblast differentiation in the bone marrow.

SUMMARIO IN INTERLINGUA

Es describite un technica de culturnation in vitro pro le studio del maturation de reticulocytos. Le methodo ha resultatos que es reproducibile. Illo se provava de valor in le studio comparative del maturation de reticulocytos in disordines hematologic. Illo ha essite usate pro demonstrar que variationes del maturation de reticulocytos in vitro curre parallel a simile variationes que es presente in vivo.

Esseva investigate le maturation de reticulocytos ab patientes con varie differente typos de anemia. In certe anemias le maturation in vitro del reticulocytos esseva prolongate, non solmente proque plus juvene reticulocytos esseva presente in le sanguine sed etiam proque le rapiditate del disparition del substantia reticular esseva reduce. Isto esseva particularly evidente in le anemia de uremia chronic, in anemia de Cooley, e in anemia perniciose in relaso. Il esseva solmente in casos sporadic de spherocytosis hereditari e de anemia hemolytic auto-immun que le maturation reticulocytic monstrava...
grados moderate de retardo. In pacientes con anemia a carentia de ferro o con anemia por sanguinacion, illos esseva semper normal.

Super le base de iste constatationes, le sequente conclusiones esseva formu-
late:

1. Le numero del reticulocytos in le sanguine circulante es le resultante de tres variabiles. Istos es (a) le rapiditate del liberation de nove reticulocytos per le medulla ossee, (b) le stadio maturational al qual le reticulocytos es livrate a in le circulation peripheric, e (c) le rapiditate del disparition del substantia reticular.

2. Le numero del reticulocytos in le sanguine circulante non pote esser usate directemente como indice del production erythrocytic.

3. Il existe bon rationes pro le conception que un defecto in le rapiditate maturational del reticulocytos in le sanguine circulante es un indice de un simile defecto in le proceso de differentiation erythroblastic in le medulla ossee.

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The Maturation Rate of Reticulocytes

MARIO BALDINI and IVO PANNACCIULLI