Energy Metabolism in the Erythrocytes of Premature Infants Compared to Full Term Newborn Infants and Adults

By Ruth T. Gross, Eleanor A. R. Schroeder and Susan A. Brounstein

The erythrocytes of premature infants have a shortened life span compared to the erythrocytes of full term newborn infants and of adults, as measured by chromium $^{51}$.1,2 The key role of adenosine triphosphate (ATP) generation and utilization in the maintenance of normal erythrocyte life span has been emphasized by others.3,4 It has also been reported that in the erythrocytes of normal adults the content of ATP decreases with in vivo aging of the cells.5-7 In the mature red blood cell, lacking intact Krebs cycle activity and an intact cytochrome system, ATP is generated only at the substrate level via the Embden-Meyerhof pathway5,4 (glucose + 2 ADP + 2 $pi$ $\rightarrow$ 2 lactate + 2 ATP).

The purpose of the present study was to examine the red blood cells of premature infants for alterations in energy metabolism which might be responsible for the shortened erythrocyte life span. Presented herein are the levels of activity of the enzymes involved in the generation of ATP and of adenosine diphosphate (ADP) as well as the content of these adenine nucleotides in the erythrocytes of premature infants compared to full term newborn infants and adults.

Material and Methods

The young subjects were healthy infants from the premature and full term nurseries of the Bronx Municipal Hospital Center. The adult subjects were hospital and laboratory personnel. The premature infants ranged in weight from 1040 to 2180 Gm. and in age from 2 days to 7 weeks. The full term newborn infants ranged in age from 1 to 7 days.

Measurements of enzymatic activity: Erythrocytes were separated from whole blood, washed and lysed as previously described8 except that the anticoagulant was acid citrate dextrose (1 part to 4 parts blood) and the cells were washed and resuspended in 0.15 M sodium chloride which was adjusted to pH 7.4 with sodium hydroxide.

The enzymatic assays were performed according to published technics with the following considerations. Since the original assays were designed for tissues other than erythrocytes, care was taken to determine that the conditions of each assay were optimal for the erythrocyte enzyme. In each instance the rate of enzyme activity was found to be constant for an initial period of 10 minutes. Enzyme activity was proportional to the concentration of hemoglobin in the assay system. Optimal substrate concentrations were determined, and an excess of auxiliary enzymes was employed. Thus, the following enzymatic activities were measured according to the technics indicated, and the optimal amounts of substrate and of hemolysate (expressed as total amount of hemoglobin) are stated: Hexokinase (Stein et
al.\textsuperscript{9})-glucose 20 \(\mu\)moles, hemoglobin 1.6 mg. Phosphofructokinase (Wu and Racker\textsuperscript{10})-fructose-6P 3.4 \(\mu\)moles, hemoglobin 0.5 mg. Phosphoglyceric acid kinase (Wu and Racker\textsuperscript{10})-3-phosphoglyceric acid 5 \(\mu\)moles, hemoglobin 0.05 mg. Pyruvate kinase (Bücher and Pfleiderer\textsuperscript{11})-phosphoenol pyruvate 1 \(\mu\)mole combined with 8 \(\mu\)moles of magnesium sulphate and 75 \(\mu\)moles of potassium chloride, hemoglobin 0.5 mg. The final volume in the cuvette for each assay was 1.0 ml. Readings were obtained with a Zeiss PMQII spectrophotometer at 340 nm. All enzyme activities were expressed as \(\mu\)moles Gm. hemoglobin minute. Hemoglobin was measured in duplicate for each sample by the cyanmethemoglobin method.\textsuperscript{12} Auxiliary enzymes were obtained commercially from C. F. Boehringer & Sons and Sigma Chemical Co.

For the measurement of phosphofructokinase activity, but not for the other enzymes, the concentration of the red blood cell suspension prior to lysis by freezing and thawing was found to be critical. Erythrocyte suspensions containing less than 100 mg. of hemoglobin per ml. before lysis exhibited loss of enzyme activity. Increasing the concentration of hemoglobin above 100 mg. per ml. did not lead to increased enzyme activity. Following lysis, the hemolysate could be diluted as much as desired for the purpose of the assay without loss of enzyme activity. In these studies all determinations of phosphofructokinase activity were obtained from hemolysates having a hemoglobin concentration of 100 mg./ml. or greater prior to lysis.

\textbf{Measurement of ATP and ADP:} In order to obtain measurements which would correspond as closely as possible to the levels of ATP and ADP under normal in vivo conditions, the following precautions were observed. Care was taken to choose healthy infants in the controlled environments of the hospital nurseries. Samples were obtained at the same time each day and always 3 hours after the last feeding; however, it was later determined that the levels of the two adenine nucleotides were not influenced by feeding or fasting the subject. Hospital and laboratory personnel were chosen as the adult controls in an effort to obtain vigorous, healthy subjects. In order to minimize continued metabolic activity in the blood in vitro, the samples were immediately deproteinized and chilled. Separation and washing of the erythrocytes were intentionally avoided. Heparinized whole blood\textsuperscript{*} was added immediately to an equal volume of cold perchloric acid (6 per cent weight/volume) and a 1/5 volume of 1.0 M magnesium chloride. The mixture was stirred and allowed to stand in an ice bucket for 45 minutes. It was then centrifuged for 5 minutes at 1000 x g. The supernatant fluid was divided into two aliquots. One was used without further treatment for the assay for ATP. The second aliquot was combined and mixed with \(1/4\) of its volume of 1.0 M triethanolamine hydrochloride and 1.3 M potassium carbonate buffer, pH 9.2, allowed to stand in an ice bucket for 15 minutes, filtered and then used for the enzymatic assay of ADP. The two filtrates could be frozen and stored at \(-10\) C. for a period up to 10 days without loss of ATP and ADP.

ATP was measured enzymatically by the method described by Stein et al.\textsuperscript{9} for the measurement of hexokinase activity. In the present assay hexokinase was added in excess so that the rate of the reaction was dependent upon the amount of ATP provided by the filtrate. An appropriate amount of filtrate for this assay was generally 0.1 ml. Activity was proportional to the amount of ATP in the filtrate over the range of 0.010 to 0.055 \(\mu\)moles. When known ATP was added to the blood, 97 per cent of the added compound was recovered.

ADP was measured enzymatically by the method described by Bücher and Pfleiderer\textsuperscript{11} for the measurement of pyruvate kinase activity.\textsuperscript{†} Pyruvate kinase was added in excess so that the rate of the reaction was dependent upon the amount of ADP in the filtrate. Activity was proportional to the quantity of ADP in the filtrate over the range of 0.002

\textsuperscript{*}The adenine nucleotides in whole blood are derived almost entirely from the erythrocytes. The contributions of both plasma\textsuperscript{13} and other cellular elements\textsuperscript{14} are negligible.

\textsuperscript{†}A commercially available kit for this determination may be obtained from C. F. Boehringer & Sons.
The significance of the difference between the means was established by the "student's" t distribution test.

Table 1.—Erythrocyte Enzyme Activity

<table>
<thead>
<tr>
<th></th>
<th>Adults</th>
<th>Full Term Newborn Infants</th>
<th>Premature Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase‡</td>
<td>(13)†</td>
<td>(12)</td>
<td>(13)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.95</td>
<td>1.73</td>
<td>1.85</td>
</tr>
<tr>
<td>Range</td>
<td>0.63–2.10</td>
<td>1.14–2.17</td>
<td>1.48–2.75</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.088</td>
<td>0.087</td>
<td>0.092</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>(13)</td>
<td>(11)</td>
<td>(16)</td>
</tr>
<tr>
<td>Mean</td>
<td>6.63</td>
<td>4.95</td>
<td>5.27</td>
</tr>
<tr>
<td>Range</td>
<td>4.98–7.97</td>
<td>4.02–5.85</td>
<td>3.92–7.23</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.25</td>
<td>0.18</td>
<td>0.19</td>
</tr>
<tr>
<td>PGA Kinase†</td>
<td>(14)</td>
<td>(12)</td>
<td>(13)</td>
</tr>
<tr>
<td>Mean</td>
<td>108.</td>
<td>151.</td>
<td>154.</td>
</tr>
<tr>
<td>Range</td>
<td>96–145.</td>
<td>120–180.</td>
<td>130–235.</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>3.29</td>
<td>5.48</td>
<td>7.70</td>
</tr>
<tr>
<td>Pyruvate Kinase</td>
<td>(28)</td>
<td>(21)</td>
<td>(19)</td>
</tr>
<tr>
<td>Mean</td>
<td>8.92</td>
<td>11.83</td>
<td>11.62</td>
</tr>
<tr>
<td>Range</td>
<td>5.35–15.31</td>
<td>7.35–19.29</td>
<td>3.24–17.93</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.36</td>
<td>0.52</td>
<td>0.51</td>
</tr>
</tbody>
</table>

The age of the full term newborn infants was 1 to 7 days; of the premature infants, 2 days to 7 weeks.

†Enzyme activity is expressed as μM Gm. hemoglobin minute.
‡Numbers in parentheses = number of subjects studied.
§Phosphoglyceric acid kinase.

The levels of activity of the two enzymes which catalyze the reactions for the generation of ATP, phosphoglyceric acid kinase and pyruvate kinase, were significantly increased in the erythrocytes of premature infants (p < .001) and full term newborn infants (p < .001) compared to adults. No significant differences were observed between the full term newborn and premature infants (table 1).

Of the two reactions in which ATP is utilized and ADP is generated, the level of hexokinase activity was found to be significantly increased in the erythrocytes of premature infants (p = < .001) and full term newborn infants (p = < .001) compared to adults; the level of phosphofructokinase activity was found to be significantly decreased in the erythrocytes of premature infants (p = < .001) and full term newborn infants (p = < .001) compared to adults.
Table 2.—Erythrocyte Content of ATP and ADP *

<table>
<thead>
<tr>
<th></th>
<th>Adults</th>
<th>Full Term Newborn Infants</th>
<th>Premature Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>(31)†</td>
<td>(33)‡</td>
<td>(21)‡</td>
</tr>
<tr>
<td>Mean</td>
<td>3.86</td>
<td>4.38</td>
<td>5.68</td>
</tr>
<tr>
<td>Range</td>
<td>2.33–4.76</td>
<td>2.92–5.52</td>
<td>4.07–7.08</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.13</td>
<td>0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>ADP</td>
<td>(30)</td>
<td>(33)</td>
<td>(15)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.34</td>
<td>0.40</td>
<td>0.38</td>
</tr>
<tr>
<td>Range</td>
<td>0.18–0.57</td>
<td>0.14–0.64</td>
<td>0.24–0.51</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>ATP + ADP</td>
<td>(30)</td>
<td>(31)</td>
<td>(15)</td>
</tr>
<tr>
<td>Mean</td>
<td>4.21</td>
<td>4.75</td>
<td>5.75</td>
</tr>
<tr>
<td>Range</td>
<td>2.65–5.25</td>
<td>3.14–6.03</td>
<td>4.36–7.02</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.14</td>
<td>0.14</td>
<td>0.20</td>
</tr>
<tr>
<td>ADP × 100</td>
<td>(30)</td>
<td>(31)</td>
<td>(15)</td>
</tr>
<tr>
<td>ATP + ADP</td>
<td>(30)</td>
<td>(31)</td>
<td>(15)</td>
</tr>
<tr>
<td>Mean</td>
<td>8.1%</td>
<td>8.5%</td>
<td>6.6%</td>
</tr>
<tr>
<td>Range</td>
<td>3.7–14.3</td>
<td>5.1–12.0</td>
<td>3.8–8.3</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>0.43</td>
<td>0.27</td>
<td>0.30</td>
</tr>
</tbody>
</table>

The age of the full term newborn infants was 1 to 7 days; of the premature infants, 2 days to 7 weeks.

*Content is expressed as μmoles Gm. hemoglobin; ATP = adenosine triphosphate; ADP = adenosine diphosphate.
†Numbers in parentheses = number of subjects studied.

to adults. No significant differences were observed between the premature and full term newborn infants (table 1).

The content of ATP was significantly greater in the erythrocytes of the full term newborn (p < 0.001) and premature infants (p < 0.001) than in adults. In addition, the content of ATP in the erythrocytes of prematures was significantly greater (p < 0.001) than in full term newborns (table 2).

The content of ADP was increased in the erythrocytes of the full term newborn infants compared to adults (p < 0.05). In the group of premature infants, the level of erythrocyte ADP was not significantly increased compared to either the full term newborns or the adults.

The total content of ATP plus ADP was significantly increased in the erythrocytes of full term newborns (p < 0.01 > 0.005) and prematures (p < 0.001) compared to adults. The mean value for premature infants was significantly increased over that of full term newborns (p < 0.001).

The percentage content of ADP \( \frac{\text{ADP}}{\text{ADP + ATP}} \times 100 \) did not differ significantly in the erythrocytes of full term newborn infants from the adults.* In the erythrocytes of premature infants the percentage content of

*All comparisons of ATP and ADP levels, as well as the combined values for the two compounds, were based upon determinations obtained from the same sample of blood in each subject.
ADP was significantly reduced ($p = < 0.01 > 0.005$), compared to the newborns and to the adults.

**DISCUSSION**

Previous investigations of the levels of activity of erythrocyte enzymes directly involved in either the Embden-Meyerhof pathway or the hexose monophosphate shunt have revealed increased activity in the red blood cells of young subjects (glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and aldolase$^{15}$). The present studies demonstrate increased levels of activity in three additional enzymes in the erythrocytes of newborn and premature infants: hexokinase, phosphoglyceric acid kinase and pyruvate kinase.

Of particular interest is the finding of decreased activity of the erythrocyte enzyme phosphofructokinase in both full term newborn and premature infants compared to adults. This is the first report, to our knowledge, of decreased activity in an enzymatic step essential for the metabolism of glucose in erythrocytes of young subjects. The extent to which phosphofructokinase activity may be a rate limiting factor in the metabolism of erythrocytes is not known. Several observations indicate, however, that the activity of this enzyme may play a significant role in the control of glycolysis in other tissues.$^{16, 24}$ Although glucose utilization by the erythrocytes of newborn infants has been found to equal$^{25}$ or exceed$^{26}$ that of adults, these observations could be accounted for by increased activity of the hexose monophosphate shunt pathway in the young subjects' erythrocytes. The possibility that decreased activity of phosphofructokinase may be a rate limiting factor in the metabolism of glucose via the Embden-Meyerhof pathway is currently being studied.

Evidence for an alteration in metabolic activity in the erythrocytes of premature infants compared to full term newborn infants and to adults is provided by the data regarding the adenine nucleotides. The content of erythrocyte ATP is markedly increased over the level in the red blood cells of newborn infants which is in turn significantly greater than in the erythrocytes of adults. In the premature infants the increase in erythrocyte ATP is not accompanied by a relative increase in ADP. The percentage content of ADP is significantly reduced compared to both full term newborns and adults.

The increase in erythrocyte ATP in premature infants is seen to be even more striking when one removes from consideration the observations obtained from four infants whose birth weights were greater than 2000 Gm. Among the remaining 17 infants, ranging in birth weight from 1000 to 2000 Gm. and in age from 2 days to 7 weeks, the level of erythrocyte ATP in each instance is seen to be greater than one standard deviation above the mean value for full term newborns (fig. 1).

The altered ADP:ATP + ADP ratio in the blood of premature infants cannot be explained by the levels of activity of the enzymes hexokinase, phosphofructokinase, phosphoglyceric acid kinase and pyruvate kinase. Although significant differences were observed in the levels of activity of these enzymes in the red blood cells of the young subjects compared to adults, no such differences existed between the premature and full term newborn infants. Furthermore,
Fig. 1.—Content of erythrocyte adenosine triphosphate (ATP) in premature infants weighing 1000–2000 Gm. plotted against age and also compared to mean level of erythrocyte ATP in full-term newborn infants. ATP content is expressed as μmoles Gm. of hemoglobin. (Note that the Y axis does not start at 0.)

The measurements of enzyme activity were obtained in an in vitro cell-free system under optimal conditions. Thus, the observed levels of activity are an expression of the maximal potential velocity of these reactions and do not necessarily reflect the intracellular rates of ATP and ADP generation.

Reports by others have provided conflicting conclusions regarding the quantity of adenine nucleotides in the red blood cells of young infants compared to adults.27-29 These differences, we believe, are related largely to variations in both the handling of the blood samples and the technics of measurement. In this laboratory it has been found that separation and washing of erythrocytes, a process of approximately 2 hours duration, results in a loss of ATP of variable magnitude. Stave and Cara,25 using methods similar to those reported here, found levels of ATP and ADP comparable to the present data.

The possibility must be considered that the increased content of ATP in the blood of the premature infants may reflect the presence of an increased number of reticulocytes and erythroblasts, both of which cells possess pathways for ATP generation which are not present in the mature erythrocyte,3,4 or merely a younger mean cell age in these samples. Reticulocyte counts were not obtained on the blood of these subjects. However, Stave and Cara28 found no correlation between the level of ATP and the per cent of reticulocytes in the blood of premature infants. In the present study we did not find, in either the premature or full term newborn subjects, that the levels of erythrocyte ATP decreased in the first few days after birth, whereas the number of reticulocytes and erythroblasts are known to diminish sharply during this period.30,31

The finding of increased content of ATP without a relative increase in ADP in the erythrocytes of premature infants may reflect either an increased rate of generation or a relative block in the utilization of ATP. It cannot be de-
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termined from these data whether this difference in energy metabolism is the result of decreased erythrocyte life span and decreased mean cell age or whether it represents a significant biochemical defect in the erythrocytes of premature infants.

Summary

1. The following aspects of energy metabolism have been compared in the erythrocytes of premature infants, full term newborn infants, and adults: levels of activity of the enzymes involved in the generation and utilization of ATP, and measurements of the content of ATP and of ADP.

2. The levels of activity of hexokinase, phosphoglyceric acid kinase and pyruvate kinase are significantly increased in the erythrocytes of premature and full term newborn infants compared to adults.

3. The level of activity of phosphofructokinase is significantly decreased in the erythrocytes of premature and full term newborn infants compared to adults.

4. In the erythrocytes of premature infants the content of ATP is significantly increased compared to both full term newborns and adults. The content of ADP is not increased. The percentage content of ADP is significantly below the values found in full term newborn infants and adults.

5. In the erythrocytes of full term newborn infants the content of both ATP and ADP is significantly greater than in adults. The ratio of ADP to the total amount of ATP plus ADP does not differ from the adult value.

Summary in Interlingua

1. Le sequente aspectos del metabolismo de energia esseva comparate inter le erythrocytos de infantes prematur, de neonatos a termino, e de adultos: Nivellos de activitate del enzymas participante in le generation e utilisation de triphosphato de adenosina e concentrationes de triphosphato de adenosina e de diphosphato de adenosina.

2. Le nivellos de activitate de hexocinase, cinase de acido phosphoglyceric, e cinase de pyruvato es significativemente augmentate in le erythrocytos de neonatos prematur e a termino in comparation con adultos.

3. Le nivello del activitate de de phosphofructocinase es reducite significativamente in le erythrocytos de neonatos prematur e a termino in comparation con adultos.

4. In le erythrocytos de infantes prematur, le contento de triphosphato de adenosina es augmentate significativamente in comparation con neonatos a termino e con adultos. Le contento de diposphato de adenosina non es augmentate. Le contento procentual de diposphato de adenosina es significativamente infra le valores trovate in neonatos a termino e in adultos.

5. In le erythrocytos de neonatos a termino, le contento de triphosphato de adenosina e de diposphato de adenosina es significativamente plus grande que in adultos. Le proportion de triphosphato de adenosina in le total de triphosphato e diposphato de adenosina non difere ab le valor trovate in adultos.
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