Glycogen Metabolism in Glycogen-rich Erythrocytes

By Shimon W. Moses, Nava Bashan, Alisa Gutman, and Per Arne Ockerman

High concentrations of red blood cell glycogen were visualized by electron microscopy and demonstrated biochemically in amylo-1,6-glucosidase- and phosphorylase-deficient red blood cells. Glycogen concentration decreased as a function of cell age. Similar incorporation rates of \(^{14}\text{C}-\text{U-glucose}\) into glycogen were observed in amylo-1,6-glucosidase-deficient and normal erythrocytes, characterized by an initial rise, followed by a plateau formation reflecting a steady state between glycogen synthesis and breakdown. A different pattern of kinetics was observed in phosphorylase-deficient cells, which in view of the lack of the degradative enzyme showed a continuous linear increase in radioactive glycogen formation leveling off only after exhaustion of substrate. Evidence that in amylo-1,6-glucosidase-deficient red blood cells the main metabolic activity affects the outer branches of the glycogen molecule was obtained directly by \(\beta\)-amylolytic degradation of the radioactive glycogen molecule and indirectly by a chase experiment substituting radioactive with nonlabeled glucose. Normal glycogen synthetase activity was found in all cases of amylo-1,6-glucosidase examined except in one family in which an unexpected low affinity of the enzyme to glycogen was found. The observation that both amylo-1,6-glucosidase- and phosphorylase-deficient red blood cells retain the capacity to incorporate glucose into glycogen indicates that glycogen synthesis in erythrocytes proceeds along the UDPG glycogen synthetase pathway and is not a result of a reverse activity of any of the degradative enzymes.

THE NORMAL HUMAN erythrocyte has no carbohydrate stores, and it is, therefore, completely dependent on environmental glucose for its metabolic activity. It has recently been shown that the lack of glycogen accumulation in normal human red blood cells is not due to an incomplete enzymatic complement catalyzing glycogen metabolism, but rather to the kinetic characteristics of UDPG glycogen synthetase, which exists only in a strictly glucose-6-phosphate-dependent form. The activity of glycogen synthetase is particularly low in this tissue as a result of the high Ka for glucose-6-phosphate. In erythrocytes the enzymes degrading the glycogen molecule, amylo-1,6-glucosidase (the debrancher), and phosphorylase are relatively more active than glycogen synthetase and therefore, under normal conditions, glycogen does not accumulate in this tissue (Fig. 1). However, a deficiency in either one of the degrading enzymes results in a new steady state, leading to a marked accumulation of glycogen in affected red blood cells.
The accumulation of glycogen does not lead to major changes in the red blood cell. Erythrocyte survival, reticulocyte counts, and osmotic fragility are within the normal range. The glycolytic activity both along the Embden Meyerhof pathway and the phosphogluconic shunt are slightly increased.6

The prevalence of cases with amylo-1,6-glucosidase deficiency in Israel and with phosphorylase deficiency in Sweden provided an opportunity to study glycogen metabolism in red blood cells from these patients.

MATERIALS AND METHODS

The various enzymes, coenzymes, and glycolytic intermediates were obtained from Boehringer, Mannheim, Germany, or Sigma Chemicals, St. Louis, Mo.

Diazyme reagent was obtained from Miles Chemicals, Elkhart, Ind. Radioactive 14C-U-glucose and UDPG (U14C) were obtained from the Radiochemical Center, Amersham, England.

Preparation of Erythrocytes

Fresh blood was obtained from healthy laboratory personnel or patients with deficiencies within the amylo-1,6-glucosidase or phosphorylase system diagnosed by accepted enzymatic methods.7,8 The blood samples were drawn into heparinized tubes and the red blood cells (RBC) sedimented by centrifugation in the cold. After aspiration of the supernatant plasma and buffy coat, the remaining blood was pressed twice through a cotton sieve, as described by Busch.9 With this technique 95% of all thrombocytes and 99% of all leukocytes are removed. The red blood cells were subsequently washed three times with 10 volumes of cold 0.15M NaCl before their use for various studies.

Analytic Methods and Incubation Conditions

Glucose-6-phosphate dehydrogenase activity was measured according to Beutler.10 Glucose was determined by the glucose oxidase method.11,12 Maltose was measured by the Somogy and
Glycogen metabolism

Nelson method. Erythrocyte glycogen concentration was determined by the addition of two volumes of 20% trichloroacetic acid to 2 ml of washed RBC. Glycogen was extracted from the supernatant and determined as described by Steinitz. Erythrocytes were separated according to their density by centrifugation of washed RBC at 4°C, 25,000 g for 60 min. The upper layer was obtained by careful aspiration of the (approximately 10%) top fraction of spun RBC. The lower layer was obtained by the free flow of the (approximately 10%) lowest fraction from the bottom of punctured centrifuge tube.

\(^{14}\)C-U glucose incorporation into glycogen was measured in an incubation mixture prepared by the addition of two volumes of reaction mixture containing 135 mM glycyl-glycine buffer, pH 7.8, 2mM glucose, 2.5 µCi \(^{14}\)C-U glucose to one volume of packed RBC. This mixture was incubated in a shaking water bath (37°C). The reaction was stopped by the addition of trichloroacetic acid and carrier glycogen, and the polysaccharide was isolated as previously described. Radioactivity measurements were made with a Packard Tricarb liquid scintillation spectrometer.

Analysis of the distribution of \(^{14}\)C-U glucosyl units between the outer branches and the limit dextrin fraction of the glycogen was carried out after β-amylolysis. Maltose, liberated by β-amylase, was separated from other compounds by descending paper chromatography on Whatman No. 1 paper. The solvent used was butanol-pyridine-water (3:0.2:5:1.5 by v/v).

Conditions of the chase experiment included initial incubation of \(^{14}\)C-U-glucose, as described above. After 1 hr of incubation, RBC were washed in 0.15M NaCl and reincubated in an identical incubation mixture as before, but without added radioactivity.

Glycogen synthetase activity was determined by incubating 0.5 ml hemolysate (prepared by the addition of equal volumes of water to packed RBC) in a final volume of 1.5 ml containing 1 µmoles UDP-(U-\(^{14}\)C) glucose (7000 counts per minute), 8 mg glycogen, 40 µmoles glycyl-glycine buffer, pH 7.5, 10 µmoles EDTA, and 10 µmoles glucose-6-phosphate. Incubation was performed at 37°C for 30 min. The reaction was stopped with 20% trichloroacetic acid, and the glycogen was isolated and its radioactivity determined as described above.

RESULTS

Figure 2 shows that red cell glycogen concentration was above normal in all cases of amylo-1,6-glucosidase and phosphorylase deficiency, though individual differences were noticeable. This deposition of glycogen was not only detectable by enzymatic methods, but could also be visualized by electron microscopy (Fig. 3), in which glycogen particles represented by black dots were seen in all red blood cells.

It had been previously assumed that the glycogen present in this cell repre-
Fig. 3. Electron microscopic picture of characteristic black dots representing glycogen particles in red blood cells of a patient with GSD III A, compared to normal B. Magnification × 50,000.
in a vestigial, metabolically inactive form, residing in the cell from an early developmental stage. In order to test this hypothesis, two experiments were performed. Glycogen content was measured in amylase-1,6-glucosidase-deficient erythrocyte populations of different cell age separated by centrifugation. Glucose-6-phosphate dehydrogenase activity, known to decrease with cell age, served as an indicator of the effectiveness of cell separation. Figure 4 shows that glycogen concentrations are higher in the cell fraction containing predominantly younger cell populations than in the higher-density fractions containing mainly older cells, in which, however, glycogen was still demonstrable.

In addition, the erythrocyte glycogen concentration was examined in three cases of amylase-1,6-glucosidase deficiency both in the fasting and in the post-absorptive state. In the erythrocytes of one of these patients, marked changes in glycogen levels were observed, as shown in Table 1.

In view of these findings and our previous observation that amylase-1,6-glucosidase-deficient cells show a progressive decrease in the length of the outer branches on incubation in the absence of substrate, it became apparent that erythrocyte glycogen undergoes dynamic changes in response to various conditions.

Table 1. Erythrocyte Glycogen Content in a Case of Amylase-1,6-glucosidase Deficiency in the Fasting and the Post-absorptive State

<table>
<thead>
<tr>
<th>Condition of Patient</th>
<th>Erythrocyte Glycogen Concentration (µg/g HB)</th>
<th>Blood Glucose Concentration (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>164</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>118</td>
<td>40</td>
</tr>
<tr>
<td>Postabsorptive</td>
<td>600</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>406</td>
<td>90</td>
</tr>
</tbody>
</table>
tions. It was, therefore, decided to study several parameters of glycogen synthesis and breakdown in greater detail.

A similar incorporation rate of glucose-$^{14}$C into erythrocyte glycogen was observed in normal, amylase-1,6-glucosidase-, and phosphorylase-deficient cells during the first hour of incubation. Subsequently, the labeling of glycogen slowed down and eventually declined in normal and amylase-1,6-glucosidase-deficient cells, whereas a different pattern was observed in phosphorylase-deficient cells: Incorporation rates were characterized by a continuous rise in the radioactivity of erythrocyte glycogen, which came to a plateau only after the exhaustion of substrate (Fig. 5). It had been previously shown in normal red blood cells that radioactive glucose was predominantly incorporated into the outer branches, indicating that this is the mobile fraction of the erythrocyte glycogen molecule. A similar distribution of radioactivity within the glycogen molecule was found in amylase-1,6-glucosidase-deficient cells after exposing the radioactive glycogen extracted from erythrocytes to $\beta$-amylolysis (Table 2).

Another indication that the metabolic activity resided mainly in the outer branches was obtained from the chase experiment. After 1 hr of incubation with

### Table 2. Distribution of Incorporated $^{14}$C-U-Glucose Between Outer Tiers and Limit Dextrin Fractions in Red Blood Cells of Normal and GSD Type III Patients

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$C-U-Glucose ($\mu$moles/gHb) Incorporated Into</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycogen</td>
<td>Outer Branches</td>
</tr>
<tr>
<td>Cont. 1</td>
<td>17.3</td>
<td>16.9</td>
</tr>
<tr>
<td>Cont. 2</td>
<td>16.4</td>
<td>14.8</td>
</tr>
<tr>
<td>A.Y.</td>
<td>16.83</td>
<td>16.34</td>
</tr>
<tr>
<td>A.J.</td>
<td>16.1</td>
<td>15.3</td>
</tr>
<tr>
<td>B.Y.</td>
<td>15.9</td>
<td>14.1</td>
</tr>
</tbody>
</table>

Incorporation of $^{14}$C-U-glucose and subsequent separation of outer branches from the limit dextrin fractions were performed as described in Materials and Methods.
GLYCOGEN METABOLISM

Reincubation with glucose-U-14C followed by reincubation with cold glucose. Pulse studies of incorporation into glycogen in normal and amylo-1,6-glucosidase-deficient patients. Incubation condition is the same as in Methods.

14C-U-glucose, the erythrocytes were washed and reincubated with nonlabeled glucose. The rate of decrease of radioactivity in erythrocyte glycogen was similar in amylo-1,6-glucosidase-deficient and in normal cells (Fig. 6).

Glycogen synthetase activity was studied in normal and amylo-1,6-glucosidase-deficient cells. In both types of cells, the enzyme was strictly glucose-6-phosphate dependent, and no interconversion to another form with different kinetic properties was demonstrable in this tissue.

The maximal glycogen synthetase activity (Vmax, 1.5 ± 0.3 μmole/g Hb/hr), the Km for UDPG (0.2 mM), and the Ka for glucose-6-phosphate (6.31 mM) in amylo-1,6-glucosidase cells did not differ from normal erythrocytes.3

Both in normal and in amylo-1,6-glucosidase-deficient erythrocytes, glycogen synthetase activity was glycogen concentration dependent, reaching a plateau at glycogen concentrations in excess of 5 mg/ml. An exception was found in two cases, belonging to the same family, whose red cell glycogen synthetase activity was low in the absence of added glycogen, and was only slightly increased by raising the glycogen concentration from 1 to 10 mg/ml (Fig. 7).

DISCUSSION

The marked variations in glycogen content in the various cases studied have been documented before by Sidbury and Cornblath.1 These variations cannot be explained by differences in the rate of incorporation of glucose into glycogen or the activity of glycogen synthetase. The effect of blood glucose levels on erythrocyte glycogen concentration found in one case, but not in others, can serve as an indication for the wide variability in the dynamics of RBC glycogen metabolism encountered among cases of amylo-1,6-glucosidase deficiency. It would seem that at least in some cases erythrocyte glycogen can function as a carbohydrate store which can respond to metabolic demands imposed on the erythrocyte by providing the cell with glucosyl moieties for energy metabolism.
Labeling of erythrocyte glycogen in cells incubated in the presence of radioactive glucose resulted in both amylo-1,6-glucosidase-deficient cells and in normal cells in a curve demonstrating two kinetic patterns. An initial linear increase of radioactivity, representing mainly synthesis of the radioactive glucosyl moieties into the glycogen molecule, and a plateau, reached after 2 hr, indicate a steady state between glycogen synthesis and breakdown. The decrease of radioactivity observed after 3 hr is a result of exhaustion of substrate.

It is evident from the chase study that the rate of glycogen breakdown found in amylo-1,6-glucosidase-deficient cells was similar to the rate observed in normal cells. The distribution of label indicated that during the short-term experiments most metabolic activity was confined to the outer branches in both normal and amylo-1,6-glucosidase-deficient cells. It was, therefore, not surprising to find similar kinetics in both types of cells. In order to explain the presence of glycogen accumulation in debrancher-deficient erythrocytes in spite of normal activity of glycogen synthetic enzymes and a normal rate of incorporation of glucose into glycogen, the following in vivo mechanism can be postulated: During the synthetic phase an outer tier elongates on which the brancher enzyme forms a new outer branch. This new branch cannot, in view of the debrancher enzyme defect, be broken down. As a consequence, a slowly progressive irreversible incorporation of glucosyl units into the glycogen core in vivo occurs; this incorporation is not reflected in the kinetic pattern of the short-term in vitro experiments herein described. In contrast, different kinetics were expected and observed in phosphorylase-deficient cells. The continuous linear increase in radioactivity during the whole period of the experiment until the exhaustion of substrate became limiting represented the synthetic phase only, since phosphorylase deficiency prevented any breakdown of the glycogen molecule.

The kinetics of glycogen synthetase indicate that, as in normal cells, the
absolute dependency of enzyme activity on glucose-6-phosphate limits the metabolic capacity of this pathway. Thus, glycogen does not accumulate in red blood cells unless a genetic deficiency of one of the degradative enzymes exists, leading to the accumulation of this polysaccharide.

The kinetics of glycogen synthetase did not differ between normal and amylase-1,6-deficient cells. An exception was found in two cases belonging to one family who presented with a different kinetic pattern of glycogen synthetase. However, the endogenous concentrations of erythrocyte glycogen even in glycogen-rich erythrocytes is less than 1 mg/ml RBC, this abnormal kinetic pattern is not expected to affect the in vivo incorporation rate of glucose into glycogen in these cells. Whether this represents a primary double-enzyme defect or some secondary effect of the debrancher enzyme deficiency on the glycogen synthetase is not clear. Such an occurrence is not unique in this disease since in amylase-1,6-glucosidase-deficient patients a regulatory deficiency of liver glucose-6-phosphatase has been described. However, the nature of the effect of one enzyme deficiency on other enzymes remains to be elucidated.

The small concentration of glycogen found in normal red blood cells and the markedly rudimentary pattern of this cell in respect to several metabolic pathways justify the question of whether the active glycogen metabolism present in this cell utilizes all the normal enzymatic reactions of glycogen synthesis and breakdown.

From the study of various types of enzyme-deficient cells the following conclusion can be drawn. The fact that the initial rates of incorporation of $^14$C-U-glucose into glycogen were similar in normal, debrancher, and in phosphorylase-deficient cells suggests that in the erythrocyte the incorporation of glucose into glycogen proceeds via the glycogen synthetase step and is not a result of reversal of either the amylase-1,6-glucosidase or the phosphorylase-catalyzed reactions. Similarly, the different kinetics of glycogen label in phosphorylase-deficient cells serves as an indirect indicator that glycogen breakdown proceeds in the normal cell via the phosphorylase step and not by alternative routes. These data represent an example whereby enzyme-deficient red blood cells can serve as a model providing information about the normal pathway of glycogen metabolism in this tissue.

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