

Glycogen Metabolism in the Normal Red Blood Cell

By Shimon W. Moses, Nava Bashan, and Alisa Gutman

Evidence for active glycogen metabolism in normal mature red blood cells (RBC) is presented. Initial rates of ^{14}C -U-glucose incorporation into erythrocyte glycogen were found to be independent of substrate concentration over a range of 3.3–16.6 mM. Incorporation of label into glycogen was initially linear but reached a plateau after a variable period of time that was inversely related to RBC concentration in the medium. The major part of the incorporated radioactivity resided in the outer branches of the glycogen

molecule. The optimum pH for ^{14}C -U-glucose incorporation into glycogen was pH 7.6. Replacing the radioactive glucose employed for incorporation after 1 hr of incubation with nonlabeled glucose resulted in a gradual loss of radioactivity from erythrocyte glycogen. In normal cells, glycogen synthesis and breakdown do not result in any significant accumulation of glycogen, whereas in erythrocytes with enzyme defects affecting glycogen breakdown, substantial deposition of glycogen may be observed.

THE MATURE HUMAN ERYTHROCYTE depends on environmental glucose to meet its energy requirements.^{1,2} The normal red blood cell (RBC) has no significant glycogen stores,³ although enzymes catalyzing glycogen synthesis and breakdown have been shown to be present.⁴⁻⁸ In contrast, glycogen deposition has been demonstrated in erythrocytes of patients affected with certain types of glycogen-storage disease (type III and type VI).^{9,10} Sidbury et al. assumed that the glycogen in affected red blood cells is a vestigial remnant from an early stage in the development of the erythrocyte that, in view of the absence of an active glycogen metabolism, is not broken down to any significant degree in the mature cell.⁹ It is also possible that the normal mature erythrocyte maintains an active glycogen metabolism in which the steady state favors, in the presence of normal enzyme activities, glycogen breakdown, whereas glycogen accumulates unless either amylo-1,6-glucosidase or phosphorylase is missing.

The present study was undertaken to investigate glycogen metabolism in the mature red blood cell, measuring rates of glycogen synthesis and degradation under varying incubation conditions and determining the distribution of radioactivity within the glycogen molecule after incorporation of radioactive glucose into glycogen.

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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 ^{14}C -U-glucose was obtained from the Radiochemical Center, Amersham, England.

Preparation of Erythrocytes

Fresh blood was obtained from healthy laboratory personnel. The blood samples were drawn into heparinized tubes, and the erythrocytes (RBC) were sedimented by centrifugation in the cold.

After aspiration of the supernatant and the buffy coat, the remaining blood was pressed through a cotton-wool sieve as described by Busch and Pelz.¹¹ Two passages of the red blood cells suspension through the syringe containing the cotton wool soaked in 0.15 M NaCl solution were found to remove 99% of leukocytes and 98% of thrombocytes originally present.

The erythrocytes subsequently were washed three times with 10 vol of cold 0.15 M NaCl before their use in the various studies.

Analytical Methods

Glucose was determined by the glucose oxidase method.^{12,13} Maltose was measured by the Somogy and Nelson method,¹⁴ and glycogen was measured with diazyme reagent.¹⁵

^{14}C -U-Glucose incorporation into glycogen was measured in an incubation mixture containing 30% RBC suspension, 6.6 mM glucose, 2.5 μCi ^{14}C -U-glucose, 15 mM NaHPO_4 , 120 mM glycylglycine buffer, pH 7.8, in a final volume of 6 ml. This mixture was incubated in a shaking water bath at 37°C. The reaction was stopped by the addition of trichloroacetic acid and carrier glycogen, and the polysaccharide was isolated as previously described.¹⁶ Radioactivity measurements were made with a Packard Tricarb liquid scintillation spectrometer.

Analysis of the distribution of ^{14}C -U-glycosyl units between the outer branches and the limit dextrin fraction of the glycogen was carried out after β -amylolysis.¹⁷

Maltose, liberated by β -amylase, was separated from other compounds by descending paper chromatography utilizing Whatman No. 1 paper. The solvent used was butanol-pyridine-water (3:2.5:1.5 by v/v).

Separation of glycogen from other polysaccharides was performed as follows: after ^{14}C -U-glucose was incorporated into intact RBC as described above, an equal volume of 6% HgCl_2 was added to precipitate the protein. Nonlabeled glycogen was added to the supernatant. The total glycogen was subsequently extracted by repeated alternate precipitations with alcohol and resuspension in water as previously described.⁷ The polysaccharide suspension was subsequently run through a Biogel-200 column. Eluting phase was water. Glycogen and radioactivity were determined in each of the 2-ml fractions collected by a fraction collector.

RESULTS

Incorporation of Glucose Into Glycogen

Incubation of normal RBC with ^{14}C -U-glucose resulted in the appearance of a radioactive material. This material was identified as glycogen by the appearance of radioactivity in a fraction that sedimented at 60% ethanol concentration. This material was further characterized by separation on a Biogel-200 column (Fig. 1), which showed that the peaks of the radioactive polysaccharide extracted from erythrocytes were identical with the peaks of the nonlabeled glycogen added. In addition, most of the radioactivity incorporated could be recovered as maltose on β -amylolysis (Fig. 2).

The rate of incorporation of radioactivity of ^{14}C -U-glucose into erythrocyte glycogen was 0.04 ± 0.01 μmoles glucose incorporated/g Hb per hr. This rate could not have been due to contamination of the RBC by leukocytes or thrombocytes. The maximum rate of glucose incorporation attributable to the number of leukocytes and thrombocytes remaining in the cell suspension after the removal procedure would have been 0.05 $\text{m}\mu\text{moles}^{18}$ and 0.03 $\text{m}\mu\text{moles}$, respectively.¹⁹ The initial linear slope represents the net rate of the incorporation reaction. As shown in Fig. 2, the curve approaches a plateau after 2 hr, indicating a steady state that is the result of an equilibrium state between the rates of incorporation into and loss of radioactivity from glycogen. On examining the distribution of radioactivity within the glycogen molecule, an increase of radioactivity is observed mainly in the outer branches and to a small extent (about 10%) in the limit dextrin fraction (core). To exclude the possibility that the radioactivity detected in the core resulted from physical trapping of maltose within the glycogen molecule during the chromatographic separating procedure, radioactive maltose was first mixed with cold glycogen, and subsequently the mixture was carried through a similar chromatographic procedure. No radioactivity was found in the glycogen and all radioactivity was detected in the maltose spot.

No difference in the initial rate of incorporation of ^{14}C -U-glucose into glycogen was observed when medium glucose concentration was varied from 3.3 to 16.6 mM (Fig. 3). After 2-hr incubation in the low glucose medium (3.3 mM), glucose had almost disappeared (as shown in the lower part of the figure), and a progressive decrease in radioactivity from erythrocyte glycogen was noted.

Further evidence for the dynamic nature of glycogen metabolism in red blood cells was obtained in experiments that showed that replacement of radioactive glucose by nonlabeled glucose after 1 hr of incubation resulted in a gradual decrease of radioactivity from RBC glycogen (Fig. 4).

The incorporation of glucose into glycogen was shown to be pH dependent, maximum activity being reached at pH 7.6, which is very near to the pH maximum for glycolytic activity.

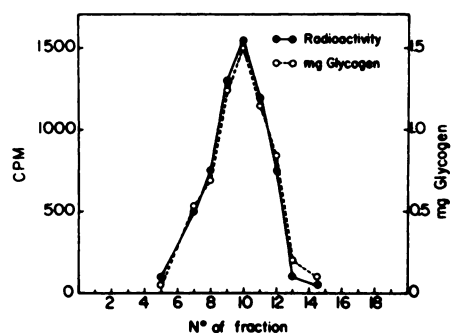


Fig. 1. Comparison of peaks of added cold glycogen with radioactive erythrocyte glycogen after passage through Biogel-200 column. Incubation of RBC ^{14}C -U-glucose was performed as described. After denaturation with cold TCA, 20 mg of glycogen were added to supernatant. Glycogen was separated from radioactive glucose by repeated precipitations with alcohol. Precipitated glycogen was resuspended in 5 ml of water and applied to a Biogel-200 column 60×3 elution phase-water. Samples of 2 ml were collected in which radioactivity and glycogen were determined.

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It is apparent from Fig. 5 that at low concentrations of RBC in the incubation medium the incorporation rate of ^{14}C -U-glucose into glycogen was linear with time up to 2 hr. However, when the concentration of cells was increased, the rate of incorporation tended to decrease as a function of time. This phenomenon could not be ascribed to depletion of substrate or other changes in the composition of the incubation medium, since replacing the incubation mixture with a fresh one had no effect on this phenomenon, whereas incubating fresh cells in an incubation medium in which other cells had previously been incubated resulted in a normal rate of incorporation.

Fig. 2. Distribution of incorporated ^{14}C -U-glucose between outer tiers and limit dextrin fraction. Aliquots of glycogen were exposed to β -amylolysis. Subsequently, separation of outer branches from the limit dextrin fractions (core) was performed by paper chromatography, and radioactivity of the various fractions was determined.

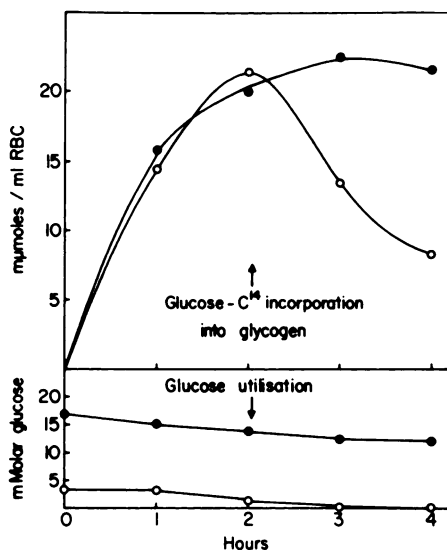
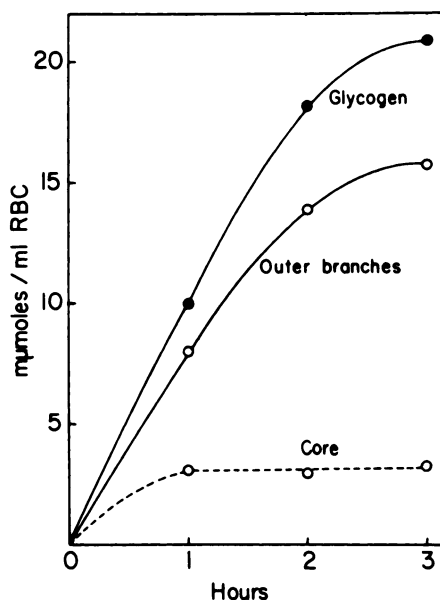


Fig. 3. ^{14}C -U-Glucose incorporation into glycogen and glucose utilization with two substrate concentrations. One milliliter of RBC was suspended in 2 ml of incubation medium containing 3.3 (open circles) or 16.6 (closed circles) mM glucose. Samples of medium were removed at specified intervals and were analyzed for glucose and for the radioactivity incorporated into glycogen.

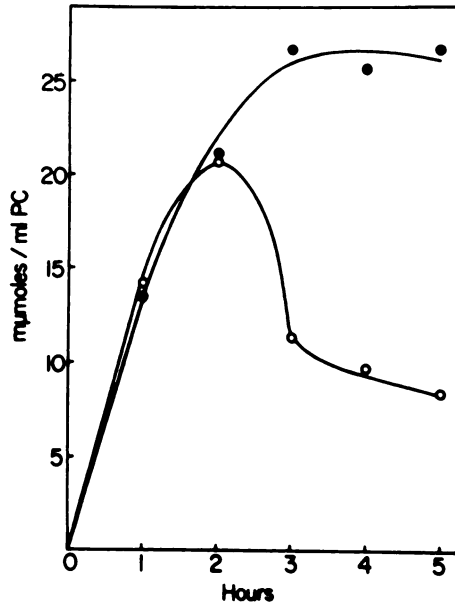
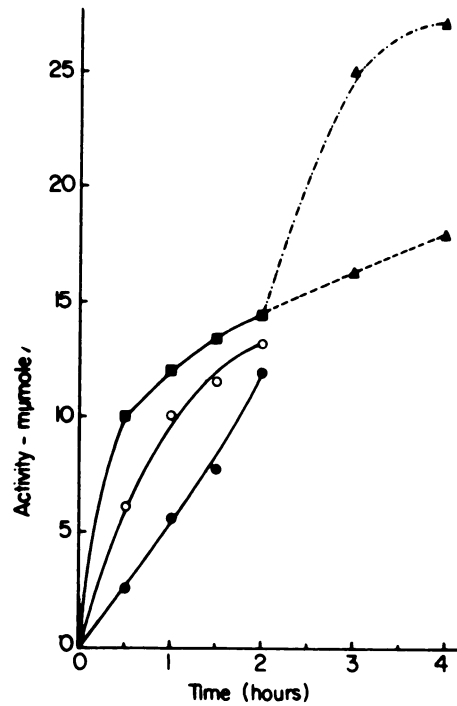


Fig. 4. Pulse studies of glucose incorporation into glycogen in normal erythrocytes. Initial incubation condition as in Methods. After 1 hr of incubation, red blood cells were washed in 0.15 *M* NaCl and reincubated in an incubation mixture as before, but without added radioactivity. Open circles represent cells reincubated in non-radioactive incubation mixture. Black dots represent controls.

Fig. 5. Effect of RBC concentration on rate of glucose incorporation into glycogen. RBC suspensions of different concentrations were incubated with ^{14}C -U-glucose. After 2 hr, cells from tube containing highest RBC concentration were separated from medium and washed with cold 0.9% NaCl solution. (A) Cells were subsequently reincubated in fresh incubation medium. (B) Separated incubation medium was used for incubation with fresh RBC. Black circles, 0.5 ml RBC; open circles, 1.0 ml RBC; black squares; 2.0 ml RBC. (A), - - - - -; (B) - · - · - · - · - · - ·. Final volume of incubation mixture was 4 ml. Composition of incubation mixture as described in methods.



To establish that the glucose incorporation into glycogen is also a function of the mature erythrocyte and not only of reticulocytes present in the red blood cell suspension, the following experiments were performed: red blood cell suspension was exposed to centrifugation for 1 hr in order to separate the younger erythrocytes, which are known to be of lower density, from the older cell population. The fraction of younger cells in which high reticulocyte counts were observed incorporated ^{14}C -U-glucose into glycogen more readily than higher density RBCs comprising the older cells. However, even this last fraction that was devoid of reticulocytes did show incorporation, indicating that the capacity of incorporation is not only a function of reticulocytes but of mature red blood cells, as well (Table 1). Further evidence to support this conclusion was presented by incorporation studies performed on blood stored for 24 hr in ACD in which no reticulocytes were found; yet this stored red blood cell suspension had the capacity to incorporate glucose into glycogen.

DISCUSSION

This report presents evidence indicating that the mature erythrocyte maintains an active glycogen metabolism that can not be attributed to other formed elements present in the blood. Both column separation experiments and enzymatic breakdown studies indicate that the incorporation of radioactive glucose is indeed into glycogen and not into other components of the cell.

Most of the label is found in the outer tiers of the glycogen molecule, whereas the limit dextrin fraction (core) contains only small amounts of radioactivity. The initial rate of disappearance of radioactivity from erythrocyte glycogen is similar to the rate of incorporation, as shown by the pulse experiment (Fig. 4).

The steady state, reached after an initial linear increase of ^{14}C -U-glucose incorporation into glycogen, represents an equilibrium between entry and exit of radioactive glycosyl units from erythrocyte glycogen. The plateau is approached after 30 min of incubation in the presence of 50% RBC concentration, whereas with 12.5% RBC concentration incorporation is linear as a function of time for 2 hr. The factors responsible for this phenomenon

Table 1. Rates of Incorporation of ^{14}C -U-Glucose Into Glycogen in Red Blood Cells Separated According to Their Relative Densities or Stored

	Reticulocytes (%)	Rates of Incorporation Activity (m μ mole/ml RBC/hr)
Unspun RBC	1.3	20.1
Upper layer of spun RBC*	3.9	42
Lower layer of spun RBC*	0	12.5
RBC stored for 24 hr in ACD	0	12.1

*Centrifugation was performed at 4°C, 25,000 g for 60 min. Upper layer was obtained by careful aspiration of approximately 10% of top fraction of spun RBC. Lower layer was obtained by free flow of lowest fraction from bottom of punctured centrifugation tube.

are not immediately apparent. The observation that the time required to reach a plateau was a function of RBC concentration does not support the possibility that a time-dependent running down of the synthetic mechanism of the cells was responsible for the observed phenomenon.

The fact that most of the radioactivity resided in the outer tiers of the glycogen molecule suggests that under the conditions of the experiment very little branching of the newly formed chains took place. The limitation imposed on *de novo* glycogen synthesis by the rate of branching may possibly contribute to the slowing down of synthesis but can not explain the dependence of this phenomenon on the concentration of RBC in the incubation mixture.

The accumulation of an inhibitory factor in the incubation medium could not be implicated in the observed decrease in the rate of incorporation in concentrated cell suspension. It has, therefore, to be assumed that a change in the intracellular environment takes place during incubation that is accelerated by increasing cell concentration. Differences in oxygen saturation of RBC hemoglobin could possibly change intracellular pH and might be an important factor regulating the relative activities of enzymes involved in glycogen metabolism.

The separation and aging studies establish that glycogen metabolism is not only a function of reticulocytes but of mature erythrocytes, as well. This is of special interest in view of the well-known presence of an active glycogen metabolism in young nucleated red cell precursors that seems to be retained in a vestigial yet functional form in the mature red blood cell.

The steady state present in the normal red blood cell does not lead to glycogen accumulation. This may be related to the minimal functional activity of glycogen synthetase,²⁰ in contrast to markedly higher activities of the enzymes catalyzing glycogen breakdown.⁸ In abnormal conditions, namely in the absence of erythrocyte amylo-1,6-glucosidase and of phosphorylase, the normal steady state of glycogen synthesis and breakdown is upset, resulting in glycogen accumulation that may reach several hundred times the normal level.

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