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Enzyme Loading of Erythrocytes

(sphingolipidoses/Gaucher's disease/β-glucocerebroside/β-glucocerebrosidase/spherocytes)

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ABSTRACT **We demonstrated that β-glucosidase and β-galactosidase can be trapped inside erythrocytes by rapid hemolysis of the cells in the presence of these enzymes. Enzyme enters only during hemolysis, and optimum uptake occurs within 60 sec. There is no loss in cell number after hemolysis-induced enzyme uptake, and the ghosts have only a slightly increased mean cell volume. Smaller proteins enter more readily than larger proteins, although enzymes with a molecular weight of at least 180,000 can be readily entrapped by erythrocytes. This finding may provide a useful approach to the problem of enzyme replacement in certain diseases, including Gaucher's disease.**

Enzyme therapy for certain diseases has attracted increasing attention recently. For example, modest success in treatment of certain asparaginase-sensitive leukemias (1) has been achieved by parenterally administered asparaginase. In addition, several human diseases are caused by the partial or complete absence of a particular enzyme activity. Presumably the consequences of these diseases could be alleviated if replacement enzymes could be introduced into such individuals to catalyze the missing reaction.

However, the injection of free enzyme may be unsatisfactory in general because of the possibility of either an unfavorable immune response or because the enzyme will be rapidly cleared from the blood. An appreciation of these difficulties has led to the notion of encapsulating enzymes in some kind of semipermeable envelope that would sequester or retain enzyme while allowing substrates to enter (2). An additional problem in some cases may be the requirement that the enzyme reach an intracellular substrate. For example, Gaucher's disease is characterized by an accumulation of β-glucocerebroside in cells of the reticuloendothelial system, including spleen, kidney, and bone marrow. The disease is due to an inherited deficiency in the enzyme β-glucocerebrosidase (3); membranous, glucocerebroside-rich storage deposits accumulate as a result of the incomplete catabolism of membrane glycolipids of erythrocytes and leukocytes (4, 5).

We have found that β-glucosidase and β-galactosidase can be trapped within sealed erythrocyte ghosts. When a hypotonic solution is added to erythrocytes, they hemolyse, and soluble intracellular contents including hemoglobin leave the cell. Depending upon conditions, the cell may be either reversibly or irreversibly ruptured. In the reversible case, pores of sufficient size to allow the escape of hemoglobin molecules open in the membrane and then close again. The resulting erythrocyte ghost is osmotically competent (6). These properties suggested to us that soluble enzyme might be able to enter the cell while the pores are open, providing a way of trapping the enzyme inside the cell. We suggest that cells loaded with β-glucocerebrosidase might be useful for treatment of Gaucher's disease because the erythrocytes will be phagocytized, thus introducing the enzyme into cells of the reticuloendothelial system.

Abbreviations: MeUGal, 4-methyl umbelliferyl-β-D-galactopyranoside; MeUGlu, 4-methyl umbelliferyl-β-D-glucopyranoside.

MATERIALS AND METHODS

Determination of Enzyme Uptake by Erythrocytes. Fresh erythrocytes from one of us (R.H.G.) were washed four times (1000 × *g*, 5 min) with a 0.9% NaCl solution. An aliquot (0.02 ml) of the packed cells was diluted with 0.20 ml of soluble β-galactosidase and β-glucosidase of rat kidney (glycosidase preparation). After 5 min at 22°, 0.9-ml aliquots of various sodium chloride solutions (0–0.9%) were added to the erythrocyte–enzyme suspension; after 30 sec, 5 ml of 1.2% NaCl solution was rapidly pipetted into the medium. Erythrocytes, recovered as a pellet after five washings with 6.5 ml of 0.9% NaCl solution, were finally lysed by suspension in 0.3 ml of distilled water. Enzyme uptake by cells was measured by assay of aliquots (20–40 μl) of the lysate for β-galactosidase or β-glucosidase activity with the synthetic substrates 4-methyl umbelliferyl-β-D-galactopyranoside (MeUGal) and 4-methyl umbelliferyl-β-D-glucopyranoside (MeUGlu), respectively, by the fluorescent assay (7). Incubations were at 37° and contained the following components in a final volume of 0.08 ml: MeUGlu or MeUGal (0.625 mM), cutscum (Fisher) (0.6%), and sodium acetate (pH 5.9, 0.125 mM). Assays were done for 15–20 min and were linear with time and enzyme concentration. One unit of enzyme is defined as that quantity of enzyme that hydrolyzes 1 nmol of substrate per hr.

Preparation of Rat Kidney Glycosidase. A soluble preparation of β-galactosidase and β-glucosidase was obtained as follows: rat kidneys were homogenized for 30 sec in a Waring Blendor with eight volumes of ice–cold medium containing 0.30 M sucrose, 0.05 M Tris·HCl (pH 7.5) and 0.3 mM EDTA. The resulting homogenate was then centrifuged for 60 min at 80,000 × *g*. The high-speed supernatant fraction was dialyzed exhaustively against 0.9% NaCl at 4°.

Protein Was Determined by the procedure of Lowry *et al.* (8), with human-serum albumin as a standard.

Preparation of ^{35}S-Labeled Escherichia coli Proteins. Logarithmic phase *E. coli* (K12) cells were grown at 37° for two generations in a sulfur-deficient medium containing the following components in 1 liter: 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 3 g of

TABLE 1. *Hemolysis-induced uptake of glycosidases by erythrocytes*

	β-Glucosidase		β-Galactosidase	
Salt concentration (%)*	0.2	0.9	0.2	0.9
Amount of enzyme (units)				
Added	6650	6650	3340	3340
Cell uptake	15.8 (0.24%)§	<0.5	6.79 (0.20%)	<0.15
Enzyme concentration† (units/ml)				
Added	2200	2200	1112	
Inside cell‡	627 (29%)	<20	270 (24%)	<6
Hemolysis (%)	86	3–5	86	3–5

Washed erythrocytes (0.02 ml, 15.8 mg of protein per ml) were subjected to hemolysis with 0.2% NaCl solution, and enzyme uptake was determined. Hemolysis was determined by absorbance at 550 nm after removal of erythrocyte ghosts by centrifugation. Physiological saline (0.9% NaCl) was used in place of 0.2% NaCl solution as a control.

* NaCl concentration (%) at the time of hemolysis; 0.9% saline solution served as a control in which <5% hemolysis occurred.

† Enzyme concentration (units/ml) at the time of hemolysis.

‡ Calculated assuming a mean cell volume of 140 μm^3 (see Fig. 2) and 180,000 erythrocytes per incubation.

§ The numbers in parentheses indicate percent uptake and equilibration of enzyme relative to added enzyme.

NaCl, 20 g of glycerol, 60 mg of $MgCl_2 \cdot 6H_2O$, 7 mg of $MgSO_4$, and 2 g of NH_4Cl. The medium was supplemented with $Na_2{}^{35}SO_4$ (4 mCi/liter). Washed cells were lysed by sonication. After centrifugation (60 min, 100,000 × *g*) and ammonium sulfate fractionation (0–60% saturation), soluble proteins were subjected to chromatography on DEAE-cellulose (10 mM Tris·HCl–5 mM $MgCl_2$, pH 7.4). Labeled proteins eluting with 0.05–0.14 M KCl were dialyzed exhaustively against 0.9% NaCl.

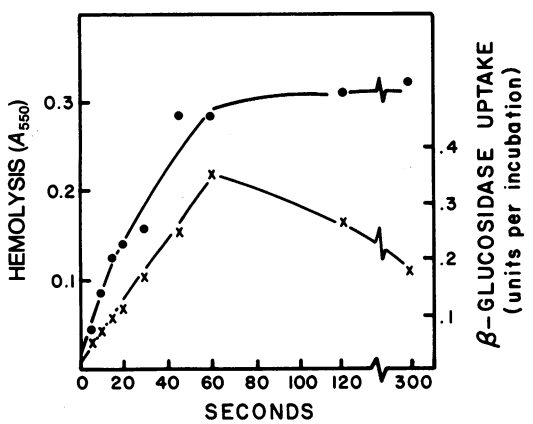

FIG. 1. Time-course of uptake of β-glucosidase by erythrocytes. Erythrocytes were subjected to hemolysis in hypotonic saline (0.2% NaCl) in the presence of the glycosidase preparation (2.2 mg of protein per ml) for various periods of time. The NaCl concentration at the time of hemolysis was 0.26%. Enzyme uptake (X——X) is expressed as units of β-glucosidase taken up per incubation; hemolysis (●——●) was measured by absorbance at 550 nm after removal of erythrocyte ghosts by centrifugation.

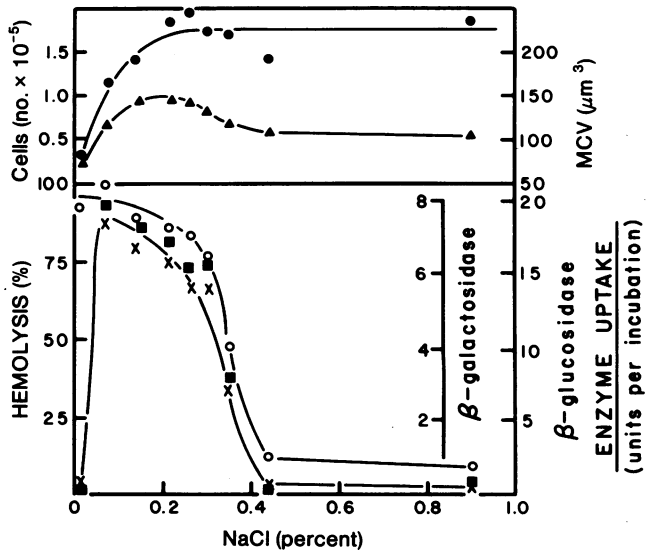

FIG. 2. Effect of NaCl concentration on uptake of enzyme by erythrocytes. Erythrocytes were suspended in the glycosidase preparation and subjected to hemolysis for 30 sec with various concentrations of NaCl solution as described in Table 1. Enzyme uptake was determined. β-glucosidase (×——×); β-galactosidase (■——■). Hemolysis (○——○) was determined as described in Table 1. Number of cells (●——●) and their mean cell volume (*MCV*, ▲——▲) were obtained with a model S Coulter counter.

RESULTS AND DISCUSSION

Hoffman (6), Baker (10), Seeman (11), and Marsden and Ostling (12) have shown that high-molecular-weight substances, including hemoglobin, ferritin, dextran, and colloidal gold, can enter erythrocytes during hemolysis. These workers proposed that during hemolysis pores transiently open in the erythrocyte membrane, allowing two-way exchange of material. After hemolysis, the pores close and the resulting hemoglobin-deficient ghosts return to an osmotically competent state in which high-molecular-weight substances are excluded. Electron microscopic examination of the resultant ghosts provided direct evidence that ferritin and colloidal gold were sequestered inside the cell. Also, electron micrographic evidence for the existence of 200–500 Å transient pores was obtained by Seeman (11), who fixed erythrocytes with glutaraldehyde during hemolysis.

We have extended this work by demonstrating that enzymes can be entrapped within erythrocytes and that they remain active after the cells are lysed. Both β-glucosidase and β-galactosidase are entrapped only when hypotonic medium (0.2% NaCl) is added to the suspension of cells and enzyme (Table 1). Negligible enzyme uptake is observed when physiological saline solution is substituted for hypotonic salt solution. The trapped enzymes are not removed when the cells are washed repeatedly (seven times) with physiological saline, suggesting the they are not merely adsorbed to the surface of the cell. Furthermore, when the enzyme-loaded cells are completely lysed by transfer to distilled water, more than 99% of the β-galactosidase and glucosidase activity is recovered in soluble form in the supernatant fraction obtained after centrifugation for 15 min at 15,000 × *g*; the pellet fraction

containing erythrocyte membrane is devoid of the two glycosidase activities. This conclusion is in agreement with the observations of Baker (10) and Seeman (11), who directly demonstrated by electron microscopy that ferritin was entrapped with erythrocytes during hemolysis.

Entry of β-glucosidase parallels the escape of hemoglobin (Fig. 1) for 60 sec after addition of hypotonic medium. Maximum enzyme uptake occurs at 60 sec, after which time the amount of trapped enzyme decreases. Seeman (11) also observed that maximum uptake of ferritin occurred 60 sec after initiation of hemolysis.

Enzyme entry and hemoglobin escape also proceed in parallel when the cells are added to solutions of decreasing NaCl concentration (Fig. 2). Entry of both enzymes reaches a maximum at 0.12% NaCl and decreases to zero at salt concentrations less than 0.007%, presumably because of irreversible lysis of the cell membrane. Erythrocytes can be loaded with enzymes at salt concentrations as low as 0.18% NaCl without decreasing the number of intact cells. The mean cell volume of the resulting enzyme-loaded cells is increased from 102 to 139 μm^3.

Using extracellular $^{59}Fe^{II}$-labeled hemoglobin, Hoffman (6) showed that after hemolysis, the concentration of labeled hemoglobin was equal in the intracellular and extracellular compartments. The concentration of intracellular enzyme obtained by us is 24–29% of the concentration of extracellular enzyme, after a 30-sec hemolysis time (Table 1), and about 60% under optimal conditions (Fig. 2). Failure to achieve 100% equilibration may be due to the molecular sieving properties of the membrane pores since both β-galactosidase and β-glucosidase have molecular weights of about 180,000, which is considerably larger than that of hemoglobin (68,000). In support of this idea, we find that smaller proteins are in fact preferentially trapped by erythrocytes. Using a crude extract of soluble ^{35}S-labeled *E. coli* proteins, we compared the molecular weight distribution of the added proteins with that of the trapped proteins (Fig. 3). Whereas the added labeled proteins are found predominantly in the 200,000 to 300,000-dalton range by molecular-sieve chromatography, the peak of radioactivity for the trapped proteins is about 90,000 daltons. A similar sizing effect was reported by Marsden and Ostling (12) using dextrans of various molecular weights. Despite this sizing effect, it is possible for proteins of very high molecular weight to enter, since we have trapped *E. coli* β-galactosidase (540,000 daltons) in erythrocytes by this procedure.

We believe that these findings may provide a basis for treatment of various diseases, particularly those of the sphingolipidoses class such as Gaucher's disease in which catabolic enzyme deficiencies exist (13, 14). Gaucher's disease involves accumulation of β-glucocerebroside in cells of the reticuloendothelial system as a consequence of incomplete catabolism of the glycolipids of engulfed leukocytes and erythrocytes. If the enzyme-loaded erythrocyte is sufficiently damaged (deliberately or otherwise) it will be phagocytized relatively quickly, largely by the spleen. Thus the β-glucocerebrosidase is contained in the very cells whose incomplete degradation gives rise to the disease and will concomitantly be delivered to the affected organs. We have estimated that only a small volume of erythrocytes at a normal hematocrit would be required to provide an amount of enzyme equal to that contained in a normal human spleen. We do not yet know the fate of the enzyme after phagocytosis, but we believe that a significant fraction might escape destruction and remain functional within the cell. If so, enzyme therapy might be feasible.

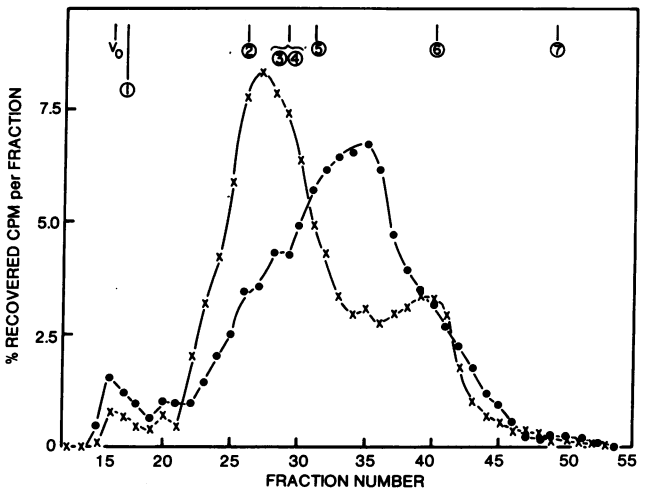

FIG. 3. ^{35}S-Labeled *E. coli* proteins taken up by erythrocytes by gel-filtration chromatography. Washed erythrocytes (0.05 ml, 0.45 × 10^6 cells) were incubated for 3 min with 0.20 ml of a solution of ^{35}S-labeled *E. coli* proteins. The cells were subjected to hemolysis by addition of 0.9 ml of 0.2% NaCl solution. After 30 sec, 4 ml of 1.2% NaCl solution was added. The cells were then washed five times with 7 ml of 0.9% NaCl solution and lysed by suspending the pelleted cells in distilled water. The lysate (●——●), containing 45,000 cpm, was subjected to chromatography on a 1.4 × 44-cm agarose column (Biogel, 1.5 Å) equilibrated with 0.01 M sodium phosphate (pH 7.6), and fractions (50 drops each) were collected. The original solution of ^{35}S-labeled proteins (2.68 × 10^6 cpm) was also passed over the same column (×——×). Recovery of radioactivity from the column was more than 95%. The extent of hemolysis in this experiment was about 60%. Radioactivity measurements were done as described (9). Results are expressed as the percent of radioactivity in each fraction relative to the total amount of radioactivity recovered from the column. The following proteins (2 mg) with the indicated molecular weights were used to calibrate the column: (*1*) *E. coli* β-galactosidase (540,000); (*2*) catalase (244,000); (*5*) human immunoglobulin (160,000); (*6*) human-serum albumin (68,000); and (*7*) equine cytochrome *c* (11,700). Rat kidney β-glucosidase (*3*) and β-galactosidase (*4*) eluted from the column in fraction 29.

Since factors that influence erythrocyte survival have been extensively studied, and because the amount of hemoglobin remaining in the cell can be controlled by addition of extracellular high-molecular-weight substances such as dextran (12), it may be possible to prepare enzyme-loaded cells that will survive in the circulation for extended periods of time. Provided the substrate for a particular enzyme can penetrate the erythrocyte membrane, these cells may also be useful for enzyme therapy in those diseases involving circulating small molecules. We believe enzyme-loaded erythrocytes may have therapeutic possibilities for several diseases.

NOTE ADDED IN PROOF

It should be possible to target erythrocytes for destruction specifically in either the spleen or the liver (and perhaps the kidney as well) by controlled doses of sulfhydryl-reactive reagents or by heating (reviewed by Rifkind, R. A. (1966) *Amer. J. Med.* **41**, 711–722). Thus a certain degree of organ specificity is possible.

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