REFERENCES AND NOTES

1. Beutler, E., Kuhl, W., Trinidad, F., Tepliz, R., and Nadler, H.: β-Glucosidase activity in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. Amer. J. Hum. Gen., *23:* 62 (1971).
2. Brady, R. O., Kanfer, J. N., Mock, M. B., and Fredrickson, D. S.: The metabolism of sphingomyelin. II. Evidence of an enzymatic deficiency in Niemann-Pick disease. Proc. Nat. Acad. Sci. U.S.A., *55:* 366 (1966).
3. Callahan, J. W., Khalil, M., and Gerrie, J.: Isoenzymes of sphingomyelinase and the genetic defect in Niemann-Pick disease, type C. Biochem. Biophys. Res. Commun., *58:* 384 (1974).
4. Callahan, J. W., Khalil, M., and Philippart, M.: Sphingomyelinases in human tissues. II. Absence of a specific enzyme from liver and brain of Niemann-Pick disease, type C. Pediat. Res., *9:* 908 (1975).
5. Callahan, J. W., Pinsky, L., and Wolfe, L. S.: G_{M1}-gangliosidosis (type II): Studies on a fibroblast cell strain. Biochem. Med., *4:* 295 (1970).
6. Crocker, A. C.: The cerebral defect in Tay-Sachs disease and Niemann-Pick disease. J. Neurochem., *7:* 69 (1961).
7. Epstein, C. J., Brady, R. O., Schneider, E. L., Bradley, R. M., and Shapiro, D.: In utero diagnosis of Niemann-Pick disease. Amer. J. Hum. Gen., *23:* 533 (1971).
8. Fredrickson, D. S., and Sloan, H. R.: Sphingomyelin lipidoses: Niemann-Pick disease. In: J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson: The Metabolic Basis of Inherited Disease, Chapt. 35, p. 783 (McGraw-Hill Book Co., New York, 1972).
9. Lowry, O. H., Rosebrough, N. L., Farr, A. L., and Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265 (1951).
10. Lynn, R., and Terry, R. D.: Lipid histochemistry and electron microscopy in adult Niemann-Pick disease. Amer. J. Med., *37:* 987 (1964).
11. Robins, E., Hirsch, H. E., and Emmons, S. S.: Glycosidases in the nervous system. I. Assay, some properties and distribution of β-glucuronidase and β-glucosidase. J. Biol. Chem., *243:* 4246 (1968).
12. Schneider, P. B., and Kennedy, E. P.: Sphingomyelinase in normal human spleens and in spleens from subjects with Niemann-Pick disease. J. Lipid Res., *8:* 202 (1967).
13. Sloan, H. R., Uhlendorf, B. W., Kanfer, J. N., Brady, R. O., and Fredrickson, D. S.: Deficiency of sphingomyelin-cleaving enzyme activity in tissue culture derived from patients with Niemann-Pick disease. Biochem. Biophys. Res. Commun., *34:* 582 (1969).
14. Thomas, G. H., Taylor, H. A., Reynolds, L. W., and Miller, C. S.: Mucolipidosis III (Pseudo-Hurler polydystrophy): Multiple lysosomal enzyme abnormalities in serum and cultured fibroblast cells. Pediat. Res., *7:* 751 (1973).
15. A detailed clinical description of this patient is in preparation and will be presented elsewhere.
16. St. Louis, Mo.
17. Colnbrook, U.K.
18. Bromma, Sweden.
19. The authors are indebted to Dr. M. Buchwald and Mrs. Nancy Allen of the Department of Medical Genetics for the fibroblast cultures and Dr. P. Quinn, Department of Bacteriology, who carried out the mycoplasma determinations.
20. Dr. J. W. Callahan is a Medical Research Council of Canada scholar.
21. This research was supported by Grant MA-4873 from the Medical Research Council of Canada.
22. Requests for reprints should be addressed to: J. W. Callahan, Ph.D., Hospital for Sick Children, 555 University Ave., Toronto, Ontario, M5G 1X8 Canada.
23. Accepted for publication July 25, 1975.

Pediat. Res. *9:* 918–923 (1975)

Erythrocyte entrapment
Fabry disease
Gaucher disease
β-glucuronidase
lysosomal storage disease

Enzyme Therapy. V. *In Vivo* Fate of Erythrocyte-entrapped β-Glucuronidase in β-Glucuronidase-deficient mice

SUZANNE R. THORPE, MORRIS B. FIDDLER, AND ROBERT J. DESNICK[40]

Departments of Pediatrics, Laboratory Medicine, and Pathology and The Dight Institute for Human Genetics, University of Minnesota Medical School, Minneapolis, Minnesota, USA

Extract

The use of erythrocyte entrapment as a strategy to deliver and protect exogenously administered enzymes for replacement therapy in selected genetic diseases has been evaluated in a mammalian model system. The uptake, tissue distribution, intracellular localization, and *in vivo* lifetime of erythrocyte-entrapped bovine β-glucuronidase were determined by a selective thermal inactivation assay after intravenous administration into β-glucuronidase-deficient mice. The exogenous activity was cleared from the circulation with a half-life of about 20 min and was no longer detectable at 2 hr. A concomitant uptake of the injected enzyme was observed in murine tissues, primarily the liver; approximately 30% of the bovine activity was recovered at 30 min and maximal hepatic uptake, 71% of dose, was detected at 2 hr. Hepatic recovery of the bovine activity was observed to decrease in a biphasic pattern to nondetectable levels by 5 days. The recovery of the entrapped activity was characterized by a latency of detection in hepatic tissue up to 13 hr postinjection. At each time point more than 80% (84–100%) of the recovered bovine activity was detected in the lysosomally enriched hepatic subcellular fraction. Maximal recoveries of 10 % and 15% of administered dose were observed in splenic and renal tissues, respectively, soon after enzyme administration. In comparison to results obtained after intravenous administration of unentrapped bovine β-glucuronidase, erythrocyte-entrapped activity was retained fourfold longer in the circulation, fivefold longer in hepatic tissue, and was more efficiently delivered to a variety of tissues.

Speculation

Entrapment in autologous erythrocytes may provide an effective means to optimize the delivery and protection of exogenous enzymes for the treatment of selected lysosomal storage diseases, such as Fabry and type 1 Gaucher diseases, by enzyme replacement therapy. Furthermore, efficient delivery to specific target sites of pathology may be accomplished by the entrapment of other therapeutic agents within these biodegradable vesicles.

Exploratory trials of enzyme therapy have been stimulated by advances in the elucidation and characterization of the specific enzymatic defects in an ever increasing number of inborn errors of metabolism (9, 26). Since 1964, when Baudhuin and coworkers (3)

dministered an α-glucosidase preparation from *Aspergillus niger* o a patient with Pompe's disease, the lysosomal storage diseases ıave been the prime focus of enzyme replacement endeavors. The herapeutic concept was based on the rationale that administered xogenous enzyme could gain access to the intracellular environs y endocytosis and that subsequent fusion with the lysosomal ıpparatus would make the active enzyme available for metabolism f its accumulating substrate within lysosomes. Recent trials of nzyme replacement *in vitro* and *in vivo* support the feasibility of his rationale. When the appropriate purified enzyme was incuated in the media of cultured fibroblasts obtained from enzyme-leficient patients (1, 15, 19, 20, 22, 18), or more importantly, when ighly purified enzymes from human sources were administered ntravenously to patients with selected lysosomal storage diseases 6, 7, 8), the capacity of the exogenous enzyme to catabolize ccumulated substrates was demonstrated.

Critical review of the recent and earlier human trials has identified the major obstacles which must be overcome if enzyme replacement is to become an effective therapeutic modality for selected inherited metabolic diseases (10). The difficulties in these pilot trials included the short circulating and intracellular half-lives of the administered enzymatic activity, immunologic complications, and the inability to serially evaluate the physiologic and biochemical factors affecting the fate of the administered enzyme. Thus, effective enzyme therapy requires the development of strategies designed to satisfy the following requisites: (*1*) techniques for enzyme administration which afford maximal protection to the exogenous enzyme from potential physiologic and immunologic inactivation, as well as (*2*) delivery of the enzyme to the specific tissue and subcellular sites of pathologic substrate accumulation, and (*3*) the development of mammalian models to systematically assess the *in vivo* effectiveness of these strategies prior to human trials.

Within this perspective, we recently reported the development of a murine model system designed to serially evaluate and maximize methods for the delivery and protection of exogenous bovine β-glucuronidase in β-glucuronidase-deficient mice (27). Intravenously administered enzymatic activity was cleared rapidly from the circulation with a $t_{0.5}$ of 3 min and was recovered almost exclusively in hepatic tissue, comparable with the results of recent human trials of direct enzyme administration (6, 7, 18). Although 70% of the dose was recovered in the liver at 30 min, the administered exogenous enzymatic activity gradually decreased and was no longer detectable by 24 hr postinjection. These studies reflect the need to develop strategies to deliver and protect administered enzymes in order to maximize their catalytic effectiveness.

The recent report by Ihler *et al.* (16) demonstrating the entrapment of enzymes in human erythrocytes suggested the intriguing possibility that these biodegradable vesicles may be useful as *in vivo* carriers of exogenous enzyme; therefore, we have explored the feasibility of enzyme entrapment in autologous murine erythrocytes (11) as vehicles for efficient enzyme delivery in our model system. The present study was undertaken to serially evaluate the effectiveness of erythrocyte entrapment as a strategy to meet the above requisites for effective enzyme therapy. We report here the uptake, tissue distribution, intracellular localization, and *in vivo* lifetime of erythrocyte-entrapped bovine β-glucuronidase in β-glucuronidase-deficient mice as determined by both enzymatic assay and electrophoretic visualization of the administered activity.

MATERIALS AND METHODS

PREPARATION OF BOVINE β-GLUCURONIDASE

Bovine liver β-glucuronidase (30) was further purified 15-fold by Sephadex G-200 (31) chromatography (0.02 M potassium phosphate buffer, pH 6.5) and had a specific activity of approximately 10,000 U/mg protein; this preparation gave a single band of enzymatic activity when electrophoresed on polyacrylamide disc gel by the method of Ganschow and Bunker (12).

ENZYME ASSAY

Blood was collected in heparanized pipettes and centrifuged at 580 × *g* for 15 min to separate plasma and erythrocytes from C3H/HeJ *Gus*^{h} male mice, 10–14 weeks old, weighing 23–28 g (32); mouse liver, spleen, lungs, and kidneys were removed immediately, rinsed well in 0.15 M NaCl, and homogenized in a Potter-Elvehjem homogenizer for 2 min at 500 rpm in 4 volumes (w/v) of 0.3 M sucrose for subsequent assay.

A selective thermal inactivation assay was used to determine the bovine (thermolabile) and residual murine (thermostable) β-glucuronidase activities (5–10% of normal murine tissue activity); this method reliably and sensitively discriminated and quantitated these activities in murine tissue homogenates obtained after enzyme administration as documented previously (27). Total β-glucuronidase activity was determined in the standard reaction mixture which contained the following in a final volume of 350 μl: 50 μl enzyme source in 0.05 M sodium acetate buffer (pH 4.6) and 300 μl of 2.50 mM 4-methylumbelliferyl-β-D-glucuronide substrate (33) in 0.05 M acetate buffer (pH 4.6) containing 0.1% Triton X-100. Partially purified bovine β-glucuronidase was assayed in the presence of 0.02% bovine serum albumin. All reaction mixtures were incubated at 37° for 30 min. Incubations of purified enzyme or tissue homogenates were terminated by the addition of 4.65 ml 0.1 M ethylenediamine, pH 11.4. Fluorescence of liberated umbelliferone was measured on a Turner model 111 fluorometer (34) with an excitation filter, Turner 7-60 (365 nm), emission filters, Turner 48 and 2A (450 nm), and a 1% neutral density filter. All assays were carried out in triplicate and were linear with time and protein concentration. A unit of enzymatic activity was defined as that amount of activity which hydrolyzed 1 nmol substrate/hr.

Thermostable murine β-glucuronidase activity from various sources was determined by preincubation of the enzyme source (adjusted to 50 μl with acetate buffer) in screw-capped test tubes at 60° for 30 min, immediately followed by immersion in an ice bath for 2 min, and then assayed for residual thermostable activity by the above standard procedure. Thermolabile bovine activity was calculated as the difference between the total and thermostable activities.

β-Glucuronidase activity in packed murine erythrocytes, whole blood, or plasma was determined in a 20-μl aliquot after a 1:4 (v/v) dilution of the initial sample with 0.05 M sodium acetate buffer, pH 4.6, in the above standard assay. Incubations were terminated by the addition of 50 μl of 21% trichloroacetic acid and centrifuged at 500 × *g* for 5 min to pellet precipitated protein. After the addition of 4.6 ml of 0.1 M ethylenediamine to the supernatant, the fluorescence was measured as described above. The average endogenous β-glucuronidase activities in murine erythrocytes and whole blood was 16 U/ml and 40 U/ml, respectively, determined as previously described for murine plasma (27).

ENZYME ENTRAPMENT

Bovine β-glucuronidase was entrapped in autologous murine erythrocytes essentially by the hypotonic exchange method of Ihler *et al.* (16) with the following modifications. Immediately before enzyme entrapment, freshly isolated murine erythrocytes were washed three times with 10 volumes of 0.15 M NaCl, and the buffy coat was removed and discarded. In a typical loading experiment, bovine enzyme (62,000 U, 3.6 mg protein) in 0.6 ml in 0.02 M potassium phosphate buffer, pH 6.5, was mixed with 0.4 ml packed, washed erythrocytes. Hypotonic exchange was induced by the rapid addition of 5.0 ml distilled water and terminated after 60 sec by the addition of 1.3 ml of 0.83 M NaCl to restore isotonicity. The enzyme-containing erythrocytes were recovered by centrifugation at 600 × *g* for 20 min at 4°; the cells were then washed repeatedly with 25 volumes of 0.15 M NaCl until no further

activity was detected in the supernatant. The β-glucuronidase activity was then determined in an aliquot of the enzyme-loaded erythrocytes.

To determine the effect of the hypotonic exchange procedure on endogenous erythrocyte β-glucuronidase activity, murine erythrocytes were exchange loaded with 0.02 M potassium phosphate buffer only. Assessment of possible nonspecific binding of the bovine enzyme to murine erythrocytes was accomplished by subjecting the erythrocytes to the enzyme-loading treatment using only isotonic solutions, to prevent exchange.

IN VIVO EXPERIMENTS

Bovine enzyme (500–1,300 U) entrapped in murine erythrocytes or unentrapped (native) enzyme (900 U, 0.05 mg protein) and/or buffer-loaded erythrocytes in a final volume of 0.2 ml 0.15 M NaCl, was injected into the tail vein of individual mice. The intravenous administrations were well tolerated and mice were killed at the desired intervals. Bovine and murine β-glucuronidase activities were determined in murine tissue homogenates after sonication of 1-ml aliquots for four consecutive 15-sec periods (with 30 sec between) at 4° using a Branson sonifier (35) equipped with a microtip. Polyacrylamide disc gel electrophoresis of hepatic tissue obtained 2 hr after enzyme administration was accomplished by the method of Ganschow and Bunker (12). Recovered bovine and endogenous murine β-glucuronidase activities were also determined in hepatic subcellular fractions obtained by differential centrifugation (14). Bovine β-glucuronidase activity recovered from murine plasma or whole blood was determined by subtracting the average endogenous activity from the total recovered β-glucuronidase activity (27). Leakage of entrapped β-glucuronidase from the hypotonically exchange-loaded erythrocytes could not be accurately assessed, particularly since released activity is rapidly cleared from the circulation (27). Therefore, estimates of *in vivo* leakage were determined by assaying murine plasma obtained at 5 and 60 min after intravenous administration of 920 U enzyme activity in 0.2 ml loaded erythrocytes.

RESULTS

ENTRAPMENT OF BOVINE β-GLUCURONIDASE IN MURINE ERYTHROCYTES

Table 1 shows the recovery of β-glucuronidase activity in murine erythrocytes from a typical hypotonic exchange-loading experiment. Approximately 4% of added enzymatic activity was routinely entrapped in the erythrocytes; the activity remained entrapped after three washes with either 0.15 M or 0.3 M NaCl, but was readily released (95–100%) into solution when the cells were resuspended in 0.15 M NaCl, sonicated for 2 sec, and centrifuged at 15,000 × *g* for 15 min. In contrast, when the loading procedure was carried out in isotonic solutions to prevent exchange, the β-glucuronidase activity recovered in the erythrocyte pellet was equivalent to the average endogenous activity obtained from untreated mouse erythrocytes (Table 1). When murine erythrocytes were subjected to hypotonic exchange in the absence of added enzymatic activity, the endogenous β-glucuronidase activity was no longer detectable in the erythrocyte pellet.

IN VIVO FATE OF ERYTHROCYTE-ENTRAPPED BOVINE β-GLUCURONIDASE

Figure 1 shows the time course for the blood clearance and tissue uptake after intravenous administration of erythrocyte-entrapped bovine β-glucuronidase into β-glucuronidase-deficient mice. Total recovery of administered activity from murine blood and tissues accounted for approximately 95% of dose at 1 and 2 hr postinjection. The activity was cleared from the circulation with a half-life of about 20 min and was no longer detectable at 2 hr. A concomitant uptake of the injected enzyme was observed in murine tissues, primarily the liver; approximately 30% of the bovine activity was recovered at 30 min and maximal hepatic uptake, 71% of dose, was detected at 2 hr. Notably, the recovered exogenous activity was maintained in hepatic tissue at this level from 2 to 13 hr. The hepatic recovery of the bovine activity was then observed to decrease in a biphasic pattern characterized by an initial drop from the maximal level to 25% of dose at 20 hr followed by subsequent retention of 10–20% of dose for up to 5 days. Significant levels of bovine activity were recovered routinely in the kidneys, 13 and 15% of dose, only at 1 and 2 hr, respectively; low levels, less than 10% of dose, were found in the spleen, whereas no exogenous activity was recovered in the lungs, bone marrow, or brain after administration.

Table 1. *Recovery of β-glucuronidase activity in hypotonic exchange-loaded murine erythrocytes*[1]

Procedure	Packed erythrocytes, U/ml: Bovine enzymatic activity added	Packed erythrocytes, U/ml: Total enzymatic activity recovered	% entrapped
Hypotonic exchange	155,000	6,900	4.4
Hypotonic exchange	0	<0.01	0
Isotonic control	232,000	20	0
Packed erythrocytes (untreated)	0	16.5[2]	0

[1] All values represent the mean of triplicate determinations.

[2] Mean endogenous β-glucuronidase activity in washed, packed murine erythrocytes (n = 12) determined as described in *Methods*.

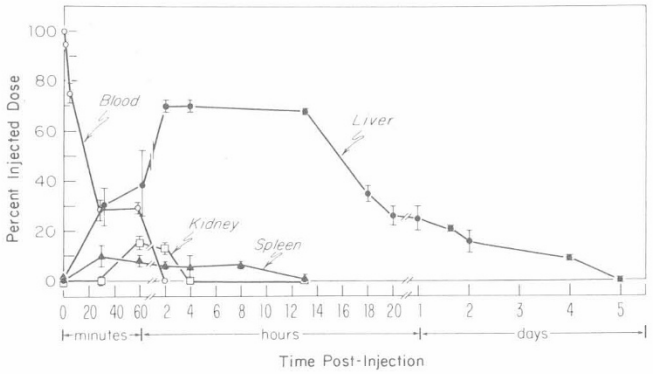

Fig. 1. Fate of erythrocyte-entrapped bovine β-glucuronidase activity administered intravenously to β-glucuronidase-deficient mice. Bovine β-glucuronidase activities recovered from murine blood, and organs after sonication, were determined as described in *Materials and Methods* and expressed as percentage of injected dose. Indicated values represent the mean and range of three to four mice at each time point.

LATENCY OF RECOVERED ENZYMATIC ACTIVITY

Figure 2 shows the effect of sonication on the recovery of exogenous activity in hepatic tissue. A marked latency in the detection of administered activity was observed when hepatic tissue homogenates obtained at sampling intervals up to 13 hr were not sonicated before enzyme assay. This latency was unique since the standard assay is accomplished in the presence of detergent and under hypotonic conditions. In contrast, sonication did not increase the amount of exogenous activity recovered in all homogenates obtained 13 hr after administration. A similar latency was observed after the sonication of splenic tissue obtained up to 4 hr after administration; however, no increase in bovine activity was found in sonicated kidney homogenates. The levels of endogenous β-glucuronidase activity in untreated and treated mice as well as the bovine activity *in vitro* were not affected by sonication.

SUBCELLULAR LOCALIZATION OF BOVINE ACTIVITY IN MURINE LIVER

Subcellular fractionation of hepatic tissue was carried out to determine the intracellular site of administered enzyme uptake. Table 2 shows the results of the subcellular fractionation of hepatic tissue into nuclear, mitochondrial-lysosomal, and soluble (including microsomal) fractions at various intervals after administration of erythrocyte-entrapped enzyme. Total fractionated murine and bovine activities recovered were 105, 95, 98, and 97% of that in the whole homogenate at 4, 13, 24, and 96 hr postinjection, respectively. At each time point, more than 80% (84–10) of the recovered bovine activity was detected in the lysosomally enriched fraction. In addition, sonication of the lysosomally enriched fraction resulted in the release of all the bovine activity into the supernatant after centrifugation at 14,000 × *g* for 30 min. Furthermore, when an *in vitro* control mixture of erythrocyte-entrapped enzyme and murine liver homogenate was fractionated, 70% of the bovine activity was recovered in the nuclear fraction, supporting the *in vivo* specificity of the lysosomal apparatus for erythrocyte-entrapped enzyme uptake.

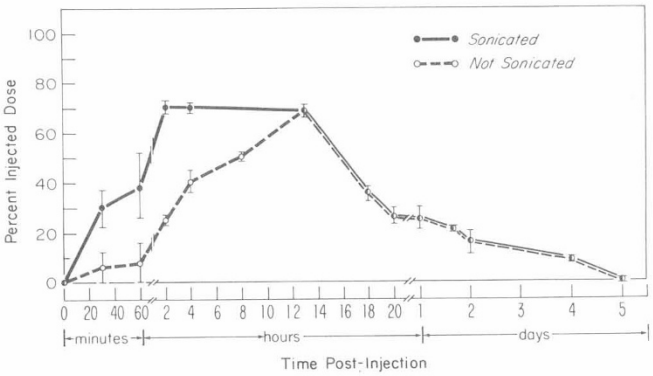

Fig. 2. Effect of sonication on the hepatic recovery of erythrocyte-entrapped bovine β-glucuronidase. Bovine β-glucuronidase activities recovered from hepatic tissue, before and after sonication, were determined as described in *Materials and Methods* and expressed as percentage of injected dose. Indicated values represent the mean and range of three to four mice at each time point.

Table 2. *Hepatic subcellular distribution of recovered erythrocyte-entrapped bovine* β-glucuronidase[1]

Time postinjection, hr	Activity administered, U	Source[1]	Recovered enzymatic activity: Total homogenate, U	Total fraction, U	% subcellular fraction recovered of total fractions[2]: N	M + L	S
4	1,000	Bovine	720	760	10	90	0
		Murine	3,040	3,240			
13	800	Bovine	617	550	0	87	13
		Murine	1,720	1,700			
24	834	Bovine	248	275	16	84	0
		Murine	2,540	2,730			
96	1330	Bovine	132	129	0	100	0
		Murine	2.500	2,390			
In Vitro control[3]	768	Bovine	704	705	71	0	29
		Murine	2,620	2,260			

[1] Bovine and murine sources designate thermolabile and thermostabile activities recovered, respectively.

[2] Subcellular fractions obtained by differential centrifugation as described in *Methods*; N: nuclear (600 × *g* pellet), M + L: mitochondrial-lysosomal (14,000 × *g* pellet), and S: soluble fraction (14,000 × *g* supernatant). Percentage of recovered of total fractions for murine activities (mean and range): N = 24% ± 6%, M + L = 46% ± 4%, S = 30% ± 2%.

[3] Erythrocyte-entrapped activity (768 U) and a liver homogenate were mixed *in vitro*, incubated at 37° for 10 min, and then fractionated.

COMPARISON OF HEPATIC RECOVERY OF UNENTRAPPED AND ERYTHROCYTE-ENTRAPPED ACTIVITY

Figure 3 compares the hepatic recovery of intravenously administered erythrocyte-entrapped bovine β-glucuronidase activity with the recoveries of unentrapped bovine activity, and a mixture of unentrapped bovine activity and buffer-loaded murine erythrocytes. The time course for the hepatic recovery of unentrapped enzyme injected alone, or of the mixture of unentrapped activity and buffer-loaded erythrocytes was essentially identical but was distinctly different than that observed after administration of erythrocyte-entrapped enzyme. No thermolabile activity, characteristic of the bovine activity, was detected in hepatic tissue when only buffer-loaded erythrocytes were injected. Furthermore, leakage of enzyme from the loaded erythrocytes, assessed at 5 and 60 min postinjection, was less than 3.5 and 1.3% of the injected dose, respectively. These *in vivo* control experiments demonstrated that the pattern of hepatic uptake and retention of erythrocyte-entrapped activity resulted from the incorporation of enzyme in the erythrocytes and intracellular uptake and processing of the enzyme-loaded cells; furthermore, the experiments indicated that the *in vivo* observations were not due to enzyme adsorption on the membrane surface or uptake of enzyme released to the circulation from leaky erythrocytes.

The electrophoretic separation of bovine and residual murine β-glucuronidase activities provided a further comparison of the hepatic recovery of unentrapped vs. entrapped activity. As shown in Figure 4, polyacrylamide disc-gel electrophoresis readily distinguished the exogenous bovine (*gel 1*) and endogenous murine hepatic (*gel 2*) activities when equal amounts of each were mixed *in vitro* (*gel 3*). Electrophoresis of hepatic tissues obtained 2 hr after injection of unentrapped (*gel 4*) and erythrocyte-entrapped (*gel 5*) bovine enzyme was accomplished; the relative difference in the staining intensity of the entrapped vs. unentrapped activity corresponds with the 2-fold greater recovery of entrapped activity detected by the thermal inactivation assay at 2 hr postinjection.

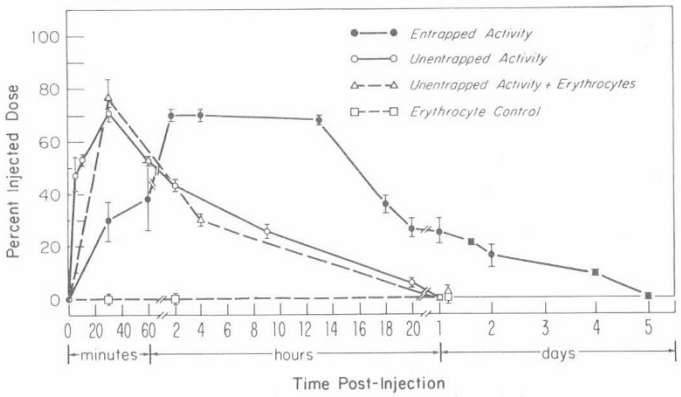

Fig. 3. Comparison of the hepatic recoveries of erythrocyte-entrapped bovine β-glucuronidase activity, unentrapped bovine activity, and a mixture of bovine activity and buffer-loaded murine erythrocytes from β-glucuronidase-deficient mice. No thermolabile activity was detected after injection of only bufferloaded erythrocytes. Bovine β-glucuronidase activities recovered from hepatic tissues, as described in *Materials and Methods*, are expressed as percentage of injected dose (unentrapped dose = 900 U; entrapped dose = 500–1300 U). Indicated values represent the mean and range of three to four mice at each time point.

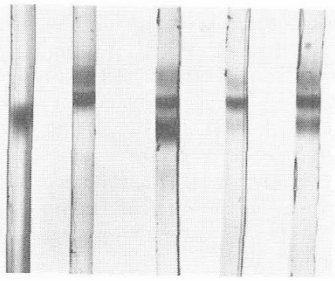

Fig. 4. Polyacrylamide disc-gel electrophoresis of bovine and murine hepatic β-glucuronidase. Sample preparation, electrophoresis, and staining for β-glucuronidase activity were performed as described in *Materials and Methods*. *Gel 1:* partially purified bovine liver β-glucuronidase; *gel 2:* murine liver homogenate; *gel 3:* equal mixture of bovine and murine hepatic β-glucuronidase activities; *gel 4:* murine liver homogenate obtained 2 hr after administration of 3,800 U unentrapped bovine enzyme; *gel 5:* murine liver homogenate obtained 2 hr after administration of 3,800 U bovine enzyme entrapped in murine erythrocytes.

DISCUSSION

Effective enzyme therapy for selected inherited metabolic diseases requires the development of strategies, evaluated in model systems, to deliver sufficient quantities of the specific, active isozyme to target tissue and subcellular sites for efficient substrate metabolism. Entrapment in vesicles for administration may provide a strategy to protect the enzyme from biodegradation and inactivation in the circulation and enhance its delivery to critical sites of substrate pathology (8, 11, 13, 16). Recently, erythrocytes have been suggested as carrier vesicles for enzyme therapy (11, 16); the ready availability, autologous origin, biodegradability, and relative ease of entrapment make these natural vesicles attactive carriers of therapeutic agents.

Under our conditions, approximately 4% of available bovine β-glucuronidase (mol wt 280,000 (21)) was routinely entrapped in the murine erythrocytes by hypotonic exchange (Table 1); the enzyme-loaded erythrocytes retained normal discoid morphology and mean corpuscular volume. The percentage of entrapment and the morphologic characteristics compare favorably with similar findings reported for the entrapment of enzymes and other proteins in human erythrocytes (16, 29). The amount of protein entrapped has been shown to be a function of the hypotonic exchange conditions, including degree and duration of hypotonicity, concentration of various ions, temperature, and the molecular weight or size of the molecule to be entrapped (2, 4, 16). Although the dilution factor required to induce hypotonic exchange establishes a maximal efficiency of entrapment, unincorporated molecules can be recovered readily by gel filtration and recycled for subsequent entrapment.

When erythrocyte-entrapped bovine β-glucuronidase was intravenously administered to the β-glucuronidase-deficient mice, significant differences were observed compared with the fate of unentrapped enzyme (Fig. 3). Several features of the recovery of enzyme administered in erythrocytes demonstrated that this strategy of administration provided efficient enzyme delivery, which may be more effective than the administration of unentrapped activity for enzyme therapy. In contrast to the rapid hepatic uptake and linear decline of unentrapped activity, erythrocyte-entrapped activity attained comparable hepatic levels, about 70% of dose, but maintained this level for 11 hr and retained significant activity up to 5 days. Furthermore, entrapped activity in the circulation was detectable four-fold longer than unentrapped activity and was also more effectively delivered to other tissues, *i.e.*, spleen and kidney (27).

Initial experiments monitoring the time course of recovery of erythrocyte-entrapped bovine β-glucuronidase revealed an unaccountable discrepancy in the pattern of blood fall off and maximal hepatic recovery of administered enzyme (11). These findings suggested a latency for the detection of the entrapped bovine activity. As shown in Figure 2, latency was indeed observed after brief sonication of liver homogenates obtained at early time points. In fact, significantly increased activities (up to three fold greater levels) were detected after sonication compared with unsonicated levels in hepatic tissue homogenates at time points up to 12 hr. The latency for detection of enzymatic activity presumably reflected the intracellular release of enzyme from erythrocytes as they were being processed within lysosomes (note lysosomal recovery of bovine activity at 4 hr, Table 2). Demonstration of a latency of *in vivo* substrate metabolism would support the value of erythrocytes to provide a time-release effect for enzyme availability. Even though ultrastructural examination of hepatic tissue from these mice did not reveal substrate accumulation in lysosomes (23), it will be important to assess the effect of erythrocyte entrapment and other enzyme delivery strategies on the turnover of endogenous β-glucuronyl-containing glycosaminoglycans.

Another unique feature of the fate of erythrocyte-entrapped activity was the gradual and biphasic decline of administered activity recovered in hepatic tissue. Since the decline of unentrapped activity was linear and more rapid (Fig. 3), it is likely that the unique pattern was the result of erythrocyte entrapment and not a property of the bovine enzyme. The biphasic pattern may reflect at least two subpopulations of enzyme-loaded erythrocytes (4), one of which was more slowly processed, resulting in a prolonged intracellular retention of entrapped activity. Alternatively, the biphasic fall-off may have resulted from a differential uptake, processing, and/or turnover of erythrocyte-entrapped activity by hepatocytes and Kupffer cells.

The tissue and subcellular distribution of the administered enzyme most likely reflects the processing of the exchange-loaded erythrocytes. Presumably, the major sites of erythrocyte uptake occur in cells of the reticuloendothelial system. The absence of sinusoid morphology (24) may account for the low uptake of entrapped activity in the murine spleen; therefore, these data may provide a minimal estimate of the uptake of autologous enzyme-loaded erythrocytes by the human spleen. Since entrapped activity was localized primarily to the lysosomally enriched subcellular fraction (Table 2), required lysosomal isozymes administered in autologous erythrocytes may provide an effective approach for the direct treatment of appropriate patients with selected lysosomal storage diseases without neural involvement (*e.g.*, Gaucher type 1 and Fabry diseases). In addition, specific chemical modification of the erythrocyte surface (10, 17) may prove a feasible approach to target enzyme-loaded erythrocytes for uptake by other tissue and cell sites.

Autologous erythrocytes should afford protection of the exogenous enzyme from potential biodegradative processes and immunologic inactivation induced by contact with the recipient's immunocompetent cells (5). The minimal leakage of entrapped activity *in vivo* and *in vitro* (29) and the lack of detectable exogenous activity on the erythrocyte surface should minimize these potential deleterious effects on the administered enzyme and, more importantly, to the recipient. In addition, the use of allogeneic enzymes may prolong the intracellular retention of enzymatic activity and further minimize potential antigenicity. Moreover, stabilizing allogeneic enzymes by cross-linking reagents (25) might decrease the rate of enzyme inactivation and/or degradation and thereby extend the interval between administrations.

Although encouraging progress has been reported in recent human trials of direct enzyme replacement (6, 7), the evaluation of enzyme therapeutic strategies in model systems before human trials, as demonstrated here for erythrocyte-entrapped β-glucuronidase, should optimize the effectiveness of future clinical endeavors.

SUMMARY

Bovine β-glucuronidase was entrapped in autologous murine erythrocytes by hypotonic exchange loading and intravenously administered to β-glucuronidase-deficient mice to determine the *in*

vivo fate of the entrapped enzymatic activity. Compared with the administration of unentrapped enzyme, erythrocyte-entrapped activity was retained in the circulation 4 times longer. Maximal hepatic recovery of the administered activity was approximately 70% of dose and was retained 5 times longer than unentrapped activity. A latency of detection in hepatic tissue up to 13 hr postinjection was observed for entrapped activity and more than 80% of the recovered activity was localized in the lysosomally enriched hepatic subcellular fraction between 1 and 96 hr. In contrast to the uptake of unentrapped enzyme, erythrocyte-entrapped activity was more efficiently delivered to renal and splenic tissues. These experiments demonstrate the effectiveness of erythrocyte entrapment as a means to optimize the delivery and protection of exogenous enzymes for the effective treatment of selected inherited metabolic diseases by enzyme replacement therapy.

REFERENCES AND NOTES

1. Bach, G., Friedman, R., Weissman, B., and Neufeld, E. F.: The defect in Hurler and Scheie syndrome: Deficiency of α-iduronidase. Proc. Nat. Acad. Sci. U. S. A., *69:* 2048 (1972).
2. Baker, R. F.: Entry of ferritin into human red cells during hyptonic haemolysis. Nature, *215:* 424 (1967).
3. Baudhuin, P., Hers, H. G., and Loeb, H.: An electron microscopic and biochemical study of type II glycogenosis. Lab. Invest., *13:* 1139 (1964).
4. Bodemann, H., and Passow, H.: Factors controlling the resealing of the membrane of human erythrocyte ghosts after hypotonic hemolysis. J. Membrane Biol., *8:* 1 (1972).
5. Boyer, S. H., Siggers, D. C., and Krueger, L. J.: Caveat to protein replacement therapy for genetic disease: Immunological implications of accurate molecular diagnosis. Lancet, *ii:* 654 (1973).
6. Brady, R. O., Penchev, P. G., Gal, A. E., Hibbert, S. R., and Dekaban, A. S.: Replacement therapy for inherited enzyme deficiency: Use of purified glucocerebrosidase in Gaucher's disease. New Engl. J. Med., *291:* 989 (1974).
7. Brady, R. O., Tallman, J. F., Johnson, W. G., Gal, A. E., Leahy, W. R., Quirk, J. M., and Dekaban, A. S.: Replacement therapy for inherited enzyme deficiency: Use of purified ceramidetrihexosidase in Fabry's disease. New Engl. J. Med., *289:* 9 (1973).
8. Chang, T. M. S.: Immobilization of enzymes, adsorbents or both within semipermeable microcapsules (artificial cells) for clinical and experimental treatment of metabolite related disorders. In: R. J. Desnick, R. W. Bernlohr, and W. Krivit: Enzyme Replacement for Genetic Diseases. Birth Defects: Original Article Series. Vol. IX, p. 66. (The Williams & Wilkins Co., Baltimore, 1973).
9. Desnick, R. J., Bernlohr, R. W., and Krivit, W.: Enzyme Therapy in Genetic Diseases. Birth Defects: Original Article Series, Vol IX (The Williams & Wilkins Co., Baltimore, 1973).
10. Desnick, R. J., Krivit, W., and Fiddler, M. B.: Enzyme therapy in genetic diseases: Progress, principles, and prospects. In: A. Milunsky: The Prevention of Mental Retardation and Genetic Disease, p. 317 (W. B. Saunders Co., Philadelphia, 1975).
11. Fiddler, M. B., Thorpe, S. R., Krivit, W., and Desnick, R. J.: Enzyme therapy: III. *In vivo* fate of erythrocyte entrapped β-glucuronidase. In: J. M. Tager, G. J. M. Hooghwinkel, and W. T. Daems: Enzyme Therapy in Lysosomal Diseases, p. 182 (North Holland Publishing Co., Amsterdam, 1974).
12. Ganschow, R., and Bunker, B.: Genetic control of glucuronidase in mice. Biochem. Genet., *4:* 127 (1970).
13. Gregoriadis, G., and Ryman, B. E.: Lysosomal localization of β-fructofuranosidase-containing liposomes injected into rats: Some implications in the treatment of genetic disorders. Biochem. J., *129:* 123 (1972).
14. Gregoriadis, G., and Sourkes, T.L.: Intracellular distribution of copper in the liver of the rat. Can. J. Biochem., *45:* 1841 (1967).
15. Hall, C. W., Cantz, M., and Neufeld, E. F.: A β-glucuronidase deficiency mucopolysaccharidosis: Studies in cultured fibroblasts. Arch. Biochem. Biophys., *155:* 32 (1973).
16. Ihler, G. M., Glew, R. H., and Schnure, F. W.: Enzyme loading of erythrocytes. Proc. Nat. Acad. Sci. U. S. A., *70:* 2663 (1973).
17. Jacob, H. S., and Jandl, J. H.: Effects of sulfhydryl inhibition on red blood cells II. Studies *in vivo*. J. Clin. Invest., *41:* 1514 (1962).
18. Johnson, W. G., Desnick, R. J., Long, D. M., Sharp, H. L., Krivit, W., Brady, B., and Brady, R. O.: Intravenous injection of purified hexosaminidase A into a patient with Tay-Sachs disease. In: R. J. Desnick, R. W. Bernlohr, and W. Krivit: Enzyme Therapy in Genetic Diseases. Birth Defects: Original Articles Series, Vol IX, p. 120 (The Williams & Wilkins Co., Baltimore, 1973).
19. Lagunoff, D., Nicol, D. M., and Pritzl, P.: Uptake of β-glucuronidase by deficient human fibroblasts. Lab. Invest., *29:* 449 (1973).
20. O'Brien, J. S., Miller, A. L., Loverde, A. W., and Veath, M. L.: Sanfilippo disease type B: Enzyme replacement and metabolic correction in cultured fibroblasts. Science, *181:* 753 (1973).
21. Plapp, B. Y., and Cole, R. D.: Purification and characterization of bovine liver β-glucuronidase. Arch. Biochem. Biophys., *116:* 193 (1966).
22. Porter, M. T., Fluharty, A. L., and Kihara, H.: Correction of abnormal cerebroside sulfate metabolism in cultured metachromatic leucodystrophy fibroblasts. Science, *172:* 1263 (1971).
23. Sharp, H. L., and Desnick, R. J.: Unpublished results.
24. Snook, T.: A comparative study of the vascular arrangements in mammalian spleens. Amer. J. Anat., *87:* 31 (1950).
25. Snyder, P. D., Jr., Wold, F., Bernlohr, R. W., Dullum, C., Desnick, R. J., Krivit, W., and Condie, R. M.: Enzyme therapy. II. Purified human α-galactosidase A: Stabilization to heat and protease degradation by complexing with antibody and by chemical modification. Biochim. Biophys. Acta, *350:* 432 (1974).
26. Tager, J. M., Hooghwinkel, G. J. M., and Daems, W. Th.: Enzyme Therapy in Lysosomal Storage Diseases (North Holland Publishing Co., Amsterdam, 1974).
27. Thorpe, S., Fiddler, M. B., and Desnick, R. J.: Enzyme therapy: IV. A method for determining the *in vivo* fate of bovine β-glucuronidase in β-glucuronidase deficient mice. Biochem. Biophy. Res. Commun. *61:* 1464 (1974).
28. von Figara, K., and Kresse, H.: Quantitative aspects of pinocytosis and the intracellular fate of *N*-acetyl-α-D-glucosaminidase in Sanfillipo B fibroblasts J. Clin. Invest., *53:* 85 (1974).
29. Whitaker, T. R., Sartiano, G. P., Hamelly, L. J., Jr., Scott, W. L., and Glew, R. H.: Hypotonic exchange loading of human erythrocytes with ^{59}Fe-labeled rabbit hemoglobin. J. Lab. Clin. Med., *84:* 879 (1974).
30. Type B-1, Sigma, St. Louis, Mo.
31. Pharmacia, Piscataway, N. J.
32. Jackson Laboratories, Bar Harbor, Me.
33. Research Products International, Elk Grove Village, Ill.
34. G. K. Turner Associates, Palo Alto, Calif.
35. Heat System-Ultrasonics, Plainville, N. Y.

36. This work was presented in part at the Annual Meeting of the American Pediatric Society, Denver, Colorado, April 16–19, 1975 (Pediat. Res., *9:* 312 (1975)).
37. The authors wish to acknowledge the expert technical assistance of Ms. Linda Walling for the electrophoretic studies and Ms. Ardys Ferman for excellent clerical assistance.
38. Dr. Thorpe is a recipient of National Institutes of Health Postdoctoral Training Fellowship IT22 GM 00025, Mr. Fiddler is a recipient of a National Science Foundation Predoctoral Fellowship, and Dr. Desnick is a recipient of a National Institutes of Health Research Career Development Award K04 AM 00042.
39. This work was supported in part by Grant 1-273 from The National Foundation-March of Dimes, Grant AM 15174 from the National Institutes of Health, and Grant 74-915 from the American Heart Association.

40. Requests for reprints should be addressed to: R. J. Desnick, M.D., Box 231 Mayo, University of Minnesota Hospitals, Minneapolis, Minn. 55455 (USA).

41. Accepted for publication July 28, 1975.