(1983); see also R. A. Roth *et al.*, *Endocrinology* **112** (Suppl.), 313 (1983).
11. M. Kasuga, Y. Fujita-Yumaguchi, D. L. Blythe, C. R. Kahn, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1538 (1982).
12. E. Van Oberghen and C. R. Kahn, *Mol. Cell. Endocrinol.* **22**, 277 (1981).
13. E. Karnieli *et al.*, *J. Biol. Chem.* **256**, 4772 (1981).
14. S. W. Cushman, D. Noda, L. B. Salans, *Am. J. Physiol.* **240**, E166 (1981).
15. M. Kasuga, C. R. Kahn, J. A. Hedo, E. Van Oberghan, K. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6917 (1981).
16. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
17. J. A. Hedo and I. A. Simpson, in preparation.
18. Y. Zick *et al.*, *J. Biol. Chem.*, in press.
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Insulin: Carrier Potential for Enzyme and Drug Therapy

Abstract. *Several carrier systems and targeting agents have been considered as means of delivering enzymes and drugs to specific tissues or cells. In this report insulin is shown to be effective in delivering enzyme-albumin conjugates to cells and tissues rich in insulin receptors. The complex is transported into cells by a process that resembles receptor-mediated endocytosis and can be identified in a lysosomal fraction. The enzyme-albumin-insulin complex retains its enzymatic activity and its ability to bind antibodies to insulin. It also has a hypoglycemic effect; however, plasma glucose concentrations can be maintained by glucose administration.*

The use of enzymes to treat certain genetic and metabolic diseases is limited because repeated injections may induce hypersensitivity reactions and because it often is difficult to direct the enzyme to the site of enzyme deficiency or substrate accumulation (*1–3*). Attempts at treating Tay-Sachs disease with hexosaminidase A resulted in a lowering of ganglioside levels in the liver but no penetration of the central nervous system and did not prolong life (*4*). Administration of α-1,4-glucosidase to patients with type 2 glycogenosis lowered glycogen levels in liver cell lysosomes but not in muscle cells of cardiac and respiratory tissue, where glycogen accumulated dramatically, resulting in failure of these organs (*5*).

Most carrier molecules, some of which are highly immunogenic, are recognized by the reticuloendothelial system and are rapidly removed from the circulation after intravenous administration. Attempts at using antibodies directed against cell surface–specific antigens as targeting agents for drugs or enzymes and carrier systems such as liposomes show some promise, but require specific antigens (*6*). Lectins have also been proposed as possible targeting agents, with good binding characteristics but poor in vivo performance of attached enzymes (*2*). The question of cellular uptake of the targeted complex after binding is an important one. In the case of targeted liposomes, stable adsorption without internalization of entrapped drug or enzyme has been demonstrated in nonphagocytic cells (*7*).

We report here on the use of insulin as a targeting agent for the delivery of α-1,4-glucosidase to muscle cells, hepatocytes, and lymphocytes. The high density of insulin receptors on muscle cells makes the insulin molecule capable of delivering enzymes or drugs to a degree not obtainable with other carriers or with free enzyme. The demonstration that the insulin molecule may be internalized after binding to its receptor (*8*) makes it an excellent candidate for the delivery of enzymes to muscle cell lysosomes, the major site of accumulating glycogen in patients with type 2 glycogenosis. We attempted to determine whether the enzyme-insulin complex is in fact bound and internalized by muscle cells both in vitro and in vivo.

Conjugating an enzyme to an excess of homologous albumin renders the enzyme both nonantigenic and nonimmunogenic (*9*). Insulin was conjugated either to native α-glucosidase or to enzyme-albumin polymers (see legend to Fig. 1). After conjugation the enzyme-albumin-insulin polymer retained 70 percent of α-glucosidase activity toward the substrate maltose and 60 percent toward the natural substrate glycogen. In vitro, both enzyme and enzyme-albumin bound to chick embryonic muscle cells and to mouse spleen cells when conjugated with an excess of bovine insulin (Table 1).

The enzyme-insulin conjugate had a mole ratio of 1:15 and the enzyme-albumin-insulin conjugate had a mole ratio of 1:10:60, as estimated from the starting cross-linking ratios and from the molecular weights of the conjugates determined by gel chromatography (*10*). The enzyme-albumin-insulin polymer had an average molecular weight of 1.1×10^6, with more than 80 percent of the conjugate eluting with molecular weight equivalents between 8.5×10^5 and 1.25×10^6.

Lowering the incubation temperature to 10°C slightly decreased the percentage of enzyme bound, while the percent degradation, as measured by the production of free ^{125}I, fell to virtually zero for all preparations both in the presence and absence of chloroquine. This suggests that internalization and degradation through lysosomal processes follows binding. Chloroquine increased binding of the insulin-containing conjugates (Table 1) and inhibited the rate of degradation of these polymers (Table 2), but had no effect on the enzyme alone or on enzyme-albumin, again suggesting that an internalization step follows the binding of enzyme-insulin (*11*). Unlabeled insulin alone had no significant effect on insulin conjugate binding, although unlabeled conjugate (enzyme-insulin or enzyme-albumin-insulin) was an effective inhibitor, indicating that the process is saturable. The insulin conjugates may

Table 1. Binding of enzyme conjugate to cultured cells. α-Glucosidase from yeast or human placenta was labeled with ^{125}I. Chick embryonic myoblasts (2×10^6) isolated from 14-day embryonic chick pectoral muscle were incubated in 1.5 ml of phosphate-buffered saline (PBS) with 0.05 μg of labeled enzyme—either free or conjugated with albumin (0.30 μg)—or with both albumin (0.30 μg) and insulin (0.150 μg). Binding was measured at 37°C after a 30-minute incubation. Isolated spleen cells (1×10^7) from BALB/cCr mice were incubated in 2 ml of PBS with 0.2 μg of labeled enzyme, either free or conjugated with 1.0 μg of albumin or 0.6 μg of insulin or with both. Binding was measured at 37°C after a 60-minute incubation. Cells were then washed seven times and radioactivity was counted. Cells receiving chloroquine (0.1 m*M*) were incubated for 10 minutes at 37°C before the labeled enzyme was added (*11*).

Preparation of $[^{125}I]$α-glucosidase	Source of enzyme	Binding (percent)		
		Chick muscle cells	Mouse spleen cells	
			Without chloroquine	With chloroquine
Free enzyme	Yeast	8.7	8.1	6.0
Enzyme-albumin (1:10)	Yeast	4.6	4.0	5.0
Enzyme-insulin (1:15)	Yeast		21.5	38.0
Enzyme-albumin-insulin (1:10:60)	Yeast	30.1	22.0	35.4
Free enzyme	Placenta	8.1		
Enzyme-albumin-insulin (1:10:60)	Placenta	31.9		

have a higher affinity for the insulin receptor than free insulin (*12*). Cross-linked conjugates of insulin competed with the binding of enzyme-albumin-insulin but only at a concentration 100 times higher than that of enzyme-insulin.

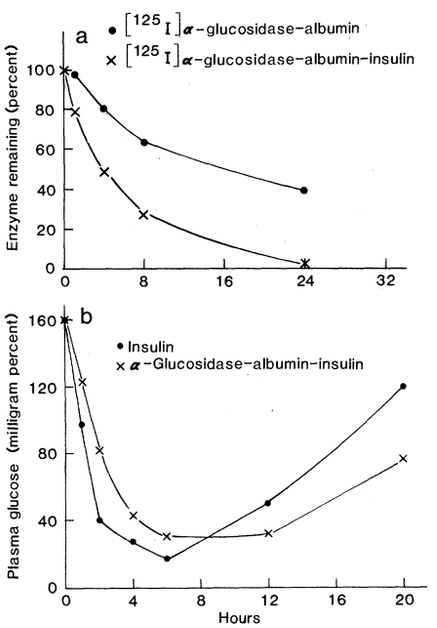

Fig. 1. (a) Clearance of α-glucosidase-albumin and α-glucosidase-albumin-insulin polymers from the circulation of 250-g female Sprague-Dawley rats after intravenous injection. Enzyme (2 mg) from yeast or human placenta was added to 25 mg of human or rat albumin in 3 ml of PBS (0.067*M* potassium phosphate, *p*H 6.8) containing 6 mg of *p*-nitrophenylglucopyranoside (yeast enzyme) or 6 mg of maltose (human enzyme). Cross-linking was carried out for 3 hours at 4°C by the addition of 50 μl of 25 percent glutaraldehyde and stopped by the addition of 30 mg of glycine followed by dialysis against 0.5 percent NaCl and 0.5 percent glycine. The polymer was further purified by ultrafiltration with Amicon XM300 or by gel chromatography. The α-glucosidase-albumin-insulin polymer was obtained by cross-linking the purified enzyme-albumin with insulin. Glutaraldehyde was used as the cross-linking agent under the same conditions as described above, except that 20 mg of enzyme-albumin was reacted with 10 mg of bovine insulin (Connaught Laboratories) at 4°C for 2 hours, at which time the reaction was stopped and the product purified. For preparations made with human placental α-glucosidase, the enzyme polymers were tested at *p*H 4.8 with maltose or glycogen as the substrate. The polymeric form retains its activity toward the natural substrate glycogen (from rat liver). (b) Comparison of the hypoglycemic effect of free insulin with that of equivalent amounts of insulin conjugated with enzyme-albumin polymer. Preparations of insulin (0.2 μg, in the free or polymeric form) were injected at zero time into the tail veins of BALB/cCr mice, and plasma glucose was measured by standard procedures. The data are means for three mice per group. (All chemicals and enzymes were purchased from Sigma, except for human placental α-glucosidase, which was prepared in our laboratory.)

Addition of insulin or cross-linked insulin separately from enzyme-albumin did not stimulate enzyme uptake beyond control values.

Attempts at down-regulation of the insulin receptors (*13*) on cells in vitro were not impressive. Incubating mouse spleen cells for 18 hours at 37°C in the presence of $10^{-7}M$ insulin decreased subsequent binding of labeled insulin by only 10 percent. A similar decrease in binding of enzyme-albumin-insulin was also detected but was not sufficient to indicate that the polymeric complex was binding to the cells at the insulin receptor. The relatively high background binding of α-glucosidase alone is unique for this enzyme. Under identical conditions, [^{125}I]L-asparaginase showed only 0.5 percent binding to mouse spleen cells but showed 24.2 percent binding when conjugated to albumin and insulin.

Table 3 shows the results of subcellular fractionation of chick embryonic muscle cells after their incubation at 10°C and 37°C with enzyme-albumin-insulin (*14*). After a 60-minute incubation at 37°C a significant amount of labeled enzyme was found to be associated with a lysosomal fraction. Lowering the incubation temperature to 10°C did not appreciably alter the binding to intact cells or to the plasma membrane fraction, but the radioactivity associated with the lysosomes was much reduced. It was possible to demonstrate a time-dependent transfer of label from the membrane fraction to the lysosome fraction at 37°C between 10 and 60 minutes of incubation. This did not occur for incubations at 10°C. Identical results were obtained whether the ^{125}I was on the enzyme, albumin, or insulin portions of the conjugate. No significant amount of uptake or internalization could be detected in the absence of conjugated insulin. Antibodies to insulin bound to the enzyme-albumin-insulin conjugate after it was dialyzed (molecular weight cutoff, 30,000) for 48 hours. The polymer was not degraded by proteolytic enzymes in fresh serum (*10*).

Other results further support the conclusion that the conjugated insulin maintains at least some of the properties of native insulin and delivers the enzyme-albumin conjugate to insulin receptors. The insulin-conjugated polymer cleared more rapidly from the circulation than equivalent amounts of the polymer without attached insulin (Fig. 1a). About twice as much insulin polymer was associated with a hepatocyte fraction of the liver as control polymer after 90 percent clearance of the polymers (*10*). Some 2 to 3 percent of the injected dose of insulin polymer was associated with the total muscle mass, compared to only 0 to 0.5 percent of control polymer (*15*). Conjugated insulin retained its hypoglycemic properties to almost the same degree as an equivalent amount of free insulin (Fig. 1b). (The similarity might be coincidental, since the clearance rates of free and conjugated insulin differ greatly.)

These findings strongly suggest that the insulin conjugate is rapidly cleared from the circulation to tissue rich in insulin receptors and that it retains at least its hypoglycemic effect. The hypoglycemic effect can be countered by the simultaneous injection of a glucose solution, so it may not be a significant drawback to the use of insulin as a targeting agent. Since the conjugate was stable and was subjected to extensive dialysis and ultrafiltration, it is unlikely that the hypoglycemic effect was due to contamination from free insulin.

Table 2. Degradation of yeast α-glucosidase after its internalization in mouse spleen cells. Binding conditions were as described in Table 1. Each group of 1×10^7 cells was incubated for 90 minutes at 37°C with ^{125}I-labeled enzyme (0.5 mCi/mg). The percentage of enzyme degraded was determined by measuring the amount of ^{125}I in the supernatant after precipitation with trichloroacetic acid.

Preparation of [^{125}I]α-glucosidase	Percentage of enzyme degraded	
	Without chloroquine	With chloroquine
Free enzyme	2.1	1.8
Enzyme-albumin (1:10)	1.6	1.1
Enzyme-insulin (1:15)	16.0	4.5
Enzyme-albumin-insulin (1:10:60)	17.8	3.0

Table 3. Cellular localization of enzyme-albumin-insulin. Chick embryonic muscle cells (8×10^6) were incubated for 60 minutes at 37°C or 10°C with 0.5 μg of [^{125}I]α-glucosidase (0.5 mCi/μg) conjugated to albumin and insulin at a mole ratio of 1:10:60. The cells were then washed five times in ice-cold saline; homogenized (Dounce); centrifuged at 2000*g* to remove nuclei, intact cells, and debris; and subjected to Percoll density gradient centrifugation (*14*). Data are expressed as a function of micrograms of protein in each fraction.

Fraction	Activity (count/min per microgram of protein)
Intact cells (37°C)	1500
Membrane (37°C)	1840
Lysosome (37°C)	3985
Mitochondria (37°C)	300
Intact cells (10°C)	1650
Membrane (10°C)	2085
Lysosome (10°C)	640
Mitochondria (10°C)	350

Antibodies, lectins, lipoproteins, and other biologically active proteins have been proposed as carriers of drugs and enzymes (*2*). Insulin might have specific advantages for delivering enzymes (and perhaps certain drugs) to muscle tissue because of the high density of insulin receptors on these cells. That other cells, such as hepatocytes and blood cells, also have numerous insulin receptor sites is not a serious problem, since the administered enzyme would not be expected to be toxic to normal tissue.

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References and Notes

1. R. J. Desnick, S. R. Thorpe, M. B. Fiddler, *Physiol. Rev.* **56**, 57 (1976).
2. M. J. Poznansky and L. G. Cleland, in *Drug Delivery Systems*, R. Juliano, Ed. (Oxford Univ. Press, New York, 1980), pp. 253–315.
3. G. A. Grabowski and R. J. Desnick, in *Enzymes as Drugs*, J. C. Holcenberg and J. Roberts, Eds. (Wiley Interscience, New York, 1981), pp. 167–208.
4. R. O. Brady, in *Metabolic Control and Disease*, P. K. Bondy and L. E. Rosenberg, Eds. (Saunders, Philadelphia, 1980), chapter 9; R. O. Brady, P. G. Pentchev, A. E. Gal, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 1310 (1975).
5. G. Hug and W. K. Schubert, *J. Cell Biol.* **35**, C1 (1967); G. Hug, *Pharmacol. Rev.* **30**, 565 (1979).
6. Y. Tsukada *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7896 (1982).
7. R. E. Pagano and J. N. Weinstein, *Annu. Rev. Biophys. Bioeng.* **7**, 435 (1978).
8. J. Schlessinger, Y. Schechter, M. C. Willingham, I. Pastan, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2659 (1978); P. F. Pilch, M. A. Schia, R. J. Benson, R. E. Fine, *J. Cell Biol.* **93**, 133 (1983).
9. M. H. Remy and M. J. Poznansky, *Lancet* **1978-II**, 68 (1978); M. J. Poznansky, M. Shandling, M. A. Salkie, J. Elliott, E. Lau, *Cancer Res.* **42**, 1020 (1982); M. J. Poznansky, *Pharmacol Ther.*, in press; T. Yagura, Y. Kamisaki, H. Wada, Y. Yamamura, *Arch. Allergy Appl. Immunol.* **64**, 11 (1981).
10. M. J. Poznansky and D. Bhardwaj, *Can. J. Physiol. Pharmacol.* **58**, 322 (1980); *Biochem. J.* **196**, 89 (1981).
11. A. Gonzalez-Noriega, J. H. Grubb, V. Talkad, W. S. Sly, *J. Cell Biol.* **85**, 839 (1980).
12. M. Wilchek, T. Oka, Y. J. Topper, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1055 (1975); P. Cuatrecasas, M. D. Hollenberg, K. J. Chang, V. Bennett, *Recent Prog. Horm. Res.* **31**, 37 (1975).
13. P. Gorden, J. L. Carpentier, P. Freychet, L. Orci, *Diabetologia* **18**, 263 (1980); J. R. Gavin, J. Roth, D. M. Neville, P. De Meyts, B. N. Buell, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 84 (1974).
14. H. Pertoft, B. Warmegard, M. Hook, *Biochem. J.* **174**, 309 (1978). Subcellular fractionation of 14-day chick embryonic muscle cells was carried out on Percoll density gradients. Fractions were identified by densities and enzyme activity, with 5′-nucleotidase being used as a marker for the plasma membrane, succinate dehydrogenase as a marker for mitochondria, and acid phosphatase as a marker for lysosomes. With 35 percent Percoll in 0.25*M* sucrose the plasma membrane peak occurred at density 1.040, the lysosome peak at 1.079, and the mitochrondrial peak at 1.101. Subcellular fractionation of cells after incubation of enzyme-albumin conjugates without attached insulin yielded low counts in both the membrane and lysosome fractions. With cells in tissue culture the absolute quantity of enzyme activity taken up into a lysosomal fraction was below our level of enzymatic detection. Absolute α-glucosidase activity in a rat liver lysosome preparation increases after administration of the conjugate in vivo (M. J. Poznansky, unpublished results).
15. Muscle-to-plasma ratios of labeled enzyme were approximately eight times higher when insulin was conjugated to the enzyme complex than when it was not. Corrections were made for plasma contamination of muscle tissue. These corrections were important since the counts for control conjugate (no insulin) in muscle tissue were very low. Mice receiving labeled enzyme-albumin-insulin (10^5 count/min) showed 700 count/min per gram of muscle and a maximum blood contamination of 80 count/min per gram. Mice receiving labeled enzyme-albumin (10^5 count/min) showed 115 count/min per gram of muscle and a maximum blood contamination of 60 count/min per gram.
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People with Absolute Pitch Process Tones Without Producing a P300

Abstract. *The P300 is a positive-going component of the event-related potential. In subjects with absolute, or "perfect," pitch, the P300 elicited by the less frequent of two auditory probes is small or absent. In these subjects, visual probes elicit a normal P300. These results support the view of P300 as a manifestation of the updating of working memory.*

Some individuals can name the tones produced by a large variety of musical instruments. People with this "perfect" or "absolute" pitch (AP) are able to label correctly upward of 50 different pitches (*1*), although they do not have superior auditory discrimination skills (*1*). The weight of the evidence suggests that individuals with AP have access to a set of internal "standards" that allows them to fetch the name of a tone without comparing the representation of the tone they have just heard with a recently fetched representation of a standard (*2*). If so, those with AP may not need to maintain, or update, in their working memory the representations of infrequently occurring events.

The P300 is a positive-going component of the event-related brain potential (ERP) that may be a manifestation of the processes of maintaining or updating working memory. It tends to be large at the parietal electrode. It is quite easily obtained in the "oddball" procedure, in which two discrete stimuli (one frequent and one rare) are presented in a Bernoulli sequence; the subject counts the rare stimulus, which elicits a large P300. A consideration of the variables that control the amplitude and latency of P300 has led Donchin and his colleagues (*3, 4*) to suggest that the component is the manifestation of a subroutine invoked whenever there is a need to update the model of the environment in working memory. If AP subjects process acoustic stimuli without reliance on such schema, they should not emit a P300 in response to novel tones. We have confirmed this prediction. We predicted little, if any, difference in the visually elicited P300 of subjects with and without AP; but we predicted that rare auditory stimuli would elicit a much smaller P300 in the AP subjects than in individuals lacking AP.

Table 1. Correlation (r) of AP score with ERP measures. For both amplitude and area, the difference between the coefficients was significant ($P < 0.05$).

Measure	Auditory	Visual
Amplitude	−0.63*	−0.11
Area	−0.64*	−0.13

*$P < 0.05$.

All subjects were students of music at the University of Illinois (eight males and six females). We assayed their ability to discriminate tones by a method adapted from Lockhead and Byrd (*5*). Each subject heard a series of pure tones generated by a programmable oscillator (*6*). The 81 tone pitches represented the fundamentals of the piano, ranging from 63 to 4186 Hz. The different tones were presented in a random sequence. The subject was instructed to identify the octave number and the name of the pitch (such as B flat or C) associated with each tone. Each tone was sounded until the subject responded. After 500 msec, the next tone was presented. In session 1, ten blocks (with 25 tones per block) were used for practice, and the subject was informed of the correct response after each identification. Ten more blocks were presented without feedback in session 2. The error distributions for session 2 are shown in Fig. 1.

Subjects who described themselves as having the AP skill (AP group) made fewer errors than did those who reported having "normal" pitch discrimination (control group) (Fig. 1). The errors that the AP subjects did make were "octave" errors—that is, they identified the pitch correctly, but they assigned it to a higher