Botstein, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5375 (1980); I. S. Sigal, B. G. Harwood, R. Arentzen, *ibid.* **79**, 7159 (1982); G. Winter, A. R. Fersht, A. J. Wilkinson, M. Zoller, M. Smith, *Nature (London)* **299**, 756 (1982); J. E. Villafranca *et al.*, *Science* **222**, 782 (1983).
2. F. M. Richards and H. W. Wyckoff, in *The Enzymes*, P.D. Boyer, Ed. (Academic Press, New York, ed. 3, 1971), vol. 4, p. 647; P. Blackburn and S. Moore, *ibid.*, vol. 15, p. 317 (1982); R. J. MacDonald, S. J. Stary, G. H. Swift, *J. Biol. Chem.* **257**, 14582 (1982).
3. M. D. Edge *et al.*, *Nature (London)* **292**, 756 (1981); J. Smith *et al.*, *Nucleic Acids. Res.* **10**, 4467 (1982).
4. For discussions of recombinant DNA techniques, see R. Wu, Ed., *Methods Enzymol.* **68** (1979).
5. F. M. Richards and P. J. Vithayathil, *J. Biol. Chem.* **234**, 1459 (1959); T. Takahashi, M. Irie, T. Ukita, *J. Biochem. (Tokyo)* **65**, 55 (1969).
6. T. H. Gawronski and F. Wold, *Biochemistry* **11**, 442, 449 (1972).
7. K. L. Agarwal *et al.*, *Angew. Chem. Int. Ed. Engl.* **11**, 451 (1972); M. J. Gait, in *Polymer-Supported Reactions in Organic Synthesis*, R. D. Hodge and D. C. Sherrington, Eds. (Wiley, London, 1980) p. 435.
8. S. L. Beaucage and M. H. Caruthers, *Tetrahedron Lett.* **22**, 1859 (1981).
9. S. A. Benner, U.S. patent application, December 1982.
10. Many different conditions and supports were used and compared (S. A. Benner *et al.*, in preparation).
11. A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980).
12. E. L. Brown, R. Belagage, M. J. Ryan, H. G. Khorana, *ibid.* **68**, 109 (1979).
13. A modification of the method of D. Hanahan (*J. Mol. Biol.*, in press) with sulfolane substituted for dimethyl sulfoxide.
14. H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, F. M. Richards, *J. Biol. Chem.* **245**, 345 (1970); S. Capaso, F. Giordano, C. A. Mattia, L. Mazzarella, A. Zagari, *Biopolymers* **22**, 327 (1983); A. L. Fink and G. A. Petsko, *Adv. Enzymol. Relat. Areas Mol. Biol.* **52**, 177 (1981); G. A. Petsko, personal communication.
15. J. J. Beintema and J. A. Lenstra, in *Macromolecular Sequences in Systematics and Evolutionary Biology*, M. Goodman, Ed. (Plenum, New York, 1981); personal communication.
16. K. Hofmann, J. P. Visser, F. M. Finn, *J. Am. Chem. Soc.* **92**, 2900 (1970); A. Komoriya and I. M. Chaiken, *J. Biol. Chem.* **257**, 2599 (1982); A. M. Labhardt and R. L. Baldwin, *J. Mol. Biol.* **135**, 231 (1979).
17. R. R. Matheson, Jr., and H. A. Scheraga, *Macromolecules* **11**, 819 (1978).
18. J. F. Brandts, H. R. Halvorson, M. Brennan, *Biochemistry* **14**, 4953 (1975).
19. T. E. Creighton, *J. Mol. Biol.* **113**, 329 (1977); R. R. Hantgan, G. G. Hammes, H. A. Scheraga, *Biochemistry* **13**, 3421 (1974); I. Kato and C. B. Anfinsen, *J. Biol. Chem.* **244**, 1004 (1969).
20. S. E. Zale and A. M. Klibanov, *Biotech. Bioeng.* **25**, 2221 (1983).
21. M. Kimura, *Nature (London)* **217**, 624 (1968); *ibid.* **267**, 275 (1977); *Sci. Am.* **241** (No. 5), 98 (1979); T. H. Jukes, *Science* **210**, 973 (1980).
22. C. C. Levy and T. P. Karpetsky, in *Enzymes as Drugs*, J. S. Holcenberg and J. Roberts, Eds. (Wiley, New York, 1981), pp. 103–166.
23. Supported by the Dreyfus Foundation, the Milton Fund, the Petroleum Research Fund, Alfred Bader, Harvard University, NSF grants PCM-8111659 (D.M.S.) and CHE 8213267 (J.K.E.), NIH grant 1 RO1 GM 30110-01A2 (J.S. and K.P.N.), and the Anna Fuller Fund (W.P.K.). We thank R. Meyers, J. Lingappa, and T. Maniatis for gifts of reagents, F. H. Westheimer and K. E. Bloch for equipment, and E. Brown and O. Makabe for helpful discussions. Address correspondence to S.A.B.

8 November 1983; accepted 27 January 1984

## Insulin Receptor Phosphorylation May Not Be a Prerequisite for Acute Insulin Action

Abstract. *An antiserum to the insulin receptor mimicked insulin's acute actions on glucose transport, phosphorylation of integral membrane proteins, and internalization of the insulin receptor in isolated rat adipose cells. These insulinomimetic actions of the antiserum occurred without the equivalent increase in phosphorylation of the β subunit of the insulin receptor observed with insulin. Thus, a role of receptor phosphorylation in acute insulin action is now questioned.*

Since the initial observations by Kasuga *et al.* (*1*) that insulin can induce the phosphorylation of the β subunit (molecular weight, ≃ 95,000) of its own receptor in both IM-9 lymphocytes and H-35 hepatoma cells, there has been considerable speculation about the potential role of this phosphorylation in insulin action. Further studies have now extended the number of cell types in which this phenomenon can be shown (*2–5*). In addition, in common with the receptors for epidermal growth factor (EGF) (*6, 7*) and several other growth factors (*8, 9*), the partially purified insulin-receptor complex itself appears to mediate both the specific autophosphorylation of a tyrosine residue in its own β subunit (*3*) and the phosphorylation of tyrosine residues in exogenous protein substrates (*10, 11*). However, in the intact H-35 hepatoma cell, insulin induces multisite phosphorylations of the β subunit, with serine residues predominating (*1*).

Häring *et al.* (*2*) have demonstrated an insulin-induced increase in the phosphorylation of the β subunit of the insulin receptor in both intact rat adipose cells and a crude plasma membrane fraction prepared from these cells. This cell type is exquisitely sensitive to insulin and, consequently, has been extensively used for studies of insulin action.

In the present study, we used the rat adipose cell in an attempt to correlate insulin action with receptor phosphorylation. More specifically, we compared the action of insulin with that of a polyclonal antiserum (B-10) against the insulin receptor. This antiserum was derived from a patient with severe insulin resistance and acanthosis nigracans. Such antisera have pronounced insulinomimetic actions including both acute actions, such as inhibition of lipolysis and stimulation of glucose transport in adipose cells, and more long-term actions, such as stimulation of protein, RNA, and DNA synthesis (*12*). In contrast to agents such as $H_2O_2$, vitamin $K_5$, and spermine, these antisera appear to exert their effects through direct interactions with the insulin receptor. Although the immunoglobulin G (IgG) fraction from a rabbit polyclonal antiserum against the insulin receptor in rat liver membrane has been shown to stimulate insulin receptor autophosphorylation in a partially purified receptor preparation from 3T3-L1 cells (*4*), none of these antisera has been investigated with respect to insulin receptor phosphorylation in intact cells.

Isolated rat adipose cells were prepared as described (*13*). Glucose transport activity was measured by the 3-*O*-methylglucose uptake technique (*13*) and insulin binding, by the method of Cushman *et al.* (*14*). To assess the effects of insulin and antiserum B-10 to the insulin receptor on insulin receptor phosphorylation, cells were incubated for 2 hours at 37°C with [$^{32}$P]orthophosphate in a phosphate-free Krebs-Ringer buffer containing bicarbonate (10 m*M*), Hepes (30 m*M*), and 1 percent untreated bovine serum albumin. The cells were then incubated for 1 hour at 37°C with either no addition (basal) insulin or with heat-inactivated antiserum B-10. After incubation the cells were washed and homogenized, and plasma membranes and high- and low-density microsomes were prepared as described (*13*). The membrane fractions were solubilized in 1 percent Triton X-100, and the receptors quantitatively immunoprecipitated with antiserum B-2 to the insulin receptor (1:200 dilution) and staphylococcal protein A (*15*). The immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (*15*). Control precipitations were performed with pooled normal human serum.

At the highest concentration of antiserum tested (1:10 dilution), glucose transport was stimulated to 93 ± 3 percent of the value obtained with a saturating concentration of insulin (Fig. 1). At this same concentration of antiserum, insulin binding was also completely inhibited. Half-maximal effects were observed at antiserum dilutions of 1:200 to 1:100 for both parameters. Figure 2A shows that both insulin and antiserum B-10 increased the phosphorylation of proteins of molecular weight ≃ 115,000 (115K), 43K, and 35K in the low-density microsomes and decreased the phosphorylation of a 26K protein in the plasma membranes. In fact, all of the changes in protein phosphorylation induced by insulin were mimicked by the antiserum, although quantitative differences be-

tween the two agents are apparent. Thus, antiserum B-10 mimics both insulin's stimulatory action on glucose transport activity and insulin's effects on membrane protein phosphorylation.

In parallel with the results previously reported by Häring *et al.* (*2*) using whole cell extracts, insulin treatment of intact adipose cells induced a 1.7- to 2-fold increase in the level of phosphorylation of the 95K receptor subunit in the plasma membranes compared to that observed in the basal state (Fig. 2B). In contrast, antiserum B-10 induced a 30 ± 4 percent decrease. When the intracellular membrane fractions were examined, markedly different phosphorylation patterns were observed. In the high-density microsomes, 4.4-fold and 2.1-fold increases were observed, respectively, in the cells treated with the insulin and antiserum B-10 compared to basal cells, whereas 5.6-fold and 2.6-fold increases were observed in the corresponding low-density microsomes. Thus, antiserum B-10 did not stimulate, but rather decreased, phosphorylation of the β receptor subunit in plasma membranes. Furthermore, while both insulin and antiserum B-10 increased phosphorylation of the β receptor subunit in the two intracellular membrane fractions, the increases induced by antiserum B-10 were only 50 percent of those induced by insulin.

In a study in which we used lactoperoxidase to $^{125}$I-iodinate insulin receptors on the surface of the rat adipose cell and the current fractionation procedure to examine the subcellular distribution of the receptors, we found that insulin induced a rapid, insulin concentration–dependent internalization of its own receptors (*17*). In basal cells, the ratio of insulin receptors among the plasma membranes (PM), high-density microsomes (HDM), and low-density microsomes (LDM) was approximately 1:0.15:0.05, a distribution directly paralleling those of plasma membrane marker enzymes (*17*) and that of the phosphorylated β receptor subunit (Fig. 2B). At steady state after exposure of cells to a saturating concentration of insulin, this ratio went to approximately 0.75:0.3:

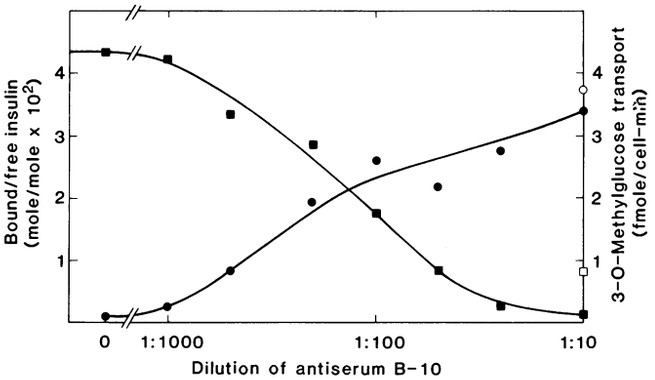


Fig. 1 (above). The concentration dependence of the stimulation of glucose transport and the inhibition of insulin binding by antiserum B-10. Isolated adipose cells, prepared by the method of Karnieli *et al* (*13*), were suspended in a Krebs-Ringer buffer containing bicarbonate (10 m*M*), Hepes (30 m*M*), *p*H 7.4, and 1 percent untreated bovine serum albumin and incubated for 1 hour at 37°C with no additions (0), 700 n*M* insulin (○), or increasing concentrations of heat-inactivated antiserum B-10 (●, ■). After incubation, triplicate samples of cells were assayed for glucose transport activity (●, ○) using the 3-*O*-methylglucose uptake technique (*13*). Additional triplicate samples of cells were assayed for insulin binding (■, □) as follows. The cells were washed three times at 16°C in incubation buffer, *p*H 7.4, supplemented with bacitracin (5 mg/ml) and resuspended to a final volume of 1.0 ml. Tracer $^{125}$I-insulin (100 μCi/μg) was added to a final concentration of 0.1 ng/ml and the cells were incubated for a further 2 hours at 16°C. Samples (200 μl) were removed and centrifuged through oil, and the cells and medium were separated and counted in a Packard gamma counter (*14*). All binding data (■) were corrected for nonspecific binding (□) measured in the presence of 1 μ*M* native insulin. Results are representative of those obtained in at least three separate experiments.

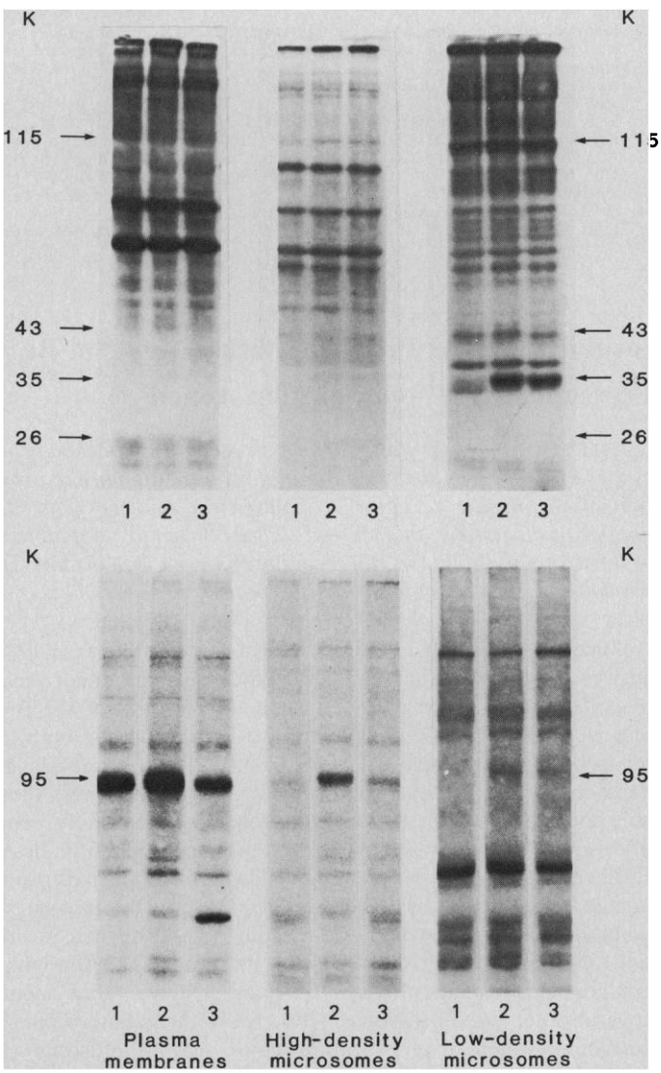


Fig. 2 (right). Comparison of the ability of insulin and antibody B-10 to induce phosphorylation of integral membrane preparations and the insulin receptor. The isolated adipose cells from 48 rats were incubated for 2 hours at 37°C in a phosphate-free Krebs-Ringer buffer containing bicarbonate (10 m*M*), Hepes (30 m*M*), *p*H 7.4, 1 percent untreated bovine serum albumin, and 10 mCi of $NaH^{32}PO_4$. After incubation, the cells were divided into three 30-ml samples and incubated for one more hour at 37°C with no additions (lane 1), 700 n*M* insulin (lane 2), or a 1:10 dilution of heat-inactivated antiserum B-10 (lane 3). The cells were then washed with a tris buffer containing EDTA, sucrose (20, 1, and 225 m*M*, respectively), *p*H 7.4, containing 10 m*M* NaF and 10 m*M* sodium pyrophosphate, and homogenized at 16°C. Plasma membranes and high- and low-density microsomes were prepared as described (*13*). Results are representative of those obtained in at least three separate experiments. (A) Samples (50 μg) of each membrane fraction were subjected to SDS-PAGE (9 percent) gel electrophoresis as described by Laemmli (*16*). The gels were stained, destained, dried, and autoradiographed, and protein molecular weights were calculated with standards as described (*15*). (B) The remaining 2 mg of plasma membranes, 1 mg of high-density microsomes, and 2 mg of low-density microsomes were solubilized in 2 ml of a 1 percent Triton X-100 solution containing 2 m*M* phenylmethylsulfonyl fluoride and aprotinin (1 trypsin inhibitor unit per milliliter). The insulin receptors were then quantitatively immunoprecipitated with antiserum B-2 (1:200 dilution) or control serum and staphylococcal protein A (*15*). The precipitates were then subjected to SDS-PAGE (7.5 percent) and the gels were processed as described for (A). Quantitation (reported in text) was performed by densitometric scanning with a Beckman DU-8 spectrophotometer. The values shown in this representative experiment did not differ in other experiments by more than ±10 percent.

0.15 (PM : HDM : LDM). Thus, insulin induced a 25 percent decrease in the number of receptors initially present in the plasma membranes, and caused corresponding twofold and threefold increases in their numbers in the high- and low-density microsomes, respectively.

When the effects of insulin on phosphorylation of the β receptor subunit (Fig. 2B) are adjusted for receptor internalization as measured previously (*17*), we observe the following relations. (i) Insulin increases the level of phosphorylation of the β receptor subunit in the plasma membranes by 2.3-fold (compared to 1.7-fold without adjustment for receptor number). (ii) Assuming that insulin's stimulatory effect on receptor phosphorylation occurs in the cell's plasma membrane prior to receptor internalization and that receptor internalization per se is not accompanied by any further change in the receptor's phosphorylation state, one would predict 4.6-fold (2 × 2.3-fold) and 6.9-fold (3 × 2.3-fold) increases in the levels of phosphorylation of the β receptor subunit in the high- and low-density microsomes, respectively, from insulin-stimulated cells. These predicted values are only slightly greater than those actually observed (4.4-fold and 5.6-fold, respectively). (iii) Assuming that antiserum B-10 does not effect insulin receptor phosphorylation but does induce a degree of receptor internalization comparable to that induced by insulin, one would predict a 25 percent decrease in phosphorylation of the receptor subunit in the plasma membranes, and two- and threefold increases in the levels of phosphorylation of the receptor subunit in the high- and low-density microsomes, respectively, in antiserum B-10–treated cells. Again, these predicted values are comparable to those observed (0.7-fold, 2.1-fold, and 2.6-fold respectively).

Figure 3 illustrates the results of an experiment in which insulin receptor internalization in response to antiserum B-10 was examined directly. Compared to control serum, antiserum B-10 induced an approximately 20 percent decrease in the levels of both the 135K α and 95K β receptor subunits in the plasma membranes, and concomitant 2.2- and 2.5-fold increases in the levels of both receptor subunits in the high- and low-density microsomes, respectively. This internalization and the levels of phosphorylation were observed consistently (±10 percent) within at least three experiments. Thus, saturating concentrations of insulin and antiserum B-10 appear to induce comparable degrees of insulin receptor internalization in this cell type. Furthermore, the phosphorylation state of the insulin receptor appears to remain unaltered during the internalization process.

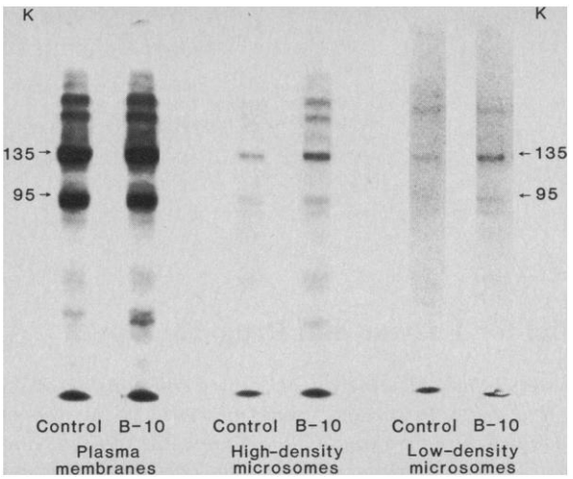


Fig. 3. Insulin receptor internalization in response to antiserum B-10. The isolated adipose cells from 30 rats were suspended in 40 ml of a phosphate-free Krebs-Ringer buffer containing bicarbonate (10 m*M*), (30 m*M*), *p*H 7.4, and iodinated for 30 minutes by means of the $Na^{125}I$ (10 mCi) and lactoperoxidase technique as described (*17*). Iodination was terminated by washing the cells three times with the above buffer supplemented with 1 percent untreated bovine serum albumin. The iodinated cells were divided into two 30-ml samples and incubated for 1 hour at 37°C with either control serum (*Control*) or antiserum B-10 at dilutions of 1:10. The cells were then washed and homogenized, and plasma membranes (1 mg), high-density microsomes (0.6 mg), and low-density microsomes (0.85 mg) were prepared, solubilized in 1 percent Triton X-100, and processed as described for Fig. 2. Quantitation (reported in text) was performed by densitometric scanning with a Beckman DU-8 spectrophotometer or by excision of the labeled bands and direct gamma counting.

These data demonstrate, therefore, that antiserum B-10 does not induce detectable changes in the phosphorylation state of the β subunit of the insulin receptor in the rat adipose cell, despite its clear insulin-mimetic action, as illustrated by its effects on (i) glucose transport, (ii) the phosphorylation state of a variety of integral membrane proteins, (iii) the internalization of the insulin receptor, and (iv) insulin binding. Thus receptor phosphorylation may not be required for acute insulin action in this cell type. This view is further supported by a survey by Zick and co-workers (*18*) of four different antisera to the insulin receptor, including an IgG fraction prepared from antiserum B-10. Although all four antisera inhibited insulin binding to its receptor, immunoprecipitated soluble insulin receptors, and mimicked insulin's stimulatory effect on lipogenesis in rat adipose cells, only antisera B-8 and B-d mimicked insulin's stimulatory effect on the tyrosine kinase activity of a partially purified rat liver membrane insulin receptor preparation tested with either the receptor itself or casein as the substrate for phosphorylation. Antiserum B-2 and the IgG fraction from antiserum B-10 had no detectable effects on receptor tyrosine kinase activity. Finally, Häring *et al.* (*2*) have noted that receptor phosphorylation in the rat adipose cell is at least an order of magnitude less sensitive to insulin than is either the stimulation of glucose transport or the inhibition of lipolysis. However, a low level of receptor occupancy (< 10 percent) is required by insulin to achieve its maximal effects on glucose transport and lipolysis in this cell type. Therefore, these results cannot completely rule out the potential role of small changes in receptor phosphorylation or receptor kinase activity in the mechanism in insulin's acute actions that were not detected in the experiments with antisera to the receptor. Furthermore, the present results do not preclude the possibility that the acute actions of insulin are mediated through the activation of a receptor-associated kinase and subsequent phosphorylation of indigenous substrates other than the receptor itself, or that the longer term growth-promoting effects of insulin may require receptor phosphorylation.

IAN A. SIMPSON
*Cellular Metabolism and Obesity Section, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, Maryland 20205*
JOSÉ A. HEDO
*Diabetes Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases*

### References and Notes

1. M. Kasuga, F. A. Karlsson, C. R. Kahn, *Science* **215**, 185 (1982).
2. H. U. Häring, M. Kasuga, C. R. Kahn, *Biochem. Biophys. Res. Commun.* **108**, 1538 (1982).
3. J. Avruch *et al.*, *J. Biol. Chem.* **257**, 15162 (1982).
4. L. M. Petruzzeli *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6792 (1982).
5. M. Crettaz and C. R. Kahn, *Endocrinology*, in press.
6. M. Chinkers and S. Cohen, *Nature (London)* **290**, 516 (1981).
7. S. Cohen, H. Ushiro, C. Stoscheck, M. Chinkers, *J. Biol. Chem.* **257**, 1523 (1982).
8. K. Glenn, D. W. Bowen-Pope, R. Ross, *ibid.*, p. 5172.
9. F. H. Reynolds, Jr., G. J. Todaro, C. Fryling, L. R. Stephenson, *Nature (London)* **292**, 259 (1981).
10. R. A. Roth and D. J. Cassel, *Science* **219**, 299

(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.
19. We thank S. W. Cushman, J. Roth, and Y. Zick for helpful comments, M. J. Zarnowski for technical assistance, and L. Zalc for typing the manuscript.

6 October 1983; accepted 14 December 1983

## 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 $1.1 \times 10^6$, with more than 80 percent of the conjugate eluting with molecular weight equivalents between $8.5 \times 10^5$ and $1.25 \times 10^6$.

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 ($2 \times 10^6$) 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 ($1 \times 10^7$) 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 | | |