Glycosylated Insulin Complexed to Concanavalin A

Biochemical Basis for a Closed-Loop Insulin Delivery System

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SUMMARY

The oligosaccharides maltose, maltotriose, mannotriose, and mannotetrose have been chemically attached to insulin molecules. Incubation of oligosaccharide and insulin at different molar ratios, with or without addition of cyanoborohydride, showed a nearly linear increase in carbohydrate attachment over time. The intravenous $t_{1/2}$ of ^{125}I-labeled sugar-insulin derivatives was identical to that of unmodified insulin (3.0 min). Biologic activity of these derivatives, assessed in rats by use of a blood glucose depression assay, did not differ significantly from control.

These glycosylated insulin molecules are reversibly bound to the glucose-binding lectin Concanavalin A (Con A). Such sugar-insulin/lectin complexes serve as an insulin reservoir from which sugar-insulin molecules are displaced by glucose. Release of sugar-insulin molecules is a function of the particular sugar-insulin and of the ambient glucose concentration. Glucose displacement of glycosylated insulin complexed to Con A is in direct proportion to the amount of glucose present in the surrounding fluid. At each glucose concentration, the relative binding affinity of the maltotriose derivative is less than that of the mannotriose derivative, while the relative binding affinity of both maltotriose and mannotriose are less than that of the mannotetrose derivative.

Prolonged incubation at 37°C causes sugar-insulin, like unmodified insulin, to spontaneously aggregate into high-mol-wt, nondiffusable complexes. This aggregation phenomenon was found to be markedly inhibited when glycosylated insulins were synthesized utilizing partially sulfated insulin.

Results from the studies described in this report provide the biochemical basis for a closed-loop, glucose-controlled insulin delivery system, utilizing glycosylated insulin complexed to Con A. DIABETES 32:499–504, June 1983.

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Injectable insulin has been available for the treatment of diabetes for more than fifty years. The simple replacement of the hormone by this method has not been able to prevent the devastating pathologic sequelae associated with this disease, however, because injected hormone is released at a fixed rate which is not dependent on the body's physiologic state. Most diabetes investigators now believe that the development of the life-threatening sequelae of diabetes would be prevented if exogenous insulin release could be in keeping with the fluctuating metabolic requirements of the body.[1]

The ultimate goal for diabetes therapy, then, is the development of a simple insulin delivery system directly governed by glucose levels. We have previously demonstrated the feasibility of using oligosaccharide-linked insulin molecules bound to lectins as a biochemical basis for developing an implantable insulin delivery system that could release appropriate amounts of insulin into the blood in response to fluctuating glucose levels.[2] This is shown schematically in Figure 1. The principle is to synthesize insulin derivatives that have attached to each insulin molecule a sugar moiety complementary to the major combining site of such plant-derived carbohydrate binding proteins as Concanavalin A (Con A). These sugar-insulin molecules can then be reversibly bound. Such sugar-insulin molecules are displaced by glucose. These complexes would be enclosed by a membrane having pores which permit passage of glucose and displaced oligosaccharide-insulin, but which prevent passage of the much larger lectin molecules. Initial experiments with the readily available disaccharide maltose have shown that biologically active semisynthetic sugar-insulin derivatives complementary to the major combining site of the lectin Concanavalin A could be synthesized. These maltose-insulin derivatives were reversibly bound to Con A, and most importantly, their displacement by glucose was proportional to

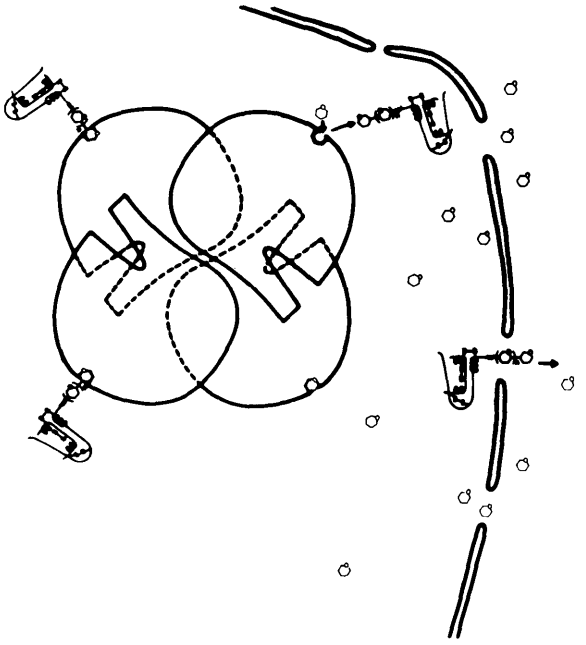

FIGURE 1. Schematic representation of glycosylated insulin bound to Con A. Binding site on lower right subunit of the lectin is shown occupied by a glucose molecule. At the upper right binding site, a glucose molecule is shown displacing a glycosylated insulin molecule. Pores in the membrane permit passage of glucose molecules and displaced oligosaccharide-insulin molecules.

the amount of glucose present in the surrounding fluid.[2] The absolute amount of maltose-insulin released for each given blood glucose level, however, exceeded the physiologic range. Since release of sugar-insulin molecules is a function both of glucose concentration and of each particular sugar-insulin's binding constant to the lectin, other sugar-insulins having different glucose displacement characteristics from complexes with Con A have now been synthesized and characterized, in an attempt to provide levels of insulin release compatible with physiologic requirements. In this communication, we describe the synthesis, chemical characterization, pharmacokinetics, and biological activity of these glycosylated insulins. Comparative data on glucose displacement of glycosylated insulins complexed to Con A are presented, and the effect of various factors on membrane diffusability of displaced insulin is evaluated.

MATERIALS AND METHODS

Synthesis and chemical characterization of glycosylated insulins. Mannose-oligosaccharides were prepared from yeast mannan by a modified acetolysis procedure,[3] followed by deacetylation and gel filtration on BioGel P2. Ten grams of yeast mannan (Sigma Company, St. Louis, Missouri) were dissolved in a mixture of 400 ml acetic anhydride, 400 ml glacial acetic acid, and 40 ml concentrated H_2SO_4. Acetolysis was carried out at 37°C for 18 h. The reaction was stopped by adding 130 ml pyridine. The mixture was evaporated to dryness and 400 ml CCH_3Cl were added. After washing with distilled H_2O (400 ml × 6) to remove salt, the CCH_3Cl was evaporated, methanol (400 ml) and sodium methoxide (40 ml) were added, and the mixture was allowed to stand for 1 h at room temperature. Dry ice was added until the pH was 7.4 and the mixture was then evaporated to dryness. Aliquots of this mannose-oligosaccharide mixture were placed on a column (2.5 × 185 cm) of BioGel P2, −400 mesh, equilibrated with glass distilled water at 65°C. Elution was carried out with glass distilled water at a flow rate of 25 ml/h, and 2.5 ml fractions were collected. Carbohydrate analysis was performed on aliquots of these fractions using the phenol-H_2SO_4 method.[4] The column was calibrated using maltotriose, maltose, and glucose as standards. P2 fractions containing mannotriose and those containing mannotetrose were pooled separately, lyophilized, and stored at 4°C in a dessicator. The oligosaccharides maltose and maltotriose were obtained from Sigma.

Oligosaccharide-insulin derivatives were synthesized using previously described methodologies.[2,5] Oligosaccharides, selected according to their differential affinities for binding to Con A, were incubated with porcine insulin (Eli Lilly and Company, Indianapolis, Indiana) at molar ratios ranging from 12:1 to 600:1 for periods of 1–12 days at 37°C, in 0.05 molar acetate buffer, 1 mM NaN_3, pH 8.2. Incubations were carried out both in the presence and the absence of 0.4 M sodium cyanoborohydride ($NaCNBH_3$). Unreacted oligosaccharides were removed by gel filtration on BioGel P6 (BioRad Company, Richmond, California) and unreacted insulin was separated by affinity chromatography on Concanavalin A coupled to Sepharose 4B (Sigma Company, St. Louis, Missouri). Carbohydrate analysis of these sugar-insulin derivatives was performed by gas-liquid chromatography (Varian Gas Chromatograph, Model 3700) after derivatization with trimethylsialane.[6] Protein content was determined by the Bradford assay.[7]

Selected samples of unreduced sugar-insulin derivatives were incubated with 12 mM NaB^3H_4 for 1 h at 23°C, then extensively dialyzed for 3 days against 0.05 M acetate buffer to remove unbound NaB^3H_4. Aliquots of these samples were hydrolyzed in 6 N HCl in sealed tubes under nitrogen at 105°C for 16 h. Portions were analyzed on a Beckman amino acid analyzer, model 119C (Beckman Instruments, Fullerton, California). The effluent was partitioned by a stream divider so that 90% was collected for determination of radioactivity. In these experiments, tritiated standards of the three possible glycosylated amino acids (glycine, phenylalanine, and lysine) were chromatographed separately to correctly identify the amino acids glycosylated on the insulin. These standards were prepared by incubating L-[^{3}H]glycine, L-[^{3}H]phenylalanine and L-[^{3}H]lysine (New England Nuclear, Boston, Massachusetts), respectively, with a 50 M excess of maltose in 0.1 M acetate buffer, pH 8.2, containing 0.4 M $NaCNBH_3$. After incubation for 18 h at 37°C, unreacted sugar was removed by chromatography on columns (1.5 × 24 cm) of Dow 50-X8, 200–400 mesh, equilibrated with glass distilled water. After washing with 3 column volumes of distilled water, the mixture of each glycosylated and nonglycosylated amino acid standard was eluted with 4 column volumes of 0.5 N NH_4OH. The ammonia was removed by lyophilization, and the amino acids were then applied to columns (1.0 × 25 cm) containing m-amino phenylboronic acid immobilized on BioGel P6 (Affigel 601, BioRad Laboratories), equilibrated in 0.025 M sodium phosphate buffer, pH 9.0, with 1 mM NaN_3. This affinity column selectively retains glucosylated amino acids and peptides.[8] Glucosylated amino acid standards

were recovered by elution with 0.125 M HCl, and subjected to hydrolysis with 6 M HCl in sealed vacuum tubes at 105°C for 8 h prior to chromatography on the amino acid analyzer.

Pharmacokinetic studies. Unmodified insulin and oligosaccharide-insulin derivatives were radioiodinated with ^{125}I using the ICl method.[9] For each milligram of insulin, 200 μCi of ^{125}I-Na (New England Nuclear) were added, followed by the addition of 66 nmol of ICl in 0.4 ml of 0.15 M NaCl. The reaction was stopped after 5 min by the addition of 160 μl of 0.1 M Na_2SO_3. The iodinated derivatives were separated from free iodide on columns (1.5 × 40 cm) of BioGel P2 (BioRad) equilibrated with 0.1 M acetate buffer, pH 7.8. The preparations contained from 0.4 to 0.8 mol iodine/mol insulin. Equal amounts of radioactivity (3.6×10^6 cpm) were injected through the tail vein into female rats of the CD strain weighing from 100 to 150 g. Blood samples (75 μl) were obtained at various time points after injection and the amount of radioactivity in the sera was determined in a Packard Auto-Gamma Scintillation Spectrometer, model 578. The data were plotted on a semilogarithmic scale, and linear regression analysis was used to obtain the value for the rate of disappearance of radioiodinated insulins.

Assessment of biologic activity. Biologic activity of each oligosaccharide-derivative was assessed in rats by use of a blood glucose depression assay.[10] After an overnight fast, each animal received 0.025 mg of protein per 100 g body wt by subcutaneous injection. Each of the animals served as its own control, receiving oligosaccharide-insulin derivative on day 1 and unmodified insulin on day 2. Blood glucose depression was determined 60 min after injection.

Glucose displacement of glycosylated insulins complexed to lectin. Oligosaccharide-insulin derivatives were applied to columns of Con A immobilized on Sepharose 4B (Sigma Company). None of these derivatives were eluted by washing with buffer, while complete displacement from Con A binding sites could be achieved by using 0.2 M α-methyl mannoside. Glucose displacement characteristics of oligosaccharide-insulins were evaluated by using 2.0-ml pulses of sodium phosphate buffered saline (PBS), pH 7.4, containing different concentrations of glucose. Fractions (0.8 ml) were collected at a rate of 30 ml/h and the amount of protein present was determined by the Bradford assay.[7]

Insulin diffusion studies. Insulin at high concentrations (12.5 mg/ml unless otherwise indicated) was incubated with different concentrations of the detergents sodium dodecyl sulfate, deoxycholate, and triton X-100; polyethylene-polypropylene glycol (Genapol, kindly supplied by Hoechst Pharmaceuticals, Frankfurt, FRG); 6 M guanidine hydrochloride, with or without a subsequent dialysis against 60 mM bicarbonate; and 2% human serum. These samples, as well as sulfated porcine sodium insulin, were dialyzed for 8 h against a 1000-fold excess of PBS at 37°C, using dialysis membrane having a molecular weight cutoff of 50,000. Protein content was determined before and after dialysis,[7] and the percentage of dialyzable insulin was calculated.

RESULTS

Synthesis and chemical characterization of glycosylated insulins. The maltose insulin derivative was selected for detailed investigation. Under nonreducing conditions, a Schiff base adduct forms between maltose and insulin, which subsequently undergoes an Amadori rearrangement to form the 1-deoxy-fructosyl-α-1,4-glucopyranoside. In the presence of sodium cyanoborohydride, the imminium moiety is reduced to the corresponding glycitol. Both of these insulin derivatives have a terminal α-D-glucose residue complementary to the binding site of Con A.[11] Analogous reactions occur under reducing conditions with the oligosaccharides maltotriose, mannotriose, and mannotetrose.

Under all incubation conditions studied, the amount of carbohydrate that reacted with insulin increased linearly with time after the first day (Figure 2). Incubation of insulin with a carbohydrate to protein molar ratio of 25:1 in the presence of cyanoborohydride (Figure 2, lower panel) gave time-course values that were nearly identical to those obtained with incubation at a molar ratio of 600:1 without cyanoborohydride (Figure 2, upper panel). Increasing the carbohydrate to insulin molar ratio of the cyanoborohydride incubation fourfold increased the rate of adduct formation only slightly (Figure 2, lower panel). Repeated analyses of duplicate samples of reduced sugar-insulin derivatives after storage for 7 wk at 24°C gave nearly identical values.

Samples of maltose insulin having 1 mol carbohydrate/mol insulin were reduced with NaB^3H_4 and prepared for amino acid analysis. The resultant chromatogram (Figure 3) showed that 49% of the carbohydrate was attached to the N-terminal phenylalanine of the B chain, and 29% to the epsilon amino group of B29 lysine. Twenty-two percent was attached to the N-terminal glycine of the A chain.

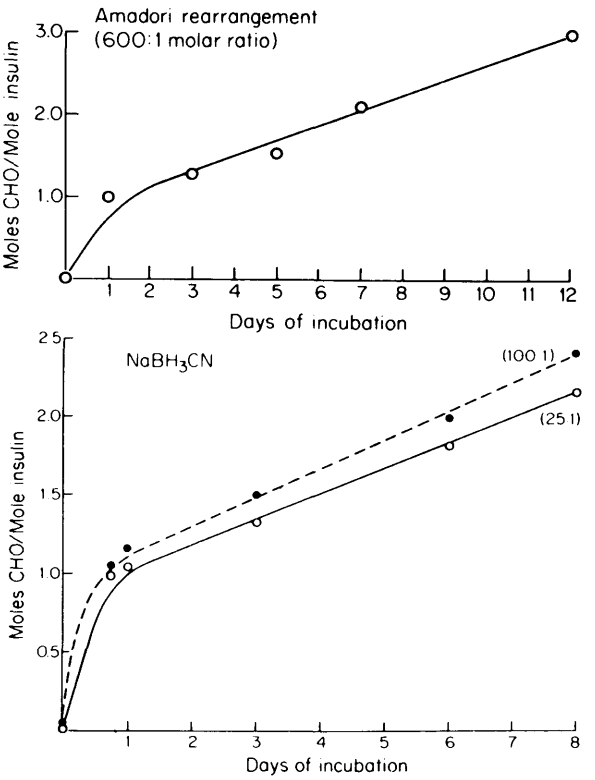

FIGURE 2. Comparison of maltose/insulin molar ratios after varying lengths of incubations with (lower panel) and without (upper panel) addition of cyanoborohydride. The ratios of carbohydrate to protein in the initial incubation mixtures are indicated in parentheses.

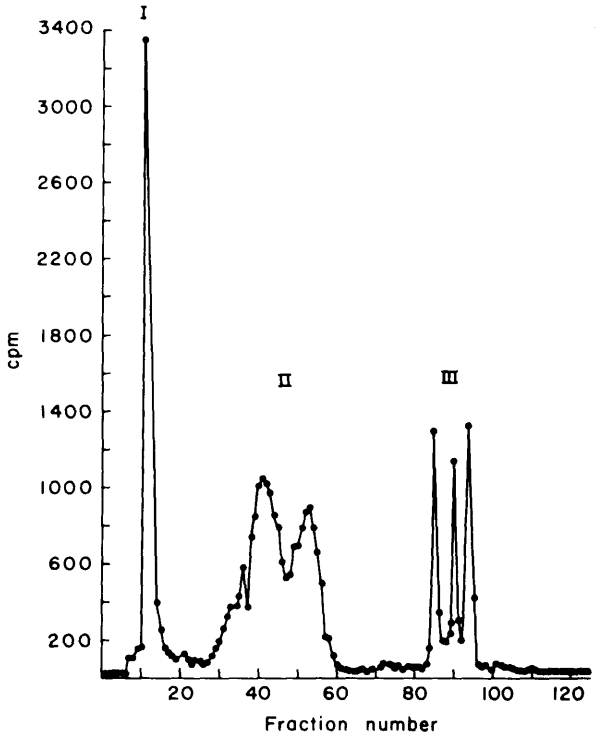

FIGURE 3. Chromatogram of the amino acid analysis of maltose-insulin having 1 mole of carbohydrate per mole of insulin. The sample was reduced with NaB^3H_4 and prepared for amino acid analysis as described in METHODS. I indicates the elution position of hydrolyzed, glycosylated L-[^{3}H]glycine, prepared as described in METHODS. II and III indicate the elution position of glycosylated L-[^{3}H]phenylalanine and [^{3}H]lysine and their hydrolysis rearrangement products, respectively.

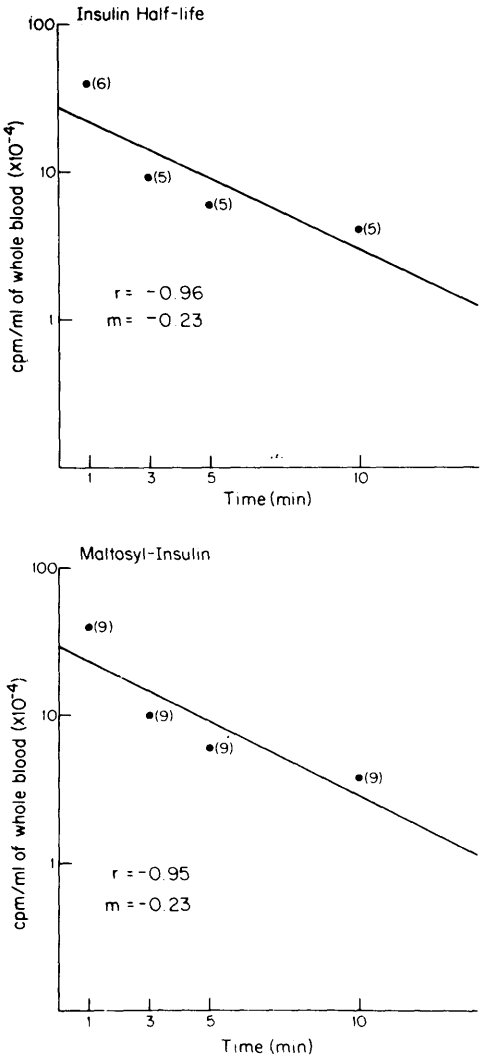

FIGURE 4. Comparison of the intravenous disappearance rate of unmodified insulin and maltose-insulin. Equal amounts of radioactivity (3.6×10^6 cpm) were injected through the tail vein into rats. Blood samples were obtained at the indicated times after injection and counted in a double channel gamma spectrometer. The number of animals at each time point is indicated in parentheses. The data are plotted on a semilogarithmic scale and linear regression analysis was used to obtain values for the rate of disappearance of radioiodinated insulin.

Pharmacokinetic studies. Disappearance of intravenously injected radioiodinated sugar-insulin derivatives was compared with the disappearance rate of unmodified insulin to evaluate potential changes in insulin metabolism induced by the introduction of each different carbohydrate moiety.[12] Equal amounts of radioactivity were injected into rats and the disappearance of radioactivity in the blood was followed. Although the disappearance of radioactivity followed at least two exponential processes, the early time points have recently been shown to represent the true disappearance of insulin.[13] Linear regression analysis of these data (Figure 4) showed that the $t_{1/2}$ of both the unmodified insulin and the maltose-insulin derivative equaled 3.0 min. Similar analyses showed that the $t_{1/2}$ of all oligosaccharide-insulin derivatives tested was essentially unchanged.

Assessment of biologic activity. We have previously reported that the maltose-insulin derivatives are reversibly bound to Con A, and most importantly, that their displacement by glucose is proportional to the amount of glucose present in the surrounding fluid. However, the absolute amount of maltose-insulin released for each given blood glucose level exceeds the physiologic range.[2] Since release of sugar-insulin molecules is a function both of glucose concentration and of each particular sugar insulin's binding constant to the lectin, other sugar-insulins having different glucose displacement characteristics from complexes with Con A were synthesized in an attempt to provide levels of insulin release compatible with physiologic requirements (Figure 5). Maltotriose was the only α-1,4-glucose oligosaccharide selected for study, since higher polymers of this series do not show any further increases in binding to Con A. In contrast to the malto-oligosaccharide series, however, the series of manno-oligosaccharides containing α-1,2 linked mannopyranoside residues shows increased binding as the number of sugar units increases from 1 to 5.[11] For this reason, mannotriose and mannotetrose were both used for synthesis of new derivatives.

Bioactivity of each oligosaccharide-insulin derivative was assessed in rats using a blood glucose depression assay. With sugar-insulin derivatives having 1 mol carbohydrate/mol protein, the bioactivity of each did not differ significantly from control (Table 1). This finding is consistent with previous observations that substitution of only 1 of the 3 primary amino groups of insulin causes little change in hormone activity.[14]

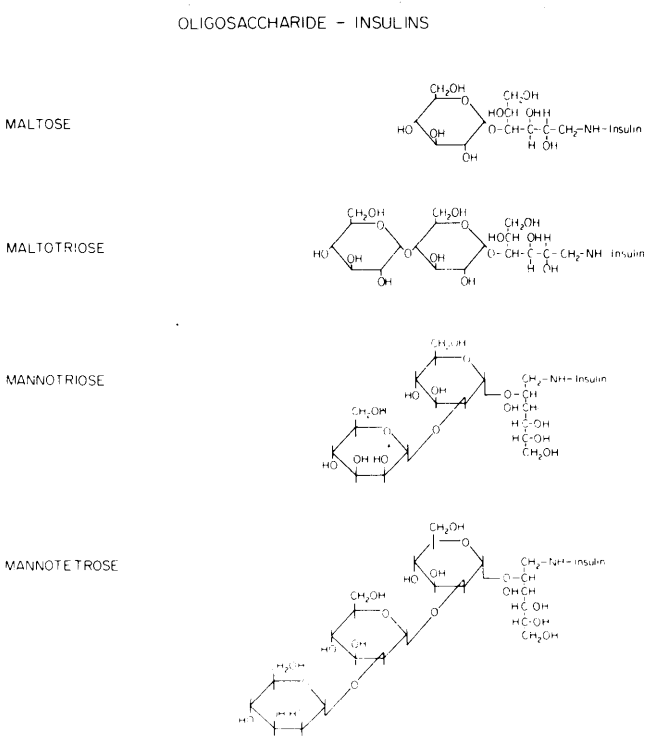

FIGURE 5. Structure of oligosaccharide-insulins synthesized and characterized in these studies. All derivatives were synthesized in the presence of cyanoborohydride as described in METHODS.

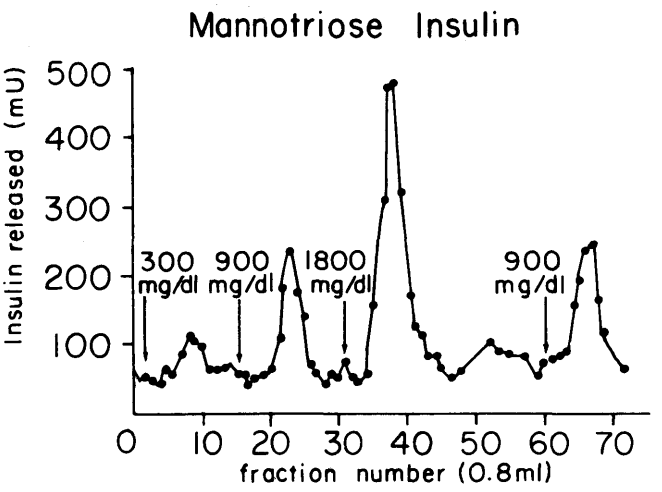

FIGURE 6. Glucose displacement of mannotriose-insulin complexed to Con A. Glucose displacement characteristics were evaluated by using 2.0-ml pulses of PBS containing varying concentrations of glucose. These are indicated in the figure by labeled arrows. The amount of insulin released was determined by the Bradford protein assay.

Glucose displacement of glycosylated insulins complexed to lectin. The method by which glucose displacement characteristics were evaluated for each oligosaccharide-insulin derivative complexed to Con A is illustrated in Figure 6. In this experiment, displacement of mannotriose insulin was accomplished by using 2.0-ml pulses of buffer containing different concentrations of glucose. These data demonstrate that the new sugar-insulin derivatives are reversibly bound to the glucose-binding lectin Con A, and that sugar-insulin displacement from Con A is in direct relation to the amount of glucose present in the surrounding fluid. A comparison of the amount of each new oligosaccharide-insulin derivative displaced from binding sites on immobilized Con A by identical pulses of glucose at two different concentrations is shown in Table 2. At each glucose concentration, it is apparent that the relative binding affinity of the maltotriose derivative is less than that of the mannotriose derivative, while the relative binding affinity of both the maltotriose and mannotriose derivatives is less than that of the mannotetrose derivative.

TABLE 1
Bioactivity of each oligosaccharide-insulin derivative, assessed in rats using a blood glucose depression assay, compared with control insulin values*

Derivative	Blood glucose depression (mg/dl)		
	Glycosylated insulin	Control insulin	P
Maltose-insulin	73.25 ± 6.6	72.5 ± 2.8	NS
Maltotriose-insulin	52.10 ± 2.6	47.22 ± 3.2	NS
Mannotriose-insulin	56.4 ± 4.7	47.22 ± 3.2	NS
Mannotetrose-insulin	75.0 ± 4.0	71.0 ± 4.1	NS

*Each of the animals served as its own control, receiving oligosaccharide-insulin derivative on day 1 and unmodified insulin exactly 24 h later. Blood glucose depression was determined in each case 1 h after injection. Eight to ten animals were used for each assay.

Insulin diffusion studies. Displacement experiments performed at 37°C over longer periods of time indicated that sugar-insulin molecules, like unmodified insulin,[15] spontaneously aggregate into high-mol-wt complexes. Since this spontaneous aggregation of sugar-insulin molecules into high-mol-wt complexes might impair diffusion of displaced sugar-insulins from a membrane-enclosed implantable insulin delivery device, the effects of a variety of preventative maneuvers suggested in the literature were evaluated.[15,16] These data are summarized in Table 3. At the high concentrations of insulin necessary for use in implantable devices, results from pretreatment with a number of detergents, as well as polyethylene-polypropyleneglycol, were uniformly disappointing. Six molar guanidine incubation followed by dialysis against 60 mM bicarbonate gave slightly better results, which could be duplicated by treating with 2% human serum. Sodium porcine insulin (Eli Lilly and Company) was tested because of its superior solubility properties compared with zinc insulin. Controlled insulin sulfation using a newly developed organic synthetic method (Pongor, S., Brownlee, M., and Cerami, A., manuscript in preparation) allowed nearly complete diffusion across a 50,000-dalton dialysis membrane, while causing only minimal change in insulin bioactivity.

TABLE 2
Glucose pulse experiments

Glucose (mg/dl)	Insulin released (mU)		
	Maltotriose	Mannotriose	Mannotetrose
300	675	394	278
900	2268	732	544

TABLE 3
Prevention of insulin aggregation*

Insulin treatment	Percent dialyzable (mol wt < 50,000)
Porcine insulin (20 mg/ml) control	0%
1% DOC	5%
0.5% Triton X-100	26%
15 mg/ml Genapol†	36%
0.5% NP-40	53%
6 M Guanidine pH 9	30%
6 M Guanidine 60 mM HCO_3^-	52%
2% Serum	54%
Na-Insulin (20 mg/ml)	36%
Sulfated-Na-Insulin	92%

*Insulin concentration was 12.5 mg/ml unless otherwise indicated.
†Genapol is a polyethylene-polypropylene glycol derivative kindly provided by Hoechst Pharmaceuticals, Frankfurt, Federal Republic of Germany.

DISCUSSION

The studies presented here demonstrate that stable, oligosaccharide-insulin derivatives can be synthesized using standard methods for coupling aldehydes to amines. In every case, the attachment of carbohydrate to insulin increased linearly with time, after an initial period of rapid glycosylation. From these experiments, synthetic conditions were identified which would result in the attachment of 1 carbohydrate unit per insulin molecule. Under these conditions, 49% of this carbohydrate was attached to the N-terminal phenylalanine of the B chain, and 29% to the epsilon amino group of B29 lysine. Oligosaccharide-insulins were prepared using the α-1,4-linked glucose polymers maltose and maltotriose, and the α-1,2-linked mannose polymers mannotriose and mannotetrose. These sugars were selected on the basis of published relative binding affinities to the lectin Con A.[11] Both the intravenous $t_{1/2}$ and the biologic activity of all monoglycosylated insulin derivatives tested were essentially identical to those of unmodified insulin. Insulin derivatives having more than 1 carbohydrate unit attached per molecule were not used in these experiments, since substitution of all three primary amino groups has been shown to result in approximately 90% loss of hormone activity.[14]

Initial experiments with the readily available disaccharide maltose showed that maltose-insulin derivatives were reversibly bound to Con A, and most importantly, their displacement by glucose was proportional to the amount of glucose present in the surrounding fluid. However, the absolute amount of maltose-insulin released for each given blood glucose level exceeded the physiologic range.[2] The series of new oligosaccharide-insulin derivatives described in this report interact with the lectin Con A in a qualitatively similar manner. However, at each different glucose concentration, the relative displacement of the maltotriose-insulin derivative from the lectin complex is greater than that of the mannotriose derivative, which in turn is greater than that of the mannotetrose derivative.

An implantable closed-loop insulin delivery system using glycosylated insulin complexed to Con A would have to function at 37°C for considerable periods of time. Under these conditions, sugar-insulin molecules, like unmodified insulin,[15] spontaneously aggregate into high-mol-wt, nondiffusable complexes. This aggregation phenomenon was found to be markedly inhibited when glycosylated insulins were synthesized using insulin partially sulfated by a newly developed organic synthetic method.

Results from the studies described in this report provide the biochemical basis for a closed-loop, glucose-controlled insulin delivery system using glycosylated insulin complexed to Con A.

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