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Enzymatically controlled drug delivery

(insulin/trilysyl insulin/polymeric controlled-release system/feedback control/glucose-sensitive polymers)

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ABSTRACT **An approach for providing feedback control for polypeptide drugs in a polymeric controlled-release system uses a trigger molecule and a polymer-bound enzyme that, in the presence of that trigger molecule, will cause an acid or a base to form. When the pH inside the polymer system changes, the solubility of the drug shifts dramatically, which changes the diffusion or dissolution driving force, and hence the release rate changes correspondingly. This concept was tested using a controlled-release system of ethylene/vinyl acetate copolymer containing insulin and immobilized glucose oxidase. The enzymatic reaction of glucose to gluconic acid reduces the pH in the polymer microenvironment. Since insulin solubility increases with decreasing pH (at physiologic pH, this is true for an insulin with an isoelectric point of 7.4 or higher), the release of insulin increases in response to glucose concentration. The feasibility of this concept has been shown using trilysyl insulin with an isoelectric point of 7.4. Multiple exposures to buffered glucose solutions over several weeks caused insulin release to reversibly increase during each exposure. Polymer-implanted diabetic rats infused with glucose solutions showed a significant increase in insulin concentration in 30 min—an effect not observed in three different sets of control rats.**

We present here an approach for feedback control of polypeptides incorporated within polymeric drug delivery systems. This approach is based on the observation that changes in pH can cause dramatic shifts in the solubility of polypeptide drugs; solubility is one of the prime determinants of release rate in any diffusion (1, 2), dissolution (3, 4), or osmotic (5) controlled release system. The system components involve an external trigger molecule and a polymer-bound enzyme that, in the presence of the trigger molecule, will cause an acid or a base to form. To test this concept, we used insulin as a drug and diabetic rats as the animal model. Implantable, glucose-dependent systems have been an attractive field of research for diabetic treatment; research in this area includes implanting an artificial pancreas (6), synthesizing glucose-dependent insulin (7, 8), and preparing glucose-sensitive membranes (9, 10).

We chose to adapt a biocompatible (11) ethylene/vinyl acetate polymeric insulin delivery system capable of treating diabetic rats for over 100 days (12). To establish feedback we used the fact that insulin solubility is pH dependent and that, in the presence of glucose oxidase, glucose is converted to gluconic acid (13). Thus, when this enzyme is incorporated within a controlled-release polymer matrix, external glucose should theoretically reduce the pH in the polymer microenvironment. Since the isoelectric point of insulin is 5.3 (14), when the polymer is exposed to the physiological pH of 7.4, a decrease in insulin solubility and release rate is expected. This undesired effect is overcome by using a modified insulin that contains more basic groups and thus has a higher isoelectric point. Trilysyl insulin with an isoelectric point of 7.4 (15) was used for this purpose. The feasibility of this enzyme-mediated feedback mechanism was investigated by three sets of experiments: (*i*) the effect of glucose on the pH in the microenvironment of the polymer, (*ii*) the effect of glucose on insulin release *in vitro*, and (*iii*) the effect of glucose on insulin release *in vivo*.

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MATERIALS AND METHODS

Insulin. Regular insulin, a zinc–porcine insulin, was a gift from Eli Lilly. Trilysyl insulin was prepared by the method of Levy and Carpenter (16), with some modifications. Porcine insulin (1.8 g) in 95 ml of dimethylsulfoxide and 0.42 ml of triethylamine were reacted with 0.8 g of bis(*tert*-butoxycarbonyl)lysine-*N*-hydroxysuccinimide ester for 48 hr at room temperature, with stirring. Acetic acid (pH 5) was then added, and the mixture was dialyzed against deionized water. The pH was adjusted to 4.8, and the precipitate was isolated by centrifugation, washed with water, and lyophilized. Batches (600 mg) of the residue were dissolved in 10 ml of trifluoroacetic acid. After 1 hr at room temperature, the solutions were concentrated *in vacuo*, and the trilysyl insulin was precipitated with ether, centrifuged, washed several times with ether, and dried over KOH *in vacuo*. The product was dissolved in ammonium bicarbonate and was chromatographed in three batches on Sephadex G-25 in 0.05 M ammonium bicarbonate (pH 8).

Polymer Matrices. Ethylene/vinyl acetate polymer matrices containing insulin and glucose oxidase were prepared according to previous methods (17), with some modifications to incorporate the enzyme. Glucose oxidase (G-2133, Sigma) was incorporated into the polymer by coupling it to Sepharose beads using cyanogen bromide (18). Briefly, Sepharose beads (CL-6B, Pharmacia) were washed with distilled water, dried, weighed, and then transferred gradually to a solution of 60% acetone in water. One milliliter of a solution of 60% acetone in water was added per gram of beads, and the solution was placed in a cold slurry at −12°C to −15°C. A 0.25-ml aliquot of 1 M cyanogen bromide (in dimethylformamide) was then added per gram of beads, with stirring, followed by an equal volume of 1.5 M triethanolamine added over 2 min. The activated beads were poured into 100 ml of 0.1 M HCl at 0°C, and their activity was measured by the König assay (18). The beads were then washed with distilled water at 0°C until the pH of the slurry was ≈5.8. One milliliter of an enzyme solution at 10 mg/ml (pH 8) was added per gram of beads, and the solution was shaken overnight on an aliquot mixer (Miles Scientific, Naperville, IL) at 4°C. The following day, the beads were washed with 100 ml of 0.5 M phosphate buffer at pH 8 and then with 100 ml of 0.1 M phosphate buffer at pH 6.5. The beads were then

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washed with distilled water and lyophilized (at −40°C, 1–5 μm Hg, Labconco freeze dryer 8) to give a fine powder.

Polymer matrices were prepared by a solvent-casting method. Ethylene/vinyl acetate copolymer (Elvax 40P, DuPont) was washed in an extraction system with acetone and distilled water (19). After drying, the polymer was dissolved in methylene chloride (10% wt/vol). Powders of insulin and immobilized glucose oxidase were sieved to <150 μm using US standard sieves. The particles were then mixed and added to the polymer solution (weight percents of insulin, immobilized enzyme, and polymer were 30%, 20%, and 50%, respectively). The mixture was vortexed for 30 sec to give a uniform suspension and poured into a glass mold (4 × 4 × 1 cm) that had been precooled to −80°C by placing it on a leveled slab of dry ice for 10 min. The frozen polymer matrix was removed from the mold using a spatula and dried (2 days in a freezer at −20°C and 2 days at room temperature under vacuum). The matrices were kept at 0°C until used.

Animals. Diabetes was induced in female Sprague–Dawley rats (200–250 g) by an intravenous injection of streptozotocin (U9889, Upjohn; 65 mg/kg of body weight). Animals were regularly checked for urine and blood glucose, and diabetes was considered to be established after the blood glucose level exceeded 375 mg/dl for three separate readings over a period of one week. The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal, 35 mg/kg), and the left jugular vein was cannulated according to the method of Weeks and Davis (20) using silastic tubing (catalog no. 602-135, Dow). The catheter was kept open by a daily injection of 0.5 ml of heparin (1000 units/ml; Elkins-Sinn, Cherry Hill, NJ). Two polymer matrices (1.6 × 0.8 × 0.3 cm) containing either trilysyl insulin plus enzyme, regular insulin plus enzyme, or insulin without enzyme were implanted subcutaneously in the lower back area using a technique previously described (21). The animals were allowed at least 3 days to recover from the surgery and were kept in metabolic cages.

Measurement of Insulin Solubility at Different pH Values. Saturated solutions of insulin were prepared by equilibrating an excess amount of insulin in 600 μl of 9.5 mM phosphate buffer (PBS) at different pH values. All solutions were left overnight to reach equilibrium. On the following day, the suspensions were centrifuged for 3 min to separate the undissolved insulin. The supernatant was collected and diluted by a factor of 5 to 30, and the insulin concentration was measured spectrophotometrically by UV absorption at 220 nm.

Effect of Glucose on the pH in the Microenvironment of the Polymer. The pH inside the polymer matrix was measured using a microelectrode (model SA-1, World Precision Instruments, New Haven, CT). The pH electrode was inserted into the matrix (2–3 mm from the surface), and the pH was recorded as the matrix was exposed to PBS at pH 7.4 containing either glucose at 1000 mg/dl or no glucose.

Effect of Glucose on Insulin Release *in Vitro*. The *in vitro* experiments were conducted in PBS (pH 7.4) at 25°C. Polymer matrices (0.5-cm diameter, 0.3-cm height) containing glucose oxidase and insulin (regular or trilysyl) were held in 3 ml of buffer by a 23-gauge needle (Becton Dickinson) glued to the cap of the vial. They were exposed daily in 2-hr intervals to buffer solutions with (1000 mg/dl) and without glucose, and the insulin released in each sample was measured. The pH of the solution was constant during the experiments as measured by a pH meter (model 601A, Orion Research, Cambridge, MA).

Effect of Glucose on Insulin Release *in Vivo*. The experiment was performed on diabetic rats with different types of implanted polymer matrices or with no polymer matrices implanted. Two blood samples were taken—one at 15 min before and one just before the glucose solution was infused. A 36% (2 M) glucose solution in water was then infused at 4 ml/hr through the left jugular vein using a MicroPerpex pump (2132-001, LKB) for 30 min. Blood samples were taken every 15 min from the tail vein, and the serum was analyzed for glucose and insulin.

Insulin Assay. In the *in vitro* experiments, regular insulin was assayed by using radioactive [^{14}C]insulin (New England Nuclear), and trilysyl insulin was assayed by HPLC. HPLC was performed on a liquid chromatograph (HP1090, Hewlett Packard) using a reverse-phase silica-based column (Vydac C_4 column, Separation Group, Hesperia, CA). The mobile phase was 90% acetonitrile/10% distilled water. The flow rate was 2 ml/min, and the injection volume was 25 μl. In the *in vivo* experiments, insulin was assayed by a double-antibody technique (22). Other reagents were all of analytical grade and were purchased from Sigma.

RESULTS

Insulin Solubility Dependence on pH. The solubility profiles for regular and trilysyl insulin are shown in Fig. 1. Regular insulin shows an isoelectric point of about 5.3, as reported (14), whereas the addition of three lysyl groups shifts the isoelectric point to about 7.4.

Glucose Decreases the pH in the Microenvironment of the Polymer. When the polymer matrices containing insulin and glucose oxidase were exposed to buffer solutions containing glucose at 1000 mg/dl, a decrease of nearly 0.5 pH units inside the matrix was recorded after 4 min; the pH then stabilized. The effect was shown to be reversible. The pH returned to its original value when the solution was replaced with fresh buffer containing no glucose (Fig. 2*A*). Control polymer matrices containing no enzyme did not show any changes in pH after exposure to glucose solutions (Fig. 2*B*). The lower pH recorded inside the polymer in the absence of glucose, as compared to the bulk solution at pH 7.4 may be a result of the Donnan equilibrium, which is frequently observed in charged polymer matrices (23).

Insulin Release Rate Increases as Polymer Matrices Are Exposed to Glucose *in Vitro*. The results of insulin release from different groups of polymer matrices before, during, and after exposure to glucose are shown in Fig. 3. The results were analyzed by the method of analysis of variances with repeated measurements. When polymer matrices containing enzyme and trilysyl or regular insulin were exposed to glucose, the average increase in the release rate of trilysyl

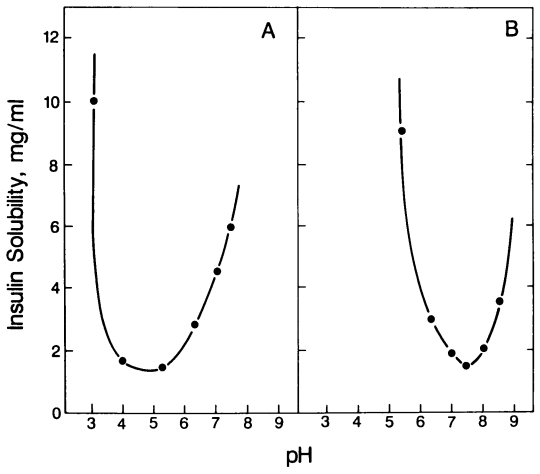

FIG. 1. Solubility dependence on pH for regular (*A*) and trilysyl (*B*) insulin. Each solution was prepared by adding insulin above its saturation level to PBS at different pH values. The solutions were centrifuged, and the concentration of insulin in each supernatant was measured spectrophotometrically.

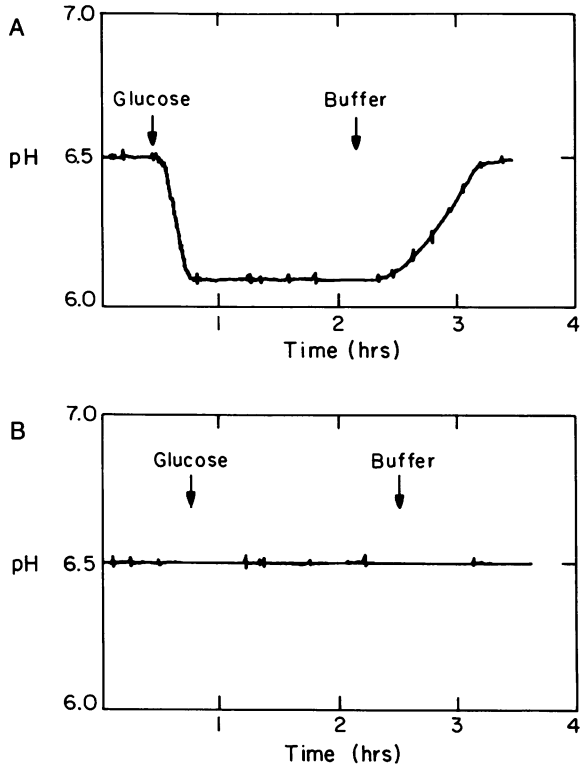

FIG. 2. Changes in pH in the microenvironment of the polymer matrix as matrices are exposed to PBS containing glucose at 1000 mg/dl and to PBS without glucose. (*A*) Polymer matrix containing insulin and enzyme. (*B*) Polymer matrix containing insulin and Sepharose beads (no enzyme).

insulin was 19% ($P \leq 0.001$) (Fig. 3*C*), and the average decrease in the release rate of regular insulin was 29% ($P \leq 0.005$) (Fig. 3*A*). Control polymers containing either regular or trilysyl insulin with no enzyme showed no response to external glucose ($P > 0.5$ in both cases) (Fig. 3 *B* and *D*). The observed decrease in the baseline release rate over the time of the experiment is a characteristic of diffusion systems with a slab geometry (2).

Insulin Release Rate Increases as Polymer Matrices Are Exposed to Glucose *in Vivo*. The results of serum insulin levels in different groups of diabetic rats before and during the infusion of glucose is shown in Fig. 4. A significant increase in serum insulin level ($P \leq 0.001$) was observed when the diabetic rats implanted with polymer matrices containing trilysyl insulin and enzyme were infused with glucose, as compared to the insulin levels before glucose infusion. Similar to the previous *in vitro* results, a decrease in insulin release rate ($P \leq 0.025$) was observed when the diabetic rats implanted with polymer matrices containing regular insulin and enzyme were infused with glucose. The control diabetic rats implanted with polymers containing no enzyme or with no polymer implanted showed no insulin response to glucose infusion ($P > 0.5$ in both cases) (Fig. 4).

DISCUSSION

The above experiments suggest the feasibility of an enzyme-responsive polymeric delivery system. The mechanism is based on a decrease in pH inside the polymer matrix as it is exposed to an external molecule, in this case, glucose (Fig. 2). When the polymer matrices are exposed to the physiological pH of 7.4, a decrease in pH causes an increase or decrease in the solubility of the incorporated substance to be released—i.e., trilysyl or regular insulin (Fig. 1). Since release rate is a function of the drug's solubility (1–5), the release rate of trilysyl or regular insulin is shown to increase or decrease, respectively, upon exposure to glucose. There is a time delay for the release rate to go back to its initial

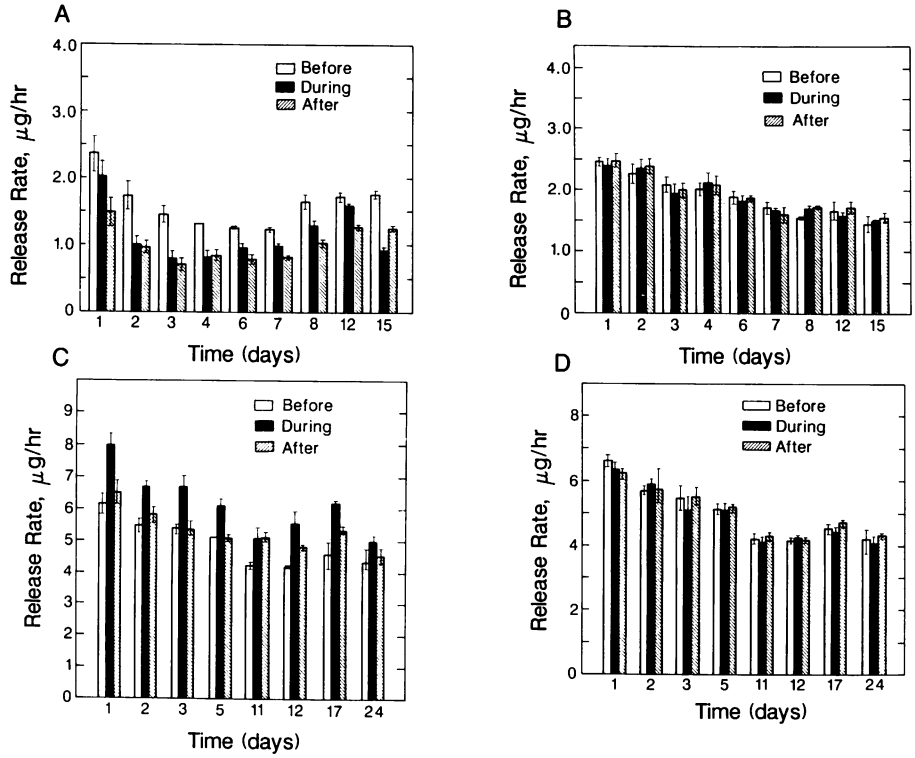

FIG. 3. Average release rate of insulins. Polymer matrices were exposed daily at 2-hr intervals to PBS at pH 7.4 (before), PBS containing glucose (1000 mg/dl) at pH 7.4 (during), and PBS at pH 7.4 (after). Each bar graph shows the mean and the SEM for four polymer matrices over a 2-hr period. (*A*) Polymer matrices containing regular insulin and immobilized glucose oxidase. (*B*) Control for *A*. Polymer matrices contain regular insulin but no enzyme. (*C*) Polymer matrices containing trilysyl insulin and immobilized glucose oxidase. (*D*) Control for *C*. Polymer matrices contain trilysyl insulin but no enzyme.

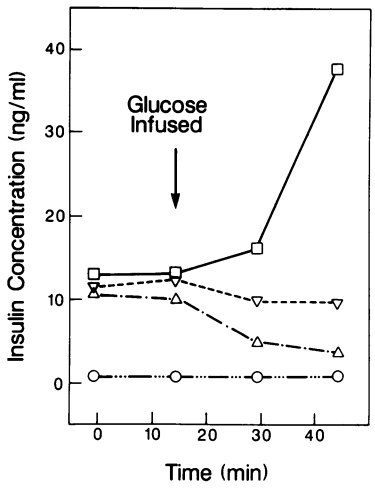

FIG. 4. Changes in serum concentrations of insulin in polymer-implanted diabetic rats. A 36% glucose solution was infused for 30 min through the left jugular vein of the rats, and blood samples were collected from the tail vein every 15 min. The flow rate was 4 ml/hr. The contents of the polymer matrices are as follows: trilysyl insulin and immobilized enzyme (□—□) (n = 7, SEM = 3–15 ng/ml depending on the time points), regular insulin and immobilized enzyme (△-·-△) (n = 3, SEM = 2–5 mg/ml depending on the time points), insulin and Sepharose beads without enzyme (▽---▽) (n = 3, SEM = 2–7 ng/ml), and no polymer (○·····○) (n = 3, SEM = 1–2 ng/ml).

value, which may be due to the diffusion of molecules inside the polymer matrix.

The *in vivo* experiments further confirm the *in vitro* results. An increase in serum insulin is observed in diabetic rats implanted with polymer matrices containing enzyme and trilysyl insulin, and the opposite effect is observed when regular insulin is used.

Various approaches might be used to further augment the response of insulin to external glucose. These include (*i*) incorporating substances like polyglycolic acid inside the polymer, which degrade faster at decreased pH (24), to achieve a cascade effect where an initial decrease in pH due to gluconic acid would further catalyze degradation of polyglycolic acid to glycolic acid; (*ii*) using more pH-sensitive insulins; (*iii*) optimizing the distance between insulin and the enzyme and optimizing the loading levels of insulin and enzyme; and (*iv*) binding the enzyme directly to the polymer. Although such approaches remain speculative, the simplicity of using drug solubility as a method for feedback control offers an approach that could be readily incorporated into different existing controlled-release systems to achieve self-regulated drug delivery.

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