ments using the albumin acetylated with aspirin (VII) also led to a similar conclusion concerning the reactive site for I.

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Pharmaceutical Approach to Subcutaneous Dosage Forms of Insulin

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Abstract □ The present studies were undertaken to describe the dynamic nature of the degradation and absorption of insulin in the subcutaneous injection site and to develop agents which would stabilize this dosage form. [^{125}I]Insulin with 0.2-U/kg of unlabeled insulin in 10 μl of aqueous solution was injected subcutaneously in rats under the depilated skin of the back. At various times, radioactive skin tissue was extracted and assayed for insulin and/or its metabolites by gel filtration. Using these data, absorption and degradation rate constants of these substances were estimated according to a one-compartment model. Absorption rate constants of insulin and its metabolite of low molecular weight (monoiodotyrosine) were 0.021 min^{-1} and 0.107 min^{-1}, respectively, while the degradation rate constant for insulin to monoiodotyrosine was 0.013 min^{-1}. Thus, the bioavailability of insulin injected subcutaneously was lower than expected, suggesting the necessity of stabilizing methods. The protection of insulin from degradation at the site of injection was examined by the addition of various peptides. It was found that benzyloxycarbonyl-Gly-Pro-Leu-Gly was a good stabilizing agent, and remarkably inhibited insulin degradation. This inhibition was confirmed by the increase of immunoreactive insulin level and the decrease of the blood glucose level. We postulated that this peptide protects the injected insulin from degradation by inhibiting the peptidase present in subcutaneous tissue.

Keyphrases □ Insulin—absorption and degradation at the subcutaneous injection site, stabilizing agents for the subcutaneous dosage form □ Stabilizing agent—for insulin, subcutaneous dosage forms, use of the peptide benzyloxycarbonyl-Gly-Pro-Leu-Gly □ Subcutaneous injection—absorption and degradation of insulin, use of benzyloxycarbonyl-Gly-Pro-Leu-Gly peptide for stabilization

Endogenous peptides having specific biological effects have attracted attention as new drugs; however, effective dosage forms of these peptides for clinical usage have been difficult to assess in any but the parenteral dosage forms. Insulin, administered subcutaneously, has been the primary treatment for severe diabetes for several years, even though effective methods have been described for oral (1–3), nasal (4), aerosol (5, 6), and rectal (7, 8) administration. In previous attempts to assess the absorption of insulin from its subcutaneous depot, radioiodinated insulin preparations were injected and the disappearance of the radioactivity from the injection site was measured by an external gamma-counter (9, 10). The stability and bioavailability of insulin administered subcutaneously are not well understood since the metabolites of insulin could not be measured. The kinetics of insulin in the blood after subcutaneous injection are the result of many factors including absorption and degradation in the injection site (11).

Recently, Berger reported that aprotinin increased the absorption rate of subcutaneously injected insulin and amplified its biological effect by inhibiting the local degradation of exogenous insulin at the injection site (12). Little else has been reported about the degradation and absorption of insulin at the injection site, although the absorption characteristics of insulin and other drugs from intramuscular injection sites were investigated (13, 14). The present studies were undertaken to investigate the dynamic nature of the degradation and the absorption of insulin in the subcutaneous injection site and to develop new stabilizing agents for this dosage form.

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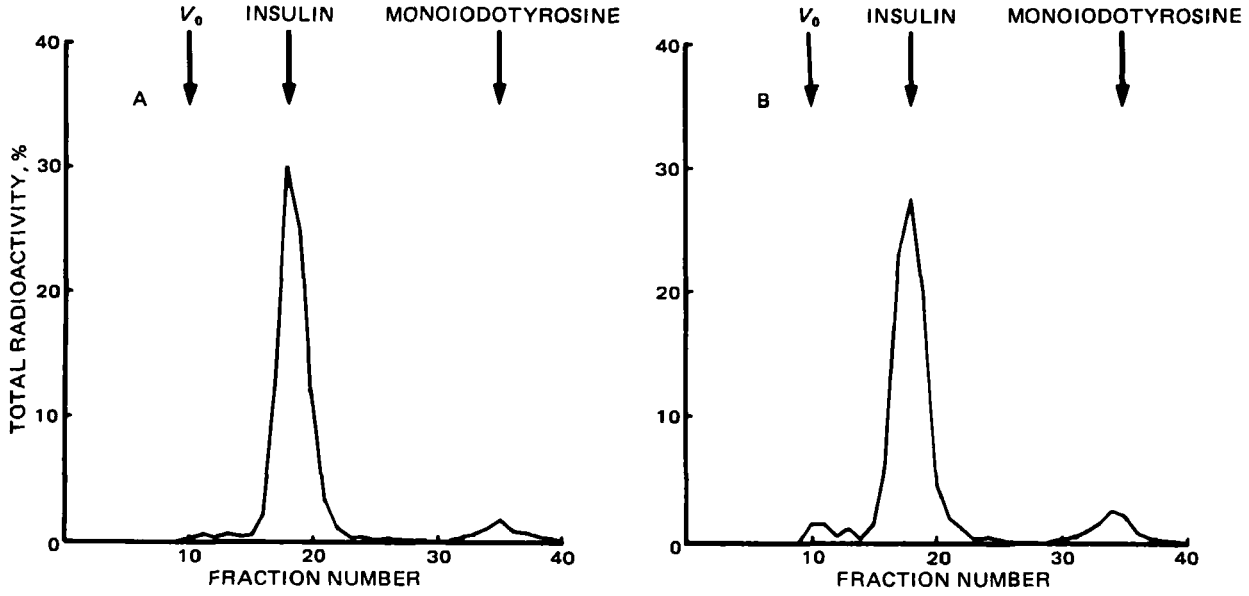


Figure 1—*Gel filtration of the radioactivity extracted from subcutaneous tissue samples 5 (A) and 10 (B) min after insulin injection of 0.2 U/kg sc.*

EXPERIMENTAL

Materials—Monocomponent porcine insulin (26.0 U/mg)[1] was used. [^{125}I]Insulin was prepared by the chloramine T method as described by Hunter and Greenwood (15). The labeled insulin was purified by gel filtration twice using a dextran gel[2]. Benzyloxycarbonyl-Gly-Pro-Leu-Gly (II)[3], benzyloxycarbonyl-Gly-Pro-Leu (III)[3], dinitrophenyl-Pro-Leu-Gly (IV)[3], benzyloxycarbonyl-Gly-Pro(V)[3], and all other chemicals were obtained from commercial sources and were analytical reagent grade.

Animals—Male Wistar rats weighing 125–150 g were used. For the experiment in diabetic rats, diabetes was induced in the rats by femoral-vein injection of streptozotocin after a 16-hr fast, as previously described (16). The streptozotocin was dissolved in acidified 0.9% NaCl (saline), pH 4.5, and given in a dose of 50 mg/kg. Control animals received no injections. The diabetic rats displayed polyuria, glycosuria, failure to gain weight, and significant hyperglycemia (mean blood sugar, 450 mg/dl) at the time of experiment (24–48 hr after the injection of streptozotocin).

Procedure—Rats were anesthetized with pentobarbital (40 mg/kg) and maintained under anesthesia during the experiment. The dorsal skin was depilated and the site for injection was demarcated. Using a thin needle (o.d. = 0.2 mm, i.d. = 0.1 mm)[4], 10 μl of drug solution containing unlabeled insulin (0.2 and 1.0 U/kg) and [^{125}I]insulin, with or without additives, were injected subcutaneously. The rectal temperature was monitored and maintained constant (36.5 ± 0.5°) by keeping the rats in a temperature-constant room. At various times, a skin sample and subcutaneous tissue around the injection site (~4 cm^2 area) was taken for analysis. To estimate the degradation rate of insulin at the injection site when its absorption would be negligible, degradation in a cardiac-arrested rat was investigated. This condition was induced by direct injection of pentobarbital (200 mg/kg) to the heart 5 sec before the experiment. For the experiment conducted to measure the immunoreactive insulin or blood glucose, unlabeled insulin with or without additives was injected.

Analytical Methods—After measurement of the residual amount of radioactivity, the sample was homogenized with 6 *M* guanidine hydrochloride in 2.4 *M* formic acid. The homogenate was centrifuged at 1500×*g* for 10 min to separate the extract. The extract then was applied to a gel filtration column (hydrophilic vinyl gel[5]) that was eluted with 3 *M* guanidine hydrochloride in 2.4 *M* formic acid at 4°. The column size was 1.5 × 48 cm and flow rate was 1 ml/min. Three-milliliter fractions were collected and the radioactivity in each fraction was counted. The column was calibrated with high molecular dextran[6] as a void volume marker (V_0) and with standard insulin and monoiodotyrosine (I). For the measure-

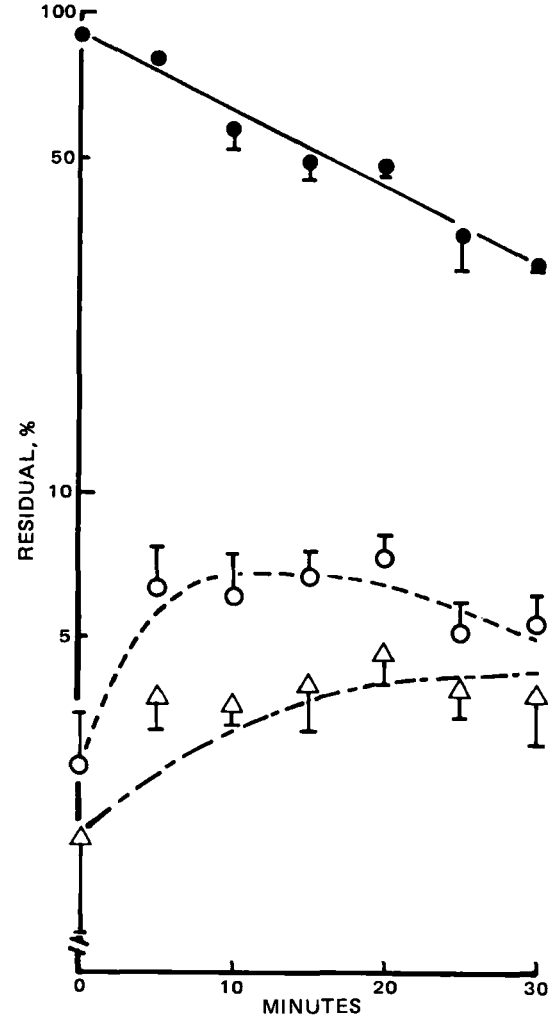


Figure 2—*Disappearance of [^{125}I]insulin from the subcutaneous injection site. Each point represents the mean value of five experiments; vertical bars indicate* SEM. *Each line represents the curve fitted with the one-compartment open model. Key: (●) intact insulin, (○) low molecular weight product (I), and (△) high molecular weight product.*

[1] Novo Industry Co., Copenhagen, Denmark.
[2] Sephadex G-25 & G-50, Pharmacia Fine Chemicals, Uppsala, Sweden.
[3] Protein Research Foundation, Osaka, Japan.
[4] N-733, Hamilton Co., Reno, Nev.
[5] Blue Dextran 2000, Pharmacia Fine Chemicals, Uppsala, Sweden.
[6] Toyopearl HW-55F, Toyo Soda Manufacturing Co., Tokyo, Japan.

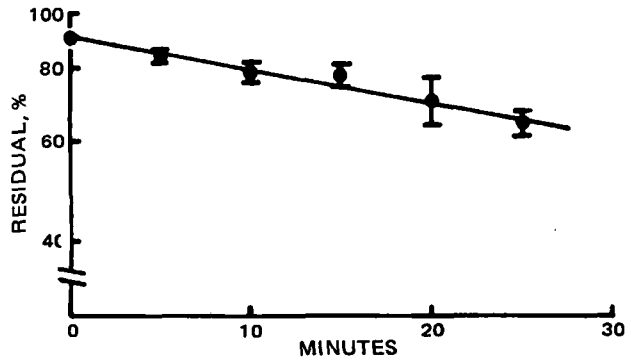
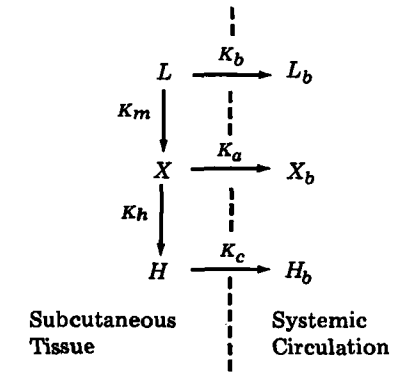


Figure 3—*Degradation of 0.2 U/kg insulin in the subcutaneous injection site of cardiac-arrested rats. The cardiac-arrested condition was induced by intracardiac injection of 200 mg/kg sodium pentobarbital 5 sec before the experiment. Each point represents the mean value of three to five experiments; vertical bars indicate* SEM.

ment of hypoglycemic effect, blood samples were collected from the jugular vein at timed intervals and glucose levels were determined in the serum by the modified method of Hyvärinen (17). Immunoreactive insulin in the plasma was determined by radioimmunoassay (18).

RESULTS AND DISCUSSION

Absorption and Degradation of Insulin in the Injection Site—The drug solution (pH 7.0) containing insulin and [^{125}I]insulin was injected subcutaneously, and the subcutaneous tissue was analyzed. Figure 1 shows the gel filtration pattern of radioactivity extracted from tissue samples obtained 5 and 10 min postinjection. Most of the radioactivity eluted at the position of intact insulin, with a small peak at the void volume (V_0) and at the position of I. The presence of a peak at V_0 indicated the production of a high molecular weight substance, as previously reported in the blood (19). It was also observed that the small peak increased gradually and the main peak (intact insulin) decreased with time.



K_a = absorption rate constant of insulin, K_b = absorption rate constant of I, K_c = absorption rate constant of high molecular weight product, K_m = degradation rate constant of insulin, K_h = aggregation rate constant of insulin, X = amount of insulin in injection site, L = amount of I in injection site, H = amount of high molecular weight product in injection site, X_b = amount of insulin in the body, L_b = amount of I in the body, and H_b = amount of high molecular weight product in the body.

Scheme I

These results suggested that subcutaneously injected insulin was metabolized at the injection site before being absorbed into the systemic circulation.

To clarify this phenomenon, time courses for the clearance of insulin and the formation of its metabolites at the subcutaneous injection site were investigated. Figure 2 shows a logarithmic plot of the insulin, I, and high molecular weight product levels remaining at the injection site as a function of time over a 30-min period. Since a straight line was obtained for the intact insulin, a first-order process seems to be predominant for the clearance of the insulin from the subcutaneous injection site. As the

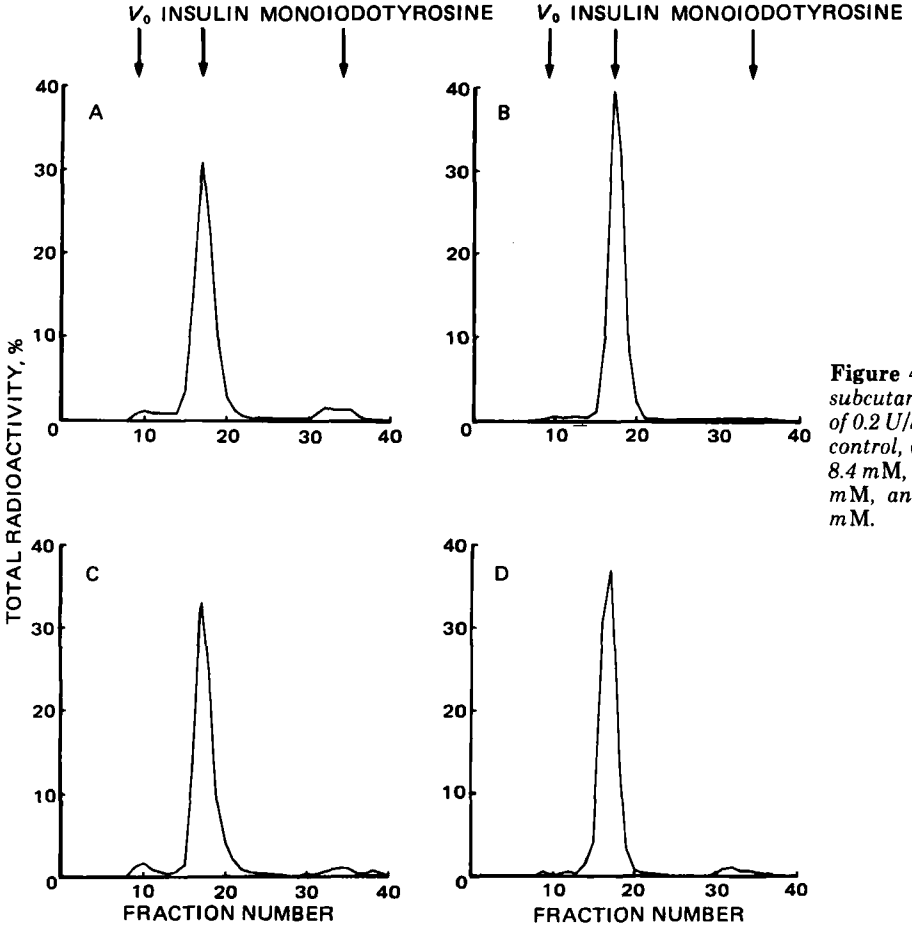


Figure 4—*Gel filtration of radioactivity extracted from subcutaneous tissue samples 5 min after insulin injection of 0.2 U/kg sc with or without stabilizing peptides. Key: (A) control, (B) with benzyloxycarbonyl-Gly-Pro-Leu-Gly(II) 8.4 m*M, *(C) with benzyloxycarbonyl-Gly-Pro-Leu(III) 8.4 m*M, *and (D) with dinitrophenyl-Pro-Leu-Gly(IV) 4.4 m*M.

Table I—Effect of Various Peptides on the Kinetic Parameters for Insulin in the Subcutaneous Injection Site

Compound Injected	Concentration	Kinetic Parameters, min^{-1}				
		K_a	K_b	K_c	K_m	K_h
Insulin	(0.2 U/kg)	0.0208	0.107	0.0113	0.0131	0.0022
Insulin + II	(0.2 U/kg + 8.4 m*M*)	0.0357	0.107	0.0113	0.0008	0.0017
Insulin + III	(0.2 U/kg + 8.4 m*M*)	0.0466	0.130	0.0113	0.0033	0.0025
Insulin + IV	(0.2 U/kg + 4.4 m*M*)	0.0229	0.109	0.0113	0.0069	0.0012
Insulin + V	(0.2 U/kg + 8.4 m*M*)	0.0324	0.201	0.0113	0.0131	0.0028

line curved for I, the main metabolite (20), a fast disappearance rate could be postulated. Meanwhile, the appearance of a high molecular weight product slowly increased. In previous papers (14, 21, 22), it was demonstrated that the rate of drug absorption from the muscle was proportional to the amount remaining in the injection site; drug absorption from the subcutaneous tissue was not demonstrated clearly to be a first-order process. From these results, the absorption mechanism for a subcutaneously injected drug would not be very different from that for an intramuscularly injected drug.

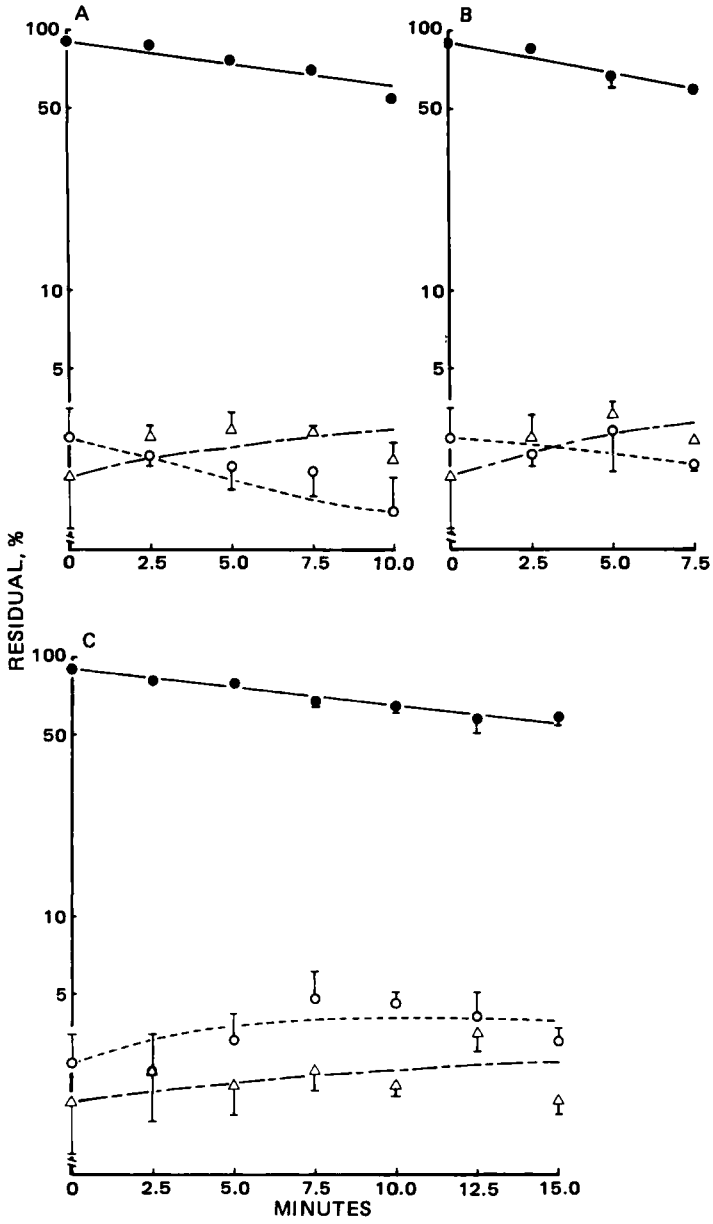


Figure 5—*Disappearance of 0.2 U/kg insulin with stabilizing peptides from the subcutaneous injection site. Each point represents the mean value of three or four experiments; vertical bars indicate* SEM. *Each line represents the curve fitted with the one-compartment open model. Key: (A) with II 8.4 m*M, *(B) with III 8.4 m*M, *and (C) with IV 4.4 m*M, *(●) intact insulin, (○) low molecular weight products (I), and (△) high molecular weight product.*

The disposition of insulin in the subcutaneous tissue was analyzed as assuming a one-compartment open model as shown in Scheme I. Insulin can be biotransformed to a low molecular weight product (I) and to a high molecular weight product, with a degradation rate constant of K_m and a formation rate constant of K_h, respectively. Compound I, insulin, and the high molecular weight product are absorbed independently with absorption rate constants of K_b, K_a, and K_c, respectively. X, L, and H were expressed by (23):

$$X = X_0 e^{-Kt} \quad \text{(Eq. 1)}$$

$$L = \frac{X_0 K_m}{K - K_b}(e^{-K_b t} - e^{-Kt}) + L_0 e^{-K_b t} \quad \text{(Eq. 2)}$$

and

$$H = \frac{X_0 K_h}{K - K_c}(e^{-K_c t} - e^{-Kt}) + H_0 e^{-K_c t} \quad \text{(Eq. 3)}$$

where X_0 is the initial amount of insulin, L_0 is the initial amount of I, H_0 is the initial amount of high molecular weight product, $K = K_a + K_m + K_h$, and t is the sampling time. Data of X, L, and H were fitted to Eqs. 1–3 by nonlinear least-squares regression (24). Refined estimates of K_a, K_b, K_m, and K_h were then obtained. K_c was determined by the subcutaneous injection of the high molecular weight product. Calculated K_a and K_h were 0.0208 and 0.0022 min^{-1}, respectively. The degradation rate constant of insulin to I (K_m) was calculated to be 0.0131 min^{-1}, a value similar to the absorption rate constant. The degradation of insulin in cardiac-arrested rats was investigated to confirm the calculated value. No absorption was observed since the systemic circulation was completely disrupted. Figure 3 shows the plot of the nondegraded insulin level at the injection site of cardiac-arrested rats as a function of time. The decrease of insulin by degradation at the injection site seems to be linear in this semilogarithmic plot. The degradation rate constant (K_m) in this experiment was 0.0137 min^{-1} which was almost the same as the calculated K_m. As K_m was close to K_a, the bioavailability of insulin injected subcutaneously could be lower than expected. The species or age variation for insulin degradation in the subcutaneous tissue will be investigated in future work, since the insulin degrading activity of muscle from rats varies with age (25).

Stabilization of Insulin Injected Subcutaneously—The results obtained in this study prompted the search for methods of stabilizing subcutaneously injected insulin to increase the bioavailability. The

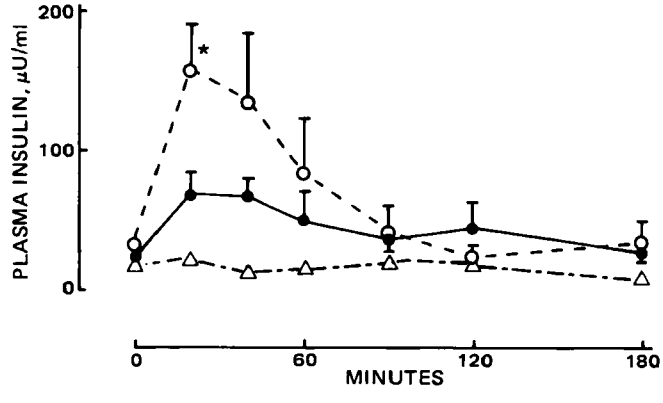


Figure 6—*Effect of II on the increase of plasma insulin levels after insulin injection of 0.2 U/kg sc in normal rats. Each point represents the mean value of four experiments. * Indicates statistical significance ($p < 0.05$), insulin* versus *insulin with II; vertical bars indicate* SEM. *Key: (●) insulin, (○) insulin with II 8.4 m*M, *and (△) II 8.4 m*M.

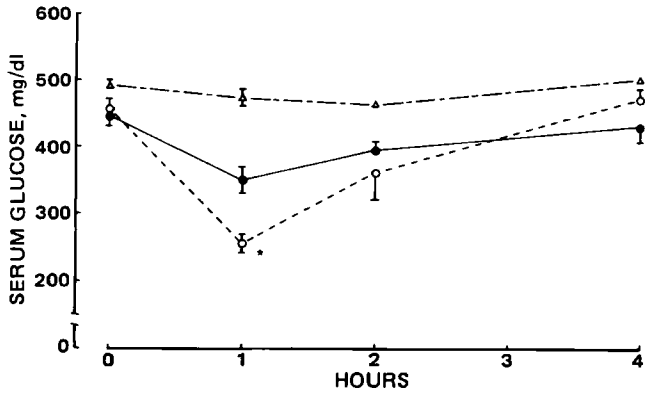


Figure 7—*Effect of II on serum glucose levels after insulin injection of 1.0 U/kg sc in diabetic rats. * Indicates statistical significance* ($p < 0.02$), *insulin* versus *insulin with II; vertical bars indicate* SE. *Key: (●) insulin, (○) insulin with II 8.4 m*M, *and (△) II 8.4 m*M.

concurrent use of peptides and amino acids as stabilizing agents of insulin degradation at the injection site was examined. Among the various peptides tested in this study, benzyloxycarbonyl-Gly-Pro-Leu-Gly (II), benzyloxycarbonyl-Gly-Pro-Leu (III), and dinitrophenyl-Pro-Leu-Gly (IV) exhibited a remarkable protection of insulin from degradation. Figure 4 demonstrates the gel filtration pattern of the radioactivity extracted from subcutaneous tissue sampled 5 min after the injection of insulin with or without these stabilizing peptides. The concentration of I at the injection site was comparatively low when these peptides were coadministered with insulin.

Many amino acids, dipeptides, and aprotinin failed to stabilize insulin at the injection site (data not shown). Aprotinin was examined as an inhibitor of peptidase. Coadministration of aprotinin with insulin led to the fast entry of insulin into the circulation and an accelerated onset of the hypoglycemic action (12). However, it appears from our studies that aprotinin does not stabilize insulin at the subcutaneous injection site; however, it could inhibit insulin degradation after entry into the systemic circulation. The stabilizing effect of these peptides was confirmed by the gel filtration pattern using cardiac-arrested rats. For kinetic analysis, the time course of insulin and its metabolites after the subcutaneous injection of insulin with or without these peptides was investigated. As shown in Fig. 5, the stabilizing effect of these peptides was clearly demonstrated. Using these data, kinetic parameters of the one-compartment model in the presence of stabilizing peptides were estimated, and are listed in Table I. The degradation rate constant of insulin (K_m) in the presence of II at the injection site was 0.0008 min^{-1}, which was less than one-sixteenth of the control. Compounds III and IV were less effective than II. Thus, it is conceivable that the degradation rate of insulin coinjected with II was negligible compared with its absorption rate. Consequently, this peptide can be regarded as a valuable stabilizing agent for insulin in a subcutaneous dosage form.

A significant increase in the absorption rate constant for intact insulin was also observed by the concurrent use of II, III, and benzyloxycarbonyl-Gly-Pro (V), but this effect was not observed using IV. It might be postulated that the amino acid sequence of V could influence this process in a variety of possible ways, such as alterations in the local circulation or possibly by changes in the permeability.

To confirm that II inhibits the degradation of insulin, plasma levels of immunoreactive insulin and serum levels of glucose were analyzed. Figure 6 reveals that the immunoreactive insulin in the plasma was significantly increased when insulin was injected with this peptide, demonstrating the inhibition of insulin degradation at the injection site. Furthermore, the hypoglycemic effect of insulin coinjected with this peptide was investigated in diabetic rats. As shown in Fig. 7, the decrease in serum glucose levels in the diabetic rats after the subcutaneous administration of insulin (1.0 U/kg) was significantly greater in the presence of this stabilizing peptide. The potent effect of II for the insulin administered subcutaneously was clearly demonstrated. We suggest that this peptide protects the injected insulin from being degraded by inhibiting the peptidase localized in subcutaneous tissue.

The study of the degradation and absorption of insulin at the subcutaneous injection site has provided a good understanding of the phenomenon involved, leading to the exploration of stabilizing agents. Further research of the insulin stabilization with other derivatives of this peptide (II) is now in progress. Although a potential effect of these peptides for insulin stabilization in the subcutaneous injection site was demonstrated, additional studies must be carried out before this methodology is useful clinically.

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