Pharmaceutical Research, Vol. 6, No. 2, 1989

Report

Synthesis of Palmitoyl Derivatives of Insulin and Their Biological Activities[1]

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Received May 10, 1988; accepted September 7, 1988

In order to improve the lipophilicity of peptides, bovine insulin was chosen for the chemical modification using palmitic acid. The *N*-hydroxysuccinimide ester of palmitic acid was used for attachment to terminal amino groups, and *p*-methoxybenzoxycarbonyl azide was used for protection of the glycine-A1 amino terminus. We obtained two purified derivatives of insulin, B1-monopalmitoyl- and B1,B29-dipalmitoyl-insulin, which were confirmed to be more lipophilic than the parent insulin on high-performance liquid chromatography (HPLC). The hypoglycemic effects of both products were measured in rats after intravenous and intramuscular injections. The mono derivative was more active than the di derivative and produced a longer effect duration than the native insulin after intravenous injection. The derivatives were also shown to be less immunoreactive as judged by an enzyme immunoassay.

KEY WORDS: insulin; diabetes; palmitoyl derivatives; lipophilic peptide; lipophilicity.

INTRODUCTION

The delivery of polypeptides is critical to their successful clinical application (1). Most of the polypeptides are highly polar, large molecules that are readily digested and poorly absorbed when administered orally or transdermally. Parenteral administration is also problematic because of the short half-lives of the drugs.

Chemical modification of polypeptides may be one of the tools to overcome these limitations. The chemical and biological properties (2–5) of insulin have been studied extensively. It is a rather polar peptide with an isoelectric point at pH 5.5 (6). Since lipophilicity is required to cross membrane barriers, one may improve the transport properties of insulin by means of lipophilic modifications.

On the other hand, chemical modification of insulin often decreases biological activity (4). Lindsay and Shall (3) reported that substitution at the glycine-A1 amino group by acetoacetyl or thiazalidine-carbonyl greatly decreased the biological activity, while modification of the lysine-B29 or phenylalanine-B1 amino groups with these reagents did not affect the biological activity. In the present paper, we describe the synthesis and biological evaluation of palmitoyl derivatives of insulin.

MATERIALS AND METHODS

Materials

Crystalline bovine insulin (25 IU/mg) was purchased from Sigma Chemical Company (St. Louis, Mo.), and *p*-methoxybenzoxycarbonyl azide (pMZ-azide) was purchased from Watanabe Chemical Industry (Hiroshima, Japan). Palmitic acid, t-butoxycarbonyloxyimino-2-phenylacetonitrile (BOC-ON), *N*-hydroxysuccinimide (NHS), *N,N*-dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF), and all other chemicals used were purchased from Nakarai Chemical Co. (Kyoto, Japan) and were of reagent-grade quality.

Synthetic Method for Palmitoyl-Insulins

For the acylation of insulin, the amino groups of phenylalanine-B1 and of lysine-B29 were designated to be modified with palmitic acid among three free amino groups of insulin. The synthetic route is outlined in Fig. 1.

Synthesis of the N-*hydroxysuccinimide Ester of Palmitic Acid (pal-osu) (Step 1)*

Pal-osu was synthesized by the method of Lapidot *et al.* (7). Palmitic acid (50 m*M*;I) was added to a solution of NHS (50 m*M*) in ethyl acetate (150 ml). A solution of DCC (50 m*M*) in ethyl acetate (15 ml) was then added, and the reaction mixture was stirred overnight at 4°C. Dicyclohexylurea was removed by filtration, and the filtrate was concentrated under reduced pressure. Ethanol was added to the crude material and the mixture was left at 4°C until the solution became turbid. Filtration of the crystalline material's recrys-

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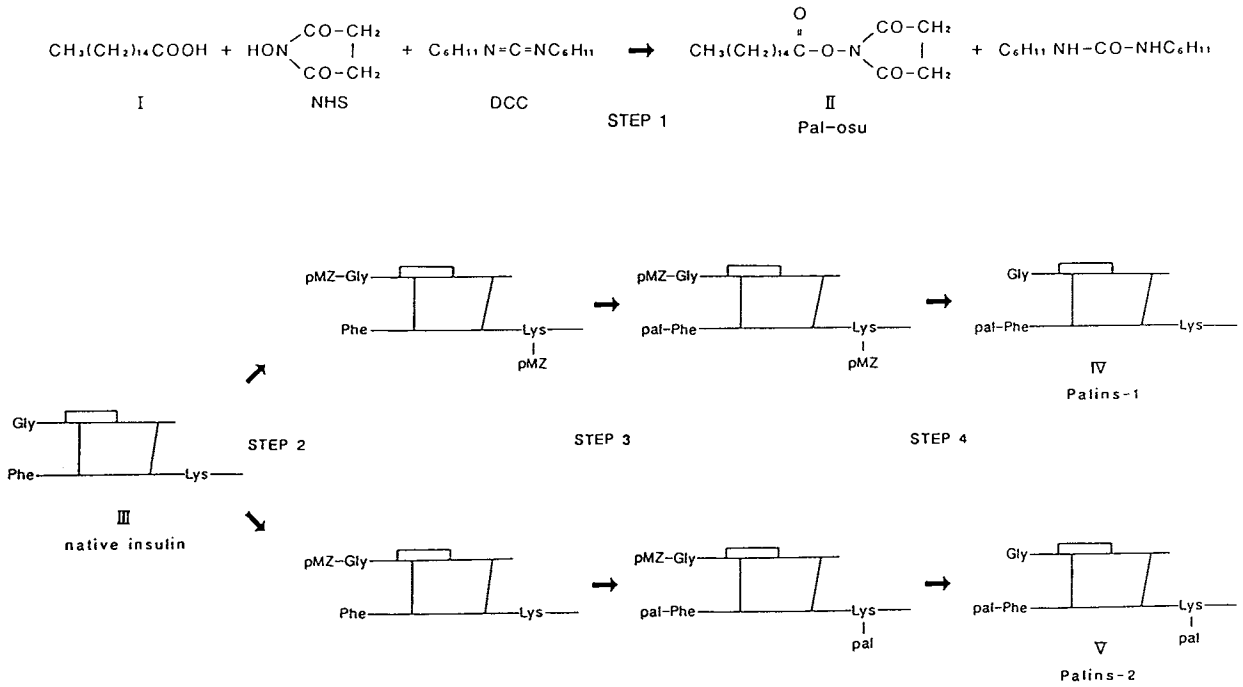

Fig. 1. Scheme depicting the synthesis of the *N*- hydroxysuccinimide ester of fatty acid (Step 1) and palmitoyl-insulins (Step 2 to Step 4).

tallization from ethanol gave colorless needles of pal-osu (II).

pMZ-Insulins (Step 2)

Bovine insulin (200 mg, 38 μM; I) and pMZ-azide (152 μ*M*) were dissolved in 1 *N* $NaHCO_3$:H_2O:DMF (2:3:4; 10 ml), and the solution was stirred for 2.5 hr at room temperature. After the reaction, 50% acetic acid was added to the mixture and evaporated under reduced pressure. The residue was rinsed with ether and a 1% acetic acid aqueous solution, dissolved in 50% acetic acid, and freeze-dried to yield the pMZ-modified insulin.

Reaction between pMZ-Insulins and pal-osu (Step 3)

The above freeze-dried pMZ-insulin was dissolved in DMF; a 50 times amount (mol) of pal-osu was added to the solution, and the mixture was stirred for 2.5 hr at room temperature.

Palmitoyl-Insulins (Step 4)

After the above reaction, DMF was evaporated under reduced pressure. The residue was treated with trifluoroacetic acid (TFA) in the presence of anisole (2 mol or more) in an ice bath for 60 min to remove the pMZ group. After evaporation of TFA *in vacuo* at 30°C, the residue was treated with dry ether.

Chromatographic Purification of the Palmitoyl-Insulins

The palmitoyl-insulins (IV and V) were collected on a column (2.5 × 5.7 cm) of Sephadex G-25 (Pharmacia Co.). The palmitoyl-insulins were dissolved in 1 *N* acetic acid and the column was developed at a flow rate of 4.6 ml/min. The fractions (10 ml each) corresponding to the main peak (fractions 13–16, monitered by UV absorption measurement at 254 nm) were pooled and the protein was freeze-dried.

Final purification was carried out by preparative HPLC. The residue was dissolved in acetonitrile:0.3% TFA (2:3) and the solution was applied to a TSK ODS-120T column (2.15 × 30cm), which was eluted with a gradient of acetonitrile (20–100%, 45 min) in 0.3% TFA at a flow rate of 0.7 ml/min. The eluate corresponding to the two main peaks (monitered by UV absorption measurement at 254 nm) were collected. The solvent was removed by evaporation, and each residue was lyophilized to give a white powder.

Deamination of Palmitoyl-Insulin Derivatives

The final products were deaminated in acetic acid with sodium nitrite. Each product (3 mg) was dissolved in 50% acetic acid (5 ml). Sodium nitrite (300 mg) dissolved in 1 ml of water was added to each solution over a period of 35 min at room temperature with stirring. The reaction was subsequently diluted with water (10 ml) and lyophilized. Purification of the product was effected by chromatography on a Sephadex G-25 (superfine) column (2 × 40 cm), eluting with 0.01 *M* ammonium acetate (pH 8.5). This product was characterized by amino acid analysis. For comparison, a duplicate deamination reaction was performed on a native bovine insulin sample.

Amino Acid Analysis

Samples were hydrolyzed in evacuated sealed glass tubes with 6 *N* HCl at 110°C for 24 hr. The hydrolysate was analyzed on an Hitachi L-3500 amino acid analyzer.

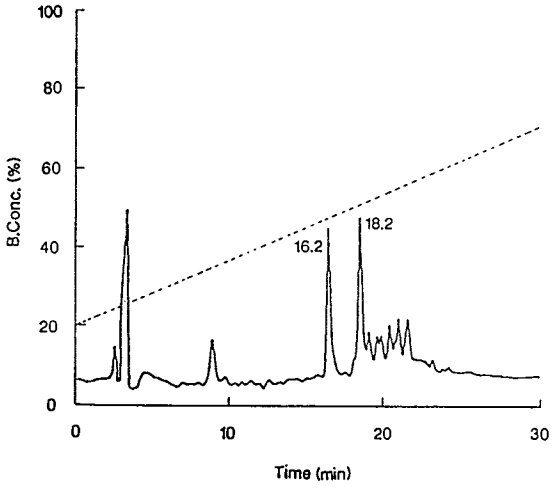

Fig. 2. Reversed-phase HPLC of crude pMZ-insulins with 0.3% TFA on a column (2.15 × 30 cm) of TSK gel 120T. Detection at 254 nm (——); acetonitrile gradient (B; - - - - -).

Preparation of Test Solutions for Biological Experiments

In all the test solutions, samples of native or palymitoyl-insulins were dissolved in 1 *N* HCl under vigorous stirring. The concentration of each insulin was 25 U/ml. Dipalmitoyl-insulin was dissolved in a 1 *N* HCl aqueous solution with a slight turbidity, although native insulin and monopalmitoyl-insulin were completely dissolved.

Animal Experiment

Male Wistar albino rats weighing 200–250 g were fasted for 20–24 hr before the experiment (but given water ad libitum) and anesthetized with an intraperitoneal injection of sodium pentobarbital during the experiment. The test solutions (100 μl each) were administered intravenously into the femoral vein and intramuscularly into the thigh muscle. Blood samples were collected periodically through polyethylene tubing cannulated into the carotid artery and then centrifuged at 1500*g* for 5 min. Plasma glucose concentrations were measured at 505 nm by a glucose-oxidase method using the glucose B test (Wako Pure Chemical Co.) (8).

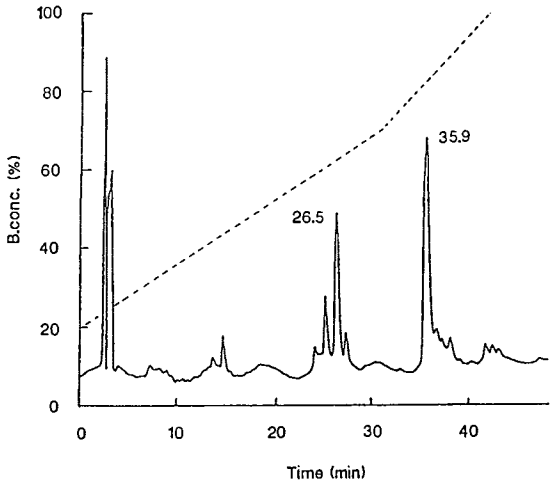

Fig. 3. Reversed-phase HPLC of crude palmitoyl-insulins. Column conditions are the same as for Fig. 2.

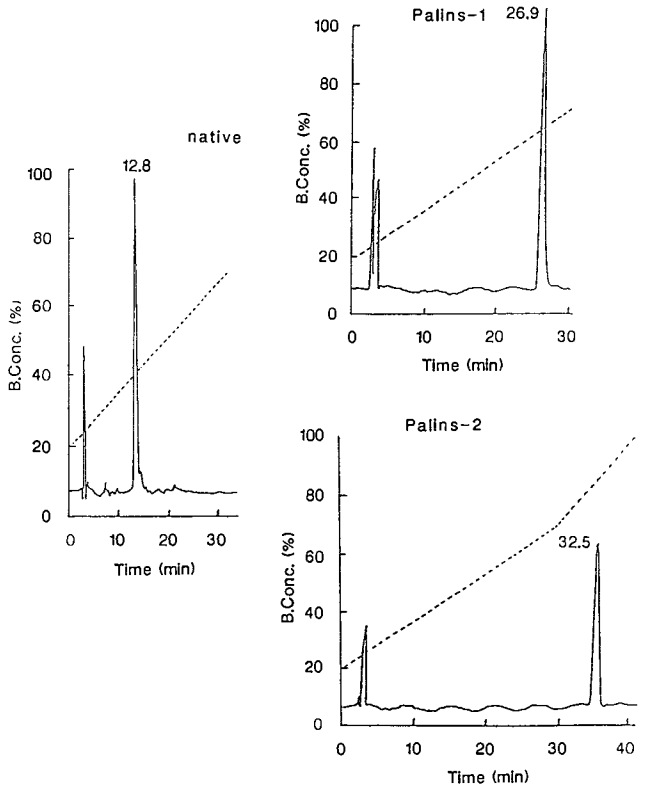

Fig. 4. Reversed-phase HPLC of native insulin and palmitoyl-insulin after gel filtration on Sephadex G-25 and preparative HPLC. Conditions are the same as for Fig. 2.

Pharmacologic Evaluation

The pharmacological activities of new insulin derivatives were evaluated by measuring the hypoglycemic effects.

Table I. Amino Acid Ratios After 6 *N* HCl Hydrolysis of Palmitoyl-Insulin Derivatives

	Insulin			Deaminated palmitoyl-insulins	
	Calc.	Native	Deaminated	Palins-1	Palins-2
Asp	3	2.93	3.03	3.47	3.12
Thr	1	0.94	0.96	0.97	1.00
Ser	3	2.54	2.71	3.05	2.80
Glu	7	7.32	7.50	8.25	7.39
Pro	1	1.15	1.23	1.00	1.09
Gly	4	4.05	3.36	3.28	3.26
Ala[a]	3	3.00	3.00	3.00	3.00
Val	5	3.30	3.70	4.87	4.19
Ile	1	0.31	0.28	0.62	0.53
Leu	6	5.42	5.56	6.47	5.81
Phe	3	2.58	2.21	2.94	2.83
Lys	1	0.95	0.09	0.05	0.69
His	2	1.96	1.93	2.09	1.94
Arg	1	1.10	1.09	1.92	1.89

[a] Diagnostic amino acid.

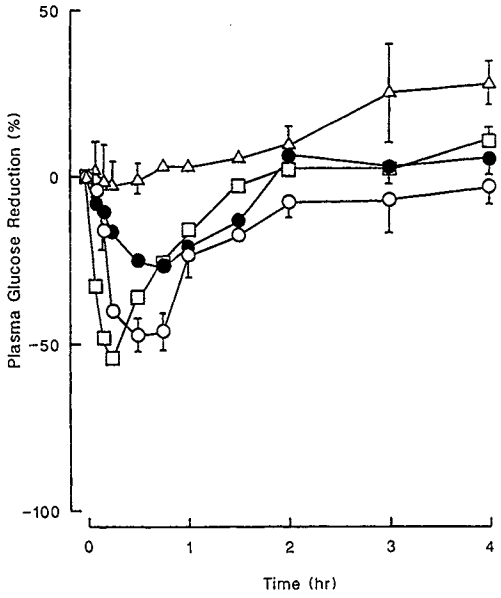

Fig. 5. Change in plasma glucose level following intravenous injection to normal rats. The administration dose was 100 μl containing 2.5 U insulin in 1 *N* HCl (10 U/kg body weight). (△) 1 *N* HCl (sham operation); (○) Palins-1; (●) Palins-2; (□) native insulin (control). Each point represents the mean ± SE of three or four experiments.

The area under the curve (AUC; % glucose reduction × min), the maximum glucose reduction (C_{max}), and the time of maximum effect (T_{max}) were estimated according to Ritschel *et al.* (9).

Immunoreactivity

The relative immunoreactivities of insulin and palmitoyl-insulin derivatives were determined by enzyme immunoassay using the insulin B test (Wako Pure Chemical Co.) (10).

RESULTS AND DISCUSSION

Chemistry

N-Hydroxysuccinimide esters of fatty acids have been previously used for the incorporation of fatty acid into peptides (7). We first synthesized the succinimide derivatives, pal-osu, for attachment to the terminal amino groups of insulin (Step 1). For the selective acylation of insulin, the A1 amino terminus can be protected with butoxycarbonyl azide (BOC-azide) (5). Here we used pMZ-azide as a mild protecting reagent for the same reaction (Step 2). Heterogeneous pMZ-insulin derivatives were obtained in this step. HPLC on a TSK gel ODS-120T column showed two major fragments (peaks at 16.2 and 18.2 min in Fig. 2) which may correspond to mono-pMZ-A1-insulin and di-pMZ-A1,B29-insulin. Thus amino groups at phenylalanine-B1 and lysine-B29 were reactive to the coupling of reagent.

The pMZ-modified insulin products were reacted with 50 times the molar amount of pal-osu (Step 3). Upon further treatment with TFA, the pMZ protecting groups were removed (Step 4), yielding the palmitoyl-insulins.

The final products were purified by gel filtration on Sephadex G-25 and, further, by HPLC on a TSK-gel ODS-120T using gradient elution with acetonitrile in 0.3% TFA. Two major fragments, B1-mono-palmitoyl-insulin (Palins-1;IV), and B1,B29-dipalmitoyl-insulin (Palins-2;V), were obtained on HPLC (Fig. 3), which eluted at 26.5 and 35.9 min, respectively. The purified and isolated compounds showed single peaks at different positions than native insulin on HPLC (Fig. 4), indicating that both derivatives were homogeneous compounds. The retention times of these compounds were ranked as follows—Palins-2 > Palins-1 > native insulin—suggesting that the palmitoyl derivative enhanced the lipophilicity of insulin.

The free amino groups in both derivatives were deaminated with sodium nitrite in order to ascertain the location of the introduced palmitoyl groups. The results of the deamination of Palins-1 and Palins-2 as well as native insulin are shown in Table I. Deamination of native insulin followed by amino acid analysis resulted in the loss of the amino groups of glycine, phenylalanine, and lysine, while Palins-1 lost two amino groups (glycine and lysine) and Palins-2 only the amino group of glycine. Therefore the palmitoyl positions were B1-phenylalanine for Palins-1 and B1-phenylalanine and B29-lysine for Palins-2.

Biological Activities

The hormonal activities of Palins-1 and Palins-2 were

Table II. Plasma Glucose Kinetic Parameters Obtained After Intravenous and Intramuscular Administration of Insulin Derivatives[a]

Route of parenteral administration	C_{max} (%)[b]	T_{max} (min)	$AUC^{0\rightarrow 2\ hr}$[c]	Pharmacological availability (%)
Intravenous				
Native	52.1 ± 2.9	15	2323.5	100
Palins-1	46.2 ± 3.9	30	3106.4	133
Palins-2	25.2 ± 3.5	45	1837.2	79.1
Intramuscular				
Native	29.5 ± 5.4	60	2657.3	114 (100)[d]
Palins-1	14.5 ± 19.1	30	513.1	22.1 (19)
Palins-2	—	—	0	0 (0)

[a] Administered doses corresponded to 10 U of native insulin/kg body weight of rat.
[b] Each value represents the mean with standard error of a group of three or four animals.
[c] AUC represents the area of blood glucose reduction versus time for 120 min. The values were calculated from the mean of the blood glucose reduction of three or four animals.
[d] Each value in parentheses represents the relative availability to the intramuscularly administered native insulin.

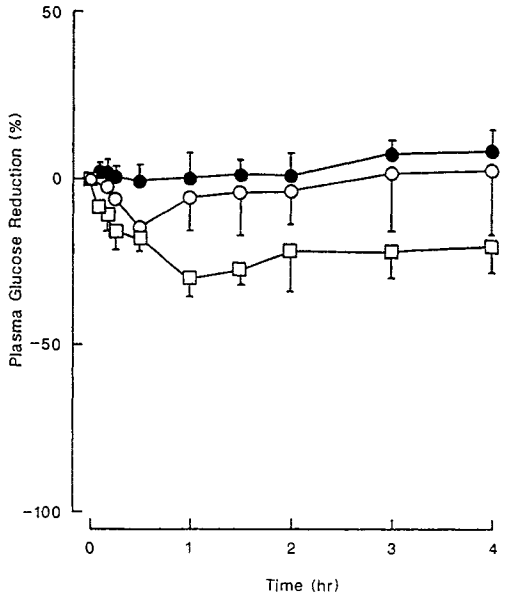

Fig. 6. Change in plasma glucose level following intramuscular injection to normal rats. The administration dose was 100 μl containing 2.5 U in 1*N* HCl (10 U/kg body weight). (O) Palins-1; (●) Palins-2; (□) native insulin (control). Each point represents the mean ± SE of three or four experiments.

tested by measuring the hypoglycemic effect in male rats. Samples (25 U/ml as insulin equivalent) were prepared in 1 *N* HCl and injected intravenously and intramuscularly and the results were compared with those for native insulin.

Figure 5 shows the time courses of plasma glucose concentrations after intravenous injections of the test compounds. Control animals (injection of 1 *N* HCl only) showed no change in plasma glucose concentration for 2 hr following dosing, but then the glucose concentration tended to increase above the baseline. The effect of native insulin was the highest (52% reduction in glycemia), but it was shorter than those of palmitoyl-derivatives. The effect of Palins-1 was higher (nearly equal to native insulin) than that of Palins-2.

Pharmacological availability has been defined as the ratio of the area under the curve (AUC) in glucose reduction versus time of new derivatives to the AUC of the standard, corrected for body weight and insulin dose by Ritschel *et al.* (9).

Table III. Comparison of Biological Activities and Immunoreactivities with Insulin Derivatives

Derivative	Biological activity (%)[a]	Pharmacological availability (%)	Immunoreactivity (%)[b]
Native	100	100	100
Palins-1	88	133	50
Palins-2	48	79	30

[a] C_{max} percentage of native insulin.

[b] The reactivities of duplicate samples of each 25 U/ml insulin derivative were measured by enzyme immunoassay. The values are expressed as the percentage of native insulin.

$$\text{Pharmacological availability} = \frac{\text{AUC (\% response} \times \text{time) i.v. or i.m. of derivatives}}{\text{AUC (\% response} \times \text{time) i.v. or i.m. of native insulin}} \times \frac{\text{(weight/dose) i.v. or i.m. of native insulin}}{\text{(weight/dose) i.v. or i.m. of derivatives}}$$

The AUC over 2 hr was estimated from the plots presented in Fig. 5, because the glucose concentration–time curve exceeded the baseline level after 2 hr in most cases. Further, the maximum glucose reduction (C_{max}) and the time of the maximum effect (T_{max}) were estimated, and their values are given in Table II.

Figure 6 presents the changes in plasma glucose concentration after intramuscular injection. Only a short hypoglycemic effect of Palins-1 was observed, but that of Palins-2 was negligible by the intramuscular administration. The pharmacological parameters obtained from these results are also listed in Table II. After intravenous injection of Palins-1, the highest AUC was obtained with a prolonged effect duration and a pharmacological availability of 133%, while intramuscular injection of Palins-1 showed only 19% of the availability. Palins-2 was less effective but still had 80% of the availability relative to insulin upon intravenous injection.

Table III shows the results of an enzyme immunoassay to test for the immunoreaction to the insulin derivatives. Relative immunoreactivities against native insulin were 50% for Palins-1 and 30% for Palins-2. Therefore, both insulin derivatives cause a marked decrease in immunoreactivity, which is consistent with the results of acylation of the B1 amino group (3). Both derivatives reported here involve the glycine-A1 amino group of insulin. It was shown that substitution at the lysine-B29 or phenylalanine-B1 amino group with the palmitoyl group had little effect on the hypoglycemic activity. Tripalmitoyl insulin, which was obtained as a by-product of the synthesis reaction, had no hypoglycemic activity (data not shown).

The effects of the site of palmitoylation of insulin activity agree with the effects of acetylation reported by Lindsay and Shall (3). As the palmitoyl-insulin analogues described here maintain an insulin activity and exhibit a lower immunoreactivity, they may become useful therapeutically. Fur-

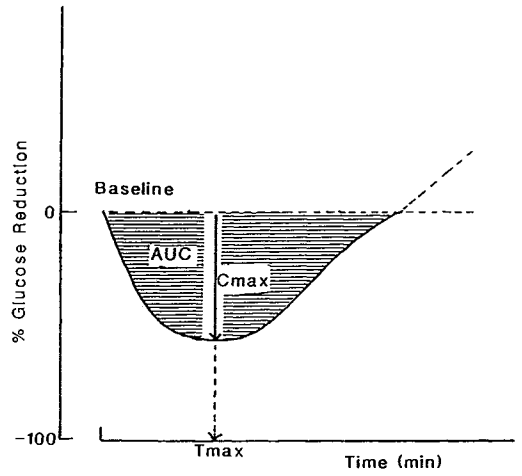

Fig. 7. Schematic representation of analysis of glucose level–time data obtained after administration of insulin derivatives. AUC represents the area of the blood glucose reduction curve under baseline vs time.

thermore, palmitoyl derivatives are predominantly lipophilic and, have thus, physicochemical properties quite different from those of native insulin.

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