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Study of Enamine Derivatives of Phenylglycine as Adjuvants for the Rectal Absorption of Insulin

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Four phenylglycine enamines were synthesized by reacting sodium phenylglycinate and β-diketones, ethyl acetoacetate, and diethyl ethoxymethylenemalonate, ethoxyethyl acetoacetate, and 3-acetylbutyrolactone (abbreviated as PG-EtAA Na, PG-DEMM Na, PG-EthoxyEtAA Na, and PG-AcBu Na, respectively). Increase in the immunoreactive serum insulin levels and decrease in the serum glucose levels were observed in rabbits and dogs following the rectal administration of insulin suppositories in the presence of most PG-enamines. However, PG-AcBu Na failed to promote the rectal absorption of insulin or inulin. The ability of PG-enamines to interact with Ca^{2+} was found to be closely correlated with their promoting efficacies on the rectal absorption of insulin in terms of the maximum response to glucose levels and on the rectal absorption of inulin in terms of the AUC value of serum levels. The lack of promoting efficacy of PG-AcBu Na was explained on the basis of its poor ability to interact with Ca^{2+}.

Keywords——insulin; inulin; suppository; adjuvants enamine derivatives; Ca^{2+}; chelating ability

1,3-Dicarbonyl compounds form enamine derivatives with amines, including amino acids.[1] To protect the active amino group, enamine derivatives have been used for the synthesis of peptides[2] and amino acid-like penicillins.[3] Recently, enamine derivatives of phenyl propanolamine were synthesized and tested as potential drugs in a search for novel amine prodrug derivatives.[4] In a series of experimental studies on enamine prodrugs of antibiotics,[5] we found that the enamine derivatives of amino acid-like β-lactam antibiotics were easily absorbed through the gastrointestinal and rectal tracts and were rapidly hydrolyzed to the parent antibiotics and diketones during the absorption process and in the blood stream in rabbits. Enhanced rectal absorption of the antibiotics was also observed upon concomitant administration of enamine derivatives of amino acids.

The enamine derivatives of amino acids are routinely synthesized under mild conditions and obtained as stable and crystalline compounds.[5] They are easily hydrolyzed to the parent compounds in aqueous solution. Their hydrolysis rates increase in acidic solution and vary widely with change of the enamine structure. Many of them are rapidly hydrolyzed during the absorption and distribution processes and the fraction of enamine derivatives found in the blood stream is not significant.

Insulin, used for the treatment of diabetes mellitus, has been administered by subcutaneous, intramuscular, or intravenous injection since its discovery. Although insulin offers many advantages in the treatment of diabetes, it also has a number of disadvantages. The obvious shortcoming of insulin therapy is the necessity for administration by injection. Many attempts have been made to administer insulin by the oral route through the gastrointestinal tracts[6–11] and by inhalation through the nasal mucosa.[12–14] The gastrointestinal absorption of insulin has been reported to be increased by the coadministration of proteolytic enzyme inhibitor, resulting in a significant reduction in blood sugar levels in mammals,[9] but these results, which were obtained by using insulin in large doses, are considered to be insufficient as a basis for

oral administration of insulin for clinical purposes. The nasal administration of insulin brought about a reduction in the blood sugar level and an increase in the serum level of immunoreactive insulin, but the dose was still much larger than that required for intramuscular injection.

Recently, Nishioka *et al.*,[15–17] Touitou *et al.*,[18] and Shichiri *et al.*[19] reported that insulin formulated in suppository and rectal capsules could be absorbed through the rectal mucosa in the presence of nonionic surfactants. However, further work is still necessary before clinical application can be considered. The development of suitable amino acid enamines for use as absorption adjuvants for the rectal administration of insulin and the mechanism of their promoting activity were the major concerns of study.

Experimental

Materials——Enamine derivatives of DL-phenylglycine tested in this study were synthesized in a manner described by Dane and Dockner[3] (Table I). Commercial crystalline beef insulin (zinc content 0.5% w/w on a dry basis, 24.3 I.U./mg; Commonwealth Serum Laboratories, Australia) and inulin (E. Merck a.-G., Darmstadt, Germany) were used in these experiments. For the study of intramuscular administration of insulin, insulin zinc suspension J.P. IX (Shimizu Pharmaceutical Co. Ltd.) was used. Other reagents used were of analytical reagent grade.

Preparation of Suppositories——Suppositories were prepared by melting suppository base (Witepsol H-15; Dynamit Novel Chemicals, Troisdorf-Oberlat, West Germany) at 40°C on a hot plate and then mixing the ingredients into the melt. The molten mass was poured into disposable plastic molds (Nichi Packing Co., Ltd., Osaka, Japan). They were allowed to solidify at 10°C and stored in well-closed containers until use. Prior to use, all suppositories in plastic molds were allowed to stand for 2 h at room temperature. Each suppositories was conical in shape with a round apex, measured 25 mm in length and 7 mm in maximum diameter, and weighed approximately 1 g. The insulin potency in each preparation was determined by a radioimmunoassay[20] and was found to remain unchanged over 3 months at 10°C. The content of insulin or inulin in any one suppository was within ±3% of the specified content.

***In Vivo* Studies**——Male white rabbits weighing 2.0 to 2.5 kg were used. They were fasted for 16 h before the experiment but water was given freely. One ml of control blood sample was collected from a marginal ear vein before rectal administration of the suppository. The suppository was warmed and liquefied at 40°C before the experiment and 0.5 was taken into a one ml disposable plastic syringe. After insertion of the tip of the barrel (7 mm long) into the anus, the liquefied suppository was injected into the rectum. After rectal administration, the anus was closed with a plastic clip. This procedure resulted in no detectable leakage of rectal contents during the experiment. Blood samples were taken at intervals for 3 h. Each sample was centrifuged for 5 min at 3000 rpm and the serum portion was then frozen until used for assays of glucose and insulin or inulin.

For the study of dogs, male beagles weighing 12.0 to 13.0 kg were used. They were fasted for 16 h before the experiment but water was given freely. Each dog was then given 0.5 g of suppository by insertion into the rectum and returned to an individual cage. Blood collections were made from the jugular vein. Other procedures were the same as those for rabbits except that a clip was not used.

***In Vitro* Studies**——Male Sprague-Dawley strain rats, weighing 250 to 300 g, were fasted for 24 h prior to the experiment. Water was given freely. After decapitation and excision of the rectum, the gut with the anus was washed with Krebs–Ringer's solution containing 0.3% glucose (pH 7.4). A rectal segment 2 cm long including the anus was used. The segment was ligated at the anus and 0.1 g of liquefied suppository was placed inside the sac. The sac was placed into a test tube containing 40 ml of Krebs–Ringer's solution with 0.3% glucose. The solution was kept at 37°C under continuous bubbling with oxygen–carbon dioxide (95: 5). The concentrations of inulin and diketone in the solution were assayed periodically.

Interaction of Enamine with Ca^{2+}——The interactions of enamines with Ca^{2+} at pH 7 to 8 were difficult to determine accurately. Thus, to compare the interactions of enamines with Ca^{2+}, measurements were made at pH 10. The interaction of enamines with Ca^{2+} was studied by the turbidimetric titration method of Ogino *et al.*[21] with some modifications. Ten ml of 0.1% solution of enamine derivatives was taken into a 50 ml glass flask and an equal volume of 0.1% solution of sodium dodecyl sulfate was added. The solution was kept at 37°C under mild stirring with a magnetic stirrer. It was titrated with a solution of calcium chloride (equivalent to 0.1% $CaCO_3$) until the solution became hazy due to the precipitation of calcium dodecyl sulfate. A 0.02 M NH_4Cl–NH_4OH buffer solution at pH 10.0 was used.

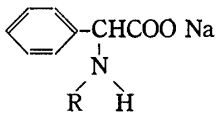
Analytical Method——The serum glucose level was determined by the *o*-toluidine–boric acid method[22] employing a Glucose-Test Kit (Wako Pure Chemicals Co., Ltd., Japan). The serum insulin level was determined by the radioimmunoassay method[22] employing a Phadebas Insulin-Test Kit (Daiichi Radioisotope Lab., Ltd., Japan). The concentrations of inulin in serum and urine were determined following the method[23]

of Schreiner. The concentration of diketone was determined by the following method; 0.5 ml of a sample solution was mixed with 4.5 ml of water and 0.5 ml of a 1% aqueous solution of $Fe(NO_3)_3$ and measured spectrophotometrically at 485 nm. This method was developed in our laboratory.

Results and Discussion

As a preliminary experiment, liquefied suppositories containing either 6 I.U. of insulin/g or 100 mg of PG–EtAA Na/g were administered to rabbits at a dose of 0.5 g of suppository per body. Control experiments were performed by administering suppositories which contained insulin and sodium phenylglycinate or insulin and ethyl acetoacetate (Table II); all results for suppression of glucose levels were negative. The initial slight increase in the levels was considered to be caused by physiological response to the stress of insertion of the suppository.

Table I. Enamine Derivatives studied



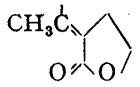
Abbreviation of enamine	β-Diketone used	R
PG–EtAA Na	Ethyl acetoacetate	$CH_3C(=)CHCOC_2H_5$
PG–DEMM Na	Diethyl ethoxymethylene malonate	$C_2H_5OOCC(=CH)COOC_2H_5$
PG–EthoxyEtAA Na	Ethoxyethyl acetoacetate	$CH_3C(=)CHCOOC_2H_4OC_2H_5$
PG–AcBu Na	3-Acetylbutyrolactone	

Table II. Control Experiments in Rabbits

Suppository	Serum glucose level, mg/100 ml, (S.D.)							
	0	0.25	0.5	0.75	1.0	1.5	2.0	2.5 (h)
Witepsol H-15 alone	102.9 (11.4)	135.8 (14.1)	135.8 (21.4)	132.9 (13.0)	127.0 (16.3)	119.3 (20.1)	120.0 (8.5)	109.2 (10.2)
+Insulin (6 I.U./g)	105.1 (11.3)	116.7 (10.2)	124.5 (4.6)	120.8 (13.6)	137.3 (16.7)	112.6 (10.4)	118.4 (3.8)	104.3 (13.5)
+Insulin (6 I.U./g)+PG Na[a] (10%)	103.8 (19.2)	105.2 (12.3)	96.2 (11.6)	90.4 (10.2)	94.3 (11.1)	104.3 (6.7)	92.8 (11.7)	101.4 (10.2)
+Insulin (6 I.U./g)+EtAA[b] (10%)	99.8 (10.4)	102.3 (12.1)	109.6 (8.9)	118.7 (6.8)	104.6 (13.5)	108.2 (11.7)	100.6 (10.6)	103.8 (9.2)
+PG–EtAA Na (10%)	111.3 (14.2)	129.5 (18.3)	115.6 (20.4)	119.8 (15.3)	121.6 (8.6)	116.8 (10.5)	118.9 (11.3)	112.3 (11.5)

Each value is the mean of 6 to 10 experiments.
a) Sodium phenylglycinate.
b) Ethyl acetoacetate.
The results for suppositories containing insulin with or without PG Na or EtAA, or containing PG-EtAA Na alone or Witepsol H-15 alone are not significantly different, statistically.

The mean serum levels of glucose and immunoreactive insulin in six rabbits and four dogs are presented in Fig. 1 following the administration of liquefied suppository at a dose of 0.5 g/body, which was equivalent to doses of 3 I.U. of insulin and 50 mg PG–EtAA Na. The administration of the suppository to rabbits caused a rapid increase in the serum insulin level to a peak level at 30 min; it then decreased gradually to the normal level within 3 h after the

administration. In dogs, the serum level of insulin reached the peak at 30 min. These results indicate that PG–EtAA Na significantly promotes the rectal absorption of insulin both in rabbits and dogs.

To study the dose-response profiles of insulin, suppositories of 0.5 g containing 0.5—12 I.U. of insulin and 10% PG–EtAA Na were administered to rabbits (Fig. 2). The response to insulin was expressed in terms of the maximum change in glucose level (per cent of the starting level). In the rectal administration of insulin in the presence of 10% PG–EtAA Na, 5 I.U. of insulin was required to reduce the glucose level to half the normal level in rabbits. It should be noted that 3 I.U. of semi-lente insulin, which is a very popular insulin product in the clinical field, was required to produce the same suppression of glucose level by intramuscular administration to rabbits (Fig. 2).

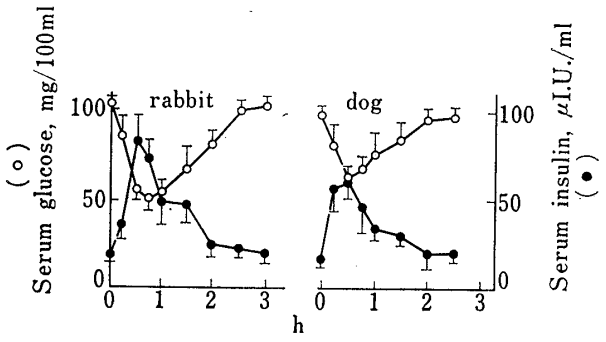


Fig. 1. Change in the Serum Levels of Glucose and Insulin in Rabbits and Dogs

Values represent the means ± S.D. of 6 rabbits or 4 dogs. Suppositories containing 6 I.U. of insulin/g and 10% PG-EtAA Na were administered at a dose of 0.5 g of suppository/body in liquefied form for rabbits and in solid form for dogs.

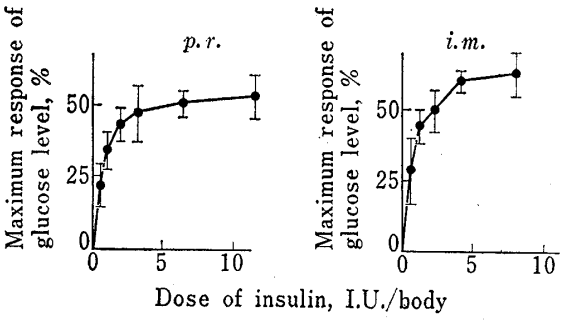


Fig. 2. Dose-response Profiles of Insulin in Rabbits administered Liquefied Suppositories containing 1—12 I.U. of Insulin/g and 10% PG–EtAA Na at a dose of 0.5 g Suppository/Body (*p.r.*) or injected with 0.5—8 I.U. of Semi-lent Insulin/Body intramuscularly (*i.m.*) in Terms of the Maximum Change in Glucose Level as Per Cent of the Starting Level

Each value represents the mean ± S.D. of 6 experiments.

To study the effect of PG–EtAA Na concentration on the absorption of insulin in rabbits, suppositories containing 6 I.U. of insulin/g and 0.5 to 20% PG–EtAA Na were administered at a dose of 0.5 g, and the maximum response and the peak serum insulin level were measured (Fig. 3). It was noted that the peak serum insulin level increased sharply with increase in the concentration of PG–EtAA Na. Similar results were obtained with dogs. Further study is required to clarify the present finding that the response of glucose level reached a limit of around 50% regardless of the gradual increase in the insulin levels.

To study the effect of the ketone on the promotive efficacy of PG-enamines, suppositories containing 6 I.U. of insulin/g and 1% of one of the PG-enamines shown in Table I were administered to rabbits (Fig. 4). It appears that 1% enamine in a suppository might be enough to provide significant adjuvant ability from the results shown in Fig. 3. PG–AcBu Na failed to decrease the glucose level even at the concentration of 10%.

Many workers have demonstrated that many water-soluble drugs including ionized forms are normally poorly absorbed from the intestinal mucosa, but the coadministration of EDTA is known to enhance the absorption of hydrophilic substances.[24] Salicylic acid is known to be absorbed from the intestinal[25] and rectal[26] tracts mainly in the ionized state and the mechanism has been partly explained on the basis of the salicylate-Ca^{2+} interaction. Enamine derivatives of amino acids and β-diketones are considered to interact with metal ions. The ability of enamines and ketones to interact with Ca^{2+} was therefore studied (Table III). The β-diketone moiety of PG-enamine may interact with Ca^{2+}.

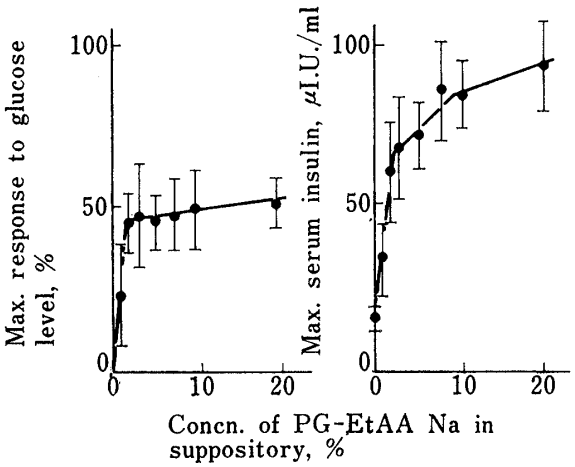


Fig. 3. Effect of the Concentration of PG–EtAA Na on the Maximum Response in Terms of Glucose Level (Per Cent of the Starting Level) and the Peak Serum Level of Insulin in Rabbits

Each value represents the mean ± S.D. of 6 experiments. The dose of insulin was 3 I.U./body.

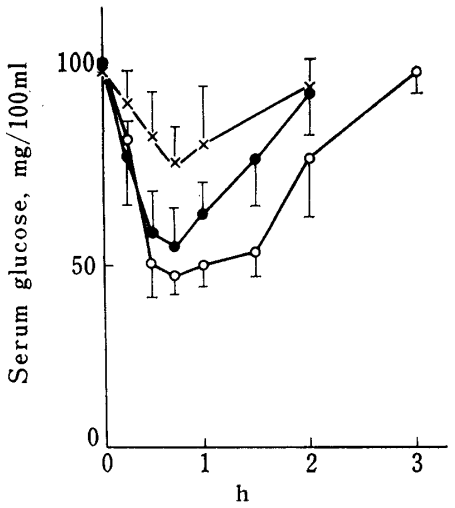


Fig. 4. Effect of the Diketone Moiety on the Promotive Efficacy of PG-enamines in Rabbits

Each value represents the mean ± S.D. of 6 animals. Suppositories containing 1% PG-enamines and 6 I.U. of insulin/g were administered at a dose of 0.5 g of suppository/body.
● PG-EtAA Na,
○ PG-DEMM Na,
× PG-Ethoxy EtAA Na.

It was interesting that the interacting ability of PG-enamines was relatively weak but it was linearly correlated with the response of glucose level (Fig. 5). Recently, Nishihata *et al.*[27] reported that an acetoacetic acid promoted the distribution of drugs to red blood cells in the blood and suggested that its effect was due to the interacting ability with Ca^{2+}. The negative result with PG–AcBu Na may be explained on the basis of its poor interacting ability with the Ca ion (Table III).

Recently, absorption of high molecular weight drugs such as insulin from the digestive tract has been studied. In our laboratory, the absorption of some high molecular weight

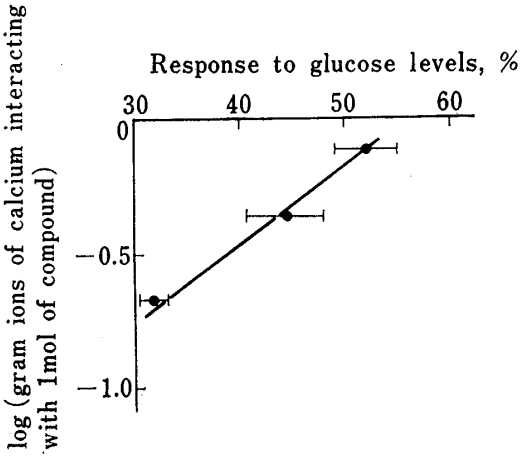


Fig. 5. Relationship between the Interacting Ability of PG-enamines with Ca^{2+} and the Maximum Response in Terms of Glucose Level as Per Cent of the Starting Level in Rabbits

Each value represents the mean ± S.D. of 6 experiments.

Table III. Ability of PG-enamines to interact with Ca^{2+} at pH 10.0 and 25 °C

Compound	Ca^{2+} gram ions/mol of compound
PG–EtAA Na	0.42
PG–DEMM Na	0.77
PG–EthoxyEtAA Na	0.20
PG–AcBu Na	0.06
EtAA	0.49
EDTA	1.75
Citric acid	1.15

drugs (lysozyme, heparin, *etc.*,) was found to be promoted by the addition of PG-enamine derivatives in a suppository. A similar linear correlation exists for the serum level of insulin. However, insulin is known to be enzymatically degraded rapidly in the living body and it is difficult to determine the actual amount absorbed through the rectal tract. Thus, inulin was used as a control because of its absence of the biotransformation and complete urinary excretion in the living body.

The serum levels of inulin were determined following rectal administration to rabbits of 0.5 g of liquefied suppository which contained 25 mg of inulin and 1% PG-enamines (Fig. 6). PG–AcBu Na failed to promote the rectal absorption of inulin. The results in terms of AUC of the time course of serum levels for 0 to 2 h were plotted against the degree of interaction of the enamines to Ca^{2+} (Fig. 7).

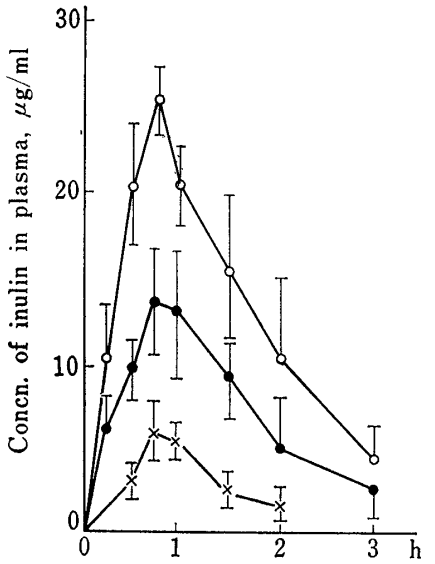


Fig. 6. Effect of PG-enamines on the Rectal Absorption of Inulin in Rabbits

Values represent the means ± S.D. of 6 rabbits. Suppositories containing 50 mg of inulin/g and 1% PG-enamines were administered at a dose of 0.5 g of suppository/body.
● PG-EtAA Na,
○ PG-DEMM Na,
× PG-Ethoxy EtAA Na.

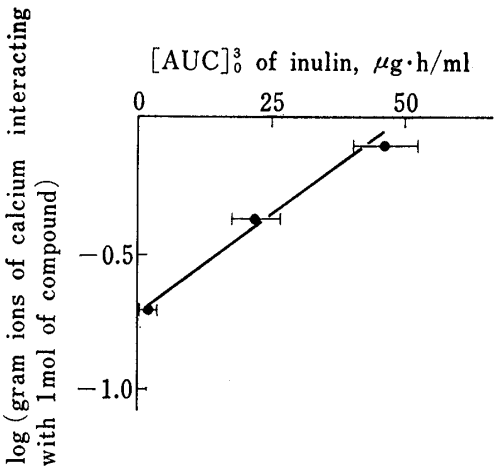


Fig. 7. Relationship between the Interacting Ability of PG-enamines with Ca^{2+} and the AUC of Inulin in Rabbits

The molecular weight of inulin is about 5000 and that of insulin is about 12000 or more in aqueous solution.[28,29] The effective molecular weight of insulin involved in the rectal absorption is not known, but the promoting effects of the enamines are not considered to be specific for insulin.

The cumulative recoveries of inulin in urine during 2.5 h after the administration of 1% PG–EtAA Na were 20.0±3.3% and 16.4±2.5% for rabbits (n=6) and dogs (n=4), respectively.

The difference of promoting efficacy between PG-enamines for the rectal absorption of insulin may be partly explained on the basis of their interaction abilities with Ca^{2+} (Table III). The disodium salt of EDTA, a typical chelating agent, interacts more strongly with Ca^{2+} than the PG-enamines studied, but the promoting efficacy of EDTA for the rectal absorption of insulin was not remarkable (Table IV). Furthermore, EtAA possessed the same to interact with Ca^{2+} as PG–EtAA Na but it failed to promote the rectal absorption of inulin. To clarify these observations, permeation of PG-enamines and inulin through the rectal membrane was studied by employing an excised sac rat rectum. One-tenth gram of liquefied suppository containing 2% each of PG-enamine and inulin was placed in the rectum sac and the cumulative amounts of compounds that permeated through the rectal membrane were measured. The

Table IV. The Effect of EDTA on the Serum Glucose Levels in Rabbits after Rectal Administration of 0.5 g of Liquefied Suppository containing 10 I.U. of Insulin/g and 20 mg of EDTA 2Na/g

Serum glucose levels, mg/100 ml							
0	0.25	0.5	0.75	1.0	1.5	2.0	2.5 (h)
105.2 ±18.6	118.5 ±16.8	124.2 ±21.5	113.5 ±17.4	109.8 ±20.3	110.4 ±11.3	112.5 ±8.6	112.8 ±15.2

Each value is the mean ± S.D. of 6 rabbits. The differences between the results for each sampling time after administration of liquefied suppository and the starting serum glucose level are not statistically significant.

Table V. Permeation of PG-enamines and Inulin through a Rectal Sac Preparation from Rats

	$k^{a)}$ ($\times 10^2$ min^{-1})	Recovery (%)	Recovery of inulin (%)
PG–EtAA Na	4.5±0.5	74.9±3.4	26.1±3.1
PG–DEMM Na	4.0±0.2	71.0±1.4	31.4±3.5
PG–EthoxyEtAA Na	2.1±0.2	62.2±4.9	15.3±1.9
EtAA	0.82±0.05	35.7±1.5	Negligible

One-tenth gram of suppository containing 2% each of PG-enamine and inulin was used.
a) k is the apparent first order rate constant. Each result is the mean ± S.D.

results are presented in Table V in terms of apparent first order permeation rate constants, and cumulative amounts of PG-enamine and inulin that permeated through the membrane during the 1.5 h experimental period. For comparison, the results with EtAA are also presented.

The differences between PG–EtAA Na and PG–DEMM Na were not significant but the results with PG–EthoxyEtAA Na were poorer than those of the other enamines. EtAA failed to promote the permeation of inulin regardless of its interacting ability with Ca^{2+} (Table III). This result may be partly explained on the basis of its poor water solubility, resulting in restricted release of EtAA from the suppository base.

On the basis of these results, it may be considered that adjuvants for rectal absorption of insulin should possess chelating ability for metal ions important for the membrane barrier function as well as the ability to permeate through the membrane, which was mainly dependent on their water solubility and partitioning properties. Their promoting effect appears only in the period during which they permeate through the membrane.

In conclusion, it should be noted that the response of glucose level to insulin treatment in diabetic patients is considered to be quite different from normal. Further, possibility of using insulin suppositories in clinical treatments should be checked on the basis of safety for long-term administration. Thus, further work is necessary before enamine derivatives of amino acids can be applied clinically as adjuvants for the promotion of rectal absorption of insulin in diabetic patients.

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