Chem. Pharm. Bull. 28(2) 379—386 (1980)

Studies on Monocomponent Insulin. IV.[1] Subcutaneous Absorption of Insulin from Preparations with Protracted Action[2]

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(Received December 11, 1978)

Since purity and source species might influence the absorption of a long-acting monocomponent insulin (MC-insulin) preparation, studies were carried out with a preparation of mixed bovine and porcine insulin obtained by conventional recrystallization, a specially prepared preparation of mixed MC grade bovine and porcine insulin, a specially prepared preparation composed of recrystallized porcine insulin alone, and MC-insulin. These were injected subcutaneously into rabbits, and their absorption were examined using hypoglycemia as an index.

The preparation composed only of porcine insulin gave a higher maximum hypoglycemic rate than the mixed bovine and porcine insulin preparation, but the blood glucose recovered to the original level earlier, indicating its more rapid absorption. This phenomenon was more strinking at larger test doses. However, there was no difference in absorption between preparations of different purity. Furthermore, the mixed MC-grade bovine and porcine insulin preparation and MC-insulin were subcutaneously administered to an in situ animal experimental model to determine the blood insulin course. MC-insulin was again found to be absorbed more rapidly. It is suggested that this occurs because porcine insulin crystals dissolve more rapidly than bovine insulin crystals. Such a difference in the solubility of the insulin crystals may be caused by configurational differences between porcine and bovine insulin, arising from the differences in the amino acid residues at positions 8 and 10 of the A-chain.

Keywords——insulin; monocomponent insulin; absorption; blood glucose; immunoreactive insulin; solubility of insulin crystal

Ever since Banting and Best[4] adminidtrated insulin for the first time to diabetics, with spectacular effect, insulin has been considered essential for the treatment of diabetes. However, early preparations tended to produce allergic reactions, required frequent infusions, *etc.* To cope with these problems, new preparations were developed, *e.g.* combinations of protamine and insulin, suspensions of insulin crystals, and suspensions of crystalline insulin and amorphous insulin at a certain ratio. It also became possible to adjust the timing of insulin action. At the same time, efforts were made to improve the purification of insulin during its extraction from animal glands. Meanwhile, Mirsky and Kawamura[5] showed by disc electrophoresis that insulin preparations marketed in the early 1960's contained various impurities. Most of these were identified as insulin-like protein impurities, after Steiner[6] discovered proinsulin. On the other hand, the development of radioimmunoassay by Berson and Yalow[7] made it possible to analyze trace amounts of various peptide hormones. Thus,

1) Part III: T. Kasama, M. Uchida, S. Yonezawa, N. Tamura, and K. Yokoi, *Tounyobyo.*, **19**, 680 (1976).
2) A part of this work was presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1977.
3) Location: *a) 2 Kanda Sakuma-cho, 3-chome, Chiyoda-ku Tokyo 101, Japan; b) 5-6-1 Mitahorahigashi, Gifu 502, Japan.*
4) F.G. Banting and C.H. Best, *Can. Med. Ass. J.*, **12**, 141 (1922).
5) I.A. Mirsky and K. Kawamura, *Endocrinol.*, **78**, 1115 (1966).
6) D.F. Steiner, O. Hallund, A. Rubenstein, S. Cho, and C. Bayliss, *Diabetes*, **17**, 725 (1968).
7) S.A. Berson and R.S. Yalow, *Adv. in Biol. and Med. Physics.*, **6**, 350 (1958).

it has been found that although insulin is a rather low molecular weight peptide, its antibody exists in the blood of patients taking the drug. This has become a therapeutic problem.

Schlichtkrull[8] assumed that the formation of antibody was due to the existence of protein impurities in conventional insulin preparations, and succeeded in obtaining a highly purified insulin by eliminating such impurities. Such highly purified insulin were named "monocomponent insulin" (hereinafter referred to as MC-insulin). Conventional insulins are purified by repeated recrystallizations, while MC-insulins are purified by eliminating impure proteins by means of gel filtration and ion-exchange chromatography. Moreover, Schlichtkrull tried to reduce the antigenicity of MC-insulin preparations further by switching the raw material source from bovine insulin to porcine insulin, because the latter has an amino acid composition which is closer to that of human insulin.

The therapeutic effect of MC-insulin is not different from that of conventional insulin. In our previous report,[9] however, we suggested that when administered to animals, the action time of a long-acting MC-insulin preparation was shorter than that of conventional insulin, and that must be a difference between the two as regards subcutaneous absorption. Since this is clinically important in the control of diabetic conditions, further studies were planned. This difference in the duration of action might be chiefly due to the difference in purity and also animal source, so we investigated the subcutaneous absorption of a long-acting MC-insulin preparation (insulin "NOVO MONOTRAD") in comparison with that of a recrystallized insulin preparation composed only of porcine insulin (100% porcine insulin LENTE), an MC-grade insulin preparation composed of a mixture of bovine and porcine insulin "MC-LENTE", and a conventional recrystallized insulin preparation composed of a mixture of bovine and porcine insulin (insulin "NOVO LENTE").

Materials and Methods

Materials——The samples of insulin NOVO LENTE (a suspension of crystalline bovine insulin, 70%, and amorphous porcine insulin, 30%; 400U/10 ml/vial) were purchased in the usual way (hereinafter referred to as C-L). The sample of 100% porcine insulin LENTE (a suspension of crystalline porcine insulin, 70%, and amorphous porcine insulin, 30%; 400U/10 ml/vial) and MC-LENTE (a suspension of purified crystalline bovine insulin, 70%, and purified amorphous porcine insulin, 30%; 400U/10 ml/vial) were prepared at the Novo Research Institute, Copenhagen, for use in the present study. These preparations are hereinafter referred to as C-PL and MC-L, respectively. A commercial preparation of insulin Novo Monotard (a suspension of purified crystalline porcine insulin, 70%, and purified amorphous porcine insulin 30%; 400 U/10 ml/vial) available in Europe was also used; this is hereinafter referred to as MC-M.

Experimental Animals——Male white Japanese rabbits, weighting 2.3—2.8 kg, were used.

Assay for Insulin——a) Blood Glucose Level: The rabbits were maintained in standardized environmental conditions for more than 1 week, and divided into four groups of 3 animals each. Group 1 was injected subcutaneously with 0.25 U/kg of C-L, group 2 with 0.25 U/kg of C-PL, group 3 with 0.25 U/kg of MC-L, and group 4 with 0.25 U/kg of MC-M. One-tenth ml of blood was sampled from each animal from the marginal ear vein at prescribed intervals, and the blood glucose content was assayed. These experiments were performed by the cross-over technique, shifting the preparations at 1-week intervals, so that every animal received all four kinds of preparations. Similar experiments with test doses of 0.5 U/kg and 1.0 U/kg were then performed on the same animals. The blood glucose was determined by the *o*-toluidine-borate method. The animals were fasted for 16 hr before each experiment.

b) Immunoreactive Insulin (IRI): The rabbits were fasted for 16 hr, then the pancreas of each animal was ligated under Nembutal anesthesia, and the abdominal aorta was cannulated for collection of blood samples at prescribed intervals after the administration of insulin for the determination of blood IRI. Considering the sensitivity of the IRI determination, the dose of insulin to be administered was chosen to be 5 U/kg, and in order to prevent hypoglycemia, each animal was infused with 10% glucose solution at the rate of 0.3 ml/min through the jugular vein. The determination of blood IRI was performed with a two-antibody radioimmunoassay kit from Eiken Kagaku Co. Ltd. (Tokyo) using 5 μ/mCi labeled insulin.

8) J. Schlichtkrull, J. Brange, H. Christiansen, O. Hallund, L.G. Heding, and K.H. Jorgensen, *Diabetes.* **21** (suppl. 2) 649 (1972).
9) T. Kasama, H. Nakajima, S. Yonezawa, T. Iiyama, M. Uchida, and S. Nakagawa, *Tounyobyo.*, **18**, 29 (1975).

Determination of the Physical Characteristics of the Preparations——Since the physical characteristics of the four preparations used in the experiments will affect their absorption, the form, size and amount of the insulin crystals contained in the preparations, and also the amount of zinc in them, were determined by the methods prescribed in the Pharmacopoeia of Japan and also as described in our previous paper.[1]

Determination of Purity of the Preparations——The purity of each of the four preparations used in the experiments was confirmed by disc electrophoresis, following the method described in our previous paper.[1]

Results

The purity of each of the four preparations used in the experiments is shown in Fig. 1. MC-L and MC-M, which are purified insulin preparations, gave only the main band of insulin, while C-L and C-PL also gave bands of impurities, such as proinsulin, arginine insulin, and deamidoinsulin, in addition to insulin. Photographs of the form and size of insulin crystals suspended in the preparations are shown in Fig. 2. The form was rhombohedral in all of one hundred insulin crystals extracted at random from C-L. C-PL, MC-M and MC-L,respectively, and the crystal sizes were 26±5, 28±6, 26±4, and 27±5 μm, respectively. There was no significant difference among the four preparations ($p>0.05$). The amounts of the crystals and of zinc are shown in Table I. The differences are small among the four preparations.

The absorption of insulin from each of the preparations was then studied using hypoglycemia, *i.e.*, their major biological activity, as an index. As shown in Fig. 3, C-PL and MC-M, which are suspensions of procine insulin crystals (porcine group) exhibited similar patterns on absorption when administered at a dose of 0.25 U/kg; the maximum hypoglycemic effect occurred 1 hr after the administration of either preparation. The maximum hypoglycemic rate was about 55% in both cases, followed by rapid recovery, with the blood glucose reaching 90% of the initial level 4 hr after administration. These findings suggest that these two preparations are absorbed very rapidly and metabolized rapidly. C-L and MC-L, which are both suspensions of bovine insulin crystals (the bovine group), also gave similar hypoglycemic curves. However, the maximum hypoglycemic rate with these preparations was significantly lower (p 0.05) than that after administration of the porcine preparations. Hypoglycemia induced with bovine preparations lasted longer, with the blood glucose recovering to 90% of the initial level 7 hr after administration. Thus, the bovine group preparations were absorbed slowly compared with the porcine group preparations. This was more evident with larger doses of the preparations. When the preparations were administered at a dose of 0.5 U/kg, as shown in Fig. 4, it took the blood glucose 10 hr after administration of the bovine group of preparations to return to 90% of the initial level, which it took only 4 hr after the administration of the porcine group to return to the same level. This difference was more apparent when the preparations were administered at a dose of 1.0 U/kg. The blood glucose returned only to about 80% of the initial level at 10 hr after the administration of the bovine group preparation, while it had returned to the initial level at 7 hr after the administration of the porcine group, as shown in Fig. 5.

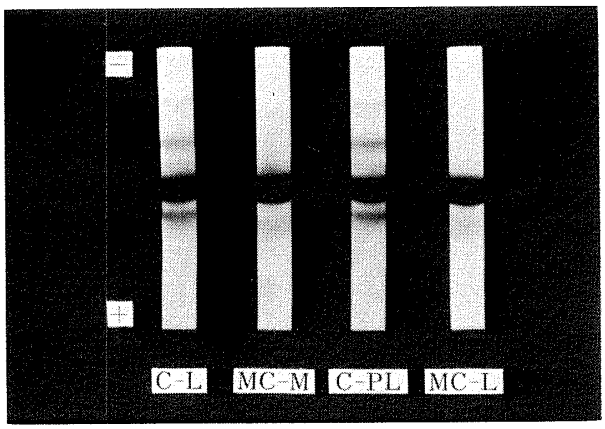

Fig. 1. Polyacrylamide Disc Gel Electrophoregrams of Tour Insulin Preparations

C-L: Conventional recrystallized insulin (bovine insulin crystals 70%; porcine amorphous insulin 30%).
MC-L: Highly purified MC- grade insulin (bovine insulin crystals 70%; porcine amorphous insulin 30%).
C-PL: Conventional recrystallized insulin (porcine insulin crystals 70%; porcine amorphous insulin 30%).
MC-M: Monocomponent insulin (porcine insulin crystals 70%; porcine amorphous insulin 30%).

These results suggest that the species difference of the source animals might be responsible for the difference in absorption between these preparations. To confirm this, MC-L and MC-M were administered to rabbits subcutaneously and the blood IRI was determined. These results are presented in Fig. 6. The peak blood IRI appeared at 4 hr after the administration of MC-M and then decreased, while the peak blood IRI occurred at 7—8 hr after the administration of MC-L. Furthermore, when MC-M and MC-L dissolved in 0.1 N hydrochloric acid, were injected intravenously into rabbits, the two preparations exhibited similar hypoglycemic curves (Fig. 7). These observations suggested that porcine crystalline insulin is absorbed more rapidly than bovine crystalline insulin.

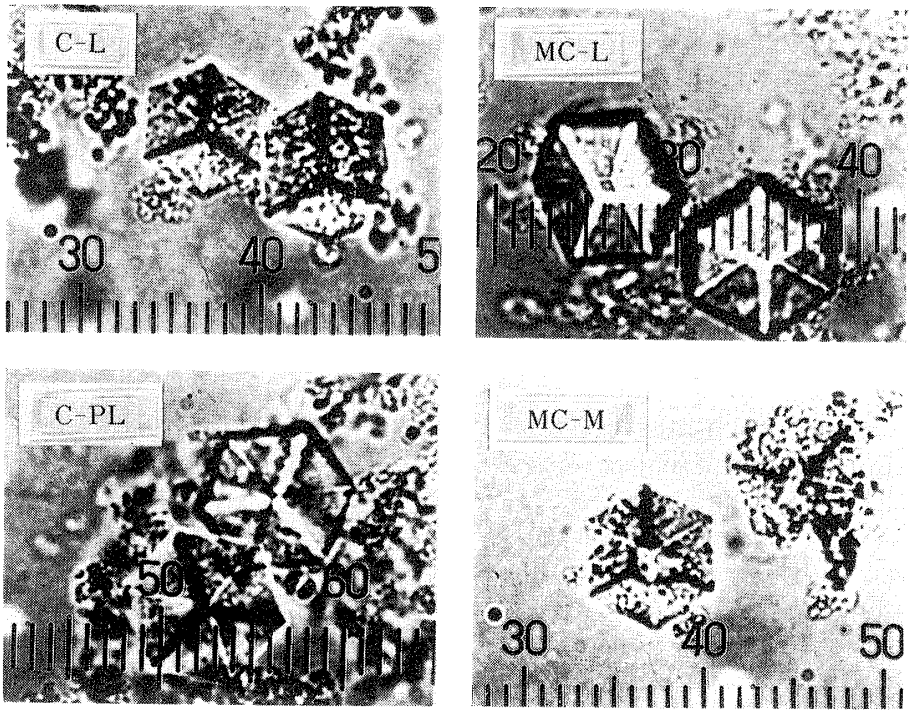

Fig. 2. Photomicrographs of Insulin Crystals in the Four Insulin Preparations

One small scale interval indicates 3 μm. Designations are the same as in Fig. 1.

Table I. Amounts of Crystalline Insulin and Zinc contained in the Four Insulin Preparations

Sample	No.	Nitrogen (mg/100 U)	Crystalline insulin (%)	Mean (%)	No.	Sample volume (ml)	Zn (mg/100 U)	Mean
C-L	1	0.387	69.00		1	5	0.213	
	2	0.385	68.66	68.66	2	5	0.211	0.212
	3	0.383	68.33					
MC-L	1	0.379	70.32		1	5	0.208	
	2	0.379	70.32	69.58	2	5	0.208	0.208
	3	0.367	68.09					
C-PL	1	0.394	66.79		1	5	0.222	
	2	0.394	66.79	66.96	2	5	0.222	0.222
	3	0.398	67.38					
MC-M	1	0.348	65.05		1	5	0.204	
	2	0.343	64.11	64.25	2	5	0.202	0.203
	3	0.340	63.59					

Designations are the same as in Fig. 1

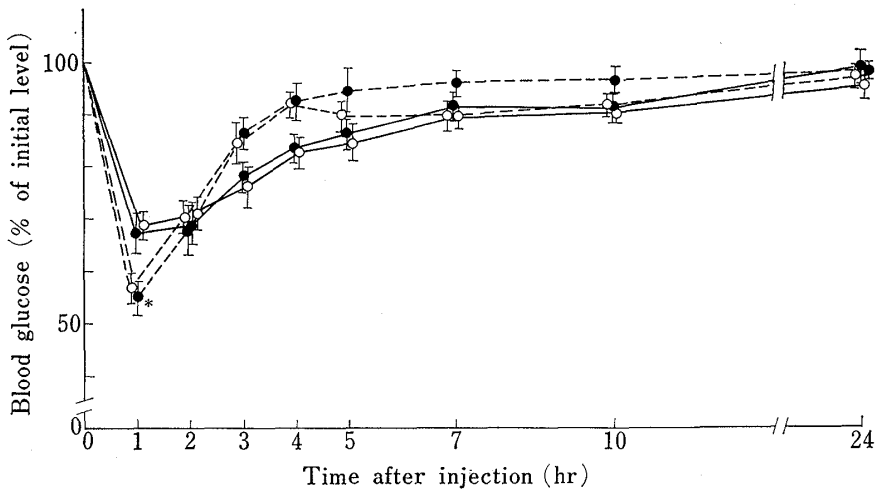

Fig. 3. Blood Glucose Curves after Subcutaneous Injection of 0.25 U/kg of Various Insulin Preparations

C-L: —●—, MC-M: —○—, C-PL: —●—, MC-L: —○—. Designations are the same as in Fig. 1. Vertical bars represent standard errors of the means (n=12). *$p < 0.05$ relative to the group injected with bovine-porcine insulin.

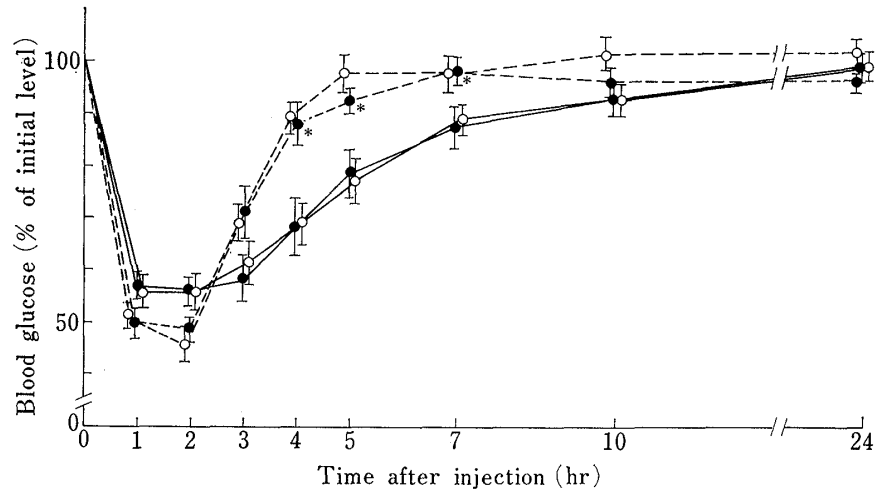

Fig. 4. Blood Glucose Curves after Subcutaneous Injection of 0.5 U/kg of Various Insulin Preparations

C-L: —●—, MC-M: —○—, C-PL: —●—, MC-L: —○—. Designations are the same as in Fig. 1. Vertical bars represent standard errors of the means (n=12). *$p < 0.05$ relative to the group injected with bovine-porcine insulin.

Discussion

The insulin zinc aquosuspensoid injection developed by Hallas-Moller[10] is a long-acting preparation. However, its subcutaneous absorption *in vivo* is influenced by various factors, such as the size and amount of insulin crystals suspended in the fluid, the amount of zinc, the purity, and the species of source animal. This problem suddenly became a major issue when MC insulin, which is a highly purified preparation composed only porcine insulin, was

10) K. Hallas-Moller, K. Petersen, and J. Schlichtkrull, *Science*, **116**, 349 (1952); K. Hallas-Moller, *Lancet*, **267**, 1029 (1954).

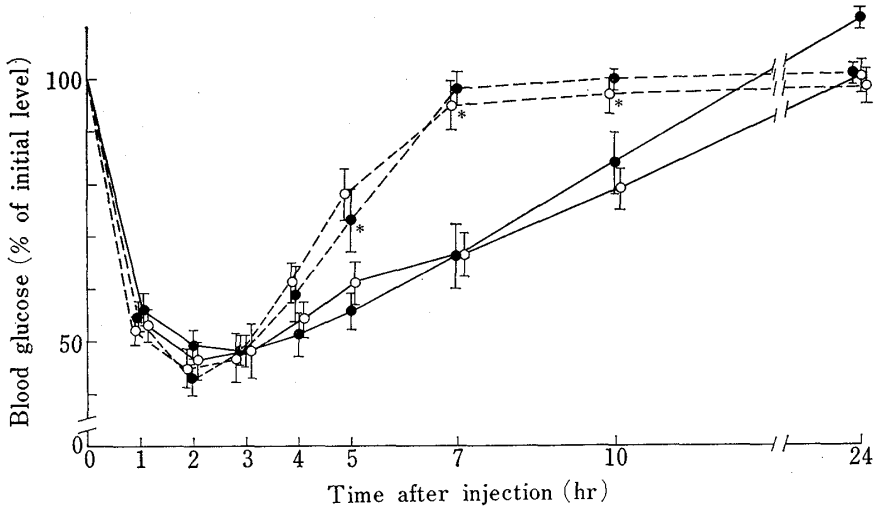

Fig. 5. Blood Glucose Curves after Subcutaneous Injection of 1.0 U/kg of Various Insulin Preparations

C-L: —●—, MC-M: --○--, C-PL: --●--, MC-L: —○—. Designations are the same as in Fig. 1. Vertical bars represent standard errors of ths means ($n=12$). *$p<0.05$ relative to the group injected with bovine-porcine insulin.

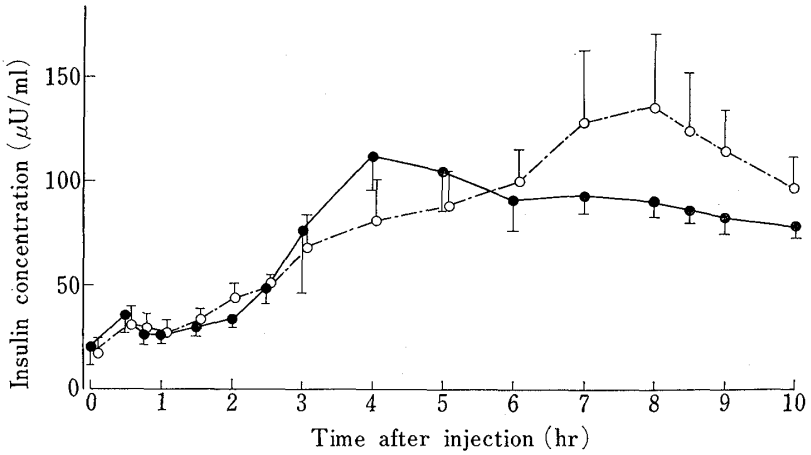

Fig. 6. Time Courses of Plasma Immunoreactive Insulin Concentration after Subcutaneous Injection of 5.0 U/kg of Various Insulin Preparations

MC-M: --○--, MC-L: —○—. Pancreases of experimental animals were ligated, and blood samples were collected from the abdominal aorta at intervals.
Designations are the same as in Fig. 1. Vertical bars represent standard errors of the means ($n=3$).

developed. As described in our previous paper,[5] the "long-acting" MC insulin is absorbed rapidly, compared with conventional recrystallized insulin, although it has the same physical characteristics as the latter. We therefore performed the present experiments with four preparations which differed in purity and source.

It was found that insulin was more rapidly absorbed from the suspended preparation of porcine insulin cystals than from that of bovine insulin crystals. This was more marked with 0.5 and 1.0 U/kg of the preparations; the blood glucose was significantly slower to recover after the administration of the bovine insulin preparatilon. It is well known that the main insulin-degrading enzymes are insulinase and glutathion-insulin-transhydrogenase in various

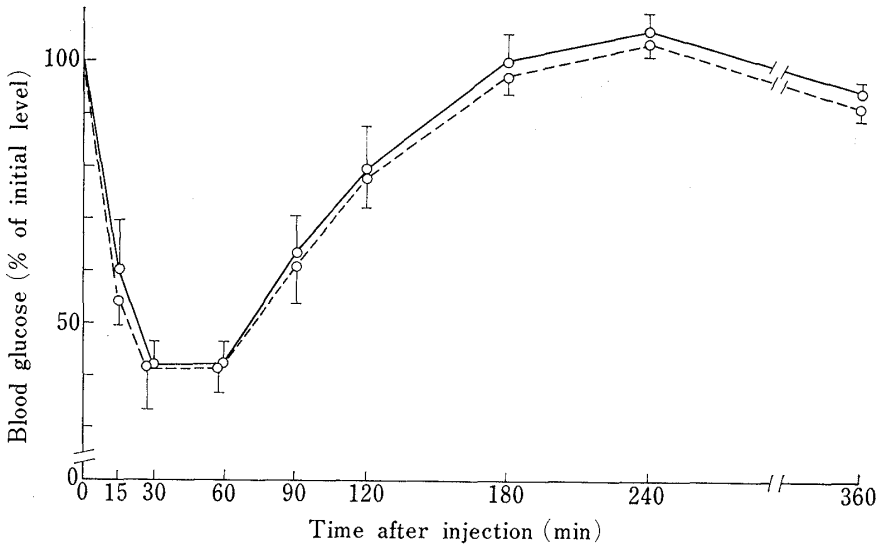

Fig. 7. Blood Glucose Curves after Intravenous Injection of 0.25 U/kg of Various Dissolved Insulin Preparations

MC-M: —○—, MC-L: —○—. Insulin crystals of both preparations were solubilized with 0.1 N hydrochloric acid. Designations are the same as in Fig. 1. Vertical bars represent standard errors of the means ($n=4$).

animals.[11] Considering their characteristics. It is unlikely that these enzymes act only on the insulin of a specific animal. Freychet *et al.*[12] reported that the binding capacities of bovine, porcine and human insulin to receptors were the same, so there may be no difference in biological activity between porcine and bovine insulin. This view is supported by the results of the present experiments, showing that the hypoglycemic effects of MC-M and MC-L were the same when MC-M and MC-L, in which crystalline and amorphous insulins were solubilized with 0.1 N HCl, were injected intravenously. Therefore, it is suggested that the difference of subcutaneous absorption observed in the present study arises because porcine insulin crystals dissolve more rapidly than bovine insulin crystals. Schlichtkrull[13] stated that the solubility of insulin crystals varies with pH and also with the amount of zinc present; he showed that porcine insulin crystals are far more soluble than bovine insulin crystals, especially at pH 7.4 at 20°, and in the presence of less than 1 μg/U of zinc, and that the solubility of porcine insulin becomes the same as that of bovine insulin upon the addition of more than 1 U/kg zinc. The insulin crystals used in our present study contained about 2 μg/U of zinc. Therefore, these preparations should have the same solubility according to Schlichtkrull's report. However, because his results were obtained from *in vitro* experiments, the effects of body temperature and body fluid were not considered. These factors appear to be responsible for the difference in the solubility of bovine and porcine insulin crystals.

Bovine and porcine insulin differ in structure only in the amino acid residues at positions 8 and 10 of the A-chain. According to Hodgkin,[14] however, positions 6—10 of the A-chain form an intramolecular ring and are distorted in the direction of the B-chain adjacent to them, playing an important role in maintaining the stereic configuration of the insulin molecule. Hence, it may be surmised that differences in the amino acid residues at these positions do affect the molecular configurations, and thus presumably influence the solubility of the crystals, leading to the difference in absorption between bovine and porcine insulin.

11) I.A. Mirsky, *Diabetes*, **13**, 225 (1964).
12) P. Freychet, J. Roth, and D. Neville, *Proc. Natl. Acad. Sci. USA*, **68**, 1833 (1971).
13) J. Schlichtkrull, "Insulin Crystals," Novo Research Institute, Copenhagen, 1961, p. 61.
14) D.C. Hodgkin, *Diabetes*, **21**, 1131 (1972).

Regarding the purity of insulin, Mitsuhashi and Takeo[15] stated that purified insulin is absorbed and metabolized more rapidly than conventional insulin, but the results of our present study indicate that there is no difference in the rates of absorption and metabolism of these insulins at any of the doses studied. Shimizu[16] reported that there is no difference in the duration of effect in terms of the daily profile of diabetics between the long-acting MC-insulin preparation and the conventional LENTE insulin. However, this finding may be a result of additional clinical factors such as pathology and food intake.

It has been shown that the long-acting preparation of MC-insulin prepared from porcine insulin alone has a short duration of effect compared with the conventional bovine and porcine insulin preparations, and it is suggested that this is due to the high solubility of porcine insulin crystals compared with that of bovine insulin crystals. However, porcine insulin is preferable to bovine insulin in having a low antigenicity, and MC insulin may be clinically preferable, depending on the objective.

Acknowledgement The authors are grateful to Prof. Shoichi Nakagawa of the Second Department of Internal Medicine, Hokkaido University, School of Medicine, Sapporo, for advice, and also to Dr. J. Schlichtkrull at the Novo Research Institute, Copenhagen, for the supply of samples of various insulin preparations.

15) K. Mitsuhashi and K. Takeo, *Tounyobyo*, **17** (Suppl.), 229 (1974).
16) N. Shimizu, *Tounyobyo*, **20**, 168 (1977).