rate was linear from 1 mg to 4 mg of tissue, and the reaction proceeded linearly with time for 15 min at 37 °C.

The rate of tyrosine hydroxylase activity in rat cerebellum was about half of that in the frontal cerebral cortex (table), which is similar to the results reported by Croll et al.[4]. However, as shown in the table, the sensitivity (activity/blank) in the present assay is 3-7 times higher than that in the radiometric assay[4]. Although comparable enzyme activity was also measured in mouse and rabbit brain tissues, the sensitivity with mouse hippocampus enzyme was low (1.75). It may be due to the lower enzyme activity. According to the histochemical and biochemical studies of catecholamines, many investigators indicate that there are catecholaminergic neurons in the cerebrum. But the activities of the catecholamine synthesizing enzymes in the cerebral cortex have been too low to study the properties of the enzymes. Using the highly sensitive assay method described here, we could study the kinetics of the tyrosine hydroxylase in rat frontal cerebral cortex. The K_m-value (mean ± SEM) for L-tyrosine in the presence of 350 µmoles/l $6MPH_4$ was 166 ± 32 µmoles/l (fig. A) and that for $6MPH_4$ was 176 ± 15 µmoles/l in the presence of 0.2 mmoles/l L-tyrosine (fig. B). These data in the frontal cerebral cortex are similar to those in rat striatum[6]. Interestingly, tyrosine hydroxylase activity in the cortex, measured using this standard assay system, was inhibited by high concentrations of $6MPH_4$ such as 3 mmoles/l, while when a higher concentration of ferrrous ammonium sulfate (1 mmole/l) was used instead of 0.1 mmoles/l, the inhibition by the higher concentration of $6MPH_4$ disappeared. The above phenomenon has also been reported by Ellenbogen et al.[7].

The present assay method for tyrosine hydroxylase using high-performance liquid chromatography with voltammetry is highly sensitive and, could be used to study the kinetics of tyrosine hydroxylase in any tissues.

1 T. Nagatsu, M. Levitt and S. Udenfriend, J. biol. Chem. *239*, 2910 (1964).
2 T. Nagatsu, K. Oka and T. Kato, J. Chromat. *163*, 247 (1979).
3 G.N. Wilkinson, Biochem. J. *80*, 324 (1961).
4 N.E. Croll, J.P. Lewis, M. Reddington, A.K. Prince and P. T-H Wong, J. Neurochem. *29*, 743 (1977).
5 T. Yamauchi and H. Fujisawa, Analyt. Biochem. *89*, 143 (1978).
6 T. Lloyd and B.J. Ebersole, J. Neurochem. *34*, 726 (1980).
7 L. Ellenbogen, R.J. Tayler, Jr, and G.B. Brundage, Biochem. biophys. Res. Commun. *19*, 708 (1965).

Semisynthesis and biological properties of the [B24-leucine]-, [B25-leucine]- and [B24-leucine, B25-leucine]-analogues of human insulin

K. Inouye[1], K. Watanabe, Y. Tochino, T. Kanaya, M. Kobayashi[2] and Y. Shigeta[2]

Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553 (Japan), and The Third Department of Internal Medicine, Shiga University of Medical Science, Ohtsu, Shiga 520-21 (Japan), 18 November 1980

Summary. Trypsin-catalyzed coupling of porcine desoctapeptide-insulin with synthetic octapeptides produced the [Leu^{B24}]- (I), [Leu^{B25}]- (II) and [Leu^{B24}, Leu^{B25}]- (III)analogues of human insulin. I, II and III displayed respectively 20-30%, 1-2% and 0.5% of the receptor binding activity of the normal hormone. Biological activities of these analogues seemed to be proportional to their binding potencies when assayed in vitro, while in an in vivo assay analogue I was fully active and II exhibited 10-20% of normal activity. III was less active than II in all assays tested.

Occurrence of a new type of human diabetes has recently been reported[3,4]. The patient secretes an abnormal insulin, which shows decreased affinity to insulin receptors and markedly low biological activities, together with an equal amount of normal hormone. The abnormal insulin isolated was found to contain a leucine residue substituted for phenylalanine at position 24 or 25 of the B-chain[3]. Very recently Tager et al.[5] have suggested that the [Leu^{B24}]-variant can be the abnormal insulin, based on the finding that the [Leu^{B24}]-, but not the [Leu^{B25}]-, analogue of porcine insulin is an active antagonist of insulin action. Now we report here the semisynthesis and partial biological characterization of the [Leu^{B24}]-, [Leu^{B25}]- and [Leu^{B24}, Leu^{B25}]-analogues of human hormone.

In a previous communication we described a semisynthesis of insulin which involved a trypsin-catalyzed coupling of desoctapeptide(B23-B30)-insulin (DOI) with a synthetic octapeptide corresponding to the sequence B23-B30 of insulin[6]. This technique was successfully applied in the present work to the preparation of the human insulin analogues.

The octapeptide derivatives, H-Gly-X-Y-Tyr-Thr-Pro-Lys(Boc)-Thr-OH (I: X = Leu, Y = Phe; II: X = Phe, Y = Leu; III: X = Y = Leu), were synthesized by the conventional solution method. Z-Thr-Pro-Lys(Boc)-N_3 derived from the corresponding hydrazide by the treatment with an alkyl nitrite[7] was coupled with H-Thr-OBzl and the resulting tetrapeptide was hydrogenolyzed over palladium to give H-Thr-Pro-Lys(Boc)-Thr-OH. This was then coupled with Z-Gly-X-Y-Tyr-N_3 to give Z-Gly-X-Y-Tyr-Thr-Pro-Lys(Boc)-Thr-OH, from which octapeptides I, II and III were derived by catalytic hydrogenolysis; $[\alpha]_D^{22}$ −38.8°, −36.0°, and −49.2° (c 1.0, acetic acid) for I, II, and III, respectively. These octapeptides were found to be homogeneous in TLC and in high-pressure liquid chromatography (HPLC) and completely hydrolyzed by aminopeptidase M (Pierce) to give the amino acid ratios expected by theory.

$N^{\alpha A1}$,$N^{\alpha B1}$-$(Boc)_2$-DOI (100 mg)[6,8] derived from porcine insulin (kindly supplied by Eli Lilly & Co., Lot 1FJ91) and octapeptide I (200 mg) were dissolved in a mixture of N,N-dimethyl-formamide (DMF)-1,4-butanediol-H_2O (35:35:30 by volume, 1.5 ml) containing 0.25 M tris(hydroxymethyl) aminomethane (Tris) acetate. To this was added L-1-tosylamido-2-phenylethyl chloromethyl ketone(TPCK)-treated trypsin (10 mg)[9] and the reaction mixture (pH 6.4) was incubated at 37 °C for 20 h. The reaction was stopped by the addition of acetic acid (1 ml). HPLC showed that 64% of the DOI had been converted into insulin. The entire material was subjected to gel filtration on a Sephadex LH-20 column (3.6 × 41 cm) with DMF-0.5 M acetic acid (1:1 by volume) to recover excess octapeptide (152 mg). The resulting material was treated

for deprotection with trifluoroacetic acid at 0 °C for 60 min in the presence of anisole. The product was applied to a Sephadex G-50 column (superfine, 3.3 × 140 cm) with 0.5 M acetic acid as solvent. The insulin isolated was further purified on a QAE-Sephadex A-25 column (1.8 × 20 cm) with 0.06 M Tris-HCl buffer (pH 8) containing 60% ethanol and 0.08–0.15 M NaCl according to Schlichtkrull et al.[10], giving a preparation of [Leu^{B24}]-human insulin in 36% yield (41 mg) as based on the amount of $(Boc)_2$-DOI used. [Leu^{B25}]-insulin and [Leu^{B24}, Leu^{B25}]-insulin were prepared in exactly the same manner as above, except for using II and III, respectively, in place of octapeptide I. The semisynthetic insulins thus obtained were found to be homogeneous in HPLC. Figure 1 is an elution profile which shows good separation of a mixture of these insulins. Amino acid ratios in acid hydrolysate (6 M HCl, 110 °C, 24 h in the presence of phenol): [Leu^{B24}]-insulin: Lys 1.10, His 1.94, Arg 1.00, Asp 3.15, Thr 2.60, Ser 2.13, Glu 6.99, Pro 1.20, Gly 4.30, Ala 1.17, Val 3.88, Ile 1.73, Leu 7.00, Tyr 3.97, Phe 2.01; [Leu^{B25}]-insulin: Lys 1.08, His 1.94, Arg 1.00, Asp 3.07, Thr 2.59, Ser 2.19, Glu 6.96, Pro 1.08, Gly 4.26, Ala 1.19, Val 3.86, Ile 1.79, Leu 7.00, Tyr 4.02,

Hypoglycemic activity of semisynthetic preparations of human insulin and its analogues

Material	Dose (μg/10 g b.wt)	Blood glucose level (mg%)
Saline	-	150.7 ± 5.6
Insulin (normal)	0.1	113.3 ± 3.8
	0.5	80.0 ± 4.0
[Leu^{B24}]-insulin	0.1	121.8 ± 8.8
	0.5	73.2 ± 4.5
Saline	-	150.2 ± 7.8
Insulin (normal)	0.25	114.0 ± 6.9
	1.0	50.3 ± 5.4
[Leu^{B25}]-insulin	1.0	100.0 ± 6.1
	10.0	56.5 ± 5.8
Saline	-	151.0 ± 2.9
Insulin (normal)	0.25	127.2 ± 4.0
	1.0	79.2 ± 6.9
[Leu^{B24}, Leu^{B25}]-insulin	1.0	143.2 ± 5.1
	10.0	108.0 ± 5.7

Male DS/Shi mice weighing 25–30 g (6 weeks old) were starved (8.30–13.00 h), then injected with insulin (dissolved in saline, 0.1 ml/10 g b.wt) through the tail vein. After 45 min, blood samples were collected from the orbital sinus and their glucose level was measured by the glucose oxidase method using the Blood Sugar-GOD-Period-Test kit (Boehringer-Mannheim). Data represent the mean ± SD of 6 determinations.

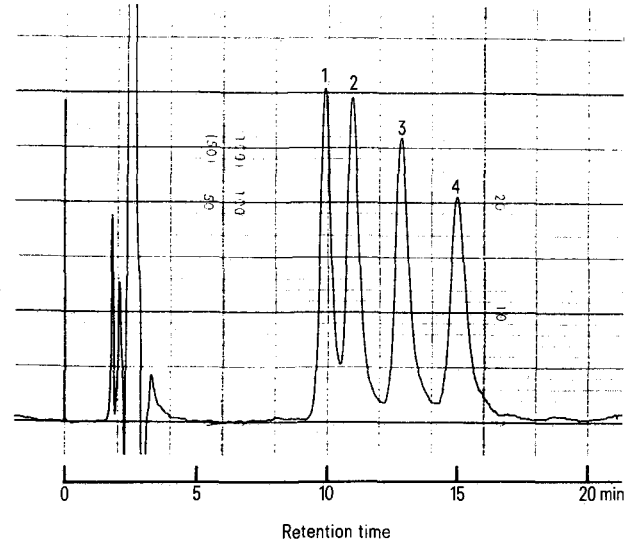

Figure 1. HPLC of semisynthetic preparations of human insulin and its analogues. Approximately the same amounts of [Leu^{B25}]-insulin (1), [Leu^{B24}, Leu^{B25}]-insulin (2), normal insulin (3), and [Leu^{B24}]-insulin (4) in 0.1 M acetic acid were combined and subjected to HPLC under the following conditions: column: Nucleosil $5C_{18}$, 0.4 × 25 cm; eluant: 0.05 M phosphate buffer (pH 3.0) containing n-$C_4H_9SO_3Na$ (5 mmoles/l), Na_2SO_4 (50 mmoles/l), and 28% CH_3CN, 1 ml/min; detection: at 220 nm (range, 0.04).

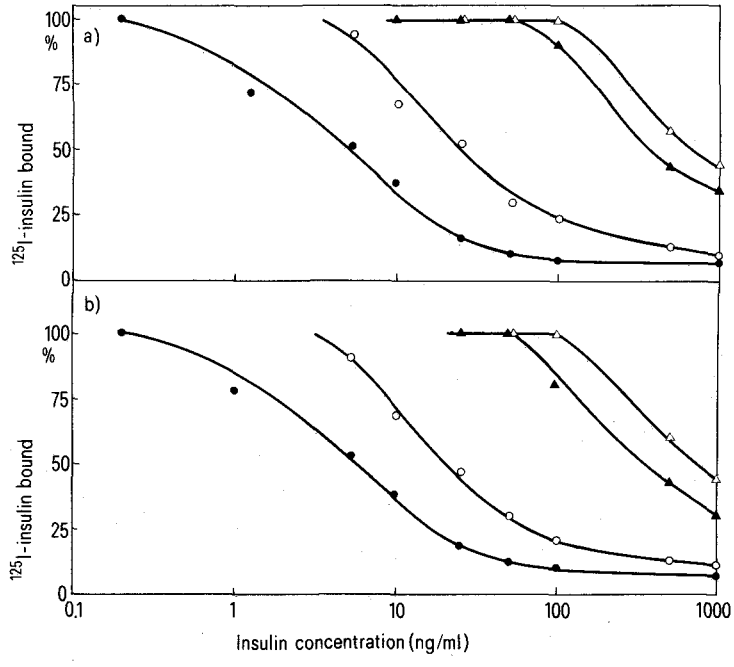

Figure 2. Ability of human insulin and its analogues to displace [^{125}I]porcine insulin from insulin receptors on: a) human placenta membranes and b) isolated rat adipocytes. ●—●, Normal insulin; ○—○, [Leu^{B24}]-insulin; ▲—▲, [Leu^{B25}]-insulin; △—△, [Leu^{B24}, Leu^{B25}]-insulin. Human placenta membranes prepared according to the method of Posner[11] were suspended in an N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid(Hepes)-Tris buffer containing 0.1% bovine serum albumin (pH 7.6) and incubated at 15 °C for 90 min with 33 pM [^{125}I]insulin (0.2 ng/ml) plus unlabeled human insulin or analogues to give the indicated total insulin concentrations. Membrane-bound insulin was separated from free insulin by centrifugation. All data were corrected for nonspecific binding by subtracting the amount of radioactivity remaining bound at an insulin concentration of 200 μg/ml. Non-specific binding was 6–10% of the total amount bound. The total specific binding of [^{125}I]insulin (0.2 ng/ml) was defined as 100%. Binding experiments with isolated rat adipocytes were performed as described elsewhere[12], in which bound insulins were separated from free insulin by the oil flotation method[13]. Data represent the mean of 3 separate experiments.

Phe 1.99; [Leu^{B24}, Leu^{B25}]-insulin: Lys 1.15, His 2.08, Arg 1.00, Asp 3.15, Thr 2.72, Ser 2.20, Glu 7.08, Pro 1.12, Gly 4.30, Ala 1.19, Val 3.86, Ile 1.72, Leu 8.00, Tyr 4.16, Phe 1.05.

The insulin analogues obtained were then compared with semisynthetic human insulin[6] in terms of some biological properties. Figure 2 illustrates the ability of human insulin and the 3 analogues to bind to isolated rat adipocytes and human placenta membranes. The displacement curves of these analogues are not strictly parallel to that of normal insulin. The cause for this is not clear, though it might be ascribed to some change in the site-site interaction induced by these analogues. However, based on the concentrations required to displace 50% of the specifically bound [^{125}I]-insulin, the binding activities of [Leu^{B24}]-, [Leu^{B25}]- and [Leu^{B24}, Leu^{B25}]-insulins are estimated to be 20–30%, 1–2% and 0.5%, respectively, of that of normal insulin. Figure 3 shows the stimulation of 2-deoxyglucose uptake by human insulin and its analogues in rat adipocytes. The activities of these analogues at relatively low insulin concentrations seem to be fairly proportional to their binding activities, although they elicit full agonist activity at high concentrations. These analogues of human insulin were also compared with normal hormone in terms of the hypoglycemic effect in normal mice. As shown in the table, [Leu^{B24}]-insulin is as active as normal hormone, whereas the activity of [Leu^{B25}]-insulin is 10–20% of normal and that of [Leu^{B24}, Leu^{B25}]-insulin is still lower.

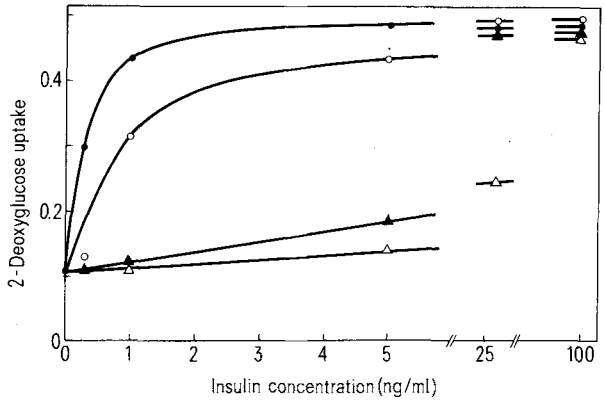

Figure 3. Ability of human insulin and its analogues to stimulate 2-deoxyglucose uptake in isolated rat adipocytes. ●—●, Normal insulin; ○—○, [Leu^{B24}]-insulin; ▲—▲, [Leu^{B25}]-insulin; △—△, [Leu^{B24}, Leu^{B25}]-insulin. Isolated rat adipocytes were incubated at 24 °C for 90 min with human insulin or analogues at the indicated concentrations in a Krebs-Ringer bicarbonate buffer containing 1% bovine serum albumin (pH 7.4). Then, these cells were incubated with 0.1 mM 2-deoxy-D-[^{3}H]glucose for 3 min. The cells separated from the media by the oil flotation method[13] were solubilized and the radioactivity was measured in a scintillation counter. The amount of sugar trapped in the extracellular water space of the cell layers was determined by using L-[^{14}C]glucose. Extracellular water space was measured in each experiment and all data for sugar uptake were corrected for this factor. Data represent the mean of 5 experiments in terms of nmoles 2-deoxyglucose per 3 min per 2×10^5 cells.

The [Leu^{B24}]- and [Leu^{B25}]-analogues of human insulin and the corresponding analogues of porcine insulin have also been prepared by Gattner et al.[14] and by Tager et al.[5], respectively, basically following the procedure of Inouye et al.[6]. They showed that [Leu^{B24}]-insulin and [Leu^{B25}]-insulin had circa 10% and 1%, respectively, of the receptor binding activity of porcine insulin[5], and that [Leu^{B24}]-insulin and [Leu^{B25}]-insulin were 12% and 3.4%, respectively, as active as bovine insulin when assayed for lipogenesis in isolated rat adipocytes[14]. These data as well as ours indicate that the biological activity decreases in the order: normal insulin > [Leu^{B24}]-insulin > [Leu^{B25}]-insulin, although some discrepancy exists among the relative potencies observed.

The amino acid sequence, Gly-Phe-Phe-Tyr (B23-B26), in the insulin molecule, has been firmly conserved through animal evolution and, because of this as well as for other reasons, the residues B24, B25, and B26 have been regarded as part of the active site or receptor binding region of the hormone[15]. These residues have also been suggested to be responsible for inducing negative cooperativity[16]. Although the new analogues of human insulin have not yet been fully characterized, the results obtained in the present work are consistent with a model, in which the residues B24 and B25 are involved in receptor binding of insulin. As has been seen above, the Leu for Phe substitution at position B25 produced a much greater decrease in binding potency than that produced by the same substitution at B24. These 2 positions are, therefore, not biologically equivalent and the specific importance of phenylalanine-B25 should be stressed. The decreased binding potency was generally paralleled by a decrease in biological activity, although the substitution at B24 did not seem to affect the activity in vivo greatly. The effect of the [Leu^{B24}]- and [Leu^{B25}]-analogues of human insulin on the action of normal insulin is under investigation.

1 To whom correspondence should be addressed.
2 Shiga University of Medical Science.

3 H. Tager, B. Given, D. Baldwin, M. Mako, J. Markese, A. Rubenstein, J. Olefsky, M. Kobayashi, O. Kolterman and R. Poucher, Nature *281*, 122 (1979).
4 B.D. Given, M.E. Mako, H.S. Tager, D. Baldwin, J. Markese, A.H. Rubenstein, J. Olefsky, M. Kobayashi, O. Kolterman and R. Poucher, N. Engl. J. Med. *302*, (1980).
5 H. Tager, N. Thomas, R. Assoian, A. Rubenstein, M. Saekow, J. Olefsky and E.T. Kaiser, Proc. natl Acad. Sci. USA *77*, 3181 (1980).
6 K. Inouye, K. Watanabe, K. Morihara, Y. Tochino, T. Kanaya, J. Emura and S. Sakakibara, J. Am. chem. Soc. *101*, 751 (1979).
7 J. Honzl and J. Rudinger, Colln Czech. chem. Commun. *26*, 2333 (1961).
8 D. Levy and F.H. Carpenter, Biochemistry *6*, 3559 (1967).
9 V. Kostka and F.H. Carpenter, J. biol. Chem. *239*, 1799 (1964).
10 J. Schlichtkrull, J. Brange, A.H. Christiansen, O. Hallund, L.G. Heding and K.H. Jørgensen, Diabetes *21*, suppl. 2, 649 (1972).
11 B.I. Posner, Diabetes *23*, 209 (1974).
12 M. Kobayashi, M. Iwasaki and Y. Shigeta, J. Biochem. (Tokyo) *88*, 39 (1980).
13 J. Gliemann, K. Østerlind, J. Vinten and S. Gammeltoft, Biochim. biophys. Acta *286*, 1 (1972).
14 H.-G. Gattner, W. Danho, C. Behn and H. Zahn, Hoppe-Seyler's Z. physiol. Chem. *361*, 1135 (1980).
15 T.L. Blundell, G.G. Dodson, D.C. Hodgkin and D.A. Mercola, Adv. Protein Chem. *26*, 279 (1972); R.A. Pullen, D.G. Lindsay, S.P. Wood, I.J. Tickle, T.L. Blundell, A. Wollmer, G. Krail, D. Brandenburg, H. Zahn, J. Gliemann and S. Gammeltoft, Nature *259*, 369 (1976).
16 P. De Meyts, E. Van Obberghen, J. Roth, A. Wollmer and D. Brandenburg, Nature *273*, 504 (1978).