direct correlation between its serum levels and the already described changes induced by it in brain tissue. A similar approach was tried before[17] in Gunn rat sucklings, but the authors reported a lack of relationship between cerebellar bilirubin content and serum concentrations of the unbound fraction. Among other possibilities, the different results reported here could be explained either by the fact that whole brain was used or by the possibility that suckling could affect the transport of bilirubin across the blood-brain barrier.

The brain bilirubin uptake, as shown in this paper, is a biphasic process. The amount of the pigment entering the brain per mg increase of total serum bilirubin is 2.75 times higher when the total serum levels are above 20 mg/dl. This can be seen by the slope change in the curve. When the serum unbound bilirubin concentrations are in the range of 1.7 µg/dl there is also an abrupt change in the slope of the line, enhancing the uptake by a factor of 5.35.

These data taken together suggest that the risk of bilirubin encephalopathy may be directly linked to serum levels of unbound bilirubin.

It is noteworthy that infants with serum indirect bilirubin at the levels of 11 and 22 mg/dl had serum unbound bilirubin of 0.77 and 1.87 µg/dl, respectively[18] which are very similar to the values reported in the present paper.

It has been reported that infants with early signs of kernicterus had serum unbound bilirubin of 1.75 µg/dl[19] and 1.17 µg/dl[20]. These values are very close to the figures shown in the present work, suggesting that they may be indeed the values of risk.

It should be pointed out that although the serum levels reported above are usually accompanied by early signs of kernicterus, lower values are responsible for biochemical changes which may not necessarily be accompanied by clinical signs. This would make them valuable as a guide for preventive therapy.

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A sensitive and inexpensive high-performance liquid chromatographic assay for tyrosine hydroxylase

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Summary. We describe a highly sensitive assay method for tyrosine hydroxylase (TH) using high-performance liquid chromatography with amperometric determination. This assay method could be applicable to any tissues with low enzyme activity, such as rat cerebellum. We also describe the kinetic properties of TH in rat cerebral cortex.

Tyrosine hydroxylase (L-tyrosine, tetrahydropteridine: oxygen oxidoreductase; E.C. 1.12.16.2) catalyzes the conversion of tyrosine to DOPA[1], the rate-limiting step in the biosynthesis of catecholamines. A highly sensitive assay method for tyrosine hydroxylase activity is frequently required for physiological and pathological studies.

The sensitive radiometric assays in current use for tyrosine hydroxylase activity measure the radioactive DOPA or water produced from labelled tyrosine. Recently, we developed a non-radiometric assay for tyrosine hydroxylase activity by high-performance liquid chromatography with an amperometric detector[2]. But the sensitivity is still not enough to measure the activity in the cerebral cortex.

In this communication, we describe an improved assay method for tyrosine hydroxylase activity which can be applied to any tissues containing low enzyme activity such as rat cerebellum. We also describe the kinetic properties of tyrosine hydroxylase in rat frontal cerebral cortex.

Materials. Male Sprague-Dawley rats weighing 200–250 g were killed by decapitation and the brains were rapidly removed. Rat frontal cerebral cortex, cerebellum, and hippocampus were collected, homogenized with 4 vol. of 0.32 moles/l sucrose, and the homogenates were frozen at −80 °C until use. Brain tissues from adult male SRJ:CD-1 (ICR) mice and New Zealand albino rabbits were also obtained by the method described above.

Experimental procedures. The standard incubation mixture consisted of the following components in a total volume of 500 µl (final concentrations in parentheses): 50 µl of 0.5 moles/l-potassium phosphate buffer, pH 6.64 (50 mmoles/l), 50 µl of 0.1 moles/l ascorbic acid (10 mmoles/l), 50 µl of 10 mmoles/l 6-methyl-5,6,7,8-

Tyrosine hydroxylase activity in rat, mouse, and rabbit brain tissues

	Brain tissue (n animals/n determinations)	Activity mean ± SD (nmole/min/g tissue)	Activity/blank
	Rat		
The data presented here	Frontal cerebral cortex (6/12)	0.431 ± 0.060	5.00
	Cerebellum (3/6)	0.201 ± 0.059	5.82
	Hippocampus (3/6)	0.280 ± 0.051	4.35
	Mouse		
	Frontal cerebral cortex (5/10)	0.319 ± 0.085	5.05
	Cerebellum (3/6)	0.299 ± 0.039	10.1
	Hippocampus (3/6)	0.107 ± 0.021	1.75
	Rabbit		
	Frontal cerebral cortex (3/6)	0.476 ± 0.016	6.55
The data presented by Croll et al.[4]	Rat		
	Frontal cerebral cortex (1/6)	0.73 ± 0.03	1.7
	Cerebellum (1/6)	0.33 ± 0.03	0.8

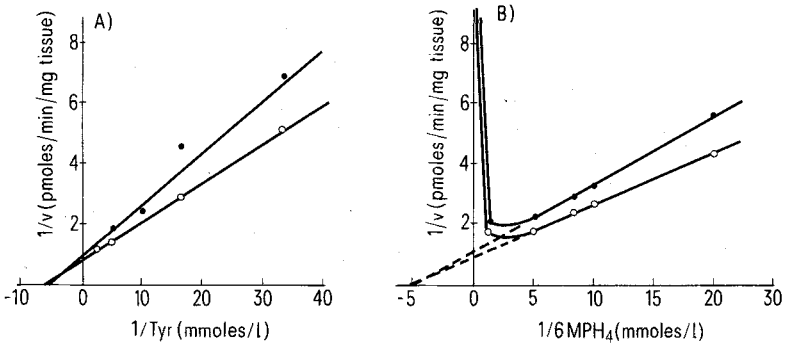
tetrahydropterin ($6MPH_4$) (1 mmole/l or various concentrations for kinetic studies) in 0.01 moles/l hydrochloric acid, 50 µl of 1 mmole/l ferrous ammonium sulfate (0.1 mmoles/l), 60 µl of 4 mg/ml catalase, 50 µl of 1 mmole/l L-tyrosine (0.1 mmoles/l or various concentrations for kinetic studies), and 190 µl of enzyme plus water. For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine and 100 pmoles of DOPA were added to another blank incubation as an internal standard for DOPA. After incubation at 37 °C for 10 min, the reaction was stopped with 4.5 ml of ethanol solution containing 100 pmoles of α-methyl-DOPA as an internal standard and 100 µl of 0.2 moles/l EDTA, and then centrifuged at 3000 rpm for 30 min. To the supernatant was added 500 µl of 2.5 moles/l Tris-HCl buffer (pH 8.6) and 100 µl of 10% sodium metabisulfite. The mixture was passed through a column containing 100 mg of alumina (12.5 × 0.4 cm inside diameter) at room temperature. The column was washed with 1.5 ml of 0.5 moles/l Tris-buffer (pH 8.6) twice, 1.5 ml of water, and 100 µl of 0.5 moles/l HCl. DOPA and α-methyl-DOPA were eluted with 250 µl of 0.5 moles/l HCl. A 50 µl aliquot of the eluate was injected for high-performance liquid chromatography [Yanaco L-2000 pump, Yanaco VMD-100 voltammetric detector, and Yanapak ODS-T column 25 × 0.4 cm inside diameter] (Yanagimoto Manufacturing Co., Fushimi-ku, Kyoto, Japan). The mobile phase was 0.1 moles/l phosphate and methanol (100:6.5 v/v) with a flow rate of 0.7 ml/min, and the temperature of the column was at 24 °C. The detector potential was set at 600 mV against the Ag/AgCl electrode. Under these conditions the retention times were: solvent front, 2.0 min; DOPA, 5.5 min; α-methyl-DOPA, 12.6 min. The sensitivity of this method in terms of the amount of DOPA which could be detected per injection of pure solutions and/or samples from enzymatic reactions was 0.1 pmoles. DOPA and α-methyl-DOPA showed the same recovery over the alumina column procedure (between 70 and 75%). The DOPA formed enzymatically by tyrosine hydroxylase was calculated using the following term

$$\frac{R(L)-R(D)}{R(D+S)-R(D)} \times 100 \text{ pmoles}$$

where R is the ratio of peak heights (peak height of DOPA/peak height of α-methyl-DOPA), R(L) being that from the L-tyrosine incubation, R(D) from the D-tyrosine incubation, and R(D + S) that of D-tyrosine plus DOPA (internal standard, 100 pmoles). The K_m-values and maximal velocities were determined from Lineweaver-Burk plots using Wilkinson's program[3].

Results and discussion. Using the present assay method, tyrosine hydroxylase activity in 1 mg of rat frontal cerebral cortex could be easily measured. As shown in table, the activity/blank ratios in rat brain tissues in this assay were larger than those in any other assays[4,5].

In our previous assay system, we could not measure tyrosine hydroxylase activity in the cerebral cortex because the blank value was very high compared with that in the radiometric method, and was half of the experimental one[2]; the blank value was rather high due to the excess tyrosine still present in the reaction mixture. In the new procedure, excess tyrosine was precipitated together with proteines by the addition of a large volume of ethanol after incubation. In aqueous ethanol, tyrosine is much less soluble than DOPA. Therefore, the present assay method is applicable to any tissues containing not only higher enzyme activity such as striatum but also lower one such as cerebellum. Using rat frontal cerebral cortex as enzyme, the reaction



Lineweaver-Burk plots illustrating the effect of tyrosine (A) or 6-methyltetrahydropterin (B) on the rate of DOPA formation by tyrosine hydroxylase in rat cerebral cortex. Samples of 3 mg of the tissues were used as the enzyme. Each point was determined by duplicate assays. *A* Concentrations of 6-methyl-5,6,7,8-tetrahydropterin were 1×10^{-4} moles/l (●——●) and 3.5×10^{-4} moles/l (○——○). *B* Concentrations of tyrosine were 1×10^{-4} moles/l (●——●) and 2×10^{-4} moles/l (○——○).

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.

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

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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