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OCCURRENCE AND SOME PROPERTIES OF A PROTEIN-LIKE INHIBITOR OF DOPAMINE β-HYDROXYLASE IN RAT LIVER

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Abstract—Evidence of endogenous inhibitors of dopamine β-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate:O_2 oxidoreductase (hydroxylating), EC 1.14.2.1] (DBH) in rat liver was obtained. At least two kinds of endogenous inhibitors were present in the 105,000 *g* supernatant of rat liver homogenate and their molecular weights were shown to be about 40,000 and 1200 by gel filtration.

The higher molecular weight inhibitor was partially purified by ammonium sulfate fractionation, column chromatography of Sephadex G-100 or Sephadex G-200, and DEAE-Sephadex(A-25) treatment. The activity of this inhibitor was not diminished by boiling for 5 min, but was lost completely when the inhibitor was treated with trypsin, suggesting that it may be a protein. The inhibition of DBH by this protein-like inhibitor in the liver was completely protected by the addition of *N*-ethylmaleimide, indicating that it may contain functional sulfhydryl groups. The inhibition by this protein-like inhibitor was of the noncompetitive type with both the substrate and with ascorbic acid, one of the cofactors in this reaction.

The existence of naturally occurring inhibitors of dopamine β-hydroxylase[3,4-dihyroxyphenylethylamine, ascorbate:O_2 oxidoreductase (hydroxylating), EC 1.14.2.1] (DBH) was first described by Levin *et al.* [1]. The occurrence of inhibitors of DBH has been reported in various tissues and organs and in various subcellular fractions of different species [2–5]; such an inhibitor was partially purified from bovine heart [6] and adrenal medulla [7]. It has also been shown that these endogenous inhibitors are heat-stable and dialyzable low molecular weight substances (less than 5,000) which may contain sulfhydryl groups as the functional residue in the molecule [2–4, 6–7]. However, the properties of these inhibitors differed slightly from one another in different tissues.

Our serial experiments in search of inhibitors of DBH [4] showed that at least two kinds of endogenous inhibitors of DBH were present in the soluble fraction of the liver and in some other organs of rats and other animals but the molecular weights of these inhibitors differed extremely from one another. The present paper describes a method for the isolation of inhibitors from rat liver and some of the characteristics of the higher molecular weight inhibitor. The low molecular weight inhibitor will not be referred to in this paper, since endogenous inhibitors of low molecular weight have been reported previously by many investigators [3–7].

MATERIALS AND METHODS

Materials. Trypsin inhibitor, trypsin, ovalbumin, bovine serum albumin, cytochrome *c*, glucagon, vitamine B_{12}, and catalase were purchased from Sigma Chemical Co., St. Louis, U.S.A. Tyramine hydrochloride was the product of Daiichi Kagaku Yakuhin Co. Ltd., Tokyo.

Preparation of native and boiled supernatants. All experiments were carried out at 0–4° unless described otherwise. Male rats of Sprague–Dawley strain [CLEA, Japan], weighing between 200–250 g, were used. The liver was quickly removed from rats sacrificed by decapitation, perfused with ice-cold 50 mM Tris–HCl buffer (pH 7.4) to remove blood and then homogenized in the same buffer as above by a Waring Blendor (20 sec, two times) and then by a Teflon homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min to remove cell debris. The resulting supernatant was centrifuged at 105,000 *g* for 60 min to prepare a soluble fraction which was designated as the native supernatant. The native supernatant was heated in a boiling water bath for 5 min. The resulting precipitate was removed by centrifugation at 10,000 *g* for 20 min. The supernatant fraction obtained by heat treatment was designated as the boiled supernatant. Activity of the native and the boiled supernatant in reducing the activity of DBH was measured by adding each, separately, to the DBH assay system.

Preparation of dopamine β-hydroxylase. DBH was prepared from bovine adrenal medulla according to the method of Foldes *et al.* [8]. The pure chromaffin granules were isolated from fresh bovine adrenal medulla using a sucrose density gradient. DBH was solubilized from purified chromaffin granules by using Triton X-100 and then the enzyme was purified using DEAE-cellulose column chromatography. The partially purified DBH preparation thus obtained was used throughout this work.

Assay of dopamine β-hydroxylase. The enzyme activity was assayed by measuring the amount of octopamine formed from tyramine according to the pro-

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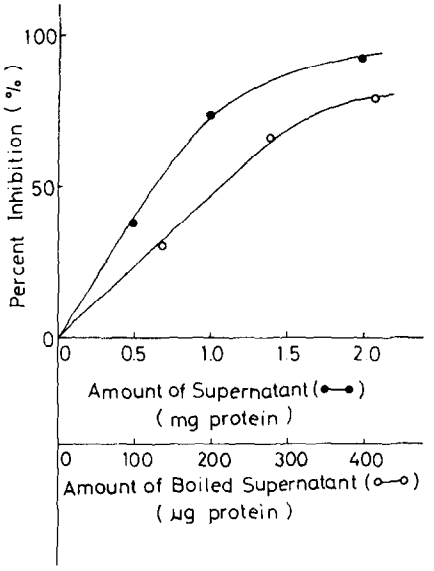

Fig. 1. Effect of boiled supernatant on DBH. Boiled supernatant was prepared by heating 105,000 *g* supernatant in a boiling water bath for 5 min. To the reaction mixture, this boiled supernatant was added as indicated in the figure. Other assay conditions for DBH were those described in Methods. Inhibition rate was expressed as per cent.

cedure of Van der Schoot *et al.* [9], as previously described [10]. One ml of the reaction mixture contained potassium phosphate buffer (pH 5.5) (100 μmoles), ascorbic acid (10 μmoles), fumaric acid (10 μmoles), tyramine hydrochloride (10 μmoles), catalase (200 Sigma units), and partially purified enzyme preparation. The reaction mixture was preincubated without tyramine for 5 min at 37°. The reaction was then initiated with the addition of tyramine, maintained for 15 min at 37° in air and terminated with the addition of 4 N ammonium hydroxide. The complete reaction mixture which terminated the reaction

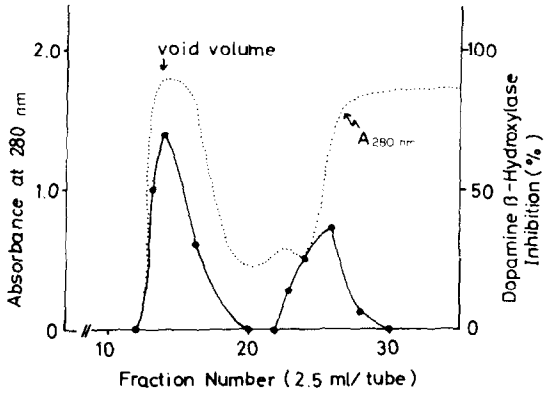

Fig. 3. Gel filtration of the boiled supernatant from bovine adrenal medulla on Sephadex G-25. Gel filtration on a Sephadex G-25 column chromatography was performed in the same manner as in Fig. 2. (---) Absorbance at 280 nm; (●) inhibitory activity on DBH.

immediately after preincubation was used as the control run. The amount of octopamine formed was determined after conversion of octopamine to *p*-hydroxybenzaldehyde which was measured at an absorbancy of 330 nm.

Protein determination. The protein content was determined by the methods of Lowry *et al.* [11], using crystalline bovine serum albumin as a standard.

RESULTS

Examination was made of the inhibitory effect of the boiled supernatant on DBH. As shown in Fig. 1, DBH was inhibited in proportion to the amount of the boiled supernatant added to the reaction mixture.

The boiled supernatant fraction, which was lyophilized and dissolved in 0.05 M Tris–HCl buffer (pH 7.4), was applied on Sephadex G-25 (1.5 cm × 43 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.4)

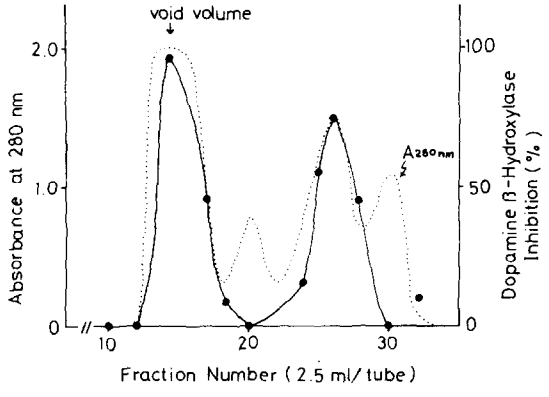

Fig. 2. Gel filtration of the boiled rat liver supernatant on Sephadex G-25. Thirty mg of protein of the boiled supernatant from rat liver was applied on a Sephadex G-25 column (1.5 cm × 43 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.4). The column was eluted at 4° with 50 mM Tris–HCl buffer (pH 7.4), and fraction of 2.5 ml were collected. Assay conditions were those described in Methods. (---) Absorbance at 280 nm; (●) inhibitory activity on DBH.

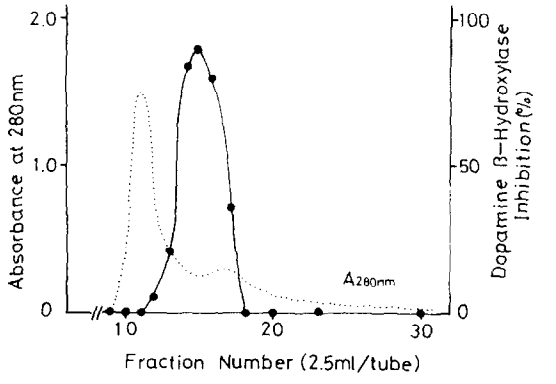

Fig. 4. Gel filtration of DBH inhibitor from rat liver on Sephadex G-100. Twenty mg of protein from the ammonium sulfate fraction were applied on a Sephadex G-100 column (1.5 cm × 43 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.4) and fractions of 2.5 ml were collected. Assay conditions were those described in Methods. An aliquot of each fraction (0.1 ml) was used for the assay of the inhibitory activity on DBH. (---) Absorbance at 280 nm; (●) inhibitory activity.

Table 1. Purification of the protein-like inhibitor of DBH from rat liver*

Fraction	Protein	Specific activity	Recovery	Purification factor
	mg	unit/mg	%	
105,000 *g* Super	11,256	1.1	100	1
105,000 *g* Boiled super	3,266	3.25	85.7	3
Ammonium sulfate (0–80% saturation)	640	7.4	38.3	6.7
Sephadex G-200	42.4	23.0	7.4	20.9
DEAE-Sephadex	13	38.5	3.1	35

* The purification procedure is described in the text. Ammonium sulfate fraction was fractionated with Sephadex G-200 instead of Sephadex G-100 column in this case. One unit of inhibitor is defined as the amount of inhibitor which is required to inhibit DBH activity by 50 per cent.

prior to use and eluted with the same buffer. As illustrated in Fig. 2, the boiled supernatant contained two kinds of inhibitors; activity of one was found at void volume of this column chromatography as a symmetrical single peak, and activity of the other at the position in which low molecular weight substances (less than 1,500) were eluted. As can be seen in Fig. 3, a similar inhibitory pattern was also obtained in the preparation of bovine adrenal medulla.

The boiled supernatant was fractionated by ammonium sulfate to purify the higher molecular weight inhibitor; solid ammonium sulfate was added to give 80% saturation. The precipitates were collected by centrifugation, dissolved in 0.05 M Tris–HCl (pH 7.4), and subjected to dialysis against the same buffer (ammonium sulfate fraction). After dialysis, this ammonium sulfate fraction was applied on Sephadex G-100 (1.5 cm × 43 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.4) and eluted with this same buffer; the elution profile is shown in Fig. 4. The active fractions (fractions 14–16) were combined and lyophilized (Sephadex G-100 fraction). The Sephadex G-100 fraction dissolved in 0.05 M Tris–HCl buffer (pH 7.4) was passed through a DEAE-Sephadex (A-25) column (1.5 cm × 43 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.4). This treatment helped to remove contaminants, since these were adsorbed on DEAE-Sephadex and the inhibitor was not. The inhibitor was not adsorbed on CM-Sephadex (C-25) or on P-cellulose equilibrated with 0.01 M potassium phosphate buffer (pH 6.5). Similar results were obtained in the preparation of bovine medulla.

Table 2. Stability of the protein-like inhibitor at 4°*

Duration of storage at 4° (hr)	Specific activity (unit/mg)
0	27.6
24	21.3
48	17.3
72	14.3

* The inhibitor fraction from the Sephadex G-100 column was stored at 4° and 0.1 ml of the fraction was used for the determination of inhibitory activity at different time intervals. The assay conditions were the same as those described in Methods.

The results of a typical purification are summarized in Table 1. The ammonium sulfate fraction was fractionated with Sephadex G-200 instead of Sephadex G-100. As shown in this Table, the inhibitor was purified about 35-fold and the yield was 3 per cent, as calculated from crude extracts. Since the yield of the inhibitor prepared by these procedures was low, its stability was tested at 4°. Fractions No. 14–16 from the Sephadex G-100 column, shown in Fig. 4, were combined and stored at 4°, and the inhibitory activity determined at different time intervals. As shown in Table 2, 72 hr of storage resulted in a 50 per cent decrease of the original inhibitory activity. These results may account for the failure to obtain the high inhibitory activity of the inhibitor after the purification procedure.

The molecular weight of the endogenous inhibitor from the rat liver was determined according to the method of Andrews [12], using Sephadex G-100 column chromatography. Cytochrome *c* (12,750), trypsin inhibitor (21,500) and ovalbumin (45,000) were used as reference standards. As shown in Fig. 5, the molecular weight of the inhibitor appears to be about 40,000.

When the boiled supernatant was lyophilized and fractionated with Sephadex G-100 column, the inhibitory activity was found in the fraction in which a substance of molecular weight of about 17,500 was eluted. Lyophilization seemed to have caused a decrease of the molecular weight of the inhibitor from

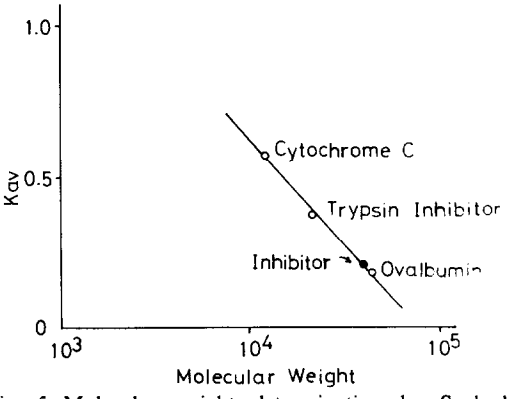

Fig. 5. Molecular weight determination by Sephadex G-100. A Sephadex G-100 column (1.5 cm × 43 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.4) was used to determine the molecular weight of the inhibitor.

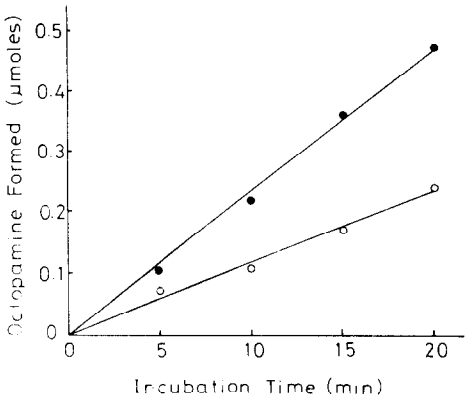

Fig. 6. Time course of octopamine formation by DBH in the presence and absence of the inhibitor. Assay conditions were the same as those described in Methods. Five μg of the DBH preparation were used for an assay system. Thirty seven μg of the inhibitor fraction prepared by the column chromatography of Sephadex G-100 were added on use. (○) In the presence of the inhibitor; (●) in the absence of the inhibitor.

40,000 to 17,500. Though causes of this decrease in molecular weight were not made clear with further analytical experiments, lyophilization might have caused dissociation of the inhibitor into subunits.

As shown in Figs 6 and 7, octopamine was formed from tyramine in proportion to the duration of incubation and to the amount of DBH regardless of the presence or absence of the inhibitor.

When the inhibitor was treated with trypsin, the activity of the inhibitor was completely lost (Table 3). Thus the inhibitor seems to be a protein-like substance or a substance which associates firmly with some protein.

As DBH may be non-specifically inhibited by such proteins as trypsin inhibitor, ovalbumin, and bovine serum albumin, the molecular weights of which are around 40,000, effects of these proteins on DBH were examined. As shown in Table 4, those proteins did

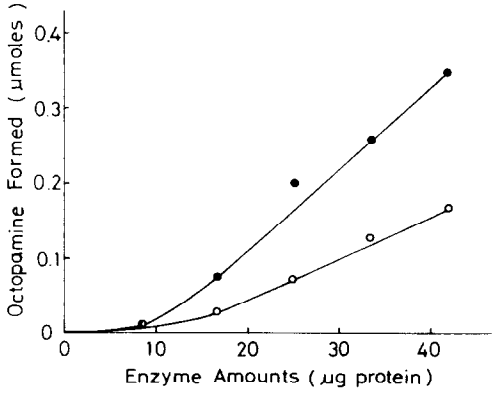

Fig. 7. Relationship between octopamine formation and DBH in various amounts in the reaction mixture. Other assay conditions were described in Methods. Thirty seven μg of the inhibitor fraction were added on use. (○) In the presence of the inhibitor; (●) in the absence of the inhibitor.

Table 3. Effect of trypsin treatment on DBH inhibitor*

Trypsin (μg/0.5 ml)	Inhibitory activity (% inhibition)
0	38.1
10	44.6
20	0
40	0
80	0

* Forty μg of the inhibitor were incubated with various amounts of trypsin in an incubation mixture of 0.5 ml, containing 100 mM of Tris–HCl (pH 7.4), 0.1 mM $MnCl_2$, for 60 min at 30°. Trypsin digestion was terminated by the addition of soybean trypsin-inhibitor (3 times the amount of trypsin used). The activity of the trypsin-treated inhibitor was assayed by the method described in Methods.

not inhibit DBH at any concentrations tested. Therefore, the inhibition of DBH by the inhibitor seems to be due to a specific action. Since Nagatsu *et al.* [2, 13] found endogenous inhibitors in the adrenal gland and the brain of rats to be sulfhydryl compounds, we investigated whether the protein-like inhibitor reported here also contains sulfhydryl groups as a functional group. As shown in Table 5, the inhibitory activity was lost by the addition of *N*-ethylmaleimide to the reaction mixture. This indicated that the protein-like inhibitor may contain a sulfhydryl group as an essential active residue. The possibility that the protein-like inhibitor produces such a proteolytic action on DBH as does catepsin may be ruled out, because the rate of inhibition did not increase by prolonging the time of preincubation of the inhibitor with DBH (Fig. 8).

As shown in Figs 9 and 10, kinetic studies on the inhibition of DBH by the protein-like inhibitor revealed that the type of inhibition was noncompetitive both with tyramine (the substrate) and ascorbic acid (a cofactor in this enzyme reaction).

The protein-like inhibitor reported in this paper could be found not only in rat liver (Fig. 2), but also

Table 4. Effect of several proteins on DBH*

Additions		Relative activity of DBH (%)
None		100
Trypsin inhibitor	12.5	107
(mol. wt 21,500)	25	118.1
	50	103.2
Ovalbumin	12.5	108
(mol. wt 45,000)	25	101.6
	50	109.6
Bovine serum albumin	12.5	95.9
(mol. wt 67,000)	25	105.2
	50	90.8
Protein inhibitor	10	23.7
(mol. wt 40,000)		

* Trypsin inhibitor, ovalbumin, or bovine serum albumin were added to the DBH assay system instead of the protein inhibitor. Other conditions for the assay of the inhibitory activity were the same as those described in Fig. 1.

Table 5. Effect of *N*-ethylmaleimide on the inhibition of DBH by the protein-like inhibitor*

Addition		Relative activity of DBH (%)
NEM (M)	Protein inhibitor	
non	non	100
non	37.5 μg	63
1×10^{-5}	non	98.6
1×10^{-5}	37.5 μg	83.7
1×10^{-4}	non	101.2
1×10^{-4}	37.5 μg	95.6
1×10^{-3}	non	103.2
1×10^{-3}	37.5 μg	116.1

* The activity of the protein inhibitor prepared by Sephadex G-100 column chromatography was assayed in the presence or absence of *N*-ethylmaleimide. Other assay conditions were the same as those described in Fig. 1.

in bovine adrenal medulla (Fig. 3), rat brain, bovine brain, monkey brain, and monkey liver (data not shown). This fact suggests that the inhibitor may have some important role in controlling the synthesis of catecholamines in various mammalian tissues.

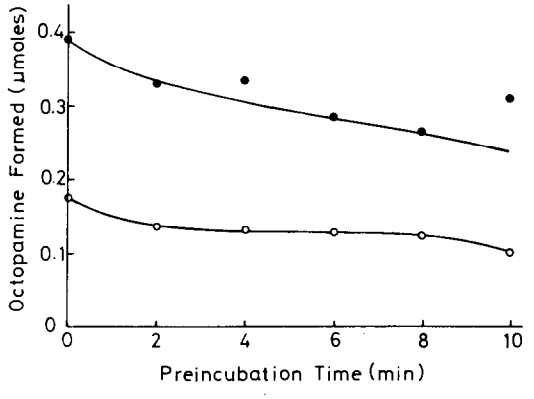

Fig. 8. Effect of pre-incubation time at 37° on DBH in the presence or absence of the inhibitor. DBH (4 μg as protein) was pre-incubated in the absence (●) or in the presence (○) of the inhibitor (37 μg as protein).

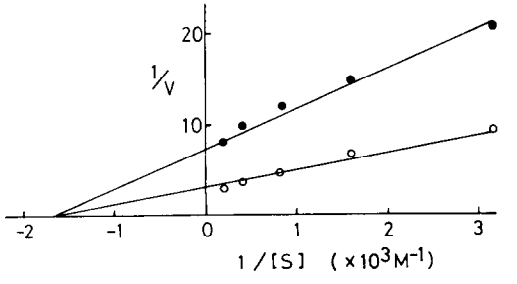

Fig. 9. Lineweaver–Burk plot of the tyramine concentration against the rate of hydroxylation in the presence or absence of the inhibitor. The assay was carried out in the same manner as that as described in Methods. The initial velocity of the reaction was determined as μmoles of octopamine formed for 15 min per 5 μg of the enzyme protein at 37°. (○); In the presence of the inhibitor; (●); in the absence of the inhibitor (37 μg as protein).

DISCUSSION

The experimental results presented in this paper show the occurrence of an endogenous protein-like inhibitor of DBH in rat liver. Although there have been several papers reporting low molecular weight endogenous inhibitors of DBH from various tissues [2–4, 6–7], there has been nothing published about a protein-like inhibitor of DBH. The present result is the first which confirms the presence of a protein-like inhibitor of DBH in several mammalian tissues.

DBH is known to be present in blood, and it has been considered that DBH may be released from the adrenal medulla and sympathetic postganglionic nerve fibers and enter into the circulation [14–16]. If DBH in blood has any significance in the production of norepinephrine, it may be that the protein-like inhibitor found by us has a physiological significance in the control of norepinephrine synthesis.

The inhibition of DBH by the protein-like inhibitor was diminished by *N*-ethylmaleimide added to the incubation medium (Table 5). Hence, the protein-like inhibitor may contain a sulfhydryl group as does the low molecular weight inhibitor in the soluble fraction of the adrenal medulla reported by Nagatsu *et al.* [2] and Duch *et al.* [7]. However, the question is left unsolved as to whether the protein-like inhibitor is specific for DBH, because we have not tested the inhibitory effect of the protein-like inhibitor on other copper enzymes.

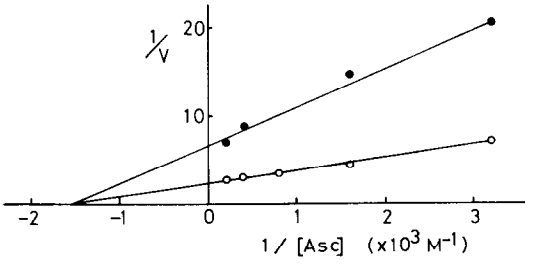

Fig. 10. Lineweaver–Burk plot of the ascorbic acid concentration against the rate of hydroxylation in the presence or absence of the inhibitor. The assay was carried out as described in the legend to Fig. 9. (○); in the presence of the inhibitor; (●) in the absence of the inhibitor (37 μg as protein).

As can be seen from Figs 9 and 10, kinetic analysis of the reaction shows that the inhibition of the protein-like inhibitor is noncompetitive with substrate (tyramine) and co-substrate. This is consistent with the finding concerning the inhibition of DBH by cysteine [2]. The K_m for tyramine did not change in the presence of the protein-like inhibitor; the inhibitor did not decrease the affinity of the enzyme for the substrate. Furthermore, the inhibition of DBH by the protein-like inhibitor was not time-dependent (Fig. 8). Therefore, the possibility may be excluded that a proteolytic process is involved in this inhibition.

It has been reported that the partial purification of whole homogenates of animal tissues results in the appearance of DBH activity, though crude homogenates show no or little enzyme activity [1, 3]. Our present results make it reasonable to assume that the protein-like inhibitor may mask DBH in tissue homogenates. Further investigation is needed to reveal a possible role of the protein-like inhibitor in the synthesis of catecholamines.

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