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INHIBITION OF DOPAMINE β-HYDROXYLASE BY FUSARIC ACID (5-BUTYLPICOLINIC ACID) *IN VITRO* AND *IN VIVO*

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Abstract—Fusaric acid (5-butylpicolinic acid) was found to be a potent inhibitor of dopamine β-hydroxylase *in vitro* and *in vivo*. Fusaric acid inhibited the enzyme by 50 per cent at a concentration of 3×10^{-8} M. Kinetic studies with purified dopamine β-hydroxylase showed that the inhibition by fusaric acid was of the uncompetitive type with the substrate and of the mixed type with a cofactor, ascorbic acid. Fusaric acid lowered endogenous levels of norepinephrine and epinephrine in brain, heart, spleen and adrenal glands. Maximum depletion of norepinephrine and epinephrine was observed between 3 and 6 hr after the administration of fusaric acid. Since dopamine β-hydroxylase activity in adrenal medulla was found to be inhibited *in vivo* after the administration of fusaric acid, the decrease in the catecholamine levels is attributed to the inhibition of norepinephrine synthesis at the dopamine β-hydroxylase stage.

FUSARIC acid (5-butylpicolinic acid), which is an antibiotic produced by fungus, has been found to be a potent hypotensive agent and a potent inhibitor of dopamine β-hydroxylase by Hidaka *et al.*[1] Since dopamine β-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (hydroxylating), EC 1.14.2.1][2,3] catalyzes the final step in the biosynthesis of norepinephrine, the effect of fusaric acid on the endogenous levels of norepinephrine and epinephrine is of interest. The present communication describes kinetic studies on the inhibition of dopamine β-hydroxylase *in vitro* and the depletion of norepinephrine stores in sympathetically innervated tissues by the compound. By using a new method for the assay of dopamine β-hydroxylase in homogenate of adrenal medulla,[4] the enzyme activity *in vivo* also was found to be inhibited after the administration of fusaric acid.

MATERIALS AND METHODS

Fusaric acid was prepared from a culture filtrate of a fungus of Fusarium Species as reported previously.[1]

Dopamine β-hydroxylase was prepared from the soluble fraction[4] of bovine

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adrenal medulla according to the procedure of Friedman and Kaufman.[3] The detergent "Cutscum" (isooctylphenoxypolyethoxyethanol containing detergent) was not used for the solubilization. Assays of the enzyme activity during the purification procedure were carried out using tyramine as substrate in the presence of *N*-ethylmaleimide as reported previously.[4] Most experiments were carried out on the eluate from calcium phosphate gel.

The reaction mixture for the enzymic assay (final volume, 1·0 ml) contained (in μmoles): fumarate, 10; potassium phosphate (pH 6·5), 100; ascorbate, 10; an appropriate amount of the enzyme (usually 9 μg of protein); and enough catalase to give maximum stimulation; and substrate (tyramine or dopamine), 10. This incubation mixture was preincubated for 8 min at 37°, and the reaction was started by adding

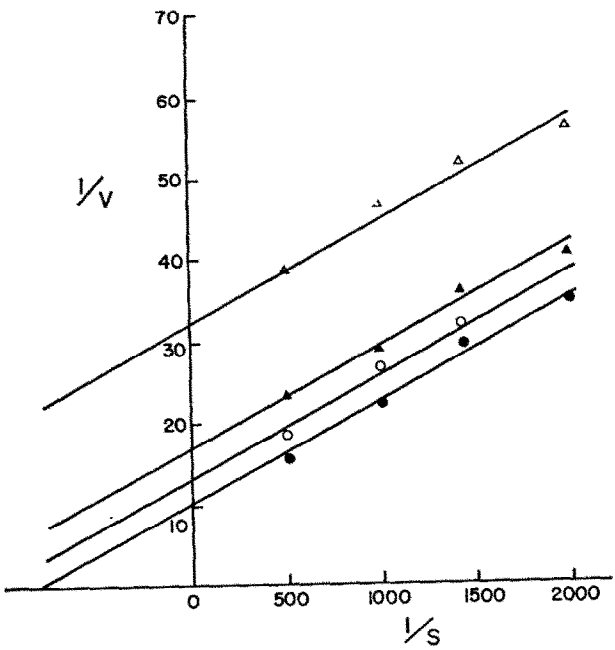

FIG. 1. Lineweaver–Burk plots of tyramine concentration against rate of hydroxylation with and without fusaric acid. Tyramine was added after preincubation of enzyme with fusaric acid. Incubation was for 30 min. The assay was carried out as described in Materials and Methods. The velocities are expressed as μmoles of norsynephrine formed from tyramine per 30 min. The substrate concentration is expressed in moles. ●——●, enzyme alone; ○——○, enzyme with 5×10^{-9} M fusaric acid; ▲——▲, enzyme with 3×10^{-8} M fusaric acid; △——△, enzyme with 1×10^{-7} M fusaric acid.

substrate. The reaction mixture was incubated for 30 min at 37° in air. The enzymic activity was measured by two methods. (1) When tyramine was the substrate, the conversion of tyramine to norsynephrine was followed according to the spectrophotometric procedure of Creveling *et al.*[5] The reaction was stopped by the addition of 0·2 ml of 3 M trichloroacetic acid. As a control, the reaction mixture without tyramine was incubated at the same time, and the substrate was added after stopping the reaction. Tyramine and norsynephrine were adsorbed on an Amberlite CG-120,

H^+ column and eluted with NH_4OH. The norsynephrine formed from tyramine was assayed on an aliquot of the column eluate by periodate oxidation and measurement of absorbance at 330 mμ of the *p*-hydroxybenzaldehyde formed. (2) When dopamine was used as substrate, the reaction was stopped by the addition of 0·2 ml of 0·6 M trichloroacetic acid. A 10 μl-aliquot of the acidified reaction mixture was added to 1·99 ml of 1 M acetate buffer (pH 6·5). Norepinephrine formed from dopamine was assayed according to the fluorometric procedure of Von Euler and Floding.[6]

Dopamine β-hydroxylase activity of rabbit adrenal medulla after the injection of fusaric acid was measured as described in our previous report.[4]

The concentrations of dopamine, norepinephrine and epinephrine in tissues were determined by a sensitive method for the simultaneous estimation of norepinephrine and dopamine in tissue by Hoagans.* Catecholamines were extracted by using phosphate buffer from tissue homogenates in dry butanol. To 2 ml of phosphate buffer extract (0·1 M, pH 6·5), 1·0 ml of 4% EDTA was added. Exactly 2 min after the addition of 0·2 ml of iodine solution (4·8 g KI and 0·25 g sublimed iodine in 100 ml water), 0·5 ml of alkaline sulfite (5 ml 12·6% anhydrous sodium sulfite made up to 25 ml with 5 N NaOH) was added. Exactly 2 min later 0·6 ml of 5 N acetic acid was added. The solutions were heated in a boiling water bath for 5 min, then cooled rapidly by immersion in cold water. The fluorescence intensities in the resulting solutions were determined in an Aminco–Bowman Spectrophotofluorometer at the following wavelengths (excitation, mμ/fluorescence, mμ): dopamine, 310/365; norepinephrine, 385/480; and epinephrine, 410/500. Assay values were corrected to 100 per cent recovery. In the case of adrenals, epinephrine plus norepinephrine were assayed, and the results were calculated as epinephrine.

Catalase activity was measured by a spectrophotometric method.[7] Tyrosine hydroxylase activity was assayed fluorometrically[8] using partially purified enzyme preparations from bovine adrenal medulla. Fe^{2+} ion (2×10^{-3} M) was included in the incubation mixture. Monoamine oxidase activity was assayed by following the disappearance of kynuramine by the method of Weissbach *et al.*[9] Rat liver mitochondria served as enzyme preparation. Aldehyde dehydrogenase activity was measured by the method of Racker.[10] The high-speed supernatant of guinea-pig kidney was used as enzyme preparation.

RESULTS

Studies in vitro

1. *Inhibition of purified dopamine β-hydroxylase by fusaric acid.* As shown in Table 1, fusaric acid inhibited dopamine β-hydroxylase at concentrations as low as 10^{-8} M. At a concentration of 3×10^{-8} M, the inhibition was about 50 per cent. Picolinic acid also inhibited the enzyme activity, but it was a less potent inhibitor than fusaric acid. Preincubation of dopamine β-hydroxylase with fusaric acid did not affect the extent of inhibition.

Fusaric acid inhibited the enzymic formation of norepinephrine from dopamine also, but to a slightly lesser extent than that of norsynephrine from tyramine as shown in Table 2.

Fusaric acid did not inhibit catalase at a concentration of 10^{-4} M. Therefore, the

*A. F. Hoagans, A sensitive method for the simultaneous estimation of norepinephrine and dopamine in tissue, personal communication.

inhibition was not due to the interaction of fusaric acid with catalase in the incubation mixture and the subsequent inactivation of the enzyme by H_2O_2 which is produced by non-enzymic autoxidation of the cofactor, ascorbic acid.[2]

2. *Mechanism of the effect on purified dopamine β-hydroxylase.— Reversibility of the inhibition by fusaric acid.* The inhibition produced by fusaric acid was completely reversed by dialysis against phosphate buffer (Table 3). Since dopamine β-hydroxylase

TABLE 1. INHIBITION OF DOPAMINE β-HYDROXYLASE BY FUSARIC ACID AND PICOLINIC ACID

Inhibitor	Concentration (M)	Dopamine β-hydroxylase (% of control activity)
———(Control)	———	100
Fusaric acid	1×10^{-9}	93
	3×10^{-9}	88
	5×10^{-9}	80
	1×10^{-8}	73
	3×10^{-8}	55
	5×10^{-8}	42
	1×10^{-7}	31
	3×10^{-7}	16
	5×10^{-7}	11
	1×10^{-6}	8
Picolinic acid	5×10^{-7}	45
	5×10^{-6}	17

TABLE 2. COMPARISON OF INHIBITION OF DOPAMINE β-HYDROXYLASE BY FUSARIC ACID FOR DIFFERENT SUBSTRATES

Substrate	Concentration of fusaric acid (M)	Inhibition of dopamine β-hydroxylase (%)
Tyramine	1×10^{-8}	21
	3×10^{-8}	45
	7×10^{-8}	56
Dopamine	1×10^{-8}	15
	3×10^{-8}	23
	7×10^{-8}	32

is a copper enzyme, the possibility that fusaric acid may inhibit the enzyme by the chelation with copper was examined. As shown in Table 4, the inhibition was not reversed significantly by the addition of copper.

Kinetic studies on the inhibition by fusaric acid. Kinetic studies were made to determine the type of inhibition involved in the action of fusaric acid. The results of these studies are expressed in Lineweaver–Burk plots. As shown in Fig. 1, inhibition of dopamine β-hydroxylase by fusaric acid was found to be of the uncompetitive type, implying that this compound affects the enzyme–substrate complex.[11] The inhibition

of fusaric acid was of the mixed type in the presence of the cofactor, ascorbic acid, as shown in Fig. 2. The extent of the inhibition of fusaric acid did not depend significantly on the fumarate concentration between 1×10^{-2} M and 5×10^{-4} M (Table 5).

Effect of fusaric acid on tyrosine hydroxylase, monoamine oxidase and aldehyde dehydrogenase. To investigate the specificity of the inhibitory activity of fusaric acid

TABLE 3. REVERSAL OF FUSARIC-ACID INHIBITION OF DOPAMINE β-HYDROXYLASE BY DIALYSIS

	Dopamine β-hydroxylase* (% of control activity)	
	Before dialysis	After dialysis
Control	100	100
Fusaric-acid treated enzyme	9	92

*Enzyme was incubated with fusaric acid (1×10^{-6}M) for 10 min at 37°, and then dialyzed against 200 volumes of 0·05 M phosphate buffer (pH 6·5) at 5° for 24 hr. Buffer was changed three times.

TABLE 4. EFFECT OF Cu^{2+} ON FUSARIC-ACID INHIBITION OF DOPAMINE β-HYDROXYLASE

Cu^{2+} Concentration (M)	Dopamine β-hydroxylase* (% of control activity)			
	Enzyme alone	Enzyme plus 1×10^{-8} M fusaric acid	Enzyme plus 3×10^{-8} M fusaric acid	Enzyme plus 1×10^{-7} M fusaric acid
0	100	72	49	27
1×10^{-5}	15	16	16	14
5×10^{-6}	21	16	18	13
1×10^{-6}	102	74	51	26
1×10^{-7}	100	75	54	29

*Fusaric acid and Cu^{2+} were preincubated at 37° for 8 min. Complete incubation mixture was then added, and the reaction was carried out at 37° for 30 min.

on dopamine β-hydroxylase, the effects of this compound on some other oxidoreductases were examined. At a concentration of 10^{-5} M, fusaric acid did not inhibit tyrosine hydroxylase, monoamine oxidase, and aldehyde dehydrogenase. At a concentration of 10^{-4} M and without adding Fe^{2+} to the incubation mixture, tyrosine hydroxylase activity was inhibited significantly.

Studies in vivo

(1) *Estimation of the inhibition of dopamine β-hydroxylase activity by fusaric acid in the adrenal medulla* in vivo. The simplified assay procedure of dopamine β-hydroxylase in crude tissue preparations[4] permitted to estimate the degree of inhibition of fusaric acid on dopamine β-hydroxylase *in vivo*. Dopamine β-hydroxylase activity was assayed in homogenates of the adrenal medulla after administration of fusaric acid

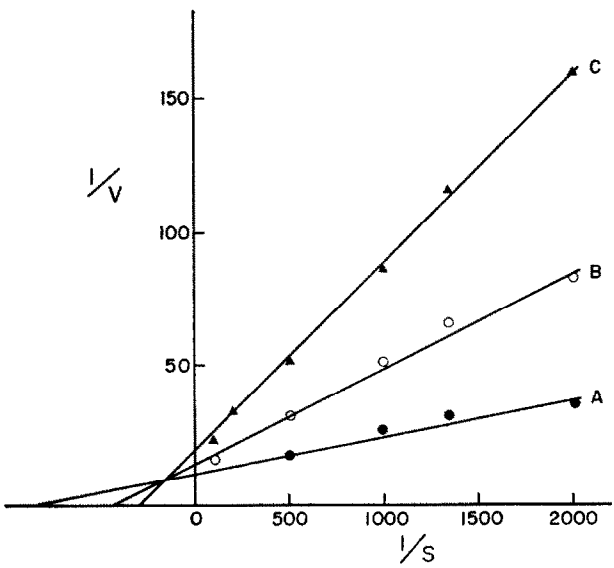

FIG. 2. Lineweaver–Burk plots of ascorbic acid concentration against rate of hydroxylation with and without fusaric acid. Fusaric acid and ascorbic acid were added simultaneously. Incubation was for 30 min. The assay was carried out as described in Materials and Methods. The velocities are expressed as μmoles of norsynephrine formed from tyramine for 30 min. The substrate concentration is expressed in moles. ●——●, enzyme alone; ○——○, enzyme with 3×10^{-8} M fusaric acid; ▲——▲, enzyme with 7×10^{-8} M fusaric acid.

TABLE 5. EFFECT OF FUMARATE CONCENTRATION ON FUSARIC ACID INHIBITION OF DOPAMINE β-HYDROXYLASE

Fumarate Concentration (M)	Dopamine β-hydroxylase (% of control activity)			
	Enzyme alone	Enzyme plus fusaric acid (M)		
		1×10^{-8}	3×10^{-8}	1×10^{-7}
1×10^{-2}	100	78	65	36
1×10^{-3}	89	68	60	32
5×10^{-4}	83	65	52	31
0	73	55	48	29

to intact rabbits. As shown in Table 6, marked inhibition of the activity was observed in the adrenal medulla homogenate 3 hr after administration of fusaric acid. The real extent of the inhibition *in vivo* may be even higher, since the dilution of the tissue homogenate during the assay procedure will cause dissociation of the inhibitor–enzyme complex.

(2) *Effect of fusaric acid on the levels of catecholamines in tissues of rats after administration of a single dose of fusaric acid.* Above results showed that fusaric acid is a very potent inhibitor of dopamine β-hydroxylase both *in vitro* and *in vivo*. Experiments

TABLE 6. DOPAMINE β-HYDROXYLASE ACTIVITY IN HOMOGENATE OF RABBIT ADRENAL MEDULLA AFTER ADMINISTRATION OF FUSARIC ACID

	Dopamine β-hydroxylase activity (μmoles/30 min/g)
Control	169 ± 61
Experiment No. 1	39
No. 2	24

Two rabbits were injected i.p. with fusaric acid (100 mg/kg). The animals were sacrificed after 3 hr and the enzyme activity in adrenal medulla was determined by a method for the assay of the activity in the homogenate.[4] Results in control were expressed as the mean ± S.E.M. of four rabbits.

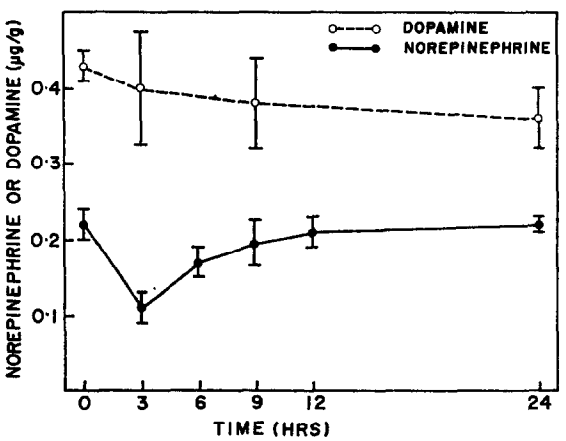

FIG. 3. Levels of norepinephrine and dopamine in brains of rats after administration of a single dose of fusaric acid (100 mg/kg i.p.). Each value represents the mean value (vertical line represents ± 1 standard error) obtained from three to five rats.

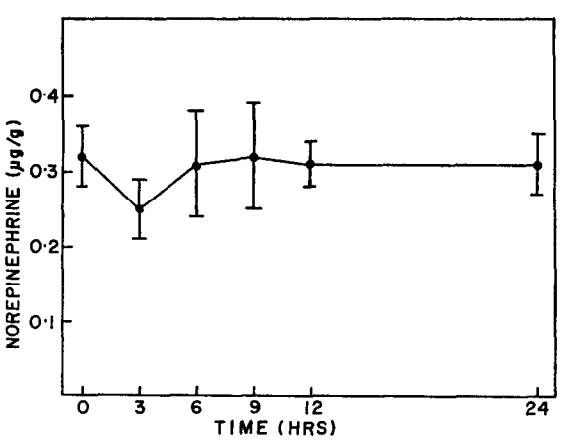

FIG. 4. Levels of norepinephrine in spleens of rats after administration of a single dose of fusaric acid (100 mg i.p.). Each value represents the mean value (vertical line represents ± 1 standard error) obtained from three to six rats.

were carried out to see whether enzyme inhibition can lead to depletion of norepinephrine in sympathetically innervated tissues. As shown in Fig. 3, norepinephrine levels in brain decreased 3 hr after administration of fusaric acid, and returned to normal levels after 12 hr. In contrast, dopamine levels did not change significantly. Norepinephrine in spleen decreased slightly after 3 hr and returned to normal levels after 6 hr (Fig. 4). Marked reduction in norepinephrine levels was observed in heart as shown in Fig. 5. The depletion was by 66 per cent after 3 hr, by 53 per cent after 6 hr, and the levels returned to the control values after 12 hr. Epinephrine levels in adrenals also decreased greatly (Fig. 6). The depletion continued up to 9 hr, and the

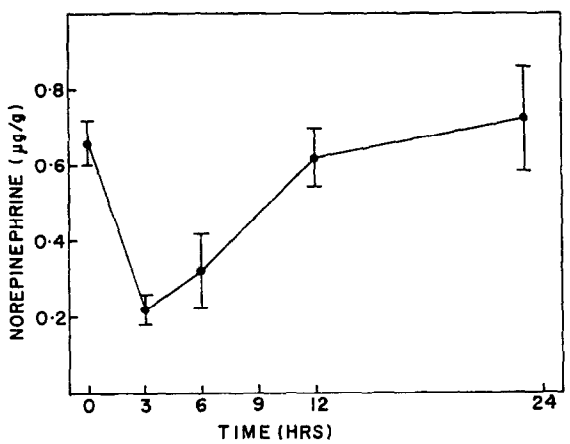

FIG. 5. Levels of norepinephrine in hearts of rats after administration of a single dose of fusaric acid (100 mg/kg i.p.). Each value represents the mean value (vertical line represents ± 1 standard error) obtained from three to six rats.

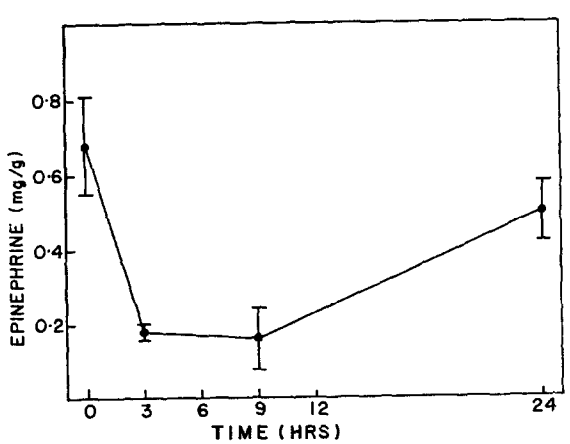

FIG. 6. Levels of epinephrine (including norepinephrine) in adrenals of rats after administration of a single dose of fusaric acid (100 mg/kg i.p.). Each value represents the mean value (vertical line represents ± 1 standard error) obtained from three rats.

levels returned to the control values after 24 hr. Significant amounts of dopamine were not detected in spleen and heart after administration of fusaric acid. However, the concentrations of dopamine in adrenals increased (control, 10 μg/g; 3 hr after administration, 30 μg/g).

DISCUSSION

Fusaric acid was found to be a very potent inhibitor of dopamine β-hydroxylase. At a concentration of 3×10^{-8} M, the inhibition was 50 per cent. Picolinic acid was an effective inhibitor also, but the introduction of 5-butyl group increased the inhibitory activity. The picolinic acid structure is an essential part for the effect. Detailed study on the relationship between structure and inhibitory activity of pyridine derivatives will be reported elsewhere.

The most potent inhibitors of dopamine β-hydroxylase previously reported were benzyloxyamines[12] which are isosteres of phenylethylamine. They inhibited the enzyme by about 60 per cent at a concentration of 1×10^{-5} M *in vitro*. The other type of potent inhibitors of dopamine β-hydroxylase *in vitro* and *in vivo* are copper chelating compounds such as diethyldithiocarbamate[13] or phenylethyldithiocarbamate.[14] These compounds inhibited the enzyme at concentrations between 10^{-5} M and 10^{-6} M *in vitro*.[15] Therefore, fusaric acid is the most potent inhibitor of dopamine β-hydroxylase which has been reported so far.

The mechanism of inhibition of fusaric acid is characteristic. Since fusaric acid can produce chelation with metals, the inhibitory mechanism was initially expected to be due to chelation of the copper atom at the active site of the enzyme. However, the experimental results indicated that the inhibition may not be due to simple chelation. The inhibition by chelating agents such as sulfhydryl compounds[16] could be reversed by Cu^{2+} ion or by extensive dialysis, and was of non-competitive type with substrate and cofactors. Therefore, the extent of inhibition was unrelated to the concentration of the substrate, ascorbate or fumarate. On the contrary, the inhibition by fusaric acid was of uncompetitive type with the substrate, indicating that this compound affects the enzyme–substrate complex.[11] Fusaric acid exhibited a mixed type of inhibition with a cofactor, ascorbate. The inhibition by fusaric acid could be reversed by extensive dialysis, but the addition of copper did not reverse the inhibition. These results suggest that the inhibition by fusaric acid is not due to simple chelation, but due to complex interactions with the enzyme–substrate complex.

The decrease in the endogenous levels of norepinephrine in heart, brain, and spleen and of epinephrine in adrenals is attributed to the inhibition of catecholamine biosynthesis *in vivo* at the dopamine β-hydroxylase stage. This interpretation is supported by the decrease of norepinephrine in heart, brain, and spleen and of epinephrine in adrenals without any marked decrease in dopamine concentrations. Fusaric acid did not alter the uptake or release of norepinephrine in mouse heart (unpublished results). Dopamine in brain did not show any increase, but that in adrenals increased. A similar elevation of dopamine in rat adrenals after the administration of inhibitors has been reported with diethyldithiocarbamate[17] and phenylethyldithiocarbamate.[14]

As reported by Hidaka *et al.*,[1] fusaric acid is a potent hypotensive agent in various mammals. From the present results it appears that the hypotensive action of fusaric acid is due to the decrease of endogenous norepinephrine concentrations in the cardiovascular system by the inhibition of dopamine β-hydroxylase.

It was reported by Holtz *et al.*[18] and Kumagai *et al.*[19] that incubation of dopamine with monoamine oxidase *in vitro* produced tetrahydropapaveroline which is a potent hypotensive agent. The inhibition of dopamine β-hydroxylase *in vivo* may facilitate the monoamine oxidase pathway for dopamine metabolism to produce tetrahydropapaveroline. However, the formation of this compound in rats after administration of fusaric acid could not be demonstrated.

Fusaric acid did not inhibit tyrosine hydroxylase at a concentration sufficient for complete inhibition of dopamine β-hydroxylase. It has been established that tyrosine hydroxylase is the rate-limiting step in the biosynthesis of norepinephrine.[20] However, such a potent inhibitor of dopamine β-hydroxylase as fusaric acid would be able to reduce endogenous levels of norepinephrine, though the enzyme is not the rate-limiting step. Since fusaric acid is a potent and specific inhibitor of catecholamine biosynthesis at the dopamine β-hydroxylase stage, the compound would be a valuable tool in pharmacological studies.

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