FORMATION OF HYDROXYTYRAMINE FROM
TYRAMINE IN THE ADRENAL GLAND
PHYSIOLOGICAL MEANING OF TYRAMINE AS
A PRECURSOR OF ADRENALINE

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The possibility that adrenaline may arise from tyramine in adrenal gland was first postulated by Ewins and Laidlaw (1) in 1910. This was supported by the perfusion data of Nikolaeff (2) and the slice experiment of Schuler (3), Devine (4) and Holtz (5) which demonstrated the conversion of adrenaline from tyramine, but not from tyrosine, dihydroxyphenylalanine (dopa), or phenylalanine. However, since the experiment of Halle (6) in 1906, Guggenheim (7), Knoop (8), Rosenmund and Dornsaft (9) asserted that tyrosine might be an effective precursor of adrenaline. In 1938, previous studies (10, 11) in our laboratory presented the evidence for the existence of this pathway, and later this was confirmed by the isotopic data of Udenfriend *et al.* (12) which established that adrenaline was formed from tyrosine, but not from tyramine *in vivo*. Recently the reaction of hydroxytyramine to *nor*-adrenaline has been demonstrated to be catalysed by an enzyme requiring FAD and Fe^{++} (13). Thus it is generally assumed at present that the following reaction sequence takes place as the main pathway to form adrenaline.

Tyrosine $\longrightarrow$ Dopa $\longrightarrow$ Hydroxytyramine $\longrightarrow$ *Nor*adrenaline $\longrightarrow$ Adrenaline

More recently it has been found in our laboratory that tyramine was oxidized to hydroxytyramine in blood and liver of rabbits (14, 15). This observation suggests the possibility that tyramine might be also a precursor of adrenaline, if the same oxidation process would be demonstrated to occur in adrenal gland. In the present paper the results of a preliminary survey of this metabolic pathway are described, together with the properties of the enzyme responsible for the oxidation of tyramine to hydroxytyramine in adrenal gland.

I. Oxidation of Tyramine to Hydroxytyramine in Adrenal Gland

The oxidation of tyramine to hydroxytyramine in adrenal gland was followed colorimetrically by two reactions, Folin-Cannon-Dennis's reaction (16) and a modification of Vulpian's reaction (17). For the purpose to remove catecholamine present in adrenal gland,

In the isotopic experiment, radioactive tyramine was added to the reaction mixture as the substrate, and the radioactivity of the products was shown on paperradioautography.

Method

Enzyme preparation: The bovine adrenal glands were minced and homogenized in 0.25 M sucrose solution with Potter-Elvehjem homogenizer. The homogenate was centrifuged with International refrigerated centrifuge at $20,000\times g$ for 60 minutes. The supernatant fluid and the precipitate were dialyzed separately against running water for 18 hours in order to remove catecholamine contained in adrenal gland tissue. All procedures were carried out at 0–5°C.

Flask content: Each flask containing 10 ml of enzyme preparation, 1.5 ml of M/5 phosphate buffer adjusted to pH 7.2, 0.05 ml of octylalcohol, and 0.5 ml of M/20 tyramine, was incubated at 37°C for 2 hours.

Folin's reaction: Reactions were stopped by the addition of an equal volume of 20 % trichloroacetic acid to the flask and the filtrate obtained was used for the analyses. To 5 ml of the filtrate, 0.5 ml of Folin's reagent, and 10 ml of 15 % Na_2CO_3 were added and color development was read at 660 m μ in Coleman spectrophotometer.

Vulpian's reaction: The protein free filtrate was adjusted to pH 6.8. To 5 ml of this filtrate, 0.2 ml of 0.1 N iodine solution was added and after 5 minutes 0.5 ml of 0.1 N- $\text{Na}_2\text{S}_2\text{O}_3$ was more added. Readings were taken at 530 m μ .

Paperchromatography: The protein fraction which precipitated at concentration of 20 to 40 per cent of ammonium sulfate was used as enzyme preparation for this purpose. After 2 hours of incubation at 37°C, 2 volumes of acidic alcohol (10 N- H_2SO_4 2.5 ml + 99 % ethanol 100 ml) was added to the reaction mixture and the deproteinized filtrate was dried in vacuum. The dried residue was dissolved in 20 ml of distilled water and treated with alumina as follows according to Euler's method (18); one gram of alumina was added to the solution and the mixture was adjusted to pH 8.0–8.5 with 1 N-NaOH. After shaking for 15 min, this alumina was collected by centrifugation and washed 3 times with 10 ml of distilled water, and then it was eluted three times with 5 ml of 0.25 N- H_2SO_4 . The three eluates were combined and adjusted to pH 4.0 with 10 % NaHCO_3 , and dried in vacuum. The resulting residue was extracted three times with 5 ml of *n*-butanol and the butanol layer was chromatographed with *n*-butanol-acetic acid-water (4:1:1) for 18 hours at room temperature. Materials were located by spraying with Folin's reagent or ammoniacal AgNO_3 .

Paperradioautography: Each flask contained 2 ml of 20 % bovine adrenal-gland homogenate, 2 ml of M/20 phosphate buffer (pH 7.2), 1 ml of M/100 $\alpha\text{-C}^{14}$ -tyramine, 0.05 ml of octylalcohol, and ATP and methionine (both added to give final concentration of M/1000). After 2 hours of incubation at 37°C, the reaction of mixture was treated with alumina according to Euler's method as described above. The eluate from alumina was chromatographed and the radioactive materials on paper were visualized by pressing the chromatograms to Fuji industrial X-ray film type 400 for 2 months in the dark cold room. adrenal gland homogenates were dialyzed and partially purified. The reaction products were assayed by paperchromatography after the treatment with alumina.

Results

TABLE 1. Increase of optical density values of color reaction by addition of tyramine.

Fraction	optical density value			
	Folin's reaction		Vulpian's reaction	
	Control	Tyramine added	Control	Tyramine added
Supernatant	0.07	0.21	0.04	0.16
Precipitate	0.12	0.35	0.06	0.20

As shown in Table 1, the optical density values determined by both color reactions were increased by the addition of tyramine, suggesting the formation of catecholamine, and this increment was observed in both fractions, supernatant and precipitate. The values of the control may be due to the remaining catecholamine originally contained in adrenal gland. Attempts to extract the active fraction from the precipitate by using *n*-butanol, pyrophosphate or by oscillation and autolysis have been unsuccessful, then the supernatant was fractionated by ammonium sulfate and dialyzed against running water.

As shown in Table 2, the highest activity was found in the precipitate at 20 to 40 per cent of ammonium sulfate, which was used as enzyme preparation for all the following experiments.

Paperchromatographic data using the active fraction of the supernatant were shown in Fig. 1.

The reaction product was adsorbed on alumina, and gave both positive Folin's reaction and AgNO_3 -test. The R_f value

TABLE 2. Activity of each fraction after the supernatant was fractionated with ammonium sulfate.

Saturation of ammonium sulfate (%)	Optical density value (Folin's reaction)	
	Control	Tyramine added
0—30	0.08	0.22
20—40	0.07	0.34
30—50	0.06	0.21
40—60	0.06	0.21

FIG. 1. Paperchromatogram of reaction product located by spraying with ammoniacal AgNO_3 or Folin's reagent.

- 1) Enzyme preparation + Hydroxytyramine 0°C
 - 2) Enzyme preparation + Tyramine 0°C
 - 3) Enzyme preparation + Tyramine 37°C
 - 4) Enzyme preparation + Tyramine 37°C
- (Products were treated with alumina)

of the reaction product on paperchromatogram was 0.43, suggesting that hydroxytyramine was formed.

In the isotopic experiment, C^{14} -tyramine was incubated with bovine adrenal grand homogenate, and the formed products were adsorbed on alumina, chromatographed and located

by spraying with ammoniacal AgNO_3 . The R_f values of the formed compounds were 0.27 and 0.39 corresponding to those of *noradrenaline* and hydroxytyramine respectively, as shown in Fig. 2.

FIG. 2. Radioautography and paperchromatography of reaction products when radioactive tyramine was incubated with homogenate of adrenal gland.

- 1) Homogenate + Hydroxytyramine + *Noradrenaline* 0°C
- 2) Homogenate + Tyramine 37°C
- 3) Homogenate + Tyramine 37°C

FIG. 3. Optimal pH of tyramine oxidase.

These results supported the assumption that hydroxytyramine might be formed from tyramine in adrenal gland.

II. Properties of Tyramine Oxidase

1) *Optimal pH*: The enzyme was most active at pH 6.8 to 7.2.

0.3 ml of M/20 tyramine was incubated with 3 ml of enzyme preparation partially purified through the ammonium sulfate fractionation in the presence of 0.3 ml of phosphate buffer (M/5) having indicated pH and 0.05 ml of octylalcohol. The reaction mixture was incubated for 20 minutes at 37°C, and determined for hydroxytyramine by the Folin's procedure.

2) *Influence of various buffers*: Phosphate, veronal or borate buffer at pH 7.2 was added to the reaction mixture at the final concentration of M/60. But the activity of the enzyme was not affected by any one of these buffers.

3) *Michaelis constant*: Michaelis constant of this enzyme seemed to be about $4 \times 10^{-4} \text{M}$ as shown in Fig. 4.

4) *Thermostability*: The enzyme activity was lost completely on exposure to 70°C for 30 minutes, or to 20°C for 24 hours.

5) *Substrate specificity*: Among various monophenol tested as substrate, only tyramine was oxidized by this enzyme while other compounds were not attacked as shown in Table 3.

6) *The activity of this enzyme as polyphenol oxidizing system*: The conventional Warburg's apparatus was used for the measurement of oxygen consumption.

FIG. 4. Measurement of Michaelis constant of this enzyme.

TABLE 2. Substrate specificity of this enzyme.

Substrate	Folin's reaction (optical density)
None	0.03
Tyramine	0.28
<i>p</i> -Nitrophenol	0.03
Tyrosine	0.06
Sympathol	0.08
Phenol	0.04

Each flask contained 1.9 ml of enzyme preparation, and 0.4 ml of M/5 phosphate buffer adjusted to pH 6.6 as well as 0.05 ml of octylalcohol in the main chamber, and 0.4 ml of M/40 tyramine or hydroxytyramine in the side arm. Center well contained 0.3 ml of 20% of KOH.

The pH of the reaction mixture was adjusted slightly acidic to avoid the non-enzymatic autoxidation of formed hydroxytyramine and under these conditions the possibility was studied that tyramine or hydroxytyramine could be oxidized by this enzyme preparation.

As shown in Fig. 5, oxygen consumption was observed only in the presence of tyramine added as substrate, but not demonstrated when hydroxytyramine was incubated instead of tyramine.

From these results, it seems unlikely that this enzyme has activity as the polyphenol

FIG. 5. Activity of this enzyme as polyphenol oxidizing system.

- 1) E + P.B. + Oct. + Tyramine
- 2) E + P.B. + Oct. + Hydroxytyramine
- 3) E + P.B. + Oct. + Aq. dest.
- 4) Aq. dest. + P.B. + Oct. + Hydroxytyramine

P.B.: Phosphate buffer

Oct.: Octylalcohol

E: Enzyme solution

oxidizing system.

7) *Hydrogen acceptor*: Methylene blue or 2,6-dichlorophenol indophenol was added as hydrogen acceptor instead of oxygen in Thunberg's tube but decolorization was not observed at all.

8) *Influences of chelating agents and p-chloromercuribenzoate on the enzyme activity*: The enzyme activity was determined by oxygen consumption in the Warburg's apparatus and by colorimetric method of Folin. Various chelating agents were added to give a final concentration of M/5000.

As shown in Table 4, the addition of KCN, diethyldithiocarbamate or oxine, resulted the decrease of oxygen uptake and the values of Folin's method, suggesting the cupric ion might be required for the reaction. In the presence of *o*-phenanthroline which chelates iron and zinc and not copper, an inhibition was observed by Folin's method but oxygen consumption increased. Moreover, an inhibition of *p*-chloromercuribenzoate seemed to indicate that sulfhydryl groups were essential for the enzymatic activity.

TABLE 4. Influences of chelating agents and *p*-chloromercuribenzoate on the activity of this enzyme.

Addition	Folin's R. (Opt. density)	Oxygen uptake (μ l)
None	0.32	29
KCN	0.21	20
<i>o</i> -Phenanthroline	0.23	35
Diethyldithiocarbamate	0.18	15
Oxine	0.25	24
EDTA	0.29	26
Pyrophosphate	0.32	28
PCMB	0.19	19

9) *Reactivation by the addition of metal ions after dialysis against chelating agents*: Various chelating agents were added to the enzyme preparation at the final concentration of M/5000 and after 3 hours the mixture were dialyzed against the solution dissolving the same chelating

TABLE 5. Reactivation of the activity of this enzyme by the addition of metal ions.

Addition	Folin's R. (Opt. dens.)	Oxygen uptake (μ l)	Dialysis against
None	0.28	21	<i>o</i> -Phenanthroline
FeCl ₂	0.36	14	"
FeCl ₃	0.33	16	"
ZnCl ₂	0.28	—	"
None	0.18	24	Diethyldithiocarbamate
CuCl ₂	0.12	30	"
None	0.27	—	EDTA
MgCl ₂	0.28	—	"
SnCl ₂	0.27	—	"
NiCl ₂	0.29	—	"

agent at the concentration of $10^{-4}M$ overnight, moreover against deionized water for 18 hours. Then the enzymatic activity was determined in the presence of various metal ions added at the final concentration of $M/60,000$.

With ferro or ferric ion, Folin's procedure indicated the stimulation of the enzymatic reaction but oxygen consumption was decreased on the contrary. In connection with this finding it was demonstrated that the autoxidation rate of hydroxytyramine increased in the presence of *o*-phenanthroline and ferrous ion added to the solution depressed the autoxidation velocity of hydroxytyramine. Therefore on the basis of these evidences together with the facts that hydroxytyramine chelates ferrous ion but not tyramine and that the oxygen consumption observed above was partly due to the nonenzymatic oxidation of hydroxytyramine, a possible explanation for results shown in Table 5 on the behavior of ferrous ion seemed to be furnished. The presence of ferrous ion may inhibit the autoxidation of hydroxytyramine but have no effect on the formation of hydroxytyramine from tyramine.

With respect of the cupric ion, further experiments were carried out as shown in Fig. 6.

FIG. 6. Influences of cupric ion on the activity of this enzyme.

- 1) Enzyme + Phosphate Buffer + Octylalcohol + Tyramine + $CuCl_2$ ($M/192000$).....0.16*
- 2) E. + P.B. + Oct. + Tyr. + $CuCl_2$ ($M/9600$).....0.14*
- 3) E. + P.B. + Oct. + Tyr. + $CuCl_2$ ($M/4800$).....0.06*
- 4) E. + P.B. + Oct. + Tyr. + Aq. dest.....0.17*

*Values of Folin's reaction at 120 minutes

At a certain concentration of cupric ion, the oxygen consumption was found to be large, but Folin's procedure did not show the increase of its value. This results would be explained from the increased rates of this enzyme activity and of the autoxidation of hydroxytyramine by the presence of cupric ion.

From this results and the data described in above chapter, it would be assumed that cupric ion may be essential for the activity of this enzyme.

DISCUSSION

On the basis of the data described in Chapter I, it has been shown that hydroxytyramine was formed from tyramine in bovine adrenal gland homogenates. This finding seems to lead immediately the concept that tyramine would be an effective precursor of adrenaline in adrenal gland *in vivo*. However this would not be a case, as indicated by the isotopic data by Udenfriend *et al.* (13), which showed that the radioactive adrenaline was not found in adrenal gland of rats after the injection of radioactive tyramine. At present the following reaction sequence is generally believed to be the main pathway to form adrenaline *in vivo*.

West (19) has observed that the amount of catecholamine present in adrenal gland was not decreased even under the condition that the activity of dopa-decarboxylase was low,

suggesting that the physiological meaning of the pathway of adrenaline formation through dopa might not be a great. This finding reported by West would be explained by the slow turnover of adrenaline formation in adrenal gland, but another explanation could be furnished by an alternative pathway to arise adrenaline, such as a route from tyramine, which would function under the condition that the main pathway from tyrosine is blocked. In the experiments which have asserted the tyrosine pathway, rats were used as the enzyme source while in other experiments to support tyramine pathway, guinea pigs, beeves and pigs were used. The difference of animal species may also be importance in these experiments. It would be presumed that the pathway to form adrenaline *in vivo* is not only one but that various alternative pathways may be operating under the different physiological condition or in different animal species.

Some properties of this enzyme responsible for the oxidation of tyramine to hydroxytyramine were described. This enzyme seems to require cupric ion for its activity as tyrosinase docs, but oxidized only tyramine among mono- and polyphenol derivatives tested as substrates. Coloring matter as inactive as hydrogen-accepter of this reaction, and oxygen was essential for its activity. The presence of TPNH or DPNH seems not to be required. The requirement of cupric iron was also suggested by the method of chelating agents of the enzymatic activity, but whether this enzyme is a real copper enzyme or not is as yet undecided. Such studies are now in progress in our laboratory.

SUMMARY

1. An enzyme, which oxidizes tyramine to hydroxytyramine, has been found to exist in the soluble and the precipitate fraction of bovine adrenal gland homogenates.
2. This enzyme which we named tyramine oxidase, seems to require cupric ion for its activity.
3. The enzyme oxidizes only tyramine among mono- and polyphenols tested as substrates.
4. The possibility that tyramine might be an effective precursor of adrenaline is discussed.

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