TYROSINASE AND PHENOLIC PRESSOR AMINES

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Recent studies of the mechanisms involved in the production of experimental renal hypertension show that humoral pressor agents are involved. It appears that either one or both of two types of pressor substance may be liberated from the kidney itself, but that a single type of substance acts to produce the peripheral vasoconstriction. One type of substance that may be liberated from the kidney has the properties of a non-dialyzable protein, like the renin of Tigerstedt and Bergmann (1). This substance reacts with certain serum constituents to produce a dialyzable amine type of pressor substance, according to Page, Koehlstaedt, and Helmer (2, 3), who called this substance angiotonin, and according to Braun-Menendez and coworkers (4), who called it hypertensin. Another type of substance that may be liberated from the kidney has the properties of a dialyzable pressor amine that is very possibly tyramine or hydroxytyramine, as developed by the work of Holtz (5, 6) and of Bing (7, 8) and their coworkers. Whatever the substances liberated by the kidney may be, it appears that the humoral agent producing a peripheral vasoconstriction is an amine in type, and very possibly a phenolic amine.

The pressor effect of renin can be inactivated by tyrosinase preparations in the presence of oxygen, and the product of its interaction with serum, angiotonin or hypertensin, can be similarly inactivated, as shown by Schroeder and Adams (9) and confirmed by Croxatto and Croxatto (10). The latter workers also showed that hypertensin could be readily inactivated by amine oxidase preparations in the presence of oxygen. Schroeder and Adams studied the effects of injected tyrosinase preparations on the blood pressures of animals with experimental renal hypertension, and also of persons having arterial hypertension (11). Falls in blood pressure and other signs of clinical improvement were obtained, and the conclusion was drawn that it was probable that some phenolic substance acting as a humoral agent was altered. A recent note by Schroeder (12) reports that the injection of amine oxidase preparations lowered the blood pressure of animals with experimental renal hypertension.

The tissues of mammals, as studied by Bhagvat and Richter (13), do not normally contain any considerable amounts of tyrosinase or other phenoloxidases. The physiological inactivation of phenolic amines in

man appears to be largely carried out by some esterifying mechanism, as shown by Richter and Richter and MacIntosh (14, 15). It is therefore of considerable interest to value the kinetics of phenoloxidase-catalyzed oxidations of a number of phenolic pressor amines, under approximate physiological conditions, in order to estimate the rôle any normally occurring amounts could play in the inactivation of such compounds in the body. Such data also furnish bases for consideration of the possible effects of injected amounts of tyrosinase or other phenoloxidase preparations.

Basic to the consideration of the action of tyrosinase on the oxidation of phenolic pressor amines are the observations of Keilin and Mann (16) and of Nelson and his coworkers (17–19) that show that different preparations may vary considerably in their relative actions on monophenols and *o*-diphenols. Both of these types of activity appear to belong to the same enzyme complex, as they bear a proportionality to the same copper content. However, since the activities vary with the purity and method of purification, each enzyme preparation must be defined in terms of both monophenolase and *o*-diphenolase activities. This was done in the present studies, and modifications of previously described preparative methods were required to retain a reasonable proportioning of such activities in purified preparations.

Purified Tyrosinase Preparations

The methods described by Nelson and coworkers (17–19) were first tried, but met with little success, owing to rapid inactivation and processing losses. The methods of Keilin and Mann (16) were then tried, and after some modification it was possible to obtain quite good yields of stable preparations which were 30 to 40 times more active per unit weight than crude aqueous extracts. In our experience, only a white cultivated variety of the common mushroom gave good results, a spotted brown and white variety gave fair results, while a brown variety gave precipitates that were difficult to handle and high inactivation and processing losses occurred.

The tyrosinase activity of the preparations was valued by methods similar to those of Adams and Nelson (17), but modified by use of available equipment. The assays were made with an Aminco Warburg apparatus, with seven 15 ml. vessels shaken at 120 oscillations per minute in a 30.0° water bath. In the body of the flasks were placed 1.0 ml. of 0.4 M sodium phosphate buffer, pH 7.0, containing 2.5 mg. of gelatin, and 0.5 ml. of enzyme preparation at a dilution to cause an oxygen uptake of about 5 microliters per minute. In the side arm was placed either 0.5 ml. of a solution containing 0.1 mg. of catechol and 5 mg. of hydroquinone per ml. for catecholase activity determination or 0.5 ml. of 0.04 M *p*-cresol for cresolase activity determination. The solutions were attemperated for

15 minutes with shaking at a 2 cm. stroke, and then mixed and shaking continued at a 4 cm. stroke. For catecholase activity, readings were made at 2 minutes, while for cresolase activity, readings were made at 5 minute intervals. In either case, a unit (catecholase = Ca. u., cresolase = Cr. u.) is defined as that which causes an uptake of 10 microliters of O_2 per minute under the conditions used. The unit was found to be seven-twelfths that of the unit similarly defined by Adams and Nelson for their equipment.

Crude Extract—5 pounds of fresh mushrooms were ground twice, mixed with 200 ml. of water, and then pressed in a canvas bag. Regrinding with sand and 400 ml. of water, repressing, then another grinding with sand and 600 ml. of water gave about 2700 ml. of reddish brown liquid.

Non-dialyzable solids, 6.52 mg. per ml., 5 Ca. u. per mg., 7 Cr. u. per mg. Total Ca. u., 92,000; total Cr. u., 123,000.

Ammonium Sulfate Precipitation, Followed by Dialysis—Crude extract adjusted with N acetic acid to pH 5 was treated with 700 gm. per liter of ammonium sulfate and the pH adjusted to 4.8, with stirring for 2 hours. Centrifugation in a Sharples machine at 25,000 R.P.M. completed the solid separation in an hour, while filtration attempts were discouraging. The solid was resuspended in 450 ml. of water with stirring for 0.5 hour, and the mixture then dialyzed within 36/32 Visking cellophane tubing by shaking in running tap water for 24 to 40 hours. Insoluble material was centrifuged out and the centrifugate made up to 1000 ml.

Non-dialyzable solids, 4.09 mg. per ml., 34 Ca. u. per mg., 29 Cr. u. per mg. Total Ca. u., 139,000;[1] total Cr. u., 118,000.

Lead Acetate Treatment—Aliquots of 20 ml. were taken and treated with 0.6 to 1.4 ml. of 0.05 saturated lead acetate solution to determine the approximate amount required to effect removal of color to a green filter reading of about 300 on a Klett colorimeter. The total batch had to be treated with a 20 to 25 per cent greater ratio of the lead acetate solution to obtain equivalent decolorization, and was then centrifuged.

Non-dialyzable solids, 1.47 mg. per ml., 57 Ca. u. per mg., 41 Cr. u. per mg. Total Ca. u., 83,000; total Cr. u., 59,000.

Clarification with Calcium Phosphate—Treatment of the extract with some freshly precipitated calcium phosphate at pH 7.0, by stirring for 0.5 hour

[1] Assay shows an increase in total Ca. u. during ammonium sulfate precipitation followed by dialysis, and this was shown by several, but not all, the preparations made. Keilin and Mann (16) also observed such an increase and postulated the presence of a proenzyme that was activated during dialysis.

and then allowing it to stand overnight, gave a clear solution after centrifugation, about two-thirds as colored as after the lead acetate treatment.

Non-dialyzable solids, 1.07 mg. per ml., 76 Ca. u. per mg., 55 Cr. u. per mg. Total Ca. u., 80,000; total Cr. u., 58,000.

The calcium phosphate was prepared by mixing equal volumes of 0.15 M calcium acetate and 0.1 M disodium phosphate and adjusting the pH to that at which the suspension was to be used. 1 ml. of this is equivalent to 15 mg. of tricalcium phosphate.

Calcium Phosphate Adsorption and Elution—80 per cent of the activity was adsorbed by adjustment of the pH to 6.6 and treatment with 1 gm. of calcium triphosphate in suspension at pH 6.6 for each 20,000 Ca. u. by stirring for 0.5 hour. The adsorption precipitate was centrifuged off with the Sharples machine, and then stirred for an hour into a suspension with 75 ml. of 0.5 M potassium phosphate buffer at pH 7.5. The eluate was centrifuged off and elution repeated twice. The combined eluates were dialyzed against running tap water for 40 hours.

Non-dialyzable solids, 1.41 mg. per ml., 140 Ca. u. per mg., 90 Cr. u. per mg. Total Ca. u., 51,000; total Cr. u., 32,000.

By treatment of such solutions with small amounts of 0.01 saturated lead acetate and with calcium phosphate suspensions, about half of the enzymic activity could be obtained in preparations showing about 210 Ca. u. per mg. with 110 Cr. u. per mg.

For use in experimental renal hypertension in animals, or in arterial hypertension in man, more concentrated solutions of the calcium phosphate eluate preparations were desired. These were prepared by precipitating relatively large volumes of eluate preparations with 700 gm. per liter of ammonium sulfate and adjusting the pH to 4.8. The precipitate was taken up with 0.9 per cent sodium chloride solution at pH 7.0, and made 1:50,000 in phenylmercuric acetate for preservation. Such solutions contained non-dialyzable solids, 3.72 mg. per ml., 300 Ca. u. per mg., 90 Cr. u. per mg. The stability of the more highly purified preparations, when kept in the refrigerator at about 5°, was very satisfactory. One preparation that was tested almost weekly for 5 months showed no change in either catecholase or cresolase activity. Another preparation carefully restandardized after 3 months showed no change in either type of activity.

These tyrosinase preparations could be rapidly inactivated by heating above 60°, with the attendant precipitation of coagulated proteins. Study of this heat inactivation showed that by keeping the preparations at 60° for 40 minutes, 95 per cent or more of the catecholase and cresolase activity was destroyed. Such heating did cause precipitation of some material which almost completely dissolved on cooling and shaking.

Tyrosinase Oxidations of Phenolic Amines

The relative activity of tyrosinase preparations when tested upon monophenols and *o*-diphenols may not be reflected in their relative activity when tested upon monophenolic and *o*-diphenolic amines. This question was especially studied, since it appeared possible that, although preparations might vary considerably in their relative catecholase to cresolase activity, certain generalizations might be made for the action of tyrosinase on types

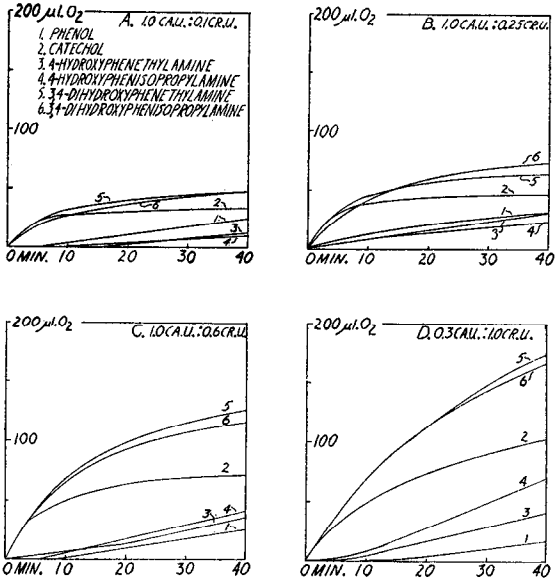

FIG. 1. Oxygen uptake in microliters against time in minutes for preparations having different ratios of catecholase to cresolase activity. *A* and *B* may be considered high catecholase, *C* and *D* high cresolase preparations. The experiments were carried out at 30° with 0.01 M amine hydrochloride substrates in 0.2 M sodium phosphate buffer, pH 7.0, and 0.5 ml. of enzyme of the concentration shown per ml. used in a 2 ml. total volume. The curve numbers in all four sections correspond to the substrates listed in *A*.

of phenolic amines, and particularly on phenolic pressor amines that might occur in the body under physiological conditions. Confirmation of this idea was indeed obtained by the study of four enzyme preparations, varying widely in their relative catecholase to cresolase activity upon the same series of six amines. Three of these amines were monophenolic; the other three were diphenolic and derivatives of catechol.

Three enzyme preparations were made by following the previously outlined methods, and represented a range from a relatively high catecholase to cresolase ratio of 10:1 through a ratio of 10:2.5, and finally of 10:6.

The oxidation rates were studied for these three preparations at 30°, with 0.5 ml. of enzyme preparation containing 1.0 Ca. u. per ml. and attendant cresolase unitage, and 0.5 ml. of 0.04 M phenolic amine, in a total volume of 2.0 ml. Curves showing the results are given as *A*, *B*, and *C* of Fig. 1. A high cresolase preparation was also made specially for this comparative study by fractional ammonium sulfate precipitation of a crude extract of brown mushrooms. This preparation showed a catecholase to cresolase ratio of 10:33, and 0.5 ml. of enzyme preparation containing 1.0 Cr. u. per ml. and but 0.3 Ca. u. per ml. was used for the studies shown in Fig. 1, *D*.

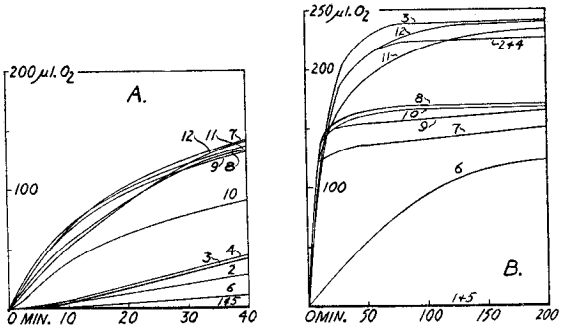

FIG. 2. Oxygen uptake in microliters against time in minutes, at 30° with 0.01 M amine hydrochloride substrates in 0.2 M sodium phosphate buffer, pH 7.0, and 0.5 ml. of enzyme used in a 2 ml. total volume. In *A* the enzyme solution used contained 1.0 Ca. u. and 0.6 Cr. u. per ml., and in *B* the enzyme concentration was 24 times greater. Curve 1, 3-hydroxyphenethylamine; Curve 2, 4-hydroxyphenethylamine; Curve 3, 4-hydroxyphenethylmethylamine; Curve 4, 4-hydroxyphenisopropylamine; Curve 5, 3-hydroxyphenethanolmethylamine; Curve 6, 4-hydroxyphenethanolmethylamine; Curve 7, 3,4-dihydroxyphenethylamine; Curve 8, 3,4-dihydroxyphenethylmethylamine; Curve 9, 3,4-dihydroxyphenisopropylamine; Curve 10, 3,4-dihydroxyphenethanolamine; Curve 11, *dl*-3,4-dihydroxyphenethanolmethylamine; Curve 12, *l*-3,4-dihydroxyphenethanolmethylamine.

The results from monophenolic and diphenolic amine substrates fall into separate groups with each of the several enzyme preparations. The principal point of difference in the action of the four preparations is the greater spread of the curves with increased cresolase activity. The cresolase activity appears to potentiate the catecholase activity of the preparations of high cresolase activity with respect to all six of the substrates studied.

To further the idea that oxidation behavior of phenolic pressor amines may be classified on the basis of the number and position of the benzene ring substituents, additional studies were carried out on a rather varied series of phenolic and *o*-diphenolic amines. For the initial study, a single

tyrosinase preparation with a catecholase to cresolase ratio of 10:6 was used, with enzyme and substrate concentrations as in the studies of *A*, *B*, and *C* of Fig. 1. A second study was carried out with enzyme concentrations 24 times as great and with longer periods of observation, to indicate the total extent of oxidation of these compounds by tyrosinase. The results of these studies are shown in Fig. 2, and it should be noted that here again the *initial* rates of the effects upon monophenolic and diphenolic amines are separable into groups, with the exception that the 3-hydroxyphenylalkyl- (or alkanol-) amines are not oxidized at all. Another point of interest is that the ethanolamine side chain appears to inhibit oxidation in both series, though not notably with adrenalin.

The total extent of tyrosinase oxidation of these various substrates, as shown by Fig. 2, *B*, indicates that the course of the oxidation, aside from phenolic oxidation, may differ greatly among the various compounds. This is perhaps related to the findings of Beyer (20), using certain phenolic pressor amines and crude potato tyrosinase preparations, that total oxidation may involve the splitting of ammonia from the molecule, as well as the production of indole derivatives and melanins, as found by Duliere and Raper (21).

Dissociation Constants of Tyrosinase and Phenolic Pressor Amines

The kinetic dissociation constants of tyrosinase with some typical phenolic pressor amine substrates were determined. These substrates were chosen because of their physiological or pharmacological interest. Although calculations were planned to be made that would apply to body temperatures, the studies were carried out in the Warburg apparatus at 25°. This was done in order to obtain sufficient experimental precision, since the rates determined must be those during the first few minutes of oxidation, and differences between bath and room temperatures of as much as 5° caused manometer shifts affecting the reading for the 1st minute owing to the removal of the vessels from the bath for mixing. Substrate concentrations varied from 0.002 to 0.02 molal. Enzyme concentrations were 3 Ca. u. per ml. with 1.8 Cr. u. per ml. for the monophenolic substrates, and 0.6 Ca. u. per ml. with 0.36 Cr. u. per ml. for the diphenolic substrates. Determinations were repeated in most cases at one-half these enzyme concentrations, and checks were obtained. For the monophenolic amines, the rates used were the maximum rates obtained over a 5 minute period after the initial lag period had ceased, and usually occurred between 8 and 13 minutes, often continuing constant for an additional 5 minutes. For the diphenolic amines, the oxidation rates of which rapidly decrease with time, the rates chosen were the maximum rates observed over a 1 minute interval, usually falling within the 1st or 2nd minute interval. By plotting $1/V$, where V = microliters of O_2 uptake per minute, against

$1/S$, where S = molal substrate concentration, the ordinate intercept $1/V_{max.}$ and the slope of the straight line $K_s/V_{max.}$ were determined, as suggested by Lineweaver and Burk (22). K_s, the dissociation constant of the intermediate enzyme-substrate compound, is then easily calculable.

DISCUSSION

The data presented with regard to the interaction of oxygen and the phenolic pressor amines in the presence of tyrosinase permit some calculations to be made with respect to the likelihood that tyrosinase plays any physiological rôle in the inactivation of these compounds in the body. Further, such calculations can indicate the order of magnitude of the amounts of tyrosinase that would have to be introduced into the body from the outside to exert an effect comparable to normal rates of destruction of these phenolic pressor amines.

By way of example, consider the oxidation rate of tyramine in a concentration of 10^{-5} molal with an enzyme concentration of 3.0 Ca. u. and 1.8 Cr. u. per ml. by substitution of the data given in Table I for these conditions into the rate relation $1/V = K_s/V_{max.}(S) + 1/V_{max.}$ Such substitution gives $1/V$ to be about 24, or V to be about 0.04 microliter of O_2 per minute, or about 0.02×10^{-7} mole of O_2 per minute. On the basis that physiological inactivation would occur with an uptake of 1 mole of O_2 per mole, 10^{-6} mole of tyramine under these conditions would require about 500 minutes to be inactivated with an enzyme concentration of 3.0 Ca. u. and 1.8 Cr. u. per ml. Such conditions of amounts and concentrations of tyramine would be approximated in the blood stream of an experimental animal immediately following the intravenous injection of 10^{-6} mole per kilo (0.18 mg. per kilo of hydrochloride) of tyramine. The studies of Clark and Raventos (23) on the relationship between dosage and duration of physiological actions of tyramine in cats and in man showed that a dosage of 10^{-6} mole of tyramine per kilo is inactivated in less than 20 minutes.

A similar calculation made from the data obtained on *l*-adrenalin in a concentration of 10^{-7} molal with an enzyme concentration of 0.6 Ca. u. and 0.36 Cr. u. per ml. would give V to be about 2×10^{-4} microliter of O_2 per minute, or about 10^{-11} mole of O_2 per minute. On the basis that physiological inactivation would occur with an uptake of 0.5 mole of O_2 per mole, 10^{-8} mole of adrenalin under these conditions would require about 500 minutes to be inactivated with the enzyme concentration of 0.6 Ca. u. and 0.36 Cr. u. per m'. Such conditions of amounts and concentrations of adrenalin would be approximated in the blood stream following the intravenous injection of 10^{-8} mole per kilo (0.0018 mg. per kilo) of adrenalin. The duration of pressor and other responses in cats from such a dosage is less than 5 minutes, and is most commonly 2 to 3 minutes.

Certainly, no such considerable amounts of tyrosinase as 3.0 or even 0.6 Ca. u. per ml. exist normally in the blood or tissues of animals yet studied, as demonstrated by Bhagvat and Richter (13). If a tyrosinase-catalyzed oxidation were the principal oxidative mechanism, 25 to 100 or more times these concentrations of enzyme would be required. Although tyrosinase is thus indicated as being an unimportant mechanism normally, it might be suggested that its injection could be resorted to in order to increase the capacity of the body to inactivate phenolic pressor amines. So far as such compounds are represented by tyramine and adrenalin, amounts of tyrosinase approximating 25 to 100 or more times 3.0 or even 0.6 Ca. u. per ml. of body fluid would be required to be injected to approach de-

TABLE I

Tyrosinase Oxidations of Phenolic Amines

The experiments were carried out at 25° in 0.2 M sodium phosphate buffer, pH 7.0, with substrate concentrations of from 0.002 to 0.02 molal.

Phenolic amine	Enzyme concentration		$\frac{1}{V_{max.}}$	$\frac{K_s}{V_{max.}}$	K_s
	Ca. u. per ml.	*Cr. u. per ml.*		$\times 10^{-4}$	
4-Hydroxyphenethylamine (tyramine)	3.0	1.8	0.104	2.42	0.0024
	1.5	0.9	0.206	4.03	0.0020
4-Hydroxyphenisopropylamine (paredrine)	3.0	1.8	0.051	3.44	0.0067
	1.5	0.9	0.100	6.00	0.0060
3,4-Dihydroxyphenethylamine (hydroxytyramine)	0.6	0.36	0.040	2.00	0.0050
	0.3	0.18	0.076	4.02	0.0053
3,4-Dihydroxyphenisopropylamine (hydroxyparedrine)	0.6	0.36	0.047	1.54	0.0033
	0.3	0.18	0.085	2.90	0.0034
dl-3,4-Dihydroxyphenethanolmethylamine (*dl*-adrenalin)	0.6	0.36	0.045	3.24	0.0072
	0.3	0.18	0.090	6.80	0.0075
l-3,4-Dihydroxyphenethanolmethylamine (*l*-adrenalin)	0.6	0.36	0.035	4.60	0.0131

struction rates comparable to those normally possessed by the body. These amounts are very large and are in excess of those that were used by Schroeder and Adams (9, 11) for the therapy of hypertension in animals and man, and it would seem probable that explanations other than increased destruction of phenolic pressor amines are required for the effects they reported.

SUMMARY

1. Methods were developed for the purification of extracts of common white mushrooms, giving good yields of stable tyrosinase preparations.

2. The ratio of catecholase to cresolase activities of such preparations was not greatly altered in the course of such purification.

3. The initial oxidation rates of diphenolic amines that were derivatives of catechol were greater than those of monophenolic amines with tyrosinase preparations, whether they are high or low in relative catecholase to cresolase activity.

4. Tyrosinase preparations oxidize a large number of monophenolic and *o*-diphenolic pressor amines, with the exception of those substituted with a single hydroxyl group in the 3 position relative to the side chain.

5. The extent of total oxidation of a number of phenolic pressor amines is quite variable, probably due to different pathways of the oxidation of the side chain with its amino group.

6. The kinetic enzyme dissociation constants of tyrosinase-amine combinations were found to be of the same order for the monophenolic and diphenolic amine types studied.

7. The dissociation constants and the oxidation rates of tyrosinase-amine combinations are such that this oxidation mechanism cannot account for any considerable part of the inactivation of tyramine or epinephrine in normal animals, and such oxidation is not more probable for related phenolic pressor amines.

8. Injections of large amounts of highly active tyrosinase preparations into animals would be required to produce rates of oxidation comparable to normal inactivation rates.

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