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PROPERTIES OF AN ENZYME FROM BANANAS (*MUSA SAPIENTUM*) WHICH HYDROXYLATES TYRAMINE TO DOPAMINE

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Abstract—An enzyme system isolated from the pulp of banana fruit (*Musa sapientum*) was partially purified and characterized. The enzyme was capable of catalysing the hydroxylation of the monophenol, tyramine, to the diphenol, dopamine (3,4-dihydroxyphenylethylamine). Unlike some tyrosinases, the reaction was not stimulated by catalytic amounts of diphenolic reaction product. Ascorbic acid, however, reduced the initial lag period in the oxidation of tyramine, stimulated the reaction rate and promoted the accumulation of dopamine during the first few minutes of the reaction. The hydroxylation of tyramine was apparently dependent upon molecular oxygen. On the basis of these observations it is tentatively suggested that the enzyme is a tyramine hydroxylase which may be responsible for the formation of dopamine in the banana.

INTRODUCTION

THE BIOSYNTHESIS of phenolic compounds has been studied by many workers,[1-7] but the factors which regulate phenol formation and accumulation have not been elucidated. One phenolic compound, dopamine (3,4-dihydroxyphenylethylamine), occurs at high levels (1–2 mg/g fr. wt.) in an uncombined form in the peel of the banana fruit[8] and is the primary substrate in the browning reactions of this tissue.[9] Palmer[8] reported a polyphenol oxidase prepared from the pulp of the banana fruit which catalysed the oxidation of a variety of *o*-dihydric phenols. Dopamine was the most effective of the substrates tested and exhibited a K_m ($6{\cdot}3 \times 10^{-4}$ M) two orders of magnitude less than that of the related compound, DOPA (3,4-dihydroxyphenyl-L-alanine). A number of tyrosinases oxidize monophenols to the corresponding *o*-dihydric phenols[2] but the banana enzyme was inactive towards monophenols such as tyramine, tyrosine, *o*-cresol or *p*-cresol. Thus, although the banana accumulates large amounts of a free *o*-dihydric phenol and thereby should constitute an ideal tissue in which to study factors that regulate phenol biosynthesis, a system for the formation of dopamine in the banana was not readily apparent.

In mammalian systems dopamine is formed by the decarboxylation of DOPA which is formed from tyrosine by a tyrosine hydroxylase.[4] Studies[4] indicated that this latter reaction

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TABLE 1. PURIFICATION OF A TYRAMINE HYDROXYLASE FROM THE PULP OF BANANA FRUIT

Fraction	Description	Total volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg protein)	Total (units)	Recovery (%)
I	Crude extract	230	3·4	6	1·7	1380	100
II	pH 5·5, protamine sulfate and $CaCl_2$	200	1·5	—	—	—	—
III	1·5 vol. acetone added to II, resuspended pellet	62	14·4	12·4	0·86	770	56
IV	30–60% $(NH_4)_2SO_4$ fraction of III	5·5	25·4	140	5·5	770	56
V	Fraction IV stored at −10° 2 days, and centrifuged at 20 kg = 20,000 *g* 15 min	5·0	14·1	200	14·1	1000	72
VI	Effluent from Bio-Gel P-100 column, precipitated with acetone, 0·8–1·2 vol. fraction	8·5	3·0	80	26·4	680	49

The units of activity and details of the purification are given in the Experimental.

was apparently a true aerobic hydroxylation[10] in that it required molecular oxygen and an external electron donor such as tetrahydropteridine. In the banana tissue, in contrast, tracer studies[11] suggested that tyramine was oxidized directly to dopamine and that DOPA probably was not an intermediate in dopamine biosynthesis. We now report the isolation and partial characterization of an enzymic system from bananas which converts tyramine to dopamine. This enzyme is tentatively referred to as a 'tyramine hydroxylase'. Most of the assays for the tyramine hydroxylase were based on the oxidation of the reaction product, dopamine, to 2,3-dihydroindole-5,6-quinone[8] by the polyphenol oxidase present in all of the hydroxylase preparations. A preliminary report of this work has appeared elsewhere.[12]

RESULTS

Purification of a Tyramine Hydroxylase

An active tyramine hydroxylase preparation was obtained from an alcohol–acetone powder of banana pulp and purified 15-fold by the procedure summarized in Table 1 and described more fully in the Experimental. Final recovery of the activity present in the initial crude extract was usually about 50 per cent. Fraction VI, the most purified fraction obtained, was essentially free of contaminating, viscous, non-proteinaceous materials (presumed to be polysaccharides) which made Fractions I–III difficult to work with. Attempts to obtain an active tyramine hydroxylase from fresh banana pulp using Palmer's method[8] for banana polyphenol oxidase were only partially successful and not consistently reproducible. Therefore, all work reported here was done with the enzyme obtained from the dehydrated powder and purified through to Fraction VI (Table 1).

Substrate Specificity

Most studies of tyrosinases employ substrates such as *o*-cresol or catechol which do not occur to a significant extent in most plant tissues. In the present study we were interested in the synthesis of dopamine which presumably was formed ultimately from tyrosine either by

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[11] T. BUCKLEY, unpublished observations.
[12] H. V. MARSH, JR. and W. DEACON, *Plant Physiol.* **42**, S-16 (1967).

the hydroxylation of tyramine or the decarboxylation of DOPA. In either event, a critical reaction would be the initial *o*-hydroxylation of the mono-hydric compound and therefore the efficacy of tyramine and tyrosine as substrates for the banana enzyme was tested. As shown by the data presented in Table 2, the purified extract oxidized both monophenols in addition to dopamine, but the maximum velocity with tyramine was 3·5-fold greater than that observed when tyrosine was the substrate. In other experiments, DOPA was found to be oxidized at about the same rate as tyramine.

Optimum pH and Enzyme Stability

The optimum pH for the oxidation of tyramine as determined in phosphate buffer over the range of 5·7–8·1 was found to be 6·0. At pH 5·2 in citrate buffer the activity was less than 10 per cent of that at pH 6·0 in phosphate buffer. The activity toward tyramine decreased

TABLE 2. RATES OF OXIDATION OF SEVERAL PHENOLIC AMINES FOUND IN THE BANANA

Compound	Rate (units/mg protein)
Tyramine	1·50*
Tyrosine	0·44*
Dopamine	26·30*

The reaction mixture (3·0 ml) contained 80 μmoles potassium phosphate, pH 6·0, enzyme and either 10 μmoles tyramine, 10 μmoles tyrosine or 1 μmole dopamine.

* Each value is the average of at least four determinations.

rapidly above pH 6·0 and at pH 7·0 was but 50 per cent of that at 6·0. This is in marked contrast to the pH 7·0 optimum for the banana polyphenol oxidase employing dopamine as substrate observed by Palmer.[8] Optimum conditions for the oxidation of substrates other than tyramine were not determined in the present work.

The tyramine hydroxylase activity was 50 per cent inactivated by holding the extract at pH 4·0 for 30 min and completely inactivated after 30 min at pH 3·5. There was no appreciable loss of hydroxylase activity after 3 days storage at 0° and only an 11 per cent loss during 2 months storage at −20°. All activity was lost in extracts boiled for 10 min.

Products of Tyramine Hydroxylase Reaction

Dopamine was postulated to be a primary reaction product in the oxidation of tyramine. Fluorometric analyses confirmed this supposition. The curves shown in Fig. 1 illustrate the activation and emission spectra of an authentic sample of dopamine and an enzymic reaction mixture incubated 4 min at 30° with 10 μmoles of tyramine. Both the activation and emission spectra of the two samples were essentially identical (with the exception of an activation peak of an unknown compound discussed below). The activation spectrum of an authentic dopamine sample peaked at 327–330 nm with a minor shoulder at 300 nm. The emission spectrum had but one peak with maximum at 372–390 nm. Control samples of tyramine and tyrosine did not fluoresce at these wavelengths. Contrary to an earlier report,[13] activation spectra of the fluorophores of dopamine and DOPA were similar but not identical and could be readily distinguished.

[13] A. CARLSSON and B. WALDECK, *Acta Physiol. Scand.* **44**, 293 (1958).

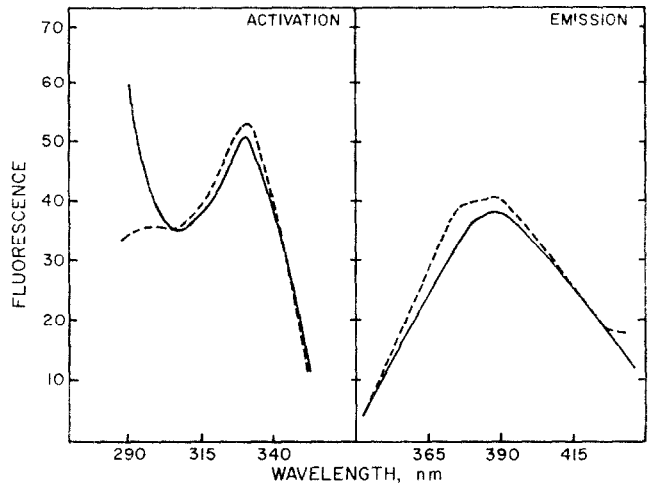

FIG. 1. ACTIVATION AND EMISSION SPECTRA OF DOPAMINE AND THE PRODUCTS OF TYRAMINE OXIDATION.
When the activating wavelength was varied, the emission wavelength was set at 372·5 nm. When the emission wavelength was varied, the activating wavelength was set at 329·8 nm. Fluorescence was measured in relative units. Two μg/ml dopamine (- - - - -); standard reaction mixture with 10 μmoles tyramine (————).

Dopamine apparently was not the sole reaction product in the oxidation of tyramine. A second fluorescence peak with maximum at 280 nm was evident in the activation spectra in all experiments employing freshly prepared enzyme (Fig. 2). The identity of the compound responsible for this second peak was not established. Storage of the enzyme preparation for 2 months at −20° reduced tyramine hydroxylase activity by 11 per cent. In contrast, the unknown compound was not formed at all by this aged preparation.

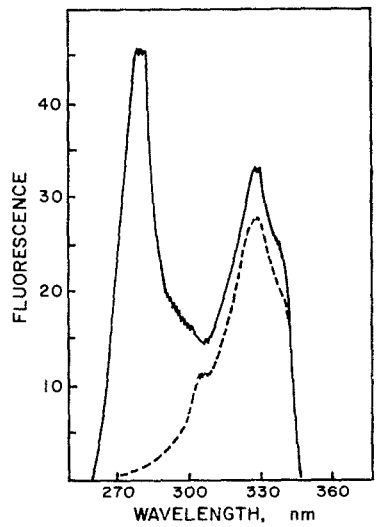

FIG 2. THE ACTIVATION SPECTRA OF THE REACTION PRODUCTS FORMED BY A FRESHLY PREPARED ENZYME AND ONE AGED 2 MONTHS AT −20°.
————; fresh enzyme. - - - - -; aged enzyme.

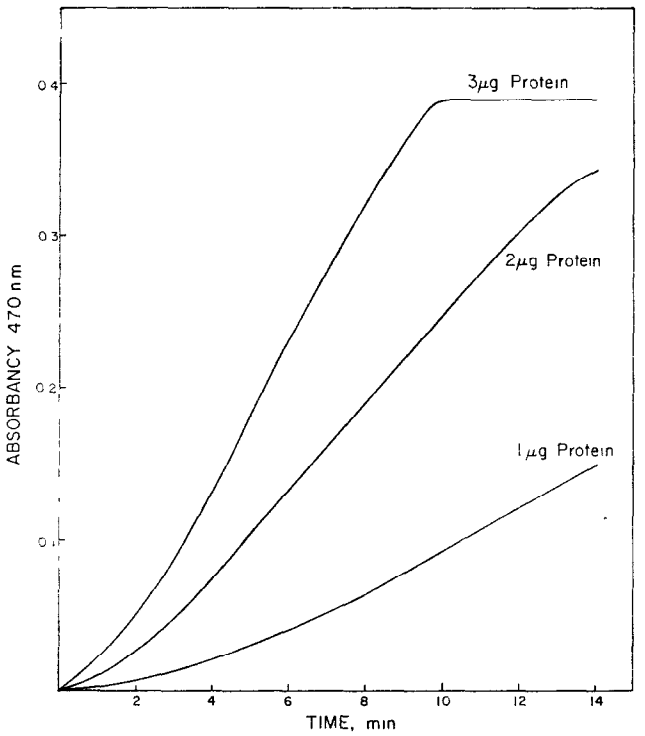

FIG. 3. EFFECT OF ENZYME CONCENTRATION ON THE DURATION OF THE LAG PERIOD IN THE OXIDATION OF TYRAMINE.

Reaction Kinetics

The oxidation of dopamine proceeded without a detectable lag (see Fig. 8). The tyramine hydroxylase reaction, however, exhibited a lag or induction period, the duration of which was inversely proportional to the amount of enzyme added (Fig. 3). As shown in Fig. 4, the addition of trace amounts (0·1 μmole) of the diphenolic reaction product shortened but did not eliminate the lag period. The induction period, however, was greatly reduced, if not eliminated, by the addition to the reaction mixture of ascorbic acid at a final concentration of $6{\cdot}6 \times 10^{-5}$ M (Fig. 5). Higher concentrations of ascorbate (5×10^{-4} M) temporarily prevented the accumulation of the quinone pigments due to the non-enzymatic reduction of the quinone.

Evidence that the ascorbic acid was oxidized in this system was obtained spectrophotometrically by following the decrease in absorbancy at 265 nm.[8] As shown in Fig. 6 there was a low level of ascorbic acid oxidase-like activity present in the extract which catalysed the slow oxidation of the ascorbic acid. However, when tyramine was added to the reaction mixture the ascorbic acid was rapidly oxidized, but only after a lag period similar to that observed in the formation of the quinone pigments from tyramine in the absence of any exogenous hydrogen donor.

In another series of experiments dopamine was determined directly by fluorometric analysis in order to establish whether the ascorbic acid was affecting the initial hydroxylation reaction, or the subsequent oxidation of dopamine. In the absence of ascorbic acid, dopamine

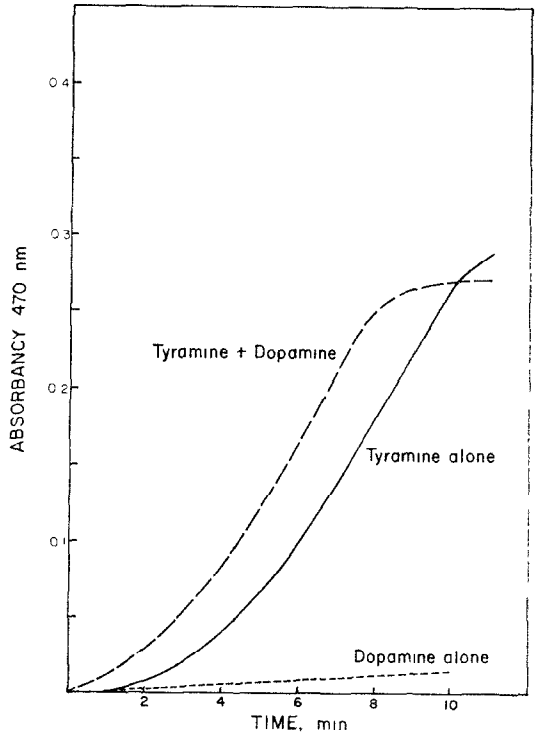

FIG. 4. TYRAMINE OXIDATION IN THE PRESENCE AND ABSENCE OF TRACE AMOUNTS OF THE REACTION PRODUCT, DOPAMINE.

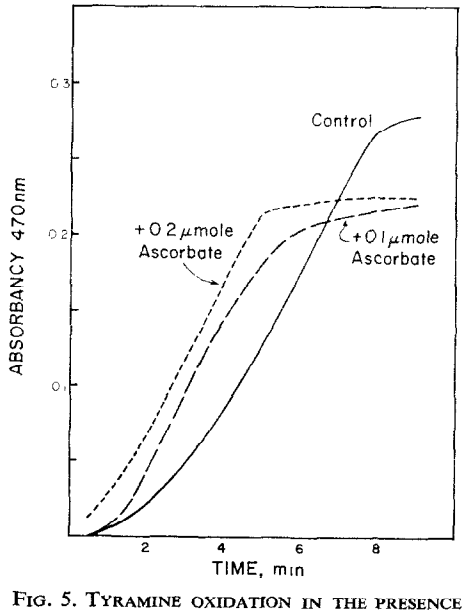

FIG. 5. TYRAMINE OXIDATION IN THE PRESENCE AND ABSENCE OF ASCORBATE.

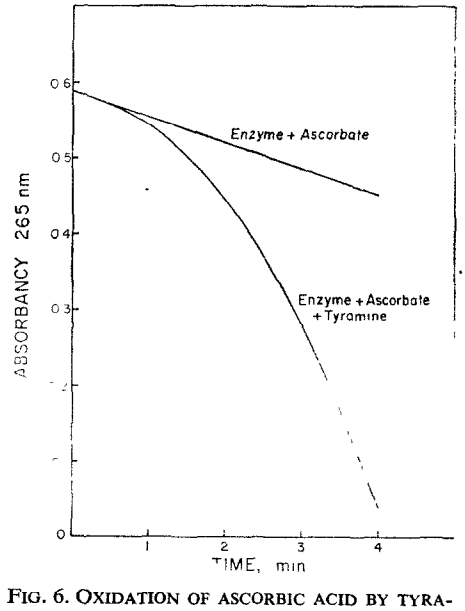

FIG. 6. OXIDATION OF ASCORBIC ACID BY TYRAMINE HYDROXYLASE PREPARATION IN THE PRESENCE AND ABSENCE OF TYRAMINE.

slowly accumulated to the level of 3·0 μg/ml after 10 min (Fig. 7). However, in the presence of 0·67 μM ascorbic acid there was a rapid accumulation of dopamine to over 3·8 μg/ml in the first 4 min, after which the level of dopamine remained constant.

Reaction Inhibition and Substrate Depletion

The oxidation of both dopamine and tyramine to the dihydroindolequinone stopped after a few minutes. In the case of dopamine this cessation of activity was apparently due to an inactivation of the enzyme *per se*: the rate of formation of the quinone gradually decreased and in time eventually stopped. Addition of more substrate had no effect whereas addition of fresh enzyme caused the reaction to proceed again at a rate approaching the initial rate (Fig. 8).

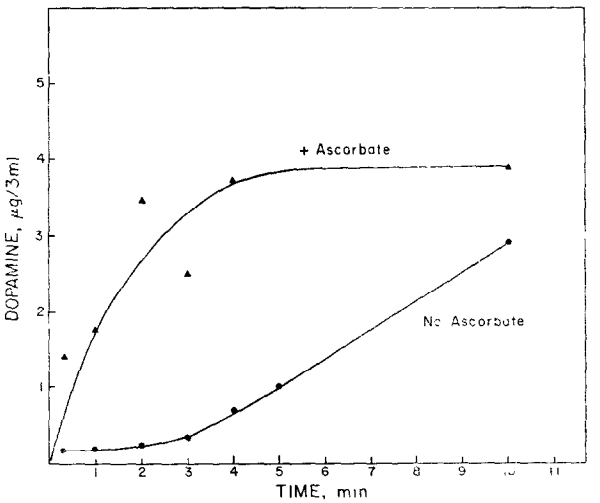

FIG. 7. TIME COURSE OF DOPAMINE ACCUMULATION IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID. Dopamine was measured directly by fluorometric analysis.

The time course for the oxidation of tyramine, in contrast, was markedly different. As shown in Fig. 9 the formation of the dihydroindolequinone proceeded after the initial lag at a rapid rate and then abruptly ceased. Addition of fresh enzyme to the reaction mixture with gentle stirring had little effect. However, when the reaction mixture was vigorously agitated without the addition of fresh enzyme, the reaction began again at the initial rate. This process could be repeated a number of times without any significant loss of hydroxylase activity.

DISCUSSION

In contrast to the banana polyphenol oxidase reported by Palmer,[8] the enzyme system reported here was capable of catalysing the oxidation of the monophenols tyramine and tyrosine. A product of the tyramine oxidation reaction was shown by fluorometric analysis to be dopamine. Although the partially purified enzyme used in the present study was not specific for tyramine, under the conditions employed it was 3·5-fold more active toward tyramine than toward tyrosine. The oxidation of tyramine was apparently absolutely dependent upon molecular oxygen as evinced by the abrupt cessation of the reaction and subsequent reactivation upon aeration of the reaction mixture. In the absence of an external

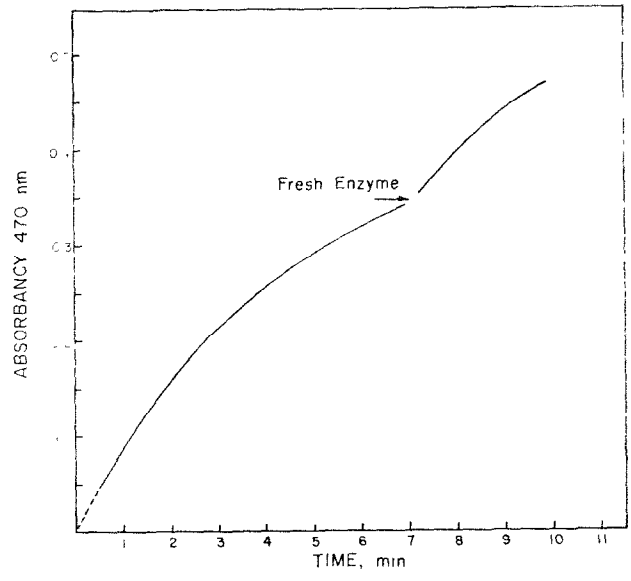

FIG. 8. AUTO INACTIVATION OF THE DOPAMINE OXIDIZING SYSTEM.

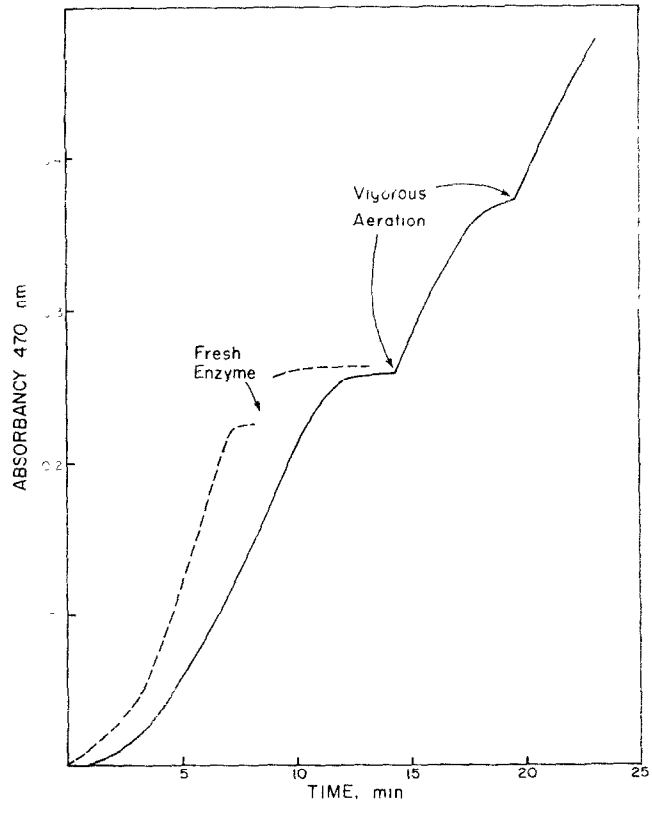

FIG. 9. EVIDENCE FOR THE OXYGEN REQUIREMENT OF THE TYRAMINE HYDROXYLASE.

hydrogen donor, the reaction exhibited a definite lag period. Ascorbic acid added to the reaction mixture greatly reduced this lag period and simultaneously was itself oxidized. These observations all taken together suggest that the present system contains a true tyramine hydroxylase[10] capable of forming dopamine from tyramine.

Tyramine occurs in the banana,[14] but no report of DOPA in this tissue has been found. Radioactive tyramine has been shown to be converted to dopamine in banana pulp.[11] In light of the results reported here, therefore, we suggest that in the banana dopamine biosynthesis proceeds via the decarboxylation of tyrosine to tyramine followed by the hydroxylation of this monophenolic amine to dopamine. Hydroxylation of tyramine to dopamine and norsynephrin has been demonstrated in mammalian systems,[15,16] but to our knowledge a tyramine hydroxylase from plant tissue has not been reported previously.

The formation of a second, unidentified compound from tyramine as indicated by the activation fluorescence maximum at 280 nm merits comment. Formation of noradrenalin from dopamine has been reported in homogenates prepared from banana peel, pulp, stalk and rind.[17] Although in the present study the compound formed by the freshly prepared enzyme was not identified, it may well have been noradrenalin. It is interesting that aging the frozen enzyme solution destroyed its capacity to form the unidentified compound which suggests that different enzymes or active sites function in the formation of dopamine and this unknown compound.

The characteristic lag in the oxidation of tyramine followed by a rapid reaction suggest an autocatalytic process in which some factor produced in the reaction stimulated the reaction rate. It is generally accepted[10] that the conversion of a monophenol to a diphenol is an oxidation–reduction reaction requiring both hydrogen and oxygen donors. Molecular oxygen appears to be the source of oxygen in most oxygenase systems[10] and would appear to function here. It has been suggested[10,18] that trace amounts of the *o*-dihydric phenolic reaction product serves as the hydrogen donor. This mechanism would seem plausible and would serve to explain the apparently autocatalytic reactions characteristically observed in tyrosinase reactions. The concept assumes, however, that either there is not an obligatory requirement for a hydrogen donor to initiate the oxidation of the monophenol, or else that trace amounts of a hydrogen donor remain even in a highly purified tyrosinase system.

The results reported here support the premise that a hydrogen donor is required for the conversion of tyramine to dopamine. As already noted, the lag period was markedly reduced by ascorbic acid. However, in contrast to the mammalian tyrosinase studied by Pomerantz[18] the addition of trace amounts of dopamine caused only a slight reduction in the lag period. On the basis of these observations it seems unlikely that free dopamine functions as the hydrogen donor in this system. However, it is well known that proteins readily bind phenolic compounds and it seems quite plausible in the present case that enzyme-bound dopamine (or other dihydric phenol) serves as the initial hydrogen donor. The fact that passage of the enzyme preparation through Bio-Gel P-100 (a treatment which should have removed free small molecules) did not confer a requirement for added hydrogen donor supports this premise.

Our present data do not indicate whether the tyramine hydroxylase and the polyphenol oxidase of Palmer[8] are structurally related or not. Nor do we have any evidence for a

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[17] W. J. Smith and N. Kirshner, *J. Biol Chem.* **235**, 3589 (1960).
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mechanism which would account for the accumulation of large amounts of dopamine by an enzyme system which *in vitro* is simultaneously capable of catalyzing its formation and oxidation. Logically, the two activities must be either physically separated in the cell, or alternatively, the oxidase activity must normally be inactive in the uninjured cell. Support for the latter possibility comes from the observation of a latent polyphenol oxidase in *Vica faba* which can be activated following extraction from the plant tissue.[19] It is possible that the typical polyphenol oxidases, including the banana enzymes, are much more readily activated by injury than the enzyme from *Vica faba* and that normally *in vivo* it is only the hydroxylase activity which is functional.

EXPERIMENTAL

Preparation of banana tyramine hydroxylase. The pulp of overmature bananas (*Musa sapientum*), purchased from a local market, was homogenized with cold (−10°) 95% ethanol (1 g/4 ml) in a chilled Waring blendor for 1 min. The homogenate was filtered and the residue extracted three more times with cold acetone. The powder was dried at room temp. and stored at −10° under which condition it was stable for at least 4 months. A crude enzyme extract was prepared by suspending the dehydrated powder in 0·04 M potassium phosphate, pH 6·0 (1 g/20 ml). The slurry was stirred for 30 min before centrifuging for 30 min at 37,000 *g*. The pH of the supernatant fluid, which comprised the crude extract (Fraction I), was adjusted to 5·5 with 1 N H_3PO_4. Protamine sulfate, suspended in 0·2 M NaOAc, pH 5·5, was added with constant stirring to the extract at the rate of 1 mg/5 ml extract. Next, 0·1 vol. of 1% $CaCl_2$ solution were added to the extract and after a 15 min equilibration period it was centrifuged for 1 hr at 37,000 *g*. The sediment was discarded and the supernatant fluid was taken as Fraction II. The protein (and other materials) in this supernatant fluid was precipitated by the addition of 1·5 volumes of cold (−10°) acetone. Following a 15 min equilibration period with constant stirring, the gummy precipitate which had formed was collected by centrifugation at 5000 *g* for 10 min. The precipitate was suspended in a minimum volume of 0·04 M potassium phosphate, pH 6·0, stored overnight at 0° and then centrifuged at 37,000 *g* for 1 hr. The supernatant fluid (Fraction III) was further fractionated by $(NH_4)_2SO_4$ precipitation taking the material insoluble between 30 and 60% saturation. This material was collected by centrifugation, suspended in 0·04 M potassium phosphate, pH 6·0, and after being clarified by centrifugation was referred to as Fraction IV. This fraction was stored at −20° for 2 days, thawed and insoluble materials were removed by centrifugation. The supernatant fluid was then termed Fraction V. The enzyme was further purified by passage through a column of Bio-Gel P-100 spheres suspended in 0·04 M potassium phosphate pH 6·0. The effluent fractions with the highest specific activities toward tyramine were combined and precipitated with cold (−10°) acetone, taking the material which was insoluble between 0·8 and 1·2 vols. The precipitate was collected by centrifugation and resuspended in a minimal volume of the dilute phosphate buffer. This preparation comprised Fraction VI and was routinely used in all assays. All the preparative manipulations were performed at 0–4°.

Enzyme assay. The tyramine hydroxylase assay was adapted from the banana polyphenol oxidase assay of Palmer.[8] The standard reaction mixture contained in 3 ml: 80 μmoles of potassium phosphate, pH 6·0, 10 μmoles of tyramine and sufficient enzyme to cause at 30° an increase in absorbancy at 470 nm of 0·02–0·07 in 1 min. One unit of activity was defined as that amount of enzyme sufficient to cause an increase in absorbancy at 470 nm of 0·100 in 1 min when the reaction was proceeding at the maximum rate (see Fig. 3).

Fluorometric analysis. The fluorophores of DOPA and dopamine were prepared according to the procedure of Carlsson and Waldeck.[13] Activation and emission spectra were obtained on a Turner "Spectro" and on an Aminco-Bowman spectrophotofluorometer. Wavelengths used in obtaining activation and emission spectra were 372·5 nm and 329·8 nm, respectively, on the Turner and 380 nm and 315 nm on the Aminco-Bowman instrument.

Protein estimation. All protein determinations were performed using the method of Warburg and Christian.[20]

Acknowledgements—We are indebted to Professor Jack Francis of the Food Science and Technology Department, University of Massachusetts, and to the Chemistry Department, University of Massachusetts for use of the Turner "Spectro" and Aminco-Bowman spectrophotofluorometers, respectively.

[19] T. SWAIN, L. W. MAPSON and D. A. ROBB, *Phytochem.* **5**, 469 (1966).
[20] O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1942).