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Metabolism of Tyramine by Smooth Muscle Preparations *in vitro*.* (26595)

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The metabolism of amines, particularly sympathomimetic amines, has been of considerable interest in recent years. In smooth muscle 2 pathways by which these amines are known to be inactivated are oxidative deamination and O-methylation(1,2). Evidence will be presented here for the presence of a new metabolic pathway in smooth muscle which converts p-hydroxy-phenylethyl amines to catecholic compounds. These results were obtained from a study of the metabolism of tyramine by rabbit aorta and stomach muscle.

Materials and methods. Fresh rabbit aorta (descending thoracic and abdominal) was rinsed with buffer, trimmed, and cut into longitudinal pieces. Strips of stomach muscle were prepared by making parallel incisions through the muscle layers only and gently teasing the muscle away from the submucosa. All procedures were carried out at 2-4°C. Incubation flasks (25 ml Erlenmeyer), containing 200 μM oxygenated Na phosphate buffer, pH 7.4, and 20 μM tyramine in a total volume of 2.0 ml, were mounted in a rack and oxygenated by bubbling 100% O_2 below the surface of the fluid for 5 minutes. Strips of tissue were added (100-200 mg wet weight), and the flasks securely stoppered and incubated at 38° with shaking. The incubation was terminated by addition of HCl (final concentration 0.2 N) after removing the tissue (usually after 240 min). Acidified samples were used directly for all chemical and chromatographic analyses. Flasks incubated without substrate or without tissue were included as controls in each experiment.

All spectrophotometric determinations were made with the Beckman DU spectrophotometer fitted with a diaphragm and microcells. For ultraviolet absorption spectra, solutions were read in 0.3 N HCl and 0.3 N NaOH at a dilution sufficient to obtain an optical density of 0.200-0.800.

Procedures used for determination of amine(3) and p-hydroxyphenol(4) groups have previously been described. In the latter method, tyramine (Mann), tyrosine (Fisher), N-methyltyramine (Smith, Kline and French), p-hydroxyphenylacetic acid (Aldrich) and p-hydroxyphenylethanol (synthesized according to Ferber)(5) gave approximately the same molar extinction coefficient(ϵ). p-Hydroxyphenylethanolamine (Winthrop-Stearn), p-hydroxymandelic acid‡ and p-hydroxybenzaldehyde (Eastman) gave about 20% of the color obtained with tyramine. Epinephrine, norepinephrine, cobefrine, 3-hydroxytyramine, and neosynephrine (all from Winthrop-Stearn) gave no significant color under these conditions.

For chromatographic analysis, aliquots of acidified samples (10-2000 μl) were put directly on Whatman #1 filter paper, 5-15 μl at a time. The following solvents were used: 1) n-butanol-glacial acetic acid-water, 4:1:1;

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2) isopropanol-conc. NH_4OH-water, 8:1:1; and 3) chloroform-glacial acetic acid-water, 2:1:1, organic layer. The spray reagents(6) were: for phenols, diazotized sulfanilic acid (DSA); for amines, ninhydrin in butanol; for catechols, ethylene diamine; and for aldehydes, 2,4-dinitrophenylhydrazine (Di-NO_2-PH).

Properties of p-hydroxyphenylacetaldehyde (HPA-ald). To determine the chromatographic and ultraviolet absorption characteristics of HPA-ald, a series of experiments were carried out using a partially purified amine oxidase (AO) preparation from steer plasma. This enzyme, which catalyzes the oxidative deamination of numerous amines including tyramine, has been measured by the ultraviolet absorption (240-280 mμ) of the aldehydes formed from benzylamine, furfurylamine and homosulfanilamide(7). AO§ (2 mg in 0.5 ml H_2O) was added to 150 μM Na phosphate buffer, pH 7.2, 4 μM tyramine, and 1 unit purified catalase in a volume of 1.5 ml. After oxygenation and incubation for 240 min, approximately 40% of the tyramine had been metabolized. Values for the p-hydroxyphenol group did not change significantly during the incubation. Addition of Di-NO_2-PH (0.1% in 2 N HCl) produced a small amount of a yellow precipitate, and the clear red solution obtained after NaOH had an absorption peak at 480 mμ, which is typical of carbonyl compounds(8,9).

The ultraviolet absorption spectrum of the incubation mixture revealed one new peak at 330 mμ in addition to the tyramine peak at 295 mμ. Both the ultraviolet-absorbing material and the Di-NO_2-PH-reacting material could be extracted quantitatively into organic solvents from an acidic solution (<pH 7) but not from an alkaline solution (>pH 10). Evaporation of the solvent layer left a film of sweet smelling, yellowish oil. The increases in optical density of the incubation mixture at 330 mμ and the Di-NO_2-PH color at 480 mμ are given in Table I. The ratio of these absorptions remained constant during the incubation time. Chromatograms showed only one phenolic spot (R_f's of .96, .94 and .92 in solvents 1, 2 and 3, respectively) in addition to tyramine (R_f's of .37, .78 and .00, respectively). The former spot was also detected after spraying with Di-NO_2-PH.

TABLE I. Increase in Optical Densities during Incubation of Tyramine with AO.

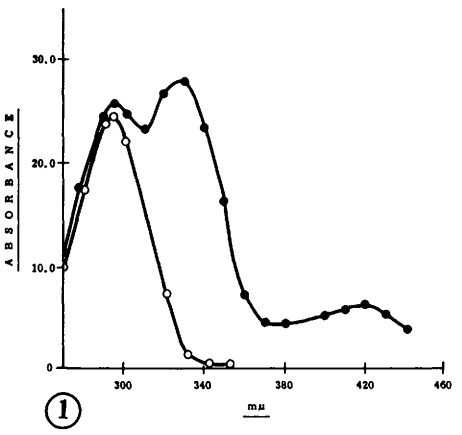
At each time indicated, 18 μl samples were diluted to 200 μl by addition of 0.3 N NaOH for the 330 mμ absorption and by addition of Di-NO_2-PH and 2 N NaOH for the Di-NO_2-PH color. The latter was read at 480 mμ. Each value represents the difference between the optical density of the incubated sample and the zero time sample.

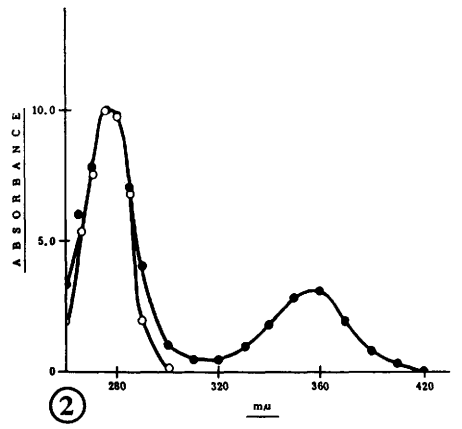
Absorption	Min. of incubation 60	180	240
A. 330 mμ	.191	.595	.815
B. Di-NO_2-PH	.067	.206	.276
A/B	2.85	2.88	2.92

The oxidation of this aldehyde to HPA-acid was demonstrated by reincubating a portion of the ether-soluble fraction of the AO incubation mixture with 100 mg rabbit aorta. This fraction was prepared by extracting the incubation mixture with ether, evaporating with a stream of N_2, and redissolving the residue in 2 ml phosphate buffer, pH 7.4. The optical density at 330 mμ decreased from an initial reading of 0.610 to 0.197 in 4 hours, while the hydrazone color decreased from a reading of 0.215 to 0.066 (330 mμ O.D./Di-NO_2-PH = 2.94). The total p-hydroxyphenol did not change. Whereas the chromatograms of the solution before incubating with aorta showed a single fast-moving phenolic spot in all 3 solvents (see above), after incubation the intensity of this spot decreased and a second phenol, identified as HPA-acid, was found (R_f's of .78, .44 and .82 in solvents 1, 2 and 3, respectively). These R_f's were identical to those found with the authentic acid run under the same conditions, and after addition of the known compound to the incubation mixture this area remained as a single spot in each solvent.

On the basis of this evidence it was concluded that the product of AO action on tyramine, HPA-ald, has a strong ultraviolet absorption peak at 330 mμ and gives R_f's of .96, .94 and .92 in solvents 1, 2 and 3, respectively.

§ AO (Stage 4, containing 5 manometric units/mg) was kindly furnished by Dr. Herbert Tabor, Nat. Inst. Health, Bethesda, Md.





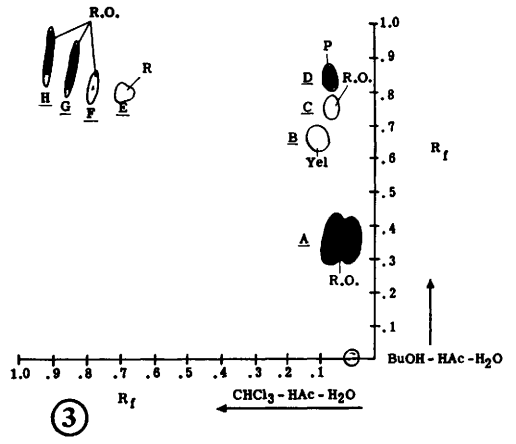


FIG. 1. Absorption spectrum of incubation mixture in alkali. Absorption spectrum of incubation mixture was obtained after diluting an aliquot with 0.3 N NaOH. Optical density in figure is calculated back to original concentration of incubation mixture. Tyramine, ○—○; incubation mixture, ●—●.

FIG. 2. Absorption spectrum of incubation mixture in acid after prior treatment with alkali. Absorption spectrum of incubation mixture was obtained by first diluting an aliquot in 0.3 N NaOH, then acidifying the solution (final concentration approximately 0.3 N HCl excess). Optical density as in Fig. 1. Tyramine, ○—○; re-acidified incubation mixture, ●—●.

FIG. 3. Two-dimensional chromatogram of a tyramine-aorta incubation mixture. Spray reagent, DSA and Na_2CO_3. Tyramine, A; HPA-acid, G; HPA-ald, H; tentatively identified as 3,4-dihydroxyphenylacetic acid, D; unknown, B, C, E, and F. Colors: R.O., red-orange; R, red; P, purple; Yel, yellow.

Results. In a series of 8 experiments with aortic strips, the average rate of tyramine disappearance by amine determination was 67.9 (range 55-80) μequivalents (μeq)/g wet weight aorta per 4 hr. The p-hydroxyphenol reaction disappeared at an average rate of 45.9 (range 31-75) μeq/g per 4 hr. The values obtained with stomach muscle were approximately the same as those with aorta. No disappearance of amine or p-hydroxyphenol reaction was observed in flasks containing boiled tissue (5 min at 100°). No evidence could be found for conjugation of the p-hydroxyphenol group, accumulation of drug or metabolites in muscle water, or metabolism of HPA-acid. Purified catalase (1 unit) added to incubation flasks did not alter the amine or p-hydroxyphenol disappearance. There was no apparent metabolism of tyramine in 100% N_2, and the rate in air for these reactions was 20-25% of the rate in oxygen.

A few preliminary experiments were done with homogenates of rabbit aorta. During the incubation, amine disappeared and aldehyde was formed (positive 2,4-dinitrophenylhydrazone test). This activity was found primarily in the residue obtained from centrifugation at 6000 × g for 30 min in the cold. No change in the p-hydroxyphenol reaction could be demonstrated in these homogenates.

During this investigation, it was observed that addition of NaOH to a colorless incubation sample produced a bright yellow solution. The ultraviolet spectrum of this alkaline sample revealed 2 absorption peaks in addition to that of tyramine.

The absorption spectrum of tyramine in 0.3 N HCl shows a single peak at 275 mμ (ϵ = 1200); in 0.3 N NaOH the peak is found at 295 mμ (ϵ = 2500). (Fig. 1). The

spectrum of closely related p-hydroxyphenyl compounds including tyrosine, N-methyltyramine, p-hydroxyphenylacetic acid, p-hydroxyphenylethanol, N-methyl-p-hydroxyphenylethanolamine, and p-hydroxyphenylethanolamine was almost identical to that of tyramine. Epinephrine, norepinephrine, 3-hydroxytyramine, epinine, cobefrine, and neosynephrine also gave a similar spectrum in acid solution; however, with the exception of neosynephrine, these latter compounds were unstable in alkali.

Fig. 1 shows the absorption spectrum of an incubated sample. In addition to the tyramine peak at 295 mμ, 2 new peaks were observed with maxima at 330 and 420 mμ. The increase in optical density at 330 mμ was proportional to the increase in Di-NO_2-PH color of the incubation mixture. The compound giving the peak at 330 mμ has been identified as HPA-ald (see *Methods*). The nature of the compound giving the 420 mμ peak is not known. Since alkaline solutions containing only HPA-ald do not show any absorption in this region, this peak may represent a previously unknown metabolic product of tyramine.

The absorption spectrum of an incubated sample in acid is identical with the tyramine curve except for some nonspecific absorption at the lower wavelengths. If a sample is reacidified after alkaline treatment, however, the original acid spectrum is not reproduced. In addition to the original absorption peak at 275 mμ (tyramine and HPA-ald), a new peak was observed at 360 mμ (Fig. 2). A second addition of NaOH reproduced the original alkaline spectrum (Fig. 1).

A typical chromatogram of an incubation mixture is reproduced in Fig. 3. The R_f region between .75 and .90 in solvent 1 was resolved into at least 6 phenolic compounds after chromatographing in solvent 3. Spot "A" represents unchanged tyramine. "G" is HPA-acid, identified by comparison with the known compound (R_f's = .78, .42 and .82 in solvents 1, 2 and 3, respectively; when added to the incubation mixture, the authentic acid and "G" remained as a single spot). "H" is probably HPA-ald (R_f's = .96, .93, .90, respectively, see *Methods*). The spot labelled "D" may be 3,4-dihydroxyphenylacetic acid, since the R_f's found in the 2 solvents agree with those reported in the literature (R_f of .85 in solvent 1 and .09 in solvent 3)(10,11), and the purple color is typical of catechols. The red-orange color of "C", "E", and "F" obtained with the DSA reagent has been seen with many p-hydroxyphenyl compounds, while the yellow color of "B" has been seen with many m-hydroxyphenyl compounds. The R_f's of these compounds do not correspond to any reported in the literature. In incubation mixtures containing boiled tissue, only unchanged tyramine could be detected.

Discussion. The disappearance of the p-hydroxyphenol reaction of tyramine reported here for rabbit aorta and stomach muscle has been previously observed in rabbit heart and intestine and in dog aorta(12,13). These authors suggested that this disappearance was due to a cleavage of the phenyl ring of tyramine. Such a reaction might produce compounds similar to the unsaturated aliphatic acids which are formed from other phenolic and catecholic compounds(14,15,16). However, preliminary studies indicated that when tyramine-8-C^{14} was incubated with rabbit aorta, the pattern of spots obtained from radioautographs of the chromatograms was not significantly different from that found on the DSA-sprayed chromatograms, which reveals only phenols.

Since all of the compounds tested which contain a p-hydroxyphenylethyl nucleus gave essentially the same amount of color in the procedure used (see *Methods*), the disappearance of p-hydroxyphenol groups probably results from formation of new compounds which produce less color in the analytical method. At least 5 new phenolic compounds have been found in the medium after incubation of tyramine with aorta. Since no metabolic products are found in presence of boiled tissue, these new compounds may be the result of new and uncharacterized enzyme systems in smooth muscle. One of these compounds has been tentatively identified as 3, 4-dihydroxyphenylacetic acid.

Pisano, Creveling and Udenfriend(17) have recently reported the β-hydroxylation of tyramine to norsynephrine in adrenal me-

dulla slices and other tissues. Although norsynephrine *per se* was not one of the unknown compounds found in the incubation mixture, perhaps Spot B, which gives color reactions similar to norsynephrine (*i.e.*, yellow with DSA), represents a metabolite of this compound. In the present study it was impossible to separate HPA-ald from p-hydroxyphenylethanol chromatographically so that the presence or absence of the alcohol could not be determined. Goldstein *et al.*(18) reported the appearance of 3-methoxy-4-hydroxyphenylethanol as an end product of dopamine metabolism.

The rate of disappearance of the p-hydroxyphenol reaction is only slightly less than that observed for amine disappearance (monoamine oxidase activity), which suggests that this pathway is important in the metabolism of tyramine by smooth muscle. It also supports the hypothesis that the pharmacological action of tyramine may result from both a direct effect on the receptor sites and an indirect effect by conversion to a more potent sympathomimetic amine, such as norsynephrine or dopamine(19).

Summary. During incubation of tyramine with rabbit aorta or stomach muscle, tyramine is metabolized as evidenced by a significant decrease in the amount of p-hydroxyphenyl and primary amine groups of tyramine and by concomitant changes in the ultraviolet absorption spectrum of the incubation mixture. Chromatographic analysis revealed the appearance of p-hydroxyphenylacetaldehyde and p-hydroxyphenylacetic acid as well as 5 unknown phenolic spots. One of these spots has been tentatively identified as 3,4-dihydroxyphenylacetic acid. The decrease of p-hydroxyphenyl groups of tyramine is probably accounted for by its conversion to dihydroxy or β-hydroxy compounds which given less color in the analytical procedure used.

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