A Study of the Formation of Fluorescent Derivatives of 4-Hydroxyphenethylamine (Tyramine), 4-Hydroxy-3-methoxyphenethylamine (3-Methoxytyramine) and Related Compounds by Reaction with Hydrazine in the presence of Nitrous Acid

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A reaction is described that allows the preparation of fluorescent derivatives of a group of related compounds with the basic 4-hydroxyphenethylamine structure. Examination of the reaction shows that it takes place in two stages, which can be considered separately. (1) Reaction of hydrazine with nitrous acid: this is instantaneous at room temperature and involves the reaction of 1 mol of hydrazine with 2 mol of nitrous acid. (2) Reaction with 4-hydroxyphenethylamine compounds: this occurs slowly at room temperature but the rate of reaction is significantly increased at higher temperatures. Ammonium sulphamate is added to remove excess of nitrous acid, found to be detrimental to the reaction. Examination of reagent concentrations necessary for maximum fluorescence yield demonstrated the need for a 40-fold molar excess of the reagent formed in the first stage. The derivatives fluoresce in alkaline solution, the fluorescence of derivatives of 4-hydroxy compounds being stable for over 1 h at room temperature, those of 4-hydroxy-3-methoxy compounds being slightly less stable. The derivatives were distinguishable from their parent compounds by t.l.c.

The fluorescence spectra of tyramine (4-hydroxyphenethylamine), tyrosine and related compounds have been described (Teale & Weber, 1956; White, 1959). This native fluorescence is weak and extremely pH-dependent. It is therefore not satisfactory for use in the accurate determination of these compounds in biological materials. Various methods have been adopted to produce derivatives of sufficiently high fluorescence intensity to allow determination in the presence of substantial concentrations of other organic molecules. Lowe, Robins & Eyerman (1958) describe the production of a fluorescent product when tyramine, tyrosine and other amino acids are incubated at 60°C with ninhydrin at pH 9.4. The low fluorescence yield of these derivatives of tyramine and tyrosine compared with that of phenylalanine precludes the use of this reaction for determinations other than phenylalanine, where the fluorescence is enhanced by the presence of the peptide L-leucyl-L-alanine (McCaman & Robins, 1962). Incubation with ninhydrin has been used successfully to give fluorescence suitable for determination of 5-hydroxytryptamine (serotonin) (Vanable, 1963; Snyder, Axelrod & Zweig, 1965).

Waalkes & Udenfriend (1957) have made use of the reaction of tyrosine with 1-nitroso-2-naphthol

to produce a fluorescent derivative applicable to quantitative determination. This has been applied to the determination of tyrosine in plasma (Udenfriend, 1962), since the major 4-hydroxy component is tyrosine. Fluorescence is also produced under similar conditions with tyramine (Wong, O'Flynn & Inouye, 1964) and 4-hydroxyphenylpyruvic acid.

Fluorescent derivatives of amines and amino acids, including tyramine and tyrosine, can be prepared by using DNS chloride, which reacts with free amino and phenolic groups (Gray & Hartley, 1963). They are resistant to acid hydrolysis and have an intense yellow fluorescence. As the reagent is relatively non-specific the production of the derivatives is usually followed by electrophoresis or t.l.c. (Crowshaw, Jessup & Ramwell, 1967) to facilitate separation.

The usual method used for the production of fluorophores of the 4-hydroxy-3-methoxy compounds metadrenalin and normetadrenalin involves oxidation to adrenolutin and noradrenolutin. It is generally assumed that oxidative demethylation precedes the formation of adrenochrome or noradrenochrome and subsequent alkaline conversion into the fluorescent lutines which form the basis of the adrenaline and noradrenaline methods

(Bertler, Carlsson & Rosengren, 1959). The stability of the fluorophores appears to depend on the pH of the final solution. If this is near neutrality the fluorescence is stable for 1 h (Smith & Weil-Malherbe, 1962; Weil-Malherbe & Smith, 1966), but if it becomes unduly alkaline the fluorescence becomes unstable (Brunjes, Wybenga & Johns, 1964; Weil-Malherbe & Smith, 1966).

While searching for a more satisfactory fluorescent derivative of tyrosine it was found that in the presence of nitrous acid, hydrazine reacts with tyrosine to give a strongly fluorescent product. Further examination showed that if the tyrosine is replaced by tyramine, metadrenalin or normetadrenalin the reaction also produces fluorescent derivatives of these. The reaction conditions necessary to give the maximum fluorescence yield and the nature and stability of the fluorescence obtained were studied.

EXPERIMENTAL

The reaction of hydrazine with tyramine, tyrosine and related compounds was studied by using the following basic method with the variations stated in the text. Into test tubes (75 mm $\times$ 12 mm) were placed 100 μ l of 80 mM- NaNO_2 and 100 μ l of 60 mM-hydrazine hydrochloride in 1 M-HCl. After mixing the solutions, 40 μ l of 1 M-ammonium sulphamate was added to each reaction mixture followed by 20 μ l of tyramine solution (or related compound, usually 0.1 g in 1 litre of 0.01 M-HCl). After further mixing the reaction was incubated at 55°C for 10 min. The solutions were cooled and made alkaline by addition of 1 ml of 0.5 M- Na_2CO_3 . The resulting fluorescence, obtained on excitation with light of wavelength 320 nm, was observed at 410 nm for 4-hydroxy compounds and at 425 nm for 4-hydroxy-3-methoxy compounds (Fig. 1). The instrument used for all measurements was an Aminco-Bowman spectrofluorimeter fitted with a xenon source.

T.l.c. of the reaction mixtures was performed after the final volumes from the above reaction had been evaporated to dryness on a rotary evaporator. The residues were taken up in the minimum volume of propan-2-ol-water (1:1, v/v) and applied to a t.l.c. plate formed of a 0.05 mm layer of silica gel G (Macherey, Nagel and Co., supplied by Camlab, Cambridge, Cambs., U.K.). Plates were developed in a solvent system of methanol-ethyl acetate (1:1, v/v), the tank being saturated with NH_3 vapour. Fluorescent and u.v.-absorbing areas were detected by viewing the plate under u.v. light while it was still wet. The plate was dried and sprayed with diazotized sulphanilic acid (Bray, Thorpe & White, 1950) to locate the 4-hydroxy compounds. Finally, after drying again, the plate was sprayed with ninhydrin in acetone reagent to locate amines and amino acids on the chromatogram.

To ascertain the part played by the nitrous acid in the reaction, the stage at which the ammonium sulphamate was added was varied from that in the basic method. The concentration was also varied from 0–0.22 M. Ammonium sulphamate was added to the solution of

Fig. 1. Fluorescence spectra of the hydrazine derivatives of tyramine (□, excitation; ■, emission) and 3-methoxytyramine (○, excitation; ●, emission) in aqueous solution, pH 9.0.

NaNO_2 before the addition of the hydrazine hydrochloride. This must compete with the hydrazine for the nitrous acid produced if this is the initial reaction. The concentration of the ammonium sulphamate was increased over the molar range stated so that in the initial reaction the proportion of nitrous acid reacting with the hydrazine should decrease. This should show itself as a decreasing fluorescence yield on subsequent reaction with tyramine. When the ammonium sulphamate is added after reaction of the hydrazine and nitrous acid then addition of increasing amounts of reagent should have no effect on the final yield of fluorescence, provided that the ammonium sulphamate itself has no quenching effect on the fluorescence.

A series of twelve solutions of hydrazine hydrochloride were prepared, ranging in concentration from 3.0–39.0 mM. A sample of 100 μ l of each was treated with 100 μ l of 20 mM- NaNO_2 in the absence of ammonium sulphamate. The same procedure was repeated using 40 mM- and 60 mM- NaNO_2 . The samples were then treated with tyrosine and the resulting fluorescences determined. Those solutions that did not fluoresce had a yellow colour, the absorption of which was read at its peak of 435 nm, with a Unicam SP.600 instrument. Thus the number of μ mol of nitrous acid reacting with 1 μ mol of hydrazine was determined.

The second stage of the reaction, that between the modified (diazotized) hydrazine and tyramine was first observed at room temperature. Subsequently it was examined at temperatures of 29, 39, 53 and 65°C to find the optimum temperature for the reaction. Standard curves were constructed with various concentrations of reactants to find their optimum concentrations.

Fluorescence is developed by making the final solution alkaline. A study was made of the effect of H^+ concentration on the fluorescence. After reaction of the 'diazotized' hydrazine and tyramine, 1 ml of 0.1M-sodium phosphate buffer, pH approx. 7.0, was added. The pH was adjusted to the required value with 2.0M-HCl or 2.0M-NaOH by using a Radiometer pH meter fitted with a capillary glass pH electrode and a calomel reference electrode with an open liquid junction. After adjustment of the volumes of the solutions the relative fluorescence intensities were determined. The pH range 3.0–14.0 was covered for the derivatives of tyramine, tyrosine and metadrenalin.

The stability of the fluorescence of the derivatives of tyramine, tyrosine, 3-methoxytyramine (4-hydroxy-3-methoxyphenethylamine), metadrenalin and normetadrenalin was examined by using first 1 ml of 0.5M- Na_2CO_3 as final diluent and then 1 ml of 2.5M-NaOH in place of the Na_2CO_3 . The fluorescence intensities in the resulting solutions were read at 10 min intervals, the solutions being kept at room temperature.

RESULTS AND DISCUSSION

A method is described above for the preparation of fluorescent derivatives of tyramine, tyrosine, 3-methoxytyramine and related compounds. Attempts to prepare similar derivatives of phenylalanine, 4-hydroxyphenylpyruvic acid, 3-hydroxytyramine and 3,4-dihydroxyphenylalanine resulted in no detectable fluorescence. The reaction is not restricted to primary amines, since the secondary amines *N*-acetyltyrosine and metadrenalin yield the fluorescent derivatives. Relative fluorescence-intensity values for the hydrazine derivatives formed from this group of compounds are shown in Table 1.

The reaction used for the preparation of the derivatives is in two stages. When ammonium sulphamate is added to the sodium nitrite before addition of the hydrazine there is a decrease in

the final fluorescence, which is more marked as the concentration of sulphamate increases. If, however, the ammonium sulphamate is added after the reaction of hydrazine and nitrous acid there is a negligible decrease in the fluorescence. Ammonium sulphamate does not quench the final fluorescence. The first stage is then the direct reaction of nitrous acid with hydrazine to form a product that is a specific entity within the reaction. The reaction is instantaneous at room temperature (19°C) and at this temperature the product is stable for at least 45 min. After this time the final fluorescence yield gradually diminished.

Further examination shows that in the absence of ammonium sulphamate two reactions are possible. With excess of hydrazine fluorescence is produced, but with excess of nitrous acid there is no fluorescence. With tyrosine to form the fluorophore, solutions giving no fluorescence because of excess of nitrous acid are coloured yellow. At 435 nm the colour is proportional to the concentration of the tyrosine present. It is likely that the colour is due to the formation of 4-hydroxybenzaldehyde from tyrosine by the action of nitrous acid. Further to this, it is found that if the concentration of hydrazine in the reaction is varied, fluorescence begins to appear when it reaches half the molar concentration of sodium nitrite (Fig. 2). If the proportion of sodium nitrite is greater no fluorescence results. If the molar ratio of hydrazine to nitrite is greater than 1:2 the fluorescence yield increases slightly (it is maximum when the molar ratio is 3:4) and then falls giving a yield of half the maximum yield when the molar ratio is 7:4.

The second stage of the reaction is optimum at 55°C, when it reaches completion after 5 min incubation. Temperatures higher than this can be used, but prolonged incubation caused decomposition with subsequent loss of fluorescence yield. The reaction only proceeds in acid solution. It is therefore important to minimize the amount of ammonium sulphamate added in the first stage.

Fluorescence is developed by making the final solution alkaline (Fig. 3). With tyramine it is maximum by pH 7.5 but with tyrosine and metadrenalin the maximum fluorescence yield is only achieved at pH 9.0. It appears satisfactory to adopt the procedure described in the method above, the addition of 0.5M-sodium carbonate, to produce fluorescence of all derivatives described. In this solution the fluorescence of 4-hydroxy derivatives is stable for over 1 h. Fluorescence of 4-hydroxy-3-methoxy derivatives is slightly less stable. If fluorescence is developed by addition of 2.5M-sodium hydroxide it is far less stable in all cases.

The resultant fluorescence is directly proportional to the tyramine concentration provided that there

Table 1. *Fluorescences of the hydrazine derivatives of compounds related to 4-hydroxyphenethylamine, relative to that of tyramine*

These were prepared by the method described in the text. All were used at concentrations of 0.1 mM.

	Fluorescence (scale readings)	Wavelengths (nm)	
		Excitation	Emission
Tyramine	152	320	410
Tyrosine	135	320	410
Tyrosine ethyl ester	131	320	410
<i>N</i> -Acetyltyrosine	82	320	410
Metadrenalin	150	320	425
Normetadrenalin	141	320	425
3-Methoxytyramine	81	320	425
3-Methoxytyrosine	92	320	425

Fig. 2. Reaction of hydrazine with nitrous acid, the reaction mixture containing 4 μ mol of nitrous acid and various amounts of hydrazine. After subsequent reaction with tyrosine, fluorescence (▲, excitation 320 nm; emission 410 nm) and E_{435} (●) of the solutions were determined.

Fig. 3. Effect of pH on the final fluorescence of hydrazine derivatives of tyramine (●), tyrosine (▲) and met-adrenalin (□).

is a 40-fold molar excess of reagent from the first stage. If this excess decreases, then it quickly becomes the limiting factor. At low concentrations of reagent (below that giving a fivefold molar

excess) gross deviations from linearity are observed. The range over which concentration is directly proportional to fluorescence intensity of the derivative has been determined for tyrosine and is 0.1–150 μ g/ml of final solution.

That the derivatives are distinguishable from their parent compounds can be shown by t.l.c. The reaction mixtures are applied to the t.l.c. plate and chromatographed under the conditions described above. When the developed plate is viewed under u.v. light two areas show a blue fluorescence. These correspond to the hydrazine derivatives of tyramine and tyrosine, the former having R_F 0.41 and the latter showing negligible migration from the origin. In each case these are only present in reaction mixtures that contain nitrous acid and excess of hydrazine. Also detected under u.v. light is a u.v.-absorbing area with R_F 0.80. This only appears in the reaction mixture containing tyrosine, hydrazine and excess of nitrous acid. It corresponds to the position of migration of the 4-hydroxybenzaldehyde standard, both giving a yellow colouration on spraying with diazotized sulphanilic acid.

The positions of tyramine and tyrosine on the chromatogram are located by using ninhydrin reagent. Their R_F values are 0.75 and 0.26 respective-

ly. The derivatives are more polar than the parent compounds, migrating shorter distances in the solvent system used. Neither of the derivatives give any colouration with diazotized sulphanilic acid or ninhydrin reagents, which demonstrates that the 4-hydroxy position on the ring and the amine group in the side chain of the parent compounds are deactivated in derivative formation. This is in agreement with the earlier observation that fluorescence is obtained only with those compounds that possess the 4-hydroxyphenethylamine structure. The fact that fluorescence is obtained after acetylation of the amino group does suggest, however, a less fundamental part for the group in the subsequent production of fluorescence.

Elution of the fluorescent areas of the chromatogram with 0.5M-sodium carbonate followed by spectral scanning showed that both areas had peak excitation and emission wavelengths of 320nm and 410nm respectively.

It is suggested that a diazonium-type reaction occurs between hydrazine and nitrous acid, the product of which reacts with the phenolic amine in a coupling reaction. Deviations from linearity at low concentrations of reagents suggest that the reaction is not a simple bimolecular one but may be a more complex dimerization reaction.

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