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Fluorescence of Catechol Amines and Related Compounds Condensed With Formaldehyde[1,2]

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The noradrenaline cells in the adrenal medulla become fluorescent when briefly fixed in formalin [6,7], but the nature of the reaction has not been elucidated. Recently Eränkö [8] and Falck and Torp [10] found that a very intense fluorescence develops in the noradrenaline cells when freeze-dried sections are exposed to formaldehyde vapour. As a great need exists for highly sensitive histochemical methods for demonstration of mono-amines—*i.a.* for localization of the brain catechol amines—it was thought worth while to study the reaction between formaldehyde and catechol amines in model systems and to isolate and examine the reaction products.

MATERIAL AND METHODS

Fluorescence microscopy. The amines (usually as hydrochlorides) and amine-formaldehyde condensation products were dissolved in 1% aqueous solutions of human serum albumin, gelatin, gliadin (wheat), or sucrose. One microliter spots were placed on object glasses and dried at room temperature or at 50°C. After microscopy the spots were treated with the vapour from a 35% solution of formaldehyde (Merck p.a.) for varying periods of time either at room temperature or at 50°C. In the latter case the glasses were prewarmed at 50°C to prevent water condensation on the spots.

The light from an Osram XBO 2001 high-pressure xenon lamp was passed through a water-cooled 1 mm Schott BG 38 filter (for heat absorption) and then through a Zeiss M4 QII monochromator whose exit slit was projected in the diaphragm of the condenser of a fluorescence microscope. To prevent stray light in the visible region from the monochromator reaching the microscope a suitable filter (usually a 2 mm Schott BG 12) was placed before the condenser. Exchangeable stop filters were placed in the microscope tube (usually a Schott GG 4 and above it a Wratten gelatin filter No. 15).

Preparation of amine-formaldehyde condensation products. Dopamine, noradrenaline, and adrenaline were treated with an excess of formaldehyde either in acid or at pH about 5.

a) An amount of the amine hydrochloride corresponding to 25 mg of base was dissolved in 4.5 ml 0.1 *N* H_2SO_4 (or HCl). After addition of 0.5 ml 35% formaldehyde the solution was heated at 100°C for 20–30 minutes, then cooled and neutralized to about pH 5 with K_2CO_3.

b) The amine hydrochloride was dissolved in water and—where necessary—neutralized to about pH 5 with K_2CO_3. Formaldehyde (about pH 6) was added and the solution heated at 50°C for 20 minutes.

The reaction mixture was transferred to a cation exchange column (Amberlite XE-64, 22×0.55 cm, equilibrated with 1 *M* ammonium acetate of pH 6). Elution (5 ml fractions) was performed with 0.02 and 0.1 *M* ammonium acetate (pH 6 and pH 5, respectively) and 0.1 *N* HCl, and was followed by determination of the ultraviolet absorption of the fractions.

The main part of the reaction products from the catechol amines (80–90%) was eluted in a single peak with the 0.1 *M* buffer. The ultraviolet absorption and the fluorescence spectra of the fractions (at pH 2, 5, and 6.5) were taken, and spots containing 0.02–1 μg of the compounds in 1% serum albumin were examined in the fluorescence microscope as described above. Finally the main fractions were pooled and evaporated to a small volume *in vacuo* at 40°C (rotating evaporator), dissolved in water, and stored at −30°C.

No unchanged catechol amines were found on paper chromatography or on spectrophotofluorimetric examinations according to Carlsson and Waldeck [4], or Bertler, Carlsson and Rosengren [2].

Preparation of 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline. Dopamine was condensed with acetaldehyde according to the procedure of Kovács and Fodor [17], which gives a good yield of the tetrahydroisoquinoline. The substance was crystallized twice from alcohol-ether. No unchanged dopamine was found on paper chromatography.

Paper chromatography. The amine-formaldehyde products were examined by ascending paper chromatography using *n*-butanol-glacial acetic acid-water (4:1:5) as the solvent system. After drying, the papers were examined in

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TABLE 1

FLUORESCENCE OF CATECHOL AMINES AND RELATED COMPOUNDS INCLUDED IN A DRIED SERUM ALBUMIN FILM AFTER TREATMENT WITH FORMALDEHYDE VAPOUR AT 50°C FOR 15 MINUTES

	Amount	Fluorescence
	μg	
Phenylalanine	1	0
Tyrosine	1	0
Meta-tyrosine	1	4+
Dopa (3,4-dihydroxy-phenyl-alanine)	0.2	6+
α-Methyl-dopa	1	4+
β-Phenylethylamine	1	0
Tyramine (4-hydroxy-phenyl-ethylamine)	1	0
Meta-tyramine (3-hydroxy-phenylethylamine)	0.2	4+
Octopamine (4-hydroxy-phenyl ethanolamine)	1	0
Dopamine (3,4-dihydroxy-phenylethylamine)	0.2	6+
Noradrenaline	0.2	6+
Adrenaline	1	3+
Methoxy-tyramine (3-methoxy-4-hydroxy-phenylethylamine)	1	3+
Normetanephrine (3-methoxy-4-hydroxy-phenylethanolamine)	1	0
Phenylephrine (3-hydroxy-N-methyl-phenylethanolamine)	1	+
Epinine (3,4-dihydroxy-N-methyl-phenylethylamine)	1	3+
Hordenine (4-hydroxy-N,N-dimethyl-phenylethylamine)	1	0
3,4-Dihydroxyphenylacetic acid	1	0

ultraviolet light and then sprayed with $K_3Fe(CN)_6$ according to James [16].

Fluorimetry. The fluorescence spectra of the amines and their formaldehyde condensation products (1–10 μg/ml), before and after oxidation with H_2O_2 according to Hess and Udenfriend [15], were determined at various pHs with the Aminco-Bowman spectrophotofluorometer. The wavelengths given for activation and fluorescence are the uncorrected instrument values.

RESULTS

Fluorescence of amines in model systems: The catechol amines and related compounds examined (see Table 1) showed no visible fluorescence in dried spots containing 1 μg of the substances in serum albumin. After exposure of the spots to formaldehyde vapour some of them were transformed into products which fluoresced intensely green to yellow. The activation peak lies at 420–440 mμ. Serum albumin itself—after treatment with formaldehyde—showed a very faint dirty-green fluorescence.

In most instances a maximum fluorescence was obtained when the formaldehyde treatment was performed at 50°C for 15 minutes. An exposure at this temperature for a longer time usually weakened or even destroyed the fluorescence, but at about 20°C an exposure time of up to 24 hours could be used.

TABLE 2

COMPARISON OF FLUORESCENCE INTENSITIES DEVELOPED FROM CATECHOL AMINES AND SOME RELATED COMPOUNDS INCLUDED IN A DRIED SERUM ALBUMIN FILM EXPOSED TO FORMALDEHYDE VAPOUR AT 50°C FOR 5 OR 15 MINUTES

	5 Min			15 Min		
	0.2	0.02	0.01	0.2	0.02	0.01
	μg			μg		
Meta-tyrosine	+	0	0	2+	0	0
Dopa	5+	3+	2+	6+	4+	3+
α-methyl-dopa	+	0	0	2+	0	0
Meta-tyramine	3+	0	0	4+	+	0
Dopamine	5+	3+	2+	6+	4+	3+
Noradrenaline	5+	3+	2+	6+	4+	3+
Adrenaline	0	0	0	+	0	0
Epinine	0	0	0	+	0	0
Methoxy-tyramine	0	0	0	+	0	0

As seen from Tables 1 and 2 there are certain structural requirements for the fluorescence reaction. Of the phenylalanine and phenylethylamine derivatives used, a very intense fluorescence is given only by those that are primary amines and also have hydroxyl groups at the 3 and 4 positions (dopa, dopamine, noradrenaline). An interesting exception is α-methyl-dopa (see Discussion). The catechol amines, which are secondary amines (adrenaline and epinine), give a much weaker fluorescence, which furthermore develops more slowly in the formaldehyde treatment. Thus it is possible to distinguish between noradrenaline and adrenaline in sections of adrenal medulla by means of the formaldehyde reaction [8,10]. Some data illustrating the sensitivity and the rate of the reaction are found in Table 2.

The results obtained favour the view that the 3-OH group is essential for the fluorescence reaction. Thus the compounds with no hydroxyl group or only one in the position *para* to the side chain seem to be non-reactive, whereas the *meta* isomers of tyrosine and tyramine show a fairly pronounced fluorescence. Furthermore the substitution of the 3-OH group by a methoxy group in dopamine and noradrenaline dramatically inhibits the reaction.

The fluorescent products formed from the primary amines in dried spots of serum albumin were quite stable for days at room temperature. The fluorescence did not disappear if the spots were further dried *in vacuo* over P_2O_5 (2 days) or by heating at 150°C (1 hour). No material decrease of the fluorescence was found after extraction of the spots with water, 0.1 *N* HCl, ethyl alcohol, xylene, or benzene, or at acid hydolysis (0.1 *N* HCl, 100°C, 10 min).

The amines were also examined enclosed in dried spots of gelatin, gliadin, or sucrose. The fluorescence obtained in gelatin was of somewhat lower intensity, and in gliadin or sucrose much weaker than that developed in serum albumin. The products formed in gelatin, and especially in gliadin, were also to a higher extent extractable with water.

Since the high water content of the formaldehyde vapour used may cause a diffusion of catechol amines in tissue sections, paraformaldehyde was tried instead of formaldehyde. When the reaction was performed at 100°C on models, a fairly intense fluorescence was obtained from dopamine and noradrenaline.

Examination of the amine-formaldehyde reaction: Dopamine, noradrenaline, and adrenaline in solution were treated with an excess of formaldehyde under mild conditions (50°C, pH 5, 20 min). The products formed were isolated by ion exchange chromatography and some of their properties were studied. Since no pure crystalline products have so far been obtained, a more definite characterization has not been possible.

The main products (80–90%) were eluted from the column in a single peak containing no detectable unchanged amine (see Methods). As shown by paper chromatography, however, at least two compounds were present with R_f values differing only slightly from those of the original amines. Neither of the spots was fluorescent when examined in ultraviolet light, but gave somewhat different colours on oxidation with ferricyanide. On spraying the papers with 0.1 *M* sodium molybdate the spots immediately turned brown, indicating that the O-dihydroxy grouping was still present (cf. [20]).

A fluorimetric examination showed that the products had retained the characteristic fluorescence of the catechols (activation at 285 mμ, fluorescence at 335 mμ) and in fact had activation and fluorescence spectra identical with the original amines. Unexpectedly, the products showed no fluorescence at all in the visible region. After oxidation with H_2O_2 (cf. [15]) only a weak new fluorescence developed (activation at 340–360 mμ, fluorescence at 410–440 mμ, pH dependent), and the original fluorescence disappeared almost completely.

The ultraviolet spectra of the products were very similar to those of the original amines, and the molar extinction coefficients at 250 and 280 mμ were practically unchanged. However, the spectra of the compounds formed from the three amines all showed the same displacement of the maximum and minimum to somewhat (3–4 mμ) higher wavelengths.

The products from dopamine and noradrenaline—just as the original amines—were easily oxidized at pH 6 with iodine or ferricyanide (or above pH 7 with O_2) to red compounds which rapidly turned brown to black.

Since it seems probable (see Discussion) that the catechol amines condense with formaldehyde in acid at higher temperatures to tetrahydroisoquinoline derivatives, the products formed from dopamine and noradrenaline in 0.1 *N* H_2SO_4 (or HCl) at 100°C were isolated and compared with those obtained at pH 5.

Paper chromatography showed the presence of at least two compounds similar to those obtained at pH 5. In solution they exhibited no visible fluorescence, but the original catechol amine fluorescence (285/335 mμ) was unaffected. The ultraviolet spectra showed the same characteristic displacement to somewhat higher wavelengths as described above.

When 1 μl spots containing 1 μg of the compounds formed from dopamine and noradrenaline at pH 5 or in acid were examined in the fluorescence microscope before drying, only a very weak greenish fluorescence was seen. After drying, however, the residue showed a fairly intense green-yellow to yellow fluorescence which for a few seconds disappeared almost completely when the water content of the spot was momentarily increased by breathing on it. The amine-formaldehyde products—although practically non-fluorescent in the visible region in solution—thus have a fairly intense fluorescence above 500 mμ in the dry state.

TABLE 3

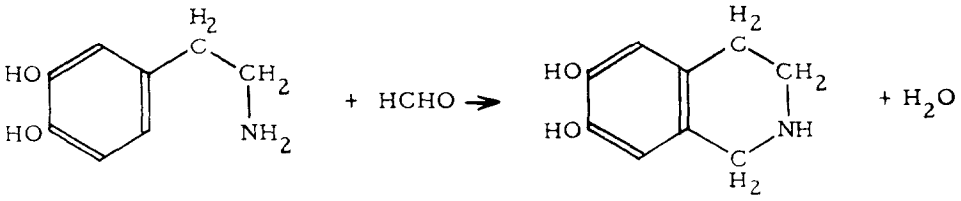
FLUORESCENCE OF ISOLATED CONDENSATION PRODUCTS INCLUDED IN A DRIED PROTEIN FILM BEFORE AND AFTER TREATMENT WITH FORMALDEHYDE VAPOUR AT 50°C FOR 15 MINUTES

	Before Treatment with Formaldehyde Vapour		After Treatment with Formaldehyde Vapour	
Condensation Product	0.2	0.02	0.2	0.02
	μg		μg	
Dopamine-formaldehyde	2+	0	6+	4+
Noradrenaline-formaldehyde	2+	0	6+	4+
1-Methyl-6,7-dihydroxy-tetrahydroisoquinoline	+	0	6+	4+

This seemed to explain the fluorescence reaction found in the model systems. However, the fluorescence intensity of the compounds in dried serum albumin spots was very much (probably more than 20 times) lower than that of the amine-formaldehyde products directly produced in the spots by use of formaldehyde vapour (Table 3). This unexpected finding is not easily explained. It would seem that the amines present in a nearly dry protein film react with formaldehyde in another way than amines in solution. The insolubility of the fluorescent products formed in the film might support this view. It was found, however, that the condensation products formed in solution (both at pH 5 and in acid) when dried in spots of serum albumin developed the same intense fluorescence as that of the amines themselves on treatment of the spots with formaldehyde vapour (Table 3), and at the same time became insoluble. This finding may provide a clue to the understanding of the formaldehyde reaction (see Discussion).

The formation of insoluble, highly fluorescent products may possibly be due to a polymerisation of the amines in the nearly dry state. This was tested by exposing a thin layer of dopamine hydrochloride to formaldehyde vapour at 50°C for 30 to 60 minutes. The condensation products were quite water soluble, however, and showed properties very similar to those of the amine-formaldehyde compounds formed in solution. Especially noteworthy is that they developed an intense fluorescence in dried serum albumin spots first after treatment with formaldehyde vapour.

A comparison of the fluorescence properties of the amine-formaldehyde compounds described above with those of a known 6,7-dihydroxytetrahydroisoquinoline is of considerable interest. Since dopamine is easily condensed with acetaldehyde to 1-methyl-6,7-dihydroxytetrahydroisoquinoline, this substance was prepared and examined (see Methods). As seen from Table 3 it showed only a weak fluorescence in dried serum albumin spots, but a very intense fluorescence developed after treatment with formaldehyde vapour. At the same time the fluorescent prod-



Dopamine 6,7-Dihydroxy-1,2,3,4-tetrahydroisoquinoline

FIG. 1

ucts became insoluble. Thus this tetrahydroisoquinoline derivative behaves just as the condensation products of dopamine (and noradrenaline) with formaldehyde.

DISCUSSION

Phenylalanine, tyrosine, β-phenylethylamine, and mescaline condense with formaldehyde to form 1,2,3,4-tetrahydroisoquinoline derivatives when acid is used as catalyst [5, 19, 22]. Undoubtedly the same type of condensation occurs when dopamine, noradrenaline, and dopa are treated with formaldehyde in mineral acid (Fig. 1).

The present work shows that dopamine and noradrenaline easily react with formaldehyde even under mild conditions (pH 5, 50°C). The findings support the view that the reaction products here, too, are tetrahydroisoquinoline derivatives. Thus the products formed both in acid and at pH 5 have the same ultraviolet absorption and fluorescence spectra, and on paper chromatography* showed similar R_f values and colour on ferricyanide oxidation. In both cases the catechol nucleus seems to be intact (see below). Finally they have some interesting fluorescence properties in common: no fluorescence in the visible region in aqueous solution, but a greenish yellow to yellow fairly intense fluorescence in the solid state; the formation of insoluble, highly fluorescent products when exposed in a dried protein film to formaldehyde vapour.

There are several indications that the hydroxy groups at the 3 and 4 positions are not attacked when the catechol amines are condensed with formaldehyde. The fact that the condensation products are very easily oxidized to coloured compounds—just as are the amines themselves—and the ultraviolet absorption and fluorescence spectra favour this view. It has further been shown [12] that phenolic groups, e.g. in tyrosine, do not react with formaldehyde at neutral or slightly acid reaction.

Although definite proof is lacking, the following discussion will be based on the assumption that the main condensation products formed from dopamine and noradrenaline under mild conditions (pH 5, 50°C) have a tetrahydroisoquinoline structure. The validity of this assumption is strongly supported by current views on the biosynthesis of isoquinoline alkaloids (cf. [18]) and by the findings that dopamine, epinine, and 3-hydroxy-4-methoxy-phenylethylamine easily condense with acetaldehyde at pH 5 and 20°C to tetrahydroisoquinolines [17,21]. Finally, strong support is provided by the observation in the present work that 1-methyl-6,7-dihydroxy-tetrahydroisoquinoline included in a protein film when treated with formaldehyde vapour develops the same intense fluorescence as that obtained from the catechol-amine-formaldehyde condensation products.

It was unexpectedly found that the isolated tetrahydroisoquinoline derivatives included in a protein film have a much weaker fluorescence in the visible region than that of the products formed when the catechol amines themselves in such a film are treated with formaldehyde vapour. It thus seems that the amines present in a nearly dry protein film react with formaldehyde in another way than that of amines in solution. However, the isolated derivatives—including 1-methyl-6,7-dihydroxy-tetrahydroisoquinoline—when dried in spots of serum albumin developed the same very intense fluorescence when treated with formaldehyde vapour as that of the amines themselves. This finding indicates that the catechol amines, both in solution and in a protein film, react with formaldehyde in essentially the same manner, but also that the tetrahydroisoquinoline derivatives primarily formed must be involved in a secondary reaction with formaldehyde to become highly fluorescent and at the same time insoluble.

This secondary reaction might take place in several ways. It could simply be a polymerization of the primary condensation products, but this hypothesis does not seem able to explain the role of the proteins. Furthermore, no insoluble polymers or highly fluorescent products were obtained when dopamine hydrochloride (in substance) was exposed to formaldehyde vapour.

The fact that the isolated tetrahydroisoquinoline derivatives included in a protein film become at the same time highly fluorescent and insoluble may suggest that a binding to the proteins is responsible for the increased fluorescence. Although there is no definite proof it seems highly probable that these secondary amines become bound to the proteins under the conditions used in at least two ways: aminomethylol groups in the proteins may condense with

CH reactive groups in the amines or aminomethylol groups in the amines may condense with amide, guanidyl, phenol, imidazole, or indole groups in the proteins (cf. [11, 13, 14]). Such linkages are resistant to acid hydrolysis [14], and in accordance with this it was found that the fluorescent products were not extracted at such treatment. The finding that the products formed from amines in gelatin or gliadin have not the same intense fluorescence as those formed in serum albumin may be explained on this basis since the latter protein has a considerably higher content of potential binding groups. This hypothesis may also explain why secondary amines, such as adrenaline and epinine, develop only a weak fluorescence. The tetrahydroisoquinoline derivatives formed are tertiary amines and thus no aminomethylol groups can be formed.

It is difficult to reconcile how, in itself, the formation of such linkages with methylene bridges between the tetrahydroisoquinoline derivatives and the proteins could increase the fluorescence. It may be more probable that the bindings, for instance, only help to orient and pack the molecules so

*The finding that apparently two compounds were present may have several explanations. For instance, methylol groups may have been introduced at various positions in the tetrahydroisoquinolines (cf. [11]).

that they may interact, and that this is the real basis for the increased fluorescence. Several examples exist where an intense fluorescence develops first after aggregation of the molecules or after adsorption of the molecules to a solid (cf. [1,23]). This could explain why fluorescence is also increased (although to a much lesser extent) in a sucrose film.

It is possible, however, that another mechanism also operates. The 6,7-dihydroxy-tetrahydroisoquinolines—just as the 3,4-dihydroxy-phenylethylamines—are very easily oxidized by molecular oxygen, probably to quinones. By analogy to the well known spontaneous rearrangement of the non-fluorescent noradrenochrome to the highly fluorescent noradrenolutin (cf. [9]), it might be thought that the quinones of the tetrahydroisoquinolines also easily undergo a similar intramolecular rearrangement with the formation of double bonds in the heterocyclic ring. That oxidative reactions occur when the tetrahydroisoquinoline derivatives in a protein film are exposed to formaldehyde vapour seems likely since it was found that the spots turned yellow to brown with this treatment if they contained larger amounts of the derivatives.

This hypothesis provides a good explanation of the results obtained in the study of different—though structurally closely related—amines. The fact that only the amines with a 3,4-dihydroxy grouping give an intense fluorescence reaction is now explained. But it is also possible to explain why α-methyl-dopa (in contrast to dopa) and adrenaline and epinine (in contrast to the primary amines) give only a very weak reaction: the formation of double bonds in the heterocyclic ring is restricted in these cases. The deleterious effect of the substitution of the 3-OH by a methoxy group may be understood, but the finding that a 3-OH group is essential may also have another explanation; namely, that a cyclization does not easily occur under mild conditions if this group is absent [17].

Another histochemical method, based on the principle that certain catechol amines may be converted to highly fluorescent trihydroxyindoles, has recently been developed in these laboratories [3]. It shows good specificity and has a much higher sensitivity for adrenaline and to a lesser degree noradrenaline than hitherto known methods, but does not demonstrate dopamine. When compared with that method the formaldehyde fluorescence method certainly has the highest sensitivity for noradrenaline and very low sensitivity for adrenaline and to this adds its ability also to demonstrate dopamine. The two methods are thus complementary and it seems probable that their sensitivity is high enough to permit a study of the cellular localization of the catechol amines in the brain.

SUMMARY

The reaction under mild conditions between formaldehyde and phenylalanine and phenylethylamine derivatives has been studied. When the amines included in a dried protein film were exposed to formaldehyde vapour a very intense green to yellow fluorescence was given only by those that as well as being primary amines also have hydroxyl groups at the 3 and 4 positions (3,4-dihydroxyphenylalanine, dopamine, noradrenaline). The 3-OH group seems to be essential for the reaction. The catechol amines, which are secondary amines (adrenaline, epinine), gave a much weaker fluorescence that developed more slowly.

The results obtained on further examination of the reaction favour the view that the amines primarily condense with formaldehyde to 1,2,3,4-tetrahydroisoquinolines which are involved in a secondary reaction to become highly fluorescent and at the same time insoluble. This secondary reaction may be a binding to protein, an oxidation with the formation of double bonds in the heterocyclic ring, or both.

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