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DETECTION OF INDOLEAMINES AND CATECHOLAMINES ON CHROMATOGRAMS BY HEATING WITH PARAFORMALDEHYDE*

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SUMMARY

Primary and secondary indoleamines and catecholamines, heated with paraformaldehyde at 100–155°, produce highly fluorescent products. The procedure permits the detection of 1 ng of indoleamine on thin-layer plates and 10–25 ng on paper chromatograms. It is somewhat less sensitive for catecholamines.

INTRODUCTION

For the detection and estimation of amines and indole derivatives, treatment with formaldehyde to yield highly fluorescent products is a sensitive procedure. Thus, tryptamine and tryptophan in solution are determined quantitatively by condensation with formaldehyde in the presence of sulfuric acid, followed by oxidation, to yield β-carbolines[1,2]. Indole derivatives on paper and thin-layer chromatograms produce intense fluorescence when heated moderately after being sprayed with the Procházka formaldehyde–acid reagent[3–5]. FALCK and co-workers[6,7] have developed a procedure for detecting and distinguishing catecholamines and serotonin in thin sections of tissue. These workers report[6] that practically no fluorescence could be obtained when catecholamines were treated on paper with aqueous or gaseous formaldehyde, but the primary amines were found to yield intensely fluorescent products in formaldehyde gas, under very mild conditions, provided the reaction took place in the presence of dry protein.

On the other hand, heat alone (perhaps with aldehyde groups in the paper) yields fluorescent products from amino acids on paper chromatograms[8–10]. Hence, we have investigated the possibility of dispensing with both the protein of the FALCK

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procedure and the acid of the PROCHÁZKA spray, by heating catecholamines and indoleamines on paper or thin-layer chromatograms, with paraformaldehyde, at temperatures above 100° to insure the anhydrous conditions stipulated by FALCK.

We find that, indeed, such a procedure serves to visualize 10–25 ng of serotonin on paper, and as little as 1 ng on thin-layer chromatograms. With catecholamines, the method is somewhat less sensitive on paper chromatograms (25–50 ng), and gives negative results with nanogram quantities on thin-layer plates.

EXPERIMENTAL

Paper chromatography

The ascending technique was used, as previously described[11]. After development (with 2-propanol–15 *M* ammonia–water (8:1:1, by vol.) for serotonin; 70 % aqueous ethanol for the catecholamines) the chromatogram was dried in air, or in an oven at 50–60°.

Thin-layer chromatography

Micro plates. Microscope-slide plates were prepared by dipping pairs of slides in a chloroform–Silica Gel G slurry. Samples of serotonin and ω-N-methylserotonin were applied to the separated plates as 0.5 or 1-λ portions of solutions. The chromatograms were developed with 1-propanol–15 *M* ammonia (19:1, by vol.).

Macro plates. The catecholamine experiments were carried out with 20 × 20 cm Silica Gel G plates, prepared in the usual way. The developing solvent was 1-butanol–glacial acetic acid–saturated aqueous SO_2 (4:1:5, by vol.: upper layer).

Formaldehyde treatment

The dried chromatograms of either type were placed in suitable loosely-covered jars or tanks having a small amount of paraformaldehyde powder sprinkled on the bottom. These were heated in an oven—in earlier experiments at 110–130° for 1–2 h, in later experiments, at 140–155° for 30 min.

Observation and photography. Chromatograms were illuminated from above or below with either long or short-wavelength ultraviolet lamps ("Mineralight").

Sharp cutoff glass filters were usually used between the eye or camera and the chromatogram, to increase contrast between the background and the fluorescent spots. Corning No. CS3-69 was especially suitable for the 5-hydroxytryptamines, and No. CS3-70 for the catecholamines.

All photographs were taken with Eastman Panatomic-X film (ASA 32), at f/1.8.

Fluorescence spectra

Fluorescence spectra were obtained initially by means of a Beckman Fluorescence Attachment, used with either a Beckman DK-2A or Beckman DU spectrophotometer. Normally, a long-wavelength (F4T5/BL) ultraviolet lamp was used for excitation, so that it was suitable for use with either paper chromatogram sections or solutions in the regular Vycor sample tubes. A Schott UG-11 primary filter was used.

For later fluorescence and excitation spectra, an Aminco-Bowman Spectrophotofluorometer was employed.

Spectra of solutions. Spectra were taken on samples eluted from paper chromatograms by 12 to 24 hours' standing with 3-ml portions of solvent (glacial acetic acid).

Spectra of paper chromatogram spots. A 12 × 35-mm strip of chromatogram paper, bearing a fluorescent spot, was fastened with pressure-sensitive tape across the window of the exciting-lamp chamber of the Beckman fluorescence attachment. A like-sized strip of polished aluminum metal was placed in the paper-strip holder (Beckman No. 75120). Thus, the exciting light passed through the paper strip; and residual exciting light, together with fluorescent light from the spot, was reflected into the monochromator–detector system by the aluminum mirror.

For the Aminco-Bowman Spectrofluorometer, a 14 × 35-mm strip of paper bearing the fluorescent spot was inserted diagonally in the rectangular cuvette.

RESULTS AND DISCUSSION

Chromatography

With the 1-propanol–ammonia solvent, serotonin and ω-N-methylserotonin were separated cleanly on a silica gel layer during a solvent front advance of 6 cm. Interestingly, here the serotonin ran ahead of the N-methylserotonin, whereas the reverse is true with the 1-butanol–ammonia–water solvent on a silica gel layer, or with 2-propanol–ammonia–water on paper.

Formaldehyde treatment

Effects of temperature and time. More intense fluorescence was obtained with both types of amines when they were heated for 30 min at temperatures of 140–155°, than at 110–130° for as long as 2 h.

TABLE I

FLUORESCENCE[a] OF SPOTS ON PAPER, TREATED[b] WITH VARIOUS ALDEHYDES

	Serotonin	*Norepinephrine*	*DOPA*
Paraformaldehyde	+	+++	+++
Butyraldehyde	++	+++	+++
Piperonal	+	+	+
2,4-Dichlorobenzaldehyde	+	+	++
Cinnamaldehyde	+	+	+
p-Nitrobenzaldehyde	o	o	+
Crotonaldehyde	++	+++	++
Acetaldehyde	+	+++	+
Furfuraldehyde	+	+++	++
p-Tolualdehyde	+	++	++
p-Anisaldehyde	+	++	++
Salicylaldehyde	+	++	++
Phenylacetaldehyde	++	++	++
Benzaldehyde	+	++	+++
Paraldehyde	++	o	+++
Trioxane	+	+++	+
No aldehydes (heat only)	+	+	+

[a] Illuminated with short (254 mμ) U.V. light. o = no, + = low, ++ = medium, +++ = high fluorescence.

[b] Approximately 5 γ of compound (in spots on Whatman No. 1 paper), heated at 110° for 30 min in a loosely closed jar, with a small amount of aldehyde.

Effect of heat alone. As is indicated in Table I, heating for 30 min at 100–110°, in the absence of formaldehyde, produced fluorescent products with serotonin, norepinephrine and DOPA, on paper. Serotonin heated on silica gel at 140–155° also yielded strongly fluorescent spots. In all cases, however, the fluorescence was enhanced by the use of formaldehyde.

Observation and photography of spots. For both visual observation and photography, best results were obtained with ultraviolet illumination from behind the paper or glass plate. Short-wavelength (254 mμ) light gave best results with paper chromatograms, whereas long-wavelength (360 mμ) light was required to penetrate the thin-layer plates.

In all cases, contrast between the fluorescent spots and the background was greatly improved by the use of sharp-cut yellow filters (*e.g.*, Corning CS3-69) between the chromatogram and the eye or the camera.

Sensitivity of the procedure. Fig. 1, representing an experiment with 2–5 ng of serotonin and ω-N-methylserotonin on thin-layer plates, shows clearcut results with 3 ng and positive results with 2 ng of these substances. (Visual observation detects as little as 1 ng.)

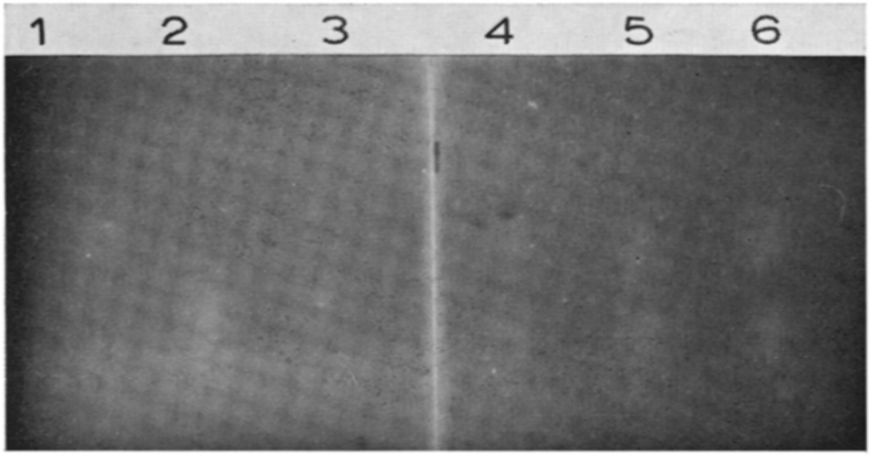

Fig. 1. Fluorescence of nanogram quantities of formaldehyde-treated 5-hydroxyindoles on microscope-slide thin-layer plates. 1 = 5 ng of serotonin; 2 = 5 ng of ω-N-methylserotonin; 3 = 2 ng of each; 4 = 3 ng of each; 5 = 4 ng of each; 6 = 5 ng of each. Illuminated from below with the 254 mμ light. Photographed through Corning CS3-69 filter. Photographic exposure 15 sec.

Fig. 2, representing a paper chromatogram of 10–500 ng of serotonin and epinephrine, indicates the sensitivity when the method is applied to paper chromatograms. (Visual observation here reveals a minimum of 10–25 ng.)

Spectra

Experimental results. For obtaining fluorescence spectra of spots on paper, with the Beckman fluorescence attachment, the method involving trans-illumination and reflection from a mirror at an angle proved to be considerably more effective than the procedure of placing the chromatogram strip itself at an angle in the paper strip holder. With the Aminco-Bowman instrument also, results were not good when the paper strip was placed at an angle in the cuvette.

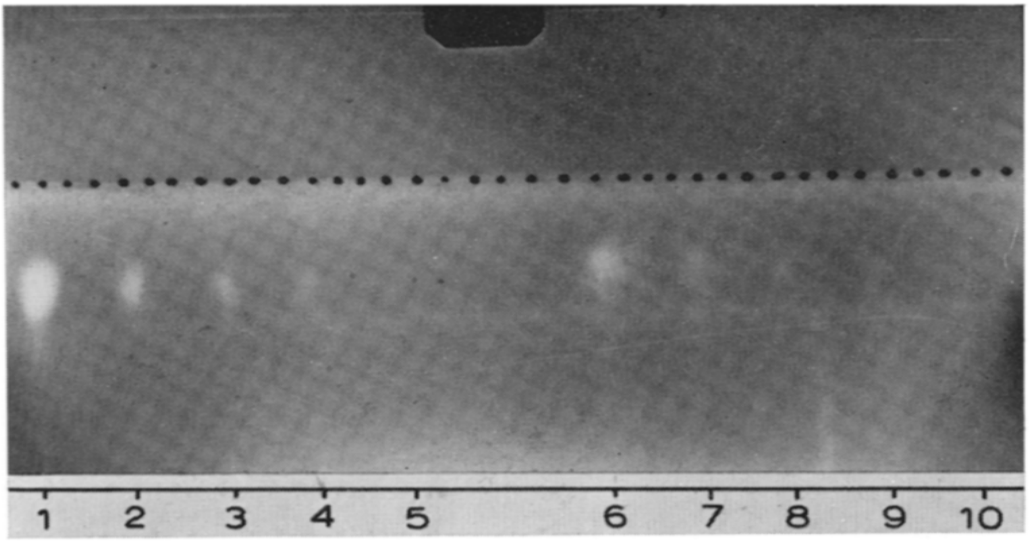

Fig. 2. Fluorescence of formaldehyde-treated serotonin (Nos. 1–5) and epinephrine (Nos. 6–10) on Whatman No. 1 paper. 1,6 = 500 ng; 2,7 = 100 ng; 3,8 = 50 ng; 4,9 = 25 ng; 5,10 = 10 ng. Illuminated from below with 254 mμ light. Photographed through Corning CS3-69 filter. Photographic exposure 1 min.

In all experiments directly involving paper strips, background fluorescence of the paper caused severe difficulty in obtaining spectra for small amounts of fluorescent products. Results were not materially improved by washing of the paper with developing solvent before application of samples for chromatography.

Less difficulty was encountered with solutions obtained by elution of spots from chromatograms, although the background fluorescence was still substantial.

On the other hand, elution (with glacial acetic acid) was far from complete, although somewhat better from thin-layer plates than from paper, and the fluorescent products from nor-epinephrine were extracted more easily than those from serotonin. The serotonin–formaldehyde products from heating at 140–155° were not eluted from paper to any extent.

Some characteristics of the fluorescence and excitation spectra of the products from serotonin and nor-epinephrine are given in Table II.

Interpretation of spectra. The emission spectra of the serotonin–formaldehyde products are consistent with the formation of a 3,4-dihydro-β-carboline (emission max. 530 mμ[6,12]) at 110–130° (on paper), and dehydrogenation of this at 140–155° (on paper or thin layers), to yield the β-carboline (emission max. 470 mμ[12]). That the initial reaction is the expected Mannich condensation[2] is further substantiated by the facts that the ω-N-methylserotonin gives results essentially identical with those from serotonin, whereas the tertiary amine, bufotenine, yields no appreciable fluorescent product.

The emission maxima at 475–490 mμ for the nor-epinephrine–formaldehyde product may indicate formation of the 3,4-dihydroisoquinoline (emission max. 480 mμ[6]).

Results with various aldehydes and various compounds. Table I indicates results obtained by treatment of serotonin, nor-epinephrine and DOPA, with a variety of aldehydes under the conditions used for the formaldehyde treatment. In general, the more complex aldehydes produced no better results than formaldehyde.

TABLE II

FLUORESCENCE AND EXCITATION SPECTRA

	Samples heated with formaldehyde		*Samples heated without formaldehyde*	
	Emission maxima ($m\mu$)	*Excitation maxima* ($m\mu$)	*Emission maxima* ($m\mu$)	*Excitation maxima* ($m\mu$)
Spectra of acetic acid extracts				
(*a*) *From thin-layer plates*				
Serotonin (140–155°)	480	320, 410	525 (weak)	410, 310 (shoulder)
Nor-epinephrine (140–155°)	430	360, 265 (shoulder)	490	320, 255 (wea 355 (shoulder
(*b*) *From paper chromatograms*				
Serotonin (110–130°)	500–520	———[a]	470	———[a]
Serotonin (140–155°)	———[b]		———[b]	
Nor-epinephrine (110–130°)	430–450	———[a]		
Nor-epinephrine (140–155°)	490 (weak)		490 (weak)	
Spectra of spots on paper				
Serotonin (110–130°)	500–525	———[a]		
Serotonin (140–155°)	475 (weak shoulder)[c]		475 (weak shoulder)[c]	260 (weak), 350 (shoulder) 400 (shoulder)
	560 (weak)	340, 375, 395, 420 (shoulder), 465		
Nor-epinephrine (110–130°)	475, 510	———[a]		

[a] Spectrum taken with constant-wavelength exciting lamp.
[b] Spectrum not different from blank (emission maximum at 425 $m\mu$); spots poorly eluted from pape
[c] Sample emission nearly swamped by background emission or reflection from paper.

Fig. 3 shows results obtained by formaldehyde treatment of approximately 5-γ quantities of sixteen compounds, on paper (without chromatographic development). Eight compounds (Nos. 2,4,5,6,8,9, 14 and 15) produced strong fluorescence of various colors. In all these compounds, a side-chain bearing a terminal primary or secondary amino group is attached to either an indole group or a benzene nucleus with a 3-hydroxyl substituent. These structural features permit reaction with formaldehyde and facilitate ring closure to the carbon adjacent to the side-chain. Compounds lacking these features show negative results (Nos. 1 and 3) or simply absorption of light (Nos. 10, 12, 16). Bufotenin (No. 7), with the terminal amino group fully alkylated, melatonin (No. 11), with the terminal amino group acetylated, and histamine (No. 13),

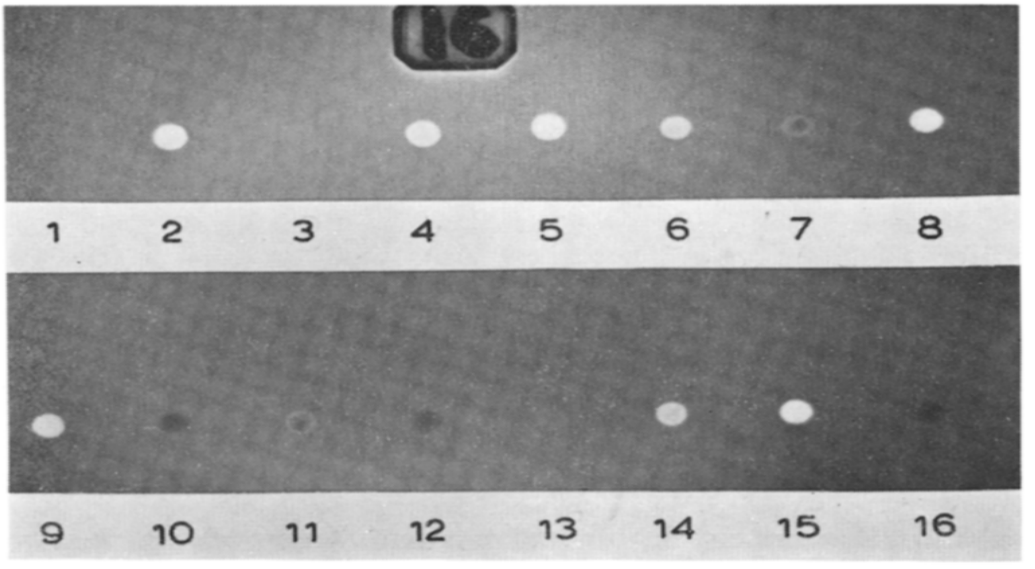

Fig. 3. Fluorescence of 1 γ quantities of various compounds, treated on paper with formaldehyde (110°, 30 min). Photographic exposure 45 sec. 1 = DL-Phenylalanine; 2 = L-tryptophan; 3 = DL-tyrosine; 4 = L-epinephrine; 5 = DL-norepinephrine; 6 = serotonin (creatinine sulfate); 7 = bufotenin (monooxylate hydrate); 8 = DL-DOPA; 9 = dopamine (HCl); 10 = DL-norsynephrine (HCl); 11 = melatonin; 12 = DL-synephrine; 13 = histamine (2 HCl); 14 = 3-indoleacetic acid; 15 = tryptamine (HCl); 16 = DL-metanephrine (HCl).

with the imidazole nucleus, produced only weak fluorescence. These structural requirements are somewhat similar to those found by MAICKEL AND MILLER[13] for the wet reaction of indole derivatives with *o*-phthalaldehyde.

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