SEPARATION AND DETECTION OF BIOGENIC AMINES BY THIN-LAYER CHROMATOGRAPHY

MICRO-ANALYSIS OF TISSUE AMINES AND OF ENZYMES INVOLVED IN THEIR METABOLISM*

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SUMMARY

Simple and sensitive methods for the separation and detection of biogenic amines by thin-layer chromatography have been developed. Nanogram amounts of these compounds can be detected by exposing the thin layer to an *o*-phthalaldehyde spray or paraformaldehyde gas, thereby converting the amines into highly fluorescent derivatives. The methods have been successfully applied to the micro-analysis of tissue amines and adapted for microradiometric enzyme assays with a high degree of sensitivity and specificity.

INTRODUCTION

Current interest in the function and metabolism of biogenic amines has been stimulated by the recent development of simple and sensitive methods for the chemical and histochemical detection of these compounds. One of the difficulties encountered in investigations on the cellular localization and turn-over of biogenic amines has been inadequate separation and discrimination of the amines themselves and of various precursors and metabolites. During the last few years thin-layer chromatography has become an indispensable analytical tool in most fields of biochemistry, and methods have also been described for the separation and detection of biogenic amines, permitting the rapid isolation and identification of minute amounts of these compounds[1–24]. The present report gives additional information on solvent systems and detection reagents, which have been found useful in the study of imidazoles, indoles and phenols. The sensitivity of these techniques is high; nanogram amounts of histamine and biogenic monoamines as well as some of their precursors and metabolites can be detected. The methods have been successfully applied to the analysis of tissue amines and to the development of simple radiometric assays for several enzymes involved in amine metabolism.

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EXPERIMENTAL

Glass plates (5 × 20 cm, 20 × 20 cm or microscope slides, 2.5 × 7.5 cm) were coated with thin layer (250 μ) of silica gel (Kieselgel G and Kieselgel H, Merck, Darmstadt) or cellulose powder (MN 300 HR, Macherey, Nagel and Co., Düren). The layer was applied as a slurry consisting of 30 g of silica gel suspended in 70 ml redistilled water or 15 g of cellulose powder in 90 ml water. The chromatoplates were dried in an oven at 100° for 1–2 h and stored in a desiccator until used. With non-aqueous solvent systems the plates were re-activated at 100° for 30 min before use. Compounds to be studied were all obtained from commercial sources with the exception of N-acetyl histamine (a generous gift from Dr. R. W. SCHAYER). Solutions of the compounds (usually 1 mg/ml) were applied to the chromatoplate with a capillary approximately 2 cm from the base. The time of development varied with the solvent system used but never exceeded 3 h for a height of 12 cm. After development, the plates were dried in an oven at 50–80°.

Detection reagents

(1) *o-Phthalaldehyde (OPT)*[17]. The pH of the thin-layer material affects the reaction with OPT. After drying, the plates were first sprayed with a solution of glacial acetic acid in acetone (1–2 drops per 10 ml) or with a solution of sodium hydroxide in methanol (1–2 drops of 10 *N* sodium hydroxide per 10 ml). After a few minutes in the oven, the plates were then sprayed with a 0.02 % solution of OPT in acetone and viewed over U.V.-light.

(2) *Paraformaldehyde gas*[25–27]. When thoroughly dried, the plates were exposed to the fumes of paraformaldehyde (70 % relative humidity) at 80° for 1 h. After this treatment they were viewed over the U.V.-light and all fluorescent spots were outlined.

(3) *Other fluorescent detection reagents*. In a series of experiments other fluorescence detection reagents were tested to compare the sensitivity of various techniques: for catecholamines iodine fumes and ammonia as described by ANGELAKOS AND KING[28], ethylenediamine according to the method of SCHNEIDER AND GILLIS[11], and finally ninhydrin and acetic acid as described by JEPSON AND STEVENS for the detection of 5-hydroxyindoles[29]. In some experiments using tissue extracts the conventional ninhydrin reagent[30] was applied after reaction with OPT and/or formaldehyde. For visualizing certain acid metabolites of biogenic amines conventional staining techniques (*e.g.* Pauly's reagent for imidazoles and phenols[30], Gibb's reagent for phenols and catechols[30], and Ehrlich's reagent for indoles[30]) had to be used for lack of more sensitive detection methods. In general, aqueous spray-reagents are unsatisfactory because of their tendency to cause diffusion of the spots.

Extraction of tissue amines

In a series of studies on the amine content of the rat pineal the following procedure was adopted: Adult female rats (170–200 g) were killed by decapitation at 10 a.m. and the pineals were removed. One or two pineals were placed on a microscope slide together with a few drops of acidified acetone (95 % acetone + 5 % 0.1 *N* HCl) or ethanol (95 % ethanol + 5 % 0.1 *N* HCl) and squashed thoroughly with a glass rod. The debris was washed several times with small amounts of the organic solvent and the extract was spotted onto the plate.

For analysis of mast cell amines, peritoneal saline washings[31] of the rat and the hamster were used. 5 ml of 0.9 % saline, containing small amounts of heparin, were injected into the peritoneal cavity of a decapitated animal. The abdominal wall was rubbed and squeezed gently for approximately 5 min. After this treatment, the abdominal wall was cut open and the saline was collected and centrifuged at low speed for a few minutes at room temperature. The sediment obtained from one animal was homogenized in 0.5–1 ml of acidified acetone or ethanol and recentrifuged. The volume of the supernatant was reduced by evaporation before separation by thin-layer chromatography.

RESULTS AND COMMENTS

Detection reagents

OPT was found to react with a number of compounds, forming fluorescent condensation products with both imidazoles and indoles. However, optimal fluorescence intensity was obtained for some compounds after pretreatment with acetic acid, for others after spraying with sodium hydroxide (see Table I). OPT is known to

TABLE I

FLUORESCENCE CHARACTERISTICS OF IMIDAZOLES AND OTHER COMPOUNDS ON THIN LAYERS AND IN SOLUTION AFTER OPT-TREATMENT

The fluorescence properties of all compounds tested were recorded after separation in chloroform–methanol–ammonia (12 : 7 : 1). Color characteristics are given only for those compounds that show fluorescence at a concn. of 1 μg.

	Thin layer (color)		*Test tube* (E_{max}/F_{max})[e]	
	Acid[a]	*Alkaline*[b]	*Acid*[c]	*Alkaline*[d]
Histidine	Yellow	Yellow		350/450
1-Methylhistidine	Strong yellow	Yellow		350/450
N-Acetylhistidine	Orange yellow	Light blue		350/450
Carnosine	Faint blue	Light blue		
Anserine	Faint blue	Faint yellow		
Histamine	Blue	Dull yellow	360/460	
1-Methylhistamine	Light blue	Yellow		
N-Acetylhistamine	Orange yellow	Light blue		
Imidazoleacetic acid	Yellow	Blue		
Imidazolelactic acid	Orange yellow	Blue		
1-Methylimidazoleacetic acid	No fluorescence			
Spermine	No fluorescence			
Spermidine	Faint blue		350/400	
Arginine	No fluorescence			350/440
Agmatine	No fluorescence			350/440
5-Hydroxytryptophan	Orange red	Dull red	see Ref. 39	
5-Hydroxytryptamine (5 HT)	Yellow	Yellow	see Ref. 39	
Melatonin	Light blue		see Ref. 39	
5-Hydroxyindoleacetic acid	No fluorescence			
Tryptophan	No fluorescence			
Tryptamine	No fluorescence			

[a] Before OPT-treatment the thin layer was sprayed with acidified acetone (see EXPERIMENTAL).
[b] Before OPT-treatment the thin layer was sprayed with alkaline ethanol (see EXPERIMENTAL).
[c] Reaction and assay conditions as described for histamine by SHORE, BURKHALTER AND COHN[33].
[d] Reaction and assay conditions as described for agmatine by COHN AND SHORE[35].
[e] Excitation and fluorescence maxima are given as uncorrected instrumental values.

react with all compounds having an amino group, but fluorophores are formed only with a limited number of substances. The formation of fluorescent OPT conjugates under test tube conditions has been studied extensively[32–39]. In some instances the conditions necessary for optimal fluorescence on thin layer are quite different from those in solution (Table I): Some compounds, such as carnosine, anserine and 1-methylhistamine, which do not form fluorophores with OPT under test tube conditions, reacted with this reagent on thin layer, emitting a weak to fairly strong fluorescence. On the other hand, arginine and agmatine, which form highly fluorescent condensation products with OPT in the test tube[35] gave no fluorescence with this reagent on thin layer. 5-Hydroxyindoles in solution form fluorophores with OPT only at high temperatures and under extremely acid conditions[39]. On thin layers these compounds reacted readily, emitting a characteristic reddish yellow light. Histamine fluoresced strongly after exposure to OPT. Acid conditions proved to be optimal for the histamine fluorescence and pretreatment with acetic acid was routinely used[40]. The fluorescence intensity of the histidine–OPT conjugate was somewhat favored by alkaline pH. Under acid conditions the fluorescence of the histidine–OPT conjugate was considerably less intense than that exhibited by the histamine fluorophore. The sensitivity of the OPT-spray for the detection of histamine was very high; nanogram amounts could be detected. However, the observation that several compounds, apart from histamine, are capable of forming fluorophores with OPT emphasizes the importance of concomitant chemical analysis in conjunction with the use of OPT as a histochemical detection reagent for histamine[41–43]. OPT reacts readily also with ammonia, forming a dark bluish (non-fluorescent) pigment, and great care should be taken to evaporate all ammonia originating from the solvent system before exposure to OPT and to reduce the amount of inorganic ammonium salts in the tissue extract before applying it to the chromatoplate.

Exposure to paraformaldehyde gas turned out to be a very efficient way to demonstrate the presence of tryptamines and catecholamines on both silica gel and cellulose thin layers. The sensitivity of this detection reagent was much reduced on polyamide layers (MN-Polyamid, Macherey, Nagel & Co., Düren, Germany). 5-Hydroxyindoles emitted a yellow light when viewed over the U.V.-light, the color of the catechols varied from yellow to bluish green. Histamine, which is known to react with formaldehyde[44], could not be visualized by this treatment. Exposure to paraformaldehyde permitted the demonstration of nanogram amounts of tryptamines and catecholamines. OPT and paraformaldehyde could be used consecutively. There was no evidence that pretreatment with OPT interfered with the subsequent reaction with paraformaldehyde gas. However, if the thin layer was exposed first to paraformaldehyde, treatment with OPT failed to induce fluorescence of any of the compounds tested.

The OPT-induced histamine fluorescence was markedly quenched by exposure to paraformaldehyde. This is in agreement with earlier observations on the use of OPT for the histochemical detection of histamine[42]. From test tube experiments, however, it appears that when the condensation of OPT and histamine has gone to completion, the resulting fluorophor is resistant to the addition of formaldehyde. Only when formalin is added before the final acidification step in the usual procedure (see SHORE, BURKHALTER AND COHN[33]) is the fluorescence affected, probably through a transaldimination reaction between the Schiff base of histamine and OPT on the

TABLE II

FLUORESCENCE CHARACTERISTICS OF INDOLES AND PHENOLS ON SILICA GEL THIN LAYERS AFTER TREATMENT WITH VARIOUS DETECTION REAGENTS

Compound	*Paraformaldehyde*		*Ethylenediamine*	
	Fluorescence color	*Minimum detectable amount (μg)*	*Fluorescence color*	*Minimum detectable amount (μg)*
Tyrosine	Dull yellow	3.0		
Tyramine	Dull yellow	0.3	Bluish green	1.0
3,4-Dihydroxyphenylalanine (DOPA)	Greenish yellow	0.03	Greenish yellow	0.03
Dopamine	Yellow	0.01	Yellow	0.01
Norepinephrine	Yellow	0.01	Greenish yellow	0.01
Epinephrine	Yellow	0.3	Yellow	0.03
3-Methoxytyramine	Bluish green	0.01	Yellow	10.0
Normetanephrine	Green	0.03	Yellow	3.0
Metanephrine	Greenish yellow	0.3	Yellow	3.0
Dopacetic acid	Brownish yellow	3.0	Yellow	3.0
Dihydroxymandelic acid	Brownish yellow	10.0	Brownish yellow	10.0
Homovanillic acid	Greenish yellow	10.0	Brownish yellow	10.0
3-Methoxy-4-hydroxy-mandelic acid	Greenish yellow	10.0	Brownish yellow	10.0
Tryptophan	Bluish green	0.1	No fluorescence	
Tryptamine	Greenish yellow	0.01	No fluorescence	
Indoleacetic acid	Greenish yellow	10.0	No fluorescence	
5-Hydroxytryptophan	Yellow	0.003	Greenish yellow	1.0
5HT	Yellow	0.003	Greenish yellow	0.3
5-Methoxytryptamine	Orange yellow	0.003	Bluish green	10.0
Melatonin	Greenish yellow	3.0	Bluish green	3.0
5-Hydroxyindoleacetic acid	Yellow	3.0	Bluish green	3.0

one hand, and formaldehyde on the other. It has been suggested that the final stabilizing step in the formation of the histamine–OPT fluorophor involves a cyclization of the initial Schiff base[33,36]. This step apparently makes the histamine–OPT conjugate non-reactive with formaldehyde. It was also observed, however, that the histamine–OPT fluorophor is heat-labile, and under aqueous conditions a few minutes at 100° abolishes all fluorescence. Consequently, the paraformaldehyde-induced quenching of the histamine–OPT fluorescence may be attributed to the heating involved in the process rather than to the exposure to paraformaldehyde. This conclusion is supported by the observation that spraying the thin layers with a formalin–ethanol mixture did not affect the fluorescence of the OPT–histamine conjugate and that heating alone (60 min at 100°) was found to reduce the fluorescence intensity.

The pH of the silica gel was critical for the formaldehyde condensation reaction and/or the fluorescence intensity. This was confirmed in a series of experiments in which the plates were sprayed with 0.1 *M* phosphate buffer of various pH before exposure to paraformaldehyde. Optimal fluorescence intensity was observed at neutral and slightly alkaline pH. Tryptophan and tryptamine, however, were exceptional in that the fluorescence intensity was the same also under mildly acid conditions. The relative humidity in the reaction tank was also important and 70 % was found to give optimal fluorescence intensity. The humidity, however, was not critical

as long as it exceeded 70 % and identical results were obtained by spraying the thin layer with a mixture of formalin and ethanol (1:1).

The nature of the fluorescent reaction products was studied in a series of investigations: The silica gel material containing the condensation product of dopamine and paraformaldehyde gas, was scraped off the plate and extracted with 0.1 *N* hydrochloric acid in a mechanical shaker for 4 h. The acid extract could be stored in a deep freeze (—30°) for several days. Fluorometric analysis showed maximal excitation at 400 mμ and maximal fluorescence at 480 mμ (instrumental values) which is characteristic of some dihydroisoquinolines[45]. Catecholamines and 5-hydroxytryptamine (5 HT) enclosed in a dried protein layer are known to react with gaseous formaldehyde to form fluorescent condensation products. This reaction forms the basis for the histochemical method of FALCK AND HILLARP which permits the visualization of monoamines at the cellular level[46–49]. The process is a two-step reaction: The first step involves a PICTET–SPENGLER reaction[50] resulting in the formation of a tetrahydroisoquinoline for catecholamines and a tetrahydro-β-carboline for 5HT; these derivatives are non-fluorescent. In the next step the condensates are dehydrogenated to strongly fluorescent dihydroisoquinolines and β-carbolines respectively. The presence of protein promotes the dehydrogenation catalytically[25,45,51]. The reaction is particularly fast if the monoamines are in a solid state, *i.e.* in tissue sections or in protein films. The nature of the catalytic property of protein is unknown. Some amino acids and peptides (*e.g.* glycine and glycylglycine) are also capable of promoting the reactions[51–54]. BELL AND SOMERVILLE[27] observed a strong fluorescence of catecholamines and tryptamines on paper after exposure to paraformaldehyde gas but only after pretreating the paper with glycine. There was no evidence in our experiments that spraying the thin layer with a solution of 1 % albumin or 5 % glycine promoted the reaction.

Reduction with borohydride causes a reversal of the dehydrogenation and this has been used as a specificity test in the histochemistry of monoamines. The autofluorescence of the tissue section is not affected whereas the fluorescence of the monoamine fluorophores is abolished[45,54]. On thin layer the paraformaldehyde-induced fluorescence could be quenched by spraying with a 1 % solution of potassium borohydride. The fluorescence could be induced again by renewed and prolonged exposure to paraformaldehyde gas.

Other detection reagents used included ethylenediamine[11], iodine and ammonia[28] and ninhydrin and acetic acid[29]. The ethylenediamine reagent gave intense fluorescence with several catechol derivatives and some 5-hydroxyindoles (Table II). The sensitivity of this detection reagent was lower than that of paraformaldehyde gas. The ethylenediamine reagent was found useful in conjunction with exposure to paraformaldehyde. However, the reactivity with ethylenediamine was decreased after condensation with formaldehyde and *vice versa*, and consequently the reagents cannot be used consecutively. The ninhydrin–acetic acid reagent gave fluorescence only with indolylalkylamines and their precursors. The sensitivity of this reagent was somewhat lower than that of paraformaldehyde. Exposure to iodine fumes for 5–10 min and subsequent exposure to ammonia for 10–30 sec gave fluorescence with all catecholamines; the technique was capricious, however, and the sensitivity was inferior to that of the other detection reagents.

On silica gel thin layers, but not on cellulose or polyamide layers, catechol

derivatives and, to some extent also 5-hydroxyindoles, were found to produce intensely colored spots (bluish-grey for catechols and reddish-brown for 5-hydroxyindoles) merely after heating in an oven at above 100° for 10 min. The color of the spot deepened markedly upon standing, probably as a consequence of continued oxidation. As a detection reagent this was not satisfactory since microgram amounts of the compounds were required. However, after the oxidation had taken place the compounds were non-reactive with both OPT and formaldehyde, which emphasizes the need to avoid excessive heating or other procedures which may facilitate oxidative pigment (melanin) production.

Solvent systems

Some of the solvent systems tested are listed in Table III. Silica Gel G and H gave approximately the same R_F values. R_F values were found to vary more on thin layers than on paper although the relative migration rate was fairly constant.

TABLE III

SOLVENT SYSTEMS

A = *n*-Butanol–acetic acid–water (15:3:5)
B = *n*-Butanol–pyridine–acetic acid–water (15:2:3:5)
C = *n*-Butanol–acetic acid (1:1)
D = Ethanol–diethyl ether–ammonia–water (10:10:1:4)
E = 8% Sodium chloride in water
F = Chloroform–methanol–ammonia (12:7:1)
G = Ethyl acetate–acetic acid–water (15:15:10)
H = Ethyl acetate–*n*-propanol–10% ammonia (4:3:1)

Catechol derivatives could be separated in several solvents (Table IV) but some difficulties were encountered in obtaining a complete separation of the various catecholamines. These compounds could be completely separated by two-dimensional chromatography on a cellulose thin layer (Fig. 1, see also refs. 11, 18 and 20). Catechols cannot be run in highly alkaline solvents because of rapid breakdown. Their

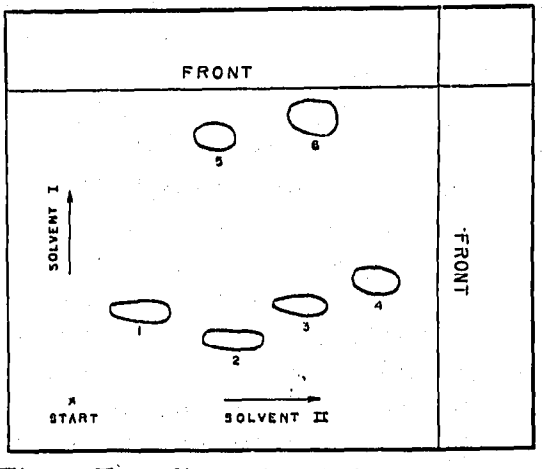

Fig. 1. Two dimensional chromatography (cellulose thin layer) of a mixture of 5 μg each of: (1) DOPA, (2) norepinephrine, (3) dopamine, (4) 3-methoxytyramine, (5) 3,4-dihydroxyphenylacetic acid, (6) homovanillic acid. Solvents: (I) *n*-butanol saturated with 0.1 *N* HCl; (II) isopropanol–5 *N* NH_4OH–H_2O (8:1:1).

O-methylated derivatives, however, had a much higher degree of stability (Table IV). Acid metabolites of catechol and indolylalkylamines were well separated from their precursors on silica gel in *n*-butanol–glacial acetic acid (1:1) (Tables IV and V). Acid imidazoles could be isolated from histamine in ethyl acetate–glacial acetic acid–water (15:15:10). Chloroform–methanol–ammonia (12:7:1) proved to be a good solvent for the separation of imidazoles and indoles on silica gel (Tables V and VI). Histamine and methylhistamine are traditionally difficult to separate; with the chloroform–methanol–ammonia solvent the separation of these two compounds was excellent.

TABLE IV

R_F VALUES OF CATECHOLAMINES, PRECURSORS AND METABOLITES (SILICA GEL)

R_F values refer to the front line of the spots.

	Solvent system[a]						
	A	*B*	*C*	*D*	*E*	*F*	*G*
DOPA	0.29	0.35	0.46				0.82
3-Hydroxytyramine, dopamine	0.45	0.61	0.35				0.81
Norepinephrine	0.44	0.59	0.36				0.79
Epinephrine	0.21	0.53	0.19				0.54
3-Methoxytyramine	0.52	0.62	0.22	0.27	0.71	0.84	0.88
Normetanephrine	0.56	0.60	0.24	0.41	0.90	0.91	0.87
Metanephrine	0.44	0.52	0.16	0.18	0.75	0.80	0.75
Dopacetic acid	0.98	0.84	0.90				0.99
Dihydroxymandelic acid	0.72	0.46	0.77				0.95
Homovanillic acid	0.98	0.85	0.96				0.99
3-Methoxy-4-hydroxymandelic acid	0.73	0.51	0.70				0.93
Tyrosine							0.93
Tyramine	0.63	0.70	0.20				0.90
p-Hydroxyphenylacetic acid	0.98	0.90	0.98		0.88		0.99

[a] For key, see Table III.

TABLE V

R_F VALUES OF INDOLEAMINES, PRECURSORS AND METABOLITES (SILICA GEL)

R_F values refer to the front line of the spot.

	Solvent system[a]							
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Tryptophan	0.55	0.56		0.51	0.75	0.65	0.80	0.16
Tryptamine	0.66	0.75	0.13	0.48	0.75	0.87	0.79	0.70
Indoleacetic acid	0.98	0.90	0.90		0.74		0.99	
5-Hydroxytryptophan	0.50	0.43		0.47	0.90	0.13	0.74	0.14
5HT	0.59	0.64	0.18	0.30	0.90	0.65	0.73	0.57
5-Methoxytryptamine	0.56	0.68			0.63		0.81	0.65
Melatonin	0.90	0.88			0.47		0.92	0.82
5-Hydroxyindoleacetic acid	0.98	0.85	0.86		0.85	0.30	0.98	0.19
Methionine[b]	0.47	0.52			0.78		0.75	
S-Adenosylmethionine[b]	0.08	0.11			0.68		0.12	

[a] For key, see Table III.

[b] Methionine and S-adenosylmethionine were included in their capacity of methyl donors for the N- and O-methylation of biogenic amines.

TABLE VI

R_F VALUES OF HISTAMINE, PRECURSORS AND METABOLITES (SILICA GEL)

Other biogenic amino compounds, besides imidazoles, are included in this Table because of their known capacity to form fluorophores with OPT in the test tube or on the thin layer. R_F values refer to the front line of the spot.

	Solvent system[a]			
	E	*F*	*G*	*H*
Histidine	0.76	0.06	0.30	0.07
1-Methylhistidine	0.72		0.36	0.06
N-Acetylhistidine	0.71	0.16	0.63	0.08
Histamine	0.68	0.40	0.18	0.30
1-Methylhistamine	0.42	0.65	0.16	0.23
N-Acetylhistamine	0.55	0.84	0.48	0.56
Carnosine	0.77	0.04	0.31	0
Anserine	0.61	0.07	0.17	0
Imidazoleacetic acid	0.77	0.15	0.80	0.32
Imidazolelactic acid	0.79	0.10	0.54	0.09
1-Methylimidazoleacetic acid				0.34
Spermine	0.79	0	0.10	0
Spermidine	0.83	0	0.16	0
Arginine	0.83	0		0
Agmatine	0.75	0		0

[a] For key, see Table III.

Biological application

The sensitivity and efficiency of the detection reagents used in this investigation was illustrated by the results of analysis of the amine content of the rat pineal and of the mast cells of the rat and hamster. The rat pineal is a rich source of 5HT[55, 56] and the acetone extract (see EXPERIMENTAL) of one single pineal had enough 5HT to permit its detection on the thin-layer chromatogram after exposure to paraformaldehyde gas. There was no evidence of any other 5-hydroxyindole or tryptamine derivative, and there were no ninhydrin positive spots using the conventional ninhydrin reagent. Several OPT-reactive compounds of unknown nature were observed. The identity of these substances will be discussed in a separate publication (AURES, HÅKANSON AND OWMAN, to be published).

The cell suspension obtained by washing the peritoneal cavity of rats and hamsters with saline (see EXPERIMENTAL) is known to contain large numbers of mast cells[31]. This was confirmed by staining smears of the cell suspension according to the method of WRIGHT[57] and of BLOOM AND KELLY[58]. For purposes of chromatography the cells were spun down and the sediment was extracted with a small volume (usually 0.5 ml) of acidified acetone or ethanol (see EXPERIMENTAL). Aliquots of this extract (usually 0.1 ml) were reduced in volume by a stream of cold nitrogen and spotted onto a silica gel thin layer which was developed in chloroform–methanol–ammonia or in 8 % sodium chloride. Histamine could be demonstrated in high concentrations in peritoneal saline washings of both rat and hamster. 5HT on the other hand was found only in extracts from the rat mast cells. Acidified acetone was found to be very efficient in extracting indoles and catechols; ethanol was superior in extracting histamine.

Some of the solvent systems described have been used successfully for radio-

metric enzyme assays[59–61]. Others can easily be adapted for that purpose as was illustrated by the development of a simple and sensitive technique for the assay of DOPA decarboxylase and 5-hydroxytryptophan decarboxylase (see Fig. 2): Small amounts of an extract of rat pineal, which is known to contain aromatic L-amino acid decarboxylase[60,62,63] were incubated in a glass capillary with ^{14}C-labeled DL-DOPA (2.5 mC/mmole; Nuclear Chicago) or ^{14}C-labeled DL-5-hydroxytryptophan (6.8 mC/mmole; Calbiochem). After incubation the mixture was applied onto a silica gel thin layer, which was developed in *n*-butanol–pyridine–acetic acid–water (15:2:3:5), dried in an oven and exposed to paraformaldehyde gas. The dopamine or 5HT spot was scraped off and the silica gel material was transferred to a counting vial with 0.1 ml water added as eluant. The radioactivity was measured by liquid scintillation counting after the addition of 15 ml dioxane–phosphor mixture[64]. The details of two such experiments are given in Figs. 2 and 3.

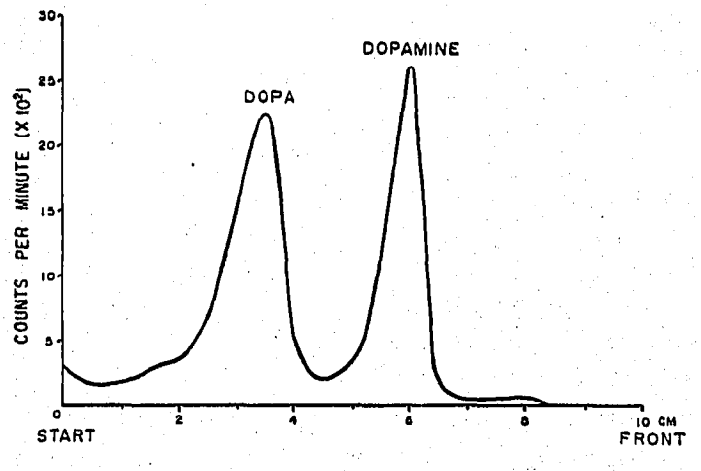

Fig. 2. Radioactivity profile of a silica gel chromatogram: 3 μl of a phosphate buffer (0.1 *M*) homogenate of rat pineal, corresponding to 100 μg pineal tissue, was incubated with DL-3,4-dihydroxyphenylalanine-2-^{14}C (400 μg/ml) and pyridoxal-5′-phosphate (1 μg/ml) at pH 7.0 in a total volume of 4 μl in a glass capillary for 1 h at 40°. The incubation mixture was then spotted onto a thin layer. After development in *n*-butanol–pyridine–acetic acid–water (15:2:3:5) for about 2 h, fractions of 4 mm of the silica gel were collected and counted by liquid scintillation counting. The positions of DOPA and dopamine were detected by paraformaldehyde treatment. Heated enzyme blanks showed only traces of radioactivity in the area corresponding to the location of dopamine.

A simple, rapid and sensitive method for the assay of monoamine oxidase was also developed: One rat pineal was homogenized in 50 μl 0.1 *M* phosphate buffer, pH 6.8. The homogenate was incubated with 2 μg ^{14}C-5HT (free base) (39.7 mC/mmole; Radiochemical Centre, Amersham) in a total volume of 60 μl, under oxygen at 37°. Incubation was stopped after 1 h by the addition of 1 ml ethyl acetate–acetic acid (95:5). Precipitated proteins were spun down and 0.5 ml of the clear supernatant was evaporated to dryness under reduced pressure. The dry material was extracted with a drop of the ethyl acetate–acetic acid mixture and spotted onto a silica gel thin-layer plate (microscope slide). The reaction product (5-hydroxyindoleacetic acid) was separated from residual 5HT in less than 15 min with *n*-butanol–acetic acid (1:1). The thin-layer material containing 5-hydroxyindoleacetic acid was scraped off and transferred to a counting vial containing 0.1 ml water

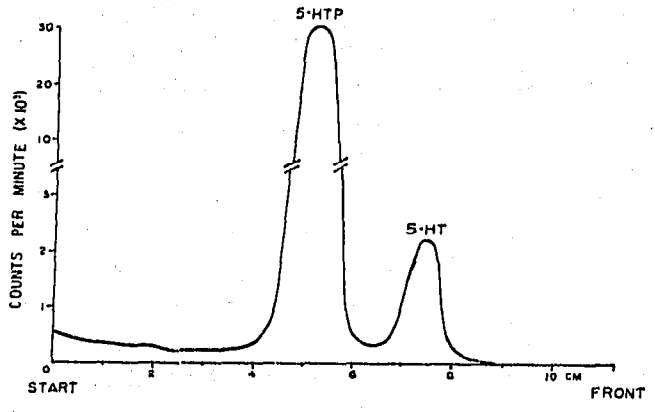

Fig. 3. Radioactivity profile of a silica gel chromatogram: 3 μl of a phosphate buffer (0.1 *M*) homogenate of rat pineal, corresponding to 20 μg pineal tissue, was incubated with DL-5-hydroxytryptophan-3-^{14}C (400 μg/ml) and pyridoxal-5'-phosphate (1 μg/ml) at pH 7.5 in a total volume of 4 μl in a glass capillary for 20 min at 40°. The incubation mixture was then spotted onto a thin layer. After development in *n*-butanol–pyridine–acetic acid–water (15:2:3:5) for about 2 h, fractions of about 4 mm of the silica gel were collected and counted by liquid scintillation counting. The positions of 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT) were detected by paraformaldehyde treatment. Heated enzyme blanks showed only traces of radioactivity in the area corresponding to the location of 5-hydroxytryptamine.

as eluant; the radioactivity was recorded by liquid scintillation counting. One such experiment is shown in Fig. 4.

The corpus striatum has a high dopamine content[65]. The uptake and metabolism of dopamine in this tissue was studied by incubating slices of rat caudate nucleus with ^{14}C-dopamine (22 mC/mmole, Nuclear, Chicago) in a Krebs medium (pH 7.4) at 37° for 30 min in an atmosphere of 95 % O_2 and 5 % CO_2. The slices were removed from the incubation medium, rinsed and homogenized in acidified methanol. The protein precipitate was spun down and an aliquot of the supernatant was spotted on a cellulose thin layer together with reference compounds. After development in the two-dimensional solvent system (Fig. 1) the compounds were visualized by exposure to paraformaldehyde gas and subsequent scanning in U.V. light. The spots

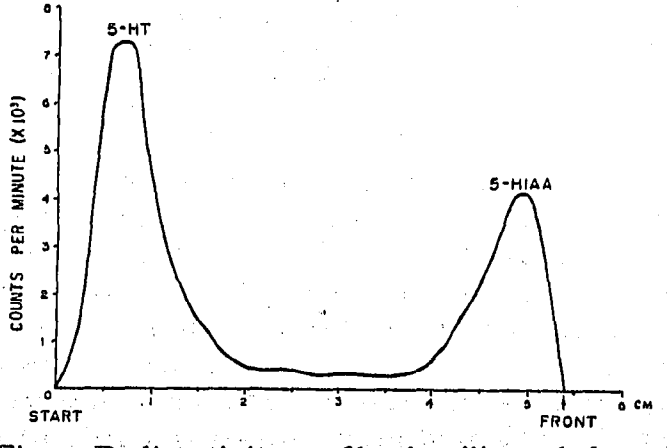

Fig. 4. Radioactivity profile of a silica gel chromatogram: 50 μl of a rat pineal homogenate (0.1 *M* phosphate buffer, pH 6.8) corresponding to 1 mg pineal tissue, was incubated with ^{14}C-5-hydroxytryptamine-3'-^{14}C (33 μg/ml) in a total volume of 60 μl for 1 h under oxygen. An aliquot of the incubation mixture was extracted with ethyl acetate–acetic acid (95:5) (see text) and spotted onto a thin layer. The ethyl acetate–acetic acid mixture extracts 5-hydroxyindoleacetic acid (5-HIAA) quantitatively but only a fraction of 5-hydroxytryptamine (5-HT).

TABLE VII

RADIOACTIVE METABOLITES IN RAT BRAIN CAUDATE NUCLEUS SLICES INCUBATED WITH ^{14}C-DOPAMINE

Compound	*Radioactivity (c.p.m.)*[a]
Dopamine	917
Norepinephrine	14
3-Methoxytyramine	3
Dopacetic acid	289
Homovanillic acid	19

[a] Approx. 50 mg rat caudate nucleus was incubated with 0.1 μC of dopamine-2-^{14}C in 2 ml of a 1% D-glucose fortified Krebs–Ringer bicarbonate solution[66] (pH 7.4). The tissue was extracted with acidified methanol, and aliquots, containing 1600 c.p.m., were used for the analysis. The recovery of radioactivity from known amounts of ^{14}C-dopamine and ^{14}C-norepinephrine subjected to the same treatment (two-dimensional separation on cellulose thin layer) was 70–80%.

were scraped off and the radioactivity was determined by liquid scintillation counting (Table VII). Dopacetic acid was found to be the major dopamine metabolite in the rat caudate nucleus. The present results confirm earlier observations by GOLDSTEIN *et al.*[67].

Further details of these and other methods for microradiometric enzyme assays will be presented elsewhere.

DISCUSSION

On thin layers OPT was found to form fluorophores with a large number of compounds, mostly imidazoles and indoles. It cannot be established at present whether all compounds which react on thin layers also react in tissue sections. This is of major importance in connection with the use of OPT as a histochemical detection reagent for histamine[41–43]. On thin layers the reaction conditions can be adjusted to provide a greater specificity to the OPT-reagent: histamine reacts with optimal fluorescence under slightly acid conditions whereas the fluorescence of histidine is somewhat more intense at alkaline pH.

Reaction with paraformaldehyde gas is the most sensitive spot test available for the demonstration of catecholamines and tryptamines on thin layers. The similarities observed in the characteristics of the formaldehyde-induced fluorescence in tissue sections and on thin layer may suggest that reactive groups in the silica gel and cellulose powder can substitute for protein in catalyzing the dehydrogenation reaction and that consequently the fluorophores are identical. However, there are some notable discrepancies between the results obtained with tissue sections and thin layers. While 3-methoxycatecholamines are reported to give no or only very slight fluorescence in tissue sections (and dry protein films) after exposure to paraformaldehyde[45], these compounds have a fairly strong fluorescence on a thin layer. Melatonin, indoleacetic acid and 5-hydroxyindoleacetic acid on thin layer also form fluorophores with paraformaldehyde but the fluorescence intensity is very low and of no practical usefulness. The chemical basis for the reaction between formaldehyde and the acid metabolites of tryptamines is not known.

Apparently the reaction of arylalkylamines with formaldehyde may result in a

number of different fluorophores, depending upon the reaction conditions (see STAHL AND KALDEWEY[2] and SEILER AND WIECHMANN[68]), and it is not possible at present to say with any certainty that the condensation products observed on thin layers after exposure to paraformaldehyde gas are identical with those appearing in tissue sections. It may for instance be pointed out that formaldehyde is capable of causing a quite different kind of condensation reaction resulting in polymerization of the amines[69].

Thin-layer chromatography provides rapid and efficient separation of biogenic amines and their precursors and metabolites. This has been utilized in the development of radiometric microassays for several enzymes involved in the metabolism of biogenic amines (see also refs. 60 and 61) and the principle introduced should be of general usefulness. Incubation of microliter volumes of enzyme–substrate mixtures in glass capillaries and subsequent separation of the reaction product by thin-layer chromatography will permit the rapid determination of enzyme activities in submicrogram amounts of tissue. Work is in progress to develop such techniques for all known enzyme reactions pertinent to the formation and degradation of biogenic amines.

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