Journal of Chromatography, 97 (1974) 47–55

CHROM. 7655

THIN-LAYER CHROMATOGRAPHIC FLUORIMETRY OF INDOLE DERIVATIVES AFTER CONDENSATION BY A PARAFORMALDEHYDE SPRAY REAGENT

MARK I. TONEBY

Wenner-Grens Institut, Stockholms Universitet, Norrtullsgatan 16, S-113 45 Stockholm (Sweden)

(Received April 19th, 1974)

SUMMARY

A sensitive paraformaldehyde spray reagent for the thin-layer chromatographic detection of indole derivatives at the nanogram level is described. Paraformaldehyde is dissolved in slightly alkaline ethanol and the ethanolic solution is neutralized by addition of acetic acid. The fluorescence is measured spectrophotofluorimetrically on thin-layer chromatograms and observations are made visually and on photographic films exposed through filters. Excitation and emission spectra are given for nine indole derivatives reacted with the paraformaldehyde spray reagent.

INTRODUCTION

Several methods have been described for the determination of tryptophan metabolites from biological tissues, based mainly on extraction and purification procedures[1–3] followed by measurements of the fluorescence obtained in the final solution when activated under UV light[4–6]. It is often desirable to start, or to make further investigations, with chromatographic procedures[1,7]. For the development of chromatographic spots of indole substances on thin-layer chromatograms, colour reactions have been described[1,7]. The most sensitive reagents, however, are those which give fluorescent products[8,10–13]. In histochemistry, gaseous formaldehyde is used for the detection of 5-hydroxytryptamine and catecholamines[14]. In paper and thin-layer chromatography (TLC)[9,12,13], it has also been used as a chromatographic reagent for indole substances and for catecholamines. It is believed that in the first step indoleamines form weakly or non-fluorescent tetrahydro-*β*-carbolines which will be fluorescent upon oxidation. In the histochemical method, the dehydrogenation reactions are catalyzed by protein. Fluorescent products are also obtained on silica gel thin-layer chromatograms, thus indicating that the dehydrogenation step will take place readily in the presence of silica gel[15]. Björklund *et al.*[15] found that the indoleamine fluorophores formed on dry protein and those formed on silica gel are identical.

In order to obtain gaseous formaldehyde, Björklund *et al.*[15] heated solid paraformaldehyde to 80–100° at 50% humidity, whereas Cowles *et al.*[13] heated paraformaldehyde to 150° to obtain drier conditions. In earlier experiments, Prochazka[7,16] introduced formaldehyde as a spray reagent for obtaining fluorescent products of

indoles. In the method described here, paraformaldehyde was dissolved in sligthly alkaline ethanol and the ethanolic solution was then evenly distributed over the thin-layer chromatogram by spraying. The fluorescence of various indole substances after gaseous formaldehyde condensation was studied in UV light and photographed through filters which increased the sensitivity. For nine fluorophores, the excitation and emission spectra were recorded *in situ* on the chromatogram.

EXPERIMENTAL

Reagents and equipment

The following reagents were used: Ethanol, absolute, spectrograde (AB Vin- & Spritcentralen, Stockholm, Sweden). Ethanol, 95% (AB Vin- & Spritcentralen). Hydrochloric acid, Titrisol, 0.1 *M* (Merck, Darmstadt, G.F.R.). Acetic acid, glacial, p.a. (Merck). Ammonia, 25% solution, p.a., Aristar (BDH, Poole, Great Britain). Methyl acetate, zur Synthese (Merck). Ethyl acetate, p.a. (Merck). Isopropanol, p.a. (Merck). *n*-Butanol, p.a. (Merck). Paraformaldehyde, purum (Kebo, Stockholm, Sweden). Sodium hydroxide, p.a. (Eka, Bohus, Sweden). Indole derivatives, listed in Table I.

Developing solvents used were: (A) methyl acetate–isopropanol–25% ammonia (9:7:4); (B) ethyl acetate–isopropanol–25% ammonia (9:7:4); (C) isopropanol –ethyl acetate–acetic acid–water (75:25:2:3); (D) *n*-butanol–acetic acid–water (4:1:5), upper layer. Solvents A, B and C were freshly prepared before each run.

The chromatography was performed on commercially pre-coated thin-layer plates of dimensions 20 × 20 or 5 × 20 cm with a 0.25-mm layer of silica gel (Merck). The plates were purified in a Shandon chromatography tank fitted with the equipment for descending chromatography. UV light was generated by a General Electric G8T5 tube mounted in a Chromatolux (Pleuger, Wijnegem, Belgium) and filtered through a UG 5 filter, thickness 2 mm (Carl Zeiss, Oberkochen/Württemberg, G.F.R.). The photographic recording was made on Agfa Scientia 50B65 or Kodak 2484 film with a Canon FT camera, lens 50 mm/1.8 or a Minolta SRT 101, lens 55 mm/1.7. A Kodak Wratten gelatin filter No. 8, yellow, and a sky-light filter (Hoya, Tokyo, Japan) were mounted on the lenses. The emission and excitation spectra were obtained from a Perkin-Elmer spectrophotofluorimeter to which a TLC scanning attachment was connected.

Procedure

Spray reagent. In 100 ml absolute ethanol, 60 mg of sodium hydroxide were dissolved. Paraformaldehyde (2 g) was added to the alkaline ethanol and, when it had completely dissolved, 0.1 ml of glacial acetic acid was added. The reagent can be stored refrigerated for at least 2 weeks before any loss in activity occurs. For comparison, paraformaldehyde was also dissolved in lower and higher concentrations and in 95% ethanol. When higher concentrations of paraformaldehyde were used, additional sodium hydroxide had to be dissolved in the alkaline ethanol.

Purification of chromatographic plates. The commercial pre-coated chromatographic plates were purified in a continuously descending chromatography system with a Whatman chromatography paper bridge between the solvent jar and the silica gel layer. Purification was performed with solvent A. The effluent was allowed to drip

from the lower part of the chromatogram. After a run of at least 24 h, the plates were dried in air for 15 min and then heated to 110° for 30 min. The plates were stored in a desiccator over silica gel. Omission of the purification step caused severe interactions between fluorescent products below the front.

Development of spots. The substances were dissolved according to the notes in Table I. Normally 1 μl of the solutions, with substance concentrations of 200, 100, 50, ..., 3.125 and 1.6, 0.8, 0.4 and 0.2 ng/μl of base, was spotted 1.5 cm from the edge of the plate. For the spectrophotofluorimetric measurements, 5–20 μg were spotted on the base line. After elution for 10 cm, the plates were dried *in vacuo* for 15–20 min, then sprayed with 10 ml of the paraformaldehyde reagent. The sprayed plates were heated at 150° for 20 min. When the silica gel layer was wetted with the spray reagent (which is not necessary), the plates were dried in air for 3–5 min before heating.

The plates were studied under UV light generated by the 254-nm lamp, the light from which was filtered through a UG 5 filter. The recording and further investigations were made on a panchromatic black and white film. The Wratten gelatin filter No. 8 cuts off the blue-violet background light from the silica gel and its use is necessary for the photographic recording. The UV lamp was placed 35 cm and the camera 65 cm above the chromatogram. The exposure time for the Agfa film was 2 min and for the Kodak 2484 15 sec with the diaphragm set at 4.0. The Agfa film was developed in Kodak D76 for 8 min, while the Kodak film was developed in Kodak D19 for 6 min.

RESULTS

Non-eluted system

In order to establish the optimal conditions for the action of the reagent, 5-hydroxytryptamine and other indoles were spotted on purified 5 × 20 cm silica gel plates. The plates were sprayed with paraformaldehyde in concentrations ranging from 0.05 to 10% (w/v) and each plate was dried separately in the oven at 150°. Optimal fluorescence was obtained when the reagent contained 2% (w/v) of paraformaldehyde in absolute ethanol.

At the optimal paraformaldehyde concentration, the temperature dependence for the formation of the fluorophores was studied at 10° intervals between 110 and 160°. The highest fluorescence intensity was obtained at 150 and 160°. When the heating time at 150° exceeded 20 min, maximal fluorescence occurred, while the fluorescence intensity decreased after 30 min.

In order to investigate the influence of water on the reagent, the paraformaldehyde was dissolved in a solution in which the absolute ethanol was replaced with 95% ethanol. The sensitivity of this reagent was greatly reduced, however: only 2 ng of 5-hydroxytryptamine could be detected, while the sensitivity was as high as 0.1 ng when absolute ethanol was used.

The fluorescence with the Prochazka reagent[7,16], a mixture of formaldehyde, ethanol and hydrochloric acid, is enhanced by exposure to *aqua regia*. In order to imitate the Prochazka reagent conditions, the plates were treated with paraformaldehyde in alkaline absolute ethanol without acetic acid. They were then sprayed either with 1–5 *M* hydrochloric acid in ethanol and heated at 150° for 20 min or first dried

TABLE I

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF INDOLE DERIVATIVES AND SOME FLUORESCENCE DATA OBTAINED AFTER REACTION WITH GASEOUS FORMALDEHYDE

The sensitivities for the different derivatives are given after 10-cm elution with solvent A and development of the spots with 2% ethanolic paraformaldehyde solution. For the spectrophotofluorimetric determinations, 5–10 μg of indole substances were spotted on purified silica gel thin-layer plates and eluted with solvent A. Excitation and emission wavelength maxima for nine indole derivatives treated with the paraformaldehyde reagent are given. Wavelengths are uncorrected. When two wavelengths are given, the lower value gives the optimal fluorescence. The fluorescence of derivatives giving weak fluorescence or fluorescence not clearly distinguishable from the background (marked Weak) was measured from spots containing 20 μg/cm² of substance. Suppliers: A = Aldrich-Europe, Beerse, Belgium; C = Calbiochem, Los Angeles, Calif., U.S.A.; E = Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; F = Fluka, Buchs, Switzerland; K = K & K Labs., Plainview, N.Y., U.S.A.; M = Mann Labs., New York, N.Y., U.S.A.; R = Professor L. Reio, Food Administration, Stockholm, Sweden; S = Sigma, St. Louis, Mo., U.S.A. Solutions (solvents for the derivatives): Ac = acetone; Et = ethanol; Et/HCl = ethanol 1 *M* with respect to HCl. Solvents A, B and C: see *Reagents and equipment*.

Indole derivative	*Supplier*	*Solution*	$R_F \times 100$			*Sensitivity (ng/spot)*	*Fluorescence maxima (nm)*		*Colour*
			A	*B*	*C*		*Excitation*	*Emission*	
N-Acetyl-5-hydroxytryptamine	S	Ac	77	76	68	25	410, 465	530	Reddish yellow
N-Acetyltryptophan	R	Ac	29	—	—	Not visible	—	—	—
DL-7-Azatryptophan	S	Ac	23	22	5	12.5	300	395	Blue
5-Benzyloxygramine	F	Et	78	81	4	25	Weak	Weak	Greyish blue
5-Benzyloxytryptamine	F	Et	68	69	4	1.6	Weak	Weak	Greenish yellow

Bufotenin	R	Ac	68	71	2	--	--	--	Brown
N,N-Dimethyltryptamine	F	Ac	78	--	--	1000	Weak	Weak	Blue
5-Fluorotryptamine hydrochloride	F	Ac	72	62	Decomp.	3.125	--	--	Blue
Gramine	M	Ac	79	--	1	500	--	--	Brown
5-Hydroxyindole	R	Ac	81	--	--	--	--	--	Brown
5-Hydroxyindole-3-acetic acid	S	Ac	21	18	67	6.25	395, 460	540	Yellow
6-Hydroxymelatonin	S	Ac	68	70	63	--	--	--	Grey
5-Hydroxytryptamine creatinin sulphate	S	Ac	58	51	4	1.6	410	530	Yellow
5-Hydroxy-L-tryptophan	S	Ac	18	18	11	3.125	395	525	Yellow
Indole	E	Ac	89	--	75	1000	--	--	Bluish
3-Indoleacetaldehyde sodium hydrogen sulphite	S	Ac	Decomp.			--	--	--	Bluish
Indole-3-acetic acid	S	Et	30	26	70	25	Weak	Weak	Bluish yellow
Indole-3-pyruvic acid	R	Ac	27	--	--	--	--	--	Brown
Melatonin	C	Et	82	76	60	--	355	450, 480	Bluish yellow
5-Methoxygramine	F	Et	71	76	2	25	Weak	Weak	Brown
5-Methoxyindole-3-acetic acid	S	Et	31	28	66	12.5	Weak	Weak	Bluish yellow
5-Methoxytryptamine	F	Et	71	61	8	1.6	310, 410	490	Bluish yellow
5-Methoxytryptophan	R	Ac	23	17	--	--	--	--	Yellow
5-Methylgramine	K	Et	79	--	2	250	Weak	Weak	Greyish blue
α-Methyltryptamine hydrochloride	A	Ac	68	71	4	1.6	--	--	Blue
5-Methyltryptamine hydrochloride	K	Et/HCl	77	63	38	1.6	310, 385	460	Blue
Tryptamine hydrochloride	S	Ac	68	67	Decomp.	1.6	310, 380	440	Blue
Tryptophan	S	Ac	25	18	6	1.6	310	440	Blue

at 150° for 20 min and then sprayed with the ethanolic hydrochloric acid. In the latter instance, the plates were heated again at 150° for 10 min. The first method resulted in a considerable reduction in the sensitivity, while the second treatment reduced the detectable amount of 5-hydroxytryptamine to 4 ng. The exposure to *aqua regia* of the original or the hydrochloric acid-treated plates did not affect the fluorescence intensity. The addition of 0.1% (v/v) of glacial acetic acid to the reagent, however, increased the sensitivity above that of the alkaline paraformaldehyde reagent.

Eluted system

When the plates were chromatographed, the sensitivity of the reagent was found to depend on the solvent system. Thus, solvent D resulted in great losses in sensitivity. When an acidic solvent is desired, it is therefore recommended that solvent D be replaced with solvent C. The minimum sensitivities of the eluted substances are given in Table I.

The fluorescence spectra of a number of substances reacted with the paraformaldehyde reagent were investigated (Table I). No differences were observed for spectra recorded on non-eluted plates and on plates eluted with solvent A. Blank fluorescence was either measured between two adjacent spots or below the one measured. The fluorescence of each substance was measured on spots from three or more different experiments.

It is interesting to note that several of the substances did not give a defined peak (Table I), but very broad maxima ranging from 450 to 550 nm. The fluorescence intensity was also low and not clearly distinguishable from the background. The emission and excitation spectra of tryptamine, tryptophan, 5-hydroxytryptamine and other indole substances with blue or yellow fluorescence could easily be measured, however (Figs. 1–3).

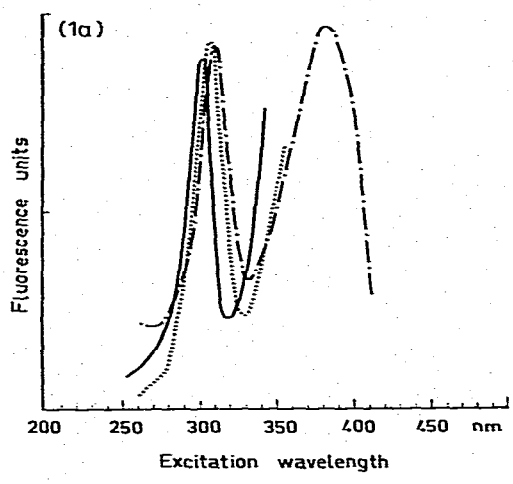
DISCUSSION

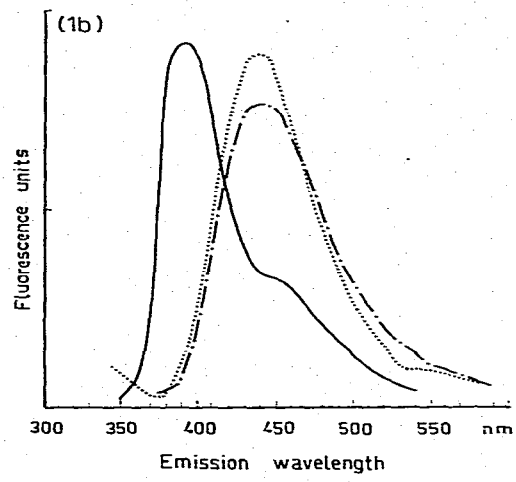
The use of paraformaldehyde as a spray reagent in the manner proposed here facilitates the rapid detection of indoles in TLC. The greatest sensitivity is obtained when spots are studied visually or by photography. The photographic recording makes it possible to measure the amounts densitometrically on the photographic

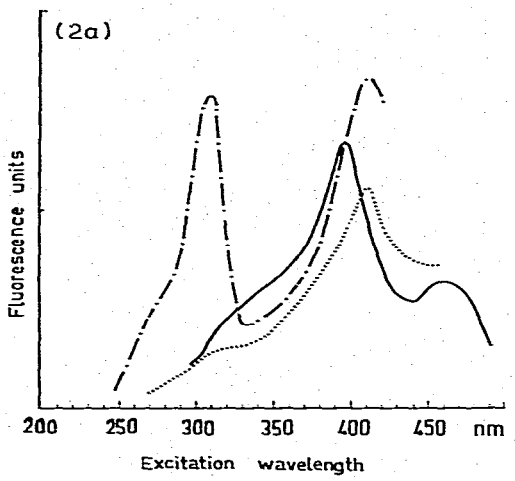
Fig. 1. (a) Excitation spectra and (b) fluorescence spectra for azatryptophan (———), tryptophan (· · · · · ·) and tryptamine (– · – · –) after reaction with the paraformaldehyde reagent. The measurements were made on spots obtained after elution with solvent A on silica gel TLC plates. Spectra are given after correction for blank fluorescence. Excitation and emission wavelengths, respectively: azatryptophan, 300 and 384 nm; tryptophan, 380 and 440 nm; and tryptamine, 310 and 440 nm. Wavelengths are uncorrected. Fluorescence is given in arbitrary units.

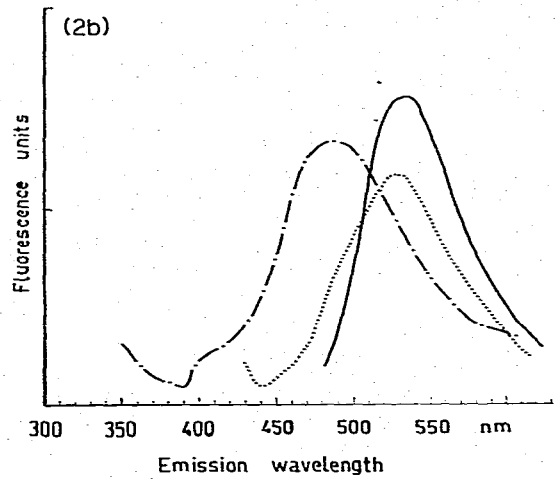
Fig. 2. (a) Excitation spectra and (b) fluorescence spectra for 5-methoxytryptamine (– · – · –), 5-hydroxyindoleacetic acid (———) and 5-hydroxytryptamine (· · · · · ·) after reaction with the paraformaldehyde reagent. Excitation and emission wavelengths, respectively: 5-methoxytryptamine, 310 and 480 nm; 5-hydroxyindoleacetic acid, 396 and 530 nm; 5-hydroxytryptamine, 410 and 530 nm. Other details as in Fig. 1.

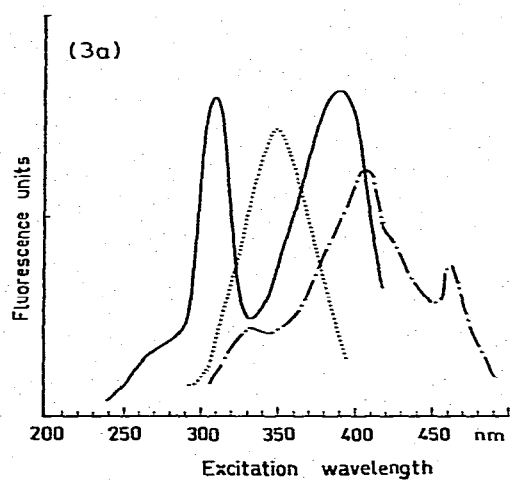
Fig. 3. (a) Excitation spectra and (b) fluorescence spectra for 5-methyltryptamine (———), melatonin (· · · · · ·) and N-acetyl-5-hydroxytryptamine (– · – · –) after reaction with the paraformaldehyde reagent. Excitation and emission wavelengths, respectively: 5-methyltryptamine, 310 and 455 nm; melatonin, 348 and 450 nm; N-acetyl-5-hydroxytryptamine, 400 and 530 nm. Other details as in Fig. 1.

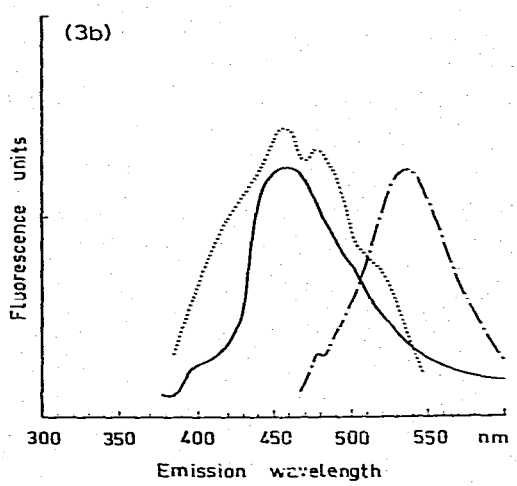

(1a)
Fluorescence units
200 250 300 350 400 450 nm
Excitation wavelength


(1b)
Fluorescence units
300 350 400 450 500 550 nm
Emission wavelength


(2a)
Fluorescence units
200 250 300 350 400 450 nm
Excitation wavelength


(2b)
Fluorescence units
300 350 400 450 500 550 nm
Emission wavelength


(3a)
Fluorescence units
200 250 300 350 400 450 nm
Excitation wavelength


(3b)
Fluorescence units
300 350 400 450 500 550 nm
Emission wavelength

negative. The use of easily obtainable gelatin filters increases the sensitivity of the method considerably.

Commercially pre-coated silica gel thin-layer plates must be purified thoroughly before use. This procedure is time consuming, however, but the use of a descending system is necessary only when two-dimensional chromatography is to be used. For one-dimensional chromatography, it is sufficient to purify the plates by running the solvent to the upper edge, preferably twice with a 30-min drying period at 110° in between.

The main choice of solvent is the alkaline solvent A and the acidic solvent C. The sensitivity of the reagent is higher when the acidic solvent is used, presumably owing to less degradation during elution. The use of solvent D is not recommended, as the loss in activity of the reagent is high. Solvent B is essentially identical with A, except that methyl acetate is replaced with ethyl acetate.

The heating times and temperatures for the development of the fluorophores are similar to those proposed by Cowles *et al.*[13]. In the method developed by Aures *et al.*[12], the temperature used is much lower (80°). As noticed by both groups of workers, the fluorescence of some of the fluorophores formed from indole derivatives is difficult to measure. This was also found in the present investigation (Table I). The spectra in Figs. 1–3 represent substances with mainly blue or yellow fluorescence.

The fluorescence of several of the derivatives listed in Table I is weak or not clearly distinguishable from the background when the measurements are made with the spectrophotofluorimeter. On the other hand, the visible fluorescence is clear and is brown-red, which is to be expected for a mixture of several wavelengths with broad peaks of equal intensities. It is considered that this phenomenon is due to the formation of different fluorophores from each substance. The difference between the fluorescence spectra reported earlier[13-15] and the present spectra is further evidence for the formation of several fluorophores. It has also been reported that changes in pH give additional fluorescent peaks with indoles[17]. If different fluorophores are formed, their formation might be explained in terms of polymerization products of dimers and trimers[17,18], in various combinations depending of the reaction conditions. One must also consider the degradation of the fluorophores with time and temperature.

Minor changes to the substituents on the indole nucleus cause shifts in excitation and emission wavelengths, which shows that spectra are easily affected by minor modifications of the fluorophores. For indole and gramines, no or extremely weak fluorescence is to be expected, as the side-chain is lacking or shorter than that for tryptophan derivatives. Thus β-carbolines cannot be formed. It has been shown that the gramines, however, become more fluorescent on prolonged oxidation, *e.g.*, on storage at room temperature for 1–2 days. The tryptamine and tryptophan derivatives, with the exception of N,N-dimethyltryptamine, readily form fluorophores at low concentrations.

The paraformaldehyde spray reagent has been used successfully in our laboratory for several years for the detection of tryptophan, tryptamine and 5-hydroxytryptamine in biological tissues, mainly from extracts of sea urchin larvae[19] and in model systems in which rat brain extracts were used. The sensitivity is in the nanogram range for indoles that fluoresce blue or yellow when activated in UV light. The method is a simple and useful complement to other analytical methods for the detection of indole derivatives in biological tissues.

ACKNOWLEDGEMENTS

This investigation was partly supported by grants from the Swedish Natural Science Research Council.

The author is indebted to Professor G. Widmark, Department of Analytical Chemistry, University of Stockholm, Sweden, for providing the spectrophotofluorimeter. He also thanks Professor L. Reio, Food Administration, Stockholm, Sweden, for valuable discussions as well as for the gift of a number of indole derivatives. Thanks are due to Professor T. Gustafson, Wenner-Gren Institute, Department of Developmental Physiology, University of Stockholm, Sweden, for stimulating discussions of the application of the method for the separation of derivatives from biological tissues. The valuable technical assistance of Mrs. Anna-Lena Kullgren is gratefully acknowledged.

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