CHROM. 3887

INDOLES AND AUXINS

II. NON-DESTRUCTIVE DETECTION OF INDOLES BY ELECTRON ACCEPTORS*

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SUMMARY

The color reactions of several electron acceptors with 48 indoles and related compounds have been studied. The advantages of these complexing spray reagents are that they: (i) are non-destructive (*i.e.* indoles can usually be regenerated); (ii) give an indication of the type of ring substitution (electron-donating or -withdrawing groups); (iii) react with 2,3-disubstituted indoles. 2,4,7-Trinitro-9-fluorenone is the generally most useful reagent. These complexing agents are less sensitive and less specific than other chromogenic reagents for indoles.

INTRODUCTION

Several spray reagents are available for the detection of indole derivatives on paper and thin-layer chromatograms (*cf.* refs. 1–3). These color reagents are sensitive, relatively specific and give a variety of colors with different indoles. For the detection and further characterization of natural or unknown indoles chromogenic sprays presently used have three major disadvantages: (i) the compound in question is chemically changed, usually in an unknown way *i.e.* regeneration of the original indole is impossible; (ii) there is no simple and logical correlation between the structure of the indole and the color produced; and (iii) 2,3-disubstituted indoles cannot be detected easily because color formation usually depends on a coupling reaction of the chromogenic reagent with either the α or β position of the indole ring system.

Indoles have electron-donor properties in charge-transfer complexes (MILLIÉ *et al.*[4] and references cited therein) and highly colored spots are produced when indoles are sprayed with solutions of electron acceptors on paper or thin-layer plates. This property of indoles has not been exploited widely. 2,4,7-Trinitro-9-fluorenone was used as a spot test reagent for the detection of six indoles[5] and recently various electron acceptors have been used to visualize peptides containing tryptophan[6].

A number of colored electron-acceptor complexes of biologically important

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indoles have been studied, mainly in solution (*cf.* refs. 7 and 8) and the properties and usefulness of pure, crystalline complexes of indole compounds are being studied in this laboratory[9]. General references on the chemistry of acceptor–donor complexes are available[7,10–14].

Electron acceptors are a useful addition to presently used indole reagents. The detection of indoles via complex is advantageous with regard to the three points mentioned above.

EXPERIMENTAL

Indoles

The majority of the indole derivatives used in this investigation were commercial samples purchased from the following companies: Aldrich, Calbiochem, Eastman, K & K, Koch-Light, Mann, Regis and Sigma. Ascorbigen A[15], 3-indole acetyl-N-L-aspartic acid[16], 3-indoleacetyl-N$^{\varepsilon}$-L-lysine[17] and N-acetylskatole[18] were prepared by methods described in the literature. Glucobrassicin was a natural sample.

Complexing agents

The following electron acceptors were used (commercial source indicated in parentheses): 1,2,4,5-benzenetetracarboxylic acid dianhydride, tetracyanoethylene, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, 4-nitrophthalic acid (Aldrich); chloranil, iodine, maleic anhydride (Anachemia); 2,4,7-trinitro-9-fluorenone, picric acid (Eastman); 2,4-dinitrobenzenesulfonic acid (K & K); 1,3,5-trinitrobenzene and 1-fluoro-2,4-dinitrobenzene (Matheson Coleman & Bell).

Color development

Indoles (10 μg, unless otherwise indicated) in methanolic or aqueous solutions were applied to silica thin-layer sheets and sprayed with 1 % solutions of the electron acceptor in chloroform or acetone. Iodine was used at very low concentration in the vapor phase. The indoles spotted on thin-layer sheets were exposed for 1–2 sec.

Chromatography

Eastman Chromagram sheets (K301R2) were used for chromatography throughout this investigation except where indicated. R_F values are not recorded when in agreement with published data[1,17]. Solvents were: (A) chloroform–methanol–acetic acid (75:20:5), (B) benzene–dioxane (55:45), (C) chloroform–methanol–carbon tetrachloride (50:40:10), (D) isopropanol–34 % ammonia–water (85:5:15), (E) ethanol–water (80:20), and (F) 1-propanol–34 % ammonia (70:30).

3-Indoleacetic acid, 3-indoleacetamide and 3-indoleacetonitrile were chromatographed on plates coated with (i) Silica Gel G (Merck), (ii) Silica Gel G impregnated with 0.2 % 2,4,7-trinitro-9-fluorenone (III)[19] and (iii) Silica Gel G with 3 % III added to the developing solvent[6]. Solvents A–E were used to develop the chromatograms.

Regeneration of indoles

After exposure to iodine. 3-Indoleacetic acid (1 mg) and 3-indoleacetamide (1 mg) were streaked on the base of a 20 × 20 cm thin-layer plate (Silica Gel G (Merck), 0.5 mm thickness) and the chromatogram was developed in solvent D. The plate was

exposed to iodine vapor, the position of the bands marked and the plate immediately placed in a desiccator over potassium hydroxide and evacuated (>0.2 mm) for 2 h. After this time, the brown color had almost completely disappeared. In a separate experiment, samples (10 μg) of tryptamine, 3-indoleacetic acid, 3-indoleacetamide and 3-indoleacetyl-N$^{\varepsilon}$-L-lysine were spotted on silica sheets, exposed to iodine vapor, marked and immediately sprayed with a solution of sodium thiosulfate (0.5 %) in ethanol–water (70:30). Silica from the marked areas in both experiments was removed, extracted with methanol or water and portions of these solutions spotted on silica sheets with corresponding standards and chromatographed in solvents C, D and F.

From complexes with 2,4,7-trinitro-9-fluorenone. The indoles were spotted on Eastman Chromagram sheets: silica (K301R2), alumina (6062), Reeve Angel paper loaded with Amberlite IRC-50 (weak acid, H^+ form) or Amberlite IR-4B (weak base, OH^- form). A 5–10 mm wide strip along the starting line was sprayed with the electron acceptor and the sheet developed in either chloroform, benzene, benzene–hexane (9:1) or ethanol containing 5, 25 and 50 % water. The pure indole compound was eluted with methanol or methanol-34 % ammonia (95:5) after the area had been cut out, scraped off or cut into narrow strips for chromatographic elution. For the decomposition of complexes by solvation, the colored spots were removed from the plate and the silica shaken with water of different pH and dichloromethane or ethyl acetate. Ehrlich's reagent (for indoles) and 5-aminoindole (for electron acceptors) was used to locate the compounds in fractions and on chromatograms. The purity of all regenerated indoles was ascertained by chromatographic comparison with pure standards.

RESULTS AND DISCUSSION

The colors obtained from indoles after spraying with electron acceptors are shown in Table I (natural indoles and related compounds) and Table II (ring-substituted indoles and compounds chemically related to indole). The colored spots on silica sheets showed no fluorescence under long- or short-wave ultraviolet light. Generally, the colors changed little after the third day and were stable for several weeks in a laboratory atmosphere.

In addition to the electron acceptors listed in the tables 2,4-dinitrobenzenesulfonic acid, 1,2,4,5-benzenetetracarboxylic acid dianhydride, 6-nitrophthalic acid and 1-fluoro-2,4-dinitrobenzene were used as spray reagents on some indoles successfully.

Color differentiation, sensitivity and specificity

Structurally similar compounds *i.e.* indoles with the same substitution pattern (*e.g.* 3-indole–CH_2–) gave similar colors with individual spray reagents in a wide variety of shades. The color formed with ring-substituted indoles shows a clear and consistent correlation with the electron-donor or -acceptor properties of the substituent. With four complexing agents the color varies from yellow to organe, red and brown to purple and violet when to the substituent is changed from strongly electron withdrawing to electron donating (–NO_2, –CN; halogen; H; –CH_3; –OH, –NH_2) in position 5 of the indole ring (Table II; also serotonin and 5-hydroxy-3-indoleacetic acid in Table I). Electron-withdrawing groups such as –CHO and –COOH (position 3; Table I) and –CO–CH_3 (on the nitrogen; Table II) have the expected effect on the color produced with complexing reagents. The increase in complex stability with

TABLE I

COLOR[a] REACTIONS OF NATURAL INDOLES AND RELATED COMPOUNDS WITH ELECTRON ACCEPTORS

Indole	*Color development with electron acceptor*[b]													
	I		*II*		*III*		*IV*		*V*		*VI*		*VII*	
3-Indoleacetic acid	V	VBr	LGr	Br	Br	RBr	LO	LOBr	Br	Br	BrR	RBr	Bl	Br
3-Indoleacetamide	V	VBr	LGr	BrOl	Br	OBr	LO	YBr	LBr	LBr	BrR	RBr	Bl	LBr
3-Indoleacetonitrile	Br	DBr	Ol	OBr	R	BkR	Y	OY	Br	Br	O	Br	V	RO
3-Indoleacetic acid ethyl ester	O	GyO	LGr	GrY	Br	RBr	O	OY	Y	Br	O	P	Bu	LO
3-Indoleacetaldehyde	V	Br	LR	Br	O	VBr	O	GyO	Y	Br	LO	VBr	Ma	VBr
3-Indolepyruvic acid	Br	Br	Gr	Br	Br	RBr	O	GyO	Br	GyBr	Br	Br	V	VBr
3-Indolelactic acid	Br	GyBr	LGr	GyBr	Br	RBr	O	GyO	Gy	Gy	R	LRBr	Gy	GyBr
3-Indolepropionic acid	V	VBr	Gr	GyBr	DBr	BrV	O	O	Br	BrGy	R	GyV	V	VBr
3-Indolebutyric acid	LV	VBr	Gr	GyBr	DBr	BrV	DO	DO	Gy	DGy	R	GyV	DV	RV
3-Indoleglyoxylic acid	GyPk	GyPk	GyGr	Gy	O	Br	Y	YBr	Y	YBr	LGrBr	RBr	LY	LYBr
3-Indoleglycolic acid	GyV	LBr	Gr	Gr	Br	PkBr	Y	LYBr	P	VBr	GyPk	PkBr	O	YBr
3-Indolesuccinic acid	GyV	VGy	Ol	VGy	Br	PkBr	Gy	LOlBr	Gy	GyBr	GyBr	VBr	Y	YBr
3-Indoleacetone	Br	DRBr	Gr	GyRBr	Br	RBr	O	OBr	Br	Br	Br	Br	Y	YBr
3-Indolecarbinol	RBr	GyRBr	Bd	Bd	Br	DBr	O	Br	Ma	Ma	Bd	Bd	O	RBr
Gramine	V	V	LY	PkBr	Br	DRBr	O	DO	LY	LOBr	Y	RO	O	O
Tryptophol	LV	LV	LGr	OlY	Br	BrV	O	O	Bu	BuBl	O	OGy	DBu	P
Tryptophan	V	VBr	Gr	BrGy	Gy	BlV	LO	LO	LY	GyOl	O	GyPk	O	GyV
Tryptamine	Br	GyBr	Gr	BrGy	Gy	BlV	O	O	LY	OlGy	O	GyO	Bu	GyV
Serotonin	GyV	VGy	OlGy	BrGy	P	BrV	P	GyBr	Gy	BrGy	GyBr	BrGy	Gy	GyBr
5-Hydroxy-3-indoleacetic acid	P	DBrV	DGy	DGy	Gy	GyV	O	DBr	Br	BrBl	R	BrBl	Bu	DBr
3-Indolecarboxaldehyde	V	PkV	LR	LBr	Y	DY	LY	LO	Y	YGr	LO	LBr	Y	Y
3-Indolecarboxylic acid	V	V	LBr	LBr	DY	DY	LY	Y	Pk	Pk	O	RBr	O	O
3-Indoleacetyl-N-L-aspartic acid	V	Br	LGr	LBr	LBr	RBr	LO	LO	LO	LOlBr	R	RBr	O	O
3-Indoleacetyl-N$^{\varepsilon}$-L-lysine	LV	LV	LY	LBr	LBr	LRBr	LO	LO	Y	LOlBr	O	LBr	O	LPk
3-Indoleacrylic acid	Bu	BlV	P	PBr	DBr	RBr	O	Br	Br	Br	R	RBr	Bd	DBd
Indican (indoxyl sulfate)	BuGy	GyV	Gr	GyGr	LBr	LBr	LBr	Y	P	LBr	GyV	VBr	Gy	Y
Ascorbigen	WBr	WBr	LGr	LOlBr	LBr	PkBr	LO	YBr	LPk	LOl	Y	Y	LBu	Y
Glucobrassicin	WBr	WBrV	LGr	LPk	LBr	LBr	LO	YBr	LY	LY	LY	LY	LY	LY

[a] Color abbreviations: Bd = burgundy; Bk = brick; Bl = black; Br = brown; Bu = blue; Ch = charcoal; Gr = green; Gy = grey; Ma = maroon; Mv = mauve; O = orange; Ol = olive; P = purple; Pk = pink; R = red; V = violet; Y = yellow; L = light; D = dark; W = weak; - = no color development.

[b] The color was read overnight (first column for each electron acceptor) and after three days (second column). I = Chloranil; II = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, reddish background; III = 2,4,7-trinitro-9-fluorenone, brownish background after several days; IV = 1,3,5-trinitrobenzene; V = maleic anhydride; VI = picric acid, yellow background; VII = tetracyanoethylene, light yellow background.

TABLE II

COLOR[a] REACTIONS OF RING-SUBSTITUTED INDOLES AND COMPOUNDS RELATED TO INDOLE

Compound	*Color development with electron acceptor*[b]							
	III		*IV*		*V*		*VII*	
Indole	BkR	OR	Y	Y	LY	Br	DO	DO
Skatole	DBr	VBr	O	O	P	P	O	YO
2,3-Dimethylindole	P	PV	BkR	LBr	R	RDBr	Bu	Br
5-Methylindole	O	BrO	Y	Y	Mv	Mv	R	RO
5-Hydroxyindole	Br	VBr	LO	Br	LY	VBr	RBr	RBr
5-Aminoindole	P	BlV	Br	Br	Y	DBr	DBr	Br
5-Fluoroindole	O	YO	Y	LY	Y	RBr	O	YO
5-Chloroindole	DO	O	Y	GyY	LY	RBr	DO	YO
5-Bromoindole	O	O	LY	LY	LY	OBr	O	YO
5-Cyanoindole	O	YO	LY	LY	O	YO	O	Y
5-Nitroindole	O	YO	LY	LY	Y	YO	O	Y
N-Acetylskatole	Y	O	LY	Y	—	—	LY	—
2,3-Diphenylindole	Gy	Gy	Br	Br	LY	LO	LY	Y
Oxindole	Y	DY	Ch	OlBr	—	WLY	O	WLY
Isatin	Y	RBr	Y	Y	Y	Y	O	O
Indoxyl acetate	Br	DBr	Br	Br	P	P	P	P
Indoline	P	BrGyV	Mv	DBr	O	BlBr	R+Gy	DBr
2,3,3-Trimethylindolenine	P	BuBl	Gy	Br	Mv	RBr	R	RO
Pyrrole	GyBr	GyBr	Br	Br	Br	Br	Br	Br
Carbazole	BkR	BkR	Y	Y	Pk	PkGy	—	GyBr

[a,b] See footnotes to Table I.

increasing electron-donor properties of the indole is reflected in these color changes[14].

The influence of a substituent's position on the color was not investigated. With the exception of some methyl derivatives, all substituents were tested in one position of the indole ring only. From theoretical calculations and charge-transfer spectra, however, additional color changes with some substituents can be expected[4,20,21]. The shift to violet colors with methyl groups is more noticeable in position 3 than 5 with most complexes. 2,3-Dimethyl substitution gives a much larger change. This could be due to localized electron-donor abilities of indoles[22] (positions 2 and 3) and the lack of long-range electronic effects of the methyl group.

The limits of detection for indoles are higher with these complexing reagents than with most popular indole spray reagents[2,23]. Limits of detection for 3-indoleacetic acid with electron acceptors I–VII are as follows: I, II and III, > 1 μg; IV and V, 1–2 μg; VI and VII ~ 1 μg. The same order of magnitude is evident for most other indoles by qualitative observation. Reagents I, II and III are the most sensitive in all cases.

Electron acceptors I–VII give color reactions with 2,3-disubstituted indoles, compounds that cannot be detected satisfactorily with most indole sprays. 2,3-Dimethylindole gives intense brown, red and violet colors with all seven complexing reagents at 10 μg. At the same concentration only faintly blue coloration is observed with Ehrlich's reagent and no color with Salkowski's reagent.

A variety of compounds other than indoles give colors with complexing reagents I–VII. Some of these electron acceptors are actually used as spray or streak reagent for other compounds[5,6,24–27]. All good electron donors such as amines, some hetero-

cyclic and aromatic compounds (particularly when polycyclic or substituted by one or more hydroxyl or amino groups) would be expected to give colors. Spots (10 μg) of L-tyrosine, L-histidine, L-phenylalanine, L-lysine and I-naphthylacetic acid were sprayed with complexes I–VII. I–IV, VI and VII gave colored spots of lower intensity than with indoles. Maleic anhydride (V) was an exception; no color at all was produced.

Although iodine is widely used as a non-specific reagent, no color was produced when the five compounds mentioned above were exposed to iodine vapor at low concentration for 1–2 sec.

In view of the low specificity, it is advisable to preclassify *e.g.* natural extracts with conventional indole spray reagents first or ascertain the indole structure after decomposition of the complex by ultraviolet[28] or luminescence (for references see ref. 29) spectroscopy.

Non-destructive detection and regeneration of indoles from complexes

Iodine as a general staining reagent for many types of organic compounds is in common use. It has also been suggested as non-destructive color reagent for thin-layer and paper chromatograms[30]. The mechanism of color formation varies with different types of compounds, the color formed with indoles is undoubtedly due to complex formation[31].

When several indoles, on thin-layer chromatograms, were exposed to iodine vapor at low concentration for 1–2 sec, well defined brown spots were formed on a colorless background. The color disappeared completely when immediately sprayed with sodium thiosulfate solution and only a faint brown color remained when the chromatograms were evacuated over potassium hydroxide. Thin-layer chromatograms of the material extracted from these spots showed that only unchanged starting material was present in both cases.

2,4,7-Trinitro-9-fluorenone (III) was chosen as an electron acceptor for studying the recovery of indoles from complexes because: (i) it is one of the most sensitive complexing reagents; (ii) in preliminary experiments it was shown to be the most suitable of all compounds I–VII for chromatographic separation from a variety of indoles; (iii) it is very insoluble in aqueous ethanol, III precipitates almost quantitatively when the complex, extracted from silica, is heated in 50–75 % ethanol; and (iv) although aromatic nitro compounds are reactive under certain circumstances[32], permanent chemical change of indoles is much less likely than with some of the other, more reactive electron acceptors; *e.g.* tetracyanoethylene is known to react with indoles resulting in sigma bond formation[33].

Complexes of III with indoles could not be clearly separated by chromatography on activated alumina thin-layer chromatograms with benzene, although this would be expected because of the affinity of this electron acceptor for alumina. Complexes of aromatic hydrocarbons and III *e.g.* can easily be separated by washing the hydrocarbon through an alumina column. 2,4,7-Trinitro-9-fluorenone remains on the top of the column in this case[34]. On the alumina thin-layer sheets used (pH 9) III travels with an R_F value of $\sim$ 0.8 in benzene. Even chromatography with aqueous ethanol is impractical, most of the complexing reagent remains on the starting line but a small amount seems to travel in complexed form.

One representative each of an acidic (3-indoleacetic acid; IAA), basic (tryptamine; trypt.) neutral (3-indoleacetamide; IAM) and water-soluble (3-indoleacetyl-

N^{ε}-L-lysine; IAA-lys.) indole was used for chromatographic separation from their 2,4,7-trinitro-9-fluorenone complex. IAA was quantitatively separated from III on paper loaded with anion-exchange resin. IAA remains on the starting line and can be extracted (5 % ammonia in methanol) after the paper has been developed and III thereby removed from the complex with benzene–hexane (9:1). (IAA streaks in higher concentration.) Similarly trypt. can be recovered from the starting spot (5 % ammonia in methanol) after the complex has been irrigated with chloroform on paper loaded with cation exchange resin. IAM also remains on the starting line when the complex is chromatographed on silica with benzene. IAA-lys. can be eluted from the complex with 50 % ethanol, in this case III remains on the origin. Most acidic, basic and water-soluble indoles are expected to behave similarly to the compounds tested. Some neutral indoles will probably require different systems.

Most indoles can easily be regenerated from complexes by solvation. Use was made of POWELL's[35] separation scheme for acidic, basic, neutral and water-soluble indoles. The compounds mentioned above were used and in some cases ethyl acetate was found to be preferable to dichloromethane. The complexing agent III remained with the neutral fraction (IAM), it had to be separated by the chromatographic method described above. Complexes of IAA, trypt. and IAA-lys. with III were also decomposed individually by shaking with ethyl acetate and water of the appropriate pH.

Decomposition of complexes is not necessary in some instances, mass spectra of more volatile indoles can be obtained on the complex directly[9,36].

Chromatography on layers treated with complexing reagent

Silica impregnated with aromatic nitro compounds proved successful for separating aromatic hydrocarbons[19,37,38]; no advantage for the separation of indoles over regular thin-layer chromatography was found in this investigation. On treated plates, spots were more diffuse and R_F values similar to untreated plates with solvents A–E. Addition of 3 % complexing reagent to the developing solvent[6] also showed no advantages.

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