

CHROM. 5938

INDOLES AND AUXINS

XIII. IDENTIFICATION AND ANALYSIS OF NATURALLY OCCURRING
INDOLES VIA ELECTRON DONOR-ACCEPTOR COMPLEXES*

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(Received January 26th, 1972)

SUMMARY

The formation of electron donor-acceptor complexes of some representative indoles with a number of electron acceptors has been investigated with particular emphasis on qualitative and quantitative aspects of analysis involving thin-layer chromatography (TLC). This non-destructive method shows a detection limit for indoles, after chromatography, from 0.5 to 1 μg , depending on the structure of the indole and the electron acceptor employed. An *in situ* method was developed for quantitation of indoles on the sprayed chromatograms and a linear relationship between concentration of the indole and detector response of a TLC scanner for reflectance at the charge transfer band maxima was observed in the range of 0.5–10 μg . With the same electron acceptor, charge transfer band maxima from complexes on a layer of silica gel shift to longer wavelength when substituents on the indoles are changed from electron withdrawing (*e.g.* $-\text{CN}$) to electron donating (*e.g.* $-\text{OH}$).

Separate mass spectra for indoles and complexing reagents can be obtained directly from the complex, as previously observed, at different temperatures. Some of the newer electron acceptors having low vapor pressures allow application of this method at much higher temperatures, *i.e.* less volatile indoles can be identified in this manner.

INTRODUCTION

The indole nucleus, like related " π excessive" heteroaromatic systems, has been shown to act as a good electron donor in charge transfer complexes. A considerable amount of data involving charge transfer properties of indoles is available because several biologically important compounds possess the indole ring system and charge transfer phenomena have been implied in explaining their mode of action. Because of their pronounced donor abilities, indoles have also been used for the theoretical study of electron donor-acceptor complexes. Most of the relevant literature has been

* Issued as NRCC No. 12387.

cited in previous papers in this series^{1,2}. General references on charge transfer complexing are cited in ref. 3.

Intense colours are usually associated with the charge transfer complexing, particularly in the solid state, and this property has been used as a detection method for indoles on paper and thin-layer chromatograms^{1,4,5}.

In this paper, new complexing agents for the detection of indoles are introduced which are superior for subsequent identification by mass spectrometry (*cf.* ref. 2). These new and some previously used electron acceptors have also been used to develop a quantitative *in situ* reflectance method and to investigate the correlation between structure and λ_{\max} . of the charge transfer band of species adsorbed on silica gel and cellulose.

EXPERIMENTAL

Chemicals

Hydroxyskatoles^{6,7} and hydroxyindole-3-carboxylic acids⁸ were prepared by methods described in the literature. All other indoles were obtained from commercial sources as listed in ref. 1.

Electron acceptors

The following electron acceptors were used for this study: (1) tetracyanoethylene (TCNE)*, (2) 2,4,5,7-tetranitro-9-fluorenone (TetNF)*, (3) 9-dicyanomethylene-2,4,7-trinitrofluorene (CNTNF)**, (4) 9-dicyanomethylene-2,4,5,7-tetranitrofluorene (CNTetNF)***, (5) 4,5,7-trinitro-9-fluorenone-2-carboxylic acid (TNF-COOH)***, (6) fluoranil*, (7) tetrabromo-*o*-benzoquinone (TBQ)*, (8) 1,3,5-trinitrobenzene (TNB)**, (9) 2,4,7-trinitro-9-fluorenone (TNF)*, (10) tetracyanoquinodimethane (TCNQ)**, (11) 1,3,5-trichloro-2,4,6-trinitrobenzene (bulbosan)[†], (12) mellitic trianhydride*.

4,5,7-Trinitro-9-fluorenone-2-carboxylic acid was prepared by the method of SULZBERG AND COTTER⁹.

9-Dicyanomethylene-2,4,5,7-tetranitrofluorene was prepared in the following manner (*cf.* refs. 10 and 11): A mixture of 2,4,5,7-tetranitro-9-fluorenone (3.6 g), malonitrile (2 g), piperidine (3 drops) and methanol (400 ml) was heated under reflux for 20 min. 3.7 g of CNTetNF, pure enough for use as spray reagent, were collected from the cooled mixture. A sample recrystallized from acetonitrile-dioxane did not melt below 400° (ref. 11). M^+ 408.0077 \pm 0.0012. Calcd. for C₁₆H₄N₈O₈: 408.0091. Analysis of C₁₆H₄N₈O₈ — Calcd.: C, 47.06; H, 0.98; N, 20.59%. Found: C, 47.36; H, 0.93; N, 20.66%.

Colour development and chromatography

Indoles dissolved in methanol, acetone or water were spotted (10 μ g, unless otherwise indicated; spot diameter 5 mm) on Eastman Chromagram Silica (6061) and

* Aldrich Chemical Co.

** Eastman Organic Chemical Co.

*** Synthesized in this laboratory.

† Hoechst Chemical Co.

Cellulose (6064) thin-layer sheets and sprayed with a 1% solution of the complexing reagent in acetone or methylene chloride.

For detection limits and quantitative measurements 3-indoleacetic acid and 5-hydroxyindoleacetic acid (0.25, 0.5, 1, 2, 5, and 10 μg) were spotted on Merck precoated silica gel thin-layer plates (0.25 mm) and the plates developed with isopropanol-ammonia-water (85:15:10) until the solvent had travelled 10 cm.

Mass spectrometry

Mass spectra were obtained with the DuPont/CEC 21-110B instrument using the standard probe for direct introduction of the sample into the ion source. The spectra were obtained either from the solid complex or from the complex formed by spraying the indole on cellulose thin-layer plates with complexing reagent. In the latter case, cellulose carrying the coloured complex was directly transferred into the mass spectrometer sample tube.

Reflectance spectra and quantitative measurements

Reflectance spectra were recorded on a Farrand uv-vis chromatogram analyzer¹² by plotting reflectance with reference to background *versus* wavelength at 10 nm intervals between 420 and 650 nm using the single beam mode of operation. The light source was a xenon lamp and a 1P28 photomultiplier tube was the detector.

The monochromator was removed from the analyzer leg for these measurements, as the monochromator on the exciter leg provided monochromatic light. An auxiliary filter (No. 3-73, sharp-cut at 400 nm) was used in the analyzer leg, with suitable slit aperture reducers appropriate to the photo tube response at the various wavelengths (an 0.031-mm reducer was used in the range of optimum response, approximately 450–600 nm, while 0.062 and 0.125 reducers were used outside this range). The 3/16–11/32 slit set was used for all measurements.

Evaluation of chromatographed indoles after complexing to determine the potential for quantitative analysis was performed in the double beam mode at the wavelengths of maximum absorption, using the instrumental parameters described above.

Preparation of complexes

Complexes of indoles with electron acceptors were prepared by mixing equimolar, boiling solutions of both components in methylene chloride or acetone, as previously described².

RESULTS AND DISCUSSION

The colours obtained from the various indoles after spraying with electron acceptors are shown in Table I (natural indoles and related compounds) and in Tables II and III (ring-substituted indoles). The colours showed little change for several days after spraying.

Colour differentiation, sensitivity and specificity

The variations in shades of colour for structurally similar indoles with individual spray reagents (electron acceptors) have been described previously¹ and similar behaviour was observed with the acceptors tested in the present study.

TABLE I

COLOUR REACTIONS OF NATURAL INDOLES AND RELATED COMPOUNDS WITH ELECTRON ACCEPTORS ON CELLULOSE THIN LAYERS

Colour code used: Brn = brown; Gy = grey; O = orange; Ye = yellow; P = purple; Pk = pink; Bu = blue; R = red; Br = brick; Mv = mauve; Cm = cream; W = white; Grn = green; Ol = olive; V = violet; Bk = black; Ro = rose; l = light; d = dark; t = trace; - = no colour.

Indole	Colour development with electron acceptor									
	TNF-COOH	CNTNF	TeNF	CNTeNF	Fluoranil	TBQ	Balbosen	Mellitic trianhydride		
3-Indoleacetic acid	Brn	Bu-Gy	dBrn	V	Brn	lMv	T	Gy-Brn		
3-Indoleacetonitrile	Brn	Brn	dBrn	V-Gy	Brn	lBr	Ye	Brn		
3-Indolepyruvic acid	Gy-Brn	Bu	dBrn	P-Gy	Gy-Brn	Cm	lBrn	Bu-Gy		
3-Indolelactic acid	Gy	IV	Gy-Brn	V	lBu	W	-	lBrn		
3-Indoleacetone	Brn	Gy	Brn	Gy	Gy-Bu	Cm	lYe-Brn	P		
3-Indolecarbinol	Brn	lBrn	Brn	Gy	Gy-Brn	lR	lYe	P		
3-Indolecarboxylic acid	O	R	lO	V	Brn	P	-	Brn		
3-Indolecarboxaldehyde	Ye	R	lO	lR	Ye	P	lYe	Brn		
3- β -Indoleacrylic acid	Brn	Ol	Gy-Brn	V	Gy	Cm	lYe	Brn		
Ethyl 1-indolecarboxylate	Ye-Brn	O	O	Brn	O	Brn	lGrn	O		
5-Hydroxyindole-3-acetic acid	P	P-Gy	lP-Gy	V	P	Brn	T	Brn		
Cramine	Brn	P-Gy	dBrn	dGrn	Gy-P	Cm	-	lYe		
Melatonin	P	P	P-Gy	dBu	dBu	W	T	Gy-Brn		
Tryptamine	Brn	Bu-Gy	dP-Gy	Gy	Gy-Bu	lBu	lYe	lBrn		
Tryptophol	Brn	P-Gy	dBrn	V	dGy-Bu	lR	Ye	Brn		
L-Tryptophan	Brn	lGy	lBrn	Gy-Grn	Gy-Bu	Bu	-	lBrn		
5-Hydroxytryptamine	Gy-Brn	dP-Gy	Gy-Brn	IV	Gy	Brn	lYe	Brn		
5-Hydroxytryptophol	Gy-Brn	Bu-Gy	dGy	Gy	dBu	lO	lYe	Brn		
DL-5-Hydroxytryptophan	Gy-Brn	lGy	Gy	Gy-Grn	Gy-Bu	Gy-Brn	T	lBrn		
N-Acetylserotonin	Brn	Bu-Gy	Gy	Gy	dBu	lO	lBrn	Brn		

TABLE II

COLOUR REACTIONS OF INDOLES SUBSTITUTED IN POSITION 5

For colour code, see Table I.

Indole	Colour development with electron acceptor							
	TNF-COOH	CNTNF	TetNF	CNTetNF	Fluoranyl	TBQ	Bulbosan	Mellitic tri-anhydride
5-Fluoroindole	O-Brn	R-Brn	Brn	P-Gy	Brn	dBu	Ye-Brn	Gy
5-Chloroindole	O-Brn	R-Brn	Brn	P-Gy	Brn	dBu	Ye-Brn	R-Brn
5-Bromoindole	R-Brn	R-Brn	Brn	P-Gy	Brn	dBu	Ye-Brn	R-Brn
5-Nitroindole	dYe	Brn	Ye-Grn	Ol	Brn	Ye-Brn	Ye-Grn	Ye-Grn
5-Cyanoindole	lO	Br-Brn	lR	lBrn	lBrn	P	lYe	lYe-lBrn
5-Hydroxyindole	Gy-Br	P-Gy	Brn	P	dBu	lBrn	lYe	dBu
5-Aminoindole	dGy	dBu	P-Gy	dBu	dBu	dBr	Br-R	lBrn

Detection limits after chromatography were generally found to be 0.5–1.0 μg , although individual complexes could vary slightly on either side of these values. Although the polynitrofluorenes have been found to be better acceptors for the charge transfer chromatography of polyaromatics^{13–15}, crystal structure studies have shown that TNB has a very favourable overlap with indole¹⁶. This could explain why detection limits with TNB and the various quinoid acceptors tested are as good as those obtained with the various polynitrofluorenes which are generally regarded as better electron acceptors.

Reflectance spectra

The reflectance spectra of the complexed indoles provide useful information both from qualitative and quantitative analytical standpoints, and also with regard to a basic understanding of parameters influencing complexation.

The influence of various substituents on complex formation is illustrated by the reflectance spectra of a series of 5-substituted indoles complexed with TNF, shown in Fig. 1. These substituents are –CN, –Cl, –H, –OH, and –NH₂. The strongly electron-withdrawing –CN group produces a complex with the narrowest band, lowest intensity and with maximum absorption at lowest wavelength. As the substituents are changed toward the strongly electron-donating hydroxyl and amino groups, the maxima move to higher wavelengths and the bands become broader and more intense. The influence of various substituents is thus shown to be quite pronounced.

The TNF complexes were chosen for this comparison because of the good colour differentiation they display. Whilst good colour differentiation could also be obtained using TCNE as the complexing reagent, preliminary mass spectral studies carried out in this laboratory have shown this to be due to the formation of tricyanovinyl adducts and not π -complexes (*cf.* ref. 17).

Quantitative analysis

Various acceptors were evaluated for *in situ* quantitative analysis of the complexed indoles on the thin-layer chromatograms by reflectance spectroscopy. While *in situ* fluorescence measurements have a much greater sensitivity^{18, 19} the quantitative

TABLE III

COLOUR REACTIONS OF RING-SUBSTITUTED INDOLES

For colour code, see Table I.

Indole	Colour development with electron acceptor									
	TNF-COOH	CNTNF	TetNF	CNTetNF	Fluoranil	TBQ	Bulbosan	Mellitic trianhydride		
Indole	R-Brn	dBrn	IMv	Brn	Brn	Bu-Grn	Ye	Bu-Gy		
1-Methylindole	Brn	dGy	Gy-Brn	IMv	Gy	IO	Ye	dGy		
2-Methylindole	Gy	dGy	Gy-Brn	IMv	IBu	Gy	Ye	dGy		
3-Methylindole	Gy-Brn	dGy	Gy	IMv	Gy	R-O	IO	Gy		
4-Methylindole	Brn	dGy	Gy-Brn	IMv	Gy-P	P	Ye	Gy		
5-Methylindole	R-Brn	dGy	R-Brn	IMv	P	Bu-Grn	Ye	dGy		
6-Methylindole	Gy	dGy	Brn	IGy	Gy-Bu	Ro	Ye	Gy-Brn		
7-Methylindole	Brn	dGy	Brn	IMv	Brn	dBu	Ye	dGy		
1,2-Dimethylindole	Gy	dGy	Gy	IMv	dBu	Cm	O	dBu-Gy		
2,3-Dimethylindole	Gy	dGy	Gy	Gy	Gy-Bu	Cm	Brn	Gy		
2,5-Dimethylindole	Brn	dGy	Brn	Ye-Grn	P	Brn	Ye-Brn	P		
2,3,5-Trimethylindole	Gy	dGy	dGy	Gy	dBu	Cm	Brn	P		
4-Hydroxyindole-3-COOH	Gy	Gy	Gy	IGy	Gy-Bu	IBu	t	t		
5-Hydroxyindole-3-COOH	Brn	P	Brn	Gy	Brn	Gy	-	t		
6-Hydroxyindole-3-COOH	IBrn	Gy	IBrn	Gy	Brn	Gy	-	t		
7-Hydroxyindole-3-COOH	IBrn	Gy	IGy-Bu	Gy-Brn	Gy-Brn	Gy-Brn	-	IPk		
4-Hydroxyskatole	Gy-Bu	P	Bu	Gy-Grn	Gy-Grn	W	IBrn	Grn		
5-Hydroxyskatole	P	Bu	Bu	Bu	P	O	t	Bu		
6-Hydroxyskatole	Gy-Brn	P	Gy-Brn	Gy-Grn	Gy	W	t	Bu		
7-Hydroxyskatole	Gy-Brn	Gy-P	Brn	Gy-Bu	Bu	IBu	lYe-Brn	Gy-Bu		

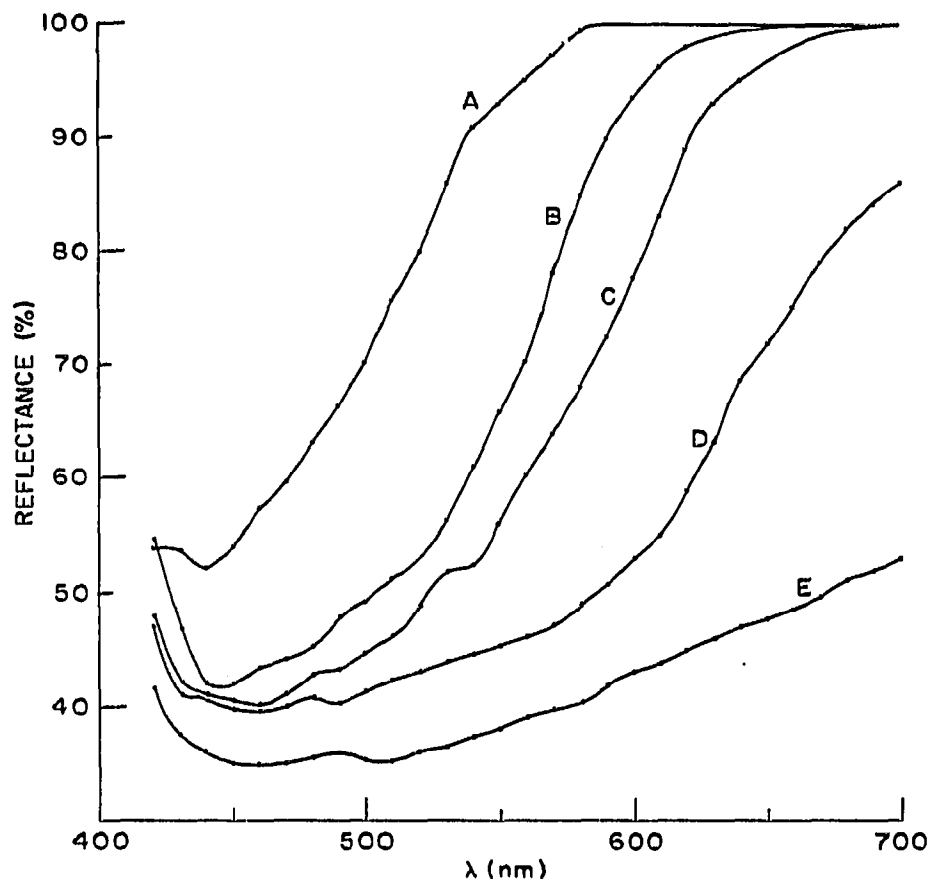


Fig. 1. Reflectance spectra for π -complex of TNF with: (A) 5-cyanoindole, (B) 5-chloroindole, (C) indole, (D) 5-hydroxyindole, and (E) 5-aminoindole on silica gel.

evaluation of complexed indoles by reflectance has advantages in certain circumstances, as, for example, when a mass spectrum of the complex is to be obtained.

For the quantitative evaluation, complexes of 3-indoleacetic acid and 5-hydroxy-3-indoleacetic acid were chosen. The complexing agents TNB and TNF were chosen for their good background and detection limits, while CNTetNF was chosen for its suitability for mass spectral analysis. With TNF as acceptor and two representative indoles (3-indoleacetic acid and 5-hydroxy-3-indoleacetic acid) a linear relationship was observed between amount of indole and detector response in the range 0.5–10 μg .

The use of the double beam mode in these determinations minimizes the problem of poor background uniformity, so that any of these complexing agents should be suitable for quantitative evaluations in cases where it might be desirable for reasons of differentiation.

Mass spectrometry

It was previously shown in these laboratories that mass spectrometry can positively identify microgram amounts of indoles²⁰ and that this information can be obtained from the colored complex² after detection with "electron acceptor" sprays¹.

For this identification, the indole complex can be directly transferred into the mass spectrometer sample tube after visualization on the thin-layer plate. The complex, when heated in the source of the mass spectrometer dissociates thermally

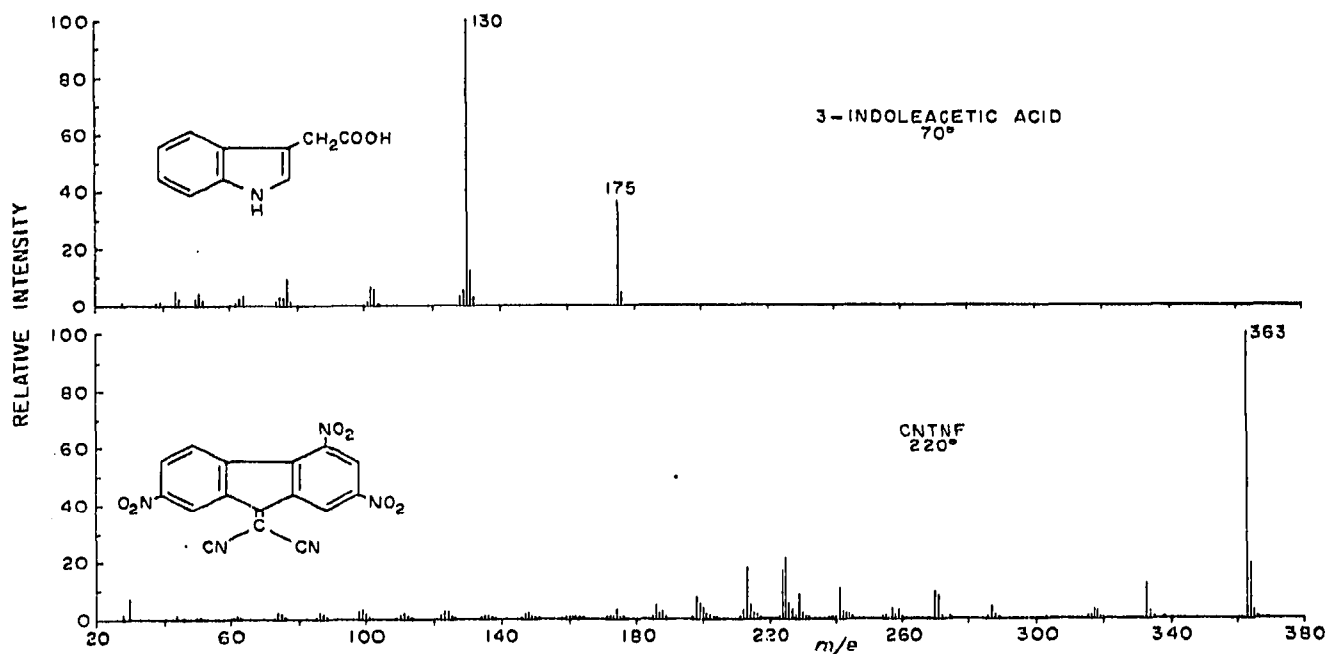


Fig. 2. 70-Volt mass spectra of a 3-indoleacetic acid-CNTNF complex at sample temperatures indicated.

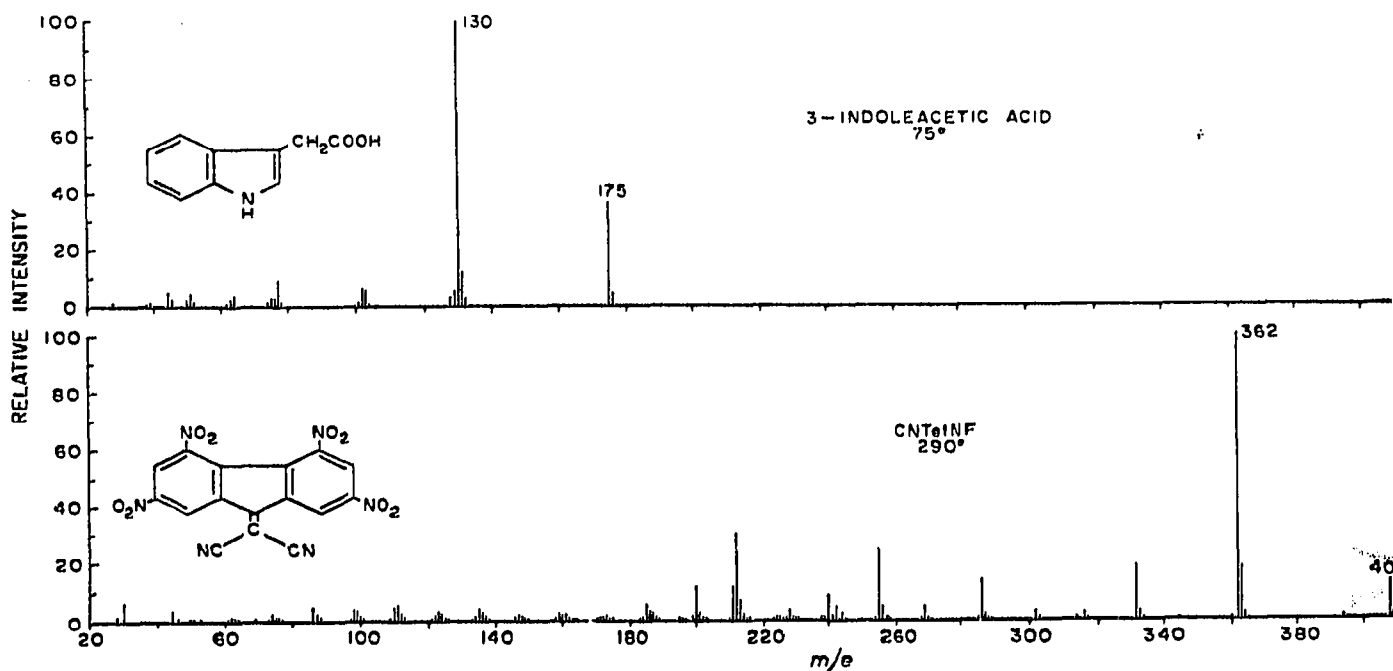


Fig. 3. 70-Volt mass spectra of a 3-indoleacetic acid-CNT₄NF complex at sample temperatures indicated.

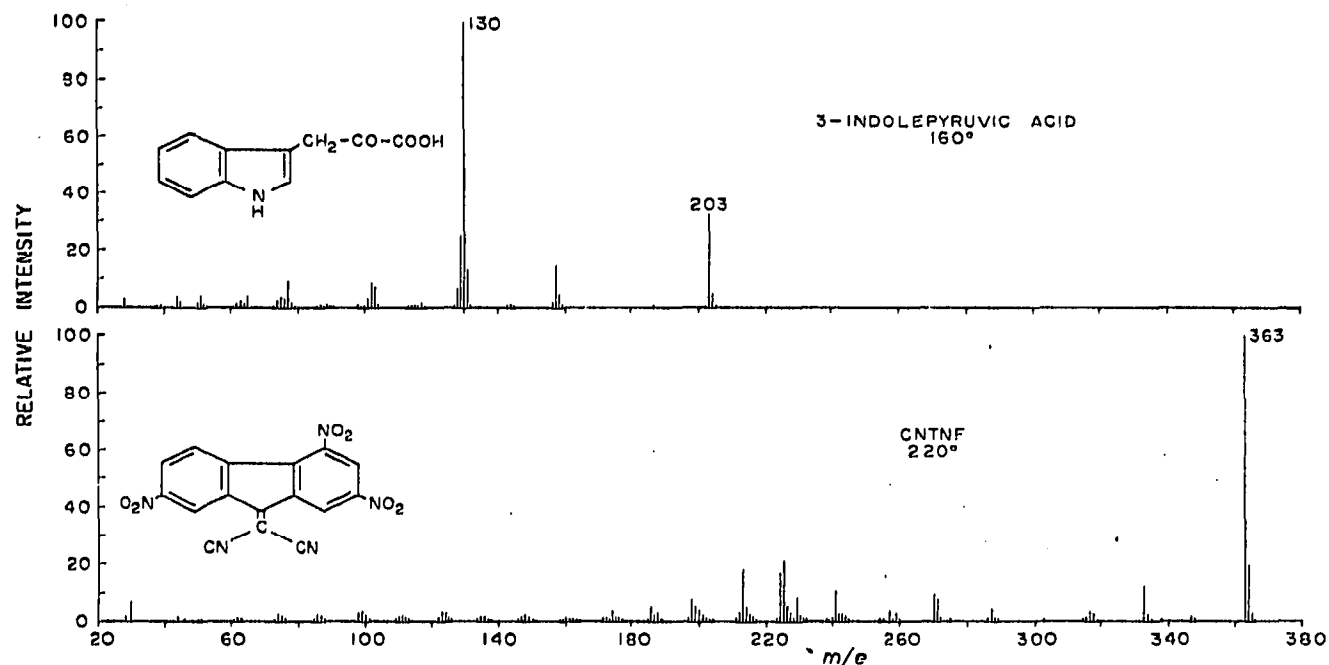


Fig. 4. 70-Volt mass spectra of a 3-indolepyruvic acid-CNTNF complex at sample temperatures indicated.

and useful spectra of the indole, which is generally more volatile than the electron acceptor, can be obtained. In previous work² polynitro-9-fluorenone derivatives were used and although "clean" indole spectra were obtained with most indole complexes, electron acceptors with lower vapor pressure would be advantageous as complexing agents for certain indoles having lower volatility (*e.g.* 3-indolepyruvic acid²).

Of all the complexing agents tested, the two 9-dicyanomethylenepolynitrofluorenes 3 and 4 were shown to be the most useful for direct mass spectrometric analysis of indoles. For example, no interfering ions appear in the spectrum of complexes of these reagents with 3-indoleacetic acid (Fig. 2 and 3) and even a spectrum of 3-indolepyruvic acid can be obtained without interference (Fig. 4). The complexing reagents 3 and 4 do not sublime significantly in the ion source of the mass spectrometer at temperatures at which the spectra of simple indoles are usually observed. CNTetNF with the lowest volatility of any electron acceptor tested so far appears to be the most useful complexing reagent for direct mass spectrometric identification of indoles since it is also one of the strongest acceptors.

Solid complexes

Similar to most indole complexes with electron acceptors such as picric acid and trinitrobenzene^{21,22}, styphnic acid²³, picryl halides²⁴, 1-fluoro (and chloro)-2,4-dinitrobenzene^{25,26}, 2,4,7-trinitro-9-fluorenone and 2,4,5,7-tetranitro-9-fluorenone², the acceptors 3 and 10, containing dicyanomethylene groups, give stoichiometric 1:1 complexes easily. The complexes formed by these acceptors with 3-indoleacetic acid are violet to black needles. Mass spectra of the individual components can be obtained on heating the complex in the mass spectrometer ion source; no evidence for reaction products was obtained.

Analyses. 9-Dicyanomethylene-2,4,7-trinitrofluorene 3-indoleacetic acid com-

plex, m.p. 190–192°. Analysis — Calcd. for $C_{26}H_{14}N_6O_8$: C, 58.00; H, 2.62; N, 15.61. Found: C, 58.36; H, 2.40; N, 15.66%. Tetracyanoquinodimethane 3-indoleacetic acid complex; m.p. 174–176°. Analysis — Calcd. for $C_{22}H_{13}N_5O_2$: C, 69.65, H, 3.45; N, 18.46. Found: C, 69.31; H, 3.23; N, 18.81%.

ACKNOWLEDGEMENTS

We thank Dr. W. D. JAMIESON AND Mr. D. J. EMBREE for the mass spectra. An instrument loan (uv-vis chromatogram analyzer) from the Farrand Optical Co. Ltd. is greatly appreciated.

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