THE DETECTION OF SOME INDOLES AND RELATED COMPOUNDS ON PAPER CHROMATOGRAMS*

A. B. DURKEE

Food Research Institute, Canada Department of Agriculture, Ottawa (Canada)

AND

J. C. SIROIS

Plant Research Institute, Canada Department of Agriculture, Ottawa (Canada)

(Received May 17th, 1963)

The condensation of pyrroles and indoles with aldehydes is a well known reaction which was studied extensively at the turn of the century and which aided HOPKINS AND COLE[1] in the discovery of tryptophan. Later on, the indole–aldehyde reaction was studied by BURR AND GORTNER[2] in connection with humin formation in protein hydrolysates. Since then, the reaction has been widely used to detect indoles, ureides and aromatic amines on paper chromatograms[3,4]. The detection of simple indoles has depended largely on the Ehrlich color reaction, using *p*-dimethylaminobenzaldehyde[4–6]. Recently HARLEY-MASON AND ARCHER[7] reported that *p*-dimethylaminocinnamaldehyde (DMCA) was ten times more sensitive for indole and tryptophan than *p*-dimethylaminobenzaldehyde (Ehrlich reagent).

The great interest in the biochemistry of naturally occurring indole derivatives in both plant and animal tissues, especially in connection with indole-3-acetic acid and serotonin, warrants further study on the methods of isolation and identification of these substances. The present report deals with the chromatographic behavior of twenty-seven indoles and eighteen related compounds. Ultra-violet fluorescence before spraying and the color reactions obtained after spraying with the two aldehyde reagents are reported and the relationship of structure to color reaction is discussed briefly.

MATERIALS AND METHODS

Materials

Indole, skatole, gramine, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, tryptophan, tryptamine, the aromatic amines, urea, thiourea, citrulline and quinaldic acid were obtained through the usual commercial sources. The 5-benzyloxyindoles, 1,2-dimethylindole, 2,3-dimethylindole, 7-azaindole, indole-3-aldehyde and indole-3-acetonitrile were obtained from Aldrich Chemical Co., Milwaukee, Wisc. 5-Hydroxytryptophan, 5-hydroxyindole-3-acetic acid (cyclohexylamine salt), kynurenine, kynurenic acid and xanthurenic acid were obtained from the California Foundation for Biochemical Research, Los Angeles, Calif. Serotonin or 5-hydroxytryptamine (creatinine sulfate complex) was kindly supplied by Vismara Terapeutici.

* Contribution No. 10 Food Research Institute and Contribution No. 338 Plant Research Institute, Canada Department of Agriculture, Ottawa.

The phenols were kindly supplied to us by Dr. H. MORITA of the Soils Research Institute, Canada Agriculture, Ottawa.

Indole-3-glyoxylic acid, indole-3-glyoxyl chloride, indole-3-glyoxylamide and methyl indole-3-glyoxylate were synthesized from indole by the methods of SHAW *et al.*[8]. 3-Cyanoindole, indole-3-aldoxime, indole-3-carboxylic acid and indole-3-acrylic acid were synthesized from indole-3-aldehyde by the methods of SHAW *et al.*

Indole-3-carboxylic acid, prepared by the carbonation of the Grignard reagent formed with ethyl magnesium bromide and indole[9] and recrystallized from aqueous ethanol was not chromatographically pure. On the other hand this acid prepared by hydrolysis of crude 3-cyanoindole by SHAW's method did not require further recrystallization and gave only one spot in both isopropanol–ammonia–water (8:1:1) and butanol–acetic acid–water (4:1:1). It decomposed at 246° (uncorr.). The product from the Grignard reaction decomposed at 220°–225°.

8-Hydroxyquinaldic acid was prepared by a four-step synthesis from *o*-anisidine and crotonaldehyde in good yield by the method of IRVING AND PINNINGTON[10,11].

Most of the substances from commercial sources were chromatographically pure and gave sharp melting points. 2,3-Dimethylindole, gramine and 5-benzyloxygramine yielded more than one spot which may have been due to decomposition during chromatography. Catechol and 3-methoxycatechol were unstable giving spots and streaks on the paper. The latter compound became discolored even when stored in the refrigerator in 95 % ethanol.

Methods

The chromatography was carried out on Whatman No. 1 paper using the ascending method in butanol–acetic acid–water (4:1:1) and isopropanol–ammonia–water (8:1:1). The spray reagents used were: (a) *Ehrlich aldehyde reagent*, prepared by dissolving 2 g *p*-dimethylaminobenzaldehyde in a mixture of 80 ml of 95 % ethanol and 20 ml of 6 *N* HCl[12]. (b) *p-Dimethylaminocinnamaldehyde* (DMCA) reagent, prepared according to HARLEY-MASON AND ARCHER[7] by dissolving 2 g of this aldehyde in a mixture of 100 ml of 95 % ethanol and 100 ml of 6 *N* HCl.

The chromatograms were equilibrated with the solvent for 2 h and developed overnight (16 h). After air-drying, they were sprayed with one or the other reagent, dried in front of a fan and then placed in an oven set at 70° for 5 min. After this treatment they were allowed to remain at room temperature for 48 h. Papers sprayed with DMCA turned very dark after 24 h, but the strong spots remained outlined on the paper. Papers sprayed with the Ehrlich reagent had very little background color. All papers were examined under ultra-violet light using a Blak-ray, long wave U.V. lamp before and after spraying with color reagent.

For chromatography, the substances were dissolved in a suitable solvent, generally 95 % ethanol or acetone and made up quantitatively to a concentration of 1 mg per ml, and 10 μl were spotted at the starting line with a micro-pipette. Because some of the substances did not react at this level some chromatograms were run using 20–30 μl per spot.

RESULTS AND DISCUSSION

The color reactions, R_F values, U.V. data, etc. for the indole derivatives and related metabolites are given in Tables I, II and III. Equations and formulae referred to throughout the text are found in Figs. 1 and 2.

TABLE I

DETECTION OF INDOLES

Compound	R_F*		Color with Ehrlich reagent**				Color with DMCA**				
	Ip–NH_3	Bu–Ac acid	Color	Intensity	Heating and standing	Time	Color	Intensity	Heating and standing	Time	Misc. (U.V. reaction)
Indole	0.91	0.92	Pi-R	3	R (fades)	F	G	4–3	B	F	
Skatole	0.92	0.92	P	3	P	F	P-B	4–3	—	F	
1,2-Dimethylindole	0.89	0.92	R	3	R-P	F	Pi	1	G	F	
2,3-Dimethylindole	—	—	Unstable, breakdown products in chromatography								
5-Benzyloxyindole	0.93	0.90	R-P	3	P	F	G	4–3	B	F	
7-Azaindole	0.83	0.83	—	—	—	—	—	—	—	—	B after spray
Indole-3-acetic acid	0.45	0.87	P-R	3	P	M	B	4	B	F	
Indole-3-propionic acid	0.50	0.86	P	3	P	M-F	B	4	B	F	
Indole-3-butyric acid	0.56	0.90	PB	3	B	F	B	4	B	F	
Indole-3-acrylic acid	0.43	0.86	G	4	B	M-F	B	3	B	F	
Indole-3-carboxylic acid	0.35	0.90	B-P	3	B-P	S	—	—	No reaction		
5-Benzyloxyindole-3-acetic acid	0.52	0.87	B	2	B	M	B	3	B	F	
Indole-3-aldehyde	0.90	0.86	—	1	lt B	SS	—		B	SS	
Indole-3-aldoxime	No immed. reaction										
Indole-3-acetonitrile	0.90	0.92	Pi	1	Br	SS	P-R	3	R-P	F	
3-Cyanoindole	—	—	No immed. reaction								
Tryptophan	0.36	0.42	P	2	Y (24 h)	M	P	3	P-B	F	
Tryptamine	0.78	0.69	P(R)	4	P-R	F	P	4	—	F	
5-Hydroxytryptophan	0.18	0.15	P	4–3	—	M	B	4–5	—	F	
5-Hydroxytryptamine	0.59	0.40	P	3	—	M	B	4–5	—	F	
5 Hydroxyindole-3-acetic acid	0.24	0.70	P	3	—	M	B	4–5	—	F	
Gramine											
5-Benzyloxygramine	—	—	—	—	—	—	—	—	—	—	
Indole-3-glyoxylic acid***	0.45	0.68	—	No reaction		—	—	No reaction		—	Y fluorescence
Indole-3-glyoxylchloride***	—	—	Y	1	—	SS	—	No reaction		—	
Indole-3-glyoxylamide***	0.81	—	Y	1	—	SS	Pi	3		S	
Methyl indole-3-glyoxylate***	—	—	Y	1	—	SS	—	No reaction		—	

* Ip–NH_3 = isopropanol–ammonia–water (8:1:1); Bu–Ac acid = butanol–acetic acid–water (4:1:1).

** B = blue; Br = brown; G = green; O = orange; P = purple; Pi = pink; R = red; Y = yellow; lt = light. Color intensity: 5 = very intense; 1 = very faint. Rate of color development: F = fast; M = medium; S = slow.

*** All the glyoxylic derivatives require at least 30 μg per spot to give a faint color with either reagent. The methyl ester and acid chloride are transformed to indole-3-glyoxylamide during chromatography in isopropanol–ammonia–water (8:1:1).

TABLE II

DETECTION OF AROMATIC AMINES, UREIDES AND QUINOLINES

Compound	R_F*		Color with Ehrlich**				Color with DMCA**				
	Ip-NH_3	Bu-Ac acid	Color	Intensity	Heating and standing	Time	Color	Intensity	Heating and standing	Time	U.V. reaction
Anthranilic Acid	0.45	0.85	Y	5	Y	F	R-Br	4	R-P	F	B fluorescence
p-Aminobenzoic acid	0.20	0.80	Y	5	Y	F	P	5	R-P	F	No fluorescence
Urea	0.45	0.47	Y	2	—	M	R	3	—	F	
Thiourea	0.50	0.47	Y	1	—	S	P	1	—	S	
Citrulline	0.10	0.16	Y	2	—	M	R	2	—	M	
Kynurenine	0.28	0.30	Y	4	—	F	Gr	2	P	F	Strong fluorescence
8-Hydroxyquinaldic acid	0.67	0.14	Yellow color seen before spraying								R fluorescence
Quinaldic acid	—	—	No reaction				No reaction				No fluorescence
Kynurenic acid	0.45	0.45	No reaction				No reaction				B fluorescence
Xanthurenic acid	0.10	0.48	No reaction				No reaction				B fluorescence

* Ip–NH_3 = isopropanol–ammonia–water (8:1:1); Bu–Ac acid = butanol–acetic acid–water (4:1:1). B = blue; Br = brown; Gr = gray; P = purple; R = red; Y = yellow. Color intensity: 5 = very intense; 1 = very faint. Rate of color development: F = fast; M = medium; S = slow.

TABLE III

DETECTION OF PHENOLS

Compound	R_F*		Color with Ehrlich**				Color with DMCA*				U.V. reaction
	Ip-NH_3	Bu-Ac acid	Color	Intensity	Heating and standing	Time	Color	Intensity	Heating and standing	Time	
Eugenol		—	—	No reaction			—	No reaction			
Catechol		—	—	Decomposition				No reaction			
Gallic acid		—	—	No reaction			—	No reaction			
Syringic acid		—	—	No reaction			—	No reaction			
Phloroglucinol		0.60	R-P				P				
3-Methoxycatechol		0.77	—	Decomposed in refrigerator			— Decomposed in refrigerator				
2,5-Dihydroxyphenylacetic acid			—	No reaction				No reaction			
2,5-Dihydroxybenzoic acid	0.58	0.75	—								Strong blue fluorescence

* Ip–NH_3 = isopropanol–ammonia–water (8:1:1); Bu–Ac acid = butanol–acetic acid–water (4:1:1).
** P = purple; R = red.

In general the new aldehyde reagent, DMCA, reacted faster and gave more intense colors with indole compounds than the Ehrlich reagent. Indole-3-acetic acid could be detected on paper chromatograms in concentrations as low as 0.5 μg per spot. The reaction of DMCA with aromatic amines and ureides was disappointing .Red and purple colors were given which would make difficult their distinction from indole derivatives (Table II). One exception to the non-selective action of DMCA is the color reactions given by indole and skatole (Table I). Since these compounds have approximately the same R_F value in both solvents, they can be identified by the fact that indole gives a distinct green color with DMCA whereas skatole gives a purple spot. Indole-3-acrylic acid may be easily detected, if both sprays are used. This substance gives a green spot with the Ehrlich reagent and a blue spot with DMCA. Indole-3-acetonitrile reacts immediately and strongly with DMCA, but the reaction with the Ehrlich reagent is very slow and rather weak. Indole-3-carboxylic acid does not react with DMCA but gives a clear blue-purple spot with Ehrlich reagent after drying for about 10–15 min.

The chromatographic behavior of the indole-3-glyoxylic acid derivatives is interesting. The acid itself does not react with either aldehyde but does fluoresce under ultra-violet light (Table I). Under the conditions of chromatography in isopropanol–ammonia–water (8:1:1) the methyl ester and acid chloride are transformed to the amide which gives a faint pink spot with DMCA and a faint yellow spot with the Ehrlich reagent.

Of the phenols tested, only phloroglucinol reacts with the aldehyde reagents and gives a red-purple color with the Ehrlich reagent and a purple color with DMCA. The quinolines did not react but fluoresced under ultra-violet (Table III).

The most significant observation in this particular study is that many 3-substituted indoles either do not react with these aldehydes or react so poorly that the color reactions cannot be used with confidence in their identification. 3-Substituted indoles in which a carbonyl or nitrile group is attached directly to the β-carbon atom of the pyrrole ring are poor reactors. If the carbonyl or nitrile group is separated by a methylene group the reaction is greatly improved. For example, 3-cyanoindole does not give a color with DMCA or Ehrlich reagent but indole-3-acetonitrile reacts rapidly with DMCA and slowly with Ehrlich reagent. Indole-3-glyoxylic acid (two carbonyl groups) does not react but the reaction occurs to some extent when $-NH_2$ is substituted for $-OH$ (indole-3-glyoxylamide). Indole-3-aldehyde and indole-3-carboxylic acid react faintly with both aldehydes but indole-3-acetic, indole-3-propionic and indole-3-butyric acids react strongly. Worthy of mention is the fact that in this homologous series the longer the chain the stronger and more rapid is the reaction.

On the basis of the above observations, these differences in reactivity can be explained by reference to the structural and electronic formulae of these indoles (Fig. 1). If the condensation takes place at the 2-position the electron-attracting CN and C=O groups tend to decrease the electron density around the 2-carbon atom, making that position positive and repelling the attack of the electrophilic aldehyde. When these electrophilic groups are separated by methylene groups there is no longer a conjugated system and therefore no electromeric effect. The presence of tertiary nitrogen in the gramines and 7-azaindole may explain their failure to react, because under the acid conditions of the test, the nitrogen becomes quaternary due to salt formation and becomes an electron-attracting group as well.

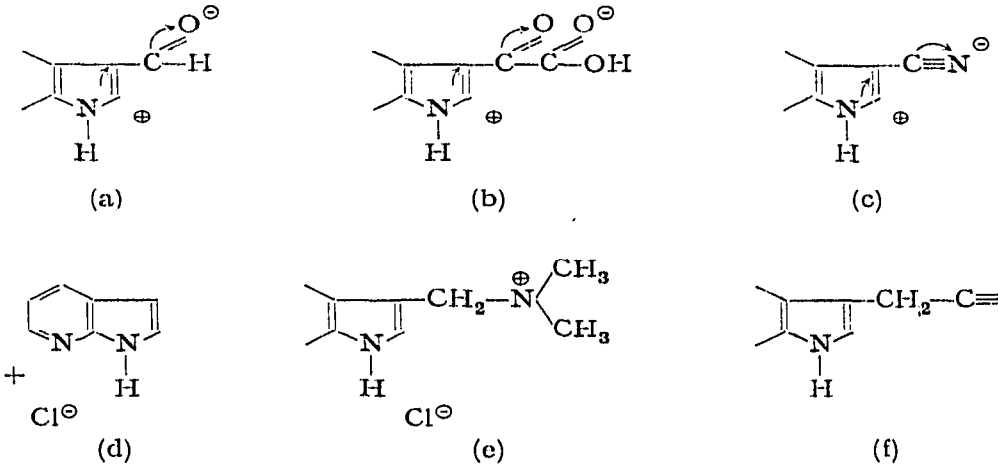

Fig. 1.

Substituents on the benzene ring in the 5-position had very little effect on the color reaction except perhaps to enhance it. Indole and 5-benzyloxyindole gave different colors with both aldehydes than the 3-substituted derivatives. The former gave a green color with DMCA and red-purple with Ehrlich reagent and the 3-substituted derivatives were mostly purple with Ehrlich reagent and blue with DMCA. FEIGL[13] suggests that indole and pyrrole react with the aldehyde in the ratio of 1 mole for 1 mole to form a colored complex of the indolidene-methane structure (B) (Fig. 2) and BURR AND GORTNER[2] have isolated colored salts of several 2-substituted indoles with this structure.

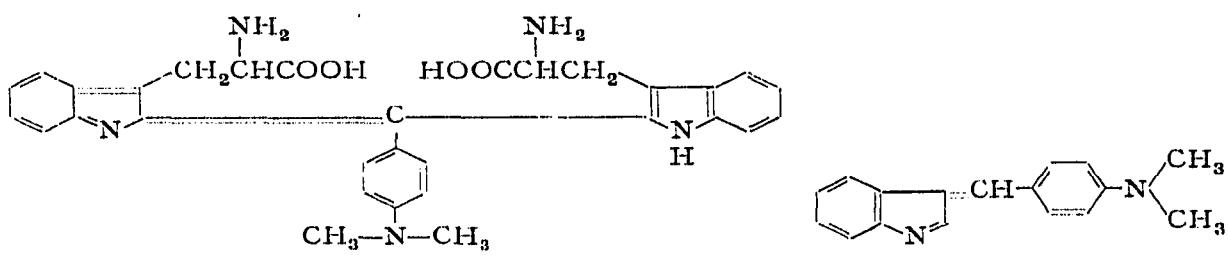

Fig. 2.

GHIGI[14] studied the reaction of *p*-dimethylaminobenzaldehyde with tryptophan and assumed that this reaction took place in the ratio of 2 moles of the indole for 1 mole of aldehyde to form a colored complex of the type (A) (Fig. 2).

It is probable that indoles with no substituents in the 3-position react 1 mole for 1 mole according to FEIGL and to BURR AND GORTNER whereas the 3-substituted compounds react in the manner described by GHIGI and that the condensation takes place on the pyrrole ring at position 2. The difference in color mentioned above and the decreased reactivity of 3-substituted indoles with carbonyl and nitrile groups attached directly to the 3-carbon atom lend support to the hypothesis that condensation takes place at the 2-position and that the colored compounds are different. However, the reaction mechanism requires further study before the nature of the colored products can be readily explained.

SUMMARY

1. *p*-Dimethylaminocinnamaldehyde (DMCA) is a more sensitive reagent, in most instances, than the Ehrlich reagent but is less selective in its color reaction and would be rather a poor chromatographic spray when both indoles and aromatic amines are present.

2. DMCA should be used if skatole or indole is assumed to be present on the chromatogram and in conjunction with the Ehrlich reagent if indole-3-acetonitrile and indole-3-acrylic acid are thought to be present.

3. The decreased reactivity of substituted indoles with carbonyl or nitrile groups attached directly to the 3-carbon atom suggest that the condensation takes place at the 2-position.

4. The differences in color of indole and benzyloxyindole from the 3-substituted derivatives, indole-3-acetic acid and tryptophan, etc., suggest that their condensation products are of different structure.

REFERENCES

1 F. B. HOPKINS AND S. W. COLE, *J. Physiol. (London)*, 27 (1902) 418.
2 G. O. BURR AND R. A. GORTNER, *J. Am. Chem. Soc.*, 46 (1924) 1224.
3 R. J. BLACK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New York, 1958, p. 313.
4 IVOR SMITH, *Chromatographic Techniques*, W. Heinemann Medical Books Ltd., London, 1958 p. 114.
5 A. J. VLITOS AND W. MEUDT, *Contrib. Boyce Thompson Inst.*, 17 (1953) 197.
6 K. K. REDDI AND E. KODICEK, *Biochem. J.*, 53 (1953) 286.
7 J. HARLEY-MASON AND A. A. P. G. ARCHER, *Biochem. J.*, 69 (1958) 60 pp.
8 K. N. F. SHAW, A. MCMILLAN, A. G. GUDMUNDSON AND M. D. ARMSTRONG, *J. Org. Chem.*, 23 (1958) 1171.
9 F. P. DOYLE, W. FERRIER, D. O. HOLLAND, M. D. MEHTA AND J. H. C. NAYLOR, *J. Chem. Soc.*, (1956) 2853.
10 A. B. DURKEE, *J. Agr. Food Chem.*, 6 (1958) 194.
11 H. IRVING AND A. R. PINNINGTON, *J. Chem. Soc.*, (1954) 3782.
12 M. D. ARMSTRONG, K. N. F. SHAW, M. J. GORTATOWSKI AND H. SINGER, *J. Biol. Chem.*, 232 (1958) 17.
13 F. FEIGL, *Spot Tests in Organic Analysis*, 6th Ed., Elsevier, Amsterdam, 1960, p. 289.
14 E. GHIGI, *Gazz. Chim. Ital.*, 64 (1933) 411.