THE HISTOCHEMICAL DEMONSTRATION OF INDOLE DERIVATIVES BY THE ROSINDOLE REACTION OF E. FISCHER

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The discrete localization of indole derivatives in tissue section became a reality with the advent of the post-coupled p-dimethylaminobenzylidene (18), the xanthydrol (29) and the N-(1-naphthyl) ethylenediamine (5) reactions. Of these the most sensitive is the post-coupled benzylidene reaction. However, this reaction demands that a freshly prepared diazonium salt (S-acid) be introduced during the procedure for coupling with the initial reaction product. Therefore, for use in routine histopathological investigations, the post-coupled benzylidene reaction presents moderate technical difficulties. With the introduction of the rosindole reaction of E. Fischer high sensitivity, discrete localization and great specificity are maintained. To these attributes is added one other: technical simplicity.

Rationale: During the development of the post-coupled benzylidene reaction, the striking similarity between the reactivity of indole with benzaldehyde and the known formation of the triphenylmethane dyes became evident (9, p. 893). Therefore, an attempt was made to produce a histochemical rosaniline type condensation of tissue indole derivatives with p-dimethylaminobenzaldehyde. The attempt met with immediate success using various oxidizing agents. A survey of the literature revealed the discovery of this reaction by Emil Fischer in 1886 (10, 12). It then became readily apparent that a reaction utilized by Kaufmann (22, 23) and others was a minor modification of Fischer's rosindole reaction. As we were aware of the conditions required to produce excellent localization of indole derivatives in a strongly acid environment (18, 28, 29), it was felt that an effort should be made to establish this method as a true histochemical procedure i.e. one having high sensitivity, specificity, and permanence and above all sharp localization of reactant compounds.

Historical: A review of the historical evolution of the benzylidene condensation reaction of indole derivatives has been presented previously (18) and only a few further comments will be added. Biochemical application of this reaction began with the works of Reichl (34) and Ehrlich (8). Following the work of Fischer the condensation of aromatic aldehydes with indole derivatives and the subsequent oxidation of the reaction product to triphenylmethane type dyes became a frequent empirical biochemical "discovery" (2, 16, 25, 40, 43) and many variations of the prototype reaction were proposed (3, 31, 33, 37). Numerous aromatic aldehydes have been utilized as the benzylidene component in the rosindole reaction (11, 15, 26, 35, 36, 37). Also, sundry oxidants for the production of the rosindole dye have been advocated by various investigators (6, 17, 21, 34, 35, 41). Several authors have described the use of perchloric acid as a condensing agent in the benzylidene reaction (4, 13).

The first application of the rosindole reaction in histochemistry occurred in 1890 when Mikosch (32) applied the technique of Reichl (35) to histologic sections of plant tissue. The other histochemical applications of the benzylidene condensation from 1890 to 1956 were the methods of Klein (24, p. 1058), Lison (30, p. 160) and Kaufman (23, 24), all of which failed to fulfill the salient criteria of a histochemical procedure.

TECHNIC

Tissues are fixed at 25°C for 6 hours in 10% calcium acetate formalin (27). Bouin's picroformol fixative may be used if the picric acid is removed from the section by a ten minute treatment with lithium carbonate saturated in 70% ethanol. The tissues are carried through the routine dehydration procedures and imbedded in paraffin. Sections are cut at 5μ, mounted and carried through xylene to absolute alcohol.

The rosindole staining technique is then carried out.

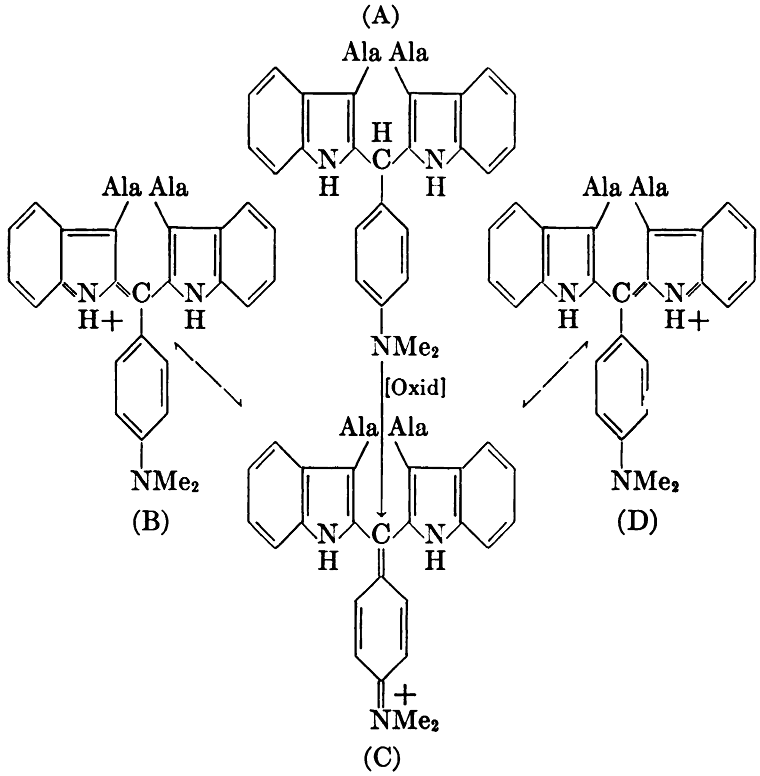
1. Dry slides for 30 seconds and immerse for 3 minutes at 25°C in solution of 1 gram of p-dimethylaminobenzaldehyde in 5 cc 60% perchloric acid, 1 cc concentrated HCl, and 34 cc glacial acetic acid.
2. A solution of 35 cc glacial acetic acid and 5 cc concentrated HCl is added to a Coplin jar layered previously with 500 mg $NaNO_2$. Transfer slides immediately to this solution. Let stand 1 minute at 25°C.
3. Wash 2 × in glacial acetic acid 1 minute each and carry through 50% glacial acetic/xylene, 20% glacial acetic/xylene, xylene 3–4×.
4. Mount in cellulose caprate.

Sites containing indole derivatives stain various intensities of blue depending on the local concentration of these compounds. Using perchloric acid as the condensing agent, complete benzylidene condensation is visualized in from 2 to 3 minutes thereby decreasing the time of exposure of tissue sections to the acid solutions. Exposure of the sections to the oxidizing solution produces an immediate rosindole reaction that is complete in one minute. Excessive exposure to the nitrous acid oxidant causes bleaching of the rosindole dye. The entire procedure thus requires at the most only 15 minutes to perform. No diffusion artiact is noted and positive sites of reaction are discrete.

MECHANISM OF THE REACTION

Tryptophan is used as the prototype compound. The benzylidene condensation of tryptophan with p-dimethylaminobenzaldehyde occurs in the presence of perchloric acid to form a phenylindolyl-methane compound (4, 13) which is probably in equilibrium (18) with the leuco-compound (A). Oxidation of the leuco-compound affords a phenylindolyl-indodylidene-methane product (15), the rosindole dye, which has three resonance forms (B), (C), and (D). The use of p-dimethylamino-benzaldehyde increases the chromogenicity due to the auxochromic effect of the "amidinium ion", $>C{=}\overset{+}{N}<$ (4). Since the feeble basicity of the indole rings favors resonance to the quinonoid structure (C) and the formation of the "amidinium ion", "degeneracy" is marked and a stable deep color is conferred (4). The rosindole reaction product is very stable (39), and is found to be sufficiently fast to light for use in the dye industry (42, p. 730).

Proof of the specificity of the reaction: The rosindole reaction was tested for chemical specificity by the following methods: 1) spot tests; 2) gelatin impregnated filter paper models; 3) use of specific blocking agents on tissue section; 4) comparison of sites of tissue staining with the xanthydrol reaction (29).



Spot tests: The method of spot-testing used has been previously described (18). It was extremely difficult to determine the limit of reactivity in these spot tests since the yellow-brown color produced by $NaNO_2$ in the glacial acetic acid/HCl solution obscured slight color changes. Positive results are tabulated in Table I. Negative results were obtained with these chemical compounds: proline, hydroxyproline, serine, alanine, threonine, glycine, arginine, aspartic acid, glutamic acid, valine, methionine, leucine, cysteine, cystine, adrenaline, dihydroxyphenylalanine, hydroquinone, p-quinone, phenol, o-aminophenol, picric acid, histamine, histidine, phenylalanine, glucosamine, toluidine, orcinol, p-cresol and catechol.

Negative results were obtained with the following indole derivatives: isatin, 2-hydroxytryptophan, 2-phenyl tryptophan, 2-carbethoxytryptophan, 1,2,3,4-tetrahydronorharman, and 2-phenylindole.

Negative results were obtained with these proteins: zein, gelatin, and insulin (glucagon free).

Gelatin filter paper models: Rosindole reactions paralleled those of the post-coupled benzylidene reaction. The reactions using these two methods coincided with all substances examined, but in some cases moderate to marked differences in color were noted (see 18).

Comparison with the xanthydrol reaction. All sites noted to be positive in the xanthydrol reaction were stained by the rosindole reaction even though the xanthydrol reaction has a wider range of reactivity with indole compounds (29). The rosindole reaction, having a greater sensitivity, revealed sites only poorly visualized in the xanthydrol reaction e.g., alpha cell reactivity in the islets of Langerhans. Consequently, it can be stated that in those sites visualized in tissue sections the two methods have a common reactivity.

Blocking reactions: Blocking reactions as performed with dinitrofluorobenzene,

TABLE I

Spot Test Reactions

Chemical Compound	10 mg	1 mg	Limit of Reactivity	*Chemical Compound*	10 mg	1 mg	Limit of Reactivity
Aniline		P++++	100 γ	Glucagon	B++++	B++++	10 γ
Dimethylaniline		P+++	100 γ	Lysozyme	NB++++	NB++++	100 γ
α-Naphthol		BN++++	10 γ	Pepsin	BG++++	PV++	100 γ
β-Naphthol		RV±	10 γ	Chymotrypsin	B++++	B++++	10 γ
α-Naphthylamine		RV+++	1 γ	Trypsin	NV+++	PV++	100 γ
Pyrogallol		R++++	10 γ	Beef platelets	NV+++	V++	1 mg
Phloroglucinol		R++++	10 γ	Formaldehyde precipitate			
				10% CaAc			
Resorcinol		RV++++	100 γ	Tryptamine + formalin 10′		NB++++	100 γ
Carbazole		B++++	1 γ	Tryptamine + formalin 60′		NB++++	100 γ
DL-Tryptophan		BG++++	100 γ	Tryptamine + formalin 2 hr.		NB++	100 γ
Tryptamine		B++++	100 γ	Tryptamine + formalin 3 hr.		—	—
Serotonin		BV++++	100 γ				
Indole		B++++	1 γ				
Pyrrole		RV++++	1 γ				
Skatole		NG++++	10 γ				
2-Methylindole		G+++	10 γ				

Key: Table I & II:

P = Purple
B = Blue
R = Red
V = Violet
G = Green
N = Black

F = Brown
Pk = Pink
Y = Yellow
— = No reaction
± = Barely visible reaction
+ to ++++ = Graded.

TABLE II
Rosindole Reactions of Aromatic Aldehydes

Aldehyde	Reaction	Aldehyde	Reaction
p-Dimethylaminobenzaldehyde	B++++	m-Hydroxybenzaldehyde	BV±
p-Diethylaminobenzaldehyde	B+++	2-Hydroxy-3-methoxybenzaldehyde	PB±
Benzaldehyde	B+++	4-Hydroxy-3-methoxybenzaldehyde	RV±
2,3-Dimethoxybenzaldehyde	B++	p-Hydroxybenzaldehyde	Pk±
m-Nitrobenzaldehyde	B++	2,4-Dihydroxybenzaldehyde	Pk±
p-Nitrobenzaldehyde	BG++	Naphthaldehyde	G±
o-Hydroxybenzaldehyde	BV+	2-Furfuraldehyde	Y±
o-Methoxybenzaldehyde	RV+	2-Hydroxy-3-naphthaldehyde	—

ninhydrin, chloramine-T, iodine, bromine, performic acid, peracetic acid, periodic acid, and sodium nitrite paralleled both the xanthydrol and post-coupled benzylidene reactions. The results and interpretations have been previously discussed (18).

Variations of the rosindole reaction: The use of aromatic aldehydes other than p-dimethylaminobenzaldehyde (Ehrlich's reagent) produced in tissue sections various color reactions of differing intensity when substituted in the rosindole reaction (Table II). None had the chromogenicity of p-dimethylaminobenzaldehyde. Michler's ketone (42, p. 728) as well as benzoyl peroxide, the peracid of benzaldehyde, (16, 38, 39) were employed without success.

The use of oxidizing agents other than sodium nitrite in acid solution produced varying results depending upon exposure time and concentration. Positive reactions (a blue color) were obtained when aqueous solutions of $KMnO_4$, $FeCl_3$, $K_2Cr_2O_7$, $K_3Fe(CN)_6$ were substituted for $NaNO_2$.

DISCUSSION

Since the only blue to black products resulting from the rosindole reaction are those of indole compounds (excepting α-naphthol and carbazole), it is logical to assume that in mammalian tissue the probable compounds demonstrable are tryptophan, tryptamine, serotonin, indole, and skatole.

The rosindole reaction applies to a benzylidene condensation of an aromatic aldehyde with indole derivatives to form an equilibrium leuco-compound. This compound is then oxidized to a chromogenic dye, rosindole. A comparison of this reaction with those of Adamkiewicz (1) and Hopkins and Cole (20) is edifying in that these reactions all depend on the condensation of an indole with an aldehyde (either aromatic or aliphatic) and its subsequent oxidation (7, 17, 19, 33) to form an indolylindolylidene-methane type dye. An analogy with the oxidation of bilirubin to biliverdin is not inappropriate (14 p. 627).

The rosindole reaction of E. Fischer as applied in histochemistry for the demonstration of indole derivatives adds another specific, chromogenic, and localizable reaction to those already described. The application of this reaction to tissue sections, as previously stated, dates from 1890, but the use of new condensing agents and reacting solutions now permits an accurate and facile histochemical localization of indole derivatives not previously possible. In a comparison of relative chromogenicity and sensitivity in tissue sections it was found that the post-coupled p-dimethylaminobenzylidene reaction was only slightly more sensitive and chromogenic than the rosindole reaction. However, the technical practicability of the rosindole reaction makes it more applicable to routine histochemistry. Both of these reactions are found to be superior in chromogenicity and sensitivity to the perhaps simpler xanthydrol reaction.

SUMMARY

1. The application of the rosindole reaction of E. Fischer to the histochemical localization of indole derivatives is described. This method demonstrates predominantly the tryptophan moiety of proteins and it therefore depends on the degree of protein fixation produced by formaldehyde.

2. The specificity of the rosindole reaction is established and the comparison is made between it and both the xanthydrol and post-coupled benzylidene reactions.

3. The facility of the histochemical technique as well as its specificity, sensitivity, and sharp localizability recommends the use of the rosindole reaction in routine histopathological investigation.

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ADDENDUM

Since the submission of the above manuscript for publication, an almost identical histochemical modification of the rosindole reaction has been published by C. W. M. Adams (J. Clin. Path. **10**: 65–61, 1957) who, however, failed to recognize the chemical basis of the reaction employed. The failure of the above author to stain the alpha cells of the islets of Langerhans and to obtain consistently negative results in elastic tissue is probably related to the dissolution of reactive tissue components by the author's use of concentrated hydrochloric acid as the sole reaction medium with subsequent loss of precise localization.