THE CHARACTERIZATION OF SUBSTITUTED NITROIMIDAZOLES ON PAPER AND THIN LAYER CHROMATOGRAPHY BY COLORIMETRIC REACTIONS

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The diazo coupling reaction is the classical method used for the detection of compounds containing the imidazole nucleus. However, since the reaction is inhibited by N-substitution or the presence of strong electronegative substituents on the imidazole ring, other methods have been used to detect such imidazoles. Nitroimidazoles, for example, can be detected by the BRATTON AND MARSHALL method following reduction of the nitro group to the corresponding amine[1]. This is a sensitive reaction, but it is of little value in differentiating between various substituted nitroimidazoles since all nitroimidazoles yield the same red color.

In connection with studies of the metabolism of nitroimidazoles, it was found that mixtures of nitroimidazoles could be separated by paper and thin layer chromatography and tentatively identified by a series of colorimetric reactions. These reactions depended upon reduction of the nitroimidazole to the corresponding aminoimidazole, followed by coupling with either diazotized sulfanilic acid, *p*-dimethylaminobenzaldehyde or ninhydrin.

A comparison of the rate of reaction and sequence of color development of known nitroimidazoles with these reagents enabled us to tentatively identify certain substituents on a nitroimidazole ring. In particular, it was found that a 2-methyl group could be characterized by the use of these three reagents. An analysis of the mechanism of these reactions further aided in characterizing other group substituents and in identifying the substitution pattern of nitroimidazoles.

EXPERIMENTAL

Materials

Imidazole and 2-methylimidazole were obtained commercially. 2-Hydroxymethylimidazole was prepared by heating benzylimidazole[2] with formaldehyde at 140° for 6 h, followed by debenzylation as described by JONES[3]. 1-(2-Hydroxyethyl)--2-methyl-5-nitroimidazole (metronidazole) was supplied by G. D. Searle and Co.

4 (5)-Nitroimidazole was prepared by the slow addition of a mixture of 68 g of imidazole and 202 g of potassium nitrate to 200 cc of concentrated sulfuric acid with stirring. The resultant syrupy mixture was poured over ice, and the residue was wash-

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ed with water, dried, and taken up in concentrated hydrochloric acid. The acid solution was clarified with charcoal, filtered and the desired product was precipitated by the addition of water (yield: 58 %) (m.p. 308–310°). 2-Methyl-4(5)-nitroimidazole was prepared by the nitration of 2-methylimidazole in the same manner. Neutralization of the reaction mixture resulted in the precipitation of the desired product, which was clarified with charcoal and recrystallized from water (yield: 47 %) (m.p. 260–262°). 2-Hydroxymethylimidazole was nitrated with the same reaction mixture; the mixture was kept below 50° during the nitration and was heated 15 min in a water bath after the reaction was complete. The resultant mixture was extracted with ethyl acetate and evaporated to dryness to give a yellow oil. The oil was taken up in acetone, treated with charcoal, and filtered. 2-Hydroxymethyl-4(5)-nitroimidazole crystallized from the acetone on standing overnight in the cold (m.p. 156–158°) (lit. 157–159°)[4,5].

1-(2-Hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole and 1-(2-hydroxyethyl)--5-nitroimidazole were prepared by refluxing 2-hydroxymethyl-4(5)-nitroimidazole and 4(5)-nitroimidazole, respectively, with an excess of ethylene chlorohydrin[6]. 1-(2-Hydroxyethyl)-4-nitroimidazole and 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole were prepared by the same method from 4(5)-nitroimidazole and 2-methyl-4(5)-nitroimidazole, respectively, except that the ethylene chlorohydrin was heated in alcohol in the presence of sodium ethylate[7].

1-Methyl-2-methyl-5-nitroimidazole was synthesized by the methylation of 2-methyl-4(5)-nitroimidazole with dimethyl sulfate as described by BHAGWAT AND PYMAN[8]. 1-Methyl-5-nitroimidazole was similarly prepared by the methylation of 4(5)-nitroimidazole[9]. 1-Acetic acid-2-methyl-5-nitroimidazole was prepared by the oxidation of 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole with chromic acid solution in a manner similar to that described by INGS *et al.*[10].

Paper chromatography

The imidazoles (10γ) were spotted on Whatman No. 1 paper strips and descending chromatograms were developed in the following solvent systems: (a) butanol saturated with water, (b) *n*-propanol–ammonia (70:30), (c) butanol–acetic acid–water, (120:30:50). The strips were developed over a period of 18 h to 50 cm, air dried and examined under the ultraviolet light for absorption bands, indicating the presence of a nitro group. The strips were then sprayed with 1.5 % $TiCl_3$ in 10 % acetic acid to reduce the nitro group and oversprayed with one of the chromogenic reagents. The sequence of color formation for each reagent was observed as the strips dried.

Thin layer chromatography

The imidazoles (5γ) were also spotted on thin layer plates precoated with microcrystalline cellulose (Avicel) to a thickness of 250 μ, and the plates were chromatographed at room temperature by ascending technique to 12–15 cm (3–5 h) in Universal chromatography tanks containing 200 cc of solvent: (a) butanol saturated with water, (b) isopropanol–ammonia–water (160:8:32), (c) butanol–formic acid–water (154:20:26). The plates were air dried, and absorption areas were marked under the ultraviolet light. The plates were sprayed with the titanium trichloride solution and oversprayed with the various color reagents. Since the colors were observed to change on standing overnight, permanent records of the plates were made by marking the spots with colored pencils as they appeared after drying.

TABLE I

R_F VALUES AND COLORIMETRIC REACTIONS OF CERTAIN IMIDAZOLES IN VARIOUS SOLVENT SYSTEMS

	Compound	*U.V. light*	*Paper chromatography solvent**			*Thin layer Avicel solvent**			*Colorimetric reactions***		
			A	*B*	*C*	*A*	*D*	*E*	*1*	*2*	*3*
1	Imidazole	—	0.84	0.80	0.46	0.16	0.35	0.83	—	orange	—
2	4(5)-Nitroimidazole	abs.	0.69	0.70	0.74	0.76	0.72	0.73	yel.-or.-pink	blue-purple	—
3	2-Methylimidazole	—	0.83	0.83	0.55	0.22	0.53	0.88	—	yellow	—
4	2-Methyl-4(5)-nitroimidazole	abs.	0.80	0.75	0.78	0.90	0.88	0.77	pink	tan	green
5	1-(2-Hydroxyethyl)-5-nitroimidazole	abs.	0.75	0.78	0.77	0.80	0.78	0.80	yel.-or.-pink	purple	—
6	1-(2-Hydroxyethyl)-4-nitroimidazole	abs.	0.64	0.72	0.67	0.62	0.74	0.77	yellow	yellow	—
7	2-Hydroxymethylimidazole	—	0.66	0.78	0.50	0.16	0.41	0.78	—	red	—
8	2-Hydroxymethyl-4(5)-nitroimidazole	abs.	0.77	0.85	0.82	0.86	0.87	0.84	yel.-or.-pink	blue-purple	gray
9	1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazole	abs.	0.80	0.85	0.80	0.84	0.82	0.84	pink	tan	green
10	1-(2-Hydroxyethyl)-2-methyl-4-nitroimidazole	abs.	0.67	0.76	0.77	0.72	0.78	0.81	yellow	yellow	—
11	1-(2-Hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole	abs.	0.72	0.80	0.74	0.72	0.69	0.80	yel.-or.-pink	purple	yellow
12	1-Methyl-4(5)-nitroimidazole	abs.	0.79	0.85	0.84	0.86	0.85	0.84	yel.-or.-pink	purple	—
13	1-Methyl-2-methyl-5-nitroimidazole	abs.	0.83	0.85	0.84	0.90	0.85	0.85	pink	tan	green
14	1-Acetic acid-2-methyl-5-nitroimidazole	abs.	0.14	0.63	0.70	0.43	0.82	0.49	pink	tan	green

* Solvent systems used: A = butanol saturated with water; B = *n*-propanol–ammonia (70:30); C = butanol–acetic acid–water (120:30:50); D = butanol–formic acid–water (154:20:26); E = isopropanol–ammonia–water (160:8:32).

** Colorimetric reagents used after reduction with 1.5% $TiCl_3$ in 10% acetic acid: 1 = 0.5% *p*-dimethylaminobenzaldehyde (w/v) in ethanol–HCl–*n*-butanol (30:3:80) (Ehrlich's reagent); 2 = 0.25% diazotized sulfanilic acid (w/v) in 10% sodium carbonate (Pauly's reagent); 3 = 0.2% ninhydrin (w/v) in acetone.

RESULTS

The ultraviolet absorption, R_F values in five solvent systems, and colorimetric reactions for certain substituted imidazoles are listed in Table I. The solvent systems used were the ones found to be most useful in the separation of mixtures of the compounds listed. It was found that paper chromatography was more useful for separating nitroimidazoles, while thin layer chromatography gave better separation of alkylimidazoles from nitroimidazoles. Although satisfactory separation of imidazoles was obtained on one-way chromatograms, mixtures of imidazoles could be more effectively separated in these solvent systems on two-dimensional thin layer chromatograms.

It was observed that 2-methyl-4(5)-nitroimidazoles after reduction yielded a pink color with *p*-dimethylaminobenzaldehyde, gave a tan color with diazotized sulfanilic acid, and exhibited a green color with ninhydrin. The green color was observed either within 2 min after heating the chromatograms in an oven at 60° or after standing for several hours at room temperature. 2-Hydroxymethyl-4(5)-nitroimidazoles as well as nitroimidazoles not substituted in the 2-position gave a blue to purple color with Pauly's reagent, a characteristic yellow-orange color which slowly changed to pink with Ehrlich's reagent, and a yellow color with ninhydrin. Substituted 4-nitroimidazoles, on the other hand, gave a yellow color with Ehrlich's as well as with Pauly's reagent, but failed to react with ninhydrin.

When the nitroimidazoles were reduced with zinc in 10 % hydrochloric acid and the neutralized solutions were chromatographed on paper strips, the corresponding aminoimidazoles could not be detected. However, the aminoimidazoles could be located by rapid runs of these solutions on thin layer plates and spraying the plates with the above chromogenic reagents. The aminoimidazoles apparently are quite labile and are destroyed over a period of hours[11].

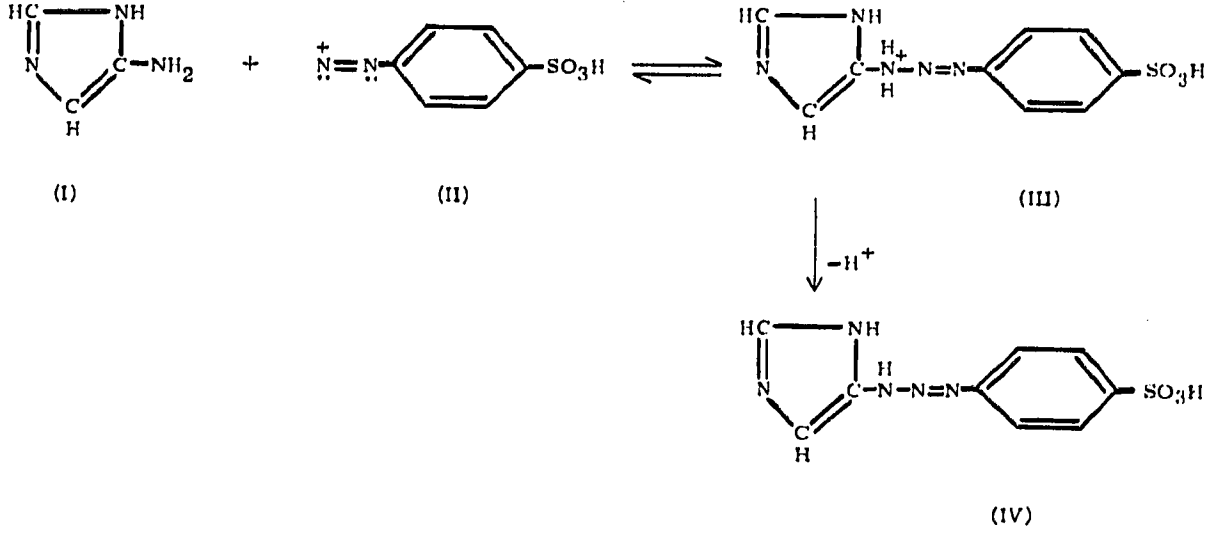
DISCUSSION

The solvent systems used in this study allowed for the effective chromatographic separation of the various nitroimidazoles as well as an evaluation of their acidic and basic properties. A comparison of the rate and sequence of color reaction with diazotized sulfanilic acid, *p*-dimethylaminobenzaldehyde and ninhydrin further aided in the characterization of these compounds. Although the mechanism of reaction of these chromogenic reagents with other compounds appears in the literature, no discussion concerning their reactions with aminoimidazoles has been reported. Sufficient evidence was obtained from this study to discuss the probable nature of the underlying mechanism for each of these reactions.

The mechanism of the diazonium coupling reaction in an alkaline medium involves an attack of a diazonium cation at a center of high electron density on an aromatic nucleus[12]. Diazotized sulfanilic acid reacts with imidazoles in this manner to form C-azo derivatives that are orange-red in color[13,14]. The reaction apparently depends upon resonance within the imidazole nucleus to form electron-rich centers on the ring carbon atoms at which the coupling takes place. N-substitution and strong electron withdrawing groups, such as a nitro group, on the imidazole nucleus decrease the availability of the ring electrons for resonance and thereby inhibit the reaction.

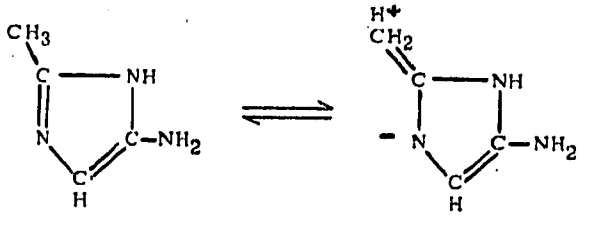
In our study 4(5)-aminoimidazole was observed to couple with diazotized

sulfanilic acid in an alkaline medium to give a blue color that slowly changed to purple. The reaction is essentially a coupling process between a diazonium cation and a primary aromatic amine, and the coupling apparently occurs primarily on the amino group, the center of highest electron density, rather than on the imidazole nucleus. Thus the mechanism for this reaction is similar to the diazo reaction with aromatic amines and involves a nucleophilic attack on the negative amine to form a diazoammonium ion (III)[15]. This initial condensation is followed by the loss of a proton to form the more stable arylazo derivative which is blue in color (IV). However, since resonance within the imidazole ring would form negative ring centers at which the coupling could also take place, the overall reaction probably involves a competition between substitution on the amine and a secondary reaction involving direct coupling with the imidazole nucleus. Nuclear substitution and the formation of a red C-azo derivative in the presence of the blue azo dye could account for the purple color observed in the overall reaction.



(I) (II) (III) (IV)

Substitution of a methyl group on the 2-position of the aminoimidazole ring was observed to alter the diazo reaction. 2-Methyl-4(5)-aminoimidazole coupled with diazotized sulfanilic acid to form a tan azo derivative, suggesting that the primary coupling did not take place on the amino group. Since 2-substituted imidazoles are known to couple at the free 4(5)-ring position, the most likely structure for this product would be a 4(5)-arylazo-5(4)-aminoimidazole. However, 2-hydroxymethyl-4(5)-aminoimidazole reacted with this reagent to give the same blue-purple color sequence observed with 4(5)-aminoimidazole, indicating that this coupling also took place

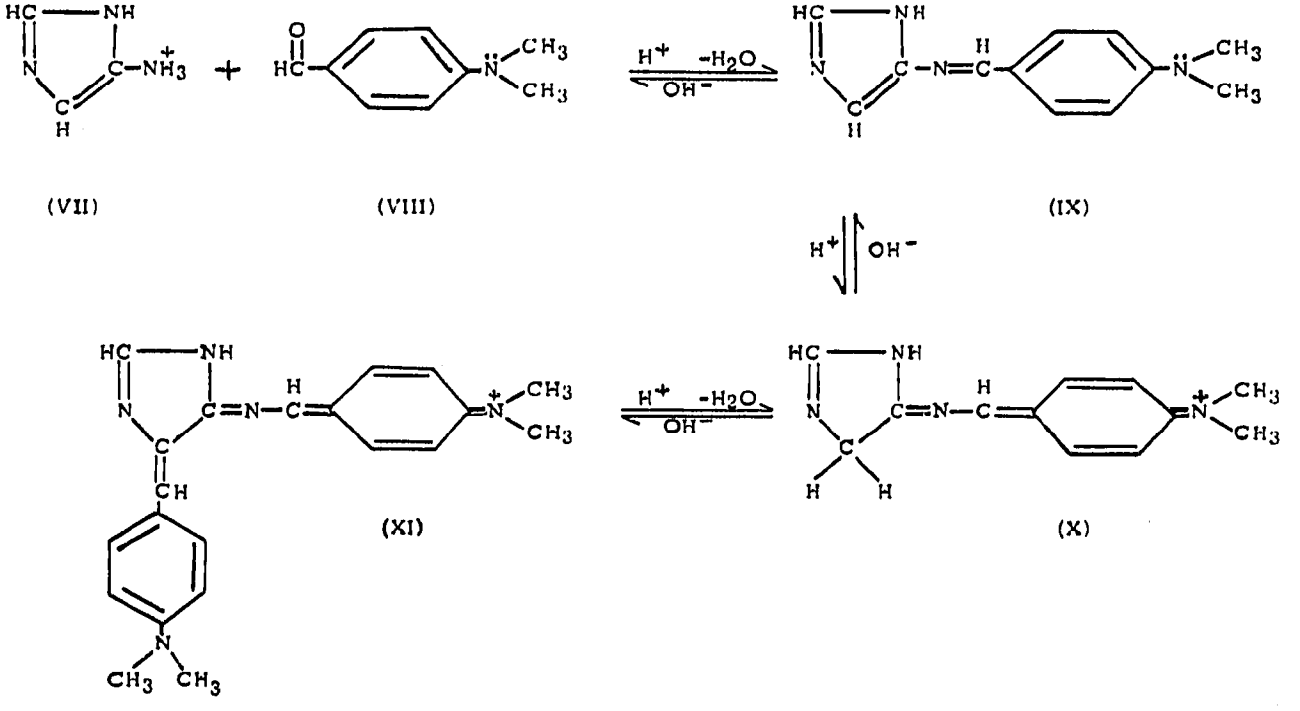


(V) (VI)

primarily on the amino radical. Since both the methyl and the hydroxymethyl groups are capable of donating electrons to the imidazole ring, the formation of the tan 2-methyl derivative is not simply due to an electropositive substituent at the 2-position. With a methyl group on the 2-position of the imidazole ring, hyperconjugation could occur to form a negative center on the adjacent ring nitrogen(s), thereby decreasing the relative electronegativity of the amino group (VI)[14]. The effect of hyperconjugation would be greater than the inductive effect of the amine; and for this reason, the tan color observed may represent an attack of the diazonium cation at the negative ring nitrogen to form an N-azo derivative.

Substitution of an alkyl group on the ring nitrogen in the above compounds did not alter the observed diazo reaction when the amine was located on the 5-position. Thus 1-substituted-5-aminoimidazoles and 1-substituted-2-hydroxymethyl-5-aminoimidazoles reacted purple with this reagent, indicating that the reaction also occurred primarily on the amino group. On the other hand, 1-substituted-2-methyl-5-aminoimidazoles were observed to give a tan reaction. In the latter compounds C-substitution is effectively inhibited due to the effect of hyperconjugation and also because the 4-position is *meta* to the 2-methyl group. However, in the 1-substituted-2-hydroxymethyl compounds coupling at the 4-position can occur since the directive influence of the amino group is greater in these compounds than it is in those with a 2-methyl substituent.

When the amine was located on the 4-position in N-alkyl imidazoles a yellow color was observed with diazotized sulfanilic acid, and the reaction was useful in differentiating 4- from 5-aminoimidazoles. Substitution of the amine in the 4-position limits ring resonance, giving these compounds aliphatic properties. Therefore, this reaction appears simply to involve a condensation of the diazonium ion on the amine to form 1-substituted-4-arylazo derivatives that are yellow in color. Reaction at the adjacent 5-position probably does not occur since ring resonance is inhibited.



p-Dimethylaminobenzaldehyde reacted with 4(5)-aminoimidazoles in an acid medium to form derivatives that were yellow to red in color. This reaction apparently involves an attack of the aldehyde anion on the electropositive NH_3^+ radical to form a Schiff base by the splitting out of a molecule of water (IX)[16]. Due to the influence of the strong electron donating properties of the dimethylamino group, the primary condensate undergoes electron rearrangement forming an active methylene group on the 4-ring position (X). The reaction proceeds to completion by the addition of another molecule of the reagent at the active $-CH_2-$center (XI).

The rate of formation of the quinoidal structure was observed to depend upon the nature of other substituents on the aminoimidazole ring, especially the substituent on the 2-position. When 4(5)-aminoimidazole was coupled with *p*-dimethylaminobenzaldehyde, the color sequence was observed to proceed slowly from yellow to orange to red. Substitution of a hydroxymethyl group in the 2-position did not alter the sequence of color formation, but the reaction was observed to take place at a faster rate. However, a 2-methyl group further increased the rate of reaction so that a faint yellow color was followed by an immediate formation of the red quinoidal structure. This increased rate of reaction may be related to hyperconjugation of the methyl group which would increase the relative positivity of the amino group and thereby enhance the reaction rate.

N-substituted-5-aminoimidazoles reacted with *p*-dimethylaminobenzaldehyde to give the same color sequence, and the rate of reaction was also found to be dependent upon the nature of the 2-substituent. N-substituted-4-aminoimidazoles, however, reacted with this reagent to give a yellow color similar to that observed with aliphatic amines. This reaction apparently involves formation of only the primary Schiff base, since further substitution at the 5-ring position would be inhibited by the bulk of the adjacent substituents.

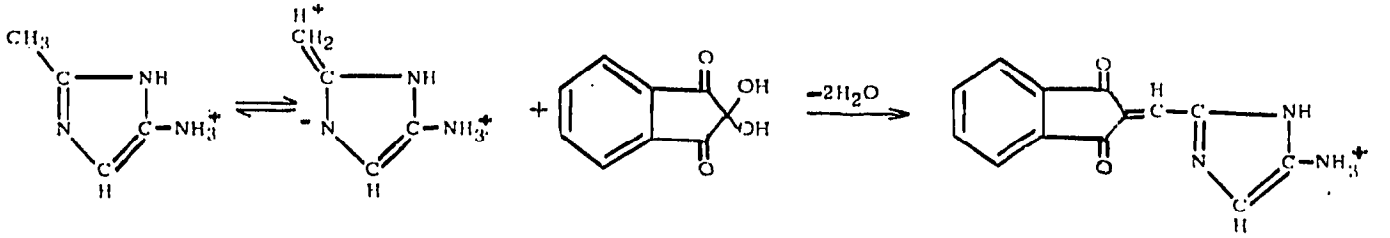
When the chromatograms containing the colored Ehrlich derivatives were made alkaline by dipping the strips or spraying the plates with ammonia or sodium methylate solution, the colors immediately changed from red to yellow and then slowly disappeared. The yellow 4-amine derivatives also were observed to disappear slowly in the basic medium. When the chromatograms were then made acidic the reaction proceeded at the same rate and in the same color sequence to form the red quinoidal structure as observed initially. This observation indicated that the red quinoidal cation is apparently stable in acid but that the reaction is reversed in an alkaline medium to form the Schiff base. The Schiff base should be more stable in an alkaline medium but in the presence of strong base hydrolysis apparently takes place to yield the free aldehyde and amine.

Since only 2-methyl-4(5)-nitroimidazoles gave a green color with ninhydrin, the formation of this color was considered to be tentative proof of a methyl group on the 2-position. A green color obtained with ninhydrin is unusual since amino acids and aromatic amines characteristically yield a blue-purple color with this reagent. The mechanism for the ninhydrin reaction is not completely known, but condensation either directly with the aromatic nucleus or with a breakdown product has been reported to occur. The nuclear reaction involves a primary condensation on a ring imino group to form a yellow derivative, followed at higher temperatures by further substitution on the aromatic nucleus to give a purple color. In compounds containing a free amino group, the reaction involves oxidative-deamination of the amino group

followed by reaction of ninhydrin with the liberated ammonia to give a blue violet dye[17].

Certain conclusions regarding the mechanism of reaction of ninhydrin with 2-methyl-4(5)-nitroimidazoles were reached from the following observations. When 2-methyl-4(5)-nitroimidazole was heated for 30 min at 60° in an acid medium containing ninhydrin, the solution turned light green. Addition of zinc to the reaction medium turned the solution dark green. Chromatograms of this solution in several solvent systems on thin layer plates revealed that a single green derivative had formed which was basic in nature. Further heating of the solution at 100° for one hour turned the solution black. Chromatograms of this solution showed that in addition to the green derivative, a small amount of purple dye had now formed which had acidic properties. 4(5)-Nitroimidazole, when treated in the same manner, failed to react with ninhydrin at 60°, but did yield a purple color after reduction and heating to 100° for 1 h. On chromatograms this purple color was found to be a single substance and was similar to the acidic derivative obtained with 2-methyl-4(5)-aminoimidazole.

The colors observed with ninhydrin were found to depend upon the amount of heat added to the reaction medium. In the case of 2-methyl-4(5)-nitroimidazole a weak reaction took place in solution without reduction of the nitro group to the amine. This reaction appears to depend upon hyperconjugation of the methyl group followed by condensation of the ninhydrin molecule on the resulting $-CH_2-$ radical. Since the nitro group would facilitate hyperconjugation the reaction could occur to some extent, but the strong electronegative nitro group would prevent the loss of a proton from the $-CH_2-$ group and would thereby tend to limit the reaction. Reduction of the nitro group and formation of the NH_3^+ radical would also facilitate hyperconjugation due to the electron withdrawing effect of this radical on the imidazole ring (XIII). This group would, however, enhance the overall reaction since resonance into the amino radical would not occur as with the nitro group, and condensation on the $-CH_2-$ group could readily take place by the splitting out of two molecules of water (XV). This latter reaction should require less energy, and on chromatograms amine formation was observed to be necessary for the reaction to occur at the low temperatures involved.



(XII) (XIII) (XIV) (XV)

The formation of the purple dye in solution observed with 2-methyl-4(5)-aminoimidazole or 4(5)-aminoimidazole and ninhydrin depended upon both the addition of heat and the presence of the amine. The reaction apparently involves oxidative-deamination of the free amine as described above.

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SUMMARY

A method is described for the separation and characterization of certain substituted nitroimidazoles by paper and thin layer chromatography. The colorimetric reaction of these compounds with *p*-dimethylaminobenzaldehyde, diazotized sulfanilic acid, and ninhydrin are discussed and a plausible mechanism for each reaction has been proposed. 2-Methyl-4(5)-nitroimidazoles reacted with ninhydrin to yield a characteristic green color. This is a new color reaction for this reagent and appears to involve condensation of the ninhydrin molecule on an active methylene group on an imidazole ring.

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