development with solvent III in the event that spot formation with the iodine spray was poor.

Sensitivity of the diazonium spray and iodine spray vary with the nature of the imidazole compound, but an order of magnitude may be indicated. The diazonium spray is capable of detecting $10^{-3}$ micromoles of imidazole and the iodine spray is capable of detecting $10^{-2}$ micromoles. Whether maximum sensitivity with the iodine spray occurs with the transient color in ordinary light, or with the ultraviolet light, depends on the nature of the compound. For example, the transient spot in ordinary light was the most sensitive indicator of 1-methylimidazole whereas the corresponding spot with 4(or 5)-bromoimidazole was weak. The latter compound gave a strong spot when viewed with ultraviolet light.

The nature of the reaction with the iodine spray is not known (*2*). Substitution on the imidazole ring is unlikely since imidazoles which bear substituents on the ring nitrogen are reported not to undergo iodination (*3*). Addition of iodine to the unsaturated imidazole ring is unlikely also in view of the known resistance of the imidazole ring to this type of reaction. Possibly a reversible physical combination of iodine with the imidazole compound occurs. This is indicated by the fact that the colored spots formed with most imidazole compounds rapidly faded, but could be revived repeatedly by further application of the iodine spray. Highly-colored unstable products were observed by Brunings (*3*). He has proposed that these products are iodinated on the ring nitrogen at position No. 3.

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# Colorimetric Method for Analysis of Histidine and Certain Related Imidazole Compounds

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**A colorimetric method is described for the quantitative analysis of histidine and certain of its derivatives, histamine and imidazole. Phenols and aromatic bases which interfere with other colorimetric procedures for histidine did not interfere with this method unless present in relatively large amounts.**

THE Pauly (*6*) diazo reaction has long been used for the colorimetric determination of imidazole compounds. This reaction between imidazoles and diazotized aromatic amines in alkaline solution leads to the formation of azo dyes. This is the basis of the classic Koessler-Hanke procedure (*5*) for histidine. A more specific method for histidine is the Kapeller-Adler method (*3*). This method is based on the observation of Knoop (*4*) that a heated solution of histidine in bromine water yields a reddish-colored product. Both of these methods suffer from lack of specificity, and many compounds other than imidazoles contribute color. These include phenols, aromatic amines, pyrroles, and indoles.

A more specific method was desired by the author in order to analyze for certain imidazole compounds in the presence of other imidazoles and other compounds known to interfere with the above methods. A number of reactions that might lead to colored products were investigated. Of these, the most promising reaction was based on the Bamberger degradation (*1*) of imidazoles with benzoyl chloride in alkali. In order to obtain a colored product, *p*-nitrobenzoyl chloride was substituted for benzoyl chloride (Equation 1). Conditions were devised so that the amount of light absorbed at the wave length of maximum absorption by (I) was proportional to the concentration of the imidazole compound. (See structural Reaction I.)

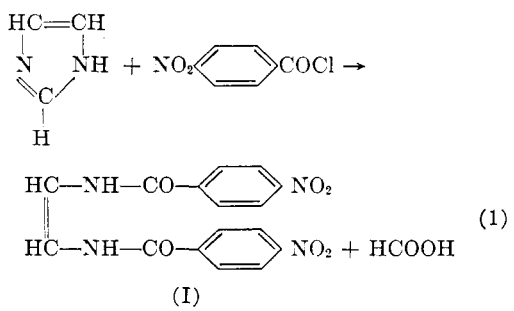
The identity of the colored product of the reaction with imidazole was established to be 1,2-di(*p*-nitrobenzamido)ethylene-(I). Properties of the colored product from the procedure for the colorimetric analysis proved to be identical with those of a purified preparation of 1,2-di(*p*-nitrobenzamido)ethylene.

The specificity of the colorimetric method was tested with 16 imidazole compounds. Of these, only imidazole, histamine, histidine, and derivatives of histidine were reactive. Other compounds such as tryptophane, tryosine, arginine, or phenol did not interfere with this procedure unless present in relatively large amounts.

## EXPERIMENTAL DETAILS

**Procedure for Colorimetric Analysis.** STOCK REAGENTS Aqueous sodium bicarbonate, 1.0*M*, and 1.0*N* aqueous sodium hydroxide.

*p*-Nitrobenzoyl chloride, 0.06*M*, in acetone. The *p*-nitrobenzoyl chloride from Eastman Kodak Co., was used without purification. The *p*-nitrobenzoyl chloride from Matheson Co. was crystallized from petroleum ether (boiling point 60° to 70° C.) before use. The acetone solution of *p*-nitrobenzoyl chloride



(I) (1)

is unstable and should be prepared no more than a few minutes before use.

PROCEDURE. An amount of sample up to 1.0 ml. is placed in a colorimeter tube calibrated for 10-ml. volume. The volume of the sample is adjusted to 1.0 ml. by addition of water, to which 0.1 ml. of 1*M* sodium bicarbonate and then 5.0 ml. of 0.06*M* *p*-nitrobenzoyl chloride are added. The mixture is vigorously swirled and kept at room temperature for 10 minutes.

Two milliliters of 1*N* sodium hydroxide is added, the mixture is vigorously swirled again, and is allowed to stand for 5 minutes.

The mixture is diluted to 10 ml. with water. After 30 minutes the color intensity is measured in a colorimeter with a filter of maximum transmittance at 420 mμ.

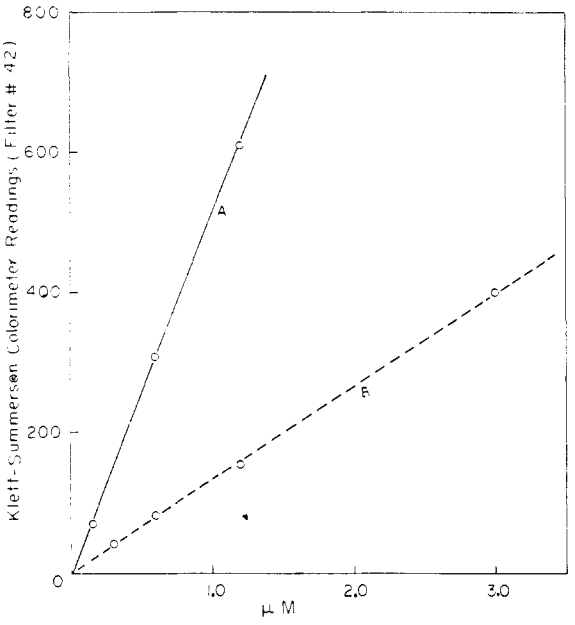



**Figure 1. Standard curves for analysis of imidazole and histidine**

*A.* Imidazole
*B.* Histidine

A standard curve should be made from aliquots of a standard solution of the same imidazole compound that is present in the sample for analysis. This standard curve was found to be linear for all compounds tested (Figure 1).

**Nature of Colored Product of Reaction.** SYNTHESIS OF 1,2-DI(*p*-NITROBENZAMIDO)ETHYLENE (I). Two grams (0.03*M*) of imidazole was dissolved in 40 ml. of 2*N* sodium hydroxide, and 5.5 grams (0.03*M*) of *p*-nitrobenzoyl chloride was added with stirring at 0° C. The mixture was maintained at 0° C. for 6 hours and stored overnight at 20° C. The mixture then was extracted with 100 ml. of benzene. The benzene extract was discarded. The pH of the aqueous mixture was adjusted to 2 to 3 with concentrated hydrochloric acid, and 50 ml. of water was added. The slurry of yellow crystals was extracted with 50 ml. of ether and 50 ml. of benzene. Both extracts were discarded. The yellow crystals were filtered and washed with water. Weight of the dried product was 3.6 grams; this was a 68% yield, based on the *p*-nitrobenzoyl chloride used.

**Table I. Compounds Not Yielding Colored Products with *p*-Nitrobenzoyl Chloride Reagent**

1-Methylimidazole
4(or 5)-Bromoimidazole
4(or 5)-Chloromethylimidazole
4(or 5)-Hydroxymethylimidazole
4(or 5)-Imidazolecarboxyaldehyde
4(or 5)-Imidazolecarboxylic acid
Methyl ester of 4(or 5)-imidazolecarboxylic acid
2,4,5-Tribromoimidazole
2,4,5-Trimethylimidazole
Pilocarpine
Ammonia
Phenol
Pyridine

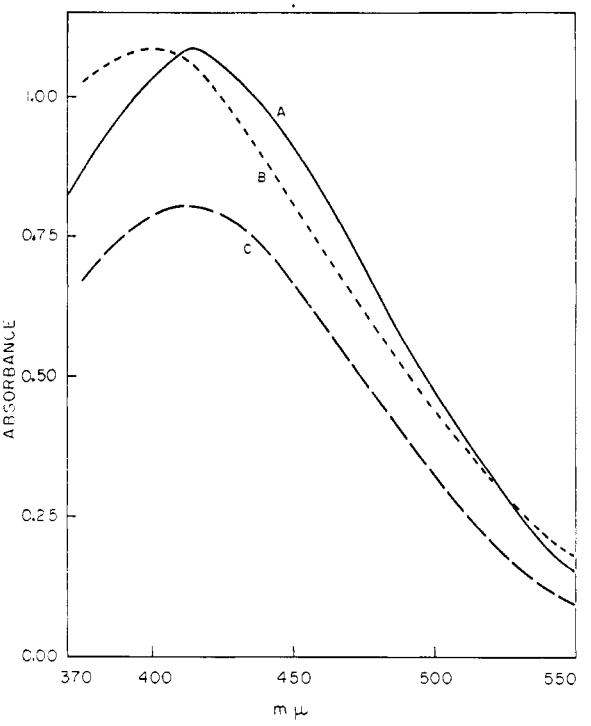



**Figure 2. Absorption spectra of products of modified *p*-nitrobenzoyl chloride procedure**

Histidine and imidazole were treated with 1.0 ml. of *p*-nitrobenzoyl chloride reagent. All other conditions were in accordance with the prescribed colorimetric procedure. The spectrophotometer was adjusted to 100% transmittance with a reagent blank.
*A.* $1 \times 10^{-4}M$ 1,2-di(*p*-nitrobenzamido)ethylene in aqueous alkali (instrument adjusted to 100% transmittance with water)
*B.* 5.5 Micromoles of histidine
*C.* 1.5 Micromoles of imidazole

**Table II. Compounds Yielding Colored Products with *p*-Nitrobenzoyl Chloride Reagent**

| Compound | Micromoles of Compd. for Equiv. Color Intensity at 420 mμ |
|---|---|
| Imidazole | 1.0 |
| Histidine | 3.4 |
| Phthalyl histidine | 3.4 |
| Methyl ester of histidine | 7 |
| Histamine | 8 |
| Benzimidazole | >100 |
| Aniline | 25 |
| Arginine | >100 |
| Hydrazine | 1.3 |
| Hydroxylamine | 18 |
| Phenylalanine | 50 |
| Tryptophane | 45 |
| Tyrosine | 100 |

The product was recrystallized from ethyl alcohol and again from acetone. Decomposition point was 256° C.

| Element Analysis, % | Calcd. for $C_{16}H_{12}O_6N_4$ | Found |
|---|---|---|
| Carbon | 53.93 | 53.81 |
| Hydrogen | 3.40 | 3.56 |

The absorption spectrum of the compound in aqueous alkali had a single peak at 417 mμ; the molar absorption coefficient was $1.104 \times 10^4$ at 417 mμ (Figure 2).

ISOLATION OF COLORED PRODUCT FROM PROCEDURE FOR COLORIMETRIC ANALYSIS OF IMIDAZOLE. In 18 ml. of water 0.12 gram of imidazole was dissolved. Then 1.8 ml. of 1*M* sodium bicarbonate followed by 90 ml. of 0.06*M* *p*-nitrobenzoyl chloride in acetone were added. The mixture was kept at room temperature for 30 minutes. To this 36 ml. of 2.0*N* sodium hydroxide and 34 ml. of water were added. The clear red solution was extracted with ether. The ether extract was dried over calcium sulfate and evaporated to leave 0.2 gram of yellow powder. The yield as 1,2-di(*p*-nitrobenzamido)ethylene was 32%. The

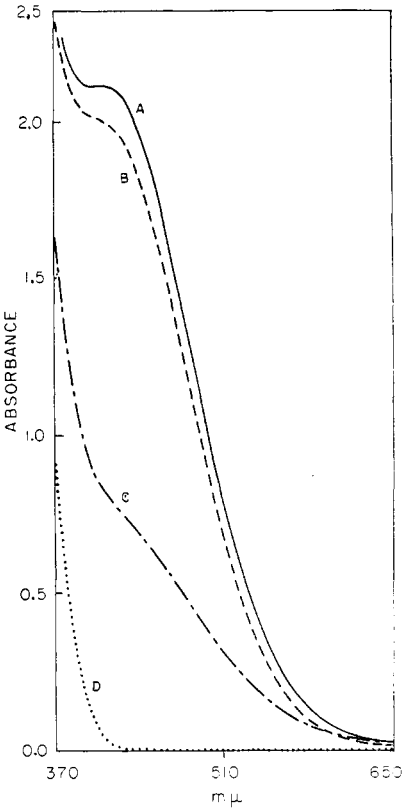



**Figure 3. Absorption spectra of products of *p*-nitrobenzoyl chloride procedure**

All samples were subjected to prescribed colorimetric procedure.
*A.* 2 Micromoles of 1,2-di(*p*-nitrobenzamido) ethylene
*B.* 2 Micromoles of imidazole
*C.* 5 Micromoles of histidine
*D.* Reagent blank

product was recrystallized from ethyl alcohol. Decomposition point was 254° C. This point was not lowered when an intimate mixture of this product and 1,2-di(*p*-nitrobenzamido)ethylene prepared above were heated together. The absorption spectrum of this product coincided with that of the 1,2-di(*p*-nitrobenzamido)ethylene.

## DISCUSSION

Histidine and its derivatives, histamine and imidazole, gave colored products with the *p*-nitrobenzoyl chloride reagent. Eight other imidazole compounds tested (Table I) failed to give colored products. Several compounds listed in Table II other than imidazoles gave colored products, but the concentrations of these compounds required for equal color intensity were at least tenfold greater than those for the reactive imidazole compounds. Exceptions were hydrazine and hydroxylamine. The sensitivity of the method to $1 \times 10^{-6}$ gram of hydrazine may make the method of some value for the analysis of hydrazine. However, this sensitivity for hydrazine is no greater, and in the case of hydroxylamine is less, than methods now in the literature (*2*, *7*).

From Figure 1, the method is capable of detecting 0.1 micromole of histidine when a colorimeter is used. By use of a more sensitive spectrophotometer at 417 mμ the sensitivity of the method is increased to 0.025 micromole of histidine or 4 γ.

Routine analyses of imidazole and histidine over a period of several months gave a standard deviation ($\sigma$) of ±5.0%. Accuracy of the method will be considered in subsequent papers on the applications of the method to the analysis of histidine in urine, and of carnosine and anserine in muscle tissues.

Comparison of absorption spectra of purified 1,2-di(*p*-nitrobenzamido)ethylene and of colorimetric analysis mixtures was difficult because of the high absorption of the latter mixtures below 400 mμ (Figure 3). This absorption below 400 mμ was due to *p*-nitrobenzoic acid from the hydrolysis of excess *p*-nitrobenzoyl chloride reagent. A better comparison of spectra could be obtained by development of the color with one fifth of the prescribed amount of *p*-nitrobenzoyl chloride reagent, and by adjustment of the spectrophotometer to 100% transmittance with a reagent blank. This comparison is made in Figure 2. The spectral peaks of 1,2-di(*p*-nitrobenzamido)ethylene and of imidazole color product occur at 417 mμ. The peak for the histidine color product appears to be at 400 mμ.

A few comments on details of the procedure should be made. The method gives erratic results if sodium bicarbonate is omitted or replaced by sodium acetate. The amount of sodium bicarbonate added is not sufficient to neutralize all hydrochloric acid liberated by reaction of the *p*-nitrobenzoyl chloride present. However, addition of greater amounts of sodium bicarbonate than are called for in the procedure was unsatisfactory. The ratio of acetone to water during the reaction of sample and *p*-nitrobenzoyl chloride is critical. Greater or lesser amounts of acetone led to erratic results. Neither *m*-nitrobenzoyl chloride nor 3,4-dinitrobenzoyl chloride could replace *p*-nitrobenzoyl chloride. The time of reaction with *p*-nitrobenzoyl chloride prior to addition of alkali is not critical, and a longer period than 10 minutes is without harm. The color produced after addition of alkali is stable for several hours.

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