THE ULTRAVIOLET ABSORPTION SPECTRA OF HISTAMINE, HISTIDINE, AND IMIDAZOLE; EFFECT OF pH AND CERTAIN FOREIGN IONS ON THE SPECTRUM OF HISTAMINE

by

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TABLE OF CONTENTS

INTRODUCTION

The purpose of this research has been to establish the ultraviolet absorption spectra of imidazole and certain of its derivatives, and, if possible, to apply the experimental results to theoretical considerations of molecular structure and to the quantitative determination of these compounds.

Special attention has been given to the molecular structure of imidazole, since it forms the fundamental structural feature of its various derivatives that are of great practical interest (see Table I for the structural formulas of the compounds studied). In addition, the spectral properties of histamine have been studied extensively, as this compound is ultimately of the greatest practical significance in establishing the mechanisms of the biological reactions associated with anaphylaxis and allergy.

Histamine has long been recognized as the agent immediately responsible for the occurrence of allergic reactions (5, 6, 21, 27, 64). Within recent years, the development of the familiar antihistamines (substances that are antagonists to the effects of histamine) has provided added impetus to investigations into the underlying principles of the interactions of histamine with various substances of biological interest. In particular, Tolstoouhov (69) has suggested that ultimately any evaluation of the biochemical reactions involved in anaphylaxis must arise from the application of physical chemical principles. Thus from both the theoretical and practical viewpoints it is

important to investigate thoroughly the most fundamental properties of histamine and its parent compound, imidazole. This added knowledge may then help lead eventually to an understanding of the allergic reaction and its effective control.

LITERATURE REVIEW

Theory and Applications of Ultraviolet Absorption Spectra

Few areas of scientific research have been so fruitful, or had so many practical applications, as the general field of spectroscopy. In particular, spectroscopy is one of the most direct and precise means of measuring the various internal degrees of freedom of atoms and molecules, i.e. the configurations and energies involved in rotational, vibrational, and electronic transitions.

The basic theory of spectroscopy involves the assumption that a given molecule may interact with electromagnetic radiation in such a manner that various transitions occur whose energies are proportional to the frequency of the radiation. (The principles involved in ultraviolet spectroscopy to be presented here are discussed at length in two important references: the survey by Friedel and Orchin (28), and the excellent review by Braude (12).)

If we consider only the absorption of radiant energy by a molecule as the only form of interaction, as distinct from diffraction and other phenomena, then this radiant energy causes the formation of an activated or excited molecule,

which exists in a state of higher energy than in its original normal, or unexcited, state. The difference between the energies of the two states is a function of the frequency of the radiation absorbed; this is stated mathematically by the following relationship where E^1 is the energy of the molecule

$$E^1 - E^0 = h\nu = hc/\lambda$$

in its excited state; E^0 is the energy associated with the original, or ground state, of the molecule; h is Planck's constant; ν is the frequency of the radiation; c is the velocity of light; and λ is the wavelength of the radiation.

In these basic principles, no mention is made of the manner in which the absorbed radiation is accommodated by the molecule; it is found, however, that the radiation in certain regions of the electromagnetic spectrum are effective in producing unique types of excitation in molecules. Thus rotational-energy transitions require low energies, and hence are to be observed in a region where the frequency of the radiation is low, or where the wavelengths are rather long. Similarly, vibrational transitions require somewhat higher energies, and the radiation necessary to produce vibrational transitions have correspondingly higher frequencies or shorter wavelengths. Typical vibrational-rotational spectra are observed in the infrared region of the spectrum.

Absorption in the visible and ultraviolet regions results in electronic transitions within the molecule; for absorption

in the near ultraviolet region (2000 - 4000 Å), the excitation energy is within the range 70 - 140 kcal./mole, which is higher than the energy required for rotational and vibrational transitions but is lower than the energy required for the rupture of most covalent bonds.

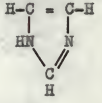
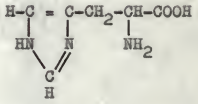
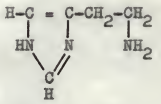
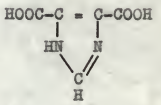
There are two types of electronic structures that may account for the absorption of ultraviolet radiation by a molecule: the unpaired electrons of free radicals, and the non-localized electrons that make up conjugated unsaturated bonds. By far the most common source of ultraviolet absorption is the presence of conjugated double bonds within the molecule. Thus, for example, the typically "aromatic" compounds are usually characterized by strong absorption bands in the ultraviolet region.

The rigorous interpretation of ultraviolet absorption data into useful information concerning the structures of molecules is often a complex procedure, and involves the application of results arising from the theoretical consideration of the molecular orbitals of electrons. Such a treatment is beyond the scope of this thesis; however, it is possible from the data obtained to deduce some items of a more general nature that may give indications of molecular structure. These conclusions will be dealt with in a later section.

De Gouveia et al. (18), and Saidel et al. (65) have previously reported the ultraviolet absorption spectrum of histidine; the spectra of imidazole and histamine, however,

have not been reported in the literature. Thus the experimental results presented in this thesis represent largely a new contribution to this aspect of their chemistry. Earlier workers (10, 14, 24, 25, 33, 42) had attempted to obtain the

Table 1. Structural formulas of the compounds studied.

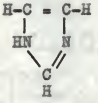
Compound	Structure
Imidazole	
Histidine	
Histamine	
Imidazole-4,5-dicarboxylic acid	

ultraviolet spectra of the imidazole derivatives, but the realization of this goal had to await the development of instruments capable of responding to wavelengths below 220 mμ.

This requirement has now been met to a large extent, and although no completely satisfactory instrument is in wide use for this purpose at the present time, the spectrophotometer employed in this work was found to be adequate.

The Molecular Structure of Imidazole

Of all heterocyclic nitrogen-containing compounds, imidazole has traditionally been one of the most difficult to characterize in terms of molecular structure. Ordinarily, the structure of imidazole is written in the form first proposed by Japp (44) in 1882, viz.



However, there are many unique properties of imidazole, both chemical and physical, that cannot be adequately explained by this structure.

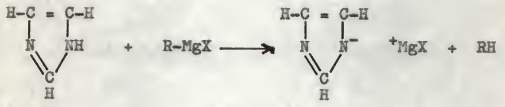
For example, the melting point (90°) and the boiling point (256°) of imidazole are remarkably high for a compound of its molecular weight of 68. No less striking is its apparently high degree of association in non-polar solvents: in particular, one attempt to obtain the molecular weight of imidazole by ebulliometry in boiling benzene (38) resulted in an apparent formula weight of 250, nearly four times the accepted value. On the other hand, the Victor Meyer procedure (32, 39, 40, 74),

gave the normal value, indicating that imidazole exists as the monomer in the vapor state.

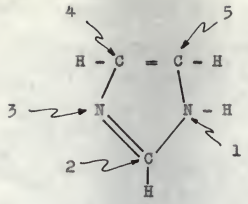
Imidazole is very slightly soluble in non-polar solvents, but is so soluble in water that the upper limit of its solubility in water has not been established (38).

Many unusual chemical properties of imidazole have also been reported. For example, it is found to be aromatic in character, undergoing typical aromatic substitution reactions rather than addition to the unsaturated bonds (38). It is resistant to quite drastic treatment with acids and bases, and is inert to many oxidizing agents, although it can be oxidized readily by potassium permanganate and hydrogen peroxide with the formation of oxamide (38).

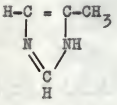
Of particular interest is the pseudoacidic nature of the imino hydrogen atom, which can be displaced under certain conditions. Thus imidazole is capable of acting as a very weak acid, whose pK has recently been reported (73) to be 14.52. Further evidence of this behavior is the ability of imidazole to decompose Grignard-type compounds (56, 57):



Finally, it should be noted that attempts to synthesize imidazole derivatives with substituents in either the 4- or 5- positions,

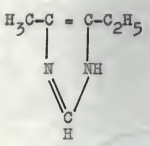


result in the formation of only one isomer, whereas two would be expected to appear (29, 34, 43, 63). Thus the two nitrogen atoms in the imidazole nucleus are apparently equivalent; this seemingly anomalous behavior has led to the somewhat ambiguous nomenclature associated with the naming of imidazole derivatives. Suppose, for example, it is desired to name the compound



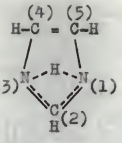
Since by convention the secondary nitrogen atom is the 1-position in the ring, and the numbering proceeds in such a way that the tertiary nitrogen is given the smallest possible number, the proper name here should be 5-methylimidazole. Yet if it is found that 4-methylimidazole and 5-methylimidazole are the same compound because the two nitrogen atoms are indistinguishable, then the exact definition of the secondary nitrogen becomes impossible and the compound might also be correctly named 4-methylimidazole. The common practise is to

recognize this situation by expressing both names; hence in the present case the proper name would be 4(or 5)-methyl-imidazole. Likewise, the compound



is named 4(or 5)-methyl-5(or 4)-ethylimidazole. Such a naming system does not necessarily imply uncertainty in the structural formula; rather it may be regarded as the recognition of the fact that the two nitrogen atoms are equivalent, which has a deeper significance.

The whole of this discussion leads ultimately to the conclusion that the imidazole nucleus is not only conjugated with respect to its electronic structure, but also that the imino hydrogen atom is tautomeric in nature. Thus the true structure of imidazole may be represented as a resonance and tautomeric hybrid of a variety of structures. While it is not intended to represent the true structure of the imidazole molecule, the following is presented as a modification of the classical structure, in an attempt to recognize the tautomeric nature of the imino hydrogen:



This structure has been confirmed by several spectroscopic techniques. Donohue et al. (19, 20) have determined by means of x-ray diffraction measurements that the lengths of the $C^{(2)}\text{-}N^{(3)}$ and $C^{(2)}\text{-}N^{(1)}$ bonds are of equal length in histidine hydrochloride monohydrate. Studies of the Raman spectra of imidazole by Edsall et al. (23, 30) and by Shigorin and Syrkin (66), and the infrared spectral studies of Otting (58) have confirmed this result. A theoretical treatment by Tsitsishvili (71) also leads to the conclusion that the imino hydrogen is not localized on either nitrogen atom.

In keeping with the accepted methods of expressing the structural formulas of imidazole and its derivatives, the classical structure will be used in this thesis as a matter of convenience, with the understanding that it is valid in the sense that it represents an admissible canonical form for the molecule.

Analytical Methods

The methods available for the quantitative analysis of the imidazole derivatives are numerous. Basically, however, it can be said that each compound is ordinarily determined by

virtue of a unique structural feature of the molecule. For example, histidine lends itself to some of the techniques commonly applied to the analysis of amino acids, and the specific physiological action of histamine has been developed into a biological method for its determination. Only a very few reactions are common to imidazole, histamine, and histidine; notable examples are the Kjeldahl method and the widely-used diazonium coupling reaction.

It would be appropriate at this point to review briefly some of the methods that have been proposed and are being used for the quantitative estimation of these substances, and to examine critically some of their respective advantages and limitations. To begin with, it is fair to say that absolutely reliable methods for the determination of these substances are rare indeed; the various techniques that may be applied quite often give reproducible results only under carefully standardized conditions, with complex procedures, or with rather elaborate experimental equipment.

It may be suggested that since imidazole, histamine, and histidine are nitrogenous materials, they might be analyzed by the common Kjeldahl or Van Slyke procedures. However, the refractory nature of the imidazole ring does not readily allow its quantitative reduction to any of the common nitrogen compounds, although Mazadro (55) and Fontana (26) have reported that satisfactory results can be obtained by modifications involving, respectively, complete ashing with sulfuric acid,

and by catalysis with selenium or mercuric sulfate. Yamagishi and Yokoo (76) have applied azometry to the determination of histamine. In a recent article by Alford (2), it is reported that good precision and accuracy can be obtained in the high-temperature catalytic oxidation of the imidazole derivatives, the oxidation products being reduced by copper to nitrogen and carbon dioxide and analyzed as such. Despite the advantages claimed by these authors, however, it cannot be said that any one of these techniques is entirely suitable for the rapid and convenient determinations required in routine work, although they may be extremely valuable in providing satisfactory routes to the elemental analysis of the substances in question, and hence a basis upon which to evaluate the more convenient and reproducible methods.

One of the most widely used methods for determining histamine is the biological assay of Barsoum and Gaddum (8), in which a dose of an unknown histamine solution is applied to the isolated ileum or intestine of the guinea pig or rabbit, and the concentration of the solution ascertained by the magnitude of the response of the tissue. This method is probably the most specific, precise, and sensitive of all tests available for histamine; in fact, it has been reported (1, 31) that with suitable modifications of procedure and instrumentation it is possible to determine as little as one part per million of histamine in an aqueous solution. However, the necessity of sacrificing experimental animals, as well as the inconveni-

ence involved in the raising and caring of them, accounts for the obvious reluctance of workers in the non-medical sciences to adopt this procedure for routine work.

Histidine, although formally an amino acid, does not undergo many of the stoichiometric reactions by which this class of compounds is ordinarily determined. The failure of histidine to behave as a conventional amino acid in this respect may be attributed to the presence of either or both of the nitrogen atoms in the imidazole ring, which have a marked effect on the reactions of the $-CH(NH_2)COOH$ residue. For example, it has been reported (45, 50) that the formol titration of histidine reveals somewhat less than the theoretical amount present; this is due to the anomalous reaction of histidine with formaldehyde, whose mechanism is more complex than that of the usual formaldehyde - amino acid interaction. Furthermore, the titration method of Popovici and Radelescu (60), in which most amino acids are titrated successfully in dilute dioxane, gives only 59 per cent of the theoretical amount of amino nitrogen in histidine. On the other hand, McGurdy and Galt (52) were able to titrate histidine hydrochloride conductometrically with perchloric acid in a mixture of formic acid and dioxane.

Both histamine and histidine have been studied chromatographically by means of the ninhydrin reaction (62, 70). This technique, aside from the time required for the chromatography process, is one of the simplest methods reported from the

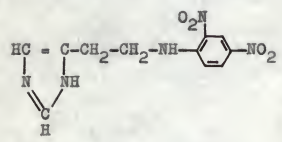
standpoint of ease of operation.

A unique reaction of histidine with bromine was developed by Kapeller-Adler (46) for its analysis. Here histidine is treated with bromine, the solution made strongly alkaline with ammonium carbonate, and the resulting blue-violet color used as an index of the histidine concentration. Madame Kapeller-Adler reported that the reaction is specific for histidine, in the sense that only its methyl ester and histamine give color reactions under the conditions employed, with those colors being much weaker than the intense blue-violet due to histidine; furthermore, its high sensitivity allowed the detection of as little as twenty parts per million of histidine. Numerous modifications of this method have been proposed (41), but the stoichiometry of the reaction has not been established, and it suffers from the familiar hazards associated with working with bromine.

Probably the most frequently used method for the determination of histamine, histidine, and imidazole involves the coupling of a diazonium cation with the imidazole nucleus to form azo dyes whose intensities are then measured colorimetrically (4, 37, 47). In spite of the widespread popularity of the method, it is noteworthy that it suffers from two basic and serious difficulties, namely that the structures of the dyes have never been firmly established, and that the dyes themselves tend to be unstable, fading after passing through an intensity maximum and thereby leading to uncertainties in

the colorimetric measurements in many cases. The common procedure is to treat the alkaline unknown solution with p-diazobenzenesulfonic acid and to read the maximum color intensity colorimetrically or spectrophotometrically at various time intervals until a maximum reading is obtained; this maximum intensity is then taken as a measure of the concentration of the unknown constituent. In view of the side reactions that may occur, and the inherent instabilities of the dyes, a surprising feature of the method is that it is highly reproducible and precise under carefully controlled conditions and within certain concentration limits. It has been suggested that the technique might be made more reliable by the addition of antioxygens (36) and by the extraction of the dyes into a nonaqueous solvent (22, 51) in order to stabilize them, but these modifications add little to the merits of the method.

McIntire and co-workers (53) have proposed a technique for the analysis of histamine that has become accepted as the standard procedure in many laboratories. This is based upon the reaction of histamine with 2,4-dinitrofluorobenzene to produce N^{α}-(2,4-dinitrophenyl) histamine,



which is then purified and determined spectrophotometrically.

McIntire reports that this derivative may be obtained in excellent yield under proper conditions; that it is very stable; that as little as 0.5 microgram of histamine may be determined with good accuracy; and that the individual determinations are highly reproducible. Thus the procedure meets the requirements of a good analytical method, and therefore deserves consideration in research work where the routine determination of histamine is required.

Various other methods have been proposed for the analysis of histidine, for example the precipitation of the mercuric sulfate complex and weighing as such (61); the ultraviolet spectrophotometric determination of its copper complex (15); the formation of various organic derivatives (9, 13, 16, 48, 54, 72, 75); and the action of various bacterial preparations (11, 35, 68).

MATERIALS AND METHODS

Instrumental

The principal instrument used throughout this work has been the Beckman Model DU Spectrophotometer, equipped with a photomultiplier tube designed to increase the sensitivity of the instrument at wavelengths as low as 200 mμ. Correspondence with the manufacturer brought assurance that experimental data taken at these short wavelengths are reliable when this instrument is operated at an appropriate sensitivity. It should be noted that this instrument is among the most up-to-

date and reliable spectrophotometers presently available. Its modifications allow a widely extended range of operation in the ultraviolet region and a much higher degree of stability over the older models which were equipped with less sensitive receptors and with battery-operated electrical components.

One-centimeter matched fused silica cuvettes were used as sample cells in all cases; the temperature of the cell compartment was maintained at 25° ± 0.2° C by means of thermospacers, through which water was circulated from a thermostated water bath. The radiation source was a hydrogen discharge lamp, Beckman Part No. 2230, and the entire spectrophotometer assembly was powered by means of a line-operated Beckman DU Power Supply, Beckman Part No. 13400.

The silica cuvettes were kept scrupulously clean by storing them when not in use in the cleaning solution recommended by the manufacturer, consisting of equal volumes of 3N hydrochloric acid and 80 per cent ethanol. Before each series of spectral measurements they were rinsed thoroughly with distilled water, and the optical surfaces dried with soft tissue.

In order to insure maximum reliability of the absorbance measurements, all readings were corrected for the absorbance of the blank at every wavelength studied, and individual absorbance measurements were repeated until consistent values were obtained. The instrument was operated according to the "maximum sensitivity" procedure recommended by the manufacturer, as described in the instruction manual accompanying the spec-

trophotometer.

All pH measurements were made on a Leeds and Northrup Type 7663 Universal pH Meter, using Leeds and Northrup glass electrodes. The literature accompanying this instrument indicates that the maximum limit of error is ± 0.05 pH unit, and that individual measurements are reproducible to within 0.02 pH unit. Before each series of measurements, the meter was standardized with 0.05 M potassium hydrogen phthalate having a pH of 4.01 at 25°.

Reagents

Attempts were made to used only materials of the highest available purity whenever possible. Imidazole, histidine, and imidazole-4,5-dicarboxylic acid were obtained from Eastman Organic Chemicals. Histamine was purchased from Eastman and from the Fisher Scientific Company; the material obtained from the Fisher Company carried a stated quality of Reagent Grade, and was used without further purification except for drying. Histamine samples obtained from the Eastman Company gave comparable results with those found for the Fisher material.

Of potential difficulty in all cases was the lack of assurance of extreme purity of the materials; however, these reagents gave consistent experimental results both after weighing *in vacuo* and after desiccation for a period of several days over Anhydrone (anhydrous magnesium perchlorate). The materials were stored under refrigeration at all times in order to prevent decomposition.

No completely satisfactory method is known for the purification of histamine. Correspondence with the manufacturers concerned brought no assurance of the purity of the product or of the means of purification. It is possible to recrystallize histamine effectively from benzene; however, the low recovery (approximately 20 per cent) renders this technique economically prohibitive when considered in terms of the initial cost of the compound (17). Thus it was necessary to report all experimental data on the assumption of high purity after drying.

Perchloric acid and the metal perchlorates were purchased from the G. F. Smith Chemical Company; these reagents are all of the highest available purity. The metal perchlorates were found by standard analytical procedures to be in excess of 99 per cent pure as the respective hydrates.

The hydrochloric acid and other inorganic reagents were Baker and Adamson Reagent Grade materials, and were used without further purification.

EXPERIMENTAL

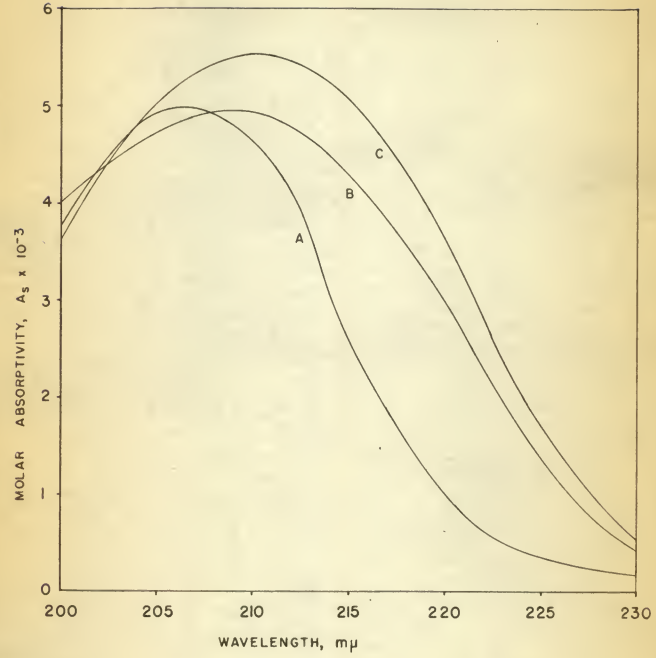
Ultraviolet Spectra in Pure Aqueous Solution

The ultraviolet absorption spectra of histamine, histidine, and imidazole in pure aqueous solution are shown in Plate I. The basic similarity of these spectra indicates that the imidazole nucleus is the primary absorbing species. The respective wavelengths of maximum absorbance, and the corresponding molar absorptivities, are as follows: imidazole,

PLATE I

Ultraviolet absorption spectra: A, imidazole; B, histamine; C, histidine.

PLATE I


6
5
4
3
2
1
0
MOLAR ABSORPTIVITY, $A_s \times 10^{-3}$
200
205
210
215
220
225
230
WAVELENGTH, mμ
A
B
C

206 mμ, 4.99 x 10^3 liters-mole^{-1}-cm.$^{-1}$; histamine, 209 mμ, 4.94 x 10^3 liters-mole^{-1}-cm.$^{-1}$; histidine, 210 mμ, 5.52 x 10^3 liters-mole^{-1}-cm.$^{-1}$.

In all cases, Beer's Law is obeyed remarkably well; the absorbance was found to be a linear function of the concentration within the range 2 x 10^{-5} to 2 x 10^{-4} molar. The Beer's Law plot for histamine is shown in Plate II as a representative example.

Effect of pH

It was found that adjustment of the pH to below 2.0 by the addition of an acid to the system invariably caused a bathochromic shift (i.e., a shift toward longer wavelengths) in the absorption spectrum of histamine, with a corresponding increase of approximately 11 per cent in the molar absorptivity. The spectra of imidazole and histidine, on the other hand, were not shifted by changes in pH, but in each case the absorptivity was enhanced approximately 5 per cent at low pH values.

For purposes of comparison, the relative effects of hydrochloric and perchloric acid upon the spectra were determined. The results were practically identical within the limits of experimental error. With the pH adjusted to below 2.0, the following wavelengths of maximum absorbance and the corresponding molar absorptivities were found for each compound: imidazole, 206 mμ, 5.05 x 10^3 liters-mole^{-1}-cm.$^{-1}$;

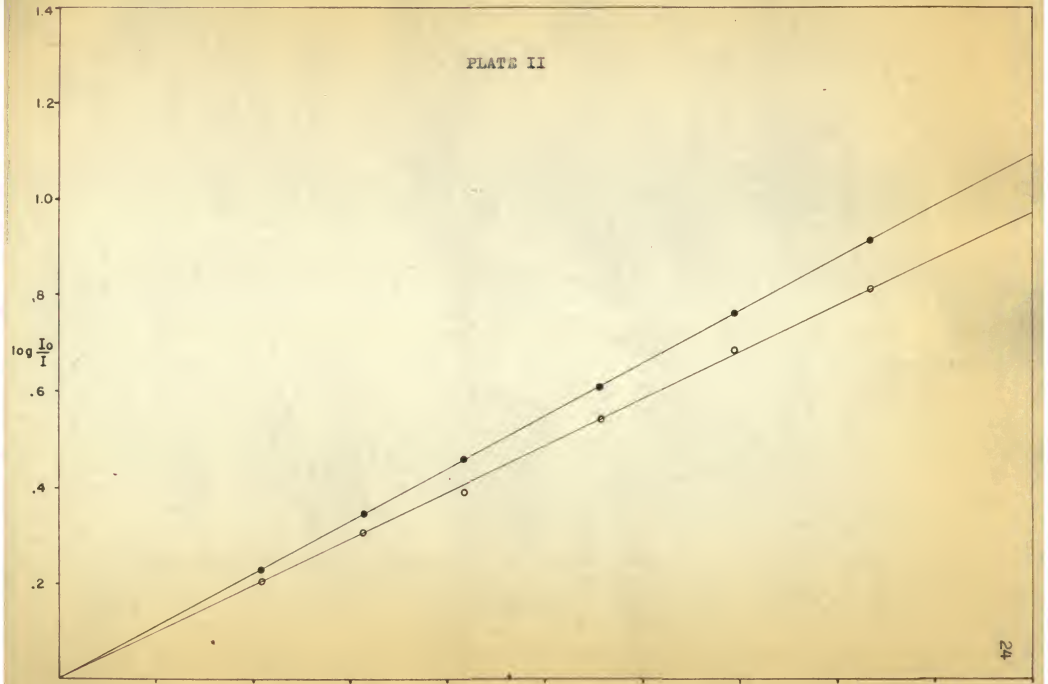
PLATE II

Adherence of histamine to Beer's Law at 209 mμ.

• pH 2.0

o pH 7-9

PLATE II


1.4
1.2
1.0
.8
log Io/I
.6
.4
.2
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
CONCENTRATION, MOLES—LITER -1 x 10^4

histamine, 211 mμ, 5.47×10^3 liters-mole^{-1}-cm.$^{-1}$; histidine, 210 mμ, 5.56×10^3 liters-mole^{-1}-cm.$^{-1}$.

It was not possible to determine the spectra of these substances under conditions of high pH because of the high degree of interference of the carbonate ion, and the corresponding danger of carbonate formation by the absorption of atmospheric CO_2 by a hydroxyl base.

Effects of Interfering Ions

Among the common anions studied, only chloride and perchlorate were found to be completely satisfactory with regard to non-interference in the spectra of histamine, histidine, and imidazole. The acetate, sulfate, and phosphate ions begin to absorb strongly below 210 mμ, and the strong absorbance of the carbonate ion below 250 mμ also led to difficulties from interference. The nitrate ion has an extremely wide absorption band between 200 and 270 mμ, rising again to a maximum at about 300 mμ; the band below 270 mμ was found to be so intense that it completely interfered with attempts to determine spectra in its presence.

In sharp contrast to the interference by anions just mentioned, a wide range of choice was found to be possible in the cations that may be present without causing appreciable interference in the spectra. Among the common cations studied, only copper (II) was found to exhibit a unique absorption spectrum below 230 mμ. Sodium and potassium had no effect

upon the spectra; the influence of hydrogen ions has been discussed previously.

It was suspected that the transition metals might cause interference by forming coordination complexes with histamine, histidine and imidazole, as well as by exhibiting their own unique absorbance; if such were the case, the absorption spectra might thus be considerably altered. Furthermore, it was suggested that this effect could be reversed under conditions of low pH, where the metal complexes would be less likely to form because of the intensive competition of the overwhelming number of protons for coordination on the Lewis base sites of the molecules.

These assumptions were verified to some extent in the case of histamine. The effects of cobalt, nickel, and copper in typical determinations of the histamine spectrum are shown in Tables 2-4. While the presence of these ions does not produce profound changes in the spectrum, and while the effect is not the same in all cases, it is evident that these metals do affect the absorbance readings to an appreciable extent, and that each effect is counteracted by the addition of perchloric acid to the system.

It should be noted that the effect of a given complexing metal ion cannot be predicted a priori; this fact is well demonstrated in the present case. Cobalt enhances the absorbance, while nickel depresses it slightly; in addition to forming a complex with histamine, copper has the added effect

Table 2. Determination of the effect of cobalt (II) on the spectrum of histamine.

(Hn) x 10^5	($HClO_4$)	(Co^{++}) x 10^4	A_{209}	A_{210}	A_{211}	A_{212}
7.19	0.000	0.00	.351	.352	.350	.346
7.19	0.018 N	0.00	.388	.393	.396	.394
7.19	0.000	0.72	.356	.355	.354	.350
7.19	0.018	0.72	.384	.391	.394	.392
7.19	0.000	2.16	.357	.357	.355	.349
7.19	0.018	2.16	.386	.392	.395	.394

Table 3. Determination of the effect of nickel (II) on the spectrum of histamine.

(Hn) x 10^4	($HClO_4$)	(Ni^{++}) x 10^4	A_{209}	A_{210}	A_{211}	A_{212}
1.11	0.000	0.00	.549	.548	.545	.536
1.11	0.018 N	0.00	.595	.605	.608	.607
1.11	0.000	0.92	.544	.544	.540	.534
1.11	0.018	0.92	.595	.605	.610	.609
1.11	0.000	9.20	.543	.545	.542	.538
1.11	0.018	9.20	.596	.607	.611	.609

Table 4. Determination of the effect of copper (II) on the spectrum of histamine.

$(Hn) \times 10^5$	$(HClO_4)$	$(Cu^{++}) \times 10^5$	A_{209}	A_{210}	A_{211}	A_{212}
9.85	0.000	0.00	.485	.486	.483	.476
9.85	0.018 N	0.00	.552	.562	.567	.565
0.00	0.000	3.15	.046	.044	.043	.041
9.85	0.000	3.15	.570	.572	.571	.570
9.85	0.018	3.15	.581	.587	.589	.586
0.00	0.000	6.29	.093	.089	.086	.083
9.85	0.000	6.29	.646	.650	.653	.653
9.85	0.018	6.29	.627	.632	.633	.628

of increasing the absorbance by virtue of its own spectrum in this region. Quite good agreement can be seen in the absorbance readings taken at low pH values with those that are predicted by adding the contributions of the various absorbing components of the system.

Application to Quantitative Analysis

The fact that histamine, histidine, and imidazole obey Beer's Law under conditions of controlled pH, would suggest the possibility that these compounds could be determined quantitatively by means of their respective absorption spectra. Indeed, attempts to perform such analyses have met with success, both with each compound taken separately and with synthetic mixtures of imidazole and histidine.

Typical results for the determination of these compounds are shown in Tables 5-8. The concentrations of the unknown solutions were calculated from the familiar Beer-Lambert-Bouguer Law,

$$A = \sum_{i=0}^{i} A_{s_i} \; bc_i$$

where

- A is the measured absorbance of the solution at a particular wavelength.
- A_{s_i} is the molar absorptivity of the ith component, equivalent to the absorbance of a one-molar solution of the component.

b is the length of the light path through the solution; in the present case b is constant and equal to 1 cm.

c_i is the concentration of the ith component, in moles per liter.

Table 5. Quantitative determination of histamine; $A_s = 4.94 \times 10^3$ at 209 mμ.

mg taken	mg found	mg reported	recovery
26.9	27.3 27.2 26.9 26.5 26.2	26.8	99.6%
20.9	22.5 22.0 21.6 19.3 19.6	21.0	100.5%
90.1	92.8 92.1 88.5 92.1 87.7	90.6	100.6%
79.0	76.6 77.9 79.6	78.0	98.7%

Table 6. Quantitative determination of imidazole; $A_s = 4.99 \times 10^3$ at 206 mμ.

mg taken	mg found	mg reported	recovery
58.5	59.2 58.3 58.9	58.8	100.5%
27.5	27.4 27.5 27.4	27.4	99.6%
23.4	23.7 23.6 23.6	23.6	100.8%

Table 7. Quantitative determination of histidine; $A_g = 5.52 \times 10^3$ at 210 mμ.

mg taken	mg found	mg reported	recovery
97.8	96.7 97.0 96.4	96.7	98.9%
38.9	39.1 39.3 39.3	39.3	101.0%
26.3	26.5 26.2 25.9	26.2	99.6%

Table 8. Quantitative determination of a synthetic mixture of imidazole and histidine (see text for details of calculations).

Im(mg.)taken	Im(mg.)found	Hd(mg.)taken	Hd(mg.)found
78.8	81.9	75.5	72.8
	84.6		68.1
	79.4		77.5
	76.5		81.0
	76.9		82.2

mg. imidazole reported: 79.9; recovery: 101.4%
mg. histidine reported: 76.3; recovery: 101.1%
per cent imidazole taken: 51.07%; reported: 51.15%

As a typical example, it was desired to find the concentration of histamine in an unknown solution. The solution as received was stated to be approximately 2×10^{-3} M, having a pH above 8. Accordingly, five 4:100 dilutions were prepared in order to yield a final concentration in the useful range for the histamine analysis, and the respective absorbance values measured at 209 mμ. These values were found to be 0.604,

0.543, 0.526, 0.500, and 0.491. The corresponding concentrations, multiplied by the dilution factor of 25 in order to represent the original concentration, were calculated to be 2.458×10^{-3}, 2.448×10^{-3}, 2.420×10^{-3}, 2.385×10^{-3}, and 2.355×10^{-3} molar. The average value was reported to be 2.413×10^{-3}, and the true value was then revealed to be 2.421×10^{-3} molar. In terms of weight units, the solution was found to contain 26.8 mg per 100 ml; this represents a recovery of 99.6 per cent when compared to the true weight of 26.9 mg.

Recently Sternberg, Stillo and Schwendeman (67), and Barnett and Bartoli (7) have reported successful results in determining the various constituents of multicomponent systems by spectrophotometric measurements. These methods depend in general upon the solution of n simultaneous equations involving the concentrations of n components from absorbance measurements taken at n wavelengths where the molar absorptivities of the components are known. Thus

$$A^{\lambda_1} = A_{s_1}^{\lambda_1} C_1 + A_{s_2}^{\lambda_1} C_2 + \cdots + A_{s_i}^{\lambda_1} C_i$$

$$A^{\lambda_2} = A_{s_1}^{\lambda_2} C_1 + A_{s_2}^{\lambda_2} C_2 + \cdots + A_{s_i}^{\lambda_2} C_i$$

$$\cdots$$

$$A^{\lambda_i} = A_{s_1}^{\lambda_i} C_1 + A_{s_2}^{\lambda_i} C_2 + \cdots + A_{s_i}^{\lambda_i} C_i$$

where A^{λ_i} is the measured absorbance of the system at the wavelength λ_i.

$A_{s_i}^{\lambda_i}$ is the molar absorptivity of the ith component at the ith wavelength.

C_i is the concentration of the ith component.

In this system of equations the various molar absorptivities, $A_{s_i}^{\lambda_i}$, are known from previously determined spectra of the pure components taken over the entire spectral region in question. The absorbance values A^{λ_i} are measured experimentally, hence all terms are known except the concentrations of the n components, which can then be determined by the solution of the n equations.

It was thus suggested that this type of approach might be applicable to the simultaneous determination of various mixtures of histamine, histidine, and imidazole. However, it was found that only the imidazole-histidine system produced satisfactory results. The imidazole-histamine system gave very poor agreement with the theoretical amounts of the components taken, revealing 133 per cent of the true amount of imidazole and only 24 per cent of the histamine present. This behavior may be attributed to the extremely close values for their respective molar absorptivities, and to the fact that their spectral curves are not sufficiently well separated to allow such a treatment. This would also suggest that similar difficulties would arise in any attempt to determine histamine and histidine simultaneously by this method.

The experimental results for the simultaneous determina-

tion of imidazole and histidine are shown in Table 8. Here the system of equations used to calculate their respective concentrations is

$$A_{206} = (4.99 \times 10^3)(Im) + (5.29 \times 10^3)(Hd)$$

$$A_{210} = (4.65 \times 10^3)(Im) + (5.52 \times 10^3)(Hd)$$

Solution of these equations leads to the result

$$(Im) = \frac{(5.52)A_{206} - (5.29)A_{210}}{2981},$$

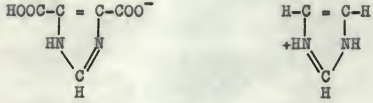
which allows the calculation of the imidazole concentration from absorbance measurements taken at 206 and 210 mμ. This value is then substituted into either of the two original equations to yield the concentration of histidine.

Imidazole-4,5-dicarboxylic Acid

In many respects, imidazole-4,5-dicarboxylic acid (see Table 1 for structural formula) was the most unique compound studied. Its melting point, 288°, is remarkably high for a compound of its molecular weight (156.10). It was found to be virtually insoluble in water, dilute mineral acids, and all of the common organic solvents. These observations indicate that the compound, in the solid state, is characterized by very strong intermolecular bonding. It was seen to be inert to the action of bromine and potassium permanganate, but dissolved readily in dilute sodium hydroxide solution, presumably forming

the monosodium salt (49). Upon boiling with 6N sulfuric acid the compound underwent decarboxylation to produce imidazole itself (38).

An interesting derivative of imidazoledicarboxylic acid was prepared by dissolving the acid in a solution of imidazole. The product recrystallized readily from water, forming large quantities of needle-like crystals. Upon filtering and drying, the derivative was reduced to a solid plastic mass. This derivative was presumed to be the compound imidazolium hydrogen imidazole-4,5-dicarboxylate,



The compound corresponding to this formula has been reported by Pauly and Ludwig (59); the melting point of the present material agreed well with their value of 245°. Elemental analysis* also indicated that this formula is correct for the derivative obtained.

For purposes of comparison, the spectra of this derivative and the potassium salt of imidazole-4,5-dicarboxylic acid were determined; these spectra are shown in Plate III. The values for the molar absorptivity of the imidazole derivative were

*Performed by the Microanalytical Laboratory, 164 Banbury Road, Oxford, England.

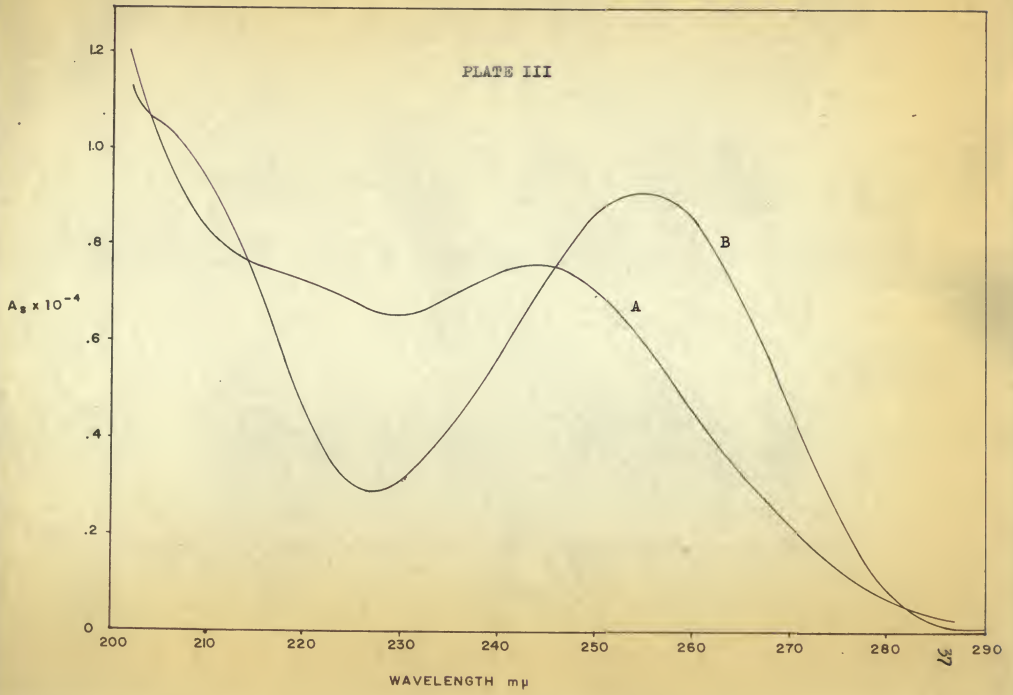
PLATE III

Ultraviolet absorption spectra of salts of imidazole-4,5-dicarboxylic acid.

A. Potassium salt

B. Imidazole salt

PLATE III

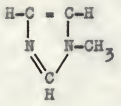

1.2
1.0
.8
.6
.4
.2
0
$A_s \times 10^{-4}$
A
B
200
210
220
230
240
250
260
270
280
290
WAVELENGTH mμ

calculated on the basis of the postulated formula of the derivative. The similarity of the two curves indicates that this hypothesis is valid.

Imidazoledicarboxylic acid was also found to dissolve readily in histamine solution, presumably with the formation of the histamine salt. Unlike the imidazole derivative, however, it was not possible to recrystallize this salt in order to study its spectral properties.

1-Methylimidazole

It was suggested that the number of possible resonance and tautomeric structures of imidazole might be greatly reduced by replacement of the imino hydrogen atom with an alkyl group, as in the case of 1-methylimidazole:



Such substitution should have the effect of completely inhibiting tautomerism within the imidazole nucleus, and thereby causing a profound change in the observed ultraviolet spectrum. If 1-methylimidazole should prove to exhibit a significantly different absorption spectrum from that of imidazole, then, the tautomeric nature of the imino hydrogen would be proven.

However, it was found that this imidazole derivative is

not available commercially, and attempts to prepare it by the direct methylation of imidazole were unsuccessful. It was not possible to confirm or deny this hypothesis.

DISCUSSION OF RESULTS

Reliability of the Spectral Measurements

It is realized that valid questions may be raised with regard to the spectral data taken at wavelengths as low as 200 mμ, the extreme lower limit of operation of the spectrophotometer. However, several significant points may be cited here in support of these experimental results. First, it should be recalled that the present instrument is among the most reliable and precise spectrophotometers available for work in the near ultraviolet region at the present time. In fact, the modifications that have been incorporated into the design of this instrument render it so sensitive in the short-wavelength region that at no time was it found necessary to operate at settings even approaching the maximum limit of its sensitivity. Admittedly, the ultimate confirmation or refutation of these data depends upon further studies in the vacuum ultraviolet region, for which equipment was not available in the present work.

Second, it should be noted that absorption spectra for the imidazole derivatives should be expected to appear in this region. Braude (12) reports that most isolated double bonds are responsible for the absorption of radiation in the region

150 to 200 mμ, and that conjugated systems begin to absorb above the longer wavelength. Hence, if conjugated double bonds are present in the imidazole nucleus, then one would reasonably expect to observe a well-defined absorbance curve at least above 200 mμ.

Finally, the spectrum of histidine found in the present study (A_s = 5.56 x 10^3 at λ_{max} = 210 mμ in acid solution) is in excellent agreement with that reported previously by Saidel and his co-workers (65) (A_s = 5.70 x 10^3 at λ_{max} = 212 mμ in acid solution). Thus the data reported here are confirmed to this degree by published results.

Interpretation of the Spectra

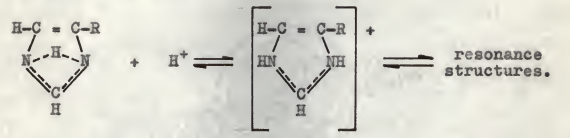
The basic similarity of the ultraviolet spectra of imidazole, histamine, and histidine would indicate that the heteronuclear, unsaturated imidazole ring is the primary absorbing species in each case. Furthermore, the side-chain groups present in the histamine and histidine molecules have apparently little effect upon the electronic structure of the imidazole nucleus, and hence only minor interactions occur between the electrons of the side-chain amino and carboxyl groups and those of the ring. At first, this may seem to be rather unexpected behavior, since these two groups are reportedly strongly auxochromic in character (28), i.e. they are substituents that do not themselves absorb radiation, but whose unshared electrons interact with an already conjugated system

to increase the absorption by that system. The absence of such a strong effect in the case of these imidazoles may be explained on the basis of the insulating effect of the aliphatic side-chain, which almost completely inhibits the electronic interactions that would be required for noticeable shifts in the spectra. On the other hand, the strongly auxochromic effect of the carboxyl groups attached directly to the imidazole nucleus is immediately seen in the spectrum of 4,5-imidazole-dicarboxylic acid, in which the two -COOH groups, being substituents of olefinic carbon atoms, provide the ideal structural conditions for a high degree of resonance.

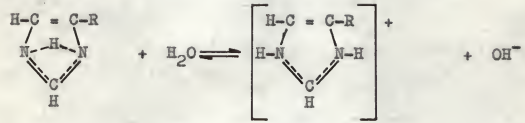
The energies found for the electronic transitions in imidazole, histamine, and histidine are in the range 135-139 kcal. per mole. This energy requirement is again rather high, indicating that although the imidazole nucleus does possess some degree of resonance character, this resonance is not nearly so great as in many other aromatic compounds, such as the derivatives of benzene, naphthalene, and pyridine.

It is significant that the result of protonating the imidazole nucleus, under conditions of low pH, is not to produce marked changes in the observed spectra. Of the compounds studied, only histamine was found to undergo a shift in the absorption spectrum, and that shift was only two millimicrons in magnitude. This may be explained in two ways: first, if the imidazole nucleus is symmetric, as the evidence for its tautomeric character would suggest, then the imidazolium ion

would quite logically possess the same symmetry, and the same resonance forms would be possible:



If such is the case, the spectrum would not be expected to be appreciably affected. Alternatively, it may be suggested that since these spectra were studied exclusively in aqueous solution, the imidazolium ion may be the principal species present, due to the hydrolysis reaction



Hence further protonation would only produce more of the same absorbing molecule and the same spectrum should be observed. This latter explanation is quite plausible when applied to the case of imidazole; however, it does not agree well with the experimental results found for histamine and histidine. It has been stated previously that the imidazole nucleus is the portion of the molecule that is most probably responsible for the appearance of the spectra. Yet the equilibrium constant of the reaction

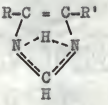
$$Im + H^+ \rightleftharpoons ImH^+ \qquad (\log K = 7.06)$$

is small enough (38) to indicate that the tertiary nitrogen atom in the imidazole ring is only weakly basic, hence is not appreciably protonated above pH 8-9. Furthermore, the primary amine group in the side chains of histamine and histidine is so basic that it is nearly completely protonated below pH 10, and its competition for protons is so great that an insignificant number of ring nitrogens in these molecules undergo hydrolysis above the pH range 8-9. Thus, since the spectra of histamine, histidine, and imidazole are so nearly the same, it follows that the same ring structure is present in all of these molecules at this high pH range, viz. the unprotonated, tautomeric structure.

The increase in the molar absorptivities of histamine, histidine, and imidazole under conditions of low pH may be readily understood if one considers that the absorptivity is a measure of the probability of the occurrence of a given electronic transition. This probability is in turn determined basically by the "effective cross-sectional area" of the absorbing portion of the molecule (12). For many molecules, e.g. the derivatives of benzene and pyridine, whose electrons are non-localized and free to resonate over the entire molecule, a comparatively large area is available for the collision of a photon with the electrons to be excited. Such a collision is then more efficient in producing an electronic transition, and the absorptivity is correspondingly increased. Thus in many aromatic compounds it is not uncommon to encounter molar

absorptivities well above 10^4 in magnitude (28). The spectrum of 4,5-imidazoledicarboxylic acid is a case in point. Here the two carboxyl groups not only increase the degree of conjugation within the molecule, thereby lowering the activation energy and shifting the absorbance curve to longer wavelengths but also increase the effective cross-sectional area of the molecule to such an extent that its molar absorptivity is nearly twice that of imidazole itself. Similarly, the protonation of the imidazole ring can be seen to reflect itself in a slightly larger area for the collision with a photon, and hence a correspondingly higher absorptivity.

In view of this evidence, then, the following is proposed as the basic structure of the compounds studied:

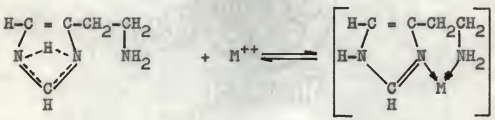


Here R=H, and R' may be H, $CH_2CH_2NH_2$, or $CH_2CH(NH_2)COOH$ in the case of imidazole, histamine, and histidine, respectively. For 4,5-imidazoledicarboxylic acid, both R and R' are COOH; however, it should be understood that this compound is characterized by strong inter- and intramolecular hydrogen bonding, which is not indicated here.

Effect of Complexing Cations

The basic idea behind the study of the effect of metal

ions upon the spectrum of histamine was to confirm the tautomerism of the imino hydrogen by localizing the bonds within the ring, and thereby altering the observed spectrum. Thus if a metal ion were to coordinate with histamine through the tertiary nitrogen atom, the imino hydrogen would no longer be tautomeric and the conjugated electrons would become localized:



Unfortunately, however, the experimental results are not conclusive at this point. A fundamental problem associated with this particular phase of the work was the lack of a suitable buffer that would provide a constant pH without at the same time interfering with the spectral measurements. Thus the question certainly deserves further attention in future work: suggestions for such a study are discussed at greater length in a later section.

Analytical Results

Attempts to analyze for histamine, histidine, and imidazole by virtue of their absorption spectra have been quite successful. The results of the determinations of the individual compounds were consistently well within two per cent of the true value, based upon the weights of the sample taken; and quite satisfactory results were obtained for the simul-

taneous determination of imidazole and histidine.

It must be emphasized that although the method used here is both rapid and precise, a consistent operating procedure is essential for its success. Several opportunities for error present themselves throughout the procedure, and these can only be minimized by following a standard routine. For example, once a Beer's Law curve is constructed for a given substance, it is important to duplicate as closely as possible such operations as weighing, diluting the samples for analysis, and especially the detailed manipulations involved in the operation of the spectrophotometer. If these precautions are properly taken, it is maintained that the spectrophotometric analysis of the imidazole derivatives will be entirely satisfactory.

PROPOSED EXTENSIONS

It has obviously not been possible to provide solutions to all of the problems that have been suggested by the results of this work. It would be appropriate, then, to enumerate several experimental approaches that may be of value in clarifying some of the molecular properties of the imidazole derivatives that are not well understood.

1-Methylimidazole

The spectral properties of 1-methylimidazole have been suggested earlier. However, it has not been possible to confirm this hypothesis because the compound was not available. These properties must then remain only in the state of specula-

tion until actual experimental work is performed. This imidazole derivative may eventually yield a conclusive answer to the question, whether the tautomeric nature of the imidazole nucleus contributes to the ultraviolet absorption spectrum of the molecule, or whether the observed imidazole spectrum is to be interpreted only in terms of the conjugated double bonds of the primary canonical structure (Table 1).

Determination of pK Values

The effect of pH on the ultraviolet spectra of the imidazole derivatives presents an interesting allusion to the possibility of determining the respective equilibrium constants for their proton-binding reactions. An experimental approach along this line has been proposed by Andon, Cox, and Herington (3), who have determined the pK values of a number of pyridine derivatives by taking spectrophotometric measurements of the pyridine solutions under conditions of varying pH. The equation used here to calculate the pK of a given base B is

$$pK_A = pH + \log_{10} \frac{\epsilon_2 - \epsilon_3}{\epsilon_1 - \epsilon_2} + \log_{10} \gamma_{BH^+}$$

where

pK_A is the negative logarithm of the thermodynamic dissociation constant of the equilibrium

$$BH^+ \rightleftharpoons B + H^+$$

ϵ_1 is the molar absorptivity of the protonated molecule in an acid solution

ϵ_2 is the molar absorptivity of the base in an alkaline solution

ϵ_3 is the molar absorptivity of the partially protonated molecule in a buffered solution

γ_{BH^+} is the activity coefficient of the base cation BH^+, calculated from the approximate Debye-Huckel equation

$$\log_{10} \gamma_{BH^+} = \frac{-0.509\, z^2 \sqrt{I}}{1 + \sqrt{I}}$$

where Z is the valency of the base

I is the ionic strength of the medium.

From the value of pK_A, it is then possible to calculate the thermodynamic dissociation constant K_B for the equilibrium

$$B + H_2O \rightleftharpoons BH^+ + OH^-$$

from the expression

$$pK_B = pK_W - pK_A$$

with the assumption that $pK_W = 14.00$ at 25° C.

Such a treatment may well lend itself to a study of the imidazole derivatives, where the base B would be imidazole, histamine, or histidine. However, some discretion would be necessary in the choice of the ionic background of the systems studied: such a choice would require careful consideration of the material employed to maintain a constant ionic strength, as well as the substances used to buffer the system at intermediate pH values, since, as was previously reported, some ions

have a pronounced effect on the spectra.

Determination of Stability Constants of Metal Complexes

A similar technique may be suggested for an extensive study of various metal-ion complexes of histamine, histidine, and imidazole. Here the method of continuous variations might be applicable, wherein the combining ratios and the stability constants of the complexes are determined by observing changes produced in the absorption spectra of the bases upon addition of increments of the metal ions. Here again it would be necessary to consider carefully the systems to be used, since it has been shown that the formation of a complex is dependent upon the pH; the problems associated with the interfering absorbance of a buffer and the hydrolysis of the metal ions to form insoluble hydroxides or hydrous oxides would need to be overcome at high pH values.

Determination of Molecular Configurations

A rigorous study of the structures of the metal complexes of the imidazole derivatives in the solid state may be possible by the application of x-ray diffraction methods. Here the configurations of the atoms within the crystal lattice may be determined in terms of their positions, bond lengths, etc. This valuable information could then be used to interpret the exact nature of the coordination that occurs in such complexes, and thus to indicate the species present in aqueous solution.

The complexes could conceivably be isolated by crystallization from non-aqueous solvents, e.g. alcohols, ethers, and ketones, in which the parent compounds are soluble but the complexes are not.

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THE ULTRAVIOLET ABSORPTION SPECTRA OF HISTAMINE, HISTIDINE, AND IMIDAZOLE; EFFECT OF pH AND CERTAIN FOREIGN IONS ON THE SPECTRUM OF HISTAMINE

by

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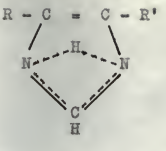
The ultraviolet absorption spectra of histamine, histidine, and imidazole in aqueous solution have been established. Of these compounds, only the spectrum of histidine has been reported previously; the results of this work are in excellent agreement with the published data. The wavelengths of the maximum absorbance, and the corresponding molar absorptivities of these compounds, are: histamine, 209 mμ, 4.94×10^3 liters per mole-cm; histidine, 211 mμ, 5.52×10^3 liters per mole-cm; imidazole, 206 mμ, 4.99×10^3 liters per mole-cm.

These values correspond to the ultraviolet spectra obtained in pure aqueous solution; under conditions of low pH the molar absorptivities of these compounds are increased slightly, and the maximum in the absorbance curve for histamine is shifted to 211 mμ. The significance of this pH effect, as well as the effect of certain interfering ions, are discussed, with emphasis on the indications of molecular structure revealed by such changes in the spectra.

The basic similarity of the spectral curves of imidazole, histamine, and histidine suggests that the imidazole nucleus is the principal absorbing species, and that the side-chain groups present in the histamine and histidine molecules affect the electronic structure of the heteronuclear ring only slightly. Based upon published evidence obtained by other experimental approaches, and upon the results of the present study, the following molecular structure is proposed for the compounds studied:



Here R is -H, and R' may be -H, $-CH_2CH_2NH_2$, or $-CH_2CH(NH_2)-COOH$ in the case of imidazole, histamine, and histidine, respectively. For 4,5-imidazoledicarboxylic acid, both R and R' are -COOH; however, in this latter compound, it must be recognized that strong intra- and intermolecular bonding occurs, as indicated by its physical properties.

Attempts to determine histamine, histidine, and imidazole quantitatively in pure aqueous solution by means of their absorption spectra have been entirely successful. Beer's Law is obeyed by each of these compounds in the range 2×10^{-5} to 2×10^{-4} molar, and the results of individual determinations are well within two per cent of the true value taken by weighing. It was also possible to determine imidazole and histidine simultaneously by absorbance measurements taken at 206 and 211 m . It was not possible, however, to perform a simultaneous analysis of imidazole and histamine, presumably because their respective wavelengths of maximum absorbance and molar absorptivities are so nearly the same; it is suggested that the same situation would apply to systems of histamine and histidine.