# Reaction of Histidine with Methyl Linoleate: Characterization of the Histidine Degradation Products[1]

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## ABSTRACT

Efforts have been made to characterize the products that result from interactions between L-histidine (free base) and peroxidizing methyl linoleate (ML) in a model system consisting of reactants dispersed on a filter paper. Imidazole lactic acid and imidazole acetic acid are identified as breakdown products when histidine is incubated with ML, methyl linoleate hydroperoxide (MLHPO), or n-hexanal over a period of 3 weeks. Two other reaction products are found to give back histidine upon acid hydrolysis. These products are though to be Schiff's base compounds which result from the condensation of the histidyl $\alpha$-amino group and carbonyl groups of reactive aldehydes formed during ML peroxidation. Most of the detectable reaction products have the imidazole moiety intact indicating the high relative reactivity of the functional groups, especially the amino group, associated with the $\alpha$-carbon. Such high reactivity provides an explanation for the low concentrations of ninhydrin-positive free amino compounds that are, at best, barely detectable on thin layer chromatography.

Free radicals, carbonyls, and other reactive species that may interact with proteins in a number of ways are formed during the oxidation of unsaturated lipids (1-3). To date, observations of the reactions between proteins and peroxidizing lipids have included the loss of enzyme activities (4-8), decreased solubility of proteins because of complex formation and polymerization (4,9-11), polypeptide chain scission (12), accelerated formation of brown pigments (13,14), and the destruction of labile amino acid residues – namely, histidine, lysine, cysteine, and methionine (15-20). In the presence of peroxidizing lipids, methionine residues are oxidized to methionine sulfoxide (21,22), lysyl $\epsilon$-amino groups readily form Schiff's base condensation products with aldehydes (20,23,24), and cysteinyl sulfhydryl groups add to the double bonds in linoleate hydroperoxides via a free radical mechanism (25) or to aldehydes via condensation reactions (26).

Little is known, however, about the reactions leading to the degradation of histidyl residues by peroxidizing lipids. Histidine is an essential amino acid for infants (27) and has been observed to exhibit relatively strong antioxidative activities in emulsified or lyophilized model systems (28,29). Moreover, because of the acid-base characteristics of the imidazole side chain at physiological pH, histidyl residues are often associated with the active sites of enzymes (30,31) and, at the same time, are important metal-binding groups in many metalloproteins (32). Thus, elucidation of the mechanisms leading to the degradation of histidyl residues by peroxidizing lipids will have important nutritional as well as biological implications.

This paper reports the characterization of products that are formed when histidine (free base) is reacted with peroxidizing methyl linoleate (ML) or its oxidation products–namely, methyl linoleate hydroperoxide (MLHPO) and n-hexanal–in a simple, low-moisture model system using filter paper as the solid support to simulate the hydrophilic porous structure of freeze-dried foods (33).

## EXPERIMENTAL PROCEDURES

### Model System Preparation

Except for MLHPO, which was prepared from ML according to the method of Banks et al. (34), reagent-grade chemicals were used in this work without further purification.

The reaction system consisted of an 11-cm-diameter filter paper (Whatman No. 42, Ashless Grade) on which the reactants were dispersed in the following manner. Supersaturated 10% aqueous solution of L-histidine (Eastman Kodak Co., Rochester, NY), adjusted from pH 7.2 to pH 7 with a few drops of dilute HCl, was applied dropwise to wet the entire filter paper. After lyophilization at room temperature for 1 hr in a Virtis 10 MRTR Freeze-Dryer, ML (NuCheck Prep, Inc., Elysian, MN) dissolved in hexane was applied dropwise on the filter paper to achieve an approximately 1:1 molar ratio of reactants. The amount of histidine ranged between 100 and 140 mg, and the amount of ML ranged between 210 and 240 mg per filter paper. After the evaporation of hexane, the filter papers, which contained 1-2% moisture, were incubated in the dark at 25 or 51 C over desiccant for periods of up to 3 weeks. In some systems, ML was replaced with MLHPO or n-hexanal (J.T. Baker Co., Phillipsburg, NJ). A molar ratio of 1:1 was adopted because a preliminary study showed that molar excess of one reactant over another, as much as 10 to 1, results in no observable changes in the pattern of breakdown products upon thin layer chromatogram (TLC) analysis. In order to determine whether similar reaction products were formed by agents other than peroxidizing ML or its autoxidation products, histidine was degraded by reaction with 30% hydrogen peroxide (saturated solution) and by irradiation with short-wave UV light onto filter papers containing L-histidine. Reaction between histidine and n-hexanal was also carried out in a stirred Erlenmeyer flask containing 1 g of each reactant to determine whether the pattern of degradation products differed in this system as compared with the reaction on a filter paper.

### Analytical Methods

Two-dimensional TLC analysis was used to provide a handy and sensitive method of monitoring the formation of reaction products in our systems. At the end of an incubation period, each filter paper was cut into small pieces and extracted with 5 ml of $H_2O$-methanol (1:1, v/v) solution. After filtration to remove cellulose fines, ca. 35 $\mu$l of the extract was spotted on a ready-coated, 160-$\mu$-thick cellulose TLC plate (Eastman Kodak,

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TABLE I

Rf Values of Histidine Breakdown Products and Some Standards

| Compounds | Solvents[a] | | |
|---|---|---|---|
| | $S_I$ | $S_{II}$ | $S_{III}$ |
| | Rf values (average of 3 runs) | | |
| I | 0.48 | 0.39 | 0.42 |
| II | 0.46 | 0.54 | 0.57 |
| III | 0.86 | 0.80 | 0.66 |
| IV | 0.87 | 0.87 | 0.90 |
| V | 0.97 | 0.97 | 0.95 |
| Imidazole lactic acid | 0.50 | 0.40 | 0.42 |
| Imidazole acetic acid | 0.46 | 0.53 | 0.57 |
| Imidazole pyruvic acid | 0.39 | 0.42 | 0.43 |
| 4-Hydroxymethyl imidazole | 0.17 | 0.57 | 0.57 |
| Urocanic acid | 0.49 | 0.68 | 0.64 |
| Histamine | 0.74 | 0.35 | 0.23 |
| Imidazole | 0.88 | 0.62 | 0.59 |

[a] $S_I$ = $NH_4OH$:$H_2O$:2-propanol (2:1:8, v/v), $S_{II}$ = acetic acid:$H_2O$:acetone:n-butanol (1:1:2:2, v/v), $S_{III}$ = formic acid:$H_2O$:2-propanol (2:10:40, v/v).

Rochester, NY) with a disposable 50-$\mu$ micropipette.

In cases where histidine was degraded by n-hexanal or 30% hydrogen peroxide in a stirred Erlenmeyer flask, reaction mixtures were diluted with aqueous methanol to achieve reaction product concentrations similar to those observed in the filter paper extract. After sample applications, the plates were developed for 8½ cm in one direction with the basic solvent system (Table I). The plates were then dried and developed with one of the acidic solvent systems (Table I) in a direction perpendicular to that of the first development. The developed and dried plates were then sprayed with ninhydrin (0.5% in acetone) to enable the detection of free amino compounds or with Pauly's reagent (diazotized sulfanilic acid) to give orange/red spots with imidazoles lacking a substituted imino nitrogen or a carboxyalkyl substituent and having at least one unsubstituted ring carbon atom (35). Iodine solution (1% in methanol) was also used as a nonspecific reagent to elucidate all organic compounds on developed TLC plates. This test, however, was much less sensitive than the two described previously because of the background resulting from the residual chemicals used in developing TLC plates.

A general outline of the procedure used to identify reaction products is illustrated in Figure 1. Reaction products for structural analysis were obtained by reacting ML and histidine in the previously described model system at 51 C in order to accelerate product formation because the pattern of reaction products in systems reacted at 25 C and 51 C does not differ qualitatively. Efforts have been concentrated on the identification of polar compounds that are soluble in aqueous methanol because TLC separation followed by iodine spray yielded the same two spots when ether extracts from systems containing peroxidized ML control and ML plus histidine were analyzed. These spots migrated close to the solvent front in all the solvent systems listed in Table I and showed a negative reaction to both ninhydrin and the Pauly's reagent. The observation that no nitrogen could be detected in the ether extract from reaction systems containing histidine analogues (N-benzoylhistidine, 4-methylimidazole) and ML (unpublished observation) indicates that the ether-soluble fraction contains mostly compounds arising from peroxidizing ML.

After precipitating excess unreacted histidine by increasing the ratio of methanol to water, the yellowish supernatant, after decoloration with Norit and concentration under vacuum, was subjected to preparative TLC

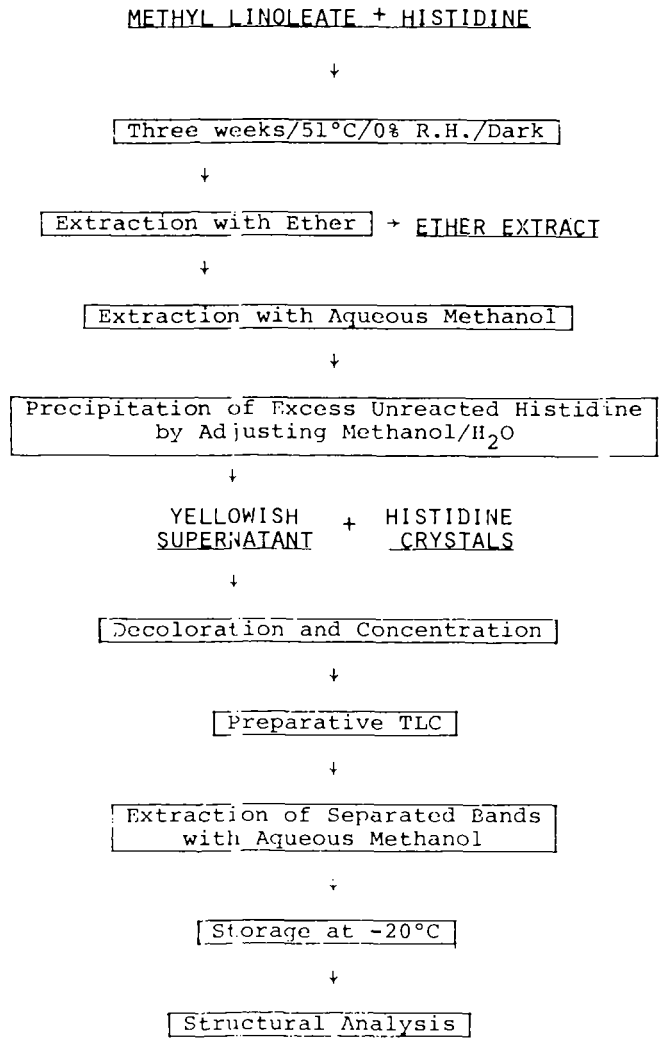



FIG. 1. Outline of procedures used in the identification of the reaction products.

separation on 500-$\mu$-thick microcrystalline cellulose plates (Analtech, Inc., Newark, DE), which were developed by formic acid-water-2-propanol (2:10:40, v/v) in one direction for 17-18 cm. Pauly's reagent was sprayed onto a small section of each developed plate while the majority of the plate was covered and protected from the reagent. No effort was made to obtain ninhydrin-

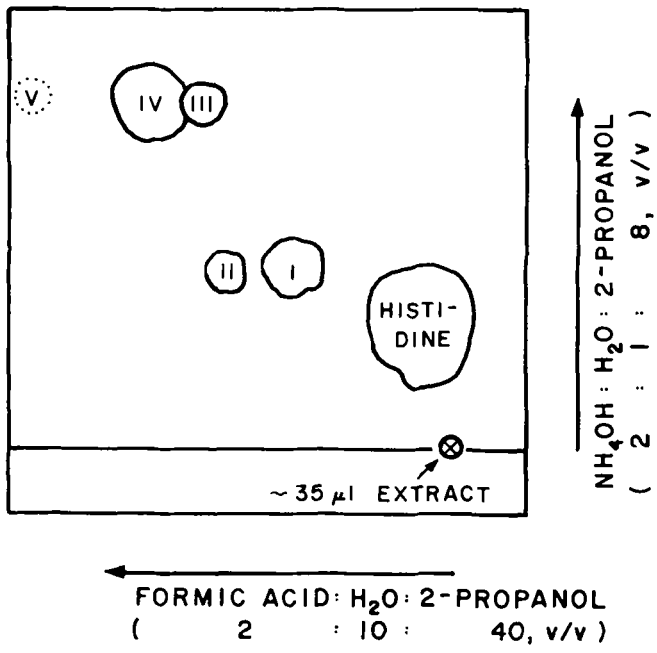



FIG. 2. Major degradation products detected by Pauly's reagent on cellulose TLC.

positive reaction products because these compounds gave very diffuse and barely detectable spots when product formation was monitored with the two-dimensional TLC analysis described above. The separated bands were scraped off the plates and extracted with aqueous methanol. These extracts were filtered through a sintered glass funnel with ultrafine pore size to remove cellulose fines and stored under nitrogen at 20 C (in the dark) prior to analysis.

Identification of products was based primarily on nuclear magnetic resonance (NMR) data because this method gives the most valuable structural information when one has some idea about the structure of the unknown compounds. Since the reaction products were soluble in either eqeuous methanol or water, their NMR spectra – as well as those of the standards – were obtained in 99.8% deuterium oxide (Stohler Isotope Chemicals, Azusa, CA) with tetramethylsilane as external reference, after the removal of solvent aqueous methanol under vacuum. A Varian T-60 NMR Spectrometer was used throughout the experiment. In addition to histidine, the standards included imidazole acetic acid, imidazole lactic acid, imidazole pyruvic acid, 4-hydroxymethyl imidazole, urocanic acid, histamine, and imidazole. Except for histamine (Fisher Scientific Co., Medford, MA) and urocanic acid (Eastman Kodak, Rochester, NY), the stated chemicals were purchased from Calbiochem, San Diego, CA. These chemicals were also used as standards in two-dimensional TLC analysis.

## RESULTS AND DISCUSSION

A typical two-dimensional, thin layer chromatogram of major reaction products, which are positive to Pauly's reagent but negative to ninhydrin, is presented in Figure 2. The Rf values of these products, along with those of the standards, are given in Table I. As indicated in the table, we observed close similarities in the migration characteristics of imidazole lactic acid and reaction product I as well as of imidazole acetic acid and reaction product II.

The NMR spectral characteristics of reaction products I and II, as well as those of some standards, are given in Table II. By comparing the spectra of the unknowns with those of the standards and the two-dimensional TLC data, reaction products I and II were identified as imidazole lactic acid and imidazole acetic acid, respectively. The spectra of reaction products III and IV showed some upfield signals (multiplets centered at 1.4 ppm) that could not be observed on the spectra of the standards examined. These upfield signals corresponded to the absorption of electromagnetic radiation by hydrogen atoms in a hydrocarbon chain.

Since it is known that the Rf values of imidazoles increase with increasing basic strength of the compounds (36), reaction products III and IV, which have Rf values close to that of imidazole, were though to be Schiff's base compounds formed by amino-carbonyl condensa-

TABLE II

NMR Spectral Characteristics of L-Histidine Reaction Products and Some Standards

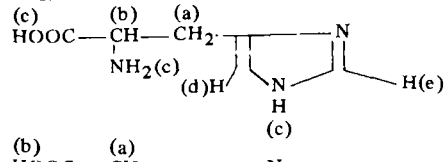
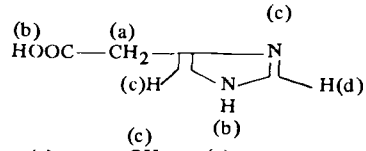
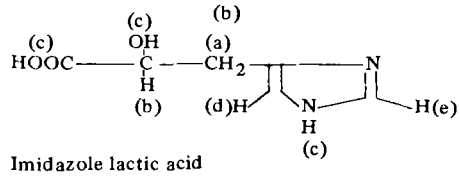
| Compounds | Spectral characteristics | Assignment |
|---|---|---|
| L-Histidine | a. 3.2 ppm (2 proton intensity doublet)<br>b. 4 ppm (1 proton intensity multiplet)<br>c. 4.8 ppm ($H_2O$ and exchangeable protons)<br>d. 7.1 ppm (1 proton intensity singlet)<br>e. 7.7 ppm (1 proton intensity singlet) |  |
| Imidazole acetic acid | a. 3.7 ppm (2 proton intensity singlet)<br>b. 4.8 ppm ($H_2O$ and exchangeable protons)<br>c. 7.3 ppm (1 proton intensity singlet)<br>d. 8.6 ppm (1 proton intensity singlet) |  |
| Imidazole lactic acid | a. 3.1 ppm (2 proton intensity doublet)<br>b. 4.3 ppm (1 proton intensity multiplet)<br>c. 4.8 ppm ($H_2O$ and exchangeable protons)<br>d. 7.3 ppm (1 proton intensity singlet)<br>e. 8.6 ppm (1 proton intensity singlet) |  <br>Imidazole lactic acid |
| Compound I | a. 3.1 ppm (2 proton intensity doublet)<br>b. 4.5 ppm (1 proton intensity multiplet)<br>c. 4.8 ppm ($H_2O$ and exchangeable protons)<br>d. 7.3 ppm (1 proton intensity singlet)<br>e. 8.6 ppm (1 proton intensity singlet) | |
| Compound II | a. 3.7 ppm (2 proton intensity singlet)<br>b. 4.8 ppm ($H_2O$ and exchangeable protons)<br>c. 7.3 ppm (1 proton intensity singlet)<br>d. 8.6 ppm (1 proton intensity singlet | Imidazole acetic acid |

TABLE III

Degradation of Histidine by Autoxidizing Lipids on Filter Paper

| Reactants | Temperature | Time (hr) 10 | 40 | 100 | 250 | 500 |
|---|---|---|---|---|---|---|
| Histidine | 51 C | none | none | none | none | none |
| | 25 C | none | none | none | none | none |
| Methyl linoleate & histidine | 51 C | none | I,II,III, IV,V | I,II,III, IV,V | I,II,III, IV | I,II,III, IV,V |
| | 25 C | none | none | I,II,IV, V | I,II,III, IV,V | I,II,III, IV,V |
| Methyl linoleate hydroperoxide & histidine | 51 C | I,II | I,II | I,II,IV, V | I,II,IV | |
| | 25 C | none | none | I | I,IV | |
| N-Hexanal & histidine | 51 C | none | I,II | I,II | I,II | I,II |
| | 25 C | none | none | I,II | I,II,IV | I,II,IV |

TABLE IV

Degradation of Histidine by UV-Irradiation, $H_2O_2$, or N-Hexanal (in Bulk)

| Reactants | Time (days) 1/2 | 3 | 7 | 12 | 18 | 30 | 36 |
|---|---|---|---|---|---|---|---|
| N-Hexanal & histidine (stirred in bulk at room temperature) | IV | IV | IV | IV | IV | | IV,I,II |
| Short-wave UV & Histidine (on a filter paper) | | I,II | | I,II | | I,II | |
| $H_2O_2$ & histidine (saturated solution in 30% $H_2O_2$) | | I,II and other compounds | | I,II and other compounds | | | |

tions involving histidyl α-amino groups and carbonyls from peroxidizing lipids. Since these reaction products migrated closely on TLC plates in different solvent systems, they were expected to have rather similar structures. Solutions containing these compounds were thus pooled and subjected to acid hydrolysis in 1 N HCl for 15 and 75 min with two-dimensional TLC to monitor the products. Upon hydrolysis, we observed that histidine, imidazole acetic acid, and, to a lesser extent, imidazole lactic acid were formed. When the time of hydrolysis was lengthened, the amounts of histidine and imidazole acetic acid, as determined by spot sizes, increased while the amounts of III and IV decreased. These results support the postulation that reaction products III and IV are Schiff's base adducts formed via amino-carbonyl condensations.

The time-dependent formation of histidine reaction products under different reaction conditions is summarized in Tables III and IV. We found that imidazole lactic acid and imidazole acetic acid were the major detectable degradation products in all the reaction systems investigated, except those in which histidine was reacted directly with n-hexanal in a stirred Erlenmeyer flask. In this system, limitations in the availability of oxygen might have contributed to the accumulation of reaction product IV. We also observed that compounds III and IV appear only in systems containing carbonyls. The appearance of these compounds, however, did not necessarily precede that of imidazole lactic acid and imidazole acetic acid, indicating that different pathways may be involved in the formation of the former compounds as compared with the latter ones.

Reaction pathways leading to the formation of histidine degradation products are postulated in Figure 3. Since imidazole lactic acid and imidazole acetic acid were formed when histidine was exposed to short-wave UV light or to hydrogen peroxide (where degradation proceeds via free radical processes), we suspect that free radical reactions were involved in the formation of these compounds when histidine was reacted with peroxidizing ML or its autoxidation products. Such a hypothesis is supported by the fact that the autoxidation of ML, MLHPO, and n-hexanal is a free radical process in which reactive alkyl, alkoxy, and peroxy radicals are generated in abundance (2,37,38). Furthermore, the formation of carbon-centered free radicals primarily associated with the α-carbon of the amino acid has been observed by electron spin resonance techniques when histidine was reacted with peroxidizing ML in a low-moisture model system (39). Along with the experimental evidence indicating that extensive deamination of the α-amino group takes place when histidine is degraded via free radical processes by high-energy irradiations (40-42), this observation can explain the formation of imidazole lactic acid and imidazole acetic acid in our model system through free-radical-mediated deamination and decarboxylation reactions.

Although the reactions involving the α-carbon functional groups of histidine may have some bearing on its antioxidative activity, they tell very little about the destruction of the histidyl residue in a protein molecule because the α-carbon functional groups participate in polypeptide linkages. Moreover, the side chain functional groups, not the amide linkages, are expected to be the primary sites for interactions between peroxidizing lipids and proteins. Therefore, research using the histidine analogues 4-methylimidazole and N-benzoylhistidine — which have the reactive α-amino group absent and blocked, respectively — is underway to elucidate the degradative reactions initiated by peroxidizing lipids on the histidyl imidazole side chain.

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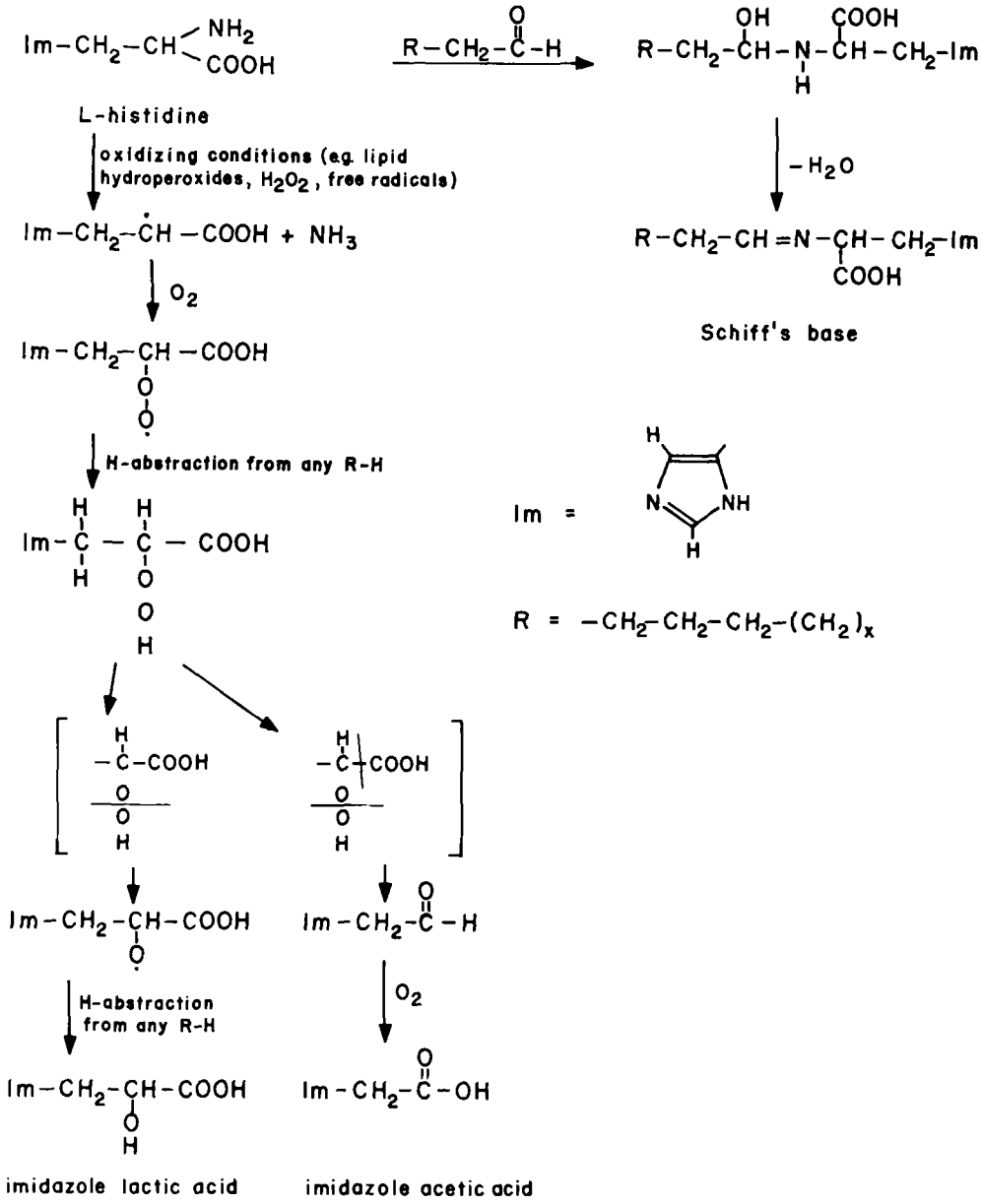


FIG. 3. Formation of imidazole lactic acid and imidazole acetic acid from histidine.

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