The Identification of 4(5)-Iodohistidine as the Product of the Limited Iodination of Histidine*

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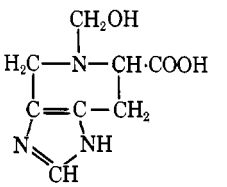
ABSTRACT: The limited iodination of histidine results in the formation of only one of the two possible isomers of monoiodohistidine. The results obtained by nuclear magnetic resonance (nmr) spectroscopy prove that this isomer is 4(5)-iodohistidine. A chemical method was developed for determining the location of the iodine atom in monoiodohistidine to aid in the identification of the monoiodohistidine isomer isolated in small amounts from iodinated proteins or other biological material. A. Neuberger [*Biochem. J. 38*, 309 (1944)] showed that the aminomethylol compound, formed by the reaction between formaldehyde and the α-amino group of histidine, undergoes an intramolecular displacement reaction at C-4(5) of the imidazole ring to yield 3,4-imidazole-1,2,5,6-tetrahydropyridino-6-carboxylic acid. Treatment of the 4(5)-iodohistidine isomer with formaldehyde gives the same product (iodine being liberated during the electrophilic displacement), which may be identified chromatographically by comparison with Neuberger's compound. In addition, we have demonstrated by nuclear magnetic resonance spectroscopy that the reductive removal of iodine from triiodoimidazole gives 4(5)-iodoimidazole rather than 2-iodoimidazole as reported previously [Pauly, H., and Arauner, E. (1928), *J. Prakt. Chem. 118*, 33].

There are two possible isomers of monoiodohistidine (MIH)[1] which could result from the limited iodination of histidine. However, when MIH is prepared by the method of Brunings (1947), only one isomer has been detected either by two-dimensional chromatography (Bensusan and Scanu, 1960) or chromatography in five different solvents (Perlgut and Wainio, 1966). When collagen was iodinated in the presence of low iodide concentrations, the MIH, obtained from alkaline hydrolysates, was chromatographically identical with the synthetic product (Bensusan, 1966). Perlgut and Wainio (1966) have isolated a phosphoriodohistidine from mitochondria which, after the removal of the phosphate, also was identical with the synthetic MIH.

Pauly and Arauner (1928) prepared monoiodoimidazole by the reduction of diiodoimidazole with an excess of sodium sulfite. The stable iodine substituent was found to be at C-2 by reason of the fact that the dibromo derivative had different physical characteristics from a known sample of 4(5)-iodo-2,5(4)-dibromoimidazole. In a similar manner, they found that the limited iodination of 4(5)-methylimidazole gave 2-iodo-4(5)-methylimidazole. Brunings (1947), without further evidence, assumed on the basis of the reported results with imidazole that the limited iodination of histidine resulted in the formation of the 2-iodohistidine isomer.

Since the unequivocal identification of the normal isomer is important in our studies on the cross-linking in collagen and in the identification of possible intermediates in oxidative phosphorylation, we have developed a simple chemical technique which has provided the proof of the location of the iodine atom. Neuberger (1944) demonstrated that, after the fast initial formation of an aminomethylol compound from formaldehyde and the α-amino group of histidine, a slower reaction occurs involving the aminomethylol group in an intramolecular cyclization by an electrophilic displacement reaction at C-4 of the imidazole ring to yield 3,4-imidazole-1,2,5,6-tetrahydropyrido-6-carboxylic acid. An additional mole of formaldehyde is added to the compound to give a methylol derivative (Neuberger's compound A). The methylol group is labile and may be removed by dimedone in dilute acid. The position of this group was uncertain since it could be located either at the pyridine or an imidazole nitrogen. However, since N-substituted imidazoles do not give a positive Pauly's test (Ames and Mitchell, 1952) and Neuberger's compound A does give a positive Pauly reaction, we have placed the methylol group at the pyridine-ring nitrogen as shown



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[1] Abbreviations used: MIH, monoiodohistidine; DIH, diiodohistidine.

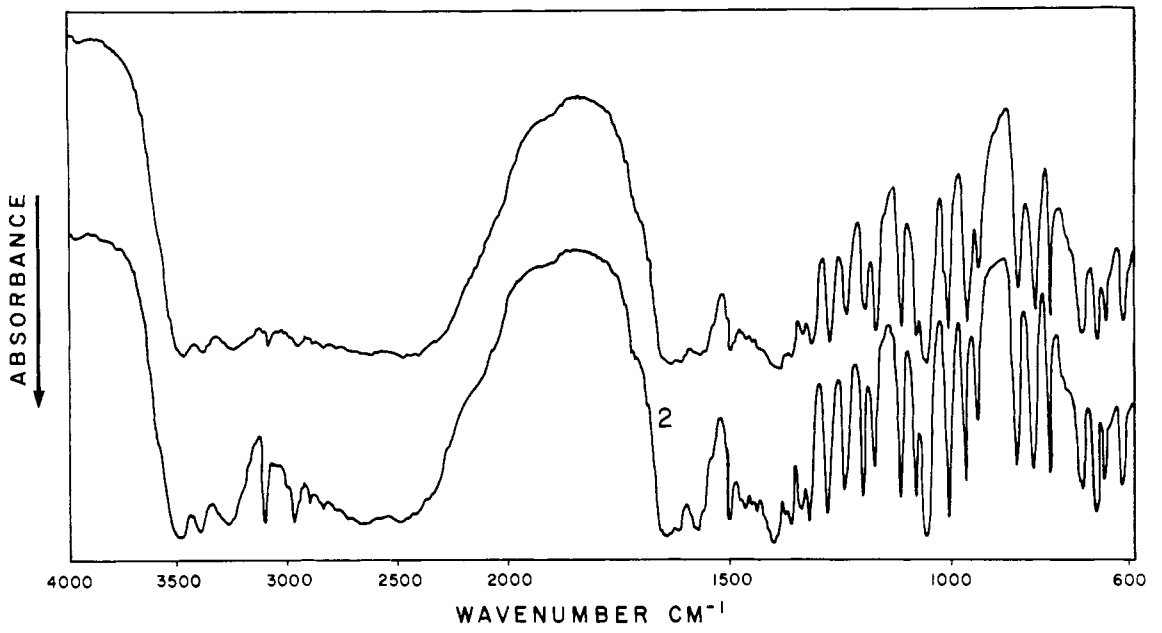

FIGURE 1: Infrared spectra of the products of the reaction between formaldehyde and histidine (curve 1) and monolodohistidine (curve 2). Except for the poorer resolution in curve 1 owing to the greater light scattering in this KBr pelliet, the spectra appear to be identical.

The reaction described above can be used to determine the position of the iodine atom in MIH. Thus, if the iodine atom is in the 4(5) position,[2] it should be removed by the electrophilic substitution reaction to yield compound A plus iodine. If, on the other hand, the iodine is located at C-2 of the imidazole ring, a new product containing iodine should be formed. This paper will demonstrate that 4(5)-iodohistidine is the isomer obtained from the limited iodination of histidine.

Methods

MIH and diiodohistidine (DIH) were synthesized according to Brunings (1947). Less darkening occurs during the preparation of MIH if 2 M NaOH is used instead of 0.2 M NaOH. The MIH melted at 210–215° (reported mp 204–206°). *Anal.* Calcd for $C_6H_9ClIN_3O_2$: C, 22.7; H, 2.85; I, 40.0; N, 13.2. Found: C, 22.5; H, 2.79; I, 39.5; N, 13.0.

Monoiodoimidazole, reported to be 2-iodoimidazole, was prepared from triiodoimidazole (Brunings, 1947) according to the method used by Pauly and Arauner (1928), who started with diiodoimidazole. Our reaction was carried out by refluxing a mixture of 2.5 g of triiodoimidazole and 10 g of sodium sulfite in 30% ethanol for 24 hr. The product was isolated and recrystallized as reported previously. The product melted at 137–138° (reported mp 136–137°). *Anal.* Calcd for $C_3H_3IN_2$: C, 18.6; H, 1.56; I, 65.4; N, 14.4. Found: C, 18.7; H, 1.69; I, 65.4; N, 14.5.

Compound A was prepared according to Neuberger (1944). 2-Methylhistidine was synthesized according to Ono and Hirohata (1956). All other chemicals and reagents were obtained from commercial sources. MIH and DIH were treated with formaldehyde as described by Neuberger for the preparation of compound A from histidine except that the iodine liberated during the course of the reaction was reduced with thiosulfate.

Infrared spectra were obtained with the Beckman IR8 spectrophotometer with the samples in pressed pellets of KBr. Nuclear magnetic resonance (nmr) spectra were obtained with the Varian Associates A-60 spectrometer with the samples dissolved in D_2O. Descending paper chromatograms were developed with butanol–acetic acid–water (40:6:15). The products were located by spraying with diazotized sulfanilic acid according to Ames and Mitchell (1952). Iodine-containing compounds were detected by their ability to catalyze the redox reaction of $Ce(SO_4)_2$–$HAsO_2$ as described by Bowden and Maclagan (1954).

Results and Discussion

As stated in the Methods section, the iodine liberated in the reactions of MIH and DIH with formaldehyde was reduced with thiosulfate using a starch indicator. In both reactions, iodine liberation ceased after about 1 hr. We have found that the reaction between histidine

[2] The numbering system for the imidazole ring places the nitrogens in positions 1 and 3. Owing to the fact that the nitrogens are identical and that the ring is symmetrical, a substituent in one of the two adjacent carbons can be numbered either 4 or 5.

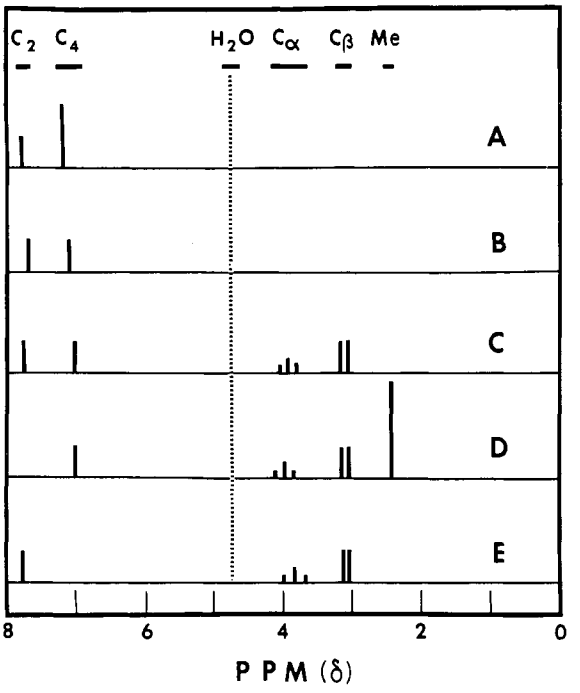

FIGURE 2: The nmr spectra of various imidazoles at pD 8.0–8.5 in D_2O. (A) Imidazole; (B) 4(5)-iodoimidazole (see Discussion); (C) histidine; (D) 2-methylhistidine; (E) 4(5)-iodohistidine (see Discussion). Trimethylsilyl propane-3-sulfonate was the internal reference standard. The height of the lines marking the chemical shifts indicates the relative intensities.

and formaldehyde is incomplete even after 48 hr, indicating that the electrophilic displacement of an iodine atom is very much faster than that of an hydrogen atom. A similar relationship of reaction rates was noted when histidine and MIH were treated with 5-(hydroxymethyl)-2-furfural (H. B. Bensusan, S. D. McKnight, and M. S. R. Naidu, to be published). The reaction of DIH with formaldehyde resulted in a spectrum of products, as shown by paper chromatography, and all of them contained iodine atoms. Therefore, it appears reasonable to assume that in addition to the intramolecular reaction an intermolecular reaction (or reactions) has taken place between the methylol derivative of one DIH molecule and C-2 of a second DIH molecule.

The product obtained from the reaction of MIH with formaldehyde melted at 208–210° (uncor) as compared with the melting point of 210–215° reported and found for compound A. A mixture melting point showed no depression. *Anal.* Calcd for $C_8H_{11}N_3O_3 \cdot H_2O$: C, 44.6; H, 6.10; N, 19.5. Found for compound A from MIH: C, 44.1; H, 6.10; N, 19.3. The MIH reaction product exhibited the same R_H value as authentic compound A (Table I) and contained no iodine, as determined by the sensitive $Ce(SO_4)_2$–$HAsO_2$ method. Comparison of the infrared spectra showed that MIH and histidine gave the same product when

TABLE I: Chromatography of Histidine, Iodohistidines, and Their Derivatives.

Compound	R_H[a]	Iodine Test
Histidine	1.00	Negative
MIH	1.55	Positive
DIH	5.1	Positive
Compound A	1.35	Negative
MIH + formaldehyde	1.30	Negative

[a] R_H is the distance of migration compared with that of histidine.

treated with formaldehyde (Figure 1). Studies in the ultraviolet region of the spectrum showed that compound A prepared from both MIH and histidine absorbed maximally at 215 mμ with a molar extinction coefficient of 5500. These results prove that MIH, as synthesized by the iodination of histidine, is 4(5)-iodohistidine.

The nmr spectra (Figure 2) are particularly interesting. The intensities of the two bands clearly identify the chemical shifts of the hydrogens attached to the C-2 and the symmetrical C-4 and C-5 positions in the imidazole rings. Therefore, the presence of the two bands of equal intensity in the spectrum of monoiodoimidazole clearly demonstrates that the isomer which we prepared is 4(5)-iodoimidazole and not the 2-iodoimidazole reported by Pauly and Arauner (1928). Although we prepared our sample from triiodoimidazole rather than from diiodoimidazole, the method of removing the iodine was the same in both preparations and the melting point of our sample was the same as reported by Pauly and Arauner. Therefore, it appears that these investigators have made an error in the identification of the product. Work is now in progress involving a complete reinvestigation of the products obtained by the halogenation of imidazole.

Our identification of the chemical shifts of the various hydrogens of histidine (Figure 2) is the same as reported by McDonald and Phillips (1963). Therefore, the presence of a single band in the spectrum of MIH at the position of the chemical shift of the hydrogen in the C-2 position indicates that the iodine is at C-4(5). The only possibility that would lead to a mistake would be if an iodine substituent at C-2 could cause a sufficient downfield shift of the C-4 hydrogen to coincide with the chemical shift of the C-2 hydrogen in imidazole. Such a downfield shift is shown to be very unlikely since the iodine in 4(5)-iodoimidazole results in a slight upfield shift of the two hydrogens.

Although the nmr spectra alone clearly establish the position of the iodine atom in MIH, we have included the chemical results to aid in the identification of MIH isolated in small quantities from biological

materials. Thus, as shown here, the unknown material may be treated with formaldehyde, dried under vacuum to remove the excess formaldehyde, and chromatographed. If the product has the same R_H value as compound A, is positive to the Pauly reagent, and contains no iodine, this should now constitute reasonable proof that it is 4(5)-iodohistidine. In the absence of a sample of 2-iodohistidine we can only deduce what would result from the treatment of this isomer with formaldehyde. Compound A containing an iodine substituent would result from the intramolecular condensation. On the other hand, since the electrophilic displacement of an iodine atom appears to take place faster, an intermolecular condensation could also occur to link the α-amino group of one MIH molecule to the C-2 carbon of another molecule through a methylene bridge. Since a reactive C-2 with an iodine substituent remains from this reaction, further intermolecular reactions can occur. Therefore, one can expect that polymers of various sizes which still contain one iodine atom at the terminal imidazole group would result. Thus, we conclude that the treatment of 2-iodohistidine with formaldehyde would show several spots on chromatography, each giving a positive iodine test.

Acknowledgment

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Identification of Mitochondrial Iodohistidine and Phosphoriodohistidine on a Sephadex G-10 Column*

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ABSTRACT: Monoiodohistidine and phosphoriodohistidine have been previously separated and identified on paper chromatograms [Perglut, L. E., and Wainio, W. W. (1966), *Biochemistry 5*, 608]; however, attempts at confirmation of this identification *via* ion-exchange column chromatography have been only partially successful. A Sephadex G-10 column produced separation and identification of the monoiodohistidine, phosphoriodohistidine, thyroxine, and triiodothyronine present in alcohol extracts of beef heart mitochondria Synthetic and mitochondrial monoiodohistidine, as eluted from the column, possessed identical ultraviolet absorption spectra; each contained labile iodine and produced comparable R_F values on paper chromatograms. ^{32}P-labeled mitochondrial extracts, when eluted from the column, produced a radioactive peak that coincided with that of synthetic phosphoriodohistidine.

We have previously reported separation and identification of mitochondrial monoiodohistidine (MIH)[1] and phosphoriodohistidine (PIH) by paper chromatography; however, ion-exchange column chromatography did not produce a positive identity (Perlgut and Wainio, 1966). We now wish to report separation and identification of both MIH and PIH, as well as thyroxine and triiodothyronine, on a Sephadex G-10 column.[2]

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[1] The following abbreviations will be used: monoiodohistidine, MIH; phosphoriodohistidine, PIH; adenosine triphosphate, ATP; thyroxine, T_4; triiodothyronine, T_3; FFCA, ferric ferricyanide–arsenious acid (Postmes, 1963); FFC, FFCA without the arsenious acid.

[2] Pharmacia Fine Chemicals, New Market, N. J.