[CONTRIBUTION FROM THE WILLIAM H. NICHOLS LABORATORY OF NEW YORK UNIVERSITY]

Preparation and Properties of the Iodohistidines[1]

BY KARL J. BRUNINGS

This paper reports the preparation of 2,5-diiodohistidine and 2- or 5- monoiodohistidine (111 and 11, Chart 1) and a study of certain of their properties. These compounds are of interest as radiopaque substances, carriers of radioactive iodine, growth promoters and possibly as substances with thyroid activity. The peculiarities of the reaction between iodine and histidine will be shown to shed light on the mechanism of the iodination of the imidazole ring, while the acid–base behavior of the iodohistidines gives new information on the fine structure of histidine and its salts. Finally, the acid-base constants of the iodohistidines may be used to explain the titration curves of the iodoproteins.[2]

Although most compounds containing the imidazole ring yield iodo derivatives readily[3,4] the preparation of the iodohistidines has not been heretofore achieved. The absorption of iodine by histidine in basic solutions has been reported several[3] times and recently a study of the kinetics of the assumed iodination has been reported by Li.[5] The results reported in the present paper indicate that the reasons for the difficulty of preparing these compounds are: (1) the peculiar resistance of histidine to form a carbon–iodine bond under conditions where colorless iodoimidazoles are obtained in good yield from the simpler derivatives; and (2) the solubility of the iodohistidines in aqueous solution containing iodide and tri-iodide ion.

The first difficulty was overcome and colorless iodohistidines obtained by iodinating aqueous solutions of histidine in the presence of much larger concentrations of sodium hydroxide than are necessary in the case of imidazole, 4-methylimidazole, histidine anhydride and the imidazole carboxylic acids. Consideration of the acidic dissociation constants and probable structure of histidine salts provides a possible explanation for the higher alkalinity needed, and is discussed below in conjunction with the acid constants of the iodohistidines. The exclusion of iodide ion from the final reaction medium has been brought about by the use of a new iodination technique which is believed to have general application to this and similar problems of iodination and particularly in the case of proteins.[6] Aqueous alkaline solutions of histidine are stirred vigorously with pure hexane and calculated volumes of iodine–hexane solution added. In this way the iodide ion content is maintained at the minimum allowed by the substitution reaction. After the reaction is complete the iodide produced by substitution is converted to iodine by adding potassium iodate solution and acid. The iodine is then extracted with hexane. In a pH range 4–2.5 diiodohistidine precipitates almost quantitatively from saturated salt solutions. The monoiodohistidine obtained by iodinating very dilute solutions at relatively lower alkalinity is isolated from salt residues by extraction with ethanol-hydrochloric acid and precipitation by neutralization with aniline. Analogous to the iodotyrosines the iodohistidines are more soluble in alcohol–water mixtures than in either pure alcohol or water.[7]

The position of iodine in monoiodohistidine has not been proved but is inferred to be in position 2 in analogy to monoiodoimidazole and monoiodo-4-methylimidazole.[3c]

The above iodination technique has been applied to other imidazole derivatives with good results. Most noteworthy is a comparison between histidine and histidine anhydride toward iodination. The diketopiperazine in which the carboxyl and α-amino groups are bound in cyclic amide formation requires only a slight amount of alkali for rapid and complete iodination. The same is true for imidazole and imidazole carboxylic acids.

Dilute solutions of histidine containing from one to three moles of sodium hydroxide take on a deep brown color as iodine is added. After one mole of iodine has been added and on standing, these solutions become almost black, and from them, ill defined amorphous compounds which contain variable amounts of iodine may be obtained. As yet these substances have not been purified sufficiently to give reliable analyses. Strongly alkaline solutions of these compounds are stable but react with iodine and lose their deep brown color. These substances are believed to be N-iodo derivatives of histidine and iodohistidine and their possible conversion to colorless iodohistidines is being investigated. The formation of these substances can only be prevented by the use of large excesses of alkali.

The dissociation constants of colorless iodohistidines have been determined and compared to histidine (Table I and Fig. 1). The conclusions to be drawn from these constants and the results summarized above appear in the discussion below.

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[1] A portion of this paper was presented at the Atlantic City Meeting of the American Chemical Society, April 1946.

[2] A. Neuberger, *Biochem. J.*, **28**, 1982 (1934); E. J. Cohn and J. T. Edsall, "Proteins and Amino Acids, etc.," Reinhold Publishing Co., New York, N. Y., 1943.

[3] (a) Pauly and Gundermann, *Ber.*, **41**, 3999 (1909); (b) Pauly, *ibid.*, **43**, 2243 (1910); (c) Pauly and Arauner, *J. prakt. Chem.*, **118**, 33 (1928).

[4] Bauer, Strauss and Maschman, *Ber.*, **68B**, 1108 (1935).

[5] C. H. Li, THIS JOURNAL, **66**, 225 (1944).

[6] The application of this technique to hemoglobin and globin has been studied in this Laboratory and will be reported later.

[7] Winnek and Schmidt, *J. Gen. Physiol.*, **19**, 773 (1936).

Experimental Section

1. **Preparation of 2,5-Diiodohistidine.**—A solution of 1 g. of 1-histidine monohydrochloride (Merck) in 300 cc. of 0.5 *N* sodium hydroxide was placed in a 1 liter, three-necked flask equipped with an efficient electrical stirrer and a 250 cc. separatory funnel. One hundred cc. of pure hexane was added and the flask chilled in an ice-bath. When the solution had cooled to 10°, 260 cc. (2 moles + 10%) of 0.1 *N* iodine hexane solution was run in from the separatory funnel at a slow rate such that the iodine color in the emulsion was not excessive. The mixture was stirred vigorously enough to ensure emulsification throughout. In one and one-half hours the addition was complete and after stirring for an additional half hour, the solution was acidified with 15 cc. of concentrated hydrochloric acid. Forty-five hundredths gram of potassium iodate in 50 cc. of water was then added while the solution was stirred vigorously. The mixture was allowed to separate and the hexane–iodine layer removed. At least two additional 200 cc. hexane extractions were needed to remove most of the iodine. The complete extraction of the iodine was found to be unnecessary because the last traces were lost in the ensuing vacuum evaporation. The solution was evaporated at 50–60° *in vacuo* to a volume of about 50 to 100 cc. and then cautiously neutralized to a *p*H of 4–2 with ammonia. The resulting solution containing the precipitate diiodohistidine was allowed to stand in the cold overnight to ensure complete precipitation. About 2 g. (94% of the theoretical) of an amorphous white solid was obtained. This material was purified by suspending 1.5 g. in 10 cc. of warm water and adding enough concentrated hydrochloric acid with a medicine dropper to ensure complete solution. Neutralization with ammonia, pyridine or aniline gave rapid precipitation. The precipitate was washed several times with a few cc. of cold water to remove salts; recovery 75–80%. The purified compound melted with decomposition at 220° when the temperature was raised rapidly. Before melting, the compound turned brown and after melting effervesced strongly, giving off iodine. Analysis and titration curves showed the product to be the monohydrochloride of 2,5-diiodohistidine.

Anal. Calcd. for $C_6H_8N_3O_2I_2Cl$: N, 9.48; I, 57.24; Cl^-, 8.00. Found: N, 9.44 (Kjeldahl and Dumas); I, 56.67 (±0.5%, Pregl, dry combustion—volumetric titration of iodine); Cl^-, 8.02.

2. **Preparation of Monoiodohistidine.**—Three grams of 1-histidine monohydrochloride in 500 cc. of 0.2 *N* sodium hydroxide was iodinated as above in (1) with 330 cc. of 0.1 *N* iodine–hexane (1 mole). The addition required about three hours. After the iodide ion had been removed the aqueous solution was evaporated to dryness *in vacuo* below 50° and the salt residue was extracted by boiling with 150 cc. of ethyl alcohol containing 2 cc. of 11 *N* hydrogen chloride–ethanol solution for fifteen minutes. Considerable darkening occurred and the refluxing should perhaps be supplanted by shaking. The solution was filtered and the residue washed with 25 cc. of alcohol. After neutralization with aniline, 1.7 g. of a brownish precipitate (A) was filtered off. The filtrate was then evaporated down to less than 50 cc. and allowed to stand in the cold several days. Filtration yielded 2.35 g. of fairly white material B. The crude precipitates A and B were recrystallized separately by suspending in a small amount of water (about 10 cc.) and adding concentrated hydrochloric acid until solution was complete. The solutions were then cleared with Norite and filtered, washing flasks and Norite with small volumes of water. The filtrates were diluted with two volumes of alcohol, neutralized with aniline and allowed to stand several days in the cold. The recrystallization of crude precipitate B yielded 0.55 g. of white crystalline material and on further standing an additional 0.46 g. The solution of crude precipitate A yielded first brownish, low melting material which was discarded, and then 600 mg. of pure white crystalline compound. Total yield of once recrystallized compound was 1.60 g. or 32% of the theoretical. All precipitates were washed with cold ethanol. The analysis and titration curves showed the compound to be monoiodohistidine hydrochloride. *Anal.* Calcd. for $C_6H_9N_3O_2ICl$: N, 13.24; I, 39.97. Found: N, 13.14 (Kjeldahl); I, 40.28 (±0.5%) (Pregl—dry combustion). The compound melted with effervescence at 204–206° without giving off iodine. A mixed melt with diiodohistidine gave practically no depression, while a mixed melt with histidine gave a depression. Mixed melt of diiodohistidine with histidine gave dec. pts. within 5° of the dec. pt. of the monoiodohistidine. On the suspicion that a 50–50 mixture of diiodohistine and histidine was at hand, mixtures of these two were recrystallized. The two did coprecipitate, but on suspending the crystals thus obtained in pure water, the diiodohistidine remained insoluble. Monoiodohistidine is very soluble in pure water. Finally, comparison of titration curves of monoiodohistidine with mixtures of mono and diiodohistidine proved that the monoiodohistidine was not a mixture.

3. **Iodination of Imidazole.**—Two-tenths gram of imidazole in 130 cc. of 0.02 *N* sodium hydroxide was iodinated as above in (1) with 196 cc. of 0.9 *N* iodine–hexane (3 moles); 140 cc. of 0.1 *N* sodium hydroxide were run in concurrently with the iodine–hexane to prevent precipitation of the product. Without removing the iodide ion the aqueous solution was brought to *p*H 5. After standing several hours 1.0 g. of triiodoimidazole was obtained. Decomposition point of product, recrystallized once from alcohol was 190–192°.[3b]

4. **Application of Iodination Procedure to Imidazole Carboxylic Acids and Histidine Anhydride.**—Slight modifications of Procedure 3 were applied to these derivatives and in all cases colorless iodo compounds were obtained in satisfactory yields. The concentration of sodium hydroxide needed to bring about rapid iodination of the imidazole dicarboxylic acids was slightly more than that required to neutralize the acids.

A 0.01 *M* solution of histidine anhydride may be iodinated as above in (1) by using just enough sodium hydroxide to neutralize the acid produced by the substitution reaction. The tetraiodohistidine anhydride which corresponded to that reported by Pauly[3b] was obtained by adjusting the *p*H of the aqueous solution to 5. Yield was 70–75% of theoretical which was better than that obtained using the excess base recommended by Pauly. When more than four moles of iodine was added, reddish colored products were produced which contained more than four equivalents of iodine. An ill defined compound with about 6 g. atoms of iodine per mole of histidine was isolated. In these polyiodo compounds the iodine is loosely bound and tetraiodohistidine anhydride may be formed by bubbling sulfur dioxide through a suspension of the polyiodo compounds in water.

These colored polyiodo compounds bring to mind the work of Bauer and Strauss[8] who obtained two types of iodo-proteins, the so-called N-iodo and C-iodo fractions. The former were highly colored and contained a greater percentage of iodine than the latter. The N-iodo fraction could be converted to the C-iodo by treatment with sulfur dioxide. The present work shows that the imidazole nucleus is capable of combining with indefinite amounts of loosely bound iodine. It therefore seems improbable that reliable conclusions can be drawn from the iodine content of the colored N-iodoproteins.

5. **Titration Curves for Mono and Diiodohistidine.** (a) **Monoiodohistidine Hydrochloride.**—Five cc. of 0.1 *M* solutions of the purified compound were made up and titrated with 1 *M* sodium hydroxide and 1 *M* hydrochloric acid, respectively. The addition was carried out from a weight buret into a titration cell which was maintained at 25 ± 0.2°. The cell consisted of a 15 cc. weighing bottle imbedded in a large can by means of a rubber stopper. 25° water was rapidly circulated through the can from a large thermostat. The *p*H was read with a Beckman meter using an ordinary glass electrode. Sodium ion corrections were made in the basic range.

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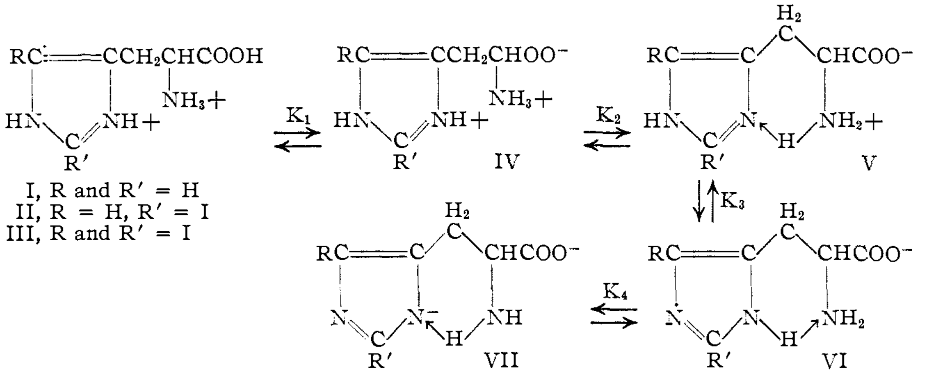
[8] Bauer and Strauss, *Biochem. Z.*, **284**, 197 (1936).

CHART I



(b) **Diiodohistidine Hydrochloride.**—One-tenth molar solution of the compound could be used only in determining the pK's in the basic range where the compound was soluble. The pK_2(2.72) was determined from 0.01 M solutions and 0.1 M sodium hydroxide. The insolubility of the hydrochloride made it impossible to determine accurately a constant for the COOH group (pK_1).

(c) **Calculations.**—Determination of the pKs from titration data was carried out by substituting values in the region of half-equivalence into the equations

$$pK_a' = p_{aH^+} - \log \frac{[\text{NaOH}] - [\text{OH}^-]}{[\text{Acid}] - ([\text{NaOH}] - [\text{OH}^-])}$$

$$pK_a' = p_{aH^+} + \log \frac{[\text{HCl}] - [\text{H}^+]}{[\text{Acid}] - ([\text{HCl}] - [\text{H}^+])}$$

The H^+ and OH^- concentrations were obtained by dividing the observed activity of the hydrogen and hydroxyl ions by activity coefficients selected from standard data on activity coefficients of these ions in inorganic salt solutions of the same total ionic strength as the titration solutions. For determining pKs which were relatively close together and for checking points far from the half-equivalence regions, general equations involving all constants were used. The author is indebted to Professor John E. Ricci[9] of this department for supplying these equations.

The titration curves are shown in Fig. 1 and the calculated pK's compared with the accepted values of histidine[10] in Table I.

TABLE I

	Histidine[a]	2- or 5-Monoiodohistidine	Diiodohistidine
pK_1' COOH	1.82	1.65 (γ_{H^+} = 0.77) 1.72 (γ_{H^+} = 1.00)	..
pK_2' ImH	6.00	4.18	2.72
pK_3' NH₂	9.17	8.62	8.18
pK_4' Im	..	(12.4)	9.76

[a] Reference 10.

Discussion

The general shift of the pK_a values of the iodohistidines to the acid side with respect to those of histidine is an expected result of the substitution of iodine on the basis of the effect of iodine on the dissociation of phenols[11] and phenylacetic acids.[12] The very large shift of 2 pK units in pK_2 for monoiodohistidine and a further shift of 1¹/₂ units for diiodohistidine are evidence in support of the assignment of pK_2 to the dissociation of the imidazolium ion (IV, Chart I), since the iodine would be expected to exert its strongest influence on the hydrogens of the imidazole ring.

The slight change in the pK_1 value for the carboxyl group is also to be expected. The relatively large shift in pK_3 (NH_3^+), however, is surprising if the effect of substitution is assumed to be transmitted through the side chain. When the reasonable assumption is made that the structure of the ampholyte of histidine contains a six-membered chelate ring (V) involving the pyridine nitrogen of the imidazole ring and the α-ammonium ion of the side chain, the striking effect of substitution of iodine on this group becomes obvious. For the sake of precision it might be argued that the new structure of the ampholyte does not permit us to assign pK_2 either to the imidazole =NH or to the α-ammonium group since chelation is the result in either case. The appearance of a fourth

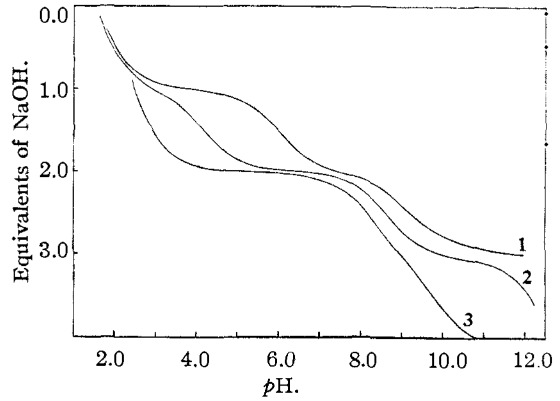


Fig. 1.—Experimental titration curves of: (1) histidine, (2) 2- or 5-monoiodohistidine, and (3) 2,5-diiodohistidine. Acid range pH 3.9 to 1.5 for histidine was calculated from the accepted value 1.82 for pK_1 (Schmidt[10]). Curves are drawn through experimental points.

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[9] Unpublished work.

[10] Schmidt, Appleton and Kirk, *J. Biol. Chem.*, **85**, 137 (1929).

[11] Bennett, Brooks and Glasstone. *J. Chem. Soc.*, 1821 (1935).

[12] Dippy, *et al.*, *ibid.*, 161, 1888 (1934); 343 (1935); 644 (1936).

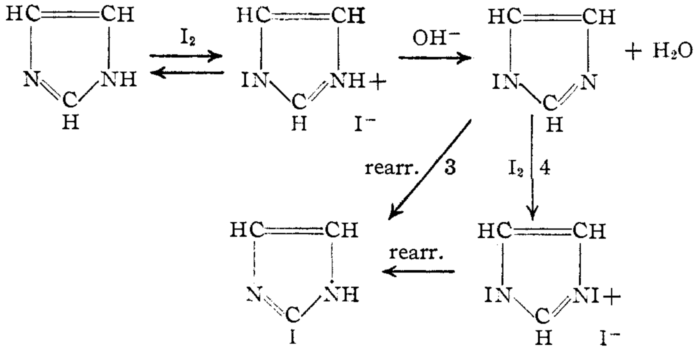

CHART II

constant in the mono and diiodohistidine and a shift of this constant by 2 pK units in diiodohistidine is in agreement with the well-known fact that the haloimidazoles form salts with bases.[13] The negative basic forms (VI and VII) are assumed also to involve chelate rings. The chelate ring involving the amino group instead of the carboxylate ion of the side chain in the mono negative ion (VI) is selected due to the stability of the six-membered ring.

It has been reported that N-substituted imidazoles do not form colorless iodo derivatives.[13] The relative resistance of histidine toward C-iodination compared, for example, to its dimeric anhydride which contains no free NH_3^+ or COO^- groups, could be explained, therefore, by assuming that the tendency of chelation of the side chain with the imidazole ring is another case of N-substitution. It must be pointed out, however, that this N-substitution involves the 3- or pyridine nitrogen while the cases thus far reported involve the 1- or pyrrole nitrogen. Thus, it may be assumed that the mechanism of iodination in solutions of low pH requires that both nitrogens in the imidazole be free.

Since mono iodination of imidazole occurs in the 2-position while bromination takes place at the four (5) position[3c] it has been assumed that iodination is not a simple electrophilic substitution on carbon but involves complex formation with subsequent rearrangement. A mechanism of normal imidazole iodination requiring the participation of both nitrogens to produce a hypothetical N-iodo intermediate is given in Chart II. Steps 3 and 4 are alternate paths to stable carbon iodination. The occurrence of carbon iodination in histidine at high pH may result either from a different mechanism, *e. g.*, direct substitution, or from the low pH mechanism, by overcoming the deactivating effect of chelation.

The isolation of monoiodohistidine at high pH is not in agreement with the observations of Li[5] on iodine absorption at low pH which indicate absorption of two moles of iodine per mole of histidine in a reaction which is first order with respect to iodine and histidine. Attempts by the present author to isolate the colorless diiodohistidine at low pH failed, but instead highly colored amorphous materials containing amounts of iodine varying from one to two atoms per molecule of histidine were obtained. The iodine in these compounds was not titrable with thiosulfate which may account for the kinetic data cited. Since the colorless mono- and diiodohistidine have been isolated, these highly colored compounds are believed to be N-iodohistidines. Analysis of purified samples of these substances and a study of the possibility and rate of their conversion to colorless iodohistidine should contribute to the mechanism of imidazole iodination.

Summary

1. 2- or 5-monoiodohistidine and 2,5-diiodohistidine have been prepared.
2. A new technique of iodination has been devised and its general applicability to imidazole compounds has been demonstrated.
3. The conditions necessary for converting histidine to colorless iodohistidines have been compared to those required for the preparation of iodoimidazoles from simpler derivatives.
4. The acidic dissociation constants of the iodohistidines have been determined, and a comparison of their values with those of histidine indicates a chelate ring in the dipolar ion of histidine. The comparison also provides evidence for the assignment of pK_2' 6.00 to the imidazolium ring in histidine hydrochloride.
5. An explanation of the effect of chelation on the iodination of histidine is offered and this effect, together with other observations on the iodination of imidazole derivatives, is correlated in a new proposed mechanism.

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[13] Meyer and Jacobson, "Lehrbuch der organischen Chemie," Vol. III, Berlin, 1920, p. 450.