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Studies on Iodinated Compounds. I. Improved Procedure for Preparation of Monoiodohistidine

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In order to obtain highly purified monoiodohistidine (MIH) in high yield, the optimum iodinating conditions for histidine were examined by HPLC. The isolation and purificaton of MIH from the reaction mixture were attempted by liquid chromatography (LC). The yields of iodohistidines [MIH and diiodohistidine (DIH)] were found to be dependent on the amount and type of the iodinating reagents; that is, the molar ratio of histidine to iodinating reagent (I_2) should be exactly 1:1 for MIH and 1:2 for DIH for the maximum yield. Purification of MIH was successfully carried out by LC on Dowex 50 (0.05 M 2,6-lutidine), SEP-PAK C_{18} cartridge (Waters Associates), and Sephadex G-10 (0.1 N AcOH). The overall yield of MIH·HCl obtained by the present method was 57%, which is the highest so far reported.

Keywords——monoiodohistidine; diiodohistidine; HPLC; iodination; LC; Dowex resin; Sephadex; SEP-PAK cartridge

It is generally known that iodinated amino acids in the animal body are mostly present in the forms of thyroxine (T_4), triiodothyronine (T_3), monoiodotyrosine (MIT) and diiodotyrosine (DIT). On the other hand, iodinated histidine such as monoiodohistidine (MIH) and diiodohistidine (DIH) are also found in the animal body as minor components and their physiological functions require clarification.[1] However, pure MIH and DIH are difficult to obtain because no synthetic method with high yield is available, and because of the difficulty in purifying them.

A method of synthesizing iodohistidines was reported by Brunings[2] in 1947, and the method is still the most generally employed one. In order to use MIH for biological studies in our laboratory, we attempted to synthesize MIH by the method of Brunings,[2] but we found the following shortcomings, that is, i) low yield, ii) difficulty in complete removal of unreacted histidine and side products, and iii) unremovable colored material formed during the reaction.

Thus, we investigated the optimum synthetic condition by high performance liquid chromatography (HPLC), and a novel method of purification of the desired product by means of LC was developed to obtain highly pure MIH in the best yield so far reported.

Results and Discussion

1. Examination of Iodination of Histidine by HPLC

Brunings[2] performed the iodination of histidine in 0.2 N NaOH solution with 0.1 N I_2–hexane solution (molar ratio of histidine to I_2, 1:1.1). The final yield of MIH·HCl was reported to be 32%, but the effectiveness of the synthetic procedure is difficult to judge, because direct recovery of MIH from the reaction mixture was not reported in his paper. Wolf and Covelli,[3] on the other hand, reported the iodination of histidine with $K^{131}I_3$ in Tris–HCl buffer, pH 8.5, and showed that the yields of the products, MIH and DIH, were dependent on the molar ratio of histidine to iodine used, that is, 2 mol of iodine relative to histidine gave MIH and 4 mol of iodine gave DIH.

We considered that the procedure described by Wolff and Covelli[3] should be examined in

detail in order to optimize MIH formation. In the present study, the iodinating reagent was added to histidine in 0.2N NaOH solution in a stepwise manner, and the iodohistidines formed were analyzed by HPLC. Iodinating reagents[4] used in this experiment were as follows: 0.1M I_2-EtOH,[1a] 0.025M I_2-hexane,[2] 0.05M I_2-KI,[1b] 0.1M ICl-EtOH, and 0.1M ICl-hexane.[5]

Analyses of the reaction mixture were performed by HPLC with a ultraviolet (UV) detector set at 254 nm. The reversed-phase column was a micro BONDAPAK C_{18} and the mobile phase was a mixture of NaH_2PO_4 solution and MeOH. Histidine has no absorption at 254 nm in this solvent, whereas iodohistidines do, and thus only the products could be detected. The effects of the amounts of MeOH and NaH_2PO_4 on the separation of iodohistidines are shown in Figs. 1 and 2. Peak 1 in Fig. 1 may be I^- (or I_3^-) formed during the iodination reactions.[6] On addition of NaH_2PO_4, a sharp MIH peak was obtained, and by reducing the amount of MeOH, retention of DIH on the column was increased (Fig. 2). As a reasonable compromise between efficiency of separation and time of analysis, a mixture of 0.005M NaH_2PO_4/MeOH (80/20) was chosen as the mobile phase for the present purpose (Fig. 1, B).

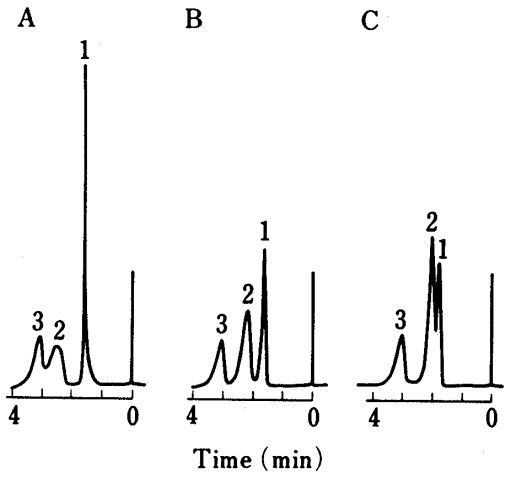

Fig. 1. Effect of NaH_2PO_4 Concentration on the Separation of Iodination Reaction Mixture

Sample: reaction mixture (histidine iodinated with 0.1 M I_2-EtOH (molar ratio of His: I_2=1:1) in 0.5 N NaOH; see "Experimental" for details).
Mobile phase: A, H_2O/MeOH (80/20);
B, 0.005 M NaH_2PO_4/MEOH (80/20);
C, 0.1 M NaH_2PO_4/MeOH (80/20).
Peak identity: 1, I^- (I_3^-); 2, MIH; 3, DIH.

Typical chromatographic patterns of the iodination products obtained with various molar ratios of iodine to histidine are shown in Fig. 3. Figure 4 shows the yields of MIH and DIH obtained with various iodinating reagents.

Except in the case of 0.1M ICl-hexane (Fig. 4 E) as the iodinating reagent, MIH was obtained in the best yield with 1:1 histidine and I_2 (ICl), and DIH with 1:2.

On the basis of these results, iodohistidine formation may proceed as follows: iodine attacks in the first place at position 4 (or 5) of the imidazole ring of histidine to form MIH, then position 2 is attacked by the iodinating reagent to form DIH. If the reaction proceeds completely, 1 mol of histidine reacts with 1 mol of I_2 (ICl) to form

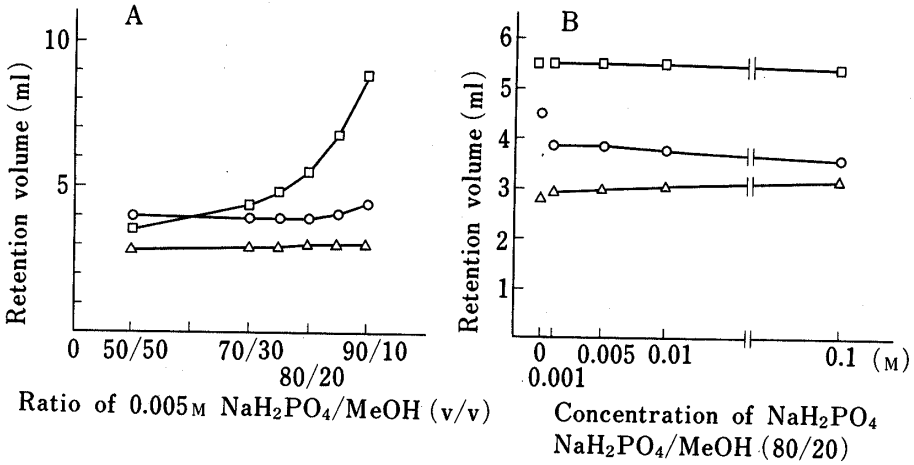

Fig. 2. Effects ot the Concentration of MeOH and NaH_2PO_4, on Retention Volumes MIH (○), DIH (□), and I^- (I_3^-) (△)

MIH because of the electrophilic nature of the iodination by HOI, then MIH reacts with another mol of I_2 (ICl) to form DIH in a stepwise manner. The present results, shown in Figs. 3 and 4, support this mechanism. While the present reaction was performed in 0.2N NaOH solution, Wolff and Covelli[3] performed the iodination under milder conditions, that is,

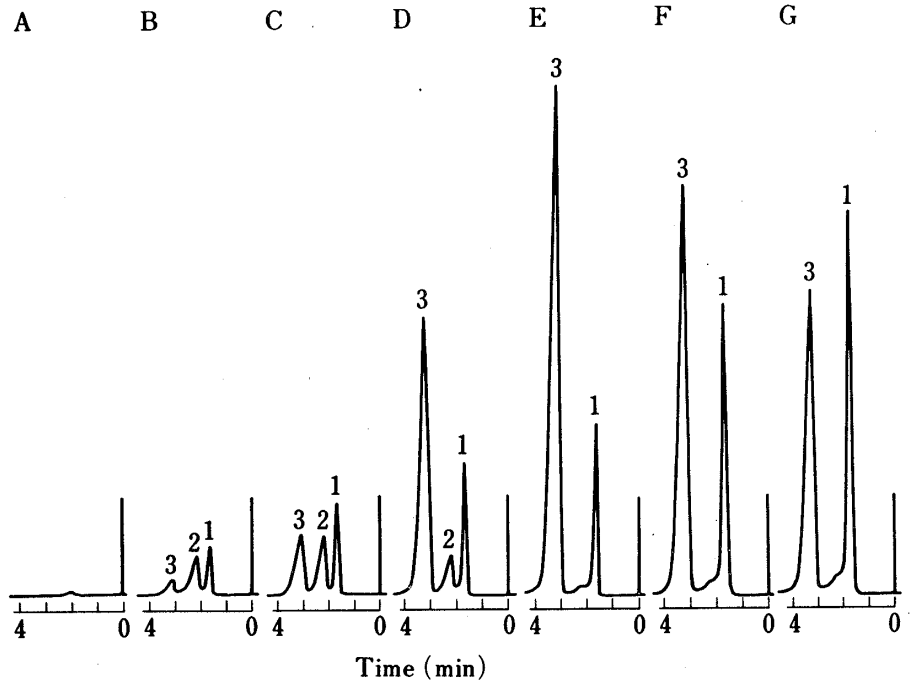

Fig. 3. Effect of the Molar Ratio of Iodine to Histidine on the Formation of MIH and DIH

Sample: reaction mixture (histidine iodinated with 0.1 M I_2–EtOH in 0.2N NaOH). His/I_2 (molar) A, 1/0; B, 1/0.5; C, 1/1.0: D, 1/1.5; E, 1/2.0; F, 1/2.5; G, 1/3.0.
Peak identity: 1, I^- (I_3^-); 2, MIH; 3, DIH.

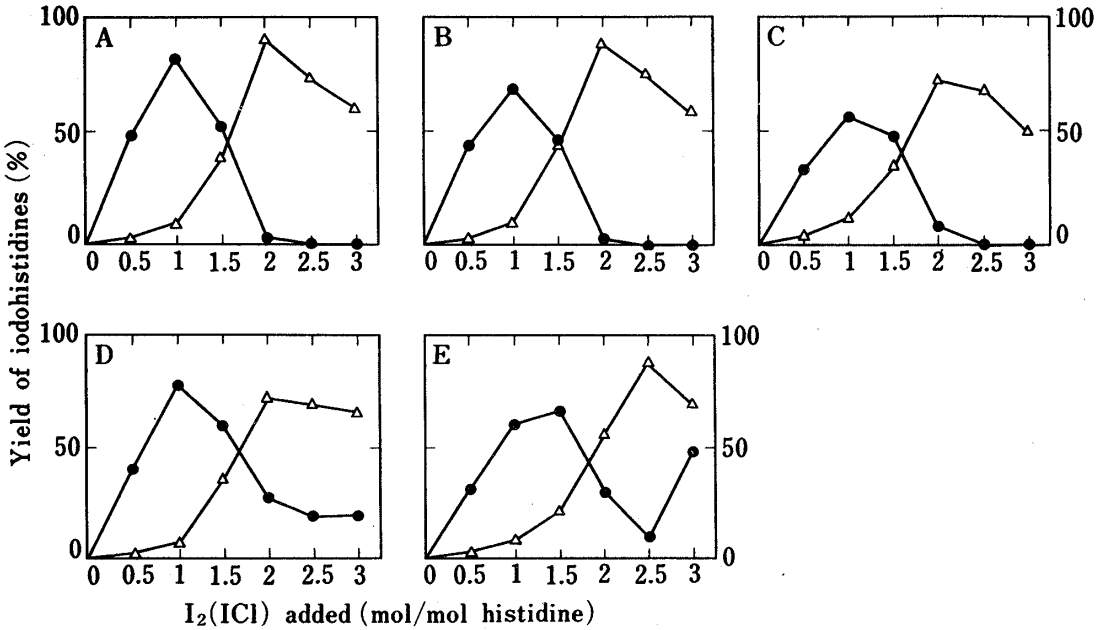

Fig. 4. Profile of Yields of MIH (●) and DIH (△) under Various Reaction Conditions

Iodinating reagents: A, 0.1M I_2–EtOH; B, 0.025M I_2–hexane; C, 0.05M I_2–KI; D, 0.1M ICl–EtOH; E, 0.1M ICl–hexane.

in Tris-HCl buffer, pH 8.5, with 1 mol of histidine and 2 mol of I_2 to obtain MIH in the maximum yield. Their conditions seem to be less efficient in terms of iodination, so that the reaction should be performed in a strongly alkaline medium. When more than 2 mol of I_2 per mol of histidine was used, the yield of DIH was lowered, suggesting oxidative decomposition of DIH by excess I_2.

Although the efficiencies of iodination of histidine with various iodinating reagents cannot be simply compared because of the use of different concentrations of reagents, it seemed that I_2 was better than ICl and that EtOH was better than hexane as a solvent. I_2–EtOH may be prepared readily with a high concentration of I_2, but I_2–hexane takes more time to prepare, and a larger amount of hexane is required. Iodination with 0.1 M ICl–hexane was the least efficient, and more iodine was required than in other procedures to obtain MIH. MIH was still detectable, although only as a minor product, by HPLC and thin layer chromatography (TLC), when more than twice as much ICl as histidine was used, which was not the case when I_2 was used.

Brunings' method was examined based on the above findings, and a ratio of 1:1.1 of histidine to I_2 was concluded to be optimal; HPLC assay showed that MIH and DIH were obtained in yields of 78 and 11%, respectively, in this case.

2. Isolation and Purification of MIH by LC

1) Ion-exchange Chromatography———Brunings[2] reported the purification of synthesized MIH by extraction from the reaction mixture, treatment with active charcoal and then recrystallization. The purified MIH thus obtained was insufficiently pure for our purposes as stated previously. Complex procedures might be one of the factors resulting in the low yield of MIH. Therefore, LC was used to isolate and purify MIH from the reaction mixture in the present work.

Suitable conditions to separate histidine, MIH and DIH in the reaction mixture by using Dowex 50 cation exchange resin were examined.

In order to recover the desired product (MIH) by a simple procedure, volatile 2,6-lutidine was used to elute the adsorbed compounds. As shown in Fig. 5, MIH was most efficiently separated from other compounds by using 0.05 M 2,6-lutidine as the eluent.

To examine the applicability of the separation system for preparative purposes, the iodination reaction mixture containing 1.5 g of histidine was loaded on a Dowex 50 W column (Fig. 6).

Although the MIH and DIH fractions overlapped slightly, unreacted histidine was well separated. Since I^- and Cl^- were eluted before the MIH fraction and Na^+ separated from MIH, the primary purpose of desalting was accomplished. Colored material was eluted mostly before the MIH fraction, on the basis of the absorbance at 410 nm.

In another trial, Dowex 1 anion exchange chromatography with the use of acetic acid was examined for the separation of MIH from the reaction mixture, but the colored material was strongly adsorbed on the resin and re-activation of the resin was inconvenient, so that this combination was not used in the present study.

The components of the reaction mixture were eluted in the order MIH, DIH and histidine from the Dowex 50 column, and histidine, MIH and DIH from the Dowex 1 column. If the basicity of the imidazole ring is decreased by iodination, the elution from Dowex 50 with lutidine should be in the order DIH, MIH and histidine. Reduction of basicity of the imidazole ring by iodination as well as increase of the hydrophobicity might cause the unexpected elution sequence.

2) Decolorization of MIH Fraction with a SEP-PAK C_{18} Cartridge———The MIH fraction obtained by chromatography on Dowex 50 was still colored. In order to remove the colored material, the fraction was passed through a SEP-PAK C_{18} cartridge (Waters Associates). Most of the colored material was adsorbed on the cartridge, while complete recovery of MIH and DIH was achieved by washing the cartridge twice with 10 ml of water (Fig. 7). Spec-

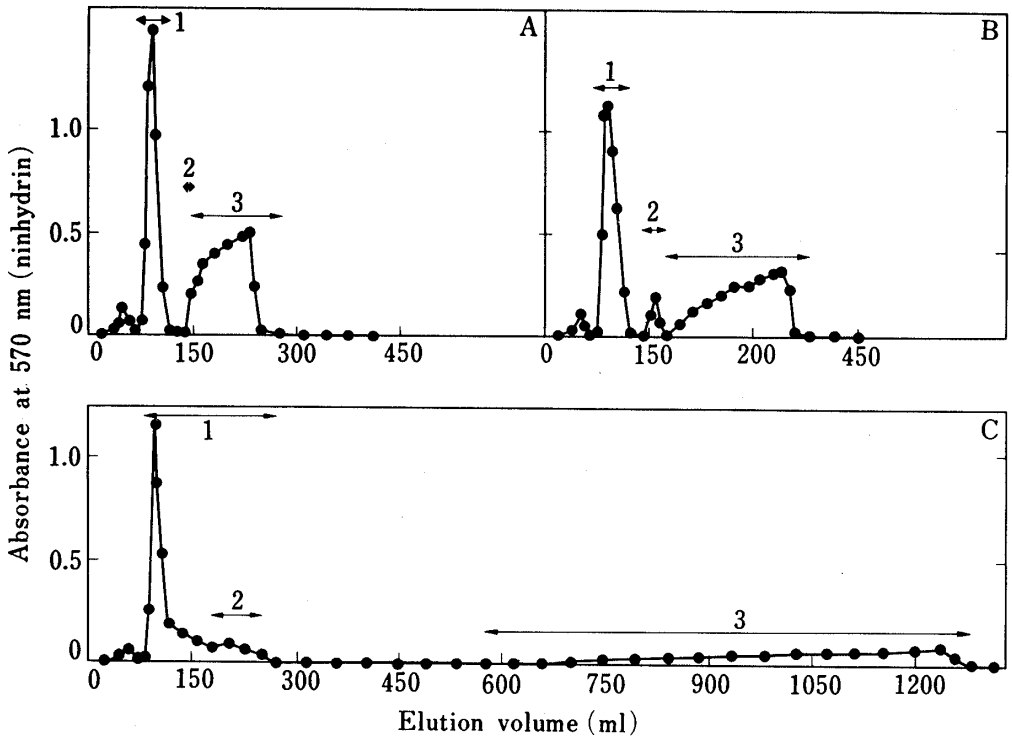

Fig. 5. Separation of Histidine, MIH and DIH from the Reaction Mixture by Dowex 50 Column Chromatography

Column: Dowex 50 W×4 (200—400 mesh) (1.8×25 cm).
Detection: Ninhydrin reaction.
Sample: Iodination of His·HCl·H_2O (2.5 g) was performed according to the method given in Ref. 1*b*). An aliquot (1/5 of the reaction mixture) was subjected to chromatography.
Peak identity; 1, MIH; 2, DIH; 3, histidine.
Eluent: A, 0.1 M 2,6-lutidine, pH 9.65; B, 0.05 M 2,6-lutidine, pH 9.60; C, 0.01 M 2,6-lutidine, pH 8.93.

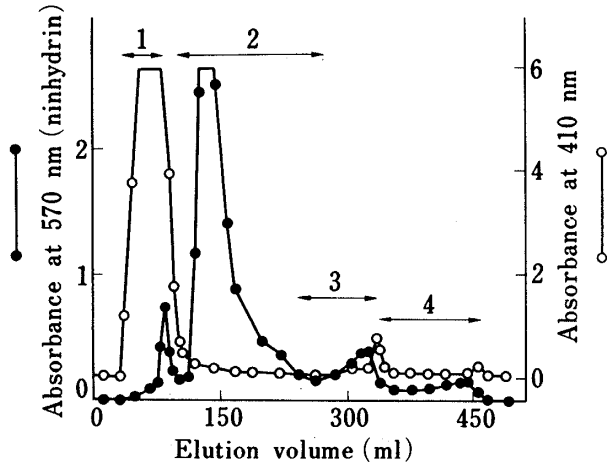

Fig. 6. Ion-exchange Chromatogram of the Reaction Mixture

Column: Dowex 50 W×4 (200—400 mesh) (2.5×25 cm).
Detection: Ninhydrin reaction.
Sample: His·HCl·H_2O (1.5 g) iodinated with 0.025 M I_2–hexane in 0.2 N NaOH.
Peak identity: 1, I^- and Cl^-; 2, MIH; 3, DIH; 4, histidine.
Eluent: 0.05 M 2,6-lutidine, pH 9.56.

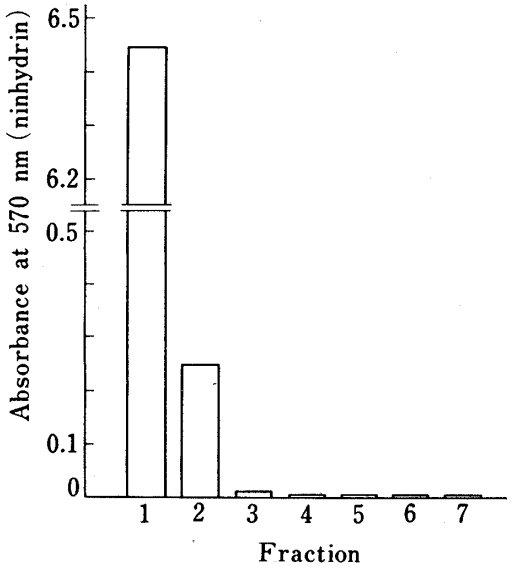

Fig. 7. Recovery of MIH fyom the SEP-PAK C_{18} Cartridge

Sample: MIH fraction collected and concentrated to 20 ml.
Detection: An aliquot (10 μl) of each fraction was reacted with ninhydrin and diluted with *n*-PrOH–H_2O (1:1).
Fraction: 1, MIH fraction passed through the cartridge; 2—6, washings of the cartridge with 10 ml of MeOH.

trophotometric confirmation of the effective removal of the colored material by SEP-PAK C_{18} is shown in Fig. 8. However, MIH was still contaminated by DIH even after this treatment.

3) Purification of MIH by Gel Filtration——In order to separate MIH from DIH, gel filtration was attempted. It was assumed that the application of gel filtration for isolation of iodohistidine[1a,7] should be based not on the molecular sieve effect but on the adsorption of the imidazole moiety by the gel material. Such a system based on Sephadex G-25 has been reported to separate amino acids with acetic acid–water as the elution medium.[8] Using this system, the elution pattern of histidine, MIH and DIH was examined (Fig. 9).

Under the conditions examined, MIH and DIH were separated well, but histidine and MIH were not separated satisfactorily. However, histidine had already been removed on Dowex 50 so that separation of histidine and MIH was not required here. Thus, the final purification of MIH was successfully performed by the use of a Sephadex G-10–0.1N acetic acid system, as shown in Fig. 10.

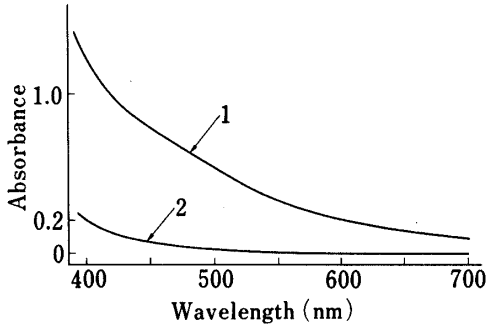

Fig. 8. Decolorization of MIH Fraction with the SEP-PAK C_{18} Cartridge

1, Before SEP-PAK C_{18} cartridge treatment; 2, after SEP-PAK C_{18} cartridge treatment. Sample: fractions 1, 2 and 3 in Fig. 7 were combined and concentrated to 20 ml.

MIH and DIH were separated well, and pure MIH was obtained. The MIH peak was split into two, but this was probably due to overloading of the sample relative to the capacity of the gel material.

4) Yield and Purity of MIH——The purified MIH was dissolved in EtOH–HCl, neutralized with aniline, and crystallized as the hydrochloride (yield: 1.10 g as crystals and then 0.20 g from the mother liquid, 1.30 g in total, 57%). The overall yield is much

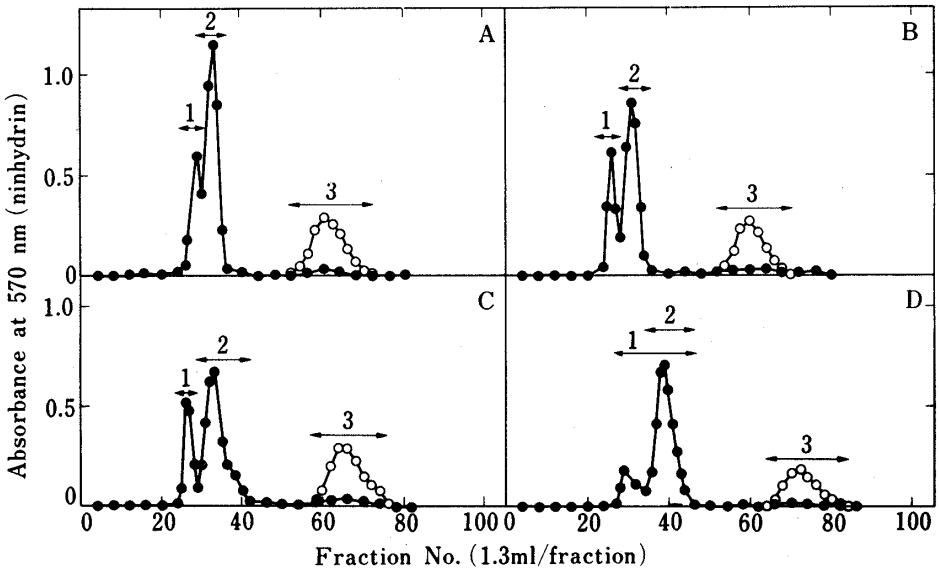

Fig. 9. Separation of Histidine, MIH and DIH on Sephadex G-10

Column: Sephadex G-10 (1.8×5 1cm).
Detection: ninhydrin reaction.
—●—, ninhydrin treatment with 10 μl aliquot; —○—, ninhydrin treatment with 100 μl aliquot.
Sample: Standard MIH·HCl (500 mg), DIH (100 mg) and His·HCl·H_2O (100 mg) were mixed, then desalted on Dowex 50 (2,6-litidine), and 1/10 of the amount was applied to a Sephadex G-10 column.
Peak identity: 1, histidine; 2, MIH; 3, DIH.
Eluent: A, 0.5N AcOH, pH 2.50; B, 0.2N AcOH, pH 2.73; C, 0.1N AcOH, pH 2.88; D, 0.01N AcOH, pH 3.40.

higher than the reported value (32%)[2] and closer to the yield of the iodination reaction (78%).

No colored material was contained in the crystallized MIH, and histidine and DIH were not detected by TLC.

Conclusion

Suitable conditions for the efficient synthesis of MIH were found, as shown in "Experimental." Because of the advantages of simplicity (easy and rapid) and efficiency, iodination was performed with iodine-EtOH with an approx. 1:1 molar ratio of histidine and iodine (I_2). Separation and purification of MIH from the reaction mixture were performed by means of serial chromatographic procedures using Dowex 50–0.05 M 2,6-lutidine, SEP-PAK C_{18} cartridge and Sephadex G-10–0.1 N AcOH.

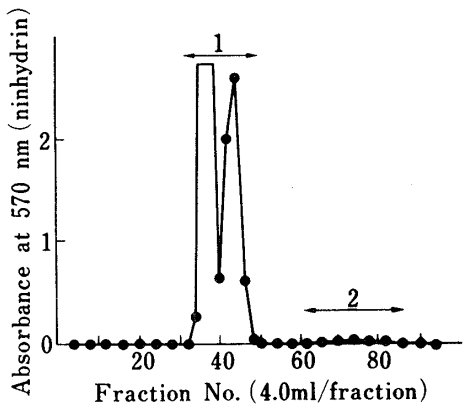

Fig. 10. Purification of MIH on Sephadex G-10

Column: Sephadex G-10 (2.5×45 cm).
Detection: ninhydrin reaction.
Sample: MIH fraction after treatment with Dowex 50 and SEP-PAK C_{18}.
Peak identity: 1, MIH; 2, DIH.
Eluent: 0.1 M AcOH, pH 2.90.

Satisfactory purity and yield of MIH was obtained by the proposed method, and the procedure is shorter and more convenient than Brunings' original method.[2]

Experimental

Reagents and Materials——L-Histidine·HCl·H_2O, I_2 and ICl, guaranteed pure grade, were products of Wako Pure Chemicals, and other reagents used were also of guaranteed pure grade. Ion-exchange chromatography was performed on Dowex 50 W×4, 200—400 mesh (Dow Chemical Co.), gel chromatography was carried out on Sephadex G-10 (Pharmacia Fine Chem.), and SEP-PAK C_{18} was obtained from Waters Associates. TLC plates were DC-Fertigplatten Cellulose (ohne Fluoreszenzindikator), 0.1 mm thickness, from Merck.

Preparation of MIH·HCl and DIH·HCl as Standards——MIH was synthesizd by the method proposed by Imai[1b] and purified on Dowex 50, Avicel and Sephadex G-10 columns, then by recrystallization from EtOH–HCl (neutralized with aniline). DIH was synthesized by Brunings' method,[2] and purified by recrystallization after being decolorized by the use of active charcoal. MIH·HCl and DIH·HCl were identified by routine procedures.

Instruments——High-performance liquid chromatography (HPLC) was performed with a Waters Associates 204 ALC/GPC machine with a Model A 6000 pump. A U6K universal injector, Model 440 UV detector and micro BONDAPAK C_{18} (4 mm ID × 30 cm) column were also used. Absorbance and visible spectra were determined on a Hitachi Type 100-50 double-beam spectrophotometer with a Type 200 recorder. Melting points were determined with a melting point apparatus (Type MP-1, Yamato Scientific Co.), and are uncorrected. NMR spectra were obtained with a JEOL LNM-FX-100 machine at 100 MHz. D_2O was used as the solvent, and TMS was used as an external standard. Elementary analyses were performed at the analytical laboratory of Hoshi College of Pharmacy.

Separation and Identification of Iodohistidines by HPLC——Histidine HCl·H_2O, 250 mg (0.0012 mol), was dissolved in 0.2 N NaOH (75 ml). To this solution, 0.0006 mol of ice-cold I_2 (ICl) was added with stirring over a period of 10 min, and the stirring was continued for further 5 min. The same procedure was further repeated 5 times; the final amount of I_2 (ICl) added was 0.0036 mol, a 3-fold excess over histidine.

An aliquot of 250 μl was withdrawn from the reaction mixture at each step, and 250 μl of 0.2 N HCl and 2 ml of H_2O were added. The solution was filtered through a 0.45 μm filter for HPLC. Using a solution of 0.005 M NaH_2PO_4/MeOH (80/20) as the mobile phase, 10 μl of the sample was developed at a flow rate of 1.8 ml/min. As reference standards, 10 μl of MIH·HCl (0.25 mg/ml H_2O), and DIH·HCl (0.2 mg/ml H_2O) were developed with the sample. The detector sensitivity was set at 0.01 AUFS for MIH and 0.05 AUFS for DIH.

Purification of MIH by LC——H-form Dowex 50 resin was saturated with 2,6-lutidine and used for the adsorption of MIH. A SEP-PAK C_{18} cartridge was pretreated with MeOH and H_2O. An aliquot of the amino acid-containing fractions, 10 μl, was colored with ninhydrin, and determination of the absorbance was

performed after diluting it with 4 ml of a diluent (*n*-PrOH: H_2O=50:50).[9] Identification of the eluted amino acids was performed by TLC (cellulose, with *n*-BuOH: AcOH: H_2O=4:1:1) with standard amino acids (*Rf*'s: His 0.05, MIH 0.16, DIH 0.40). Detection of halogens and sodium ions was performed with $AgNO_3$ test solution and potassium pyroantimonate[10] using 50 μl of sample with 1 ml of water.

Established Method for the Synthesis of MIH·HCl——L-Histidine·HCl·H_2O, 1.5 g, was dissolved in 250 ml of 0.2 N NaOH, and I_2-EtOH (I_2 2 g+EtOH 100 ml) was added with stirring under ice-cooling over a period of 2 h. Stirring was continued for further 30 min. When the reaction was complete, concentrated HCl was added to the reaction mixture to give pH 5, and the solution was concentrated under reduced pressure to approx. 20 ml. The concentrated mixture was applied to a column of Dowex 50 W ×4 (2.5×25 cm) and eluted with 0.05 M 2,6-lutidine, and fractions of 5 ml were collected. An aliquot of each fraction was subjected to TLC to examine the compositions. MIH fractions, fraction Nos. 23—50, were collected and the solvent was removed under reduced pressure. The residue was dissolved in a small amount of water, and the solution was passed through a SEP-PAK C_{18} cartridge. The cartridge was washed with 10 ml of water twice, then the washings were combined, and concentrated to 10 ml under reduced pressure. The concentrate was applied to a Sephadex G-10 column (2.5 × 40 cm) and eluted with 0.1 N AcOH, and 4 ml fractions were collected. MIH fractions (fractions 34—48) were combined, and concentrated under reduced pressure. Next, 1 ml of concentrated HCl and 100 ml of EtOH were added to the residue, and the solution was neutralized with aniline, then allowed to stand at −5° overnight to obtain crystals of MIH. Crystals were collected by centrifugation at 3000 rpm for 10 min to remove most of the solvent, and were washed with a small amount of EtOH, then dried under a vacuum. The yield was 1.20 g at this stage. The mother liquor contained additional MIH, and 0.10 g of crystals was obtained. The overall yield was 1.30 g (57%). Mp 178—180°C (dec.) (reported mp (dec.) 204—206°C,[2] 181—182°C).[1a] ^{1}H-NMR (5% solution in D_2O) δ: 3.22 (2H, d), 3.97 (1H, t), 8.35 (1H, S). *Anal.* Calcd for $C_6H_9ClIN_3O_2$: C, 22.70; H, 2.86; N, 13.23. Found: C, 22.68; H, 2.95; N, 13.32.

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