Studies on Mono- and Diiodohistidine

I. THE IDENTIFICATION OF IODOHISTIDINES FROM THYROIDAL IODOPROTEINS AND THEIR PERIPHERAL METABOLISM IN THE NORMAL MAN AND RAT

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ABSTRACT The problem as to whether iodohistidines are normally biosynthetized in thyroglobulin and thyralbumin has been examined both in man and the rat. Evidence has been obtained for the first time that diiodohistidine (DIH) is present in both species in these two iodoproteins. The biosynthesis of monoiodohistidine (MIH) in the thyroglobulin of the normal rat has been confirmed and extended to rat thyralbumin and to human thyroid iodoproteins.

The iodohistidine identification is based on five original methods including: (*a*) the preparation of stable and radioiodine-labeled iodohistidines; (*b*) the protection of the labile iodohistidines during the iodoprotein enzymatic hydrolysis; (*c*) the isolation of iodohistidines by ion-exchange resin chromatography; (*d*) their separation from each other and from iodinated cationic butanol-insoluble compounds by Sephadex G-10 chromatography; and (*e*) their purification by successive crystallizations to a constant specific activity.

Iodohistidine levels (in percent of protein radioactivity from iodide given *in vivo*) were found comparable in man and the rat. However, the values (mean ±SE) for thyroglobulin (MIH, 0.61±0.10%; DIH, 0.050±0.015%) and for thyralbumin (MIH, 2.61±0.57%; DIH, 0.28± 0.09%) differ significantly ($P < 0.05$).

Iodohistidines are stable during *in vitro* exposure to iodotyrosine dehalogenase preparations. In contrast to iodotyrosines the iodohistidines when given *in vivo* to man either orally or intravenously were in large part recovered in 24-h urines.

INTRODUCTION

The normal in vivo iodination of histidine is usually overlooked, although monoiodohistidine (MIH)[1] has been identified in the rat thyroid (1, 2). No biosynthetically formed diiodohistidine (DIH) has ever been detected. In contrast MIH and DIH are readily formed during the *in vitro* iodination of histidine either as the free amino acid (3) or as histidyl residues from proteins (1, 2, 4–13). The formation of iodohistidines in normal human thyroid glands has never been investigated. In human congenital goiters however, iodo-compounds have been isolated from thyroid (14–17), blood (16, 18, 19), and urine (14, 20, 21) as noniodide, butanol-insoluble, unknown compounds. We have identified large amounts of MIH and DIH among similar compounds in these congenital goiters (22). Therefore the question arose as to whether these findings were specific for this pathological condition.

The aims of the present study are as follows: (*a*) to develop suitable methods for the protection and the identification of the fragile iodohistidines from biologic materials, which would be superior to the classic chromatographic techniques; (*b*) to reinvestigate MIH and DIH biosynthesis in normal rat thyroid glands in comparison with normal human thyroid glands; (*c*) to study in man the in vivo metabolism of the iodohistidines.

Evidence is presented here for the first time that: (*a*) DIH is present in normal thyroglobulin; (*b*) iodohistidines are elaborated in vivo in normal thyroid albumin (Ta) to a greater extent than in thyroglobulin (Tg); (*c*) the iodohistidines are stable during the in vitro exposure to iodotyrosine dehalogenase and when given in vivo are recovered for the most part in the urine.

METHODS

The reagents and their sources are as follows: L-histidine (free base), A grade, and Pronase, B grade, Calbiochem, San Diego, Calif.; 1-methyl-2-mercaptoimidazole (Thiamazole), Diamant Laboratories, Paris; NADPH, Sigma Chemical Co., St. Louis, Mo.; ^{131}I (100 mCi/ml) and ^{125}I (10 mCi/ml), Commissariat à l'Energie Atomique, France. The ion-exchange resins were from Bio-Rad Laboratories, Richmond, Calif. Anionic Dowex AG-1-X2, 200–400 mesh, and cationic Dowex AG-50W-X2, 200–400 mesh, were regenerated by suspending in 6 N HCl, and the resins were washed with deionized water until neutral. Sephadex G-200

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[1] *Abbreviations used in this paper:* MIH, 4-monoiodo-L-histidine; DIH, 2,4-diiodo-L-histidine; MIT, 3-monoiodo-L-tyrosine; DIT, 3,5-diiodo-L-tyrosine; T_4, 3,5,3′,5′-tetraiodo-L-thyronine; T_3, 3,5,3′-triiodo-L-thyronine; Tg, thyroglobulin; Ta, iodinated albumin from the thyroid gland (thyralbumin); RISA, radioiodinated human serum albumin.

and Sephadex G-10 were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden.

Preparation of stable iodohistidines. Iodohistidines were made according to the method of Brunings (3) with the following modifications. Iodination was performed by adding dropwise and with constant stirring 1 M iodine in absolute ethanol to L-histidine as the free base, dissolved in 300 ml, of 0.2 M NaOH, and kept at 4°C. The ratios of added iodine to histidine were 2.1 and 5.0 mg at. wt/mmol for MIH and DIH synthesis, respectively. The slow iodination required about 1 h. The iodide ion was excluded from the final reaction medium, acidified to pH 1 with concentrated HCl, with a 3×30 cm Dowex 1 resin column.

DIH crystallization was obtained by reducing the volume of the iodide-free Dowex 1 direct effluent mixed with the water washes in a rotating vacuum evaporator. DIH was purified from some MIH contaminant by repeated crystallizations, as described below. Chromatographically pure DIH (see Chromatographic Systems) had the capillary melting point (mp) of 210–211°C (reported 220°C) (3). Iodine to carbon ratio (I/C) was found to be 3.44 (calculated value — 3.50).

The MIH reaction medium was first cleared of iodide on a similar Dowex 1 column and then dried. MIH from the residue was extracted with concentrated HCl-absolute ethanol (1.3% vol/vol), reduced to dryness under vacuum, and then dissolved in water. MIH was crystallized at −20°C after the addition of 1 vol of concentrated HCl and 1 vol of absolute ethanol. The highly hydroscopic MIH crystals were dried at 100°C. Capillary mp: 181–182°C (reported 204–206 [3] and 164–170 [23]). The I/C ratio was found to be 1.75 (calculated value—1.76).

Preparation of iodine-labeled iodohistidines. MIH was prepared from histidine by substitution and DIH from stable DIH by exchange reaction. Iodinating solutions were prepared in two ways: (*a*) 0.1 ml 1 N HCl and 0.1 ml KIO3 (10 mg/ml) solutions were added to 10 μl of ^{131}I or ^{125}I solutions. (*b*) 0.1 ml of Chloramine-T solution (2.18 mg/ml) was added to 10 μl of the carrier-free radioiodide solution(s). Solution *a* or *b* was added to 100 μl of a solution of L-histidine base or crystallized DIH (1.5 mg/ml). After 3 min, 0.2 ml of a 5% sodium metabisulfite solution was added, followed by 0.1 ml of a 20% KI solution.

The mixture was then chromatographed on a 0.6×5 cm Dowex 1 column and the iodohistidines were eluted with 15 ml of water. The eluate was adjusted to pH 1 with 1 N HCl then applied to a Dowex 50 column. Pre-elution with 10 ml of 1 N HCl was discarded. The iodohistidine fraction was finally collected in 15 ml of 4 N HCl.

The average yield of incorporated radioactivity was 40%. Specific activities obtained were between 5 and 500 mCi/mmol.

Purity of stable and labeled iodohistidines. This purity was tested in two systems. Ascending paper chromatography with butanol-acetic acid-water (78-5-15 vol/vol) on Whatman 1 paper for 20 h (24) gave a R_f for MIH of 0.05–0.08 and 0.19–0.23 for DIH. Thin-layer chromatography (TLC) with tertiary amyl alcohol-tertiary butanol-6 N ammonia-acetone (10-20-20-80 vol/vol) on TLC silica gel plates (Eastman type K 30 1-R2) for 2 h (25) gave a R_f for MIH of 0.50 and 0.34 for DIH. Stable compounds were developed with Pauly's reagent and labeled compounds by autoradiography. Labeled MIH was always pure while DIH was usually contaminated with MIH requiring further purification by Sephadex G-10 chromatography as described below.

Isolation of MIH and DIH from normal thyroid glands. Glands were obtained from patients undergoing thyroid surgery for cold nodules and from Wistar rats. Patients were given 200 μCi orally and the rats 1 mCi subcutaneously of ^{125}I 24–48 h before surgery. The tissues were minced, suspended in 4 vol of cold 0.9% NaCl, and extracted for 24 h at 4°C. The crude saline extracts were centrifuged at 105,000 *g* in a Spinco model L2 (rotor 50) ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). The sediment was washed twice in 0.15 M NaCl and centrifuged at 105,000 *g*. The soluble iodoproteins were purified from the total 105,000 *g* supernate by a modification of the method described by Ramagopal, Spiro, and Stanbury (26). A portion of 10–30 μCi ^{125}I in a volume of 1 ml was filtered through a 1.2×30 cm Sephadex G-200 column, suspended in 0.15 M NaCl, and eluted with the same solvent. Fractions were counted (Autogamma, Packard Instrument Co., Downers Grove, Ill.), and the top of the Tg peak of radioactivity appearing after elution with 14 ml was saved. Ta, when isolated from Tg, was monitored by the prior addition of 0.1 μCi [^{131}I-]radioiodinated human serum albumin (RISA) which gave a peak on the column appearing after 25 ml. The corresponding Ta peak was fully separated from Tg by refiltration through an identical Sephadex G-200 column. From each protein fraction, portions were taken to determine the total radioactivities and the protein content (27). Protein hydrolysis was routinely performed with Pronase at a 3 mg/ml concentration in 0.2 M Tris-HCl at a pH of 8.0 and in the presence of DIH (2.5×10^{-3} M), Thiamazole (10^{-2} M), and a few drops of toluene. The protein content was kept below 10 mg/ml. The duration of the Pronase hydrolysis was 3 days at 37°C.

Fractionation of the iodoamino acids was done on $1.5 \times$ 20-cm Dowex 1 columns. After applying the hydrolysate the columns were rinsed with 20 ml water, eluted with 0.1 N HCl-butanol (3.3% vol/vol) (28), and 2-ml fractions were collected. Peak 1, containing the iodohistidines, and two other peaks containing MIT and DIT were eluted sequentially. 3,5,3′,5′-Tetraiodo-L-thyronine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) were finally eluted with 60% acetic acid. Iodide was retained by the column. The nature of MIT, DIT, T_4, and T_3 peaks was verified by paper chromatography. The Dowex 1 peak 1 was vacuum evaporated then taken back in 1 ml of 0.2 N HCl. [^{131}I] MIH and/or DIH were added as internal indicator(s) and the mixture was applied to Sephadex G-10 columns. Two 0.6×40-cm columns, equilibrated and eluted with 0.2 N HCl, one over the other were used routinely. The flow rate was 0.1 ml/min and 0.5-ml fractions were collected.

Successive crystallizations to constant specific activity was the reference method chosen for the identification of both iodohistidines. The vacuum-evaporated active material, taken back in water, was added to a known amount of crystallized MIH or DIH (usually 500 mg). Complete dissolution in 2 ml of water required for DIH the addition of concentrated HCl and heating. MIH crystallization was obtained by adding a few drops of concentrated HCl, then 1 vol of ethanol; DIH crystallization was obtained by increasing the pH to 3 with concentrated ammonia.

After standing 24 h at −20°C mother liquors were separated by centrifugation, and the crystals were washed three times with cold absolute ethanol for MIH and cold water for DIH. Crystals were dried at 100°C to constant weight, and portions (100 mg), mother liquors, and washes were counted. Subsequent recrystallizations were done in the same manner. The volume of water was lowered in order to keep the volume of the mother liquors and that of the wet crystals in a constant proportion close to 4 to 1.

The yield of crystallization was 60–80%. Specific radioactivities were corrected for physical decay, geometry, and ^{125}I self-absorption within the crystals (a direct function of the amount of iodine) according to an experimentally determined curve. The true content of (*a*) the MIH or DIH Sephadex G-10 peaks, (*b*) the Dowex 1 peak 1, and (*c*) the original hydrolysate were recalculated stepwise from the "crystallizable" MIH or DIH radioactivities found to be constant during several crystallizations.

In vitro enzymatic deiodination of MIH (or DIH). This was investigated with tissue homogenates (29, 30). Fragments of freshly removed normal thyroid and kidney from rat and human origin were suspended in 4 vol of 0.25 M sucrose in 0.15 M Tris-HCl buffer at pH 7.4 and ground in a glass-teflon homogenizer at 0°C. Centrifugation at 700 *g* for 5 min gave a crude sediment which was discarded. 2.5-ml solutions with Tris-HCl buffer (pH 7.4) containing 0.5 ml of the homogenate, 10^{-3} M NADPH, labeled MIT, and various concentrations of cold MIT and/or MIH and DIH were prepared. The solutions were incubated with constant shaking at 37°C then 50-μl portions were chromatographed on 0.6 × 3-cm Dowex 50 columns. I^- was eluted from the columns with 15 ml of water, then MIH or DIH with 15 ml 4 N HCl, or MIT with 15 ml 2 N ammonia. The radioactivity, present in the water effluent, was further identified as iodide by paper and anion-exchange chromatography.

In vivo peripheral metabolism of MIH and DIH. This was investigated in eight normal adult volunteers. The original method as applied to iodotyrosines (31) was modified as follows. Six fasting volunteers were given a 100 mg KI solution orally. 30 min later 5–10 μCi of carrier-free-labeled MIH, DIH, or DIT (specific activity 1 mCi/mg) was given orally. Urines were collected between 0–4 and 4–24 h. The radioactivity excreted was calculated as the percent of the administered dose. Urines were hydrolyzed with HCl to a final 4 N HCl concentration, and then heated at 60°C for 30 min. Iodohistidines and iodotyrosines, recovered from hydrolyzed urines, were separated from radioiodide by Dowex 50 columns as previously described.

Two normal subjects were injected intravenously: one with [^{125}I] MIH, the other with both [^{131}I] DIH and [^{125}I] MIH. The carrier-free-labeled iodohistidines were sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.). Doses were of the order of 100 μCi (specific activity 1 mCi/mg). Iodohistidine renal clearances were calculated according to a computer program (32). Iodohistidines were separated from acid-hydrolyzed urine samples by single column Sephadex G-10 chromatography. Statistical analysis was made according to Schwartz (33).

RESULTS

Properties of iodohistidines. Iodohistidines known to be extremely soluble in water (3) were not extractable with butanol from aqueous solutions. The partition coefficient between 0.1 N HCl and *n*-butanol as determined with pure labeled MIH and DIH was about 49. This property, which differentiates iodohistidines from any other iodoamino acid, has been used during their purification.

Both anion- and cation-exchange resins easily separate iodohistidines from iodoamino acids and from

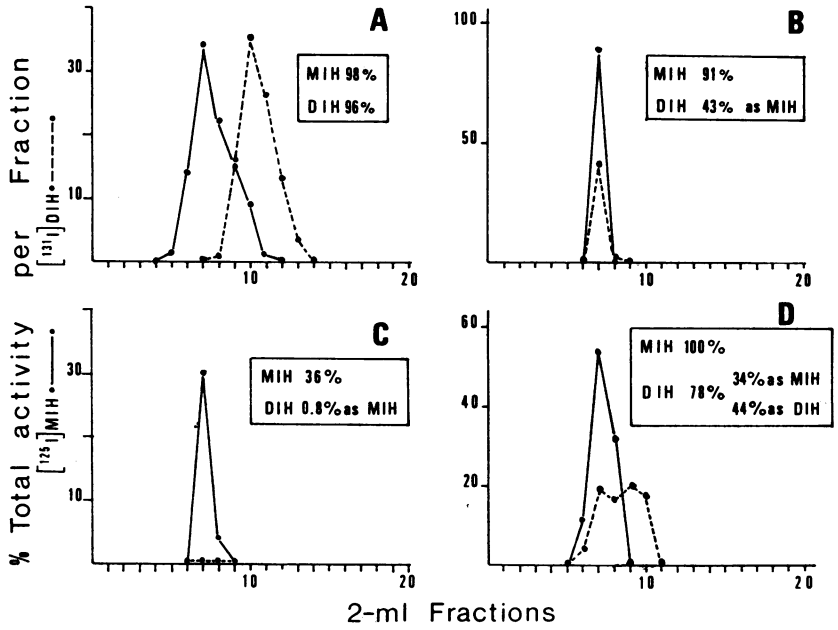

FIGURE 1 Dowex 1 chromatographic recovery of iodohistidine indicators which have been added before the following hydrolytic procedures. A, 4 N HCl-acidified urines heated 30 min at 60°C; B, buffered albumin solution, pH 8.0, incubated for 72 h at 37°C *without* hydrolytic enzymes; C, same conditions as B but with Pronase (3 mg/ml); D, same conditions as C but in the presence of 2.5×10^{-3} M DIH. Recoveries indicated in rectangles. The unaccounted for radioactivities were identified as iodide retained by the column.

iodide. Dowex 50 columns separated iodide appearing in the water washes from cationic iodohistidines which can be selectively eluted. In the 15 ml 4 N HCl eluate over short columns, the iodohistidine recovery was better than 98% with very little contamination by MIT, DIT, or iodothyronines. Pure iodohistidines were eluted from Dowex 1 columns in two overlapping peaks (Fig. 1 A). Mixed MIH and DIH from iodoprotein hydrolysates are quantitatively recovered in a single peak 1, which has been called the "crude iodohistidine fraction." Iodotyrosines, eluted later, were well separated from iodohistidines and from each other (Fig. 2).

Sephadex G-10, as shown in Fig. 3, was the only column chromatographic method found to reliably give a satisfactory separation of MIH, DIH, and I^- which appeared respectively after 19, 24, and 75 ml of elution in the conditions described previously. It is mandatory that the Sephadex G-10 gel be prepared and eluted with 0.2 N HCl.

Successive crystallizations in the purification of iodohistidines. The purification of iodohistidines obtained by successive crystallizations was demonstrated by the recovery of chromatographically pure labeled MIH and DIH. The specific activity of the crystals was constant and the calculated recovery of the radioactivity in the crystals was 101.8±2.94% for MIH and 95.6±1.38% for DIH (mean ±SE). Experiments were done to demonstrate the purification of iodohistidines from each other: (*a*) one variety of iodohistidine, MIH or DIH, was added as pure tracer to 500 mg of the other iodohistidine carrying a different label. (*b*) Under similar conditions the contaminant was added as 5% of the total iodohistidine weight. The specific activities from the contaminant decreased with first order kinetics as a function of the crystallization number. In experiment (*a*) the purification from the contaminant DIH or MIH was 38 and 40% and in experiment (*b*) was 56 and 73% per crystallization step. Therefore no effort was made to purify [^{127}I] iodohistidines before the crystallizations.

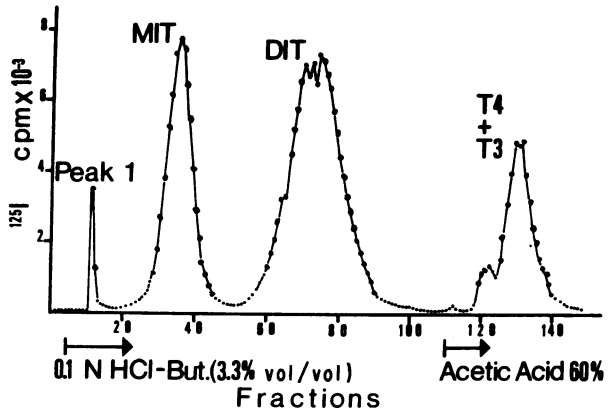

FIGURE 2 Elution profile obtained with Dowex 1 chromatography of human Tg hydrolyzed with Pronase. The narrow peak 1 in which iodohistidines, if added, could be quantitatively recovered was called "crude iodohistidine fraction."

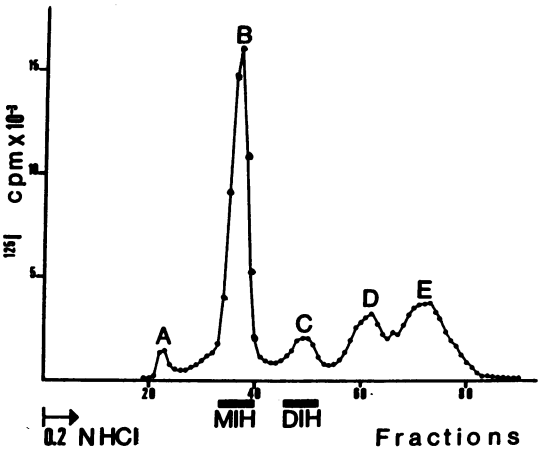

FIGURE 3 Sephadex G-10 elution profile of Dowex 1 "crude iodohistidine fraction" from normal rat Tg hydrolyzed with Pronase. Separated but incompletely purified MIH and DIH were identified respectively in peaks B and C.

Recovery of MIH and DIH after acid or enzymatic hydrolysis. The fragility of the iodohistidines during hydrolytic procedures was investigated by Dowex 1 chromatography as follows. When labeled iodohistidines were added to urines acidified to 4 N HCl concentration and heated for 30 min at 60°C, no apparent alteration of MIH or DIH was found (Fig. 1 A). Increasing the temperature to 100°C or the time to 24 h significantly decreased the tracer recovery.

DIH fragility was demonstrated by the complete conversion of DIH to MIH when incubated at 37°C in buffered albumin solutions at neutral pH for 48–72 h (Fig. 1 B). When Pronase was present denaturation of iodohistidines was more pronounced (Fig. 1 C). The addition of 2.5×10^{-3} M DIH provided complete DIH protection with the subsequent recovery of about 50% of the DIH (Fig. 1 D). Even at 10^{-5} M DIH was still partially effective. The presence of 10^{-2} M Thiamazole was ineffective alone but seemed to potentiate the action of stable DIH. The Pronase hydrolysis of thyroidal iodoproteins was thus made in the presence of 2.5×10^{-3} M DIH and 10^{-2} M Thiamazole. These conditions gave the largest recovery of MIH and DIH with the smallest degree of conversion of DIH to MIH.

Identification of MIH and DIH from human and rat thyroid glands. The previously described sequential procedure involved in isolating iodohistidines from thyroid tissue is outlined in Fig. 4. The Dowex 1 chromatographic pattern from a human Tg hydrolysate is depicted

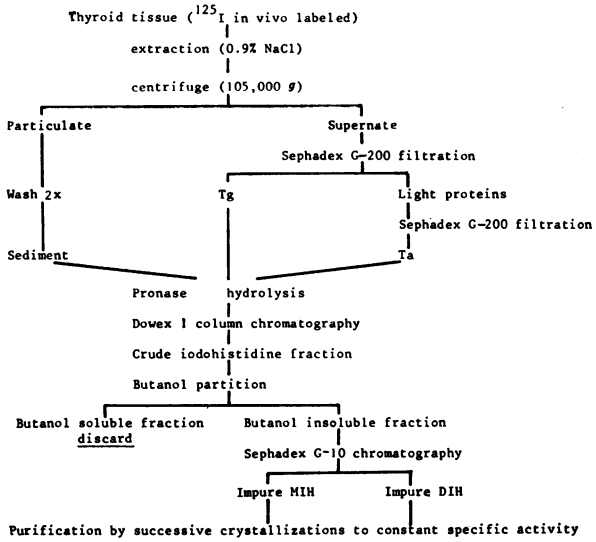

FIGURE 4 Sequential procedures involved in isolating MIH and DIH from thyroid tissue.

in Fig. 2. Iodide released during the extraction and hydrolysis procedure was retained by the column and contained 4.47±0.51% (n = 12) of the protein radioactivity. The crude iodohistidine fraction from the Dowex 1 chromatography was extracted three times with 0.1 N HCl saturated *n*-butanol in order to remove traces of iodotyrosines. The aqueous phase was pooled with the 0.1 N HCl washes of the butanol extract. This procedure permitted a 97% recovery of the iodohistidines in the control experiments. The Sephadex G-10 chromatography of the butanol-extracted crude iodohistidine fraction separated iodohistidines from each other and also from other iodinated compounds present in the iodoprotein hydrolysate (Fig. 3). Peptides were found in peak A. Peaks D and E contained compounds resistant to additional Pronase hydrolysis and that did not crystallize with MIH or DIH.

The validity of the crystallization method as described above in the purification of synthetic iodohistidines was extended to the identification of biosynthetically formed iodohistidines. Peaks from Sephadex G-10 chromatography, presumed to be [^{125}I] MIH and DIH at unknown degrees of purity, were mixed with ^{131}I and ^{127}I analogues. The ^{125}I and ^{131}I specific activities of the crystals obtained during successive crystallizations were compared. In the corresponding mother liquor and washes of the crystals, the ^{125}I/^{131}I ratios were determined as shown in Table I. The results demonstrate that: (*a*) constant specific activities were obtained since the first crystallization; (*b*) no significant changes in the ratios of the specific activities due to the ^{125}I biologic material and the ^{131}I internal indicator were observed from the first to the sixth crystallization; (*c*) ^{125}I/^{131}I ratios from the mother liquors and from the washes of the first three crystallizations were higher than that of the crystals, indicating the purification from contaminants; (*d*) these ratios approached that of the crystals at the third crystallization and thereafter remained constant and similar. Thus, a minimum of three crystallizations were made routinely to identify MIH or DIH irrespective of the origin (human or rat) or of the fraction studied (whole thyroid glands, purified Tg or Ta, 105,000 *g* particulate fractions). From these experiments, labeled impurities in the Sephadex G-10 peaks were estimated at 20% for the MIH peak 35% for the DIH peak.

TABLE I

Purification Obtained by Six Successive Crystallizations of the Free Iodohistidines Liberated by Pronase Hydrolysis from ^{125}I In Vivo-Labeled Human Thyroglobulin

Iodohistidine	Test of purification	Fraction studied	Crystallization number 1	2	3	4	5	6
MIH		Crystals weight, *mg*	489	430	355	262	221	188
	Specific activity *cpm/g*	^{125}I Biologic material	15,500	13,900	15,400	13,700	15,000	15,880
		^{131}I Internal indicator	34,400	36,400	37,700	40,500	38,880	37,900
	^{125}I/^{131}I ratios	Crystals	0.451	0.382	0.409	0.338	0.386	0.419
		Mother liquors	3.944	0.885	0.661	0.387	0.401	0.355
		Crystals washes	2.13	0.70	0.39	0.36	0.38	0.37
DIH		Crystals weight, *mg*	512	424	372	241	184	112
	Specific activity *cpm/g*	^{125}I Biologic material	7,550	7,440	7,450	7,575	7,450	8,275
		^{131}I Internal indicator	6,350	6,200	6,225	6,350	6,200	6,100
	^{125}I/^{131}I ratios	Crystals	1.19	1.20	1.19	1.19	1.20	1.36
		Mother liquors	7.25	1.57	1.35	1.23	1.23	1.21
		Crystal washes	6.35	1.37	1.12	1.26	1.09	1.17

Impure ^{125}I in vivo-labeled MIH and DIH were first recovered together in the first peak from the Dowex 1 column chromatography of the Pronase hydrolysate. Then they were separated by Sephadex G-10 chromatography. The ^{125}I-separated MIH and DIH fractions were mixed with a known amount of ^{127}I analogue and a known activity of ^{131}I-labeled analogue, added as an internal indicator, then six times recrystallized. The purification obtained was submitted to two tests: (*a*)-successive specific activities, (*b*)-^{125}I/^{131}I ratios in the mother liquors as compared with that of the crystals.

TABLE II

Comparison of Iodohistidine Fractions from Normally Biosynthetized Thyroglobulin and Thyralbumin from Normal Man and Rat

Iodoproteins		Iodohistidine fractions		
		Crude iodohistidine fraction	Crystallizable MIH	Crystallizable DIH
Nature	Origin	percent of [^{125}I] hydrolysate radioactivity		
Thyroglobulin (Tg)	Normal man A	0.75	0.36	0.030
	Normal man B	1.32	0.52	0.026
	Normal rat A	2.8	0.80	0.055
	Normal rat B	2.9	0.77	0.094
	Mean	1.94	0.61	0.050
	±SE	0.537	0.105	0.015
Thyralbumin (Ta)	Normal man A	5.5	2.2	0.12
	Normal man B	4.98	1.2	0.31
	Normal rat A	5.8	3.49	
	Normal rat B	8.3	3.57	0.42
	Mean	6.14	2.61	0.28
	±SE	0.738	0.566	0.087
Probability levels, difference of paired means (Ta) minus (Tg)		<0.001	<0.01	<0.05

Since crystallizable MIH and DIH together amounted to about 1% of the iodoprotein-hydrolysate radioactivity, the question arose as to whether this small iodohistidine fraction might have been formed by in vitro iodination, or by the isotopic exchange of iodine. The former might occur during the 24 h extraction of the minced thyroid in the presence of labeled iodide. This was excluded by the following experiment. 10 μCi of carrier-free ^{125}I, 5×10^{-4} M histidine and [^{131}I] MIH (internal indicator) were added to 700 mg of unlabeled minced human thyroid tissue in 4 ml of 0.15 M NaCl. After the extraction and identification of iodohistidines in the usual manner, no ^{125}I was found in the MIH or DIH crystals. The second possibility of isotopic exchange of iodine which might take place during the prolonged Pronase hydrolysis in which stable DIH was added was excluded by the following experiment. 10 μCi of carrier-free [^{125}I] and [^{131}I] MIH were added to a soluble extract of an unlabeled human thyroid gland. Once again, after hydrolysis and purification of iodohistidines in the usual manner, no ^{125}I was found in the MIH or DIH crystals.

Comparison of the iodohistidine content from normal thyroglobulin and thyralbumin. Thyroglobulin (Tg) and thyralbumin (Ta) (34) have been prepared from the thyroids of two normal men and two rats. The purified Ta fraction obtained amounted to about 1% of the total radioactivity of the soluble iodoproteins.

The crystallizable MIH and DIH were calculated and the results summarized in Table II. Since no obvious species difference has been observed from such small groups, the four sets of paired data have been used to compare the Tg and Ta results. The mean values of the iodohistidine fractions purified from normal Tg, expressed in percent of the protein radioactivity, were respectively: crude iodohistidine fraction 1.94±0.54%; crystallizable MIH 0.61±0.105%; and crystallizable DIH 0.05±0.015%.

The observed mean levels of every iodohistidine fraction purified from normal Ta were: crude iodohistidine 6.14±0.74%; MIH 2.61±0.57%; DIH: and 0.28±0.09%. They were higher than those obtained from normal Tg. The differences between Ta and Tg results were highly significant: $P < 0.001$ from the crude iodohistidine fractions; $P < 0.01$ from crystallizable MIH; $P < 0.05$ from crystallizable DIH.

Stability of MIH and DIH during in vitro exposure to iodotyrosine deiodinase. MIH and DIH were found to be unaffected by exposure to human thyroid and kidney homogenates whereas iodotyrosines were deiodinated (Fig. 5). Similar results were obtained with rat thyroid and kidney homogenates. In addition, the possible interference of iodohistidines on the MIT deiodination has been studied by routine enzyme kinetics. In a Lineweaver-Burke plot of the MIT deiodination data, both the slope of the regression line and the K_m for MIT values were unaffected by the presence of various concentrations of MIH or DIH. This lack of competitive inhibition on MIT deiodination is shown in Fig. 6 where

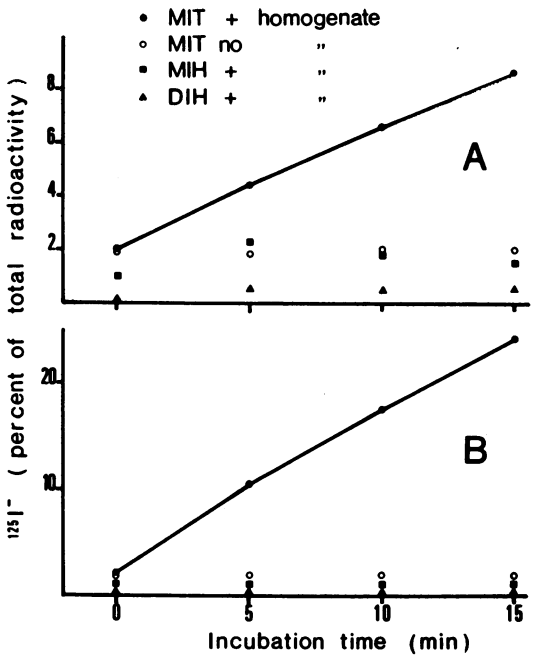

FIGURE 5 Stability of MIH and DIH during exposure to iodotyrosine deiodinase preparations made from human *thyroid* homogenate (A) and from human *kidney* homogenate (B).

MIH is at a concentration 1000 times that of the K_m for MIT.

In vivo peripheral metabolism of MIH and DIH in normal men. After oral administration of MIH and DIH, their urinary excretion was studied and compared with that of DIT (Table III). 50–65% of the iodohistidines were recovered in the 24-h urines. The nearly complete absence of peripheral MIH and DIH deiodination is best indicated by a radioiodide fraction in 24-h urines as low as 2.9% of the radioactivity (percent of administered dose) from MIH and 10.2% from DIH, as opposed to 97% from DIT. In addition the urinary excretion patterns from MIH and DIH differ significantly.

These results were confirmed by studying the metabolism of intravenously injected MIH and DIH. The renal clearances were approximately 35 ml/min for MIH and 700 ml/min for DIH. More than 90% of the MIH and DIH radioactivities were recovered in 72-h urines. Two-thirds of the activity from MIH was recovered as free crystallizable MIH. Sephadex G-10 fractionation of successive urine samples gave 67% of the DIH activity excreted as DIH and 20% excreted as MIH (Fig. 7).

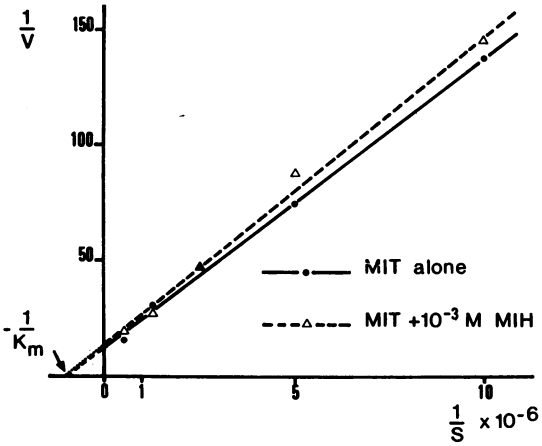

FIGURE 6 MIT deiodination kinetics. The calculated K_m for MIT—1.02×10^{-6} M was not found to be significantly modified (0.97×10^{-6} M) by the presence of 10^{-3} M MIH. V is the deiodinating velocity in μM I^- produced/min per mg of proteins. S is MIT concentration in moles per liter.

DISCUSSION

The present study describes the preparation of stable and radioiodine-labeled MIH and DIH. Their properties are described, and the method of purification of trace amounts by crystallizations to constant specific activity is detailed. Iodohistidines were isolated from normal thyroidal iodoproteins from both man and the rat. The MIH and DIH content in Ta is significantly greater than that in Tg. Iodohistidines, in contrast to iodotyrosines, are stable during in vitro exposure to iodotyrosine deiodinase preparations and when given in vivo these iodohistidines are recovered in the urine.

The normal metabolism of MIH and DIH have not been previously reported. Apart from the spontaneous conversion of some DIH to MIH, no enzymatic deiodination was apparent. Iodohistidines, although not de-

TABLE III
Comparative Recovery in Urines of Labeled MIH, DIH, and DIT Administered as Carrier-Free-Labeled Molecules to Normal Men

Tracer	Normal volunteers	Urine samples 0–4 h %D	Urine samples 4–24 h %D	Total 0–24 h %D
		mean ±SD	*mean ±SD*	*mean ±SD*
MIH	6	16.1±3.9*	40.1±7.1‡	56.2±3.9§
DIH	6	46.3±8.7*	21.4±7.4‡	67.7±4.4§
DIT	20	1.5±1.3	1.1±0.2	2.6±1.4

* Comparison of mean $P < 0.001$.
‡ Comparison of mean $P < 0.002$.
§ Comparison of mean $P < 0.002$.

tected in normal human urine (unpublished data), were identified in the urine of patients with congenital goiters (22).

No information is as yet available on the presence of iodohistidines in human thyroidal proteins. Traces of labeled MIH have been identified in native Tg from rats and dogs (1). In equilibrium-labeled Tg from rats, 0.22% (2) and 0.4% (35) of the iodine has been characterized as MIH. No DIH was detected. Evidence is presented here that DIH is a normal constituant of iodoproteins. Detection of DIH may be attributed to new approaches to the three major technical problems in any iodohistidine investigation, namely: recovery, purification, and identification.

The iodohistidine recovery from iodoprotein hydrolysates has not been fully investigated. During alkaline or acid hydrolysis, DIH is completely and MIH partially destroyed (4, 6). Enzymatic hydrolysis with pancreatin (7), trypsin (1) or Pronase (12) have been reported to denature the fragile iodohistidines as opposed to the more stable iodotyrosines (36). 5 h Pronase incubation has been reported to give the maximal digestion with minimal deiodination (10). Control experiments in these conditions have found a 50% conversion of DIH to MIH. Addition of stable DIH prevents most of this iodohistidine degradation and allows longer and more complete hydrolysis (37) to occur.

Iodohistidines, when prepurified by anion-exchange resin chromatography on Dowex 1 appeared in the first peak of acid elution which is known to contain iodopeptides (28). In control experiments, iodohistidine peptides such as iodocarnosine were recovered in this fraction. Iodohistidines seemed to be loosely bound to the resin by their carboxylic group since iodohistamines were not retained on the column. The acid elution of DIH after that of MIH is in agreement with the downward shift of the carboxylic pK by one more iodine substitution (3). The separation of MIH from iodide by filtration over Sephadex G-10 columns equilibrated in neutral phosphate buffer has been reported (38) and denied (23). In our hands the separation of MIH, DIH, and I^- from each other by Sephadex G-10 columns was obtained only by 0.2 N HCl elution, and this finding indicates that it is due to reversible adsorption rather than true filtration.

MIH has thus far been isolated from iodoprotein hydrolysates by quantitative paper chromatography in multiple solvent systems either alone (1, 2) or after Dowex 50 column chromatography followed by TLC on silica gel or cellulose plates (35). Because of their low specific activity iodohistidines from normal human iodoproteins could not have been detected by these

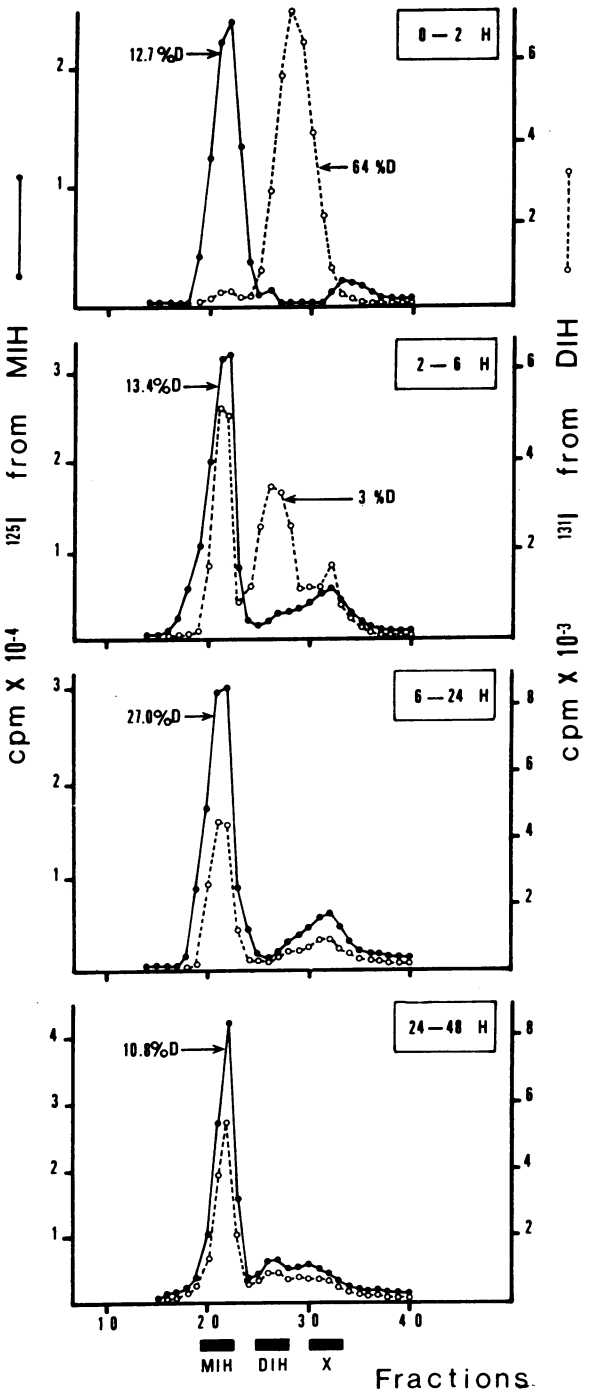

FIGURE 7 Sephadex G-10 chromatography of four successive urine samples collected after [^{125}I] MIH and [^{131}I] DIH intravenous injection in man. The in vivo conversion of DIH to MIH is apparent. A urinary metabolite of MIH has been indicated as *X*. %D represents percent of administered dose.

methods. On the contrary, crystallizations to constant specific activity is the method of choice insuring sensitivity and specificity. Large amounts of stable iodohistidines were synthetized and identified by their nuclear magnetic resonance spectrum. The data obtained confirmed that the iodine atom of MIH was substituted to the proton attached at the imidazolic C_4 only (39) and that DIH was 2,4-diiodo-L-histidine (3).

Iodohistidines have been identified from human thyroid glands that were normal on macroscopic and routine microscopic examinations. These gave normal DIT/MIT ratios between 1.5 and 1.9. Iodohistidines have been detected by their radioactivity after in vivo radioiodide labeling. The true [^{127}I] iodohistidine content of the human iodoproteins cannot be assessed. Only with rats could accurate determination be made at isotopic equilibrium.

The high iodohistidine content of Ta as compared with Tg is not explained by the calculated tyrosyl/histidyl residue ratios which, in man, have been found respectively to be 1.0 for Tg and 0.9 for Ta (40). This may be due to known variations in reactivity of the histidyl residue from different proteins (2). Both for Tg and Ta the amount of iodohistidine present is so small that it is usually disregarded as a side product of the normal iodination and hormonogenesis. However, after in vivo and in vitro iodination, an inverse relationship between the iodothyronine and the iodohistidine content of Tg has been reported (35). The biosynthesis of iodothyronines has been attributed to thyroid peroxidase-mediated iodination (41). Since iodohistidines are probably iodinated in vivo by this enzyme and because the reactivity of the labile iodine atom attached at the DIH carbon 2 is not known the participation of DIH (and/or MIH) in normal hormonogenesis as a slow iodine donor is at least a theoretical possibility.

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