Chemical Determination of Iodinated Compounds in Human Thyroid

TOSHIRO NAKASHIMA*, AKIYO SHIROOZU, KEN OKAMURA,
KENJIRO INOUE** AND TERUO OMAE

*Second Department of Internal Medicine, Faculty of Medicine
Kyushu University, Fukuoka 812, Japan*

Synopsis

As a tool with which to detect iodinated compounds in human thyroid specimens, we have reevaluated a nonincineration technique which has so far been employed in the determination of thyroxine-iodine in peripheral blood.

The catalytic action of iodoamino acids in the Ce-As reaction was enhanced by a small amount of Cl_2 . On the contrary, a large amount of Cl_2 inhibited the reaction unexpectedly. Among iodide, iodotyrosine and iodothyronine, iodide was the most effective catalyst in the Ce-As reaction and iodothyronine was the least effective one. Protein seemed to inhibit this reaction of thyroglobulin. But the result of iodine content in thyroglobulin by this technique agreed well with that by incineration when measured ^{127}I was corrected by percent activity of dialyzable part of the total activity of ^{131}I -thyroglobulin with the same protein concentration, after the NaClO treatment.

The results of human thyroid specimens were as follows: the thyroglobulin content of five normal subjects was $8.0 \pm 1.5\%$ of wet thyroid weight. That of Hashimoto's disease was significantly decreased which seemed compatible with the decrease in iodine content of thyroglobulin, whereas thyroglobulin content of Graves disease treated with 1-methyl, 2-mercaptoimidazole followed by a large dose of iodide was well preserved in spite of a lower degree of iodination of thyroglobulin. As for the distribution of iodoamino acids-iodine in normal thyroid, T_4 was $20.5 \pm 0.7\%$.

This technique ultimately looks promising as a tool with which to study intra-thyroidal iodine metabolism in human.

The intrathyroidal iodine metabolism in human has been mainly studied so far on newly iodinated compounds using radioactive iodine. But also the study of iodine metabolism in equilibrium, in other words, study of ^{127}I metabolism should be essential in the whole aspect of metabolism.

It was, however, uncommon that the

papers concerned with ^{127}I metabolism in human were written. This seems to be due to complicated methods of analysis rather than to little opportunity of obtaining specimens.

The nonincineration technique for thyroxine- (T_4) -iodine by Pillegi and Kessler (1968) simplified the procedure by avoiding ashing. We intended to extend this simple technique for the determination of other iodinated compounds, monoiodotyrosine(MIT), diiodotyrosine(DIT), triiodothyronine(T_3) and thyroglobulin(Tg).

The present study is mainly concerned

Received April 8, 1977.

* Present address; Department of Pharmacology,
The University of Texas, Health Science Center
at Dallas, 5323 Harry Hines Blvd., Dallas, Texas,
U.S.A.

** To whom reprint requests should be addressed.

with the basic one of this method and the clinical application to normal subjects, Hashimoto's disease and Graves disease.

Materials and Methods

0.1 N arsenious acid reagent

With the aid of heat, 4.95 g of As_2O_3 (E. Merck A.G.) was dissolved in 25 ml of 4% NaOH. This was added to 300 ml of distilled water and then slightly acidified with approximately 4 ml of 7 N H_2SO_4 . This acidified solution was finally brought to 1,000 ml with distilled water.

0.02 N ceric reagent

12.65 g of ceric ammonium sulfate, dihydrate (G. Frederick Smith Chemical Co.) was dissolved in 500 ml of distilled water. This was added to 230 ml of 7 N H_2SO_4 and brought to 1,000 ml with distilled water.

0.01 N NaClO

Sodium hypochlorite solution of estimated concentration 2.8 M (Wako Pure Chemical Co.) was diluted 280 times with distilled water.

Iodoamino acids and iodide standards

Stock solutions of MIT, DIT, T_3 , T_4 and KI; 10 mg equivalent compounds in terms of iodide were dissolved in each 100 ml of distilled water with weak alkaline. Working standards were prepared freshly once a month, by diluting stock solutions 50 times with ethanol. A working standard of KI for measuring iodine content of Tg was prepared to contain 1 μg of iodide per milliliter by diluting the stock with distilled water.

Human thyroid tissue

Thyroid tissues were obtained by surgery or autopsy.

Labeled rat thyroid

One Sprague-Dawley male rat fed on iodine sufficient diet (Oriental MF pellet) was injected with 500 μCi of carrier-free ^{131}I ip. One day after the injection the thyroid gland was taken out of the injected rat after killed by ether.

Homogenization of human thyroid tissue

200–300 mg of chopped thyroid tissue was suspended in 4 ml of cold saline 0.01 M phosphate buffer (pH 6.8). Homogenization was performed in a conial, glass motor-driven tissue grinder of 5 ml capacity in

cold. After removing cell debris by centrifugation at 2,000 rpm for 10 min, the supernatant was centrifuged at $105,000 \times g$ for 60 min. A portion of the clear supernatant was adjusted to contain 0.9% sodium chloride and 0.3 mM Tris-HCl (pH 8.5), and 0.05 M 1-methyl, 2-mercaptoimidazole for the hydrolysis. The other portion was used for the study by sucrose density gradient ultracentrifugation.

Homogenization of labeled rat thyroid gland

Procedures employed for homogenization, centrifugation and pH adjustment were the same as those for human thyroid specimens.

Pronase hydrolysis

Various amounts of human thyroid homogenate in relation to estimated iodine content were put into Thunberg tube and added to approximately 150,000 cpm of ^{131}I -labeled rat thyroid homogenate which had apparently no effect to the chemical analysis of iodine and then added to 0.1 vol. of 5% pronase (Kaken Chemical Co.) solution. Anaerobic hydrolysis was undertaken at 37°C for 16 hr (Inoue and Taurag, 1968). When the concentration of iodine in a sample was not enough for measurement, such a sample was concentrated after digestion under negative pressure with N_2 gas charging so as to contain at least 0.6 μg of the total iodine in 80 μl of hydrolysate. As a control, the same volume of saline 0.01 M phosphate buffer instead of human homogenate was dealt with in the same way as a thyroid sample.

Chromatography

At the end of the digestion period, 50–80 μl of each digestion mixture corresponding to 0.6–0.8 μg of iodine was delivered on a paper strip of Whatman 3 MM for ascending chromatography with n-butanol-ethanol-0.5 N NH_4OH (5 : 1 : 2) solvent for 14 hr.

Cutting and counting

The paper strip was then exposed to X-ray film for 24 hr in order to determine the location of iodide compounds and cut into fractions according to radioautogram. The radioactivity of each fraction was counted in a well-type scintillation counter (Aloka) before and after extraction.

Extraction from paper

Paper fractions were cut into small fragments and extracted in glass stoppered extraction tubes of 30 ml capacity with 12 ml of 50% acetic acid, 4 ml of 2 N HCl and 4 ml of 0.01 N NaClO at 37°C for 30 min. Radioactivity of an aliquot of each fraction was counted, as mentioned above, to determine extraction efficiency, which was used for a correction of measured ^{127}I data.

Sucrose density gradient ultracentrifugation

Sucrose density gradient was made in a nitrocellulose tube of 5 ml capacity with each 2.3 ml of 20% sucrose in saline 0.01 M phosphate buffer (pH 6.8) and 5% one. A portion of the clear supernatant of the human thyroid sample (0.3 ml containing less than 2.0 mg of protein) with $100\text{--}200 \times 10^3$ cpm of the clear supernatant of labeled rat thyroid homogenate was applied on the top of the sucrose density gradient. Three tubes were centrifuged simultaneously in a Hitachi swinging rotor (Model-40RPS) at 25,000 rpm for 13 hr. After centrifugation, the bottom of the tube was punctured and consecutive fractions were collected, each consisting of 10 drops. After radioactivities of samples had been counted, the same fractions were then used for the protein measurement (Lowry *et al.*, 1951).

Purification of Tg

The samples of each three fractions of main protein peak around 19S were collected into Visking tube and dialyzed against 2,000 ml of cold saline for 12 hr twice. Otherwise, the clear supernatant was purified by ammonium sulfate fractionation (Ui and Tarutani, 1961). The supernatant of 1.55 M ammonium sulfate was dialyzed against 1,000 ml of cold saline for 6 hr three times.

Iodine determination

Each 5-ml extract was transferred into a test tube of 12 ml capacity, containing 0.5 ml of arsenious acid reagent, and then these were well mixed. Finally 1 ml of ceric reagent was added to the mixture and the mixture was incubated at 25°C for 20 min. At the end of incubation, the reaction was stopped by adding 2 drops of 0.2 N HgNO_3 . Samples for a standard curve were added with 3 ml of 50% acetic acid, 1 ml of 2 N HCl and 1 ml of 0.01 N NaClO, and then this was dealt with in the same way as extract. Per cent transmittance (T%) of sample was read at 420 nm under the light path of 0.1 mm.

Tg content

This was calculated from the following factors, wet tissue weight, protein concentration of the clear supernatant and per cent of Tg of thyroid soluble protein.

Results

Incubation temperature and time

Various amounts of iodide in a volume of 1 ml were added to a tube containing 3 ml of 50% acetic acid, 1 ml of 2 N HCl, 1 ml of 0.01 N NaClO, 0.5 ml of 0.1 N. As

reagent and 1 ml ceric ammonium.

As shown in Fig. 1, the catalytic action of iodide with the reduction of ceric ammonium was accelerated in proportion to incubation temperature.

As we could obtain enough sensitivity at 25°C which was almost equal to room temperature, we conducted all experiments at this temperature for 20 min.

Fig. 1. Incubation temperature and time. The catalytic action of iodide was accelerated in proportion to incubation temperature.

The effect of DIT on the catalytic reaction

In order to test if the other iodoamino acids than T_4 could catalyze As-Ce reaction in this system, we examined the effect of DIT. As shown in Fig. 2, DIT catalyzed the reduction of ceric ammonium by itself in an acid solution, furthermore the catalytic action of DIT was apparently enhanced by adding a small amount of NaClO.

This enhancement seemed to be owing to a small amount of Cl_2 produced by HCl and NaClO (Pileggi and Kessler, 1968).

The effect of various concentrations of NaClO on the catalytic action of DIT

As shown in Fig. 3 where the molar concentration of NaClO was expressed in

Fig. 2. DIT measured by the nonincineration technique (solid line) in contrast to the control (broken line). Catalytic action of DIT was apparently enhanced by adding NaClO. This was supposed to be owing to Cl_2 produced by HCl and NaClO.

Fig. 3. Evaluation of Cl_2 for this assay. Concentration of NaClO was expressed in molar concentration of NaClO solution. Relatively low concentration of Cl_2 looks favorable for this assay.

molar concentration of the reagents, the plateau of enhancement was observed between 0.01 M and 0.05 M. On the other hand, T(%) at 1.0 M was rather lower than that at zero point, suggesting that the reaction was apparently inhibited by an excessive amount of Cl_2 . Ultimately 0.01 M NaClO was employed for all experiments.

The determination of iodoamino acids

Equivalent iodoamino acids in terms of iodide content were determined by the non-incineration technique. As shown in Fig. 4, there was practically no difference between MIT and DIT, and also it was the same with T_3 and T_4 .

T(%) of iodotyrosine was higher than that of iodothyronine in the same iodine basis, indicating that iodotyrosine was more an effective catalyst in this reaction than iodothyronine. As shown in Fig. 5, T(%) of iodide was the highest among these three

Fig. 4. Determination of iodoamino acids. T(%) of iodotyrosine was higher than that of iodothyronine.

Fig. 5. Determination of iodide and iodoamino acids. T(%) of iodide was the highest.

kinds of compounds, indicating that iodide was the most effective catalyst.

Extraction from paper

Iodide and iodoamino acids were extracted from paper fragments in a mixture of 6 ml of 50% acetic acid, 2 ml of 2 N HCl and 2 ml of 0.01 N NaClO. Extraction efficiencies were calculated from ^{131}I activities before and after the extraction. Iodide and iodoamino acids on paper were sufficiently extracted almost 100%.

Recovery of iodide

Experiments were conducted with each 0.50 μg of iodide. Chemical purity of ^{127}I was $94.9 \pm 1.3\%$ by paper chromatography. Determined ^{127}I expressed in per cent of expected ^{127}I on paper fragment was $99.0 \pm 1.1\%$, suggesting that there was practically no interfering substance of the reaction in extracts. The final recovery of ^{127}I was $93.9 \pm 1.0\%$.

Iodide determination of Tg

Iodide content of Tg was determined in the same way as iodoamino acids or iodide. As shown in the top of Fig. 6, measured ^{127}I of purified Tg was lower than expected ^{127}I which was previously determined by the incineration method (Barker, 1964). It was assumed that protein might inhibit the reaction. In fact, bovine serum albumin (BSA) inhibited iodide determination of Tg. Iodide determination of 13.2 μg of Tg was inhibited 7%, 9% and 15% by adding

Fig. 6. Top of the Fig. shows iodine determination of a human Tg purified by sucrose density gradient ultracentrifuge. Measured ^{127}I was lower than expected ^{127}I . Various amounts of BSA were added to ^{131}I -labeled rat Tg purified by sucrose density gradient ultracentrifuge. These were dealt with an acid mixture of 50% acetic acid, 2N HCl and 0.01N NaClO and then dialyzed against saline. ASA inhibited a conversion of ^{131}I -Tg into a dialyzable form shown in the bottom.

50 μ g, 100 μ g and 150 μ g of BSA, respectively. When ^{131}I labeled Tg was dealt with a mixture of 50% acetic acid, 2 N HCl and 0.01 N NaClO and then the mixture was dialyzed against saline, most radioactivity was found in the sample outside of dialysis bag. But this conversion of ^{131}I -Tg into some dialyzable form in the presence of an acid mixture was also inhibited by adding various amounts of BSA into the mixture, shown in Fig. 6, bottom. In addition, the degree of this inhibition by BSA was almost equal to that of inhibition of iodide determination of Tg by BSA. Therefore, iodine in Tg is probably changed by an acid mixture, into some dialyzable form; iodide, iodoamino acids and small peptide including iodide to catalyze As-Ce reaction.

As was mentioned above, protein seemed to inhibit the iodide determination of Tg. However, after correcting nonincineration's data by the percent activity of dialyzable

part of the total activity of standard ^{131}I -Tg with same protein amount under the NaClO treatment, in other words, correcting the data by the inhibiting factor relating to protein amount, the results of iodine content of Tg by the nonincineration technique agreed well with those by Barker's incineration method.

Intrathyroidal distribution of iodine compound in normal and patients' thyroid

As shown in Table 1, Tg content of euthyroid subjects was $8.0 \pm 1.5\%$ of wet thyroid weight. That of Hashimoto's disease patients was decreased. But that of Graves disease patients treated with 1-methyl, 2-mercaptoimidazole and a large dose of iodide was not significantly decreased. The degree of iodination of Tg from euthyroid subjects was $0.49 \pm 0.03\%$. Tg from Hashimoto's disease patients and treated Graves disease patients were poorly iodinated.

Table 1. Intrathyroidal distributions of iodoamino acids-iodine in normal subjects, treated Graves disease patients and Hashimoto's disease patients.

Diagnosis	Age	Sex	content* Tg	I content of Tg**	Origin	DIT	MIT	I ⁻	T ₄	T ₃
1. Oesophagus carcinoma	64	M	7.1	0.52	7.5	45.1	25.8	2.0	18.9	2.1
2. Gastric ulcer	82	M	6.1	0.46 (0.50)	5.6	41.0	27.0	2.0	19.8	2.8
3. Nodular goiter; normal part	25	F	8.0	0.45 (0.47)	5.6	41.2	28.2	1.7	20.2	2.7
4. Nodular goiter; normal part	44	F	8.5	0.51	7.0	40.9	24.6	1.4	23.0	2.9
5. Nodular goiter; normal part	25	F	10.0	0.50	5.9	42.2	27.2	1.3	20.8	2.5
Mean			8.0	0.49	6.7	42.1	26.6	1.7	20.5	2.6
±			±	±	±	±	±	±	±	±
SD			1.5	0.03	1.1	0.8	0.6	0.3	0.7	0.3
6. Graves disease treated by MMI and I ⁻	28	F	5.0	0.03	12.6	21.2	47.6	5.3	4.1	9.0
7. Graves disease treated by MMI and I ⁻	39	F	4.1	0.09 (0.10)	3.4	27.3	29.7	3.1	32.4	2.5
8. Hashimoto's disease focal type	39	F	3.4	0.23	5.7	40.8	34.5	5.6	12.5	0.9
9. Hashimoto's disease diffuse type	50	F	0.8	0.08 (0.07)	3.1	47.9	35.4	1.3	6.0	6.1

* Purified Tg and thyroid homogenate were used for iodine content of Tg and the other iodine compounds, respectively. Tg content was expressed in per cent of wet thyroid weight and other data were also expressed in per cent.

** Results of I content of Tg in parentheses were obtained with samples prepared by ammonium sulfate fractionation.

The ratios of iodoamino acids in euthyroid subjects were as follows: 0.63 ± 0.5 for MIT/DIT, 0.49 ± 0.05 for T_4 /DIT and 0.13 ± 0.01 for T_3/T_4 . And T_4 -iodine was $20.5 \pm 0.7\%$ of total iodine in the thyroids from euthyroid subjects.

On the other hand, the total iodothyronines were decreased in most patients with poorly iodinated Tg.

Discussion

Simplicity of the nonincineration technique for the chemical determination of T_4 is more than a match for the alkaline-ashing method. It is quite natural that we should try to apply this technique for only T_4 -iodine to the determination of the other iodinated compounds of the thyroid.

In fact, we observed that DIT also catalized the reduction of ceric ammonium in the presence of NaClO. Although DIT catalized the reaction in an acid solution by itself, its catalyzing ability was apparently enhanced by the NaClO treatment.

When equivalent potassium iodide and iodoamino acids in terms of iodide amount were determined, there was no difference between the values from MIT and DIT, and it was the same with T_3 and T_4 . $T(\%)$ value of iodothyronine was, however, lower than that of iodotyrosine. And $T(\%)$ of iodoamino acids was lower than that of inorganic iodide. These results suggest that tyrosine and thyronine might suppress the reaction of those iodinated compounds in this assay system. Practically, it is necessary for us to prepare the standard curves of these three kinds of iodine compounds, that is inorganic iodide, iodotyrosine and iodothyronine when we actually determine samples.

As was above mentioned, the NaClO treatment enhanced the catalytic activity of DIT. This seemed to depend on iodide oxidatively released from tyrosine by the

action of Cl_2 since it had been described by Pileggi and Kessler (1968). In our experiment by paper chromatography, however, iodide fraction was not so much increased after the NaClO treatment of iodoamino acids or Tg. The reason for this was not known. But it was reported that the catalytic activity of T_4 in the ceric arsenite reaction resulted from the release of iodide from T_4 molecule by the action of ceric ion (Bowden *et al.*, 1955). At this point, the NaClO treatment might readily make iodoamino acids susceptible to release much iodide by ceric ion.

As for the extraction method, Mandl and Block described a high yield of iodine, about 98% when iodo compounds in a proteolytic digest of iodo proteins were extracted by n-butanol (Mandl and Block, 1959). Also, in the present experiment, extraction efficiencies of iodoamino acids from papers were almost 100%, indicating that correction of measured ^{127}I by extraction efficiency was practically unnecessary. However, extraction efficiency fluctuated to a very small extent in assays. Subsequently, if one would like to have very precise data, it is necessary to correct the results with extraction efficiencies.

In determination of Tg-iodine, measured ^{127}I was lower than expected one. This was presumed to be an inhibition by protein. In order to study this question, the effect of BSA on the determination of Tg-iodine was examined. And BSA actually inhibited the determination of Tg-iodine.

Therefore, we should take account for the inhibitory action of protein in this assay. Supposedly tyrosine and/or thyronine might suppress the reaction. But it is considered that there is practically no problem, because the results by this technique agreed well with those by incineration if measured ^{127}I was corrected by the percent activity of dialyzable part of ^{131}I -Tg with the same protein concentration after the NaClO treatment. Also it would be a good way

Table 2. Per cent distribution of iodoamino acids-iodine in normal subjects.

		I ⁻	DIT	MIT	T ₄	T ₃	MIT/DIT	T ₄ /DIT	T ₃ /T ₄
Arosenius (1964)	6	*	35	33	24	8	0.88	0.69	0.34
Braasch <i>et al</i> (1955)	5	6	25	17	35	8	0.68	1.40	0.23
Dimitriadou <i>et al</i> (1966)	5	*	41	38	— 22 —		0.93	—	—
Ermans <i>et al</i> (1968)	7	10	33	33	16	8	1.00	0.48	0.50
Rolland <i>et al</i> (1970)	?	6**	27	49	16	5	1.83	0.59	0.18
		(0.26)	(1.9)	(4.9)	(1.0)	(0.2)			
Present data	5	2	42	27	21	3	0.64	0.50	0.14

* Not written in the papers.

** These were calculated from values expressed in mole in parentheses.

to determine Tg-iodine in samples with a fixed amount of protein.

As shown in Table 2, the present data with intrathyroidal distribution of iodoamino acids-iodine of normal subjects are significantly different from those of other authors except T₄ (Arosenius, 1964; Braasch *et al.*, 1955; Dimitriadou *et al.*, 1966; Ermans *et al.*, 1968; Rolland *et al.*, 1970). MIT/DIT and T₃/T₄ ratios of our data are significantly lower than those by the other authors. There seems to be a difficult problem to compare our data with those from other countries because people in Japan take sea weed frequently, which is known to be rich in iodine. Nagataki *et al.* (1972) also reported low T₃/T₄ ratio in normal Japanese.

Consequently higher ratios of MIT/DIT and T₃/T₄ of the other authors might suggest the relatively iodine deficient state in those countries. On the other hand, T₄/DIT of ours was almost the same to the others' data.

As for inorganic iodide fraction, we recognized significantly lower values comparing to others' data in spite of the determination after digestion. This seems to be owing to the anaerobic technique of hydrolysis described by Inoue and Taurog (Inoue and Taurog, 1968).

Decrease in Tg content of the thyroid of Hashimoto's disease seems to be compatible with decrease in iodine content of Tg. Tg content of the thyroid of treated

Graves disease, however, was well preserved in spite of low iodine content of Tg. This might be owing to a large dose of exogenous iodide which supposedly inhibit proteolysis of Tg.

In most patients with poorly iodinated Tg, decreases in the total iodothyronines and much higher ratios of T₃/T₄ were observed. One case of Graves showed, however, a rather higher value of T₄ in spite of a low degree of iodination of Tg with higher MIT/DIT. This case took a long term and a large dose of 1-methyl, 2-mercaptoimidazole to obtain the euthyroid state, whereas another case of Graves disease with lower T₄ had rather become hypothyroidism medicamentosa in a short period. It seems that at least some case of Graves disease might have a strong ability of T₄ formation. This technique ultimately looks promising as a tool with which to study intrathyroidal iodine metabolism in human.

Acknowledgement

We are greatly indebted to Prof. K. Tanaka of the Department of Pathology, Kyushu University for his kind supply of thyroid glands. We are also indebted to Prof. A. Taurog of Southwestern Medical School, the University of Texas for his helpful discussions to prepare the manuscript.

References

- Arosenius, K. E. (1964). *Scand. J. Clin. Lab. Invest.* 16, 440.
- Barker, S. B. (1964). *Biochem. J.* 90, 214.
- Bowden, C. H., N. F. MacLagan and J. H. Wilkinson (1955). *ibid.* 59, 93.
- Braasch, J. W., A. Albert, F. R. Keating and B. M. Black (1955). *J. Clin. Endocr. Metab.* 15, 732.
- Dimitriadou, A., R. Suwanik, T. R. Fraser and J. D. Pearson (1966). *J. Endocr.* 34, 23.
- Ermans, A. M., J. Kinthaert, C. Delcroix and J. Collard (1968). *J. Clin. Endocr. Metab.* 28, 169.
- Inoue, K. and A. Taurog (1968). *Endocrinology* 83, 816.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). *J. Biol. Chem.* 193, 265.
- Mandl, R. H. and R. J. Block (1959). *Arch Biochem. Biophys.* 81, 25.
- Nagataki, S., H. Uchimura, Y. Matsuyama, K. Nakao and K. Ito (1972). *J. Clin. Endocr. Metab.* 35, 18.
- Pileggi, V. J. and G. Kessler (1968). *Clin. Chem.* 14, 339.
- Rolland, M., R. Aquaron and S. Lissitzky (1970). *Analyt. Biochem.* 33, 307.
- Ui, N. and O. Tarutani (1961). *J. Biochem.* 50, 508.