mentioned above, the titre of thorium nitrate varies in accordance with the amount of fluoride present.

After much experience with the method described we have encountered only one natural water in which the estimation could not be satisfactorily carried out. This sample contained a relatively high concentration of aluminium, sufficient to give a visible precipitate of aluminium hydroxide on the addition of alkali. This sample was, however, satisfactorily analysed and showed good recovery of added fluoride by our modification of the colorimetric method of Elvove (1933).

Out of 295 samples of domestic water in Great Britain only seven were found to contain more than 1 p.p.m. of fluorine.

SUMMARY

1. A method for the estimation of fluoride in natural waters by titration with thorium nitrate at pH 3.3, using sodium alizarinsulphonate as indicator, is described.

2. Interfering anions are first removed by addition of barium chloride and filtration.

3. Calcium and magnesium in concentrations found in hard waters do not interfere.

This work was in part financed by an expenses grant from the Medical Research Council, for which we wish to record thanks.

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3:5:3'-Triiodothyronine

1. ISOLATION FROM THYROID GLAND AND SYNTHESIS

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(Received 5 August 1952)

In the course of an investigation on thyroid metabolism with the aid of radioactive iodine (Gross, Leblond, Franklin & Quastel, 1950; Gross & Leblond, 1951*a, b*), an unknown radioactive compound (unknown 1) was detected in extracts of thyroid gland, plasma, tissues and faeces of animals which had been injected with radioactive iodide or with thyroxine labelled with radioactive iodine. This compound was later found in human plasma after the administration to patients of therapeutic doses of radioactive iodide (Gross & Pitt-Rivers, 1951). Evidence will now be presented identifying unknown 1 as 3:5:3'-triiodothyronine, and its isolation from thyroid gland and synthesis will be described.

Unknown 1 was originally detected by autoradiographs of two-dimensional chromatograms of butanol extracts of tissues without previous hydro-

lysis; the distribution of the radioactive spots was the same as that shown in Fig. 1*a*; this demonstrates the presence of unknown 1 in butanol extracts of a tryptic hydrolysate of radioactive rat thyroid; the existence of this compound in thyroid tissue itself suggested that it might be possible to isolate it from the thyroid gland. Accordingly, large amounts (5 kg. batches) of ox thyroid gland were digested with trypsin and the acidified hydrolysate was extracted with chloroform repeatedly, to remove fat. Butanol extracts were then made and their contents analysed by paper chromatography using butanol-acetic acid and butanol-dioxan-ammonia as the solvents and developing the spots with diazotized sulphanilic acid (Gross & Leblond, 1951*b*). In the first crude preparations, minute amounts of unknown 1 were detectable, and a considerable degree of purification was achieved by elution from a large number of papers; this laborious method was, however, unsatisfactory, in that it still yielded relatively impure fractions. It was nevertheless possible to demonstrate that the substance was

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acidic, that it gave a positive, though feeble colour with nitrous acid, and, from the ninhydrin reaction, that it contained an amino group. Its position on paper chromatograms compared with the positions of a number of thyroxine peptides and other compounds containing the diphenyl ether grouping

Fig. 1. (a) Autoradiograph of the paper chromatogram of the butanol extract from a tryptic hydrolysis of the thyroids of rats given Na^{131}I . The black spots indicate the positions of the radioactive components in the extract. (b) Photograph of the paper chromatogram showing the positions of known carrier substances added with the radioactive material. The carriers were visualized by spraying with diazotized sulphanilic acid and the correspondence between the radioactive spot unknown 1 and triiodothyronine is evident. Origin is at the lower left-hand corner; the solvents were butanol-acetic acid vertically and butanol-dioxan-ammonia horizontally.

indicated that unknown 1 was likely to possess a diphenyl ether structure; further, the purple colour obtained with diazotized sulphanilic acid also lent support to this idea, since all the tyrosine derivatives already tested had given an orange colour with this reagent.

The possibility that unknown 1 might be diiodothyronine had already been excluded by Gross *et al.* (1950); triiodothyronine was then considered,

and its synthesis was attempted. Roche, Lissitzky, Michel & Michel (1951) had shown that iodination of tyrosine with small amounts of iodine led to the formation of considerable amounts of monoiodotyrosine, and Pitt-Rivers (unpublished) had used monoiodination of tyrosine in ammonia as a method of preparing monoiodotyrosine; considerable purification of the product was necessary since it was contaminated with both diiodotyrosine and unchanged tyrosine. Monoiodination of 3:5-diiodothyronine under similar conditions followed the same pattern; triiodothyronine was the main reaction product, but it contained both thyroxine and unchanged diiodothyronine.

The purification of triiodothyronine consisted essentially of two processes, crystallization of the hydrochloride from aqueous hydrochloric acid, which largely eliminated the contaminating thyroxine and completely eliminated unchanged diiodothyronine, and separation on a kieselguhr column using 0.5N-sodium hydroxide as the stationary phase and 20% chloroform in butanol (v/v), equilibrated with 0.5N-sodium hydroxide, as the mobile phase. The iodophenols in the effluent fractions were identified by the colour reaction with nitrous acid followed by alkali. A typical run of 50 mg. crude triiodothyronine on a 50 g. column is shown in Fig. 2a. The fractions corresponding to the large peak were pooled and the product obtained converted to the hydrochloride. By very slow cooling of the hydrochloric acid solution, long birefringent needles separated out (Pl. 3 of Addendum). X-ray diffraction patterns of single crystals were obtained (Pl. 3j of Addendum) and from measurements of the unit cell dimensions and the density of the crystals the molecular weight of the compound was calculated to be 689: 3:5:3'-triiodothyronine hydrochloride has a molecular weight of 687.5. Elementary analysis of the purified triiodothyronine hydrochloride agreed with the calculated values. This material behaved chromatographically in two dimensions similarly to unknown 1 isolated from the thyroid gland (Fig. 1a, b), and identically with the radioactive unknown 1 present in rat-thyroid hydrolysate and in human plasma (Gross & Pitt-Rivers, 1952).

The identification of triiodothyronine in human plasma with the aid of chromatography and autoradiography has already been described (Gross & Pitt-Rivers, 1951, 1952); a knowledge of its chemical properties now made it more practicable to isolate it in a pure state from thyroid gland. Batches of ox thyroid (5 kg.) were hydrolysed either with trypsin or with barium hydroxide and extracted with butanol; the purification of the crude butanol extracts will be described fully in the experimental section. The final purification consisted in separation of tarry impurities, thyroxine and (pre-

Fig. 2. Chromatographic separations on 50 g. kieselguhr columns with 0.5N-NaOH as the stationary phase and 20% CHCl_3 in *n*-butanol (v/v) as the mobile phase. Each fraction contained 14.9 ml. The iodophenols in the effluent were detected by the colour obtained with HNO_3 followed by alkali. (a) Separation of 50 mg. of crude synthetic L-triiodothyronine hydrochloride. The triiodothyronine is present in the effluent represented by the third peak; contaminating thyroxine is indicated by the second peak; the first peak contains unidentified material believed to be an artifact of the separation (cf. (b) below and fig. 2c in Gross & Pitt-Rivers, 1952). (b) Position of 10 mg. triiodothyronine isolated from tubes 65-85 of a run similar to that shown in (a). The difference in the shape and position of the two peaks is due to overloading in column (a). (c) Separation of the precipitate obtained from the butanol extract of tryptic digests of fresh thyroid tissue. The material in the third peak was isolated for identification.

sumably) triiodothyronine on kieselguhr columns as described for the synthetic material; a typical run is shown in Fig. 2c. The lack of correspondence between the positions of synthetic triiodothyronine (Fig. 2a) and the natural compound is due to overloading in Fig. 2a which causes deformation of the

peak; that this was so was demonstrated by isolating synthetic triiodothyronine from tubes 65-85 of a 100 mg. run and re-running 10 mg. of this material on another column. A diagram of this run is shown in Fig. 2b, where there is good correspondence between the peak and that of Fig. 2c. The effluent corresponding to the third peak in Fig. 2c was concentrated to dryness and the residue converted to the crystalline hydrochloride (Pl. 3h, i of Addendum); iodine analysis and melting point of this material agreed closely with the values obtained for the synthetic material; it was, however, difficult to get large enough crystals for full X-ray analysis, and much material was wasted during these attempts.

Powder X-ray diffraction analysis of this biologically derived compound showed a pattern identical with that obtained from the synthetic triiodothyronine hydrochloride (see Addendum). Similarly, the single crystal X-ray pictures agreed, thus confirming the identity of the thyroid derivative with 3:5:3'-triiodothyronine.

An attempt was made to estimate the relative amounts of thyroxine and triiodothyronine present in the thyroid glands used in these experiments. From 500 g. of fresh thyroid 30.4 mg. thyroxine and 1.25 mg. triiodothyronine were isolated. These figures probably represent minimal values, since possible losses in the course of isolation were not controlled.

EXPERIMENTAL

Synthesis of 3:5:3'-triiodo-L-thyronine

3:5-Diiodo-L-thyronine (1.05 g.) was dissolved in methanol (40 ml.) and concentrated NH_3 (40 ml.) and iodinated slowly with 3.5 ml. (1.75 atomic prop.) N-I_2 in KI at room temperature; most of the methanol and NH_3 was then removed under diminished pressure, the solution was diluted with water to the original volume, warmed to about 60° and brought to pH 4-5 with acetic acid. A crystalline precipitate separated immediately; after chilling in an ice bath, it was collected, washed with water and dissolved in the minimum of boiling 2N-HCl (about 300 ml.). The boiling solution was filtered to remove a small amount of relatively insoluble thyroxine hydrochloride and brought to pH 4-5 with NaOH solution. After several hours at 4° the crystalline precipitate was collected, washed with water and dried. The yield was 70-80% of the theoretical.

Equally good yields of triiodothyronine were obtained by iodinating diiodothyronine with N-I_2 at room temperature in 33% (w/v) ethylamine or in N-NaOH solution, and purifying in the manner described above. At this stage the product contained about 10% thyroxine; this was removed by separation on a kieselguhr column, using 20% CHCl_3 in *n*-butanol (v/v) equilibrated with 0.5N-NaOH (Fig. 2a). The pure L-triiodothyronine so obtained had m.p. $233-234^\circ$ (decomp.). (Found: C, 28.0; H, 2.6; N, 2.2; I, 58.1; $\text{C}_{15}\text{H}_{12}\text{O}_4\text{NI}_3$ requires C, 27.6; H, 1.8; N, 2.2; I, 58.5%.)

L-Triiodothyronine hydrochloride. L-Triiodothyronine (100 mg.) was dissolved in boiling 2N-HCl (50 ml.) and

allowed to cool very slowly; long needles (Pl. 3f, g) of the hydrochloride separated in 65–70% yield; m.p. 202–203° (decomp.). $[\alpha]_D^{20} + 21.5^\circ$ in 4.75% solution in a mixture of 1 vol. *n*-HCl and 2 vol. ethanol. (Found: C, 25.9; H, 2.8; N, 1.9; I, 54.7. $C_{15}H_{11}O_4NI_3$. HCl requires C, 26.2; H, 1.9; N, 2.0; I, 55.4%.)

DL-Triiodothyronine. This was prepared from DL-diiodothyronine in the same way as the L compound; it had m.p. 233–234° (decomp.). The hydrochloride melted at 200–201° (decomp.).

D-Triiodothyronine. This was prepared from D-diiodothyronine (26 mg.) in the same way as the L and DL amino-acids; it was not purified on a kieselguhr column and contained about 10% thyroxine.

Separation procedure on kieselguhr columns

The chromatographic procedure used in these experiments is a modification of that described by Grönkvist & Hellberg (1951). The stationary phase on the kieselguhr column was 0.5*N*-NaOH and the mobile phase was 20% $CHCl_3$ in *n*-butanol (v/v).

Fig. 3. Standard curves of the colour reaction of thyroxine and triiodothyronine treated with HNO_3 followed by alkali.

Prior to use, the kieselguhr was heated for 4 hr. at 400–500°. After cooling, the powder was mixed with a large volume of 6*N*-HCl and left to settle overnight. The acid was decanted, and the residue was washed with water until the washings were no longer acid, and dried. For the preparation of a column 40 ml. of 0.5*N*-NaOH were thoroughly mixed with 50 g. of kieselguhr, and $CHCl_3$ saturated with 0.5*N*-NaOH was added until a thin slurry was obtained; the slurry was degassed by evacuation on a water pump. Using a plunger with a perforated disk at the end, this mixture was packed into a glass column of 3 cm. internal diameter and 65 cm. in length. Degassed *n*-butanol containing 29% $CHCl_3$ (v/v) equilibrated with 0.5*N*-NaOH was then passed

through the column. Complete replacement of the first solvent by the second was indicated by a transitory opalescence in the effluent. Asbestos (3 g.) was then packed on to the top of the kieselguhr column. The mixtures to be separated were dissolved in 0.5*N*-NaOH (1–2 ml.) (the lower phase of the equilibration mixture with 20% $CHCl_3$ in butanol) and added to the asbestos packing. Kieselguhr (40 g.) containing 0.5*N*-NaOH (32 ml.) was added to the equilibrated 20% $CHCl_3$ -butanol mixture to form a thin slurry which was then poured on to the top of the asbestos. This second lot of kieselguhr was necessary to prevent removal of the stationary phase. A constant head of 20% $CHCl_3$ in *n*-butanol was maintained on the column, and the effluent was fractionated by a siphon of 14.9 ml. capacity mounted on an automatic fraction-cutter. Detection of the iodinated phenols in the effluent was carried out as follows: to 1 ml. samples of the fractions were added 95% ethanol (3 ml.), 11*N*-HCl (0.1 ml.) and freshly prepared 5% (w/v) $NaNO_2$ (0.5 ml.); after mixing, the tubes were immersed in a boiling-water bath for 30 sec. and then allowed to stand at room temperature for 10 min.; those samples which contained thyroxine or triiodothyronine showed a yellow colour which changed to a brownish orange after the addition of 40% (w/v) NaOH (0.1 ml.). This colour showed a maximum between 460 and 470 $m\mu$. in the Unicam spectrophotometer. Standard curves at a wavelength of 465 $m\mu$. were made for thyroxine and triiodothyronine (Fig. 3), and were almost linear over the range of concentrations tested. When the amount of thyroxine or of triiodothyronine was very small, pooled fractions were evaporated to dryness and the residue was dissolved in acid-ethanol (4 ml.) and determined in the usual way. The standard curves obtained in the absence of $CHCl_3$ and butanol were similar to those shown in Fig. 3.

Identification of radioactive 3:5:3'-triiodothyronine in rat-thyroid hydrolysates

Four male hooded rats weighing 200–250 g. were each injected subcutaneously with 100 μ c. ^{131}I and 5 days later were anaesthetized with ether and killed by exsanguination. The thyroid glands from all four animals were removed and ground up together in 0.5 ml. NH_4 buffer at pH 8.4 (Sumner & Somers, 1943); urea (20 mg.) and powdered crude trypsin (1 mg.) were added and the mixture was incubated at 38° for 2 days; during the incubation, two further additions of trypsin (1 mg. and 0.25 mg.) in buffer were made. The hydrolysate was then acidified to pH 2 with dilute HCl and extracted once with 2 vol. and twice with 1 vol. *n*-butanol. The pooled butanol extracts were concentrated to dryness under diminished pressure without washing, the residue was placed on Whatman no. 1 or no. 2 paper, together with synthetic triiodothyronine (100 μ g.) and run two-dimensionally in *n*-butanol-dioxan-ammonia and *n*-butanol-acetic acid. Autoradiographs of the chromatograms were made and the carrier triiodothyronine was visualized with diazotized sulphuric acid (see Gross & Leblond, 1951 b). Five experiments of this type were carried out; a sample is shown in Fig. 1a, b.

Isolation of 3:5:3'-triiodothyronine from ox thyroid glands

(a) *Tryptic hydrolysis.* At first, considerable difficulty was encountered in working up the products of enzymic hydrolysis owing to the large amounts of fat and fatty acid present,

and many experiments were done yielding only minute amounts of triiodothyronine. The method described below was finally chosen because of its relative simplicity, although considerable losses of both thyroxine and triiodothyronine probably occur during the process of isolation.

Method. Fresh, frozen thyroid gland (5 kg.) was minced and suspended in 15 l. water containing 810 g. urea, 875 g. NH_4Cl and sufficient conc. NH_3 to bring the pH to 8. The suspension was kept at 4° for 16 hr. and then filtered through a large wire kitchen sieve to remove the fat. Powdered trypsin (70 g.) was made into a paste and added to the filtrate and the mixture incubated under toluene at 37° for 6–8 days. The solution was brought to pH 4 with conc. HCl and chilled at 4° for 16 hr. The precipitate was collected, and while wet was extracted repeatedly with CHCl_3 to remove fat and brown tarry impurities; it was then dissolved in 1 l. 2N-NaOH and extracted twice with 1 l. *n*-butanol. The combined butanol extracts were evaporated to dryness under diminished pressure, the residue dissolved in 250 ml. water and brought to pH 3–4 with HCl. The precipitate obtained was collected by centrifuging, washed with water, shaken repeatedly with CHCl_3 and then with light petroleum (b.p. 40–60°) and dried; 1.52 g. were obtained. This precipitate was extracted five times with dry ether leaving 1.14 g. powdery material. In order to remove most of the thyroxine, the powder was extracted twice with boiling 2N-HCl (about 50 ml.) and filtered; the filtrate was brought to pH 3–4 with 5N-NaOH and after chilling for 16 hr. the precipitate was collected, washed with water and dried; 23.2 mg. material containing much tarry impurity and still much thyroxine was obtained. Two-dimensional paper chromatography of 1 mg. of the material, as previously described, revealed at this stage the presence of small amounts of triiodothyronine. The tryptic digestion and purification process was repeated until about 80 mg. of crude thyroxine-triiodothyronine precipitate had been collected, and the whole lot was then separated on a kieselguhr column (Fig. 2c). The triiodothyronine-containing fraction of the effluent was concentrated to dryness, the residue dissolved in dilute NH_3 (about 0.1N), centrifuged to remove a little insoluble material from the column, warmed to 60° and brought to pH 4–5 with HCl and acetic acid. After chilling for 72 hr. the precipitate which had separated was collected, washed with water and dried. The free amino-acid was converted to the hydrochloride in the same way as the synthetic material and had m.p. 201–203° (decomp.); the mixed melting point with synthetic L-triiodothyronine hydrochloride was not depressed.

(b) *Barium hydroxide hydrolysis of fresh, frozen thyroid.* Minced thyroid (2 kg.) was boiled under reflux for 17 hr. with water (800 ml.) and $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ (1 kg.), the mixture was filtered hot and the precipitate was extracted once with 1 l. 2N- NH_3 at the boiling point. After filtering again the insoluble barium precipitate containing much fat and also thyroxine was discarded. The two filtrates were combined and brought to pH 3–4 with HCl. After cooling, the reaction mixture was extracted once with an equal volume of butanol which was evaporated to dryness. The residue was dissolved in water and brought to pH 4 with 4N-NaOH and chilled for several days. The precipitate which separated was then collected, washed and dried; it weighed 1.8 g. This precipitate was dissolved in 150 ml. 2N-NaOH, boiled with excess Na_2SO_4 to remove Ba, filtered and extracted with 2 vol. and then 1 vol. *n*-butanol.

The combined butanol extracts yielded 0.28 g. material insoluble at pH 3.5–4 which was shown chromatographically to contain much tarry material and thyroxine; extraction with 2N-HCl (30 ml.) and neutralization of the acid-soluble fraction to pH 3.5–4 with 4N-NaOH gave 28 mg. of material containing much chromatographically detectable triiodothyronine. The $\text{Ba}(\text{OH})_2$ hydrolysis was repeated until enough of this fraction had been collected for purification on the kieselguhr column. Colorimetric determination of the amount of triiodothyronine in four pooled hydrolyses showed the presence of about 12 mg. of which 7 mg. could be isolated as the hydrochloride. The natural DL-triiodothyronine hydrochloride so obtained had m.p. 197–199° (decomp.). The mixed melting point with synthetic DL-triiodothyronine hydrochloride was not depressed. (Found: I, 55.4. $\text{C}_{15}\text{H}_{12}\text{O}_4\text{NI}_3 \cdot \text{HCl}$ requires I, 55.4 %.)

Determination of the relative amounts of thyroxine and triiodothyronine in fresh thyroid gland

Fresh, frozen thyroid (500 g.) was dissolved with heating in 500 ml. 4N- NH_3 and cooled. A large amount of fat which separated was then skimmed off. The solution was strained through butter muslin, brought to pH 4–5 with HCl and treated with 2 vol. acetone. The precipitate so obtained was washed with several volumes 80% acetone and finally with dry acetone, and dried. Yield, 41 g., containing 0.49% I.

The defatted thyroid powder (40 g.) was hydrolysed as before with 400 ml. water and 200 g. crystalline $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$. After hydrolysis, the insoluble barium material was worked up for thyroxine isolation by Harington's (1926) method, giving a crude yield of acid-insoluble material of 9 mg. (fraction A). The filtrate was worked up as before and gave 46 mg. crude acid-insoluble material containing both thyroxine and triiodothyronine (fraction B); extraction of this with boiling 2N-HCl gave 8 mg. of material from which 1.25 mg. pure triiodothyronine were obtained; the residue, together with fraction A, yielded after chromatographic purification 30.4 mg. thyroxine; the triiodothyronine content of this sample of thyroid powder was therefore about 4% of its thyroxine content.

DISCUSSION

Concurrently with these investigations, Roche, Lissitzky & Michel (1952*a, b*) have synthesized triiodothyronine from diiodothyronine, and have also identified radioactive triiodothyronine in hydrolysates of the thyroids of rats which had been given Na^{131}I ; the chromatographic techniques are somewhat different from those described in the present paper, but the results obtained in each afford mutual confirmation.

The possibility that triiodothyronine might be derived from thyroxine as an artifact of alkaline hydrolysis was considered, but was rendered improbable by the following control experiment: thyroxine was boiled together with casein for 8 hr. in 50% (w/v) barium hydroxide solution; no triiodothyronine could be detected among the hydrolysis products.

Further, triiodothyronine was not formed from thyroxine on the kieselguhr column; this was demonstrated in a previous paper (Gross & Pitt-Rivers, 1952).

Triiodothyronine is not likely to be an artifact of tryptic hydrolysis of the thyroid gland; it is known to occur among the other iodinated free amino-acids extractable from unhydrolysed thyroid tissue (Gross & Leblond, 1951*b*) which suggests that it is continuously being liberated from peptide linkage, together with thyroxine, by the proteolytic enzymes present in the gland.

These findings, when considered together, indicate that triiodothyronine is a true constituent of the thyroid gland.

SUMMARY

1. 3:5:3'-Triiodothyronine has been isolated from ox thyroid gland and has been identified in tryptic hydrolysates of thyroids from rats previously injected with sodium [¹³¹I] iodide.

2. The synthesis of L-, DL- and D-triiodothyronine, together with the method of purification and X-ray crystallographic characteristics, are described.

We wish to thank Dr B. A. Hems of Glaxo Laboratories Ltd. for generous gifts of L- and D-diiodothyronine and Dr D. Findley of Burroughs Wellcome Ltd. for a gift of desiccated thyroid. We also wish to thank Mr P. Bovill for help with much of the experimental work, and Mr C. Sutton and Mr E. Hitchcock for the autoradiographs and photographs.

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ADDENDUM

A Crystallographic Examination of Triiodothyronine and some Related Compounds

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(Received 29 August 1952)

A verification of the identity of the substance isolated by Pitt-Rivers & Gross was undertaken. Since unknown 1 was available in powder form only, a comparison was made of its X-ray powder diffraction photograph with those of 3:5-diiodo-L-thyronine, L-triiodothyronine, L- and DL-thyroxine. The interplanar spacings and approximate relative intensities of the reflected X-rays are shown in Table 1. Spacings greater than 6 Å. are omitted. The data for L-diiodothyronine and DL-thyroxine agree well with those of Spiegel-Adolf, Hamilton & Henny (1942). The unknown 1 had to be recrystallized several times before any characteristic crystal pattern could be observed. This was consistent with that of L-triiodothyronine, but resolution was too poor for unequivocal identification.

The powder photographs were all taken with a cylindrical camera of 6 cm. diameter, using copper radiation. The samples were enclosed in thin-walled Lindemann glass capillary tubes of 0.2 mm.

diameter and weighed about 10 µg. Measurements of the interplanar spacings were accurate to about 2 %.

In powder photographs much of the available information is lost as the diffraction patterns of a large number of crystallites are superimposed. A few dozen rings only can be resolved instead of the hundreds of distinguishable diffraction spots in photographs from single oriented crystals.

Single crystals of unknown 1 could not be prepared, but more success was attained with the hydrochloride, which grew as needles 5 µ. thick. These were accordingly compared with synthetic triiodothyronine hydrochloride which was obtained as four-sided needles 2–20 µ. thick (Pl. 3*f-i*). Examined between crossed Nicols they were both birefringent with the fast ray, of refractive index 1.660, vibrating parallel to the needle axis. The lattice constants of the synthetic material were found approximately from moving-film and full-

Table 1. *X-ray diffraction patterns of iodinated aromatic amino-acids*(Interplanar spacings in Ångström units. Intensity scale: *S*, strong; *M*, medium; *W*, weak.)

L-Diiodothyronine	L-Triiodothyronine	L-Triiodothyronine hydrochloride*		L-Thyroxine	DL-Thyroxine
		Synthetic	Natural		
1.88 <i>W</i>	2.04 <i>W</i>	2.07 <i>W</i>	2.07	1.59 <i>W</i>	1.49 <i>W</i>
2.04 <i>W</i>	2.16 <i>W</i>	2.36 <i>W</i>	2.36	1.70 <i>W</i>	1.56 <i>W</i>
2.11 <i>M</i>	2.24 <i>W</i>	2.76 <i>W</i>	2.78	1.77 <i>W</i>	1.73 <i>M</i>
2.31 <i>W</i>	2.44 <i>W</i>	3.28 <i>M</i>	3.29	1.90 <i>W</i>	1.77 <i>M</i>
2.39 <i>M</i>	2.66 <i>W</i>	3.79 <i>S</i>	3.80	2.09 <i>W</i>	1.82 <i>W</i>
2.49 <i>W</i>	2.82 <i>M</i>	4.26 <i>S</i>	4.31	2.41 <i>W</i>	1.89 <i>W</i>
2.62 <i>M</i>	3.03 <i>W</i>	4.67 <i>W</i>	4.70	2.53 <i>W</i>	1.99 <i>M</i>
2.97 <i>M</i>	3.23 <i>W</i>	5.10 <i>W</i>	5.13	2.70 <i>M</i>	2.09 <i>W</i>
3.07 <i>M</i>	3.49 <i>S</i>	5.61 <i>W</i>	5.68	3.09 <i>M</i>	2.15 <i>M</i>
3.29 <i>M</i>	3.69 <i>W</i>			3.29 <i>W</i>	2.31 <i>M</i>
3.48 <i>S</i>	3.93 <i>S</i>			3.49 <i>W</i>	2.41 <i>M</i>
3.82 <i>S</i>	4.21 <i>S</i>			3.74 <i>S</i>	2.54 <i>W</i>
4.06 <i>S</i>	4.46 <i>W</i>			4.11 <i>W</i>	2.71 <i>S</i>
4.27 <i>W</i>	4.77 <i>M</i>			4.58 <i>M</i>	2.81 <i>S</i>
4.55 <i>W</i>	5.22 <i>W</i>			5.99 <i>W</i>	3.05 <i>W</i>
5.47 <i>S</i>	6.37 <i>W</i>				3.25 <i>S</i>
5.91 <i>W</i>					3.55 <i>S</i>
					3.95 <i>S</i>
					4.37 <i>M</i>
					4.72 <i>M</i>
					5.10 <i>W</i>

* The interplanar spacings for synthetic DL-triiodothyronine hydrochloride are identical with those obtained for the synthetic L isomer.

rotation photographs, and determined more accurately using the ' θ ' method (Weisz, Cochran & Cole, 1948). The unit cell dimensions are:

$$\begin{aligned} a &= 16.69 \pm 0.05 \text{ Å.} & c &= 28.64 \pm 0.05 \text{ Å.} \\ B &= 5.14 \pm 0.02 \text{ Å.} & \beta &= 114^\circ 49 \pm 6'. \end{aligned}$$

The density of the crystals was measured by centrifuging in benzene-bromoform mixtures of known density until a match was obtained. The density determined by this method was 2.05 g./ml. Crystals which had been allowed to stand for several days increased in density and darkened in colour. A similar effect was observed on exposure to X-rays when the crystals rapidly darkened and gave much weakened diffraction spots. The molecular weight, calculated from the unit cell volume and density, assuming four molecules per unit cell, was 689. The error of this value should not exceed 2%. The molecular weight calculated for $\text{C}_{15}\text{H}_{12}\text{O}_4\text{NI}_3 \cdot \text{HCl}$ is 687.5.

The space group was established using Weissenberg zero and higher layer line photographs about the principal axes and about [101]. The cell type is pseudo *A* face-centred, but the presence of some weak (*hkl*) reflexions with *k*+1 odd indicates a primitive lattice. Odd orders of (0*k*0) absent. The probable space group is therefore $P2_1$. The unit cell can accommodate the four molecules in two sets of general positions.

The crystals of unknown 1 hydrochloride were

too small to permit full determination of the lattice constants. However, the repeat along the needle axis was found to be 5.14 Å. and the spacings and intensities of the distinguishable spots were in good agreement with those of the synthetic hydrochloride. The plane of symmetry perpendicular to the *b* axis was also evident. The optical properties, density and Debye-Sherrer powder photographs were all identical (Table 1). Pl. 3*a*, *b*, shows a composite photograph, the upper half from unknown 1 hydrochloride and the lower from synthetic triiodothyronine hydrochloride. The diffraction patterns of L and DL-triiodothyronine hydrochloride are indistinguishable showing that on crystallization resolution into D and L forms has occurred.

Further crystallographic work on triiodothyronine and related compounds is in progress.

SUMMARY

1. The Debye-Sherrer powder photograph of unknown 1 was compared with those of 3:5-diiodo-L-thyronine, L-triiodothyronine, L-thyroxine and found to be consistent with L-triiodothyronine.

2. Unknown 1 hydrochloride was found by single crystal diffraction photographs, powder photographs, optical properties and density to be identical with synthetic triiodothyronine hydrochloride.

3. The unit cell and space-group of triiodothyronine hydrochloride were determined.

REFERENCES

- Spiegel-Adolf, M., Hamilton, R. H. jun., & Henny, G. C. (1942). *Biochem. J.* **36**, 825.
- Weisz, O., Cochran, W. & Cole, W. F. (1948). *Acta cryst., Camb.*, **1**, 83.

EXPLANATION OF PLATE

Powder X-ray diffraction photographs of: (a) synthetic L-triiodothyronine hydrochloride, (b) hydrochloride of pure compound isolated from thyroid gland, (c) L-thyroxine, (d) L-triiodothyronine, (e) L-diiodothyronine. Note the correspondence between patterns of (a) and (b); this is also shown quantitatively in Table 1. (f) Crystals of L-triiodothyronine hydrochloride photographed with ordinary light; (g) polarized light; (h) crystals of hydro-

chloride of compound isolated from the thyroid gland photographed with ordinary light; (i) polarized light. The direction of extinction in both the natural and synthetic crystals is parallel to the needle axis. (j) Single crystal X-ray diffraction photograph of synthetic triiodothyronine hydrochloride. Crystal oscillated 15° round the *b*-axis. Filtered Cu radiation.

3:5:3'-Triiodothyronine

2. PHYSIOLOGICAL ACTIVITY

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(Received 5 August 1952)

On establishing the identity of 3:5:3'-triiodothyronine as a normal body constituent, it was decided to investigate its biological activity. Preliminary experiments on its effect in preventing thiouracil-induced goitre (Gross & Pitt-Rivers, 1952*b*) and on human myxoedema (Gross, Pitt-Rivers & Trotter, 1952), indicated that triiodothyronine possessed marked thyroid-hormone like properties.

The present paper describes a more comprehensive survey of the biological activity of 3:5:3'-triiodo-L-thyronine both in the goitre prevention test and in the growth and organ weights of thyroidectomized rats.

Dempsey & Astwood (1943) showed that the thiouracil-induced hyperplasia of the thyroid gland was quantitatively inhibited by graded doses of thyroxine; subsequently, Reineke, Mixner & Turner (1945) demonstrated that this response could be closely correlated with the standard metabolism tests. For this reason, and also because of its simplicity, this test was chosen to determine the activity of triiodothyronine relative to thyroxine as well as to examine the activities of the DL and D forms of the new amino-acid.

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METHODS

Goitre prevention assay

Male hooded rats, bred in the National Institute for Medical Research, weighing 100 ± 15 g. were housed two in a cage and fed the stock diet 41 for rats and mice (Bruce & Parkes, 1949) *ad lib.*; this diet contains approximately 1 μ g. I/g. Thiouracil was given in 0.1% solution in the drinking water. Groups of six to eight animals were used to test each dose. The test compounds (thyroxine and triiodothyronine) were dissolved in 0.1 N-NaOH and diluted with 0.9% NaCl to give the desired dose in 0.1 ml. solution; the final concentration of alkali was of the order of 0.001 N. Similar solutions omitting the test substance were used for the control groups. The doses were injected subcutaneously in the scruff of the neck. In the assay comparing the relative effectiveness of injected and oral doses the latter were diluted with 15% sucrose solution, the dose being contained in 0.2 ml. which was fed to the animals from a graduated syringe; the doses injected for comparison were given in 0.1 ml. solution.

After ten daily doses, the animals were killed with CHCl_3 , weighed, their thyroids dissected from the trachea and weighed on a torsion balance to the nearest 0.1 mg. In the first experiments the pituitaries were also removed, fixed in Susa fluid, sectioned and stained with Masson's trichrome stain.

Using the procedure described by Finney (1951), the slopes of the curves and the potencies were calculated. The potencies were expressed as percentages of the activity of equimolar amounts of L-triiodothyronine. The limits of error at the 5% level are also given for each result, for