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The Oxidation of Diiodotyrosine Derivatives

By ROSALIND PITT-RIVERS, *National Institute for Medical Research, Hampstead, London, N.W. 3*

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It is now well established that when diiodotyrosine is incubated under mildly alkaline conditions, thyroxine is formed (von Mutzenbecher, 1939; Block, 1940; Johnson & Tewkesbury, 1942; Harington & Pitt-Rivers, 1945); under the most favourable conditions, however, the yields of thyroxine are small, and a large proportion of the diiodotyrosine is lost, giving rise to tarry by-products, inorganic iodide and ammonia. So far, no compound has been isolated after such an incubation which would indicate through what intermediate step thyroxine is synthesized; nor has the side chain, which is removed from one molecule of diiodotyrosine during the coupling of two molecules to give thyroxine, or a recognizable derivative thereof, been isolated, although Johnson & Tewkesbury (1942) claim to have detected pyruvic acid in the reaction mixture when thyroxine is formed under these conditions.

It seemed possible that further investigation of the products of alkaline oxidation of diiodotyrosine might reveal some compound which would give a clue to the mechanism of the reaction. It had been shown by Harington & Pitt-Rivers (1945) that the pH at which diiodotyrosine is incubated exerts a marked effect both on the amount of thyroxine formed and on the extent to which diiodotyrosine itself is oxidized. At a high pH (13 or 14) no thyroxine was obtained by these authors, but instead another crystalline iodine-containing compound was isolated, which was not characterized at the time. This compound has now been re-examined and has been identified as 3:5-diiodo-4-hydroxybenzaldehyde. Further examination of the products of incubation of diiodotyrosine at pH 10 has shown that at this pH the aldehyde is formed at the same

time as thyroxine; at lower pH (7.8–8.8) none of the aldehyde is obtained, while the yields of thyroxine are very small.

The simultaneous formation of 3:5-diiodo-4-hydroxybenzaldehyde and thyroxine from diiodotyrosine led first to the hypothesis that the aldehyde might be an intermediate in thyroxine synthesis, and attempts were made to demonstrate this by incubating diiodotyrosine in the presence of the aldehyde, in the hope that increased yields of thyroxine might result. These attempts have proved abortive, and in all the experiments done the yield of thyroxine was unchanged and the aldehyde was recovered at the end of the reaction. Further, Dakin's (1909) observation that 3:5-diiodo-4-hydroxybenzaldehyde is unaffected by alkaline hydrogen peroxide, while the dichloro and dibromo analogues give more or less good yields of the corresponding quinols, has been confirmed. Only under the most drastic conditions may the diiodo aldehyde be oxidized, and the product of oxidation is 2:4:6-triiodophenol. These facts have, therefore, led to the abandonment of the hypothesis that the aldehyde is an intermediate in thyroxine synthesis, and it is now thought that its formation from diiodotyrosine is due to a different mechanism from that which leads to thyroxine.

The mode of formation of 3:5-diiodo-4-hydroxybenzaldehyde from diiodotyrosine is itself of some interest. It has been found that, when the aldehyde is obtained by incubating diiodotyrosine at pH 14, an amount of oxalic acid can be isolated as the calcium salt from the reaction mixture which is molecularly equivalent to the amount of aldehyde isolated; this leads to the suggestion that the

reaction entails, first, oxidation on the β carbon atom of the diiodotyrosine side chain. Further, when diiodotyrosine is incubated at pH 10, with the simultaneous formation of thyroxine and the aldehyde, oxalic acid is again obtained. In this instance the amount of oxalic acid appeared, in two experiments, to be molecularly equivalent to the sum of thyroxine and diiodo-*p*-hydroxybenzaldehyde isolated. This observation suggested that the aldehyde and thyroxine might be obtained by a mechanism whose initial steps were similar, but which, under the influence of pH or other factors (Pitt-Rivers, 1947), led to different end products. Even if we suppose that the apparent equivalence, mentioned above, of the oxalic acid formed with the sum of aldehyde and thyroxine produced at pH 10 is a genuine phenomenon, the observation is of little value in relation to the essential question of the form in which the side chain of one of the reacting molecules of diiodotyrosine is eliminated; oxalic acid is the common end product of oxidation of many 2- and 3-carbon aliphatic compounds, and in the present experiments its appearance could be explained equally well by the stepwise degradation of the side chain involving primary scission of the two terminal carbon atoms, or by secondary oxidation following elimination of the whole side chain as a 3-carbon fragment.

From what has already been said, it appears that the formation of 3:5-diiodo-4-hydroxybenzaldehyde from diiodotyrosine depends upon a certain degree of alkalinity, and in this connexion the formation of acid-insoluble iodine-containing compounds other than thyroxine during the preparation of artificial thyroproteins is called to mind.

During the preparation of such proteins by the method of Ludwig & von Mutzenbecher (1939), later developed by other workers (Reineke & Turner, 1942; Pitt-Rivers & Randall, 1945), the initial pH of the protein solution in bicarbonate buffer is about 8 and may rise to 9 during the iodination; it may also be remembered that Deanesly & Parkes (1945) showed that the method of assay as used for thyroid powder (determination of the acid-insoluble iodine content) cannot be applied to the artificial thyroproteins, since they contain large amounts of acid-insoluble iodine-containing products which are biologically inactive. The inactivity of 3:5-diiodo-4-hydroxybenzaldehyde as measured by the tadpole-metamorphosis test, together with the effect of alkaline conditions on aldehyde production from diiodotyrosine, suggested that this aldehyde might be isolated from the products of baryta hydrolysis of such a thyroprotein. This has proved to be so, and in one instance the amounts of aldehyde and thyroxine isolated were in the ratio of 10:1. No aldehyde has been isolated following baryta hydrolysis of desiccated thyroid powder.

The instability of diiodotyrosine itself is so great that it does not permit readily of its use in determining in what form the 3-carbon side chain is eliminated during thyroxine synthesis. In a further attempt to elucidate this point, a method of stabilizing the side chain has therefore been sought, and it was thought probable that acetylation of the amino group might achieve this result. Preliminary experiments on the alkaline incubation of *N*-acetyl-L-diiodotyrosine in solutions with a pH range of 7-8.5, followed by alkaline hydrolysis and butanol fractionation of the products, led to the isolation of thyroxine in small yields. During these incubations, however, it was noticed that a crystalline salt separated. This proved to be the sodium salt of *N*-acetyl-L-thyroxine, and from it the free acid could be obtained in yields of about 25%, calculated on the amount of unchanged starting material recoverable at the end of the experiments. *N*-Acetyl-L-diiodotyrosine has been titrated potentiometrically, and the pH optimum for acetyl thyroxine formation has been determined. Acid hydrolysis of acetyl thyroxine in a mixture of acetic acid and concentrated hydrochloric acid yielded L-thyroxine with $[\alpha]_D^{25} - 5.42^\circ$ in 3% solution in a mixture of two parts ethanol and one part *N*-NaOH.

In the thyroid gland itself, thyroxine must, in part, be formed from diiodotyrosine molecules, which are joined through both their amino and carboxyl groups in peptide linkage; it appeared, therefore, to be of interest to discover whether a diiodotyrosine derivative, in which both these groups are blocked, would lead *in vitro* to the formation of a corresponding thyroxine derivative, and *N*-acetyl-DL-diiodotyrosylglutamic acid was prepared for this purpose. Incubation of the acetyl peptide at pH 7.2 in dilute sodium hydroxide solution led to no insoluble salt of a thyroxine derivative, but alkaline hydrolysis of the reaction mixture did yield a small amount of thyroxine. If the incubation of the acetyl peptide were carried out at pH 7.2 in baryta, however, a partly crystalline barium salt separated which, after decomposition with acid, yielded *N*-acetyl-DL-thyroxyl-L-glutamic acid. This thyroxine derivative has been obtained in yields up to 36% net, and conversion of the diiodotyrosine derivative to the thyroxine derivative is particularly good in this instance, gross yields being of the order of 20%. Acid hydrolysis of acetylthyroxylglutamic acid led to the isolation of thyroxine and glutamic acid.

The good yields of acetylthyroxine and the acetylthyroxine peptide obtained in these experiments, together with the relative freedom of the reaction mixtures from tarry products, have led to the hope that the diiodotyrosine side chain which is detached might be isolated, either as an acetamido compound or, after hydrolysis, as an amino-acid. After 2 or 3 weeks' incubation of acetyldiiodotyrosine in

baryta and removal of acetylthyroxine and of as much as possible of the starting material, the residue was hydrolyzed with sulphuric acid and freed from diiodotyrosine, iodide and sulphate. The residual solution contained an amino-acid (positive ninhydrin reaction) and chromatographic examination, kindly carried out by Dr R. L. M. Synge, revealed in two separate experiments the presence of small amounts of alanine. Further experiments are being carried out in an effort to isolate this amino-acid, and to discover whether this represents the diiodotyrosine side chain which is split off. Serine and glycine have been looked for, but Dr Synge has reported that there is no evidence of their presence.

EXPERIMENTAL

Isolation and identification of 3:5-diiodo-4-hydroxybenzaldehyde from L-diiodotyrosine after oxidation at pH 14 with H₂O₂ (hot)

L-Diiodotyrosine. 2H₂O (28.2 g.) was dissolved in a mixture of 12 ml. 10N-NaOH and 100 ml. 2N-NaOH, and heated on a boiling water bath; 20 vol. H₂O₂ (60 ml.) were added, and after the vigorous reaction had subsided the solution was cooled and extracted with an equal volume of *n*-butanol; these operations were repeated three times. The pooled butanol extracts were washed once with half their volume of 2N-NaOH and concentrated to dryness. The crystalline residue was dissolved in water (200 ml.) and acidified at the boiling point with 14N-H₂SO₄. A white crystalline precipitate immediately appeared which was collected, washed with water and dried *in vacuo* over P₂O₅. The yield was 1.11 g. After recrystallization from benzene, 0.71 g. of the product was obtained (prisms), and had m.p. 194–196° (decomp.) not depressed in admixture with authentic 3:5-diiodo-4-hydroxybenzaldehyde. (Found: C, 22.6; H, 1.2; I, 68.0. Calc. for C₇H₄O₂I₂: C, 22.5; H, 1.07; I, 67.9%.)

Reduction of the above compound. 0.42 g. was dissolved in 7 ml. N-NaOH and 20 ml. water, and hydrogenated in the presence of 0.25 g. palladized strontium carbonate. At the end of 2 hr. the reduction was complete and 40 ml. of hydrogen had been taken up. The reaction mixture was filtered, treated with 1.4 ml. 5N-H₂SO₄ and concentrated to dryness. The residue was crystallized from the minimum amount of hot water. The aqueous mother liquor was again concentrated to dryness and crystallized from water; 90 mg. product were obtained with m.p. 111–112°, and were recrystallized from water in needles with m.p. 114–115°. The addition of *p*-hydroxybenzaldehyde did not depress the melting point.

The product formed a semicarbazone with m.p. 218–220° which, with *p*-hydroxybenzaldehyde semicarbazone, had m.p. 219–220°. (Found: N, 23.0. Calc. for C₈H₈O₂N₃: N, 23.5%.)

Isolation of 3:5-diiodo-4-hydroxybenzaldehyde and oxalic acid after incubation of L-diiodotyrosine for 14 days at pH 14

Diiodotyrosine. 2H₂O (46.9 g.) was dissolved in a mixture of 300 ml. N-NaOH and 20 ml. 10N-NaOH, and incubated for 14 days, after which the solution was extracted once with

an equal volume of butanol. After washing the butanol extract with about 50 ml. N-NaOH it was concentrated to dryness. It yielded 0.308 g. crystalline product with m.p. 184–190° (decomp.) with previous darkening. The product was then extracted repeatedly with hot 20% sodium metabisulphite solution, decomposition of which, with mineral acid, gave 0.235 g. of 3:5-diiodo-4-hydroxybenzaldehyde, m.p. 198–200° (decomp.); the mixed melting point with the authentic aldehyde was not depressed. The aqueous fraction was freed from butanol by a short distillation under diminished pressure, and the pH was brought up to 4.3 by addition of conc. HCl. The diiodotyrosine which separated was allowed to crystallize at 4° during 4–5 hr. and was then collected, washed with a little water and air-dried. Recovered: diiodotyrosine, 44.8 g.

The filtrate was brought to pH 5.5 with NaOH and treated with 2 ml. 5M-CaCl₂ solution. After a short time calcium oxalate began to separate, and crystallization was completed by leaving at room temperature for 15 hr. The precipitate was collected on the centrifuge, washed with water and determined by titration with 0.1N-KMnO₄. Oxalic acid found: 47 mg. The volume of the solution from which the calcium oxalate had separated was 850 ml. Calcium oxalate is soluble in water at 25° to the extent of 0.7 mg./100 ml., so a correction for this was made bringing the oxalic acid to 51.2 mg. (0.57 mmol.). The amount of diiodo-*p*-hydroxybenzaldehyde found (0.235 g.) was 0.63 mmol.

Formation of oxalic acid during incubation of diiodotyrosine at pH 10

(a) Diiodotyrosine. 2H₂O (46.9 g.) was incubated at pH 10 as described by Harington & Pitt-Rivers (1945). The butanol-soluble fraction yielded acid-insoluble material weighing 1.08 g. This was extracted three times with about 25 ml. ether, then twice with 20 ml. ethyl acetate and again with ether. The ether-insoluble portion, on dissolving in boiling 0.1N-Na₂CO₃ and working up as usual gave 0.12 g. thyroxine.

The ether-ethyl acetate-soluble portion after removal of solvent weighed 0.82 g. Extraction with hot 20% sodium metabisulphite solution, until no further material would dissolve, gave in all 0.46 g. diiodo-*p*-hydroxybenzaldehyde, m.p. 196–198° (decomp.).

From the aqueous fraction, 41.68 g. diiodotyrosine were recovered. 5M-CaCl₂ solution was added to the filtrate and the precipitated oxalate was collected, redissolved in acid, filtered from a small amount of brownish material, reprecipitated, collected by centrifuging and washed with water. The calcium oxalate was then dissolved in N-H₂SO₄ and the solution was divided into halves. A determination of oxalate by permanganate titration was made on the one half, while the other half was extracted many times with ether. The ether extract on evaporating to dryness gave crystals which on recrystallization from water had m.p. 100–101°, not depressed in admixture with oxalic acid dihydrate. The permanganate titration showed that there was present 0.190 g. calcium oxalate (1.46 mmol.). Thyroxine isolated was 0.12 g. (0.155 mmol.); diiodo-*p*-hydroxybenzaldehyde was 0.46 g. (1.23 mmol.).

(b) A repetition of the above experiment on 35.2 g. diiodotyrosine. 2H₂O with 7 days' incubation gave thyroxine 39 mg. (0.05 mmol.), diiodo-*p*-hydroxybenzaldehyde 0.28 g. (0.75 mmol.) and oxalic acid 0.78 g. (0.87 mmol.).

Incubation of diiodotyrosine at pH 7.8

This was done in exactly the manner described by Harington & Pitt-Rivers (1945). The butanol extract gave an acid-insoluble crude product weighing 69 mg. After extraction with ether, this fraction weighed 15 mg., from which 8 mg. pure thyroxine were obtained. The ether extract on being concentrated to dryness and treated with metabisulphite gave no aldehyde which could be weighed, although a trace of material (less than 1 mg.) was seen in the acidified metabisulphite solution which crystallized in the characteristic prisms of diiodo-*p*-hydroxybenzaldehyde. The recovered diiodotyrosine weighed 8.09 g., which gave a net yield of thyroxine of 0.74%.

Products obtained from the hot peroxide oxidation of diiodotyrosine

L-Diiodotyrosine (23.5 g.) was dissolved in 0.96 N-NaOH (99 ml.) and heated on a boiling water bath. Butanol (150 ml.) was added, and then 20 vol. H_2O_2 (150 ml.) was added in portions with continuous vigorous shaking. When there was no further effervescence the reaction mixture was cooled and the butanol layer separated. This was repeated twice, using 250 ml. butanol for the subsequent extractions. The pH was kept just alkaline to phenolphthalein throughout the reaction by small additions of N-NaOH; each butanol extract was washed with 100 ml. N-NaOH and the pooled washings were re-extracted with 250 ml. butanol.

The butanol-soluble fraction contained 3.18 g. of acid-insoluble material. This was extracted seventeen times by grinding with ether. The ether-insoluble portion gave 0.25 g. thyroxine after purification through the sodium salt.

The ether extract was concentrated to dryness. Repeated extraction with hot 20% sodium metabisulphite gave 0.29 g. of a crystalline compound, m.p. 198–199° (decomp.), mixed melting point with 3:5-diiodo-4-hydroxybenzaldehyde, 200–201° (decomp.). The residue after bisulphite extraction was steam distilled; the steam-volatile material weighed 0.144 g. and had m.p. 154–156° not depressed by mixing with 2:4:6-triiodophenol. (Found: I, 80.5 and 81% on duplicate analysis. Calc. for $C_8H_5OI_3$: I, 80.7%.)

The aqueous residue after butanol extraction was added to the butanol washings and acidified at the boiling point. A dark-brown tar separated which solidified on cooling; it was ground with water, collected and dried, and weighed 7.45 g. It contained no steam-volatile material.

The tar (5 g.) was dissolved in N-KOH (165 ml.) and reduced with hydrogen in the presence of palladized strontium carbonate (15 g.). At the end of the reduction the solution was filtered from catalyst, concentrated to a low volume and acidified with 5N-HCl; 0.75 g. of insoluble material was precipitated and was extracted several times with ether. The ether extract on evaporating to dryness gave a solid residue which, on crystallization from water, yielded 35 mg. of a crystalline compound with m.p. 208°. The melting point of this compound was not depressed by mixing with *p*-hydroxybenzoic acid with m.p. 211–213°.

Of the original unreduced tar, 0.4 g. was extracted with warm saturated sodium acetate solution. The extract was diluted with two parts of water and acidified at the boiling point with conc. HCl. Sheaves of needles separated on cooling and weighed 30 mg. They had m.p. about 220°

(decomp.) with loss of iodine above 200°. Mixed melting point with 3:5-diiodo-4-hydroxybenzoic acid was not depressed.

*Preparation of biologically active iodinated casein and isolation therefrom of thyroxine and diiodo-*p*-hydroxybenzaldehyde*

Casein (Kahlbaum nach Hammarsten) (50 g.) was suspended in water (21.) and dissolved with the aid of 2N-NaOH. $NaHCO_3$ (15 g.) was added and the pH was brought back to 8 by addition of 2N-HCl; 19 ml. of a 4.16N- I_2 solution were added over a period of 2.5 hr. while the solution was mechanically stirred at 40°. The temperature was then raised to between 60 and 70° and maintained there for 5 hr., at the end of which the pH was 8.9 (British Drug Houses Ltd. capillator).

The iodinated casein was then precipitated by bringing the reaction mixture to pH 4 by addition of HCl. The separated protein was redissolved in the same volume of water by addition of alkali, reprecipitated with acetic acid, collected, washed with about 450 ml. N/700 acetic acid and air-dried. After some accidental loss the yield was 38 g.

Of this preparation 35 g. were boiled for 20 hr. under reflux with 500 ml. water and 200 g. recrystallized $Ba(OH)_2$. The hydrolysate was filtered hot, and the decomposed barium salt was combined with the acid-insoluble material in the filtrate. This product weighed 0.82 g.; it was extracted twice with ether, once with ethyl acetate and then three times again with ether. The residue weighed 0.78 g., and yielded in all 108 mg. pure thyroxine, from which the thyroxine content of the protein was 0.31%. The pooled ether and ethyl acetate extracts were combined and evaporated to dryness, and the crystalline residue was purified by extraction with hot 10% sodium metabisulphite. The crystalline compound obtained by decomposition of this extract weighed 15 mg. and had m.p. 197–198° (decomp.). The mixed melting point with authentic 3:5-diiodo-4-hydroxybenzaldehyde was not depressed.

Isolation of 3:5-diiodo-4-hydroxybenzaldehyde from iodinated proteins with low biological activity

The method was as follows: 50–100 g. protein were boiled with 20 parts 2N-NaOH for 18 hr., and then cooled, strained through glass wool if necessary, and extracted once with an equal volume of butanol. The butanol fraction was worked up as described in the previous hydrolysis.

Iodinated casein (PB11) DT/5/829 contained 8.9% total I and 3.3% acid-insoluble I (see Deanesly & Parkes, 1945); 60 g. yielded 23 mg. of pure thyroxine (giving 0.038% thyroxine) and 45 mg. (0.075%) pure diiodo-*p*-hydroxybenzaldehyde.

Iodinated casein (PB6) DT/5/823 contained 7.4% total I and 2.3% acid-insoluble I (see Deanesly & Parkes, 1945). Hydrolysis of 50 g. of this preparation for 7 hr. with 4N-NaOH, followed by butanol extraction, yielded no thyroxine and only 5–6 mg. pure aldehyde.

Hydrolysis of a mixture of iodinated caseins PB6 and PB11 with fractionation of acid-insoluble I

PB6 (50 g.) + PB11 (15 g.) were boiled under reflux for 16.5 hr. with 1 l. 2N-NaOH. After cooling, the hydrolysate was extracted with an equal volume of butanol. This gave

an acid-insoluble precipitate of 1.05 g. containing 27.8% I, and after purification through the usual processes gave thyroxine (3.0 mg.) and 3:5-diiodo-4-hydroxybenzaldehyde (30 mg.). The aqueous fraction contained 6.77 g. acid-insoluble material containing 5.51% I, from which no crystalline compound could be obtained.

Under these conditions of hydrolysis, it therefore appears that: (i) of the acid-insoluble iodine found (665 mg.) 56% is not soluble in butanol, and (ii) that of the butanol-soluble acid-insoluble iodine, less than 1% could be accounted for as thyroxine, while 7% was accounted for as diiodo-*p*-hydroxybenzaldehyde; 92% of this butanol-soluble iodine was not characterized.

Hydrolysis of desiccated thyroid powder B 8

Defatted thyroid powder (200 g.) was boiled under reflux with 2 l. 2*N*-NaOH for 17 hr., and fractionated into butanol as described above. The acid-insoluble residue from the butanol fraction weighed 5.6 g. This fraction was composed largely of fat and was largely soluble in ether. The ether-soluble material was boiled three times with 20% sodium metabisulphite solution, and each extract was cooled before filtering (since the bisulphite compound of 3:5-diiodo-4-hydroxybenzaldehyde is soluble in cold water, this procedure did not risk the loss of aldehyde). Decomposition of the bisulphite solution with mineral acid led to no insoluble material, so that no aldehyde appeared to be present. Since it was possible that the aldehyde might not be extractable from the fat with any ease, about 1 mg. authentic aldehyde was added to it: one bisulphite extract easily removed it.

Experiments on the incubation products of *N*-acetyl-L-diiodotyrosine

N-Acetyl-L-diiodotyrosine. This compound has already been prepared by Myers (1932), but the method is not suitable for large amounts. Moreover, the compound is unstable and tends to become coloured during recrystallization. The following method gives a product which may be used for incubation experiments without further crystallization. *ON*-Diacetyl-L-diiodotyrosine (Myers, 1932) (25.85 g.) was dissolved in 150 ml. cold *N*-NaOH. After 10 min. at room temperature, the solution was filtered, diluted to 600 ml. with water, warmed to 75° and acidified by addition of 170 ml. *N*-HCl with stirring; the product began to crystallize immediately and the solution was then cooled rapidly to 0°. After 2 hr. in the ice chest the crystals were collected, washed with water and dried *in vacuo* over P₂O₅; the yield was 22.5 g., m.p. 125°, $[\alpha]_D^{18} + 49.7^\circ$ in 2% solution in 2*N*-NaOH. (Found: C, 27.3; H, 2.6; I, 52.9. Calc. for C₁₁H₁₁O₄NI₂·½H₂O: C, 27.3; H, 2.5; I, 52.5%.)

Apparent dissociation constant of the diiodophenolic group of N-acetyl-L-diiodotyrosine. The monosodium salt of *N*-acetyl-L-diiodotyrosine was titrated potentiometrically in 0.05*M*-solution at 23 and 37°. At 23° the apparent p*K* was 6.95; at 37° it was about 6.75, but at the higher temperature the compound was not stable, and the p*K* values calculated are not as constant as those obtained from the curve at 23°. This result is in fairly close agreement with the values calculated by Winnek & Schmidt (1935) for the apparent dissociation constant of the diiodophenolic group of diiodotyrosine at different temperatures. These authors found that the apparent p*K* calculated from solubility determinations was 6.53 at 0°, 6.48 at 25° and 6.45 at 40°.

Formation of thyroxine during aerobic incubation of N-acetyl-L-diiodotyrosine. *N*-Acetyl-L-diiodotyrosine (9.5 g.) was suspended in 100 ml. water, and dissolved by adding 37 ml. *N*-NaOH; the initial pH was 7.6. After 14 days at 37° the reaction mixture was made 4*N* with respect to NaOH and boiled under reflux for 1.5 hr. The cooled solution was diluted with an equal volume of water and extracted twice with an equal volume of butanol. Purification of the butanol-soluble fraction in the usual manner (Harington & Pitt-Rivers, 1945) yielded 144 mg. pure thyroxine; 5.25 g. diiodotyrosine was recovered from the aqueous fraction. The net yield of thyroxine was therefore 4.7%.

Effect of pH on thyroxine formation. *N*-Acetyl-L-diiodotyrosine was dissolved in water and different amounts of alkali, the initial pH of each solution being calculated from the titration curve. After 14 days at 37°, the thyroxine formed and residual diiodotyrosine were separated as described above. The results are given in Table 1.

Table 1. *Formation of thyroxine from N-acetyl-L-diiodotyrosine incubated at different pH*

Acetyl-diiodotyrosine incubated (g.)	pH	Thyroxine isolated (mg.)	Diiodotyrosine recovered (g.)	Net yield of thyroxine (%)
7.13	7.15	70	2.67	2.0
6.5	7.25	122	2.73	4.3
9.5	7.45	162	5.58	5.9
9.5	7.70	144	5.25	4.7
9.5	8.0	84	6.96	4.2
9.5	8.35	74	6.72	3.4

Isolation of N-acetyl-L-thyroxine from the aerobic incubation products of N-acetyl-L-diiodotyrosine. *N*-Acetyl-L-diiodotyrosine (9.5 g.) was dissolved in 100 ml. 0.05*M*-borate buffer at pH 7.6 and 17.8 ml. 2*N*-NaOH; the initial pH was 7.6. After 2 days at 37° a crystalline sodium salt began to separate, and this slowly increased in amount. After 12 days' incubation, the sodium salt was separated on the centrifuge and washed with a little water. Decomposition of this salt with dilute HCl led to the isolation of a crystalline compound weighing 290 mg. Recrystallization by the method of Ashley & Harington (1929) gave a product which crystallized in long thin prisms and which had m.p. 232–234° (decomp.); $[\alpha]_D^{25} + 25.7$ in 4% solution in a mixture of equal volumes of ethanol and *N*-NaOH. (Found: I, 62.0. Calc. for *N*-acetylthyroxine, C₁₇H₁₃O₅NI₂: I, 62.0%.) From the mother liquor 8.25 g. *N*-acetyl-L-diiodotyrosine was recovered; the net yield of the acetylthyroxine was therefore 27%. Incubations of *N*-acetyl-L-diiodotyrosine in baryta solution at pH 7.6 led to the separation of the barium salt of *N*-acetyl-L-thyroxine in net yields of 15–20% calculated on the recovered starting material.

L-Thyroxine from *N*-acetyl-L-thyroxine

N-Acetyl-L-thyroxine (1.64 g.) was boiled under reflux for 1 hr. with 25 ml. acetic acid and 15 ml. conc. HCl. The solution was then concentrated to dryness, and the residue was dissolved in boiling 0.1*N*-Na₂CO₃ from which the sodium salt of thyroxine separated. After recrystallization, the thyroxine weighed 1.25 g. and had m.p. 232° (decomp.);

$[\alpha]_D^{21} - 5.40^\circ$ in 3.35% solution in two parts ethanol and one part π -NaOH. Since this rotation was considerably higher than that found for L-thyroxine by Harington & Pitt-Rivers (1945), it was thought that the L-thyroxine obtained above might be contaminated by a small amount of *N*-acetyl-L-thyroxine. A second hydrolysis with the same acid mixture was done for 2 hr. The L-thyroxine obtained had $[\alpha]_D^{21} - 5.42^\circ$ in 3.28% solution in the same solvent. The specific rotation of the L-thyroxine was then determined in 3.25% solution in a mixture of 24 g. 0.5 *N*-NaOH and 56 g. ethanol, and was found to be $[\alpha]_D^{17.5} - 4.62^\circ$. This is in good agreement with the value obtained by Foster, Palmer & Leland (1936) for L-thyroxine from the thyroid gland; these authors found $[\alpha]_D - 4.4^\circ$ in the same solvent.

Attempted identification of the side chain of diiodothyroxine which is eliminated during thyroxine synthesis

N-Acetyl-L-diiodothyroxine (96 g.) was dissolved in 1.12 l. dilute baryta solution containing 1.78 equiv. $\text{Ba}(\text{OH})_2/\text{mol}$. acetyldiiodothyroxine. The initial pH was 7.6 as measured by cresol red (British Drug Houses Ltd. capillator). After 3 weeks at 35° the barium salt of acetylthyroxine, which had separated, was collected on the filter, and after decomposition with acid 6.23 g. acetylthyroxine were obtained. The mother liquor was just acidified with dilute H_2SO_4 . Much unchanged acetyldiiodothyroxine was precipitated together with BaSO_4 , from which it was separated by solution in dilute alkali and reprecipitation with acid. The recovered acetyldiiodothyroxine weighed 63.0 g., whence the net yield of acetylthyroxine was nearly 22%. The filtrate was extracted with ether several times to remove some acetyldiiodothyroxine which has a significant water solubility. It was concentrated somewhat and made 2*N* with respect to H_2SO_4 and boiled under reflux for 1 hr. to hydrolyze any acetyl amino-acid present. The solution was then freed from H_2SO_4 with $\text{Ba}(\text{OH})_2$ and concentrated to about half its volume. Addition of Ag_2SO_4 in excess precipitated the inorganic iodide liberated by decomposition of acetyldiiodothyroxine during the incubation. Sulphate ions were again removed with $\text{Ba}(\text{OH})_2$, and the filtrate was concentrated to 200 ml. At this point, the HNO_3 reaction for *o*-diiodophenolic groups was positive, as was the ninhydrin reaction. It was therefore suspected that diiodothyroxine was present; so the solution was treated with excess lead acetate and allowed to stand for 12 hr. The bulky precipitate was then separated on the filter and the filtrate freed from Pb with H_2S . After concentration to a small volume, crystals separated which had m.p. $215-216^\circ$ (decomp.). These crystals gave positive ninhydrin, Millon and HNO_3 reactions. The mixed melting point with authentic L-monoiodothyroxine was not depressed. The presence of this amino-acid is attributed to incomplete iodination of the tyrosine from which the *N*-acetyl-L-diiodothyroxine was prepared. Since monoiodothyroxine is a fairly soluble amino-acid, it was thought that its removal would best be effected by its hydrogenation to tyrosine, which was accordingly done using palladized strontium carbonate as catalyst. After the reduction the solution was acidified with H_2SO_4 and freed from iodide with Ag_2SO_4 ; it was then freed from sulphate ions, and concentrated to about 15 ml. After several days in the ice chest 0.52 g. of tyrosine had separated. This was removed, and the mother liquor (16 ml.) was

examined by Dr R. L. M. Synge by paper chromatography. The predominant amino-acid was alanine; a little tyrosine was also present. Attempts to crystallize the alanine by concentrating the solution to a low volume and distilling in ethanol were unsuccessful.

In a second experiment, 26.85 g. *N*-acetyl-L-diiodothyroxine were incubated at 35° in 415 ml. baryta solution at pH 7.6 for 4 weeks. At the end of this time 1.69 g. acetylthyroxine were obtained, and after removal of unchanged starting material (15.6 g.) the filtrate was treated as in the first experiment. No monoiodothyroxine was detected. When the volume of the mother liquor had been concentrated to 25 ml., this was again examined by Dr Synge who found as before that alanine was the predominant amino-acid present. Attempts to isolate alanine as the azobenzenesulphonic acid salt also failed.

Experiments on the incubation products of N-acetyl-DL-diiodothyrosyl-L-glutamic acid

N-Acetyl-DL-diiodothyrosyl-L-glutamic acid. 2-Methyl-14(3':5'-diiodo-4'-acetoxybenzyl) oxazolone (Gell, Harington & Pitt-Rivers, 1946) (15.5 g.) was dissolved in 120 ml. acetone and mixed with a solution of glutamic acid (7.35 g.) in 100 ml. *N*-NaOH. After 12 hr. at room temperature 31 ml. 2*N*-NaOH were added to the solution to hydrolyze the *O*-acetyl group. After 1 hr. acetone was removed under diminished pressure, the solution was diluted with water and acidified with 3*N*-HCl; the product, which crystallized in small prisms, was recrystallized by dissolving in warm methanol and diluting with water to turbidity; the yield was 14.2 g. (76% of the theoretical). The compound had m.p. $212-214^\circ$ (decomp.). (Found: C, 30.8; H, 3.47; I, 40.9. $\text{C}_{16}\text{H}_{18}\text{O}_7\text{N}_2\text{I}_2 \cdot \text{H}_2\text{O}$ requires C, 30.9; H, 3.22; I, 40.8%.) On attempting to dehydrate this compound by drying *in vacuo* at 100° , about 5% of its weight was lost, and there was some decomposition.

The isolation of N-acetyl-DL-thyrosyl-L-glutamic acid from the incubation products of the above peptide. Some preliminary incubations were done on *N*-acetyldiiodothyrosylglutamic acid in dilute NaOH solution at pH 7.2 and hydrolysis of the reaction mixture yielded some thyroxine; however, no insoluble sodium salt of the thyroxine derivative separated during the incubations. *N*-acetyl-DL-diiodothyrosyl-L-glutamic acid (11.8 g.) was dissolved in 155 ml. 0.341 *N*-Ba(OH)₂; the pH was 7.4 as measured with indicators. After a few hours at 37° a partly crystalline barium salt had started to separate and this greatly increased in the course of time. After 4 weeks, the barium salt was collected by filtration, washed with water and decomposed with dilute HCl. The product (2.3 g.) was crystallized by dissolving in cold methanol and diluting with water to turbidity, and separated in minute prisms which had m.p. $190-200^\circ$ (decomp.). (Found: C, 27.8; H, 2.77; I, 52.5. *N*-acetylthyrosylglutamic acid, $\text{C}_{22}\text{H}_{26}\text{O}_9\text{N}_2\text{I}_2 \cdot \text{H}_2\text{O}$ requires C, 27.3; H, 2.3; I, 52.6%.) Attempts to dehydrate this compound at 100° led to some decomposition and loss of iodine. This thyroxine derivative does not form an insoluble sodium salt. Of *N*-acetyl-DL-diiodothyrosyl-L-glutamic acid 3.44 g. were recovered from the mother liquor, whence the net yield of the thyroxine derivative was 36%.

Acid hydrolysis of N-acetyl-DL-thyrosylglutamic acid. The peptide (1.93 g.) was boiled under reflux for 1.5 hr. with acetic acid (25 ml.) and conc. HCl (15 ml.). The solution was concentrated to dryness and the residue was extracted with

dilute HCl. The insoluble thyroxine was collected and purified in the usual manner. After crystallization the yield of thyroxine was 0.77 g. and it had m.p. 235° (decomp.). The filtrate was concentrated to a low volume and brought to pH 3.2 with NH_3 . After a short time, triangular prisms began to separate. After 24 hr. in the ice chest these were collected, and the mother liquor was concentrated when more of the same crystals separated. The total yield of this compound was 0.163 g. After crystallization from water the compound had m.p. 204° (decomp.). The mixed melting point with authentic L-glutamic acid was not depressed. (Found: C, 40.9; H, 6.0; N, 9.7. Calc. for $\text{C}_9\text{H}_9\text{O}_4\text{N}$: C, 40.85; H, 6.1; N, 9.5%.)

DISCUSSION

The isolation of the side chain of diiodotyrosine as a 2-carbon residue (oxalic acid), after the formation of 3:5-diiodo-4-hydroxybenzaldehyde during oxidation at high pH values, suggests immediately the conclusion that the first step in the degradation of the amino-acid side chain under these conditions entails oxidation on the β carbon atom, with the formation of 3:5-diiodo-4-hydroxyphenylserine. By analogy with the finding of Bettzieche (1925), that phenylserine is broken down in alkaline solution to give benzaldehyde and glycine, the above hydroxy acid would, under the same conditions, give 3:5-diiodo-4-hydroxybenzaldehyde and glycine.

Now it is a little difficult to believe that the glycine molecule thus produced would, under the mild oxidative conditions prevailing during aerobic incubation at 38°, be oxidized quantitatively to oxalic acid (cf. Dakin, 1905-6, on the formation of glyoxylic acid and oxalic acid by the action of hydrogen peroxide on glycine). It is, therefore, suggested that the 3:5-diiodo-4-hydroxyphenylserine undergoes further oxidation on the α or β carbon atom, and subsequent fission to the aldehyde, oxalic acid and ammonia:

With regard to the formation of 3:5-diiodo-4-hydroxybenzaldehyde during the preparation of artificial thyroproteins, this may be simply a function of the pH of the reaction. The absence of this aldehyde from the thyroid is exceedingly probable, but has not yet been demonstrated conclusively, since the sample of B.P. thyroid used for an attempted isolation had been defatted, and any aldehyde present would be removed by any fat solvent. The experiment with thyroid powder was done in order to find out whether the aldehyde obtained from the iodinated casein might be an artifact of alkaline hydrolysis from diiodotyrosine present in the protein, and the negative result with thyroid powder showed that this was not so. For other reasons, however, it is unlikely that the aldehyde is formed in the thyroid gland *in vivo*: first, because the pH of the tissues is below that at which aldehyde formation occurs during the incubation of diiodotyrosine; and secondly, because Harington & Randall (1929) were able to account for all the acid-insoluble iodine in the thyroid as being thyroxine iodine.

The formation of 2:4:6-triiodophenol from diiodotyrosine after hot peroxide oxidation calls for some comment. It has been found, during a repetition of Dakin's (1909) experiments on the formation of quinols from hydroxybenzaldehydes, that 3:5-diiodo-4-hydroxybenzaldehyde, while it gives no quinol with hydrogen peroxide, can, under vigorous conditions of oxidation, lose the formyl group with the production of 2:4:6-triiodophenol besides much free iodine and iodoform. No triiodophenol is formed from diiodotyrosine under mild oxidative conditions, which indicates that the triiodophenol obtained from diiodotyrosine during hot peroxide oxidation is a secondary breakdown product formed from the aldehyde; the formation of this compound is, therefore, not considered to have any importance in elucidating the mechanism of thyroxine synthesis.

The original suggestion by Harington & Barger (1927) that thyroxine was formed by the coupling of two molecules of diiodotyrosine was amplified first by Johnson & Tewkesbury (1942). These authors postulated that during incubation of diiodotyrosine under von Mutzenbecher's (1939) conditions hypoiodite is formed. This oxidizes one molecule of diiodotyrosine to a quinonoid intermediate possessing a

free radical, which unites with a further molecule of diiodotyrosine with univalent oxygen, the side chain being split off as pyruvic acid and ammonia. Later Harington (1944) developed a theoretical mechanism for thyroxine synthesis based on electronic considerations, and leading to the same type of intermediate as that suggested by Johnson & Tewkesbury (1942).

The chromatographic identification of small amounts of alanine among the reaction products obtained by acid hydrolysis of the mother liquors, after acetylthyroxine has been formed from acetyldiiodotyrosine, may lead to a different mechanism. If we suppose that this alanine is derived from the side chain which has been eliminated from one molecule of acetyldiiodotyrosine during the coupling, then the overall reaction may be graphically represented as a dismutation.

No oxidative process is required for this representation, although it is known (Harington & Pitt-Rivers, 1945) that thyroxine is not formed from diiodotyrosine under anaerobic conditions, and iodine is, in fact, liberated from acetyldiiodotyrosine during the reaction. This can be demonstrated by the presence of inorganic iodide in the reaction mixture, even in experiments where the net yields of acetylthyroxine are high and the recovered acetyldiiodotyrosine is free from tarry products (i.e. incubation in borate buffer).

The presence of alanine and the absence of serine (and glycine) during thyroxine synthesis recall the metabolism of tryptophan by *Escherichia coli*, elucidated by Dawes, Dawson & Happold (1947). These authors have identified the tryptophan side chain chromatographically as alanine when indole is formed by *Esch. coli* in the presence of the retarding agent mepacrine, and no serine has been found. It is interesting to speculate whether the two reactions, resulting in thyroxine on the one hand, and indole on the other may not follow the same metabolic

pathway, although the causative factor is different in the two cases, the former being chemical, the latter enzymic. Acetylalanine might, on the other hand, be obtained as a by-product of an entirely different reaction. If we assume that Johnson & Tewkesbury's (1942) mechanism of thyroxine synthesis is correct, the side chain would be eliminated from acetyldiiodotyrosine as pyruvic acid and acetamide. Now Knoop's theory (Knoop, 1910; Knoop & Kertess, 1911) to explain the formation of amino-acids from keto acids *in vivo* postulated the intermediate formation of an acylated amino-acid:

This theory was based on Knoop's own findings from *in vivo* experiments and was supported by the work of Erlenmeyer & Kunlin (1899, 1902) and de Jong (1900), who studied the *in vitro* reaction with many keto acids; in particular, when pyruvic acid and ammonium carbonate reacted together in aqueous solution, acetylalanine was formed in good yield. The theory was much later re-established by the work of du Vigneaud & Irish (1938) in experiments on the transformation in dogs of one amino-acid isomer into its enantiomorph through the intermediate keto acid.

If this be the source of acetylalanine in the reaction at present being studied, it must be assumed that the acetamide liberated during deamination of the side chain is hydrolyzed to acetic acid and ammonia, since Herbst (1939) has shown that the product of condensation of pyruvic acid and acetamide itself is of an entirely different nature. One experimental result, however, can decide upon the genesis of the alanine which has been detected: Acetylalanine obtained *in vitro* from pyruvic acid and ammonia would be optically inactive, whereas alanine split off as acetylalanine from *N*-acetyl-L-diiodotyrosine would be optically active, as is the *N*-acetylthyroxine formed. Efforts are now being made to isolate either acetylalanine or alanine itself in sufficient quantity to determine this point. Until this is done, the mechanism of the formation of alanine during thyroxine synthesis cannot profitably be discussed.

SUMMARY

1. 3:5-Diiodo-4-hydroxybenzaldehyde and oxalic acid have been isolated in equimolecular amounts after aerobic incubation of diiodotyrosine at pH 14. These findings are discussed.

2. The above aldehyde and thyroxine are formed together during aerobic incubation of diiodotyrosine at pH 10.

3. 2:4:6-Triiodophenol, the aldehyde and thyroxine are all formed during the hot peroxide oxidation of diiodotyrosine.

4. 3:5-Diiodo-4-hydroxybenzaldehyde is formed during the iodination of proteins to give products with thyroid-like activity; the aldehyde itself has no biological activity as measured by the tadpole metamorphosis test.

5. *N*-Acetyl-L-thyroxine is formed in substantial yield during aerobic incubation of *N*-acetyl-L-diiodotyrosine. Acid hydrolysis of the thyroxine derivative yields L-thyroxine.

6. *N*-Acetyl-DL-diiodotyrosylglutamic acid has been prepared. Aerobic incubation of this peptide leads to the formation of good yields of *N*-acetyl-

DL-thyroxylglutamic acid which, on acid hydrolysis, yields thyroxine and glutamic acid.

7. Alanine has been chromatographically identified among the reaction products obtained during thyroxine synthesis from diiodotyrosine. This finding is briefly discussed.

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The Steroids of Pregnant Mares' Urine

1. A METHOD FOR THE EXTRACTION OF STEROID SULPHATES AND THE ISOLATION OF *allo*PREGN-16-EN-3(β)-OL-20-ONE SULPHATE*

By W. KLYNE, B. SCHACHTER AND G. F. MARRIAN
Departments of Biochemistry, Universities of Toronto and Edinburgh

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In a previous paper (Schachter & Marrian, 1938) dealing with the isolation of oestrone sulphate from the urine of pregnant mares, the isolation from the same source of what appeared to be the sulphate of a non-phenolic steroid was briefly noted. Fuller details were not reported at that time since concordant analyses upon the new sulphate and the parent steroid could not be obtained, and since there

was some evidence to suggest that the material isolated might be a mixture of closely related sulphates varying in composition from batch to batch. More recently, it has been possible to commence a reinvestigation of the conjugated steroids in pregnant mares' urine, and in the present paper the methods used for the extraction and concentration of a steroid-sulphate fraction and the isolation and identification of one pure component of this fraction are described.

* For preliminary accounts of this work see *Biochem. J.* 1945, **39**, xlv; 1946, **40**, lv; 1948, **42**, i.