The enantiomorphs showed identical x-ray powder diffraction patterns.[14]

(14) We are indebted to Dr. A. V. Guzzo of this laboratory for the x-ray comparison.

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Reaction of 4-Hydroxy-3,5-diiodophenylpyruvic Acid with 3,5-Diiodotyrosine

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4-Hydroxy-3,5-diiodophenylpyruvic acid readily reacts with 3,5-diiodotyrosine and with *N*-acetyl-3,5-diiodotyrosine to give thyroxine and *N*-acetylthyroxine, respectively. The presence of an oxidizing agent is required.

In 1939 von Mutzenbecher[1] reported that incubation of weakly basic solutions of 3,5-diiodotyrosine at 38° gave rise after a few days to small amounts of thyroxine. Part of the interest in this reaction has been based on the reaction's potential for the synthesis of thyroxine and its analogs; part of the interest has been based on the speculation that knowledge of the *in vivo* formation of thyroxine might be gained by an understanding of this *in vitro* reaction.

Among the postulates on the course of the reaction has been the suggestion that a molecule of 3,5-diiodotyrosine is first converted to 4-hydroxy-3,5-diiodophenylpyruvic acid. This is then followed by reaction of such a molecule with another molecule of 3,5-diiodotyrosine to form thyroxine.

Hillmann[2] investigated the possibility of such a coupling and reported that under his unspecified conditions, a yield of about 3% was obtained when 4-hydroxy-3,5-diiodophenylpyruvic acid was allowed to react with either 3,5-diiodotyrosine or *N*-acetyl-3,5-diiodotyrosine. Interestingly, Hillmann reported that oxygen was detrimental to the reaction. Since it had already been shown[3] that oxygen was necessary for the conversion of 3,5-diiodotyrosine to thyroxine, Hillmann's work implied that the conversion of 3,5-diiodotyrosine to 4-hydroxy-3,5-diiodophenylpyruvic acid occurred by oxidation, but that the subsequent coupling did not entail oxidation. The subsequent report by Pitt-Rivers and James[4] on the isolation of ϵ-*N*-hydroxypyruvyl-α-acetyllysine from an incubation of ϵ-*N*-(N-acetyl-3,5-diiodotyrosyl)-α-*N*-acetyllysine, strengthened our opinion that the coupling step was an oxidative process. We, therefore, decided to carry out a coupling of 3,5-diiodotyrosine with 4-hydroxy-3,5-diiodophenylpyruvic acid.

When we added 4-hydroxy-3,5-diiodophenylpyruvic acid to 3,5-diiodotyrosine in a borate buffered (*p*H 7.8) solution kept saturated with oxygen, within a very short time, we obtained 10% yields of crude thyroxine. If either the pyruvic acid or the oxygen was omitted, no product was obtained. When the addition of the 4-hydroxy-3,5-diiodophenylpyruvic acid was made in small portions or dropwise, as a 1-butanol solution, and when a second phase such as 1-butanol, chloroform, carbon tetrachloride, or toluene was present, yields of crude thyroxine well in excess of 20% could be obtained. The instability of 4-hydroxy-3,5-diiodophenylpyruvic acid under the conditions of the reaction, and the need for sufficient oxygen to be present for the occurrence of the desired reaction, are probably the reasons for the increased yield on the portionwise addition of the pyruvic acid. Because of the instability of the 4-hydroxy-3,5-diiodophenylpyruvic acid in solution and the resulting losses, an excess was desirable. The maximum yield was obtained when approximately 40% excess of 4-hydroxy-3,5-diiodophenylpyruvic acid was used.

Some oxidants other than oxygen were also tried. Potassium ferricyanide in the place of oxygen, or in addition to oxygen, was unsatisfactory. Iodic acid at *p*H 6.5–7, however, could take the place of oxygen, although the yield was decreased. Similarly, hydrogen peroxide or *t*-butyl peroxide could be used in place of oxygen, but again at the expense of some of the yield. When, however, *t*-butyl peroxide was used in catalytic amounts in addition to oxygen, yields of almost 30% were obtainable.

The literature reports[5] that incubations in which *N*-acetyl-3,5-diiodotyrosine alone is used, usually

(1) P. von Mutzenbecher, *J. physiol. Chem.*, **261**, 253 (1939).

(2) G. Hillmann, *J. Naturforschung*, **11b**, 474 (1956).

(3) C. R. Harington and R. V. Pitt-Rivers, *Biochem. J.*, **39**, 157 (1945).

(4) R. V. Pitt-Rivers and A. T. James, *Biochem. J.*, **70**, 173 (1958).

(5) R. V. Pitt-Rivers, *Biochem. J.*, **43**, 223 (1948).

result in better yields than when 3,5-diiodotyrosine itself is used. In our work with 4-hydroxy-3,5-diiodophenylpyruvic acid, however, the *N*-acetyl-3,5-diiodotyrosine did not give as good yields as the unacetylated compounds. This could be interpreted to mean that in the usual incubation using only 3,5-diiodotyrosine or *N*-acetyl-3,5-diiodotyrosine, the *N*-acetyl derivative is so much more satisfactory for conversion to the pyruvic acid that it more than compensates for the subsequently poorer coupling step.

In these laboratories we found that the presence of different salts had various effects on the yields of incubations and that the direction of the effects varied with the particular diiodotyrosine derivative or analog employed. Thus, manganese sulfate gave better yields than magnesium sulfate or sodium sulfate when *N*-acetyl-3,5-diiodotyrosine was used. When 4-hydroxy-3,5-diiodophenylacetic acid was used, manganese sulfate gave only 4-hydroxy-3,5-diiodobenzaldehyde, whereas magnesium and sodium sulfates gave 3,5-diiodo-4(4′-hydroxy-3′,5′-diiodophenoxy)phenylacetic acid.

In the coupling of 4-hydroxy-3,5-diiodophenylpyruvic acid with 3,5-diiodotyrosine or *N*-acetyl-3,5-diiodotyrosine, sodium and magnesium sulfate, when present to the extent of 4–5% in the aqueous phase, were alike in their effect on the yield. Manganese sulfate was tried only with *N*-acetyl-3,5-diiodotyrosine and proved less satisfactory than either sodium or magnesium sulfate.

Incubation of 3,5-diiodotyrosine alone gave an optimum yield at a higher *p*H (*p*H 10) than did incubation of *N*-acetyl-3,5-diiodotyrosine (*p*H 7.5).[3] In reactions using 4-hydroxy-3,5-diiodophenylpyruvic acid, the yield at *p*H 7.4–7.9 was better than at 6.4–6.7 or at 9.7–10.2. Poor solubility of 3,5-diiodotyrosine at low *p*H values, and alkaline instability of 4-hydroxy-3,5-diiodophenylpyruvic acid, limit the possibility of much *p*H variation.

Temperature variation between 4° and 50° had little effect in our work in contrast to literature reports on incubations.[6] Again, if one accepts that the von Mutzenbecher reaction proceeds *via* a pyruvic acid intermediate, this would suggest that the conversion to the pyruvic acid is the temperature dependent part of the reaction.

Under the conditions of the reaction, 4-hydroxy-3,5-diiodophenylpyruvic acid formed the diphenyl ether not only with 3,5-diiodotyrosine and with *N*-acetyl-3,5-diiodotyrosine but also with 4-hydroxy-3,5-diiodobenzoic acid, with 4-hydroxy-3,5-diiodophenylacetic acid, and with 4-hydroxy-3,5-diiodophenylpropionic acid. These latter reactions were not studied in depth. The crude products were identified by paper partition chromatography and small purified samples were identified by mixed melting points with authentic samples.

(6) P. Z. Anthony, D. R. Borgen, and L. G. Ginger, U. S. Patent 2,803,654, August 20, 1957.

The limitations of the structure required for reaction of 4-hydroxy-3,5-diiodophenylpyruvic acid are still not known. It should be noted, however, that 4-hydroxyphenylpyruvic acid is much more stable than the iodinated compound, and that neither 4-hydroxyphenylpyruvic acid nor 3,5-diiodo-4-methoxyphenylpyruvic acid reacts to give diphenyl ethers.

4-Hydroxy-3,5-diiodophenylpyruvic acid was prepared in two ways. The first procedure used was based on the method of Bergmann, Zervas, and Lebrecht for the preparation of α-keto acids.[7] 3,5-Diiodotyrosine was *N*-chloroacetylated and then treated with acetic anhydride and pyridine to give the desired product after hydrolysis. A more satisfactory procedure, however, is that of Tong, Taurog, and Chaikoff,[8] who hydrolyzed the azlactone obtained by condensation of 4-hydroxy-3,5-diiodobenzaldehyde with aceturic acid. The procedure of Matsuura and Cahnmann[9] for the preparation of 4-hydroxy-3,5-diiodobenzaldehyde, however, was superior to the method used by Paal.[10]

EXPERIMENTAL[11]

N-Chloroacetyl-3,5-diiodotyrosine[12] was prepared according to the literature in 98% yield based on unrecovered 3,5-diiodotyrosine. Our crude material, satisfactory for further use, melted at 212–214°, reported 221° dec.

4-Hydroxy-3,5-diiodobenzaldehyde[9] was prepared according to the literature in 94% yield. Our crude material suitable for further use, melted at 196–198°, reported 199–200°.

4-(4-Acetoxy-3,5-diiodobenzal)-2-methyl-5-oxazolone. (a)[13] A mixture of 60 g. (0.162 mole) of 4-hydroxy-3,5-diiodobenzaldehyde, 150 ml. of acetic anhydride, 19.5 g. (0.162 mole) of aceturic acid, and 13.5 g. (0.162 mole) fused sodium acetate was heated on a steam bath for 2 hr. The reaction mixture was cooled and allowed to stand in an ice chest overnight. The precipitate was filtered, washed well with water and then with petroleum ether (b.p. 60–71°). The product was recrystallized from a mixture of benzene and petroleum ether to give 56 g. (69%) of product which melted at 231–233°. The crude material is reported[8] to melt at 200–210°.

Anal. Calcd. for $C_{13}H_9O_4NI_2$: C, 31.41; H, 1.82, Found: C, 31.47; H, 2.07.

(b)[7] *N*-Chloracetyl-3,5-diiodotyrosine (51 g., 0.1 mole) was dissolved in 250 ml. of pyridine. Acetic anhydride (500 ml.) was added dropwise while keeping the temperature between 15° and 20°. After addition was completed, the reaction mixture was stirred for 45 min. and then poured onto ice. The resultant precipitate was collected and used without further purification or drying.

4-Hydroxy-3,5-diiodophenylpyruvic acid.[8] (a) The above product (b), 600 ml. of acetic acid and 300 ml. of 6*N* hydrochloric acid, were kept at reflux for 6 hr. The reaction mixture

(7) M. Bergmann, L. Zervas, and F. Lebrecht, *Ber.*, **64**, 2315 (1931).

(8) W. Tong, A. Taurog, and I. L. Chaikoff, *J. Biol. Chem.*, **207**, 59 (1954).

(9) T. Matsuura and H. J. Cahnmann, *J. Am. Chem. Soc.*, **81**, 871 (1959).

(10) C. Paal, *Ber.*, **28**, 2407 (1895).

(11) Melting points were taken on a Fisher-Johns melting point block and temperatures are uncorrected.

(12) E. Ronwin, *J. Org. Chem.*, **18**, 1546 (1953).

(13) E. L. Bennett and E. Hoerger, *J. Am. Chem. Soc.*, **74**, 5975 (1954).

was allowed to stand overnight and the resultant precipitate collected and recrystallized from acetic acid to give 16 g. of product melting at 238–241°. The melting point varies greatly with rate of heating. The reported melting point is 215–220° dec. with vigorous evolution of gas. The over-all yield for the two steps was 37%.

Anal. Calcd. for $C_9H_6O_4I_2$; C, 25.02; H, 1.40; I, 58.76. Found: C, 24.96; H, 1.41; I, 59.22.

(b) A solution of 40 g. (80 mmoles) of purified azlactone in 1600 ml. of acetic acid and 400 ml. of 6*N* hydrochloric acid was kept at reflux for 4 hr. and then placed in an ice chest for 48 hr. The precipitate was collected, triturated with petroleum ether and placed in a desiccator over the weekend. Final drying at 78° gave 27.7 g. (80%) of the acid which melted at 240–242°.

3,5-Diiodoanisaldehyde[14] was prepared according to the literature from 4-hydroxy-3,5-diiodobenzaldehyde in over-all yield of 25%.

4(4-Methoxy-3,5-diiodobenzal)-2-methyl-5-oxazolone. A mixture of 13.5 g. (35 mmoles) of the above product, 4.1 g. (35 mmoles) of aceturic acid, 35 ml. of acetic anhydride, and 2.9 g. (35 mmoles) of fused sodium acetate was heated on a steam bath for 2 hr. The reaction mixture was then cooled in an ice box overnight. A small amount of cold acetic acid was added to the thick precipitate. The filtered product, after recrystallization from benzene and petroleum ether weighed 7.8 g. (48%) and melted at 169.5–171°.

Anal. Calcd. for $C_{12}H_9O_3NI_2$: C, 30.73; H, 1.93; I, 54.12. Found: C, 30.77; H, 2.06; I, 54.40.

4-Methoxy-3,5-diiodophenylpyruvic acid. A solution of 7.0 g. (14.9 mmoles) of the above azlactone, 300 ml. of acetic acid, and 75 ml. of 6*N* hydrochloric acid was kept at reflux for 4 hr. The reaction mixture was then allowed to stand at room temperature overnight and then cooled in an ice bath. The filtered product weighed 6.2 g. (93% yield) and melted at 192–193°.

Anal. Calcd. for $C_{10}H_8O_4I_2$: C, 26.93; H, 1.81; I, 56.91. Found: C, 26.95; H, 1.83; I, 56.95.

Thyroxine. (a) The *p*H of a suspension of 2.17 g. (4.64 mmoles) of L-3,5-diiodotyrosine dihydrate, 50 ml. of 0.2*M* borate buffer solution (*p*H 7.6), 17.5 ml. of saturated solution of sodium sulfate, and 17.5 ml. of 1*N* sodium hydroxide was adjusted to 7.6 by the addition of 4*N* hydrochloric acid and 25 ml. of chloroform was added. Oxygen was vigorously bubbled through the mixture, while 1.8 g. (4.16 mmoles) of 4-hydroxy-3,5-diiodophenylpyruvic acid was added in small portions (*ca.* 80 mg.) at 5-min. intervals. During the addition, vigorous stirring was maintained and the *p*H of the reaction mixture was kept between 7.4 and 7.8 by the addition of 2*N* sodium hydroxide. Addition of the pyruvic acid required 1.75 hr. A slight temperature rise resulted from the heat generated by the magnetic stirrer.

After completion of the addition, the mixture was stirred for an additional 10–15 min. The chloroform was removed under water pump vacuum. The residue was filtered to give 750 mg. of crude product. On circular paper partition chromatography using 6*N* ammonia-*t*-amyl alcohol, this material showed itself to consist almost solely of thyroxine. This represents a yield of 23% based on the pyruvic acid used.

When the ratio of reactants of 4.6 mmoles of 3,5-diiodotyrosine to 6.5 mmoles of pyruvic acid was used in this type of experiment, the yield based on pyruvic acid did not change but the yield based on 3,5-diiodotyrosine was increased to 36%.

(b) A mixture of 2.17 g. (4.64 mmoles) of L-3,5-diiodotyrosine dihydrate, 50 ml. of 0.2*M* borate buffer (*p*H 7.6), 17.5 ml. of 1*N* sodium hydroxide, and 17.5 ml. of saturated solution of sodium sulfate was adjusted with 4*N* hydrochloric acid to *p*H 7.6. A solution of 0.1 g. of *t*-butyl hydroperoxide (Union Bay State Co., Cambridge, Mass.) (available active oxygen 10%) was added in 10 ml. of 1-butanol and oxygen gas was bubbled through the rapidly stirred mixture, accompanied by the dropwise addition of a solution of 2.8 g. (6.5 mmoles) of the 3,5-diiodo-4-hydroxyphenylpyruvic acid in 50 ml. of 1-butanol. Addition required about 90 min. and the *p*H of the reaction mixture was kept between 7.4 and 7.8 by addition of 2*N* sodium hydroxide. The temperature rose slightly due to heat effect of the magnetic stirrer.

The reaction mixture was allowed to stir an additional 10 min. after completion of the addition of the pyruvic acid. The 1-butanol layer was removed under water pump vacuum and the volume restored by addition of water. The suspended precipitate was filtered and washed with water. The precipitate was triturated with 4*N* hydrochloric acid, filtered, washed with water and dried to give a crude yield of 1.05 g. of L-thyroxine. Circular paper partition chromatography showed only slight contamination with 3,5-diiodotyrosine.

This represents a yield of 29% based on 3,5-diiodotyrosine. When a ratio of reactants of 4.64 mmoles of 3,5-diiodotyrosine to 2.3 mmoles of 4-hydroxy-3,5-diiodophenylpyruvic acid was used in this type of experiment, the yield was 29% based on the pyruvic acid.

An idea of the purity and further proof of identity of the crude product was obtained by conversion to 3,5-diiodo-L-thyronine.[15] The yield (80–85%) was the same as is ordinarily obtained from commercial sodium thyroxine. The melting point, paper partition chromatography, and optical rotation were also satisfactory.

(c) A mixture of 2.17 g. (4.64 mmoles) of 3,5-diiodotyrosine dihydrate, 17.5 ml. of 1*N* sodium hydroxide, 17.5 ml. of saturated sodium sulfate solution, and 50 ml. of 0.2*M* borate buffer was adjusted from *p*H *ca.* 10 to *p*H 7 by hydrochloric acid. The addition of 1 g. (4.7 mmoles) of potassium iodate was followed by the dropwise addition of 1 g. (2.3 mmoles) of 4-hydroxy-3,5-diiodophenylpyruvic acid in 30 ml. of 1-butanol over a period of 90 min. The *p*H was maintained at 6.5–7. The mixture was vigorously stirred but no oxygen was passed through the reaction mixture. An hour after the addition was completed, the solution (made basic, *p*H 10.5) was diluted with an equal volume of water and extracted with 1-butanol. Evaporation of the washed 1-butanol extract, gave 370 mg. of material. By circular paper partition chromatography and a ninhydrin spray, thyroxine was shown to be the only amino acid present and was present in fairly pure state.

N-Acetyl-L-thyroxine. In preparations such as described under the thyroxine preparation (a) yields of about 10% were obtained. The presence or absence of chloroform did not greatly affect the yield and neither did a lower *p*H of 6.4–6.6. The product was identified by circular paper partition chromatography and by conversion in 80% yield to 3,5-diiodo-L-thyronine by treatment with hydriodic acid in acetic acid. The 3,5-diiodo-L-thyronine was identified by circular paper partition chromatography and had a satisfactory optical rotation. The solvent used in the chromatography was 6*N* ammonia-*t*-amyl alcohol.

MORRIS PLAINS, N. J.

(14) J. H. Wilkinson, *J. Chem. Soc.*, 2370 (1949).

(15) NOTE ADDED IN PROOF. The hydriodic acid conversion of the tetraiodo compounds to the diiodo compounds was similar to that recently revealed by P. Z. Anthony, U. S. Patent 2,950,315. In our work acetic acid was added as solvent.