[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

Model Reactions for the Biosynthesis of Thyroxine. I. Structural Influence of the Side Chain in Analogs of Diiodotyrosine on their Conversion to Analogs of Thyroxine[1]

BY TERUO MATSUURA[2] AND H. J. CAHNMANN

RECEIVED AUGUST 21, 1958

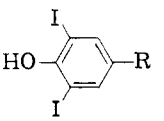
In a search for a simple model reaction that would permit the study of the reaction mechanism of the biosynthesis of thyroxine, the non-enzymic conversion of a number of desamino side chain analogs of 3,5-diiodotyrosine to the corresponding analogs of thyroxine under mild conditions (*p*H 7.5, 37°) has been investigated. The propionic acid analog (diiododesaminotyrosine) was found to be the best model substance for this reaction.

Introduction

A complete elucidation of the biosynthetic mechanism by which two molecules of 3,5-diiodotyrosine combine to form thyroxine cannot be expected as long as the enzyme systems involved remain unknown. It is, however, possible to test by means of simple non-enzymic model reactions the validity of some of the proposed hypotheses[3-8] concerning the reaction mechanism.

Such a model reaction was provided by the discovery that a small amount of thyroxine is formed *in vitro* when an alkaline solution of diiodotyrosine is incubated at 37° in the absence of enzymes.[9] Unfortunately, diiodotryrosine is a poor model substance. It is practically insoluble at a physiological *p*H. At *p*H 8 where most model reactions were carried out, the yield of thyroxine is only about 0.2% of the theory.[3,9-11] The *p*H optimum was found to be 10.[12] A much better model substance was found in N-acetyl-3,5-diiodotyrosine. The *p*H optimum for its conversion to N-acetylthyroxine is about 7.5 and the yield of thyroxine is 2–3%.[13] This considerable increase in yield when the free amino group of diiodotyrosine is blocked prompted us to investigate the formation of analogs of thyroxine in the *in vitro* incubation of a number of diiodotyrosine analogs which do not contain an amino group. Although relatively poor yields of analogs of thyroxine have been reported in the incubation of desamino analogs of diiodotyrosine (less than 1% for the acetic and propionic acid analogs[14] and less than 2% for the lactic acid analog),[15] it was thought that a systematic investigation of such compounds might lead to a better model substance for the further study of the biosynthesis of thyroxine, and at the same time permit the determination of the structural requirements for the *in vitro* conversion of an analog of diiodotyrosine in the corresponding analog of thyroxine.

Solutions of the 3,5-diiodotyrosine analogs Ia through Ih and of N-chloroacetyl-3,5-diiodo-L-tyrosine (Ii), which served as a reference substance, were incubated aerobically at 37°.



Ia, R = COOH
Ib, R = CH_2COOH
Ic, R = $(CH_2)_2COOH$
Id, R = $(CH_2)_3COOH$
Ie, R = $CH_2CH(CH_3)COOH$
If, R = $CH_2CH(C_6H_5)COOH$
Ig, R = $CH{=}CHCOOH$
Ih, R = $CH_2CH(OH)COOH$
Ii, R = $CH_2CH(NHCOCH_2Cl)COOH$

The initial *p*H was adjusted with sodium hydroxide to about 7.5 or slightly higher whenever the starting material was not completely soluble at *p*H 7.5. After 4–7 days (32 days in the case of Ia) the reaction mixtures were analyzed for the corresponding analogs of thyroxine as well as for other reaction products. The following analytical methods were used: elemental analysis and infrared spectroscopy whenever enough material could be isolated, and paper chromatography and high voltage paper electrophoresis for small amounts of reaction products. The methods of working up the incubation mixtures varied depending on the behavior of the various starting materials during the incubation (see Experimental Part).

Methods

Melting points were taken in capillary tubes and are uncorrected. Infrared spectra were determined in a Perkin-Elmer recording spectrophotometer, model 21, equipped with sodium chloride optics.

High voltage electropherograms were obtained with a Wieland-Pfleiderer perograph[16] in an electric field of about 75 volts/cm. A buffer of *p*H 6.5 (pyridine–acetic acid–water, 10:1:89) was used.

Chromatograms were made using both the ascending and the descending method (Whatman paper 3 MM.). Chromatographic solvents were: (1) 1-butanol–2 *N* ammonia; (2) 1-butanol–dioxane–2 *N* ammonia (4:1:5); (3) *t*-amyl alcohol–2 *N* ammonia; (4) 1-butanol–water; (5) 1-butanol–butanol–pyridine–water (12:1:12). These solvents form two phases. The upper phase was used; a beaker containing some of the lower phase was placed in the bottom of the tank. The following color reagents were used for the detection of substances on paper chromatograms and on pherograms: (1) diazotized N^1,N^1-diethylsulfanilamide. The paper was first sprayed with a solution prepared according to Winikoff and Trikojus,[17] then with a 2.5% solution of Na_2CO_3. (2) 2,4-Dinitrophenylhydrazine. The paper was first sprayed with a saturated solution in 1 *N* HCl, then with 2 *N* NaOH. Reagent 1 was used for the detection of phenolic compounds, reagent 2 for the detection of ketonic compounds. Chromatographic and electrophoretic spots

(1) A preliminary report of part of this work was made recently (*Biochem. Biophys. Acta*, **29**, 216 (1958)).

(2) Visiting Scientist from Osaka City University, Japan.

(3) T. B. Johnson and L. B. Tewkesbury, Jr., *Proc. Nat. Acad. Sci., U. S.*, **28**, 73 (1942).

(4) C. R. Harington, *J. Chem. Soc.*, 193 (1944).

(5) A. Neuberger, *Ann. Rev. Biochem.*, **18**, 243 (1949).

(6) S. Bouchilloux, D. Kertesz and S. Lissitzky, *Compt. rend. soc. biol.*, **150**, 399 (1956).

(7) G. Hillman, *Z. Naturforsch.*, **11b**, 424 (1956).

(8) J.-G. Lunggren, *Acta Chem. Scand.*, **11**, 1072 (1957).

(9) P. von Mutzenbecher, *Z. physiol. Chem.*, **261**, 253 (1939).

(10) P. Block, *J. Biol. Chem.*, **135**, 51 (1940).

(11) A. E. Barkdoll and W. F. Ross, THIS JOURNAL, **66**, 898 (1944).

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

(13) R. Pitt-Rivers, *ibid.*, **43**, 223 (1948).

(14) E. Frieden, H. M. Walborsky and J. E. McRae, *Science*, **125**, 887 (1957).

(15) J. A. Saul and V. M. Trikojus, *Biochem. J.*, **42**, 80 (1948).

(16) T. Wieland and G. Pfleiderer, *Angew. Chem.*, **67**, 257 (1955).

(17) D. Winikoff and V. M. Trikojus, *Biochem. J.*, **42**, 475 (1948).

were identified by means of authentic samples. Reference substances not commercially available were synthesized.

All commercially available starting materials were checked for purity by paper chromatography in different solvents.

Solutions to be incubated were prepared by suspending the analog of diiodotyrosine in about 2 ml. of water per mmole of substance and adding the minimum amount of 1 *N* NaOH required for complete dissolution, then 1 *N* HCl until the *p*H was about 7.5 or until a faint cloudiness appeared,[18] and finally enough water to obtain a 0.24–0.25 *M* solution. The incubations were carried out at 37° in loosely covered, partially filled Erlenmeyer flasks or culture bottles to provide a large liquid surface. Changes of the *p*H were recorded from time to time.

Experimental[19]

Incubation of 3,5-Diiodo-4-hydroxybenzoic Acid (Ia).—A solution of 39.0 g. (0.1 mole) of Ia (Eastman Kodak) was incubated. After one week, crystals began to appear. The incubation period was therefore extended to 22 days, after which time the reaction mixture (*p*H 8.3) was cooled in an ice-bath, then filtered; 0.23 g. of crystals. After two days' standing at 2°, the filtrate was divided into two equal portions. One of these was adjusted to *p*H 7.5 with 1 *N* HCl; then both portions were again incubated for another 10 days. As no more crystals had formed, both portions (*p*H 8.0 and 8.5) were recombined and extracted three times with *n*-butanol[20] after the addition of enough NaOH to make the solution about 1 *N* with respect to NaOH. The combined extracts were washed with 1 *N* NaOH and with water, then dried over Na_2SO_4 and evaporated *in vacuo*. Upon acidification of a hot ethanolic solution of the residue with dil. HCl crystals formed; yield 0.35 g. These were dissolved in hot dil. ethanol together with the 0.23 g. of crystals obtained before. The solution was decolorized with Norit A. Upon cooling, white needles, m.p. 159–160°, were obtained which showed no m.p. depression with 2,4,6-triiodophenol.

Anal. Calcd. for $C_6H_3I_3O$: C, 15.27; H, 0.64; I, 80.69. Found: C, 15.89; H, 0.83; I, 80.37.

The infrared spectrum (KBr) was identical to the one of authentic 2,4,6-triiodophenol.

Chromatography of the mother liquor from the 0.35 g. of crude triiodophenol (solvents 1, 2, 3) gave a strong yellowish brown and a weak purple spot. R_F-values and colors were identical with those obtained with authentic samples of 2,4,6-triiodophenol and of 3,5,3′,5′-tetraiodothyroformic acid, respectively.[21] Acidification of the combined aqueous layers obtained in the butanol extraction yielded 36.5 g. of crude starting material.

3,5-Diiodo-4-hydroxyphenylacetic Acid (Ib).—A solution of 15.2 g. (0.12 atom) of iodine and 15 g. of potassium iodide in 50 ml. of water was added during 20 minutes to a stirred solution of 4.56 g. (0.03 mole) of 4-hydroxyphenylacetic acid (Aldrich Chemical Co.) in 66 ml. of aqueous (20%) methylamine. Stirring was continued for another 30 minutes. Acidification with dil. HCl yielded 11.9 g. (98.2%) of 3,5-diiodo-4-hydroxyphenylacetic acid. After recrystallization from ethyl acetate and from aqueous ethanol; white needles, m.p. 219° dec. were obtained; lit.[22] m.p. 218–219°.

Incubation of 3,5-Diiodo-4-hydroxyphenylacetic Acid.—A solution of 2.02 g. (5 mmoles) of Ib was incubated. After a few hours, a crystalline precipitate of crude sodium 3,5,3′,5′-tetraiodothyroacetate began to form and the solution turned brown. After 5 days the precipitate was filtered and washed with a small amount of ice-cold water; yield 110 mg. (5.8%). The free acid obtained by acidification with dil. HCl was recrystallized from benzene containing a small amount of ethanol; white needles, m.p. 228–230° dec. (m.p. of authentic 3,5,3′,5′-tetraiodothyroacetic acid 229–230°; mixed m.p. 228–230°).

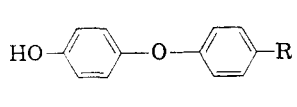
Anal. Calcd. for $C_{14}H_8I_4O_4$: C, 22.48; H, 1.08; I, 67.89. Found: C, 22.65; H, 1.40; I, 67.96.

The infrared spectrum (KBr) was identical to the one of authentic 3,5,3′,5′-tetraiodothyroacetic acid. Chromatography (solvents 1–5) also proved the identity of the two substances.

Chromatography (solvents 1, 2, 4, 5) of the filtrate from the sodium tetraiodothyroacetate after acidification followed by dilution with ethanolic ammonia revealed three major spots, two of them red and one yellow. The red spots had the same R_F values as 3,5-diiodo-4-hydroxyphenylacetic acid and 3-iodo-4-hydroxyphenylacetic acid, respectively. In order to identify the yellow spot, the solution was acidified and the precipitate formed removed by filtration. Continuous extraction of the filtrate with ether, followed by evaporation of the ether extract, yielded an oily residue consisting mainly of the material giving a yellow spot on chromatograms. It was identified by high voltage electrophoresis as 3,5-diiodo-4-hydroxyphenylglycolic acid containing a small amount of 3,5-diiodo-4-hydroxyphenylglyoxylic acid and traces of other unidentified substances. Some 3,5-diiodo-4-hydroxyphenylglycolic acid was also found to be present in the precipitate. Repeated crystallizations of the precipitate from ethyl acetate and from isooctane–ethyl acetate led to its enrichment in the final mother liquors. Chromatography (solvents 1, 2) of the more soluble crystalline fractions obtained in these fractional crystallizations and spraying with color reagent 2 revealed the presence of 3,5-diiodo-4-hydroxybenzaldehyde.[23]

3,5-Diiodo-4-hydroxyphenylglyoxylic Acid.—A solution of 3.25 g. (0.02 mole) of iodine monochloride in 5 ml. of 20% HCl was added within 5 minutes to a stirred solution of 1.66 g. (0.01 mole) of *p*-hydroxyphenylglyoxylic acid[24] in 14 ml. of 20% HCl and 120 ml. of water.[25] Stirring was continued for another 2 hr. The precipitate formed was filtered and washed with water (3.53 g.). It was combined with a second crop (0.30 g.) separating from the filtrate on standing overnight (92% yield). Recrystallization from water yielded almost white crystals, m.p. 164–166° dec., which on standing decomposed and turned brown. Paper chromatography (solvents 2, 5) and high voltage electrophoresis revealed the presence of a contaminant, probably a degradation product as its amount increased when the crystals were permitted to stand several days at room temperature in an open container.

3,5-Diiodo-4-hydroxyphenylglycolic Acid.—A reduction of 2.5 g. (0.015 mole) of *p*-hydroxyphenylglyoxylic acid[24] with sodium amalgam was carried out as described in the literature.[26] The reaction mixture was neutralized (congo-violet) with concd. HCl, then 10 ml. of 20% HCl and 18 ml. of water was added. A solution of 4.9 g. (0.030 mole) of iodine monochloride in 10 ml. of 20% HCl was then added dropwise with stirring. After about 15 minutes needles began to separate. After 3.5 hr., the mixture was put in the refrigerator (2°). The crystals formed overnight were filtered off and dried; yield 5.37 g. (86%); m.p. 148–151°.



IIa, R = COOH
IIb, R = CH_2COOH
IIc, R = $(CH_2)_2COOH$
IId, R = $(CH_2)_3COOH$
IIe, R = CH=CHCOOH
IIf, R = $CH_2CHOHCOOH$

(18) β-(3,5-Diiodo-4-hydroxyphenyl)-D,L-lactic acid was completely soluble at *p*H 6.5. In this case the *p*H was adjusted to 7.5 with more NaOH.

(19) The microanalyses were made by Dr. W. C. Alford and his associates of the Analytical Service Laboratory of this Institute, the infrared spectra by Mr. H. K. Miller, also of this Institute. For *p*H changes of the various solutions during incubation, consult Table I and Fig. 1, for R_F values and colors of chromatographic spots, Table II. The colors refer to those obtained with reagent 1 unless reagent 2 is mentioned specifically.

(20) J. P. Leland and G. L. Foster, *J. Biol. Chem.*, **95**, 165 (1932).

(21) The following nomenclature is used: *p*-(*p*-hydroxyphenoxy)-phenylacetic acid = thyroacetic acid (IIb); β-[*p*-(*p*-hydroxyphenoxy)-phenyl]-propionic acid = thyropropionic acid (IIc); γ-[*p*-(*p*-hydroxyphenoxy)-phenyl]-butyric acid = thyrobutyric acid (IId); β-[*p*-(*p*-hydroxyphenoxy)-phenyl]-acrylic acid = thyroacrylic acid (IIe); β-[*p*-(*p*-hydroxyphenoxy)-phenyl]-lactic acid = thyrolactic acid (IIf); *p*-(*p*-hydroxyphenoxy)-benzoic acid = thyroformic acid (IIa). This name for IIa is a logical extension of the names used for other analogs of thyroxine and therefore more appropriate and also more descriptive than the name thyroxylic acid suggested by K. Tomita and H. A. Lardy, *J. Biol. Chem.*, **219**, 595 (1956).

(22) D. Papa, E. Schwenk, H. Breiger and V. Peterson, THIS JOURNAL, **72**, 2619 (1950).

(23) 3,5-Diiodo-4-hydroxybenzaldehyde gives only a faint color with color reagent 1.

(24) M. Businelli, *Farm. Sci. tec.*, **5**, 522 (1950); m.p. 175–177° dec.

(25) *Cf.* M. Dohrn and P. Diedrich, U. S. Patent 2,116,104 (1938).

(26) A. Ellinger and Y. Kotake, *Z. physiol. Chem.*, **65**, 402 (1910).

3,5-Diiodo-4-hydroxyphenylglycolic acid exists in two polymorphic forms. The crystals melting at 148–151°, on recrystallization from water, yielded needles melting at 203° (dec.). When a few drops of dil. HCl were added to a hot aqueous solution of either these crystals or those melting at 148–151°, the white needles that separated on cooling melted at 152–154°. However, when no HCl was used in the recrystallization, white needles melting at 203° (dec.) were obtained in both cases. Both substances have the same electrophoretic mobility and nearly the same elemental composition corresponding best to $C_8H_6I_2O_4 \times {}^1/_2\ H_2O$.

Anal. Calcd. for $C_8H_6I_2O_4 \times {}^1/_2\ H_2O$: C, 22.40; H, 1.64; I, 59.16. Found for the low melting compound: C, 22.48; H, 1.68; I, 58.93. Found for the high melting compound: C, 22.18; H, 1.66; I, 59.08.

β-(*p*-Hydroxyphenyl)-propionic Acid (Phloretic Acid).—A modification of the procedure of Karasch, *et al.*,[27] was used. Raney alloy[28] instead of Raney nickel was used for the hydrogenation of the *p*-hydroxycinnamic acid. Phloretic acid was obtained in 66% yield; m.p. 128–129°; lit.[27] m.p. 129–130°.

Incubation of β-(3,5-Diiodo-4-hydroxyphenyl)-propionic Acid (3,5-Diiodophloretic Acid) (Ic).—A solution of 5.02 g. (12 mmoles) of Ic, m.p. 165–167° dec.,[29] was incubated. After a few hours, a precipitate of crude sodium 3,5,3′,5′,-tetraiodothyropropionate began to form and the solution turned greenish brown, later dark brown. After 7 days, the precipitate was separated by centrifugation followed by filtration and washed with a small amount of ice-cold water; yield 0.55 g. (12%). Acidification of a solution of the sodium salt in dil. NH_4OH yielded 0.44 g. (10%) of the free acid. Traces of resinous material were removed by filtration of a solution of the acid in acetone. Recrystallization from benzene gave 0.21 g. of almost white crystals. A second recrystallization after decolorization with Norit SG yielded white needles, m.p. 214–216° dec. The melting point of authentic tetraiodothyropropionic acid, m.p. 217–218°, was not depressed.

Anal. Calcd. for $C_{15}H_{10}I_4O_4$: C, 23.65; H, 1.32; I, 66.63. Found: C, 24.38; H, 1.59; I, 65.53.

The infrared spectrum (KBr) was identical to the one of authentic 3,5,3′,5′-tetraiodothyropropionic acid.

Acidification of the filtrate from the sodium tetraiodothyropropionate with dil. H_2SO_4 gave an oily precipitate that solidified upon standing at 2°. Filtration yielded 3.92 g. of crude 3,5-diiodophloretic acid contaminated with 3-iodophloretic acid (chromatographic analysis with solvents 1, 2, 3, 4, 5). After several recrystallizations from isooctane–ethyl acetate, 1.95 g. of pure diiodophloretic acid was obtained. The filtrate from the iodinated phloretic acids was concentrated *in vacuo* to 30 ml. A small amount of insoluble material was filtered off and the filtrate was extracted with ether in a continuous extractor. Chromatography (solvents 1, 2) and electrophoretic analysis (pherogram) of the oily residue obtained after evaporation of the ether revealed the presence of much 3-iodophloretic acid, a small amount of phloretic acid and traces of 3,5-diiodophloretic acid and of an unidentified substance (yellow spot).

Mixture of β-(3-Iodo-4-hydroxyphenyl)-propionic Acid (3-Iodophloretic Acid) and β-(3,5-Diiodo-4-hydroxyphenyl)-propionic Acid (3,5-Diiodophloretic Acid).—A mixture of mono- and diiodophloretic acid containing slightly more of the monoiodo compound was prepared by iodination of 1 g. (6 mmoles) of phloretic acid. The procedure was the same as for the preparation of diiodophloretic acid[29] except that only 1.85 g. (14.6 milliatoms) of iodine was used. The incubation was carried out with the precipitate obtained by acidification of the reaction mixture, without further purification.

Incubation of a Mixture of 3-Iodophloretic Acid and 3,5-Diiodophloretic Acid.—A solution of 1.70 g. (approx. 5.4 mmoles) of iodinated phloretic acids was incubated. It darkened much less than a solution of 3,5-diiodophloretic acid alone and no precipitate formed. After 7 days, enough 10 *N* NaOH was added to make the solution 2 *N* with respect to NaOH. The solution was then extracted once with 20 ml. and once with 10 ml. of 1-butanol. The combined extracts were washed with 10 ml. of 2 *N* NaOH and with 10 ml. of water, then evaporated *in vacuo*. Chromatography of the residue (solvents 2 and 3) revealed the presence of 3,5,3′,5′-tetraiodothyropropionic acid and of a small amount of 3,5,3′-triiodothyropropionic acid. (When a butanol extract of an incubation mixture of 3,5-diiodophloretic acid alone was chromatographed in the same manner, only a trace of triiodothyropropionic acid was detected.)

γ-(*p*-Hydroxyphenyl)-butyric Acid.—This acid was prepared by a Clemmensen reduction (Martin modification)[30] of 4 g. (21 mmoles) of β-(*p*-hydroxybenzoyl)-propionic acid, m.p. 156–158°.[31] Recrystallization of the crude acid from benzene–petroleum ether yielded 2.45 g. (66%) of γ-(*p*-hydroxyphenyl)-butyric acid, m.p. 105–107°; lit.[32] m.p. 107–108°.

γ-(3,5-Diiodo-4-hydroxyphenyl)-butyric Acid (Id).—The same method as described for the preparation of Ib was used, except that the reaction mixture was cooled in an ice-bath during the addition of the iodine solution. The crude acid was obtained in a 93% yield. Recrystallization from isoöctane gave white needles, m.p. 105–107°; lit.[22] m.p. 105–106°.

Incubation of γ-(3,5-Diiodo-4-hydroxyphenyl)-butyric Acid.—A solution of 2.16 g. (5 mmoles) of Id was incubated. The solution turned slowly brown on standing but no precipitate formed. After 4 days, chromatograms (solvents 1, 2, 3) showed a strong and a weak red spot (Id and γ-(3-iodo-4-hydroxyphenyl)-butyric acid) and a brown streak apparently formed from polymerized material. After the addition of enough 10 *N* NaOH to make the solution 2 *N* with respect to NaOH, the incubation mixture was extracted twice with 20 ml. of 1-butanol. The combined extracts were washed with 20 ml. of 1 *N* NaOH and with 10 ml. of water, then evaporated *in vacuo*. Chromatography of the residue (solvents 1, 2, 3) gave a strong and a weak purple spot (3,5,3′,5′-tetraiodo- and 3,5,3′-triiodothyrobutyric acid) in addition to a red spot (Id).

Incubation of 3,5,3′,5′-Tetraiodothyrobutyric Acid.—This acid was prepared from a commercial sample (Cyclo Chemical Corp.)[33] which was contaminated with 3,5,3′-triiodothyrobutyric acid. After paper chromatography of the crude product (solvent 3), the band of tetraiodothyrobutyric acid was cut out and eluted with solvent 1. Evaporation of the solvent *in vacuo* yielded pure 3,5,3′,5′-tetraiodothyrobutyric acid. Three incubation experiments were carried out. In experiment 1, a mixture of 3 mg. of 3,5,3′,5′-tetraiodothyrobutyric acid and 8 ml. of water was adjusted to *p*H 7.5 by the careful addition of dil. NaOH. In experiment 2, the same amount of tetraiodothyrobutyric acid was mixed with 8 ml. of 0.2 *M* phosphate buffer, *p*H 7.5. Experiment 3 was done like experiment 2, except that a phosphate buffer of *p*H 7.0 was used. All three solutions were incubated for 4 days, after which time the *p*H in experiment 1 had risen to 7.7. The reaction mixtures were extracted with 1-butanol in the usual manner. Chromatography of the butanol extracts (solvents 1, 2, 3) gave in all experiments a single purple spot corresponding to 3,5,3′,5′-tetraiodothyrobutyric acid.

α-Methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic Acid (Ie).—α-Methyl-β-(*p*-hydroxyphenyl)-propionic acid[22] was iodinated according to the procedure used in the preparation of Id. Recrystallization from chloroform–petroleum ether and from isoöctane–benzene gave white crystals melting at 113–115° (82% yield); lit.[22] m.p. 118–119°.

Incubation of α-Methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic Acid (3,5-Diiodo-α-methylphloretic Acid).—A solution of 6.48 g. (15 mmoles) of Ie was incubated. After a few hours, the solution turned brown and a precipitate began to form. After 5 days, the light brown, amorphous precipitate was separated by centrifugation (0.40 g.). Chromatography of the dark brown supernatant (solvents 1, 2, 3) revealed the presence of Ie and of α-methyl-β-(3-iodo-4-hydroxyphenyl)-propionic acid. The precipitate was acidified, filtered and washed with water. Chromatog-

(27) N. Kharasch, S. H. Kalfayan and J. D. Arterberry, *J. Org. Chem.*, **21**, 925 (1956).

(28) E. Schwenk, D. Papa, B. Whitman and H. F. Ginsberg, *ibid.*, **9**, 175 (1944).

(29) J. H. Barnes, E. T. Borrows, J. Elks, B. A. Hems and A. G. Long, *J. Chem. Soc.*, 2824 (1950).

(30) E. L. Martin, THIS JOURNAL, **58**, 1438 (1936).

(31) Obtained by recrystallization of a crude material kindly supplied by the Cyclo Chemical Corp.

(32) N. Kharasch and S. Kalfayan, *J. Org. Chem.*, **21**, 929 (1956).

(33) Kindly supplied by Warner Chilcott Research Labs.

raphy of the amorphous powder obtained by reprecipitation from hot benzene (solvents 1, 2, 3) gave only brown streaks having their highest density at or near the origin. They were probably formed by polymerized material. When the benzene mother liquor was chromatographed, an unidentified strong orange-brown spot and a strong purple spot, probably[34] 3,5,3′,5′-tetraiodo-α-methyl-thyropropionic acid, were observed in addition to streaks. The same two spots were found in chromatograms of a 1-butanol extract (0.07 g.) of an alkaline suspension (2 *N* NaOH) of the amorphous precipitate formed during the incubation of Ie. Acidification of the dark brown supernatant mentioned above and filtration of the precipitate formed, followed by an extraction of the filtrate with ether in a continuous extractor and evaporation of the ether yielded an oily residue whose pherogram revealed the presence of 3-iodo-α-methylphloretic acid and of a small amount of α-methylphloretic acid.

Incubation of α-Phenyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic Acid (3,5-Diiodo-α-phenylphloretic Acid) (If).—A solution of 6.66 g. (13.5 mmoles) of If[35] was incubated. After a few hours, the solution began to darken and an amorphous precipitate began to form. After 4 days the precipitate was centrifuged off. Chromatography of the dark brown supernatant (solvents 1, 2, 3) revealed the presence of some α-phenyl-β-(3-iodo-4-hydroxyphenyl)-propionic acid in addition much If. The precipitate was acidified, then fractionated by reprecipitation from hot benzene in a similar manner as described for the amorphous precipitate obtained in the incubation of Ie. The fraction soluble in cold benzene was further fractionated by precipitation with increasing amounts of isoöctane.[36] All fractions were chromatographed (solvents 2, 3). All chromatograms showed the presence of If and of an unidentified compound giving a strong orange-brown spot. Small amounts of two additional unidentified substances were present in the most soluble fraction. No analog of thyroxine was detected in any of the fractions.

3,5-Diiodo-4-hydroxybenzaldehyde.—*p*-Hydroxybenzaldehyde (Eastman Kodak) was iodinated according to the procedure described for the preparation of 3,5-diiodo-4-hydroxyphenylglyoxylic acid; m.p. 199–200°; lit. m.p. 198–199°[37] and 200–201°.[38]

3,5-Diiodo-4-hydroxycinnamic Acid (Ig).—The procedure of Paal and Mohr[39] for the synthesis of this acid from 3,5-diiodo-4-hydroxybenzaldehyde[40] was used with the following modifications. The reaction mixture was stirred at 140–150° for 26 hr. The crude reaction product was heated with 10% NaOH on a steam-bath for 1 hr. in order to hydrolyze the acetoxy group. After acidification, the crude 3,5-diiodo-4-hydroxycinnamic acid was recrystallized from ethanol. Fine white crystals, m.p. 280° dec., were obtained in a 13% yield; lit. m.p. 245°[39,41] and 247°.[41,42]

Anal. Calcd. for $C_9H_6I_2O_3$: C, 25.99; H, 1.45; I, 61.02. Found: C, 26.20; H, 1.54; I, 61.08.

The infrared spectrum (KBr) is consistent with the structure of a *trans*-cinnamic acid. It shows strong bands at 1689 cm.$^{-1}$ characteristic for the carboxyl group of an α,β-unsaturated aromatic acid and at 1625 cm.$^{-1}$ caused by the conjugated C–C double bonds.[43]

Incubation of 3,5-Diiodo-4-hydroxycinnamic Acid.—A solution prepared from 2.91 g. (7 mmoles) of Ig was incubated. The initial *p*H was 8.0.[44] After a few hours, the solution began to darken. After 4 days, a chromatographic analysis (solvents 1, 2, 3) of the dark brown solution revealed the presence of Ig and of some apparently polymerized material forming streaks with their highest density very close to the origin. Chromatography (solvents 1, 2, 4) of the red 1-butanol extract prepared in the usual manner from the solution showed one main spot (Ig) and three minor spots. One of these (light brown) is possibly due to 3-iodo-4-hydroxycinnamic acid. Another appeared bright red after spraying of the paper with Na_2CO_3 but did not change its color after spraying with diazotized N¹,N¹-diethylsulfanilamide. The nature of this spot and of another faint yellow spot was not investigated. Starting material (Ig) could not be removed from the butanol extract by washing with strong alkali (8 *N* NaOH). None of the chromatograms showed a purple spot with an R_F value expected for 3,5,3′,5′-tetraiodothyroacrylic acid.

β-(*p*-Hydroxyphenyl)-D,L-lactic Acid.—This acid was obtained in yields of 81–95% by the reduction of *p*-hydroxyphenylpyruvic acid (Nutritional Biochemicals Corp.) with sodium amalgam.[15] The white needles sintered at 117–120°, then solidified again completely and melted at 135°. After a few days' standing they melted at 143–145° without sintering; lit.[15] m.p. 137–139°.

β-(3,5-Diiodo-4-hydroxyphenyl)-D,L-lactic Acid (Ih).—To a stirred solution of 3.36 g. (18.4 mmoles) of β-(*p*-hydroxyphenyl)-D,L-lactic acid in 15 ml. of 20% of HCl and 100 ml. of water was added within 5 minutes 6.01 g. (37 mmoles) of iodine monochloride dissolved in 10 ml. of 20% HCl. After 2 hr. standing, the precipitate formed was filtered off and recrystallized from water; yield 4.8 g. (60%) of white needles melting at 165°; lit.[38] m.p. 163–164°. In an experiment on a larger scale (22 g.) a yield of 71% was obtained.

Incubation of β-(3,5-Diiodo-4-hydroxyphenyl)-D,L-lactic Acid.—A solution of 37.0 g. (85.3 mmoles) of Ih was incubated for 6 days. The crude sodium 3,5,3′,5′-tetraiodothyro-D,L-lactate formed was filtered off and washed with a small amount of ice-cold water. Chromatography of the light brown crystals (solvents 1, 2, 3) revealed the presence of 3,5,3′,5′-tetraiodothyro-D,L-lactic acid contaminated with some starting material (Ih). Chromatography of the filtrate (solvents 1, 2, 3) gave a strong red spot (Ih) and a weak pink spot (β-(3-iodo-4-hydroxyphenyl)-D,L-lactic acid). Acidification of the sodium salt yielded 0.93 g. (2.8%) of the free acid. After two recrystallizations from ethyl acetate–benzene white needles, m.p. 207–208° dec., were obtained; it.[15] m.p. 176–177°.

Anal. Calcd. for $C_{15}H_{10}I_4O_5$: C, 23.16; H, 1.30; I, 65.26. Found: C, 23.15; H, 1.42; I, 65.23.

Partial Iodination of Analogs of Tyrosine.—3-Iodo-4-hydroxyphenylacetic acid, β-(3-iodo-4-hydroxyphenyl)-propionic acid (3-iodophloretic acid), γ-(3-iodo-4-hydroxyphenyl)-butyric acid and α-methyl-β-(3-iodo-4-hydroxyphenyl)-propionic acid were prepared by partial iodination of the corresponding desiodo compounds. The procedure was the same as described for the preparation of 3,5-diiodo-4-hydroxyphenylacetic acid except that only 2 atoms of iodine was used for each mole of starting material. The crude iodination products were used as reference compounds without further purification. They were contaminated with starting material and with the corresponding 3,5-diiodinated compounds.

Partial Deiodination of Analogs of 3,5-Diiodotyrosine.—α-Phenyl-β-(3-iodo-4-hydroxyphenyl)-propionic acid and 3-iodo-4-hydroxyphenyl-D,L-lactic acid were prepared by partial hydrogenation of the corresponding 3,5-diiodinated compounds. Solutions containing 0.5–1 mmole of starting material in ethyl acetate were hydrogenated slightly above atmospheric pressure in the presence of 50 mg. of 10% Pd on charcoal until 1 mole of hydrogen per mole of starting material was taken up. This took about 50 minutes in the case of If and about 3 minutes in the case of Ih. The crude semi-deiodination products were used as reference compounds without further purification. They were contaminated with starting material and with the corresponding completely deiodinated compound.

Incubation of N-Chloroacetyl-3,5-diiodo-L-tyrosine (Ii).—A solution of 5.09 g. (10 mmoles) of N-chloroacetyl-3,5-

(34) No authentic reference substance was available.

(35) Kindly supplied by the Schering Corp. (Trade name: Iodoalphionic acid.)

(36) No butanol fractionation was carried out because 1-butanol extracts a considerable amount of If from its solution in 2 *N* NaOH.

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

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

(39) C. Paal and L. Mohr, *Ber.*, **29**, 2302 (1896).

(40) When *p*-hydroxycinnamic acid was treated with iodine monochloride according to the procedure described for the preparation of 3,5-diiodo-4-hydroxyphenylglycolic acid, the precipitate formed was 3,5-diiodo-4-hydroxybenzoic acid.

(41) The substance described in the literature may actually have been 3,5-diiodo-4-acetoxycinnamic acid, which would explain the discrepancy between the prevously reported m.p. and ours.

(42) H. L. Wheeler and C. O. Johns, *Am. Chem. J.*, **43**, 11 (1910).

(43) *Cf.* J. Lecombe and J. Guy, *Compt. rend., Paris*, **227**, 54 (1948), and Z. Horii, I. Ninomiya and Y. Tamura, *Pharm. Bull. (Tokyo)*, **5**, 6 (1957).

(44) Ig was not completely soluble at a lower *p*H.

TABLE I

REACTION PRODUCTS OBTAINED IN THE INCUBATION OF ANALOGS OF 3,5-DIIODOTYROSINE

Starting material	*p*H Initial	*p*H Final	Incubation period (days)	Yield of thyroxine analog (% of theory)	Other identified reaction products[a]
R = COOH	7.4	8.5	32	Trace	2,4,6-Triiodophenol (1.2 mmoles from 0.1 mole of starting material)
R = CH_2COOH	7.6	7.4	5	6%	3-Iodo-4-hydroxyphenylacetic acid; 3,5-diiodo-4-hydroxyphenylglycolic acid; 3,5-diiodo-*p*-hydroxybenzaldehyde; 3,5-diiodo-4-hydroxyphenylglyoxylic acid (trace)
R = $(CH_2)_2COOH$	7.7	7.9	7	11%	β-(3-Iodo-4-hydroxyphenyl)-propionic acid; β-(4-hydroxyphenyl)-propionic acid (trace); 3,5,3′-triiodothyropropionic acid (trace)
R = $(CH_2)_3COOH$	7.5	7.0	4	Small amount[b]	γ-(3-Iodo-4-hydroxyphenyl)-butyric acid; 3,5,-3′-triiodo thyrobutyric acid (trace); γ-(4-hydroxyphenyl)-butyric acid (trace)
R = $CH_2CH(CH_3)COOH$	7.6	7.8	5	Small amount[b]	α-Methyl-β-(3-iodo-4-hydroxyphenyl)-propionic acid; α-methyl-β-(4-hydroxyphenyl)propionic acid (trace)
R = $CH_2CH(C_6H_5)COOH$	7.6	7.8	4	Not detected	α-Phenyl-β-(3-iodo-4-hydroxyphenyl)-propionic acid
R = CH=CHCOOH	8.0	9.4	4	Not detected	
R = $CH_2CH(OH)COOH$	7.5[c]	7.2	6	3%	β-(3-Iodo-4-hydroxyphenyl)-lactic acid
R = $CH_2CH(NHCOCH_2Cl)COOH$	7.4	5.9	7	1%	Not investigated

[a] Starting material, always recovered in large amounts, is not included. [b] Probably not more than 1% on the basis of the intensity of the color of the corresponding chromatographic spot. [c] In another run (incubation period 4 days) the initial *p*H was 8.5 and the final *p*H, 8.4.

diiodo-L-tyrosine[45] was incubated. The initial *p*H was 7.4. After 7 days, enough 10 *N* NaOH was added to the dark brown reaction mixture to make it 2 *N* with respect to NaOH. This solution was then extracted twice with 40 ml. of 1-butanol. The combined butanol extracts were washed with 2 *N* NaOH and with water, then evaporated *in vacuo* to dryness. After acidification with dil. HCl, the sticky residue was separated from the supernatant by decantation, then washed and dried; 60 mg. (1.5%) of crude N-chloroacetyl-L-thyroxine. Chromatography (solvents 1, 2, 3) showed a strong purple spot (N-chloroacetyl-L-thyroxine) and a faint brown streak.

N-Chloroacetyl-L-thyroxine.—A suspension of 0.70 g. (0.79 mmole) of L-thyroxine–sodium pentahydrate[46] in about 1 ml. of ethanol was acidified with a few drops of glacial acetic acid, then evaporated *in vacuo* to dryness. A solution of 0.4 ml. (5.3 mmoles) of chloroacetyl chloride in 40 ml. of ethyl acetate was added, and the mixture was refluxed for 15 minutes. The turbid solution was evaporated *in vacuo* to dryness. A solution of the residue in 10 ml. of ethyl acetate was filtered to remove insoluble material. The filtrate was concentrated *in vacuo* to about 1 ml. and then 10 ml. of benzene was added. The crystals formed after one day's standing at 2° (0.41 g.; 61%) melted at 223° dec. Two recrystallizations from ethyl acetate–benzene gave colorless needles, m.p. 229° dec.

Anal. Calcd. for $C_{17}H_{12}O_5ClI_4N$; C, 23.93; H, 1.42; I, 59.49. Found: C, 24.18; H, 1.56; I, 58.1.

Results and Discussion

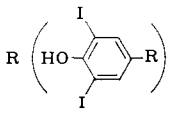
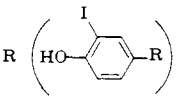
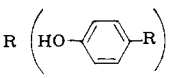
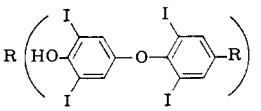
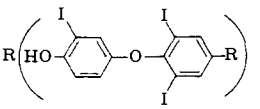
The degree of conversion of analogs of diiodotyrosine to the corresponding diphenyl ethers under the mild conditions of our experiments is very much dependent on the structure of the aliphatic side chain (Table I). By far the highest yield is obtained with diiodophloretic acid (diiododesaminotyrosine). Incubation of the acetic and lactic acid analogs of diiodotyrosine yield considerably less analog of thyroxine. In the case of the acetic acid analog, the presence of a highly active CH_2– group leads to extensive oxidative degradation, mainly with the formation of the glycolic acid analog, followed by oxidative decarboxylation leading to 3,5-diiodo-*p*-hydroxybenzaldehyde. Lengthening of the side chain of the analog of diiodotyrosine (butyric acid analog) or branching (α-methylpropionic acid analog) leads to very poor yields of analogs of thyroxine. In the latter case, much of an unidentified, apparently polymerized, material is formed. Branching with the bulkier phenyl group (α-phenylpropionic acid analog) or introduction of a double bond in conjugation with the aromatic ring (acrylic acid analog) seems to suppress entirely the formation of an analog of thyroxine. Traces of these analogs may, however, have escaped detection, since in these two cases, no fractionation with *n*-butanol was possible (see Experimental Part). In the latter case, a pigment was formed which is bright red in alkaline medium and light yellow in acid medium. A major reaction product in the incubation of α-methyldiiodophloretic acid, judging from the intensity of the orange-brown chromatographic spot obtained upon staining with color reagent 1, is a substance whose R_F value (0.35 in solvent 1; 0.51 in solvent 2; 0.21 in solvent 3) corresponds neither to 2,4,6-triiodophenol or 3,5-diiodo-4-hydroxybenzaldehyde nor to the formic, glycolic or glyoxylic acid analogs of diiodotyrosine. As all other conceivable degradation products of α-methyldiiodophloretic acid should have lower R_F values, it is likely that the substance is a condensation product of the starting material. A similar but not identical substance is formed in the incubation of α-phenyldiiodophloretic acid. (R_F 0.55 in solvent 2; 0.20 in solvent 3). The formic acid

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

(46) Kindly supplied by Smith, Kline and French Labs.

TABLE II

COLORS OBTAINED WITH DIAZOTIZED N^1,N^1-DIETHYLSULFANILAMIDE AND R_F VALUES

Substance	Color	R_F[a] in solvent 1	2	3	4	5
						
COOH	Yellow	0.04	0.10	0.01	..	0.63
CH_2COOH	Red	.07	.15	.02	0.64	.62
$(CH_2)_2COOH$	Red	.12	.23	.03	.75	.74
$(CH_2)_3COOH$	Red	.14	.26	.03	..	..
$CH_2CH(CH_3)COOH$	Red	.15	.28	.05	.85	.77
$CH_2CH(C_6H_5)COOH$	Red	.25	.35	.10	.85	.83
$CH{=}CHCOOH$	Brown	.09	.21	.02	.56	.50
$CH_2CH(OH)COOH$	Red	.06	.17	.06	.42	.43
$CH_2CH(NHCOCH_2Cl)COOH$	Red	.08	.19	.06	..	..
CH(OH)COOH	Yellow	..	.07	..	..	.43
COCOOH	Brownish yellow	..	.10	..	..	.3–.73[b]
CHO	Light brown[c]	.83	.79	.38	.94	.93
I	Brownish yellow	.72	.83	.55	..	..
						
CH_2COOH	Red	0.12	0.31	0.03	..	..
$(CH_2)_2COOH$	Red	.21	.33	.12	0.75	0.74
$(CH_2)_3COOH$	Red	.27	.47	.09	..	..
$CH_2CH(CH_3)COOH$	Red	.27	.48	.11	..	..
$CH_2CH(C_6H_5)COOH$	Red	.43	.61	.22	..	..
$CH_2CH(OH)COOH$	Red	.14	.31	.05	..	..
						
CH_2COOH	Orange red	0.12	0.20	0.04	..	..
$(CH_2)_2COOH$	Orange red	.22	.34	.09	0.63	0.62
$(CH_2)_3COOH$	Orange red	.28	.36	.11	..	..
$CH_2CH(CH_3)COOH$	Orange red	.31	.37	.12	..	..
						
COOH	Purple[d]	0.45	0.53	0.21	..	..
CH_2COOH	Purple[d]	.37	.46	.18	..	..
$(CH_2)_2COOH$	Purple[d]	.45	.48	.16	..	..
$(CH_2)_3COOH$	Purple[d]	.44	.54	.20	..	..
$CH_2CH(CH_3)COOH$	Purple[d]	.44	.55	.27	..	..
$CH_2CH(OH)COOH$	Purple[d]	.33	.46	.22	..	..
$CH_2CH(NHCOCH_2Cl)COOH$	Purple[d]	.28	.46	.13	..	..
						
$(CH_2)_2COOH$	Purple[d]	0.59	0.64	0.37	..	..
$(CH_2)_3COOH$	Purple[d]	.60	.65	.46	..	..

[a] Mean values of several runs. [b] Elongated spot. [c] Color reagent 2 gives a brown spot. [d] When a high concentration is used the spot obtained has a brown center and a purple periphery.

analog of diiodotyrosine formed very slowly a small amount of 3,5,3′,5′-tetraiodothyroformic acid. The main reaction product in this case is 2,4,6-triiodophenol formed by reaction of the formic acid analog with iodine furnished by deiodination of part of the starting material.

In all incubations, a large amount of starting material was recovered. Partial deiodination to the corresponding analog of monoiodotyrosine was observed in most cases. Relatively much of the monoiodo compound was detected in the incubation of the acetic, propionic, butyric and α-methyl-

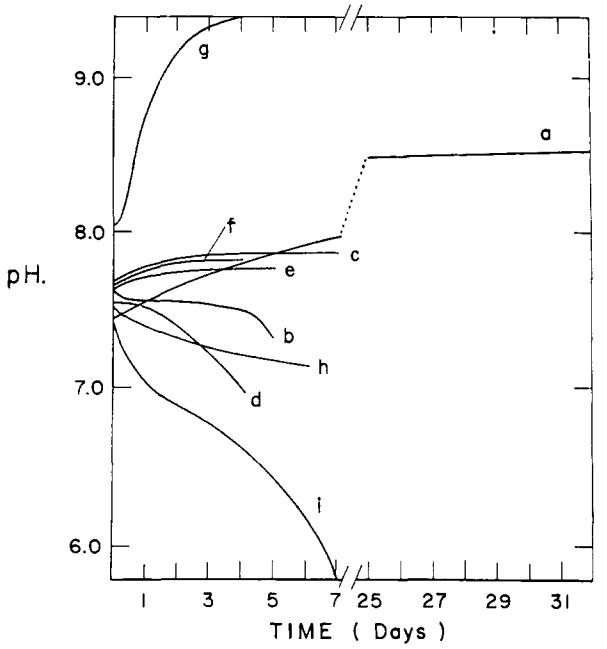
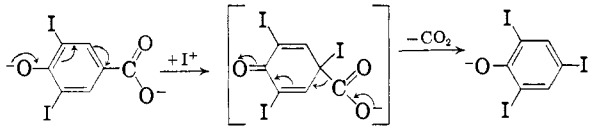


Fig. 1.—Change of *p*H during the incubation of analogs of 3,5-diiodotyrosine. a, 3,5-diiodo-4-hydroxybenzoic acid; b, 3,5-diiodo-4-hydroxyphenylacetic acid; c, β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid; d, γ-(3,5-diiodo-4-hydroxyphenyl)-butyric acid; e, α-methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid; f, α-phenyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid; g, 3,5-diiodo-4-hydroxycinnamic acid; h, β-(3,5-diiodo-4-hydroxyphenyl)-D,L-lactic acid; i, N-chloroacetyl-3,5-diiodo-L-tyrosine.

propionic acid analogs. In the latter three cases, a trace of the corresponding completely deiodinated analog of tyrosine was also found.



When the reaction mixtures obtained in the incubations of the propionic and butyric acid analogs of diiodotyrosine were extracted with 1-butanol traces of the corresponding analogs of triiodothyronine were detected together with the analogs of thyroxine. In order to determine whether the analogs of triiodothyronine were formed by partial deiodination of the corresponding analogs of thyroxine or by condensation of one molecule of an analog of monoiodotyrosine with one molecule of an analog of diiodotyrosine, two experiments were carried out. In the first experiment, 3,5,3′,5′-tetraiodothyrobutyric acid was incubated in a similar manner as the butyric acid analog of diiodotyrosine. Only starting material was recovered; no analog of triiodothyronine was formed. In the second experiment, a mixture of 3-iodophloretic acid and of 3,5-diiodophloretic acid was incubated under the same conditions as 3,5-diiodophloretic acid alone. The mixture yielded more 3,5,3′-triiodothyropropionic acid. Both experiments permit the conclusion that the analogs of triiodothyronine were not formed by deiodination of the corresponding analogs of thyroxine, but by direct synthesis.

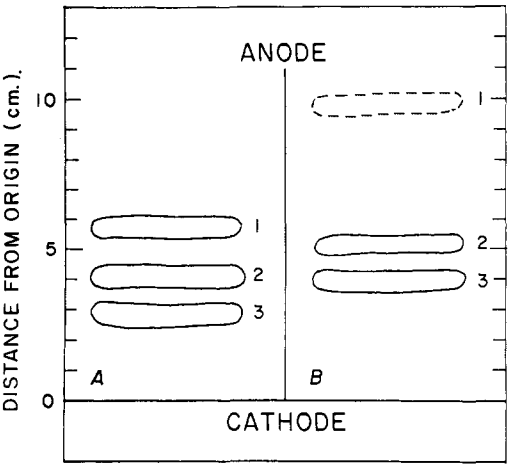


Fig. 2.—Electrophoretic separation (A) of β-(*p*-hydroxyphenyl)-propionic acid (1), β-(3-iodo-4-hydroxyphenyl)-propionic acid (2) and β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid (3); and (B) of an unidentified contaminant of 3,5-diiodo-4-hydroxyphenylglyoxylic acid (1), 3,5-diiodo-4-hydroxyphenylglyoxylic acid (2) and 3,5-diiodo-4-hydroxyphenylglycolic acid (3). Duration of the electrophoresis: A = 3 hours; B = 4 hours.

A temperature of 37° and a *p*H as close to 7.5 as possible were chosen for the incubation in order not to deviate much from physiological conditions. The yields of the analogs of thyroxine thus formed are not necessarily optimal. They might be increased by changing the experimental conditions, *e.g.*, by working at a higher temperature, by bubbling oxygen through the reaction mixture or by adding catalysts.[12,15,47] An extension of the incubation period beyond the one shown in Table I did not increase the yield of the analogs of thyroxine but only of oxidative degradation products and of polymerized material. An experiment in which diiodophloretic acid was incubated aseptically showed that the formation of analogs of thyroxine is not due to the presence of bacteria in the incubation mixture. The *p*H of the incubated solutions changed in a manner characteristic for each analog of diiodotyrosine (Fig. 1). In most instances it increased or decreased first rapidly, then gradually more slowly.

Table II shows the R_F values of the starting materials and of the identified reaction products in various solvents. Also listed are the colors obtained with color reagents 1 and 2. Reagent 1 gave in most instances more intense colors than the frequently used diazotized sulfanilic acid. The colors produced by reagent 1 are rather characteristic for certain types of compounds. Thus phenols with a saturated aliphatic acid side chain give a red color, unless the carbon atom adjacent to the aromatic ring carries an oxygen function (–OH, =O, –COOH) in which case a yellow color is produced. Phenol ethers (analogs of thyroxine) give purple spots.

High voltage electrophoresis frequently permits the unequivocal identification of a mixture of compounds when paper chromatography fails to do so.

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

Thus phloretic acid, 3-iodophloretic acid and 3,5-diiodophloretic acid could not be separated from each other satisfactorily by chromatography. In solvents 1, 2 and 3 phloretic acid and 3-iodophloretic acid have almost identical R_F values and in solvents 5 and 6, the two iodinated phloretic acids move together. High voltage electrophoresis achieves an excellent separation of these three substances. Similarly, 3,5-diiodo-4-hydroxyphenylglycolic acid and 3,5-diiodo-4-hydroxyphenylglyoxylic acid can be much better separated by electrophoresis than by chromatography (Fig. 2 and Table II).

The present investigation demonstrates that the non-enzymic conversion of analogs of diiodotyrosine to the corresponding analogs of thyroxine is extremely sensitive to small structural changes in the aliphatic side chain. In the incubation of the propionic acid analog (diiododesaminotyrosine) the corresponding analog of thyroxine is obtained in particularly good yield and only small amounts of side products are formed. This should make the propionic acid analog of diiodotyrosine an excellent model substance for the study of the mechanism of the conversion of diiodotyrosine to thyroxine.[48]

(48) The fate of the aliphatic side chain that is lost in the model reaction with diiodophloretic acid will be reported in a forthcoming paper.

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY I AND THE INSTITUTE OF CELL RESEARCH AND GENETICS, KAROLINSKA INSTITUTET]

Chemical Synthesis of Adenosine 5′-Phosphosulfates[1]

BY PETER REICHARD AND N. R. RINGERTZ

RECEIVED MAY 27, 1958

Adenosine 5′-phosphosulfate was prepared by the carbodiimide route in a final yield of 20–25% (based on adenosine 5′-phosphate). By-products of the reaction, containing sulfate groups attached to ribose, were separated by zone electrophoresis on a cellulose column and characterized. Snake venom (*Crotalus adamanteus*) and extracts of human prostate were able to cleave phospho–sulfate but not ribose–sulfate linkages. Sulfate groups attached to the ribose of adenosine 5′-phosphate inhibited the phosphatase activity of snake venom but not that of the prostatic extract.

Introduction

It was originally demonstrated by Bernstein and McGilvery[2] and De Meio, *et al.*,[3] that sulfate must be "activated" with ATP before further participating in enzymic reactions. The mechanism of the activation was recently clarified by Robbins and Lipmann[4] and Bandurski, *et al.*,[5] who showed that a two-step reaction is involved. Thus during the first step adenosine 5′-phosphosulfate (APS) is formed from ATP[6] and sulfate (ATP sulfurylase)

$$\text{sulfate} + \text{ATP} \rightleftharpoons \text{adenosine 5′-phosphosulfate} + \text{pyrophosphate}$$

The second step involves the conversion of APS to the biologically active sulfate donor, adenosine 3′-phosphate-5′-phosphosulfate (adenosine-phosphosulfate kinase).

The present paper is concerned with the chemical synthesis of APS and some compounds closely related to it. Chemical synthesis of APS has recently been carried out by sulfurylation of AMP with the SO_3 complex of pyridine by Baddiley, *et al.*[7] In preliminary reports[8] we have described a one-step synthesis of APS and other compounds containing both AMP and sulfate by treating a mixture of AMP and concentrated sulfuric acid in aqueous pyridine with an excess of dicyclohexylcarbodiimide (DCC). This paper is a complete report of these studies.

Carbodiimide has previously been used for the synthesis of biologically important anhydrides containing pyrophosphate groups[9] and also for the synthesis of sulfonic acid anhydrides.[10] Therefore, it seemed likely that mixed anhydrides between a phosphate group and sulfuric acid could be synthesized with the aid of DCC. We found that the reaction took place only when the volume of the reaction mixture was kept very small. However, other compounds were also formed, which contained sulfate esterified to the ribose of AMP. Thus, a mixture of AMP-monosulfates, AMP-disulfates and AMP-trisulfates was obtained. However, by choosing the proper conditions it was possible to transform about 50% of the AMP to APS with the formation of only small amounts of sulfate esters. After preparative zone electrophoresis[11] APS was obtained in a final yield of 20–25%.

(1) This paper describes the synthesis of compounds containing sulfate attached to the ribose and/or phosphate moieties of adenosine 5′-phosphate (AMP). Such compounds are named AMP-sulfates. The term adenosine 5′-phosphosulfates is restricted to compounds, specifically containing an anhydride linkage between phosphate and sulfate.

(2) S. Bernstein and R. W. McGilvery, *J. Biol. Chem.*, **198**, 195 (1952); **199**, 745 (1952).

(3) R. H. DeMeio and L. Tkacz, *ibid.*, **195**, 175 (1952); R. H. DeMeio, M. Wizerkaniuk and E. Fabriani, *ibid.*, **203**, 257 (1953).

(4) P. W. Robbins and F. Lipmann, THIS JOURNAL, **78**, 2652 (1956); **78**, 6409 (1956); *J. Biol. Chem.*, **229**, 837 (1957).

(5) R. S. Bandurski, L. G. Wilson and C. C. Squires, THIS JOURNAL, **78**, 6408 (1956).

(6) The following abbreviations are used: AMP and ATP for adenosine-mono- and triphosphate, APS for adenosine-5′-phosphosulfate, SAP for adenosine 2′-(and -3′-)-sulfate-5′-phosphate, DCC for dicyclohexylcarbodiimide and tris-(hydroxymethyl)-aminomethane.

(7) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, 1067 (1957).

(8) (a) P. Reichard and N. R. Ringertz, THIS JOURNAL, **79**, 2026 (1957). (b) N. R. Ringertz and P. Reichard, *Acta Chem. Scand.*, **11**, 1081 (1957).

(9) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954); G. W. Kenner, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 2843 (1954).

(10) H. G. Khorana, *Can. J. Chem.*, **31**, 585 (1953).

(11) J. Porath, *Biochem. Biophys. Acta*, **22**, 151 (1956).