Riboflavinyl Glucoside: a New Derivative of Riboflavin

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Homogenates of rat liver and aqueous extracts of acetone powders prepared from rat liver were shown to produce an unknown flavin, when incubated with riboflavin; it was reported that the reaction was enzymic (Whitby, 1950). The preparation and characterization of this new compound, which has been identified as a glucoside of riboflavin, is described here.

EXPERIMENTAL

Methods

Detection of enzymic activity. The production of riboflavinyl glucoside is detected by paper chromatography (Consden, Gordon & Martin, 1944; Whitby, 1950). Samples are prepared for chromatography by Crammer's (1948) method. The compound is not found in control experiments (samples taken at the beginning of an incubation, or after incubating riboflavin with liver preparations previously heated for 5 min. at 100°). The amount formed in any experiment is determined semi-quantitatively by visual comparison of the intensity of fluorescence with that of known amounts of riboflavin run simultaneously on the chromatograms; or, quantitatively, by separating a band of the mixed flavins and measuring the absorption at 450 mμ. in a Beckman spectrophotometer after elution of the glucoside with water, suitable correction being made for impurities in the filter paper.

Criteria of purity. (1) Separation from all contaminants which could be detected on chromatograms by their fluorescence in ultraviolet light was determined in several solvents (see Table 1). (2) An absorption spectrum of constant shape (aqueous solution). (3) A constant empirical mol.wt., as determined by the light absorption at 450 mμ. (Absorption coefficient, $\beta = 2{\cdot}80 \times 10^7$ cm.^{2}/mol.) (4) A sharp m.p.; this was determined with the Kofler micro-m.p. apparatus (A. H. Thomas Co., Philadelphia, U.S.A.) and is a corrected value.

Preparation of the compound

An acetone powder of rat liver was made as soon after death as possible: twenty livers were blended with 400 ml. of acetone, at 0°, for 1 min. in a Waring blender; the suspension was filtered rapidly on a Büchner funnel, and the solid pressed between filter papers. The solid was broken up, blended again with 400 ml. of acetone at 0°, filtered, pressed between filter papers, dried in a vacuum desiccator over H_2SO_4 and NaOH, and ground to a powder in a coffee mill. In all, 120 rats were killed and 180 g. of powder obtained; 5 g. batches were extracted by grinding in a mortar with 250 ml. of distilled water. This preparation was as active a source of the enzyme as the equivalent amount of liver homogenate, prepared according to Hunter's (1949) conditions.

The enzymic solution was incubated in lots of 1·5 l. (30 g. powder) with 300 mg. of riboflavin at 37°, in darkness for 6 hr. The solution was then deproteinized and desalted by a technique which is a large-scale development of Crammer's (1948) method: 100 ml. of trichloroacetic acid (50% w/w) were added, and the suspension cooled rapidly to 0°; 800 g. of $(NH_4)_2SO_4$ were added and the precipitate centrifuged off; the yellow solution was shaken with 300 ml. of aqueous phenol in a separating funnel, when the flavins were concentrated in the phenol.

Six batches were prepared in this way and the phenol concentrates combined (vol. 2 l., including washings). A sample of this phenolic solution was analysed by chromatography, and the main flavin components (riboflavin and the new flavin) were estimated by their light absorption at 450 mμ. (Table 2, *A*).

Table 1. *R_F values of flavins*

(Whatman no. 1 paper.)

Solvent systems*	*n*-Butanol, 4; acetic acid, 1; water, 5†	*n*-Butanol, 2; *n*-propanol, 2; water, 1	*n*-Butanol, 77; formic acid, 10; water, 13	Water, saturated with *iso*amyl alcohol‡
	R_F			
Riboflavin	0·30	0·20	0·13	0·4
Flavin mononucleotide	0·10	0·04	0·02	0·85
Flavin-adenine dinucleotide	0·03	0	0	0·9
Riboflavinyl glucoside	0·20	0·10	0·04	0·5
Lumiflavin	0·40	0·30	0·28	0·25
Lumichrome	0·70	0·55	0·50	0·1

* Composition of solvents is given in terms of volume ratios.
† Upper layer used as mobile phase.
‡ Ascending chromatogram.

Table 2. *Stages in the isolation of riboflavinyl glucoside*

	Yield of glucoside (mg.)	Ratios of light absorption at different wavelengths: 375 mμ./450 mμ.	275 mμ./450 mμ.	260 mμ./450 mμ.	Empirical mol.wt.
(*A*) Phenol concentrate	280 (+1·6 g. riboflavin)	—	—	—	—
(*B*) After shaking phenol concentrate with water and ether	230 (+300 mg. riboflavin)	0·86	—	7·0	—
(*C*) Effluent from first series of paper columns	200 (traces of riboflavin, etc.)	0·86	2·65	3·38	—
(*D*) Effluent from second series of paper columns	160 (chromatographically pure)	0·85 (4)	2·07	2·37	—
(*E*) Crystalline material	120	0·85 (2)	2·03	2·25	579
(*F*) Recrystallization from water	80	0·85 (2)	2·03	2·25	564
(*G*) Recrystallization from 80% (v/v) aqueous ethanol	60	0·85 (2)	2·03	2·25	540
(*H*) Second recrystallization from 80% (v/v) aqueous ethanol	40	0·85 (2)	2·03	2·25	540

Purification of the compound

It was not practicable to separate the compounds present in the phenol concentrate directly on paper columns, because of the small capacity of these columns for flavins, and the preponderance of riboflavin was reduced by shaking the phenol concentrate with 200 ml. water and 2 l. ether. Most of the new flavin was extracted into the aqueous phase, but relatively little of the riboflavin (240 mg. glucoside; 500 mg. riboflavin). The flavins were re-extracted into 200 ml. aqueous phenol; this phenol solution was shaken with 20 ml. water and 200 ml. ether, and the composition of the aqueous layer is shown in Table 2, Stage *B*. The limit of this technique had now been reached, and the flavin was next purified by chromatography.

Paper columns were made with Brown's 'Solka Floc' (B.W. Grade, 200 mesh). The paper was washed thoroughly before use by boiling for 0·5 hr. under reflux with water, then with 5% (w/v) acetic acid, water, 7% (w/v) K_2CO_3 to remove peptide material (Hird, 1950), water, *n*-butanol, water and finally acetone; it was spread on trays to dry. The columns were made in long glass tubes (5 cm. diam.), which were drawn out at one end; a plug of glass wool was put in the tapered end to support the column. The paper was poured on as a slurry in acetone, in accordance with the method of Campbell, Work & Mellanby (1951). No pressure was applied to the columns at any stage (cf. Peterson & Reineke, 1949). The rate of flow was controlled by a needle valve.

The separation of the new flavin from riboflavin on a paper column was accompanied by so great a broadening of the individual bands that it was necessary to select a solvent in which the R_F value of the new flavin was greater than the R_F value of riboflavin. This limited the choice of solvent to salt solutions and to water; it was advisable to add *iso*amyl alcohol to improve the separations effected by these solvents (Carter, 1950), but the separations were not as sharply defined as those obtained with solvents which were dependent upon partition mechanisms for their action. Water, saturated with *iso*amyl alcohol, was the solvent chosen for the first series of columns.

After packing, each column was washed with 50% (v/v) aqueous ethanol, and then with water. A washing with 5% glycine was included at this stage, to avoid the decomposition of flavins which otherwise occurred on the column. The glycine was washed out with water, and the columns equilibrated with the solvent. The capacity was very small owing to the low solubility of the flavins; a column built from 600 g. of paper could separate 15 mg. of the new flavin from 20 mg. of riboflavin. The rate of flow was adjusted to 25 ml./hr., and the separation followed by means of an ultraviolet lamp. Although this phase was tedious, riboflavin was almost completely removed from the mixture.

The effluent from the columns was extracted with ether (to recover the *iso*amyl alcohol), and the flavin solution evaporated to dryness in the dark, at 45°, under reduced pressure, and in an atmosphere of nitrogen. Despite these precautions, there was some decomposition with the formation of lumichrome. Besides this impurity, small amounts of riboflavin and of flavin mononucleotide were present. Although this series of columns had greatly decreased the content of impurities which absorbed light below 300 mμ. (Table 2, *C*), it was still possible to remove impurities which absorbed light in this region. These impurities could not be identified by the technique of Markham & Smith (1949), because the flavin trailed seriously on the chromatograms at concentrations which were too low to allow of the detection of purines, pyrimidines, etc., by this method, even if the total impurity had been due to a single compound.

The solvent system, *n*-butanol, 77; formic acid, 10; water, 13, was used on a second batch of columns, as trial chromatograms had shown that this solvent effected the greatest reduction in the light absorption below 300 mμ., relative to the 450 mμ. absorption. The solvent was theoretically capable of resolving all the fluorescent components of the mixture and the R_F value of the new flavin in this solvent differed from the R_F values of a wide range of derivatives of nucleic acids (Markham & Smith, 1949; Smith & Markham, 1950).

The columns were made as previously described; after glycine had been removed by washing with water, the columns were washed with 50% (v/v) aqueous ethanol, then with 85% (v/v) aqueous ethanol, before being finally equilibrated with the solvent (the components were distilled to remove their non-volatile residue). The flavin mixture was

dissolved in the minimum amount of solvent, and the band of the new flavin was collected in fractions; the solvent was removed by distillation under reduced pressure (in darkness, at 45°, and in an atmosphere of nitrogen), and all fractions which had a ratio of light absorption at 260 mμ.: 450 mμ. greater than 2·40 were repurified on a later column (Table 2, *D*). The overall yield up to this stage was about 55%. Paper chromatography showed that the compound had been freed from all fluorescent contaminants, and that little further alteration in the absorption spectrum could be effected.

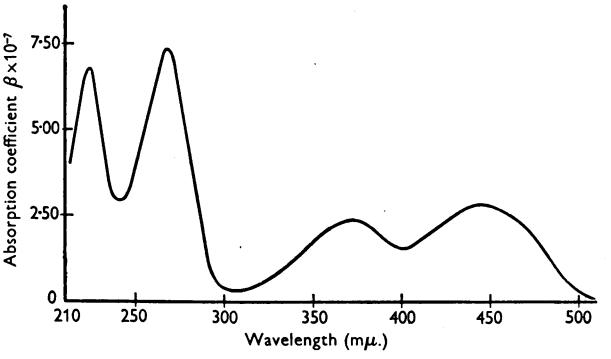


Fig. 1. Absorption spectrum of riboflavinyl glucoside in water.

Crystallization. The flavin was crystallized by evaporating an aqueous solution (about 20 mg./ml.) under reduced pressure at room temperature, a current of N_2 being made to impinge upon the surface of the solution. About 0·5 mg. of dry crystalline material was dissolved in 25 ml. water; the density of the solution at 450 mμ. was measured and the empirical mol.wt. calculated (Table 2, *E*). The compound was recrystallized as shown in Table 2, and 10 mg. of the final preparation (stage *H*) were recrystallized from acetic acid; the empirical mol.wt. of the specimen was 554, but the solution smelt slightly of acetic acid and attempts to remove it were prevented by shortage of material. No preparation was obtained with an empirical mol.wt. lower than 540±3 and this material (Table 2, stage *H*) was used for determining the m.p. and elementary composition.

The new compound had m.p. 247–248° (the crystals turned light brown in colour at 246°). Only carbon, hydrogen, nitrogen (and oxygen) could be detected. (Found: C, 51·1; H, 5·6; N, 10·3. $C_{23}H_{30}O_{11}N_4$ requires C, 51·3; H, 5·6; N, 10·4%.)

Molecular weight. An estimate of the mol.wt. was obtained with a micro-modification of Rast's method (Reilly & Rae, 1948). The compound had only slight solubility in camphor, so the molecular weight obtained by this method was approximate. The value was 500±50. $C_{23}H_{30}O_{11}N_4$ requires mol.wt. 538·5.

Characterization of the new compound

Stability. An aqueous solution is stable at 100°, but N-sodium hydroxide at 100° rapidly destroys the flavin by attacking the *iso*alloxazine nucleus. Lumiflavin is formed under the alkaline conditions described by Warburg & Christian (1938). The compound is hydrolysed by strong acids, with the production of riboflavin; the reaction is completed within 2·5 hr. in N-hydrochloric acid at 100°.

Absorption spectrum (Fig. 1). The molecular absorption coefficient at 450 mμ. is the same as the value reported for riboflavin by Singer & Kearney (1950); this value, which is about 8% higher than the value reported by Warburg & Christian (1938), has been confirmed with a potentiometrically pure sample of riboflavin. This sample was also found pure by chromatography. It had m.p. 280–282°. (Found C, 54·2; H, 5·3; N, 15·0. Calc. for $C_{17}H_{20}O_6N_4$: C, 54·3; H, 5·3; N, 14·9%.)

The positions of the maxima and minima in the absorption spectrum of the new flavin coincide with the values for riboflavin; at the same molar concentration, their absolute heights coincide, except in the region 440–310 mμ., where the spectrum of the new flavin is consistently lower, the difference being very small at the extremes of this range, but gradually increasing to a difference of 3% at 400 mμ. and of 2% at 375 mμ.; the rest of the spectrum coincides with the spectrum of riboflavin within the limits of experimental error. The reasons for the difference between the spectrum of the new flavin and the spectrum of riboflavin are unknown.

The identity of the attached group was first investigated by chromatography after hydrolysis. Riboflavin interfered on chromatograms because of its intense yellow colour, but this interference was eliminated by dissolving the compound in ammonia (sp.gr. 0·880) and refluxing at 100° for 30 min.; riboflavin was treated similarly as a control. Ammonia was removed *in vacuo*, and the solid residue was dissolved in 2N-hydrochloric acid and heated for 2 hr. at 100°, after which hydrochloric acid was removed *in vacuo*. Samples of the hydrolysate were run on chromatograms, and glucose was identified as a product of hydrolysis with four different solvent systems, two of which were mixtures recommended by Jermyn & Isherwood (1949). Glucose was not released by refluxing with ammonia, as glycosidic links are stable to alkali.

Table 3. *Manometric estimation of* D-*glucose released from riboflavinyl glucoside by hydrolysis with acid*

(Manometer contents; main compartment: 2 ml. N-HCl; 0·25 ml. NaOH (40 g./100 ml. water); 0·5 ml. M-phosphate buffer (pH 6·0); 25 mg. ethanol; side bulb: 0·2 ml. notatin (400 μg./ml.); 0·1 ml. catalase.)

Amount hydrolysed (mg.)	Uptake of oxygen (μl.)	Glucose (mg.)			Recovery (% theory)
		Observed	Corrected*	Theoretical	
3·348	128	1·03	1·07	1·12	95·5
2·701	104·5	0·84	0·87	0·90	97

* Correction applied for loss of glucose during hydrolysis.

Estimation of D-*glucose.* The presence of D-glucose in the new derivative of riboflavin was proved, and the amount estimated manometrically, by means of notatin (Keilin & Hartree, 1948).

The compound was hydrolysed at 100° with N-hydrochloric acid for 2·5 hr., in a Warburg manometer cup fitted with an air condenser; the completeness of the hydrolysis was checked by chromatography. The acid was neutralized and D-glucose estimated manometrically. Control experiments were performed with known amounts of glucose and riboflavin; even with these mild conditions of hydrolysis there was a significant loss of D-glucose, amounting to 4%. The results of this experiment are shown in Table 3.

The point of attachment of D-*glucose to riboflavin; reaction with sodium metaperiodate.* The uptake of periodate was estimated by the method of Forrest & Todd (1950). The progress of the reaction is shown in Fig. 2; the uptake was 4 mol. periodate/mol. flavin.

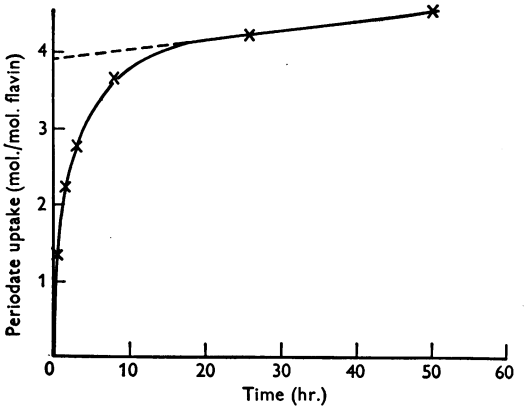
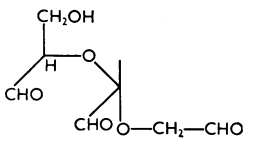


Fig. 2. Reaction of riboflavinyl glucoside with sodium metaperiodate.

Formaldehyde was estimated among the reaction products by the method of Boyd & Logan (1942); the quantity was less than one-quarter of the amount which would have been formed if the molecule had contained the structure CH_2OH—CHOH—.

Glucose is therefore attached either to the 4′ or 5′ position of riboflavin. The uptake of periodate shows that the formula must be either 6-(4′D-riboflavinyl)-D-glucopyranose or 5′D-riboflavin-D-glucopyranoside, i.e. riboflavinyl glucoside. The former alternative is excluded because glucose would be linked to riboflavin by an ether linkage, which would be stable to hydrolysis; the latter is held to be the correct alternative, because the glycosidic link is stable to hydrolysis with alkali and labile to hydrolysis with acid, and these are the observed stabilities of the linkage.

In riboflavinyl glucoside, 2 molecules of periodate are taken up by the glucose residue and 2 molecules by the riboflavin residue. The small amount of formaldehyde detected among the products of the reaction of the flavin with periodate is thought to arise from overoxidation of the end product (I):



(I)

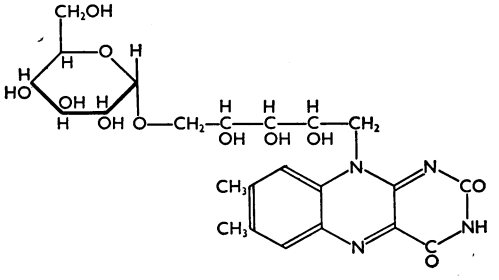
Overoxidation by periodate has been observed with similar compounds (Lindstedt, 1945; Halsall, Hirst & Jones, 1947), and a secondary breakdown of this type would produce, from this hypothetical end product, a compound capable of reacting further with periodate to form formaldehyde. The presence of formaldehyde among the products of the periodate titration could then be explained, as well as the slow reaction with periodate which occurs after the initial, comparatively fast, uptake of 4 mol./mol. flavin has been completed.

The proposed formula, riboflavinyl glucoside, was confirmed by the negative result given by the compound with the dinitrosalicylic acid reagent of Sumner & Sisler (1944), which indicated that the reducing group of glucose was involved in the linkage to riboflavin.

The configuration of the glucosidic link. This has not been definitely established, but in view of the following evidence it is suggested that the compound is an α-glucoside: (1) Sweet almond emulsin had no effect upon riboflavinyl glucoside. The very active β-glucosidase in this preparation hydrolyses

a wide range of β-glucosides (Pigman, 1944). (2) A preparation of α-glucosidase from dried baker's yeast (Krieble, Skau & Lovering, 1927) caused a slow breakdown of riboflavinyl glucoside to riboflavin; this preparation also hydrolysed maltose and α-methylglucoside, but was not shown to be entirely devoid of β-glucosidase activity. Different yeasts, grown under different conditions, produce α-glucosidases which between them can hydrolyse a number of α-glucosides (Gottschalk, 1950), but no single α-glucosidase has been prepared which is free from β-glucosidase and which has a wide range of action on α-glucosides. (3) The enzyme which produces riboflavinyl glucoside could be precipitated from the aqueous extract of the acetone powder of rat livers by half-saturation with ammonium sulphate; the precipitate dissolved in water, but the solution had no enzymic activity, unless an extract made by boiling a rat liver with water was added to the solution. Activity could also be restored by an aqueous solution of maltose or of glycogen, but not of glucose; α-glucose-1-phosphate inhibited the reactivation (preliminary experiments indicate that this inhibition is competitive). It is concluded that the enzyme catalyses a transglucosidation from maltose or glycogen to riboflavin. No transglycosidation has been reported which involves inversion of the configuration at the glycosidic link of the transferred carbohydrate residue (Bell, 1947).

The proof of the configuration of the glucosidic link may have to await chemical synthesis of the α- and β-glucosides. Meanwhile, the formula (II) is proposed as the probable structure of this new derivative of riboflavin.



II. 5′D-Riboflavin-D-glucopyranoside (riboflavinyl glucoside).

DISCUSSION

Riboflavinyl glucoside is a new derivative of riboflavin; this derivative of the vitamin was not observed by Crammer (1948) in any of the tissues which he investigated, but the reason may have been limitations in chromatographic technique. Riboflavin has been detected by the author in a concentrated extract of rat liver, although Crammer (1948) only detected riboflavin in preparations from spleen; further concentration of tissue extracts may reveal the natural occurrence of this new flavin. Bessey, Lowry & Love (1949) reported the presence of small amounts of riboflavin in various tissues, but their fluorimetric procedure was not designed to distinguish between riboflavin and its glucoside. Riboflavinyl glucoside has not been detected in urine; it would therefore appear to be different from a urinary flavin, the existence of which was postulated by Koschara (1935).

No function has so far been ascribed to the compound. It may undergo further metabolism and become incorporated into new nucleotides; flavin-X is a flavin dinucleotide of unknown structure (Sanadi & Huennuekens, 1950) and it is conceivable that riboflavinyl glucoside forms part of the molecule of flavin-X.

It should be possible to assess the significance of riboflavinyl glucoside when quantitative studies of the enzymic reaction have been completed, and the enzymic specificity determined. The combination of riboflavin with glucose may be one of a range of transglycosidations catalysed by the enzyme; the specific role of riboflavin will be tested by replacing riboflavin with some of the synthetic *iso*alloxazine derivatives as acceptors of glucose in the enzymic reaction.

SUMMARY

1. A new derivative of riboflavin, prepared by incubation of riboflavin with an enzyme obtained from rat liver, is described.

2. The elementary composition of the substance is $C_{23}H_{30}O_{11}N_4$; it has been identified as 5′D-riboflavin-D-glucopyranoside (riboflavinyl glucoside).

3. It is suggested that the glucosidic linkage has the α-configuration.

4. Preliminary investigations of the enzymic reaction indicate that the enzyme catalyses a transglycosidation of D-glucose from maltose or glycogen to riboflavin.

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Note on the Synthesis of the Acetic Acid Analogue of Thyroxine

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The acetic acid analogue of thyroxine has been prepared in order to determine its biological importance; biological experiments will be described in a later paper.

EXPERIMENTAL

Melting points are not corrected for emergent stem.

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*benzyl alcohol.* 3:5-Diiodo-4-(4′-methoxyphenoxy)benzaldehyde (Harington & Barger, 1927) (6·5 g.) was boiled under reflux with benzene (15 ml.) and 26 ml. of a solution of aluminium *iso*propoxide in *iso*propanol made from 5·5 g. Al and 200 ml. *iso*propanol. When a test portion of the reaction mixture no longer gave a precipitate with 2:4-dinitrophenylhydrazine, the product was extracted with dilute HCl, and the crystalline residue was collected and crystallized from 70% (v/v) ethanol. The yield was almost quantitative; after a further crystallization from benzene, the compound had m.p. 111–112°. (Found: I, 52·4. $C_{14}H_{12}O_3I_2$ requires I, 52·7%.)

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*benzyl chloride.* The above alcohol (3·6 g.) was dissolved in $CHCl_3$ (20 ml.) and cooled in ice-salt, and powdered PCl_5 (1·83 g.) was added in small portions. At the end of the addition, the solution was kept at room temperature for 1 hr., washed with water, dilute $NaHCO_3$ solution, and water again, and dried over $CaCl_2$; on concentrating the solution, the product separated and was collected and crystallized from ethanol. The yield was 3·6 g. (96%). After distillation of a small amount *in vacuo* and crystallization from acetic acid, the compound had m.p. 96·5–97°. (Found: I, 50·7. $C_{14}H_{11}O_2ClI_2$ requires I, 50·8%.)

3:5-*Diiodo*-4-(4′*methoxyphenoxy*)*benzyl cyanide.* The preceding compound (2·25 g.) was dissolved in ethanol (13·5 ml.) containing a trace of KI and boiled under reflux for 4 hr. with KCN (0·45 g.) in water (0·9 ml.); the solution was concentrated to a low volume under diminished pressure and diluted with water, when the product separated. The yield was 2·025 g. (91%). After crystallization from acetic acid, the compound had m.p. 140–142°. (Found: N, 2·7; I, 52·3. $C_{15}H_{11}O_2NI_2$ requires N, 2·9; I, 51·8%.)

3:5-*Diiodo*-4-(4′-*hydroxyphenoxy*)*phenylacetic acid.* The nitrile (1·525 g.) was boiled under reflux for 1 hr. with red P (1 g.) and a mixture of acetic acid (10 ml.) and hydriodic acid, sp.gr. 1·7 (12 ml.). After removal of P the solution was concentrated to dryness, treated with water containing a little bisulphite and concentrated again. The product was dissolved in hot 0·1 N-Na_2CO_3 solution, filtered and acidified at the boiling point with dilute HCl. On cooling, 1·235 g. (80%) of the acid separated; after crystallization from 50% (v/v) acetic acid it had m.p. 214–216·5°. (Found: I, 50·8. $C_{14}H_{10}O_4I_2$ requires I, 51·2%. 9·0 mg. of the acid required 1·74 ml. of 0·0106 N-NaOH for neutralization to phenolphthalein, whence mol.wt. = 490; calc. mol.wt., 496.)

3:5-*Diiodo*-4-(3′:5′-*diiodo*-4′-*hydroxyphenoxy*)*phenylacetic acid.* The iodination offered some difficulty at first, as the conditions used for the preparation of thyroxine from diiodothyronine gave only gummy products which could not be purified; eventually success was achieved by the following method: the diiodo acid (25 mg.) was dissolved in methanol