S 45. *The Synthesis of Thyroxine and Related Substances. Part IV. Polarographic Determination of Thyroxine.*

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A polarographic procedure for the determination of small amounts of thyroxine in iodinated casein preparations has been developed. The polarographic behaviour of 23 related aromatic iodo-compounds was examined and the conditions for the hydrolysis of iodinated casein have been studied. It is shown that chemical methods for the determination of thyroxine based on Leland and Foster's butanol extraction procedure give high results.

In Part I (this vol., p. S 185) we stated our intention to study the preparation of thyroxine by: (*a*) chemical synthesis, and (*b*) oxidation of 3 : 5-di-iodotyrosine and its derivatives, both free and when bound in proteins. For work on the latter problem a reliable method was needed for estimating small quantities of thyroxine in the presence of excess of 3 : 5-di-iodotyrosine and other iodo-amino-acids.

The various biological, chemical, and physical methods proposed for the determination of thyroxine have been critically examined. The xenopus tadpole test developed by Deansley and Parkes (*J. Endocrin.*, 1944—6, **4**, 324) was studied in our Pharmacology Unit; it was not possible to attain the accuracy claimed, and the test will not be discussed in this paper.

In Kendall's original chemical method (*J. Biol. Chem.*, 1914, **19**, 251) the total iodine in desiccated thyroid glands was determined and considered to be a measure of thyroid activity. Harington and Randall (*Quart. J. Pharm.*, 1929, **2**, 501), however, showed that desiccated

thyroid powder contained appreciable quantities of di-iodotyrosine and that, by subjecting the powder to mild alkaline hydrolysis and acidifying the hydrolysate, two fractions, a soluble fraction containing di-iodotyrosine and an insoluble one containing thyroxine, could be obtained. An iodine determination on the latter fraction was believed to constitute a reliable thyroxine assay. Doery (*ibid.*, 1945, **18**, 384), who used the method for thyroid tablets containing lactose, found that the reliability of the method depended on a compromise between loss of thyroxine in the acid and deposition of some di-iodotyrosine on the precipitated thyroxine. Some inorganic iodide may have been adsorbed on the precipitate.

A new approach to the problem was made when Leland and Foster (*J. Biol. Chem.*, 1932, **95**, 165) determined the partition coefficients of thyroxine, di-iodotyrosine, and inorganic iodide between butanol and alkali and showed that butanol could extract thyroxine almost quantitatively. They recommended that thyroid powder should be hydrolysed by refluxing with 2N-sodium hydroxide for 18 hours. Blau (*ibid.*, 1935, **110**, 351) claimed that a better separation was obtained by refluxing with 8% baryta solution for 6 hours, extracting the hydrolysate at pH 3·5—4·0 with butanol, and washing the butanol extract with alkali. The iodine content of the butanol extract was taken as a measure of the thyroxine originally present. Reinecke, Turner, Kohler, Hoover, and Beezley (*ibid.*, 1945, **161**, 599), who first applied the technique to iodinated casein, found it necessary to reflux with 40% baryta for 20 hours and to extract the butanol phase twice with alkali. In this way they obtained results that agreed well with biological assay values determined by the increase in metabolic rate of guinea pigs.

Other methods based on absorption spectra (Reinecke and Turner, *Res. Bull. Agric. Exp. Station, Coll. Agric., Univ. Missouri*, No. 355, 1942), the red colour formed with sodium nitrite in ammoniacal solution (Roche and Michel, *Biochim. Biophys. Acta*, 1947, **1**, 335), the red colour formed with diazobenzenesulphonic acid in sodium carbonate solution (Moser, *Experientia*, 1947, **3**, 119), or the purple colour formed with diazotised N^1-diethylsulphanilamide in alkaline solution (Winikoff and Trikojus, *Biochem. J.*, 1948, **42**, 475) have been suggested.

We investigated the method of Reinecke *et al.*, and found that, in order to comply with their conditions for the hydrolysis, it was necessary to concentrate the filtered incubates under reduced pressure to such a volume that a gum tended to separate out. In order to eliminate this stage and its inherent losses due to foaming, the iodinated casein was precipitated at pH 3·8. After the solution had been cooled for two hours, the precipitate was isolated as a friable powder, suitable for hydrolysis, by centrifuging, washing, recentrifuging, and drying in a vacuum over phosphoric oxide. Control experiments in which the iodinated caseins were freeze-dried showed that no thyroxine had been lost during the precipitation and subsequent isolation.

The procedure for estimating iodine was examined with a view to simplifying Kendall's method. Leland and Foster's sodium hydroxide fusion technique was found to be more satisfactory than Waters and Beal's potassium carbonate fusion method (*J. Amer. Pharm. Ass.*, 1945, **34**, 297). The after-blueing of the starch indicator, observed by the latter authors, did not cause us any trouble.

In an effort to avoid the complicated fusion step, we successfully applied Harington's hydrogenolysis procedure (*Biochem. J.*, 1926, **20**, 300) to the butanol extract. Palladised calcium carbonate was used as catalyst to hydrogenolyse thyroxine in alkaline solution to thyronine, and the liberated sodium iodide was determined in acid solution by the Volhard method. We found that the butanol fraction could be hydrogenolysed directly at room temperature and pressure by mixing with ethyl alcohol and aqueous alkali. Before titration, the mixture was homogenised by the addition of more alcohol. The great dilution made the Volhard end-point rather indistinct, but this difficulty was partly overcome by practice.

The hydrogenolysis and fusion methods were compared and found to give almost identical results. The advantages of the fusion method, *e.g.*, the well-defined end-point and the high factor (*i.e.*, 1·00 ml. of 0·1N-sodium thiosulphate equivalent to 0·00324 g. of thyroxine) as compared with that of the Volhard titration (*i.e.*, 1·00 ml. of 0·1N-silver nitrate equivalent to 0·0194 g. of thyroxine), appeared to outweigh its disadvantages.

In view of the unsatisfactory nature of the above methods. we have re-examined Simpson and Traill's polarographic procedure (*ibid.*, 1946, **40**, 116). These authors showed that thyroxine can be determined polarographically in the presence of di-iodotyrosine in a 40% alcoholic 0·5N-sodium carbonate solution containing 1% of either tetramethylammonium iodide or bromide, and subsequently Simpson, Johnston, and Traill (*ibid.*, 1947, **41**, 181) devised a method for determining thyroxine in the acid-insoluble fractions of iodinated " Ardein " (ground-nut protein), casein, and thyroid powder. 100 G. of the protein were hydrolysed by baryta, and about 1 g. of a partly purified acid-insoluble active fraction was isolated according to the method

of Ludwig and von Mutzenbecher (*Z. physiol. Chem.*, 1939, **258**, 195). The thyroxine content of this fraction as determined polarographically was found to agree fairly well with that calculated from the acid-insoluble iodine value, all the iodine being assumed to be thyroxine iodine. This observation is at variance with that reported by Deansley and Parkes (*J. Endocrin.*, 1944—6, **4**, 356), who found that there was ten times as much acid-insoluble iodine in iodinated casein as in a dried thyroid preparation showing the same biological activity.

We have studied the polarographic procedure in greater detail and have tried to adapt the technique for the estimation of smaller quantities of thyroxine than those studied by Simpson *et al.*

It should be remembered that, unless special precautions are taken, different results are to be expected from the biological, chemical, and physical methods. The first methods assay all substances with thyroid activity regardless of chemical composition, the second determine total butanol-soluble iodine, and the third determines total iodinated diphenyl ethers.

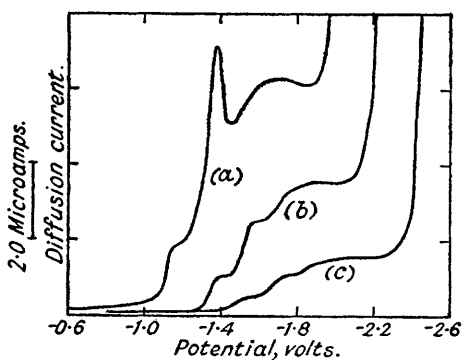
Experimental.

A standard Cambridge polarograph was employed; it had been calibrated to read directly in microamps. All potentials were measured against the saturated calomel electrode. The dropping-mercury capillary had the following characteristics: at a pressure of 55·5 cm. of mercury, the drop-time (t) on open circuit in 0·1N-potassium chloride at 25° was 3·13 secs., the weight of mercury dropping per second (m) was 1·82 mg., and $m^{2/3}t^{1/6}$ was 1·80.

The thyroxine preparations were tested in a 20% *iso*propyl-alcoholic solution containing 1·0% of tetramethylammonium bromide and 2·65% (0·5N) sodium carbonate (pH 11·3). Freshly distilled *iso*propyl alcohol was used, since many laboratory batches of ethyl alcohol contained traces of aldehydes or ketones that interfered with the thyroxine step. Tetramethylammonium bromide is much more soluble in water than the iodide and was thus more convenient for this type of work. All reagents were tested to ensure that they did not contain impurities that would interfere with the thyroxine step. Oxygen was removed from all solutions by bubbling with nitrogen for 10 minutes.

When examined in this way, thyroxine gave a series of three polarographic steps (cf. Fig. 1). The second step for solutions containing more than 0·025% of thyroxine was surmounted by a distinct maximum, which could be suppressed by adding 0·05% of gelatin to the solution. Concentrated 3 : 5-di-iodotyrosine solutions did not form a maximum. The first thyroxine step with a half-wave potential of −1·12 v. (versus the saturated calomel electrode) was the characteristic step for thyroxine, and, since it was not masked by the first di-iodotyrosine step (*i.e.*, half-wave potential of −1·51 v.), it could be used for the estimation of thyroxine in the presence of di-iodotyrosine. A better separation of the thyroxine and the di-iodotyrosine step was obtained by extending the potential scale of the polarograph in the ratio of 2 : 3 (cf. Werthessen and Baker, *Endocrinology*, 1945, **36**, 351).

Fig. 1.



Polarograms for: (a) 0·05% *thyroxine*, (b) 0·025% *thyroxine*, (c) 0·010% *thyroxine*.

A calibration curve connecting the heights of the first and of the total thyroxine step with concentration over the concentration range in the final solution of 0·00006—0·0012M (0·05—1·0 mg. per ml.) is shown in Fig. 2. Over this range, the relationship between diffusion current (*i.e.*, step height) and concentration was approximately linear. A similar calibration curve for di-iodotyrosine is shown in Fig. 3.

Polarography of Substances related to Thyroxine.—In addition to thyroxine, 23 related substances were examined polarographically in order to find out whether they would interfere with our assay procedure. These substances, which had the properties listed below, were tested at a concentration level of 0·025—0·050% in a 20% *iso*propyl-alcoholic solution containing 1·0% of tetramethylammonium bromide and 2·65% of sodium carbonate (pH 11·3). Nitrogen was bubbled through the solutions to remove oxygen, and the measurements were taken when the polarograph cell was immersed in a thermostat maintained at 25° ± 0·1°.

(i) DL-Tyrosine, m. p. 288—289° (decomp.).

(ii) DL-*N*-Benzoyltyrosine ethyl ester, m. p. 122—123° (Curtius and Donselt, *J. pr. Chem.*, 1917, **95**, 349).

(iii) DL-3 : 5-Di-iodotyrosine, m. p. 195—196° (decomp.) (m. p. 195°; Bauer and Strauss, *Ber.*, 1936, **69**, 245).

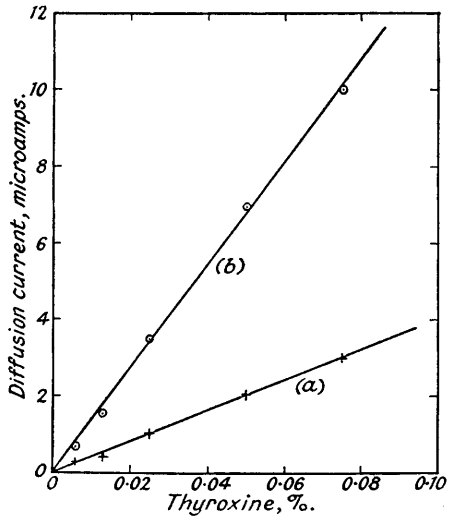
(iv) DL-*N*-Acetyl-3 : 5-di-iodotyrosine, m. p. 216—217° (decomp.) (Found : C, 27·8; H, 2·6; N, 3·0; I, 54·0. Calc. for $C_{11}H_{11}O_4NI_2$: C, 27·8; H, 2·3; N, 2·9; I, 53·5%) (m. p. 205—206°, decomp.; Myers *J. Amer. Chem. Soc.*, 1932, **54**, 3718).

(v) DL-*N* : 4-Diacetyl-3 : 5-di-iodotyrosine, m. p. 208—209° (Found : C, 28·8; H, 2·7; N, 2·9; I, 49·0. Calc. for $C_{13}H_{13}O_5NI_2,0{\cdot}5H_2O$: C, 29·6; H, 2·7; N, 2·7; I, 48·5%) (m. p. 186°; Myers, *loc. cit.*).

(vi) DL-N-*Acetyl*-3 : 5-*di-iodotyrosine* n-*butyl ether*, micro-crystals, m. p. 157—159° (Found : C, 34·0; H, 2·8; N, 2·67; I, 47·2. $C_{15}H_{19}O_4NI_2$ requires C, 33·9; H, 3·5; N, 2·65; I, 47·8%). The L-form of this substance has been described by Woolley (*J. Biol. Chem.*, 1946, **164**, 11).

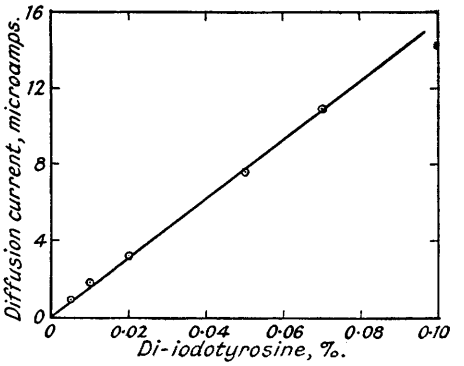
(vii) The corresponding *benzyl ether*, micro-crystals m. p. 100—102° from benzene (Found : N, 2·15; I, 39·6. $C_{18}H_{17}O_4NI_2,C_6H_6$ requires N, 2·16; I, 39·6%). The L-form of this substance has been described by Woolley (*loc. cit.*).

FIG. 2.



Calibration curves for thyroxine: (a) *First thyroxine step.* (b) *Total thyroxine step.*

FIG. 3.



Calibration curve for di-iodotyrosine.

(viii) The analogous 4′-*nitrobenzyl ether*, needle crystals, m. p. 115° (Found : N, 5·2; I, 40·5. $C_{18}H_{16}O_6N_2I_2$ requires N, 4·6; I, 41·7%). The L-form of this substance has been described by Woolley (*loc. cit.*).

(ix) DL-Thyronine, m. p. 254—256° (decomp.) (m. p. 253—254°, decomp.; Harington, *Biochem. J.*, 1926, **20**, 300).

(x) DL-3 : 5-Di-iodothyronine, m. p. 256—257° (decomp.), prepared by the method described in Part III (this vol., S 199).

(xi) DL-Thyroxine, m. p. 234—235° (decomp.), prepared by the method described in Part III.

(xii) DL-*N*-Acetylthyroxine, m. p. 214—215° (decomp.) (m. p. 210—215°; Ashley and Harington, *Biochem. J.*, 1928, **22**, 1436).

(xiii) Benzoic acid, m. p. 121—122°.

(xiv) *o*-Iodobenzoic acid, m. p. 161° (m. p. 162°; Cohen and Raper, *J.*, 1904, **85**, 1271).

(xv) *m*-Iodobenzoic acid, m. p. 187° (m. p. 187—188°; *idem, ibid.*).

(xvi) *p*-Iodobenzoic acid, m. p. 270° (m. p. 270°; Flaschner and Rankin, *Monatsh.*, 1910, **31**, 44).

(xvii) 4-Hydroxybenzoic acid, m. p. 215° (m. p. 214—215°; Willstätter and Mieg, *Annalen*, 1915, **408**, 76).

(xviii) 3 : 5-Di-iodo-2-hydroxybenzoic acid, m. p. 220° (m. p. 220—230°, decomp.; Anschütz, Robitsek, and Schmitz, *ibid.*, 1906, **346**, 330).

(xix) 3 : 5-Di-iodo-4-hydroxybenzoic acid, m. p. 239° (m. p. 237°; Paal and Mohr, *Ber.*, 1896, **29**, 2302).

(xx) 3 : 5-*Di-iodo*-4-*acetoxybenzoic acid*. Acetic anhydride (1·35 ml.) was added slowly to a solution of 3 : 5-di-iodo-4-hydroxybenzoic acid (5 g.) in 2N-sodium hydroxide (20 ml.). The mixture was shaken for 10 minutes and then acidified with hydrochloric acid. The precipitate was filtered off, dried, and crystallised first from aqueous alcohol, then from glacial acetic acid. The *acid* (3·95 g.; 71%) melted at 224° (Found : C, 25·2; H, 1·6; I, 59·6. $C_9H_6O_4I_2$ requires C, 25·0; H, 1·4; I, 58·8%).

(xxi) 3 : 5-Di-iodo-4-methoxybenzoic acid, m. p. 260° (m. p. 255—256°, decomp.; Wheeler and Liddle, *Amer. Chem. J.*, 1909, **42**, 458).

(xxii) 3 : 5-Di-iodo-4-(4′-methoxyphenoxy)benzoic acid, m. p. 232—234° (cf. Part III).
(xxiii) 3 : 5-Di-iodo-4-(4′-hydroxyphenoxy)benzylhydantoin (cf. Part III).
(xxiv) 3 : 5-Di-iodo-4-(4′-methoxyphenoxy)benzylhydantoin, m. p. 213—215° (cf. Part III).

Our polarograms for derivatives of benzoic acid and tyrosine (Table I) and for diphenyl ethers (Table II) confirm that iodine-free acids are not reduced at the dropping-mercury electrode and show that acids containing one, two, or four iodine atoms yield one, two, or three polarograph steps, respectively. However, the two single steps for di-iodo-substances such as 3 : 5-di-iodotyrosine frequently merge into one double step with a half-wave potential of about −1·60 v. Each single step, except the second step formed by thyroxine and *N*-acetylthyroxine, probably corresponds to the reduction of one iodine atom, *i.e.*, a two-electron change. The second step for thyroxine and *N*-acetylthyroxine corresponds to the reduction of two iodine atoms, *i.e.*, a four-electron change.

TABLE I.

*Polarograms for Derivatives of Benzoic Acid and Tyrosine.**

Substance.	Molar concn. (× 10⁴).	First step.		Second step.		Third step.	
		P.	*C.*	*P.*	*C.*	*P.*	*C.*
Benzoic acid	8·0	No steps	—	—	—	—	—
o-Iodobenzoic acid	20	—	—	−1·50	8·0	—	—
m-Iodobenzoic acid	20	—	—	−1·44	7·5	—	—
p-Iodobenzoic acid	20	—	—	−1·44	8·4	—	—
4-Hydroxybenzoic acid	18	No steps	—	—	—	—	—
3 : 5-Di-iodo-2-hydroxybenzoic acid	12·8	−1·25	4·5	−1·50	3·2	—	—
3 : 5-Di-iodo-4-hydroxybenzoic acid	6·2	—	—	−1·55	1·96	−1·72	1·66
3 : 5-Di-iodo-4-acetoxybenzoic acid	5·8	−1·08	1·63	−1·25	1·79	−1·63	0·48
3 : 5-Di-iodo-4-methoxybenzoic acid	12·5	−1·20	3·8	−1·50	3·5	—	—
Tyrosine	14	No steps	—	—	—	—	—
N-Benzoyltyrosine ethyl ester	3·2	No steps	—	—	—	—	—
3 : 5-Di-iodotyrosine	11·4	—	—	−1·51	3·7	−1·72	3·9
N-Acetyl-3 : 5-di-iodotyrosine	10·5	—	—	−1·47	2·85	−1·67	3·35
N : 4-Diacetyl-3 : 5-di-iodotyrosine	4·8	—	—	−1·48	1·15	−1·68	1·91
N-Acetyl-3 : 5-di-iodotyrosine *n*-butyl ether	9·5	−1·16	3·28	−1·42	2·70	—	—

* *P* = half-wave potential, in volts; *C* = diffusion current, in microamps.

TABLE II.

*Polarograms for Diphenyl Ethers.**

Substance.	Molar concn. (× 10⁴).	First step.		Second step.		Third step.	
		P.	*C.*	*P.*	*C.*	*P.*	*C.*
3 : 5-Di-iodo-4-(4′-methoxyphenoxy)-benzoic acid	5·0	−1·11	1·30	−1·30	1·40	—	—
N-Acetyl-3 : 5-di-iodotyrosine benzyl ether	8·9	−1·09	2·55	−1·33	2·73	—	—
N-Acetyl-3 : 5-di-iodotyrosine 4′-nitrobenzyl ether	8·2	−0·78	5·40	−0·94	2·45	−1·34	2·75
Thyronine	8·9	No steps	—	—	—	—	—
3 : 5-Di-iodothyronine	9·5	−1·18	2·71	−1·37	2·56	—	—
Thyroxine	3·1	−1·12	1·02	−1·30	1·60	−1·51	0·90
N-Acetylthyroxine	3·0	−1·10	0·90	−1·29	1·40	−1·50	0·60
4-(4′-Hydroxyphenoxy)-3 : 5-di-iodo-benzylhydantoin	9·5	−1·19	3·10	−1·39	2·50	—	—
4-(4′-Methoxyphenoxy)-3 : 5-di-iodo-benzylhydantoin	9·0	−1·10	2·56	−1·30	2·66	—	—

* *P* = half-wave potential, in volts; *C* = diffusion current, in microamps.

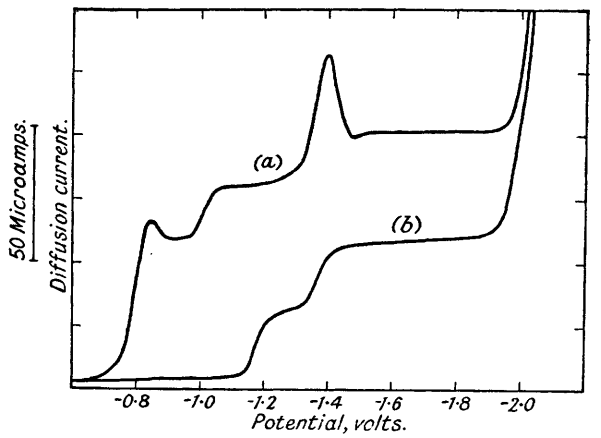
All the steps are well defined and are suitable for the determination of the substances in simple solutions. Some characteristic polarograms for *N*-acetyl-3 : 5-di-iodotyrosine 4′-nitrobenzyl ether and 3 : 5-di-iodothyronine are reproduced in Fig. 4. The maxima that surmount the steps formed by substances such as the 4-nitrobenzyl ether are readily suppressed by the addition of 0·05% of gelatin.

Substitution in the 4-hydroxy-group of 3 : 5-di-iodobenzoic acid and 3 : 5-di-iodotyrosine facilitates the reduction of the acids, and the derivatives so formed, *e.g.*, 3 : 5-di-iodo-4-(4′-methoxyphenoxy)-benzoic acid and 3 : 5-di-iodothyroxine, will interfere with the polarographic determination of thyroxine. Acetylation at the *N*-position of tyrosine and thyroxine has no effect on their reduction potentials. The large step formed by *N*-acetyl-3 : 5-di-iodotyrosine 4′-nitrobenzyl ether at −0·78 v. is probably a six-electron step and is to be associated with the reduction of the nitro-group.

It is noteworthy that 3 : 5-di-iodo-4-acetoxybenzoic acid was hydrolysed in the alkaline polarographic solution (pH 11·3). After such a solution had stood at room temperature for six hours, the steps at −1·08 and −1·25 v. disappeared and were replaced by one double step at −1·63 v. *N* : 4-Diacetyl-3 : 5-di-iodotyrosine did not form a large step at −1·10 v., and it is conjectured that at pH 11·3 it is hydrolysed more rapidly than the corresponding benzoic acid derivative.

Determination of Thyroxine in Iodinated Casein Hydrolysates.—By direct polarographic examination, it was possible to estimate thyroxine in simple mixtures containing a ten-fold excess of 3 : 5-di-iodotyrosine, but for mixtures containing a higher proportion of di-iodotyrosine (*e.g.*, iodinated casein hydrolysates) the height of the first thyroxine step could not be measured with the desired precision. The excess of di-iodotyrosine was removed from such samples by Leland and Foster's method. The thyroxine was extracted by means of *n*-butanol from a slightly acid solution (pH 3·5) of the hydrolysate, and the extract washed with a mixture of sodium hydroxide and sodium hydrogen carbonate to eliminate di-iodotyrosine. Leland and Foster claimed that *n*-butanol eluted about 92% of the thyroxine and only 2·5% of the di-iodotyrosine from a solution containing 0·24 mg. and 1·6 mg. respectively per ml., but our polarographic results showed that, whereas their data for thyroxine are approximately correct, about 12% of the di-iodotyrosine was extracted. Although the amount of di-iodotyrosine left behind was sufficient to impair the accuracy of the chemical assay, it did not interfere with the recording of a polarogram containing a well-defined thyroxine step that was suitable for analytical purposes.

FIG. 4.



Polarograms for: (a) 0·050% N-*Acetyl*-3 : 5-*di-iodotyrosine 4'-nitrobenzyl ether.* (b) 0·050% 3 : 5-*Di-iodothyronine.*

In order to check the butanol extraction technique, small quantities of synthetic thyroxine (5·0—6·0 mg.) were added to 100-ml. portions of iodinated casein hydrolysates, the solutions were extracted, and the amount of thyroxine was determined, with reasonable recoveries (cf. Table IV).

Comparison with the Chemical Method of Reinecke et al.—A number of *n*-butanol extracts of hydrolysed casein were polarographed, and the results compared with those obtained by our modification of the method of Reinecke *et al.* The values for the true thyroxine content, as calculated from the height of the first polarographic step at −1·12 v., were about one-third to one-half of those obtained chemically (cf. Table III) but, if the thyroxine data were calculated from the height of the total step at −1·70 v., no di-iodotyrosine being assumed to be present, the polarographic figures (" total ") showed better agreement with the chemical assay values. This result is not unreasonable, when it is remembered that for the chemical assay we assumed that all the iodine in the *n*-butanol extract is derived from thyroxine, whereas we have shown (see above) that *n*-butanol extracts appreciable quantities of di-iodotyrosine.

TABLE III.

Comparison of Chemical and Polarographic Assays for Iodinated Casein Hydrolysate.

	Concn. of thyroxine, mg./ml.				Concn. of thyroxine, mg./ml.		
No. of solution.	Chem. assay.	Polarographic assay. True.	" Total."	No. of solution.	Chem. assay.	Polarographic assay. True.	" Total."
11	0·15	0·07	0·20	19	0·09	0·04	0·11
12	0·25	0·09	0·27	21	0·09	0·04	0·10
15	0·25	0·09	0·27	23	0·12	0·03	0·10
16	0·18	0·05	0·13	NCB 1 & 2	0·15	0·08	0·16
17	0·15	0·05	0·15	S	0·15	0·05	0·15
18	0·15	0·05	0·12				

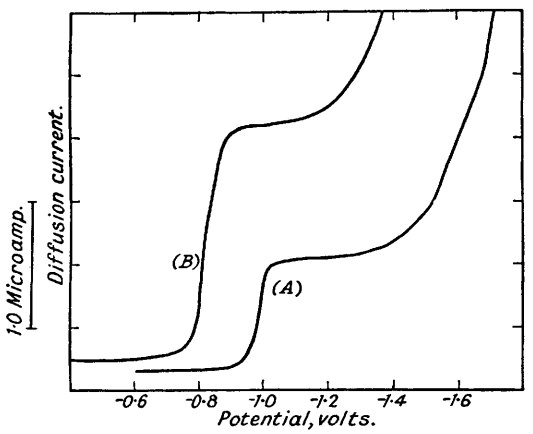
Hydrolysis of Iodinated Casein.—Experiments were undertaken on a special batch of iodinated casein to find the best hydrolytic procedure. The problem had previously been attacked by several groups of investigators; Reinecke *et al.* had recommended the use of 40% barium hydroxide for this purpose.

Known amounts of synthetic thyroxine and di-iodotyrosine were added to samples of casein and iodinated casein, the mixtures were hydrolysed for 6 hours with 10-ml. portions of alkali, and the hydrolysates were examined for thyroxine polarographically; the results (Table IV) confirmed that 40% barium hydroxide and 1·0N-sodium hydroxide are the best hydrolysing agents. The percentage of recovered thyroxine decreased as the quantity of added thyroxine was increased. Sodium carbonate and sodium hydroxide solutions below 1·0N gave low thyroxine recoveries.

An attempt to improve the recovery of thyroxine by hydrolysis in an atmosphere of nitrogen was unsuccessful. Larger quantities of alkali and hydrolysis for longer periods also gave lower yields of thyroxine.

We were surprised to find that when the time of hydrolysis with 1·0N-sodium hydroxide was reduced below 3 hrs., and the partial hydrolysate examined directly in the usual *iso*propyl-alcoholic solution, the polarogram had a well-defined step at −0·8 v. A casein solution that had been partly hydrolysed with sodium hydroxide gave a similar step (cf. Fig. 5), which disappeared if the hydrolysis was continued for longer. A further study of this step should be of interest.

FIG. 5.



(*A*) *Two g. of casein refluxed with* 10 *ml. of* 1·0N-NaOH *for* 5 *hrs. and then made up to* 20 *ml. with* 2·0 *ml. of* 10% *tetramethylammonium bromide and* 4·0 *ml. of* iso*propyl alcohol.*

(*B*) *Two g. of iodinated casein refluxed with* 10 *ml. of* 1·0N-NaOH *for* 1 *hr. and then made up to* 20 *ml. with* 2·0 *ml. of* 10% *tetramethylammonium bromide and* 4·0 *ml. of* iso*propyl alcohol.*

TABLE IV.

Recovery of added Thyroxine.

Material.	Wt. of material, g.	Wt. of added thyroxine, mg.	Method of hydrolysis.	Thyroxine recovered, mg.	Added thyroxine recovered, %.
Casein	2·0	0	1·0N-NaOH	0·4	—
	2·0	5·0	,,	5·0	92
	2·0	10·0	,,	8·4	80
Iodocasein	1·0	0	40% $Ba(OH)_2$	3·3	—
	1·0	5·0	,,	8·4	102
	1·0	10·0	,,	11·4	81
	2·0	0	1·0N-NaOH	6·0	—
	2·0	5·0	,,	10·0	80
	2·0	10·0	,,	13·4	74

Our recommended procedure for the estimation of thyroxine in iodinated casein containing large amounts of di-iodotyrosine is as follows. Hydrolyse 1·0 g. of iodinated casein (containing about 5 mg. of thyroxine) by means of 40% baryta according to the method of Reinecke *et al.* (*loc. cit.*). Adjust the pH value of the resulting 100 ml. of solution to 3·5 by means of dilute hydrochloric acid, and extract the solution with one 50-ml. portion followed by two 25-ml. portions of *n*-butanol. Wash the butanol extract with two 50-ml. portions of a solution containing 16% of sodium hydroxide and 5% of sodium hydrogen carbonate, and evaporate to dryness under reduced pressure. Dissolve the residue in 10 ml. of 1·0N-sodium carbonate, transfer to a 20-ml. graduated flask, add 2·0 ml. of 10% tetramethylammonium bromide and 4·0 ml. of *iso*propyl alcohol, and make the solution up to 20 ml. with distilled water. Polarograph a 3·0-ml. oxygen-free portion of the final solution over the potential range of −0·6 to −2·0 v. Measure the height of the first thyroxine and the combined thyroxine and di-iodotyrosine steps.

For preparations containing more than 0·5% of thyroxine, the accuracy of the method is about ±10%.

Discussion.—Our polarographic procedure is suitable for the determination of much smaller amounts of thyroxine than the method described by Simpson, Johnston, and Traill, but is less sensitive than those of Reinecke *et al.*, and Roche and Michel. The smallest quantity that can be detected polarographically is about 0·1% of thyroxine in 1·0 g. of iodinated protein, whereas Reinecke *et al.* claim the assay of much smaller amounts.

The polarographic method has the advantage that it can differentiate between thyroxine and 3 : 5-di-iodotyrosine and does not depend on the non-extraction of di-iodotyrosine by *n*-butanol.

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