THE DETERMINATION OF THYROXINE WITH SPECIAL REFERENCE TO TABLETS

By D. C. M. Adamson, A. P. Domleo, J. P. Jefferies and W. H. C. Shaw

From Glaxo Laboratories, Ltd., Greenford, Middlesex

Received July 3, 1952

Chemical methods for the determination of thyroxine are generally based on measuring the organically combined iodine. As a rule organic matter is destroyed by ignition with one or more of the alkali carbonates[1] (cf. the methods specified for thyroid in the British Pharmacopœia, 1948, and in the United States Pharmacopœia XIV). The iodide resulting from the ignition is determined directly[2,3] or, more frequently, by subsequent oxidation to iodate with potassium permanganate,[4,5] hypochlorite[1,6] or bromine[7]; after acidification and removal of excess of oxidising agent, iodide is added, the liberated iodine being determined volumetrically or colorimetrically. Various other methods have been suggested[8,9,10] to avoid the preliminary ignition.

Although capable of high accuracy, determination of thyroxine by ignition with alkali suffers from interference by inorganic iodides and other organic iodine compounds, so that with impure materials preliminary separation of the thyroxine is usually necessary.[11,12,13,14] Moreover, the method is tedious for large numbers of determinations.

The recent commercial availability of synthetic L-thyroxine[15] led us to investigate other possible methods of determination, particularly those of potential value for the analysis of tablets. Since the activity of L-thyroxine is extremely high, the amounts available for analysis are often small (e.g., 50 to 100 μg. of L-thyroxine per tablet) and a satisfactory method must be of high sensitivity. Three methods have been studied: ultra-violet absorption, polarography and a colorimetric procedure based upon the orange colour developed by treating thyroxine with nitrous acid and then with ammonia.[16]

Preparation of purified sodium L-thyroxine.—Specially purified sodium L-thyroxine, prepared by the method of Chalmers *et al.*,[15] was dried at 40° C. Found: Na, 2·34; I, 57·2; H_2O, 10·2 per cent. $C_{15}H_{10}O_4NI_4Na$, $5H_2O$ requires Na, 2·58; I, 57·2; H_2O, 10·1 per cent.

Ultra-violet Absorption

The ultra-violet absorption of thyroxine has been studied by a number of workers,[17,18,19,20] and curve A in Figure 1 is in close agreement with the results recently published by Reinecke and Turner[21] for wavelengths above 260 mμ. Besides the maximum at 325 mμ a second much larger maximum occurs at approximately 227 mμ ($E_{1\text{ cm.}}^{1\text{ per cent.}} = 620$), but this is of limited value, since interference by irrelevant absorption is likely at such wavelengths and similar maxima are exhibited by closely related substances (B and C, Fig. 1).

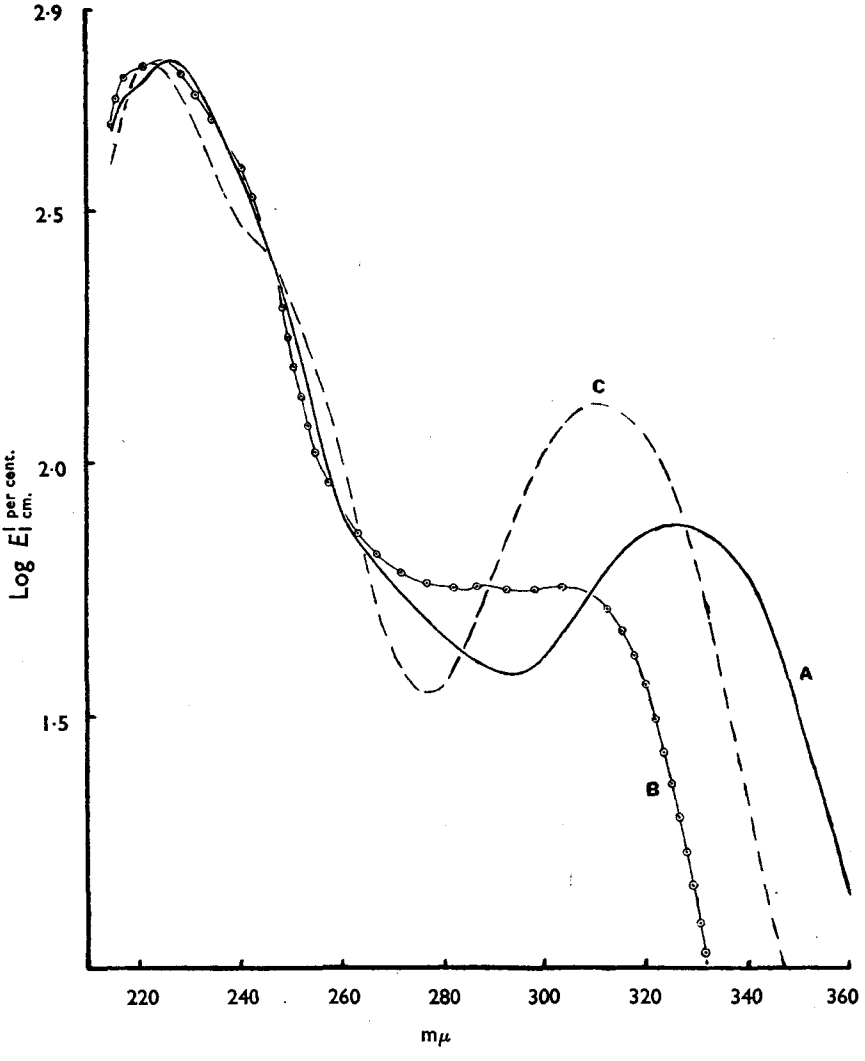

FIG. 1. Ultra-violet absorption spectra in 0·1 N sodium hydroxide.
A, sodium L-thyroxine. B, 3;5-diiodo-L-thyronine. C, 3;5-diiodo-L-tyrosine.

In practice, ultra-violet absorption has not been found of great value for the analysis of thyroxine, though the ratio of the extinction at 325 mμ (maximum) to that at the minimum near 295 mμ provides useful information. Any marked deviation from a value of 1·98 for this ratio suggests the presence of an impurity. The extinction of sodium L-thyroxine at 325 mμ is relatively low ($E^{1\%}_{1cm.} = 76$); nevertheless, this property may be made a basis for determination in suitable preparations, though interference by tablet excipients limits the value of the method.

POLAROGRAPHY

The polarographic reduction of thyroxine was first reported by Simpson and Traill,[22] who used a base solution containing 1 per cent. of tetramethylammonium bromide in a mixture of ethanol (2 vols.) and 0·5 N sodium carbonate (3 vols.). Borrows, Hems and Page[23] retained the tetramethylammonium bromide, but used a final concentration of 0·5 N sodium carbonate in 20 per cent. v/v *iso*propanol. They reported three steps having half-wave potentials at − 1·12 V, − 1·30 V and − 1·51 V measured against a saturated calomel electrode, the second step being surmounted by a prominent maximum. The height of either the first or the total step could be used for quantitative purposes, though the first was preferred, because it was not affected by the presence of 3:5-diiodotyrosine. 3:5-Diiodothyronine, however, interfered, giving steps at − 1·18 V and − 1·37 V.

Preliminary trials with the method using commercial samples of sodium L-thyroxine disclosed considerable discrepancies in the ratios of the first to the total step height. This was apparently due to the presence of maxima of varying heights on the first step. Specially purified samples gave polarograms with a pronounced maximum on the first and a large one on the second step (A, Fig. 2). Further investigation into the effect of differences in composition of the base solution appeared desirable.

A Cambridge pen-recording polarograph was used throughout. The capillary constants in 0·1 N potassium chloride at 25° C. on open circuit were $m = 1{\cdot}83$ mg./sec., $t = 3{\cdot}15$ sec., and all polarograms were recorded at 25 (± 0·1) °C. against a saturated calomel electrode. Oxygen was removed from all solutions by thorough bubbling with nitrogen. Purified sodium L-thyroxine prepared as described above was used as standard.

(*a*) iso*Propanol concentration.*—*iso*Propanol was purified by distillation over sodium hydroxide and zinc dust. Differences in concentration in the base solution affected the height of both the first and total steps to the same extent, as shown in Table I, where 20 per cent. v/v is regarded as standard.

(*b*) *Tetramethylammonium bromide concentration.*—Omission of this ingredient caused much distorted polarograms (E, Fig. 2): but between 0·75 and 1·25 per cent. w/v the concentration is not critical.

(*c*) *Sodium carbonate concentration.*—Variations in strength from 0·4 to 0·6 N had no effect on the shape of the polarogram nor on the step height.

(*d*) *Suppression of the maxima.*—By addition of graded amounts of gelatin, the maxima on both first and second steps may be suppressed (B, C and D, Fig. 2). However, the amount required must be very carefully adjusted, since too little gives a fictitiously high first step and too much causes distortion. The variations in the maxima observed on the first steps given by commercial preparations of sodium L-thyroxine may well be due to traces of impurities acting in a similar manner. Although the shape of the unsuppressed first step may be of possible value as a guide to quality, it has been found of little value for quantitative

purposes. Figure 2 and Table I show the effects of varying the concentration of gelatin. Methylcellulose, carboxymethylcellulose, α-naphthol alizarin, methyl red and bromophenol blue were tried as suppressors but all were inferior to gelatin.

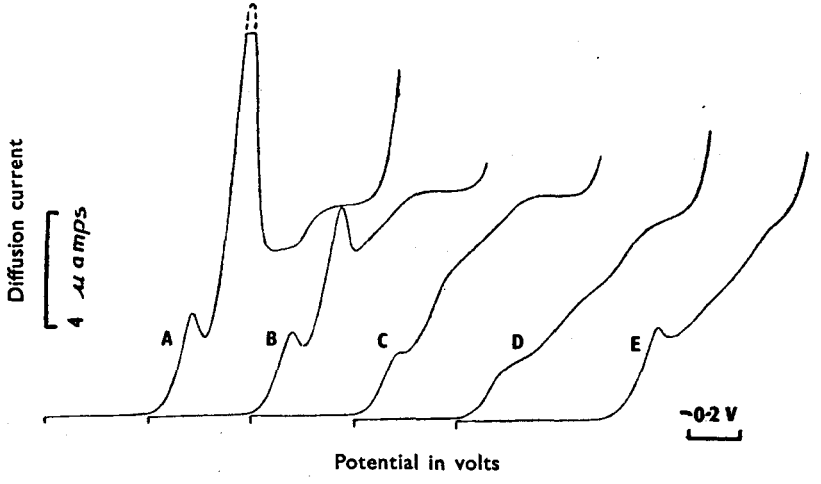

FIG. 2. Polarograms for sodium L-thyroxine, 0·06 per cent. w/v at *p*H 11·6.
A, without gelatin. B, 0·0025 per cent. C, 0·0065 per cent. D, 0·030 per cent. of gelatin. E, without tetramethylammonium bromide.
All polarograms start at −0·6V.

(*e*) *Variation in* p*H*.—The sodium carbonate base solution recommended by Borrows *et al.*[23] has *p*H 11·6. Since the height of the first step at that *p*H is affected by the presence of a maximum, and suppression is extremely critical, the total step height is preferably used for quantitative purposes. The effect of varying the *p*H (Fig. 3) shows that at *p*H 10

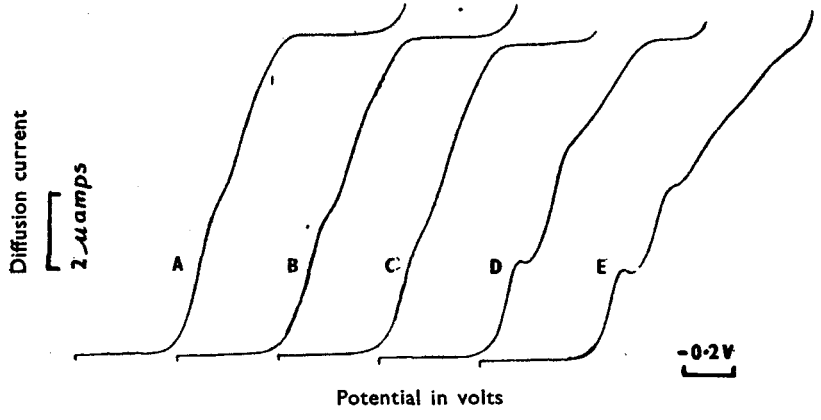

FIG. 3. Effect of variation of *p*H on polarograms for sodium L-thyroxine (0·06 per cent. w/v).
A, *p*H 9·4. B, *p*H 10·0. C, *p*H 10·3. D, *p*H 11·6. E, *p*H 13·2.
All polarograms start at −0·6V.

thyroxine gives only two steps with half-wave potentials at — 1·11 V and — 1·32 V. This first step is also unsuitable, but the total step is more satisfactory than the one occurring at *p*H 11·6.

The following base solution has been found satisfactory:—

	ml.
2·5 N Sodium carbonate	5·0
1·0 N Sodium bicarbonate	12·5
*iso*Propanol	10·0
Tetramethylammonium bromide solution (10 per cent. w/v) ..	5·0
Gelatin solution (0·1 per cent. w/v)	2·0
Water .. .. to	50·0

Note.—The sample (about 30 mg. of sodium L-thyroxine pentahydrate) should be dissolved in the ingredients (other than the sodium bicarbonate) diluted with about 10 ml. of water. The sodium bicarbonate is added and the solution is then diluted to volume.

Variation of any constituent of the above solution gives results similar to those in Table I for a base solution of *p*H 11·6, but the concentration of gelatin may be varied between 0·002 per cent. and 0·010 per cent. w/v without significantly affecting the step height.

TABLE I

EFFECT OF VARIATION IN CONCENTRATION OF *iso*PROPANOL AND GELATIN

*iso*Propanol concentration per cent. v/v	Gelatin concentration per cent. w/v	Percentage of standard step height*	
		First step	Total step
10	0·0065*	123	124
15	0·0065	108	111
20	0·0065	100*	100*
25	0·0065	92	92
30	0·0065	85	86
20	nil	125	97
20	0·0015	115	103
20	0·0025	110	104
20	0·0045	105	101
20	0·0065	100*	100*
20	0·010	99	99
20	0·030	(not measurable)	approx. 89

* 20 per cent. v/v of *iso*propanol and 0·0065 per cent. w/v of gelatin yield the most satisfactory polarograms at *p*H 11·6 and the step heights at these concentrations are here regarded as 100.

Application to tablets.—The polarographic method with the base solution given above has been successfully adapted to the analyses of thyroxine tablets, and good agreement with other methods has been obtained (Table II).

Preliminary separation from the excipients is usually necessary, since common ingredients such as magnesium stearate or buffering compounds interfere. Although satisfactory separations can be devised for tablets of known composition, the scope of the method is limited since it appears that each formulation would require separate consideration.

TABLE II

ANALYSES OF SODIUM L-THYROXINE TABLETS

Sample	Labelled strength (per tablet)	Thyroxine content (mg. anhydrous sodium L-thyroxine per tablet)		
		By iodine determination	Polarographic method	Colorimetric method
A	0·1 mg.	0·097 0·097	0·098 0·103	0·098 0·097
B	0·1 mg.	0·099 0·100	0·100 0·102	0·101 0·100
C	0·1 mg.	0·094 0·095	0·096 0·098	0·095 0·093
D	0·05 mg.	0·048 0·049	0·049 0·048	0·049 0·048

COLORIMETRIC DETERMINATION

Like many other 2:6-diiodophenols, thyroxine gives a red colour when treated first with nitrous acid and then with ammonia. This reaction, originally noted by Kendall and Osterberg,[24] was used for the photometric determination of thyroxine and diiodotyrosine by Morton and Chaikoff.[25] It has recently been the subject of a detailed study by Roche and Michel[10] for use in the analyses of thyroid gland and iodinated proteins. Other colorimetric methods of determination are based upon the red colour developed by treatment with freshly-prepared diazobenzenesulphonic acid in alkaline solution,[26] and a similar method makes use of the diazo derivative of *N'*-diethylsulphanilamide.[27] Thyroxine gives no colour with Millon's reagent, which may therefore be used for determining other reactive phenols in the presence of thyroxine.[16] However, thyroxine is converted to a reactive phenol by prolonged treatment with alkaline stannite and a colorimetric method based on this procedure has been suggested.[28]

Of these methods, the one based on the nitrite-ammonia reaction appeared to be the most suitable for the determination of synthetic sodium L-thyroxine and of particular value for application to tablets. Although not as selective for thyroxine as that described by Winikoff and Trikojus,[27] the method is simple, rapid and sufficiently sensitive. The procedure detailed by Roche and Michel[16] was found essentially satisfactory for maximum colour development, as shown by the results in Table III.

We prefer, however, to replace the prescribed sodium hydroxide-hydrochloric acid reagent by an equivalent amount of sodium chloride and to increase the proportions of hydrochloric acid and of ammonia: any buffering effect of tablet excipients can then be ignored. In addition, 20 minutes contact with nitrous acid is more satisfactory. The colour produced by thyroxine is photolabile, particularly after addition of ammonia, and all operations should be carried out in diffuse light. Although unlikely to be encountered in synthetic sodium L-thyroxine, 3:5-diiodotyrosine gives a more intense colour than thyroxine,[10] while we have found that 3:5-diiodothyronine gives about 65 per cent. of the colour of thyroxine.

TABLE III

THE EFFECT OF VARIATION IN CONDITIONS FOR DEVELOPMENT OF NITRITE-AMMONIA COLOUR

Condition varied		Percentage of maximum colour
1. Concentration of hydrochloric acid at nitrosation stage	approximately 0·035N	73
	" 0·07N	100*
	" 0·10N	100
	" 0·50N	100
	" 1·00N	98
2. Concentration of sodium chloride at nitrosation stage	approximately 0·75N	100
	" 1·5N	100*
	" 2·25N	102
3. Concentration of ethanol (95 per cent.) in final solution	15·0 per cent. v/v	93
	20·0 " " "	97
	25·0 " " "	99*
	27·5 " " "	100
	30·0 " " "	100
	32·5 " " "	98
	35·0 " " "	97
	37·5 " " "	93
4. Volume of sodium nitrite solution (1 per cent. w/v) used	1·0 ml.	99
	2·0 ml.	100*
	3·0 ml.	100
5. Time of contact with sodium nitrite solution (2 ml. 1 per cent. w/v)	2·5 minutes	69
	5 "	96
	10 "	98*
	15 "	100
	20 "	100
	25 "	100
6. Stability of colour from time of preparation	2 minutes	100
	8 "	100
	20 "	99

* Conditions prescribed by Roche and Michel.[16]

Extraction of the thyroxine from tablets in a form suitable for colorimetric estimation usually presents little difficulty. Most of the commonly used excipients are either soluble in the reagents without interfering or can be removed by some simple extraction and filtration technique. The colorimetric method is thus more rapid and selective for thyroxine than determination of the organically combined iodine and is more accurate and less likely to suffer from interference by tablet excipients than the polarographic method. It is also more sensitive than either procedure and gives a linear calibration over the range 0 to 0·8 mg. of sodium L-thyroxine.

The following method has been found satisfactory for commercial sodium L-thyroxine tablets:—

Reagents

(1) *Sodium chloride reagent.*—Dissolve 170 g. of sodium chloride (A.R.) in sufficient N hydrochloric acid to produce 1000 ml.

(2) *Sodium nitrite solution.*—1 per cent. w/v, freshly prepared.

Weigh and powder a sufficient number of tablets. Transfer an accurately weighed quantity of the powder, equivalent to 1·5 to 2·0 mg. of anhydrous

sodium L-thyroxine, to a 50-ml. graduated flask. Add 17·5 ml. of ethanol (95 per cent.) and 25 ml. of sodium chloride reagent. Heat the flask in a boiling water bath until the solution boils, cool and dilute to volume with sodium chloride reagent. Filter and collect 35 ml. of filtrate. Transfer 15·0 ml. of clear filtrate to a 20-ml. graduated flask, add 2·0 ml. of sodium nitrite solution, mix and allow to stand in the dark for 20 minutes. Dilute to volume with strong ammonia solution (32·5 per cent. w/w of NH_3), mix and determine the optical density of the solution in a 2-cm. cell using spectrum blue (602) filters. Carry out a blank determination with 2·0 ml. of water in place of the 2·0 ml. of sodium nitrite solution and apply the necessary correction to the optical density.

Calculate the sodium L-thyroxine content of the tablets by reference to a calibration graph prepared with known amounts of pure sodium L-thyroxine (0·5 mg. normally gives an optical density of about 0·33).

SUMMARY

1. Methods for the determination of sodium L-thyroxine have been examined, particularly those of potential value for the analysis of tablets prepared from synthetic material.

2. Ultra-violet absorption, polarography and a colorimetric method based upon nitrosation of thyroxine have been applied. Of these, the colorimetric method has been found the most satisfactory.

REFERENCES

1. Hunter, *J. biol. Chem.*, 1910, **7**, 321.
2. Hilty and Wilson, *Industr. Engng. Chem., Anal. Ed.*, 1939, **11**, 637.
3. Sappington, Halperin and Salter, *J. Pharmacol.*, 1944, **81**, 331.
4. Fabre and Pénau, *C.R. Soc. Biol.*, 1922, **87**, 1026.
5. Pickworth, *Biochem. J.*, 1925, **19**, 768.
6. Beal and Skalkowski, *J. Amer. pharm. Ass.*, 1935, **24**, 742.
7. Schulek and Stasiak, *Pharm. Zentralh.*, 1928, **69**, 513.
8. Shahrokh, *J. biol. Chem.*, 1943, **147**, 109.
9. Paal and Motz, *Biochem. Z.*, 1935, **279**, 106.
10. Nicklaus and Tippett, *J. Amer. pharm. Ass., Sci. Ed.*, 1940, **29**, 124.
11. Harington and Randall, *Quart. J. Pharm. Pharmacol.*, 1929, **2**, 501.
12. Leland and Foster, *J. biol. Chem.*, 1932, **95**, 165.
13. Blau, *ibid.*, 1933, **102**, 269.
14. Blau, *ibid.*, 1935, **110**, 351.
15. Chalmers, Dickson, Elks and Hems, *J. chem. Soc.*, 1949, 3424.
16. Roche and Michel, *Biochim. Biophys. Acta*, 1947, **1**, 335.
17. Hicks, *J. chem. Soc.*, 1925, **127**, 771.
18. Abderhalden and Haas, *Z. physiol. Chem.*, 1927, **166**, 78.
19. Heidt, *J. biol. Chem.*, 1936, **115**, 223.
20. Ungande, *J. Amer. chem. Soc.*, 1941, **63**, 2091.
21. Reinecke and Turner, *Res. Bull. Agric. Exp. Station Coll. Agric. Univ. Missouri*, No. 355, 1942.
22. Simpson and Traill, *Biochem. J.*, 1946, **40**, 116.
23. Borrows, Hems and Page, *J. chem. Soc.*, 1949, S204.
24. Kendall and Osterberg, *J. biol. Chem.*, 1919, **40**, 265.
25. Morton and Chaikoff, *ibid.*, 1943, **147**, 1.
26. Moser, *Experentia*, 1947, **3**, 119.
27. Winikoff and Trikojus, *Biochem. J.*, 1948, **42**, 475.
28. Brand and Kassell, *J. biol. Chem.*, 1939, **131**, 489.

DISCUSSION

The paper was presented by MR. D. C. M. ADAMSON.

The CHAIRMAN remarked that it was interesting to see how close were the results given by the three methods of determination, namely, ultra-violet absorption, polarography and colorimetry.

DR. F. WOKES (King's Langley) said that in Figure 1 the absorption band due to thyroxine was overlapped to a considerable extent by the band for 3:5-diiodo-L-tyrosine. The spectrographic method might possibly still be employed if, instead of using the minimum at 295 mμ (as compared with the maximum at 325 mμ), the authors used the top part of the curve and applied a correction. They could then work with a much narrower band.

MR. T. D. WHITTET (London) asked whether the authors had tried tests on the new substance triiodothyronine which had been found to be more active than thyroxine. It had been detected polarographically in thyroid extracts.

MR. M. DOMBROW (London) said it was difficult to reconcile the half-wave potentials recorded with the actual figures in the diagrams. He asked how the *p*H was determined. Was it measured with a *p*H meter or was it calculated from a mixture of known buffers?

DR. G. E. FOSTER (Dartford) enquired whether thyroid tablets had been assayed by the method described.

DR. N. EVERS (Hertford) said that if Mr. Adamson and his colleagues had found a method which would replace the present B.P. method they deserved thanks, because there was no method which took up so much time and gave such unsatisfactory results in the hands of different analysts.

MR. D. C. M. ADAMSON, in reply, said there had been a plea for spectrophotometric methods with the Morton correction. Ultra-violet spectrophotometry had found its place in industrial and other laboratories in recent years, but those with experience of the difficulty of determining vitamin A would not wish to become involved with a Morton's correction for another substance. The method had not been applied to triiodothyronine. For routine use he preferred to increase the height of the wave at higher potentials—more than half-wave. It was not claimed that the method worked with thyroid tablets; it might do so, but probably it would be necessary to extract the thyroxine.