A COMPARISON OF THE CALORIGENIC POTENCIES OF \( l \)-THYROXINE, \( dl \)-THYROXINE, AND THYROID GLAND

WITH A NOTE ON THE THYROXINE CONTENT OF THE ACID-SOLUBLE FRACTION OF THE PEPTIC DIGEST OF THYROID PROTEIN

BY G. L. FOSTER, WALTER W. PALMER, AND JESSICA P. LELAND

(From the Department of Biological Chemistry and the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York)

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In a previous paper (1) it was shown that (a) the calorigenic activity of desiccated thyroid preparations is proportional to the thyroxine content of the gland as determined by the method of Leland and Foster (2), and (b) that the calorigenic activity of a certain dosage of thyroxine in the form of thyroid gland is nearly twice as great as the activity of the same dosage of pure racemic thyroxine, when both are administered orally. It was suggested that this difference may be due to the greater biological activity of the naturally occurring \( l \)-thyroxine. In this paper experiments are presented which show that this is the case, and that the calorigenic activity of a certain dosage of pure \( l \)-thyroxine is quantitatively equal to the activity of the same amount of thyroxine in the form of thyroid gland substance.

EXPERIMENTAL

Isolation of \( l \)-Thyroxine—\( l \)-Thyroxine was isolated from hog thyroids as follows: Approximately 9 kilos of fresh thyroid were minced, suspended in 20 liters of water, and heated to 90° by steam. A large amount of fat rose to the surface and was skimmed off after cooling. To the suspension of coagulated thyroid substance was added a solution of 100 gm. of Merck's pepsin, and, after being adjusted to pH 1.5 with hydrochloric acid, the whole was incubated...
at 38° for 3 days, pH 1.5 being maintained by the addition of hydrochloric acid from time to time. The digest was then adjusted to pH 5 with sodium hydroxide and filtered by gravity through coarse fluted filters. Filtrate A was saved.

The material insoluble at pH 5 was washed with warm alcohol which removed most of the fatty material. The products so obtained from two such peptic digests of 9 kilos each were combined, suspended in 6 liters of water, mixed with 50 gm. of Fairchild's trypsin, adjusted to pH 7.5 with sodium carbonate, and incubated at 38° for 30 hours, being kept at pH 7.5. The digest was acidified to pH 5 with acetic acid; the precipitate was collected by filtration and washed with water.

The fraction insoluble at pH 5 was suspended in 1200 cc. of water and digested 24 hours at 38° and pH 7.5 with a glycerol extract of pig intestinal mucosa. The digest was acidified to pH 5 with acetic acid, and after about 20 hours the precipitate was collected by centrifugation.

To complete the hydrolysis the acid-insoluble fraction was refluxed 10 hours with 20 times its weight of 2 N sulfuric acid. The hydrolysate was adjusted to pH 5; the dark colored precipitate was collected and washed with water on the centrifuge, and dissolved in 2 liters of water with the help of a few cc. of ammonium hydroxide. The solution was warmed to 60° and treated with a slight excess of warm barium hydroxide solution, which caused precipitation of most of the dark colored impurities. The solution was filtered quickly and the pale yellow filtrate acidified to pH 5 with acetic acid, which caused the separation of a light cream-colored precipitate. The insoluble barium compounds from the foregoing treatment were suspended in water, treated with a few cc. of ammonium hydroxide, and stirred with a slight excess of sodium sulfate. The barium sulfate was filtered off; the filtrate was diluted to 2 liters, again treated with a slight excess of barium hydroxide, filtered quickly, and acidified to pH 5, when a small second crop of insoluble substance was obtained. The two acid-insoluble precipitates were combined and washed with water.

1 The filtration was troublesome because of the large amount of fat still present. It would undoubtedly have been better to have started the digestion with defatted material.

2 Kindly given to us by Professor Max Bergmann.
Further purification was effected (as was done by Harington (3)) by crystallization of the monosodium salt, for which purpose the material was warmed with 0.5 per cent sodium carbonate solution, the insoluble impurities were separated by centrifuging, and on cooling in the ice box the salt of thyroxine separated in crystalline form. Crystallization in this manner was repeated (three times) until the substance was pure white, whereupon it was dissolved in 80 per cent alcohol containing sodium hydroxide and acidified with acetic acid, which caused the separation of the free acid in pure form.

The yield of pure thyroxine was 104 mg., only a small fraction of the total amount of thyroxine one would expect to be present in 18 kilos (wet weight) of average hog glands.

The polariscopic examination was made with a high grade Schmidt and Haensch half shadow polarimeter; the light source was an electric sodium vapor lamp.

100 mg. of substance dissolved in 3 cc. of a solution composed of 24 gm. of 0.5 N sodium hydroxide and 56 gm. of absolute alcohol; tube length, 1 dm.

Ten readings on the solution yielded a mean value of $-0.178^\circ$, with an average deviation of $0.013^\circ$. Six readings with an empty tube gave a mean of $-0.031^\circ$, with an average deviation of $0.005^\circ$. Hence the observed rotation was $-0.147^\circ$; whence $[\alpha]_b = -4.4^\circ$.

The specific rotation of our material is slightly greater than that observed by Harington and Salter (4) who reported $-3.8^\circ$ both for the material obtained by resolution of synthetic thyroxine and for thyroxine isolated from the thyroid gland. The difference between our specific rotation and that of Harington and his collaborators may be even slightly greater, because Harington used the mercury green light, which commonly gives somewhat higher values than does sodium light.

Whether the difference between our rotation and that of Harington is significant is uncertain, because the observed rotations were small and the errors consequently large.

The substance was recovered after polariscopic examination and analyzed for iodine and nitrogen.

*Analysis*—C$_{12}$H$_{17}$O$_4$N$I_1$. Calculated. N 1.80, I 65.4

*Found.* “1.82, “65.1, 65.0
This specimen was used for the bioassays reported here.

A second preparation obtained in the same manner, except that the starting material was 2.2 kilos of commercial desiccated and defatted thyroid powder, yielded 28 mg. of analytically pure thyroxine. The mean observed rotation was $-0.055^\circ$ for a 0.93 per cent solution in a 1 dm. tube. Analysis showed 65.0 per cent iodine.

**Note on Thyroxine Content of Acid-Soluble Fraction after Peptic Digestion**

It has been assumed by some investigators (cf. Salter and Pearson (5)) that the acid-soluble fraction after peptic digestion of thyroid protein is free from thyroxine. This is far from being the case. Very large losses of thyroxine resulted in the isolation described above from discarding the acid-soluble fractions after each step in the hydrolysis. Filtrates A from the two peptic digests described above contained 2.64 and 2.70 gm. of total iodine (approximately half of the total iodine in the thyroid material used). Of this 0.170 and 0.155 gm. (6.4 and 5.7 per cent respectively) were present as thyroxine as determined by the method of Leland and Foster.

Some experiments performed on one of these peptic digest filtrates are of interest in connection with the recent paper of Salter and Pearson (5), and are briefly reported here.

A portion of Filtrate A which contained 5.7 per cent of its iodine in the form of thyroxine was boiled for 5 minutes to inactivate any remaining pepsin and was then concentrated under reduced pressure to one-tenth of its volume. An aliquot of the concentrate was mixed with pepsin and incubated at pH 1.5 for 24 hours. It was then diluted with water to its original volume and adjusted to pH 5 with sodium hydroxide. A precipitate separated which when washed and dried was found to contain 0.284 per cent total iodine, of which 15 per cent was in the form of thyroxine. This confirms the observation of Salter and Pearson. However, we cannot accept the interpretation of these investigators, who neglected the presence of thyroxine in their filtrates and suggested that the

3 The corresponding specific rotation is $-5.9^\circ$, but, since the observed rotation is so small, the measurement has scarcely more than qualitative significance.
pepsin added to the concentrates caused the synthesis of thyroxine or some closely related (and presumably, active) substance. We have found that a similar partially selective precipitation of the thyroxine-containing peptides is obtained in the absence of pepsin when egg albumin is used in its place. The denatured egg albumin precipitates at pH 5 and apparently carries down somewhat selectively the thyroxine-containing peptides. The precipitate contained 0.20 per cent total iodine, of which 18.5 per cent was in the form of thyroxine.

Pepsin which had been boiled 5 minutes gave a product having 0.16 per cent total iodine, of which 15.5 per cent was as thyroxine.

The peptic digest filtrate obtained at pH 5 if acidified more strongly (to about pH 3) yields a precipitate of protein material with relatively high phosphorus content (2.6 per cent), which probably represents a salt of peptone and nucleic acid. This precipitate was found to have 0.42 per cent total iodine with 21 per cent of it as thyroxine. It appears, therefore, that a protein precipitate forming in such a solution carries down with it somewhat selectively the thyroxine-containing peptides which obviously are not completely removed from a peptic digest at pH 5.

No attempt was made to isolate thyroxine from the acid-soluble products of peptic digestion. However, in the second preparation of thyroxine, from the commercial thyroid powder, the material soluble at pH 5 after tryptic digestion yielded 33 mg. of pure l-thyroxine after the hydrolysis was completed by boiling with acid, clearing with barium hydroxide, and purifying through the monosodium salt, as described above. This is actually more than was obtained from what was regarded as the main thyroxine fraction. However, yields in these preparations have no quantitative significance.

Biological Assays—The biological assays were based on the measurement of oxygen consumption of guinea pigs according to the method of Krogh and Lindberg (6).

Two groups of seven guinea pigs each received subcutaneously the sodium salt of l-thyroxine every other day over a period of 14 days. Daily determinations of the increase over the normal oxygen consumption were made. Details of the routine have been described previously (1).

Great care was taken to prevent the racemization of the l-
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Iodine content</th>
<th>Dose administered*</th>
<th>Increase in O\textsubscript{2} consumption</th>
<th>Average weight loss on 14th day of dosage period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>mg. I per sq. m.</td>
<td>Mean of last 3 days of individual animals</td>
<td>Mean of group</td>
</tr>
<tr>
<td>(\text{\textit{l}})-Thyroxine, Group 1, 7 animals</td>
<td>65.1</td>
<td>0.262 as disodium salt injected subcutaneously</td>
<td>50.1</td>
<td>40.3</td>
</tr>
<tr>
<td>(\text{\textit{l}})-Thyroxine, Group 2, 7 animals</td>
<td>65.1</td>
<td>„ „</td>
<td>39.5</td>
<td>37.6</td>
</tr>
<tr>
<td>(\text{\textit{dl}})-Thyroxine, 6 animals</td>
<td>64.9</td>
<td>„ „</td>
<td>24.5</td>
<td>23.1</td>
</tr>
<tr>
<td>Burroughs Wellcome commer- cial thyroid preparation as standard, 6 animals</td>
<td>0.254 (Total iodine)</td>
<td>1.13 total I</td>
<td>35.8</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td>0.0593 (Thyroxine iodine)</td>
<td>0.262 thyroxine iodine by mouth</td>
<td>38.6</td>
<td>35.1</td>
</tr>
<tr>
<td>(\text{\textit{dl}})-Thyroxine, 6 animals</td>
<td>63.3</td>
<td>0.262 as disodium salt by mouth</td>
<td>23.2</td>
<td>17.9</td>
</tr>
</tbody>
</table>

* Administered every other day for 14 days.
† As determined by the method of Leland and Foster.
thyroxine. Samples of approximately 1.5 mg. were weighed into sterile beakers, dissolved in 4 drops of sterile 0.1 N sodium hydroxide, and made up to volume with sterile distilled water. The standard dose, 0.262 mg. of thyroxine iodine per sq. m., in a volume of approximately 1 cc. was injected immediately without heating. Fresh solutions were prepared for each of the seven injections received by the animals.

For an exact comparison with L-thyroxine, equal doses of pure racemic thyroxine were administered in an identical manner to a group of six guinea pigs.

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Comparison of the increases in oxygen consumption produced by L-thyroxine and by dL-thyroxine.

The results of these experiments are shown in Table I and Fig. 1. Composite Curves 1 and 2 of Fig. 1 show the results obtained with L-thyroxine. Each point was determined by averaging the increase in oxygen consumption over the normal of the seven animals of the group. For comparison of different curves a mean value was obtained by averaging the last 3 days of the 14 day period. This was regarded as the full effect of the dose. As shown in Table I, the mean value of the last 3 days of Group 1 of animals receiving L-thyroxine was 40.3 per cent, while that of Group 2 was 37.6 per cent, which is within the limit of error of this method.
The results obtained when racemic thyroxine was injected under identical conditions (Fig. 1, Curve 3) are markedly lower over the entire 14 day period. The mean of the last 3 days of the group was only 23.1 per cent as compared with 37.6 and 40.3 per cent obtained with l-thyroxine. From this it appears that the l form is much more active than the d form.

For comparison of our assays of l-thyroxine with our previous studies (1) of racemic thyroxine and of thyroid gland, some data previously published are presented again in Fig. 2 and in Table I.

![Graph showing comparison of oxygen consumption increases](http://www.jbc.org/)

Two composite curves are shown in Fig. 2, each curve the mean of a group of six animals. The upper curve shows the effect observed when Burroughs Wellcome thyroid powder, used as a standard, was administered by mouth. A 35.3 per cent increase over the normal oxygen consumption was found when the results of the last 3 days were averaged. The results shown in the lower curve (Fig. 2) were obtained when an amount of the sodium salt of racemic thyroxine equivalent to the thyroxine content of the standard (as determined by the method of Leland and Foster) was administered in a similar manner. The mean of the last 3 days of the group in
this case was only 17.9 per cent. It should be noted (Table I) that one of the six animals included in the group was relatively insensitive to the dose, which tended to lower the group mean. The average of the remaining five animals was 19.8 per cent. Approximately 50 per cent only of the increase in oxygen consumption which was obtained with the standard thyroid powder was observed with racemic thyroxine. Subcutaneous injection of the same dose over a second 14 day period failed to raise the figure significantly.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Comparison of the increases in oxygen consumption produced by the standard thyroid preparation (Burroughs Wellcome) and by \( l \)-thyroxine. \( Th.I \) = thyroxine iodine; \( T.I \) = total iodine.

Fig. 3 shows the two \( l \)-thyroxine curves together with that of the standard thyroid substance of Fig. 2 in equi-thyroxine doses. Throughout the entire 14 day period the three curves follow one another so closely that the response must be considered the same for all three within the limits of error of the method. The conclusion may safely be drawn that the calorigenic activity of pure thyroxine is fully equivalent to that of an equi-thyroxine dose of thyroid gland, provided the thyroxine is administered as the naturally occurring isomer.
DISCUSSION

Our data permit some comment on the question of the relative activities of d- and l-thyroxine. As shown in Fig. 3, the calorigenic activity of a certain parenteral dose of l-thyroxine (0.262 mg. of thyroxine iodine per sq. m. every 2nd day) is fully equal to that of the same amount of thyroxine in the form of thyroid substance given by mouth. Exactly the same dose of dl-thyroxine (Curve 3, Fig. 1, this paper) had the same calorigenic effect as was reported previously ((1) Table I) for one-half that dosage (i.e., 0.131 mg. per sq. m. every 2nd day) of thyroxine iodine as thyroid. The mean increases of oxygen consumption above the normal periods were 23 per cent for the dosage of 0.262 mg. of thyroxine iodine as dl-thyroxine and 22 per cent for 0.131 mg. of thyroxine iodine as thyroid substance ((1) Table I). It thus appears that the calorigenic activity of dl-thyroxine is ascribable, within the rather wide limits of accuracy of our method, solely to the l component. The observation by Gaddum (7) of some activity in d-thyroxine may perhaps be attributed to incomplete resolution in his sample, which possessed an appreciably lower specific rotation than that of the natural product used in our experiments.

Salter, Lerman, and Means (8) administering the same preparations as used by Gaddum to myxedema patients failed to find any difference between the two forms.

SUMMARY

1. l-Thyroxine ([α]_D = -4.4°) was isolated from thyroid glands and its calorigenic activity compared with that of pure dl-thyroxine and equi-thyroxine doses of thyroid substance.

2. l-Thyroxine was found to have a calorigenic effect on normal guinea pigs approximately twice as great as that of racemic thyroxine.

3. The calorigenic activity of a certain dosage of thyroid gland is quantitatively accounted for by the l-thyroxine which it contains.

4. The acid-soluble fraction of a peptic digest of thyroid protein contains an appreciable amount of thyroxine, presumably still in peptide linkage. From such a solution, the thyroxine-containing peptides are somewhat selectively carried down on various protein precipitates which may be caused to form in the solution.
Foster, Palmer, and Leland

BIBLIOGRAPHY


G. L. Foster, Walter W. Palmer and Jessica P. Leland


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