Thyroxin–Triiodothyronine Concentrations in Thyroid Powders

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Eleven thyroid powders were assayed for their respective goiter prevention activities in thiouracil treated rats. These activities were compared with the corresponding triiodothyronine and thyroxin levels of the powders as determined by a chemical method. A comparison between pronase, enzyme P, and an erepsin–trypsin mixture was made with respect to their effectiveness in releasing thyroxin and triiodothyronine from commercial thyroid. Pronase was demonstrated to be preferable. There was a consistent relationship between the triiodothyronine levels of the powders and the goiter prevention activities. This relationship was independent of the thyroxin level. Bioassay of diets containing measured amounts of thyroxin and triiodothyronine confirmed the validity of the chemical analyses and the preponderant contribution of the triiodothyronine component to goiter prevention by this assay.

OF ALL the iodine-containing substances in thyroid powders, it is believed that triiodothyronine (T_3) and thyroxin (T_4) account for the total biological activity (1). Reported discrepancies in activity between thyroid preparations (2, 3) might therefore be attributed to varying amounts of T_3 and T_4 even though the total iodine remains constant. Correlation between the iodothyronine content and biological activity has been hampered mainly by difficulties in chemical procedures which must take into account such factors as (*a*) iodothyronine instability in solution (4–6), and (*b*) the slowness of iodothyronine release from thyroid protein by most proteolytic agents (7, 8). In this report, the hydrolytic actions of pronase, enzyme P, and an erepsin–trypsin mixture have been compared with respect to their effectiveness in releasing T_4 and T_3 from thyroid powders. The T_4 and T_3 concentrations of thyroid powders were compared with their corresponding goiter prevention activities. The activities of diets containing measured amounts of T_4 and T_3 were employed to confirm the analyses.

METHODS

Iodoamino Acid Determination.—A quantity of the thyroid powder, containing 50–200 mcg. of iodine, was placed in 2 ml. of 0.05 *M* Tris buffer at pH 8.5. One milligram of pronase[1] or enzyme P[2] was added, and the mixture was incubated at 39° for periods of time ranging from 6–96 hr. For the erepsin–trypsin[3,4] digests, 10 mg. of each enzyme was added at the beginning of the digestion time and 5 mg. of erepsin was added daily thereafter. Occasional gentle shaking during the first hour of incubation was helpful in dispersing the powder. Butanol extraction, paper chromatography, and measurement of the thyronines were performed as previously described (7). U.S.P. reference standard triiodothyronine (liothyronine) and thyroxin (B grade, Calbiochem, Los Angeles, Calif.) were employed throughout as thyronine standards. The iodothyronine values reported were the average of 4 separate determinations.

Goiter Prevention Assay.—The biological activities of the thyroid samples were determined by the goiter prevention assay employing thiouracil-treated adult female rats as the test animals (9). For the preparation of diets containing T_3 and T_4, solutions of each thyronine were made up in acid ethanol (90 ml. of ethanol plus 10 ml. of 2 *N* H_2SO_4) at a concentration of 100 mcg./ml. The required volumes of the thyronine solutions were mixed in a mortar with 2-Gm. amounts of casein, then dried in a stream of nitrogen and dispersed in ground Fox Cubes.[5]

RESULTS AND DISCUSSION

Rate of Release of the Iodothyronines, T_3 and T_4, During Proteolysis in Tris Buffer.—In Fig. 1 the effectiveness of pronase and enzyme P in releasing iodothyronines from commercial thyroglobulin (sample 6, Table 1) is compared with that of erepsin and trypsin in combination. Similar peaks of recovery of T_3 and T_4 were obtained in both enzyme P and pronase digests. The largest amount of T_3 was recovered following 6 hr. digestion, and the amount of T_4 recovered reached a near maximum level at 24 hr. Pronase was considered to be superior because a greater quantity of the iodothyronines was recovered. The reduced recoveries of the T_3 and T_4 with longer digestion periods in enzyme P and pronase digests were apparently due to iodothyronine instability in this buffer medium. A similar decline in recoverable T_3 and T_4 from enzymatic hydrolysates as the period of digestion was lengthened, has been reported by others (10, 11). Since the stability of T_3 relative to T_4 in this medium is not known with precision, it is not possible to say that pronase favors the release of T_3 over

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[1] Calbiochem, Los Angeles, Calif.
[2] Biddle-Sawyer Co., New York, N. Y.
[3] Erepsin, Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] Trypsin, Difco 1:250.
[5] Maple Leaf Mills, Toronto, Ontario, Canada.

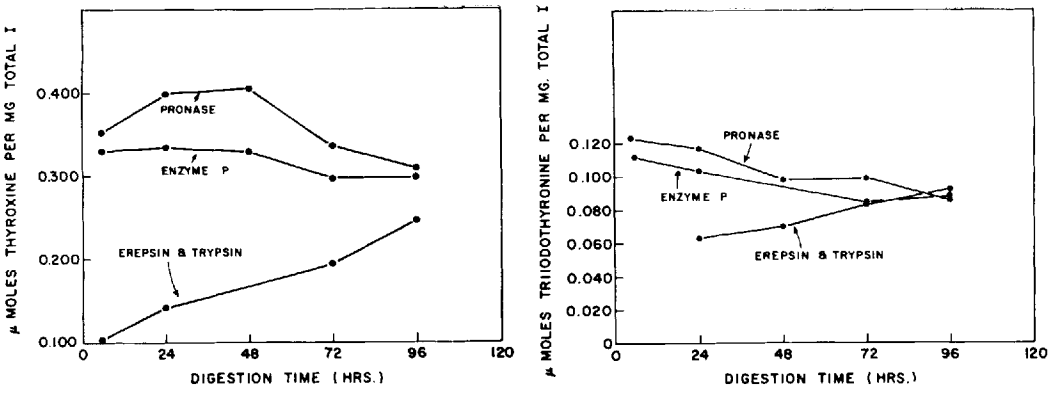

Fig. 1.—The recovery of thyroxin and triiodothyronine from enzymic digests of commercial hog thyroglobulin (1.02% iodine). Digestion was performed in 0.05 *M* Tris (hydroxymethyl aminomethane) buffered at pH 8.5. Butanol extracts of the hydrolysate were taken to dryness and the residue redissolved in ammoniacal alcohol immediately before application to paper chromatograms.

TABLE I.—IODOTHYRONINE CONTENT OF COMMERCIAL THYROID POWDERS

Sample	Species of Origin	Geographic Origin	Total Iodine, %	mcg./mg. Iodine T_4	mcg./mg. Iodine T_3	$\frac{I T_4}{I \text{ Total}} \times 100$	$\frac{I T_3}{I \text{ Total}} \times 100$	*M* Ratio $T_4:T_3$
1	...	...	0.63	114	41	7.5	2.4	2.3
2	Beef	U.S.	0.62	226	49	14.8	2.9	3.9
3	Beef	U.S.	0.55	294	50	19.2	2.9	4.9
4	Beef	...	0.44	348	60	22.7	3.5	4.8
5	Sheep	U.K.	0.48	280	73	18.3	4.3	3.2
6	Hog	U.S.	1.02	274	75	17.9	4.4	3.1
7	Hog	U.S.	0.73	268	78	17.5	4.6	2.9
8	Beef	U.K.	0.35	340	83	22.2	4.9	3.4
9	Hog	U.S.	0.20	210	87	13.7	5.1	2.0
10	Hog	U.S.	0.86	268	95	17.5	5.6	2.4
11	Hog	U.K.	0.60	256	100	16.7	5.9	2.1

T_4 in the early stages of hydrolysis as might be deduced from Fig. 1. There was a steady increase in iodothyronine recoveries up to 96 hr. in the erepsin–trypsin digests which indicated that the rate of their release from the thyroid protein under these conditions exceeded the rate of degradation. The greater bulk constituted by the erepsin–trypsin mixture in the hydrolysate medium may have conferred a measure of stability to the free iodothyronines in solution. In this connection, Rosenberg has observed that the rate of disappearance of T_4 is less in Viokase thyroid hydrolysates when compared with pronase hydrolysates (11). Although the recoveries of T_3 and T_4 continued to increase during the digestion period with the erepsin–trypsin mixture in Tris buffer, the final amounts of T_3 and T_4 actually obtained were less than the peak levels found with the other enzymes.

In a previously reported investigation (7) comparable peak recoveries of T_3 and T_4 were achieved in 96-hr. erepsin–trypsin digests when performed in borate buffer. However, the short digest periods required by pronase action reduced risk of losses by degradation or microbiological contamination. Also Tris buffer provided a better buffering capacity than the borate medium. Therefore, optimum conditions for the proteolytic removal of T_3 and T_4 from commercial thyroid powders has been defined as overnight digestion (15–18 hr.) in the presence of 1 mg. of pronase per 2 ml. of Tris buffer.

Iodothyronines in Commercial Thyroid Powders.—Table I summarizes the results of analyses of the T_3 and T_4 content in 11 thyroid powders obtained from 4 different laboratories. Sample *9* was diluted with filler of unspecified composition to meet the requirements of the "United States Pharmacopeia" (12). Sample *1* possessed an exceptionally low iodothyronine level which was reflected in bioassay experiments here and elsewhere. Samples comparable in quality and activity to sample *1* have been judged "clinically defective" in human therapy trials.[6] In general, the beef thyroid powders had a lower per cent of total iodine than the hog powders.

Excluding the defective sample, the per cent of the total iodine accounted for by the T_3 component ranged from 2.9–5.9. The per cent of the total iodine accounted for by the T_4 component ranged from 13.7–22.7. The molar ratios ranged from 3.4–4.9 in the beef powders and from 2.0–3.1 in the hog powders. This species difference in $T_4:T_3$ *M* ratios seems to be a characteristic which results from simultaneous elevated T_4 levels and reduced T_3 levels in the beef thyroids when compared with those originating from the hog. Pileggi *et al.* (13) have reported essentially the same range of T_3 concentrations as listed in Table I but slightly lower T_4 levels in a number of commercial thyroid powders of unspecified species origin. The single sheep sample

[6] Private communication from Dr. L. Meister, Veteran's Administration Hospital, Long Beach, Calif.

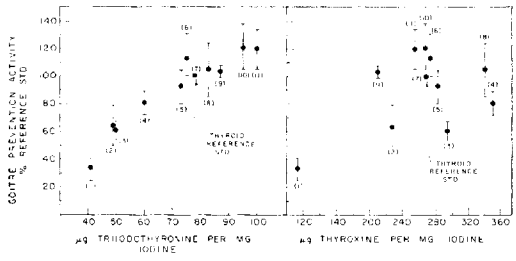

Fig. 2.—The relation between triiodothyronine and thyroxin concentrations in thyroid powders and goiter prevention activity in thiouracil-treated rats. The numbers in parentheses correspond with thyroid sample numbers listed in Table I. Vertical bars represent the 95% confidence limits for each estimate of potency.

reported in Table I had a molar ratio of 3.2; 22.6% of the total iodine was accounted for by the T_3 and T_4 components. In these respects, it was therefore intermediate between the hog and beef thyroid powders.

Goiter Prevention Versus T_3 Content in Orally Administered Thyroid.—In the goiter prevention assay the dose of desiccated thyroid was adjusted according to the total iodine content. Experiments employing this bioassay suggested that the T_3 component probably accounted for most of the response (14). To test this hypothesis, the individual T_3 and T_4 concentrations expressed as mcg./mg. total iodine in the thyroid powders were plotted against the respective goiter prevention activities (Fig. 2). All activities were expressed as a percentage of that possessed by the reference standard (sample 7, Table I). It was noted that, with the exception of sample 8, all thyroids possessing activity of more than 100 by this system of comparison originated from the hog. The T_4 levels within this group varied from 210–340 mcg./mg. iodine, and did not correlate well with the observed activities. However, in spite of the fluctuating T_4 levels, there remained a consistent relationship between the T_3 concentrations and the respective goiter prevention activities.

It was desirable also to compare the level of goiter prevention activity of thyroid powder with that of a synthetic mixture containing similar amounts of T_3 and T_4. If the same level of activity could be demonstrated in this manner, it would, in addition to lending support to the validity of the chemical analysis, show that neither the union of the hormones with thyroid protein at the time of administration nor the presence of other components normally present in thyroid powders contribute to the response. Consequently, the 3 diets listed in Table II were prepared. Diet *A* contained the reference thyroid powder while diet *B* contained a mixture of T_4 and T_3 in the same proportions as diet *A*. Diet *C* had the same content of T_3 as diets *A* and *B* but had no T_4. Diet *D* had T_4 only in a concentration which would reduce the gland weight in the assay to a suitable level for comparison with the other diets. For purposes of calculating the relative potency it was supposed that the added thyronines provided the same fraction of the total iodine content as the reference powder. The activities of the the powder and the corresponding thyronine mixture were approximately the same. The artificial mixture of T_4 and T_3 in diet *B* possessed only slightly higher activity than that of the reference powder. If the accuracy of the chemical analysis is accepted, this small discrepancy may be due to incomplete intestinal hydrolysis of the orally ingested powder and consequent reduced absorption of T_4 and T_3. This possibility has been raised by Levy and Knox (15) who observed a discrepancy of similar magnitude between the activities of a thyroid powder and its hydrolysate. Diet *C* which contained the same amount of T_3 as in the reference thyroid produced essentially the same activity as that of the powder itself. Diet *D*, with more than 5 times the thyroxin present in the reference powder diet mix, was only about one-seventh as active. The minor contribution of T_4 relative to T_3 under these conditions of assay is therefore confirmed. These findings are consistent with the relationships illustrated in Fig. 2.

It is acknowledged that the metabolic effectiveness of T_4 may be impaired in thiouracil-treated animals (16), and therefore in this assay the contribution of T_3 relative to T_4 will be somewhat exaggerated. However, the essential agreement between the activity of the powder and its corresponding T_3–T_4 mix suggests at least the validity of the T_3 measurement. It has now been deduced that the T_3 component of many thyroid tablets when administered orally during clinical therapy probably accounts for the greater part of the response (25).

The widely varying levels of T_4 and T_3 which have been reported for thyroid (17, 18) have often been attributed to the physiological state of the

Table II.—The Goiter Prevention Activities of Thyroxin–Triiodothyronine Mixtures Relative to a Corresponding Thyroid Powder

Diet	Material Added to Diet	Dose/100 Gm. Diet[a] μm. T_3	Dose/100 Gm. Diet[a] μm. T_4	Relative Potency with 95% Confidence Limits
A	Thyroid powder	0.004	0.013	1.0
		0.007	0.022	
B	$T_4 - T_3$	0.004	0.013	1.10 (0.99–1.25)
		0.007	0.022	1.11 (1.00–1.23)
C	T_3	0.004	...	1.03 (0.84–1.28)
		0.007		
D	T_4	...	0.096	0.14 (0.12–0.21)
			0.145	

[a] Two dosage levels of the thyroactive diets were administered in all bioassays. The amounts listed indicate the ratio present. Each dose group comprised 8 animals.

TABLE III.—T_4 AND T_3 LEVELS IN 1-gr. U.S.P. THYROID (65 mg. 0.2% IODINE)

Species	Sample[a]	mcg./gr. U.S.P. Thyroid T_4	T_3	*M* Ratio T_4:T_3
Beef	*2*	29	6.4	
Beef	*3*	38	6.5	
Beef	*4*	45	7.8	
Beef	*8*	44	10.8	
Av.		39	7.9	4.0
Hog	*6*	36	9.8	
Hog	*7*	35	10.1	
Hog	*9*	27	11.3	
Hog	*10*	35	12.4	
Hog	*11*	33	13.0	
Av.		33	11.3	2.5

[a] Sample numbers correspond to those appearing in Table I.

animals from which the glands were taken (19). Thyroid powders prepared on the North American continent for pharmaceutical use usually originate from gland pools representing large numbers of healthy animals. In those instances where the thyroid preparation had obviously low clinical activity (20), the possibility of losses or degradative changes during processing must be considered as a cause. Fresh thyroid tissue is known to possess autolytic activity (21) which could bring about selective losses of iodine-containing constituents, if, for example, the tissue were not processed or frozen immediately following collection. Also the unusual prospect of a thyroid powder acquiring a gain in activity during processing operations has been raised by Braverman and Ingbar (22). They have treated patients with commercial thyroid powder standardized to U.S.P. specifications which gave P.B.I. levels and other clinical responses indicative of markedly increased amounts of T_3 or some other equally potent thyroactive substance. Alternatively, this phenomenon might also be caused by a selective loss of T_4 during processing operations.

U.S.P. thyroid is required to contain 0.2 ± 0.03% iodine in thyroid combination (12). In Table III the levels of T_3 and T_4 have been calculated for U.S.P. thyroid as they would exist when prepared by dilution of the powders listed in Table I. From the average values, 1 gr. of a composite sample of the 4 beef thyroid samples would contain 39 mcg. of T_4 and 7.9 mcg. of T_3. Similarly, 1 gr. of U.S.P. thyroid prepared from the hog powders would have 33 mcg. of T_4 and 11.3 mcg of T_3. There is significantly less T_3 in the beef thyroid and this would account for previously reported lower levels of activity in beef thyroid when assayed by this method (14).

The present study suggested that the oral administration of desiccated thyroid reduced thiouracil-induced goiter in rats to an extent which was comparable to that brought about by an equivalent mixture of T_3 and T_4. In view of such factors as diet and species which are known to affect the availability of orally ingested T_4 (23, 24) the fraction of activity contributed by the T_4 component in a T_4–T_3 mixture may be influenced by the conditions of the experiment. Lavietes and Epstein (25) have demonstrated the difficulties in following the course of thyroid therapy from observation of serum P.B.I. levels when the T_4:T_3 ratio of the administered U.S.P thyroid is abnormally low. Clinicians have noted also some qualitative differences between the responses of T_3 and T_4 when administered separately (26, 27). In any event, an expression of potency for a thyroid powder will depend not only on its T_3 and T_4 content but also on the physiological effect selected as the response metameter.

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