THE CHEMICAL DETERMINATION OF LIOTHYRONINE AND THYROXINE IN ENZYMIC HYDROLYSATES OF PORK THYROID

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Maximum amounts of liothyronine and thyroxine were released from pork thyroid by the proteolytic action of erepsin and trypsin after incubation for 96 hr. at 39°. The iodinated thyronines were extracted from the hydrolysate with butanol, separated chromatographically, eluted from the paper and measured quantitatively by a chemical method in which the reduction of ceric ion by arsenious acid is catalysed by the iodine containing compounds. The iodine found in liothyronine and thyroxine accounted for 18 to 25 per cent of the total organic iodine in pork thyroid. It is postulated that pork thyroglobulin contains approximately 1 molar residue of liothyronine and 2 molar residues of thyroxine.

THYROID preparations are standardised chemically by measuring either the total organic iodine (Pharmacopeia of the United States XVI) or "thyroxine" iodine (British Pharmacopoeia 1958, Addendum 1960). The latter method is based on a procedure originally described by Harington and Randall (1929) and assumes that thyroxine in an alkaline hydrolysate of desiccated thyroid is precipitated quantitatively at pH 3·5, while the former method presumes a quantitative relationship between biologically active iodine and the total iodine. However, recent publications have cast some doubt on the adequacy of these chemical assays (Stasilli and Kroc, 1956; Taylor, 1961; Sturnick and Lesses, 1961; *Pharm. J.*, 1961).

Since thyroid contains liothyronine (3,5,3′-triiodothyronine) in addition to thyroxine (Gross and Pitt-Rivers, 1953) any chemical procedure for determining the potency of desiccated thyroid should provide a quantitative measure of both of these biologically active iodinated thyronines.

Mandl and Block (1959) described a method for the quantitative determination of the various iodinated compounds in a commercial sample of thyroglobulin. Since there appeared to be some variation between the estimates of liothyronine and thyroxine reported by Mandl and Block, modifications of their conditions of enzymic hydrolysis and extraction were investigated in our laboratory. In addition, a study was made of methods of chromatographic resolution and quantitative measurement of liothyronine and thyroxine employed by others (Leffler, 1954; Maclagan, Bowden, and Wilkinson, 1956; Pind, 1957). We have made a comparison of the rate of release of thyroxine and liothyronine from pork thyroid powder by the action of trypsin and erepsin both alone and in combination. Evidence is presented which suggests that under the conditions described, the amounts of liothyronine and thyroxine released by enzymic hydrolysis of thyroid approach maximum values. Recovery experiments have shown that losses which may occur during the determination have been reduced to a minimum.

EXPERIMENTAL

Reagents

All reagent solutions were prepared from analytical grade chemicals and water distilled from an all-glass apparatus over alkaline potassium permanganate.

Borate buffer. 0·05M adjusted to pH 8·5.

Arsenious acid solution. Sodium arsenite (13·0 g. $NaAsO_2$) was dissolved in 500 ml. water and the volume made up to 1000 ml. with 30 per cent sulphuric acid. In this solution was dissolved 0·5 g. recrystallised sodium chloride.

Stock ceric sulphate solution. (Leffler, 1954.) In a beaker, were placed 6·5 g. ceric sulphate ($Ce(SO_4)_2$), 6·0 ml. concentrated sulphuric acid and 5·0 ml. water. The mixture was heated to just below boiling for 30 min., then cooled and made up to a volume of 70 ml. The solution was filtered and stored in a refrigerator.

Ceric sulphate solution. The stock ceric sulphate was diluted (usually about 1:20) by the addition of 2N sulphuric acid so the reagent control tube described under "quantitative measurement" would give an absorbance between 0·700 and 0·800 at 420 mμ in a spectrophotometer cell of 1·0 cm. light path.

Ceric sulphate arsenious acid reagent for staining. The stock ceric sulphate was diluted with two volumes 2N sulphuric acid and mixed with an equal volume of a 1:3 dilution of the arsenious acid solution immediately before use.

Ammoniacal alcohol. 5 per cent concentrated ammonia in methanol.

Standard solutions. An accurately weighed amount of the sodium salt of either liothyronine or thyroxine was dissolved in a 1:10 dilution of 20 per cent acetic acid in methanol to provide a concentration of 100 μg. of the free dehydrated form per ml. Although liothyronine and thyroxine are not readily soluble in this solvent, they will dissolve completely when shaken gently for an hr. or more. Once in solution, they remain stable for at least 7 days when kept at 4°.

Mercuric nitrate. (Strickland and Maloney, 1957.) Two g. mercuric nitrate were dissolved in 100 ml. water to which a few drops of concentrated nitric acid had been added.

Enzyme Hydrolysis

In a 15 by 150 mm. test-tube were placed approximately 25 mg. of thyroid, accurately weighed, 10 mg. trypsin (Difco 1:250), 10 mg. erepsin (Nutritional Biochemicals Corporation), and 2 ml. borate buffer containing one drop of thiomersal (Eli Lilly and Company—solution No. 45). The suspension was thoroughly mixed and placed in the dark at 38–40° for 96 hr. The mixture was shaken occasionally. After the first 4 hr. the pH was adjusted to 8·5 with 0·2N NaOH. At daily intervals thereafter, 5 mg. erepsin were added, with gentle shaking.

Extraction

At the end of the incubation period, the pH was adjusted to 3·0 with N sulphuric acid. The acidified digest was extracted four times with

3 ml. volumes of n-butanol which had been previously equilibrated with 0·1N sodium thiosulphate. The extracts were combined and the solvent removed under reduced pressure at 60°. The dried residue may be stored for at least 1 week in the cold under nitrogen.

Paper Chromatography

The dried residue was dissolved in sufficient ammoniacal alcohol (usually less than 1·0 ml.) to permit the application of an aliquot containing about 4 μg. total organic iodine to each of three 1 in. Whatman No. 1 paper strips, assuming for this calculation that all of the iodinated compounds have been extracted by the butanol. The first strip was used to locate the liothyronine and thyroxine areas, and the other two strips for duplicate measurement of each of the thyronines. On respective strips of Whatman No. 1 paper were spotted 0·2, 0·5, and 1·0 μg. liothyronine, and 0·5, 1·0 and 2·0 μg. thyroxine. A marker strip was included for each standard. These quantities of standard and hydrolysate have been chosen so that calibration and measurement may be made on eluates which have been treated in an identical manner. This allows compensation for any possible deterioration of liothyronine and thyroxine in the ammoniacal alcohol. Chromatographic examination has not indicated any observable deterioration of these compounds when dissolved in ammoniacal alcohol for up to 2 hr. The chromatograms were allowed to develop overnight in a descending t-pentanol : N-ammonia (2 : 1) solvent system (Gleason, 1955).* The solvent fronts were marked and the strips were stained by the method of Bowden, Maclagan, and Wilkinson (1955). As soon as the strip was removed from between the glass plates, it was drawn once through 2N ammonia and then dried in an oven. This treatment provides a stained chromatogram which, if kept in the dark, remains in good condition for several weeks.

Quantitative Measurement

The areas on the unstained chromatograms corresponding to liothyronine and thyroxine were cut out and placed in 25 ml. glass stoppered flasks containing 5 ml. ammoniacal alcohol. The flasks were shaken gently for 30 min. to elute the iodinated thyronines.

For the determination of liothyronine, 3 ml. aliquots were withdrawn from both the standard and hydrolysate eluates, transferred to 10 × 125 mm. test-tubes,† and taken to dryness under a stream of nitrogen. For the measurement of thyroxine, 1 ml. aliquots of the appropriate eluates were taken to dryness in a similar manner. To all tubes and one reagent control tube were added 3 ml. of water and 2 ml. arsenious acid solution. The contents were mixed thoroughly and the tubes were placed in a water bath at 30° for 15 min. At carefully timed intervals, 1·0 ml. of the ceric sulphate solution was added to each tube,

* When developed at room temperature (21–23°) the solvent front advanced about 10 in. from the line of application, giving R_F values for iodide, thyroxine and liothyronine of 0·15, 0·25 and 0·53 respectively.

† These tubes were cleaned with hot nitric acid to remove traces of mercury salt, and were discarded after several weeks' use (Strickland and Maloney, 1957).

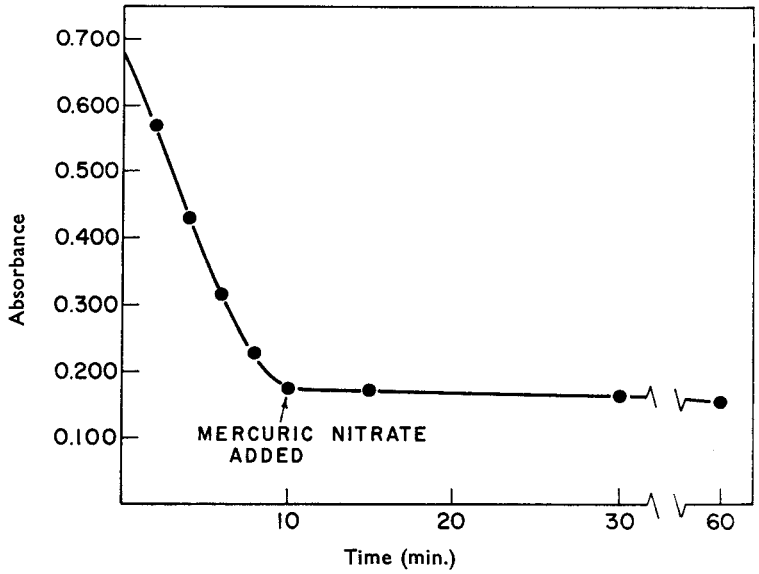

FIG. 1. The effect of the addition of mercuric nitrate on the rate of reduction of ceric ion by arsenious acid catalyzed by iodine.

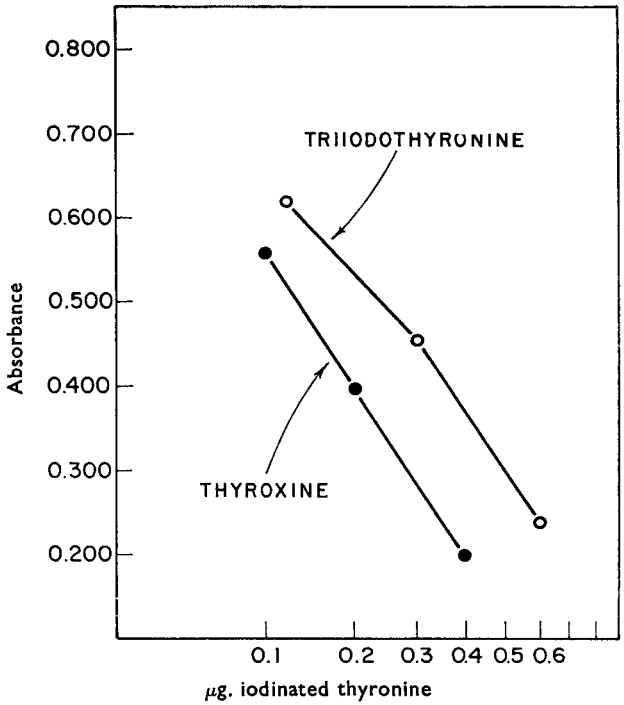

FIG. 2. The calibration curves for liothyronine and thyroxine.

and exactly 10 min. later, the reaction was stopped by the addition of 2 drops of mercuric nitrate solution (Strickland and Maloney, 1957). Fig. 1 illustrates the effect of mercuric nitrate on the rate of ceric ion reduction under these conditions. The absorbance of each solution at 420 mμ was recorded promptly against a water blank. Fig. 2 shows the standard curves obtained by plotting absorbances of the reference materials on semilogarithmic paper, employing the relation described by Acland (1957). The amounts of liothyronine and thyroxine in the hydrolysates were estimated directly from the calibration curves. It has been found advisable to calibrate for each set of determinations.

The arsenious acid and ceric sulphate reagents employed for the final measurement were of high purity. The reagents were considered satisfactory if the absorbance decrease of the reagent control at 420 mμ did not exceed 0·050 during the 10 min. catalytic reduction period of ceric sulphate.

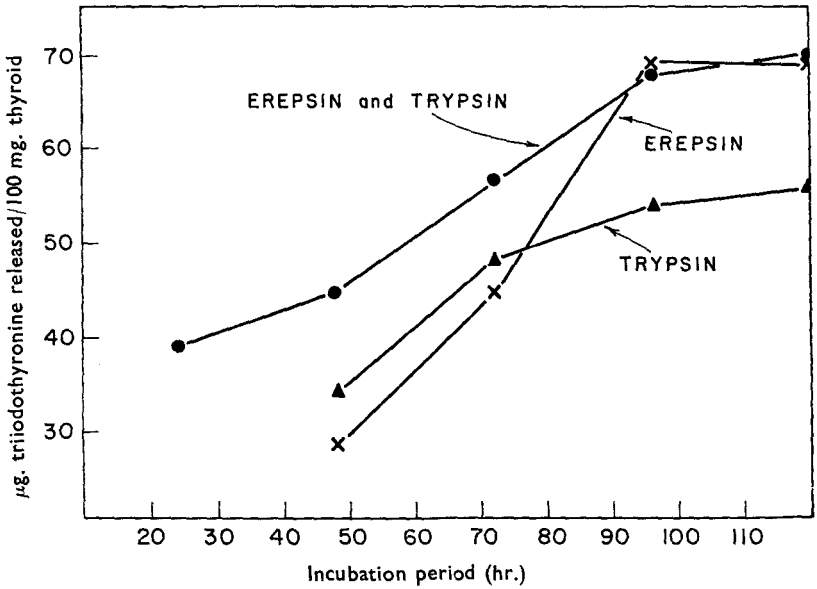

FIG. 3. The rate of release of liothyronine from pork thyroid by proteolytic hydrolysis.

Erepsin vs. Trypsin Hydrolysis

Although erepsin alone usually released maximum amounts of liothyronine and thyroxine (Figs. 3 and 4) over 96 hr., trypsin was added at the beginning of the digestion period because more consistent results were thus obtained. Crude trypsin alone, under the same conditions, was not capable of hydrolysing the maximum amounts of liothyronine and thyroxine from thyroid and was markedly inferior to erepsin in releasing thryoxine (Fig. 4). The trypsin activity appears to have more access to the liothyronine linkage with thyroglobulin than to the thyroxine

linkage. A similar result was obtained with a pancreatin preparation (Pancreatin N.F.—Fisher Scientific Co.). Increased enzyme concentration did not affect the rate of release. In erepsin digests, it has been noted that the drop in buffer pH was gradual over the first 48 hr. whereas when trypsin was present, the increase in acidity was rapid and required adjustment to pH 8·5 after 4 hr. digestion.

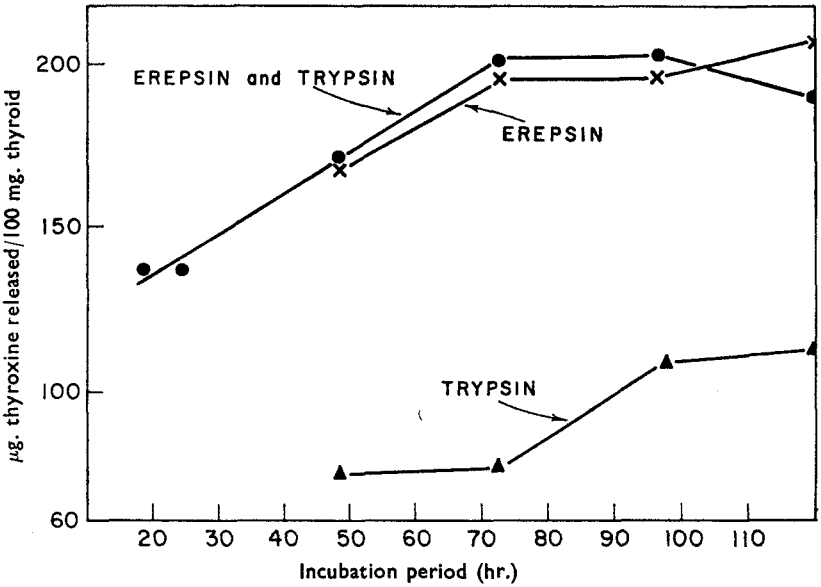

FIG. 4. The rate of release of thyroxine from pork thyroid by proteolytic hydrolysis.

TABLE I

RECOVERY OF LIOTHYRONINE AND THYROXINE FROM THYROID HYDROLYSATES

	Liothyronine				Thyroxine			
Digest	From thyroid μg.	Added standard μg.	Measured total μg.	Per cent recovery	From thyroid μg.	Added standard μg.	Measured total μg.	Per cent recovery
1	0·133	0·20	0·343	105	0·502	1·0	1·408	91
2	0·136	0·20	0·338	101	0·514	1·0	1·428	91
3	0·134	0·20	0·347	107	0·563	1·0	1·565	100

Recoveries

To demonstrate recovery values, quantities of liothyronine and thyroxine were added to digests of thyroid at the beginning of the incubation period to provide final concentrations which would approximate those obtained when thyroid alone was processed. The values shown in Table I indicate that extraction with butanol provided satisfactory recovery of liothyronine and thyroxine. In addition, other amino-acids or peptides released during hydrolysis did not appear to interfere with the quantitative measurement of the iodinated thyronines. The slight increase in per cent recovery

of liothyronine suggests the possibility that some of the thyroxine may be converted to liothyronine at some stage during the analysis.

Liothyronine and Thyroxine in Thyroid

The per cent composition of five samples of pork thyroid is listed in Table II. Samples A, B and E were desiccated thyroid while C and D

TABLE II

PER CENT BY WEIGHT COMPOSITION OF PORK THYROID

Thyroid	Iodine per cent	Moisture per cent	Thyroxine per cent	Liothyronine per cent
A	0·23	6·4	0·047 ± 0·002*	0·019 ± 0·001
B	0·79	8·8	0·212 ± 0·007	0·073 ± 0·002
C	0·86	6·5	0·230 ± 0·005	0·108 ± 0·005
D	1·02	7·2	0·243 ± 0·004	0·090 ± 0·002
E	0·63	7·8	0·146 ± 0·003	0·075 ± 0·002

* S.E.

TABLE III

THE RELATION OF LIOTHYRONINE, THYROXINE AND "THYROXINE" IODINE (B.P.) CONCENTRATION IN THYROID TO TOTAL IODINE

Thyroid	mg. T−4 per 100 mg. I	mg. T−3 per 100 mg. I	Molar ratio T−3: T−4	$\frac{I_{T-4} + I_{T-3} \times 100}{I_{Total}}$	$\frac{\text{"Thyroxine" iodine} \times 100}{I_{Total}}$
A	20·4	8·3	0·48	18·2	27·4
B	26·8	9·2	0·42	22·9	32·4
C	26·7	12·5	0·57	24·8	33·6
D	23·8	8·8	0·44	20·8	33·0
E	23·2	11·9	0·61	22·2	30·2

T−4 = thyroxine. T−3 = liothyronine.

were commercial pork thyroglobulin preparations. The samples were of North American origin with the exception of E which was obtained from the United Kingdom. The per cent total iodine was determined by the oxygen flask method (Johnson and Vickers, 1959), which gives values that are usually slightly higher than those obtained by the procedure described in the Pharmacopeia of the United States XVI.

In Table III, the concentrations of liothyronine and thyroxine are expressed as mg. of thyronine per 100 mg. of thyroid iodine. The variation in the per cent of the total iodine found in the liothyronine plus thyroxine fraction shown in Table III, suggests that the total iodine is related to the biologically active iodinated thyronine content in an approximate manner only. In addition, this relation is complicated by the fact that the ratio of liothyronine to thyroxine varies significantly.

Also from Table III, the per cent of the total iodine found in the "thyroxine" iodine fraction as measured by the method in the British Pharmacopoeia was consistently higher than that estimated from the liothyronine plus thyroxine content. When the material precipitated from an alkaline hydrolysate of thyroid at pH 3·5 was chromatographed on paper, using the t-pentanol: N-ammonia system, thyroxine as well as liothyronine and other unidentified iodinated compounds were detected.

Molar concentrations of Liothyronine and Thyroxine

The samples available for this investigation were obtained from four different sources and it is assumed that each sample was taken from a pool collected from large numbers of animals. In Table III it is noted that pork thyroid is iodinated to a degree where the molar ratio of liothyronine to thyroxine for all samples is close to 0·5. The molar residues for the two pork thyroglobulin samples, C and D, have been calculated and are shown in Table IV. Apparently, for each mole of thyroglobulin, assuming a molecular weight of 650,000 (Derrien and others, 1949) there is approximately 1 mole of liothyronine and 2 moles of thyroxine.

TABLE IV

MOLAR RESIDUES OF LIOTHYRONINE AND THYROXINE IN COMMERCIAL HOG THYROGLOBULIN

Thyroid	Residues thyroxine per mole thyroglobulin	Residues liothyronine per mole thyroglobulin
C	2·1	1·2
D	2·2	1·0

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