THE RELATIVE POTENCIES OF THYROXINE AND LIOTHYRONINE BY ORAL AND SUBCUTANEOUS ADMINISTRATION IN THE RAT

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In goitre prevention assays in adult female rats, thyroxine was only one-fifth to one-sixth as active by mouth as by subcutaneous injection whereas liothyronine had about the same biological activity for the two routes of administration. These findings provide an explanation for the fact that thyroxine was one-sixth to one-seventh as active as liothyronine when injected subcutaneously but only one-twentieth to one-thirtieth as potent when the hormones were administered orally. Chemical examination of three commercially available thyroxine samples which were labelled as "chromatographically pure" revealed that one of them contained approximately 10 per cent liothyronine. The presence of this contaminant had a marked influence on the biological responses of the test animals to this preparation.

Recently Wiberg, Devlin, Stephenson, Carter and Bayne (1962) demonstrated that the liothyronine (tri-iodothyronine) content of orally administered desiccated thyroid accounted for most of the biological activity as measured by the goitre-prevention response in adult female rats treated with thiouracil. This conclusion was based on the results of a series of bioassays of pork, beef, and sheep thyroid preparations for which the content of liothyronine and thyroxine had been determined by the method of Devlin and Stephenson (1962). A possible reason for this observation could be that the availability of an oral dose of liothyronine is substantially greater than that of thyroxine (Gross and Pitt-Rivers, 1953). Inasmuch as the parenteral potency of liothyronine is known to be 5–7 times that of thyroxine (Danowski, 1962), the oral administration of these substances would effect a still greater disparity in the comparative biological activity.

The present investigation was designed to secure quantitative proof of this contention. The availability of liothyronine and thyroxine from the oral route has now been measured and in addition, the relative potency of the two hormones by subcutaneous injection and gastric intubation has been compared.

Methods

Three different samples of "pure" sodium L-thyroxine pentahydrate purchased directly from the manufacturers were examined. The liothyronine was kindly supplied by Smith, Kline and French Co. Ltd. as well as a thyroxine preparation which served as a chemical standard.*

* Data accompanying the Smith, Kline and French samples indicated that for their sodium liothyronine salt ("Cytomel" sample RM 3678) 1·137 mg. was equivalent to 1·0 mg. of the free base, and that for their sodium L-thyroxine pentahydrate preparation (Elthrin sample BS 7861) 1·123 mg. was equivalent to 1·0 mg. of the free base.

Chromatographic analyses of the various preparations were made by the method of Devlin and Stephenson (1962) and the total iodine content of the thyroxine samples were determined by the oxygen flask method of Johnson and Smith (1961).

The thyroid hormones were dissolved in a solution containing 95 per cent ethanol (9 vols.) and 20 per cent acetic acid in water (1 vol.), such that each ml. contained either 100 μg. thyroxine or 10 μg. liothyronine. Final dilutions were made from these stock solutions with 1 per cent sodium bicarbonate immediately before dosing.

Adult female rats, weighing 150–160 g., derived from an inbred Wistar Strain were used. They were fed *ad libitum* a diet of ground chow containing 3 per cent maize oil and 0·3 per cent thiouracil. Each dose group contained eight animals. The dose-response relation for six doses, separated by a dose interval of 1·35 was investigated. The doses (1·0 ml./rat) were adminstered for 14 consecutive days, orally by a blunted No. 17 gauge $2\frac{1}{2}$ in. hypodermic needle, or subcutaneously by an interscapular injection. At the end of this period, the animals were killed and the relative thyroid weights determined.

Log dose-response curves for each substance were plotted and only those doses which produced a response lying on the linear portion of the curve were used in subsequent calculations. The relative potency and confidence limits were calculated by conventional statistical procedures (Bliss, 1952; Finney, 1952). The simultaneous determination of the oral and subcutaneous log dose-response lines for each substance permitted an evaluation of the availability from the gut of the various thyroactive preparations. The oral and subcutaneous potencies of liothyronine relative to each thyroxine sample were determined in a separate series of assays.

RESULTS

A comparison of the effectiveness of thyroxine and liothyronine by the oral and subcutaneous routes is presented in Table I. Thyroxine

TABLE I

A COMPARISON OF THE BIOLOGICAL AVAILABILITY OF VARIOUS THYROXINE SAMPLES AND A LIOTHYRONINE PREPARATION FROM THE ORAL ROUTE IN ADULT FEMALE RATS USING THE GOITRE PREVENTION RESPONSE

Sample	Type of assay	Availability from the oral route with 95 per cent confidence limits (Subcutaneous potency = 100)	Index of Precision
Thyroxine A ..	3 × 2	21·8 per cent 20·6–23·6 per cent	0·034
Thyroxine B ..	3 × 3	16·8 " 15·5–18·2 "	0·054
Thyroxine C ..	2 × 3	30·3 " 27·8–32·9 "	0·054
Liothyronine .. Experiment 1	3 × 3	103·6 " 95·9–112·3 "	0·060
Liothyronine .. Experiment 2	2 × 3	74·0 " 63·9–85·5 "	0·050

samples A and B were only one-fifth to one-sixth as active by the oral route as when injected whereas thyroxine C retained one-third of its parenteral potency. The availability of liothyronine by the oral route

was even greater. In the first experiment, liothyronine had the same level of biological activity for each of the two routes of administration but in a second experiment the oral dose possessed 26 per cent less activity than the equivalent dose administered subcutaneously. The weight of the thyroid gland in the goitre prevention assay has an upper and lower limit and the slope of the log dose response line is steep. Thus it is necessary to use a small dose interval between successive doses in order to have two or three responses fall on the linear portion of the curve. Therefore slight variations in sensitivity between groups of test animals to a thyroactive substance can produce significant differences in the estimated potency in replicate assays of the same materials. However, the experiments show that from 75 to 100 per cent of an oral dose of liothyronine is available. These results are of the same order of magnitude as those of Gross and Pitt-Rivers (1953) who estimated an oral dose of liothyronine to be 86 per cent as active as the comparable subcutaneous dose. The availability of an oral dose of thyroxine on the other hand is considerably less.

The potency of liothyronine relative to the three thyroxine samples by the subcutaneous route is recorded in Table II, and the estimates are in

TABLE II

SUBCUTANEOUS POTENCY OF LIOTHYRONINE RELATIVE TO VARIOUS THYROXINE SAMPLES BY THE GOITRE PREVENTION ASSAY

Sample	Type of assay (S × U)	Relative potency with 95 per cent confidence limits*		Index of Precision
Thyroxine A		1·00	—	
Liothyronine	3 × 3	6·25	5·86–6·68	0·043
Thyroxine B		1·00	—	
Liothyronine	3 × 3	6·97	6·45–7·59	0·046
Thyroxine C		1·00	—	
Liothyronine	3 × 3	5·24	4·82–5·71	0·053

* Potencies were computed on an equimolar basis.

good agreement with the results of other workers, i.e., that liothyronine is from 5 to 7 times as potent as thyroxine on a molar basis (Danowski, 1962). However, when the oral activity of liothyronine is compared to that of the three thyroxine samples (Table III) a vastly different relationship was observed. Here liothyronine was much more active than any of the thyroxine preparations investigated: thus, it was 23 times as potent as thyroxine "A", 30 times more potent than thyroxine "B", and 12 times as effective as thyroxine "C". These estimates of oral activity were in general agreement with those found in a second series of bioassays also shown in Table III.

As the same liothyronine preparation was used in each of the assays, it would seem that some of the thyroxine samples must contain other active components, or conversely, inert materials to account for these marked differences in oral potency between the three products. Accordingly the purity of the thyroxine samples was checked by total iodine analysis and paper chromatographic examination. The results of

these investigations are presented in Table IV. Total iodine determinations did not reveal any great variation between the three samples, certainly not enough to be detected by biological assay. However examination of the paper chromatograms showed that thyroxine "C" contained liothyronine. Quantitative elution of "liothyronine" and "thyroxine" spots from the three thyroxine samples and their subsequent chemical

TABLE III

ORAL POTENCY OF LIOTHYRONINE RELATIVE TO VARIOUS THYROXINE SAMPLES BY THE GOITRE PREVENTION ASSAY

Sample	Type of assay (S × U)	Relative potency with 95 per cent confidence limits*		Index of Precision
Thyroxine A	3 × 2	1·0	—	0·057
Liothyronine		23·2	21·7–25·0	
Thyroxine A	3 × 2	1·0	—	0·064
Liothyronine		21·2	18·6–23·2	
Thyroxine B	3 × 3	1·0	—	0·063
Liothyronine		30·1	28·4–34·1	
Thyroxine B	3 × 2	1·0	—	0·058
Liothyronine		24·0	22·0–26·2	
Thyroxine C	3 × 3	1·0	—	0·044
Liothyronine		12·2	11·2–13·3	
Thyroxine C	3 × 3	1·0	—	0·052
Liothyronine		11·9	11·0–12·8	

* Potencies were computed on an equimolar basis.

assay against the standards also revealed that thyroxine "A" contained slightly more thyroxine than did samples B and C. It also showed that that thyroxine "C" was not pure but that it contained approximately 10 per cent liothyronine. Since it has been found that liothyronine is 20 to 30 times more active orally than thyroxine, then paradoxically this contaminant would account for a major proportion of the biological activity of thyroxine "C" at least when given by mouth to rats.

TABLE IV

CHEMICAL ANALYSIS OF COMMERCIAL THYROXINE SAMPLES

Sample	Total iodine		Chromatographic analysis	
	per cent found	per cent of theory*	Thyroxine (T_4) per cent recovered†	Liothyronine (T_3) per cent recovered†
Thyroxine "A" ..	57·3	100·4	109	—
Thyroxine "B" ..	56·4	98·8	101	—
Thyroxine "C" ..	56·0	98·1	102	10

* Pure sodium thyroxine pentahydrate contains 57·10 per cent iodine.
† These values represent the amount of T_4 and T_3 eluted from chromatograms of the various samples in comparison to the chemical standards which underwent identical treatment.

DISCUSSION

Thyroxine "C" will not be considered in the first part of this discussion since it contained liothyronine.

Quantitative proof has been obtained that thyroxine is much less active by the oral route than by subcutaneous injection in rats. This

loss of biological activity by thyroxine could result from various mechanisms. The simplest and a frequently advanced explanation is that of incomplete absorption of thyroxine from the gastrointestinal tract (Clayton, Free, Page, Sommers and Woollett, 1950; Albert, Tenney and Lorenz, 1952; Levy and Knox, 1961). However proof of this hypothesis would be difficult since a number of complications are involved. Thyroxine in rats undergoes an entero-hepatic circulation (Albert and Keating, 1952; Pitt-Rivers and Tata, 1959), hence studies of faecal thyroxine levels will not provide conclusive evidence of incomplete absorption. Furthermore, certain dietary components, including ground chow, may increase the faecal loss of thyroxine (van Middlesworth, 1957; Beck, 1958). Also, Stasilli, Kroc and Edlin (1960) have reported that thiouracil increases the faecal thyroxine level above that of control animals. Acceptable evidence for the incomplete absorption of thyroxine from the gut would have to make allowance for these factors.

Alternative explanations for the loss of activity after oral ingestion of thyroxine include such possibilities as metabolic transformation of the hormone by the intestinal flora, e.g., deiodination or decarboxylation; chemical degradation at the alkaline pH of the intestinal tract or perhaps racemisation. Whatever the mechanism or mechanisms involved, an oral dose of liothyronine does not seem to be subject to the same influences as those acting on thyroxine. The availability of these hormones from the gastrointestinal tract can be discussed without specifying a particular mechanism and this we have done.

Probably one-sixth or one-quarter of the oral dose of thyroxine is available whereas at least three-quarters and perhaps the entire oral dose of liothyronine is available. Bioassays confirmed that liothyronine is 5 to 7 times as active as thyroxine by the subcutaneous route. Consequently if the factors of gastrointestinal availability and parenteral potency operate in conjunction, then the oral activity of liothyronine in rats could range from 20 to 40 times that of thyroxine. The results obtained with thyroxine samples A and B fully support this conclusion.

This marked difference in the oral potencies of liothyronine and thyroxine has immediate relevance to the biological activity of desiccated thyroid. Analyses made in this laboratory have shown that the molar ratio of thyroxine to liothyronine usually varies from 2:1 to 3:1 for samples of pig, ox and sheep thyroid (Devlin and Stephenson, 1961; Wiberg and others, 1962)*. Provided there is no interaction between liothyronine and thyroxine, it is obvious that the greater part of the activity of thyroid powder, by mouth, is due to the liothyronine content and not to thyroxine. The data leading to these conclusions were obtained with the goitre-prevention response in adult female rats and may not be applicable to man. Nevertheless, in man, thyroxine has been reported to be less active by mouth than by the parenteral route (Thompson, Thompson and Dickie, 1933; Blackburn and Keating, 1954),

* Investigations made by one of us (W. F. D.) now include more than 25 different samples of thyroid powder from these three species and the molar thyroxine: liothyronine ratio has never been greater than 3:1.

whereas liothyronine has about the same activity for both routes (Lerman, 1953; Blackburn and Keating, 1954). The literature comparing the physiological responses of man to thyroxine and liothyronine is extensive and not always in agreement (Starr and Liebhold-Schuek, 1953, Selenkow and Asper, 1955; Strisower, Gofman, Strisower and deLalla, 1958; Kyle, Canary, Meyer and Pac, 1958; Wellby, Good, Charnock and Hetzel, 1960). However the validity of any comparison is dependent upon the purity of the hormones used.

With commercially available sodium thyroxine, we have observed some discrepancies in the total iodine content which could be due to varying water content, other inert material, or iodinated substances. Sodium thyroxine pentahydrate and anhydrous sodium liothyronine have virtually the same theoretical iodine content: 57·10 per cent for the thyroxine salt and 57·07 per cent for the liothyronine salt. Therefore total iodine analysis of a supposedly pure sample of sodium thyroxine pentahydrate will not be altered by the presence of liothyronine. Similarly contamination of sodium liothyronine by the thyroxine salt will not change the iodine content. Accordingly, an alternative method for assessing the purity of the iodothyronines must be used.

Paper chromatographic procedures for the resolving of liothyronine, thyroxine and other iodinated compounds are available and would appear to be the method of choice for establishing purity but for this purpose they are subject to the limits of sensitivity in detecting contaminants. Not all methods are equally sensitive in detecting small amounts of iodinated substances. Provided a solvent system has been used which separates liothyronine and thyroxine, the extremely sensitive Bowden, Maclagan, Wilkinson (1955) staining procedure (in which iodinated compounds act *catalytically* in the reduction of ceric sulphate by arsenious acid reagent) would be superior to a stoichiometric chemical reaction such as the diazotisation stain of Gross and LeBlond (1951). Considering the ceric sulphate-arsenious acid stain alone, (*a*) the relative concentrations of the ceric ion and arsenious acid, (*b*) the acidity of reagents, and (*c*) the reaction time, can be varied to reach a sensitivity of detection of 0·05 μg. iodinated thyronine. Further modification, such as that suggested by Stolc (1958), which involved spraying the paper wtih fluorescein and subsequent examination under ultraviolet light, can be used to obtain greater sensitivity. Similarly, Gawienowski (1957) advocated spraying with brucine sulphate and Gmelin and Virtanen (1959) have employed a "ferrichloride-ferricyanide-arsenic acid" spray to increase the sensitivity. Consequently the term, "chromatographically pure" applied to thyroxine and liothyronine preparations depends upon the methods used.

The presence of liothyronine in thyroxine sample C appreciably affected its biological activity, especially by mouth. Thus the apparent oral potency of liothyronine relative to thyroxine was reduced about two-fold. As would be expected biological assays of the three thyroxine samples indicated that thyroxine "C" was much more potent orally than thyroxine samples A and B.

Wiberg and Stephenson (1961) noted earlier that the slope of the log dose-response curve for L-thyroxine in the goitre prevention assay was significantly less steep than that for desiccated thyroid. The thyroxine preparation used in those studies was thyroxine sample A. The slope of log dose-response lines for thyroxine sample C in similar tests was steeper than that for thyroxine samples A and B and approached that obtained for desiccated thyroid in the goitre prevention assays.

The choice of a satisfactory solvent for stock solutions of liothyronine and thyroxine is of importance. Traditionally aqueous solutions of sodium bicarbonate or carbonate have been used, since the pH of the solution is suitable for parenteral adminstration and the chance of racemisation is reduced. However, in our experience, the liothyronine and thyroxine preparations lose some of their activity in these media. For example, a sample of thyroxine lost 33 per cent of its biological activity over the 14-day dosing schedule when administered in sodium bicarbonate compared to the same substance dissolved in the acetic acid-ethanol solvent. In addition, Maclagan, Bowden and Wilkinson (1957) report that thyroxine undergoes chemical decomposition in an aqueous solution of sodium carbonate. Chromatographic studies in this laboratory not only confirmed this observation but also indicated that samples of liothyronine and thyroxine dissolved in acetic acid-ethanol were stable up to two weeks.

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