Quantitation of Extrathyroidal Conversion of L-Thyroxine to 3,5,3'-Triiodo-L-Thyronine in the Rat

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A B S T R A C T   Studies of the rate of extrathyroidal conversion of thyroxine (T4) to 3,5,3'-triiodo-L-thyronine (T3) were carried out in rats. Total body homogenates were prepared and extracted with ethanol 48, 72, and 96 hr after the intravenous injection of ^{125}I-T4. ^{131}I-T3 was added, and the paper chromatographic purification of T3 was effected by serial elution and rechromatography in three paper and one thin-layer cycles. The ratio of ^{131}I-T3 and ^{125}I-T3 counting rates in the final chromatograms, which was identical in three different paper chromatography systems, was used to calculate the proportion of ^{125}I-T3 to ^{125}I-T4 in the original homogenates. In order to discount the effects of in vitro monodeiodination of T4 during extraction and chromatography, we killed control animals immediately after injection of ^{125}I-T4 and processed them in a similar fashion to the experimental groups. The average ratio of ^{125}I-T3 to ^{125}I-T4 in carcass extracts of animals killed between 48 and 96 hr after isotopic injection was 0.08 whereas the average ratio of ^{125}I-T3 to ^{125}I-T4 in chromatograms of control animals was 0.01. On the basis of the proposed model, calculations indicated that about 17% of the secreted T4 was converted to T3. Assuming values cited in the literature for the concentration of nonradioactive T3 in rat plasma, these findings would suggest that about 20% of total body T3 is derived by conversion from T4. Moreover, since previous estimates have suggested that in the rat, T3 has about 3 to 5 times greater biologic activity than T4, these results also raise the possibility that the hormonal activity of T4 may be dependent in large part on its conversion to T3.

A necessary assumption in calculating T4 to T3 conversion in this and other studies is that the 3' and 5' positions are randomly labeled with radioiodine in

phenolic-ring iodine-labeled T4. Evidence supporting this assumption was obtained in the rat by comparing the amount of labeled T3 produced after injection of phenolic and nonphenolic-ring iodine–labeled T4.

INTRODUCTION

Since the initial identification of triiodothyronine (T3) in human plasma by Gross and Pitt-Rivers (1), a number of studies have dealt with the source of this hormone. In laboratory animals, the concentration of T3 is higher in the thyroid venous effluent than in the arterial blood, suggesting its secretion by the gland (2, 3). On the other hand, a number of studies have focused on the possibility of the extrathyroidal conversion of thyroxine (T4) to T3 (4–7). A claim that T4 was converted to T3 in man (8) was subsequently retracted (9) and the likelihood of metabolically significant conversion of T4 to T3 was generally discounted.

More recently, the possibility of peripheral conversion of T4 to T3 was revived by the report of Braverman, Ingbar, and Sterling (10) that demonstrated the presence of T3 in the plasma of athyreotic human subjects maintained on constant doses of T4. Both labeled and nonradioactive T4 were given by oral and intravenous routes. Subsequently, Sterling, Brenner, and Newman (11) and Pittman, Chambers, and Read (12) have reported the conversion to T3 of isotopically labeled T4 administered intravenously to normal human volunteers.

In spite of these findings, there has been considerable uncertainty about the extent of metabolic conversion. Difficulty in making precise quantitative assessments of T4 to T3 conversion is due largely to the fact that recent studies have been based exclusively on analysis of plasma samples. In plasma, the concentration of T4 is manyfold greater than that of T3; T3 is primarily an intracellular hormone (13). Thus, after the injection of labeled T4 in man, only about 0.5–3.0% of the plasma radioactivity

is in the form of T3 (11, 12). Clearly, even a small overlap of radioactivity from the T4 to the T3 region of the chromatogram or any difficulty in making a precise estimate of the "paper background" radioactivity will introduce a substantial error in any estimate of conversion. Moreover, the possibility that a small proportion of radioactive T4 is converted to T3 in vitro during extraction and chromatography has not been rigorously excluded. In this connection, Dussault and Fisher (14) have recently reported that when appropriate account was taken of artifactual in vitro conversion, no metabolic transformation of T4 to T3 could be demonstrated in the sheep.

Because of the technical problems inherent in the analysis of plasma, we attempted to base a quantitative estimation of T4 to T3 conversion on measurements of rat carcass after injection of labeled T4. The results of these studies will be described.

METHODS

Sprague-Dawley rats, weighing approximately 130 g, were maintained on Wayne Lab diet and water ad lib. Commercially obtained ¹²⁵I-labeled thyroxine (¹²⁵I-T4) was repurified by paper chromatography. Contaminating ¹²⁵I-triiodothyronine (¹²⁵I-T3) ranged from 0.4 to 0.8%. 30–50 μc of the ¹²⁵I-T4 in 4% albumin were injected via the tail vein; 0.5 mg of iodide were administered subcutaneously daily in order to prevent thyroidal reutilization of radioactive iodide. 4 rats were killed at 48 hr, 6 at 72 hr and 1 at 96 hr after injection. In order to correct for the possibility of artifactual conversion of ¹²⁵I-T4 to ¹²⁵I-T3 during the analytical procedures, animals were similarly injected and killed within 2 min. Intestinal contents were removed since intraluminal radioactivity was considered not to contribute to exchangeable hormonal pools (13). The total carcass[1] was then homogenized with added water. Two 5-g portions of the homogenate were extracted with 5-ml volumes of 95% ethanol until at least 85% of the counts had been removed. Purified ¹³¹I-T3 was added to the combined ethanolic extracts in amounts equivalent to approximately 3–5% of the ¹²⁵I activity present. This served as a radioactive marker for T3, allowed appropriate corrections for losses during the procedure, and made possible the determination of the ¹²⁵I-T3 to ¹³¹I-T3 ratio as a rigorous criterion for the identification of any T3 derived from the injected ¹²⁵I-T4. The extracts were concentrated in vacuo and applied, along with carrier T4 and T3, to Whatman 3MM paper. Descending chromatography was carried out overnight in tert-amyl alcohol: 2 N ammonia: hexane (15). The T3 area was visualized under ultraviolet (UV) light and eluted with methanol: ammonia (95:5). The eluates were concentrated and rechromatographed in the same solvent system. This procedure was repeated once again for a total of three cycles of paper chromatography (Fig. 1). The eluates of the third chromatogram were applied to silica gel thin layer chromatography plates (Eastman Kodak Co.) and chromatographed in a solvent system consisting of toluene: acetic acid: water (2:2:1) (16). In this system, T4, T3, and

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[1] In contrast to a previous study in which rat carcass was studied after removal of liver, kidney, and plasma (13), carcass in the present experiments included these tissues.

iodide remain at the origin while tetraiodothyroacetic acid has an R_f of approximately 0.6. The origin area was eluted with methanol: ammonia. Equal portions were chromatographed on paper in three solvent systems: (a) tert-amyl alcohol: 2 N ammonia: hexane; (b) butanol: dioxane: 2 N ammonia (4:1:5), descending; and (c) butanol: ethanol: 0.5 N ammonia (5:1:2), ascending. After drying and staining with diazotized sulfanilic acid (17) to visualize the T4 and T3 areas, we cut the T3 area into 0.5 cm sections and counted them in a 2-channel Packard Autogamma (Model 410A). The counting rates for the two isotopes were corrected for isotopic decay during the time that elapsed since the initial extraction of the carcass homogenate. Corrections were made for spillover from the ¹³¹I channel to the ¹²⁵I channel (average 11%). The ratio of ¹²⁵I to ¹³¹I in the T3 peak was determined. Recovery of added ¹³¹I-T3 varied from 50 to 60%.

Since the losses of T3 carrying either radioactive label can be assumed to be proportional, the per cent of total ¹²⁵I counts in the carcass present as T3 is

$$= \frac{r \times {}^{131}I\text{-}T3 \times 100}{{}^{125}I}$$

where r is the ¹²⁵I to ¹³¹I ratio in the T3 area of the final chromatograms; ¹³¹I-T3, the known amount of counts per minute added to the original carcass extract; and ¹²⁵I is the total counts per minute of this isotope in the original carcass extract.

Estimation of the fraction of radioactivity representing inorganic iodide was made from chromatography of a small portion of the carcass ethanol extracts. The fraction of radioactivity in the initial extract representing T4 was taken as the difference between the total radioactivity and the ¹²⁵I-T3 and ¹²⁵I-. The method employed did not allow quantitation of ¹²⁵I-tetraiodothyroacetic acid. Since studies by Pitt-Rivers and Rall (18) and Heninger, Larson, and Albright (19) using the technique of isotopic equilibrium did not show significant quantities of this compound in the rat, it is likely that this would not lead to significant error in our estimates of conversion. The fractional turnover of T4 was calculated from the product of the per cent T4 and the residual carcass radioactivity determined from a weighed portion of the carcass homogenate (13).

An important assumption in calculating the T4 to T3 conversion (see below) is that phenolic-ring iodine–labeled T4 as commercially prepared (20) carries only a single radioactive atom of iodine randomly distributed between the 3' and 5' positions. In order to test the validity of this assumption, three animals were injected with a combined dose of phenolic-ring labeled ¹³¹I-T4 and nonphenolic-ring-labeled ¹²⁵I-T4 synthesized by the method of Surks and Shapiro (21). The animals were killed 48 hr later. Homogenization, extraction and chromatographic analyses were carried out as described above.

Kinetic Analysis. After the injection of tracer T4, the rate of change of tracer T3 in the body represents the net effect of conversion of T4 to T3 and removal of T3. This relationship can be formalized by the following differential equation

$$\frac{d(T3)^*}{dt} = k(T4)^* - \lambda_3(T_3)^* \tag{1}$$

where $(T4)^*$ and $(T3)^*$ represent the pools of labeled T4 and T3 at any time t following the injection of labeled T4, k, the fractional rate of conversion of labeled T4 to T3, and λ_3, the
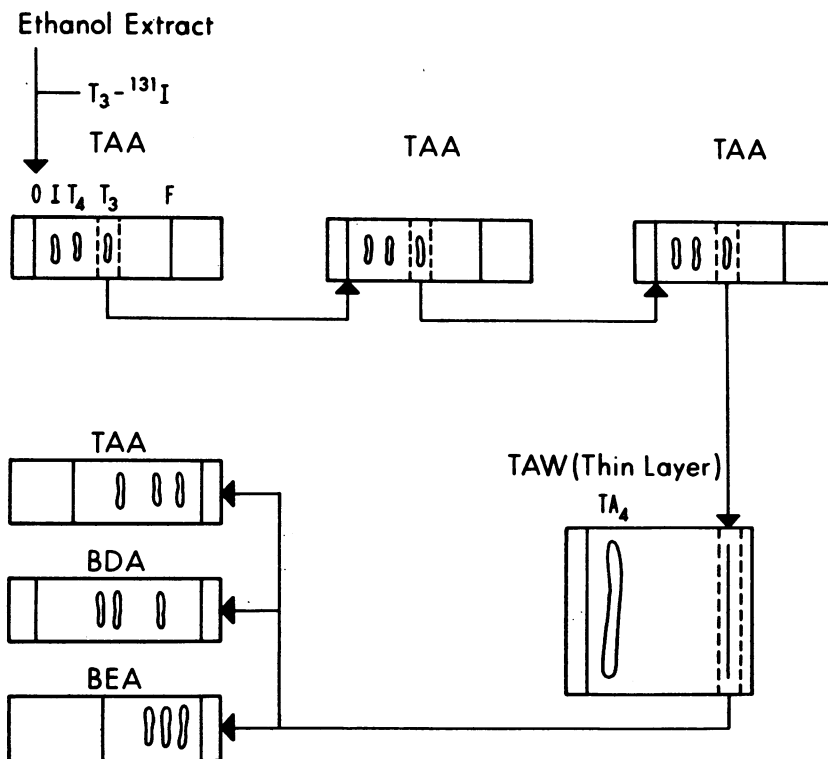
Ethanol Extract

FIGURE 1 Scheme for chromatographic separation of radioactive T3 in tissue homogenates. See text for details. TAA, *tert*-amyl alcohol:2 N ammonia:hexane; TAW, toluene:acetic acid:water; BDA, butanol:dioxane:ammonia; BEA, butanol:ethanol:ammonia.

fractional irreversible removal rate of T3. The fractional irreversible rate of removal of T4 can also be represented by the following first order process

$$\frac{d(T4)^*}{dt} = -\lambda_4 (T4)^* \qquad (2)$$

where λ_4 is the fractional rate of removal of T4.

Differential equations 1 and 2 can be solved by standard methods. If at t = 0, T4 = 1, it can be shown that at any time t

$$(T3)^* = \frac{k}{\lambda_3 - \lambda_4} (e^{-\lambda_4 t} - e^{-\lambda_3 t}) \qquad (3)$$

$$(T4)^* = e^{(-\lambda_4 t)} \qquad (4)$$

Dividing Equation (3) by Equation (4)

$$\left(\frac{T3^*}{T4^*}\right) = \frac{k}{\lambda_3 - \lambda_4} [1 - e^{-(\lambda_3 - \lambda_4)t}] \qquad (5)$$

Thus, on rearranging

$$k = \frac{\left(\frac{T3^*}{T4^*}\right)(\lambda_3 - \lambda_4)}{1 - e^{-(\lambda_3 - \lambda_4)t}} \qquad (6)$$

It is generally assumed that positions 3' and 5' are randomly labeled in [125]I- and ^{131}I-phenolic-ring–labeled T4. Thus, for every molecule of ^{125}I-T4 monodeiodinated, a molecule of unlabeled T4 must also be converted to T3. The fractional conversion of unlabeled T4 to T3 therefore is 2k, where k, as used in the above equations, represents the fractional conversion of T4 labeled in the phenolic ring. Experimental evidence supporting the use of the factor 2 is presented in Results.

A conversion ratio (CR) representing the percentage contribution of T4 to T3 conversion to the net turnover of T4 can be defined and by use of equation 6, evaluated as follows

$$CR = \frac{2k(100)}{\lambda_4} = \frac{200 \left(\frac{T3^*}{T4^*}\right)\left(\frac{\lambda_3}{\lambda_4} - 1\right)}{1 - e^{-(\lambda_3 - \lambda_4)t}} \qquad (7)$$

This equation was used to calculate the final term entered into Table I.

Since λ_3 is greater than λ_4, at time t = ∞, Equations 5 and 7 can be simplified to the following expressions.

$$\left(\frac{T3^*}{T4^*}\right)_\infty = \frac{k}{\lambda_3 - \lambda_4}$$

$$(CR)_\infty = 200 \left(\frac{T3^*}{T4^*}\right)\left(\frac{\lambda_3}{\lambda_4} - 1\right)$$

Thus, a state of secular equilibrium between (T3)* and (T4)* will supervene.

RESULTS

Chromatography in three serial paper and one thin-layer silica gel systems in conjunction with the addition of authentic ^{131}I-labeled T3 to the homogenate, facilitated both the rigorous identification of ^{125}I-T3 and a precise estimate of the percentage of T3 in the carcass. As illustrated in Fig. 2, chromatography of the final thin-layer eluate in three different solvent systems resulted in ^{131}I and ^{125}I peaks in the T3 area with similar (^{125}I/^{131}I) ratios. Inspection of the curves illustrated in Fig. 2 will indicate that the ratio of ^{125}I to ^{131}I is constant throughout the individual peaks, another indication of chemical identity.

One of the principal problems encountered in previous efforts to determine the percentage conversion of T4 to T3 was uncertainty about the extent of in vitro monodeiodination during extraction and chromatography. Our results would support the possibility that monodeiodination can occur in vitro. In one study in which the injected dose was rechromatographed by the same procedure applied to the total body homogenate, the T3/T4 ratio found was 0.0039, whereas the corresponding ratio in three animals injected with this dose and killed immediately varied between 0.0059 and 0.0079. Despite the likelihood of monodeiodination, the T3/T4 ratio found in the homogenates 48, 72, and 96 hr after injection (Table I) exceeded the average T3/T4 ratio in animals killed immediately after injection by a factor of 8.1, 7.7, and 8.0 respectively. Clearly, the high T3/T4 ratio could not be accounted for by artifactual in vitro conversion of T4 during the process of extraction and separation.

In order to calculate the percent of secreted T4 which is converted to T3 a number of assumptions are made.

(a) In the model describing the conversion of T4 to T3 it is assumed that rapid exchangeability occurs both with respect to T4 and T3 generated from T4 within the volume of distribution of injected T4 and T3, respectively.

(b) As indicated in equation 7, the fractional turnover of T4 (λ_4) and T3 (λ_3) must be known. The fractional turnover of T4 can be determined from the data at hand (Table I). The mean fractional turnover of T3 (0.0933/hr) was obtained from previous studies in similar rats (13).

(c) The true T3/T4 ratio used was assumed to represent the difference between the observed T3/T4 ratio and the mean T3/T4 ratio observed in control animals. This difference may be an underestimate since the control T3/T4 value may include small amounts of residual contaminating T3 in the T4 preparation injected.

(d) Another assumption used in this and previous analyses is that in commercial preparation of ^{125}I-T4 the 3' and 5' positions are randomly labeled with iodine. This would imply that for every radioactive T3 molecule
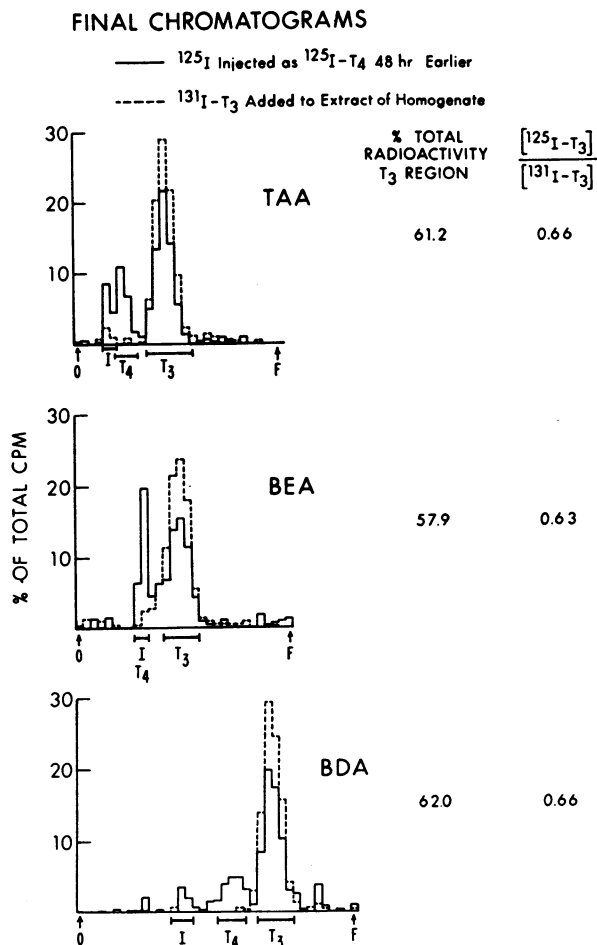
FINAL CHROMATOGRAMS

FIGURE 2 The identity of ^{125}I-T3 is apparent from the parallel radioactivity curves of ^{125}I and ^{131}I from authentic ^{131}I-T3 as well as the similarity of ^{125}I/^{131}I ratios in three different chromatographic systems. Abbreviations as Fig. 1. Approximately 200 cpm both of ^{131}I and ^{125}I were added to each of three chromatograms.

generated, a nonradioactive T3 molecule is also produced. If this is correct, twice as many labeled T3 molecules should be generated after injection of T4 labeled in the nonphenolic ring than after injection of phenolic ring–labeled T4. When a combined dose of nonphenolic (^{125}I)- and phenolic (^{131}I)-ring–labeled T4 was injected into three rats, the average ratio ^{125}I-T3/^{131}I-T3 generated at 48 hr was 2.18. The deviation from the theoretical value of 2 could be attributed to experimental error.

As indicated in Table I results of our analysis show that the calculated percentage conversion at 48 hr is 18.5, at 72 hr 15.9, and at 96 hr, 16.6. Statistical comparisons indicate that these values are not significantly different from each other. These data are thus compatible with the assumed model.

TABLE I

Conversion of ^{125}I-T4 to ^{125}I-T3 after Injection of a Single Dose of ^{125}I-T4

Condi- tions	Animal No.	Radioactive composition of carcass			(T3/T4)*		t₁ of T4	Conver- sion ratio‡
		I⁻	T4	T3	Observed	Corrected		
		%	%	%				
Control§								
	Mean	5.10	93.70	1.20	0.0127	—	—	—
	±SEM	1.10	1.00	0.21	0.0022			
48 hr	1	35.98	59.07	4.95	0.0838	0.0711	13.5	13.4
	2	29.00	64.61	6.39	0.0989	0.0862	15.2	20.0
	3	19.96	71.49	8.55	0.1196	0.1069	14.5	22.9
	4	32.98	60.46	6.56	0.1085	0.0958	13.3	17.6
	Mean	29.48	63.91	6.61	0.1027	0.0900	14.1	18.5
	±SEM	3.48	2.78	0.74	0.0076	0.0076	0.44	2.0
72 hr	1	53.96	42.40	3.68	0.0858	0.0731	13.0	11.6
	2	45.73	49.52	4.75	0.0959	0.0832	13.0	13.2
	3	41.82	52.22	5.96	0.1141	0.1014	14.5	20.0
	4	51.33	43.67	5.00	0.1144	0.1017	14.0	18.7
	5	49.91	46.00	4.09	0.0889	0.0762	15.0	16.1
	6	53.55	42.68	3.77	0.0883	0.0756	15.0	16.1
	Mean	49.38	46.08	4.54	0.0979	0.0852	14.1	15.9
	±SEM	1.94	1.64	0.36	0.0053	0.0053	0.37	1.3
96 hr	1	35.4	58.63	5.97	0.1018	0.0891	14.1	16.6

* Corrected T3/T4 determined from the difference of T3/T4 in specific experimental animal and the mean T3/T4 of control group.

‡ Conversion ratio: Per cent of secreted T4 converted to T3 calculated from Equation 7. The t₁ of T3 was assumed to be 7.43 hr from studies in reference 13.

§ 9 control animals sacrificed within 2 min after injection.

DISCUSSION

The results of this study confirm a number of recent reports that significant conversion of T4 to T3 occurs during the course of the in vivo metabolism of T4 (10, 11, 12). Furthermore, by using tissue rather than plasma samples, some of the technical problems inherent in quantitation of this conversion were obviated. Our findings indicate that between 48 and 96 hr after injection of ^{125}I-T4 the concentration of ^{125}I-T3 found in total body homogenates is about 8 times that which could be attributed to artifactual in vitro conversion. Moreover, addition of authentic ^{131}I-T3 to the homogenate and serial chromatography of tissue extracts in paper- and thin-layer systems facilitated rigorous radiochemical identification of the ^{125}I-T3.

Our results are at variance with over-all implications of a recent report by Dussault and Fisher (14) who were unable to demonstrate significant conversion of T4 to T3 in sheep. Although a species difference cannot be excluded, it appears probable that the degree of in vitro conversion of T4 to T3 in the column chromatography used by these authors was too large to allow reliable detection of the small quantities of T3 in plasma which reflect metabolic conversion. It should be reemphasized that since T3 is largely intracellular in location and is metabolically cleared from the body at a faster rate than T4, the proportion of T3 to T4 in plasma will be very small after injection of labeled T4.

One of the major assumptions in our quantitative analysis of the conversion of T4 to T3 is that the T3 which is generated in tissues is readily exchangeable with the entire T3 pool. This assumption is supported by the finding that our model system is consistent with the experimental results obtained. Another consideration which militates against the possibility of significant pools of nonexchangeable T3 is the following. In the presence of hypothetical nonexchangeable tissue pools the concentration ratio of total T3 between tissues and plasma should be greater than the corresponding exchangeable hormone concentration ratio determined isotopically after injection of labeled hormone. This, however, does not appear to be the case. In long-term iodine

equilibrated rats studied by Heninger, Larson, and Albright (19), the concentration ratio (tissue/plasma) was 7.6 for kidney and 2.9 for liver. In recent distribution studies in our laboratory (13), the equilibrium value of the distribution ratio of isotopically labeled T3 between kidney and plasma was 8.3, and between liver and plasma 5.3. In our studies the corresponding carcass/plasma [2] ratio was 1.52. Although Heninger et al. do not provide a similar ratio for carcass, their value for muscle, 1.22, may perhaps serve as a representative approximation. Thus, under no circumstances is the T3 tissue/plasma ratio derived from equilibration studies higher than the corresponding ratio determined after injection of isotopically labeled hormone. In these calculations we have omitted consideration of nonextractable iodine containing compounds which probably account for no more than 10% of either tissue or plasma iodine (22).

The results of experimental findings and calculations suggest that an average of 17% of secreted T4 is converted to T3. In previous studies we have shown that approximately 47% of T4 is metabolized by deiodination (13). Thus, 36% of T4 deiodination proceeds through T3. The estimate of 17% conversion must be considered an approximation for the following reasons. Individual values for fractional turnover of T3 were not measured; assumptions were made regarding the adequacy of the compartmental analysis; and lastly, the control T3/T4 ratio which was subtracted from the observed carcass T3/T4 ratio may have been an overestimate, since no systematic effort was made to determine the precise T3 contamination in the purified dose. The last consideration, therefore, would lead to an underestimation of the metabolic conversion.

It is possible to calculate the proportion of total body T3 which is due to metabolic conversion from T4, if we assume that the nonradioactive T4 and T3 concentrations determined by isotopic ^{125}I equilibration in the rat are 33.5 ng/ml and 1.15 ng/ml respectively (19). Let us at the same time assume an extrathyroidal T4 space of 16.4 ml/100 g, an extrathyroidal T3 space of 165 ml/100 g, mean fractional T4 and T3 removal rates of 1.032/day and 2.064/day (13). The total turnover of T4 will be 567 ng/day per 100 g body weight ($= 33.5 \times 16.4 \times 1.032$). Thus, 95.8 ng of T4 ($= 0.169 \times 567$) are converted to T3. This will be equivalent to 80.3 ng T3. The total turnover of T3 in the rat will be 392 ng/day per 100 g body weight ($= 1.15 \times 165 \times 2.0664$). Thus, 20.5% ($= 100 \times 80.3/392$) of total T3 turnover is due to the conversion of T4 and the remaining 79.5 ng presumably being secreted by the thyroid.

Of particular interest is the fact that T3 has been estimated to be from 3 to 5 times more metabolically active

than T4 in the rat (23, 24, 25). Thus, it would appear that from 50–85% of the metabolic potency of T4 could be due to its conversion to T3. Since our estimate of conversion may be somewhat low, the possibility should be considered that T4 has no metabolic activity other than that arising through the formation of T3. Under any circumstances, the findings in this and other reports strongly point to the predominant role of T3 in determining the thyroidal status of the organism.

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[2] Carcass is defined in our previous study as residual tissue after exclusion of liver, kidney, plasma, and gut contents.

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