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Studies of the Peripheral Metabolism of Triiodothyronine and Reverse Triiodothyronine in the Rat: A Comparison of Extraction and Chromatographic Methods of Analysis*

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ABSTRACT. Studies were performed of the peripheral metabolism of radioiodine-labeled T_3 and rT_3 in the rat. Two methods of analysis were employed to determine the residual concentrations of labeled hormones after their single iv injection: trichloroacetic acid-ethanol extraction, as employed by others, and a recently described technique for the chromatography of plasma on columns of Sephadex G-25. In the case of both T_3 and rT_3, Sephadex chromatography regularly revealed the appearance in plasma of labeled iodoprotein, radioiodide, and a peak of unidentified radioiodinated materials that eluted just before T_3 (pre-T_3). Radioiodine in the pre-T_3 zone, whether generated from T_3 or rT_3, proved to be almost completely TCA-precipitable and ethanol-extractable. Consequently, for both T_3 and rT_3, plasma disappearance curves derived by the precipitation-extraction technique were at all time points higher than were curves representing chromatographically isolated administered hormone. In the case of rT_3, and to a lesser extent T_3, this discrepancy was further accentuated by the inclusion in the precipitable-extractable counts of a significant fraction (about 5%) of the very large proportion of inorganic radioiodine that appeared in the plasma soon after administration of labeled hormone.

As would be expected, metabolic clearance rates of the two hormones calculated by a multicompartmental technique were much higher when based on data generated chromatographically than when based on data obtained by the precipitation-extraction technique. For T_3, values of the metabolic clearance rates were 17.7 and 14.9 ml/100 g BW/h, respectively. For rT_3, corresponding values were 250 and 171 ml/100 g BW/h. The more rapid clearance of rT_3 than of T_3 from plasma could not be explained by less intense binding of the rT_3 to plasma proteins; on the contrary, the percentage of free rT_3 in rat plasma was found to be only half that of free T_3. It is inferred, therefore, that the difference resides in the ability of one or more tissues to take up and degrade rT_3 more rapidly than T_3. (*Endocrinology* **102:** 1247, 1978)

IT HAS been generally assumed that labeled iodide and iodoprotein are the principal radioiodinated products that appear in the serum of animals or man after the administration of radioiodine-labeled thyroxine (T_4) or T_3 (1, 2). It is recognized, furthermore, that these products must be excluded from measurements of the concentration of residual labeled T_4 or T_3 in plasma if accurate measurements of their metabolic clearance rates (MCR) are to be obtained from analyses of plasma disappearance curves (3). This consideration is far more applicable to T_3 than to T_4, owing to the much more rapid peripheral deiodination of the former hormone and the resulting greater accumulation in plasma of the products of peripheral hormone metabolism.

In almost all previous studies of the peripheral metabolism of T_3, one of two methods has been employed to separate T_3 from iodide and iodoprotein. One is trichloroacetic acid (TCA) precipitation of serum proteins, followed by ethanol extraction of the TCA precipitate, the assumption being that iodide is almost completely excluded from the TCA precipitate and that iodoprotein is completely excluded from the ethanol extract (3). The second method employs ion-exchange column chromatography of serum to separate iodide and iodoproteins from iodothyronines, though mixtures of the latter class of compounds are not resolved into their individual components (4).

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Recent investigations have demonstrated, however, that after the iv injection into man of radioiodine-labeled T_3 or rT_3 and, to a far lesser extent, T_4, there appears in serum a group of radioiodinated compounds comprising a variety of mono- and diiodothyronines and sulfo- and glucurono-conjugates thereof, which rapidly come to constitute a substantial proportion of the total radioiodine-labeled iodothyronines present (5–7). These compounds can be separated from the parent T_3 or rT_3 by chromatography on columns of Sephadex G-25, but not by the precipitation-extraction or the ion-exchange chromatographic methods mentioned above (6). Because of this shortcoming, use of the latter two methods in the processing of plasma samples introduces systematic errors into the measurement of the MCR of T_3 or rT_3 in man. However, these methods do have the advantage of simplicity and rapidity, whereas the Sephadex column chromatographic method is time consuming and laborious.

The present studies were undertaken to ascertain whether labeled products other than iodide and iodoprotein, analogous to those seen in man, also appear in the plasma of the rat after administration of labeled T_3. The extent to which such products influence the measured MCR of T_3 has been assessed by comparing the value of the MCR based on the use of TCA precipitation-ethanol extraction of plasma with that based on the use of Sephadex chromatography to isolate labeled T_3, specifically. In addition, in view of recent interest in the physiology of rT_3 and inasmuch as the peripheral metabolism of rT_3 in the rat has apparently not been studied, we have made similar observations in the case of rT_3, making possible a comparison of the MCR of the two hormones for the rat.

Materials and Methods

Studies were conducted in male Sprague-Dawley rats weighing 125–150 g and maintained on Purina laboratory chow and tap water *ad libitum*. Thirty minutes before initiation of turnover studies, animals were given 1 mg KI sc to block thyroid uptake of radioiodine liberated from administered labeled hormones. At 0-time, animals were given iv injections of 6 μCi [^{131}I]T_3 (85 μCi/μg) mixed with 6 μCi [^{125}I]rT_3 (724 μCi/μg) or 6 μCi [^{125}I]T_3 (50–100 μCi/μg) alone.[1] All labeled hormones had been dissolved in a 1% solution of human serum albumin in saline. At carefully timed intervals between 2 min and 24 hr after injection, animals were exsanguinated by aortic puncture with the use of heparinized syringes. Plasma was quickly separated and frozen until used. Each plasma sample was then analyzed by two methods: TCA precipitation-ethanol extraction and Sephadex chromatography.

TCA precipitation-ethanol extraction

The technique employed was almost identical to that used in other laboratories for the analysis of serum from animals or patients given radioiodine-labeled T_3 (1). Samples of rat plasma were enriched with carrier NaI (1 mM) and methimazole (5 mM). Measured aliquots in scintillation-counting tubes were combined with outdated blood bank plasma to a final volume of 2 ml and treated with 10 ml 20% TCA. After centrifugation, precipitates were washed twice with 10 ml 1% TCA and were then extracted four times with 10 ml absolute ethanol. Recovery experiments using labeled T_3 and rT_3 added to rat plasma indicated that more than 90% of each hormone was recovered in the ethanol extract. Counts for ^{125}I and/or ^{131}I were obtained in the whole plasma, the unextracted TCA precipitate, and in the ethanol-extracted precipitate. All counts were measured in a two-channel γ-scintillation counter, with correction of the ^{125}I counts for spillover of ^{131}I into the ^{125}I channel. In all instances, an aliquot of the administered dose of the appropriate hormone was subjected to TCA-precipitation and the TCA-precipitate was employed as a counting standard. TCA-precipitable, ethanol-extractable (hereafter referred to as ethanol-extractable) radioactivity was calculated as the difference between counts obtained in the precipitate before and after ethanol extraction; this has been considered to represent radioactivity in iodothyronines, as radioiodine in iodide and iodoprotein has been essentially excluded (1).[2]

[1] [^{131}I]T_3 was purchased from Industrial Nuclear Company, St. Louis, MO and [^{125}I]labeled T_3 and rT_3 from Abbott Laboratories, North Chicago, IL.

[2] It is not entirely clear from the reference cited whether, in the measurement of TCA-precipitable, ethanol-extractable radioiodine, TCA-precipitation and ethanol-extraction were performed sequentially on the same aliquot of serum as in the present studies or on separate aliquots, one of which was TCA-precipitated and the other ethanol-extracted. For this reason, a comparison was made of the results yielded by the two analytic techniques. In duplicate aliquots of the same serum, the two methods yielded results that agreed closely.

Sephadex chromatography

Samples of rat plasma (2–4 ml) were diluted with an equal volume of a solution of 8-anilino-1-naphthalene sulfonic acid (9 mg/ml), and 4–8 ml of the resulting mixture were analyzed by chromatography on columns (35 × 2.5 cm) of Sephadex G-25 fine, in a modification (6) of the method described by Green (8). Columns prepared from Sephadex G-25 fine that had been swollen overnight in 5 vol solution containing 0.5 M NaCl and 0.020 M $Na_2S_2O_5$, adjusted to pH 13 with concentrated NaOH, were washed with the same solution. After application of the sample, elution at a rate of 0.8–1.0 ml/min was begun with 300 ml solution of 0.005 M NaCl and 0.020 M $Na_2S_2O_5$, pH 11.7. After the void volume of 50 ml had passed, 50 successive 5-ml fractions were collected which contained labeled iodoprotein and iodide. The eluting solution was then adjusted to pH 12.0 and an additional 180 fractions that contained labeled T_3 and rT_3, and labeled metabolites thereof, were collected. In analyses from animals given [^{125}I]T_3 alone, [^{131}I]T_3 was often added as a marker. For each of the compounds separated by chromatography, its fractional contribution to the total radioactivity eluted was determined, and its concentration in plasma was calculated by determining the product of this fraction and the total plasma radioactivity.

Metabolic clearance rates

Values of the MCR of T_3 and rT_3 were calculated from data obtained by both the TCA precipitation-ethanol extraction and by the Sephadex chromatography techniques. In the former technique, the values employed for plasma T_3 or rT_3 concentration were those for the ethanol-extractable fractions, whereas in the latter technique they were those of the administered compound specifically isolated.

Two animals were exsanguinated at each time point and specimens of plasma obtained. Ethanol-extractable radioactivity was measured separately in the two sera and the mean value was employed in calculating the MCR yielded by this technique. In the case of column chromatographic analysis, larger quantities of sera were required for isolation of the specific compound administered. Therefore, equal volumes of the two sera obtained at each time point were mixed together and the resulting pool was subjected to analysis.

To describe the disappearance curves in the case of T_3, plasmas obtained at 2, 6.5, and 30 min and at 1, 2, 3, 6, 8, 16, 20, and 24 h were analyzed. In the case of rT_3, plasma samples obtained at 2, 6, 5, and 30 min and at 1, 2, 3, 3.5, and 6 h were studied. Hence, data relative to T_3 metabolism were derived from a total of 22 rats, and those relative to rT_3 metabolism from 16 rats.

Composite, rather than individual, disappearance curves were obtained so that at each time point there would be sufficient volumes of plasma available to permit resolution of iodinated compounds contained therein by column chromatography. Metabolic clearance rates were determined by a multicompartmental technique. Areas under the plasma disappearance curves were determined as the direct integral of a multiexponential function obtained by computer-assisted curve-fitting, with the use of a least squares technique. The best-fit disappearance curves for T_3 and rT_3 were generated from equations with three and two exponential terms, respectively. These equations were determined by a peeling technique to obtain initial estimates, followed by Gauss-Newton iteration to obtain those coefficients which produced the best fit (least squares) of observed values and the calculated curve. The area under the disappearance curve was determined mathematically as the integral of the function between 0-time and infinity. Values of the MCR were then calculated as the quotient of the administered dose (100%) divided by the area under the disappearance curve.

Binding of T_3 and rT_3 in plasma

Values for the percentage of free T_3 and rT_3 in rat plasma were determined by equilibrium dialysis of diluted (1:25) plasma to which tracer concentrations of labeled T_3 or rT_3 had been added (9).

Results

Control experiments

A variety of studies was performed to assess the efficacy of the several analytic techniques used and the purity of the injected materials. In the case of Sephadex chromatography, more than 95% of the total counts added to the column in plasmas of animals given labeled T_3 or rT_3 was recovered in the fractions eluted from the columns. When purified compounds were added to the columns, no artifactual deiodination during chromatography could be detected. Chromatography of the initially injected doses revealed more than 98% of the radioactivity to be in the form of T_3 or rT_3. Sephadex column chromatography of plasma indicated that after administration of T_3, and especially rT_3, iodide came to con-

stitute a major proportion of plasma radioactivity. Experiments were conducted, therefore, to determine the extent to which inorganic iodide is included in the ethanol-extractable fraction. In accord with the findings of Oppenheimer *et al.* (9), in 10 samples of outdated blood bank plasma to which labeled I^- had been added, 4.0 ± 0.01% added I^- was recovered in the washed TCA precipitate, and this radioactivity was entirely ethanol-extractable.

To assess the reproducibility of results obtained by the Sephadex method of analysis, duplicate samples of serum obtained after the administration of labeled T_3 or rT_3 were subjected to analysis. In two such studies of serum containing [^{125}I]T_3, the values for the [^{125}I]T_3, as a percentage of total eluted ^{125}I, were 92.6 *vs.* 92.0 and 45.6 *vs.* 47.4. In five paired analyses of sera containing [^{125}I]rT_3, comparable values were 89.9 *vs.* 92.0, 74.0 *vs.* 72.0, 65.3 *vs.* 63.6, 57.7 *vs.* 57.1, and 11.2 *vs.* 11.7.

Metabolism of radioiodine-labeled T_3

Chromatographic analyses of serum from animals given radioiodine-labeled T_3 (or rT_3) revealed three peaks of radioactivity in addition to the peak of administered hormone (Fig. 1). These comprised peaks corresponding sequentially to iodoprotein and iodide, followed by a peak that eluted just before T_3 itself (pre-T_3). Although the pre-T_3 peak is very small in the 2-min sample shown in Fig. 1, it grew

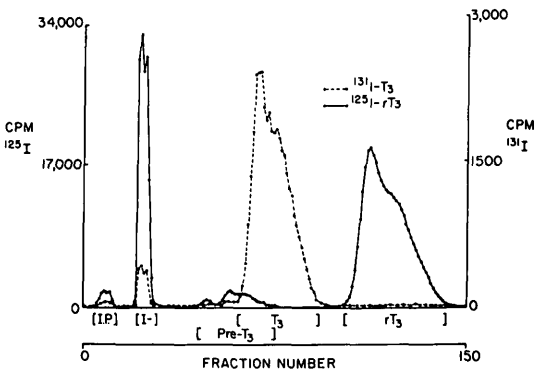

FIG. 1. Sephadex G-25 chromatography of pooled plasmas obtained from two rats 2 min after iv administration of ^{131}I-labeled T_3 and ^{125}I-labeled rT_3. Administered compounds were at least 98% pure as judged from similar chromatographic analyses. I.P., iodoprotein.

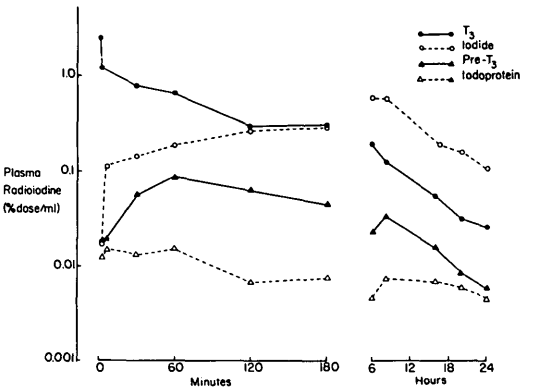

FIG. 2. Disappearance of radioiodine-labeled T_3 and appearance of its labeled metabolites in the plasma of rats given labeled T_3 iv at time 0. In this and subsequent figures, each point represents the results obtained in the pooled plasma from two rats.

relatively larger and, therefore, easier to measure separate from T_3 in succeeding samples (Fig. 2). When calculated as a fraction of the administered dose of radioiodine, the concentration of T_3 declined progressively with time, whereas the concentration of iodoprotein, iodide, and pre-T_3 first increased and then diminished (Fig. 2). From 6 to 8 h after injection, concentrations of T_3, iodide, and pre-T_3 maintained relatively constant ratios to one another. During this period of near or apparent equilibruim, iodide comprised approximately 75% of total plasma radioactivity and the pre-T_3:T_3 ratio was approximately 0.27.

Plasma disappearance curves for T_3 specifically isolated by Sephadex chromatography and for T_3 measured as ethanol-extractable radioactivity are compared in Fig. 3. At each time point studied, concentrations of radioiodine were greater in the ethanol-extractable fraction than in the Sephadex isolate, and from about 8 h onward, the two curves were essentially parallel.

As would be expected from the foregoing plasma disappearance curves, the calculated value of MCR was greater when measured by the Sephadex method (17.7 ml/100 g/h) than by the ethanol-extraction technique (14.9 ml/100 g/h).[3]

[3] The SD from regression for the T_3 curves derived from Sephadex and ethanol-extraction data were 0.034 and 0.033% dose/ml, respectively.

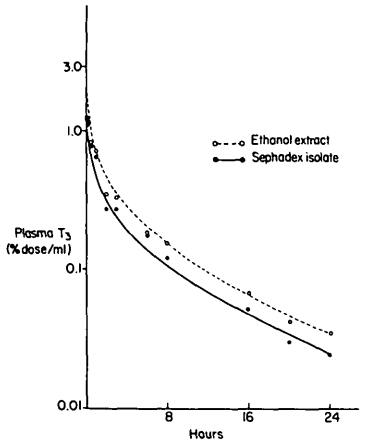

FIG. 3. A comparison of the curves of disappearance of radioiodine-labeled T_3 from the plasma of rats after a single iv injection, as judged from the results of trichloroacetic acid-ethanol extraction and Sephadex chromatography.

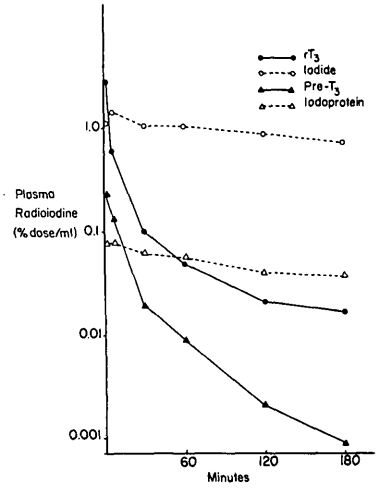

FIG. 4. Disappearance of radioiodine-labeled rT_3 and appearance of its labeled metabolites in the plasma of rats given labeled rT_3 iv at time 0.

Metabolism of ^{125}I-labeled rT_3

In addition to the residual labeled rT_3, Sephadex chromatography of serum revealed radioactive peaks in the iodoprotein, iodide, and pre-T_3 zones analogous to those seen after the administration of labeled T_3. Relatively high concentrations of iodide, iodoprotein, and pre-T_3 appeared with exceeding rapidity and were clearly evident in the 2-min sample (Fig. 1); over the next 3 h, concentrations of the three products decreased with greater or lesser rapidity (Fig. 4). As the figure demonstrates, curves for the disappearance of rT_3 and pre-T_3 are not parallel as would be expected, and values for the pre-T_3:rT_3 ratio never reached constancy. This was probably due to the very low levels of radioactivity present in the pre-T_3 zone during the latter portion of the study despite the relatively large volumes of serum analyzed.

Concentrations of rT_3 (Fig. 5) and T_3 (Fig. 3) in plasma at the 2-min time interval did not differ appreciably. Thereafter, however, rT_3 disappeared much more rapidly than T_3 did (note difference in time scales); the concentration of rT_3 at 3 h after injection was 0.017% dose/ml and that of T_3 was 0.27 percent dose/ml. In samples of plasma obtained more than 3 h after injection of [^{125}I]rT_3, there was insufficient radioactivity in the rT_3 and pre-T_3 zones to define significant peaks and, accordingly, the concentration of [^{125}I]rT_3 in plasma could not be determined. In contrast, labeled iodide was present at 3 h in a concentration of nearly 1.0% dose/ml (Fig. 4). A 5% recovery of plasma iodide in the ethanol-extractable fraction, as indicated by the preliminary experiments, would contribute 0.05% of the administered dose/ml, a value three times the concentration of rT_3 itself at that time point.

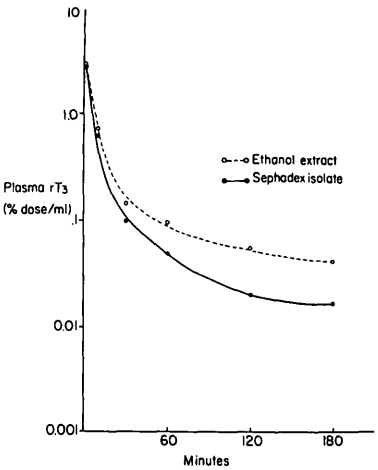

FIG. 5. A comparison of the curves of disappearance of radioiodine-labeled rT_3 from the plasma of rats after a single iv injection, as judged from the results of trichloroacetic acid-ethanol extraction and Sephadex chromatography.

As would be expected from the foregoing

considerations, the concentration of ethanol-extractable ^{125}I was at each time point much higher than that of the rT_3 isolated by Sephadex chromatography (Fig. 5). Readily measurable concentrations of ethanol-extractable ^{125}I were present at 6 h (not shown), long after rT_3 was no longer demonstrable by Sephadex chromatography. As a consequence, calculated values of the MCR of rT_3, when assessed by the chromatographic method (250 ml/100 g/h), were far in excess of those measured by the TCA-precipitation, ethanol-extraction technique (171 ml/100 g/h).[4]

Binding of T_3 and rT_3 in plasma

Values for the percentage of free T_3 and rT_3 in rat plasma were 0.73 and 0.33, respectively.

Discussion

In the present studies, we have demonstrated that in the rat, as in man (7), labeled products of peripheral hormone metabolism other than and in addition to iodide and iodoprotein appear in the plasma after administration of radioiodine-labeled T_3 or rT_3. Although these products in the rat have not been specifically identified, it is likely that they are the same as those that have been found in the plasma of man (5). In man, labeled T_3 gives rise to labeled 3,3′-diiodothyronine and 3′-monoiodothyronine, whereas labeled rT_3 yields both of these compounds and 3′,5′-diiodothyronine as well. Glucurono- and sulfo-conjugates of each of these iodothyronines are also formed (7).

Because these products are TCA-precipitable and ethanol-extractable, their presence causes values of the MCR of T_3 based on plasma disappearance curves generated by precipitation-extraction of plasma to be significantly in error. In the case of T_3, the MCR was 15% lower when plasma disappearance curves represented ethanol-extractable radioactivity than when they represented T_3 specifically isolated by Sephadex chromatography. This difference was even greater in the case of rT_3 (30%), owing to the fact that rT_3 disappeared from plasma much more rapidly than T_3 did, whereas labeled iodide rapidly appeared in a high relative concentration. Hence, although this was the case, even the small recovery of labeled iodide in the ethanol extract represented a concentration of radioactivity several times that of specifically isolated rT_3. This would explain why, 6 h after rT_3 administration, ethanol-extractable radioactivity was present in plasma although no rT_3 could be demonstrated by chromatography.

The present studies point to an additional error in previous measurements of the MCR of T_3 in the rat which have usually been calculated by single compartment analysis (1, 10). For compounds that are metabolized rapidly in relation to their rate of distribution, non-compartmental or multicompartmental analysis yields values that are lower and more nearly accurate than single compartment analysis does, even when a terminal disappearance slope can be defined (11, 12). In addition, an examination of the present plasma disappearance curves for T_3 (Fig. 3) and rT_3 (Fig. 5) reveals the absence of a linear terminal slope regardless of whether data were generated by TCA-precipitation and ethanol-extraction or by Sephadex chromatography. Hence, it is clear that single compartment analysis of plasma disappearance curves cannot yield accurate values for the MCR of T_3 or rT_3 in the rat.[5]

It would appear from the foregoing, therefore, that multicompartmental analysis of plasma disappearance curves representing T_3 specifically isolated by chromatography would yield values for the MCR of this hormone (or of rT_3) more nearly accurate than those ob-

[4] The SD from regression for the Sephadex and ethanol-extraction rT_3 curves were 0.0062 and 0.0078% dose/ml, respectively.

[5] Despite these limitations, we have attempted to make gross estimates of the MCRs of T_3 and rT_3 by the single compartment technique, applying a visual best fit to obtain a linear slope for the terminal portions of the disappearance curves. As would be expected, values for the MCR of T_3 and rT_3 were about 25 and 45% greater, respectively, than those derived from multicompartmental analysis. In a single study of T_3 clearance in the rat in which non-compartmental analysis was employed, values of the MCR obtained were similar to those we have found by using the Sephadex and multicompartmental techniques (13). However, results of the two studies are difficult to compare because in the previous study an entirely different method of separating administered T_3 from its products was employed.

tained by methods previously applied.[5] By using this approach, the MCR of T_3 in the 125–150 g rat has been found to be approximately 18 ml/100 g/h; that of rT_3 is remarkably high, 250 ml/100 g/h, and the deiodination of rT_3 is exceedingly rapid. A similarly rapid removal of rT_3 from plasma has been noted in man (14–16).

In the rat, the MCR of rT_3 proved to be some 14 times that of T_3 despite the fact that overall intensity of binding of rT_3 in rat plasma, as judged from the percentage free, was two times that of T_3. Hence, the observed difference in their rates of metabolism must rest upon intrinsic differences in their cellular uptake and degradative metabolism within one or more organs. Such seeming discrepancy between binding to plasma proteins and the rate of peripheral turnover is not without precedent. In man, for example, the percentage of free tetraiodothyroacetic acid in serum is substantially lower than that of T_4, but the rate of turnover is much more rapid (17).

Acknowledgments

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