

**Concentration of l-Thyroxine and l-Triiodothyronine Specifically Bound to Nuclear Receptors in Rat Liver and Kidney:  
*QUANTITATIVE EVIDENCE FAVORING A MAJOR ROLE OF T<sub>3</sub> IN THYROID HORMONE ACTION***

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To estimate the relative contribution of l-triiodothyronine (T<sub>3</sub>) and l-thyroxine (T<sub>4</sub>) to thyroidal effects, we have measured the concentration of iodothyronine bound to specific hepatic nuclear receptor sites by three different techniques: (a) specific radioimmunoassay after separation of T<sub>3</sub> and T<sub>4</sub> by preparative paper chromatography; (b) in vivo kinetic approaches as reported previously; and (c) isotopic equilibration. By these three methods, receptor concentration of T<sub>3</sub> and T<sub>4</sub> in liver was 0.51±0.19 (SD) and 0.08±0.06; 0.52±0.12 and 0.08±0.02; and 0.50±0.13 and 0.10±0.03 pmol/mg DNA, respectively. The percentage contribution of T<sub>3</sub> and T<sub>4</sub> to total receptor iodothyronine was thus 86.8±9.0 and 13.2±9.4; 86.3±3.5 and 13.7±3.5; and 83.7±5.6 and 16.3±5.6%, respectively. In kidney, specifically bound nuclear T<sub>3</sub> and T<sub>4</sub> were estimated both by isotopic equilibration and by in vivo kinetic techniques to be 0.28±0.11 and 0.03±0.01 pmol/mg DNA, respectively. Thus, T<sub>3</sub> constituted 89.4±3.2% of total receptor iodothyronine in this tissue. No other iodothyronines or analogs were bound to the nuclear sites in either tissue. Kidney and liver nuclear T<sub>3</sub> concentrations also were identical to values previously reported with in vivo kinetic techniques. Other studies from this laboratory have suggested that thyroid effect is related to the molar concentration of iodothyronine bound to specific nuclear sites, that the sites are similar in various tissues, and that iodothyronine in plasma is in equilibrium with nuclear [...]

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# Concentration of L-Thyroxine and L-Triiodothyronine Specifically Bound to Nuclear Receptors in Rat Liver and Kidney

## QUANTITATIVE EVIDENCE FAVORING A MAJOR ROLE OF T<sub>3</sub> IN THYROID HORMONE ACTION

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**ABSTRACT** To estimate the relative contribution of L-triiodothyronine (T<sub>3</sub>) and L-thyroxine (T<sub>4</sub>) to thyroidal effects, we have measured the concentration of iodothyronine bound to specific hepatic nuclear receptor sites by three different techniques: (a) specific radioimmunoassay after separation of T<sub>3</sub> and T<sub>4</sub> by preparative paper chromatography; (b) *in vivo* kinetic approaches as reported previously; and (c) isotopic equilibration. By these three methods, receptor concentration of T<sub>3</sub> and T<sub>4</sub> in liver was  $0.51 \pm 0.19$  (SD) and  $0.08 \pm 0.06$ ;  $0.52 \pm 0.12$  and  $0.08 \pm 0.02$ ; and  $0.50 \pm 0.13$  and  $0.10 \pm 0.03$  pmol/mg DNA, respectively. The percentage contribution of T<sub>3</sub> and T<sub>4</sub> to total receptor iodothyronine was thus  $86.8 \pm 9.0$  and  $13.2 \pm 9.4$ ;  $86.3 \pm 3.5$  and  $13.7 \pm 3.5$ ; and  $83.7 \pm 5.6$  and  $16.3 \pm 5.6\%$ , respectively. In kidney, specifically bound nuclear T<sub>3</sub> and T<sub>4</sub> were estimated both by isotopic equilibration and by *in vivo* kinetic techniques to be  $0.28 \pm 0.11$  and  $0.03 \pm 0.01$  pmol/mg DNA, respectively. Thus, T<sub>3</sub> constituted  $89.4 \pm 3.2\%$  of total receptor iodothyronine in this tissue. No other iodothyronines or analogs were bound to the nuclear sites in either tissue. Kidney and liver nuclear T<sub>3</sub> concentrations also were identical to values previously reported with *in vivo* kinetic techniques. Other studies from this labo-

ratory have suggested that thyroid effect is related to the molar concentration of iodothyronine bound to specific nuclear sites, that the sites are similar in various tissues, and that iodothyronine in plasma is in equilibrium with nuclear T<sub>3</sub>. If these relationships are assumed, T<sub>3</sub> contributes between 85 and 90% of thyroidal effects in the euthyroid rat. The remaining 10–15% of thyroidal effect appears to result from the intrinsic activity of T<sub>4</sub>.

### INTRODUCTION

The demonstration by Braverman et al. (1) that L-thyroxine (T<sub>4</sub>)<sup>1</sup> was converted to L-triiodothyronine (T<sub>3</sub>) peripherally has stimulated an extensive reconsideration of the role of T<sub>3</sub> and T<sub>4</sub> in determining hormonal status. Specifically, attention has been directed to the determination of the proportion of hormonal effects of T<sub>4</sub> that are mediated by conversion to T<sub>3</sub> and those due to the intrinsic potency of T<sub>4</sub> (2–6). The recent recognition of T<sub>3</sub> receptors in the nuclei may be of potential use in the resolution of these problems (7, 8). A number of studies with thyroid hormone analogs in rats have suggested a very good correlation between hormonal effects in liver and pituitary and the mass of iodothyronine bound to these sites (9–11). To date no compound that binds to these sites but fails to exert the expected hormonal activity has been identified. Moreover, since the

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<sup>1</sup> Abbreviations used in this paper: T<sub>3</sub>, L-triiodothyronine; T<sub>4</sub>, L-thyroxine; triac, 3,5,3'-triiodothyroacetic acid; TSH, thyroid stimulating hormone; rTSH, rat thyroid stimulating hormone.

apparent association constant of the receptor site from various tissues appears to be the same (12), and since iodothyronine in plasma appears to be in equilibrium with the nuclear sites (8), it appears reasonable to assume that the sites in different tissues are identical. Thus, if the nuclear sites are the principal points of initiation of thyroid hormone action in rat liver and pituitary the measurement of the relative concentration of  $T_4$  and  $T_3$  specifically bound to the nucleus should provide an index of the contribution of thyroid hormones to net hormonal effects in the animal.

With this purpose in mind, studies were undertaken to measure the concentration of iodothyronines bound to specific nuclear receptor sites. Although we had previously provided estimates of the  $T_4$  and  $T_3$  concentration at these sites (8, 12), these were calculated values based on *in vivo* kinetic relationships and measurements of plasma hormone concentration only. With improved methodology, we now present direct radioimmunoassay measurements of  $T_4$  and  $T_3$  at the nuclear sites. In addition, the possibility that other iodothyronines such as 3,5,3'-triiodothyroacetic acid (triac) might be bound to specific receptor sites was considered. To explore this possibility animals were equilibrated with  $^{125}\text{I}$ , a procedure which also provided an additional check on the measurement of the nuclear content of  $T_3$  and  $T_4$  by radioimmunoassay and kinetic techniques.

## METHODS

Euthyroid male Sprague-Dawley rats were obtained from Carworth Division, Becton, Dickinson & Co., New City, N. Y. They were fed Wayne Laboratory rat diet (1  $\mu\text{g}$  iodine/g) (Allied Mills, Inc., Chicago, Ill.) and tap water *ad libitum*. Athyreotic rats used in some studies were purchased from the same supplier after surgical thyroidectomy. Upon receipt in the laboratory they were placed on a low iodine test diet (0.05  $\mu\text{g}$  iodine/g) (Nutritional Biochemicals Corp., Cleveland, Ohio) for 7 days and then injected *ip.* with 100  $\mu\text{Ci}$  Na  $^{131}\text{I}$ -iodide to complete the thyroidectomy. These rats were considered athyreotic when weight gain ceased. At that time, their serum concentrations of  $T_4$  and  $T_3$  (see below) were undetectable as assessed by radioimmunoassay.

**Radioimmunoassay of nuclear  $T_3$  and  $T_4$ .** Iodothyronines were extracted from isolated hepatic nuclei and their concentration was measured by radioimmunoassay. To facilitate calculation of  $T_3$  and  $T_4$  recovery, groups of four to five euthyroid rats and one athyreotic rat were injected *i.v.* with a combined dose of  $^{131}\text{I}$ - $T_3$  (100–130 ng/100 g body weight) (Mallinckrodt Inc., St. Louis, Mo.) and chromatographically purified  $^{125}\text{I}$ - $T_4$  (5 ng/100 g body weight) (Abbott Laboratories, Chemical Div., North Chicago, Ill.). The specific activity of the  $^{131}\text{I}$ - $T_3$  was 30–50  $\mu\text{Ci}/\mu\text{g}$  and that of  $^{125}\text{I}$ - $T_4$ , 450–550  $\mu\text{Ci}/\mu\text{g}$ . The rats were killed 30 min after injection by exsanguination through the abdominal aorta under light ether anesthesia. The livers were rapidly excised, rinsed with ice-cold saline, and placed on ice. 4-g portions were taken for isolation of nuclei by the method of Samuels and Tsai (13). The liver was minced and homogenized at 0°C in 10

vol of 0.25 M sucrose, 20 mM Tris:HCl, 1.1 mM  $\text{MgCl}_2$  buffer, pH 7.85 at 25°C (STM buffer). The homogenates were centrifuged at 800 *g* for 10 min, and the resulting pellet was resuspended and centrifuged twice in 10 ml of STM-Triton buffer (0.25 M sucrose, 20 mM Tris-HCl, 1.1 mM  $\text{MgCl}_2$ , 0.5% Triton X-100, pH 7.85 at 25°C). Triton X-100 was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. The nuclei were then washed with 5 ml ice-cold water and assayed for radioactivity.

To extract the iodothyronines, the nuclei were treated three times with 5 ml 95% ethanol. The extracted nuclear pellet as well as a sample of the initial liver homogenate were frozen for subsequent measurements of DNA (14). 10 mg of 6-propyl-thiouracil (Sigma Chemical Co., St. Louis, Mo.) was added to each nuclear extract, and the volume was reduced to dryness under a stream of  $\text{N}_2$  at <50°C. The dried extracts were taken up in 2 ml  $\text{CH}_3\text{OH}:\text{NH}_3$  (95:5), and the volume of the methanolic extracts was reduced to 0.2 ml under a stream of  $\text{N}_2$  at 20°C. After addition of 25  $\mu\text{g}$  NaI, each extract was applied to a sheet (9 × 50 cm) of Whatman 3MM paper (Whatman, Inc., Clifton, N. J.). Flanking strips to which 25  $\mu\text{g}$  each of NaI,  $T_4$ , and  $T_3$  had been applied were placed on both sides of the paper with extract. Chromatograms were developed in a tertiary-amyl alcohol: 2 N  $\text{NH}_4\text{OH}$ : hexane, 5:6:1 solvent system (15) at 32°C. After 20 h, the papers were removed from the tanks and damp dried.  $T_4$  and  $T_3$  standards were localized under ultraviolet light, and the NaI standard was stained with  $\text{PdCl}_2$ . Based on the position of the standards on the flanking strips, the  $T_3$  and  $T_4$  areas were approximated, cut out, and eluted with  $\text{CH}_3\text{OH}:\text{NH}_3$  (95:5) as previously described (2). The methanolic eluates were reduced to dryness under a stream of  $\text{N}_2$  at 20°C. 1 ml of 0.3% bovine serum albumin (Sigma Chemical Co.) was added to the tubes containing  $T_3$ , and the  $T_3$  concentration was measured by radioimmunoassay as previously described (16). 0.8 ml, 0.15% bovine serum albumin was added to the tubes containing  $T_4$ .  $^{125}\text{I}$ - $T_4$ , 5,000 cpm in 0.1 ml, and 0.1 ml  $T_4$  antibody (raised in rabbits against a  $T_4$ -albumin conjugate) were added (final antibody dilution 1:6,000). After a 20-h incubation at 5°C, the bound and free fractions were separated with dextran-coated charcoal as described for the  $T_3$  radioimmunoassay (16).

Counting rates for both injected isotopes were measured on appropriate samples during the isolation of nuclei, extraction and separation of  $T_4$  and  $T_3$  and just before radioimmunoassay to determine the recovery of  $T_4$  and  $T_3$  from the isolated nuclei. Nuclear hormone concentrations were corrected for isotopic recovery and expressed as nanograms or picomoles per milligram DNA.

**Nuclear  $T_3$  and  $T_4$  by *in vivo* kinetic techniques.** The counting rates in plasma and nuclei of both injected isotopes were also used to calculate kinetic parameters as previously described (12). The concentrations of nonradioactive  $T_3$ ,  $T_4$ , and thyroid stimulating hormone (TSH) were measured in plasma samples obtained just before injection of isotopic  $T_4$  and  $T_3$ . Serum  $T_4$  was determined by the method of Larsen et al. (17), and rat TSH (rTSH) was assessed by homologous radioimmunoassay with reagents generously supplied by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolism, and Digestive Diseases. The rTSH assay was modified from the assay protocol supplied by the National Institutes of Health as previously described (18).

**Nuclear  $T_3$  and  $T_4$  measurements by isotopic equilibration.** Because of possible crossreaction with 3,5,3'-triiodothyroacetic acid (triac) in the  $T_3$  immunoassay and to determine whether other iodothyronines were bound, the endogenous  $T_3$  and  $T_4$  concentrations in nuclei isolated from

liver and kidney were also measured by isotopic equilibration techniques. Two groups of 10 euthyroid rats were fed a low iodine test diet, 0.05  $\mu\text{g}$  I/g, for 2 wk to deplete their iodine pools. The diet was then changed to a lot of low iodine test diet from the same supplier containing 0.3  $\mu\text{g}$  I/g. Control rats were allowed tap water ad libitum. The water of the experimental rats was supplemented with  $^{125}\text{I}$ -iodide (sp act = 0.08  $\mu\text{Ci}/\text{ng}$ ) at a concentration of 0.625  $\mu\text{Ci}/\text{ml}$ . The rats remained on this dietary regimen for 35 days. At that time, the specific activity of their iodine pools was within 90% of equilibrium with the specific activity of the ingested iodine (19).

Iodine equilibrated and control groups were killed by exsanguination through the abdominal aorta under light ether anesthesia. Plasma was set aside for measurements of  $\text{T}_4$ ,  $\text{T}_3$ , and TSH as described above with  $^{131}\text{I}$ -labeled hormones as tracers. The thyroid gland was carefully removed, weighed, and one lobe from each rat was fixed in neutral formalin for histologic examination. Liver and kidney were excised, rinsed with ice-cold saline, and weighed. Nuclei were isolated from a 4-g portion of liver and the entire kidney as previously described (7). After isolation they were suspended in STM-Triton to remove nonspecifically bound hormone and pelleted at 800  $g$  for 10 min. They were then resuspended in ice-cold water (5 ml) and pelleted by centrifugation at 800  $g$  for 10 min, and the hormones were extracted with 95% ethanol. The ethanolic extracts were taken to dryness as described above. The solvent partition procedure described by Flock and Bollman (20) was employed to remove excess lipids and salts. Briefly, the dried ethanolic extracts were taken up in 2 ml *n*-butanol saturated with 5% HCl, and the butanol was extracted three times with an equal volume of Blau's solution (5% anhydrous  $\text{Na}_2\text{CO}_3$  in 4 N NaOH). 2 ml  $\text{CHCl}_3$  was then added to the butanol and the hormones were back-extracted into 2 N  $\text{NH}_4\text{OH}$ . Trial experiments showed that the recovery of isotopically labeled  $\text{T}_4$ ,  $\text{T}_3$ , and triac (synthesized in our laboratory [21]) added to 95% ethanol was greater than 90% in this procedure. The 2 N  $\text{NH}_4\text{OH}$  was evaporated at  $<50^\circ\text{C}$  under a stream of  $\text{N}_2$ , and the hormones were redissolved in 0.5 ml  $\text{CH}_3\text{OH}:\text{NH}_3$ . 50  $\mu\text{g}$  each of  $\text{T}_4$ ,  $\text{T}_3$ , iodide, and triac were added, and 0.475 ml was applied to Whatman 3MM paper (16  $\times$  50 cm). Chromatography was carried out as described above for 48 h, and the solvent was allowed to run off the paper. Papers were dried, and the standards localized under ultraviolet light and stained with diazotized sulfanilic acid as described previously (22). The papers were cut up into 1-cm strips which were assayed for radioactivity in a Packard Tricarb Autogamma spectrometer (Packard Instrument Co., Inc.). A 25- $\mu\text{l}$  portion of each extract was also applied to silica gel thin layer sheets (Eastman Kodak Co., Rochester, N. Y.) and developed in a toluene:acetic acid:water; 2:2:1 solvent system (22).

Although urine had been obtained from the isotopically equilibrated rats near the end of the study for determination of the endogenous iodine specific activity, these specimens were inadvertently heavily contaminated with non-radioactive iodine after collection. The specific activity of endogenous iodine was, therefore, determined from the counting rates of TCA precipitates of plasma and the plasma  $\text{T}_4$  and  $\text{T}_3$  concentrations. The specific activity for plasma iodothyronine iodine in the five equilibrated rats varied within a narrow range, mean  $3,375 \pm 120$  (SD) cpm/ng I. The iodine content of nuclei isolated from 1 g liver of kidney was then calculated: nuclear counting rate/iodine specific activity. The nuclear iodothyronine concentration was calculated from the nuclear iodine, the fraction of total  $^{125}\text{I}$  represented by  $\text{T}_4$  plus  $\text{T}_3$  on the paper chromatograms (0.73

$\pm 0.063$  for liver and  $0.71 \pm 0.12$  for kidney), and the chromatographic  $\text{T}_3/\text{T}_4$  ratio.

*Other studies.* The fraction of endogenous  $\text{T}_3$  and  $\text{T}_4$  bound specifically to nuclei isolated by the two methods employed (see above) and the loss of specifically bound hormones during treatment of isolated nuclei with water was also determined. Doses of 10 and 10,000 ng/100 g body weight of purified  $^{125}\text{I}$ - $\text{T}_3$  and  $^{125}\text{I}$ - $\text{T}_4$  were separately injected into groups of four euthyroid rats. Animals were killed 30 min later by bleeding through the abdominal aorta. Two 4-g portions of each liver were rinsed with ice-cold saline and placed on ice. Nuclei were isolated from one portion by the STM-Triton method (13) and from the second portion by centrifugation through 2.4 M sucrose (7). Nuclei which were isolated from the second portion were then resuspended in STM-Triton for 10 min and pelleted at 800  $g$ . After resuspension in 5 ml ice-cold water, nuclei prepared by both methods were reisolated by centrifugation. Samples of the isolated nuclei before and after treatment with Triton X-100 and water were taken for determination of the counting rates of isotopically labeled  $\text{T}_3$  and  $\text{T}_4$  and the concentration of DNA.

One additional experiment was carried out to determine whether  $\text{T}_4$  specifically bound to nuclear sites was released during the isolation of nuclei. Liver nuclei were isolated (7) 30 min after i.v. injection of  $^{125}\text{I}$ - $\text{T}_4$ , 50 ng/100 g body wt. Radioactively labeled nuclei equivalent to 1 g of liver were incubated at  $5^\circ\text{C}$  with nonradioactive cytosol obtained from 1 g liver from separate rats. Incubations were terminated after 0, 30, 60, 90, and 120 min by pelleting the nuclei at 10,000  $g$  for 5 min. The pellet was resuspended twice in 0.32 M sucrose, 3 mM  $\text{MgCl}_2$ , and 0.2% Triton X-100 and centrifuged at 10,000  $g$  for 5 min. The counting rates of the nuclear pellet containing specifically bound  $^{125}\text{I}$ - $\text{T}_4$  and samples from all other fractions were then determined.

Data are expressed as mean  $\pm$  SD throughout the manuscript. Student's *t* test was used to analyze the data statistically. Linear regression and correlation coefficients were also employed for some variables (23).

## RESULTS

As shown in Table I, specifically bound  $\text{T}_3$  in hepatic nuclei isolated through 2.4 M sucrose (0.1970 – 0.0234 = 0.1736% injected dose/mg DNA) did not differ significantly from bound  $\text{T}_3$  in the same nuclei after treatment with Triton X-100 (0.1781% dose/mg DNA) or from specifically bound  $\text{T}_3$  in nuclei isolated from the same liver by the STM-Triton method (0.1896% dose/mg DNA). Similarly, specifically bound  $\text{T}_4$  (Table I) in nuclei isolated through 2.4 M sucrose (0.00743 – 0.00488 = 0.00255% injected dose/mg DNA) was not significantly different from specifically bound  $\text{T}_4$  assessed in the same nuclei after treatment with Triton X-100 (0.00218% dose/mg DNA). In accord with previous experiments (8) 88–90% of nuclear  $\text{T}_3$  and 29–34% of nuclear  $\text{T}_4$  appear specifically bound when nuclei are isolated by centrifugation through 2.4 M sucrose.

Treatment with ice-cold water resulted in a significant decrease in specifically bound  $\text{T}_4$  ( $P < 0.025$ ) and  $\text{T}_3$  ( $P < 0.05$ ). The mean percent decrease in specifically bound hormone as a consequence of the water

TABLE I  
Specific and Nonspecific Binding of  $^{125}\text{I-T}_3$  and  $^{125}\text{I-T}_4$  by Hepatic Nuclei

Isolation of nuclei	Treatment	$^{125}\text{I-T}_3$		$^{125}\text{I-T}_4$	
		10	10,000	10	10,000
		ng/100 g body weight		ng/100 g body weight	
Sucrose, 2.4 M	None	0.1970±0.0160	0.0234±0.0029	0.00743±0.00018	0.00488±0.00071
	STM-Triton	0.1781±0.0211	0.0041±0.0014	0.00218±0.00081	0.00087±0.00021
	Water	0.1704±0.0162	0.0040±0.0011	0.00181±0.00073	0.00078±0.00023
STM-Triton	None	0.1896±0.0183	0.0043±0.0008	0.00336±0.00093	0.00102±0.00018
	Water	0.1739±0.0281	0.0038±0.0007	0.00308±0.00047	0.00088±0.00020

Entries are mean percent injected dose per milligram DNA±SD for groups of four euthyroid rats. Nuclei were isolated by the two indicated methods from duplicate 4-g portions of each liver.

treatment was 4.3 and 8.3% for  $T_3$  and 16.9 and 8.3% for  $T_4$  in nuclei isolated through 2.4 M sucrose or by the STM-Triton method, respectively. Thus, hormone present in nuclei at the stage of ethanolic extraction represents predominantly hormone that is specifically bound to nuclear binding sites.

**Radioimmunoassay measurements of nuclear  $T_3$  and  $T_4$ .** Hepatic nuclear  $T_3$  and  $T_4$  concentrations were measured by radioimmunoassay in 13 euthyroid and 3 athyreotic rats. The efficiency of extraction of  $T_3$  and  $T_4$  from the nuclei was approximately 90% (Table II), and the mean recovery at the time of radioimmunoassay was 36.1 and 41.6% for  $T_4$  and  $T_3$ , respectively. The low recoveries were due to eluting a purposefully narrow  $T_3$  and  $T_4$  area to minimize contamination with the other iodothyronine. The separate  $T_3$  and  $T_4$  eluates from the paper chromatograms were

minimally contaminated (5–10%) with the other iodothyronine. This degree of contamination did not significantly affect the precision of hormone measurements since the cross-reaction of the  $T_3$  antibody with  $T_4$  is less than 0.1% and that of the  $T_4$  antibody with  $T_3$  is 8%. Nuclear  $T_3$  concentration was corrected for  $T_3$  which was present as a consequence of the  $T_3$  mass in the low specific activity  $^{131}\text{I-T}_3$  that was injected. The mass of  $T_3$  in the nuclei due to  $^{131}\text{I-T}_3$  was calculated as the product: mean nuclear  $T_3$  concentration of similarly injected athyreotic rats,  $0.06\pm 0.01$  ng/mg DNA, and the recovery of  $T_3$  for each of the euthyroid rats.  $T_4$  was not detected in the athyreotic rats which were injected with isotopically labeled  $T_3$  and  $T_4$ . The hepatic nuclear  $T_3$  concentration greatly exceeded that of  $T_4$  (Table II). The molar ratio,  $T_3:T_4$ , was 6.56. Thus,  $T_3$  constituted  $86.8\pm 9.0\%$  and  $T_4$  constituted  $13.2\pm 9.4\%$  of the total radioimmunoassayable nuclear iodothyronine.

**Nuclear  $T_3$  and  $T_4$  by in vivo kinetic techniques.** Nuclear iodothyronine concentrations determined by radioimmunoassay were virtually identical to mean nuclear  $T_3$  and  $T_4$  concentrations calculated from in vivo kinetic parameters and plasma hormone concentrations (Table III). By in vivo kinetics, the  $T_3:T_4$  molar ratio was 5.78.  $T_3$  thus constituted  $86.3\pm 3.5\%$  of the total nuclear iodothyronine. In addition to the similarity of the mean hormone concentrations, individual measurements by radioimmunoassay and in vivo kinetic parameters were significantly correlated ( $r = 0.735$ ,  $P < 0.01$  for  $T_3$ ; and  $r = 0.553$ ,  $P < 0.05$  for  $T_4$ ).

**Nuclear  $T_3$  and  $T_4$  by isotopic equilibration techniques.** Studies in rats equilibrated with  $^{125}\text{I-iodide}$  yielded essentially the same results. The isotopically equilibrated rats did not appear to be iodine deficient or have radiation thyroiditis. Equilibrated rats had the same growth rate, thyroid weight, and histology as controls fed the same diet. Moreover, the mean serum concentrations of  $T_4$ ,  $T_3$ , and TSH

TABLE II  
Hepatic Nuclear  $T_3$  and  $T_4$  Concentrations Measured by Radioimmunoassay

Extraction of nuclei*	Recovery*†	Nuclear $T_3$ and $T_4$			
		DNA	DNA	Total pmol	
%	%	ng/mg	pmol/mg	%	
$T_3$	89.8±3.5	41.6±7.5	0.33±0.12	0.51±0.19	86.8±9.0
$T_4$	93.1±1.0	36.1±5.9	0.06±0.04	0.08±0.06	13.2±9.4

Each entry is the mean±SD for 13 euthyroid rats. The mass of injected  $^{131}\text{I-T}_3$  recovered in the nuclei was subtracted from the individual  $T_3$  measurements. The mass of  $^{131}\text{I-T}_3$  was calculated as the product:  $T_3$  recovery × mean nuclear  $T_3$  concentration of three athyreotic rats,  $0.06\pm 0.01$  ng/mg DNA. As assessed from the injected  $^{131}\text{I-T}_3$  dose and kinetic parameters (see Table III), the nuclear  $T_3$  measured in the athyreotic rats was fully attributable to the nonradioactive  $T_3$  in the injected  $^{131}\text{I-T}_3$  dose. Nuclear  $T_4$  was not detectable in the athyreotic rats.

\* Calculated from the cpm of  $^{131}\text{I-T}_3$  and  $^{125}\text{I-T}_4$  in the nuclei before extraction and the separate counting rates in the ethanolic extract.

† Determined before radioimmunoassay.

in equilibrated rats were also similar to controls;  $T_4$ ,  $4.8 \pm 0.7$  and  $4.6 \pm 0.3$   $\mu\text{g/dl}$ ;  $T_3$ ,  $67 \pm 7$  and  $74 \pm 5$   $\text{ng/dl}$ ; and TSH,  $624 \pm 23$  and  $599 \pm 17$   $\text{ng/ml}$ , respectively.

Approximately 90% of the radioactivity of liver nuclei and 85% of kidney nuclei was extracted by ethanol treatment. The average recovery before chromatography was 70% of the initial nuclear radioactivity in liver and 67.5% in kidney. For both liver and kidney, the recovery of counts applied to the chromatograms was nearly complete; average 94% for liver and 96% for kidney. 72 to 74% of applied radioactivity occurred in two peaks which co-chromatographed with authentic  $T_3$  or  $T_4$  standards (Fig. 1). The remaining counts were found at the point of application or were  $^{125}\text{I}$ -iodide. There was no suggestion of other radioactive peaks in any chromatogram. In parallel studies, purified  $^{125}\text{I}$ - $T_4$  was added to isolated nuclei before extraction. Conversion to  $^{125}\text{I}$ - $T_3$  during extraction and chromatography could not be demonstrated.

The concentration of  $T_3$  and  $T_4$  in hepatic nuclei calculated from the  $T_3:T_4$  ratio on each chromatogram (Table IV) was similar to that determined by radioimmunoassay (Table II) or by *in vivo* kinetics (Table III). The  $T_3:T_4$  molar ratio in liver nuclei was 5.79. Thus,  $T_3$  constituted  $83.7 \pm 5.6\%$  of the total nuclear iodothyronine by this method (Table IV). In kidney, the nuclear  $T_3$  and  $T_4$  concentrations were smaller than in liver nuclei. The  $T_3:T_4$  molar ratio was 9.33, significantly greater than 5.79 and 6.56 in liver,  $P < 0.025$ . Thus, in kidney  $89.4 \pm 3.2\%$  of nuclear iodothyronine was  $T_3$ . Although there was no radioactive peak corresponding to triac or 3,3',5'-triiodothyronine on the paper chromatograms, the nuclear extracts were also chromatographed on silica gel thin-layer sheets in

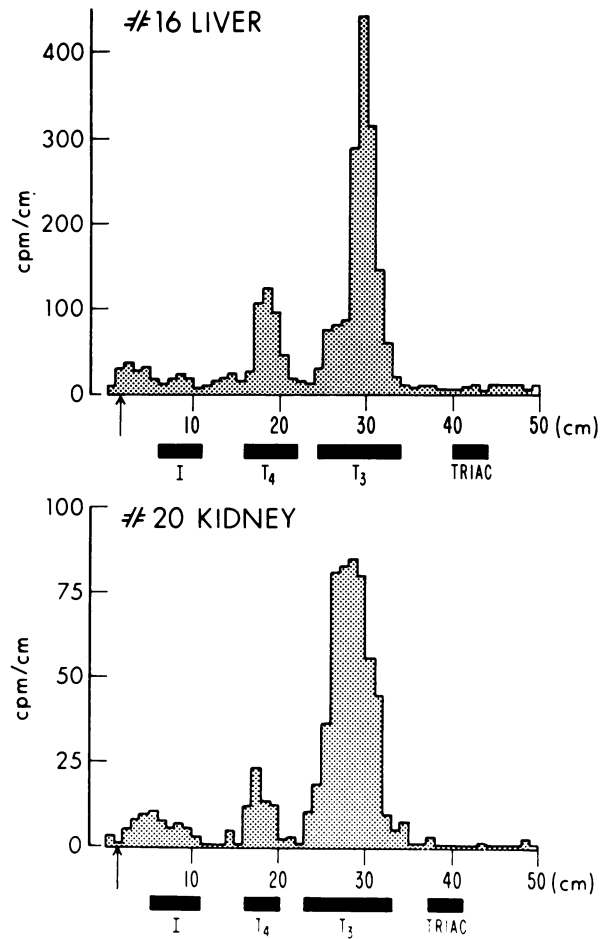


FIGURE 1 Representative paper chromatograms of extracts of liver and kidney nuclei from  $^{125}\text{I}$ -iodide equilibrated rats. 50  $\mu\text{g}$  each of nonradioactive I,  $T_4$ ,  $T_3$ , and triac were added to the extract before application to the paper. Chromatograms were developed in a tert-amyl alcohol: 2 N  $\text{NH}_4\text{OH}$ :hexane, 5:6:1 solvent system (15) for 48 h at  $32^\circ\text{C}$ . The solvent was allowed to run off the paper. Point of application is indicated by the vertical arrow and the position of I,  $T_4$ ,  $T_3$ , and triac by the horizontal black bars. I was stained with  $\text{PdCl}_2$ ;  $T_4$ ,  $T_3$ , and triac were visualized under ultraviolet light and stained with diazotized sulfanilic acid. The distribution of  $^{125}\text{I}$ -iodine radioactivity is illustrated. In both liver (upper panel) and kidney (lower panel) a large peak co-chromatographed with the  $T_3$  standard and smaller peaks comigrated with  $T_4$  and I.

TABLE III

Hepatic Nuclear  $T_3$  and  $T_4$  Concentrations Measured by *In Vivo* Kinetic Techniques

Plasma concentration	Nuclear $T_3$ and $T_4$				Total pmol
	N/P	DNA	DNA	%	
ng/ml	ng/ml	ng/mg	$\mu\text{mol/mg}$	%	
$T_3$	$1.21 \pm 0.07$	$0.452 \pm 0.103$	$0.34 \pm 0.08$	$0.52 \pm 0.12$	$86.3 \pm 3.5$
$T_4$	$43.8 \pm 6.5$	$0.0021 \pm 0.0003$	$0.06 \pm 0.02$	$0.08 \pm 0.02$	$13.7 \pm 3.5$

Each entree is the mean  $\pm$  SD for 13 euthyroid rats. N/P is the nucleus:plasma activity ratio for the injected  $^{125}\text{I}$ - $T_3$  and  $^{125}\text{I}$ - $T_4$  (percent dose per gram nuclei per percent dose per milliliter plasma (12)). Nuclear  $T_3$  or  $T_4$  was calculated as the product:  $\text{N/P} \times \text{plasma concentration}$ . Plasma  $T_3$  or  $T_4$  concentration was the sum of the individual immunoassayable hormone concentration and hormone contributed by the injected isotopically labeled iodothyronines. The mass of injected  $^{125}\text{I}$ - $T_3$  recovered in the nuclei was subtracted from the calculated nuclear  $T_3$ . The mass of nuclear  $^{125}\text{I}$ - $T_3$  was  $0.06 \pm 0.01$   $\text{ng/mg}$  DNA as calculated from the N/P and plasma  $T_3$  concentration in the athyreotic rats. Nuclear  $T_4$  was not detectable in the athyreotic rats.

a solvent system that resolves triac and 3,5,3',5'-tetraiodothyroacetic acid from other iodothyronines (Fig. 2). Less than 1% of the applied radioactivity occurred in the triac area and this appeared to be the end of a forward trail from the single peak consisting of 98% of applied radioactivity which remained at the point of application. Thus, neither triac nor 3,3',5'-triiodothyronine are bound to the nuclear receptor in the euthyroid rat.

Since the low nuclear  $T_4$  concentrations could result

TABLE IV  
Nuclear T<sub>3</sub> and T<sub>4</sub> Concentrations Measured  
by Isotopic Equilibration

	Chromatographic distribution	Nuclear T <sub>3</sub> and T <sub>4</sub>		
		DNA	DNA	Total pmol
		% of applied cpm	ng/mg	pmol/mg
Liver				
T <sub>3</sub>	58.9±8.7	0.33±0.09	0.50±0.13	83.7±5.6
T <sub>4</sub>	14.8±4.3	0.07±0.02	0.10±0.03	16.3±5.6
Kidney				
T <sub>3</sub>	62.1±11.8	0.18±0.07	0.28±0.11	89.4±3.2
T <sub>4</sub>	9.6±2.6	0.02±0.01	0.03±0.01	10.6±3.2

Entrees are mean±SD for five rats (liver) or four rats (kidney). Nuclear iodothyronine concentration was calculated from the individual T<sub>3</sub>/T<sub>4</sub> molar ratios from the chromatograms (cpm T<sub>3</sub> peak/cpm T<sub>4</sub> peak) × 4/3 and the iodine concentration of the isolated nuclei (cpm/g nuclei) ÷ (specific activity of iodine).

from a disproportionate loss from the nuclear binding sites of T<sub>4</sub> compared to T<sub>3</sub> during the isolation of nuclei, we measured the rate of release of T<sub>4</sub> which was specifically bound to hepatic nuclear sites under conditions which simulated those of nuclei after tissue homogenization. Specifically bound nuclear T<sub>4</sub> was released at a rate of 8.85% per h. Since nuclei are in the presence of cytosol for a maximum of 30 min during the isolation procedures employed, the T<sub>4</sub> concentration measured in these studies could be underestimated by only 4–5%. Release of T<sub>3</sub> from nuclear sites during isolation of nuclei has previously been shown to be insignificant (8).

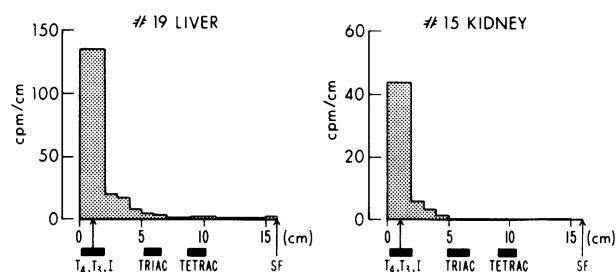


FIGURE 2 Representative silica gel thin-layer chromatograms of extracts of liver and kidney nuclei from <sup>125</sup>I-iodide equilibrated rats. Authentic standards including 3,5,3',5'-tetraiodothyroacetic acid were added as described in the legend to Fig. 1. Chromatograms were developed in toluene:acetic acid:H<sub>2</sub>O, 2:2:1 (22) for 1 h at 20°C. The vertical arrow on the left indicates the point of application, that on the right the solvent front (SF). Position of authentic compounds depicted as black horizontal bars. For nuclei from both liver (left panel) and kidney (right panel) the major radioactive peak remained at the application point. Radioactive peaks were not observed for triac or 3,5,3',5'-tetraiodothyroacetic acid.

## DISCUSSION

Our results suggest that the molar concentration of T<sub>3</sub> specifically bound to hepatic nuclear receptor sites is sixfold greater than that of T<sub>4</sub>. By the methods employed, T<sub>3</sub> appears to constitute an average of 85.8% of iodothyronine bound to the nuclear receptor sites. The remaining 14.2% appears to be T<sub>4</sub>. The internal consistency of these T<sub>3</sub> and T<sub>4</sub> concentrations measured by three independent methods was striking. Measured by radioimmunoassay, *in vivo* kinetic parameters, and isotopic equilibrium, mean nuclear T<sub>3</sub> concentration was 0.51, 0.52, and 0.50 pmol/mg DNA and T<sub>4</sub> was 0.08, 0.09, and 0.10 pmol/mg DNA, respectively. The insignificant difference between mean nuclear T<sub>3</sub> concentration determined by *in vivo* kinetic techniques compared to determinations by radioimmunoassay and isotopic equilibration, moreover, provides strong support for the precision of the calculated nuclear T<sub>3</sub> concentrations in previous studies reported from this laboratory (8, 12) in which *in vivo* kinetic methods were exclusively employed. For example, the previously reported mean hepatic nuclear T<sub>3</sub> concentration determined by *in vivo* kinetic methods in euthyroid rats was 0.43±0.15 pmol/mg DNA (12), a value which is 85% of that measured in the current studies. In another report, calculated T<sub>3</sub> and T<sub>4</sub> concentrations specifically bound to chromatin were 0.52 and 0.06 pmol/mg DNA, respectively (24). These values are not significantly different from those in the present report. Although the kinetics of specific nuclear T<sub>4</sub> binding have not been reported, the similarity of the nuclear T<sub>4</sub> concentration measured by isotopic equilibration to those measured by radioimmunoassay and by *in vivo* kinetic parameters suggests that injected T<sub>4</sub> is fully equilibrated with specifically bound nuclear T<sub>4</sub> 30 min after injection and that, in liver and kidney nuclei, specifically bound T<sub>4</sub> and T<sub>3</sub> is fully exchangeable with the iodothyronines in plasma.

Hormone measurements in kidney nuclei were carried out only by the isotopic equilibration method. Mean T<sub>3</sub>:T<sub>4</sub> molar ratio, 9.33, was significantly greater than that of liver nuclei. Since this finding is based on only four measurements in kidney, we feel that the greater T<sub>3</sub>:T<sub>4</sub> molar ratio in kidney should not be over-interpreted until it is confirmed by different methods and in larger groups of rats. Even if taken at face value, the molar ratio of 9.33 indicates that 90.3% of total nuclear iodothyronine is T<sub>3</sub> in kidney whereas in liver nuclei, the T<sub>3</sub> contribution to total nuclear iodothyronine, 85.8%, is only slightly smaller. Similar to measurements in liver nuclei, mean kidney nuclear T<sub>3</sub>, 0.28±0.11 pmol/mg DNA was identical to reported values measured by *in vivo* kinetic techniques, 0.28±0.03 pmol/mg DNA (12).

The  $T_3:T_4$  molar ratios reported herein may possibly be affected by two processes which could alter the nuclear concentration of  $T_4$ . It is possible that  $T_3$  and  $T_4$  might dissociate from the nuclear sites and be redistributed to other cellular constituents during homogenization and nuclear isolation. We have previously shown (8) that significant amounts of  $T_3$  are not released from the nuclear receptor during isolation of nuclei from liver homogenates. Because of a much greater ratio of nonspecific:specific binding similar studies have not been carried out for  $T_4$ . The affinity of the nuclear receptor for  $T_4$  is approximately 10-fold less than for  $T_3$ . The present studies show that  $T_4$  which is specifically bound to nuclear receptors is released at a rate of 8.5%/h when isolated nuclei are incubated with cytosol. From this experiment we estimate that the release of  $T_4$  during nuclear isolation could result in underestimation of nuclear  $T_4$  by approximately 5%. If so, the  $T_3:T_4$  molar ratio would be minimally smaller than that observed. It should be noted, however, that this estimate of loss of specifically bound  $T_4$  may not adequately reflect the loss of  $T_4$  that occurs during isolation of nuclei. Also, significant losses of specifically bound hormone during repetitive washes of nuclei have been reported by Spindler et al. (25).

In our view, an underestimation of the observed  $T_3:T_4$  molar ratio is more likely the case because of incomplete removal of  $T_3$  and  $T_4$  bound nonspecifically to nuclei. Since nonspecific binding of  $T_3$  was approximately 10% of the total nuclear  $T_3$  whereas nonspecifically bound  $T_4$  constitutes about 70% of hormone bound to nuclei (Table I), incomplete removal of nonspecifically bound hormone would result in a negligible change in nuclear  $T_3$  but an overestimation of specifically bound  $T_4$ . The observed  $T_3:T_4$  molar ratio would therefore be underestimated. If, for example, 10% of nonspecifically bound  $T_4$  remained with the nuclei, the  $T_3:T_4$  molar ratio would be 7.0 rather than the observed ratio, 6.0.

Properties of the nuclear iodothyronine binding sites which indicate that the relative concentrations of specifically bound  $T_3$  and  $T_4$  are proportional to their biological activity in the intact animal were summarized in Introduction. Of particular importance to this formulation is the assumption that the nucleus is the only site of hormonal initiation. Although specific binding sites in extranuclear fractions have not been demonstrated either with *in vivo* studies in the rat (7) or in cultured  $GH_1$  cells (13), recent reports by Tata (26) and Sterling and Milch (27) describe *in vitro* studies in which limited binding capacity, high-affinity sites are described in other subcellular fractions (26) or in fractionated mitochondria (27). The physiologic relevance of these sites remains uncertain. In previous studies by Dillman et al. (28), the

limited-capacity binding of  $T_3$  by cytosol which was demonstrated had an equilibrium association constant 200 to 400-fold smaller and a binding capacity 170-fold greater than that of the nuclear sites. Moreover, the affinity of cytosol for different iodothyronine analogs was markedly different from the nuclear sites. Re-examination of the hepatic mitochondrial fraction in *in vivo* studies with hypothyroid animals<sup>2</sup> failed to show displacement of tracer  $^{125}I$ - $T_3$  from the mitochondria after injection of a molar excess of non-radioactive  $T_3$ .

Of special interest, the present studies clearly show that triac was not present in the nucleus. Triac is avidly bound by the nuclear receptors (9, 21) but has only one-third the metabolic activity of  $T_3$  (29, 30). Reports from this laboratory have indicated that this discrepancy between nuclear binding and biological activity results from the more rapid metabolism of triac compared to  $T_3$  (21). It was also shown that triac was not derived from  $T_3$ , but other sources were not explored. The current studies also show that 3,3',5'-triiodothyronine does not occur in the nucleus but the presence of other iodothyronines such as 3-monoiodothyronine, 3,5- or 3,3'-diiodothyronine, compounds which neither cross-react in the immunoassays employed nor have significant calorogenic activity, cannot be excluded. The only biologically active iodothyronines bound to the nuclear receptor sites appear to be  $T_4$  and  $T_3$ .  $T_3$  is bound to 85–90% of the occupied nuclear receptor sites and hence as a consequence of the properties of the nuclear sites cited above and the assumption that the nuclear sites are the only cellular sites for hormonal initiation,  $T_3$  contributes between 85 and 90% of thyroidal activity in the euthyroid rat. The remaining 10–15% of biological activity due to the thyroid hormones results from the intrinsic activity of  $T_4$ .

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