Limited Binding Capacity Sites for L-Triiodothyronine in Rat Liver Nuclei

NUCLEAR–CYTOPLASMIC INTERRELATION, BINDING CONSTANTS, AND CROSS–REACTIVITY WITH L–THYROXINE

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A B S T R A C T Further studies have been performed to define the kinetic characteristics of nuclear triiodothyronine (T_3) binding sites in rat liver (*J. Clin. Endocrinol. Metab.* 1972. **35**: 330). Sequential determination of labeled T_3 associated with nuclei and cytoplasm over a 4-h period allowed analysis of the relationship of T_3 in nuclear and cytoplasmic compartments. A rapid interchange of hormone between nuclei and cytoplasm was demonstrated, and in vitro incubation experiments with nuclei yielded no evidence favoring metabolic transformation of T_3 by the nuclei. In vivo displacement experiments were performed by subcellular fractionation of liver $\frac{1}{2}$ h after injection of $[^{125}I]T_3$ with increasing quantities of unlabeled T_3. The nuclear binding capacity for T_3 could be defined (0.52 ng/mg DNA). Analysis of these experiments also allowed an estimation of the association constant of nuclear sites for T_3 (4.7 × $10^{11}M^{-1}$). The affinity of these sites for T_3 was estimated to be 20–40 fold greater than for thyroxine (T_4). Chromatographic analysis of the nuclear radioactivity after injection of labeled T_4 indicated that the binding of T_4 by the nucleus could not be attributed to in vivo conversion of T_4 to T_3 but reflected intrinsic cross-reactivity of the two iodothyronines at the nuclear binding sites.

INTRODUCTION

We have previously demonstrated limited capacity nuclear binding sites for L-triiodothyronine (T_3) [1] in rat liver and kidney nuclei (1). Our findings were based on in vivo displacement studies and suggested a high degree of specificity for T_3, since thyroxine (T_4) appeared to be much less avidly bound to nuclei and the degree of displacement of radioactive T_4 by nonradioactive T_4 was far less pronounced than in the case of T_3. Since Tata and Widnell (2) had previously shown that pulse injection of T_3 into thyroidectomized rats stimulates nuclear RNA metabolism before biochemical changes in other subcellular fractions, the finding of limited capacity nuclear sites suggested to us the possibility that such sites might represent hormonal "receptors" responsible for the initiation of the hormonal message. Accordingly, we initiated a series of studies designed to explore the nature and significance of these sites in greater detail. In the present communication, we have addressed ourselves to the following quantitative and kinetic problems: (*a*) the compartmental relationship between T_3 in nuclei and T_3 in cytoplasm of liver cells; (*b*) measurement of the nuclear binding capacity of T_3 per gram of liver; and (*c*) the extent of cross-reactivity of these sites between T_3 and T_4.

METHODS

The methods for measuring nuclear binding have been briefly reported in our previous communication (1). As

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indicated above, this technique depends upon displacement of [^{125}I]T$_3$ by nonradioactive T$_3$ in vivo. Male Sprague-Dawley rats weighing between 150 and 250 g were obtained from Carworth Div., Becton, Dickinson & Co., New City, N. Y., and were maintained on a Wayne Lab Rat diet (1 μg I/g) (Wayne Kerr Lab Ltd., Chessington, Surrey, England). Animals were used within 2 wk of shipment. Rats were injected through the tail vein with doses of isotopic and nonisotopic iodothyronines in a 1% albumin solution. [^{125}I]T$_3$ (sp act approximately 60–80 μCi/μg) and [^{125}I]T$_4$ (sp act approximately 60–80 μCi/μg) were obtained from Abbott Laboratories (North Chicago, Ill.). Nonradioactive T$_4$ and T$_3$ were obtained from Mann Research Labs, Inc., New York. Unless otherwise indicated, animals were killed 30 min after injection by exsanguination under light ether. Blood was obtained from the abdominal aorta, after which the liver was removed, blotted, and processed at 0°C. Approximately 4 g of liver was weighed and homogenized in 3.0 vol of 0.32 M sucrose containing 2 mM MgCl$_2$ in a mechanically driven Teflon pestle. The homogenate was brought to 10 vol of 0.32 M sucrose and centrifuged at 700g for 10 min. The 700g pellet was resuspended in 2.4 M sucrose and subjected to an additional centrifugation at 28,000g for 45 min in a Spinco 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (3). The purity of the nuclear preparations was checked by electron and phase microscopy. Radioactive measurements were made in a Packard automatic well scintillation counter with standard techniques with a statistical counting error of less than 5% (Packard Instrument Co., Inc., Downer's Grove, Ill.). In the case of T$_3$, chromatographic studies indicated that in the time interval studied, over 95% of the radioactivity in both the total liver homogenate and the purified nuclear pellet was in the form of T$_3$. After injection of T$_4$ the nuclei selectively bound the small quantity of radioactive T$_3$ derived both from the in vivo T$_4$ to T$_3$ conversion (4) and the trace contamination (up to 2% of total radioactivity) in the doses of radioactive T$_4$ used (5). The problem created by the selective retention of T$_3$ by nuclei will be discussed in detail in the Results section. Ethanolic extract of liver homogenate and nuclear pellets were subjected to paper chromatography in t-amyl alcohol: 2 N ammonia: hexane (6). Plasma samples were precipitated with trichloracetic acid to remove radioiodide. DNA determinations on total liver homogenate and nuclear pellets were performed according to the method of Burton (7). Plasma T$_3$ in a group of normal rats was measured by radioimmunoassay (8). In order to assess the possibility that nuclei can metabolize T$_3$, liver nuclei were incubated for 1 h at 37°C with tracer [^{125}I]T$_3$ in a Tris buffer (pH 7.4) containing 1 mM MgCl$_2$, 10^{-3} M ATP, and a NADPH-generating system of 10^{-3} M NADH, 0.1 M glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. Calculations used in the analysis will be discussed in conjunction with the results of individual experiments.

RESULTS

Compartmental relationship between T$_3$ in the nucleus and T$_3$ in the cytoplasm. Previous studies have indicated that T$_3$ is bound to nuclear constituents by noncovalent linkage, since the hormone can be easily extracted with ethanol. It was not clear, however, whether in the cell nuclear T$_3$ is readily exchangeable with T$_3$

in the cytoplasm (= extranuclear cellular T$_3$) under in vivo conditions. In an effort to answer this question, we analyzed the concentration of [^{125}I]T$_3$ in the nucleus and cytoplasm (= homogenate radioactivity − nuclear radioactivity) in livers of animals killed 5 min, 15 min, 30 min, 1.5 h, 2.5 h, and 4 h after the injection of tracer hormone.[a] Four animals were used at each time point. Paper chromatography of ethanolic extracts of nuclear pellets and total liver homogenates indicated that over 95% of the radioactivity was in the form of T$_3$. The dose of T$_3$ injected (3.85 ng T$_3$/100 g body wt) was determined to be in the true tracer range by establishing that smaller quantities of hormone injected produced identical distribution of radioactivity among plasma, liver, and nuclei. The largest increments of T$_3$ in plasma due to injected hormone were achieved in animals killed 5 min after the injection and averaged 0.016 ng/ml, a value only 2% of the estimated normal T$_3$ plasma concentration. In other experiments we have shown that when the plasma T$_3$ concentration is increased by less than 20% of its normal concentration, no alterations in the parti-

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[a] In this and following analyses we have assumed that the partition of T$_3$ between specific nuclear sites and the remainder of the cell as determined by differential centrifugation provides an accurate representation of the relationship that exists in the intact cells. This assumption appears reasonable since at 0°C there is extremely poor exchange of T$_3$ between specific nuclear sites and the cytoplasm in vitro. Thus, nuclear binding of tracer T$_3$ added to the liver homogenate at 0°C is much weaker than binding observed after in vivo injection of isotope (1). Conversely, we have added as much as 200 μg T$_3$/g liver to the final homogenate without displacing in vivo injected tracer T$_3$. Moreover, when the final nuclear pellet is recentrifuged through sucrose, less than 2% of the radioactivity is lost. Finally, an experiment was carried out to determine whether dilution of the liver homogenate in increasing volumes of sucrose would promote the dissociation of T$_3$. The liver homogenate was brought up to 5, 10, and 20 vol of 0.32 M sucrose and centrifuged at 700g for 10 min. The pellet was resuspended and centrifuged at 28,000g in heavy sucrose as described above. No consistent effect of dilution was noted (1:5, 0.32% dose in the nuclear fraction per gram liver; 1:10, 0.34%; 1:20, 0.32%).

The possibility that nuclear T$_3$ is instantaneously lost when the homogenate is placed in hypertonic sucrose should be considered. Although losses due to this mechanism cannot be easily estimated experimentally, theoretical considerations can serve to set an upper limit. Assume that 20% of hepatocellular binding is due to "specific" nuclear sites. If these are saturated, the liver: plasma ratio is reduced from about 10 to 8, barely detectable given biological variations (1). If, on the other hand, we assume that 40% of liver binding was due to specific nuclear sites, then saturation would reduce the L/P ratio from 10 to 6, which should be easily detected. In the absence of an observable reduction in the L/P ratio with saturating doses, one must consider that any possible losses due to "osmotic shock" are less than 50% of the amount originally bound to the limited-capacity nuclear sites.
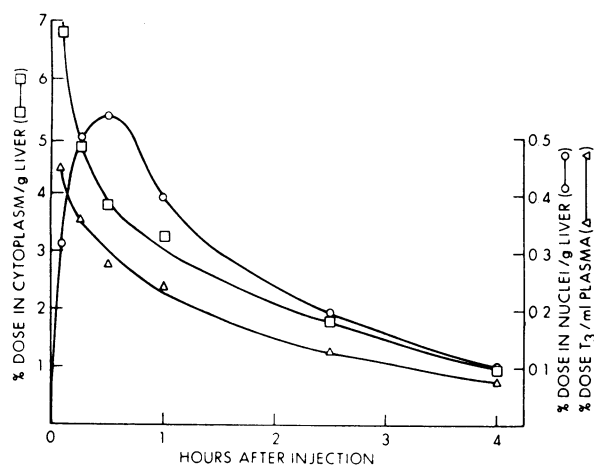
FIGURE 1 Time-course of concentration of radioactive T₃ in plasma, cytoplasm, and nuclei after intravenous injection of a single dose of tracer T₃.

tion of hormone among nuclei, cytoplasm, and plasma are detectable.

The results of sequential analyses of radioactive T₃ distribution are illustrated in Fig. 1. A progressive fall in the concentration of radioactive T₃ in plasma and cytoplasm was noted from the earliest point of observation (5 min). The concentration of nuclear [^{125}I]T₃ rose to a peak at 30 min and thereafter appeared to approach a fractional decay rate similar to that of cytoplasm and plasma. The ratio between T₃ in plasma and cytoplasm was constant throughout the experiment, a
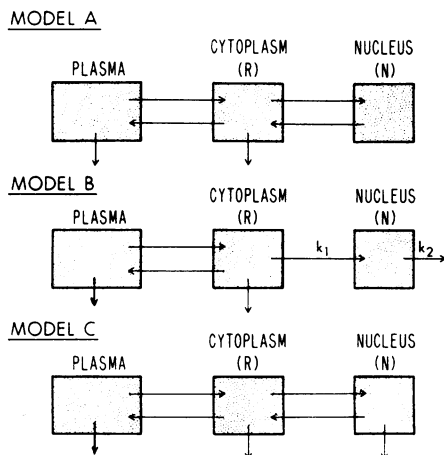

FIGURE 2 Three model systems describing the possible relationships between cytoplasm and nuclei. On the basis of data contained in Fig. 1, k_1 and k_2 were evaluated for Model B. It was determined that the calculated value of k_2 led to an estimate of irreversible clearance rate of T₃ from the nuclei several times larger than entire metabolic clearance rate of the animal. Since Model B is not possible, there must be a return of T₃ from nuclei to cytoplasm as indicated in Model A or Model C. See text for details.

TABLE I

Nuclear Binding of T₃ in Euthyroid Animals

Exp.	Dose T₃ injected	Hepatic T₃ concn.	$\left(\dfrac{100N}{L}\right)$	$\left(\dfrac{100N}{L}\right)_c$	T₃ bound to specific nuclear sites
	ng/100 g body wt	ng/g liver			ng/g liver
11	65	11.2	8.56	7.53	0.96
	120	16.2	6.64	5.61	0.94
	176	17.5	6.72	5.69	1.00
	232	21.2	5.32	4.29	0.91
	344	33.6	3.99	2.96	0.99
	1,961	150.1	1.79	0.76	1.14
	10,049	825.0	1.03	—	——
					av. 0.99
16	79	16.0	6.85	5.85	0.94
	148	18.9	6.99	5.99	1.13
	288	32.4	5.42	4.42	1.43
	2,100	180.0	1.76	0.76	1.37
	9,767	667.0	1.02) 1.00	—	——
	20,919	1,844.0	0.98)	—	
					av. 1.22
25A	5	9.4	12.54	10.68	1.01
	230	24.7	5.89	4.03	1.00
	575	41.0	4.73	2.87	1.18
	2,047	149.0	2.82	0.96	1.43
	9,798	614.0	1.96) 1.86	—	——
	20,092	1,263.3	1.77)	—	
					av. 1.16
27A	5	8.7	10.73	9.47	0.82
	50	13.1	8.92	7.66	1.00
	245	26.2	5.75	4.49	1.17
	10,212	642.1	1.25) 1.26	——	——
	40,011	2,341.0	1.28)		
					av. 1.00
28B	5	9.50	10.96	9.46	0.90
	51	13.05	9.65	8.16	1.07
	208	16.27	6.20	4.76	0.77
	10,058	642.8	1.51	——	——
	20,069	1,436.0	1.07) 1.49		
	40,092	2,526.0	1.88)	——	——
					av. 0.91
30B	5	8.16	9.75	8.71	0.71
	51	11.06	7.85	6.81	0.75
	196	21.86	4.23	3.69	0.81
	579	47.04	3.98	2.94	1.38
	10,092	331.9	1.24) 1.04	——	——
	20,033	603.8	0.83)		
					av. 0.91

Each entry represents the average of four animals.

Abbreviations: $\dfrac{100N}{L} = \dfrac{100 \times \% \text{ dose nuclear fraction/g liver}}{\% \text{ dose/g liver}}$

$\left(\dfrac{100N}{L}\right)_c = \dfrac{100N}{L}$, corrected for nonspecific binding as determined by minimal values for $\dfrac{100N}{L}$ found when the dose injected equaled or exceeded 10,000 ng/100 g body wt. When more than one "loading" dose was used, the average value for $\dfrac{100N}{L}$ for the high doses indicated by brackets, was subtracted from the uncorrected $\dfrac{100N}{L}$ at the low dose. Each entry represents the mean of four values. The average coefficient of variation of $\left(\dfrac{100N}{L}\right)_c$ was 12.5%.

TABLE II

Specific Activity of Nuclei and Total Liver Homogenate $\frac{1}{2}$ h after Injection of $[^{125}I]T_3$

Rat	Homogenate			Nuclei			Sp act Nuclei
	Dose in extract	T_3 in extract	Sp act	Dose in extract	T_3 in extract	Sp act	Sp act Homogenate
	%	ng	% dose/ng T_3	%	ng	% dose/ng T_3	
1	2.49	2.40	1.04	0.836	0.623	1.34	1.29
2	1.91	2.58	0.740	0.616	0.687	0.897	1.21
3	3.46	1.96	1.77	1.24	0.733	1.64	0.95
4	2.03	3.54	0.575	0.616	1.143	0.539	0.94
Mean	2.47	2.62	1.031*	0.827	0.796	1.117*	1.10

Four rats were injected with 2 ng/100 g body weight $[^{125}I]T_3$ and killed 30 min thereafter. Nuclei were prepared as described in Methods. The nuclear pellet was resuspended in 2.0 ml sucrose, (0.32 M), and the resuspended nuclei as well as a sample of the original homogenate were extracted with 95% ethanol. Four extractions with 4 vol of ethanol were sufficient to remove 95% of the counts. These ethanolic extracts were taken to dryness in a flash evaporator in vacuo at a temperature less than 45°C. Evaporation flasks were then washed with 2% bovine serum albumin. Three 1-ml washes were required to remove 95% of the $[^{125}I]T_3$ counts. The counting rates of these solutions were then determined and portions were taken for measurement of nonradioactive T_3 by radioimmunoassay (9).
* Specific activity of nuclear T_3 is not significantly different from specific activity of T_3 in total homogenate as determined by paired t test.

finding indicating that the equilibrium between the cytoplasm and plasma is rapidly established.

Three possible compartmental models can be proposed to account for these relationships between T_3 in nucleus and cytoplasm (Fig. 2). Model A assumes that there is a two-way interchange between T_3 in cytoplasm and nuclei. Model B assumes that once T_3 has been transported from the cytoplasm to the nucleus, it does not return as such to the cytoplasm but is irreversibly degraded by nuclear metabolism. Model C is a combination of A and B and assumes both backward movement of T_3 and irreversible metabolism of T_3 from the nuclei. It is possible to exclude Model B for the following reason. If we assume that all T_3 reaching the nucleus is irreversibly metabolized, it is possible to calculate the metabolic clearance rate attributable to the postulated nuclear metabolism (Appendix A). This clearance value would be 72 ml/hr/100 g. The total metabolic clearance rate of T_3 in these animals, however, varies between 15.3 and 26.0 ml/hr/100 g body wt, as determined in two previous studies (10, 11). Thus, the estimated unidirectional nuclear clearance rate is three- to five-fold greater than the metabolic clearance rate, clearly an impossible situation. The concept that T_3 once delivered to the nucleus, is removed exclusively by irreversible processes (Model B) must, therefore, be rejected.

We have also performed in vitro experiments in which isotopically labeled T_3 in tracer quantities has been incubated with nuclei in the presence of ATP and cofactors at 37°C for 1 h. Chromatographic analysis of the nuclei and medium showed no metabolic transformation

of T_3. Thus, no support could be elicited to favor the concept that nuclei metabolize T_3.

On the basis of these kinetic considerations and the negative biochemical studies, it appears reasonable to conclude that nuclear T_3 must exchange with cytoplasm (Models A or C). The possibility that some nuclear metabolism occurs (Model C) cannot be rigorously excluded because of the inherent limitations posed by the application of results from any in vitro model to the intact organism.

Measurement of the nuclear binding capacity of T_3 (Tables I and II, Fig. 3). Theoretical considerations applied to the experiments illustrated in Fig. 1 suggest that the "equilibrium time point" at which the specific activity of nuclear T_3 is equal to the specific activity of extranuclear T_3 occurs $\frac{1}{2}$ h after the intravenous injection of tracer into normal animals with a presumed steady state level of nonradioactive T_3.[*] This assumption

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[*] Pearson, Veall, and Vetter (12) have shown that in a two-compartment system there exists a single time point when the specific activity in both compartments is the same. Thus, in the example under consideration, after the injection of tracer T_3, the specific activity in the nuclear pool is at first less than that of the cytoplasmic and plasma T_3. From a kinetic point of view, the liver cytoplasm and the plasma can be considered to be a single compartment, since there is no significant time lag in equilibration (Fig. 1). Subsequently, the specific activity of the nuclear T_3 will be greater than that of the extranuclear T_3. According to the principles of the analysis by Pearson et al., the single point at which the specific activity into the two kinetic pools is the same can be determined by the time point at which the rate of change of nuclear T_3 is 0. This occurs $\frac{1}{2}$ h after injection (Fig. 1) (12).
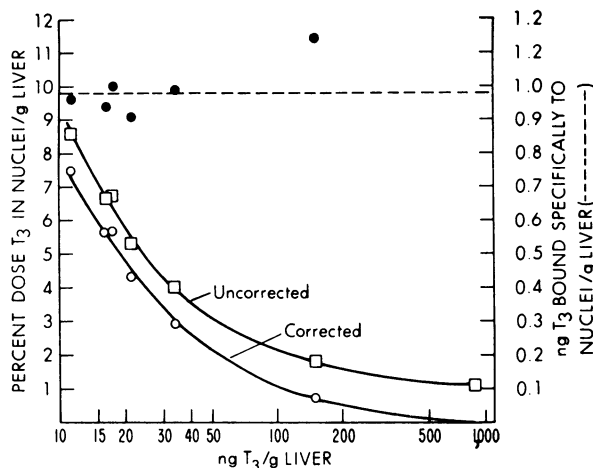
FIGURE 3 Calculation of nuclear binding capacity (Exp. 11). The uncorrected percent of the dose of T_3 per gram liver (= 100N/L) and the corresponding corrected value $(100N/L)_c$, determined by subtracting nonspecific binding, are plotted as a function of the concentration of T_3 in liver. The product of $(100N/L)_c$ and the concentration of T_3 in liver shows that the amount of T_3 specifically bound to the nuclei is remarkably constant. It would thus appear that the limited capacity binding sites are close to saturation at endogenous concentration of T_3. The first point represents the injection of trace quantities of T_3.

was confirmed in experiments in which the specific activity of nuclear and cytoplasmic T_3 was measured by radioimmunoassay of T_3 (Table II). Accordingly, all radioactive measurements for assessing nuclear T_3 pools were made $\frac{1}{2}$ h after injection.

As the dose of injected T_3 was increased, a progressive decrease was observed in the percent of total liver radioactivity associated with the nuclear pellet (designated 100 N/L). These results thus confirmed our previous observations (1). For a given group of animals, the observed 100 N/L with tracer levels of injected T_3 assumed a value ranging from 7 to 13 with a mean of 9.3. As the injected dose of T_3 was increased above 10,000 ng/100 g body wt, 100 N/L remained constant (see Table I). It appeared reasonable, therefore, to assume that when 100 N/L had reached minimal values, the specificially bound material would become a negligible proportion of the total. This lower limit for 100 N/L was therefore considered to represent largely nonspecific binding. Additional support for the concept of specific and nonspecific binding is derived from the demonstration that specifically bound nuclear T_3 is largely extracted with 0.4 M KCl, whereas nonspecifically bound T_3 is not (13).

In order to quantitate the amount of T_3 bound to specific T_3 nuclear sites, the nonradioactive concentration of T_3 in the liver was determined by multiplying the liver/plasma (L/P) concentration ratio of radioactive T_3 by the estimated total plasma T_3 concentration. The

latter value was determined from the sum of the endogenous T_3 concentration, 0.84 ng/ml[4] and the exogenous T_3 as calculated from the specific activity of the injected T_3 and the counting rate of trichloroacetic acid-precipitated serum. The product of the estimated liver T_3 concentration and the difference between the 100 N/L value observed at a particular dose and the 100 N/L value at the loading dose of T_3 (10,000 ng/100 g or greater) yielded the calculated mass of T_3 specifically bound to nuclei for a particular dose of injected T_3.

Results of six individual experiments are summarized in Table I. The binding capacity achieved in these experiments varied between 0.91 and 1.22 ng/g liver with a mean value of 0.99 ng/g. The average concentration of DNA in the final nuclear pellet determined in 32 animals was 1.7 mg/g liver. In these animals the average recovery of DNA from the homogenate was 62%. Thus the nuclear binding capacity per gram of tissue can be estimated to be approximately 1.60 ng T_3. A graphic representation of one of these studies is presented in Fig. 3. These results indicate a sharply defined binding capacity over a wide range of injected T_3, from 5 to 2,000 ng/100 g body wt. The average pool of exchangeable T_3 in the rat can be estimated to be approximately 130 ng/100 g body wt. When 2,000 ng/100 g body wt are injected, the plasma and total liver concentrations are observed to increase about 15-fold over the endogenous levels of T_3. Since the endogenous concentration of T_3 was not individually determined in these experiments, only a gross approximation can be offered of the percentage saturation of the nuclear sites under physiological conditions. Inspection of Table I, however, does suggest that over 70% of the sites are normally saturated.

Relationships of binding constants for T_3 and T_4. In our previous studies (1), we had demonstrated that nuclear binding sites showed a substantially greater avidity for T_3 than for T_4. To provide some quantitative estimate of the relative strength of association of T_3 and T_4, the following studies were undertaken. An additional point requiring resolution was whether the apparent cross-reactivity between T_3 and T_4 could be attributed to either in vivo conversion of T_4 to T_3 or to the existence of contaminating T_3 both in radioactive and nonradioactive preparation of T_4 used. As previously discussed, one could postulate that because of selective nuclear binding of T_3, any tracer T_3 derived from radioactive contamination of T_3 or metabolic conversion from labeled T_4 would be concentrated by the nucleus. Loading quantities of T_4 would present large amounts of nonradioactive T_3 to the nucleus and displace the tracer T_3 present.

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[4] Based on the mean value determined in the serums of 52 rats by a radioimmunoassay technique (8). The standard error was ±0.04 ng/ml.

In order to assess the binding characteristics of T_3, saturation experiments were analyzed according to expressions derived from the law of mass action. The ratio of T_3 bound nonspecifically to cytoplasm (T_{3R} = total hepatocellular T_3 — specific nuclear T_3) to specifically bound nuclear T_3 (T_{3N}) was plotted as a function of T_{3R} on the abscissa. From Eq. 6 (Appendix B) it is apparent that the slope of the resulting function is $(1/M)$ where M is the nuclear binding capacity and intercept on the ordinate $1/Mk_3$, where k_3 is the equilibrium constant governing the interaction of T_3 between cytoplasmic and nuclear sites as defined in Eq. 5 (Appendix B). The value of k_3 can therefore be calculated. When experiment 11 was analyzed in this fashion (Fig. 4) the following results were obtained. $k_3 = 1.4 \times 10^8$ kg/mol and $M = 1.8$ mol/kg.

Experiments were also carried out to assess the equilibrium constant governing the interaction of T_4 between cytoplasmic and specific nuclear sites. This was accomplished by determining the effect of increasing doses of unlabeled T_4 on the distribution of tracer [^{125}I]T_3 between cytoplasm and specific nuclear sites. From Eq. 11 (Appendix B) it is apparent that when (T_{3R}/T_{3N}) is plotted as a function of T_{4R}, the concentration of T_4 in the cytoplasm, the resulting function will have a limiting slope of (k_4/k_3) which will extrapolate to an intercept on the ordinate with a value of $(1/k_3 M)$. In order to evaluate T_{4R}, tracer [^{131}I]T_4 was injected together with tracer [^{125}I]T_3 and unlabeled T_4. T_{4R} was calculated from the L/P concentration ratio of [^{125}I]T_4 and the known
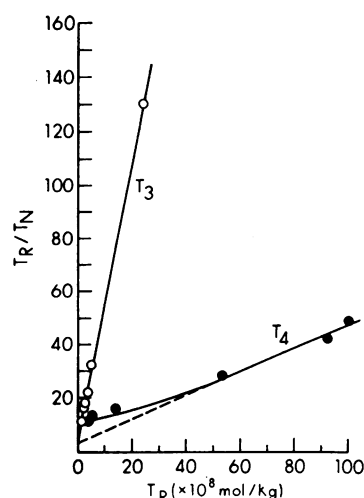

FIGURE 4 T_R, the molar concentration of T_3 or T_4 in the cytoplasm, is plotted against the ratio of T_R to T_N, the molar concentration of iodothyronine specifically bound in the nuclei. In the case of T_3, there is a linear relationship, consonant with theoretical expectation for a single set of nuclear binding sites. In the case of T_4, the relationship is not linear at the lower doses of T_4 injected, since the predominant effect on partition between nucleus and cytoplasm is determined by the endogenous levels of T_3 in the cell. Euthyroid animals were used in these studies and each point represents the mean of four animals. See the text for details of design, calculations, and interpretation of results.

plasma concentration of T_4. The results are illustrated in Fig. 4 in an experiment with the same group of animals used in estimating k_3. It was estimated that $k_4 = 1.1 \times 10^7$ mol/kg and that the ratio k_3 to k_4 was 13. In this analysis the assumption is made that the equilibrium time point for T_4 is the same as for T_3. Serial measurements of nuclear [^{131}I]T_4 have verified this assumption, with maximal radioactivity occurring between 15 and 30 min after intravenous injection of tracer.

The following experiments were carried out to evaluate the possibility that the observed cross-reactivity between T_4 and T_3 was due exclusively to contamination of the T_4 preparation with T_3 or the conversion of T_4 to T_3 in vivo. In Table III we have summarized two experiments in which tracer amounts of radioactive T_4 were injected alone as well as with loading doses of T_4 and T_3. With loading doses of either T_4 or T_3, approximately 33% and 40% of the nuclear radioactivity at tracer levels was displaced in Exp. 31 and 22, respectively. Chromatographic analysis of radioactivity of nuclear extracts from animals injected with tracer T_4 only (Exp. 31, group A), however, showed that only 7% of nuclear radioactivity was in the form of T_3. The remainder was T_4. Thus, on the basis of T_3 contamination or conversion, one could expect only a 7% decrease in

TABLE III

Displacement of Labeled T_4 by Loading Doses
of Iodothyronines

Exp	Iodothyronine injected	$\left(\dfrac{100N}{L}\right)$
	μg/100 g body wt	
31	A. tracer T_4 only	1.43
	B. tracer T_4 + 80 μg T_4	0.91
	C. tracer T_4 + 20 μg T_3	0.99
	D. specifically bound	0.48
	$= \left(A - \dfrac{B + C}{2} \right)$	
22	A. tracer T_4 only	1.19
	B. tracer T_4 + 1.2 μg T_4	0.82
	C. tracer T_4 + 24.2 μg T_4	0.73
	D. tracer T_4 + 20 μg T_3	0.74
	E. specifically bound T_3	0.48
	$= \left(A - \dfrac{C + D}{2} \right)$	

For definition of $\dfrac{100N}{L}$, see legend to Table I.

the counting rate of nuclear radioactivity with loading doses of T_4. Therefore, intrinsic cross-reactivity by nuclear sites for T_3 and T_4 exists and only a fraction of the observed cross-reactivity can be attributed to T_4 contamination or conversion to T_3.

The data in Table III can also be used to provide an independent estimate of the ratio of k_3 to k_4. Thus, from the law of mass action, it can be shown that:

$$\left(\frac{k_3}{k_4}\right) = \frac{(T_{3N}/T_{3R})}{(T_{4N}/T_{4R})}. \tag{13}$$

If we assume from Table I that the average (corrected) percent bound to the nucleus is 8.61, then it follows that (T_{3N}/T_{3R}) is equal to $8.61/91.39 = 0.0942$. From Table III, similar calculations of specific binding would indicate that the corresponding ratio for T_4 is 0.00472. Hence, k_3/k_4 is equal to 20. This contrasts with the value of 13 calculated from the experiments illustrated in Fig. 4. Whereas the agreement between the two values is not ideal, the estimates have the same order of magnitude and are useful approximations of the relative avidity of the nuclear sites for T_3 and T_4 in cytoplasm.

It should be emphasized that the values for k_3 and k_4 provided in these studies are not association constants since the equilibrium assessed was between the nuclei and cytoplasm, not between the nuclei and an aqueous medium. Nevertheless, if one knows the strength of protein binding as determined by equilibrium dialysis studies for T_3 and T_4, one can calculate the order of magnitude of the apparent association constants. The approximate dialyzable fraction for T_3 (DF_3) is 0.003 and that for T_4 (DF_4) is 0.0003 (11). If we designate k'_{3N} as the apparent association constant

$$\frac{k'_{3N}}{k_{3N}} = \frac{(L/P)}{(DF_3)} = \frac{10}{0.003}. \tag{14}$$

if $k_{3N} = 1.4 \times 10^8$, $k'_{3N} = (10/0.003) \times (1.4 \times 10^8) = 4.7 \times 10^{11}$ liters/mol. Similarly, since $DF_4 = 0.0003$ and the average $(L/P)_4 = 0.6$, $k'_{4N} = 0.6/0.003 \times 1.1 \times 10^7 = 2.2 \times 10^{10}$. The ratio of k'_3 to k'_4 is therefore about 21. Alternatively, if one accepts the ratio of $(k_3/k_4) = 20$ as derived from Eq. 12, then similar considerations can be applied to show that $(k'_3/k'_4) = 33.3$

DISCUSSION

The demonstration of specific nuclear binding sites for T_3 derives potential significance, not only from previously cited data by Tata and associates indicating early nuclear events after pulse injection of T_3, but also from rapidly accumulating evidence, that a number of steroid hormones exert their action at a nuclear level (14, 15). The hypothesis has been advanced by several investigators that progesterone, estradiol, dihydrotestosterone, and cortisol are first bound to a cytosol receptor and

that the cytosol receptor hormone complex in turn is transferred to the nucleus. There remains considerable controversy as to whether the steroid receptor in the nuclear chromatin fraction is the same as that in the cytosol.

These considerations raise the problem as to whether specific cytosol receptors also exist for T_3. This problem is currently under intensive study in our laboratory. A number of investigators have reported T_3 and T_4 cytosol-binding proteins (16–19) but the role of these proteins has not been well defined. In previous studies with tracer and loading doses of T_3, we have analyzed the distribution of T_3 in cytosol and other subcellular fractions. No evidence was found for appreciable quantities of specific binding sites other than those in nuclei. It is possible, however, that the concentration of cytosol binding sites was too small to allow detection. Regardless of whether T_3 in the cytoplasm is bound specifically or nonspecifically, the kinetic data presented in the current report indicate a relatively rapid two-way shuttle of T_3 between the nucleus and the cytoplasm. One-way transport of T_3 from cytosol to nucleus is not possible, because a model with one-way transport leads to an estimate of the hypothetical unidirectional clearance rate by nuclei several times greater than the total metabolic clearance rate of the entire rat. Moreover, no evidence was found in experiments in vitro to support the concept that nuclei can metabolize T_3.

Our data indicate that only 9–12% of cellular radioactivity is recovered with the nuclear pellet. On the basis of determinations of DNA in the tissue homogenate and in the nuclear pellet, we estimate that approximately 38% of the nuclear fraction is lost in the purification process. Thus, our data suggest that from 14–19% of the total radioactivity in the cell is associated with the nucleus. These findings explain the relative stability of the total L/P concentration ratio with increasing doses of injected T_3. Since no more than 19% of total cellular binding is due to specific nuclear binding sites, one would anticipate that maximal doses of T_3 would reduce the total L/P ratio to 81% of the baseline value. Inherent biological variation makes it difficult to detect changes in this range unless large numbers of animals are used.

Siegel and Tobias (20) have reported preferential localization of [^{125}I]T_4 to the nuclei of kidney tumor cells grown in tissue culture. No studies were carried out with [^{125}I]T_3. Saturation studies were not performed, and no information was provided about possible losses of radioactivity from the cells during fixation and radioautography. The concentration of T_4 in the culture medium was at least 50 times the plasma concentration of T_4 in mammalian serum. Griswold, Fischer, and Cohen (21) have recently reported temperature-dependent intracellular distribution of T_4 in amphibian liver. After in-

jection of T_4 at 25°C, from 40–50% of tadpole liver T_4 was associated with nuclei. The molar concentration of T_4 bound to nuclei was approximately 10,000 times greater than the molar concentration of iodothyronine bound to specific nuclear sites in the rat. Again, no saturation studies were carried out. It is therefore difficult to compare the findings in these reports to the results of our studies.

If we estimate that each gram of liver contains 3.34×10^8 nuclei (22) and if we assume that there has been a 38% loss of nuclear material during preparation, we can calculate from the nuclear binding capacity (0.99 ng T_3/g liver) that each nucleus contains approximately 4,400 molecules of T_3. If we further assume that each nucleus contains 254-cm double-stranded DNA (23) we can also estimate that on the average there is one molecule of T_3 for every 575 μm of DNA. It will be of interest to determine whether similar quantitative relationships exist between T_3 and DNA in other tissues.

The results of the present set of experiments indicate that the nuclear sites are not absolutely specific for T_3 since they also interact with T_4. We had previously queried whether the slight depression in the nuclear binding of labeled tracer T_4 by the injection of large doses of nonradioactive T_4 might not have been caused by contaminating T_3 in the T_4 preparations used and the conversion of T_4 to T_3 in vivo (1). The chromatographic studies summarized in Table III effectively exclude this possibility. On the other hand, the current data suggest that the affinity of nuclear sites for T_3 is at least 20 times greater than their affinity for T_4. The relative specificity of nuclear sites for T_3 supports recent metabolic studies which have suggested that the hormonal action of T_4 is derived largely, if not exclusively, from its conversion to T_3 (4, 10, 24). The concept that T_3 is the "active" hormone was first proposed by Gross and Pitt-Rivers (25) but subsequently rejected on the basis of a report by Lassiter and Stanbury (26) negating T_4 to T_3 conversion in man. The concept of T_4 to T_3 conversion was subsequently revived by Braverman, Ingbar and Sterling (28) who found significant conversion of T_4 to T_3 in athyreotic subjects maintained on T_3.

If one assumes that metabolic effects of T_4 and T_3 are equivalent, molecule-for-molecule bound to the specific nuclear sites, it is possible to provide an estimate of the overall intrinsic metabolic contributions of T_3 and T_4 (independent of its conversion to T_3) both in rat and in man. On the basis of the isotopic measurements and the known concentration of T_3 and T_4 in plasma, it is possible to calculate that the quantity of T_3 bound specifically to nuclei per gram of rat liver is 20.3×10^{-13} mol, whereas the corresponding mass of T_4 specifically bound is 2.83×10^{-13} mol. Thus one would estimate that the contribution of T_3 to hormonal effects of T_3 exceeds that

of T_4 by a factor of 7.1. It is possible to provide a similar estimate in man, since in man the ratio of T_4/T_3 plasma binding ($= 10$) and the approximate molar ratio of total T_4/T_3 ($= 45$) are known, and the ratio of the association constants of T_3 to T_4 is probably in the same range as in the rat ($= 21$–33). Calculations based on these data suggest that in normal man the contribution to hormonal effect derived from the total blood production of T_3 exceeds the intrinsic contribution of T_4 by a factor in the range of 4.7–7.3. In other words, from 12 to 18% of the total metabolic effect of the iodothyronines may be due to T_4. Thus, a change in one μg/100 ml T_4 would be approximately equivalent to 2.6–3.7 ng T_3/100 ml.

The demonstration of limited-capacity high-affinity T_3 binding sites per se does not prove the biological relevance of such sites. Nevertheless, on the basis of Tata's demonstration of early nuclear response to T_3, the role of nuclei in mediating the effects of other hormones, and the existence of nuclear T_3 sites in a wide variety of tissues, it appears highly likely that these sites are responsible for initiating biochemical reactions that result in the expression of hormonal activity. If so, our data raise an interesting set of questions about the quantitative relationship between the concentration of the T_3-nuclear complex and the hormonal effect. Our data suggest that at least 70% of the nuclear sites are occupied at endogenous concentrations of plasma T_3. For hyperthyroidism one one or more of the following would have to occur: (a) that the occupancy of the residual sites exerts a proportionately greater quantitative effect, (b) that there are qualitative or quantitative differences in nuclear T_3 binding in hyperthyroidism, (c) that the transition from the hypothyroid to the euthyroid state is mechanically different from the transition from the euthyroid to the hyperthyroid state. Further experiments are required to resolve these issues.

Lastly, it is important to emphasize the limitations in our analysis. The numercial values for the binding capacity and association constants are approximations only, since their determination is limited by kinetic assumptions, mean group values for plasma hormone concentrations rather than individual animal assays, and the experimental error inherent in the techniques used. Thus, in this study, we did not correct for DNA losses in the preparation of individual nuclear pellets, but simply assumed a mean loss. Also, the possibility that some T_3 dissociates immediately from the nucleus as soon as it is placed in hypertonic sucrose cannot be excluded nor easily tested by available techniques. Moreover, it is important to emphasize that the concept of nuclear binding as used in this study is operationally defined as the mechanism for the selective concentration of T_3 and related compounds at special nuclear sites. Thus, while an analogy is implicit between this process and more con-

ventional examples of binding, such as the binding of hormones by plasma proteins, care should be exercised not to endow kinetic characteristics with specific mechanistic interpretations. For example, we have not excluded the possibility that either the association or dissociation of T_3 with the sites requires the expenditure of metabolic energy.

APPENDIX A

It follows from the definitions of the model system B (Fig. 1) that

$$N_t = k_1 \int_0^t R dt - k_2 \int_0^t N dt, \qquad (1)$$

where N_t equals the percent of the dose injected in the nuclei per gram tissue at the time t in hours after the injection; R, the percent of the injected dose per gram tissue in the cytoplasm, k_1 the fractional rate (h^{-1}) of transfer of T_3 from compartment R to N; and k_2, the fractional irreversible rate of disposition of T_3 from the nuclei (h^{-1}). Eq. 1 simply states that the nuclear content of T_3 at any time t represents the difference between the total amount of radioactivity brought to the nuclei and the total amount of T_3 removed from the nuclei.

Similarly, it follows that the difference in nuclear counts between two time intervals after the injection, t and t', is given by the expression

$$N_t - N_{t'} = k_1 \int_{t'}^t R dt - k_2 \int_{t'}^t N dt. \qquad (2)$$

The rate constants k_1 and k_2 can be evaluated by solution of simultaneous equations 1 and 2. Thus, let t' equal 0.5 h and t, 4.0 h. The integral expressions in Eqs. 1 and 2 can be evaluated graphically. The value of both $N_{0.5}$ and N_4 are known. Solution of the equations will indicate that k_1 equals 0.94/h and k_2 equals 8.53/h. The specific activity of T_3 is uniform throughout the system under consideration at approximately 30 min, the time at which nuclear radioactivity is maximal, i.e., at the time that the rate of delivery of radioactivity to the nucleus equals the rate of removal from the nucleus. From this information we can calculate the unidirectional clearance ($_NCl_{T_3}$) of T_3 from the nuclei. Thus,

$$_NCl_{T_3} = \rho(N/P)k_2, \qquad (3)$$

where (N/P) = ratio of nuclei T_3 per gram liver to the concentration of T_3 per milliliter of plasma and ρ = liver weight in grams per 100 g of body weight. On the basis of previous studies with similar rats in this laboratory (10), ρ has been estimated to be 4.36. N/P at 30 min is 1.93 and k_2 8.53/h. Thus $_NCl_{T_3} = 71.8$ ml/h/100 g body wt.

APPENDIX B

In analyzing the interactions of increasing concentrations of T_3 with nuclear sites, the contributions of endogenous T_4 can be disregarded as a first approximation. Thus,

$$T_{3R} + N \rightleftharpoons T_{3N}, \qquad (4)$$

where T_{3R} is the concentration of T_3 in cytoplasm; T_{3N}, the concentration of T_3 in nuclei; and N, the concentration

of unoccupied nuclear binding sites. From the law of mass action one can define an equilibrium constant k_3 such that

$$k_3 = \frac{T_{3N}}{(N)(T_{3R})}. \qquad (5)$$

Since $N = M - T_{3N}$, where M is the nuclear binding capacity, one can substitute into Eq. 5 and rearrange terms so that

$$\left(\frac{T_{3R}}{T_{3N}} \right) = T_{3R} \left(\frac{1}{M} \right) + \frac{1}{k_3 M}. \qquad (6)$$

From the slope and ordinate intercept of a plot of (T_{3R}/T_{3N}) as a function of T_{3R}, M, and k_3 can be determined.

In a system in which the contributing T_4 cannot be ignored, i.e. one in which progressively increasing doses of T_4 are injected, the relationship between the tracer distributing [^{125}I]T_3 in cytoplasm and nucleus T_{3R}/T_{3N} can be related to the concentration of T_4 in cytoplasm (T_{4R}) as follows. Since both T_3 and T_4 occupy the same sites it is apparent that

$$N = \frac{T_{3N}}{(T_{3R})k_3} = \frac{T_{4N}}{(T_{4R})(k_4)}, \qquad (7)$$

where the subscript 4 refers to T_4, in a notation analogous to that for T_3.

From Eq. 7 it follows that

$$k_4 = \frac{T_{4N}}{T_{4R}(M - T_{4N} - T_{3N})}. \qquad (8)$$

Rearrangement of Eq. 8 leads to

$$\left(\frac{T_{4R}}{T_{4N}} \right) = \frac{\left(\dfrac{1}{k_4} \right) + T_{4R}}{M - T_{3N}}. \qquad (9)$$

From Eq. 7 it is apparent that

$$\left(\frac{T_{4R}}{T_{4N}} \right) = \frac{T_{3R}}{T_{3N}} \cdot \frac{k_4}{k_4}. \qquad (10)$$

Substituting into Eq. 9,

$$\left(\frac{T_{3R}}{T_{3N}} \right) = \frac{\dfrac{1}{k_3} + \dfrac{k_4}{k_3}(T_{4R})}{M - T_{3N}}. \qquad (11)$$

As the mass of injected T_4 is progressively increased T_{3N} approaches 0 and

$$\left(\frac{T_{3R}}{T_{3N}} \right) \approx \frac{1}{k_3 M} + \frac{(k_4/k_3)(T_{4R})}{M}. \qquad (12)$$

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