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Research Article

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In Vitro Binding of L-Triiodothyronine to Receptors in Rat Liver Nuclei

KINETICS OF BINDING, EXTRACTION PROPERTIES, AND LACK OF REQUIREMENT FOR CYTOSOL PROTEINS

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ABSTRACT Isolated hepatic nuclei from euthyroid rats were incubated with tracer [^{125}I]L-triiodothyronine (T_3) and increasing doses of nonradioactive T_3 for 30 min at 37°C. The T_3 bound specifically to nuclear sites increased with increasing T_3 doses to a plateau, which represented the nuclear binding capacity, M. Addition of 1 mM KCN, NaF, dinitrophenol, or iodoacetate did not affect nuclear binding, indicating that active metabolism was not required. Kinetic studies showed that the nuclear sites were equilibrated with T_3 within 30 min of incubation (one-half maximal binding at 3 min) and that the rate of release of T_3 in vitro (0.058 min^{-1}) was the same for endogenous T_3 or for T_3 bound to nuclei in vitro. Nuclear T_3 resisted extraction with 0.14 M NaCl buffered at pH 7.5, but both endogenous hormone and T_3 bound in vitro were readily extracted by 0.4 M KCl at pH 8.0. The elution profiles of endogenous and in vitro-bound T_3 from Sephadex G-100 columns showed a common protein peak with a molecular weight of 60–65,000, assuming a globular protein. Scatchard analysis of in vitro displacement studies showed a single class of binding sites. Mean $M = 0.23 \times 10^{-9}$ M or 0.85 ng T_3 for nuclei isolated from 1 g of liver. Mean M closely corresponded to that anticipated from reported in vivo studies. The apparent association constant K_a for the nuclear sites, 5.55×10^8 M^{-1}, was lower than in studies in vivo, probably attributable to the different ionic milieu of nuclei in the incubation buffer and in the intact cell. Thus, the identity of the nuclear T_3 binding sites studied in vitro to those reported for endogenous hormone is demonstrated by similar binding capacities, release rates, analogue binding affinities (previously reported), and localization to chromatin nonhistone proteins of comparable molecular weight. The role of cytosol protein in nuclear binding was assessed by comparing binding parameters for extensively washed nuclei and nuclei incubated either with contaminating or added cytosol. No difference in K_a or M was found. Moreover, it was unlikely that specific cytosol proteins were already present in nuclei and functioned during incubation as a shuttle for T_3, since K_a and M for nuclei obtained from athyreotic rats were similar to K_a and M for nuclei from euthyroid animals. Thus, an initial interaction between T_3 and specific cytosol proteins does not appear to be a prerequisite for translocation of T_3 to nuclear sites.

INTRODUCTION

Using in vivo displacement techniques, we have demonstrated the presence of limited-capacity binding sites for L-triiodothyronine (T_3)[1] in the nuclei of rat liver and kidney (1). T_3 bound to hepatic nuclear sites exchanged rapidly with T_3 in the cell cytosol (2). These techniques thus allowed measurement of the nuclear binding capacity (M) for T_3 as well as the apparent association constant of the hepatic nuclear sites. Several lines of evidence suggest that the nuclear sites represent the

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[1] *Abbreviations used in this paper:* HSA, human serum albumin; M, nuclear binding capacity; T_3, L-triiodothyronine; T_4, L-thyroxine.

cellular receptors for thyroid hormones and that binding of T_3 to these sites is a prerequisite for the expression of hormonal activity: First, the nuclear sites appeared relatively specific for T_3, since L-thyroxine (T_4) was bound to a much lesser degree. The apparent association constant (K_a) of the nuclear sites for T_3, 4.7×10^{11} M^{-1}, was estimated to be about 20-fold greater than that of T_4 (2). Second, in vivo competition studies of various analogues of iodotyrosines and iodothyronines and of T_3 for the nuclear sites of both liver and heart showed that when analogue distribution and metabolism were considered, the displacement activities of the analogues were closely correlated with their reported thyromimetic activities (3). Third, examination of T_3 binding to nuclei of a number of rat tissues showed limited-capacity sites with K_a similar to that of liver in all tissues studied (4). Moreover, there was a good correlation between tissue M and its reported biological response to thyroid hormones. For example, the M of spleen and testis, tissues considered unresponsive to thyroid hormones, were substantially less than those of liver, kidney, heart, and anterior pituitary. Fourth, studies of the intranuclear distribution of T_3 showed that the limited-capacity binding sites were localized to the chromatin fraction (5). The binding sites were further characterized as chromatin-associated nonhistone proteins. The identification of the T_3 nuclear receptors as nonhistone chromatin proteins is of special interest, since this class of nuclear proteins is considered intimately related to the control of DNA transcription (6). Moreover, some of the earliest detectable cellular biochemical changes that follow T_3 administration are the augmentations in DNA-dependent RNA polymerase activity and the rate of nuclear RNA synthesis (7).

These observations have been confirmed in part by DeGroot and Strausser (8) in rat studies, and Samuels and Tsai have demonstrated limited binding capacity nuclear sites for iodothyronines in pituitary GH_1 cells in tissue culture (9) and in intact lymphocytes (10).

More recently, we have demonstrated limited-capacity, high-affinity binding of T_3 to isolated rat liver nuclei in vitro (11). Binding of T_3 to limited-capacity nuclear sites in vitro has been separately reported by Samuels and Tsai (9, 12). Our preliminary observations have indicated that the nuclear sites demonstrated in vitro have similar characteristics to those previously described in in vivo studies (2, 3). The present report describes the kinetics of binding and the extraction properties of T_3 bound to the nuclear receptor sites in vitro. In addition, data are presented that suggest that cytosol proteins are not required to effect the translocation of T_3 from cytosol to the nucleus.

METHODS

Male Sprague-Dawley rats, weighing between 150–200 g, were obtained from Carworth Div., Becton, Dickinson & Co., New City, N. Y., and maintained on a Wayne Laboratory rat diet (Allied Mills, Inc., Chicago, Ill.) (1 μg iodine/g) and tap water ad lib. In several experiments athyreotic animals were used. Surgical thyroidectomy was performed by the supplier. On receipt in the laboratory, thyroidectomized animals were placed on Low Iodine Test Diet fortified with vitamins (Nutritional Biochemical Corporation, Cleveland, Ohio) for 2 wk and then injected i.p. with 100 μCi of [^{131}I]sodium iodide. Body weight was measured twice a week, and the animals were considered athyreotic only after their body weights had remained constant for 3 wk. The serum T_3 concentration of these rats was undetectable (less than 2 ng/100 ml) as measured by radioimmunoassay (13).

Methods for the preparation of the nuclei have been detailed in previous reports (2, 14). After centrifugation through 2.2 M sucrose and 3 mM $MgCl_2$, the nuclear pellet obtained contained highly purified nuclei, as demonstrated by electron microscopy (average protein/DNA ratio was 1.84; average RNA/DNA ratio was 0.36). The nuclei were resuspended in 4 ml of 0.32 M sucrose and 3 mM $MgCl_2$, so that 1 ml of the nuclear suspension contained nuclei recovered from 1 g of liver. These nuclear suspensions were called unwashed nuclei.

Nuclei were further purified in several experiments to remove the small amount of cytosol protein (see Results) that might contaminate the nuclear pellet. The resuspended nuclei were centrifuged at 700 *g* for 10 min. The pellet was again suspended in 4 ml 0.32 M sucrose and 3 mM $MgCl_2$ and centrifuged at 700 *g* for 10 min. This procedure was carried out once more, and the resulting nuclei were suspended in 4 ml 0.32 M sucrose and 1 mM $MgCl_2$ (nuclei recovered from 1 g liver per ml). These preparations were called washed nuclei.

Cytosol was obtained by centrifugation of the initial 700 *g* supernate at 130,000 *g* for 60 min in a Spinco 40 angle rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Incubation of the nuclei was carried out as follows: T_3 labeled either with ^{125}I (sp act, 33 μCi/nM), or with ^{131}I (sp act 28 μCi/nM) was obtained from Abbott Laboratories, North Chicago, Ill. [^{125}I]T_3 was added to 0.2 M Tris buffer, pH 7.0, at a concentration of 10 pmol/ml. Human serum albumin (HSA) (Albutein, Abbott Scientific Products Div., Abbott Laboratories, South Pasadena, Calif.) was added where indicated (0.3 or 3%). A series of tubes containing various concentrations of T_3 was then prepared by adding appropriate quantities of nonradioactive T_3 (free acid, Sigma Chemical Co. Inc., St. Louis, Mo.), dissolved in 0.1 N NaOH. 0.2 ml nuclear suspension, equivalent to nuclei from 200 mg of liver, was then incubated with either tracer T_3 solutions alone or with increasing amounts of nonradioactive T_3 in a volume of 1 ml incubation medium. The final concentration of the constituents of the incubation medium were: T_3, 1–10,000 pmol/ml in 0.32 M sucrose, 3 mM $MgCl_2$, 20 mM Tris, and 1 mM NaOH, pH 7.0, at 37°C (incubation medium) in a total volume of 1 ml. The incubations were generally carried out for 30 min at 37°C, after which 1 ml of 1% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) in 0.32 M sucrose and 3 mM $MgCl_2$ was added. After standing on ice for 15 min, the nuclei were recovered by centrifugation at 10,000 *g* for 10 min. The supernate was removed by gentle suction, and the counting

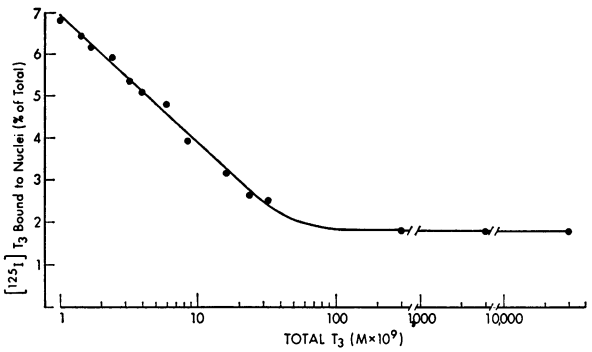

FIGURE 1 Displacement of tracer [^{125}I]T_3 bound to isolated hepatic nuclei by increasing amounts of nonradioactive T_3. Hepatic nuclei incubated with tracer [^{125}I]T_3 (1 pmol/ml) and increasing amounts of nonradioactive T_3 (up to 30,000 pmol/ml). The percentage of [^{125}I]T_3 falls progressively with increasing T_3 doses and reaches a nadir of 1.8% at 300 pmol/ml. Greater doses of T_3 incubated did not further reduce the percentage of [^{125}I]T_3 bound.

rate of the nuclear pellet was determined in a Packard Autogamma Spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.). Sufficient counts were accumulated to limit counting error to less than ±5%. Measurements of the percent of incubated [^{125}I]T_3 bound to the Triton X-100-treated nuclei were performed in triplicate at each dose of T_3 incubated.

To examine the nature of the [^{125}I]T_3 bound to the nuclear sites in vitro, nuclear T_3 was extracted with either 0.14 M NaCl buffered with 0.01 M Tris at pH 7.5, or 0.4 M KCl in 0.01 M Tris and 0.001 M EDTA, at pH 8.0. After incubation with [^{125}I]T_3, the nuclei were pelleted by centrifugation for 10 min at 10,000 *g* and then suspended by gentle homogenization into 2 ml of the indicated salt solutions. After 30 min at 0°C, the nuclei were pelleted by centrifugation at 10,000 *g* for 5 min. The supernate was removed and the nature of the radioactivity was examined by Sephadex gel filtration. Two different-sized columns were employed. To determine whether the extracted [^{125}I]-T_3 was protein-bound or free hormone, 0.5 ml of the 0.14 M NaCl or the 0.4 M KCl extracts were applied to 5 ml Sephadex G-50 medium columns (1.2 × 5.5-cm) equilibrated with the same buffer used for extraction. 1-ml fractions were collected. Protein-bound [^{125}I]T_3, eluted at the void volume, and nonprotein-bound [^{125}I]T_3 was recovered somewhat after the total column volume. Recovery of the applied radioactivity was 80–110%. The 0.4 M KCl extracts were studied further by filtration on large columns of Sephadex G-100. The columns (100 × 1.3-cm) with a total vol of 90–100 ml were packed with Sephadex G-100 equilibrated with 0.4 M KCl, 0.01 M Tris, 0.001 M EDTA, and 0.1% sodium azide, pH 8.0, and then washed extensively with the same buffer. 0.5–1 ml of the nuclear 0.4 M KCl extracts were applied. The flow rate was maintained at 15 ml/h. Fractions of 1.3 ml were collected, and the elution profile of radioactivity was compared to that of dextran blue (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and proteins with known molecular weights (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.).

Analytical procedures were as follows: DNA was determined by the method of Burton (15) with calf thymus DNA (Sigma Chemical Co.) as a standard; proteins were measured by the method of Lowry, Rosebrough, Farr, and Randall (16), with bovine serum albumin as a standard. Equilibrium dialysis was carried out as described previously (17). The nature of the radioactivity in various preparations was assessed by paper chromatography on Whatman 3 MM paper in a tert-amyl alcohol: 2 N NH_4OH: hexane solvent system. Details of the methods are reported elsewhere (18).

RESULTS

Characteristics of the in vitro incubation system. After incubation of isolated liver nuclei with tracer [^{125}I]T_3 (1–2 pmol/ml) 6–14% (35 experiments) of the incubated [^{125}I]T_3 was recovered in the Triton X-100-washed nuclear pellet. When increasing amounts of nonradioactive T_3 were incubated, a progressive decrease in the percentage of bound [^{125}I]T_3 was observed. In the representative experiment shown in Fig. 1, the percentage of [^{125}I]T_3 bound to nuclei incubated with tracer [^{125}I]T_3, 6.8, progressively decreased to a nadir of 1.8 when the nuclei were incubated with 300 pmol/ml T_3. Since incubation with higher concentrations of T_3 (3,000 or 30,000 pmol/ml) did not result in a further decrease in the percentage of [^{125}I]T_3 bound, the 1.8% value was considered to represent nonspecific binding and was subtracted from the percentage of [^{125}I]T_3 bound at the lower ligand concentrations. The corrected percentage of [^{125}I]T_3 bound was considered equivalent to [^{125}I]T_3 specifically bound to the nuclei. The product of the specific percent [^{125}I]T_3 bound and the total T_3 incubated represents T_3 specifically bound to nuclear sites. When

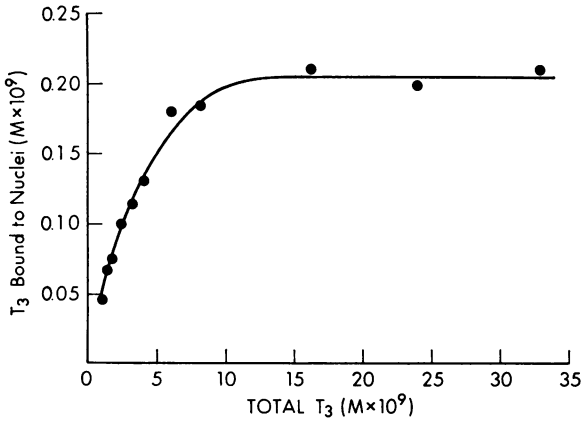

FIGURE 2 Specific binding of T_3 by isolated hepatic nuclei. Data are taken from the experiment illustrated in Fig. 1. The percent total binding of [^{125}I]T_3 minus the percent [^{125}I]T_3 bound nonspecifically (1.8%) was equivalent to the percent specific [^{125}I]T_3 binding. The percent specific [^{125}I]T_3 bound multiplied by total T_3 incubated was equal to T_3 bound specifically to nuclei. The plot shows that T_3 bound specifically to nuclear sites increases with increasing T_3 doses incubated until a plateau is reached that represents the maximum binding capacity, M, of the incubated nuclei.

plotted as a function of the total T_3 in the incubation mixture (Fig. 2), T_3 bound specifically to nuclear sites approaches a plateau that represents the binding capacity, M, of the nuclear sites. Thus, as in previously reported in vivo studies (2), the binding of T_3 to isolated nuclei in vitro is characterized by a well-defined binding capacity.

To examine further the characteristics of binding of T_3 to isolated nuclei, experiments were first carried out to study the stability of the in vitro system. Fig. 3 shows a time course of nuclear T_3 binding after incubation of both tracer (1 pmol/ml) and a molar excess (3,072 pmol/ml) of [^{125}I]T_3. With the molar excess of [^{125}I]T_3, approximately 1% of the incubated hormone was bound nonspecifically at all time intervals examined. After correction for nonspecific binding, the percentage of [^{125}I]T_3 specifically bound increased from 0.4 at 1 min of incubation to 4.9 at 10 min. Thereafter, the percentage of [^{125}I]T_3 specifically bound remained between 4.3 and 4.7 through the 60 min of incubation. Thus, binding to specific nuclear sites as well as nonspecific sites appeared relatively constant for 60 min of incubation. In other studies, however, (Table I) in which nuclear suspensions were preincubated for either 30 or 45 min at 37°C before addition of tracer or loading doses of [^{125}I]T_3, there appeared to be a moderate decrease in the binding of tracer [^{125}I]T_3 with increasing time of preincubation. Nonspecific binding remained essentiallly unchanged. Specific nuclear binding decreased, from 4.28% for nuclei not preincubated, to 3.50 and 2.93% for nuclei preincubated at 37°C for 30 and 45 min, respectively, before incubation with [^{125}I]T_3.

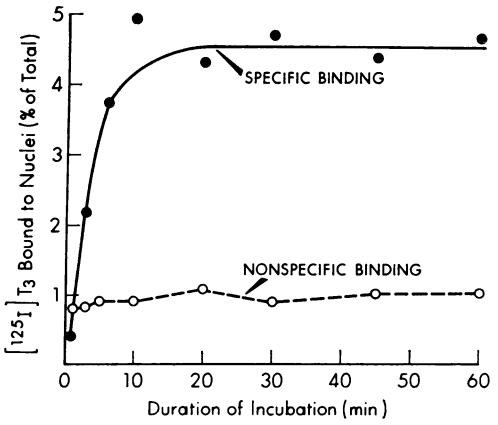

FIGURE 3 Time course of [^{125}I]T_3 binding by isolated nuclei. Nuclei were incubated with tracer [^{125}I]T_3 (1 pmol/ml) or a loading dose of [^{125}I]T_3 (3072 pmol/ml). Samples were removed at the designated time intervals for the determination of total [^{125}I]T_3 binding (tracer [^{125}I]T_3) and nonspecific binding (loading dose of [^{125}I]T_3). Plotted are the curves for nonspecific and specific (total − nonspecific) [^{125}I]T_3 binding.

TABLE I

Effect of Preincubation and Addition of Metabolic Inhibitors on the Nuclear Binding of T_3

Preincubation	Additions	Nuclear binding of [^{125}I]T_3		
		Tracer dose	Loading dose	Specific
min		*% of total* [^{125}I]T_3		
—	—	6.42	2.14	4.28
30	—	5.42	1.92	3.50
45	—	4.82	1.89	2.93
—	KCN	6.12	1.84	4.28
—	NaF	6.36	1.89	4.47
—	Dinitrophenol	6.84	1.87	4.97
—	Iodoacetate	6.75	1.85	4.90

Incubations of isolated nuclei recovered from 0.2 g liver in 1 ml 0.32 M sucrose, 0.02 M Tris, 3 mM $MgCl_2$, 0.03% human serum albumin, pH 7.0, with tracer [^{125}I]T_3, 1.54 pmol/ml and a loading dose [^{125}I]T_3, 3,000 pmol/ml. Specific binding was calculated as the percentage binding at tracer dose (total binding) minus the percentage binding at loading dose (nonspecific binding). Metabolic inhibitors were added at concentrations of 10^{-3} M. All flasks were incubated for 30 min at 37°C after addition of [^{125}I]T_3. Each entree represents the average of three determinations.

The possibility that the modest decrease in specific nuclear binding with increasing time of incubation was due to a requirement for active metabolism and that essential metabolic substrates might be depleted during prolonged incubation appeared to be excluded, since addition of different metabolic inhibitors did not affect nuclear T_3 binding significantly (Table I). Since the cause of the moderate decrease in nuclear binding during prolonged incubation was not determined, the duration of incubation in all subsequent studies was limited to 30 min.

To determine whether specific binding was proportional to the number of nuclear sites in the incubation mixture, increasing volumes of the nuclear suspension were incubated with either tracer or a molar excess of [^{125}I]T_3 (Fig. 4). An increase in the percentage of [^{125}I]T_3 bound was observed for both tracer and loading doses. The [^{125}I]T_3 bound specifically was proportional to the concentration of nuclei incubated.

Kinetics of nuclear binding of T_3 in vitro. The time course of specific nuclear T_3 binding is illustrated in Fig. 3. Whereas nuclear binding appeared to plateau at 10 min in this experiment, the percentage of [^{125}I]T_3 specifically bound to nuclei generally increased rapidly for 10 min, more slowly for 20–30 min, and occasionally declined gradually during the next 30 min (see Fig. 5). One-half maximal binding was attained at approximately 3–5 min of incubation.

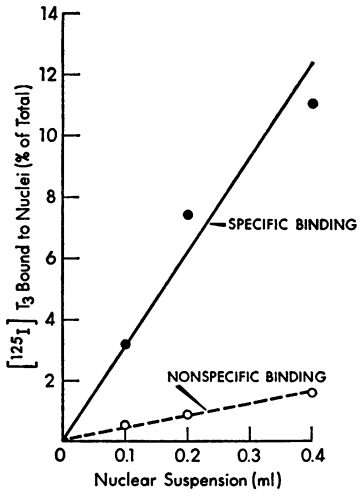

FIGURE 4 Linear relationship between specific [^{125}I]T_3 binding to isolated nuclei and the concentration of nuclei incubated. [^{125}I]T_3 incubated as per the legend to Fig. 3 with an increasing concentration of hepatic nuclei (from 0.1 to 0.4 ml suspension of isolated nuclei) equivalent to nuclei harvested from 0.1 to 0.4 g liver in 1 ml incubation medium. Specific nuclear binding is proportional to the nuclear concentration.

The release rate of T_3 bound to nuclear sites was determined both for endogenous hormone and for T_3 bound during incubation in vitro (Fig. 5). A tracer dose of [^{125}I]T_3 (5 ng/100 g body wt) was injected i.v. 30 min before the animals were killed. During this interval the specific nuclear sites are maximally labeled in vivo (2). Nuclei were then harvested and incubated with tracer [^{131}I]T_3 (1 pmol/ml). Incubation mixtures were sampled at 2-min intervals to determine specific nuclear binding. At 9.5 min of incubation, the mixtures were divided. A molar excess of T_3 (3,000 pmol/ml) was added to one portion to prevent detectable further nuclear binding of [^{125}I]T_3 from the incubation medium, and the vehicle in which the loading dose was dissolved was added to the other portion. The curves in Fig. 5 show that the release rate of nuclear T_3 was the same for endogenous T_3 ($t_{\frac{1}{2}} = 14.0$ min) as for T_3 bound to specific nuclear sites in vitro ($t_{\frac{1}{2}} = 14.5$ min). The lack of significant change in the maximal percentage of [^{131}I]T_3 bound specifically in vitro when vehicle was added indicated that the vehicle did not perturb the system. In two other experiments, similarly designed, the $t_{\frac{1}{2}}$ of release of nuclear T_3 was 10.0 and 12.2 min. The similarity in the rates of release in vitro of T_3 bound to the nuclear sites in vivo and in vitro provides further evidence that T_3 binding to isolated nuclei occurs at the same sites as previously reported in studies in vivo.

The average $t_{\frac{1}{2}}$ of 12 min for T_3 bound to nuclear sites also allows estimation of the effect of endogenous T_3 released from the nuclei upon subsequent calculations. As determined by in vivo displacement studies (4), the mean M is 1.77 ng T_3 for nuclei derived from 1 g of liver (2.72 pmol/g). In euthyroid rats, the mean percentage saturation of these sites is 47% (4), and the mean recovery of nuclei based on DNA measurements is 59%. Thus, the nuclear suspensions used in these studies contain approximately 0.151 pmol T_3/0.2 g liver nuclei ($2.72 \times 0.47 \times 0.58 \times 0.2$). Based on the observed release rates, the main portion (82%) of the endogenous T_3 will be released from the nuclei during 30 min of incubation. Since 1 pmol/ml of [^{125}I]T_3 is the lowest concentration of T_3 incubated in vitro, the released endogenous T_3 will augment by 15% the medium hormone concentration at tracer [^{125}I]T_3 levels and will make a progressively smaller contribution to the T_3 concentration when increasing doses of hormone are incubated. The resulting change in the calculation of bound T_3 is so small that it is within the error of determination of specific T_3 binding. Thus, the contribution of endogenous T_3 to total incubated hormone concentration has been discounted in subsequent calculations.

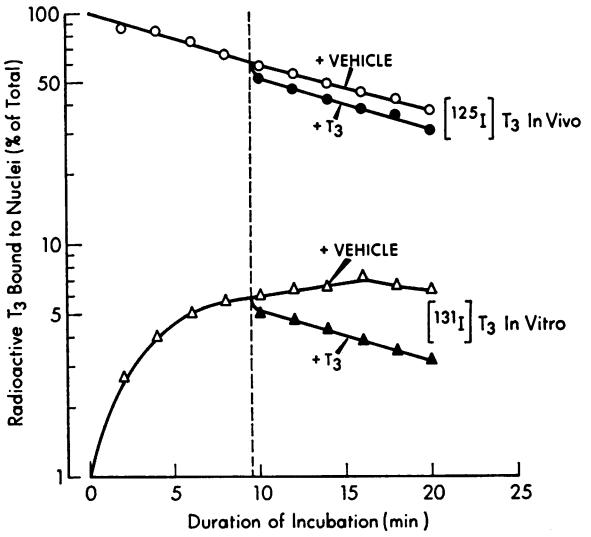

FIGURE 5 Similar rate of release for T_3 bound to specific nuclear sites in vitro and for endogenous nuclear T_3 upon incubation of isolated nuclei in vitro. Endogenous nuclear T_3 was labeled by injection of [^{125}I]T_3 (5 ng/100 g body weight) 30 min before the rats were killed and the liver nuclei harvested. The nuclear suspensions were then incubated in vitro for 10 min with [^{131}I]T_3 (1 pmol/ml) and a loading dose of T_3 (not illustrated). Samples were withdrawn at the indicated time intervals to determine the percent of in vitro-bound [^{131}I]T_3 (open triangles) and endogenous [^{125}I]T_3 (open circles) bound to specific nuclear sites. At 9.5 min of incubation (vertical interrupted line) the incubation mixture was divided. Nonradioactive T_3 (3,000 pmol/ml) was added to one portion (filled circles or triangles) to prevent reuptake by nuclei of [^{125}I]- or [^{131}I]T_3 from the incubation buffer and vehicle (open circles or triangles) was added to the other portion.

Additionally, since an average of 47% of the nuclear sites are occupied under endogenous conditions, calculations based on the observed release rate of nuclear T_3 indicate that 80–95% of the nuclear sites will be accessible for hormone binding in vitro during a 30-min incubation. Thus, the nuclear binding capacity determined in vitro should reflect adequately that measured in vivo.

Characterization of T_3 bound to isolated nuclei. Chromatographic studies were performed to determine the nature of the T_3 bound to isolated nuclei for comparison to previously reported data when nuclei were labeled with radioactive T_3 in vivo (5). Rats were injected with [^{131}I]T_3 i.v., 50 ng/100 g body wt, 30 min before they were killed. Nuclei were harvested and incubated either with a low dose (1 pmol/ml) or a molar excess (3,000 pmol/ml) [^{125}I]T_3 in vitro for 10 min at 37°C. After incubation, one portion was taken for measurement of total nuclear T_3 radioactivity and another was treated with Triton X-100, 0.5% in 0.32 M sucrose and 3 mM $MgCl_2$. Pelleted nuclei from a third and fourth portion were treated separately with buffered 0.14 M NaCl or 0.4 M KCl. As indicated in Table II, 50.2–59.5% of the nuclear [^{131}I]T_3 bound in vivo remained with the nuclei after incubation with either a low or high dose of T_3. In contrast, the 22.1% of [^{125}I]T_3 bound to nuclei in vitro after incubation with a tracer dose of [^{125}I]T_3 and the 16.2% upon incubation with a molar excess of [^{125}I]T_3 was substantially decreased by Triton X-100 treatment to 7.4 and 1.2% of the added [^{125}I]T_3, respectively.

TABLE II

Extraction by Buffered 0.14 M NaCl or 0.4 M KCl of Nuclear T_3 Bound after in Vivo Injection of [^{131}I]T_3, Followed by in Vitro Incubation with [^{125}I]T_3

Treatment	Source of radioactive T_3 bound to nuclei			
	In vitro incubation ([^{125}I]-T_3)		In vivo injection ([^{131}I]-T_3)	
	Low dose	High dose	Low dose	High dose
	% of added [^{125}I]T_3		*% of initial [^{131}I]T_3*	
None (total binding)	22.1	16.2	59.5	51.7
0.5% Triton, 0.32 M sucrose,	7.4	1.2	54.5	50.2
Extracted radioactivity, *% of nuclear radioactivity*				
0.14 M NaCl, 10 mM Tris, pH 7.8	12.8	15.0	6.4	6.1
0.4 M KCl, 10 mM Tris, pH 8.0	52.7	47.3	43.1	40.8

Endogenous nuclear T_3 was labeled in vivo by injection of [^{131}I]T_3, 50 ng/100 g body wt, 30 min before rats were killed. After isolation, the nuclei were incubated in vitro with either a low (1.0 pmol/ml) or high dose (3,000 pmol/ml) [^{125}I]T_3 for 10 min at 37°C. The values presented are representative of three experiments. The small difference between the percentage of initial [^{131}I]T_3 recovered in the nuclei is not significant statistically.

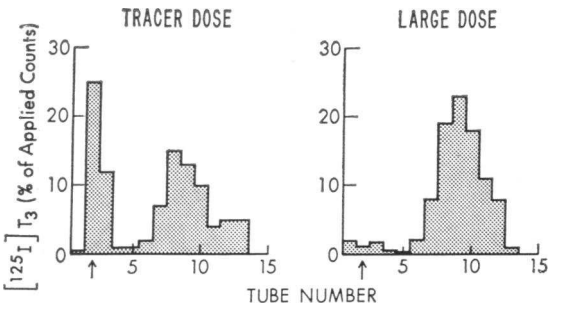

FIGURE 6 Sephadex G-50 chromatography of 0.4 M KCl extracts of nuclei incubated in vitro with tracer [^{125}I]T_3 (1 pmol/ml) or a large dose (3,000 pmol/ml) [^{125}I]T_3. 0.5 ml nuclear extract was applied, and 1-ml fractions were collected. The arrow designates the void volume as determined by the elution of dextran Blue. Recovery of applied counts was between 90–110%.

Extraction of the incubated nuclei with 0.14 M NaCl buffered at pH 7.5 removed 13–15% of the [^{125}I]T_3 bound in vitro and a smaller fraction of the [^{131}I]T_3 bound in vivo. Upon filtration of these extracts on small Sephadex G-50 columns, 97–99% of the applied radioactivity eluted as free hormone.

Treatment with 0.4 M KCl buffered at pH 8.0 extracted 47–53% of the nuclear [^{125}I]T_3 bound in vitro and 41–43% of the in vivo-bound [^{131}I]T_3. Consonant with previous stuides (5), 80% of the [^{131}I]T_3 bound specifically to the nuclei in vivo appeared in the void volume and was considered protein-bound. The elution profiles of the 0.4 M KCl extracts of [^{125}I]T_3 bound to the nuclei in vitro are illustrated in Fig. 6. After incubation with a low dose of T_3, 37% of the KCl-extractable [^{125}I]T_3 eluted in the void volume (protein-bound). The remainder eluted as free or nonprotein-bound hormone. After incubation with a molar excess of T_3, virtually all of the KCl-extractable [^{125}I]T_3 eluted as free hormone. The nuclear radioactivity in the 0.4 M KCl extracts, the 0.14 M NaCl extracts, the unextracted nuclei, and the Triton X-100-treated nuclei was shown to be authentic T_3 by paper chromatographic procedures.

Since an appreciable amount (37%) of the [^{125}I]T_3 bound to specific nuclei sites in vitro was recovered as protein-bound hormone, this extract was analyzed in greater detail on a Sephadex G-100 column (Fig. 7). The [^{131}I]T_3 incorporated in vivo showed an elution profile virtually identical to that reported previously (5). The large peak of radioactivity observed at the void volume could represent either T_3 protein complex still in association with DNA or aggregates of the T_3 nucleoprotein complex. A second peak observed at tube 29 is T_3 bound to nucleoprotein. If its structure is globular, the molecular weight of this protein, determined by comparison to the elution profiles of marker proteins

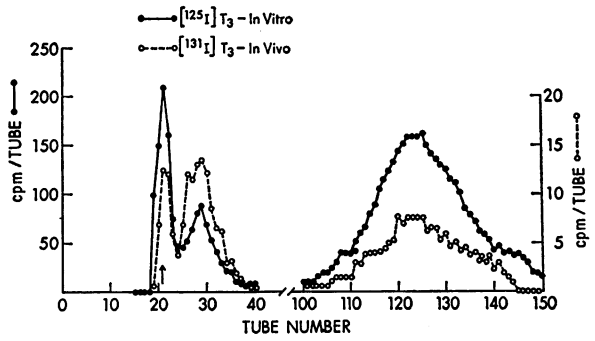

FIGURE 7 Radioactivity elution profile of 0.4 M KCl extracts of nuclei with specific T_3 binding sites labeled in vivo with ^{131}I-T_3 and in vitro with ^{125}I-T_3. The arrow designates the void volume as determined from the elution of Dextran Blue. Each tube contained 1.3 ml.

with known molecular weights, was 60–65,000, as observed previously (5). The remainder of the radioactivity eluted after tube 100 and represented free hormone. An almost identical elution profile was observed for the [^{125}I]T_3 bound to nuclear sites in vitro. The distribution of radioactivity among the peaks was somewhat different, however, with a greater fraction of radioactivity appearing as free hormone. This is probably due to the fact that treatment with 0.4 M KCl extracts some [^{125}I]T_3 bound nonspecifically as well as that bound to specific sites during in vitro incubation (Table II). This would not occur when the nuclear sites were labeled in vivo, since only 10% of the nuclear T_3 is bound nonspecifically after injection of a tracer dose of T_3 (1, 2). The virtual identity of the elution profiles of in vivo and in vitro-bound nuclear T_3 suggests that T_3 is bound by isolated nuclei to the same chromatin-associated nonhistone protein as previously shown for endogenous T_3 in vivo (5).

Apparent equilibrium assoication constant and binding capacity. After it was established that equilibrium conditions prevailed when nuclei were incubated at 37°C for 30 min, displacement studies, as depicted in Fig. 1, were plotted according to Scatchard to determine the apparent K_a for the specific nuclear sites and T_3, and the nuclear binding capacity (M) (Fig. 8). These plots revealed a single class of specific nuclear binding sites for T_3. In the illustrated experiment, carried out with unwashed nuclei incubated in the presence of 0.3% HSA, $K_a = 4.28 \times 10^9$ M^{-1}. Mean K_a for 13 studies[2] performed under these conditions was 5.55×10^9 M^{-1} (range = $1.9 - 12.3 \times 10^9$ M^{-1}) (Table III). The possibility that another class of binding sites with a higher affinity for T_3 might have been overlooked is unlikely, since no significant change in K_a or M was noted in several studies in which the tracer dose of [^{125}I]T_3 was reduced to 0.1 or 0.01 pmol/ml. HSA was employed to insure solubility of the higher doses of T_3 in the incubation medium. Since it was possible, however, that the added 0.3% HSA might extensively bind the T_3 in the incubation buffer, the free T_3 concentration in the incubation buffer might be overestimated, and the calculated K_a from Scatchard plots underestimated. This possibility was considered unlikely, since the K_a was not significantly different from the K_a of the 0.3% HSA incubation in studies in which either unwashed nuclei were incubated in buffer supplemented by a 10-fold lower HSA concentration (0.03%) or washed nuclei were incubated in buffer without addition of exogenous protein (Table III). The degree of protein binding of T_3 was also studied by equilibrium dialysis. The dialysis fraction of incubation buffer with tracer [^{125}I]T_3 supplemented with 0.3% HSA was 0.84, and that of 0.3% HSA alone was 0.87. These studies showed that, at most, only 16% of T_3 in the incubation medium could be protein-bound. Thus, K_a determined by these methods was not significantly influenced by protein binding of T_3 in the incubation medium.

The Scatchard plots of these incubations also enabled estimation of the M of the nuclear sites for T_3. Mean M for 19 experiments[2] was 0.23×10^{-9} M (Table III) and was equivalent to 0.3–0.5 ng T_3/mg DNA. The T_3 M determined in these experiments was in good agreement with that anticipated for the nuclear receptor sites from measurements of M in in vivo displacement studies (4). The mean M in in vivo studies is 1.77 ng T_3 for nuclei derived from 1 g liver (range = 1.02–2.35 ng/g). Since the mean recovery of hepatic nuclei isolated by centrifugation through 2.2 M sucrose is 59%,[3] a mean M of 1.04 ng T_3/g liver nuclei (range = 0.60 — 1.37 ng T_3/g) would be anticipated in vitro. Mean M isolated was 0.85 ng T_3/g isolated liver nuclei (range = 0.46 — 1.30 ng) for all experimental conditions. The modest reduction in binding capacity in washed nuclei may result from a small decrease in the recovery of nuclei during the washing procedures. The agreement in M determined in vitro with that predicted from measurements in vivo provides further evidence that the nuclear T_3 binding site studied in vitro is probably the same as in the intact animal.

Role of cytosol protein. A significant role for specific cytosol proteins in effecting the translocation of T_3 from the incubation medium to the nuclear receptor site was considered unlikely, since nuclear binding of T_3 to sites quantitatively and physicochemically similar to those

[2] Most of these determinations were performed in the course of other investigations.

[3] The mean 59% recovery of hepatic nuclei was determined in in vivo studies (4). A similar recovery was found in the present studies based on DNA measurements of the liver homogenate and isolated nuclei.

studied in vivo could readily be demonstrated in vitro. Nevertheless, the possibility existed that a small amount of cytosol protein contaminating the isolated nuclear pellet might be critical for nuclear binding in vitro. In a representative experiment, the total protein content of hepatic nuclei recovered from 0.2 g liver was 1.780 mg. After suspension in 1 ml incubation medium, nearly 10% of the nuclear protein, 0.166 mg, was found in the medium as soluble protein. The nuclear protein at the start of incubation was 1.614 mg (1.780–0.166). After a 30-min incubation at 37°C, the protein content of the incubation medium increased to 0.212 mg. Thus, only 0.046 mg or 2.8% $[(0.212 - 0.166)/1.614] \times 100$ of the initial nuclear protein was released during incubation. We considered, therefore, that the major portion of the protein found in the incubation medium at the start of incubation might be cytosol protein contaminating the nuclear pellet. The potential role of such cytosol contamination in nuclear binding was assessed in experiments employing nuclei extensively washed before incubation. In one study, K_a of nuclei incubated in buffer alone was 1.5×10^8 M^{-1}. Addition of the protein recovered from the supernate of the nuclear washes ("contaminating cytosol") at a concentration of 0.11 mg/ml did not result in any change in K_a. Similar results were obtained for nuclei incubated with or without washing after a 30-min preincubation at 37°C. In a third experiment, K_a for extensively washed nuclei and T_3 was 1.70×10^8 M^{-1}. Addition of separately isolated hepatic cytosol to the incubation medium at a concentration of 1 mg/ml resulted in a minimal change in K_a to 1.53×10^8 M^{-1}. Finally, a single batch of nuclei was divided, one portion being extensively washed and the other simply resuspended in incubation medium containing contaminating cytosol protein. The K_a was 6.2×10^8 M^{-1} and 8.0×10^8 M^{-1} and M was 0.16×10^{-9} M and 0.14×10^{-9} M, respectively. Thus no significant changes in nuclear binding resulted from extensive washing.

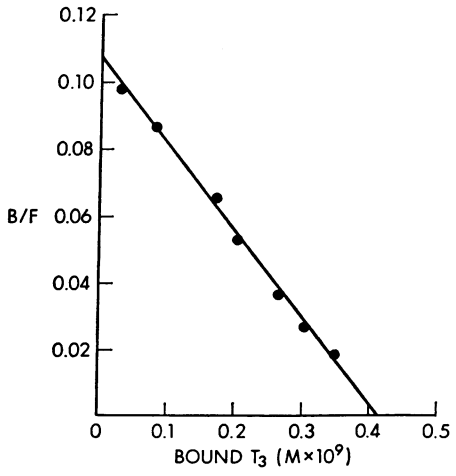

FIGURE 8 Scatchard plot of T_3 bound to specific sites of isolated hepatic nuclei in vitro. In this experiment, nuclei were incubated directly after isolation without further purification in the presence of 0.3% HSA. $M = 0.42 \times 10^{-9}$ M, equivalent to 1.37 ng T_3 per g liver nuclei. K_a is 4.28×10^8 M^{-1}.

TABLE III

Apparent K_a and Binding Capacity (M) for Isolated Hepatic Nuclei Incubated with T_3 under Different Conditions

Incubation conditions	n	K_a	M	
		$\times 10^{-8}$ M^{-1}	$\times 10^9$ M	ng/g liver nuclei
Unwashed nuclei +0.3% HSA	13	5.55 (1.9–12.3)	0.22 (0.13–0.40)	0.85 (0.46–1.37)
Unwashed nuclei +0.03% HSA	2	3.10 (2.3–3.9)	0.33 (0.28–0.38)	1.07 (0.91–1.24)
Washed nuclei	4	3.48 (2.1–4.5)	0.21 (0.11–0.36)	0.72 (0.54–1.17)

Unwashed nuclei were isolated through 2.2 M sucrose and 3 mM $MgCl_2$ and resuspended in incubation buffer. Washed nuclei were similarly isolated but suspended in 0.3 M sucrose, 3 mM $MgCl_2$, and reisolated by centrifugation three times before suspension in incubation buffer. The entrees are the mean values. n refers to the number of experiments. Numbers in parentheses indicate the range of values. A 20% suspension of nuclei were incubated in all experiments except three in the 0.3% HSA group, in which a 10% suspension was used.

Since nuclei isolated from euthyroid rats were used in all of these studies, it remained possible that endogenous T_3 bound to specific cytosol proteins required for translocation of T_3 to the nuclei receptor site were already present within the nuclei at the start of incubation. These proteins might then be released into the medium during incubation, bind exogenous T_3, and transport the hormone back into the nucleus to the receptor site in vitro. If this formulation were correct, it would not be possible to demonstrate specific nuclear binding in vitro when nuclei isolated from athyreotic rats were used. In such nuclei there is no T_3 bound to the nuclear receptor site at the beginning of incubation. Fig. 9 illustrates a Scatchard plot of an in vitro displacement study employing nuclei obtained from athyreotic rats. The calculated K_a and M are in the range of these parameters measured in nuclei from euthyroid animals (Table III). In the study illustrated, the K_a was 4.27×10^8 M^{-1}, and M was 0.35×10^{-9} M (1.14 ng/g liver nuclei). Thus, the demonstration of specific binding of T_3 by isolated nuclei from athyreotic rats suggests that the existence of intranuclear cytosol protein that facilitates T_3 translocation is unlikely.

DISCUSSION

Since many effects of iodothyronines reported in vitro have subsequently been shown to be unrelated to physio-

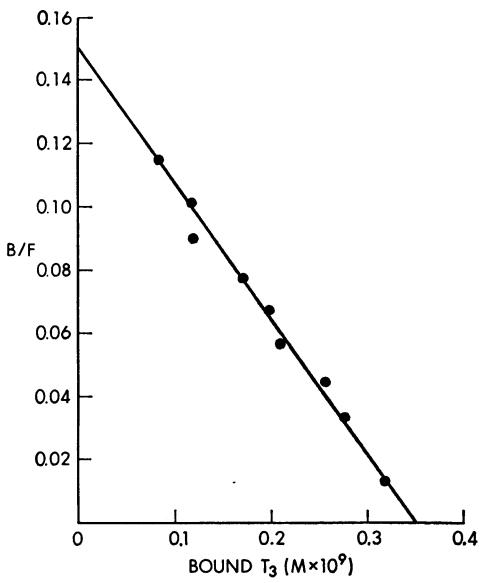

FIGURE 9 Scatchard plot of T_3 bound to specific sites of isolated nuclei obtained from athyreotic rats. The isolated nuclei were further purified by three washing procedures, as described in Methods, and incubated without the addition of exogenous protein. $M = 0.35 \times 10^{-9}$ M (1.14 ng T_3/g liver nuclei). K_a is 4.27×10^8 M^{-1}.

logical events in the intact animal, a major focus of the current studies has been to assess the relevance of T_3 binding to nuclei in vitro to nuclear binding of endogenous T_3 in vivo (1, 2). Thus, after the initial demonstration of specific T_3 binding to limited-capacity high-affinity sites in isolated hepatic nuclei (11, 12), considerable effort was expended to ascertain whether nuclear binding of T_3 in vitro occurred at the same site as endogenous T_3 in the intact rat. Separate lines of evidence indicate that this is indeed the case. First, the concentration of nuclear sites determined in vitro, 0.23×10^{-9} M, was virtually indistinguishable from the concentration of nuclear T_3 binding sites reported in vivo (2, 4), when corrected for recovery of nuclei during the isolation procedures. Similar results had been reported in separate studies by Samuels and Tsai (12). From published in vivo data, the predicted nuclear T_3 binding capacity in vitro was 1.04 ng T_3 (range 0.60–1.37) for nuclei derived from 1 g of liver or 0.48 ng T_3/mg DNA. The binding capacity observed in the present studies was 0.85 ng T_3 (range $0.46 - 1.30$)/g liver nuclei ($0.30 - 0.71$ ng T_3/mg DNA), values not significantly different from those predicted. Second, T_3 bound to the nuclear sites in vitro and endogenous T_3 labeled in vivo were released from the nuclear sites at the same rate in vitro. The kinetics of in vitro nuclear binding indicate that the in vitro incubation system does not conform to a simple two-compartmental model. Calculation of the rate constants suggests that the rate constant of T_3 binding to the nuclear receptor is several orders of magnitude too small to be compatible with a K_a in the range of 10^8 M^{-1}. An unknown nuclear compartment, perhaps the nuclear outer membrane, appears to delay translocation of T_3 from the incubation buffer to specific sites within the nuclei. Third, the physical characteristics of the nuclear T_3 binding sites demonstrated in vitro appear identical to the reported characteristics of the nuclear T_3 binding site in vivo (5). Thus, T_3 bound to the nuclear site in vitro resists extraction by low salt solutions but is solubilized readily by treatment with 0.4 M KCl buffered at pH 8.0. The column chromatographic studies of the extracted T_3 indicate that binding occurs to a protein with the same apparent molecular weight, if the protein is globular, as observed in in vivo studies, and suggest that the extracted hormone, similar to endogenous T_3, appears bound to a chromatin-associated nonhistone protein. Fourth, the structural requirements for nuclear binding in vitro appear strikingly similar to those for nuclear binding in vivo. This has been demonstrated by measurements of the capacity of a series of iodothyronine analogues to compete with T_3 for the nuclear sites. Data demonstrating similar avidity of nuclear sites for a series of T_3 analogues has been reported previously both in intact animals (3) and in the in vitro incubation system (11).

The only difference observed between T_3 binding by nuclei in vitro and T_3 binding in vivo is that the binding affinity of the nuclear sites was substantially lower (1,000-fold) in vitro than in the living animal (2). The current studies show that specific nuclear binding in vitro does not depend on active metabolic processes, since T_3 binding was unaffected by addition of different metabolic inhibitors. The difference in binding affinity, therefore, probably results from conditions of incubation of the isolated nuclei vastly different from the intracellular milieu. In this regard, a sixfold decrease in binding affinity but no change in total sites has been reported by Samuels and Tsai (12) for T_3 binding by nuclei isolated from cultured pituitary GH_1 cells and rat liver, compared to T_3 binding by nuclei in intact GH_1 cells and human lymphocytes (10). Thus, the bulk of evidence indicates that in vitro binding of T_3 occurs at the same chromatin protein site as endogenous hormone. The ability to study nuclear-T_3 interactions in vitro should facilitate a detailed analysis of this process.

Although it is clear that the T_3 receptor is a chromatin-associated nonhistone protein, which can be demonstrated both in vivo and in vitro, the mechanism by which T_3 enters the nucleus from the cytosol and binds to the receptor remains obscure. The present studies show that active metabolic processes are not required for binding to nuclear sites. That the translocation of

steroid hormones to chromatin receptor sites appears to require initial binding to specific cytosol proteins, which are translocated into the nucleus and bind to the chromatin, raises the question whether analogous events obtain for T_3. Cytosol proteins that bind both T_3 and L-thyroxine have been reported previously from a number of laboratories (19–23), but the role of these proteins in effecting the translocation of T_3 to the nucleus was not studied by these investigators. Recently, however, Dillman, Surks, and Oppenheimer (24) have compared the quantitative aspects of binding of T_3 and iodothyronine analogues by cytosol proteins to those of the nuclear receptor sites. Although a class of limited-capacity, high-affinity binding sites for T_3 was demonstrated in the cytosol, marked differences were observed between these sites and those of the nuclear receptor. The apparent association constant for cytosol T_3 binding sites was approximately 1/200th that of the nucleus, and the binding capacity of the cytosol T_3 binding sites was nearly 250 times that of the nuclear sites. Moreover, less than 1% of the cytosol sites were saturated, compared to 47% saturation of nuclear sites at euthyroid endogenous T_3 concentrations (4). Furthermore, the spectrum of binding affinities of the cytosol sites for T_3 analogues was grossly different from that of the nuclear receptor. Thus, unless radical changes occur in the quantitative and qualitative binding characteristics of cytosol proteins upon entry into the nucleus, these major differences between cytosol protein and nuclear receptor binding of T_3 suggested that an interaction between T_3 and specific cytosol proteins was not a prerequisite for binding of T_3 to the nuclear receptor. The present studies, which demonstrate that T_3 is translocated in vitro from an aqueous medium to nuclear sites that are qualitatively and quantitatively similar to the nuclear receptor sites of the intact rat, strongly support this conclusion. Addition of small amounts of cytosol protein did not enhance translocation of T_3 into the nucleus. In contrast, incubation of isolated nuclei directly in cytosol decreased nuclear binding (11). Finally, the possibility was tested that intranuclear T_3 bound to a specific cytosol protein was already present in the isolated nuclei and then acted like a shuttle, binding hormone at the nuclear membrane and transporting it to the chromatin receptor. This possibility appears quite unlikely since the T_3 binding characteristics in vitro for nuclei obtained from athyreotic rats were similar in all respects to those obtained from euthyroid animals.

Thus, in contrast to steroid hormones, T_3 does not appear to require an initial interaction with a specific cytosol protein to effect translocation into the nucleus. A different provisional model for nuclear T_3 binding may then be proposed. T_3 bound to plasma proteins exchanges rapidly with cytosol proteins and cellular organelles. Once in the cell, bound T_3 readily dissociates and either returns to the plasma compartment or may be translocated into the nucleus as free hormone with subsequent binding to the chromatin nonhistone receptor. Binding to the receptor may then result in an increased rate of transcription of DNA or the transcription of new DNA loci, which eventuate in the expression of hormone action.

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