Specific Binding of Thyroid Hormones by Hepatic Nuclei
Early in Amphibian Development

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SUMMARY

Low doses of [^{125}I] thyroxine or [^{125}I] triiodothyronine were injected intraperitoneally into Rana catesbeiana tadpoles, and hepatic cell fractions were isolated. An unsaturable binding to nuclei, probably due to low levels of contamination by other cellular organelles, and a saturable binding were observed. The saturable binding was pronounced 15 minutes after intraperitoneal injection of triiodothyronine and possibly thyroxine. Nuclei were saturated with thyroid hormones at injection doses per tadpole above 30 pmoles for thyroxine and less than 5 pmoles for triiodothyronine even though about 80% more thyroxine than triiodothyronine was present in the tissue. In both cases, of the order of 3500 hormone molecules/nucleus are accumulated 150 minutes after intraperitoneal injection. Analogues of thyroxine compete with labeled triiodothyronine for saturable binding to cell nuclei, and their competitive ability parallels their metamorphic potency.

INTRODUCTION

Thyroid hormones are involved in the regulation of both metabolic rate and differentiation and development of higher eukaryotic organisms¹. Induction of de novo synthesis of some enzymes has been demonstrated. A particularly favorable model system for studying developmental changes which are induced by thyroid hormone is amphibian differentiation during metamorphosis. (For reviews of the biochemical changes associated with metamorphosis, see 2-5).

In a previous study⁶ dl-[¹⁴C] thyroxine was found to bind to nuclei in a temperature-dependent step three hours after its intraperitoneal injection into tadpoles. More recently we have observed⁷ that thyroxine or triiodothyronine increases the activity of the soluble enzyme ornithine decarboxylase less than five hours after its intraperitoneal injection. We now have extended our earlier study involving injection of dl-[¹⁴C] thyroxine in several ways. We have used l-[¹²⁵I] thyroxine with a specific activity 3000x that of the [¹⁴C] thyroxine in order to investigate its subcellular distribution at concentrations in the physiological range. The present study has demonstrated that radioactivity can be coisolated with liver nuclei less than 15 min after intraperitoneal injection of [¹²⁵I] triiodothyronine into tadpoles. Furthermore, the ability of large doses of nonradioactive thyroid hormone analogues coinjected with [¹²⁵I] triiodothyronine, to diminish the binding of radioactivity to nuclei, parallels their metamorphic potency. These data provide further evidence that cell nuclei are a primary site of action of thyroid hormones during amphibian differentiation and development and are consistent with recent observations from this laboratory that regulation of RNA polymerase activity is involved⁸.

METHODS AND MATERIALS

Rana catesbeiana tadpoles of stages X-XIII⁹ were obtained from Lemberger Company, a subsidiary of Mogel Ed. Tadpoles were fed ground spinach on alternate days before experiments but were fasted during both the two day equilibration period immediately before experiments and during the experimental period.

Solutions of [¹²⁵I] thyroxine (or its analogues) in 0.1 ml of 10 mM NaOH were injected intraperitoneally into each of eight tadpoles. After the incubation period at 25°C, tadpoles were chilled by packing in ice, and the livers were excised, weighed and homogenized by means of a glass-teflon homogenizer in 10 volumes of Buffer A (30% glycerol (v/v), 5 mM MgCl₂, 10 mM KCl, 10 mM CaCl₂, 50 mM Tris-Cl, pH 7.5 at 4° C). The crude filtrate, obtained by filtering the homogenate through three layers of gauze was centrifuged at 620 x g_{max} for 10 min. The pellet was gently resuspended in 10 ml Buffer A and centrifuged again at 620 x g_{max} for 10 min. The crude nuclear pellet was resuspended in 16 ml of a 2.4 M sucrose solution which contained 5mM MgCl₂, 10 mM KCl, 10 mM CaCl₂, and 50 mM Tris-Cl, pH 7.5 at 4°C, and centrifuged at 130,000 x g_{max} for 45 min in a Spinco SW-27 rotor. After removal of the pellicle it was resuspended in 1 ml Buffer A by manual homogenization in a glass-teflon homogenizer.

The postnuclear supernatant (from the first centrifugation) was filtered through three layers of gauze and centrifuged again at 620 x g_{max} for 10 min to remove further traces of nuclei. The supernatant was centrifuged at 140,000 x g_{max} for 1 hr in a Spinco 50-Ti rotor. The supernatant, called the cytosol, was filtered through three layers of gauze. The precipitate after the high speed centrifugation was resuspended in 7 ml Buffer A by manual operation of a glass-teflon homogenizer, centrifuged at 140,000 x g_{max}

for 1 hr and resuspended in 1 ml Buffer A.

The binding of radioactivity during the isolation procedure itself was monitored by the identical fractionation of the crude filtrate, to which [^{125}I] thyroid hormone had been added. In these experiments, called in vitro experiments, the tadpoles had received a prior sham intraperitoneal injection.

The radioactivity and protein concentrations were assayed in quadruplicate for four cell fractions: (1) crude filtrate, (2) purified nuclei, (3) washed particulate membranes and (4) cytosol. Protein concentrations were assayed according to a variation¹⁰ of the procedure described by Lowry et al.¹¹ with bovine serum albumin as a standard. Radioactivity was measured as follows: to 50 λ aliquots scintillation vials were added 300 λ NCS Solubilizer (Amersham-Searle) and 10 ml toluene scintillation fluid containing 40 mg 2,5-diphenyloxazole and 2 mg 1,4-bis-2-(5-phenyloxazole)-benzene. Each sample was counted at an efficiency of 82% for at least 5 min in a Model 3375 Packard Tri-Carb Scintillation Counter.

[^{125}I]-L-Thyroxine (58 Ci/mmol) and [^{125}I]-L-triiodothyronine (46 Ci/mmol) were obtained from Abbott Labs. Nonradioactive thyroxine and its analogues were obtained from Sigma Chemical Company.

The specific levels of hormone associated with each cell fraction are expressed in the units of pmoles [^{125}I]/mg protein. We define the subcellular distribution index of radioactivity for a cell fraction (DI(fraction)) as the ratio of the specific level of radioactivity for that fraction as compared to that of the crude filtrate x 100.

RESULTS

Accumulation in Liver

The accumulation of radioactivity by hepatic tissue 150 min after intraperitoneal injection of [^{125}I] thyroxine into tadpoles is depicted in Figure 1a, and the corresponding accumulation after injection of [^{125}I] triiodothyronine in Figure 1b. In both cases the amount of radioactivity accumulated is directly proportional to the amount of [^{125}I] hormone injected in the range of 3 pmoles - 60 nmoles/tadpole. The higher levels of radioactivity observed in the crude hepatic filtrate for an injection of [^{125}I] thyroxine as compared with injection of [^{125}I] triiodothyronine is consistent with the data of Ashley and Frieden¹² for whole liver.

The accumulation of thyroid hormones by liver at times earlier than 150 min is shown in Table I. A substantial accumulation of both hormones was observed as early as 15 min after intraperitoneal injection.

Accumulation in Cell Fractions

Liver was fractionated by differential centrifugation to yield cytosol, nuclei and an intermediate fraction, which we have called the particulate fraction. The distribution index (see Methods and Materials) of radioactivity for the three cell fractions is tabulated in Table II. The particulate fractions contain the greatest and the nuclei the smallest levels of radioactivity/mg protein. The particulate and nuclear fractions contain relatively higher levels of radioactivity from triiodothyronine than from thyroxine.

The distribution index of radioactivity associated with purified nuclei in in vitro experiments was calculated as 7.7 for thyroxine and 12.3 for

triiodothyronine and was independent of the dose of hormone which had previously been added to the crude filtrate. However, after in vivo administration of either hormone, the distribution index for purified nuclei was higher at low concentrations of hormone. The difference between the level of radioactivity associated with nuclei in in vivo and in vitro experiments at equivalent doses of hormone in the homogenate has been defined as the specific level of nuclear binding. The total, in vitro and specific levels of nuclear binding are shown for thyroxine and triiodothyronine in Figures 2 and 3, respectively.

The specific binding of each thyroid hormone to nuclei is saturable, and the limiting level of radioactivity associated with the nuclei is comparable: approximately 130 fmoles [^{125}I] per mg nuclear protein. If we assume a nuclear content of 15 pg DNA per nucleus (the value for Rana pipiens)¹³ and a 3:1 nuclear protein: nuclear DNA ratio,¹⁴ then approximately 3500 molecules containing [^{125}I] bind specifically to hepatic cell nuclei. However, considerably higher levels of radioactivity must be present in the tissue before nuclei are saturated: > 3 pmoles [^{125}I] from thyroxine per mg tissue protein compared to < 0.3 pmoles [^{125}I] from triiodothyronine per mg tissue protein. These levels are equivalent to injected doses of >30 pmoles thyroxine per tadpole and < 5 pmoles triiodothyronine per tadpole.

The accumulation of radioactivity by nuclei at shorter times after the injection of [^{125}I] thyroxine or [^{125}I] triiodothyronine is shown in Table III. As early as 15 min after injection of triiodothyronine there is a concentration dependent level of radioactivity associated with cell nuclei.

Hormonal Specificity of Nuclear Binding

Figure 4 demonstrates the linear relationship between $\log [DI(\text{nuclei})]$ and $\log [\text{total hormone injected}]$. The absolute value of the slope in such a plot is a measure of the potency of a nonradioactive hormone to diminish the association with purified nuclei of radioactivity derived from injected radioactive hormone. In this case the slope of triiodothyronine of -0.99 is considerably greater than the value of -0.58 for thyroxine. Cross-competition experiments between labeled triiodothyronine and other unlabeled thyroid hormone analogues of known metamorphic competence¹⁵ were performed. The slopes through the data points for each of the analogues are listed in Table IV. Those thyroid hormone analogues of low metamorphic potency are poor competitors for the specific binding of radioactivity to nuclei, whereas those analogues of high metamorphic potency are the best competitors.

DISCUSSION

In vivo binding of thyroid hormones to microsomes from rat liver has been reported¹⁶. Our data indicate that the levels of radioactivity associated with the particulate fraction (mitochondria and microsomes) were proportional to the total amount of radioactivity in the tissue. Recent experiments in our laboratory (R. F. Brucker, unpublished observations) have indicated that nuclei purified according to the isolation procedure outlined above contain 2 - 5% microsomal contamination. Therefore, the unsaturable binding of thyroid hormones to nuclei in experiments with either large doses of hormones or only an in vitro exposure to the hormone could reflect low levels of contamination of the nuclei with some of the particulate fraction. In both cases of unsaturable binding 55 - 60% more radioactivity is associated with

the cell fraction after an injection of triiodothyronine than after a similar injection of thyroxine. A contamination of nuclei with 4.8% particulate protein would completely account for the unsaturable component of binding.

Tata has demonstrated¹⁷ that whole Xenopus laevis embryos contain sites which are saturable for thyroxine and triiodothyronine. These sites are specific for thyroid hormones active in inducing metamorphosis and are not present until just before tadpoles are competent to respond to thyroid hormones. Our data for competition between triiodothyronine and its analogues for binding to nuclei suggest that at least one of the subcellular sites in amphibian liver is in the nucleus.

One characteristic which a subcellular binder must have to be identified as a receptor is that it be saturated at physiological concentrations of the hormone. We have observed that the injection of pmolar amounts of the hormones is sufficient to fill the saturable sites of hepatic cell nuclei. The corresponding concentrations of thyroid hormones within tadpole livers which are needed to saturate these sites can be calculated, assuming a protein content of 50 mg/g tissue and a density of 1.1 g tissue/ml, to be greater than 700 nM for an injection of thyroxine and less than 70 nM for triiodothyronine. Although no data are available for either the tissue or serum concentrations of thyroid hormones in R. catesbeiana during natural metamorphosis, the physiological concentration of protein bound iodide of the serum of R. pipiens has been shown to rise to as high a level as 2 μ M during metamorphic climax¹⁸. If one assumes comparable physiological levels in R. catesbeiana, this concentration should suffice to saturate hepatic nuclei.

Recent experiments of Oppenheimer et al.¹⁹ have indicated saturable binding sites for triiodothyronine in the cell nuclei of adult rat liver but none in the cytosol. Tissue culture experiments with mammalian cells²⁰⁻²² have corroborated these results. The data in the present study indicate similar saturable sites for thyroid hormones in cell nuclei but not in the cytosol of amphibian liver. Thus it becomes necessary to distinguish between the mechanism of action of thyroid hormones in the regulation of calorogenesis and in the differentiation of higher eukaryotes. Several thyroid hormone analogues have been tested in rats²³ and tadpoles (Table IV) for their ability to compete with [¹²⁵I] triiodothyronine in binding to cell nuclei. Of those analogues which have been tested in both organisms triiodothyronine has greater biological potency than thyroxine which in turn is more biologically potent than diiodotyrosine for both development and calorogenesis⁵. Tetraiodothyroacetic acid on the other hand is a better inducer of metamorphosis in tadpoles but a less effective regulator of calorogenesis in rats than is thyroxine¹⁵. The nuclear binding in all four cases parallels the biological activity in that organism. However, until the metabolic effects of tetraiodothyroacetic acid have been determined in both organisms, it would be premature to conclude that the mechanisms of action of thyroid hormones in calorogenesis and differentiation and development are different. Further comparisons of the mechanisms of action of thyroid hormones in calorogenesis and in differentiation and development will have to await the determination of the active metabolite and the molecular target of thyroxine in tadpole liver nuclei. Such information has recently been reported for rat liver²⁴.

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TABLE I. UPTAKE OF RADIOACTIVITY BY LIVER AFTER ADMINISTRATION OF [^{125}I]
 THYROID HORMONES TO TADPOLES IN VIVO

<u>Time</u>	<u>Percentage of Total Radioactivity</u>	
	<u>Thyroxine</u>	<u>Triiodothyronine</u>
15 min	5.5 ± 1.4 (4) ^a	3.0 ± 2.5 (4)
45	9.9 ± 0.7 (4)	6.2 ± 3.0 (4)
75	10.0 ± 3.5 (4)	5.6 ± 1.0 (4)
150	5.8 ± 2.0 (18)	3.2 ± 0.8 (21)

^aEach number in parenthesis represents the number of experiments.

TABLE II. DISTRIBUTION INDEX OF RADIOACTIVITY IN LIVER FRACTIONS IN VIVO
(150 MIN AFTER ADMINISTRATION OF [125 I] THYROID HORMONES TO TADPOLES) AND
IN VITRO (AFTER ADDITION OF [125 I] HORMONES TO CRUDE FILTRATES).

		Concentration of Radioactivity in Crude Filtrate					
		<u>Thyroxine</u>			<u>Triiodothyronine</u>		
<u>Cell fraction</u>		<u><2.5 units^a</u>			<u><2.5 units</u>		
		<u>>10 units</u>			<u>>10 units</u>		
Cytosol	<u>in vivo</u>	70 $\pm$ 9	(11) ^b	60 $\pm$ 6	(7)	71 $\pm$ 15	(15) 56 $\pm$ 8 (8)
	<u>in vitro</u>	73 $\pm$ 4	(2)	78 $\pm$ 4	(4)	63 $\pm$ 1	(2) 52 $\pm$ 7 (4)
Particulate	<u>in vivo</u>	99 $\pm$ 24	(3)	113 $\pm$ 13	(6)	141 $\pm$ 14	(2) 163 $\pm$ 65 (6)
	<u>in vitro</u>	150 $\pm$ 9	(2)	162 $\pm$ 9	(3)	240 $\pm$ 30	(2) 273 $\pm$ 10 (3)
Nuclei	<u>in vivo</u>	11.9 $\pm$ 2.9	(11)	6.3 $\pm$ 1.1	(7)	52 $\pm$ 18	(15) 11 $\pm$ 2 (8)
	<u>in vitro</u>	9.2 $\pm$ 0.4	(2)	7.0 $\pm$ 1.4	(4)	12 $\pm$ 3	(2) 15 $\pm$ 2 (4)

^a 1 unit = 1 pmole [125 I] per mg protein.

^b Numbers in parenthesis represent the number of experiments.

TABLE III. UPTAKE OF RADIOACTIVITY BY NUCLEI AFTER ADMINISTRATION OF [^{125}I]
THYROID HORMONES TO TADPOLES IN VIVO.

Concentration of Radioactivity in Crude Filtrate				
Time	Thyroxine DI (nuclei)		Triiodothyronine DI (nuclei)	
	<2.5 units ^a	>10 units	<2.5 units	>10 units
0 ^b min	9.2 $\pm$ 0.4 (2) ^c	7.0 $\pm$ 1.4 (4)	12 $\pm$ 3 (2)	15 $\pm$ 2 (4)
15	11.5 $\pm$ 0.4 (2)	8.7 $\pm$ 1.6 (2)	32 $\pm$ 3 (2)	15 $\pm$ 2 (2)
45	9.8 $\pm$ 2.7 (2)	7.1 $\pm$ 1.2 (2)	40 $\pm$ 6 (2)	11 $\pm$ 5 (2)
75	14.5 $\pm$ 0.5 (2)	8.7 $\pm$ 1.0 (2)	45 $\pm$ 7 (2)	20 $\pm$ 2 (2)
150	11.9 $\pm$ 2.9 (11)	6.3 $\pm$ 1.1 (7)	52 $\pm$ 18 (15)	11 $\pm$ 2 (8)

^a1 unit = 1 pmole [^{125}I] per mg protein.

^bIn vitro experiment.

^cNumbers in parenthesis represent the number of experiments.

TABLE IV. COMPETITION OF ANALOGUES WITH TRIIODOTHYRONINE
FOR BINDING TO LIVER NUCLEI^a

Hormonal Analogue	Slope	Relative Metamorphic Potency
3',3,5-Triiodothyropropionic acid ^b	-1.0 ^c (6) ^d	30,000
3',5',3,5-Tetraiodothyroacetic acid	-0.8 (6)	500-1000
3',3,5-Triiodothyronine	-1.0 (13)	500
Thyroxine	-0.7 (9)	100
3,5-Diiodothyronine	-0.4 (8)	40
3,5-Diiodotyrosine	-0.2 (8)	0

^aAll assays were performed 150 min after injection of tadpoles.

^bAll compounds are the l-enantiomorph.

^cThe slope is derived from a plot of log{specific DI (nuclei)}
vs log{total hormone injected}.

^dNumbers in parenthessse indicate the number of data used to derive the
least squares straight line.

Fig. 1.

The effect of various doses of [^{125}I] thyroid hormones on the accumulation of radioactivity in tadpole hepatic tissue 150 min after intraperitoneal injection. Aliquots of [^{125}I] thyroid hormones with or without unlabeled hormone were injected intraperitoneally into each of eight tadpoles. After the tadpoles had been maintained at 25° C for 150 min, the livers were excised, weighed, homogenized in 10 vol of homogenization buffer, and filtered through three layers of gauze. Quadruplicate aliquots (50 λ) of the crude filtrate were assayed for radioactivity. Numbers in parentheses indicate the number of experiments. The [^{125}I] hormones were: (a) thyroxine and (b) triiodothyronine.

Fig. 2.

The effect of the level of [125 I] thyroxine in liver on the accumulation of radioactivity in cell nuclei 150 min after intraperitoneal injection.

x, total radioactivity in the cell nuclei; —, nonspecifically bound radioactivity; o, difference between total and nonspecific radioactivity; --o--o--, difference between total and non-specific radioactivity.

Fig. 3.

The effect of the level of [^{125}I] triiodothyronine in liver on the accumulation of radioactivity in cell nuclei 150 min after intraperitoneal injection. x, total radioactivity in the cell nuclei; —, nonspecifically bound radioactivity; o, difference between total and nonspecific radioactivity; ---o---o---, difference between total and non-specific radioactivity.

Fig. 4.

The effect of injection dose of [^{125}I] thyroid hormones upon the accumulation of specifically bound radioactivity in liver nuclei. Each set of experiments was normalized such that the $\text{DI}(\text{nuclei}) = 100.0$ for an injection dose of 11.7 pmole/tadpole for thyroxine and 6.45 pmole/tadpole for triiodothyronine. Numbers in parentheses indicate the number of experiments. The [^{125}I] thyroid hormones were (a) [^{125}I] thyroxine and (b) [^{125}I] triiodothyronine.