Temperature-Dependent Intracellular Distribution of Thyroxine in Amphibian Liver

(isotope injection/cell fractionation/accumulation in nuclei/thyroxine transport/chromatin-bound)

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ABSTRACT Rana catesbeiana tadpoles were injected with [14C]thyroxine, and the subcellular distribution of the labeled hormone was determined. At 25° the amount of isotope found in the liver was maximal after 1-2 hr and then rapidly decreased to a relatively constant value. A large percentage of the hormone was found associated with the purified nuclei isolated 24 hr after injection of [14C]thyroxine. Injection of [14C]thyroxine into tadpoles maintained at 5° resulted in a much slower but constant accumulation of isotope in the liver, with virtually no movement of thyroxine into the cell nucleus. Thyroxine was bound very tightly to the chromatin fraction of the nucleus, but extraction and chromatography revealed no chemical modification of the thyroxine itself. These results suggest the presence of two temperature-dependent processes: one concerned with the transport of thyroxine into the liver cell and a second concerned with the transport of the intracellular thyroxine into the cell nucleus. It is proposed that the latter process is involved in the low-temperature inhibition of thyroxine-induced metamorphosis.

The consequences of the action of thyroxine in amphibian liver tissue are similar to those of the action of steroid hormones on their respective target tissues in that RNA polymerase activity is increased, the RNA product is altered both qualitatively and quantitatively, and eventually de novo protein synthesis results (1-4). The action of steroid hormones on their target tissues involves intracellular receptor proteins that strongly and specifically bind the hormone and transport it into the nucleus, where the hormone-receptor complex interacts with components of the chromatin (5, 6). For rat uterus treated with estrogen this transport to the nucleus appears to be a temperature-dependent process (5, 6). In contrast, very little is known about the intracellular transport of thyroxine or the ultimate subcellular target of the hormone in amphibians.

The induction of amphibian metamorphosis by exogenous thyroxine exhibits a very pronounced low-temperature inhibition (5, 7). Tadpoles treated with thyroxine and maintained for months at 5° will show no response to the hormone (2). If at any time during this period the water temperature is raised to 25°, a response is apparent with a very short lag phase (2). The mechanism involved in this temperature sensitivity and the nature of its reversibility are unknown. The change from 25 to 5° has little effect on total protein and RNA synthesis in the tadpole (8). The experiments on Rana catesbeiana liver reported here indicate that a hormone-transport process may be involved in this temperature sensitivity, and suggest that the initiation of metamorphosis is dependent on the movement of thyroid hormone into the cell nucleus.

MATERIALS AND METHODS

Tadpoles of Taylor-Kollros stages XII-XIV (9) were divided into two groups, one of which was maintained for 24 hr at 25° and the other at 5°. After the animals were maintained at the respective temperatures, each tadpole was injected with labeled thyroxine (New England Nuclear, DL-[2-14C]thyroxine 18.1 Ci/mol), and each group was maintained at the respective temperature for a defined time period. The livers were then removed, pooled, homogenized, and separated into subcellular fractions by differential centrifugation. The nuclear fraction was further purified by centrifugation at 50,000 X g in 2.4 M sucrose as described (10). The distribution of the radioactivity was determined by dissolving 10 μl of each fraction in NCS solution (Nuclear-Chicago Tissue Solubilizer, Nuclear Chicago Corp.) and toluene scintillation fluid. The radioactivity of these samples was then determined in a Packard Scintillation Spectrometer.

Liver nuclei from animals injected with [14C]thyroxine were extracted with 0.3 M KOH, and aliquots were subjected to descending chromatography on Whatman 3MM paper. The liquid phase was butanol–ethanol–0.3 M NaOH 5:1:2 or butanol–acetic acid–water 4:1:1.

Thyroxine bound to macromolecules was separated from free hormone by both polyacrylamide columns and a charcoal technique. BioGel P30, 50–150 mesh, was swollen overnight at 5° in Buffer A (50 mM Tris·HCl-25 mM KCl-5 mM MgCl₂, pH 7.5, to which 1/5 volume of glycerol was added). A fraction (0.2 ml) of the cytosol was placed on the 1.2 × 15 cm column and eluted with Buffer A. Water (0.5 ml) was added to each fraction, and the absorbance of each diluted fraction was measured at 280 nm. The radioactivity of the diluted fractions was assayed in Bray's solution (11).

In the charcoal binding technique, an equal volume of a charcoal–dextran solution, 100 mg washed Norit/ml H₂O–10% Rheodox 10:1, was added to an aliquot of the cytosol (12). The mixture, in conical centrifuge tubes, was agitated vigorously on a Vortex mixer, maintained at 0° for 10 min, and centrifuged for 10 min at 600 X g in an International Centrifuge. An aliquot of the supernatant was assayed for radioactivity in Bray's solution.

RESULTS

Fig. 1 illustrates the uptake of labeled thyroxine into liver at the various time periods after injection. It is apparent that at 25° the uptake is rapid with a gradual leveling off, while at 5° the isotope is taken into the liver more slowly, but the concentration gradually rises. After 48 hr the concentration of
[14C]-thyroxine in the liver of tadpoles at 5° exceeds that of the tadpoles at 25°. Yamamoto et al. (13) obtained similar results when determining the uptake of thyroxine or triiodothyronine into tissues of intact tadpoles that were immersed in a solution containing the labeled hormone.

Fig. 2 illustrates the intracellular distribution of the labeled thyroxine. At 25° the hormone is rapidly transported into the nucleus. At 5° there is no accumulation of hormone in the nuclei, and the radioactivity is found associated with the microsomal, mitochondrial, and cytosol fractions of the cell. It is apparent that at 5° the movement of thyroxine into the nucleus is inhibited.

Fig. 2 also illustrates that at 5° the concentration of isotope associated with nonnuclear elements of the cell gradually increases. At 25° the total amount of thyroxine found associated with these fractions steadily decreases. It again appears that thyroxine is moving through the cell and into the nucleus at 25°, but that at 5° transport has been interrupted. In the time periods studied, a large percentage of the total liver [14C]-thyroxine is found in the nucleus. Fig. 3 illustrates that in this particular experiment between 45 and 55% of the thyroxine present in the original homogenate is in the nucleus, while at 5° this value is less than 5%. In some experiments, more than 80% of the radioactivity in the liver could be recovered in the nuclear fraction from tadpoles kept at 25°.

Temperature inhibition of the accumulation of thyroxine in the nucleus is reversible, as shown in Fig. 4A. Tadpoles that had been injected with isotope and maintained at 5° were placed in water at 25°. After 24 hr at 25°, 85% of the radioactivity was found in the purified nuclei, while no transport took place if the tadpoles were maintained at 5°.

In order to demonstrate that the temperature-dependent uptake of hormone from the intraperitoneal cavity is a process that is distinct from the temperature-dependent movement of intracellular thyroxine into the nucleus, tadpoles were injected with [14C]-thyroxine at 25° and maintained at this temperature for short time periods and then maintained at 5°. The results, shown in Fig. 4B, reveal that even though the total thyroxine in the liver had reached its maximum concentration, the movement of thyroxine into the nucleus is a separate temperature-dependent process and could still be inhibited if incubation at 5° followed the pulse of [14C]-thyroxine in the liver.

The [14C]-thyroxine is tightly bound in the nuclei. If unlabeled nuclei are suspended in a solution of dL-[14C]-thyroxine in vitro, some radioactivity is found in the nuclei after they are removed from the solution by centrifugation. This radioactivity is diffusible in that much of the [14C]-thyroxine (70-80%) can be washed out if the nuclei are washed with a buffer solution. However, if the [14C]-thyroxine is injected into vivo the radioactive label in the recovered purified nuclei of the liver is not freely diffusible and is tightly bound to the chromatin.
fractions. More than 90% of the original radioactivity can be recovered in the chromatin fraction after these nuclei are washed in buffer, broken in EDTA, and washed in 0.3 M KCl. We investigated the possibility that the thyroxine is bound to the outer nuclear membrane by washing nuclei labeled in vivo in Buffer A that contained 0.05% Triton-X-100 (Sigma Chemical Co.). This detergent failed to solubilize any of the bound thyroxine.

In order to determine if any chemical modification of the injected thyroxine had occurred, we extracted the radioactivity from the various fractions and chromatographed the samples on paper. It was found that more than 95% of the radioactivity could be extracted from all fractions in 0.3 M KOH. The extracts were prepared for paper chromatography after neutralization with HClO₄, centrifugation, and application on Whatman 3MM paper according to Dratman et al. (14). The results in Table 1 show that most of the labeled material has an Rf value similar to the standard unlabeled and radioactive thyroxine. It thus appears that no extensive modification of the injected thyroxine has taken place.

In order to determine the amount of thyroxine bound to macromolecules in the cytosol, we used a polyacrylamide column and a charcoal technique. The first peak, which was eluted from the column at its void volume and contained most of the material absorbing at 280 nm, was macromolecule-bound thyroxine. The second peak was eluted at or after the elution volume of the column and corresponded to free thyroxine. Because the charcoal binding assay was considerably easier to perform and showed the same amount to be bound as the column assay, the charcoal binding assay was used routinely for the determination of bound and unbound thyroxine.

Cytosols of liver from tadpoles injected with [14C]thyroxine were analyzed for macromolecule-bound thyroxine (Fig. 5). The data show that the amount of [14C]thyroxine bound to macromolecules at either 5 or 25° in the cytosol parallels the total amount of thyroxine present in the cytosol.

We have further explored the movement of thyroxine into nuclei in vitro. After nuclei from untreated tadpoles were incubated at 25° for 1 hr with the cytosol from tadpoles that had been injected previously with [14C]thyroxine, the nuclei

![Table 1. Descending chromatography of thyroxine](image)
were washed twice and assayed for radioactivity. It was found that the amount of \(^{14}C\)thyroxine that was bound tightly to the nuclei (5-15% of the \(^{14}C\)thyroxine in the cytosol) was not linearly related to the amount of cytosol. Furthermore, a 300-fold excess of unlabeled exogenous \(L\)-thyroxine added to the incubation mixture did not decrease the amount of \(^{14}C\)thyroxine that was bound to the nuclei.

**DISCUSSION**

The initial (0.25-2 hr) large pulse of radioactivity found in the liver of tadpoles maintained at 25° reflects the rapid uptake of hormone from the intraperitoneal cavity and transport into the liver. Several observations suggest that most of the hormone during this time period is intracellular. First, if the livers are homogenized in 10 mM unlabeled thyroxine, there is no alteration of the subcellular distribution of radioactivity; this would be expected to occur if the \(^{14}C\)thyroxine were extracellular and became associated with the subcellular fractions after tissue disruption. Williams and Gorski (15), doing similar studies, showed that if rat uteri were homogenized in a medium combining a large excess of unlabeled estrogens, there was reduction in the amount of radioactivity that was actually bound to cytosol components but there was no change in the total amount of \(^{3}H\)estrogen in the nuclear fraction.

The second observation supporting the early intracellular distribution of thyroxine is that if tadpoles are injected with \(^{14}C\)thyroxine at 25° and kept at that temperature for 1-2 hr, to achieve maximum incorporation of isotope into the liver, and then cooled to 5° for 16 hr, the initially high concentration of thyroxine in the liver is maintained. Finally, if the amount of protein-bound thyroxine in the cytosol is determined by the charcoal–dextran method, most of the thyroxine in the liver during this time period is not bound to protein. If the same determination is performed at longer time intervals after injection (3-72 hr), the percentage of macromolecular-bound thyroxine is much higher and approaches 50% of total thyroxine. It is possible that this initial pulse of thyroxine saturates existing cytosol binding sites, and the excess hormone is quickly catabolized or removed from the cell. There is no direct evidence for specific thyroxine receptor proteins in amphibian liver cytosol. However, macromolecule-thyroxine binding has been observed for liver cytosols in the present study. While the binding sites for thyroxine in nuclei are saturable, a large excess of exogenous unlabeled thyroxine had no effect on the in vitro transport of \(^{14}C\)thyroxine from cytosol (labeled in vivo by injection) into nuclei. Therefore, we must conclude that at least some of the binding of cytosol thyroxine to nuclei involves macromolecule-bound thyroxine.

The results of this preliminary study suggest that the underlying mechanism responsible for the low-temperature inhibition of thyroxine-induced metamorphosis is the failure of operation of a temperature-sensitive system for transport of thyroxine into the nucleus. The large and rapid accumulation of unaltered thyroxine in the cell nucleus of tadpole liver at 25° implicates the nucleus as the major, if not the exclusive, target site for the action of this hormone in this organ.

These and other results also suggest some parallels in the mechanism of thyroxine induction of metamorphosis and the action of estrogens in rat uterus (for a review, see ref 5). The ultimate effect of both hormones on their respective target tissues is an alteration of the activity of RNA polymerase and transcription, as a result of which there is increased protein synthesis and \(de novo\) induction of protein synthesis (5, 7, 10, 16). The similarity also extends to the temperature-dependent transport of hormone molecules into the cell nucleus (6, 16). It is thus possible that the reported estrogen receptor proteins have their counterparts in thyroxine-binding receptors in amphibian liver. Tata (17) has reported that the simultaneous binding of thyroid hormone and acquisition of metamorphic competence in *Xenopus* larvae is temperature-dependent. It is thus likely that the acquisition of competence involves the development of a system for transport of thyroxine into the cell nucleus.

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