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3,5,3′-Triiodo-L-thyronine and L-Thyroxine Uptake into Red Blood Cells of Rainbow Trout, *Oncorhynchus mykiss*

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Uptake of the thyroid hormones (TH) 3,5,3′-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) by trout red blood cells (RBC) was studied by incubating washed RBC in a balanced salts medium containing glucose and [^{125}I]TH at the fish acclimation temperature of 12°. RBC were separated from the medium by centrifugation through silicone oil and glycine buffer (pH 10.5). Maximal [^{125}I]T_3 uptake occurred by 10–15 min, but not by 60 min for [^{125}I]T_4. First-order uptake was measured at 30 sec for T_3 and at 90 sec for T_4. Total T_4 uptake was enhanced ≈15-fold from pH 8 to 6 and was affected most below pH 7.2; total T_3 uptake was maximal between pH 6.4 and 7.0, but was relatively insensitive to pH. At 0.2 n*M,* nonsaturable uptake of T_3 exceeded that of T_4 3- to 6-fold, accounting for 3% (T_3) and 50% (T_4) of total uptake. Saturable TH uptake was described by Michaelis–Menten kinetics. The saturable transport system for T_3 had an apparent K_t (carrier affinity) of 70–119 n*M* and J_{max} (maximal uptake velocity) of 540–1116 pmol · 10^6 cells^{-1} · min^{-1}. A saturable system was also found for T_4, with an apparent K_t of 99 p*M*–1.1 n*M* and J_{max} of 8–77 fmol · 10^6 cells^{-1} · min^{-1}. Saturable uptake of both TH depended on temperature. Activation energies for the nonsaturable component were 48 (T_4) and 64 (T_3) *KJ* · mol^{-1} over the range 0–21°. Activation energies for the saturable components were 52 *KJ* · mol^{-1} (T_4, 0–21°), 52 *KJ* · mol^{-1} (T_3, 0–10°), and 3 *KJ* · mol^{-1} (T_3, 10–21°). During a 16-month study saturable and nonsaturable uptake of both TH increased, probably due to fish age. We conclude that in trout RBC, rapid T_3 uptake by a pH- and temperature-sensitive saturable carrier greatly exceeds T_4 uptake. The rate of T_3 uptake exceeds by 100- to 1000-fold that of mammals and amphibia, and in contrast to those taxa some saturable T_4 uptake also occurs.

L-thyroxine (T_4) appears to be the primary form of thyroid hormone (TH) secreted by the thyroid gland of rainbow trout, *Oncorhynchus mykiss.* It may be converted subsequently in extrathyroidal tissues to the more physiologically active form, 3,5,3′-triiodo-L-thyronine (T_3) (Eales and Brown, 1993). Both T_3 and T_4 are present in approximately equal concentrations in rainbow trout plasma, reversibly bound to plasma proteins or as free hormone. Less than 1% of the total plasma TH is present as the free form, with a lower proportion of free T_3 than free T_4 (Eales and Shostak, 1985). Since it is the free TH which is believed to exchange between the plasma and the tissues, the properties of the plasma TH-binding proteins exert a major influence on the flux of TH across capillaries. However, TH may also enter the red blood cell (RBC), and this TH pool also has the potential to exchange with and influence the free TH concentration in plasma.

RBC-associated T_3 represents 23% of the total blood T_3 in rat, and the concentration of T_3 inside the RBC is 25 times the external free T_3 concentration (Francon *et al.,* 1990). Human, rat, and frog RBC take up T_4 solely by a nonsaturable mechanism, but possess a saturable, carrier-mediated transport mechanism for T_3 (Docter *et al.,* 1982; Galton *et al.,* 1986; Osty *et al.,* 1988; Zhou *et al.,* 1990). Uptake of T_3 into the RBC is therefore significant, and it has the

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potential to be regulated in these species. T_3 and T_4 are taken up by RBC of some fish (Leloup and Fontaine, 1960). However, the mechanism and characteristics of this uptake have not previously been investigated.

We have examined T_3 and T_4 uptakes into RBC of rainbow trout and, for both TH, observed concentrative, saturable, temperature-sensitive, and pH-sensitive carrier-mediated transport systems, the capacities of which increased with age.

METHODS

Fish. Yearling rainbow trout were obtained from the Rockwood Experimental Hatchery (Balmoral, Manitoba) and held in 2300-liter fiberglass tanks for 1 to 3 years. They were maintained on a 12-hr light:12-hr dark photoperiod at 12° and fed a commercial diet (Martin Feed Mills, Elmira, Ontario; trout feed pellets, 3.2-mm diameter) at a ration of 1% body weight once daily. Trout were weighed during September to December 1994 and ranged between 450 and 2200 g, with the majority between 800 and 1000 g. Individual fish were sampled repeatedly throughout the study, but never more frequently than once in 2 weeks, and usually at much longer intervals. Fish were bled between 0800 and 0900 hr, between 20 and 22 hr after feeding.

RBC preparation. Fish were netted and anesthetized in unbuffered MS222 (tricaine methanesulfonate, 0.07 g/liter; Syndel Labs, Vancouver, BC). Blood, usually 3 ml, 2–11% of estimated blood volume (Sefkow *et al.,* 1996), was removed from caudal vessels into a sodium heparinized syringe, and then was held on ice. Fish were returned to the holding tank after weighing.

Blood was transferred into plastic centrifuge tubes and centrifuged at 127*g* for 5 min at 4°. Plasma and the buffy layer containing white cells were removed and 8 to 10 ml of suspension buffer was added. Suspension buffer, which was also used to measure uptake, consisted of 124 m*M* NaCl, 3.4 m*M* KCl, 0.9 m*M* $CaCl_2$, 0.9 m*M* $MgSO_4$, 2.56 m*M* NaH_2PO_4, and 10 m*M* Hepes. This was gassed with 99% O_2:1% CO_2 for 30 min on the morning of the experiment. Glucose, Na^+–pyruvate, and $NaHCO_3$ were added to this solution to give final concentrations of 2.8, 4.0, and 30 m*M*, respectively. The pH was adjusted to 7.5 and then 0.1 mg/ml ammonium heparin was added.

The RBC were resuspended by inversion of the tube and recentrifuged. The cell suspension was washed (8–10 ml) twice more to remove all plasma proteins. After the final centrifugation, cells were resuspended in 8 or 9 ml of suspension buffer, counted on a hemacytometer, and tested for viability by trypan blue (0.4% suspension; Sigma Chemical Company, St. Louis, MO) exclusion. Cells were maintained on ice until just prior to incubation. Cells were used on the day they were collected, and, unless otherwise stated, the experiment was completed within 8 hr of the final cell resuspension.

Hormone uptake by RBC. Uptake assays were modified from Riley and Eales (1993) and were similar for both T_3 and T_4. Unless otherwise stated, all assays were performed at 12°. Flasks and test tubes were siliconized prior to use (Sigmacote; Sigma).

Assays were performed in 50-ml flasks, which were gassed with 99% O_2:1% CO_2 and shaken at 150 rpm. The final incubate consisted of 0.5 ml of cell suspension, [^{125}I]TH (75–120 nCi for T_3, 195–235 nCi for T_4) combined with sufficient radioinert TH to give the appropriate final TH concentration, and sufficient suspension buffer to give a final volume of 6 ml. Incubation was begun by addition of hormone. However, to facilitate instantaneous sampling for the zero time point for T_4, incubation was begun by addition of cells.

After incubation, 500 μl was removed and added to 250 μl of ice-cold ''stop'' solution (suspension buffer containing 150 μM appropriate TH). An aliquot of 600 μl was removed and added to a microcentrifuge tube which had been prepared according to Riley and Eales (1993), containing 150 μl of alkaline glycine buffer plus 50 μM hormone and 350 μl silicone oil. Transfer to the microcentrifuge tube was done as rapidly and consistently as possible, since a small amount of TH uptake continued even after addition of the incubation mixture to the stop solution. Cells were not floated on the upper silicone oil layer, as in Riley and Eales (1993), because this extended the time that cells were in stop solution. The 600-μl aliquot of cells was pipetted directly into the oil to remove external TH and centrifuged immediately (17,000*g,* for 5 min at room temperature). Subsequent washing and counting procedures followed those of Riley and Eales (1993).

Uptake analysis. Data analysis was based on the equation

$$J = \frac{J_{max} \cdot [TH]}{K_t + [TH]} + K_n[TH],$$

where J is the total uptake, J_{max} is the maximal uptake velocity of the saturable system, K_t is the Michaelis constant, K_n is the nonsaturable uptake constant, and [TH] is the concentration of TH.

Saturable uptake was determined by subtracting nonsaturable uptake from total uptake at each concentration of TH. The J_{max} and apparent K_t were calculated by linear regression of the means of the Eadie–Hofstee plots of saturable transport.

Nonsaturable uptake was calculated by extrapolating from the origin to a single high TH concentration (10 μM). The slope of the line (shown to be linear by prior investigation of intermediate TH concentrations) provided an estimate of K_n.

HPLC. Three fish were sampled to assess possible metabolism of T_3 and T_4 by RBC. HPLC radioiodine profiles of cell pellet and supernatant were obtained after 2- and 30-min incubations with [^{125}I]T_3 or [^{125}I]T_4. To the final washed cell pellet, 500 μl of ethanol containing 1 mM methylmercaptoimidazole (MMI) were added and mixed. A volume of 300 μl of initial supernatant was mixed with 300 μl of ethanol/MMI. Cell pellet and supernatant extracts were evaporated to dryness and then reconstituted in HPLC solvent. Samples of 200 μl were injected onto an Econosil column [Alltech 60146 (Alltech Associates, Inc., Deerfield, IL), 250 × 4.6 mm, C_{18}, 10 μm, 30°] employing an isocratic solvent system (40% acetonitrile:58% water containing 0.1% trifluoroacetic acid) run at 1 ml/min. The HPLC system was as described by Sweeting and Eales (1992).

ATP measurement. The concentration of ATP in RBC was measured by the method of Adams (1963), using a kit from Sigma (Procedure 366-UV).

Statistics. The main effects were evaluated by the General Linear Model (SAS; SAS Institute, Inc., Cary, NC). When significant main effects were obtained, Tukey's test was used to establish where statistical differences occurred between means ($P < 0.05$). Differences between kinetic parameters on different dates were evaluated using t tests or, when distributions were found to depart from normality, the Wilcoxon Rank Sum test (SAS) was used.

RESULTS

RBC Viability

RBC viability as assessed by trypan blue exclusion was usually 100% and never below 97%. Cells held at 12° for over 1 hr showed some agglutination, although cell viability by trypan blue exclusion was not diminished. Cells held on ice until just prior to use showed no hemolysis or agglutination.

RBC were prepared with or without glucose in the suspension buffer, held on ice, and assayed 5 hr later (Day 1) at 12°. There were no significant effects of glucose deprivation on cell viability, RBC total uptake of TH (0.2 nM), or RBC ATP content (data not shown). The same cell suspensions were then held at 4°, and after 24 hr the RBC were resuspended in fresh glucose-free suspension buffer and reassayed (Day 2) at 12°. Again there were no effects of glucose deprivation on any measured parameter. Furthermore, values between Day 1 and Day 2 did not differ significantly. Overall means (±SEM) were 1.8 ± 0.09 pmol · 10^6 cells^{-1} · min^{-1} for total T_3 uptake, 85 ± 7 fmol · 10^6 cells^{-1} · min^{-1} for total T_4 uptake, and 0.7 ± 0.05 μmol · 10^6 cells^{-1} for ATP concentration, and the mean cell number was $7.5 \times 10^5 \pm 6.6 \times 10^4$ cells · ml^{-1}.

Cell Concentration

The concentration of assayed cells ranged from 3.0×10^5 to 1.5×10^6 cells · ml^{-1}. Dilutions of cells to cover this range showed no significant effect of cell concentration on T_3 and T_4 uptake (data not shown).

HPLC Analyses

HPLC profiles of RBC and supernatant were obtained after 2- and 30-min incubations. Aside from a small radioiodide peak due to radioiodide contamination of labeled T_3 or T_4, the only radioiodine peak detected at 2 or 30 min in either cell or supernatant fractions corresponded to the administered TH (data not shown).

Time Course of TH Uptake

T_3 uptake at 0.2 nM reached an equilibrium value of 781 fmol · 10^6 cells^{-1} between 10 and 15 min (Fig. 1). Initial uptake was approximately linear for at least 45 sec; an incubation time of 30 sec was chosen for subsequent studies. T_3 uptake extrapolated close to the origin, indicating negligible instantaneous adsorption to the RBC membrane.

T_4 uptake at 0.2 nM was slower than T_3 uptake and had not attained equilibrium at 60 min (uptake at 60

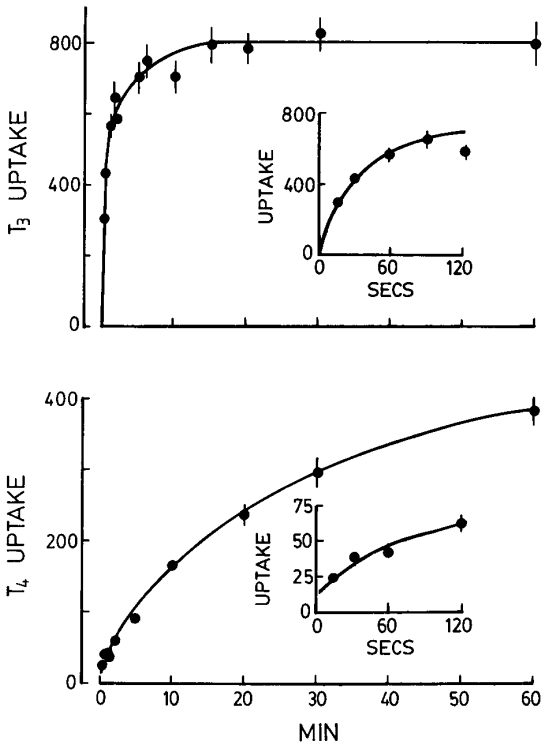

FIG. 1. Long and short (inset) time courses for the uptake at 12° of 0.2 n*M* T_3 and T_4 into rainbow trout RBC. Each point represents the mean (±SEM where symbol size is exceeded) of 4–5 determinations for T_3 and 6–17 determinations for T_4. Units of uptake are fmol · 10^6 cells^{-1} · min^{-1}. Experiments were conducted in September (T_4) and October (T_3) 1993.

min, 382 ± 48 fmol · 10^6 cells^{-1}) (Fig. 1). Initial uptake was approximately linear for at least 5 min; an incubation time of 90 sec was chosen for subsequent studies. T_4 uptake did not extrapolate through the origin, indicating some instantaneous adsorption of T_4 to the RBC membrane. Therefore zero-time values were measured in all subsequent T_4 assays and subtracted from a 90-sec value to obtain the true uptake.

Uptake Kinetics

Saturability of TH transport was demonstrated by measuring uptake of [^{125}I]TH in the presence of excess unlabeled TH (500 n*M*). The initial velocity of T_3 uptake was inhibited 76% (from 30.9 ± 2.6 to 7.1 ± 1.0 fmol · 10^6 cells^{-1} · min^{-1}) by excess T_3, and that of T_4 was inhibited 61% (from 3.0 ± 0.07 to 1.2 ± 0.11 fmol · 10^6 cells^{-1} · min^{-1}) by excess T_4, indicating saturable uptake components for both TH. At 0.2 n*M*, the TH concentration used for most of the assays, nonsaturable uptake accounted for approximately 50% of total T_4 uptake, but only 3% of total T_3 uptake.

Saturable uptake conformed to Michaelis–Menten kinetics. Kinetic parameters for T_3 were measured in November 1993, February 1994, and September 1995 (Fig. 2) and for T_4 in October 1993 (Fig. 3) and February 1994. For T_3, the apparent K_t was higher in November than in February or September, while the J_{max} was higher in

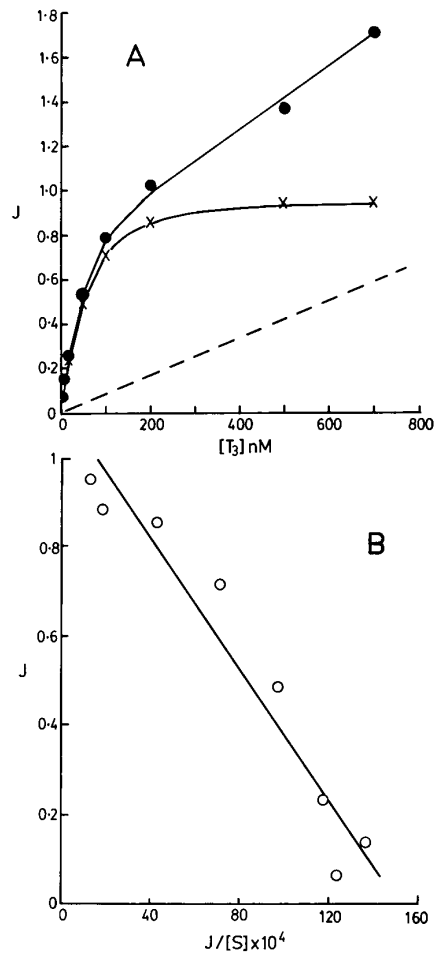

FIG. 2. (A) The effect of T_3 concentration on T_3 instantaneous uptake into trout RBC at 12°, measured in September 1995. Saturable uptake (×) was calculated by subtracting nonsaturable uptake (– –) from total uptake (●) at each concentration of T_3. Each point represents the mean of seven determinations. Units of uptake are nmol · 10^6 cells^{-1} · min^{-1}. (B) Eadie–Hofstee plots of saturable uptake involved plotting J (nmol · 10^6 cells^{-1} · min^{-1}) ainst J/S (fmol · 10^6 cells^{-1} · min^{-1} · fM^{-1}). Slopes and intercepts were estimated by linear regression.

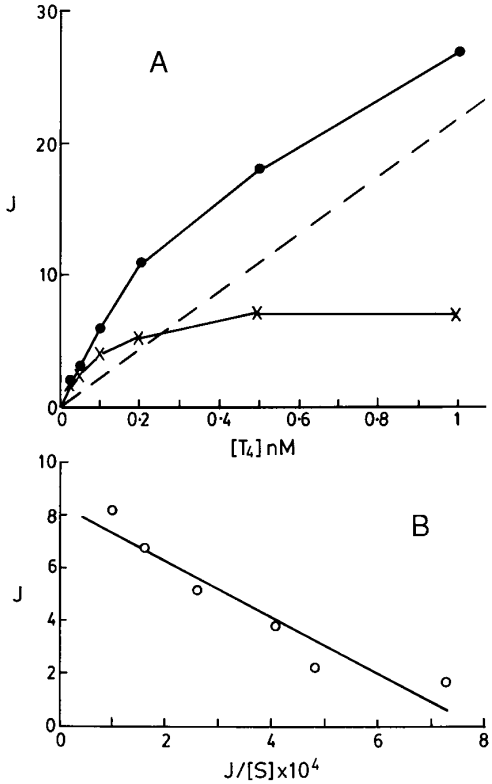

FIG. 3. (A) The effect of T_4 concentration on T_4 instantaneous uptake into trout RBC at 12°, measured in October 1993. Saturable uptake (×) was calculated by subtracting nonsaturable uptake (– –) from total uptake (●) at each concentration of T_4. Each point represents the mean of five determinations. Units of uptake are fmol · 10^6 cells^{-1} · min^{-1}. (B) Eadie–Hofstee plots of saturable uptake involved plotting J (fmol · 10^6 cells^{-1} · min^{-1}) against J/S (fmol · 10^6 cells^{-1} · min^{-1} · fM^{-1}). Slopes and intercepts were estimated by linear regression.

September than in November or February (Table 1). For T_4, values for both parameters were significantly greater in February than in November.

pH

Total uptake of T_3 and T_4 was measured between pH 6.1 and 8.1 at 12° (Fig. 4). Uptake of T_4 decreased as pH increased, with the major decrease between 6.4 and 7.2. No maximum was established because of marked hemolysis below pH 6.0. The optimum pH for total T_3 uptake lay between 6.4 and 7.0. T_3 uptake decreased significantly from pH 6.4 to 8.1. The degree of ionization of the 4′-hydroxyl (4′–OH) group of T_4 (pK_a = 6.7) and T_3 (pK_a = 8.45) may influence the TH interaction with transport proteins and account for the pH effect observed. An approximately linear relationship was found between T_4 uptake and the calculated percentage of hormone in the 4′–OH undissociated (electroneutral) form (Fig. 4, inset). T_3 uptake increased curvilinearly with the percentage of T_3 in the undissociated form to the optimum of pH 6.4.

Temperature

Rising incubation temperature increased saturable and nonsaturable uptake of both TH (Fig. 5). The Arrhenius plot for saturable T_3 uptake was inflected at 9.7°, but the plot for saturable T_4 uptake was linear over the range 0–21° (Fig. 6). Apparent activation energies ($KJ \cdot mol^{-1}$) were calculated as follows: saturable T_3 from 0 to 9.7°, 51.7; saturable T_3 from 9.7 to 21°, 3.2; nonsaturable T_3 from 0 to 21°, 64.0; saturable T_4, 52.2; and nonsaturable T_4, 48.3.

Temporal Variations

Total uptake of T_3 and T_4 increased gradually and significantly from September/October 1993 to December 1994 (Fig. 7). No relationship between fish weight and TH uptake was found in the period for which fish weights were available, September to December 1994 (data not shown). Based on analyses at designated times, changes occurred in both the saturable and the nonsaturable components (Fig. 8). Since repeated sampling and handling of the fish might be responsible for the increase, we measured TH uptake for a sample of five ''virgin'' fish (mean weight, 230 g) held under the same conditions but not previously bled. These values were compared with those of experimental fish, bled within the 2 weeks prior to (group 1) or 2 weeks after (group 2) the virgin fish. The K_n (mean ± SE) for group 1, virgin, and group 2 fish were, respectively, for T_4 71 ± 14, 42 ± 9, and 72 ± 8 $\mu l \cdot 10^6$ cells^{-1} · min^{-1}; and for T_3 287 ± 49, 304 ± 39, and 231 ± 50 $\mu l \cdot 10^6$ cells^{-1} · min^{-1}. Saturable transport values (mean ± SE) for group 1, virgins, and group 2 were, respectively, for T_4 25 ± 6, 19 ± 2, and 18 ± 2 fmol · 10^6 cells^{-1} · min^{-1}; and for T_3 1721 ± 158, 1160 ± 200, and 1499 ± 210 fmol · 10^6 cells^{-1} · min^{-1}. No significant differences were found between virgins and experimental fish for any of these parameters.

TABLE 1
Kinetic Parameters for T_3 and T_4 Instantaneous Uptake into Rainbow Trout RBC

		Transport[a]		
		Nonsaturable		
Hormone	Date	K_n[b]	J_{max}[c]	K_t[d]
T_3	Nov	165 ± 16	591 ± 61	119 ± 10***
	Feb	268 ± 46	540 ± 49	79 ± 6
	Sep	419 ± 110	1117 ± 109**	72 ± 8
T_4	Oct	22 ± 1.4*	0.008 ± 0.0005*	0.099 ± 0.009*
	Feb	80 ± 4	0.077 ± 0.015	1.10 ± 0.030

[a] Mean ± SEM; $n = 5$ except Sep 1995 (T_3) where $n = 7$.
[b] $\mu l \cdot 10^6$ cells$^{-1} \cdot$ min^{-1}.
[c] pmol $\cdot 10^6$ cells$^{-1} \cdot$ min^{-1}.
[d] n*M*.
* Significantly different from February, $P < 0.05$.
** Significantly different from November and February, $P < 0.05$.
*** Significantly different from February and September, $P < 0.05$.

DISCUSSION

Trout RBC appeared viable for at least 29 hr after sampling. They showed no changes in trypan blue exclusion, ATP content, or total T_3 and T_4 uptake. However, to minimize pipetting problems due to cell agglutination, assays were run routinely within 8 hr, and RBC were held on ice until just prior to assay.

HPLC analyses of cell and supernatant extracts indicated no chemical conversions of either T_3 or T_4 by trout RBC over 30 min. This is consistent with the observed absence of deiodinating systems in rainbow trout RBC (MacLatchy and Eales, 1992). Thus uptake of TH into RBC was not confounded by any intracellular conversions.

Apparent affinity constants for T_3 transport were approximately 70 and 119 n*M*, corresponding closely with those for RBC of other species: 124 n*M* in humans (Docter *et al.,* 1982), 130 n*M* (Osty *et al.,* 1988) and 46 n*M* (Galton *et al.,* 1986) for rat, and 57 and 45 n*M*, respectively, for frog and tadpole (Galton *et al.,* 1986). However, our J_{max} values were considerably higher than those recorded for mammals, which ranged from a low of 3 fmol $\cdot 10^6$ cells$^{-1} \cdot$ min^{-1} for human RBC (Zhou *et al.,* 1990) to a high of 4.9 pmol $\cdot 10^6$ cells$^{-1} \cdot$ min^{-1} for rat RBC (Osty *et al.,* 1988). Values of J_{max} for both frog (286 fmol $\cdot 10^6$ cells$^{-1} \cdot$ min^{-1}) and tadpole (592 fmol $\cdot 10^6$ cells$^{-1} \cdot$ min^{-1}) RBC (Galton *et al.,* 1986) were also much lower than those for the trout, indicating that a high J_{max} is not necessarily an ectothermic feature. The T_3 J_{max} for trout RBC is also greater than that for hepatocytes of trout (Riley and Eales, 1994) and rat (Blondeau *et al.,* 1988). In general, J_{max} for T_3 transport into trout RBC is several orders of magnitude greater than that of other vertebrates.

We identified a saturable T_4 transporting system in trout RBC, with an apparent K_t ranging from 99 p*M* to 1.1 n*M* and a J_{max} ranging from 8 to 77 fmol $\cdot 10^6$ cells$^{-1} \cdot$ min^{-1}. These T_4-transporting sites had a higher affinity and lower capacity than transporting sites for T_3. Our results contrast with those for human, rat, and frog, in which RBC T_4 uptake is entirely nonsaturable (Galton *et al.,* 1986; Osty *et al.,* 1988).

We examined T_3 and T_4 uptake from 0 to 21°, corresponding to the temperature range that trout may experience in nature. Temperature influenced both nonsaturable and saturable uptake. Apparent activation energies for T_4 saturable uptake (0–21°) and T_3 saturable uptake (0–10°) were similar (52 *KJ* $\cdot$ mol^{-1}), and in the range usually associated with carrier-mediated active transport (LeCam and Freychet, 1977). Above 10°, a temperature corresponding approximately to the acclimation temperature of the trout, the apparent activation energy for T_3 fell to 3 *KJ* $\cdot$ mol^{-1}. The break in the slope of the Arrhenius plot probably results from lipid phase transitions, which would affect the function of the transporter in the RBC membrane (Schroeder and Sweet, 1988). The occurrence of an inflexion point for

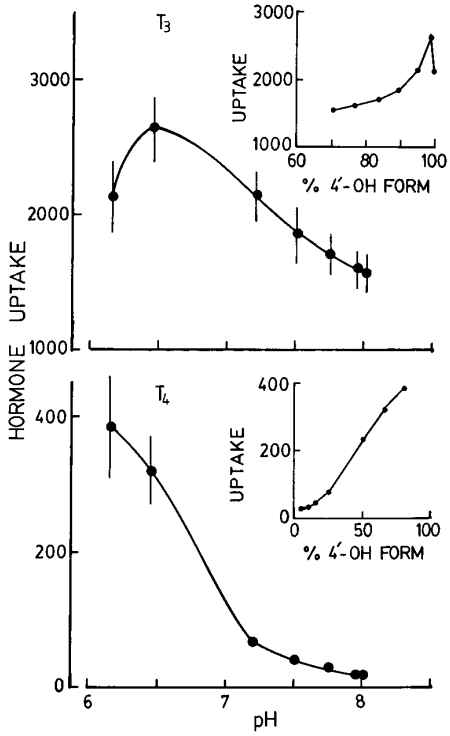

FIG. 4. Effect of pH on T_3 and T_4 instantaneous total uptake by RBCs of rainbow trout, measured at 0.2 n*M* hormone (mean ± SEM, $n = 3–9$, units of uptake are fmol · 10^6 cells^{-1} · min^{-1}). pH values are the means of measured values for each determination. The insets show uptake of TH as a function of calculated percentage of TH in the deionized form. Experiments were conducted in November 1993.

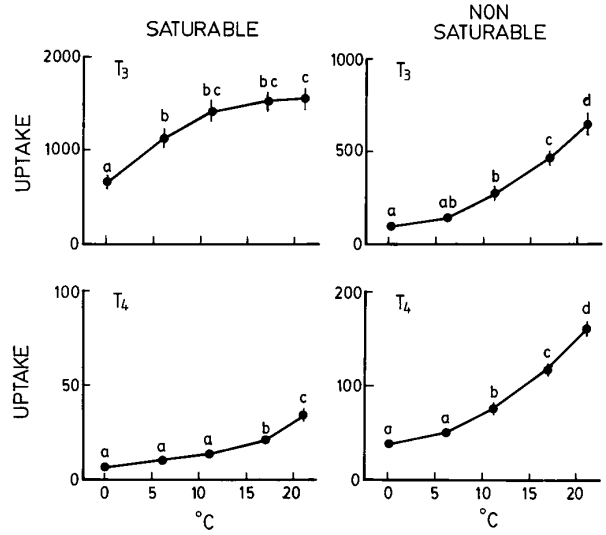

FIG. 5. Effect of assay temperature on T_3 and T_4 saturable instantaneous uptake (in fmol · 10^6 cells^{-1} · min^{-1}) and nonsaturable uptake constant (in μl · 10^6 cells^{-1} · min^{-1}) into RBCs of rainbow trout (mean ± SEM, $n = 5$). Means with the same letter are not significantly different ($P > 0.05$). Experiments were conducted in January 1994.

T_3 but not T_4 uptake suggests that the two TH do not share the same transporter.

Apparent activation energies for nonsaturable transport were 64 *KJ* · mol^{-1} for T_3 and 48 *KJ* · mol^{-1} for T_4, again suggesting carrier-mediated transport. Apparent activation energies for nonsaturable and saturable transport of various amino acids into mouse brain slices are similar to each other and within this same range, suggesting that these nonsaturable systems might represent a high capacity facilitated diffusion (Cohen, 1975).

T_3 uptake approximately halved from pH 6.4 to pH 8, but T_4 uptake was 15-fold lower at pH 8 than at pH 6. The approximate linearity between T_4 uptake and degree of ionization of the 4′−OH group suggests preferential uptake of the undissociated form. The more pronounced curvilinearity of T_3 uptake against 4′−OH ionization suggests a more complex influence of pH on transport of T_3, possibly involving the ionization state of the transport protein. T_3 transport declined only 13% from pH 7.2 to 7.5, while T_4 uptake dropped 37% over the same range. Thus T_3 uptake was affected less than T_4 uptake by pH changes in the range of plasma pH

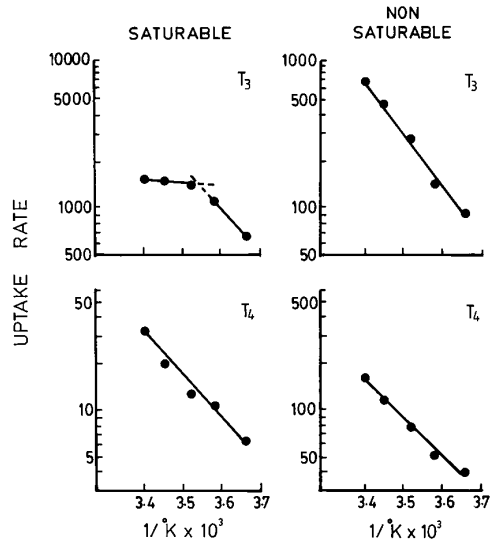

FIG. 6. Arrhenius plots of T_3 and T_4 saturable instantaneous uptake (in fmol · 10^6 cells^{-1} · min^{-1}) and nonsaturable uptake constant (in μl · 10^6 cells^{-1} · min^{-1}) of trout RBC (means, $n = 5$). Lines were fitted by linear regression of the means. °K, absolute temperature.

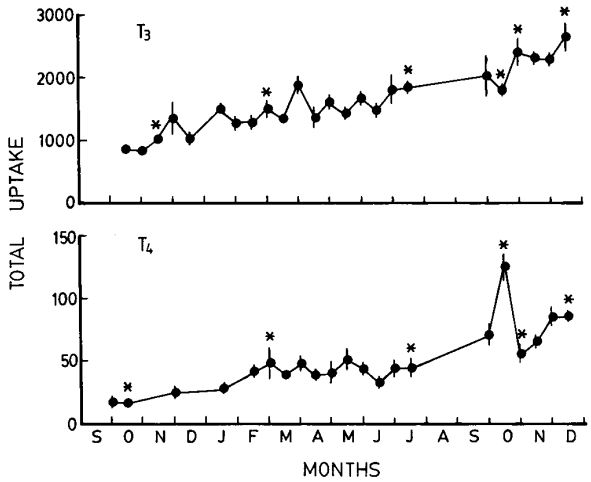

FIG. 7. Total instantaneous uptake (in fmol · 10^6 cells^{-1} · min^{-1}) at 12° of T_3 and T_4 by rainbow trout RBCs measured from September 1993 to December 1994. Mean values (±SEM; n = 3–12) were calculated semimonthly (1st to 15th, inclusive, and 16th to month end). Asterisk indicates the sampling times represented in Fig. 8.

perturbations normally tolerated by trout *in vivo*. Local pH changes in the unstirred layer of the RBC due to cellular activity may also occur. This relative stability in RBC T_3 uptake in the face of a pH challenge may permit the RBC to contribute more effectively to regulation of plasma T_3.

Both saturable and nonsaturable T_3 and T_4 uptake increased progressively from September/October of 1993 to December of 1994. Trout were fed a constant ration and held in the same water supply at 12° under a constant photoperiod, suggesting little influence of these variables. Uptake was measured over 16 months and there was no evidence of a circannual rhythm. These changes therefore suggest progressive alteration with age. Serial blood sampling could also induce a stress response at the level of the individual RBC or by modifying properties of the RBC population. However, when previously sampled trout were held for 2 months without sampling, TH uptake did not decline. Furthermore, previously unsampled (''virgin'') fish of the same brood stock and held under the same conditions did not differ in TH uptake from serially sampled fish. Nevertheless, we cannot eliminate the possibility that serial sampling stimulated erythropoiesis and increased the proportion of circulating immature RBC. Sullivan *et al.* (1987) found that 24 days after bleeding rainbow trout, hematocrit and total number of RBC had returned to control levels, but the proportion of immature RBC was elevated. The immature cells had a much higher level of putative TH receptors and thus may also have a higher TH uptake into the cell.

Our study indicates that the flux of TH from a protein-free solvent into RBC of trout exceeds that of mammals and amphibians measured under similar protein-free conditions. *In vivo* flux rates will differ from those measured *in vitro* since TH-binding plasma proteins will influence the free TH levels available for transport. However, levels of free T_3 and T_4 in salmonid blood (≈5 fmol · ml^{-1}) are comparable to those of other vertebrates (Eales and Shostak, 1985). Thus we predict that under *in vivo* conditions the flux of TH into the RBC of the trout will be greater than that for other vertebrates studied to date.

Under steady-state conditions, the net TH efflux will equal the net influx, since there is no evidence for TH metabolism within the RBC. Therefore, the RBC of trout may constitute a dynamic pool of TH in rapid exchange with plasma and important in buffering variations in

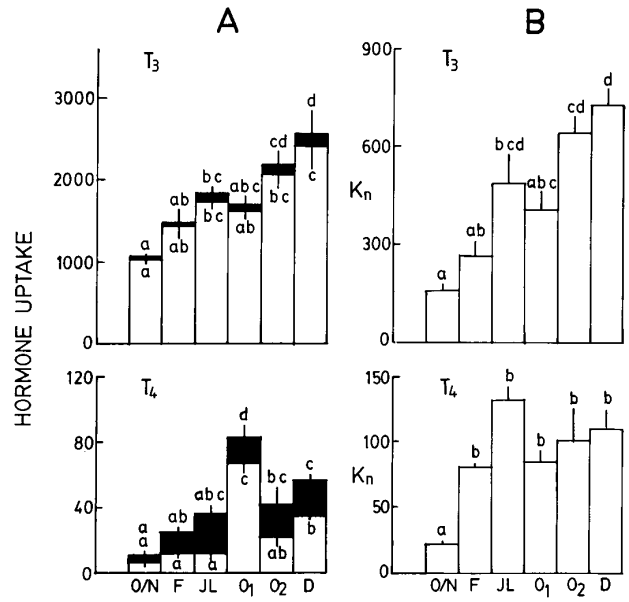

FIG. 8. T_3 and T_4 instantaneous uptake at 12° by rainbow trout RBCs at designated periods through the study [O/N, October (T_4) or November (T_3) 1993; F, February 1994; JL, July 1994; O1, October 1–15, 1994; O2, October 16–31, 1994; D, December 1994]. (A) Relative contributions (mean ± SEM, n = 5) of saturable (□) and nonsaturable (■) uptake to total uptake, measured at 0.2 nM hormone. (B) Constants for nonsaturable transport (K_n; means ± SEM, n = 5). Means with the same letters are not significantly different ($P > 0.05$). Units of uptake are fmol · 10^6 cells^{-1} · min^{-1}; units of nonsaturable uptake constant are μl · 10^6 cells^{-1} · min^{-1}.

plasma TH levels. It is significant that plasma T_4, which exchanges much more slowly than T_3 with the RBC, exhibits marked short-term fluctuations in response to stress, nutrient intake, and the diel light cycle (Eales and Brown, 1993). Plasma T_3, in contrast, tends to be refractory to such factors, and this may be due in part to a more rapid buffering exchange with the RBC T_3 compartment.

In summary, the RBC of rainbow trout possess saturable and nonsaturable uptake systems for both T_3 and T_4. T_3 uptake is much more rapid than T_4 uptake. The transport systems for TH are pH and temperature sensitive and subject to change over time. Thus RBC may take part in the equilibrium of TH, particularly T_3, in peripheral tissues. The presence of saturable transport systems suggests that this mechanism has the potential to be regulated.

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