Characterization of plasma triiodophenol binding proteins in vertebrates and tissue distribution of triiodophenol in *Rana catesbeiana* tadpoles

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Abbreviations: HDL, high-density lipoprotein; IC_{50}, 50% inhibitory concentration; i.p., intraperitoneally; K_d, dissociation constant; T_3, 3,3′,5-triiodo-L-thyronine; T_4, L-thyroxine; TBG, thyroxine-binding globulin; TH, thyroid hormone; THBP, TH-binding protein; TIP, 2,4,6-triiodophenol; TTR, transthyretin; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean.

Abstract

We investigated the interaction of 2,4,6-triiodophenol (TIP), a potent thyroid hormone disrupting chemical, with serum proteins from rainbow trout (*Onchorhynchus mykiss*), bullfrog (*Rana catesbeiana*), chicken (*Gallus gallus*), pig (*Sus scrofa domesticus*), and rat (*Rattus norvegicus*) using a [^{125}I]TIP binding assay, gel filtration chromatography, and native polyacrylamide gel electrophoresis. [^{125}I]TIP bound non-specifically to proteins in trout serum, specifically but weakly to proteins in bullfrog serum, and specifically and strongly to proteins in chicken, pig, and rat serum samples. Candidate TIP-binding proteins included lipoproteins (220–320 kDa) in trout, albumin in bullfrog, albumin and transthyretin (TTR) in chicken and pig, and TTR in rat. TTR in the chicken, pig, and rat serum samples was responsible for the high-affinity, low-capacity binding sites for TIP (dissociation constant 2.2–3.5 × 10^{-10} M). In contrast, a weak interaction of [^{125}I]TIP with tadpole serum proteins accelerated [^{125}I]TIP cellular uptake in vitro. Intraperitoneal injection of [^{125}I]TIP in tadpoles revealed that the radioactivity was predominantly accumulated in the gallbladder and the kidney. The differences in the molecular and binding properties of TIP binding proteins among vertebrates would affect in part the cellular availability, tissue distribution and clearance of TIP.

Keywords: binding proteins; serum; thyroid hormone homeostasis; tissue distribution; transthyretin; triiodophenol; uptake

1. Introduction

An increasing number of chemicals released into the environment have been detected in the air, soil, sediment, and water of, and in organisms living in, contaminated habitats. Although the concentrations of most chemicals found in contaminated habitats are not apparently toxic, such chemicals accumulate in organisms at concentrations several orders of magnitude higher than those measured in the environment (Vallack et al., 1998). The accumulation of such chemicals is due to their lipophilic nature and to their movement through food chain. Consequently, the bioaccumulation of environmental chemicals can interfere with the endocrine system through many mechanisms of action (Boas et al., 2006).

The bioavailability and bioactivity of the environmental chemicals in vertebrates are affected by how these chemicals are transported in the bloodstream (Nagel et al., 1997). Plasma proteins that have significant binding affinity for environmental chemicals include the thyroid hormone-binding proteins (THBPs). For example, highly lipophilic polyhalogenated aromatic hydrocarbons, such as dioxins, benzo[*a*]pyrene, and polychlorinated biphenyls, bind non-specifically (i.e., non-saturable and non-displaceable) to lipoproteins, and specifically, but with low affinity in vivo and in vitro, to albumin (Borlakoglu et al., 1990; Monteverdi and Di Giulio, 2000), whereas relatively lipophilic chemicals with polar groups (e.g., hydroxyl groups) bind specifically to albumin (Sheehan and Young, 1979), and TTR (Van den Berg, 1990; Lans et al., 1993; Ishihara et al., 2003).

2,4,6-triiodophenol (TIP), an halogenated phenol, is an environmental chemical with the potential for bioactivity in vertebrates. Either released into the environment from industrial, medical and agricultural runoffs or forming in the environment when iodine reacts with phenol to produce iodophenols or converts chlorophenols to iodophenols (Patnaik and Khoury, 2003), TIP has been detected at concentrations of 0.53 μg/L or less in the surface water of rivers in the USA (Kannamkumarath et al., 2004). Previously, we demonstrated that TIP interfered with TH-binding to TTR and disrupted thyroid hormone activity in both *Xenopus laevis* cell culture system and tadpoles (Kudo and Yamauchi, 2005). In other studies, the binding affinity of TIP for human TTR was the highest of the halogenated phenols tested (McKinney et al., 1985; Van den Berg, 1990) and was 3.8 times greater than that for TH (McKinney et al., 1985).

Species-specific differences in TIP binding to plasma proteins, and the subsequent cellular uptake, of and cellular response to TIP, is possible (Morgado et al., 2007) as the THBPs and their binding affinities and capacities for THs differ among vertebrates. More than half of THs are bound to lipoproteins, predominantly high-density lipoprotein (HDL) in rainbow trout (*Oncorhynchus mykiss*) (Babin, 1992). In adult bullfrogs (*Rana catesbeiana*), major THBP is albumin, whereas in tadpole bullfrogs, TTR also participates in TH binding as high-affinity binding sites (Yamauchi et al., 1993). Albumin and TTR are the

major THBPs in birds and rodents (Schreiber and Richardson, 1997), whereas albumin, TTR and thyroxine-binding globulin (TBG) are the major THBPs in large eutherians. In humans, 70-80% of protein-bound THs are transported by TBG, due to its high affinity for THs (Robbins, 1996).

The tendency of environmental chemicals to accumulate primarily in aquatic ecosystems from industrial residues, medical and agricultural runoffs pose a threat to vertebrates living in water-side habitats. In this study, we investigated which plasma proteins interact with, and their affinity for, TIP in serum from trouts and bullfrogs (ectotherms), and chickens, pigs, and rats (endotherms), the effect of tadpole and rat serum proteins on the uptake of TIP into in vitro, and the tissue distribution of TIP after administration to tadpoles.

2. Materials and Methods

2.1. Reagents

Radiolabeled iodine ($Na^{125}I$; 629 GBq/mg as iodine) was purchased from PerkinElmer (Waltham, MA, USA). L-Thyroxine (T_4; ≥98% purity), 3,3',5-triiodo-L-thyronine (T_3; ~98% purity), all-*trans*-retinoic acid (≥98% purity), and pentachlorophenol (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ioxynil (3,5-diiodo-4-hydroxybenzonitrile, analytical standard, 99% purity) was purchased from Riedel-de Haën Fine Chemicals (Seelze, Germany) and TIP (98% purity) was from Wako Pure Chemical Industries (Tokyo, Japan). Diethylstilbestrol (>98% purity) and tetrabromobisphenol A (>98% purity) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Sodium *p*-toluenesulfonchloramide trihydrate (chloramine-T; >98% purity) and chromatography-grade methanol were obtained from Kanto Chemical (Tokyo, Japan).

All chemicals tested as binding competitors were dissolved in dimethyl sulfoxide to concentrations of 2–10 mM, except for retinoic acid, which was dissolved in phosphate-buffered saline to a concentration of 10 mM. All chemicals were diluted with an appropriate buffer to give less than 0.4% (v/v) solvent. A control assay without the test chemicals was performed in the presence of the solvent at a concentration of less than 0.4% (v/v). The solvent did not affect the competitive [^{125}I]TIP binding assays.

2.2. Radiolabeling

2,4,6-Triiodophenol was radiolabeled by iodine exchange reaction using the chloramine-T method (Greenwood et al., 1963), with some modifications. An aliquot of the diluted TIP solution (660–1320 pmols) was mixed with $Na^{125}I$ (7.4 MBq), 280–560 pmols of unlabeled NaI, and 525 nmols of chloramine-T in 53 μL of 20 mM sodium phosphate, pH 7.2. After incubating the mixture for 10 min, the radiolabeling was stopped by the addition of 16 μL of 4 mM sodium disulfite. The reaction mixture was extracted with 300 μL of chloroform:acetic acid (99:1). The organic phase was collected and then evaporated with a test tube evaporator (type TVE-1000, EYELA, Tokyo, Japan). The residue was reconstituted in an appropriate volume of methanol:distilled water:acetic acid (74:25:1), and purified by high-performance liquid chromatography on a reverse-phase C_{18} analytical column (Mightysil RP-18 GP, 250 mm × 4.6 mm, 5 μm particle diameter; Kanto Chemical), with an isocratic mobile phase (methanol:distilled water:acetic acid, 74:25:1) at a flow rate of 1.0 mL/min. The concentration of chemical was monitored by absorbance at 254 nm. Both radiolabeled and unlabeled TIPs were eluted at 17.4 min. The respective fractions were collected and radioactivity was measured in a γ-counter (Auto Well Gamma System ARC-380CL, Aloka; Tokyo, Japan). The specific activity of TIP was estimated from the amount of

and the radioactivity of the purified TIP, which was 3.4–8.9 TBq/mmol (0.043-0.11 radioactive atom/TIP molecule), with a yield of approximately 40%. The purity of the isolated TIP was assessed by thin-layer chromatography. Aliquots of the radiolabeled TIP were loaded on a thin-layer chromatography plate (PE SIL G/UV, Whatman, Maidstone, Kent, England, UK) and run for 1.0–1.5 h in chloroform/acetic acid (99/1) solvent. The amount of purified TIP was estimated from a calibration line of standard TIP at defined amounts. The purified TIP was stored in dimethyl sulfoxide at 4°C until used.

2.3. Biological materials

Serum samples were collected from rainbow trouts (*Oncorhynchus mykiss*; males $n = 10$, females $n = 10$), bullfrogs (*Rana catesbeiana*; adult males $n = 3$, adult females $n = 3$, tadpoles $n = 50$), chickens (*Gallus gallus*; adult males $n = 3$, adult females $n = 3$, juveniles $n = 5$), pigs (*Sus scrofa domesticus*,adult male $n = 3$), Sprague-Dawley rats (*Rattus norvegicus*; adult male $n = 4$), and C3H/HeJ mice (*Mus musculus*; adult male $n = 4$). Blood was collected from sexually mature trouts at the Fuji Trout Hatchery in the Shizuoka Prefectural Research Institute of Fishery, Fujinomiya, Shizuoka. Tadpole and adult bullfrogs were obtained from Saitama Amphibian Institute, Saitama, Japan. Tadpoles (8–18 g body mass) were anesthetized by immersion in 0.2% (w/v) ethyl 3-aminobenzoate methanesulfonic acid (Sigma-Aldrich), whereas adult bullfrogs (170–200 g body mass) were anesthetized by injecting ethyl 3-aminobenzoate methanesulfonic acid then pithed. Tadpole and adult bullfrog blood were collected as described previously (Yamauchi et al., 1993). Tadpole red blood cells were prepared as described previously (Yamauchi, 1989). Chicken and pig blood were collected from mature animals at the Swine and Poultry Research Center in the Shizuoka Prefectural Research Institute of Animal Industry, Kikugawa, Shizuoka, Japan. Blood was also collected from 60-day-old chickens at a local abattoir in Shizuoka, Japan. Rat and mouse blood were kindly provided by Dr T. Koike, Department of Biological Science, Faculty of Science, Shizuoka University. Serum was separated from blood cells by centrifugation at $400 \times g$ for 15 min at 4°C. Serum samples were used immediately or stored at –20°C until required.

The care and treatment of animals used in this study were in accordance with the Guidelines for Proper Conduct of Animal Experiments, Japan.

2.4. Binding assay

Serum or TTR, purified as described previously (Eguchi et al., 2008), was incubated with 0.5 nM [^{125}I]TIP (6×10^4 dpm) in 250 μL of buffer containing-100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, 20 mM HEPES, pH 7.5 (Buffer A) in the presence or absence of 5 μM unlabeled

TIP for 1 h at 4°C. Competitive [^{125}I]TIP binding was performed with solvent only or increasing concentrations of unlabeled competitors. Protein-bound [^{125}I]TIP was separated from free [^{125}I]TIP by the Dowex method (Lennon,1992). Radioactivity was measured in a γ-counter. The amount of non-specifically bound [^{125}I]TIP (in the presence of 5 μM unlabeled TIP) was subtracted from that of total bound [^{125}I]TIP (in the absence of 5 μM unlabeled TIP) to give the value of specifically bound [^{125}I]TIP. Background radioactivity, which was estimated using tubes containing no serum proteins, was also subtracted. The K_d for TIP binding to proteins was determined from curvilinear Scatchard plots (Rosenthal, 1967).

2.5. Gel filtration column chromatography

After incubating serum with 0.7 nM [^{125}I]TIP (7×10^4 dpm) for 1 h at 4°C, 0.5 mL of each sample was applied to a Cellulofine GCL 1000-sf column (1.5 × 87 cm; Seikagaku Kogyo, Tokyo, Japan), and eluted with 20 mM sodium phosphate buffer, pH 7.4, at a flow rate of 7.8 mL/h. Protein concentrations were monitored by absorbance at 280 nm and the radioactivity was measured in a γ-counter. The column was calibrated with the following standards: apoferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), obalbumin (45 kDa), and myoglobin (17 kDa). The void volume of the column was determined by the elution volume of blue dextran (~2000 kDa).

2.6. Native polyacrylamide gel electrophoresis (PAGE)

For analysis of TIP-binding proteins, 10 μL of serum was incubated with 10 nM [^{125}I]TIP (2×10^4 dpm) in the presence of 10 μM competitors or solvent alone for 1 h at 4°C, followed by electrophoresis in a native 10% polyacrylamide gel in 0.05 M Tris-HCl, pH 8.6, at 4°C (Richardson et al., 1994). To improve the separation of TTR from albumin, the gel buffer was pH 8.3 for mouse serum and pH 9.3 for pig serum. Two aliquots of serum, 5 μL and 2 μL, were analyzed for the detection of [^{125}I]TIP-binding proteins by a phosphorimager (Personal Molecular Imager FX^{TM}, Bio-Rad, Hercules, CA, USA) and for the detection of proteins by staining with Coomassie Brilliant Blue R-250, respectively. An additional 2 μL of trout serum was analyzed for detection of lipoproteins by staining with Sudan Black B (Blom et al., 2003). Albumin was identified as the most abundant protein in each serum sample. TTR was identified from its mobility relative to albumin (Thomas et al., 1990; Duan et al., 1995; Ishihara et al., 2003; Kato et al., 2009).

2.7. Uptake of [^{125}I]TIP into tadpole red blood cells

[^{125}I]TIP (5×10^4 dpm) was pre-incubated with tadpole or rat serum (at final concentrations of 1,

4 and 10%) in 200 μL of Buffer A containing 5.6 mM glucose for 30 min at 4°C. Uptake of [^{125}I]TIP was initiated by mixing the [^{125}I]TIP solution (final 0.5 nM) with 50 μL of the tadpole red blood cell suspension (5.0×10^6 cell/tube), prepared using Buffer A containing 5.6 mM glucose, at 25°C. In each experiment, red blood cells were freshly prepared from three to four tadpoles at premetamorphic stages. The final concentration of DMSO was less than 0.1%. After two minutes, the cell-associated [^{125}I]TIP were separated from free [^{125}I]TIP by the oil-centrifugation method (Yamauchi et al., 1989). The tip of the polyethylene tube (0.4 μL microcentrifuge tube, Porex Bio Products, CA, USA) containing the cell pellet was cut off. Radioactivity of the cell-associated [^{125}I]TIP was determined in a γ-counter.

2.8. Tissue distribution of [^{125}I]TIP in tadpoles

[^{125}I]TIP (6.0×10^5 dpm) was administered intraperitoneally (i.p.) at a dose of 10 pmols (in 20 μL of Frog Ringer) to three groups consisting of five tadpoles at stages X-XIII. Blood, liver, gallbladder, stomach, intestine, and kidney were removed and weighed after 1, 4 and 22 hours. The radioactivity of these samples and the remaining carcasses was counted by a γ-counter. This experiment was repeated twice.

2.9. Statistical analysis

The data presented are the mean ± standard error of the mean (SEM, $n = 3$), unless otherwise noted. Differences between groups were analyzed by a paired Student t test, or a one-way analysis of variance, with the Fisher least-significant-difference test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. **Results**

3.1. Interaction of [^{125}I]TIP with serum proteins

The interaction between [^{125}I]TIP and serum proteins in the rainbow trout and bullfrog serum samples was weaker than those in the chicken, pig, and rat serum samples (Fig. 1). Most of the protein-bound [^{125}I]TIP in trout serum was non-specifically bound, whereas about 20% of the protein-bound [^{125}I]TIP in bullfrog serum was non-specifically bound. In the endothermal serum samples, especially in rat serum, small amounts (< 10%) of [^{125}I]TIP were non-specifically bound. The amounts of protein-bound [^{125}I]TIP that were specifically and non-specifically bound did not differ between serum samples from male and female trouts, bullfrogs, or chickens, at defined concentrations.

The molecular size of the [^{125}I]TIP-binding proteins in trout serum differed between sexes (males, ~320 kDa; females, 220–280 kDa) and from those in serum samples from the other species tested (58–68 kDa) (Table 1). The percentage of [^{125}I]TIP that was distributed in the protein fractions after gel chromatography was 46–67% for trout serum, 15–37% for bullfrog serum, including tadpole serum, and ≥ 66% for the endotherm serum samples (Table 1). The [^{125}I]TIP binding capacity of the serum samples was estimated by chromatography to be similar to that estimated by the binding assay (Fig. 1). There were no differences in the elution pattern of the [^{125}I]TIP-binding proteins between serum samples from male and female bullfrogs or chickens, although the amounts of protein-bound [^{125}I]TIP were higher in the female bullfrog than in the male bullfrog.

[^{125}I]TIP-binding proteins were detected in all serum samples tested. The [^{125}I]TIP-binding proteins in trout serum (lanes 1, 2, 12 and 13, Fig. 2B) corresponded partially to lipoproteins (lanes 12 and 13 in Fig. 2A). The mobilities of the [^{125}I]TIP-binding proteins differed between serum samples from male and female trouts. The major [^{125}I]TIP-binding protein in tadpole and bullfrog serum corresponded to albumin (lanes 3–5 and 14 in Fig. 2B). In chicken serum, two distinct [^{125}I]TIP-binding proteins corresponding to TTR and albumin were detected (lanes 6-8, Fig. 2B). [^{125}I]TIP-binding was also detected as a slow-migrating broad band in female chicken serum (lane 8, Figs. 2B and 2C) but as a narrow band in juvenile and male chicken serum in the presence of excess unlabeled TIP (lanes 6 and 7, Fig. 2C). TTR was the predominant [^{125}I]TIP-binding protein in the juvenile and male, but not in the female, chicken serum. The major [^{125}I]TIP-binding protein (pH 8.6) detected in pig and mouse serum corresponded somewhat to albumin (lanes 9 and 11, respectively, Fig. 2B). However, native PAGE of pig serum at pH 9.3 resulted in two distinct bands (lane 15, Fig. 2B) that corresponded to albumin (fast-migrating band) and TTR (slow-migrating band). Native PAGE of mouse serum at pH 8.3 separated the major [^{125}I]TIP-binding protein from albumin, which did not interact with [^{125}I]TIP (lane 16, Fig. 2B). This result for mouse serum

was similar to that for rat serum (native PAGE at pH 8.6; lane 10, Fig. 2B). The addition of unlabeled TIP (10 μM) barely inhibited the binding of [^{125}I]TIP to the putative lipoproteins in trout serum, and to albumin in the other serum samples. However, it strongly inhibited the binding of [^{125}I]TIP to TTR in chicken, pig, rat, and mouse serum (Fig. 2C).

3.2. Characterization of [^{125}I]TIP binding to serum proteins in the chicken, pig and rat serum

[^{125}I]TIP binding to serum proteins in the presence of unlabeled T_3 and T_4 was assessed using the ~60 kDa fraction from the gel filtration chromatography of serum from male chickens, pigs, and rats. TIP, rather than T_3 and T_4, was the most potent competitor of [^{125}I]TIP binding in the ~60 kDa fractions. The 50% inhibitory concentration (IC_{50}) for TIP was 1.4 ± 0.2 nM (n = 3) for chicken, 7.4 ± 1.0 nM (n = 4) for pig, and 1.2 ± 0.1 nM (n = 3) for rat serum (Fig. 3). The IC_{50} for T_3 was one order of magnitude lower than that for T_4 for chicken serum, but one order of magnitude higher than that for T_4 for pig serum. T_3 was nearly as potent as T_4 in the 60 kDa fraction from rat serum.

The IC_{50} for TIP binding to chicken and rat TTRs [1.5 ± 0.3 nM (n = 3) and 1.2 ± 0.1 nM (n = 3), respectively] was similar to that for TIP binding in the chicken and rat serum ~60 kDa fractions (Fig. 3). However, the IC_{50} for TIP binding to purified pig TTR [1.8 ± 0.2 nM (n = 6)] was four times lower than that for TIP binding to the pig serum ~60 kDa fraction ($P < 0.01$).

Scatchard plots of [^{125}I]TIP binding to chicken, pig, and rat TTRs indicated two classes of TIP binding sites (Fig. 4). The K_d values of TIP for the high and low affinity sites were 0.29 ± 0.14 nM and 4.78 ± 2.07 (n = 3) for chicken TTR, 0.35 ± 0.01 nM and 9.02 ± 1.79 nM (n = 6) for pig TTR, and 0.22 ± 0.01 nM and 14.90 ± 3.52 nM (n = 3) for rat TTR, respectively.

3.3. Effects of environmental chemicals on [^{125}I]TIP binding to serum proteins from the endotherms

In male chicken serum (Fig. 5A), TIP was the most, and ioxynil the second most, potent competitor for [^{125}I]TIP binding to TTR. The addition of TIP, ioxynil, tetrabromisphenol A, or T_3 increased the amount of [^{125}I]TIP bound to albumin. Competition with [^{125}I]TIP binding to TTR or albumin was weak or not detected for the other chemicals tested. Similar results were obtained in female chicken serum (data not shown). In pig serum (Fig. 5B), the amount of [^{125}I]TIP bound to TTR decreased, but the amount of [^{125}I]TIP bound to albumin hardly changed with the addition of the various chemicals. The rank order potency of the chemicals tested for the displacement of [^{125}I]TIP from TTR were TIP > ioxynil > pentachlorophenol, T_4, and retinoic acid > tetrabromobisphenol A, diethylstilbestrol, and T_3. In rat serum (Fig. 5C), TIP was the most potent competitor for [^{125}I]TIP binding to TTR. Competition with [^{125}I]TIP

binding to TTR or albumin was weak for the other chemicals tested. However, the addition of TIP, T_3, or T_4 increased the amount of [^{125}I]TIP bound to albumin.

3.4. Effect of serum on uptake of [^{125}I]TIP into tadpole red blood cells

The uptake of [^{125}I]TIP into tadpole red blood cells in the absence (0%) and presence of rat and tadpole serum (1%, 4% and 10%; Fig. 6) was examined. [^{125}I]TIP uptake into the cells reached a plateau after 2 min. The percentage of [^{125}I]TIP associated with the cells in the absence of serum varied from 11% to 12% (control, data not shown). Rat and tadpole serum inhibited the uptake of [^{125}I]TIP into the cells in a concentration-dependent manner. Inhibition of [^{125}I]TIP uptake was significantly greater for rat serum than for tadpole serum (1%, $P < 0.01$; 4% and 10%, $P < 0.05$).

3.5. Tussue distribution of [^{125}I]TIP in tadpoles

The distribution of [^{125}I]TIP at 1, 4 and 22 h after i.p. administration of [^{125}I]TIP in tadpoles was assessed. The highest amount of radioactivity was detected in the kidney, followed by the intestine and liver, gallbladder, and stomach (Fig. 7A). The amount of radioactivity in all tissues investigated, except for the intestine and gallbladder, had significantly decreased at 22 h from 4 h. The accumulation of radioactivity, estimated as the tissue-to-blood concentration ratio of the radioactivity, was highest in the kidney at all time points tested (Fig. 7B). Radioactivity accumulated gradually in the gallbladder and intestine with time.

4. Discussion

4.1. Species-specific TIP-binding proteins in plasma and their diversity during vertebrate evolution

Findings in our study suggest that TIP interacts with serum proteins from rainbow trouts, bullfrogs, chickens, pigs and rats in three distinct modes: (1) non-specific binding to putative lipoproteins of 220–320 kDa (e.g., in trout serum), (2) specific binding to low affinity sites on albumin (e.g., in bullfrog, chicken, and pig serum), and (3) specific binding to high affinity sites on TTR (e.g., in chicken, pig, and rat serum). These distinct modes suggest an evolutionary change in the interaction between TIP and plasma proteins. Whereas lipoproteins may play an important role for TIP binding in the plasma of fish, the onset of TTR synthesis and the structural changes of albumin and TTR during tetrapod evolution may have increased TIP binding sites in plasma by providing an abundant, but low affinity, binding site (albumin) and a high affinity binding site (TTR). In support of this, we found that the amount of TIP bound to serum proteins was greater in serum samples from endotherms than those from ectotherms. The change in the affinity of TTR for TIP seems to be correlated with that in the affinity for THs from T_3 to T_4 (Yamauchi et al., 1993; Richardson, 2007).

Our results suggested that candidates for [^{125}I]TIP-binding proteins in trout serum were lipoproteins, of which HDL is a dominant lipoprotein (Bavin and Vernier, 1989). The binding properties, and differences between sexes in the molecular size, of the trout TIP-binding proteins partially resembled those of a major THBP (~150 kDa) detected in trout serum (Cyr and Eales, 1992) and 2,3,7,8-tetrachlorobenzo-*p*-dioxin-binding proteins detected in mummichogs serum (*Fundulus heteroclitus*) (Monteverdi and Di Giulio, 2000). These proteins were putatively identified as HDLs in male mummichog (~350 kDa) and trout serum (Babin, 1992). Collection of the trout serum during the spawning season may have contributed to the sex-specific differences detected in TIP-binding proteins.

Albumin was the sole TIP-binding protein identified in adult and tadpole bullfrog serum. Although tadpole serum contains TTR (Yamauchi et al., 1993), we did not detect TIP binding to TTR in our native PAGE experiment of tadpole serum. Compared with rat serum, in which TIP binds to albumin and TTR, tadpole serum also had a reduced capacity to inhibit [^{125}I]TIP uptake into tadpole red blood cells. This result suggests that the concentration of free TIP in tadpole blood is likely to be high, which may result in increased uptake and activity of TIP within tadpole cells and a high clearance rate of TIP. As such, tadpoles could be a good model to investigate the bioactivity and bioavailability of TIP.

The difference in serum TIP-binding proteins between female and male chickens may influence TIP plasma kinetics or its uptake into tissues. However, TTR in serum from female chickens appeared to bind less TIP than TTR in serum from juvenile and adult male chickens as the unidentified TIP-binding

protein in female chicken serum may lower the amount of TIP bound to TTR. Alternatively, the concentration of TTR in female chicken serum may be lower than those in juvenile and adult male chicken serum, particularly given that TTR expression in the marine fish *Sparus aurata* and the toad *Xenopus laevis* was found to be suppressed by estrogens (Funkenstein et al., 2000; Urbatzka et al., 2007). The observation of TIP binding as a third broad band in the female serum and a narrow band in the juvenile and male serum suggests the potential for several molecular species of TIP-binding protein, especially in female serum. Whether these species are homologous to TIP-binding proteins detected in trout is unclear.

4.2. Factors influencing TH disruption in plasma by TIP

The extent with which TIP disrupts TH distribution in plasma may be influenced by the following factors: (i) whether TIP interacts with a specific or several THBPs, (ii) whether TIP interacts with a major THBP , (iii) whether TIP's binding mode is specific or non-specific, and (iv) whether the THBP has high- or low-affinity sites for TIP. Of the serum samples investigated in our study, TIP is most likely to interfere with TH distribution in adult rat plasma. Although TTR was the sole TIP-binding protein detected, TTR is the predominant THBP in adult rat plasma (Thomas et al., 1990), even though TBG has been detected in serum from developing and aged rats (Savu et al., 1987; Savu et al., 1991). If TIP competes strongly with TH binding to TTR *in vivo*, this may increase the free concentration of THs in plasma and the plasma clearance rates for THs. This scenario has been reported when flavonoids, such as F21388, were administered into rats (Lueprasitsakul et al., 1990). Such TTR-mediated adverse effects on the thyroid system may not be applicable to large eutherians, such as pigs and humans, because TBG, which has a higher affinity for THs than TTR (Janssen et al., 2002), is the major THBP in blood.

TIP is likely to interfere with T_4-binding to TTR in male chickens and rats at concentrations less than plasma T_4 levels as the K_d values of TTR for TIP were 10^{-10} M. Plasma concentrations of THs in rainbow trout, chicken, pig and rat are about 10^{-8} for T_4 and 10^{-9} M for T_3 (Nejad et al., 1975; Yen and Pond, 1985; Pavlidis et al., 1991; Sechman et al., 2003). THs in plasma from adult bullfrog were undetectable (White and Nicoll, 1981) and, in particular, the plasma T_4 concentrations in the endotherms were in the same range as the K_d values of TTR for T_4 (Duan et al., 1995; Chang et al., 1999).

4.3 Cellular uptake and tissue-distribution of TIP

The accumulation of TIP or its metabolites in the kidney and liver of tadpoles suggests that specific uptake mechanisms for TIP exist in these tissues. The increase in the tissue-to-blood concentration ratio of radioactivity in the gallbladder, but not the liver, suggests the presence of a strong efflux system to

avoid the accumulation of TIP or its metabolites in the liver and to accelerate their biliary excretion. TIP was eliminated more rapidly from the blood of bullfrog tadpoles than from human plasma (Troconiz et al., 2006). This rapid elimination may be in part due to a weak interaction of TIP with serum proteins.

5. Conclusions

The findings in our study demonstrated that TIP is differently bound to serum proteins in a species-specific manner: non-specifically to lipoproteins in trout, specifically to low-affinity, high capacity sites on albumin in bullfrog and chicken, and specifically to high-affinity, low capacity sites on TTR in chicken, pig, and rat. Low TIP binding activity of tadpole serum accelerated cellular uptake of TIP in vitro, compared with TIP binding activity of rat serum. [^{125}I]TIP administrated to tadpoles was eliminated rapidly from the blood. The differences in the molecular and binding properties of TIP binding proteins among vertebrates would affect in part the cellular availability, tissue distribution and clearance of TIP.

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Figure legends

Figure 1. Binding of [^{125}I]-2,4,6-triiodophenol (TIP) to proteins in (*A*) serum (0.8–3.2 μL) from male (M) and female (F) rainbow trouts (*Oncorhynchus mykiss*), bullfrog (*Rana catesbeiana*) tadpoles, and male and female adult bullfrogs, and (*B*) serum (0.004–0.032 μL) from male and female chickens (*Gallus gallus)*, male pigs (*Sus scrofa domesticus*), and male rats (*Rattus norvegicus*). Serum samples were incubated with [^{125}I]TIP (6×10^4 dpm) in 250 μL of buffer in the presence or absence of 5 μM unlabeled TIP for 1 h at 4°C. Open bars represent specific [^{125}I]TIP binding; solid bars represent non-specific [^{125I}]TIP binding ([^{125I}]TIP binding non-displaceable with 5 μM TIP). Experiments were repeated three times, using serum samples from different individuals of each species.

Figure 2. Native polyacrylamide gel electrophoresis (PAGE) of [^{125}I]-2,4,6-triiodophenol (TIP) binding proteins in serum from rainbow trouts, bullfrogs, chickens, pigs, rats, and mice. *(A)* Proteins (Coomassie Brilliant Blue; lanes 1–11) or lipoproteins (Sudan Black; lanes 12–13) in the serum samples; *(B* and *C)* [^{125}I]TIP bound proteins (lanes 12–14, exposure length four times that of lanes 1–3) in the serum samples. Serum samples were incubated with [^{125}I]TIP (2×10^4 dpm) in the (*A* and *B*) absence or (*C*) presence of 10 μM unlabeled TIP for 1h at 4°C. Proteins were separated by native PAGE at pH 8.6 (lanes 1–14), pH 9.3 (lane 15), or pH 8.3 (lane 16). White arrow head = albumin; Black arrow head = transthyretin. Experiments were repeated three times, using serum samples from different individuals of each species.

Figure 3. Inhibition of [^{125}I]-2,4,6-triiodophenol (TIP) binding to serum proteins or transthyretin (TTR) from male (*A*) chickens, (*B*) pigs, and (*C*) rats. Serum proteins in the ~60 kDa gel filtration chromatography fractions (———) or TTR (– – – – –) were incubated with 0.5 nM [^{125}I]TIP (6×10^4 dpm) in the presence or absence (control) of TIP (●, ♦), T_3 (▲) and T_4 (■), at various concentrations, for 1 h at 4°C. Non-specific binding, which was less than 15% of total binding, was subtracted from total binding to give values for specific binding.

Figure 4. Scatchard plots of [^{125}I]-2,4,6-triiodophenol (TIP) binding to transthyretin (TTR). TTR purified from the male (*A*) chicken, (*B*) pig, and (*C*) rat serum were incubated with 0.5 nM [^{125}I]TIP (6×10^4 dpm) in the presence of unlabeled TIP, at various concentrations, for 1 h at 4°C. Non-specific binding, which was less than 15% of total binding, was subtracted from total binding to give values for specific binding. Broken lines represent the Scatchard plots resolved into two lines showing the high-affinity and low-affinity sites.

Figure 5. Effect of various compounds on [^{125}I]-2,4,6-triiodophenol (TIP) binding to proteins in serum (10 μL) from male (*A*) chickens, (*B*) pigs, and (*C*) rats. Serum samples were incubated with [^{125}I]TIP (2×10^4 dpm) in the absence (lane 1) or presence (lane 2) of 10 μM unlabeled TIP, ioxynil (lane 3), tetrabromobisphenol A (lane 4), diethylstilbestrol (lane 5), pentachlorophenol (lane 6), triiodothyronine (lane 7), thyroxine (lane 8), and retinoic acid (lane 9) at 4°C for 1 h. Proteins were separated by native polyacrylamide gel electrophoresis at pH 9.3 (pig serum) or pH 8.6 (chicken and rat serum samples) and detected by autoradiography. White arrow head = albumin; Black arrow head = transthyretin.

Figure 6. Dose-dependent inhibition of [^{125}I]-2,4,6-triiodophenol (TIP) uptake into tadpole red blood cells by serum proteins. Saturable initial uptake of [^{125}I]TIP into tadpole red blood cells was examined in the HEPES buffer in the presence or absence (control) of tadpole and rat serum samples (final 1%, 4% and 10%). Each point represents the mean ± SEM from triplicate determinations. Statically significant difference between tadpole and rat serum was detected: **, $P < 0.01$; *, $P < 0.05$. Experiments were repeated three times.

Figure 7. Tissue distribution of [^{125}I]-2,4,6-triiodophenol (TIP) after i.p. administration of [^{125}I]TIP into bullfrog tadpoles. At 1, 4 and 22 h after [^{125}I]TIP administration, the radioactivity in each tissue was measured. (*A*) Percentage of dose of [^{125}I]TIP in each organ. In the blood samples, this value was represented as a percentage of dose/100 μL. Each column represents the mean ± SEM for 9-10 animals. (*B*) Tissue-to-blood concentration ratio of [^{125}I]TIP in various organs after i.p. administration of [^{125}I]TIP into bullfrog tadpoles. The data were converted from Fig. 7A. Each column represents the mean ± SEM for 9-10 animals. Statically significant differences were detected: **, $P < 0.01$; *, $P < 0.05$.

Figure 1

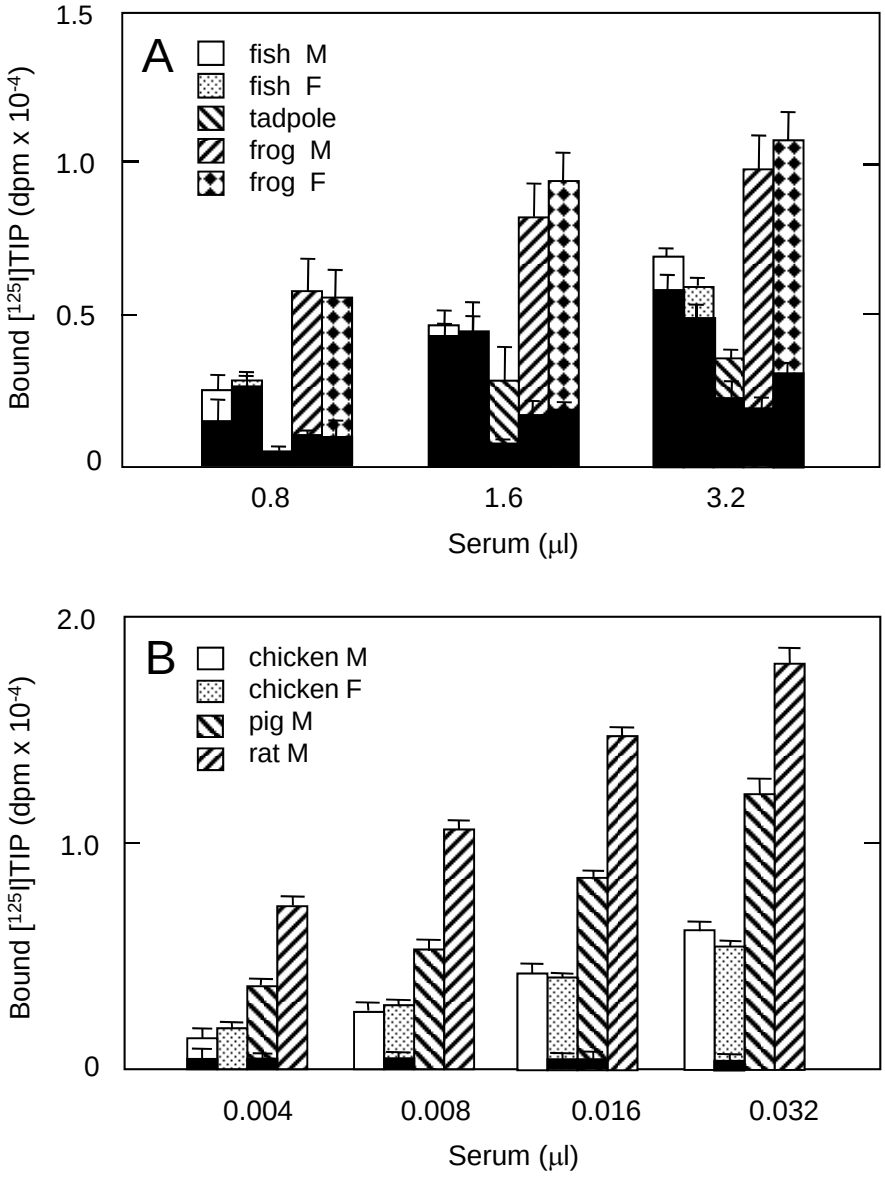

A
fish M
fish F
tadpole
frog M
frog F
Bound [125I]TIP (dpm x 10-4)
1.5
1.0
0.5
0
0.8
1.6
3.2
Serum (μl)
B
chicken M
chicken F
pig M
rat M
Bound [125I]TIP (dpm x 10-4)
2.0
1.0
0
0.004
0.008
0.016
0.032
Serum (μl)

Figure 2

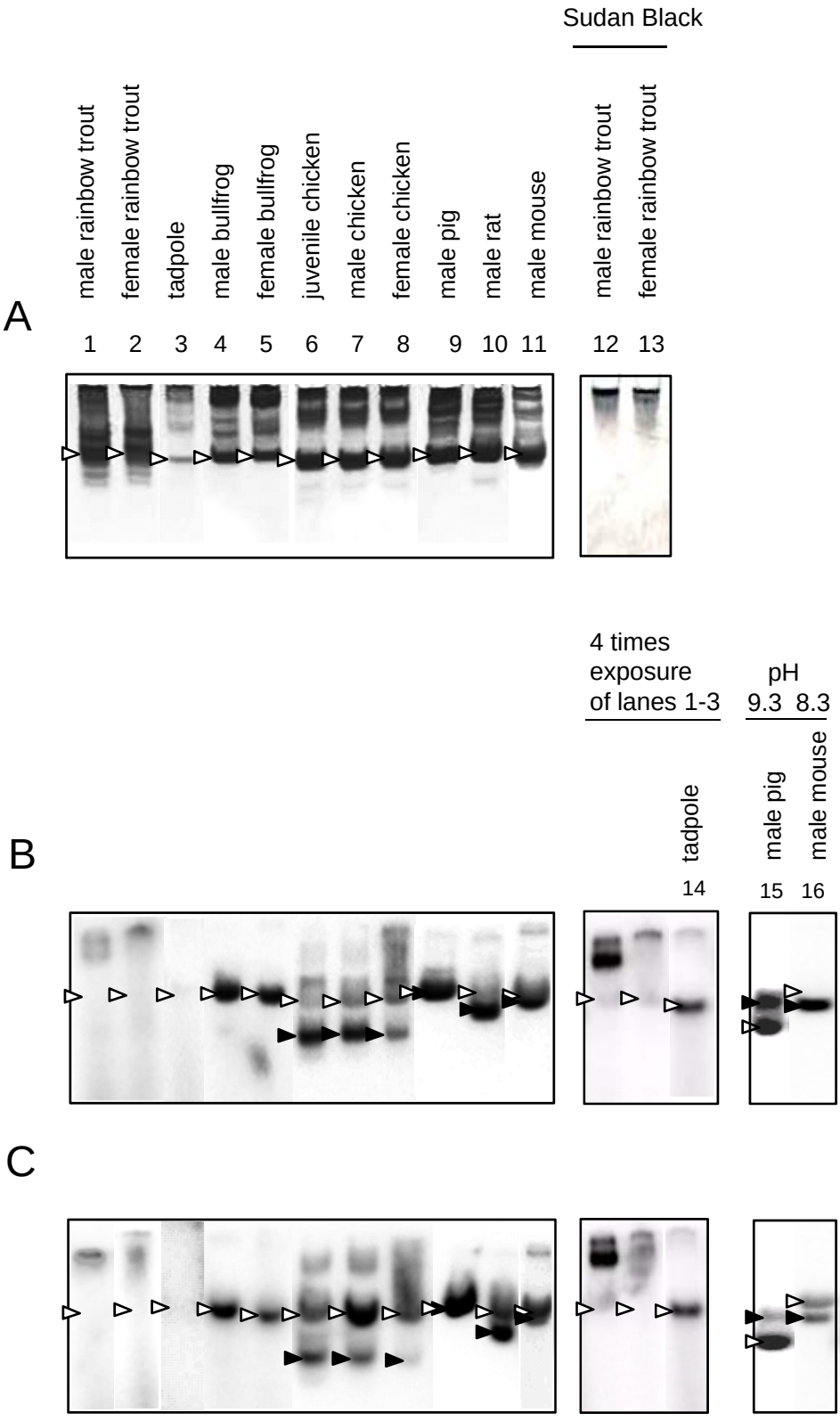

Sudan Black
male rainbow trout
female rainbow trout
tadpole
male bullfrog
female bullfrog
juvenile chicken
male chicken
female chicken
male pig
male rat
male mouse
male rainbow trout
female rainbow trout
1 2 3 4 5 6 7 8 9 10 11 12 13
A
4 times exposure of lanes 1-3
pH 9.3 8.3
tadpole
male pig
male mouse
14 15 16
B
C

Figure 3

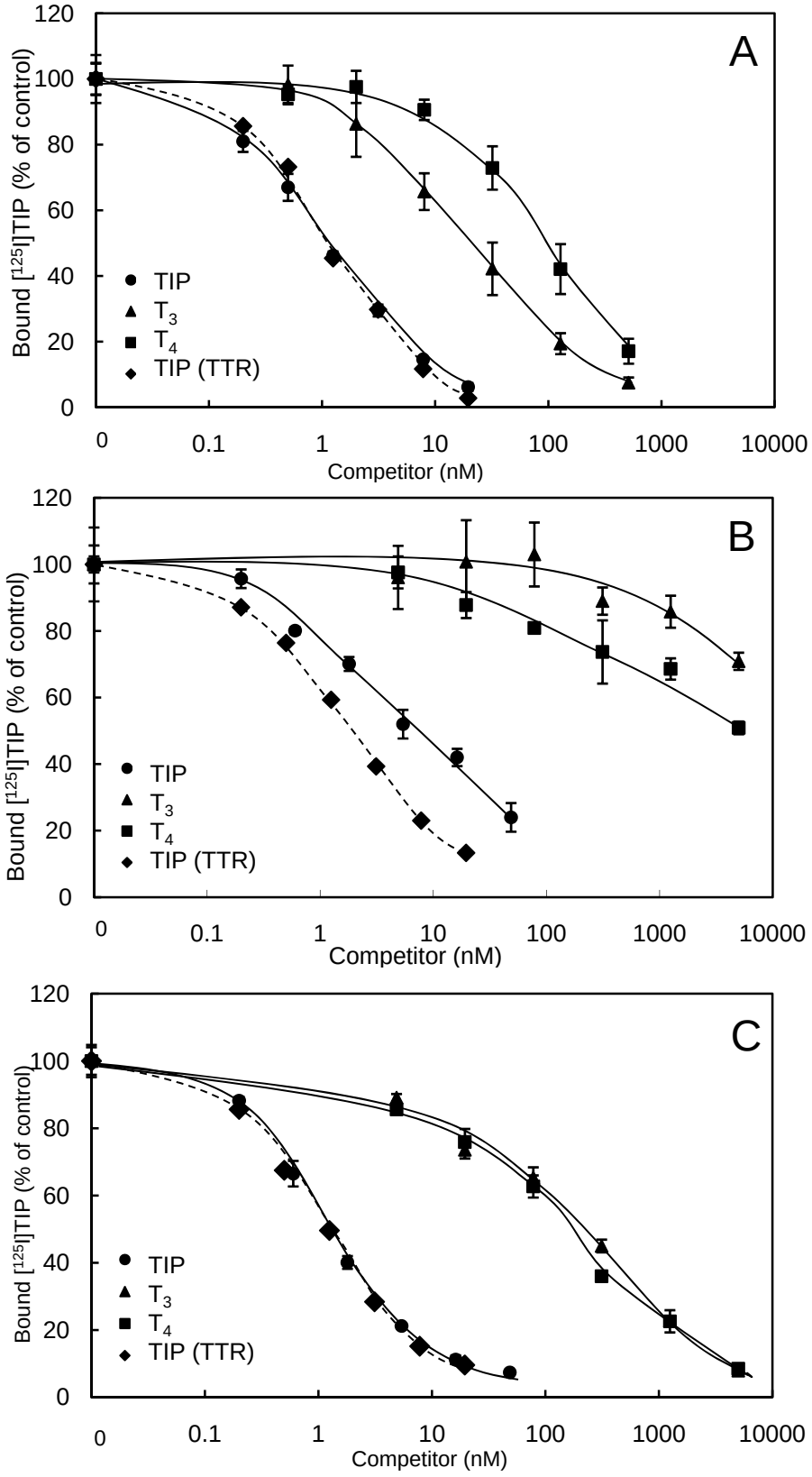

A
B
C
Bound [125I]TIP (% of control)
Competitor (nM)
TIP
T3
T4
TIP (TTR)
0
20
40
60
80
100
120
0
0.1
1
10
100
1000
10000

Figure 4

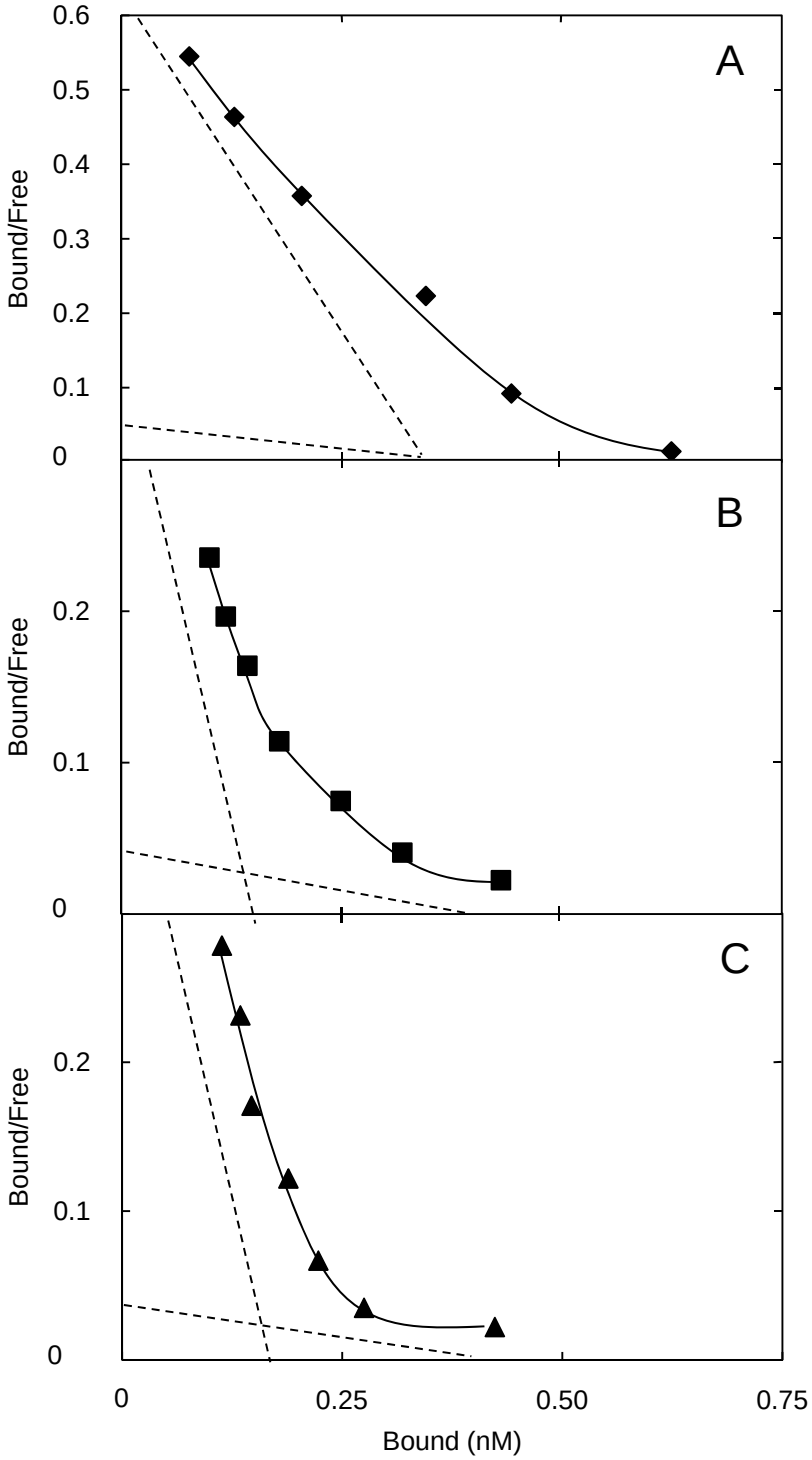

A
B
C
Bound/Free
Bound/Free
Bound/Free
0.6
0.5
0.4
0.3
0.2
0.1
0
0.2
0.1
0
0.2
0.1
0
0
0.25
0.50
0.75
Bound (nM)

Figure 5

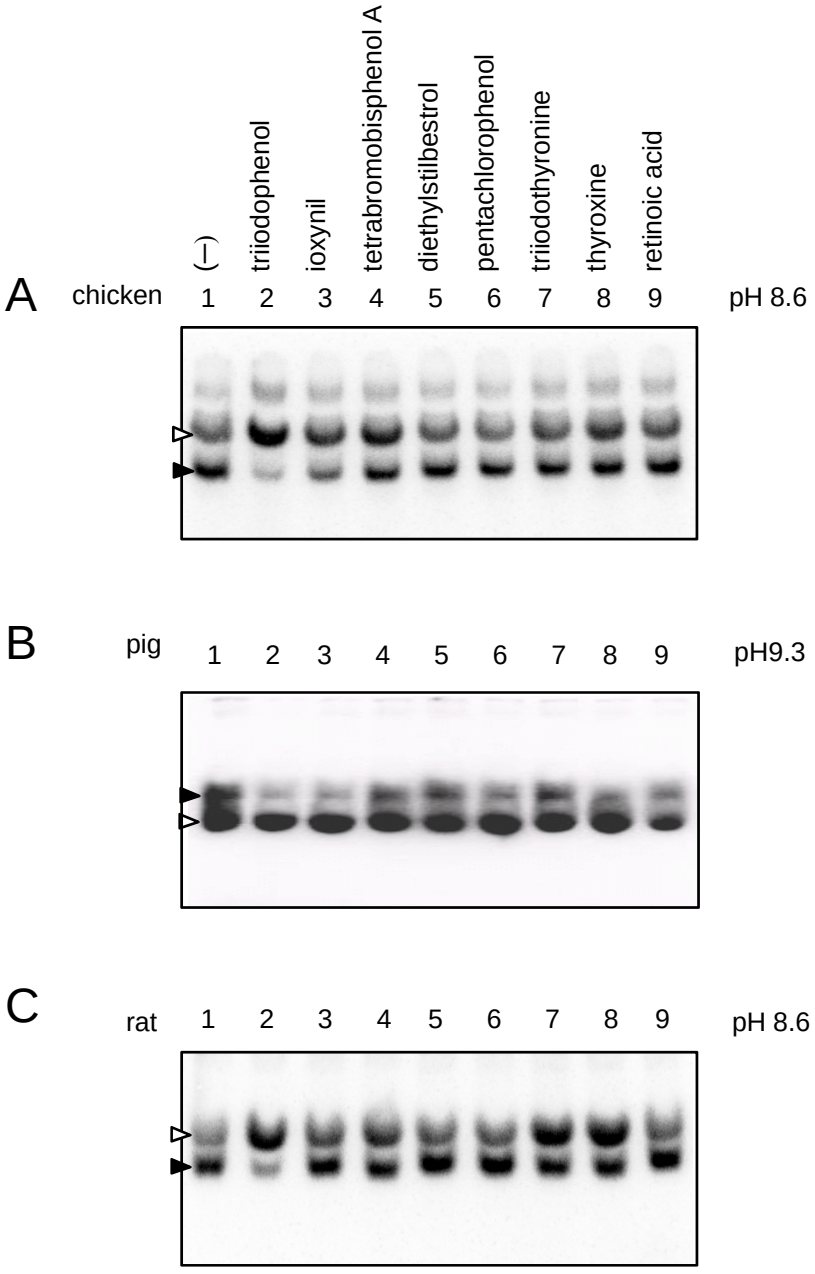

A
chicken
(–)
triiodophenol
ioxynil
tetrabromobisphenol A
diethylstilbestrol
pentachlorophenol
triiodothyronine
thyroxine
retinoic acid
1 2 3 4 5 6 7 8 9
pH 8.6
B
pig
1 2 3 4 5 6 7 8 9
pH9.3
C
rat
1 2 3 4 5 6 7 8 9
pH 8.6

Figure 6

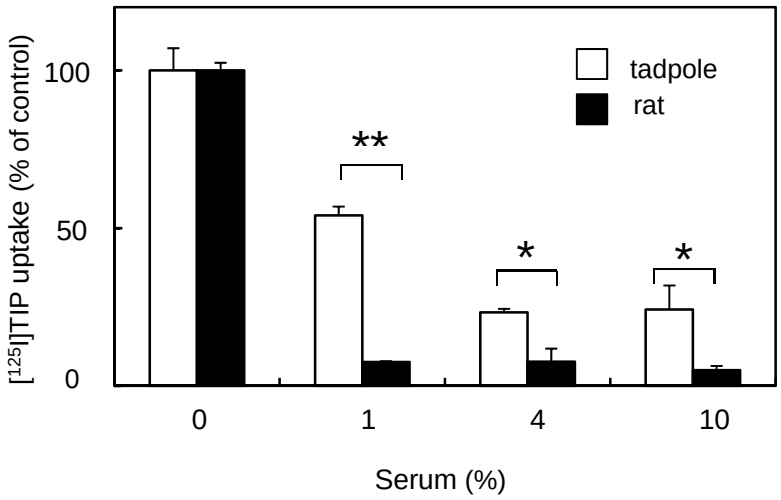
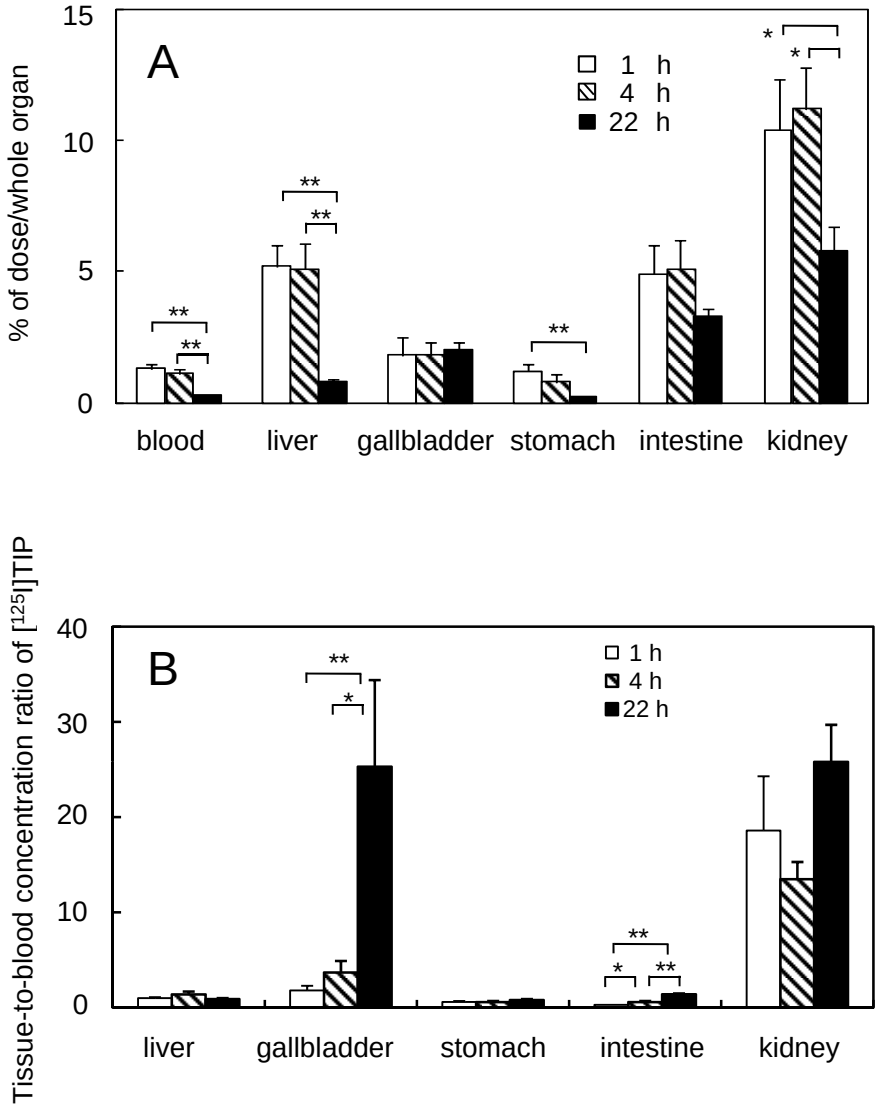


Figure 7


A
1 h
4 h
22 h
% of dose/whole organ
15
10
5
0
blood
liver
gallbladder
stomach
intestine
kidney
B
1 h
4 h
22 h
Tissue-to-blood concentration ratio of [125I]TIP
40
30
20
10
0
liver
gallbladder
stomach
intestine
kidney