



Published in final edited form as:

*Toxicol Appl Pharmacol.* 1999 February 15; 155(1): 24–31. doi:10.1006/taap.1998.8611.

## Inhibition by Lead of Production and Secretion of Transthyretin in the Choroid Plexus: Its Relation to Thyroxine Transport at Blood–CSF Barrier

Wei Zheng<sup>\*,†,1</sup>, William S. Blaner<sup>‡</sup>, and Qiuqu Zhao<sup>\*</sup>

<sup>\*</sup>Division of Environmental Health Sciences, School of Public Health, Columbia University, New York, NY 10032

<sup>†</sup>Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, NY 10032

<sup>‡</sup>Department of Medicine, Columbia University, New York, NY 10032

### Abstract

Long-term, low-dose Pb exposure in rats is associated with a significant decrease in transthyretin (TTR) concentrations in the CSF. Since CSF TTR, a primary carrier of thyroxine in brain, is produced and secreted by the choroid plexus, *in vitro* studies were conducted to test whether Pb exposure interferes with TTR production and/or secretion by the choroid plexus, leading to an impaired thyroxine transport at the blood-CSF barrier. Newly synthesized TTR molecules in the cultured choroidal epithelial cells were pulse-labeled with [<sup>35</sup>S]methionine. [<sup>35</sup>S]TTR in the cell lysates and culture media was immunoprecipitated and separated by SDS-PAGE, and quantitated by autoradiography and liquid scintillation counting. Pb treatment did not significantly alter the protein concentrations in the culture, but inhibited the synthesis of total [<sup>35</sup>S]TTR (cells + media), particularly during the later chase phase. Two-way ANOVA of the chase phase revealed that Pb exposure (30  $\mu$ M) significantly suppressed the rate of secretion of [<sup>35</sup>S]TTR compared to the controls ( $p < 0.05$ ). Accordingly, Pb treatment caused a retention of [<sup>35</sup>S]TTR by the cells. In a two-chamber transport system with a monolayer of epithelial barrier, Pb exposure (30  $\mu$ M) reduced the initial release rate constant ( $k_r$ ) of [<sup>125</sup>I]T<sub>4</sub> from the cell monolayer to the culture media and impeded the transepithelial transport of [<sup>125</sup>I]T<sub>4</sub> from the basal to apical side of epithelial cells by 27%. Taken together, these *in vitro* data suggest that sequestration of Pb in the choroid plexus hinders the production and secretion of TTR by this tissue. Consequently, this may alter the transport of thyroxine across this blood-CSF barrier.

### Keywords

lead; transthyretin; thyroxine; choroid plexus; blood-CSF barrier; pulse-chase

The choroid plexus resides within brain ventricles and separates two distinct body fluids: the blood and cerebrospinal fluid (CSF) (Davson and Segal, 1996; Johanson, 1995; Smith, 1991). Aside from its primary role in CSF production and regulation, the choroid plexus manufactures and secretes the proteins for the extracellular compartment in the central nervous system (CNS). Of the proteins in the CSF, transthyretin (TTR or prealbumin) is exclusively produced and secreted by the choroid plexus (Aldred *et al.*, 1995; Herbert *et al.*, 1986; Nilsson *et al.*, 1992). TTR is a 55,000-Dalton protein consisting of four identical subunits in a tetrahedral symmetry. Per unit of weight, rat choroid plexus contains 10 times more TTR mRNA than liver, and per gram of tissue synthesizes TTR 13 times faster than the liver, which is the major organ producing serum TTR (Dickson *et al.*, 1985; Schreiber *et al.*, 1990). CSF TTR (~15 µg/ml) makes up 25% of total CSF protein (Aldred *et al.*, 1995). In humans, TTR serves as a major thyroid hormone binding protein in the CNS and conveys about 60–80% of CSF thyroxine (Hagen and Elliott, 1973; Herbert *et al.*, 1986; Larsen and DeLallo, 1989). The binding of thyroxine to CSF TTR allows a fine control of the levels of thyroid hormones in the CSF and in the extracellular space of the brain. Recent evidence has suggested that repression of TTR in CSF and/or choroid plexus may lead to an alteration of the balance of thyroid hormones in the brain (Chanoine *et al.*, 1992; Southwell *et al.*, 1993).

Thyroid hormones have striking effects on the CNS, particularly during the developmental period (Dussault and Ruel, 1987). Deficiency of thyroid hormones during this period produces multiple morphological, biochemical, and electrophysiological alterations of neurons and neuroglia (Dussault and Ruel, 1987; Farsetti *et al.*, 1991; Legrand, 1984; Ruiz-Marcos *et al.*, 1979). In children, deprivation of thyroid hormones causes irreversible mental retardation (Smith *et al.*, 1957; Glorieux *et al.*, 1983; Legrand, 1984). Recently, Thompson (1996) has isolated and identified the genes that are expressed in response to thyroid hormones in developing rat brain. Notably, the TTR gene in the choroid plexus is expressed early in the fetal development, a phenomenon consistent with the importance of the thyroid hormones in embryonic brain development (Thomas *et al.*, 1989; Cavallaro *et al.*, 1993; Schreiber *et al.*, 1995). Thus, the choroid plexus, by manufacturing and regulating CSF TTR, appears to play a crucial role in regulating and mediating the delivery of thyroid hormones to the developing brain.

Previous studies have shown that the choroid plexus sequesters lead (Pb) to an extraordinary degree following Pb exposure (Friedheim *et al.*, 1983; Zheng *et al.*, 1991, for review, see Zheng, 1996). Our recent work demonstrates that disposition of Pb in rat choroid plexus is directly associated with a significant reduction in CSF concentration of TTR (Zheng *et al.*, 1996). The synthesis of TTR by the choroidal epithelia has been suggested to mediate the transport of thyroid hormones from the blood to CSF (Chanoine *et al.*, 1992; Dratman *et al.*, 1991; Schreiber *et al.*, 1990; Southwell *et al.*, 1993). Thus, alteration of TTR concentration in CSF and/or choroid plexus by Pb exposure may influence the transport of thyroxine at the blood-CSF barrier, leading to an altered brain economy of thyroid hormones.

The purpose of this study was to test the hypothesis that Pb exposure impairs TTR production and/or secretion in the choroid plexus, which may affect the transepithelial transport of thyroxine at the blood-CSF barrier. We conducted pulse-chase experiments using [<sup>35</sup>S]methionine to label the newly synthesized proteins in the cultured choroidal

epithelial cells. The effect of Pb on the production and secretion of TTR was investigated by estimating the amounts of [<sup>35</sup>S]TTR that were selectively precipitated by specific TTR antibody and separated by SDS PAGE. To investigate thyroxine transport, we adapted a two-chamber transport model which was originally developed by Southwell *et al.* (1993). The model allows the culture media in both chambers to be separated by a barrier of confluent epithelial cells grown on a permeable membrane. By monitoring [<sup>125</sup>I]T<sub>4</sub> in both chambers, we examined the effect of Pb exposure on transport kinetics of thyroxine across the choroidal epithelial barrier.

## MATERIALS AND METHODS

### Materials and animals

Chemicals were obtained from the following sources: Pb acetate, epidermal growth factor (EGF), fibroblast growth factor (FGF), gentamycin, phenylmethyl sulfonyl fluoride (PMSF), aprotinin, leupeptin, laminin, prostaglandin E<sub>1</sub> (PE<sub>1</sub>), and cycloheximide from Sigma Chemical Co. (St. Louis, MO); Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), and antibiotic-antimycin from Gibco Laboratories (Grand Island, NY); pronase from Cal-Biochem-Novabiochem (La Jolla, CA); [<sup>125</sup>I]thyroxine (specific activity: 4.4 Ci/μmol), SOLVABLE, and EN<sup>3</sup>HANCE autoradiography enhancer from Du Pont (Boston, MA); L-methionine, L-glutamine, and [<sup>35</sup>S]methionine (specific activity: 1218 Ci/mmol) from ICN Biomedicals (Aurora, OH); transwell-COL culture wells from Costar (Cambridge, MA). All reagents were of analytical grade, HPLC grade, or the best available pharmaceutical grade.

### Preparation of TTR antibody

Purified rat plasma TTR and monospecific rabbit anti-rat TTR polyclonal antibody were prepared according to the methods previously described (Navab *et al.*, 1977; Blaner, 1990). The purified rat serum TTR used as immunogen ran as two bands on an overloaded (50 μg protein) Coomassie-stained SDS-PAGE, one at approximately 14 KDa (corresponding to the TTR monomer) and one at 28 KDa (corresponding to the TTR dimer). Collected rabbit antiserum was divided into aliquots and frozen at -80°C until used in the present and earlier studies (Herbert *et al.*, 1986; Episkopou *et al.*, 1993; Tsutsumi *et al.*, 1992; Zheng *et al.*, 1996, 1998). The TTR antiserum was diluted (1:300) prior to use in this study.

### Choroidal epithelial cell culture

Choroidal epithelial cells were cultured using the method established in this laboratory (Zheng *et al.*, 1998). In short, plexus tissues were collected from Sprague-Dawley rats (4–6 weeks old, both sexes), which were purchased from Harlan Inc. (Indianapolis, IN). The plexuses were dissected, washed in DMEM, chopped with scissors, and digested in Hank's buffer containing 0.2% pronase at 37°C for 5 min. The cells were further mechanically dissociated by 7–8 forced passages through a 20-gauge needle. The dissociated cells were washed in medium A (DMEM with 100 units/ml each of penicillin, streptomycin, and gentamycin, and 0.25 μg/ml amphotericin B) twice and resuspended in normal growth medium (medium A supplemented with 10% FBS and 10 ng/ml EGF). The cells were plated in 35-mm Petri dishes (2–3 × 10<sup>5</sup> cells per dish) and cultured in a humidified incubator with

95% air–5% CO<sub>2</sub> at 37°C. The growth medium was replaced two days after initial seeding and every other day thereafter. The culture from 5-week-old rats showed a dominant polygonal type of epithelial cells for at least 7–10 days with a doubling time about 3–4 days. A 6–8-day culture was used in this study.

For two-chamber transport studies, permeable membranes attached to the Transwell-COL culture wells were pretreated with laminin (14 µg/ml) for 10 min and allowed to air dry for at least 45 min prior to cell seeding. Aliquots (0.5 ml) of cell suspension were plated into 12-mm laminin-coated culture wells ( $2 \times 10^5$  cells per well). This was designated as the inner (apical) chamber. The inner chambers were then inserted into the outer (basal) chambers which contained 1 ml of culture medium. The cultures continued for 48 h and the medium was changed every 2 days thereafter. The formation of an impermeable confluent monolayer occurred within 5 days after seeding and was verified by the presence of a steady electrical resistance across the membrane ( $120 \pm 10$  SD ohm per cm<sup>2</sup>) (Southwell *et al.*, 1993; Zheng *et al.*, 1998).

### Pulse-chase study of TTR synthesis and secretion

Prior to Pb exposure, the cultured epithelial cells were washed 3 times with medium A. The cells were then exposed to Pb (as Pb acetate) dissolved in medium A at a final concentration of 30 µM at 37°C for 4 h. The concentration of Pb (30 µM) was chosen because preliminary studies under this experimental condition had shown that Pb at this concentration caused the maximum inhibition of T<sub>4</sub> transport and minimum cytotoxicity. At the end of exposure, the cells were rinsed and incubated for 40 min in medium B (serum- and methionine-free medium A) to deplete the intracellular methionine pool (Wang *et al.*, 1994). The cultured cells were pulse-labeled for 2 h in a pulse medium (medium B supplemented with 100–150 µCi/ml [<sup>35</sup>S]methionine). Following the pulse treatment, the cells were washed 3 times with the chase medium (medium B supplemented with 10 mM L-methionine and 2 mM L-glutamine). The chase phase commenced by adding 1 ml of the chase medium to each group. At the times selected, the media were removed. The cells were washed with lysis buffer (see below) and harvested using a rubber policeman.

The harvested cells were immediately mixed with 200 µl of warm lysis buffer that consisted of 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 1% Triton X-100, 1% SDS, and 0.1% BSA. The mixture was incubated at 70°C for 15 min to ensure complete lysis. The culture medium was centrifuged at 10,000g for 10 min to remove cell debris, after which aliquots (300 µl) of medium were mixed with 100 µl of 4× lysis buffer and incubated at 70°C for 15 min. Both cell and medium lysates were then diluted 10-fold with 150 mM NaCl and 0.1% BSA in 10 mM Tris buffer (pH 8.0) to achieve a 0.1% SDS final concentration. The diluted solutions were used for separation and quantitation of [<sup>35</sup>S]TTR described below. Another aliquots (50 µl) of cell and medium lysates were diluted with distilled, deionized water and used for protein determination.

## Immunoprecipitation and SDS-PAGE

Aliquots (300  $\mu$ l) of diluted cell lysate and culture medium were incubated with rabbit anti-rat TTR antiserum (1:300) at room temperature for 2 h. The TTR-antibody complexes were precipitated by adding 100  $\mu$ l of Protein A agarose beads (Bio-Rad) in 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 0.05% Triton X-100 and incubating at 4°C overnight with constant shaking. Following centrifugation at 10,000g for 10 min, the pellets were dissolved and incubated in eluting buffer containing 0.1 M Tris (pH 6.8), 5% SDS, 5% 2-mercaptoethanol, 10 mM DTT, and 10% glycerol at 95°C for 5 min. The mixture was further centrifuged at 13,000g for 10 min to separate the eluted proteins from the beads. The supernatant containing primarily [<sup>35</sup>S]TTR underwent further purification.

Aliquots (20  $\mu$ l) of the supernatant, purified rat TTR standard, and protein molecular weight markers were applied to two identical 8.5% SDS-polyacrylamide gels and electrophoresized under constant current of 30 mA per gel. One gel was stained with Coomassie brilliant blue reagent for 4–6 h. The bands corresponding to TTR standard and molecular weight at 14 kDa were excised and dissolved in 0.5 ml of SOLVABLE solution at 50°C for 3 h. Incorporation of [<sup>35</sup>S]methionine into newly synthesized TTR was then determined by scintillation counting in a Packard Tri-Carb Model 2100TB liquid scintillation counter. The other gel was immersed in 100 ml of EN<sup>3</sup>HANCE solution for 1 h for enhancement and completely dried using a Model Gel Dryer (Bio-Rad) at 70°C for 2 h. The dried gels were then exposed to Kodak Biomax MR film for 4–7 days using an intensifying screen.

## Transport of [<sup>125</sup>I]-T<sub>4</sub> at two-chamber culture system

Choroidal epithelial cells were plated onto a laminin-coated permeable membrane, which was on the bottom of the inner chamber. The inner chamber was immersed in the medium of the outer chamber. The formation of a confluent monolayer occurred normally within 5 days after initial plating. The cells under this condition displayed a similar morphology to that observed in plastic culture dishes and survived for at least 2 weeks (Southwell *et al.*, 1993; Zheng *et al.*, 1998). Upon the formation of an impermeable monolayer at Day 7–8, the cells cultured on the Transwell-COL wells were washed twice with medium C (serum-free DMEM supplemented with 5  $\mu$ g/ml each of insulin and transferrin, 5 ng/ml each of sodium selenite and FGF, 10 ng/ml EDF, and 25  $\mu$ g/ml PE<sub>1</sub>). The cells were then exposed to 30  $\mu$ M Pb in medium C of both chambers for 4 h. At the end of Pb exposure, [<sup>125</sup>I]T<sub>4</sub> was added into the outer chamber to the final concentration of 40 pM (0.18  $\mu$ Ci/ml). A volume (5  $\mu$ l) of media in both chambers was removed at various times and determined for radioactivity using a Packard model Cobra-II gamma counter.

The method of Bradford (1976), using bovine serum albumin as the reference, was used for all protein determinations.

## Statistics

Concentrations of TTR and proteins in cells and culture media as affected by time and Pb treatment were analyzed by two-way analysis of variance (ANOVA). When ANOVA revealed an overall treatment effect, contrast analyses were performed at individual time points using Scheffe's multiple comparison test (Scheffe, 1967).

## RESULTS

### Expression of TTR by Cultured Epithelial Cells

When the newly synthesized proteins were pulse-labeled with [<sup>35</sup>S]methionine, <sup>35</sup>S-labeled species that were selectively immunoprecipitated showed two major bands on SDS-PAGE gels with the molecular weight equivalent to 14,000 and 43,000 Dalton (Fig. 1). Since TTR is a 55,000-Da protein consisting of four identical subunits in a tetrahedral symmetry, the bands at 14,000 Da in fact represent a single dissociated chain of TTR subunits. As the bands at 43,000 Da were usually poorly displayed in the medium preparations, the bands may represent a nonspecific species which was present in, but not secreted by, the cells. Therefore, we chose 14,000-Da bands to monitor [<sup>35</sup>S]TTR in the subsequent studies. As shown in Fig. 1, after the pulse treatment, the newly synthesized [<sup>35</sup>S]TTR molecules increased in the culture media, while decreased in the cultured cells, suggesting that the choroidal epithelial cells in our primary culture possessed the ability to synthesize and release TTR.

### Effect of Pb on Protein and Total TTR Synthesis

When cell lysates and culture media were assayed for protein concentrations, Pb treatment had no significant effect on the concentrations of total proteins presented in either the cell lysates or culture media (Table 1).

The total [<sup>35</sup>S]TTR production was estimated by summation of radioactivity in 14,000-Da bands in both cell lysates and media, and was normalized for protein concentration. Two-way ANOVA of the data presented in Fig. 2 revealed a marginal overall inhibitory effect of Pb on total TTR ( $p < 0.074$ ). However, Pb significantly inhibited total [<sup>35</sup>S]TTR by 37% ( $p < 0.01$ ) at 2 h and 34% ( $p < 0.01$ ) at 3 h, respectively, following the chase (Fig. 2). It is noteworthy that the onset of Pb action appeared in a delayed temporal pattern. There was no significant change in total [<sup>35</sup>S]TTR until the cells were chased for more than 1 h (Fig. 2).

### Effect of Pb on Intracellular TTR

Figure 3A delineates the time courses of intracellular newly synthesized [<sup>35</sup>S]-TTR in cell lysates. Intracellular [<sup>35</sup>S]TTR in the control group appeared to decline over the 3-h chase period, indicating either a continuous secretion of [<sup>35</sup>S]TTR molecules from the intracellular space to extracellular media or the destruction of [<sup>35</sup>S]TTR. By linear regression of the terminal phase (0.5–3 h) of TTR concentration-time curve, the intracellular half-life ( $t_{1/2}$ ) of the newly synthesized [<sup>35</sup>S]TTR approximated 2.6 h in the control group (Fig. 3A). In contrast, the concentrations of cellular [<sup>35</sup>S]TTR in the Pb-treated group were maintained at a relatively stable level. While Pb exposure resulted in an increase in overall cellular concentrations of [<sup>35</sup>S]TTR in comparison to the controls, this was not statistically significant by two-way ANOVA ( $p < 0.0929$ ). However, the cellular concentrations of [<sup>35</sup>S]TTR at the later times of the chase phase were significantly higher in the Pb-treated group than those in the controls ( $p < 0.01$ ) (Fig. 3A). Autoradiography of TTR bands on the gel also shows that Pb treatment caused an intracellular retention of [<sup>35</sup>S]TTR (Fig. 3B).

### Inhibition of TTR Secretion by Pb

Pb exposure suppressed the secretion of newly synthesized [<sup>35</sup>S]TTR from epithelial cells to the culture media. Two-way ANOVA revealed that there was an overall significant statistical difference in [<sup>35</sup>S]TTR of culture media between the Pb-treated group and the control group ( $p < 0.05$ , Fig. 4A). The difference was greatest at later times. For example, TTR concentration in medium at 3 h following the chase was 7.5-fold lower in Pb groups than in the controls. As depicted by autoradiography in Fig. 4B, the densities of TTR bands in Pb-treated groups were accordingly lower than those in the corresponding controls.

It is noteworthy that Pb treatment at this concentration did not significantly alter the cell viability, nor did it induce any visible morphological alterations in cultured cells (data not shown). In addition, we found that the same Pb treatment did not significantly inhibit the uptake of [<sup>35</sup>S]methionine by the cultured choroidal epithelial cells ( $p > 0.1$ ,  $n = 3$ ).

### Alteration by Pb of T<sub>4</sub> Transepithelial Transport at the Blood-CSF Barrier

We further established a two-chamber system to study the transport kinetics of [<sup>125</sup>I]T<sub>4</sub> across the epithelial barrier by continuously monitoring the radioactivity in both chambers. Our earlier studies (Zheng *et al.*, 1998) have shown that in the absence of cultured cells, addition of [<sup>125</sup>I]T<sub>4</sub> to the outer chamber results in a concentration-driven diffusion of [<sup>125</sup>I]T<sub>4</sub> between two chambers. The radioactivity eventually reaches the same level in both chambers, indicating an equilibrium status. In the presence of cells, when [<sup>125</sup>I]T<sub>4</sub> was added to the outer chamber (contacting the basement of epithelial cells), the radioactivity migrates from the outer to inner chamber (contacting apical surface of epithelial cells) to a higher concentration in the inner chamber. The current work was carried out under similar experimental conditions to our previous study.

As shown in Fig. 5A, after addition of [<sup>125</sup>I]T<sub>4</sub> into the outer chamber, the radioactivity in the inner chamber of the control group rose steadily at the early stage. There was a linear increase in radioactivity in the inner chamber during the first 6 h. This was followed by a slower ascending phase until the plateau was reached (Fig. 5A). In the Pb-treated group, the steady-state concentration ( $C_{ss}$ ) of radiolabels in the inner chamber was 27% lower as compared to the control (Table 2).

In parallel with the changes of radioactivity in the inner chamber, [<sup>125</sup>I]T<sub>4</sub> in the outer chamber declined in the initial phase, indicating an uptake of [<sup>125</sup>I]T<sub>4</sub> by epithelial cells (Fig. 5B). The initial uptake rate constant ( $k_u$ ) of [<sup>125</sup>I]T<sub>4</sub> in the control group approximated  $0.15 \text{ h}^{-1}$ , while in the Pb-treated group, the rate constant was reduced to  $0.10 \text{ h}^{-1}$ , about 33% decrease (Table 2). The steady state concentration of radiolabels in the outer chamber of the Pb-treated group was 41% higher than that of the controls. These data are consistent with the notion that exposure of the cells to Pb in the culture medium repressed the transport of [<sup>125</sup>I]T<sub>4</sub> radioactivity from the outer to the inner chamber.

## DISCUSSION

Our previous studies by RT-PCR analysis (reverse-transcriptase polymerase chain reaction) and immunocytochemistry have demonstrated that the cultured choroidal epithelial cells

established in this laboratory possess specific TTR mRNA and synthesize and secrete TTR proteins (Zheng *et al.*, 1998). The pulse-chase studies presented in this report further corroborate that these cultured epithelial cells dynamically produce and secrete TTR (Fig. 1). Our observations support the view that the choroid plexus epithelia serve as an important site of TTR production in the CNS (Aldred *et al.*, 1995; Herbert *et al.*, 1986; Schreiber *et al.*, 1995).

Our initial studies in rats suggest that the sequestration of Pb in the choroid plexus following chronic Pb exposure accompanies a diminished CSF TTR (Zheng *et al.*, 1996). The present studies extend our previous observations and further show that Pb exposure significantly inhibits the total TTR (cells + media) production by cultured choroidal epithelial cells (Fig. 2), while the concentrations of total protein in both cultured cells and media were not significantly altered by Pb exposure (Table 1). The cytotoxicity of Pb has been associated with the suppression of biosynthesis of a number of cellular proteins, largely owing to the strong binding of the metal to cellular sulfhydryl groups (Goering, 1993). As we did not examine the expression of TTR mRNA before and after Pb treatment, the questions as to how and at what stage Pb influences TTR biosynthesis, as well as possible questions regarding gene regulation processes in the cultured cells, remain unanswered.

Pb exposure significantly inhibits the rate and amount of newly synthesized [<sup>35</sup>S]TTR secreted into the cultured media (Fig. 4). The effect of Pb on TTR secretion could be due to a direct interaction of Pb ions on TTR secretion processes. The decline of intracellular TTR concentration could occur as the results of a continuous outflow of the newly synthesized [<sup>35</sup>S]TTR to the culture medium, coupled with intracellular enzymatic or nonenzymatic degradation of TTR. Accordingly, a blockage of TTR secretion should bring about the retention of TTR within the cells. This, indeed, appears to be the case in our studies. While Pb treatment decreased the secretion rate of new [<sup>35</sup>S]TTR molecules into the extracellular space (Fig. 4A), the intracellular TTR following Pb exposure remained at a constant level (Fig. 3A), which reflects a retarded outflow for newly synthesized [<sup>35</sup>S]TTR to exit to the culture media.

Whereas the internalization of TTR is reportedly mediated by TTR receptors on the cell surface (Divino and Schussler, 1990), the processes that govern TTR secretion from the plexus to the CSF are as yet unknown. Accordingly, the mechanism(s) whereby Pb interacts with TTR secretion remains a subject for speculation. We recently observed that Pb potently inhibited the activity of mitochondrial complex-I (NADH-ubiquinone reductase), the enzyme that is critical to the energy production in mitochondrial respiratory chain (unpublished data). Others have reported that Pb inhibits cellular ATPases (Chanez *et al.*, 1986; Rajanna *et al.*, 1991). In addition, we have demonstrated that Pb exposure promotes the translocation of protein kinase C (PKC) from the cytosol to membrane in the choroid plexus (Zhao *et al.*, 1998). We do not know whether the alterations in energy production or PKC activity by Pb underlie, either directly or indirectly, the inhibitory action of Pb on TTR secretion. It is noteworthy, however, that PKC plays a crucial role in a variety of cellular functions such as in transducing cellular signals, in regulating membrane ion channels, and in controlling phosphorylation of key enzymes and proteins (Nishizuka, 1986; Bressler *et al.*, 1996).



The reduction in [<sup>35</sup>S]TTR secretion could also be attributable to an arrested *de novo* biosynthesis or an increased rate of intracellular degradation of TTR in Pb-exposed cells. The lack of sufficient intracellular TTR would then lead to less TTR molecules available for secretion. As shown in Fig. 2, Pb treatment inhibited the total TTR, particularly at later time. Interestingly, this delayed onset of Pb effect on TTR concentration (Fig. 2) coincided with a greater suppression of TTR secretion observed at later times of the chase phase (Fig. 4A). Thus, the inhibitory effect of Pb on TTR synthesis might contribute, at least in part, to the diminished secretion of TTR. In addition, the possibility that Pb may directly bind to TTR molecules needs to be considered and substantiated in future studies.

The intracellular [<sup>35</sup>S]TTR present in Pb-treated cells, however, did not seem to decline greatly, but rather stayed in a relatively constant level (Fig. 3). This was expected. If Pb had only suppressed the secretion process without influencing the TTR production, the cellular TTR would have continuously built up and led to a time-related increase in intracellular [<sup>35</sup>S]TTR. Based on the results in Fig. 3, a constant level of cellular TTR in the Pb-treated group may reflect a compromised effect of Pb on the inhibition of TTR secretion (resulting in cellular TTR increases) and the repression of TTR production (resulting in cellular TTR decreases). Taken in conjunction, these studies establish that the abnormal dynamics in TTR secretion and/or production in the choroid plexus occurs as a consequence of Pb sequestration in this blood-CSF barrier.

The bulk of evidence from the past two decades suggests that TTR is importantly involved in the transport of thyroxine from the blood to the cerebral compartment (Chanoine *et al.*, 1992; Ingenbleek and Young, 1994; Nilsson *et al.*, 1992; Schreiber *et al.*, 1990, 1995). Chanoine *et al.* (1992) have shown that in wild-type rats, the injection with a competitive inhibitor for T<sub>4</sub>-TTR binding results in a significant reduction in the percentage of injected [<sup>125</sup>I]T<sub>4</sub> in the choroid plexus, CSF, cerebral cortex, and cerebellum. Nilsson *et al.* (1992) reported that rats administered with cycloheximide (a protein synthesis inhibitor) displayed an altered pattern of T<sub>4</sub> distribution in the choroid plexus and other brain regions. As the choroid plexus expresses TTR genes early in the fetal development (Thomas *et al.*, 1989; Cavallaro *et al.*, 1993; Schreiber *et al.*, 1995), expression of TTR in this tissue may help to control thyroid hormone homeostasis in the CNS from early in fetal development throughout life and play a fundamental role in brain development. This possibility is argued against by the works of Palha *et al.* (1994), which indicate that TTR-deficient mice are euthyroid and “phenotypically normal.” However, it is possible that these congenitally TTR-deficient mice may not serve as an appropriate model for study of thyroid hormone delivery to the brain, since expression of the thyroid-binding globulin gene may seem to be turned on at early stage of embryo development to compensate for the loss of TTR expression. Whether this process in TTR knock-out *mice* would also occur in wild-type *rats* born with developed thyroid regulatory system but under excess Pb exposure is unknown. It is also possible that other compensatory mechanisms might give rise to the “normal” phenotype of these mice. Notably, whether this mutant strain of mice are “phenotypically normal” with regard to the behavioral/neurologic parameters remains uninvestigated. It is not clear whether the absence of TTR in mice has more subtle and still unexplored effects on brain thyroid hormone economy similar to those observed in rats by Chanoine *et al.* (1992) and Nilsson *et al.*

(1992). Such subtle differences in brain thyroid hormone economy may underlie the cognitive and/or behavioral deficits not being investigated in the TTR-deficient mice by Palha *et al.* (1994).

The data from our chamber study, which is a reproduction of that of Southwell *et al.* (1993), showed that the presence of Pb in the culture medium markedly inhibited the transport of [<sup>125</sup>I]T<sub>4</sub> across the choroidal epithelial barrier (Fig. 5 and Table 2). The reduction of TTR production/secretion caused by Pb treatment (Figs. 2–4) could explain the effect of Pb on T<sub>4</sub> transport at this barrier. Hence, these studies are consistent with the hypothesis that normal synthesis and secretion of TTR by the choroid plexus epithelia are needed to maintain normal T<sub>4</sub> transport at the blood-CSF barrier. If this *in vitro* observation can be extended to the *in vivo* situation, then distortion of TTR production/secretion in the choroid plexus by Pb exposure would be expected to impair the transport of thyroid hormones from the blood to the cerebral compartment. Such an impairment could account for the known loss of cognitive abilities observed in Pb-poisoned children. This notion, however, will require further experimental proof before it can be accepted.

In conclusion, the results of current pulse-chase studies revealed that *in vitro* Pb exposure reduced the production and secretion of TTR by cultured choroid plexus epithelial cells. Experiments using a two-chamber polarized cell culture model of the blood-CSF barrier further demonstrated that this reduced production and secretion of TTR upon Pb exposure is coincident with reduced transport of [<sup>125</sup>I]T<sub>4</sub> across the model choroidal epithelial barrier. This association between lessened synthesis and secretion of TTR and lessened T<sub>4</sub> transport at the blood-CSF barrier, along with the question of whether the possible impaired transport of T<sub>4</sub> via TTR ultimately affects whole brain economy of thyroid hormones which these observations raise, deserves further investigation.

## Acknowledgments

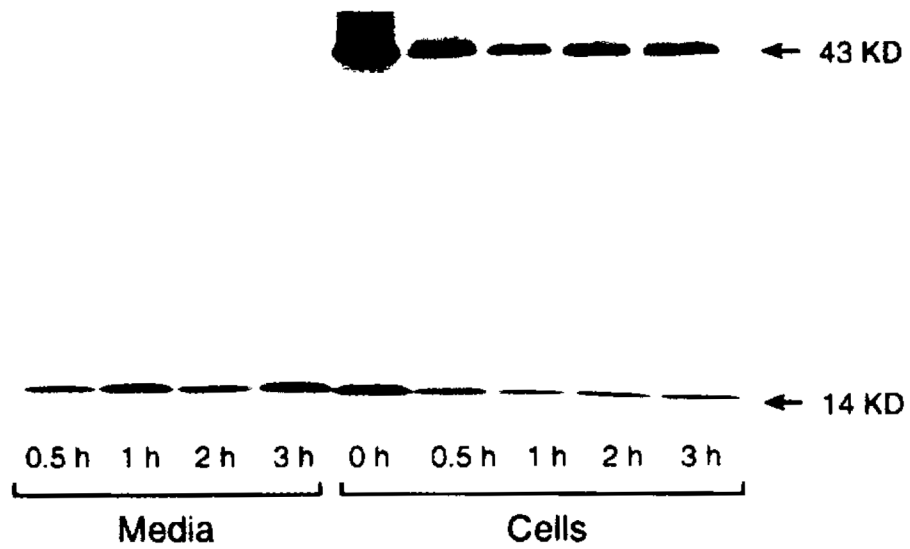
We are greatly indebted to Dr. Bridget R. Southwell for her critical review and many helpful suggestions for this manuscript. This research was supported by National Institute of Environmental Health Sciences Grant ES08146.

## References

- Aldred AR, Brack CM, Schreiber G. The cerebral expression of plasma protein genes in different species. *Comp Biochem Physiol.* 1995; 111B:1–15.
- Blaner WS. Radioimmunoassays for retinol-binding protein, cellular retinol-binding protein, and cellular retinoic acid-binding protein. *Methods Enzymol.* 1990; 189:270–281. [PubMed: 1963461]
- Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
- Bressler JP, Belloni-Olivi L, Forman S, Goldstein GW. Distinct mechanisms of neurotransmitter release from PC12 cells exposed to lead. *J Neurosci Res.* 1996; 46:678–685. [PubMed: 8978502]
- Cavallaro T, Martone RL, Stylianopoulou F, Herber J. Differential expression of the insulin-like growth factor-II and transthyretin genes in the developing rat choroid plexus. *J Neuropathol Exp Neurol.* 1993; 52:153–162. [PubMed: 8440997]
- Chanez C, Giguere JF, Flexor MA, Bourre JM. Effect of lead on Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in the developing brain of intra-uterine growth-retarded rats. *Neurochem Pathol.* 1986; 5:37–49. [PubMed: 3031558]

- Chanoine JP, Alex S, Fang SL, Stone S, Leonard JL, Korhle J, Braverman LE. Role of transthyretin in the transport of thyroxine from the blood to the choroid plexus, the cerebrospinal fluid, and the brain. *Endocrinology*. 1992; 130:933–938. [PubMed: 1733735]
- Davson, H.; Segal, MB. *Physiology of the CSF and Blood-Brain Barrier*. CRC Press; New York: 1996.
- Dickson PW, Aldred AR, Marley PD, Tu GF, Howlett GJ, Schreiber G. High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. *Biochem Biophys Res Commun*. 1985; 127:890–895. [PubMed: 3838668]
- Divino CM, Schussler GC. Receptor-mediated uptake and internalization of transthyretin. *J Biol Chem*. 1990; 265:1425–1429. [PubMed: 2153133]
- Dratman MB, Crutchfield FL, Schoenhoff MB. Transport of iodothyronines from bloodstream to brain: Contributions by blood:brain and choroid plexus: cerebrospinal fluid barrier. *Brain Res*. 1991; 554:229–236. [PubMed: 1933305]
- Dussault JH, Ruel J. Thyroid hormones and brain development. *Annu Rev Physiol*. 1987; 49:321–334. [PubMed: 3551803]
- Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME, Robertson EJ. Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proc Natl Acad Sci USA*. 1993; 90:2375–2379. [PubMed: 8384721]
- Farsetti A, Mitsuhashi T, Desvergne B, Robins J, Nikodem VM. Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. *J Biol Chem*. 1991; 266:23226–23232. [PubMed: 1720778]
- Friedheim E, Corvi C, Graziano J, Donnelly T, Breslin D. Choroid plexus as protective sink for heavy metals? *Lancet*. 1983; i(8331):981–982. [PubMed: 6132283]
- Glorieux J, Dussault JH, Letarte J, Guyde H, Morissette J. Preliminary results on the mental development of hypothyroid children detected by the Quebec Screening Program. *J Pediatr*. 1983; 102:19–22. [PubMed: 6848724]
- Goering PL. Lead-protein interactions as a basis for lead toxicity. *Neurotoxicology*. 1993; 14:45–60. [PubMed: 8247411]
- Hagen GA, Elliott WJ. Transport of thyroid hormones in serum and cerebral spinal fluid. *J Clin Endocrinol Metab*. 1973; 37:415–422. [PubMed: 4206491]
- Herbert J, Wilcox JN, Pham KC, Fremeau RT, Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Zimmerman EA, Roberts JL, Schon EA. Transthyretin: A choroid plexus-specific transport protein in human brain. *Neurology*. 1986; 36:900–911. [PubMed: 3714052]
- Ingenbleek Y, Young V. Transthyretin (prealbumin) in health and disease: Nutritional implications. *Annu Rev Nutr*. 1994; 14:495–533. [PubMed: 7946531]
- Johanson, CE. Ventricles and cerebrospinal fluid. In: Conn, PM., editor. *Neuroscience in Medicine*. Lippincott; Philadelphia, PA: 1995. p. 171-196.
- Larsen PD, DeLallo L. Cerebrospinal fluid transthyretin in the neonate and blood-cerebrospinal fluid barrier permeability. *Ann Neurol*. 1989; 25:628–630. [PubMed: 2742364]
- Legrand, J. Effect of thyroid hormones on central nervous system development. In: Yanai, J., editor. *Neurobehavioral Teratology*. Elsevier; New York: 1984. p. 331-363.
- Navab M, Smith JE, Goodman DS. Rat plasma prealbumin. Metabolic studies on effects of vitamin A status and on tissue distribution. *J Biol Chem*. 1977; 252:5107–5114. [PubMed: 559674]
- Nilsson C, Lindvall-Axelsson M, Owman C. Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Res Rev*. 1992; 17:109–138. [PubMed: 1393190]
- Nishizuka Y. Studies and perspectives of protein kinase C. *Science*. 1986; 233:305–312. [PubMed: 3014651]
- Palha J, Episkopou V, Maeda S, Shimada K, Gottesman ME, Saraiva MJM. Thyroid hormone metabolism in a transthyretin-null mouse strain. *J Biol Chem*. 1994; 269:33135–33139. [PubMed: 7806543]
- Rajanna B, Chetty CS, Stewart TC, Rajanna S. Effects of lead on pH and temperature-dependent substrate-activation kinetics of ATPase system and its protection by thiol compounds in rat brain. *Biomed Environ Sci*. 1991; 4:441–451. [PubMed: 1664209]

- Ruiz-Marcos A, Sanchez-Toscano F, Morreale de Rey F, Morreale de Escobar G. Severe hypothyroidism and the maturation of the rat cerebral cortex. *Brain Res.* 1979; 162:315–329. [PubMed: 761091]
- Scheffe, H. *The Analysis of Variance*. John Wiley & Sons; New York: 1967.
- Schreiber G, Aldred AR, Jaworowski A, Nilsson C, Achen MG, Segal MB. Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. *Am J Physiol.* 1990; 258:R338–R345. [PubMed: 2309926]
- Schreiber G, Southwell BR, Richardson SJ. Hormone delivery systems to the brain-transthyretin. *Exp Clin Endocrinol Diabet.* 1995; 103:75–80.
- Smith DW, Blizzard RM, Wilkins L. The mental prognosis in hypothyroidism of infancy in childhood. *Pediatrics.* 1957; 19:1011–1022. [PubMed: 13441356]
- Smith QR. The blood-brain barrier and the regulation of amino acid uptake and availability to brain. *Adv Exp Med Biol.* 1991; 291:55–71. [PubMed: 1927691]
- Southwell BR, Duan W, Alcorn D, Brack C, Richardson SJ, Kohrle J, Schreiber G. Thyroxine transport to the brain: Role of protein synthesis by the choroid plexus. *Endocrinology.* 1993; 133:2116–2126. [PubMed: 8404661]
- Thomas T, Schreiber G, Jaworowski A. Developmental patterns of gene expression of secreted proteins in brain and choroid plexus. *Dev Biol.* 1989; 134:38–47. [PubMed: 2471663]
- Thompson C. Thyroid hormone-responsive genes in developing cerebellum include a novel synaptotagmin and a hairless homolog. *J Neurosci.* 1996; 16:7832–7840. [PubMed: 8987811]
- Tsutsumi C, Okuno M, Tannous L, Piantedosi R, Allen M, Goodman DS, Blaner WS. Retinoids and retinoid-binding protein expression in rat adipocytes. *J Biol Chem.* 1992; 267:1805–1810. [PubMed: 1370481]
- Wang H, Yao Z, Fisher EA. The effects of n-3 fatty acids on the secretion of carboxyl-terminally truncated forms of human apolipoprotein B. *J Biol Chem.* 1994; 269:18514–18520. [PubMed: 8034600]
- Zhao Q, Slavkovich V, Zheng W. Lead exposure promotes translocation of protein kinase C activities in rat choroid plexus *in vitro*, but not *in vivo*. *Toxicol Appl Pharmacol.* 1998; 149:99–106. [PubMed: 9512732]
- Zheng, W. The choroid plexus and metal toxicities. In: Chang, LE., editor. *Toxicology of Metals*. CRC Press; New York: 1996. p. 609-626.
- Zheng W, Perry DF, Nelson DL, Aposhian HV. Protection of cerebrospinal fluid against toxic metals by the choroid plexus. *FASEB J.* 1991; 5:2188–2193. [PubMed: 1850706]
- Zheng W, Shen H, Blaner SB, Zhao Q, Ren X, Graziano JH. Chronic lead exposure alters transthyretin concentration in rat cerebrospinal fluid: The role of the choroid plexus. *Toxicol Appl Pharmacol.* 1996; 139:445–450. [PubMed: 8806863]
- Zheng W, Zhao Q, Graziano JH. Primary culture of rat choroidal epithelial cells: A model for *in vitro* study of the blood-cerebrospinal fluid barrier. *In Vitro Cell Biol Dev.* 1998; 34:40–45.



**FIG. 1.** Presence of newly synthesized TTR in cells and media from choroid plexus epithelial cell culture. Cultures were incubated with [<sup>35</sup>S]methionine for 2 h. At the times shown, cell lysates and culture media were collected and immunoprecipitated with TTR antibodies and protein A beads. Aliquots (20  $\mu$ l) of TTR immunoprecipitate were separated by SDS PAGE and exposed to X-ray film for 7 days. TTR migrates with apparent MW of 14 KDa.

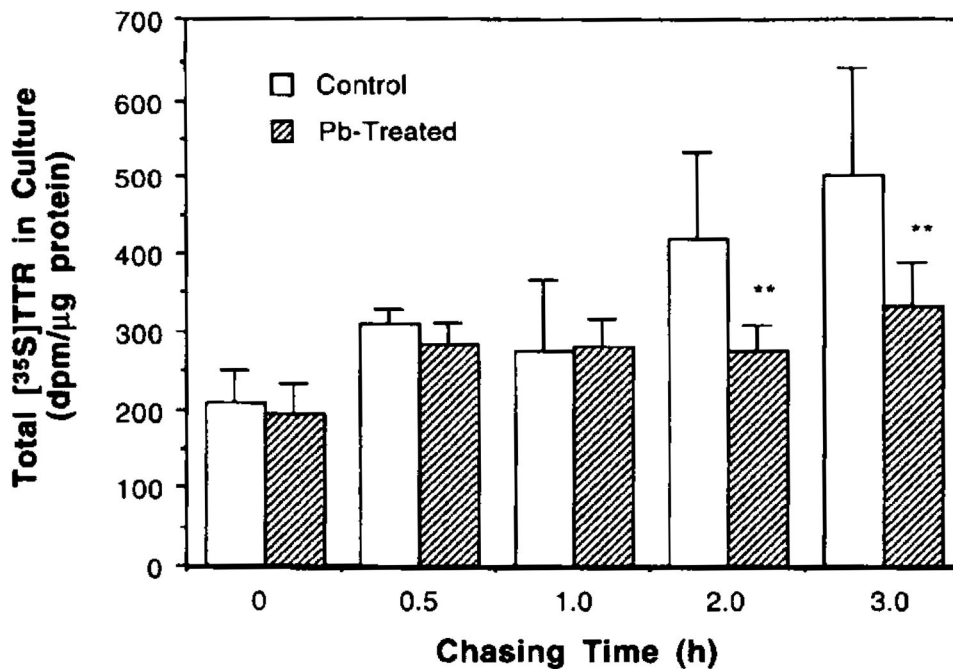
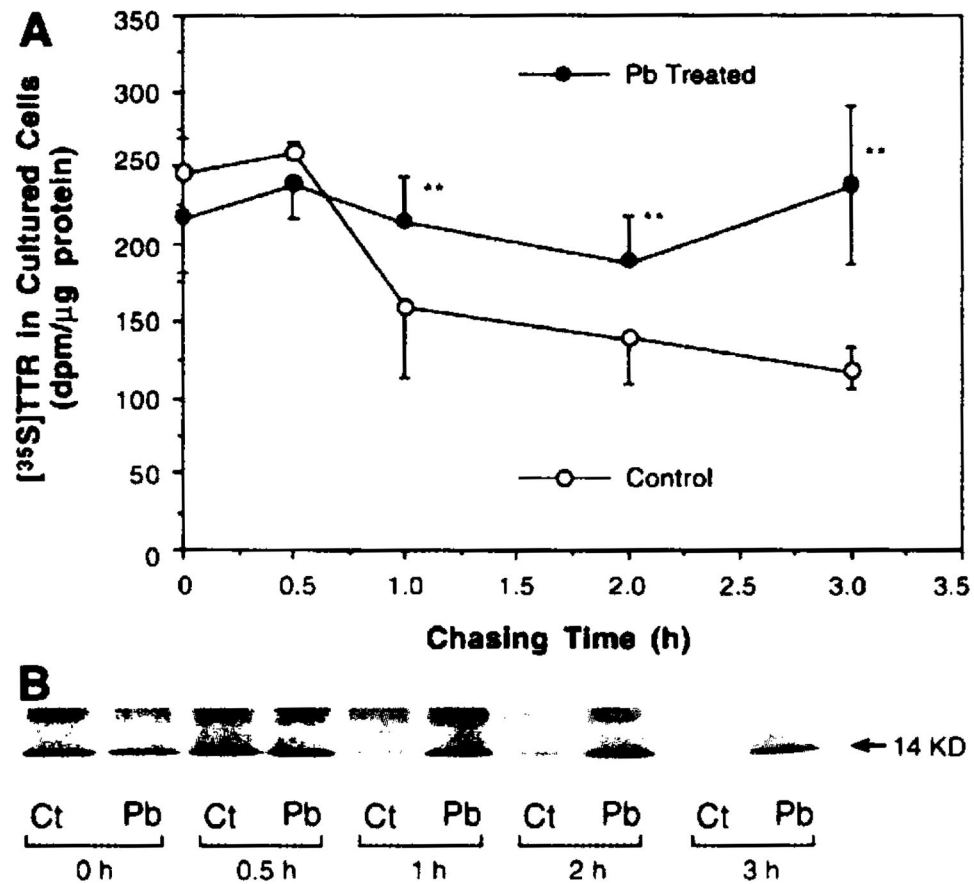
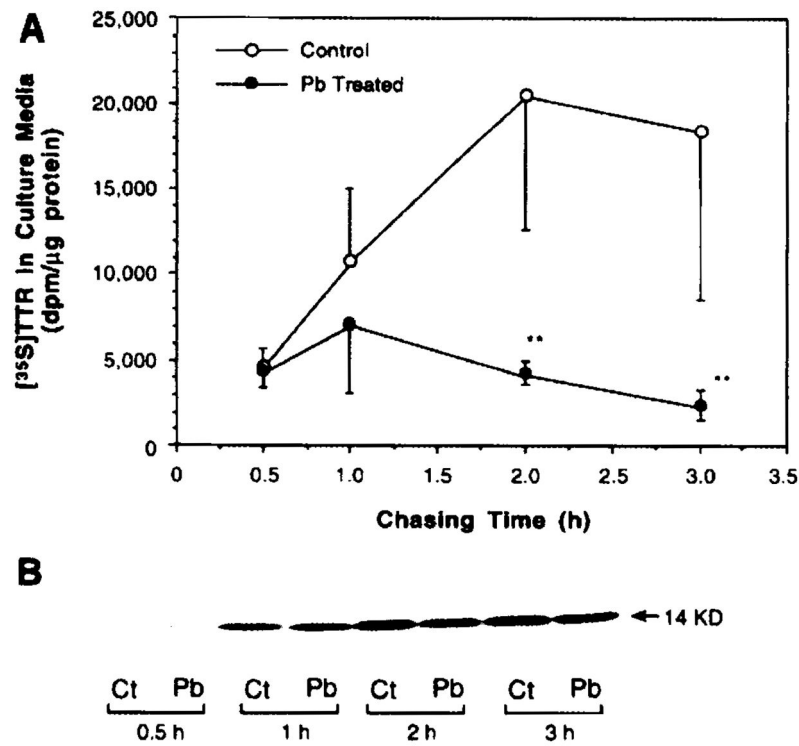


FIG. 2.

Effect of Pb on total [ $^{35}\text{S}$ ]TTR production. Total [ $^{35}\text{S}$ ]TTR was estimated by summation of radioactivity in 14-kD bands derived from both cell lysates and media and normalized for protein concentrations, except for time 0 where only cell lysate data are presented. Data represent means  $\pm$  SE ( $n = 5$ ).  $p = 0.074$  when the data in total of Pb-treated groups were compared to total of control groups by two-way ANOVA. \*\* $p < 0.01$ .

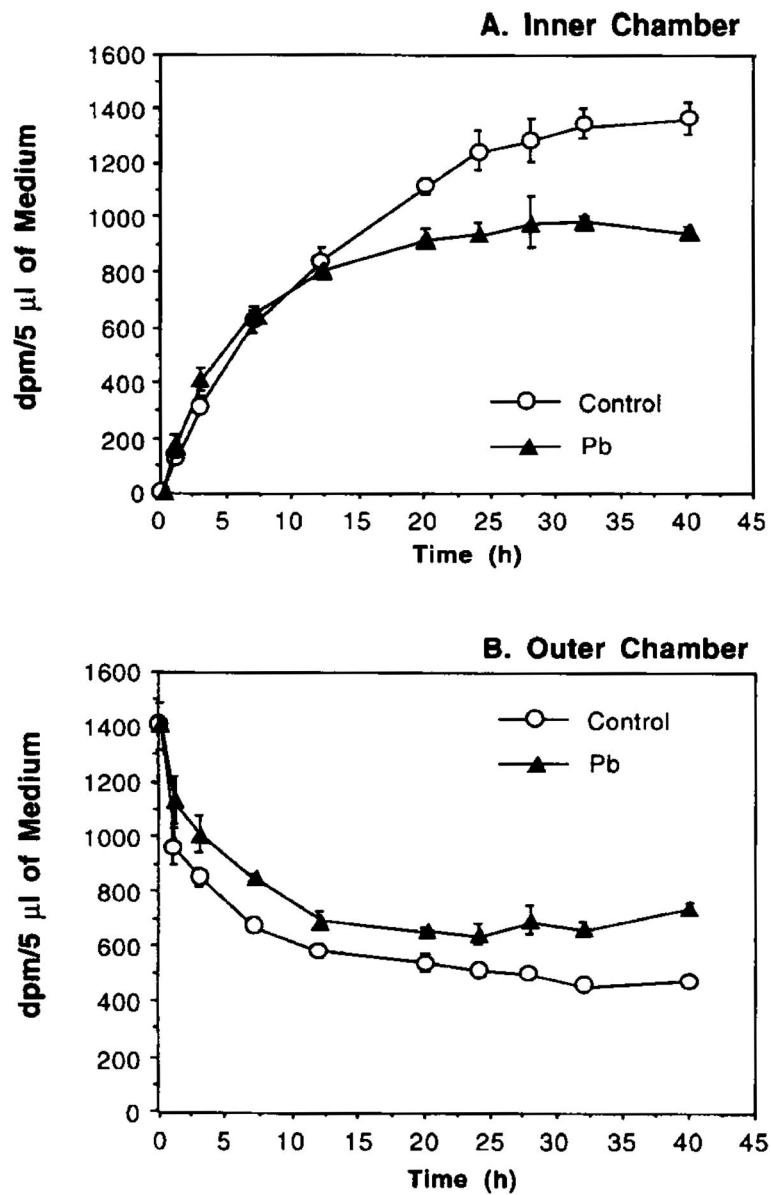


**FIG. 3.** Effect of Pb exposure on the newly synthesized [ $^{35}\text{S}$ ]TTR in the cultured cells. The cultured cells were lysed and immunoprecipitated. Aliquots ( $20\ \mu\text{l}$ ) of TTR were immunoprecipitated and electrophoresized on SDS-PAGE gels. (A) The 14-kD bands corresponding to TTR were excised from the gels for determination of [ $^{35}\text{S}$ ] radioactivity. Data represent means  $\pm$  SE ( $n = 4$ ).  $**p < 0.01$  as compared to the controls by Scheffe's method. (B) The gels were enhanced, dried, and exposed to X-ray films for 5 days. The bands represent the fragment of [ $^{35}\text{S}$ ]TTR with the molecular weight equivalent to 14 kDa at various chase times. Ct, control group; Pb, Pb-treated group.



**FIG. 4.** Effect of Pb exposure on secretion of [ $^{35}\text{S}$ ]TTR into the culture media. The culture media were immunoprecipitated. Aliquots ( $20\ \mu\text{l}$ ) of TTR immunoprecipitate were electrophoresized on SDS-PAGE gels. (A) The 14-kDa bands corresponding to TTR were excised from the gels for determination of [ $^{35}\text{S}$ ] radioactivity. Data represent means  $\pm$  SE ( $n = 4$ ). \* $p < 0.01$  as compared to the controls by Scheffe's method. (B) The gels were enhanced, dried, and exposed to X-ray films for 5 days. The bands represent the fragment of [ $^{35}\text{S}$ ]TTR with the molecular weight equivalent to 14 kDa at various chase times. Ct, control group; Pb, Pb-treated group.





**FIG. 5.** Inhibition by Pb of the transepithelial transport of  $[^{125}\text{I}]\text{T}_4$  in a two-chamber model of blood-CSF barrier. Cells were exposed to Pb ( $30 \mu\text{M}$ ) for 4 h.  $[^{125}\text{I}]\text{T}_4$  ( $40 \text{ pM}$ ,  $0.18 \mu\text{Ci/ml}$ ) was added into the outer chamber at time 0. Data represent means  $\pm$  SD ( $n = 3$ ).

**TABLE 1**

Effect of Pb Exposure on Protein Concentrations in Cultures of Choroidal Epithelia in the Pulse-Chase Studies

Chasing time (hour)	Control ( $\mu\text{g/ml}$ )	Pb-treated ( $\mu\text{g/ml}$ )
Cell lysate		
0.0	839.4 $\pm$ 228.4	588.1 $\pm$ 69.4
0.5	573.0 $\pm$ 62.3	550.9 $\pm$ 43.6
1.0	603.1 $\pm$ 67.0	557.3 $\pm$ 75.5
2.0	630.5 $\pm$ 110.8	553.2 $\pm$ 72.9
3.0	485.0 $\pm$ 30.5	443.4 $\pm$ 53.6
Culture medium		
0.0	2.43 $\pm$ 0.80	2.74 $\pm$ 0.85
0.5	2.80 $\pm$ 1.01	2.54 $\pm$ 0.65
1.0	3.32 $\pm$ 1.20	2.56 $\pm$ 0.67
2.0	3.15 $\pm$ 1.34	3.53 $\pm$ 0.86
3.0	3.47 $\pm$ 1.17	5.20 $\pm$ 1.19

*Note.* Protein concentrations in cell lysate or culture medium were determined by BioRad assay kit using BSA as standard. Data represent means  $\pm$  SE ( $n = 5$ ). Pb treatment did not significantly alter protein concentrations in cell lysate or in culture medium by two-way ANOVA.

**TABLE 2**Effect of Pb Exposure on Transport of [<sup>125</sup>I]T<sub>4</sub> at the Cultured Choroidal Epithelial Layer

	Blank (no cells)	Control (with cells)	Pb (30 μM with cells)
Inner chamber			
$C_{\max}$ (dpm/5 μl)	838 ± 16.4	1368 ± 61.1	992 ± 12.8*
$k_r$ (h <sup>-1</sup> )		0.25	0.21
Outer chamber			
$C_{\max}$ (dpm/5 μl)	739 ± 2.6	462 ± 21.5	650 ± 39.1*
$k_u$ (h <sup>-1</sup> )		0.15	0.10

Note. Data present means ± SD,  $n = 3$  separate assays. \* $p < 0.05$  as compared to the control group.  $k_r$ , initial release rate constant;  $k_u$ , initial uptake rate constant.