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Thiamine Analysis in Fish Tissues[1]

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Abstract.—Thiamine pyrophosphate, thiamine monophosphate, and thiamine were measured by reversed phase high-performance liquid chromatography in tissues of lake trout *Salvelinus namaycush* and alewife *Alosa pseudoharengus*. Mean assay sensitivity for thiamine and its phosphates was 0.012 pmol. Average recoveries of low and high doses of thiamine compounds added to tissue samples ranged from 91.4 to 104.5%. Average coefficients of variation for between assay reproducibility ranged from 4.8 to 12.8%. The predominant form of vitamin B_1 was unesterified thiamine in eggs and plasma of lake trout. Thiamine pyrophosphate was the predominant form in red blood cells, liver, muscle, and kidney. The stability of thiamine forms in fish tissues was temperature and species dependent. Thiamine levels were markedly depressed in lake trout collected from Lake Ontario relative to levels in fish captured from Lake 468 at the Experimental Lakes Area in northwestern Ontario.

Many physiological processes depend on adequate levels of vitamin B_1 (thiamine and its phosphorylated forms). Thiamine is a cofactor for several important enzymatic pathways in carbohydrate and amino acid metabolism, and it is vital for nerve function (Combs 1992). Thiamine deficiency in fish is characterized by poor appetite, muscle atrophy, convulsions, loss of equilibrium, edema, poor growth, and increased sensitivity to physical disturbance or light (Halver 1972; Morito et al. 1986). Low levels of egg thiamine are linked with the occurrence of a maternally related yolk sac and swim-up stage mortality in salmonids from the Finger Lakes (Fisher et al. 1996) and in the Laurentian Great Lakes (Fitzsimons 1995; Fitzsimons and Brown 1996). This type of mortality is widespread, affecting Atlantic salmon *Salmo salar*, lake trout *Salvelinus namaycush*, brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss*, coho salmon *O. kisutch*, and chinook salmon *O. tshawytscha* from various regions in the Great Lakes basin (Marcquenski 1996). The ailment has been referred to as Cayuga syndrome in the Atlantic salmon from the Finger Lakes region (Fisher et al. 1995) and early mortality syndrome (EMS) in the salmonids from the lower Great Lakes (Marcquenski 1996). Moreover, a similar thiamine-related embryonic mortality called M74 affects feral Atlantic salmon stocks in the Baltic Sea (Bylund and Lerche 1995; Johansson et al. 1995; Amcoff et al. 1996). To enhance the current understanding of these thiamine-related embryonic syndromes, it is essential to determine the cause(s) of the low levels of thiamine. An analytical method to measure quantities of thiamine and its active forms in various fish tissues and other biota is an important prerequisite to a quantitative description of thiamine dynamics in these syndromes.

In animal tissues, thiamine is found principally in its phosphorylated forms (Combs 1992), with the coenzyme, thiamine diphosphate, representing the predominant form (about 80%). In contrast, the major form of thiamine found in lake trout eggs from certain locations was free thiamine (this study). To detect and quantify thiamine and its different phosphorylated forms, these compounds were first extracted in trichloroacetic acid, converted to their respective thiochromes, and then assayed by reversed phase high-performance liquid chromatography (HPLC). The tissue extraction and assay procedures were modified from those described by Warnock (1982), Sanemori et al. (1980), and Kawasaki and Sanemori (1985). We used this protocol to assess the levels of thiamine in tissues of feral collections of lake trout from Lake Ontario, where EMS is known

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to occur (Fitzsimons 1995), and at the Experimental Lakes Area in northwestern Ontario, where embryonic survival is high (Delorme 1995).

Methods

Fish Sampling and Sample Storage

Near spawning broodstock lake trout (weight, 850–3,320 g; length, 41–65 cm) were collected in September and October from Lake Ontario at Port Weller (near St. Catharines) and from Lake 468 at the Experimental Lakes Area, near Kenora in northwestern Ontario (Cleugh and Hauser 1971). Lake trout were captured either by gill nets examined for fish at 30-min intervals or by overnight trap net. Alewife *Alosa pseudoharengus* (weight, 14.2–33.2 g; length, 9.5–14.5 cm) were captured by trawl in the western basin of Lake Ontario. Only freshly caught live individuals were used for analysis. Before sampling, lake trout were anesthetized in water containing tricaine methanesulfonate (0.76 mmol/L) solution neutralized to ambient pH with ammonium hydroxide and NaCl (150 mmol/L) approximately isoosmotic with fish plasma. Immobilization was complete within 1–2 min. Blood was then removed from the caudal vessels using preheparinized 3- to 5-mL syringes with 18-gauge needles. Plasma and red blood cells were separated by centrifugation, placed into polyethylene vials, and immediately frozen on dry ice. Tissues were quickly dissected, weighed, packaged in Whirl-Pac® bags, and frozen between slabs of dry ice. Whole alewife specimens were quickly frozen. Samples were then transported to the laboratory on dry ice and stored at temperatures of less than −90°C. Samples were analyzed within 3 months of collection.

Reagents

Thiamine HCl (TH), thiamine pyrophosphate chloride (TPP), and thiamine monophosphate chloride (TMP) were obtained from ICN Biomedicals (Montreal, Quebec, Canada). American Chemical Society-grade sodium hydroxide, potassium ferricyanide, and trichloroacetic acid were purchased from Sigma Chemical Co. (Mississauga, Ontario, Canada). Potassium phosphate (HPLC-grade) was obtained from Fisher Scientific (Edmonton, Alberta, Canada). The distilled in glass *N*,*N*-Dimethylformamide (DMF) was obtained from Caledon (Edmonton, Alberta, Canada). We used distilled deionized water (DDW, MilliQ®, Millipore, Bedford, Massachusetts) to prepare stock solutions and buffers.

Apparatus

Gradient reversed phase chromatography was performed using a Hamilton PRP-1 column (150 × 4.1 mm; 5-mm mesh size; Alltech, Deerfield, Illinois) with attached guard column (25 × 2.3 mm; 12- to 20-mm mesh size) as stationary phase. The HPLC system consisted of two model 302 solvent pumps, a model 231 automatic sample injector, a model 704 system controller, and a four-channel model 620 data module (Gilson Medical Electronics, Middleton, Wisconsin). A Shimadzu (Columbia, Maryland) model RF-535 fluorometric detector was set at 375-nm excitation wavelength and 433-nm emission wavelength for thiochrome detection. The column thermostat was set at 30°C. The mobile phase consisted of 25 mM potassium phosphate (pH 8.4) and was applied for the first 4 min after sample injection. From 4 to 4.1 min, the mobile phase was changed to 3% DMF, 25 mM potassium phosphate (pH 8.4), and this was run until 8 min to elute thiamine pyrophosphate and thiamine monophosphate. Then between 8 and 8.1 min, the mobile phase was changed to 20% DMF, 25 mM potassium phosphate (pH 8.4) to elute thiamine. After 13.0 min, the mobile phase was returned to the initial conditions to equilibrate the column for the next sample. The flow rate was 1.0 mL/min and the total run time was 20 min.

Statistics

Significant differences between groups were determined by *t*-tests for independent samples or paired *t*-test for dependent samples using the Systat statistical package (Wilkinson et al. 1992).

Assay Procedure

The tissue extraction and assay procedures were modified from those described by Warnock (1982) and Kawasaki and Sanemori (1985). All thiamine standards were freshly prepared in DDW and used immediately. Aliquots of standards were subjected to the same extraction and washing procedures as the tissue samples.

Tissue Extraction

1. While tissue is frozen, finely chop approximately 500 mg and place it into a chilled, preweighed centrifuge tube. Reweigh the tube to obtain exact tissue weight.

2. Add 1.5 mL of ice-cold 2% trichloroacetic acid (TCA) and homogenize for 1–2 min either by hand or with a low-speed tissue grinder. Tis-

sue homogenization must be thorough and carefully conducted. Variable results will be obtained unless the close fitting homogenizer (0.02 cm difference between the diameter of pestle and the inside diameter of the tube) is used. If recovery of thiamine is low or variable, grind twice, once when tissue is placed in TCA and a second time after the boiling step. High-speed and ultrasonic homogenization caused losses and changes in the proportions of different thiamine compounds (Figure 1) and is not recommended.

3. Place tubes in a boiling water bath for 10 min, then remove tubes and briefly spin the mixture. Cool tubes for 10 min on ice. Preliminary experiments indicated an optimal heating time of between 5 and 10 min (Figure 2).

4. Add 1.5 mL of ice-cold 10% TCA. Homogenize sample briefly to mix.

5. Centrifuge the tubes at approximately 14,000 g for 15 min and transfer the supernatant to a clean container. The thiamine compounds in the sample supernatant are now stable for at least 72 h when stored at 4°C (Figure 3).

6. Wash the supernatant four times with an equal volume of ethyl acetate:hexane (3:2, volume per volume). This step removes most TCA and raises the pH of the supernatant to approximately 5.

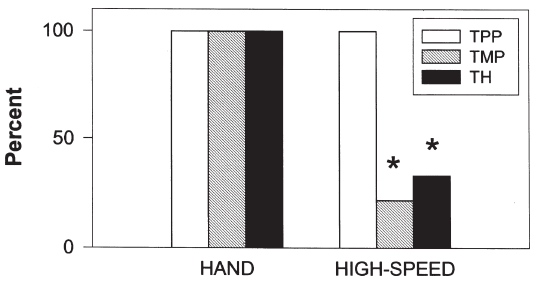

FIGURE 1.—The percentage recovery of thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), and thiamine (TH) in red blood cells after hand or high-speed homogenization. Values are the mean of duplicate determinations from each of two samples. Asterisks indicate significant differences from hand homogenization.

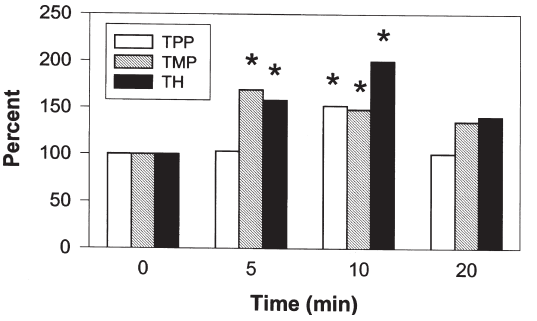

FIGURE 2.—The percentage recovery of thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), and thiamine (TH) in eggs after heating at 100°C for up to 20 min. Values are the mean of duplicate determinations from each of two samples. Asterisks indicate differences from time 0.

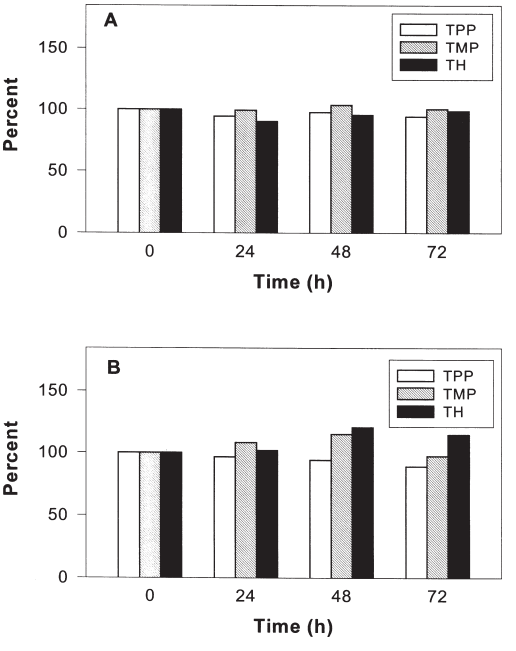

FIGURE 3.—The percentage recovery of thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), and thiamine (TH) from extracted standards (**A**) and extracts of egg tissue (**B**) after storage of supernatants from the 10% TCA precipitation step at 4°C for up to 72 h. Values are the mean of duplicate determinations from each of two samples.

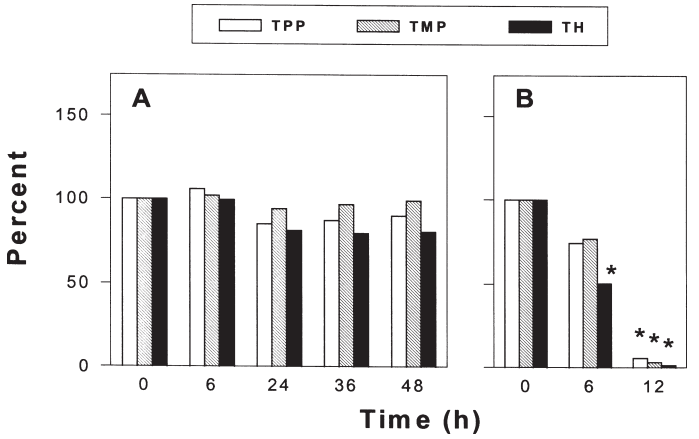

FIGURE 4.—The percentage recovery of thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), and thiamine (TH) after room temperature storage of thiochrome solutions of samples in the dark (**A**) and in the light (**B**). Values are the mean of duplicate determinations from each of two samples. Asterisks indicate differences from time 0.

Thiochrome Preparation

1. Transfer 425 μL of the washed aqueous supernatant to a clean microcentrifuge tube and mix with 45 μL of 1.2 N NaOH and 30 μL of 0.1% potassium ferricyanide (prepared fresh daily). A final pH of greater than 8 is necessary for maximal fluorescence of thiochrome and its phosphates (Kawasaki and Sanemori 1985). When protected from light, alkaline solutions (approximately pH 9) of thiochrome were stable for 48 h (Figure 4).
2. Chromatograph the oxidized thiamine supernatant (20–100 μL) by reversed phase HPLC. Blanks are obtained by substituting distilled water for NaOH.

Results and Discussion

Assay Characteristics

Thiochrome fluorescence of extracted thiamine standards eluted with characteristic retention times that did not differ between preparations (Figure 5). Typical standard curves for phosphorylated thiamine

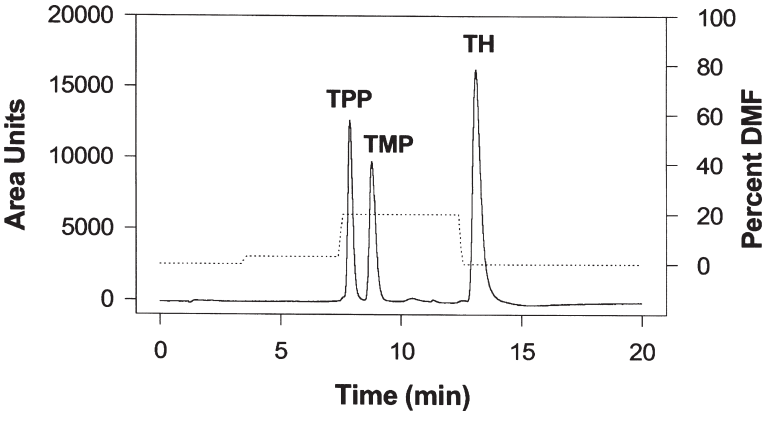

FIGURE 5.—The solid line indicates thiochrome fluorescence (375-nm excitation wavelength and 433-nm emission wavelength) after chromatography of a standard sample containing 8.4 pmol of thiamine pyrophosphate chloride (TPP), 10.3 pmol of thiamine monophosphate chloride (TMP), and 10.7 pmol of thiamine HCl (TH) on a Hamilton PRP-1 column (150 × 4.1 mm; 5-mm mesh size). The dotted line indicates the percentage of *N*,*N*-dimethylformamide (DMF) in the mobile phase (25 mM potassium phosphate, pH 8.4).

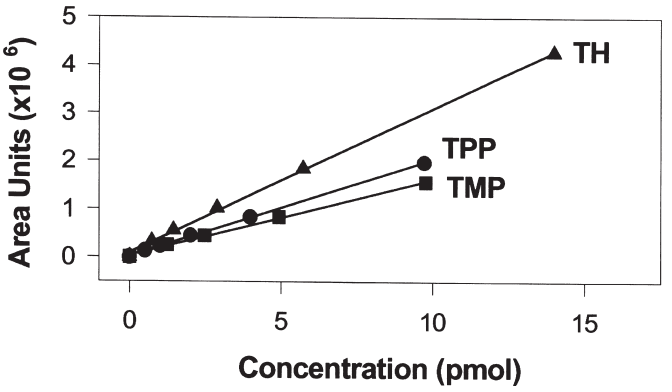

FIGURE 6.—Standard curves for thiamine pyrophosphate chloride (TPP), thiamine monophosphate chloride (TMP), and thiamine HCl (TH). Fluorescence detector response was linear ($r^2 = 0.99$ for TPP, TMP, and TH). Amounts injected ranged from 0.5 to 14.0 pmol. Values are the mean of triplicate determinations.

(TPP and TMP) and thiamine (TH) were linear ($r^2 \geq 0.99$; Figure 6). The detection limit for thiamine and its phosphates averaged 0.012 pmol per injection loop (20–100 μL). Dilutions of extracts of liver or egg tissue, ranging from 25 to 175 mg, gave parallel standard curves with $r^2 > 0.98$ for thiamine pyrophosphate, thiamine monophosphate, and thiamine in liver tissue and $r^2 > 0.96$ for thiamine pyrophosphate, thiamine monophosphate, and thiamine in eggs. Recovery of low (1 nmol/g) and high doses (50 nmol/g) of added thiamine compounds from 10 samples averaged 104.5 ± 2.8, 98.8 ± 1.9, and 91.4 ± 1.6% for thiamine pyrophosphate, thiamine monophosphate, and thiamine, respectively. Repeated measures (six repetitions) on extracts of the same egg homogenate or liver sample gave coefficients of variation of 8.1, 12.4, and 5.5% for thiamine pyrophosphate, thiamine monophosphate, and thiamine,

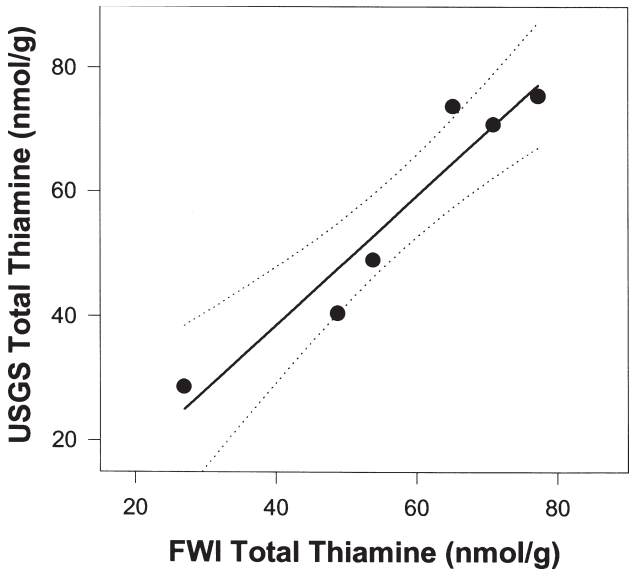

FIGURE 7.—The solid line shows the linear relationship ($r^2 = 0.916$, slope = 1.037) between total thiamine levels measured in six different lake trout egg samples at the U.S. Geological Survey Laboratory (USGS), Wellsboro, Pennsylvania, and at the Freshwater Institute Science Laboratory (FWI), Winnipeg, Manitoba. The dotted lines indicate the 95% confidence range.

respectively. Repeated measures (12 repetitions) on a tissue extract from a reference pool of lake trout eggs, analyzed on different days, gave coefficients of variation of 4.8, 7.8, and 12.8% for thiamine pyrophosphate, thiamine monophosphate, and thiamine, respectively. Using the protocol described above (see "Assay Procedure"), six different egg batches were measured at both the U.S. Geological Survey Research and Development Laboratory (Wellsboro, Pennsylvania) and the Freshwater Institute Science Laboratory (Winnipeg, Manitoba, Canada; Figure 7). The r^2-values for the correlation between laboratories were 0.928, 0.834, 0.924, and 0.916 for thiamine pyrophosphate, thiamine monophosphate, free thiamine, and total thiamine, respectively.

Stability of Thiamine in Fish Samples

The stability of thiamine forms in liver and muscle samples of trout and alewife were species and temperature dependent (Table 1). When liver tissue from lake trout was held at room temperature, the total thiamine levels were unchanged. Initially, thiamine pyrophosphate predominated; however, there was a gradual shift between the levels of thiamine pyrophosphate and free thiamine such that free thiamine was the predominant form after 24 h. When the tissues were maintained at 4°C, changes in the type of thiamine were not apparent in the first 4 h. After 24 h at 4°C, there was some evidence of conversion of thiamine monophosphate to free thiamine in trout liver. Maintaining muscle tissue from lake trout at room temperature resulted in lower thiamine pyrophosphate levels at 24 h. This decline was offset by a corresponding increase in the level of thiamine monophosphate. Samples of alewife muscle were unstable at room temperature, and the levels of all thiamine forms declined sharply after 1 h. Refrigeration at 4°C stabilized the total thiamine levels in alewife but did not prevent a shift from thiamine pyrophosphate to thiamine monophosphate after 4 h. Reanalysis of tissue samples stored at −90°C for 1 year gave results similar to those from fresh tissue. However, if the sample thaws and is refrozen, the analysis of extracts of the refrozen tissue will reflect losses and changes in the forms of thiamine present.

Although our information about thiamine stability was derived from laboratory experiments, the findings are relevant to thiamine measurements in fish collected in field surveys. Measurements in fish that were not freshly caught and sampled would lead to erroneous assessments of the proportions and quantities of the different thiamine forms. Species such as alewife and smelt that contain thiaminase activity (Gnaedinger 1964) seem particularly vulnerable. Furthermore, the degenerative changes occur at a faster rate when temperatures are higher. Appropriate protocols for collection and storage of the samples are critical. A procedure in which fresh samples from live specimens are quickly frozen is necessary to obtain an accurate representation of thiamine forms.

TABLE 1.—Percentage total thiamine compounds for thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), and free thiamine (TH) and percentage recovery of total thiamine in liver or muscle samples held at 20 or 4°C for up to 24 h before analysis. Values represent the mean of duplicate measurements. Differences from 0 h are indicated by z.

Time	Percentage total thiamine			Percentage recovery
	TPP	TMP	TH	
Lake trout liver (20°C)				
0 h	85.2	14.0	0.8	100.0
1 h	75.8	18.7	9.2	103.8
4 h	62.6z	12.4	24.2z	99.3
24 h	21.5z	7.1	83.9z	112.5
Lake trout liver (4°C)				
0 h	81.9	17.0	1.1	100.0
1 h	88.3	20.1	2.7	111.1
4 h	85.7	21.6	5.4z	112.7
24 h	81.5	12.1	16.2z	109.9
Lake trout muscle (20°C)				
0 h	86.6	12.6	0.9	100.0
1 h	81.1	9.6	0.5	91.2
4 h	92.0	15.6	1.2	108.8
24 h	23.2z	50.7z	6.5z	80.4z
Alewife muscle (20°C)				
0 h	76.9	18.4	4.7	100.0
1 h	78.2	26.3	5.2	109.7
4 h	3.5z	7.8z	0.5z	11.8z
24 h	2.2z	0.0z	0.0z	2.2z
Alewife muscle (4°C)				
0 h	71.2	24.3	4.5	100.0
1 h	70.2	35.6	2.6	108.5
4 h	53.7z	44.0z	3.1	100.8
24 h	23.4z	69.6z	6.7	99.6

Thiamine Levels in Lake Trout Tissues

Similar to sea urchin eggs (Shimada et al. 1993), in lake trout eggs the major form of thiamine was free thiamine (Figure 8A). Total thiamine levels in spawning female fish were lowest in plasma (Table 2). Although plasma levels of phosphorylated forms were detectable, free thiamine was the predominant form. Free thiamine may be able to cross cell mem-

branes more readily than esterified forms (Combs 1992). Moreover, the possible presence of a female-specific vitamin transport protein for thiamine similar to that reported in birds and amphibians (Adiga and Murty 1983; White 1987) warrants investigation as a mechanism for the incorporation of the large amounts of free thiamine detected in oocytes. As is generally found in other animal species (Combs 1992), thiamine in red blood cells, liver, and kidney of lake trout was predominantly the metabolically functional enzyme cofactor (TPP; Figure 8B, Table 2). In female lake trout collected from Lake 468 at the Experimental Lakes Area, levels of total thiamine were comparable with levels reported in liver of feral bream *Abramis brama*, roach *Rutilis rutilus*, and pike *Esox lucius* (Malyarevskaya and Karasina 1992). Red blood cell and liver thiamine pyrophosphate levels of female lake trout from Lake 468 (Table 1) were also similar to concentrations reported for juvenile rainbow trout fed a commercial diet supplemented with thiamine (Masumoto et al. 1987). Depending on the tissue examined, total thiamine levels of lake trout collected from Lake Ontario were only 10–50% of concentrations found in fish from Lake 468 (Table 2). The low levels of thiamine found in red blood cells and liver from lake trout collected in Lake Ontario (Table 2) were comparable with those found in juvenile rainbow trout exhibiting overt signs of thiamine deficiency after consuming a thiamine-deficient diet (Masumoto et al. 1987). Levels of free thiamine in eggs showed the greatest difference between locations. The low thiamine levels found in salmonid eggs from areas in the Laurentian Great Lakes have been implicated as a possible cause of early mortality syndrome and recent reproductive failures (Fitzsimons 1995; Fisher et al. 1996; Marcquenski 1996).

Acknowledgments

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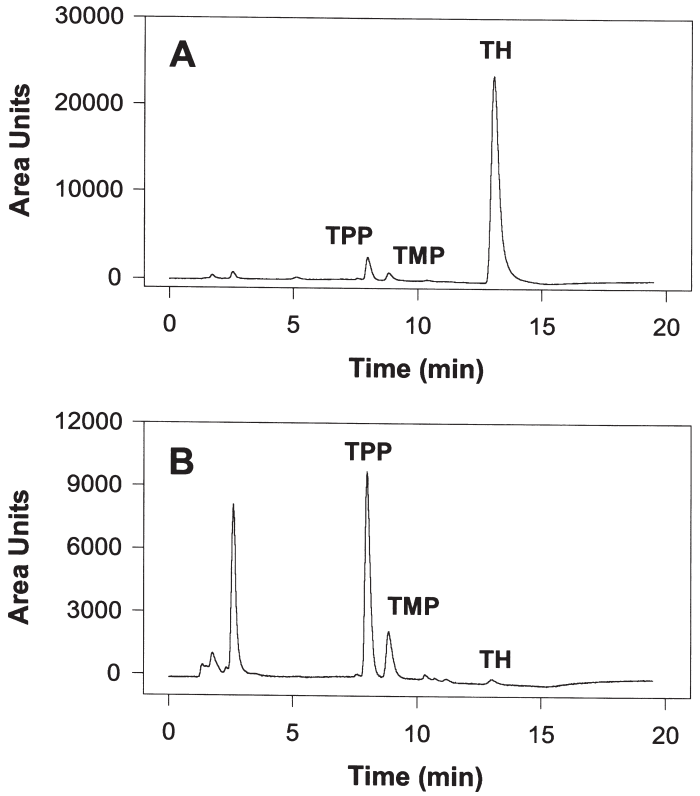

FIGURE 8.—Thiochrome fluorescence after chromatography of tissue extracts prepared from eggs (**A**) and liver (**B**) from a female lake trout captured in Lake 468 at the Experimental Lakes Area. Identified peaks co-elute with thiochromes of thiamine pyrophosphate chloride (TPP), thiamine monophosphate chloride (TMP), and thiamine HCl (TH).

TABLE 2.—Thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), free thiamine (TH), and total thiamine (TTH) (nmol/g) in selected tissues of female lake trout collected from Lake 468 at the Experimental Lakes Area and from Lake Ontario near Port Weller. Values represent the mean (SE) of duplicate measurements from three or four fish.

	Lake 468				Lake Ontario			
Tissue	TPP	TMP	TH	TTH	TPP	TMP	TH	TTH
Eggs	1.843	0.840	16.954	19.637	0.723	0.353	0.951	2.027
	(0.068)	(0.013)	(0.197)	(0.278)	(0.076)	(0.090)	(0.544)	(0.706)
Plasma	0.036	0.039	0.072	0.148	0.013	0.024	0.036	0.073
	(0.010)	(0.018)	(0.013)	(0.035)	(0.004)	(0.005)	(0.003)	(0.009)
Red blood cells	1.170	0.168	0.038	1.376	0.213	0.104	0.080	0.398
	(0.184)	(0.046)	(0.004)	(0.217)	(0.036)	(0.041)	(0.010)	(0.078)
Liver	8.501	2.954	0.247	11.702	5.142	0.493	0.147	5.782
	(1.753)	(0.243)	(0.038)	(1.809)	(0.418)	(0.077)	(0.142)	(0.629)
Kidney	9.153	3.405	2.419	14.976	3.346	0.392	0.181	3.920
	(1.046)	(0.923)	(0.548)	(2.191)	(0.503)	(0.051)	(0.042)	(0.584)

of lake trout and alewife from Lake Ontario. We gratefully acknowledge the helpful criticisms of K. Mills, R. Hunt, and C. Haux on a previous version of the manuscript.

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