Utilization of the Bone/Liver Alkaline Phosphatase Activity Ratio in Blood Plasma as an Indicator of Ascorbate Deficiency in Salmonid Fish (43990)

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Abstract. **The goal of this study was to test the hypothesis that the ratio of liver to bone alkaline phosphatase in blood plasma reflects the ascorbate status in scurvy-prone teleost fish (rainbow trout [*Oncorhynchus mykiss*]). The studies focused on finding a method for distinguishing bone alkaline phosphatase present in blood plasma from other alkaline phosphatase isoforms. We tested temperature optima and thermostability of liver, kidney, gill cartilage, and intestinal alkaline phosphatases. We did not observe differences among liver, bone, and kidney enzymes with respect to temperature optima and thermostability. We partially purified alkaline phosphatase from juvenile rainbow trout vertebrae and liver using *n*-butanol solubilization and ammonium sulfate fractionation. We found a difference between bone alkaline phosphatase, which precipitated in 0%–20% ammonium sulfate saturation, and liver enzyme, which required 40%–50% ammonium sulfate saturation for precipitation. We conducted a series of urea inactivation studies on partially purified enzymes from liver and vertebrae. Urea differentially inhibited the enzymes with $t_{1/2}$ = 1.1 and 0.4 min, for bone and liver, respectively. Subsequently, we subjected blood plasma alkaline phosphatase to urea inhibition, and using regression analysis we calculated the ratio of liver to bone alkaline phosphatase. We found that thus obtained ratios of bone enzyme in blood plasma correlated with liver ascorbate concentration. Bone alkaline phosphatase declined in ascorbate deficiency 10-fold, whereas low ascorbate status resulted in a 3.5-fold decrease. In order to draw a general conclusion on the linearity of the response of blood plasma/bone alkaline phosphatase as an indicator of ascorbate deficiency in fish, further studies must include analysis of individual fish followed in the process of developing avitaminosis.** [P.S.E.B.M. 1996, Vol 212]

Clinical symptoms of scurvy are likely to be preceded by subclinical changes which, once identified, would provide useful markers for assessment of ascorbate status. Liver ascorbate concentration (1), vertebral collagen content (2), and hydroxyproline to proline ratio (3) are among the most reliable and most used indicators in teleost fish. These methods require sacrifice of experimental animals, however, preventing detailed pharmacokinetic studies over time. This is particularly relevant in respect to fish, a group that expresses especially high variability among individuals.

Blood plasma ascorbate levels do not reflect the true ascorbate status, owing to their dependence on immediate dietary supplementation (4) and to possible interference from hemoglobin (5). However, ascorbic acid is also involved both in collagen (6) and vitamin D hydroxylation (7). Vitamin C and bone metabolism are closely related, hence the use of associated enzymes as potential indicators of ascorbate status. Proper collagen hydroxylation depends on lysine and proline hydroxylases which require ascorbic acid as a cofactor

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(8). Proline hydroxylase, however, has not been detected in blood plasma (9).

Alkaline phosphatase plays an essential role in bone calcification (10). Moreover, its plasma levels closely reflect osteoblastic activity (11). Studies on guinea pigs (12–14) revealed that ascorbate deficiency is mirrored by decline in alkaline phosphatase activity in blood plasma. Studies of the correlation between total plasma alkaline phosphatase and ascorbate status in other animals, however, have provided contradictory results (15–17).

Recent studies by Johnston *et al.* (18) confirmed that in salmonid fish several tissue isoenzymes are represented in blood plasma and their proportion change with physiological state. Brown and Lewis (19) demonstrated that bone and liver alkaline phosphatase isoforms can be distinguished from one another by differential inhibition with urea. Our study sought to apply this technique to rainbow trout blood plasma. We correlated the changes in liver to bone alkaline phosphatase ratio to the ascorbate status of the fish. Using this approach, we then attempted to evaluate the usefulness of the measurements of alkaline phosphatase isoenzymes in blood plasma as a routine analytical method for evaluating ascorbate status *in vivo* in teleost fishes and possibly scurvy-prone primates. Bone alkaline phosphatase should decline in scurvy fish as bone metabolism declines. Therefore, we hypothesized that the measurement of the liver to bone isoenzymes ratio of alkaline phosphatase activity in blood plasma could potentially be used as clinical indicator of vitamin C deficiency.

Materials and Methods

Materials. Vertebrae, gills cartilage, livers, kidneys, and blood plasma were obtained from healthy, juvenile (20–40 g) rainbow trout (*Oncorhynchus mykiss*) fed a diet supplemented with ascorbate above the requirement level (20). Tissues were dissected and stored at −85°C. Blood was centrifuged and its plasma stored at −85°C.

Blood plasma was collected from juvenile rainbow trout after 18 weeks on diets differing in ascorbate content (21). Fish were divided into eight experimental groups (24 tanks, three replicate tanks per dietary group, 50 fish per tank). Diets contained ascorbate polyphosphate equimolar to 0, 20, 40, 80, 160, 320, and 1280 mg ascorbic acid/kg diet or ascorbate monophosphate equimolar to 320 mg ascorbic acid/kg diet. A group of fish of similar size and age was fed a commercial diet (28–261 mg AA/kg; Bioproducts, Warrenton, OR) and served as normal, healthy controls (3–8 fish per dietary treatment were analyzed). All animal procedures and handling were conducted with special care in compliance with the guidelines of the Institutional Animal Care and Use Committee, The Ohio State University.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were analytical grade.

Tissue Extract Preparation. Liver, kidney, and intestine were homogenized in 10 m*M* Tris/HCl buffer, pH 7.4, containing 1% Triton X-100 and 2 m*M* phenylmethylsulfonyl fluoride (PMSF), using a Teflon homogenizer. The homogenates were centrifuged at 8700*g* for 10 min. Supernatants were collected and frozen at −85°C for further studies. Gills were homogenized in 10 m*M* Tris/HCl buffer, pH 7.4, using a Teflon homogenizer and centrifuged at 8700*g* for 10 min. Pellets, containing cartilage, were then homogenized again in 10 m*M* Tris/HCl buffer, pH 7.4, containing 1% Triton X-100 and 2 m*M* PMSF. After centrifugation at 8700*g* for 10 min supernatants were collected and stored at −85°C.

Partial Purification of Liver and Bone Alkaline Phosphatases. Vertebrae were split open and spinal cord removed. Any remains of other tissues from livers and vertebrae were removed. Vertebrae and livers collected from nine juvenile rainbow trout were then weighed and homogenized in five volumes of 50 m*M* Tris/HCl, pH 7.4, at 4°C followed by *n*-butanol extraction. *n*-butanol was gradually added to homogenates in proportion, two volumes of *n*-butanol per five volumes of homogenate at ambient temperature (22). *n*-butanol extracts were then incubated for 5 min at 36°C and centrifuged at 2000*g* (23). The aqueous phase, containing alkaline phosphatase activity, was collected and subjected to ammonium sulfate fractionation. Ammonium sulfate was added in a stepwise manner to determine the concentration at which bone and liver isoforms of alkaline phosphatase precipitate. After each ammonium sulfate addition, solutions were centrifuged, precipitates dissolved in 50 m*M* Tris/HCl buffer, pH 7.4, and alkaline phosphatase activity measured. Fractions with the highest alkaline phosphatase purification factor were pooled. The salted out enzymes were dialyzed against Tris/HCl buffer, pH 7.4, with the addition of magnesium and zinc ions. The dialyzates were divided into small portions and stored at −85°C.

Resolution of Liver and Bone Isoforms by Differential Denaturation by Urea. Patterns of liver and bone alkaline phosphatase denaturation with urea (19) were determined using the enzymes purified by the procedure described above. Urea concentrations between 2.7 and 4.0 *M* at a temperature between 33° and 37°C were used to determine the optimal assay conditions for inhibition of the rainbow trout enzyme.

The liver/bone isoenzymes ratio was determined in the blood plasma from healthy, juvenile rainbow trout based on urea inhibition patterns obtained for partially purified enzymes. Blood plasma collected from rainbow trout with different tissue ascorbate status (ranging from below 20 mg/g liver—ascorbate de-

ficiency, to 130 mg/g liver—ascorbate-saturated tissues [see Refs. 21 and 24 for justification of this assumption]) were tested for the liver/bone isoenzyme activity ratio.

Temperature Optima and Thermostability. Temperature optima for intestinal, liver, kidney, and gill cartilage alkaline phosphatase from juvenile rainbow trout were examined in the range of 3°–55°C. All assays in indicated temperatures were conducted in triplicate. Thermostability of intestinal, liver, and kidney alkaline phosphatase activity was measured after 30-min preincubation at indicated temperature, and then activities followed at 25°C.

Alkaline Phosphatase Activity. Total alkaline phosphatase activity (in blood plasma, liver, kidney, intestine, and gill cartilage) was monitored spectrophotometrically at 405 nm using 5 m*M* *p*-Nitrophenylphosphate in 100 m*M* diethanolamine (DEA)/HCl buffer, pH 9.8, in the presence of 5 m*M* $MgCl_2$ at 25°C. In the case of urea inhibition studies, the reaction mixture was composed of 100 m*M* DEA/HCl buffer, pH 9.8, 1 mM $MgCl_2$, and 2.7, 3.0, 3.5, and 4.0 *M* urea at 33°, 35°, and 37°C. Reaction in each condition was followed for 20 min and the absorption measured every 10 sec. For determination of liver/bone ratios in blood plasma, the reaction was conducted in the presence of 3.5 *M* urea at 33°C for 20 min and absorption measured in 10-sec intervals.

Calculation of Half-Lives of Purified Bone and Liver Isoforms and Their Activities in Blood Plasma. Half-life times of the liver and bone alkaline phosphatases in various conditions (temperature and urea concentration) were estimated by the curvilinear regression of measured activities of the purified enzymes.

The decay of individual isoenzymes in a mixture is a first-order process and residual total activity (C) is a sum of exponentials (19):

$$C = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} + \ldots + C_n e^{-k_n t} + C_0 \quad [1]$$

where C_n is the initial activity of the nth species of isoenzyme and k_n represents the corresponding decay constant. From the above equation we can calculate the half-life of the nth species:

$$t_{1/2n} = \ln 2/k_n \quad [2]$$

Calculations of liver and bone alkaline phosphatase activity in blood plasma were based on the assumption that there are two decaying isoforms with known half-lives and a stable component. The residual activity after, for instance, 0.33, 2, and 10 min can be described by the following system of linear equations:

$$C_{0.33} = C_{B*}\exp(-\ln 2/t_{B1/2} * 0.33) + C_{L*}\exp(-\ln 2/t_{L1/2} * 0.33) + C_0 \quad [4]$$

$$C_2 = C_{B*}\exp(-\ln 2/t_{B1/2} * 2) + C_{L*}\exp(-\ln 2/t_{L1/2} * 2) + C_0 \quad [5]$$

$$C_{10} = C_{B*}\exp(-\ln 2/t_{B1/2} * 10) + C_{L*}\exp(-\ln 2/t_{L1/2} * 10) + C_0 \quad [6]$$

where $C_{0.33}$, C_2 and C_{10} are the remaining activity after 0.33, 2, and 10 min, respectively.

Ascorbate Analysis. Dissected livers were stored at −85°C and then homogenized in deproteinizing solution containing 50 g/l trichloroacetic acid, 250 m*M* perchloric acid and 0.8 g/l EDTA. Homogenates were centrifuged at 29,000*g* for 30 min. Ascorbic acid concentration in thus obtained supernatants was determined according to the dinitrophenylhydrazine method modified as described by Dabrowski and Hinterleitner (25).

Statistical Analysis. Differences between groups were tested in one-way analysis of variance (ANOVA) followed by Scheffe F test (26) using the StatView statistical package for Macintosh (BrainPower, Inc., Calabasas, CA, 1986), with fish ascorbate status as the main effect. Correlation between fish ascorbate status and enzyme activities was conducted using the same statistical package.

Results

The intestine alkaline phosphatase exhibits a distinct, narrow temperature optimum of 20°–23°C and can be easily distinguished from that of liver, kidney, and gill cartilage, which have broader temperature optima of between 30° and 40°C (Fig. 1). Thermostability studies also demonstrated higher temperature susceptibility of intestinal isoenzyme than liver, gill cartilage, and kidney isoforms (Fig. 2). After 30-min incubation at 45°C, intestinal alkaline phosphatase activity declined to 50% whereas enzymes from other sources remained fully active.

Urea differentially inactivates bone and liver isoforms in human blood plasma (19). We attempted to modify this method to estimate liver and bone alkaline phosphatase activities in rainbow trout blood plasma. Because of low enzyme activity in bone, we partially purified the enzymes from both bone and liver to find urea inactivation patterns of these isoforms. The purification steps and results are summarized in Table I. We noted different ammonium sulfate precipitation patterns for bone and liver alkaline phosphatase (Fig. 3). Most of the bone enzyme was salted out in 20% ammonium sulfate, whereas the liver enzyme required 40%–50% ammonium sulfate saturation for precipitation. Dialysis and subsequent centrifugation resulted in further increase of the bone, but not the liver, isoenzyme activity. Concentration of the enzymes by ultrafiltration using Ultrafree-CL filters at 3,900*g*

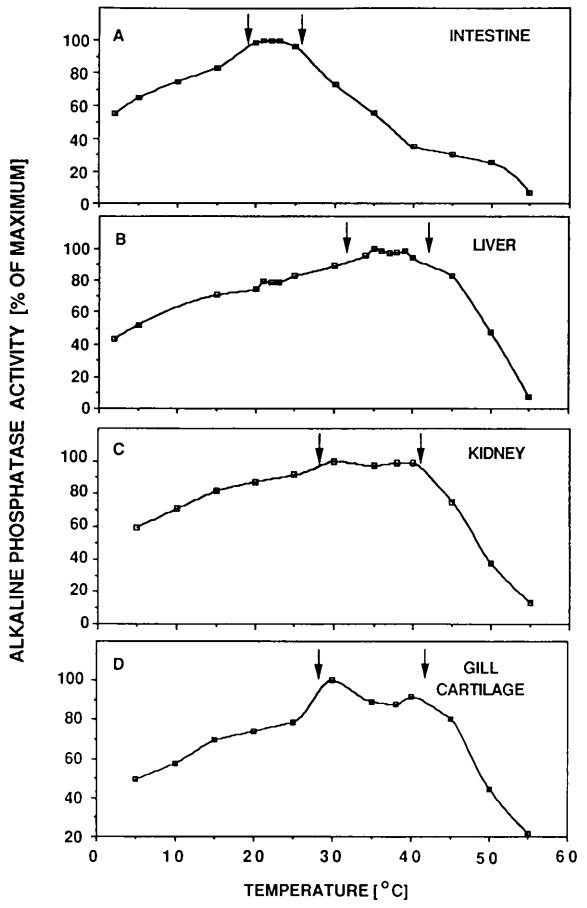

Figure 1. Temperature optima for intestinal (A), liver (B), kidney (C), and cartilage (D) alkaline phosphatase from juvenile rainbow trout, *Oncorhynchus mykiss*. Arrows indicate the temperature optima for a particular form of the enzyme. All assays, in all conditions were conducted in triplicate.

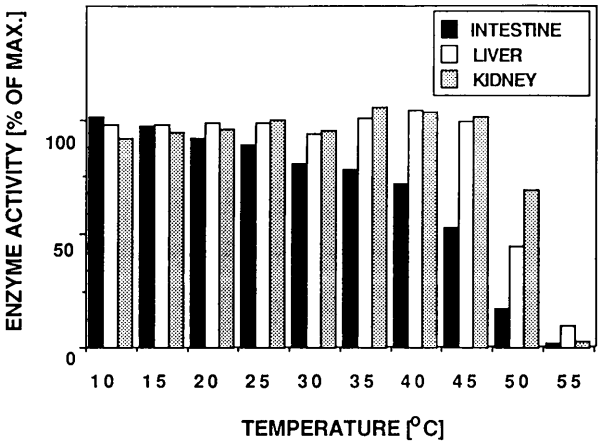

Figure 2. Thermostability of intestinal, liver, and kidney alkaline phosphatases from juvenile rainbow trout, *Oncorhynchus mykiss*. Enzymes were preincubated in indicated temperatures for 30 min before the assay and then activities followed at 25°C. Assays in all conditions were conducted in triplicate.

caused considerable losses of the enzyme, so the procedure was abandoned in further purifications.

Ascorbate deficiency in guinea pigs is accompanied by decline in total alkaline phosphatase activity in blood plasma (12, 13). Although, total blood plasma alkaline phosphatase activity differed among groups of rainbow trout fed diets supplemented with different amounts of ascorbate for 18 weeks (one-way ANOVA, Scheffe F test, $P < 0.05$; Table II), ascorbate status and total plasma alkaline phosphatase activity were not correlated ($r = 0.31$).

Temperature and urea concentration affected half-lives of partially purified bone and liver alkaline phosphatases. In general, half-lives for liver alkaline phosphatase were longer than those for the enzyme from bone. At lower temperatures, the differences between liver and bone half-lives increased as urea concentration increased. The most apparent differences were noted in 3.5 and 4.0 M urea concentrations (Table III). From these comparisons, the most suitable assay conditions were chosen. Inhibition studies with blood plasma isoenzymes were further conducted in 3.5 M urea at 33°C. Employing nonlinear regression to inhibition patterns in blood plasma, we distinguished three components: fast decaying activity from bone (Fig. 4, Line B), slower declining activity from liver (Fig. 4, Line L) and a third, constant component (Fig. 4, Line C), which was not affected by urea.

Finally, we attempted to employ the obtained half-live times to distinguish between the liver and bone alkaline phosphatases in blood plasma from fish with high, low, and deficient ascorbate status and compare the results with control fish. The estimated half-lives were $t_{B1/2} = 1.1$ min and $t_{L1/2} = 0.4$ min. We substituted these values into Equations 4, 5, and 6 from Materials and Methods and obtained the following equation system:

$$C_{0.33} = 0.812 * C_B + 0.586 * C_L + C_0 \quad [7]$$

$$C_2 = 0.284 * C_B + 0.031 * C_L + C_0 \quad [8]$$

$$C_{10} = 0.002 * C_B + 3\text{ E-}9 * C_L + C_0 \quad [9]$$

where coefficients at C_B and C_L represent remaining activity after a given time.

After 0.33 min, bone activity decreased to 81.2% of the initial activity and liver to 58.6%. After 2 min, bone and liver activity further decreased to 28.4% and 3.1%, respectively. After 10 min, both bone and liver activity approached zero (0.2% and $0.3 * 10^{-6}$%, respectively).

Ascorbic acid (AA) concentration in liver was used as the indicator of fish ascorbate status. Fish from the group with high ascorbate status had the same liver to bone alkaline phosphatase ratio (L/B) as the control group. Fish with very low, deficient ascorbate status (13.87 ± 9.99 mg of AA/g tissue) had significantly higher L/B ratios than the control group. The group with ascorbate liver concentrations of

Table I. Purification of Alkaline Phosphatases from Rainbow Trout Vertebrae and Liver

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Bone					
Tris/HCl extraction	10.35	490.00	0.021	100	1.0
n-butanol extraction	6.85	176.41	0.039	66	1.8
Ammonium sulfate fractionation	4.33	12.92	0.333	42	15.8
Dialysis and centrifugation	1.95	1.56	1.252	19	59.3
Liver					
Tris/HCl extraction	26.34	1260.22	0.021	100	1.0
n-butanol extraction	10.77	124.03	0.087	41	4.1
Ammonium sulfate fractionation	6.41	25.46	0.252	24	12.0
Dialysis and centrifugation	1.73	6.50	0.268	7	12.8

Note. Alkaline phosphatase activity was monitored spectrophotometrically at 405 nm using 5 m*M*-*p*-Nitrophenylphosphate in 100 m*M* diethanolamine/HCl buffer, pH 9.8, in the presence of 5 m*M* $MgCl_2$ at 25°C. Protein was measured in the Bradford method. Specific activity (U/mg) was defined as micromoles of *p*-Nitrophenyl released in 1 min by 1 mg of protein.

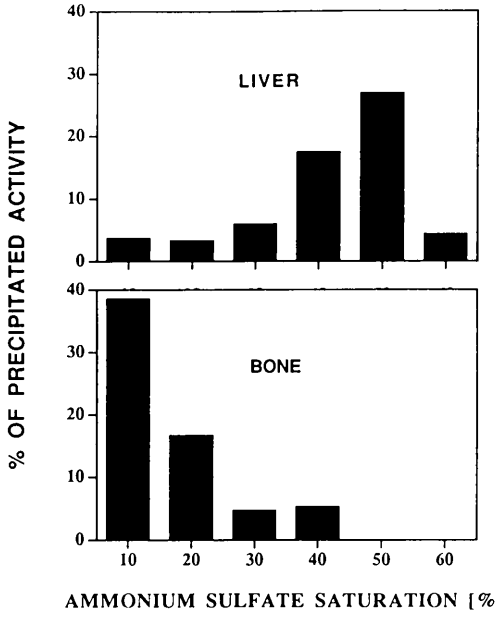

Figure 3. Amonium sulfate fractionation of liver and bone alkaline phosphatases from rainbow trout. Ammonium sulfate was added in a stepwise method. Activities were measured in 100 m*M* diethanolamine/HCl buffer, pH 9.8, containing 5 m*M* $MgCl_2$ and 5 m*M* *p*-Nitrophenylphosphate as a substrate. Reaction was followed by the absorption decline at 405 nm.

34.16–38.75 mg AA/g tissue did not, however, differ either from the control or from the scorbutic group (one-way ANOVA, Scheffe F test, $P > 0.05$; Table IV). These results seem to indicate a tendency for bone alkaline phosphatase to decline under conditions of ascorbate deficiency (correlation coefficient: $r = -0.57$; $P = 0.088$). The sample number per treatment was reduced significantly because some of the serum samples were contaminated by hemoglobin. Hemoglobin has an absorption maximum close to that of paranitrophenol. While it does not affect the total alkaline phosphatase activity measurements (22), it interferes with the assay in the presence of urea.

Discussion

Subclinical indicators of ascorbate deficiency would permit the detection of reduced ascorbate status prior to clinical symptoms. They can also be useful in the assessment of bioavailability of different ascorbate derivatives (esters) in the diet. Unfortunately, the usefulness of most of these indicators is questionable because of contradictory results obtained by various authors (2, 20, 27, 28). The comparisons between proposed indicators are difficult because of different experimental conditions used by various investigators. The species of fish on which experiments were conducted, their size, the water temperature, and algae growth in experimental tanks are among the factors that can influence the outcome of the studies on vitamin C requirements. Growth rate does not always provide useful information because a low dose of ascorbate in the diet (10–20 mg/kg) can support normal growth but fail to prevent scurvy (27). Ascorbate concentration in the anterior kidney, proposed by Halver (28) as index of ascorbate status, is generally used, although some authors have raised questions about its reliability. Lim and Lovell (2) did not detect any significant differences in ascorbate concentration in anterior kidney between the deficient group and the group with the highest ascorbate supplementation (240 mg AA/kg) in their experiment with channel catfish despite the manifestation of other deficiency symptoms. In our previous experiments (24), both kidney and liver ascorbate concentrations reflected ascorbate supplementation when ascorbate was supplied in a stable form (ascorbate monophosphate). Vertebral collagen percentage (2) and, most commonly used, liver

Table II. Total Plasma Alkaline Phosphatase Activity in Juvenile Rainbow Trout Fed for 18 Weeks Diets Supplemented with Graded Levels of Ascorbic Acid in Form of Ascorbate Polyphosphate or Ascorbate Monophosphate

Diet ascorbate*	Group							
	1	2	3	4	5	6	7	8
(mg/kg) Total AP	0	20APP	40APP	80APP	160APP	320APP	1280APP	320AMP
(U/liter)	37.46[a]	42.54[a,b]	36.05[a]	46.27[a,b]	33.60[a]	35.67[a]	45.78[a]	55.27[b]
SD	15.32	13.07	5.44	8.19	6.39	7.99	11.48	3.62

Note. $n = 3$ for all groups except Group 5, where $n = 2$; $P < 0.05$. Values in rows with the same superscripts do not differ significantly.
* Amounts of ascorbate equimolar to ascorbic acid. AMP, ascorbate monophosphate; APP, ascorbate polyphosphate; SD, standard deviation.

Table III. Effect of Urea and Temperature on Half-Lives ($t_{1/2}$) of Alkaline Phosphatase Purified from Bone and Liver

Urea conc. (*M*)	Half-life times (min)					
	Bone			Liver		
	33°C	35°C	37°C	33°C	35°C	37°C
2.7	0.49	0.48	0.35	0.44	0.45	0.47
3.0	0.44	0.50	0.34	0.65	1.05	0.62
3.5	0.40	0.65	0.96	1.10	0.85	2.49
4.0	0.94	0.96	1.31	2.18	1.29	1.18

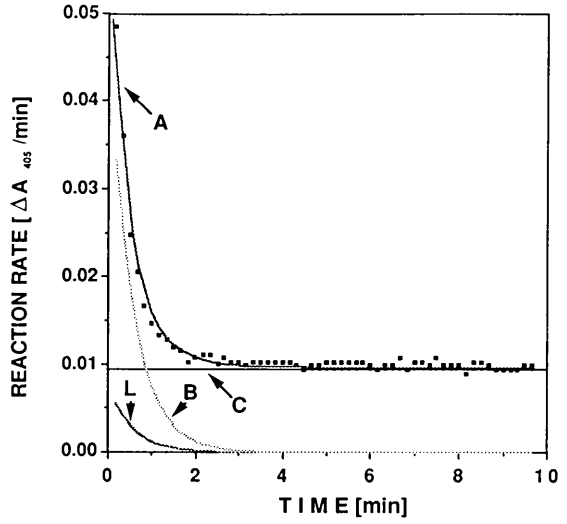

Figure 4. Alkaline phosphatase urea inhibition patterns in blood plasma from control fish. The actual activity decline is represented by closed rectangles. Line A, enzyme activity decay curve for a mixture of two unstable and one stable enzyme forms; Line B, fast decaying bone-type activity; Line L, slower declining liver-type activity; Line C, constant component, not affected by urea.

ascorbate concentration (1) are considered the most reliable indices of fish ascorbate status. The need to sacrifice a fish does not allow for continuous monitoring of the changes in the same individual and prevents any detailed pharmacokinetic studies. Blood ascorbate concentration reflects the dietary intake when sampled with care (27), but close correlations with immediate intake make it unreliable (4). Leukocyte ascorbate level is commonly considered the best indicator of tissue stores of the vitamin C in humans (5), but not enough data are available for fish, probably due to a laborious separation procedure.

In the ascorbate deficiency state, the ratio of hydroxyproline to proline decreases and collagen formation is impaired (29). Proline hydroxylation is conducted by proline hydroxylase, an enzyme that requires ascorbic acid as a cofactor (8). The reduction of collagen synthesis slows bone formation process. Osteoblastic activity correlates with bone type of alkaline phosphatase which plays an important role in calcification process (10). In human blood plasma, alkaline phosphatase can be derived from bone, liver, kidney, intestine, and placenta, and changes in enzyme levels in a particular organ are reflected in plasma level (22). As a result of the diminished bone metabolism, alkaline phosphatase level in blood plasma declines. A similar picture of blood plasma isozymes of alkaline phosphatase of different origin occurs in salmonid fish (18).

In guinea pigs fed an ascorbate-free diet, total alkaline phosphatase activity in blood plasma dropped significantly (12, 13). With other animals, however, results were equivocal. Machlin *et al.* (16) were not able to find evidence of a correlation between ascorbate deficiency and total plasma alkaline phosphatase in the rhesus monkey. Similarly, contradictory results have been reported for fish. Wilson and Poe (15) reported that in channel catfish ascorbate deficiency was accompanied by significantly lower alkaline phosphatase activity in blood plasma compared with fish fed a diet supplemented with 2000 mg AA/kg. Our present results for rainbow trout and those reported earlier (17), do not confirm the findings of Wilson and Poe (15). The deficient fish in our experiment had significantly lower alkaline phosphatase activity than fish fed the diet supplemented in 320 mg of ascorbic acid as ascorbate monophosphate. We did not, however, observe a specific tendency between increasing liver ascorbate concentration (21) and total plasma alkaline

Table IV. Bone (B) and Liver (L) Alkaline Phosphatase Activities in Blood Plasma of Rainbow Trout with Different Ascorbate Status

Ascorbate status	n	Liver AA (μg AA/g tissue)	L	B	C	L/B ratio
Control group	8	n.a.	3.5 ± 2.3	23.1 ± 14.4[a]	7.4 ± 2.0	0.21 ± 0.18[a]
Deficient fish	3	13.9 ± 10.0	4.2 ± 1.1	2.4 ± 1.8[b]	2.8 ± 0.5	2.27 ± 1.05[b]
Low ascorbate	4	35.5 ± 1.9	4.7 ± 4.4	6.5 ± 1.7[a,b]	4.4 ± 1.7	0.75 ± 1.04[a,b]
High ascorbate	4	114.9 ± 8.7	4.1 ± 3.4	21.6 ± 14.6[a]	3.7 ± 3.6	0.28 ± 0.26[a]

Note. Fish were fed for 18 weeks diets varying in ascorbate concentration and form. Bone and liver blood plasma alkaline phosphatase activities were distinguished by differential inhibition by urea. C, stable component of plasma alkaline phosphatase, not affected by urea. Values presented are averages from a given $n \pm$ SD; values in columns with the same superscripts do not differ significantly (one-way ANOVA, $P < 0.05$); n.a., not analyzed.

phosphatase activity. Wilson and Poe (15) compared only two groups of channel catfish: scorbutic fish and those fed a diet supplemented with 2000 mg AA/kg. Moreover, they tested only fish with visible deficiency signs. Therefore, from their experiment we can only conclude that total plasma alkaline phosphatase levels decline in the case of overt scurvy. No information is available on the enzyme activity before the full outbreak of scurvy. This would impose a limitation on using total alkaline phosphatase activity as a clinical indicator *in vivo*.

Several techniques have been proposed for differentiation among liver and bone forms in serum. Brown and Lewis (19) described a method based on differential inhibition by urea that has been applied to the quantification of bone and liver forms in human serum (30). Inactivation of bone, kidney, and liver alkaline phosphatase occurs during the reaction time and to different degrees typical and consistent for a particular form (31). Instead of a graphical method described by the above authors we employed curvilinear regression in our analysis. However, not all plasma samples were suitable for urea inhibition studies. Even a slight contamination with hemoglobin strongly interfered with the assay, which prevented us from using the results of all the dietary groups in which total alkaline phosphatase has been estimated. Based on the results obtained in the inhibition studies, we calculated the ratio of liver to bone alkaline phosphatase in blood plasma and tested this ratio as an indicator of ascorbate status. We observed that the decline in the liver ascorbate content was accompanied by an increase of liver/bone alkaline phosphatase ratio. The significant difference was between the groups with marginal and high liver ascorbate concentration. The proposed method requires more studies and it opens new possibilities in studying deficiency symptoms in fish and other scurvy-prone animals and humans.

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1. Hilton JW, Cho CY, Slinger SJ. Effect of graded levels of supplemental ascorbic acid in practical diets fed to rainbow trout (*Salmo gairdneri*). J Fish Res Board Can **35**:431–436, 1978.
2. Lim C, Lovell RT. Pathology of the vitamin C deficiency syndrome in channel catfish (*Ictalurus punctatus*). J Nutr **108**:1137–1146, 1978.
3. Sato M, Yoshinaka R, Ikeda S. Dietary ascorbic acid requirement of rainbow trout for growth and collagen formation. Bull Jpn Soc Sci Fish **44**:1029–1035, 1978.
4. Lovell RT. Ascorbic acid metabolism in fish. In: Wegger I, Tagwerker FJ, Moustgaard J, Eds. Ascorbic Acid in Domestic Animals. Copenhagen: The Royal Danish Agricultural Soc, pp196–205, 1984.
5. Lee W, Davis KA, Rettmer RL, Labbe RF. Ascorbic acid status: Biochemical and clinical considerations. Am J Clin Nutr **48**:286–290, 1988.
6. Hata RI, Sunada H, Arai K, Sato T, Ninomiya Y, Nagai Y, Senoo H. Regulation of collagen metabolism and cell growth by epidermal growth factor and ascorbate in cultured human skin fibroblasts. Eur J Biochem **173**:261–267, 1988.
7. Weiser H, Schlachter M, Probst HP, Kormann AW. The relevance of ascorbic acid for bone metabolism. In: Ascorbic Acid in Animal Nutrition, Proceedings of the 2nd Symposium in Zurich-Ittingen, Switzerland, pp73–95, 1992.
8. Kivirikko KI, Prockop DJ. Enzymatic hydroxylation of proline and lysine in protocollagen. Proc Natl Acad Sci USA **57**:782–789, 1967.
9. Mussini E, Hutton JJ Jr., Udenfriend S. Collagen proline hydroxylase in wound healing, granuloma formation, scurvy and growth. Science **157**:927–929, 1967.
10. Anderson HC. Biology of disease. Mechanism of mineral formation in bone. Lab Invest **60**:320–330, 1989.
11. Tietz NW, Burtis CA, Duncan P, Ervin K, Petitclerc CJ, Rinker AD, Shuey D, Zygowicz ER. A reference method for measurements of alkaline phosphatase activity in human serum. Clin Chem **29**:751–761, 1983.
12. Goldman HM, Gould BS, Munro HN. The antiscorbutic action of L-ascorbic acid and D-isoascorbic acid (erythorbic acid) in the guinea pig. Am J Clin Nutr **34**:24–33, 1981.
13. Weiser H, Probst HP. Biopotency of ascorbic acid and its derivatives determined by plasma alkaline phosphatase activity in guinea pigs. In: Ascorbic Acid in Animal Nutrition, Proceedings of the 2nd Symposium in Zurich-Ittingen, Switzerland, pp132–142, 1992.

14. Kim M, Otsuka M, Yu R, Kurata T, Arakawa N. The distribution of ascorbic acid and dehydroascorbic acid during time regeneration in wounded dorsal skin of guinea pigs. Int J Vit Nutr Res **64**:56–59, 1992.
15. Wilson RP, Poe WE. Impaired collagen formation in the scorbutic channel catfish. J Nutr **103**:1359–1364, 1973.
16. Machlin LJ, Garcia F, Kuenzig W, Brin M. Antiscorbutic activity of ascorbic acid phosphate in the rhesus monkey and the guinea pig. Am J Clin Nutr **32**:325–331, 1979.
17. Dabrowski K. Comparative bioavailability of ascorbic acid and its stable forms in rainbow trout. In: Ascorbic Acid in Animal Nutrition, Proceedings of the 2nd Symposium in Zurich-Ittingen, Switzerland, pp344–356, 1992.
18. Johnston CE, Horney BS, Deluca S, MacKenzie A, Eales JG, Angus R. Changes in alkaline phosphatase isoenzyme activity in tissues and plasma of Atlantic salmon (*Salmo salar*) before and during smoltification and gonadal maturation. Fish Physiol Biochem **12**(6):485–497, 1994.
19. Brown PB, Lewis KO. Reaction rate retardation as a method for serum alkaline phosphatase isoenzyme measurement. Ann Clin Biochem **17**:192–198, 1980.
20. Dabrowski K, Matusiewicz M, Blom JH. Hydrolysis, absorption and bioavailability of ascorbic acid esters in fish. Aquaculture **124**:169–192, 1994.
21. Matusiewicz M, Dabrowski K, Volker L, Matusiewicz K. Ascorbate polyphosphate is a bioavailable vitamin C source in juvenile rainbow trout: Tissue saturation and compartmentalization model. J Nutr **125**:3055–3061, 1995.
22. McComb RB, Bowers GN, Posen S. Alkaline Phosphatase, 1–986. New York: Plenum Press, 1979.
23. Wachsmuth ED, Hiwada K. Alkaline phosphatase from pig kidney: Method of purification and molecular properties. Biochem J **141**:273–282, 1974.
24. Matusiewicz M, Dabrowski K, Volker L, Matusiewicz K. Regulation of saturation and depletion of ascorbic acid in rainbow trout. J Nutr Biochem **5**:204–212, 1994.
25. Dabrowski K, Hinterleitner S. Simultaneous analysis of ascorbic acid, dehydroascorbic acid and ascorbic sulfate in biological material. Analyst **114**:83–87, 1989.
26. Zar JH. Biostatistical Analysis. Englewood Cliffs, NJ: Prentice-Hall, 1984.
27. Gabaudan J, Meier M, Verlhac V, Wahli T. Influence of the dietary source and dosage of vitamin C on rainbow trout (*Oncorhynchus mykiss*): metabolism, histology and immunity. In: Ascorbic Acid in Animal Nutrition, Proceedings of the 2nd Symposium in Zurich-Ittingen, Switzerland, pp357–377, 1992.
28. Halver JE. The role of ascorbic acid in fish disease and tissue repair. Bull Jpn Soc Sci Fish **38**:79–92, 1972.
29. Chen TL, Raisz LG. The effects of ascorbic acid deficiency on calcium and collagen metabolism in cultured fetal rat bones. Calcif Tiss Res **17**:113–127, 1975.
30. Miggiano GAD, Mordente A, Martorana GE, Castelli A. Time-resolved fractionation of bone and liver alkaline phosphatase activities with a "peeling-off" method. Enzyme **41**:61–67, 1989.
31. Miggiano GAD, Mordente A, Pileri M, Martorana GE, Meucci E, Castelli A. Studies on the selective chemical inhibition by urea of alkaline phosphatase isoenzymes in the reaction course. Enzyme **32**:162–169, 1984.