

AN ABSTRACT OF THE THESIS OF  
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Title: BLOOD CALCIUM AND PHOSPHORUS PERTURBATIONS IN RAINBOW TROUT  
(Salmo gairdneri) AND COHO SALMON (Oncorhynchus kisutch): SOME  
CORRELATES OF PHYSIOLOGICAL STRESS.

Abstract approved: \_\_\_\_\_

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Changes in blood calcium and phosphorus concentrations were characterized for rainbow trout (Salmo gairdneri) and coho salmon (Oncorhynchus kisutch) following intense exercise or exposure to suspended volcanic ash or topsoil. Significant elevations in blood calcium and phosphorus accompanied exercise-induced acidemia in both S. gairdneri and O. kisutch. These elevations persisted for many hours post-exercise. Previous authors have identified bone mineral as the source of the excess calcium observed during exercise-induced hypercalcemia, but data presented herein indicates that phosphorus released into the bloodstream during exercise must have an additional, non-skeletal source.

Exposure of juvenile O. kisutch to suspended Mt. St. Helens volcanic ash caused significant elevations in blood calcium and phosphorus only when fish so exposed were maintained under a regime of moderate exercise. No significant changes in blood calcium or

phosphorus occurred following exposure of unexercised fish to suspended volcanic ash, nor were they elicited in exercised fish exposed to an identical level of suspended topsoil.

The effects of exercise-induced hypercalcemia on the blood buffering capacity of S. gairdneri were also investigated. Results obtained in vitro suggest that dissolution products of bone hydroxyapatite may significantly diminish the in vivo blood buffering power of S. gairdneri, especially during exercise-induced acidosis.

Blood Calcium and Phosphorus Perturbations in Rainbow Trout  
(Salmo gairdneri) and Coho Salmon (Oncorhynchus kisutch):  
Some Correlates of Physiological Stress

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Blood Calcium and Phosphorus Perturbations in Rainbow Trout  
(Salmo gairdneri) and Coho Salmon (Oncorhynchus kisutch):  
Some Correlates of Physiological Stress.

CHAPTER I

Effects of Intense Exercise on Blood Calcium and Phosphorus

INTRODUCTION

The reliance upon anaerobic metabolism for the expedient production of energy is a phylogenetically widespread and ancient trait among the vertebrates (Ruben & Bennett, 1980). The principle end product of vertebrate anaerobiosis is lactic acid, and therefore poikilothermic vertebrates which utilize this pathway most extensively often experience a significant activity-related blood pH depression and a decreased capacity for O<sub>2</sub> transport, enzymatic function, and further activity (Bennett, 1978).

The activity metabolism of salmonid fish has been extensively studied (see Love, 1980, for review). While notable for their relatively great aerobic scope (Brett, 1972), salmonids also generate considerable quantities of lactic acid during intense exercise (Black, 1966) and experience a marked post-exercise acidosis which persists for many hours. The correlates of metabolic acidosis in salmonids are known to include hypercalcemia (Ruben & Bennett, 1981) and hyperphosphatemia (Hammond & Hickman, 1966).

The present work corroborates the findings of earlier authors that exercise is accompanied by changes in blood pH, calcium, and phosphorus levels in salmonid fish. The time courses for post-exercise acidemia, hypercalcemia, and hyperphosphatemia are characterized for Salmo gairdneri, and the relative proportions and probable sources of calcium and phosphorus released into the bloodstream of Oncorhynchus kisutch during exercise are discussed.

#### METHODS & MATERIALS

Sub-adult rainbow trout (Salmo gairdneri, Alsea River, 1979 brood; mean wt. = 150 grams; range: 120-170) and pre-smolt coho salmon (Oncorhynchus kisutch, Big Creek, 1980 brood; mean wt. = 30 grams; range: 28-32) were held in flowing dechlorinated tap water under conditions of constant temperature ( $10^{\circ} \pm 2^{\circ} \text{C}$ ) and constant dim lighting. All fish were fasted at least 96 hours prior to use in experiments.

Measurements of blood pH, serum calcium and inorganic phosphorus ( $P_i$ ), and muscle total phosphorus were made for Salmo gairdneri. For Oncorhynchus kisutch, blood pH and total blood calcium and phosphorus were determined. Post-exercise measurements of these parameters were obtained from fish individually exercised to exhaustion by hand chasing (8-10 minutes), and then recovered for various time intervals. Water conditions in the recovery tank were identical to those in the holding tank. Resting measurements were obtained from previously undisturbed individuals removed from the holding tank and

quickly stunned with a blow to the head. Struggling was minimal during this procedure, and maximum elapsed time between first contact and immobilization was less than 10 seconds.

Blood was collected by severing the caudal peduncle after carefully cleaning the area of mucus and scales. For pH determination, blood was collected into heparinized glass capillary tubes directly as it issued from the cut dorsal aorta, and was immediately analyzed on a Radiometer BMS 3 MK 2 Blood Micro System complexed with a PHM 73/Blood Gas Monitor. The pH electrode (type G299A; Radiometer) was regulated at the body temperature of the fish ( $10^{\circ}\text{C}$ ) and was calibrated prior to each measurement.

Additional blood was collected into non-heparinized plastic centrifuge tubes. For determination of total calcium, a 50  $\mu\text{l}$  aliquot of fresh, uncoagulated blood was diluted 1:20 with lanthanum oxide solution ( $3.6 \times 10^{-3} \text{ M La}_2\text{O}_3$ ;  $6.0 \times 10^{-2} \text{ M HCL}$ ) and analyzed on an Instrumental Labs IL 551 atomic absorption spectrophotometer using an air-acetylene flame. Total phosphorus was determined by mixing an additional 200  $\mu\text{l}$  aliquot of whole, uncoagulated blood with 5N sulfuric acid and then digesting, oxidizing, and analyzing for total phosphorus as described in Hawk, Oser, and Summerson (1954). The remaining blood was tightly capped and allowed to coagulate at room temperature for 20 minutes prior to centrifugation. A portion of the resultant serum was diluted 1:10 with lanthanum oxide solution and analyzed for total calcium by flame atomic absorption spectrophotometry. Another portion was analyzed for inorganic

phosphorus using a Pierce Phosphorus Rapid Stat Kit (Pierce Chemical Co., Rockford, Illinois) and a Beckman Model 24 spectrophotometer set at 690 nm.

For the measurement of muscle total phosphorus, 0.25-0.30 gram of bone- and skin-free epaxial muscle was dissected from the region immediately anterior to the dorsal fin and quickly weighed to the nearest 0.001 gram on a Mettler balance; this tissue was then completely homogenized in deionized water using a Sorvall Omnimixer. An aliquot of the resultant homogenate was mixed with 5N sulfuric acid and digested, oxidized, and assayed for total phosphorus as described in Hawk, et al., 1954.

All data were statistically analyzed using a one-way ANOVA followed by an unpaired t-test (Glantz, 1981). The P values which appear in the text correspond to the outcome of the t-test.

### RESULTS & DISCUSSION

The time courses for post-exercise changes in blood pH, and serum calcium and phosphorus of Salmo gairdneri are given in Figure 1. Following exhaustive exercise, blood pH became maximally depressed within 30 minutes (7.64 to 7.32;  $P < 0.001$ ), and then slowly returned to near normal values over the eight hour recovery period. However, at eight hours post-exercise, mean blood pH was still significantly below resting levels ( $P < 0.02$ ).

Mean serum calcium attained maximum elevation (2.49 to 3.16 mM;  $P < 0.001$ ) within 10 minutes of the end of exercise, and displayed

FIGURE 1

Time course for changes in blood pH and serum calcium and inorganic phosphorus concentrations following intense exercise and various recovery periods in Salmo gairdneri at 10° C. Each data point represents the mean ( $\pm$  S.E.M.) of at least seven different individual fish.

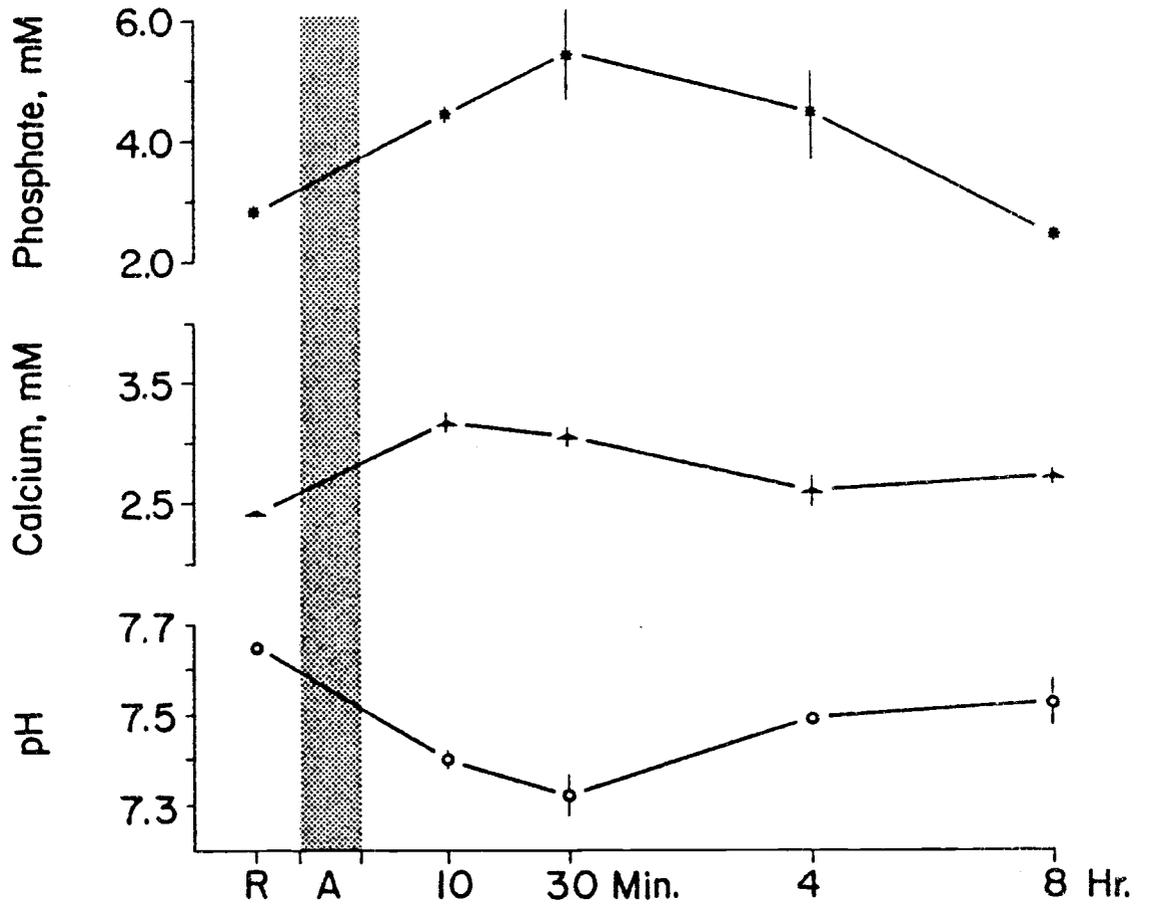


Figure 1

much more interindividual variation than did serum calcium. Following eight hours recovery, mean serum phosphorus was significantly below resting values ( $P < 0.01$ ).

Figure 2 shows the changes in whole blood total calcium and phosphorus concentrations following exhaustive exercise in Oncorhynchus kisutch. Within 10 minutes, mean total calcium had risen 24% (1.68 to 2.08 mM;  $P < 0.003$ ) and mean total phosphorus had risen 13% (41.8 to 47.4 mM;  $P < 0.003$ ).

No significant change was detected between the resting and post-exercise muscle total phosphorus content of Oncorhynchus kisutch.

It is evident from Figure 1 that the post-exercise time courses for hypercalcemia and hyperphosphatemia closely mirror that for blood pH depression. Black (1966) has also reported an exercise-induced lactacidosis of approximately eight hours for Salmo gairdneri held at a similar temperature ( $12^{\circ}\text{C}$ ). While elevations in serum calcium and inorganic phosphorus are both highly significant statistically, the rise in serum phosphate is much higher than that of serum calcium. This phenomenon probably reflects both the sources and the relative physiological significance of the two elements.

These findings supplement those of Ruben & Bennett (1981), who demonstrated that the most likely source of exercise-related hypercalcemia is dissolution of a fraction of the hydroxyapatite component of bone. Their results preclude the possibility that the excess calcium is derived from scales or soft tissues. Calcium plays an essential role in numerous physiological functions such as blood

FIGURE 2

Percent elevation in whole blood total calcium and total phosphorus concentrations immediately (0) and 10 minutes following intense exercise of Oncorhynchus kisutch at 10° C. Each data point represents the mean ( $\pm$  S.E.M.) of at least nine different individual fish.

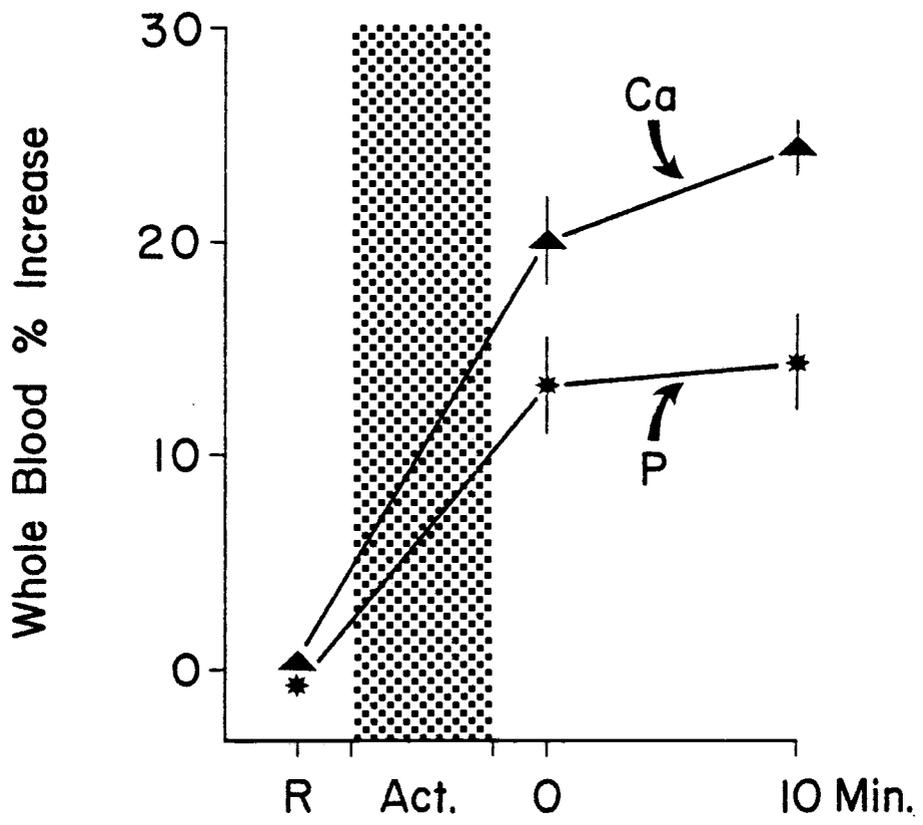


Figure 2

clotting, skeletal and cardiac muscle contraction, and neurotransmission (Parfitt & Kleerekoper, 1979a), and is expected to be closely regulated by vertebrate systems; such a significant and prolonged elevation is therefore surprising.

In addition to its role as a substrate for the phosphorylation of high energy compounds, phosphate is of primary importance as an intracellular and urinary buffer (Guyton, 1976; Lehninger, 1979). It has recently been demonstrated by Kobayashi & Wood (1980) that Salmo gairdneri can excrete an endogenously produced acid load by renal mechanisms alone, and that the trout kidney excretes hydrogen ion by the same mechanism as the mammalian kidney, i.e., by phosphate buffering of the urine. Both hypoxia (Hunn, 1969) and sustained exercise (Meyerhoff, 1981) significantly increase renal excretion of phosphate by Salmo gairdneri. This mechanism probably accounts, at least in part, for the return of blood pH and serum phosphate to near normal levels following eight hours of recovery.

The absolute, post-exercise millimolar increments in Oncorhynchus kisutch whole blood calcium and phosphorus appear in the ratio 1 Ca:14 P. This ratio is unlike that found in the major crystalline component of bone, hydroxyapatite (10 Ca:6 P; Parfitt & Kleerekoper, 1979a), and strongly indicates an additional, non-skeletal origin for phosphorus released into the bloodstream during exercise.

Locomotory skeletal muscle seems the most likely source of this excess phosphorus. Muscle contains the bulk of the phosphorus found

in the soft tissues of mammals (Parfitt & Kleerekoper, 1979b), and presumably in fish as well. This possibility is further supported by the observations in mammals that:

- i.) Asphyxic heart muscle releases inorganic phosphate into the bloodstream, and this release occurs even in the absence of red blood cells (Opie, et al., 1972);
- ii.) "fast" skeletal muscle releases more phosphate than "slow" skeletal muscle (Hudlicka, 1971); and,
- iii.) the amount of phosphate released is proportional to the frequency of contraction within a single muscle (Hilton & Vrbova, 1969).

Although no significant decrease was measured between resting and post-exercise muscle total phosphorus, the percentage of muscle phosphorus required to raise whole blood by the observed amount is so small (less than 1%; see Appendix for calculation) as to be undetectable. Intracellular and extracellular phosphate are known to be in equilibrium (Parfitt & Kleerekoper, 1979a), and in situations which mandate the net catabolism of phosphagen (e.g., hypoxia, intense exercise), inorganic phosphate should accumulate inside cells, pass down its concentration gradient through the cell membrane, and increase the  $[P_i]$  of the extracellular fluid. Increases in the free  $[P_i]$  of asphyxic and/or actively contracting skeletal and cardiac muscle cells are well documented (Aragon & Lowenstein, 1980; Feinstein, 1962; Hammond & Hickman, 1966; Hems & Brosnan, 1970; Opie, et al., 1972). It seems reasonable, therefore,

to conclude that the major portion of the excess phosphorus observed post-exercise is released from muscle, and perhaps other soft tissues as well.

The physiological consequences of acute, exercise-related elevations in blood calcium and phosphorus have yet to be investigated. In mammals, experimental or pathological hypercalcemia is associated with muscular weakness and fatigue, hypertension, bradycardia and arrhythmias, soft tissue calcification, impaired renal function, and occasionally convulsions (Epstein, 1979; Parfitt & Kleerekoper, 1979b). Hyperphosphatemia is mainly implicated in soft tissue calcification and tetany (Parfitt & Kleerekoper, 1979b). During the course of these experiments, three fish exhibited an incapacitating tetany during exercise; two subsequently died. Measurements obtained from these fish shortly after death (before rigor mortis) revealed the highest serum inorganic phosphorus concentrations (9.8 and 10.0 mM/liter) yet observed in this laboratory. An increased phosphate load is frequently associated with hypocalcemia and tetany in mammals (Parfitt & Kleerekoper, 1979b). In this regard, it is of interest that, four hours following exhaustive exercise, individual fish which exhibited the highest serum phosphate concentrations also exhibited the lowest serum calcium concentrations.

Under normal circumstances, the importance of phosphate as a blood buffer is minimal due to its low concentration (Ganong, 1979). However, it is possible that inorganic phosphorus released from soft

tissue during intense exercise could assume a heightened role in extracellular buffering. Preliminary evidence from in vitro studies suggests that calcium released from the dissolution of hydroxyapatite may antagonize blood buffering capacity (Chapter 3; J. Ruben, 1983), but this effect may be overridden in vivo by the much greater quantity of phosphate released from soft tissues during intense exercise.

Because this study involved only the measurement of total calcium concentration, the magnitude of elevations in blood calcium ion ( $\text{Ca}^{2+}$ ) remain unknown. However, such elevations must surely have occurred, because 1.) they have been measured in the blood of exercising humans (Nielsen, et al., 1977), and 2.) the  $\text{Ca}^+$  fraction of blood is increased under acidic conditions, due to the effect of elevated  $\text{H}^+$  on the  $\text{Ca}^{2+}$ --protein equilibrium (Henry, 1974; Nielsen, et al., 1977). Thus, in the absence of hyperproteinemia, an increase in total  $[\text{Ca}]$  must contain an increase in  $[\text{Ca}^{2+}]$ .

Calcium ion ( $\text{Ca}^{2+}$ ) is widely recognized as a stimulator of numerous cell processes (Jamieson, et al., 1980). Even though cytosolic calcium ion concentration is believed to be self-regulating via a plasma membrane,  $\text{Ca}^{2+}$ --activated calcium pump (Larsen & Vincenzi, 1979), it seems possible that transient elevations in intracellular  $\text{Ca}^{2+}$  could result from exercise-induced hypercalcemia. Intracellular increases in free  $\text{Ca}^{2+}$  are known to activate the ubiquitous calcium-binding protein calmodulin, which is implicated in the regulation of numerous cellular enzymes (Cheung, 1980). Intense

exercise may thereby have some important, and as yet, undescribed biochemical and physiological effects.

## CHAPTER II

### Mount Saint Helens Volcanic Ash Induced Systemic Stress in Juvenile Coho Salmon

#### INTRODUCTION

The devastating mud flows unleashed by the 18 May 1980 eruption of Mount Saint Helens deposited tons of volcanic ash and debris within the valleys of several nearby river systems (Lehre, et al., 1982). Consequently, during periods of heavy runoff, suspended volcanic material, principally ash, attains high levels especially in the Toutle River and the lower portion of its confluent, the Cowlitz River (Stober, et al., 1982). Geologists predict that both rivers will continue to carry substantial sediment loads for many years to come (Lehre, et al., 1982). The capacity of the Toutle River to support aquatic life was virtually destroyed by the eruption events (Martin, et al., 1982), but the Cowlitz River still hosts a large and economically important salmon fishery. During migration from the upper river, juvenile salmon (smolts) must traverse the often silt-laden 35 kilometers of the lower river before reaching their estuarine and oceanic feeding grounds. The consequences of this passage to the fishes' health and subsequent ability to successfully adapt to saltwater life will probably remain a major concern to fisheries managers. Due to its high content of glassy silicate particles, its angular surface characteristics, and its fineness

(Fruchter, et al., 1980), volcanic ash is highly abrasive and might be expected to have deleterious effects upon the respiratory epithelia of fish. Previous studies have demonstrated the stressful nature of suspended solids in general, and volcanic ash in particular, upon juvenile salmonids (Noggle, 1978; Redding & Schreck, 1980). However, these studies have not distinguished between the effects of suspended volcanic ash and other materials likely to be encountered by salmon, perhaps because they failed to account for higher activity levels required of the fish in nature.

The present work was based upon two hypotheses: 1.) that due to its physical characteristics, suspended volcanic ash would cause greater physiological stress (acting at the respiratory surface) than would suspended topsoil; and 2.) that activity above resting levels would compound the effects of exposure to suspended solids. Other conditions which engender systemic respiratory stress in salmonids, such as hypoxia or situations which require intense or sustained activity are known to be accompanied by increased hematocrit (Swift & Lloyd, 1974), acidemia (Black, et al., 1959), hyperphosphatemia (Hammond & Hickman, 1966), and recently, hypercalcemia (Ruben & Bennett, 1981). We chose to measure serum calcium and inorganic phosphorus concentrations because recent findings within our laboratory have established that these are closely correlated with hypoxia- and exercise-induced respiratory stress.

## METHODS & MATERIALS

Juvenile coho salmon (Big Creek x Soleduc hybrids, 1979 brood) were fasted at least 96 hours prior to and during all experiments to reduce contamination due to fecal material. Seven to twelve fish (mean wt. = 50 grams; range: 40-60) were held in 135 liter tanks containing dechlorinated tap water or water and suspended volcanic ash or topsoil at concentrations of  $1.0 \pm 0.25$  grams/liter. The water temperature was maintained at  $12 \pm 2^\circ$  C and the  $PO_2$  at  $155 \pm 5$  mm Hg throughout the experiments. Three submersible pumps maintained the suspension and created a circular current within the tanks of approximately 0.1 meter/sec. To avoid accumulation of nitrogenous waste products, fish were transferred to fresh tanks (maintained under identical conditions) at 48 hour intervals. All experimental fish which were subjected to an exercise regime were equally stimulated to moderate activity twice daily (at 1200 and 1800 hours) by hand chasing for five minutes. Experiments lasted 120 hours and were begun and terminated at 1200 hours to control for the possibility of diurnal variation in the blood parameters measured. The last exercise period occurred at least 18 hours prior to blood sampling. No significant mortality occurred during any of the experiments.

Blood from exsanguinated fish was collected into plastic centrifuge tubes and allowed to clot at room temperature for 20 minutes prior to centrifugation. 100 ul of the resultant serum was diluted 1:10 with lanthanum oxide solution ( $3.6 \times 10^{-3}$  M  $La_2O_3$ ; 6 X

$10^{-2}$  M HCL) and analyzed for total calcium by air-acetylene flame atomic absorption spectrophotometry. An additional 50  $\mu$ l of serum was analyzed for inorganic phosphorus using a Pierce Phosphorus Rapid Stat Kit (Pierce Chemical Co., Rockford, Ill.) and a Beckman Model 24 spectrophotometer set at 690 nm.

All data were statistically analyzed using a one-way ANOVA followed by a two-tailed t-test (Glantz, 1981). The P values which appear in the text correspond to the outcome of the t-test.

### RESULTS & DISCUSSION

When encountered independently, neither the exercise regime used in these experiments nor simple exposure to suspended solids caused hypercalcemia or hyperphosphatemia in juvenile coho salmon. However, when exposed to suspended volcanic ash, exercised fish experienced significant elevation of serum calcium and inorganic phosphorus concentrations (Figure 3). Mean serum calcium rose from 2.49 (control values from fish exercised in clear water) to 2.70 mM/liter ( $P < 0.02$ ), and mean serum inorganic phosphorus rose from 2.65 to 3.01 mM/liter ( $P < 0.01$ ). Following exposure to the same concentration of suspended topsoil, both mean serum calcium and inorganic phosphorus concentrations showed a trend towards elevation, but this trend was not significant ( $P > 0.05$ ; Figure 3). Neither were significant differences observed between the blood parameters of control fish and unexercised fish exposed to suspended volcanic ash ( $P > 0.2$ ; Figure 3).

FIGURE 3

Mean percent increase ( $\pm$  S.E.M.) in serum calcium and inorganic phosphorus concentrations of juvenile coho salmon (Oncorhynchus kisutch) following exposure to various suspended sediment and exercise regimes. Clear bars represent exposure of unexercised fish to suspended volcanic ash (N = 7). Colored bars represent exposure of exercised fish to suspended volcanic ash (N = 8) or topsoil (N = 12). Fish exercised in clear water served as the control group. \*\* denotes significance at  $P < 0.01$ ; \* denotes significance at  $P < 0.02$ .

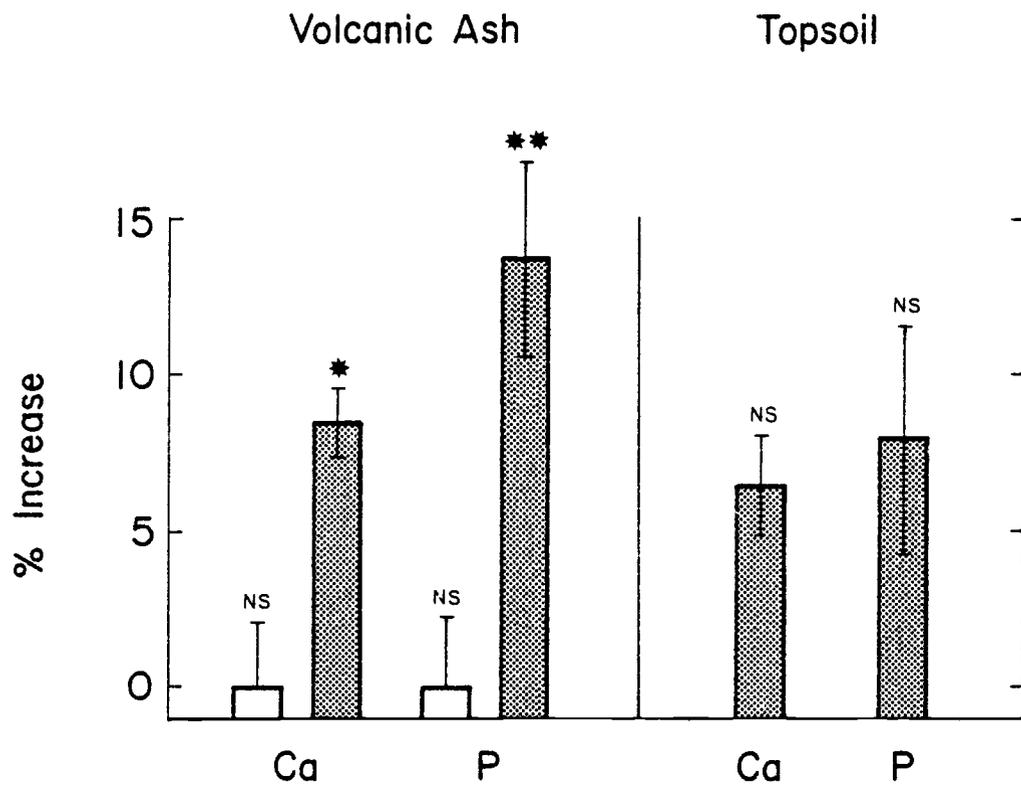


Figure 3

It seems especially noteworthy that exercised fish exhibited much more severe accumulation or "packing" of sediment in the gill lamellae than did unexercised fish. The gill lamellae of exercised fish exposed to suspended topsoil exhibited packing which was similar in degree to that observed in unexercised fish exposed to suspended volcanic ash.

Although salmonids possess relatively great aerobic scope (Brett, 1972), as poikilotherms they must rely extensively upon anaerobic glycolysis during "burst" activity (Bennett, 1978), such as probably occurs in the course of feeding, escaping predators, and negotiating difficult currents while migrating. In most vertebrates, such reliance upon anaerobic metabolism is commonly associated with the acquisition of an  $O_2$  debt (Bartholomew, 1977). It seems likely that exercised juvenile coho salmon exposed to suspended volcanic ash display altered blood parameters which are usually associated with hypoxia or intense exercise because volcanic ash interferes with the fishes' ability to either repay an  $O_2$  debt, or to avoid incurring one during exercise. The mechanism by which ash does this is unclear, but may involve a simple reduction in the respiratory surface area (via packing) or an irritation-induced reduction in vascular perfusion of the gill lamellae. Exposure to suspended topsoil seems to have the same general effect, although to a somewhat lesser degree. Because no consistent effects upon gill histology have been reported for salmonids following exposure to suspended solids, it is difficult to identify the exact mechanism by which volcanic ash

interferes with respiration. In any case, these results strongly indicate that juvenile salmonids may experience significant systemic stress both during and following exposure to ash laden waters.

### CHAPTER III

#### Influence of Exogenous Calcium Phosphate, $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ on the in vitro Blood Buffering of Rainbow Trout

### INTRODUCTION

The capacity to buffer changes in the hydrogen ion concentration  $[\text{H}^+]$  of the extracellular fluid is essential to the proper function of blood and other vertebrate tissues (Eckert, 1978; Ganong, 1979; White, 1977). Blood buffering becomes especially important during intense exercise, when production of metabolic and respiratory acid is greatly increased. There is also increased enzymatic function and  $\text{O}_2$  transport at this time, both of which are subject to disruption by the resultant acidemia (Bennett, 1978; White, 1977). Among poikilothermic vertebrates, which rely heavily upon anaerobic metabolism for the expedient production of energy, salmonid fish are notable both for their anaerobic capacity and for their sensitivity to post-exercise acidosis (Black, 1958). Blood pH values below approximately 6.90 commonly result in mortality of salmon and trout (Jonas, et al., 1962). Clearly, the blood of such fish is presented with a  $\text{H}^+$  buffering challenge of enormous magnitude during and following intense exercise. It is also of interest that salmonids display other, less well known correlates of exercise-induced acidosis to the highest degree of any vertebrate yet examined. Following severe exercise in Oncorhynchus and Salmo, mean serum calcium and inorganic phosphorus concentrations routinely rise by 25%

and 60%, respectively (Chapter I), and both have been observed to rise by as much as 100% in exhausted rainbow trout (B. Adams, unpublished observation; J. Ruben, unpublished manuscript). Such significant changes in ionic composition might be expected to alter the buffering status of the blood in some way, thus influencing the maintenance or re-establishment of normal pH.

The present work investigates the effect of addition of calcium phosphate, as hydroxyapatite,  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ , on the in vitro blood buffering capacity of Salmo gairdneri. Calcium phosphate in this chemical form was chosen because 1.) hydroxyapatite is the major crystalline component of bone mineral (Parfitt & Kleerekoper, 1979a); and 2.) bone has been identified as the most probable source of excess calcium observed during exercise-induced hypercalcemia (Ruben & Bennett, 1981). Therefore, in determining the effects of skeletal dissolution products upon blood buffering, hydroxyapatite was judged the most appropriate source of calcium and phosphate.

#### METHODS & MATERIALS

Rainbow trout (Salmo gairdneri, mean wt. = 200 grams, range: 170-250) were acclimated to 10° C flowing dechlorinated tap water for at least one month, and were maintained on a diet of chopped beef liver. All fish were fasted for at least one week prior to use in experiments.

For blood collection, previously undisturbed individuals were removed from the holding tank and quickly stunned with a blow to the

head. Struggling was minimal during this procedure, and total elapsed time between first contact and immobilization was less than 10 seconds. The caudal peduncle was severed with a sharp blade after carefully cleaning the area of mucus and scales, and blood was collected directly into a heparinized (0.2 mg/ml blood; Sigma Chemical Co.) glass beaker, covered, and stored on ice until use.

Immediately prior to treatment, the collected blood was gently but thoroughly mixed and divided into two equal portions. Calcium phosphate,  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ , was added to one portion; the other served as the control. Calcium phosphate was added in excess (approx. 25 mg/ml blood) because it was reasoned that in vivo the extracellular fluid is essential in contact with an excess of bone mineral, the soluble fraction being determined by the physico-chemical factors of the blood.

Despite the fact that in vivo exercise-induced acidemia is largely due to the production of lactic acid, the hydration of  $\text{CO}_2$  was chosen as a means of generating free  $\text{H}^+$  because the acidosis produced in this manner is readily reversible. Pure  $\text{CO}_2$  was gently bubbled through each blood sample, and pH and  $\text{PCO}_2$  were simultaneously measured on a Radiometer BMS 3 MK 2 Blood Micro System complexed with a PHM 73/Blood Gas Monitor. Both electrodes were regulated at  $10^\circ\text{C}$ . The pH electrode (type G299A) was calibrated prior to each measurement, and the  $\text{PCO}_2$  electrode prior to every third measurement. There was no noticeable drift of either electrode during the experiment.

## RESULTS & DISCUSSION

The result of addition of excess  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$  on the in vitro buffering by Salmo gairdneri blood over a physiologically realistic pH range (7.60 to 6.90) is given in Figure 4. The slope of each line represents the buffering capacity, which is here defined as the amount of added  $\text{H}^+$  required to cause a given drop in pH. In this experiment, it was assumed that  $[\text{H}^+]$  was directly proportional to the measured  $\text{PCO}_2$  of each sample.

The steeper slope of the buffer line for  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ --treated blood indicates a decreased capacity for buffering  $\text{H}^+$  produced via the hydration of  $\text{CO}_2$ , when compared to untreated blood. This difference becomes especially pronounced at higher concentrations of  $\text{H}^+$ , such as would be experienced following intense exercise. For example, at a  $\text{PCO}_2$  of 50 mm Hg, the free  $\text{H}^+$  concentration of the  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ --treated blood was 18% higher than that of the untreated blood sample.

The mechanism by which  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$  reduces blood buffering capacity is uncertain, but may involve the cation exchange of  $\text{Ca}^{2+}$  for  $\text{H}^+$  at buffering sites on hemoglobin or plasma proteins. Cation exchange is well known in the study of soil chemistry (Brady, 1974) and in biochemistry (Lehninger, 1979). Due to their imidazole groups, the 38 histidine residues of hemoglobin constitute an extremely important buffering component of vertebrate blood (Ganong, 1979); a decreased availability of these sites might significantly

alter blood buffering capacity. The exchange of  $\text{Ca}^{2+}$  for  $\text{H}^+$  would effectively increase the free  $\text{H}^+$  concentration of the  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ -treated blood, resulting in a lower pH at any given  $\text{PCO}_2$ .

FIGURE 4

Influence of addition of  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$  on in vitro blood buffering by Salmo gairdneri. The linear descriptions of these data are  $[\text{H}^+] = 1.48 + (0.17)\text{PCO}_2$  for the untreated blood (control), and  $[\text{H}^+] = 1.39 + (0.21)\text{PCO}_2$  for the blood treated with  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ . The slopes of the two lines differ significantly ( $P < 0.01$ ; t-test. Glantz, 1981).

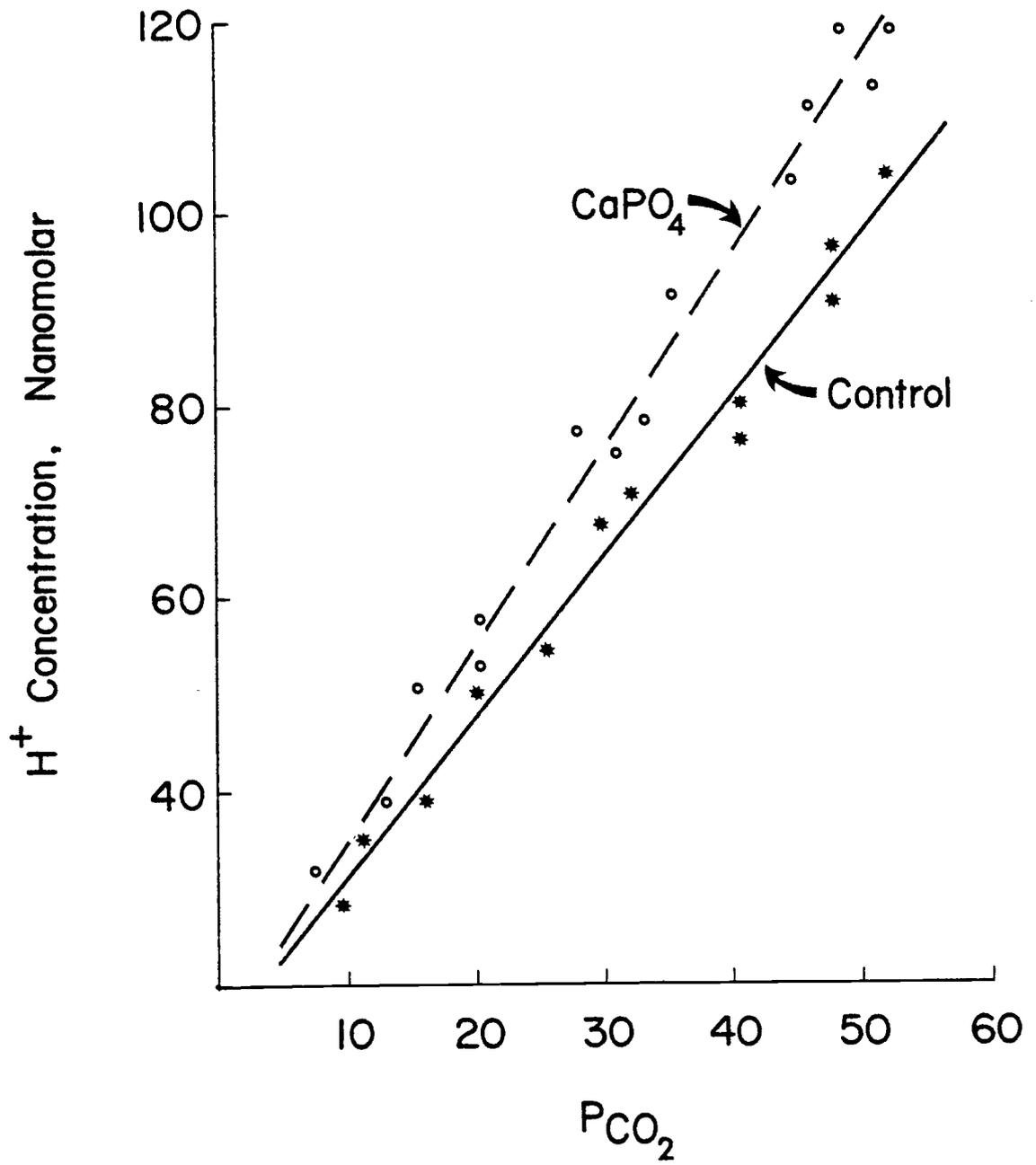


Figure 4

The dissociation of bone hydroxyapatite,  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$  should liberate equivalents of positive and negative charge as carried by  $\text{Ca}^{2+}$ ,  $\text{OH}^-$ , and  $\text{PO}_4^{3-}$ . Therefore, any buffering action that  $\text{PO}_4^{3-}$  and  $\text{OH}^-$  may have will be cancelled out by the  $\text{Ca}^{2+}$  released. However, the in vivo conditions which elicit the acidic dissolution of bone (e.g., hypoxia, intense exercise) also cause the additional release of phosphorus from non-skeletal sources (Chapter I). Absolute post-exercise increments in the serum [phosphate] and [Ca] are 2.5 and 0.7 mM/liter, respectively, and the whole blood total phosphorus concentration rises by 6 mM/liter following exhaustive exercise in the closely related genus Oncorhynchus (Chapter I). Although the chemical forms in which phosphorus enters the whole blood post-actively are not known, because the post-exercise serum phosphate increment is only 2-3 mM/liter, whereas the increment in whole blood total phosphorus is nearly 6 mM/L, it may be safely assumed that not all of the observed rise in whole blood total phosphorus is due to inorganic phosphorus alone. Thus, phosphorus may enter the bloodstream as ATP or other phosphate esters in addition to  $\text{P}_i$ , and these compounds may also contribute to blood buffering power. Consequently, phosphorus liberated in vivo during exercise may contribute more to blood buffering capacity than  $\text{Ca}^{2+}$  released from bone detracts from it.

Despite this possibility, the results depicted in Figure 1 suggest an antagonistic effect of hydroxyapatite dissolution on blood buffering capacity. If the same effect is present in vivo, this

phenomenon might seriously diminish the ability of salmonids to maintain blood pH within a tolerable range following intense exercise, and may partially account for the sensitivity of such fish to exercise-induced acidosis.

LITERATURE CITED

- Aragon, J.J., and J.M. Lowenstein. 1980. The purine-nucleotide cycle: comparisons of the levels of citric acid cycle intermediates with the operation of the purine nucleotide cycle in rat skeletal muscle during exercise and recovery from exercise. *Eur. J. of Biochem.* 110: 371-377.
- Bartholomew, G.A. 1977. Energy Metabolism. Chapter 3 in Malcolm S. Gordon, ed. *Animal Physiology*. MacMillan Publishing Company, New York.
- Bennett, A.F. 1978. Activity metabolism of the lower vertebrates. *Ann. Rev. of Physiol.* 400: 447-469.
- Black, E.C. 1958. Hyperactivity as a lethal factor in fish. *J. Fish. Res. Bd. Can.* 15: 573-586.
- Black, E.C., W. Chui, F.D. Forbes, and A. Hanslip. 1959. Changes in pH, carbonate and lactate of the blood of yearling Kamloops trout, Salmo gairdneri, following muscular activity. *J. Fish. Res. Bd. Can.* 16: 391-402.
- Black, E.C., G. T. Manning, and K. Hayashi. 1966. Changes in levels of hemoglobin, oxygen, carbon dioxide, pyruvate, and lactate in venous blood of rainbow trout, (Salmo gairdneri) during and following severe muscular activity. *J. Fish. Res. Bd. Can.* 23 (6): 783-795.
- Brett, J.R. 1972. The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. *Resp. Physiol.* 14: 151-170.
- Cheung, W.Y. 1979. Calmodulin plays a pivotal role in cellular regulation. *Science* 207: 19-27.
- Eckert, R., and D. Randall. 1978. *Animal Physiology*. W.H. Freeman and Company, San Francisco. 558 pp.
- Epstein, F.H. 1979. Signs and symptoms of electrolyte disorders. Pages 499-530 in Morton H. Maxwell and Charles R. Kleeman, eds. *Clinical disorders or electrolyte metabolism*. 3rd ed. McGraw-Hill Book Company, New York.
- Feinstein, M. 1962. Effects of experimental congestive heart failure, ouabain, and asphyxia on the high energy phosphate and creatine content of the guinea pig heart. *Circ. Res.* 10: 333-346.

- Fruchter, J.S., et al. 1980. Mount St. Helens ash from the 18 May, 1980 eruption: chemical, physical, mineralogical, and biological properties. *Science* 209: 1116-1125.
- Ganong, W.F. 1979. Review of medical physiology. Lange Medical Publications, Los Altos. 618 pp.
- Glantz, S.A. 1981. A primer of biostatistics. McGraw-Hill Book Co., New York. 352 pp.
- Guyton, A.C. 1976. Textbook of medical physiology. W.B. Saunders Co., Philadelphia. 1194 pp.
- Hammond, B.R., and C.P. Hickman, Jr. 1966. The effect of physical conditioning on the metabolism of lactate, phosphate, and glucose in the rainbow trout, Salmo gairdneri. *J. Fish. Res. Bd. Can.* 23 (1): 65-83.
- Hawk, P.B., B.L. Oser, and W.H. Summerson. 1954. Practical physiological chemistry. The Blakiston Co., New York. 1439 pp.
- Hems, D.A., and J.T. Brosnan. 1970. Effects of ischemia on content of metabolites in rat liver and kidney in vivo. *Biochem. Jour.* 120: 105-111.
- Henry, J.B. 1974. Clinical chemistry. Pages 516-656 in Israel Davidsohn and John Bernard Henry, eds. *Clinical Diagnosis*. 15th ed. W.B. Saunders Co., Philadelphia.
- Hilton, S.M., and G. Vrbova. 1970. Inorganic phosphate--a new candidate for the mediator of functional vasodilation in skeletal muscle. *J. of Physiol.* 206: 29-30P.
- Hudlicka, O. 1971. Uptake of substrates in isolated contracting slow and fast muscles in situ in relation to fatigue. Pages 215-223 in Bengt Pernow and Bengt Saltin, eds. *Advances in experimental medicine and biology*. Vol. 2: Muscle metabolism during exercise. Plenum Press, New York.
- Jamieson, G.A. 1980. Structure and function relationships among calmodulin and troponin c-like proteins from divergent organisms. Pages 1-3 in D. Martin Watterson and Frank F. Vincenzi, eds. *Calmodulin and cell functions*. *Annals of the New York Academy of Science*. Volume 356.
- Jonas, R.E.E., H.S. Sehdev, and N. Tomlinson. 1962. Blood pH and mortality in rainbow trout (Salmo gairdneri) and sockeye salmon (Oncorhynchus nerka). *J. Fish. Res. Bd. Can.* 19:619-624.

- Kobayashi, K.A., and C.M. Wood. 1980. The response of the kidney of the freshwater rainbow trout to true metabolic acidosis. *J. Exp. Biol.* 84: 227-244.
- Larsen, F.L., and F.F. Vincenzi. 1979. Calcium transport across the cell membrane: stimulation by calmodulin. *Science* 204: 306-309.
- Lehninger, A.L. 1975. *Biochemistry*. Worth Publishers, New York. 1104 pp.
- Lehre, A.K., B. Collins, and T. Dunne. 1982. Preliminary post-eruption sediment budget for the North Fork Toutle River Drainage, June 1980-May 1981, in *Proceedings from the Conference: Mount Saint Helens: Effects on water resources*, Oct. 7-8, 1981, Janzten Beach, Oregon. State of Washington Water Research Center Report No. 41, Pullman, Washington. Pages 215-234.
- Love, R.M. 1980. *The chemical biology of fishes*. Vol. 2. Academic Press, New York. 943 pp.
- Martin, D.J., L.J. Wasserman, R.P. Jones, and E.O. Salo. 1982. Effects of the eruption of Mount St. Helens on salmon populations and habitats of the Toutle River. Pages 235-252 in *State of Wash. Water Research Center Report No. 41*.
- Meyerhoff, R.D. 1981. Exercise and acid-base balance in rainbow trout. Ph.D. Thesis, Oregon State University, Corvallis, Oregon. 84 pp.
- Neilsen, S.P., T.F. Christiansen, O. Hartling, and J. Trap-Jensen. 1977. Increase in serum ionized calcium during exercise. *Clinical Science and Molecular Medicine* 53: 579-586.
- Noggle, C.C. 1978. Behavioral, physiological, and lethal effects of suspended sediments on juvenile salmonids. M.S. Thesis, University of Washington, Seattle, Washington. 96 pp.
- Opie, L.H., M. Thomas, P. Owen, and G. Shulman. 1972. Increased coronary venous inorganic phosphate concentrations during experimental myocardial ischemia. *Am. Jour. Cardio.* 30: 503-513.
- Parfitt, A.M., and M. Kleerekoper. 1979a. The divalent ion homeostatic system--physiology and metabolism of calcium, phosphate, magnesium, and bone. Pages 269-398 in Morton H. Maxwell and Charles R. Kleeman, eds. *Clinical disorders of fluid and electrolyte metabolism*. 3rd ed. McGraw-Hill Book Co., New York.

- \_\_\_\_\_. 1979b. Clinical disorders of calcium, phosphorus, and magnesium metabolism. Pages 947-1152 in ibid.
- Redding, J.M., and C.B. Schreck. 1980. Chronic turbidity and stress in juvenile coho salmon and steelhead trout. Oregon State Univ. Final report to the U.S. Forest Service. 30 June, 1980. 84 pp.
- Ruben, J.A. 1983. Am. Zool., in press.
- Ruben, J.A., and A.F. Bennett. 1980. The antiquity of the vertebrate pattern of activity metabolism. Nature 286: 886-888.
- \_\_\_\_\_. 1981. Intense exercise, bone structure, and blood calcium levels in vertebrates. Nature 291: 411-413.
- Stober, Q.J., et al., 1982. Effect of suspended volcanic sediment on coho and chinook salmon of the Toutle and Cowlitz Rivers. Pages 308-346 in State of Washington Water Research Center Report No. 41.
- Swift, D.J., and R. Lloyd. 1974. Changes in urine flow rate and hematocrit value of rainbow trout, Salmo gairdneri (Richardson) exposed to hypoxia. J. Fish. Biol. 6: 379-387.
- White, F.N. 1977. Respiration. Chapter 5 in Malcolm S. Gordon, ed. Animal Physiology. MacMillan Publishing Co., New York.

APPENDIX

- ASSUMING THAT:
- 1.) all excess phosphorus appearing in the blood following exercise is derived from muscle;
  - 2.) trout are 60% muscle by weight; therefore, a 150 gram trout contains  $(0.6 \times 150) = 90$  grams muscle;
  - 3.) a 150 gram trout contains about 10 ml extracellular fluid.

- GIVEN THAT:
- 1.) whole blood phosphorus is observed to rise by 6 uM/ml post-exercise;
  - 2.) trout muscle contains approximately 70 uM phosphorus/gram wet weight; therefore, a 150 gram trout contains  $(70 \times 90) = 6300$  uM phosphorus within its muscle tissue.

THEREFORE:  $6 \text{ uM/ml} \times 10 \text{ ml} = 60 \text{ uM}$  phosphorus must enter extracellular fluid from outside source (such as muscle) in order to cause the observed rise in whole blood. This only constitutes  $60/6300 = 0.009 \times 100 = 0.9\%$  of total muscle phosphorus.