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EFFECTS OF SUBLETHAL HEXAVALENT CHROMIUM EXPOSURE ON THE OSMOREGULATION, IMMUNE RESPONSE, AND BLOOD CHARACTERISTICS OF COHO SALMON (ONCORHYNCHUS KISUTCH)

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by

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To simulate a potential field situation in which
seaward-migrating juvenile coho salmon pass through Cr^{+6}-
polluted freshwater (FW) before being exposed to seawater (SW)
or marine pathogenic bacteria, I investigated the effects of
sublethal FW exposure to Cr^{+6} (as sodium dichromate) on
salinity tolerance and serum osmolality after transfer to SW,
and on disease resistance and serum agglutinin production
against <u>Vibrio anguillarum</u>. Additional research on the effects
of sublethal FW Cr^{+6} exposure on 12 blood characteristics
investigated the mode of Cr^{+6} toxicity and determined the
relative sensitivity of various blood tests as indicators of
sublethal Cr^{+6} toxicity.

SW survival was significantly decreased in salmon
exposed in FW to 0.23 mg Cr^{+6}/l for 4 weeks or to 0.5 mg Cr^{+6}/l
for 2 weeks and transferred to 20 or 30 ^{O}/oo SW, respectively.
Serum osmolality, measured after 2 weeks of FW exposure to 0.0
or 0.5 mg Cr^{+6}/l and after transfer to 20 or 30 ^{O}/oo SW, was
significantly higher in Cr^{+6}-exposed fish from 1/2 to 2 days

after SW transfer but not at the end of the FW exposure period or at 7 days after SW transfer.

Disease mortality, measured in salmon exposed to FW to 0.0 or 0.5 mg Cr^{+6}/l for 2 weeks and injected in subgroups with 1 of 4 graded doses of V. anguillarum (ca. 3-4 x 10^{2-5} bacteria/.1 ml), increased with dose and was significantly higher in Cr^{+6}-exposed fish. Serum agglutinin titers of salmon injected with V. anguillarum bacterin and exposed in FW to 0.0 or 0.5 mg Cr^{+6}/l for 2 weeks were undetectable after this 2 week period, equal in both groups at 4 weeks, and significantly higher in control fish at 6 weeks.

Exposure in FW to 1.0 mg Cr^{+6}/l for 1 week caused significant increases in hematocrit (Hc), methemoglobin (M-Hb), percent methemoglobin (% M-Hb), serum total protein (TP), and glucose (Glu); significant decreases in mean corpuscular hemoglobin concentration (MCHC) and erythrocyte sedimentation rate (ESR); and no significant effect on hemoglobin (Hb), complete or partial erythrocyte osmotic fragility (EOF), serum osmolality (Os), and serum lysozyme (Lys). The greatest significant change occurred in M-Hb, followed in order by % M-Hb > ESR > Hc > TP > MCHC > Glu.

These results indicated that short-term FW exposure to 0.23-1.0 mg Cr^{+6}/l caused a sublethal general stress response during FW exposure and lowered the resistance of coho salmon when exposed to the additional stressors of SW transfer or bacterial challenge.

INTRODUCTION

Hexavalent chromium (Cr^{+6}) salts are used extensively
in such industrial processes as leather tanning, pigment and
catalyst production, wood preservation, metal plating, and
corrosion inhibition (National Research Council, 1974), and
the wastes from these operations can result in substantial
water pollution. Many power plants, for instance, routinely
discharge coolant water that has been treated with 10 to 50
mg/l chromate (4.5 to 22.4 mg Cr^{+6}/l) in order to inhibit
corrosion of condenser and other metal surfaces (Becker and
Thatcher, 1973). The concentration of Cr in most unpolluted
rivers is normally between 1 and 10 µg/l (National Research
Council, 1974). There have been reports, however, of persistent
concentrations of 0.2 to 0.5 mg Cr/l in a river receiving wastes
from chromate manufacture (Breeze, 1973), and occasional
concentrations between 0.5 and 20 mg Cr/l in rivers subjected
to industrial wastes (Surber, 1959; Herbert et al., 1965;
Kelso and MacCrimmon, 1969).

Several reviews of the many acute toxicity tests with
fishes indicated that the 96 h LC-50 (median lethal concentra-
tion) ranged from 17.6 to 630 mg Cr^{+6}/l, depending on such
factors as species and age of test fish, type of Cr^{+6} salt,
temperature, pH, dissolved oxygen, and water hardness (McKee
and Wolfe, 1963; Becker and Thatcher, 1973; Eisler, 1973;
National Research Council, 1974; Eisler and Wapner, 1975).
There is less information concerning the chronic toxicity of

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Cr^{+6} to fishes, although it is clear that much lower concentrations can have detrimental effects after prolonged exposure. Olson and Foster (1956), for instance, reported that growth and survival of early life stages (embryo through 4-month-old juvenile) of chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Salmo gairdneri) were lowered by exposure to 0.013 and 0.080 mg Cr^{+6}/1, respectively. Benoit (1976) exposed rainbow trout and brook trout (Salvelinus fontinalis) to several Cr^{+6} concentrations for up to 22 months (alevin through adult), and found temporary growth retardation at 0.01 mg Cr^{+6}/1 and increased alevin mortality at 0.35 mg Cr^{+6}/1. Benoit used the latter effect to designate a maximum acceptable toxicant concentration (MATC) between 0.20 and 0.35 mg Cr^{+6}/1. McKim (1977) reviewed the published and unpublished research concerning chronic exposure of fishes to numerous water pollutants, and cited seven MATC values for Cr^{+6} ranging from a low of 0.051 - 0.105 mg Cr^{+6}/1 for rainbow trout to a high of 1.00 - 3.95 mg Cr^{+6}/1 for fathead minnows (Pimephales promelas).

The mode of Cr^{+6} toxicity to fishes at such low chronic exposure levels is not known, although Benoit (1976) states that young trout exposed to 0.35 mg Cr^{+6}/1 had slower yolk-sac absorption and eventually starved to death because they did not swim up to accept food. The absence of swim-up behavior was reported also by Olson and Foster (1956) in young chinook salmon exposed to 0.18 mg Cr^{+6}/1.

The primary sites of Cr^{+6} action at higher, acutely toxic, exposure concentrations seem to be the intestine and

kidney. Fromm and Schiffman (1958) exposed largemouth bass (<u>Micropterus</u> <u>salmoides</u>) to 94 mg Cr^{+6}/l (about 1/2 of the 48 h LC-50) and found severe histopathological changes in the epithelium of the intestine, but none in the gill. Strik <u>et</u> <u>al.</u> (1975) reported severe necrosis of renal and intestinal epithelia, and hyperplasia and hypertrophy of gill epithelium in rainbow trout surviving exposure to 10 mg Cr^{+6}/l for 15 days. The absence or relatively low degree of gill damage after acute Cr^{+6} exposure is in contrast with the effects of exposure to such metal cations as Zn^{+2}, Cu^{+2}, Cd^{+2}, and Pb^{+2} which are toxic primarily through mucus precipitation and cell destruction of the gill epithelium, with subsequent interference with branchial gas exchange (Doudoroff and Katz, 1957; Katz, 1975).

Cr^{+6}, as anionic chromate (CrO_4^{-2}) or dichromate ($Cr_2O_7^{-2}$), readily penetrates cell membranes, is a strong oxidizing agent, and reacts with organic matter whereby it is reduced to Cr^{+3}, in which valence state it binds with proteins, nucleic acids, and other biological ligands (National Research Council, 1974). Knoll and Fromm (1960) reported that the primary route of metal uptake in rainbow trout exposed for 24 days to 2.5 mg Cr^{+6}/l was through the gills and that Cr in the spleen, kidney, posterior gut, pyloric caeca, stomach and liver was accumulated to a higher concentration that that in the exposure water. All of these organs except spleen and kidney, lost most of the accumulated Cr within 25 days after transfer to clean water. In a more extensive study using the same species and Cr^{+6} concentration, Buhler <u>et</u> <u>al.</u> (1977)

found that all of the 20 measured tissues, organs and body
fluids accumulated Cr between 1.1 and 8.5 times the concentra-
tion in control fish. The Cr concentration of all except gall
bladder, bile, liver, gill, and kidney had returned to pre-
exposure levels by 17 days after transfer to clean water. The
lower rate of decrease, and an actual increase in Cr concentration
in bile, probably reflected the involvement of these organs in
excretion of this metal.

Various physiological effects have been reported for
fishes exposed to relatively high (2 - 289 mg Cr^{+6}/l) sublethal
or lethal Cr^{+6} concentrations. Abegg (1950) showed a decrease
in blood specific gravity and an increase in tissue water
content of bluegill sunfish (Lepomis macrochirus) exposed for
24 h to the 24 h LC-50 of sodium dichromate (289 mg Cr^{+6}/l)
in freshwater. He suggested that general hydration was caused
by a Cr^{+6}-induced increase in skin permeability. Fromm and
Schiffman (1958) reported a significant decrease in oxygen
consumption by largemouth bass during exposure to the acutely
lethal concentration of 94 mg Cr^{+6}/l. This effect was caused
presumably by a decrease in general metabolism, rather than
gill dysfunction, because there were no indications of gill
histopathology or respiratory distress.

Strik et al. (1975) reported that exposure of rainbow
trout to the lower concentration of 10 mg Cr^{+6}/l caused, in
addition to the histological effects mentioned previously, 25%
mortality, decreased plasma Na^+ concentration, and increased
blood glucose, hematocrit, and hemoglobin. They also treated
roach (Rutilus rutilus) with up to 10 mg Cr^{+6}/l for 39 days

and found a decrease in the liver enzyme, glutamate-oxaloacetate transaminase, but no other effects. Increased hematocrit had been reported previously by Schiffman and Fromm (1959) for rainbow trout treated with 2-4 mg Cr^{+6}/l. Exposure of rainbow trout to 2.5 mg Cr^{+6}/l caused reduction of intestinal glucose absorption (Stokes and Fromm, 1965), inhibition of Na^+,K^+-ATPase in the kidney and intestine (Kuhnert et al., 1976), and no effect on six other microsomal, mitochondrial, or soluble enzymes (Buhler et al., 1977). Fromm and Stokes (1962) investigated the effects of 39 days of exposure to 1 mg Cr^{+6}/l on rainbow trout and reported a small, but not statistically significant, increase in water content of pyloric caeca, liver, and kidney. The oxygen consumption of these tissues was not affected at this low dose.

There is only one report of statistically significant physiological effects at Cr^{+6} exposure concentrations below 2 mg/l. Hill and Fromm (1968) reported that exposure of rainbow trout to 0.02 and 0.2 mg Cr^{+6}/l for 1 week caused a significant elevation in plasma cortisol. This effect, however, was transient, as plasma levels were similar to those of control fish after 2 and 3 weeks of Cr^{+6} exposure. The authors concluded that cortisol measurement might be a useful indicator for the presence of acute, but not chronic, stressful conditions.

In his review of sublethal effects of water pollutants on fishes, Sprague (1971) emphasized the importance of relating sublethal effects to the probable success of fish populations in natural polluted environments. Short-term physiological and biochemical tests can serve to elucidate the mode of toxicity,

but often cannot be related readily to life functions at the organismal level or be used to determine a "safe" environmental concentration. Chronic bioassays, in contrast, measure such ecologically important factors as growth, reproduction, and long-term survival; but such tests are usually time-consuming and expensive, and do not measure the effect of such variables as temperature, water quality, and ecological interactions.

A useful intermediate toxicological approach is one which measures the combined effects of short-term toxicant exposure and other naturally occurring stressors in the organism's environment. Infectious disease and salinity change are two examples of stress factors to which many fish populations are exposed naturally. These two factors would be particularly important for anadromous species of fishes because they are exposed simultaneously to new pathogens and a salinity increase upon migration from freshwater (FW) to seawater (SW). Anadromous coho salmon (Oncorhynchus kisutch), the species chosen for the present research, normally migrate from FW to SW during the spring of their second year of life as juveniles in the smolt form. These fish, upon reaching SW, are exposed for the first time to one of their most serious pathogens, the halophilic bacterium Vibrio anguillarum. Numerous serious outbreaks of vibriosis disease have been reported in salmon and many other species of fishes in the marine and estuarine environment (Anderson and Conroy, 1970).

Very little is known about the effects of Cr^{+6} or any other chemical pollutant, on the salinity tolerance and disease resistance of fishes. I decided, therefore, to use juvenile

coho salmon to investigate the effects of sublethal Cr^{+6} exposure in FW on osmoregulation and survival after transfer to SW; and on acquired antibody production and disease resistance against V. anguillarum. These experiments were designed to simulate a potential field situation in which migrating coho salmon pass through Cr^{+6}-polluted FW before being exposed to SW or V. anguillarum.

Additionally, I decided to examine the effects of exposure to 1 mg Cr^{+6}/l on 12 blood characteristics of juvenile coho salmon because almost all of the previous research concerning the physiological effects of Cr^{+6} on fishes has utilized exposure concentrations greater than 2 mg Cr^{+6}/l. Further goals of this physiological research were to increase the understanding of the mode of Cr^{+6} toxicity, and to determine the relative sensitivity of various blood tests as indicators of sublethal Cr^{+6} toxicity.

EFFECTS OF Cr^{+6} ON OSMOREGULATION

INTRODUCTION

Short or long-term exposure to sublethal concentrations of heavy metals is known to cause a variety of physiological effects in fishes (Katz, 1975). One such effect of salts of Cu, Zn, Hg, and Cd is a more or less transient alteration of water and electrolyte concentrations in the blood and other tissues of FW and SW fishes (McKim et al., 1970; Lewis and Lewis, 1971; Christensen et al., 1972; Bouquegneau, 1973; Calabrese et al., 1975; Larsson et al., 1976; McCarty and Houston, 1976). This may reflect some degree of osmoregulatory dysfunction because most of the observed changes were consistent with excessive salt depletion and/or hydration in FW or excessive salt accumulation and/or dehydration in SW. Similar changes have been observed in fishes exposed to acutely lethal concentrations of Zn^{+2} and Cd^{+2} (Skidmore, 1970; Thurberg and Dawson, 1974). The ecological significance of these metal-induced osmoregulatory changes is uncertain, however, because they are often transient during chronic sublethal exposure, and not the primary cause of death during acute lethal exposure.

A low metal concentration may have little or no detectable osmoregulatory effect under stable FW or SW conditions. It is possible, however, that the same or lower concentration could impair the ability of fishes to adapt successfully to the stress of changing salinity. Such a salinity change occurs naturally during the FW to SW migration

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of juvenile coho salmon. This anadromous species migrates from FW to SW normally during the spring of its second year of life. This critical period of transition from FW to SW is accompanied by many active and passive changes in osmoregulatory function, primarily in the gills, kidney, and intestine (Motais and Garcia-Romeu, 1972). Any pollutant which impairs osmoregulatory function would be expected, therefore, to have a particularly pronounced effect during this stage in the life cycle of coho salmon and other anadromous fishes. This idea is supported by a recent report that sublethal Cu^{+2} exposure in FW had deleterious effects on the downstream migration and SW survival of juvenile coho salmon (Lorz and McPherson, 1976).

Exposure to Cr^{+6}, the subject of this study, has a number of effects on fishes which suggest the possibility of osmoregulatory impairment. Previous research with relatively high sublethal or lethal Cr^{+6} concentrations (2.5 - 289 mg/l) established that this metal accumulated in the gills, kidney, and gastrointestinal tract (Knoll and Fromm, 1960; Kuhnert et al., 1976); caused histopathological changes in these tissues (Fromm and Schiffman, 1958; Strik et al., 1975); depressed blood specific gravity (Abegg, 1950) and plasma Na^+ concentration (Strik et al., 1975); and inhibited the active sodium transport enzyme, Na^+K^+-ATPase, in kidney and intestine (Kuhnert et al., 1976).

Because this information suggested that sublethal Cr^{+6} exposure also may impair the ability of fishes to adapt to a change in salinity, I decided to examine the effects of FW

exposure to even lower sublethal Cr^{+6} concentrations on the
ability of juvenile coho salmon to osmoregulate and survive
after transfer to SW. This laboratory study was designed to
simulate a potential field situation in which migrating coho
salmon might pass through Cr^{+6}-polluted FW shortly before
they reach SW.

MATERIALS AND METHODS

Juvenile coho salmon (9-16 cm total length) were
obtained from a hatchery where they were reared from eggs in
flowing spring water (10 $\pm$ 3° C, 60 mg/l $CaCO_3$ hardness, pH
6.6) under a natural photoperiod and fed commercial salmon feed.

Flow-through FW Cr^{+6} exposures were conducted in glass
aquaria (25 x 50 x 30 cm) fitted with translucent covers and
side outlets to maintain a volume of 30 l. The aquaria were
held in a water bath and were protected from direct sunlight
and disturbance by an opaque curtain. Hatchery water flowed
through these aquaria at 12 l/h, providing 99% replacement of
the water in about 12 h (Sprague, 1969). A stock solution of
sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) dissolved in distilled water
was added to the experimental stream with a peristaltic pump
at a rate to give a nominal concentration of 0.2 or 0.5 mg
Cr^{+6}/l, depending on the experiment. Water and stock solution
were siphoned from a dilution chamber into a split-flow mixing
cell (Benoit and Puglisi, 1973), from which it flowed to each
test aquaria at an equal rate. Uncontaminated water was supplied
in an identical manner to the control aquaria. Water and stock
solution flow rates were checked daily, and Cr^{+6} concentrations

were measured every other day by the diphenylcarbazide method (American Public Health Association, 1971). Dissolved oxygen was held above 80% saturation by aeration, and temperature was maintained at $13 \pm 2^{\circ}$ C.

At the start of each Cr^{+6} exposure, previously graded fish were netted gently from hatchery holding tanks and placed at random into control and experimental aquaria until there were ten in each. The fish were fed once every other day only as much as they would consume in one feeding. At the end of the FW exposure period, the fish were transferred to the laboratory where the SW experiments on salinity tolerance and serum osmolality were conducted.

All SW exposures were conducted at $13 \pm 2^{\circ}$ C in a cold room under a photoperiod adjusted to that of the hatchery. Groups of ten control or exposed fish were placed in covered glass aquaria (30 x 60 x 40 cm) containing 60 l of natural SW adjusted to either 20 or 30 parts per thousand ($^{\circ}/oo$) salinity with distilled water. Salinity was measured with a salinity refractometer. The fish were not fed during the tests, and the water was recirculated, aerated, and filtered within each aquarium by air-lift filters.

Salinity Tolerance

Four salinity tolerance experiments were conducted to determine the effect of sublethal FW Cr^{+6} exposure on SW survival. Each experiment utilized 20 control and 20 Cr^{+6}- exposed coho salmon.

In Exp. I, salmon were exposed to 0.0 or 0.5 mg Cr^{+6}/l for 2 weeks and then transferred to 30 $^{\circ}/oo$ SW.

The fish in Exp. II were treated with 0.0 or 0.5 mg Cr^{+6}/1 for 2 weeks and then transferred to 20 ^{o}/oo SW, followed after 1 week by a second transfer to 30 ^{o}/oo SW.

In Exp. III, fish were maintained in 0.0 or 0.5 mg Cr^{+6}/1 for only 1 week and then transferred to 30 ^{o}/oo SW.

In Exp. IV, fish were kept in 0.0 or 0.2 mg Cr^{+6}/1 for 4 weeks and then transferred to 20 ^{o}/oo SW.

SW deaths in each experiment were recorded daily until no further mortalities occurred over a period of 3 days.

Serum Osmolality

Two additional experiments were conducted to measure the effects of FW Cr^{+6} exposure on serum osmolality in FW and after transfer to SW. In Exp. V, two groups of 44 fish each were exposed to 0.0 or 0.5 mg Cr^{+6}/1 for 2 weeks and then transferred to 20 ^{o}/oo SW. In Exp. VI, two groups of 30 fish each were exposed to 0.0 or 0.5 mg Cr^{+6}/1 for 2 weeks and then transferred to 30 ^{o}/oo SW. Serum osmolality was measured on blood samples taken from at least five control and five Cr^{+6}- exposed fish at the end of the FW exposure period and at intervals of 0.5, 1, 2, and 7 days in SW. At each time interval, individual fish were netted quickly and immediately killed by a blow to the head. Anesthetic, such as tricaine methanesulphonate, was not used since it may alter the blood electrolyte composition (Houston et al., 1971). After blotting the fish on absorbent paper, the tail was severed just behind the adipose fin, blotted again, and blood was collected from the exposed dorsal artery in non-heparinized microcapillary tubes. Extreme care was taken to prevent contamination of the

blood sample with water and tissue fluids. The tubes were plugged and the blood was allowed to clot for 1 h at 13° C. The samples were then centrifuged at 11,500 rpm for 5 min in a microcapillary centrifuge, and 25 µl of serum withdrawn by microsyringe was diluted exactly eight-fold with double-distilled, deionized water. The osmolality, in milliosmoles per liter (mOsm/l), of these 0.2 ml samples was then measured by freezing point depression in an osmometer. This dilution technique was necessary because of the particularly small volume of blood available from many of the Cr^{+6}-exposed fish in SW. A similar dilution technique was used by McKim et al. (1970) for individual serum osmolality determinations from brook trout exposed to Cu^{+2}.

The statistical procedures used were from Sokal and Rohlf (1969). The mean and standard error were calculated for each group of control and experimental osmolality data. Statistical significance of the difference between control and experimental osmolality was analyzed by the non-parametric Mann Whitney U test. The statistical significance of the difference between control and experimental salinity tolerance was determined from a table giving the confidence limits for percentages based on the binomial distribution. Significant differences were accepted at the $P \leq 0.05$ level.

RESULTS

Salinity Tolerance

There was no mortality in any control or experimental group during the initial Cr^{+6} exposure period in FW. The

post-SW transfer mortality data are given in Table I and Fig. 1. No control fish died after transfer to SW in any of the four experiments.

In Exp. I, exposure to 0.52 mg Cr^{+6}/l for 2 weeks resulted in 85% mortality within 5 days after transfer to 30 ^{o}/oo SW. This is significantly different from 0% control mortality (P < 0.01). Under similar Cr^{+6} exposure conditions in Exp. II (0.48 mg Cr^{+6}/l for 2 weeks), only 20% died after 1 week in 20 ^{o}/oo SW. This is not significantly different from the control (P > 0.05). An additional 30%, however, died within 1 week after transfer from 20 ^{o}/oo to 30 ^{o}/oo SW, indicating that adaptation was incomplete. The resulting 50% total mortality in this experiment is significantly different (P < 0.05) from the control. In Exp. III, exposure to essentially the same concentration of Cr^{+6} (0.51 mg Cr^{+6}/l) for only 1 week resulted in 35% mortality after transfer to 30 ^{o}/oo SW. This mortality was not significantly different (P > 0.05) from the control. Exposure for 4 weeks to 0.23 mg Cr^{+6}/l in Exp. IV resulted in 55% mortality after 4 days in 20 ^{o}/oo SW. This mortality is significantly different (P < 0.01) from the control.

More than half of the total mortality of Cr^{+6}-exposed fish in Exp. I and IV occurred by 2 days after transfer to SW (Fig. 1). A similar pattern is seen in Exp. II, where the fish were transferred to 20 ^{o}/oo SW for 1 week, followed by transfer to 30 ^{o}/oo SW. More than half of the mortalities of Cr^{+6}-exposed fish at each salinity treatment in this experiment had occurred by 2 days after each SW transfer. The final

Table I. Mortality of control and Cr^{+6}-exposed coho
salmon 1 week after transfer to SW.

Total Mortality

Experiment	Cr^{+6}	%	Control	%
I[a]	17/20	85**	0/20	0
II[b]	10/20	50*	0/20	0
III[c]	7/20	35	0/20	0
IV[d]	11/20	55**	0/20	0

a   FW exposure for 2 wk to 0.0 or 0.52 ± .06 mg Cr^{+6}/1,
    (mean ± S.D.), transfer to 30 °/oo S.W.

b   FW exposure for 2 wk to 0.0 or 0.48 ± .05 mg Cr^{+6}/1,
    transfer to 20 °/oo S.W., then 30 °/oo S.W.

c   FW exposure for 1 wk to 0.0 or 0.51 ± .08 mg Cr^{+6}/1,
    transfer to 30 °/oo S.W.

d   FW exposure for 4 wk to 0.0 or 0.23 ± .06 mg Cr^{+6}/1,
    transfer to 20 °/oo S.W.

*   Significantly different from control, $P < .05$
** Significantly different from control, $P < .01$
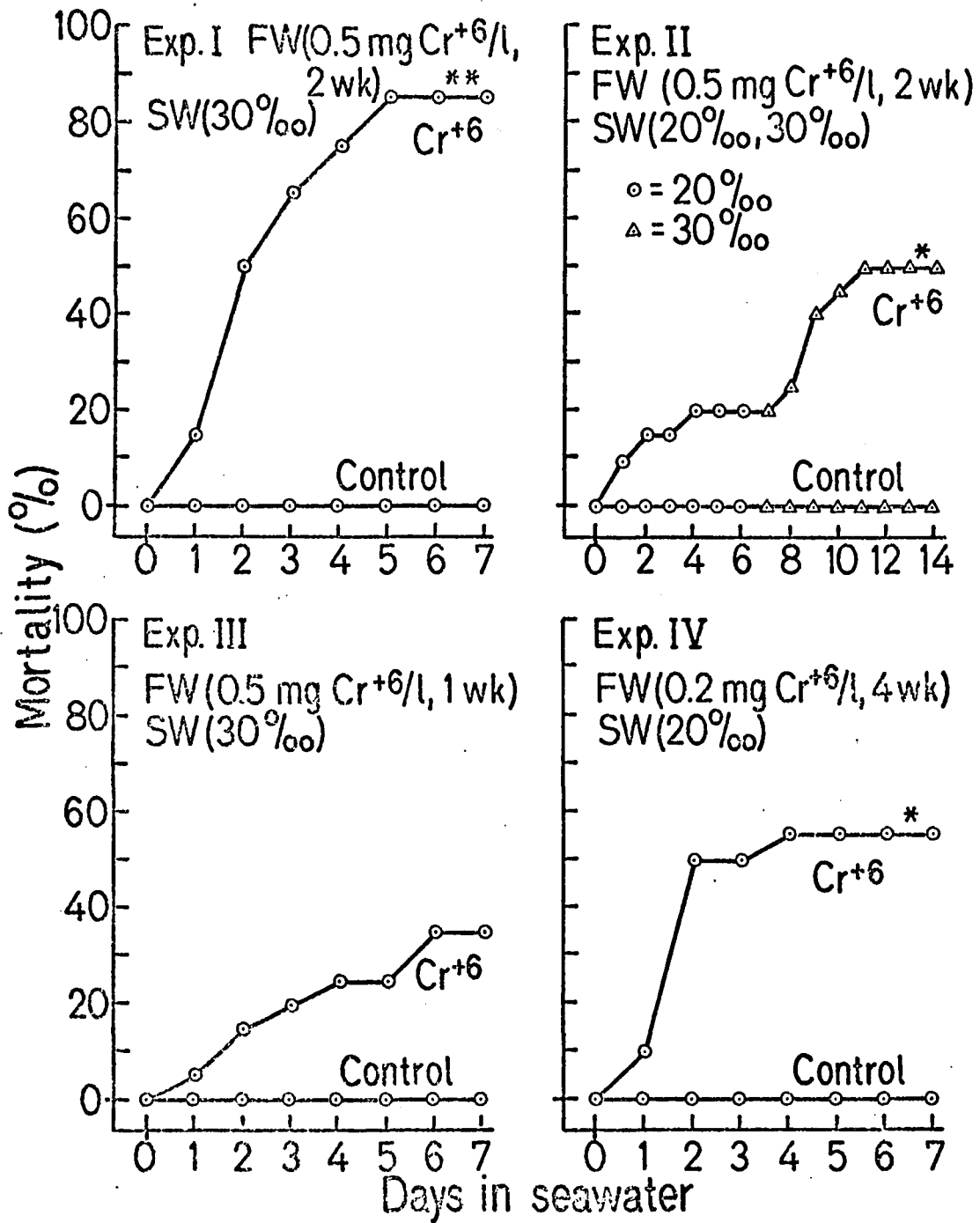
Fig. 1. Cumulative percent mortality of control and Cr^{+6}-
exposed coho salmon after transfer to SW. Each
line represents the mortality within a group of
20 fish. (* P < .05, ** P < .01)

mortality at any one salinity treatment in these three experiments was reached by 4 or 5 days after transfer. In contrast, the mortalities of Cr^{+6}-exposed fish in Exp. III occurred at a lower initial rate and continued at a nearly constant rate until 6 days after SW transfer.

The symptoms of affected coho salmon were nearly identical to those reported by Conte and Wagner (1965) for juvenile steelhead trout (Salmo gairdneri) failing to adapt to transfer from FW to 30 °/oo SW. These symptoms consisted of an initial darkening of the skin, followed in about 1 day by swimming to the surface and gulping behavior. Death usually occurred a few hours after the gulping behavior started.

Serum Osmolality

As in the previous experiments, there was no mortality during the 2 week FW exposure period in Exp. V and VI. The mean Cr^{+6} concentrations during this period were 0.54 mg/l in Exp. V and 0.49 mg/l in Exp. VI (Table II). The serum osmolality data are tabulated in Table II and plotted in Fig. 2. There were two mortalities of Cr^{+6}-exposed fish after transfer to 20 °/oo SW in Exp. V and four after transfer to 30 °/oo SW in Exp. VI. Although most of the Cr^{+6}-exposed fish in 30 °/oo SW, and some in 20 °/oo SW, showed skin darkening, no fish in the terminal gulping stage were used for determination of serum osmolality.

Fig. 2 indicates that Cr^{+6} exposure caused a greater magnitude and duration of elevated serum osmolality after transfer to 20 °/oo and 30 °/oo SW. The mean osmolality values

Table II. Serum osmolality of control and Cr^{+6}- exposed
coho salmon before and after transfer to SW.

Serum Osmolality (mOsm/l)

Days in SW	Experiment V[a]		Experiment VI[b]	
	Control	Cr^{+6}	Control	Cr^{+6}
0	288 ± 3 (12)[c] (259 - 302)	289 ± 4 (12) (254 - 301)	289 ± 8 (5) (261 - 309)	287 ± 8 (5) (255 - 305)
.5	333 ± 5 (8) (318 - 361)	369 ± 13 (8)* (330 - 429)	393 ± 11 (5) (366 - 420)	466 ± 11 (5)** (432 - 490)
1	310 ± 10 (8) (259 - 346)	395 ± 15 (8)** (330 - 435)	386 ± 9 (5) (365 - 408)	493 ± 12 (5)** (465 - 523)
2	289 ± 7 (8) (259 - 307)	331 ± 14 (8)* (289 - 410)	372 ± 9 (5) (339 - 394)	415 ± 12 (5)* (382 - 446)
7	293 ± 5 (8) (270 - 310)	298 ± 6 (6) (274 - 316)	297 ± 6 (10) (261 - 317)	307 ± 8 (6) (275 - 331)

a FW exposure for 2 wk to 0.0 or $0.54 \pm .07$ mg Cr^{+6}/l
(mean $\pm$ S.D.), transfer to 20 °/oo SW.

b FW exposure for 2 wk to 0.0 or $0.49 \pm .04$ mg Cr^{+6}/l
(mean $\pm$ S.D.), transfer to 30 °/oo SW.

c Mean $\pm$ S.E. (number of fish sampled)
    (range)

* Significantly different from control, P < .05

** Significantly different from control, P < .01
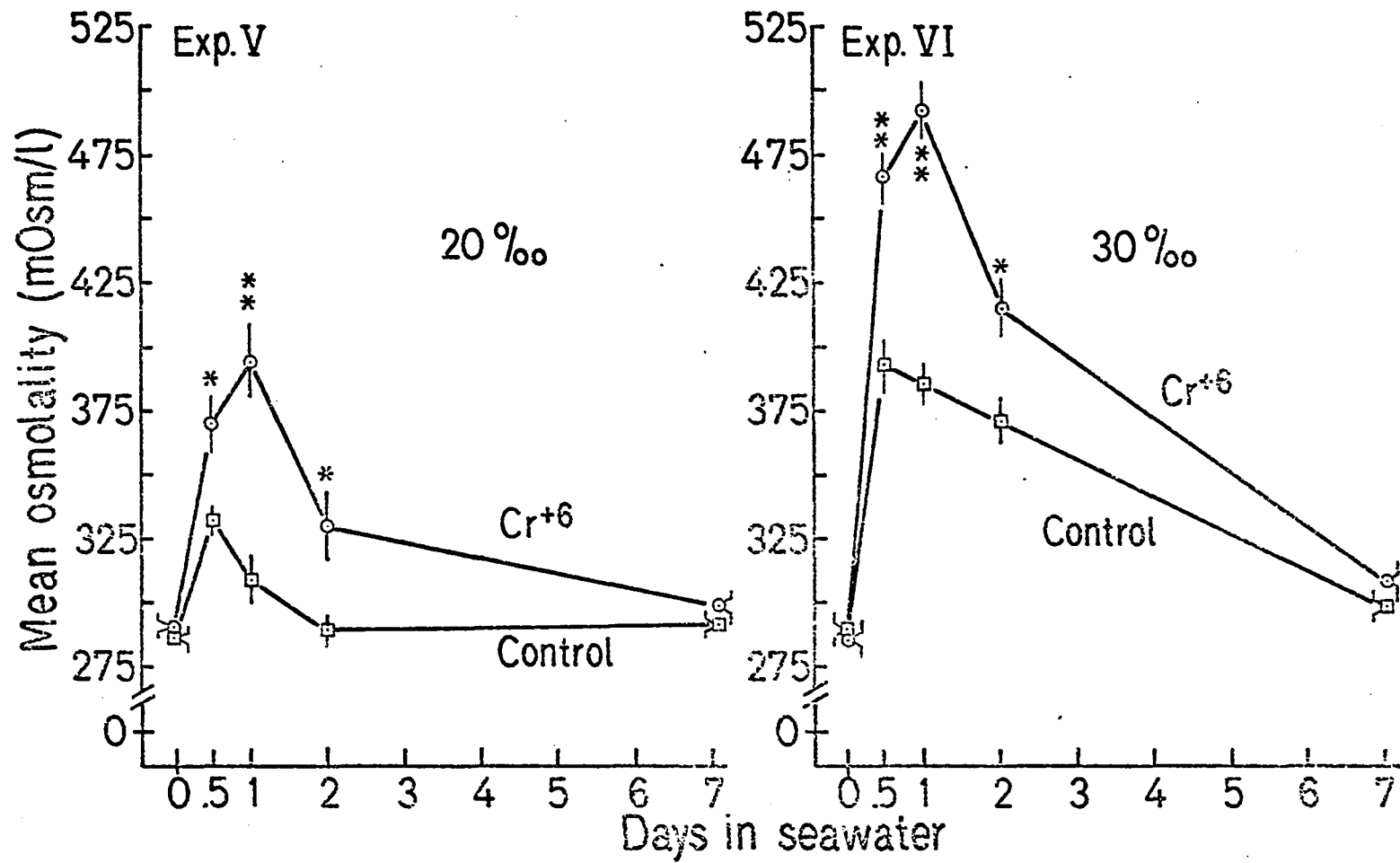
Fig. 2. Mean serum osmolality of control and Cr^{+6}-exposed coho salmon
before and after transfer to 20 °/oo SW (Exp. V) or 30 °/oo SW
(Exp. VI). Vertical bars represent the standard error of a
group of 5 to 12 fish (see Table II). (* P < .05, ** P < .01)

for control and Cr^{+6}-exposed fish in both experiments were
remarkably similar (287-289 mOsm/l) at the end of the FW
exposure period. Both Cr^{+6}-exposed groups, however, had
significantly higher osmolality values than their respective
control groups after 1/2 day in SW. Osmolality had decreased
in both control groups after 1 day, but had increased even
further in the Cr^{+6} exposed groups. The mean osmolality of
all groups dropped between 1 and 2 days, but the means of
both Cr^{+6}-exposed groups were still significantly higher than
their respective controls. Seven days after SW transfer there
was no significant difference between the mean osmolality of
Cr^{+6}-exposed and control groups in either experiment, or
between the mean osmolality of any group and its starting FW
values.

The magnitude of osmolality change for both control
and Cr^{+6}-exposed fish is greater in Exp. VI (30 °/oo SW) than
in Exp. V (20 °/oo SW), although the pattern of change is
qualitatively similar in both experiments. Control fish
transferred to 30 °/oo SW suffered no mortality and had no
individual osmolality values greater than 420 mOsm/l (Table
II). The Cr^{+6}-exposed group, however, had four mortalities,
and all fish sampled within 1 day after transfer had osmolality
values greater than 432 mOsm/l, with all but one value being
greater than 450 mOsm/l.

In experiment V, control fish transferred to 20 °/oo
SW had serum osmolality values which were never higher than
360 mOsm/l, and which returned to FW levels between 1 and 2
days after transfer without mortality. In contrast, the

magnitude of osmolality increase of Cr^{+6}-exposed fish at this salinity was even greater than the control fish in 30 ^{o}/oo SW, with a maximum value of 435 mOsm/l and two mortalities.

DISCUSSION

The salinity tolerance and osmoregulatory patterns of control fish in this study are nearly identical to those found by Conte et al. (1966) and Miles and Smith (1968), who transferred juvenile coho salmon from FW to 30 ^{o}/oo SW and 25.5 ^{o}/oo SW, respectively. In sharp contrast, sublethal Cr^{+6} exposure in FW clearly decreased the ability of juvenile coho salmon to osmoregulate and to survive within a short period after transfer to SW. These effects occur, furthermore, at a Cr^{+6} concentration which has no detectable effect on serum osmolality or survival while the fish are in FW.

Although the mechanisms by which Cr^{+6} affected SW adaptation and survival were not determined, it is probable that Cr^{+6} caused non-specific damage to the branchial, renal, and intestinal transport epithelia involved in osmoregulation. The initial osmoregulatory responses of fishes to SW transfer are primarily passive and include changes in gill permeability to water and ions, redistribution of extracellular and intra-cellular water and ions, and decreased urine volume (Houston, 1964; Motais and Garcia-Romeu, 1972). These initial responses serve to minimize excessive salt accumulation and dehydration until more active, energy-requiring, regulatory mechanisms, such as enhanced gill Na^+,K^+-ATPase activity, can become fully developed. In coho salmon, for instance, maximum activity of

gill Na^+, K^+-ATPase was not reached until 2 to 4 weeks after SW transfer (Zaugg and McLain, 1970; Giles and Vanstone, 1976). It is probable that Cr^{+6} affected one or more of the initial responses because most of the abnormal increase in serum osmolality and mortality occurred within 2 days after SW transfer. Since the volume of blood obtainable from Cr^{+6}-exposed fish was much less than control fish in both osmolality experiments, it is likely that excessive dehydration was occurring. Uncontrolled water loss, aggravated by influx of ions, could have accounted for the higher observed serum osmolality and could have resulted in a critical loss of blood volume. Conte and Wagner (1965) found a lower obtainable blood volume and higher plasma osmotic concentrations (> 500 mOsm/l) in juvenile steelhead trout failing to adapt to transfer from FW to 30 ^{o}/oo SW. They observed pronounced darkening of the skin and gulping behavior in affected trout, and suggested that this resulted from respiratory difficulty caused by inadequate blood supply to the gills and other vital organs. Because nearly identical symptoms were present in Cr^{+6}-exposed coho salmon in this study, it is probable that dehydration, followed by respiratory distress, was the proximate cause of SW death.

Because most of the normal efflux of water occurs through the gills of fishes in SW (Motais and Garcia-Romeu, 1972), the primary site of Cr^{+6} damage may be this tissue. Previous research with higher Cr^{+6} concentrations, however, indicated that Cr^{+6} may act also on the kidney and intestine. Fromm and Schiffman (1958) exposed largemouth bass to 94 mg

Cr^{+6}/l (about 1/2 of the 48 h LC-50) and found severe patho-
logical changes in the epithelium of intestine, but not gill.
Using rainbow trout, Strik et al. (1975) reported that 15 days
of FW exposure to 10 mg Cr^{+6}/l caused 25% mortality, hyperplasia
and hypertrophy in gill epithelium, and severe necrosis in
the epithelium of kidney tubules and intestine of surviving
fish.

Although histopathology at lower Cr^{+6} concentrations
has not been reported in fishes, sublethal exposure may cause
functional impairment of fish kidney and intestine. Fromm
and Stokes (1962) found that FW exposure to 1 mg Cr^{+6}/l over
a 39 day period caused a slight, but not statistically significant,
increase in water content of several tissues of rainbow trout,
and suggested that this tissue hydration was due to kidney
damage. Stokes and Fromm (1965) reported that FW exposure to
2.5 mg Cr^{+6}/l for 1 week inhibited intestinal glucose absorption
in the same species. Most recently, Kuhnert et al. (1976)
showed that 2 days of FW exposure to 2.5 mg Cr^{+6}/l significantly
inhibited Na^+, K^+-ATPase in the kidney of rainbow trout. There
was also substantial, but not statistically significant, enzyme
inhibition in the intestine. Inhibition was not detectable
in gill, which normally has a low enzyme level in FW. They
suggested that inhibition of this enzyme was in direct propor-
tion to its normal level in FW fish tissues and could partially
account for the previously reported tissue hydration and
inhibited glucose absorption because both of these processes
are linked to Na^+ transport.

Although specific Na^+, K^+-ATPase inhibition may have contributed to the post-SW transfer osmoregulatory effects observed in the present study, it is probable that Cr^{+6} acted in a more general manner on many components of transport epithelia and other tissues. Hexavalent Cr, as anionic chromate (CrO_4^{-2}) or dichromate ($Cr_2O_7^{-2}$), readily penetrates cell membranes, is a strong oxidizing agent, and reacts with organic matter whereby it is reduced to Cr^{+3}, in which valence state it binds with proteins, nucleic acids, and other biological ligands (National Research Council, 1974). In rainbow trout exposed to 2.5 mg Cr^{+6}/1, the metal is taken up primarily through the gill and accumulates above environmental levels in gill, spleen, kidney, liver, and gut; all of which except spleen seem to be capable of some Cr excretion (Knoll and Fromm, 1960; Kuhnert et al., 1976). Regardless of the particular mechanisms and sites of Cr^{+6} action in coho salmon, it is clear that sublethal FW exposure to this metal can cause osmoregulatory impairment and mortality after SW transfer, under laboratory conditions.

Direct application of laboratory results such as these to natural conditions must always be made cautiously. Although chronic FW exposure to 0.2 and 0.5 mg Cr^{+6}/1 is certainly possible in polluted rivers (Breeze, 1973), the abrupt transfer of coho salmon to 20 and 30 °/oo SW was not completely realistic. Salinity preference tests by Houston (1957) and Baggerman (1960) showed that, during the migratory period, coho smolts will move into 18-24 °/oo SW from FW within about 3 h after given the choice. Baggerman, however, found that this SW preference was

temporary, with FW preference occurring shortly before and after the spring migratory period. Although juvenile coho salmon can adapt reasonably well to SW throughout the year, optimal salinity tolerance and osmoregulation probably coincide with the migratory period (Conte et al., 1966). In addition, Zaugg and McLaine (1970) and Giles and Vanstone (1976) found that maximum FW levels of coho salmon gill Na^+, K^+-ATPase occurred during the migratory period, probably indicating some degree of physiological preadaptation in this species.

This information suggests that coho salmon with impaired or sub-optimal osmoregulatory ability, as with sublethal Cr^{+6} treatment or during non-migratory periods, would not voluntarily enter overly stressful or lethal salinities until adaptive processes could be regained. Exposure to Cr^{+6} might result, therefore, in a delay of migration into and through the estuary, rather than direct mortality. Such a delay might last only 1 week since the data in this study indicate that the serum osmolality of both Cr^{+6}-exposed and control fish is equal to the FW level within a week after SW transfer (Fig. 2). It could last longer, however, because adaptation by Cr^{+6}-exposed fish may not be complete, as shown by the additional mortalities in Exp. II when fish were transferred to 30 ^{o}/oo SW after 1 week in 20 ^{o}/oo SW (Fig. 1). If the FW Cr^{+6} pollution source is located near the entrance to an estuary, such a delay would tend to retain the fish in the most polluted area. It may be significant also that Cr bound to FW suspended sediment is most rapidly solubilized in the upper reaches of certain temperate estuaries (Groot and Allersma, 1975).

The observed effects of sublethal Cr^{+6} exposure on osmoregulation and salinity tolerance would be most likely to occur in environmental situations where relatively small polluted rivers flow abruptly into high salinity SW, as along open coasts or below certain fish ladders or dams. Fish in these situations would be less able to avoid sudden exposure to stressful or lethal salinities. Substantial Cr^{+6} pollution below a fish ladder has been reported by Capuzzo and Anderson (1973) in the estuarine portion of a small New Hampshire river (Cocheco River), indicating that such situations do exist.

Determination of the actual significance of Cr^{+6} pollution on the salinity tolerance and osmoregulation of migrating salmon requires field research in such polluted areas. The urgency for such research is emphasized by the present study and by the possibility that many other pollutants may have a similar effect on salmon and other aquatic organisms at even lower concentrations. Testing for these effects may be useful also as a sensitive indicator of sublethal toxicity in aquatic bioassays, requiring less time and expense than those chronic bioassays which measure other ecologically significant factors such as growth, reproduction, and long-term survival. Benoit (1976) reported that the maximum acceptable Cr^{+6} concentration for brook trout was between 0.20 and 0.35 mg Cr^{+6}/l, based on the criterion of long-term (8-22 months) survival. This concentration range is similar to that found in the present study to affect the osmoregulatory ability of coho salmon after only 2 to 4 weeks of exposure to Cr^{+6}. Further research on the relative sensitivity of these tests for other pollutants and aquatic species would be of great value.

EFFECTS OF Cr^{+6} ON THE IMMUNE RESPONSE

INTRODUCTION

A review by Snieszko (1974) indicated that several natural and man-made environmental stressors may be associated with outbreaks of infectious diseases in fishes. Very little is known, however, about the specific effects of heavy metal pollution on the immune responses of fishes. Laboratory exposure to sublethal concentrations of Zn^{+2} suppressed the production of serum antibody in zebrafish (Brachydanio rerio) immunized with a heat-killed bacterium (Proteus vulgaris) (Sarot and Perlmutter, 1976). Production of serum antibodies in the blue gourami (Trichogaster trichopterus) against infectious pancreatic necrosis virus and P. vulgaris was inhibited by sublethal exposure to methylmercury and Cu^{+2} (Roales and Perlmutter, 1977). Sublethal Cd^{+2} exposure caused a greater clearance rate, but a lower killing rate, of intra-cardially-injected bacteria (Bacillus cereus) in the liver and spleen of the cunner (Tautogolabrus adspersus) (Robohm and Nitkowski, 1974).

The potential impact of heavy metal pollution on disease resistance of wild fish populations is suggested by Pippy and Hare (1969) who reported that a surge of Zn and Cu pollution in a Canadian river acted synergistically with high temperature and low flow to enhance mortalities attributed to bacterial disease (Aeromonas liquefaciens) in Atlantic salmon (Salmo salar) and white suckers (Catostomus commersoni).

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Determination of the specific role of Cu and Zn was complicated by the occurrence of stressful environmental variables other than metal pollution. Further evidence for an effect of Cu on fish disease resistance is provided by Rødsaether et al. (1977), who found that seawater eels (Anguilla anguilla) died with signs of vibriosis (V. anguillarum) over a period of 50 to 120 days after transfer to FW contaminated with 30 to 60 ug Cu^{+2}/l. As no mortalities occurred in non-contaminated FW, the authors attributed the losses to vibriosis, rather than to Cu toxicity, because V. anguillarum was isolated in pure culture from all moribund fish but not from control fish.

Evidence that Cr^{+6} also may affect the immune response of fishes was provided by Benoit (1976) who exposed brook trout to several Cr^{+6} concentrations for up to 22 months to determine chronic effects on survival, growth, and reproduction. He noted in the reproduction study that four of six fish previously exposed to 0.35 mg Cr^{+6}/l died from bacterial disease (A. liquefaciens) during the spawning period, suggesting that the combination of Cr^{+6} stress with that of maturation and spawning rendered the fish more susceptible to disease. Cr^{+6} has been shown also to depress the circulating antibody response of rats repeatedly injected with 1/3 of the LD-50 (median lethal dose) of Cr^{+6} and immunized with viral antigen (Figoni and Treagan, 1975).

Because this information suggested that Cr^{+6} exposure also may impair the immune response of fishes, I decided to examine the effect of sublethal Cr^{+6} exposure on the innate disease resistance and acquired antibody production of juvenile

coho salmon against one of its most serious bacterial pathogens
(V. anguillarum). A review by Anderson and Conroy (1970)
indicated that numerous serious outbreaks of vibriosis have
occurred in salmon and many other species of fish in the marine,
estuarine, and, less often, freshwater environments. Anadro-
mous coho salmon normally spend their first year of life in
FW and then migrate to sea as juveniles in the smolt form.
This laboratory study was designed to simulate a potential
field situation in which seaward migrating coho salmon pass
through Cr^{+6}-polluted FW before being exposed to V. anguillarum.

MATERIALS AND METHODS

Juvenile coho salmon (9-16 cm total length) were
obtained from a hatchery where they were reared from eggs in
flowing spring water ($10 \pm 3^{\circ}$ C, 60 mg/l $CaCO_3$ hardness, pH
6.6) under a natural photoperiod and fed commercial salmon feed.

A virulent strain of V. anguillarum (No. 569, charac-
terized and provided by R. G. Strout, Animal Science Department,
University of New Hampshire) was used in all immunization and
challenge experiments. This strain, isolated originally from
diseased winter flounder (Pseudopleuronectes americanus), had
been passed three times through coho salmon. The organism is
a gram negative, motile, curved rod which is sensitive to the
vibriostatic agent 0/129 (2,4-diamino-, 6,7-diisopropylpteridine)
and novobiocin, requires NaCl, and has other biochemical
characteristics identifying it as V. anguillarum. Slant
cultures were maintained on trypticase soy agar supplemented
with 1.5% NaCl under mineral oil at 4° C. Bacteria to be used

for challenge were grown for 24 h at 20° C on trypticase soy agar containing 1.5% NaCl. Harvested cells were suspended in 1.5% NaCl saline to 0.3 OD at 575 nm using a spectrophotometer. Tenfold serial dilutions (10^{-4} to 10^{-7}) were then made from this suspension in 1.5% saline, and the number of viable bacteria at each of the four dose dilutions was determined by the pour plate method.

Disease Resistance

To examine the effects of sublethal Cr^{+6} exposure on the disease resistance of coho salmon, two groups of 40 fish each were exposed to either 0.0 or 0.5 mg Cr^{+6}/l (as $Na_2Cr_2O_7 \cdot 2H_2O$) for 2 weeks in flowing $13 \pm 2^{\circ}$ C hatchery water and then challenged with graded log doses of live V. anguillarum. This experiment was repeated and the two trials were designated as Exp. VII and VIII. The methods used for Cr^{+6} flow-through exposure and analysis are described on page 10.

At the end of the Cr^{+6} exposure period, the fish were transferred to a laboratory coldroom for challenge. Four groups of ten control and ten Cr^{+6}-exposed fish were anesthetized with tricaine methanesulfonate (100 mg/l) and injected subcutaneously just anterior to the dorsal fin with 0.1 ml of one of the four tenfold dilutions of bacteria. Each group of ten fish was placed in one of eight covered glass aquaria (30 x 60 x 40 cm), each containing 60 l of dechlorinated tap water ($15 \pm 1^{\circ}$ C, 56 mg/l $CaCO_3$ hardness, pH 7.3) which was aerated and filtered through activated charcoal by air lift aquarium filters. The fish were not fed during the 1 week

challenge period, and deaths were recorded at least twice daily. The anterior kidneys of dead fish were sampled aseptically within less than 6 h after death to verify the presence of V. anguillarum. Presumptive identification was based on growth on trypticase soy agar containing 1.5% NaCl, motility in 1.5% NaCl saline, immobility in distilled water, and negative gram staining.

Analysis of variance with replication on arcsin transformed mortality proportions (Sokal and Rohlf, 1969) was used to assess whether disease mortality was dependent on Cr^{+6} treatment and bacterial dose. Statistical significance was accepted at the $P \leq .05$ level. The moving average method (Thompson, 1947) was used to estimate the LD-50 for the Cr^{+6}- exposed and control fish in each test.

A negative control experiment (Exp. IX) was conducted to assess whether laboratory transfer, injection trauma, and delayed Cr^{+6} toxicity caused mortality without exposure to bacteria. Two groups of 20 fish each were exposed to 0.0 or 0.5 mg Cr^{+6}/1 for 2 weeks as in the previous experiments. The fish were transferred to the laboratory coldroom, where ten fish from each group were anesthetized and injected sub- cutaneously with 0.1 ml sterile 1.5% NaCl saline, and ten fish from each group were untreated. The fish were placed in aquaria containing dechlorinated tap water as in the previous experiments and were observed for 2 weeks.

Agglutinin Production

To assess whether sublethal Cr^{+6} exposure had an effect on the acquired immune response of coho salmon, two

groups of 35 fish each were immunized with V. anguillarum
bacterin and then exposed to either 0.0 or 0.5 mg Cr^{+6}/l for
2 weeks in flowing 13 $\pm$ 2°C hatchery spring water as in the
previous experiments. The serum agglutinin titer was measured
in at least ten control and ten Cr^{+6}-exposed fish at the end
of this 2 week period, and at the end of 2 and 4 additional
weeks of maintenance in Cr^{+6}-free flowing hatchery water. The
fish were fed once every other day over this 6 week period.
This experiment was designated as Exp. X.

Bacterin for immunization was prepared from cells
grown for 24 h at 20° C on trypticase soy agar containing 1.5%
NaCl. Harvested cells were washed twice in 0.85% saline and
suspended to a final concentration of 10 mg/ml (wet weight)
in saline. This preparation was heat-killed at 100° C for
2 h, preserved with 0.4% formalin, tested for sterility, and
stored at 4° C. Fish to be immunized were anesthetized as
before and injected intraperitoneally with 0.1 ml of the
bacterin preparation.

Procedures for measurement of serum agglutinin were
similar to those used by Paterson and Fryer (1974). Individual
fish were anesthetized, and the caudal peduncle was severed
just posterior to the adipose fin. Blood was collected from
the exposed dorsal artery with non-heparinized microcapillary
tubes, allowed to clot for 1 h at room temperature, and kept
for 12 h at 4° C. Serum was separated from blood cells by
centrifugation at 11,500 rpm for 5 min in a microcapillary
centrifuge and tested for agglutinin titer with a microtiter
apparatus using microtiter plates with "V" bottom wells

(Cook Engineering Co.). Two-fold serial dilutions of 25 ul serum were made in 0.85% NaCl saline from an initial 1:2 serum dilution. The antigen was prepared by dilution of the immunization bacterin with 0.85% NaCl saline to a spectrophotometer reading of .90D at 520 nm. Each serum dilution and a saline control received 25 ul of antigen to give final serum dilutions ranging from 1/4 to 1/4096. The microtiter plates were covered, mixed gently, and incubated for 3 h at room temperature followed by 12 h at 4° C. The maximum dilution giving a positive agglutination reaction was read at a magnification of 20x using a microscope, and the titer was recorded as the reciprocal of that dilution. A positive reaction consisted of an even dispersal of antigen over the bottom of the microtiter well. A negative reaction was indicated by the formation of a small button of antigen at the bottom of the "V" in the microtiter well.

The data were reported as the mean, standard error, and range of $\log_2$ titers for each control and Cr^{+6}-exposed group. Serum samples with no detectable agglutination were considered to have a zero titer for purposes of calculation and presentation of the mean log titer results. The statistical significance of the difference between control and experimental titers was analyzed by Students t test (Sokal and Rohlf, 1969). Statistical significance was accepted at the $P \leq .05$ level. Agglutinin titer was measured also on serum samples from 20 non-immunized fish taken from hatchery stocks in order to test for the presence of natural or previously induced agglutinins which might have cross-reacted with V. anguillarum antigen.

RESULTS

Disease Resistance

During the 2 week hatchery exposure period there was
no mortality in any group of Cr^{+6}-exposed or control fish.
The analyzed Cr^{+6} concentration (mean $\pm$ S.D.) was $0.50 \pm .049$
mg/l in Exp. VII, $0.54 \pm .051$ mg/l in Exp. VIII, and $0.47 \pm$
.06 in Exp. IX. There was no detectable Cr^{+6} in the control
aquaria in any of the three experiments. There were no
mortalities in the saline-injected or untreated fish in
negative control Exp. IX.

Analysis of variance of the mortality data for both
bacterial challenge experiments (Table III, Fig. 3) indicated
that Cr^{+6}-exposed fish were less resistant than control fish
to disease caused by injected V. anguillarum. Mortality was
significantly related ($P < .01$) to the presence or absence of
Cr^{+6} exposure and to the bacterial dose. The significant
($.01 < P < .025$) interaction between Cr^{+6} treatment and
bacterial dose indicated that these two factors acted syner-
gistically on the fish. In Exp. VII, the total mortality for
all bacterial doses combined was 47.5% in the Cr^{+6}-exposed
group and 20% in the control group. The combined total mortality
in Exp. VIII was 45% in Cr^{+6}-exposed fish and 10% in control
fish. The mortality of each individual dose in both experiments
was higher in the Cr^{+6}-exposed fish than in the respective
control fish, except at the lowest dose in Exp. VIII at which
no fish died in either group. The calculated LD-50 for Cr^{+6}-
exposed fish was lower than control fish in both experiments
(Table III).

Table III. Mortality of control and Cr^{+6}-exposed coho salmon 1 week after injection

of live Vibrio anguillarum.

Mortality at each bacteria dose

Experiment		10^{-7}	10^{-6}	10^{-5}	10^{-4}	Total (all doses)	Calculated LD-50 (bacteria/ml)
VII[a]	Cr^{+6}	2/10	4/10	4/10	9/10	19/40	1.6×10^5
	Control	0/10	1/10	2/10	5/10	8/40	7.7×10^6
VIII[b]	Cr^{+6}	0/10	2/10	6/10	10/10	18/40	2.1×10^5
	Control	0/10	1/10	0/10	3/10	4/40	1.9×10^9

a Exposure for 2 wk to 0.0 or $0.50 \pm .049$ mg Cr^{+6}/l (mean $\pm$ S.D.); estimated number of bacteria in 10^{-7} dose = 3.06×10^2/0.1 ml

b Exposure for 2 wk to 0.0 or $0.54 \pm .051$ mg Cr^{+6}/l (mean $\pm$ S.D.); estimated number of bacteria in 10^{-7} dose = 4.12×10^2/0.1 ml

Analysis of variance

Source of variation	df	SS	MS	F_s	
Subgroups	7	2.98	0.43	43	(P < .01)
Cr^{+6} Treatment	1	1.03	1.03	103	(P < .01)
Bacterial Dose	3	1.78	0.59	59	(P < .01)
Interaction	3	0.17	0.06	6	(.01 < P < .025)
Error	8	0.05	0.01		
Total	15	3.03			
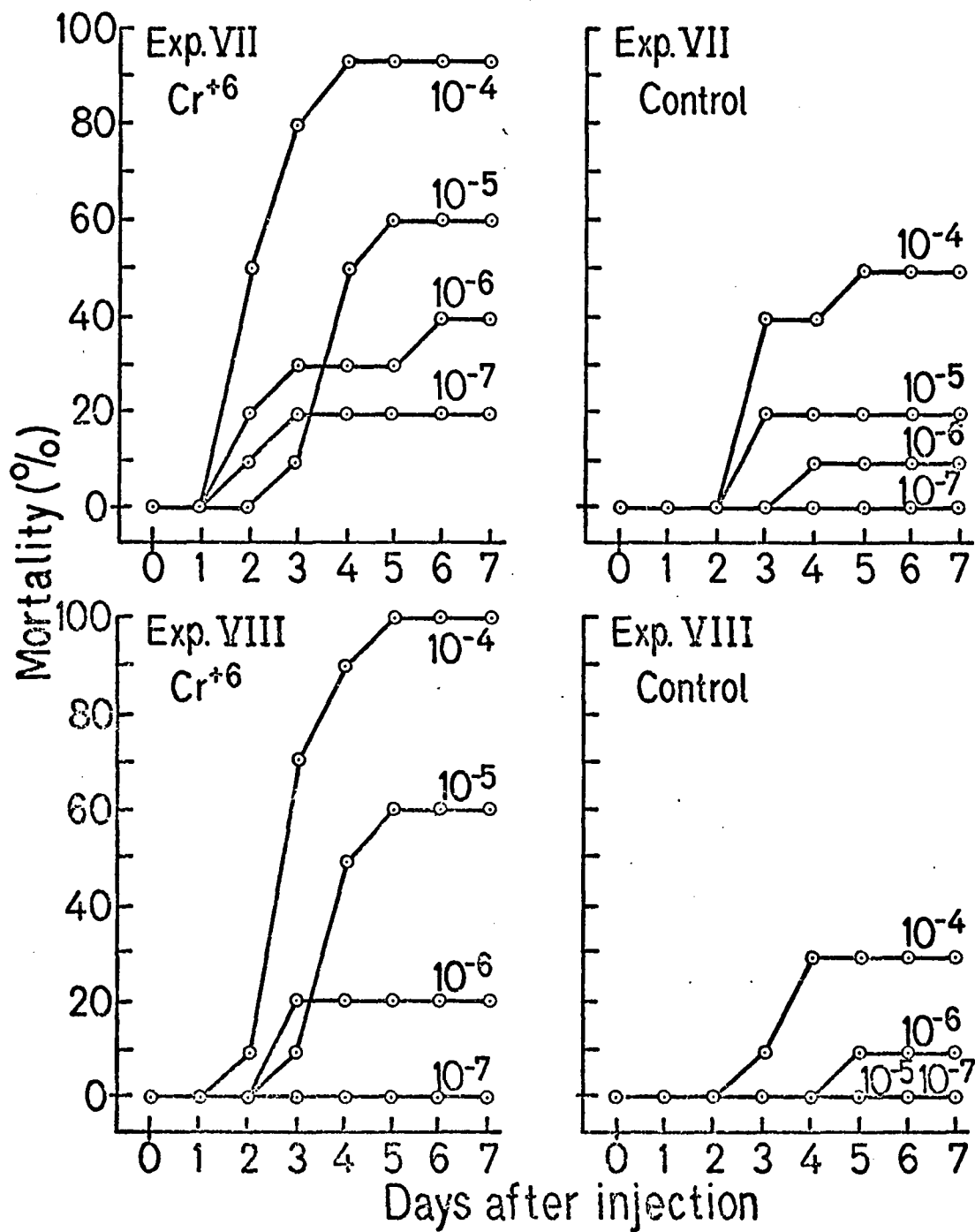
Fig. 3. Cumulative percent mortality of control and Cr^{+6}-
exposed coho salmon over 7 days after injection
with live _Vibrio anguillarum_. Each line represents
the mortality within a group of 10 fish injected
with 1 of 4 bacteria dose dilutions (10^{-4} to 10^{-7},
see text).

The cumulative daily mortality of each treatment group (Fig. 3) indicates, with one exception, that disease mortality in all Cr^{+6}-exposed groups began at least 1 day earlier than in the respective control groups. The exception is the lowest dose in Exp. VIII, where all fish survived. Initial mortality occurred most often between 1 and 2 days after injection in the Cr^{+6}-exposed groups and between 2 and 3 days after injection in the control groups. The time at which final mortality was reached ranged from 3 to 6 days after injection in those Cr^{+6}-exposed and control groups where deaths occurred. The period between initial and final mortality in these groups was no more than 3 days in any control group and no more than 5 days in any Cr^{+6}-exposed group.

The most common signs of vibriosis in diseased fish were hemorrhages at the base and between the rays of the pectoral, anal, and dorsal fins. These hemorrhages were often the only external signs of disease and occasionally were absent in fish which died. The subcutaneous injection site often was swollen, with inflammation in the underlying musculature. Other less frequent signs were petechiae in the peritoneal wall and intestine, distension of the anus, and two cases of exopthalmia. Large numbers of bacteria presumptively identified as V. anguillarum were isolated from the anterior kidney of all mortalities examined in Exp. VII and Exp. VIII. Based on uniform colony morphology, these isolates were in pure culture. The pathology, isolation of V. anguillarum, and complete survival of negative controls in Exp. IX indicate that the direct cause of death was vibriosis, and not laboratory transfer, delayed Cr^{+6} toxicity, or injection trauma.

Agglutinin Production

The analyzed Cr^{+6} concentration (mean $\pm$ S.D.) during the 2 week metal exposure period in Exp. X was $0.48 \pm .077$ mg/l. There was no detectable Cr^{+6} in the control aquaria. Three Cr^{+6}-exposed fish and two control fish died within 4 days after antigen injection. These mortalities may have been caused by toxicity of the antigen preparation or by internal damage caused by the intraperitoneal injection, a route which is more likely to cause damage than the subcutaneous route used in the previous experiments. One additional Cr^{+6}-exposed fish died in the third week of the experiment for unknown reasons.

Serum agglutinating antibody was not detected in any of the 20 non-immunized fish or in any of the ten Cr^{+6}-exposed and ten control fish sampled at the end of the first 2 week period after immunization (Table IV, Fig. 4). The mean agglutinin log titer at 4 weeks had risen to 4.60 in Cr^{+6}-exposed fish and to 4.40 in control fish. Agglutination was detected in nine of ten control fish and in all ten Cr^{+6}-exposed fish. The mean log titer of control fish was not significantly different from that of Cr^{+6}-exposed fish ($0.90 < P$; $t= 0.28$; $df= 18$), although the means of both groups showed a significant increase ($P < .001$; $t= 7.57$ or 11.50; $df= 18$) from their respective 2 week values. The mean log titer at 6 weeks was 5.18 in Cr^{+6}-exposed fish and 7.15 in control fish. The mean log titer of control fish was significantly higher ($P < .01$; $t= 2.84$; $df= 22$) than that of the Cr^{+6}-exposed fish. The increase in mean log titer between 4 and 6 weeks was significant in the control group ($P < .01$; $t= 3.81$; $df= 21$) but not in the Cr^{+6}-exposed group ($.40 < P$; $t= 0.86$; $df= 19$).

Table IV. Serum agglutinin production in control and

Cr^{+6}-exposed coho salmon after immunization

with <u>Vibrio</u> <u>anguillarum</u> bacterin

Agglutinin titer (log_2)

Weeks after[a] immunization	Control	No. fish sampled	Cr^{+6}	No. fish sampled
2	0.0 ± 0.0[b] (0.0)	10	0.0 ± 0.0 (0-0)	10
4	$4.40 \pm .581$ (0-6)	10	$4.60 \pm .400$ (3-7)	10
6	$7.15 \pm .451$* (5-9)	13	$5.18 \pm .536$ (2-8)	11

a The fish were immunized and exposed for 2 weeks to 0.0 or 0.48 $\pm$ .077 mg Cr^{+6}/1 (mean $\pm$ S.D.)

b mean $\pm$ S.E.
   (range)

* significantly greater than in Cr^{+6}-exposed fish, P < .01
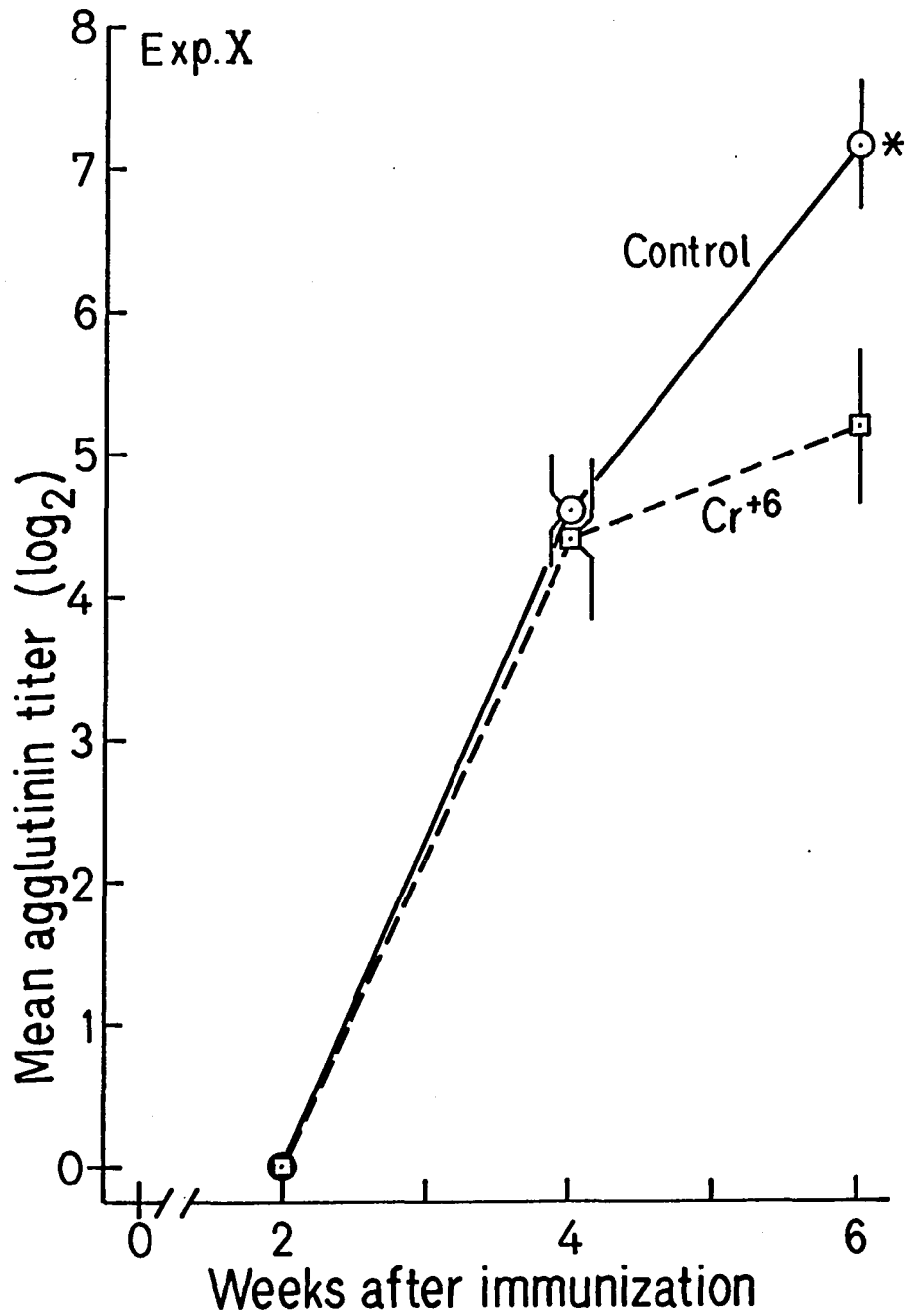
Fig. 4. Serum agglutinin production in control and
Cr^{+6}-exposed coho salmon after immunization
with _Vibrio anguillarum_ bacterin. Each point
represents the mean agglutinin log titer of
10-13 fish. Bars are standard error of the
mean. (* $P < .01$)

statistically significant in the control group (P < .01; f= 3.81; df= 21) but not in the Cr^{+6}-exposed group (0.40 < P; t= 0.86; df= 19.

DISCUSSION

The experimental results indicate that 2 weeks of sublethal exposure to Cr^{+6} impaired the disease resistance and agglutinin production of coho salmon against V. anguillarum. These respective effects on the innate and acquired immune responses could have resulted from the direct or indirect action of Cr^{+6} on several of the humoral and cellular immuno- logic mechanisms known to occur in teleost fishes (Anderson, 1974; Corbel, 1975). Cr^{+6}, as anionic chromate (CrO_4^{-2}) or dichromate ($Cr_2O_7^{-2}$), readily penetrates cell membranes, is a strong oxidizing agent and reacts with organic matter to be reduced to Cr^{+3}, in which valence state it binds with proteins, nucleic acids, and other biological ligands (National Research Council, 1974). The metal is concentrated and strongly retained by the kidney and spleen of fishes (Knoll and Fromm, 1960; Buhler et al., 1977) and, therefore, may impair the immunologic function of the reticuloendothelial system.

These binding characteristics of Cr^{+6} may have impaired disease resistance through interference with bacterial destruc- tion by phagocytic cells or through interference with the synthesis or functional activity of such immunologically important serum proteins as complement, properdin, lysozyme, and interferon; all of which have been detected in teleost fishes (Corbel, 1975). It is also possible that Cr^{+6} acted indirectly by causing a general stress response. This metal

caused elevated plasma cortisol in rainbow trout exposed to
0.02 mg Cr^{+6}/1 (Hill and Fromm, 1968) and elevated plasma
glucose both in rainbow trout subjected to 10 mg Cr^{+6}/1
(Strik et al., 1975) and in coho salmon treated with 1.0 mg
Cr^{+6}/1 (see page 51). Elevated plasma cortisol and glucose
are characteristic in fishes of a non-specific stress response,
components of which may contribute to increased susceptibility
to infectious disease (Wedemeyer, 1970).

Regardless of the mechanisms by which Cr^{+6} impaired
disease resistance in coho salmon, significantly fewer treated
fish than control fish were able to restrict the multiplication
and pathogenic effects of V. anguillarum below that critical
level required to prevent disease mortality. This conclusion
is supported by the isolation of massive numbers of bacteria
from all dying fish, and by the higher total mortality, lower
LD-50, and somewhat faster mortality rate of the Cr^{+6}-exposed
fish in both disease resistance experiments.

Impaired antibody production in Cr^{+6}-exposed coho
salmon may have resulted from similar direct or indirect effects
of Cr^{+6} on antigen processing and antibody synthesis by the
cells of the reticuloendothelial system. It is possible also
that Cr^{+6} suppressed the induction and proliferation of
antibody-producing lymphocytes, processes which are known to
occur normally in the anterior kidney and spleen of teleost
fishes after immunization (Smith et al., 1967; Chiller et al.,
1969). The pattern of serum agglutinin production in this
experiment indicated that Cr^{+6} exposure had no detectable
effect on the initial occurrence, percent response, and mean log

titer of agglutinin up to 4 weeks after vaccination. Between 4 and 6 weeks after vaccination, however, the mean log titer had increased significantly in control fish, but not in Cr^{+6}- exposed fish. This pattern may indicate that Cr^{+6} exposure had little effect on initial antigen processing or on the primary population of inducible antibody-producing lymphocytes, and a greater effect on the later stages of lymphocyte proliferation and subsequent rate of antibody synthesis.

Direct application of laboratory results such as these to natural conditions must always be made cautiously. FW exposure to 0.5 mg Cr^{+6}/1 is certainly possible in polluted rivers (Surber, 1959; Herbert et al., 1965; Kelso and MacCrimmon, 1969; Breeze, 1973). Natural exposure of wild fish to V. anguillarum, however, is improbable in FW because this bacteria has an obligate requirement for NaCl and because most reported outbreaks of vibriosis have occurred in SW (Anderson and Conroy, 1970). FW outbreaks of vibriosis in cultured fish have been reported, but they were probably due to introduction of marine vibrios via contaminated feed (Rucker et al., 1954; Evelyn, 1971). Bacterial challenge in the present study was carried out in FW, not SW, because SW transfer, by itself, was known (pages 13-17) to result in significant mortality in Cr^{+6}- exposed coho salmon. This additional stress would have compli- cated the comparison of disease resistance, although it is probable that the difference in mortality between Cr^{+6}-exposed and control fish would have been even greater under the more realistic conditions of combined SW transfer and V. anguillarum

exposure. This conclusion is supported by a recent report (Antipa and Amend, 1977) that natural $\underline{V}$. $\underline{anguillarum}$ challenge of coho salmon in SW pens can result in nearly 60% vibriosis mortality. This mortality is higher than that found even in those control fish, but not Cr^{+6}-exposed fish, injected with the highest bacterial dose in the present study, indicating that the effects of Cr^{+6} found in the laboratory may be even greater under more natural conditions.

Although Cr^{+6} exposure significantly inhibited mean agglutinin log titer when measured 6 weeks after immunization, it is unknown whether this inhibition would have continued or whether it would have resulted in lowered resistance to vibriosis. The relationship between agglutinin titer and degree of disease protection in fishes is not well understood, although Antipa and Amend (1977) reported that immunization provided significant protection against vibriosis in coho salmon under field condi- tions. These authors found that immunized groups of fish, which had mean agglutinin log titers between 7 and 9, suffered less mortality than did non-immunized groups, having mean log titers less than 2. Following natural challenge, the mean log titers of non-vaccinated fish increased to about 6 although they continued to suffer greater vibriosis mortality than vaccinated fish. These results may indicate that log titers of 6 or less are not as protective as log titers of 7 or greater under natural conditions. The Cr^{+6}-exposed fish in the present study had a mean log titer of about 5 at a time when control fish had a mean log titer of about 7, suggesting that Cr^{+6}-exposed fish would

have been more susceptible to vibriosis, if challenged at that time.

Determination of the actual significance of Cr^{+6} pollution on the immune response of coho salmon requires field research in similarly polluted areas. The urgency for this type of field research is emphasized by the present study and by the possibility that many other pollutants, at even lower concentrations, may have similar effects on salmon and other aquatic organisms. Measurement of these effects may be useful also as a sensitive indicator of sublethal toxicity in aquatic bioassays, requiring less time and expense than those chronic bioassays which measure other ecologically significant factors such as growth, reproduction, and long-term survival. Using long-term (8-22 months) survival as a criterion, Benoit (1976) found that the maximum acceptable Cr^{+6} concentration for brook trout was between 0.20 and 0.35 mg Cr^{+6}/l, which is similar to the 0.5 mg Cr^{+6}/l concentration determined in this study to affect the immune response of coho salmon after only 2 weeks of Cr^{+6} exposure. Further research on the relative sensitivity of these tests for other pollutants and aquatic species would be of great value.

EFFECTS OF Cr^{+6} ON BLOOD CHARACTERISTICS

INTRODUCTION

The measurement of physiological and biochemical changes in the blood of fishes is one of several approaches used to detect sublethal toxicity of heavy metals in the aquatic environment (Katz, 1975). Changes in one or more blood characteristics, including hematocrit, hemoglobin, glucose, protein, enzymes, and ions, have been found in fishes exposed to salts of Pb (Dawson, 1935; D'Amelio et al., 1974), Cu (McKim et al., 1970; Christensen, 1972; Christensen et al., 1972), Hg (Tamura et al., 1962; Bouquegneau, 1973), Fe (Brenner et al., 1976), Zn (Skidmore, 1970; Watson and McKeown, 1976), and Cd (Thurberg and Dawson, 1974; Larsson et al., 1976; McCarty and Houston, 1976).

Exposure to Cr^{+6}, the subject of this study, has been shown also to affect various blood characteristics of fishes at concentrations ranging from 0.02 to 289 mg Cr^{+6}/l. Abegg (1950) reported that 24 h exposure to 289 mg Cr^{+6}/l (the 24 h LC-50) caused a decrease in blood specific gravity and an increase in tissue water content of bluegill sunfish. Shiffman and Fromm (1959) found that 2-4 mg Cr^{+6}/l caused an increased hematocrit in rainbow trout. Strik et al. (1975) reported that 15-22 days of exposure to 10 mg Cr^{+6}/l caused a decrease in blood Na^+ and an increase in blood glucose, hematocrit, and hemoglobin in rainbow trout. This relatively high Cr^{+6} concentration also caused about 25% mortality during the exposure

period. The only report of significant blood effects in fishes at Cr^{+6} concentrations below 2 mg Cr^{+6}/l is that of Hill and Fromm (1968), who found a transient increase of plasma cortisol in rainbow trout exposed to 0.02 and 0.20 mg Cr^{+6}/l.

In order to extend this work, the effects of exposure to 1 mg Cr^{+6}/l on 12 blood characteristics of juvenile coho salmon were investigated. Further goals of this investigation were to increase the understanding of the mode of toxic action of Cr^{+6}, and to determine the relative sensitivity of various blood tests as indicators of sublethal Cr^{+6} toxicity.

MATERIALS AND METHODS

Juvenile coho salmon (19.5 g mean weight) were obtained from a hatchery where they were reared from eggs in flowing spring water (10 $\pm$ 3° C, 60 mg/l $CaCO_3$ hardness, pH 6.6) under a natural photoperiod and fed commercial salmon feed.

Six static Cr^{+6} exposure experiments were conducted in polyethylene drums containing 200 l dechlorinated tap water (56 mg/l $CaCO_3$ hardness, pH 7.3) in a laboratory cold room at a temperature and photoperiod adjusted to that of the hatchery. At the beginning of each experiment, a stock solution of sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) dissolved in distilled water was added to one of two tanks to give a nominal concentration of 1.0 mg Cr^{+6}/l. The actual Cr^{+6} concentration in the experimental and control tanks was measured at the beginning and end of each experiment by the diphenylcarbazide method (American Public Health Association, 1971). Dissolved oxygen

was held above 80% saturation by aeration, and the water was filtered through nylon wool with air lift aquarium filters.

In each of five experiments, 20 previously graded fish were transferred from the hatchery to the cold room where they were divided equally between one control and one experimental tank. The fish loading factor was approximately 1 1/g fish as recommended by the American Public Health Association (1971) for static bioassays. In a sixth test, a total of 30 fish were divided equally between the control and experimental tanks, giving a fish loading factor of approximately 0.7 1/g fish. At the end of each 7 day exposure period, during which the fish were not fed, individual salmon were netted alternately from each tank and stunned immediately with a blow to the head. The caudal peduncle was severed just posterior to the adipose fin, and the blood was collected from the exposed dorsal artery in heparinized microcapillary tubes. The blood samples from the control and experimental fish in each test were used for measurement of one or more of the following nine blood characteristics: hematocrit (Hc), hemoglobin (Hb), percent methemoglobin (% M-Hb), erythrocyte sedimentation rate (ESR), erythrocyte osmotic fragility (EOF), glucose (Glu), total protein (TP), osmolality (Os) and lysozyme (Lys). Hc and Hb values were used to calculate mean corpuscular hemoglobin concentration (MCHC). Hb and % M-Hb values were used to calculate the concentration of methemoglobin (M-Hb).

Hc was measured by the standard microhematocrit technique (Snieszko, 1960) in which the blood samples were centrifuged at 11,500 rpm for 5 min in a microcapillary

centrifuge and read with the aid of a microhematocrit card reader.

Hb was estimated by the cyanmethemoglobin method using Hycel reagents and standards. Fresh whole blood (0.02 ml) was added to 5.0 ml reagent, mixed, and measured in a spectrophotometer at 540 nm against a reagent blank.

MCHC was calculated from the equation: MCHC = (Hb/Hc)100.

Percent M-Hb was estimated by the spectrophotometric method of Van Kampen and Zijlstra (1965) with some modifications. Fresh whole blood (0.2 ml) was hemolyzed in 10 ml 0.0167 M phosphate buffer, pH 6.6, and divided into two equal aliquots. The absorbance of one aliquot was measured at 630 nm before and after the addition of one drop of 5% neutralized potassium cyanide, yielding respective values D_1 and D_2. The other aliquot was measured at 630 nm after adding one drop of 5% potassium ferricyanide (D_3), and then again after adding one drop of 5% neutralized potassium cyanide (D_4). All measurements were made against a phosphate buffer blank. Percent M-Hb was calculated from the equation: %M-Hb =

$$\left[(D_1-D_2)/(D_3-D_4)\right] 100$$

M-Hb concentration was calculated from the equation: M-Hb = (%M-Hb/100)Hb.

ESR was measured by the Micro-Wintrobe method (Blaxhall and Daisley, 1973) using 75 x 1.0-1.25 mm heparinized capillary tubes. Blood from the severed dorsal artery was allowed to flow about 50 mm into the capillary tube, which was then sealed and placed in vertical position for 1 h at room temperature.

The sedimentation distance was read to the nearest 0.5 mm at a magnification of 20x using a microscope. The Hc of these samples was then measured as previously described.

EOF was measured by mixing 0.02 ml aliquots of fresh whole blood with 3 ml of different NaCl solutions ranging in concentration from 0.2 to 0.6% in 0.05% increments. The highest NaCl concentrations causing complete and partial hemolysis were recorded after incubation for 15 min at room temperature. The criterion for complete hemolysis was red color without turbidity after shaking the reaction tubes. Designation of partial hemolysis was based on red color with turbidity after shaking.

Glu was estimated by a micromodification of the O-toluidine colorimetric method using the Hycel direct sugar test. Reagent (3.0 ml) was added to each fresh plasma sample (0.04 ml), incubated for 10 min at 100° C, and measured with a spectrophotometer at 590 nm against a reagent blank. Standard curves were made using the same volumes of glucose standard and reagent.

TP was measured by the colorometric method of Lowry et al. (1951) using 0.02 ml plasma samples and bovine serum albumen as a standard.

Os was measured by freezing point depression in an osmometer. Plasma samples (0.25 ml) were diluted exactly eight-fold with double-distilled deionized water and measured.

Lys was estimated by a micromodification of the nephelometric method using the Worthington lysozyme assay set. This method measures the rate at which a buffered suspension

of dried <u>Micrococcus</u> <u>lysodeikticus</u> cells decreases in turbidity
between 0.5 and 3.0 min after the addition of lysozyme.
Turbidity was measured with a spectrophotometer at 550 nm,
using 3.0 ml substrate and 0.1 ml plasma. Standard curves
were made with purified egg white lysozyme.

The mean, range, and standard error were reported for
each blood characteristic, and the statistical significance
of the difference between control and experimental values was
determined by the non-parametric Mann Whitney U test (Sokal
and Rohlf, 1969). Correlation coefficients and their
statistical significance were calculated also according to
Sokal and Rohlf (1969). Statistical significance was accepted
at the $P \leq .05$ level.

<div align="center">RESULTS</div>

There were no mortalities or signs of stress in any
group of fish during the six Cr^{+6} exposure experiments. The
initial Cr^{+6} concentrations, ranging between 0.93 and 1.04
mg $Cr^{+6}/1$ (mean 0.98 mg/l; SD 0.04 mg/l), had not changed by
the end of the 1 week exposure periods.

Hc ($P < .01$), M-Hb ($P < .01$), % M-Hb ($P < .01$), TP
($P < .01$) and Glu ($P < .05$) were increased significantly in
Cr^{+6}-exposed fish relative to control fish (Table V). MCHC
($P < .01$) and ESR ($P < .01$) showed significant decreases in
Cr^{+6}-treated fish. The correlation coefficient between Hc and
ESR was $-.74$ ($P < .01$) in Cr^{+6}-exposed fish and $-.84$ ($P < .01$)
in control fish. These correlation coefficients were not
significantly different ($P < .10$) from each other. There were

Table V. Blood characteristics of coho salmon exposed
to 0.0 or 1.0 mg Cr^{+6}/1 for 1 week.

Blood Characteristic		Control	Cr^{+6}
Exp. XI			
Hc (%)	$(10)^a$	45.3 ± 1.5^b (36 – 52)	$54.7 \pm 2.9**$ (34 – 69)
Hb (g/100 ml)	(10)	7.85 ± 0.21 (6.7 – 8.6)	7.91 ± 0.36 (6.1 – 9.6)
MCHC (%)	(10)	17.40 ± 0.40 (15.75 – 19.55)	$14.69 \pm 0.72**$ (10.89 – 18.22)
Exp. XII			
Hc (%)	(15)	33.9 ± 0.6 (31 – 40)	$40.9 \pm 0.7**$ (36 – 46)
Glu (mg/100 ml)	(15)	67.1 ± 2.1 (51 – 78)	$75.0 \pm 3.0*$ (63 – 95)
TP (g/100 ml)	(10)	2.99 ± 0.15 (2.10 – 3.86)	$3.62 \pm 0.08**$ (3.24 – 3.91)
Os (mOsm/1)	(10)	293.3 ± 3.98 (279 – 321)	290.9 ± 3.80 (276 – 314)
Exp. XIII			
EOF-compl. (% NaCl)	(10)	0.31 ± 0.006 (0.30 – 0.35)	0.32 ± 0.010 (0.30 – 0.35)
EOF-part. (% NaCl)	(10)	0.43 ± 0.010 (0.40 – 0.45)	0.40 ± 0.013 (0.35 – 0.45)

Table V. continued

Blood Characteristic		Control	Cr^{+6}
Exp. XIV			
Hb (g/100 ml)	(10)	7.75 ± 0.13 (7.4 – 8.7)	7.97 ± 0.17 (7.0 – 8.8)
% M-Hb (%)	(10)	2.45 ± 1.04 (0.0 – 11.3	16.81 ± 1.76** (8.6 – 26.9)
M-Hb (g/100 ml)	(10)	0.188 ± 0.010 (0.00 – 0.84)	1.330 ± 0.140** (0.71 – 2.15)
Exp. XV			
Hc (%)	(10)	39.0 ± 1.4 (30 – 46)	48.7 ± 1.5** (37 – 57)
ESR (mm/hr)	(10)	2.2 ± 0.2 (1.5– 3.5)	1.1 ± 0.1** (0.5 – 1.5)
Exp. XVI			
Lys (mg/100 ml)	(10)	2.94 ± 0.82 (1.1– 9.8)	3.95 ± 0.92 (1.4 – 10.7)

a   number of fish sampled in each group

b   mean ± SE
    (range)

*   significantly different from control, P < .05

**   significantly different from control, P < .01

no significant differences in Hb, EOF, Os and Lys between treated and untreated groups of fish.

The blood characteristic showing the greatest mean change in exposed fish was M-Hb, which increased 607% over the control mean (Fig. 5). The percentage change of the other significantly altered blood characteristics decreased in the order of %M-Hb (+586%), ESR (-50%), Hc (+21 to +25%), TP (+21%), MCHC (-16%) and Glu (+12%). Mean Lys of treated fish was 34% higher than that of control fish, but this difference was not statistically significant due to the wide overlap of individual enzyme concentrations in both groups. Mean values for Hb, EOF and Os in Cr^{+6}-exposed fish were within 1-7% of the control mean values.

DISCUSSION

The effect of greatest magnitude was the increased concentration of M-Hb in the blood of Cr^{+6}-exposed fish. M-Hb is the oxidized and thermodynamically more stable form of Hb. It is incapable of transporting oxygen, and excessive M-Hb (methemoglobinemia) can result in hypoxia and cyanosis. In mammals, and presumably fishes, spontaneously formed M-Hb is reduced back to Hb by a complex reductase system (Cameron, 1971). Many compounds with oxidant properties are known to cause M-Hb formation in man and animals (DeBruin, 1976). Increased M-Hb formation has been reported in fishes exposed to nitrite (Smith and Williams, 1974; Smith and Russo, 1975; Perrone and Meade, 1977) and chloramine (Grothe and Eaton, 1975; Buckley, 1976). Cr^{+6}, as chromate (CrO_4^{-2}) or
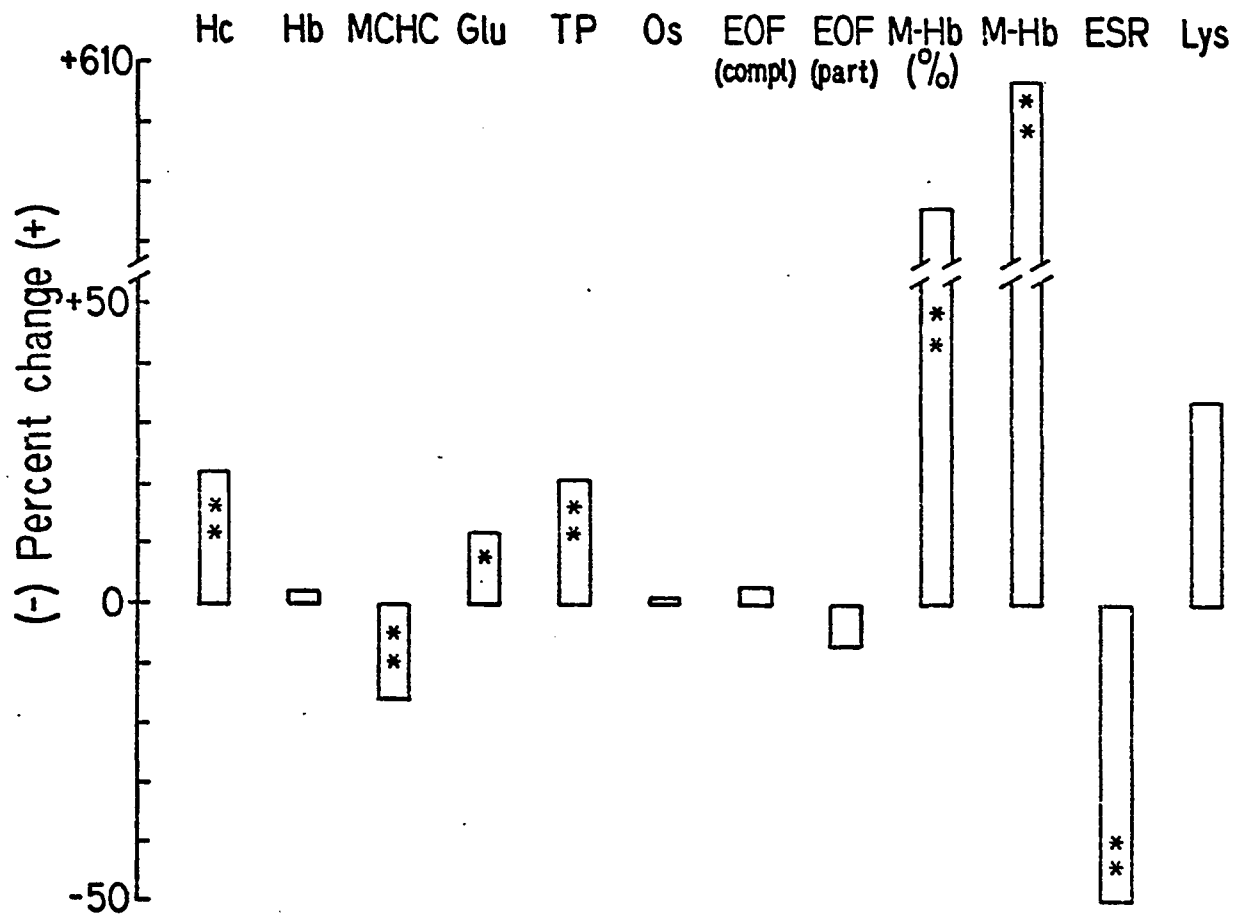
Fig. 5. Mean percent change from mean control values of 12 blood characteristics of coho salmon exposed for 1 week to 1.0 mg Cr^{+6}/l. (* P < .05, ** P < .01)

dichromate $(Cr_2O_7^{-2})$, is a strong oxidizing agent which binds readily to Hb (National Research Council, 1974) and has been shown to cause M-Hb formation in human blood treated in vitro with 30 µg Cr^{+6}/ml (Hughes-Jones and Mollison, 1956). The Cr^{+6}-induced M-Hb elevation in coho salmon may have been caused by direct oxidation of Hb to M-Hb, or by inhibition of M-Hb reduction, or both. Regardless of the mechanism of toxic action, it is probable that the oxygen carrying capacity of the blood was lowered, resulting in some degree of hypoxia.

Hypoxia is known to increase the Hc of fishes (Holeton and Randall, 1967; Soivo et al., 1974) and may have contributed to the higher Hc observed in Cr^{+6}-exposed coho salmon. Schiffman and Fromm (1959) reported that exposure to 20 mg Cr^{+6}/l for 24 h caused an increased Hc in rainbow trout and that this change could be accounted for by an increase in erythrocyte size, erythrocyte number, and a probable but unproven, decrease in plasma volume. They found also that the Hb concentration was unaffected at 20 mg Cr^{+6}/l and that 2-4 mg Cr^{+6}/l was the lowest concentration which caused an increased Hc. Strik et al. (1975) reported that exposure to 10 mg Cr^{+6}/l for 15-22 days caused an increase in both Hc and Hb in rainbow trout. They also found hyperplasia and hypertrophy of the gill lamellae in treated fish and suggested that impaired gas exchange might lead to increased pCO_2 and decreased pO_2 in the blood, both of which are known to cause erythrocyte swelling and increased Hc in fish blood (MacLeod and Smith, 1966; Holeton and Randall, 1967; Soivo et al., 1974). Depression of MCHC, as found in Cr^{+6}-exposed coho salmon in the

present study, can also be indicative of erythrocyte swelling (Soivo et al., 1974).

The highly significant negative correlation between Hc and ESR in both groups of coho salmon indicated that the lower ESR in Cr^{+6}-exposed fish was probably a result of the increased Hc in this group. This relationship between ESR and Hc is well known in clinical hematology, and corrections for Hc are made routinely in evaluating the changes in ESR which occur in humans during acute infection, nephritis, heavy metal poisoning, and other disease states (Seiverd, 1964). Increased ESR has been reported also in fishes suffering from infectious diseases (Schumacher et al., 1956; Pandey, 1974). Iwama et al. (1976) reported that blood samples from juvenile coho salmon had ESR values between 0.5 and 1.5 mm/h and Hc values between 43 and 55%. The Cr^{+6}-exposed fish in the present study had about the same range of values (ESR 0.5-1.5 mm/h; Hc 37-57%), whereas control fish had higher ESR and lower Hc values (ESR 1.5-3.5 mm/h; Hc 30-46%). These control values are probably within the range of natural variation because the "estimated normal range" for Hc in a group of more than 1000 juvenile coho salmon has been shown to be between 32.5 and 52.5% (Wedemeyer and Chatterton, 1971). All of this information suggests that the depressed ESR found in Cr^{+6}-exposed fish was caused by the increase in Hc, rather than a direct effect of Cr^{+6}.

Blood Glu is known to be elevated in fishes in response to a wide variety of environmental stressors, including physical disturbance (Wedemeyer, 1972), asphyxia (Chavin and

Young, 1970), heat shock (Wedemeyer, 1973), and sublethal exposure to such pollutants as kraft pulpmill effluent (McLeay, 1977), polychlorinated biphenyls (Johansson et al., 1972), dieldrin (Silbergeld, 1974), endrin (Grant and Mehrle, 1973), Cu^{+2} (Christensen et al., 1972), Cd^{+2} (Larsson et al., 1976), and Zn^{+2} (Watson and McKeown, 1976). The hyperglycemic stress response in fishes is a result of tissue glycogen mobilization caused in part by stimulation of the pituitary-interrenal axis and subsequently increased production of ACTH, corticosteroids, and catecholamines (Fagerlund, 1967; Wedemeyer, 1970; Watson and McKeown, 1976). Strik et al. (1975) found hyperglycemia in rainbow trout exposed for 10-15 days to 10 mg Cr^{+6}/l and suggested that this might be caused by general stress, increased metabolism, or a specific anti-insulin effect of Cr^{+6}. It is probable that the Cr^{+6}-induced hyperglycemia reported here and by Strik et al. (1975) was caused by a general stress response because Hill and Fromm (1968) reported that exposure of rainbow trout to as little as 0.02 mg Cr^{+6}/l caused an elevation in plasma "cortisol". Cortisol is the major secreted corticosteroid in salmonid fishes (Hane and Robertson, 1959; Donaldson and Fagerlund, 1972) and is elevated in response to environmental stressors (Fagerlund, 1967; Wedemeyer, 1969; Grant and Mehrle, 1973; Spieler, 1974; Donaldson and Dye, 1975).

Increased levels of serum or plasma TP have been reported previously in fishes exposed to Cu^{+2} (McKim et al., 1970) and Hg^{+2} (Calabrese et al., 1975). Rats exposed for up to 4 months of dusts of Pb, Cr, and Zn salts and oxides showed an increased level of serum TP, due to initially elevated

albumin and subsequently elevated globulin (Makarov, 1970).
Shifts in the albumin/globulin ration, with or without changes
in TP, result from exposure to many other toxicants (DeBruin,
1976). The increased TP in Cr^{+6}-exposed coho salmon may have
resulted from a similar protein shift, or it may reflect
mobilization of liver proteins into the blood (Pesch, 1970)
or hemoconcentration (Wedemeyer, 1973).

Plasma Os was measured because previous research has
shown that lethal concentrations of Cr^{+6} have osmoregulatory
effects on fishes. Abegg (1950) reported that 24 h exposure
to 289 mg Cr^{+6}/1 (the 24 h LC-50) caused a decrease in blood
specific gravity and an increase in tissue water content of
bluegill sunfish. Strik et al. (1975) found that 15-22 days
of exposure to 10 mg Cr^{+6}/1 caused 25% mortality and a decrease
in blood Na^+, among other effects, in rainbow trout. Rainbow
trout sublethally exposed to 1 mg Cr^{+6}/1 for 39 days showed
a small, but not statistically significant, increase in water
content in pyloric caeca, liver, and kidney (Fromm and Stokes,
1962). The absence of a Cr^{+6} effect on plasma osmolality in
the present study indicates that this measure of osmoregulatory
function is not useful as an indicator of sublethal Cr^{+6}
exposure. In contrast with Cr^{+6}, sublethal exposure to Cu^{+2}
(McKim et al., 1970), Cd^{+2} (Larsson et al., 1976), Hg^{+2}
(Calabrese et al., 1975), and endrin (Grant and Mehrle, 1973)
has been shown to change the blood Os of fishes.

EOF was examined because changes in this parameter
were known to be indicative of toxicant damage to the erythro-
cyte membrane (DeBruin, 1976), and because there was a report

that Cr^{+6} caused hemolysis of human blood treated with 35 µg Cr^{+6}/ml (Ebaugh et al., 1953). The lack of effect on EOF in the present study indicated that sublethal exposure to 1 mg Cr^{+6}/l has little or no effect on the integrity of the erythrocyte membrane in coho salmon.

Plasma Lys was studied because elevation of this enzyme in the blood was known to be indicative of leukocyte destruction (Hiatt et al., 1952) and because mammalian leukocytes were rendered more susceptible to lysis by increased levels of corticosteroid hormones (Dougherty, 1960). Leuko- cyte destruction and/or leukopenia in fishes has been reported after exposure to several water pollutants, including phenol (Waluga, 1966), kraft pulp mill effluent (McLeay, 1973), Zn^{+2} (McLeay, 1975), and dehydroabietic acid (Iwama et al., 1976). The lack of significant Lys change in treated fish suggests that Cr^{+6}, at this exposure level, has little or no effect on the leukocytes of coho salmon.

These results indicate that 1 week of exposure to 1 mg Cr^{+6}/l caused a significant stress response in juvenile coho salmon. The increases in Hc and Glu, accompanied by elevated M-Hb, suggest that this response may have been caused in part by Cr^{+6}-induced hypoxia. Although Cr^{+6} exposure affected the internal dynamics of coho salmon, it is unclear whether the toxicant-induced changes represented an adaptive or pathological stress response. The values for the blood characteristics which were significantly altered in Cr^{+6}-exposed fish were within the ranges reported for control coho salmon in previous studies. Wedemeyer and Chatterton (1971), for instance, gave

the following "estimated normal ranges" for blood samples from a group of more than 1000 juvenile coho salmon: Glu, 41-135 mg/100 ml; Hc, 32.5-52.5%; Hb, 6.5-9.9 g/100 ml; TP, 1.4-4.3 g/100 ml. Iwama et al. (1976) reported that the ESR and Hc ranges of control coho salmon were 0.5-1.5 mm/h and 43-55%, respectively. Previously reported mean values for M-Hb or %M-Hb in control coho salmon include 0.18 g/100 ml (Buckley et al., 1976), 3.3-4.2% (Buckley, 1976) and 27.3-28.1% (Perrone and Meade, 1977). The first two values for mean M-Hb and %M-Hb agree rather well with those found in control fish in this study. The last values are much higher and may have been caused by the relatively greater mean nitrite concentration (0.6 vs. $\leq$ 0.05 mg NO_2^--N/l) in the water to which those fish had been acclimated.

This information indicates that the altered blood characteristics in Cr^{+6}-exposed fish were within the range of values known to be compatible with continued survival in other populations of coho salmon, suggesting that the blood changes in fish exposed to 1 mg Cr^{+6}/l for 1 week represented an adaptive, rather than a pathological, stage in the stress response. Continued exposure to this Cr^{+6} concentration, however, would have resulted in greater stress and mortality because chronic exposure to as low as 0.08 mg Cr^{+6}/l has been reported to lower the survival of other salmonid fishes (Olson and Foster, 1956). Short-term exposure to 1 mg Cr^{+6}/l, although not directly lethal under the present controlled laboratory conditions, would probably lower the ability of coho salmon to survive in a natural environment, where they

would be subjected to such additional factors as competition, predation, disease, and changing water characteristics. This idea is supported by my previous findings that exposure to 0.5 mg Cr^{+6}/l for 2 weeks lowered the salinity tolerance and disease resistance of juvenile coho salmon.

The actual ecological significance of short-term exposure to 1 mg Cr^{+6}/l can only be determined after further research. The present laboratory results, however, support the conclusion that this degree of Cr^{+6} exposure caused a stress response in coho salmon, and that several blood characteristics were useful as indicators of acute sublethal Cr^{+6} exposure.

SUMMARY

The toxicity of Cr^{+6} to fishes is well established.
However, little is known about the physiological effects of
short-term sublethal Cr^{+6} exposure, or the relationship
between such effects and the ability of fishes to survive
natural stress factors such as salinity change or infectious
disease. To simulate a potential field situation in which
seaward-migrating coho salmon pass through Cr^{+6}-polluted
freshwater (FW) before being exposed to seawater (SW) or
marine pathogenic bacteria (<u>Vibrio anguillarum</u>), I investigated
the effects of sublethal Cr^{+6} exposure in FW on salinity
tolerance and serum osmolality after transfer to SW, and disease
resistance and serum agglutinin production against <u>V. anguillarum</u>.
Additional research on the effects of sublethal Cr^{+6} exposure
in FW on 12 blood characteristics investigated the mode of
Cr^{+6} toxicity and determined the relative sensitivity of various
blood tests as indicators of sublethal Cr^{+6} toxicity.

Salinity tolerance, as measured by mortality of control
and Cr^{+6}-exposed fish after transfer from FW to SW, was
significantly decreased in salmon exposed in FW to 0.5 mg
Cr^{+6}/l for 2 weeks or to 0.23 mg Cr^{+6}/l for 4 weeks and trans-
ferred to 30 or 20 °/oo SW, respectively. Exposure in FW to
0.5 mg Cr^{+6}/l for just 1 week followed by transfer to 30 °/oo
SW resulted in higher mortality which, however, was not
statistically significant. Treatment with 0.5 mg Cr^{+6}/l in
FW for 2 weeks, followed by transfer to 20 °/oo SW for 1 week
and then to 30 °/oo SW for another week, resulted in significantly

higher mortality only after the second SW transfer. No
control fish died after SW transfer in any experiment.

Serum osmolality, which was measured by freezing point
depression in serum samples from fish at the end of 2 weeks of
FW exposure to 0.0 or 0.5 mg Cr^{+6}/l and at intervals of 1/2,
1, 2, and 7 days after transfer to 20 or 30 ^{o}/oo SW, was
significantly higher in Cr^{+6}-exposed fish from 1/2 to 2 days
after SW transfer, but not at the end of the FW exposure
period or at 7 days after SW transfer. The pattern of serum
osmolality changes was similar at both salinities, although
the magnitude was greater in 30 ^{o}/oo SW.

Duplicate disease resistance experiments, in which
groups of fish were exposed in FW to 0.0 or 0.5 mg Cr^{+6}/l
for 2 weeks and then injected in subgroups with one of four
tenfold dilutions of live V. anguillarum (ca. 3-4 x 10^{2-5}
bacteria/.l ml dose), showed that disease mortality increased
with dose and was significantly greater in Cr^{+6}-exposed fish.

Serum agglutinin production was measured by a micro-
titer method on fish which were injected with V. anguillarum
bacterin and exposed in FW to 0.0 or 0.5 mg Cr^{+6}/l for 2 weeks.
Subgroup log titers were measured at the end of this 2 week
period, and after 2 and 4 more weeks of maintenance in uncon-
taminated FW. Mean agglutinin log titer, undetectable at 2
weeks, rose by 4 weeks to about 4.5 in both groups and was
significantly higher (7.15 vs. 5.18) in control fish at 6 weeks.

Exposure to 1.0 mg Cr^{+6}/l for 1 week in FW caused
significant increases in hematocrit (Hc), methemoglobin (M-Hb),
percent methemoglobin (% M-Hb), serum total protein (TP), and

glucose (Glu); significant decreases in mean corpuscular hemoglobin concentration (MCHC) and erythrocyte sedimentation rate (ESR); and no significant effect on hemoglobin (Hb), complete or partial erythrocyte osmotic fragility (EOF), serum osmolality (Os), and serum lysozyme (Lys). The percentage change from control values of the significantly altered blood characteristics decreased in the order: M-Hb > % M-Hb > ESR > Hc > TP > MCHC > Glu.

The effects of sublethal Cr^{+6} exposure on short-term SW survival and osmoregulation probably were caused by non-specific damage to the branchial, renal, and intestinal epithelia involved in the initial osmoregulatory responses of coho salmon to SW transfer. Uncontrolled water loss, aggravated by influx of ions, could have accounted for the observed effects of higher serum osmolality, lower obtainable blood volume, and signs of hypoxia in Cr^{+6}-exposed fish dying after SW transfer.

Sublethal Cr^{+6} exposure could have lowered disease resistance and serum agglutinin production directly through non-specific damage to several tissues involved in the immune response or indirectly by eliciting a general stress response, components of which are known to be immunosuppressive.

Similar direct or indirect effects of sublethal Cr^{+6} exposure could have been responsible for the observed blood changes, which represented a general stress response probably caused in part by Cr^{+6}-induced M-Hb formation and a low degree of hypoxia.

The large variety of physiological effects reported here and the known general toxicant properties of Cr^{+6} support

the conclusion that Cr^{+6} acted on many tissues of coho salmon to cause non-specific damage and a general stress response. These general effects, however, were not sufficiently severe during the FW exposure periods to cause mortality or blood changes beyond the range reported elsewhere for control coho salmon. The detrimental effects of short-term sublethal Cr^{+6} exposure in FW became apparent only after treated fish were subjected to the additional stressors of SW transfer or bacterial infection. These effects on osmoregulation and the immune response occurred, furthermore, at Cr^{+6} concentrations similar to those which required, in other studies, a much longer exposure period to elicit chronic effects on FW survival.

Because many other water pollutants may have similar stressful effects on fishes, measurement of salinity tolerance, disease resistance, and blood changes may be useful as sensitive indicators of sublethal toxicity in aquatic bioassays, requiring less time and expense than those chronic bioassays which measure other ecologically significant factors as growth, reproduction, and long-term survival.

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