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Effects of Intermittent Chlorination on Plasma Proteins of Rainbow Trout (*Salmo gairdneri*)

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The increased total residual chlorine concentration in receiving water has become a major problem. Many of the residuals are the result of power plant chlorination. These plants require enormous quantities of water to remove waste heat. Screening intake water is insufficient to remove large amounts of bacteria and blue-green algae, and resultant growth on piping walls decreases heat transfer and reduces generating efficiency. For this reason, 0.7 to 3.0 mgL^{-1} chlorine is added to maintain a total residual chlorine concentration of 0.5 to 1.0 mgL^{-1} (DICKSON et al. 1977).

Before return to receiving bodies, the now chlorinated water must be cooled, and during the cooling process chlorine is concentrated by evaporation (BASS 1975). Chlorinated water is then continuously or intermittently released to the aquatic environment. This release poses a toxic threat to non-target organisms, such as fish and invertebrates, in aquatic ecosystems. Proposed solutions to chlorine removal have proven ineffective in protecting more sensitive fish species, e.g. trout. Indeed, rainbow trout (Salmo gairdneri) have been widely used in toxicity studies due to their known sensitivity.

Until recently, chlorinated water was continuously released from cooling towers, and most of the literature concerning the impact on trout has dealt with the effects of continuous exposure (BASS & HEATH 1977). The recent shift from continuous to intermittent chlorination has resulted in problems, due to lack of information concerning impact on ecosystems.

It has been suggested that the establishment of certain baselines for normal hematological ranges could be used to diagnose disease and physiological changes induced by toxicants (BARNHART 1969). The components of fish blood most extensively studied are plasma and serum proteins. Since these proteins are responsible for such things as osmotic pressure maintenance, pH stabilization, viscosity control, and material transport, they may be the most useful parameters in impact studies (SPRAGUE 1971).

PERRIER et al. (1979) electrophoretically separated trout plasma proteins into 13 bands. LAUVER (1977) noted apparent protein profile alterations in fish subjected to chlorine pulses, such as those encountered downstream from a power plant. In the expanded study, reported here, the protein banding pattern changed significantly.

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METHODS AND MATERIALS

All experiments were conducted at 14±1°C, using a laboratory system designed by SELLERS et al. (1975). Each fish was anesthetized with a solution of 100 mgL^{-1} MS-222 and catheterized via the dorsal aorta with Clay Adams PE 50 Intramedic tubing using a procedure modified after HOUSTON (1971).

Two experimental and one control fish were placed, head upstream, in plexiglas holding chambers (60 x 10 x 13 cm) and allowed to acclimate for 4 to 6 h, while receiving 250 mL min^{-1} dechlorinated water. During the test period, the experimental chambers were supplied with chlorinated water, while the control chamber continued receiving dechlorinated water.

Chlorine from a stock solution (prepared from $Ca(OCl)_2$) was delivered to the toxicant pool by a peristaltic pump, where it was diluted to achieve total residual chlorine concentrations in the experimental chambers of 0.11 mgL^{-1} ±0.005 S.E. and 0.22 mgL^{-1} ±0.006 S.E. After a 2 h exposure, the pump was stopped, and the experimental chambers were supplied with dechlorinated water during the remaining 6 h of the test. A total of 12 chlorine treated (experimental) and 6 untreated (control) fish were tested at each concentration.

Following acclimation, but prior to chlorine exposure, a 50 µL blood sample was obtained from all 3 fish for use in establishing baseline plasma protein banding patterns. Samples were collected in 70 µL heparinized capillary tubes, centrifuged for 5 min and stored at 4°C.

During chlorine exposure, blood samples were obtained at 30, 60, 90, and 120 min from the two experimental fish, and at 60 and 120 min from the control. During the remaining 6 h of each experiment, samples were taken from all fish at 1 h intervals.

The plasma proteins were separated utilizing disc gel electrophoresis modified from DAVIS (1964). The samples used in electrophoresis contained 66.67 µg protein as determined by PERRIER et al. (1973). Following polymerization, the stacking gel was overlayed with upper buffer, and the plasma sample was placed on top of the stacking gel. All samples were then subjected to electrophoresis for 3 h at 3 ma $tube^{-1}$ at 5°C.

Following electrophoresis, the proteins were fixed in the gel with 5.0% trichloroacetic acid and 10.0% acetic acid for 8 h. The gels were then stained with 1.0% fast green in 10.0% acetic acid for 4 h (GOROVSKY et al. 1970). Gels were destained by diffusion for 48 h in 10% acetic acid in an EC destainer modified to hold gels, with only the pump operating.

Absorbency of each protein band was continuously recorded with a Gelman DCD-16 densitometer at 1.0 O.D. and 580 nm. The identity of each band was determined by comparison to those described by PERRIER et al. (1973). The percentage of each protein band in relation to the total protein content of the gel was ascertained by measuring the area under each densitometric peak with a polar planimeter. The absolute amount of protein in each band was calculated from the percentage, assuming that each gel contained a total of 66.67 µg plasma protein. Analysis of

variance was applied to the values obtained for each protein using an ANVAR 4 program.

RESULTS

Fish exposed to 0.11 mgL^{-1} total residual chlorine coughed, and coughing frequency increased during the first hour of exposure. Coughing frequency was greater in fish exposed to 0.22 mgL^{-1} chlorine. All but one experimental fish hemorrhaged subcutaneously along the back and tail, and the severity of hemorrhaging was directly related to chlorine concentration. Ventilation rates appeared to decrease with increased chlorine exposure time. Blood samples became very dark and viscous over the 8 h test period, making sampling difficult after 6 h. All fish exposed to 0.22 mgL^{-1} total residual chlorine died within 2 h following termination of the experiment.

The electrophoretic protein banding pattern of experimental fish prior to testing (Hour 0) was basically the same as that of the controls throughout the experiment (FIG. 1, H 0). Although the mobility of bands 9-12 in the control fish changed slightly during the experiment, the number of bands and protein quantity remained constant.

No variation was noted in protein bands 1-6 and 13 in individuals subjected to 0.11 mgL^{-1} chlorine; protein band 2 split into 2 bands in some individuals (FIG. 1). Protein bands 7-12 varied in all experimental fish, regardless of treatment. In fish exposed to 0.11 mgL^{-1} total residual chlorine, mobilities of protein bands 1-10 remained fairly constant, while protein bands 11 and 12 varied over the test period. In all chlorine-exposed fish, total protein appeared to increase as indicated by increased area under the densitometric profiles. This was especially noticeable in bands 2, 4, and 6.

Protein bands 1-6 and 13 demonstrated little variability in all fish prior to exposure to 0.22 mgL^{-1} total residual chlorine (FIG. 2). In contrast, proteins 7-12, and especially protein bands 10 and 12, varied considerably. All fish exposed to 0.22 mgL^{-1} demonstrated alterations in the mobilities of several bands. Protein band 12 disappeared in all experimental fish (FIG. 2, arrow), band 10 was absent in most fish (FIG. 2, arrow), and band 7 was apparent in approximately one-half the fish (deletion not illustrated). Protein bands 1-6 remained constant in all fish tested.

The protein concentration for each band from all fish tested with 0.11 and 0.22 mgL^{-1} chlorine was pooled and analyzed, using the ANVAR 4 program, to determine: 1) if a significant difference existed in protein concentration in each band between control and experimental fish, 2) if a significant difference existed between control and experimental fish over time, and 3) if a significant difference existed in the interaction of chlorine concentration and time between control and experimental fish.

Protein concentration in bands 1, 2, 4, 8, 9, 11, and 13 in fish treated with 0.11 mgL^{-1} chlorine was not significantly different from those fish treated with 0.22 mgL^{-1} chlorine

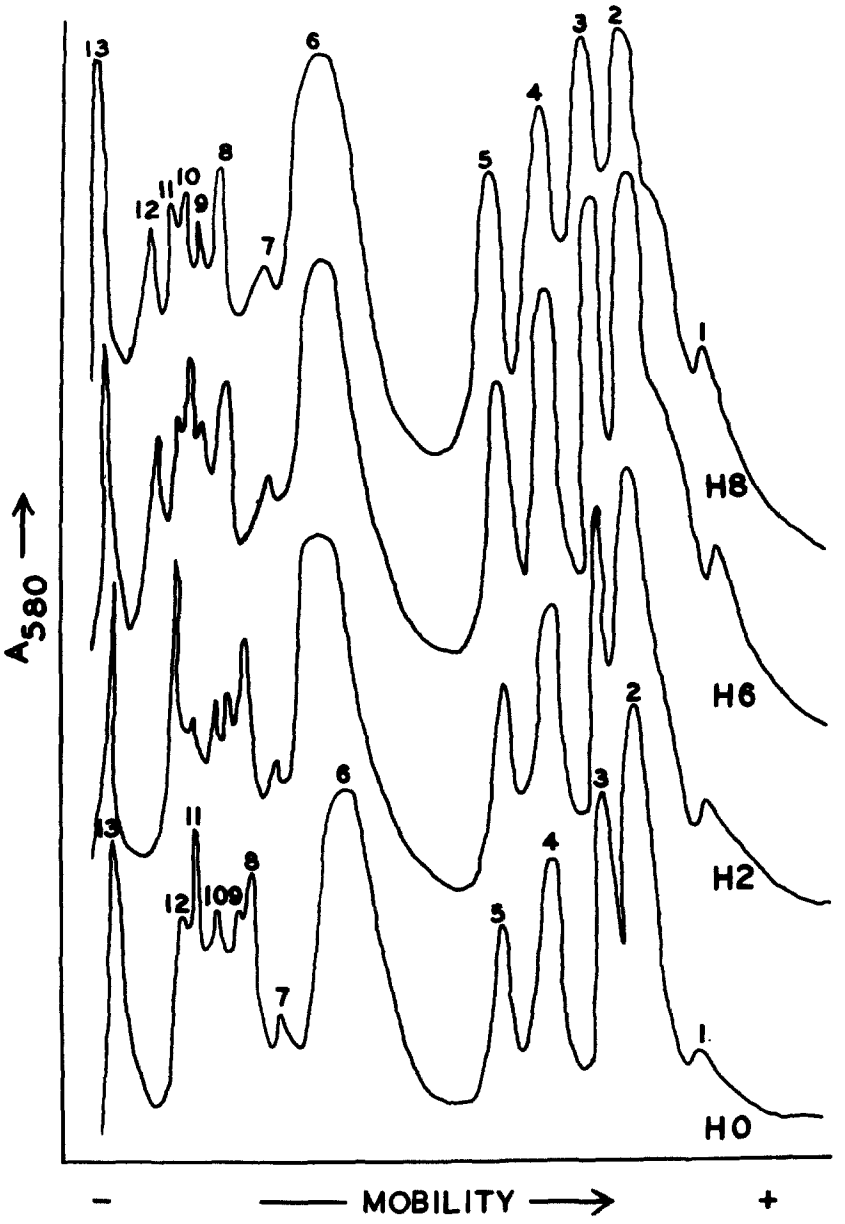

FIGURE 1. Representative densitometric plasma protein profile of experimental fish exposed to 0.11 mgL^{-1} chlorine. H0 = sample taken prior to chlorine exposure. H2-H8 = samples taken 2-8 h after 2 h chlorine pulse.

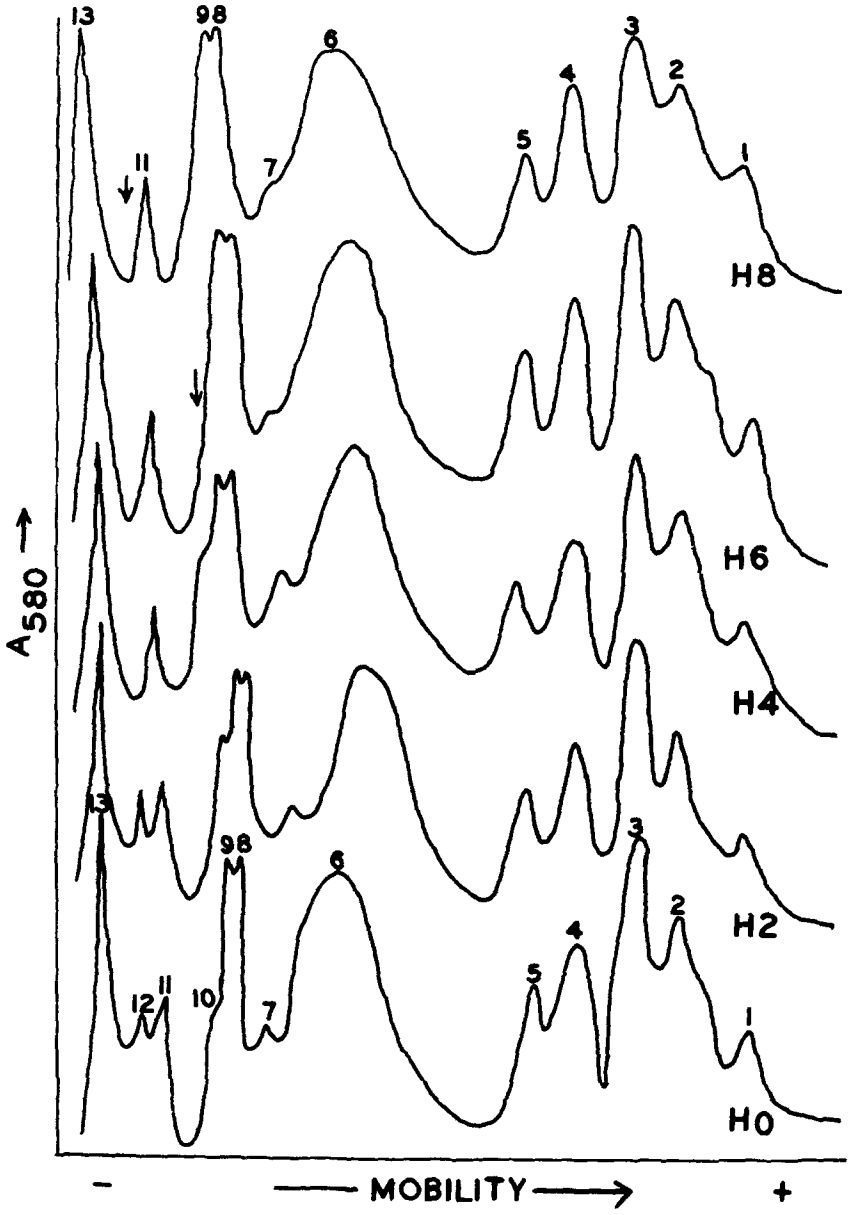

FIGURE 2. Representative densitometric plasma protein profile of fish exposed to 0.22 mgL^{-1} chlorine. H0 = sample taken prior to chlorine exposure. H2-H8 = samples taken 2-8 h after a 2 h chlorine pulse. Arrows indicate band disappearance.

(P>0.05). There was a significant difference at the 0.01 level of probability in proteins 3, 5, 6, 7, 10, and 12. When the data from the experimental and control fish were pooled, there was a significant difference in protein concentration at the 0.01 level of probability in all but band 11. More importantly, when the data from the experimental and control fish were pooled, there was a significant difference at the 0.01 level of probability over the 8 h test period in band 11. There was also a significant difference at the 0.05 level of probability in band 12 over the testing period. Protein 7 differed significantly at the 0.10 level of probability over the test period.

DISCUSSION

Prior to chlorine exposure, 13 major protein bands were ascertained in all experimental and control fish. Utilizing bands 6 and 13 as markers, all protein bands reported by PERRIER et al. (1973) could be located.

The observations made during the study appear to substantiate previous findings regarding chlorine toxicity in trout. The deletion of bands 7, 10, and 12, indicates that chlorine exposure altered blood protein composition, since no alteration was noted in control fish. The greatest effect occurred with 0.22 mgL^{-1} total residual chlorine, which appears to be the rainbow trout's lethal limit.

Exposure to 0.22 mgL^{-1} chlorine resulted in several responses previously reported. The occurrence of coughing agreed with findings by BASS (1975). Coughing was accompanied by movement of fish to the surface of the water in test chambers, and gulping for air indicated apparent hypoxia. Since water supplied to the chambers was fully aerated, it appears that chlorine exposure reduced respiratory gas exchange. Darker, more viscous blood appeared within 4 h following onset of chlorine exposure. ZEITOUN (1977) attributed darker blood to increased methemoglobin concentrations, while increased viscosity was thought to be the result of increased plasma protein concentrations.

The electrophoresis data support the theory of gas exchange alteration. Protein 7 was identified by PERRIER et al. (1974) as ceruloplasmin, a protein known to have oxidase activity and a role in copper metabolism (SCHULTZE & HEREMANS 1966). Although copper is not a hemoglobin constituent, it is necessary in hemoglobin formation. The deletion of protein 7 in fish exposed to 0.22 mgL^{-1} chlorine may indicate altered hemoglobin formation, which would inhibit oxygen transport. Since there is always a small methemoglobin percentage in erythrocytes, there are methemoglobin reductases, which maintain normal hemoglobin concentrations (HARRIS 1963). Loss of the ceruloplasmin fraction may prevent the reduction of methemoglobin to hemoglobin. This would result in increased methemoglobin and decreased oxygen carrying capacity.

In addition to alteration of the ceruloplasmin fraction, there was a general increase in proteins 1-6 in fish exposed to

0.11 mgL^{-1} chlorine. There was no significant difference in proteins 1-6 as a function of time, possibly because total protein concentration remained elevated for the duration of the experiment. Band 4 was reported to be transferrin (PERRIER et al. 1977), a protein responsible for iron transport. Since iron is a major hemoglobin component, there may be a link between this protein increase and the oxidation of hemoglobin. Accelerating hemoglobin production could represent the animal's response to increased methemoglobin concentrations. Assuming bands 4 and 7 are transferrin and ceruloplasmin, respectively, chlorine's effect on these proteins may in part explain previous findings of hypoxia in chlorine-exposed fish.

Protein bands 2 and 3, identified as albumins, which function in oncotic pressure regulation (HARROW & MAZUR 1966), also increased markedly. An increase in these bands may represent altered oncotic pressure regulation. The observed albumin increase may be a homeostatic mechanism to compensate for this osmotic pressure aberration. Alternatively, hemoglobin, released by chlorine-induced erythrocyte hemolysis, may have co-electrophoresed with one or both bands, resulting in an apparent increase.

Since the liver is the synthesis site for ceruloplasmin, transferrin, and albumin, any change in these fractions is probably tied to hepatic damage. Liver damage has been reported to result in decrease or total inhibition of protein synthesis (HESS & HENLEY 1963). BASS (1975) confirmed hepatic injury in rainbow trout exposed to intermittent chlorination.

Of the proteins originating in the liver, fibrinogen has been shown to be continuously produced, due to high turnover rate and utilization in clotting (SCHULTZE & HEREMANS 1966). PERRIER et al. (1974) identified protein 12 as fibrinogen. The severe hemorrhaging noted in fish exposed to 0.22 mgL^{-1} chlorine was closely correlated with the alteration of the fibrinogen fraction, which was deleted from the densitometric profile.

These data indicate that chlorine does not act on one specific parameter, but rather on several targets resulting in a cumulative toxic effect. This work has confirmed that rainbow trout protein banding patterns should play an important role in toxicant studies. It is hoped that the current study will stimulate research utilizing blood proteins as indicators of environmental alteration.

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