Promotor: dr. J.H. Koeman, hoogleraar in de toxicologie

An assessment of the environmental toxicity of hexavalent chromium in fish

Iksan van der Putte

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PROEFSCHRIFT
ter verkrijging van de graad van
doctor in de landbouwwetenschappen
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
hoogleraar in de veeteeltwetenschappen,
in het openbaar te verdedigen
op woensdag 21 oktober 1981
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISN: 1465 90 - 03

Stellingen

1. Een adequate beoordeling van de toxicologische risico's van chroom in het Nederlandse oppervlaktewater wordt bemoeilijkt door het ontbreken van relevante informatie over de chemische vorm waarin het metaal voorkomt.

Dit proefschrift.

2. De invloed van de zuurgraad op de acute toxiciteit van zeswaardig chroom voor de regenboogforel is voorspelbaar op basis van een strikt additieve toxiciteit van hydrochromaat- en chromaat-ionen.

Dit proefschrift.

3. De door Payan en Matty ontwikkelde kieuwperfusietechniek biedt een goede mogelijkheid om na te gaan welke in oppervlaktewater aanwezige metaal-ionen-species beschikbaar zijn voor opname door vissen.

P. Payan and A.J. Matty, J. comp. Physiol. 96 (1975) 167-184.
Dit proefschrift.

4. De conclusie van Schubert e.a. dat de mutageniteit van zeswaardig chroom is terug te voeren op het intracellulair gevormde driewaardig chroom is voorbarig gezien de bevindingen van Polnaszek.

J. Schubert, F.J. Teslik, S.K. Wiederhold and J.M. Gentile,
Environ. mutagenesis 1 (1979) 164.
C.F. Polnaszek, Fed. Proc. 40 (1981) 715.

5. De classificatie van sommige kieuwepitheelcellen op basis van hun cytoplasmatische dichtheid in "dark cells" en "dense cells" wijst op de noodzaak van voortgaand onderzoek naar celdifferentiatie processen in dit weefsel.

C.M. Morrison, J. Fish Biol. 15 (1979) 601-605.
M. Morgan and P.W.A. Tovell, Z. Zellforsch. 142 (1973) 147-167.

6. Bij de bestudering van het respiratiegedrag van vissen in continu doorstroomde systemen dient het stromingspatroon te worden gekarakteriseerd.

7. De door Marking beschreven discrepantie tussen de toxiciteits- en dissociatiecurve van antimycine dient primair te worden toegeschreven aan de chemische degradatie van antimycine in alkalisch milieu en houdt in mindere mate verband met een foutieve bepaling van de dissociatieconstante.

L.L. Marking, J. Fish. Res. Board Can. 32 (1975) 769-773.

8. De voorschriften die op het ogenblik tot stand komen op het gebied van toxiciteitsonderzoek zijn teveel gericht op een routinematige screening waardoor het gevaar ontstaat dat onvoldoende inzicht wordt verkregen in werkingsmechanismen, welke noodzakelijk is voor een goede risico-evaluatie.

Commissie Toelating Bestrijdingsmiddelen. Aanvraag tot toelating bestrijdingsmiddelen, onderdeel E. Jan. 1981.
OECD-guideline for testing chemicals. Feb. 1981.

9. Het ware wenselijk de mogelijkheid te hebben evenals bij straling het geval is, de toegevoegde risico's van mutagenen en carcinogenen te relateren aan de "natuurlijke" achtergrond.

10. Gezien het toenemende multiculturele karakter van de Nederlandse samenleving en de geringe kans op terugkeer van etnische minderheden naar hun land van herkomst, verdient intercultureel onderwijs de prioriteit boven bicultureel onderwijs.

11. In het kader van interlandelijke adoptie heeft de overheid van ontvangende landen de plicht op effectieve wijze te voorkomen dat aspirant adoptiefouders een kind dat op illegale wijze is verworven, gaan verzorgen en adopteren.

12. Deeltijdarbeid is niet altijd gedeelde arbeid.

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An assessment of the environmental toxicity of hexavalent chromium in fish
Wageningen, 21 oktober 1981

There are a number of levels of biological integration and each level finds its explanation of mechanisms in the levels below, and its significance in the levels above.

G. A. Bartholomew

Aan Gerja en Pepijn

Voorwoord

Aan de totstandkoming van dit proefschrift hebben vele personen een bijdrage geleverd.

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Contents

General introduction

Occurrence of chromium in the aquatic environment

At present chromium is a common contaminant in surface waters in many countries. Various sources contribute to the contamination. These include atmospheric emissions from industry and households and runoff from agriculture. However, the most important source is the waste water from industrial and domestic origin fed directly into the aquatic system (Förstner and Wittmann, 1979; NRCC, 1976). In the Netherlands the amounts of chromium introduced by the rivers Rhine and Meuse constitute the major sources of chromium pollution of surface waters. An additional amount is discharged by industries (Table I).

TABLE I

Estimated amounts of chromium (tons/year) discharged in the Netherlands. Source: IMP (1975; 1981).

Year	Total discharge	Industrial discharge	Released into surface waters	Introduced by Rhine and Meuse
1973	---	---	---	2000 - 3000
1975	400	390	350	---
1978	---	---	---	2600
1980	150	140	110	---
1985	120[a]	110[a]	100[a]	---

[a] Prognosis

Since its discovery by the French chemist Vauquelin in 1797, world's usage of chromium has increased up to 17.10^5 tons/year (Förstner and Wittmann, 1979) and the element is now the most ubiquitous of all metals used in industrial activities (Dean et al., 1972). Chromium salts are used extensively in the metal finishing industry (electroplating), the leather industry (tanning), the textile industry (mordants) and in catalytic manufacture.

They are also present in cooling waters, pigments and primer paints, and in fungicides and wood preservatives. A survey of various industries discharging chromium waste water in the Netherlands is given in Table II. It is obvious that the total industrial discharge has been reduced markedly in the period between 1975 and 1980. In the next period of five years (1980-1985) the reduction in chromium discharge is predicted to be much smaller, although continuing progress is being made in reducing or recycling the waste water in certain industries e.g. the leather (IMP, 1981) and metal finishing industry (Eversteijn and Van Deursen, 1979).

TABLE II

Estimated amounts of chromium (tons/year) discharged by various industries in the Netherlands. Source: IMP (1981).

Industry	1975	1980	1985[a]
Textile	3.6	1.4	1.4
Leather tanning	36	36	11
Photography and printing	2.0	1.4	1.2
Fertilizers	20	15	12
Chemical raw material	70	56	55
Chemical products	110	7.6	6.2
Basic steel works	20	6.0	6.0
Basic non-ferrous metal works	80	--	--
Metal products	48	18	12
Others	1.2	1.1	1.1
Total	390	140	110

[a] Prognosis

In Dutch surface waters total chromium concentrations have been reported to range from 9 to 88 µg/l (Strik et al., 1975). Sometimes local values of 240 µg/l (RIZA, 1978, in correspondence) to 1000 µg/l (Strik et al., 1975) are found.

Values for total and dissolved chromium concentrations at some sampling locations in 1980 are given in Table III.
Kopp and Kroner (1969) found dissolved chromium in 24.5% of the water samples taken from rivers and lakes in the United States; the mean concentration was 9.7 µg/l, and the range was 1 to 121 µg/l. Natural background levels are estimated to be <1 µg/l (NRCC, 1976; Förstner and Wittmann, 1979).

TABLE III
Total and dissolved chromium concentrations in Dutch surface waters at some sampling locations in 1980. After Rijkswaterstaat (1980).

	Total chromium concn. µg/l Cr		Dissolved chromium concn. µg/l Cr	
Location	mean	range	mean	range
Rhine - (Lobith)	20	7 - 36	5	0 - 13
IJssel - (Kampen)	15	9 - 26	4	0 - 6
Meuse - (Eysden)	10	5 - 38	2	0 - 6
West Scheldt- (Schaar Van Ouden Doel)	20	8 - 28	2	0 - 5

Aquatic chemistry of chromium

The most important oxidation states, in which chromium is used by industries and in which it is present in surface waters are the trivalent and hexavalent states. These two oxidation states are the most stable forms of the element, that may have oxidation states ranging from -2 to +6 (Cotton and Wilkinson, 1972).

Trivalent chromium, Cr(III), is a positively charged ion with a strong tendency to form stable complexes with negatively charged organic or inorganic species. Hence it is unlikely that appreciable quantities of uncomplexed Cr(III) will be found in water containing any anionic dissolved or particulate matter (e.g. decaying plant or silt or clay particles). Even if no anionic spe-

cies are present, Cr(III) can react with water itself to form colloidal hydrous oxides (U.S. EPA, 1978; Cotton and Wilkinson, 1972; NRCC, 1976).

Hexavalent chromium, Cr(VI), in aqueous solution almost exclusively exists in the form of the oxo-anions chromate (CrO_4^{2-}), hydrochromate ($HCrO_4^-$) and dichromate ($Cr_2O_7^{2-}$). The proportion of Cr(VI) present in each of these forms depends on pH. In strongly basic and neutral solutions the chromate form predominates. As pH is lowered, the hydrochromate concentration increases. At very low pH the dichromate species predominates. In the pH ranges encountered in natural waters the proportion of dichromate ions is relatively low. In the acid portion of the environmental range, the predominant form is hydrochromate ion (65% at pH 6.0 to 6.2); in the alkaline portion of the range, the predominant form is chromate ion (96% at pH 8.5 to 7.8) (Trama and Benoit, 1960). As Cr(VI) species are negatively charged, they do not complex with anionic particulate matter. Hence Cr(VI) is more mobile than Cr(III) which is largely associated with particulate matter and is subject to sedimentation or filtration (NRCC, 1976). Cr(VI) is a powerful oxidizing agent especially in acidic solutions. The tendency is strong to react with reducing agents to form Cr(III). Nevertheless, when the concentration of reducing agents is low hexavalent chromium is stable in natural waters and will persist for a long period of time.

Aspects of chromium toxicity in fish and other aquatic organisms

Mean acute toxicity values of Cr(VI) for various species of aquatic organisms are given in Table IV. The species mean acute values for five of the six invertebrate species are less than that of any fish species, showing that invertebrate species are generally more sensitive for Cr(VI) than fish species. For Cr(III) both the most sensitive and the least sensitive species are invertebrates (U.S. EPA, 1980).

When fish are irritated by toxic levels of Cr(III), they secrete large amounts of mucus which complexes with the metal ion and thus reduces the ionic diffusion through the skin (Carpenter, 1927). The main toxic action of Cr(III) is presumed to arise from the coagulation or cross-linking of the mucus secreted by the gills, or from direct damage to the gill tissue which in turn interferes with respiratory function, resulting in death by suffocation (Doudoroff and Katz, 1953). Olson (1958) exposed a large number of chinook salmon fingerlings to low levels of Cr(III) and Cr(VI) for 12 weeks. Under the test conditions (water hardness 70 mg/l, pH 7.7 - 8.0, tempera-

ture 8 - 16 °C) 0.2 mg/l Cr(III) did not increase the mortality over controls (0.8% mortality), whereas 0.2 mg/l Cr(VI) caused a 53% mortality.

TABLE IV

Mean acute toxicity values for hexavalent chromium in various species. Figures represent mean 96-h LC50 values (a); mean 48-h EC50 values (b), and mean 48-h - 96-h LC50 values (c). Sources: U.S. EPA (1980) and Federal Register (1980).

Species	Mean acute toxicity value (µg/l CrVI)
Largemouth bass (Micropterus salmoides)	195,000 (a)
Bluegill (Lepomis macrochirus)	134,000 (a)
Goldfish (Carassius auratus)	120,000 (a)
Rainbow trout (Salmo gairdneri)	69,000 (a)
Midge (Tanytarsus dissimilis)	59,900 (a)
Brook trout (Salvelinus fontinalis)	59,000 (a)
Fathead minnow (Pimephales promelas)	43,100 (a)
Striped bass (Morone saxatilis)	30,400 (a)
Guppy (Poecilia reticulata)	30,000 (a)
Snail (Physa heterostropha)	25,000 (a)
Rotifer (Philodina roseola)	6,800 (b)
Cladoceran (Daphnia magna)	6,400 (b)
Rotifer (Philodina acuticornis)	3,100 (b)
Scud (Gammarus pseudolimneus)	67 (c)

General symptoms of Cr(VI) poisoning in fish are decreased food intake, lack of activity and severe alterations in internal organs. It is somewhat uncertain whether Cr(VI) also causes damage to the gil tissue; This was found by Strik et al. (1975) in rainbow trout but not by Frómm and Schiffman (1958) in largemouth bass acutely exposed to the metal.

Knoll and Fromm (1960) studied the accumulation and elimination of Cr(VI) by rainbow trout. They showed that the gills function as the major route of uptake of the metal and that Cr levels in the blood were always lower than

those in the surrounding water. They concluded that Cr(VI) probably entered through the gills by passive diffusion. Kidney, liver and intestinal tract accumulated the metal in considerable amounts. After exposure stopped, chromium was eliminated rapidly from the various tissues except from spleen and kidney.

Various environmental factors may influence the toxicity of metal ions to aquatic organisms and especially water characteristics such as hardness and pH have been considered of primary importance. Trama and Benoit (1960) suggested that a change in pH alters the distribution of Cr(VI)-species in water and therefore influences the availability and toxicity of Cr(VI) to fish. However, no studies have been made to test this hypothesis. A review of the toxic effects of chromium (U.S. EPA, 1980) indicates that hardness has an insignificant influence on the toxicity of Cr(VI) in fresh water in contrast with the toxicity of Cr(III). Furthermore the data indicate that Cr(VI) is far more toxic than Cr(III) to aquatic organisms. Finally it can be mentioned that Cr(III) is an essential trace element (Mertz, 1969), and therefore should not be considered as a xenobiotic compound. These findings are also reflected in the water quality criteria given by U.S. EPA (1980):
1) For total recoverable Cr(VI) the criterion to protect freshwater aquatic life is 0.29 µg/l as a 24-h average and the concentration should not exceed 21 µg/l at any time; 2) For freshwater aquatic life the concentration (in µg/l) of total recoverable Cr(III) should not exceed the numerical value given by $e^{(1.08\ (\ln \text{hardness}) + 3.48)}$ at any time. For example at hardnesses of 50, 100, and 200 mg/l as $CaCO_3$, the concentration of total recoverable Cr(III) should not exceed 2,200, 4,700 and 9,900 g/l respectively.
In the Netherlands no specific water quality criteria for chromium and aquatic organisms are given yet. A preliminary quality objective of 50 µg/l based on total chromium as a maximum permissible concentration has been recommended (IMP, 1975; 1981).

Objectives of the present study

The aim of the investigations presented in this thesis was to make an in-depth study of the toxicology of Cr(VI) in fish. Experiments were carried out at different pH values because a change in pH may alter the distribution of Cr(VI)-species in water and therefore at the same time its availability and toxicity to fish. Another reason was that hexavalent chromium is also a strong oxidizing agent and its oxidizing action is dependent on pH. As hard-

ness has little effect on Cr(VI) toxicity, this parameter was not considered in detail in the present study.
Rainbow trout (Salmo gairdneri) was used as the test-species. Appropriate radio-tracer techniques, including autoradiography, were employed to ascertain how and where the metal is concentrated within the animal. The oxidizing action was studied by electron spin resonance (ESR) and the distribution of Cr(VI)-species in water was assessed by equilibrium calculations. Hematological, histological and biochemical techniques were used to obtain qualitative data on the toxic action of Cr(VI).

Chapter 1 reports on the effect of pH on uptake, tissue distribution and retention of Cr(VI) in trout. The effect of pH on the acute toxicity of Cr(VI) and the role of different Cr(VI)-species are dealt with in chapter 2. Besides their respiratory function, the gills play an important role in osmoregulation. They are the main "porte d'entrée" for dissolved chemical compounds, including Cr(VI). Experiments were conducted to study the transfer of oxygen and chromium in isolated perfused gills of trout exposed to Cr(VI) at different pH values. Results of these experiments are presented in chapter 3. Chapter 4 describes the effects of Cr(VI) on respiration and osmoregulation at different pH values. The last chapter deals with the toxic effects of Cr(VI) after prolonged exposure to the metal.

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Chapter 1

EFFECT OF pH ON UPTAKE, TISSUE DISTRIBUTION AND RETENTION OF HEXAVALENT CHROMIUM IN RAINBOW TROUT (SALMO GAIRDNERI)[a]

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SUMMARY

Uptake, distribution and retention of chromium in rainbow trout (Salmo gairdneri) was studied after short-term (2-4 days) exposure to $^{51}CrO_4^{2-}$-containing Na_2CrO_4 solutions of different concentrations (2-50 mg/l Cr) and pH (7.8 and 6.5). At pH 7.8, highest contents of chromium were found in gill, liver, kidney, and digestive tract of the trout. Chromium was not distributed evenly among the different subcellular fractions of the tissues, but was concentrated in the nuclear fraction of the gill tissue and in the soluble fraction of the kidney and liver tissue. Upon transfer of exposed fish to tap-water, chromium was rapidly eliminated from blood, gill and digestive tract. However, chromium contents tended to remain high in kidney and liver. When the pH was decreased from 7.8 to 6.5, the lethal action of hexavalent chromium increased and a different pattern of accumulation and elimination of chromium was observed. The major differences were found in the gills, which concentrated significantly more chromium at pH 6.5 than at pH 7.8, irrespective of the exposure time and concentration. As an electron-spin-resonance signal characteristic for trivalent or pentavalent chromium was detected in the gills, the differences must have been at least partly due to the higher oxidizing action of hexavalent chromium at the lower pH.

Key words: chromium; pH; rainbow trout; uptake; distribution; retention

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[a] Aquat. Toxicol. 1(1981), 3-18

INTRODUCTION

When fish are exposed to hexavalent chromium, Cr(VI), or other heavy metals dissolved in the water, the gills function as the major route for uptake of these compounds (Knoll and Fromm, 1960; Olson et al., 1973). The gill tissue rapidly accumulates most heavy metals in such a manner that its content, at least initially, far exceeds that in other tissues. This has been shown for copper (Sellers et al., 1975), zinc (Matthiesen and Brafield, 1973, 1977), cadmium (Sangalang and Freeman, 1979) and mercury (Olson et al., 1973; Lock, 1979). The rapid accumulation in gill tissue is usually accompanied by deleterious effects to gill structure and may therefore interfere with its respiratory and osmoregulatory function. Indeed, many workers have attributed acute heavy-metal toxicity in fish to the impairment of one of these main functions of the gills (Skidmore, 1970; Lewis and Lewis, 1971; Burton et al., 1972).

However, it has been indicated that Cr(VI) behaves toxicologically in a manner quite different from most heavy metals (Doudoroff and Katz, 1953). In aqueous solution, Cr(VI) almost exclusively exists in the form of oxoanions (CrO_4^{2-}, $HCrO_4^-$, $Cr_2O_7^{2-}$), which have been observed to pass readily through the gill membrane and to accumulate in various tissues and organs (Knoll and Fromm, 1960). Thus the hexavalent metal could elicit its toxic effect at some internal site. Direct evidence of an internal site for Cr(VI) toxicity has been reported by Fromm and Schiffman (1958), who observed severe pathological changes in the intestines immediately behind the pyloric caeca in largemouth bass exposed to Cr(VI) at 96 mg/l. Also Kuhnert et al. (1976) showed that the Na^+, K^+-ATPase activity of kidney and intestine, but not of gill, significantly decreased in rainbow trout exposed to 2.5 mg/l. However in most of the studies in which the effects of Cr(VI) on fish have been investigated, the pH of the exposure solutions was not carefully controlled.

Trama and Benoit (1960) suggested that a change in pH alters the distribution of the Cr(VI)-species and therefore influences the availability of Cr (VI) to fish. Hexavalent chromium is also a strong oxidizing agent and its oxidizing action is dependent on pH (Cotton and Wilkinson, 1972). Thus the pH of water probably plays a major role in the toxicity of Cr(VI) in fish.

In this study an attempt was made to obtain additional information on the mode of action of Cr(VI). The aim was to learn more about the sites of chromium accumulation and retention in rainbow trout during and after exposure

to Cr(VI) at different pH values. Three series of experiments were conducted, concerned with the tissue and subcellular distribution of chromium and the oxidizing action of Cr(VI), assessed with electron spin resonance (ESR); the relation between lethality and uptake of Cr(VI); and the dynamics of uptake and elimination of chromium, respectively.

MATERIALS AND METHODS

Fish and water characteristics

Yearling and fingerling rainbow trout (Salmo gairdneri), cultured in the laboratory from a stock originally obtained from a commercial hatchery, were reared in 300-l fibreglass tanks receiving a continuous supply of tap-water. The chemical characteristics of the tap-water are presented in Table I.

TABLE I
Chemical characteristics of the tap-water used as dilution water for all experimental work.

Characteristic	Unit	Mean	Range
Ammonia	mg/l NH_4^+	< 0.01	-
Nitrite	mg/l NO_2^-	< 0.01	-
Nitrate	mg/l NO_3^-	0.2	0.1 - 0.4
Sodium	mg/l Na^+	5.0	4.5 - 5.2
Potassium	mg/l K^+	0.6	0.5 - 0.6
Chloride	mg/l Cl^-	6.1	6.0 - 6.5
Dissolved oxygen	mg/l O_2	8.5	7.6 - 10.5
Free carbon dioxide	mg/l CO_2	1.5	1.0 - 3.0
pH	-	7.80	7.75 - 7.90
Alkalinity	mg/l HCO_3^-	92.0	88.0 - 98.0
Total hardness	mg/l $CaCO_3$	80.0	77.5 - 82.5
Calcium	mg/l Ca^{2+}	28.4	28.0 - 28.5
Magnesium	mg/l Mg^{2+}	2.2	2.1 - 2.4

Chemical analysis was by methods of APHA et al. (1975). Tap-water was used in the experiments at its original pH of 7.8 and at a decreased pH of 6.5, maintained within 0.1 of the required pH with an automatic pH-stat, which added dilute NaOH or HCl to the tap-water in reservoirs of 300 l. Vigorous aeration mixed the water thoroughly and removed excess CO_2 released by lowering of pH. Acidification and subsequent aeration increased the mean concentration of Cl^- in tap-water from its original value of 6.1 to 62.0 mg/l; the mean alkalinity value decreased from 92.0 to 4.0 mg/l HCO_3^-.

Yearling trout used in the first series of experiments had weights of 70 ± 12 g (mean ± SD) and total lengths of 21 ± 1 cm. Fingerling trout used in the second and third series of experiments had weights of 9.5 ± 1.4 g and 5.0 ± 1.5 g and total lengths of 11.2 ± 1.0 cm and 8.0 ± 0.8 cm, respectively. Fish were fed daily with a commercial food (Trouvit) during holding and acclimation at a rate of 20 g/kg body weight per day. From 48 h before and during exposure to Cr(VI), fish were not fed. After transfer to tap-water without chromium, fish were fed at a rate of 10 g/kg body weight per day. Fish were acclimated for 2 wk to the pH of the tap-water and for 48 h to the exposure system. The photoperiod was 12 h light/12 h dark. Water temperature was maintained at 12 °C.

Exposure system and radiotracer techniques

The exposure system consisted of six 25-l glass aquaria housed in a water bath. The exposure solutions were prepared by dissolving Na_2CrO_4 in tap-water and were aerated with an airstone. The $^{51}CrO_4^{2-}$ used as a tracer in the solutions, was obtained from The Radiochemical Centre, Amersham. To ensure that a state of equilibrium was obtained between the tracer and the dissolved Na_2CrO_4, the solutions were prepared at least 24 h before experimental use. The pH was controlled with a pH-stat, which added HCl or NaOH, both of concentrations 0.1 mol/l. The solutions were renewed daily to reduce the build-up of metabolites.

The total weight of the fish held in each aquarium never exceeded 100 g. In this manner, the NH_3 concentration was kept below 0.025 mg/l, a concentration safe for fish (Water Quality Criteria for European Freshwater Fish, 1973).

The ^{51}Cr was estimated in a gamma-ray spectrometer equipped with a well-type NaI (Tl) scintillation crystal installed in a lead chamber. The data were corrected for background and physical decay of the isotope. Tissues

and water samples with volumes up to 20 ml were counted in the well of the crystal; a Perspex container that fitted around the crystal allowed activity measurements of whole live fish. Activity measurements were made after rinsing the live fish for 10 min in tap-water. Organs and tissues were dissected after anaesthetization of the fish with tricaine (MS-222).
Blood samples were collected from the caudal artery.

Autoradiograms of whole fish were made by procedures described by Ullberg (1977). The sections made of radioactive fish had a thickness of 100 µm and were freeze-dried at -20 °C. The sections were pressed against a Structurix D 10 (Agfa-Gevaert) photographic film and exposed for 30 days at 4 °C.

Subcellular fractionation and electron spin resonance (ESR)

In order to prepare the nuclear, mitochondrial, microsomal and soluble fractions, tissues were removed from individual radioactive fish, chilled on ice and homogenized in a Potter Elvehjem homogenizer, in cold (0-4 °C) KCl solution (conc. 154 mmol/l) at a ratio of 1 volume of tissue to 2 volumes of solution. The homogenates were subjected to differential centrifugation at 600 g, 10 000 g and 105 000 g for 10,20 and 60 min, respectively.

Electron spin resonance was used to assess the valence state of chromium in tissues. The reduction of hexavalent chromium to the trivalent form, Cr(III), proceeds via the pentavalent form, Cr(V) (Wiberg and Schäfer, 1969). Due to their paramagnetic properties, Cr(III) and Cr(V) give an ESR signal with a g factor of 1.98 in contrast to the non-paramagnetic Cr(VI) (Gutierrez et al., 1976). The ESR studies were performed on an X-band (9.5 GHz) Varian E-3 spectrometer. Samples of whole tissue and subcellular fractions were studied at about 90 K as frozen cylinders in 3-mm quartz tubes using a Varian variable temperature accessory.
Routine settings of the spectrometer were: microwave power 2 mW, modulation amplitude 1 mT, field set 320 mT. The g factors were obtained using diphenyl picryl hydrazyl (DPPH) at g = 2.0036 as a reference. Tissues and subcellular fractions of unexposed fish and frozen aqueous solutions of $CrCl_3$ and Na_2CrO_4 were used as control samples.

Experimental procedure

In the first series of experiments, yearling rainbow trout were exposed

to Cr(VI) at 40.0 mg/l for 2 and 4 days at pH 6.5 and 7.8 The distribution of chromium in the fish was assessed by measuring the chromium uptake in different tissues. The subcellular distribution and the presence of an ESR signal at g = 1.98 was examined in gill, liver and kidney tissue.

For the second series of experiments, fingerling trout were exposed for 4 days at pH 6.5 and 7.8 to Cr(VI) at concentrations of 2.0, 5.0, 16.5 and 50.0 mg/l, the two higher concentrations being lethal to the fish. Survival and contents of chromium in surviving fish were determined.

In the third series of experiments, fingerling trout were exposed to Cr(VI) at 2.0 and 5.0 mg/l for 4 days with pH 6.5 and 7.8. Samples of 5 fish were removed at 24-h intervals and uptake in whole fish and tissues of the fish was measured. Whole-body autoradiograms were made as a check. The elimination rate of chromium was determined in the fish exposed to Cr(VI) at 2.0 mg/l for 4 days. After exposure, these fish were placed in tap-water of the respective pH and contents in whole fish and in tissues of the fish were determined at intervals over 11 days with samples of 5 fish.

Data were analysed for statistical difference by the Student's t-test.

RESULTS

Tissue and subcellular distribution

In yearling trout exposed to Cr(VI), lowest contents of chromium were found in white muscle and skin (Table II). Gill, liver, kidney and digestive tract contained most. The most remarkable effect of pH was in the gill tissue, which concentrated significantly more chromium at the lower pH, irrespective of exposure time. The substantially higher content in gills at the lower pH was also reflected in the distribution of chromium in the whole fish (Fig. 1). The chromium accumulated in gills accounted for 13 and 21% of the total after exposure for 2 and 4 days, respectively. By contrast, the chromium content of the gills of fish exposed at the higher pH only accounted for 8% of the total after either exposure time.

Chromium was not distributed evenly among the different subcellular fractions but was concentrated in the nuclear fractions of gill tissue and the soluble fractions of the liver and kidney tissue (Table III). Significant differences in subcellular distribution dependent on the exposure pH was evident in all tissues examined after 4 days of exposure. At the lower pH, a greater proportion of the chromium was found in the nuclear fraction of

gill, the microsomal fraction of liver and the soluble fraction of kidney. The proportion in the gill mitochondrial, microsomal and soluble fraction was significantly lower.

TABLE II

Effect of pH and exposure time on content of chromium in tissues (µg/g, wet weight) of yearling rainbow trout exposed to Cr(VI), concentration 40.0 mg/l. Values are means ± SE for 5 fish.

	2 days exposure		4 days exposure	
	pH 6.5	pH 7.8	pH 6.5	pH 7.8
Whole body	4.9 ± 0.5	3.6 ± 0.3[a]	5.9 ± 0.6	4.6 ± 0.5
Gill	67.1 ± 16.9	31.9 ± 4.3[b]	91.3 ± 14.2	27.5 ± 5.8[b]
Liver	24.7 ± 5.8	21.5 ± 2.9	23.3 ± 2.8	35.5 ± 7.5
Kidney	37.4 ± 7.1	34.9 ± 6.6	49.0 ± 7.2	39.0 ± 3.3
Stomach	19.3 ± 2.9	18.5 ± 1.8	17.6 ± 1.0	22.1 ± 3.5
Caeca	30.8 ± 4.5	28.7 ± 3.3	35.4 ± 2.7	20.8 ± 1.7[b]
Intestine	29.6 ± 5.3	36.9 ± 5.1	30.6 ± 6.7	23.5 ± 1.0
Red cells	4.5 ± 0.6	4.4 ± 0.3	6.7 ± 1.1	4.8 ± 0.3
Plasma	6.4 ± 0.7	7.2 ± 1.4	11.3 ± 1.0	9.1 ± 2.4
Heart	4.0 ± 0.5	3.0 ± 0.3	6.0 ± 0.4	5.5 ± 1.0
Brain	1.0 ± 0.2	0.8 ± 0.2	1.3 ± 0.2	0.9 ± 0.1
Bile	7.7 ± 1.7	4.7 ± 1.0	15.5 ± 4.2	11.3 ± 3.9
Opercular bone	6.8 ± 0.6	4.5 ± 0.6	9.2 ± 1.2	7.1 ± 0.3
Vertebral bone	3.2 ± 0.8	2.5 ± 0.2	2.8 ± 0.7	1.8 ± 0.5
White muscle	0.5 ± 0.2	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
Skin	0.8 ± 0.3	0.7 ± 0.2	1.0 ± 0.1	0.7 ± 0.1
Spleen	5.6 ± 0.7	4.2 ± 0.7	6.9 ± 1.8	4.5 ± 0.8
Gonad	2.6 ± 1.0	1.9 ± 0.7	3.5 ± 0.7	3.5 ± 1.1

[a] Significantly different at $P < 0.05$ relative to pH 6.5.

[b] Significantly different at $P < 0.01$ relative to pH 6.5.

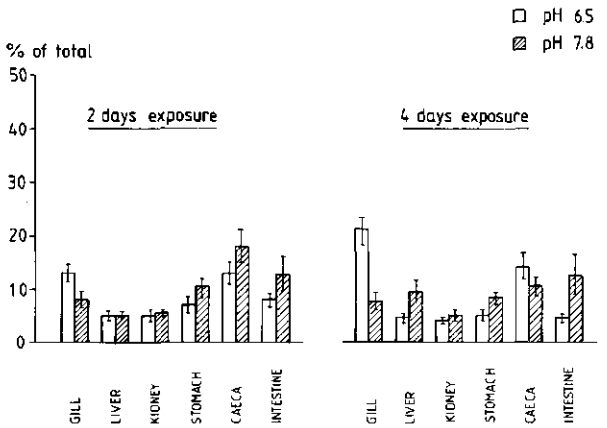

Fig. 1. Effect of pH and exposure time on percentage distribution of chromium in tissues from yearling rainbow trout after exposure to 40.0 mg/l Cr(VI). Values represent means ± SE of 5 fish.

ESR signals at g = 1.98 were observed in whole gill tissue and nuclear fractions of gills, irrespective of exposure pH and exposure time. The intensity of the signals in samples from fish exposed at pH 7.8 was somewhat lower than in those from fish exposed at pH 6.5 (Fig. 2). The content of Cr(III) and Cr(V), however, could not be estimated, mainly because of differences in ESR line shape. No signals at g = 1.98 were observed in liver and kidney of exposed fish, although the chromium content in those tissues was of the same order of magnitude as in gill. Control subcellular fractions and tissues of unexposed fish were also free of these signals.

Uptake and lethality

Exposure to Cr(VI) at 2.0 and 5.0 mg/l was not lethal to the fingerling trout at the pH and exposure time studied (Table IV). At the lower pH the whole fish and the gill tissue accumulated significantly more chromium than at the higher pH (P <0.05). In addition, the contents in gills of fish exposed at the lower pH far exceeded those in kidney, liver and digestive tract. At exposure concentrations lethal to the fish, 16.5 and 50.0 mg/l Cr(VI), the lethal action was enhanced at the lower pH. The contents in gills of fish surviving the exposure at pH 6.5 still exceeded those in other tissues. However, in fish surviving the highest exposure concentration at pH 7.8, the content in kidney, liver and digestive tract was considerably higher than that in gills. These differences in fish exposed at different pH are also reflected in the distribution of chromium in the fish (Fig. 3).

TABLE III

Effect of pH and exposure time on distribution (%) of chromium in subcellular fractions of tissues from yearling rainbow trout exposed to Cr(VI), concentration 40.0 mg/l. Values are means ± SE for 5 fish.

	2 days exposure		4 days exposure	
	pH 6.5	pH 7.8	pH 6.5	pH 7.8
Gill				
Nuclear	69.5 ± 6.4	62.8 ± 4.0	83.4 ± 0.9	62.9 ± 2.5[b]
Mitochondrial	7.0 ± 1.2	6.5 ± 0.2	2.1 ± 0.2	5.9 ± 1.5[a]
Microsomal	2.9 ± 0.4	5.3 ± 1.9	1.8 ± 0.1	3.9 ± 0.6[b]
Soluble	22.7 ± 5.0	25.8 ± 2.6	12.6 ± 0.8	27.3 ± 2.0[b]
Liver				
Nuclear	12.3 ± 2.1	14.0 ± 1.4	26.3 ± 1.2	29.3 ± 2.9
Mitochondrial	14.4 ± 1.2	10.7 ± 1.1[a]	6.9 ± 0.3	9.4 ± 1.7
Microsomal	6.9 ± 0.8	8.0 ± 1.2	12.2 ± 1.4	5.8 ± 0.8[b]
Soluble	66.4 ± 3.4	67.8 ± 2.3	54.6 ± 3.0	55.6 ± 7.6
Kidney				
Nuclear	19.3 ± 5.5	15.8 ± 1.7	25.0 ± 1.1	35.7 ± 3.6[a]
Mitochondrial	10.9 ± 0.8	9.9 ± 0.5	6.1 ± 1.1	8.5 ± 2.8
Microsomal	5.2 ± 0.3	6.1 ± 1.1	6.4 ± 1.7	4.5 ± 0.5
Soluble	62.2 ± 4.3	68.1 ± 2.0	62.3 ± 0.7	51.2 ± 4.5[a]

[a] Significantly different at $P < 0.05$ relative to pH 6.5.

[b] Significantly different at $P < 0.01$ relative to pH 6.5

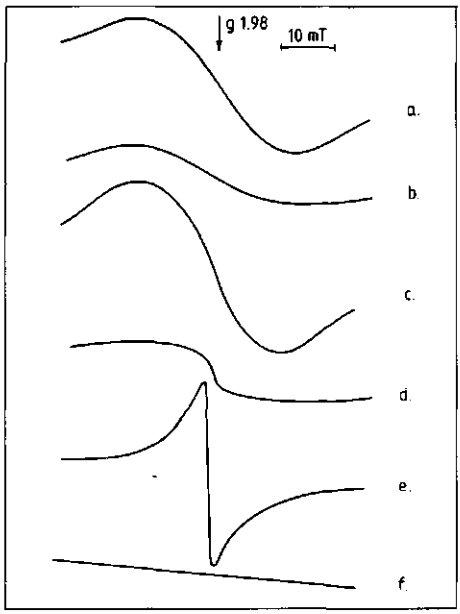

Fig. 2. ESR spectra of following samples: (a) gill tissue of fish exposed at pH 6.5; (b) gill tissue of fish exposed at pH 7.8; (c) nuclear fraction of gill tissue from fish exposed at pH 6.5; (d) nuclear fraction of gill tissue from fish exposed at pH 7.8; aqueous solutions: (e) $CrCl_3$ at pH 1.0, (f) Na_2CrO_4 at pH 8.0.

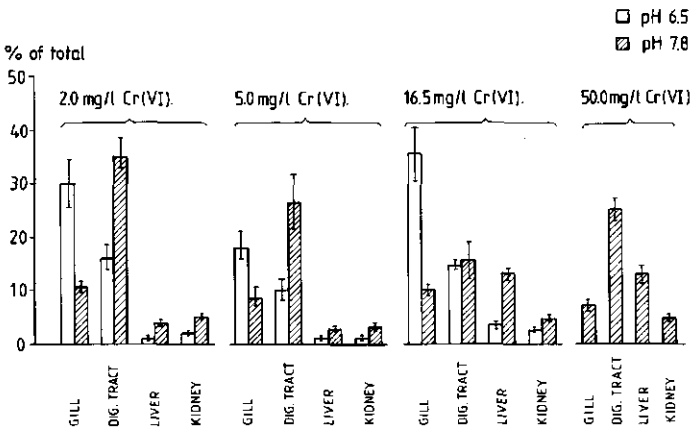

Fig. 3. Effect of pH on percentage distribution of chromium in tissues from fingerling rainbow trout after exposure to various concentrations of Cr(VI) for 4 days. Values represent means ± SE of surviving fish.

TABLE IV

Effect of pH on survival and content of chromium (means ± SE) in tissues of fingerling rainbow trout after 4 days exposure to Cr(VI) at different concentrations.

Concn. of Cr(VI) (mg/l)	pH	Survival		Content of Cr(µg/g, wet tissue)				
		Rel. (%)	Abs.	Whole body	Gill	Digestive tract	Liver	Kidney
2.0	6.5	100	9	2.0 ± 0.2	31.7 ± 6.0	6.2 ± 1.0	2.0 ± 0.3	6.7 ± 0.8
2.0	7.8	100	9	0.9 ± 0.1	5.1 ± 0.3	7.4 ± 1.5	3.4 ± 0.4	8.5 ± 1.0
5.0	6.5	100	10	5.5 ± 0.6	51.8 ± 12.3	9.5 ± 1.5	3.8 ± 0.5	10.7 ± 1.1
5.0	7.8	100	8	2.3 ± 0.2	10.6 ± 2.6	11.2 ± 2.9	5.1 ± 0.7	12.2 ± 0.8
16.5	6.5	25	5	8.7 ± 0.6	139.0 ± 13.2	23.4 ± 1.4	24.8 ± 5.6	43.2 ± 4.6
16.5	7.8	63	5	8.9 ± 1.2	35.3 ± 8.4	22.6 ± 2.9	25.9 ± 6.9	24.6 ± 2.5
50.0	6.5	0	0	-	-[a]	-	-	-	-	-	-	-	-
50.0	7.8	50	5	10.5 ± 0.5	37.6 ± 7.9	45.0 ± 2.5	85.6 ± 2.5	70.3 ± 9.5

[a] Values not determined because of zero survival.

Dynamics of uptake and elimination

The uptake rate of chromium in blood and tissues of fingerling trout was rapid at both exposure concentrations and pH levels studied (Figs. 4-6). In most tissues, contents tended to reach equilibrium within 2-4 days of exposure. Only the gill tissue of fish exposed at the lower pH showed a markedly different pattern of chromium uptake. In these tissues, there was no apparent reduction in accumulation; in fact the rate of uptake increased during 4 days of exposure. In contrast to fish exposed at pH 7.8, contents in these gill tissues far exceeded those in other tissues already after 2 days of exposure. This was also illustrated by autoradiograms (Fig. 7).

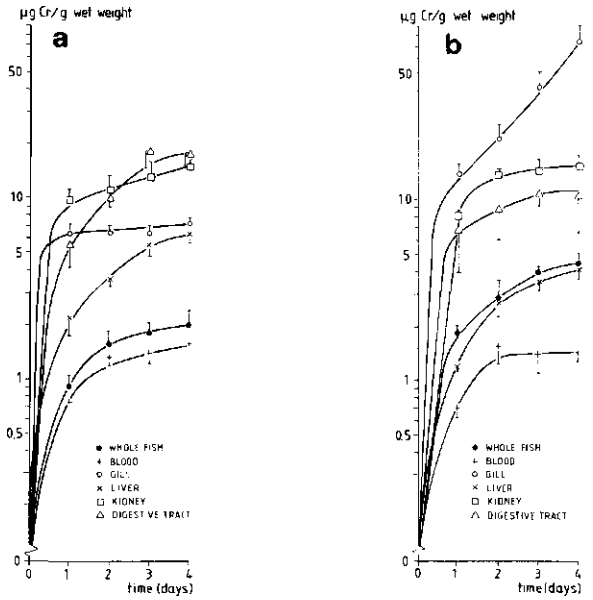

Fig. 4. Uptake of chromium in whole fish and various tissues of fingerling rainbow trout exposed to 5.0 mg/l Cr(VI) at pH 7.8 (a) and pH 6.5 (b). Values represent means ± SE of 5 fish.

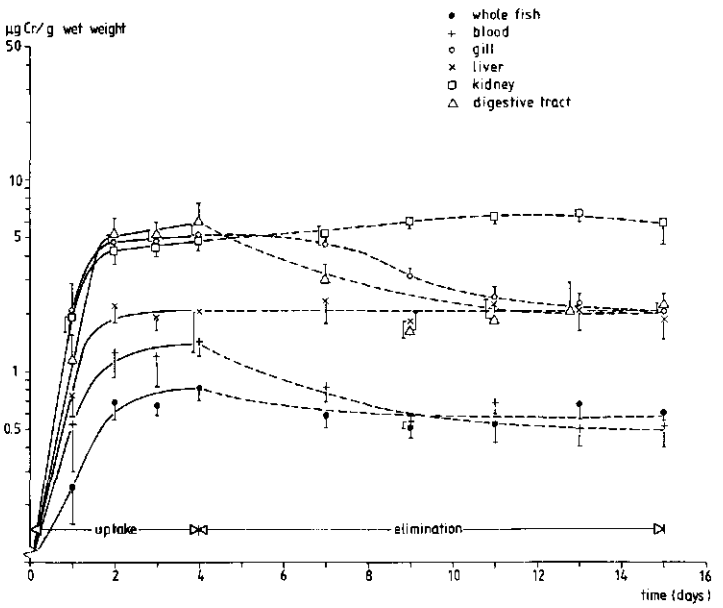

Fig. 5. Uptake and elimination of chromium in whole fish and various tissues of fingerling rainbow trout during and after exposure to 2.0 mg/l Cr(VI) at pH 7.8. Values represent means ± SE of 5 fish.

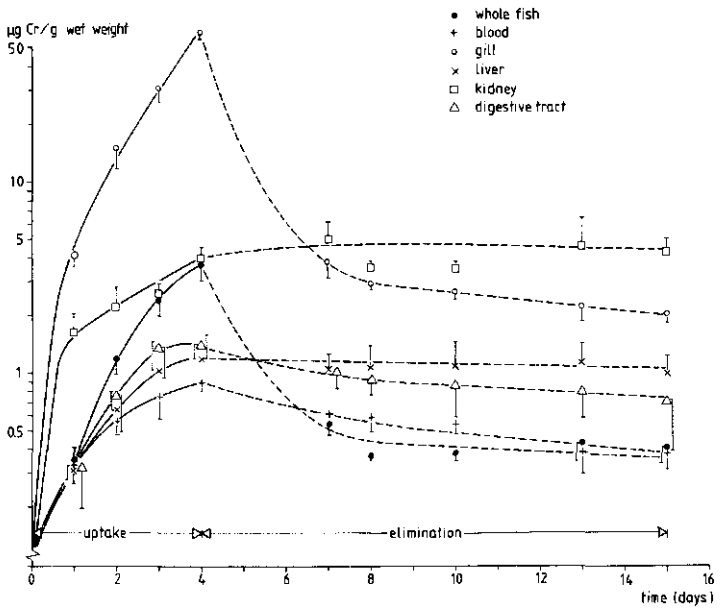

Fig. 6. Uptake and elimination of chromium in whole fish and various tissues of fingerling rainbow trout during and after exposure to 2.0 mg/l Cr(VI) at pH 6.5. Values represent means ± SE of 5 fish.

When the fingerling trout exposed to Cr(VI) were returned to tap-water without chromium, the element was rapidly lost from blood and digestive tract at both pH levels (Figs. 5 and 6). Contents tended to remain high, however, in liver and slightly increased in kidney during an elimination period of 11 days. The content in whole body and in gill of fish exposed at the lower pH declined rapidly for the first 3 days. In this period of elimination, 85% of the chromium was eliminated from whole body and 93% from gills. The decline of the chromium in whole body and in gills of fish exposed at the higher pH was very slow. In the first 3 days of elimination, only 25% of the chromium was eliminated from whole body and 12% from gills.

DISCUSSION

The present investigation has shown that the pH of water controls uptake, tissue distribution and retention of Cr(VI) in rainbow trout.

At pH 7.8, considerably more chromium was accumulated in internal organs than in gills of fingerling trout surviving the highest exposure concentration (Table IV).

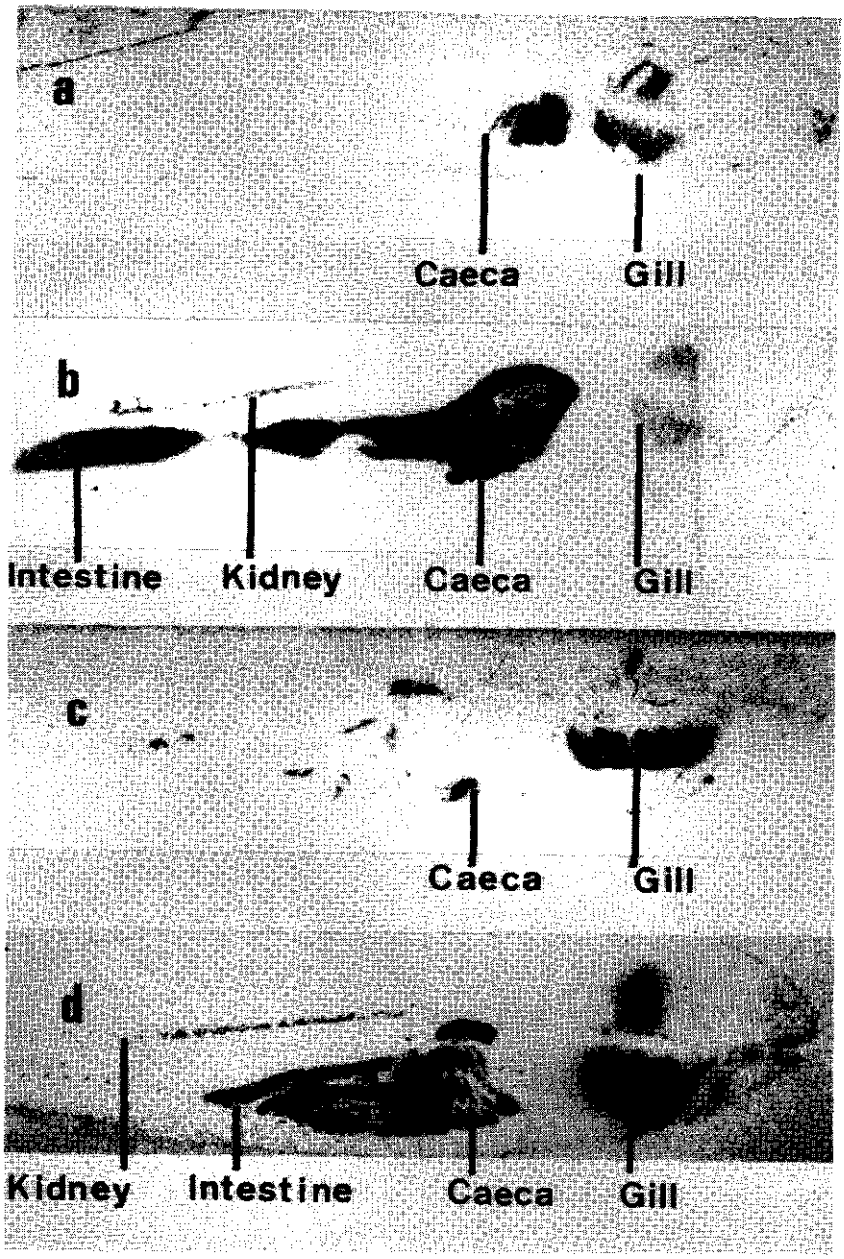

Fig. 7. Whole body autoradiograms of fingerling rainbow trout exposed to 5.0 mg/l Cr(VI) containing $^{51}CrO_4^{2-}$ (specific activity: 30 µCi/mg Cr) with different pH and exposure times: (a) pH 7.8, 2 days; (b) pH 7.8, 4 days; (c) pH 6.5, 2 days; (d) pH 6.5, 4 days. Black areas show radioactivity localization.

This supports the hypothesis that Cr(VI) elicits its toxic effect at some internal site and that the gill is not the larget organ in acute Cr(VI) toxicity (Fromm and Schiffman, 1958; Kuhnert et al., 1976). Furthermore at pH 7.8, the rate of uptake was very rapid and contents tended to reach equilibrium within 2-4 days at low exposure concentrations; similar results have been obtained by Buhler et al. (1977). The slow whole-body elimination rate of chromium at pH 7.8 is supported by the data of Ten Holder et al. (1977), who found that upon transfer of exposed rainbow trout to chromium-free water, 34% of the total amount of Cr(VI) accumulated was retained with a half-period of 1.0 day and 66% with a half-period of 25.6 days. The pH of the water in the experiments ranged from 7.8 to 8.0.

At pH 6.5, contents of chromium in gills far exceeded those in other tissues at all exposure concentrations studied. The observed higher percentage mortality of fingerling trout exposed to lethal concentrations at pH 6.5 than at pH 7.8 (Table IV) is probably due to this distinct difference in chromium uptake in gill tissue. Trama and Benoit (1960), using K_2CrO_4 as the basic salt and $K_2Cr_2O_7$ as the acidic salt, found the acute toxicity of Cr(VI) to bluegills to be accentuated at lower pH, while Strik et al. (1975) have observed histopathological changes in gill tissue of rainbow trout after exposure to acidic $K_2Cr_2O_7$. We noted that the rate of uptake of Cr(VI) in gill tissue at pH 6.5 increased throughout exposure (Figs. 4 and 6). As a rather high proportion of the chromium was accumulated in the gills, rate of uptake in the whole body of fingerling trout did not slow down. These results may explain why, in contrast to Buhler et al. (1977), Fromm and Stokes (1962) found that rainbow trout exposed to concentrations >0.05 mg/l accumulated chromium at a constant rate, but failed to achieve equilibrium during a 28-day exposure time. According to Buhler et al. (1977), the differences in uptake pattern may be due to differences in age of the rainbow trout. No attention was paid, however, to possible differences in pH of the exposure solutions.

During exposure, the chromium content in blood of experimental fish never exceeded that of surrounding water and was usually considerably less at both pH levels. Thus chromium could enter the fish by diffusion across the gill membrane as postulated by Knoll and Fromm (1960). Upon transfer of exposed fish to tap-water without chromium, blood lost chromium rapidly at both pH levels, whereas contents in liver remained high and contents in kidney even tended to increase. This may be due to the redistribution of the metal from the blood associated with the involvement of kidney and liver

in the excretion of the metal from the fish (Knoll and Fromm, 1960). Whole-body elimination rate of chromium, however, was considerably higher at pH 6.5 than at pH 7.8. This was mainly due to the rapid loss from the gills at the lower pH (Fig. 6).

The observed pH effect on uptake, tissue distribution and retention of Cr(VI) in rainbow trout may be attributed to the presence of different Cr(VI) species in the exposure solutions. The equation expressing the equilibrium between the different Cr(VI) species can be written in the following simplified form:

$$2CrO_4^{2-} + 2H^+ \rightleftharpoons 2HCrO_4^- \rightleftharpoons Cr_2O_7^{2-} + H_2O$$

At the pH and concentrations used, $HCrO_4^-$ and CrO_4^{2-} are the predominant species; a relatively small part exists as $Cr_2O_7^{2-}$ (<0.1%). At pH 7.8, the molar ratio of CrO_4^{2-} to $HCrO_4^-$ is 1:0.05. At pH 6.5, however, this ratio is 1:1, indicating that the $HCrO_4^-$ concentration increased at the same total Cr (VI) concentration (Stumm and Morgan, 1970). The pH-dependent uptake of Cr(VI) may, therefore, be related to the different ratio of $HCrO_4^-$ to CrO_4^{2-}, in which $HCrO_4^-$ is more readily taken up by the gill tissue because it is monovalent (Trama and Benoit, 1960).

A decrease in pH and therefore an increase in $HCrO_4^-$ concentration is also associated with an increased oxidizing action of Cr(VI) (Sillén and Martell, 1964). That a reduction of Cr(VI) is taking place in the gills, follows from the observed ESR signals in yearling trout (Fig. 2). These signals were also found in the nuclear fraction of the gill tissue. However, due to the method used, the nuclear fraction also contained large membrane fragments. As the gill epithelial membrane is in direct contact with the exposure solution of different pH, reduction of Cr(VI) probably took place at this site of the gill tissue. The extent of oxidizing reactions involving Cr(VI) in internal organs could not be ascertained by ESR. No signals were detected in liver and kidney in spite of the fact that chromium contents in these organs were of the same order of magnitude as those in the gills. The possibility remains that the reduced forms of Cr(VI) are bound in such a manner in these organs that no signal could be detected. It is also possible, however, that Cr(III) or Cr(V) is only present in a low concentration or not at all in these tissues. As Cr(VI) is known to cross biological membranes with relative ease in contrast with Cr(III) (Gray and Sterling, 1950), the chromium preferentially accumulated in the soluble fraction of liver and kidney of yearling trout exposed to Cr(VI) would thus mainly consist of the

soluble Cr(VI) anion.

ESR allows direct measurement in tissues without previous digestion or extraction. The latter process can alter the chemical form of chromium, and so may be the cause of contradictory data on the oxidation state of chromium in biological materials (Mertz, 1969).

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Chapter 2

EFFECT OF pH ON THE ACUTE TOXICITY OF HEXAVALENT CHROMIUM TO RAINBOW TROUT (SALMO GAIRDNERI)[a]

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SUMMARY

The acute toxicity of hexavalent chromium, Cr(VI), to rainbow trout (Salmo gairdneri) increased with decreasing pH in the range from 7.8 to 6.5. Morphological changes that could be associated with acute Cr(VI) poisoning at pH 7.8 were found in gills, kidney and stomach, whereas those at pH 6.5 appeared to be restricted to the gills only. At both pHs, however, similar alterations in plasma osmolatily and hematocrit values of blood were found in fish surviving an exposure to acute toxic concentrations.
To explain the observed effects, hydrochromate ($HCrO_4^-$) and chromate (CrO_4^{2-}) were considered as the toxic species of Cr(VI). An attempt was made to calculate the relative toxicities of these ionic species from empirical toxicity relationships for weak acids in fish, as described in the literature.

Key words: Chromium, pH, rainbow trout, toxicity, ionic species.

INTRODUCTION

It is widely accepted that water characteristics may influence the chemical form and therefore the availability and toxicity of heavy metals to aquatic organisms (Andrew et al., 1976; McCrady and Chapman, 1979). Especially hardness, pH, and alkalinity have been considered of primary importance in this respect (Andrew et al., 1977; Alabaster and Lloyd, 1980; Pagenkopf et al., 1974). However, the basic mechanism by which these

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characteristics influence the toxicity is virtually unknown. In the present study an attempt was made to identify the mechanism responsible for the toxic action of hexavalent chromium, Cr(VI), to rainbow trout, based on the presence of different ionic species of the metal in water of different pH.

The pH dependent equilibrium between the ionic Cr(VI) species formed in aqueous solution can be expressed in the following simplified form (Van der Putte et al., 1981):

$$2\ CrO_4^{2-} + 2H^+ \rightleftharpoons 2HCrO_4^- \rightleftharpoons Cr_2O_7^{2-} + H_2O$$

In the concentration range lethal to fish in acute toxicity tests (20 - 280 mg/l), and within a pH range between 6.0 and 8.8 (Trama and Benoit, 1960; Fromm and Schiffman, 1958; Pickering and Henderson, 1966), $HCrO_4^-$ and CrO_4^{2-} ions are the most common species of Cr(VI).
A relative small part exists as $Cr_2O_7^{2-}$ and other protonated forms (Stumm and Morgan, 1970). Furthermore it has been observed that the lethal action of Cr(VI) increases with decreasing water pH (Trama and Benoit, 1960; Van der Putte et al., 1981). To explain this, two hypotheses based on the assumption that acute Cr(VI) toxicity is attributable to $HCrO_4^-$ and CrO_4^{2-} species have been put forward.
The first suggests that only the availability of Cr(VI) to the fish increases with an increasing $HCrO_4^-/CrO_4^{2-}$ ratio at lower pH levels, possibly because monovalent ions tend to be more readily absorbed than divalent ions (Trama and Benoit, 1960; Becker and Thatcher, 1973). The second takes the enhanced oxidizing action of Cr(VI) at a decreased pH, which is associated with an increased $HCrO_4^-/CrO_4^{2-}$ ratio, also into consideration (Van der Putte et al., 1981). Both hypotheses suggest that the apparent toxicity of $HCrO_4^-$ is higher than that of the CrO_4^{2-} species. However, whether this is the case and to what extent has not been ascertained yet.

In this context it is worth noting that the $HCrO_4^-$ species has a weak acidic character (pK_a = 6.5) and that several empirical methods have been published to assess the relative toxicities of protonated and deprotonated forms of some weak acidic compounds. These methods have been described extensively by Broderius et al. (1977); Könemann (1979) and Sano (1976), but have not yet been applied to the $HCrO_4^-/CrO_4^{2-}$ system.

MATERIALS AND METHODS

Test fish

Rainbow trout (Salmo gairdneri) were reared in the laboratory from fertilized eggs obtained from a commercial hatchery. They were kept in tap-water with constant characteristics averaging 80 mg/l total hardness as $CaCO_3$, 92 mg/l alkalinity as HCO_3^- and pH 7.8 (see also Van der Putte et al., 1981).

In the tests on quantitative aspects of acute toxicity, trout were used of five different weight classes with mean wet weights (± SD) among survivors of 0.23 ± 0.02, 1.9 ± 0.1, 6.0 ± 1.9, 13.1 ± 2.0 and 25.0 ± 1.8 g, and ages of 4, 7, 7, 8, and 9 months respectively.

Trout used for the tests on qualitative aspects of acute toxicity were 9 months old and had mean wet weights among survivors of 16.3 ± 1.9 g. The fish were fed unrestricted rations of a commercial food (Trouvit) twice daily until 48 h before exposure to the toxicant.

The tap-water temperature during holding and testing of the trout was maintained at 12.0 ± 0.5 °C using a hot water heat exchange system.

The laboratory temperature was controlled at 12 ± 2 °C. The photoperiod was 12 h light/12 h dark, with half hour dimming and brightening within the light period provided by incandescent bulbs.

Exposure system

Tests were performed in identical diluter and test-chamber units, each including one control and five treatment chambers. The test-chambers were glass aquaria with a water volume of 60 l for fish exceeding 6.0 g, and a volume of 20 l for the smaller fish.

Tap-water was introduced from 300-l head reservoirs to 2-l mixing chambers and thereafter delivered to the 20-l and 60-l aquaria at a rate of 4.0 and 12.0 l/h respectively. Stock solutions having different concentrations of reagent grade sodium chromate (Na_2CrO_4) and adjusted to the required pH with dilute HCl were metered into the mixing chambers at a rate of 50 ml/h. Peristaltic pumps were used for the introduction of stock solution and tap-water to the mixing chambers, where mixing was accomplished by a magnetic stirrer. The mixed dilution was gravity fed into the treatment aquaria as described by Scheier and Burton (1973).

The test solutions in the aquaria were continuously aerated with air diffuser stones to achieve sufficient oxygen concentrations and thorough mixing. Replacement time for 90% of the test solution was calculated to be 12 h (Sprague, 1969).
The pH of the tap-water in each of the head reservoirs was controlled by a pH meter coupled to an electric controller. The controller activated a peristaltic pump that added dilute HCl or NaOH to the head reservoir to hold the pH at the required level ± 0.1 unit; vigorous aeration was maintained to mix the water thoroughly and to remove excess CO_2 released by lowering of pH.

Experimental procedure

Rainbow trout of the respective weight classes were preacclimated for at least two weeks to the different pH levels. For each test the fish were randomly distributed over the test chambers (12 specimens in each chamber) and were subsequently acclimated to the exposure system for 48 h before introduction of chromium. Introduction of chromium was started after dosing the aquaria with adequate stock solution to achieve the nominal test concentrations. Two different series of tests were performed to obtain quantitative and qualitative data on acute toxicity respectively.

The quantitative toxicity tests were conducted at pH 7.8, 7.0 and 6.5. Percentage fish mortality after 96-h exposure was used as the response criterion except for the test on 6.0-g trout, in which mortality percentages were monitored over periods of 24, 48, 72 and 96 h. Estimates of the concentration of Cr(VI) most likely to cause 50% mortality (LC50), their 95% confidence limits and toxicity slope functions were made by log-probit analysis (Litchfield and Wilcoxon, 1949).

In the qualitative toxicity tests, trout were exposed for 96 h at pH 7.8 and 6.5 to Cr(VI) concentrations approximating 100%, 25%, and 0% (control) of the 96-h LC50s. In these tests, after exposure five fish out of each group were anaesthetized in a solution of tricaine (MS-222) at a concentration of 150 mg/l, neutralized with 300 mg/l sodium bicarbonate. Blood samples were collected by caudal severence into heparinized test tubes and were analyzed for hematocrit and osmolality of blood plasma according to the procedures described by Wedemeyer and Yasutake (1977). The gills, liver, spleen, intestinal tract, and kidney were fixed in Bouin's solution for 24 h. Thereafter the tissues were stored in ethyl alcohol (70% ^{v}/v) until they were embedded in paraplast. The tissues were sectioned at 5 µm and stained

with Delafield's hematoxilin and eosin.

Data were analyzed for statistical difference by the Student's t-test.

Assesment of water quality parameters and Cr(VI) species

During each test dissolved oxygen (DO), pH, and Cr(VI) concentrations were checked daily in each of the test chambers; alkalinity, ammonia, and chloride concentrations were checked daily in the control chambers.
Determinations were made according to procedures described by APHA et al. (1975) except for Cr(VI), which was determined by the diphenylcarbazide colorimetric method as described by APHA et al. (1971).
Due to acidification of the tap-water the mean concentration (± SD) of Cl^- increased from its original value of 6.1 ± 0.4 mg/l at pH 7.8 to a maximum value of 62.0 ± 5.2 mg/l at pH 6.5; the mean alkalinity decreased from 92.0 ± 6.2 mg/l to a minimum of 4.0 ± 0.8 mg/l as HCO_3^-. The ammonia concentrations remained low (< 0.5 mg/l NH_4^+); dissolved oxygen was always above 90% saturation.

The molar concentration of the various Cr(VI) species in solution were calculated for each pH level using equilibrium constants at zero ionic strength and a temperature of 25 °C for the following equilibria (Sillén and Martell, 1964; 1971; Stumm and Morgan, 1970):

$$CrO_4^{2-} + H^+ \rightleftharpoons HCrO_4^- \qquad \log K_1 = 6.5$$

$$HCrO_4^- + H^+ \rightleftharpoons H_2CrO_4 \qquad \log K_{12} = -0.8$$

$$2HCrO_4^- \rightleftharpoons Cr_2O_7^{2-} + H_2O \qquad \log K_{22} = 1.52$$

$$Cr_2O_7^{2-} + H^+ \rightleftharpoons HCr_2O_7^{2-} \qquad \log K_{23} = 0.07$$

Activity and temperature corrections for equilibrium constants were neglected as approximative calculations indicated that corrected values were within the range of the experimental error induced by the pH fluctuation of 0.1 unit. Activity corrections were made according to an iteration method described by Novozamsky et al. (1976), in which the Güntelberg equation (Stumm and Morgan, 1970) was used to calculate the individual ion activity coefficients. When available, heat content changes of reactions (ΔH) were used for the estimation of temperature effects, in which the change in log K per degree was approximated by applying a ΔH of 0.0025 (Stumm and Morgan, 1970; Sillén and Martell, 1971).

RESULTS

Results of the quantitative toxicity tests are summarized in Tables I - III. In all tests the acute toxicity of Cr(VI) was affected by changes in pH, with LC50s decreasing by a factor of 2 - 4 when pH was decreased from 7.8 to 6.5 (Tables I and II). For each combination of pH and LC50, CrO_4^{2-} and $HCrO_4^-$ were calculated to be the predominant species. LC50s proved not to be correlated to the concentration of one specific ion-species, which would be expected if only one of the Cr(VI) species is responsible for the toxicity. An example of the species distribution at different LC50s and pHs is given for 0.2-g trout in Table III.

TABLE I

Effect of pH on the acute toxicity of hexavalent chromium to rainbow trout of different weight classes at 96-h exposure.

Average pH	Average fish weight (g)	96-h LC50 mg/l Cr(VI)	Slope function
7.8	0.2	12.2 (9.4 - 16.6)[a]	4.10
7.8	1.9	27.3 (17.6 - 42.2)	2.37
7.8	13.1	46.8 (34.7 - 63.3)	2.12
7.8	25.0	65.5 (47.8 - 89.7)	2.21
7.0	0.2	7.6 (4.7 - 11.6)	3.10
7.0	1.9	12.8 (8.3 - 19.8)	2.03
7.0	13.1	25.9 (18.2 - 36.8)	2.14
7.0	25.0	45.0 (30.8 - 65.7)	2.60
6.5	0.2	3.4 (2.2 - 5.3)	2.56
6.5	1.9	7.5 (4.8 - 11.9)	2.87
6.5	13.1	13.0 (8.3 - 20.2)	3.90
6.5	25.0	20.2 (11.8 - 34.7)	3.86

[a] Parentheses enclose 95% confidence limits

The quantitative toxicity data also indicate that independent of pH, smaller fish were more susceptible to Cr(VI) than larger fish (Table I), and that acute toxicity increased with increasing exposure times (Table II). At each

pH, LC50s were found to be highly correlated with fish weight and exposure times when expressed by the linear logarithmic equation:

$$\text{Log LC50} = \underline{a} + \underline{b} \text{ Log } \underline{c} \qquad (1)$$

with $\underline{a}$ and $\underline{b}$ as constants depending on pH and $\underline{c}$ as fish weight or exposure time. In these equations linear correlation coefficients determined by the standard least squares technique varied from 0.96 to 0.99.

Qualitative data of acute toxicity are presented in Table IV and Figs. 1 - 3. In blood of fish surviving a 96-h exposure to Cr(VI) concentrations approximating 100% and 25% of the 96-h LC50, plasma osmolality was decreased and hematocrit was increased at pH 7.8 as well as at pH 6.5 (Table IV).

TABLE II

Effect of pH on the acute toxicity of hexavalent chromium to 6.0-g trout at different exposure times.

Average pH	Exposure time (h)	LC50 mg/l Cr(VI)	Slope function
7.8	24	180.2 (152.1 - 213.5)[a]	1.53
7.8	48	111.1 (85.5 - 144.2)	1.70
7.8	72	70.8 (56.7 - 88.9)	2.10
7.8	96	53.2 (42.8 - 66.2)	1.84
7.0	24	136.7 (88.3 - 211.5)	1.89
7.0	48	82.6 (52.4 - 130.2)	1.81
7.0	72	52.7 (35.9 - 77.4)	2.14
7.0	96	29.5 (19.9 - 43.6)	2.35
6.5	24	90.6 (77.5 - 105.8)	1.46
6.5	48	40.1 (30.4 - 53.1)	2.01
6.5	72	24.4 (15.2 - 39.0)	1.50
6.5	96	15.6 (9.9 - 24.4)	1.67

[a] Parentheses enclose 95% confidence limits

Exposure to the lower concentration at both pHs induced only slight aberrations in gill tissue. The most notable changes occurred in the epithelium of the lamellae revealing some hypertrophy and hyperplasia.

TABLE III

Calculated concentrations of various ionic species of Cr(VI) at the 96-h LC50 for 0.2-g trout at different pH levels.

Average pH	LC50 mol/l Cr(VI) X 10^{-4}	$HCrO_4^-$ mol/l Cr(VI) X 10^{-5}	CrO_4^{2-} mol/l Cr(VI) X 10^{-5}	$Cr_2O_7^{2-}$ mol/l Cr(VI) X 10^{-8}	H_2CrO_4 mol/l Cr(VI) X 10^{-12}	$HCr_2O_7^-$ mol/l Cr(VI) X 10^{-15}
7.8	2.35	1.12	22.34	0.41	0.03	0.08
7.0	1.46	3.51	11.10	4.08	0.56	4.79
6.5	0.65	3.27	3.27	3.53	1.64	13.13

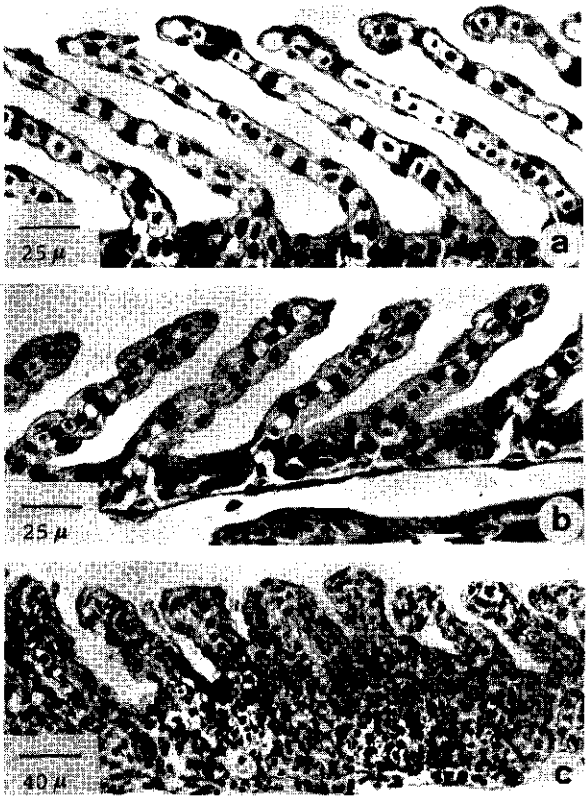

Fig. 1. Gill lamellae of rainbow trout: (a) Control fish; (b) Trout exposed to 44.8 mg/l Cr(VI) for 96 h at pH 7.8. Gill lamellae show varying but minimal epithelial hypertrophy; (c) Trout exposed to 13.1 mg/l Cr(VI) for 96 h at pH 6.5. Extensive alteration of the normal architecture of the lamellae is evident with severe epithelial hyperplasia.

TABLE IV

Survival and changes in bloodparameters of rainbow trout exposed for 96 h to acute toxic concentrations of Cr(VI) at pH 7.8 and 6.5.

Average pH	Exposure concentration % 96-h LC50	mg/l Cr(VI)	Survival Rel. (%)	Abs.	Hematocrit[a] (%)	Plasma osmolality[a] (mosmol/l)
7.8	0	0.0	100	12	34.7 ± 2.2	308.1 ± 12.4
7.8	25	13.2	92	11	40.8 ± 2.0[c]	288.4 ± 9.0[b]
7.8	100	44.8	50	6	49.7 ± 3.9[c]	271.2 ± 16.2[c]
6.5	0	0.0	100	12	36.5 ± 2.0	304.6 ± 10.6
6.5	25	3.6	75	9	42.2 ± 4.1[b]	287.4 ± 17.0
6.5	100	13.1	58	7	52.1 ± 7.5[c]	268.7 ± 17.9[c]

[a] Values are means ± SD for 5 fish

[b] Significantly different at $P < 0.05$ relative to control values.

[c] Significantly different at $P < 0.01$ relative to control values.

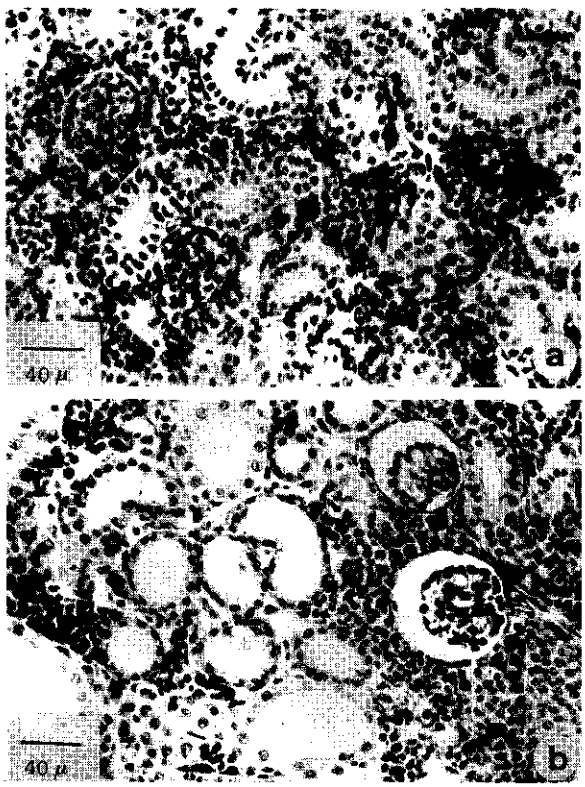

Fig. 2. Kidney of rainbow trout: (a) Control fish; (b) Trout exposed to 44.8 mg/l Cr(VI) for 96 h at pH 7.8. Dilation of lumina of tubules and increase of nucleus-to-cytoplasma ratio of the epithelium.

At the higher concentration and a pH of 6.5, these changes became more pronounced, whereas at pH 7.8 no increase in gill tissue effects was observed (Figs. 1a - c). At pH 7.8, internal organs were also affected whereas at pH 6.5, tissue damage was restricted to the gills. Histopathological effects in internal organs at pH 7.8 included degenerative changes in the kidney and the stomach. In the kidney, lumina of tubules were dilated and the nucleus-to-cytoplasma ratio in the tubular epithelium was increased (Figs. 2a - b). In the stomach, extensive hyperemia and necrotic changes in the mucous membrane and submucosa were observed (Figs. 3a - b).

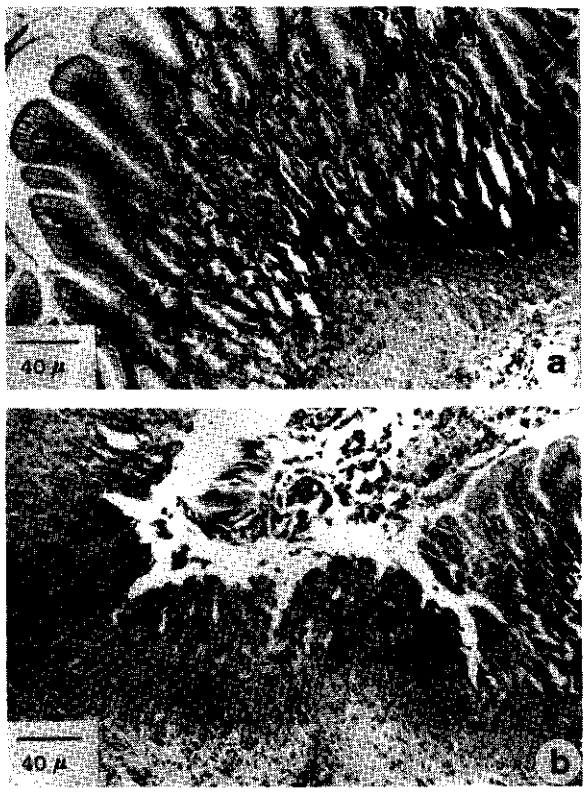

Fig. 3. Gastric mucosa and submucosa of rainbow trout: (a) Control fish; (b) Trout exposed to 44.8 mg/l Cr(VI) for 96 h at pH 7.8. Extensive hyperemia and necrotic changes in the mucous membrane and submucosa.

DISCUSSION

The results of the qualitative toxicity tests are in accordance with previous findings on the effect of pH on uptake and tissue distribution of Cr(VI) in rainbow trout. At lethal exposure concentrations and pH 7.8, considerably more chromium was accumulated in the internal organs than in the gills, whereas at pH 6.5 the reverse was observed (Van der Putte et al., 1981). In the present study acute Cr(VI) poisoning at pH 7.8 was associated with histological changes in gills, kidney and stomach, whereas those at pH 6.5 were restricted to the gills.
Consequently the assumption that Cr(VI) elicits its toxic effect at some internal site and that the gill is not the target organ in acute Cr(VI) toxicity (Fromm and Schiffman, 1958; Kuhnert et al., 1976) is only appropriate at relatively high pH levels. At pH 6.5, the gill seems to be the primary site of toxic action.

The quantitative toxicity data, including the Cr(VI) species distribution, confirm the hypothesis that acute Cr(VI) toxicity is attributable to $HCrO_4^-$ and CrO_4^{2-} species (Trama and Benoit, 1960; Becker and Thatcher, 1973; Van der Putte et al., 1981). Various methods may be applied to assess the relative toxicities of the two Cr(VI) species, because $HCrO_4^-$ may be considered as a weak acidic compound. One of the methods can be derived from the formulas given by Sano (1976) for the effect of pH on the toxicity of sulfite to guppy (Lebistes reticulatus) and goldfish (Carassius auratus).
Sano (1976) observed an increase in toxicity to the fish with decreasing pH and proposed the following formulas to express the toxicity of sulfite:

$$S_{eff} = [HSO_3^-] + \underline{f}\ [SO_3^{2-}] \qquad (2)$$

$$\text{Log } S_{eff} = \underline{a} + \underline{b}\ \text{Log } T \qquad (3)$$

where S_{eff} is the effective concentration of sulfite for toxicity, $\underline{a}$ and $\underline{b}$ are constants, T is half life time of the fish group, and $\underline{f}$ is a coefficient indicating the contribution of SO_3^{2-} toxicity to the effective toxicity of sulfite. The appropriate value for $\underline{f}$ is determined by trial and error so that, with the standard least squares technique, the best linearity is obtained in equation (3).
Using a comparable approach, the following relationships can be given for $HCrO_4^-$ and CrO_4^{2-} ions:

$$Cr_{eff} = [HCrO_4^-] + \underline{f}\,[CrO_4^{2-}] \quad (4)$$

$$\text{Log } Cr_{eff} = \underline{a} + \underline{b} \text{ Log } \underline{T} \quad (5)$$

where Cr_{eff} is the effective concentration of hexavalent chromium for toxicity, $\underline{a}$ and $\underline{b}$ are constants, $\underline{T}$ is exposure time in LC50 determinations, and $\underline{f}$ is a coefficient indicating the contribution of CrO_4^{2-} toxicity to the effective toxicity of Cr(VI).

Expressing the experimental LC50 data for 6.0-g trout as Cr_{eff} concentrations so that the best linearity was obtained in equation (5), a value for $\underline{f}$ of 0.20 was established (Figs. 4a - b).

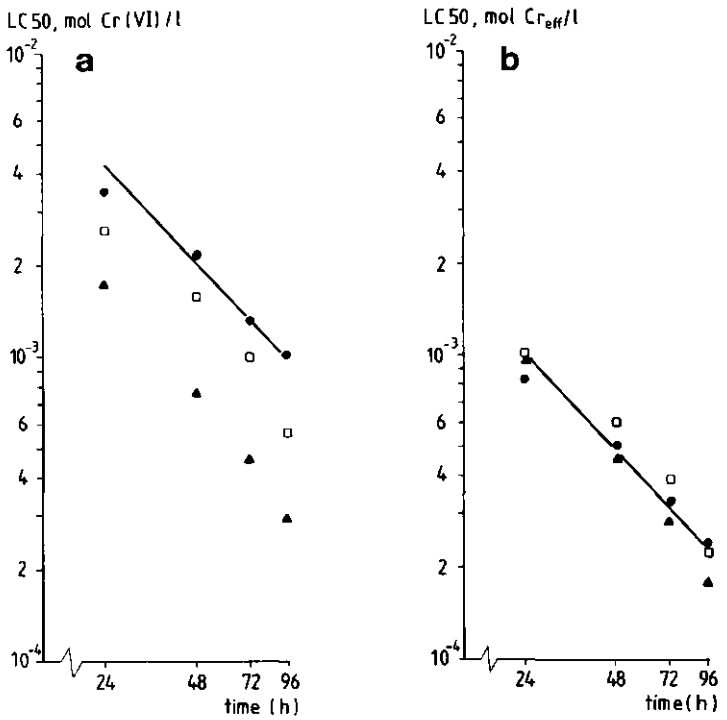

Fig. 4. Correlation between LC50 of hexavalent chromium for 6.0-g trout and time at pH 6.5 (▲), pH 7.0 (□) and pH 7.8 (●): where (a) Cr(VI) = $[HCrO_4^-]$ + $[CrO_4^{2-}]$ and (b) Cr_{eff} = $[HCrO_4^-]$ + 0.20 $[CrO_4^{2-}]$.

The linear correlation coefficient in equation (5) was calculated to be 0.97. The $\underline{f}$ value of 0.20 indicates that $HCrO_4^-$ can be considered five times as toxic as CrO_4^{2-} for 6.0-g trout over an exposure period of 24 h to 96 h.

Another empirical method to estimate the relative toxicity of protonated

and deprotonated forms of weak acidic compounds is given by Könemann (1979), who studied the effect of pH on the toxicity of chlorophenols to guppy (Poecilia reticulata). He adapted the formula originally developed by Tabata (1962) for the NH_3/NH_4^+ system and used by Broderius et al. (1977) to explain the pH effects on the toxicity of HCN/CN^- and H_2S/HS^- systems. From Broderius' formula:

$$\frac{[HA] + [A^-]}{LC50} = T_m [HA] + T_i [A^-] \qquad (6)$$

Könemann derived the following equation:

$$1/LC50 = T_m - (T_m - T_i) \frac{K_a}{[H^+] + K_a} \qquad (7)$$

where 1/LC50 is an expression for total toxicity, T_m is the toxicity of the molecular or protonated form (HA), and T_i is the toxicity of the ionized or deprotonated form (A^-) of a weak acid; K_a is the acidic ionization constant. Plotting 1/LC50 against $K_a/([H^+] + K_a)$, a linear relationship is found. The best linearity is obtained by the standard least squares technique and T_m and T_i can be calculated from the intercept and the slope of the resulting straight line.

Using equation (7) to analyze the experimental 96-h LC50 data for trout of different weight classes, it was established that $HCrO_4^-/CrO_4^{2-}$ toxicity ratios were fairly constant and ranged from 9.1 to 11.9 times that of CrO_4^{2-} over an exposure period of 96 h. An analysis of the LC50 data for 6.0-g trout by the same procedure indicates that exposure time is influencing the $HCrO_4^-/CrO_4^{2-}$ toxicity ratio (Table V).

TABLE V

$HCrO_4^-/CrO_4^{2-}$ toxicity ratios for different fish weight classes and exposure times, calculated from experimental LC50 data according to Könemann (1979).

Average fish weight (g)	LC50 exposure time (h)			
	24	48	72	96
0.2	-	-	-	11.9
1.9	-	-	-	9.1
6.0	3.6	7.1	8.0	9.2
13.1	-	-	-	10.6
25.0	-	-	-	9.9

With an exposure time decreasing from 96 h to 24 h, calculated toxicity ratios decreased from 9.2 to 3.6, and thus were more in agreement with the value determined by the method of Sano (1976). The linear correlation coefficients in equation (7) were found to vary between 0.96 to 0.99.

From the calculated $HCrO_4^-/CrO_4^{2-}$ toxicity ratios the effect of pH on the acute toxicity of Cr(VI) can easily be predicted using the above mentioned formulas or those of Broderius et al. (1977) and Tabata (1962). However, it should be recognized that all these formulas are based on the assumption that the toxicities of the protonated and deprotonated forms of a weak acid are simply additive. A priori it seems illogical to expect that this is the case with $HCrO_4^-$ and CrO_4^{2-} ions, as the kind of histopathological effects appeared to be dependent on pH and thus on the presence of each of these ions in the external solution. On the other hand these formulas have been found to work empirically very well to explain the effect of pH on Cr(VI) toxicity. In addition it was observed that independent of pH acute Cr(VI) poisoning was associated with similar alterations in the blood. This indicates that $HCrO_4^-$ and CrO_4^{2-} ions in the external solution cause common effects in the blood of fish although different organs are affected. It may be postulated that these hematological effects are simply additive and are possibly directly associated with the death of the fish. A comparable explanation for simple additive toxicity for mixtures of poisons has been given in the "toxic unit" model described by Brown (1968) and Sprague (1970).

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Chapter 3

OXYGEN AND CHROMIUM TRANSFER IN PERFUSED GILLS OF RAINBOW TROUT (SALMO GAIRDNERI) EXPOSED TO HEXAVALENT CHROMIUM AT TWO DIFFERENT pH LEVELS[a]

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SUMMARY

An in vitro study was performed on uptake and transfer of hexavalent chromium, Cr(VI), in gills of rainbow trout (Salmo gairdneri).
Gills were perfused according to the isolated head perfusion technique, and externally exposed to Na_2CrO_4 solutions containing $^{51}CrO_4^{2-}$. Experiments were conducted at a concentration of 10 mg/l Cr and at pH values of 8.1 and 6.5. The results show that the transfer of chromium is directly coupled with the transfer of oxygen from the external solution to the internal perfusion medium. Under similar conditions of oxygen transfer, however, chromium transfer was significantly more effective at pH 6.5 than at pH 8.1.
In addition more chromium was accumulated by the gill tissue at the lower pH.
Gill preparations of trout which had been pre-exposed in vivo for 4 days to 10 mg/l Cr(VI) at pH 6.5, exhibited an impaired oxygen transfer. This could be well explained by the structural alterations seen after histological examination of the perfused gills.

Key words: perfused gills, rainbow trout, chromium, uptake, pH.

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[a] Aquat. Toxicol. (In press)

INTRODUCTION

Gills of fish are organs not only involved in respiration and osmoregulation but also in nitrogen excretion and regulation of acid-base balance (Evans, 1975; Fromm and Gillette, 1968; Girard and Payan, 1980).
The various exchange functions are structurally served by large lamellar surface areas separating blood and water by thin epithelial cell layers. It is therefore not surprising that in fish, exposed to heavy metals dissolved in the water, the gills function also as the main route for uptake of these compounds (Knoll and Fromm, 1960; Olson et al., 1973).
Following uptake by the fish, the transport of the metals to other tissues is undoubtedly by way of the circulatory system. In which organs and tissues metals are accumulated and at which site toxic effects are elicited depend on the kind of metal and fish species studied (Sellers et al., 1975; Coleman and Cearly, 1974; Cearly and Coleman, 1974), the exposure time and concentration (Hughes and Flos, 1978; Matthiessen and Brafield, 1977), and the physico-chemical characteristics of the water (Van der Putte et al., 1981a; Mount, 1966).

Water pH is considered to be an important physico-chemical factor in modifying the toxic action of hexavalent chromium, Cr(VI), in fish (Trama and Benoit, 1960; Becker and Thatcher, 1973). It has been shown that exposure of trout to acutely toxic concentrations of Cr(VI) at a pH of 6.5 leads to a preferential accumulation of the metal in the gills, whereas at a pH of 7.8 relatively more chromium is accumulated by internal organs (Van der Putte et al., 1981a).
This difference in accumulation pattern is also reflected in the histopathological effects which are induced by Cr(VI) (Van der Putte et al., 1981b).

In the present investigation uptake and transfer of Cr(VI) in gills of rainbow trout was studied in vitro. A gill perfusion technique was used as described by Pärt and Svanberg (1981) and Payan and Matty (1975).
The aim was to learn more about the uptake mechanisms of chromium in fish exposed to Cr(VI) solutions at pH 6.5 and pH 8.1. Special attention was paid to the relation between the transfer of oxygen and the transfer of chromium from the external exposure solution to the internal perfusion medium.

MATERIALS AND METHODS

Fish and water characteristics

Rainbow trout (Salmo gairdneri), 165 - 200 g, were used in all experiments. They were obtained from a commercial hatchery and kept in aerated Uppsala tap-water at a temperature of 10°C - 12°C for at least 3 months before experimentation. Tap-water characteristics averaged 320 mg/l total hardness as $CaCO_3$, 262 mg/l alkalinity as HCO_3^- and pH 7.6 (see also Pärt and Svanberg, 1981). Water pH was adjusted in the experiments to pH 8.1 ± 0.1 and pH 6.5 ± 0.1 by manual addition of NaOH or HCl. Vigorous aeration was maintained to remove excess CO_2 (Van der Putte et al., 1981a). Fish were acclimated for at least 2 days to the respective pHs.
During the holding and acclimation period fish were fed daily with a commercial trout food (Trout feed axtra, Astra-Ewos, Sweden). From 48 h before and during the pre-exposure experiment to Cr(VI) fish were not fed. The photoperiod was 12 h light/12 h dark.

Gill preparation and perfusion

Gills were prepared and perfused according to the isolated head perfusion technique described by Pärt and Svanberg (1981) and Payan and Matty (1975). Briefly, the fish were decapitated behind the opercula following heparinization, and the ventral and dorsal aortas were cannulated with polyethylene catheters (Intramedic PE 90 and PE 190, Clay-Adams Inc., USA). The eight gill arches were simultaneously perfused by connecting the ventral aortic catheter to the perfusion pump delivering the perfusion medium. A pulsatile perfusion pump with variable stroke volume and frequency was used (Pärt and Svanberg, 1981). The perfusion flow, 1.8 - 2.5 ml/min, was adjusted by changing the stroke volume with the frequency kept at 50 beats/min. The pressure in the ventral aorta was measured by a pressure transducer (P 23 Db, Gould Statham Instruments Inc., USA) connected to a potentiometric recorder (Servograph Radiometer, Denmark). The dorsal aortic cannula was connected to a chamber which was equipped with an oxygen electrode to measure P_{O_2} levels in the perfusion medium leaving the gills. The oxygen electrode (model "Clark") was constructed on basis of the electrode described by Solymar et al. (1971).

During perfusion the isolated head was placed in a cylindrical box permit-

ting circulation of external solution over the gills without leakage at a rate of 1.5 ml/min, which was maintained by a rotary pump (Eheim). A similar model "Clark" oxygen electrode was used to measure P_{O_2} levels in the external solution. Both the external solution and the perfusion medium were maintained at 10°C by a thermostat unit (Heto, Denmark).

The surgical procedures performed before perfusion were carried out with the gills irrigated by aërated tap-water. Average preparation time for each of the 18 preparations was 15 min, measured from catching the fish in the aquarium until the start of perfusion. The gills were not irrigated for less than 1 min during this time.

Perfusing medium and external solution

The perfusion medium was Cortland salmonid saline (Wolf, 1963) containing 40 g/l of PVP-40 (Polyvinylpyrrolidone, MW = 40 000, Sigma Inc., USA) and 5000 IE/l heparine (Sodium-heparine, Vitrum, Sweden). It was gassed with a gas mixture (5.00% O_2; 0.9% CO_2; 94.1% N_2) at room temperature (20°C). After gassing the pH of the perfusion fluid was 7.5 at 10°C, the P_{O_2} was 31 - 35 mmHg and the P_{CO_2} was 2 - 4 mmHg. These values are comparable with those obtained for venous blood in vivo (Holeton and Randall, 1967; Haswell et al., 1978). Before use the medium was filtered through a 1.2 µm filter (Millipore Inc., USA), followed by the addition of adrenalin (L-epinephrine, Sigma Inc., USA) to a final concentration of 1. 10^{-6} mol/l.

The external exposure solutions were prepared by adding 5 ml of a Na_2CrO_4 stock solution containing $^{51}CrO_4^{2-}$ (Radiochemical Centre, Amersham) to 200 ml tap-water, which was adjusted to pH 8.1 or 6.5. The final concentration was 10 mg/l Cr(VI) with a specific activity approximating 50 µCi/mg Cr.

During perfusion the pH of the external solutions was controlled within 0.1 unit by manual addition of dilute HCl or NaOH; the oxygen concentration was maintained near saturation levels by pressurized air.

Histology

Pieces of the first right gill arch were cut from perfused heads and fixed in 2.5% glutaraldehyde in 0.1 mol/l Na-phosphate buffer (pH = 7.4).

For scanning electron microscopic examination the gills were postfixed in 1% osmium tetroxide in 0.1 mol/l Na-phosphate buffer (pH = 7.4), dehydra-

ted in a graded ethanol series, and after critical point drying with liquid CO_2, sputter coated with gold. A JSM-35c scanning electron microscope was used, operated at 25 keV.

For light microscopic examination the gills were dehydrated in ethanol, followed by methylbenzoaat and benzene, and thereafter embedded in paraplast. The tissues were sectioned at 5 µm and stained with Delafield's hematoxilin and eosin.

Experimental procedure

Three series of perfusion experiments were performed. Two series were conducted at pH 6.5 and 8.1 respectively using trout from the stock tanks supplied with tap-water. A third series was conducted at pH 6.5 using trout which had been pre-exposed for 4 days to a stable Na_2CrO_4 solution at a concentration of 10 mg/l Cr and a pH of 6.5. Pre-exposure was performed under static conditions in a 200-l tank at a temperature ranging from 10 - 12°C, with oxygen concentrations maintained near saturation levels by pressurized air.

The isolated trout heads were perfused for 10 - 15 min before ^{51}Cr exposure to obtain a blood-free preparation. Each ^{51}Cr uptake experiment lasted 50 min, divided in 10 min periods. During each of these 10-min-periods one 10 ml sample of the perfusion medium containing ^{51}Cr was collected from the dorsal aorta with the perfusion flow being determined gravimetrically. The ^{51}Cr was concentrated to a volume of 1 ml by precipitation. This was accomplished by addition of 0.1 ml of a $FeCl_2$ solution (conc. 1 mol/l) acidified by HCl (0.12N) followed by addition of 1 ml NaOH (1N). The precipitate was centrifuged for 10 min at 900 g. The recovery was 98 ± 5% (± SD; n = 10). The radioactivity of the external solution was checked by 0.5 ml samples taken at the start of exposure and at the end of each of the 10-min-periods.

P_{O_2} of the external solution and of the perfusion medium leaving the gills was recorded continuously together with the pressure levels in the ventral aorta. After exposure, the gills were rinsed for 2 min in Cr-free tap-water. Tissue samples were taken for histological examination and for the measurement of Cr-accumulation.
Water and tissue samples were counted in a scintillation counter (Model 1185, Nuclear Chicago, USA) for 10 000 counts.

Calculation of Cr-influx and oxygen transfer

The influx of chromium from the external solution into the perfusion medium was calculated from:

$$F_{Cr} = \frac{^{51}Cr_i \cdot Q \cdot 60}{^{51}Cr_e^*}$$

F_{Cr} = influx of Cr in mole . h^{-1} (100 g fish)$^{-1}$

$^{51}Cr_i$ = ^{51}Cr in perf. medium in dorsal aorta (cpm . ml^{-1})

Q = perfusion flow in ml . min^{-1} (100 g fish)$^{-1}$

$^{51}Cr_e^*$ = specific activity of ^{51}Cr in external solution (cpm . $mole^{-1}$)

From the oxygen tension in the external solution ($Pext_{O_2}$) and the oxygen tensions in the perfusion medium entering and leaving the gills (Pv_{O_2} and Pa_{O_2} respectively) the following parameters could be determined (see also Jones et al., 1970):

Mean oxygen tension gradient over the gills (dP_{O_2}) = $Pext_{O_2} - \frac{(Pa_{O_2} + Pv_{O_2})}{2}$ (mmHg)

Oxygen uptake in perfusion medium (V_{O_2}) = $(Pa_{O_2} - Pv_{O_2}) \cdot a \cdot Q$

(µl O_2 min^{-1} (100 g fish)$^{-1}$

Oxygen transfer factor (T_{O_2}) = $\frac{V_{O_2}}{dP_{O_2}}$

(µl O_2 min^{-1} (100 g fish)$^{-1}$ $mmHg^{-1}$)

All P_{O_2} values in mmHg; a = oxygen solubility constant in the perfusion medium at 10°C (µl O_2 ml^{-1} $mmHg^{-1}$); Q = perfusion flow in ml min^{-1} (100 g fish)$^{-1}$.

In the calculation of dP_{O_2} it was assumed that the oxygen tensions of "inspired" and "expired" water (Pi_{O_2} and Pe_{O_2}) in the original equations given by Jones et al. (1970) did not differ from each other and were equal to $Pext_{O_2}$. This was considered reasonable as the gills were irrigated at a high rate (1.5 ml/min) and as the oxygen uptake from the external solution was low.

Data were analyzed for statistical difference by Wilcoxon's two sample rank test (Snedecor and Cochran, 1967).

RESULTS

Oxygen and Cr-transfer

Results of the perfusion experiments conducted with trout which had not been pre-exposed to Cr(VI) are given in Table I and Figs. 1 and 2 respectively. From the P_{O_2} recordings it could be calculated that the addition of chromium to the external medium did not affect the oxygen transfer factor (T_{O_2}) in perfused gills. T_{O_2} values also remained constant during the exposure period. A state of equilibrium in Cr-influx (F_{Cr}) was reached after the first 10 min of exposure (Fig. 1).

The mean equilibrium T_{O_2} and F_{Cr} values at pH 8.1 were not statistically different from those at pH 6.5 ($P > 0.1$) (Table I). On the other hand, as shown in Fig. 2, the influx of chromium was always higher at pH 6.5 than at pH 8.1 under similar conditions of oxygen transfer. In addition it can be seen that F_{Cr} tends to increase linearly with respect to T_{O_2}. The slopes of the regression lines determined by the method of least squares were 90 and 101, and linear correlation coefficients were 0.96 and 0.98 at pH 8.1 and pH 6.5 respectively.

Correspondingly uptake of Cr by the perfused gills after the exposure period of 50 min was significantly higher at the lower pH (Table I).

TABLE I

Cr-tissue concentrations (TC), and equilibrium values for Cr-influx (F_{Cr}) and oxygen transfer (T_{O_2}) in perfused trout gills exposed for 50 min to 10 mg/l Cr(VI) at different pHs. Values are means ± SE for 6 fish.

pH	$F_{Cr} \cdot 10^{-7}$	T_{O_2}	TC
	$mole^{-1}\ h^{-1}\ (100\ g\ fish)^{-1}$	$\mu l\ O_2\ min^{-1}\ (100\ g\ fish)^{-1}\ mmHg^{-1}$	µg Cr/g, wet tissue
8.1	3.92 ± 0.89	0.051 ± 0.009	0.56 ± 0.12
6.5	4.70 ± 0.53	0.036 ± 0.005	3.85 ± 1.21[a]

[a] Significantly different at $P<0.025$ relative to pH 8.1

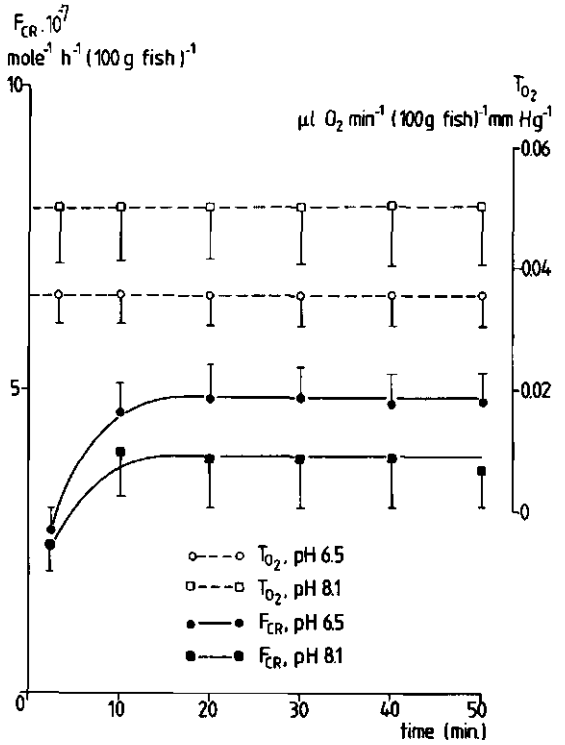

Fig. 1. Relation between time, oxygen transfer (T_{O_2}) and Cr-influx (F_{Cr}) in perfused trout gills exposed to 10 mg/l Cr(VI) at two different pH levels. Values represent means ± SE of 6 fish.

TABLE II

Cr-tissue concentrations (TC^x, tentative), and equilibrium values for Cr-influx (F_{Cr}^x, tentative) and oxygen transfer (T_{O_2}) in perfused gills of "pre-treated" trout exposed for 50 min to 10 mg/l Cr(VI) at pH 6.5. Values are means ± SE for 6 fish.

$F_{Cr}^x \cdot 10^{-7}$	T_{O_2}	TC^x
mole^{-1} h^{-1} (100 g fish)$^{-1}$	µl O_2 min^{-1} (100 g fish)$^{-1}$ mmHg^{-1}	µg Cr/g, wet tissue
5.98 ± 0.71	0.014 ± 0.005	6.44 ± 0.89

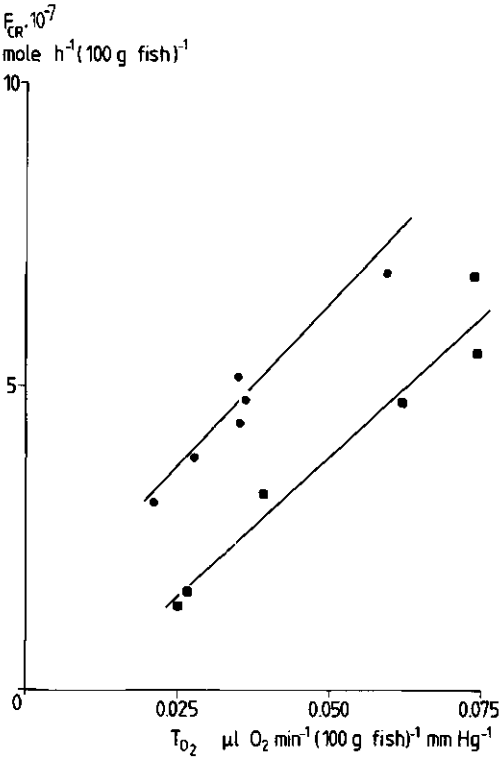

Fig. 2. Relation between equilibrium values for oxygen transfer (T_{O_2}) and Cr-influx (F_{Cr}) in perfused trout gills exposed to 10 mg/l Cr(VI) at pH 6.5 (●) and at pH 8.1 (■).

Results of the perfusion experiments conducted with pre-exposed trout at pH 6.5 are given in Table II and Figs. 3 and 4. Cr-influx ($F_{Cr}^{\times}$) and Cr uptake values ($TC^{\times}$) in the gills of these trout must be regarded as tentative, because calculations did not include corrections for exchange reactions, which probably occurred between stable Cr accumulated by the gills during pre-exposure, and ^{51}Cr from the external solution during perfusion. For this reason F_{Cr} and TC are indicated as $F_{Cr}^{\times}$ and $TC^{\times}$ to mark the uncertainty which might be involved. It is clear, however, that $F_{Cr}^{\times}$ values in these experiments exhibited a comparable relationship with time and T_{O_2} values as in gills of trout which had not been pre-exposed to Cr(VI). A

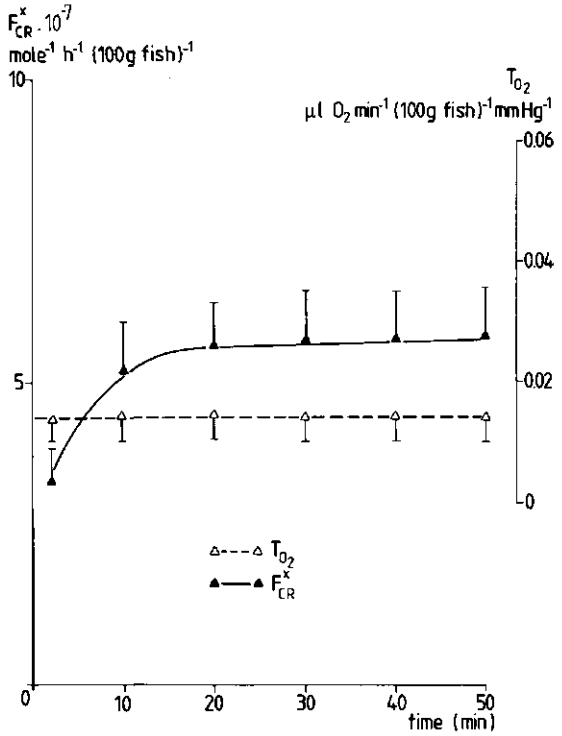

Fig. 3. Relation between time, oxygen transfer (T_{O_2}) and Cr-influx ($F_{Cr}x$, tentative) in perfused gills of "pre-treated" trout, exposed to 10 mg/l Cr(VI) at pH 6.5. Values represent means ± SE of 6 fish.

conspicuous finding was that T_{O_2} values of pre-exposed trout gills were significantly lower than those of the other groups ($P<0.05$). Under these circumstances also the addition of chromium during perfusion did not influence the levels of T_{O_2}.

Histology and ventral aortic pressure

Recordings during the perfusion experiments showed that the perfusion pressure, which is analogous to the ventral aortic pressure, remained stable at a level of 25 - 35 mmHg. No rise in pressure, which would have been indicative of a non-viable preparation (Pärt and Svanberg, 1981), was observed. It has been shown by Pärt and Svanberg (1981), that the perfusion technique used, induces no changes in the normal structure of the gills. No

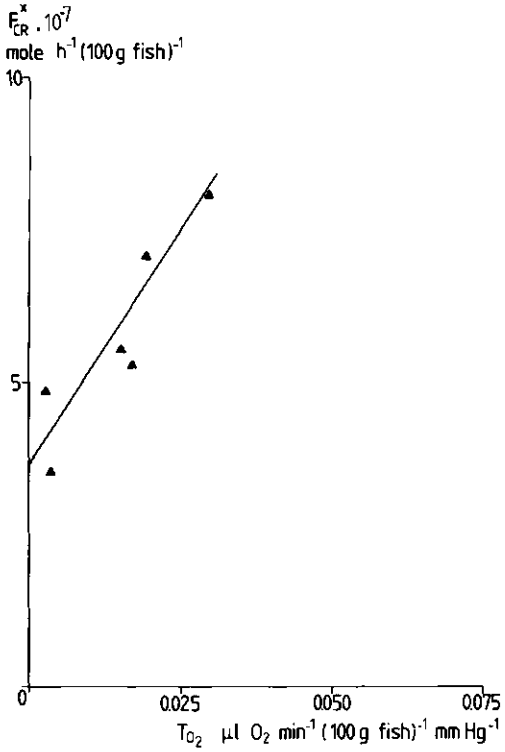

Fig. 4. Relation between equilibrium values for oxygen transfer (T_{O_2}) and Cr-influx (F_{Cr}^{x}, tentative) in perfused gills of "pre-treated" trout, exposed to 10 mg/l Cr(VI) at pH 6.5.

structural damage was seen either in gills of trout which had not been pre-exposed to Cr(VI) in the present experiments (Figs. 5A-B).
The structure of these gills was similar to those described for unperfused gills, except for the absence of blood cells in the blood channels (Newstead, 1967; Kendall and Dale, 1979).
However, gills of trout which had been pre-exposed to Cr(VI) for 4 days at pH 6.5, had undergone structural alterations including hyperplasia of the lamellar epithelium, partly resulting in fusion of the secondary lamellae, swollen epithelial cells, and edema in the primary filament (Figs. 5C-D).
It is clear that all these alterations are adversely affecting the effective surface area of the gills.

DISCUSSION

A commom method to study heavy metal uptake by freshwater organisms is to analyze the metal content in blood and tissues after exposure. However, the concentration in the organism, being the net sum of uptake and elimination, is subject to large individual variations. Therefore, small changes in the rate of uptake due to differences in the availability of the metal from the external solution, cannot be detected easily. The use of isolated organ technology, in this case the perfused gill preparation of trout, may remove some of the variables and thus smaller differences may be discerned.

As has been pointed out in previous work concerning Cr(VI) toxicity to fish (Van der Putte et al., 1981a; 1981b), water pH may not only change the availability of the metal, due to its influence on metal speciation, but may also change the oxidizing action of the metal, which increases at lower pHs. These processes are thought to be responsible for the specific accumulation of chromium in gills of trout exposed to Cr(VI) in vivo at a relatively low pH of 6.5 (Van der Putte et al., 1981a). A similar observation was made in the present investigation, where perfused gills accumulated significantly

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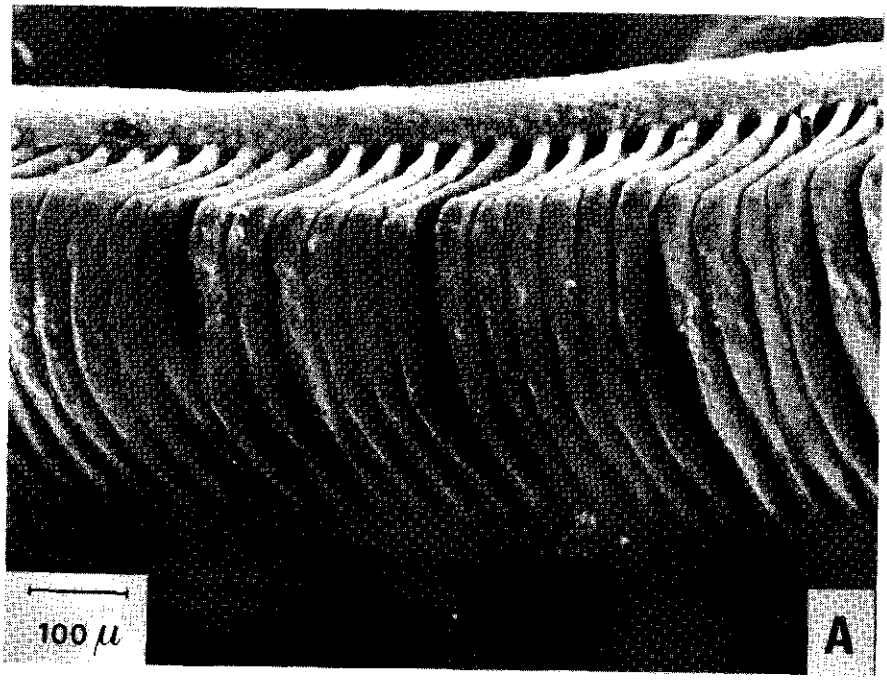
Fig. 5. Scanning electron micrographs (SEM) and light micrographs (LM) of perfused trout gills exposed for 50 min to 10 mg/l Cr(VI) at pH 6.5.

(A) Perfused gills with a normal structure; Not "pre-treated" trout;
T_{O_2} = 0.037 µl O_2 min^{-1} (100 g fish)$^{-1}$ $mmHg^{-1}$; SEM.

(B) Perfused gills with a normal structure; Not "pre-treated" trout;
T_{O_2} = 0.059 µl O_2 min^{-1} (100 g fish)$^{-1}$ $mmHg^{-1}$; LM.

(C) Perfused gills of "pre-treated" trout; Note hyperplasia of the lamellar epithelium and swollen epithelial cells;
T_{O_2} = 0.003 µl O_2 min^{-1} (100 g fish)$^{-1}$ $mmHg^{-1}$; SEM.

(D) Perfused gills of "pre-treated" trout; Note edema in the primary filament;
T_{O_2} = 0.002 µl O_2 min^{-1} (100 g fish)$^{-1}$ $mmHg^{-1}$; LM.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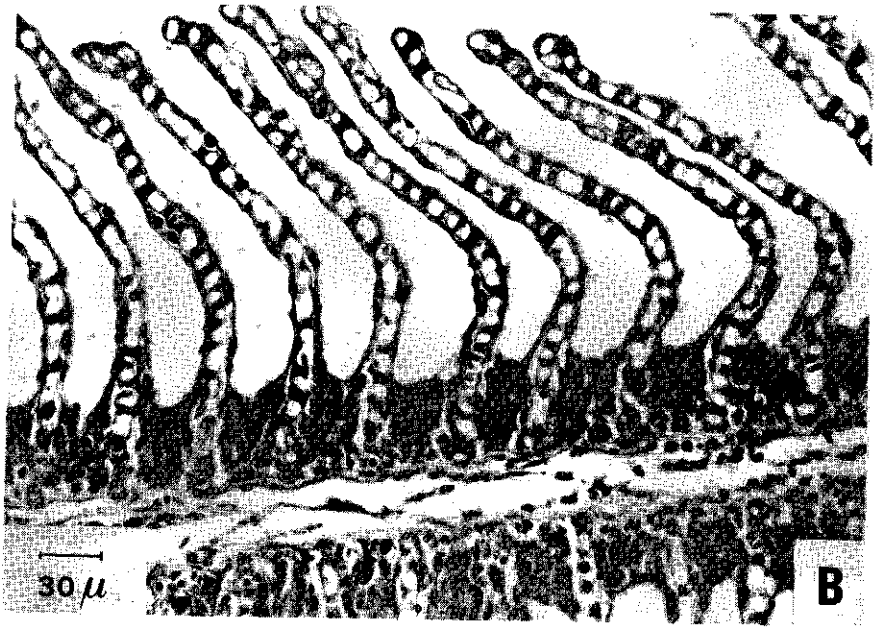
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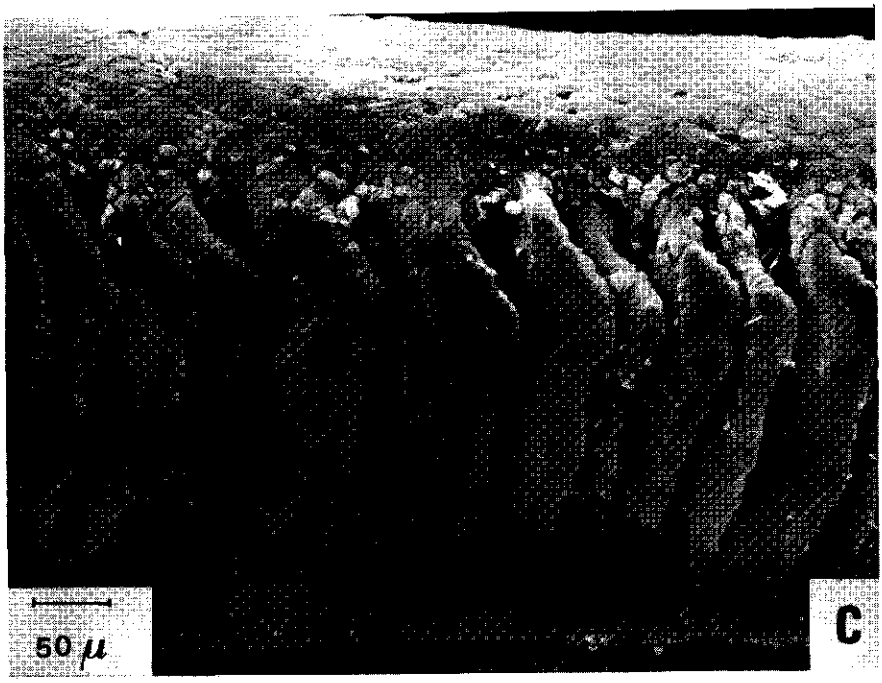
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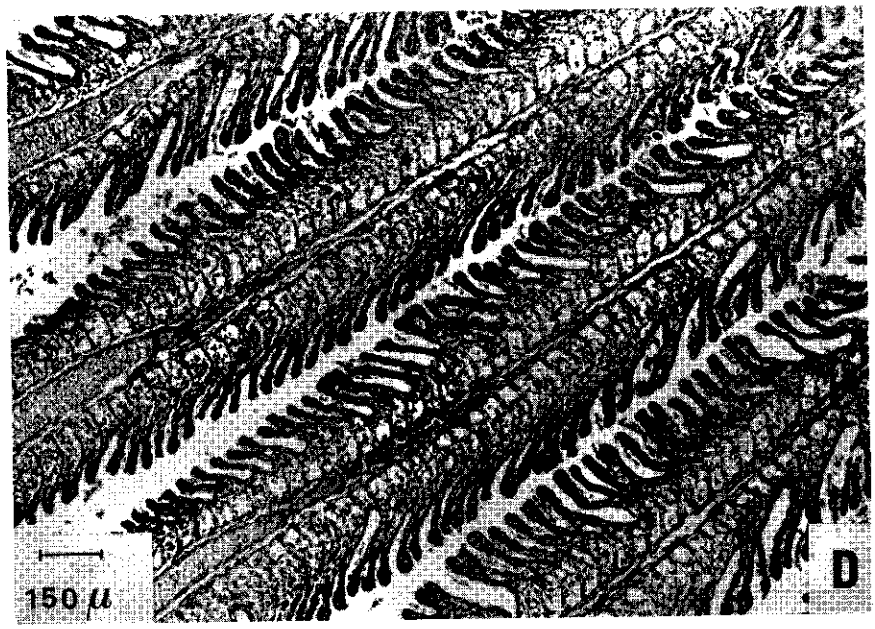
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more chromium at the lower pH, after exposure to Cr(VI) at pH 6.5 and 8.1 respectively. In addition, the positive linear correlation between Cr-transfer and O_2-transfer, may suggest that uptake mechanisms of these compounds are similar. This would indicate that chromium is taken up by the blood by passive diffusion from the external solution across the epithelium of the secondary lamellae. When it is assumed that Cr-transfer is completely coupled with O_2-transfer, and $F_{Cr} = 0$ when $T_{O_2} = 0$ (Fig. 2), it can be estimated by linear regression (Snedecor and Cochran, 1967) that Cr-transfer at pH 6.5 is 1.6 times more effective than at pH 8.1. Indeed, this small increase would be difficult to detect by in vivo experiments.

Theoretically, the pH effect on Cr-transfer may not solely be attributed to different concentrations of metal species available to the fish, but also to a change in the permeability of the gill epithelium. This has also been suggested by Hodson et al. (1978) in their study on pH-induced changes in blood-lead of lead exposed rainbow trout. On the other hand it has recently been indicated that acute acid stress in isolated perfused gills of trout, did not affect the diffusive permeability of the gill epithelium per se for tritium labelled water (Jackson and Fromm, 1980).

A positive linear correlation between O_2 and metal transfer is not found in perfused gills exposed to Cd (Pärt, unpublished results), nor are equilibrium levels in Cd-transfer reached as fast as found for Cr(VI) in the present investigation (Pärt and Svanberg, 1981).

These findings support the general idea that Cr(VI), as an oxo-anion, readily passes through membranes and behaves toxicologically in a manner quite different from most other cationic heavy metals (Gray and Sterling, 1950; Knoll and Fromm, 1960; Doudoroff and Katz, 1953).

The large variation in T_{O_2} and F_{Cr} values between perfusion experiments is probably an effect of variations in functional gill surface area.

Within the animal this factor may vary due to a change in the number of secondary lamellae being perfused at a given time (Randall, 1970; Hughes, 1972) or by changes in the relative degree of perfusion of lamellar exchange versus intralamellar bypass channels (Steen and Kruysse, 1964; Richards and Fromm, 1969). Vasoactive agents such as adrenaline and acetylcholine participate in the regulation of perfusion flow, but the exact nature of this regulation remains uncertain (Booth, 1979b). However, it has recently been established that adrenaline, which has been added to the perfusion medium in the present investigation to prevent vasoconstriction, at least increases the proportion of secondary lamellae receiving blood in intact unrestrained

trout (Booth, 1979a).

The low T_{O_2} values in perfused gills of rainbow trout that had been pre-exposed to Cr(VI) at pH 6.5 do not seem to be related only to differences in lamellar recruitment. It seems that the observed structural alterations in the gill tissue impede gas exchange by an increased diffusion distance between water and perfusion medium, and also by an ineffective irrigation of the secondary lamellae. Experimental evidence for an impaired oxygen uptake as a result of gill damage has also been given for trout exposed in vivo to Zn (Skidmore, 1970).

The tentative figures for Cr-transfer in pre-exposed gills, indicate that a similar relationship exists between Cr-transfer, time, and T_{O_2} values as found for gills which had not been pre-exposed to Cr(VI).

This would indicate that the uptake pathway of Cr(VI) in gills of trout which have been exposed during a longer period to the metal is probably not drastically changed.

Acknowledgements

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Chapter 4

RESPIRATION AND OSMOREGULATION IN RAINBOW TROUT (SALMO GAIRDNERI) EXPOSED TO HEXAVALENT CHROMIUM AT TWO DIFFERENT pH LEVELS[a]

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SUMMARY

Recordings were made of the ventilation frequency, coughing rate and oxygen uptake rate of rainbow trout (Salmo gairdneri) subjected for 4 days to sublethal levels of hexavalent chromium. Experiments were conducted at pH values of 7.8 and 6.5 and concentrations ranging between 5 - 50 and 1 - 10 mg/l Cr respectively.
During exposure no significant effect of chromium on oxygen uptake rate was detected. The ventilation frequency and coughing rate increased proportionally to an increase in metal concentration, with fish being more susceptible at the lower pH.

Alterations in blood and plasma variables determined after exposure included a significant dose-dependent decrease in plasma osmolality and electrolyte concentrations, and an increase in hemoglobin, hematocrit, plasma glucose and lactate levels. The pattern of these changes was dependent on pH and exposure concentration, and seemed to be related with chromium-induced histological alterations. The results indicate, that at pH 7.8 as well as at pH 6.5 both an osmoregulatory and respiratory dysfunction are part of the physiological mechanism of hexavalent chromium toxicity.

Key words: chromium, pH, rainbow trout, respiration, osmoregulation

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[a] Submitted to Aquat. Toxicol.

INTRODUCTION

Several studies have shown that exposure of fish to heavy metals leads to a number of disturbed physiological processes (Alabaster and Lloyd, 1980). Some work has been focussed on the respiratory changes induced by these compounds. This approach was stimulated by the suggestion that the action of metals might be primarily on the gill surface and might consequently affect gaseous exchange (Jones, 1947; Skidmore, 1970; Sellers et al., 1975). Other studies have emphasized the effect of metals on water- and ion-balance in fish (McKim et al., 1970; Lewis and Lewis, 1971; Larsson et al., 1981; Lock et al., 1981). Certainly, it has frequently been observed that acute or subacute exposure to metals causes histological alterations in organs involved in respiration (gills) and osmoregulation (gills, kidney and intestine). This has been reported for metals like cadmium (Gardner and Yevich, 1970), zinc (Skidmore and Tovell, 1972), copper (Baker, 1969) and mercury (Wobeser, 1975). What molecular and biochemical changes precede such cellular and systemic effects is not yet fully understood. Nor is it clear what relative importance an impairment of respiration or osmoregulation has for the toxic mechanism of a metal, and what factors influence the induction of this impairment (Hughes and Flos, 1978).

Exposure of fish to hexavalent chromium, Cr(VI), also has a number of effects suggestive of an osmoregulatory and/or respiratory impairment. It has been established that the metal is accumulated in gills, kidney and intestine (Knoll and Fromm, 1960; Van der Putte et al., 1981a), causes histopatholocigal changes in these tissues (Strik et al., 1975; Van der Putte et al., 1981b), adversely affects salinity tolerance (Sugatt, 1980), inhibits Na^+, K^+-ATPase in kidney and intestine (Kuhnert et al., 1976) and decreases plasma osmolality in blood of rainbow trout (Van der Putte et al., 1981b). However, at which site accumulation and histological alterations occur mainly, viz. in gills and/or internal organs, is largely dependent on water pH (Van der Putte et al., 1981a; 1981b).

The present study was performed to investigate the alterations in respiratory activity of rainbow trout during a short-term exposure to Cr(VI) at different pH values. After exposure, effects on some hematological variables, carbohydrate metabolism, plasma osmolality and electrolyte concentrations were examined to provide information on the pattern of a possible osmoregulatory and respiratory distress. Ion-regulating tissues were studied histologically in an attempt to correlate the findings with chromium-induced structural alterations.

MATERIALS AND METHODS

Fish source and maintainance

Rainbow trout (Salmo gairdneri) were reared in the laboratory from fertilized eggs obtained from a commercial hatchery. They were kept in tap-water with constant characteristics averaging 80 mg/l total hardness as $CaCo_3$, 92 mg/l alkalinity as HCO_3^- and pH 7.8 (see also Van der Putte et al., 1981a).

Fish selected for experiments were 1 to 2 year old, had weights of 195 ± 65 g (mean ± SD) and total lenghts of 27 ± 5 cm. They were fed each day a maintainance ratio of a commercial food (Trouvit). The feeding times were randomized to prevent a conditioning of the fish to a particular time schedule (Davis and Bardach, 1965; Sellers et al., 1975). Fish were not fed for 72 h before exposure to Cr(VI), in order to place them in a post-absorptive state (Beamish, 1964b; O'Hara, 1971b). The tap-water temperature during holding and testing of the trout was maintained at 13.0 ± 0.5 °C using a hot water heat exchange system. The laboratory temperature was controlled at 13 ± 2 °C. A photoperiod of 12 h light, ran from 09.00 to 21.00 h.

Exposure system

Tests were performed in an electrode-chamber system, including one control and four treatment chambers (Fig. 1). Each chamber was enclosed in a screen to shield the fish from outside visual disturbance.

Tap-water was introduced from a 100-l head reservoir via 3-l mixing vessels into the respective electrode chambers at a rate of 15 l/h. Stock solutions having different concentrations of reagent grade sodium chromate (Na_2CrO_4) and adjusted to the required pH with dilute HCl were metered into the mixing vessels at a rate of 50 ml/h. Peristaltic pumps were used for the introduction of stock solution to the mixing vessels; mixing was accomplished by a magnetic stirrer. Tap-water was delivered by a constant pressure pump with the flow being controlled by a flow meter. The pH of the tap-water in the head reservoir was kept at the required level ± 0.1 U as described by Van der Putte et al. (1981b); vigorous aeration was maintained to mix the water thoroughly and to remove excess CO_2 released by lowering of pH.

The design of the electrode chambers was based on that of Spoor et al.

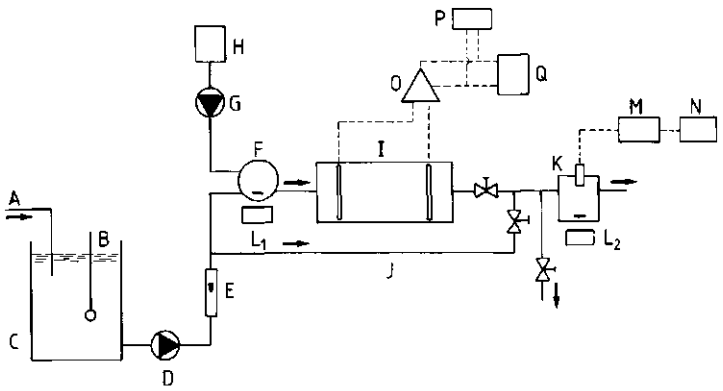

Fig. 1. Diagram of the electrode chamber in the experimental setup. (A), water supply; (B), air supply; (C), reservoir; (D), constant pressure pump; (E), flow meter; (F), mixing vessel; (G), peristaltic pump; (H), Cr stock solution; (I), electrode chamber; (J), by-pass with taps; (K), oxygen sensing probe; (L_{12}), magnetic stirrer; (M), oxygen meter; (N), recorder; (O), differential amplifier; (P), oscilloscope; (Q), visicorder.

(1971) and Roberts (1964). They consisted each of a rectangular glass aquarium (52 cm long x 15 cm wide x 10 cm high) that could be closed airtight at the top by a plexiglass cover. Water entered the chambers through a nozzle and a flow-straightening baffle similar to that of Poels (1975) and left through a perforated plate. These adaptations were necessary to prevent short-circuiting of incoming water and resulted in plug flow characterized by an axial dispersion number of approx. 0.05 determined after Levenspiel (1962) and Levenspiel and Bisschoff (1963). Recording electrodes consisting of rectangles of stainless steel mesh were placed at each end of the fish compartment and covered the total cross section of the chambers. The potential change between the electrodes induced by the ventilatory movements of the fish (Spoor et al., 1971; Drummond et al., 1973) was amplified by a differential amplifier (Frederick Haer and Co., model 74/20/1) operated at a gain of approx. 10 000. Filters were used to limit the frequency response of the amplification system to 0.5 - 5 Hz. The amplified ventilatory signals, including the ventilation frequency and coughing rate, were displayed on an oscilloscope (Hewlett-Packard, model 1223A) and on an oscillograph visicorder (Honeywell, model 1706).

The airtight construction of the electrode chamber enabled oxygen uptake measurements as well. This was accomplished by the determination of oxygen

levels in the water before and after passage through the test chambers as described by O'Hara (1971a). Oxygen determinations were made with a WTW model oxi-56 oxygen meter and records were made on a strip chart recorder. The tap-water flow rate of 15 l/h allowed the fish to lower the oxygen content in the water by 1 - 2 mg/l. The oxygen levels in the electrode chambers remained therefore above 8.0 mg/l at all times.

Experimental procedure

Individual fish were put into the electrode chambers and allowed to acclimate for 2 wk to the exposure system and water pH.
Two series of tests were performed in which the fish were exposed to Cr(VI) for 4 days. One series was conducted at pH 7.8 and nominal concentrations of 0 (control), 5, 10, 25, and 50 mg/l Cr(VI). Another series was conducted at pH 6.5 and nominal concentrations of 0 (control), 1, 2, 5, and 10 mg/l Cr(VI). Each test was replicated five times and thus 6 fish were studied at each concentration. The test concentrations varied by not more than 10% of the scheduled nominal values, as determined by daily measurements using the diphenylcarbazide colorimetric method (APHA, 1971).

Recordings of the ventilation frequency and coughing rate were made during the light period starting at 09.30 h and 2, 4, 6, and 10 h later. A 5-min section of the records was used to calculate individual group mean data. During the same period oxygen uptake by the fish was monitored twice for 1 h. The monitoring of these ventilatory and respiratory parameters was initiated 24 h before the start of Cr(VI) exposure to provide a background for the test results. The background period is referred to as day 0. Beginning with the first 24-h of exposure, test days are referred to as day 1, 2 etc.

After exposure, fish were anaesthetized within 1 min in a solution of tricaine (MS-222), neutralized with sodium bicarbonate according to the procedure described by Wedemeyer and Yasutake (1977). Blood was drawn by a heparinized syringe from the caudal vessels and analyzed for its composition. Immediately determined were hematocrit and hemoglobin according to the methods of Wedemeyer and Yasutake (1977), plasma glucose according to the enzymatic GOD-Perid method and plasma lactate according to the enzymatic lactate test-UV method (Boehringer Mannheim Corp.).
The rest of the blood was rapidly centrifuged. Plasma was immediately deep-frozen and stored at -20 °C. Later, the plasma sodium and potassium

concentrations were determined by flame atomic absorption (Perkin-Elmer, model 51 Ca). Plasma osmolality was measured by freezing point depression with a Vogel Micro Osmometer.
Histological preparations were made of gill, kidney and intestine according to the procedure described by Van der Putte et al. (1981b).

Statistics

At each pH, a two-way analysis of variance (experimental period on 6 levels vs. Cr(VI) concentrations on 5 levels) was performed on blood, plasma, respiratory and ventilatory data respectively. The mean daily increase in oxygen uptake rate, ventilation frequency and coughing rate for each fish during the exposure period of 4 days was used as the respiratory and ventilatory response in the analysis. This approach was employed because of the great individual variability in the basic ventilation and respiration pattern of unexposed fish.

The possible effects of Cr(VI) and experimental period on the responses were assumed to be additive, i.e. without interaction. Thus variances were estimated with 20 degrees of freedom (Snedecor and Cochran, 1967).
Statistical inference on the effects of Cr(VI) was carried out by considering the differences between each of the 4 treatments (Cr(VI)-exposure) and the control. Furthermore effects of Cr(VI) were decomposed in linear (first degree) and residual (second, third, and fourth degree) components by the use of orthogonal polynomial regresssion equations (Sokal and Rohlf, 1969). In all cases the critical level (P) of the appropriate t- or F- test is compared with the usual significance levels (0.1 - 0.001).

RESULTS

Alterations in respiratory activity

Analysis of variance did not show that the performance of experiments at consecutive periods of time influenced the respiratory and ventilatory response of the fish to a specific Cr(VI) concentration ($P > 0.1$).

The mean daily ventilation frequencies and coughing rates of different groups of rainbow trout exposed to Cr(VI), followed the trends shown in Figs. 2 and 3 respectively.

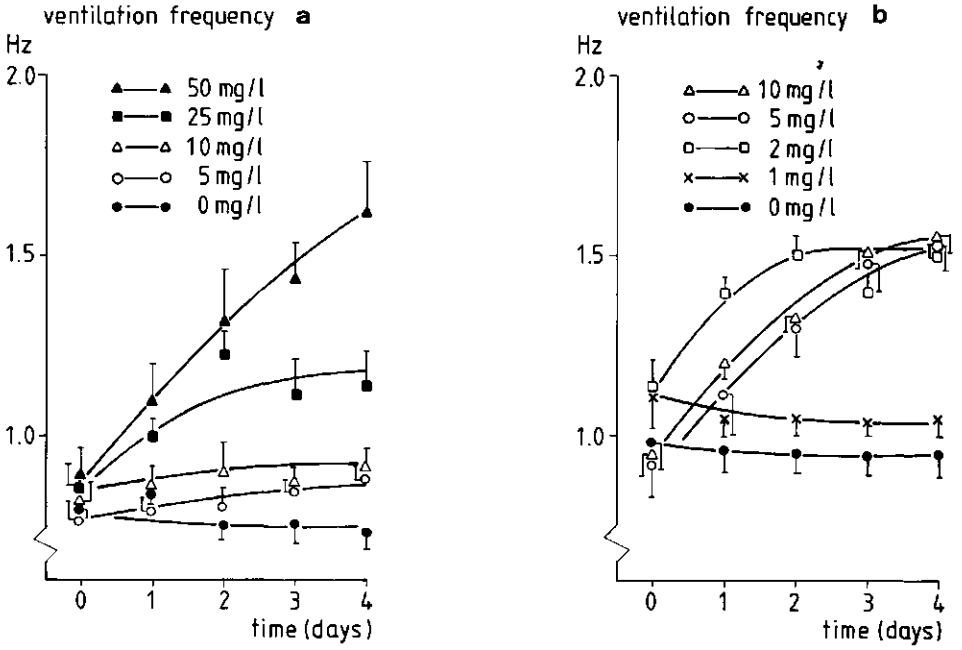

Fig. 2. Ventilation frequency of trout during exposure to various concentrations of Cr(VI) at (a), pH 7.8 and (b), pH 6.5. Values are means ± SE for 6 fish.

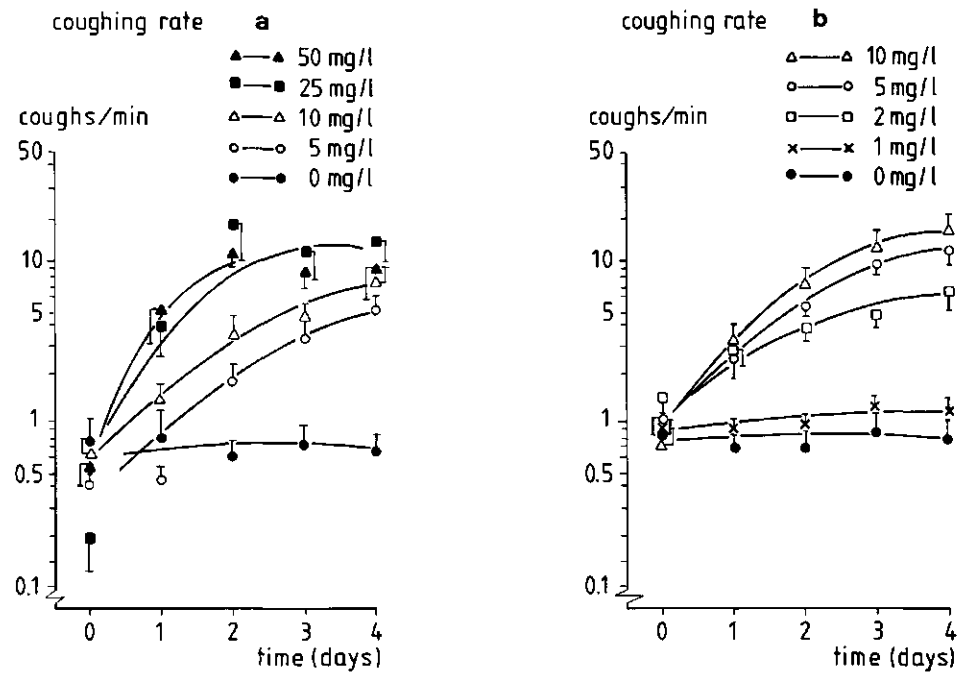

Fig. 3. Coughing rate of trout during exposure to various concentrations of Cr(VI) at (a), pH 7.8 and (b), pH 6.5. Values are means ± SE for 6 fish.

There was an overall increase in response with increasing exposure time and concentration. Exceptions are the coughing rates of fish exposed to 25 and 50 mg/l Cr(VI) at pH 7.8, that seemed to decline after exposure for 2 days. Ventilation frequencies and coughing rates of unexposed fish remained at a stable level during the whole exposure period.

The notorious variability in respiratory activity of individual fish (Sellers et al., 1975) was also reflected in the results of the present investigation. Basal ventilation frequencies ranged from 0.6 to 1.5 Hz, whereas basal coughing rates ranged from 0.1 to 2.3 coughs/min. The mean background oxygen uptake rate of the different groups of trout was 104 mg 0_2/kg fish.h and ranged from 62 to 184 mg 0_2/kg fish.h for the individual fish. It is known that due to this individual variability the absolute data cannot be used to determine the respiratory and ventilatory response of fish to a toxicant. Several methods have therefore been proposed in the literature to overcome this problem (Ultsch et al., 1980; Drummond et al., 1974). The mean daily increase in ventilation frequency, coughing rate and oxygen uptake rate, used in this study as response criteria, are presented in Fig. 4.

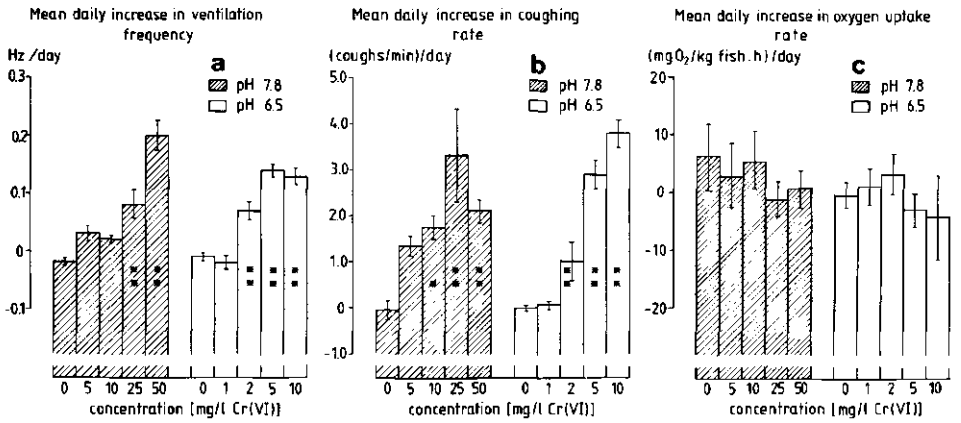

Fig. 4. Mean daily increase in (a), ventilation frequency, (b), coughing rate, and (c), oxygen uptake rate of trout during exposure for 4 days to various concentrations of Cr(VI) at pH 7.8 and pH 6.5. The columns represent mean values ± SE for 6 fish. One asterisk indicates significant difference from the control group at $\underline{P}$ <0.05, two asterisks at $\underline{P}$ <0.01.

At pH 6.5, Cr(VI) induced an increase in ventilation frequency and coughing rate at much lower concentrations than at pH 7.8. At both pH values this increase was linearly related with the exposure levels ($P < 0.001$). The oxygen uptake rate was not significantly affected by exposure to Cr(VI), although a slight decrease in the mean values was noted at pH 6.5 and concentrations of 5 and 10 mg/l Cr(VI).

Alterations in blood and plasma variables

The effects of the period of experimentation on the blood and plasma variables of rainbow trout were also found to be non significant ($P > 0.1$).

Osmotic values, electrolyte, glucose and lactate concentrations of the plasma at various Cr(VI) concentrations are presented in Table I. Both at pH 7.8 and pH 6.5 a decrease in plasma osmolality was induced by Cr(VI), but the dosage required for a significant decrease was much lower at pH 6.5.

TABLE I

Osmolality, electrolyte, lactate, and glucose concentrations of blood plasma of rainbow trout exposed for 4 days to various concentrations of Cr(VI) at pH 7.8 and pH 6.5.

pH	Cr(VI) (mg/l)	Osmolality[a] (mOsmol/l)	Na^+ [a] (meq/l)	K^+ [a] (meq/l)	Glucose[a] (mmol/l)	Lactate[a] (mmol/l)
7.8	0	292.3 ± 0.8	155.7 ± 1.9	2.5 ± 0.3	3.1 ± 0.2	1.4 ± 0.4
7.8	5	294.2 ± 1.6	141.8 ± 2.5[c]	1.8 ± 0.2[c]	3.7 ± 0.6	1.6 ± 0.2
7.8	10	293.2 ± 0.7	140.2 ± 1.5[c]	1.1 ± 0.1[c]	3.5 ± 0.4	1.8 ± 0.4
7.8	25	275.3 ± 2.8[c]	131.0 ± 1.9[c]	1.2 ± 0.1[c]	6.8 ± 0.8[b]	2.6 ± 0.2
7.8	50	263.3 ± 2.5[c]	126.8 ± 2.0[c]	1.3 ± 0.2[c]	15.2 ± 4.1[c]	5.0 ± 1.3[c]
6.5	0	298.5 ± 3.9	146.5 ± 1.7	2.1 ± 0.2	1.9 ± 0.3	1.0 ± 0.2
6.5	1	292.5 ± 5.0	151.0 ± 2.3	1.9 ± 0.2	2.6 ± 0.3	1.4 ± 0.1
6.5	2	300.5 ± 7.9	143.5 ± 5.4	1.7 ± 0.1	3.0 ± 0.3	2.8 ± 0.6[c]
6.5	5	292.3 ± 6.7	144.5 ± 4.8	1.8 ± 0.2	2.9 ± 0.2	3.3 ± 0.2[c]
6.5	10	268.2 ± 8.5[c]	134.3 ± 3.9[b]	2.2 ± 0.3	5.5 ± 1.0[c]	4.1 ± 0.3[c]

[a] Values are means ± SE for 6 fish

[b] Significantly different from control at $P < 0.05$

[c] Significantly different from control at $P < 0.01$

Furthermore, a significant decrease in plasma osmolality coincided with a decrease in sodium concentrations, and at pH 7.8 also the potassium concentrations were depressed. Glucose and lactate concentrations were increased by Cr(VI), especially at the lower pH. Except for the decrease in sodium concentration at pH 6.5, the decrease in plasma osmolality and electrolyte concentrations were linearly dependent on the exposure levels ($P < 0.001$). This linear dependency was also found for the increase in plasma glucose and lactate concentrations.

Finally it was a remarkable finding that at pH 7.8 lactate concentrations changed significantly at much higher Cr(VI) concentrations than osmolality values and electrolyte concentrations. The reverse is the case at pH 6.5. These data indicate that the pattern of alterations of plasma variables is dependent on pH.

Hematocrit values, hemoglobin and mean corpuscular hemoglobin concentrations in the blood of trout at various exposure concentrations are presented in Fig. 5.

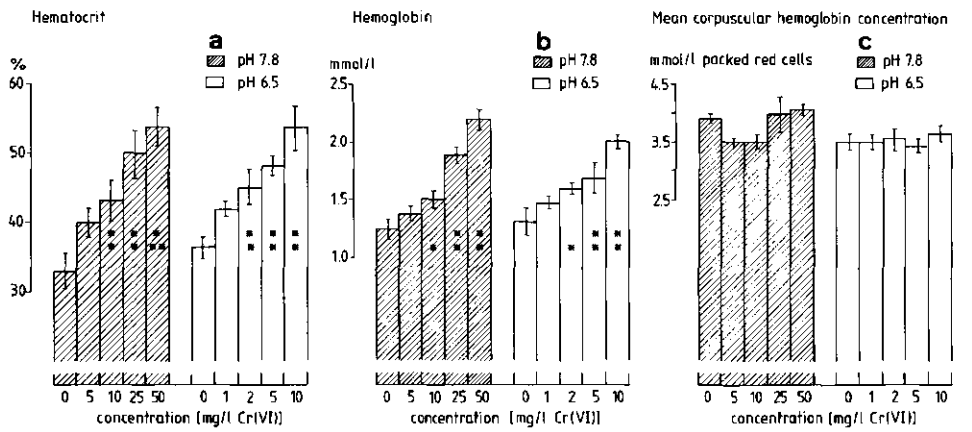

Fig. 5. (a), Hematocrit values, (b), hemoglobin, and (c), mean corpuscular hemoglobin concentrations of blood of trout exposed for 4 days to various concentrations of Cr(VI) at pH 7.8 and pH 6.5. Symbols as in Fig. 4.

At both pH values hematocrit and hemoglobin concentrations increased linearly with the exposure levels ($P < 0.001$). Significant effects were induced by much lower concentrations of Cr(VI) at pH 6.5 than at pH 7.8. The mean corpuscular hemoglobin concentration, which was calculated by divi-

ding the hemoglobin concentration x 100 by the hematocrit, did not change significantly. This indicates that the rise in hematocrit values is probably a result of an increase in number, and not in volume of red cells.

Histological alterations

Structural changes induced by exposure to Cr(VI) at different pH values in these experiments were found to be largely similar to those previously reported by Van der Putte et al. (1981b). At pH 6.5 changes were restricted to the gills and included hypertrophy and hyperplasia of the lamellar epithelium with some detachment of epithelial cells. These changes were more pronounced with increasing exposure concentrations. At pH 7.8 and Cr(VI) concentrations of 25 and 50 mg/l, lumina of kidney tubules were dilated and the nucleus-to-cytoplasma ratio in the tubular epithelium was increased. Hypertrophy and some hyperplasia of the gill lamellar epithelium occurred only at an exposure concentration of 50 mg/l Cr(VI). No histological alterations in the intestinal tract were found.

DISCUSSION

The results of the present experiments show that sublethal concentrations of Cr(VI) affect the respiratory activity and osmoregulatory function of trout at pH 7.8 as well as at pH 6.5. However, when the severity of effects is considered, Cr(VI) is more toxic at pH 6.5 than at pH 7.8: The dosage required to induce comparable effects on plasma osmolality, ventilation frequency and coughing rate was roughly 2 - 5 times higher at pH 7.8 than at pH 6.5. This is in accordance with the previously reported enhancement of the lethal action of Cr(VI) by a decrease in pH (Van der Putte et al., 1981a; 1981b).

The induced ventilatory alterations are not specific for Cr(VI), but have also been recorded in fish exposed to mercury (Drummond et al., 1974), zinc (Hughes and Adeney, 1977), copper (Drummond et al., 1973), kraft mill effluent (Walden et al., 1970) and organochlorine pesticides (Lunn et al., 1976). However, the cause of these alterations is not yet fully understood.

Heath (1973) and Bramford (1974) concluded that the arterial oxygen tension is the primary controlling factor for the ventilation frequency in fish. In the present study gill epithelial damage and increased levels of

plasma lactate suggest a blockage of oxygen uptake in the gills and a shift to some anaerobic metabolism. Indeed elevated levels in ventilation frequency, hematocrit, plasma glucose and lactate, which have been observed in the present experiments, have also been found in fish subjected to severe hypoxic conditions (Heath and Pritchard, 1965; Holeton and Randall, 1967). Direct evidence for a link between an increase in ventilation frequency and a decrease in arterial blood oxygen tension and/or increase in blood lactate concentrations has been provided for trout exposed to chlorine (Bass and Heath, 1977) and zinc (Skidmore, 1970; Sellers et al., 1975).
Another finding in support of the assumption that Cr(VI) induces a respiratory dysfunction that is at least partly due to gill damage, has recently been obtained in gill-perfusion experiments. In these experiments it was noted that the transfer of oxygen in perfused gills of trout which had been pre-exposed for 4 days to 10 mg/l Cr(VI) at pH 6.5 was greatly impaired (Van der Putte and Pärt, 1981, submitted).

The alteration of the other ventilatory parameter measured, coughing rate, is probably not caused by a decrease in arterial blood oxygen tension (Hughes, 1975). As suggested by Bass and Heath (1977) for chlorine, which is also an oxidizing agent, a general irritation of the gill membranes seems to be a better explanation for the increased coughing rate. On the other hand, it is to be expected that a drastic increase in coughing rate adversely affects the efficiency of oxygen transport as the coughing reflex involves water flow reversals at the gill surface (Davis, 1973). Oxygen deficiency in trout exposed to Cr(VI) may therefore have been caused by gill epithelial damage in conjunction with a high coughing rate.

The rate of oxygen uptake did not alter during exposure to Cr(VI), although an induced increase in ventilatory activity involved a greater energy expenditure. Possibly, alterations in oxygen uptake were masked by variations in spontaneous activity of the fish. On the other hand, the mean oxygen uptake rate value of 104 mg 0_2/kg fish.h in our experiments was only slightly higher than the standard rate of oxygen uptake at 15 °C recorded by Beamish (1964a) and Job (1955) for brook trout of the same size. It is therefore probable that the spontaneous activity of fish in the electrode chambers was generally quite low.
In addition it has been shown by Skidmore (1970) that rainbow trout with a reduced arterial oxygen tension caused by exposure to zinc, exhibited a normal rate of oxygen uptake. This is also in accordance with the observation by Holeton and Randall (1967) that the rate of oxygen uptake by trout

did not alter during progressive deoxygenation of the environment, although lactate concentrations in blood were elevated.
These data support the idea that fish exposed to Cr(VI) had to work increasingly hard to maintain an adequate rate of oxygen uptake.

The observations in the present investigation indicate that not only a respiratory impairment but also an osmoregulatory dysfunction is part of the physiological mechanism of Cr(VI) toxicity. At pH 7.8 the decrease in plasma osmolality at the higher Cr concentrations coincided with a decrease in plasma sodium and potassium concentrations, whereas at pH 6.5 only the sodium concentration was found to be depressed. This difference seems to be related with the structural alterations induced in both kidney and gills at pH 7.8 and in gills only at pH 6.5. It has been shown by Gardner and Yevich (1970) that a euryhaline teleost (Fundulus heteroclitus) exposed to cadmium also suffered from pathological alterations in renal tubules. A depression in plasma potassium concentration in flounder (Platichtys flesus L.) exposed to cadmium, has been suggested to be associated with kidney damage (Larsson et al., 1981). In parallel with the latter suggestion, it may be that a defective renal function induced by Cr(VI) at pH 7.8, resulted in an impaired reabsorption of potassium and sodium ions in the renal tubules. This is supported by the results of Kuhnert et al. (1976) indicating that at least Na^+, K^+-ATPase activity in kidney is inhibited in rainbow trout by exposure to Cr(VI) (as chromate). Na^+, K^+-ATPase activity in gills was not found to be significantly inhibited although the influence of pH had not been studied.
At the lower pH it may be that Cr(VI) changed the permeability characteristics of the gills for water, as shown for mercury in trout (Lock et al., 1981). As a consequence a greater renal loss of sodium ions will occur by an enhanced urine flow, which is not fully compensated by an increased uptake of these ions via the damaged gills.

Finally the results show that the pattern of alterations in plasma variables were dependent on pH. This would also indicate that the underlying mechanisms of chromium-induced alterations are different at pH 6.5 and 7.8, although the ultimate physiological effects, viz. osmoregulatory and respiratory dysfunction, are similar.

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Chapter 5

EFFECTS OF HEXAVALENT CHROMIUM IN RAINBOW TROUT (SALMO GAIRDNERI) AFTER PROLONGED EXPOSURE AT TWO DIFFERENT pH LEVELS[a]

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SUMMARY

Toxic effects were studied in rainbow trout (Salmo gairdneri) exposed to Na_2CrO_4 solutions of different concentrations (0.02, 0.2, and 2.0 mg/l Cr) and pH (7.8 and 6.5). The study involved an embryo-through-juvenile and an alevin-through-juvenile exposure for 32 wk, and an exposure of yearling trout for 1, 3, 6, and 12 wk respectively. The experiments were started at the same time of the year and were carried out concurrently.

When survival was used as the criterion, Cr was more toxic at pH 6.5 than at pH 7.8 in all life stages studied. For trout in the embryo-through-juvenile exposure study the lowest concentrations inducing an increase in mortality were 0.2 mg/l Cr at pH 6.5 and 2.0 mg/l Cr at pH 7.8. Embryo hatchability was only affected at the highest exposure concentration at a pH of 6.5. No growth retardation was detected at the termination of the experiments.

In addition to survival and growth, biochemical, hematological and histological changes were studied as indicators of toxicity in yearling trout. These parameters also showed that fish were more susceptible to Cr at the lower pH. The observed effects are discussed in relation to previously reported findings in short-term toxicity tests.

Key words: chromium, pH, prolonged exposure, rainbow trout

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INTRODUCTION

The toxicity of hexavalent chromium, Cr(VI), to fish has frequently been studied in short-term experiments with a duration of one to four days. Little information is available on the toxic effects due to prolonged exposure to the metal. Most of it concerns results from partial and complete life cycle tests in which reproduction and/or hatchability, growth and survival were used as criteria for toxic effect: Olson (1958) and Olson and Foster (1956, 1957) exposed chinook salmon (Oncorhynchus ishawytscha) and rainbow trout (Salmo gairdneri) at early life stages to Cr(VI); Benoit (1976) examined the chronic effects of Cr(VI) on brook trout (Salvelinus fontinalis) and rainbow trout; Sauter et al. (1976) studied the effects of Cr(VI) on eggs and alevins of seven fish species; and Pickering (1980) investigated the chronic toxicity of Cr(VI) to the fathead minnow (Pimephales promelas). In these studies the lowest concentrations causing mortality varied from 0.08 mg/l Cr(VI) for chinook salmon and rainbow trout (Olson and Foster, 1956) to 3.95 mg/l Cr(VI) for the fathead minnow (Pickering, 1980). Growth retardation occurred at even lower concentrations, but was shown to be only temporary for the species studied by Benoit (1976) and Pickering (1980). In all tests survival and/or growth of alevins were affected at concentrations lower than those that did affect hatchability or reproduction.

It is known from acute exposure studies that pH is one of the most important water characteristics modifying Cr(VI) toxicity to fish (Trama and Benoit, 1960; Van der Putte et al., 1981a; 1981b). Nevertheless, the effect of pH has not yet been studied under conditions of prolonged exposure.
The main objective of the present study was to investigate the effects of Cr(VI) on rainbow trout during exposure at different pH values for periods of up to 32 weeks. In addition to hatchability, survival and growth, attention was paid also to a number of biochemical, hematological and histological parameters.

MATERIALS AND METHODS

Fish and water characteristics

Tests were initiated with rainbow trout (Salmo gairdneri) as yearlings, swim-up alevins and eyed embryos. All trout were obtained from the same commercial hatchery as fertilized eggs. They were reared in tap-water with

constant characteristics averaging 80 mg/l total hardness as $CaCO_3$, 92 mg/l alkalinity as HCO_3^- and pH 7.8.
Tap-water was used in the tests at its original pH of 7.8 and at a decreased pH of 6.5, maintained within 0.1 U of the required pH with an automatic pH-stat which added dilute NaOH or HCl to the tap-water in reservoirs of 300 l (see also Van der Putte et al., 1981a).

Trout at the various developmental stages were acclimated to the pH of the tap-water and to the exposure system for at least 2 wk before exposure. Alevins were 5 wk old and embryos were in the late eyed stage at the start of exposure. Yearling trout were 14 mth old, had initial weights of 45 ± 10 g (mean ± SD) and total lengths of 17 ± 1 cm. Fish were fed with a commercial food according to the manufacturers scheme (Trouvit, Putten, The Netherlands). The frequency of feeding was reduced from 6 times daily for swim-up alevins to 2 - 3 times daily for trout exceeding an age of 10 wk.

The tap-water temperature during holding and testing of the trout was maintained at 12.0 ± 0.5 °C using a hot water heat exchange system. The laboratory temperature was controlled at 12 ± 2 °C. The photoperiod was 12 h light/12 h dark, with 0.5 h dimming and brightening within the light period provided by incandescent bulbs.

Exposure system

Tests were performed in identical diluter and test-chamber units, each including one control and three treatment chambers. The systems for pH control, tap-water and toxicant delivery were similar to those described by Van der Putte et al. (1981b).

Three different types of test-chambers were used depending on the developmental stages reached by the various fish groups during exposure. Glass aquaria with a water volume of 80 l were used for yearling trout and trout that had reached the 12 wk - old juvenile stage. Alevins were held in plexiglass aquaria with a water volume of 20 l, equipped with stainless steel mesh screens at the bottom. These aquaria were designed to direct the flow of test-water up through the fish compartments. Embryos were kept until 1 wk after hatch in 0.5-l chambers of a similar design, that were inserted in the water-flow system between the diluter and alevin holding chambers.

Test-water flow rates averaged 80 l/h for aquaria containing yearlings and juveniles and 16 l/h for those containing embryos and alevins to meet a flow criterion of 2 - 3 l/g of fish per day. Replacement times for 95% of the

test solutions were calculated to be less than 4 h (Sprague, 1969). The test solutions were aerated gently throughout exposure to keep the oxygen concentration above 90% saturation; debris were siphoned daily. All chambers were enclosed in a screen to shield the fish from outside visual disturbance. Embryos and alevins up to 4 wk old were protected from direct light by sheets of black plastic.

Experimental procedure

Tests were performed at nominal concentrations of 0 (control), 0.02, 0.2, and 2.0 mg/l Cr(VI) and pH values of 7.8 and 6.5 respectively. The test concentrations varied by not more than 5% of the scheduled nominal values as determined by weekly measurements using the diphenylcarbazide colorimetric method as described by APHA (1971). The pH was checked daily and was always within 0.1 U of the required value.

The exposures of trout at the various developmental stages were started simultaneously in April 1980. The embryo-through-juvenile exposure lasting 32 wk, was initiated with 100 eyed embryos at each concentration. After hatch, the number of alevins were randomly reduced to 50 per concentration.

The 32-wk alevin-through-juvenile exposure was initiated with 50 alevins per concentration. Mortality in embryos, alevins and juveniles was recorded daily. After exposure total wet weights of individual surviving fish were measured. Length measurements were made during the embryo-through-juvenile exposure at intervals of 6, 10, 16, 24, and 32 wk after the start of exposure using the photographic method described by Martin (1969).

Exposure of yearling trout was initiated with 25 fish per concentration. At intervals of 1, 3, 6, and 12 wk of exposure 5 fish out of each group were sampled and anaesthetized in a solution of tricaine (MS-222) neutralized with sodium bicarbonate, according to the procedure described by Wedemeyer and Yasutake (1977). Total lengths and wet weights were determined, and blood was drawn from the caudal vessels by a heparinized syringe and subsequently analyzed for its composition. Hematocrit and hemoglobin were determined immediately according to the methods of Wedemeyer and Yasutake (1977). Plasma glucose was measured according to the enzymatic GOD-Perid method and plasma lactate according to the enzymatic lactate test-UV method (Boehringer Mannheim Corp.). The remainder of the blood was centrifuged. Plasma was deep-frozen immediately and stored at

-20 °C. At a later stage, the plasma sodium concentrations were determined by flame atomic absorption (Perkin-Elmer, model 51Ca). Plasma osmolality was measured by freezing point depression with a Vogel Micro Osmometer.

Histological preparations were made of gill and kidney according to the procedure described by Van der Putte et al. (1981b). Na^+/K^+-ATPase activity in the microsomal fraction of gill and kidney tissues was determined according to the procedure described by Lock et al. (1981).

A statistical analysis of the data was carried out by testing control values against treatment values according to the Student's t-test and Wilcoxon's two sample rank test.

RESULTS

Embryo-through-juvenile exposure

Hatchability, survival and growth data are presented in Table I. Percentage mortality and total lengths of fish at different time intervals of exposure are given in Figs. 1a-b and 2a-b respectively.

At pH 7.8 embryo hatchability was not affected by Cr(VI) as 98 - 100% of the embryos hatched successfully at all concentrations tested including the control (Table I). Survival and growth of fish from exposed embryos were adversely affected at a concentration of 2.0 mg/l. In this group mortality increased progressively until 68% had died at the termination of the test; mortality in the control group amounted to 19% (Fig. 1a).
In addition yolk absorption was slower than in the control and the smallest fish were observed to be always the first to die. They were lethargic, did not swim up to accept food and eventually starved to death. Although growth in length was significantly retarded after 16 and 24 wk of exposure ($P<0.01$, Wilcoxon's two sample rank test), no significant difference in growth relative to the controls was observed at the termination of exposure. This was probably caused by the fact that only the larger fish had survived the exposure to 2.0 mg/l Cr(VI). Survival and growth of fish exposed to concentrations of 0.2 and 0.02 mg/l Cr(VI) at pH 7.8 were similar to those of the control fish.

At pH 6.5 embryo hatchability was slightly affected at a concentration of 2.0 mg/l Cr(VI). Only 84% of the embryos hatched successfully at this concentration in contrast to 98 - 100% at all other concentrations tested including the control (Table I). Within 3 wk of exposure to 2.0 mg/l Cr(VI) all

TABLE I

Hatchability, survival and wet weight of rainbow trout exposed for 32 wk to various concentrations of Cr(VI) at pH 7.8 and pH 6.5.

pH	Exposure concn. (mg/l CrVI)	Embryo-juvenile exposure			Alevin-juvenile exposure	
		Hatchability (%)	32-wk survival (%)	32-wk wet weight[a] (g)	32-wk survival (%)	32-wk wet weight[a] (g)
7.8	0.0	98	82	15.0 ± 5.6 (41)[b]	79	17.5 ± 6.5 (39)[b]
7.8	0.02	99	78	15.3 ± 5.0 (39)	75	18.3 ± 9.4 (37)
7.8	0.2	98	76	10.8 ± 4.0 (38)	76	18.4 ± 9.0 (38)
7.8	2.0	100	32	12.6 ± 5.3 (16)	44	18.0 ± 4.2 (22)
6.5	0.0	98	82	15.4 ± 5.2 (41)	78	19.6 ± 9.1 (39)
6.5	0.02	98	80	14.2 ± 6.0 (40)	75	20.1 ± 9.6 (37)
6.5	0.2	100	40	15.8 ± 6.9 (20)	72	19.5 ± 9.6 (36)
6.5	2.0	84	0	---	0	---

[a] Values are means ± SD

[b] Numbers of fish in parenthesis

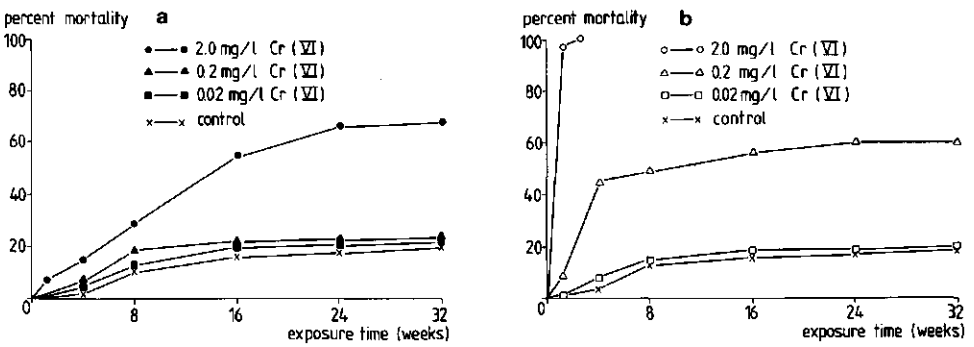

Fig. 1. Percentage mortality of rainbow trout during the embryo-through-juvenile exposure to various concentrations of Cr(VI) at pH 7.8 (a) and pH 6.5 (b).

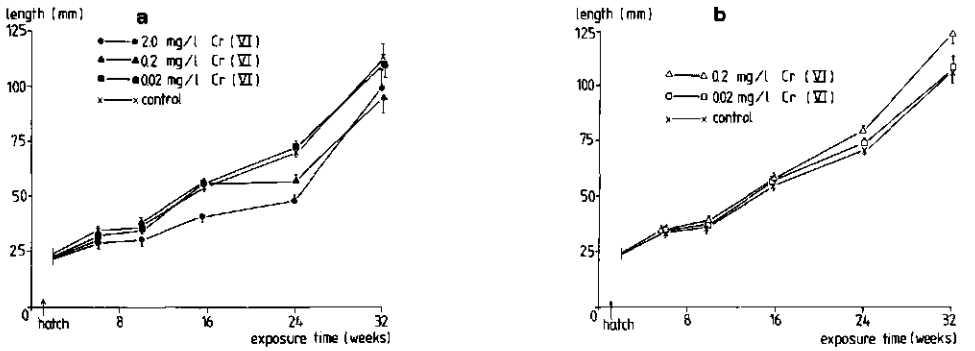

Fig. 2. Total lengths of rainbow trout during the embryo-through-juvenile exposure to various concentrations of Cr(VI) at pH 7.8 (a) and pH 6.5 (b). Values are means ± SE for 15 fish.

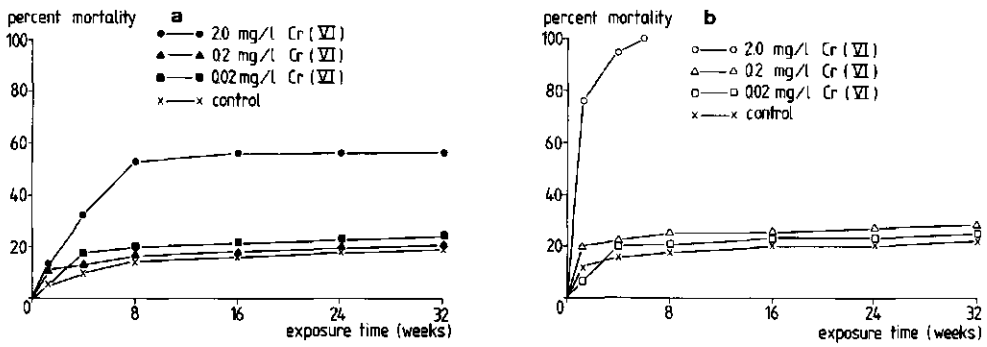

Fig. 3. Percentage mortality of rainbow trout during the alevin-through-juvenile exposure to various concentrations of Cr(VI) at pH 7.8 (a) and pH 6.5 (b).

fish had died. At pH 6.5 fish survival was also affected by exposure to 0.2 mg/l Cr(VI). At this concentration and pH mortality was 43% and 60% after exposure for 4 wk and 32 wk respectively. During the same period mortality of control fish increased from 4% to 18% (Fig. 2b). Growth at pH 6.5 was not affected by Cr(VI) at all exposure concentrations tested. Survival of fish at 0.02 mg/l Cr(VI) was also similar to that of the control fish.

Alevin-through-juveline exposure

Survival and growth data at the termination of the 32 wk alevin through juvenile exposure at different pH values are presented in Table I.
Mortality of fish at different time intervals of exposure is given in Fig. 3a-b.

At pH 7.8 as well as at pH 6.5 only survival was observed to be affected during the first 8 wk of exposure to 2.0 mg/l Cr(VI). After this 8 wk exposure period complete mortality had occurred at pH 6.5, whereas the mortality percentage at pH 7.8 was only 56%. Mortality of the control group was 15%. The difference in mortality between controls and groups exposed to 0.2 and 0.02 mg/l Cr(VI) respectively was less than 5%.

Exposure of yearling trout

Mortality of yearling trout occurred only in the group exposed to a concentration of 2.0 mg/l Cr(VI) at pH 6.5. In this group 3 out of 10 fish died in the period from 6 - 12 wk after the start of the experiment. Data on growth and chromium induced changes in blood parameters after 1, 3, 6 and 12 wk of exposure are presented in Tables II and III for pH 7.8 and pH 6.5 respectively. Data on Na^+/K^+-ATPase activity of kidney and gill tissue after 12 wk of exposure are presented in Table IV.

None of the exposure concentrations tested did affect growth, gill Na^+/K^+-ATPase activity, plasma glucose and plasma lactate concentrations.

At pH 7.8 a transient increase occurred in hematocrit values during the first week of exposure to 0.2 and 2.0 mg/l Cr(VI). Kidney Na^+/K^+-ATPase activity was increased at a concentration of 2.0 mg/l Cr(VI) after 12 wk of exposure.

At pH 6.5 chromium-induced changes in blood parameters were observed at 2.0 mg/l Cr(VI) and were not found to be of a transient nature. These changes included a significant increase in hematocrit values accompanied by an increase in hemoglobin concentrations after 3, 6, and 12 wk of exposure,

TABLE II

Blood and plasma variables, total length and wet weight of yearling trout exposed to various concentrations of Cr(VI) at pH 7.8 for 1 wk (A), 3 wk (B), 6 wk (C) and 12 wk (D)[a].

	Exposure concn. mg/l Cr(VI)	Plasma osmolality (mOsm/l)	Plasma Na^+ (meq/l)	Plasma glucose (mmol/l)	Plasma lactate (mmol/l)	Hematocrit (%)	Hemoglobin (mmol/l)	Length (cm)	Weight (g)
A	0.0	300±14	145±11	2.1±0.8	1.3±0.6	37±3	1.2±0.2	18±1	57± 6
	0.02	294±15	146± 8	2.0±0.3	1.2±0.5	38±5	1.1±0.1	18±2	56±10
	0.2	295±13	147±11	2.2±0.3	1.3±0.8	40±1*	1.1±0.1	18±1	52±10
	2.0	305±15	142±10	2.2±0.5	1.7±0.7	41±3*	1.1±0.1	18±1	51±10
B	0.0	305± 8	148± 5	3.9±1.1	1.0±0.4	39±6	1.2±0.1	19±1	82± 8
	0.02	303±18	153± 5	3.5±0.6	1.0±0.4	38±2	1.0±0.2	19±1	80±10
	0.2	300±14	143± 4	3.6±1.0	1.1±0.8	41±3	1.1±0.1	19±1	79±11
	2.0	306±15	149±14	3.5±1.0	0.9±0.6	39±3	1.0±0.2	18±3	71±21
C	0.0	308± 8	149± 9	1.5±0.4	1.0±0.3	43±2	1.4±0.2	21±1	97± 9
	0.02	307±20	147± 3	1.7±0.4	0.6±0.2	40±3	1.5±0.1	20±2	89±16
	0.2	289±33	150± 7	1.9±0.2	1.0±0.3	40±7	1.4±0.1	20±1	85±12
	2.0	305± 7	148±10	2.1±0.6	1.2±0.9	42±3	1.5±0.2	20±2	94±27
D	0.0	294±13	149± 3	2.0±0.4	2.5±2.0	37±3	1.3±0.2	22±2	115±60
	0.02	295±20	147± 4	2.2±1.2	1.7±0.5	37±4	1.2±0.1	22±2	131±19
	0.2	302±34	147± 2	2.0±0.9	1.6±1.0	41±4	1.4±0.2	22±1	132±37
	2.0	309±20	149±11	2.6±0.3	2.7±1.7	36±6	1.3±0.3	21±2	121±29

[a] Values are means ± SD for 5 fish; Significantly different from control at *$P<0.05$ (Student's t-test)

TABLE III

Blood and plasma variables, total length and wet weight of yearling trout exposed to various concentrations of Cr(VI) at pH 6.5 for 1 wk (A), 3 wk (B), 6 wk (C) and 12 wk (D)[a].

	Exposure concn. mg/l Cr(VI)	Plasma osmolality (mOsm/l)	Plasma Na^+ (meq/l)	Plasma glucose (mmol/l)	Plasma lactate (mmol/l)	Hematocrit (%)	Hemoglobin (mmol/l)	Length (cm)	Weight (g)
A	0.0	299±20	148± 6	2.3±0.7	1.0±0.5	37±1	1.2±0.1	18±1	60± 7
	0.02	295±14	148±12	2.2±0.4	1.6±0.4	38±1	1.1±0.2	17±2	55±17
	0.2	286± 8	154± 9	1.9±0.3	1.3±0.6	38±6	1.2±0.2	17±1	53±12
	2.0	264±14**	136± 5**	2.4±0.6	1.6±0.5	46±4**	1.2±0.2	18±1	58± 9
B	0.0	297± 9	148± 6	3.1±1.0	1.3±0.5	37±3	1.1±0.2	19±2	78±16
	0.02	298± 6	149± 4	3.5±0.9	1.0±0.6	40±3	1.1±0.2	20±1	82± 4
	0.2	304± 7	144±12	3.6±0.6	1.5±1.3	40±3	1.1±0.2	18±2	67±16
	2.0	298±12	150±10	2.9±1.2	0.8±1.0	44±6**	1.3±0.1*	17±3	61±28
C	0.0	300±15	146± 6	1.9±0.5	0.8±0.3	37±5	1.4±0.1	19±2	79±16
	0.02	310±15	145± 6	2.0±0.8	0.9±0.5	44±5	1.4±0.1	20±2	96±13
	0.2	299±13	144± 7	2.2±1.4	1.6±2.2	44±5	1.4±0.2	20±1	85±10
	2.0	276±23*	135±14*	3.6±3.3	0.6±0.2	56±9**	1.6±0.2*	19±3	79±25
D	0.0	299±10	148± 5	2.0±0.6	2.0±0.5	37±1	1.3±0.2	21±2	112±20
	0.02	290±10	145± 7	2.1±0.4	2.6±1.5	35±3	1.2±0.2	21±1	115±41
	0.2	283±18	146± 6	2.0±0.2	1.4±0.9	35±4	1.1±0.2	22±3	123±42
	2.0	241±23**	134± 5**	2.0±0.5	3.2±2.0	43±7**	1.7±0.2*	20±1	85±24

[a] Values are means ± SD for 5 fish; Significantly different from control at *$P<0.01$ and *$P<0.05$ (Student's t-test).

TABLE IV

Na^+/K^+-ATPase activity in the microsomal fraction of the gills and kidney of yearling trout exposed for 12 wk to various concentrations of Cr(VI) at pH 7.8 and pH 6.5.

pH	Exposure conc. (mg/l Cr(VI)	Na^+/K^+-ATPase activity[a]	
		Gills	Kidney
7.8	0.0	0.68 ± 0.32	2.48 ± 1.61
7.8	0.02	0.57 ± 0.29	3.78 ± 2.12
7.8	0.2	0.73 ± 0.21	4.62 ± 2.48
7.8	2.0	0.95 ± 0.63	5.02 ± 1.06**
6.5	0.0	0.52 ± 0.27	1.93 ± 0.73
6.5	0.02	0.40 ± 0.29	3.20 ± 1.38
6.5	0.2	0.55 ± 0.21	5.41 ± 1.55**
6.5	2.0	0.47 ± 0.63	4.32 ± 1.86**

[a] Activity is expressed in µmol ATP hydrolyzed per mg protein per hour at 37 °C (mean ± SD for 5 fish); Significantly different from control at **$P<0.01$ (Student's t-test).

and a significant decrease in plasma osmolality and sodium concentrations after 1, 3, and 12 wk of exposure. Histological alterations were found in the gills of fish exposed to 2.0 mg/l Cr(VI) at pH 6.5. These alterations included hypertrophy and hyperplasia of the gill epithelium, that increased in severity with increasing exposure times. A significant increase in Na^+/K^+-ATPase activity in kidney tissue was observed after 12 wk exposure to concentrations of 0.2 and 2.0 mg/l Cr(VI) at pH 6.5

DISCUSSION

When survival was used as a criterion of toxicity, Cr(VI) was more toxic at pH 6.5 than at pH 7.8. The lowest concentrations that induced an increase in mortality relative to the controls, were 0.2 mg/l Cr(VI) at pH 6.5 and 2.0 mg/l Cr(VI) at pH 7.8 for trout in the embryo-through-juvenile

exposure study. Hatchability was only affected at the highest concentration (2.0 mg/l CrVI) at pH 6.5.

Pickering (1980) found that chronic exposure of fathead minnow to Cr(VI) at a concentration of 3.95 mg/l adversely affected survival of both first- and second-generation fish whereas the hatching success of embryos was not affected. Sauter et al. (1976) reported on the toxicity of chromium to the eggs and alevins of seven fish species. In all tests survival and/or growth of alevins were affected at concentrations lower than those that affected hatchability. This is in accordance with the results obtained during the embryo-through-juvenile exposure in the present investigation.

Based on survival it was found that at pH 6.5 fish from exposed embryos in the embryo-through-juvenile exposure were more susceptible to Cr(VI) than fish from unexposed embryos in the alevin-through-juvenile exposure. This has also been reported for rainbow trout chronically exposed to lead (Davies et al., 1976). At pH 7.8 this difference in susceptibility was negligible and this seems to be in accordance with the effects of Cr(VI) on brook trout as reported by Benoit (1976).

Chromium-induced mortality in trout at early life stages occurred within the first 24 wk of exposure, after which no further increase in mortality was observed. Growth of these fish at the end of the exposure period of 32 wk was not found to be significantly retarded. However, especially at the highest exposure concentration and a pH of 7.8, it could clearly be seen that the smaller fish were the first to die. This observation was also made by Pickering (1980) in studying the effects of Cr(VI) on the fathead minnow. Growth effects at the higher exposure concentrations in the present study could therefore have been masked by an increased mortality of the fish.

In yearling trout only at a concentration of 2.0 mg/l Cr(VI) and a pH of 6.5 effects were found that were not of a transient nature. These effects included histological alterations in gills, increased hematocrit and hemoglobin values, decreased plasma osmolality and sodium concentrations, and mortality at the last stage of exposure.

Fish gills are involved in both osmoregulation and respiration, and results from acute exposure studies suggest that an impairment of these two functions are part of the physiological mechanism of Cr(VI) toxicity in fish (Van der Putte et al., 1981b; 1981c). The increased hemoglobin and hematocrit values may therefore reflect a hypoxic stress resulting in secondary polycythemia. Plasma glucose and lactate levels were not elevated by the

tested Cr(VI) concentrations indicating that hypoxic stress, if it occurred, was at least not detrimental to the fish after prolonged exposure. However, plasma osmolality and sodium concentrations seemed to decrease with increasing exposure time, indicating that trout were not able to compensate adequately for a loss of plasma ions. Mortality of yearling trout after 6 - 12 wk of exposure to 2.0 mg/l Cr(VI) at pH 6.5 was probably caused primarily by a disturbed osmotic and ionic balance in the fish.

Gill Na^+/K^+-ATPase activity in yearling trout was not affected by Cr(VI), whereas the activity in kidney increased at the higher exposure concentrations. Kuhnert et al. (1976) also found that exposure of rainbow trout to 2.5 mg/l Cr(VI) for 48 h did not affect gill Na^+/K^+-ATPase activity significantly. On the other hand kidney Na^+/K^+-ATPase activity in their study was inhibited which is in contrast with our findings after 12 wk exposure to approximately the same concentration.

Inhibition of Na^+/K^+-ATPase activity in the ion-regulating tissues could therefore not have been the direct cause for a decrease in plasma osmolality and sodium concentrations in yearling trout exposed for 12 wk to 2.0 mg/l Cr(VI) at pH 6.5. It may be that Cr(VI) at the lower pH increased the permeability of the gills for water, as shown for mercury in trout (Lock et al., 1981). Consequently a greater renal loss of ions may occur by an enhanced urine flow, which is not fully compensated by an increased uptake of these ions via kidney and/or damaged gills.

In the present series of experiments test concentrations lower than 2.0 mg/l Cr(VI), did not induce permanent alterations of the blood and plasma parameters of yearling trout. Survival of trout exposed at early life stages to Cr(VI) was therefore a more sensitive indicator of toxicity. It can be estimated that the concentrations affecting trout survival during the embryo-through-juvenile exposure lie between 0.2 and 2.0 mg/l Cr(VI) at pH 7.8 and between 0.02 and 0.2 mg/l Cr(VI) at pH 6.5 respectively. Previously reported 96-h LC50 values for rainbow trout (Van der Putte et al., 1981b) ranged from 12.2 to 65.5 mg/l Cr(VI) at pH 7.8 and from 3.4 to 20.2 mg/l Cr(VI) at pH 6.5 for fish weighing from 0.2 to 25.0 g.

These data clearly show that pH should be considered as an important factor in the evaluation of the toxicity of Cr(VI) to fish in both acute and prolonged exposure studies.

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General summary and conclusions

At present chromium is a common contaminant in surface waters in many countries. In water the metal may be present in the trivalent form (CrIII) or in the hexavalent form (CrVI), the latter of which is more toxic to aquatic organisms.

The investigations presented in this thesis were aimed at a thorough understanding of the mechanism of action of hexavalent chromium in fish. The process of uptake and elimination was studied in detail, while special attention was paid to physiological and histological alterations underlying the toxic effects. Water pH was taken into consideration as a varying environmental factor, because of its known influence on the oxidizing action of Cr(VI) and on the distribution of the ionic species of Cr(VI) in water. Rainbow trout (Salmo gairdneri) was used as the test-species.

Chapter 1 describes the effect of pH on uptake, tissue distribution and retention of Cr(VI) in trout. In trout exposed to Cr(VI) at pH 7.8 for 2-4 days the highest contents of chromium were found in gill, liver, kidney and digestive tract. Upon transfer of exposed fish to tap-water, chromium disappeared rapidly from blood, gill and digestive tract, whereas chromium contents in liver tended to remain high and in kidney even tended to increase. When the pH was decreased from 7.8 to 6.5, a different pattern of accumulation and elimination of chromium was observed. The major differences were found in the gills which concentrated significantly more chromium at pH 6.5 than at pH 7.8, irrespective of the exposure time and concentration. As the intensity of the electron-spin-resonance signal characteristic for trivalent or pentavalent chromium in the gills was somewhat higher at pH 6.5 than at pH 7.8, the differences must have been at least partly due to the higher oxidizing action of Cr(VI) at the lower pH.

Chapter 2 deals with the effect of pH on the acute toxicity of Cr(VI) to trout. The lethal action of the metal increased with decreasing pH in the range from 7.8 to 6.5. Morphological changes that could be associated with acute Cr(VI) poisoning at pH 7.8 were found in gills, kidney and stomach, whereas those at pH 6.5 appeared to be restricted to the gills. These findings are in accordance with the results obtained in the Cr-uptake experiments. Consequently, the general assumption that Cr(VI) elicits its toxic effect in some internal organ and that the gill is not the target organ in acute Cr(VI) toxicity, is only appropriate at relatively high pH levels. At pH 6.5 the gills seems to be the primary target organ.

To explain the acute toxic effects, hydrochromate ($HCrO_4^-$) and chromate (CrO_4^{2-}) were considered as the toxic species of Cr(VI). The relative toxicities of these ionic species were calculated from empirical toxicity relationships for weak acids in fish, as described in the literature. By this calculation it was found that the relative toxicity of $HCrO_4^-$ to trout was from 3.6 to 11.9 times greater than that of CrO_4^{2-}, depending on exposure time and fish weight.

Chapter 3 reports on an in vitro study on transfer of oxygen and chromium in gills of trout. Gills were perfused according to the isolated head perfusion technique and externally exposed to Na_2CrO_4 solutions containing $^{51}CrO_4^{2-}$.
The results show that the transfer of chromium is directly coupled with the transfer of oxygen from the external solution to the internal perfusion medium. Under similar conditions of oxygen transfer, however, chromium transfer was significantly more effective at pH 6.5 than at pH 8.1.
The data suggest that chromium is taken up by the blood by passive diffusion from the external solution across the epithelium of the secondary lamellae. In addition it is indicated that the availability of the metal to the fish increases with decreasing pH.
Gill preparations of trout that were structurally damaged by pre-exposure in vivo to Cr(VI), exhibited an impaired oxygen transfer.

Chapter 4 describes the effect of Cr(VI) on respiration and osmoregulation in trout. Recordings were made of the ventilation frequency, coughing rate and rate of oxygen uptake in trout subjected to sublethal concentrations of Cr(VI) for 4 days at pH 7.8 and 6.5.
During exposure no significant effect of chromium on oxygen uptake rate was detected. The ventilation frequency and coughing rate increased proportionally to an increase in metal concentration, with fish being more susceptible at the lower pH.

Alterations in blood and plasma variables determined after exposure indicated a significant dose-dependent decrease in plasma osmolality and electrolyte concentrations, and an increase in hemoglobin, hematocrit, plasma glucose and lactate levels. The pattern of these changes was dependent on pH and exposure concentration, and seemed to be related with chromium-induced histological alterations. The results indicate, that at pH 7.8 as well as at pH 6.5 both an osmoregulatory and respiratory dysfunction are part of the physiological mechanism of hexavalent chromium toxicity.

In chapter 5 toxic effects of Cr(VI) are described in trout after pro-

longed exposure at different pH values for periods of up to 32 weeks. Different developmental stages were studied.
In all stages tested, fish were more susceptible to Cr(VI) at pH 6.5 than at pH 7.8. Fish in the embryo-through-juvenile stage were the most susceptible to Cr(VI). For this developmental stage it can be estimated that concentrations affecting survival lie between 0.2 and 2.0 mg/l Cr(VI) at pH 7.8 and between 0.02 and 0.2 mg/l Cr(VI) at pH 6.5.
Finally, the observed effects of a prolonged exposure are discussed in relation with the results of the short-term toxicity tests described in the preceeding chapters.

The toxicological evaluation of chromium in surface waters in the Netherlands is hampered by a lack of information on the proportion of trivalent and hexavalent forms of chromium in the total chromium content. Until now only total chromium contents have been determined at various sampling locations. If one compares the total chromium levels in Dutch surface water with the lowest toxic concentrations of Cr(VI) in trout, it can not be excluded that chromium concentrations may locally exceed toxic levels for fish. However, a final conclusion can only be drawn when additional information on the chemical status of the metal in the aquatic environment in the Netherlands has been made available.

Algemene samenvatting en conclusies

Chroom komt in veel landen als contaminant voor in het oppervlaktewater. Het metaal kan in water in de driewaardige vorm, Cr(III), en/of in de zeswaardige vorm Cr(VI), aanwezig zijn. Het zeswaardige chroom is toxischer voor aquatische organismen dan de driewaardige vorm.

Het in dit proefschrift beschreven onderzoek had tot doel een diepgaand inzicht te verkrijgen in het werkingsmechanisme van zeswaardig chroom bij vissen. De opname en eliminatie van chroom werden grondig bestudeerd, terwijl tevens uitgebreide aandacht besteed werd aan de fysiologische en histologische veranderingen, die aan de toxische effecten ten grondslag liggen. De pH van het water werd als een variërende milieufactor in het onderzoek betrokken, daar het bekend is dat deze parameter van invloed is op de oxiderende werking van Cr(VI) alsmede op de verdeling van de Cr(VI)-ionenspecies. Als proefdier werd de regenboogforel (Salmo gairdneri) gebruikt.

In hoofdstuk 1 wordt het effect van de pH op de opname, weefselverdeling en retentie van Cr(VI) bij de forel beschreven. Het bleek dat in forellen, die gedurende 2-4 dagen aan Cr(VI) bij een pH van 7,8 blootgesteld waren, de hoogste concentraties chroom voorkwamen in kieuw, lever, nier en darmstelsel. Na de overbrenging in leidingwater van de blootgestelde vissen namen de chroomconcentraties in bloed, kieuwen en darmstelsel snel af, terwijl de chroomconcentratie in de lever hoog bleef en die in de nier zelfs toenam.

Bij een verlaging van de pH van 7,8 tot 6,5 werd een ander accumulatie- en eliminatiepatroon van chroom waargenomen. De belangrijkste verschillen traden op in de kieuwen, die significant meer chroom accumuleerden bij pH 6,5 dan bij pH. 7,8, onafhankelijk van de blootstellingsduur en de concentratie. In het kieuwweefsel werd tevens een electron-spin-resonantiesignaal waargenomen, dat karakteristiek was voor driewaardig of vijfwaardig chroom. De intensiteit van dit signaal was bij vissen, die waren blootgesteld bij pH 6,5 enigszins hoger dan die welke waren blootgesteld bij pH 7,8.

Hieruit kan worden geconcludeerd dat de waargenomen verschillen op zijn minst ten dele veroorzaakt zijn door de grotere oxiderende werking van Cr(VI) bij de lagere pH waarde.

In hoofdstuk 2 wordt de invloed beschreven van de pH op de acute toxiciteit van Cr(VI). De letale werking van het metaal nam toe met een dalende pH (7,8 naar 6,5). Morfologische veranderingen traden na een acute

Cr(VI)-vergiftiging bij pH 7,8 op in de kieuwen, nier en maag, terwijl deze bij pH 6,5 alleen werden waargenomen in de kieuwen.
Deze bevindingen kwamen overeen met de resultaten, welke waren verkregen in de Cr-opname experimenten. Op basis van deze gegevens kan men concluderen dat de algemeen verbreide opvatting, dat Cr(VI) zijn toxische werking in één der interne organen uitoefent en de kieuw niet het primaire effector-orgaan is bij een acute Cr(VI) vergiftiging, slechts bij relatief hoge pH waarden geldig is. De resultaten wijzen erop dat de kieuw bij pH 6,5 het primaire effector-orgaan is. Ter verklaring van de pH afhankelijke toxiciteit werd aangenomen, dat hydrochromaat ($HCrO_4^-$) en chromaat (CrO_4^{2-}) als de toxische species van Cr(VI) beschouwd kunnen worden. De relatieve toxiciteit van deze ionenspecies werd berekend de hand van empirische toxiciteits-relaties, zoals die in de literatuur voor zwakke zuren bij vissen beschreven zijn. Door middel van deze methode werd berekend dat de relatieve toxiciteit van $HCrO_4^-$ voor de forel, afhankelijk van de blootstellingsduur en het gewicht van de vissen een factor 3,6 tot 11,9 groter was dan die van CrO_4^{2-}.

In hoofdstuk 3 wordt een in vitro onderzoek beschreven naar de overdracht van zuurstof en chroom in de kieuwen van de forel. Kieuwen werden geperfuseerd volgens de geïsoleerde kop perfusietechniek en vervolgens extern blootgesteld bij pH 8,1 en 6,5 aan Na_2CrO_4 oplossingen, gemerkt met $^{51}CrO_4^{2-}$. De overdracht van chroom bleek direct gekoppeld te zijn aan de overdracht van zuurstof uit de externe oplossing naar het interne perfusiemedium. De overdracht van chroom was echter significant effectiever bij pH 6,5 dan bij pH 8,1 en dit bij identieke omstandigheden van zuurstofoverdracht. Structureel beschadigde kieuwpreparaten afkomstig van forellen, die in vivo waren blootgesteld aan Cr(VI), vertoonden een geremde zuurstofoverdracht.
De resultaten ondersteunen de hypothese dat chroom via een passief diffusieproces uit de externe oplossing door het epitheel van de secundaire lamellen in het bloed wordt opgenomen. Bovendien wijzen de bevindingen erop dat de beschikbaarheid van het metaal voor de vis toeneemt bij een dalende pH.

In hoofdstuk 4 wordt het effect van Cr(VI) op de respiratie en osmoregulatie bij de forel beschreven. De ademhalings- en hoestfrequentie en de snelheid van zuurstofopname werden geregistreerd bij forellen die gedurende 4 dagen aan subletale Cr(VI)-concentraties werden blootgesteld bij pH 7,8 en 6,5. Tijdens de blootstelling werden geen significante effecten van

chroom op de zuurstofopnamesnelheid waargenomen. De ademhalings- en hoestfrequentie namen proportioneel toe met een toenemende metaalconcentratie, terwijl de gevoeligheid van de vissen groter was bij de lagere pH waarde. De veranderingen in bloed- en plasmaparameters, die na de blootstelling werden bepaald, bestonden uit een significante en dosis-afhankelijke afname in osmolaliteit en elektrolytconcentraties en een toename in hemoglobine-, hematocriet-, glucose- en lactaatwaarden. Het patroon waarin deze veranderingen optraden was afhankelijk van de pH en de blootstellingsconcentratie en scheen gerelateerd te zijn aan de door chroom geïnduceerde histologische veranderingen. De resultaten wijzen erop, dat een verstoring van respiratie- en osmoregulatieprocessen, zowel bij pH 7,8 als bij pH 6,5 deel uitmaken van het toxische werkingsmechanisme.

In hoofdstuk 5 wordt een beschrijving gegeven van de toxische effecten van Cr(VI) bij de forel na langdurende blootstelling bij verschillende pH waarden. Verschillende leeftijdsstadia werden onderzocht met een maximale blootstellingsduur van 32 weken.
Er werd waargenomen dat alle onderzochte leeftijdsklassen gevoeliger waren voor chroom bij pH 6,5 dan bij pH 7,8. Vissen in het embryo-juvenile stadium bleken echter het gevoeligst te zijn. Concentraties die de overleving in dit leeftijdsstadium beïnvloedden, lagen naar schatting tussen 0,2 en 2,0 mg/l Cr(VI) bij pH 7,8 en tussen 0,02 en 0,2 mg/l Cr(VI) bij pH 6,5. In de discussie van dit hoofdstuk worden de effecten van langdurende blootstelling vergeleken met de effecten na kortdurende blootstelling, die in de voorgaande hoofdstukken zijn beschreven.

Tenslotte kan worden gesteld dat de toxicologische evaluatie van chroom in het Nederlandse oppervlaktewater wordt bemoeilijkt door het gebrek aan informatie over de relatieve concentraties waarin Cr(III) en/of Cr(VI) voorkomen. Tot nu toe is uitsluitend het totale chroomgehalte op verschillende monsterpunten bepaald.
Bij een vergelijking van de totale chroomgehaltes in het Nederlandse oppervlaktewater met de laagste Cr(VI)-concentraties, welke toxisch zijn voor de forel, kan het niet uitgesloten worden geacht dat de toxische niveaus voor vis incidenteel worden overschreden. Een definitieve uitspraak kan echter pas worden gedaan, wanneer meer informatie over de chemische status van het metaal in het aquatische milieu in Nederland beschikbaar komt.

Curriculum vitae

Iksan van der Putte werd op 25 februari 1952 te Bermi (Indonesië) geboren. Na het behalen in 1970 van het diploma gymnasium β aan het Erasmus Lyceum te Almelo, begon hij in datzelfde jaar met de studie in de richting Milieuhygiëne aan de Landbouwhogeschool te Wageningen.
Het ingenieursdiploma, met als hoofdvakken waterzuivering en microbiologie en als bijvak toxicologie behaalde hij in januari 1978.
Van april 1978 tot april 1981 was hij als promotie-assistent verbonden aan de vakgroep Toxicologie van de Landbouwhogeschool, waar het in dit proefschrift beschreven werk is verricht.