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INFLUENCE OF SALINITY AND SULFATE ON THE TOXICITY OF CHROMIUM(VI) TO THE ESTUARINE DIATOM *THALASSIOSIRA PSEUDONANA*[1]

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ABSTRACT

The acute toxicity of Cr(VI) to the diatom Thalassiosira pseudonana *(Hasle and Heimdal) clone 3H was determined in artificial media of 3.2 and 0.32 ppt salinity and with variations of sulfate concentration in the media independent of salinity. Inhibitory concentrations of Cr(VI) ranged from 6.6 μM for growth rate and 4.9 μM for cell yield at 3.2 ppt salinity and 2.8 mM sulfate to 0.04 μM for growth rate and 0.02 μM for cell yield at 0.32 ppt salinity and 0.019 mM sulfate. The inhibition by Cr(VI) was a function of the ratio of Cr(VI) to sulfate. Inhibition occurred when this ratio exceeded about 500:1. It is suggested that the mechanism for the toxicity of Cr(VI) to diatoms and perhaps other aquatic organisms involves a site at which sulfate and chromate compete.*

Key index words: chromate; chromium; diatoms; sulfate; Thalassiosira pseudonana; *toxicity*

Hexavalent chromium is toxic to freshwater algae at concentrations between 1 and 10 μM (Hervey 1949, Wium-Anderson 1974, Patrick et al. 1975). The toxicity of Cr(VI) to marine algae has not been thoroughly examined; however, studies demonstrate that it is relatively non-toxic in marine waters (Hollibaugh et al. 1980, Thomas et al. 1980). Frey et al. (1983) found that the inhibitory concentration of Cr(VI) to natural phytoplankton populations varied from 1.9 μM in water of 0.04 ppt salinity to greater than 19 μM in seawater of 32.5 ppt salinity in the Yaquina Estuary, Oregon. In addition, the inhibitory concentration of Cr(VI) to the diatom *Thalassiosira pseudonana* clone 3H was less than 0.38 μM in water of 0.03 ppt salinity and greater than 3.8 μM in water of 2.1 ppt salinity.

The present study explores the possibility that Cr(VI) toxicity is related to competition of chromate for sulfate in some aspect of cellular metabolism and that the salinity dependence of Cr(VI) toxicity observed by Frey et al. (1983) is related to the concentration of sulfate in estuarine waters.

MATERIALS AND METHODS

Culture. A unialgal culture of *Thalassiosira pseudonana* clone 3H was obtained from Mark Brzezinski at Oregon State University; it originated from the collection of R. R. L. Guillard at Woods Hole Oceanographic Institution. The clone 3H was isolated from an estuarine environment, and a primary consideration in selecting this clone for study was its broad salinity tolerance (Guillard and Ryther 1962). For routine maintenance and Cr(VI) toxicity experiments, cultures were grown in an artificial estuarine water made up by diluting Standard Ocean Water (SOW) (Anonymous 1971) with the major salts of the nutrient enriched artificial freshwater medium WC (Guillard and Lorenzen 1972). The medium was autoclaved prior to the addition of trace metals, Fe-EDTA and vitamins at f/2 levels (Guillard and Ryther 1962). This medium is silicon limiting to *T. pseudonana* when growth is unchecked by Cr toxicity. For routine maintenance cultures were kept in 1 ppt salinity SOW-WC (3 parts SOW and 97 parts WC). Modifications of both WC and SOW in which sulfate was eliminated by substitution of chloride salts in molar equivalents (designated SOW-SO4 and WC-SO4) were used in Cr(VI) toxicity experiments. To produce media with variable sulfate and Cr(VI) concentrations at fixed salinity, first SOW and WC with normal sulfate concentrations were mixed to produce 1 L of SOW-WC with normal sulfate concentration of the desired salinity. Similarly, from WC-SO4 and SOW-SO4 2 L of SOW-WC of the same salinity without sulfate were produced. These two solutions were mixed to make five 250 mL aliquots of media containing 5, 10, 20, 50, and 100% of the sulfate of theoretical estuarine water of that salinity, from which seven 30 mL experimental samples for the Cr(VI) addition series were taken. The pH of the medium was ca. 8.5 at the beginning of an experiment and rose to 9.5 during rapid growth. Experimental cultures were grown in acid-washed Siliclad coated autoclaved 50 mL Pyrex test tubes containing 30 mL of medium. Experimental cultures were maintained in a water bath regulated at 20 ± 1° C and irradiated with Cool-White fluorescent lights at 170 $\mu E \cdot m^{-2} \cdot s^{-1}$ on a light/dark cycle of 16/8 h.

Experimental treatments. Experiments were carried out at two salinities, 3.2 ppt and 0.32 ppt, with variations in the sulfate concentration as described above. In the 3.2 ppt salinity experiment Cr(VI) additions of 10, 5, 2, 1, 0.5, 0.2, and 0.1 μM were tested at each sulfate level, while in the 0.32 ppt salinity experiment Cr(VI) concentrations of 1, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 μM were tested at each sulfate concentration. Cr(VI) was added as $K_2Cr_2O_7$ dissolved in distilled deionized water.

A rapidly growing culture in SOW-WC at the experimental salinity was used as the inoculum. To initiate the growth experiments 0.1 mL of the inoculum culture was added to the 30 mL of experimental medium. Thus the addition of sulfate from the inoculum was small even in the cultures with 5% of the maximum sulfate. The experimental tubes with 3.2 ppt salinity water were inoculated with 2×10^4 cells and the tubes with 0.32 ppt with 6×10^4 cells.

Determination of cell response. Cell growth was monitored daily using in vivo fluorescence using a model 10 Turner Designs fluorometer equipped with a red sensitive photomultiplier tube and large cuvette holder. The fluorometer was calibrated daily using a solution of 5 μg/L coproporphyrin as an arbitrary standard. The cultures were agitated to suspend the cells and the entire culture was inserted in the fluorometer. The fluorescence yield was taken to be the highest fluorescence recorded for an individual culture over the course of the experiment. Growth rate was calculated from the regression of log transformed fluorescence vs. time. Although fluorescence per cell can vary depending on environmental conditions, it has been shown that for logarithmic growth in clonal cultures the increase in fluorescence closely approximates the increase in cell number (Brand and Guillard 1981).

The Cr(VI) concentration estimated to cause 50% inhibition

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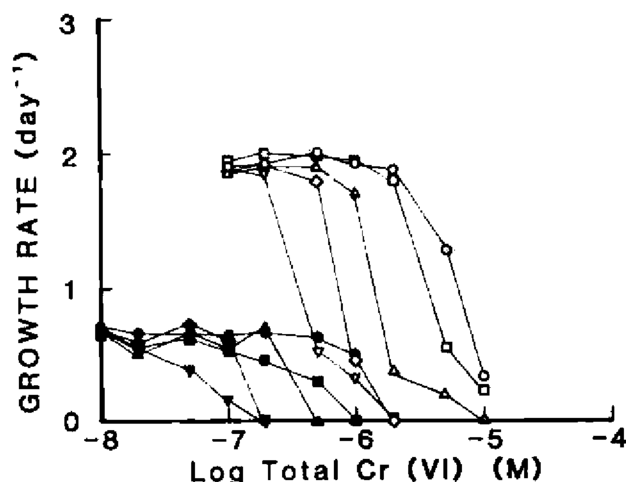

FIG. 1. Growth rate of *T. pseudonana* clone 3H plotted vs. total Cr(VI) concentration in artificial estuarine water with various combinations of sulfate and salinity. Symbols designate different treatment series. ○ = 2.90, □ = 1.45, △ = 0.58, ◇ = 0.29, ▽ = 0.15 mM sulfate in 3.2 ppt salinity. ● = 0.38, ■ = 0.19, ▲ = 0.08, ◆ = 0.04, ▼ = 0.02 mM sulfate in 0.32 ppt salinity. For clarity only the lowest concentration in each series that showed no growth is shown on the ordinate.

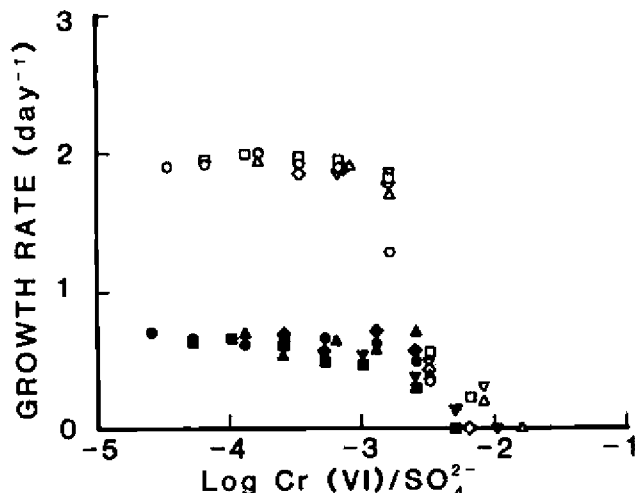

FIG. 2. Growth rate of *T. pseudonana* clone 3H plotted vs. the ratio of Cr(VI) to sulfate in artificial estuarine water of various combinations of sulfate and salinity. Symbols as in Figure 1.

of both growth rate ($EC50_r$) and fluorescence yield ($EC50_y$) was calculated by the maximum likelihood method of Finney (1971), using a FORTRAN program for the iterative calculation (Davies 1971).

RESULTS

The growth rate response of *T. pseudonana* to Cr(VI) at both 3.2 and 0.32 ppt salinity and at various concentrations of total sulfate is shown in Figure 1. The growth rate of *T. pseudonana* clone 3H was considerably less in the 0.32 ppt experiment than in the 3.2 ppt experiment; however, the maximum yields were the same in either medium (not shown). For both the 3.2 ppt and the 0.32 ppt salinity experiments Cr(VI) exerts toxicity at lower concentrations as the sulfate concentration is reduced. At 0.32 ppt salinity Cr(VI) is more toxic at equal fractions of sulfate than at 3.2 ppt salinity.

The same growth rates plotted against the ratio of Cr(VI) to sulfate in the test media are shown in Figure 2. In this plot it can be seen that for both experiments inhibition of growth rate was within a relatively narrow envelope centered around a Cr(VI) to sulfate ratio of approximately 1:500. Similar results were found for fluorescence yield (not shown). Table 1 shows the $EC50_r$ and $EC50_y$ values calculated for each sulfate salinity combination series using both the total Cr(VI) concentration and the Cr(VI)/SO_4^{2-} ratio as the independent variable. Over the entire range of salinities and sulfate concentrations, the mean $EC50_r$ based on the ratio Cr(VI)/SO_4^{2-} was $2.28 \pm 0.15 \times 10^{-3}$, while the mean $EC50_y$ was $1.61 \pm 0.16 \times 10^{-3}$ (mean ±1 SE).

DISCUSSION

The hypothesis that Cr(VI) toxicity for phytoplankton might be strongly related to the sulfate concentration of the water in which they grow was predicted on the basis of chemical similarities between the sulfate ion and the chromate ion, as well as on known biological interactions of chromate and sulfate. Both sulfate and chromate are doubly charged anions with four tetrahedral oxygens. Chromate is the larger of the ions, with a crystal lattice bond length of 0.165 nm compared to 0.154 nm for sulfate. In ion exchange reactions sulfate and chromate are strongly competitive (Pankow and Janauer 1974). Biochemical competition between chromate and sulfate in a variety of contexts is well established (see below).

Inorganic chemistry of chromium. The expected equilibrium distribution of aqueous chromium species

TABLE 1. *Calculated concentration estimated to cause 50% inhibition of growth rate ($EC50_r$) and of fluorescence yield ($EC50_y$) for all combinations of sulfate concentration and salinity tested. They are expressed both as a function of total Cr(VI) and as the Cr(VI) to sulfate ratio.*

			Total[Cr(VI)]		[Cr(VI)]/[SO_4^{2-}]	
Salinity (ppt)	SO_4^{2-} (%)	SO_4^{2-} (mM)	$EC50_r$ (µM)	$EC50_y$ (µM)	$EC50_r$ ($\times 10^{-3}$)	$EC50_y$ ($\times 10^{-3}$)
3.20	100	2.90	6.56	4.94	2.23	1.70
3.20	50	1.45	3.80	2.56	2.50	1.76
3.20	20	0.58	1.44	0.99	2.45	1.63
3.20	10	0.29	0.66	0.58	2.25	1.98
3.20	5	0.15	0.40	0.34	3.02	2.38
0.32	100	0.38	0.75	0.73	1.97	1.92
0.32	50	0.19	0.23	0.11	1.22	0.55
0.32	20	0.08	0.19	0.11	2.49	1.40
0.32	10	0.04	0.10	0.06	2.59	1.63
0.32	5	0.02	0.04	0.02	2.05	1.17

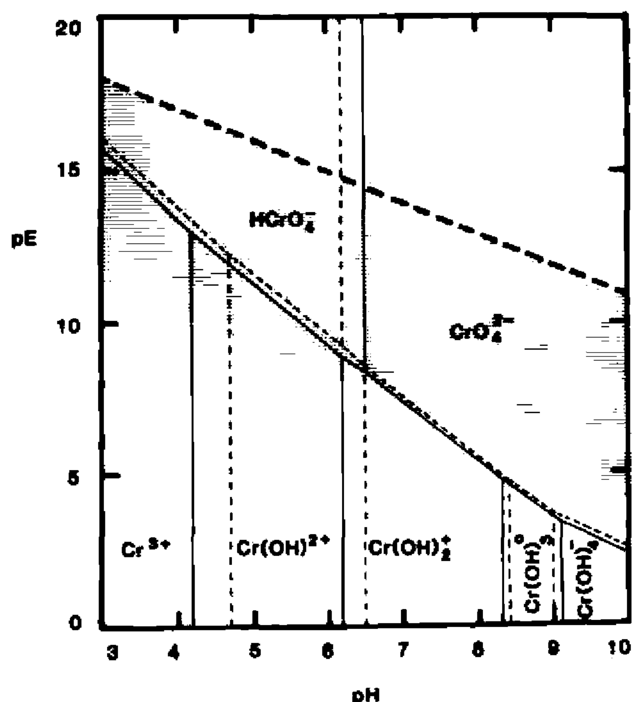

FIG. 3. Calculated equilibrium distribution of aqueous chromium species as a function of pE, pH and ionic strength (I) at 25°C. Assumes infinite dilution of chromium species (i.e. no solids or polymers). Solid lines for I = 0, dashed lines for I = 0.07 (ca. 3 ppt salinity). The heavy dashed line shows the theoretical pE of water in equilibrium with atmospheric oxygen (Sillen 1967). The shaded area gives the range of pE values proposed to be the functional pE of oxic water; the lower boundary comes from steady state peroxide concentrations measured in seawater (Moffet and Zika 1983). Chromium(III) pH equilibrium constants from Kragten (1978), and redox equilibrium and Cr(VI) pH equilibrium constants from Ingle et al. (1978). Single ion activity coefficients calculated using the Davies' formula (Stumm and Morgan 1971). Ion pairing of Cr(III) and Cr(VI) species with specific ions in seawater other than hydroxide has not been considered, as many ion pairing constants for Cr(VI) are not available. Cr(VI) is known to form complexes with sulfate and chloride at low pH and high concentrations (Sillen and Martell 1971); however, these forms are insignificant fractions of the total Cr(VI) at the experimental conditions.

as a function of redox potential (pE) and pH is shown in Figure 3. Although the effective redox potential in oxic solution is as yet a matter of controversy, it is clear that at the pH of these experiments (ca. 8.5), chromate ion is predicted to be the dominant form of chromium. The observed distribution and speciation of chromium in the ocean also shows that Cr(VI) is dominant except in reducing environments (Cranston and Murray 1978).

Because Cr(VI) species are anionic and do not bind standard culturing chelators such as EDTA, the concentration or activity of chromate cannot be regulated independently of total Cr(VI), as has been done with cationic trace metals such as Cu (Sunda and Guillard 1976), Zn (Anderson et al. 1978), and Fe (Anderson and Morel 1983). The question of the stability of Cr(VI) in the experimental cultures is somewhat unclear. Equilibrium calculations suggest that even relatively strong chelators of Cr(III) such as the EDTA added to these culture media cannot substantially stabilize Cr(III) if the pE is 12.5 as predicted by equilibrium between oxygen and water. However, equilibrium with oxygen may not be approached and reduction by organic compounds may be favored by kinetics. For example, photochemical reduction of Fe^{3+} in the presence of EDTA and other chelators has been shown to alter the biological activity of Fe (Anderson and Morel 1983). Experiments using *s*-diphenyl carbazide (a compound which reacts with Cr(VI) but not Cr(III) to form a colored product) has shown that during growth experiments such as these, less than 1% of the total (Cr(VI) is lost from solution, either by cellular uptake or reduction to Cr(III) (unpublished data).

Biological interaction of chromate and sulfate. Sulfate is utilized by phytoplankton for the synthesis of sulfur containing amino acids, various metabolic intermediates, and sulfonated polysaccharides. Sulfate uptake in algae is known to be an active process, and internal concentrations of sulfate are regulated when external concentrations are varied. There may be multiple systems for sulfate uptake with different half-saturation constants (Raven 1980). Several studies have shown that chromate acts to inhibit sulfate uptake by algae and higher plants, though at chromate to sulfate ratios ranging from 1:10 to 10:1 (Smith 1976, Coughlan 1977, Jeanjean and Broda 1977, Deane and O'Brien 1981). Conversely, in rat liver mitochondria, chromate uptake inhibition by sulfate has been demonstrated (Alexander et al. 1982). The low ratio of chromate to sulfate at which *T. pseudonana* clone 3H is inhibited argues against sulfate deficiency due to reversible competition between chromate and sulfate for uptake as the cause of the inhibition as it would require the sulfate uptake site to have an affinity for chromate about two orders of magnitude greater than for sulfate, its natural substrate.

Previous discussions of the toxicity of Cr(VI) have stressed its oxidizing nature (Mertz 1969). However, the oxidizing strength of Cr(VI) is strongly dependent on pH, having stronger oxidizing power at low pH (Fig. 3). Because of the high pH in the experimental media and in estuaries, Cr(VI) under these conditions would be in a relatively non-reactive form. However, some localized compartments within phytoplankton cells, such as vacuoles and thylakoids, have pH values of 4–5 (Wheeler and Hellebust 1981).

One mechanism by which Cr(VI) could be toxic at very low chromate to sulfate ratios could involve these pH gradients and the Cr(VI) pH dependent equilibria. If chromate is transported as a sulfate analogue into low pH sites within the cell, an accumulation of $HCrO_4^-$ in such areas may take place due to the effect of the pH gradient and the chromate-hydrogen chromate equilibrium (Fig. 3). The

action of Cr(VI) as a strong oxidizing agent in an acidic environment might then be responsible for the toxic effect. Furthermore, Cr(III) would be accumulated internally as a consequence of Cr(VI) oxidation. Cr(III) might also have a toxic effect as a competitive inhibitor in the metabolism of trace elements such as Fe^{3+}. The rate of uptake of Cr(VI) would be regulated by the cells' sulfate uptake rate and the competition of chromate and sulfate for uptake.

There is an alternative mechanism for chromate toxicity consistent with sulfate competition and the low chromate to sulfate ratio at which inhibition occurs. The sulfate uptake site might again bind chromate as a sulfate analogue, but in this case irreversibly, initiating reduction of the chromate and oxidation of the sulfate uptake site. The toxic effect in this case would result from sulfate limitation when sufficient sulfate uptake sites had been oxidized.

Both of these mechanisms require that chromate act as a sulfate analogue for the sulfate uptake transport system, and that Cr(VI) should ultimately be reduced to Cr(III) by the phytoplankton cells. The uptake of a trace metal as an anion and its subsequent reduction to a chelatable cation would be an unusual process, although a similar path exists in tunicates which take up vanadate, V(V), from seawater into specialized blood cells containing V(III) (Dingley et al. 1981).

Of the transition elements that exist predominantly as anions in seawater and for which vertical concentration profiles have been produced in the open ocean (vanadium and molybdenum, Morris 1975; and chromium, Cranston and Murray 1978) chromium is unique in that it shows a substantial surface water depletion, indicative of significant rates of incorporation into particles, probably phytoplankton. Selenate, another trace metal anion whose uptake has also been tentatively linked to sulfate uptake by algae (Wheeler et al. 1982), also shows considerable surface water depletion (Measures and Burton 1980).

The effects of a variety of trace elements on the growth and physiology of marine and estuarine phytoplankton have been investigated in some detail. Cationic trace elements have been more intensively studied (e.g. Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+}), and generally serve as cofactors in enzymes when acting in a beneficial role, or compete with other trace elements and/or bind to cellular ligands for which they have no proper function when acting in a negative role. These elements may have a few common sites of toxic effect that depend on their binding to cellular ligands, as is suggested by the high correlation between acute toxicity and their respective binding constants for ligands (Fisher and Jones 1981), and by competitive interactions found between elements (e.g. Cu-Zn, Rueter and Morel 1981; Cu-Mn, Sunda et al. 1981). Anionic trace elements such as MoO_4^{2-}, SeO_4^{2-}, SeO_3^{2-}, $VO_3(OH)^{2-}$, AsO_3^{2-}, AsO_4^{2-} and GeO_4^{4-} have not yet received as much attention as cations, but in several cases their uptake by organisms has been linked to their acting as analogues for chemically similar anionic nutrients (e.g. GeO_4^{4-} and SiO_4^{4-}, Azam and Volcani 1981; AsO_4^{3-} and PO_4^{3-}, Rothstein 1963; SeO_4^{2-} and SO_4^{2-}, Wheeler et al. 1982). The results of the experiments presented here suggest a similar relationship exists between CrO_4^{2-} and SO_4^{2-}.

Environmental significance. Sulfate is a major conservative constituent of seawater ($[SO_4^{2-}] = 29$ mM at 33 ppt), and a lower and variable constituent of freshwater. Therefore, the concentration of sulfate in estuarine waters varies as a function of salinity. If the sulfate dependence of Cr(VI) toxicity found for *T. pseudonana* clone 3H in this study is found in most algae, estuaries can be expected to be areas where the sensitivity to Cr(VI) should change uniformly in response to salinity. From a pollution standpoint, a source of Cr(VI) in the upper (freshwater) portion of an estuary might have far more severe effects than the same source at the seaward end, at least as far as the algae are concerned. Organisms other than algae have also been shown to decrease their sensitivity to Cr(VI) in response to increased salinity (Fales 1978, Olson and Harrel 1973), an indication that the effect of sulfate on Cr(VI) toxicity might be quite general. Sulfate concentrations are variable in freshwater depending on the nature of the surrounding soil and other factors, and the Cr(VI) sensitivity of algae might be found to vary accordingly.

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