Table II. Hydrogen Bond Distances and Angles in Aspartame

D-H		A[a]	D-A	H-A	D-H-A
N1	HN11	OD1[1]	2.99	2.40	112
		OX3[1]	3.04	1.97	166
		OX3[2]	2.86	2.06	128
N1	HN12	OD1[4]	3.00	2.41	110
		OD2[4]	2.76	1.85	159
N1	HN13	OD1[6]	2.79	1.91	161
OX3		OD1[1]	2.61		
		OX3[5]	2.56		
OX3		OD1[2]	2.80		
		OD1[3]	2.99		

[a] Symmetry code: (1) x, y, z; (2) $-y, x, z + {}^1/_4$; (3) $-x, -y, z + {}^1/_2$; (4) $y, -x, z + {}^3/_4$; (5) $-x, -y, z - {}^1/_2$; (6) $x, y, z + 1$.

that the four symmetry-related positions of the water molecules are too close to exist simultaneously in one unit cell. Thus, in any given unit cell either one pair or the other is present. This is the reason for the water molecule having 50% occupancy. In either case the hydrogen-bonding scheme obviously is equivalent. As can be seen in Figure 5, the water molecules apparently are involved in seven hydrogen-bonding contacts. The water molecule acts as a donor in four instances (to three different OD1 atoms and to the neighboring water oxygen atom through bifurcated hydrogen bonds) and as an acceptor in three other directions (from two N1 positions and the other neighboring water molecule). The only other available donor is the terminal $-NH_3^+$ group and it participates in six hydrogen bonds (to three different OD1 atoms, one water molecule and to an OD2 atom through bifurcated hydrogen bonds). One of the hydrogen bonds between N1 and OD1 is an intramolecular hydrogen bond. Thus, the extensive and elaborate hydrogen bonding is interconnecting aspartame molecules to form a long column with hydrophobic surface. Distance and angles associated with all of the hydrogen bonds are reported in Table II.

At the present time little is known about the sweet receptors on the human tongue, and nothing is known about what form of aspartame is recognized by these receptors. But intense efforts are being directed toward the chemical modification of aspartame and the design of aspartame-like molecules not only to understand the chemical and structural determinants of the sweet taste but also to find a better sweetener. The detailed three-dimensional structure of aspartame and its columnar polymeric arrangement provide a reasonable explanation for unexpected low water solubility and fibre-forming tendency of the molecule, and it may provide some important new insights for these purposes.

Acknowledgment. The work reported here has been supported by NIH Grant NS15174 and the aspartame sample was kindly provided by G. D. Searle & Co., Skokie, IL. We thank Dr. Fred Hollander of the University of California, Berkeley, for the data collection.

Registry No. Aspartame, 22839-47-0.

Supplementary Material Available: A listing of thermal parameters of aspartame molecules (2 pages). Ordering information is given on any current masthead page.

In Vitro Reaction of the Carcinogen Chromate with Cellular Thiols and Carboxylic Acids

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Abstract: Since a key step in the carcinogenicity and toxicity of chromium(VI) compounds is intracellular reduction to chromium(III), we have examined the interaction of chromium(VI) with a series of low molecular weight cellular reductants and model compounds at physiological pH. The reduction of chromium(VI) at physiological pH was found to be under kinetic rather than thermodynamic control. Only ascorbate and those reductants containing a thiol group were capable of reducing chromium(VI) at a significant rate at pH 7.4 (1 M Tris-HCl). The kinetics describing the reaction of chromium(VI) with the various thiols could be separated into four categories: (a) glutathione showed clear spectral evidence for the formation of a chromium(VI) thioester in a rapid pre-equilibrium step, followed by a slower redox step involving reaction of the chromium(VI) thioester with a second molecule of thiol; (b) cysteine ethyl ester, cysteine, cysteamine, coenzyme M, homocysteine, *N*-acetylcysteine, coenzyme A, mercaptoethanol, and thioglycolate showed formation of a chromium(VI) thioester followed by a redox step involving reaction of the thioester with a second molecule of thiol with kinetics consistent with the steady-state approximation; (c) penicillamine, dithiothreitol, 2,3-dimercaptosuccinate, thiolactate, and thiomalate showed rate-determining formation of the chromium(VI) thioester; and (d) unithiol and dihydrolipoate showed the formation of a chromium(VI) thioester in a rapid pre-equilibrium step, followed by a unimolecular redox reaction of the thioester, although there was no spectral evidence for thioester formation. Of possible biological importance is the fact that glutathione, the most abundant intracellular thiol, appeared in the first category. The rapid formation of the chromium(VI) glutathione thioester, followed by its slow reduction, may well prolong the lifetime of chromium(VI) in the cell and promote its interaction with cellular macromolecules. Brønsted plots showed that the second-order rate constants for the first step of the thiol-chromium(VI) reaction, formation of the chromium(VI) thioester, were inversely related to the pK_a's of the thiol groups. At pH 7.4, it appears that chromate, CrO_4^{2-}, is attacked by the un-ionized thiol which transfers a proton to an oxygen atom of the chromate as part of the rate-limiting step and that the subsequent loss of the hydroxide ligand, OH^-, is facilitated by protonation in the case of thiols containing a free amino group.

In recent years, several studies have reviewed the toxicity and carcinogenicity of chromium compounds.[2-4] Epidemiological and animal studies, as well as in vitro mutagencity assays,[5] indicate that chromium(VI) compounds pose serious dangers to biological

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systems, whereas chromium(III) compounds are relatively nontoxic. An uptake–reduction model has been proposed to explain this phenomenon.[6,7] Chromium(VI) crosses the cell membrane and oxidizes cellular components in a process which leads to cellular damage, including interference with the genetic machinery. The chromium(III) produced intracellularly upon reduction of chromium(VI) binds to macromolecules, e.g., DNA and protein, or small molecules, e.g., glutathione and nucleotides, thereby interfering with their normal cellular function.

Possible candidates for chromium(VI)–reductase activity in the cell range from small molecules in the cytoplasm to complex membrane enzyme systems. The endoplasmic reticulum has been identified as a site for chromium(VI) metabolism within the cell. Rat liver microsomes were found to possess a NAD(P)H-dependent chromate–reductase activity which involved the cytochrome P-450 electron-transport system.[8,9] Furthermore, the rat liver microsomal system was capable of catalyzing the formation of Cr–DNA and Cr–DNA–protein complexes in vitro.[10] There is also evidence for uptake and reduction of chromate by rat liver mitochondria.[7,11,12] In addition to these organelles, many purified proteins have been examined for their ability to reduce chromium(VI). However, of the multitude of proteins tested, only certain heme proteins and flavoproteins were active.[7]

Many small molecules found within the cell are redox active and should be able to reduce chromium(VI) based on their redox potentials. These compounds may be important to the cellular metabolism of chromium(VI). Extrapolation to pH 7.4 using the Nernst equation gives a redox potential of +0.52 V for the chromium(VI)–chromium(III) half-reaction:[13]

$$CrO_4^{2-} + 4H_2O + 3e^- = Cr(OH)_3 + 5OH^-$$

However, most studies of chromium(VI) oxidations of small molecules have been carried out under acidic conditions.[14,15] Therefore, we have undertaken a study of the reactions between chromate and various low mol. wt. cellular constituents under conditions of physiological pH. These studies show that chromate reduction by small molecules is kinetically controlled and only thiols and ascorbate react as a significant rate at pH 7.4.

Experimental Procedures

Materials. Sodium formate, sodium oxalate, and potassium dichromate (1000 ppm atomic absorbance reference solution) were purchased from Fisher Scientific Co., Pittsburg, PA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Determination of Chromium(VI) and Thiol Concentrations. The concentration of chromium(VI) was determined by measuring absorbance at 372 nm in 0.1 M KOH (ϵ_{372} = 4810 M^{-1} cm^{-1}).[16] The concentration of the thiols was measured at 412 nm after reaction with Ellman's reagent (ϵ_{412} = 13 700 M^{-1} cm^{-1}).[17]

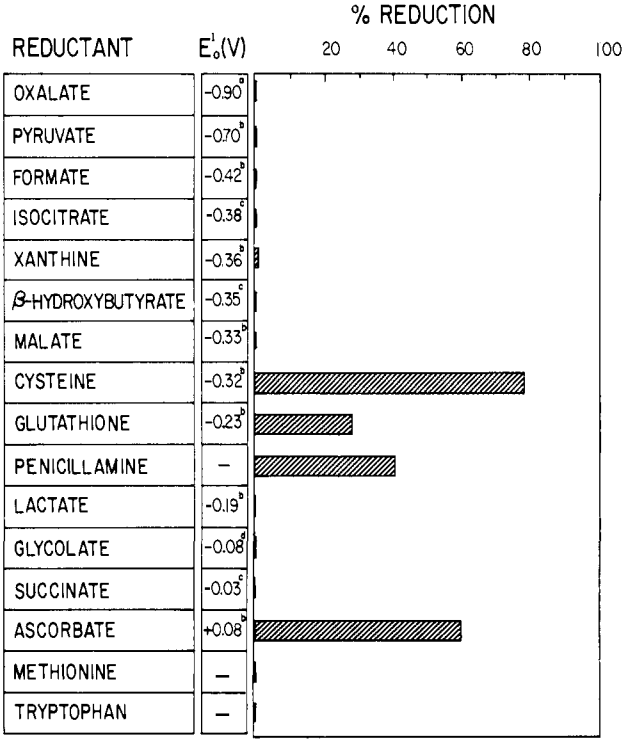

Figure 1. The extent of reaction of chromium(VI) with various reductants after 1 min in 1 M Tris–HCl, pH 7.4. Reactions were carried out at 25 °C for all reductants except methionine, tryptophan, glycolate, and xanthine which were at 37 °C. Initial [Cr^{VI}] = 3.7 × 10^{-4} M and [reductant] = 2.5 × 10^{-2} M, except [xanthine] = 6.3 × 10^{-3} M. References for the reduction potentials are (a) ref 13, (b) ref 46, (c) ref 47, and (d) ref 48.

Determination of the pK_a's of Hydrogen Chromate and Various Thiols. The pK_a of hydrogen chromate at 25 °C, 0.5 M KCl, was determined spectrophotometrically at 370 nm.[18] The pK_a's of the –SH group of cysteine ethyl ester, cysteine, cysteamine, homocysteine, coenzyme M, *N*-acetylcysteine, and thioglycolate at 25 °C, 0.5 M KCl, were determined spectrophotometrically at 230 nm under anaerobic conditions.[19]

Determination of Chromate Reduction. The reduction of chromium(VI) (3.7 × 10^{-4} M) by various reductants in 1 M Tris–HCl, pH 7.4, was monitored by following the absorbance of chromium(VI) at 372 nm as a function of time with a Cary 219 spectrophotometer. Kinetic data obtained from monitoring the disappearance of chromium(VI) at 372 nm were consistent with kinetic data obtained from monitoring the appearance of chromium(III) products at 550–650 nm and from monitoring the appearance and disappearance of the chromium(VI) thioester intermediates at 420–440 nm. Little or no chromium(V) was observable by EPR spectroscopy under the experimental conditions used, indicating that changes in absorbance at 372 nm reflected changes in chromium(VI) concentration without interference from chromium(V).

Under these experimental conditions (pH 7.4, I = 0.4–0.5 M, 25 °C) the ratio of $HCrO_4^-$ to CrO_4^{2-} is 0.038, based on a pK_a($HCrO_4^-$) = 5.98 (see above). Thus, 96.3% of the chromium(VI) is in the form of chromate, CrO_4^{2-}, in these studies. Reactions were carried out at 25 °C except for reactions with methionine, tryptophan, glycolate, and xanthine which were carried out at 37 °C. Initial studies comparing the reactivity of chromium(VI) with various reductants were carried out under pseudo-first-order reaction conditions at a reductant concentration of 2.5 × 10^{-2} M, except in the case of xanthine where a concentration of 6.3 × 10^{-3} M was used because of solubility problems. Over the course of the reactions the pH was constant to within 0.1 pH unit. All of the thiols were found to be stable over the time course of the kinetics measurements.

Apparent second-order rate constants for the reaction of chromium(VI) with ascorbate and with various thiols were determined from plots of pseudo-first-order rate constants, k_{obsd}, vs. reductant concentration (either [ascorbate] or [thiol], was varied from 0.01 to 0.14 M). In the case of linear plots of k_{obsd} vs. [reductant], the apparent second-order rate

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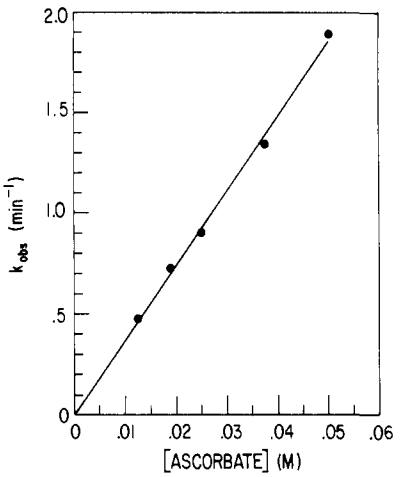


Figure 2. Linear plot of the pseudo-first-order rate constant, k_{obsd}, vs. ascorbate concentration for the disappearance of chromium(VI) at 25 °C in 1 M Tris–HCl, pH 7.4. Initial [Cr^{VI}] = 3.7×10^{-4} M; slope = 37 M^{-1} min^{-1}; r = 0.998.

constant was determined from the slope of the line, and in the case of curved plots, the apparent second-order rate constant was calculated from a computer fit of the data with use of either the program PHASIC or BIPHASIC.[20]

Results

Reaction of Various Reductants with Chromium(VI). The reaction of chromium(VI) with various reductants is shown in Figure 1. The only reductants with significant reactivity toward chromium(VI) at pH 7.4 were ascorbate, cysteine, glutathione, and penicillamine. Although oxalate, pyruvate, formate, isocitrate, xanthine, β-hydroxybutyrate, and malate have lower redox potentials than the thiols, and lactate, glycolate, and succinate have lower redox potentials than ascorbate, no significant rate of chromium(VI) reduction was observed with these compounds.

Reaction of Ascorbate with Chromium(VI). Since the pK_a's of ascorbic acid are 4.3 and 11.82,[21] the major species at pH 7.4 is the ascorbate anion (HA^-). Plots of k_{obsd} vs. [ascorbate] (Figure 2) were linear with slope (apparent second-order rate constant) = 36 ± 1 M^{-1} min^{-1} and intercept = zero consistent with the following expression:

$$k_{obsd} = a[\text{ascorbate}][Cr^{VI}] \quad (1)$$

A possible mechanism which would account for the observed kinetics involves the reaction of ascorbate with chromium(VI) to form a chromium(VI) ester which then undergoes a unimolecular redox reaction. The level of the chromium(VI) ester intermediate may reach a steady-state concentration in which case the following equations would apply:

$$HA^- + Cr^{VI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HACr^{VI} \quad (2)$$

$$HACr^{VI} \xrightarrow{k_3} Cr^{IV} + A \quad (3)$$

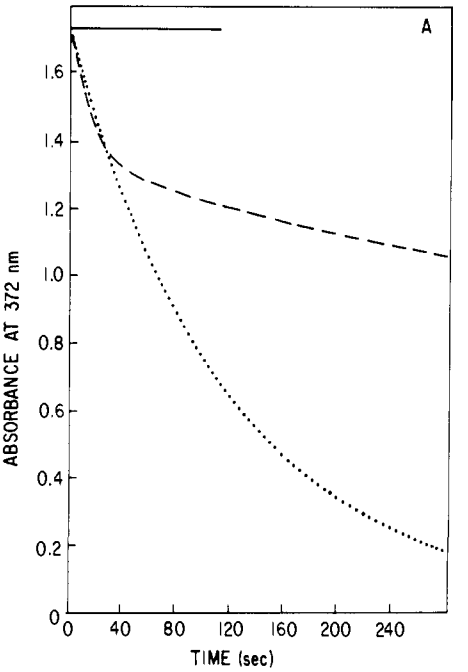
where A is dehydroascorbate. The rate law consistent with this mechanism is

$$-d[Cr^{VI}]/dt = k_3k_1[\text{ascorbate}][Cr^{VI}]/(k_{-1} + k_3) \quad (4)$$

Thus the a parameter of eq 1 corresponds to $k_3k_1/(k_{-1} + k_3)$. If the redox reaction is rapid ($k_3 \gg k_{-1}$, the formation of the chromium(VI) ester will be rate determining and the a parameter corresponds to k_1.

Reaction of Thiols with Chromium(VI). The stoichiometry of the reaction of chromium(VI) with cysteine, cysteamine, penicillamine, mercaptoethanol, 2,3-dimercapto-1-propanesulfonate (unithiol), and dihydrolipoate was ~3–4 thiol per chromium(VI). This is consistent with three thiol molecules providing three electrons to reduce chromium(VI) to chromium(III), and it indicates that only one sulfhydryl group of the dithiols (unithiol and dihydrolipoate) participates in the redox reaction. In contrast, the stoichiometry of the reaction of chromium(VI) with dithiothreitol was only ~1.5–2 dithiothreitol per chromium(VI). Since dithiothreitol is a dithiol, this result is consistent with participation of both sulfhydryl groups in the redox reaction.

The rate of chromium(VI) reduction by penicillamine was characteristic of a reaction first order in chromium concentration,



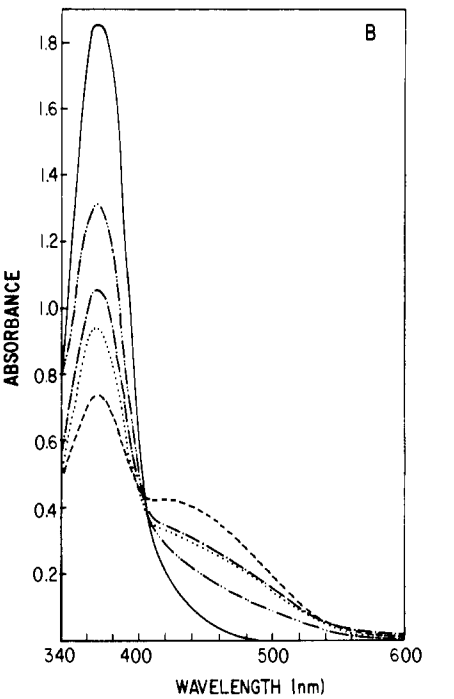


Figure 3. (A) Change in absorbance at 372 nm with time during the reaction of chromium(VI) with glutathione (- - -) and penicillamine (···) at 25 °C in 1 M Tris–HCl, pH 7.4. (—) Buffer only. Initial [Cr^{VI}] = 3.7×10^{-4} M and [thiol] = 2.5×10^{-2} M. (B) Absorption spectrum of chromium(VI) (—) 2.2 min after the addition of glutathione (- - -), *N*-acetylcysteine (- · -), coenzyme M (···), or coenzyme A (- · · -), at 25 °C in 1 M Tris–HCl, pH 7.4. Initial [Cr^{VI}] = 3.9×10^{-4} M and [thiol] = 4.0×10^{-2} M. Scans were run from 600 to 340 nm at a scan speed of 10 nm/s.

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Table I. Second-Order Rate Constants for the Reaction of Chromium(VI) with Thiols and Dithiols at 25 °C in 1 M Tris–HCl, pH 7.4 (Values Represent Mean ± Standard Error of 2–4 Independent Determinations)

thiol	k_1, M^{-1} min^{-1}	a/b, M^{-1} min^{-1}	k_1',[a] M^{-1} min^{-1}	b, M^{-1}	log k_1'	pK_a
1. cysteine ethyl ester	135 ± 6[b]	147 ± 4[c]	244	49 ± 6[c]	2.39	7.58[d]
2. cysteine	78[b,e]	109 ± 13[c]	120	65 ± 4[c]	2.08	8.40[d]
3. cysteamine	71 ± 1[b]	84 ± 11[c]	93	21 ± 2[c]	1.97	8.37[d]
4. glutathione	38 ± 1[b]	12 ± 2[c]	39	21 ± 7[c]	1.59	8.93[f]
5. penicillamine	21 ± 2[g]		23.5		1.37	8.32[h]
6. homocysteine	14.2 ± 1[b]	14.6 ± 2[c]	14.8	38 ± 7[c]	1.17	9.27[d]
7. coenzyme M	13.4 ± 0.2[b]	12.4 ± 0.7[c]	12.7	15 ± 1[c]	1.10	9.06[d]
8. thioglycolate		8.8 ± 0.6[c]	8.8	39 ± 5[c]	0.95	10.07[d]
9. dithiothreitol	8.7 ± 0.1[g,i]		8.6		0.95	9.14[j]
10. *N*-acetylcysteine	6.6 ± 0.7[b]	6.3 ± 0.6[c]	6.3	8 ± 2[c]	0.80	9.55[d]
11. coenzyme A	5.2 ± 1.1[b]	4.9 ± 0.8[c]		38 ± 13[c]		
12. mercaptoethanol	5.1 ± 0.1[b]	7.7 ± 1.0[c]	7.8	40 ± 2[c]	0.89	9.48[k]
13. thiolactate	3.1 ± 0.2[g]		3.1		0.49	10.27[k]
14. thiomalate	2.0 ± 0.1[g]		2.0		0.30	10.94[l]
15. 2,3-dimercaptosuccinate	1.9 ± 0.1[g,i]		1.9		0.28	10.79[l]
16. unithiol		1.43[m]		60.4[m]		
17. dihydrolipoate		0.67[m]		15.6[m]		

[a] Apparent second-order rate constant corrected for actual [RSH]. [b] Determined from initial rates. [c] Determined by using the program BIPHASIC,[20] a/b corresponds to k_1 and b corresponds to k_2/k_{-1}, except in the case of glutathione where a/b corresponds to k_2 and b corresponds to K. [d] This work. [e] One determination. [f] Reference 42. [g] Determined from linear fit. [h] Reference 43. [i] Calculated on the basis of [-SH]. [j] Reference 44. [k] Reference 45. [l] Reference 21. [m] Determined by using the program PHASIC,[20] a/b corresponds to k_3 with units of min^{-1} and b corresponds to K.

whereas the reduction by glutathione was clearly biphasic in character (Figure 3A). Plots of ln $(A - A_f)_{372}$ (where A is the absorbance at any time during the reaction and A_f is the absorbance at the end of the reaction) vs. time were linear for reactions of chromium(VI) with ascorbate, penicillamine, dithiothreitol, 2,3-dimercaptosuccinate, thiolactate, thiomalate, 2,3-dimercapto-1-propanesulfonate (unithiol), and dihydrolipoate but were curved for the reaction of chromium(VI) with cysteine ethyl ester, cysteine, cysteamine, glutathione, 2-mercaptoethanesulfonate coenzyme M (CoM), homocysteine, *N*-acetylcysteine, coenzyme A (CoA), mercaptoethanol, and thioglycolate. The formation of a chromium(VI) thioester intermediate was apparent for these thiols, since an increase in absorbance at 420–440 nm was observed immediately after initiating the reaction (Figure 3B). Plots of reciprocal absorbance vs. time did not produce linear fits of the data, indicating that these reactions were not second order in chromium(VI).

A general mechanism for the reaction of chromium(VI) with thiols involves the formation of a chromium(VI) thioester followed by either a redox reaction involving a second molecule of thiol or a unimolecular redox reaction of the thioester (which leads to Cr(V) with monothiols and Cr(IV) with dithiols which are capable of forming an intramolecular disulfide bond):

$$RSH + Cr^{VI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RSCr^{VI} \quad (5)$$

$$RSCr^{VI} + RSH \xrightarrow{k_2} Cr^{IV} + RSSR \quad (6)$$

$$RSCr^{VI} \xrightarrow{k_3} Cr^{V} + RS\cdot \quad (7)$$

$$\begin{matrix} RSCr^{VI} \\ | \\ SH \end{matrix} \xrightarrow{k_4} Cr^{IV} + \begin{matrix} R{-}S \\ \diagdown\ \diagup \\ S \end{matrix} \quad (8)$$

If the chromium(VI) thioester reaches a steady-state concentration, the rate law consistent with this general mechanism is

$$\frac{-d[Cr^{VI}]}{dt} = \frac{(k_2k_1[RSH]_t^2 + k_3k_1[RSH]_t)[Cr^{VI}]}{(k_{-1} + k_3 + k_2[RSH]_t)} \quad (9)$$

A two-step mechanism involving the formation of a chromium(VI) thioester and its subsequent reaction with a second molecule of thiol (eq 5 and 6) is consistent with the observed rate law for cysteine ethyl ester, cysteine, cysteamine, glutathione, CoM, homocysteine, *N*-acetylcysteine, CoA, mercaptoethanol, and thioglycolate. Values of k_{obsd} for these thiols were determined from initial rates and, therefore, the slopes of plots of k_{obsd} vs. total thiol concentration, $[RSH]_t$ (Table I) correspond to k_1, the rate constant for the formation of the chromium(VI) thioester (eq 5).

For these ten thiols k_{obsd} was also determined from the linear portion of ln $(A - A_f)$ vs. time plots. These data lead to plots of k_{obsd} vs. $[RSH]_t$ (Figure 4) which fit the following expression:

$$k_{obsd} = a[RSH]_t^2/(1 + b[RSH]_t) \quad (10)$$

indicating that the unimolecular redox pathway (eq 7) was negligible ($k_3 \ll k_2[RSH]_t$). The a and b parameters of eq 10 were determined from the fit of k_{obsd} vs. $[RSH]_t$ with the program BIPHASIC.[20] For all thiols except glutathione (and cysteine which had only one determination of k_1 from initial rate data) the value of the apparent second-order rate constant (a/b) determined from the BIPHASIC fit was identical with the k_1 determined from initial rate data (Table I). Thus the data are consistent with the following rate law:

$$\frac{-d[Cr^{VI}]}{dt} = \frac{k_2k_1[RSH]_t^2[Cr^{VI}]}{(k_{-1} + k_2[RSH]_t)} \quad (11)$$

and the a and b parameters correspond to the following expressions:

$$a = k_2k_1/k_{-1},\ b = k_2/k_{-1},\ a/b = k_1 \quad (12)$$

For glutathione the apparent second-order rate constant determined from the BIPHASIC fit was ~3 times smaller than k_1 determined from initial rate data (Table I). Therefore, it appears that formation of the chromium(VI) thioester with glutathione occurs in a rapid pre-equilibrium step which is followed by a slower redox step involving a second molecule of glutathione. Under these conditions $[RSCr^{VI}]/[Cr^{VI}]_t = K[RSH]_t/(1 + K[RSH]_t)$. Thus, the rate law consistent with the kinetic data for the reaction of chromium(VI) with glutathione is

$$\frac{-d[Cr^{VI}]_t}{dt} + \frac{k_2K[RSH]_t^2[Cr^{VI}]_t}{1 + K[RSH]_t} \quad (13)$$

where K is the equilibrium constant (k_1/k_{-1}) for the reaction of glutathione with chromium(VI) (eq 5) and $[Cr^{VI}]_t$ is the total concentration of chromium(VI). Thus, in the case of glutathione the a and b parameters of eq 10 correspond to the following expressions:

$$a = k_2K,\ b = K,\ a/b = k_2 \quad (14)$$

Apparent second-order rate constants for reaction of thiolactate, thiomalate, penicillamine, dithiothreitol, and 2,3-dimercaptosuccinate were calculated from the slope of k_{obsd} vs. $[RSH]_t$ plots (Figure 5) which were linear. The kinetics observed for thiolactate, thiomalate, penicillamine, and 2,3-dimercaptosuccinate are explained by a mechanism where the rate-determining step is the reaction of the thiol with chromium(VI) to form a chromium(VI)

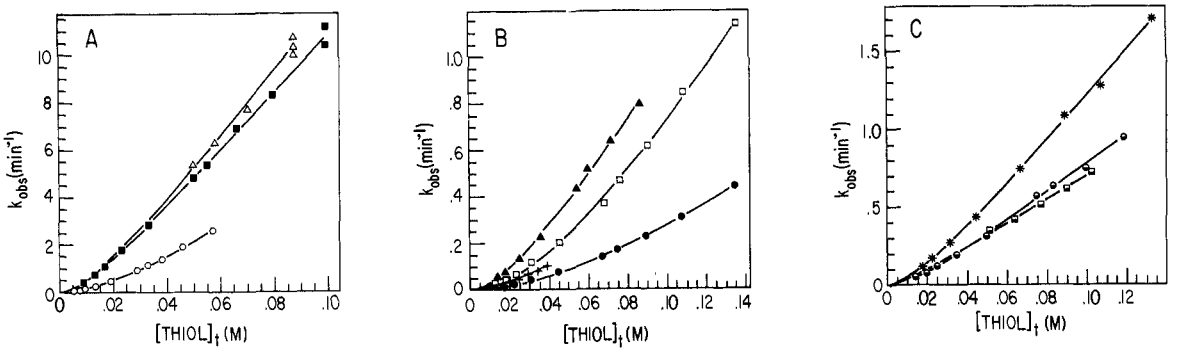


Figure 4. BIPHASIC plots of the pseudo-first-order rate constants, k_{obsd}, vs. total thiol concentration for the disappearance of chromium(VI) at 25 °C in 1 M Tris–HCl, pH 7.4: (A) cysteine ethyl ester (△) (a/b = 142 M^{-1} min^{-1}), cysteine (■) (a/b = 124 M^{-1} min^{-1}), and cysteamine (○) (a/b = 80 M^{-1} min^{-1}); (B) glutathione (▲) (a/b = 13.2 M^{-1} min^{-1}), coenzyme M (□) (a/b = 15.3 M^{-1} min^{-1}), *N*-acetylcysteine (●) (a/b = 7.3 M^{-1} min^{-1}), and coenzyme A (+) (a/b = 4.7 M^{-1} min^{-1}); (C) homocysteine (*) (a/b = 14.8 M^{-1} min^{-1}), thioglycolate (◒) (a/b = 7.8 M^{-1} min^{-1}), and mercaptoethanol (◙) (a/b = 9.7 M^{-1} min^{-1}).

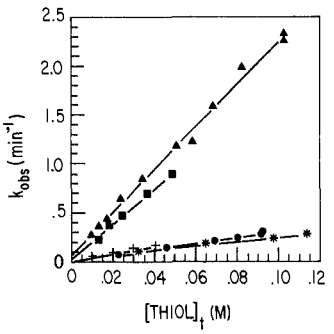


Figure 5. Linear plots of the pseudo-first-order rate constants, k_{obsd}, vs. total thiol concentration for the reaction of chromium(VI) with penicillamine (▲) (slope = 22 M^{-1} min^{-1}, r = 0.997), dithiothreitol (■) (slope = 17.7 M^{-1} min^{-1}, r = 0.997), 2,3-dimercaptosuccinate (+) (slope = 3.9 M^{-1} min^{-1}, r = 0.998), thiolactate (●) (slope = 3.1 M^{-1} min^{-1}, r = 0.996), and thiomalate (*) (slope = 2.2 M^{-1} min^{-1}, r = 0.997) at 25 °C in 1 M Tris–HCl, pH 7.4.

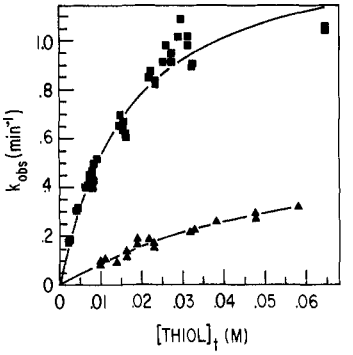


Figure 6. PHASIC plots of the pseudo-first-order rate constants, k_{obsd}, vs. total thiol concentration for the reaction of chromium(VI) with unithiol (■) (a/b = 1.43 min^{-1}) and dihydrolipoate (▲) (a/b = 0.67 min^{-1}) at 25 °C in 1 M Tris–HCl, pH 7.4.

thioester (eq 5) which then undergoes a rapid redox reaction. The simplified form of the rate law consistent with the data is

$$-d[Cr^{VI}]/dt = k_1[RSH]_t[Cr^{VI}] \quad (15)$$

In the case of dithiothreitol, the formation of the chromium(VI) thioester intermediate (eq 5) may be followed by a unimolecular redox reaction as represented in eq 8. Assuming the reaction of the chromium(VI) thioester with a second thiol (eq 6) is negligible ($k_2[RSH]_t \ll k_3$ or k_4), the rate law for such a mechanism would be

$$-d[Cr^{VI}]/dt = k_3k_1[RSH]_t[Cr^{VI}]/(k_{-1} + k_3) \quad (16)$$

Furthermore, since dithiothreitol can form an intramolecular disulfide bond, whereas 2,3-dimercaptosuccinate cannot, the redox step (eq 8) may be rapid (i.e., $k_4 \gg k_{-1}$), in which case eq 15 is obtained.

In contrast to the linear plots observed for the dithiols 2,3-dimercaptosuccinate and dithiothreitol, the plot of k_{obsd} vs. $[RSH]_t$ for unithiol and dihydrolipoate (Figure 6) fit the following expression:

$$k_{obsd} = a[RSH]_t/(1 + b[RSH]_t) \quad (17)$$

If k_{-1} of eq 5 is large, the chromium(VI) and chromium(VI) thioester are in equilibrium ($K = k_1/k_{-1}$) and the redox step (eq 7 or 8) is rate determining. The corresponding rate law is

$$\frac{-d[Cr^{VI}]_t}{dt} = \frac{k_3K[RSH]_t[Cr^{VI}]_t}{1 + K[RSH]_t} \quad (18)$$

According to this mechanism the a and b parameters of eq 17, determined from the fit of k_{obsd} vs. $[RSH]_t$ data with the program PHASIC,[20] correspond to the following expressions:

$$a = k_3K,\ b = K, \text{ and } a/b = k_3 \quad (19)$$

However, k_{obsd} was determined from the disappearance of chromate at 372 nm and there was no spectral evidence for the formation of a chromium(VI) thioester (normal absorbance maximum at 420–440 nm, see Figure 3B) with unithiol and dihydrolipoate. It is possible that the absorbance of the intermediates with unithiol and dihydrolipoate are very similar to that of chromate, since they may form chelates in which both sulfhydryl groups are bound to chromium(VI). In this case the absorbance at 372 nm reflects total chromium(VI) concentration and eq 18 applies.

The apparent second-order rate constants, k_1, determined for the reaction of chromium(VI) with various thiols are listed in Table I. Since the apparent second-order rate constants were derived from plots by using the total thiol concentration, $[RSH]_t$, and since significant ionization to RS^- would occur at pH 7.4 with some of the thiols tested, the constants were recalculated on the basis of [RSH] according to the following expression:

$$[RSH]_t = (K_a/[H^+] + 1)[RSH] \quad (20)$$

where K_a is the acid dissociation constant for the thiol group. These corrected values, k_1', are listed in Table I. A plot of log k_1' vs. the pK_a of the thiol (Figure 7) showed an inverse relationship (slope = −0.68, r = 0.957). Thus, the rate of ligand substitution on chromium(VI) by the thiol is faster the lower the pK_a of the thiol group. The dependence of the log k_1' on pK_a could be considered separately for thiols which contain a source of protons, 1–6 in Table I, and those thiols without a protonated group at pH 7.4, 7–10 and 12–15 in Table I. There was approximately a twofold difference in the slopes for these two sets of thiols, −0.71 (r = 0.966) vs. −0.42 (r = 0.989), respectively. Since the observed rate constant for the reaction of penicillamine with chromium(VI) was anomalously low when compared to the other thiols containing the $-NH_3^+$ group and the observed rate constant for the reaction of thioglycolate with chromium(VI) was anomalously high when

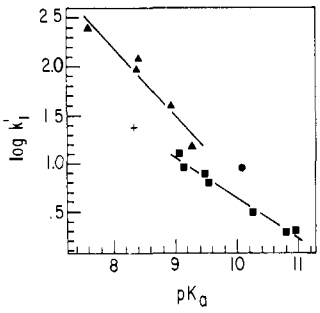


Figure 7. Plot of the log of the apparent second-order rate constant, k_1', for the reaction of chromium(VI) with various thiols at 25 °C in 1 M Tris–HCl, pH 7.4, vs. the pK_a of the thiol. Slope = –0.71, r = 0.966 for the five thiols containing $-NH_3^+$ groups (▲) and slope = –0.42, r = 0.989, for the seven thiols lacking the $-NH_3^+$ functional group (■). Penicillamine (+) and thioglycolate (●) were excluded from the linear regression calculations.

compared to the nonprotonated thiols, they were excluded from the calculation of the slopes.

Discussion

Under conditions of physiological pH, we have shown that only ascorbate and thiols react at a significant rate with chromium(VI). These results are consistent with previous studies showing oxidation of ascorbate to dehydroascorbate by chromium(VI) over the pH range of 5.6 to 6.6[22] and reaction of chromium(VI) with penicillamine at pH 7.0.[23] The fact that oxalate (E_0' = –0.9 V) did not reduce chromium(VI) and ascorbate (E_0' = +0.08 V) rapidly reduced chromium(VI) indicates that the reduction of chromium(VI) at pH 7.4 is controlled by kinetic rather than thermodynamic factors. The *cis*-diol of ascorbate and the –SH of the thiols appear to be important groups in the reaction of chromium(VI) with biological reductants. The fact that all of the reductants which failed to reduce chromium(VI), including those known to reduce chromium(VI) at low pH, e.g., lactate,[24] oxalate, and formate,[25] had attacking functional groups with pK_a < 6 suggests that the reductant reacts with chromium(VI) at a significant rate only when protonated or that the reductant must supply chromium(VI) with a proton. Methionine was found to react with chromium(VI) under acidic conditions[26] and under neutral conditions after prolonged incubation at 37 °C.[27] Lactate and tryptophan were also found to react with chromium(VI) at pH 7 after prolonged incubation at 37 °C.[27] Since we found no significant reaction with methionine, lactate, or tryptophan at short reaction times at pH 7.4, these compounds are not likely to be important reactants for chromium(VI) in biological systems.

The stoichiometry for reaction of chromium(VI) with various thiols was ~3 thiol molecules per chromium(VI) except in the case of dithiothreitol which required only ~1.5 molecule per chromium(VI). Previous studies have shown a thiol–chromium(VI) ratio of 3:1 with penicillamine,[23,28] glutathione,[28] and cysteine.[29] These results are consistent with an overall reaction (see below) in which chromium(VI) is reduced to chromium(III) and the thiol is oxidized to the disulfide (an intramolecular disulfide in the case of dithiothreitol).

$$n\mathrm{H^+} + 2\mathrm{Cr^{VI}} + 6\mathrm{RSH} \rightarrow 2\mathrm{Cr^{III}} + 3\mathrm{RSSR}$$

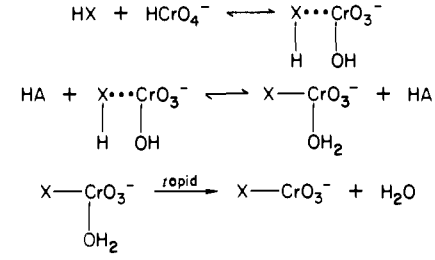
The apparent second-order rate constants for the reaction of chromium(VI) with thiols and dithiols (calculated on the basis of –SH concentration) varied by a factor of ~77 from cysteine ethyl ester to 2,3-dimercaptosuccinate. Assuming these rate constants refer to the formation of the chromium(VI) thioester, the reaction under consideration at pH 7.4 would be a ligand-substitution reaction on the tetrahedral chromate anion (see below):

$$\mathrm{CrO_4^{2-}} + \mathrm{RSH} \xrightarrow{k_1} \mathrm{RSCrO_3^-} + \mathrm{OH^-}$$

The thiols containing positively charged $-NH_3^+$ functional groups had the largest values of k_1, whereas the thiols containing two negatively charged $-COO^-$ functional groups had the lowest values. The electrostatic interaction between the thiol and chromate would be expected to affect the rate in the manner observed. However, the rate for cysteine, which carries a net charge of zero, is faster than the rate for cysteamine (net +1 charge). Thus, the presence of the $-NH_3^+$ group appears to be as important as the net charge on the thiol with respect to the rate of thiol attack on chromate. The distinct plot of log k_1' vs. pK_a for these thiols (vs. the thiols without the $-NH_3^+$ moiety) also suggests an important role for the $-NH_3^+$ group in the rate of formation of the chromium(VI) thioester.

It is likely that formation of the chromium(VI) thioester is rate determining ($k_1 \ll k_2$[RSH] or k_3) in the case of the sterically hindered penicillamine, for the sterically hindered and negatively charged thiolactate (–1), thiomalate (–2), and 2,3-dimercaptosuccinate (–2), and for dithiothreitol which should undergo a rapid redox reaction due to the formation of an intramolecular disulfide bond. The apparent second-order rate constants for the dithiols (calculated on the basis of –SH concentration) are not abnormal when compared to the monothiols, thus it appears that the second thiol group does not confer any advantage with respect to the initial attack on chromate. It is possible that under conditions where the redox step is rate limiting the dithiols which can form an intramolecular disulfide bond (dithiothreitol and dihydrolipoate) would react much faster than monothiols or dithiols (unithiol and 2,3-dimercaptosuccinate) which are expected to form cyclic dimers with intermolecular disulfide bonds.[30]

The general decrease in k_1 with increasing pK_a of the thiol indicates that the ionization of the thiol plays an important part in determining the rate of formation of the chromium(VI) thioester. Rate-determining proton transfer has been proposed in substitution reactions of hydrogen chromate with acidic ligands. In both uncatalyzed and acid catalyzed reactions of hydrogen thiosulfate,[31] hydrogen thiocyanate,[32] and hydrogen chromate[33] and in acid catalyzed reactions of dihydrogen phosphate[34] and dihydrogen phosphite[35] with hydrogen chromate it was proposed that the rate of substitution depended on the transfer of a proton to the hydroxy ligand on the hydrogen chromate either from the weakly associated acidic substituting ligand (HX) or from a proton in solution:



where HA is an acid catalyst or solvent. However, Haim[36] pre-

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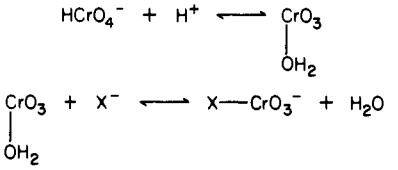
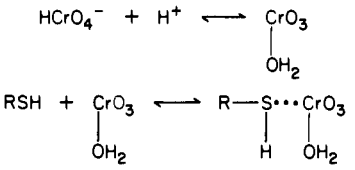
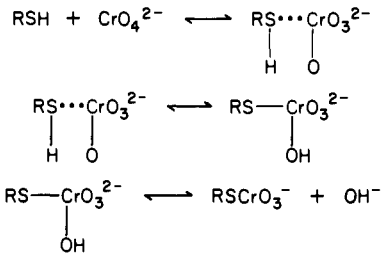
ferred a dissociative mechanism, in which protonation of the OH^- leaving group facilitates its departure over the limiting associative (rate-determining proton transfer) mechanism to explain the very weak bonding of chromium(VI) with the substituting ligand in the transition state:



The preferred mechanism of Haim[36] was modified so that water loss occurred more rapidly than proton transfer in order to explain the acid catalyzed rates for the reaction of hydrogen chromate with penicillamine, cysteamine, and cysteine:[37]



It is likely that under our basic conditions the acid catalyzed reaction pathway is negligible. Attack of chromate by the protonated thiol will be favored over attack by the thiolate for electrostatic reasons. Since hydroxide ion is the leaving group, the transition state occurs later along the reaction pathway (Cr–OH bond breaking is rate determining) for the thiols not containing the $-NH_3^+$ group. It is possible that the thiols containing the $-NH_3^+$ group may facilitate ligand loss by protonation of the leaving group and, therefore, attack by the thiol (Cr–SR bond making which also involves RS–H bond breaking) becomes rate determining. The following mechanism involving five-coordinate chromium(VI) is proposed for the ligand-substitution reactions of chromate with thiols under basic conditions:



The redox reaction must be considered in the mechanism for the reaction of cysteine ethyl ester, cysteine, cysteamine, glutathione, homocysteine, 2-mercaptoethanesulfonate (coenzyme M), coenzyme A, thioglycolate, mercaptoethanol, and *N*-acetylcysteine with chromate. Little steric hindrance is expected with these thiols since they all contain $R-CH_2-SH$ groups. The net charge varies from +1 to −1 for these thiols. The chromium(VI) thioester was present as an intermediate in the reaction of all these thiols with chromate. However, in the case of glutathione the chromium(VI) thioester clearly formed in a pre-equilibrium step prior to the redox step, indicating that $k_{-1} \gg k_2[RSH]$. Thus, the reaction of glutathione with chromate comes closer than any of the other thiol–chromate reactions to exhibiting the kind of behavior at high pH that has been observed at low pH for penicillamine, glutathione, cysteamine, and cysteine.[28,29,37]

The fact that with glutathione the redox step is considerably slower than the formation of the chromium(VI) thioester has important biological implications. The normal intracellular concentration of glutathione in mammalian tissues ranges from 0.8 to 8.0 mM[38] and is higher than any other intracellular thiol concentration. Although the rate of chromium(VI) thioester formation with glutathione is 2–3 times lower than that for other intracellular thiols, e.g., cysteine and cysteamine, these thiols are present at ~10–1000 times lower intracellular concentrations.[39,40] The rate of reaction of chromate with glutathione is comparable to that with ascorbate which is present only at micromolar concentrations in the cell.[41] The slower rate of the subsequent reduction of the chromium(VI)–glutathione thioester compared to the chromium(VI) (thio)esters with other cellular thiols and ascorbate might allow interaction of the chromium(VI)–glutathione thioester with cellular components which would be unreactive toward chromate or the chromium(III) final products of reduction. A cellular component containing a thiol group may react more readily with the chromium(VI)–glutathione thioester than with chromate. Formation of chromium(VI)–glutathione thioester may prolong the lifetime of chromium(VI) inside the cell, allowing its transport to critical targets such as DNA in the nucleus.

These studies suggest that cellular thiols may play an important role in the metabolism of carcinogenic chromium(VI) compounds. Whether these reactions represent possible detoxification pathways or activation pathways is uncertain. It is possible that the stabilization of chromium in the +6 oxidation state by formation of a thioester, particularly by glutathione, may greatly enhance its ability to damage critical cellular macromolecules.

Acknowledgment. This work was supported by the donors of the Petroleum Research Fund, administered by the American Chemical Society, by Grant BC-320 from the American Cancer Society, by PHS Grant CA34869 awarded by the National Cancer Institute, DHHS, and by an A. P. Sloan Research Fellowship. The helpful advice of Walter H. Stockmayer in interpretation of the kinetics was invaluable and is gratefully acknowledged. The assistance of Thomas H. Hampton in all aspects of computerized data collection and analysis is gratefully acknowledged.

Registry No. Ascorbic acid, 50-81-7; glutathione, 70-18-8; cysteine ethyl ester, 3411-58-3; cysteine, 52-90-4; cysteamine, 60-23-1; coenzyme M, 45127-11-5; homocysteine, 6027-13-0; *N*-acetylcysteine, 616-91-1; coenzyme A, 85-61-0; mercaptoethanol, 60-24-2; thioglycolic acid, 68-11-1; penicillamine, 52-67-5; dithiothreitol, 3483-12-3; 2,3-dimercaptosuccinic acid, 2418-14-6; thiolactic acid, 79-42-5; thiomalic acid, 70-49-5; unithiol, 4076-02-2; dihydrolipoic acid, 462-20-4; chromate, 13907-45-4.

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