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Studies on the genotoxicity of chromium: from the test tube to the cell

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Abstract

A critical review of the relevance of in vitro chemical studies to Cr-induced cancers is given. In particular, the nature of the most likely reductants, the species that stabilise intermediates and the mechanisms of DNA damage and damage to transcription proteins are discussed. It is concluded that the major damaging species responsible for the genotoxicity include Cr(V), Cr(IV) and/or reactive organic intermediates. These studies on the chemistry are also related to spectroscopic and other studies on mammalian cells.

Keywords: Cell permeability; Cellular genotoxicity; Chromium; DNA-damage; Hydroxy radical; Intracellular spectroscopy; Mechanisms of reduction; Organic radicals

1. Introduction

Chromium was first identified as a potential occupational carcinogen over a century ago and Cr(VI) compounds were amongst the earliest chemicals to be classified as human carcinogens [1,2]. Such Cr(VI)-induced occupational cancers are of considerable concern because of the widespread industrial use of Cr and the epidemiological evidence that has established links between exposure and respiratory cancers [1,3]. Since Cr(VI)-induced carcinogenicity has been established, a robust debate has developed regarding the species most likely to be responsible for cellular damage, and the mechanism(s) involved in genotoxic damage. Chromium(VI) alone does not react with DNA in vitro, or isolated nuclei. In the presence of reductants, however, it causes a wide variety of DNA lesions, including Cr–DNA adducts, DNA–DNA crosslinks, DNA–protein crosslinks, apyrimidinic/apurinic (AP) sites, and oxidative damage [4–6]. In vivo and in vitro studies on cultured cells have shown that Cr(VI) induces chromosomal aberrations and the formation of micronuclei, sister-chromatid exchanges, DNA strand breaks and unscheduled DNA synthesis [7,8]. These results are explained by the uptake-reduction model of Cr-induced cancers [9]. This model initially assumed that all chromates were taken up via SO_4^{2-} and HPO_4^{2-} channels, but insoluble chromates are absorbed by cells via phagocytosis, whereby the absorbed particles become

engulfed in acidic vacuoles (ca. pH 4) [10]. Moreover, there is a need to consider the uptake of species generated by the extracellular reduction of chromates and also to gain an appreciation of the redox chemistry that can occur at the cell membranes via vitamin E redox chemistry. To take account of the increased complexity outlined above, we have modified the uptake-reduction model as illustrated in Fig. 1. This modified model also allows for the uptake of extracellular Cr in oxidation states other than Cr(VI), since certain Cr(V) and Cr(III) complexes that could be generated extracellularly also have high permeabilities. The intracellular incorporation of such species from the extracellular environment has been largely ignored in the past in terms of their relevance to Cr genotoxicity.

The complexity of the biochemistry of Cr(VI), illustrated by Fig. 1, requires an in-depth investigation of the chemistry of Cr in the extracellular environment, membranes, and the diverse range of intracellular chemical environments, before an appreciation of the nature of the initial molecular-level genotoxic events can be garnered. Our approach to this problem has been to characterise biotransformation reactions at a fundamental level and to couple this with in vitro studies of genotoxicity and permeability, and intracellular spectroscopic studies. In doing so, the 'test-tube chemistry' has been related to that which is likely to ensue in the cell.

Although research in this area has resulted in a very rich and often controversial literature, given the space limitations, this review is confined mainly to studies conducted within our research group. However, we have also addressed these controversies in the context of both our research and those from other laboratories. The review describes the factors that stabilise different Cr species and non-metal radicals in the diverse biological environments, the kinetics and mechanisms of their

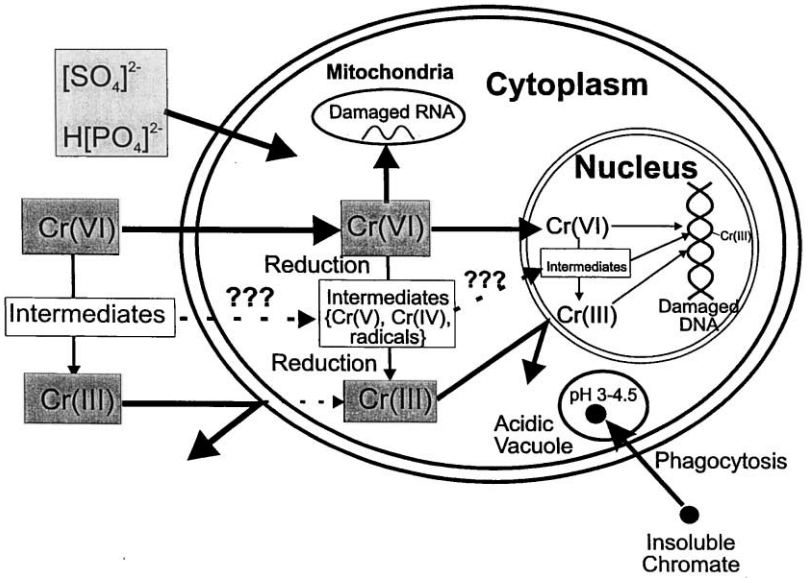


Fig. 1. The uptake-reduction model for Cr(VI)-induced carcinogenesis.

reactions and the methods for their characterisations. This has then enabled an evaluation of the species that are most likely to lead to genotoxic events and how they are likely to impart the damage to the genetic apparatus that ultimately leads to cancers.

2. Characterisation of the potential genotoxic intermediates

2.1. Chromium(VI)

The postulate that Cr(VI) is transported into the cell through channels that exist for the transfer of the isostructural and isoelectronic endogenous anions, such as SO_4^{2-} and HPO_4^{2-}, was confirmed by Beyersmann et al. [11] and Arslan et al. [12]. They showed that Cr(VI) uptake was reduced significantly in the presence of reagents known to block such anion-transport channels (e.g. 4,4′-diisothiocyanostilbene-2,2′-disulfonate) [11,12]. The fate of Cr(VI) in vivo is intimately linked to its intracellular redox behaviour, where the reduction potential of CrO_4^{2-} at physiological pH values ($E' = 0.52$ V) is sufficiently positive to ensure the oxidation of a range of biological molecules. This section of the review describes the nature of Cr(VI) species likely to be found in vivo.

In aqueous solution at pH values between 3 and 11, Cr(VI) is present as CrO_4^{2-}, $Cr_2O_7^{2-}$ and $HCrO_4^-$. While there is some contention regarding the existence of the $HCrO_4^-$ anion [13], elegant work by Brasch et al. characterised this species by electronic absorption and ^{17}O NMR spectroscopies [14]. They tentatively attributed the fine structure observed in the electronic absorption spectrum of an acidified solution of Cr(VI) (0.208 mM) to the lowering of the symmetry from T_d in CrO_4^{2-} to C_{3v} in $HCrO_4^-$ [14]. At normal physiological pH values (~ 7.4), however, the concentration of $HCrO_4^-$ is small, with Cr(VI) existing predominantly ($\sim 96\%$) as the CrO_4^{2-} anion [15]. Glutathione is present in vivo at relatively high (millimolar) concentrations and is likely to play an important role in the intracellular metabolism of Cr(VI). Several studies have focused on species formed between Cr(VI) and glutathione itself [15] and smaller thiolate reductants [15–17]. A Cr(VI)–thiolate ester has been observed by Brauer and Wetterhahn in the reaction between $K_2Cr_2O_7$ and glutathione at $pH \leq 6$ [16]. More recently, complexes between Cr(VI) and γ-glutamylcysteine, *N*-acetylcysteine and cysteine, formulated as $[RSCr^{VI}O_3]^-$, have been characterised in solution by Brauer et al. using ^1H-, ^{13}C- and ^{17}O-NMR spectroscopies [17]. Kinetic analyses on the Cr(VI)/cysteine system at ca. pH 7, indicate that cysteine acts as a bidentate chelate [18]. Unlike other thiols, glutathione forms a complex with Cr(VI) in a rapid equilibrium step, followed by a slow reduction of Cr(VI) [15]. Thus, glutathione can act as an intramolecular stabiliser of Cr(VI) via the formation of a thiolate ester. The importance of glutathione with respect to the intracellular metabolism of Cr(VI) has been illustrated by Borges et al. [19], who showed that Cr(VI) in the presence of glutathione yielded Cr(V) species and altered DNA conformation. In addition, Kortenkamp et al. [20] showed PM2 DNA breaks occurred in the presence of

Cr(VI) and glutathione. It is uncertain whether Cr(VI)–thiolate ester complexes can damage DNA, or whether the damage is due to other species.

2.2. Chromium(V)

Following the discovery of a long-lived EPR-active Cr(V) species formed upon the reduction of Cr(VI) by microsomes in the presence of NADPH [21], attention became focused on the possible role(s) played by Cr(V) species in Cr(VI)-induced carcinogenesis. Chromium(V) complexes with several ligand-types have been isolated and characterised by X-ray crystallography [22] and X-ray absorption fine structure (XAFS) analyses [23,24]. The discussion here will focus on complexes with an oxo group and six-coordinate catecholato complexes. Complexes with nitrido ligands will be mentioned briefly, since they are not likely to be biologically relevant.

2.2.1. Characterisation of Cr(V) complexes

The most common means of characterising Cr(V) complexes in solution is EPR spectroscopy ($Cr(V) = d^1$), where strong isotropic signals are observed at room temperature in X-band spectra. A typical EPR spectrum of a Cr(V) complex is centred at $g_{iso} \approx 1.98$ and exhibits an intense narrow line (1–5 G), due to the ^{n}Cr ($n = 50$, 52, 54; $I = 0$) isotopes, with the appearance of a quartet of hyperfine satellites (^{53}Cr $A_{iso} \approx 16–18 \times 10^{-4}\,cm^{-1}$) due to the ^{53}Cr isotope (natural abundance = 9.55%; $I = 3/2$). The nature of the coordinating ligands can be analysed further by the splitting of the central signal resulting from superhyperfine coupling with hydrogen (^{1}H $a_{iso} \approx (0.5–1.0) \times 10^{-4}\,cm^{-1}$, $I = 1/2$) or nitrogen (^{14}N $a_{iso} \approx (2.0–2.5) \times 10^{-4}\,cm^{-1}$, $I = 1$) atoms. An empirical relationship between the nature and number of donor groups and the EPR spectral parameters of Cr(V) complexes has been established [25]. The g_{iso} and ^{53}Cr A_{iso} values in five-coordinate Cr(V) species are higher and lower, respectively, than the corresponding parameters in similar six-coordinate species [25]. Such empirical analyses are extremely useful in the assignment of the structures of new oxoCr(V) species. Chromium(V) species also have intense charge transfer bands in their electronic absorption spectra that often overlap with those of Cr(VI), but have a weak band at ~ 700 nm characteristic of Cr(V) [22].

2.2.2. 2-Hydroxyacids

By far the most extensively studied Cr(V) compounds are Cr(V)–2-hydroxyacid complexes (Fig. 2). Roček and co-workers successfully isolated $K[CrO(hmba)_2]\cdot H_2O$ (hmba = 2-hydroxy-2-methylbutanoato(2−)) using both an aqueous [26] and a non-aqueous route [27]. Chromium(V) complexes with *tert*-2-hydroxyacids are easily isolated, air stable and have half-lives ranging from minutes to days in protic solvents (depending on the pH value and temperature) and about 1 year in aprotic solvents, such as DMSO (ambient temperature, in the absence of light) [28]. The X-ray crystal structures of $K[CrO(hmba)_2]\cdot H_2O$ (**Ia**) [26] and $Na[CrO(ehba)_2]\cdot 1.5H_2O$ (ehba = 2-ethyl-2-hydroxybutanoato(2−)) (**Ib**) [28] have

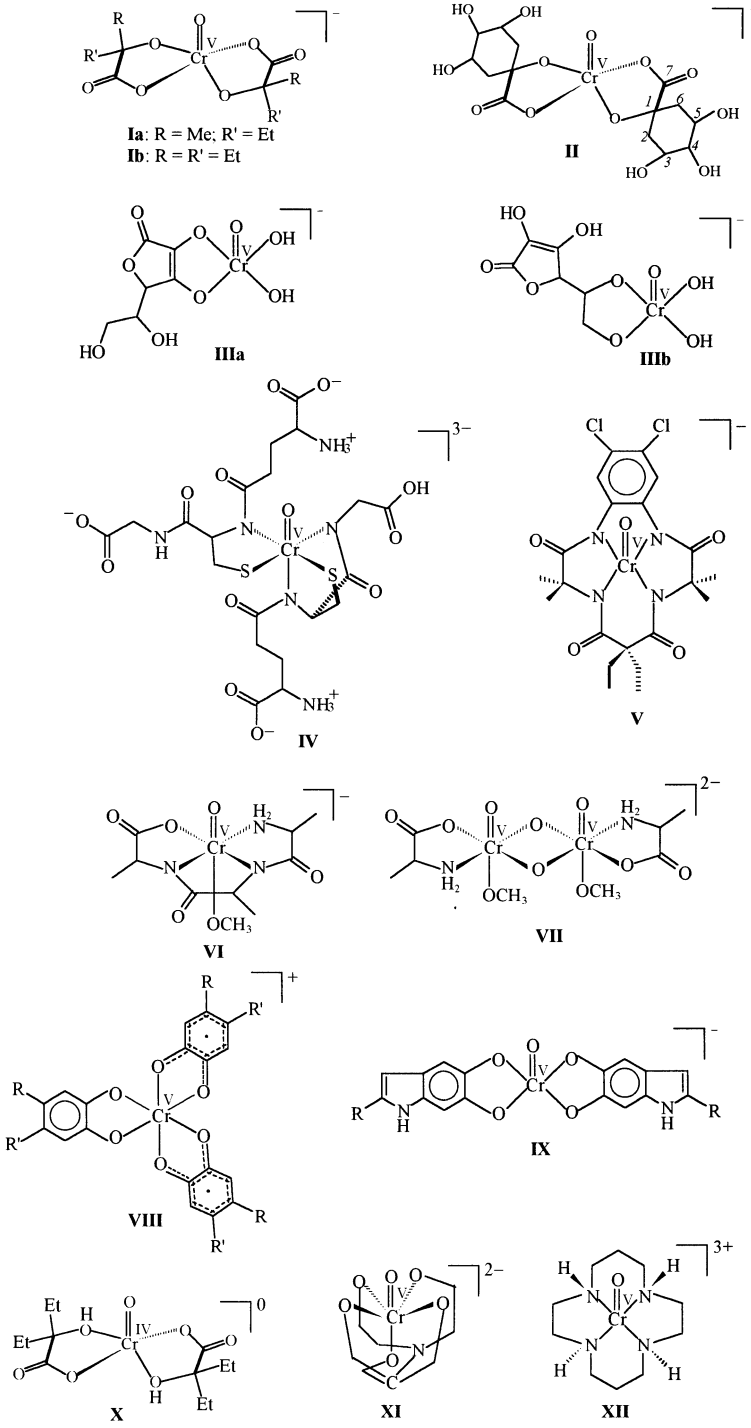


Fig. 2. Structures of Cr(V) and Cr(IV) complexes.

similar distorted trigonal bipyramidal coordination geometries, where the axial and equatorial sites are occupied by the carboxylato and alcoholato donors, respectively, with the remaining equatorial site being occupied by an oxo ligand. The additional species that are evident from the ^{53}Cr-hyperfine structure in EPR spectra from solutions of **Ia**,**b**, have been ascribed by Bramley et al. to the presence of more than one geometrical isomer [29]. This is in agreement with subsequent ^{1}H ENDOR studies by Branca et al. [30].

A novel Cr(V)–2-hydroxyacid complex has recently been isolated featuring the multifunctional ligand quinic acid (1*R*,3*R*,4*R*,5*R*-1,3,4,5-tetrahydroxycyclohexanecarboxylic acid, qaH_5) [31]. The structure of the complex, $K[CrO(qaH_3)_2]\cdot H_2O$ (**II**), recently solved by analysis of XAFS data [23] (Fig. 3), shows a coordination geometry analogous to that of **Ia**,**b**.

In addition to the *tert*-2-hydroxyacid group (O^1,O^7), quinic acid has a triol group in which one diol pair is oriented in a *cis*-fashion (O^3,O^4) and the other in a *trans*-fashion (O^4,O^5). Therefore, a total of six linkage isomers are possible within the Cr(V)/quinic acid system: $[CrO(O^1,O^7\text{-}qaH_3)_2]^-$, $[CrO(O^1,O^7\text{-}qaH_3)(O^3,O^4\text{-}qaH_2)]^{2-}$, $[CrO(O^1,O^7\text{-}qaH_3)(O^4,O^5\text{-}qaH_2)]^{2-}$, $[CrO(O^3,O^4\text{-}qaH_2)(O^4,O^5\text{-}qaH_2)]^{2-}$, $[CrO(O^3,O^4\text{-}qaH_2)_2]^{3-}$ and $[CrO(O^4,O^5\text{-}qaH_2)_2]^{3-}$. In aqueous solutions at pH < 4.0, **II** gives two EPR signals ($g_{iso} = 1.9787$, ^{53}Cr $A_{iso} = 17.2 \times 10^{-4}$ cm^{-1}; $g_{iso} = 1.9791$, ^{53}Cr $A_{iso} = 16.4 \times 10^{-4}$ cm^{-1}). The relative intensities of the signals are independent of $[qaH_5]$:[Cr(V)], and of increasing $[qaH_5]$ and [Cr(V)] at constant

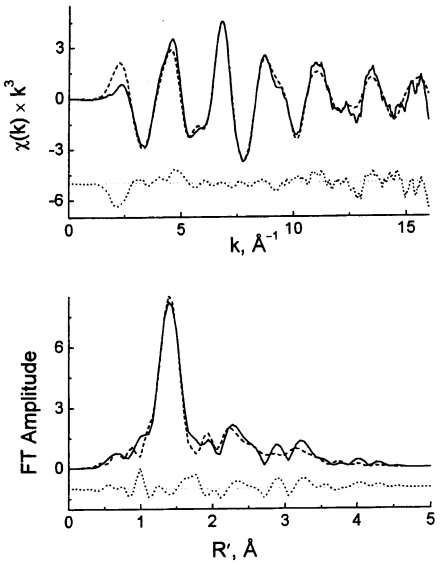


Fig. 3. Results of multiple-scattering calculations of XAFS spectra of $K[CrO(qaH_3)_2]$ (qaH_3 = quinato(2 −); solid; 10 K). Solid lines are the observed spectra, dashed lines are the calculated spectra, and dotted lines are the fit residues. (Reproduced with permission from Ref. [23]. Copyright American Chemical Society.)

[qaH_5]:[Cr(V)] and pH values. These signals (Table 1) are consistent with those found with well-characterised Cr(V)–2-hydroxyacid complexes and are assigned to two geometric isomers of the [$CrO(O^1,O^7\text{-}qaH_3)_2]^-$ linkage isomer. The diolato functionality of quinic acid is able to coordinate to the Cr(V) ion at higher pH values (>5.0), as shown by solution EPR spectroscopic studies [31], which yields an equilibrium mixture of species with coordination of Cr(V) by either the 2-hydroxyacid and/or diolato motifs. The EPR spectra from Cr(V)–quinic acid solutions have been deconvoluted into signature spectra for each individual linkage isomer (Fig. 4) and the equilibrium constants describing the relative stabilities of the isomers were determined [31]. The value of K_{eq} for [$CrO(O^1,O^7\text{-}qaH_3)(O^3,O^4\text{-}qaH_2)]^{2-}$ (10^{-7} M), is about an order of magnitude greater than that for [$CrO(O^1,O^7\text{-}qaH_3)(O^4,O^5\text{-}qaH_2)]^{2-}$ (10^{-8} M), which is consistent with preferential binding of *cis*-diolato rather than *trans*-diolato groups to Cr(V) [32,33]. It is noteworthy that the generation of Cr(V)–diolato species, as described above, requires the presence of glutathione as a reductant, since without glutathione the

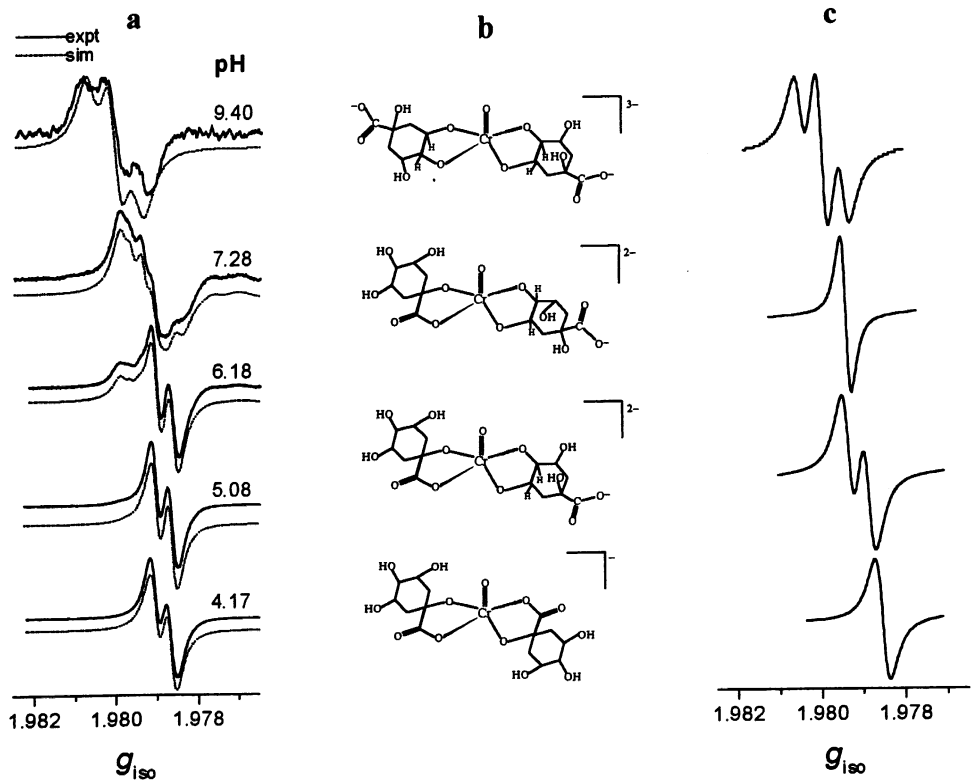


Fig. 4. Room-temperature X-band EPR spectra (solid line: experimental; dotted line: simulation) of the Cr(V) intermediates in the reaction of Cr(VI) (40 mM) with GSH (2 mM) in the presence of qaH_5 (100 mM) at pH 4.17, 5.08, 6.18, 7.28 or 9.40 (a). The Cr(V)–quinic acid linkage isomers comprising the equilibrium mixtures are shown in (b), which are aligned with the individual signature EPR spectra (c).

Table 1
EPR spectroscopic parameters (g_{iso}, ^{53}Cr A_{iso}) and line widths (LW) for $Na[CrO(hmba)_2]\cdot H_2O$ (**Ia**), $Na[CrO(ehba)_2]\cdot H_2O$ (**Ib**) and $K[CrO(qaH_3)_2]\cdot H_2O$ (**II**) in aqueous solution and in the solid state

Complex	Solution						Solid	
	Species 1			Species 2			g_{iso}	LW (G)
	g_{iso}	LW (G)	$10^4 \times {}^{53}Cr$ A_{iso} (cm^{-1})	g_{iso}	LW (G)	$10^4 \times {}^{53}Cr$ A_{iso} (cm^{-1})		
$[CrO(hmba)_2]^-$ (**Ia**)	1.9785	0.91	17.4	1.9785[a]	0.91[a]	16.3	1.9790	14.6
$[CrO(ehba)_2]^-$ (**Ib**)	1.9784	0.78	17.2	1.9784[a]	0.78[a]	16.1	1.9783	7.8
$[CrO(qaH_3)_2]^-$ (**II**)	1.9787	0.49	17.2	1.9791	0.45	16.4	1.9803	30.4

[a] The g_{iso} values and the line widths of the individual species cannot be distinguished.

concentration of Cr(V) species formed is very small. The reduction potentials of glutathione and diol and hydroxyacid ligands, however, are similar [15], which indicates, in agreement with previous work by Connett and Wetterhahn [15], that Cr(VI) reduction is under kinetic rather than thermodynamic control.

2.2.3. Ascorbic acid, sugars and diols

At least seven Cr(V) signals are observed, in addition to the signal for the ascorbate radical, in the EPR spectra from Cr(VI) and ascorbate reaction solutions [34]. The nature of the Cr(V)–ascorbate species formed is dependent upon both the [Cr(VI)]:[ascorbate] and pH value. The most abundant signals ($g_{iso} = 1.9791$) were attributed to monoligated oxo–Cr(V)–ascorbate complexes involving binding from either the enediolato moiety (**IIIa**) or the diolato moiety from the aliphatic side chain of the ligand (**IIIb**).

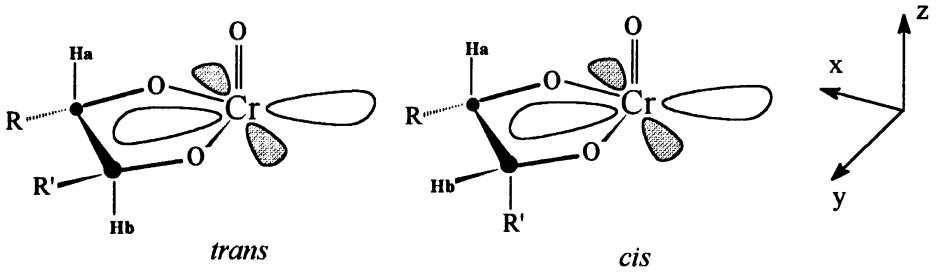
In the presence of O_2, EPR spectroscopic signals ascribed to Cr(V)–ascorbate–peroxo complexes were observed; however, these species were undetectable in the presence of catalase [34]. The nature of the Cr(V)–ascorbate–H_2O_2 complexes has been confirmed in experiments with added H_2O_2. In aerated ascorbate solutions, H_2O_2 is formed during the autooxidation of ascorbate [34]. The nature of the Cr(V)–ascorbate species formed was also investigated as a function of buffer [35]. The previously observed Cr(V)–ascorbate species were present in all the buffers examined with new Cr(V) species noted ($g_{iso} = 1.9765$ and $g_{iso} = 1.9781$) in Tris (tris(hydroxymethyl)aminomethane) buffer, assigned as a mixed-ligand Cr(V)–ascorbate–tris complex [35].

The biological relevance of the interaction of Cr(V) with diolato groups was first indicated by the work of Goodgame et al. who showed that Cr(V) complexation occurred with single ribonucleotide units but not with the analogous deoxyribonucleotides [36]. This suggested that the *cis*-diolato group of the ribose unit was involved in Cr(V) coordination. Chromium(V) species formed with ribonucleotides and with D-ribose 5′-monophosphate have been characterised recently and yield EPR spectroscopic parameters ($g_{iso} \approx 1.979$ and ^{53}Cr $A_{iso} \approx 16.3 \times 10^{-4}\,cm^{-1}$) indicative of Cr(V)–diolato binding [37]. The Cr(VI) oxidation of a variety of carbohydrate ligands, such as D-galacturonic acid [38], D-galactono-1,4-lactone [39], 2-acetoamido-2-deoxy-D-glucose [40], methyl α-D-glucose [41], and D-glucose [25,32,33,42,43], has been examined. In the reduction of Cr(VI) to Cr(III) by D-glucose, it is proposed that the formation of intermediate Cr(V)–D-glucose complexes results from the reaction between a Cr(VI) ion and a glucose radical [42]. This study was conducted under conditions where the rate of mutarotation is slower than the Cr(VI)–D-glucose redox reaction, and enabled the speciation between Cr(V) and α- or β-D-glucose to be examined. The conclusions were consistent with those drawn from earlier work that noted a preference for *cis*-diolato rather than *trans*-diolato coordination in Cr(V)–D-glucose complexes [25,32,33]. The complexity of the EPR signals obtained from Cr(V)–D-glucose solutions has been addressed by using Q-, X- and S-band EPR spectroscopies and partially or fully deuterated D-glucose ligands [25,32,33]. The Q-band EPR spectra of Cr(V)/$^2H_7$–D-glucose-*C* solutions were shown to contain at least six Cr(V)

species [32,33]. One of the most important observations of recent times with regard to Cr(V)–diolato complexes is that the multiplicity of the signal in the EPR spectrum is dependent upon whether the ligand is cyclically strained (e.g. sugar) or not (e.g. linear diol). Seminal work conducted by Irwin on species formed between Cr(V) and *cis*- or *trans*-1,2-cyclohexanediol established that the strain of the ring system imparts inequivalence to the magnetic environment of the protons in the second coordination sphere [32]. The signals in the EPR spectra of [CrO(*cis*-1,2-cyclohexanediolato)$_2$]$^-$ or [CrO(*trans*-1,2-cyclohexanediolato)$_2$]$^-$ exhibit a triplet and a singlet, respectively. In the latter complex, no protons lie in the Cr(V)–ligand plane and, therefore, there is minimal overlap between the proton orbital and the Cr(V) orbital containing the unpaired electron density (d_{xy}). In the case of [CrO(*cis*-1,2-cyclohexanediolato)$_2$]$^-$, however, two protons (one from each chelate ring) are lying in the Cr(V)–ligand plane, which gives rise to a triplet in the EPR spectrum (Scheme 1) [32].

This work has been corroborated by solution EPR spectroscopy of Cr(V) complexes formed with quinic acid and shikimic acid (3*R*,4*R*,5*R*-3,4,5-trihydroxy-1-cyclohexene-carboxylic acid) [31]. The bis-chelate Cr(V)–shikimato complexes are formed from coordination via either the *trans*-(O^4,O^5) or *cis*-(O^3,O^4)-diolato donor groups and have signature isotropic EPR signals of a singlet or a triplet, respectively [31]. The Cr(V)–shikimato linkage isomer involving coordination of the *cis*-diolato group is preferentially formed compared with the *trans*-diolato isomer, as evidenced by the dominance of a triplet signal in the EPR spectra of Cr(V)–shikimato solutions [31]. Several studies have interpreted a triplet signal in the EPR spectra of Cr(V)–sugar complexes to be due to a mono-Cr(V) complex with a single bidentate chelate, containing two magnetically equivalent protons [43–45]. Based on the current knowledge of the subtle difference in the proton environments in cyclic-diols and that these studies use high [ligand]:[Cr(VI)] (e.g. 25–250) [43], it has been established that the triplets in these cases are due to bis-chelate complexes with *cis*-diolato ligation [31–33].

In Cr(V) complexes in which the ligands are not conformationally constrained, such as [CrO(ed)$_2$]$^-$ (ed = 1,2-ethanediolato(2 −)), dynamic processes impart magnetic equivalence to the system and the axial or equatorial protons are indistin-



Scheme 1. Orbital overlap between a Cr(V) ion and cyclically strained diolato ligands oriented in a trans- or cis- fashion.

guishable in isotropic EPR spectra [46]. The products from ligand-exchange experiments between **Ib** and 1,2-ethanediol, $[CrO(ehba)(ed)]^-$ and $[CrO(ed)_2]^-$, give rise to five- or nine-line spectra, respectively, due to the ^1H-superhyperfine coupling associated with the coordination of either one or two ed ligands [46]. The analogous products, $[CrO(ehba)(ox)]^-$ and $[CrO(ox)_2]^-$ (ox = oxalato(2 −)), have been observed from ligand-exchange experiments between **Ib** and oxalic acid [47]. Measurements of isotropic and anisotropic ^1H ENDOR spectra of a series of Cr(V)–diolato complexes are able to distinguish the magnetic environment of the protons in bis-Cr(V)–diolato (diol = linear) complexes with superhyperfine coupling contants $^1H_{eq}$ a_{iso} and $^1H_{ax}$ a_{iso} equal to 0.81×10^{-4} and 0.37×10^{-4} cm^{-1}, respectively [48]. Similar ligand-exchange reactions were noted in bis-tris (bis(hydroxyethyl)amino-tris(hydroxymethyl)methane) buffer solutions used to study in vitro DNA damage caused by **Ib** [49].

It is becoming more apparent that diol ligands may play an important role in the stabilisation of Cr(V) species. A recent study by Liu et al. [50] used EPR spectroscopy to examine the metabolism of Cr(VI) in whole live mice and assigned the EPR signal to a Cr(V)–diolato species formed with NADP. It may be more appropriate to think of the small-molecule Cr(VI) reducing agents, such as glutathione and ascorbate, as detoxifying agents, where the ultimate genotoxic agents are species formed between Cr(V) and diolato or sugar-like molecules. Some Cr(V)–sugar species are very stable at physiological pH values, with an EPR signal detectable after 48 h of the initiation of the reaction [32,33].

2.2.4. Amino acids, peptides and peptide models

The formation of two major Cr(V) species ($g_{iso} = 1.996$ and $g_{iso} = 1.985$) during the reduction of Cr(VI) by glutathione in neutral aqueous media was first observed by O'Brien et al. [51]. They also isolated a Cr(V)-containing material (solid state EPR signal at $g = 1.996$) from the reaction between Cr(VI) and glutathione [52]. The Cr(V) intermediates from the reactions between Cr(VI) and glutathione and a series of thiols with both free amine and *N*-acetyl substituted (amide) groups were studied [53]. The reaction between Cr(VI) and excess *N*-acetylcysteine yielded Cr(V) species with similar EPR spectroscopic parameters ($g_{iso} = 1.995$; $g_{iso} = 1.985$) that were observed in the Cr(VI)/glutathione system. Reactions between Cr(VI) and cysteine or related thiols with free amine groups yielded very small concentrations of Cr(V) species, which indicates that the deprotonated amide N atom is a stronger Cr(V) donor than the free amine [53]. Levina et al. [54] have further studied the Cr(V)-containing material, isolated by the method of O'Brien et al. [52] using new techniques, such as electrospray mass spectrometry and XAFS spectroscopy. Based on these results and previous EPR spectroscopic data, the main Cr(V) intermediate formed in the Cr(VI)/glutathione reaction is proposed as $[CrO(GS)_2]^{3-}$ (GSH = glutathione) (**IV**).

Two Cr(V) complexes have been isolated and characterised with macrocyclic ligands that mimic non-sulfur-containing proteins, namely $[CrO(mampa)]^-$ (mampa = 5,6 - (4,5 - dichlorodibenzo) - 3,8,11,13 - tetraoxo - 2,2,9,9 - tetramethyl-

12,12-diethyl-1,4,7,10-tetraazacyclotridecane(4 −)) (**V**) and $[CrO(mac)]^{-}$ (mac = 3,6,9,12,14-pentaoxo-2,2,5,5,7,7,10,10-octamethyl-13,13-diethyl-1,4,8,11-tetraazacyclotridecane(4 −) [55]). The EPR spectra of **V** and $[CrO(mac)]^{-}$ exhibit nine-line signals centred at $g_{iso} = 2.006$ and $g_{iso} = 1.999$, respectively, each with well resolved superhyperfine coupling from four equivalent N donor atoms (^{14}N $a_{iso} = 2.43 \times 10^{-4}\,cm^{-1}$) in addition to four ^{53}Cr satellite signals (^{53}Cr $A_{iso} = 16.6 \times 10^{-4}\,cm^{-1}$) [55].

Cr(V) species formed with the tripeptides, GlyGlyGly (Gly_3; Gly = glycine) or AlaAlaAla (Ala_3; Ala = alanine), have been characterised in solution following the oxidation of the Cr(III)–tripeptide species by PbO_2 or the reduction of Cr(VI) in methanol [56]. The reduction of Cr(VI) by Ala_3 in methanol in the presence of fluorescent light yielded a quintet in the EPR spectrum ($g_{iso} = 1.9827$, ^{14}N $a_{iso} = 2.35 \times 10^{-4}\,cm^{-1}$, ^{53}Cr $A_{iso} = 17.82 \times 10^{-4}\,cm^{-1}$) indicative of two equivalent N donors (proposed structure **VI**) [56]. Similar spectral parameters ($g_{iso} = 1.9824$; ^{14}N $a_{iso} = 2.44 \times 10^{-4}\,cm^{-1}$) were observed for a Cr(V)–Gly_3 species [56]. The use of photoreduction for the formation of Cr(V)–amino acid complexes was first noted by Bolte et al. [57], who observed an EPR signal ascribed to a Cr(V)–Gly complex from a photoreduced solution of Cr(VI) and Gly. The PbO_2 oxidation of Cr(III)–Gly_4 or Cr(III)–Gly_5 complexes has also been investigated and yields a more complex mixture of Cr(V) species. On the basis of an X-ray absorption spectroscopy (XAS) study [58], a dinuclear Cr(V) species (**VII**) has been proposed for a Cr(V)–Ala complex that is EPR silent in the solid state [56]. Cr(V) complexes with peptide-like ligands have also been isolated [59] following the oxidation of the Cr(II) analogues with more biologically relevant oxidants, such as organic peroxides. The generation of Cr(V)–peptide species from the oxidation of the Cr(II) or Cr(III) analogues has important implications with respect to the potential carcinogenicity of Cr(III) compounds in vivo (see Section 2.4).

2.2.5. Peroxides

The X-ray crystal structure of $K_3[Cr(O_2)_4]$ has recently been re-determined by Wood et al. [60] and discussed in terms of the applicability of bond valence sum (BVS) analysis to Cr(n)–O_m (n = II, III, IV, V, VI; m = 3–6) complexes. Three new Cr(V) species were observed ($g_{iso} = 1.9820$; $g_{iso} = 1.9798$, ^{53}Cr $A_{iso} = 16.3 \times 10^{-4}\,cm^{-1}$; $g_{iso} = 1.9764$, ^{53}Cr $A_{iso} = 18.1 \times 10^{-4}\,cm^{-1}$) in addition to the well-characterised $[Cr(O_2)_4]^{3-}$ species ($g_{iso} = 1.9723$, ^{53}Cr $A_{iso} = 18.4 \times 10^{-4}\,cm^{-1}$) from the reaction between Cr(VI) and H_2O_2 [61]. The pH and $[H_2O_2]$ dependencies of the Cr(VI) + H_2O_2 reaction were studied and these results, taken together with the results of analogous V(V)/H_2O_2 [62] chemistry, led to the assignment of the species at $g_{iso} = 1.9764$, $g_{iso} = 1.9798$ and $g_{iso} = 1.9820$ as $[Cr(O_2)_3(OH)]^{2-}$, $[CrO(O_2)_2(OH)]^{-}$ and $[CrO(O_2)(OH)]^{+}$, respectively [61]. The reaction between Cr(VI) and H_2O_2 produces intermediates capable of inducing DNA cleavage in vitro [63,64], and while it is possible that the complexes responsible for DNA damage may be Cr(V)–peroxo derived, it is more likely that alternative Cr(V) and/or Cr(IV) species are the relevant genotoxic agents in vivo, due to intrinsic enzyme systems that keep the concentration of H_2O_2 very low (on the order of nanomolar) [5,65].

2.2.6. Catechol(amine)s and oxidised proteins

The importance of catecholato (1,2-benzenediolato) groups as Cr(V) stabilisers in biological systems has been overlooked for a long time. The main biological sources of catechol moieties are: (i) neurotransmitters and their precursors, such as L-3,4-dihydroxyphenylalanine (DOPA) and dopamine; (ii) hormones, such as adrenaline and noradrenaline; (iii) the skin pigment, melanin, which is formed by oxidation and polymerisation of catecholamines and contains some non-oxidised catecholic groups [66]; (iv) products of oxidative damage of proteins, containing protein-bound DOPA [67]; and (v) products of partial enzymatic oxidation of polyaromatic compounds, contained in air pollution and cigarette smoke [68]. The redox chemistry of Cr–catecholato species is particularly complex owing to the redox-active nature of the ligands, which can exist in catecholato, semiquinone or quinone forms, thus making the redox state of Cr ambiguous [69].

Detailed EPR spectroscopic studies of the Cr(VI) reductions with catechol, adrenaline, noradrenaline, DOPA and their derivatives in aqueous solutions (pH 3.5–7.5) have been performed [70]. In the presence of excess reductant, all of the substrates yield Cr species with signals at $g_{iso} \approx 1.972$. These species, which were previously considered as monoanionic Cr(III) complexes [69,71], have been re-assigned, on the basis of EPR and X-ray absorption spectroscopic studies of a model Cr–catecholato complex [72], as octahedral monocationic desoxoCr(V) complexes (e.g. $[Cr(semiquinone)_2(catecholate)]^+$ (**VIII**)), possessing electron delocalisation between the Cr ion and the ligands. Other major and long-lived EPR signals, at $g_{iso} \approx 1.980$, were detected in the reactions of catecholamines with excess Cr(VI), and assigned to the Cr(V) species, $[CrO(L)_2]^-$, where L is a cyclised catecholamine, bound to Cr via the catecholato moiety (**IX**). Similar EPR signals of Cr(V) species were observed during the reactions of Cr(VI) with enzymatically oxidised proteins [73].

2.2.7. N-Heterocycles

Complexes with *N*-heterocycles are of interest in terms of both understanding the potential interactions of Cr(V) with biological *N*-heterocycles and also because they can be generated from Cr(III) precursors that are mutagenic. Oxochromium(V)–salen (salen = (*N*,*N*′-ethylenebis(salicylideneaminato)(2 −)) complexes result from iodosylbenzene or *m*-chloroperbenzoic acid [74,75], or PbO_2 [76,77] oxidations of the corresponding Cr(III)–salen complexes. An intense band in the infrared spectrum of $[CrO(salen)]^+$ at $1000\,cm^{-1}$ is characteristic of the Cr=O stretch in oxoCr(V) complexes. The EPR spectrum of the complex exhibits a quintet (^{14}N $a_{iso} = 2.01 \times 10^{-4}\,cm^{-1}$) centred at $g_{iso} = 1.978$ [75]. The reaction between the corrole-type ligand MEC (2,3,17,18-tetramethyl-7,8,12,13-tetraethylcorrolato(3 −)) and Cr(II) yielded [CrO(MEC)], which showed well-resolved superhyperfine coupling to four equivalent N donor atoms (^{14}N $a_{iso} = 3.3 \times 10^{-4}\,cm^{-1}$) in the EPR spectrum ($g_{iso} = 1.987$) [78]. Stable [CrN(ttp)] (ttp = 5,10,25,20-tetra-*p*-tolylporphyrinato(2 −)) and [CrN(oep)] (oep = 2,3,7,8,12,13,17,18-octaethylporphyrinato(2 −)) complexes have also been prepared and characterised [79,80]. The 11-line signal in the isotropic EPR spectrum in [CrN(ttp)] and [CrN(oep)] indicates

the magnetic equivalence of the five nitrogen atom donors (^{14}N $a_{iso} = 2.52 \times 10^{-4}\,cm^{-1}$). The inequivalence between the pyrrole N atoms and the axially coordinated nitrido atom becomes discernible from anisotropic EPR and ^{14}N ENDOR spectroscopic measurements [80]. The nitrido Cr(V) complex, [CrN(bpb)] (bpb = N,N′-bis(pyridine-2-carbonyl)-*o*-phenylenediamido(2 −)), was isolated and characterised crystallographically following photolysis of the Cr(III)–azido complex Na[Cr(bpb)(N_3)$_2$] [81], and has more recently been examined by electronic absorption and EPR spectroscopy [82]. Nitrido Cr(V)–porphyrinates are more stable than the cationic oxo Cr(V) analogues, with the latter complexes being easily reduced to the neutral oxo Cr(IV)–porphyrinates [83].

2.3. Chromium(IV)

Compared with the current knowledge of the chemistry of Cr(V) intermediates from Cr(VI) reductions, fewer studies have focused on Cr(IV) species for the following reasons: (i) Cr(IV) complexes are generally less stable in aqueous media than the corresponding Cr(V) complexes [84]; and (ii) EPR spectroscopy, which is a very powerful tool in Cr(V) chemistry, is not applicable for Cr(IV) complexes under ambient conditions [22,25].

The relatively stable intermediates with $\lambda_{max} \approx 460$ nm formed in the reactions of Cr(VI) with glutathione and other thiols in acidic aqueous media (pH 1–6) have been attributed by most authors to Cr(VI) thioesters [16,17,85–87]. Bose and co-workers [88,89], on the basis of dynamic magnetic susceptibility measurements, argued that these intermediates are in fact Cr(IV)–thiolato complexes. However, these measurements do not unambiguously distinguish between Cr(V) (d^1) or Cr(IV) (d^2) intermediates, and mixtures of Cr(VI) (d^0) and Cr(III) (d^3) species. Recent studies by electrospray mass spectrometry confirmed the formation of relatively stable Cr(VI) thioesters in the Cr(VI) + thiol reactions, and failed to produce any evidence for the formation of Cr(IV)–thiolato complexes [90].

As the above results [88,89] show, reports by Shi and co-workers [91–93] of the isolation of a stable Cr(IV)–glutathione complex from the reaction of Cr(VI) with two mole equivalents of glutathione in aqueous media, in addition to the isolation of a water-stable (pH 7.0) 2,4-dimethyl-2,4-pentanediol Cr(IV) complex by the reaction of Cr(VI) with the ligand [94,95], are erroneous. The results of magnetic susceptibility measurements are attributed to Cr(VI) + Cr(III) mixtures, and the broad EPR signals with $g \approx 1.98$ are typical for octahedral Cr(III) complexes [96].

Many authors have used indirect evidence to postulate the formation of Cr(IV) intermediates, using Mn(II) as a selective trap for Cr(IV). This approach is based on the following: (i) many Cr(VI) reductions in strongly acidic media are inhibited by Mn(II), presumably due to Mn(II) trapping of Cr(IV) [97,98]; and (ii) Mn(II) catalyses disproportionation of the relatively stable Cr(IV) complex, [CrO(ehbaH)$_2$]^0 (**X**), but does not affect the disproportionation rate of the related Cr(V) complex, **Ib** [99]. Kwong and Pennington [100] studied the influence of Mn(II) on the kinetics of the Cr(VI) + cysteine reaction at ca. pH 7. Unexpectedly, it was found that Mn(II) catalyses rather than inhibits the reduction of Cr(VI). However,

no definitive conclusions about the reaction mechanisms could be made, as speciation and reactivity of Mn(II) in neutral media are significantly different than those in strongly acidic media [100]. Stearns and Wetterhahn [101] and Kortenkamp et al. [102] used the decrease in intensity of Mn(II) EPR signals during the reactions of Cr(VI) with ascorbate or glutathione as evidence for the formation of Cr(IV) species, which selectively react with Mn(II), but Cr(V)–ascorbato, Cr(V)–ascorbato–peroxo, and Cr(V)–glutathione complexes are able to oxidise Mn(II) under the same conditions [35,53]. Inhibition by Mn(II) of the oxidative cleavage of DNA and nucleotides with **Ib**, or with the Cr(VI)/reductant systems, was used as an argument for a key role of Cr(IV) intermediates in DNA cleavage [101–104]. However, the amounts of Mn(II) required to inhibit the DNA cleavage with **Ib** are about an order of magnitude higher than those required to suppress significantly the amount of Cr(IV) formed during the decomposition of **Ib** [105]. Thus, Mn(II) is likely to inhibit the DNA cleavage through reduction of oxidised DNA intermediates, rather than by reaction with Cr(IV) [105]. In summary, Mn(II) cannot be used as a selective trap for Cr(IV) to provide conclusive evidence for the role of Cr(IV) intermediates in the mechanisms of Cr(VI) reactions with biological reductants, or in the oxidative DNA damage induced by these reactions.

Relatively stable Cr(IV)–2-hydroxyacid complexes are generated in aqueous media (pH 2–4) by reactions of Cr(VI) with As(III) in the presence of excess ligand [106,107]. The initially generated Cr(IV) complexes undergo ligand-exchange reactions with certain ligands, such as oxalic acid or picolinic acid, to form new Cr(IV) species [107]. Complexes of Cr(IV) with some ligands, such as quinic acid or oxalic acid, are surprisingly stable at near physiological pH values ($t_{1/2} = 1–1.5$ h at pH 5–6; [Cr] = 0.1 mM; 25°C) [107]. The concentrations of these Cr(IV) complexes in solutions can be readily determined from their intense electronic absorbances at 400–600 nm ($\varepsilon_{max} = (2–4) \times 10^3\ M^{-1}\ cm^{-1}$; characteristic electronic absorption spectra of Cr(VI/V/IV/III) species are shown in Fig. 5), although the nature of the

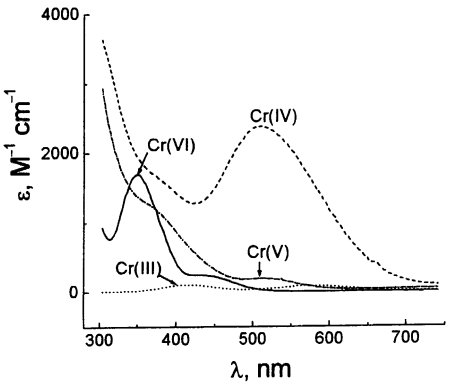


Fig. 5. Typical UV–visible spectra of different oxidation states of Cr in 250 mM ehbaH_2/ehbaH buffer, pH 3.5. Main absorbing species (> 90%) are: Cr(VI) = $[HCrO_4]^-$; Cr(V) = $[CrO(ehba)_2]^-$; Cr(IV) = $[CrO(ehbaH)_2]^0$; Cr(III) = $[Cr(ehbaH)_2(OH_2)_2]^+$.

Cr(IV) charge transfer band in the visible region is yet to be assigned [106,107]. These findings enable direct studies of Cr(IV) reactions with DNA and other biomolecules to be performed [108–110]. Biologically relevant ligands, such as glutathione and 1,2-diols (including carbohydrates), did not form stable Cr(IV) species in ligand-exchange reactions with Cr(IV)–2-hydroxyacid complexes [107]. This further supports the notion that the purported Cr(IV) complexes with similar ligand types isolated by Shi and co-workers [91–95] are mixtures of Cr(VI) and Cr(III) species.

The structure of a relatively stable Cr(IV)–ehba complex, which has been studied in detail with respect to its reactivity with biomolecules [108,109], has been the subject of considerable debate [111,112]. The structure of this complex, together with those of the Cr(V)–ehba and Cr(III)–ehba complexes, has been determined from the analysis of XAS data (frozen aqueous solutions, 10 K) [24,113]. The structure of the Cr(IV) complex **X** (at pH 3.5 and in the presence of excess ligand) is similar to that of the Cr(V) complex **Ib** (distorted trigonal bipyramidal [28]), and very different to that of the Cr(III) complex $[Cr(ehbaH)_2(OH_2)_2]^+$ (octahedral [114]). The main difference between the structure of **X** and that of **Ib** is the protonation of alcoholato groups of the ligands, leading to a significant elongation of the corresponding Cr–O bonds (2.00 Å in **X** versus 1.80 Å in **Ib**) [24,113]). This structural distinction is reflected in the differences in chemical properties between **X** and **Ib**, including deprotonation [107,109], easy ligand exchange [107], and the ability to cleave DNA in the presence of excess ligand [105]. This also explains why Cr(IV), unlike Cr(V), is not stabilised by 1,2-diolato ligands, including carbohydrates [107]. Determination of the solution structure of the Cr(IV)–ehba complex solves the controversy over the mechanisms of electron transfer in the Cr(V/IV/III)–ehba redox series [112,115].

2.4. Chromium(III)

Stable cationic Cr(III) complexes interact electrostatically with negatively charged phosphate groups of DNA [116]. Cr(III) salts, such as $CrCl_3$ or $Cr(NO_3)_3$, bind strongly to DNA in neutral aqueous media [117] and, recently, the coordination mode involving phosphate groups and N-7 atoms of guanine residues has been established [118]. From the observation that Cr(VI) (in the form of $[CrO_4]^{2-}$) does not bind to isolated DNA, but Cr(III) ($CrCl_3$) does, Beyersmann and Köster [117] concluded that Cr(III) is the ultimate carcinogenic form of Cr. Kortenkamp et al. [119] argued that the reactions of strongly acidic Cr(III) complexes, such as $CrCl_3$, with isolated DNA are unlikely to be relevant to the in vivo Cr–DNA interactions and suggested that the intracellular reduction of Cr(VI) in the vicinity of DNA can lead to the formation of reductant–Cr(III)–DNA complexes [120]. Zhitkovich et al. [121] found that Cr(III)–amino acid complexes, which are likely to form during intracellular reduction of Cr(VI), are also capable of binding to DNA. The formation of stable (due to the kinetic inertness of Cr(III) [84]) DNA–Cr(III)–protein and DNA–Cr(III)–DNA crosslinks during the reduction of Cr(VI) has been well documented in both in vitro and in vivo systems [121–123] and regarded

as one of the major reasons of Cr(VI) genotoxicity [5]. Among possible genotoxic properties of Cr(III)–DNA complexes are: enhancement of RNA synthesis [124]; alteration of endonuclease function [125]; and arrest of DNA replication by polymerases [122,126]. In addition, low molecular weight Cr(III) complexes have been shown to catalyse phosphorylation of proteins [127] and to interact with cell membranes [128].

Another possible mechanism by which Cr(III) complexes can cause genotoxicity is their involvement in redox reactions in cells. Sugden et al. [129] suggested that Cr(III) complexes with aromatic imines, possessing relatively high Cr(III/II) redox potentials, can be reduced intracellularly and form DNA-damaging hydroxyl radicals, via Fenton-like reactions (Eqs. (1) and (2)). It was proposed that this reaction course explained the observed mutagenicity of these Cr(III) complexes in bacterial assays [129]:

$$\mathrm{Cr(III)} + \mathrm{Red} \rightarrow \mathrm{Cr(II)} \tag{1}$$

$$\mathrm{Cr(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Cr(III)} + {}^{\bullet}\mathrm{OH} + \mathrm{OH^-} \tag{2}$$

Similar mechanisms (involving $^{\bullet}OH$ as the ultimate DNA-damaging species) were proposed for DNA cleavage in vitro by the Cr(III)/H_2O_2 or Cr(III)/O_2/reductant systems [130–135]. Some Cr(III) complexes (such as *cis*-$[Cr(phen)_2(OH_2)_2]^{3+}$ (phen = 1,10-phenanthroline) and *trans*-$[Cr(salen)(OH_2)_2]^{+}$) cause mutations in bacterial and mammalian cells. This correlates with their ability to be oxidised to Cr(V) complexes under mild conditions by oxygen donors (such as PbO_2 or PhIO) [76,77]. It was also shown that the Cr(V)-containing oxidation products were able to cleave DNA in vitro [77]. This led to the suggestion [76,77] that Cr(III) complexes accumulated in Cr(VI)-treated cells can be enzymatically re-oxidised to Cr(V/IV) complexes, which in turn can cause DNA damage (see also Section 4.1). Such re-oxidation under biological conditions has previously been considered impossible [136]. However, this possibility is supported by some recent findings, such as the intramolecular conversion of a biomimetic Cr(III)–peroxo–L (L = 1,4,8,11-tetraazacyclotetradecane) complex into an oxoCr(V)–L complex [137]. Re-oxidation of accumulated Cr(III) may explain the long-term health effects in workers previously exposed to Cr(VI) [138].

2.5. Reactive oxygen species and organic radicals

Oxidative stress, caused by uncontrolled reduction of O_2 with the participation of catalytic metal ions, leads to the formation of reactive oxygen species (ROS) and organic radicals and is well known to be a major source of mutagenesis and a starting point of many human diseases [139]. Whether the mutagenicity and carcinogenicity of Cr(VI) can be fully or partially explained by this mechanism is under debate [65,140]. Sugden et al. [141] and Kortenkamp et al. [102] have shown that O_2 is required for Cr(VI)-induced mutations in bacterial cells and for the in vitro DNA cleavage by the Cr(VI)/reductant systems. Based on these and similar observations, many authors [142–144] concluded that ROS (with $^{\bullet}OH$ as the most

powerful O_2-derived oxidant [139]), are the ultimate genotoxic species formed in Cr(VI)-exposed cells. Shi et al. [145] are strong advocates of this hypothesis and propose a number of pathways in which $^{\bullet}OH$ can be formed in Haber–Weiss and Fenton-like reactions with the participation of different oxidation states of Cr.

However, several lines of evidence contradict the proposed role of $^{\bullet}OH$ in Cr(VI)-induced genotoxicity. The products of oxidative DNA damage, induced by the Cr(VI)/reductant systems, are different from those caused by the $^{\bullet}OH$ sources, such as γ-radiation [5,146]. The O_2-dependence of Cr(VI/V/IV)-induced DNA cleavage reactions is explained by the reactions of O_2 with DNA radicals, formed from direct DNA oxidation by Cr species [108,147]. Finally, Cr(VI/V/IV) species mimic ROS in the reactions with spin traps and oxidant-sensitive dyes [148–150], which are usually used to gain indirect evidence for the involvement of ROS in DNA damage [145].

Thus, the current knowledge suggests that the genotoxic effects of Cr cannot be fully explained by the action of ROS or more specifically $^{\bullet}OH$. Nevertheless, the possible role of ROS in these effects cannot be disregarded [151]. The reactions of Cr(VI) with biological reductants, such as ascorbate and thiols, are accompanied by formation of organic radicals, which in turn cause O_2 activation and formation of ROS [109]. Another possible source of ROS is the uptake of insoluble chromate particles by phagocytosis, which is accompanied by oxidative stress [10]. Both events can trigger apoptosis [10,152].

Oxygen- or sulfur-based radicals have been observed (by EPR and/or electronic absorption spectroscopic techniques) during the reactions of Cr(VI/V/IV) with biological reductants, including thiols [153–155], ascorbate [34,101,156], catecholamines [70] and vitamin E (α-tocopherol) or its analogue, Trolox (*R*- or *S*-6-methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) [109]. Carbon-based radicals have been observed during the in vitro reduction of Cr(VI) by ascorbate [101], as well as in the bile of rats, treated with Cr(VI) (possibly originating from the bile acids) [157,158]. These radical species can participate in DNA damage directly [155] or through the formation of ROS [109]. Organic radicals, however, are unlikely to play a major role in Cr-induced genotoxicity, since these species feature in normal metabolic pathways [139,159].

3. Kinetic and mechanistic studies

3.1. General mechanisms of Cr(VI) reduction to Cr(III): formation of Cr(V/IV) intermediates

Experiments that focus on the mechanistic pathways of reactions involving Cr(V/IV) and free radicals are essential to understand the possible mechanisms of Cr(VI)-induced carcinogenicity. Reduction of Cr(VI) to Cr(III) is a three-electron process, whereas most organic reductants act as one- or two-electron donors, so their reactions with Cr(VI) will necessarily pass through Cr(V/IV) intermediates [97]. The suggestion of Roček and co-workers, that one-step three-electron reduc-

tion of Cr(VI) is possible under certain conditions (mainly in co-oxidation of two reductants) [160,161], has been disputed by other authors [162].

A vast literature has been devoted to the mechanisms of Cr(VI) reduction. The great majority of these studies, however, were carried out under strongly acidic conditions ($pH \leq 2$), and thus have limited implications to biological systems. The most extensive and systematic mechanistic studies of Cr(VI) reductions have been performed by Westheimer [97], Beattie and Haight [98], Roček and co-workers [161,163], Espenson and Bakac [164,165], Mitewa and Bonchev [162], and Gould (reactions of Cr(V/IV) complexes) [111,166]. Here, we will briefly describe the most general mechanistic schemes proposed for Cr(VI) reduction to Cr(III) by one- or two-electron donors.

Reductions with one-electron donors proceed via three consecutive steps; this mechanism is most common for transition metal ions (Eqs. (3)–(5)) [164]:

$$\mathrm{Cr(VI)} + \mathrm{M}^{n+} \rightarrow \mathrm{Cr(V)} + \mathrm{M}^{(n+1)+} \tag{3}$$

$$\mathrm{Cr(V)} + \mathrm{M}^{n+} \rightarrow \mathrm{Cr(IV)} + \mathrm{M}^{(n+1)+} \tag{4}$$

$$\mathrm{Cr(IV)} + \mathrm{M}^{n+} \rightarrow \mathrm{Cr(III)} + \mathrm{M}^{(n+1)+} \tag{5}$$

More complex schemes are applicable to those reductants that act as either one- or two-electron donors. Generally, the first step is the formation of a Cr(VI)–substrate complex (Eq. (6)) [97,162] followed by two-electron (Eq. (7)) [97] or one-electron transfer (Eq. (8)) [167,168]:

$$\mathrm{Cr(VI)} + \mathrm{S} \rightleftharpoons \mathrm{Cr(VI)\text{–}S} \tag{6}$$

$$\mathrm{Cr(VI)\text{–}S} \rightarrow \mathrm{Cr(IV)} + \mathrm{P} \tag{7}$$

$$\mathrm{Cr(VI)\text{–}S} \rightarrow \mathrm{Cr(V)} + \mathrm{R}^{\bullet} \tag{8}$$

where S is the substrate, P is the product and $R^{\bullet}$ is a free radical.

Most of the proposed mechanisms of Cr(VI) reductions differ mainly in the reactions of Cr(IV) intermediates, formed in Eq. (7). They can undergo (i) comproportionation with Cr(VI) (Eq. (9)) [97], (ii) disproportionation (Eq. (10)) [169], or (iii) reduction by the substrate with a subsequent formation of Cr(V); the latter can be performed in one-electron (Eqs. (11) and (12)) [170] or two-electron (Eqs. (13) and (14)) [171,172] pathways:

$$\mathrm{Cr(IV)} + \mathrm{Cr(VI)} \rightarrow 2\mathrm{Cr(V)} \tag{9}$$

$$2\mathrm{Cr(IV)} \rightarrow \mathrm{Cr(V)} + \mathrm{Cr(III)} \tag{10}$$

$$\mathrm{Cr(IV)} + \mathrm{S} \rightarrow \mathrm{Cr(III)} + \mathrm{R}^{\bullet} \tag{11}$$

$$\mathrm{Cr(VI)} + \mathrm{R}^{\bullet} \rightarrow \mathrm{Cr(V)} + \mathrm{P} \tag{12}$$

$$\mathrm{Cr(IV)} + \mathrm{S} \rightarrow \mathrm{Cr(II)} + \mathrm{P} \tag{13}$$

$$\mathrm{Cr(VI)} + \mathrm{Cr(II)} \rightarrow \mathrm{Cr(V)} + \mathrm{Cr(III)} \tag{14}$$

Thus, at least five different mechanisms for the formation of Cr(V) intermediates have been proposed, dependent on the substrate and reaction conditions. One of the major factors, affecting the reaction mechanism, is the ability of the substrate to stabilise Cr(V/IV) intermediates [173]. For example, the reaction in Eq. (9) is proposed as the preferred route for Cr(V) formation during the Cr(VI) reduction by diols in aqueous acidic media, based on the fast rate with which a transient Cr(IV)–aqua complex reacts with Cr(VI) [162]. The rate of a reaction between Cr(VI) and a relatively stable Cr(IV)–oxalato complex is slow, and, therefore, the reaction sequence in Eqs. (11) and (12) was proposed as a main route for the formation of Cr(V)–oxalato complexes in the Cr(VI) reduction by oxalic acid [174].

In most of the earlier studies a two-electron reduction process (Eq. (15)) was considered as a main route for Cr(V) decay [162]:

$$\mathrm{Cr(V)} + \mathrm{S} \rightarrow \mathrm{Cr(III)} + \mathrm{P} \tag{15}$$

However, more recent studies with the use of relatively stable Cr(V)–2-hydroxy-acid complexes have shown that many potential two-electron reductants (such as ascorbate or hydroquinone) react with Cr(V) through one-electron pathways, with the formation of Cr(IV) intermediates [111].

The reaction schemes in Eqs. (3)–(15) consider only changes in the Cr oxidation states, and ignore the complexation of Cr(V/IV/III) species with reduced or oxidised forms of the substrate, or with other components of the reaction media (e.g. the buffer). Such simplification is appropriate for many reactions in strongly acidic media, where Cr(V/IV/III) aqua complexes are dominant [172,173]. However, the lack of information on the structures and reactivities of intermediate Cr complexes remains the main obstacle for detailed mechanistic studies of Cr(VI) reductions under physiological pH values [18].

3.2. Reactions of Cr(VI/V/IV) with biological reductants

Connett and Wetterhahn [15], on the basis of in vitro kinetic studies, have shown that among low molecular weight intracellular reductants, ascorbate and thiols (such as glutathione or cysteine) are most likely to reduce Cr(VI) at significant rates. The kinetics and mechanisms of Cr(VI) reduction by glutathione, cysteine and related thiols have been studied extensively [15–18,44,51,52,85–89,100,168,175–185]. Usually, the kinetics of these reactions were followed spectrophotometrically from the decrease in Cr(VI) absorbance at 350 nm or 372 nm (for $HCrO_4^-$ or CrO_4^{2-}, respectively), and were described by the following rate law:

$$-\frac{\mathrm{d[Cr(VI)]}}{\mathrm{d}t} = \frac{a[\mathrm{Cr(VI)}][\mathrm{RSH}]^2}{1 + b[\mathrm{RSH}]} \tag{16}$$

This led to the proposal of a general mechanism of Cr(VI) reduction by thiols [15,100,182] involving the formation of a Cr(VI) thioester (Eq. (17)), which can either react with a second thiol molecule forming a Cr(IV) intermediate and a disulfide oxidation product (Eq. (18)), or undergo an intramolecular redox reaction to form a Cr(V) intermediate and a thiyl radical (RSH = thiol) (Eq. (19)):

$$Cr(VI) + RSH \rightleftharpoons Cr(VI)SR \quad (17)$$

$$Cr(VI)SR + RSH \rightarrow Cr(IV) + RSSR \quad (18)$$

$$Cr(VI)SR \rightarrow Cr(V) + RS^{\bullet} \quad (19)$$

Formation and decay of the Cr(VI) thioester can also be followed from the absorbance of the intermediate ($\lambda_{max} \approx 460$ nm) [87,178]. Mechanisms of further reactions that lead to the Cr(III) products are not clear, as these reactions do not affect the rate law [100].

O'Brien and co-workers [168,176] studied the kinetics of formation and decomposition of Cr(V) intermediates in the Cr(VI) + glutathione reaction (following the absorbance of Cr(V) species at 600–650 nm or by EPR spectroscopy). Two main pathways (Eqs. (19) and (10)) were proposed for the formation of different types of Cr(V) species in these reactions. The relative importance of the one-electron pathway (Eq. (19)) increases with decreasing $[RSH]_0$ [168]. Perez-Benito et al. [87,179] suggested the importance of a pathway involving Cr(II) formation (Eq. (13)) in the Cr(VI) reduction by thiols under biological conditions. It was proposed that rapid reaction of Cr(II) with O_2 led to the generation of a Cr(III)–peroxo complex, which was detected from its characteristic absorbance at $\lambda_{max} \approx 290$ nm [179].

Complex dependencies of the Cr(VI) + RSH reaction kinetics on the nature and concentration of the buffer were evident [180], and, under certain conditions (pH < 6, acetate buffer), Cr(VI) reductions by cysteine or glutathione are catalysed by Zn(II) [180,181]. Reductions of Cr(VI) by glutathione in the presence of carbohydrates, such as D-glucose, lead to the formation of Cr(V)–carbohydrate intermediates, which are thermodynamically more stable than the Cr(V)–thiol species [32,33,44].

Kinetic studies of the Cr(VI) reactions with ascorbate are much less numerous than those with thiols [15,101,186–188]. In all such studies, the kinetics of Cr(VI) decay was described by a simple rate law (RH^- = ascorbate):

$$-\frac{d[Cr(VI)]}{dt} = a[Cr(VI)][RH^-] \quad (20)$$

where the rate coefficient a is pH dependent [186]. The formation of intermediates in this reaction is not apparent from the time-dependent changes in the electronic absorption spectra. EPR spectroscopy, however, reveals the formation of several Cr(V) intermediates, in addition to ascorbate anion-radicals and other carbon-based radicals [34,35,101]. Stearns and Wetterhahn [101], on the basis of kinetic and EPR spectroscopic studies, have proposed the following general mechanism of Cr(VI) reduction by ascorbate in neutral aqueous solutions:

$$Cr(VI) + RH^- \rightleftharpoons Cr(VI)\text{–}RH \quad (21)$$

$$Cr(VI)\text{–}RH \rightarrow Cr(IV) + R \quad (22)$$

$$Cr(IV) + RH^- \rightarrow Cr(III) + R^{-\bullet} \quad (23)$$

$$Cr(IV) + Cr(VI) \rightarrow 2Cr(V) \quad (24)$$

$$Cr(V) + R \rightarrow \text{carbon-based radicals} \quad (25)$$

where Eq. (22) is the rate-limiting step, and the Cr(VI)–RH intermediate is formed in small steady-state concentrations. The relative importance of the proposed pathway leading to Cr(V) and carbon-based radicals (Eqs. (24) and (25)) increases with increasing $[Cr(VI)]_0$:$[RH^-]_0$ [101]. Others suggested that the rate-limiting stage of this reaction is the formation of a Cr(VI) ester (Eq. (21)) [187], or emphasised the potential importance of one-electron reductions of Cr(VI/V/IV) with ascorbate or its anion-radical [186].

The important role of nicotinamide adenine dinucleotides ($NAD(P)H/NAD(P)^+$) (which exist as cofactors of many redox enzymes) in the biological reduction of Cr(VI) has been suggested [21,189], and NADH-dependent Cr(VI) reductases have been isolated from bacteria [190,191]. The mechanism of Cr(VI) reduction by NAD(P)H is affected by the ability of the substrate to stabilise Cr(V) at $pH \geq 7$ via the *cis*-1,2-diolato moiety of the ribose ring [21]. It has been suggested that a Cr(V) intermediate is formed in a fast one-electron reduction of Cr(VI) (Eqs. (6) and (8)) and is reduced to Cr(III) in a slower two-electron process (Eq. (15)) [190]; however, no mechanistic details of Cr(VI) reduction by NAD(P)H are known, as yet. Studies of the Cr(VI) reduction by a NAD(P)H model, 10-methyl-9,10-dihydroacridine (which is a chain reaction), show the formation of Cr(V), Cr(IV) and organic radical intermediates [192], but this is not biologically relevant because of the use of strongly acidic media [192] and the inability of the substrate to stabilise Cr(V).

Catecholamines and catechol groups of oxidised proteins may also be significant intracellular reductants of Cr(VI) [70]. Reactions of Cr(VI) with catecholamines in vitro produce Cr(V) and organic radical intermediates [70] and induce O_2 activation and DNA cleavage [193,194]. However, detailed kinetic and mechanistic studies of these reactions are complicated by the production of melanin.

Vitamin E, the most common lipophilic biological reductant, has been observed to act both as an anti-oxidant [195] and as a pro-oxidant [196] in Cr(VI)-treated mammalian cell cultures. The reductions of Cr(VI) by vitamin E [197,198], or its water-soluble analogue, Trolox [199], proceed via the formation of relatively stable Cr(V) complexes of the oxidised substrates. Reduction of Cr(VI) by Trolox in the presence of carbohydrates leads to the formation of Cr(V)–carbohydrate complexes [197,198]. Trolox is oxidised efficiently in neutral aqueous solutions by relatively stable Cr(V/IV)–ehba complexes (**Ib** and **X**), probably via a series of one-electron transfers, since relatively stable Trolox anion-radicals could be observed directly during these reactions from their characteristic electronic absorption spectra [109]. No detailed mechanistic studies of Cr(VI) reduction by vitamin E or its analogues have been performed as yet.

Biological Fe(II) complexes, primarily haemproteins, may participate in intracellular Cr(VI) reductions [145], as was suggested when erythrocytes, rather than blood plasma, were shown to be responsible for the formation of Cr(V) complexes in the blood of Cr(VI)-exposed rats [200], although other intracellular species may have been the reductants. No mechanistic studies of Cr(VI) interactions with haem

Fe(II) are available at present, but the kinetics of the cytochrome *c* reaction with **Ib** at pH 4–5 (which sufficiently stabilises Cr(V) [163]) have been studied [201]. In this reaction, an irreversible outer-sphere one-electron transfer from Fe(II) to Cr(V) is preceded by a precursor complex formation [201].

Photochemical reduction of Cr(VI) in polypeptide gelatine gels is used widely in imaging technology [202,203] and may have analogies in Cr(VI) reduction by animal skin. Kinetic studies by Lafond et al. [203] have shown that the first fast step in Cr(VI) photoreduction by gelatine is the formation of Cr(V)–peptide complexes, followed by their slow conversion to Cr(III) products. Reaction of Cr(VI) with the skin of living rats has also been shown to produce Cr(V) intermediates, although the influence of light on this reaction has not been studied [204].

Hydrogen peroxide is often considered to be among the important biological reductants of Cr(VI), but this is unlikely because of its very low (on the order of nanomolar) concentrations in the cells [5,65]. The mechanism of the Cr(VI) + H_2O_2 reaction in neutral and slightly acidic aqueous media is complex and involves the formation of several Cr(VI)–H_2O_2 and Cr(V)–H_2O_2 intermediates [61,205]. At ca. pH 7, Cr(VI) acts as a catalyst of H_2O_2 disproportionation [205].

The kinetics and mechanisms of Cr(VI) reduction by some amino acids (serine [206,207], or methionine [208]) and carbohydrates [39–43,209] have been studied in acidic aqueous media. However, these reactions are unlikely to have any biological relevance, as the rates of Cr(VI) reduction with these substrates under physiological pH conditions are negligible compared with those of more powerful biological reductants, such as glutathione, ascorbate, vitamin E or catechols. This does not exclude the potential role of carbohydrates and amino acids as stabilisers of Cr(V) intermediates formed during the reactions of Cr(VI) with intracellular reductants [31–33,56,210].

3.3. Activation of molecular oxygen

Significant consumption of O_2 [91,211], and promotion of alkene epoxidation [177] in the Cr(VI)/reductant/O_2 system provide evidence for involvement of O_2 in the mechanisms of Cr(VI) reactions with biological reductants. On the other hand, the presence of O_2 does not affect the kinetics of the Cr(VI) + ascorbate reactions in demetallated buffers [187]. The earlier data, which showed the inhibition of this reaction by O_2 [186], were explained by rapid autooxidation of ascorbate in buffers containing traces of Fe(III) and Cu(II) [187].

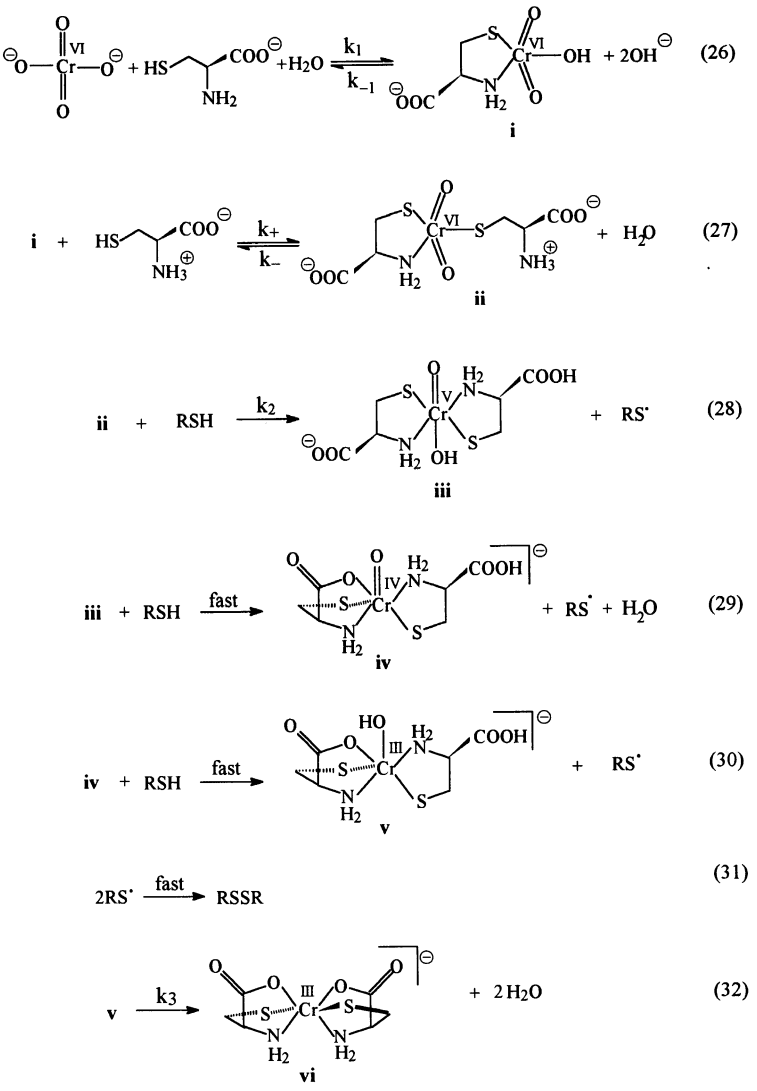
Systematic studies of the roles of O_2 in reactions of Cr(VI/V/IV) with major intracellular reductants and their models were conducted [109,110]. Reactions of Cr(VI), Cr(V) (**Ib**), or Cr(IV) (**X**) with ascorbate, glutathione or cysteine in neutral aqueous solutions were accompanied by significant O_2 consumptions, but no such consumptions were observed for the reactions with Trolox. Comparison of the kinetics of O_2 consumption (measured by an oxygen electrode) with that of Cr(VI/V/IV) reduction (followed by time-dependent electronic absorption spectroscopy) led to the proposal of O_2 activation mechanisms that involve chain reactions of O_2 with organic radicals formed during the reduction of Cr(VI/V/IV)

[109]. Alternative mechanisms of O_2 activation, such as the formation of Cr(V)–peroxo or –superoxo complexes [212], or formation of Cr(II) species and their reactions with O_2 [179], were ruled out on the basis of stoichiometric and kinetic studies [109]. This study has shown that Cr(V/IV) species, formed during the intracellular reductions of Cr(VI), can act as initiators of radical chain reactions, leading to oxidative stress and mutagenesis [157]. This does not exclude the possibility of direct reactions between Cr(V/IV) species and DNA [9]. The lack of significant H_2O_2 accumulation as a result of O_2 consumption during the reduction of Cr(VI/V/IV) by cysteine, glutathione or ascorbate (determined with catalase [109]) supports the suggestion of Kortenkamp et al. [5] that Fenton-like reactions do not play a significant role in the DNA damage by the Cr(VI)/reductant/O_2 systems in the absence of added H_2O_2.

3.4. Application of global kinetic analysis to mechanistic studies

Almost all the kinetic studies of Cr(VI) reductions mentioned in this review were performed spectrophotometrically, by following the absorbance changes at a single wavelength. The development of computing techniques at the beginning of the 1990s gave rise to the practical application of global kinetic analysis methods for the processing of three-dimensional data sets (e.g. absorbance–wavelength–time) [213–215]. This method allows: (i) fitting of kinetic models to multi-wavelength data sets; (ii) the determination of a number of spectrally different components in the reaction mixture (by singular value decomposition technique [215]); and (iii) the estimation of spectra of short-lived intermediates.

The first application of the global kinetic analysis approach to the studies of Cr(VI) reductions resulted in the proposition of a new mechanism (Scheme 2) of Cr(VI) reduction by L-cysteine in neutral aqueous solutions (used as a model of the biologically more important Cr(VI) reduction by glutathione) [18]. Based on electronic absorption and CD spectroscopic data, a Cr(III)–cysteine reaction product was observed that was consistent with earlier studies [100], and identified as the crystallographically characterised compound sodium *N*(*cis*),*O*(*cis*),*S*(*trans*)-bis(L-cysteinato(2 −))chromate(III) (**vi** in Scheme 2) [216]. The structures of kinetically inert Cr(III) products of Cr(VI) reductions provide important information about the structures of kinetically labile Cr(V/IV) intermediates [173]. Multi-wavelength kinetic analysis revealed three distinct stages of the reaction (only one stage was observed in single-wavelength studies [100]). These steps were determined from: (i) rate laws of each stage; (ii) estimated spectra of the intermediates; (iii) comparison with the kinetics of Cr(VI) reduction by related thiols (2-mercaptoethylamine and 3-mercaptopropionic acid); and (iv) comparison with the kinetics of cysteine reaction with **Ib**. Contrary to the previously suggested mechanism of Cr(VI) reduction by thiols (Eqs. (17)–(19)) [15,100,182], sequential one-electron reduction ($Cr(VI) \rightarrow Cr(V) \rightarrow Cr(IV) \rightarrow Cr(III)$) was most consistent with the kinetic data (Scheme 2). Formation of the Cr(V)–$(cysteinato)_2$ complex involves three molar equivalents of cysteine (26–28 in Scheme 2), and a further two equivalents are spent in the formation of Cr(IV)– and Cr(III)–$(cysteinato)_2$ complexes, respectively, (29



Scheme 2. Proposed mechanism of Cr(VI) reduction to Cr(III) by excess cysteine in neutral aqueous solutions. (Reproduced with permission from Ref. [18]. Copyright American Chemical Society.)

and 30 in Scheme 2). This explains the observed stoichiometry (1:5) of Cr(VI) reduction by cysteine in neutral media [100], and the formation of a Cr(III)–(cysteinato)$_2$ complex as the only detected product. By contrast, the previously proposed mechanism (Eqs. (17)–(19)) suggests the formation of Cr(V/IV)–aqua complexes, which is unrealistic for neutral media [173].

Until recently, kinetic studies on Cr(VI) reductions concentrated on changes of Cr oxidation states and not on the structures of the reacting species [162]. The main

problems in the mechanistic studies of these reactions are: (i) the complexity of the chemistry, involving both oxidation–reduction and complexation reactions; and (ii) often very low steady-state concentrations of the intermediates. The recent development of analytical techniques enables more thorough investigations of Cr(VI) reductions. These techniques include: (i) multi-wavelength kinetic analysis [18,109,110]; (ii) determination of reaction products by electronic absorption and CD spectroscopies [18,110] and electrospray mass spectrometry [110]; and (iii) EPR spectroscopy [105] or O_2 consumption [109,110] used for studying reaction intermediates. Several studies have focused on the reactions of relatively stable Cr(V/IV) complexes with the same reductant [18,109–111,166].

4. In vitro studies of DNA damage and interference with transcription

4.1. DNA damage

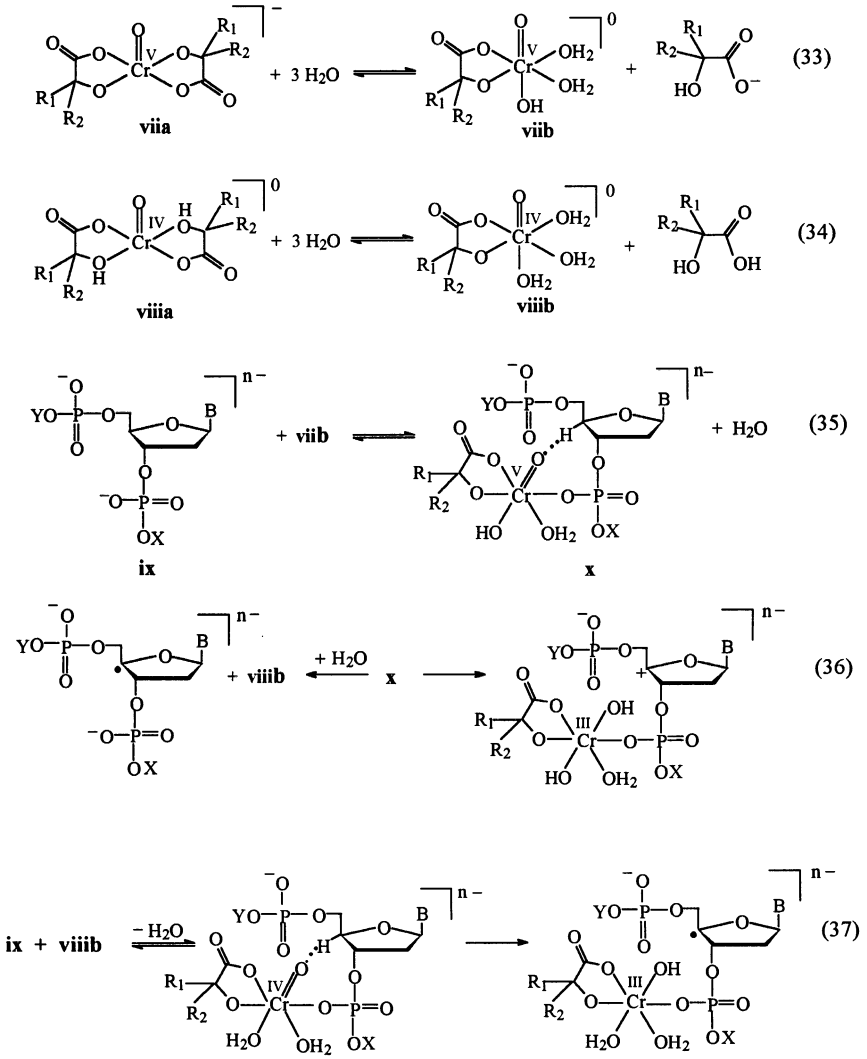
Stearns and Wetterhahn [6] and Kortenkamp et al. [5] showed that the reactions of isolated DNA with the Cr(VI)/glutathione or Cr(VI)/ascorbate systems in the presence of O_2 yield a similar spectrum of DNA damage (including strand breaks, alkali-labile sites, and Cr(III)–DNA complexes) as that found in cell cultures and living organisms exposed to Cr(VI) [138]. Mechanistic studies of the in vitro $Cr(VI) + reductant + DNA + O_2$ reactions have recently been reviewed [5,6,217,218]. The main conclusion from these studies is that a multitude of reactive species, including Cr(V/IV) complexes with reductant- and O_2-derived ligands, as well as free radicals, can be involved in DNA damage; no single reactive species can be attributed to a particular type of DNA damage [5]. Some of the major problems in gaining mechanistic insights into the DNA damage induced by Cr(VI/V/IV) species are the following: (i) many commonly used inhibitors of DNA damage interact with Cr (such as mannitol, capable of stabilising Cr(V) [219]); and (ii) in most of the Cr(VI)/reductant systems, the Cr(IV) intermediates cannot be observed directly (unlike the Cr(V) intermediates, which can be easily followed by EPR spectroscopy) [103].

Developments in the chemistry of relatively stable Cr(V/IV) complexes [22,27,107,111] allowed detailed studies of the DNA reactions with these 'unusual' oxidation states of Cr. Following the first observation of the in vitro plasmid DNA cleavage induced by **Ib** in slightly acidic media (pH 3.8–4.8) [220], several research groups [49,104,105,108,147,221–223] showed that this Cr(V) complex is able to induce oxidative cleavage of DNA or nucleotides at physiological pH values. This is one of the few metal complexes that is known to induce oxidative DNA cleavage in the absence of peroxides [224,225]. Sugden and Wetterhahn [104,147,221], on the basis of product studies, found that the reactions of DNA and model nucleotides with **Ib** (pH 5.5–7.5, acetate or phosphate buffers) follow two main pathways: (i) O_2-dependent deoxyribose ring cleavage through hydrogen atom abstraction at the C-4′ position; and (ii) O_2-independent base release. Direct reaction of Cr species with DNA through the binding to the DNA phosphate groups has been suggested

[104]. Furthermore, inorganic phosphates and pyrophosphates form complexes with **Ib**, as shown by EPR spectroscopy [226].

The first evidence that transient Cr(IV)–ehba complexes are more powerful DNA-cleaving species than the parent Cr(V) complex was found during the reaction of **Ib** with V(IV) in the presence of DNA [227]. Sugden and Wetterhahn [104], on the basis of Mn(II) inhibition studies, suggested that Cr(IV)–ehba intermediates are the main DNA-cleaving species in the buffer solutions of **Ib**. Subsequent work, however, has shown that Mn(II) cannot be used as a selective Cr(IV) trap in this system ([105]; see also Section 2.3). The first direct Cr(IV)-induced DNA cleavage [108] was studied using well-characterised Cr(IV)–2-hydroxyacid complexes [106,107]. A general mechanism of DNA damage, induced by Cr(V/IV)–2-hydroxyacid complexes, was proposed [105,108] (Schemes 3 and 4) based on the following: (i) detailed quantitative studies of the dependences of Cr(V/IV)-induced DNA cleavage levels on the reaction conditions [105]; (ii) kinetic investigation of the decomposition of **Ib** under the conditions of DNA cleavage, and comparison of the kinetics of Cr(V) decomposition and DNA cleavage [108]; (iii) DNA cleavage product studies under similar conditions [147]; and (iv) generally accepted mechanisms of oxidative DNA cleavage [224,225]. Binding of the Cr(V/IV) complexes to DNA is facilitated by their partial aquation with the formation of monochelated species [108,220] (33, 34 in Scheme 3); however, bis-chelated Cr(V) species can also bind to DNA at $pH \geq 7$ [105]. Hydrogen abstraction from the deoxyribose ring is facilitated by H-bond formation with the oxo groups of Cr(V/IV) complexes (35, 37 in Scheme 3), so the preferential oxidation site is determined by the geometry of the Cr(V/IV) complex. Different intramolecular redox reactions ($Cr(V) \rightarrow Cr(IV)$, $Cr(V) \rightarrow Cr(III)$, $Cr(IV) \rightarrow Cr(III)$) lead to a mixture of DNA radicals and cations (36, 37 in Scheme 2), some of them retaining bound Cr(III) complexes (their dissociation is likely to be slow in the time scale of the DNA cleavage [108,173]). These DNA-derived species are further oxidised through several O_2-dependent and -independent pathways (Scheme 4). Organic molecules, such as alcohols, carboxylic acids, DMSO, or common buffers (Tris, HEPES) efficiently inhibit the DNA cleavage by **Ib** at ca. pH 7 [108], presumably through the thermodynamically favourable repair reactions (40 in Scheme 4) [228] (none of these inhibitors reacts significantly with Cr(V)). Importantly, kinetically inert Cr(III)–DNA complexes are formed even in the presence of inhibitors (40 in Scheme 4). Therefore, formation of Cr(III)–DNA complexes during the reactions of Cr(V/IV) with DNA are likely to be more damaging in vivo than the DNA strand breaks caused by Cr(V/IV), as the latter are easily eliminated by the presence of common organic substances, which are abundant in the intracellular environment.

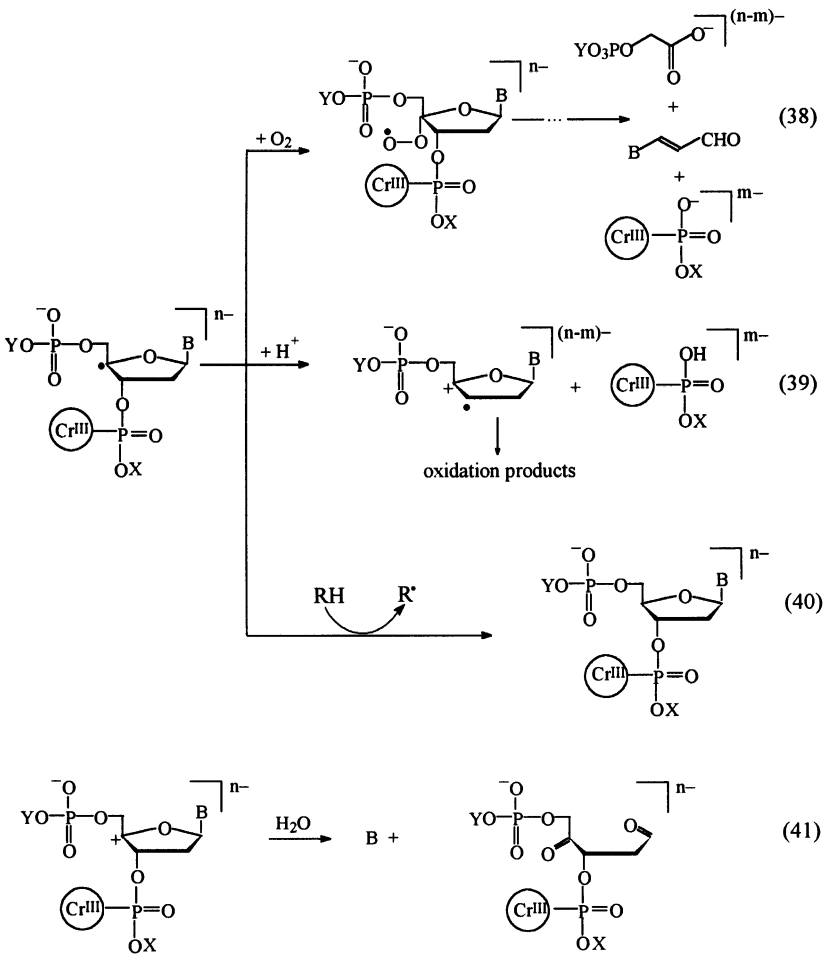
The effect of the structure of the Cr(V) complex on the mechanism of DNA cleavage has been shown by Bose and co-workers [49,222,223]. These authors studied the reactions of DNA with $[CrO(bis\text{-}tris)]^{2-}$ (**XI**), which is formed in situ by the reaction of **Ib** with excess bis-tris ligand [229]. This Cr(V) complex causes hydrogen abstraction primarily at the C-1′ and C-5′ positions of the deoxyribose ring. The formation of complexes between **XI** and the phosphate groups of DNA has been observed directly by EPR spectroscopy [49].



Scheme 3. Proposed mechanism of DNA interactions with Cr(V/IV)–2-hydroxyacid complexes. (Reproduced with permission from Ref. [108]. Copyright American Chemical Society.)

Dillon et al. [230] and Bakac and Wang [231] studied the reactions of plasmid DNA with macrocyclic Cr(V) complexes (**V**, **XII**). Unlike **Ia**,**b**, these complexes are substitutionally inert [230], so their direct binding to the phosphate groups of DNA (Scheme 3) is improbable. A possible mechanism for oxidative DNA cleavage by macrocyclic Cr(V) complexes may involve DNA intercalation and hydrogen atom abstraction or oxo group transfer reactions [230].

The DNA interactions of the Cr(V) complexes *cis*-$[Cr(O)_2(phen)_2]^+$ and $[CrO(salen)_2]^+$ (formed in vitro by PbO_2 oxidation of the Cr(III) complexes) were



Scheme 4. Proposed mechanism of oxidative DNA damage induced by Cr(V/IV)–2-hydroxyacid complexes. (Reproduced with permission from Ref. [108]. Copyright American Chemical Society.)

also studied [77]. The Cr(V) complex $[Cr(O)_2(phen)_2]^+$ induced almost complete nicking of supercoiled pUC9 DNA to relaxed and linear DNA at pH 3.3 (0.5 mM Cr) and $\geq 80\%$ nicking at pH 7.4 (2 mM Cr). The Cr(V) complex $[CrO(salen)]^+$ (0.5 mM) caused the total disappearance of supercoiled DNA at pH 3.3 and substantial cleavage. Oxidation of *trans*-$[Cr(salen)(OH_2)_2]^+$ by iodosylbenzene in phosphate buffer at pH 7.4 produced short-lived Cr(V) species that caused DNA smearing on agarose gels. Importantly, only weak interactions were observed for the Cr(III) complexes, compared with the Cr(V) analogues at pH 7.4 [77].

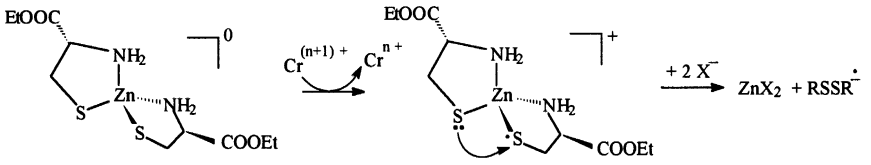
Detailed studies of DNA cleavage induced by model Cr(V/IV) complexes [105,108] revealed significant differences between the mechanisms of DNA interactions with isolated Cr(V/IV) complexes or the Cr(VI)/reductant/O_2 systems [5,6].

First, DNA cleavage is observed during the reactions of Cr(VI) with excess reductants that could be expected to act as potent cleavage inhibitors (40 in Scheme 4). Second, DNA cleavage induced by **Ib** is unaffected by catalase, whereas those induced by the Cr(VI)/reductant/O_2 systems are strongly inhibited by this selective peroxide scavenger [5,193]. In an attempt to explain the participation of both Cr(V/IV) and O_2-derived species in the mechanisms of in vitro DNA damage by the Cr(VI)/reductant/O_2 systems, a role for the Cr(V)–reductant–H_2O_2($O_2^{\cdot -}$) complexes as the ultimate DNA-cleaving species [34,146,193,212] has been suggested (H_2O_2 and $O_2^{\cdot -}$ are formed during the auto- or Cr(VI)-promoted oxidation of the reductants by O_2 [34,109,193]). However, this hypothesis is unlikely to explain the mechanisms of DNA damage by the Cr(VI)/reductant systems in vivo, where the concentrations of H_2O_2 and $O_2^{\cdot -}$ are negligibly low due to scavenging by catalase and superoxide dismutase, respectively [232,233]. It is clear that further studies, including those with mono- and oligo-nucleotide models, are required to clarify the nature of reactive species and the mechanisms of DNA damage in the Cr(VI)/reductant/O_2 systems. Another important direction of future work is the study of DNA reactions with Cr(V)–carbohydrate complexes, which are the likely dominant hypervalent Cr species in vivo [32,33], and have been shown to damage DNA in preliminary studies [197,198].

4.2. Transcription factor damage

Compared with the amount of work focused on Cr(VI/V/IV)–DNA reactions, Cr(VI)-induced damage of nuclear transcription factors (which may well be synergistic with Cr–DNA interactions) has received relatively little attention. Disruption of transcription factor functions induced by transition-metal ions can block normal DNA replication [234], or prevent DNA repair [235]. Zinc finger proteins form the largest family of transcription factors [236]; some of them, such as p53, are thought to be critically important in carcinogenesis [237,238]. Biological activity of zinc finger proteins is dependent on the formation of Zn(II)–S bonds with the cysteine residues of the protein, and the oxidative damage of these bonds leads to the loss of protein–DNA binding ability [239,240]. There are several conflicting reports on suppression [241,242] or activation [152,243] of the DNA binding abilities of different transcription factors in the presence of Cr(VI/V/IV). O'Connell and co-workers [244,245] have shown a decrease in the DNA binding ability of a zinc finger protein, GATA-1, treated by **Ib**. Depending on the oxidant and conditions used, the oxidation of zinc finger proteins or model peptides can lead to the formation of S–S bonds [246] or thiosulfinate groups [247]. A simple model complex, $[Zn(SR)_2]^0$ (where RSH = *O*-ethyl-L-cysteine) [248], that mimics a typical tetrahedral (2*S*,2*N*) binding site of zinc finger proteins [236], was used to study the mechanisms of Cr(VI/V/IV) reactions with biologically important Zn(II)–S bonds [110]. These reactions led to the formation of S–S and Cr(III)–S bonds. In contrast to the reactions of free thiols [109], reductions of Cr(V/IV) with $[Zn(SR)_2]^0$ were not accompanied by a significant O_2 consumption, which points to an intramolecular mechanism of disulfide formation (Scheme 5) [110]. Similar mechanisms of oxida-

tion of Zn(II)–S bonds have been suggested as the basis of regulatory and antioxidant activities of zinc metallothioneins [249–251], and as the mechanism of oxidative damage of zinc finger proteins [252], but these studies on the Zn model complexes were the first to provide such evidence.



Scheme 5. Proposed mechanism of intramolecular S–S bond formation during the reactions of Cr(VI/V/IV) with $[Zn(SR)_2]^0$ (RSH = *O*-ethyl-L-cysteine). (Reproduced with permission from Ref. [110]. Copyright American Chemical Society.)

5. Cell studies

5.1. Bacterial mutagenicity and toxicity

Soluble Cr(VI) complexes are well established as bacterial mutagens [2]. In the absence of microsomes/S9 liver homogenate, $Na_2Cr_2O_7{\cdot}2H_2O$-, $K_2Cr_2O_7$-, $CaCrO_4$-, $(NH_4)_2CrO_4$-, CrO_3-, $ZnCrO_4{\cdot}Zn(OH)_2$- and CrO_2Cl_2-induced mutations in various strains of *Salmonella typhimurium* (TA1537, TA1438, TA97a, TA98 and TA100) [253,254]. The mutagenic profile exhibited by $[Cr_2O_7]^{2-}$ in *S. typhimurium* increases in the following order: TA98 (423 ± 5 revertants/μmol Cr), TA100 (967 ± 26 revertants/μmol Cr), TA97a (2055 ± 37 revertants/μmol Cr), TA102 (3504 ± 94 revertants/μmol Cr) [77]. The greatest mutagenicity observed in TA102 indicates the ability of Cr(VI) to induce oxidative mutations [255,256]. A decreased mutagenic response was observed when human liver S9 fractions or rat tissue S9 fractions were added owing to the reduction of Cr(VI) to Cr(III) by reducing metabolites [253,256,257]. Whereas insoluble Cr(VI) complexes were generally non-mutagenic in most strains, increased mutagenicity (*S. typhimurium* TA100) of $PbCrO_4$ and $PbCrO_4{\cdot}PbO$ were reported when the solubilities of the complexes were increased by the addition of nitrilotriacetic acid or NaOH to the test medium [258].

The mutagenicity of Cr(V) complexes was initially established with **Ia**,**b** in *S. typhimurium* TA100 [220]. Complex **V**, which is stable in bacterial growth medium, is mutagenic in *S. typhimurium* TA100 and TA102, implicating oxidative mechanisms in the mutagenic initiation [230,256,259].

Generally, Cr(III) complexes are not mutagenic [2,260]; however, Cr(III) complexes containing aromatic imine ligands, *cis*-$[Cr(phen)_2Cl_2]^+$, *cis*-$[Cr(bipy)_2Cl_2]^+$ (bipy = 2,2′-bipyridine) and $[Cr(bipy)_2(ox)]^+$, are mutagenic in *S. typhimurium* (TA92, TA98 and TA100) [260]. The bacterial mutagenicity of the phen-containing complexes cannot be attributed directly to the ligand (i.e. phen is not mutagenic in *S. typhimurium* [261,262]), although it is believed that the lipophilic nature of the

phen and bipy ligands increases the permeabilities of the complexes, leading to the observed genotoxicities [117]. It has recently been shown that *trans*-[Cr(salen)$(OH_2)_2]^+$ is also mutagenic in *S. typhimurium* TA97a (13 ± 9 revertants/μmol Cr), TA98 (66 ± 11 revertants/μmol Cr), TA100 (37 ± 9 revertants/μmol Cr) and TA102 (440 ± 40 revertants/μmol Cr), whereas the salen ligand is not mutagenic. In bacterial mutagenicity assays, $[Cr(phen)_2(OH_2)_2]^{3+}$ and $[Cr(salen)(OH_2)_2]^+$ exhibited similar mutagenic responses to their Cr(V) analogues, which was believed to be due to the instabilities of the Cr(V) complexes in the bacterial growth medium [77]. Importantly, $[Cr(phen)_2(OH_2)_2]^{3+}$ and $[Cr(O)_2(phen)_2]^+$ exhibited greatest mutagenicity in the bacterial strains most susceptible to oxidative damage (TA100 and T102), which implicates an oxidative mechanism for mutagenesis. This result is consistent with reports that the mutagenicity of Cr(III)–bipy was O_2 dependent [141].

A series of Cr(III)–amino acid and –peptide complexes (with ligands: Ala_3, Aib_3 (AibH = α-aminoisobutanoic acid), Gly_3, Gly_4 and Gly_5) and the Cr(V) complexes of Gly_4 and Gly_5 were not mutagenic in *S. typhimurium*; however, the Cr(V) complexes of Gly_3, Ala_3 and Gly were mutagenic towards *S. typhimurium* with a broad spectrum of activity. The Cr(V)–Ala_3 complex was the most active complex, followed by the Cr(V)–Ala and Cr(V)–Gly complexes, whereas the Cr(V)–Gly_3 complex exhibited the lowest level of activity [56,263].

5.2. Mammalian cell studies

5.2.1. Metabolism: EPR spectroscopy and micro-X-ray absorption spectroscopy (XAS)

Conventional EPR spectroscopy is an effective tool for monitoring intracellular Cr(V), e.g. a Cr(V) signal at $g_{iso} = 1.989$ and a Cr(III) signal at $g_{iso} = 2.02$ occurs following treatment of V79 Chinese hamster lung cells with 200 μM Na_2CrO_4 for 2 h [264]. The Cr(V) signal decayed over the period of 1 h following the removal of Cr(VI). Pretreatment of the cells with biological reductants, such as α-tocopherol and ascorbic acid, resulted in a smaller Cr(V) signal, whereas pretreatment with riboflavin enhanced the level of the intermediate [264]. Correlations were observed between the cellular levels of Cr(V) and the levels of chromate-induced DNA single-strand breaks, alkali-labile sites and enzyme inhibition, implicating Cr(V) as the damaging intermediate [264].

Chromium(VI) reduction to Cr(V) has also been measured on the skin of living rats using a loop resonator extended from an EPR spectrometer operating at 1.2 GHz. The production of a Cr(V) signal at $g = 1.979$ (width = 2.5 G) occurred within minutes of the administration of Cr(VI) to the skin and continued for at least 60 min before it decayed [204].

In an intriguing experiment [200], a Cr(V) signal was monitored in the circulating blood from a rat that had been treated intravenously with saline solutions of $K_2Cr_2O_7$ or $Na_2Cr_2O_7$. The Cr(V) complexes appeared to correlate with the $[CrO(OR)_4]^-$ signal obtained from possible reactions with ascorbic acid and also $[CrO(SR)_2(SR')_2]^-$, indicative of glutathione or 2,3-dimercapto-1-propanesulfonic

acid binding. Interestingly, different kinetics were observed for the K^+ and Na^+ salts of Cr(VI).

Ueno et al. [265] showed that both Cr(V) and Cr(III) complexes were detected in the livers of mice that had been treated with an intraperitoneal injection of $K_2Cr_2O_7$ (10–40 mg Cr/kg). A Cr(V) signal comprising three main Cr(V) species centred at $g_{iso} = 1.984$ was detected within 15 min of the administration and persisted for 12 h. A concomitant broad EPR signal of Cr(III) that was observed during the same period was similar to that observed following treatment of V79 cells with Na_2CrO_4 [265]. These EPR results are consistent with the reduction of Cr(VI) to stable Cr(III) via Cr(V) intermediates, as proposed in the uptake model [9].

Importantly, Cr(III), Cr(IV), Cr(V) and Cr(VI) complexes exhibit distinct X-ray absorption near-edge structure (XANES) spectra [24,113,266–268]. Cr(VI) spectra characteristically show relatively large pre-edge peaks due to the symmetry-forbidden $1s \rightarrow 3d$ transition, which achieves considerable intensity from mixing of the p and d orbitals due to the tetrahedral symmetry of the complexes and the strong π bonding involved in the Cr=O bonds. Although Cr(V) XANES spectra are similar to those of Cr(VI), their pre-edge peaks are smaller [113,268]. The XANES spectra of Cr(IV) and Cr(III) complexes commonly show small and very small pre-edge peaks, respectively, and are approximately 4 eV and 5 eV lower in energy respectively than those of Cr(VI) complexes [113,267]. Microprobe XAS (200 μm diameter beam) was used for the analysis of V79 Chinese hamster lung cells that had been treated with Cr(VI) and Cr(V) complexes [268]. The Cr(VI) and Cr(V) complexes were reduced completely ($> 90\%$) to Cr(III) within a 4 h cell exposure (Fig. 6) [268]. Micro-XAS provides unequivocal evidence for the hypothesis that these genotoxic oxidants react rapidly with intracellular reductants, as the spectra (unlike EPR spectroscopy) include contributions from all Cr species present.

5.2.2. Permeability: radioactive tracers, atomic absorption spectroscopy and X-ray analysis

There have been numerous investigations into the permeabilities of Cr(III) and Cr(VI) complexes in mammalian cells using a multitude of techniques, including: radioactive tracer analysis [12,269–271], atomic absorption spectroscopy (AAS) [272,273] and gas–liquid chromatography (GLC) [274]. The rate and extent of Cr(VI) entry into cells is much higher than that for Cr(III) and the cellular uptake of Cr is dependent on its chemical and physical state [270,275,276], the extracellular concentration [274], and the exposure period [270,271]. More recently, Cr(III), Cr(V) and Cr(VI) permeabilities have also been studied using micro-particle-induced X-ray emission (micro-PIXE) analyses, nano-synchrotron-radiation-induced X-ray emission (nano-SRIXE) analyses, and graphite furnace atomic absorption spectroscopy (GFAAS) [76,259,277].

The most cited Cr permeability studies were performed in 1950 on human erythrocytes that had been exposed to solutions of $[^{51}CrO_4]^{2-}$ [270]. It was shown that the intracellular Cr levels increased asymptotically with time until a saturation level was reached at 2 h. In comparison, the cellular content of Cr was insignificant following treatment with equimolar solutions of the Cr(III) compound,

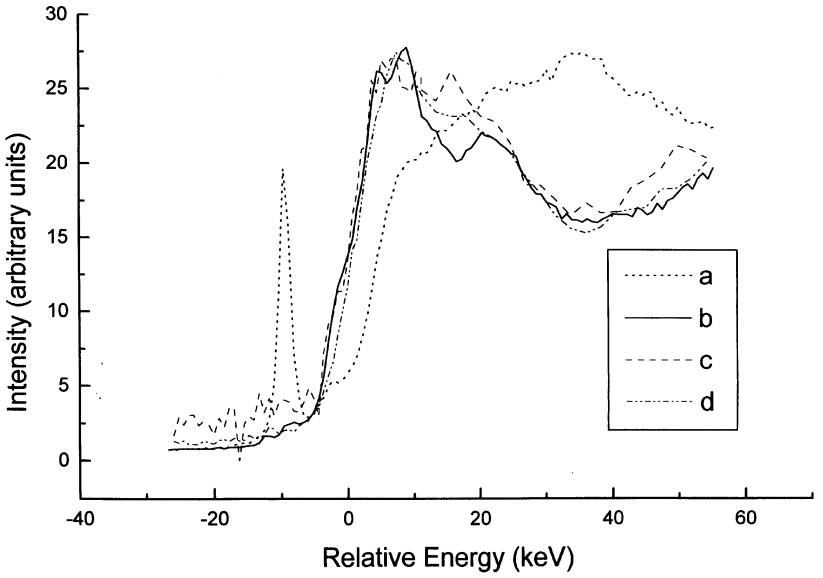


Fig. 6. XAS spectra of (a) a Cr(VI) standard, (b) a Cr(III) standard, and (c) Cr(VI)- or (d) Cr(V)-treated V79 Chinese hamster lung cells. (Reproduced with permission from Ref. [268]. Copyright American Chemical Society.)

$^{51}CrCl_3 \cdot 3H_2O$ [270]. Buttner and Beyersmann [278] showed that the uptake of $^{51}CrO_4^{2-}$ (0.001–1 mM) by erythrocytes reached a saturation plateau within 30 min at 37°C and that the total amount of Cr that entered the cell was dependent on the initial concentration of Cr in the cell medium. Furthermore, Cr accumulated within red blood cells, presumably due to the unidirectional flow of Cr into the cells imposed by the reduction of intracellular Cr(VI) to impermeable Cr(III) [278].

The intracellular Cr detected in human erythrocytes (2×10^8 cells) following exposure to Cr(III) complexes (1 mM for 1 h) was approximately $(0.5–2.0) \times 10^{-18}$ mol/cell [273]. GFAAS analyses showed that Cr concentrations in whole blood were similar when human erythrocytes were exposed to the following Cr(III) complexes: $[Cr(phen)_2Cl_2]^+$, $[Cr(bipy)_2Cl_2]^+$, $[Cr(2,4\text{-pentanedione})_3]$, $[Cr(Gly)_3]$, $[Cr(glutathione)_2]^-$ and $[Cr(cysteine)_2]^-$. It was concluded that no distinction could be made concerning the permeability properties of the complexes based on charge.

AAS analysis of acid-digested V79 cell pellets showed that Cr(VI) entry (CrO_3, $BaCrO_4$, $PbCrO_4$ and $K_2Cr_2O_7$) was greater than Cr(III) entry ($CrCl_3$) following a 12 h exposure [272]. Extracellular Cr(III) concentrations approximately 300 times those of Cr(VI) were necessary before similar intracellular levels were detected [272].

GFAAS analyses of V79 cells (10^6 cells) that had been treated with $[Cr(Gly_2)_2]^-$, $[Cr(en)_3]^{3+}$, $[Cr(phen)_2(OH_2)_2]^{3+}$ or $[Cr(salen)(OH_2)_2]^+$ (0.4 mM for 4 h), showed no significant increase in the cellular Cr above the control levels (control cells = $4.7 \pm 0.8 \times 10^{-15}$ g/cell; $[Cr(Gly_2)_2]^- = 8.2 \pm 1.7 \times 10^{-15}$ g/cell; $[Cr(en)_3]^{3+} =$

$17.3 \pm 1.8 \times 10^{-15}$ g/cell; $[Cr(phen)_2(OH_2)_2]^{3+} = 5.6 \pm 1.4 \times 10^{-15}$ g/cell; $[Cr(salen)(OH_2)_2]^{+} = 13.3 \pm 3.3 \times 10^{-15}$ g/cell) [76]. An increase in intracellular Cr was observed, however, for the Cr(V) analogue of $[Cr(phen)_2(OH_2)_2]^{3+}$ ($65.7 \pm 32.5 \times 10^{-15}$ g/cell), although $[CrO(salen)]^{+}$ exhibited a similar permeability to that of the Cr(III) complexes ($10.3 \pm 1.5 \times 10^{-15}$ g/cell). It is believed that the increased permeability of $[Cr(phen)_2(OH_2)_2]^{3+}$ was due to the increased stability of the complex with respect to intracellular reduction compared with that of $[CrO(salen)]^{+}$ [76]. While the Cr(III)–amino acid complexes $[Cr(Aib)_3]$ and $[Cr(Ala)_3]$ were relatively impermeable to V79 cells (GFAAS analysis of 10^6 cells), an increase in Cr uptake was observed for the Cr(V)–Ala complex. Furthermore, the Cr(III)– and Cr(V)–peptide complexes Cr(III)–Gly_2, Cr(III)–Gly_3, Cr(III)–Gly_4 and Cr(V)–Gly_3, Cr(V)–Gly_4, Cr(V)–Gly_5 exhibited similar permeabilities to those of Cr(VI). Most interestingly, Cr(V)–Ala_3 was four to five times more permeable than Cr(VI) [56].

Some of the first permeability studies of Cr(V) complexes were performed using micro-PIXE. Single cells that had been treated with 0.1 mM Cr for 4 h were analysed (ca. five per dose), using a 1 μm focused beam, and the Cr uptake was found to increase in the following order: $[Cr(phen)_2(H_2O)_2]^{3+} <$ **Ib** $<$ **V** $< [Cr_2O_7]^{2-}$ [259].

The uptake of Cr has also been monitored by a number of cell-imaging experiments employing X-ray emission techniques. Transmission electron microscopy (TEM) analysis of the phagocytosis of insoluble Cr(VI) as $PbCrO_4$ by human lung small airway epithelial cells showed that the lead chromate particles were present in cytoplasmic vacuoles [279]. Furthermore, energy-dispersive X-ray analysis (EDXA) spectra performed at point locations showed that Cr and Pb were present in the nucleus [279]. The micro-PIXE elemental maps of a $[Na_2Cr_2O_7]$-treated V79 cell obtained using a 1 μm diameter scanning proton beam (Fig. 7) show that Cr was dispersed throughout the cells following the 4 h treatment [259,277]. Similar results were also obtained for cells treated with **Ib** or **V** [259]. Nano-SRIXE analyses provided more informative maps from V79 cells treated with Cr(VI), because the increased sensitivity and resolution imparted by the X-ray beam permits elemental mapping of thin-sections at 0.3 μm resolution. The existence of Cr localisations on P-enriched regions at the centre of the cell showed that: (i) Cr did not remain bound at the cell wall, but did, in fact, pass through the cell membrane; (ii) Cr travelled some distance within the cell; and (iii) Cr was most likely bound to DNA [281,282].

5.2.3. Cytotoxicity and genotoxicity

It is believed that chromates potentiate cell death by apoptosis. Blankenship et al. [283] showed that, following exposure of Chinese hamster ovary (CHO) cells to Na_2CrO_4 (150 μM and 300 μM) for 2 h, there was 19–30% inhibition of DNA synthesis within 1 h of treatment, which did not recover until 4 days. Similarly, protein synthesis was inhibited by 52–60% within 1 h of treatment, but recovery was observed at 24 h. Analysis of chromate-treated cells by TEM showed features characteristic of apoptosis, including chromatin margination and fragmentation and cytoplasmic condensation with intact membrane and organelle structure [283].

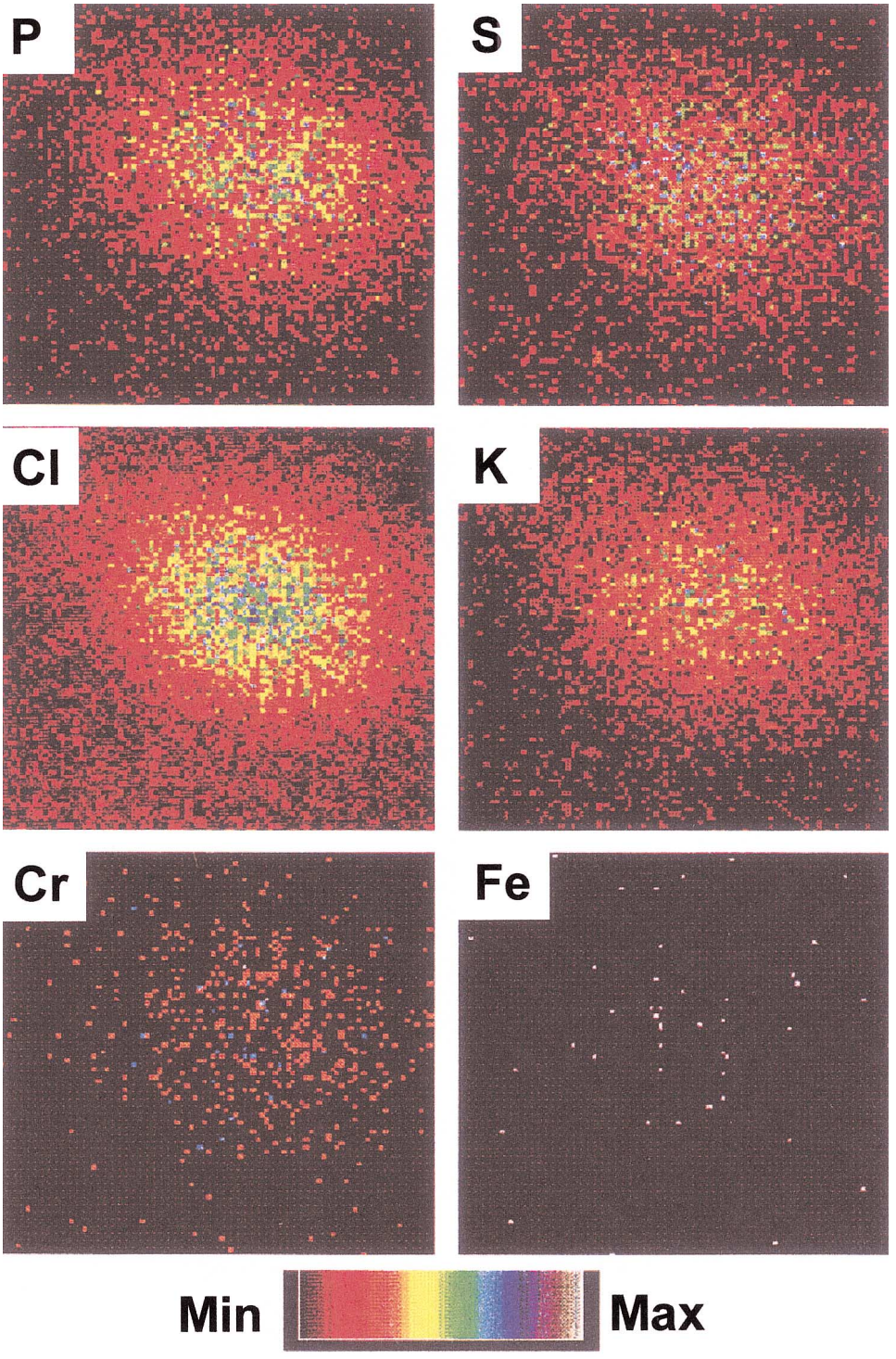


Fig. 7. Micro-PIXE elemental maps (map dimensions: $20 \times 20\ \mu m^2$) of a Cr(VI)-treated V79 cell. (Reprinted with permission from Ref. [280]. Copyright Elsevier Science.)

The cytotoxicities of soluble and insoluble Cr(VI) complexes (zinc and lead chromate, industrial and laboratory-synthesised pigments) were investigated in cultured Syrian hamster embryo cells [284]. The cytotoxicity was highly dependent on the solubility of the complexes; the most solubilised complexes (Zn^{2+}, Ca^{2+} and Sr^{2+} chromates) were eightfold more toxic than the less soluble Pb^{2+} and Ba^{2+} chromates [284].

In clonal assays performed with V79 cells, solutions of Cr(V) complexes (**Ib** and **V**) and Cr(VI) ($[Cr_2O_7]^{2-}$) exhibited LD_{50} values in the range 0.01–0.2 mM Cr [259], whereas the LD_{50} values of Cr(III) complexes ($[Cr(Gly_2)_2]^-$, $[Cr(en)_3]^{3+}$, $[Cr(salen)(OH_2)_2]^+$ and $[Cr(phen)_2(OH_2)_2]^{3+}$) occurred within the range 1–6 mM [76]. There was no evidence that the cytotoxicity of the Cr(III) complexes was enhanced by the more anionic species and the imine complexes were only slightly more toxic than the aliphatic complexes [76].

Soluble $CaCrO_4$ induced a dose-dependent mutation to 6-thioguanine resistance in CHO cells following 5 and 24 h treatments [285]. In vitro micronucleus assays using CHO cells treated with CrO_3 and $K_2[Cr_2O_7]$ showed a dose-dependent increase in the incidence of micronuclei formation in binucleated (BN) cells for Cr(VI) concentrations of 10^{-8} to 10^{-6} M [286].

The genotoxicities of a number of Cr(V) complexes were compared with those of Cr(VI) using the micronucleus (MN) assay. This assay (Fig. 8) employs the cytokinesis block method to measure chromosome breaks and damage to the spindle apparatus in BN cells [56,259]. Complex **Ib** (133 ± 10 MN/1000 BN/μmol Cr) and **V** (152 ± 20 MN/1000 BN/μmol Cr) exhibited similar genotoxic responses to Cr(VI) (182 ± 5 MN/1000 BN/μmol Cr) [259]. Importantly, comparisons of the genotoxicity data with the permeability data showed that the Cr(V) complexes were as genotoxic, if not more genotoxic, than Cr(VI) [259].

The Cr(V) complexes of Gly_3 (1.4 ± 0.1 MN/1000 BN cells/μmol Cr), Gly_4 (0.8 ± 0.1 MN/1000 BN cells/μmol Cr), Gly_5 (25.1 MN/1000 BN cells/μmol Cr), Ala_3 (51.5 ± 1.8 BN cells/μmol Cr) and Ala (48.3 ± 3.0 BN cells/μmol Cr) were all genotoxic in V79 Chinese hamster cells, although they all displayed a lower level of activity compared with Cr(VI) and the Cr(V) complexes **Ib** and **V** [56,259].

Although most Cr(III) complexes were reportedly non-mutagenic, a distinct difference in the genotoxicities was reported for Cr(III)-containing aromatic imine ligands. *cis*-$[Cr(phen)_2Cl_2]^+$ induces mutations at the hypoxanthine–guanine phosporibosyl transferase (HGPRT) locus in V79 cells [117]. Whereas the Cr(III) complexes containing the aliphatic ligands $[Cr(Gly_2)_2]^-$ and $[Cr(en)_3]^{3+}$ were non-genotoxic in the micronucleus assay, the Cr(III) imine complexes ($[Cr(phen)_2(OH_2)_2]^{3+}$ and $[Cr(salen)(OH_2)_2]^+$) that could be oxidised to Cr(V) complexes showed significant increases in micronuclei formation ($[Cr(Gly_2)_2]^- = 0.01$ MN/1000 BN cells; $[Cr(en)_3]^{3+} = 0.1971$ MN/1000 BN cells; $[Cr(salen)(OH_2)_2]^+ = 13.56$ MN/1000 BN cells; $[CrO(salen)]^+ = 18.18$ MN/1000 BN cells; $[Cr(phen)_2(OH_2)_2]^{3+} = 3.29$ MN/1000 BN cells; $[Cr(O)_2(phen)_2]^+ = 125$ MN/1000 BN cells). The more potent genotoxic character exhibited by $[Cr(O)_2(phen)_2]^+$ was attributed to the greater stability of the Cr(V) complex and the greater cellular uptake of the complex compared with those displayed by $[CrO(salen)]^+$ [76,77].

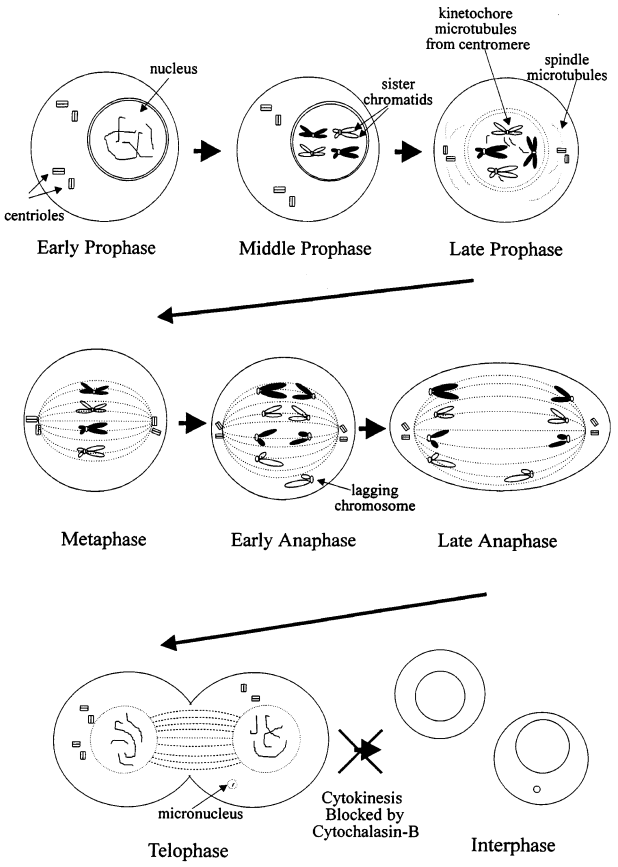


Fig. 8. The mitosis events leading to micronuclei formation during the in vitro micronucleus-block assay.

Damage by $[Cr(phen)_2(OH_2)_2]^{3+}$ was also evident from the in vitro reactions with supercoiled pUC9 DNA (see Section 4.1). Consequently, the increased permeability, genotoxicity and DNA damage caused by $[Cr(phen)_2(OH_2)_2]^{3+}$ raises concerns regarding the potential damage caused by Cr(III) complexes that may be oxidised by two-electron oxidants, such as oxo-transfer enzymes (either directly or indirectly) and organic peroxides found in vivo.

6. Conclusions

As outlined in this review, over recent years considerable progress has been made in understanding the diversity of mechanisms involved in the reduction of soluble chromates by different biomolecules, the natures of the intermediates formed, and the factors that stabilise such intermediates. This has also led to an unravelling of the likely genotoxic species and the mechanisms by which they damage DNA and transcription factors. As a result, $OH^{\bullet}$ radicals are all but ruled out as likely

intermediates, although a range of other species, Cr(V), Cr(IV), organic radicals and peroxides, may all contribute. Despite the considerable progress in this area, much is still to be learnt about the relative importance of these mechanistic pathways in vivo, and this will require detailed competition experiments coupled with information obtained from intracellular spectroscopic techniques and other cell biology experiments. One area, in particular, that has been poorly explored is the chemistry of insoluble chromates that ensues after these particles are taken up by phagocytosis. This is a rich area for further research.

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