

STUDIES OF CHROMIUM AND IRON COMPLEXES
OF BIOLOGICAL AND MEDICAL RELEVANCE.

BY

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Abstract

Part 1

It has been known for some time that chromium, in the +6 oxidation state is both mutagenic and carcinogenic. Reported in this thesis are the results of studies of the interaction of biologically relevant molecules, such as GSH, ascorbate, carbohydrates and ribonucleotide derivatives, with Cr(VI) under physiologically relevant conditions.

The production of intermediate Cr(V) species in the above reactions was monitored by X-band epr spectroscopy, possible structures of these complexes being proposed by interpretation of the observed nuclear hyperfine structure.

A large number of relatively stable Cr(V) complexes were formed under these experimental conditions, several being present at a steady state concentration for a number of weeks.

Part 2

A range of transition metal complexes, primarily of iron, have been synthesised and tested for their radiosensitizing and toxic action towards oxic and hypoxic V79 cells.

Results of experiments using ferrocenium salts showed them to be good sensitizers of hypoxic, and to a lesser extent, oxic cells. The degree of sensitization observed under hypoxia was of the order of 10x greater than that of misonidazole under the same conditions.

Cellular survival curves parallel to the N_2 line were observed with these complexes at all drug concentrations under hypoxia, suggesting a mechanism of action other than that of a simple electron-affinic radiosensitizer.

Gross thiol-depletion and production of H_2O_2 were shown not to contribute to the mechanism of action.

A medium-dependent toxicity was encountered with the ferrocenium drugs, almost zero toxicity being observed with MEM medium compared to significant values when in pbs.

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To my Family

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PART 1

STUDIES INTO THE PRODUCTION OF RELATIVELY
LONG LIVED Cr(V) SPECIES ON REACTION OF
CARCINOGENIC Cr(VI) WITH BIOLOGICALLY
RELEVANT MOLECULES.

Abbreviations

5'-AMP = adenosine-5'-monophosphate

BSO = buthionine sulfoxime

CHO = Chinese hamster ovary

5'-CMP = cytidine-5'-monophosphate

DMF = dimethyl formamide

DMSO = dimethyl sulphoxide

DNA = deoxyribonucleic acid

DRM = d-ribose-5-monophosphate

EDTA = ethylenediaminetetraacetic acid

EHBA = 2-ethyl-2-hydroxybutyric acid

ENDOR = electron nuclear double resonance

epr = electron paramagnetic resonance

5'-GMP = guanosine-5'-monophosphate

GSH = reduced glutathione

GSSG = oxidised glutathione

GTF = glucose tolerance factor

H₂A = ascorbic acid

2-HBA = 2-hydroxybenzyl alcohol

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HMPA = hexamethylphosphoramide

NAC = N-acetyl-L-cysteine

NADPH = β -nicotinamide adenine dinucleotide phosphate
(reduced form)

PSH = D.L-penicillamine

ptp = peak-to-peak

TMSPS = 3-(trimethylsilyl)propanesulphonic acid

TRIS = tris(hydroxymethyl)aminomethane

CHAPTER 1. INTRODUCTION.

1.1. Background

Over the past few years there has been a growing awareness of the importance of a wide range of metallic and non-metallic elements in the functioning of biologically important systems [1,2].

Twenty five elements are now considered essential to warm blooded animals. Of the metallic elements, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sn and Mo are classified as trace ions whereas Na, K, Mg and Ca are bulk components. In many cases the exact role of the trace elements is still not completely understood but the great majority function as key components of molecules performing essential biological operations, for example, in enzyme systems.

In addition to being essential for life, several heavy metals have been found to possess undesirable biological properties when present in higher than normal quantities. In-vitro examination of the acute and chronic effects of heavy metals on mammalian cells has established the following order of toxicity; $Pb < Mn < Hg < Ni < As < Cd < Cr(VI)$ [2], although many more metals than are given here are known to have high toxicity. Chromium in particular has received extensive study due to its adverse biological behaviour and Part (I) of this thesis covers work carried out with this metal.

There is conclusive evidence suggesting that chromium, especially in the +6 oxidation state is both toxic and carcinogenic [3-8]. This is of particular importance since exposure to chromate is a major problem in industry and the

occurrence of cancer, particularly of the lung, is frequent among the workers involved. The main sources of environmental Cr today are in the paint [7], leather and cement industries as well as the increasingly widespread use of Cr compounds as anti-corrosion additives in a variety of cooling systems [5].

1.2. Chromium biochemistry

In 1955 it was discovered that Cr is an essential trace element for rats [9], and in 1975 it was demonstrated that Cr was also essential for humans [10]. The experiments with rats showed that an impairment of intravenous glucose tolerance could be reversed on treatment with Cr(III) compounds, no other metals having this effect. This action could be produced naturally by the action of brewer's yeast and the unknown species responsible was termed the glucose tolerance factor (GTF).

The Cr(III) complex present in GTF has not yet been characterised and its mode of action is not fully understood. However, recent work has shown that GTF isolated from yeasts often contains nicotinic acid and either reduced glutathione or its constituent amino acids. This stimulated a great deal of work preparing and characterising Cr(III) complexes with relevant ligands. Complexes have been isolated with GSH [11], L-cysteine [12], D-penicillamine [13], aspartic acid [14] and L-histidine [15] to name but a few.

A limited number of clinical trials using Cr(III) to combat diabetes have been done [16] and in several cases favourable responses were observed.

A comprehensive review covering the occurrence and function of chromium in biological systems has been published [17] and a more recent report concentrates specifically on the role of GTF [18].

Laboratory work established some time ago that Cr in the +6 oxidation state is both toxic and mutagenic towards bacterial and mammalian cell systems [8]. However, introducing Cr(III) into exactly the same test systems did not show the same damaging effects; indeed, recent in-vivo studies with rats have shown that animals treated with Cr(III) survived a given experimental period whereas rats given Cr(VI) all died within 60 days [19].

The difference in activity between Cr(VI) and Cr(III) in cellular systems has been explained by the "uptake-reduction" model of Cr(VI) carcinogenicity [20]. In this scheme, the inactivity of Cr(III) is due to its inability to cross the cell membrane barrier and enter the cell, whereas Cr(VI) is able to make this crossing [20]. It is still not clear what molecular processes are involved in this differentiation, but it has been shown that heteroaromatic ligands such as bipy and o-phen do facilitate Cr(III) uptake [21].

Once in the cell, the chromate can react with a wide variety of cellular components ultimately giving potentially harmful Cr(III) complexes with small cellular molecules, protein and DNA [22]. It is believed that these complexes then inhibit normal cellular enzyme function and disrupt replication, transcription and translation processes of DNA. This is presumably the primary cause of chromosome aberrations as well as genetic mutations.

Cr(VI) has been shown to cause DNA strand breaks and DNA-protein crosslinks in CHO cells [23] and DNA cross links in rat

liver and kidney [24]. The degree of strand breakage was found to increase in proportion to chromate concentration and is probably initiated via oxidation processes. The CHO cross links were saturated at a Cr(VI) concentration of 0.1 mM and appear to be produced by a different mechanism.

Cr(VI) is unreactive to DNA at pH 7.4 in the absence of a metabolizing system [25]. However, in the presence of NADPH and microsomes reaction does occur to form stable Cr-DNA complexes. Similarly, the roles of GSH and cytochrome P450 in the Cr(VI)-induced damage of DNA in chick embryo hepatocytes was studied. Both were found to be important in the metabolism of Cr(VI) and in the production of Cr species which damage the DNA [26].

Although Cr(III) is the ultimate form of the metal bound in the cells, there is evidence accumulating that the intermediate oxidation states of +5 and to a lesser extent, +4, may be important in the reaction sequences leading to the adverse biological behaviour of Cr(VI).

Cr(V) is a potentially damaging species and, should stabilization of this oxidation state occur within the cell, it could facilitate its transport to the critical cellular sites. Any Cr(III) species formed en-route are unlikely to interact directly with DNA due to their kinetic inertness and resistance to ligand substitution reactions. In addition, any Cr(V) species formed may be able to react directly with certain cellular components which would otherwise not react with chromate.

With this in mind, a great deal of recent research has focussed on detection and characterization of relatively stable

Cr(V) complexes with ligands of biological relevance formed under physiological conditions.

Wetterhahn first noticed the production of relatively stable (lifetime of approx. 1 hour) Cr(V) species on the incubation of chromate with rat liver microsomes and NADPH [27]. In our laboratories it has been shown that Cr(VI) produces relatively long lived Cr(V) species on reaction with nucleotides [28], reduced glutathione [29] and ascorbate [30] in aqueous solution, these being biologically relevant molecules.

The work reported in this thesis is a continuation of this field of study, investigating the production of Cr(V) species on reaction of Cr(VI) with relevant systems, and the possibility of such species to be transported within the cellular environment where they may have a damaging effect.

1.3. Chromium chemistry

Chromium exists in a number of oxidation states in aqueous solution, some more stable than others, and a knowledge of their chemistry and relative stabilities under physiological conditions is necessary for a good understanding of the metabolism of chromate in cellular systems.

1.3.1. Chromium(VI)

In aqueous solution, there are three main chromate species which can exist, the major component at any one time depending upon solution pH and chromate concentration (table 1.1).

<u>Species</u>	<u>pH</u>	<u>Conc.(M)</u>
$\text{Cr}_2\text{O}_7^{2-}$	<7	$>10^{-2}$
HCrO_4^-	<6	$<10^{-2}$
CrO_4^{2-}	>6	low
	>7	high

Table 1.1 The forms of chromate present in aqueous solution

Hence, under physiological pH conditions (7.4), the major form of Cr(VI) is CrO_4^{2-} , a small percentage also existing as HCrO_4^- . The presence of the latter could be important since its oxygen atoms are more labile to substitution than with CrO_4^{2-} [31].

Dichromate ($\text{Cr}_2\text{O}_7^{2-}$) is a much stronger oxidizing agent than CrO_4^{2-} ($E_0 = +1.33\text{V}$ at pH=0, $E_0 = -0.12\text{V}$ at pH=14 respectively), and it is for this reason that Cr(VI) oxidations of organic substrates are carried out in strongly acidic solution.

1.3.2. Chromium(V)

The heavier triad elements have a much more extensive +5 chemistry than Cr, including a number of biologically interesting Mo compounds.

Cr(V) species were first discovered as intermediates in the oxidation, by chromate, of organic substrates such as oxalic acid [32]. These species were very short lived and could only be observed by epr spectroscopy. Until fairly recently, the only known Cr(V) complexes which had been isolated and characterised were the fluoride, oxohalide and peroxide type species, and no stable complexes with organic ligands were known.

However, Rocek [33] showed that stable Cr(V) complexes could be obtained with a selection of tertiary-hydroxy acids. In one case, using 2-ethyl-2-hydroxybutyric acid, a crystalline sample

was prepared and X-ray crystal structure determination showed the Cr(V) to be in a square pyramidal, oxygen donor environment with two coordinating ligand molecules and a central Cr=O unit (fig.1.1). This confirmed earlier predictions made from epr data.

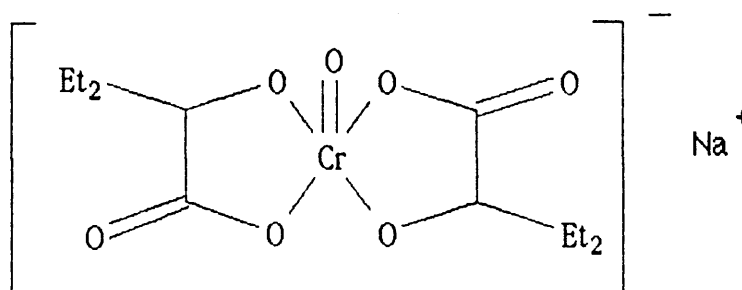


Fig.1.1. Structure of sodium bis(2-hydroxy-2-ethylbutyrate)oxochromium(V).

Aqueous solutions of these tert-hydroxy Cr(V) complexes are found to be stable for extended periods of time. This is in direct contrast to the usual instability of Cr(V) in water, rapid disproportionation to Cr(III) and Cr(VI) normally taking place.

Experimental data show that, in general, the best complexation agents for Cr(V) are O-containing ligands, specifically 1,2-dihydroxy derivatives capable of forming 5-membered chelate rings.

Much work has been done investigating the most suitable organic solvents for the formation and stabilization of Cr(V) species. Studies with oxygen, sulphur and phosphorus donor ligands have shown that the best solvents are DMF, HMPA and DMSO [34]. It was found that the solvents played an important role in the reactions by initially reducing Cr(VI) to Cr(V) which was

then stabilized as complexes by the relevant ligands.

These results prompted the use of both DMF and DMSO in as much of the work reported here as possible, their use being limited by poor ligand solubility.

A comprehensive review on the coordination chemistry of Cr(V) has recently been published [35] and reference should be made to this for more detail.

1.3.3. Detection of Cr(V)

The $3d^1$ outer electron configuration of Cr(V) makes it an ideal species for study by electron paramagnetic resonance (epr) spectroscopy [36]. The epr spectrum of a Cr(V) species usually gives a characteristically sharp (peak-to-peak band width 2 to 10 G) signal in the $g=1.9$ region, four nuclear hyperfine resonances due to ^{53}Cr ($I=3/2$, 9.55% abundant) surrounding the central band. The position of the signal in conjunction with the observed ^{53}Cr spacing (a_{Cr}) can be used as a guide to the coordination environment around the metal centre.

Due to the sharp nature of the Cr(V) signals, nuclear superhyperfine structure due to splitting by adjacent ligand atoms (eg. protons $I=1/2$) can often be seen, thus yielding information as to the mode of binding. Were the main signal much broader, this splitting would not be easily seen and valuable structural information would be lost.

It is with epr signals such as those described above that the ENDOR (electron nuclear double resonance) epr technique is at its most effective. Unfortunately, during the course of these studies this facility was not available. However, its use in the future

would allow much more thorough interpretation of the proton super hyperfine splittings observed in this work, yielding more information on the Cr(V) coordination environment.

Most of the Cr(V) species generated from Cr(VI) reduction are thought to possess a central Cr=O bond in the axial position. As would be expected, this exhibits a characteristic absorption in the IR spectrum in the $\nu=900$ to 1050 cm^{-1} region, the position of which could be affected by the coordination environment.

The Cr(V) ion exhibits a characteristic absorbance in the visible spectrum in the $\lambda_{\text{max}}=750\text{nm}$ region which has been used in some kinetic studies of Cr(V) formation or depletion during Cr(VI) reduction [37]. However, this band is usually simple in nature and little structural information can be gained from it.

Large ($\sim 100\text{ppm}$) paramagnetic shifts of nmr resonances are known with Ni and Co complexes [38]. Work is reported in this thesis (chapter 4) investigating the possible application of this technique to study the Cr(V) centre.

Since most of the Cr(V) species produced in this work are present at very low concentrations (μM), epr spectroscopy is the most satisfactory method for their study due to its great sensitivity and the availability of structural information. In addition, epr facilitates the calculation of absolute Cr(V) concentrations via computer double integration techniques.

Where Cr(V) was produced in higher than normal concentrations, IR spectroscopy was also used in characterization.

1.3.4. Chromium(IV)

The major isolable Cr(IV) species are peroxo and fluoro complexes, treatment of $[\text{Cr}(\text{O}_2)_4]^{3-}$ with warm aqueous ammonia giving the Cr(IV) complex $[\text{Cr}(\text{O}_2)_2(\text{NH}_3)_3]$, exhibiting a pentagonal bipyramidal structure with peroxo groups filling four of the planar positions.

The chemistry of Cr(IV) in aqueous solution is very limited. The presence of this oxidation state has been proposed for the reaction of Cr(VI) with cellular reductants, although there has been little direct evidence of its production. A recent paper described the formation of Cr(IV) from the reaction of Cr(VI) with ascorbate [113].

1.3.5. Chromium(III)

This is the most stable and best known oxidation state of Cr. Thousands of stable Cr(III) complexes have been prepared and the vast majority are hexa-coordinate.

One of the more important aspects of Cr(III) chemistry, with respect to its biological action, is the kinetic inertness of Cr(III) to ligand substitution reactions in aqueous solution. This is due to the half-filled t_{2g} level of an octahedral d^3 configuration.

The electronic and magnetic properties of Cr(III) complexes have been extensively studied. Cr(III) gives rise to broad epr absorptions as observed in this work. However due to the excessive line width, information regarding the modes of ligand coordination cannot be extracted from nuclear superhyperfine structure, in contrast to the case with Cr(V) complexes.

CHAPTER 2. PRODUCTION OF RELATIVELY LONG LIVED Cr(V) SPECIES ON THE REACTION OF Cr(VI) WITH THIOL-CONTAINING CELLULAR REDUCTANTS.

2.1. Introduction

Reduced glutathione (GSH) is a sulphydryl-containing tripeptide (γ -L-glutamyl-L-cysteinylglycine) (fig.2.1).

GSH is the most abundant, naturally occurring, low molecular weight thiol, being present in all mammalian cells at intracellular concentrations ranging from 0.8 to 8.0 mM [39].

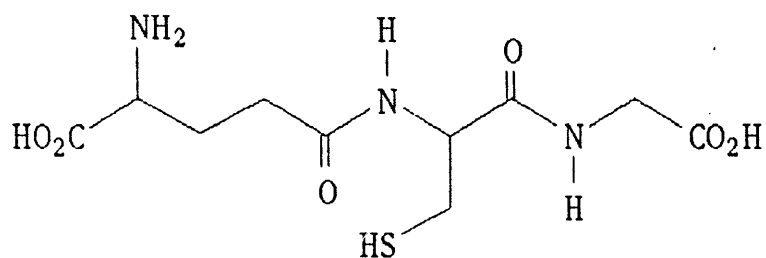
The role of GSH within the cell is not fully understood but some of its functions include maintenance of protein sulphydryl groups in the reduced state, contributions to the cellular redox state and protection against various oxidants, including oxygen. Another role of GSH is in the repair of radiation-induced cell damage on the molecular level. (This is discussed in much more detail in part 2 of this thesis).

Glutathione occurs in two oxidation states; reduced glutathione (GSH) and oxidized glutathione (GSSG) with a redox reaction as shown.

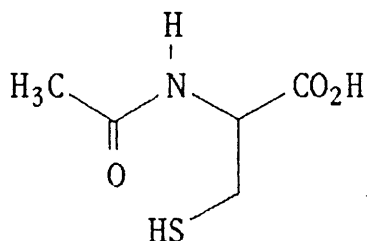


GSH is therefore well suited for its role as an anti-oxidant and once oxidized to GSSG is readily regenerated within the cell by glutathione reductase. This system is extremely efficient and enables the normal GSH : GSSG ratio of about 1000 : 1 to be maintained even in the presence of powerful oxidants.

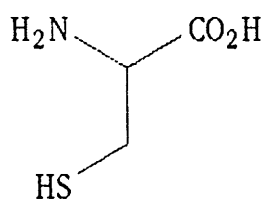
The role of GSH in the carcinogenicity of Cr(VI) has received extensive study [27,40]. It was found that alteration of the cellular levels of GSH by treatment with N-acetylcysteine or



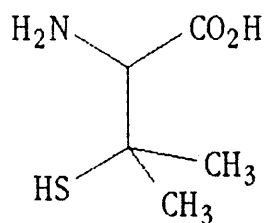
Reduced glutathione (GSH)



N-acetylcysteine (NAC)



Cysteine



Penicillamine (PSH)

Fig. 2.1. Structure of the reductants used for reaction with Cr(VI) in aqueous solution.

buthionine sulfoxime (BSO) had profound effects on the degree of cellular damage [41]. On increasing the GSH level there was a dramatic increase in Cr(VI)-induced DNA strand breaks. However, on GSH depletion, little or no DNA strand breaks or DNA interstrand cross-links were observed after Cr(VI) treatment.

Incubation of human red blood cells with Na_2CrO_4 (10mM) was found to decrease the GSH content of the cells by 90% [40], this reduction being very similar to that obtained with diethylmaleate (DEM) a well known GSH depleting agent. In addition, studies with radioactive $^{51}\text{Cr(VI)}$ suggest that complexes of low weight cellular fractions with Cr do occur, these probably being Cr-GSH complexes. This is indicative of a role of GSH in the intracellular reduction of Cr(VI) to Cr(III)

It was suggested that the reaction of Cr(VI) with GSH could take place in the cytoplasm of Cr(VI) exposed cells since it is found in reactive concentrations in this area, as are several other reducing species [42].

Recent research has therefore been directed at investigating the reaction of Cr(VI), in the form of chromate, with GSH and other relevant cellular thiols to probe the possible mechanisms of cellular reduction.

Results obtained from kinetic studies [43,44] have shown that the reaction of Cr(VI) with GSH in aqueous solution at physiological pH follows a mechanism common to all monothiol (fig.2.2). In this scheme, the initial formation of a Cr(VI) thioester complex (1) is followed by either a redox reaction involving a second molecule of thiol (2) or a unimolecular redox

reaction of the thioester (3). With monothiols, the latter reaction would give rise to a Cr(V) complex but with dithiols a Cr(IV) complex is formed due to the possibility of forming an intramolecular disulphide bond (4).

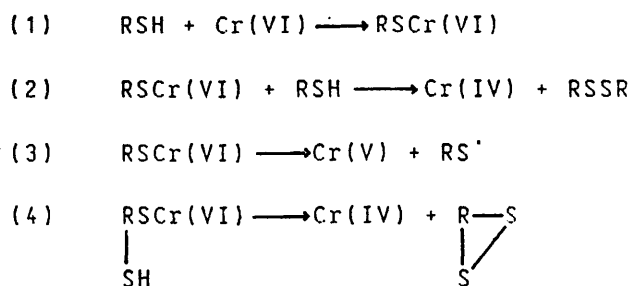


Fig.2.2. Reaction scheme for Cr(VI) / thiol interaction

Kinetic data obtained by following the rate of decay of the $\lambda_{\text{max}} = 372\text{nm}$ chromate band with time indicated that in the case of GSH (also N-acetylcysteine, cysteine and penicillamine) equations (1) and (2) are the valid routes, whereas reaction (3) was shown to be negligible. In addition, it was found that with GSH, the formation of the Cr(VI) thioester takes place in a rapid pre-equilibrium step followed by a slower redox step involving a second molecule of GSH.

The overall stoichiometry of the reaction of Cr(VI) with thiols was found to be three thiol molecules per Cr(VI) centre [45], this being consistent with a reaction scheme in which Cr(VI) is ultimately reduced to Cr(III).

Step (2) being considerably slower than the initial formation of the Cr(VI) thioester with GSH could have important biological implications. The slow redox reaction may allow

interaction of the Cr(VI) thioester with important cellular components which otherwise would not react with Cr(VI) or the Cr(III) final products. Formation of the thioester may therefore prolong the lifetime of Cr(VI) allowing its transport to the critical sites within the cell where it will have its ultimate damaging effects.

Step (3) shows the unimolecular redox reaction of the thioester to give a Cr(V) species and the RS' radical. Now, under the reaction conditions employed in the kinetic studies [43,44], little or no Cr(V) was observable by epr spectroscopy. However, as outlined previously, the production of intermediate complexes in the biological reduction of Cr(VI) may be of great importance with respect to the transport of damaging forms of Cr within the cell.

It has been recently shown both in our laboratory [29] and elsewhere [37] that interaction of Cr(VI) with GSH does indeed give rise to relatively stable Cr(V) species, as observed by epr spectroscopy.

The work reported here [29] looks at the reaction of Cr(VI) with GSH but in greater detail than reported elsewhere. Reactions were done under a wide range of conditions including solution pH and reactant ratio variation. The reactions of chromate with L-cysteine, N-acetyl-L-cysteine and DL-penicillamine were also investigated. These are all thiol-containing biomolecules and have been shown to react with chromate via the mechanism as described previously. Formation of relatively long-lived Cr(V) species with these reductants may also be important, since in the case of L-cysteine and N-acetyl-L-cysteine they are good models

for the reactive sites of GSH. DL-penicillamine is of interest due to its possible role as a detoxifying agent in chromate poisoning.

The production of Cr(V) complexes was monitored by epr spectroscopy in both mobile and frozen solution, the results obtained compared, contrasted and related to the mechanism of Cr(VI) carcinogenicity.

2.2. Experimental

The general experimental methods set out below for the aqueous reaction of chromate with the cellular reductants discussed in this chapter were also used for the studies in later chapters.

2.2.1. Mobile solution studies

To a solution of potassium dichromate in water (0.032M, 2ml) was added a solution of the required amount of cellular reductant in H₂O to give reaction solutions with a variety of reactant ratios. The pH of the resultant solution was adjusted to the desired value by the dropwise addition of 0.5M KOH or HCl (aq.).

The X-band epr spectra of the solutions in an Heraeus quartz flat cell were recorded immediately and at timed intervals thereafter at room temp. (24°C).

2.2.2. Frozen solution studies

On selected systems, frozen aq. solution studies were made and Cr(V) concentration calculated. The reaction solutions were prepared as described above. Once the desired pH was achieved,

the sample was transferred to a quartz epr tube, sealed and then quickly frozen in liquid N₂ (77K). The X-band epr spectrum was recorded with the sample tube immersed in liq. N₂ in a quartz finger dewar, the microwave cavity being purged with N₂ gas to remove oxygen.

The spectra were recorded onto a computer disc and this then used for the concentration calculation via a double integration technique.

The execution of the double integration program yields two parameters, the integration intensity (II) and the relative intensity (RI). RIxII gives I in the equation below used for the concentration calculation.

$$[Ex] = [Std] \times \frac{I_{ex}}{I_{Std}} \times \left[\frac{SW_{ex}}{SW_{Std}} \right]^2 \times PGF$$

Ex = experimental system
 Std = standard Cu(II) solution
 SW = sweep width of spectrum
 PGF = power and gain factor

PGF takes into account the different spectrometer settings for the standard and experimental spectra. To help with the reproducibility of results, care was taken to ensure that in all double integrations the same quartz finger dewar was used and that its orientation in the cavity was constant.

It is necessary to have a standard solution of known concentration for an absolute determination. Cu(II) was chosen, an aq. solution (5mM) prepared by dissolving CuSO₄·5H₂O (0.125g, 5x10⁻⁴ mol) and EDTA (1.861g, 5x10⁻³ mol) in H₂O (100ml). The frozen solution epr spectrum was recorded, the required

parameters determined and used in the calculation.

The possibility of using one of the stable Cr(V) tertiary hydroxy acid complexes as the standard in place of Cu(II) was considered, this being a much more representative of the systems under study. This was rejected however for two main reasons; firstly, the epr signals of Cr(V) complexes are extremely narrow and to allow their measurement by the spectrometer, the solutions would have to be quite dilute, thereby reducing the accuracy with which the initial solution could be prepared. Secondly, Cr(V) complexes disproportionate in aqueous solution. With the tert-hydroxy acid complexes this decreases with concentration and for the dilute solution required here, decomposition would occur within a few hours, hence giving a false standard concentration.

For both mobile and frozen solution studies a Varian E12 X-band (approx. 9.5 GHz) spectrometer was used. Frequency calibration was made with a Sanders WM16 wavemeter and field calibration with a Varian E500 nmr gaussmeter. All reagents were used as supplied from Hopkin and Williams Ltd. ($K_2Cr_2O_7$) and Sigma Chemical Co. Ltd. or Aldrich Chemical Co. Ltd. (reductant).

2.3.. Results

The structures of the thiol-containing reductants used in these studies are given in the introduction (fig.2.1).

2.3.1: GSH

Reactions with GSH were studied from pH 6 to 8 with Cr(VI):GSH reactant ratios of 1:10 to 2:1.

Fig.2.3. shows the X-band epr spectrum of an aqueous solution of Cr(VI) and GSH at a 1:1 ratio at pH 7.0 (unbuffered). The first spectrum was measured 5 min. after mixing, the other spectra being obtained at 253 sec. scan intervals.

In each case the spectrum is dominated by a characteristically sharp Cr(V) signal (1.2 gauss peak-to-peak derivative width) at $g=1.985$. The four weak nuclear hyperfine bands marked * are due to the naturally present ^{53}Cr (9.55% abundant, $I=3/2$) and show a spacing of 8.5 G.

Also observable under these conditions were additional weak bands at $g=1.995$, 1.977, 1.972 and 1.970. All of the signals decayed with time, the central band with a half-life ($T_{1/2}$) of approx. 10 min. (as measured from the rate of change of peak intensity) this still being observable after 30 min.

As the GSH excess was increased (pH remaining at 7.0) the $g=1.985$ signal decreased in relative intensity while a new band at $g=1.995$ grew and, by a Cr(VI):GSH ratio of 1:5 had become the dominant feature (figs. 2.4, 2.5). The $g=1.995$ band was broader than that at $g=1.985$ (6G peak-to peak derivative width) and ^{53}Cr hyperfine bands were no longer resolved. The $g=1.977$, 1.972 and 1.970 bands decreased in relative intensity with decreasing Cr(VI):GSH ratio and were replaced by a new small band at $g=1.98$.

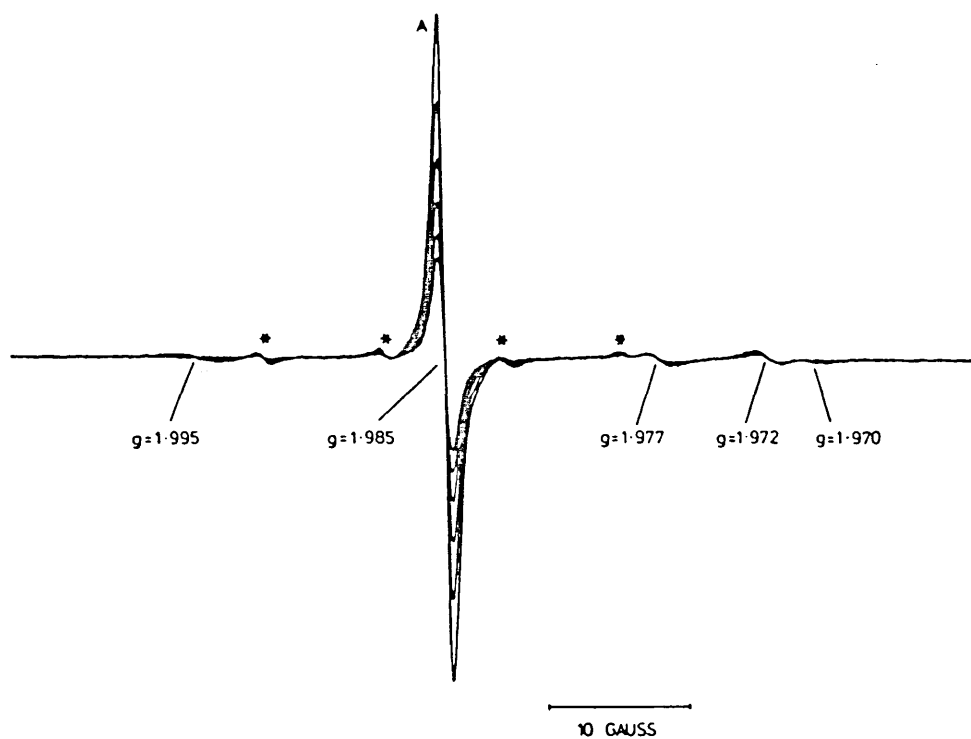


Fig. 2.3. X-band epr spectra of an aqueous solution (pH 7.0) of GSH + Cr(VI) in 1:1 mole ratio. Spectra taken at 253s scan intervals.

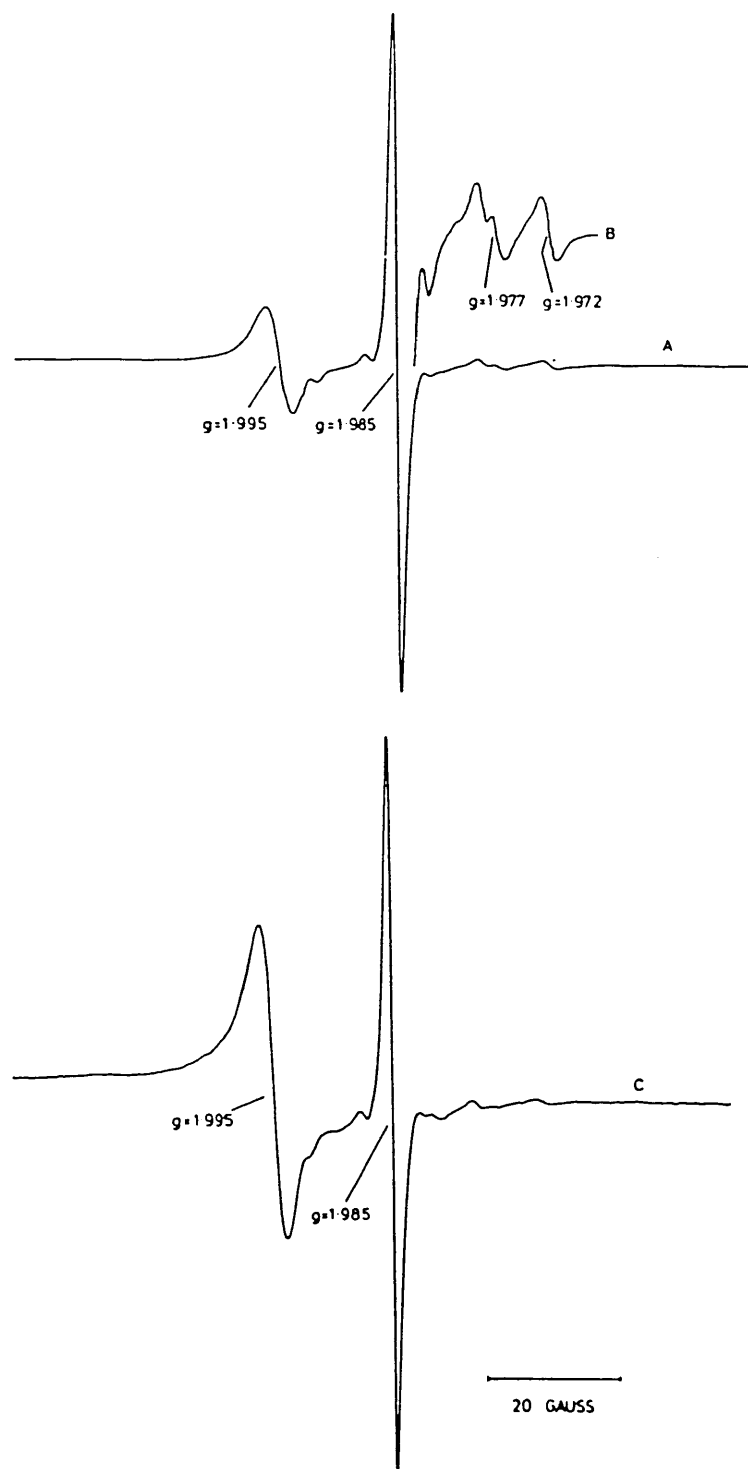


Fig. 2.4. X-band epr spectra of aqueous solutions (pH 7.0) of GSH + Cr(VI) at; (A) 2:1 mole ratio
 (B) $g=1.97$ - 1.98 region of (A) at increased gain.
 (C) 4:1 mole ratio

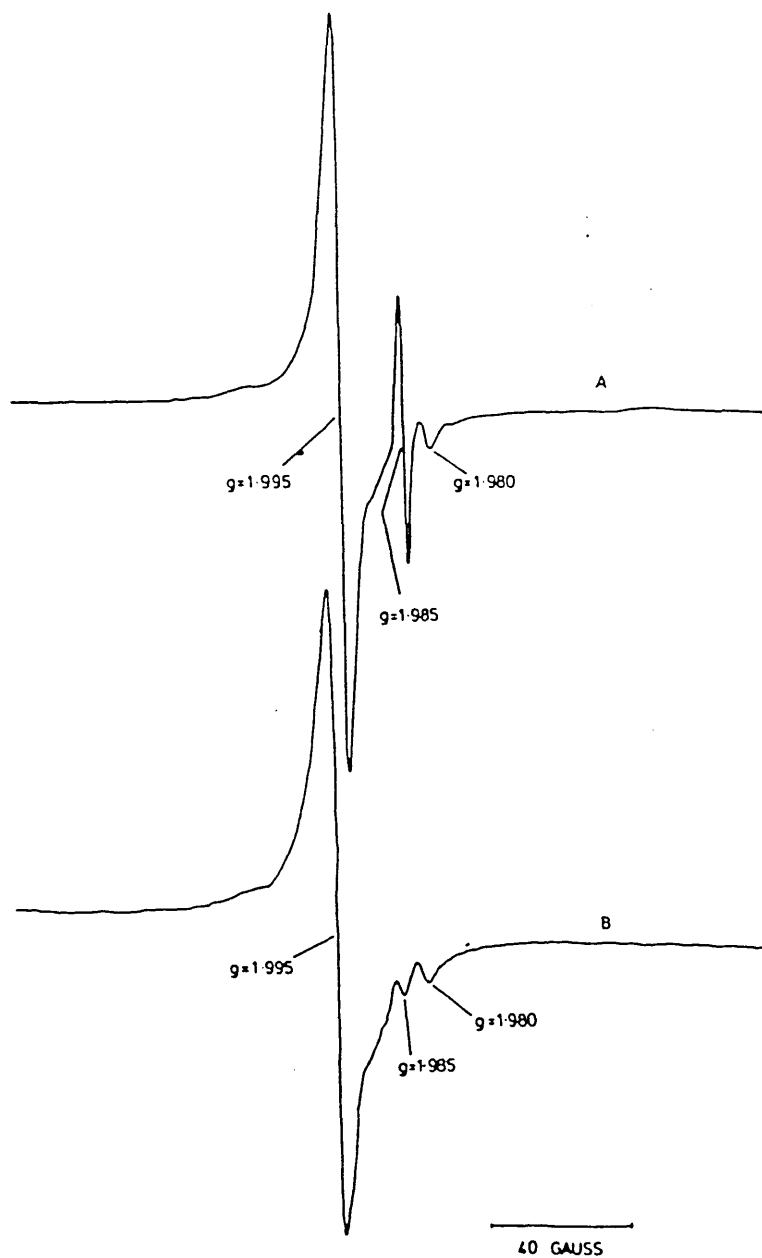


Fig. 2.5. As fig. 2.4. but with GSH:Cr(VI) ratios of (A) 5:1 and (B) 10:1

In the cases where both the $g=1.995$ and 1.985 bands were present at an observable concentration (eg. $\text{Cr(VI)}:\text{GSH} = 1:4$), the rate of decay appeared to be very similar for both species.

As the $\text{Cr(VI)}:\text{GSH}$ ratio was decreased, the rate of signal decay increased and at a 1:10 ratio the signal (at $g=1.995$) was observable for only 10 min. An excess of Cr(VI) over GSH (2:1) resulted in total removal of the signal at $g=1.995$. The $g=1.985$ band was still present but was now of lower intensity relative to the $g=1.977$, 1.972 and 1.970 bands.

Alteration of the solution pH in the range 6 to 8 yielded very similar results to those described at pH 7.0, two main bands at $g=1.995$ and 1.985 being produced under varying ratios. The major difference on pH change was that at pH 8.0, the rate of decay of the Cr(V) signal was very rapid indeed and was difficult to follow. At pH 6.0 the initial intensity of the Cr(V) band was greatly reduced and the rate of signal decay was slower. Measurement of solution pH after recording the spectra showed a relatively small pH variation (approx. $1/2$ pH unit) to have taken place. It was therefore felt that the use of a buffer was not essential. Selected reactions were carried out in TRIS.HCl buffer solution however, in response to results reported in a recent paper [46]. The use of this particular buffer was not desirable since it has been shown (Chapter 3.) that in several cases it can interfere with the production of Cr(V) .

Reactions in the presence of TRIS.HCl were done under the following conditions;

Cr(VI) + GSH (1:1) in 25mM TRIS.HCl pH 7.0

Cr(VI) + GSH (1:1) in 25mM TRIS.HCl pH 8.0

Cr(VI) + GSH + TRIS.HCl (1:1:1) pH 8.0

Cr(VI) + GSH + TRIS.HCl (1:1:5) pH 8.0

Under these conditions the main Cr(V) band observable was exactly the same as that formed in unbuffered solution at this reactant ratio. However, the initial intensity of the Cr(V) band was very much lower and the rate of signal decay greater than when unbuffered.

Aqueous reactions of Cr(VI) and GSH at 1:1 and 1:5 ratios in the presence of an excess or equivalent of GSSG gave spectra identical in all respects to those without GSSG. A control reaction of Cr(VI) with GSSG (xs) yielded no epr observable species.

Frozen solution X-band epr spectra were recorded for the reaction of Cr(VI) with GSH at 1:1 and 1:10 ratios at pH 7.0 (fig.2.6). Spectrum (B) recorded at 1:10 ratio is typical of a Cr(V) complex in an axially distorted ligand field, $g_{\parallel}=2.014$ and $g_{\perp}=1.987$ giving a weighted average of $g_{av}=1.996$.

A computer simulation of spectrum B was carried out (fig 2.9B). This showed that the signal is in fact slightly rhombic in character, 3 g-values at 2.015, 1.988 and 1.987 being resolved. (The slight discrepancy of these positions compared to those obtained experimentally is due to field-measurement inaccuracies)

A more complex spectrum (C) was obtained at the 1:1 ratio. As with (a) an anisotropic signal is observed and at least four

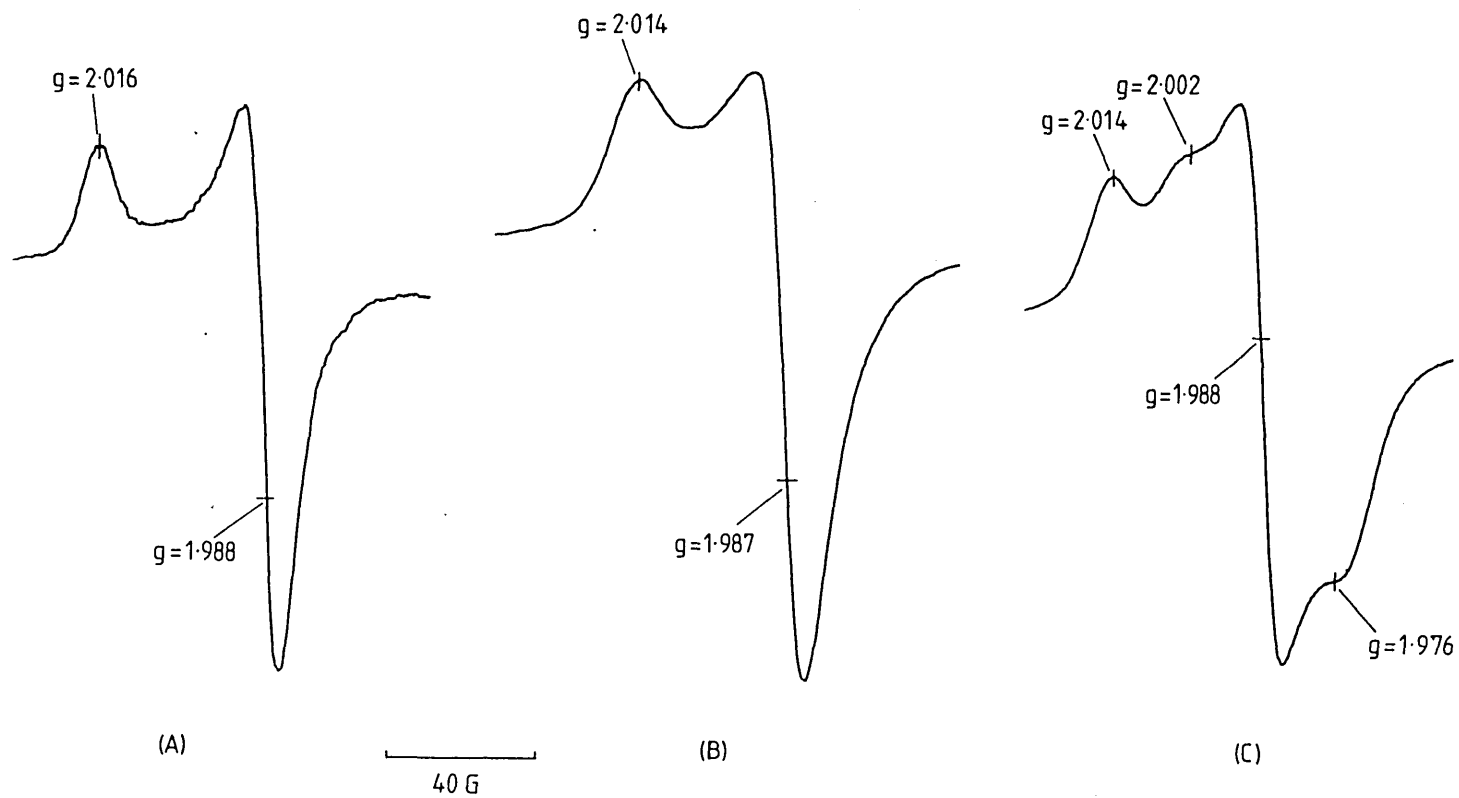


Fig. 2.6. X-band epr spectra of frozen (77K) aqueous solutions of;
 (A) Cr(VI) + NAC (1:10) pH 7.0.
 (B) Cr(VI) + GSH (1:10) pH 7.0.
 (C) Cr(VI) + GSH (1:1) pH 7.0.

distinct g-values can be measured at $g=2.014$, 2.002 , 1.988 and 1.976 . The $g=2.002$ and 1.976 bands appeared symmetrical about the $g=1.988$ band, giving for these three signals $g_{av}=1.988$.

Double integration of spectra (a) and (b) showed the amount of Cr(V) present to be 2.25% and 0.5% respectively, when expressed as a percentage of the original Cr(VI) concentration.

In all aqueous, mobile reactions of Cr(VI) with GSH, the pH of the resultant solution immediately after mixing was approx. 4, there being no obvious solution colour change. On raising the pH towards neutrality however, the solution rapidly turned dark red - brown. In all cases, the ultimate colour of the reaction solution was purple (due to formation of Cr(III)), this being obtained within an hour at a Cr(VI):GSH ratio of 1:10 and over a longer time period (10-15 hours) at a 1:1 mole ratio.

Attempts to follow the production of Cr(V) with a greater than ten-fold excess of GSH were unsuccessful, the signal decaying too rapidly.

2.3.2. N-acetyl-L-cysteine (NAC)

Reaction of Cr(VI) with NAC (1:1, pH 7.0) in unbuffered aqueous solution gave the typically sharp Cr(V) epr signal at $g=1.985$ as observed with GSH; the ^{53}Cr spacing = 8.8 G. The signal was decreasing in size with time ($T_{1/2}=20\text{min.}$)

At a 1:5 Cr(VI):NAC ratio (pH 7.0) the epr spectrum showed two Cr(V) bands at $g=1.985$ and 1.996 , the second being much broader (6G ptp). No ^{53}Cr hyperfine bands could be seen. Both signals were decaying with time at a very similar rate, this being slightly greater than at 1:1.

Increasing the excess of NAC further to 1:10 gave a spectrum

in which the $g=1.996$ band was now dominant, although the $g=1.985$ band was still quite large. Both bands were decreasing in size more rapidly with time ($T_{1/2} < 4$ min.) both apparently at the same rate. Lowering the reaction solution pH to 6.0 slowed down the rate of decay of the Cr(V) species and raising it to 8.0 increased the rate of decay.

The weak bands seen at $g=1.977$, 1.972 and 1.970 with GSH were not produced in these reactions.

Frozen solution studies gave very similar epr spectra to those with GSH under exactly the same conditions. At Cr(VI):NAC = 1:10 (pH 7.0) an apparently axial spectrum was seen, $g_{\parallel}=2.015$, $g_{\perp}=1.988$, $g_{av}=1.997$ (fig.2.6 (A)). At 1:1 ratio four g values could be measured at 2.016, 2.004, 1.990 and 1.978. Double integration of both of these spectra showed a Cr(V) yield comparable to that with GSH.

A computer simulation of spectrum A was carried out (fig. 2.9A). This showed the signal to be slightly rhombic in character, 3 g -values at 2.018, 1.989 and 1.987 being resolved. Again there are discrepancies between these values and those obtained experimentally due to field-measurement inaccuracies.

2.3.3. L-cysteine

The reaction of Cr(VI) with L-cysteine (1:1, pH 7.0) in unbuffered aqueous solution showed no Cr(V) epr band immediately after mixing, a broad featureless band centred at $g=1.99$ being the only component. The reaction was repeated at pH 6.0 in an attempt to slow down the rate of decay of any Cr(V) species possibly being formed. At this pH a very small signal was seen,

its rate of decay being too rapid for satisfactory recording. At pH 5.3 (unchanged from mixing) the Cr(V) signal was initially quite large but rapidly decaying. This band was broader (ptp=10G) than that observed with GSH or NAC at the same reactant ratio but it exhibited a similar g-value of 1.987. Attempts to resolve any nuclear super hyperfine structure possibly causing the observed line broadening were made, but these were unsuccessful. At no time could the ^{53}Cr hyperfine bands be resolved, the signal-to-noise ratio being too low. An excess of Cr(VI) produced little Cr(V).

The frozen solution epr spectrum of Cr(VI) + L-cysteine (1:1, pH unchanged) showed Cr(V) in an axially distorted ligand field, $g_{\parallel}=2.015$, $g_{\perp}=1.984$ giving $g_{av}=1.994$ (fig.2.7 (A)). The signal band width was almost double that of GSH (1:10) or NAC, computer double integration showed 0.31% of total chromium to be Cr(V).

2.3.4. DL-Penicillamine (PSH)

At a Cr(VI) : PSH ratio of 1:1 at pH 7.0 no Cr(V) species were observed in aqueous solution. At pH 5.0 (unchanged from mixing) a very small Cr(V) band was seen but this decayed too rapidly for satisfactory recording. Repeating the reaction at the same pH but with a ten-fold excess of Cr(VI) slowed down the Cr(V) decay sufficiently for a satisfactory epr spectrum to be recorded (fig.2.8). A relatively broad (ptp 6G) Cr(V) signal centred at $g=1.985$ was obtained, ^{53}Cr hyperfine bands not being resolved. The important feature of this spectrum was the presence of nuclear super hyperfine structure of the central Cr(V) band. There is no obvious order to the observed structure and it did

not change as the signal decayed.

The frozen solution spectrum of Cr(VI) + PSH (10:1, pH unchanged) gave a Cr(V) signal exhibiting axial distortion (fig.2.7 (B)) with $g_{11}=2.004$ and $g_{1r}=1.976$ giving $g_{av}=1.985$, exactly the same position as in mobile solution. The g_{11} component was poorly resolved in comparison to the other reductants, the band width increasing as with L-cysteine. Double integration of this spectrum gave a Cr(V) yield of 0.68% of original [Cr(VI)].

The results of the epr studies done are summarized below in table 2.1.

<u>Reductant(R)</u>	<u>Cr(VI):R</u>	<u>pH</u>	<u>mobile</u>		<u>frozen</u>	
			<u>g</u>	<u>g_{11}</u>	<u>g_{1r}</u>	<u>g_{av}</u>
GSH	1:10	7.0	1.995	2.014	1.987	1.996
GSH	1:1	7.0	1.985	-	-	-
NAC	1:10	7.0	1.995	2.016	1.988	1.997
NAC	1:1	7.0	1.985	-	-	-
L-cysteine	1:1	5.0	1.987	2.015	1.984	1.994
PSH	10:1	5.3	1.985	2.004	1.976	1.985

Table 2.1 Summary of epr parameters of Cr(V) species obtained on reaction of Cr(VI) with thiols.

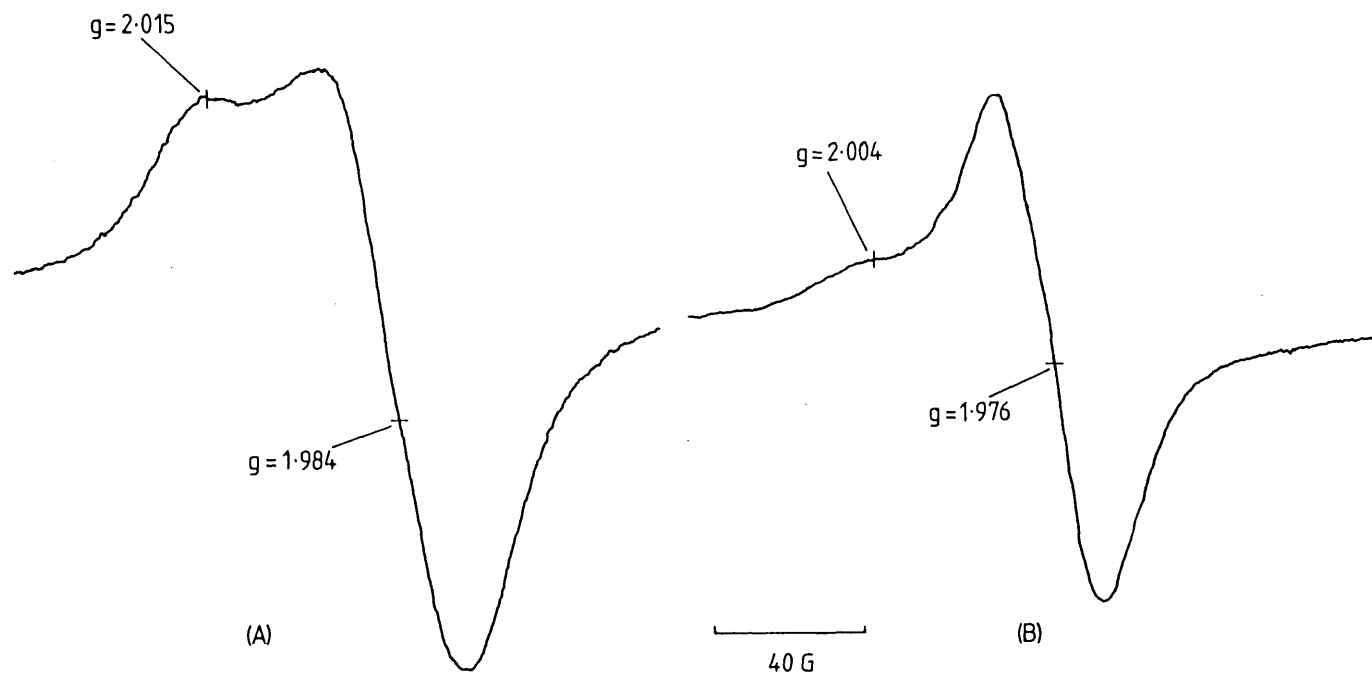


Fig. 2.7. X-band epr spectra of frozen aqueous solutions of;
(A) Cr(VI) + L-cysteine (1:1) pH unchanged from mixing.
(B) Cr(VI) + PSH (10:1) pH unchanged from mixing.

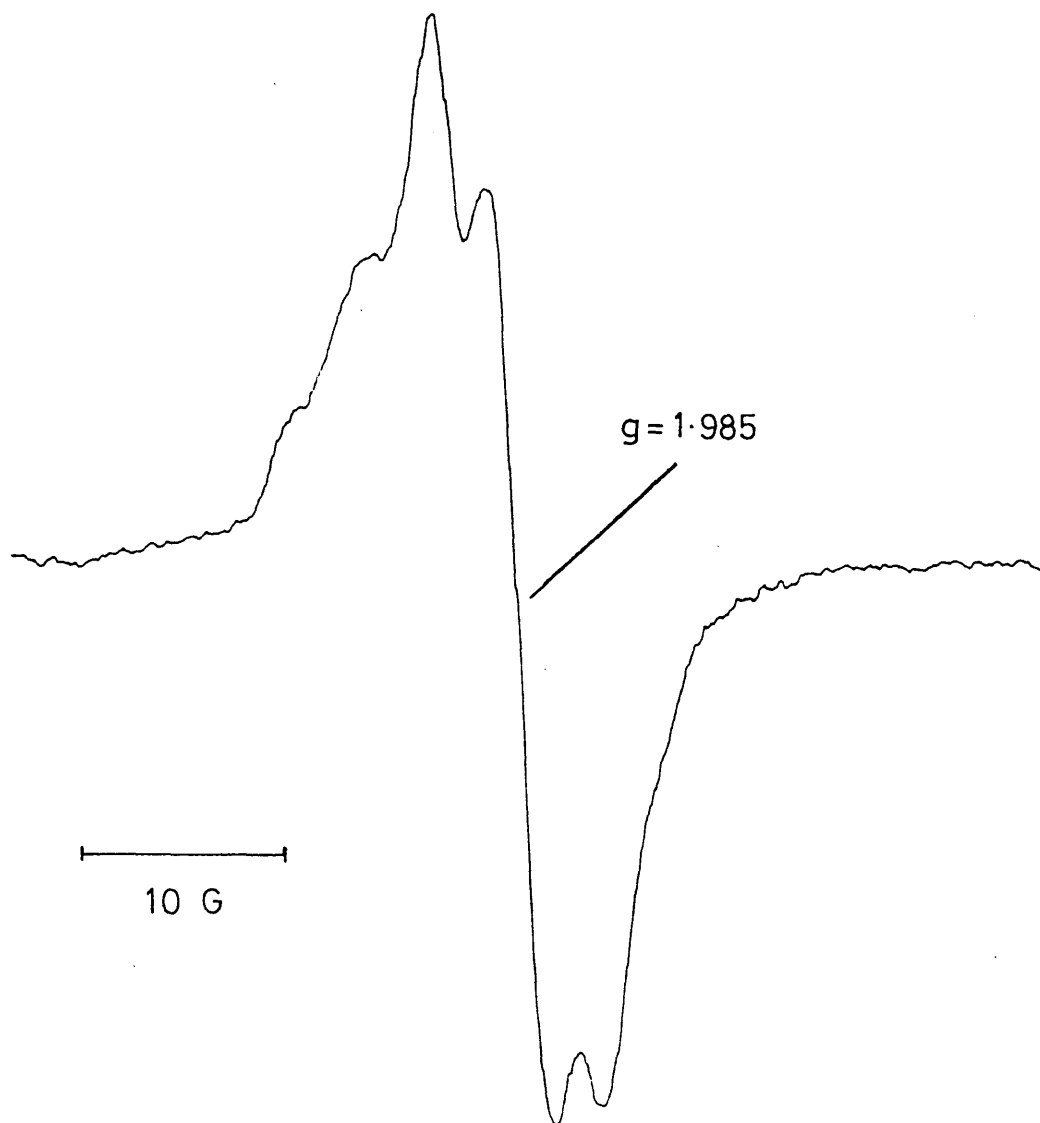


Fig. 2.8. X-band epr spectrum of an aqueous solution of Cr(VI) + PSH (1:10) pH unchanged from mixing.

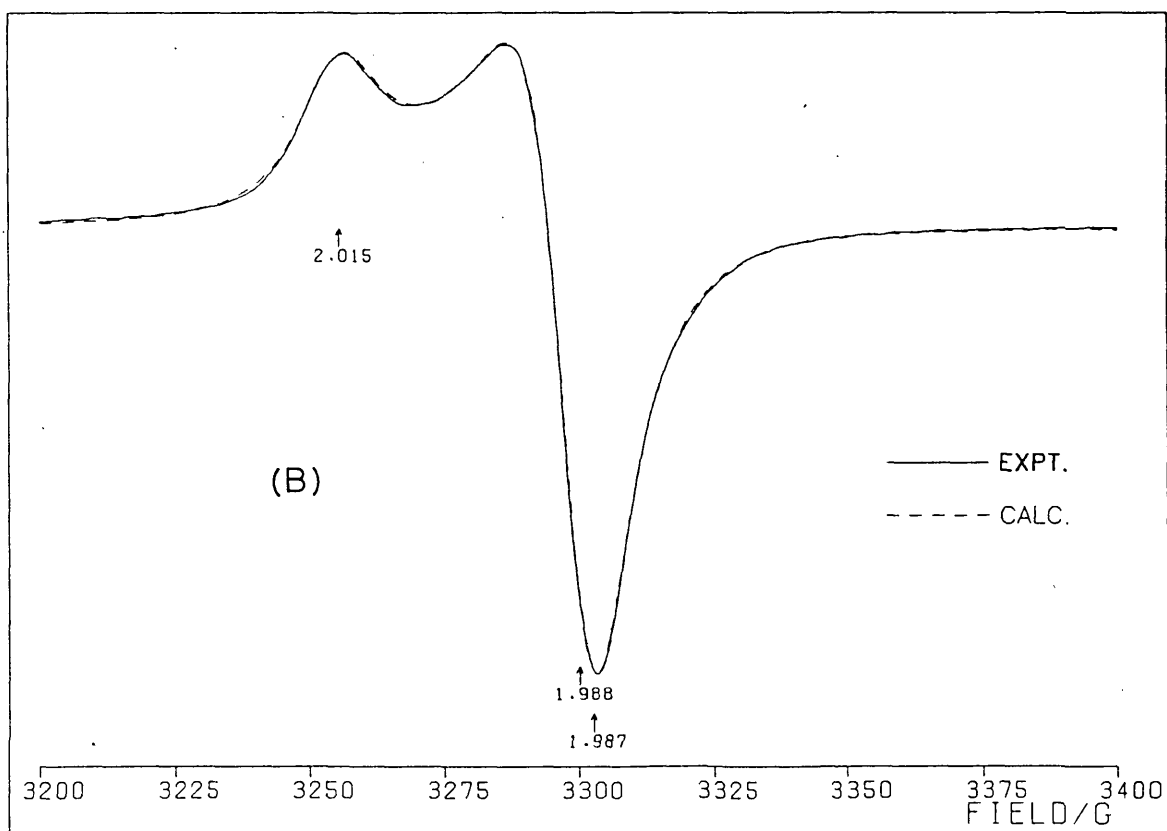
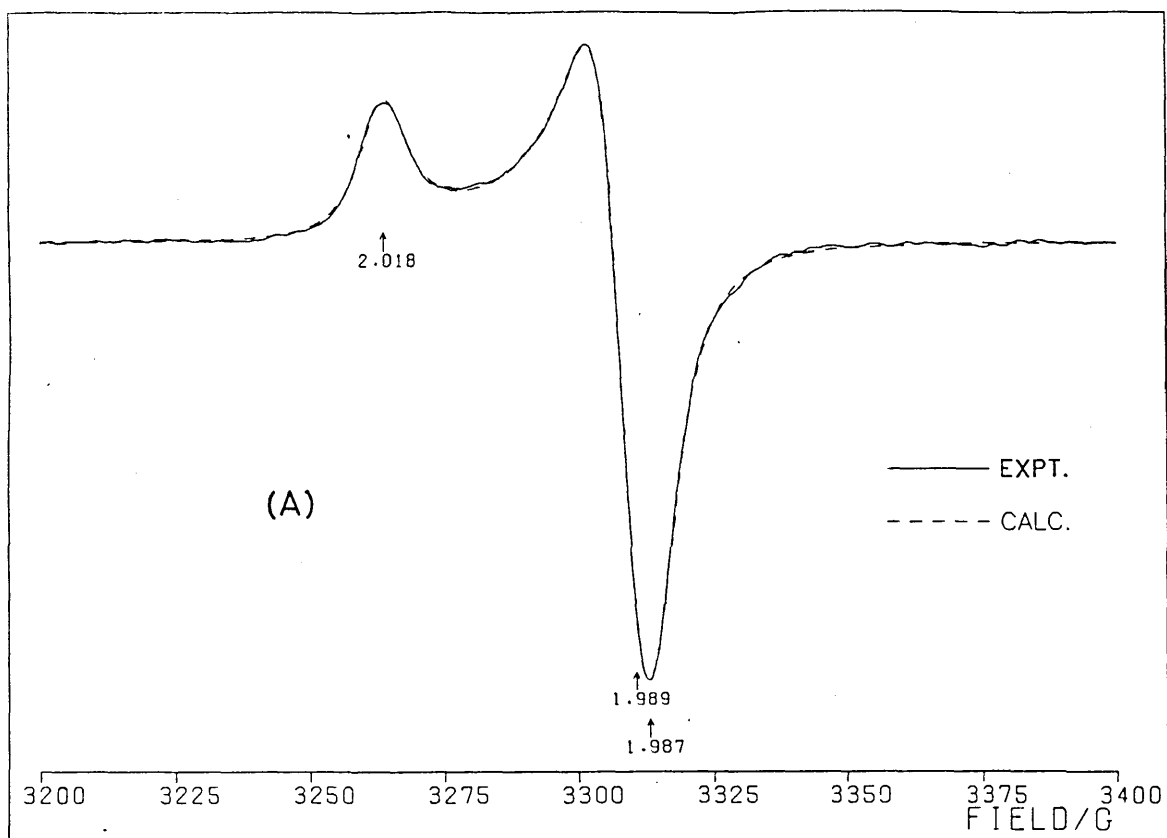


Fig. 2.9. Experimental and computer simulated X-band epr spectra of frozen aqueous solutions of:
 (A) Cr(VI) + NAC (1:10 ratio, pH 7.0)
 (B) Cr(VI) + GSH (1:10 ratio, pH 7.0)

2.4. Discussion

The biological importance of GSH has prompted a great deal of study into its ability to form complexes with a variety of transition metals. Complexes with GSH and GSSG have been reported for iron [47,48], zinc [49], cobalt [50], manganese [50] copper [51,52], vanadium [53] chromium [11] and osmium [54].

With Fe it was demonstrated that two Fe-GSH stoichiometries, 1:1 and 1:2 are formed. The 1:1 complex contains two different Fe(II) environments, one five and the other six coordinate. From IR data there was evidence for Fe-S and Fe-O (from C-O (peptide) and CO_2^- groups) bonds and some evidence for a Fe-N bond (from the glutamyl residue). The 1:2 complexes contain only one Fe(II) site in a distorted octahedral 6-coordinate environment. There is evidence of O, N and S donation, O coming from the CO_2^- group of the glycine residue, the N from the glutamyl and perhaps the peptide bond.

EPR and NMR studies have been made into the interaction of GSH with Cu(II) in aqueous solution. It was found that in basic solution, one GSH molecule combines with two Cu(II) ions, one Cu coordinated to the cysteinyl -SH and the deprotonated N atom of the peptide linkage (between the cysteine and glutamic acid residues). The other Cu combines with the terminal groups of the glutamic acid residue. At no point was the glycine residue involved in the coordination.

Studies with Zn suggest that it coordinates to GSH via the cysteinyl S and amino group. With a 1:1 Zn(II)-GSSG complex formed, coordination takes place via the amino and carbonyl groups of the glutamyl residues.

Reaction of OsO_4 with GSH in aqueous acetone yields a brown complex of 1:2 Os:GSH stoichiometry [54]. It was suggested from IR data that each ligand is terdentate with coordination of one O atom from CO_2^- and of the S and amide N. The paramagnetism of the complex suggested that Os was in the +4 oxidation state.

During the course of this work, a paper was published reporting the results of studies into the reaction of Cr(VI) with GSH [37]. The reactions were done at a Cr(VI):GSH ratio of 1:10 and relatively long-lived Cr(V) species were observed by epr spectroscopy. A Cr(V) signal centred at $g=1.995$ was seen and this was identical to spectrum (B) in fig.2.5 obtained here. The band decayed with time with a first order rate constant $k=2.39 \times 10^{-3} \text{ s}^{-1}$ calculated by measuring the rate of change of signal intensity with time. It is felt that following the change of signal intensity with time is not a very satisfactory method of rate constant determination, the decay rate being quite sensitive to very small changes in reaction conditions and even spectrometer settings. It is for this reason that approximate half-lives alone are quoted in the results reported here.

O'Brien [37] also reported evidence of ^{53}Cr hyperfine bands associated with the $g=1.995$ signal, although no spacing was quoted. At no time were these seen in the present study, the broad "wings" of the main Cr(V) band obscuring them. It would appear as though the bands at $g=1.985$ and 1.980 were wrongly assigned to be the ^{53}Cr bands, when in fact this work has shown the $g=1.985$ signal to be a separate Cr(V) species.

By adjusting the relative reactant ratios it was indeed found

that two major Cr(V) complexes can be produced on the reaction of Cr(VI) with GSH. At 1:1 stoichiometric ratio the major epr band encountered in mobile solution is at $g=1.985$. Of note here is that the value of the ^{53}Cr hyperfine spacing (a_{Cr}) is 8.5 G. This compares with $a_{\text{Cr}}=18$ G reported previously for the Cr(V) species produced on the reaction of Cr(VI) with nucleotides [55], in which it was proposed that the Cr(V) centre was in a square-pyramidal oxygen donor environment. Reduction of a_{Cr} suggests that the Cr(V) d^1 electron is being pulled from the metal onto the ligands. This greater bond covalency between metal and ligand is consistent with the coordination of other atoms such as sulphur in addition to oxygen (the difference between O and N is low in this respect [56]).

A clue to possible S-donation in the Cr(V) complex produced at high GSH ratios is the observed g -value of 1.995. This compares to values of 1.994 and 1.996 found with some Cr(V)-dithiolate complexes in which the principal donor atoms are S [35]. Cr(V) in a totally N environment exhibited g -values of 1.982 and 1.987 whereas those with O-donors are usually in the 1.980 region.

Also present at the 1:1 reactant ratio were some additional bands at $g=1.977$, 1.972 and 1.970 decaying at different rates to the principal band. To investigate the possible origin of these bands, the reaction of Cr(VI) and GSH in the presence of excess GSSG were carried out. If GSSG was involved in the formation of these bands then its presence in excess would alter the relative ratios of the signals produced and may change their rate of decay. Under all conditions employed there were no changes to any

of the bands on addition of GSSG, thus suggesting that its coordination is not involved in the production of Cr(V) complexes.

On increasing the GSH excess to 5:1 the second, broader band becomes dominant, the weaker bands apparently disappearing. When present together, the $g=1.995$ and 1.985 signals decay at very similar rates. This suggests that the two Cr(V) complexes are in equilibrium, the relative reactant ratios determining its position. Were this not so, their rates of decay would be expected to be independent.

The frozen solution epr spectrum at 1:10 Cr(VI):GSH ratio gave an apparently axially distorted spectrum with $g_{av}=1.996$. This is essentially the same as that found in mobile solution ($g=1.995$) suggesting that the Cr(V) signal is due to only one Cr(V) species. However, at the 1:1 ratio a more complex spectrum is produced, the presence of several g -values suggesting that there are at least two Cr(V) complexes in solution.

Double integration of these spectra showed the total yield of Cr(V) in these reactions to be very small. It had been reported that with some Cr(V) complexes of square pyramidal geometry, it was possible to stabilize the complex by solvent coordination into the vacant axial site [35]. Attempts to stabilize the Cr(V) complexes produced here, by addition of DMSO, did not have any noticeable effect on complex yield or stability. Reactions could not be done in totally non-aqueous media due to reductant insolubility.

There was a marked similarity between the reactions of Cr(VI)

with GSH and NAC, their spectral characteristics being the same in both mobile and frozen solution. Hence it seems highly probable that the mode of ligand coordination to Cr(V) is the same in both cases.

Cr(V) complexes with the ligand salen (N,N'-ethylenebis(salicylideneaminato)) have been prepared elsewhere [56]. These complexes possess a square pyramidal coordination sphere with an axial Cr=O bond and a cis-N₂O₂ ligand arrangement. They show a Cr(V) epr signal at $g=1.978$ ($a_{Cr}=20G$) with very clearly defined super nuclear hyperfine structure due to the N donor atoms ($I=1$). ($a_N=2.15G$). No N hyperfine structure has been seen in the spectra recorded in the reactions with GSH suggesting that the coordinating atoms do not include N.

To date, most Cr(V) complexes with two coordinating ligand molecules are square pyramidal in nature with a central Cr=O bond. It is felt that this is the likely form of the Cr(V) complexes formed in this work, although the only real evidence to prove this assumption comes from the axially distorted, frozen solution spectra.

Fig. 2.10 shows that there are two possible modes of coordination, not involving N, common to GSH and NAC; both show coordination of S⁻. In (A) the second coordinating atom is the O⁻ of the glutamyl residue (for GSH), formed by tautomerism of the peptide linkage. This would form a seven membered chelate ring. Mode (B) invokes coordination of O from the cysteine residue carboxylic acid group (NAC) or the tautomerized peptide bond (GSH) to form a six membered chelate ring. The second alternative has the advantage of possible complex-stabilization

by the glutamyl O and a more favourable ring size.

Now, with both GSH and NAC, the nature of Cr(V) species formed is dependent upon the relative reactant ratios. This suggests that the two major Cr(V) species may be a 1:1 Cr(V):GSH ($g=1.985$ band) complex formed under 1:1 stoichiometric conditions or a 1:2 Cr(V):GSH complex ($g=1.995$) formed with an excess of GSH.

Studies elsewhere [53] have shown vanadium (IV) (isoelectronic with Cr(V)) to form an oxovanadium-(GSH)₂ complex. The V=O moiety is analogous to the Cr=O bond already described, two GSH molecules coordinating to V in a square-planar fashion via the carboxylate groups of the glycine and glutamyl residues. This mode of attachment could therefore be considered for Cr(V) and GSH. However, the lack of both of these carboxylate groups in NAC removes this possibility.

From the hard-soft, acid-base (HSAB) point of view, oxygen is a much more favourable donor atom to Cr(V) than is S, since both can be classed as hard centres, S being much softer. This helps to explain the relative stabilities observed for purely O-donor environments (eg. with carbohydrates, chapter 4.) compared to the results obtained here. Cr(V) species with O-donors are often stable in solution for several weeks whereas those produced here decay in tens of minutes, again suggesting that with GSH, other donor atoms apart from O are in operation.

From the results obtained, it is proposed that at high GSH levels, a Cr(V)(GSH)₂ complex is formed exhibiting an epr signal at $g=1.995$. Coordination may take place via the

deprotonated -SH group and $-O^-$ of the cysteinyl residue (fig.2.10.(B)) of two GSH ligands, a mode of coordination similar to that found in a Fe-GSH 1:1 complex [47]. The obvious problem arising from this proposed structure is that the Cr(V) would be in a rhombic donor environment, but the frozen solution epr spectrum is apparently axial. A possible explanation for this is that the $g_{\perp r}$ rhombicity is very small in comparison with that of $g_{\parallel}$. The axial distortion itself is relatively small and the rhombic distortion may well be hidden within the $\perp_r$ tail of the spectrum.

Indeed, the computer simulations carried out on the Cr(V) epr spectra produced on reaction of Cr(VI) with GSH and NAC (fig. 2.9 A and B) do show that there is rhombic distortion in both cases, confirming the above suggestions.

The nature of the major Cr(V) species formed at 1:1 stoichiometric reactant ratios is not clear, coordination of GSH may be via the same atoms as above, the glutamyl O acting to stabilize the complex. The observed Cr(V) band position of $g=1.985$ is lower than that found previously with S-donor ligands, this may be due to coordination of only one GSH group via S thus reducing its influence.

The similarity of the reaction of Cr(VI) with GSH and that with NAC suggests that the Cr(V) complexes formed in both cases are very similar.

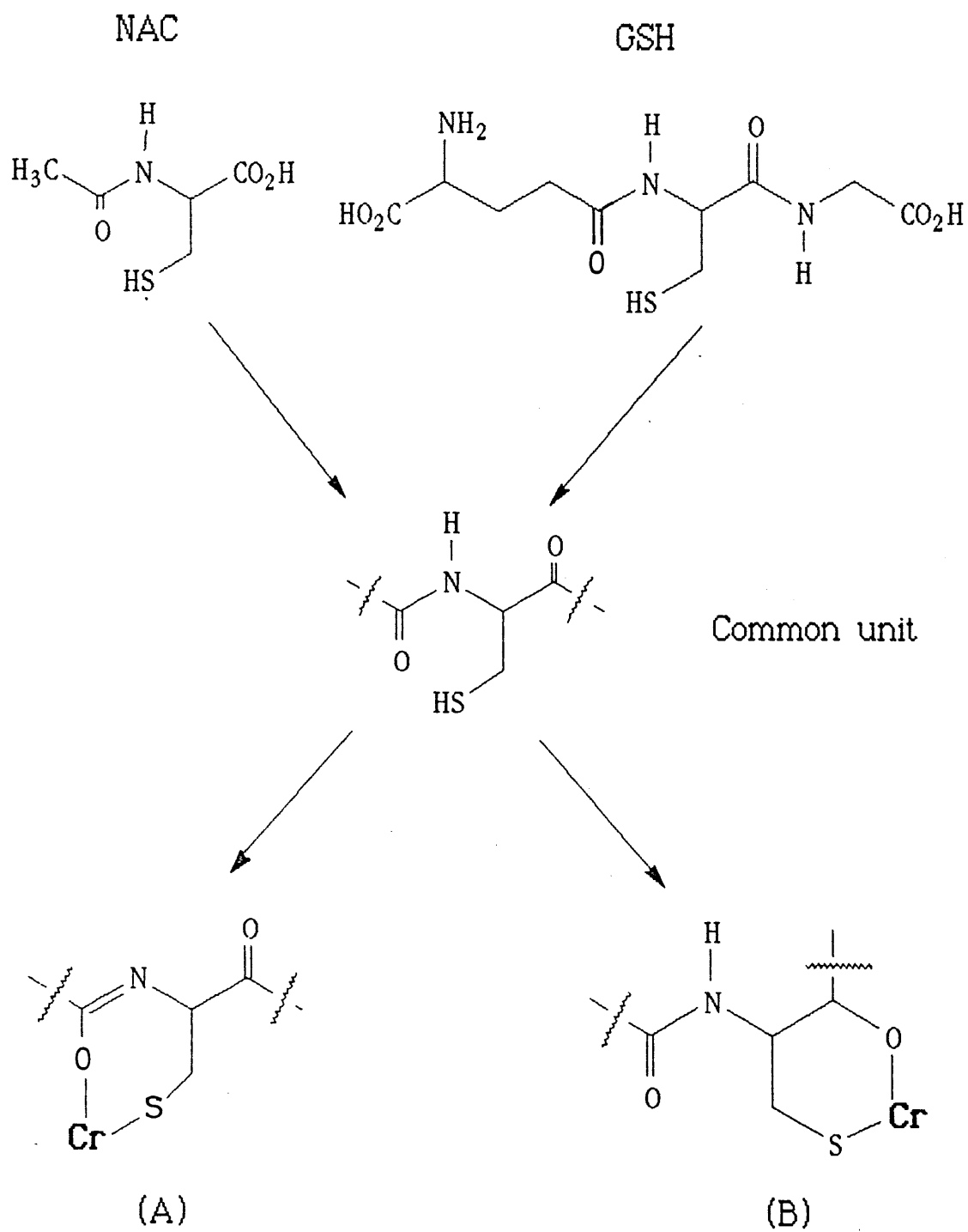


Fig. 2.10. Modes of binding to Cr(V) common to both GSH and NAC.

The reaction of Cr(VI) with L-cysteine and PSH gave rise to very different epr spectra to those found above. The most notable difference was the much faster Cr(V) species decay than found with GSH or NAC, L-cysteine being the faster. The stability of a Cr(V) complex depends not only upon ligand structure but also on its reducing ability. Ligands with a low oxidation potential are not able to stabilize Cr(V) even when the structure is reasonable. It is probably for this reason that the Cr(V) complexes produced on reaction with thiols decay within tens of minutes. The much more rapid decay of the Cr(V)-L-cysteine complex compared to say GSH, is to be expected from its lower reduction potential of -0.32V ($E' = -0.24\text{V}$ for GSH).

The rapid rate of decay with L-cysteine and PSH may also have its origin in the absence of the potentially stabilizing influence of the glutamyl O, which is present in both GSH and NAC.

The speed of reduction prevented detailed analysis of the Cr(V) species formed, but its position ($g=1.987$) suggests it may be similar to the 1:1 Cr(V)-GSH complex already encountered. However, a value of $g_{av}=1.994$ does show that there are more than one Cr(V) species present ($g=1.987$ in mobile solution).

With PSH it was necessary to have an excess of Cr(VI) to facilitate Cr(V) signal observation for a reasonable period. The Cr(V) signal observed was very interesting in that it was the only example of the four thiols studied in which nuclear super hyperfine structure of the main band was present. The value of $g_{av}=1.985$ is exactly the same as that found in mobile solution suggesting that there is only one Cr(V) species present.

Observation that the structure remained constant throughout the signal decay agrees with the above.

The origin of this splitting is not clear and due to the rapid signal decay it was difficult to record in detail. The observed spacing was of the order of 3G which would appear to be a little high for splitting by adjacent protons ($a_H=1G$ with sugars, chapter 4.). Repeating the reaction in D_2O had no effect showing that exchangeable protons were not responsible. The magnitude of splitting is very similar to that of the oxochromium(V)salen complex (2.15G) [56], and the relative peak ratios do bear a slight resemblance to those reported for that compound. It therefore seems that with PSH, a N-donor atom may well be present.

The question now arises; why should the Cr(V)-PSH complex show splitting by N whereas that with L-cysteine does not? The only difference between the two is that PSH possesses two additional CH_3 groups. The Cr(V)-L-cysteine complex can only be observed as a very small epr signal and may in fact be split by N, but in this case it is poorly resolved. With PSH and L-cysteine the potential N-donor is a primary amine, whereas in GSH and NAC the equivalent position is a secondary amine. This difference could explain the lack of N-donation in the latter.

A Cr(V) complex with two coordinated PSH molecules has been postulated elsewhere in the reaction of Cr(VI) with PSH [57]. One PSH molecule acted to reduce Cr(VI) to Cr(V) then two more coordinated to the Cr(V) centre. However, no attempt was made to determine the structure of this complex.

With both L-cysteine and PSH, the frozen solution epr spectra showed the signals broadened compared to those seen with GSH and NAC. There may be several sources of this effect but a possible explanation is that the more rapid reduction by L-cysteine and PSH produces a significant amount of paramagnetic Cr(III) before the sample can be frozen, thus resulting in signal broadening.

Cr(III) complexes have been isolated and characterized with GSH [11] L-cysteine [12] and PSH [57]. In all cases the Cr(III) is in the expected octahedral environment, two ligand molecules coordinating each via N, S and O donor atoms. With GSH it was proposed that coordination takes place via the S of the cysteinyl group and the adjacent N and O atoms of the glycine residue. NAC does not possess the glycine unit and as such, this mode of coordination is not likely with Cr(V).

The major stumbling blocks to determining the structure of the Cr(V) complexes produced here are the very low amounts of Cr(V) present at any one time and their relative instability. In all cases, the yield of Cr(V) was less than 1% of the total chromium content and it is only due to the signal sharpness and epr sensitivity that they are seen at all. A much higher Cr(V) yield with possible isolation would be necessary for a true structure determination, but all efforts to achieve this were unsuccessful.

The reactions of Cr(VI) with GSH in TRIS.HCl buffer were carried out because of its use in a recent paper examining the mechanism of DNA cleavage by chromate [46]. In this report a Cr(V) species was seen in the epr spectrum at $g=1.987$ with

$a_{Cr} = 18.2G$ ($T_{1/2} = 20min.$). The reactant ratios employed were not clear from the text but the ^{53}Cr hyperfine spacing was in contradiction to the observations found here ($a_{Cr} = 8.5G$ for $g = 1.985$ band). Hence the reactions were repeated under similar conditions to those described in [46]. In all cases the a_{Cr} value obtained was $8.5G$ thus nullifying their observations.

In addition, DNA strand breakage experiments apparently showed the $Cr(V)$ complex formed on reaction of $Cr(VI)$ with GSH to have no DNA damaging ability. However, since the " $Cr(V)$ " species in [46] is clearly different to those found in the present study, the validity of the above statement may be in doubt.

In the kinetic studies of the $Cr(VI)$ - GSH reaction [43], the possible production of $Cr(V)$ was acknowledged but never observed and hence was deemed to be negligible. The failure to see $Cr(V)$ was probably because the reactions were done under pseudo first order conditions with a 100 fold excess of GSH. At this high level the rate of $Cr(V)$ decay would be very rapid indeed.

It has been shown here that the $Cr(V)$ production is significant at lower GSH levels and must therefore be taken into consideration in the kinetic schemes.

The reaction (fig.2.1, eqn.(3)) which produces $Cr(V)$ from the $Cr(VI)$ -thioester also yields the $RS\cdot$ radical. Attempts were made to detect such radicals by epr spin-trapping methods but these gave no evidence of $RS\cdot$ production.

From a biological point of view, the important result from this work is that interaction of $Cr(VI)$ with cellular thiols, in

particular GSH, generates relatively long-lived Cr(V) species. These transient complexes may afford a mechanism by which potentially harmful Cr can be transported to the critical sites within the cell, similar to that proposed for the Cr(VI)-thioester species. Another important consequence of Cr(V) production may be the reaction of the reactive Cr(V) species with additional cellular constituents, these normally reacting with Cr(VI) only slowly if at all.

This last phenomenon has in fact been observed with nucleotides and carbohydrates, the results from these studies are reported in chapter 5.

CHAPTER 3. STUDY OF THE Cr(VI) AND RADICAL SPECIES PRODUCED IN THE REDUCTION OF Cr(VI) BY ASCORBATE.

3.1. Introduction

The importance of L-ascorbic acid (Vitamin C, H_2A) (fig.3.1) in the treatment and prevention of the toxic effects of chromate has been known for some time. Acute chromate toxicity in rats could be prevented by prompt administration of ascorbic acid, leading to a rapid dose-dependent reduction of Cr(VI) to Cr(III) [58]. The reduction of Cr(VI) was increased and accelerated by H_2A and the resulting non-toxic Cr(III)-protein complexes could be excreted in the urine [59].

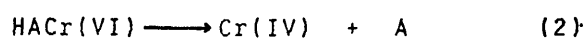
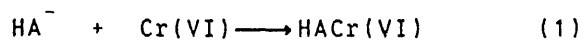
Ascorbic acid, as a 10% aqueous solution was found to be effective in protecting chromate-sensitive workers in the printing and lithographing industries [58]. Similarly, impregnation of H_2A into face-mask filters was effective in protecting workers from inhalation of chromic acid mist [60].

Experiments with rats demonstrated that injection of a lethal Cr(VI) dose followed within two hours by H_2A resulted in a 93% survival rate [61], and further experiments showed that dichromate administration to rats led to a decrease in the H_2A levels of the kidney [62], the latter suggesting the importance of ascorbate within the body.

In Chinese hamsters injected with $K_2Cr_2O_7$ and H_2A , the number of chromosome aberrations in bone marrow cells was approx. 50% of that with $K_2Cr_2O_7$ alone, showing H_2A to have a protective effect against Cr(VI) mutagenicity [63]. In addition, H_2A was found to decrease the growth inhibition and partially protect DNA from single-strand breakage caused by exposure to chromate on CHO

cells [64]. H_2A has also found a use in the ^{51}Cr (as $^{51}CrO_4^{2-}$) labelling of mice fibrosarcoma cells [65].

Since H_2A is present in mammalian cells, albeit at much lower concentrations than for eg. GSH, it has received attention with respect to its possible role in the reduction of $Cr(VI)$ within the cellular environment. Chemical kinetic studies of the $Cr(VI)$ -ascorbate (HA^-) reaction have shown an apparent second order rate constant and the proposed mechanism is given below [43], $HACr(VI)$ being present at a steady state concentration.

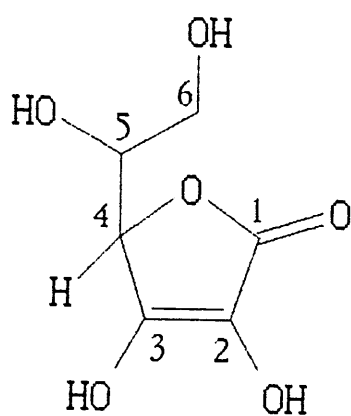


A =dehydroascorbic acid

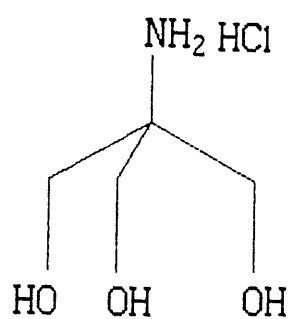
HA^- =ascorbate anion

In this scheme, $Cr(VI)$ reacts with HA^- (at physiological pH ascorbic acid is in the deprotonated form) to give the chromate ester species $HACr(VI)$ in the rate determining step. $HACr(VI)$ then undergoes a unimolecular redox reaction to give $Cr(IV)$ and A . No suggestion was made as to the fate of $Cr(IV)$ and of note in the context of this thesis is the apparent non-participation of $Cr(V)$.

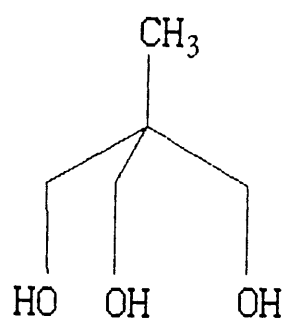
After one minute of reaction (pH 7.4, 1M TRIS.HCl buffer, $[Cr(VI)] = 3.7 \times 10^{-4} M$, $[HA^-] = 2.5 \times 10^{-2} M$), 60% chromate reduction was observed and it was suggested that the cis-diol unit of the ascorbate was the important grouping [43].



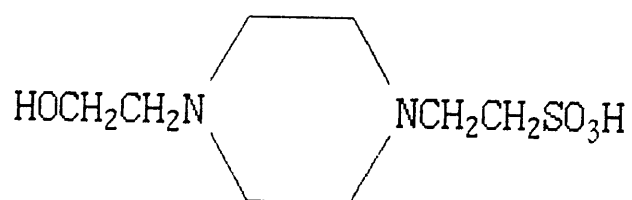
L-Ascorbic acid



TRIS. HCl



THME



HEPES

Fig. 3.1. Structures of the reagents used in these studies.

Considering the low levels of cellular H_2A , the reactions quoted above were carried out with an unrealistically high ascorbate excess (100 fold) if results were to be related to the situation encountered within the body. For this reason, and the potential of ascorbate to form relatively long-lived Cr(V) species, the reactions of Cr(VI) with HA^- under physiologically relevant conditions were carried out here, the relative reactant ratios being much closer to stoichiometry.

Reactions were carried out in both buffered (HEPES or TRIS.HCl)(fig.3.1) and unbuffered aqueous solution, the formation of transient Cr(V) or radical species being followed by epr spectroscopy [30].

The aqueous chemistry of the ascorbate ions is complex resulting in few isolations of analytically pure metal-ascorbate complexes [66]. Spectroscopy is the primary method of complex characterization, studies having been made with a wide selection of metals including Cr, Mo, V, Os, W and Ti [67]. Complexes of the type $M(HA)_n \cdot xH_2O$ have been characterized [(M=Cr, n=3, x=6) and (M=Mn, Co, Ni, Zn, n=2, x=4)]. It was suggested that in these complexes ascorbate is acting as a bidentate ligand [68,69]. Mixed ligand complexes with ascorbate and amino acids have also been formed with Co [70].

The first transition metal complexes to yield complete structural characterization are a series of ascorbate complexes with the cis-diamine Pt(II) unit. These proved to be unique because they contain Pt bound directly to C(2) and the deprotonated hydroxyl group on C(5) of ascorbate. They are of interest due to their potential use as second generation Pt anti-

cancer drugs, in-vivo studies having shown promise in this area [71].

NMR studies have shown that the reaction of HA^- with $[\text{Pt(II)(en)(H}_2\text{O)}_2]^{2+}$ in aqueous solution gives three species in equilibrium ; (i) $[\text{Pt(II)(en)(H}_2\text{O)(HA)}]^+$ (ii) $[\text{Pt(II)(en)(HA)}_2]$ and (iii) $[\text{Pt(II)(en)(A)}]$. In (i) and (ii) HA^- coordinates via O(3) and in (iii) ascorbate is acting as a bidentate ligand coordinating via O(2) and O(3) [72].

Ascorbate complexes with Sn, Ti and Fe have also shown promise as anti-cancer agents. Treating $\text{Ti(Cp)}_2\text{Cl}_2$ or R_2SnCl_2 (R=Et, Me) with sodium ascorbate gives $(\text{Cp})_2\text{Ti(HA)}_2$ and $\text{R}_2\text{Sn(A)}$, the ligand coordinating as the monoanion to Ti but as the dianion to Sn [73].

Similarly, it was found that an Fe(II)-ascorbate complex exhibited a greater cytotoxicity towards human carcinoma than ascorbic acid alone, a simple mixture of Fe(II) and H_2A being less effective. The prolonged half-life of chelated ascorbate within the cell was given as a possible explanation [74].

Metal complexes of ascorbic acid have found other uses apart from anti-cancer agents. A recent review outlines the uses of Fe(II)-ascorbate complexes as deodourizers, preservatives and micro-organism inhibitors in food packaging [75]. Zinc ascorbate has been used in skin lightening cosmetics [76] and many transition metal ascorbates are useful as catalysts [77].

3.2. Experimental

Reactions in unbuffered, aqueous solution were carried out as described previously (Chapter 2). For reactions under pH control,

the required amount of sodium ascorbate was dissolved in aqueous buffer solutions prepared with TRIS.HCl (tris(hydroxymethyl)aminomethane.HCl) (1M) or HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) (0.1M). When using TRIS.HCl the $K_2Cr_2O_7$ was initially dissolved in 0.5M KOH aq. (2ml) to neutralize the acid.

The reactions of Cr(VI) with THME (tris(hydroxymethyl)ethane) were done in unbuffered aqueous (H_2O and D_2O) solution over the pH range 7.0 to 8.5

The X-band epr spectra of all reaction solutions were recorded immediately after mixing in mobile or frozen solution, computer double integration being performed on the latter.

3.3. Results

Studies were made varying the Cr(VI):ascorbate mole ratios within the range 2:1 to 1:5 at various pH values between 7.0 and 8.5 and employing both unbuffered and buffered (TRIS.HCl or HEPES) solutions. Under all conditions used, mixing the reagents resulted in an immediate solution colour change from yellow to green.

Depending on solution pH, relative mole ratio of reactants and the buffer system used (if any), the Cr(VI)-ascorbate reaction gave four types of mobile solution, X-band epr spectra labelled A, B, C and D (figs. 3.2 and 3.3). A summary of the results obtained is given in table 3.1.

Unbuffered reaction solutions containing equimolar amounts of Cr(VI) and ascorbate at pH 7.0 gave spectra of type A, comprising a typically sharp (peak-to-peak derivative width 1.5G) epr signal at $g=1.979$ characteristic of Cr(V), with the four ^{53}Cr hyperfine bands clearly visible at 17.7G spacing.

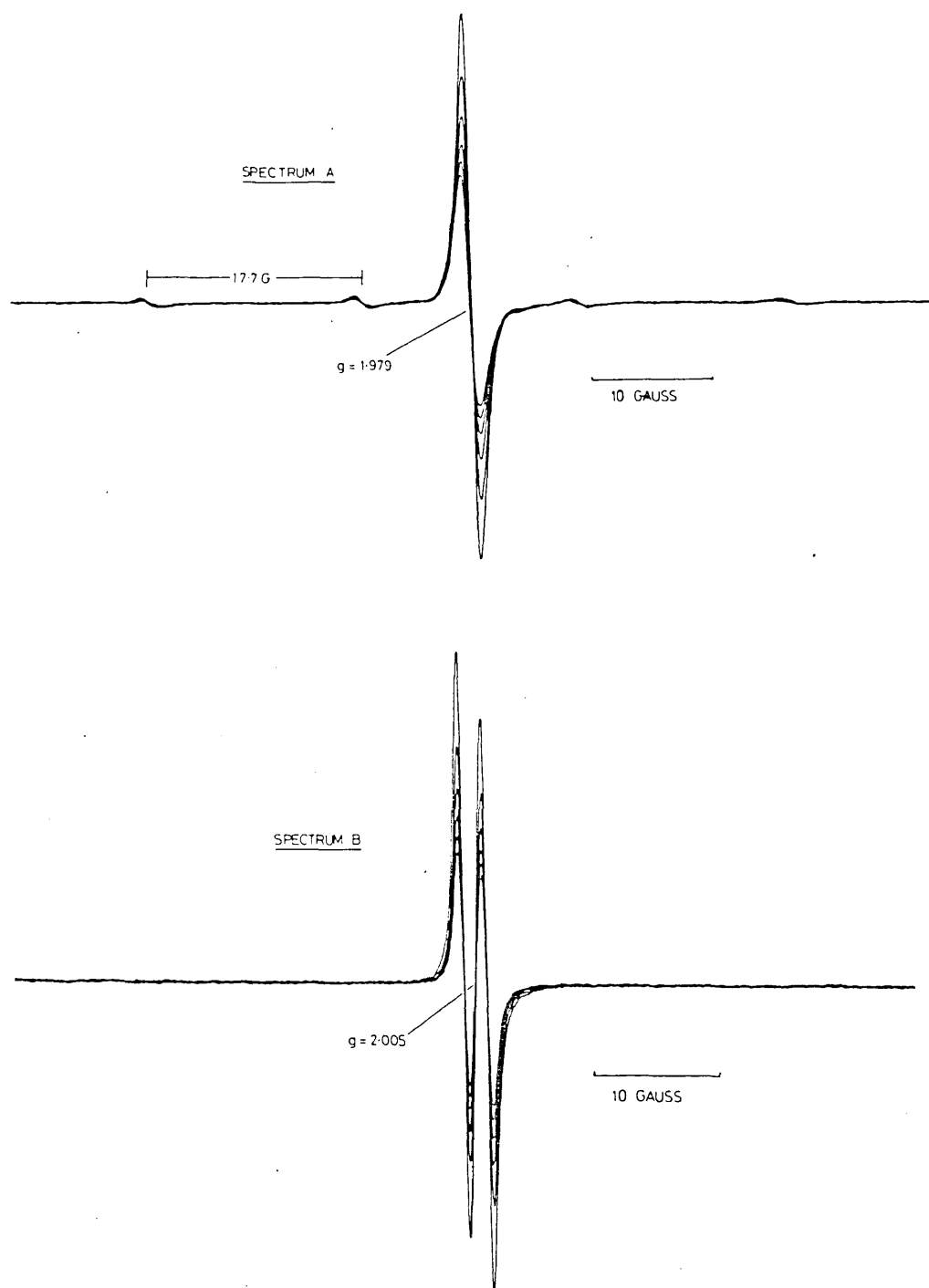


Fig. 3.2. X-band epr spectra of an aqueous solution of Cr(VI) + ascorbate at (A) 2:1 ratio pH 7.0 (B) 1:1 ratio pH 8.0 spectra taken at 253sec. scan intervals.

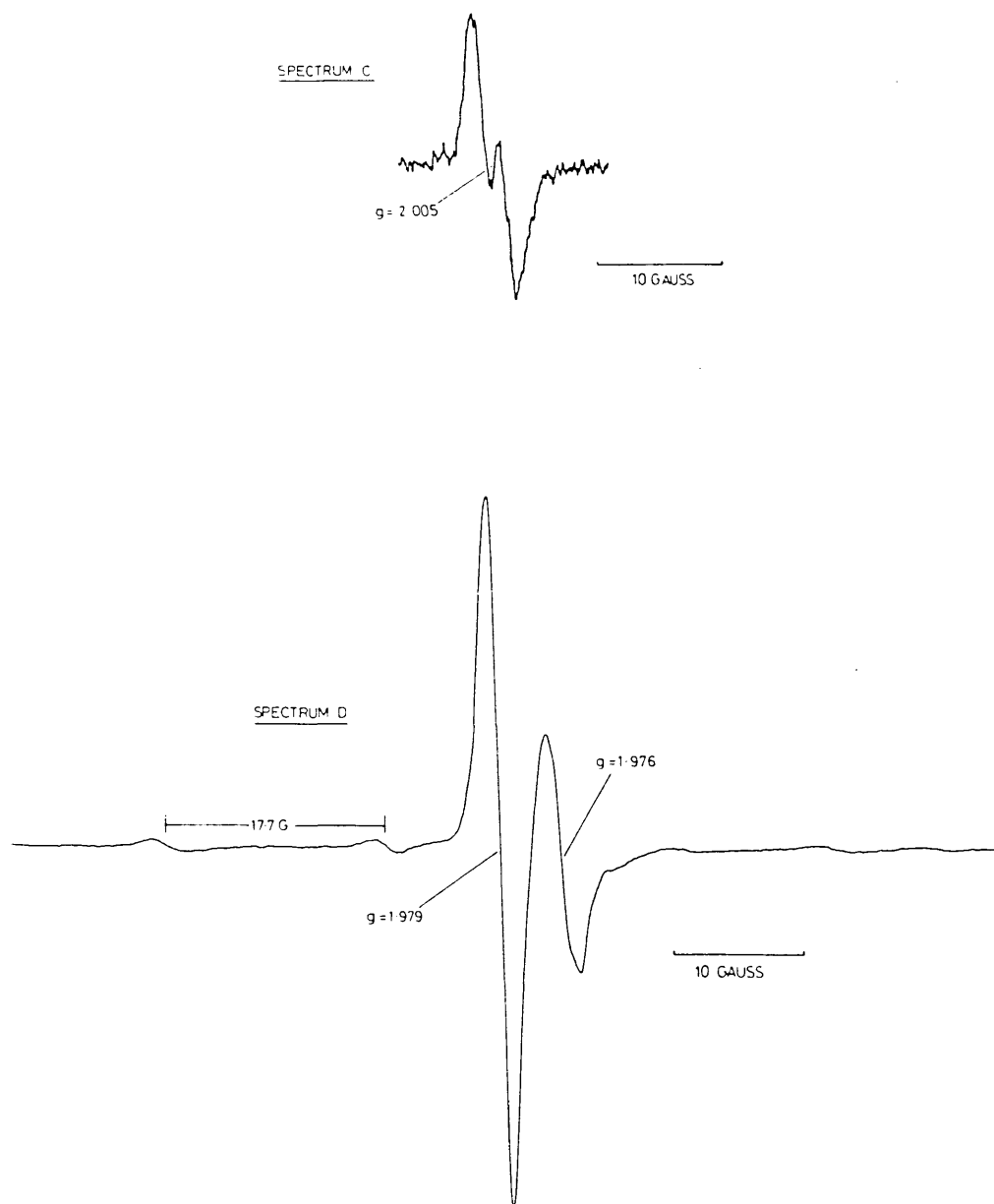


Fig. 3.3. X-band epr spectra of aqueous solutions of Cr(VI) + ascorbate at (C) 1:5 ratio pH 7.0 (D) 2:1 ratio pH 7.0 TRIS.HCl buffered (1M).

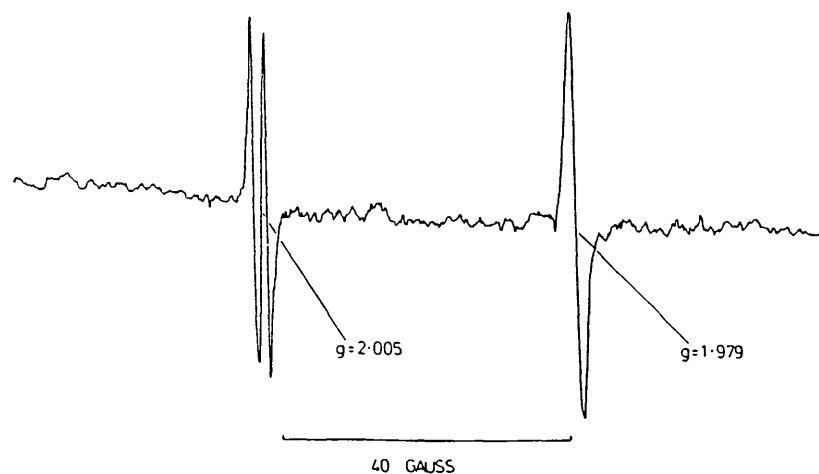


Fig. 3.4. X-band epr spectrum of an aqueous solution (pH 8.5, HEPES buffered) of Cr(VI) + ascorbate (1:1 mole ratio).

The intensity of these Cr(V) signals decreased with time with an approximate half-life ($T_{1/2}$) of 15 minutes.

On raising the solution pH to 7.5 (still unbuffered), the Cr(V) signal was replaced by a sharp doublet centred at $g=2.005$. This doublet signal corresponds to the ascorbate radical species [78,79] and exhibits a hyperfine splitting (due to ring proton on C(4)) of 1.7G. The signal intensity decreased with time eg. $T_{1/2} =$

15min. (pH 8.0, unbuffered. Spec. B).

The Cr(V) and ascorbate radical signals were present simultaneously only over a very narrow pH range just below 7.5.

Variation of the Cr(VI):ascorbate mole ratio at pH 7.0 gave a Cr(V) signal (Spec.A.) when Cr(VI) was in excess, but not for Cr(VI):ascorbate ratios of 1:2 or 1:5. In both of these cases the spectrum obtained was of type C, comprising the ascorbate radical signal centred at $g=2.005$ (as spec.B) but with an associated line broadening resulting in apparent signal cancellation in the central region.

The spectral observations for reactions with pH control were dependant upon the buffer system used. The use of HEPES gave very similar results to those obtained in unbuffer solution, except that the Cr(V) and ascorbate radical species co-existed over a wider pH range. Thus, when HEPES was used with Cr(VI):ascorbate in 1:1 mole ratio, Cr(V) and radical signals were both seen in the pH range 8.0 to 8.5 (fig.3.4) but only Cr(V) bands were observed in the range 7.0 to 8.0.

On carrying out the above reactions in 1M TRIS.HCl buffer in place of HEPES, quite different spectra were obtained. Under conditions where, with unbuffered solutions and with HEPES buffer, type A spectra were obtained, the use of TRIS.HCl gave an extra Cr(V) signal at $g=1.976$ in addition to the $g=1.979$ band (Spec D. fig.3.3). With a 1:1 Cr(VI):ascorbate ratio at pH 8.0 in TRIS.HCl an ascorbate radical spectrum of type C was observed. This contrasts with the situation in HEPES or unbuffered where the spectra obtained were A+B and B respectively.

[Cr(VI)]: [ascorbate]	Buffer ^a	pH	Spectral ^b type
1:1	H	7.0	A
1:1	H	7.5	A
1:1	H	8.0	A+B
1:1	H	8.5	A+B
2:1	H	7.0	A
1:2	H	7.0	C
1:5	H	7.0	C
1:1	N	7.0	A
1:1	N	7.5	B
1:1	N	8.0	B
2:1	N	7.0	A
1:2	N	7.0	C
1:5	N	7.0	C
1:1	T	7.0	D
1:1	T	7.5	D
1:1	T	8.0	C
2:1	T	7.0	D
1:2	T	7.0	C

a H=HEPES, N=no buffer, T=TRIS.HCl

b see figs. 3.2 - 3.4. for spectral types

Table 3.1. Summary of types of mobile solution epr spectra observed in the Cr(VI)-ascorbate reaction.

Control reactions of Cr(VI) with HEPES (0.1M) and TRIS.HCl (1.0M) in the absence of ascorbate yielded no Cr(V) signals over the pH range covered in these studies.

The frozen solution (77K) spectrum of Cr(VI)+ascorbate (1:1, pH 7.0, HEPES buffer) showed the presence of a broad Cr(III) band centred at $g=1.98$ with a small axial component at $g=4.7$. In the centre of the main Cr(III) band was a sharp, apparently isotropic Cr(V) signal at $g=1.979$. Double integration of the Cr(V) band alone showed 0.025% of the initial Cr(VI) concentration was Cr(V), the bulk of Cr being present as Cr(III) (fig.3.5.).

Reactions of Cr(VI) with a five-fold excess of THME in unbuffered aqueous solution were done over the pH range 7.0 to

8.5. No Cr(V) species were seen in the epr spectrum immediately after mixing and monitoring the reaction for seven days showed no Cr(V) or Cr(III) formation.

Repeating the reaction of Cr(VI) + ascorbate (2:1, pH 7.0) in an unbuffered aqueous solution of THME (1.0M) gave two Cr(V) signals at $g=1.979$ and 1.976 (signals (a) and (b) fig.3.6). Signal (a) exhibited no structure of the central band, in contrast to signal (b) which possessed super nuclear hyperfine structure with a relative band ratio of 1:2:1. This structure was also visible in the associated ^{53}Cr hyperfine bands ($a_{\text{Cr}}=19.4\text{G}$), the hyperfine bands of signal (a) being obscured by other signals.

Bands (a) and (b) decayed in size with time and in the case of (b), the super hyperfine structure changed from the 1:2:1 ratio observed initially to a more complex set of bands (Spec (c), fig.3.7). Re-recording the spectrum after six hours gave very small Cr(V) signals and these disappeared after 20 hours.

On repeating the above reaction in D_2O , signal (b) still possessed its super hyperfine structure.

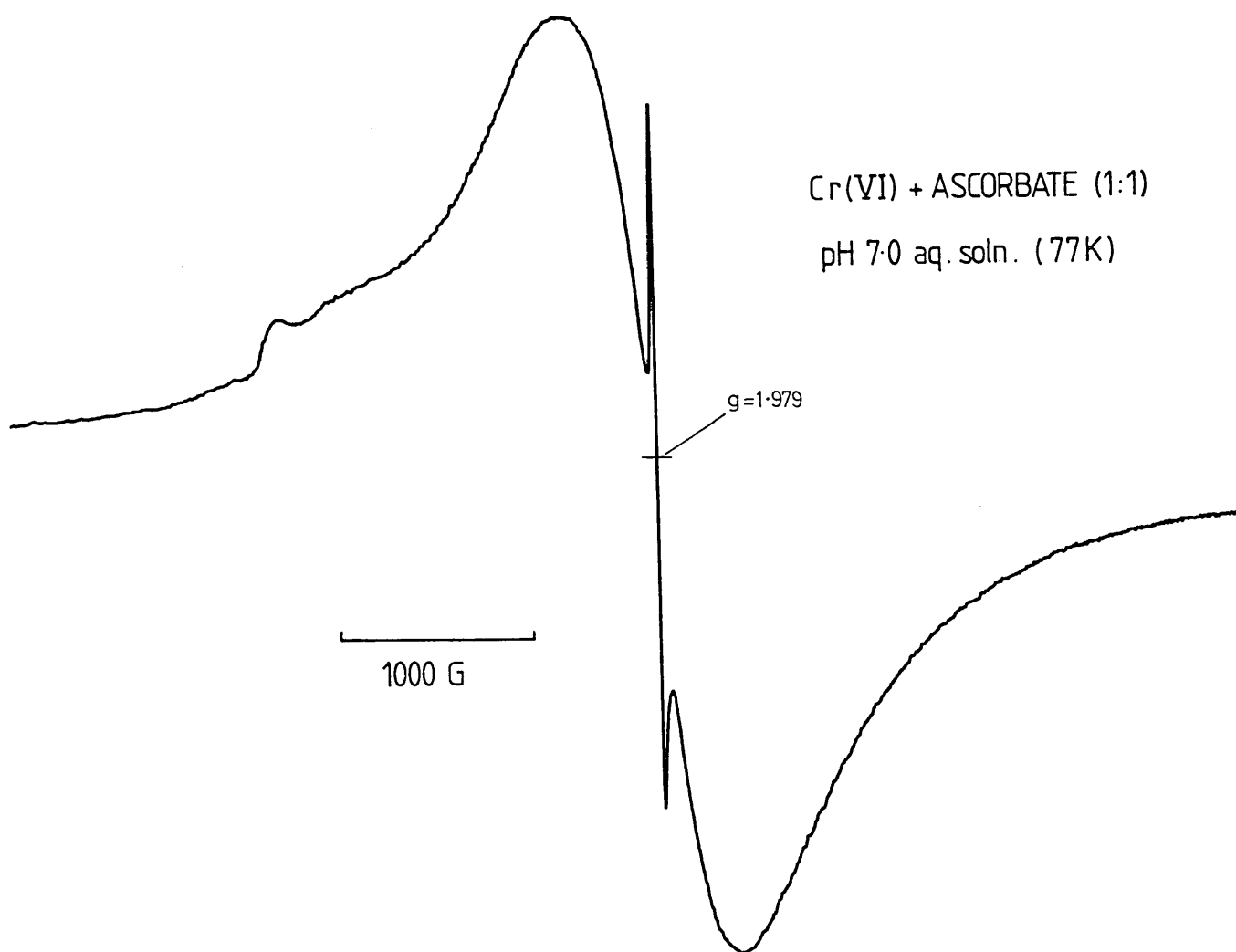


Fig. 3.5. X-band epr frozen aqueous solution (77K) spectrum of Cr(VI) + ascorbate (1:1 ratio, pH 7.0)

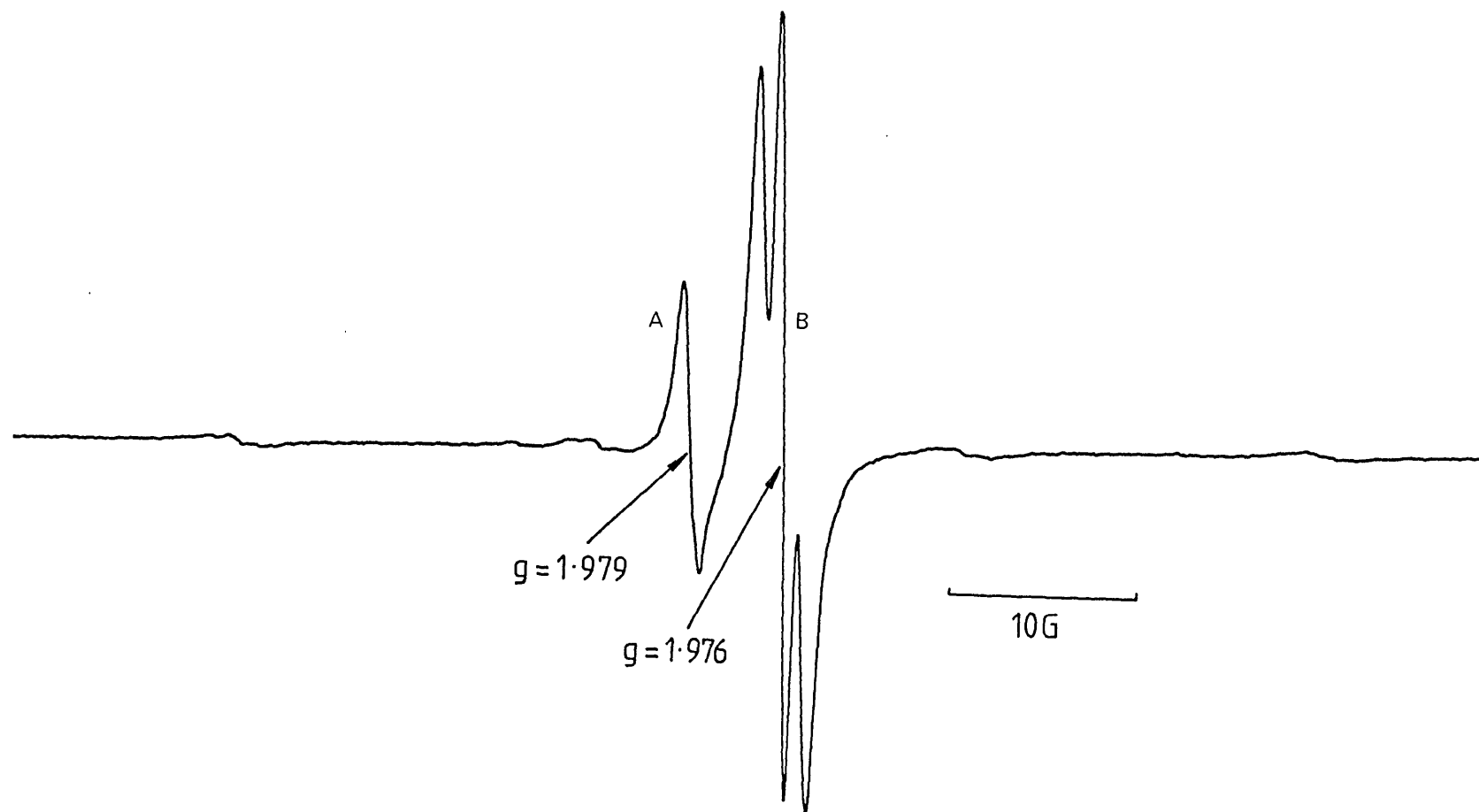


Fig. 3.6. X-band epr spectrum of an aqueous solution of Cr(VI) + ascorbate (2:1 ratio, pH 7.0) in THME (1M) Spectrum taken immediately after mixing.

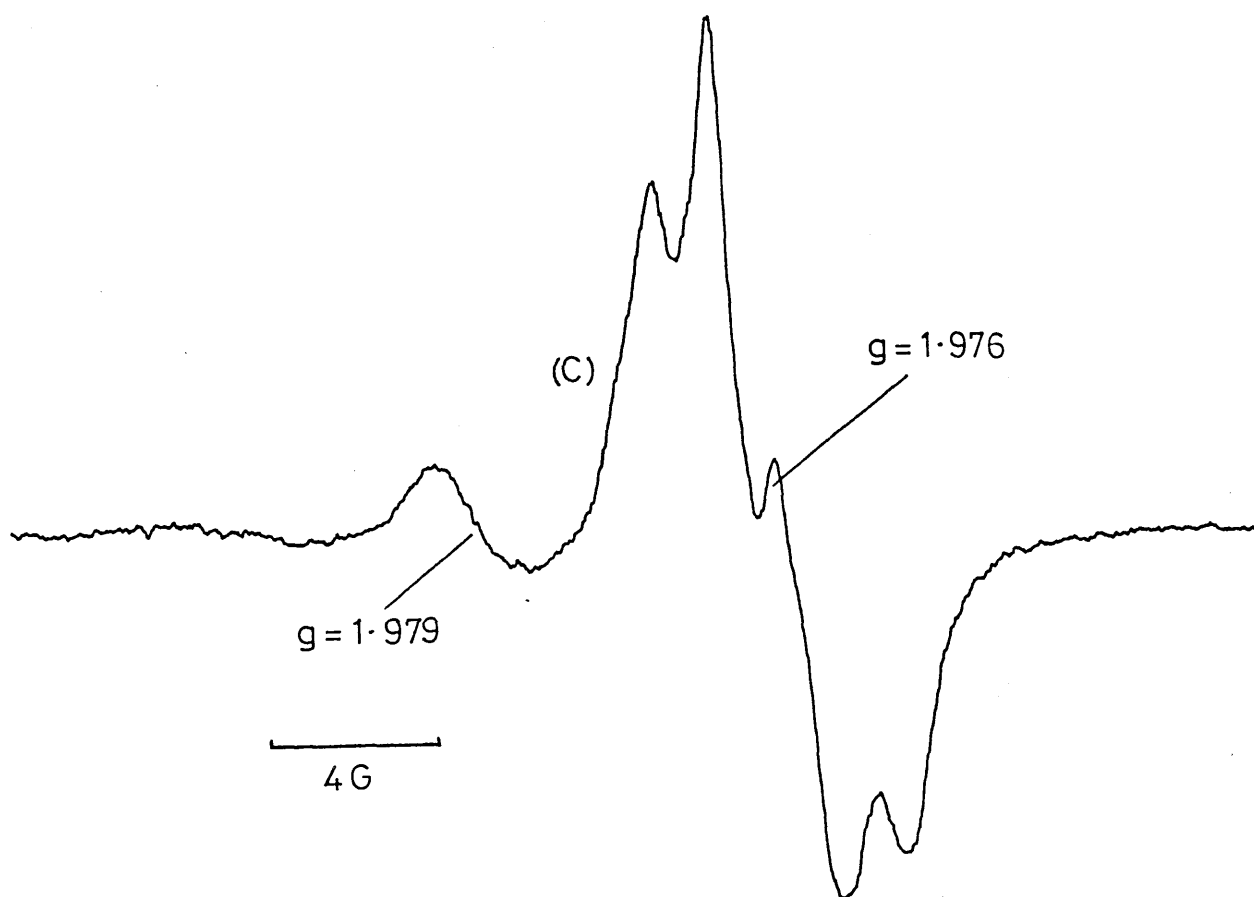


Fig. 3.7. X-band epr spectrum of an aqueous solution of Cr(VI) + ascorbate (2:1 ratio, pH 7.0) in THME (1M) Spectrum taken after two hours.

3.4. Discussion

The Cr(V) signal (Spec A) observed on reaction of Cr(VI) with ascorbate is very similar in position ($g=1.979$) and ^{53}Cr nuclear hyperfine spacing ($a_{\text{Cr}}=17.7\text{G}$) to that obtained previously by Hayman on reaction of Cr(VI) with nucleotides [28]. Both parameters are consistent with the Cr(V) centre in a square pyramidal, oxygen donor environment. The value of a_{Cr} is in contrast to that produced on reaction of Cr(VI) with GSH and NAC ($a_{\text{Cr}}=8.5\text{G}$, chapter 2.) in which coordinated sulphur atoms are probably involved.

The lack of nuclear super hyperfine structure of the Cr(V) band suggests that there are no protons adjacent to the donating O groups. Hence, coordination of ascorbate probably takes place via the deprotonated hydroxyl groups on C(2) or C(3) (or both) rather than the side-chain hydroxyls on C(5) and C(6), proton hyperfine structure being expected in the latter. Little useful information was forthcoming from the frozen solution spectrum of the Cr(V) signal except that the percentage of Cr(V) present was very low (0.025% total Cr).

From the knowledge that the most favourable mode of coordination around Cr(V) is an oxygen environment with five-membered chelate rings and a central Cr=O bond, it seems most likely that the mode of attachment of ascorbate to Cr(V) is as a bidentate ligand via the deprotonated -OH groups on C(2) and C(3), with two ligand molecules per Cr(V) (fig.3.8). This is similar to the modes found elsewhere for ascorbate complexes of Mn, Co, Ni and Zn [68,69].

As with GSH, the very low amount of Cr(V) at any one time

prohibits complex isolation and the sensitivity of the epr technique is the only reason that they can be seen at all.

Raising the solution pH to 7.5 and above (unbuffered) resulted in production of the ascorbate radical anion signal at $g=2.005$ (Spec. B). The doublet nature of this signal is due to the ring proton on C(4), the hyperfine splitting of 1.7G being in agreement with the major radical species observed by Laroff et al. [79] in which the radical electron is spread over the highly conjugated tricarbonyl system of the radical anion. This is in contrast to the formation of Cr(V) species on reaction of Cr(VI) with GSH in which raising the pH merely resulted in a faster rate of Cr(V) signal decay.

On increasing the excess of ascorbate over Cr(VI) the ascorbate radical signal was again produced (Spec. C) but in this case it was of lower intensity and the signals were broader than in Spec. B. The line broadening of the ascorbate radical anion has been observed elsewhere on the rapid exchange of Ca^{2+} between the bulk and coordination sites of the ascorbate radical. The mechanism of line-broadening was explained in terms of a combination of a g and hyperfine shift upon M ion coordination [80].

In unbuffered solution, the only time in which Spec. C was observed was in the presence of an excess of ascorbate, the normal unbroadened radical signal B being seen on all other occasions. The reasons for this apparent selectivity are not known and in the case of Spec. C, the origin of the broadening is uncertain, it is probably due to interaction of the ascorbate

radical with a metal centre, but the oxidation state(s) of Cr responsible is not known.

The spectral observations for reactions under pH control were found to depend upon the buffer reagent used. HEPES appeared to be relatively inert in the reactions and for this reason is probably the best choice for future work. However this too may be problematic in some areas, a recent paper showing HEPES to interact directly with Cu(II) [81].

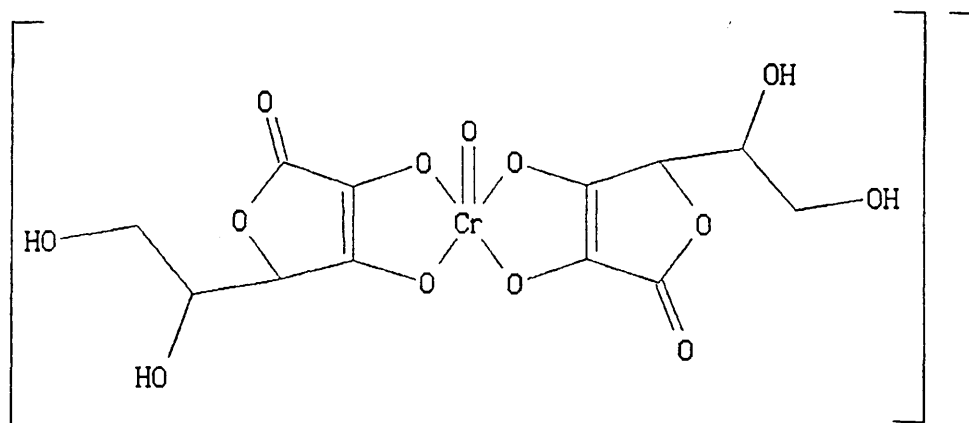


Fig. 3.8. Proposed structure of the Cr(V)-ascorbate complex.

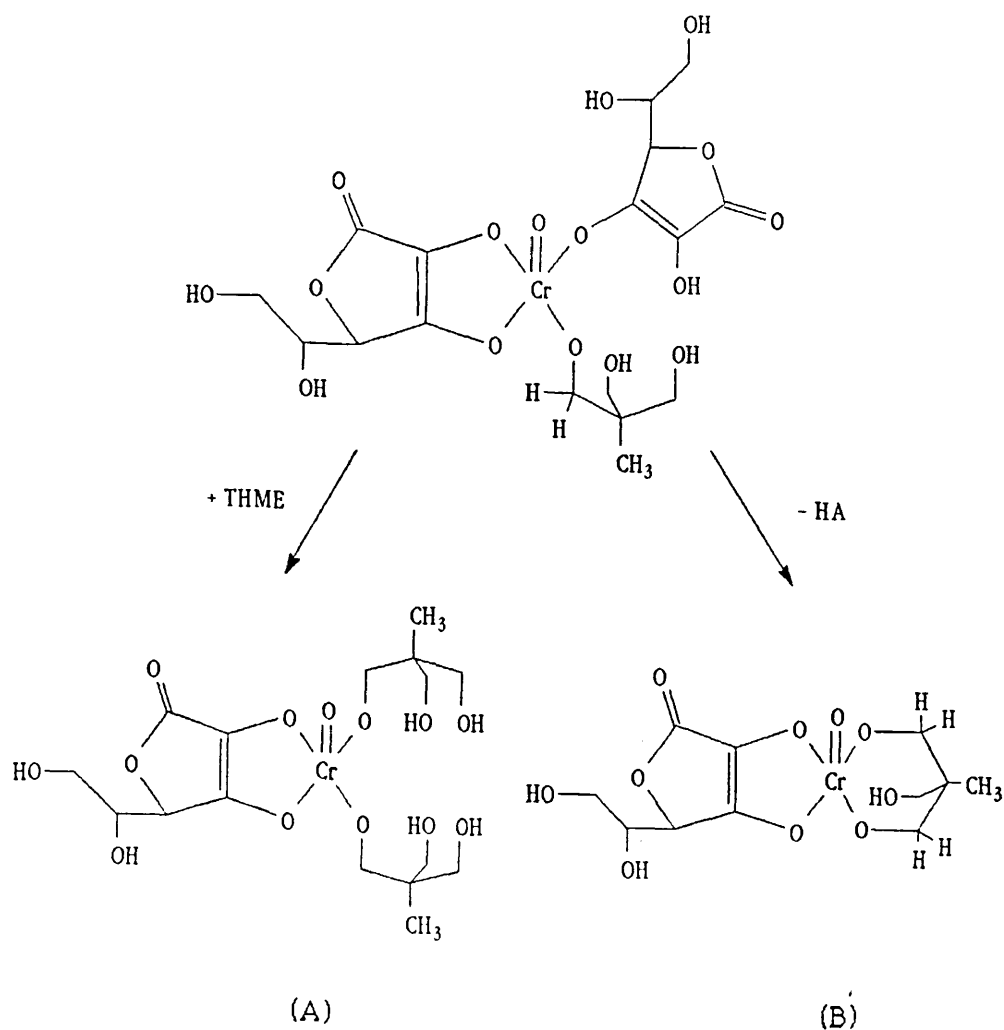


Fig. 3.9. Possible modes of coordination of THME to Cr(V).

With TRIS.HCl (1M) a new Cr(V) signal appeared at $g=1.976$ (Spec. D) under conditions where in unbuffered solution only one Cr(V) band at $g=1.979$ was observed (Spec. A). The species responsible for this extra signal is probably a Cr(V) complex involving TRIS, the hydroxy groups of this molecule being potential donors to Cr(V). Of note here is the lack of Cr(V) species production in the control reaction of Cr(VI) and TRIS.HCl

alone. This suggests that TRIS cannot react directly with Cr(VI) and must go via either the Cr(V)-ascorbate or HACr(VI) ester complexes.

To gain an insight into the mode of coordination of TRIS to Cr(V), the reactions of Cr(VI) with THME were carried out in the presence and absence of ascorbate under similar conditions to TRIS, THME being a model for the hydroxy portion of the TRIS molecule.

As with TRIS, THME did not react with Cr(VI) alone but the reaction of Cr(VI) with ascorbate in 1M THME (pH 7.0, 2:1 reactant ratio) produced two bands at $g=1.979$ and 1.976 as seen with TRIS. However, in this case the $g=1.976$ band possessed proton super hyperfine structure in a 1:2:1 ratio ($a_H=1G$), this being absent with TRIS. The central Cr(V) signal appears to be split by two equivalent protons, the most probable source of which is via coordination of one of the deprotonated THME hydroxyl groups in place of one of the ascorbate donating groups, the adjacent CH_2 unit causing the splitting (fig.3.9). As can be seen from fig.3.9, the ascorbate hydroxyl group on C(2) detaches to allow THME coordination. This is the probable mode since this -OH group has a $pK_a = 11.7$ compared to 4.17 for the -OH on C(3) [82]. Running the reaction in D_2O did not remove the structure showing the responsible protons to be non-exchangeable.

With time, the super hyperfine splitting changed its structure (fig.3.7) suggesting that at least one more deprotonated OH group of THME may be coordinating. Whether this additional coordination is due to the formation of a THME chelate or the attachment of another THME molecule is not known, but there would

appear to be an equilibrium being established between several Cr(V) species in solution, not unlike the situation reported for the reaction of cis-Pt(II)(diamine) with HA^- in aqueous solution [72].

Since the Cr(V) signal at $g=1.976$ decays in size with time, further changes in the proton super hyperfine structure cannot be observed and it is not known if further THME coordination to Cr(V) takes place. However, the species exhibiting the $g=1.976$ signal does probably still contain ascorbate in a ternary Cr(V)/ascorbate/THME complex.

Relating these findings to those with TRIS, the position of this extra band is the same in both cases suggesting similar coordination environments. The puzzling observation in this respect however, is the lack of signal structure with TRIS, the source of this difference being unknown.

This evidence for the direct involvement of TRIS in a Cr(VI)/Cr(V) redox system is important as TRIS.HCl is a commonly used buffer system in biological studies. Indeed, the recently reported kinetic study of the Cr(VI)-ascorbate reaction [43] was carried out in 1M TRIS.HCl buffer. From the results reported here, TRIS actually interferes with the sequence of reactions and should therefore be regarded as a non-innocent buffer reagent in systems of this type.

The mechanism proposed by Connett and Wetterhahn [43] for the reaction of Cr(VI) with ascorbate at pH 7.4 (1M TRIS.HCl) involves the initial formation of a HACr(VI) ester species eventually giving Cr(IV) and dehydroascorbic acid. Similarly, a

recent theoretical study of the general oxidation mechanism of ascorbic acid invokes the formation of the ascorbate radical as the initial product in neutral or basic solutions [83].

Our results suggest that under the conditions employed here, which are close to those of physiological relevance, the mechanism of Cr(VI) reduction by ascorbate can involve both Cr(V) and ascorbate radical species. It must be remembered that ascorbate is present in cells at micro-molar concentrations and any interaction in-vivo is likely to produce Cr(V) species at least as an initial step before radical production occurs.

A much more complex situation than either of the two mechanisms described by Connett and Wetterhahn [43] or Abe [83] is therefore acting. Hence, these additional species should be taken into account in assessing the possible mechanism(s) for the Cr(VI)-ascorbate reaction and in the likely generation of Cr(V) from Cr(VI) by ascorbate in biological systems.

CHAPTER 4. PRODUCTION OF LONG-LIVED Cr(V) SPECIES ON REACTION OF Cr(VI) WITH CARBOHYDRATES AND DERIVATIVES.

4.1. Introduction

A variety of carbohydrates such as mono-, di- and poly-saccharides and inositols are present in the aqueous biological fluids within the body. The poly-hydroxy nature of these compounds provides a very attractive coordination environment for formation of relatively long-lived Cr(V) species.

Recent work by Hayman et al. [55] showed that interaction of Cr(VI) with nucleotides in aqueous solution near neutral pH produced Cr(V) species, as observed by epr spectroscopy. Similar reactions with the equivalent 2-deoxynucleotides gave rise to no such species, hence it was suggested that the presence of the cis-diol unit of the sugar group was necessary to facilitate complex formation via diol chelation.

This work by Hayman prompted a more thorough investigation of the interaction of Cr(VI) with carbohydrates under physiologically relevant conditions, with the aim of showing if stabilization of Cr(V) could occur within the cellular environment, and thereby provide a means of transporting potentially damaging forms of Cr to the critical areas within the cell.

Reported here are the results obtained on reaction of Cr(VI) with a wide range of sugars, inositols and other poly-hydroxy molecules (fig.4.1) in aqueous solution. Some reactions to extend those carried out by Hayman are also described.

The formation of Cr(V) complexes was followed by epr

spectroscopy and comparisons made between the spectra observed in each case, the aim being elucidation of the mode and position of binding of the metal to the carbohydrate, and determination of selection rules for Cr(V) complex formation with these ligands.

In organisms, polysaccharides have two major functions; as storage forms of energy-yielding fuels eg. starch and glycogen and as extracellular structural elements such as cellulose. Starch and glycogen are made up of repeating units of D-glucose joined via their anomeric centres and D-glucose itself acts as a precursor of other sugars such as D-fructose, D-mannose and sucrose.

D-glucose and D-fructose are the most abundant monosaccharides in nature, D-ribose and 2-deoxyribose being the sugar components of RNA and DNA respectively. The most abundant disaccharides are sucrose, lactose and maltose.

Complexes between sugars and metal cations are known and there is evidence to show that in aqueous solution these complexes are formed reversibly. The interest in such complexes stems from the fact that these poly-hydroxy compounds and metal ions co-exist in body fluids where complex formation could take place [84].

The metals of interest in this area are usually from the alkaline and alkaline-earth metal groups. Complexes have been observed with Na^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} and Ba^{2+} and in these complexes coordination takes place via three protonated hydroxyl groups [84]. A great deal of work has been carried out to determine the most suitable steric arrangement of the hydroxy groups to facilitate cation coordination [85,86]. From this it

was found that sugars containing an axial-equatorial-axial sequence of hydroxy groups on a six-membered ring, in its chair form, readily form complexes with metal ions in aqueous solution. Similarly, a cis-cis sequence in a five-membered ring preferentially forms metal complexes.

A variety of physical methods have been used to study complex formation between sugars and metal ions; two most useful methods being electrophoresis and nmr spectroscopy.

Nmr techniques have shown that complexation of sugars and metal ions in aqueous solution changes the tautomeric equilibrium positions of the sugar involved. For example, the tautomeric equilibrium of D-allose in aqueous solution is altered by the presence of some cations which form complexes with the α -pyranose and α -furanose but not with the β -pyranose and β -furanose forms. Hence, on addition of a complexing cation, some of the α -pyranose will be removed from the equilibrium as a metal complex and the equilibrium will shift in favour of the α -pyranose form. The proportion of the two forms can be determined by integration of the ^1H nmr spectra, the anomeric proton signals of the four tautomeric forms being well separated. The limitation of this method is that only diamagnetic cations can be used, excessive line-broadening occurring with paramagnetic centres [87].

More recent studies have been made into the complexation of Cr^{3+} with sugars, this having relevance to the possible action of the glucose tolerance factor [88]. All of the sugars studied reacted fairly rapidly with Cr(III) to produce optically active complexes and it was suggested that a bidentate mode of

coordination was acting, rather than the fac-tridentate mode encountered with the other metal cations. These reactions were carried out in non-aqueous solution, no stable Cr(III) - sugar complexes being found in aqueous solution.

Recent interest in magnetic resonance imaging has produced a need for certain metal ion chelates to act as contrasting agents for image enhancement. In this context it was reported that Cr³⁺ complexes to cis-inositol and this may be a precursor for new contrasting agents that can be developed using inositols as starting materials [89]. It was suggested that the mode of coordination of Cr³⁺ was via the three axial hydroxyl groups, this providing a binding site for metals of ionic radii ~0.8 Å, whereas larger metal ions would chelate via the ax-eq-ax arrangement described earlier.

Inositol complexes with Mn²⁺ and Cu²⁺ are known, but these undergo very rapid exchange in solution and do not make good contrasting agents.

Inositols themselves are available biologically, myo-inositol being present in nearly all cells. In plants, inositol is a component of galactinol which may be a specific precursor of cell-wall polysaccharides.

Various phosphate esters of inositol occur in nature and, in particular, inositol triphosphate is involved in the transport of calcium and functions as a messenger in cellular signal transduction [90,91]. Similarly, myo-inositol is a membrane phospholipid. Inositol triphosphate may also be implicated in carcinogenesis [92].

Of medical significance amongst the sugar-metal complexes are

a group of osmium-carbohydrate polymers called osmarins, these possess anti-inflammatory properties especially in the area of reducing inflammation caused by rheumatoid arthritis [93,94].

The preparation of a 1:1 osmium-arabinose complex has been reported [95]. In this it was proposed that the sugar coordinates in a bidentate fashion via two adjacent, deprotonated hydroxyl groups.

A recent report [96] outlined an unusually high mortality in cattle grazing in a field next to a dump containing chromium compounds as waste material from a chemical incinerator. The chemical waste spillage was reported to contain very high concentrations of Cr, inferred to be Cr(VI), and relatively high Cr concentrations (eg. 127-138 ppm.) were found in the pasture soil near the dump.

As stated [96], one cannot necessarily conclude that such pollution contributed to the cattle death, and alternative assessments of the cause, and of the amounts of Cr released, particularly Cr(VI), have been advanced elsewhere [97].

However, high values of chromium (1300 ppb, stated normal < 20 ppb) were also reported in the milk of cows grazing on the field in question. Soil ingestion during grazing has been advanced as a possible explanation.

Although no information is available as to the oxidation state(s) of the Cr in the milk samples cited above, work is reported here studying the interaction of Cr(VI) with fresh pasteurized milk and some of its components, relatively stable Cr(V) species being observed by epr spectroscopy.

This represents a continuation of work carried out in this laboratory investigating the potential environmental hazards of Cr(VI) and the intermediate Cr(V) species [98,99].

In diamagnetic liquids, proton resonance lines are extremely sharp and protons in different chemical environments show small shifts in their resonance positions owing to the magnetic shielding effects of the surrounding groups.

However, in paramagnetic materials the magnetic effects of the unpaired electrons often obscure the fine structure of the resonance lines, and it has been shown that resonances of nuclei in atoms or molecules which are bound to paramagnetic ions or radicals, can show large shifts, arising partly from the delocalisation of the unpaired spin over the bound groups.

Shifts in proton resonances of up to ± 200 ppm have previously been observed with Co^{2+} complexes and some Ni^{2+} -amino acid complexes.

The observation of these shifts prompted exploratory work to be carried out here to evaluate the use of the nmr method as a tool for studying Cr(V) complexes in solution.

Initially, studies were made with the well characterized sodium bis(2-ethyl-2-hydroxybutyrate)oxochromate(V) $[\text{Cr(V)EHBA}_2]$, and, if this were successful, it would be extended to Cr(VI)-reductant systems producing long lived Cr(V) species.

4.2. Experimental

4.2.1 Reactions in solution

All reactions of Cr(VI) and carbohydrates (fig.4.1) in mobile aqueous solution were carried out as described previously

(chapter 2). A range of Cr(VI):carbohydrate mole ratios were employed and a ratio of 1:5 was found to be best for Cr(V) signal observation in most cases. Care was necessary in the reaction of Cr(VI) with α -D-methylglucopyranoside to avoid acid hydrolysis of the glycoside bond. To this end, the reactant solution pHs were adjusted to 7.0 or above before mixing.

No buffer systems were employed in any of the reactions, as the solution pH was found to remain nearly constant for long periods of time.

The X-band epr spectra were recorded immediately after mixing and pH adjustment to 7.0, further spectra being recorded at timed intervals thereafter. In most cases, the spectra were re-recorded several weeks and months after mixing.

All reactions were repeated in D_2O to investigate the origin of the observed superhyperfine structures.

Optical rotation studies were made on the reaction of Cr(VI) with D-glucose, the solutions being prepared as above, any changes in specific rotation of the sugar with time being monitored.

In the reactions of Cr(VI) with milk, a typical measurement would comprise $K_2Cr_2O_7$ (10mg in 0.5 ml H_2O) added to fresh pasteurized milk (2ml) the epr spectrum being recorded immediately and at timed intervals.

A time-course study on the degree of Cr(VI) reduction in the milk sample was made by daily measurement of the amount of Cr(III)+Cr(V) in solution. This was achieved by freezing the solution (77K) followed by double integration of the frozen solution epr spectrum, the concentration of Cr(III)+Cr(V) could

then be calculated as described previously (chapter 2). The samples were stored at room temperature in sealed epr tubes between spectrum measurement.

The ^1H nmr study of the stable Cr(V) complex was made with bis(hydroxybutyrate)oxochromate(V) $(\text{Cr(V)}(\text{EHBA})_2)$, the preparation of which is described elsewhere [33]. In these studies, solutions were prepared in D_2O to a concentration of 0.1M, and the ^1H nmr spectra were recorded at room temperature (250MHz, -100 to +100ppm and 0 to +200ppm). Where applicable, the reference employed was trimethylsilyl-1-propanesulphonic acid (TMSPS).

The ^{13}C nmr spectrum of Cr(V)EHBA_2 (0.1M) in D_2O was also investigated.

4.2.2 Preparation of cis-1,3-cyclohexanediol

To a solution of cis-1,3-(dibenzoate)cyclohexane (0.5g, 1.57×10^{-3} mol) in dry MeOH (10ml) was added 2 drops of a saturated solution of freshly prepared NaOMe in MeOH. The resultant solution was refluxed under N_2 for 2 hours after which the reaction was complete, as followed by tlc (silica gel plates, toluene eluent).

The solvent was removed from the reaction mixture under reduced pressure to yield a white solid. To this solid was added 40-60 petroleum ether (5 x 10ml) to remove methylbenzoate. The solid was then left for 12 hours at room temp. under 40-60 pet. ether. Agitation of this mixture dispersed a clean, white solid which was collected, washed with 40-60 pet. ether (2 x 10ml) and recrystallized from dry acetone to give a pure white product.

mpt of solid = 83-85°C (lit 85°C [100])

Elemental analysis;	Expected	%C = 62.02	%H = 10.43
	Found	%C = 61.50	%H = 10.20

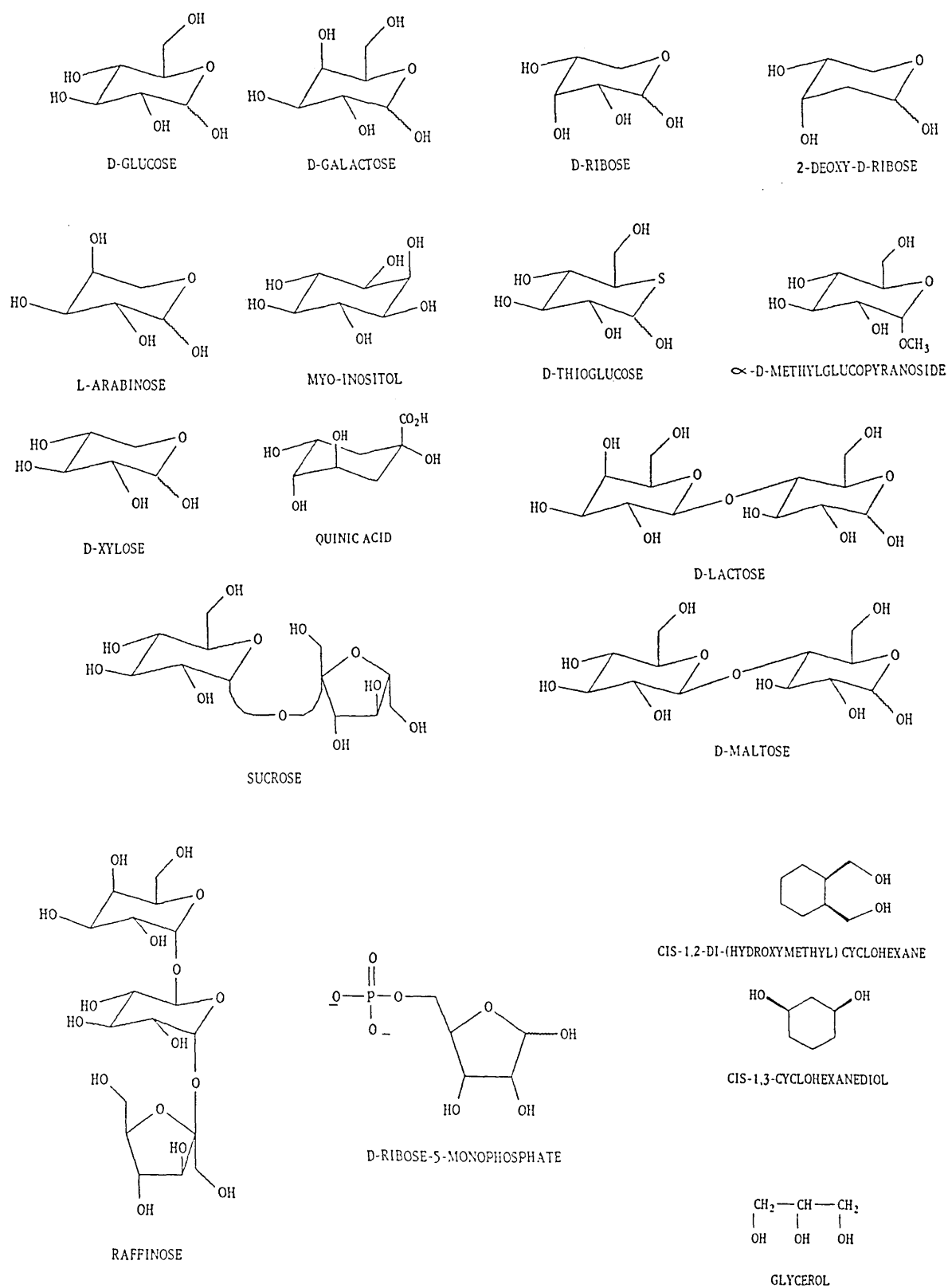


Fig.4.1 Structure of the carbohydrates and derivatives employed in these studies.

4.3. Results

The reagents employed in these studies can be grouped by the structure of the carbohydrate and the nature of the Cr(V) epr signal observed.

4.3.1. Nucleotides, D-ribose and derivatives

The reactions of Cr(VI) with 5'-AMP, 5'-GMP and 5'-CMP (1:5 mole ratio, pH 7.0) in aqueous solution all exhibited the same mobile solution epr spectrum (fig.4.2, Spec A). The signal observed was a characteristically sharp Cr(V) band centred at $g=1.979$. This band exhibited proton super hyperfine structure ($a_{Cr}=1G$) without a recognisable relative peak height ratio. ^{53}Cr hyperfine bands were also present ($a_{Cr}=18 G$).

Initially the Cr(V) signal intensity was small but within a few hours had grown considerably up to a maximum after approx. 24 hours. After this time, the signal size remained constant for several weeks.

The reaction of Cr(VI) with adenosine under exactly the same conditions as the nucleotides also produced Spec. A., its behaviour mimicing that described above. The H_2O insolubility of guanosine prevented its reaction with Cr(VI) in aqueous solution.

The interaction of Cr(VI) with D-ribose-5-monophosphate (DRM) (1:5, pH 7.0) gave rise to a Cr(V) signal as shown (fig.4.2 Spec. B). The general structure of this band was very similar to that with the nucleotides except for the absence of one super hyperfine band (marked *). The ^{53}Cr hyperfine bands were seen ($a_{Cr}=17.7 G$) and the signal intensity increased up to a maximum which remained stable for several weeks.

D-ribose produced a Cr(V) epr signal as above on reaction with Cr(VI) but the super hyperfine structure was poorly resolved. Similarly, reaction with 2-deoxy-D-ribose also produced a Cr(V) signal but in this case no structure was visible. Both of these signals exhibited ^{53}Cr hyperfine lines ($a_{\text{Cr}}=17.7 \text{ G}$).

Recording the epr spectra of the reaction in D_2O did not produce any changes in the observed super hyperfine structure.

4.3.2. D-glucose, D-galactose, D-lactose, D-maltose, raffinose and myo-inositol.

The reactions of Cr(VI) with the above reagents in aqueous solution all exhibited very similar spectral characteristics. Immediately after reagent mixing and pH alteration to 7.0, a small Cr(V) signal with apparently simple superhyperfine structure was observed ($a_{\text{H}}=1 \text{ G}$). The signal intensity was increasing with time and, in all cases except raffinose, the structure of the Cr(V) signal was also changing.

This change is shown sequentially for the reaction of Cr(VI) with D-glucose (1:5 mole ratio, pH 7.0) (fig.4.3). As fig.4.3 shows, an initially simple 1:2:1 type structure soon changed its relative peak intensities and, after 48 hours, the structure became much more poorly resolved, this being the final form of the Cr(V) signal.

The final structure of the Cr(V) band for each of the sugars in this section is given in fig.4.4 .

In all cases, the Cr(V) signals grew in intensity for approx. 24 hours after which they appeared to be present at a constant intensity. The superhyperfine structure of the Cr(V) band was observed to change for up to six hours, then remained unchanged

for the lifetime of the Cr(V) species. The Cr(V) epr signals were observable three months after solution mixing at an almost unchanged intensity.

As before, all of these Cr(V) signals were centred at $g=1.979$ and exhibited ^{53}Cr hyperfine bands with 18 G spacing.

Repeating the reaction of Cr(VI) with the above sugars (and also those in section 4.3.3) but with the sugar pre-dissolved in water several hours before reactant mixing produced exactly the same Cr(V) epr signals as when freshly dissolved, the superhyperfine structure initially being of a simple form.

Optical rotation studies of aqueous solutions of Cr(VI) with D-glucose showed there to be no change in the rate of attainment of configurational equilibrium of the sugar. Similarly the specific rotation eventually obtained was unchanged from that of the sugar alone.

4.3.3. D-xylose and L-arabinose

Reaction of Cr(VI) with the above sugars (1:5 mole ratio, pH 7.0) produced a small, growing Cr(V) epr signal immediately after mixing. These bands were centred at $g=1.979$ but, in contrast to those in the previous sections, the superhyperfine structure was very poorly resolved, shoulders being present where definite peaks were seen with, for example, D-glucose. ^{53}Cr hyperfine bands were observable with 18 G spacing (fig.4.5).

The growth of the signal continued for 24 hours up to a constant intensity, during which time the structure of the Cr(V)-L-arabinose epr signal had changed very little. However, with D-xylose, the observed structure became quite complex although the

bands were still poorly resolved.

D-ribose could also be included in this section since its molecular structure and type of Cr(V) epr signal are similar to L-arabinose and D-xylose.

4.3.4. Sucrose, α -D-methylglucopyranoside, α and β -cyclodextrins, cis-1,3-cyclohexanediol, cis-1,2-di(hydroxymethyl)-cyclohexane.

Reaction of Cr(VI) with the above reactants at a range of mole ratios and pHs produced no Cr(VI) reduction to Cr(V) or Cr(III) immediately after mixing or after four weeks at room temperature.

4.3.5. β -D-Thiogluucose.

The mobile solution X-band epr spectrum recorded immediately after mixing Cr(VI) with β -D-thiogluucose (Na salt, 1:1 mole ratio, pH unchanged from mixing ~4) in aqueous solution is shown in (fig.4.6). The spectrum comprises a typically sharp (peak-to-peak 2 G) Cr(V) band at $g=1.986$. The signal decreased in intensity with time ($T_{1/2}=5$ min.) and did not exhibit nuclear superhyperfine structure, although ^{53}Cr hyperfine bands ($a_{\text{Cr}}=18.2$ G) were seen.

Also present were two additional smaller bands at $g=1.984$ and $g=1.982$. These bands exhibited no structure or ^{53}Cr hyperfine bands and decayed in at a similar rate to the central signal.

Immediately after mixing there was no colour change of the reaction solution. However, after five minutes, a brown colouration appeared which became lime green after 15 minutes.

Repeating the reaction as above with a five-fold excess of β -

D-thiogluco^{se} resulted in the production of the Cr(V) signal at $g=1.986$ as seen at the 1:1 mole ratio. However, here the signal intensity diminished very rapidly ($T_{1/2} < 1$ min.) and the ^{53}Cr hyperfine bands were no longer observable. At this reactant ratio the solution colour rapidly became lime green.

4.3.6. Glycerol

The reaction of Cr(VI) with glycerol in aqueous solution (1:5 mole ratio, pH 7.0) gave rise to a slowly growing Cr(V) signal at $g=1.980$ ($a_{\text{Cr}}=18$ G) exhibiting superhyperfine structure. This structure was not changing with time and, in contrast to the final Cr(V) signals seen with most of the mono and disaccharides, it was symmetrical about the centre of the Cr(V) band (fig.4.7).

This symmetry prompted a computer simulation of the spectrum to determine the source of the splittings. The result of the simulation (fig.4.7) shows the presence of nine superhyperfine bands the relative intensities of which approximate very well to those expected from interaction with eight equivalent protons. The position of these bands is indicated by the scale in the centre of fig.4.7. Also shown are the experimental and calculated second derivative spectra of the Cr(V) band. This shows that there is only one major Cr(V) species producing the observed structure.

The Cr(V) signal was still present, at a slightly reduced intensity, three months after solution mixing, the structure being identical to that described above.

4.3.7. Quinic acid

The X-band epr spectrum of a mobile aqueous solution of Cr(VI) + quinic acid (1:5 mole ratio, pH 7.0) showed a complex spectrum immediately after mixing. A characteristically sharp Cr(V) signal was observed at $g=1.979$, its intensity increasing and superhyperfine structure changing with time. The Cr(V) signal was observable for a week but no ^{53}Cr hyperfine bands could be resolved.

The reaction of Cr(VI) + quinic acid (1:2 mole ratio) in anhydrous acetone produced a yellow precipitate after shaking at room temperature for one day. This solid was recovered and dried, and its X-band epr spectrum recorded.

This spectrum showed a very strong, isotropic Cr(V) signal centred at $g=1.979$ (peak-to-peak 34 G). A solution of the solid in H_2O gave the mobile solution epr spectrum shown in fig.4.8. consisting of two closely overlapping, sharp (peak-to-peak ~ 2 G) Cr(V) signals at $g \sim 1.979$, each with associated ^{53}Cr hyperfine bands with slightly different spacings. The intensity of all the signals decreased with time and no superhyperfine structure of either band was seen at any time.

The frozen (77K) aqueous solution spectrum of the solid at a known concentration gave an apparently isotropic Cr(V) signal at $g=1.979$. Computer double integration of this spectrum gave a Cr(V) content of approx. 1% (assuming a 1:2 Cr(V):quinic acid complex).

On storing the solid under aerobic conditions in the light for two months, the pale yellow solid gained a tan

colouration. Dissolution of the solid in H_2O produced exactly the same spectrum as that seen with the freshly prepared sample (fig.4.8).

4.3.8. Milk

The interaction of Cr(VI) with milk at room temperature gave, within a few minutes, a small, sharp Cr(V) epr signal centred at $g=1.980$. This signal grew in intensity up to a maximum after approximately one day, the signal size remaining nearly constant over a period of several weeks.

The central Cr(V) band exhibited nuclear super hyperfine structure with $\sim 1G$ spacing (fig.4.9). The four ^{53}Cr hyperfine bands could be clearly seen with 17.9 G spacing. There was also a further set of at least six bands (two of these being weak shoulders of the central band), again with $\sim 1G$ spacing, centred at $g=1.977$. These additional bands were all of similar intensity.

The time dependence of the reduction of Cr(VI) to the eventual Cr(III) form by milk was followed by computer double integration of the Cr(III)/Cr(V) signals over a timed period (fig.4.10). Under the experimental conditions employed, the total conversion of Cr(VI) to Cr(III) is quite slow, with 50% conversion after five days.

In frozen solution, the Cr(V) signal was apparently isotropic and much sharper than the broad Cr(III) band.

4.3.9. NMR studies

The position of the resonances obtained in these studies are given in table 4.1.

In all cases, a large sharp signal was observed at $\delta = 4.9$ ppm corresponding to HOD; where no reference was employed, the position of the other signals was measured against this peak.

The ^1H nmr spectrum of Cr(V)(EHBA)_2 alone (a) showed several relatively small signals. Comparison of these with those obtained from Cr(V)(EHBA)_2 with excess EHBA (f) showed the bands at $\delta = 0.76, 1.61$ and 1.78 ppm to be of uncomplexed EHBA. Closer inspection of (f) showed very small signals at $\delta = 0.98, 2.10$ and 2.40 ppm. These also compared well with those seen in (a) suggesting that they also originate from the ligand.

The spectrum of EHBA in D_2O (e) showed the documented spectrum with well defined structure. However, on addition of a small amount of Cr(V)(EHBA)_2 , the fine structure was totally removed giving two broad, featureless bands with unchanged position (f).

Similarly, with TMSPS the ^1H nmr spectrum showed sharp bands with structure (c), these also being removed on addition of Cr(V)(EHBA)_2 (d). Comparison of spectra (d) and (f) with that of $\text{Cr(V)(EHBA)}_2 + \text{TMSPS}$ (b) enabled assignment of the observed bands of (a) as originating from broadened TMSPS or uncomplexed EHBA resonances. At no time were any significant paramagnetic shifts recorded, resonance broadening being the only noticeable effect of Cr(V)(EHBA)_2 addition.

A timed series of ^1H nmr spectra for the reaction of Cr(VI) with 5'-AMP (large excess of 5'-AMP) in D_2O were recorded over 12 1 hour intervals. Hopefully the large nucleotide excess would allow observation of $\text{Cr(V)(AMP)} / 5'\text{-AMP}$ ligand exchange if this

were taking place. At no time were there any changes of the original nucleotide spectrum, the amounts of Cr(V) produced being insufficient to give a noticable effect.

The ^{13}C nmr spectrum of Cr(V)(EHBA)_2 (0.1g) in D_2O was also investigated. In this case, the paramagnetic broadening was such that no signals were observed at any time.

<u>System</u>	<u>^1H nmr resonances (ppm)</u>
(a) Cr(V)(EHBA)_2	0.76(m), 0.96(w), 1.65(w), 1.78(w), 2.25(w), 2.56(vw).
(b) Cr(V)(EHBA)_2 + TMSPS	0.00(s), 0.62(w), 0.83(m), 0.98(w), 1.56(w), 1.75(w), 2.21(w), 2.53(vw), 2.90(w). (all ns).
(c) TMSPS	0.00(s), 0.63(m,st), 1.78(m,mu), 2.90(m,st).
(d) Cr(V)(EHBA)_2 + xs TMSPS	0.00(s), 0.63(m,ns), 1.78(m,ns), 2.90(m,ns).
(e) EHBA	0.68(s,t), 1.54(m,mu), 0.98(vw,ns).
(f) Cr(V)(EHBA)_2 + xs EHBA	0.8(s,ns), 1.7(m,ns).

vw = very weak, w = weak, m = medium, s = strong,
st = structure, ns = no structure, mu = multiplet.

TMSPS = 3-trimethylsilyl-1-propanesulphonic acid
EHBA = 2-ethyl-2-hydroxybutyric acid.

Table 4.1. ^1H nmr parameters of Cr(V)(EHBA)_2 in D_2O .

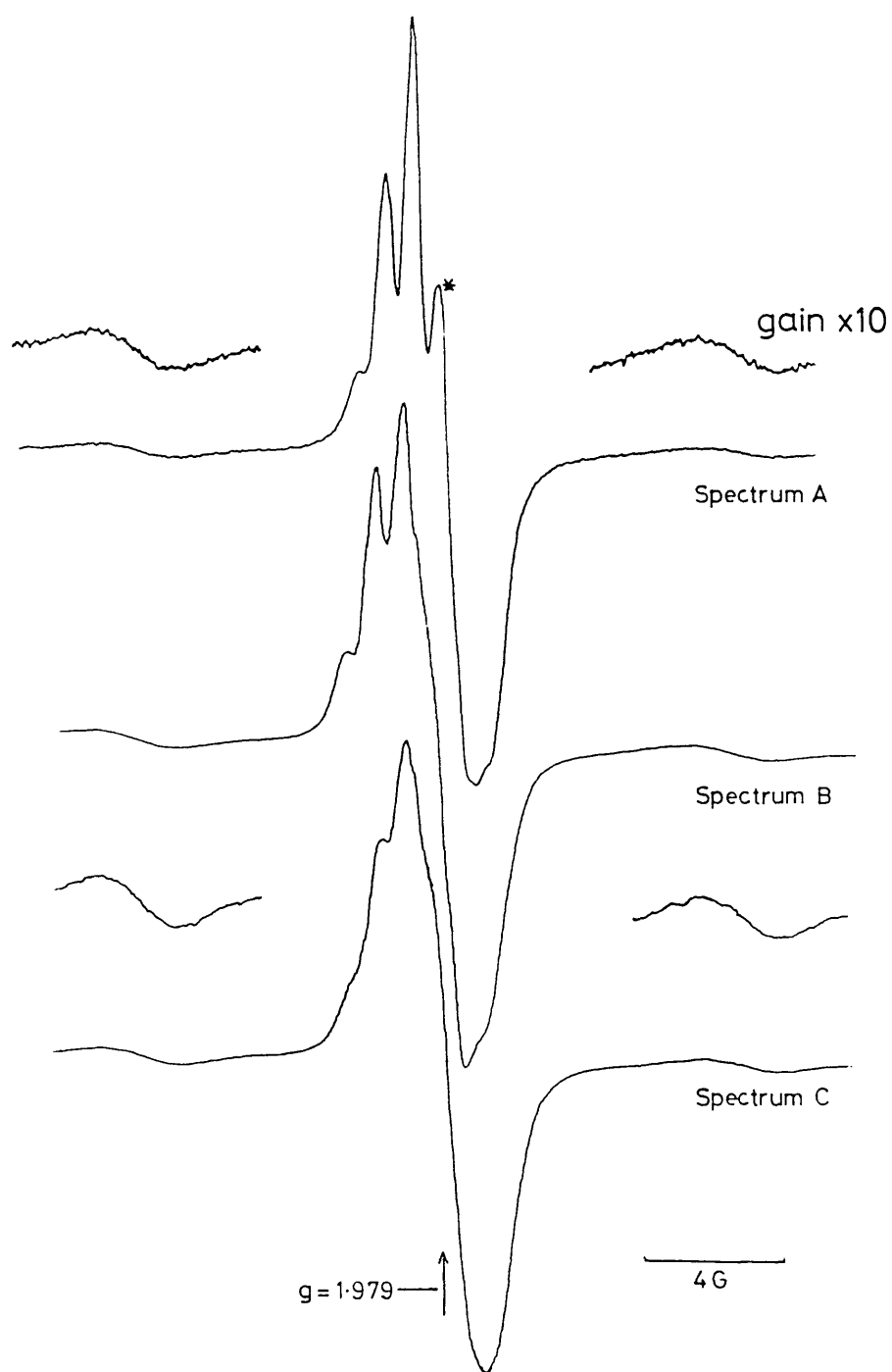
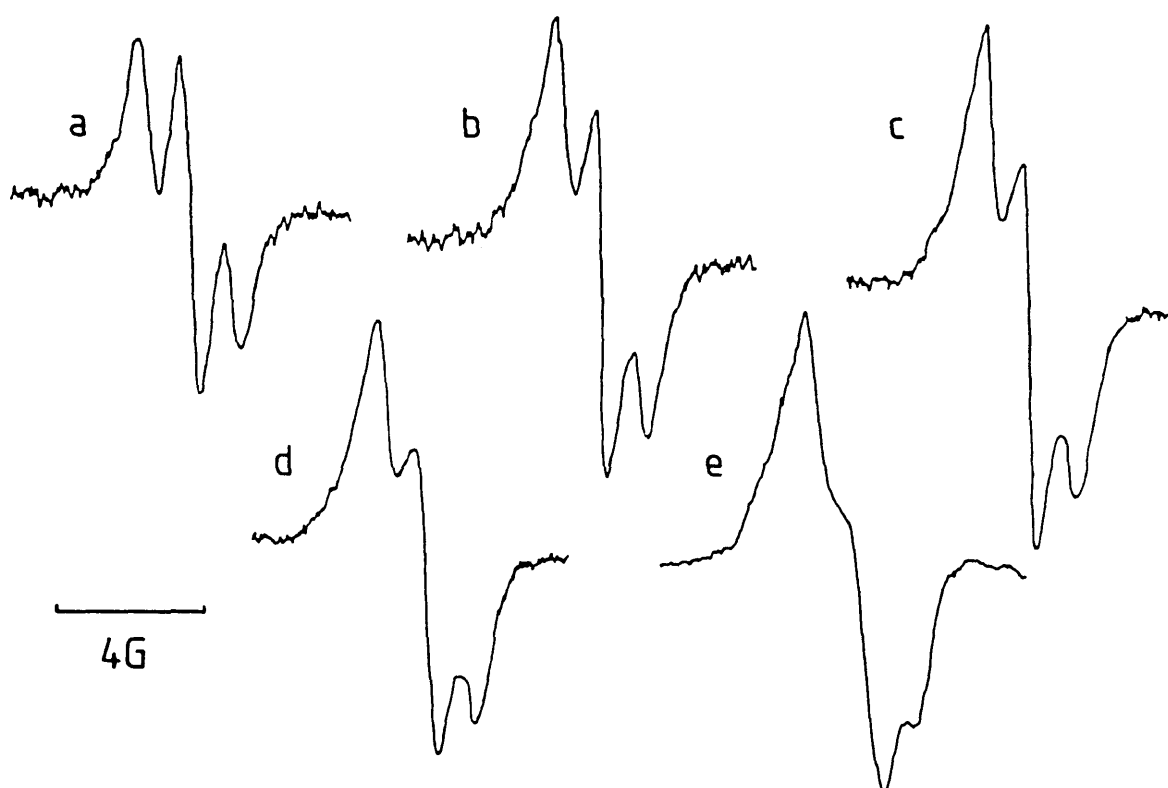
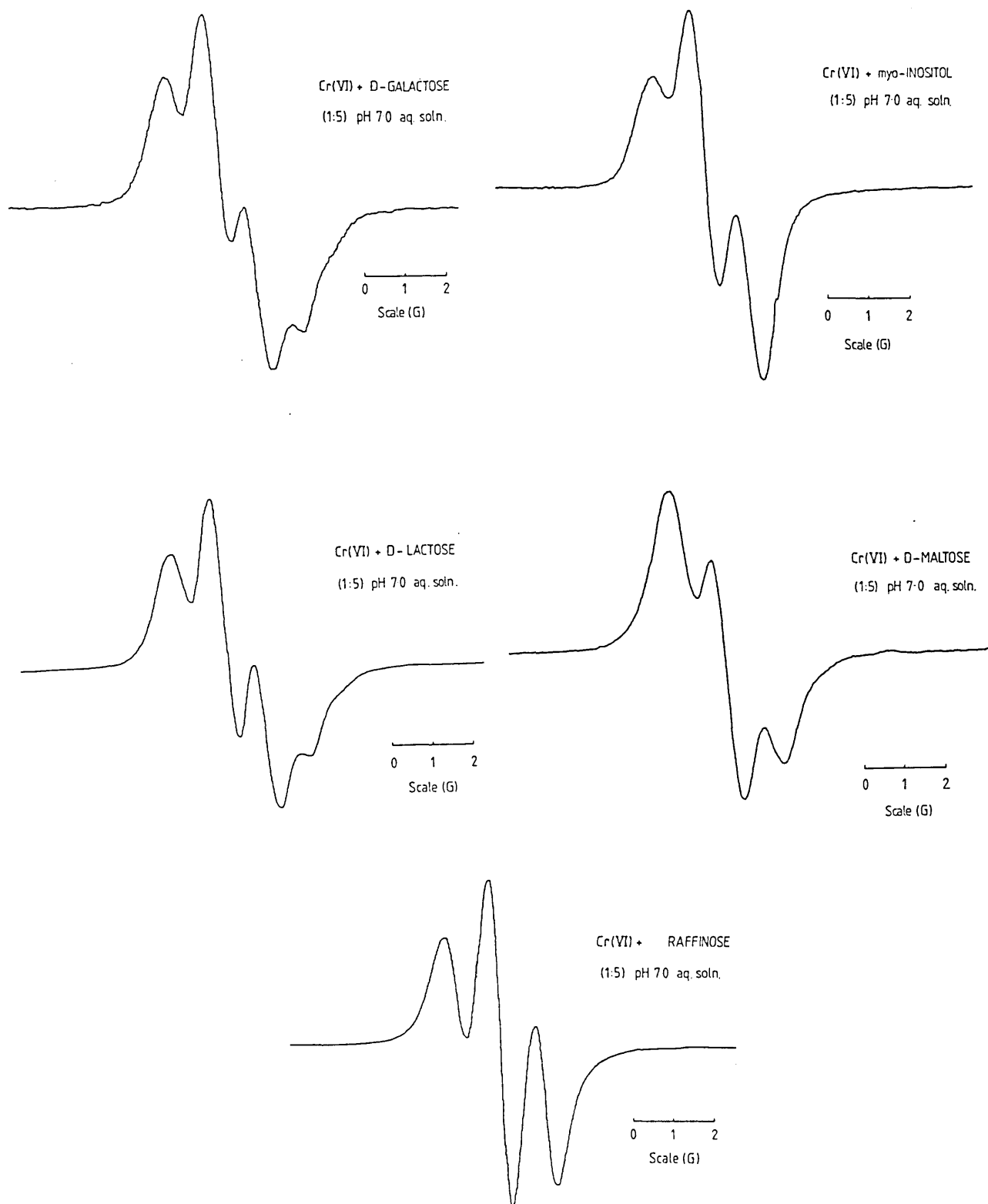


Fig. 4.2. X-band epr spectra observed after several hours on the reaction of Cr(VI) with (A) 5'-AMP (1:5 ratio, pH 7.0) (B) DRM (1:5 ratio, pH 7.0) (C) D-ribose (pH 7.0, 1:5 mole ratio) in aqueous solution.



Cr(VI) + D-Glucose, Spectral variation with time. (pH 7.0.aq)
 (a-d) 10min intervals after mixing, (e) after 48 hrs.

Fig. 4.3. A timed series of mobile solution epr spectra of the reaction of Cr(VI) with D-glucose (pH 7.0, 1:5 mole ratio).



All signals centred at $g=1.980$.

Fig.4.4. Diagram showing the final form of the Cr(V) epr spectra obtained on reaction of Cr(VI) with a range of carbohydrates in aqueous solution.

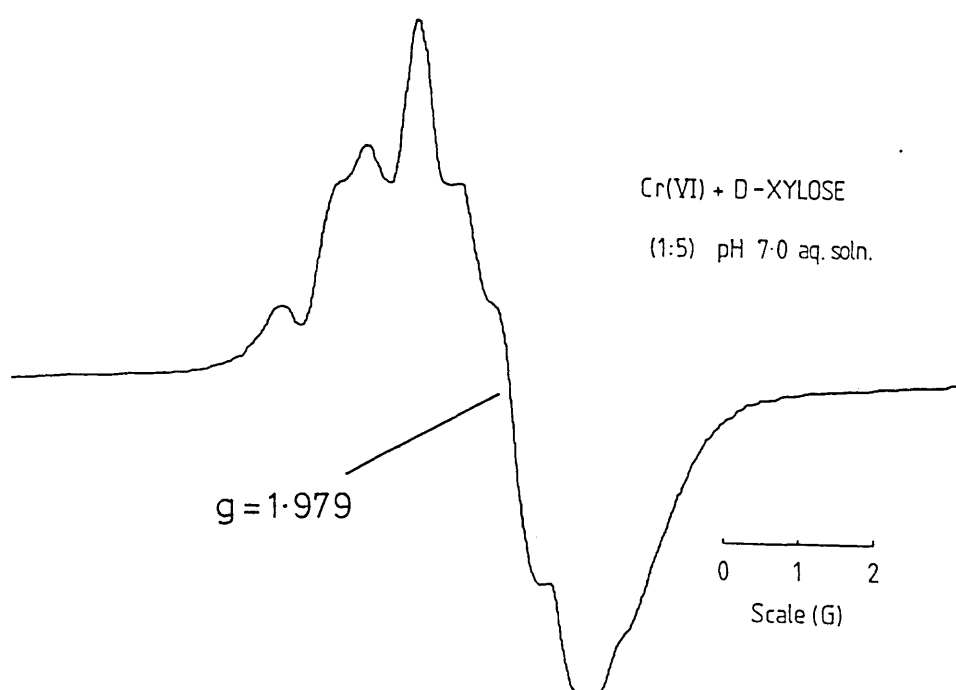
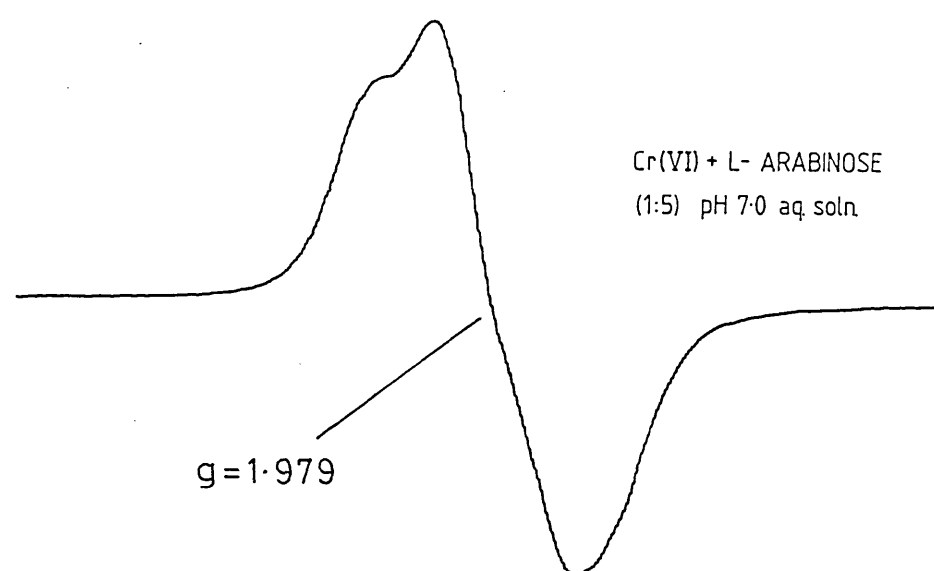


Fig.4.5. Cr(V) epr spectra seen on reaction of Cr(VI) with D-xylose and L-arabinose (pH 7.0, 1:5 mole ratio) in aqueous solution.

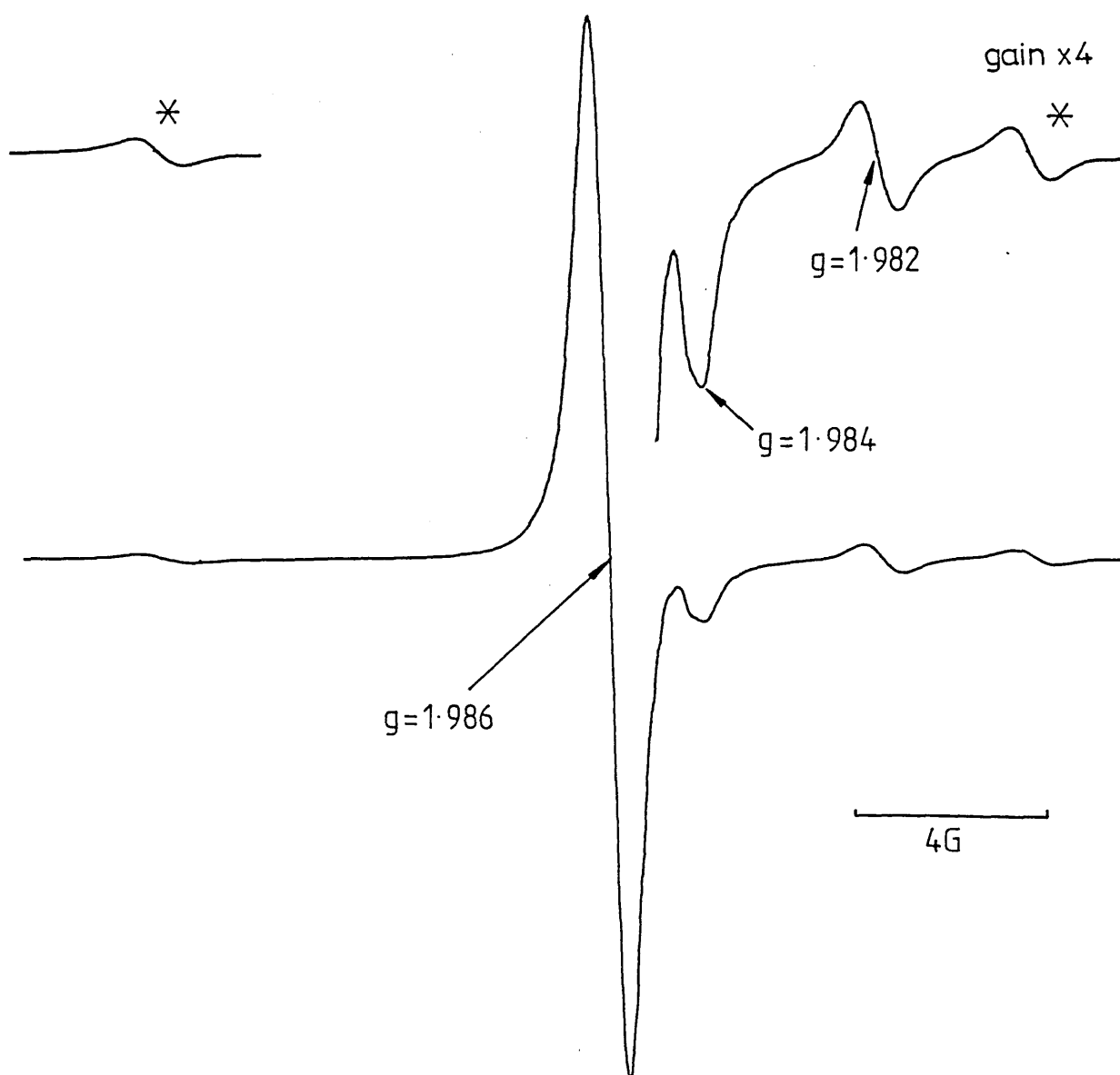
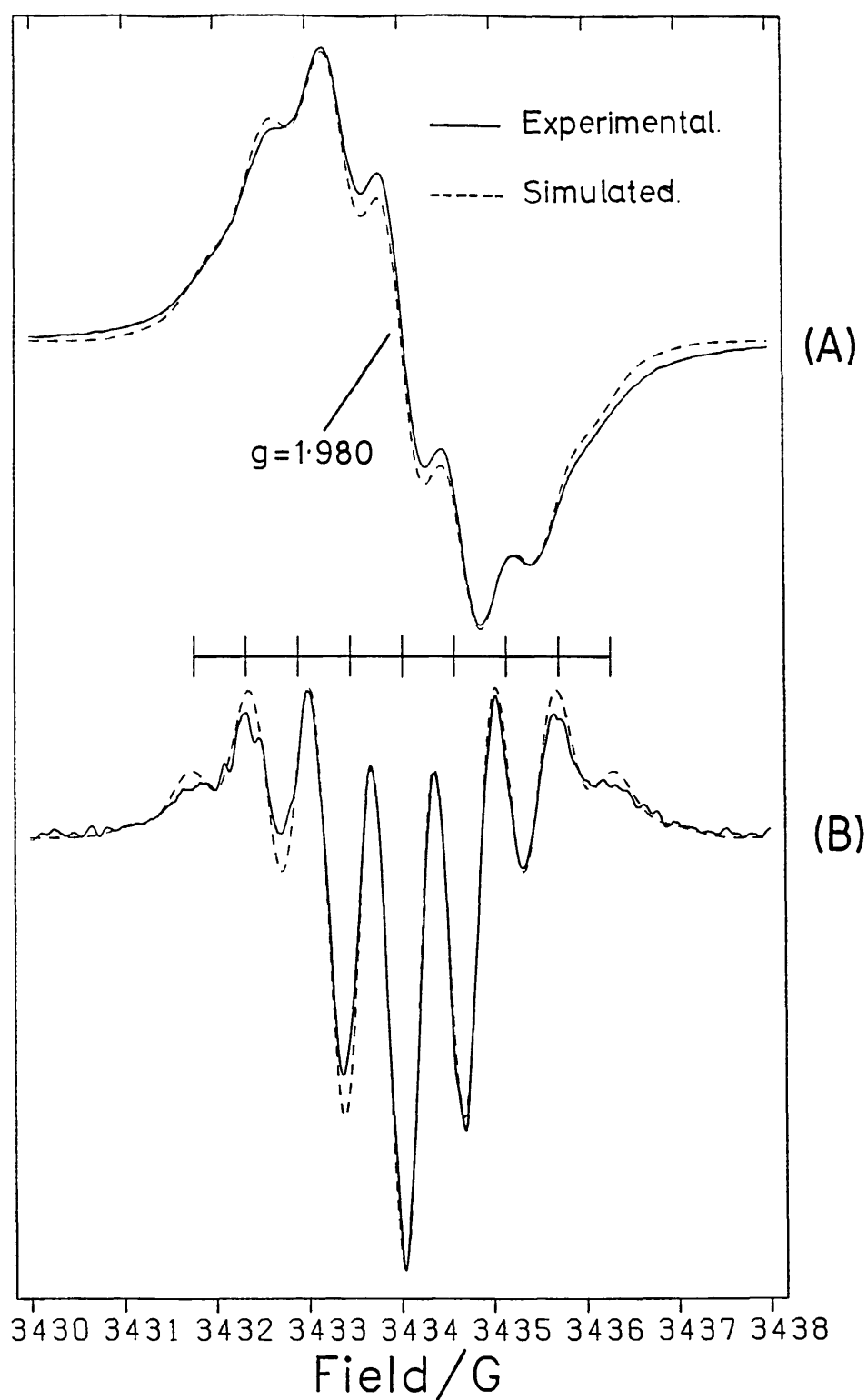


Fig.4.6. Cr(V) epr spectrum recorded immediately after mixing of the reaction of Cr(VI) and β -D-thioglucose in aqueous solution (pH ~4, 1:1 mole ratio).



(A) = first derivative spectrum.
 (B) = second derivative spectrum.

Fig.4.7. Experimental and computer simulated epr spectra of the Cr(V) species produced on reaction of Cr(VI) and glycerol in aqueous solution (pH 7.0, 1:5 mole ratio).

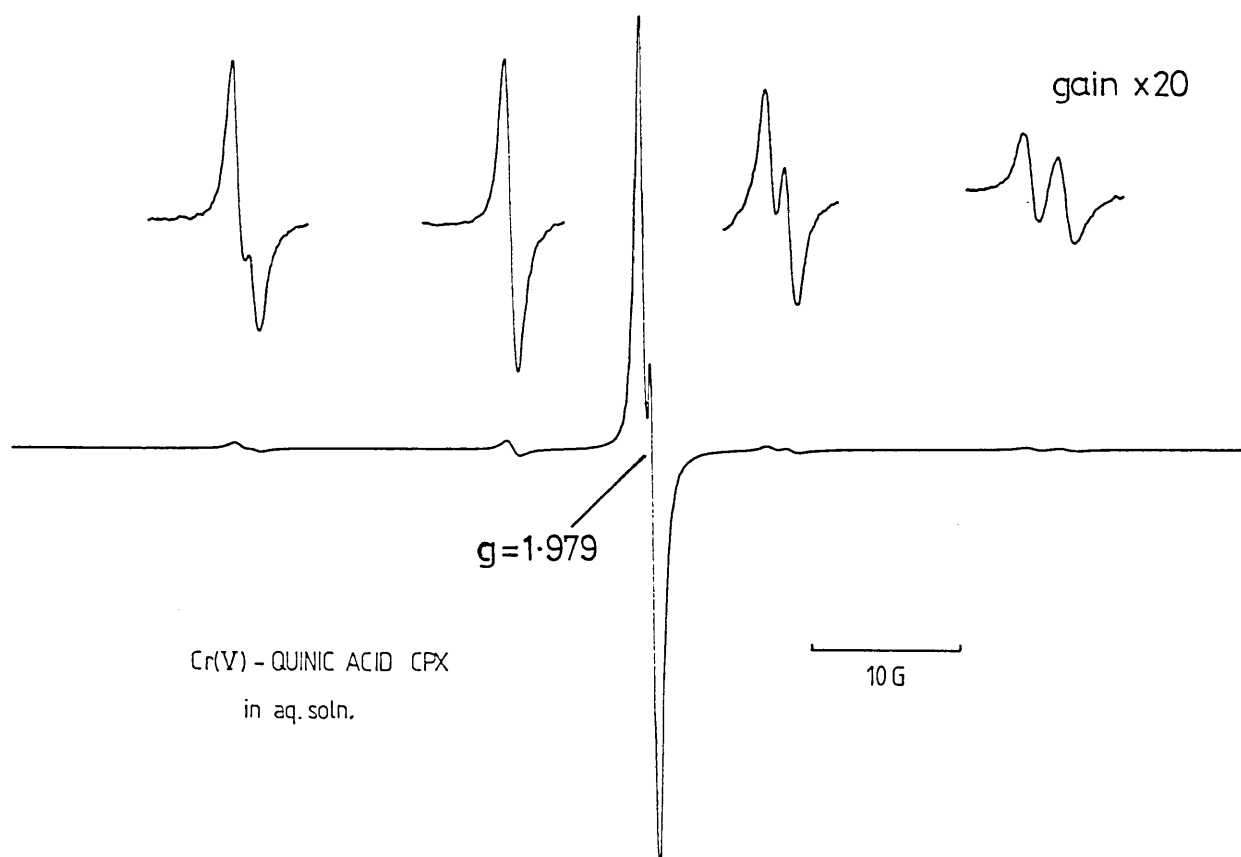


fig.4.8. Mobile aqueous solution epr spectrum of the solid isolated from the reaction of Cr(VI) and quinic acid in anhydrous acetone.

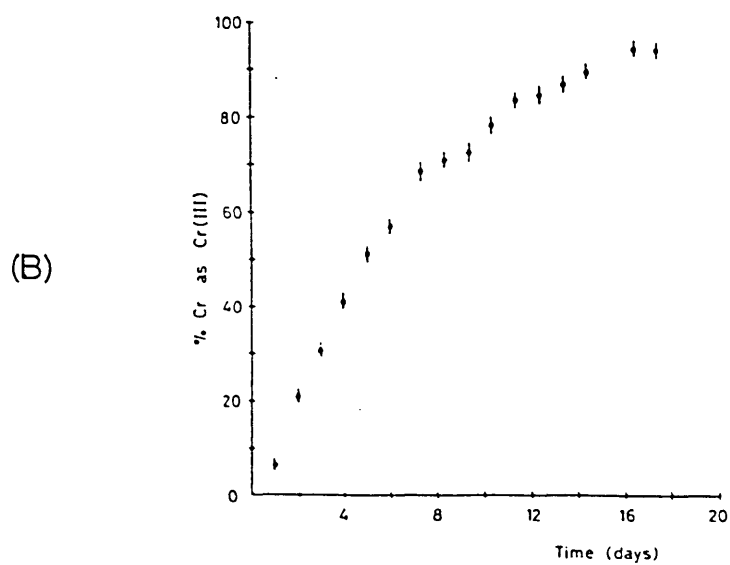
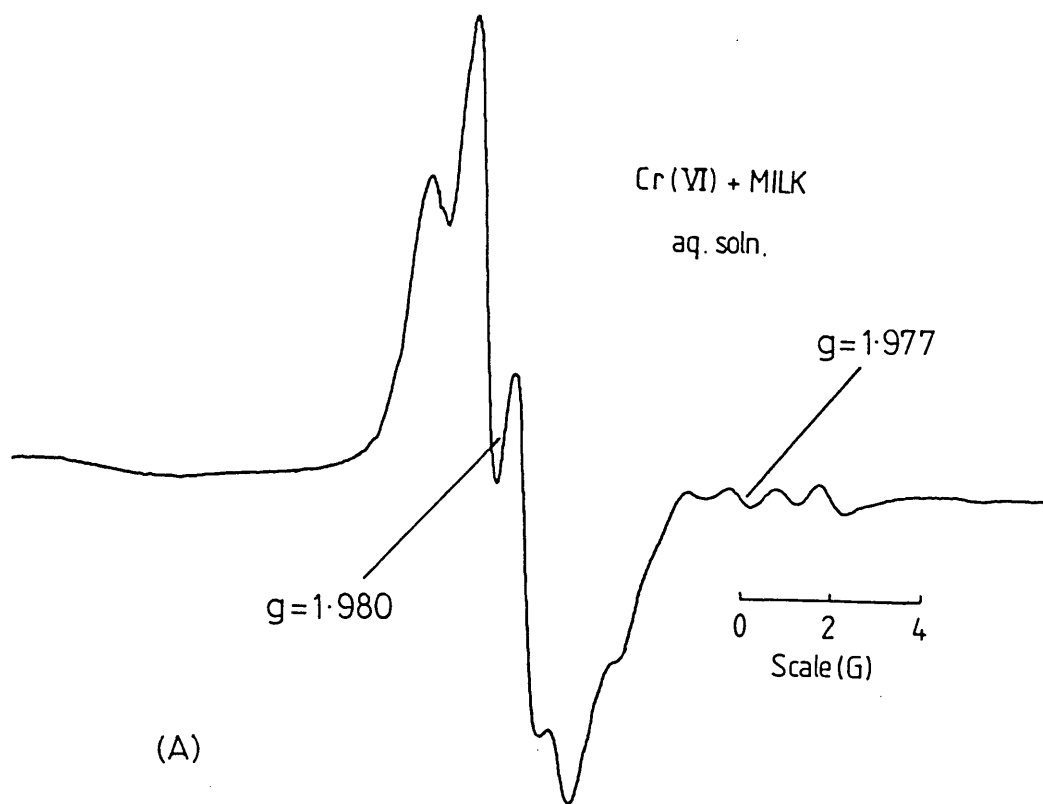


Fig.4.9. (A) Epr spectrum of a solution of Cr(VI) and fresh pasteurised milk, recorded two hours after mixing.

(B) Time dependence of the conversion of Cr(VI) to Cr(III) and Cr(V) by milk as measured by double integration of the combined Cr(III)/Cr(V) epr absorption.

4.4. Discussion

The Cr(V) epr spectra obtained on reaction of Cr(VI) with 5'-AMP, 5'-GMP and 5'-CMP in aqueous solution are the same as that obtained by Hayman [28] for the reaction with 5'-AMP under similar experimental conditions. Hayman proposed that the observed superhyperfine structure of the central Cr(V) band was due to interaction of two equivalent base nitrogen atoms of the two coordinating nucleotides with the unpaired Cr(V) electron. The result of this being a five band splitting pattern, two such Cr(V) species being present producing overlapping signals.

In the studies made here, the nucleotides employed possess a range of base units but the same spectrum is obtained in all cases suggesting that interaction with N is not the cause of the observed structure.

The reaction of Cr(VI) with adenosine produced the same Cr(V) spectrum as with the nucleotides showing that the phosphate group is probably not involved in coordination with the Cr(V). Similarly, the reaction of Cr(VI) with D-ribose, the sugar unit of the nucleotides, gave a Cr(V) epr signal with superhyperfine structure. This confirmed the suggestion by Hayman [28] that the nucleotide coordinates to Cr(V) via the sugar unit, but that the observed superhyperfine structure, albeit more poorly resolved than with the nucleotides, is due to ring-bound, non-exchangeable protons rather than the base nitrogens.

One problem with D-ribose is that in aqueous solution it is present mainly in its pyranose ring form, the furanose form being present in the nucleotides. This will therefore give rise to

discrepancies in their respective hydroxyl group conformations. To overcome this, the reaction of Cr(VI) with D-ribose-5-monophosphate was investigated, D-ribose being in its furanose form in this state and more accurately modelling the situation present in the nucleotides. The observation of a Cr(V) epr signal with much better structure resolution than D-ribose confirmed that the furanose form was probably the predominant binding of the nucleotides to the Cr(V) centre.

Apart from myo-inositol and raffinose, all of the sugars used in section 4.3.2. can be classified as reducing sugars, that is they all have one potentially free carbonyl group capable of being oxidized in the anomeric position, both myo-inositol and raffinose being non-reducing. Similarly, all of the reagents in section 4.3.4. are non-reducing, the anomeric positions (if present) being blocked.

Now, if the interaction of Cr(VI) with sugars to give Cr(V) species proceeds via a mechanism involving the anomeric centre, it could be expected that the Cr(V) species would be formed with the reducing sugars and not the non-reducing sugars. This is indeed the case except for the reaction of Cr(VI) with raffinose. Here a Cr(V) signal with unchanging superhyperfine structure is produced, despite the fact that all anomeric centres are blocked and there are no cis-diol groups in the five-membered ring unit. The intensity of this signal is too great for it to be produced by a small quantity of impurity within the raffinose sample and the mechanism of complex formation in this case is not understood.

Sucrose is a non-reducing sugar and does not produce any Cr(V) species on reaction with Cr(VI). The sucrose molecule has a D-glucose and a furanose unit joined via the anomeric centre. Now, from section 4.3.2. it was shown that D-glucose readily forms Cr(V) species on reaction with Cr(VI). This evidence shows that in the case of D-glucose (and probably the other reducing sugars) the formation of a Cr(V) complex must involve interaction with the anomeric OH, despite the presence of additional OH groups elsewhere in the sugar molecule.

The lack of reaction of Cr(VI) with both α - and β -cyclodextrins (six- and seven-membered rings of D-glucose units joined via their anomeric centres) also indicated the importance of the anomeric centre for Cr(V) complex production. The cyclodextrins closely mimic the structure of cellular polysaccharides, such as glycogen, thereby suggesting that Cr(V) stabilization by coordination to such species is not probable in the cellular environment.

The reaction of Cr(VI) with α -D-methylglucopyranoside also showed no Cr(V) formation, the anomeric centre being blocked by -OMe. This again shows the requirement of a free anomeric position for the production of Cr(V) species.

The question now arises: if the anomeric centre is necessary for the formation of a Cr(V)-sugar complex, by what mechanism are Cr(V) complexes formed on reaction of Cr(VI) with myo-inositol, and raffinose, inositol being a cyclohexane derivative and raffinose a non-reducing sugar?

To look more closely at the former, the reactions of Cr(VI) with some other cyclohexane derivatives were carried out. With

both cis-1,3-cyclohexanediol and cis-1,2-di(hydroxymethyl)-cyclohexane no Cr(V) species were seen to be formed. However, rather than being due to the lack of an anomeric centre, these observations are probably caused by the fact that the most stable conformation of sugars in solution is that in which the largest number of hydroxyl groups are in the equatorial position. In both of the cases cited here, with the substituents in the more stable configuration, the OH groups are much too far apart to be capable of simultaneous binding to the metal centre. It would appear that the driving force of complexation by chelation is not enough to overcome this barrier.

A possible explanation of why raffinose produces a Cr(V) complex is that in aqueous solution, it could undergo hydrolysis to give its constituent sugars which can then interact with Cr(VI) themselves to give the Cr(V) species. However, this argument falls down for two reasons; (1) no formation of Cr(V) is observed with sucrose under exactly the same experimental conditions as raffinose, hydrolysis being possible in both cases; (2) the Cr(V) epr signal obtained with raffinose is unlike those observed with its individual constituent sugars and, in addition, the superhyperfine structure of the Cr(V) band was observed to remain constant with time, unlike those seen with other mono and di-saccharides.

An observation common to many of the reactions of Cr(VI) with saccharides is the formation of an initially simple superhyperfine splitting pattern associated with the Cr(V) epr signal, which then changes over a period of several hours eventually to

give complex patterns.

In most cases, this initial splitting was a 1:2:1 pattern indicative of interaction of the Cr(V) with two equivalent protons. The time period over which the structure was observed to change was very similar to that taken by sugars to achieve conformational and configurational equilibrium in solution.

Hence the optical rotation studies were carried out to see if the Cr(V) species being formed were dependent upon the equilibrium composition of the sugar in solution, and if the said equilibrium was at all affected by the Cr(V) complex formation.

Similarly, if the attainment of structural equilibrium of the sugars influenced the observed Cr(V) signal structure, pre-dissolution of the sugar several hours before reaction with Cr(VI) should immediately produce the Cr(V) signal with more complex splitting.

The optical rotation studies showed that the specific rotation of the sugar (D-glucose tested) did not alter from the usual equilibrium position on reaction with Cr(VI). Reactions with pre-dissolved sugars did not give rise to the complex splitting patterns expected immediately after reactant mixing, thus showing that the changes in Cr(V) signal structure are not due solely to the sugar attaining configurational equilibrium in solution, the Cr(V) species being formed depending upon this equilibrium.

The initially simple structure observed suggests that there is probably only one Cr(V) species present at this time. However, after several hours, at least 2 Cr(V) species are probably present, each with slightly differing splitting patterns, giving

rise to the complex spectra.

The origin of these various complexes has not been established, but it may be related to the attainment of conformational equilibrium by the sugar, an eventual steady state Cr(V) species mixture being reached after several hours.

The reactions of Cr(VI) with D-xylose and L-arabinose were carried out to investigate the role of the CH₂OH group of the C(5) sugar position in Cr(V) complex formation. In both of these sugars the CH₂OH group is absent.

Both sugars yielded relatively stable Cr(V) species on reaction with Cr(VI) in aqueous solution. However, the Cr(V) epr signals observed possessed very complex super hyperfine splitting patterns immediately after solution mixing. This is in contrast to the situation encountered in the reaction of Cr(VI) with, for example, D-glucose. With the latter, a relatively simple 1:2:1 superhyperfine splitting pattern is seen immediately after mixing, which slowly changes to a more complicated spectrum after several hours.

It therefore appears as though CH₂OH may play some part in the initial Cr(V) complex formation with sugars possessing this group, although it is not essential for complex formation since both D-xylose and L-arabinose react to give Cr(V) species. D-ribose may also be included in this group since it too lacks a CH₂OH group and gives a complicated spectrum immediately after mixing.

The reaction of Cr(VI) with quinic acid is of interest since it contains two potential sites for Cr(V) coordination. At one

end there are three OH groups, as seen in most of the sugars and inositols already described. A tert-hydroxy carboxylic acid group, the only organic molecule grouping known to form stable, isolable Cr(V) complexes is also present.

The reactions of Cr(VI) with this molecule in aqueous solution showed the formation of several Cr(V) species resulting in a complex epr signal. However, in non-aqueous acetone a solid containing a relatively high proportion of Cr(V) complex was formed. The aqueous solution epr spectrum of this solid showed the presence of two Cr(V) species with slightly differing spectral characteristics. It seems probable that the Cr(V) complexes formed in the latter case are due to interaction of the metal centre with the carboxylic acid group. The main reason behind this suggestion is that no proton superhyperfine structure of the Cr(V) signals is seen in aqueous solution, this being expected if complexation was via the ring-hydroxyl groups.

The presence of H₂O in the reaction mixture has a complicated effect on the Cr(V) species produced, as could be reasonably expected from the known Cr(V) - H₂O reactivity. However, the apparent stability of the Cr(V) complexes in solid form would suggest the possibility of eventually isolating the complexes but all attempts at this during the present work were unsuccessful.

The exact nature of the two Cr(V) complexes in the solid are not known. Their very similar epr spectra suggest a close resemblance in their structures, a possible cause being conformational or configurational differences between the two.

The reaction of Cr(VI) with β-D-thioglucoose gave some very interesting results. Under conditions which gave rise to a

relatively long-lived Cr(V) species, the epr signal observed was centred at $g=1.986$, and ^{53}Cr hyperfine bands with 18.2 G spacing were also present. The position of this band was very similar to that seen earlier (chapter 2) on reaction of Cr(VI) with reduced glutathione at a 1:1 relative mole ratio. With GSH, it was proposed that the ligand atoms donating to the Cr(V) centre included sulphur, thus producing the change in g -value from the normal region with oxygen only donors. However, the Cr(V)-GSH complex exhibited ^{53}Cr hyperfine bands with 8.6 G spacing, but with β -D-thioglucose the equivalent spacing was 18.2 G. These observations appear to be giving contradictory results, the g -value suggesting possible S-donation and the hyperfine spacing suggesting an oxygen only environment. In addition, the central Cr(V) band seen with β -D-thioglucose showed no nuclear superhyperfine structure as seen with all other sugar systems investigated. This suggests that there are no non-exchangeable ring protons interacting with the Cr(V) centre or that if S is involved in the binding process, it is removing the said structure. Support for the latter suggestion is provided by the lack of structure in the epr bands of the Cr(V)-GSH complexes, despite the presence of adjacent non-exchangeable protons.

If the S is coordinating to the Cr(V) centre, it is probably doing so via the open chain form of the sugar, thioether ligands being known to show little or no Cr(V) complex formation [22]. This now prompts the question, could this open chain mode of attachment be taking place in Cr(V) complex formation with the

normal sugars? This seems unlikely since Cr(V) complexes giving very similar epr spectra are readily formed with inositols which do not possess hetero-ring atoms. A more likely explanation of the situation is that the thioether linkage is more labile than its oxygen counterpart, thereby allowing complex formation via this route. In fact, changing the hetero atom of D-glucose from oxygen to sulphur has a marked effect on the rate of production and subsequent decay of the Cr(V) species. The formation of the Cr(V) complexes with the former took days to reach a steady concentration but with sulphur, pHs above 4 or any excess of β -D-thiogluco^{se} resulted in extremely rapid production and decay of the Cr(V) complex.

From all of the evidence collected and outlined above concerning the reaction of Cr(VI) with sugars and their derivatives, it is possible to say that the anomeric centre of the sugar is important for the formation of Cr(V) complexes in aqueous solution. There are some notable exceptions to this rule however, the reaction with inositols being in this category. The fundamental rules governing Cr(V) formation and the modes of ligand binding have not yet been determined. In order to gain the additional information required for a more complete description of Cr(V) formation, alternative physical techniques must be employed. The most promising of these would be the ENDOR epr method, its attributes being well suited for study of epr bands of very narrow band width, as present in Cr(V) signals. Future study in this area should include ENDOR studies which would hopefully reveal the nature of binding of Cr(V) to sugars and their derivatives.

A paper was published some time ago describing the formation of a relatively stable Cr(V) species on reaction of Cr(VI) with glycerol [112]. That report showed the formation of the Cr(V) complex via epr spectroscopy. However, the description given made no mention of the observation of nuclear super hyperfine structure of the central Cr(V) band - as would be expected from the structure of glycerol (fig.4.1.).

This reaction was repeated here and as expected, a stable Cr(V) epr signal possessing superhyperfine structure was observed. The structure appeared to be symmetrical about the centre, a feature not seen with the Cr(V)-sugar complexes, and so a computer simulation of the spectrum was recorded. This showed that the central Cr(V) was being split into nine bands by the interaction with eight $I=1/2$ nuclei, ie. eight adjacent protons. The only feasible ligand coordination to give this form of spectrum is given in fig.4.10. In this proposal two glycerol molecules coordinate per Cr(V) centre, the relevant protons being on the CH_2 groups.

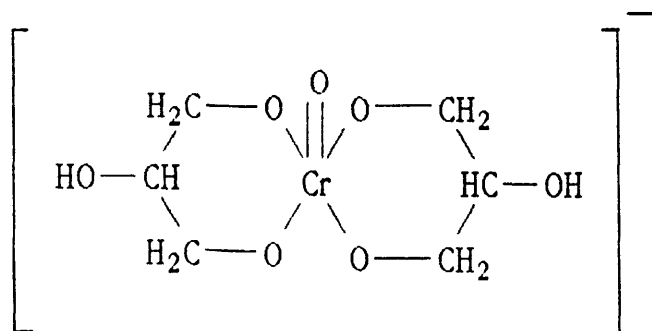


Fig. 4.10. Proposed structure of the Cr(V)-glycerol complex.

Cr(V) complexes with other diol ligands have been shown to produce epr signals with symmetrical superhyperfine structure [35], the splittings being attributed to the equivalent protons to those responsible with glycerol. However, the work here is the first to show the splittings by glycerol and confirm their origin by computer simulation.

Reaction of Cr(VI) with milk

The reaction of Cr(VI) with fresh, pasteurized milk was carried out in response to a paper published outlining the potential lethality of environmental Cr(VI) towards grazing cattle [96].

On performing this reaction the results obtained showed that a relatively long-lived Cr(V) complex was formed. The nature of this Cr(V) signal was very similar to those found on reaction of Cr(VI) with the sugar systems. It is known that cow's milk contains a relatively high proportion (~5 % by weight) of the disaccharide lactose (milk sugar).

Indeed, comparison of the Cr(V) epr spectra obtained with milk and lactose respectively showed them to be very similar, suggesting that it is lactose which forms the Cr(V) species on reaction with milk. The origin of an additional set of weak epr signals on the high-field side of the main Cr(V)-milk signal is not clear, although they may arise from interaction of Cr(VI) with one of the many minor components present in milk. Ascorbic acid is present in milk but at a relatively low concentration (~16mg per L). Chapter 3 investigated the interaction of Cr(VI)

with ascorbate, and at no time were epr signals observed at g-values as low as those of the additional bands above. It is probable therefore that Cr(V)-ascorbate complexes are not important in this system.

The reduction of Cr(VI) by milk results in its eventual conversion to Cr(III). By double integration of the epr signal of the Cr(III) so produced it was possible to measure the time-dependence of the production of Cr(III). It appears that under the experimental conditions used here, the total conversion of Cr(VI) to Cr(III) is quite slow, with only ca. 50% conversion after 5 days.

The environmental consequences of Cr(VI) ingestion by cattle will depend upon the rate at which Cr is transported to the milk within the body and on the extent to which biological reduction to Cr(III) takes place en route. At first glance it would appear very unlikely that the Cr would enter the milk in its +6 form, prior reduction being probable. This said, should any Cr(VI) reach the milk, then its rate of reduction once there would be relatively slow, thus allowing the possibility of potentially harmful effects to anybody ingesting the contaminated milk samples.

CHAPTER 5. THE REACTION OF Cr(VI) WITH BIOLOGICALLY RELEVANT MOLECULES IN THE PRESENCE OF GSH OR L-CYSTEINE.

5.1. Introduction

Chapters 2 and 4 have shown that relatively long-lived, or at least a steady state concentration, of Cr(V) species, can be produced on reaction of Cr(VI) with cellular reductants under physiologically relevant conditions.

The question must now be asked; if these reactive Cr(V) species are produced within the cellular environment, can they then react further with other cellular constituents to form potentially more damaging or longer lived Cr(V) species, these then being transported to the critical sites within the cell? The same could also be applied to the Cr(VI)-ester species initially produced on the reduction of Cr(VI) by, for example, cellular thiols, as has been suggested elsewhere [22]. In addition, could the Cr(V) species produced subsequently react with, and form new Cr(V) complexes of, cellular constituents which would not otherwise react with Cr(VI)?

The most abundant naturally occurring cellular reductant in the body is reduced glutathione (GSH). Reaction of this with Cr(VI) in aqueous solution gives rise to Cr(V) species which persist for up to one hour (chapter 2). GSH is therefore one of the more likely molecules to react with Cr(VI) very soon after entering the cell, the Cr(V) species so produced possibly reacting with other molecules.

The reactions reported here are of Cr(VI) with GSH in the presence of a third component, and can be conveniently grouped into two sections; (i) The reactions of Cr(VI) with GSH in the

presence of another component which itself reacts with Cr(VI), albeit at a much slower rate than with GSH, and (ii) reactions in the presence of a molecule which would not normally react with Cr(VI) under the reaction conditions employed here to give Cr(V) species.

In (i), reactions were studied in the presence of sugars and nucleotides and in (ii), reactions with amino acids, sucrose, DNA and α and β -cyclodextrins were studied.

In 1964, Samitz and Katz [101] showed that out of twenty amino acids tested, only L-cysteine and methionine were capable of reducing chromate after 48 hours at pH 7.0 (phosphate buffer). These reactions have been repeated here to confirm the findings of Samitz [101] and subsequently repeated in the presence of GSH to investigate the possibility of their reaction with the Cr(V)-GSH complex, rather than Cr(VI).

Similarly, as other work (chapter 4) has shown that there was no interaction of Cr(VI) with α and β -cyclodextrins or sucrose in aqueous solution at pH 7.0, the possible reaction of these with the Cr(V)-GSH complex was also investigated here.

5.2. Experimental

The general methods of solution preparation and reaction monitoring have been described earlier (chapter 2).

Reactions were carried out in aqueous, unbuffered solution at physiological pH. The production of Cr(V) species was followed by recording the mobile solution epr spectrum immediately after mixing and pH alteration, and at times intervals thereafter.

The additional components used in these studies are D-ribose,

D-glucose, sucrose, 5'-AMP (adenosine 5'-monophosphate), 5'-GMP (guanosine 5'-monophosphate), DNA (calf thymus), α and β -cyclodextrins and a wide selection of common, non-thiol amino acids. These components were initially dissolved in the same solution as the GSH (or L-cysteine), the relative reactant mole ratios being 1:1:5 (Cr(VI):GSH:component). (50mg of DNA were used)

5.3. Results

In agreement with the findings of Samitz and Katz [101], the present work showed no reaction of Cr(VI) with amino acids in aqueous solution at pH 7.0 in the absence of GSH. Re-recording the epr spectrum over seven days after mixing still showed no Cr(V) or Cr(III) complex formation.

On repeating these reactions in the presence of GSH, the only signal present was that of a quickly decaying ($T_{1/2} \sim 15$ min.), characteristically sharp Cr(V) band at $g=1.985$, with four weak ^{53}Cr hyperfine bands of 8.5 G spacing. This signal corresponds to the Cr(V) species observed earlier (chapter 2) on reaction of Cr(VI) with GSH alone (1:1).

A similar situation to this was found on reaction of Cr(VI) with GSH in the presence of α or β -cyclodextrin or sucrose, the only epr signal seen at $g=1.985$ being due to the Cr(V)-GSH complex. With the cyclodextrins, a very small additional signal was observable close to $g=1.985$ causing a very slight asymmetry in the main Cr(V) band.

The reaction of Cr(VI) with GSH in the presence of DNA (50mg) gave no additional signals to those outlined above.

On reaction of Cr(VI) with GSH + D-ribose, D-glucose, 5'-AMP,

or 5'-GMP (all 1:1:5 mole ratio, pH 7.0) the major epr signal observed immediately after mixing was that at $g=1.979$ due to the Cr(V) complex with the sugar or nucleotide (e.g. fig.5.1). In all cases, the initial size of this signal was comparable to that obtained after two days of reaction in the absence of GSH, its intensity slowly increasing and reaching a constant size after approx. one hour.

All of the above signals exhibited the same proton superhyperfine structure as described earlier (chapter 4), the ^{53}Cr hyperfine bands also being clearly seen ($a_{\text{Cr}} \sim 18 \text{ G}$). (fig.5.1)

Also present were two additional signals at $g=1.985$ and 1.987 , the intensity of which decreased rapidly with time. There was evidence of ^{53}Cr hyperfine bands associated with the $g=1.985$ band ($a_{\text{Cr}} \sim 8.5 \text{ G}$).

Repeating the above reactions with L-cysteine in place of GSH resulted in the rapid production of the Cr(V) signal at $g=1.979$. The intensity of this band increased and reached a maximum after 20 to 25 min. At no stage could a signal be observed corresponding to the Cr(V)-L-cysteine complex and there was no additional band at $g=1.987$.

The reaction of Cr(VI) with D-glucose and an excess of GSH (1:5:5, pH 7.0) produced four epr signals at $g=1.996$, 1.987 , 1.985 and 1.979 , immediately after mixing. Of these, the signals at $g=1.996$, 1.987 and 1.985 possessed no structure and decayed rapidly with time ($T_{1/2}$ approx. 4, 5 and 8 min. respectively). The band at $g=1.979$ did exhibit structure, and with time, slowly increased in intensity, reached a maximum after 20 min. and then

slowly decreased.

In no reaction was any solid isolated from the aqueous reaction mixtures. However, the reaction solution colour was observed to become green with time, the formation of Cr(III) being confirmed by the presence of a broad epr signal at $g=1.98$.

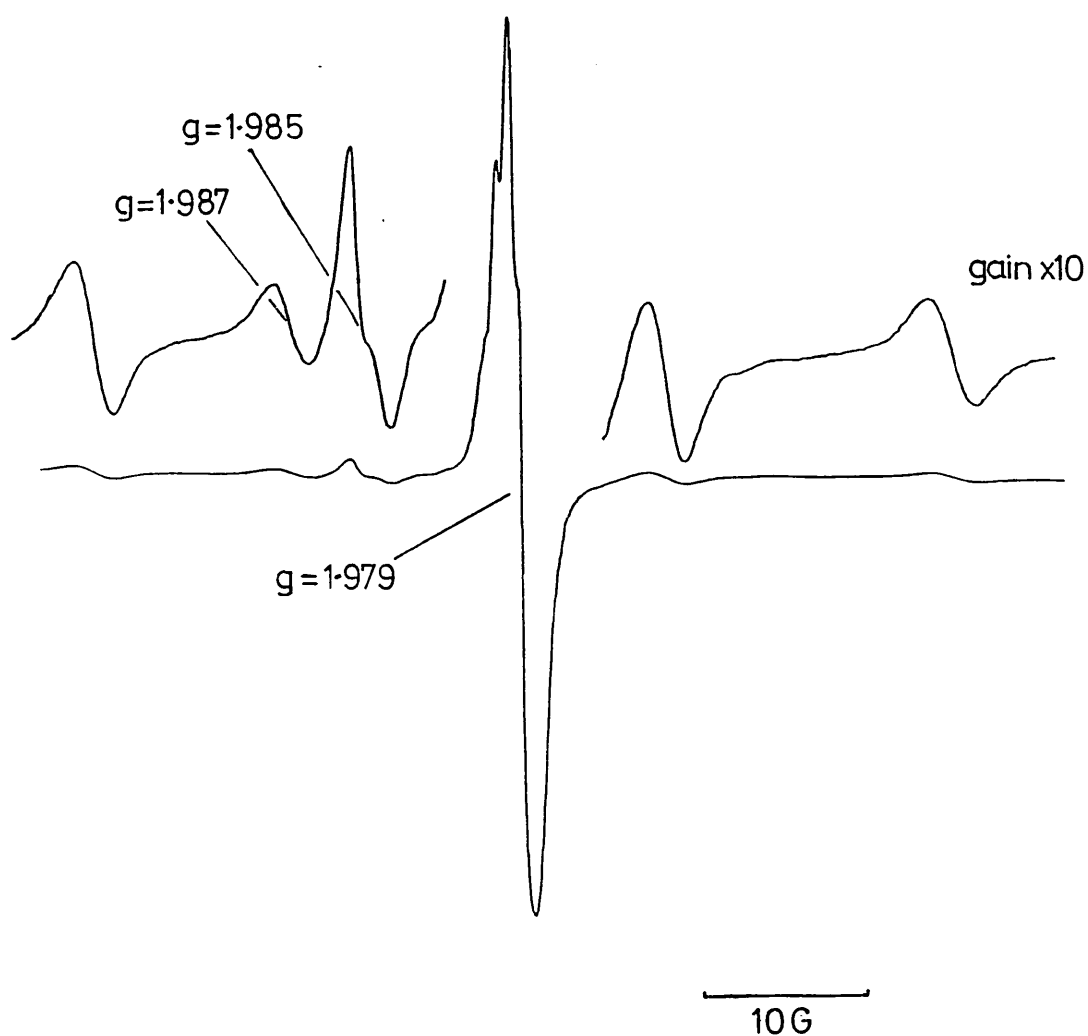


Fig. 5.1. X-band epr spectrum of an aqueous solution of Cr(VI) + GSH + 5'-AMP (1:1:5 mole ratio) recorded immediately after mixing.

5.4. Discussion

Although the line of work studied in this chapter is far from complete because of time limitations, the results obtained may have important biological implications.

As observed previously by Samitz et al. [101] and confirmed here, there is no reaction of Cr(VI) with non-S containing amino acids in aqueous solution at pH 7.0. Similarly, the presence of GSH, which produces intermediate Cr(V) species, does not allow reaction with the same amino acids, suggesting that probably they are not implicated in the possible stabilization of Cr(V) within the cell. The same is true with sucrose and α and β -cyclodextrins, the latter being reasonable model systems for the polysaccharide components of cells, e.g. glycogen.

On reaction of Cr(VI) with GSH in the presence of DNA, (which does not interact directly with Cr(VI) the only Cr(V) species observed were those with GSH as described previously (chapter 2). This is in agreement with the results obtained by Kawanishi et al. [46], who reported that the Cr(V)-GSH complex formed on interaction of Cr(VI) with GSH is not capable of causing DNA strand breakage. However, as stated in chapter 2, the Cr(V) complex reported by Kawanishi [46] exhibited conflicting spectral parameters ($a_{Cr}=20$ G) to those obtained here ($a_{Cr}=8.5$ G).

The reactions of Cr(VI) with GSH in the presence of D-glucose, D-ribose, 5'-AMP and 5'-GMP all gave rise to the much longer-lived Cr(V) species of the additional component in preference to the Cr(V)-GSH complex. Of interest here is that the rate of Cr(V) complex formation with, for example, 5'-AMP, is

much greater than that in the absence of GSH. Without GSH it would take several hours for the Cr(V)-5'-AMP species concentration to grow, and it would attain its steady state concentration only.

However, in the presence of GSH, this maximum concentration is reached within one hour. In both cases, the resultant Cr(V)-5'AMP complex can be seen in the epr spectrum at a constant concentration for several weeks. Frozen solution epr studies showed that the maximum yield of Cr(V) species was independent of the absence or presence of GSH, this holding true with all of the other sugars and nucleotides tested.

In the above reactions, it would appear as though the Cr(V) species initially formed with GSH ($g=1.985$) is able to undergo a rapid ligand-exchange reaction with the third component molecule, hence giving its respective Cr(V) complex much more rapidly than could be achieved on reaction with Cr(VI) alone. The presence of a small, rapidly decaying Cr(V) species at $g=1.985$ tends to support the proposal that the Cr(V)-GSH complex is the initial product, this then reacting further.

An additional band at $g=1.987$ was observed in all of these reactions but its origin is unknown. The position corresponds to a ligand environment with additional donor atoms other than oxygen.

The Cr(V)-GSH species is effectively acting as an intermediate in the ultimate Cr(V) stabilization by the sugar containing molecules. Were this mechanism of rapid ligand exchange to take place within the cellular environment, it would

provide a real opportunity for Cr(V) stabilization and subsequent transportation to the critical sites within the cell.

The reactions of Cr(VI) with the sugars or nucleotides in the presence of a five-fold excess of GSH (over Cr(VI)) were carried out to investigate the possibility of further reduction of the Cr(V)-sugar/nucleotide complex. The epr spectrum showed four Cr(V) signals. Those at $g=1.985$ and 1.996 have been seen previously and are due to complex formation with GSH, the origin of the $g=1.987$ signal being unknown. The Cr(V)-sugar/nucleotide band at $g=1.979$ reached its maximum intensity after ~ 20 min. compared to ~ 1 hour with only one equivalent of GSH. Subsequently, the signal size slowly decreases with time due to further reduction by GSH.

As already stated, the results reported in this chapter could have important biological implications with respect to the production and stabilization of Cr(V) species within the body.

In addition, there may be the potential for using a similar strategy for the production, and possible isolation, of Cr(V) complexes of ligands other than those which react with Cr(VI) directly, initial reaction with GSH yielding a substitutionally labile Cr(V) complex.

CHAPTER 6. PRODUCTION OF Cr(V) ON REACTION OF Cr(VI) WITH
PYRIDOXINE (VITAMIN B₆).

6.1. Introduction

Vitamin B₆ (pyridoxine, 3-hydroxy-4,5-dihydroxymethyl-2-methylpyridine, fig.6.1.) is an essential component of our diet, and is widely distributed in foods [102].

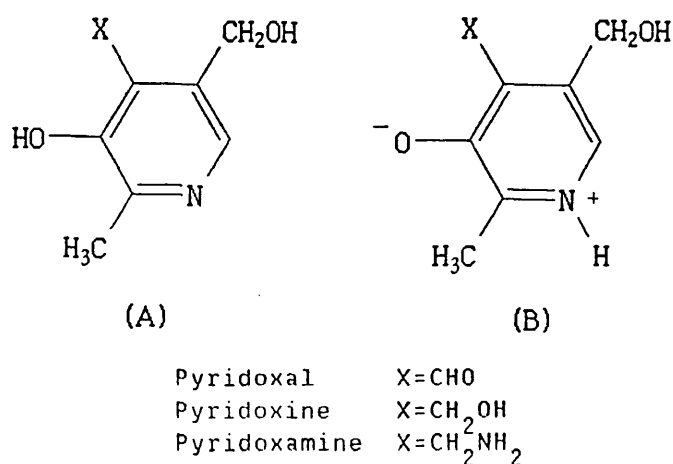


Fig.6.1. The structure and tautomeric forms of the B₆ vitamins.

It was recognised that vitamin B₆, as the coenzyme pyridoxal-5-phosphate, is concerned with a wide variety and a vast number of enzyme systems, associated almost entirely with nitrogen metabolism.

The most striking symptom of severe vitamin B₆ deficiency in man is the onset of convulsions, although with rats, the occurrence of a facial dermatitis has also been observed.

In animal tissues and products, pyridoxine is largely in the form of pyridoxal, pyridoxamine (fig.6.1) and their phosphate esters, whereas plant tissues contain mostly pyridoxine which acts as root growth hormone.

The CHO and CH₂NH₂ groups of pyridoxal and pyridoxamine are

labile, and excessive exposure to heat or light can result in destruction of the molecule [102]. The more stable pyridoxine is not so sensitive, although rapid decomposition by light does occur in neutral or alkaline solution [103].

In aqueous solution, pyridoxine exists mainly in the zwitterionic form (B) (fig.6.1), as a consequence of the relative pK_a values of the ring N ($pK_a=8.79$) and the phenol OH ($pK_a=8.20$) [104]. This form of pyridoxine is often encountered in its metal complexes, many of which have been prepared and characterized.

Complexes of the type $[Co(bipy)_2L](ClO_4)_2$ (I) and $[Cu(bipy)-(HL)Cl](ClO_4).H_2O$ (II), (HL=pyridoxine), have been prepared and characterized [105]. Both structures were found to be monomeric with octahedral geometry around Co(III) and distorted square pyramidal around Cu(II). In (I), two atoms from the phenolic and 4-(hydroxymethyl) (4-HM) groups of L and two N atoms from each of the two bipy groups form the coordination sphere. In (II), one HL and one bipy coordinate with the same atoms as (I) acting as bidentate chelates in the basal plane, the Cl occupying the apical site. In both (I) and (II) HL and L exist in the tautomeric form in which the pyridine N is protonated and phenolic OH deprotonated. A novel feature of (I) is that L is anionic due to the deprotonation of the 4-HM group.

With vanadium [106] a dimeric complex bis(μ -pyridoxinato)-(dioxovanadium(V)) is formed with pyridoxine. Two VO_2 fragments are connected by two chelate bridging pyridoxinato anions in which N is not coordinated but is protonated, the OH and 4-HM groups both being deprotonated.

Two complexes; $\text{Cu}(\text{HL})_2(\text{NO}_3)_2$ (III) and $\text{Cu}(\text{L})(\text{HL})(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$ (IV) are produced on reaction of $\text{Cu}(\text{NO}_3)_2$ with pyridoxine [107]. In (III) the Cu has square planar coordination with the phenol OH and the 4-HM groups of two HL ligands in their zwitterionic form. Complex (IV) is polymeric with the HL ligand as the zwitterion as in (III), and the L^- ligand acting as a tridentate bridge through its O(3), O(4) and N(1) atoms [107].

Mixed Cr(III)-oxalato complexes with pyridoxine have been prepared and characterized, the ligands acting in a bidentate (deprotonated) fashion [108].

Complexes of the type $[\text{Zn}(\text{HL})_2(\text{OAc})_2] \cdot 6\text{H}_2\text{O}$ (V), $[\text{Zn}(\text{HL})_3] \cdot (\text{SCN})_2 \cdot \text{H}_2\text{O}$ (VI) and $\text{ZnL}_2 \cdot 4\text{H}_2\text{O}$ (VII) have been prepared on reaction of Zn salts with pyridoxine. In (V) and (VI), pyridoxine is bidentate with coordination through the phenolic O and the protonated 4-HM group. The coordination of (VII) is as for (V) and (VI) except that the phenolic groups are deprotonated [109].

The synthesis and IR spectra have been reported of Cd, Mn and Zn complexes containing the family of B_6 vitamins in various protonation states [110]. In the complexes with neutral HL, the ligand exists in the zwitterionic form with the ring N protonated. The M ions are chelated through the usual phenol and 4-HM groups.

The crystal and molecular structure of $\text{Cd}(\text{HL})_2\text{Cl}_2$ has been found to consist of infinite chains with Cd bridged by Cl atoms. Cd has an octahedral coordination being bound to three Cl atoms and three O atoms of HL, HL being bidentate through two O atoms. At pH 7.0, HL reacts with Cd as the zwitterion to give the 1:1 chelate [111].

6.2. Experimental

In these studies, the pyridoxine.HCl adduct was used in place of the free base and the source of Cr(VI) was anhydrous $\text{Na}_2\text{Cr}_2\text{O}_7$.

In all cases, care was taken to avoid excessive exposure of reaction solutions or solids to light due to the sensitivity of pyridoxine.

Reactions in solution were carried out in H_2O or DMSO using a variety of reactant ratios and pHs (aqueous). After mixing the reactant solutions, the X-band epr, mobile solution spectrum was immediately recorded and at time intervals thereafter. The general procedures for solution preparation and spectrum measurement have been given previously (chapter 2).

On carrying out the reactions in DMSO, solids were isolated by the addition of toluene, removal of the supernatant liquid and washing with acetone. These solids were analysed by solid state epr spectroscopy, but elemental microanalysis did not yield consistent results.

Repeating the Cr(VI)-pyridoxine.HCl (1:2) reaction in acetone gave a high yield of Cr(V)-containing solid, the method of its preparation is given below.

To finely ground, anhydrous $\text{Na}_2\text{Cr}_2\text{O}_7$ (30mg, 1.15×10^{-4} mol) in anhydrous acetone (5ml) was added pyridoxine.HCl (94mg, 4.58×10^{-4} mol). The sealed vessel was then shaken in the dark for six hours after which a fine brown precipitate had been deposited, the supernatant solution being dark red in colour.

The brown solid was quickly filtered off, washed with dry

acetone and air dried in the dark. The IR spectrum ($4000-400\text{cm}^{-1}$, KBr plates, nujol mull) was then recorded as well as the X-band epr spectrum both as a solid and dissolved in H_2O , D_2O or DMSO (10mg in 2ml)(room temp.). The frozen solution (77K) spectrum was recorded in H_2O with subsequent double integration .

Double integration of the solid phase spectrum was also carried out, the Cr(V) concentration being calculated by comparison with a standard Cr(V) concentration obtained with a known weight of sodium bis(2-hydroxy-2-ethylbutyrato)oxochromium-(V).

The reactions of Cr(VI) with 2-hydroxybenzyl alcohol (2-HBA) were carried out in both H_2O and anhydrous acetone as described for pyridoxine.HCl.

With both pyridoxine.HCl and 2-HBA, attempts were made to precipitate the Cr(V) complex from solution by the addition of excess PF_6^- or Cl^- , neither was successful in giving a solid sample.

6.3. Results

Reaction of Cr(VI) with pyridoxine.HCl in aqueous solution (1:5 mole ratio, pH7.0) produced a very small Cr(V) signal in the mobile solution X-band epr spectrum. The signal appeared to exhibit nuclear superhyperfine structure but the low signal to noise ratio coupled with rapidly decreasing signal size prevented an accurate observation.

Repeating the reaction without pH alteration from mixing (~3) gave rise to a much stronger Cr(V) signal at $g=1.976$. ^{53}Cr hyperfine bands were seen ($a_{\text{Cr}}=20\text{ G}$) as was super nuclear

hyperfine structure with a 1:2:1 peak height ratio (Spec.A,fig.6.2.). With time, this signal appeared to be losing the structure resolution and after four hours the structure was still present but was no longer in an ordered ratio (Spec.B, fig. 6.2.). At this stage the signal as a whole was slowly decaying in intensity.

Re-recording the spectrum after 24 hours showed no Cr(V) signal, only a broad Cr(III) band centred at $g=1.98$. The solution was then a strong green colour, in contrast to the original orange colour which gained a brown colouration soon after mixing (20 min.).

The reaction was repeated under the above conditions but in D_2O . The mobile solution spectrum recorded immediately after mixing showed a Cr(V) signal with the 1:2:1 super hyperfine structure. After 90 minutes, the splitting had become less well resolved and after 180 minutes the structure had totally disappeared from the Cr(V) signal, an isotropic band at $g=1.975$ (peak-to-peak 2.8 G) then being present. After this time the reaction solution had turned from brown to green.

This reaction was repeated with pyridoxine.HCl pre-dissolved in D_2O and left for 18 hours in the dark before mixing. The epr spectrum of the mobile solution immediately after mixing showed the Cr(V) signal with 1:2:1 superhyperfine structure, the splittings being removed within three hours.

At a 1:1 mole ratio in aqueous solution, the Cr(VI)/pyridoxine.HCl reaction gave similar results to those at the 1:5 ratio except that the Cr(V) signal observed was of lower intensity.

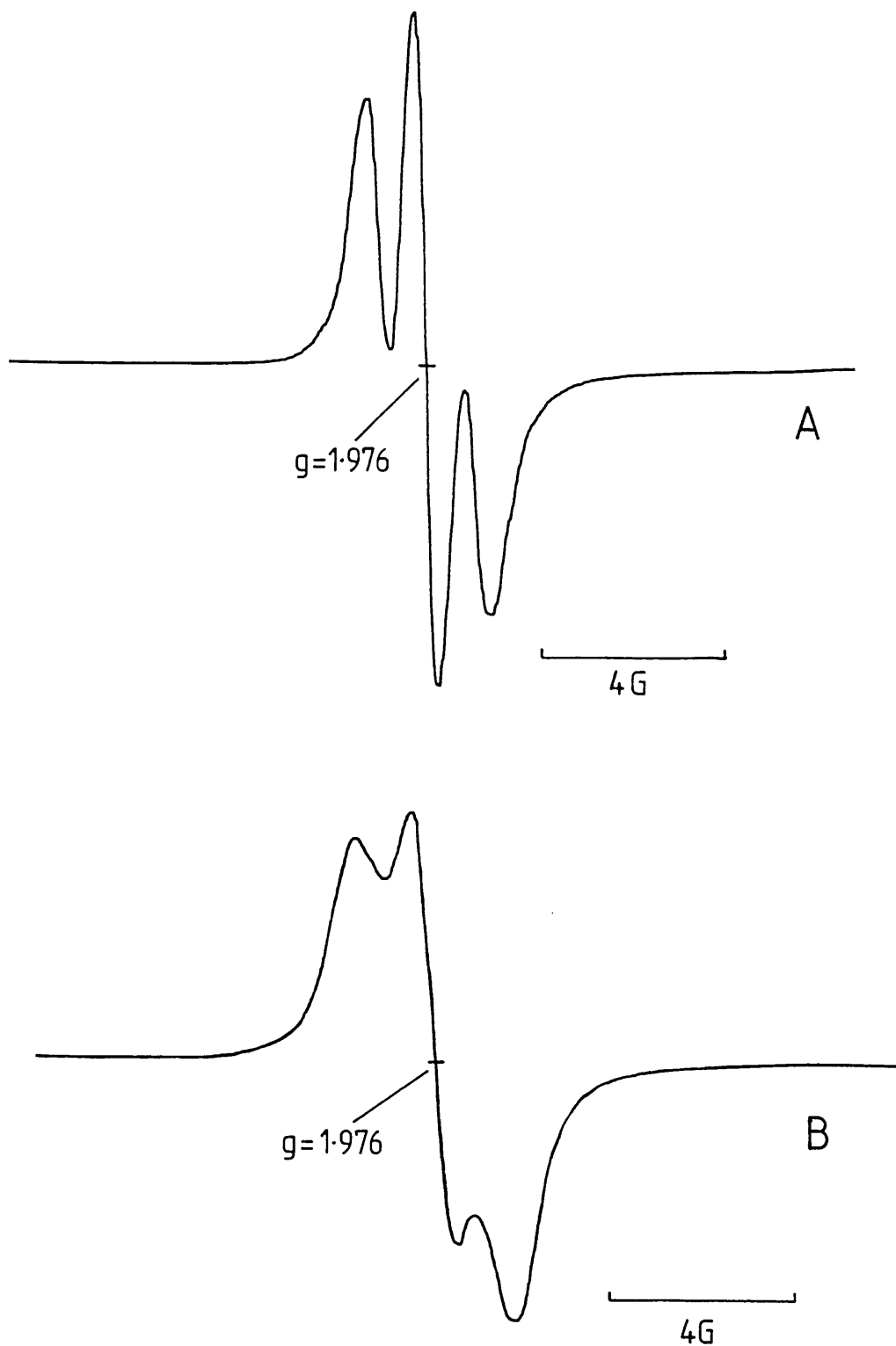


Fig. 6.2. X-band epr spectra of an aqueous mobile solution of Cr(VI) + pyridoxine.HCl (pH 3)
Spec. A immediately after mixing,
Spec. B four hours after mixing.

In DMSO, the 1:1 reaction gave a strong Cr(V) signal at $g=1.974$ immediately after mixing. The signal exhibited super nuclear hyperfine structure as seen in aqueous solution after four hours (Spec. B). This structure did not change with time, the Cr(V) signal increasing in intensity. The originally orange solution quickly turned brown, this colouration remaining on leaving for three days in the dark. After this time, no Cr(V) species remained in solution.

Addition of toluene to this solution separated a dark brown oil. Removal of the supernatant followed by washing with acetone yielding a brown solid, which was collected, washed and air dried. The Q-band ($\sim 34\text{GHz}$) epr spectrum of this solid showed the presence of a single broad Cr(III) band (peak-to-peak 524 G) centred at $g=1.977$. No further work was carried out on this compound.

The reaction of Cr(VI) with pyridoxine.HCl in DMSO at a 1:2 ratio gave rise to a strong Cr(V) signal at $g=1.974$ with structure very similar to that of Spec. B. The signal grew with time and, as at the 1:1 ratio, the reaction colour changed from orange to brown. However, on leaving in the dark for 18 hours, the solution had turned green, no Cr(V) species being present, only a broad Cr(III) epr band.

Addition of toluene to this solution deposited a green oil/syrup, removal of the supernatant followed by washing with acetone producing a green solid. On allowing to stand in the air for a short time, this solid quickly reverted to the syrup, rewashing with acetone recovering the solid.

Attempted recrystallizations of the solids produced in DMSO at both 1:1 and 1:2 mole ratios were unsuccessful.

The reaction of Cr(VI) with pyridoxine.HCl (1:2) in anhydrous acetone produced a fine brown solid. The X-band epr spectrum (RT) of this solid showed a very strong Cr(V) signal centred at $g=1.974$ (peak-to-peak 32.8 G). There was no superhyperfine structure of this signal and no ^{53}Cr hyperfine bands could be seen.

On leaving the solid in the dark for 24 hours a distinct colour change from brown to green occurred. The epr spectrum of this solid sample showed the presence of a broad Cr(III) band at $g=1.98$. Storing the solid under a nitrogen atmosphere overnight did not prevent the observed colour change from taking place. No further studies were made on this sample.

Dissolution of the original solid in H_2O followed by immediate measurement of the epr spectrum (mobile) showed a strong Cr(V) signal at $g=1.974$ exhibiting superhyperfine structure with a 1:2:1 peak height ratio. ^{53}Cr hyperfine bands were observed with 20 G spacing. The Cr(V) signal intensity was slowly decreasing with time, the solution colour changing from brown to yellow/green. The 1:2:1 structure remained unchanged for the period of observation of the Cr(V) signal (~3 hours).

When in D_2O , the solid exhibited the Cr(V) signal with 1:2:1 structure immediately after dissolution. Re-recording the spectrum after 90 minutes showed the signal to be unchanged except for its lower intensity. After 180 min. the spectrum was very small indeed but the 1:2:1 structure was still present.

The frozen solution (77K) spectrum of a known concentration

of solid in H_2O showed an apparently isotropic signal at $g=1.976$. Computer double integration of this spectrum showed approximately 25% of the solid sample to be Cr(V). Double integration of the Cr(V) signal of a known weight of solid, followed by comparison with a standard Cr(V) solid spectrum showed approximately 40% of the solid sample to be in the form of Cr(V).

The mobile solution epr spectrum of the solid in DMSO gave a Cr(V) signal of type B., its intensity decreasing with time.

The IR spectrum of the solid ($4000cm^{-1}$ to $400cm^{-1}$) showed a sharp band at $\nu=1535cm^{-1}$ representative of a protonated ring nitrogen, the position of the equivalent band in pyridoxine.HCl being $1550cm^{-1}$. Similarly, two bands at $\nu=1630$ and $1620 cm^{-1}$ were present in the sample compared to only one sharp band at $\nu=1630cm^{-1}$ in the ligand. There were no additional IR bands in the complex spectrum in the Cr=O region ($\nu=900$ to $1050 cm^{-1}$).

The reaction of Cr(VI) with 2-hydroxybenzyl alcohol (2-HBA) in aqueous solution (1:5, pH 7.0) gave no Cr(V) species immediately after mixing or after several hours at room temperature. Repeating the reaction without changing the pH from that on mixing (~ 4.5) or raising the pH to 9.0 also produced no reaction. After three days there was no evidence for Cr(VI) reduction.

On mixing Cr(VI) with 2-HBA in anhydrous acetone, the solution turned brown within ten minutes. The mobile solution, X-band epr spectrum of this solution showed the presence of a strong Cr(V) signal at $g=1.975$ (peak-to-peak 4 G). There was no sign of super hyperfine structure of this signal but ^{53}Cr

hyperfine bands were observed, with 20 G spacing.

This signal was apparently constant in size with time and re-recording the spectrum after three hours showed no change. Leaving the solution in the dark for seven days showed the presence of the Cr(V) band in the centre of a broader Cr(III) band.

In the reactions with both pyridoxine.HCl and 2-HBA which produced larger than normal amounts of Cr(V), attempts to precipitate the Cr(V) complexes by the addition of the anions PF_6^- and Cl^- and were unsuccessful.

6.4. Discussion

Depending on the reactant ratios employed, the reaction of Cr(VI) with pyridoxine.HCl in DMSO produced one of two Cr(III) complexes on extraction from solution. On employing a 1:1 reactant ratio, a brown solid exhibiting a broad Cr(III) epr resonance was formed. However at a Cr(VI):pyridoxine.HCl ratio of 1:2 the complex isolated was green and apparently hygroscopic.

No further studies were made on these complexes but it would be reasonable to suggest that at the 1:1 ratio a 1:1 metal-ligand complex is being produced, and that on increasing the amount of pyridoxine.HCl a 1:2 complex is formed. The hygroscopic nature of the latter caused difficulties in its study. The first of these may be polymeric in nature, similar to the Cu(II) complexes mentioned previously [107].

The reaction of Cr(VI) with pyridoxine.HCl (1:5, pH 3.0) in aqueous solution gave rise to a Cr(V) species exhibiting a characteristically sharp epr signal with super hyperfine

structure of 1:2:1 relative peak heights. The observed ^{53}Cr hyperfine spacing of 20 G and band position of $g=1.976$ are consistent with the Cr(V) centre being in an oxygen donor environment, as previously encountered with sugars and ascorbate. Of note here is the lack of superhyperfine structure with 1:1:1 peak ratios as would be expected if the ring nitrogen was one of the coordinating atoms.

The 1:2:1 peak ratios are consistent with the Cr(V) centre interacting with two equivalent protons ($a_H=1$ G). With time, the structure changed its form until, after four hours, spec. B was observed, in which there was no longer any order in the relative peak heights.

Both of these results mimic quite closely the observations made on the reaction of Cr(VI) with a series of sugars (chapter 4). With the sugars, it was suggested that the spectral changes were due to formation of additional Cr(V) species resulting from changes in the conformational equilibrium of the sugars on complexation. This is unlikely to be the case with pyridoxine since it does not exist in such an equilibrium. Another difference is that the structure of the Cr(V)-sugar signal was due to non-exchangeable protons, whereas with pyridoxine, the spectral structure was removed on running the reaction in D_2O showing it to be a result of adjacent exchangeable protons, probably on the pyridoxine hydroxyl groups.

The loss of this 1:2:1 pattern in H_2O after four hours is probably due to the formation of an additional Cr(V) species, the structure of which is unknown.

On repeating the above reactions in DMSO, a Cr(V) species

showing the more complex structure of spec.B was immediately produced. This suggests that in H_2O (or D_2O), the solvent is acting to stabilize the Cr(V) complex formed initially, or to prevent the rapid formation of the additional Cr(V) species. In DMSO, this suppression of reaction is not occurring.

Acetone was the only solvent which allowed the rapid formation of a solid on reaction of Cr(VI) with pyridoxine.HCl, the proportion of Cr(V) present in this solid being two or three orders of magnitude greater than that produced in any of the previous reactions in aqueous solution. Of note here is that acetone is the solvent employed by Rocek [33] in the preparation of the isolable bis(2-ethyl-2-hydroxybutyrate)oxochromium(V).

Dissolution of the solid produced here in H_2O gave a Cr(V) band with 1:2:1 superhyperfine structure in the mobile solution epr spectrum. However, unlike the situation observed in the reaction of Cr(VI) with pyridoxine.HCl in H_2O , the structure of this band did not change with time, exactly the same signal being present (of much lower intensity) after four hours. This suggests that the changes of the 1:2:1 spectrum in the reaction solution are due to further redox changes, these not being possible with the complex alone in solution.

The amount of Cr(V) present in the solid was determined by double integration of the frozen solution (77K) and solid sample (R.T.) epr spectra. In solution, it was found that 20% of the total Cr content was present as Cr(V). However, it was felt that this value may actually be lower than that actually present, the Cr(V) signal size decreasing with time immediately after

dissolution suggesting Cr(V) disproportionation. Hence a double integration calculation was made on the solid for comparison, no such decomposition being significant in this form. This yielded a Cr(V) content of 40% and it is felt that further reactions with this system may eventually produce a truly isolable Cr(V) complex with a biologically relevant ligand, the first such complex.

The Cr(V) signal of the solid complex was considerably broader (peak-to-peak 32.8 G) than those observed in solution. This is most probably caused by the high Cr(V) concentration in the solid, resulting in paramagnetic interactions between the Cr(V) centres, which is observed as a removal of band structure and band broadening.

The frozen solution epr spectrum did not give much structural information about the Cr(V) complex, an apparently isotropic signal similar to those obtained with the sugars being seen.

Of interest here is the observation that the X-band epr spectrum of a solution of the solid in D₂O (R.T.) gave the 1:2:1 type signal. However, this structure was not removed, as would be expected due to H-D exchange, on leaving for three or more hours. This suggests that non-exchangeable protons are responsible for the splitting, which is in direct contradiction to the results obtained on running the Cr(VI)-pyridoxine.HCl reaction in D₂O which showed exchangeable protons to be responsible.

The reason for this difference is not clear but it may be related to the fact that while in the reaction solution, the Cr(V) is probably at a steady state concentration, in a constant state of decay and production. The disappearance of the super

hyperfine structure is therefore a consequence of the time taken to deuterate the relevant pyridoxine hydroxyl groups before complexation to the metal centre. This is not possible with the isolated Cr(V) complex since reaction will not continue on dissolving in H₂O, deuteration of the bound hydroxyl groups apparently being more difficult than in the free ligand.

One problem with this proposed mechanism is the observation that pre-dissolution of pyridoxine in D₂O 18 hours before reaction with Cr(VI) still produced the Cr(V) signal exhibiting the 1:2:1 structure. If the above argument was true, this time delay should have allowed enough time for the H-D exchange of the ligand hydroxyl groups. The Cr(V) signal observed immediately after mixing should then exhibit no proton super hyperfine structure.

The IR spectrum of the isolated solid showed the presence of a band at $\nu=1535\text{cm}^{-1}$. A strong peak in the $\nu=1525$ to 1545cm^{-1} area is indicative of the presence of a protonated ring N [110]. If this peak were missing, the pyridoxine would be either in the anionic form or in a tautomeric state not involving the pyridinium ion.

This band is also present in pyridoxine.HCl itself but at $\nu=1550\text{cm}^{-1}$, the observed shift to lower wavenumber possibly being due to coordination to Cr(V) via oxygen, the N remaining protonated.

The lack of absorption at $\nu=1550\text{cm}^{-1}$ suggests that the brown solid isolated does not contain any unreacted pyridoxine.HCl. Unfortunately, no firm evidence was found from the IR spectrum of

the presence of a Cr=O unit.

From the experimental data obtained, it is proposed that the structure of the Cr(V)-pyridoxine complex is as shown in fig.6.3.

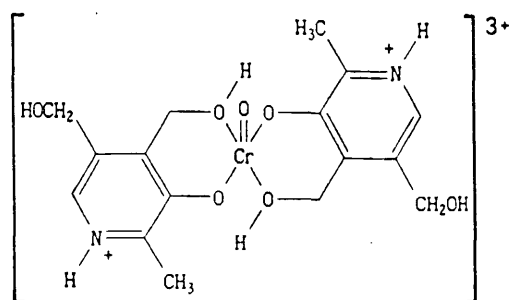


Fig.6.3. Proposed structure of the Cr(V)-pyridoxine complex.

In this proposal the ligand is coordinating in its neutral, zwitterionic form, the ring N being protonated. Coordination takes place via the phenol and 4-hydroxymethyl groups of two ligands to give the usual square pyramidal, oxygen donor environment. Deprotonation of the 4-hydroxymethyl group has not occurred and it is proposed that these two protons are responsible for the 1:2:1 super hyperfine structure of the Cr(V) band.

This form of pyridoxine binding has been observed previously with many metals, examples of these being given in the introduction to this chapter.

Studies with 2-hydroxybenzyl alcohol were made since its substitution pattern is the same as that involved in the proposed mode of pyridoxine coordination. It was hoped that using a model compound would give information of the mode of binding and possibly allow the isolation of a pure Cr(V) complex. Neither of these hopes were actually fulfilled. There was no reaction at all

between Cr(VI) and 2-HBA in aqueous solution at a variety of pH values and reactant ratios. However, repeating the reaction in anhydrous acetone gave a relatively high Cr(V) yield.

Why there should be no reaction in aqueous solution is puzzling and the reason behind the initiation of reaction in acetone unknown. One interesting observation was the lack of superhyperfine structure of the observed Cr(V) band, suggesting both coordinating hydroxyl groups to be deprotonated, in contrast to the mode seen with pyridoxine itself.

The high yields of Cr(V) isolated on reaction of Cr(VI) with pyridoxine.HCl in anhydrous acetone suggests that it may be possible to prepare pure Cr(V) complexes with members of the B6 family of vitamins. This would be of great interest since to date, there have been no successful isolations of Cr(V) complexes with biologically relevant ligands.

From a biological point of view, the stabilization of Cr in the +5 oxidation state is an important observation. Pyridoxine is not so widespread in the body as, for example, glutathione, but it is present in many different forms which are crucial to the correct functioning of bodily functions. Hence, reaction with Cr(VI) forming Cr(V) and Cr(III) complexes could be of great importance in the damaging action of chromate.

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PART 2

STUDIES INTO THE USE OF TRANSITION METAL
COMPLEXES AS HYPOXIC CELL RADIOSENSITIZERS.

Abbreviations

bipy = bipyridine

BSA = bovine serum albumin

CAT = catalase

CHO = Chinese hamster ovary

DMF = dose modification factor

EDTA = ethylenediaminetetra-acetic acid

ER = enhancement ratio

Fc = ferrocene

fcs = foetal calf serum

Fc⁺ = ferrocenium

GSH = reduced glutathione

MEM = minimum essential medium + 10% fcs

METRO = metronidazole

MISO = misonidazole

NADPH = β -nicotinamide adenine dinucleotide phosphate
(reduced form)

NPSH = non-protein thiol

o-phen = 1,10-phenanthroline

pbs = phosphate buffered saline

SF = surviving fraction

t-pFc = tert-pentyl ferrocene

TCA = trichloroacetate

7.1 INTRODUCTION.

7.1.1. Background

Cancer, in a variety of forms, is a major cause of death in today's society, second only to heart disease.

At present there are three main approaches to its treatment. These are listed below and can be administered individually or in combination.

- (1) Surgery
- (2) Chemotherapy
- (3) Radiotherapy

Surgery is self explanatory and involves the physical removal of the cancerous tissue. Should any of the tumour cells remain however, the tumour could possibly re-grow; hence to be effective it is often used in conjunction with (2) or (3).

Chemotherapy requires the administration of a cytotoxic drug, i.e. one that kills the diseased cells and destroys the tumour.

There are vast numbers of organic compounds which have been tested for anti-tumour activity. Metal complexes have also been found to be effective, the most notable of these being cis-platin (cis-diamminedichloroplatinum(II)) which is being used successfully to combat ovarian, testicular and bladder cancers [1].

Radiotherapy [2,3] involves the selective killing of localized tumour cells by means of focussed beams of ionizing radiation, usually gamma-radiation or X-rays. It is this form of cancer therapy which has been studied in this section of work and what follows is a description of the technique, outlining its

associated problems and devised solutions, along with the cellular mechanisms proposed in this form of treatment.

7.1.2. Radiation damage to tissue

Any beam of radiation used in radiotherapy, of whatever type, either charged particles or high energy photons, passes through body tissue and interacts with the constituent atoms and molecules. The absorbed dose measured in grays (1 Gy = 100 rad = 1 J/Kg) accumulates when energy is transferred from the radiation beam to the tissue, leading to excitation and ionization of the target biomolecule. The most important consequence of ionizing radiation on a tumour is the destruction of individual cancer cells, this cell killing being the biological consequence of a complex series of reactions occurring at the physical, chemical, biochemical and cellular level [4]. Radiation biological events start at the time of irradiation with energy deposition followed by physical effects ($<10^{-13}$ sec.), chemical reactions of the primary and secondary free radicals and excited species ($<10^{-3}$ sec.), biochemical effects of metastable products (hours) and finally biological effects, for example, cancer (years).

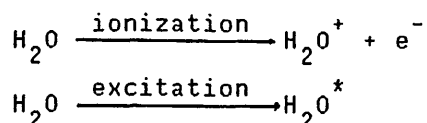
7.1.3. Mechanisms of tissue damage

Radiation damage to biological materials can arise from either direct or indirect action, the former being energy absorption by the tissue biomolecules themselves, the latter being absorption by the surrounding H_2O , the reactive species

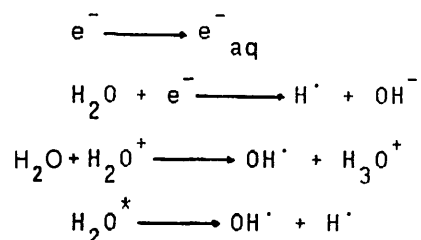
thus produced then causing the damage.

Water is the main constituent of most living organisms comprising 65% of cellular and multicellular biological material. A knowledge of the effects of ionizing radiation on the aqueous portion of the cell is therefore fundamental to an understanding of the biological effects of radiation by indirect action [5].

The deposition of energy in H_2O by a beam of ionizing radiation has the following effects,



Three highly reactive primary species result, solvated electrons (e^-_{aq}), atomic hydrogen ($H^\cdot$) and hydroxyl radicals ($OH^\cdot$)



In aerated solution, both reducing species e^-_{aq} and $H^\cdot$ react at diffusion controlled rates with O_2 producing the superoxide radical. Although all of the species produced are very reactive towards many biological molecules, there is evidence that only the $OH^\cdot$ oxidizing species is important in initiating damage in biological systems, its damage resulting from either H-abstraction or $OH^\cdot$ addition to the target molecule producing reactive redox intermediates, and ultimately giving rise to DNA strand breakage.

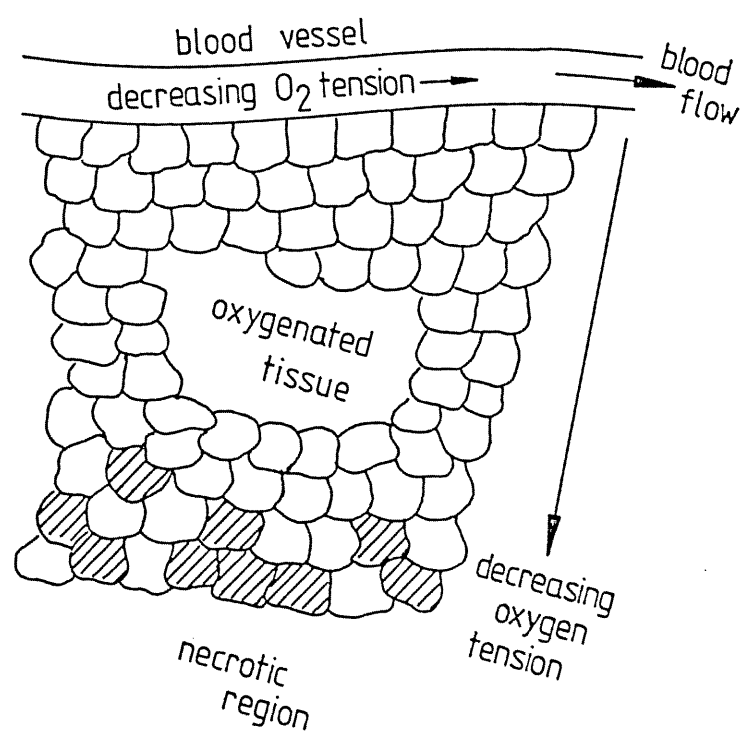
It is the prevailing view that damage to the DNA structure of cells is of much greater importance than the damage which may occur to other cellular components since it seems probable that events such as mutagenesis, carcinogenesis and lethality have their molecular basis in DNA modification.

A recent series of papers by Symons et al. [6-9] has investigated the effects of ionizing radiation on DNA samples under a variety of conditions and in the presence of selected radiation modifiers. Symons subscribes to the view that the actual concentrations of DNA in the nuclei of mammalian cells do not approximate to a dilute aqueous solution, as would be necessary for indirect damage to be dominant, and that direct ionization of DNA, or in the sheath of water molecules surrounding DNA, may constitute a major source of damage. Results from his work suggest that irradiation of DNA samples in frozen aqueous solution gives rise to localization of separated charges on the guanine (+ve) and thymine (-ve) base units of the DNA, these being the primary products of direct damage.

7.1.4. The problem of tumour hypoxia

A comprehensive review of the impact of tumour hypoxia on cancer therapy has recently been published [10]. The description given here covers the important aspects of the problem but reference should be made to [10] for a more detailed account.

It is believed that many solid tumours have necrotic regions and adjacent areas of hypoxic cells (fig.7.1). These hypoxic



○ = Oxic cells
 ◐ = Hypoxic cells

Fig. 7.1. Model of hypoxic cell distribution in tumours.

cells arise because the rate of growth of the tumour outstrips the growth of its associated vascular supply. As a consequence, there can be areas of tumour that are oxygen deficient, that is hypoxic. This is supported by measurements of average oxygen tension of tumours showing it to be lower than those of normal tissue.

The problem which is now encountered is that these hypoxic cells have been found to be approximately three times more radiation resistant than oxic cells. Hence if any hypoxic tumour cell should survive the radiation treatment and then become re-oxygenated, it could lead to a recurrence of the disease.

This radiation resistance of hypoxic cells in tumours is one of the major limitations on the success of radiotherapy. In addition, it is believed that these areas of hypoxia may also contribute to the resistance to some chemotherapeutic agents, e.g. bleomycin [11] and adriamycin [12].

A proposed mechanism underlying the apparent radiation-sensitivity of well oxygenated, relative to hypoxic cells, is given below (fig.7.2)[13].

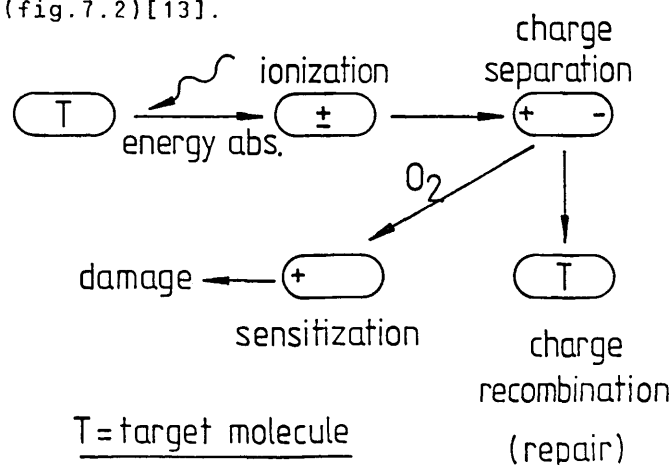


Fig. 7.2. Mechanism of sensitization of hypoxic cells

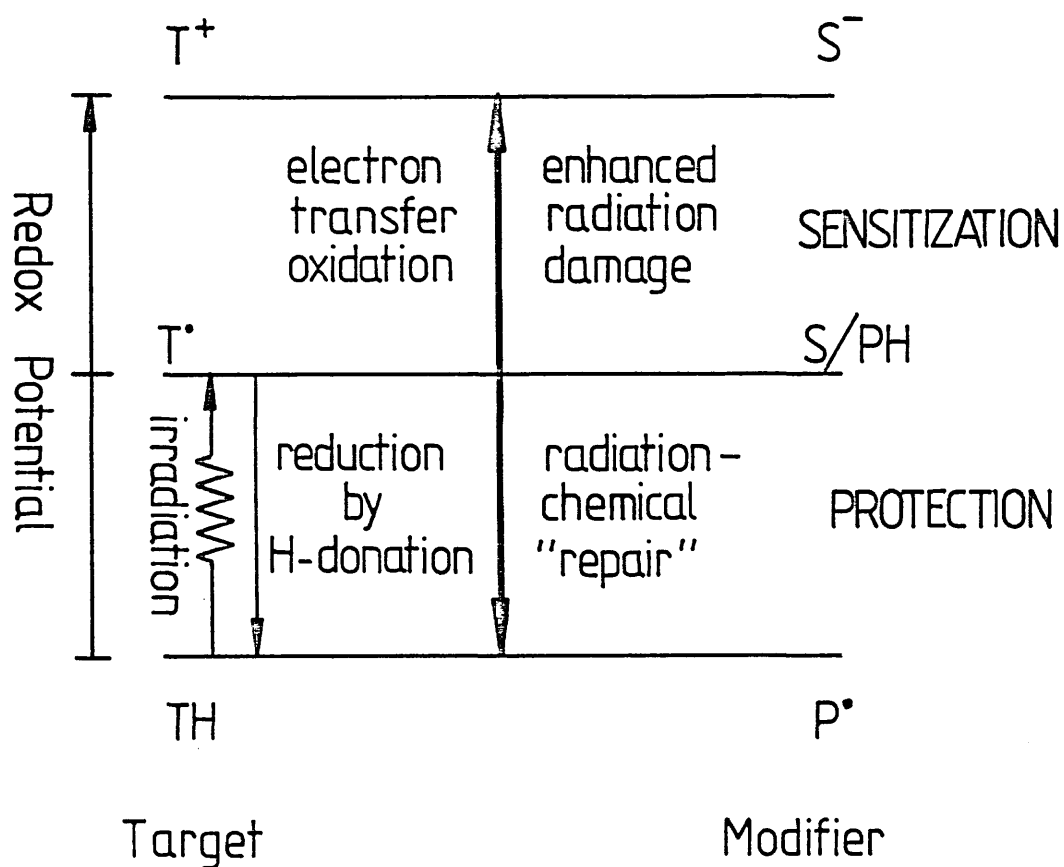
Direct energy absorption from the radiation by the target molecule takes place producing an ion pair. This may then separate, the individual charges localizing at favourable positions elsewhere in the molecule, which if DNA, would likely be the G⁺ and T⁻ positions, as found by Symons. Now, if a sensitizer of sufficiently high electron-affinity was nearby, in this case oxygen, then charge transfer from the polarized target molecule to the sensitizer could occur. This prevents the possibility of charge recombination and subsequent repair, thereby causing damage to the DNA.

Model chemical studies were undertaken [3] to probe the molecular mechanisms and chemical kinetics of radiation damage via free radical reactions, which ultimately lead to cell killing.

The kinetic scheme proposed from these studies describes the mechanism of action of chemical radiation sensitizers and protectors in terms of free radical redox reactions and is given below (fig.7.3 ,from ref.[3])

In this scheme, the target biomolecule (TH) is excited on irradiation and undergoes H-abstraction to give the radical T'. One of two events can now occur depending upon the cellular status.

In hypoxic cells the target molecule is regenerated via H-donation from available radioprotectors yielding TH and P', the latter being relatively unreactive species. In most cases, these protecting species are cellular reductants, in particular thiol molecules.



S = Sensitizer

PH = Protector

TH = Target biomolecule

Fig. 7.3. A redox model for modification of radiation damage

In oxic cells however, (or in the presence of a radiation sensitizer) the radical $T^{\cdot}$ can react rapidly with available oxygen to give T^{+} and superoxide. In other words, the initial damage to the target molecule has been fixed by electron transfer oxidation to the sensitizer and, since this is irreversible, the cell has been killed. The oxygen has acted as a radiosensitizer by enhancing the fixation of potentially harmful radical damage.

The scheme also shows that the redox potential of the modifier has a bearing on the sensitization or protection observed, the more positive the potential the more efficient the drug action thereby altering the concentration of drug required to cause the damage.

7.1.5. Overcoming tumour hypoxia

The only way to improve local tumour control by radiation lies in seeking ways to enhance selectively tumour cell killing with respect to normal tissue. Three principal approaches have been employed to improve this local control and combat the problem of hypoxia.

- (1) Hyperbaric oxygen treatment where the oxygen tension within the cells is boosted prior to irradiation.
- (2) High L.E.T. (linear energy transfer) therapy in which the differential radiosensitivity of oxic and tumour cells is reduced.
- (3) Chemical Radiosensitization in which oxygen mimetic drugs are administered prior to irradiation to selectively sensitize the hypoxic tumour cells.

Hyperbaric oxygen treatment attempts to produce an increase in

the oxygen tension in the blood and enable it to reach previously hypoxic regions. The patient breathes oxygen at a higher than atmospheric pressure. The levels of success obtained with this treatment have been limited [14] and the practical difficulties involved in conjunction with undesirable side-effects limit its widespread use.

High L.E.T. radiation treatment [15] uses the observation that the oxygen enhancement ratio decreases with increasing ionization intensity of radiation, with, for example, neutrons. This results in removal of the oxic / hypoxic cell differential. Thus the protective effect of the tumour hypoxia is reduced. This technique is limited by the necessity for specialized and hence expensive equipment.

The third approach is receiving most attention at present on the grounds of simplicity and economy, is the use of hypoxic cell radiosensitizers.

To be useful clinically, a hypoxic cell radiosensitizer should be effective at a dose level which is relatively non-toxic to normal tissue. Similarly, the sensitization and/or toxicity in aerobic cells should either be minimal or should become minimal at a dose level at which hypoxic cell sensitization remains effective.

7.1.6. Quantification of a radiosensitizer's efficiency

Fig. 7.4 shows the cellular survival curves obtained on irradiation studies of mammalian cells in-vitro under oxic (O_2) and hypoxic (N_2) conditions. In both situations, cell survival as a function of radiation dose is characterised by an initial

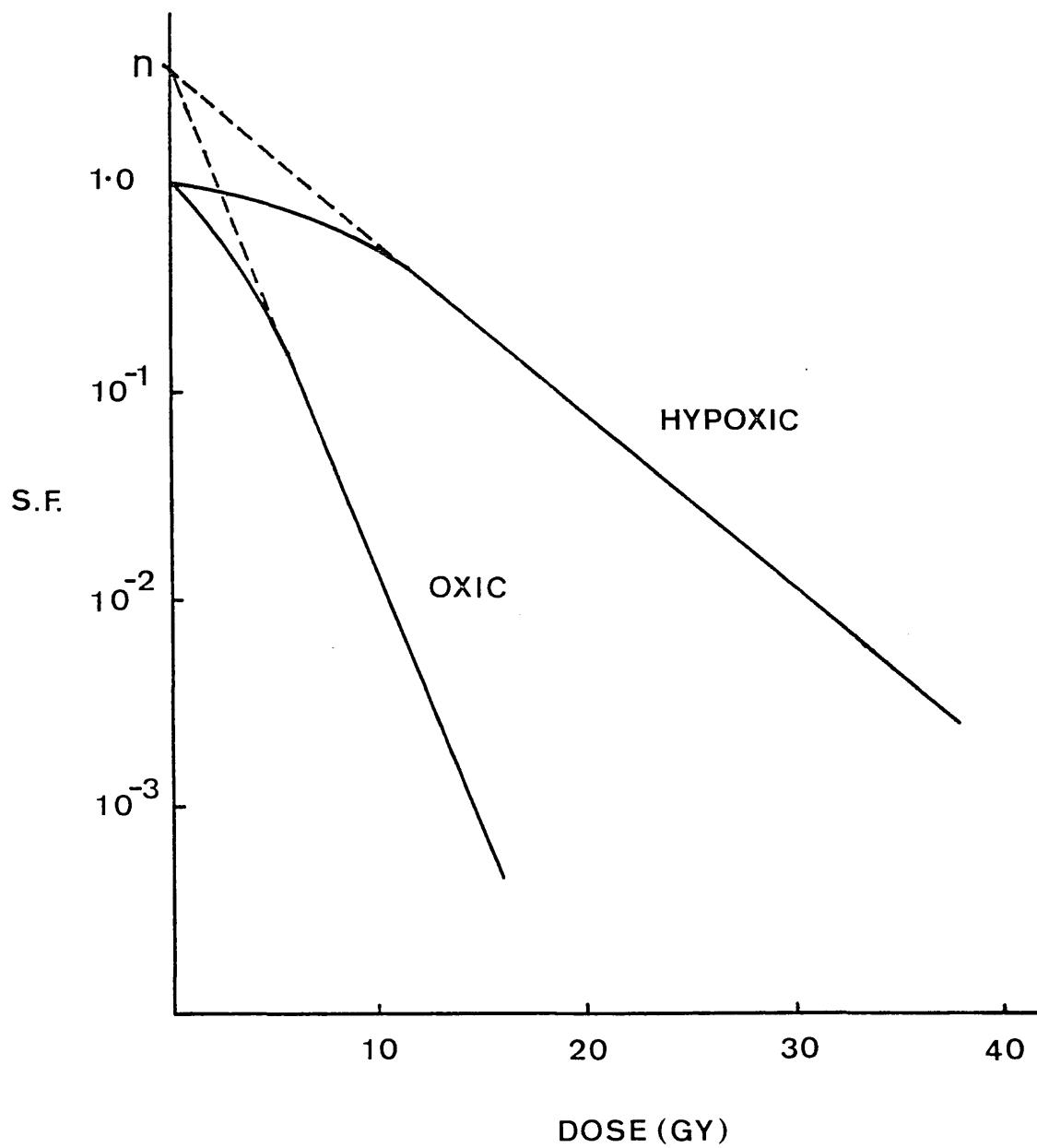


Fig. 7.4. Survival curves showing the radiation sensitivities of oxic and hypoxic cells.

broad shoulder region. This reflects the response of the cells to low level radiation exposure and the operation of internal cellular repair processes, in particular DNA strand break repair.

Beyond these low radiation doses the cell killing increases exponentially with radiation dose and the survival curve becomes linear, as plotted on a logarithmic cell surviving-fraction (S.F.) scale.

It should be noted that extrapolation of the linear part of the survival curves towards the S.F. axis results in both lines intercepting the S.F. axis at the same point, given by the extrapolation number n . Generally, this parameter n is independent of the presence or absence of O_2 and can be described by; $SF = 1 - (1 + e^{-kD})^n$ (k =slope, D = a measure of shoulder width).

The radiosensitization efficiency of a given drug is expressed by the Sensitizer Enhancement Ratio (S.E.R.) or Enhancement Ratio (E.R.) which is defined as the ratio of the slopes of the exponential portions of the cellular survival curves in the absence and presence of sensitizer. The survival curve with drug present would lie between the oxic and hypoxic curves, the higher the value of E.R. the greater the sensitizing ability.

Since the observed sensitization may be dependent upon the drug concentration, an E.R. must be quoted with respect to a given drug concentration; inter-drug comparisons may then be made directly. In addition, calculation of an E.R. is dependant upon the survival curves having the same extrapolation number. Should this not be the case, a radiation dose modification factor (DMF) must be quoted. DMF is the ratio of radiation doses without

and with drug necessary to achieve a specified S.F.

Generally, oxygen exhibits an E.R. of 3.0, indicating that hypoxic cells are three times as radiation resistant as oxic cells. Studies with new sensitizing drugs aim to achieve E.R.s of this order, oxygen being considered the "best" radiosensitizer.

7.1.7. Structure activity relationships of radiosensitizers

Effort in recent research has been to correlate the chemical and physical properties of known sensitizers, such as structure and reactivity, with their efficiency as chemical radiosensitizers or oxygen mimics. Their findings are summarized in this next section.

7.1.7a. The electron affinic relationship

The early classes of compounds found to be radiosensitizers included conjugated ketones and quinones [13]. It was soon found however that other oxidizing agents such as nitro compounds and in particular, nitroheterocyclic compounds were much more efficient.

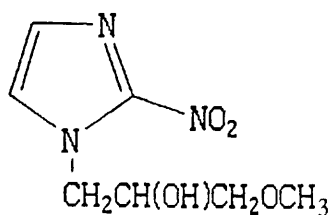
In each case, it was found that the important feature was the nitro-aromatic structure. Nitro compounds are well known for their ease of reduction and hence the redox properties of these molecules were determined [16].

It was found that there was a direct correlation between the electron affinity of a sensitizer (as quantified by its one-electron reduction potential under relevant conditions) and its sensitizing ability.

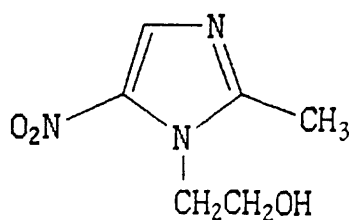
A range of potentials exists for a given class of compounds

and this reflects the influence of additional substituents on the aromatic ring other than NO_2 . Two compounds in particular, misonidazole (MISO) and metronidazole (METRO), have received a great deal of attention, MISO having recently undergone advanced clinical trials.

MISO is a 2-nitroimidazole and METRO a 5-nitroimidazole as shown below, each containing solubilizing side-chain substituents



MISO



METRO

Both MISO and METRO have been found to be active radiosensitizers in-vitro and in-vivo, MISO being more promising clinically. They differ in one-electron redox potential by approx 100mV (E'_7 MISO = -389mV, E'_7 METRO = -486mV) and this is reflected in a greater sensitizing ability of MISO.

Substitution of a powerful electron-withdrawing group (e.g. CHO) at the C-5 position of a 2-nitroimidazole greatly increases the reduction potential consequently, a lower drug concentration is required to result in a given level of ER.

Having a desirable electron affinity is one pre-requisite for a radiosensitizer but there are other chemical and physical properties to be considered. For example, some nitro-benzenes and 5-nitro-furans have been found to be good sensitizers in-vitro,

but on testing in-vivo showed a reduced effectiveness due to their poor metabolic stabilities. Certain properties of the drugs other than electron affinity must be acting and knowledge of these is necessary for a complete understanding.

7.1.7b. Cellular thiol depletion

It has been known for some time that exogenous reduced-glutathione (GSH) protects animals and cultured cells against radiation damage. Thus it was suggested that endogenous GSH must confer a degree of natural radiation-resistance on mammalian cells [17].

If this were true, it should be possible to sensitize mammalian cells to radiation by depletion of available cellular GSH. This approach has indeed been employed and over 20 years ago it was found that cellular thiol depletion using N-ethylmaleimide (N.E.M.) markedly enhanced the radiosensitivity of cells held at low oxygen tensions. In contrast, addition of exogenous thiols results in radiation resistance both in aerobic and hypoxic conditions [18].

These observations were consistent with the proposal that oxygen and thiols compete for reaction with radiation induced lesions on target molecules, oxygen reacting rapidly to render the damage permanent and thiols reacting more slowly to repair it.

Reference to fig. 7.3 shows that the radioprotecting species P is actually GSH which reacts by H-donation to repair the T' radical species.

Two approaches to hypoxic cell sensitization are therefore

available, oxygen mimetic radiosensitizing drugs whose action is determined in part by electron affinity, and drugs which selectively remove cellular thiols, primarily GSH [19,20].

The ideal drug would act as an oxygen mimetic radiosensitizer and as a thiol depletion agent, thereby giving an observed radiosensitization in-vitro greater than its electron affinity would predict. Examples of such compounds are 5-substituted-4-nitroimidazoles, 2-nitroimidazole-5-carboxaldehyde, 8-nitro - caffeine and some 2-nitrobenzimidazoles [2].

Major differences between in-vitro and in-vivo responses for such compounds are to be expected however due to differing GSH availability. The problem of selective thiol depletion in tumours or hypoxic cells is that a patient might have about 0.1 mol. GSH available for reaction, whereas a single dose of drug, approximately as toxic as MISO might be restricted to 5 mmol. resulting in <5% thiol depletion, if the reaction is of 1:1 stoichiometry.

One possible solution is the use of buthionine sulfoxime (BSO) which inhibits γ -glutamylcysteine synthetase [21] and consequently GSH biosynthesis.

Experiments of BSO with hamster cells resulted in upto 45% GSH depletion having a large effect on, for example, MISO sensitization [22].

7.1.7c. Drug lipophilicity

The distribution and rate of passage of a molecule across any biological phase boundary, for example in aqueous phases separated by a lipid membrane, will be influenced by the

equilibrium partition properties of the molecule.

As a quantitative guide to the lipophilicity of a drug, a partition coefficient P is defined as the ratio of the equilibrium concentrations of the compound in the lipid phase, (e.g. octanol) and the aqueous phase (e.g. pH 7.4 buffer) when distributed in such a biphasic system, the higher the value of P , the greater the compound's lipophilicity.

It was found that variation of P over a wide range in-vitro apparently had little effect on radiosensitization, this is almost certainly not the case in-vivo however, where variations of P would influence uptake, distribution, metabolism and excretion as well as affecting the concentration of the compound at the active site(s) within the tumour cell. Indeed it was recognised that compounds of low P tended to be excluded from the brain and perhaps from peripheral neural tissue, the dose limiting site. As a result of this, it was found that a 2-nitroimidazole an order of magnitude less lipophilic than MISO showed a much lower toxicity. This may result from both decreased neural tissue penetration and decreased drug half-life. Hydrophilic SR2508 (1-[N-(2-hydroxyethyl)acetamido]-2-nitroimidazole) with redox properties very similar to MISO, shows differences in therapeutic ratio, arising not from free radical reactivity but phase distribution differences.

The magnitude of P in, for example, the nitroimidazoles will thus be influenced by (i) the position of the NO_2 group on the ring and (ii) other ring-bound substituents. Similarly, for metal complex radiosensitizers the nature and position of the ligands and their substituents will determine P .

7.1.7d. Drug cytotoxicity

One of the major problems of drug development for clinical use is toxicity of the administered compound, directly, or as a consequence of metabolism.

The drugs used as clinical sensitizers, MISO and METRO, exhibit neurotoxic [23] and carcinogenic [24] side-effects respectively. With MISO, the peripheral neuropathy often encountered in its clinical use limits the total drug dose given during fractionated radiotherapy to 12 g/m^2 . This is below that required for maximum sensitization to be observed.

It has been observed [25] that some nitro-aromatic and nitro-heterocyclic compounds actually possess differential cytotoxicity towards hypoxic cells. This would be desirable in radiation treatment since the very cells are killed which limit the therapeutic use of radiotherapy.

For a range of nitro compounds [26], a correlation was found between drug toxicity under hypoxic conditions, and electron affinity, the higher the electron affinity (more +ve redox potential) the greater the toxicity. This would not be unexpected if reduction of the nitro group is important for toxicity.

However, although they show similar electron affinity correlations, the mechanisms of toxicity and sensitization are probably not the same [27].

7.1.8. The search for clinically useful sensitizers

A lot of work and effort has therefore gone into the development of drugs superior to MISO and METRO.

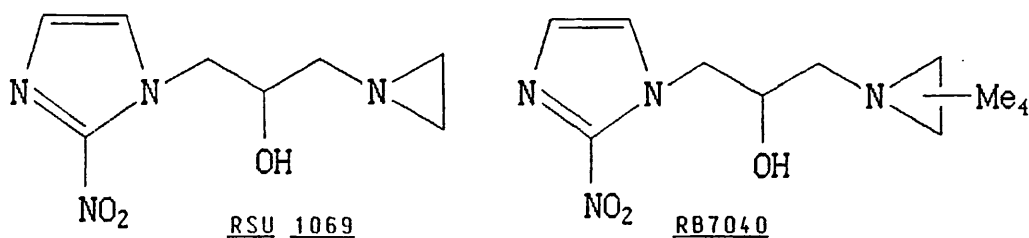
This work includes;

- (i) The design and synthesis of new radiosensitizers based upon the basic nitroimidazole nucleus or other related organic molecules,
- or (ii) The preparation of totally novel radiosensitizers such as transition metal complexes in the hope that if chosen on sound reasoning, they may show activity.

It is the second of these which is being investigated in this work, a series of transition metal complexes having been prepared and tested in-vitro and in-vivo for sensitizing activity.

7.1.9. Organic radiosensitizers

The most promising nitroimidazole derivative at present with respect to sensitization is the compound RSU 1069 [28,29]. It has the same basic structure as MISO except that an aziridine ring has been substituted for the OCH_3 group on the side chain.



RSU 1069 has the same electron affinity as MISO but is a substantially more efficient radiosensitizer of hypoxic mammalian cells both in-vitro and in-vivo. For example, in-vitro it shows an E.R. of 3.0 compared with 1.6 for MISO, both at a concentration of 0.5 mmol/dm^3 [30]. In-vivo studies [31] of

tumour radiosensitization and toxicity of RSU 1069 showed it to be more cytotoxic than MISO but its sensitizing effect on hypoxic tumour cells was also greater. Further, there appeared to be little effect of RSU 1069 on well perfused oxic tumour cells.

On a concentration basis, RSU 1069 showed 100 fold greater toxicity towards hypoxic relative to oxic cells in-vitro. This is substantially higher than MISO [32] which is only a factor of ten. Reductive processes were found to be important for its hypoxic toxicity and it was deduced that under oxic conditions, RSU 1069 functions as a typical monofunctional alkylating agent via the aziridine ring. In hypoxia however, reduction of the NO₂ group provides an additional alkylating species converting it into a bifunctional alkylating agent.

The introduction of alkyl groups onto the aziridine ring [33] of RSU 1069 resulted in reduction of the drug toxicity and greater sensitizing activity in-vitro and in-vivo. In particular, the tetramethyl analogue RB7040 was found to be a much more efficient radiosensitizer than RSU 1069 especially at lower concentrations. An explanation of this difference is given in terms of the drugs' relative basicity. RB7040 has a pKa greater than the average intracellular pH thereby enabling easier uptake into the cells. This gives a greater intracellular drug concentration for the same extracellular concentration, resulting in a greater sensitizing efficiency. The pKa of RSU 1069 is lower than the cellular pH, thus restricting its uptake.

Several variations of the basic nitroimidazole structure have been found to exhibit hypoxic cell sensitizing ability. Examples of such compounds are nitrothiazoles [34] and nitropyrazines

[35]. In addition, it has been suggested that due to the capacity of nitroxyl spin labels to selectively sensitize hypoxic cells to X-rays, they may have potential use in radiotherapy [36].

7.1.10. Metal complex radiosensitizers

The use of metal complexes as radiosensitizers can offer some advantages over their organic counterparts;

(1) Most metals are themselves redox centres offering a wide range of one-electron reduction potentials. Hence they are ideally equipped to stabilize an additional electron captured during irradiation. Within a series of complexes of a given metal, the redox potential could be fine tuned by systematic variation of the ligands. Theoretically, it should be possible to fine tune the redox potential so that the drug is only effective under certain desirable conditions, for example in hypoxic rather than oxic cells. This has been proposed for the antitumour activity of Ru [37].

(2) Rigid ligand configurations can be maintained about a metal centre. This could be very useful for making a drug more specific in its action. The best example of this type of specificity is provided by the observation that cis-platin is an effective antitumour agent whereas trans-platin is not.

(3) As explained, drug lipophilicity is very important for the efficiency of a radiosensitizer in-vivo. The solubility of organic compounds is altered by substitution of solubilising groups. Unfortunately, this may have the undesirable effect of altering, among other properties, the redox potential. For example, the 2-nitroimidazoles with the CHO and CH₂OH groups in

the 5'-position, have very similar lipophilicity effects but show markedly different redox properties. It should be much easier to alter the solubility of metal complexes however, subtle changes in ligand substitution being possible without an associated redox potential change.

The major disadvantage of metal complex drugs is the well known metal toxicity giving rise to undesirable side-effects, this being well documented for the Pt(II) drugs. It is not necessary to persist with these toxic metals however and other systems with less damaging properties must be a possibility.

Radiosensitization has been observed for a variety of metals in a wide range of oxidation states. A review of this topic has recently been published [38] and what follows here is an outline of some of the observed effects.

The first metal complex anti-tumour drug to be used in the clinic is the well known cis-diamminedichloroplatinum(II) (cis-platin) [39].

In 1976 [40] it was discovered that cis-platin, and to some extent, its second generation analogues [41,42] can also act as radiosensitizers of hypoxic tumour cells. The mechanism of interaction of radiation with Pt(II) complexes is not fully understood but it is assumed to be related to the known DNA binding properties of the complexes during irradiation [43,44, 45].

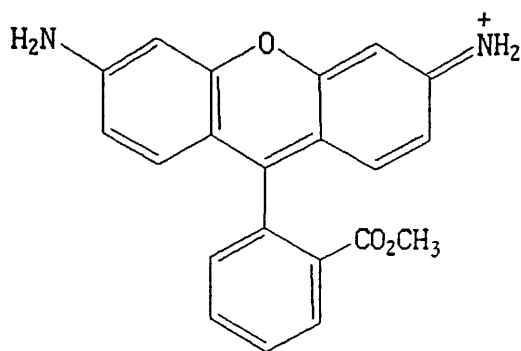
The ERs achieved with these complexes are low, of the order of 1.3, but they may still have potential use in the clinic due to their cytotoxic properties [46].

Of interest here is the finding that both the cis and trans isomers are active radiosensitizers, (the trans isomer being more effective) whereas only the cis isomer is active chemotherapeutically. This difference is ascribed to the lower toxicity of the trans isomer allowing a higher intracellular drug concentration. It has been suggested that the specific DNA lesions produced in cis-platin cytotoxicity are not required for radiosensitization. Recent findings suggest that sensitization by trans-platin involves the OH' radical whereas cis-platin does not, again suggesting the operation of different mechanisms [47].

A recent approach to the design of novel Pt(II) sensitizing drugs involves coordination of the traditional nitroimidazole drugs to the Pt centre, hopefully resulting in more efficient targeting of the drug to the site of action. In addition, it was hoped that the cytotoxic properties of Pt would also be observed, thereby facilitating a double-barrelled attack.

A wide range of this type of complex has been prepared [48, 49,50,51] with two nitroimidazole ligands per Pt centre, but conflicting results were obtained [52]. In some cases the sensitization observed was less than that of the ligand alone [53]. More recent studies suggest that these (nitroimidazole)₂-PtCl₂ type complexes do not actually bind to DNA and that a series of compounds containing only one nitroimidazole ligand may be better in this respect [54].

[Rhodamine 123]₂PtCl₄.4H₂O is the first tetrachloroplatinate (II) salt to exhibit anti-tumour activity [55]. In addition, it has been shown to possess radiosensitizer activity.



Rhodamine 123

A range of $[\text{Rh(II)(carboxylate)}_2]_2$ complexes have been examined for their hypoxic cell radiosensitization, their anti-tumour potential having been studied previously [56]. They were found to sensitize in both air and N_2 . A major component of the mechanism of action was attributed to intracellular thiol depletion by the Rh(II) species [57].

Among the series of carboxylates studied, an order of sensitization ability was found to be dependant upon the R group of the carboxylate ligand; $\text{R} = \text{butyrate} > \text{propionate} > \text{acetate} > \text{methoxyacetate}$ in order of decreasing effect. These differences were attributed to their abilities to interact with cellular thiols and intracellular uptake changes related to drug lipophilicity. A combination of Rh(II) carboxylate with MISO in-vitro showed an additive radiation response, but this was not reproducible in-vivo.

As with the Pt(II) complexes, recent work has studied the sensitizing potential of Rh(II) carboxylates with coordinated nitroimidazole ligands [58]. In-vitro results showed sensitization of V79 cells under hypoxic ($\text{ER} = 1.7 @ .02\text{mM}$) and, to a lesser extent, oxic conditions. Substantially lower concentrations of these complexes were required compared to the

free nitroimidazoles to achieve a given effect, efficiencies upto 50 x greater being obtained.

Combination experiments of Rh(II) acetate + MISO gave higher sensitization than could be achieved with either individually, the new complex operating more efficiently than its component parts.

High sensitization towards bacteria has been observed by Rh(III) complexes of ethylenediamine and nitrate [54] however, when medium rather than buffer is used this effect is abolished. No sensitization of mammalian cells was shown with these drugs.

Co(III) complexes are attractive as drugs due to their kinetic inertness preventing cellular degradation. A series of complexes of the type $\text{Co}(\text{NH}_3)_x(\text{NO}_2)_{6-x}^{n+}$ have also been prepared, the number of nitro ligands varying from 0 to 6 [59], and their sensitization properties investigated. The hexa-nitro, tetra-nitro, tri-nitro and trans-dinitro complexes were very active sensitizers with DMFs of 3.0, 2.7, 3.0 and 2.8 respectively.

Vitamin B12 (cyanocobolamin 1.0mM) was found to sensitize V79 plateau cells in hypoxia (ER=1.3), no effect being seen on spores [60].

$[\text{Fe}(\text{CN})_6]^{-3}$ was chosen for testing as a sensitizer because of its thiol binding properties and an ER of 2.4 was found with E-coli at 1mM. No enhancement was observed at 0.1mM with CHO cells [61]. Nitroprusside $[\text{Fe}(\text{CN})_5\text{NO}]^{-2}$ (0.01mM) gives radiation sensitization enhancement under both oxic and hypoxic conditions with V79 and CHO cells in-vitro. A problem of in-vivo use of this compound however, is its known ability to slow blood flow.

The work reported in this thesis covers the study of some Co(III) and Fe(II)/(III) complexes as radiation sensitizers. Most of the work was carried out on ferrocenium complexes (fig.7.5) which have recently been shown to possess anti-tumour properties associated with low toxicity [62].

The experiments were done exclusively in-vitro but preliminary in-vivo results carried out in collaboration with Dr. I.J. Stratford are also reported.

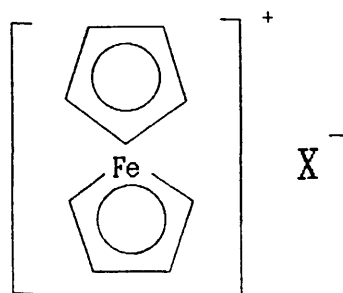


fig.7.5. Structure of the ferrocenium complex.

7.2. EXPERIMENTAL

7.2.1. Preparation of drugs for testing

In all cases, compounds were characterized by I.R. spectroscopy, elemental microanalysis and, where appropriate, melting point determination or ^1H nmr spectroscopy.

All microanalytical data are summarized in table 7.1.

Starting materials were supplied by Aldrich Chemical Co. Ltd.

Ferrocenium hexafluorophosphate. (Fc^+PF_6^-)

Ferrocene (200mg, 1.075×10^{-3} mol) was dissolved in 98% sulphuric acid (0.4ml). This solution was left at room temperature for 2 hours to facilitate complete reaction. The resulting dark blue solution was carefully diluted by the addition of ice-cold water (20ml). To this filtered solution of ferrocenium sulphate was added NH_4PF_6 (0.175g, 1.075×10^{-3} mol) in water (5ml) with swirling. A blue precipitate was quickly formed.

After leaving at 4°C for 18 hours, the solid was filtered off, washed with a minimum of cold H_2O at 0°C and dried in vacuo over P_2O_5 for 3 hours.

Ferrocenium trichloroacetate. ($\text{Fc}^+(\text{CCl}_3\text{CO}_2^-) \cdot 2\text{CCl}_3\text{CO}_2\text{H}$)

Ferrocenium sulphate was prepared as for Fc^+PF_6^- . To this solution was then added pre-dried $\text{CCl}_3\text{CO}_2\text{H}$ (1.23g, 7.525×10^{-3} mol) (dried in vacuo over P_2O_5 at 30°C for 24 hours) with stirring. The solution was left in the dark for 7 days at room temp. to facilitate solvent evaporation. This yielded dark blue crystals of the desired compound which were recovered, washed with a minimum of cold H_2O and dried over P_2O_5 in vacuo for 3 hours.

Mpt. 134-136°C (lit. 135-138°C [63])

Attempts were made to prepare the ferrocenium picrate and tetrachloroferrate complexes according to literature procedures [64]. Neither of these was successful, the products being very difficult to purify.

tert-pentylferrocenium hexafluorophosphate. ($\text{FcC}(\text{CH}_3)_2\text{CH}_2\text{CH}_3^+\text{PF}_6^-$)

The method used here was as for Fc^+PF_6^- but tert-pentyl ferrocene was used in place of ferrocene.

Hydroxymethylferrocene. (FcCH_2OH)

This was prepared by direct reduction of ferrocene carboxaldehyde.

To a solution of FcCHO (0.26g, 1.21×10^{-3} mol) in methanol (5ml) was added NaBH_4 (0.061g, 1.61×10^{-3} mol). There was an immediate solution colour change from red to yellow indicating reduction. The solution was stirred until effervescence had stopped. Evaporation of the solvent under reduced pressure and recrystallization from hot H_2O yielded the desired product.

Mpt. 74-76°C (lit. 76°C [65])

Acetylferrocene. (FcCOCH_3)

Ferrocene (3.0g, 1.6×10^{-2} mol) was added to acetic anhydride (10ml) moisture being excluded by a calcium chloride guard tube. To this was added orthophosphoric acid (2ml) dropwise with stirring. The mixture was then heated on a steam bath for 20 min. and then poured onto crushed ice (80g) with stirring. Once the ice had melted, NaHCO_3 was added to neutralize the solution,

cooling this to 0°C for 20 min. followed by filtration yielded a brown solid.

Drying the solid for 18 hours in-vacuo followed by recrystallization from cyclohexane gave the desired product as dark brown crystals.

Mpt. 84-86°C (lit. 85-86°C [65])

1-ferrocenylethanol. (FcCH(OH)CH₃)

To a solution of acetylferrocene (1.0g, 4.39×10^{-3} mol) in 1:3 H₂O : Ethanol (v/v) (20ml) was added a solution of NaBH₄ (0.8g 2.1×10^{-2} mol) in water (4ml) with stirring. A pale yellow solution resulted after 15 min.

Water (100ml) was added and this solution extracted with diethylether (2x20ml). Drying the organic extracts over anhydrous MgSO₄ followed by solvent removal under reduced pressure and recrystallization of the product from 40-60°C petroleum ether gave the product as brown crystals.

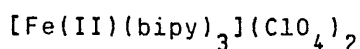
Mpt. 74-77°C (lit. 77-78°C [65])

Attempts were made to synthesise the ferrocenium analogues of FcCH₂OH, FcCOCH₃ and FcCH(OH)CH₃ but these were not successful. In the case of acetylferrocene a dark blue solution was formed on oxidation indicating the formation of a ferrocenium specie but on dilution with water an immediate red/brown precipitate of acetylferrocene was formed.

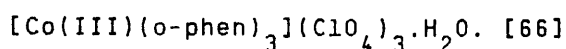
[Fe(II)(o-phen)₃](ClO₄)₂

To a solution of FeSO₄.7H₂O (100mg, 3.597×10^{-4} mol) in water (2ml) was added a solution of 1,10-phenanthroline (194mg,

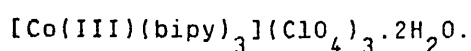
1.079×10^{-3} mol) in methanol (1ml). A blood-red solution immediately resulted. After 5 min. HClO_4 (1ml, 60%) was added, a maroon solid immediately precipitated. The solid was washed with H_2O and dried in vacuo over P_2O_5 for 12 hours.



This was prepared as above, 1,2-bipyridine (168mg, 1.079×10^{-3} mol) being used in place of 1,10-phenanthroline. No further use was made of this compound due to its aqueous insolubility.



To a solution of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (200mg, 8.4×10^{-4} mol) in water (5ml) was added a solution of 1,10-phenanthroline (500mg, 2.55×10^{-3} mol) in methanol (1ml). To this was added bromine water (2ml) and the whole refluxed for 30 min. HClO_4 (60%, 2.5ml) was then added to the hot solution depositing yellow crystals of the desired complex. The suspension was cooled in ice and the product collected and recrystallized from hot H_2O .



As for the o-phen complex above but bipyridine (400mg, 2.25×10^{-3} mol) was used in place of o-phen.

Preparation of cyclodextrin-ferrocene inclusion complexes

The method of Harada was used for the attempted preparation of both α - and β -cyclodextrin inclusion complexes of Fc [84]. To an aqueous solution of cyclodextrin at 60°C was added fine crystals of freshly sublimed Fc with stirring. After a few hours a pale yellow solid was deposited in all cases; these were then analysed by IR and nmr spectroscopy and elemental microanalysis.

At no time could the desired products be isolated and attempted recrystallization from hot H₂O as given in [84] were unsuccessful. The reactions were repeated several times, all attempts failing.

<u>Compound</u>	<u>Analysis</u>		
	<u>%C</u>	<u>%H</u>	<u>%N</u>
FcPF ₆	36.06 (36.28)	2.95 (3.05)	- -
FcTCA	27.89 (28.43)	1.86 (1.79)	- -
t-pFcPF ₆	44.93 (44.90)	4.99 (5.03)	- -
Fc(CH ₂ OH)	60.34 (61.14)	5.63 (5.61)	- -
Fc(COCH ₃)	62.98 (63.21)	5.20 (5.26)	- -
Fc(CH(OH)CH ₃)	62.74 (62.66)	6.01 (6.09)	- -
[Fe(II)(o-phen) ₃](ClO ₄) ₂	49.65 (49.81)	3.19 (3.35)	11.60 (11.61)
[Co(III)(o-phen) ₃](ClO ₄) ₃ ·H ₂ O	46.96 (47.19)	2.61 (2.86)	9.17 (9.17)
[Co(III)(bipy) ₃](ClO ₄) ₃ ·2H ₂ O	41.55 (41.79)	3.13 (3.28)	9.70 (9.74)

Table 7.1. Microanalysis results for drugs synthesised.
Figures in parentheses show theoretical values.

7.2.2. Biological studies

7.2.2a Cells

For all experiments, chinese hamster V79-379A cells were used, the parental V-line being originally isolated from the lung tissue of a young adult male chinese hamster in 1958.

The cells were used in their exponential growth phase for all experiments. The growth of cells in air is characterized by an initial lag-phase, after which the cells enter exponential growth. Once the cell number approaches 1×10^6 cells per ml, the growth begins to slow down and reaches a plateau or stationary phase (fig. 7.6.)

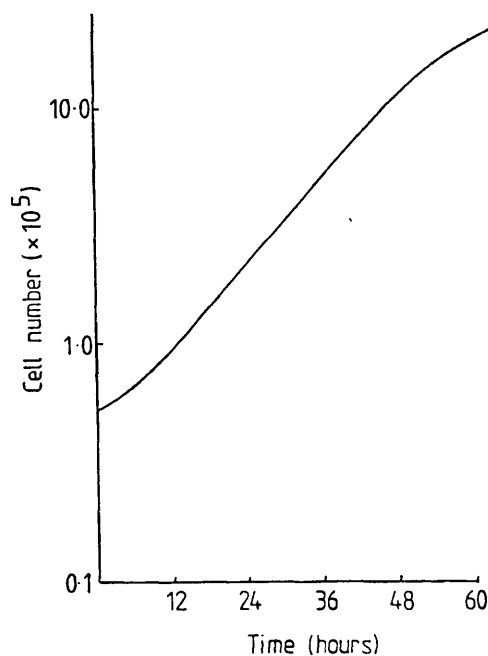


Fig. 7.6. Growth characteristics of V79 cells in suspension at 37°C in air.

The cells were maintained in the exponential phase of growth by diluting daily with MEM to 2×10^5 cells per ml, this number doubling every 10-11 hours.

7.2.2b. Preparation of drug solutions

For radiation experiments, the drugs were dissolved in pbs to an initial concentration of 1 mmol/dm^3 , sonication being employed to facilitate complete dissolution where necessary. Before addition to the cells, the solutions were filtered through a $0.4 \mu\text{m}$ millipore cellulose acetate filter to remove any contaminating bacterial components. These solutions were then serially diluted to give the desired drug concentrations.

In the case of toxicity experiments, drug solutions were prepared in pbs or MEM. The concentration of drug in solution was checked by absorbance measurement at $\lambda_{\text{max}} = 617 \text{ nm}$.

7.2.2c. Preparation of materials

Rigorous precautions were necessary to avoid contamination of experimental materials; not achieving this would inhibit cell growth. All glassware was extensively washed, dried and then sterilised in an autoclave. Similarly, stock reagents such as pbs were sterilised in the autoclave before use, MEM being sterilised by filtration.

7.2.2d. Composition of reagents

Cell Growth Medium

The growth medium used for all cell culture experiments was Eagle's Minimum Essential Medium supplemented with foetal calf serum. The composition of the medium is shown in table 7.2.

Composition of Eagle's Minimum Essential Medium

<u>Components</u>	<u>Conc</u> (mg per litre)
<u>Amino Acids</u>	
L-arginine	126.40
L-cystine disodium salt	28.42
L-glutamine	292.30
L-histidine hydrochloride	41.90
L-isoleucine	52.50
L-leucine	52.50
L-lysine hydrochloride	73.06
L-methionine	14.90
L-phenylalanine	33.02
L-threonine	47.64
L-tryptophan	10.20
L-tyrosine	36.22
L-valine	46.90
<u>Vitamins</u>	
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
L-inositol	2.00
Nicotinamide	1.00
Pyridoxyl hydrochloride	1.00
riboflavin	0.10
Thiamine hydrochloride	1.00
<u>Inorganic Salts</u>	
$\text{CaCl}_2 \cdot \text{H}_2\text{O}$	264.90
KCl	400.00
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200.00
NaCl	6800.00
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	158.30
<u>Miscellaneous</u>	
Glucose	1000.00
Sodium Phenol Red	17.00

Table 7.2.

Phosphate buffered saline

Pbs was prepared from buffer tablets of the composition given in table 7.3. to give a solution of pH 7.4.

<u>Component</u>	<u>Conc. (g per Litre)</u>
NaCl	8.00
KCl	0.20
Na ₂ HPO ₄	1.15
KH ₂ PO ₄	0.20

Table 7.3.

Trypsin

Trypsin detaches the cells from the surfaces of the culture vessels and has the composition as shown in table 7.4.

<u>Component</u>	<u>Conc. (mg per ml)</u>
Glucose	1000.0
KCl	400.0
KH ₂ PO ₄	60.0
NaCl	8000.0
Na ₂ HPO ₄	47.5
NaHCO ₃	350.0
Trypsin	25000.0

Table 7.4.

7.2.2e. Radiation experiments in monolayer

To study the radiosensitizing effects of the drugs under consideration, cells were attached to sterile glass petri dishes, plastic dishes being unsuitable for hypoxic irradiation studies due to dissolved oxygen in the plastic matrix [86].

Cells were removed from culture suspension, counted using a

hemocytometer viewed through a microscope, and then serially diluted in MEM to give the desired cell concentrations ranging from 10^2 to 10^5 cells per ml. Aliquots of cell suspension (1ml) were then pipetted into the petri dishes in triplicate, each dish already containing MEM (3ml). The number of cells plated per petri dish was determined by estimating the number of cell colonies to survive any given radiation dose. This figure was then used so as to achieve a final colony count of between 20 and 150 per plate, this range allowing accurate counting.

The dishes were gently agitated by swirling to disperse the cells and incubated at 37°C in an atmosphere of air + 5% CO_2 to allow cell attachment. After two hours, the medium was removed and replaced with a solution of the drug in pbs at the required concentration (2ml).

The dishes, without lids, were then placed in vessels designed for cell irradiation in monolayer under controlled gas conditions. These durals (see plate 7.1.) are made of an aluminium / steel alloy and each can hold four dishes. Each dural has an inlet and an outlet port through which they may be purged with the desired gas.

After tightly clamping the lid onto an O-ring seal by three thumb screws, the whole vessel was then purged with moist oxygen-free N_2 for hypoxic experiments for exactly one hour, this length of time being sufficient to achieve radiobiological hypoxia. After this the durals were sealed and the samples irradiated with the desired gamma-ray dose from a ^{60}Co source, the position of



Plate 1. The aluminium dural used for irradiation experiments

Plate 2. The dural on the 12cm jig prior to irradiation.

the dural relative to the source being kept constant throughout by the use of a 12cm jig. (see plate 7.2), thus ensuring a uniform dose across the whole vessel.

Immediately after irradiation the petri dishes were removed from the durals and the drug solution removed, this then being replaced by fresh MEM (3ml). The cells were then incubated at 37°C under a humidified air / 5% CO₂ atmosphere for seven days to allow for colony formation. After this time, the colonies were stained and counted.

A series of irradiation experiments were carried out in the presence of either catalase (CAT) or bovine serum albumin (BSA). In these, the required amount of CAT or BSA was pipetted onto the plates (0.1ml per plate) at the same time as the drug solution.

This additional solution volume would slightly lower the overall drug concentration and hence, to be consistent, pbs (0.1ml) was added to those plates to which CAT or BSA had not been included.

7.2.2f. Three hour drug toxicity experiments

Cells were plated in 100ml flat glass bottles to a concentration of approx. 1.5×10^6 cells per bottle in a 5ml solution volume.

The cells were incubated at 37°C in an air / 5% CO₂ atmosphere allowing growth to confluence. The growth medium was then removed and replaced by a solution of drug in MEM or pbs at the desired concentration. The cells were then incubated for a further 3 hours.

For air toxicity experiments, the atmosphere above the cells

during drug exposure was the standard air / 5% CO₂ mixture. For hypoxic toxicity, a mixture of N₂ + 5% CO₂ was flowed over the surface of the drug solution via a gas manifold, two syringe needles in each securely fastened bottle-top acting as inlet and outlet ports. The gas flow rate was monitored by a water bubbler attached to one of the outlet ports.

After incubation, the drug solution was removed and the cells washed with pbs (2x5ml). The cells were then detached from the surface of the glass bottles by addition of 1xtrypsin in pbs (5ml per bottle). After 5 min. MEM (5ml) was added to halt the trypsinisation. At this point the cell density was determined by counting on a hemocytometer.

The cell samples were then serially diluted in pbs to give a range of cell concentrations depending on the level of survival expected.

Aliquots of 1ml were pipetted onto sterile plastic or glass petri dishes followed by MEM (3ml), the numbers plated being such as to give between 20 and 100 colonies per plate. After gentle agitation to facilitate cell dispersal, the cells were incubated for 7 days at 37°C in the standard air / 5% CO₂ atmosphere, after which the colonies were stained and counted.

7.2.2g. One hour drug toxicity experiments

Cells were plated as described for the radiation experiments, 2×10^2 cells being plated per petri dish. After the initial incubation period, the culture medium was removed and replaced with a solution of the drug at the desired concentration in either MEM or pbs (2ml).

As was the situation with the radiation experiments, CAT and BSA were used, the protocol used as already described. Additional experiments were carried out in the presence of dimethylsulphoxide (DMSO) or highly irradiated (HR) cells. The amount of DMSO added per plate was so as to give a final DMSO concentration of 0.1, 0.5 or 1.0 mol/dm³, this being added to the plates at the same time as the drug solution. HR cells were prepared by subjecting a sample of cell suspension to a high dose (200 Gy) of radiation. These then being plated at a density of 10⁵ cells per plate at the same time as the non-irradiated cells.

After addition of drug solution to the dishes they were left in the flow-cabinet at room temperature for one hour. The lights in the cabinet were switched off during this exposure since it had been found previously that the light emitted can adversely affect cell survival[67].

After one hour, the drug solution was removed and replaced with MEM, the plates then being incubated at 37°C in an air/5%CO₂ atmosphere for 7 days. The surviving cell colonies were then stained, counted and cell-survival calculated.

7.2.2h. Viable cell-colony staining and counting

After incubation, the dishes were removed from the incubator and the medium poured off. To each plate was then added 70% ethanol (0.5ml) to fix the cell colonies. The ethanol was quickly removed and replaced with methylene blue diluted in 70% ethanol (approx. 2.5ml per plate.)

After 10 min. the stain was removed and the dishes rinsed under a flow of water and then left to dry at room temp.

The number of colonies present was counted using a pressure activated colony counter. For the purposes of radiation and toxicity experiments, a colony is a group of 50 or more cells produced from a single cell under incubation over a 7 day period.

7.2.2i. Calculation of cell surviving fraction (SF)

This method was used for both irradiation and toxicity studies.

The plating efficiency of the experiment (P.E.) is defined as

$$P.E. = \frac{\text{No. colonies counted on control dishes}}{\text{No. colonies plated on control dishes}}$$

This is then used to calculate the surviving fraction as below.

$$SF = \frac{\text{No. colonies counted}}{\text{No. cells plated}} \times \frac{1}{P.E.}$$

The surviving fraction is the quantity plotted for survival curves.

7.2.2j. Non-Protein Thiol (NPSH) Depletion Experiments

The method used here is that described by Griffith [68].

V79 cells were harvested from spinner culture in aliquots of approx. 20ml with $>10^7$ cells per aliquot. Each of these was centrifuged in plastic universal vessels (800 rpm, 10 min.) to form a pellet which was washed with pbs (x2) to remove thiols present in the medium. The pellet was resuspended in a solution of drug at the required concentration in MEM, a control sample being prepared with MEM alone.

These suspensions were then put into a blood rotator at 37°C and rotated at 20 rpm for 1 hour. After incubation, the cells

were pelleted (800 rpm, 10 min.) and washed with pbs (x2). The cell pellet was then lysed for 5 min. in water (0.75ml), addition of sulphosalicylic acid (0.25ml, 12%) precipitating cellular protein this being removed by centrifugation (microcentrifuge, 12000g, 20sec).

At this point, the sample could be frozen for assaying at a later date. The total NPSH present in the sample supernatant was assayed with the mixture shown in table 7.5.

NPSH assay mixture

<u>Component</u>	<u>Conc.</u>	<u>Vol. added</u>
Triethanolamine.HCl buffer. (pH 8.0)	1mol/dm ³	0.30ml
NADPH	0.3mmol/dm ³	2.00ml
Glutathione- Reductase	0.5 units	0.03ml
DTNBA *	6mmol/dm ³	0.30ml
Supernatant	-	0.30ml

* DTNBA = 5,5'-dithiobis(2-nitrobenzoic acid)

table 7.5.

The reagents were prepared in 0.125 mol/dm³ sodium phosphate buffer containing 6.3 mmol/dm³ EDTA (pH 7.5).

The absorbance of 2-nitro-5-thiobenzoic acid at 412nm was monitored spectrophotometrically (quartz cells, 1cm path length) this providing a measure of GSH concentration. The amount of GSH present was expressed as a percentage of the control sample value.

7.2.2k. Drug Lipophilicity

This was determined by monitoring the distribution of drug between 1-octanol (lipid phase) and pH 7.4 pbs (aqueous phase) at room temperature.

Pbs was pre-equilibrated with 1-octanol by shaking for 20 min. and then allowing phase separation overnight. This solution was then used for the preparation of all drug solutions and as the blank in spectrophotometric determinations.

For each drug, a solution of approx. 0.5mmol/dm^3 was prepared, its absorbance at a characteristic wavelength being recorded.

Aliquots (3ml) of the drug solutions were then added to 1-octanol (3ml) in triplicate. These were then shaken at room temp. for 100 min. to facilitate drug partition. The resultant emulsions were broken up by centrifugation, the aqueous phases carefully removed and their absorbances re-measured.

The percentage of drug in the 1-octanol phase can be calculated from the decrease in absorbance and from this, the partition coefficient (P) is derived as follows.

$$P = \frac{\% \text{ Drug in 1-octanol}}{\% \text{ Drug in pbs}}$$

7.2.3. Determination of the aqueous solubility of ferrocene

Finely ground, freshly sublimed ferrocene (200mg , $1.08 \times 10^{-3}\text{mol}$) was added to water (200.0ml) in a volumetric flask. The solution was then stirred vigorously at room temp. (3 hours). Any remaining solid was removed by filtration and the filtrate

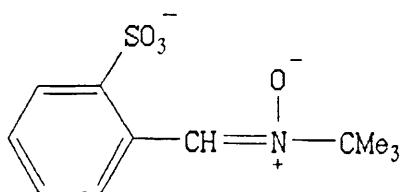
extracted with freshly distilled cyclohexane (1x50.0ml). The organic extract was then passed through a phase-separation filter paper to remove traces of water. Spectrophotometric determination of the absorbance of this solution at $\lambda_{\text{max}} = 440\text{nm}$ (quartz cells, 1cm path-length) was then used to calculate the concentration of ferrocene from the Beer-Lambert law. ($\epsilon_{440\text{nm}} = 102 \text{ M}^{-1} \text{ cm}^{-1}$ in cyclohexane [69]). The determination was carried out three times, a mean result being quoted.

Sources of error in this experiment include ferrocene particle size variability, loss of solution during manipulations and the presence of traces of water in the organic extract.

7.2.4. Radical trapping studies

To a solution of ferrocenium complex (1×10^{-5} mol) in pbs (0.1ml) was added a solution of GSH (6.2mg, 2×10^{-5} mol) and 2-SSPBN (28mg, 1×10^{-4} mol) in pbs (0.1ml) at room temp. In each case, a yellow precipitate of ferrocene was quickly formed (as characterized by ^1H nmr) which was removed by filtration through a cotton wool stopper. The room temp. X-band epr spectrum of the filtrate was then recorded, with the sample in an Heraeus quartz flat cell. Care was necessary to avoid power-saturation of the signal, a microwave power of 20dB being used throughout.

A control experiment was done for each drug in which the GSH was omitted from the reaction solution, no radical signals were seen in these tests.



2-SSPBN = sodium 2-sulphonatophenyl tert-butyl nitronium [70].

7.2.5. Stabilization of ferrocenium in aqueous solution

These reactions were done on a purely qualitative level.

To a solution of ferrocenium salt (10mg) in 40% EtOH (2ml) was added a solution of amino acid or glucose (10 equiv.) also in 40% EtOH (2ml). The pH of the resultant solution was adjusted by the dropwise addition of 0.5M KOH, the solution colour and deposition of solid being monitored by eye. When required, the solutions were made more acidic by the addition of 0.5M HCl.

7.2.6. Decomposition of ferrocenium in aqueous buffer

Experiments were carried out on 1.0 and 0.01mM solutions of FcPF₆ and FcTCA. The drug solutions were prepared in pH 7.4 pbs, the resultant pH being adjusted to this level by the addition of 0.5M KOH. The rate of decomposition was studied by following the disappearance of the absorption band at $\lambda_{\text{max}} = 617\text{nm}$ with time. (quartz, 1cm path length cells). At the higher drug concentrations small amounts of EtOH were added to the reaction mixture to dissolve ferrocene produced in the process, this not being necessary at lower drug levels.

An alternative method, removing the necessity for EtOH, involved measuring the absorbance immediately after mixing, leaving the solution for one hour and then re-measuring the absorbance after centrifugation to remove ferrocene. Both methods gave very similar results.

7.3. RESULTS

The drugs under investigation were subjected to a preliminary irradiation experiment as a guide to their radiosensitizing ability. The results of these one-point survival curves are given in table 7.6. The decision about further work was made from these figures, those with favourable toxicity and sensitizing properties being chosen.

The bipy and o-phen complexes all exhibited very good toxicity characteristics but showed negligible sensitizing ability at the drug concentrations used.

Of the ferrocene derivatives, $\text{Fc}(\text{CH}_2\text{OH})$ appeared the most promising giving an ER of 1.90 at 1mmol/dm^3 . At this drug level however, the toxicity would be unacceptably high, an SF of 0.87 being found at a drug concentration of 0.1mmol/dm^3 .

Ferrocene itself and $\text{FcCH}(\text{OH})\text{CH}_3$ both had very low aqueous solubility hence saturated solutions were used. An approximate figure for the concentration of Fc in a saturated aqueous solution at room temperature was determined as 9mg/dm^3 .

Fc and $\text{FcCH}(\text{OH})\text{CH}_3$ showed relatively small sensitization but quite high toxicity. Problems were encountered with FcCOCH_3 which, after dissolution, quickly decomposed yielding a dark brown precipitate; no further experiments were therefore attempted.

Of the drugs tested, the ferrocenium complexes appeared most promising. FcPF_6 and FcTCA both exhibited good sensitization with low toxicity at concentrations, much lower than, for example, MISO for the same ER. The (tert-pentyl) FcPF_6 complex was far too toxic at these concentrations giving no cell survival.

It was decided that further work, probing the mechanisms of action and quantifying sensitization and toxicity parameters should be carried out on these ferrocenium complexes, comparisons being made between them. Despite the high toxicity of (tert-pentyl)FcPF₆, it too was studied, the aim being possible correlation of the cyclopentadienyl ring substitution with the drug's toxicity and sensitization action.

<u>Drug</u>	<u>Toxicity</u>	<u>ER</u>
Ferrocene	SF=0.55 @ ss.*	1.20 @ ss.
Fc(CH ₂ OH)	SF=1.00 @ 0.05mM SF=0.87 @ 0.10mM	1.90 @ 1.0mM 1.22 @ 0.5mM
Fc(CH(OH)CH ₃)	SF=0.75 @ ss.	1.30 @ ss. 1.10 @ ss/2
FcPF ₆	SF=1.00 @ 0.02mM (in MEM) SF=0.55 @ 0.01mM (in pbs)	2.14 @ 0.02mM 1.40 @ 0.01mM
FcTCA	SF=1.00 @ 0.01mM	1.50 @ 0.01mM
t-pFcPF ₆	SF=0.00 @ 0.02mM	No result
[Fe(II)(bipy) ₃](ClO ₄) ₂	SF=1.00 @ 0.02mM	1.25 @ 0.02mM 1.18 @ 0.01mM
[Co(III)(bipy) ₃](ClO ₄) ₃	SF=1.00 @ 0.02mM	1.25 @ 0.02mM 1.10 @ 0.01mM
[Co(III)(o-phen) ₃](ClO ₄) ₃	SF=0.80 @ 0.02mM	1.38 @ 0.02mM

* ss. = saturated solution

Table 7.6. Results of preliminary sensitization and toxicity studies.

7.3.1. Irradiation Studies

The results obtained from the irradiation experiments with the ferrocenium complexes are given numerically in table 7.7.

<u>Drug</u>	<u>Concn. (mM)</u>	<u>Atm.</u>	<u>DMF</u>
FcPF ₆	0.001	N ₂	1.09
"	0.002	"	1.12
"	0.005	"	1.54
"	0.010	"	2.03
"	0.020	"	2.20
FcPF ₆ + CAT	0.010	"	1.90
FcTCA	0.010	"	1.50
FcTCA + CAT	0.010	"	1.64
FcPF ₆	0.010	Air	1.42

Table 7.7. Radiation Dose Modification Factors for SF=0.1

Fig. 7.7. shows the cellular survival curves resulting from hypoxic irradiation studies of FcPF₆ at 0.010 and 0.005 mmol/dm³ concentrations. In both cases, sensitization of cell kill is observed with respect to the hypoxic (N₂) line, the higher the concentration the greater the sensitization. A range of drug concentrations was investigated, these all gave survival curves showing sensitization of hypoxic cell kill but are not given graphically.

Of note is that the survival curves in fig. 7.7. and those not drawn were essentially parallel to the N₂ line, exhibiting an extrapolation number of approx. 1.0 rather than 4.0 to be expected from the action of the cellular repair

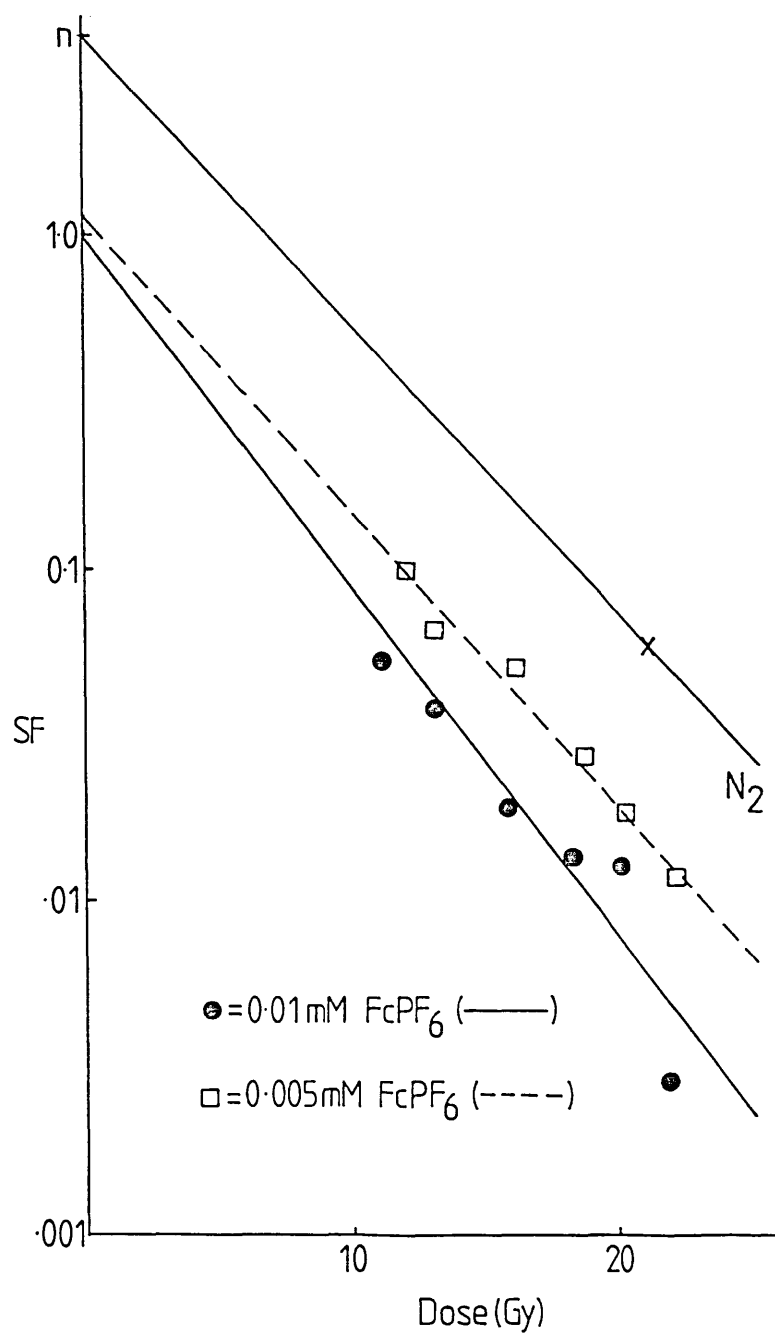


Fig.7.7.7. Cellular survival curves for cells irradiated under hypoxic conditions in the presence of FcPF_6 .

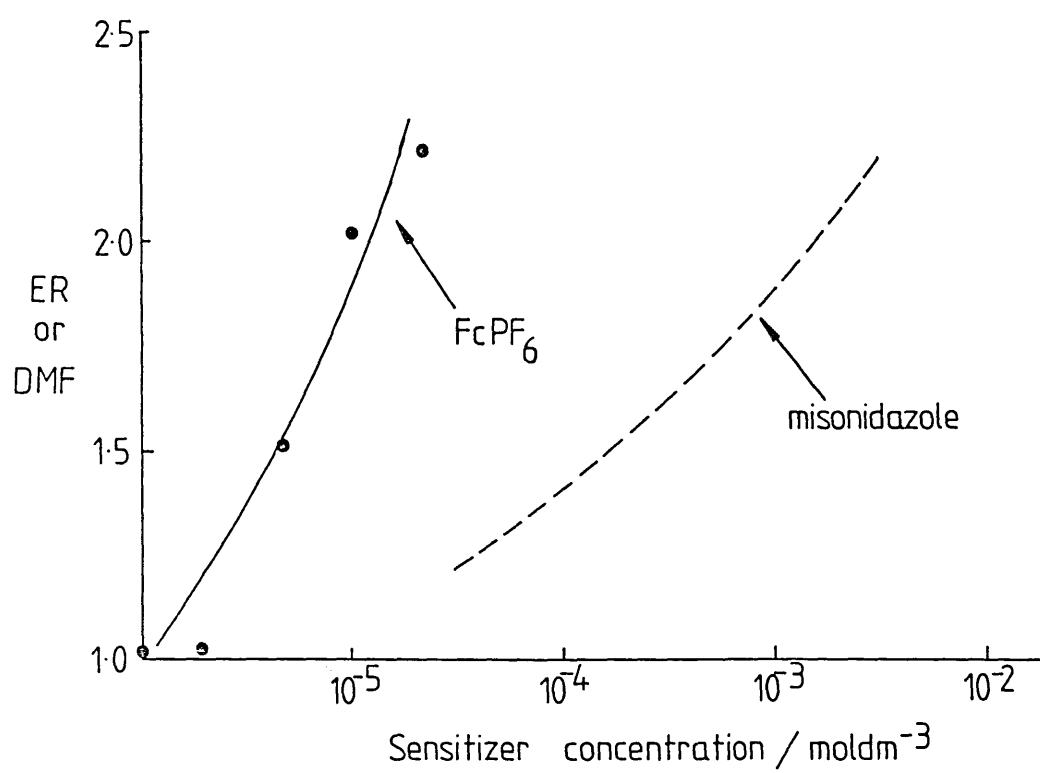


Fig.7.8. Variation of ER or DMF with sensitizer concentration under hypoxic conditions.

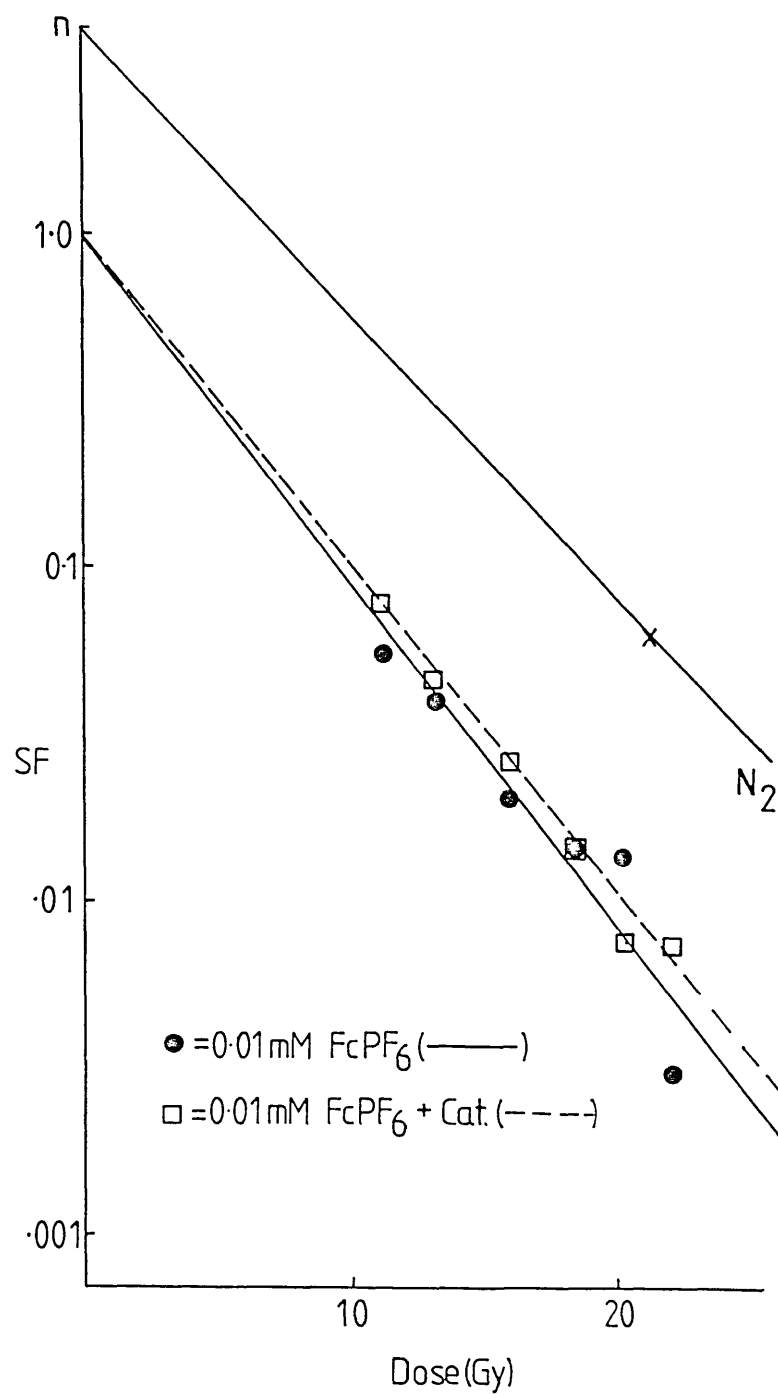


Fig.7.9. Cellular survival curves for cells irradiated under hypoxic conditions in the presence of FcPF_6 (0.01mM) with and without CAT (20,000 units per plate).

mechanisms (section 7.1.6.). This difference in extrapolation number (n) means that an enhancement ratio (ER) cannot be quoted. A Dose Modification Factor (DMF) for a cell surviving fraction of 0.10 is therefore given. Fig. 7.8. shows graphically the variation of DMF for a range of FcPF_6 concentrations, a trend of increasing DMF with concentration being clearly seen and is compared with the variation of ER with concentration for MISO [71].

Direct comparison of the FcPF_6 survival curve (0.01mmol/dm^3) with that obtained under exactly the same conditions in the presence of catalase (CAT) (2×10^4 units per plate) is made in fig. 7.9.

Addition of CAT apparently had little effect on the degree of hypoxic cell sensitization, a DMF of 1.90 being found in the presence of CAT compared to 2.03 without. The survival curve remained parallel to the N_2 line with CAT present.

Irradiation experiments in the presence of Bovine Serum Albumin (BSA) at an equivalent concentration per plate to the protein in CAT, gave a survival curve with FcPF_6 (0.01mmol/dm^3) identical to that with CAT, no changes in sensitization or extrapolation number being seen.

Not shown graphically but given in table 7.7. are the sensitization results for FcTCA at 0.01mmol/dm^3 with and without CAT. In both cases the survival curves were parallel to the N_2 line with an extrapolation number of approx. 1.0. Sensitization of hypoxic cell kill by FcTCA is lower (DMF=1.50) than that of FcPF_6 (DMF=2.03) both at 0.01mmol/dm^3 .

Fig. 7.10 shows the survival curve of 0.01mmol/dm^3 FcPF_6 under aerobic conditions. An enhancement of cell kill is observed ($\text{DMF} = 1.42$) and in this case the curve has an extrapolation number nearer the value of 4.0.

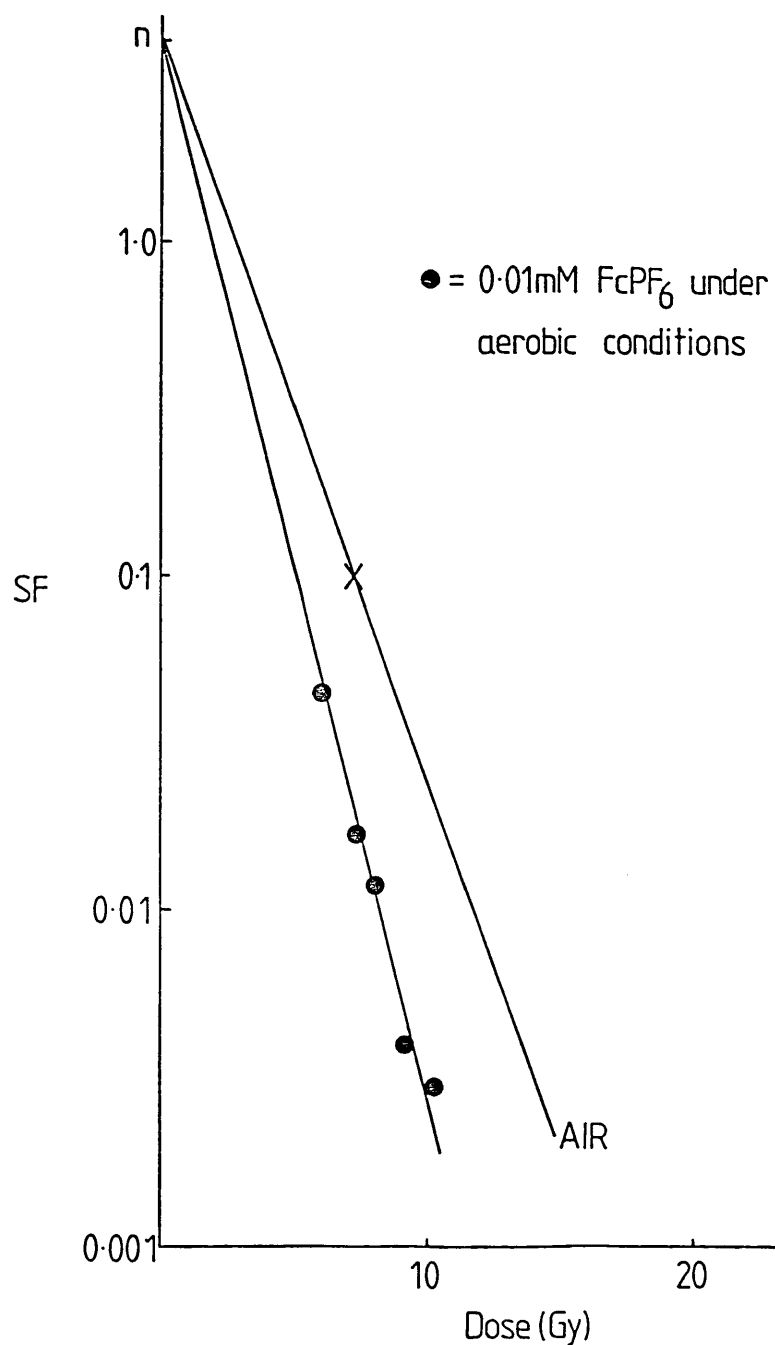


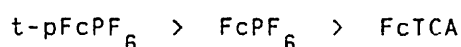
Fig. 7.10. Cellular survival curve for cells irradiated under oxic conditions in the presence of FcPF_6 (0.01mM).

7.3.2. Toxicity Studies

Two types of toxicity experiments were used. One-hour, room temperature experiments were carried out to compare directly the toxicity of the three ferrocenium complexes, at differing concentrations in pbs or MEM under aerobic conditions. The maximum drug concentrations achieved in this method was 0.03mmol/dm^3 . Beyond this, the three-hour bottle toxicity method was used, the experiments being done at 37°C under oxic or hypoxic conditions, with drug solutions being prepared in MEM to a maximum concentration of 2mmol/dm^3 .

The one-hour toxicity results are shown graphically in fig.7.11 and quantified in table 7.8.

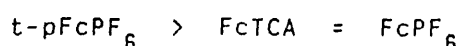
An order of drug toxicity under these conditions was determined as,



$t\text{-pFcPF}_6$ was by far the most toxic, this agreeing with the preliminary investigations into sensitizing ability (table 7.6.).

In each case, addition of CAT (2×10^4 units per plate) reduced the drug's toxicity, the extent of which is expressed as a CAT Protection Factor (CPF) in table 7.8, although the order of drug toxicity remained constant.

The CPF tells us that if one unit of FcPF_6 , say, gave a surviving fraction of 0.1, then in the presence of CAT (2×10^4 units), 1.48 units of FcPF_6 may now be used to give the same SF. An order of CAT protection is shown below, the $t\text{-pFcPF}_6$



analogue being protected most.

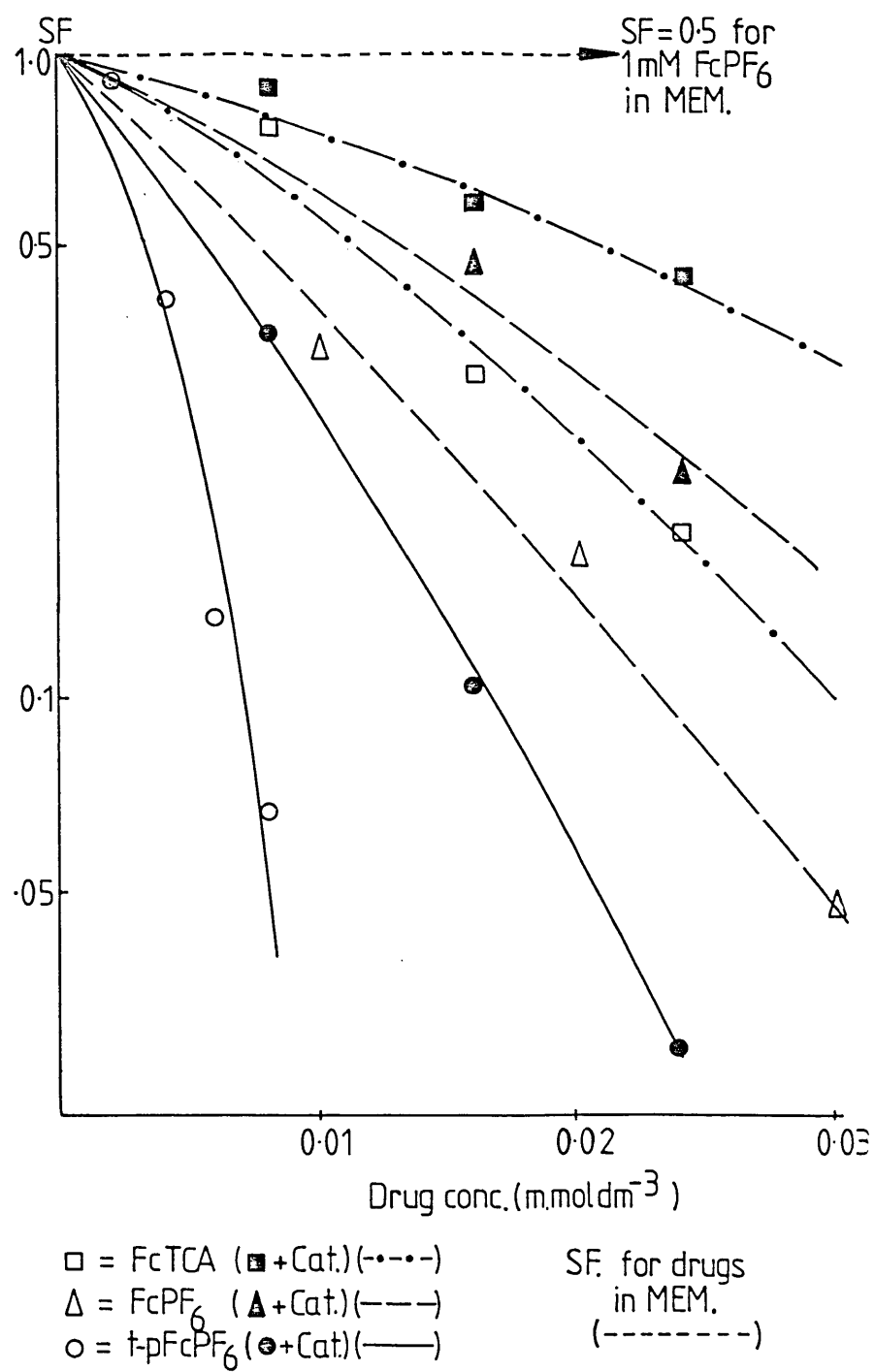


Fig. 7.11. Cellular survival curves obtained from one hour, aerobic toxicity experiments.

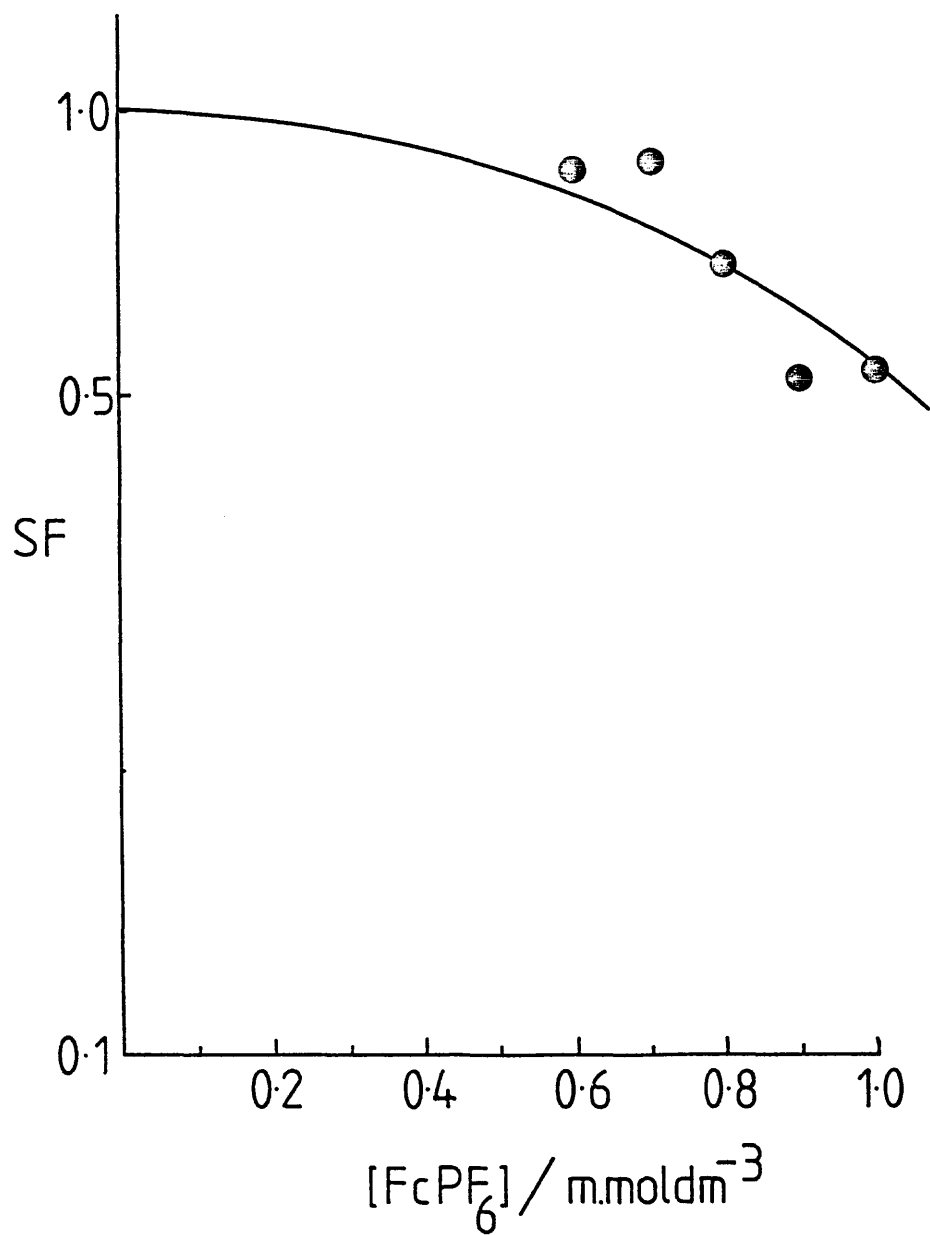


Fig. 7.12. Three hour aerobic toxicity of FcPF_6 in MEM at 37°C .

On repeating the above experiment but with 2×10^3 units CAT per plate, no reduction in drug toxicity was observed.

<u>Drug</u>	<u>[Drug]/mM for</u> <u>SF = 0.1</u>	<u>CAT protection</u> <u>factor</u>
FcPF ₆	0.0234	1.00
FcPF ₆ + CAT	0.0346	1.48
FcTCA	0.0318	1.00
FcTCA + CAT	0.05	1.57
t-pFcPF ₆	0.0068	1.00
t-pFcPF ₆ + CAT	0.0166	2.44

Table 7.8. Drug toxicity to V79 cells (1 hour contact in air at RT in pbs)

Of note here is that on preparing the drug solutions in MEM rather than pbs, all three drugs exhibited 100% cell survival at the concentrations covered. Three-hour experiments were done for FcPF₆ in MEM at concentrations up to 2 mmol/dm^3 under oxic conditions, the results are shown in fig. 7.12.

Even at a drug concentration of 1 mmol/dm^3 the SF was 0.5. Above this level however, no meaningful results were obtained since at these concentrations the cells lysed during incubation.

The toxicity of FcPF₆ under hypoxia was not greatly changed from that under aerobic conditions. (SF=0.806 compared to 0.90 under aerobic conditions both at 0.5 mmol/dm^3)

One-hour toxicities for FcPF₆ over a range of concentrations were determined in the presence of DMSO at 0.1, 0.5 and 1.0 mol/dm^3 . These experiments were hampered by the toxicity of DMSO itself, normalization of the results compensating for this.

Little change in drug toxicity was found at 0.1 and 0.5 mol/dm³ DMSO levels, at 1mol/dm³ however an increase in SF was observed (fig.7.13).

Experiments in the presence of highly irradiated cells were not successful, little if any cell colony formation being observed on the plates containing the HR cells. These experiments were carried out to investigate the possibility of high cellular concentrations giving rise to inconsistent results, as obtained with some of the three hour toxicity studies.

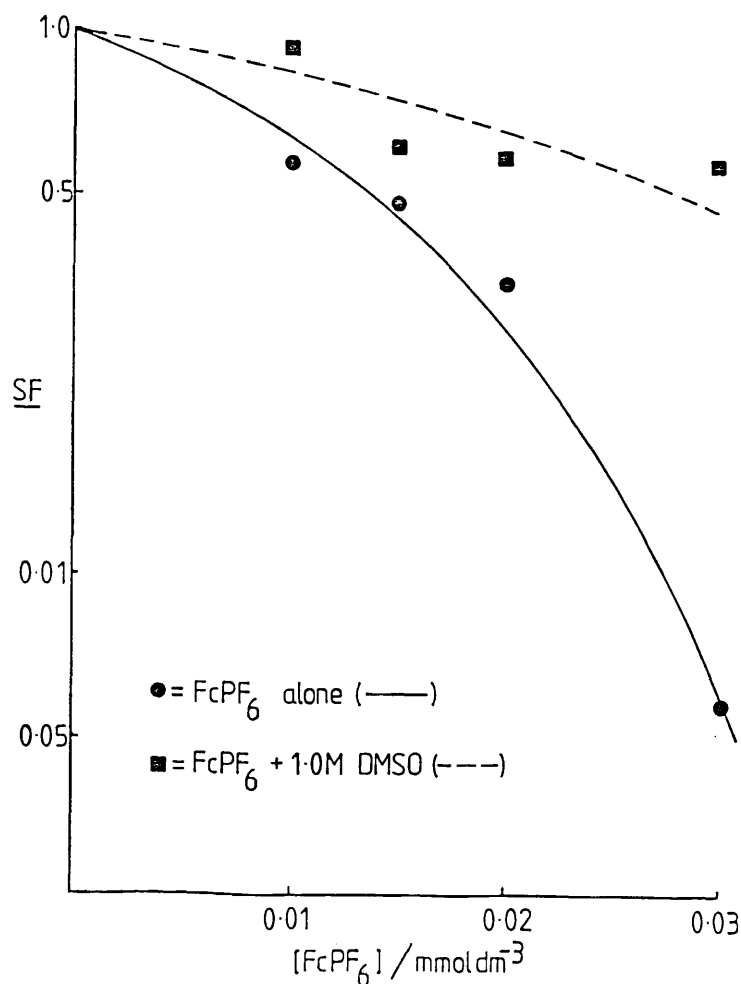
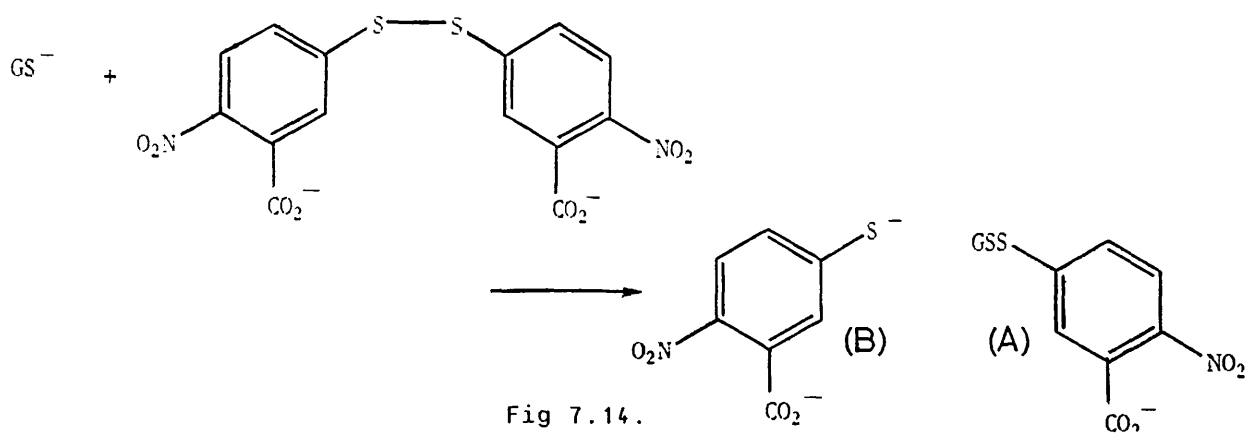


Fig. 7.13. One hour aerobic toxicity of FcPF₆ with and without DMSO.

7.3.3. Non-protein thiol depletion

The mechanism of reaction of DTNBA with NPSH is as follows.



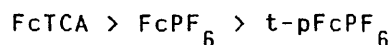
(B) is a highly coloured species exhibiting an electronic absorption at $\lambda_{\text{max}} = 412 \text{ nm}$ ($\epsilon = 13600 \text{ M}^{-1} \text{ cm}^{-1}$). In the method used here [68], an enzymatic-cycling process takes place in which the NPSH is sequentially oxidized by DNTBA and then reduced by NADPH in the presence of GSH-reductase. The extent of 2-nitro-5-thiobenzoic acid formation is monitored at 412nm and this compared with a control sample prepared and measured simultaneously.

Thiol depletion experiments were carried out on the three ferrocenium complexes each at a concentration of 0.01 mmol/dm^3 , the absorbance being measured ten minutes after reaction initiation; the results are given in table 7.9.

<u>Drug</u>	<u>ABS(412nm)</u>	<u>NPSH as % of control</u>
Control	1.037	-
FcPF ₆	0.661	63.74
FcTCA	0.529	51.01
t-pFcPF ₆	0.987	95.18

Table 7.9. Results of the thiol depletion experiments

In each case, a depletion of cellular thiol content by drug is observed. A tentative order of depletion is shown below, more



data being necessary for a rigorous quantitative assessment..

7.3.4. EPR Spin Trap Studies

The ferrocenium complexes all gave an X-band epr spectrum as shown (fig.7.15) on reaction with GSH in the presence of a spin trap species.

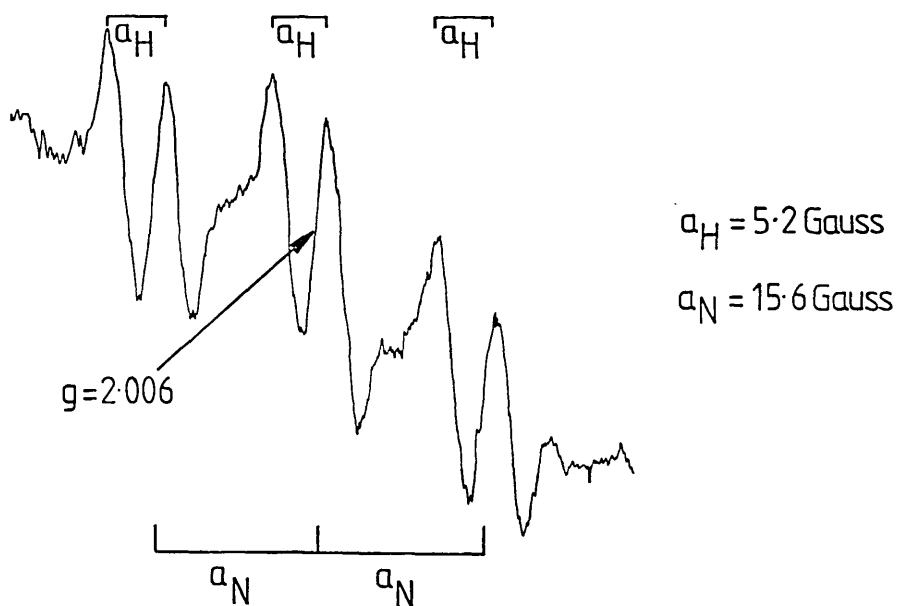


Fig. 7.15. X-band epr spectrum (RT) of the spin-trapped $\text{OH}^\cdot$ radical produced in the ferrocenium-GSH reaction in pbs.

The signal corresponds to the spin trapped $\text{OH}^\cdot$ radical, the nuclear hyperfine coupling constants a_H and a_N being the same as those assigned previously [70]. The position of the centre of the signal at $g=2.006$ confirms the organic radical nature of the

paramagnetic centre.

In all cases, the signal intensities were small and could not be used for determination of the radical yield. The spin-trapped species were quite stable however, being epr observable for several hours.

7.3.5. Drug Lipophilicity

Determinations were carried out with all three ferrocenium complexes. In each case, the characteristic absorbance at $\lambda_{\text{max}} = 617\text{nm}$ was followed.

Ten minutes after starting the shaking period it was found that for all drugs, the aqueous phase had become much paler in colour and the 1-octanol phase had become pale yellow. Shaking was continued for a total of 100min. after which the aqueous phase had become colourless and the 1-octanol markedly yellow. The absorbance of the aqueous phase was measured showing no ferrocenium complex to be present.

7.3.6. Aqueous solubility of ferrocene

The determination was done twice, a mean value of the solubility being calculated using the Beer-Lambert law. The results obtained (table 7.10) were reasonably consistent, an approximate solubility being required.

	Abs(440nm)	[Fc]/mol dm ⁻³	[Fc]/g dm ⁻³
1st run	0.017	4.2×10^{-5}	8×10^{-3}
2nd run	0.022	5.4×10^{-5}	10×10^{-3}

(for ferrocene $\epsilon_{440} = 102.0 \text{ M}^{-1} \text{ cm}^{-1}$ [69])

Table 7.10. Results of aqueous solubility determination of ferrocene.

7.3.7. Stabilization of Ferrocenium in aqueous solution

Stabilization of Fc^+ was investigated with the following amino acids; DL-alanine, L-leucine, DL-valine and DL-threonine. Studies were also made with glucose, a major component of MEM. All three ferrocenium complexes were used in these studies.

On mixing the reaction solutions the blue colour of the ferrocenium complex was present, raising the pH towards neutrality however, gradually changed the colour to yellow. This became dominant above pH 7 and alteration upto pH 8.0 was possible before solid started to precipitate out of solution. At pHs upto this point the original blue Fc^+ could be regenerated by the addition of HCl (aq).

Addition of GSH (aq) to the reaction solutions while at pH 8.0 quickly deposited ferrocene (characterized by ^1H nmr and mp determination).

7.3.8. Decomposition of Ferrocenium in pbs

These studies were hampered throughout by the formation of solid ferrocene from the decomposition. To overcome this a small amount of EtOH was added to dissolve the solid, it is however possible that this may affect the rate or degree of decomposition.

FcPF_6 and FcTCA were studied and in both cases a decrease in drug level of approx. 10% was observed after 1 hour at 1mmol/dm^3 . Reduction of the concentration to 0.01mmol/dm^3 resulted in negligible decomposition occurring over the same time period.

7.3.9. In-vivo experiments

The survival curve obtained on the irradiation of KHT sarcoma in the presence of FcTCA (100mg/Kg administered dose, irradiation 90 min. after injection) is shown in fig.7.16. An ER of 1.3 is found, this being similar to the degree of sensitization observed in-vitro at a drug concentration of 0.01mM.

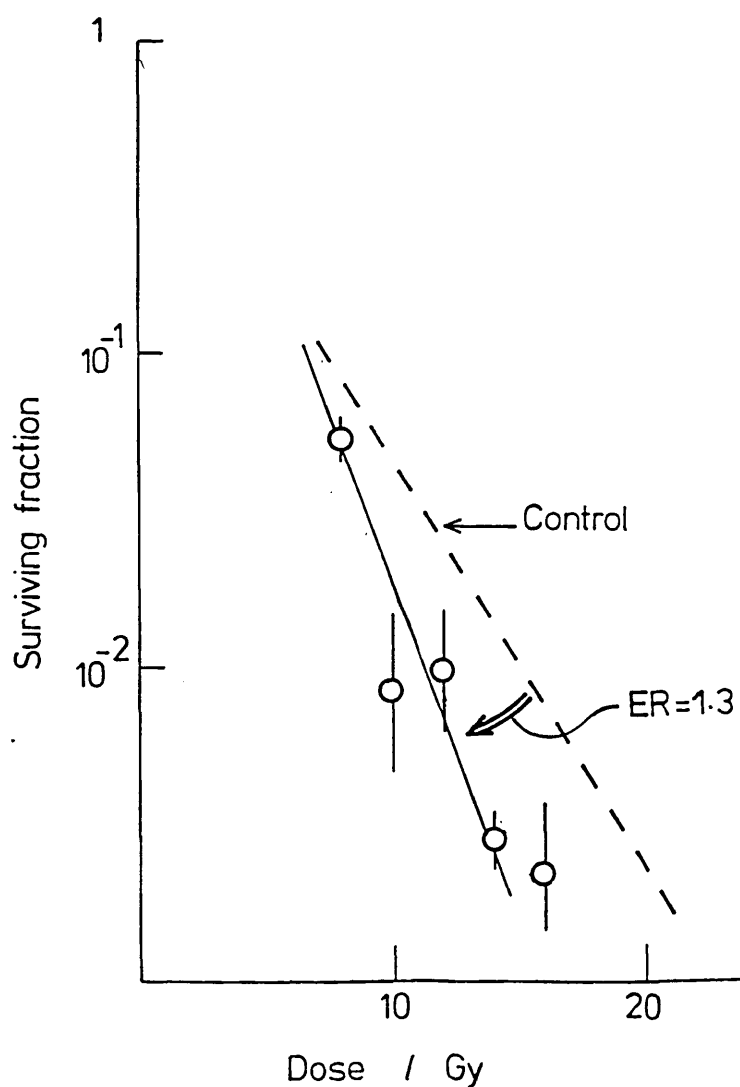


Fig. 7.16. Cellular survival curve of the sensitization of KHT sarcoma by FcTCA at a drug dose of 100mg/Kg, administered 90 min. before irradiation.

7.4. DISCUSSION

The discovery by Kopf-Maier of the anti-tumour properties of ferrocenium complexes [62] prompted us to consider the possible radiosensitizing ability of this type of compound.

Ferrocenium salts possess high aqueous solubility and, on a purely electron-affinic basis, would appear promising as radiosensitizers. The one-electron reduction potential of $\text{Fc}^+ + \text{e}^-$

Fc is +0.4V in aqueous media [71], making it very much more electron affinic than, for example, MISO (-0.389V).

A major consideration for a drug's clinical use is its toxicity. With the Fc^+ complexes this was reported to be relatively low [62], representing a clear advantage over the better known metal-based drugs which are often very toxic.

Substitution of alkyl groups onto the Cp rings alters the ferrocenium reduction potential [72]. Hence the tert-pentyl analogue was prepared to determine what influence, if any, this had on sensitizing ability. Unfortunately, its high toxicity prevented the measurement of any sensitization.

Results of experiments with V79 cells using the ferrocenium salts FcPF_6 and FcTCA showed them to be good sensitizers of hypoxic and to a lesser extent, oxic (only FcPF_6 studied) cells. The degree of sensitization achieved with both complexes under hypoxia was approximately 10 x greater on a concentration basis than that of MISO (fig.7.8).

An important observation related to the action of both complexes is that at all drug concentrations in hypoxia, the cellular survival curves were parallel to the nitrogen line exhibiting extrapolation numbers of approximately 1.0.

This effective removal of the initial broad shoulder region implies that the internal cellular repair processes are being suppressed, exponential cell kill being attained at very low radiation doses. The Fc^+ complexes appear to be operating via a different mechanism to that of the simple electron-affinic drugs with which the shoulder region is unchanged.

Removal of cellular repair processes has been observed previously, a notable example being in the sensitizing action of diazenedicarboxylic acid bis(N,N'-dimethylamide) [diamide] [73]. Diamide readily reacts with thiols and it was suggested that its mode of action was due to intracellular thiol depletion, effectively removing radioprotection of the cells and resulting in enhanced cell killing.

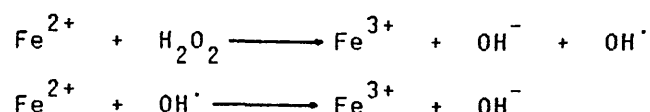
The observation of sensitization by FcPF_6 in oxic conditions also suggests that an alternative sensitizing mechanism is in action. If the mechanism was that of a typical electron-affinic sensitizer such as O_2 or certain nitroimidazoles, no enhancement of cell kill beyond the fully oxic line would be observed, oxygen-mimetic drugs tending to the same maximum ER of 3.0. Oxic sensitization is observed however with a DMF = 1.42.

The possible role of GSH depletion in the Fc^+ mechanism was therefore investigated. Both FcPF_6 and FcTCA gave approx. 50% cellular thiol depletion at 0.01mmol/dm^3 . A negligible reduction was observed with $t\text{-pFcPF}_6$. The important deduction to be made from these results is that gross thiol depletion is not taking place, as would be necessary to cause appreciable sensitization as seen with diamide. This suggests that GSH depletion is not a

major factor in the Fc^+ mechanism of action.

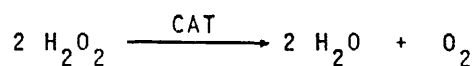
It had previously been observed that $[\text{Rh(II)(methoxyacetate)}_2]_2$ sensitizes both hypoxic and oxic V79 cells, reduction of the shoulder size being observed. Here it was suggested that sensitization was not caused by lowering of cellular NPSH levels, but was in some way related to inhibition of one or more biochemical repair mechanisms. This conclusion [57] may also be proposed as a possible contributory factor in the sensitizing ability of the Fc^+ complexes, since these too show similar properties.

It was suggested that Fenton chemistry may be of some relevance to the mode of sensitization of the ferrocenium complexes [74]. This involves the reaction of ferrous salts with H_2O_2 as shown below [75] and, to be relevant here would necessitate reduction of Fe(III) to Fe(II) either before, or during irradiation.



Removal of H_2O_2 at the time of irradiation would therefore alter the observed enhancement of cell kill if Fenton chemistry was important in the sensitization process.

This was investigated via irradiation experiments in the presence of catalase (CAT). CAT is a hemoprotein which catalyses the decomposition of toxic H_2O_2 within the cellular environment to give harmless products. (H_2O_2 attacks unsaturated fatty acid components of membrane lipids thus damaging the membrane structure)



Irradiation experiments with FcPF_6 and FcTCA in the presence of CAT showed little change in the observed sensitization (table 7.7). Hence it is suggested that Fenton chemistry is not important in the radiosensitizing action of ferrocenium salts. In addition, it can be deduced that damaging amounts of H_2O_2 are not being produced during irradiation this then leading to greater cell kill. Control experiments with bovine serum albumin did not change the sensitization thus showing that protein was not important in this process.

Toxicity studies of the ferrocenium complexes gave very interesting results. The (tert-pentyl) FcPF_6 drug was by far the most toxic, so much so that no irradiation experiments were successful. Previous work [57] had shown that for a series of rhodium carboxylate complexes, the drug toxicity was greatly affected by the alkyl portion of the carboxylate ligands. It was found that the more lipophilic the substituent, the greater the drugs overall toxicity. This trend was ascribed to the more lipophilic drugs having a higher cellular uptake, thereby giving a higher intracellular drug concentration and subsequent toxicity. This mode of action was considered for the greater toxicity of t-p FcPF_6 over the unsubstituted drugs, and lipophilicity experiments were done comparing the three ferrocenium salts. No conclusive results were obtained from these studies, however, since under the experimental conditions employed, all three complexes appeared to partition totally into the 1-octanol phase giving yellow solutions. The change of

colour on dissolution of ferrocenium salts in certain organic solvents has been described elsewhere [76], but in the context of this work, it adds another variable to the possible mode of action of this type of compound.

Drug uptake studies would be the best method of reliably determining the intracellular drug concentrations.

The most interesting finding from the toxicity studies was that in pbs, all three drugs showed a marked toxicity to V79 cells at relatively low ($0.01-0.03 \text{ mmol/dm}^3$) concentrations (fig.7.11 and table 7.8). As stated previously, the greatest effect was seen with t-pFcPF₆, FcTCA being least toxic. The difference between FcTCA and FcPF₆ is probably due to differences in interaction of the two anions with biological molecules, TCA apparently being less toxic.

In MEM however, the drugs all gave 100% cell survival up to a concentration of 0.03 mmol/dm^3 and with FcPF₆, a concentration 30 x greater gave SF = 0.5 (fig.7.12). From these observations it is clear that some component of the culture medium, absent from pbs, is interacting with the drugs' mechanism of toxicity. There are two possible pathways for this interaction; firstly, reduced toxicity may be due to direct interaction of the drug with biomolecules present in MEM thereby inhibiting its damaging action. Secondly, reactions of the drugs within the cells may give rise to potentially damaging species, e.g. OH[•] and these are then neutralized by one or more components of the medium, before damage to the cells can take place.

To investigate the second possibility, 1 hour toxicity experiments were carried out in the presence of DMSO. DMSO is a

radioprotecting agent which acts by trapping $\text{OH}^\cdot$. The high reactivity of the hydroxyl radical requires the DMSO to be in very large excess, hence the high concentrations used.

At 0.1 and 0.5 mol/dm³ DMSO concentrations, no reduction in FcPF_6 toxicity was observed, at 1mol/dm³ however, an effect was seen (fig.7.13). The problem encountered here was the high toxicity of DMSO itself, probably due to its known ability to transport drugs into cells giving rise to high intracellular concentrations and related toxicity. Normalization of the results was necessary to counter this. Even so, the cell colony count after exposure was very low and the reduction of FcPF_6 toxicity observed must therefore be regarded with care.

As an additional check for the production of hydroxy radicals, the reactions of the ferrocenium complexes with GSH in the presence of a radical spin trap were studied by epr spectroscopy. The spectrum obtained in all cases was that of the spin-trapped hydroxyl radical (fig.7.14) as characterized by the nuclear hyperfine splitting parameters [70]. The reagent concentrations for these reactions were higher than those of clinical relevance, in order to facilitate signal observation. Despite this, the yield of radical species was very small, suggesting that under the more dilute physiological conditions, $\text{OH}^\cdot$ production would be minimal, although this could still be important depending on where the radicals are being produced.

It is not at all clear how the reaction of FcPF_6 with GSH could produce the hydroxyl radical without the involvement of another molecule. It has been shown that thiol depletion of up to

50% is caused by the Fc^+ complexes and, although this is not high enough to give radiation sensitization, it is significant if damaging hydroxy radicals are being produced.

As with the irradiation studies, the possible role of H_2O_2 in the toxicity of these drugs was investigated. One hour toxicity experiments in the presence of CAT (2×10^4 units per plate) showed increased cell survival for all three ferrocenium complexes (table 7.8). At a lower CAT level (2×10^3 units pp.) no increased survival was observed. As before, control experiments using pure protein (BSA) in place of CAT were carried out. These studies gave increased cellular survival to exactly the same degree as that found with CAT, demonstrating conclusively that it was not the action of catalase itself which caused the toxicity reduction, but the protein with which it was supplied.

It therefore appears that the ferrocenium complexes must be interacting with protein in some manner, thus restricting their toxicity action.

The observation that in the presence of protein (from either CAT or BSA), sensitization enhancement is unchanged but drug toxicity is reduced, suggests that these actions do not operate by the same mechanism. A reduction in sensitization would be expected in the presence of protein relative to drug alone if they were the same.

A recent paper reported that the cytotoxic potency to tumour cells in-vitro of auranofin, an anti-arthritic gold(I) drug, was reduced ten-fold on the addition of foetal calf serum (fcs) to the culture medium, this being a result of gold binding to the serum proteins [77].

In the toxicity experiments reported here, the culture medium contained 10% fcs and the possibility therefore arises that a similar drug-protein interaction is taking place with the ferrocenium complexes, resulting in reduced toxicity.

Inspection of the components of the cell culture medium (table 7.2) shows that there is a wide range of molecules present which might interact with Fc^+ . It has been reported that under aqueous conditions, EDTA and several other carboxylic acids form stable chelate species with ferrocenium complexes [78]. These chelate species are stable at higher than physiological pHs and the ferrocenium complexes can be regenerated as discrete complexes by the addition of acid (non-oxidizing).

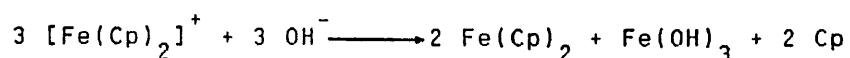
As there is a plentiful supply of amino acids in MEM and the cellular environment, a series of qualitative reactions were done here in which the stabilization of ferrocenium salts by a variety of amino acids was investigated. The results obtained showed that the ferrocenium complexes are indeed stabilized by the amino acids, regeneration from solution pHs as high as 8.0 being achieved. To show that it is actually the ferrocenium complex present at these elevated pHs, an excess of GSH was added to the solution, immediately precipitating ferrocene as the reduction product (as characterized by ^1H nmr spectroscopy and mpt.). This reduction could not have taken place had there been no oxidizing ferrocenium present. This reaction also shows that stabilization of ferrocenium to alkaline pH does not stabilize it to reduction by GSH.

Stabilization studies were carried out in the presence of D-

glucose, a major component of the culture medium. These showed no stabilization of ferrocenium at higher pHs.

The mode of binding responsible for the ferrocenium stabilization is not fully understood. ^1H nmr studies would be useful in gaining an insight but these are not possible due to the paramagnetic nature of the Fe(III) centre causing line broadening.

The stability of ferrocenium complexes at high pHs in the presence of amino acids and other carboxylic acids is important since in alkaline solution ferrocenium complexes decompose as shown below, the process being irreversible under physiological conditions [79].



Stabilization would allow transport of the drug to the crucial areas within the cell in the Fc^+ form.

The rates of decomposition of FcPF_6 and FcTCA in aqueous buffer solutions, at physiological pHs were studied in this work by following the rate of decrease of absorbance of the $\lambda_{\text{max}} = 617\text{nm}$ band with time, this being a characteristic ferrocenium moiety transition [63]. Studies were very much hampered by the presence of ferrocene, one of the products of the Fc^+ decomposition, as it precipitated in the quartz cells.

The results obtained showed the Fc^+ complexes to reduce in concentration by approx. 10% in 1 hour at 1mmol/dm^3 . At drug levels used in the biological studies the rate of decomposition was very low, suggesting that there would be negligible drug decomposition in solution before administration.

In the presence of amino acids however, the solution colour had changed at these pHs and no results were forthcoming from this wavelength.

From the evidence collected, it seems likely that in MEM, Fc^+ may be interacting with both fcs and amino acids, not to mention the other carboxylic acid components also present.

The result of this is reduced drug toxicity and drug stabilization towards decomposition at physiological pHs, the latter being important in relation to transport of the drug within the cell.

Of note here is that MEM is a much more representative model of the situation actually encountered in the body, hence the large reductions of toxicity in this medium are very encouraging with regard to the clinical potential of these drugs.

Preliminary In-vivo studies of FcTCA on KHT-sarcoma tumours gave very promising results. At a drug concentration of 0.01mmol/dm^3 an ER of 1.3 was obtained, comparing favourably with the classic sensitizing drugs. The observed toxicity at this level was relatively low which tends to support the results of toxicity experiments seen in-vitro.

Although very promising results have been obtained from radiosensitizing experiments with the ferrocenium complexes, a great deal of work still needs to be done to understand fully the mechanism of action of these compounds. It is not yet certain as to the true nature of the actual sensitizing species. Is Fc^+ the active agent or is it possible that interaction with cellular constituents forms new species, these then being the active

agents? Similarly, could Fc^+ be reduced soon after administration, by for example GSH, Fc then being the sensitizer?

It has already been shown that the first proposal is a possibility. The second alternative was studied by determining the degree of sensitization by Fc and a series of more water soluble derivatives. Poor sensitization was exhibited by these compounds and this coupled with their relatively high toxicity suggests that they are probably not the active agents (table 7.6).

A third alternative is that the drug itself, in whichever form it exists at the time, interacts with radiation directly forming a new active species. Previous studies on irradiation of solid Fc have shown the formation of several radical species at 77K, but these disappear on warming [80]. Radiation studies of solutions of Fc in cyclohexane [81] suggest that Fc is not consumed on irradiation and radical attack is not of great importance. Aqueous solutions of ferrocenium sulphate have shown the production of Fc on irradiation, the yield increasing with radiation dose but slightly decreasing in oxic relative to hypoxic solutions [81]. However these studies were carried out at pH 1.0 and may not be relevant to the physiological situation.

The ideal situation would be to test simultaneously a water soluble ferrocene complex and its ferrocenium analogue, a direct comparison then being made. Attempts to prepare such systems were made but none could be found in which both iron oxidation states were stable in aqueous solution.

Recent research has found that an efficient method of giving drugs a high degree of aqueous solubility is to encapsulate them

into a cyclodextrin ring system [82]. Cyclodextrins are cyclic oligosaccharides consisting of six, seven or eight glucose units joined via their anomeric centres. They are known to form inclusion complexes with smaller molecules which can fit into the central cavity. Thus, a drug normally aqueous insoluble could be rendered soluble by this mechanism. This is indeed the case and with the second generation platinum anticancer drug carboplatin, [83] a soluble form has been prepared with α -cyclodextrin and clinical tests have shown that "complexed" carboplatin is at least as effective at fighting cancer as the drug alone.

It was with this in mind that attempts were made to prepare the known cyclodextrin complexes with Fc [84], with the aim of preparing a water soluble Fc drug to be tested for sensitizing ability. Unfortunately, all attempts at this preparation failed and no biological experiments were done.

The bipy and o-phen complexes gave disappointing sensitization results. It was hoped that the known intercalating and DNA strand breaking properties of complexes of these ligands [85] would target the metal centre to DNA, the believed site of action, thereby giving more efficient sensitization. As with the Cp rings of the Fc^+ complexes, these ligands possess a delocalised electronic structure capable of stabilizing an additional electron. Further work in this area could involve the functionalization of the ligands with for example, electron withdrawing NO_2 groups, the complexes then being tested for activity.

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