CHROMIUM (III) COMPLEXES AND THEIR RELATIONSHIP TO THE GLUCOSE TOLERANCE FACTOR

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ABSTRACT

To forms of the dinicotinate complex $Cr(nic)_2(H_2O)_3OH$ were formed which were yellow and blue, respectively. For the yellow form the nicotinic acid ligands were coordinated via the pyridine ring nitrogen atom but this complex was biologically inactive, while for the blue form nicotinic acid was coordinated via the carboxylate group and this compound was biologically active. Only Cr(III) formed a stable carboxylate coordinated dinicotinate complex. No stable complexes were formed with Fe(III) and Mn(III) due to significant olation, even at acidic pH's, and the complexes of nicotinic acid with Cr(II), Mn(II), Co(II) and Ni(II) were all pyridine nitrogen atom coordinated and biologically inactive.

Several chromium (III) complexes with amino acids possessed biological activity also, and these included the α-carboxylate coordinated species $Cr(gly)_n(H_2O)_{6-n}^{3+}$ and $Cr(glu)_n(H_2O)_{6-n}^{3+}$, the bidentate coordinated $Cr(gln)_2(H_2O)_2^+$ complex, and the NH_4OH- eluted complexes obtained when $Cr(gly)_2(H_2O)_2^+$ and $Cr(glu)_2(H_2O)_2^+$, but not $Cr(cys)_2(H_2O)_2^+$, were eluted from a DOWEX 50W-X12 cation-exchange column (loss of the α-amino coordination was postulated to have occurred).

The biologically active mixed ligand complex postulated as $Cr_2(nic)_4(gly)_2(OH)_2$ was prepared and found to be stable at neutral pH as a result of coordination of the glycine ligands.

The activity of the chromium (III) complexes in the yeast fermentation assay suggested that similar effects would be found in mammalian systems. The yeast assay system was found to be a simple, quick and reproducible method of determining biological activity.

All of the active chromium (III) complexes prepared were found to be similar, in structure, to the diguanide compound

1,4-diguanidinobutane which is known to lower blood sugar levels in mammals. This similarity in structure suggested a similar function might be possessed by the complexes reported in this thesis.

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SECTION ONE

INTRODUCTION

It was in 1957 that Schwarz and Mertz (1957) first reported the existence of a dietary factor, known as the glucose tolerance factor (GTF), which was required for the maintenance of normal glucose tolerance in rats. A deficiency in GTF was associated with delayed removal rates of excess glucose from the bloodstream during intravenous glucose tolerance tests and this was observed in laboratory rats fed on a stock diet (Torila yeast) which was deficient in GTF. For these rats the clearance rate of excess glucose in the bloodstream was 2.8% per minute compared with a normal clearance rate of about 4.0% per minute. However, these effects of impaired glucose tolerance could be reversed by GTF preparations from naturally occurring sources, such as Brewer's yeast, or from acid hydrolysates of pork kidney powder fed to rats as a single stomach-tubed dose (50-100 µg/100g body weight (Schwarz and Mertz 1959).

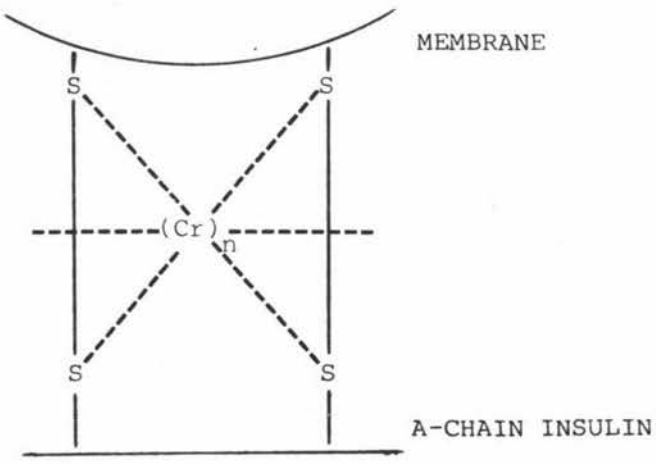
Tri-valent chromium was identified as the active ingredient in GTF by Schwarz and Mertz (1959) and it was also reported that the GTF-deficient rats were in fact chromium-deficient. The effect of chromium (III) ions, in various complex forms, on the uptake of glucose by epididymal fat tissue of rats was then investigated by Schwarz et al (1961). Great care was taken by Schwarz to maintain the low chromium (III) environment which is necessary to induce a chromium-deficient state; the rats were fed a diet known to be GTF deficient and contamination by chromium was minimised wherever possible. A dose of 1mg chromium (III) per 100g of body weight, as the hexa-urea complex, was used in the rat's diet, and in their in vitro tests chrome alum ($Cr_2(SO_4).K_2SO_4.24H_2O$) was used. In the

GTF deficient rats the glucose removal rate diminished (3.7 ± 0.5% per minute) but was restored after five weeks on a ration containing 100µg of hexa-urea complex per 100g of diet to a normal glucose removal rate of 4.7 ± 0.5% per minute. In the in vitro test using epididymal tissue a pronounced increase of glucose uptake was obtained with 0.1µg of chromium (III) and at this level an optimal level of added insulin was found. An increase in glucose uptake could be demonstrated in vivo as well as in vitro. In both cases chromium (III) stimulates the insulin effect but has no influence by itself, however, insulin does have an effect by itself since at high levels of insulin the effect of chromium (III) diminishes.

Many experiments have been carried out in vivo to observe possible interactions between chromium, glucose and insulin using a variety of laboratory animals fed on both normal and low-chromium diets. Schroeder (1966) examined the serum glucose levels of chromium-deficient rats and claimed that these rats which had an average blood serum glucose level of 137.2 ± 6.8mg/100cm^3 were hyperglycemic while those rats which were receiving a chromium (III) supplementation only had an average blood serum glucose level of 116.6 ± 1.7mg/100cm^3. This does not prove that these diabetic symptoms are caused by chromium-deficiency as other factors could also have been involved. The in vitro experiments used by many researchers have been developed into assay systems and these are discussed in Section 5.1. Since insulin is an essential part of the epididymal rat fat pad assay attempts have been made to demonstrate an interaction between chromium (III) and insulin. Evans et al (1972) used GTF preparations from Brewer's yeast as well as synthetic, biologically-active chromium (III) preparations and has shown that binding of these samples to insulin does occur. Anderson and Brantner (1977) also found that insulin binds to chromium (III)-containing GTF preparations but the form of the chromium (III) is not known.

It has become evident that only certain forms of chromium (III) are biologically active since inorganic chromium compounds display little or no in vitro insulin potentiating activity but upon conversion to certain (not yet characterised) organic chromium complexes appear to acquire significant insulin potentiating activity. The effect of chromium (III) on the polarography of mitochondria (Christian et al 1963) was consistent with the hypothesis that chromium can form a ternary complex between insulin and tissue insulin receptors, as shown in Figure 1.1. The model involves coordination of chromium to two sulphydryl groups and the two sulfur atoms of the A-chain disulphide bridge of insulin, however, no account is made of the two remaining coordination sites of chromium (III) (which usually forms octahedral complexes). Christian postulated that it was the unknown ligands coordinated to those two remaining sites that accounted for the difference between the relatively small effect of a variety of synthetic chromium compounds and the much stronger glucose tolerance factor activity associated with yeast extracts. It was not known what ligands were coordinated to the chromium (III) atom necessary to form the ternary complex between insulin and tissue receptors. It must be emphasized that such an hypothesis should not rule out the possibility of other modes of action at different sites, for example participation in redox reactions ($Cr^{2+} \rightleftharpoons Cr^{3+}$), or electron transport along chains of olated chromium complexes, but such possibilities have not been proved to be important within the cell.

FIGURE 1.1: HYPOTHETICAL TERNARY COMPLEX OF CHROMIUM AT SITE OF ACTION



Chromium is a transition metal and can exist in one of three common valence states, II, III, and VI, but only the trivalent state has strong coordinating tendencies. The chromous ion is a strong reducing agent and, unless protected by the coordination of strong ligands, is unstable in air and rapidly oxidises to the trivalent state. It is unlikely that it exists in biological systems, except as part of a $Cr^{2+} \rightleftharpoons Cr^{3+}$ redox system involving the more stable chromic ion. The trivalent state is the most stable and is regarded as the most important one from a biological point of view. The majority of complexes are octahedral and chromium (III) is characterised by a strong tendency for coordination. In the aqueous solutions of numerous salts, chromium (III) is present in the form of $Cr(H_2O)_6^{3+}$, however, this ion does not exist in biological systems because it undergoes olation or polymerisation with bridging hydroxide ions at neutral pH. It is necessary therefore if chromium is to have a biological effect that under physiological conditions, chromium (III) is complexed in some way so that it can remain in solution.

Many attempts have been made towards the isolation and identification of compounds which exhibit glucose tolerance factor activity from Brewer's yeast (Mertz et al 1974, Toepfer et al 1977 and Anderson et al 1978), however only partially purified GTF preparations have been obtained to date. Recently Haylock et al (1982) have published a preparation scheme for the isolation of all the chromium-containing fractions in brewer's yeast extract as a first step towards identifying the nature of GTF. Therefore work was carried out, in this thesis, to try and prepare biologically active, synthetic chromium (III) compounds in an attempt to gain insights into the structure and possible metabolic function of biologically active chromium (III) complexes.

SECTION TWO

NICOTINIC ACID COMPLEXES WITH CHROMIUM AND OTHER TRANSITION METALS

2.1 INTRODUCTION

The glucose tolerance factor, GTF, has been defined as a chromium-containing compound which is essential for the normal maintenance of glucose tolerance in mammals (Schwarz and Mertz, 1957). During the purification of natural GTF fractions from Brewer's yeast a close correlation between the biological activity of the preparations and an absorption maximum at 262nm has been observed (Mertz et al, 1974). Since the presence of a pyridine ring structure has been inferred from mass spectrometric analysis of the preparations, it has been suggested that nicotinic acid is the compound which is responsible for the 262nm absorption. It was also noticed by Mertz et al (1974) that when chromic chloride was mixed with nicotinic acid in aqueous solution and heated at pH 3.2, a chromium complex, or mixture of complexes, which exhibited GTF-like biological activity could be isolated from the mixture by ion exchange chromatography. Mertz postulated that a tetra-aquo-dinicotinic acid complex of chromium was the active compound by analogy with similar nicotinic acid complexes which were known for other transition metals.

Although several nicotinic acid complexes with transition metals are known and have been characterised (Anagnostopoulos et al 1972, Kleinstein and Webb 1971, Allen et al 1977, Chiacchierini et al 1977) little is known of chromium nicotinate complexes. If Mertz's suggestion is valid knowledge about the structure of the chromium dinicotinic acid complex would be helpful in understanding the possible biological activity of this complex, and also that of any naturally-occurring chromium complexes. Nicotinic acid possesses two functional groups both of which are suitable for coordination to Cr(III) therefore two modes of coordination are possible;

(i) coordination through the carboxylate group, and
(ii) coordination through the nitrogen atom of the pyridine ring.

The major aim in this study was to determine the exact structure of the biologically active chromium nicotinic acid complex described by Mertz, and to see whether biological activity was restricted to chromium (III) complexes.

2.2 TRANSITION METAL CHEMISTRY

2.2.1 Introduction

All transition metals possess partially filled d shells and the elements of the first transition series, which include Cr, Mn, Fe, Ni, and Co all have partly filled 3d shells. The d orbitals project well out to the periphery of the atoms, or ions, so that the electrons occupying them are strongly influenced by the surroundings of the ion, and in turn, are able to influence the electron orbitals of the ligands surrounding the metal ion very significantly. Thus many of the properties of an ion with a partly filled d shell are quite sensitive to the number and arrangement of the d electrons present. The electronic configurations of chromium and some other transition metal ions are shown in Table 2.1. One electron may help to determine the orbital occupied by another electron by direct interaction between the electrons. It is the net effect of all the forces:

(i) nuclear-electronic attraction,
(ii) shielding of one electron by others,
(iii) interelectronic repulsions and
(iv) exchange forces that determine the stabilities of an electronic configuration.

TABLE 2.1: ELECTRONIC CONFIGURATION OF METAL ATOMS

Metal	4s electrons	3d electrons
Cr	1	5
Mn	2	5
Fe	2	6
Co	2	7
Ni	2	8

2.2.2 Magnetic Properties

Most compounds of the transition metals possess paramagnetism, caused by the presence of ions, atoms, or molecules which have unpaired electrons (a paramagnetic substance is one which is attracted into a magnetic field). The electrons determine the magnetic properties of matter in two ways. Firstly, the electron is itself a magnet and as the electron spins on its axis it produces a magnetic moment called the spin moment of the electron. Secondly, as the electron orbits the nucleus, it produces a magnetic moment called the orbital moment of the electron. Together the spin and orbital contributions determine the magnetic properties of any individual atom or ion. The magnetic moment μ_s of a single electron being given by the equation:

$\mu_s = g\sqrt{s(s+1)}$ Bohr Magnetons (B.M.) Equation 2.1 in which s is the absolute value of the spin quantum number and g is the gyromagnetic ratio. The quantity $\sqrt{s(s+1)}$ is the value of the angular momentum of the electron and for the free electron g = 2.00, thus for a single electron equation 2.1 becomes:

$$\mu_s = 2\sqrt{\tfrac{1}{2}(\tfrac{1}{2}+1)} = 1.73 \text{ B.M.}$$

The spin quantum number of the whole ion, S is the sum of the spin quantum numbers, $s = \frac{1}{2}$, for the individual electrons. The "spin only" moment is found by substituting S for s in equation 2.1 to give:

$$\mu_S = g\sqrt{S(S+1)} \text{ B.M.} \qquad \text{Equation 2.2}$$

The values of μ_S for all possible real cases are shown in Table 2.2. Discrepencies between the observed and calculated magnetic moments arise from the orbital contribution of the unpaired electrons since, in general, the transition metal ions in their ground states (D or F being the most common) possess orbital angular momentum. If the orbital contribution is included, the total magnetic moment is given by the expression:

$$\mu_{S+L} = \sqrt{4S(S+1) + L(L+1)} \qquad \text{Equation 2.3}$$

in which L is the angular momentum quantum number for the ion.

TABLE 2.2: "SPIN ONLY" MAGNETIC MOMENTS FOR VARIOUS NUMBERS OF UNPAIRED ELECTRONS

Number of unpaired electrons	S	μ_s(B.M.)
1	1/2	1.73
2	1	2.83
3	3/2	3.87
4	2	4.90
5	5/2	5.92
6	3	6.93
7	7/2	7.94

In Table 2.3 are listed the observed magnetic moments for several transition metal ions, together with the calculated values of μ_S and μ_{S+L} and it is apparent that the observed magnetic moments frequently exceed μ_S but are seldom as high as μ_{S+L}. The reason that the observed magnetic moment is usually less than μ_{S+L} resides in the fact that the electric fields of the surrounding ligands restrict the orbital motion of the electrons, partially "quenching" the

orbital angular momentum and hence the orbital moments. In the d^3 case, such as for Cr^{3+} in an octahedral environment, the orbital contribution is introduced in opposition to the spin contribution and moments slightly less than μ_S are observed.

TABLE 2.3: THEORETICAL AND EXPERIMENTAL MAGNETIC MOMENTS FOR VARIOUS TRANSITION METAL IONS

Ion	Ground State quantum number		Spectroscopic Symbol	μ_S (B.M.)	μ_{S+L} (B.M.)	Observed Moments (B.M.)
	S	L				
Ni^{2+}	1	3	^3F	2.83	4.47	2.8-4.0
Cr^{3+}	3/2	3	^4F	3.87	5.20	$\simeq$ 3.8
Co^{2+}	3/2	3	^4F	3.87	5.20	4.1-5.2
Fe^{2+}	2	2	^5D	4.90	5.48	5.1-5.5
Co^{3+}	2	2	^5D	4.90	5.48	$\simeq$ 5.4
Mn^{2+}	5/2	0	^6S	5.92	5.92	$\simeq$ 5.9
Fe^{3+}	5/2	0	^6S	5.92	5.92	$\simeq$ 5.9

The magnetic susceptibility of a substance can be measured experimentally and is commonly used to calculate the magnetic moment of the paramagnetic ion. In the Faraday method, the magnetic susceptibility χ of a sample compound is compared with that of a standard sample with a known magnetic susceptibility (χ_{STD}) using the equation:

$$\chi_{SAMPLE} = \chi_{STD} \times \frac{\Delta W_{SAMPLE}}{\Delta W_{STD}} \times \frac{W_{STD}}{W_{SAMPLE}} \qquad \text{Equation 2.4}$$

where ΔW is the change in weight upon immersion in a magnetic field and W is the weight of the sample. The observed magnetic moment is then given by the equation:

$$\mu_{eff} = 797.9 \sqrt{\chi_M^{CORR} . T} \qquad \text{Equation 2.5}$$

where χ_M^{CORR} is the molar susceptibility after correction for diamagnetism and T is the temperature (Cotton and Wilkinson, 1972).

2.2.3 Electronic Structures and Spectral Properties

A transition metal ion possesses five d-shell orbitals designated the d_{xy}, d_{yz}, d_{zx}, d_{z^2} and $d_{x^2-y^2}$ orbitals. In the free metal ion all the d obitals are degenerate, however, in an octahedral complex, they are not all equivalent since the d_{z^2} and $d_{x^2-y^2}$ orbitals have lobes of electron density heavily concentrated in the vicinity of the ligands, while the d_{xy}, d_{yz} and d_{zx} orbitals have lobes which project between the ligands. Thus the coordinating ligands tend to split the d-orbitals into two groups on coordination in an octahedral environment as shown in Figure 2.1. In an octahedral field the group of orbitals d_{xy}, d_{yz} and d_{zx} are all equivalent and form the t_{2g} set while the other two orbitals d_{z^2} and $d_{x^2-y^2}$ are also equivalent and form the higher energy e_g set.

Hunds first rule, the rule of maximum multiplicity, states that: "in general if a group of n or less electrons occupies a set of n degenerate orbitals, they will spread themselves among the orbitals and give n unpaired spins" (Cotton and Wilkinson, 1972a). In transition metal ions, the electrons are distributed in minimal energy configurations in accord with Hund's rule and some of these possible ground state occupancy schemes are shown in Figure 2.2. In the d^5 and d^7 cases two configurations are possible, a high spin state which has the maximum number of unpaired electrons and a low spin state which has the minimum number of unpaired electrons determined by the magnitude of the electrostatic field and the pairing energy of the electrons occupying the same orbital.

FIGURE 2.1: Energy level diagram of an octahedral metal ion.

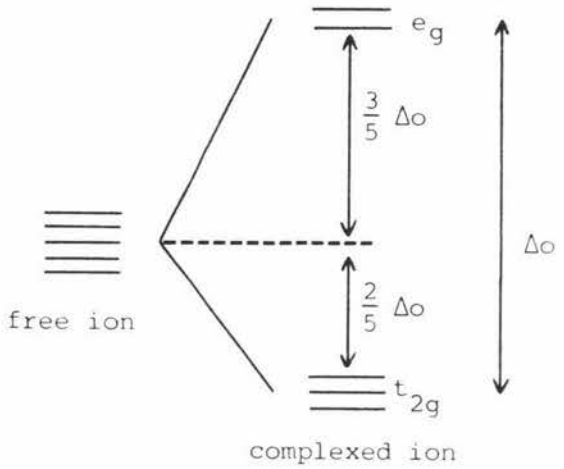
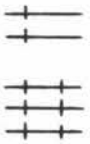


FIGURE 2.2: Ground state d-orbital configurations

Configuration	Distribution	Example
d^3		Cr^{3+}
d^7	 High Spin Low Spin	Co^{2+}
d^8		Ni^{2+}
d^5	 High spin Low Spin	Mn^{2+}

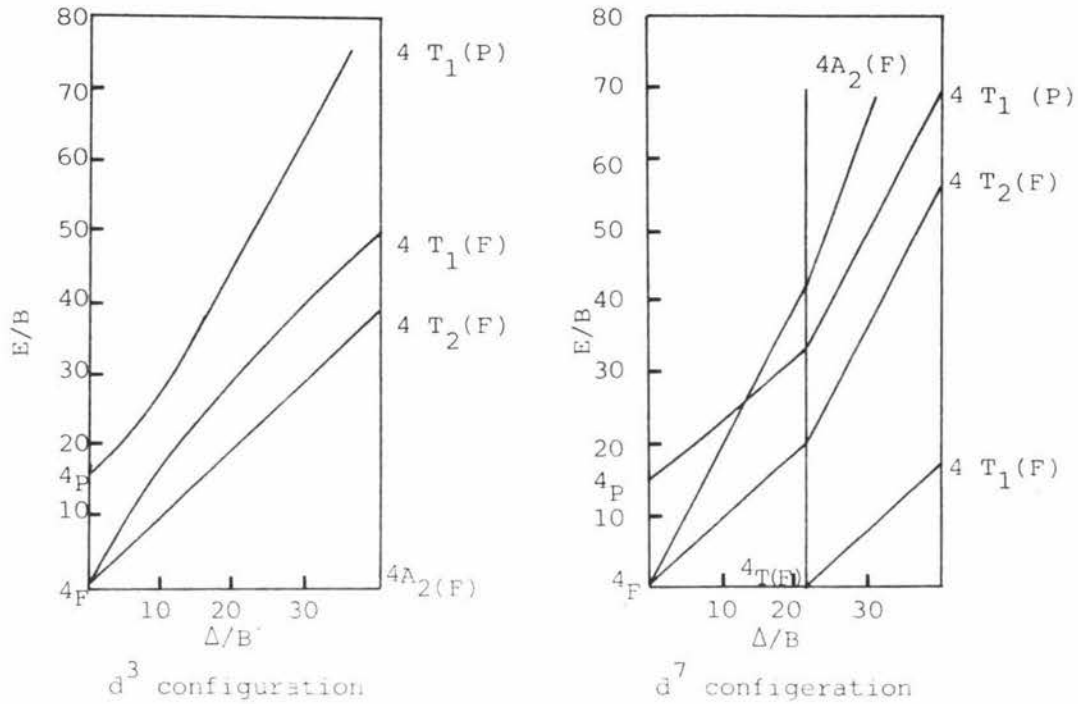
The ultraviolet-visible absorption spectra of transition metal complexes can be interpreted by using energy-level diagrams based upon the Russell-Saunders states of the d^n configurations. These are called Tanabe-Sugano diagrams and are graphs of energy (E) against the crystal field splitting energy (Δ)(determined by the ligand). At the extreme left of the energy-level diagrams are the Russell-Saunders states for the free ion, which are split in the octahedral crystal field due to the coordinated ligands into the components shown in Table 2.4. A and B are singly degenerate states, E doubly degenerate states and T designates triple degeneracy, while the left superscripts indicate spin multiplicity. The ground state in the Tanabe-Sugano diagram is always taken as the abscissa and the energy of the other states plotted against it as shown in Figure 2.3 for the d^3, d^7 and d^8 octahedral configurations.

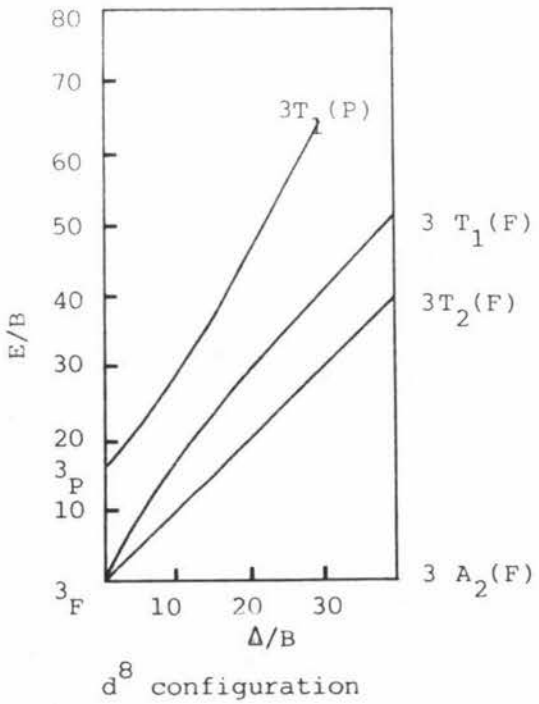
TABLE 2.4: SPLITTING OF RUSSELL-SAUNDERS STATES

State of free ion	States in complexed ion
S	A_1
P	T_1
D	$E + T_2$
F	$A_2 + T_1 + T_2$
G	$A_1 + E + T_1 + T_2$
H	$E + 2T_1 + T_2$

In order to use these energy level diagrams to predict or interpret the spectra of octahedral complexes we use the quantum-mechanical selection rule that forbids transitions between states of different spin multiplicity which simplifies the diagrams somewhat as shown in Figure 2.3. Thus the only possible transitions occur between the ground state and the higher energy states shown in Figure 2.3 (although these too are not fully allowed and typically have extinction coefficients of a few hundred 1 mol^{-1} cm^{-1}) and the greater

FIGURE 2.3: Tanabe-Sugano Diagrams





the energy difference of the transition, the lower the frequency at which such a transition is observed in the electronic spectrum.

For the d^3 configuration, as found in Cr^{3+} complexes, three transitions are expected from Figure 2.3: $^4A_2(F) \rightarrow {}^4T_1(P)$; $^4A_2(F) \rightarrow {}^4T_1(F)$ and $^4A_2(F) \rightarrow {}^4T_2(F)$ in order of increasing wavelength of the d-d transition and hence the electronic spectrum of Cr^{3+} complexes should exhibit three d-d bands. Co^{2+} has a d^7 configuration and most common ligands confer a high spin state for the ground state of the metal ion thus in this case three d-d transitions are again expected: $^4T_1(F) \rightarrow {}^4T_1(P)$, $^4T_1(F) \rightarrow {}^4A_2(F)$ and $^4T_1(F) \rightarrow {}^4T_2(F)$. The Ni^{2+} ion has a d^8 configuration and hence three d-d transitions are also expected in this case: $^3A_2(F) \rightarrow {}^3T_1(P)$, $^3A_2(F) \rightarrow {}^3T_1(F)$ and $^3A_2(F) \rightarrow {}^3T_2(F)$.

D-d transitions between states of the same spin multiplicity give rise to the spin-allowed absorption bands seen in the electronic spectra. The position of these bands is dependent on the Δo values, which are the energy differences between e_g and t_{2g} orbitals (see Figure 2.1). The dependence of the Δo values on the identity of the ligands follow a regular order known as the spectrochemical series in which various types of ligands are arranged in a series according to their capacity to cause d-orbital splitting. An example of such a series generalised from a large number of metal ions is as follows: $I^- < Br^- < Cl^- < F^- < OH^- < C_2O_4^{2-} \simeq H_2O < NCS^- < py \simeq NH_3 < en < bipy < o\text{-}phen < NO_2^- < CN^-$. Thus the greater the capacity of a ligand to cause d-orbital splitting, the greater the energies of the d-d transitions and hence the more the spectral bands are shifted to lower wavelengths.

2.2.4 Charge Transfer Spectra

There is an important class of transitions in which the electron moves from a molecular orbital centred mainly on the ligands to one centred mainly on the metal ion, or vice versa. Since the charge distribution of the complex is very different for ground and excited states, these transitions are called charge-transfer transitions. There are two broad classes: ligand-to-metal (L→M) transitions and metal-to-ligand (M→L) transitions both of which are fully allowed transitions and give strong bands (extinction coefficients of approximately 10^4 l $mol^{-1}cm^{-1}$) lying in the extreme blue region of the visible spectrum or the near ultra-violet region. They can therefore easily obscure the less intense d-d transitions of the metal ion.

Metal-to-ligand transitions can only be expected when the ligands possess low-lying empty orbitals and the metal has filled orbitals of higher energy than the highest energy ligand orbitals. Some examples have been provided by such ligands as CO, CN and aromatic amines such as pyridine which can bond to metals such as Cr^0 ($Cr(CO)_6$), Fe^{2+} ($Fe(CN)_6^{4-}$) and $Ni^{2+}(Ni(py)_4Cl_2)$.

2.2.5 Electron Spin Resonance Spectra

Electron spin resonance (ESR) is observed when metal ions containing one or more unpaired electrons are placed in a magnetic field. The unpaired electrons behave as magnets as a result of their spin and can line up either parallel or antiparallel with the external field, each alignment having a slightly different energy. The transitions between these energy levels are detected by applying the energy for the transition in the form of a radio frequency electromagnetic radiation. A single absorption is observed for the electron in an isolated metal ion but in many complexes more complicated spectra are observed depending upon the environment of the metal ion. Thus an examination of esr spectra can provide structural information about transition metal complexes.

The energy difference between the two energy levels of the unpaired electron is equal to $g\beta H$ where g is the gyromagnetic ratio, β the Bohr magneton and H is the magnetic field strength. Generally for an isolated metal ion, the unpaired electrons give rise to a g value close to 2.00 and experimentally this value of g can be calculated from the following equation:

$$g = g_{DPPH} \times (\nu_{DPPH}/\nu_{SAMPLE}) \qquad \text{Equation 2.6}$$

where ν_{DPPH} = 3220 GAUSS

g_{DPPH} = 2.0036

DPPH = diphenyl picryl hydrazyl standard

2.3 METHODS AND MATERIALS

2.3.1 Sources of Chemicals

Nicotinic acid was obtained from Roche. The transition metal complex salts were obtained as follows: $Cr(NO_3)_3.9H_2O$ (British Drug Houses LTD), $Co(NO_3)_2.6H_2O$ (Koch Light Laboratories LTD), $Ni(NO_3)_2.6H_2O$ (British Drug Houses LTD), $Fe(NO_3).9H_2O$ (Riedel-de Haen Ag) and $Mn(SO_4).4H_2O$ (Baker LTD). All other chemicals were A.R. grade.

2.3.2 Methods and Instrumentation

2.3.2.1 Micro analysis

Micro analysis for the elements carbon, hydrogen and nitrogen was kindly carried out by the Department of Chemistry at Otago University.

2.3.2.2 Chromium determination

Aqueous solutions containing chromium were analysed on a Varian Techtron AA5 atomic absorbance spectrophotometer using a slightly luminous air-acetylene flame. A spectral slit width of 0.2nm was used together with the chromium resonance band of 357.8nm. Chromium standards were prepared from a standard atomic absorption spectroscopy solution of chromium nitrate and acidified with 2.5M hydrochloric

acid to minimise olation (Rollinson et al, 1967).

2.3.2.3 Electronic, infra-red and electron resonance spectroscopy

Electronic spectra were recorded on a Shimadzu MPS 5000 spectrophotometer, infra-red spectra on a Pye Unicam SP3-300 spectrophotometer and ESR spectra were recorded at a temperature of -160°C on a Varian E-104A EPR system with a Varian E-257 Variable Temperature control using a 1,1-diphenyl-2-picryl-hydrazyl (DPPH) standard as a calibrant.

2.3.2.4 Magnetic susceptibility

Magnetic susceptibility was determined by the Faraday method using a Cahn Faraday Balance with a Cahn Electrobalance DTL model 7550. The magnetic susceptibility standard was $[Ni(en)_3]S_2O_3$ and the diamagnetic corrections used were calculated with Pascal constants (Marr & Rockett, 1972).

2.3.2.5 X-ray diffractometry

The x-ray diffraction powder patterns were recorded using a Philips PW1011 x-ray generator coupled to a Philips PW 1352 recording unit.

2.3.2.6 Absorbance, pH and conductivity measurement

Absorbance was measured on a Unicam SP500 spectrophotometer, conductivity on a Radiometer model CDM 2 e and pH measurements on a Radiometer model 28 with a combination electrode.

2.3.2.7 Ion-exchange resins

The DOWEX 50W-X12 (hydrogen ion form) resin was prepared by equilibration in 2 volumes of 2.5M HCl for 30 minutes followed by washing with 2 volumes of distilled water, twice. This cycle was repeated before the column was packed after which the resin was washed with distilled water until the effluent was neutral (pH 7.0).

The DOWEX 1-X8 (chloride ion form) resin was prepared in the same manner as the DOWEX 50W-X12 resin before the column was packed and washed with distilled water until the effluent was neutral.

2.3.2.8 Gel filtration resin

The gel filtration resin used was Sephadex G15 which was run in water and prepared according to the manufacturers instructions.

2.4 RESULTS

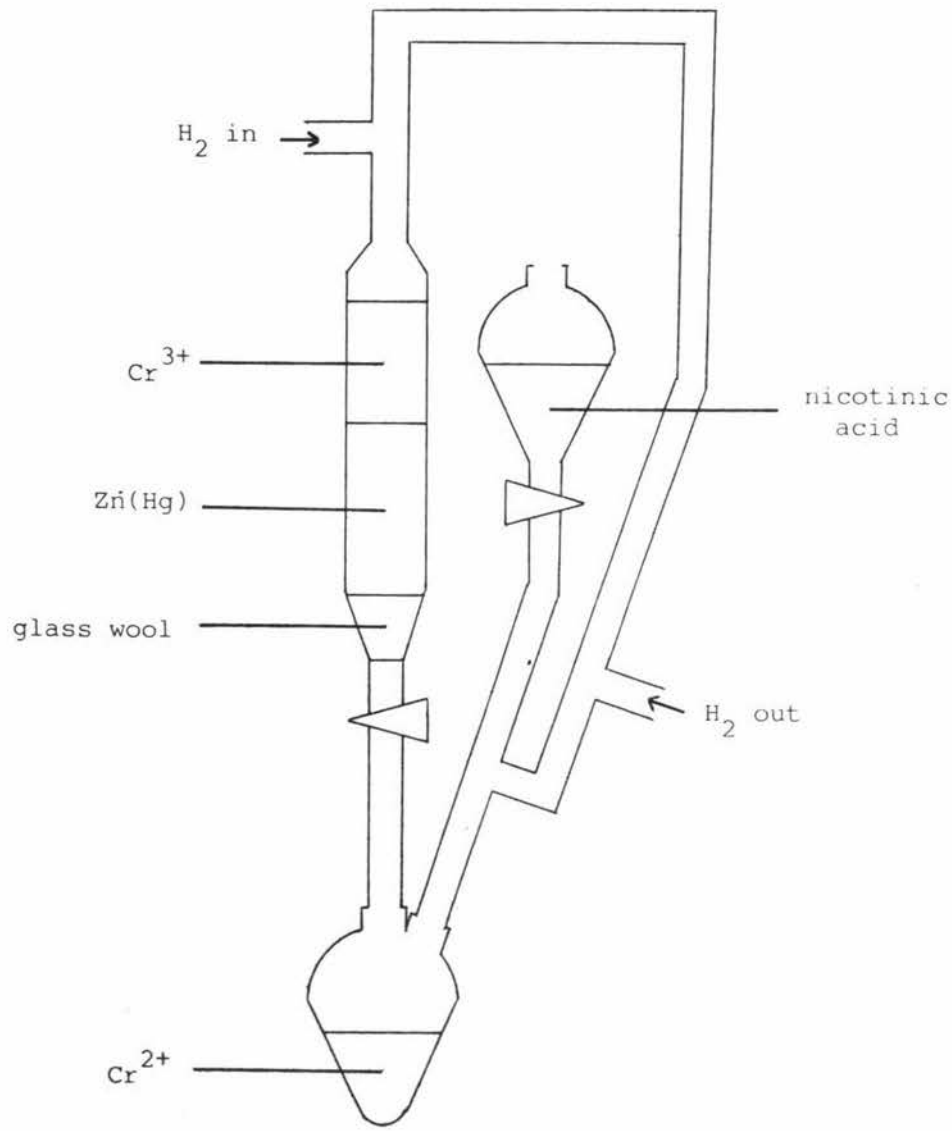
2.4.1 The Yellow Chromium (III) Dinicotinate Complex

2.4.1.1 Preparation of yellow chromium (III) dinicotinate complex

A solution of chromous ions of known concentration was prepared according to Vogel (1957). First a standard chromic solution was prepared by dissolving $K_2 Cr_2 O_7$ (2.94g) in water (50 cm^3), acidifying with concentrated sulphuric acid (2.8 cm^3) and then reducing the dichromate ion to chromic ion by slowly adding in several portions (approximately 8 cm^3) of pure 30% H_2O_2. The solution was heated just to boiling until the evolution of O_2 gas ceased (about 15 to 20 minutes) and then cooled to room temperature before transferring to a 250 cm^3 volumetric flask and making up to the mark with water. The concentration of the chromic solution was 8.0×10^{-2} mol l^{-1}. The chromic solution was run through a zinc-mercury amalgam [1] (50g) column and reduced to the chromous solution which was immediately reacted with nicotinic acid by means of the apparatus shown in Figure 2.4.

Footnote 1: Preparation of the zinc-mercury amalgam catalyst. Granulated zinc (50g A.R. grade) was covered with a solution of Hg_2Cl_2 (100 cm^3 of 2% concentration) and stirred for ten minutes. The zinc-mercury amalgam was then washed with water five times by decantation until the amalgam had a bright silvery lustre.

FIGURE 2.4: Apparatus for preparation and reaction of Cr^{2+}



A solution containing nicotinic acid (0.59g, 0.0048 moles) in water (10 cm^3) at a pH of 6.0 was placed into the reaction flask. Thirty cubic centimetres of the light blue chromous solution was then run into the reaction flask in 5 cm^3 portions and an immediate pink precipitate formed but this gradually changed to a yellow compound. The yellow precipitate was filtered off and washed with H_2O, EtOH and acetone. If the pH of the nicotinic acid solution was less than 5.0 no yellow product was obtained. The yellow compound was insoluble in all solvents tried, stable in air and the elemental analysis was consistent with either $Cr(nic)_2(H_2O)_4$ or $Cr(nic)_2(H_2O)_3OH$ (see Table 2.5). The yellow colour was unusual since all other chromium (III) complexes studied were purple, blue or green.

TABLE 2.5: ANALYTICAL DATA OF NICOTINIC ACID COMPLEXES

	Observed (%)			Calculated (%)		
	C	H	N	C	H	N
blue $Cr(nic)_2(H_2O)_4$	38.80	4.54	7.25	39.13	4.38	7.61
or $Cr(nic)_2(H_2O)_3OH$				39.25	4.12	7.63
yellow $Cr(nic)_2(H_2O)_4$	38.35	4.57	7.33	39.13	4.38	7.61
or $Cr(nic)_2(H_2O)_3OH$				39.25	4.12	7.63
$Co(nic)_2(H_2O)_4$	39.27	4.66	7.17	38.42	4.30	7.47
$Ni(nic)_2(H_2O)_4$	37.64	4.59	7.27	38.44	4.30	7.47
$Mn(nic)_2(H_2O)_4$	42.92	4.09	8.14	43.00	3.61	8.36

2.4.1.2 X-ray powder diffractometry

The x-ray diffraction powder pattern of a powdered sample of the yellow chromium (III)-nicotinic acid complex was recorded. A complex of known crystal structure, namely $Co(nic)_2(H_2O)_4$ (Anagnostopoulos et al, 1969), prepared as described in Section 2.4.2.1, was powdered and its x-ray diffraction powder pattern recorded. The diffraction patterns of the yellow chromium (III) complex and $Co(nic)_2(H_2O)_4$ were compared as shown in Table 2.6.

The infra red spectrum of the yellow chromium (III)-nicotinic acid complex and $Co(nic)_2(H_2O)_4$ were recorded and are shown in Figures 2.5(a) and 2.5(b) together along with the spectrum of nicotinic acid in Figure 2.5(c).

2.4.1.3 Electronic Spectra and Magnetic Properties

The electronic spectrum of the yellow chromium-nicotinic acid complex did not display the d-d absorption bands in the visible spectrum characteristic of Cr(III) octahedral complexes but only one very intense absorption band was observed at approximately 340 nm (see Table 2.7). This could have obscured the less intense d-d bands, and be responsible for the observed yellow colour. When the complex was dissolved in NaOH(2M), a brown solution formed which rapidly changed to green with precipitation of chromium hydroxide. The electronic spectrum of the alkaline solution now showed the two d-d bands expected for Cr(III) at 405nm and 600nm. The ultra-violet spectrum of a nujol mull of the yellow chromium nicotinic acid complex exhibited a very intense absorption maximum at 266nm with very pronounced shoulders. The spectrum was similar to that obtained for nicotinic acid in a basic solution.

The observed magnetic moment of μ_{eff} = 3.18 B.M. (Table 2.8) was unusual since it was much less than the moment expected for a monomeric Cr(III) complex ($\simeq$3.88 B.M.).

FIGURE 2.5:

Infra red absorption spectrum using a KBr pellet of (a) yellow $Cr(nic)_2(H_2O)_3OH$, (b) $Co(nic)_2(H_2O)_4$, and (c) nicotinic acid.

FIGURE 2.5

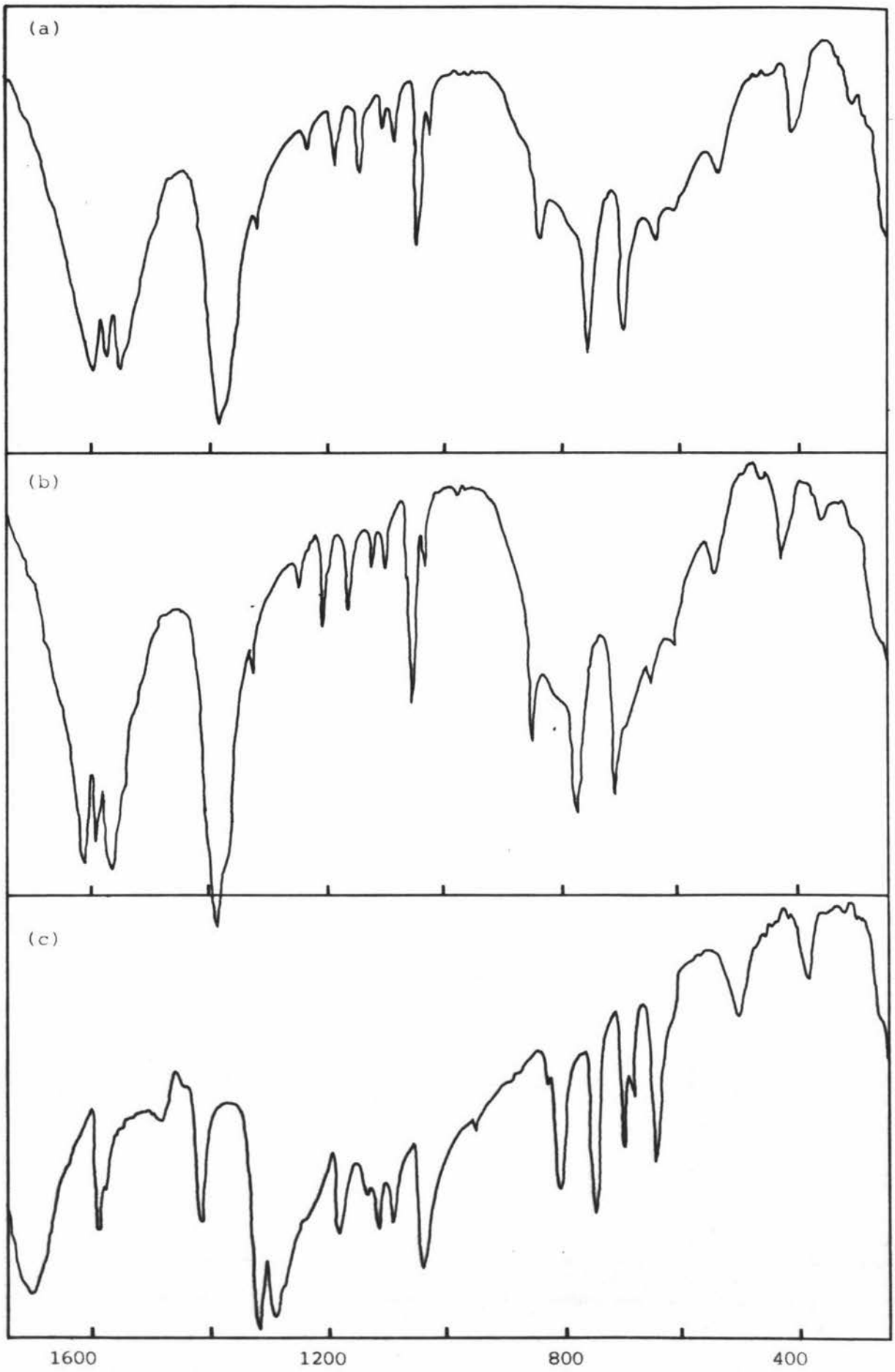


TABLE 2.6: X-RAY DIFFRACTION POWDER PATTERNS OF YELLOW $Cr(nic)_2(H_2O)_3OH$ and $Co(nic)_2(H_2O)_4$

$Cr(nic)_2(H_2O)_3OH$		$Co(nic)_2(H_2O)_4$	
d (Å)	Relative Intensity	d (Å)	Relative Intensity
7.50	14	7.44	18
6.61	100	6.55	100
6.23	sh	6.20	sh
6.07	62	6.00	26
5.35	9	5.31	5
4.25	22	4.18	20
3.96	28	3.95	21
3.92	sh	3.87	9
3.58	7	3.55	3
3.47	58	3.43	25
3.36	22	3.33	15
3.30	sh	3.26	5
3.15	7	3.12	3
3.07	13	3.04	5
3.04	18	3.01	8
2.82	5	2.80	3
2.68	18	2.68	6
-	-	2.65	12
2.59	5 br	2.58	3
		2.56	4
2.49	10 br	2.48	5
		2.46	3
2.39	5	2.37	3
2.18	12	2.18	12
2.14	5	2.13	4
2.02	4	2.02	3
1.98	6	1.97	3
1.95	9	1.94	10
1.78	4 br	1.79	2
		1.77	3

sh = shoulder
br = broad

TABLE 2.7: ELECTRONIC ABSORPTION BANDS OF NICOTINIC ACID COMPLEXES

Compound	Ultraviolet band λmax (nm)	d-d transitions λmax (nm)(d)	State of Compound
blue-$Cr(nic)_2(H_2O)_3OH$	263(a)	420 575	Solid (nujol mull)
blue-$Cr(nic)_2(H_2O)_4^{3+}$	262(vi)	425(35) 575(26)	2M HNO_3 solution
blue-$Cr(nic)_2(H_2O)_5^{2+}$	262(vi)	417(20) 570(27)	aqueous solution
yellow $Cr(nic)_2(H_2O)_3OH$	266(vi)	(b)	solid (nujol mull)
$Co(nic)_2(H_2O)_4$	260(a)	510	solid (nujol mull)
$Ni(nic)_2(H_2O)_4$	260(a)	378 625	solid (nujol mull)
$Mn(nic)_2(H_2O)_2$	260(a)	(c)	solid nujol mull)

Note: (a) broad band, no distinct λmax

(b) obscured by charge transfer band, λmax = 340nm

(c) too weak to be observed

(d) figures in brackets refer to molar extinction coefficients

vi very intense

TABLE 2.8: MAGNETIC PROPERTIES OF NICOTINIC ACID COMPLEXES

Compound	μ_{eff} (B.M.)
blue $Cr(nic)_2(H_2O)_3OH$	4.88
yellow $Cr(nic)_2(H_2O)_3OH$	3.18
$Co(nic)_2(H_2O)_4$	5.10
$Ni(nic)_2(H_2O)_4$	3.36
$Mn(nic)_2(H_2O)_4$	5.91

The electronic spectra were recorded in a nujol mull due to the great insolubility of the yellow chromium-nicotinic acid complex in all solvents tried.

2.4.1.4 Electron spin resonance spectroscopy

The electron spin resonance spectrum for the yellow chromium-nicotinic acid complex was very simple and therefore of limited use since the only signal which was recorded was the signal from one unpaired electron (see Figure 2.6(a) The single maximum found at g = 1.98388 (calculated using equation 2.6) was consistent with the chromium (III) atom in an octahedral environment in the case of the yellow chromium-nicotinic acid complex and gave no evidence for interaction between chromium atoms. Indeed the characteristic Cr^{3+} e.s.r. signal (g = 1.98) has been used as an analytical tool in Cr^{3+} determinations by Bryson (1980). The presence of the trivalent chromium atom could result from the oxidation of the chromous ion after coordination of the nicotinic acid ligands.

2.4.1.5 Infra-red Spectra

The infra-red spectrum of the yellow chromium-nicotinic complex is shown in Figure 2.5(a) and the main absorption bands are compared with those of nicotinic acid in Table 2.9. In the region between 650cm^{-1} and 200 cm^{-1} the three bands at 410cm^{-1}, 535cm^{-1} and 645cm^{-1} were similar to the three bands found in nicotinic acid but with slight shifts in wavenumber while the fourth band at 310cm^{-1} was new and could be due to the mode of coordination of nicotinic acid.

The second region, 1000cm^{-1} - 650cm^{-1}, showed only two intense bands in the yellow chromium-nicotinic acid complex at 700cm^{-1} and 760cm^{-1} and these bands were found to be more intense than those of uncomplexed nicotinic acid.

FIGURE 2.6(a):
Electron spin resonance spectrum of a powdered sample of the yellow $Cr(nic)_2(H_2O)_3OH$ complex at -160°C.

FIGURE 2.6(b):
Electron spin resonance spectrum of a powder sample of the blue $Cr(nic)_2(H_2O)_3OH$ complex at -160°C.

FIGURE 2.6(a)

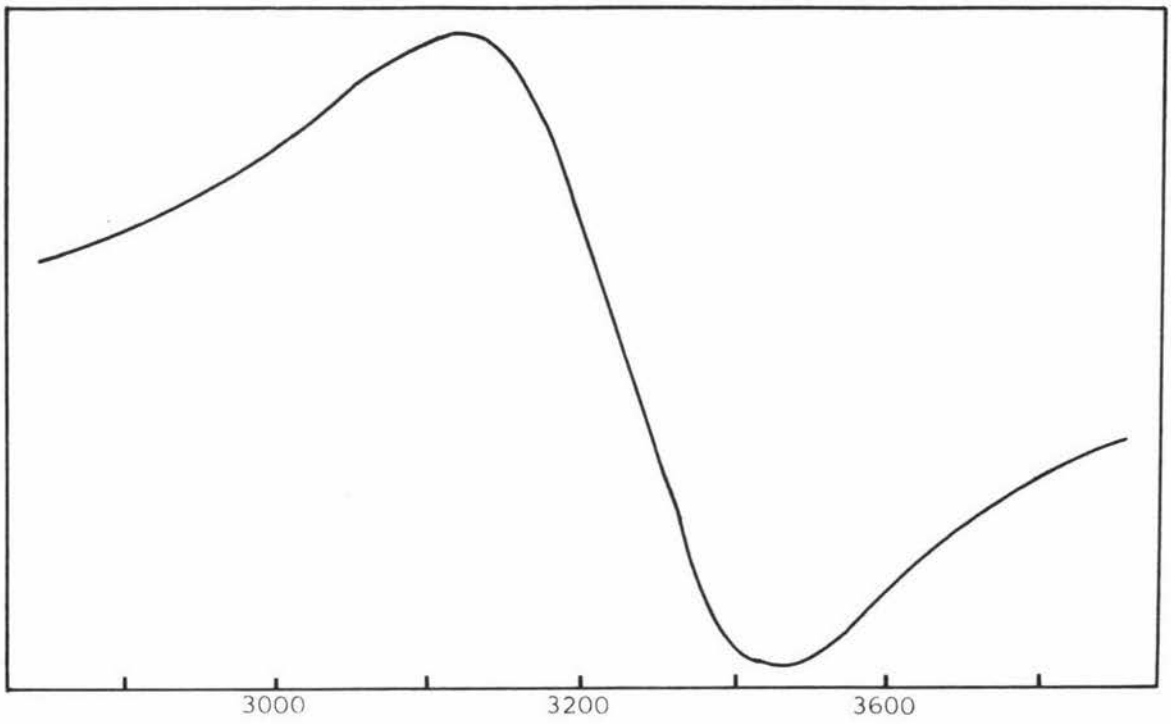


FIGURE 2.6(b)

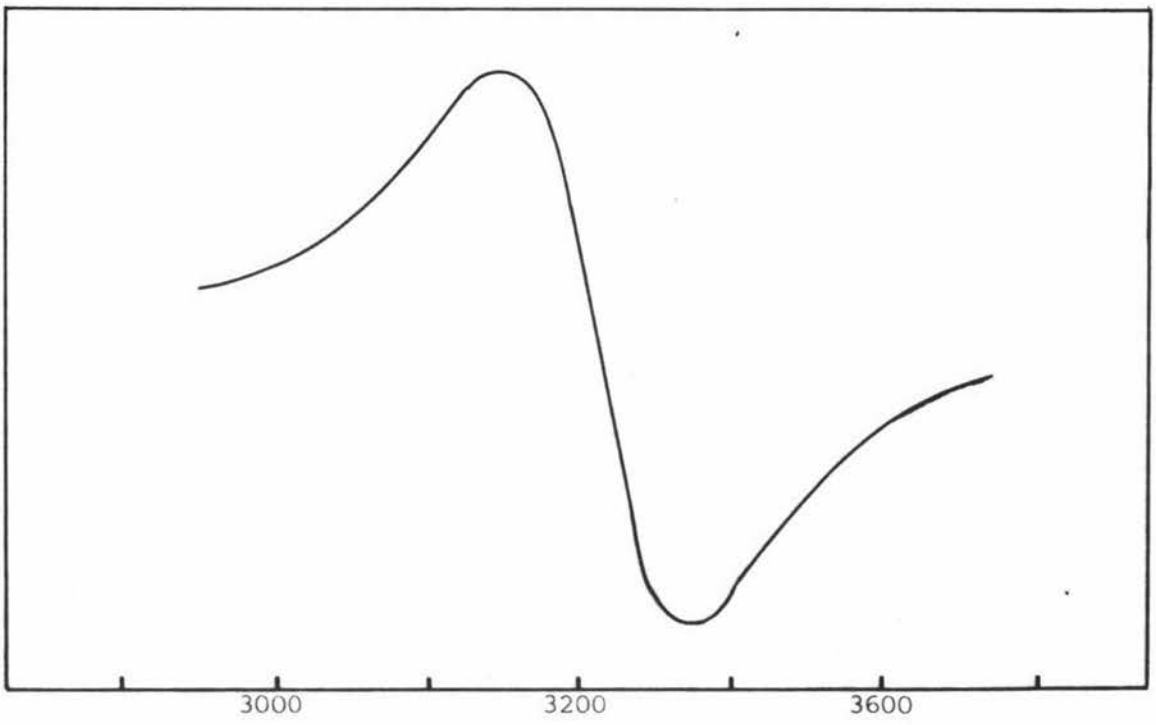


TABLE 2.9: INFRA-RED SPECTRUM OF THE YELLOW CHROMIUM-NICOTINIC ACID COMPLEX

$Cr(nic)_2(H_2O)_3OH$	Nicotinic Acid	Assignment of Bands
310m	-	chromium-nitrogen stretching vibration
410m	380m	CH out-of-plane ring bending deformation
535m	495m	carbonyl bending deformation
645m	640s	CH in-plane ring bending deformation
700vs	695s	)CH out-of-plane ring bending
760vs	745s	) deformation
840m	805s	
1030w	-	
1050s	1040s	skeletal ring stretch
1090m	1090m	)
1110m	1115m	) CH in-plane ring bending
1150m	1135m	) deformations
1190m	1180m	)
-	1290s	
1325w	1315s	hydroxyl bending deformation
1390vs	1410s	symmetrical carbonyl stretch
-	1480w	
1555s	1575s	)
1580s	1590s	) pyridine ring stretchs
1600s	1695sb	asymmetrical carbonyl stretch
3050w	3050w	CH stretch

w weak
m medium
s strong
vs very strong
sb strong broad

The greatest changes were seen between 1800cm^{-1} and 1300cm^{-1} in which the asymmetric carbonyl stretch at 1695cm^{-1} in the free ligand was shifted to a smaller wavenumber (1600cm^{-1}), and the symmetric carbonyl stretch at 1410cm shifted slightly lower (1390cm^{-1}) and intensified. The strong band at 1315cm^{-1} was attributed to the hydroxyl deformation vibration in nicotinic acid, but was absent in the complex spectrum. The pyridine ring vibrations at 1590cm^{-1} and 1575cm^{-1} were shifted only very slightly to a lower wavenumber, but as the three bands, the asymmetric carbonyl and the two pyridine ring stretching vibrations were so close together, assignments of the bands could only be tentative.

2.4.2 Other Transition Metal Nicotinic Acid Complexes

2.4.2.1 Preparation of $Co(nic)_2(H_2O)_4$

The method used was similar to that of Anagnostopoulos et al (1972). A solution of $Co(II)(NO_3)_2.6H_2O$ (1.18g, 0.004 moles) in H_2O(15cm^3) was heated to 60°C. To this solution was added (also at 60°C) a solution containing nicotinic acid (1.0g, 0.008 moles) in H_2O (15cm^3) at pH 6.0. The pink precipitate of $Co(nic)_2(H_2O)_4$ appeared immediately, and on cooling to room temperature, the precipitate was filtered, washed with H_2O, EtOH and diethyl ether, and the pink crystalline compound was finally dried in vaccuo.

2.4.2.2 Preparation of $Ni(nic)_2(H_2O)_4$

The same procedure was used as for the preparation of $Co(nic)_2(H_2O)_4$, Section 2.4.2.1. $Ni(II)(NO_3)_2.6H_2O$ (1.19g, 0.004 moles) was used as the starting material and the precipitate of $Ni(nic)_2(H_2O)_4$ formed was crystalline and blue in colour. The precipiate was filtered, washed and dried as for $Co(nic)_2(H_2O)_4$.

2.4.2.3 Preparation of $Mn(nic)_2(H_2O)_4$

A procedure similar to that for the Co(II) and Ni(II) dinicotinate complexes was used to prepare $Mn(nic)_2(H_2O)_4$ using

$Mn(SO_4).4H_2O$ (o.94g, 0.004 moles) as the starting material except that the solution was boiled for five minutes after adding the nicotinic acid. On cooling a white crystalline product precipitated from the solution and was filtered, washed and dried as described for $Co(nic)_2(H_2O)_4$.

2.4.2.4 Preparation of $Fe(nic)_2(H_2O)_3(OH)$

A solution of $Fe(No_3)_3.9H_2O$ (1.66g, 0.004 moles) in $H_2O(20cm^3)$ was heated to 60°C. Nicotinic acid (1.0g, 0.008 moles) was dissolved in $H_2O(20cm^3)$ the pH adjusted the 6.0 and the solution heated to 60°C. On addition of the nicotinic acid solution to the ferric solution, a yellow-orange precipitate formed but dissolved on heating further. The solution was boiled for five minutes, and on cooling left a dark brown solution with no visible precipitate at pH = 2.5. On dropwise addition of NaOH(2M) a brown precipitate formed at pH 8.5 - possibly an olated species similar to that found for the Cr(III) reaction. However if $NaClO_4$ (2M) was added dropwise to the dark brown solution and heated a dark brown product was formed.
The compound was washed with EtOH and diethyl ether after filtration.

2.4.2.5 Properties of the transition metal complexes

The various properties of the Co(II), Ni(II) and Mn(II) compounds were compared with those obtained for the chromium (III) complexes. The complexes prepared were $Co(nic)_2(H_2O)_4$ (pink), $Ni(nic)_2(H_2O)_4$ (blue) and $Mn(nic)_2(H_2O)_4$ (white). All were very insoluble in all common solvents, but dissolved readily in 2M HNO_3 with no changes in colour suggesting that the coordination of the ligands was unchanged in 2M HNO_3.

The preparation of $Fe(III)(nic)_2(H_2O)_3OH$ was attempted by a method similar to that used for the Cr(III) complexes, however elemental analysis of the brown precipitate obtained was not consistant with that expected for $Fe(nic)_2(H_2O)_3OH$

or its perchlorate salt.

A similar attempt to prepare $Mn(III)(nic)_2(H_2O)_3$ was also unsuccessful probably because Mn(III) is not very stable except under very acidic conditions since the tendency towards olation is too great (Cotton and Wilkinson 1972b) and hence its complexes are not generally stable in aqueous solution.

2.4.2.6 Electronic and magnetic properties

For $Ni(nic)_2(H_2O)_4$, two transitions were observed in the electronic spectrum using a nujol mull. The first transition at 378nm was assigned to the $^3A_{2g}(F) \rightarrow {}^3T_{1g}(P)$ transition and the second at 625nm was assigned to the $^3A_{2g}(F) \rightarrow {}^3T_{1g}(F)$ transition but the expected third transition $^3A_{2g}(F) \rightarrow {}^3T_{2g}(F)$ for a d^8 configuration was not observed. Similar assignments were made by Anagnostopoulos et al (1972) who also observed a third transition at 1053nm but we could not scan to this wavelength. A broad band at 260nm was observed and this was attributed to the nicotinic acid ligands. The observed magnetic moment (μ_{eff} = 3.36 B.M.) was in good agreement with the predicted value of 2.9 - 3.4 B.M. but was slightly higher than the value of Anagnostopoulos of 3.11 B.M.

The electronic spectrum for $Co(nic)_2(H_2O)_4$ showed only one d-d band at 510nm which was assigned to the $^4T_{1g}(F) \rightarrow {}^4T_{1g}(P)$ transition by Anagnostopoulos but the two other transitions which were expected for a d^7 configuration were not seen because the $^4T_{1g}(F) \rightarrow {}^4A_{2g}(F)$ transition is usually close to but much weaker than the 510nm transition and the $^4T_{1g}(F)$ $^4T_{2g}(F)$ transition is usually found in the near-infra red region (Cotton and Wilkinson 1972c) but was not scanned for. The ultra-violet spectrum of $Co(nic)_2(H_2O)_4$ was identical to that for $Ni(nic)_2(H_2O)_4$. The observed magnetic moment (μ_{eff} = 5.10 B.M.) was higher than the value reported by Anagnostopoulos of 4.80 B.M.

The visible spectrum of $Mn(nic)_2(H_2O)_4$ was not very useful because the d-d transition absorption bands were too weak as expected due to very low molar extinction coefficients. The observed magnetic moment (μ_{eff} = 5.91 B.M.) was very close to the spin only value (μ_s = 5.9 B.M.) and the ultraviolet spectrum was essentially the same as for the Ni(II) and Co(II) complexes as expected.

2.4.2.7 Infra-red spectra

The infra-red spectra of the series $M(nic)_2(H_2O)_4$, where M = Mn(II), Co(II) and Ni(II) were virtually identical with that of the yellow chromium-nicotinic acid complex prepared from chromous ions and nicotinic acid. The main absorption bands are summarised in Table 2.10. The only region where there are differences in the infra-red spectra for Cr(III), Ni(II), Co(II) and Mn(II) is between $650cm^{-1}$ and 200^{-1} and this region is shown in Figure 2.7. The two bands seen for nicotinic acid at $380cm^{-1}$ and $640cm^{-1}$ were shifted to higher wavenumbers in the yellow chromium-nicotinic acid complex ($410cm^{-1}$ and $645cm^{-1}$), in $Co(nic)_2(H_2O)_4$ ($415cm^{-1}$ and $630cm^{-1}$) and in $Ni(nic)_2(H_2O)_4$ ($420cm^{-1}$ and $630cm^{-1}$) while for all complexes an entirely new band was observed which decreased in wavenumber for Ni($365cm^{-1}$) to Co ($350cm^{-1}$) to Cr ($310cm^{-1}$). Over the rest of the spectrum the absorption bands were identical with those of the yellow chromium-nicotinic acid complex.

2.4.2.8 X-ray diffraction powder patterns

The x-ray diffraction powder patterns of the yellow chromium-nicotinic acid complex, $Co(nic)_2(H_2O)_4$ and $Ni(nic)_2(H_2O)_4$ were virtually identical, while the diffraction pattern for $Mn(nic)_2(_2O)_4$ was slightly different (possibly due to nicotinic acid impurities). The main diffraction maxima are shown in Tables 2.6 and 2.11.

FIGURE 2.7:

Infra red absorption spectrum using a KBr pellet of (a) $Mn(nic)_2(H_2O)_4$, (b) nicotinic acid, (c) $Ni(nic)_2(H_2O)_4$, (d) $Co(nic)_2(H_2O)_4$ and (e) yellow $Cr(nic)_2(H_2O)_3OH$

FIGURE 2.7

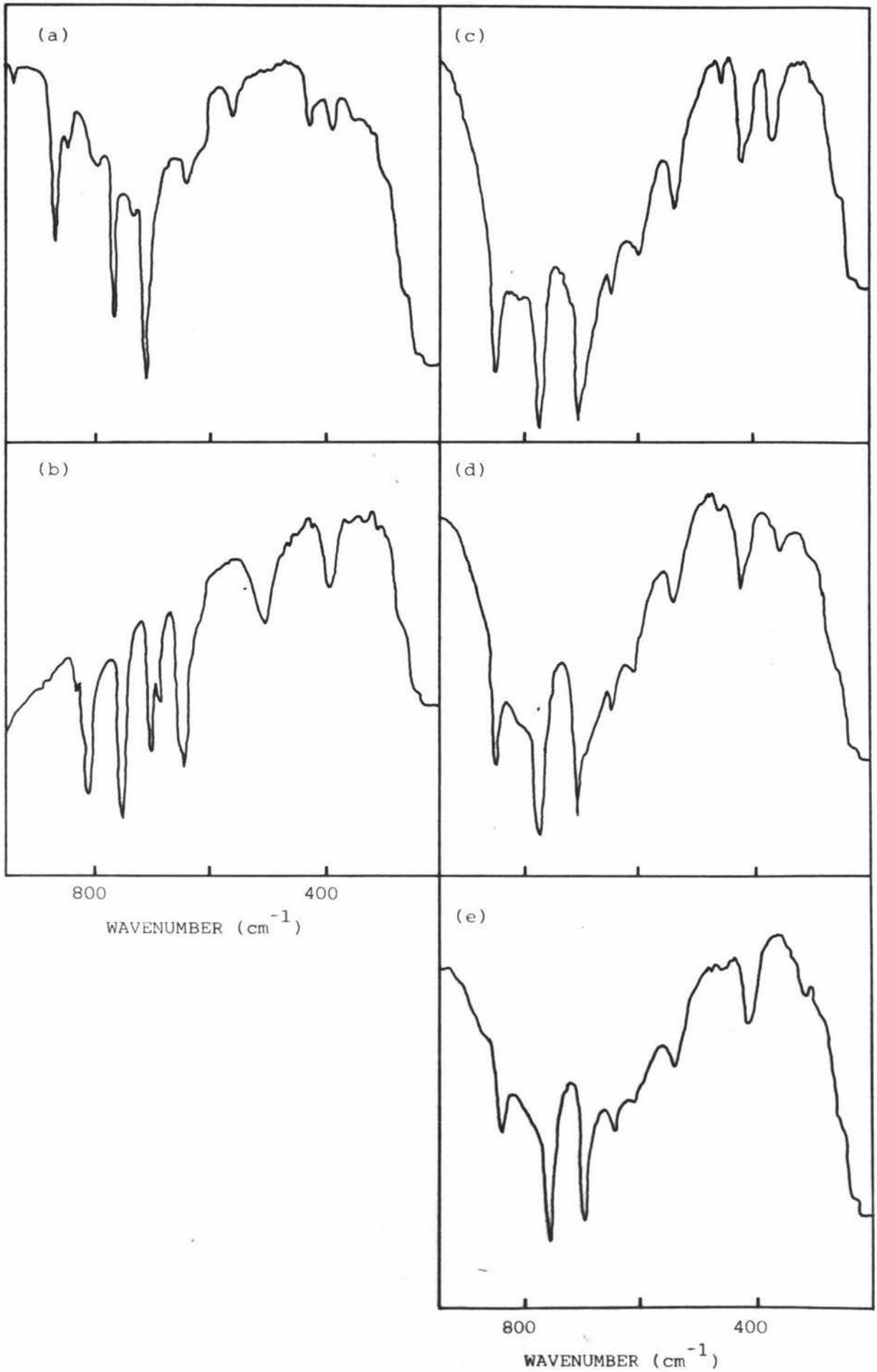


TABLE 2.10: INFRA-RED SPECTRA OF NITROGEN COORDINATED DI-NICOTINIC ACID COMPLEXES

Nicotinic Acid	$Cr(nic)_2(H_2O)_3OH$	$Co(nic)_2(H_2O)_4$	$Ni(nic)_2(H_2O)_4$	Assignment
-	310m	350m	365m	metal-nitrogen stretch
380m	410m	415m	420m	CH out-of-plane ring deformation
495m	535m	530m	530m	carbonyl deformation
640s	645m	630m	630m	CH in-plane ring deformation
695s	700vs	700vs	700vs	CH out-of-plane ring deformation
745s	760vs	770vs	770vs	
805s	840m	840m	845m	
-	1030w	1030w	1030w	
1040s	1050s	1050s	1050s	skeletal ring stretch
1090m	1090m	1090m	1095m	CH in-plane ring deformation
1115m	1110m	1115m	1120m	
1135m	1150m	1155m	1160m	
1180m	1190m	1200m	1200m	
1290s	-	-	-	
1315s	1325w	1320w	1320w	hydroxyl bending deformation
1410s	1390vs	1380vs	1380vs	symmetrical carbonyl stretch
1480w	-	-	-	
1575s	1555s	1560s	1565s	pyridine ring stretches
1590s	1580s	1585s	1590s	
1695sb	1600s	1605s	1610s	asymmetrical carbonyl stretch
3050w	3050w	3050w	3050w	CH stretch

w = weak
m = medium
s = strong
vs = very strong
sb = strong broad

TABLE 2.11: X-RAY DIFFRACTION POWDER PATTERNS OF $Ni(nic)_2(H_2O)_4$ AND $Mn(nic)_2(H_2O)_4$

$Ni(nic)_2(H_2O)_4$		$Mn(nic)_2(H_2O)_4$	
d(Å)	Relative Intensity	d(Å)	Relative Intensity
7.39	25	8.52	100
6.51	92	6.51	39
6.12	sh	5.11	32
6.02	100	4.49	10
5.28	19	4.36	60
4.22	43	3.93	11
3.92	49	3.68	27
3.85	24	3.50	49
3.54	10	3.46	16
3.43	83	3.30	14
3.31	32	3.26	19
3.24	3	3.14	10
3.11	9	2.97	40
3.02	18	2.71	10
3.00	29	2.57	12
2.78	10	2.18	23
2.67	13	1.93	10
2.64	24		
2.57	6		
2.55	11		
2.47	14		
2.46	9		
2.36	6		
2.16	30		
2.12	6		
2.00	6		
1.96	10		
1.93	13		
1.77	8br		

sh = shoulder

br = broad

2.4.3 The Blue Chromium Dinicotinate Complex

2.4.3.1 Preparation of blue chromium dinicotinate complex

The blue dinicotinate complex of chromium (III) was prepared by dissolving $Cr(NO_3)_3.9H_2O$ (2.0g, 0.005 moles) in H_2O ($20cm^3$) and warming on a hot-plate to approximately 60°C. A solution containing a two fold excess of nicotinic acid (1.23g, 0.010 moles) was prepared in H_2O ($20cm^3$) and the pH adjusted to 6.0 with NaOH (2M) before warming to approximately 60°C. On addition of the nicotinic acid solution to the Cr^{3+} solution a green colour developed, but this changed to blue on boiling and when the solution was cooled a blue precipitate formed. The resulting powder was filtered and washed with H_2O, EtOH and diethyl ether before drying under vacuum. The blue powder continued to precipitate slowly from the blue reaction mixture for 48 hours. Further purification of the blue powdered solid was carried out by extraction of any nicotinic acid impurity with sodium dried diethyl ether in a soxhlet apparatus. The pH of the reaction solution decreased from 6.0 to 2.5 as the blue solution formed indicating that protons were released into the solution.

The blue solid formed in the reaction of chromium (III) with nicotinic acid was analysed for C, H and N, and the results which are shown in Table 2.5 were consistent with an empirical formula of $Cr(nic)_2(H_2O)_3OH$.

2.4.3.2 Electronic spectra and Magnetic properties

The electronic spectrum of the blue solid was determined by the reflectance method in a nujol mull and is shown in Table 2.7. The spectrum contained two d-d transitions, one at 420nm assigned to the $^4A_2(F) \rightarrow {}^4T_1(F)$ transition and one at 575nm assigned to the $^4A_2(F) \rightarrow {}^4T_2(F)$ transition by reference to the Tanabe-Sugano diagram (Figure 2.3) and assuming that the lowest energy transition corresponded to the 575nm d-d band. The third transition $^4A_2(F) \rightarrow {}^4T_1(P)$

expected for a d^3 configuration (section 2.2.3) was not observed.

The ultra-violet absorption spectrum of the blue coloured chromium-nicotinic acid complex showed only a single broad band, at 263nm with slight shoulders presumably associated with the nicotinic acid ligands. (This could also be an effect due to the lack of crystalline structure or a matrix effect of the nujol mulling agent).

The value of the observed magnetic moment of the blue powdered solid, (μ_{eff} = 4.88 B.M.) shown in Table 2.8 was higher than that expected for Cr(III) complexes (μ_s = 3.87 from Table 2.3). The reason for this deviation is not known.

2.4.3.3 Infra red spectra

The infra-red spectrum of a KBr pellet of the blue complex was recorded over the range 4000cm^{-1} to 200cm^{-1} and is shown together with that of nicotinic acid in the same wave number range in Figure 2.8(a) and (b). A summary of the main absorption bands is shown in Table 2.12 and it is apparent that the spectrum of $Cr(nic)_2(H_2O)_3OH$ is very different from that of uncomplexed nicotinic acid. However some of the bands are coincident and any changes in the spectrum can be related to coordination of the ligand. The main regions of interest were;

(i) 650cm^{-1} - 200cm^{-1};

(ii) 1000cm^{-1} - 650cm^{-1}; and

(iii) 1800cm^{-1} - 1300cm^{-1}.

In uncomplexed nicotinic acid two bands occurred at 495 cm^{-1} and 640cm^{-1}, both of medium intensity and they were also observed for the blue chromium-nicotinic acid complex but had shifted to 520cm^{-1} and 600cm^{-1} respectively. A band at 380cm^{-1} in nicotinic acid was completely absent in the complex.

FIGURE 2.8:

Infra red absorption spectrum using a KBr pellet of (a) blue $Cr(nic)_2(H_2O)_3OH$ and (b) nicotinic acid.

FIGURE 2.8

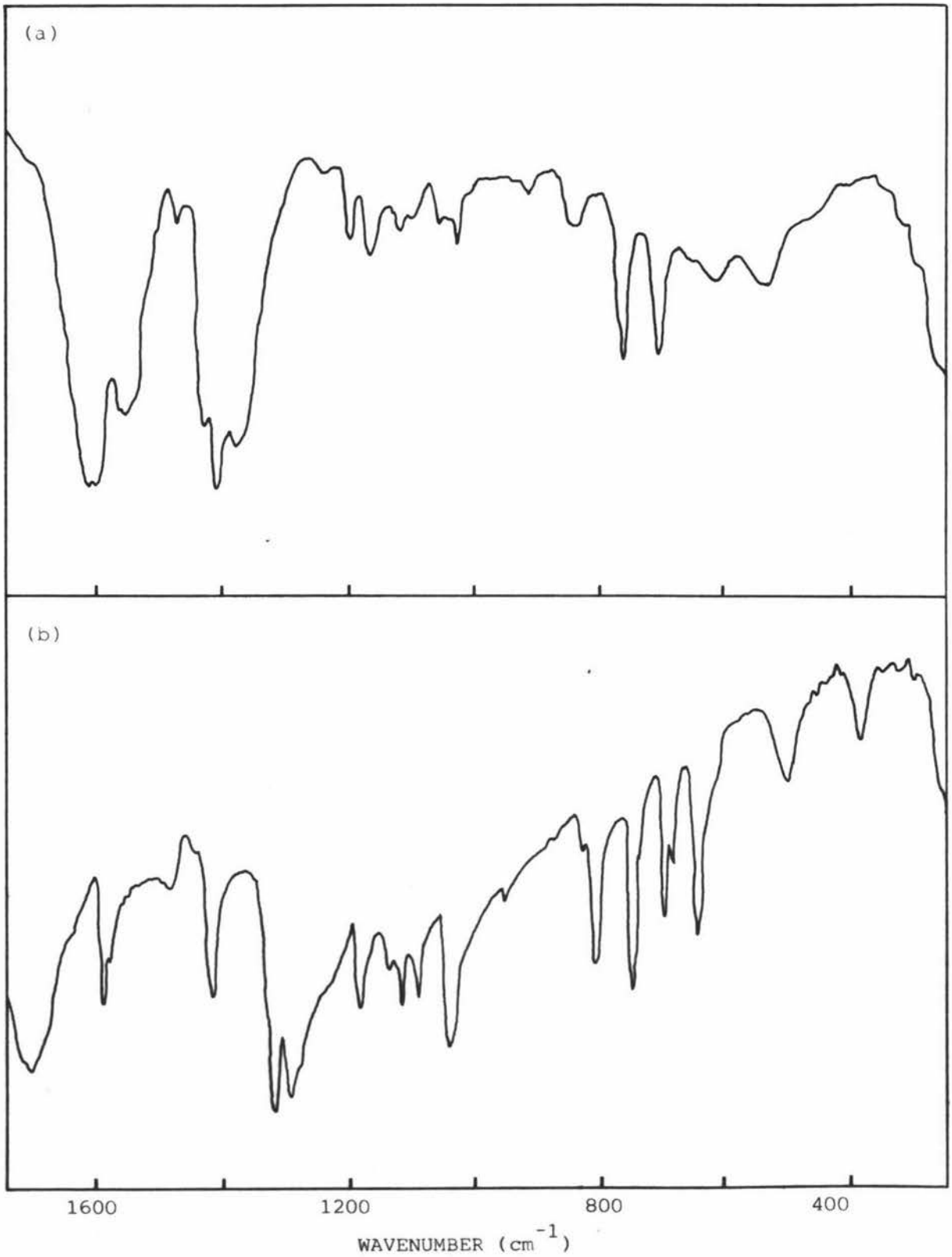


TABLE 2.12: INFRA-RED SPECTRUM OF BLUE CHROMIUM-NICOTINIC ACID COMPLEX

$Cr(nic)_2(H_2O)_3OH$	Nicotinic Acid	Assignment of bands
-	380m	CH out-of-plane ring bending deformation
520m	495m	carbonyl bending deformation
600m	640s	CH in-plane ring bending deformation
705s	695s	CH out-of-plane ring bending deformation
760s	745s	
840m	805s	
915w	-	Tentative chromium-oxygen stretch
1030m	-	
1055w	1040s	skeletal ring stretch
1095sh	1090m	CH in-plane ring bending deformation
1120w	1115m	
1165m	1135m	
1200m	1180m	
-	1290s	
-	1315s	hydroxyl bending deformation
1410vs	1410s	symmetrical carbonyl stretch
1430sh	1430sh	pyridine ring stretch
1475w	1480w	
1555s	1575s	
1595vs	1590s	
1610vs	1695sb	asymmetrical carbonyl stretch

w = weak
m = medium
s = strong
vs = very strong
sb = strong broad
sh = shoulder

The region between 650cm^{-1} and 1000cm^{-1} contained four bands originally of the same intensity, but on complexation the two inner most bands (at 705cm^{-1} and 760cm^{-1}) intensified while the outside pair (at 600cm^{-1} and 840cm^{-1}) weakened. A new band at 915cm^{-1} in the spectrum of the chromium complex was found which was not present in nicotinic acid.

Major changes in the spectra were seen in the region between 1800cm^{-1} and 1300cm^{-1} upon complexation of the nicotinic acid. In the complex the hydroxyl bending deformation band was absent, indicating the loss of the carboxylate proton upon coordination. The symmetric carbonyl stretch (1410cm^{-1} in the free ligand) was now replaced by the three strong bands centred at 1410cm^{-1}, while the asymmetric carbonyl stretching vibraton at 1695cm^{-1} was shifted to 1610cm^{-1} in the complex. The rather broad band at 1600cm^{-1} was most probably the cyclic ring stretching vibration associated with the pyridine ring and appears not to be subject to any coordination influences.

2.4.3.4 Electron spin resonance spectroscopy

The electron spin resonance spectrum of the blue chromium-nicotinic acid complex shown in Figure 2.6(b) was consistent with an octahedral Cr^{3+} ion as expected. The g value of 2.00049 calculated using Equation 2.6 was found to be close to that of three unpaired electrons as seen for the yellow chromium-nicotinic acid complex which suggested that in both cases the complexes were octahedral in structure and that the chromium atom was trivalent.

2.4.3.5 X-ray powder diffractometry

A powdered sample of the blue chromium-nicotinic acid complex did not exhibit an x-ray diffraction powder pattern, in fact it appeared to be totally amorphous with a lack of any crystalline structure. This suggested that the complex lacked groups suitable for hydrogen bonding which would have provided a more regular arrangement of the complex units and a more crystalline structure.

2.4.4 Reaction of the Blue Chromium-Nicotinic Acid Complex with Acid

2.4.4.1 Electronic spectra

When the blue chromium-nicotinic acid complex was dissolved in acid (2M HNO_3), a blue coloured solution was formed which had absorption maxima at 425nm and 575nm, with molar extinction coefficients of 35 l mol^{-1} cm^{-1} and 26 l mol^{-1} cm^{-1} respectively. The similarity of the two spectra suggested that there had been little change in the coordinated ligands upon dissolution of the blue solid and that there was no major change in structure.

The acidified solution of the blue chromium-nicotinic acid complex showed a pronounced absorption maximum in the ultraviolet region at 262nm confirming coordination of the nicotinic acid moiety. The blue solution had a chromium to metal ratio of 1:2 when the absorbance of the nicotinic acid maxima at 262nm was used to calculate the concentration of nicotinic acid as outlined below.

The concentration of the ligand was calculated using Beer's Law:

$$\varepsilon = \frac{D}{Cl}$$ where ε = molar absorption coefficient of nicotinic acid

D = optical density

C = Concentration (mol l^{-1})

l = pathlength (cm)

The ionisation of the carboxylate group is known to have little effect on the absorption spectrum of nicotinic acid (Green and Tong, 1956). Thus coordination of the carboxylate group to Cr^{3+} should not change the ultraviolet spectrum to any great extent and at acidic pH values the pyridine ring nitrogen atoms should be protonated. Therefore it was assumed that the value of ε remained unchanged upon coordination and hence the value of 5012 l mol^{-1} cm^{-1} in acid solution was used in the calculations (Hirayama 1967).

The concentration of the nicotinic acid was thus:

$$C_L = \frac{A_{262}}{\varepsilon} \qquad \text{Equation 3.7}$$

where A_{262} = optical density at 262nm

2.4.4.2 Ion-exchange and Gel filtration chromatography

The blue solution formed on dissolving the blue chromium-nicotinic acid complex in acid was titrated to pH 3.5 and loaded onto a cation-exchange column (DOWEX 50W-X12, H^+ form). However all attempts to elute this species from the resin with NaCl salt gradients and phosphate pH gradients were unsuccessful due to the very tight binding of the complex species.

Gel filtration of the acidic blue solution was carried out on a Sephadex G15 resin column (2.0cm x 60.0cm) and the blue solution was eluted after the salt peak (which was eluted at approximately the bed-volume of the column) as shown in Figure 2.9.

2.4.5 The Soluble Blue Chromium (III)-Mononicotinate Complex

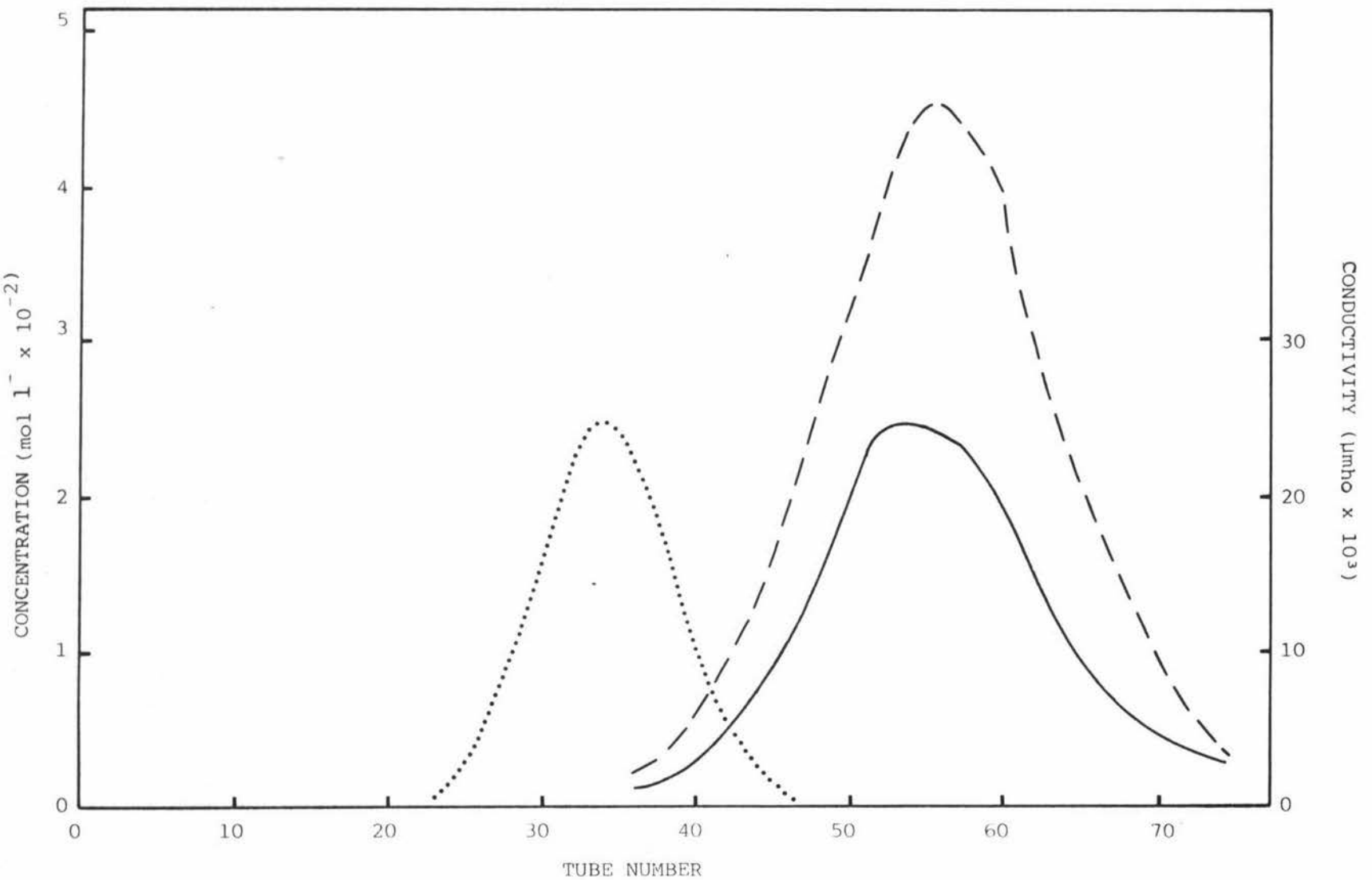
2.4.5.1 Attempted preparation of a mononicotinate complex of chromium (III)

The procedure for preparation of a mono-nicotinic acid complex with Cr(III) was similar to that employed for the solid blue complex described above. $Cr(NO_3)_3.9H_2O$ (2.0g, 0.005 moles) was dissolved in H_2O ($20cm^3$) and the pH of the solution was adjusted to 3.0 using HNO_3 (2M). An equi-molar amount of nicotinic acid (0.615g, 0.005 moles) was dissolved in H_2O ($20cm^3$) and the pH of the solution was adjusted to 3.0 using NaOH (2M). Both solutions were warmed to approximately 60°C and then mixed. On boiling, a blue coloured solution developed, but no blue coloured precipitate formed if the solution pH was less than 4.0. The blue solution was cooled in ice and the final pH of 2.25 indicated

FIGURE 2.9:

Elution profile of the blue coloured $Cr(nic)_2(H_2O)_4^{3+}$ complex on a Sephadex G15 column (2.0cm x 60.0cm). The tube volume was $2.0cm^3$ and the column was run in water as the eluent. Chromium (III) (———), nicotinic acid (-----) and conductivity (·····).

FIGURE 2.9


CONCENTRATION (mol l^{-} x 10^{-2})
CONDUCTIVITY (µmho x 10^{3})
TUBE NUMBER
0
1
2
3
4
5
10
20
30
40
50
60
70

that protons were released into the solution.

If the pH of the reacting chromium (III) and nicotinic acid solutions was greater than 4.0 the blue solid complex precipitated. However, if the pH of the chromium solution was increased higher than 5.0, chromium-hydroxide formed which is so stable that no reaction with nicotinic acid was possible.

An attempt was made to crystallise the mononicotinate complex by adding a saturated solution of $NaClO_4$ but this was unsuccessful. Since no precipitate was formed by this procedure the reaction mixture was purified by ion exchange chromatography using a DOWEX 50W-X12 cation-exchange resin (strong acid) in the H^+ form. The reaction solution was diluted until the conductivity was less than 3,000 umbo, the pH was adjusted to 3.5 and then loaded onto a cation exchange column, (3.0cm x 9.5cm) and washed with H_2O. When this column was eluted with 0.5M NaCl a blue solution was obtained with absorption bands in the visible region at 417nm and 570nm and a chromium to nicotinic acid ratio of 1:1. Upon further elution with a pH gradient set up in situ, by washing the column with disodium phosphate (0.05M) until the pH reached 9.0, a green fraction was eluted at pH 5.0 with a chromium to nicotinic acid ratio of 1:1 also. A final elution with 2.0M HCl failed to elute any further species, however from a comparison of the amount of chromium (III) originally loaded onto the column with the amount of chromium (III) actually eluted it was evident that much of the original material was still bound to the column.

Since such a large proportion of the mixture remained bound to the cation-exchange resin gel filtration was employed to purify the reaction mixture obtained in the preparation of the mononicotinate chromium (III) complex. The gel resin used was Sephadex G15 and the column size was 2.0cm x 60.0cm. The solutions to be chromographed were concentrated by rotary evaporation to a volume of less than $5.0cm^3$ and run through the column at a flow rate of $1.0cm^3$ per minute.

When the solution containing the blue chromium (III)-nicotinic acid species was run through the gel-filtration column with H_2O as the eluent three coloured bands were observed, their colours in order of elution were purple-violet, blue and blue. The elution profile was obtained (Figure 2.10) by measuring the chromium and nicotinic acid concentration and the conductivity of each fraction collected.

The purple-violet band eluted before the salt peak had a chromium to nicotinic acid ratio of 1:2. The two blue bands over-lapped and were eluted after the salt peak suggesting that interaction with the column matrix had retarded their elution. Although two seperate blue coloured bands were seen on the column only one blue fracton was collected (see Figure 2.11). The chromium and nicotinic acid peaks were not coincident for the retarded fractions which suggested that the two blue peaks had different chromium to nicotinic acid ratios.

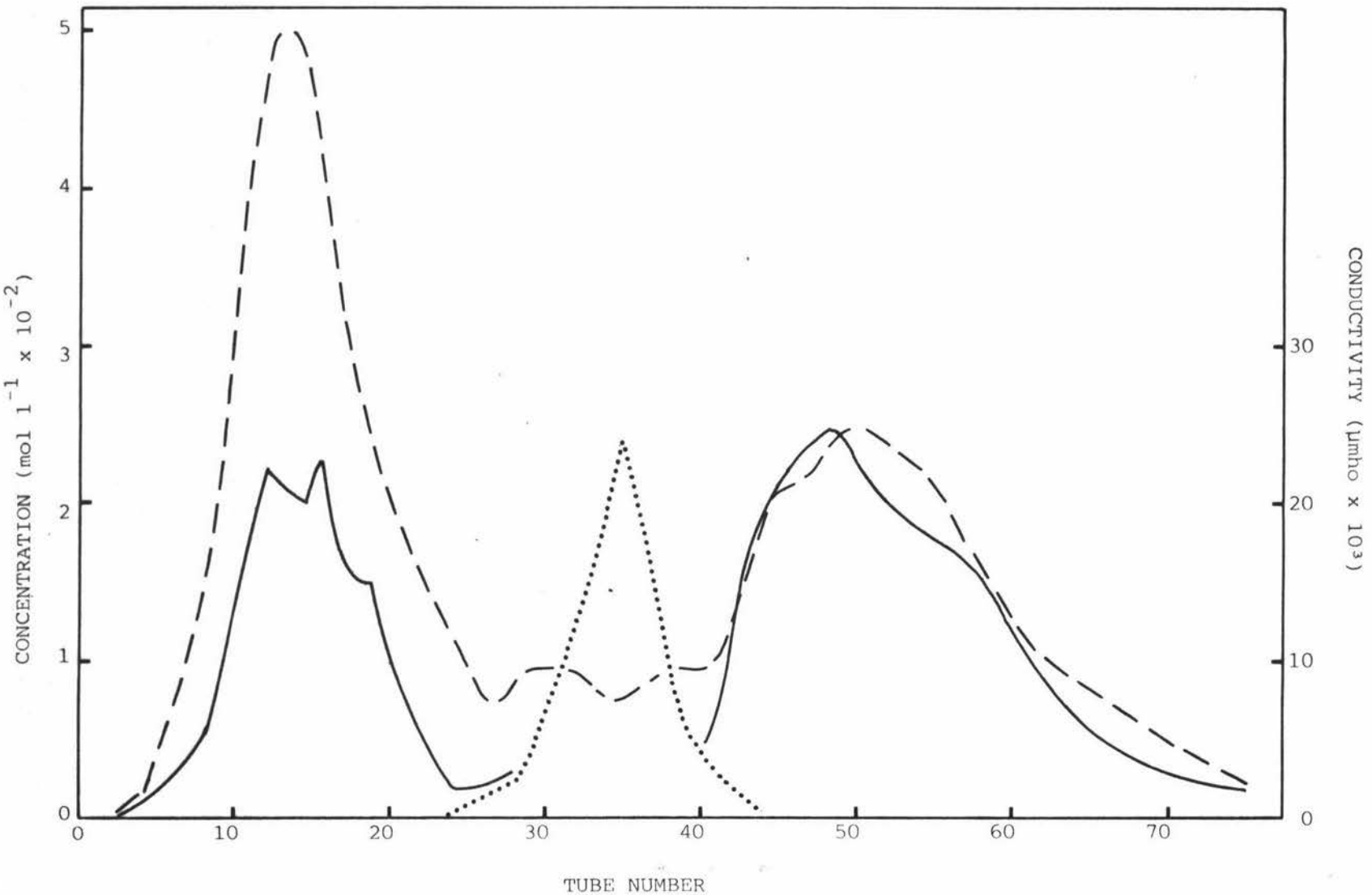
As sodium perchlorate had been added to the original blue chromium-nicotinate solution, the behaviour of a solution of $NaClO_4$ on the column was studied. Firstly the determination of the void volume of the column was carried out with both Blue Dextrans, a high molecular weight compound which was known to be larger than the exclusion limit of the G15 resin (molecular weight of approximately 1500), and also by elution of a NaCl solution. The $NaClO_4$ solution was eluted just before the bed volume, thus any complexes eluted after the salt solution were considered to have interacted with the resin matrix.

The behavior of a saturated solution of pure nicotinic acid was also studied by running the solution through the gel filtration column. The nicotinic acid was eluted just before the expected salt peak showing that no interaction had occurred with the gel matrix.

FIGURE 2.10:

Elution profile of the blue chromium (III)-nicotinic acid species on a Sephadex G15 column (2.0cm x 60.0cm). The tube volume was 2.0cm and the column was run in water as the eluent. Chromium (III) (———), nicotinic acid (------) and conductivity (·····).

FIGURE 2.10


CONCENTRATION (mol l^{-1} x 10^{-2})
CONDUCTIVITY (μmho x 10^3)
TUBE NUMBER
0
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10
20
30
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50
60
70

2.4.5.2 Electronic spectra

The soluble blue complex eluted from the cation-exchange column which was thought to be the mono-nicotinic acid Cr(III) species (because of the 1:1 ratio of chromium to nicotinic acid) had two d-d absorption bands at wavelengths slightly less than the blue solid, $Cr(nic)_2(H_2O)_3OH$. The observed maxima were at 417nm and 570nm with the first band being less intense than the second, and the molar extinction coefficients were 20 l mol^{-1} cm^{-1} and 27 l mol^{-1} cm^{-1} respectively. A similar spectrum was observed for the initial portion of the retarded species eluted from the Sephadex G15 gel filtration column. The electronic spectrum of the final portion of the retarded species eluted from the Sephadex G15 gel filtration column contained two bands at wavelengths of 425nm and 575nm.

The ultra-violet spectrum of the soluble mononicotinate complex exhibited a pronounced maximum at 262nm and similar spectra were obtained for the two species found to be retarded on the Sephadex G15 column.

2.5 DISCUSSION

2.5.1 Yellow Chromium (III)-Dinicotinate Complex

The reaction of chromous ions with nicotinic acid in a molar ratio of 1:2 formed a yellow crystalline solid which analysed as $Cr(nic)_2(H_2O)_3OH$. Since the complex is very insoluble in a variety of both polar and non-polar solvents it seems unlikely that it carries either a positive or a negative charge. Thus although the analysis figures are not accurate enough to exclude the possibility of four water molecules being coordinated to the chromium atom one of the equatorial positions is shown occupied by an OH group so that the complex would bear no charge.

The structure of the $Co(nic)_2(H_2O)_4$ complex is known to have two nicotinic acid ligands coordinated to the metal atom via the pyridine ring nitrogen atom in a trans position and four axial H_2O ligands (Anagnostopoulos et al 1969) (see Fig. 2.11). Since the x-ray diffraction powder patterns of the yellow $Cr(nic)_2(H_2O)_3OH$ and $Co(nic)_2(H_2O)_4$ were virtually identical, the indication is that they are isostructual, although comparison of the d-spacings (between diffracted maxima) and the relative intensities of the maxima suggests that they are not necessarily isomorphous structures. The structure of yellow $Cr(nic)_2(H_2O)_3OH$ was therefore also assumed to have the two nicotinic acid ligands coordinated via the pyridine ring nitrogen atom, in trans positions, with three H_2O ligands and one hydroxide ligand in a planar arrangement as shown in Figure 2.12. The axial nicotinic acid ligands, due to coordination through the nitrogen atom, are free from steric hindrance and the carboxylate groups are in positions favourable for hydrogen bonding between a carboxylate oxygen atom on one complex unit and a hydrogen atom from two H_2O ligands on another complex. This abundance of hydrogen bonding possibilities could account for the insolubility of yellow $Cr(nic)_2(H_2O)_3OH$ in all polar and non-polar solvents tried. The replacement of an H_2O ligand with an hydroxide ligand in the yellow $Cr(nic)_2(H_2O)_3OH$ complex would be expected to perturb the crystalline structure to a small extent, as observed.

FIGURE 2.11: Molecular Structure of $Co(II)(nic)_2(H_2O)_4$.

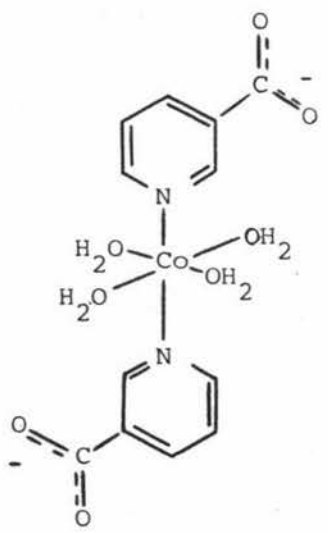
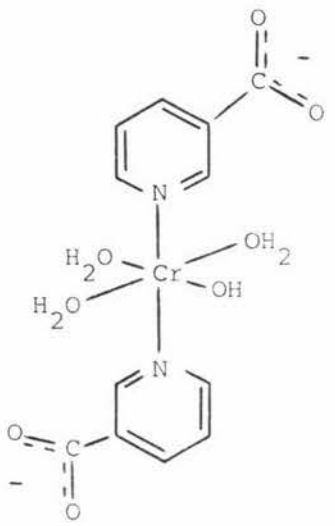


FIGURE 2.12: Structure of yellow $Cr(nic)_2(H_2O)_3OH$



The use of infra-red spectra as a method of "finger-printing" a given structure is well known, and can also be used to establish the identity of two compounds. The infra-red spectrum of yellow $Cr(nic)_2(H_2O)_3OH$ and $Co(nic)_2(H_2O)_4$ were virtually identical which again suggests that both compounds are very similar in structure and would therefore possess the same type of coordination of the nicotinic acid ligands.

The visible spectrum of the yellow $Cr(nic)_2(H_2O)_3OH$ complex was somewhat unusual because the d-d bands which are characteristic of Cr(III) were not seen in the nujol mull spectrum. Coordination with the pyridine ring nitrogen atom (which is higher in the spectrochemical series than H_2O) should displace the observed maxima to wavelengths shorter than those of $Cr(H_2O)_6^{3+}$ and hence result in a red coloured complex. Ultimately, if six nitrogen ligands were present the shift in the positions of the absorption maxima would result in a bright yellow colour as seen for $Cr(NH_3)_6(NO_3)_3$ which has λmax values of 353nm and 470nm. Therefore, a Cr(III) complex with only two nitrogen ligands and four aquo ions should have a pair of absorption maxima displaced less towards shorter wavelengths than for the hexa-ammine

complex and a colour intermediate between blue and yellow as experimentally observed.

The electronic spectrum of the yellow $Cr(nic)_2(H_2O)_3OH$ complex exhibited an intense absorption band at 340nm which was probably associated with a charge-transfer transition between the ligand and the metal ion because of its high intensity and its position in the near ultra-violet region of the spectrum would obscure the less intense d-d bands and cause the yellow colour. As the nicotinic acid ligand is probably coordinated to the Cr(III) ion by the pyridine nitrogen atom then the overall charge of the complex can be balanced by assuming negative charges on the carboxylate groups as described for the cobalt dinicotinate complex. The presence of a zwitterion type structure for the nicotinic acid ligand could result in charge transfer through the pyridine ring to the Cr(III) metal ion thus accounting for the absorption maximum at 340nm.

The yellow $Cr(nic)_2(H_2O)_3OH$ complex dissolved in both dilute acid and base but when dissolved in 2M NaOH the electronic spectrum of the resulting solution was not stable with time. Initially the spectrum was similar to the reflectance spectrum of the solid but rapidly, over a period of thirty minutes, two bands at 405nm and 600nm appeared which suggested that the complex was unstable in aqueous solution, and had re-equilibrated to the Cr^{3+} ions and free nicotinic acid. With 2M HNO_3 the reaction was similar to that of base but occurred immediately. It is apparent that the Cr-N bond is unstable in aqueous acid or base when the chromium atom has a 3+ charge as found in $Cr(nic)_2(H_2O)_3OH$.

The ultraviolet absorption spectrum of the yellow $Cr(nic)_2(H_2O)_3OH$ complex although recorded as a nujol mull was very similar to that obtained for nicotinic acid in basic solution with the shoulders on the 262nm maximum being very pronounced. This also occurs in nicotinic acid when the pyridine ring nitrogen atom is not protonated (Green and Tong, 1956). In the yellow $Cr(nic)_2(H_2O)_3OH$ complex if the Cr-N bond is

relatively weak, the nitrogen atom is effectively similar to the unprotonated nitrogen atom in nicotinic acid (in contrast with the N-H bond which is regarded as very covalent) due to the donation of a lone pair of electrons to the Cr^{3+} ion.

The unusually low value obtained for the magnetic moment of yellow $Cr(nic)_2(H_2O)_3OH$ could be explained in two ways. If the chromium atom in the $Cr(nic)_2(H_2O)_3OH$ complex had a charge of 2+ a magnetic moment of approximately 3.2 bohr magnetons would be expected since it is known that a low spin Cr(II) complex will possess a magnetic moment of approximately this value (Cotton & Wilkinson, 1972d). However such a complex normally requires a ligand with a high ligand field strength such as CN^- which forms $Cr(CN)_6^{4-}$. Such compounds with chromium stabilised in a 2+ state are rare and it seems unlikely that two trans-nicotinic acid groups would be sufficient to cause the formation of a low-spin chromium (II) complex. The second alternative is to suggest some sort of exchange interaction between two chromium atoms via hydrogen-bond linkages or a dihydroxy-bridged structural arrangement. Dihydroxy-bridged dimers of Cr(III), $[Cr(gly)_2OH]_2$, have been prepared for which Earnshaw and Lewis (1961) found a μ_{eff} value of 3.80 B.M. as expected for a dimer with no interaction between the chromium atoms. However Morishita et al (1965) found μ_{eff} = 3.37 suggesting a very significant spin coupling interaction between the two chromium atoms. This could be the case in the nicotinic acid complex, but proof would rely on a single crystal x-ray structural determination to show that the species was in fact dimeric. Another type of interaction could occur due to the non-classical zwitterian structure of the complex in which some form of interaction could occur between Cr(III) ions via hydrogen-bonding between carboxylate groups and aquo ions. The exact mechanism is however unclear.

In the infra red spectrum of the yellow $Cr(nic)_2(H_2O)_3OH$ complex the pair of ring deformations (at $410cm^{-1}$ and $645cm^{-1}$) were shifted to higher wavenumbers as compared with nicotinic acid (see Figure 2.5) as a consequence of coordination of

the nicotinic acid via the pyridine ring nitrogen atom. This would tend to alter the bond strength of the C-C and C-H bonds of the pyridine ring by changing the resonance structure to a small degree. The increase in frequency of infra-red vibrations occurs when the particular bonds become stronger (possessing more bond energy), which increases their vibrational energy. Such increases in wavenumber of these bands was observed by Clark and Williams (1965) for the series: $M(py)_2Cl_2$ where M = Co(II), Ni(II), Cr(II), Cu(II); and py = pyridine on coordination of the pyridine ring.

The O-C-O carboxylate deformation (at $535cm^{-1}$) shifted to a higher frequency in the infra-red spectrum of the yellow complex probably due to ionisation of this group which results in equivalence of the C-O bonds and an increase in the deformation energy due to ionic repulsion.

The two main bands in the region $1000cm^{-1}$ to $650cm^{-1}$ which were believed to be associated with the C-H out-of-plane pyridine ring deformation of the nicotinic acid ligand were also present for the yellow $Cr(nic)_2(H_2O)_3OH$ complex but were intensified and shifted to slightly higher wavenumbers. However these bands were in the region of $820-760cm^{-1}$ and $730-690cm^{-1}$ which have been found for other pyridine-ring containing compounds (Rao 1963). The increase in frequency of the C-H out-of plane deformation vibrations was thought to be a consequence of the altered structure of the pyridine ring due to complexation of the nitrogen atom (which would alter the resonance structure and hence the bond strength associated with the pyridine ring).

Major changes were expected for the region $1800cm^{-1}$ to $1300cm^{-1}$ due to changes in the structure of the carboxylate group. The asymmetric carbonyl stretching frequency decreased as did the symmetric carbonyl stretching frequency when the nicotinic acid was coordinated in the yellow $Cr(nic)_2(H_2O)_3OH$ complex. Since the pyridine ring nitrogen atom was coordinated to the chromium atom the changes in the

stretching frequencies of the carboxylate group reflected the proposed ionisation of the carboxylate group. In a fully ionised carboxylate group, such as $-COO^-$, the two C-O bonds are considered to be equal in strength and it is expected that the asymmetric carbonyl stretching frequency would decrease in frequency as the symmetric character of the group increased. This increase in symmetric character would therefore cause an increase in the intensity of the symmetric carbonyl stretching frequency. With the yellow $Cr(nic)_2(H_2O)_3OH$ complex the behaviour of the two carbonyl stretching vibrations confirmed that ionisation of the carboxylate group of the nicotinic acid was occurring upon coordination. Thus during the reaction with Cr^{2+}, two nicotinic acid ligands with ionised carboxylate groups would neutralise the 2+ charge to form the pink precipitate, which then oxidised with the addition of a hydroxide ligand to form the yellow complex (still with two ionised nicotinic acid ligands). Similar changes in stretching frequencies are seen when the carboxylate group is coordinated to a metal ion as in the Cu(II) complexes with glycine studied by Sweeny et al (1955) where coordination of the carboxylate group causes ionisation to occur. The absence of the hydroxyl deformation vibration in the yellow $Cr(nic)_2(H_2O)_3OH$ complex also confirmed that loss of the carboxylate proton had indeed occurred upon coordination of the nicotinic acid.

The positions of the pyridine ring stretching vibrations in the complex were shifted to lower frequencies and since these vibrations are due to the C=C and C=N bonds, this indicated the involvement of the whole pyridine ring in the coordination of the nicotinic acid with the nitrogen atom coordinated to the chromium atom (even if only weakly) the strength of the aromatic bonding would be lessened due to destabilisation of the ring structure when the nitrogen atom donates electrons to the chromium atom to form a covalent bond. In fact, due to the close proximity of the pyridine ring to the chromium ion some overlap of the ring π-orbitals and the d-orbitals of the metal would also result in changes in the ring structure. The resulting decreases

in bond energies could result in a decrease in the stretching vibrations of the affected bonds.

Hydrogen bonding in the yellow $Cr(nic)_2(H_2O)_3OH$ complex would be expected to occur between adjacent molecules from one of the carboxylate oxygen atoms to two hydrogens from two of the coordinated H_2O molecules. The strong hydrogen-bonding effects could cause the great insolubility of the complex in all polar and non-polar solvents tried. Also the infra red spectrum would be difficult to analyse because of the different degrees of hydrogen-bonding involving interactions between the $-COO^-$ and H_2O groups present.

2.5.5.1 Comparison with literature

The complexation of the pyridine carboxylic acids nicotinic acid, isonicotinic acid and picolinic acid with transition metal ions is well documented (Anagnostopoulos et al 1972, Kleinstein and Webb 1971, Allen et al 1977). Kleinstein and Webb postulated for the series $M(nic)_2$ where M = Mn(II), Co(II), Ni(II), Cu(II), Ag(II), and Zn(II) that coordination of the nicotinic acid to the metal ion was through the pyridine ring nitrogen atom and assigned an infra-red absorption band in the region $450\,cm^{-1}$ - $480cm^{-1}$ to the metal-nitrogen stretching frequency. In the case of picolinic acid chelation to the metal occurs through a pyridine ring nitrogen atom and an ortho-substituted carboxylate group and an absorption band in the region $410cm^{-1}$ - $420cm^{-1}$ was assigned to the metal-oxygen stretching frequency. The range of the infra-red spectrum recorded by Kleinstein and Webb was $1000cm^{-1}$ - $250cm^{-1}$ and two intense bands in the region $800cm^{-1}$ - $650cm^{-1}$ were observed. Similar intense bands have been observed for the blue and yellow $Cr(nic)_2(H_2O)_3OH$ complexes in the present work and it seemed as if these bands, assigned to the CH out-of-plane deformation vibrations, are a characteristic of coordinated nicotinic acid ligands.

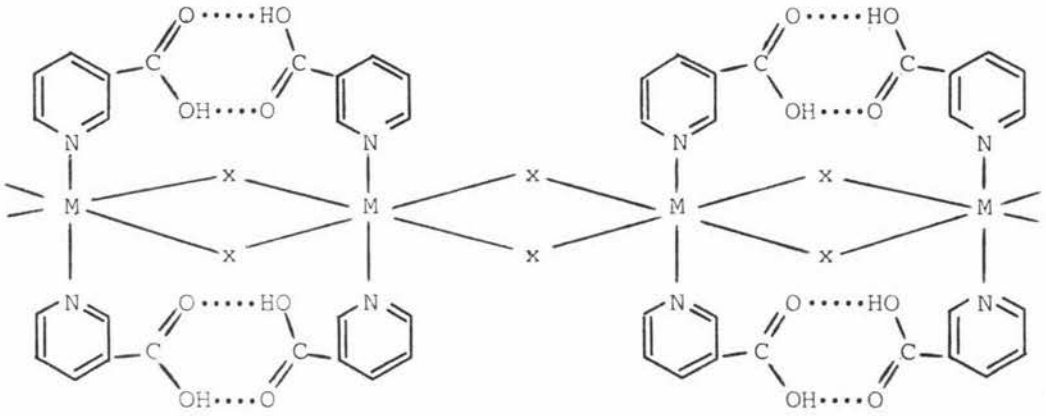
Nicotinic acid also seems to form halide bridged dinicotinic acid complexes with some first row transition metals but

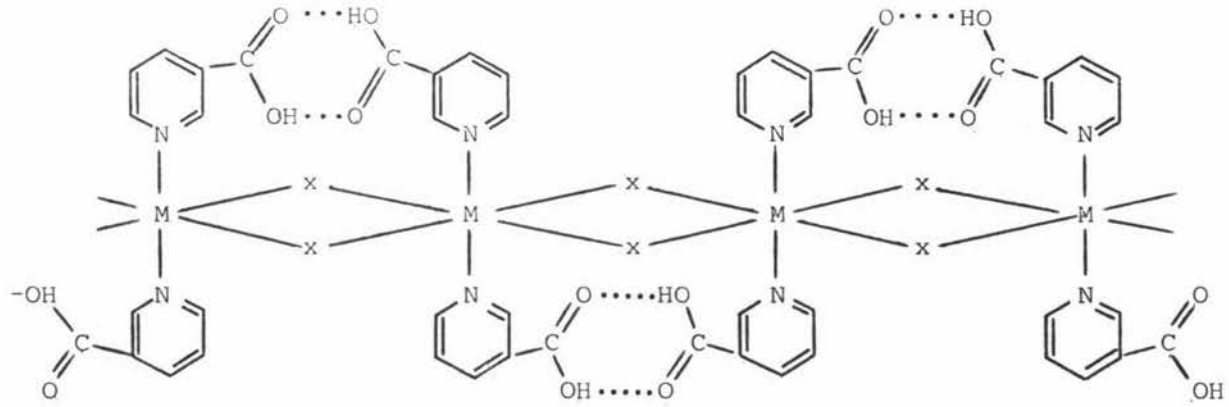
only under anhydrous conditions (Allen et al 1977). The complexes prepared by Allen et al were of the general form ML_2X_2 where M = Mn(II), Fe(II), Ni(II), Co(II), and Cu(II); L = nicotinic acid or nicotinamide; and X = Cl or Br and their infra-red spectra were recorded in the range $4000cm^{-1}$ - $200cm^{-1}$. All of the complexes were proposed to involve coordination of the nicotinic acid, through the pyridine ring nitrogen atom, in a trans position with the planar coordination sites containing bridging halide atoms. The overall structure was thus polymeric, with hydrogen bonding between adjacent carboxylate groups, explaining the poor solubility of the complexes in both polar and non-polar solvents, see Figure 2.13. The metal-nitrogen stretching vibration in the infra-red region was assigned to a single band at a frequency less than $300cm^{-1}$.

The yellow $Cr(nic)_2(H_2O)_3OH$ complex prepared in this study also contains an octahedral metal ion and trans nicotinic acid ligands with the planar positions occupied by three H_2O ligands and a hydroxide ligand. The trans position of the nicotinic acid ligands seems common to all the first row transition metals that form octahedral complexes and it is interesting to note that all such complexes have a divalent metal ion. In the preparation of yellow $Cr(nic)_2(H_2O)_3OH$ the coordination of nicotinic acid first occurs with the divalent Cr(II) ion which is then oxidised to Cr(III) ion but retains the nitrogen atom coordination which is not found in the reaction of Cr^{3+} with nicotinic acid. The nitrogen-chromium(III) bond is however thought to be unstable (Gould and Taube 1964) although the formation of hydrogen bonds similar to those found in the $M(nic)_2X_2$ complex prepared by Allen et al (1977) would confer stability in the solid form.

The assignment of the infra-red band at $310cm^{-1}$ in yellow $Cr(nic)_2(H_2O)_3OH$ to the metal-nitrogen stretching vibration is consistant with the weak nature of this bond which would tend to shift the frequency to a lower value when compared to such complexes as $M(nic)_2X_2$ (Allen et al 1977) which

FIGURE 2.13: Proposed structures of dinicotinate complex with bridging halogens





have a M-N stretching frequency in the region of approximately $300cm^{-1}$ or $M(nic)_2$ (Kleinstein and Webb 1971) which have a M-N frequency at $450cm^{-1}$ - $480cm^{-1}$ (or $410cm^{-1}$ - $420cm^{-1}$). Kleinstein's assignment of the $410cm^{-1}$ - $420cm^{-1}$ band to a M-O stretching frequency seemed to imply that the strength of the M-O bond was less than the M-N bond which could only occur if the M-N band was covalent and the M-O bond weakly ionic. A covalent M-O bond would have a stretching vibration shifted to a higher frequency and could mean that Kleinstein and Webb's assignments were in error.

Anagnostopoulos et al (1972) recorded the infra-red spectra of Co(II) and Ni(II) complexes with several pyridine carboxylates, including nicotinic acid, isonicotinic acid, pyridine-2,3-dicarbocylic acid and pyridine-2,5-carboxylic acid. They concluded that coordination of the pyridine ring nitrogen atom occurs and the ratio of M/L = 1:2 for the Mi(II) and Co(II) complexes with monocarboxylic acids. Confirmation of this proposal was established with the determination from the 3-dimensional single crystal x-ray analysis of the structure of $Co(nic)_2(H_2O)_4$ which revealed a slightly distorted octahedral environment around the cobalt atom, comprising a planar array of water molecules, and two axial nicotinic acid ligands (Anagnostopoulos et al 1969) as shown in Figure 2.12.

The reaction of chromous ions with nicotinic acid, isonicotinic acid and picolinic acid has been studied by Vrachnou-Astra and Katakis (1972) and at acidic pH values no reaction takes place between Cr(II) and nicotinic acid as expected since protonation of the pyridine ring nitrogen atom at this pH would render the ligand unavailable for coordination. In the preparation of yellow $Cr(nic)_2(H_2O)_3OH$ the pH of the nicotinic acid was found to be important and the reaction only occurred at pH 6.0 (presumably because the pyridine ring is not extensively protonated at this pH since the pk_a value of the nitrogen atom is 4.81 (Green and Tong 1956)), which explained why Vrachnou saw no reaction. However

Vrachnou did see a reaction with isonicotinic acid when the reacting solution was neutralised with alkali, the resulting red solution was postulated as an intermediate complex which was not stable but allowed for reduction of the isonicotinic acid to an aldehyde. This red intermediate complex was probably similar in structure to the pink precipitate obtained with the reaction of chromous solution with nicotinic acid ($Cr(II)(nic)_2(H_2O)_4$) which subsequently oxidised to the yellow $Cr(nic)_2(H_2O)_3OH$.

2.5.2 Other Transition Metal Complexes with Nicotinic Acid

The visible spectrum of $Co(II)(nic)_2(H_2O)_4$ was consistent with a high spin d^7 configuration. Only one d-d transition was observed due to the frequency limits of the spectrophotomer, but this transition at 510nm was similar to that observed in the hexa aquo Co(II) ion at approximately 540nm (Cotton and Wilkinson 1972e). It was noticed that the frequency of this band was shifted to a lower frequency presumably because two nitrogen atoms from the two nicotinic acid ligands were coordinated which replaced two aquo molecules which had a weaker ligand field. The rather broad, indistinct ultra-violet absorption maximum was consistent with the presence of the nicotinic acid ligands which normally exhibit a characteristic group of bands at approximately 262nm. The lack of defined structure in the ultra-violet spectrum of the complex was thought to be due to a charge transfer effect associated with the ionised carboxylate groups as discussed for the yellow chromium-dinicotinate complex.

The observed magnetic moment for $Co(nic)_2(H_2O)_4$ (5.10 B.M.) is in the region expected for high-spin octahedral complexes with a d^7 configuration (4.7 - 5.2 B.M., Cotton and Wilkinson 1972f) because tetrahedral complexes have μ_{eff} less than 4.7 B.M. and square complexes are low spin with μ_{eff} = 2.2 - 2.7 B.M. (Cotton and Wilkinson 1972f). The high magnetic

moment was greater than the value of μ_s (Table 2.3) and was probably due to the intrinsic angular momentum in the octahedral ground state, thus there is consistently a considerable orbital contribution. The significant orbital contribution was considered to be due to the interaction of the $^4A_{1g}(P)$ and $^4A_{2g}(F)$ states (Allen et al 1977).

In the $Ni(nic)_2(H_2O)_4$ complex the position and frequencies of the electronic absorption bands were also consistent with the coordination of the two nicotinic acid ligands through the pyridine ring nitrogen atoms which was responsible for the blue colour. The ultra-violet spectrum was similar to the $Co(nic)_2(H_2O)_4$ complex as expected due to the similar structure.

The observed magnetic moment of $Ni(nic)_2(H_2O)_4$ (3.36 B.M.) was in agreement with that predicted for the two unpaired electrons and effectively ruled out other types of geometry because tetrahedral complexes have μ_{eff} = 3.5 - 4.0 B.M. and planar complexes are diamagnetic (μ_{eff} = 0 B.M.) (Cotton and Wilkinson 1972g).

The visible spectrum of $Mn(II)(nic)_2(H_2O)_4$ could not be recorded since the d-d transitions were very weak as expected for a d^5 high spin configuration which gives rise to spin forbidden as well as parity forbidden transitions resulting in the pale weak colour of the complex. The electronic configuration contains five unpaired d electrons, one in each orbital in the ground state and all spins are parallel, making a spin sextuplet ground state. Only one sextuplet state is possible, thus all excited states have a different multiplicity and all transitions are spin forbidden. Although the analytical data suggested a coordination number of four, the lack of colour in the complex is only seen for an octahedral complex and not for tetrahedral complexes which are yellow-green in colour, and more intense than for octahedral complexes (Cotton and Wilkinson 1972h). The ultra-violet spectrum was essentially the same as the Co(II) and Ni(II)

complexes, therefore all three complexes were assumed to have similar structures.

The magnetic moment of $Mn(II)(nic)_2(H_2O)_4$ was 5.91 B.M. which is very close to the spin-only value of 5.9 B.M. usually observed for high spin d^5 configuration complexes. This was consistant with a high spin octahedral environment in the complex $Mn(II)(nic)_2(H_2O)_4$.

The infra-red spectra of all the complexes prepared from Mn(II), Ni(II), Co(II) and Cr(II) (which oxidised to Cr(III) after reaction) and nicotinic acid were virtually identical. They were all of the form $M(nic)_2(H_2O)_4$, excepting the yellow $Cr(nic)_2(H_2O)_4OH$. The fact that all the complexes had the same infra-red spectra again implied that all the complexes had a similar structure because similar vibrations and deformations in the bonding would be possible resulting in similar infrared bands. The only changes in the complexes were due to the type of metal atom, therefore the M-N bond would differ and thus the metal-nitrogen stretching frequency would be expected at different frequencies in the series of complexes. The coordination of the nicotinic acid ligand was found to be through the pyridine ring nitrogen atom in $Co(nic)_2(H_2O)_4$ (Anagnostopoulos et al 1969), so it was assumed that similar coordination had occurred for the other metals Mn(II), Cr(II) and Ni(II) also.

The only change seen in the various spectra for the metal complexes was in the region $600cm^{-1}$ - $200cm^{-1}$ where a new band was found in a similar place for all the complexes, its position depending on the metal ion involved. This is therefore likely to be the metal-nitrogen stretching vibration which would be dependent on the metal atom (a result of the strength of the bond). Vibrations of this type are expected to occur low in the infra-red region due to the large size of the metal atom and the weak nature of the metal-nitrogen bond (which would possess a low bond energy). The position of the new band shifted to lower

frequencies in the order Ni(II) > Co(II) > Cr(II) which suggested that the Ni-N bond was the strongest and the Cr-N bond the weakest (in fact the Cr-N bond was unstable in aqueous solution). All the other main infra red absorption bands in the spectrum of the Co(II), Ni(II), Mn(II) and Cr(III)[1] complexes were similar and were thus assigned to stretching and deformation vibrations as for the yellow $Cr(nic)_2(H_2O)_3OH$ complex.

The x-ray diffraction powder patterns of $Co(nic)_2(H_2O)_4$ and $Ni(nic)_2(H_2O)_4$ were virtually identical which suggested that both complexes had a similar complex structure with the coordination of the two nicotinic acid ligands in a trans position via the pyridine ring nitrogen atom and with four planar water molecules. However the diffraction pattern of $Mn(nic)_2(H_2O)_4$ was slightly different from the Co and Ni analogues. Many of the bands were coincident, but extra bands could only be accounted for by assuming an impurity of free ligand. This would affect the x-ray diffraction pattern greatly but would not affect the infra-red spectrum significantly which was similar to those of the other complexes with only minor changes. Essentially, then the structures of the complexes in the series $M(nic)_2(H_2O)_4$, where M = Co(II), Ni(II) and Mn (II), are identical with the structure of the yellow $Cr(nic)_2(H_2O)_3OH$ complex.

2.5.3 Blue Coloured Chromium (III)-dinicotinate Complex

When chromium (III) ions were reacted with a 2:1 molar ratio of nicotinic acid at pH values greater than 4.0 an insoluble blue solid which analysed as $Cr(nic)_2(H_2O)_3OH$ was formed. Thus the elemental analysis was consistent with the coordination of two nicotinic acid ligands in the blue complex. This empirical formula was identical with that of the yellow $Cr(nic)_2(H_2O)_3OH$ complex hence the blue and yellow forms were considered to be isomers but their structures are clearly different.

Footnote 1: Derived from Cr(II) then air oxidised to Cr(III)

With reference to the spectrochemical series given in Section 2.2.3, it can be seen that nitrogen donor ligands are higher in the series, while weakly basic oxygen ligands are lower in the series, than the H_2O ligand. The absorption maxima for the blue $Cr(nic)_2(H_2O)_3OH$ complex (420nm and 575nm) were at frequencies greater than the absorption maxima for $Cr(H_2O)_6^{3+}$ (406nm and 571nm) thus coordination by the carboxylate group of nicotinic acid appears to be responsible for the shifts in position of the d-d transition bands. The observed increase in the frequencies of the transitions compared with $Cr(H_2O)_6^{3+}$ indicates a decrease in the energies of the d-d transitions consistent with coordination of the nicotinic acid through the carboxylate group rather than via the pyridine ring nitrogen atom and this type of coordination is referred to as oxygen-type coordination rather than nitrogen-type coordination.

The broad band in the ultra-violet region of the reflectance spectrum of the blue $Cr(nic)_2(H_2O)_3OH$ complex was obviously associated with the nicotinic acid ligand, although the lack of a defined maximum with pronounced shoulders was considered to be a matrix effect. Normally the spectrum of nicotinic acid is only affected by protonation of the pyridine ring nitrogen atom (Green and Tong 1956) and when the nitrogen atom is protonated, the shoulders become less intense but in this complex the nitrogen atoms are unlikely to be protonated since if they were the protonated pyridine ring would probably render the complex soluble which is contrary to experiment.

A comparison of the main infra-red absorption bands of the free nicotinic acid ligand and the blue $Cr(nic)_2(H_2O)_3OH$ complex was useful in determining the mode of coordination of the nicotinic acid ligand, because any differences could be assigned to changes due to coordination. In the region $650cm^{-1}$ - $200cm^{-1}$ the spectra of the blue complex $Cr(nic)_2(H_2O)_3OH$ and nicotinic acid were very similar, but the absorption frequencies in the complex were shifted to either

higher or lower wavenumbers. These changes in frequency were anticipated because for pyridine the bands at 405cm^{-1} and 604cm^{-1}, which have been assigned to the out-of-plane ring deformation and the in-plane ring deformation frequencies respectively (Clark and Williams 1965), are shifted to 380cm^{-1} and 640cm^{-1} for nicotinic acid which is a 3-carboxylic acid pyridine derivative. These changes were similar to those observed for other 3-substituted pyridines for which the 405cm^{-1} band tends to shift towards lower frequencies and the 640cm^{-1} band tends to shift towards higher frequencies (Isaac and Bentley 1959) as compared with pyridine itself. It was therefore not unreasonable that coordination of the chromium (III) metal ion to the 3-carboxylate group of nicotinic acid would alter the positions of the pyridine stretching frequencies in the 600cm^{-1} and 400cm^{-1} regions. However the out-of-plane ring deformation frequency was not observed probably due to the broadening found for all of the infra red absorption bands of the complex. It was also noticed that no band was found in the 300cm^{-1} region indicating that coordination of the pyridine ring nitrogen atom had not occurred.

The extra band observed for the complex between the out-of-plane and in-plane ring deformation bands of nicotinic acid was probably a carbonyl bending vibration which had shifted to a higher frequency (495cm^{-1} to 520cm^{-1}) when the nicotinic acid was coordinated. This increase in frequency could be caused by the C-O bonds becoming more equivalent which would be expected if the metal-oxygen bond was weaker than the hydrogen-oxygen bond it had replaced and the more equally charged oxygen atoms would be repulsed due to their similar electrical charge (resulting in a higher deformation energy).

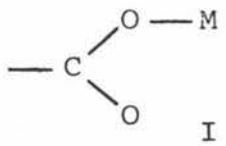
The second region from 1000cm^{-1} to 600cm^{-1} contained four main bands in the spectrum of both nicotinic acid and blue $Cr(nic)_2(H_2O)_3OH$. It is thought that the bands in the region of 820-760cm^{-1} and 730-690cm^{-1} are associated with CH out-of-plane deformations of the pyridine ring (Rao 1963), and with the blue complex $Cr(nic)_2(H_2O)_3OH$ two intense bands

in the infra-red spectrum were seen in these positions. The increase in intensity of these two bands upon coordination of the nicotinic acid to the chromium was thought to be due to changes in the bond-strengths of the aromatic heterocyclic pyridine ring CH groups because the 3-substituted group had been changed as a result of coordination to the Cr(III) ion, i.e. a change in the resonance structure of the pyridine ring. In fact this increase in intensity of these bands seemed to be characteristic of complexation of pyridine derivatives with transition metals because it was observed in many complexes in this study (yellow $Cr(nic)_2(H_2O)_3OH$, $Co(nic)_2(H_2O)_4$, $Ni(nic)_2(H_2O)_4$ and blue $Cr(nic)_2(H_2O)_3OH$). An unexpectedly new band was found at $915cm^{-1}$ in the complex and not in nicotinic acid, which although weak, could only be attributed to the chromium-oxygen bond stretching vibration. On comparison of this frequency with the frequency of the chromium-nitrogen stretching vibration found in the yellow $Cr(nic)_2(H_2O)_3OH$ comlex ($310cm^{-1}$) it is consistant with the chromium-oxygen bond being stronger and thus much more stable.

If the carboxylate group of nicotinic acid is coordinated to the Cr(III) atom major changes in the vibrational spectra associated with the carboxylate stretches would be expected. The hydroxyl deformation, the symmetrical carbonyl stretch, pyridine ring stretches and the asymmetrical carbonyl stretches are all found in the $1800cm^{-1}$ - $1300cm^{-1}$ region in both the blue $Cr(nic)_2(H_2O)_3OH$ complex and for nicotinic acid. The infra-red absorption stretching and deformation vibrations associated with the pyridine ring structure were expected to change upon coordination of the nicotinic acid to the chromium (III) ion because the pyridine ring although not directly altered structurally, was expected to influenced by the change in the substituent carboxylate group. Only small changes in the spectrum were observed in the other regions so in the region 1600 - $1400cm^{-1}$ any bands that changed to only a small degree upon coordination were assigned to pyridine ring stretching vibrations as shown in Table 2.8.

However several bands in the infra-red spectrum of nicotinic acid were drastically altered upon coordination, and this suggested that the structure of nicotinic acid has changed thus changing its vibrational spectrum. The absence of the hydroxyl deformation in the blue $Cr(nic)_2(H_2O)_3OH$ complex was important because this indicated that coordination of the carboxylate group had occurred with subsequent loss of the proton (which incidently accounted for the increased acidity of the reaction solution). This would be expected to be accompanied by changes in the intensity and positions of the symmetric and asymmetric carbonyl stretching frequencies. The asymmetric carbonyl stretching frequency in the complex was in fact shifted to a lower wavenumber ($1695cm^{-1}$ to $1610cm^{-1}$) in agreement with this expectation and suggested that the strength of the C = O bond had decreased (less energy involved with this vibration). The symmetric carbonyl stretching frequency of the nicotinic acid molecule was replaced in the complex by a more intense band in the same position with two shoulders. One shoulder (at $1340cm^{-1}$) was present in nicotinic acid and was probably a pyridine ring stretching vibration but the other two bands must have originated by a splitting of the symmetric carbonyl stretching frequency. Such a split would suggest two types of carbonyl stretching vibration present in the complex and this could occur with a significant amount of hydrogen bonding between complex units via the carboxylate groups.

The bonding situation between the carboxylate group and the chromium (III) atom was thought to be:



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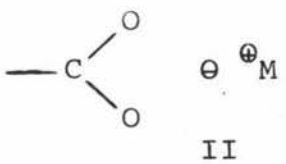
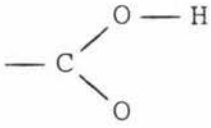
rather than:



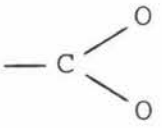
II

whereby the carbonyl group is present in a structure similar

to that found in nicotinic acid. With structure II the two oxygen atoms became identical and the asymmetric stretching frequency is therefore expected to decrease (less energy) and the symetric frequency is expected to increase (more energy). However in the infra-red spectrum of the complex although the asymmetric frequency decreases, the position of the symmetric frequency remained unchanged, suggesting that the strengths of the carbon-oxygen bonds were midway between those of the structure



and the structure



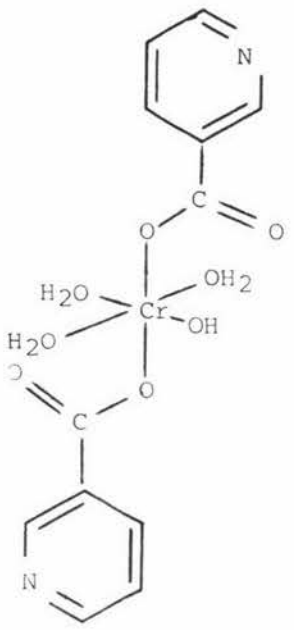
This would place the oxygen-chromium bond midway between the strong covalent bond found in the hydroxyl bond and the more ionic bond found if the carboxylate group is completely ionised.

In the blue $Cr(nic)_2(H_2O)_3OH$ complex the two nicotinic acid ligands are coordinated to the chromium atom via the carboxylate group and are most likely in a trans position to eachother because of the steric hindrance which would result if they were placed in a cis position. The remaining ligands; three H_2O molecules and one hydroxide ion would assume a planar arrangement around the chromium atom as shown in Figure 2.14. The great insolubility of the blue solid in all solvents tried was probably a result of the neutral charge on the complex and a lack of easily ionisable functional groups.

2.5.4 The Reaction of the Blue $Cr(nic)_2(H_2O)_3OH$ Complex with Acid

Although the blue insoluble species $Cr(nic)_2(H_2O)_3OH$ was very stable it dissolved very slowly in dilute acid to give a blue solution. (Reaction with base produced a green solution which was probably Cr^{3+} and free nicotinic acid and the change in colour was further evidence that the complex had decomposed).

FIGURE 2.14: Structure of the blue $Cr(nic)_2(H_2O)_3OH$ complex



The blue coloured solution had absorption maxima which were very similar to those observed in the reflectance spectrum for the solid complex and thus it is likely that the structure of the complex was unchanged by the protonation reaction. The nicotinic acid ligands are therefore probably still coordinated through the carboxylate group as found in the solid complex, the only change being protonation of the equatorial OH group (and possibly the nicotinic acid nitrogen atom) as shown in equation 3.8.

$$Cr(nic)_2(H_2O)_3OH + 3H^+ = Cr(nic)_2(H_2O)_4^{3+} \quad \text{Equation 3.8}$$

A tri-positive charge is shown for this complex since it was assumed that coordination of the carboxylate groups to the chromium (III) ion would lower the pk_a value for the carboxylate group sufficiently so even in 3M HNO_3 the carboxylate oxygen atoms would not be protonated, (this is obvious since the spectrum is not changed), however the two pyridine ring nitrogen atoms could be easily protonated.

The blue coloured solution containing $Cr(nic)_2(H_2O)_4^{3+}$ had an intense absorption maximum in the ultra-violet region of the spectrum and because the solid and solution species were thought to be structurally similar, the molar extinction coefficient of the 262nm band was used as a method of calculating the molar ratio of metal to ligand by assuming that the spectrum of the nicotinic acid (with the pyridine ring nitrogen atom protonated) was unchanged on coordination of the carboxylate group. Calculations showed that the ratio was indeed 1:2 which was consistent with the assumption that no gross structural change was occurring as a result of protonation.

Therefore the blue $Cr(nic)_2(H_2O)_4^{3+}$ complex derived from the blue solid $Cr(nic)_2(H_2O)_3OH$ by dissolving in acid was considered to be a stable species in acid. The structure of the solid and solution dinicotinate complexes were similar because two nicotinic acid ligands were coordinated to the chromium (III) atom through the carboxylate group and these ligands were most likely in trans positions across the Cr(III) atom because of the steric hindrance which would result if they were placed in a cis position. The four water ligands are arranged in a plane at the equator of the octahedral complex and along with the tri-positive charge on the complex confer solubility in H_2O. The structure of the blue $Cr(nic)_2(H_2O)_4^{3+}$ complex is shown in Figure 2.15. The action of base on this complex would be to remove a proton from a water molecule ligand and the two pyridine nitrogens and cause precipitation of the blue solid $Cr(nic)_2(H_2O)_3OH$.

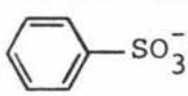
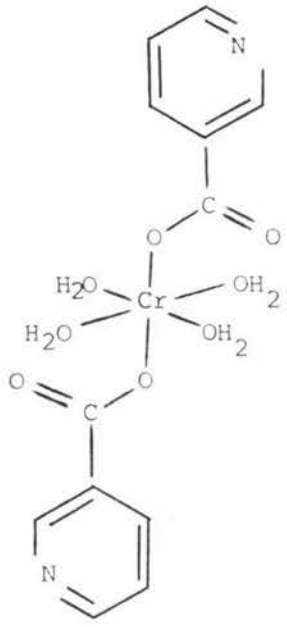
The blue solution of $Cr(nic)_2(H_2O)_4^{3+}$ could not be eluted from the cation-exchange column which suggested that some sort of non-specific adsorption was occurring such that the complex was bound very tightly to the resin. The resin used was DOWEX 50W-X12 which is composed of polystyrene beads containing the benzene sulphonic acid functional group () and it could be that some form of hydrophobic

FIGURE 2.15: Structure of the blue coloured $Cr(nic)_2(H_2O)_4^{3+}$ species in acidic solution



interaction between the complex and the ion-exchange resin was responsible for the very tight binding.

The gel filtration chromatography of the blue $Cr(nic)_2(H_2O)_4^{3+}$ solution proved interesting because this species appeared to interact with the resin so much that it was eluted after the salt peak. In gel filtration, the largest sized complexes are usually eluted first because smaller complexes tend to diffuse into the gel resin more easily, thus retarding their elution, whereas the larger complexes pass through relatively unimpeded because they are too large to diffuse into the resin. By comparison of their relative sizes, a chromium complex containing two nicotinic acid ligands should be much larger than simple salts. Thus the complex species should be eluted near the void volume whereas the salt should be eluted much later due to diffusion into the resin. However when the solid $Cr(nic)_2(H_2O)_3OH$ was dissolved

in acid, the soluble $Cr(nic)_2(H_2O)_4^{3+}$ species was in fact eluted after the bed volume of the column.

The retarded elution of $Cr(nic)_2(H_2O)_4^{3+}$ from the Sephadex G15 resin was probably due to non-specific hydrophobic interactions with the resin. Similar interactions on Sephadex resins by other aromatic compounds have been cited by Janson (1967) but the nature of such interactions is unknown. However a planar structure and an extended system of conjugated bonds in the solute favours adsorption due to a basic structural similarity with the gel matrix. The soluble dinicotinate complex of chromium (III) contains the pyridine ring structure which is a likely gel-interactive group and would account for the observed interaction on the column and subsequent retardation of elution.

2.5.5 The Soluble Blue Mononicotinate Complex of Chromium (III)

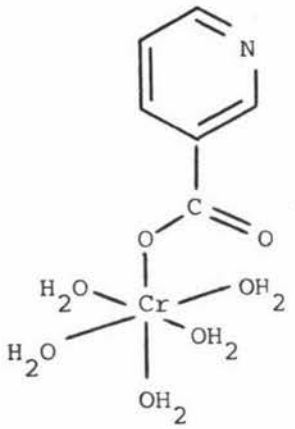
When chromium (III) ions were reacted with an equimolar solution of nicotinic acid at pH values less than 4.0 a blue solution was formed which contained a variety of species. Cation exchange chromatography yielded a blue solution on elution with a NaCl solution and a green solution was eluted with a phosphate buffer gradient but some of the chromium remained bound to the column. This bound chromium would be in the form of a complex, because uncomplexed Cr^{3+} was eluted after the blue fraction, and was probably the soluble blue $Cr(nic)_2(H_2O)_4^{3+}$ species which as we have seen binds to the cation-exchange resin very tightly.

The blue solution eluted from the cation-exchange column had a molar ratio of chromium to nicotinic acid of 1:1 and was eluted just before the $Cr(H_2O)_6^{3+}$ ions thus the effective charge on the complex was probably less than 3+. These properties would be expected for a chromium (III)-mononicotinate complex with the nicotinic acid ligand coordinated through the carboxylate group and such a complex could be thought of as $Cr(nic)(H_2O)_5^{2+}$.

For the soluble mono-nicotinate chromium (III) complex the positions of the absorption maxima lay mid-way between those found for the soluble $Cr(nic)_2(H_2O)_4^{3+}$ species and the $Cr(H_2O)_6^{3+}$ species. This observation is consistent with a complex which had only one nicotinic acid ligand bound per chromium atom. The effect of only one carboxylate group coordinated to the chromium ion would obviously be to displace the absorption maxima less towards higher wavelengths than for the dinicotinate complex. The molar extinction coefficients of the absorption bands were consistent with octahedral coordination in the complex because they were less than 40 1 mol^{-1} cm^{-1}. Since only one nicotinic acid molecule was coordinated to the chromium atom in this species the formula for the complex is most likely $Cr(nic)(H_2O)_5^{2+}$. The ultra-violet spectrum of this species ($Cr(nic)(H_2O)_5^{2+}$) was very similar to that observed for the $Cr(nic)_2(H_2O)_4^{3+}$ species. The shoulders were barely visible with λmax equalling 262nm (associated with the nicotinic acid ligand) and the ratio of chromium to nicotinic acid which was calculated by the method used for $Cr(nic)_2(H_2O)_4^{3+}$ was found to be 1:1, consistent with only one nicotinic acid molecule coordinating to the chromium (III) atom.

In $Cr(nic)(H_2O)_5^{2+}$ the nicotinic acid ligand is coordinated through the carboxylate group and since only one nicotinic acid is present in the complex only one structure is possible and this is shown in Figure 2.16. Such a structure explains the instability of this complex in basic solution because the five water ligands can be easily replaced by hydroxide ions which would result in significant olation of this species.

FIGURE 2.16: Structure of soluble blue $Cr(nic)(H_2O)_5^{2+}$



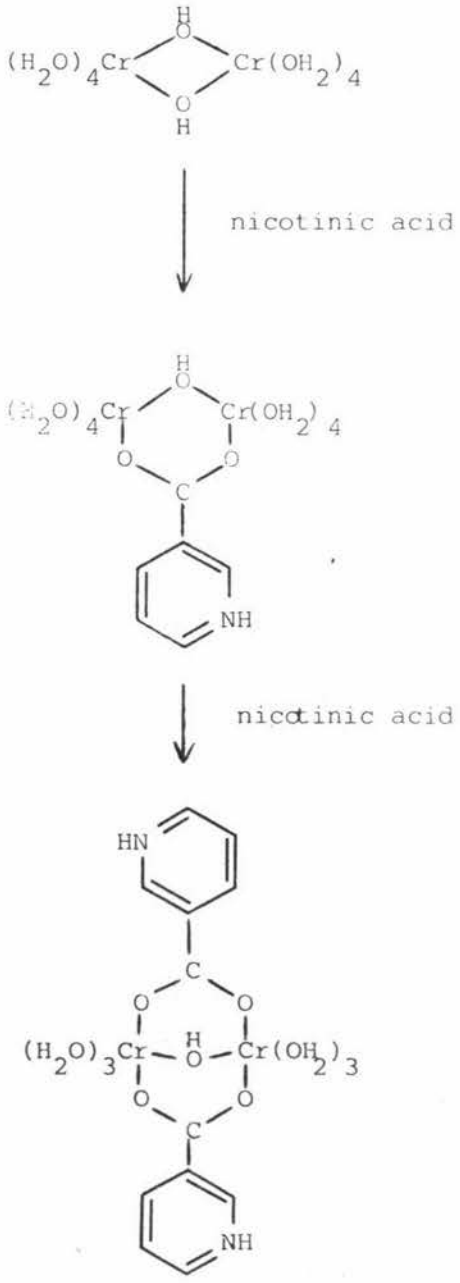
The differing ion-exchange properties meant that one species, the blue mononicotinate complex, could easily be seperated from the unreacted nicotinic acid (which was eluted before $Cr(nic)(H_2O)_5^{2+}$), Cr^{3+} (eluted after $Cr(nic)(H_2O)_5^{2+}$) and the very tightly bound $Cr(nic)_2(H_2O)_4^{3+}$ species which was lost beyond recovery.

The green fraction formed during the preparation of the mono-nicotinate complex was possibly a polymeric Cr(III) complex with nicotinic acid since the species possessed a M/L ratio of 1:1 and was green in colour instead of blue as found for the $Cr(nic)(H_2O)_5^{2+}$ species. The green colour in a Cr(III) complex is usually associated with the presence of hydroxide bridging in the complex such as found for the basic chromic polymers isolated when a solution of Cr^{3+} was boiled for several hours by Laswick and Plane (1959) and Ardon and Plane (1959) but the exact structures have not so far been elucidated. Such a polymeric complex containing one nicotinic acid ligand per chromium (III) atom would need to possess a charge greater than 3+ to remain bound to the cation-exchange resin after the elution of $Cr(H_2O)_6^{3+}$. If it was assumed that the carboxylate group was capable of attachment as a bidentate ligand to two chromium (III) atoms joined by a hydroxy-bridge then a dimeric chromium (III)-nicotinic acid complex is possible. The suggested reaction is shown in Figure 2.17 and the complex formed $(H_2O)_3Cr(nic)(OH)(nic)Cr(H_2O)_3$ has a net charge of 5+. The complex was bound to the cation-exchange resin more strongly than the similarly charged $Cr(H_2O)_6^{3+}$ ion due to hydrophobic interactions similar to those postulated for $Cr(nic)_2(H_2O)_4^{3+}$ and the greater charge.

The gel filtration chromatography of the blue solution obtained in the preparation of the mononicotinate complex succeeded in seperating three coloured fractions. The purple-violet fraction was eluted just before the salt peak with a chromium to nicotinic acid ratio of 1:2 and this fraction probably consisted of unreacted $Cr(H_2O)_6^{3+}$ (which was responsible for

the violet colour) and nicotinic acid (which was found to be eluted before the bed volume) - the similar elution positions being expected on the basis of their similar molecular weights (160 and 123 respectively).

FIGURE 2.17: Reaction scheme for dimeric chromium (III)-nicotinic acid complex.



The two blue bands which were eluted from the gel column after the salt fraction had obviously interacted with the column in some way and must have been chromium-nicotinic acid complexes due to their blue colour and the demonstrated presence of nicotinic acid. Although both bands overlapped significantly it was possible to conclude that the first band was $Cr(nic)(H_2O)_5^{2+}$ because the ratio of M/L was slightly greater than one at the beginning of the broad blue band and the second component was $Cr(nic)_2(H_2O)_4^{3+}$ because the metal to ligand ratio was less than one. This would result if the first band with a metal to ligand ratio of 1:1 overlapped a second band with a metal to ligand ratio of 1:2 as shown in Figure 2.18 and it was reasonable to assume that if the mono-nicotinic acid complex interacted with the resin while nicotinic acid did not, then the di-nicotinic acid complex would interact more and be eluted last because it contains a higher proportion of the gel-interactive group (most probably the pyridine structure).

The absorption spectrum of the final eluted fractions from the gel filtration column was similar to the $Cr(nic)_2(H_2O)_4^{3+}$ species which would account for very tight binding of some of the cationic chromium (III) complex mixture to the cation-exchange resin. The ultra-violet spectrum of the species was also very similar to the $Cr(nic)_2(H_2O)_4^{3+}$ species therefore both the mono- and di-nicotinate chromium (III) complexes were obtained during the preparation of the mononicotinate complex.

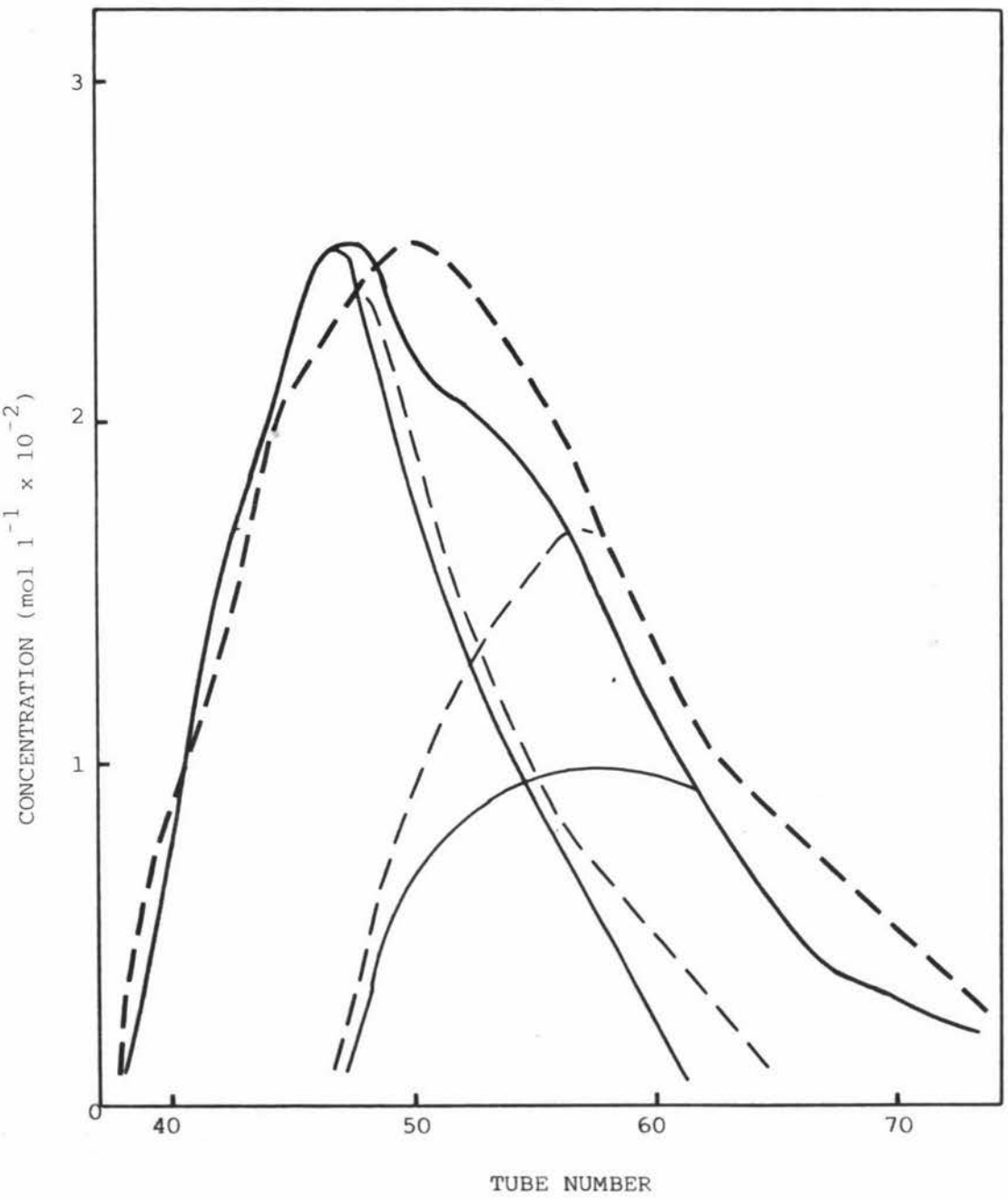
However during the preparation procedure the pH was always less than 4.0 which meant the blue solid $Cr(nic)_2(H_2O)_3OH$ was not able to precipitate due to protonation of the complex species and thus the chromium-dinicotinate complex was soluble.

The reason why the complex species interacted with the gel matrix while nicotinic acid alone did not was probably due to hydrophobic interactions of the complex species with the Sephadex resin. It is also possible to distinguish

FIGURE 2.18:

Predicted overlap of the elution profiles of the blue coloured $Cr(nic)_2(H_2O)_4^{3+}$ and $Cr(nic)(H_2O)_5^{2+}$ species on a Sephadex G15 column (2.0cm x 60.0cm) (compare with Figure 2.10). Total chromium (III) (———) and total nicotinic acid (------). Overlapping chromium (III) (———) and overlapping nicotinic acid (------).

FIGURE 2.18



between pure adsorptivity which is based on the structure of the substance and the superimposed effects such as ionic strength and pH of the eluent and the effect of carboxylic acid groups (Janson 1967). In the cases of the chromium-nicotinic acid complexes, although charged, they possess very little acidic or basic properties so they are not affected by changes in ionic strength or pH to any great extent. However the nicotinic acid molecule contains a readily ionisable group, the carboxylate group, which due to its charge would tend to be surrounded by water of hydration which would prevent much of the interaction of the molecule with the gel resin from occurring and due to the smaller size of the molecule compared with the complexes, may account for the elution of nicotinic acid before the salt peak as would have been expected.

2.5.6 Comparisons with Literature

Complexes with coordination of nicotinic acid ligands through the carboxylate group are less well known than complexes in which the pyridine ring nitrogen atom is coordinated. Chiacchierini et al (1977) studied the reaction of chromium (III) with nicotinic and isonicotinic acids and similar reactions with picolinic acids were studied by Campanella et al (1977). The results obtained indicated that Cr(III) was coordinated to nicotinic and isonicotinic acids through their carboxylate group. Chiacchierini et al found the absorption spectra of a blue chromium-nicotinate solution (λ_{max} 410nm and 568nm) correspond to Cr(III) compounds with O-M bonds which suggested that they had succeeded in preparing the $Cr(nic)(H_2O)_5^{2+}$ species only and not the $Cr(nic)_2(H_2O)_4^{3+}$ species as in this thesis. They also found ε_{max} values of the d-d bonds less than 40 l mol^{-1} cm^{-1} consistent with octahedral complexes and the acidity of the solutions of metal ion and ligands increased after the reaction which must occur if the carboxylate group is coordinated to the chromium atom resulting in the release of a proton. In this study at pH's less than 4.0 soluble, blue-coloured

species were obtained which contained $Cr(nic)(H_2O)_5^{2+}$ and $Cr(nic)_2(_2O)_4^{3+}$, however Chiacchierini et al applied Jobs method of equimolar solutions[1] and the straight line method[2] to their solutions at pH 3.0 and fitted the data to only one species with a molar ratio of chromium to nicotinic acid of 1:1. Although at higher pH values, pH 4.5 - 4.8, Chiacchierini et al found gray amorphous solutions and elemental analysis suggested a formula corresponding to $Cr(nic)_2(H_2O)_3OH$, they failed to distinguish the $Cr(nic)_2(H_2O)_4^{3+}$ species (also blue coloured) which was stable in acidic aqueous solution and formed under similar conditions in this study. The positions of the d-d bands of the di-nicotinate complexes prepared during this work were consistent with the electronic spectrum recorded by Chiacchierini et al for the gray solid $Cr(nic)_2(H_2O)_3OH$ (425nm and 575 nm).

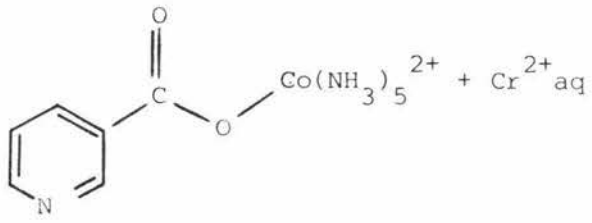
The mono-nicotinic acid complex was also obtained when cobalt (III)-pentaamine-3-pyridine carboxylic acid was reduced with $Cr(H_2O)_6^{2+}$ (Gould and Taube 1964). The reaction scheme proposed involved the formation of a nitrogen-coordinated Cr(III) product which rapidly re-equilibrated to the more

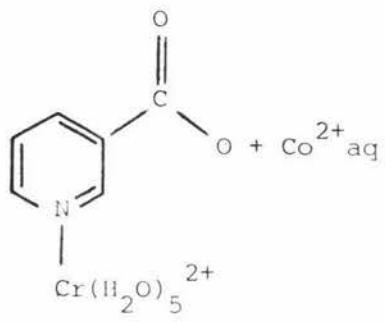
Footnotes:

1. In Jobs method of equimolar solutions, the change in absorbance ΔA of the complex M_mL_n is plotted against $\frac{C_m}{C_m + C_L}$ where C_m is the concentration of metal ions and C_L is the ligand concentration. $\Delta A \propto C_{mL}$ the concentration of M_mL_n. If m = n = 1, then ΔA has a maximum at $\frac{C_m}{C_m + C_L}$ = 0.5 and minimums when either C_m or C_L equal zero (Angelici 1969).

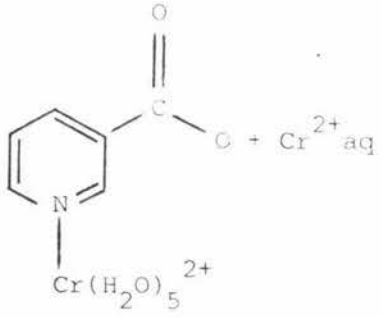
2. In the straight-line method take a series of flasks of volume V and add constant V_o of solution M and varying U of solution L. Measure the absorbance A at a suitable wavelength. For the equilibrium mM + nL = MmLn, when m = 1 and n = 1, $\frac{1}{V^n} \propto \frac{1}{A}$ therefore a plot of $1/V^n$ versus 1/A will be a straight line (Klausen and Langmyhr 1963).

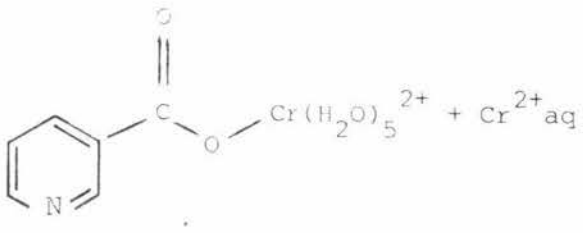
stable carboxylate-coordinated complex.





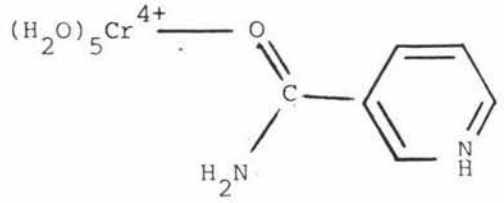
In the intermediate complex the Cr-N bond does not survive but electron transfer through the pyridine-carboxylate system results in the formation of a more stable Cr-O bond by the reaction with unreacted Cr(II).





The blue coloured product $Cr(nic)(H_2O)_5^{2+}$ had absorption maxima at 410nm and 577nm which were similar to the values obtained in both this study and that of Chiaccherini which suggested that the mono-nicotinic acid complex was very easily prepared and quite stable.

The chromous ion reaction was also used by Nordmeyer and Taube (1966) who found the reaction of Cobalt (III)-penta-ammine-nicotinamide with Cr(II) produced a blue solution with the colour being assigned to a complex with the following structure:



The infra-red spectra of metal complexes with nicotinic acid have not been studied to any great degree by other workers. The coordination of nicotinic acid to Ag(II) and Cu(II) in the formation of $M(nic)_2(H_2O)_2$ complexes was postulated to be through the carboxylate group (Fowless et al 1968), due to shifts in the infra-red spectrum of the asymmetric carbonyl stretching vibration to lower frequencies in a similar fashion to the infra-red spectral shifts observed in the blue solid $Cr(nic)_2(H_2O)_3OH$. However contradictory evidence was put forward by Kleinstein and Webb (1971), with the series $M(nic)_2$ where M = Mn(II), Co(II), Ni(II), Zn(II), Cu(II) and Ag(II), who proposed coordination of the pyridine ring nitrogen atom. It could have been that the hydrated form was similar to the blue $Cr(nic)_2(H_2O)_3OH$ complex with carboxylate coordination, but in the $M(nic)_2$ complex a polymeric form existed with both types of coordination present leading to different infra-red spectra.

2.6 CONCLUSION

Many complexes of pyridine carboxylic acid ligands such as nicotinic acid, with transition metals are known. In the case of nicotinic acid there are two possible groups suitable for coordination to the metal ion, (1) the carboxylate group and (2) the pyridine ring nitrogen atom. In the majority of complexes coordination to the metal ion is via the pyridine ring as is seen with the pyridine ligand. Transition metal atoms found to have this type of coordination prepared and studied here were Mn(II), Ni(II), Co(II) and Cr(II). The complexes formed in all cases were found to be octahedral with the coordination of two nicotinic acid ligands trans to one another. All complexes were also insoluble in all common solvents due to hydrogen-bonding

within the crystalline structure. No soluble species were isolated, and dissolving the complexes in dilute acid or base led to destruction of the complex especially with the yellow $Cr(nic)_2(H_2O)_3OH$ compound which was stable in its solid form, but in acid or base solution the Cr-N bond was unstable and consequently coordination of the nicotinic acid was lost.

The only transition metal found to have coordination of the nicotinic acid through the carboxylate group was Cr(III). The soluble species were found to be $Cr(nic)(H_2O)_5^{2+}$ and $Cr(nic)_2(H_2O)_4^{3+}$ while the insoluble form was $Cr(nic)_2(H_2O)_3OH$ and all were blue in colour. In the di-nicotinic acid complex the nicotinic acid ligands were found to occupy positions trans to one another, the complex being octahedral in structure.

The preference of either pyridine ring nitrogen or carboxylate coordination and the stability of such metal complexes seemed to follow certain trends. The theory of Hard and Soft Acids and Bases, HSAB (Huheey 1978), was found to be useful in the prediction of such trends with transition metal ions. In the HSAB theory metal ions and ligands are classified as belonging to type (a) or (b) according to their preferential bonding. Class (a) or hard acids include the lighter transition metals in higher oxidation states such as Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{3+} and H^+. Class (b) or soft acids include the heavier metals and those in lower oxidation states such as Cu^+, Ag^+, Pd^{2+}, Pt^{2+} and Hg^{2+}. Borderline acids include Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. Hard bases include RNH_2, H_2O, OH^-, ROH and CH_3COO^- while borderline bases include $C_6H_5NH_2$ and pyridine and soft bases include RS^-. The stability of most complexes can be summarised as follows:

Tendency to complex with class (a) type metal ions	Tendency to complex with class (b) type metal ions
N >> O >> S	N << O << S

A simple rule suggested by Pearson (1963) for predicting such stabilities of complexes formed between acids and bases

is: "Hard acids prefer to bind to hard bases and soft acids prefer to bind to soft bases". However such predictions are always subject to exceptions.

With reference to the HSAB theory it would be predicted that Cr^{3+}, Mn^{2+}, Fe^{3+} and Co^{3+} would prefer a hard base such as a carboxylate group in preference to a pyridine group and in support of the prediction it was observed that Cr(III) formed an oxygen coordinated nicotinic acid complex very easily which was stable under acid conditions. The Mn(II) nicotinic acid complex was shown to have coordination through the pyridine nitrogen atom in preference to the carboxylate group. The main difficulty with Fe(III), even though it has a greater affinity for oxygen ligands (Cotton and Wilkinson 1972i), was that the extent of hydrolysis was very great even under very acidic pH conditions. Therefore it was very difficult to prepare a stable nicotinic acid complex without concurrent olation occurring. Co(III) was known to show a particular affinity for nitrogen donors (Cotton and Wilkinson 1972j) but no stable nicotinic acid complex was formed in aqueous solution in this work.

Those transition metal ions that prefer a soft base such as pyridine are predicted to be Fe^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}. The Co(II) and Ni(II) complexes formed were consistent with the form $M(nic)_2(H_2O)_4$ in an octahedral structure with trans coordinated nicotinic acid ligands via the pyridine ring group. No stable Fe(II) complex was isolated in this study and Cu(II) was thought to form a square planar $Cu(nic)_2(H_2O)_2$ complex with coordination through the carboxylate group. Cr^{2+} could be classed as a borderline or soft base due to its low oxidation state, thus its preference would be for coordination to the pyridine group. The reaction of Cr(II) with nicotinic acid in fact formed the yellow $Cr(nic)_2(H_2O)_3OH$ which was shown to be coordinated through the pyridine ring and was structurally very similar to $Co(nic)_2(H_2O)_4$. It was apparent that Cr^{2+} preferred nitrogen ligands and Cr^{3+} oxygen ligands, thus the behaviour of Cr^{2+} was similar to the divalent metals Co^{2+}, Ni^{2+} and Mn(II). However, the Cr^{2+}

complex is unstable in solution and rapidly oxidises to the Cr(III) complex which is the form in which it is actually isolated. The coordinated pyridine groups remain unchanged during oxidation.

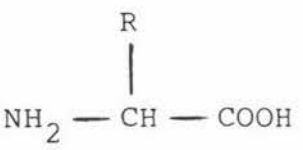
Chromium (III) seemed to differ from most other transition metals because of its ability to form carboxylate coordinated nicotinic acid complexes. The mono- and di-nicotinic acid Cr(III) complexes were the only stable soluble nicotinic acid complexes found amongst many other transition metal complexes. This seemed to be a property of Cr(III) metal ions exclusively, and thus sets Cr(III) apart as possessing inorganic chemistry different from most other first row transition metals.

SECTION THREE

AMINO ACID COMPLEXES OF CHROMIUM (III)

3.1 INTRODUCTION

Amino acids tend to complex readily with transition metal ions and many characterised complexes are already known for chromium (III) which have an octahedral structure. All amino acids have a general structure of the form:



where R may be a variety of functional groups or simply -H. Thus amino acids possess two and sometimes three suitable groups for coordination to the metal ion; these being the α-amino group, the α-carboxylate group and the functional group attached to the sidechain, which may be an amine group, a carboxylate group, a sulphydryl group or others.

In this study the structures of several amino acid complexes were characterised including glycine (-H sidechain), cysteine($-CH_2-SH$), glutamine ($-CH_2-CH_2-CONH_2$) and glutamic acid ($-CH_2-CH_2-COOH$). In this way it was hoped to study the effect of the sidechain group on the type of complexes observed.

Soluble amino acid complexes with chromium (III) are little known possibly due to the ease with which chromium (III) olates by the addition of hydroxide ions into the coordination sphere and results in the formation of insoluble dimers and hydroxy-compounds. The only soluble complexes known are $Cr(gly)_2(H_2O)_2 \cdot NO_3$, $Cr(lys)_2(H_2O)_2 \cdot Cl$ (Mizuochi et al 1971) and $NaCr(cys)_2 \cdot 2H_2O$ (Hodgson et al 1977) which have been fully characterised, the complexes with other amino-acids are either insoluble or not easily isolated.

The glucose tolerance factor, GTF, proposed by Mertz (1967) and others supposedly contains chromium (III) as the active subtsance and although the exact structure is unknown, GTF is thought to contain nicotinic acid and the amino acids glycine glutamic acid and cysteine most probably in the form of an octahedral chromium (III) complex. In Section 2, nicotinic acid complexes with chromium (III) were studied in an attempt to prepare a compound that acted in a similar fashion to the naturally occurring GTF. If amino acids are also constituents of GTF, a study of the complexation of those amino acids is important to an understanding of the possible structure of GTF. To this end the soluble chromium (III) complexes with amino acids were studied and the relationship between the structure of the complex and its biological activity investigated. It might then be possible to predict the type of amino-acid ligands that could stabiise the $Cr(nic)_2(H_2O)_4^{3+}$ structure in neutral solution and result in a stable active synthetic GTF-like complex.

3.2 METHODS AND MATERIALS

3.2.1 Sources of Chemicals

The chromium salts: $CrCl_3 \cdot 6H_2O$, $Cr(NO_3)_3 \cdot 9H_2O$ and $CrCl_3$ were obtained from BDH Chemicals Ltd. The amino acids: glycine, glutamic acid (sodium salt), cysteine·HCl, and glutamine were obtained from Prolabo, BDH Chemicals Ltd, BDH Chemicals Ltd, and Pierce Chemical Company, respectively. All other chemicals were A.R. grade.

3.2.2 Experimental Methods and Instrumentation

3.2.2.1 Cation-exchange chromatography

The cation-exchange resin used was Dowex 50W-X12 in the hydrogen ion form and was recycled and prepared as discussed in Section 2. The resin was packed into a column measuring

3.0 cm x 10.0cm and washed with H_2O until pH of the effluent reached 7.0. All samples to be loaded were titrated to pH 3.5 and diluted to a conductivity of 3,000 µmhos, or less, unless otherwise stated, to ensure optimum binding of the samples.

3.2.2.2 Anion-exchange chromatography

The anion-exchange resin used was Dowex 1-X8 in the chloride ion form and was recycled and prepared by mixing with two volumes of HCl (2.5M) for one-half hour followed by two washes with H_2O, and this process was repeated again. The resin was packed into a column measuring 3.0 cm x 10.0 cm and washed with H_2O until the pH of the effluent reached neutral. All samples to be loaded were titrated to pH 8.5 and diluted to a conductivity of 3,000 µmhos, or less, unless otherwise stated; to ensure optimum binding.

3.2.2.3 Gel filtration chromatography

The gel filtration resin used was Sephadex G15 which was prepared according to the manufacturers recommendations. The resin was packed in a column measuring 2.0 cm x 60.0 cm and water was used as the solvent in all gel filtration chromatography. The solutions to be chromatographed were freeze-dried and dissolved in a minimum amount of water and a sample volume of less than 5.0cm^3 applied to the column and eluted with water.

3.2.2.4 Electronic spectroscopy

Electronic spectra were recorded on a Shimadzu MPS 5000 spectro-photometer.

3.2.2.5 Absorbance, pH and conductivity measurement

These measurements were carried out as outlined in Section 2.3.2.6.

3.2.2.6 Chromium determination

Aqueous chromium containing solutions were analysed as in Section 2.3.2.2.

3.2.2.7 Micro analysis

Micro analysis for the elements carbon, hydrogen and nitrogen were kindly carried out by the Department of Chemistry at Otago University.

3.3 RESULTS

3.3.1 Chromium (III) Complexes with Glycine

3.3.1.1 Preparation of glycine complexes

The tris-glycinato complex $Cr(gly)_3 \cdot H_2O$ was prepared using a method similar to Hodgson et al (1973). $CrCl_3 \cdot 6H_2O$ (6.66g, 0.025 moles) and glycine (5.60g, 0.075 moles) were heated in H_2O ($30cm^3$) and NaOH (3.0g, 0.075 moles) dissolved in H_2O ($10cm^3$) was added dropwise. The green Cr^{3+} coloured solution changed to pink after 30 minutes continuous heating with stirring and a green precipitate was removed by filtration after adding three volumes of ethanol. The ethanol was removed by rotary evaporation at 35°C and the resultant red-purple solution cooled to 4°C. After 7 days bright red crystals formed which were filtered and washed with ethanol and which analysed as $Cr(gly)_3 \cdot H_2O$ (Table 3.1).

TABLE 3.1: ANALYTICAL DATA FOR AMINO ACID COMPLEXES

Compound	Observed (%)			Calculated (%)		
	C	H	N	C	H	N
$Cr(gly)_3 \cdot H_2O$	24.56	5.08	14.32	24.67	4.83	14.38
$[Cr(gly)_2OH]_2$	20.21	4.17	11.70	21.24	4.42	12.39
$NaCr(cys)_2 \cdot 2H_2O$	21.58	4.55	7.92	20.63	4.04	8.05

The bis-hydroxy dimer $[Cr(gly)_2OH]_2$ was prepared using a method similar to Bryan et al (1971). $CrCl_3 \cdot 6H_2O$ (6.66g, 0.0025 moles), glycine (5.60g, 0.075 moles) and NaOH (3.0g, 0.075 moles) were refluxed for 12 hours in H_2O (50cm^3). The pink precipitate which formed was filtered and washed with ethanol and acetone, and the elemental analysis figures were consistent with the structure $[Cr(gly)_2OH]_2$ (see Table 3.1).

A red solution was obtained during the preparation of $Cr(gly)_3 \cdot H_2O$ above which was apparently stable in acidic solution but above pH 7.0 a pink insoluble powder precipitated which was also analysed as $[Cr(gly)_2OH]_2$. Attempts to obtain crystals from the red solution were unsuccessful.

Alternatively, when a chromous solution[1] (0.0024 moles, 30cm^3) was added to glycine (0.54g, 0.0072 moles) in H_2O (10 cm^3) at pH 10.0, a red solution was formed immediately which was similar in colour to the red solution above.

A blue solution was obtained under acidic conditions (pH less than 3.0) when $CrCl_3 \cdot 6H_2O$ (6.66g, 0.0025 moles) and glycine (5.60g, 0.075 moles) were dissolved in H_2O (30 cm^3). The pH of the solution was adjusted to 2.35 and solution heated to boiling. NaOH (2M) was then added drop-wise until the solution turned blue in colour. The pH of the solution remained constant at 2.35 and on cooling gave a dark blue solution.

3.3.1.2 Ion exchange chromatography of glycine complexes

A cation-exchange resin was used to facilitate the possible separation of the soluble cationic glycine complexes with chromium. After loading the column was washed with a NaCl

Footnote 1: As prepared in Section 2

solution (0.1M, 5,000 μmhos conductivity) to remove all traces of glycine and initially the conductivity increased to approximately 15,000 μmhos but the washing continued until the conductivity returned to 5,000 μmhos.

The red Cr(III)-glycine complex was adsorbed onto the cation-exchange resin and eluted with a 0.5M NaCl solution (20,000 μmhos conductivity) after which any impurity of $Cr(H_2O)_6^{3+}$ was eluted with 1.0M NaCl (30,000 μmhos. No other species were eluted with higher concentrations of NaCl.

When either $Cr(gly)_3 \cdot H_2O$ or $[Cr(gly)_2OH]_2$ were dissolved in dilute acid (2M HNO_3), the resulting red solution was adsorbed onto the cation-exchange resin and eluted with 0.5M NaCl. The red solution formed in the reaction of glycine with chromous ions was also adsorbed onto the cation-exchange resin and a red species was eluted with 0.5M NaCl, followed by elution of $Cr(H_2O)_6^{3+}$ with 1.0M NaCl. The similar elution conditions and colour of all the adsorbed chromium-glycine complexes suggested that they were identical species.

The blue Cr(III)-glycine solution was adsorbed onto the cation-exchange resin, but upon elution with NaCl solution, only one unresolved chromium peak was eluted at a concentration of 1.0M NaCl. The separation of the individual species from each other and from $Cr(H_2O)_6^{3+}$ was not possible using this resin even when other salts such as $NaNO_3$ or $KClO_4$ were tried with conductivity gradients from 0.1M to 1.0M concentrations.

The elution of the red Cr(III)-glycine complex from the cation exchange resin with 0.5M NH_4OH was attempted, but the eluted material was now blue in colour (at pH 9.3) and was stable for no more than 24 hours. After this period of time a slight precipitate appeared and the solution turned green in colour and this change was accelerated by moderate heating. The addition of dilute acid (2M HNO_3) to the blue eluted material turned the colour to red-purple similar to the original solution which was loaded onto the resin. No reaction was observed

when the red chromium (III)-glycine solution was titrated to pH 9.5 with NH_4OH (0.5M) but a green solution developed on heating the solution moderately.

3.3.1.3 Electronic spectra

The electronic spectra of the solid complexes $Cr(gly)_3 \cdot H_2O$ and $[Cr(gly)_2OH]_2$ were recorded in a nujol mull due to the great insolubility of these compounds in all common solvents tried. In the tris-type complex, $Cr(gly)_3.H_2O$, the two bands characteristic of Cr(III) complexes were found at 388nm and 510nm, and in the compound $[Cr(gly)_2OH]_2$ at 390nm and 535nm, both examples being consistent with octahedral coordination of the Cr(III) metal ion. The electronic spectra of all the glycine complexes prepared are summarised in Table 3.2. The d-d bands appeared to be broad and unsplit, and the intensity of the first band was seen to be less than that of the second band in all of the glycine complexes.

The red chromium (III)-glycine solution exhibited two bands in the visible region at 398nm and 535nm and identical spectra were recorded for the red solutions which were formed when $Cr(gly)_3 \cdot H_2O$ or $[Cr(gly)_2OH]_2$ were dissolved in dilute acid (2M HNO_3) or when glycine was reacted with chromous ions. All d-d bands were broad and unsplit with the intensity of the first band less than the second.

The blue Cr(III)-glycine solution had two d-d bands in the visible region at 420nm and 570nm at pH 4.0 with the intensity of the first band being less than the second band. As the solution was fitrated to greater than pH = 7.0 the solution turned green and a gelatinous precipitate formed and the d-d bands were now shifted to 430nm and 580nm similar to those normally found for Cr^{3+} in basic solution.

When the red Cr(III)-glycine complex was adsorbed onto the cation exchange resin (Dowex 50W-X12) and eluted with NH_4OH (0.5M), the colour of the eluted solution was blue at pH 9.3

TABLE 3.2: ELECTRONIC SPECTRA OF GLYCINE COMPLEXES

Complex	Colour	Absorption Maxima (nm)	Physical State
$Cr(gly)_2(H_2O)_2^+$	red	398, 535	aqueous solution
$Cr(gly)_3 \cdot H_2O$	red	388, 510	solid (Nujol mull)
$[Cr(gly)_2OH]_2$	pink	390, 535	solid (Nujol mull)
$Cr(gly)_n(H_2O)_{6-n}^{3+}$ (a)	blue	420, 570	aqueous solution
$Cr(gly)_2(H_2O)_4^{3+}$ (b)	blue	405, 565	aqueous solution
$Cr(gly)_2(H_2O)_2^+$ (c)	red	398, 533	aqueous solution

(a) mixture of species, where n = 1, 2, 3, 4, 5, 6

(b) eluted from cation-exchange column with 0.5M NH_4OH

(c) derived from Cr^{2+} reaction

in contrast to the red solution which was loaded. The electronic spectrum of the blue solution exhibited two bands at 405nm and 565nm, a shift towards higher wavelenghts when compared with the original solution. When a solution of $Cr(gly)_2(H_2O)_2^+$ was titrated to pH 9.5 with ammonium hydroxide the positions of the d-d bands remained uncharged at 398nm and 535nm. However this complex was considered to be very unstable because addition of dilute acid (2M HNO_3) turned the solution red with maxima at 398 and 535nm similar to the original solution.

3.3.2 Chromium (III) Complexes with Cysteine

3.3.2.1 Preparation of cysteine complexes

The complex $NaCr(cys)_2 \cdot 2H_2O$ was prepared using a method similar to that reported by Hodgson et al (1977). $Cr(NO_3)_3 \cdot 9H_2O$

(1.00g, 0.0025 moles) and cysteine .HCl (1.18g, 0.0075 moles) were boiled in the H_2O (30cm³) and solid NaOH was added until the colour of the solution changed from red-violet to blue, at which stage the pH was 7.0. The hot solution was filtered and on cooling dark blue crystals formed which were filtered and washed with ethanol and diethyl ether and subsequently analysed as $NaCr(cys)_2 \cdot 2H_2O$. The terdentate cysteine complex $NaCr(cys)_2 \cdot 2H_2O$ dissolved readily in water to form a dark blue solution with a pH of 7.0, however, on the addition of dilute acid (2M HNO_3) the dark solution changed to red at pH 4.0, but no solid could be crystallised. Alternatively, the addition of dilute base (2M NaOH) to the blue solution changed the colour to green when the pH reached 8.5. The acid-base colour changes were reversible with the blue form intermediate between the red and green forms.

3.3.2.2 Ion exhange chromatography of cysteine complexes

The solid complex $NaCr(cys)_2 \cdot H_2O$ was dissolved in H_2O and titrated to pH 3.5 whereupon the blue solution rapidly changed colour to red. This red coloured solution was easily bound to the cation-exchange resin and was then eluted with NaCl solution (0.5M, 20,000 μmho conductivity) confirming the species to be cationic in nature. Further elution with salt concentrations up to 5.0M NaCl and pH gradients from pH 3.5 to pH 12.0 with phosphate buffers set up in situ using the resins natural acidity (firstly 0.05M Na_2HPO_4 until the pH reached 9.0 then 0.05M Na_3PO_4 until the pH reached 12.0) was unsuccessful in eluting any other fractions. Therefore it was assumed that only one species was present.

When $NaCr(cys)_2 \cdot 2H_2O$ was titrated to pH 8.5 with NaOH prior to loading onto the anion-exchange resin, the blue solution changed colour to a green solution which bound well to the resin. Elution was carried out with T.R.I.S. - $NaNO_3$ buffer (0.19M TRIS at pH 9.0, conductivity 10,000 μmho), the sole fraction eluted being green in colour, whilst further elution with higher salt concentrations and pH gradients down to pH 3.5 (with 0.5M acetic acid) failed to elute any other species.

It was observed that the green solution at a pH of 8.5 was unstable and gradually over one week changed back to the blue solution, but when left at a pH greater than 9.0 the solution remained green.

3.3.2.3 Electronic spectra of cysteine complexes

The electronic-visible spectrum of $NaCr(cys)_2 \cdot 2H_2O$ was recorded in both aqueous solution and solid state using a nujol mulling agent. Both spectra were identical but the results were considered unusual due to the presence of two pairs of bands rather than the single pair as seen for Cr(III) complexes. The first pair of d-d bands appeared at 413nm and 555nm and the second pair at approximately 450nm and 615nm (see Figure 3.1). In aqueous solution the positions of the bands were independent of pH but the relative intensities of the bands were found to be very dependent on the pH. The electronic spectra of the soluble and insoluble cysteine complexes is summarised in Table 3.3.

TABLE 3.3: ELECTRONIC SPECTRA OF CYSTEINE COMPLEXES

Complex	Colour	Absorption maxima (nm)		Physical state
		1st pair	2nd pair	
$NaCr(cys)_2 \cdot 2H_2O$	blue	413, 555	≈450, 615	Solid (Nujol mull)
$NaCr(cys)_2 \cdot 2H_2O$	blue	413, 555	≈450, 615	aqueous solution; pH 7.0
$Cr(cys)_2(H_2O)_2^+$	red	410, 550	- -	aqueous solution; pH 3.5
$Cr(cys)_2^-$	green	410, 550	≈450, 615 vi	aqueous solution; pH 8.5

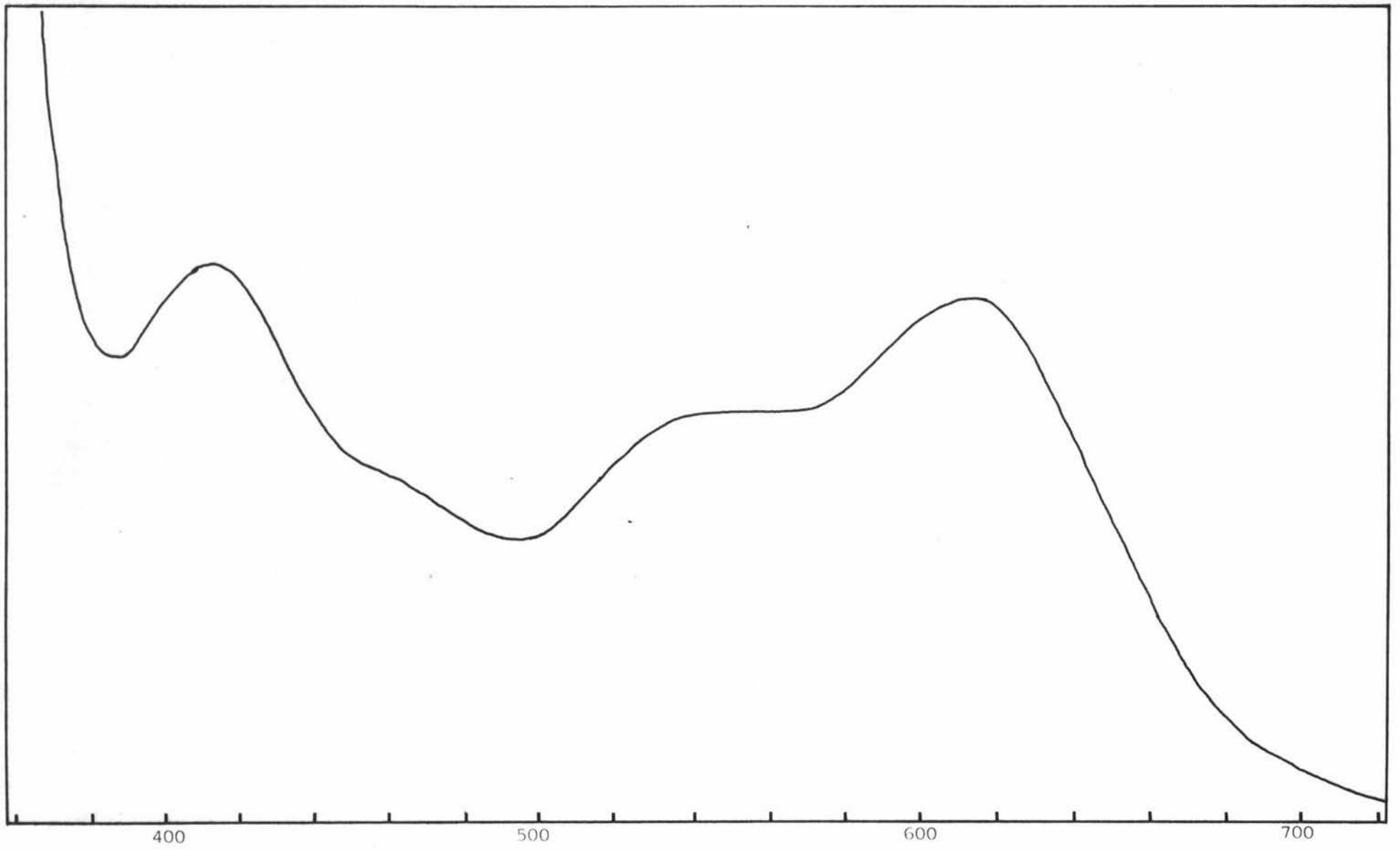
vi = very intense

In the red species the first pair of bands appeared at 410nm and 550nm but no second pair of bands were observed as long as the pH of the solution was kept at pH 3.5. Above pH 4.0 however the blue coloured species reappeared.

FIGURE 3.1:

Electronic spectrum of $NaCr(cys)_2.2H_2O$ in aqueous solution at pH 7.0 and the solid state transittance spectrum using a nujol mull.

FIGURE 3.1



The green species possessed the two pairs of bands similar to $NaCr(cys)_2 \cdot 2H_2O$ at 410,550nm and 450,615nm as long as the pH was kept at pH 8.5 to minimise formation of the blue species at the more neutral pH's or the process of olation at extremely basic pH. The intensity of the 615nm band was much greater in the green species at pH 8.5 than for the blue species pH 7.0.

A blue species at pH 7.0 derived from $NaCr(cys)_2 \cdot 2H_2O$ dissolved in H_2O, was titrated with either dilute acid (2M HNO_3) or dilute base (2M NaOH) and the visible absorption spectrum recorded at various pH values. A series of visible spectra at varying pH values is shown in Figure 3.2. As the pH was decreased from pH 7.0 to pH 3.5 the intensity of the bands at 615 and 450nm decreased to zero and the band at 413nm decreased slightly while the band at 555nm increased. These variations of extinction coefficients as a function of pH are shown in Figure 3.3.

It was decided to follow the variation in intensity with change in pH for the 413nm and 615nm bands to determine the pk value associated with the two different pairs of d-d bands to determine which ligands were responsible for these bands. Analysis of the titration curve for the 413nm band gave a pk value at approximately 4.5 and the 615nm band had a pk value of approximately 10.9. The pk of 4.5 corresponded with a visual colour change from blue to red, and at pk = 10.9 a colour change from blue to green.

3.3.2.4 Titration of $NaCr(cys)_2 \cdot 2H_2O$

A sample of $NaCr(cys)_2 \cdot 2H_2O$ was dissolved in the H_2O, and divided into two parts; one part being titrated with dilute acid (0.25M H_2SO_4) and the other with dilute base (0.5M NaOH). The pH of the system versus the amount of acid or base added was recorded and the results were combined and graphed as shown in Figure 3.4(a). Only one isoelectric point was observed for the system at pH 7.0, which correspond with the transition between the red cationic species and the green

FIGURE 3.2(a):

Series of electronic spectra obtained when an aqueous solution of $NaCr(cys)_2.2H_2O$ at pH 7.0 was titrated with dilute acid (2M HNO_3). The numbered curves indicate pH 7.0 (1), pH 5.1 (2), pH 4.6 (3), pH 4.3 (4), and pH 3.4 (5).

FIGURE 3.2(a)

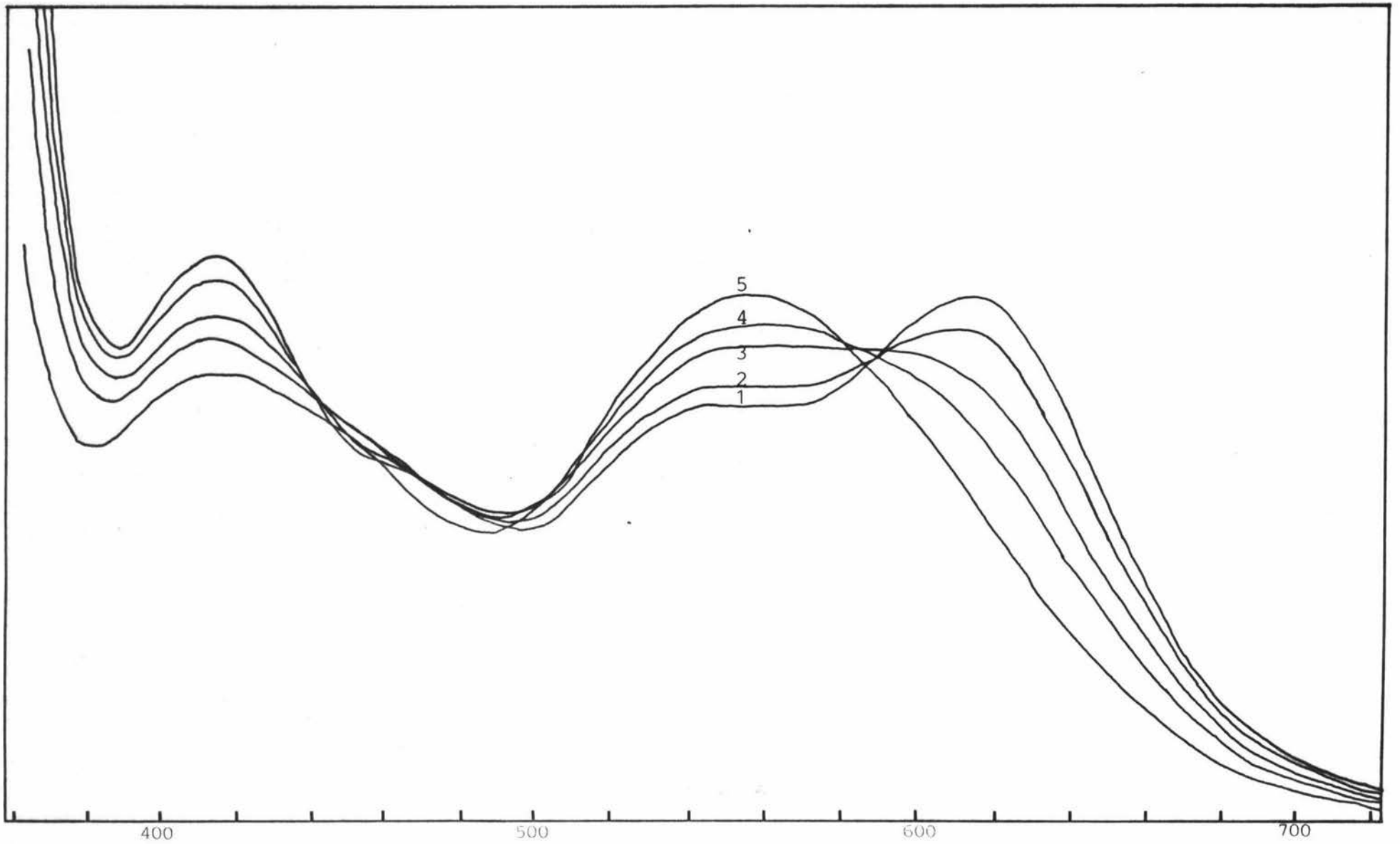


FIGURE 3.2(b):

Series of electronic spectra obtained when an aqueous solution of $NaCr(cys)_2.2H_2O$ at pH 7.0 was titrated with dilute base (2M NaOH). The numbered curves indicate pH 7.0 (1), pH 8.9 (2), pH 9.8 (3), pH 10.4 (4), and pH 11.1 (5).

FIGURE 3.2(b)

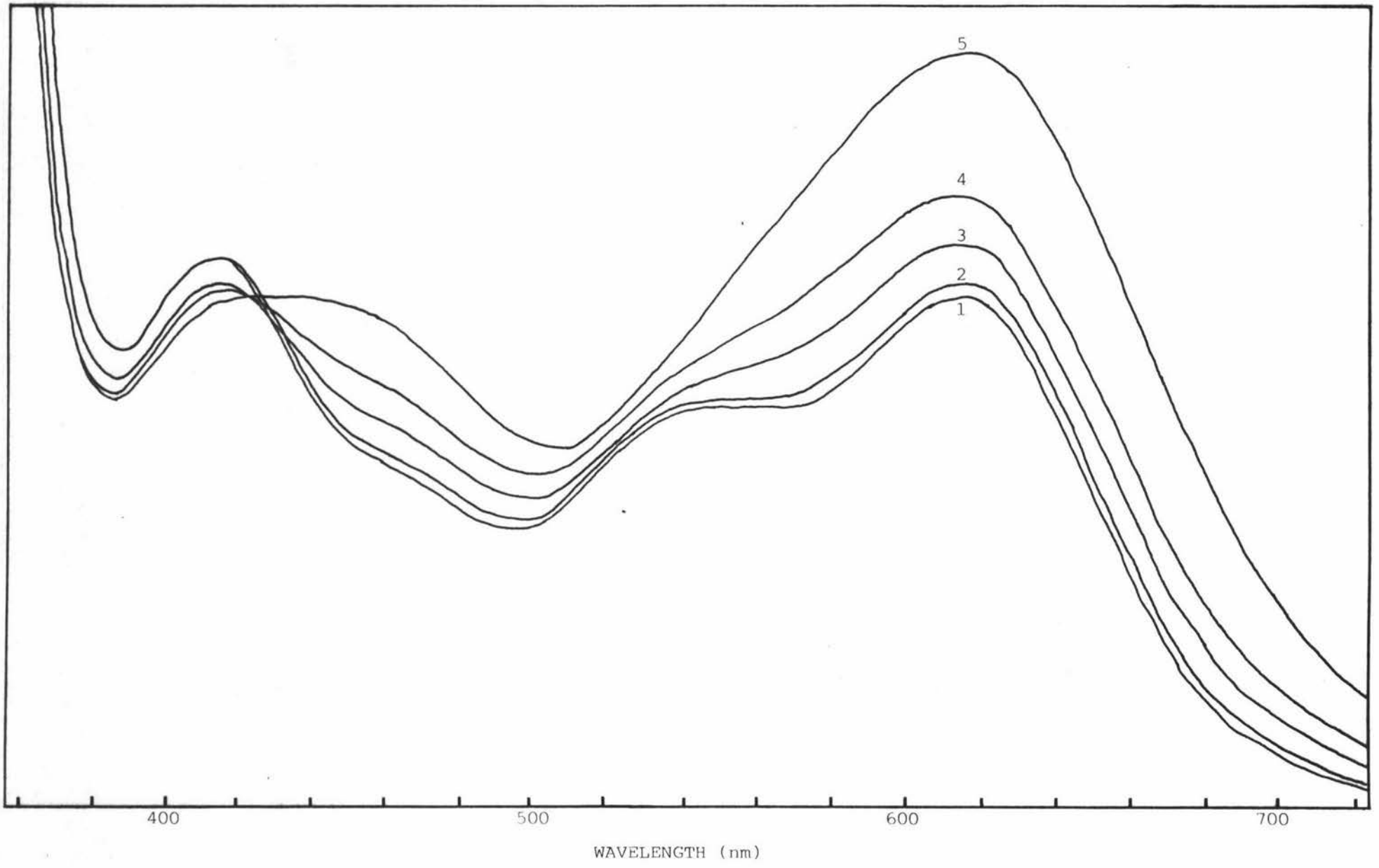


FIGURE 3.3:

Variation in intensity of the d-d bands in the visible spectrum of an aqueous solution of $NaCr(cys)_2.2H_2O$ with pH. The absorbance values were derived from Figure 3.2(a) and (b) and were followed for the wavelengths 413nm, ≃450nm, 555nm and 615nm.

FIGURE 3.3

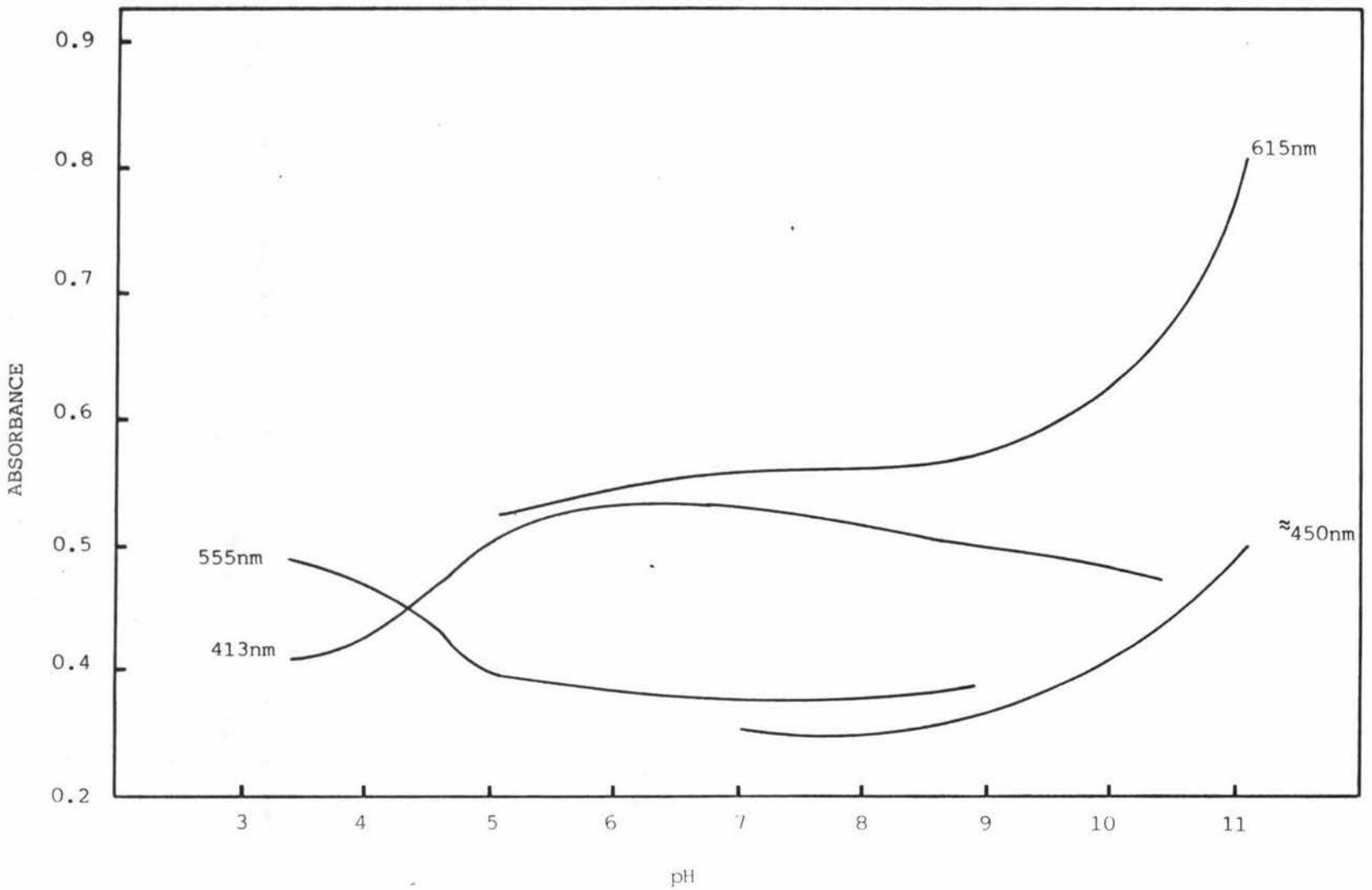

0.9
0.8
0.7
0.6
0.5
0.4
0.2
ABSORBANCE
615nm
555nm
413nm
≈450nm
3
4
5
6
7
8
9
10
11
pH

FIGURE 3.4(a):

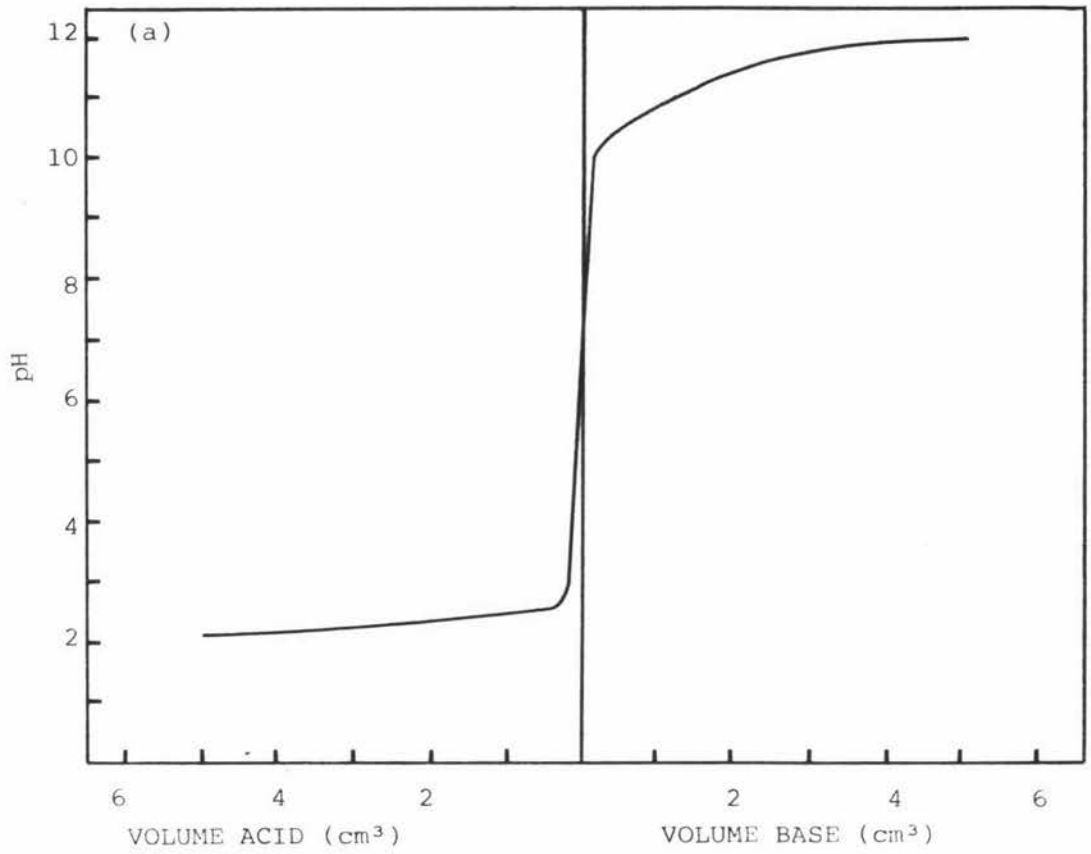
Titration curve for an aqueous solution of $NaCr(cys)_2.2H_2O$.

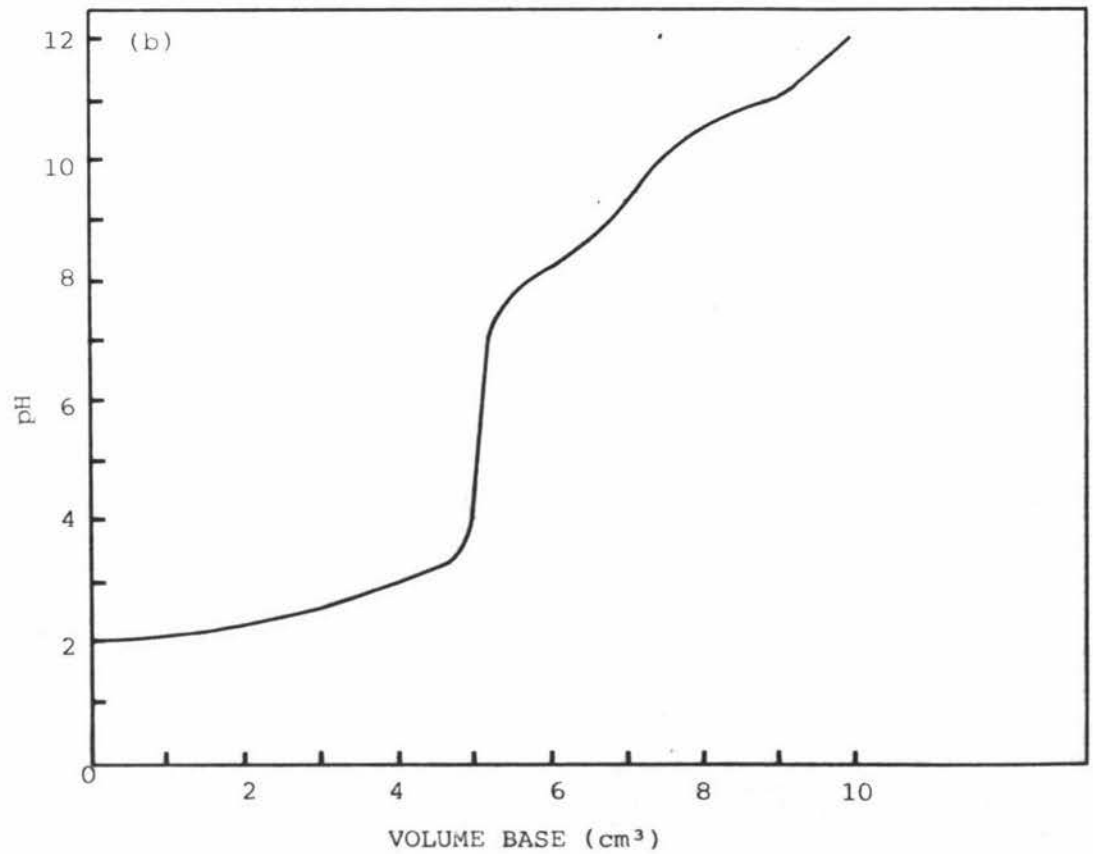
Two samples of $NaCr(cys)_2.2H_2O$ at pH 7.0 were titrated with either 0.25M H_2SO_4 or 0.5M NaOH and the results combined as shown.

FIGURE 3.4(b):

Titration curve for an aqueous solution of cysteine.HCl titrated with 0.5M NaOH.

FIGURE 3.4


(a)
12
10
8
6
4
2
pH
6
4
2
VOLUME ACID (cm³)
2
4
6
VOLUME BASE (cm³)


(b)
12
10
8
6
4
2
0
pH
2
4
6
8
10
VOLUME BASE (cm³)

anionic species suggesting one ionisable group. The plateaus in the regions pH 3.5 to pH 4.5 and pH 8.0 to pH 10.0 corresponded to the red species and the green species, respectively.

The free ligand was also titrated in a similar manner by dissolving cysteine-HCl in H_2O, and titrating with either 0.25M H_2SO_4 or 0.5M NaOH and a graph of pH versus the amount of acid or base added is shown in Figure 3.4(b). Three easily recognisable pk values were observed at pH = 2.0, 8.4 and 10.3 corresponding to the three ionisable groups -COOH, $-NH_3^+$ and -SH, respectively. These three pk's were not observed for the titration of $NaCr(cys)_2 \cdot H_2O$.

3.3.2.5 Determination of cysteine by the DTNB reaction

The reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) is known to react with the S^- group and was used here to determine the presence and concentration of the cysteine ligand. A standard solution of DTNB was prepared containing 5mM DTNB, 1mM EDTA and made up in a phosphate buffer (0.05M) at pH 7.0 according to the method of Ellman (1959). All cysteine containing solutions were made up in phosphate buffer at pH 8.0 unless otherwise specified.

The solution of DTNB was faintly yellow in colour but after reaction with any compound which contained a sulphydryl group an intense yellow colouration developed. The 2-nitro-5-thio benzioic acid anion had an extinction coefficient of 13,600 $1\ mol^{-1}cm^{-1}$ at a wavelength of 412nm when present in a solution at pH 8.0. The concentration of cysteine in a solution was found by adding $0.1cm^3$ of DTNB reagent to a $3.0cm^3$ sample solution containing the cysteine and measuring the absorbance at 412nm. The concentration of the cysteine was determined using the equation $C = \frac{D}{\varepsilon \times \ell}$ Equation 3.1 where c is the concentration of cysteine, D is the absorbance at a wavelength of 412nm, ℓ is the pathlength of the cuvette used (1.0cm),

and ε is the molar extinction coefficient of the DTNB derivative (13,600 l $mol^{-1}cm^{-1}$).

The blue compound $NaCr(cys)_2 \cdot 2H_2O$ was dissolved in a phosphate buffer at pH 8.0 and 0.1 cm^3 of DTNB reagent was added. No yellow colouration was found initially but after two hours an intense yellow colour had developed. The absorbance of the DTNB derivative was followed at a wavelength of 412nm for a period of time (3 hours) and found to vary exponentially with time, finally reaching a plateaux after two hours. The ratio of cysteine to chromium after the reaction with DTNB was calculated by weighing out a known quantity of $NaCr(cys)_2 \cdot 2H_2O$ (molecular weight 349.20) to establish the number of moles of Cr(III) present and measuring the absorbance at 412nm to determine the concentration of cysteine using equation 1. The ratio of Cr/cys equalled 1:2, thus all the coordinated sulphur had reacted with the DTNB reagent.

The red cysteine complex was prepared in a phosphate buffer at pH 4.0. When 0.1cm^3 of the DTNB reagent was added to the diluted red solution the colour of the solution remained faintly pink until the solution was titrated to pH 8.0 at which point an intense yellow colour developed immediately. The absorbance at 412nm of this solution remained constant with time and the ratio of chromium to cysteine was calculated to be 1:2 using equation 3.1. As a control a solution of phosphate buffer at pH 4.0 was prepared and 0.1 cm^3 of DTNB reagent was added, the solution remained colourless even after titration to 8.0 with dilute NaOH (2M). The reaction of the red cysteine complex species with DTNB was therefore thought to be due entirely to the reaction of $-S^-$ with the DTNB reagent.

The reaction of DTNB reagent with the free ligand cysteine dissolved in a phosphate buffer at pH 8.0 produced an intense yellow colour. It was also seen that the reaction at pH 8.5 with cysteine was instantaneous in contrast to the reaction with the blue solution obtained from $NaCr(cys)_2 \cdot 2H_2O$ which was time dependent.

Since the rate of reaction of DTNB with $NaCr(cys)_2 \cdot 2H_2O$ was able to be followed by the absorbance of 412nm (from the DTNB anion) a Guggenheim plot was derived from the plot of absorbance against time to give the rate of the cleavage of the Cr-S band. The absorbance plot is shown in Figure 3.5 and the Guggenheim plot of ln(A-A') against time is shown in Figure 3.6 where A' values were between times t = 0 sec and t = 240 secs and A values were between times t = 960 secs and t = 1200 secs (as shown in Figure 3.5). The Guggenheim plot gave an apparent rate constant $-(1.16 \pm 0.02) \times 10^{-3}$ sec^{-1}, with the negative sign due to measurement of the absorbance of the DTNB anion produced.

3.3.3 Chromium (III) Complexes with Glutamic Acid

3.3.3.1 Preparation of glutamic acid complexes

A solution containing $Cr(NO_3)_3 \cdot 9H_2O$ (1.0g, 0.0025 moles) and glutamic acid sodium salt (0.95g, 0.0050 moles) in H_2O ($30cm^3$) was heated to boiling and solid NaOH was added until firstly a blue solution formed, then a purple gel. NaOH (2M) was added dropwise until the gel dissolved to give a violet solution which, when cooled to room temperature, was found to have a pH of 10.5.

The reaction of $Cr(NO_3)_3 \cdot 9H_2O$ (1.0g, 0.0025 moles) and glutamic acid sodium salt (0.95g, 0.0050 moles) in H_2O ($30cm^3$) with the pH adjusted to 2.1 produced a blue solution upon boiling with the dropwise addition of NaOH (2M). On cooling, the pH of the blue solution was 2.5, but if dilute NaOH was added until the pH was 7.0 a blue precipitate formed.

3.3.3.2 Ion exchange of glutamic acid complexes

The violet solution prepared by neutralisation of a boiling solution of glutamic acid and Cr^{3+} was titrated to pH 8.5 with dilute NaOH (2M) and diluted to a conductivity of 3,000 µmho prior to loading onto a previously prepared Dowex 1-X8 ion-exchange column and the anionic species in solution

FIGURE 3.5:

Plot of absorbance at 412nm (for the DTNB anion) against time for the reaction of $NaCr(cys)_2.2H_2O$ at pH 8.0 with DTNB. Time interval A' was between t = 0 sec and t = 240 secs and time interval A was between t = 960 secs and t = 1200 secs.

FIGURE 3.6:

Guggenheim plot of ln(A-A') against time derived from the abosrbance plot (Figure 3.5) using time intervals A and A'. Slope of best fit line was $-(1.16 \pm 0.02) \times 10^{-3}$ sec^{-1} using least squares methods.

FIGURE 3.5

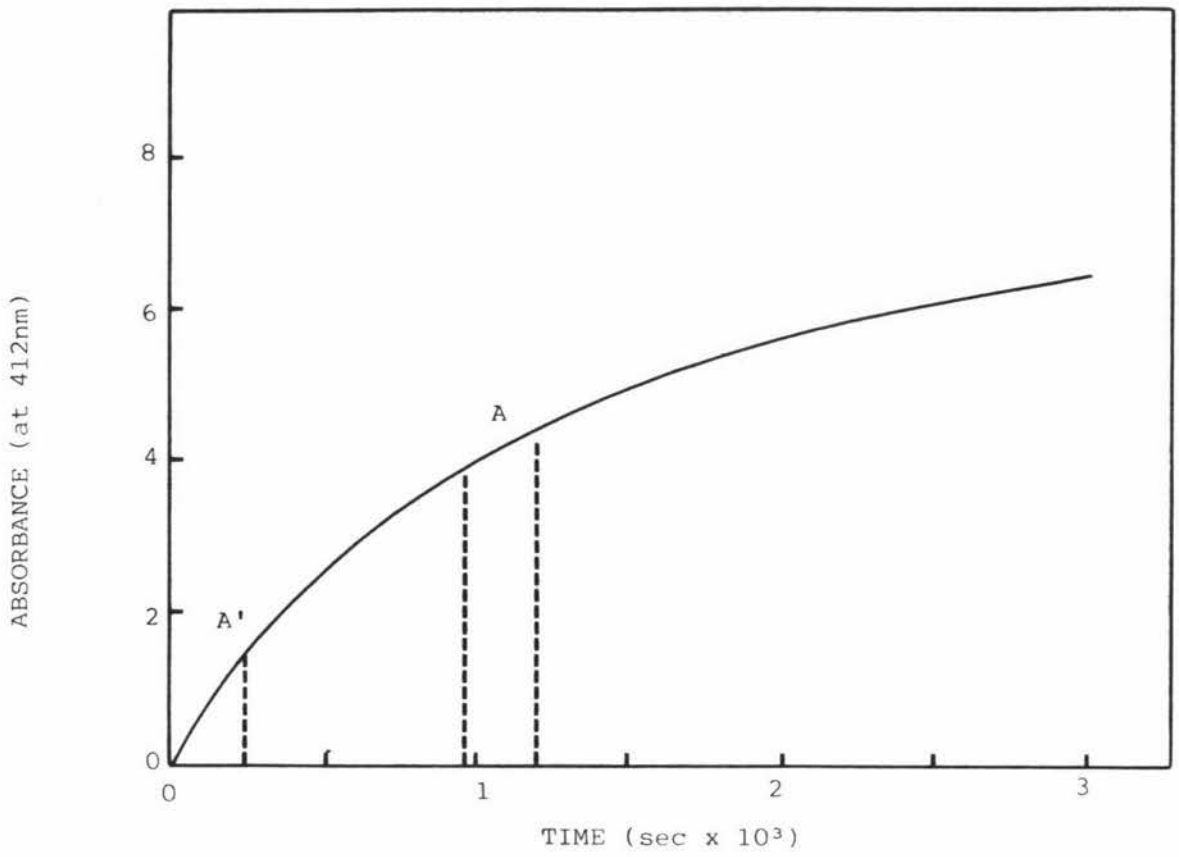
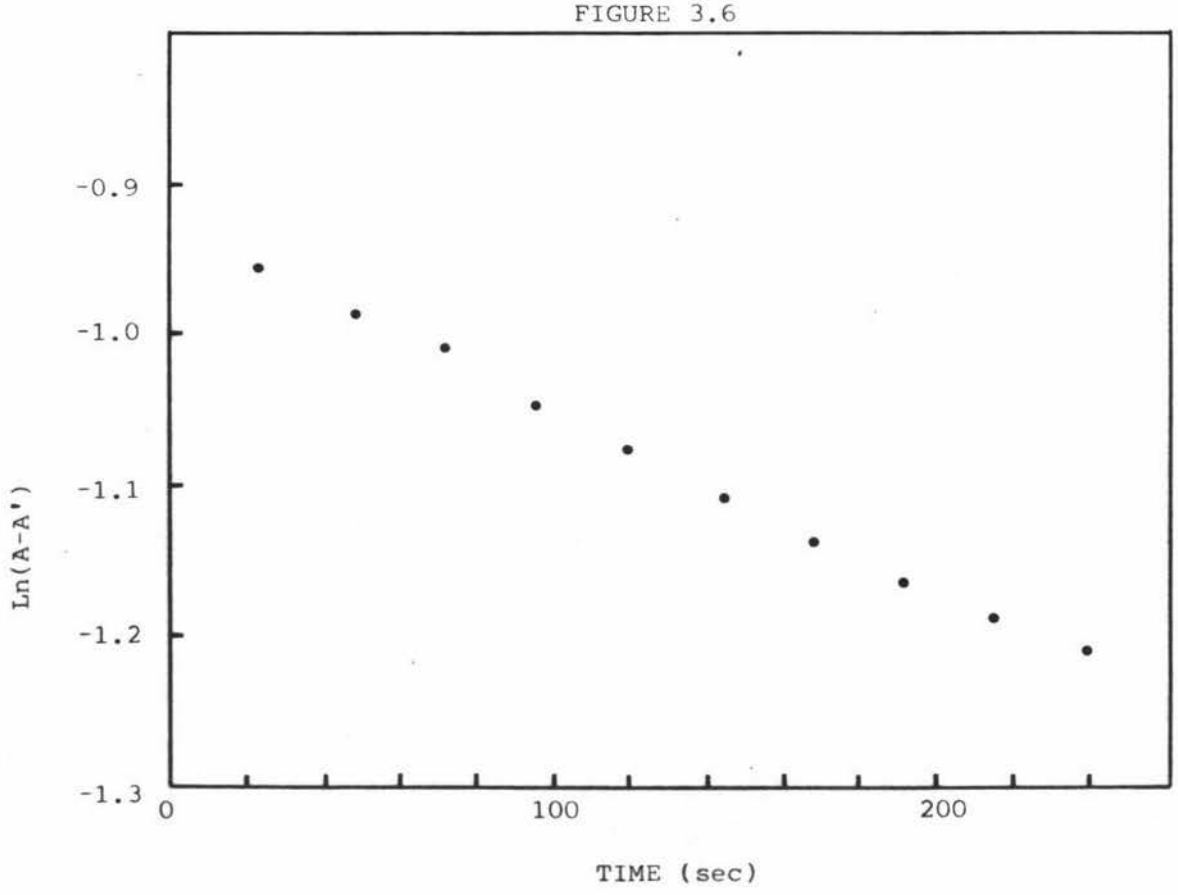


FIGURE 3.6



adsorbed. Subsequent elution with distilled H_2O failed to elute any cationic or neutral species, but when eluted with a T.R.I.S./NaCl buffer (pH 9.0; 10,000 µmho conductivity) a pink solution was collected, which was concentrated by rotary evaporation. When a pH gradient was applied by mixing T.R.I.S./NaCl buffer (pH 9.0; 10,000 µmho) and acetic acid-NaCl buffer (pH 4.0; 10,000 µmho conductivity) in a gradient mixer a purple solution was eluted. Further treatment of the column with 0.05M HCl failed to elute any other bound anionic species, so it was assumed that only the pink and purple species were present in the unpurified reaction mixture and that both species were anionic at pH 8.5.

The pink anionic species was now titrated to pH 3.0 and diluted to a conductivity of 3,000 µmho prior to loading onto a previously prepared Dowex 50-X12 ion-exchange column and was absorbed onto this column indicating a cationic character at pH 3.0. The subsequent elution with a NaCl solution (0.2M, 10,000 µmbs conductivity) produced only the pink species and no other species were found when the column was treated with higher salt concentrations or pH gradients up to pH 12.0 using phosphate buffers (0.05M NaH_2PO_4 until the pH reached 4.5, followed by 0.05M Na_2HPO_4 until the pH reached 9.0, followed by 0.05M Na_3PO_4 until the pH reached 12.0).

The other anionic species, which was purple in colour, was also titrated to pH 3.0 with nitric acid (2M) and diluted to a conductivity of 3,000 µmho before loading onto the Dowex 50-X12 column and was also adsorbed at this pH. However elution with salt gradients containing NaCl failed to elute a purple species, but at a conductivity of 50,000 µmbs (2.0M NaCl) a blue coloured solution was eluted. This elution occurred at a higher conductivity than that used to elute $Cr(H_2O)_6^{3+}$, as reported in Section 3.3.1.2. Further elution with pH gradients up to pH 12.0 failed to elute any other cationic species. The blue coloured solution very quickly formed a cloudy precipitate upon standing in H_2O at room

temperature for three days and the blue colour disappeared.

The blue solution, prepared by heating Cr^{3+} and glutamic acid in an acidic solution, was adsorbed onto a Dowex 50-X12 cation exchange at pH 3.5 and conductivity of 3,000 μ mbs. Only a single broad unresolved peak was eluted at a concentration of 1.0M NaCl (30,000 μmho conductivity), elution conditions which were similar to those used for the blue chromium (III)-glycine solution.

The effect of elution by ammonium hydroxide was studied by titrating the violet mixture (prepared in Section 3.3.3.1) to pH 3.5 with HNO (2M) and loading onto the Dowex 50-X12 cation-exchange column. On washing with 0.5M NH_4OH a blue solution was eluted at a pH of 10.0, which changed to a green solution after standing for two days. However, if dilute HNO_3 (2M) was added, the blue solution turned purple at pH 7.0, and this solution remained unchanged on standing for several weeks. The apparent change in the species bound to the resin resulting in a colour change from red-violet to blue could not be duplicated in the absence of the Dowex 50-X12 resin, the end product in this case being a green solution.

3.3.3.3 Electronic spectra

The pink anionic species obtained at pH 9.0 contained two bands centred at 385 nm and 550 nm with the intensity of the first band greater than the second band. The electronic spectrum remained unaltered after titration to pH 3.0 and was similar to the pink cationic species eluted from the Dowex 50-X12 column.

The purple coloured anionic species at pH 9.0 contained d-d absorption bands at 418nm and 555nm with intensity of the first band slightly less than the second. As with the pink species, upon titration to pH 4.0 with 2M HNO_3 the spectrum was unchanged. However because the now cationic species could

not be eluted from the cation exchange column no spectrum could be recorded after separation on the cation exchange column.

The cationic blue solution (prepared by the reaction of glutamic acid and Cr^{3+} at acidic pH) exhibited two d-d bands in the visible region at 420nm and 570nm with the intensity of the first band being less than the second band. A similar spectrum was recorded for the solution after elution from the cation-exchange column with a salt gradient containing NaCl.

The unpurified red-violet solution (thought to contain two species at least: the pink and red-purple species) when titrated to pH 3.0 and loaded onto the cation-exchange column and eluted with 0.5M NH_4OH was changed into a blue solution, which at pH 10.0 exhibited d-d bands at 405nm and 565nm with the intensity of the first band being greater than the second band. This blue solution was not very stable in aqueous solution, since if left at pH 10.0 it turned green or if a small amount of dilute HNO_3 was added the solution turned purple in a similar fashion to the soluble red glycine complex described in Section 3.3.1.2.

3.3.4 Chromium (III) Complexes with Glutamine

3.3.4.1 Preparation of $[Cr(NH_3)_6](NO_3)_3$

The hexaamine-chromium (III) complex was prepared according to the method of Oppegard and Bailar (1950). Liquid ammonia ($400cm^3$) was placed in a 1 l beaker inside a 1.5 l beaker and on adding sodium metal (0.25g) a blue colour developed which was lost when ferrous ammonium sulphate (0.1g) was added. Anhydrous $CrCl_3$ (25g) was added in 2g amounts with stirring during two hours. The supernatent liquid was then decanted from the brown residue and placed in an evaporating dish and stirred occasionally until the ammonia smell had gone, after which a bright yellow powder of $[Cr(NH_3)_6]Cl_3$

remained. The $[Cr(NH_3)_6]Cl_3$ was purified by dissolving quickly in H_2O ($75cm^3$), to which concentrated HCl ($5cm^3$) was added, at 40°C and filtered. Immediate treatment with concentrated HNO_3 ($25cm^3$) and cooling to room temperature precipitated pure $[Cr(NH_3)_6](NO_3)_3$, a bright yellow crystalline compound, which was washed with cold H_2O containing a little HNO_3, then EtOH, then diethyl-ether. The product was vacuum dried and stored in a brown bottle.

3.3.4.2 Preparation of glutamine complexes

A solution containing $Cr(NO_3)_3 \cdot 9H_2O$ (1.0g, 0.0025 moles) and glutamine (1.10g, 0.0075 moles) in distilled H_2O ($20cm^3$) was heated on a hot-plate to approximately 90°C until a purple solution formed which changed to a red solution with the dropwise addition of NaOH (0.30g, 0.0075 moles). At pH 8.5 the red solution was stable but at pH 3.5 the solution turned purple over a period of five days at room temperature. Similar results were found when freshly prepared $Cr(OH)_3$ or $CrCl_3 \cdot 6H_2O$ were used as starting materials.

An alternative method was to grind up in a mortar a mixture of $Cr(NH_3)_6 \cdot (NO_3)_3$ (0.60g, 0.0012 moles) and glutamine (0.44g, 0.0030 moles) and to place the finely divided powder in a thermostatically controlled oven at a temperature of 135°C for a reaction time of 60 minutes. The resultant purple powder was immediately dissolved in a minimum amount of distilled H_2O ($20cm^3$) to give a dark red solution which was stable at pH 8.5 but at pH 3.5 turned purple.

A solid glutamine complex, insoluble in H_2O and pink in colour, slowly formed when the $Cr(NH_3)_6$ $(NO_3)_3$ (0.60g, 0.0012 moles) and glutamine (0.44g, 0.0030 moles) were dissolved in H_2O ($20cm^3$), heated on a water bath until the colour of the solution changed to red and then allowed to cool and evaporate at room temperature. The pink compound was washed with H_2O and EtOH, vacuum dried but was not analysed.

3.3.4.3 Ion exchange of the glutamine complexes

The red solution obtained from the reaction of glutamine with the complex $Cr(NH_3)_6(NO_3)_3$ was titrated to pH 3.5 with dilute acid (2M HNO_3) and diluted to a conductivity of 2,500 µmho before loading onto the previously prepared Dowex 50W-X12 cation-exchange resin. Upon elution with the H_2O no coloured fractions were seen, but with 0.1M NaCl (5,500 µmho conductivity) the conductivity of the effluent rose markedly to approximately 17,000 µmho suggesting the elution of some material which because of its lack of colour was assumed to be unreacted glutamine. Finally the conductivity of the effluent dropped to a conductivity of 5,500 µmho and a pink fraction was eluted, which was freeze-dried ready for desalting on the gel filtration resin. Further elution of the column with a salt gradient from 0.1M NaCl to 0.5M NaCl resulted in the elution of a dark purple fraction at a conductivity of 20,000 µmbs, which was also freeze-dried in preparation for desalting. The chromium concentration in the purple fraction was twenty-five times that in the pink fraction. However since the solution originally loaded onto the column was red in colour, this suggested that the red solution had changed into the purple fraction during elution from the column.

The purified cationic pink and purple fractions were loaded onto a previously prepared anion-exchange resin at pH 8.5 and a conductivity of 2,000 µmho and found to be eluted with H_2O confirming their cationic character.

3.3.4.4 Electronic spectra

The electronic spectrum of the soluble pink species exhibited two absorption bands characteristic of chromium (III) complexes one at 395nm and one at 526nm with the intensity of the first band being less than that of the second band. Identical d-d band positions were seen for the red solution obtained from the reaction of glutamine with $Cr(NH_3)_6(NO_3)_3$ which

had absorption bands at 395nm and 526nm, also with the first band less intense than the second.

The purple fraction was eluted at a higher salt concentration than the pink fraction from the cation-exchange resin and exhibited d-d bands at 410nm and 550nm with both bands being rather broad and the intensity of the first band being approximately equal to the second. Similar spectra were seen for the purple solution obtained from boiling a solution of glutamine and $Cr(NO_3)_3 \cdot 9H_2O$, or $CrCl \cdot 6H_2O$, at neutral pH, or when the pink species seperated above was left to stand at neutral or acidic pH for several days.

3.4 DISCUSSION

3.4.1 Chromium (III) Complexes with Glycine

Chromium (III) formed several complexes with glycine, both insoluble and soluble in water, although only the soluble complexes were considered to be important in the study of chromium complexes in biological systems. However the insoluble crystalline glycine complexes could be more easily studied with respect to the type and geometry of ligand coordination enabling comparisons to be made with the less easily identified soluble glycine complexes.

3.4.1.1 The tris-glycine chromium (III) complex

When chromium (III) was reacted with glycine at a molar ratio of 1:3 in aqueous solution with the addition of no more than 3 moles of NaOH per mole of chromium an insoluble red complex was formed, as expected, which analysed as $Cr(gly)_3 \cdot H_2O$. This complex consists of three chelated glycine ligands around an octahedral chromium (III) atom as shown by an x-ray crystal structure determined by Bryan et al (1971). The type of

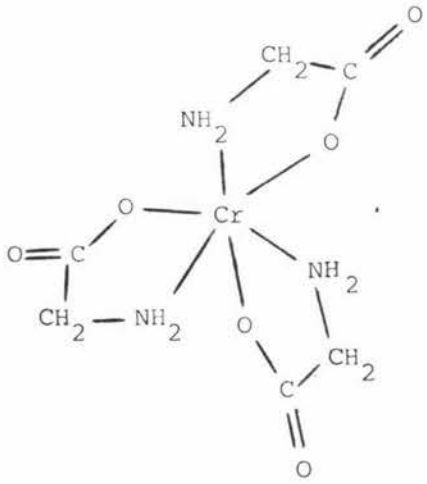
coordination found in $Cr(gly)_3 \cdot H_2O$ can be referred to as 3N, 3O, that is three nitrogen and three oxygen ligands are coordinated to the chromium (III) atom. This type of structure is reflected in the two d-d bands which were found to be shifted markedly to lower wavelengths compared to $Cr(H_2O)_6^{3+}$ due to the coordination of the nitrogen ligands which are higher in the spectrochemical series than the oxygen ligands. Only two possible geometrical isomers are known for tris-type amino acid complexes; the mer (cis, trans) and the fac (cis, cis) type with respect to the nitrogen or oxygen ligands which are coordinated to the central metal ion. The main difference between the absorption curves for these two isomers appears in the second, higher wavelength band. As seen for Co(III) complexes (Hidaka et al 1962) in the mer form this band exhibits some splitting while in the fac form no splitting occurs. The spectrum of the $Cr(gly)_3 \cdot H_2O$ solid exhibited no splitting in the d-d band at 510nm therefore the nitrogen atoms and the oxygen atoms coordinated to Cr(III) in complex were probably fac (cis, cis) arranged.

For a cis structure such as that found in $Cr(gly)_3 \cdot H_2O$ the positions of the absorption maxima can be calculated using the method discussed by Matsuoka et al (1967) for the series $[Co(O)_n(N)_{6-n}]$. The predicted shifts were calculated using the observed values of the absorption maxima for $Cr(en)_3^{3+}$ and $Cr(ox)_3^{3-}$ to define the spectral limits of the series. The $Cr(en)_3^{3+}$ complex serves to give the positions of the bands for an octahedral environment of nitrogen ligands ($\delta^{I}(N)$ and $\delta^{II}(N)$) and the $Cr(OX)_3^{3-}$ complex yields the positions of the bands for an octahedral environment of oxygen ligands ($\delta^{I}(O)$ and $\delta^{II}(O)$). The calculated position of a band in a given chromium complex is then assumed to be given by the appropriate weighting of the band positions of the two limiting complexes. For example in a fac $[Cr(N)_3(O)_3]$ complex the calculated position of the first d-d band would be

$\frac{1}{2}\ \delta^{I}(N) + \frac{1}{2}\ \delta^{I}(O)$ and the second d-d band would be expected at $\frac{1}{2}\ \delta^{II}(N) + \frac{1}{2}\ \delta^{II}(O)$.

Using the values of 353nm and 457nm for $Cr(en)_3^{3+}$ for the first and second d-d bands respectively and 420nm and 571nm for $Cr(ox)_3^{3-}$ (Oki and Otsuka 1976), the calculated positions of the complex $[Cr(N)_3(O)_3]$ were 386.5nm and 514nm. These calculated absorption maxima were very close to the experimentally observed values for $Cr(gly)_3 \cdot H_2O$ at 388nm and 510nm indicating that the structure was of the fac or cis, cis form as shown in Figure 3.7.

FIGURE 3.7: STRUCTURE OF $Cr(gly)_3 \cdot H_2O$



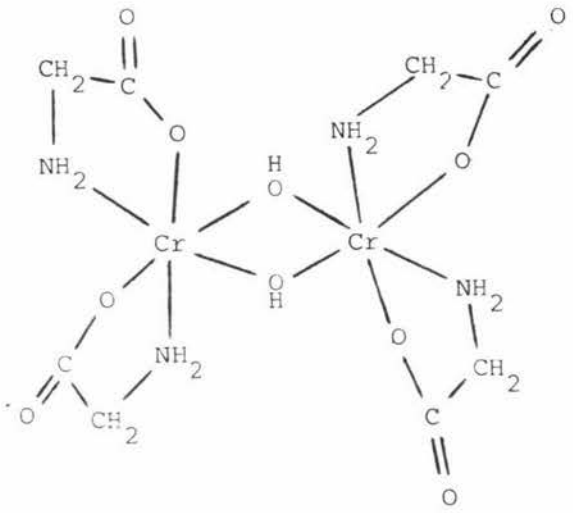
3.4.1.2 The dimeric bis-glycine chromium (III) complex

When chromium (III) and glycine were reacted in a molar ratio of 1:2 in a basic solution a pink compound was formed which was insoluble in all solvents and analysed as $[Cr(gly)_2OH]_2$. This compound was shown to be dimeric by Hodgson et al (1973) with the chelation of two glycine ligands per chromium atom and two hydroxy bridges between the two chromium (III) atoms. Such a complex can be referred to as a N_2, O_4 type complex due to coordination of two nitrogen ligands and four oxygen ligands to the chromium (III) atom.

The electronic spectrum of the $[Cr(gly)_2OH]_2$ solid was similar to that of the solid tris complex, $Cr(gly)_3 \cdot H_2O$, which was a fac isomer, in that the second absorption maximum exhibited no appreciable splitting, so the structure of this complex is probably related to the fac form. This would mean that the nitrogen atoms and the oxygen atoms of the glycines would be cis to one another. With reference to the series $[Cr(N)_n(O)_{6-n}]$ the complex $[Cr(gly)_2OH]_2$ would be referred to as cis $[Cr(N)_2(O)_4]$ and by using the predicted absorption maxima equation of Matsuoka et al (1967), the second band would be predicted to contain two components labelled $a = \frac{1}{4}\ \delta^{II}(N) + \frac{3}{4}\ \delta^{II}(O)$ and $b = \frac{1}{2}\ \delta^{II}(N) + \frac{1}{2}\ \delta^{II}(O)$ while the first band would be predicted to be at $\frac{1}{3}\ \delta^{I}(N) + \frac{2}{3}\ \delta^{I}(O)$. However because the complex contained two hydroxide ions as ligands instead of two carboxylate groups the $\delta^{I}(O)$ and $\delta^{II}(O)$ values had to be adjusted. For $[Cr(ox)_3]^{3-}$ the absorption maxima were at 420 and 571nm; while for $Cr(H_2O)_6^{3+}$ the maxima were at 408 and 575 nm. These values of $\delta^{I}(O)$ and $\delta^{II}(O)$ were averaged to give $\delta^{I}(O)' = 414$nm and $\delta^{II}(O)' = 575$ nm and used in the calculations to predict a position of 393 nm for the first band of $Cr(N)_2(O)_4$ and for the second d-d transition a split band at 544 nm and 515 nm. The split bands for the second absorption band were actually too close together to be seperated in the visible spectrum, the observed value being a broad, unsplit band centred around 529nm. The observed absorption maxima for the dimeric complex was therefore found to be very close to the predicted positions for a cis arrangement of the ligands rather than a trans arrangement which would cause a larger splitting of the second d-d transition. If the glycine ligands were in a cis position this leaves two vacant positions also cis to one another and the two hydroxy bridges would be coordinated here as in the structure shown in Figure 3.8. The hydroxy-bridged dimer was therefore two bis-complexes joined together by two hydroxy bridges and the complex seemed to be most stable in basic solution. Partial olation of the chromium (III) atom had occurred to create the hydroxy bridged complex,

but olation ceased at this point because of the stability with two chelate rings per chromium atom and also two hydroxy bridges between the chromium atoms. This seemed to represent a compromise between the chelation and olation processes.

FIGURE 3.8: STRUCTURE OF $[Cr(gly)_2OH]_2$



3.4.1.3 Monodentate glycine complexes of chromium (III)

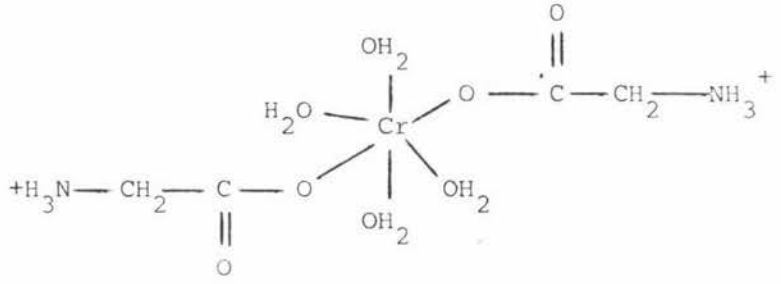
When a solution of chromium (III) was reacted with glycine in a molar ratio of 1:3 at pH values less than 4.0, a blue solution formed which was found to be stable in acidic solution. However isolation of the cationic chromium complexes was not possible using a cation-exchange resin. A blue solution was eluted from the cation-exchange column under conditions which were similar to those used for the elution of $Cr(H_2O)_6^{3+}$ which suggested that the complex species probably carried a charge of 3+. Gel filtration was also attempted but the very broad blue band which resulted could be accounted for by assuming that a series of similar complexes, differing in size by only small amounts, was present in the blue solution.

The positions of the electronic absorption bands were at higher wave-lengths than for $Cr(H_2O)_6^{3+}$ which suggested that the carboxylate group of the glycine ligand was coordinated to the chromium (III) atom. The carboxylate group, $-COO^-$, is considered to be a slightly basic oxygen type ligand which would be placed lower in the spectrochemical series than H_2O hence accounting for the observed shift from the violet $Cr(H_2O)_6^{3+}$ to the blue glycine complex. This is consistent with the known preference of Cr(III) for oxygen ligands rather than for nitrogen ligands. If the amine group had been coordinated to the chromium (III) atom, then the positions of the d-d bands would be expected to shift to higher wavelengths than for $Cr(H_2O)_6^{3+}$ because nitrogen donor type ligands are higher in the spectrochemical series than H_2O.

It is possible that due to coordination of the carboxylate group of glycine a series of complexes of the form $Cr(gly)_n(H_2O)_{6-n}^{3+}$ (n = 0,1,...,6) could be present in the blue solution. The tri-positive charge would be expected if the amine groups were protonated, instead of being coordinated, thus effectively neutralising the negative charges of the coordinated carboxylate groups in which case the overall charge would be determined by the charge on the chromium (III) ion. In a complex such as $Cr(gly)(H_2O)_5^{3+}$ only one carboxylate group would be coordinated to the central chromium atom which would tend to shift the visible absorption bands slightly to higher wavelengths than for $Cr(H_2O)_6^{3+}$. However in a complex such as $Cr(gly)_6^{3+}$ with coordination of six glycine carboxylate groups to the central chromium atom the visible absorption bands would be expected at similar positions to those found for the chromium-trioxalate complex ($Cr(ox)_3^{3-}$) (420nm and 571nm). Thus even though the postulated series of complexes $Cr(gly)_n(H_2O)_{6-n}^{3+}$ could not be seperated from each other, the electronic spectrum was in the region between that of $Cr(H_2O)_6^{3+}$

and $Cr(ox)_3^{3-}$ (408-420nm and 571-575nm) consistent with the suggestion that a mixture of complexes is present. The possibility of different isomers for some of the members of this complex series means that more than six species could be present in the blue solution, but each would have the same type of structure to that shown in Figure 3.9. It is possible that the major portions of the solution consisted of species with two or more coordinated glycine ligands since the chromium to glycine molar ratio of the original mixture was 1:3 and the absorption bands were in fact found to be similar to the bands previously observed for the $Cr(nic)_2(H_2O)_4^{3+}$ species which was known to have two carboxlylate groups coordinated.

FIGURE 3.9: STRUCTURE OF POSTULATED $Cr(gly)_2(H_2O)_4^{3+}$



The blue solution was found to be stable only in acidic corditions suggesting that chelation had not occurred. However, on the addition of base (2M NaOH) the solution became very cloudy and a light gelatinous precipitate formed at a pH value greter than 8.0. This was thought to be due to the process of olation which occurs easily with $Cr(H_2O)_6^{3+}$ but less easily with complexed Cr(III) (Rollinson et al 1967). In this process compounds are formed with bridging OH^- groups, finally resulting in the formation of polymeric complexes incorporating many coordinated ligands. Chromium (III) in

aqueous solution has a strong tendency to olate at pH's greater than 3.5 with the formation of a green solution containing basic chromic polymers and finally precipitation of insoluble chromium hydroxide occurs. The tendency to olate has been found to be lessened when Cr(III) is coordinated to strongly chelating ligands such as glycine (Rollinson et al 1967). With a monodentate coordinated species for example $Cr(gly)_n(H_2O)_{6-n}^{3+}$ the tendency to olate would be expected to decrease due to replacement of the water molecules with more strongly coordinated glycine ligands.

3.4.1.4 The soluble bis-glycine chromium (III) complex

When glycine was coordinated to Cr(III) by both the carboxylate group and the amine group, a chelated complex was formed as found in $Cr(gly)_3.H_2O$ and $[Cr(gly)_2OH]_2$. However by using different reaction conditions a soluble red solution was prepared and a summary of reaction conditions for each species obtained is shown in Table 3.4. When chromium (III) and glycine in a molar ratio of 1:2 were mixed with a pH less than 3.0 the purple colour of the solution was probably due to the $Cr(H_2O)_6^{3+}$ ion but upon the addition of aqueous alkali, the solution became blue suggesting the formation of a monodentate coordinated complex species $Cr(gly)_n(H_2O)_{6-n}^{3+}$. As the pH was increased further the colours of the solution changed to purple-red, which after ion-exchange seperation yielded a purple-red solution which was thought to contain $Cr(gly)_2(H_2O)_2^{+}$. No other glycine complexes were found in the solution. These colour changes suggest that the formation of the bis-glycine chromium complexes is a step wise process. It seems likely that the carboxylate group of the glycine molecule co-ordinates first and if the pH is sufficiently high, so that a significant fraction of the amino groups are deprotonated, is followed by coordination of the amino group. Shuttleworth and Sykes (1960) found that coordination of the carboxylate group occurred at pH values less than approximately 4.0, with coordination of the amine group following at pH 7.0, thus forming the chelated ring structure.

TABLE 3.4: REACTION CONDITIONS FOR PREPARATION OF GLYCINE COMPLEXES

Species	Colour	Reaction conditions			
		Molar ratios			pH
		Cr^{3+}	glycine	NaOH	
$Cr(gly)_n(H_2O)_{6-n}^{3+}$	blue	1	3	-	3.0
$Cr(gly)_2(H_2O)_2^{+}$	red	1	2	2	7.0
$[Cr(gly)_2OH]_2$	pink	1	2	3	8.5
$Cr(gly)_3 \cdot H_2O$	red	1	3	3	7.0

Volshtein (1956) also reported the formation of a chelate ring with amino acids and chromium (III) upon the addition of base and the cleavage of such rings on addition of acid.

The red complex which was eluted from the cation-exchange resin before the unreacted $Cr(H_2O)_6^{3+}$ under acid conditions presumably possessed a charge of less than 3+ due to its ease of elution as compared to the hexa aquo-chromium (III) complex. A soluble complex species which was also red, cationic and stable in acid medium was formed when $[Cr(gly)_2OH]_2$ was dissolved in acid or dissolved in water with heating. Both red solutions were stable at pH's below 8.5 but after several days at pH 8.5 the solutions precipitated out a pink complex which analysed as $[Cr(gly)_2OH]_2$ and since the colour of the solutions was unchanged the complex species in solution probably has a similar structure to $[Cr(gly)_2OH]_2$.

The electronic spectrum of the red solution was in fact very similar to that of the $[Cr(gly)_2OH]_2$ complex and thus both complexes were considered to be of the type $cis[Cr(N)_2(O)_4]$

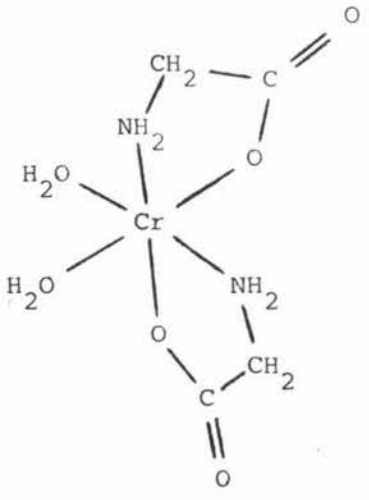
as discussed in Section 3.4.1.2. This suggested that the red species was a bis-glycine chromium (III) complex but because of its solubility could not contain the two hydroxy bridges between two chromium atoms. A possible structure is $Cr(gly)_2(H_2O)_2^+$ which contains two bidentate coordinated glycine ligands and two water ligands and the 1+ charge accounts for the easy elution of the complex from the cation-exchange column.

In basic solution the following equilibrium between the red soluble species $Cr(gly)_2(H_2O)_2^+$ and the pink insoluble species would be expected:

$$2Cr(gly)_2(H_2O)_2^+ + 2\ OH^- \rightleftharpoons [Cr(gly)_2OH]_2 + 4H_2O$$

Thus the arrangement of the glycine ligands in $Cr(gly)_2(H_2O)_2^+$ is identical to that of $[Cr(gly)_2OH]_2$, that is a cis structure with the hydroxide ions replaced by water ligands as shown in Figure 3.10.

FIGURE 3.10: STRUCTURE OF $Cr(gly)_2(H_2O)_2^+$



An interesting result was obtained when a chromous ion solution was reacted with glycine in the apparatus used in the preparation of yellow $Cr(nic)_2(H_2O)_3OH$ (see Section 2.4.1.1). The immediate product was a purple-red solution which was similar to the purple-red solution just discussed which is believed to result from the presence of $Cr(gly)_2(H_2O)_2^+$ with no intermediate product observed. This behaviour differed from the reaction of chromous ions with nicotinic acid when an intermediate complex was formed which subsequently oxidised in solution to the final product. The reaction of glycine with Cr^{2+} only occurred when the pH of the glycine solution was approximately 10.0 which is very close to the pk_2 of the $-NH_3^+$ group of glycine at which point the glycine molecule consists of 50% $NH_2-CH_2-COO^-$. Chromous ions are very reactive so it is probable that two glycine molecules chelated with the divalent Cr^{2+} ion, the charge on the Cr^{2+} ion being neutralised by the two carboxylate groups. As soon as $Cr(II)(gly)_2(H_2O)_2$ was formed it would oxidise to $Cr(III)(gly)_2(H_2O)_2^+$ to constitute the sole product of the reaction. The electronic spectrum of this solution was identical with that of $Cr(gly)_2(H_2O)_2^+$ prepared from chromium (III) as discussed above which is consistent with an identical structure for the complex in the two solutions (see Figure 3.10).

The insoluble species $Cr(gly)_3 \cdot H_2O$ and $[Cr(gly)_2OH]_2$ were both unstable in acid, undergoing some kind of acid hydrolysis or chemical reaction to bring about solubility. The absorption maxima of the red solution obtained from both the tris and dimer complexes were similar to those observed for the proposed $Cr(gly)_2(H_2O)_2^+$ species which indicated that the acid reaction was producing $Cr(gly)_2(H_2O)_2^+$. This reinforced the view that the main stable chelated complex was of the bis form with a cis nitrogen and cis oxygen structure around

the chromium (III) atom. No evidence was found that the mono-glycine complex $Cr(gly)(H_2O)_4^{2+}$ was formed in aqueous solution, even when the ratio of chromium to glycine was changed to 1:1 in the preparative procedure.

The soluble bis complex of glycine with chromium (III), $Cr(gly)_2(H_2O)_2^+$, thus possesses a cis structure and hence is not a suitable structure to allow the formation of a stable $Cr(nic)_2(gly)_2$ complex. The two vacant ligand positions containing water are in a cis position which would mean that if nicotinic acids were coordinated here they would be subjected to great steric hindrance. Such a complex would not be as stable as $Cr(gly)_2(H_2O)_2^+$ and would not form in solution since a stable dinicotinate complex would need to allow the nicotinic acid ligands to remain in a trans position.

3.4.1.5 The reaction of $Cr(gly)_2(H_2O)_2^+$ with NH_4OH

The elution of the red $Cr(gly)_2(H_2O)_2^+$ complex from the cation-exchange resin with ammonia resulted in a blue fraction, indicating that some reaction had occurred while the complex was bound to the resin. The characteristic d-d bands of $Cr(gly)_2(H_2O)_2^+$ had now shifted towards higher wavelengths, such as those observed for chromium (III) complexes with oxygen type ligands rather than nitrogen ligands. It seems likely that the eluted complex consisted of two glycine ligands coordinated only through the carboxylate group (monodentate coordination). This would partly explain why the absorption bands were observed at higher wavelengths, but not totally because the positions of the d-d bands were still too low compared to those observed for the series of $Cr(gly)_n(H_2O)_{6-n}^{3+}$ complexes. The eluted solution must therefore have been a mixture of $Cr(gly)_2(H_2O)_2^+$ (chelated) and $Cr(gly)_2(H_2O)_4^{3+}$ (unchelated).

The ion exchange behaviour was considered unusual because no similar reaction was observed in ammonia solution alone. The products in this case were green polymeric chromium hydroxide complexes and not the red, or blue, complexes found before, or after, the reaction on the resin. The action of ammonium hydroxide as a base was ruled out as a cause for the change in the complex, because all other bases tried (NaOH, LiOH and KOH) caused complex breakdown after elution from the resin resulting in a green chromium solution which was assumed to be a mixture of hydroxy-polymers. Generally a high solution pH has been shown to be essential for the formation of a chelate ring in chromium-amino acid complexes (Volshtein 1956) which suggests that glycine complexes should be stable in alkali. Since the glycine complex was not stable the cation-exchange resin was thought to be responsible for the observed reaction.

When the blue ammonia-eluted solution was left standing at pH 9.3 it gradually precipitated a green gelatinous product which was similar to chromium hydroxide. This indicated that the blue complex was not very stable at this pH, however, on dropwise addition of dilute HNO_3 the colour of the solution reverted to red-purple and the absorption maxima (at 398nm and 535nm) were identical with those for the original $Cr(gly)_2(H_2O)_2^+$ complex. It was concluded therefore that the acidity of the Dowex 50-X12 ion-exchange resin probably served to catalyse the conversion of $-NH_2$-Cr to $-NH_3^+$ which is bound to the resin. When NH_4OH is added it can partly neutralise the column and displace $-NH_3^+$ by NH_4^+ resulting in monodentate coordination of the glycine to chromium (III) via the carboxylate group and elution of the blue $Cr(gly)_2(H_2O)_4^{3+}$ solution discussed in Section 3.4.1.3.

3.4.2 Chromium (III) Complexes with Cysteine

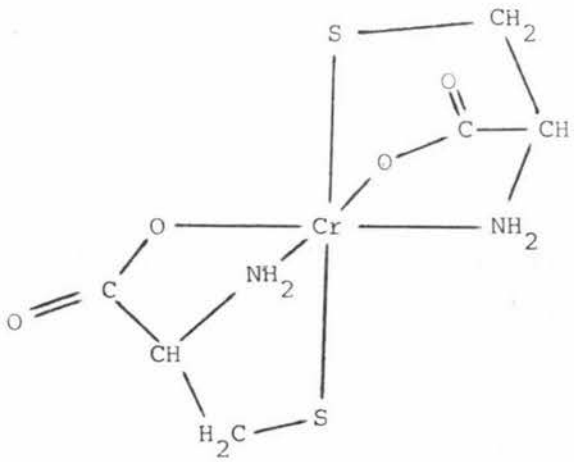
3.4.2.1 The tridentate cysteine complex with chromium (III)

When chromium (III) was reacted with cysteine in the molar ratio of 1:2 a dark blue crystalline product was formed which

analysed as $NaCr(cys)_2 \cdot 2H_2O$ and was similar to the compound prepared by Hodgson et al (1977) whose x-ray crystal structure is shown in Figure 3.11.

When the blue complex $NaCr(cys)_2 \cdot 2H_2O$ was dissolved in H_2O a blue solution was formed, and since no colour change was observed, it was assumed that no chemical reaction had occurred on dissolution of the species, which was believed to contain two terdentate cysteine ligands. The structure of such a complex would of course be identical with the octahedral geometry observed with the sodium salt $NaCr(cys)_2 \cdot 2H_2O$, as is shown in Figure 3.11. Ligand-displacement reactions of Cr(III) complexes have half-times in the range of several hours explaining the relative kinetic inertness of these complexes in aqueous solution (Cotton and Wilkinson 1972k). Thus it is likely that the blue complex would remain stable for a relatively long period of time in aqueous solution.

FIGURE 3.11: STRUCTURE OF $NaCr(cys)_2 \cdot 2H_2O$



The positions of the d-d bands in the electronic spectra of the various cysteine complexes (see Table 3.3) correlated with the observed colours and enabled the identification of which groups on the cysteine ligand were coordinated to the chromium (III) atom. The presence of four identifiable d-d bands which could be grouped in two pairs was unusual because normally only two bands are expected as in the cases of $Cr(H_2O)_6^{3+}$ and $Cr(gly)_2(H_2O)_2^+$ discussed above. The pair of bands at wavelengths similar to those of $Cr(gly)_2(H_2O)_2^+$ (413nm and 555nm for $NaCr(cys)_2 \cdot 2H_2O$) will be referred to as Pair I and the other pair of bands found at a higher wavelength (450nm and 615nm) will be referred to as Pair II. It was observed that in all of the cysteine complexes studied, the Pair I bands were always present but the Pair II bands were only observed in the complexes which were thought to have cysteine coordinated through the sulphydryl group. This is confirmed from the electronic spectrum of both solid and solution $NaCr(cys)_2 \cdot 2H_2O$ which contained both pairs of d-d bands and the complex is known to contain two cysteine ligands with coordination through the carboxylate group, the amine group and also the sulphydryl group (Hodgson et al 1977). The Pair I bands were found in positions similar to those of $Cr(H_2O)_6^{3+}$ which has six H_2O ligands with coordination through the oxygen atoms and also $Cr(gly)_2(H_2O)_2^+$ which has a mixture of coordination through both nitrogen and oxygen ligands, suggesting that these bands were associated with the coordination of the carboxylate and amine groups of the cysteine ligands. However the Pair II bands were in positions which are not usually observed in chromium (III) complexes and it seems likely that the Pair II bands are associated with coordination to the sulphur atom.

The positions of the three types of functional groups found in the cysteine molecule in relation to H_2O in the spectrochemical series are as follows: $Cl^- < S^- < C_2O_4^- < H_2O < NH_3$ (Huheey 1978a), which suggests that nitrogen coordinat-

ion causes greater splitting of the d orbitals than does coordination with H_2O molecules, hence the electronic spectrum would be shifted to lower wavelengths; coordination to the weakly basic oxygen type ligands of the carboxylate group would cause less d orbital splitting than H_2O hence a shift in the spectrum to higher wavelengths would be expected; and the effect of coordination to a sulphur atom would be to shift the electronic spectrum to even higher wavelengths than the oxygen type ligands. Thus we would expect to find the d-d transition bands due to coordination with nitrogen and oxygen atoms as being either slightly less than or slightly greater than those of H_2O ligands respectively and due to overlap of their absorption envelopes, only two bands would be observed in the electronic spectrum. However, the effect of sulphur ligand coordination would cause a large shift, toward higher wavelengths, of these two bands. The appearance of two sets of bands could indicate that two complex species were present in solution at pH 7.0, one of which involves a sulphydryl coordination to the chromium. Two sets of bands in the nujol mull spectrum of $NaCr(cys)_2 \cdot 2H_2O$ could be due to a symmetry effect of the trans-sulphur groups in the complex.

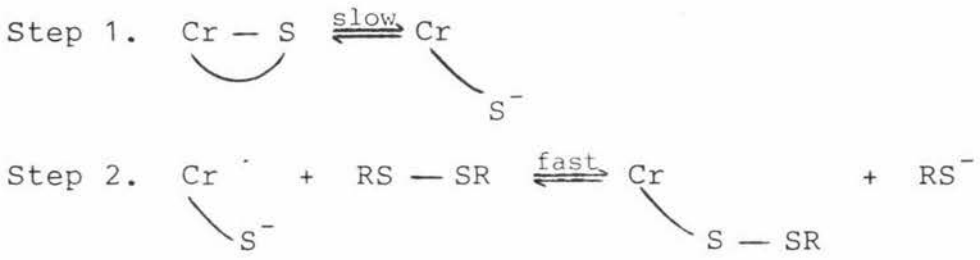
Examination of the change in intensity of the various d-d bands in the spectrum of $NaCr(cys)_2 \cdot 2H_2O$ as a function of pH (see Figure 3.2) confirmed that there were indeed two seperate sets of d-d bands present. Upon titration of the blue solution, at pH 7.0, with acid the first band of the pair I set decreased while the second band increased and analysis of the shape of the curve for the first band of Pair I as a function of pH gave a pk of approximately 4.5. This pk was thought to be due to a change in the coordination of the ligands involving an increase in the amount of the red species which did not possess coordinated sulphydryl groups, and the displaced ligand positions could have been aquated by two water ligands.

In the case of the Pair II bands, the intensity of both bands increased in a similar fashion as the pH was increased suggesting that both were affected by the same cause, that is an increase in the other green coloured species. The analysis of the shape of the curve for both bands as a function of pH gave a pk (approximately 10.9) which was very similar to that of the ionisation of the sulphydryl group in cysteine (pk = 10.28). It was obvious therefore that the Pair II bands were associated with the coordination of the sulphur atoms.

The titration curve of $NaCr(cys)_2 \cdot 2H_2O$ exhibited only one ionisable group compared with the three groups observed for the free ligand cysteine. This observation was consistent with the view that the ionisable group was the sulphydryl group and that the coordination of this group was only possible when it had been ionised through loss of a proton on neutralisation with the base present. Since the titration curve of the complex was so different from that of cysteine this was taken as proof that some of the three possible ionisable groups (-COOH, $-NH_3^+$ and -SH) were involved in coordiantion with the chromium (III) ion. In cysteine the pk at 10.3 was associated with the sulphydryl group and the close similarity with the pk of 10.9 suggested the sulphydryl group was coordinated only transiently at a basic pH to achieve stability. The pk associated with the amine group was entirely lacking in the $NaCr(cys)_2 \cdot 2H_2O$ titration indicating that this group was tightly coordinated to the chromium (III) ion and unavailable for titration with acid or base. Finally the pk associated with the carboxylate group of the amino acid was not observed, and the pk at 4.5 would be situated at too high a pH to be a titratable -COOH group, suggesting that the carboxylate group was strongly coordinated and the pk at 4.5 accountered for by assuming the coordination of two H_2O ligands at acidic pH's. The intermediate blue colour at pH 7.0 was the point when both the red species, with H_2O ligands coordinated (with pk 4.5), and the green species, with coordination of the sulphydryl groups (with

pk 10.9), were both present probably in a ratio of 1:1.

The use of the DTNB reagent also allowed the determination of both the presence and concentration of free sulphydryl groups in aqueous solution. For a solution of $NaCr(cys)_2 \cdot 2H_2O$ at pH 8.0 the reaction with DTNB was slow and obeyed first order kinetics. Such kinetic behaviour is consistent with no sulphydryl groups being available for reaction initially (due to coordination to chromium (III)) but with the DTNB reacting rapidly with any sulphydryl group which becomes available due to the slow dissociation of the CR-S bond at pH 8.0 to form a DTNB derivative. A reaction mechanism can be written as follows:



where RS - SR is the DTNB reagent

After the formation of the DTNB derivative the sulphydryl group is unable to coordinate with the chromium. This slow rate (in step 1) could be a measure of the dissociation rate for the Cr - S bond. A Guggenheim plot of Ln(A-A') against time for the absorbance at 412nm of the DTNB anion formed gave a straight line which is consistent with a rate limiting slow dissociation of the Cr - S bond. The apparent rate constant, $k = (1.16 \pm 0.02) \times 10^{-3}$ sec^{-1}, is therefore the rate of dissociation of the Cr - S bond and is slower than the very fast reaction of DTNB with cysteine which has freely available sulphydryl groups.

3.4.2.2 The reaction of $NaCr(cys)_2 \cdot 2H_2O$ with acid

The titration of the blue species, postulated as the anion $Cr(cys)_2^-$, with dilute acid formed a red solution at pH 3.5, which, as shown by its adsorption onto the cation-exchange

resin, was now cationic. It was obvious that the presence of the acid had by some type of reaction, which was probably a protonation, caused a change in the anionic complex to produce a cationic complex, consistent with this view was the observation that the colour change was reversible on addition of the base which presumably deprotonated the red species. The charge associated with this red coloured species was probably less than 3+ because it was eluted off the column under milder conditions than those used to elute $Cr(H_2O)_6^{3+}$ and was thus bound to the resin less tightly due to less charge density on the complex as a whole.

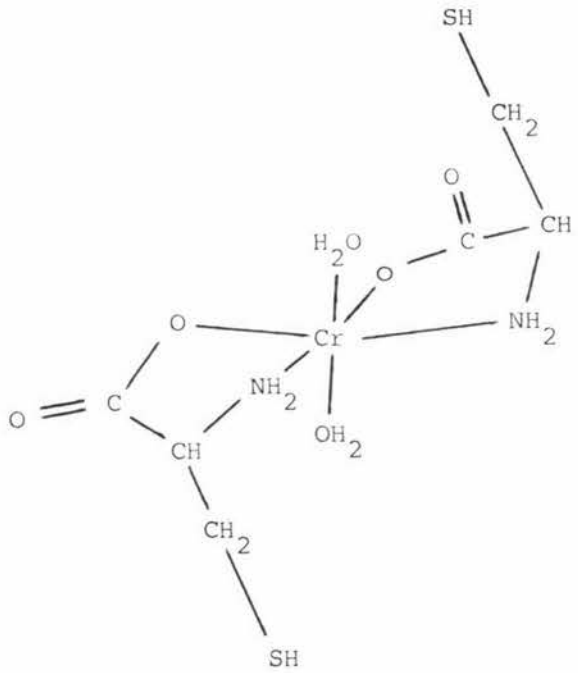
The red species of the dicysteine chromium (III) complex was only observed in acidic solution and under these conditions only the Pair I bands were observed in the electronic spectrum suggesting that no coordination of the sulphydryl group was occurring. In the acidic solution protonation of the coordinated sulphydryl groups would occur and prevent the coordination of the sulphydryl groups, necessitating the coordination of two water molecules to complete the coordination sphere of the chromium. This protonation of the sulphur group and cleavage of the Cr - S bond suggests a very labile bond compared to the Cr - N and Cr - O bonds also present (from the α-amino and α-carboxylate groups of the cysteine ligand). Chromium-sulphur bonds are more labile than Cr - N and Cr - O bonds (Dwyer and Sargeson 1959) and an investigation of the aquation kinetics of thiolatobis (ethylenediamine) chromium (III) complexes has been carried out by Weschler and Deutsch (1973), in which a reversible acid-base equilibrium was found attained via the making and breaking of the single Cr - S bond present in these complexes. However even though the rate of Cr - S band cleavage, measured by Weschler and Deutsch, was found to be approximately 10^4 times faster than the corresponding Co(III) complex, the reaction of acid with $NaCr(cys)_2 \cdot 2H_2O$ was virtually instantaneous in the present study. Weschler and Deutsch concluded that the enhanced lability of the Cr - S bond is due to a greater tendency of sulphur to be protonated when it is coordinated

to chromium. This would strongly favour the efficient acid-dependent aquation for the chromium-sulphydryl complex due to the coordinated sulphydryl group being a stronger Bronsted base. It is possible that in the dicysteine-chromium (III) complex prepared here the effect of the two trans sulphydryl groups causes a greater tendency for protonation due to a trans effect but the exact nature of such a tendency is not known.

The red complex would therefore be $Cr(cys)_2(H_2O)_2^+$ with bidentate coordination of the cysteine molecules but the overall structure would be similar to the original $Cr(cys)_2^-$ complex because no other change in the coordination of the chelated-amino-acid entity would be expected since the cleavage of these bonds (Cr - N and Cr - O) in acid is known to be extremely slow ($Cr(gly)_2(H_2O)_2^+$ is very stable in acid solutions as shown above). The probable structure for $Cr(cys)_2(H_2O)_2^+$ would therefore have the two amino-acid ligands coordinated in an equatorial position with the H_2O ligands trans to one another in axial positions (as in the solid $NaCr(cys)_2 \cdot 2H_2O$ structure) with the sulphydryl groups free as shown in Figure 3.12. This structure is different from that of the related complex $Cr(gly)_2(H_2O)_2^+$ which most likely has the nitrogen and oxygen atoms mutually cis with the H_2O ligands in a cis configuration and not trans as with $Cr(cys)_2(H_2O)_2^+$. The difference is that in the blue cysteine complex the complex is most stable with the coordinated sulphydryl groups in trans positions and on aquation to the red species this configuration is maintained.

At pH 4.0, although the sulphydryl groups will be avaialble for reaction with DTNB, no yellow colour is observable since the thiophenoxide ion is protonated at this pH. However when the solution of $Cr(cys)_2(H_2O)_2^+$ and DTNB was titrated by quickly adding the approximate amount of 2M NaOH to pH 8.0, the immediate colour change indicated either the instantaneous

FIGURE 3.12: STRUCTURE OF RED COLOURED $Cr(cys)_2(H_2O)_2^+$



deprotonation of the thiophenoxide that had already been formed at pH 4.0 (but which was colourless at that pH) or that the titrated sulphydryl group had reacted rapidly with DTNB upon loss of a proton before being able to coordinate with the chromium (III) ion. It is concluded that the sulphydryl group is more available for reaction with DTNB at pH values than 8.0, and this can only be the case if the sulphydryl group is not coordinated to the chromium (III) ion under these conditions. The control reaction of a solution of DTNB at pH 4.0 titrated to pH 8.0 did not yield an intense yellow colour since no free sulphydryl groups were present which further indicated that the reaction of the red dicysteine-chromium (III) complex with DTNB was due to uncoordinated sulphydryl groups.

The structure of the red species (shown in Figure 3.12) had a major difference compared to other amino-acid complexes with chromium (III), that is equatorial coordination of the α -amino and α-carboxylate groups with the trans positions aquated. This could conceivably allow for coordination of

other ligands in the trans position if coordination of the ligand to the chromium (III) atom was stronger than the Cr - S bond possible at pH's above 7.0. It seems likely that the red species could facilitate the preparation of a stable dinicotinate-chromium (III) complex, possibly of the form $Cr(nic)_2(cys)_2$ which would constitute a synthetic GTF-like compound.

3.4.2.3 The reaction of $NaCr(cys)_2 \cdot 2H_2O$ with base

The green species formed when the blue dicysteine complex solution was titrated to pH 8.5 with dilute base was anionic and possibly carried a charge of 1- due to the ease of elution of this species from the anion-exchange column. In a similar fashion to the red species, the green complex was easily changed to the blue species by addition of dilute acid. A good deal of structural similarity is expected between two such readily interconvertible species.

The visible spectrum of the green species was similar to that of $NaCr(cys)_2 \cdot 2H_2O$ with the presence of the two pairs of d-d bands suggesting that the sulphydryl group was again coordinated but the greater intensity of Pair II bands suggested a greater proportion of the second species. It appeared that the strength of the Cr - S interaction was very much dependent on the pH of the solution since the change in intensity of the Pair II bands associated with the sulphur ligand was very pH dependent as discussed previously. With reference to the HSAB theory explained in Section 2, the sulphur type ligands have the least tendency to complex with type (a) metals, which include Cr^{3+}, when compared with nitrogen or oxygen type ligands, so the sulphydryl group of cysteine would have less coordinating ability than the amine or carboxylate groups. Therefore only in basic solution when the sulphydryl group exists predominantly in the form of $-S^-$, and due to the increased stability of the chelating effect of three chelated rings per cysteine ligand, would a Cr - S bond exist resulting in the more intense d-d absorp-

tion bands associated with the sulphur group. The structure of the green species must involve two terdentate cysteine ligands as found in the blue solid with the sulphydryl groups in a trans position and an overall charge of 1-. The green colour of this species differs from that of the blue solution formed on dissolving $NaCr(cys)_2 \cdot 2H_2O$ due to the presence of two species in the later, that is the green and the red forms. However the blue colour of the $NaCr(cys)_2 \cdot 2H_2O$ in crystalline form could only be explained by certain solid state effects possibly resulting from the multitude of hydrogen bonds expected between $Cr(cys)_2$ units, the waters of hydration and the sodium ions present.

3.4.2.5 The pH dependent equilibrium between $Cr(cys)_2(H_2O)_2^+$ and $Cr(cys)_2^-$

Since the equilibrium between the red species $Cr(cys)_2(H_2O)_2^+$ and the green species $Cr(cys)_2^-$ was pH dependent, the protonation of the green species was considered to be the initial step in the aquation of this species. At pH 8.5 the Cr - S bond was found to be quite stable in aqueous solution, but was weaker than the Cr - N and Cr - O bond formed with the α-amino and α-carboxylate amino acid groups. Upon titration with acid the sulphydryl group is protonated and this allows cleavage of the Cr - SH bond followed by aquation of the now vacant ligand positions on the chromium atom as shown in the following mechanism:

Step 1. $Cr(NH_2CH(COO)CH_2S)_2^- + 2H^+ \rightleftharpoons Cr(NH_2CH(COO)CH_2SH)_2^+$

Step 2. $Cr(NH_2CH(COO)CH_2SH)_2^+ + 2H_2O \rightleftharpoons Cr(NH_2CH(COO)CH_2SH)_2(H_2O)_2^+$

By dissolving $NaCr(cys)_2 \cdot 2H_2O$ in water at pH 7.0 an equilibrium is set up such that both the red and green species are present giving the impression that a single blue species is present.

3.4.3 Glutamic Acid Complexes with Chromium (III)

3.4.3.1 The soluble pink glutamic acid complex with chromium (III)

Anion-exchange chromatography at pH 8.5 was successful in seperating a pink solution and a purple solution from the red-violet reaction mixture (obtained on heating Cr^{3+} and glutamic acid in a molar ratio of 1:2 and titrating the solution with NaOH until the pH = 7.0). The charge on the pink species was probably less negative than that on the purple species since the pink fraction was eluted before the purple fraction. On titration to pH 3.0 the pink fraction was also bound to a cation-exchange resin which shows that this species is amphoteric. This type of pH behaviour is expected for a complex with free sidechain carboxylate groups since a bis-type complex of the form: $Cr(glu)_2(H_2O)_2$ would have an overall charge of -1 in basic solution and an overall change of +1 in acidic solution. Such a complex would possess two uncoordinated carboxylate groups which would have pk's of approximately 2.10 (Dawson et al 1969) and consequently at a pH greater than 4.0 would be predominantly ionised while at a pH less than 3.0 they would be predominantly protonated thus explaining the amphoteric nature of the fraction. The cationic form of the pink species could be eluted from the cation-exchange column under similar conditions to those need for $Cr(gly)_2(H_2O)_2^+$ which is consistent with the suggestion that the charge of this species is +1. It therefore seems likely that the pink fraction contains a bis glutamic acid-chromium (III) complex.

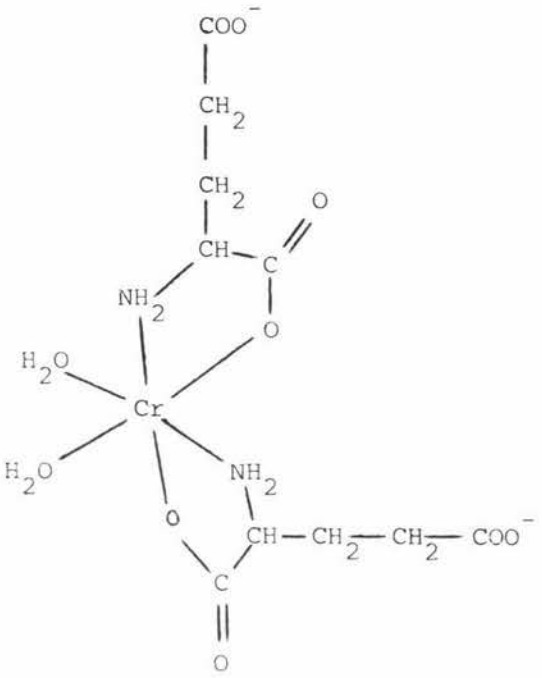
The pink species had absorption maxima at wavelengths which were less than those found for the hexa-aquo complex, $Cr(H_2O)_6^{3+}$ and the positions of the absorption maxima were infact at wavelengths which were similar to those found for the bis-type complex of chromium (III) with glycine which was of the general form $Cr(aa)_2(H_2O)_2$ where aa is bidentate coordinated glycine. The close similarity of the absorption

band positions between these complexes (with glycine and glutamic acid) is consistent with the suggestion that similar coordination of the ligands is involved. Thus the pink chromium (III)-glutamic acid species is likely to be $Cr(glu)_2(H_2O)_2^-$ at pH 9.0 (and $Cr(glu)_2(H_2O)_2^+$ at pH 3.0). The similarity of the visible spectrum of the pink glutamic acid complex with the $Cr(gly)_2(H_2O)_2^+$ complex was also extended to the lack of any splitting in the second absorption band which was consistent with a fac type structure rather than a mer type structure as discussed with the glycine complexes. The fac structure involves a cis arrangement of the glutamic acid ligands and also the water ligands cis to one another as shown in Figure 3.13.

As found for the glycine complex $Cr(gly)_2(H_2O)_2^+$, the pink $Cr(glu)_2(H_2O)_2^-$ species probably involves two bidentate glutamic acid ligands coordinated via the α-amino and α-carboxylate groups acting as ligands in the formation of a chelate ring. This very stable ring structure would be expected to confer sufficient stability on the complex to allow it to persist in the basic solution necessary for its preparation, without the formation of the insoluble olated hydroxy compounds which are normally observed for chromium (III) in basic solution (Rollinson et al 1967). The other carboxylate group was apparently not coordinated probably because coordination would mean the formation of a seven or eight membered chelate ring between either the α-amino group or the α-carboxylate group and the sidechain carboxylate group. Such seven or eight membered chelate rings are generally unstable and hence the formation of such a large chelate ring is improbable and also since the fac structure is present, the tridentate coordination found for the cysteine complex $NaCr(cys)_2 \cdot 2H_2O$ is not possible.

The possibility that the pink species was infact the tris-type complex $Cr(glu)_3^{3-}$ at pH 9.0 was considered but if this were so the positions of the absorption maxima would be

FIGURE 3.13: STRUCTURE OF $Cr(glu)_2(H_2O)_2^-$ AT pH 8.5



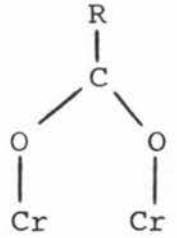
similar to those found for $Cr(gly)_3 \cdot H_2O$ (388nm and 510nm) and a brighter red colour would be expected which is contrary to experiment. In the case of the tris-coordinated complex, $Cr(glu)_3^{3-}$, the uncoordinated carboxylate groups would react with a silver nitrate solution as in the case of $Cr(asp)_3^{3-}$ (Volshtein et al 1957), but the precipitation of $Ag_3Cr(glu)_3$ was not observed confirming that the only bidentate coordinated species present was $Cr(glu)_2(H_2O)_2^-$ (the silver salt of which would be $Ag_2Cr(glu)_2(H_2O)_2^+$ which would be soluble due to the 1+ charge).

The cis, cis geometry of $Cr(glu)_2(H_2O)_2^-$ would make formation of a stable $Cr(nic)_2(glu)_2$ complex improbable for the same reasons as for the bis glycine complex with a similar structure. The only available sites for coordination of nicotinic acid are cis orientated which would cause steric hindrance between coordinated nicotinic acid ligands leading to an unstable complex.

3.4.3.2 The soluble purple glutamic acid complex with chromium (III)

The purple solution was eluted after the pink $Cr(glu)_2(H_2O)_2^-$ species from the anion-exchange column and was therefore thought to be more negatively charged than $Cr(glu)_2(H_2O)_2^-$, but whereas $Cr(glu)_2(H_2O)_2^-$ was stable in acidic solution the purple species could not be later eluted after binding to a cation exchange column. Since the purple solution could not contain bidentate coordinated glutamic acid ligands (the mono complex was unstable, the bis complex was pink in colour and the tris complex was not formed) then it is obvious that the anionic solution must contain glutamic acid coordinated to the chromium (III) in a different manner.

The purple species, however, could not be completely characterised from its electronic spectrum. The positions of the absorption bands were at wavelengths greater than those found in $Cr(H_2O)_6^{3+}$, thus the coordinated ligands must be lower in the spectrochemical series than H_2O and the only possibilities were the two types of carboxylate groups found in glutamic acid, the hydroxide ion, and of course the aquo group. The possibility of chelation through the α-amino and α-carboxylate groups of glutamic acid was ruled out because the presence of amino groups would tend to shift the positions of the absorption maxima to much lower wavelengths as seen in the pink species $Cr(glu)_2(H_2O)_2^-$. However if chelation could occur through the carboxylate group (either one) whereby two chromium (III) ions are bridged by a single carboxylate group via the two oxygen atoms a structure may be envisaged with bridging via the carboxylate group as shown:

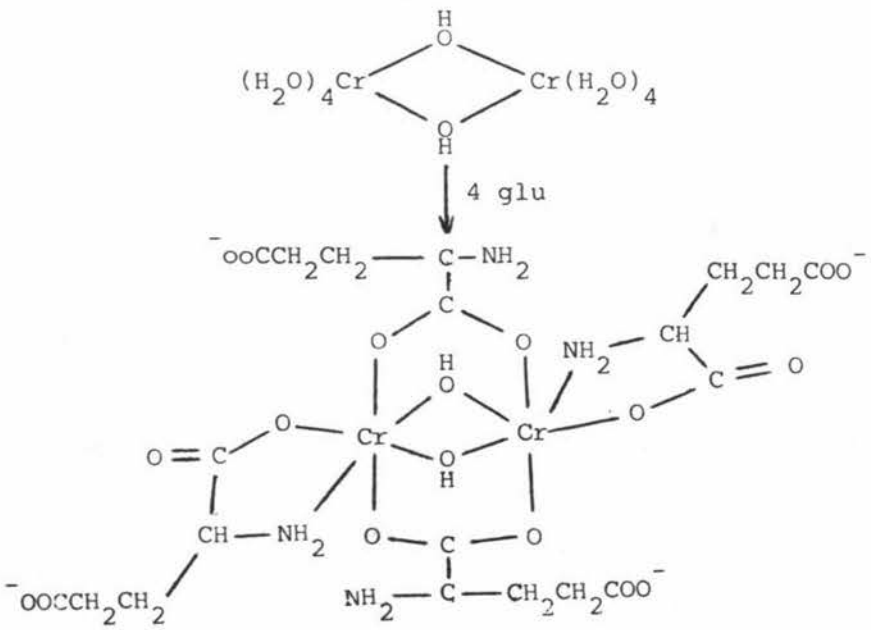


where R is the remainder of the glutamic acid molecule. Such bridging of chromium atoms by carboxylate

groups is found in basic acetate compounds such as $Cr_3O(CH_3COO)_6(H_2O)_3$ with three chromium atoms linked in an equilateral triangle (Cotton and Wilkinson 1972l). This type of coordination could only occur if a chelate ring structure was formed concurrently in order to confer some measure of stability and if a hydroxy-bridge is also included a six-membered chelate ring is formed. This type of coordination might be expected to be equally as stable as a normal chelate ring involving only one Cr(III) atom and a bidentate amino-acid, and would account for the observed spectral properties because only one nitrogen-type ligand is involved the, remaining five ligands being oxygen-type ligands. The proposed structure of this complex would have the form shown in Figure 3.14.

The rather complex glutamic-acid structure shown in Figure 3.14 would possess a 4- charge at pH 8.5 due to the uncoordinated carboxylate groups, but acidifying would probably result in a compound with a 2+ charge which would be expected to be eluted before the $Cr(H_2O)_6^{3+}$ ion. The failure to elute the purple glutamic acid could have been due to some sort of hydrophobic interaction with a chemical reaction with the resin.

FIGURE 3.14: REACTION OF CHROMIUM (III) WITH GLUTAMIC ACID UNDER ACIDIC CONDITIONS



Although the purple coloured (glu)Cr(glu)$_2$(OH)$_2$Cr(glu) complex is relatively stable in solution it has no vacant ligand positions suitable for the coordination of nicotinic acid ligands and thus is not likely to stabilise a chromium-dinicotinate complex.

3.4.3.3 The soluble blue monodentate glutamic acid complex with chromium (III)

In the preparation of the glutamic acid complexes with chromium (III) the initial blue coloured solution which was formed was very similar to the blue solution formed in the preparation of monodentate coordinated glycine complexes at acidic pH, thus it was assumed that the initial coordination of glycine and glutamic acid in acidic solutions were similar. On comparison of the pk's of the α-carboxylate group of glycine (pk_1 2.35) and glutamic acid (pk, 2.10) it seemed probable that in the region of pH 2.0 - 3.0, the carboxylate group of both amino acids would be ionised and thus available for coordination with the chromium (III) ion whereas the other ionisable groups for glycine (amine pk_2 9.78) or for glutamic acid (amine pk_3 9.47 and sidechain carboxylate pk_2 4.07) would be mainly protonated and not available for coordination. Therefore the blue coloured glutamic acid-chromium (III) species is probably monodentate and probably of the form: $Cr(glu)_n(H_2O)_{6-n}^{3+}$ at pH 3.0 as was proposed for the glycine monodentate complexes at acidic pH.

As expected, the blue species (prepared at pH below 4.0) was cationic and bound readily to the cation-exchange column but a mixture of species of the form $Cr(glu)_n(H_2O)_{6-n}^{3+}$ would be difficult to seperate due to the similarity in terms of the overall charge on the members of this series. All the glutamic acid ligands would be coordinated through the α-carboxylate group and since such a species would be vulnerable to olation, the species would only be expected to be stable in acidic aqueous solution.

3.4.3.4 The effect of ammonia on the cationic glutamic acid complexes of chromium (III)

The effect of ammonia elution on the soluble glutamic acid complexes was similar to that found for the $Cr(gly)_2(H_2O)_2^+$ species, the original bound complex was chemically altered by contact with the cation-exchange resin and dilute ammonium hydroxide. In the case of the glycine complex the ammonia solution appeared to have caused the loss of the amine coordination which was found to be temporary because titration with dilute acid (2M HNO_3 until the pH reached 7.0) could restore the amine coordination thus reforming the bidentate coordinated glycine complex. The similarity with the glutamic acid species suggested that a similar reaction had occurred on the cation-exchange column, again causing loss of amine coordination.

3.4.4 Glutamine Complex with Chromium (III)

3.4.4.1 The soluble red glutamine complex with chromium (III)

When $Cr(NH_3)_6(NO_3)_3$ and glutamine in a molar ratio of 1:3 were heated in solution and NaOH added (molar ratio to chromium 3:1) a red solution formed. Two species of glutamine complexes were seperated using ion-exchange chromatography both of which were cationic, with one being red and the other purple in aqueous solution at pH 7.0. The red species seemed unstable when left in acidic solution resulting in the formation of the purple species and this behaviour was accelerated when the red species was bound to the cation-exchange resin. The apparent change in the proportion of red and purple species after contact with the resin suggested that it had catalysed the conversion of the red species, which was eluted first and was thus less positively charged, to the purple species which was eluted at a higher salt concentration thus possessing a greater positive charge. The red species was obviously less positively charged than the tri-positive

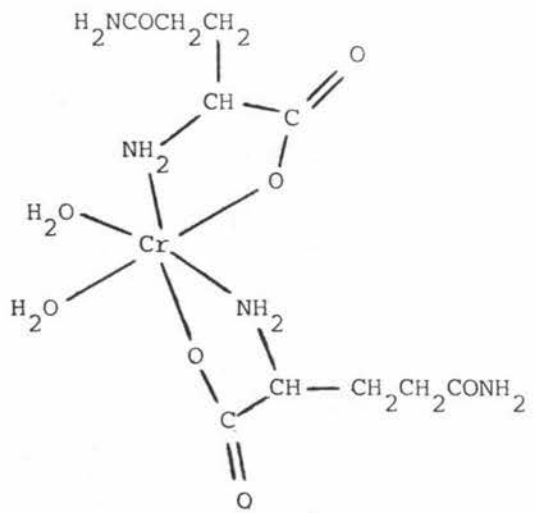
Cr(III) aqueous ions and as judged from the elution conditions probably possessed a charge of +1. At pH 3.5 any $-NH_2$ groups would be protonated and thus contribute a 1+ charge to the complex, but on titration to pH 8.5, these groups would tend to loss their protons and a decrease in positive charge should result. For a species with a charge of +1 at pH 3.5, the loss of one or more protons would confer neutral or anionic character on this species at a more basic pH and since the red species remained cationic at more basic pH it was assumed that it lacked titratable amine groups. The glutamine molecule possesses a basic α-amino group and a neutral amide sidechain. The α-amino group would normally be coordinated to the chromium atom following chelation with the carboxylate group. The amide group is part of the sidechain, and owing to the length of this sidechain coordination of this group was thought to be improbable (the resulting chelate ring would be seven or eight membered which is too large for stability). The sidechain would also be hard to protonate at acidic pH because of its low basicity and would thus not contribute to the overall charge of the complexed species on changing the pH of the solution. This leads to the proposal that the red species possesses two coordinated glutamine groups with the amide groups uncoordinated and hence on summation of the various charges leads to an assignment of a +1 (the coordinated carboxylate groups accounted for a 2- charge and the chromium (III) ion accounted for a 3+ charge).

The positions of the d-d bands in the red species were very similar to those of $Cr(gly)_2(H_2O)_2^+$ (398nm and 535nm), consistent with coordination of the glutamine ligands to the chromium (III) ion by both the caboxylate and the α-amino groups. The coordinated amino groups (which are lower in the spectrochemical series than H_2O) would account for the observed shift in the absorption maxima to lower wavelengths than for $Cr(H_2O)_6^{3+}$. The second absorption band of the red species exhibited no splitting which is consistent with a fac type structure with the coordinated glutamine ligands in a cis

arrangement. In fact the visible spectrum of the red species was so similar to that of the $Cr(gly)_2(H_2O)_2^+$ complex that a bis complex was most likely because a mono or tris complex would shift the visible spectrum to either higher or lower wavelengths compared with a bis complex. A tris glutamine complex would be similar to $Cr(gly)_3 \cdot H_2O$ in both the visible spectrum and such properties as insolubility in water due to a lack of polar groups as discussed with the tris glycine complex.

Since the bis-glycine complex was known to have a cis, cis structure and a general form $Cr(N)_2(O)_4$, the glutamine complex with similar spectral properties was inferred to have the same structure, with the sidechain $-CH_2-CH_2-CONH_2$ group uncoordinated as shown in Figure 3.15. This species because it had a positive charge and several carbonyl groups as well as the cis arranged H_2O ligands was, as expected, very soluble in H_2O. If it had the two amide groups coordinated, the positions of the d-d bands would be shifted markedly to lower wavelengths and the compound would probably be insoluble in H_2O, however a compound with tridentate coordinated glutamine ligands was never isolated.

FIGURE 3.15: STRUCTURE OF $Cr(gln)_2(H_2O)_2^+$



The tris complex with glutamine $Cr(gln)_3 \cdot 2H_2O$ has been prepared by Mizuochi et al (1971) and found to be insoluble in water with reflectance absorption maxima at 395nm and 535nm. These absorption maxima were at lower wavelengths than for the $Cr(gln)_2(H_2O)_2^+$ complex prepared in this thesis because the complex was of the form $Cr(N)_3(O)_3$ which was a direct result of chelation by three amino acid ligands. However when the complex $Cr(gln)_3 .2H_2O$ prepared by Mizuochi was dissolved in perchloric acid the absorption maxima were now similar to those of $Cr(gln)_2(H_2O)_2^+$. Thus by dissolving $Cr(gln)_3.2H_2O$ in dilute acid the stable $Cr(gln)_2(H_2O)_2^+$ species was formed and a similar reaction of $Cr(gly)_3.H_2O$ with acid was discussed above in which the stable $Cr(gly)_2(H_2O)_2^+$ species was formed.

The bis glutamine complex, $Cr(gln)_2(H_2O)_2^+$, is not likely to form a stable $Cr(nic)_2(gln)_2$ complex for the reasons discussed for the glycine and glutamic acid complexes, that is the cis position of the water molecules would hinder the coordination of two nicotinic acid ligands.

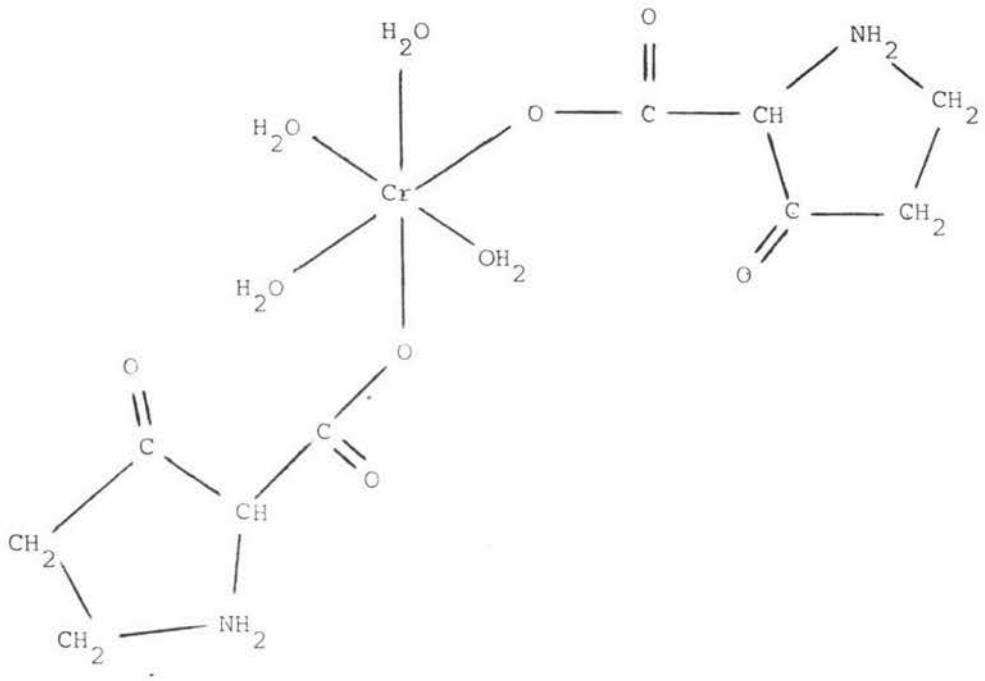
3.4.4.2 The soluble purple glutamine complex with chromium (III)

The reaction of glutamine and simple chromium (III) salts in a molar ratio of 1:3 only formed the purple solution when the solution was boiled initially in a weakly acidic aqueous media. However, it is known that glutamine is not stable under these conditions (Dawson et al 1969) and is rapidly converted in boiling neutral or weakly acidic solution to the ammonium salt of the pyrrolidone carboxylic acid as shown in the following reaction:

Under acidic conditions the glutamine could not coordinate by chelation, but the cyclised form could coordinate to chromium via the carboxylate group. At pH 8.5, the purple species seperated from the pink species on the cation-exchange column was still not anionic, which was in agreement with the suggestion that this species lacked titratable groups as a result of the cyclisation reaction. This complex can be envisaged as having two coordinated pyrrolidone carboxylic acid ligands and each would possess a secondary amide group which could not be protonated at pH 3.5. Such a species would therefore possess a charge of 1+ at pH 3.5 (the coordinated carboxylate groups would account for 2- charge and the chromium (III) ion a charge of 3+) while at more basic pH the overall charge would still be 1+ which accounts for the cationic nature at pH 3.5.

The electronic spectrum of the soluble purple complex suggested that this species was not a chelated complex because the positions of the d-d bands were very similar to those of $Cr(H_2O)_6^{3+}$, which suggested that coordination of the amino groups was not involved in this species. This observation is consistent with the coordination of the carboxylate groups of the cyclic pyrrolidone carboxylic acid as proposed. The purple species was therefore thought to have the general formula of $Cr(pyr)_2(H_2O)_4^+$ in aqueous solution and a structure as shown in Figure 3.16. The two pyrrolidone carboxylic acid ligands possessed carboxylate groups which were expected to be very similar to the original carboxylate groups in glutamine and since all amino acids have similar structures the coordination of the pyrrolidone carboxylic acid ligands would be similar to the monodentate coordination of glycine. However the positions of the d-d bands of $Cr(pyr)_2(H_2O)_4^+$ were slightly lower in wavelength suggesting that the carboxylate group in the pyrrolidone carboxylic acid was less basic than the original carboxylate group in glutamine and thus the position in the spectrochemical series was thought to be somewhere between the H_2O and amino acid α-carboxylate ligands.

FIGURE 3.16: STRUCTURE OF POSTULATED $Cr(pyr)_2(H_2O)_4^+$



Since the purple species seemed to be formed from the pink species from the cation-exchange, the process of cyclisation could occur to a coordinated glutamine ligand as well as the free glutamine. The reaction on the cation - exchange resin could be caused by the acidity of the resin catalysing the cyclisation of the glutamine molecule which would involve breaking of the Cr-α-amino bond initially. Thus cyclisation of the coordinated glutamine would not involve the carboxylate group which would remain coordinated to the chromium (III) atom. Since the pink species is believed to possess two coordinated glutamine groups then the purple species should also possess two as well, if it is assumed that cycisation of glutamine groups affected the amine groups only as discussed above. The elution of the purple species after the pink species from the cation-exchange column was thought to be due to greater interaction of the cyclised complex form with the resin but the exact nature of this was unknown.

3.5 CONCLUSION

In general, all amino acids possess two functional groups which can act as ligands in metal complexes, that is the α-carboxylic acid group and the α-amino group. It appears that chromium (III) coordinates readily with both the α-carboxylate and α-amino groups resulting in the formation of chelated complexes with the amino acid acting in fact as a bidentate ligand and a summary of the complexes formed is shown in Table 3.5. However, both groups on the amino acid were not coordinated simultaneously, a stepwise process of coordination was observed at acidic pH values. The initial coordination of the amino acid was found to occur through the carboxylate group and monodentate complexes were prepared with glycine and glutamic acid. On the addition of base up to neutral pH the amino groups coordinated resulting in chelation of the amino acid ligand. At neutral pH when both $-COO^-$ and $-NH_2$ are present and can compete for chromium it was not possible to determine which group binds first. Chromium (III) is considered to be a hard acid, according to the Theory of Hard and Soft Acids and Bases, and therefore prefers to bind to such hard bases as RNH_2 or $RCOO^{\ominus}$, however at acidic pH's of approximately 4.0 the amine group is protonated and thus is not available for coordination, whereas the carboxylate group is ionised in the form $-COO^-$ which is suitable for coordination to a metal ion. The monodentate complexes formed contained a chromium (III) ion with possibly one to six ligands depending on the concentrations of the reacting Cr^{3+} and amino acids. A high proportion of Cr^{3+} ions would result in more mono-amino acid complex species and a high proportion of amino acid would result in more highly coordinated complex species. In the aqueous solution it was convenient to consider the mixture as a whole because separation of individual species was not accomplished and the glycine and glutamic acid complexes were referred to as $Cr(gly)_n(H_2O)_{6-n}^{3+}$ and $Cr(glu)_n(H_2O)_{6-n}^{3+}$ respectively to signify a mixture of all six possible members of such a series. All of the amino acids: glycine, glutamic acid, cysteine and glutamine formed similar monodentate complexes at acid pH's with chromium (III).

TABLE 3.5: SUMMARY OF AMINO ACID COMPLEXES WITH CHROMIUM (III)

Complex	Colour	Structural features
$Cr(gly)_2(H_2O)_2^+$	red	bis coordinated glycine, fac arrangement
$Cr(glu)_2(H_2O)_2^+$	red	bis coordinated glutamic acid, fac arrangement
$Cr(gln)_2(H_2O)_2^+$	red	bis coordinated glutamine, fac arrangement
$Cr(gly)_n(H_2O)_{6-n}^{3+}$	blue	monodentate coordination
$Cr(glu)_n(H_2O)_{6-n}^{3+}$	blue	monodentate coordination
$Cr(cys)_2(H_2O)_2^+$	red	bis coordinated cysteine, trans water ligands, no sulphydryl coordination
$Cr(cys)_2^-$	green	tridentate coordinated cysteine, trans coordination of sulphydryls

The formation of a chelated chromium (III) complex was accomplished by coordination of the amino group after the initial carboxylate coordination by raising the pH of the solution. The protonated $-NH_3^+$ group was not a particularly good ligand but the addition of base de-protonated this group and together with the additional stability of a chelate ring structure, facilitated the coordination of the amine group. The most prevalent structure in solution was a bis type complex with both the nitrogen and oxygen ligands mutually cis, leaving two aquo ligands in a cis position as well, this type of arrangement was of the fac form and the visible spectrum of $Cr(gly)_2(H_2O)_2^+$, $Cr(glu)_2(H_2O)_2^+$ and $Cr(gln)_2(H_2O)_2^+$ were very similar and consistent with the assigned structure based on theoretical calculations of the predicted d-d bands expected. These bis-type complexes were stable in acidic solution,

but in basic solution olation occurred which resulted in the formation of dimeric structures such as $[Cr(gly)_2OH]_2$.

Since the bis-type amino acid complexes with chromium (III) had the two easily coordinated ligand positions (occupied by aquo groups) in positions cis to one another, the possibility of coordination by nicotinic acid ligands resulting in the formation $Cr(aa)_2(nic)_2$ complexes was considered remote. The cis position would cause considerable steric hindrance of the nicotinic acid groups which would not allow formation of a stable complex. However, the coordination of amino acid ligands to the complex species $Cr(nic)_2(H_2O)_4^{3+}$ was entirely possible in acidic pH solutions but upon raising the pH to allow coordination of the α-amino groups of the amino acid ligands could result in precipitation of the $Cr(nic)_2(H_2O)_4^{3+}$ ions as observed in Section 2 at neutral pH.

The cysteine ligand favoured a different type of chromium (III) complex not seen for the other amino acids due to the presence of a third possible coordinating ligand group (the sulphydryl group). Of the two forms found for the dicysteine complex in solution, coloured red and green, the common structural feature was chelatin of the amine and carboxylate groups in the equatorial positions around the octahedral chromium (III) ion. This left the axial trans positions free for coordination by either the sulphydryl group in the green species $Cr(cys)_2^-$ or a water molecule in the red species $Cr(cys)_2(H_2O)_2^+$ and since the two species were in an acid-base equilibrium both forms retained the planar coordinated α-amino and α-carboxylate groups. The unusual geometry of the cysteine complex was stabilised by the tridentate coordination of the cysteine ligand and was not possible for glutamic acid or glutamine because the respective side-chains were unsuitable for favourable coordination.

The dicysteine-chromium complex is most likely to allow formation of a $Cr(cys)_2(nic)_2$ complex since the trans positions are available for coordination by other ligands. Even at pH 8.0 the Cr - S bond was shown to be relatively weak by the reaction of the sulphydryl group with DTNB and at pH 3.5 the Cr - S bond was sufficiently labile to allow aquation of the trans positions upon protonation of the coordinated sulphydryl groups. The positions of the nicotinic acid ligands in a $Cr(cys)_2(nic)_2$ complex would be the same as in the $Cr(nic)_2(H_2O)_4^{3+}$ prepared but greater stabiity of the complex would be expected due to chelation of the other equatorial positions making olation of the complex at neutral pH not possible.

SECTION FOUR

MIXED LIGAND COMPLEXES WITH CHROMIUM (III)

4.1 INTRODUCTION

Chromium (III) forms complexes easily with both nicotinic acid and various amino acids, as has been demonstrated in the two previous sections of this thesis. The postulated glucose tolerance factor is thought to consist of a chromium (III) complex with nicotinic acid and several amino acids believed to be glycine, cysteine and glutamic acid (Mertz et al., 1974) but such a complex has not been seperated from natural sources, such as Brewer's yeast, or prepared and characterised by synthetic techniques up to the present time. However a chromium-dinicotinate complex structure has been put forward as the essential structural unit for a biologically active GTF analogue (Mertz et al., 1974) but the main problems with such a suggestion seemed to be the instability of such a complex at physiological pH ranges. At pH's greater than neutral, the chromium-dinicotinate complex rapidly precipitated as a grayish substance with a subsequent loss of biological activity.

Several attempts have been made to prepare a synthetic GTF-like compound using chromium (III), nicotinic acid and various amino acids but without much success (Toepfer et al., 1977 and Kienle et al., 1979). Toepfer et al prepared a mixture of complexes by dissolving Cr^{3+} and nicotinic acid in 80% alcohol and refluxing for several hours before adding successively glycine, glutamic acid and cysteine with further refluxing. Separation of a biologically active fraction from this mixture was carried out by loading the crude reaction mixture onto a DOWEX 50 cation-exchange column and eluting with NH_4OH. Further purification of the mixture was carried out using gel

filtration on a Sephadex G15 column and ascending paper chromatography using a 1-butanol/acetic acid/water solvent system but no specific complex that possessed biological activity could be isolated and characterised. Kienle et al carried out a similar preparation of a synthetic GTF-like complex and used thin layer chromatography with silica-gel and an ethanol/water solvent system to separate at least two chromium complexes from the mixture. However other attempts at isolation and purification of chromium complexes failed due to the great diversity of the compounds present.

A different approach was used by Nath and Sidhu (1979) who decided that since the amino acids glycine, glutamic acid and cysteine (thought to be present in GTF) were also the constituents of the naturally occurring tripeptide glutathione then GTF could exist as a complex of chromium (III) with nicotinic acid and glutathione. The synthetic complex preparation involved refluxing chromium (III) and nicotinic acid in 70% ethanol until a blue solution formed, adding reduced glutathione and further refluxing until a purple solution formed. Anion-exchange chromatography using IRA 400 and Sephadex G25 chromatography was used to seperate out an active compound but this compound (or mixture) was not further purified or isolated.

It seems likely that a specific chromium (III)-nicotinic acid complex does exist which can influence the metabolism of glucose in biological systems and the aim of the work reported in this section was to prepare and characterise such a complex since a dinicotinate chromium (III) complex structure is thought to confer biological activity. Attempts were made to prepare a stable mixed ligand complex with the chromium (III)-nicotinic acid structure and which also was biologically active.

4.2 METHODS AND MATERIALS

4.2.1 Source of Chemicals

The compounds: glycine, cysteine hydrochloride, glutamic acid (sodium salt of), and the complex salt $Cr(NO_3)_3.9H_2O$ were obtained as stated in Section 3.2.1. Reduced glutathione was obtained from Sigma and nicotinic acid from Roche. All other chemicals were A.R. grade.

4.2.2 Methods and Instrumentation

4.2.2.1 Chromium determination

Aqueous, chromium containing solutions were analysed as in Section 2.3.2.2.

4.2.2.2 Electronic spectroscopy

Electronic spectra were recorded on a Shimadzu MPS5000 spectrophotometer.

4.2.2.3 Absorbance, pH and conductivity measurement

These measurements were carried out as outlined in Section 2.3.2.6.

4.2.2.4 Ion-exchange resins

The DOWEX 50W-X12 (hydrogen ion form) and DOWEX 1-X8 (chloride ion form) ion-exchange resins were prepared as outlined in Section 2.3.2.7.

4.2.2.5 Gel filtration resins

The gel filtration resins used were Sephadex G10 or G15 which were prepared according to the manufacturers instructions.

4.3 RESULTS

4.3.1 Preparation of a Mixed Solution of Chromium (III) Complexes with Nicotinic acid and Glycine

In the method employed, an intermediate chromium-nicotinate complex solution was prepared first and the glycine was then added to complete the reaction. $Cr(NO_3)_3.9H_2O$ (2.0g, 0.005 moles) was dissolved in H_2O ($20cm^3$) and the pH of the solution adjusted to 3.0 with NaOH (2M). Nicotinic acid (1.23g, 0.01 moles) in H_2O ($20cm^3$) was dissolved by adjusting the pH to 3.0 with HNO_3 (2M) and both solutions were heated to 60°C and mixed. The blue chromium (III)-nicotinate solution appeared after 5 minutes boiling and glycine (0.75g, 0.01 moles) dissolved in H_2O ($10cm^3$) at pH 6.0 and a temperature of 60°C was added quickly. NaOH (2M) was added to the boiling reaction mixture until the solution turned purple and after cooling the pH was 7.4. A slight gray-blue precipitate was filtered but was not studied further.

4.3.1.1 Ion-exchange chromatography

The separation of the complex species present in the purple solution was carried out on a DOWEX 50-X12 cation-exchange column (2.5cm x 9.5cm) prepared as described previously. The solution was titrated to pH 3.5 and loaded onto the cation-exchange column at a conductivity of 3000 µmho and a green solution was eluted with water. On elution with a $NaNO_3$ salt gradient a pink solution was eluted at a conductivity of 20,000 µmho and no further species were eluted up to a conductivity of 50,000 µmho.

A pH gradient was obtained by running a solution of Na_2HPO_4 (0.05M) through the cation-exchange column until the pH reached 9.0 and a single green fraction was eluted. A further gradient was applied by mixing Na_2HPO_4 (0.05M, $100cm^3$) and Na_3PO_4 (0.05M, $100cm^3$) in a gradient mixer and a dark green fraction was eluted.

4.3.1.2 Gel filtration chromatography

The purple solution, prepared in Section 4.3.1 (1.0cm³), was run through a Sephadex G15 column (1.5cm x 38.0cm) and two coloured fractions were obtained, the first was green in colour and the second was red in colour. The elution profile, shown in Figure 4.1(a), includes the chromium concentration, the absorbance at 262nm, and the conductivity of each fraction collected. The green fraction was eluted close to the void volume of the column, while the red fraction and nicotinic acid were eluted close to the conductivity peak.

4.3.1.3 Electronic spectra

The visible spectrum of the green fraction, from the gel filtration chromatography, contained d-d bands at 417nm and 575nm with the intensity of the first band greater than the second band. The presence of a series of bands in the ultra-violet spectrum of the green fraction at 260nm indicated the presence of nicotinic acid.

The visible spectrum of the red fraction contained d-d bands at 407nm and 553nm, both of approximately equal intensity, but no absorbance was found at 260nm.

4.3.2 Preparation of a Mixed Solution of Chromium (III) Complexes with Nicotinic acid and Cysteine

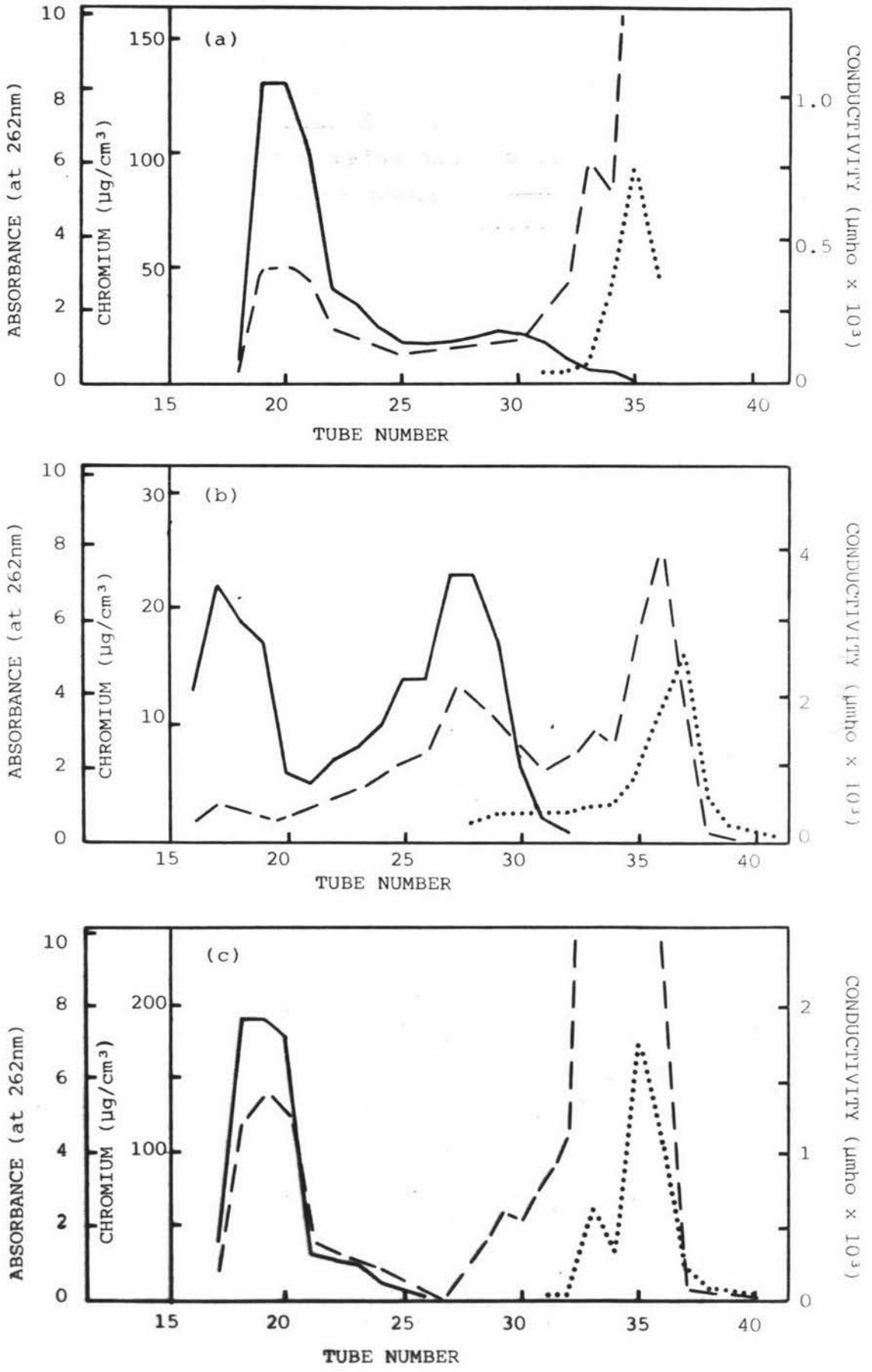
Firstly, the blue complex $NaCr(cys)_2.2H_2O$ was prepared according to the method described in Section 3.3.2.1 and 0.2g (0.0005 moles) was dissolved in water (10cm³) and titrated with HNO_3 (2M) until the pH was 4.0 to form the red species $Cr(cys)_2(H_2O)_2^+$. Nicotinic acid (0.14g, 0.001 moles) was dissolved in water (10cm³) by adjusting the pH of the solution to 4.0 and both solutions were warmed to 90°C before mixing. On boiling, a purple solution formed and NaOH (2M) was added dropwise until the pH was 7.0. The resulting slight gray/blue precipitate was then

FIGURE 4.1(a):
Elution profile of the chromium (III)-nicotinate-glycine reaction mixture on a Sephadex G15 column (1.5cm x 38.0cm). The tube volume was 2.0cm^3 and water was used as the eluent. Chromium (III) (———), absorbance at 262nm (------) and conducitivity (·····).

FIGURE 4.1(b):
Elution profile of the dicysteine chromium (III)-nicotinic acid reaction mixture on a Sephadex G15 column (1.5 cm x 38.0 cm). The tube volume was 2.0cm^3 and water was used as the eluent. Chromium (III) (———), absorbance at 262nm (------) and conductivity (·····).

FIGURE 4.1(c):
Elution profile of the chromium (III)-nicotinate-glutathione reaction mixture on a Sephadex G15 column (1.5cm x 38.0cm). The tube volume was 2.0cm^3 and water was used as the eluent. Chromium (III) (———), absorbance at 262nm (------) and conductivity (·····).

FIGURE 4.1


(a)
(b)
(c)
ABSORBANCE (at 262nm)
CHROMIUM (µg/cm³)
CONDUCTIVITY (µmho × 10³)
TUBE NUMBER

filtered off but continued to prepicipate for over one week.

4.3.2.1 Ion-exchange chromatography

The purple solution obtained above (Section 4.3.2) was titrated to pH 3.5 and loaded onto a DOWEX 50-X12 cation-exchange column (2.5cm x 9.5cm) and a purple solution was eluted with water. The column was then washed with a salt gradient using NaCl and a pink fraction was eluted at a conductivity of 20,000 µmho followed by a blue fraction at a conductivity of 30,000 µmho, however, no further fractions were eluted with higher NaCl concentrations (up to 60,000 µmho conductivity). A pH gradient was now applied by washing the column with Na_2HPO_4 (0.05M) until the pH reached 9.0 and a minor green fraction was eluted at pH 5.0.

4.3.2.2 Gel filtration chromatography

The purple solution obtained in Section 4.3.2 (1.0cm^3) was run through a Sephadex G15 column (1.5cm x 38.0cm) and a red fraction was eluted near the void volume of the column followed by a blue fraction, as shown in Figure 4.1(b). The absorbance at 262nm (characteristic of nicotinic acid) showed a maximum with the blue fraction while unreacted nicotinic acid was eluted near the salt peak. The first red fraction contained no nicotinic acid and the small absorbance at 262nm was thought to be due to coordination of the cysteine ligands.

4.3.2.3 Electronic spectra

The visible spectrum of the red fraction eluted from the Sephadex G15 column contained d-d bands at approximately 410nm and 550nm but no series of bands at 262nm (characteristic of nicotinic acid) was found in the ultra-violet

spectrum. The visible spectrum of the blue fraction eluted from the Sephadex G15 column contained a d-d band at 410nm and a very broad band at approximately 550nm to 600nm whose maximum wavelength was difficult to determine. However, an addition of HNO_3 (2M) th colour changed to red and d-d bands were now found at 410nm and 550nm. The ultra-violet spectrum of the blue fraction exhibited a series of bands at 262nm similar to nicotinic acid.

4.3.3 Preparation of Chromium (III)-glutathione Complexes

A solution containing $Cr(NO_3)_3.9H_2O$ (1.0g, 0.0025 moles) and reduced glutathione (2.30g, 0.0075 moles) in H_2O ($30cm^3$) was boiled and solid NaOH added until a purple gel formed which subsequently dissolved on addition of NaOH (2M) dropwise. The resultant red solution at pH 10.5 was stable and no change in colour was seen on addition of acid or base.

4.3.3.1 Ion exchange chromatography and electronic spectra

The red solution was titrated to pH 8.5 with HNO_3 (2M) and loaded onto a previously prepared DOWEX 1-X8 anion-exchange column at a conductivity of 3,000 µmho. The column was washed with water until no more glutathione was detected (with DTNB reagent - see Section 3.3.2.5) and then washed with a salt gradient from 5000 µmho to 30,000 µmho conductivity in a TRIS buffer at pH 9.0 (both solutions $100cm^3$ volume). A single red solution was eluted at a conductivity of 20,000 µmho which had d-d bands at 400nm and 555nm in the visible spectrum at pH 9.0.

4.3.4 Preparation of a Mixed Solution of Chromium (III) Complexes with Nicotinic acid and Reduced Glutathione

The intermediate chromium (III)-nicotinate solution was prepared as for the other mixed-ligand complexes with

$Cr(NO_3)_3 .9H_2O$ (1.60g, 0.004 moles) and nicotinic acid (0.98g, 0.008 moles). To this blue solution was added reduced glutathione (1.23g, 0.004 moles) dissolved in H_2O ($10cm^3$) at a pH of 3.0 and a temperature of 60°C. Solid NaOH was added to the boiling solution until the colour turned to purple and a violet precipitate formed which dissolved on adding more NaOH. The resultant purple solution was cooled in ice and the pH was found to be 8.4.

4.3.4.1 Gel filtration chromatography and electronic spectra

The purple solution obtained above ($1.0cm^3$) was run through the Sephadex G15 column (1.5cm x 38.0cm) and only one purple coloured fraction was eluted as shown in Figure 4.1(c). The nicotinic acid was eluted just before the salt peak and was also present in the purple fraction since it exhibited a series of bands at 262nm. The purple fraction also exhibited d-d transition bands in the visible spectrum at 404nm and 565nm at pH 8.4.

4.3.5 Preparation of a Mixed Solution of Chromium (III) complexes with Nicotinic acid, Glycine, Cysteine and Glutamic acid

The method used was a modification of the procedure published by Toepfer et al (1977) for the preparation of GTF-like synthetic chromium complexes which are formed by these reactants in the molar ratio of chromium: nicotinic acid: glycine: cysteine: glutamic acid of 1:2:2:1:1. Two modifications were employed, the first being very similar to the Toepfer method in which $Cr_2(SO_4)_3 .15H_2O$ (5.30g, 0.016 moles) was dissolved in 80% ethanol ($750cm^3$) containing glacial acetic acid ($2cm^3$) and neutralised with NH_4OH to pH 7.0. Nicotinic acid (4.00g, 0.032 moles) was added to the chromic solution in a one litre spherical flask and refluxed with stirring for 3 hours,

during which time the colour of the solution remained green. Glycine (2.40g, 0.032 moles), l-glutamic acid-sodium salt (3.00g, 0.016 moles) and cysteine (2.50g, 0.016 moles) were added separately at one-half hour intervals and finally the entire solution refluxed for 3 hours. The resulting purple solution was cooled to room temperature and the insoluble dark purple residue filtered before removing the ethanol by rotary evaportaion.

In the second modification employed in the preparation of the mixed chromium (III) complex solution, the 80% ethanol solvent was dispensed with and instead the reaction was carried out exactly as described before but in aqueous solution. The reaction of chromium (III) with nicotinic acid produced a dark blue solution which changed to dark red-purple with the addition of the amino acids. The resultant solution was reduced by one-half by rotary evaporation however no insoluble material was produced in this reaction.

4.3.5.1 Ion-exchange separation

Ion-exchange seperation of the rather complex crude purple solutions prepared above was carried out by titrating the solutions to pH 3.0 with HCl (2M) and diluting to a conductivity of 2,500 µmho before loading onto a previously prepared DOWEX 50-X12 cation-exchange resin in the hydrogen ion form (column size 2.5cm x 10.0cm). Both the preparations behaved in a similar fashion, that is the purple solution was bound to the resin and a pink solution was eluted with water comprising about one-fiftieth of the original loaded chromium (III). Conductivity gradients were now applied using NaCl solutions mixed in a gradient mixer from 2,500 µmho to 50,000 µmho conductivity and several well defined coloured fractions were eluted as shown in Figure 4.2. The first peak in the absorbance at 280nm was attributed to nicotinic acid which was eluted at 5,000 µmho and was colourless, this

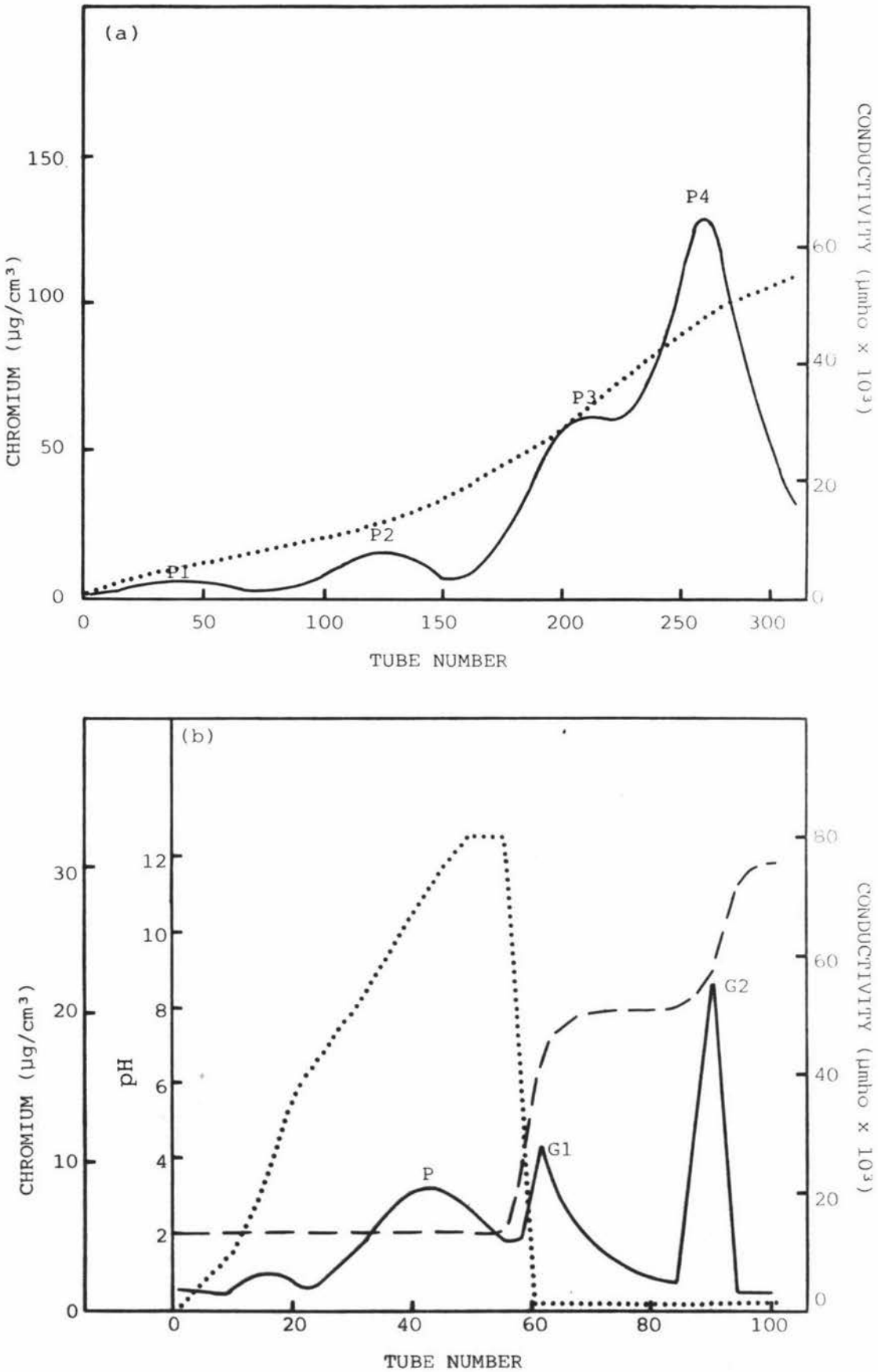
FIGURE 4.2(a):

Elution profile of the cationic chromium (III) complexes prepared in Section 4.3.5 on a DOWEX 50-X12 cation-exchange column (2.5cm x 10.0cm). The tube volume was $10cm^3$ and the eluent reagents are described in results. Chromium (III) (———) and conductivity (·····).

FIGURE 4.2(b):

Elution profile of the cationic chromium (III) complexes prepared in Section 4.3.5 on a DOWEX 50-X12 cation-exchange column (2.5cm x 10.0cm). The tube volume was $10cm^3$ and both conductivity and pH gradients were applied as described in results. Chromium (III) (———), conductivity (·····) and pH (------).

FIGURE 4.2


(a)
CHROMIUM (µg/cm³)
CONDUCTIVITY (µmho x 10³)
P1
P2
P3
P4
TUBE NUMBER
(b)
CHROMIUM (µg/cm³)
pH
CONDUCTIVITY (µmho x 10³)
P
G1
G2
TUBE NUMBER

was followed by a red fraction at 6,000 µmho, a purple fraction at 12,000 µmho, a purple fraction at 25,000 µmho and a green fraction at 45,000 µmho. A pH gradient was then applied by washing the column with Na_2HPO_4 (0.05M) and using the acidity of the column to create a pH gradient in situ up to pH 8.5. A second pH gradient was now applied by mixing Na_2HPO_4 (0.05M) and Na_3PO_4 (0.05M) in a gradient mixer and this was followed by washing with Na_3PO_4 (0.05M) until the pH of the effluent reached 12.0. Two seperate green coloured fractions were eluted with the pH gradient at pH 5.0 and pH 9.0 and no further fractions were eluted after this. The cationic fractions thus seperated are summarised in Table 4.1 together with ratio of chromium (III) to nicotinic acid calculated using the method outlined in Section 2.4.4.1.

TABLE 4.1: CATIONIC FRACTIONS ELUTED FROM DOWEX 50-X12 COLUMN

Number	Conductivity	pH	Chromium:nicotinic acid	Colour
P1	6,000		1 : 68	red
P2	12,000		1 : 2	green (purple initially)
P3	25,000		-	purple
P4	45,000		-	green
G1		7.0	1 : 1	green
G2		9.0	-	green

4.3.5.2 Gel filtration chromatography

Gel filtration was used as a method of seperating complex species from each other and from the sodium salts used in the ion-exchange chromatography with varying degrees

of success. The solutions to be chromatographed were reduced in volume by freeze-drying and were dissolved in a minimum amount of water, or by rotary evaporation and then applied to a column containing G10 Sephadex resin (2.0cm x 60.0cm). The column was then eluted with water and the eluted fractions freeze dried and if still contaminated with salt were rechromatographed.

The purple coloured fraction (P) obtained on loading the reaction mixture (prepared in Section 4.3.6) onto the cation-exchange column and eluting with NaCl (conductivity of 50,000 μmbo) was run through the Sephadex G10 column and the profile of chromium concentration and conductivity, shown in Figure 4.3(a), indicated that the major portion of this fraction was eluted very close to the salt peak. However, if the Sephadex G10 column was run in 50% EtOH a much better seperation from the salt peak occurred as shown in Figure 4.3(b).

When the green coloured fraction (G1), obtained from the above cation-exchange column by elution with Na_2HPO_4 (0.05M), was run through the Sephadex G10 column it was seperated from the salt peak, as shown in Figure 4.3(c). However, it was evident from these gel filtration columns that although good seperation of the chromium (III) complexes from the salt peak occurred there was little seperation of individual complex species from each other.

4.3.5.3 Electronic spectra

The colour of the reaction mixture, derived from Cr^{3+}, nicotinic acid, glycine, cysteine and glutamic acid, was very much dependent on the pH - at pH 3.5 the solution was red and at pH 9.5 the solution was green. Due to the similarity of these colour changes with the dicysteine complex equilibrium studied in Section 3.4.2, a pH titration curve was determined by titrating the solution from pH 3.5 with NaOH (2M) until pH 12.0, as shown in Figure 4.4. Two pk's at approximately 4.3 and 9.0 were estimated

FIGURE 4.3(a):

Elution profile of fraction P on a Sephadex G10 column (2.0cm x 60.0cm). The tube volume was $10cm^3$ and water was used as the eluent. Chromium (III) (_____) and conductivity (------). The first peak was green and the second peak purple in colour.

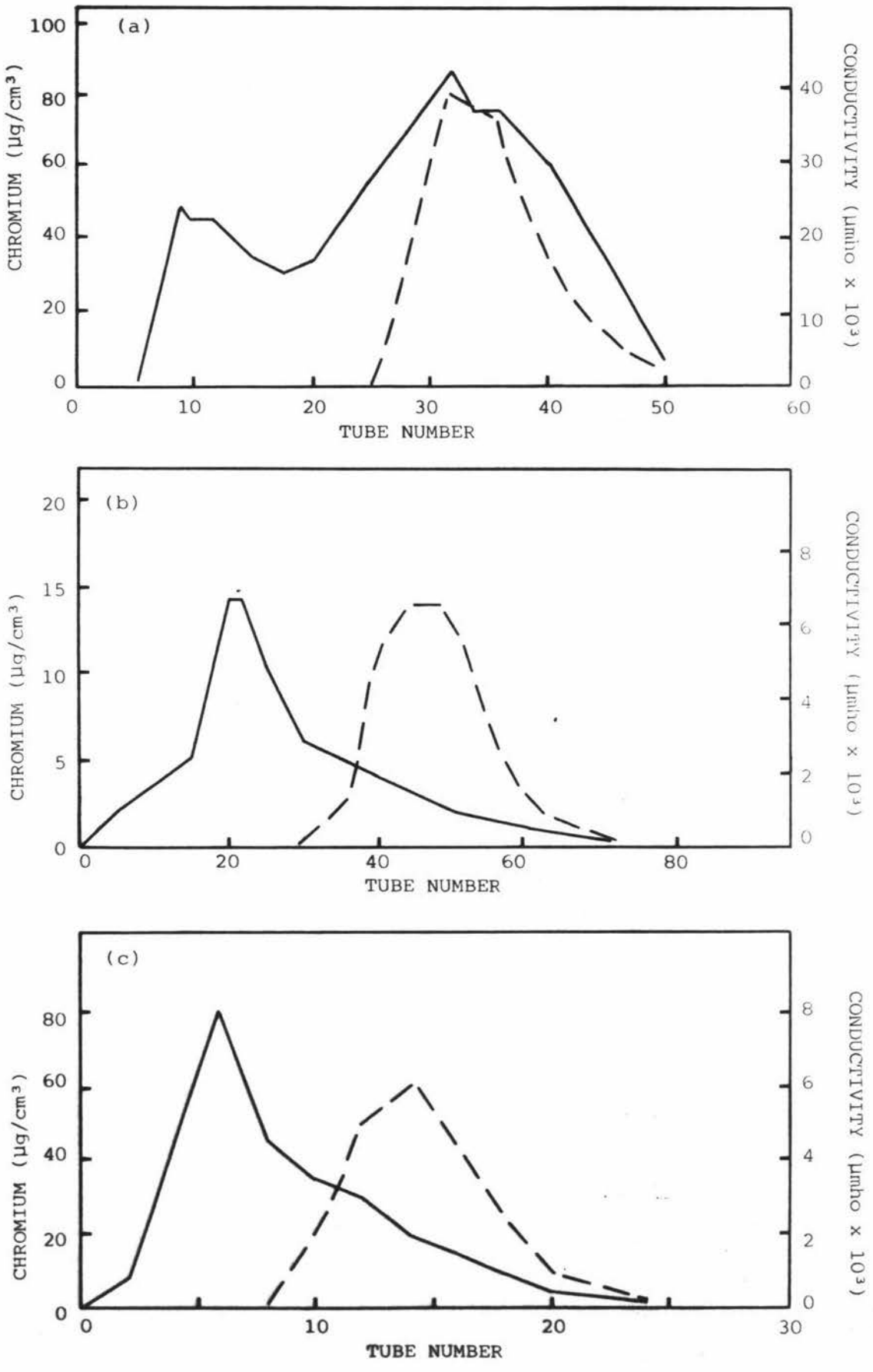
FIGURE 4.3(b):

Elution profile of fraction P on a Sephadex G10 column (2.0cm x 60.0cm). The tube volume was $10cm^3$ and the column was run using 50% EtOH solution as the eluent. Chromium (III) (_____) and conductivity (------).

FIGURE 4.3(c):

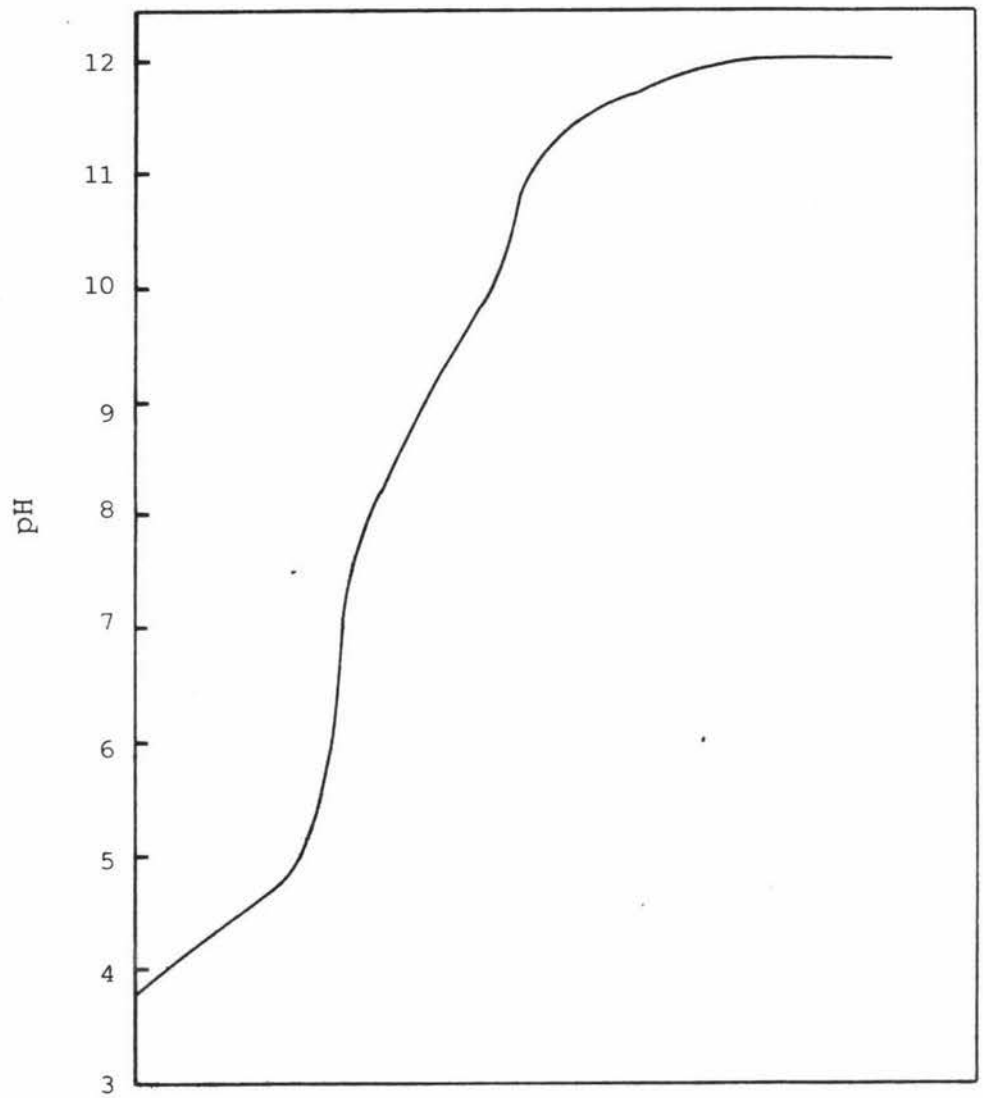
Elution profile of fraction G1 on a Sephadex G10 column (2.0cm x 60.0cm). The tube volume was $10cm^3$ and water was used as the eluent. Chromium (III) (_____) and conductivity (------).

FIGURE 4.3


(a)
CHROMIUM (µg/cm³)
CONDUCTIVITY (µmho x 10³)
TUBE NUMBER
(b)
CHROMIUM (µg/cm³)
CONDUCTIVITY (µmho x 10³)
TUBE NUMBER
(c)
CHROMIUM (µg/cm³)
CONDUCTIVITY (µmho x 10³)
TUBE NUMBER

from this titration curve.

FIGURE 4.4: pH TITRATION OF REACTION MIXTURE



The electronic spectra of the various seperated chromium (III) complexes are shown in Table 4.2. From the ultra-violet spectra, it is obvious that nicotinic acid is present in fractions P_1, P_2, P_3 and G_1, but not present in fractions P_4 and G_2. The visible spectrum of P_2 could not be recorded due to its instability in aqueous solution - concentrating by rotary evaporation turned the purple solution green in colour.

TABLE 4.2: ELECTRONIC SPECTRA OF CATIONIC FRACTIONS

Fraction	Visible bands (nm)		Ultra-violet bands (nm)
P_1	410	562.5	262
P_2	-	-	262
P_3	402	552	262
P_4	430	600	-
G_1	460	615	262
G_2	425	580	258, 290

4.3.5.4 Thin layer chromatography

Since amino acids are easily separated by thin layer chromatography (TLC), it was thought that this technique would also be useful in seperating chromium (III) amino acid complexes. Thin layer chromatography was carried out on glass plates (20cm x 10cm) with a layer of absorbing material, silica gel, suspended in water applied to the plate with a thickness of 0.25mm. The plates were activated before use by placing in an oven at 110°C for 45 minutes and allowed to cool to room temperature. The solvent system used was propan-1-ol/acetic acid/water in the volume ratio of 7:0.5:2.5 and the samples to be chromatographed were spotted onto the plate and developed for 30 minutes before spraying with ninhydrin to visualize the seperated species.

The behaviour of the amino acids: glycine, glutamic acid and cysteine and also nicotinic acid were studied with TLC since these compounds could be present as impurities in any chromium (III) complexes seperated. The migration of the compounds was compared with the distance moved by the solvent front on the TLC plate and expressed as an R_f value by using the following equation:

$$R_f = \frac{\text{distance moved by compound}}{\text{distance moved by solvent front}} \quad \text{Equation 4.1}$$

The R_f values for the amino acids and nicotinic acid are shown in Table 4.3 together with the colour with ninhydrin reagent used to help in identification of these compounds.

TABLE 4.3: THIN LAYER CHROMATOGRAPHY OF AMINO ACIDS AND NICOTINIC ACID

Compound	R_f value	Colour with ninhydrin
glycine	0.42	yellow
glutamic acid	0.14, 0.42	light yellow, purple
cysteine	0.60	yellow
nicotinic acid	0.81	red

The purple coloured fraction, obtained with the reaction mixture prepared in Section 4.3.6 was loaded onto the cation-exchange resin and eluted with NaCl (conductivity of 50,000 μmho), was applied to the TLC plate and after development in the solvent several well seperated species were found. In Table 4.4 the R_f value of the developed spots are shown together with the colour of the ninhydrin reagent and at least seven well defined compounds were present in this sample. When the ninhydrin reagent was not used it was evident that the majority of the sample contained a compound with an R_f value of 0.45.

The green coloured fraction also eluted from the cation-exchange column described above, by elution with a pH gradient obtained by washing the column with Na_2HPO_4 (0.05 M) until the pH reached 9.0, was also applied to the TLC plate and after development with the solvent at least six compounds were well seperated. The R_f values of these compounds are shown in Table 4.5 and it was seen that the majority of the sample was green in colour and did not move with the solvent front. On comparison with the

R_f values of the amino acids it seemed that this green fraction was not contaminated with amino acid impurities but consisted of several chromium (III) complexes. It was also found that the green species with R_f = 0.00 migrated with water as the solvent which suggested a very polar compound being present, however water also dislodged the gel layer from the TLC plate thus rendering it useless.

TABLE 4.4: THIN LAYER CHROMATOGRAPHY OF NON-CHROMATOGRAPHED P FRACTION

R_f value	Colour with ninhydrin	Colour without ninhydrin	Probable Compound
0.04	red		Cr(III)-complex
0.14	red-purple		Cr(III)-complex
0.25	purple		Cr(III)-complex
0.40	yellow		glycine
0.45	purple	purple	Cr(III)-complex
0.58	light yellow		cysteine
0.81	red		nicotinic acid

TABLE 4.5: THIN LAYER CHROMATOGRAPHY OF G_1 FRACTION

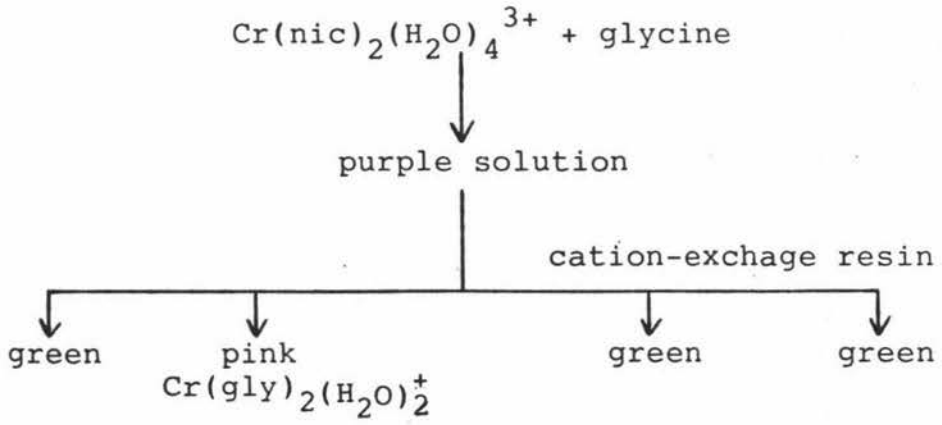
R_f value	Colour with ninhydrin	Colour without ninhydrin
0.00	green	green
0.25	red	
0.32	purple	
0.53	light purple	
0.60	purple	
0.65	red	

4.4 DISCUSSION

4.4.1 Mixed Ligand Complexes of Chromium (III) with Nicotinic Acid and Glycine

When chromium (III) and nicotinic acid were mixed in acidic aqueous solution and boiled together in a molar ratio of 1:2, a mixture of mono- and dinicotinic acid complexes was formed (see Section 2.5) and on addition of glycine, a purple solution was formed when the pH of the reacting solution was increased to 7.0. Cation-exchange chromatography of the purple solution seperated out a green fraction which was eluted with H_2O suggesting that it was a neutral or anionic charged species. A pink fraction was eluted with a salt gradient under conditions which were very similar to those used in eluting $Cr(gly)_2(H_2O)_2^+$ in section 3.3.1.2 which suggests that this fraction was the soluble bis glycine complex. Upon further elution of the cation-exchange column two green fractions were eluted which contained no nicotinic acid, (as shown by a lack of absorbance at 262nm), and must therefore be basic chromic polymers which are cationic and are presumably formed when an aqueous solution of $Cr(H_2O)_6^{3+}$ is heated to boiling. A summary of the cation-exchange chromatography is shown in Figure 4.5.

FIGURE 4.5: SUMMARY OF THE CATION-EXCHANGE CHROMATOGRAPHY OF THE PURPLE $Cr(nic)_2(H_2O)_4^{3+}$ -GLYCINE REACTION MIXTURE

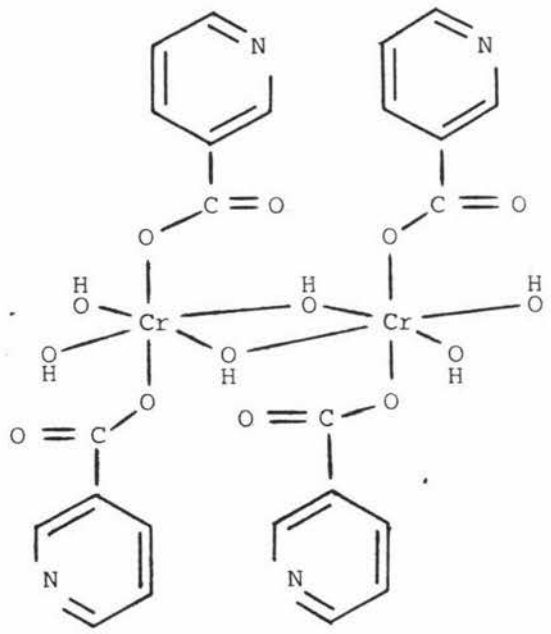


Gel filtration chromatography is useful in seperating out complex species according to molecular size and when the purple solution prepared by the reaction of glycine with $Cr(nic)_2(H_2O)_4^{3+}$ in basic solution, containing a mixture of chromium (III) complexes, was chromatographed on a Sephadex G15 column, good separation of a green species and a pink species was achieved (and found to be similar to those seen in Figure 4.5). The green species was eluted near the void volume of the column suggesting a large molecular size for this fraction and since similarly coloured fractions were eluted from the cation-exchange column, the green fraction most likely consists of polymeric chromium (III) complexes, possibly hydroxy-bridged because the solution was prepared at pH 7.0 at which pH olation is a significant factor. The d-d bands in the visible spectrum were at higher frequencies than those found for $Cr(H_2O)_6^{3+}$ which is consistent with the coordination of such ligands as OH^- which are higher in the spectrochemical series than H_2O, however, the ultra-violet spectrum indicated the presence of nicotinic acid. Since nicotinic acid is a fairly small molecule it was expected to be eluted from the Sephadex G15 column near the salt peak, as observed in Figure 4.1(a), but the presence of nicotinic acid in the green fraction suggested that there was still some coordination of nicotinic acid to chromium (III) in the green fraction. It was not possible to determine the number of nicotinic acid ligands per chromium (III) atom since the green fraction seemed to consist of a mixture of chromium (III) with nicotinic acid ligands and hydroxy-bridged chromium (III) polymers, but the nicotinic acid could be present in a hydroxy-bridged polymer structure such as shown in Figure 4.6.

The pink species which was eluted from both the cation-exchange column and the Sephadex G15 column was easy to identify since the visible spectrum of both species were similar to $Cr(gly)_2(H_2O)_2^+$ which is also pink or red in

colour in aqueous solution. This complex, being relatively small with a molecular weight of approximately 238, was expected to be eluted just before the salt peak on the Sephadex G15 column as in fact was observed and it did not contain nicotinic acid.

FIGURE 4.6: POSTULATED POLYMERIC STRUCTURE OF $[Cr(nic)_2(OH)_2]_n$



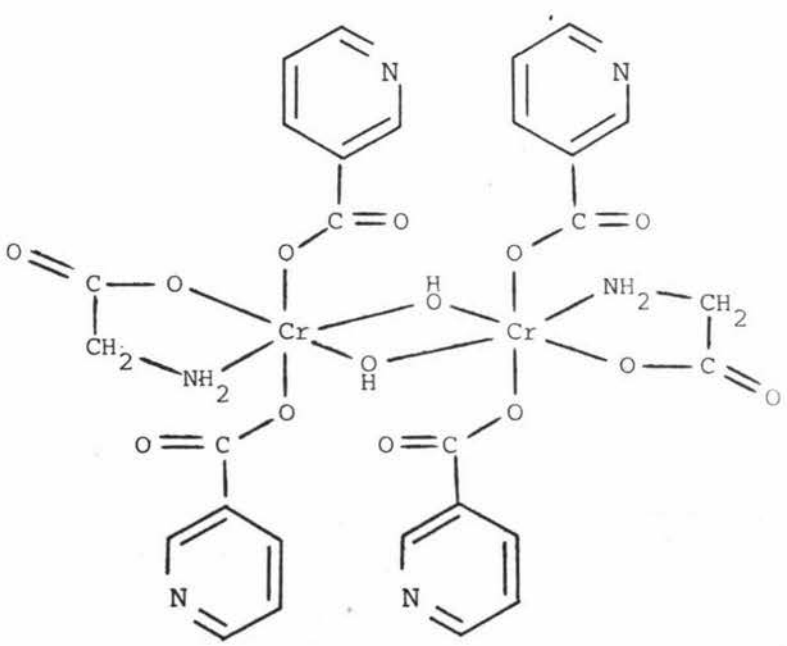
Although it was thought unlikely that nicotinic acid could coordinate to the bis-glycine complex $Cr(gly)_2(H_2O)_2^+$, since the water ligands were in cis positions (as discussed in Section 3.4.1.4), glycine could have been coordianted to $Cr(nic)_2(H_2O)_4^{3+}$ to form $Cr(nic)_2(gly)_2$ but such a compound would be expected to be eluted from the Sephadex G15 column near the salt peak as a consequence of its small molecular size. However, in a polymeric complex such as that shown in Figure 4.6, the glycine ligands could equally well be substituted in place of the bridging OH^- groups, becoming terminal chelating ligands which would stop the formation of large insoluble polymeric

compounds and resulting in the formation of a stable complex such as shown in Figure 4.7. This compound would be expected to possess biological activity, according to the criterion proposed by Mertz et al 1974, due to the trans nicotinic acid ligands, but not undergo olation due to the presence of the glycine ligands which would prevent the substitution of hydroxide ions and is a possible structure for the green complex. The suggested reaction is as follows:

$$Cr(nic)_2(H_2O)_4^{3+} + \text{glycine} \rightarrow \underset{\text{red}}{Cr(gly)_2(H_2O)_2^{+}} + Cr_2(nic)_4 -$$

$$\underset{\text{green}}{(gly)_2(OH)_2} + \text{nicotinic acid}$$

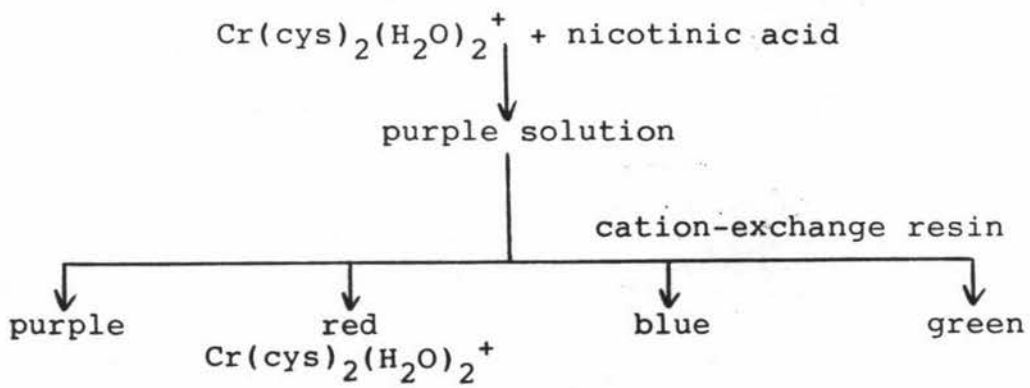
FIGURE 4.7: POSTULATED STRUCTURE OF $Cr_2(nic)_4(gly)_2(OH)_2$



4.4.2 Mixed Ligand Complexes of Chromium (III) with Nicotinic acid and Cysteine

The reaction of $Cr(cys)_2(H_2O)_2^+$ with nicotinic acid at pH 7.0 and a molar ratio of 1:2 resulted in the formation of a purple solution. When this purple solution was applied to a cation-exchange column, a purple fraction was eluted with water suggesting this to be a neutral or anionic species. The first cationic species which was eluted was red in colour and eluted under conditions which were similar to those used for the elution of the red species $Cr(cys)_2(H_2O)_2^+$ in Section 3.3.2.2 suggesting that some of the original starting material was unchanged during the reaction with nicotinic acid. A blue fraction was also eluted, but at a higher salt conductivity, which was similar to the mono-nicotinate chromium (III) complex - the dinicotinate complex, if formed, would not be expected to be eluted from the cation-exchange column due to very tight binding to the resin (see Section 2.5.4). The small green coloured fraction, which was eluted with a phosphate buffer, was very similar to the green coloured soluble hydroxy polymers of chromium (III) found when Cr^{3+} is boiled in aqueous solution and only a small amount of these complexes could be formed because the majority of the chromium (III) was in the form of stable chelated amino acid complexes. A summary of the cation-exchange chromatography is shown in Figure 4.8.

FIGURE 4.8: SUMMARY OF THE CATION-EXCHANGE CHROMATOGRAPHY OF THE PURPLE $Cr(cys)_2(H_2O)_2^+$-NICOTINIC ACID REACTION MIXTURE

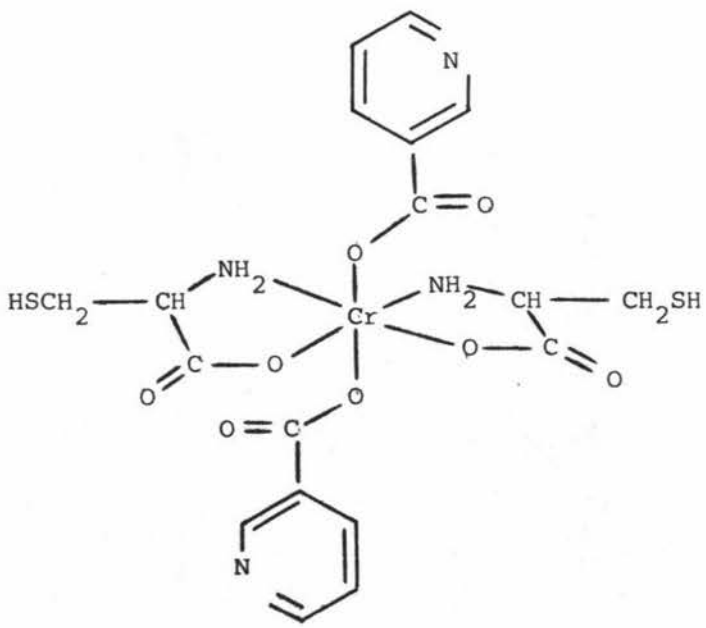


The use of Sephadex G15 columns gave an indication of the relative sizes of the complexes which were formed in the reaction of $Cr(cys)_2(H_2O)_2^+$ with nicotinic acid. Two main coloured species were eluted from the Sephadex G15 column when the reaction mixture was chromatographed. The first was red and eluted very close to the void volume of the column, however, since nicotinic acid was not present in this fraction, the species probably must consist of large (possibly polymeric) chromium (III)-cysteine complexes. The d-d bands of this red fraction were similar to those recorded for other chelated amino acid chromium (III) complexes (Section 3.4) but it was not possible to determine the exact structure of this species. It is conceivable that the sulphydryl groups could form disulphide bridges between several bis-cysteine chromium (III) complexes resulting in a polymeric structure.

The blue fraction was eluted just before the salt peak from the Sephadex G15 column, consistent with the suggestion that it contains smaller, mono-nuclear chromium (III) complexes and since what were thought to be $Cr(cys)_2(H_2O)_2^+$ and $Cr(nic)(H_2O)_5^{2+}$ have been found in the $Cr(cys)_2(H_2O)_2^+$-nicotinic acid reaction mixture, they probably make up the bulk of this fraction. The visible spectrum indicated that $Cr(cys)_2^-$ was present in the blue fraction due to rather broad band in the 550nm to 600nm region characteristic of sulphydryl coordination to chromium (III) and on addition of acid an immediate colour change to red was observed, which is normally seen for the $Cr(cys)_2^- \rightleftharpoons Cr(cys)_2(H_2O)_2^+$ equilibrium (Section 3.4.2.5). The ultra-violet spectrum of the blue fraction exhibited the strong series of bands characteristic of nicotinic acid, and since the bulk of the uncomplexed nicotinic acid was eluted with the salt peak, the presence of nicotinic acid in the larger molecular weight coloured fraction suggests the coordination of nicotinic acid. Although the dinicotinate complex is equally likely to have been formed, at pH 7.0 (conditions of preparation) this species would

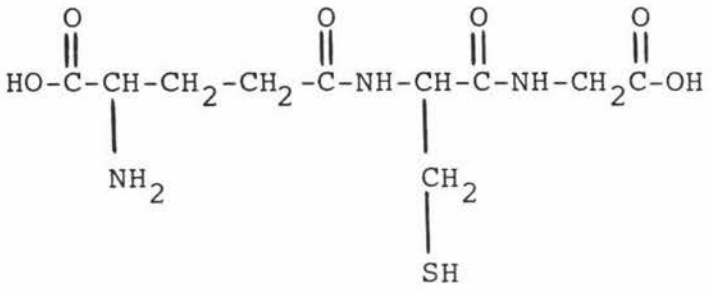
most probably have precipitated as the blue powder which was formed during and after the preparation and since no compounds were found that were eluted from the Sephadex G15 column after the salt peak, the mono-nicotinate complex could not have been formed. The blue fraction must therefore consist of a mixture of $Cr(cys)_2^-$ and mixed ligand complexes of chromium (III), cysteine and nicotinic acid. Since the dicysteine complex was used as a starting material, the complexes $Cr(cys)_2(nic)_2$ and $Cr(cys)_2(nic)(H_2O)$ are likely to be formed but due to their similarity in size they would not be easily seperated using gel filtration. These complexes, unlike the mono-and dinicotinate chromium (III) complexes, did not appear to interact with the Sephadex resin which is probably a result of the coordinated cysteine ligands increasing the size of the complex. It is therefore likely that these complexes also would not interact with the cation-exchange resin and at pH 3.5, since the pyridine ring nitrogen atoms are protonated, both would carry a charge of 1+ which suggests that the blue fraction eluted from the cation-exchange column contains a mixture of both $Cr(cys)_2(H_2O)(nic)^+$ and $Cr(cys)_2(nic)_2^+$ (which is shown in Figure 4.9).

FIGURE 4.9: POSTULATED STRUCTURE OF $Cr(cys)_2(nic)_2$



4.4.3 Chromium (III) Complexes with Glutathione

Glutathione is a tripeptide containing three amino acids and in the reduced form exists as:



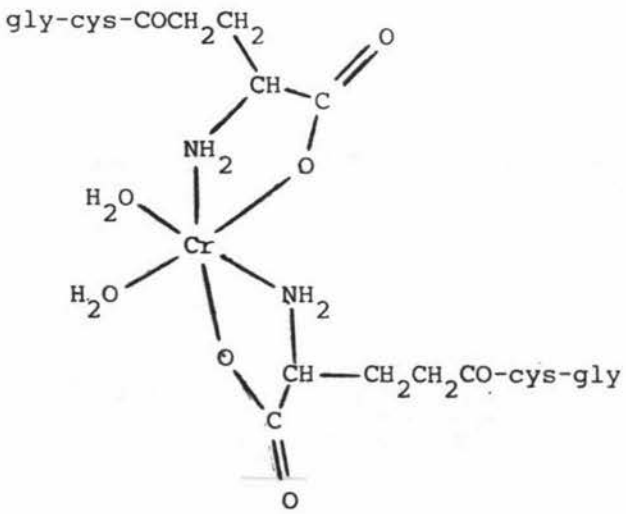
which can be written as: glu-cys-gly. Thus glutathione has two carboxylate groups, one amine group and one sulphydryl group suitable for coordination to chromium (III). A red coloured chromium (III)-glutathione complex was prepared by mixing Cr^{3+} and reduced glutathione in a molar ratio of 1:3, boiling the solution and then adding NaOH until the red solution appeared. The red coloured anionic species was eluted from the anion-exchange column using fairly mild conditions (T.R.I.S. buffer at pH 9.0 and conductivity of 20,000 µmho) which suggests a charge on the complex of 1-, and since the visible spectrum was similar to the bis-glycine complex $Cr(gly)_2(H_2O)_2^+$ a similar bis coordination via a chelated α-carboxylate and α-amino group is suggested. This is possible with glutathione since the glutamic acid residue has both an α-carboxylate and an α-amino group and if two glutathione molecules are coordinated at pH 9.0, a charge of 1- would result for the complex $Cr(glut)_2(H_2O)_2^-$ as shown in Figure 4.10

The lack of coordination of the sulphydryl group is evident since no d-d bands were observed in the 615nm range consistent with Cr-S bonds as found in the dicysteine complex at pH 8.5 ($Cr(cys)_2^-$). The lack of sulphydryl coordination is expected since the chelation of the α-carboxylate and α-amino groups has been found to be very stable and the weaker Cr-S bond can only occur in the formation of a

tridentate complex where no chelate ring is greater in size than five or six members which is not possible with glutathione since the resulting ring size would be too large (ten or eleven members).

The glutathione molecule acts in a similar fashion to single amino acids in its chelation with chromium (III) and like such amino acids as glycine, glutamine, and glutamic acid, was expected to form a bis coordinated complex with a cis geometry resulting in a cis position of the two coordinated water molecules. The differing arrangement of the chelated amino acid ligands in $Cr(cys)_2(H_2O)_2^+$ is a result of the formation of the more stable tridentate complex $Cr(cys)_2^-$ initially and in $Cr(glut)_2(H_2O)_2^-$ there was no reason to suggest a planar arrangement of the glutathione ligands around the chromium (III) atom. The structure of $Cr(glut)_2(H_2O)_2^-$ is shown in Figure 4.10 and due to its similarity with $Cr(gly)_2(H_2O)_2^+$, the coordination of nicotinic acid ligands by substitution of the two cis water ligands, resulting in a stable $Cr(nic)_2(glut)_2$ complex, is not possible due to steric hindrance, as explained in Section 3.4.1.4.

FIGURE 4.10: STRUCTURE OF $Cr(glut)_2(H_2O)_2^-$



4.4.4 Mixed Ligand Complexes of Chromium (III) with Nicotinic Acid and Reduced Glutathione

The reaction of a solution containing chromium (III)-nicotinate complexes with reduced glutathione, in a molar ratio of Cr^{3+}, nicotinic acid and glutathione of 1:2:2 resulted in the formation of a purple coloured solution. When applied to a Sephadex G15 column a single purple coloured fraction, which contained nicotinic acid (as shown by the ultra-violet absorption bands at 262nm), was eluted near the void volume of the column, suggesting a large molecular weight complex. Unreacted nicotinic acid was also eluted before the salt peak. The visible spectrum of the purple fraction was similar to that obtained for $Cr(glut)_2^-$ in Section 4.3.4 but since nicotinic acid was also present in this fraction, a complex of high molecular weight with nicotinic acid coordinated must also be present. Due to the similarity in size of $Cr(glut)_2^-$ and other possible reaction products, such as $Cr(nic)_2(glut)_2$, no seperation was possible using gel filtration techniques. It is therefore very difficult to suggest a possible structure for the chromium (III)-glutathione-nicotinic acid complex which was formed, but if two trans nicotinic acid ligands are present we would expect the compound to be biologically active.

These results were very similar to those reported by Nath and Sidhu (1979) who prepared a purple solution as well, using a similar method, but were unable to isolate a stable nicotinic acid complex. However when an attempt was made to elute the purple solution from an anion-exchange column (DOWEX 1-X8, chloride ion form) using 0.01M NH_4OH, as used by Nath and Sidhu, no chromium (III) complex was eluted, the reason for this behaviuor was not clear.

4.4.5 Mixed Ligand Complexes of Chromium (III) with Nicotinic acid, Glycine, Cysteine and Glutamic acid

The reaction of chromium (III) with nicotinic acid, glycine, cysteine and glutamic acid in the molar ratio to chromium (III) of 1:2:2:1 produced a dark purple solution which contained a great variety of chromium (III) complexes as expected. In the reaction with 80% ethanolic solution, the reaction of Cr^{3+} and nicotinic acid produced a green solution which suggested that the nicotinic acid was not coordinated since no blue solution developed. However, in aqueous solution, the blue colour indicative of $Cr(nic)_2(H_2O)_4^{3+}$ and $Cr(nic)(H_2O)_5^{2+}$ was formed which suggests that this solution would be more suitable for the preparation of a mixed ligand chromium (III)-nicotinate complex.

Ion-exchange separation of the cationic species revealed at least six different chromium (III) complexes present in the above reaction mixture. The seperation on the cation-exchange column was facilitated with a combination of the salt conductivity gradients and phosphate buffer pH gradients which had been used in isolating chromium (III)-nicotinic complexes in Section 2 and chromium (III)-amino acid complexes in Section 3. The conditions of elution of the complexes in this section were compared with those used in the described isolations described in the previous two sections which enabled the partial characterisation of these fractions. Fraction P_1 was eluted under conditions similar to those used for the elution of pure nicotinic acid and this is consistent with the high ratio of nicotinic acid to chromium which was found, however no complex of nicotinic acid or the constituent amino acids was eluted similarly. The visible absorption spectrum of P_1 suggested that this fraction was a chelated compound, due to its similarity with the spectrum of the $Cr(gly)_2(H_2O)_2^+$ species, but the exact structure is not known. Fraction P_2 was not stable in solution but was eluted and behaved in a similar fashion to that observed for the $Cr(glu)_2(H_2O)_2^+$ complex (Section

3.3.3.2) which was also unstable in acidic solution. However, it was not clear why the solution turned green apart from the possibility of a reaction with the nicotinic acid which was present due to overlap of the P_2 band with unreacted nicotinic acid eluted at a lower salt concentration. A large amount of chromium (III) was present in fraction P_3 and the purple colour (with visible bands at 402nm and 552nm) suggests the presence of chelated amino acid complexes, the most likely species being $Cr(gly)_2(H_2O)_2^+$, $Cr(cys)_2(H_2O)_2^+$, and possibly $Cr(nic)(H_2O)_5^{2+}$ (due to the band at 262nm in the ultraviolet region). Fraction P_4 was most probably a mixture of monodentate complexes but the green colour of this solution (λ max 430nm and 600nm) suggested that binuclear hydroxy complexes were present which, since no ultra-violet bands were observed, were obviously not coordinated by nicotinic acid. The green fraction G_1 was eluted at pH5 and corresponded in elution conditions to the nicotinate complex $(H_2O)_3Cr(nic)(OH)(nic)Cr(H_2O)_3$ described in Section 2.5.5 which also had a chromium to nicotinic acid ratio of 1:1. The final fraction which was eluted (G_2) did not contain nicotinic acid and is probably a mixture of basic chromic polymers similar to those prepared by Ardon and Plane (1959) but their structure is not known.

The gel filtration chromatography was successful in separating the purple, P ($P_1 \rightarrow P_4$ fractions), or green, G(G_1 and G_2) fractions from each other and the salt peak, however only broad elution profiles were obtained which suggested that all of the P fractions were of similar molecular size but were smaller than the G fractions. This was consistent with the suggestion that the P fractions comprised small chromium (III)-amino acid complexes while the G fractions consisted of larger hydroxy dimeric or polymer chromium (III) complexes.

The pH dependence of the colour of the original reaction mixture was similar to that observed for an aqueous solution of $NaCr(cys)_2 .2H_2O$ (Section 3.3.2.3). Infact both titration curves were similar, with pk_a's of approximately 3.5 and 9.5, which suggested that a large amount of the dicysteine complex was present and this is not unreasonable since the above complex is very stable in solution (Section 3.4.2.1).

Thin layer chromatography is very useful in the separation of amino-acids and peptides and was found to be successful in seperating at least four chromium (III) complexes present in the reaction mixture as well as unreacted amino acid impurities. For the purple fraction (P), the majority of the solution consisted of a purple material with R_f = 0.45, however, the structures of this and the three other chromium (III)-complexes could not be determined using this technique due to the small scale used. The presence of glycine, cysteine and nicotinic acid suggested that much of these amino acids was unreacted and could easily hinder purification of the chromium (III) complexes. The majority of the green fraction (G) consisted of a green complex which did not migrate with the solvent. This material probably consisted of the basic chromic polymers postulated above, which due to the absence of coordinated amino acids would not give a colour with the ninhydrin reagent. All of the species which were seperated on the TLC plates were thought to be amino-acid complexes due to the positive test with ninhydrin, but the great variety of complexes observed suggested that isolation with ion-exchange chromatography (as above) would not succeed in separating discrete pure chromium complexes.

The seperation of synthetic chromium (III) complexes containing nicotinic acid, glycine, cysteine and glutamic acid has also been attempted by Toepfer et al (1977), but the techniques used were very simple. The purple solution prepared by Toepfer et al was bound to a DOWEX 50 cation-exchange column but instead of using conductivity

or pH gradients to elute the cationic complexes from the resin, they washed the column with 0.1M NH_4OH. This treatment effectively wiped off all the bound material at once, as has been the case with the elution of the various chromium (III)-amino acid complexes studied in Section 3, and would result in the elution of all the bound nicotinic acid, amino acid complexes and also $Cr(nic)(H_2O)_5^{2+}$ if formed, but $Cr(nic)_2(H_2O)_4^{3+}$ would remain tightly bound to the resin. After further seperation using Sephadex G15 columns and ascending paper chromatography, a compound was isolated, reported to be a chromium containing, synthetic GTF analogue and the ultra-violet absorption spectrum and infra-red spectrum were recorded. The ultra-violet spectrum exhibited a band at 262nm thought to be due to coordinated nicotinic acid, but since unreacted nicotinic acid had not been removed during the separation procedure, its presence should have been expected. It was also apparent that the infra-red spectrum of the reported GTF analogue was identical to that of pure nicotinic acid (see Section 2.4.1.5) which suggests that Toepfer et al had succeeded in separating a material which consisted predominantly of unreacted nicotinic acid and not a chromium complex at all.

4.5 CONCLUSION

The preparation of the chromium (III)-dinicotinate complex with the nicotinic acid ligands coordinated to the chromium via the carboxylate group in a trans position was studied in Section 2, however since this complex was unstable in neutral aqueous solution, several attempts were made to prepare a more stable form. This met with limited success since the separation of such a mixed ligand complex consisting of a fundamental chromium (III)-dinicotinate unit with coordinated amino acids replacing the four aquo ions from the $Cr(nic)_2(H_2O)_4^{3+}$ species initially formed,

was very difficult. Other researchers (Toepfer et al 1977 and Kienle et al 1979) decided that since the proposed glucose tolerance factor was believed to contain a chromium (III) complex with nicotinic acid, glycine, glutamic acid and cysteine, then by mixing the constituent compounds together and boiling the required complex should result. When this was attempted a wide range of chromium (III) complexes resulted (as expected) since chromium forms such complexes very readily and since many are structurally very similar, their similar physical properties make separation very difficult. Under the reaction conditions adopted by this study and Toepfer et al (1977), the coordination of the amino acids to chromium (III) was found to be monodentate via the α-carboxylate group since chelation through the α-amino group only occurs if the pH is raised to about 7.0, however a small amount of the chelated species $Cr(gly)_2(H_2O)_2^+$, $Cr(cys)_2(H_2O)_2^+$ and $Cr(glu)_2(H_2O)_2^+$ were found to be formed as well. Thin layer chromatography was successful in separating out several chromium (III)-amino acid complexes but these could not be characterised due to the small scale involved. However this drew attention to the great diversity of both the simple amino acid complexes formed and the more complex polymeric complexes which presumably contained several different ligands. The separation of a stable dinicotinate complex from such a mixture is highly unlikely.

A better approach, from a chemical point of view, is to start with the known dinicotinate complex and try to prepare a mixed ligand complex with single ligands such as glycine, cysteine or glutathione (a tripeptide containing: glycine, cysteine and glutamic acid). With glycine, a green, possibly polymeric, compound (due to its large size) was prepared which contained nicotinic acid, was stable in neutral solution and was thought to be of the form: $Cr_2(nic)_4(gly)_2(OH)_2$. This compound could contain more hydroxide bridges between the chromium atoms, but the terminal glycine ligands would be expected to confer

stability in neutral solution by preventing olation and subsequent precipitation. The reaction of the bis dicysteine complex with nicotinic acid was not so easy to explain. When the blue complex $Cr(nic)_2(cys)_2$ was formed it could not be easily seperated from $Cr(cys)_2(H_2O)_2^+$ due to its similar molecular size but at pH 3.5, although it too possessed a positive charge, the blue species was found separated from the red dicysteine complex, by cation-exchange chromatography using a salt gradient. This complex was stable in neutral solution and did not precipitate due to the coordination of the cysteine ligands which prevented olation. A large molecular weight complex was prepared between the dinicotinate complex and glutathione, but purification of this purple solution was not successful. Although coordinated nicotinic acid was present in the solution, so was the glutathione complex $Cr(glut)_2(H_2O)_2^-$ which could not be separated from the nicotinic acid containing complex probably due to similar size and charge.

The glucose tolerance factor supposedly contains a chromium (III) dinicotinate structure but if this is so stabilisation in neutral solution can only be brought about by incorporation of amino acid ligands to prevent olation. It is likely that only two chelated amino acids can be present since monodentate coordination of a number of amino acids via only the α-carboxylate group is far less stable than bidentate chelation and only four vacant coordination sites, apart from the nicotinic acid ligands, are available. Although a mononuclear chromium (III) complex, such as $Cr(nic)_2(cys)_2$, might be expected to be biologically active, it is equally likely that larger polymeric complexes such as $Cr_2(nic)_4(gly)_2(OH)_2$ would also be active, as long as they possess the coordinated nicotinic acid ligands and are not so large as to present solubility problems in aqueous solution.

SECTION FIVE

THE BIOLOGICAL ACTIVITY OF CHROMIUM (III) COMPLEXES

5.1 INTRODUCTION

Since chromium (III) was postulated to be an essential trace element and thought to be involved in the metabolism of glucose in mammals (Schwarz and Mertz 1959), much work has been done on studying the biological activity of chromium (III) complexes. It has been reported that most inorganic chromium compounds display little or no in vitro insulin potentiating activity but upon conversion to certain organic chromium complexes acquire significant biological activity, however the exact structures of the natural occurring active chromium complexes have not been identified (Mertz et al 1974). Mertz did succeed, though, in preparing an active chromium (III)-nicotinic acid complex solution but was unable to identify the active species. What are presumed to be active chromium (III) complexes have been prepared by mixing chromium (III) salts with other ligands such as nicotinic acid and glutathione (Nath and Sidhu 1979), nicotinic acid and glycine (Anderson et al 1978) or nicotinic acid and the amino acids glycine, cysteine and glutamic acid (Toepfer et al 1977) but the biologically active compounds were not isolated.

Several different complexes of nicotinic acid with chromium (III) and other transition metals were prepared and characterised in Section 2, chromium (III) complexes with several amino acids were prepared in Section 3, and mixed ligand complexes which contained chromium (III), nicotinic acid and several amino acids were prepared in Section 4. The aim of the work reported in this section was to determine the biological activity of these complexes and to investigate the relationship between the structure of the biological forms of the complexes. In this way it was hoped to gain insights into the structure of the naturally occurring glucose tolerance factor.

Several different types of assay system have been developed and employed by various workers to measure the biological activity of natural and synthetic GTF compounds. Since natural GTF extracts were found to reverse the impaired glucose tolerance of rats which were fed on certain stock laboratory diets (Schwarz and Mertz 1959), the earliest assay systems involved the administration of biologically active samples to live laboratory animals to measure their effect. Such an assay system was employed by Tuman et al (1978) who compared the effects of synthetic and natural GTF preparations using normal and genetically diabetic strains of mice. However since assay systems with live rats or mice are far too time consuming, and labour intensive, for routine assaying of large numbers of samples, other systems have been developed. The three main assay systems in use are:

(i) the adaptation of the standard epididymal rat fat pad assay by monitoring the glucose uptake or carbon dioxide production,

(ii) the modification of the epididymal rat fat pad assay using isolated adipocytes as the tissue source, and

(iii) the measurement of the rate of carbon dioxide production during yeast fermentation of glucose, under anaerobic conditions.

The standard epididymal rat fat pad assay system was used by Mertz et al (1961) who raised the rats on a chromium-deficient diet before killing them and removing their fat tissue. The tissue was incubated in oxygenated Krebs-Ringer phosphate medium with the glucose substrate and after two hours, the glucose uptake was measured by either the difference in glucose concentration of the medium compared with the blank sample or the incorporation of radioactive ^{14}C into the fat using glucose-^{14}C. Mertz et al found that chromium (III) compounds of high structural stability,

such as the ethylenediamine complexes or chromium-acetylacetonate, were ineffective. Others showed varying degrees of biopotency depending on the ligands involved, with neutralised solutions of chrome alum, and the bis-biguanide hydroxoaquo chromium sulphate being particularly potent. However, the response to chromium was found to be dependent on the presence of small amounts of insulin. With 1 milliunit of insulin, the neutralised solution of chrome alum showed an optimum response at 0.01 to 0.1 µg of element per flask, which yielded increases in glucose uptake of 88% and 94%, respectively. Of 13 transition elements other than Cr, only manganese, in the form of $MnSO_4.H_2O$, stimulated glucose uptake of the system, but to a lesser degree.

Anderson et al (1978) used isolated adipocytes from rats fed on a low chromium diet because their insulin response to chromium is enhanced (Mertz et al 1961). The advantages over the standard fat pad assay are:

(i) all fat cells become part of an homogeneous pool thus eliminating variation between animals;

(ii) a large number of assays can be performed using few animals;

(iii) nicotinic acid, known to interfere with fat tissue and whole animal bioassays, does not interfere significantly in the fat cell bioassay; and

(iv) greater sensitivity is obtained than for other systems since less insulin was required for a given response using individual fat cells.

Anderson et al (1978) tested the insulin potentiating activity of a synthetic mixture derived from chromium (III), nicotinic acid and glycine (which gave potentiation of 2.76 compared to 1.00 for a control assay, calculated by dividing the amount of radioactive CO_2 released in the presence of insulin plus sample by that released by insulin alone); and also a mixture derived from chromium (III), nicotinic acid and glutathione (potentiation of 3.18).

No potentiation was found for $CrCl_3.6H_2O$ and the individual components of the biologically active synthetic complexes.

The third assay system uses a chromium-deficient yeast strain in a fermentation assay developed by Mirsky et al (1980). Since Brewer's yeast is claimed to be the richest source of GTF, Mirsky et al reasoned that GTF must have some biological function to perform in yeast and based an assay system on this. The assay involved comparing the rate of carbon dioxide production of a yeast sample (of known concentration), supplied with glucose and a sample of a GTF preparation, with a yeast sample supplied with glucose alone. After a lag phase of up to 50 minutes, an increase from the basal rate of carbon dioxide production was found for biologically active GTF samples and no insulin was needed to initiate such a response. Several chromium salts, such as $CrCl_3.6H_2O$, were found to be inactive in the concentration range of 6-150 ng Cr^{3+}/cm^3, however, basic chromium acetate, $[Cr_3O(O_2C_2H_3)_6(H_2O)_3]^+$ caused an enhanced rate of CO_2 production of 15-20%.

The yeast fermentation assay is a very simple method of determining biological activity and, as in the other bio-assay systems, can differentiate between inorganic chromium, which was not biologically active, and certain chromium (III) complexes which were similar in activity to naturally occurring GTF preparations. In the present study of various types of chromium (III) complexes with several amino acids and nicotinic acid, the yeast fermentation assay was used to establish which types of chromium (III) complexes possessed biological activity.

5.2 METHODS AND MATERIALS

5.2.1 Yeast culture

A strain of the yeast *Saccharomyces ellipsoideus*, kindly supplied by Dr Zvi Dori (Technion Institute, Haifa, Israel), was used in the assay system employed by Haylock (1981) who found it to be ideal as a simple, rapid, and sensitive assay.

5.2.2 Pre-assay growth on plating medium

The strain of S. ellipsoideus was grown initially by inoculating on an agar medium plate by streaking to facilitate the seperation of a single cell colony. This medium was prepared in the following manner: glucose monohydrate (1.0g), yeast extract (4.0g) and agar (3.0g) were dissolved in doubly distilled deionised water (100cm^3) and sterilised in a pressure worker at 15 lbs/sq. in. for 10 minutes before filling sterile culture plates. The inoculated plates were placed in an incubator at 30°C for 24-36 hours and afterwards stored in a refrigerator.

Storage of the yeast strain was carried out using the following media preparation: yeast nitrogen base without amino acids (1.34g) (Difco), glucose monohydrate (4.0g) and agar (3.6g) were dissolved in doubly distilled deionised water (200cm^3) and sterilised at 15 lbs/sq. in. for 10 minutes before filling sterile culture plates. The plates were inoculated with a single cell colony by streaking and incubated at 30°C for 24-48 hours before refrigeration.

5.2.3 Pre-assay growth in a liquid medium

The yeast was made chromium deficient by three successive growths in a liquid medium consisting of yeast nitrogen base without amino acids (2.0g) and glucose monohydrate (6.0g) dissolved in doubly distilled deionised water (300cm^3) placed in three conical flasks (100cm^3 in each). At the

incubation temperature of 30°C stationary aerobic growth conditions were used with each successive inoculation occurring as close to the beginning of the previous stationary growth phase as possible (about 24 hours).

5.2.4 Assay growth on a defined medium

A sample of the final pre-assay growth medium (0.1cm^3) was used to inoculate a defined growth medium consisting of sugar, vitamins and essential elements. The medium was prepared as follows: Sugar: glucose monohydrate (20.0g l^{-1}); Vitamins: thiamine hydrochloride (1.25mg l^{-1}), vitamin B_{12} (1.25mg l^{-1}), calcium panthotherate (1.25 mg l^{-1}), nicotinic acid (1.25 mg l^{-1}), pyridoxal phosphate (0.25mg^{-1}), para-aminobenzoic acid (0.25mg l^{-1}), inositol (0.25mg l^{-1}), folic acid (0.025mg l^{-1}) and biotin (0.20 mg l^{-1}); Essential Elements: $CaCl_2.6H_2O$(0.14g l^{-1}), $(NH_4)_2SO_4$ (5.0g l^{-1}), $MgSO_4.7H_2O$ (0.5g l^{-1}), NaCl (0.1g l^{-1}), KH_2PO_4 (0.875g l^{-1}) and K_2HPO_4 (0.125g l^{-1}) dissolved in doubly distilled deionised water and sterilised at 15 lbs/sq. in. for 10 minutes.

As with the other pre-assay liquid medium, the growth conditions were stationary aerobic at 30°C and to limit the yeast to the logarithmic phase the growth time was limited to 20 hours.

5.2.5 Cell harvesting and concentration determination

The yeast cells grown in the defined medium were centrifuged at 4000g for 5 minutes, with two water washes to eliminate any remaining growth medium constituents before taking up in pH 5.7 phosphate buffer (0.062M).

The assay required a constant cell concentration of 1.5 x 10^8 cells/cm^3 and this was determined from a standard curve of optical density against cell concentration established

with standard cell counting techniques on a hemacytometer as described by Haylock (1981) and shown in Figure 5.1.

5.2.6 Standard assay technique

The rate of CO_2 production of the yeast under anaerobic conditions was followed using the Warburg manometric technique. The temperature of the water bath was 30°C.

FLASK: 2.0cm³ of chromium deficient yeast (1.5×10^8 cells/cm³) in pH 5.7 phosphate buffer (0.062M).

SIDE ARM: 0.4cm³ of glucose monohydrate solution (20g l^{-1}), 0.1cm³ of the sample to be assayed.

After loading, the Warburg flask and its manometer were purged with oxygen-free nitrogen for three minutes to ensure totally anaerobic conditions and the flasks were thermally equilibrated for 15 minutes in the water bath.

The assay was initiated by tipping the sugar solution into the yeast solution and continuously agitating between manometer readings at 20 minute intervals.

5.2.7 Manometric calculations and data interpretation

The height (in cm) from the manometer reading was corrected for changes in atmospheric temperature and pressure by subtracting the thermobar values and converted to the volume of CO_2 gas produced in µmoles using the following equation 5.1.

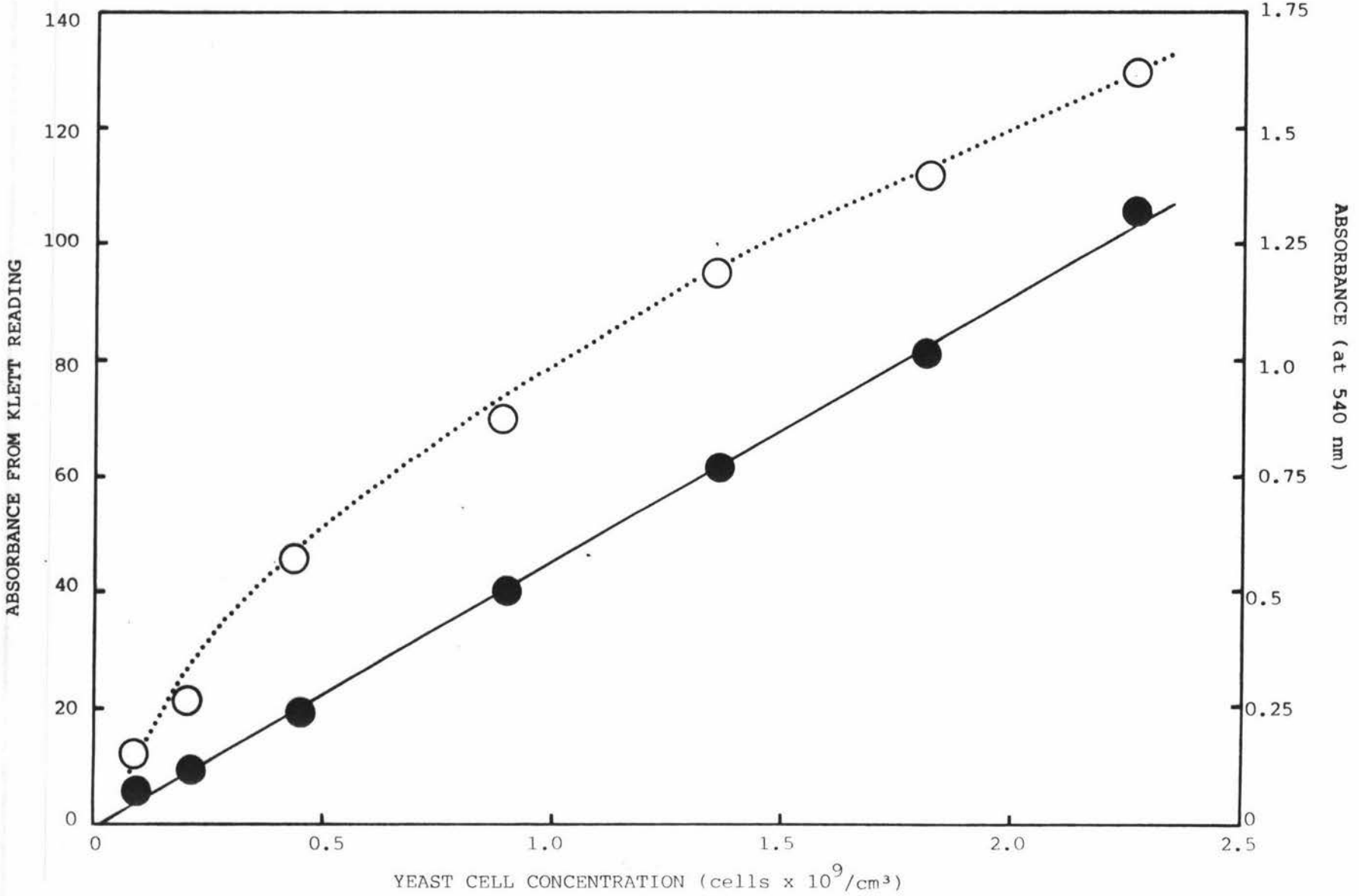
$$\mu\text{moles } CO_2 = H \times \frac{[(V_f + V_m) - V_s] \times 273/303 + (V_s \times S_{CO_2})}{22.4}$$

Equation 5.1

FIGURE 5.1:

Standard curves used in the determination of yeast cell concentration after the growth in the defined medium and prior to assay. Absorbance determined from the Klett reading (●———●) and absorbance determined on a Unicam SP500 spectrophotometer at 540nm (O·····O).

FIGURE 5.1


ABSORBANCE FROM KLETT READING
140
120
100
80
60
40
20
0
ABSORBANCE (at 540 nm)
1.75
1.5
1.25
1.0
0.75
0.5
0.25
0
0
0.5
1.0
1.5
2.0
2.5
YEAST CELL CONCENTRATION (cells x 10^9/cm³)

where H = corrected manometer height (cm)

V_f = volume of Warburg flask (cm^3)

V_m = volume of Warburg manometer (cm^3)

V_S = volume of yeast and sample solution (usually 2.5cm^3)

S_{CO_2} = solubility of CO_2 in water (0.655 at 30°C)

The volume of CO_2 evolved was graphed against time during the assay up to 300 minutes at 20 minute intervals for each sample and compared with a blank sample which contained a phosphate buffer instead of the sample (usually a chromium (III) complex) to be assayed. The slopes of the blank and sample curves were calculated during the last 100 minutes when the slope became linear and a value of the percentage enhancement was determined using equation 5.2.

$$\% \text{ Enhancement} = \frac{\mu\text{moles } CO_2/\text{minute of sample} - \mu\text{moles } CO_2/\text{minute of blank}}{\mu\text{moles } CO_2/\text{minute of blank}} \times \frac{100}{1}$$

5.2.8 Optimum assay conditions

The optimum assay conditions used in the yeast fermentation assay were determined by Haylock (1981). The yeast cell concentration was 1.5×10^8 cell/cm^3 since this concentration ensured optimum rates of evolution for both blank and test assays and was low enough to avoid any errors due to the decrease in glucose concentration during the course of the assay. The glucose solution used was set at 2% and the system buffered at pH 5.70 with a phosphate buffer. All samples to be assayed were of a low conductivity (below 5,000 µmho) and no extremes of pH were allowed.

5.3 RESULTS

Compounds which showed activity in the standard yeast assay were those which exhibited a greater rate of CO_2 evolution than the blank sample. After an initial lag phase which lasted approximately 100 minutes the active samples showed a rapid increase in CO_2 evolution before becoming linear for the final 100 minutes up to 300 minutes after which the assay was stopped. A typical curve for an active sample is compared with that for a blank sample in Figure 5.2 (the active sample used was $Cr(gly)_2(H_2O)_4^{3+}$ at a chromium concentration of 50 $\mu g/cm^3$).

The slopes of several blank samples were determined over a number of assays and found to vary between 1.31×10^{-2} μmol CO_2/min and 1.97×10^{-2} μmol CO_2/min, the mean and standard deviation of the slopes being 1.72×10^{-2} μmol CO_2/min and 0.19×10^{-2} mol CO_2/min for eight different assays. For the slope of an active sample to be considered as significantly different from the blank sample, it was decided to define any slope greater than two standard deviations of the average slope as being biologically active. Such a sample would therefore have a slope at least 22% greater than the blank sample and have a percentage activity of at least 22%.

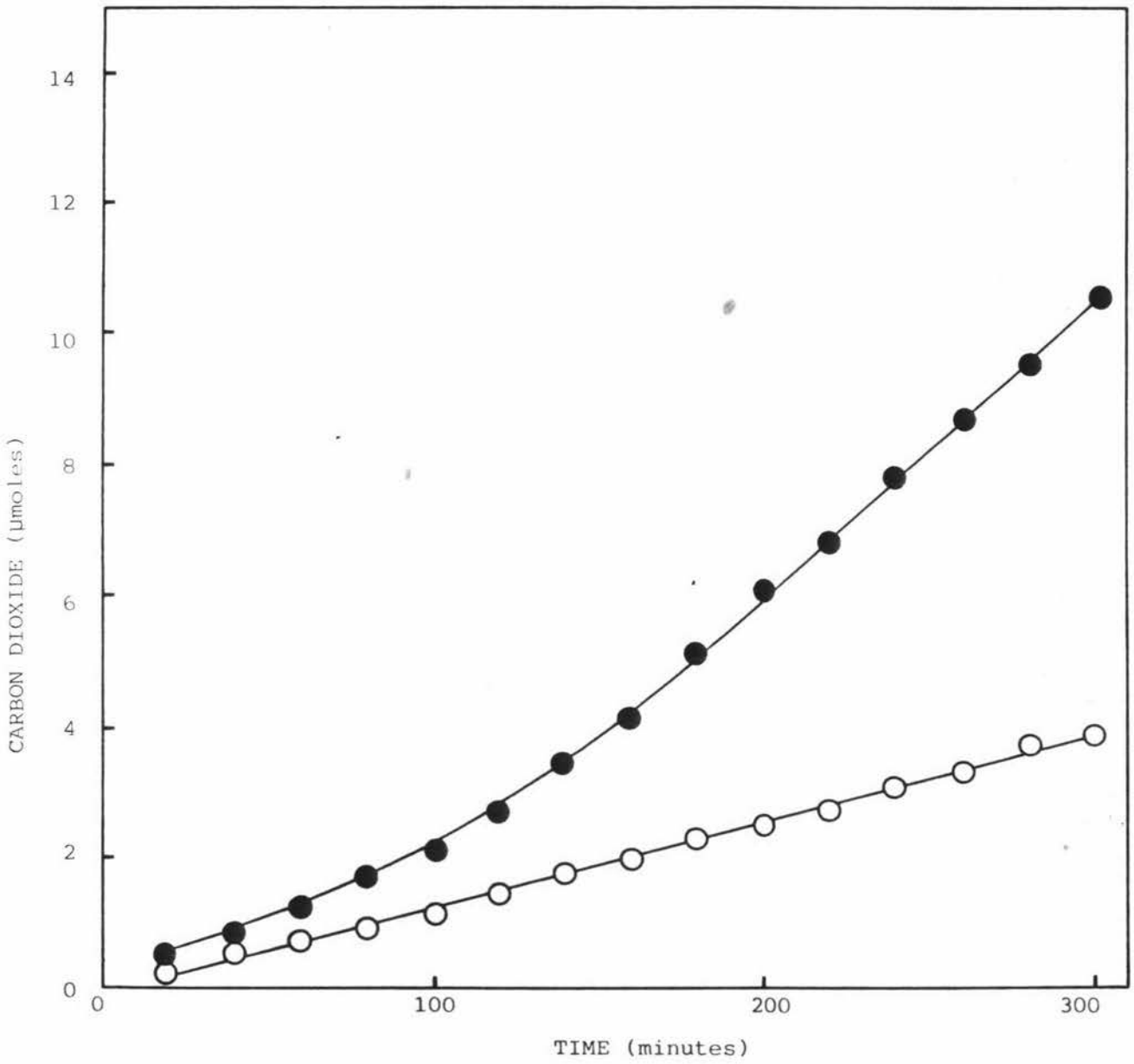
5.3.1 Nicotinic Acid Complexes with Transition Metals

A number of nicotinic acid complexes with transition metals were prepared as described in Section 2. Only the complexes which were soluble in water were assayed and these included the blue chromium (III) nicotinic acid complex species $Cr(nic)_2(H_2O)_4^{3+}$ and $Cr(nic)(H_2O)_5^{2+}$, the yellow chromium (III) nicotinic acid complex $Cr(nic)_2(H_2O)_3OH$ (dissolved in acid or base), and the dinicotinate complexes of Co(II), Ni(II) and Mn(II) dissolved in dilute acid (HNO_3). Other solutions

FIGURE 5.2:

Standard yeast assay showing the carbon dioxide production of a sample of $Cr(gly)_2(H_2O)_4^{3+}$ (●——●) and a phosphate buffer blank (O——O) against time.

FIGURE 5.2


14
12
10
8
6
4
2
0
0
100
200
300
CARBON DIOXIDE (µmoles)
TIME (minutes)

assayed were the unpurified reaction products of the reaction of nicotinic acid with Mn^{3+} or Fe^{3+} ions and pure nicotinic acid. The results of the assays are shown in Table 5.1. The results indicate that only the blue dinicotinate complex of chromium (III) is active in the assay. The dinicotinate complexes of other transition metals are inactive as is nicotinic acid alone.

TABLE 5.1: ACTIVITY OF NICOTINIC ACID COMPLEXES OF TRANSITION METALS

	Compound	Solvent (d)	pH	Conc.(a) (µg/cm³)	Activity (%)
blue	$Cr(nic)_2(H_2O)_4^{3+}$	water	4.0	50	369 ± 50
blue	$Cr(nic)_2(H_2O)_3OH$	acid	1.5	45	-17 ± 4
blue	$Cr(nic)(H_2O)_5^{2+}$	water	5.0	50	1 ± 4
yellow	$Cr(nic)_2(H_2O)_3OH$	acid	1.5	50	20 ± 11
yellow	$Cr(nic)_2(H_2O)_3OH$	base	12.5	50	16 ± 5
	$Co(nic)_2(H_2O)_4$	acid	5.0	50	26 ± 22
	$Ni(nic)_2(H_2O)_4$	acid	5.0	50	27 ± 12
	$Mn(nic)_2(H_2O)_4$	acid	5.0	10	-25 ± 6
	Fe^{3+} + nic	water	5.0	(b)	10 ± 5
	Mn^{3+} + nic	water	5.0	(b)	8 ± 6
	nicotinic acid	water	4.0	100(c)	2 ± 7

(a) concentration in µg/cm³ of metal

(b) concentration and types of species unknown

(c) concentration in µg/cm³ of nicotinic acid

(d) dissolved in water, acid (2M HNO_3) or base (2M NaOH) then titrated to pH given

5.3.2 Chromium (III) Complexes with Various Amino Acids

The amino acid complexes with chromium (III) prepared in Section 3 were also assayed for activity in the yeast fermentation assay. The amino acids involved were glycine, cysteine, glutamic acid, and glutamine and the activities of the various complexes are shown in Table 5.2. The complexes found to be active were the $Cr(gly)_n(H_2O)_{6-n}^{3+}$ mixture (with glycine coordinated to the chromium through the carboxylate group), $Cr(NH_3)_6^{3+}$ and $Cr(gln)_2(H_2O)_2^+$ which showed considerable activity. $Cr(cys)_2(H_2O)_2^+$ was not appreciably active. The activities of the constituent amino acids alone were also determined and are shown in Table 5.3. Only glutamine was found to be active, but this activity was still much lower than that of the active chromium (III)-glutamine complex which was prepared.

TABLE 5.2: ACTIVITY OF CHROMIUM (III) COMPLEXES WITH VARIOUS AMINO ACIDS

Compound	Concentration ($\mu g/cm^3$ Cr)	Activity (%)
$Cr(gly)_2(H_2O)_2^+$	40	- 5 ± 5
$Cr(gly)_n(H_2O)_{6-n}^{3+}$	50	118 ± 5
$Cr(cys)_2^-$	50	43 ± 5
$Cr(cys)_2(H_2O)_2^+$	50	51 ± 7
$Cr(glu)_2(H_2O)_2^+$	40	5 ± 7
$Cr(gln)_2(H_2O)_2^+$	6	322 ± 6
$Cr(NH_3)_6^{3+}$	50	177 ± 7
$Cr_2(SO_4)_3.15H_2O$	10	-14 ± 6

TABLE 5.3: ACTIVITY OF AMINO ACIDS

Amino acid	Concentration $\mu g/cm^3$	Activity (%)
glycine	100	37 ± 9
cysteine	100	33 ± 9
glutamic acid	100	16 ± 6
glutamine	100	90 ± 8

5.2.3 Mixed Ligand Complexes of Chromium (III)

The activities of the mixed ligand chromium (III) complexes prepared in Section 4 were also determined with the yeast assay and the results are shown in Table 5.4(a). Of the mixed ligand complexes of chromium (III)-nicotinic acid with the amino acids glycine or cysteine; or with the tripeptide glutathione, only the green polymeric chromium (III)-nicotinic acid-glycine complex showed any appreciable activity. The activity of $Cr(glut)_2(H_2O)_2^-$ was included in this section because it contains a tripeptide rather than a simple amino acid ligand, but as with the chelated amino acid complexes assayed in Section 5.3.2 it showed no activity.

The mixed ligand complexes which were prepared from a mixture of chromium (III), nicotinic acid, glycine, cysteine and glutamic acid under acidic conditions show varying degrees of activity (see Table 5.4(b)). The active fractions included the cationic fractions P_1 and P_4, and the activity of fraction P was attributed to the presence of P_1 and P_4.

TABLE 5.4(b): ACTIVITY OF MIXED LIGAND COMPLEXES OF CHROMIUM (III)

Compound	Concentration ($\mu g/cm^3 Cr$)	Activity (%)
P_1	8	43 ± 15
P_2	8	10 ± 9
P_3	8	6 ± 1
P_4	8	51 ± 9
P	8	161 ± 11
G_1	8	32 ± 13
G_2	8	-33 ± 10

TABLE 5.4(a): ACTIVITY OF MIXED LIGAND COMPLEXES OF CHROMIUM (III)

Compound	Colour	Concentration ($\mu g/cm^3 Cr$)	Activity (%)
Cr-nic-gly	green	50	128 ± 8
Cr-nic-cys	red	50	19 ± 5
Cr-nic-cys	blue	50	22 ± 4
$Cr(glut)_2(H_2O)_2^-$	red	50	20 ± 6
Cr-nic-glut	purple	50	2 ± 5

5.3.4 The Effect of Ammonium Hydroxide on Chromium (III) Complexes

It was noticed that when some cationic chromium (III) complexes which bound to the Dowex 50W-X12 cation-exchange column were eluted with ammonium hydroxide the previously inactive complex after elution possessed biological activity in the yeast fermentation assay. The samples to be assayed were obtained by loading the appropriate chromium (III) complex at pH 3.5 onto the Dowex 50W-X12 column, washing with water until no chromium could be detected in the effluent by atomic absorption spectrophotometry and finally eluting the column with ammonium hydroxide (0.05M). The ammonia was then removed by vacuum pump until the evolution of gaseous bubbles ceased (about 60 minutes).

The cationic chromium (III) complexes studied were $Cr(glu)_2(H_2O)_2^+$, $Cr(gly)_2(H_2O)_2^+$, $Cr(cys)_2(H_2O)_2^+$ and $Cr(nic)_2(H_2O)_4^{3+}$ and the results obtained are shown in Table 5.5. The $Cr(cys)_2(H_2O)_2^+$ species was still red in colour after elution from the cation-exchange column and showed no apparent increase in biological activity. The $Cr(nic)_2(H_2O)_4^{3+}$ species was bound very tightly to the cation-exchange column and the small amount of material which was eluted by NH_4OH (which could be products from the decomposition of this complex) exhibited virtually no activity. The $Cr(glu)_2(H_2O)_2^+$ and $Cr(gly)_2(H_2O)_2^+$ species after treatment with NH_4OH on the Dowex 50W-X12 column were both blue in colour (in contrast to the red species which were loaded onto the column) and both showed considerable increases in biological activity. The activity of the blue NH_4OH-eluted chromium (III)-glycine species was determined as a function of the chromium concentration and after an initial approximatley linear increase in activity, a plateau in activity was reached, as shown in Figure 5.3.

The $Cr(gly)_2(H_2O)_2^+$ and $Cr(glu)_2(H_2O)_2^+$ species were inactive (see Table 5.2) and only became active after elution with

TABLE 5.5: ACTIVITY OF CHROMIUM (III) COMPLEXES AFTER ELUTION WITH NH_4OH

Complex	Concentration ($\mu g/cm^3$ Cr)	Activity (%)	Discussion
$Cr(glu)_2(H_2O)_2^+$	50	450 ± 40	see Section 3.4.3.4
$Cr(cys)_2(H_2O)_2^+$	10	7 ± 5	
$Cr(gly)_2(H_2O)_2^+$	390	380 ± 15	see Section 3.4.1.5
	200	371 ± 30	
	100	405 ± 24	
	75	279 ± 20	
	50	171 ± 10	(a),(b),(c)
	25	101 ± 10	
	1.0	-25 ± 6	
	0.1	-17 ± 5	
$Cr(nic)_2(H_2O)_4^{3+}$.	46	13 ± 5	

(a) NH_4OH eluted solution titrated to pH 5.0 with HNO_3 (2M) at 50 $\mu g/cm^3$Cr gave 147 ± 12% activity (see section 3.4.1.5 for discussion).

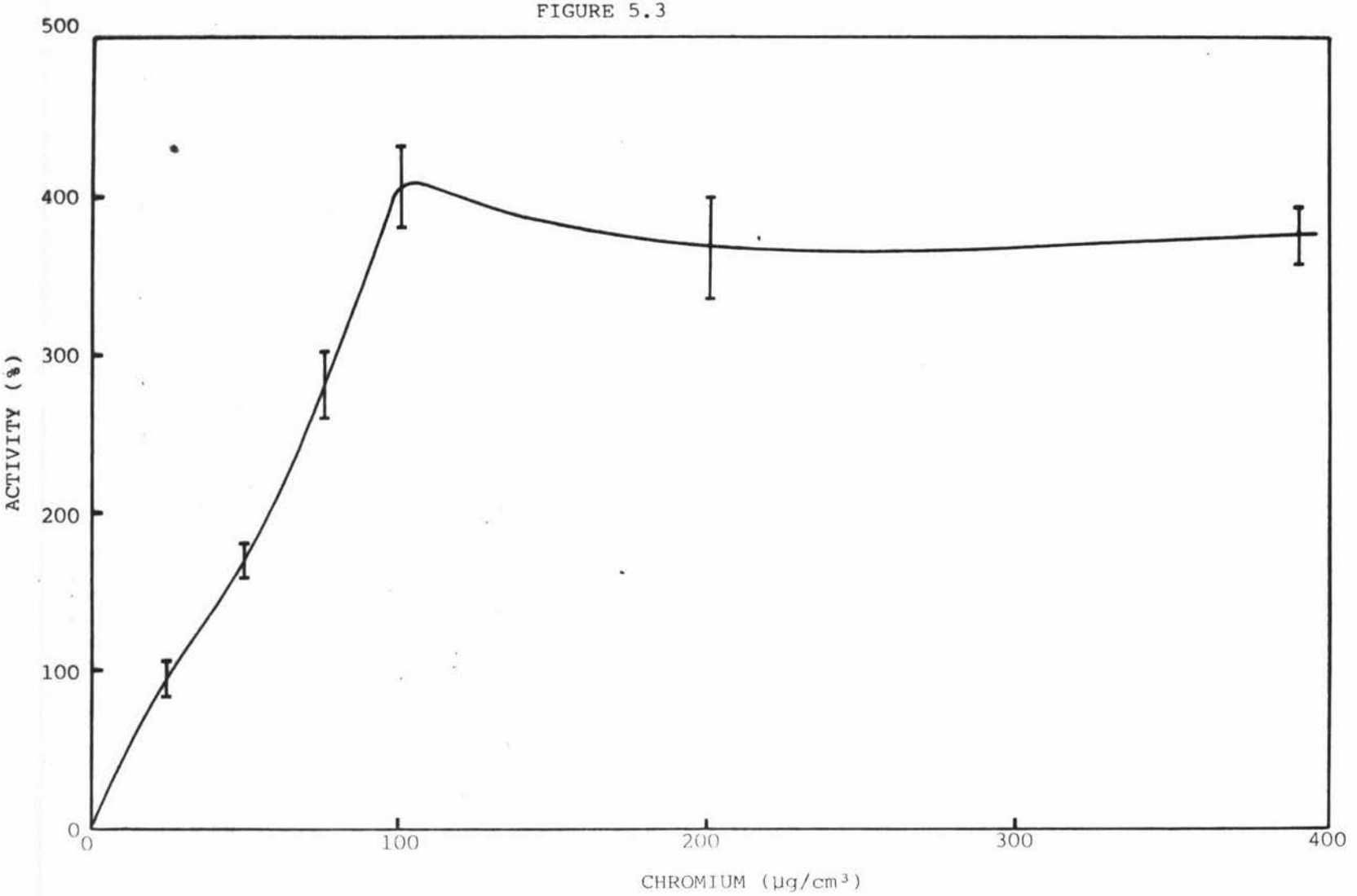
(b) as a control experiment a sample of $Cr(gly)_2(H_2O)_2^+$ was titrated to pH 9.5 with NH_4OH (0.05M) and at 50 $\mu g/cm^3$Cr gave 37% activity (see Section 3.4.1.5 for discussion).

(c) a similar experiment to (b) was performed with LiOH (0.05M) instead of NH_4OH (0.05M) and at 50 $\mu g/cm^3$ gave 37% activity

FIGURE 5.3:

Activity of the NH_4OH-eluted $Cr(gly)_2(H_2O)_2^+$ species as a function of chromium (III) concentration. The least squares standard error for each point is shown as the error bars indicate.

FIGURE 5.3


500
400
300
200
100
0
ACTIVITY (%)
0
100
200
300
400
CHROMIUM (µg/cm³)

NH_4OH from the Dowex 50W-X12 cation-exchange resin. The effect of titration with NH_4OH (0.05M) up to pH 9.5 in the absence of the cation-exchange resin did not produce any activity. The effect of titration with another base, LiOH (0.05M), gave similar results to titration with NH_4OH, that is no increase in activity in the absence of the cation-exchange resin. When the active, blue coloured, NH_4OH-eluted chromium (III)-glycine complex was titrated to pH 5.0 with HNO_3 (2M) the colour changed to red and the biological activity decreased somewhat which suggested that the red coloured solution was either less active than the blue solution or that the red solution was inactive and some of the active blue solution was still present.

The mixed-ligand chromium (III) complexes which were prepared from a mixture of Cr^{3+}, nicotinic acid, glycine, cysteine and glutamic acid (see Section 4.4.5) were loaded onto the Dowex 50-X12 cation-exchange column and also eluted with NH_4OH (0.05M), and the activity of each species before and after the ammonia elution is shown in Table 5.6. From Table 5.6 it can be seen that P1 remained active after elution with NH_4OH as did the unchromatographed P fraction. Fraction P2 remained inactive, while fractions P3 and G1 became more active. The remaining fraction P4 became less active after elution with NH_4OH.

TABLE 5.6: ACTIVITY OF MIXED LIGAND CHROMIUM (III) COMPLEXES AFTER ELUTION WITH NH_4OH

Compound	Before elution Concentration ($\mu g/cm^3 Cr$)	Activity (%)	After elution Concentration ($\mu g/cm^3 Cr$)	Activity (%)
P_1	8	43 ± 15	8	22 ± 8
P_2	8	10 ± 9	8	9 ± 7
P_3	8	6 ± 1	8	66 ± 9
P_4	8	51 ± 9	8	7 ± 6
P	8	161 ± 11	1	53 ± 4
G_1	8	32 ± 13	1	51 ± 3

5.4 DISCUSSION

5.4.1 Nicotinic acid Complexes with Transition Metals

The activity of the nicotinic acid complexes with Cr(III), Ni(II), Co(II) and Mn(II) inidcated that it was only the dinicotinate chromium (III) complex, with the nicotinic acid ligands coordinated via the carboxylate group, which was biologically active in the yeast fermentation assay. The complexes which were studied all possessed two nicotinic acid ligands coordinated to the metal atom in a trans position. Coordination via the pyridine ring of nicotinic acid was established for the yellow $Cr(nic)_2(H_2O)_3OH$ complex and the series $M(nic)_2(H_2O)_4$, where M = Ni(II), Co(II) or Mn(II), in Section 2, however the nitrogen coordinated complexes were inactive. Since the blue species $Cr(nic)_2(H_2O)_4^{3+}$ has a very different type of structure compared to the nicotinic acid complexes with Ni, Co and Mn, it seems that this unique structure is responsible for its biological activity. The attempts at preparation of dinicotinate complexes with Mn(III) and Fe(III) were unsuccessful in that no solid complexes were characterised (see Section 2). The reaction mixtures prepared were not active in the assay which therefore suggests that either no nicotinic acid complexes were formed or that any complexes formed were not in fact active. These proposals indicate that Cr(III) is the only transition metal which forms a stable biologically active dinicotinate complex.

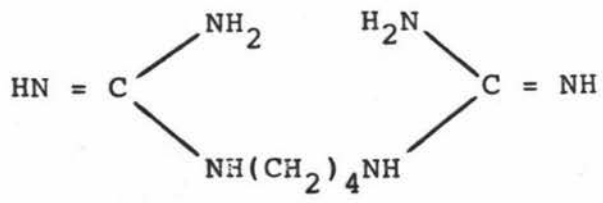
Since chromium (III) forms both mono- and di-nicotinate complexes but only the later form is biologically active this suggests that the axial positions of the ligands is important in determining whether there is any biological activity. The inactivity of nicotinic acid alone makes it unlikely that a single coordinated nicotinic acid group (such as in the mono-nicotinate chromium (III) complex) could cause activity. In the blue $Cr(nic)_2(H_2O)_4^{3+}$ complex, the coordination of the carboxylate groups in a trans

position results in the pyridine ring groups also lying trans across the chromium atom. It is this trans arrangement of the exposed pyridine rings that seems to be an essential requirement for the activity of the complex and certainly complexes of metal ions other than chromium (III), where no analogues containing this structural arrangement could be prepared, were inactive.

5.4.2 Chromium (III) Complexes with Various Amino Acids

The activity of chromium (III) complexes was not confined to the dinicotinate complex alone since the blue species $Cr(gly)_n(H_2O)_{6-n}^{3+}$ (in which glycine is believed to be coordinated in a monodentate fashion via the carboxylate groups), $Cr(gln)_2(H_2O)_2^{+}$ (with bidentate coordination of the α-amino and α-carboxylate groups) and also $Cr(NH_3)_6^{3+}$ were all found to be very active in the yeast assay. When the activity of the various bis coordinated amino acid complexes of chromium (III) were compared, only the glutamine complex showed any activity which suggests that the functional group on the amino acid sidechain is important in imparting biological activity to this complex. In the bis form, the two amide groups of the bound glutamines are at approximately opposite positions across the chromium (III) complex and the high degree of activity of this form suggests that this trans arrangement of the amide groups is responsible for the biological activity. In fact the uncoordinated glutamine also possessed significant activity, although it was much less than when coordinated to chromium (III).

It is known that certain diguanide compounds are effective in the lowering of the blood sugar content of the blood in mammals, and one such compound is 1,4-diguanidinobutane which is an animal base extracted from the mussel Noahs Ark (Grant 1969). This compound can be represented as:



and the main structural feature here seems to be the two guanidine groups at opposite ends of the molecule. In $Cr(gln)_2(H_2O)_2^+$ (Figure 3.15) the two amide groups are also at opposite ends of the complex thus it is possible that the similar arrangement of nitrogen containing groups may elicit a lowering of sugar levels in yeast in a similar fashion to that found in mammalian metabolism of sugars.

Two other complexes of chromium (III) also found to possess biological activity were $Cr(gly)_n(H_2O)_{6-n}^{3+}$ and $Cr(NH_3)_6^{3+}$ which, while not as active as $Cr(gln)_2(H_2O)_2^+$, were more active than the chromic salt $Cr_2(SO_4)_3.15H_2O$ which exists as $Cr(H_2O)_6^{3+}$ in solution. The $Cr(gly)_n(H_2O)_{6-n}^{3+}$ complex was similar in structure to the $Cr(gln)_2(H_2O)_2^+$ complex in the placement of its nitrogen containing $-NH_3^+$ groups which were not coordinated to the chromium (III) atom. It may be the similarity in the location of the nitrogen atoms in these complexes with those in the diguanide compound discussed above that confers activity on these complexes. However, with $Cr(NH_3)_6^{3+}$ the reason for its biological activity was unclear.

5.4.3 Mixed Ligand Complexes with Chromium (III)

The purpose of trying to prepare mixed ligand complexes of chromium (III) was to prepare a dinicotinate complex which was stable in aqueous solution and which also exhibited good biological activity. The most promising way of preparing such a complex with the nicotinic acid ligands in a trans position (which seemed to be essential for biological activity) was to use $Cr(cys)_2(H_2O)_2^+$ as the starting material. In this complex the H_2O ligands are in trans positions at acidic pH in aqueous solution and thus if coordination of nicotinic acid occurred it would be likely to take place in the trans positions. However, the products of the reaction of nicotinic acid with $Cr(cys)_2(H_2O)_2^+$ (an unidentified

red complex and a blue fraction consisting of $Cr(cys)_2^-$ and possibly $Cr(cys)_2(nic)_2$ and $Cr(cys)_2(nic)(H_2O))$ were not active in the assay. This result would be expected either if the dinicotinate dicysteine complex was not formed or if the free sulphydryl groups present in such a complex interfered with the activity of the trans $Cr(nic)_2$ unit in some way. In the mono-dentate amino acid complexes which were prepared by eluting bis amino acid complexes from a cation-exchange column with NH_4OH (see Section 5.4.4), the sulphydryl groups in the cysteine complex $Cr(cys)_2(H_2O)_4^{3+}$ seemed to inhibit the activity of this complex and it was noticed that if the side chain group containing the sulphydryl group was replaced by a -H group or an alkyl carboxylate group the activity increased significantly. It seems possible, in hindsight, that the $Cr(cys)_2(nic)_2$ complex, if formed, would be also inactive due to the presence of free sulphydryl groups.

The green coloured polymeric complex formed when $Cr(nic)_2(H_2O)_4^{3+}$ was reacted with glycine was active in the assay. This compound was also stable in aqueous solution at pH 7.0 which suggests that we have succeeded in preparing a stable complex with the nicotinic acid ligands presumably in a trans position since the starting material contained this structure. The activity could not be associated with the presence of free, uncoordinated amine groups in the complex because with the reaction conditions used (pH 7.0) the amine groups would also be coordinated with the formation of a chelated amino acid complex of the form $Cr_2(nic)_4(gly)_2(OH)_2$, as shown in Figure 4.7.

5.4.4 The Effect of Ammonium Hydroxide on Chromium (III) Complexes

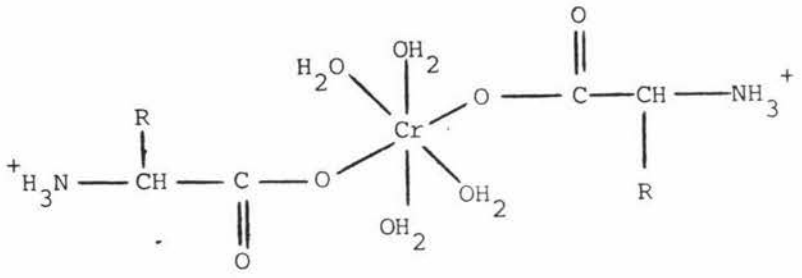
When certain amino acid complexes with chromium (III) were bound to the cation-exchange resin, Dowex 50W-X12 (hydrogen ion form), a previously inactive complex became considerably

more active in the yeast fermentation assay after elution with NH_4OH. The effect on the type of coordination of ammonia eluted complexes was discussed in Section 3.4.1.5 for $Cr(gly)_2(H_2O)_2^+$ and Section 3.4.3.4 for $Cr(glu)_2(H_2O)_2^+$ and it was concluded that the ammonia eluted complexes possessed amino-acid ligands coordinated via the α-carboxylate group only. Interaction on the column of the coordinated α-amino groups of the amino acid with the resin had somehow brought about loss of the amine coordination after elution with ammonia. Therefore the active complexes found for the glycine and glutamic acid complexes were in fact $Cr(gly)_2(H_2O)_4^{3+}$ and $Cr(glu)_2(H_2O)_4^{3+}$, respectively, and this structure was found to be active, as discussed in Section 5.4.2.

The ammonia elution had no effect on the activity of the cysteine complex, $Cr(cys)_2(H_2O)_2^+$, and the nicotinic acid complex, $Cr(nic)_2.(H_2O)_4^{3+}$, as both were inactive before and after treatment on the cation-exchange column. In the case of the nicotinic acid complex, this species was very tightly bound to the column and could not be eluted with ammonia so no conclusion can be drawn as to the effect of ammonia on this complex. For the cysteine complex however, the effect of ammonia was assumed to bring about of loss of coordination of the α-amino group, as found for the glycine complex, but no activity was observed. This suggests that although the inactive cysteine complex is similar in structure to the active glycine complex, the different side-chain group must be responsible for the lack of activity. In cysteine the side-chain is a methyl-sulphydryl group which therefore must interfere with the biological interaction which normally causes activity, perhaps by getting involved in nonproductive disulphide exchange reactions. For glutamic acid the side chain is a carboxylate group which seems to confer more activity on the structure since the activity for $Cr(glu)_2(H_2O)_4^{3+}$ (450 ± 10 % enhancement at 50 μg/cm³ concentration of chromium) is considerably higher than for $Cr(gly)_2(H_2O)_4^{3+}$

(171 ± 10 %enhancement at 50 μg/cm³ concentration). Thus although the common structure, shown in Figure 5.4, is quite active, the sidechain groups determine the level of activity - if the sidechain, R, is -H the complex in active, if R is -COOH the complex is very active, and if R is -SH the complex is inactive.

FIGURE 5.4: THE STRUCTURE OF ACTIVE MONODENTATE CHROMIUM (III)-AMINO ACID COMPLEXES



The level of activity of a complex was dependent on the concentration (see Figure 5.3). In the case of $Cr(gly)_2(H_2O)_4^{3+}$, the activity shows a fairly linear increase with concentration of this species but at a concentration of 100 μg/cm³ of chromium (III) a saturation effect becomes evident in which no further increase in activity is found. Effects such as this are common in the administration of biologically important compounds in biological systems which leads to the conclusion that this chromium (III) complex behaves in a similar fashion to naturally occurring GTF preparations.

The possible relationship between the structures of the nicotinic acid, amino acid and mixed ligand chromium (III) complexes and their activity in the yeast fermentation assay will be discussed further in Section 6.

SECTION SIX

CONCLUSION

During the course of this thesis, many stable complexes of chromium (III), with such ligands as nicotinic acid, various amino acids and glutathione, were isolated and characterised with a view to the synthesis of a chromium (III) complex which possessed glucose tolerance factor activity. In fact a number of different chromium (III) complexes were found to be biologically active in the yeast fermentation assay, that is, they increased the rate of carbon dioxide evolution presumably by an increase in the metabolism of glucose or an increase in the rate of glucose transport (Mirsky et al 1980). However, the structure of the chromium (III) complex and the nature of the ligands bound to chromium have a great bearing on the level of activity since simple chromium (III) salts, such as $Cr_2(SO_4)_3.15H_2O$, are inactive and can in fact cause a slight inhibitory effect on the yeast. Several classes of chromium (III) complexes were found to be active and these included: the blue coloured dinicotinate complex, $Cr(nic)_2(H_2O)_4^{3+}$, with the carboxylate group of the nicotinic acid ligands coordinated in a trans position; the monodentate amino acid complexes with glycine (including the species $Cr(gly)_2(H_2O)_4^{3+}$ formed during elution of $Cr(gly)_2(H_2O)_2^{+}$ from a DOWEX 50W-X12 cation-exchange column with NH_4OH); the NH_4OH eluted species $Cr(glu)_2(H_2O)_4^{3+}$; and the bis glutamine complex $Cr(gln)_2(H_2O)_2^{+}$. It was obvious that the dinicotinate complex, characterised in Section 2, was not the only chromium (III) complex to exhibit biological activity which suggests that complexes other than $Cr(nic)_2(H_2O)_4^{3+}$ could be responsible for the activity of the proposed glucose tolerance factor.

Two forms of the dinicotinate complex $Cr(nic)_2(H_2O)_3OH$ were prepared by different procedures. In the yellow form, the nicotinic acid ligands were coordinated via the pyridine

ring nitrogen atom and this only occurred through the formation of a chromous ion complex intermediate. This complex was only stable in crystalline form and when dissolved in dilute acid (2M HNO_3) or dilute base (2M NaOH) did not display biological activity in the yeast fermentation assay. Earlier, Mertz et al (1974) had postulated that a complex with this structure (see Figure 2.12) was responsible for the biological activity of chromium (III)-nicotinate complexes, although Mertz had not infact isolated or characterised any pure complex. Mertz further proposed the formation of a ternary complex between insulin and tissue membrane receptors achieved with the participation of the pyridine ring-coordinated chromium (III) dinicotinate complex if the two acidic carboxylate groups were to connect to basic groups on the insulin and tissue receptor, thus bringing both closer together. Such an arrangement is not consistent with chromium chemistry discussed in this thesis since the pyridine ring nitrogen atom-chromium (III) bonds in the yellow $Cr(nic)_2(H_2O)_3OH$ were shown to be unstable which suggests that this type of complex is unlikely to be important in biological systems.

The dinicotinate complexes, with coordination of the nicotinic acid ligands through the carboxylate group (both the mono-nuclear complex $Cr(nic)_2(H_2O)_4^{3+}$ and the bi-nuclear mixed ligand complex $Cr_2(nic)_4(gly)_2(OH)_2$), owe their activity to the trans coordinated nicotinic acid ligands, however, the mixed ligand complex is more stable at neutral pH due to the chelated glycine ligands. This biologically active complex structure is unlike the nicotinic acid complexes with other transition metals which have the nicotinic acid coordinated via the pyridine ring nitrogen atom (as seen for the complexes $M(nic)_2(H_2O)_4$, where M = Mn(II), Co(II) and Ni(II)). The importance of chromium (III) appears therefore to reside in the fact that Cr(III) combines preferentially with oxygen ligands (such as $-COO^-$) rather than nitrogen ligands as do other related transition metal ions. Thus the complex with trans arranged pyridine

ring groups forms comparatively readily. Transition metals such as Cr(II), Mn(II), Co(II) and Ni(II) preferably form nitrogen coordinated complexes which are not active. Another important factor is that while the tendency of chromium (III) to olate is large, it is significantly less than for the other metal ions such as Fe(III) and Mn(III) (which form insoluble hydroxy compounds easily) and hence nicotinic acid complexes will not be readily formed with such metals to give a simple complex that might be active.

Amino acid complexes with chromium (III) also have activity comparable with the activity of the dinicotinate complexes. The similarity in structure between these active amino acid complexes is in the coordination of at least two amino acid ligands, but the coordination may be either mono-dentate or bi-dentate. In the active mono-dentate complexes the activity may therefore be due to the presence of the free amine groups placed approximately trans across the chromium (III) atom with the level of activity depending on the sidechain group (a carboxylate group increases activity while the presence of a sulphydryl group results in an inactive complex). Thus although we can postulate that the basic α-amino groups are important for the interaction involved between the chromium (III) complex and its site of action, other groups on the amino acid can interact in a stimulatory or an inhibitory manner. The active bidentate complex, $Cr(gln)_2(H_2O)_2^+$, has the α-amino group coordinated (as well as the α-carboxylate group) but in this case the amide sidechain group apparently confers activity. Similar bidentate complexes with carboxylate or sulphydryl sidechain functional groups are virtually inactive thus confirming that the activity is due to the presence of the two uncoordinated amide groups in the bis glutamine complex.

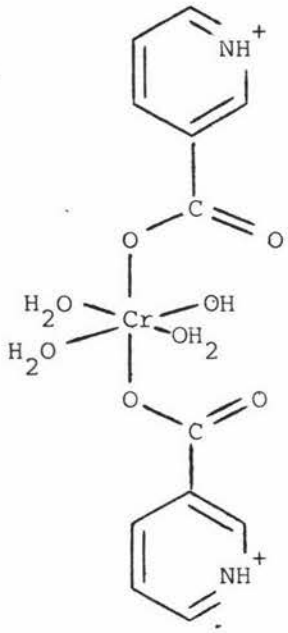
A common feature of all of the active chromium (III) complexes seems to be the need for two ligands coordinated

FIGURE 6.1:

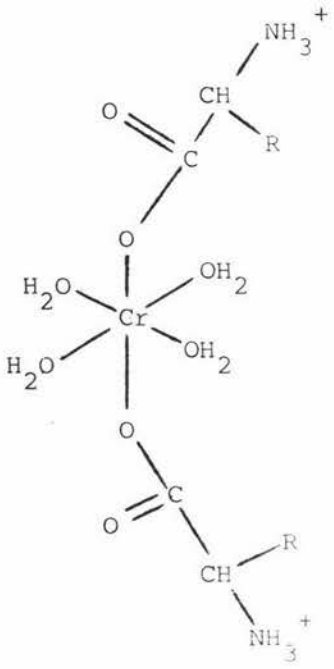
Comparison of the structures of the active chromium (III) complexes (a) blue $Cr(nic)_2(H_2O)_4^{3+}$, (b) $Cr(a\ a)_2(H_2O)_4^{3+}$, and (c) $Cr(gln)_2(H_2O)_2^{+}$ with (d) 1,4-diguanidinobutane.

FIGURE 6.1

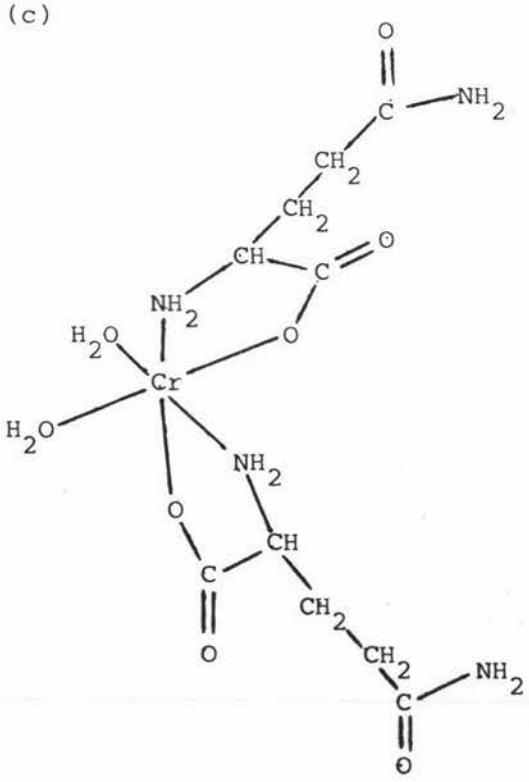
(a)



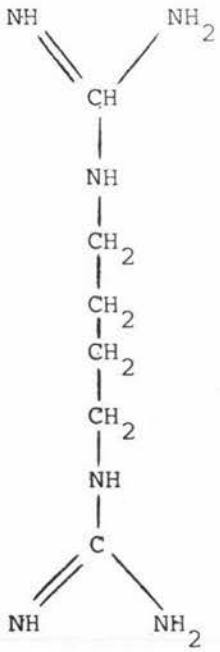
(b)



(c)



(d)



In summary, chromium (III), although probably not an essential part of the glucose tolerance factor may be able to act as a suitable template which can easily form complexes which due to structural similarities with the GTF have a similar effect in the yeast fermentation assay. Chromium (III) differs from other transition metals in that;

(i) although its tendency to olation is high, other metals, such as Fe(III) and Mn(III), olate much more easily and hence at neutral pH reasonably stable chromium (III) complexes may form; and

(ii) chromium (III) prefers to complex with oxygen donor ligands, such as the carboxylate group of nicotinic acid and amino acids, thus complexes can form easily with trans arranged nitrogen containing groups whereas Cr(II), Mn(II), Co(II) and Ni(II) prefer nitrogen ligands resulting in a trans arrangement of oxygen containing groups which lack biological activity.

This work has possible implications for the design of more effective blood glucose lowering drugs and may be of considerable value in the eventual understanding of the nature of the glucose tolerance factor.

APPENDIX

LIST OF ABBREVIATIONS

aa	amino acid
bipy	bipyridine
cys	cysteine
en	ethylenediamine
gln	glutamine
glu	glutamic acid
glut	glutathione
gly	glycine
nic	nicotinic acid
o-phen	ortho-phenanthroline
ox	oxalate ion
py	pyridine
pyr	pyrrolidone carboxylic acid
T.R.I.S.	hydroxymethyl aminomethane
Zn(Hg)	zinc/mercury amalgam

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