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Chromium(III) Complexes and their Relationship to the Glucose Tolerance Factor. Part II.* Structure and Biological Activity of Amino Acid Complexes

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A number of octahedral chromium complexes with amino acids as ligands have been prepared and their structures assigned on the basis of their chromatographic and spectral properties. These include complexes with the general structure $Cr(AA)_2(H_2O)_2$ *where the amino acids glycine, glutamic acid and glutamine act as bidentate ligands. The analogous compound with cysteine as ligand is stable at low pH, but at high pH a terdentate cysteine complex,* $Cr(cysteine)_2^-$*, is formed. These complexes, as well as a solution of monodentate glycine aquo complexes, and Cr–nicotinic acid–glycine and Cr–nicotinic acid–cysteine complexes of undetermined structure, have been assayed for glucose tolerance factor activity using a yeast assay. Only* $Cr(glutamine)_2(H_2O)_2^+$*, Cr–nicotinic acid–glycine and the mixture of complexes* $Cr(glycine)_n(H_2O)_{6-n}^{+3}$ *showed significant activity. It is proposed that a* trans *arrangement of the non-coordinated nitrogen atoms in the ligands of these complexes can mimic the structural features of the glucose tolerance factor which are essential for biological activity.*

Introduction

Glucose tolerance factor (GTF), a fraction isolated from brewer's yeast which displays biological activity in a number of assay systems [1–4], may have an important role to play in glucose metabolism. Toepfer *et al.* [2] have reported that a solution which contains chromium(III), glycine, glutamic acid and cysteine mimics the biological activity of this naturally-occurring GTF fraction. Although no chemically-defined complexes have been isolated from this synthetic solution it has been suggested [5] that the active ingredient is a nitrogen-coordinated Cr(III)-(nicotinic acid)$_2(H_2O)_4^+$ complex which is protected from olation by the presence of the amino acids. Recently however, we have shown that GTF does not contain chromium [6]. We have therefore begun a systematic study of the reaction of chromium(III) with individual components of the synthetic mixture described by Toepfer *et al.* [2] in an effort to discover which chromium(III) complexes possess GTF activity as measured by a yeast bioassay system. Active Cr(III) complexes are necessarily different chemically from the chromium-free native glucose tolerance factor, but to be active they must presumably have some general structural features which are similar to the native GTF. From such a study therefore we hope to learn something about the structure of GTF.

In a previous paper [7] we have shown that, contrary to the suggestion of Mertz [5], coordination of two nicotinic acid ligands to a chromium ion *via* the pyridine ring nitrogen atoms does not result in GTF activity. However, coordination of the nicotinic acid ligands *via* the oxygen atoms of the carboxylate groups does result in a complex which possesses good biological (GTF) activity in the yeast bioassay system [7].

In order to ascertain further the factors which give rise to GTF activity in chromium complexes we have now carried out a study of the structures, solution behaviour and biological activities of the chromium–amino acid complexes which would be expected to form in the synthetic mixture described by Toepfer *et al.* [2]. The results reported in this paper with the chromium complexes of the amino acids glycine, cysteine, glutamic acid and also glutamine show that nicotinic acid is not necessary for biological activity. On the basis of these results a rationalisation is given of the observation that chromium complexes

*The previous paper [7] is regarded as part I of this series.

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TABLE I. Electronic Absorption Spectra of Chromium–Glycine Complexes.

Complex	Colour	Absorption Maxima (nm)		Physical State	Elution Conditions
$Cr(H_2O)_6^{+3}$	purple	406	571	aqueous solution	1.0 *M* NaCl[a]
$Cr(gly)_3 \cdot H_2O$	red	388[b]	510	solid	
$[Cr(gly)_2OH]_2$	pink	390[b]	535	solid	
$Cr(gly)_2(H_2O)_2^+$	red	398[b]	535	pink or red solid dissolved in 2 *M* HNO_3	0.5 *M* NaCl[a]
$Cr(gly)_n(H_2O)_{6-n}^{+3}$	blue	420[c]	570[c]	aqueous solution	1.0 *M* NaCl[a]
chromium–glycine–nicotinic acid species		417	575	green solution	

[a]Dowex 50W-X12 cation exchange column. [b]The first absorption band was less intense than the second in each case. [c]For $Cr(oxalate)_3^{-3}$ the d–d bands are observed at 420 nm and 571 nm.

can have biological activity but that the naturally-occurring glucose tolerance factor does not contain chromium.

Experimental

Preparation of Solid Amino Acid Complexes

Tris(glycinato) Chromium(III) Monohydrate

The tris-glycinato complex was prepared using a method similar to that used by Bryan *et al.* [8] from $CrCl_3 \cdot 6H_2O$ (6.66 g, 0.025 mol) and glycine (5.60 g, 0.075 mol). After 7 days bright red crystals formed which were filtered and washed with ethanol and analysed as $Cr(gly)_3 \cdot H_2O$.

Di-μ-hydroxo-tetraglycinato Dichromium(III)

The bis-hydroxy dimer was prepared according to the procedure of Earnshaw and Lewis [9] and the resulting precipitate was washed with ethanol and acetone. The elemental analysis was consistent with the structure $[Cr(gly)_2OH]_2$.

Sodium bis(l-cysteinato) Chromium(III) Dihydrate

The bis-cysteine complex was prepared by a method similar to that reported by de Meester *et al.* [10] from $Cr(NO_3)_3 \cdot 9H_2O$ (1 g, 0.0025 mol) and *l*-cysteine hydrochloride (1.18 g, 0.0075 mol). The dark blue crystals were washed with ethanol and diethyl ether and subsequently analysed as $Na[Cr(cys)_2] \cdot 2H_2O$.

Hexaamine Chromium(III) Nitrate

The hexaamine–chromium(III) complex was prepared according to the method of Oppegard and Bailar [11] as bright yellow crystals which were dried *in vacuo* and stored until required.

Analyses

Microanalysis for the elements carbon, hydrogen and nitrogen was carried out at the Department of Chemistry at the University of Otago, Dunedin, New Zealand. Chromium determinations were carried out on a Varian Techtron AA5 atomic absorbance spectrophotometer using a slightly enriched luminous air-acetylene flame as described previously [12].

Bioassays

Bioassays were carried out as described previously [4] using a yeast fermentation assay system.

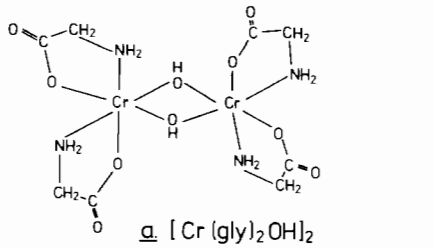
Ion-Exchange Chromatography

Ion-exchange columns were prepared using either Dowex 50W-X12 cation exchanger or Dowex 1-X8 anion exchanger and eluted as described previously [12]. Soluble chromium complexes were isolated and purified by a combination of ion-exchange and gel filtration chromatography.

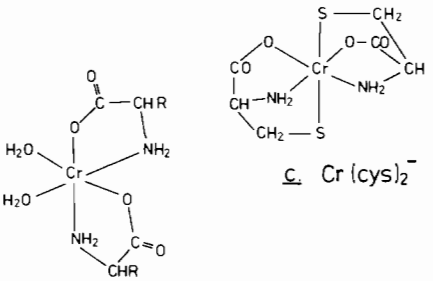
Results and Discussion

Reaction of Chromium(III) with Glycine

When a solution of chromium(III) was reacted with a three-fold molar excess of glycine at pH values between 4 and 7 a red crystalline complex which analysed as $Cr(gly)_3 \cdot H_2O$ precipitated from the solution. The red solution which remained was stable as long as the solution pH was maintained below 7, but once the pH value was increased above 7 a pink complex, which analysed as $[Cr(gly)_2OH]_2$, slowly precipitated. This latter complex was also obtained by reacting chromium(III) ions with a two fold excess of glycine and making the solution alkaline (pH 8) with NaOH according to the procedure of Earnshaw and Lewis [9]. Both the $Cr(gly)_3 \cdot H_2O$



a. $[Cr(gly)_2OH]_2$



c. $Cr(cys)_2^-$

b. $Cr(AA)_2(H_2O)^+$ R= -H, $-CH_2CH_2CO_2H$, $-CH_2CH_2CONH_2$

Fig. 1. Structures of amino acid complexes of chromium (III).

and $[Cr(gly)_2OH]_2$ complexes dissolved in the presence of 2 *M* HNO_3 to reform the red solution, which when subjected to cation-exchange chromatography on a Dowex 50W-X12 cation-exchange column produced a red species on elution with 0.5 *M* NaCl (20,000 μmho) as a single peak. The charge on the soluble red complex was less than +3 since the $Cr(H_2O)_6^{+3}$ ion was not eluted from similar columns until the NaCl concentration was increased to 1.0 *M* (30,000 μmho). The visible absorption spectrum of the soluble red complex exhibited d–d bands at 398 nm and 535 nm (Table I) irrespective of which of the three different methods by which it was produced. The two absorption bands were broad but unsplit and the intensity of the first absorption band was less than that of the second band.

The crystal structures of the $[Cr(gly)_2OH]_2$ and $Cr(gly)_3 \cdot H_2O$ complexes have recently been determined [8, 13]. The structure of the dimeric bis-glycine chromium(III) complex consists of well separated $[Cr(gly)_2OH]_2$ units in which the chromium atoms are in roughly octahedral environments. Each chromium atom has two glycine molecules coordinated in a *cis* arrangement and the other two coordination positions are occupied by bridging hydroxy groups also in a *cis* arrangement. The two chromium atoms and two hydroxyl groups of the bridging unit are strictly planar (Fig. 1a) and extensive hydrogen bonding exists within the crystal accounting for its low solubility in water and most common organic solvents. In the trimeric glycine complex of chromium(III) the third glycine is coordinated at the *cis* bridging positions [8] in a mutual *cis* arrangement (fac) of the three nitrogen atoms.

The shift of the second d–d transition of the tris glycine complex from 510 nm to 535 nm on dissolution in 2 *M* HNO_3 (Table I) is consistent with a change in the environment of the chromium atom to one in which there is a weaker ligand field as would be the case if coordination of the amino group of one of the three glycine ligands was replaced by an oxygen ligand such as a water molecule. This change is consistent with the suggestion [14] that fac-tris (amino acidato) chromium(III) complexes are unstable in acid solution. Since there was virtually no shift in the d–d bands when $[Cr(gly)_2OH]_2$ dissolved in 2 *M* HNO_3 (Table I) and the same red solution was obtained by dissolution of both $Cr(gly)_3 \cdot H_2O$ and $[Cr(gly)_2OH]_2$ in 2 *M* HNO_3 the most likely structure for the soluble red species is a bisglycinato diaquo chromium(III) complex. Furthermore, it seems likely that this bisglycinatodiaquo chromium(III) complex has a *cis* arrangement of the two glycine molecules and also a *cis* arrangement of the water molecules. (Fig. 1b, R = H). The soluble $Cr(gly)_2(H_2O)_2^+$ species is presumably obtained from the solid $[Cr(gly)_2(OH)]_2$ complex by protonation and hydrolysis which splits the dimer into two *cis* bisglycinatodiaquo chromium(III) units.

$$[Cr(gly)_2(OH)]_2 + 2H_3O^+ \rightleftharpoons 2Cr(gly)_2(H_2O)_2^+ \quad (1)$$

Glycine Complexes of Chromium(III) at pH < 4.0

When a boiling solution of chromium(III) was mixed with glycine at a 1:3 molar ratio at pH values less than 4.0, a stable blue solution was formed. Two d–d transitions were observed for this blue solution (Table I) with absorption bands at 420 nm and 570 nm (at pH 4.0) which suggested that only the carboxylate groups of the glycine ligands were coordinated to the chromium(III) ion. If the amino groups of the glycine ligands had also been coordinated then the positions of the d–d bands would be expected to shift to lower wavelengths than for $Cr(H_2O)_6^{+3}$ as found for $Cr(gly)_2(H_2O)_2^+$ (Table I). As the pH of the boiling solution was increased the solution slowly changed from blue to purple-red consistent with coordination of the α-amino groups of glycine following deprotonation to form $Cr(gly)_2(H_2O)_2^+$. Shuttleworth and Sykes [15] have previously reported a stepwise formation of chelates with coordination of the carboxylate group taking place at pH values less than 4.0 and coordination of the amino group only taking place at pH values greater than 7.0 to form the chelated ring structure.

Attempts were made to characterise the structure of the blue solution but only a single peak was eluted from a Dowex cation-exchange column under conditions which were similar to those used for elu-

tion of $Cr(H_2O)_6^{+3}$. This indicated that the complex in solution carried an overall charge of +3. Gel filtration of the blue solution resulted in a very broad blue band which could be accounted for by assuming that a series of similar complexes of the form $Cr(gly)_n(H_2O)_{6-n}^{+3}$ were present in solution. All such species would have a tripositive charge at pH < 7.0 since the α-amino groups would be largely protonated. The number of glycine ligands attached to the central chromium(III) ion could not be determined but the electronic spectrum of the solution was similar to that found previously [6] for the dinicotinate complex which contained two nicotinic acid ligands coordinated *via* the carboxyl groups. Thus the main species in the solution was probably $Cr(gly)_2(H_2O)_4^{+3}$ particularly in view of the fact that $Cr(gly)_2(H_2O)_2^{+}$ was formed on careful neutralisation of the boiling solution.*

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

Since it seems unlikely that two nicotinic acid ligands could occupy the two *cis* positions on the soluble $Cr(gly)_2(H_2O)_2^{+}$ complex (Fig. 1b, R = H), attempts to form a mixed-ligand complex of chromium(III) with nicotinic acid and glycine were made by forming initially the oxygen-coordinated *trans* Cr(nicotinic acid)$_2(H_2O)_4^{+3}$ complex. The tetraaquodinicotinate complex was prepared as described previously [7] and then after 5 minutes 2 mol of glycine (0.75 g, 0.01 mol in 10 cm^3 H_2O) per mol of complex were added to the reaction mixture at pH 6.0 and 60 °C. NaOH (2 *M*) was added dropwise to the boiling reaction mixture until it turned purple and after cooling the pH (7.4) was measured and found to be just sufficiently basic to allow chelation of the α-amino and α-carboxyl groups of glycine to the chromium.

The purple solution was obviously a complex mixture of compounds as cation exchange chromatography on Dowex 50W-X12 resin resulted in a green fraction on elution with water (suggestive of a neutral or anionic species), a pink fraction (at a conductivity of 20,000 μmho) on elution with a $NaNO_3$ salt gradient which was similar to that required for the elution of $Cr(gly)_2(H_2O)_2^{+}$, and two further cationic green fractions which were eluted when the column was subjected to a pH gradient as described previously [12]. Neither of these two green fractions contained nicotinic acid and were presumably basic chromic polymers thus they were not further investigated.

*When the cold blue solution was made basic by the addition of 2 *M* NaOH the solution became cloudy and a gelatinous precipitate formed at pH values greater than 8.0.

Since it is known [7] that oxygen-coordinated tetraaquodinicotinate complexes of chromium are bound tightly to Dowex ion-exchange resins the reaction mixture was also subjected to gel filtration to determine whether any additional complexes were present in the original solution. The purple reaction mixture yielded a green fraction and a red fraction when it was run through a Sephadex G15 column and eluted with water. The green species was eluted near the void volume suggesting that it was probably polymeric since simple monomeric nicotinic acid and glycine complexes are retarded by G15 columns. An analysis of the ratio of the number of moles of nicotinic acid per mole of chromium in the green fraction indicated that only one nicotinic acid molecule was associated with four chromium ions, again suggesting the existence of a polymeric species. As expected for such a polymeric chromium species (containing little nicotinic acid) the d–d transitions (Table I) occurred at wavelengths similar to those for $Cr(oxalate)_3^{-3}$ (Table I). The actual structure of the polymeric material was not further investigated. The pink species, which was obtained from the reaction mixture either by gel filtration or cation-exchange chromatography, was eluted as a complex with a single positive charge from the cation exchange column and on the basis of this and the visible absorption spectrum, was assigned the structure $Cr(gly)_2(H_2O)_2^{+}$. This complex was presumably formed at the expense of the original oxygen-coordinated Cr(nicotinic acid)$_2(H_2O)_4^{+3}$ complex.

Reaction of Chromium(III) with Cysteine

When $Cr(NO_3)_3 \cdot 9H_2O$ (1.0 g, 0.0025 mol) was reacted with *l*-cysteine hydrochloride (1.18 g, 0.0075 mol) according to the method of de Meester *et al.* [10] dark blue crystals were obtained which analysed as $Na[Cr(cys)_2] \cdot 2H_2O$. The terdentate complex dissolved in water to form a dark blue solution with a pH of 7.0. However, on addition of dilute acid (2 *M* HNO_3) the solution became red (at pH 4.0) whereas if the blue solution was treated with dilute base (2 *M* NaOH) the colour changed to green when the pH reached 8.5. These acid–base colour changes were rapid and completely reversible with the blue form being intermediate between the red and green foms. Ion-exchange chromatography showed that the complex was cationic (red form) at pH 3.5 and anionic (green form) at pH 8.5 and no other chromium complex was detectable in solution at pH values between 3.5 and 8.5.

The electronic spectrum of the crystalline $Na[Cr(cys)_2] \cdot 2H_2O$ complex was recorded (Table II) and this proved to be identical with the spectrum obtained when the solid $Na[Cr(cys)_2] \cdot 2H_2O$ complex was dissolved in aqueous solution at pH 7.0. In the solid state [10] the complex anion has its central Cr atom in a slightly distorted octahedral

TABLE II. Electronic Absorption Spectrum of Chromium–Cysteine Complexes.

Complex	Colour	Absorption Maxima (nm)				Physical State
		First pair		Second pair		
$Na[Cr(cys)_2]\cdot 2H_2O$	blue	413,	555	≅	450, 615	Solid (Nujol mull)
$Na[Cr(cys)_2]\cdot 2H_2O$	blue	413,	555	≅	450, 615	Aqueous solution; pH 7.0
$Cr(cysSH)_2(H_2O)_2^+$	red	410,	550			Aqueous solution; pH 3.5
$Cr(cys)_2^-$	green	410,	550	≅	450, 610 (vi)[a]	Aqueous solution; pH 8.5
Cr–cysteine–Nicotinic Acid	blue	410,	550–600[b,c]			Blue solution at pH 7.0 Red solution at pH 3.5

[a]Very intense band. [b]Bands at 410 nm and 550 nm intensified on addition of 2 *M* HNO_3. [c]Bands were also observed at 262 nm.

coordination environment with two *trans* Cr–S bonds, two *cis* Cr–O bonds and two *cis* Cr–N bonds (Fig. 1c). Since the electronic absorption spectrum of the complex was the same in solution as in the solid state (Table II) it seems reasonable to assume that the solution structure is similar to that of the solid at pH 7.0. The four identifiable d–d bonds of the complex can be grouped into two pairs. A pair of bands at 413 nm and 555 nm (Table II) which are similar to those observed for $Cr(gly)_2(H_2O)_2^+$ (Table I) and a pair of bands at 460 nm and 615 nm. A titration of the $Na[Cr(cys)_2]\cdot 2H_2O$ solution with acid resulted in an instantaneous change in colour of the solution (blue to red) and the disappearance of the second pair of bands (Table II). Reaction of the resulting complex at pH 4.0 with the sulphydryl reagent 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) followed by neutralisation to pH 8.0 gave an almost instantaneous production of the yellow colour due to the presence of the 2-nitrothiobenzoate anion which is formed when DTNB reacts with SH groups. The intensity of the yellow colour was consistent with two free SH groups per mole of complex and no such colour was observed in the absence of the complex. These results are explicable if reaction of the chromium cysteine complex with acid rapidly destroys the coordination of the sulphur atoms to produce two free SH groups as shown in eqn. 2.

$$Cr(cys)_2^- + 2H_3O^+ \rightleftharpoons Cr(cysSH)_2(H_2O)_2^+ \qquad (2)$$

As the second pair of bands disappeared (Table II) when this reaction occurred they must be associated with the coordinated sulphur ligands. When the coordination of the sulphur atoms is lost a red coloured solution results as would be expected since the environment of the resulting chromium atom (with four oxygen and two nitrogen ligands) is similar to that of the red $Cr(gly)_2(H_2O)_2^+$ complex. The protonation of the sulphur group and subsequent cleavage of the Cr–S bond suggests a very labile bond as compared with the Cr–N or Cr–O bonds.

The sulphur ligation reaction was completely reversible and when the pH of the solution was raised to 7.0, the second pair of bands (and hence the blue colour) returned instantaneously. At pH 7.0 the sulphur atoms are largely coordinated to the chromium atom but an equilibrium apparently exists between the coordinated and free thiol groups in the complex. Reaction of DTNB with the complex at pH 8.0 resulted in only a slow increase in appearance of absorbance at 412 nm due to the 2-nitrothiocarboxylate ion, whereas reaction with cysteine at this pH (which possesses free thiol anions) was apparently instantaneous. The overall absorbance change was again consistent with the presence of two thiol groups per mole of complex and, as there was no absorbance at 412 nm at time zero, most of the thiol groups must indeed be initially coordinated to the chromium atom. From the rate of increase in the absorbance at 412 nm a first order rate constant can be calculated for the process ($k = 1.16 \pm 0.02 \times 10^{-3}$ sec^{-1}) which is approximately equal to the rate of dissociation of the C–S bond at pH 8.0 (eqn. 3) since reaction of DTNB with thiol anions is fast [16].

$$\begin{aligned} &Cr(cys)_2^- \overset{\text{slow}}{\rightleftharpoons} Cr(cysS)_2^- \\ &Cr(cysS)_2^- + 2RSSR \xrightarrow{\text{fast}} Cr(cysSSR)_2^+ + 2RS^- \end{aligned} \qquad (3)$$

This rate of dissociation was too slow to account for the very fast colour change (blue to red) on addition of acid to a solution of the complex and thus the rate of dissociation of the Cr–S bond must be much greater when the sulphur atom is protonated. A

similar conclusion was reached by Weschler and Deutsch [17] who studied the aquation kinetics of thiolatobis (ethylene diamino) chromium(III) complexes and proposed that it is the greater tendency of sulphur to be protonated when it is coordinated to chromium which causes this enhanced lability.

The important feature of these results from the point of view of the structure of the glucose tolerance factor is that at pH values below about 5 the two *trans* ligand positions of the complex are potentially available for reaction with other ligands such as nicotinic acid.

Preparation of Mixed-Ligand Complexes with Nicotinic Acid and Cysteine

The red species $Cr(cysSH)_2(H_2O)_2^+$ was prepared at pH 4.0 and two mol of nicotinic acid per mol of complex (0.14 g in 10 cm^3 of H_2O at pH 4.0) were then added at 90 °C. When the solution was boiled a purple solution slowly formed. The purple solution was titrated to pH 7.0 with NaOH (2 *M*) after which a slight blue-grey precipitate formed. The presence of some unreacted $Cr(cysSH)_2(H_2O)_2^+$ in the reaction mixture was demonstrated by cation-exchange chomatography on Dowex 50W-X12. However, chromium–dinicotinate complexes tend to adhere to this matrix [7] and instead purification was carried out using Sephadex gel filtration column chromatography. The purple reaction mixture was passed down a Sephadex G15 column (1.5 cm × 38.0 cm) and a red fraction was eluted near the void volume followed later by a blue fraction which contained nicotinic acid, and finally uncomplexed nicotinic acid was eluted near the salt peak. The red complex did not contain nicotinic acid and most probably consisted of a polymeric chromium(III)–cysteine complex since the d–d transitions at 410 nm and 550 nm were similar to those observed for the $Cr(cysSH)_2(H_2O)_2^+$ complex (Table II).

The blue fraction was eluted just before the salt peak consistent with the suggestion that it contained a smaller mononuclear chromium(III) complex. The visible spectrum (Table II) indicated that $Cr(cys)_2^-$ was present in the blue solution due to the rather broad band in the region 550–600 nm characteristic of sulphydryl coordination. Also, on addition of acid the colour of the solution immediately changed to red as expected for a $Cr(cys)_2^-$ complex on protonation and loss of coordination of the sulphur atoms. The blue solution also exhibited the strong series of bands in the ultraviolet spectrum characteristic of nicotinic acid which, since uncomplexed nicotinic acid is normally eluted along with the salt peak [7], suggests the presence of coordinated nicotinic acid. Analysis of the chromium:nicotinic acid ratio from the chromium and A_{262} profiles leads to the conclusion that 2 nicotinic acid molecules are present for every chromium atom. Since it has been shown [7] that the nicotinic acid complex is eluted after the conductivity peak on Sephadex gel filtration columns the nicotinic acid which was present in the blue fraction must be considered to be coordinated to chromium as part of a more complex structure. The exact structure of the complex was not determined but the blue fraction most probably consists of a mixture of unreacted starting material $Cr(cys)_2^-$ and a mixed chromium–cysteine–dinicotinic acid complex.

Complexes with Glutamic Acid

Since glutamic acid can be regarded as a substituted glycine molecule it is to be expected that similar bis type complexes will be formed *via* the α-amino and α-carboxylate groups. The questions of interest with respect to the possible formation of a mixed glutamic acid–nicotinic acid complex are whether the two glutamic acid molecules will coordinate in a terdentate manner and if not whether the vacant ligand positions are *cis*, as in the chromium–glycine complex, or *trans*. When a solution of $Cr(NO_3)_3 \cdot 9H_2O$ (1.0 g, 0.0025 mol) and glutamic acid sodium salt (0.95 g, 0.0050 mol) in H_2O (30 cm^3) was heated to boiling and solid NaOH was added the solution turned blue initially, consistent with coordination of the carboxylate groups of glutamic acid, and finally a purple gel was formed. Dropwise addition of NaOH (2 *M*) dissolved the gel and formed a violet solution which was separated by anion-exchange chromatography on a Dowex 1-X8 resin into pink (pH 9.0 tris–NaCl buffer; 10,000 μmho conductivity) and purple fractions (pH gradient; pH 9.0 tris–NaCl buffer (10,000 μmho conductivity)/pH 4.0 acetic acid–NaCl buffer (10,000 μmho conductivity)).

The pink fraction was amphoteric as shown by the fact that it was also retained by a Dowex 50W-X12 cation exchanger at pH 3.0 and was eluted with NaCl (0.2 *M*, 10,000 μmho conductivity) as a single pink fraction. The visible absorption spectrum of the pink solution had absorption maxima at wavelengths which were lower than those obtained for the $Cr(H_2O)_6^{+3}$ species (Table III) but similar to those found for $Cr(gly)_2(H_2O)_2^+$ (Table I). Thus the pink species probably has two glutamic acid molecules coordinated through the α-amino and α-carboxylate groups in a *cis, cis* manner (Fig. 1b, R = $-CH_2CH_2CO_2H$) as in $Cr(gly)_2(H_2O)_2^+$. The γ-carboxyl group was obviously not involved in coordination to the central chromium since the spectrum did not change with pH even though the charge characteristics of the complex were different. Since the cationic form was eluted under conditions which were similar to those used for $Cr(gly)_2(H_2O)_2^+$ it is likely that the charge was also +1 for the glutamic acid complex at acidic pH values and hence the complex may be formulated as $Cr(glu)_2(H_2O)^+$. When the pH was increased to

TABLE III. Electronic Absorption Spectra of Chromium–Glutamic Acid and Chromium–Glutamine Complexes.

Complex	Colour	Absorption[a] Maxima (nm)		Physical State	Elution Conditions
$Cr(glu)_2(H_2O)_2^-$	pink	385	550	Solution (pH 9)	
$Cr(glu)_2(H_2O)_2^+$	pink	385	550	Solution (pH 3.5)	0.2 *M* NaCl[b]
$Cr(gln)_2(H_2O)_2^+$	red	395	526	Solution (pH 8.5)	0.1 *M* NaCl[b]

[a]In each case the absorption bands were unsplit and the first absorption band was less intense than the second. [b]Dowex 50W-X12 cation exchange column.

9 the complex would then become $Cr(glu)_2(H_2O)_2^-$ as a result of ionisation of the γ-carboxyl groups which is consistent with the observed ion-exchange behaviour of the complex at this pH. It should be noted that coordination of the γ-carboxyl group would have necessitated the formation of the normally unstable seven or eight membered chelate ring.

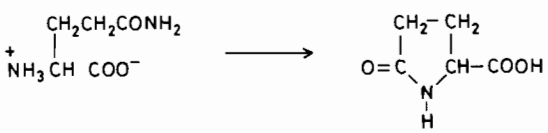
On the other hand the absorption spectrum for a terdentate complex with the structure $Cr(glu)_3^{-3}$ should have absorption maxima which were similar to those found for $Cr(gly)_3 \cdot H_2O$ (Table I), which was not the case. Furthermore, no precipitate was observed on the addition of $AgNO_3$ to an aqueous solution of the complex. For the tris complex with three uncoordinated carboxylate groups a precipitate of $Ag_3Cr(glu)_3$ would be expected [18], but not for a bis complex with the proposed structure of $Cr(glu)_2(H_2O)_2^-$ because the Ag^+ ions would form the positively-charged water-soluble complex $Ag_2Cr(glu)_2(H_2O)_2^+$.

Glutamine Complex of Chromium(III)

Glutamine–chromium complexes were prepared in order to determine the biological activity of complexes containing an amino acid with an amide side chain. The chromium(III)–glutamine complexes were prepared by grinding in a mortar, a mixture of $Cr(NH_3)_6 \cdot (NO_3)_3$ (0.60 g, 0.0012 mol) and glutamine (0.44 g, 0.0030 mol) and heating the solid mixture at 135 °C for 60 minutes. A purple powder resulted which was very soluble in water to give a dark red solution which was stable at pH 8.5. However, if the pH was reduced to 3.5 the solution slowly turned purple.

The red solution was titrated to pH 3.5 with dilute acid (2 *M* HNO_3) and diluted to a conductivity of 2500 μmho before loading onto a Dowex 50W-X12 cation exchange column. Elution of the column with 0.1 *M* NaCl resulted in a colourless fraction (unreacted glutamine) and a red fraction, while with a 0.1–0.5 *M* NaCl salt gradient a dark purple fraction was eluted. The chromium concentration of the purple fraction was 25 times that of the red fraction which suggested that, since the solution originally loaded onto the column was red, that most of the red solution was converted into the purple solution during elution from the column.

The red species was less positively charged than $Cr(H_2O)_6^{+3}$ and as judged from its elution conditions probably possessed a charge of +1 which was consistent with the presence of a bis complex. The positions of the d–d bands were very similar to those of $Cr(gly)_2(H_2O)_2^+$ (Table I and Table III) which suggested that the glutamine ligand was coordinated to the chromium(III) ion by the α-amino and α-carboxylate groups probably again in a *cis, cis* manner (Fig. 1b, R = $CH_2CH_2CONH_2$). No splitting of the second absorption band of the red species was observed which was consistent with this suggestion. Side chain amide group coordination would have resulted in the d–d bands appearing at lower wave lengths than those observed for the complex. In fact the weakly basic amide side chain group would not be expected to coordinate especially as coordination would have required the formation of an unstable seven or eight membered ring. A tris chromium–glutamine complex was not obtained in the present work, although Mizuochi *et al.* [19] prepared $Cr(gln)_3 \cdot 2H_2O$ under similar conditions. They found the tris complex to be insoluble in water but when dissolved in perchloric acid a red solution was obtained whose absorption maxima (395 nm and 535 nm) were similar to those observed for $Cr(gly)_2(H_2O)_2^+$, $Cr(glu)_2(H_2O)_2^+$ and $Cr(gln)_2(H_2O)_2^+$. Thus, as observed for $Cr(gly)_3 \cdot H_2O$, reaction with perchloric acid probably forms the bis complex which seems to be the most stable form in aqueous solution. Under acidic conditions glutamine is rapidly converted into L-2-pyrrolidone-5-carboxylic acid [20].



Thus, under these conditions the $Cr(gln)_2(H_2O)_2^+$ complex may be slowly destroyed and converted

TABLE IV. Biological Activity of Chromium–Amino Acid Complexes.

Compound	Colour	Concentration (μ g Cr/cm^3)	Activity[a]
$Cr(gly)_2(H_2O)_2^+$	red	40	−5 ± 5
$Cr(gly)_n(H_2O)_{6-n}^{+3}$	blue	50	118 ± 5
$Cr(cys)_2^-$	blue	50	43 ± 5
$Cr(glu)_2(H_2O)_2^-$	red	40	5 ± 7
$Cr(gln)_2(H_2O)_2^+$	red	6	322 ± 6
Cr–nic–gly	green	50	128 ± 8
Cr–nic–cys	blue	50	22 ± 4
$Cr_2(SO_4)_3 \cdot 15H_2O$		10	−14 ± 6
glycine		100	37 ± 9
cysteine		100	33 ± 9
glutamic acid		100	16 ± 6
glutamine		100	90 ± 8

[a]Activity is expressed as $(R_s - R_c)/R_c \times 100$ where R_s and R_c are the rates of CO_2 evolution for the sample and control respectively.

into a purple chromium–pyrrolidone carboxylate complex.

Biological Activity of Chromium Amino Acid Complexes

The biological activities of the various chromium–amino acid complexes prepared in this work were determined as described previously [7] by the yeast fermentation bioassay system [3, 4]. Each sample was titrated to pH 5.75 just prior to commencement of the assay and the concentration was so adjusted that the chromium content was as shown in Table IV. The percentage activity shown in the table is the percentage increase in the sample rate when compared with the control rate and thus when the variation in the control rate is taken into account only percentage increases of greater than 40–50% can be considered as significant.

The chromium–amino acid complexes $Cr(gly)_2(H_2O)_2^+$, $Cr(glu)_2(H_2O)_2^-$ and $Cr(cys)_2^-$ prepared in the present work would be expected to be formed in the synthetic solution described by Toepfer *et al.* [2]. However, none of these complexes were active in the yeast bioassay and thus the activity reported by Toepfer *et al.*, for their chromium–nicotinic acid–glycine–glutamic acid–cysteine solution, cannot be due to the presence of these complexes or presumably to mixed complexes such as $Cr(gly)(glu)(H_2O)_2$ which may also have been formed. Most of the activity of the synthetic mixture must therefore be attributed to the O-coordinated chromium–dinicotinic acid complex [7] although there will also be a contribution from the green, polymeric chromium–glycine–nicotinic acid

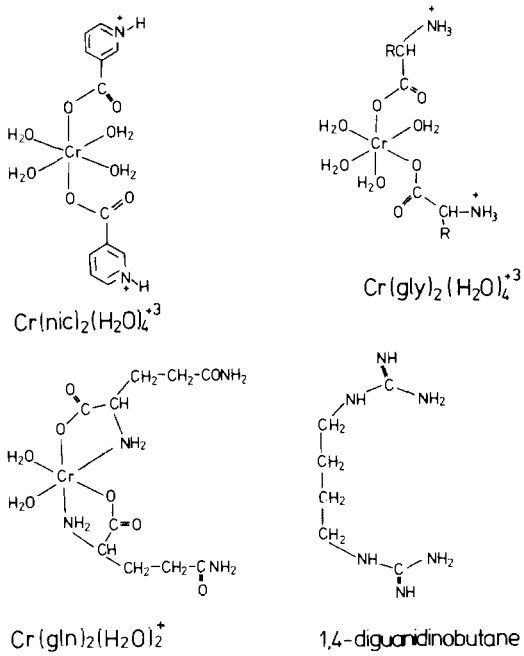


Fig. 2. Structures of biologically-active chromium(III) complexes.

complex (Table IV). Since the structure of this complex is not known the activity of this species does not lead to any conclusions about the possible structure of GTF.

Of particular interest with respect to the structure of GTF are the results obtained for the $Cr(gln)_2(H_2O)_2^+$ complex. Although the other bis coordinated amino acid complexes of chromium were

not active, even though they all have the same *cis, cis* arrangement of the α-amino and α-carboxylic acid groups, and a *trans* arrangement of the –H, $-CH_2CH_2COOH$ or $-CH_2SH$ groups respectively it is apparent that the *trans* arrangement of two amide groups (Fig. 2) does confer biological activity on the complex. (The individual amino acids do not show significant activity except for glutamine). The monodentate chromium–glycine complex (probably $Cr(gly)_2(H_2O)_4^{+3}$ also possessed biological activity and it is interesting to note (Fig. 2) that all of the active chromium complexes have a structural similarity with the O-coordinated $Cr(nic)_2(H_2O)_4^{+3}$ complex (Fig. 2) which has also been shown to be biologically active [7]. The active chromium complexes contain a pair of nitrogen atoms, *trans* with respect to the chromium and which can adopt geometries in which the distance between the nitrogen atoms is similar. The actual chemical environment of the nitrogen atoms does not seem to be important since the pyridine ring nitrogen atoms in $Cr(nic)_2(H_2O)_4^{+3}$ and the amide nitrogen atoms in $Cr(gln)_2(H_2O)_2^{+}$ give rise to similar increases in the basal yeast fermentation rate at similar concentrations of chromium in the assay. It is known that certain diguanide compounds are effective in the lowering of the sugar content of the blood in mammals and one such compound is 1,4-diguanidinobutane (Fig. 2) which is an animal base extracted from the mussel Noah's Ark [21]. This compound (Fig. 2) can also adopt a conformation with nitrogen atoms arranged in a similar manner to those in the $Cr(gln)_2(H_2O)_2^{+}$ and $Cr(nic)_2(H_2O)_4^{+3}$ complexes and it therefore seems reasonable to suggest that these structures mimic at least part of the structure of GTF much as morphine mimics part of the structure of endorphins [22]. Thus, even though the native GTF itself contains no chromium [6] (and chromium is not even an element which is essential for yeast metabolism [4]) a fortuitous structural arrangement leads to chromium complexes which are biologically active in the yeast assay.

The fact that all active chromium(III) complexes have, or can adopt, a surface topology with a similar *trans* arrangement of nitrogen-containing functional groups (Fig. 2) (which appears to be required to trigger the observed biological effects) has implications for the design of more effective oral hypoglycaemic drugs and may be of considerable interest in the eventual understanding of the nature of the glucose tolerance factor.

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