Inorganica Chimica Acta, *91* (1984) 1–9

Structure and Biological Activity of Nitrogen and Oxygen Coordinated Nicotinic Acid Complexes of Chromium

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Received July 14, 1983

A series of N-coordinated M(II)(nic)$_2$(H$_2$O)$_4$≠ complexes, where M = Co, Ni, Mn or Cr and the O-coordinated species Cr(III)(nic)$_2$(H$_2$O)$_4^+$ and Cr(III)-(nic)(H$_2$O)$_5^{+2}$ have been prepared. A single crystal X-ray analysis of the complex Cr(II)(nic)$_2$(H$_2$O)$_4$ shows that the chromium ion is in the +2 oxidation state and that two nicotinic acid ligands are coordinated in a trans arrangement via the pyridine ring nitrogen atoms. The four equatorial positions are occupied by water molecules. Biological activity measurements with a yeast bioassay system however show that none of the N-coordinated complexes possess any glucose tolerance factor (GTF-type) activity. On the other hand, the O-coordinated chromium(III)–dinicotinic acid complex is biologically active which suggests that a trans arrangement of pyridine nitrogen atoms resembles that part of the GTF structure which is recognised by the receptors or enzymes which are involved in the expression of the biological effect.

Introduction

The view that the glucose tolerance factor (GTF) which is obtained from brewer's yeast is a N-coordinated dinicotinic acid chromium(III) complex has become increasingly common in the literature [1–4] although recent work [5, 6] has cast serious doubts on this hypothesis. For example, it has been shown [5] that chromium is not an essential trace element for yeast cell growth and metabolism, and more importantly that GTF fractions isolated from brewer's yeast by a new separation scheme [6] do not contain chromium. Nevertheless, a synthetic mixture which contains chromium, nicotinic acid and the amino acids glycine, glutamic acid and cysteine (the supposed constituents of GTF [7]) and which mimics the biological effects of GTF fractions (isolated from brewer's yeast) has been reported [7]. The chemical composition of this synthetic mixture has not however been established and thus it is not clear which species in the solution are actually responsible for the biological effects.

Since the chemical identity of GTF is not yet known we have undertaken a systematic study of the reaction of chromium with nicotinic acid and amino acids in order to determine the structural features of the resulting complexes which give rise to biological activity. This information may lead to an understanding of the structure of the naturally-occurring glucose tolerance factor. We now report results obtained with chromium-nicotinic acid complexes which demonstrate that monomeric complexes with either N-coordinated or O-coordinated nicotinic acid ligands may be obtained, but only the O-coordinated dinicotinic acid complex possesses GTF activity.

Experimental

Analyses

Microanalysis for the elements carbon, hydrogen and nitrogen was carried out by the Department of Chemistry at the University of Otago, Dunedin, N.Z. Chromium determinations were carried out on a Varian Techtron AA5 atomic absorbance spectrophotometer using a slightly luminous air-acetylene flame. A spectral slit width of 0.2 nm was used together with the chromium resonance band of 357.8 nm.

Bioassays

These were performed as described previously [5] using a yeast fermentation assay system.

Column Chromatography

Ion-exchange columns were prepared using either a Dowex 50W-X12 cation exchanger or a Dowex 1-X8 anion exchanger eluted and prepared as described previously [8]. Gel filtration chromatography was carried out using Sephadex G-15 columns (2.0 cm × 6.0 cm) which were prepared according to the manufacturers instructions and eluted with H_2O.

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≠Nicotinic acid is abbreviated to nic throughout the text.

0020-1693/84/$3.00

Physical Measurements

Since the complexes obtained in this work were insoluble in polar and non-polar solvents visible and ultra violet absorption spectra were determined by the diffuse reflectance technique on a Shimadzu MPS 5000 spectrophotometer. Infra red spectra were recorded as nujoll mulls on a Pye Unicam SP 3-300 spectrophotometer. Magnetic susceptibility measurements were determined by the Faraday method with a Cahn Electro-balance DTL model 7550 system using $[Ni(en)_3]S_2O_3$ as calibrant. X-Ray powder photographs were recorded using a Phillips PW 1011 X-ray generator coupled to a Phillips PW 1352 recording unit.

Preparation of Complexes

N-Coordinated Cr(II)(nic)$_2$(H$_2$O)$_4$

Since according to the theory of hard and soft acids and bases (HSAB) enunciated by Pearson [9] the chromium(II) ion is likely to be a soft base (as a result of its low oxidation state) it is to be expected that chromous ions would coordinate preferentially to the nitrogen atom of a nicotinic acid moiety. Thus attempts were made to prepare an N-coordinated chromium-dinicotinate complex via the intermediacy of chromous ions. Chromous ions, prepared by passing an aqueous chromic ion solution (2.4×10^{-3} moles in 30 cm^3 H_2O) down a Zn-amalgam column under an atmosphere of hydrogen [10], were run into a solution of nicotinic acid (0.59 g, 4.8×10^{-3} moles in 10 cm^3) also under hydrogen. A pink precipitate formed immediately but slowly changed into a yellow crystalline compound which (after exposure to air) was finally filtered off and washed successively with water, ethanol and acetone. If the pH of the solution was less than 5.0 no yellow product was formed presumably because the nicotinic acid nitrogen atom remained completely protonated under these conditions (pK_a 4.81 [11]). The yellow complex was insoluble in both polar and non-polar solvents and the elemental analysis was consistent with either the composition $Cr(III)(nic)_2(H_2O)_3OH$ or $Cr(II)(nic)_2(H_2O)_4$. Calculated for $CrC_{12}H_{16}N_2O_8$: C, 39.13; H, 4.38; N, 7.61%. Found: C, 38.35; H, 4.57; N, 7.33%.

O-Coordinated Cr(III)(nic)$_2$(H$_2$O)$_3$OH

A blue dinicotinate complex of chromium(III) was prepared by mixing a warmed (60 °C) aqueous solution of $Cr(NO_3)_3 \cdot 9H_2O$ (2.0 g, 0.005 moles in 20 cm^3) with an aqueous solution of nicotinic acid (1.23 g, 0.01 moles in 20 cm^3) whose pH had been adjusted to 6.0 with NaOH (2*M*) before also being warmed to 60 °C. The resulting green solution changed to blue on boiling while the pH decreased from 6.0 to 2.5. On cooling, a blue precipitate formed and after 48 hours this was filtered, washed with water, ethanol and diethyl ether and dried in vacuo. Further purification of the blue powder was carried out by extracting any unreacted nicotinic acid with sodium-dried ether in a soxhlet apparatus. The elemental analysis of the blue powder was consistent with the formulation $Cr(III)(nic)_2(H_2O)_3OH$. Calculated for $CrC_{12}H_{15}N_2O_8$: C, 39.25; H, 4.12; N, 7.63%. Found: C, 38.80; H, 4.54; N, 7.25%.

Preparation of Nicotinic Acid Complexes of Other Transition Metals

Dinicotinic acid complexes of $Co(II)(NO_3)_2 \cdot 6H_2O$ (1.18 g), $Ni(II)(NO_3)_2 \cdot 6H_2O$ (1.19 g) and $Mn(II)(SO_4) \cdot 4H_2O$ (0.94 g) were prepared by the method of Anagostopoulos *et al.* [12]. In each case the elemental analysis was consistent with the structure $M(nic)_2(H_2O)_4$.

Results and Discussion

Crystal Structure of Yellow Chromium–Dinicotinate Complex

The yellow complex was isolated from aqueous solution as small straw coloured plates. Preliminary cell dimensions and space group were determined from rotation and Weissenberg photographs and the final parameters and diffractometer orientation matrix* were refined by least squares methods from the setting angles of 25 well dispersed high angle reflections. Cell Data: $CrC_{12}H_8N_2O_4 \cdot 4H_2O$, Mol wt. = 368, space group C2, a = 14.243(4) Å, b = 6.914(2) Å, c = 8.495(3) Å, β = 118.37(2)°, V = 736.16 Å^3, Z = 2, d_c = 1.66 g cm^{-3}, d_m (by flotation) = 1.69 g cm^{-3}, λ(Mo Kα) = 0.71069 Å, F(000) = 748ē, μ(Mo) = 20.48.

1162 data ($\pm h,k,l$, $3° < 2\theta < 45°$) were collected from a single, typically small crystal mounted, with the unique axis parallel to ϕ, on a Nicolet four circle diffractometer. Measurements were made with Mo Kα radiation using the $\omega/2\theta$ scan mode and backgrounds were measured at each end of the 1° scan range. Three moderately strong well separated reflections were measured every 50 data to monitor crystal movement and/or deterioration. Lorentz, polarisation and absorption corrections were applied in the usual manner and after merging equivalent reflections a unique data set of 542 structure amplitudes with $I > 3\sigma(I)$ was obtained and used for the subsequent structure solution and refinement.

All calculations were performed using the SHELX-76 structure solution package**. The structure was solved from an observed Fourier synthesis phased by

*All control and data processing routines used were those supplied with the Nicolet diffractometer.

**SHELX-76-PROGRAM for crystal structure determination.

the uniquely positioned chromium atom at 0,0,0. All non-hydrogen atoms were accorded anisotropic thermal parameters and these, together with the positional parameters, were refined by full matrix least squares. The quantity minimised was $\Sigma\omega(|F_o| - |F_c|)^2$. Toward the end of refinement peaks at locations expected for the hydrogen atoms were found in the difference synthesis and those were included (with U = 0.06 Å) in the calculations although their parameters were not refined. The final R factor $\left(= \frac{\Sigma||F_o| - |F_c||}{\Sigma|F_o|}\right)$ was 0.053 and the final difference map showed no significant maxima apart from two located close to the chromium atom. Refinement of the structure in the centrosymmetric space group C 2/m showed on the basis of the Hamilton R factor test [13] that the structure was indeed non centrosymmetric. The refined atom parameters are given in Table I, intramolecular bonds and angles in Table II and tables of observed and calculated structure factors are available from the authors.

As expected the structure consists of discrete molecules of $Cr(C_6H_4NO_2)_2 \cdot 4H_2O$ (Fig. 1) with the two nicotinate moieties bound to the chromium in a trans configuration through their pyridine ring nitrogen atoms. Each molecule has 2-fold rotational symmetry about an axis which bisects the O(3)–Cr–O(3′) angle. Like the Co(II) bis-nicotinato tetraaquo complex [14] with which it is almost isomorphous, this molecule is an unusual non-classical zwitterion with the formal positive charge residing in the centre and the negative charges at each end. Estimated standard deviations (e.s.d.) for the Cr–N and Cr–O bonds are 0.008 Å and 0.02 Å respectively while those for the light atom-light atom interactions vary between 0.01 Å and 0.02 Å. E.s.d's on interbond angles are all about 1°. The geometry of the nicotinate moiety appears reasonable with all bond lengths and angles within 3σ of their expected values. The chromium environment is a slightly distorted octahedron with the nitrogen atoms trans and the water molecules occupying the four equatorial sites. The Cr–N distance of 2.136(8) Å compares favourably with the values found by Reed *et al.* [15] for Cr(II)–pyridine bonds (2.141(8) Å, 2.121(8) Å) and is slightly longer than similar bonds determined for chromium(III) complexes (2.103(7) Å [16], 2.087(4) Å [17]). Similarly the distances for the Cr–OH_2 bonds (2.16(2) Å, 2.08(2) Å) are slightly longer than those determined for Cr(III)–OH_2 interactions (1.958(3) Å, 1.989(2) Å [18]). Interbond angles at the chromium vary between 88.4(8)° and 92.6(8)°. All hydrogens seem reasonable and those on the water molecules are all located where they can participate in intermolecular hydrogen bonds.

Within the crystals molecules are bound together by hydrogen bonds between the oxygens of the carboxyls and the water molecules attached to adjacent chromiums. Oxygen O(2) is bonded to both O(3)

TABLE I. Refined Atom Parameters for $Cr(II)(nic)_2(H_2O)_4$.

Atom	x/a	y/b	z/c	U_{11}*	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
Cr	0.000	0.000	0.000	0.012(1)	0.025(1)	0.013(1)	0.000	0.003(1)	0.000
N	0.147(1)	−0.004(5)	0.245(1)	0.036(5)	0.045(6)	0.041(5)	0.010(14)	0.019(4)	0.010(15)
C(1)	0.147(1)	−0.000(5)	0.403(2)	0.035(6)	0.054(7)	0.048(7)	0.048(12)	0.022(5)	0.032(5)
C(2)	0.240(1)	0.002(4)	0.569(1)	0.043(6)	0.045(7)	0.038(6)	−0.036(13)	0.019(5)	−0.039(6)
C(3)	0.388(1)	0.001(6)	0.569(2)	0.033(6)	0.048(7)	0.044(7)	0.012(17)	0.011(5)	0.018(16)
C(4)	0.341(1)	−0.008(6)	0.410(2)	0.031(5)	0.062(11)	0.048(7)	−0.031(17)	0.016(5)	−0.015(16)
C(5)	0.244(1)	−0.001(5)	0.250(2)	0.053(7)	0.058(9)	0.050(7)	−0.044(15)	0.032(6)	−0.047(14)
C(6)	0.231(1)	−0.004(5)	0.736(2)	0.059(8)	0.036(7)	0.042(7)	−0.023(15)	0.021(6)	−0.028(15)
O(1)	0.317(1)	0.008(4)	0.885(1)	0.065(5)	0.051(6)	0.034(4)	0.004(13)	0.004(4)	0.017(13)
O(2)	0.138(1)	0.011(5)	0.719(1)	0.054(5)	0.091(10)	0.063(6)	−0.035(13)	0.037(5)	−0.028(13)
O(3)	0.056(1)	0.224(2)	0.887(2)	0.035(8)	0.033(7)	0.019(6)	0.012(6)	0.010(5)	0.010(6)
O(4)	−0.064(2)	−0.210(3)	0.099(3)	0.053(12)	0.087(14)	0.103(15)	−0.037(13)	0.032(11)	−0.036(11)
H(1)	0.076	0.007	0.398	0.06					
H(3)	0.409	0.053	0.700	0.06					
H(4)	0.402	0.007	0.382	0.06					
H(5)	0.241	0.009	0.138	0.06					
H(6)	−0.102	0.236	0.186	0.06					
H(7)	0.875	0.309	0.019	0.06					
H(8)	0.888	0.690	0.018	0.06					
H(9)	0.901	0.830	0.179	0.06					

*Anisotropic thermal parameters are of the form: $T = \exp - 2\pi(U_{11}h^2a^{*2} + U_{22}k^2b^{*2} + U_{33}l^2c^{*2} + 2U_{12}hka^*b^* + 2U_{13}hla^*c^* + 2U_{23}klb^*c^*)$.

TABLE II. Bond Lengths and Interbond Angles for $Cr(II)(nic)_2(H_2O)_4$.

Bonds	d(Å)	Angles	Degrees	Angles	Degrees
Cr–N	2.136(8)	N–Cr–N′	178.6(8)	N–C(1)–C(2)	123.5(9)
Cr–O(3)	2.16(2)	N–Cr–O(3)	92.6(8)	C(1)–C(2)–C(3)	118.2(9)
Cr–O(4)	2.10(2)	N–Cr–O(3′)	88.4(8)	C(1)–C(2)–C(6)	118.8(9)
N–C(1)	1.34(1)	N–Cr–O(4)	90.7(9)	C(3)–C(2)–C(6)	122.9(10)
N–C(5)	1.36(1)	N–Cr–O(4′)	88.4(9)	C(2)–C(3)–C(4)	119.5(10)
C(1)–C(2)	1.41(2)	O(3)–Cr–O(3′)	88.5(10)	C(3)–C(4)–C(5)	118.8(10)
C(2)–C(3)	1.39(1)	O(3)–Cr–O(4)	176.3(10)	C(4)–C(5)–N	122.9(10)
C(2)–C(6)	1.49(2)	O(3)–Cr–O(4′)	89.8(10)	C(2)–C(6)–O(1)	117.3(10)
C(3)–C(4)	1.37(2)	O(4)–Cr–O(4′)	92.1(10)	C(2)–C(6)–O(2)	116.9(11)
C(4)–C(5)	1.41(2)	Cr–N–C(1)	120.5(6)	O(1)–C(6)–O(2)	124.9(11)
C(6)–O(1)	1.28(1)	Cr–N–C(5)	122.5(7)		
C(6)–O(2)	1.27(2)	C(1)–N–C(5)	116.9(9)		

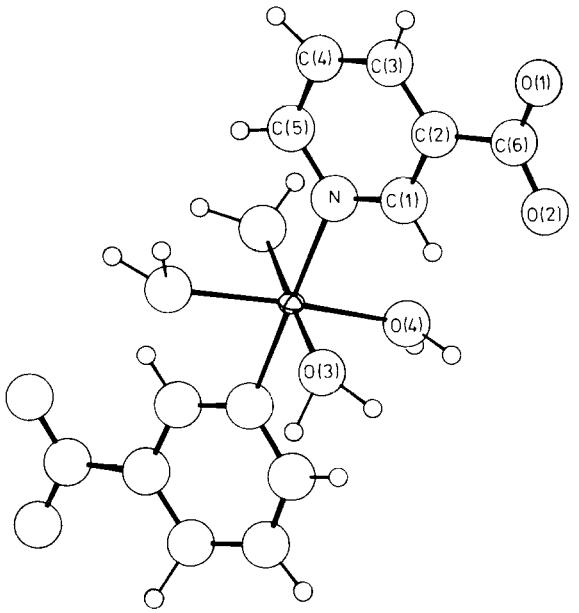

Fig. 1. Structure of $Cr(II)(nic)_2(H_2O)_4$.

and O(4) (O(2)–O(3) 2.67 Å, O(2)–O(4) 2.71 Å) of the next molecule at 0,0,1 + z to form ribbons of molecules parallel to the *ac* plane and elongated along *c*. Each ribbon is then bonded to the C-centre related ribbons at x + 1/2, y + 1/2, 0 and x + 1/2, y – 1/2, 0 by the hydrogen bonds O(1)–O(3) (2.75 Å) and O(1)–O(4′) (2.66 Å) respectively. Such an arrangement considerably reduces the charge separation of these non-classical zwitterions by bringing the carboxyl groups much closer to the formally positive chromium atoms and no doubt contributes to the stability of the crystals.

Since the basic structural unit of the crystal is $Cr(nic)_2(H_2O)_4$ with no evidence for any interspersed negative ions the chromium must be considered to be present in the +2 oxidation state. Although such a stable monomeric low-spin chromium(II)–dinicotinate species might be considered unusual, other similar structures with pyridine-type ligands are known [15]. The magnetic moment of the complex (3.18 bohr magnetons) is consistent with the proposed oxidation state since it is significantly less than the value normally expected for an octahedral monomeric chromium(III) complex (3.88 bohr magnetons) but close to the value (2.8 bohr magnetons) expected for an octahedral low spin chromium(II) complex [19]. The mechanism by which nicotinic acid stabilizes the +2 oxidation state is presently unknown but the complex exhibits remarkable stability in air, maintaining its yellow colour more or less indefinitely. Part of its long term stability undoubtedly resides in the extreme insolubility of the complex in most common solvents which presumably results from the extensive hydrogen bonding network within the crystal lattice. The complex did however dissolve in NaOH (2*M*) but the resulting electronic spectrum slowly changed with time indicating that structural changes were occurring. Initially, the electronic spectrum of the solution was similar to the reflectance spectrum of the crystals and hence no major structural change occurred on immediate dissolution of the crystals. However, two bands (at 405 nm and 600 nm respectively), similar to (but not identical with) those expected for the chromium(III) hexaquo ion (see Table III) gradually appeared. Thus, following dissolution of the crystals the stabilisation of the low spin +2 state is lost and the chromium appears to be slowly oxidised to the +3 state. For biological assay of the $Cr(II)(nic)_2(H_2O)_4$ complex it was therefore solubilised at either alkaline or acid pH and then adjusted to the pH of the assay (5.75) immediately prior to commencement of the assay.

Comparison of Dinicotinate Complexes of Cr(II), Mn(II), Ni(II) and Co(II)

A series of analogous $M(II)(nic)_2$ complexes, where M = Mn(II), Co(II) or Ni(II) were prepared according to the procedure of Anagostopoulos [12]

TABLE III. Summary of Electronic Absorption Spectra of Nicotinic Acid Complexes of Chromium.

Complex	Absorption maxima[a] (nm)			Colour and conditions of measurement
$Cr(III)(H_2O)_6^{+3}$	–	406	571	purple-red solution
$Cr(III)(nic)_2(H_2O)_3(OH)$	263	420	575	blue solid
$Cr(III)(nicH)_2(H_2O)_4^{+3}$	262	425(35)	575(26)	blue solution in 2 *M* HNO_3
$Cr(III)(nic)_2(H_2O)_4^{+}$	262	425(35)	575(26)	blue solution at pH 7
$Cr(III)(nic)(H_2O)_5^{+2}$	262	417(20)	570(27)	blue solution at pH 7
$Cr(III)(NH_3)_6^{+3}$	–	353	470	yellow solid
$Cr(II)(nic)_2(H_2O)_4$	262	340	–	yellow solid

[a]The extinction coefficient of the absorption band (where known) is given in brackets.

and their infra red spectra and X-ray powder diffraction patterns were prepared for comparison with $Cr(II)(nic)_2(H_2O)_4$. The X-ray powder diffraction patterns for the Cr(II) and Co(II) complexes were virtually identical and the fact that closely similar patterns were obtained for the Ni(II) and Mn(II) complexes suggests that all of the complexes have the same trans N-coordinated arrangement of the nicotinic acid ligands. The infra red spectra of the four complexes were also virtually identical as shown for example in Figure 2a and Figure 2b for the Cr(II) and Co(II) complexes. In each case there was an intense new band at about 1600 cm^{-1} and the quartet of peaks which occurs between 800 cm^{-1} to 650 cm^{-1} in uncomplexed nicotinic acid (Fig. 2d) was altered by a large increase in the intensity of the two innermost bands. This strengthening of the two innermost bands appears to be diagnostic for coordinated nicotinic acid ligands irrespective of the mode of coordination (see below). It is interesting to note therefore that the infra red spectrum of the GTF fraction isolated from brewer's yeast by Toepfer *et al.* [7] does not show these characteristic bands, but is identical with the spectrum of pure, uncomplexed nicotinic acid. This confirms the suggestion [8] that the material which was isolated by Toepfer *et al.* was in fact mainly nicotinic acid.

The only region of the infra red spectrum where there were differences for $Cr(nic)_2(H_2O)_4$, $Ni(nic)_2(H_2O)_4$ and $Co(nic)_2(H_2O)_4$ lay between 650 cm^{-1} and 200 cm^{-1} where a new band was observed for all of the complexes. The fact that the position of the band depended on the metal ion involved (Ni, 365 cm^{-1}; Co, 350 cm^{-1}; Cr, 310 cm^{-1}) suggests that this band is a metal-nitrogen stretching vibration and that the order of M–N bond strengths is Ni(II)–N > Co(II)–N > Cr(II)–N. Metal–nitrogen stretching frequencies in this region of the infra red spectrum have also been reported by Allen *et al.* [20] for a series of complexes ML_2X_2 where M = Mn(II), Fe(II), Ni(II), Co(II) and Cu(II); L = nicotinic acid or nicotinamide and X = Br or Cl. All of these complexes were also considered to involve co-ordination of the nicotinic acid group through the pyridine ring nitrogen atoms in a trans relationship with the equatorial coordination sites containing bridging halogen atoms. For these complexes the metal–nitrogen stretching frequency was also assigned to a single band at a frequency around 300 cm^{-1}.

The series of complexes all exhibited strong charge transfer bands in the ultraviolet region of the spectrum at about 280 nm [12] except for the chromium complex which absorbed intensely at 340 nm. This 340 nm charge transfer band obscured the much weaker d–d transition expected for the Cr(II) ion and was responsible for the yellow colour of the crystals.

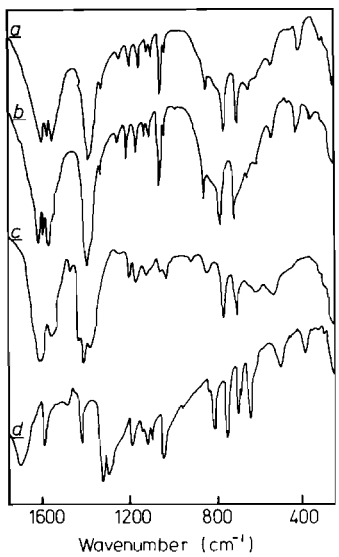

Fig. 2. Infrared absorption spectra of (a) $Cr(II)(nic)_2(H_2O)_4$, (b) $Co(II)(nic)_2(H_2O)_4$, (c) $Cr(III)(nic)_2(H_2O)_3(OH)$ and (d) nicotinic acid. All spectra were obtained as KBr pellets.

Structure of Chromium(III)–dinicotinate Complex

Since the so-called synthetic GTF mixture reported by Toepfer *et al.* [7] was prepared from chromium(III) salts rather than via the chromium(II) ion, we also prepared dinicotinate complexes of

chromium(III) by established procedures and obtained an amorphous blue solid which analysed as $Cr(III)(nic)_2(H_2O)_3OH$. The electronic spectrum of the blue solid was determined by the reflectance method in a nujol mull and contained the expected d–d transitions for a Cr(III) octahedral complex, one at 420 nm assigned to the $^4A_2(F) \rightarrow {}^4T_1(F)$ transition and one at 575 nm assigned to the $^4A_2(F) \rightarrow {}^4T_2(F)$ transition. The third transition expected for a d^3 configuration was not observed (There was no charge transfer band observable as was the case for $Cr(II)(nic)_2(H_2O)_4$.) These absorption maxima were at frequencies greater than the absorption maxima for the $Cr(H_2O)_6^{+3}$ ion (see Table III) which indicates a decrease in the energies of the d–d transitions consistent with coordination of the nicotinic acid through the carboxylate group rather than through the nitrogen atom as with the yellow $Cr(II)(nic)_2(H_2O)_4$ complex. If the nicotinic acid moiety were coordinating via the nitrogen atom it would be expected that the absorption maxima would shift to wavelengths shorter than for $Cr(H_2O)_6^{+3}$ as for example with $Cr(NH_3)_6^{+3}$ which is bright yellow (see Table III), rather than longer as observed. The suggestion that the nicotinic acid ligands are coordinated through the carboxyl oxygens is also in line with expectations based on the theory of hard and soft acids and bases and experimental support is obtained from the crystal structure of the trimeric chromium(III)–nicotinic acid complex, recently reported by Gonzalez-Vergara *et al.* [2]. This structure shows that each of the nicotinic acid ligands bridges two chromium centres through the carboxylate oxygens to form the $[Cr_3O(nic)_6(H_2O)_3]^{+7}$ ion. A similar oxygen ligation mode has also been proposed for binding of nicotinic acid to diaqua [N,N′-ethylenebis(salicylideneaminato)]chromium(III) chloride on the basis of visible and infra red spectral measurements [4].

The infra red spectrum of $Cr(III)(nic)_2(H_2O)_3(OH)$ was also consistent with the suggestion of a carboxylate oxygen ligation mode since there was no band in the region 350 cm^{-1} to 300 cm^{-1} as found for the nitrogen coordinated Cr(II), Ni(II) and Co(II) complexes. Apart from this essential difference the general features of the infra red spectrum (Fig. 2c) were similar to those observed for the yellow $Cr(II)(nic)_2(H_2O)_4$ complex although the peaks were individually less well resolved. The peak at 1315 cm^{-1} in uncomplexed nicotinic acid again became intensified but was now split into a triplet in the complex and the peak at 1695 cm^{-1} (Fig. 2d) shifted to about 1600 cm^{-1}. As for the N-coordinated chromium(II)–dinicotinate complex the quartet of peaks between 800 cm^{-1} and 650 cm^{-1} in uncomplexed nicotinic acid were altered on coordination so that the two innermost peaks were by far the most obvious.

It seems reasonable to assume that the two nicotinic acid ligands are coordinated to the chromium centre via the carboxylate oxygens in a trans arrangement since in the absence of constraining factors a trans relationship of the nicotinic acid ligands would involve the least amount of steric hinderance and should therefore be preferred. The remaining ligands would be expected to assume a planar arrangement around the chromium centre as shown in Fig. 1 for $Cr(II)(nic)_2H_2O)_4$.

Soluble Chromium(III)–Dinicotinate Complex

The blue solid dissolved in 2*M* HNO_3 to give a blue solution with absorption maxima and molar extinction coefficients typical of octahedral coordination and whose visible spectrum showed no deterioration with time, remaining similar to the reflectance spectrum of the solid. The dissolution of the solid in acid was therefore probably caused by protonation of the pyridine nitrogen atoms and ligating hydroxyl (equation 1) thereby disrupting the hydrogen bonding network which probably exists in the solid

$$Cr(nic)_2(H_2O)_3(OH) + 3H^+ \rightleftharpoons Cr(nicH)_2(H_2O)_4^{+3} \quad (1)$$

The magnitude of the positive charge on this soluble blue complex could not be confirmed by ion-exchange chromatography since the whole solution bound so tightly to a Dowex 50W-X12 cation exchange column that it could not be removed by any of the eluants used. The $Cr(nicH)_2(H_2O)_4^{+3}$ species was therefore run through a sephadex G-15 column to determine whether any other species were present in solution. However, only a single peak was observed on elution with water. Nevertheless there was obviously also some interaction between the blue species and the sephadex resin since it was eluted from the sephadex column after the conductivity peak. Similar behaviour on sephadex gels with other aromatic compounds has been cited by Janson [21] but the nature of the interactions is unknown. Free nicotinic acid itself did not show any matrix effects since it was always eluted from the sephadex column before the conductivity peak as expected. Despite the anomalous retardation of the soluble blue complex on the gel filtration column there was no evidence for the presence of any other species and determination of the chromium to nicotinic acid ratio (determined assuming that the extinction coefficient ϵ (5012 l mol^{-1} cm^{-1}) was unchanged on coordination [11]) confirmed that 2 nicotinic acid ligands were still associated with each chromium(III) ion throughout the peak (Fig. 3). Since the soluble blue complex was eluted from the gel filtration column with water the pyridine nitrogen atoms should not be protonated therefore forming $Cr(III)(nic)_2(H_2O)_4^+$. However, the visible absorption spectrum of

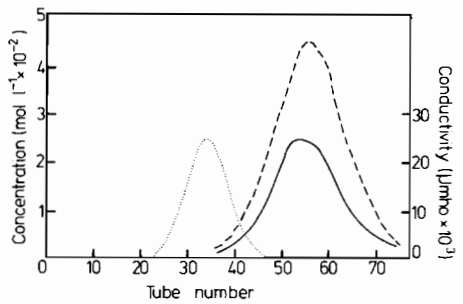

Fig. 3. Elution profile of $Cr(III)(nic)_2(H_2O)_4^+$ on a sephadex-G-15 gel filtration column. The tube volume was 2.0 cm^3 and the column was eluted with H_2O. Chromium (——), nicotinic acid (- - -), conductivity (· · ·).

this species was virtually identical with that recorded for the $Cr(III)(nicH)_2(H_2O)_4^{+3}$ complex and also for the solid $Cr(III)(nic)_2(H_2O)_3(OH)$ (see Table III). Thus the structure of all three species would seem to be the same and probably that of a trans-coordinated arrangement of two nicotinic acid residues around a central chromium(III) ion ligated via the carboxyl oxygen atoms.

Preparation of Cr(III)–Mononicotinate Complex

The procedure for preparation of a mono nicotinic acid complex of Cr(III) was similar to that described for the solid blue $Cr(nic)_2(H_2O)_3OH$ complex except that equimolar amounts of $Cr(NO_3)_3 \cdot 9H_2O$ (2.0 g, 0.005 moles) and nicotinic acid (0.62 g, 0.005 moles) were used. Attempts to crystallise the mononicotinate complex as its perchlorate salt were unsuccessful so the solution was subjected to cation-exchange chromatography on a Dowex 50W-X12 resin essentially as described by Haylock *et al.* [8]. The solution was loaded on to the column (3.0 cm X 9.5 cm) at pH 3.5 and at a conductivity of less than 3000 μmho and the column was then washed with water. Elution with NaCl (0.5 *M*) gave a blue solution with a chromium to nicotinic acid ratio of 1:1 and further elution with a phosphate pH gradient [8] yielded a green fraction at pH 5.0 which also had a chromium to nicotinic acid ratio of 1:1. Final elution with 2.0 *M* HCl failed to elute any further chromium species although from a chromium balance it was obvious that much of the original material was still bound to the column.

The blue complex was eluted off the column just prior to the marker $Cr(H_2O)_6^{+3}$ showing that the effective charge on the complex was less than 3. Since the positions of the absorption maxima lay between those observed for $Cr(nic)_2(H_2O)_4^+$ and the $Cr(H_2O)_6^{+3}$ species (Table III) and since the chromium:nicotinic acid ratio was 1:1, it seems reasonable to assume that the composition of the solution was $Cr(nic)(H_2O)_5^{+2}$ with a single nicotinic acid ligand coordinated via the carboxylate group. Green colour in a chromium(III) complex is usually associated with the presence of oxygen bridging therefore the green fraction was most probably a polymeric Cr(III)–nicotinic acid species similar to the polymeric trinuclear chromium(III)–nicotinic acid recently reported by Gonzalez–Vergara *et al.* [2].

Since such a large amount of the original blue solution remained bound to the cation-exchange column, gel filtration was used in an attempt to separate and identify the various components. The solutions to be chromatographed were concentrated to 5 cm^3 and run through the G-15 column at a flow rate of 1.0 cm^3 per minute using H_2O as the eluent. Three bands were observed, their order of elution being purple-violet, salt peak, blue and blue (Fig. 4).

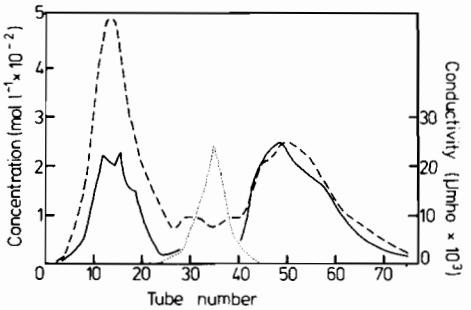

Fig. 4. Elution profile for chromium(III)–mononicotinate complex on a sephadex G-15 gel filtration column. The tube volume was 2.0 cm^3 and the column was eluted with H_2O. Chromium (——), nicotinic acid (- - -), conductivity (· · ·).

The purple-violet band consisted of unreacted $Cr(H_2O)_6^{+3}$ and nicotinic acid and although the two blue bands overlapped it was possible to conclude that the leading edge of the peak consisted largely of $Cr(nic)H_2O)_5^{+2}$ since the chromium:nicotinic acid ratio was about 1. For the second half of the peak the ratio was less than 1 which is consistent with the presence of some $Cr(nic)_2(H_2O)_4^+$ thus although the required mononicotinate–chromium(III) complex was clearly formed and isolated by ion-exchange chromatography a considerable amount of the chromium was probably converted into the dinicotinato–chromium(III) complex.

Biological Activity of Chromium–Nicotinato Complexes

The biological activity of the various complexes was determined by the yeast fermentation assay reported by Haylock *et al.* [5], and Mirsky *et al.* [22]. This assay involves comparing the rate of CO_2 production of a chromium-deficient yeast sample (of known concentration) supplied with a test sample and glucose, with an identical yeast sample supplied only with glucose. After a lag phase of about 50 minutes an increase in the basal rate of CO_2 production is found for samples with glucose tolerance factor activity.

The rates of CO_2 evolution of several blank samples were determined over a number of assays and were 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 rates was $1.72 \pm 0.19 \times 10^{-2}$ μmol CO_2/min for eight separate assays. For the rate of CO_2 evolution of an active sample to be considered as significantly different from the blank sample it was decided to accept only those rates which were greater than two standard deviations from the average rate of CO_2 evolution as being biologically active. Therefore, unless the rate of CO_2 production of an assay exceeded that of the blank by at least 22% it was not considered to represent significant biological (or GTF) activity.

The results of the bioassays for a range of N- and O-coordinated nicotinic acid complexes of chromium and other transition metals are shown in Table IV. It is apparent from the results that only the O-coordinated chromium(III)–dinicotinate complex can be considered to exhibit biological (or GTF-like) activity. Neither the O-coordinated mononicotinic acid chromium(III) complex nor any of the N-coordinated dinicotinic acid complexes of the divalent transition metals produced any significant stimulation of the basal rate of CO_2 evolution in the yeast bioassay.

TABLE IV. Bioassay Results for Nicotinic Acid Complexes of Cr(III) and Other Transition Metals.

Compound	pH[a] of sample	Concentration of metal (μg cm^{-3})	Activity[b] %
$Cr(III)(nic)_2(H_2O)_4^+$	4.0	50	369 ± 50
$Cr(III)(nic)(H_2O)_5^{+2}$	5.0	50	1 ± 4
$Cr(II)(nic)_2(H_2O)_4$	1.5	50	20 ± 11
$Co(nic)_2(H_2O)_4$	5.0	50	26 ± 22
$Ni(nic)_2(H_2O)_4$	5.0	50	27 ± 12
$Mn(nic)_2(H_2O)_4$	5.0	10	−25 ± 6
nicotinic acid	4.0	100	2 ± 7

[a]The assay conditions were pH 5.7 phosphate buffer, 2% glucose solution and 1.5×10^8 chromium-depleted yeast cells (*Saccharomyces cerevisiae*) per cm^3. The sample was prepared at the stated pH before addition to the assay mixture. [b]Activity is expressed as the percentage increase in the rate of CO_2 production for a particular sample over the control rate.

The results are therefore not consistent with the suggestion [23] that it is a pair of N-coordinated trans-arranged nicotinic acid ligands which is responsible for the biological activity in the GTF bioassay systems. Of course it must be conceded that the transition metals in the N-coordinated complexes tested in the present work are all in the +2 oxidation state (including the chromium complex) whereas Mertz [23] proposed that it was a N-coordinated chromium(III) complex which constituted the essential structural element of GTF. However, since it is the shape of the complex which is likely to be the important factor in determining the presence or absence of biological activity, and octahedral complexes are expected for both chromium(II) and chromium(III) the difference in oxidation state of the metal should not be a significant factor.

Unfortunately it was not possible to prepare stable O-coordinated dinicotinic acid complexes of any other transition metal and thus their biological activities could not be determined. Chromium(III) therefore appears to be unique among the transition metals in its ability to form O-coordinated dinicotinic acid complexes which are relatively stable at near neutral pH (olation does however occur after extended periods of time). It seems likely that it is this ability of chromium(III) to form O-coordinated complexes which has led to its suggested role in glucose metabolism, rather than anything fundamentally important about the chromium ion particularly in view of the fact that the naturally occurring GTF material does not contain chromium [6]. It is logical to assume therefore that GTF bears some overall structural resemblance to the O-coordinated $Cr(III)(nic)_2(H_2O)_4^+$ complex and we suggest that the significant factor is the trans arrangement of the pyridine nitrogen atoms which exists in the O-coordinated Cr(III)–dinicotinate complex. This arrangement must resemble the part of the GTF structure which is recognised by the receptors or enzymes involved in the expression of the biological effect.

The synthetic mixture reported by Toepfer *et al.* [7] probably contains some (soluble) $O–Cr(III)(nic)_2(H_2O)_4^+$ which is responsible for at least part of the observed biological acitivity of this mixture. Since this mixture also contained amino acids there must also have been other chromium complexes in the mixture, some of which could also be biologically active. Investigation of these chromium amino acid complexes will shed further light on the structural features of chromium complexes which are responsible for the biological acticity and hence on the possible structure of the glucose tolerance factor.

Acknowledgements

We thank Dr. W. T. Robinson of the University of Canterbury for collecting the X-ray diffraction data and Dr's E. W. Ainscough and A. M. Brodie for helpful discussions.

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