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An Investigation by Ion-exchange Chromatography of a Chromium, Amino Acid and Nicotinic Acid Mixture

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Abstract

The mixture of chromium, nicotinic acid and the amino acids glycine, glutamic acid and cysteine which stimulates the rate of CO_2 production in a yeast bioassay system was subjected to the separation scheme based on ion-exchange chromatography which has been used to separate the chromium-containing fractions in brewer's yeast, [S. J. Haylock, P. D. Buckley and L. F. Blackwell, *J. Inorg. Biochem., 18*, 195 (1983)]. Four chromium-containing fractions (C2 to C5) were obtained by salt gradients and two further fractions (G1 and G2) were obtained using a pH gradient. All were amino acid-containing complexes of chromium and all except C5 also contained nicotinic acid. However, none of the isolated chromium fractions showed any activity in a yeast bioassay. On the basis of previous work, the activity of the original mixture was attributed to the presence of an oxygen-coordinated *trans* chromium(III)-dinicotinate complex. Biologically-inactive chromium complexes such as $Cr(glu)_2(H_2O)^+_2$ and $Cr(gly)_2(H_2O)^+_2$ after elution by ammonium hydroxide from Dowex 50W-X12 cation-exchange columns, stimulated the rate of CO_2 production in the yeast bioassay. Elution with other bases, such as lithium hydroxide, potassium hydroxide and sodium hydroxide led to inactive fractions in all cases. A warning is therefore given that the use of ammonium hydroxide-elution of ion-exchange columns to isolate glucose tolerance factor fractions from biological samples (such as brewer's yeast) can lead to active fractions which do not relate to the native material.

Introduction

A mixture described by Toepfer *et al.* [1] which contains chromium, nicotinic acid (nic) and the amino acids glycine (gly), glutamic acid (glu) and cysteine (cys) has been shown [2] to stimulate the rate of CO_2 production in a yeast bioassay [1, 2] and in this respect may be said to mimic one of the effects of the putative glucose tolerance factor (GTF). The separate components of this mixture are inactive in the yeast bioassay [2] hence a chromium complex (or complexes) formed in the mixture must be responsible for the observed biological effects.

We have recently shown that a *trans* arrangement of non-coordinated nitrogen atoms in the ligands of chromium(III) complexes gives rise to biological activity in the yeast bioassay [3]. It is therefore of interest to separate any chromium-containing fractions from the chromium mixture described by Toepfer *et al.* [1] and compare the ion-exchange and spectroscopic data of any biologically-active fractions with those already obtained from parallel studies [3, 4] of the reaction of chromium(III) with each of the individual components of the mixture.

We have now applied our separation procedure [2], which resolves the chromium-containing material in brewer's yeast into eleven different fractions, to the chromium mixture described by Toepfer *et al.* [1]. The results reported in this paper show that the main active compound in the mixture is an oxygen-coordinated *trans* chromium(III)–dinicotinate complex and that inactive complexes may be converted into active complexes if elution from Dowex 50 ion-exchange columns with ammonia is employed.

Experimental

All reagents were AR grade wherever possible. The following chemicals and resins were obtained from the sources indicated: Dowex 50W-X12 cation-exchange resin (Riedel de Haen, Seelze, Hannover); Dowex 1-X8 anion-exchange resin (Bio-Rad, Richmond, CA); Sephadex G15 gel filtration resin (Sigma Chemical Co., St. Louis, MO).

Chromium analyses, electronic spectra, absorbance, pH and conductivity measurements were all

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performed as described previously [4]. The ion-exchange and gel-filtration columns were prepared and eluted also as discussed elsewhere [2, 5].

Results

Preparation of a Chromium Solution Which Stimulates the Rate of CO_2 Production in a Yeast Bioassay

This was prepared essentially as described by Toepfer *et al.* [1], except that it was carried out in totally aqueous solution. Reaction of nicotinic acid (4.0 g) with $Cr_2(SO_4)_3 \cdot 15H_2O$ (5.3 g) produced a dark blue solution (as expected if $Cr(nic)_2(H_2O)^+_4$ was being formed [4]) which changed to the red-purple colour typical of chromium(III)–amino acid complexes [3] on addition of glycine (2.4 g), glutamic acid sodium salt (3.0 g) and cysteine (2.5 g). The resulting solution was reduced to one half the initial volume before subjection to ion-exchange chromatography.

pH Behaviour and Ion-Exchange Chromatography of Chromium Solution

The colour of the reaction mixture was dependent on the pH of the solution. At pH 3.5 the solution was red, whereas at pH 9.5 the solution was green and a pH titration of the mixture (from 3.5 to 12.0) yielded two pK_a values of approximately 4.3 and 9.0, similar to those recently determined for the $Cr(cys)_2^-$ complex [3]†.

The reaction mixture was titrated to pH 3.0 and diluted to a conductivity of 2500 μmho before loading onto a Dowex 50W-X12 cation-exchange column (2.5 cm × 10.0 cm) essentially as described previously [2]. The purple solution bound to the resin and a pink solution, which comprised about 2% of the original chromium, was eluted with water. A NaCl conductivity gradient (2500 μmho to 50 000 μmho) was applied to the column and several well-defined coloured fractions were eluted. A colourless peak (Cl) containing mainly unreacted nicotinic acid was eluted at 5000 μmho, followed by a red fraction (C2) eluted at 6000 μmho, a purple fraction (C3) at 12 000 μmho, a second purple fraction (C4) at 25 000 μmho and a green fraction (C5) at 45 000 μmho. Of these fractions C4 and C5 were present in much greater amounts than either C2 or C3. Subsequent application of a pH gradient (from 3.0 to 12.0), as described previously by Haylock *et al.* [2], resulted in the elution of one green band (G1) at pH 5.0 and a second green (G2) band at pH 9.0. The positions of the d–d bands and nicotinic acid content of these fractions (as determined from the ultra-violet absorption at 262 nm) are given in Table I. The fractions were desalted using Sephadex gel filtration columns eluted with 50% aqueous ethanol, which gave maximum separation of the various chromium complexes from the salt peak. The C2–C5 fractions were all of similar size and were smaller than the G fractions.

Biological Activity of the Isolated Chromium Fractions

The biological activities of the isolated chromium fractions, as determined by their stimulation of the rate of CO_2 evolution in a yeast bioassay [6], are given in Table II. Each sample (0.1 cm^3) was prepared in phosphate pH 5.75 buffer immediately prior to the assay with chromium-depleted yeast as previously described [6]. Only rates of CO_2 evolution which were more than 40–50% enhanced over the control rate were considered to be active for the reasons previously discussed [6]. On this basis, although the original reaction mixture gave a rate of CO_2 production which was significantly greater than the control rate (Table II), none of the fractions isolated by our separation procedure was active.

†No other simple chromium complexes prepared from the individual components of the reaction mixture showed pH-dependent colour changes [2, 4].

TABLE I. Absorption Maxima of the Fractions Obtained by Elution from Dowex 50W-X12 Cation-Exchange Column.

Fraction	Colour	Visible bands (nm)	Ultra-violet bands (nm)	Cr/Nicotinic acid ratio
C1	–	–	262	–
C2	Red	410, 562.5	262	1:68
C3	Purple[a]	–	262	1:2
C4	Purple	401, 552	262	–
C5	Green	430, 600	–	–
G1	Green	460, 615	262	1:1
G2	Green	425, 580	258, 290	–

[a]The colour of this fraction was altered on standing in acidic media.

TABLE II. Biological Activity of the Chromium Fractions.

Compound, or fraction[b]	Activity[a]	Activity after treatment with NH_4OH
Reaction mixture	84	
C2	43 ± 15	22 ± 8
C3	10 ± 9	9 ± 7
C4	6 ± 1	66 ± 9
C5	51 ± 9	7 ± 6
G1	32 ± 13	51 ± 3
G2	−33 ± 10	–

[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 of the sample and control respectively. The concentration of chromium was 8 $\mu g/cm^3$ in all cases. [b]The samples indicated were bound to Dowex 50W-X12 cation-exchange columns and eluted with NH_4OH (0.5 M).

Each of the inactive cationic chromium-containing fractions was separately loaded onto Dowex 50W-X12 columns at pH 3.5, washed with water until no chromium could be detected in the effluent, and then eluted with NH_4OH (0.1 M). The ammonia was removed *in vacuo*, (or by freeze drying) until the evolution of gaseous bubbles ceased (ca. 60 min) and the eluted fractions were again assayed. After this treatment there was no significant increase in the activity of the fractions arising from C2, C3 or G1, but that arising from C4 was significantly increased, even at the 8 $\mu g/cm^3$ levels used in the assay (Table II).

Effect of NH_4OH Elution on the Activity of Characterised Chromium Complexes

The characterised complexes $Cr(glu)_2(H_2O)^+_2$, $Cr(gly)_2(H_2O)^+_2$, $Cr(cys)_2^-$ and $Cr(nic)_2(H_2O)^+_4$ previously prepared and structurally-characterised in our laboratories [3, 4] were also subjected to elution by NH_4OH from Dowex 50W-X12 cation-exchange columns.

$Cr(gly)_2(H_2O)^+_2$

When the biologically-inactive red diaquobis-glycine chromium(III) complex [3] was absorbed onto Dowex 50W-X12 resin and eluted with NH_4OH (0.5 M) a single blue fraction was obtained. The solution pH was 9.3 and the blue solution was stable at this pH for about 24 h, following which a slight precipitate appeared and the solution turned green. These changes were accelerated by moderate heating. The eluted blue solution exhibited two d–d bands in the visible region of the spectrum at 404 nm and 565 nm at pH 9.5, as compared with the d–d bands of the original red solution at 398 nm and 535 nm. Addition of 2 M HNO_3 turned the solution red again and the absorption maxima appeared to be identical with those of the original red solution. However, the reformed red solution now showed activity in the yeast bioassay at pH 5.75 (Table III). The biological activity of the $Cr(gly)_2(H_2O)^+_2$ complex after elution from the Dowex 50W-X12 column with ammonia was determined as a function of the chromium concentration (at pH 5.75). The activity showed a hyperbolic dependence on the chromium concentration with a maximum stimulation of the rate of CO_2 evolution (*ca.* 400%) being obtained at a chromium concentration of about 100 $\mu g/cm^3$ (Fig. 1).

TABLE III. Activity of Characterised Chromium(III) Compounds After Elution with NH_4OH.

Compound	Concentration ($\mu g/cm^3$ Cr)	Original activity[a]	Final activity
$CrCl_3$	10	9	32[b]
$Cr(NH_3)^{+3}_6$	50	177 ± 7	–
$(NH_4)_3PO_4$ (0.05 M)	–	54	–
NH_4OH[c]	–	inhibition	–
$Cr(gly)_2(H_2O)^+_2$	50	−5 ± 5(50)[d]	171 ± 10
$Cr(glu)_2(H_2O)^+_2$	50	5 ± 7(40)	450 ± 40
$Cr(cys)_2(H_2O)^+_2$	10	51 ± 7(50)	7 ± 5
$Cr(nic)_2(H_2O)^{+3}_2$	46	369 ± 50(50)	13 ± 5

[a]The chromium concentration used in the assay before ammonia treatment is given in brackets. Activity as defined in Table II. [b]Eluted with 0.25 M NH_4OH. [c]There was an initial increase due to evolution of NH_3. [d]A 0.1 cm^3 sample of $Cr(gly)_2(H_2O)^+_2$ titrated to pH 9.5 with NH_4OH (0.5 M) or LiOH (0.5 M) in the absence of the Dowex resin gave an activity of 37% when assayed at a chromium level of 50 $\mu g/cm^3$ and at pH 5.75.

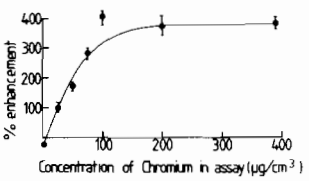

Fig. 1. Activity of the NH_4OH-eluted $Cr(gly)_2(H_2O)^+_2$ species as a function of the chromium(III) concentration. The yeast assay was performed at pH 5.75 as described previously [6] and the least squares standard error is shown for each point.

When a control experiment was carried out by titrating the red diaquobisglycine chromium(III) complex to pH 9.5 with NH_4OH in the absence of the Dowex 50W-X12 resin the colour did not change, and the titrated solution did not show any biological activity in the yeast assay system. Also, when the complex was absorbed onto a Dowex 50W-X12 column and subsequently eluted with NaOH, LiOH or KOH, only inactive green solutions were obtained.

$Cr(glu)_2(H_2O)^+_2$, $Cr(cys)_2^-$ and $Cr(nic)_2(H_2O)^+_4$

The effect of ammonia elution of the red-purple diaquobisglutamic acid chromium(III) complex (prepared as previously [3]) from the Dowex 50W-X12 cation-exchange column was identical with the behaviour observed with $Cr(gly)_2(H_2O)^+_2$. In this case the red solution resulting after neutralisation with HNO_3 (2 M) was almost 100 times as active in the yeast assay as the parent complex (Table III).

Unlike the diaquobisglycine and diaquobisglutamic acid complexes of chromium(III), the diaquobiscysteine chromium(III) complex did not stimulate the rate of CO_2 evolution after elution with NH_4OH from the Dowex 50W-X12 cation-exchange column (Table III). Changes had however taken place in the complex since the d–d bands at around 615 nm associated with coordination of the sulphur atom (see [6]) were now absent. The activity of the *trans* oxygen-coordinated $Cr(nic)_2(H_2O)^+_4$ complex (see [4]) was destroyed by the ammonia treatment but this was not unexpected since we have already demonstrated that this complex is not stable at alkaline pH values [4] and the resulting green colour was consistent with olation of the complex.

The effect of NH_4OH elution on the biological activity of a $CrCl_3$ solution was also determined and although the data (Table III) showed a small increase it was probably not significant (see [6]). The $Cr(NH_3)^{3+}_6$ complex (prepared as described previously [3]) was significantly active in the bioassay but ammonium hydroxide itself had a marked inhibitory effect.

Discussion

Reaction of chromium(III) with the mixture of nicotinic acid, glycine, cysteine and glutamic acid in a totally aqueous solution produced a dark purple solution, similar to that described by Toepfer *et al.* [1], from which a number of chromium-containing fractions were isolated by our published separation procedure [2]. Comparison with our published spectral data for characterised chromium amino-acid complexes [3] (of glycine, glutamic acid and cysteine) indicated, as expected, that chelated amino acid complexes were present in all of these fractions which also (except C5) contained nicotinic acid. For example, the d–d spectral bands observed at low pH for all the fractions isolated (except C3) were similar to those found for the $Cr(cys)_2(H_2O)^+_2$ and mixed cysteine–nicotinic acid complexes prepared previously [3] but not to the $Cr(gly)_2(H_2O)^+_2$ or $Cr(glu)_2(H_2O)^+_2$ complexes, suggesting the importance of cysteine coordination. However, since none of these isolated fractions was in fact active in the yeast bioassay further investigation of the possible modes of coordination of the amino acids and nicotinic acid in the various fractions was not considered to be warranted.

An active chromium complex must however have been present in the original reaction mixture (before ion-exchange chromatography) and must have been either too unstable to be isolated, or have remained tightly bound to the Dowex resin. Of the possible chromium–amino acid complexes or nicotinic acid-containing complexes characterised previously [3] and which would be expected to form in the mixture, only the *trans* oxygen-coordinated $Cr(nic)_2(H_2O)^+_4$ complex (as prepared elsewhere [4]) shows biological activity in the yeast bioassay [3, 4]. This complex which is relatively unstable forms readily under the reaction conditions (as shown by the initial blue colour of the reaction mixture) but binds tightly to the Dowex cation-exchange resin [4] and would therefore not be isolated by the separation procedure used in this work. The biological activity of the original reaction mixture is therefore most likely due entirely to its presence as we have suggested [3, 4].

We therefore conclude that the structure suggested by Toepfer *et al.* [1] as being responsible for the GTF-mimicking properties of their solution, namely an amino acid (unspecified)-stabilised *trans* nitrogen-coordinated nicotinic acid complex, must be considered extremely improbable both on chemical grounds and on the basis of all the experimental evidence which is now available (see for example [3] and [4] as well as the present work).

Effect of NH_4OH Elution on the Biological Activity of Chromium(III) Complexes

Most previous attempts [4, 7–9] to isolate active chromium(III) complexes from the mixture of chromium, nicotinic acid, glycine, glutamic acid and cysteine, or from biological sources, have all involved the elution of the bound chromium from a Dowex cation-exchange column with ammonia solution at some stage in the procedure. This treatment, as we have shown elsewhere [2], effectively wipes off all the bound material at once and therefore does not allow a separation of the various components as is possible with the pH and conductivity gradients used in the present work. More importantly, the elution of some chromium complexes with ammonia was found to give rise to significant increases in their biological activity as determined by the yeast bioassay system (Table III).

The ammonia effect cannot be simply an acid-base phenomenon since other hydroxides (LiOH, NaOH, KOH) do not convert inactive complexes into active ones but rather the reverse, presumably as a result of olation and the consequent production of basic chromium polymers. Hence the effect must

be related to the presence of ammonia but treatment of inactive chromium complexes with NH_4OH in the absence of the Dowex 50 resin had no effect whatsoever on the biological activity and ammonium ions themselves did not significantly stimulate the rate of CO_2 evolution in the yeast bioassay (Table III). Some specific chemical change must therefore be taking place on the ion-exchange resin in the presence of ammonia, or ammonium ions.

If ammonia was coordinated to chromium as a result of the NH_4OH elution, the increases in activity in the yeast bioassay might be explained since the chromium–hexaamine complex showed a significant ability to stimulate the rate of CO_2 production (see Table III). However, the positions of the d–d bands observed for all of the blue ammonia-eluted active solutions in this work are not consistent with coordination of basic amino groups [3, 4]. Neutralisation of these solutions with HNO_3, did however return the red colour characteristic of mixed carboxyl and amine coordination [3] but the spectral properties of the now active solutions were indistinguishable from those of the original solutions of $Cr(gly)_2(H_2O)^+_2$ and $Cr(glu)_2(H_2O)^+_2$ (see Results).

For the ammonia-eluted $Cr(gly)_2(H_2O)^+_2$ solution the dependence of the biological activity on the chromium concentration (Fig. 1) showed typical saturation behaviour, as is common for the binding of an effector molecule to a limited number of sites in a biological system. Thus the changes caused by elution with ammonia produced a species which behaved exactly like the other biologically-active chromium(III) compounds which we have reported [3, 4] all of which have had a pair of nitrogen atoms in a *trans* arrangement about a central chromium atom.

What is clear from the present study is that chromium-containing fractions which have been isolated from yeast (or other biological sources) should not be eluted from Dowex cation-exchange columns with ammonia, but rather with an alternative system such as the one which we have reported [3]. Otherwise fractions may be obtained which display apparent GTF activity but which nevertheless are unrelated to any naturally-occurring components in the yeast (or other) sample. Care must therefore be taken when interpreting the results reported in the literature [4, 7–9] where ammonia-elution of chromium-containing fractions has been used.

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References

1 E. W. Toepfer, W. Mertz, M. M. Polansky, E. E. Roginski and W. R. Wolff, *J. Agr. Food Chem., 25*, 162 (1977).
2 S. J. Haylock, P. D. Buckley and L. F. Blackwell, *J. Inorg. Biochem., 18*, 195 (1983).
3 J. A. Cooper, L. F. Blackwell and P. D. Buckley, *Inorg. Chim. Acta, 92*, 23 (1984).
4 J. A. Cooper, B. F. Anderson, P. D. Buckley and L. F. Blackwell, *Inorg. Chim. Acta, 91*, 1 (1984).
5 S. J. Haylock, P. D. Buckley and L. F. Blackwell, *J. Inorg. Biochem., 19*, 105 (1983).
6 S. J. Haylock, R. J. Thornton, P. D. Buckley and L. F. Blackwell, *Exp. Mycol., 6*, 335 (1982).
7 N. Mirsky, A. Weiss and Z. Dori, *J. Inorg. Biochem., 15*, 275 (1981).
8 R. A. Anderson, J. H. Bratner and M. M. Polansky, *J. Agr. Food Chem., 26*, 1219 (1978).
9 J. Kumpulainen, P. Koivistoinen and S. Lahtinen, *Bioinorg. Chem., 8*, 419 (1978).