component was indistinguishable from sorbitol or mannitol by our GC–MS procedures and was present in only trace amounts in most soybean extracts.

Cyclitols are known to occur in a number of plant families (Plouvier, 1958; Angyal and Anderson, 1959), however, in soybean, and in all legumes examined, the cyclitols consitute a major portion of the nonstructural carbohydrates (Smith and Phillips, 1980). In recent years there have been numerous studies published which attempted to relate nonstructural carbohydrates to some biological function in leguminous plants. Almost all of those have used methods which either failed to distinguish between th cyclitols and sugars and other carbohydrates or failed to even detect cyclitols. Thus many of these studies have attempted to relate some biological function to only a part, in some cases a small part, of the carbohydrate present.

Cyclitols may be equivalent to sugars in some biological processes but in others they are not (Dreyer et al., 1979; Talbot and Seidler, 1979) and in many biological processes the effects of cyclitols, other than *myo*-inositol, are unknown.

While there is no substitute for identification by GC–MS, NMR, etc., in species where this has not been done, there is a useful procedure for those who may not have ready access to analytical equipment and are working with species where cyclitols are known to occur. The sugars and most carbohydrates other than cyclitols can be completely removed from solution by the modification of the procedure of Roseman et al. (1952) described under Materials and Methods. The cyclitols, other sugar alcohols, and methyl glucosides remain in solution. We have used this method extensively on soybeans and other legumes and have found only small amounts of other sugar alcohols. Methyl glucosides have been found only in white clover (*Trifolium repens* L.) (Smith and Phillips, 1981). Thus, in most cases, the contribution of the cyclitols to the nonstructural carbohydrate pool can be determined directly—after removal of the sugars from the sample—by any analytical procedure which detects cyclitols.

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# Properties of a Chromium Complex from Higher Plants

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A biologically formed chromium complex has been isolated from alfalfa. It appears to be distinct from the glucose tolerance factor isolated from brewer's yeast. The in vivo and in vitro formation is described along with analysis of constituents and stability parameters affecting the complex. The chromium complex is identical regardless of the valence or mode of incorporation of the chromium. The complex is specific for chromium insertion and is extremely stable once formed. The complex is anionic and contains no peptides or deoxyribose units.

The trace element chromium has been widely studied since its definition as a micronutrient in 1959 (Schwarz and Mertz, 1959). Deficiency conditions have been described in pregnancy (Davidson and Burt, 1973), diabetes (Gurson and Saner, 1978), senescence (Levine et al., 1968), and protein-calorie malnutrition (Gurson and Saner, 1973). The form of chromium which is best absorbed in the gut has also been sought. Since chromium oxide is unusually inert, chromate and amino acids have been examined as the active gut complex. Schwarz and Mertz (1959) have proposed a glucose tolerance factor (GTF) as the biologically active complex that protects the chromium from olation, which inactivates it.

This work describes another biologically formed chromium complex distinct from GTF. This chromium complex is found in alfalfa, crested wheatgrass, beans, and wheat (Blincoe, 1974; Huffman and Allaway, 1973). Pre-

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vious studies found it to be a water-soluble, anionic complex, polydispersed with respect to the apparent molecular weight (Blincoe, 1974).

## EXPERIMENTAL PROCEDURES

**In Vitro Complex Formation.** Mature alfalfa or intact seedlings were cut into approximately 2-cm pieces and pressed at 2300 kg/cm$^2$. The expressed liquid was collected and centrifuged for 30 min at 10000$g$ at 0–4 °C. The supernatant was then removed, and an aliquot was incubated at room temperature with carrier-free $^{51}$Cr(VI) or $^{51}$Cr(III) for 30 min.

**In Vivo Complex Formation.** Alfalfa seedlings were immersed in distilled water containing $^{51}$Cr(VI) for 48 h at 4 °C. The seedlings were then rinsed 3 times with distilled water, pressed, and centrifuged as in the in vitro procedure.

Adult alfalfa plant $^{51}$Cr(VI) incorporation was as described by Blincoe (1974). Double labeling of the alfalfa with chromium and carbon was done by soil injection with $^{51}$Cr(VI) as above and liberation of $^{14}CO_2$ into an enclosed atmosphere around the alfalfa plant. The extracts were prepared and isolated as above.

**Column Chromatography.** The previously labeled samples were chromatographed on a variety of size exclusion gels including Sephadex G-25, Sephadex G-50, Bio-Gel P-4, and Bio-Gel P-6 (Bio-Rad Laboratories). Fractions were eluted with 0.01 M phosphate buffer, pH 7.00. Columns were calibrated with blue dextran, oxytocin, insulin B chain, streptomycin sulfate, and NADH. Affinity gels used included Con A–Sepharose (Pharmacia Fine Chemicals) and the dye Matrex screening kit (Amicon Corp.). These were eluted by using a 25 mM Tris–1.0 M KCl buffer, pH 7.00. Ion-exchange media included Dowex 50 X 8 ($H^+$), DEAE-cellulose, and DEAE-Sephacel. Elution gradients ranging from 0.01 to 1.0 M phosphate, pH 7.00, were used. Rechromatography of the peak fractions duplicated the original method. Columns were stored with buffer containing 0.02% sodium azide to prevent bacterial growth. Chromium-51 was monitored as previously described by Blincoe (1974), and carbon-14 was measured by liquid scintillation counting.

**Dialysis.** Alfalfa extracts were dialyzed at 4 °C in Spectrapor dialysis tubing (Spectrum Medical Industries) against selected buffers. This tubing reportedly has specific molecular weight cutoffs of 1000, 2000, and 3600.

**Competition Studies.** The point at which a competing ion was added depended on the process under study. When complex formation was under study, the competing ion in the noted concentration was added with the $^{51}$Cr(VI). If the stability of the complex was being studied, the competing ion was added to the pre-formed chromium complex. Analysis of the fractions was as noted above.

**Atomic Absorbance Analysis.** Chromium was quantitated in separated fractions with a flameless atomic absorption spectrophotometer. The manufacturer's recommended conditions for water and wastewater analysis were used (Emmel et al., 1976). Standards and unknowns containing 0.05% $HNO_3$ were run in 10-$\mu$L sample aliquots up to 10 ng/mL.

**Composition Analysis Methods.** Protein detection methods were those of Lowry (Chaykin, 1966), Coromassie blue (Bradford, 1976), fluorescamine (Undenfriend et al., 1972), and biuret (Chaykin, 1966). The iodine starch test was done according to Horowitz (1970). Anthrone analysis was done according to Roe (1955). Dithiothreitol (DTT) and mercaptoethanol buffer concentrations were 5 mM in 0.01 M phosphate buffer, pH 7.00. Buffers were added both before and after in vitro chromate-51 incorporation into the alfalfa plant extract.

Table I. Apparent Molecular Weight of Chromium Complex from Various Higher Plant Sources

| source | $M_r$[a] |
|---|---|
| alfalfa | |
| $^{51}$Cr detected | 2600 |
| total Cr detected | 1900 |
| $^{51}$Cr(VI) | 2600 |
| $^{51}$Cr(III) | 2600 |
| in vivo | 2900 |
| in vitro | 2600 |
| rechromatography of | |
| in vivo | 3100 |
| in vitro | 2900 |
| 24-h dialysis of | |
| in vivo | 3300 |
| in vitro | 3600 |
| plant sources | |
| alfalfa | 2600 |
| seedlings | 2300 |
| apples | 1700 |
| crested wheatgrass | 3000 |
| hays: alfalfa | 2900 |
| crested wheatgrass | 3000 |

[a] Standard error of the estimate is approximately ±800.

Table II. Molecular Weight Estimation by Gel Filtration

| exclusion gel | $M_r$ |
|---|---|
| Sephadex G-25 | 2600 ± 500[a] ($n$ = 12)[b] |
| Sephadex G-50 | 2600 ± 900 ($n$ = 12) |
| Bio-Gel P-6 | 1000 ± 400 ($n$ = 6) |
| | 600 ± 400 ($n$ = 6)[c] |

[a] Standard error of the mean. [b] Number of samples. [c] Requires fresh alfalfa to form.

Fractions containing peak radioactivity were dried at 90 °C and rehydrated in either distilled water or methanol for spectral analysis. These fractions were scanned in both the ultraviolet and visible ranges on a dual-beam spectrophotometer against a reference cuvette containing chromate in the same concentration as the chromium in the unknown, producing a difference (differential) spectra (Rao, 1975). This should emphasize those portions of the spectra due to the organic portion of the complex.

Acrylamide gel electrophoresis was done according to Weber and Osborn (1969) on a 5% gel. A 50-$\mu$L sample of labeled alfalfa extract was used. The gel was scanned spectrophotometrically after electrophoresis.

## RESULTS

**Identification of the Chromium Complex from Several Sources.** The approximate molecular weight of the chromium complex under study as determined by Sephadex G-25 is detailed in Tables I and II. The total stable chromium peak molecular weight coincides with the in vitro and in vivo radioactive peak molecular weight. This chromium complex yields a similar molecular weight in vitro whether the VI or III valence state of chromium is in the incubation media. The complex migrates similarly whether the $^{51}$Cr(VI) is incorporated in vitro or in vivo. These estimates are supported upon rechromatography. Other similarities between in vivo and in vitro complexes noted include removal in the same fraction from DEAE-cellulose (Blincoe, 1974) and DEAE-Sephacel with 1.0 N phosphate, pH 7.00, buffer. They both are intact after 24 h of dialysis against 0.01 N phosphate buffer (Table III). The chromium complex appears to have similar apparent molecular weight in all those higher plants examined. Chromatography on DEAE-cellulose was identical for the complex formed in vivo in alfalfa and in wheatgrass. Migration on Sephadex G-25 and DEAE-cellulose was

Table III. Stability of Alfalfa Chromium Complex

| |
|---|
| perturbation after which the $M_r$ 3000 Cr complex is isolated by gel chromatography |
| dialysis (24 h) |
| DTT and mercaptoethanol during in vitro formation (5 mM) |
| α-amylase (8 μg/mL), trypsin, and pepsin (10 μg/mL) digestion |
| centrifugation at 10000g (2 h) or 0.2-μm filter |
| drying (90 °C) |
| storage at 4 °C for 30 days |
| boiling (30 min) |
| pH shifts from 1.00 to 11.00 |
| ionic strength shifts from 0.01 to 1.0 M |
| perturbation after which inorganic chromium is isolated by gel chromatography |
| HCl digestion |
| high pH (2 h) |
| dialysis (48 h) |
| boiling (24 h) |

Table IV. Molecular Weight Dialysis[a]

| $M_r$ cutoff | % of chromium retained |
|---|---|
| 1000 | 4.4 ± 0.7[b] |
| 2000 | 3.0 ± 1.0 |
| 3600 | 0.4 |

[a] Dialyzed 24 h against 0.01 N phosphate buffer, pH 7.00. [b] Standard error of the mean.

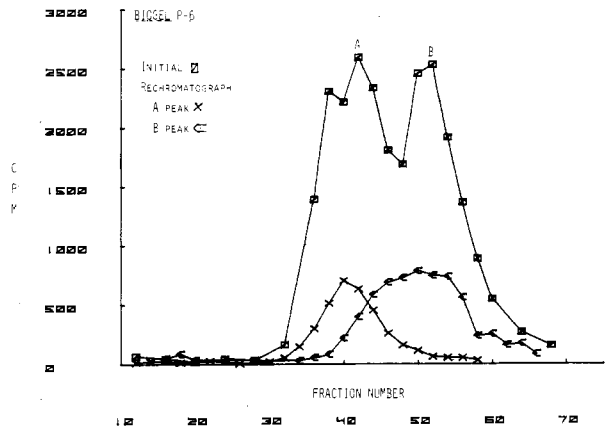


**Figure 1.** Bio-Gel P-6 chromatography of in vitro labeled $^{51}Cr(VI)$ alfalfa complex.

unaffected by drying the plant material as in hay making.

**Molecular Weight Estimates.** The molecular weight of the in vitro complex was estimated on Sephadex G-25 and Sephadex G-50 and Bio-Gel P-6. Although these columns were standardized with the same compounds, a substantial difference between the two gel types was found (Table II). In further studies, the two gel types were saturated with Cr(VI) to eliminate any transition metal affinities (Evans et al., 1979). This did not affect the molecular weight estimation for either gel. For examination of the disparate molecular weights, dialysis tubing with pore sizes at specific molecular weight cutoffs was used to dialyze in vitro labeled alfalfa extracts. As can be seen in Table IV, the complex remained only in less than 2000 molecular weight pore size tubing.

Further examination of the Bio-Gel P-6 double peak showed the rechromatography of the individual peaks supported their original molecular weight estimate (Figure 1). Further, when the fractions containing each of the individual peaks were rechromatographed on Sephadex G-50 or G-25, both yielded a molecular weight of 3000 (Figure 2).

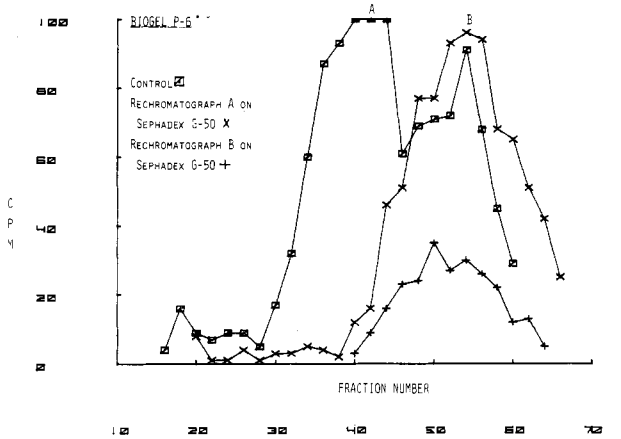


**Figure 2.** Rechromatography of Bio-Gel P-6 peaks isolated from in vitro $^{51}Cr(VI)$ labeled complex on Sephadex G-50.

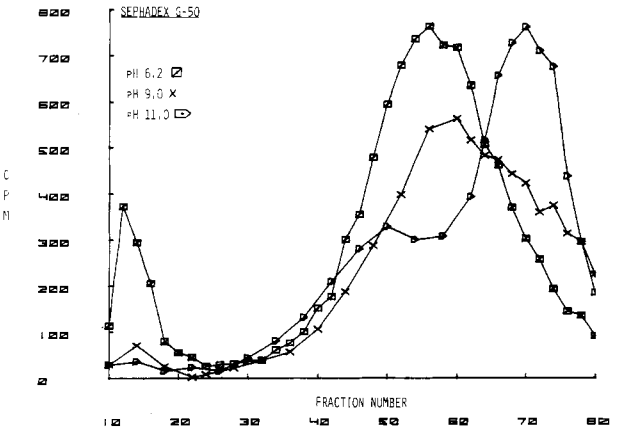


**Figure 3.** Chromatography on Sephadex G-25 of in vitro labeled $^{51}Cr(VI)$ alfalfa complexes formed at different pHs.

**Stability of Chromium Complex.** Table III contains a variety of physical parameters tested to perturb the chromium complex structure. The complex appears resistant to ionic strength and pH shifts. It is also very heat stable once formed. The complex before and after heating migrates identically on Dowex 50 X 8, Dowex 1 X 8, DEAE-cellulose, and TEAE-cellulose as well as Sephadex G-25. The complex disintegrates when heated to 90 °C for 24 h or dialyzed longer than 48 h. Only inorganic chromium was isolated after hydrochloric acid digestion.

Chromium could not be removed from the alfalfa or wheatgrass complex by either petroleum ether or by methyl isobutyl ketone at pH 2.7, 7.0, or 13. This was true both for the in vivo and in vitro labeled extracts and for the peak fractions as separated on Sephadex G-25. The chromium could not be precipitated with barium ion except on prolonged treatment at pH 13. If the radioactive complex is allowed to stand with chromic or chromate ion at 1000 times the physiological concentrations prior to Sephadex G-25 separation, no radioactivity is found to migrate with the inorganic chromium. The chromium in the complex is thus not exchangeable with either chromic or chromate ion.

The in vitro chromium insertion into the complex requires an acidic pH and is inhibited by the presence of other transition metals. As can be seen in Figure 3, alkaline pHs increased the amount of inorganic chromium isolated while decreasing the concentration of the $M_r$ 3000 chromium complex. Acidic pHs between 3.00 and 6.20 during complex formation and chromatography produced the normal elution pattern of the $M_r$ 3000 D chromium complex on Sephadex G-25. Several transition metals were

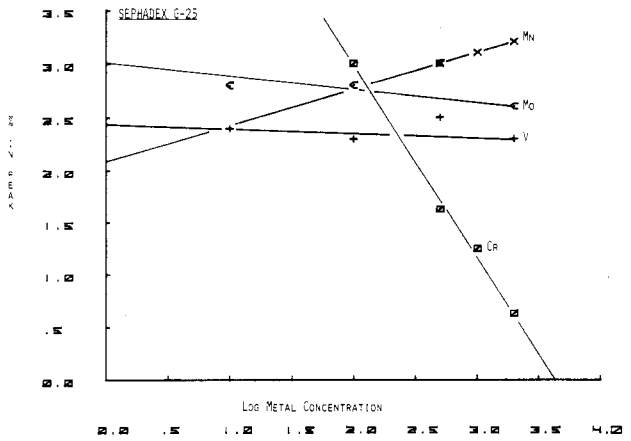


**Figure 4.** Amount of $^{51}Cr(VI)$ complex isolated after in vitro formation with various metals.

Table V. Spectral Data. Difference Spectra against $K_2CrO_4$

| solvent system | | wavelength, nm | | | |
|---|---|---|---|---|---|
| $H_2O$ | mean: | 222.6 | | 310.9 | |
| | SD: | 1.3 | | 4.4 | |
| methanol | mean: | 228.9 | 253.3[a] | 312.1 | 369.2[a] |
| | SD: | 0.8 | 8.5 | 4.8 | 1.5 |

[a] Shoulder.

examined for their ability to interfere with the chromium complex formation. Vanadium, manganese, and magnesium do not compete with the chromium in the complex formation. However, as can be seen in Figure 4, unlabeled chromium will compete with chromate-51 during in vitro formation of the complex, linearlly reducing the amount of radioactivity labeled complex detectable. DTT and mercaptoethanol were placd in the phosphate buffer to quantitatively reduce disulfide bonds. Exposure of the alfalfa extract to these compounds before and after chromium complex formation did not alter the apparent molecular weight of 3000 (Table III).

**Identification of the Functional Groups.** For examination of components of the chromium complex, a double label ($^{51}Cr$ and $^{14}C$) was supplied to the alfalfa plant soil and the atmosphere surrounding the plant, respectively. Both nuclides were incorporated into the chromium complex and were both present after rechromatography of the $^{51}Cr$ peak fractions on Sephadex G-25 and Bio-Gel P-6. This indicates that the chromium complex probably contains carbon. [$^{14}C$]Acetate in vivo and [$^{14}C$]glucose in vitro did not label the complex. In plant material doubly labeled with phosphate-32, zinc-65, or selenium-75 and chromium-51, the chromium label was easily separated from the other trace-element label by ion exchange and/or gel filtration.

Additional functional group examination by ultraviolet–visible spectral analysis yields no visible peaks. As noted in Table V, the difference spectra in a water solvent system yields maxima at 222.6 ± 1.3 and 310.9 ± 4.4 nm. The methanol solvent system produces more shoulder phenomena but still shows the major peaks of 229.8 ± 0.8 and 369.2 ± 1.5 nm. Lowry, Coomassie blue, biuret, and 280-nm absorption protein analysis and iodine starch detection have shown that none of these reactive groups exist in the complex in detectable concentrations. The chromium complex chromatographs with primary amines, as detected by fluorescamine. This activity can be removed with Dowex 50 X 8 ($NH_4^+$) treatment without apparent disturbance to the chromium complex structure. The anthrone reaction indicates that sugars are present in the complex, while α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically relayed residues by Con A–Sepharose proved negative. Further, α-amylase, trypsin, and pepsin did not alter the apparent molecular weight of the complex. The chromium complex had no affinity for the Amicon dye ligand series. The chromium complex discussed here migrates ahead of the marker bromophenol blue on Na-$DodSO_4$ gel electrophoresis. This substantiates the low molecular weight estimate of the chromium complex and that it is not associated with proteins.

## DISCUSSION

The polyfunctional, anionic complex formed in alfalfa in vivo (Blincoe, 1974) appears identical with the in vitro complex. The identification of chromium complexes from a variety of plant sources has yielded a consistent picture. The complex appears similar regardless of initial chromium valence, higher plant source, or mode of incorporation of radioactive tracer.

The double-label experiments have shown that $^{14}CO_2$ from photosynthesis processes appears in the chromium complex. The data involving Zn, Se, and P coupled with the competition data of V, Mo, and Mn detail a process of chromium insertion that is very specific for chromium alone.

The elution pattern of the chromium complex is broad from Sephadex G-25, but DEAE-cellulose and DEAE-Sephacel ion-exchange chromatography yields sharp peaks, indicative of a like-charge density on the complex. The highly charged nature of Sephadex gels probably accounts for some retardation of this chromium complex on the columns. This retardation of the complex introduces a large discrepancy into the molecular weight estimates from different gels. The Sephadex gels, either G-50 or G-25, consistently yield an average of 3000 as the molecular weight. The Bio-Gel P-6 gel yields two peaks, 1000 and 500. Neither gel's molecular weight estimate changes in the presence of saturating concentrations of chromium. Whatever affinity either of these gels has for the chromium complex is not through the transition metal itself. This is consistent with most known transition metal compounds where the metal is centrally located in the molecule. It had been previously observed that the chromium-containing complex was polydispersed with respect to molecular weight (Blincoe, 1974). This would account for the broad peaks observed in size-exclusion gel chromatography. We are dealing with a family of complexes somewhat polydispersed with respect to size but exhibiting the same charge.

An independent method of molecular weight estimation used was specific molecular weight cutoff pore size dialysis tubing. Although the pore sizes are an estimate and conformational changes in molecules can allow large molecules to dialyze out, the chromium complex was retained in the $M_r$ 2000 pore size and not in the $M_r$ 3600 tubing. This would seem to support the Sephadex molecular weight estimate of about 3000 rather than the lower molecular weight estimate from Bio-Gel P-6.

The stability of the chromium plant complex is indicative of transition metal binding in biological materials. Once formed, perterbation by heat, pH, ionic strength, and limited dialysis does not alter its molecular weight. It is not exchangeable with either chromic or chromate ions present in great excess. Extraction with methyl isobutyl ketone and barium precipitation (Blincoe, 1974) indicate that the complex is not lipid soluble over a wide pH range and that the chromium is not removable as either the chromate or the dichromate except on prolonged treatment

with strong base. Extreme methods such as hydrochloric acid digestion, prolonged treatment with strong base, extended dialysis, and long-term boiling can remove the chromium from the complex.

The insertion of the chromium into the plant complex is extremely specific for chromium. Other transition metals with like outer shell configurations do not compete with the chromium complex synthesis. The pH optimum is acidic, with increasing pH above 7.00 yielding increasing amounts of inorganic chromium. This is in direct contrast to chromium incorporation below pH 7.00 where little free chromium is detectable. Apparently, disulfide bonds are not integral to complex formation since dithiothreitol and mercaptoethanol buffers do not affect the apparent molecular weight of the complex.

Functional group analysis has yielded clues as to some of the components of the alfalfa chromium complex. After purification with two molecular sieves, two ion-exchange resins, and dialysis, the ultraviolet difference spectra is reminiscent of a furfural sugar. The difference spectra technique does, however, mask any absorbance around 269 nM. The anthrone reaction supports the existance of a sugar moiety, while the lack of affinity to Con A–Sepharose probably rules out an accessible α-D-mannopyranose or α-D-glucopyranose as the sugar. There is an amine present with the chromium complex before chromatography with Dowex 50 X 8 ($H^+$). The removal of this activity does not alter the apparent molecular weight of the alfalfa chromium complex. All other methods indicative of peptides or proteins have proved negative.

The characteristics of the alfalfa chromium complex herein described are in marked contrast to the GTF isolated by Schwarz and Mertz (1959). This chromium complex has been isolated from a variety of higher plants, including alfalfa, crested wheatgrass, and their hays (Blincoe, 1974), beans, and wheat (Huffman and Allaway, 1973). All of these plant isolates yield an anionic, polymorphic complex of approximately $M_r$ 3000. This is consistent whether an in vivo or in vitro $^{51}Cr$ incorporation is used. The best source of GTF is brewer's yeast (*Saccromyces carlesbergensis*), which contains a cationic, $M_r$ 400 D chromium complex. While either Cr(III) or Cr(VI) valence states are complexed in alfalfa, only Cr(III) has been reported to be incorporated in vivo into GTF (Kumpulainen, 1977).

The comparison of the spectral data from various chromium complexes accentuates the differences in these chromium compounds. Alanine–chromium complexes yield two peaks at 385 and 515 nm (Oki, 1977). The GTF, as reported by Toepfer et al. (1977), contains a single 262-nm absorbance. The chromium complex described here produces three major ultraviolet peaks at 222, 269, and 310 nm. Even the difference in composition is marked between the GTF and the alfalfa complexes. Toepfer et al. (1977) has reported that the GTF contains nicotinic acid, glycine, glutamic acid, and cysteine. The alfalfa chromium complex described here contains a chromium, a carbon, and possibly an amine loosely associated with it.

In conclusion, this work has described a plant chromium complex remarkably distinct from the brewer's yeast GTF. Further elucidation of the alfalfa chromium complex structure and stability will help define the parameters of chromium bioavailability in mammals.

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