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The Essential Role of Cobalt in the Inhibition of the Cytosolic Isozyme of 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate Synthase from *Nicotiana silvestris* by Glyphosate[1]

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The prime molecular target of glyphosate (*N*-[phosphonomethyl]glycine), a potent herbicidal and antimicrobial agent, is known to be the shikimate-pathway enzyme, 5-*enol*-pyruvylshikimate-3-phosphate synthase. Inhibition by glyphosate of an earlier pathway enzyme that is located in the cytosol of higher plants, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DS-Co), has raised the possibility of dual enzyme targets *in vivo*. With the recent appreciation that magnesium (and manganese) can replace cobalt as the divalent-metal activator of DS-Co, it has now been possible to show that sensitivity of DS-Co to inhibition by glyphosate is obligately dependent upon the presence of cobalt. Evidence for a cobalt(II):glyphosate complex with octahedral coordination was obtained through examination of the effect of glyphosate upon the visible electronic spectrum of aqueous solutions of cobalt(II) chloride. The presence of glyphosate increased the concentration of cobalt(II) chloride required for enzyme activity, and the concentration of cobalt(II) chloride markedly affected the concentration of glyphosate required for inhibition of DS-Co activity. The extent to which DS-Co is vulnerable to inhibition by glyphosate *in vivo* depends, therefore, upon the unknown extent to which DS-Co molecules in the cytosol might be associated with cobalt.

N-[Phosphonomethyl]glycine (glyphosate)[4] is an antimicrobial agent and herbicide that impairs the biosynthesis of aromatic amino acids. The common-pathway enzyme, 5-*enol*-pyruvylshikimate-3-phosphate (EPSP) synthase, is quite sensitive to inhibition by glyphosate (1) and is generally accepted as a primary target of antimetabolite action. Consistent with these findings are the observations that plants (2) and microorganisms (3) accumulate large amounts of shikimate and/or shikimate 3-phosphate in response to glyphosate treatment.

The potent inhibition of EPSP synthase has been attributed to the effectiveness of glyphosate as a transition-state analog of phospho*enol*pyruvate (PEP) (4). That this transition state may be unique to EPSP synthase was suggested by the finding that other PEP-utilizing enzymes were not inhibited by glyphosate (2). However, several reports of inhibition of 3-deoxy-

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[4] Abbreviations used: glyphosate, *N*-[phosphonomethyl]glycine; EPSP, 5-*enol*-pyruvylshikimate 3-phosphate; PEP, phospho*enol*pyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; Epps, 4-[2-hydroxyethyl]-1-piperazinepropanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

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D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase by glyphosate have appeared (5-7), raising the possibility that two enzyme targets in series may exist in some organisms. A property common to DAHP synthase in all three systems where glyphosate sensitivity has been reported is activation of enzyme activity by cobalt.

Higher plants contain both plastid and cytosolic isozymes of DAHP synthase (8) which differ in a number of characteristics. The cytosolic isozyme requires either magnesium, cobalt, or manganese for activity. It was designated DS-Co since only cobalt was initially recognized as an activator (6). In contrast to this glyphosate-sensitive DS-Co, the plastidial isozyme (DS-Mn) was resistant to glyphosate (6). Although DS-Co in the cytosol is several orders of magnitude less sensitive to glyphosate than EPSP synthase is in the plastid compartment, the concentration of glyphosate in the cytosol is several orders of magnitude higher than in the chloroplast (2). The substantial amounts of shikimate 3-phosphate and/or shikimate that accumulate (primarily in the vacuole) seemingly would not be generated if DS-Co is inhibited by glyphosate *in vivo*, but these accumulated molecules could originate from the plastidial compartment where DS-Mn is insensitive to glyphosate.

With the recognition that other divalent cations can substitute for cobalt as activators, it became possible to test the question of whether DS-Co is intrinsically sensitive to glyphosate or whether inhibition is obligately linked to the presence of cobalt cation as an activating moiety.

MATERIALS AND METHODS

Materials. Biochemicals, enzyme-grade ammonium sulfate, phenyl-Sepharose, Sephacryl S-200, Epps (4-[2-hydroxyethyl]-1-piperazinepropanesulfonic acid), Pipes (1,4-piperazinediethanesulfonic acid), and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. Protein assay reagent was obtained from Bio-Rad. Ultrafiltration cells and membranes were from Amicon. PD-10 (Sephadex G-25) columns were obtained from Pharmacia. Analytical-grade glyphosate (free acid), a gift of Monsanto Chemical Co., was dissolved in distilled water and adjusted to the desired pH with KOH. Growth of the suspension-cultured cell line ANS-1 from *Nicotiana silvestris* was as described by Gaines *et al.* (9).

Enzyme preparation. All procedures were carried out at 0-4°C. A crude enzyme extract was prepared by stirring 163 g of liquid nitrogen powdered cells with 163 ml of buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, and 0.1% 2-mercaptoethanol, pH 7.2). The resulting extract was clarified by centrifugation at 38,000*g* for 15 min at 4°C. Ammonium sulfate (58.5 g) was added to 280 ml of this extract to yield 35% of saturation. After stirring on ice for 30 min, the suspension was centrifuged at 38,000*g* for 15 min at 4°C. The supernate (300 ml) was brought to 55% of saturation with ammonium sulfate by the addition of a further 38.7 g. After storage on ice overnight, the suspension was centrifuged at 38,000*g* for 15 min at 4°C. The supernate was discarded and the pellets were resuspended in 10 mM K-Pipes buffer, 20% saturated with ammonium sulfate (pH 7.2), adjusted to 20 ml, and applied to a column (3 × 7 cm) of phenyl-Sepharose equilibrated in the same buffer. Nonadsorbed protein was washed through the column with 60 ml of buffer. An additional large amount of contaminating protein was removed by washing the column with 300 ml of 10 mM K-Pipes buffer, containing ammonium sulfate (pH 7.2) at 10% of saturation. DS-Co was eluted from the column with 10 mM K-Pipes buffer (pH 7.2), and four 10-ml fractions were collected. The leading fraction was discarded. The pooled enzyme-containing fractions (30 ml) were concentrated to 4.7 ml by ultrafiltration through a PM-10 membrane at 15 psi and stored at −20°C. A portion (2.0 ml) of this material was chromatographed on a Sephacryl S-200 column (1.5 × 53.5 cm) equilibrated with 50 mM K-phosphate buffer (pH 7.2) at a flow rate of 10.1 cm/h. Fractions of 1.0 ml were collected and assayed for DS-Co and absorbance at 280 nm. Fractions 34-43 were pooled (see Fig. 1), divided into aliquots, and stored at −20°C. Prior to assay, samples were desalted into 10 mM K-Pipes buffer (pH 7.2) by gel filtration on a PD-10 column.

Enzyme assay. Reaction mixtures contained 50 mM K-Epps buffer (pH 8.6), 1 mM PEP, 6 mM erythrose-4-P, divalent cation (as indicated), and enzyme in a final volume of 200 μl. Reactions were initiated by the addition of enzyme and allowed to proceed for 20 min at 37°C. For the determination of specific activity, 10 mM $MgCl_2$ was used (8). Reactions were terminated by the addition of 50 μl of 20% (w/v) ice-cold trichloroacetic acid; following centrifugation, DAHP in the supernate was determined as previously described (8).

Determination of protein concentration. Protein concentrations were determined by the method of Bradford (10), using the Bio-Rad assay reagent. Bo-

vine serum albumin (fraction V) was used as a standard.

Visible electronic spectra. Visible electronic spectra were recorded at room temperature (25°C) with a Beckman Model 25 dual-beam spectrophotometer and recorder set at 0.25 absorbance span with scanning at 50 nm/min and a chart speed of 1 in./min.

RESULTS

Preparation of the DS-Co isozyme. Purification of DS-Co by the scheme outlined under Materials and Methods led to a 37-fold purification and a specific activity of 1.12 μmol/min/mg protein with a 12% yield. The elution profile from Sephacryl S-200 of DS-Co and the position of standard proteins are indicated in Fig. 1. Calibration plots for molecular weight and Stokes radius yielded estimates of 440,000 and 65 Å, respectively, for the native DS-Co protein. Identical values have been obtained in other experiments with both crude and partially purified preparations.

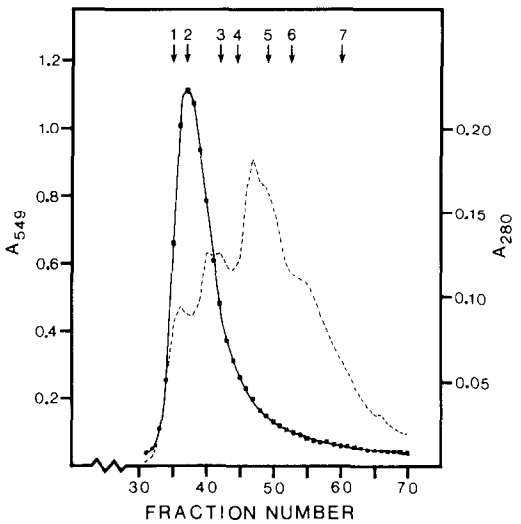

FIG. 1. Sephacryl S-200 chromatography as described under Materials and Methods. Solid boxes show DAHP synthase data points (left ordinate). Distribution of eluate protein (absorbance at 280 nm) is indicated by the broken line. Arrows mark the peak position of the standard proteins: 1, thyroglobulin (void volume); 2, apoferritin (M_r 443,000); 3, γ-globulin (M_r 205,000); 4, aldolase (M_r 158,000); 5, bovine serum albumin (M_r 66,000); 6, ovalbumin (M_r 45,000); 7, chymotrypsinogen-a (M_r 25,000).

TABLE I

GLYPHOSATE INHIBITION OF DS-Co: CATION SPECIFICITY[a]

Cation	Activity (nmol/min) −Glyphosate	Activity (nmol/min) +Glyphosate	Inhibition (%)
Cobalt	1.05	0.14	87
Manganese	0.24	0.23	4
Magnesium	0.13	0.13	0

[a] Reaction conditions were 50 mM K-Epps buffer (pH 8.0), 2 mM PEP, 2 mM erythrose-4-P, enzyme, and divalent chloride salts at 0.5 mM. Glyphosate was absent or present at a concentration of 1 mM. Values given are means of duplicate assays.

Cation specificity of glyphosate inhibition. Since the cytosolic isozyme of DAHP synthase from *N. silvestris* required a divalent-cation activator, the three effective cations (magnesium, cobalt, and manganese) were tested in combination with glyphosate. Each cation has a specific saturation profile and maximum activation ability (8), and at optimal concentrations of approximately 10 mM magnesium, 0.5 mM cobalt, and 0.5 mM manganese, relative activities were 100, 85, and 20, respectively. The K_m values of DS-Co for its substrates (erythrose-4-P and PEP) are not significantly influenced by the divalent cation used as activator. Table I shows the effect of 1.0 mM glyphosate on enzyme activity when the concentration of the divalent cation was fixed at 0.5 mM, a concentration at which maximum activity was obtained with cobalt. In controls run in the absence of glyphosate, activities with manganese and magnesium were 23 and 12% of that obtained with cobalt, respectively. Significant inhibition by glyphosate was only observed when cobalt was used as an activator.

We considered the possibility that inhibition might depend on the degree of saturation by the activator. However, it was found in a separate experiment that 0.75 mM glyphosate, while inhibiting 70% in the presence of 0.5 mM cobalt, had no effect on the enzyme assayed with 10 mM (saturating) magnesium.

Effects of cobalt and glyphosate on enzyme activity. The activity of DS-Co was assayed as a function of cobalt concentration in the presence and absence of glyphosate. Figure 2 shows the effect of glyphosate on the saturation curves obtained with respect to cobalt. Enzyme activity in the absence of glyphosate was strictly dependent on cobalt concentration, and a hyperbolic response to increasing cobalt concentration up to a maximum at 0.6 mM was observed; higher cobalt concentrations were inhibitory. When 0.5 mM glyphosate was present, enzyme activity showed a sigmoidal response to increasing cobalt up to an activity maximum at 0.8-0.9 mM cobalt. The latter activity maximum was, however, only 69% of the maximum obtained in the absence of glyphosate.

Dependence of glyphosate inhibition on the concentration of cobalt. Replotting the data in Fig. 2 as percentage inhibition obtained at a given cobalt concentration versus the concentration of cobalt in the assay shows that inhibition by glyphosate can be partially antagonized by cobalt (Fig. 3).

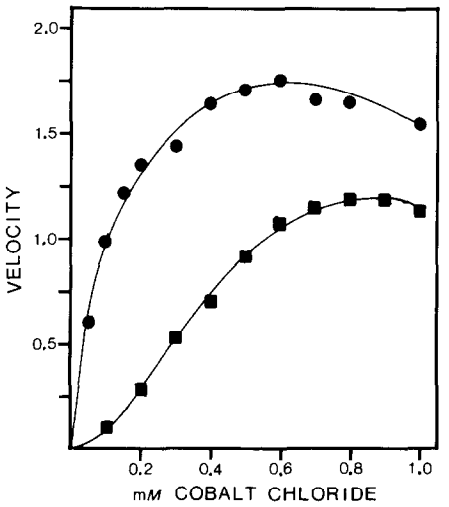

FIG. 2. Kinetic evidence for a cobalt(II):glyphosate complex: effect of 0.5 mM glyphosate on the activity of DS-Co as a function of cobalt(II) chloride concentration. Solid circles and boxes indicate activity in the absence and presence of 0.5 mM glyphosate, respectively.

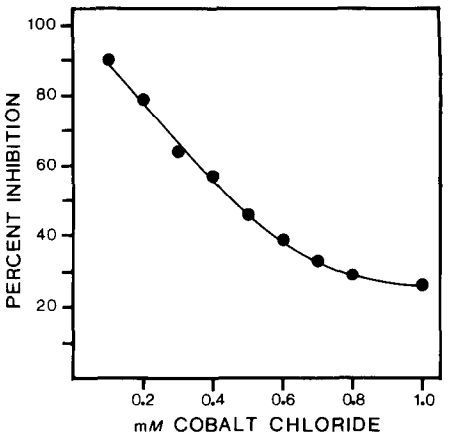

FIG. 3. Effect of cobalt concentration upon sensitivity to inhibition by 0.5 mM glyphosate. The data were calculated from those presented in Fig. 2.

Enzyme activity was also followed as a function of glyphosate concentration at two fixed concentrations of cobalt. One concentration (0.6 mM) was chosen to give optimal activity in the absence of glyphosate; the second concentration (0.1 mM) was chosen because it gave approximately half-maximal activity in the absence of glyphosate (see Fig. 2). The data were plotted as percentage inhibition (relative to uninhibited controls) versus glyphosate concentration (Fig. 4). At the optimal cobalt concentration (0.6 mM), glyphosate gave a sigmoidal inhibition curve, approaching maximal inhibition at concentrations in excess of 1.0 mM. At 0.6 mM cobalt, the concentration of glyphosate required to produce 50% inhibition was estimated to be 0.62 mM. In contrast, when DS-Co was assayed with 0.1 mM cobalt, a hyperbolic inhibition curve was obtained, with 50% inhibition occurring at 0.1 mM glyphosate.

Visible electronic spectra. Aqueous solutions of cobalt(II) chloride were prepared from a 0.1 mM solution in distilled water. Such unbuffered solutions are pink and composed primarily of octahedrally coordinated hexaquocobalt (11). At greater-than-neutral pH, the formation of hydroxides with tetrahedral coordination begins to occur, giving the solution a bluish color (11). The effect of glyphosate

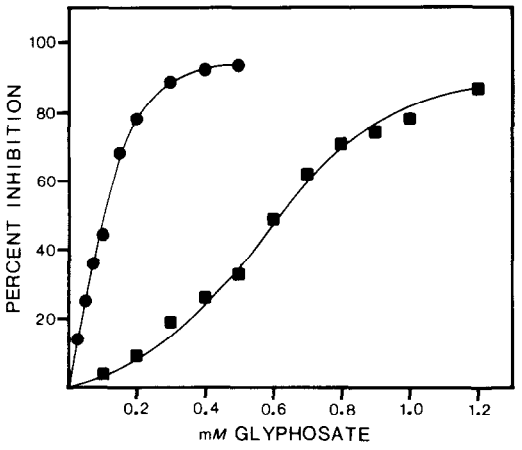

FIG. 4. Effect of different fixed cobalt(II) chloride concentrations on the glyphosate inhibition curve. Percentage inhibition is plotted as a function of glyphosate concentration. Solid circles and boxes represent data points obtained at 0.1 and 0.6 mM cobalt(II) chloride, respectively. Control activities at 0.1 mM cobalt = 1.1 mU and at 0.6 mM cobalt = 1.9 mU.

on the visible electronic spectrum of aqueous solutions of cobalt(II) chloride buffered at pH 8.6 (as in the DS-Co enzyme assay) is shown in Fig. 5. The spectrum in the absence of glyphosate was characterized by absorption maxima at 665-670, 625-640, 510, and 460-475 nm, in addition to strong absorbance at lower wavelengths. Addition of glyphosate at one-half the molar concentration of cobalt decreased the absorption of the two long-wavelength (tetrahedral coordination) peaks and perturbed the spectrum below 590 nm. Glyphosate at concentrations equimolar-or-greater with respect to cobalt(II) chloride had a marked effect on the spectrum; absorbance above 590 nm and at low wavelengths was suppressed. Peak absorption in the presence of glyphosate was at 520-525 nm with a shoulder at 460-475 nm. Solutions of cobalt(II) chloride and equimolar-or-greater concentrations of glyphosate buffered at pH 8.6 were pink in color, similar in appearance to hexaquocobalt solutions.

The complexity of spectra obtained for alkaline solutions precluded the determination of stoichiometry by spectrophotometric titration of cobalt with glyphosate at a single wavelength. However, the simpler spectra obtained at less-than-neutral pH allowed this to be accom-

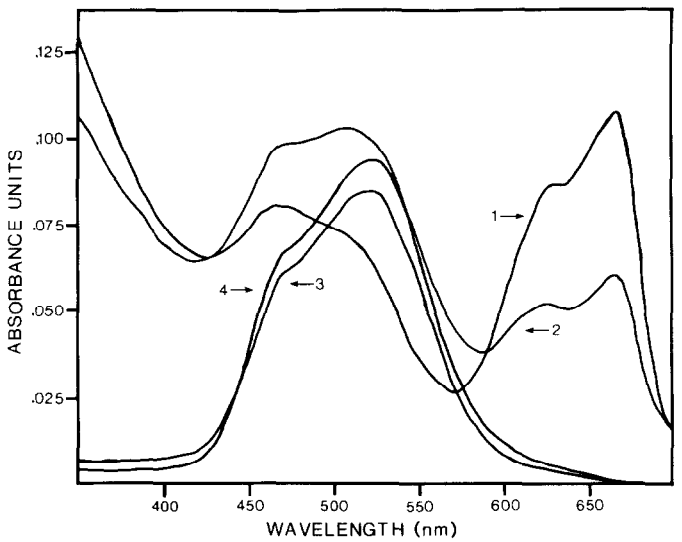

FIG. 5. Glyphosate-mediated perturbation of the visible electronic spectrum of aqueous solutions of cobalt(II) chloride buffered at pH 8.6. Solutions were prepared by dilution of 0.1 M Co(II) chloride (unbuffered, pH 6) and 0.1 M glyphosate (pH 8.6) into a final concentration of 50 mM K-Epps buffer (pH 8.6). The spectrum for 10 mM Co(II) chloride in the absence (trace 1) or presence of glyphosate at 5 mM (trace 2), 10 mM (trace 3), and 30 mM (trace 4) is shown.

plished. Figure 6 (top) shows the visible electronic spectrum of a solution of cobalt(II) chloride buffered at pH 6.8 and the enhanced absorption in the presence of glyphosate. A slight bathychromic shift induced by glyphosate is evident in the difference spectrum shown in Fig. 6 (bottom). Peak absorption of these solutions was in the range 510–520 nm with a shoulder at 460–475 nm. The above results suggested that a cobalt(II):glyphosate complex was formed at this pH also. By following the increase in absorption at 515 nm of a solution of cobalt(II) chloride at pH 6.8 as a function of glyphosate concentration, it was found that both 1:1 and 1:2 cobalt(II):glyphosate complexes could be formed (data not shown). At equimolar and higher ratios of cobalt to glyphosate, the 1:1 complex predominated. However, with a 2.2- to 2.4-fold excess of glyphosate, complexes of cobalt with two glyphosate ligands were formed.

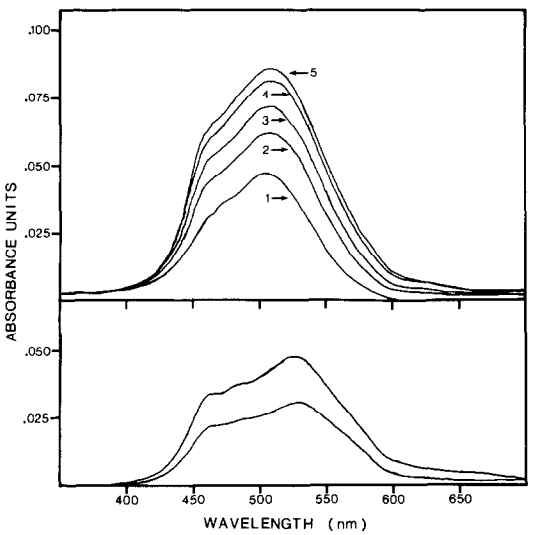

FIG. 6. Overall similarity of visible electronic spectrum found for hexaquocobalt and the cobalt(II):glyphosate complex. Solutions were prepared by dilution of 0.1 M Co(II) chloride into 50 mM K-Pipes buffer (pH 6.8). (Top) Spectrum for 10 mM Co(II) chloride in the absence (trace 1) and presence of glyphosate at 5 mM (trace 2), 10 mM (trace 3), 20 mM (trace 4), and 30 mM (trace 5). (Bottom) Difference spectrum showing slight bathychromic shift accompanying complex formation. Lower trace, 3 vs 1; upper trace, 5 vs 1.

Calculation of a dissociation constant for the cobalt(II):glyphosate complex under conditions of enzyme activity. The activity of DS-Co exhibits strict dependence upon the concentration of cobalt, and activity is influenced by the interactions between cobalt and glyphosate. These features were exploited to estimate the concentrations of free and complexed species necessary for calculation of a dissociation constant. For the equilibrium process,

$$M + PMG \rightleftharpoons MPMG,$$

where M is the cobalt cation, PMG is the ligand glyphosate, and MPMG is the cobalt(II):glyphosate complex, the following conservation equations apply:

$$M_t = M_0 + MPMG$$

$$PMG_t = PMG_0 + MPMG.$$

The total concentration of cobalt is designated M_t; the free concentration of cobalt is designated M_0; the total concentration of glyphosate is designated PMG_t; the free concentration of glyphosate is designated PMG_0; and the concentration of the cobalt(II):glyphosate complex is designated MPMG.

For calculation of the dissociation constant from the cobalt saturation data presented in Fig. 2, the following assumptions were made: (i) In the absence of glyphosate, all cobalt is free and capable of binding to DS-Co and activating it. Thus, the concentration of cobalt giving 50% of maximal activity ($A_{0.5}$) is taken as a known quantity $M_t = M_0 = 0.08$ mM. (ii) In the presence of glyphosate (0.5 mM) some cobalt is complexed and $M_t = M_0 + MPMG$. The concentration of cobalt which, in the presence of glyphosate, gave 50% of the maximal activity obtained in the absence of glyphosate ($A'_{0.5}$) is taken as equivalent to $M_t = 0.46$ mM. (iii) The cobalt is complexed primarily to one glyphosate ligand. From Fig. 2

$$M_t = M_0 + MPMG$$

$$0.46 \text{ mM} = 0.08 \text{ mM} + MPMG$$

$$0.38 \text{ mM} = MPMG$$

$$PMG_t = PMG_0 + MPMG$$

$$0.50 \text{ mM} = \text{PMG}_0 + 0.38 \text{ mM}$$

$$0.12 \text{ mM} = \text{PMG}_0.$$

Thus, the dissociation constant (K_d) is represented as

$$K_d = \frac{[\text{M}_0][\text{PMG}_0]}{[\text{MPMG}]}$$

$$K_d = \frac{[0.08 \text{ mM}][0.12 \text{ mM}]}{[0.38 \text{ mM}]}$$

$$K_d = 2.53 \times 10^{-5} \text{ M}.$$

A second, independent way of calculating the dissociation constant was based on the inhibition data presented in Fig. 4. The above assumptions and conservation equations were applied. In the presence of 0.1 mM cobalt, the concentration of glyphosate required for 50% inhibition of DS-Co was 0.1 mM. Thus, the total concentration of cobalt $\text{M}_t = 0.1$ mM, and the total concentration of glyphosate $\text{PMG}_t = 0.1$ mM. Under these conditions the residual activity would reflect the free cobalt concentration. By extrapolation from Fig. 2, the concentration of cobalt giving 50% of the activity obtained with 0.1 mM cobalt was 0.4 mM = M_0. Since

$$\text{M}_t = \text{M}_0 + \text{MPMG}$$

$$0.10 \text{ mM} = 0.04 \text{ mM} + \text{MPMG}$$

$$0.06 \text{ mM} = \text{MPMG}$$

$$\text{PMG}_t = \text{PMG}_0 + \text{MPMG}$$

$$0.10 \text{ mM} = \text{PMG}_0 + 0.06 \text{ mM}$$

$$0.04 \text{ mM} = \text{PMG}_0.$$

Thus, the dissociation constant (K_d) is represented as

$$K_d = \frac{[\text{M}_0][\text{PMG}_0]}{[\text{MPMG}]}$$

$$K_d = \frac{[0.04 \text{ mM}][0.04 \text{ mM}]}{[0.06 \text{ mM}]}$$

$$K_d = 2.66 \times 10^{-5} \text{ M}.$$

DISCUSSION

Dependence of glyphosate inhibition upon cobalt. Enzymes that catalyze reactions in which PEP is a substrate are commonly activated by exogenous divalent cations. Two well-documented examples are PEP carboxylase (12) and enolase (13). O'Leary *et al.* (12) examined the metal-ion activation of PEP carboxylase from *Zea mays* and found that of eight cations tested, manganese, cobalt, and magnesium were the three most effective. A wide range of divalent cations are capable of activating enolase, and again magnesium, cobalt, and manganese were among the best. Thus, the effects of divalent cations on the activity of DS-Co are generally consistent with a role for metal ions in catalysis by PEP-utilizing enzymes.

Only recently has the range of divalent-cation activators of DS-Co been known to include magnesium and manganese (8). Since all reports of glyphosate inhibition of DAHP synthase had been with the cobalt-activated enzyme, we examined the effect of glyphosate on the enzyme in the presence of these alternative activators. In so far as significant inhibition by glyphosate was observed only with the cobalt-stimulated activity, it seemed likely that cobalt and glyphosate interact to form a complex. It was reasoned that if glyphosate formed a complex with cobalt under conditions of enzyme assay, then the presence of glyphosate would decrease the concentration of free cobalt and should increase the total concentration of cobalt required for enzyme activity. Such an effect was apparent from the change in the cobalt saturation curve induced by glyphosate. Increasing the concentration of cobalt in the assay, however, did not completely restore full activity. This could perhaps indicate that the cobalt(II):glyphosate complex can bind tightly to the enzyme to form an inactive species.

The cobalt(II):glyphosate complex. Direct evidence for the formation of a cobalt(II):glyphosate complex was obtained. Changes in the visible electronic spectrum of aqueous solutions of cobalt(II) chloride and glyphosate point to the formation of a cobalt(II):glyphosate complex with electronic properties consistent with octahedral coordination. Cobalt complexes can

have either octahedral or tetrahedral coordination since for a d^7 ion, the ligand field stabilization energies disfavor the tetrahedral configuration to a smaller extent than for any other d^n ($1 \leq n < 9$) ion (11). In neutral and slightly acidic aqueous solution, the cobalt(II) is liganded primarily to six water molecules in an octahedral arrangement (11). Addition of glyphosate increased the absorbance but had no major qualitative effects on the spectrum, indicating a similar coordination geometry for the resulting cobalt(II):glyphosate complex. In alkaline solutions the formation of hydroxide complexes with tetrahedral coordination can occur (14); the ligand glyphosate apparently displaced the four hydroxyl ligands and freed additional coordination sites that presumably were then occupied by water, accounting for the observed shift in the spectrum toward that obtained for hexaquocobalt. Typical spectra for comparison can be seen in Cotton and Wilkinson (11).

The lack of glyphosate inhibition of DS-Co when assayed with magnesium or manganese suggested that complexes with these two divalent cations did not occur to any great extent. Explanations for these data may be found in the unique chemical properties of the three cations (11). Although manganese(II) can form many complexes, the dissociation constants are not as low as those for the succeeding main-group transition elements because manganese(II) is the largest ion of the series and has no ligand field stabilization energy in its hexaquo ion or other high-spin complexes. Of the group-II elements, only magnesium and calcium show any tendency to form complexes in aqueous solution, and these are generally weak compared to the transition elements. The formation of complexes with compounds containing methylene phosphonate functional groups has been studied (15, 16). It can be noted that the stability of the complexes increased with the number of methylene phosphonate groups per ligand. Glyphosate, with its single methylene phosphonate group, is apparently not a strong enough ligand to complex significantly with manganese or magnesium under conditions of enzyme assay.

Two reports of the formation of glyphosate complexes with divalent cations have been published recently. Glass (17) demonstrated the formation of copper(II) and cadmium(II):glyphosate complexes by a combination of polarographic, ultraviolet electronic absorption, and infrared spectral techniques. Motekaitis and Martell (18) applied potentiometric analysis to the interaction of glyphosate with 13 divalent cations. Calcium and magnesium had very little effect on the potentiometric equilibrium curve whereas cobalt markedly suppressed the curve, indicating complex formation; manganese depressed the curve slightly, indicating some tendency to form a complex with glyphosate. In light of the results of Motekaitis and Martell (18), the small amount of inhibition (4%) of DS-Co assayed with manganese (see Table I) may reflect the formation of a manganese(II):glyphosate complex to a limited extent under conditions of enzyme assay. No data have so far been reported for the coordination state of cobalt:glyphosate. Thus, our visible electronic absorption data contribute to a growing base of information on glyphosate complexes.

In vivo implications. The ultimate consequence of glyphosate has been postulated to be an energy-drain effect promoted by the uncoupling of the regulation of the DAHP synthase isozyme (DS-Mn) in the chloroplast compartment (6, 19–21). Inhibition of EPSP synthase by glyphosate lowers the intracellular level of L-arogenate which otherwise feedback inhibits the plastidial isozyme. A model of the energy-drain effect in a microbial system having an analogous arrangement of pathway regulation has been presented (3).

If DS-Co in the cytosol is inhibited by glyphosate, then the attack of two serial enzyme targets (DS-Co and cytosolic EPSP synthase) in the cytosol [where glyphosate concentration is orders of magnitude higher than in the plastid (2)], may cause drastic starvation for aromatic amino acids. However, it seems probable

that magnesium (or manganese) is the metal used by most DS-Co isozyme molecules since 10 mM magnesium or 0.5 mM manganese is consistent with the intracellular milieux, whereas 0.5 mM cobalt is not. Although it appears that the majority of DS-Co molecules will be bound with magnesium (or manganese) in the cytosol, a small fraction of molecules might be associated with cobalt instead. If these are irreversibly inactivated (as is consistent with the inability of cobalt to completely antagonize glyphosate inhibition), it is possible that progressive inactivation of the DS-Co molecule population occurs in the presence of glyphosate.

We did not find any evidence for a divalent cation requirement of EPSP synthase in *N. silvestris*. The dramatic state of activation induced in *Bacillus subtilis* EPSP synthase by monovalent cations (22) also was not seen with *N. silvestris* EPSP synthase.

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