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Metal Ion Induced Reaction Specificity in Vitamin B_6 Model Systems

The Effects of Zn^{2+} and Cu^{2+} on the 5-Deoxypyridoxal Catalyzed Reactions of α-Phenyl-α-aminomalonic Acid

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In the absence of metal ions, the reaction of 5-deoxypyridoxal (**III**) with α-phenyl-α-aminomalonic acid, (**II**; R = C_6H_5) leads to the formation of two 5-deoxypyridoxal-derived products, 5-deoxypyridoxamine (**VII**) and a dimer-like product (**VIII**) formed from the condensation of a molecule of **III** and a molecule of **VII** (J. W. Thanassi, *Biochemistry* **12**, 5109 (1973)). The addition of excess Zn^{2+} ions or limiting Cu^{2+} ions leads to the formation of **VII** only. Addition of excess Cu^{2+} ions results in an entirely different 5-deoxypyridoxal-derived product, **VI**, which is a peptide of 5-deoxypyridoxic acid and D,L-α-phenylglycine (**V**), and which is formed in an oxidative reaction. Mechanisms for the formation of these compounds are discussed in relation to reaction selectivity in vitamin B_6 catalysis.

INTRODUCTION

Vitamin B_6 is an essential cofactor for a large variety of enzymes that, for the most part, deal with the intermediary metabolism of amino acids (*2–4*). Depending on the particular enzyme, any of the four bonds attached to the α-carbon of an amino acid can participate in the bond-making and bond-breaking processes that take place at the active sites of vitamin B_6-dependent enzymes. These processes occur in a Schiff base structure, **I**, that is formed between pyridoxal phosphate and the amino acid. In transamination reactions, bonds *a* and *d* are involved; in decarboxylations, bond *c* undergoes cleavage; and in aldol-type condensations and retrocondensations, bond *b* is involved. These represent only some of the reaction types that occur in vitamin B_6-dependent enzymes (*2*).

Reports by Snell and his colleagues (*5*) and by Braunstein (*6*) provided the basis for our understanding of the mechanism of vitamin B_6 catalysis. Since then, the literature dealing with the chemistry and enzymology of vitamin B_6 has become extensive, and a

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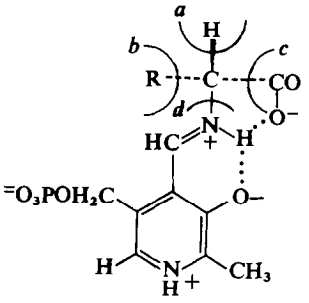


(I)

number of review articles and proceedings of symposia on these topics have appeared.[3]

In addition to the vitamin B_6-dependent enzymatic reactions of amino acids, glycogen phosphorylase has been shown to require pyridoxal phosphate, probably as a structural component (*7*), and several Cu^{2+} requiring amine oxidases have been described (*8–10*). The metal ion dependency of the latter enzymes is in contrast to other vitamin B_6-dependent enzymes.

How reaction specificity is achieved in a large number of enzymes that catalyze such a wide diversity of reactions, yet which all require the same cofactor in common, is an intriguing question. The proposals of Dunathan offer an attractive explanation for such reaction specificity (*11, 12*). Dunathan suggests a steric control mechanism in which the bond occupying position *a* in I is the reactive bond because position *a* provides the maximum overlap with the pi-system of the extended, conjugated Schiff base, thereby allowing for easier delocalization of the electron pair in bond *a* into the pi-system. Reaction specificity can therefore be achieved by having the enzyme determine which bond is to occupy the reactive position *a*. This is done by suitable rotation about the C_α-amino nitrogen bond of the amino acid at the active site of a given enzyme. Interpretation of a number of pyridoxal phosphate catalyzed reactions has been discussed in terms of this model (*12*).

As a continuation of our studies (*1, 13–15*) on the 5-deoxypyridoxal catalyzed reactions of α-aminomalonate and α-substituted-α-aminomalonates (II; R = H, CH_3, and C_6H_5), we have begun to investigate the effects of metal ions on these reactions and have found that Zn^{2+} and Cu^{2+} can cause marked changes on the course of the 5-deoxypyridoxal catalyzed reactions of α-phenyl-α-aminomalonic acid. The reaction pathways become altered and selectivity is imparted. The reactions in the presence of these two metals are not only different from a system without metal, but can be different from each other, depending on the metal ion and its concentration. These observations form the basis of this report.

[3] Literature of a review nature can be found in articles by Snell (*2*), Braunstein (*3*), Guirard and Snell (*26*) and Dunathan (*12*). Discussions of vitamin B_6 chemistry and enzymology can be found in books by Greenstein and Winitz (*27*), Meister (*4*), Bruice and Benkovic (*28*), Jencks (*29*), and in "Methods in Enzymology," Vol. 18, Part A, S. P. Colowick, N. O. Kaplan, D. B. McCormick, and L. B. Wright, Eds., Academic Press, New York, 1970. In addition, two IUB symposia on pyridoxal catalysis have been published: "Chemical and Biological Aspects of Pyridoxal Catalysis," E. E. Snell, P. M. Fasella, A. E. Braunstein, and A. Rossi-Fanelli, Eds., Pergamon, Oxford, 1963; and "Pyridoxal Catalysis: Enzymes and Model Systems," E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky, Eds., Interscience, New York, 1968.

EXPERIMENTAL

Materials and Methods

The preparation of 5-deoxypyridoxal (**III**) and the ammonium salt of α-phenyl-α-aminomalonic acid have been described (*13*, *16* and *17*). Only distilled, deionized water that had been boiled, cooled under a stream of nitrogen, and stored under nitrogen was employed in these studies. All solutions were thoroughly flushed with nitrogen. All other chemicals were the highest quality available from commercial sources, usually reagent grade. All reactions were carried out in a nitrogen atmosphere in 0.40 *M* potassium cacodylate buffer (pH 6.2) at a calculated ionic strength of 1.0 *M* (with KCl).

5-Deoxypyridoxamine (**VII**), D,L-α-phenylglycine (**V**), and the dimer-like condensation product (**VIII**) formed from a molecule each of **III** and **VII**, were identified as described previously (*1*, *14*). Copper ions were located by ultraviolet and visible absorption spectroscopy. Phenylglyoxylic acid (**IV**) was identified by comparison of its ultraviolet absorption spectrum with an authentic sample, and by the formation of a derivative with *o*-phenylenediamine (*18*). The structure of **VI**, *N*-5-deoxypyridoxoyl-α-phenylglycine, has been established by an unambiguous synthesis (*19*).

Spectrophotometric and pH measurements were made as described previously (*13*). Instrumentation used to obtain infrared, nmr, chemical ionization, and electron impact mass spectra for the purpose of product analyses have been reported elsewhere (*19*).

Carbon dioxide was measured by conventional Warburg manometry in a nitrogen atmosphere. "Total CO_2" (Fig. 4) is defined as the absolute amount of carbon dioxide released, at infinite time, over and above control reaction mixtures containing no 5-deoxypyridoxal. The reaction solutions (2.5 ml) were 1 m*M* in 5-deoxypyridoxal and 25 m*M* in α-phenyl-α-aminomalonic acid.

Ion Exchange Chromatography of Reaction Solutions

The following is a typical preparative procedure used in separating and identifying the products obtained after the reaction of 5-deoxypyridoxal with α-phenyl-α-aminomalonic acid in the presence and absence of metal ions. A solution (500 ml) that was 0.001 *M* in 5-deoxypyridoxal, 0.025 *M* in α-phenyl-α-aminomalonic acid, 0.01 *M* in $CuCl_2 \cdot 2H_2O$, and 0.40 *M* in potassium cacodylate buffer (pH 6.2; ionic strength, 1.0 *M* with KCl) was kept overnight at room temperature in the dark in a tightly stoppered polyethylene bottle in a nitrogen atmosphere. After the pH had been adjusted from 6.2 to approximately 1 with 50 ml of 6 *N* HCl, the reaction mixture was applied to a 1.9 × 58-cm column of Bio-Rad AG50Wx8 (200–400 mesh) cation exchange resin in the hydrogen form. The column was then eluted with HCl solutions of increasing concentration, and fractions were collected, as previously described (*19*). The elution profile for this reaction is provided in Fig. 1. Figure 2 shows the elution profile for a comparable but scaled-down reaction mixture in which the Cu^{2+} ion concentration is 5×10^{-4} *M*. In Fig. 3, Cu^{2+} has been replaced by Zn^{2+} at a concentration of 0.01 *M*.

RESULTS

In Figs. 1–3 are shown the elution profiles obtained upon ion-exchange chromatography of reaction mixtures containing 0.025 *M* α-phenyl-α-aminomalonate (**II**,R = C_6H_5), 0.001 *M* 5-deoxypyridoxal (**III**), and the metal ions Zn^{2+} and Cu^{2+}. The reaction

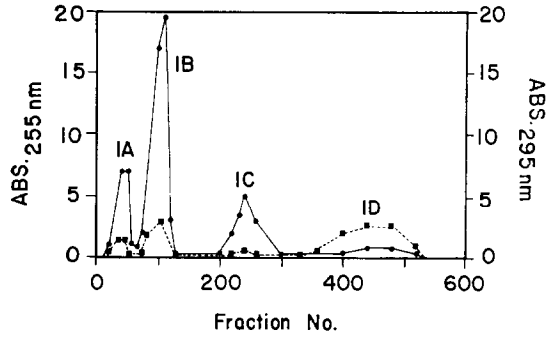


FIG. 1. Elution profile of an acidified reaction mixture containing 0.025 *M* α-phenyl-α-aminomalonic acid, 0.01 *M* $CuCl_2$, and 0.001 *M* 5-deoxypyridoxal in 0.40 *M* potassium cacodylate buffer (pH 6.2; ionic strength, 1.0 *M* with KCl). Peaks 1A-1D contain, respectively, Cu^{2+}, phenylglyoxylic acid (IV), α-phenylglycine (V), and *N*-5-deoxypyridoxoyl-α-phenylglycine (VI). Eluants: 0.5 *N* HCl, fractions 1–190; 1.0 *N* HCl, fractions 191–300; 2.0 *N* HCl, fractions 301–530; 4.0 *N* HCl, fractions 531–600. Solid line, absorbancy at 255 nm; dashed line, absorbancy at 295 nm.

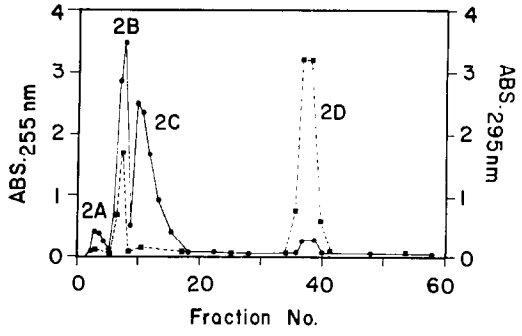


FIG. 2. As in Fig. 1, except that the Cu^{2+} concentration was 5×10^{-4} *M*. Peaks 2A-2D contain, respectively, Cu^{2+}, phenylglyoxylic acid (IV), α-phenylglycine (V), and 5-deoxypyridoxamine (VII). Eluants: 1.0 *N* HCl, fractions 1–20; 2.0 *N* HCl, fractions 21–32; 3.0 *N* HCl, fractions 33–45; 3.6 *N* HCl, fractions 46–60.

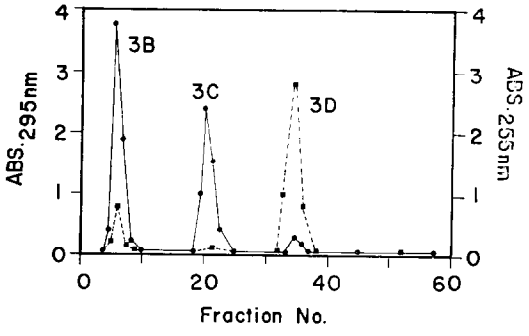
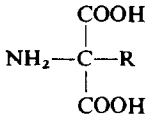
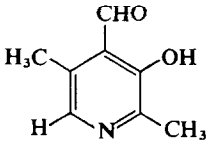


FIG. 3. As in Fig. 2, except that the metal ion was Zn^{2+} at a concentration of 0.01 *M*. Peaks 3B–3D correspond to peaks 2B–2D in Fig. 2. Eluants, as in Fig. 2.

solutions were comparable in all respects (pH 6.2; 0.4 *M* potassium cacodylate buffer; ionic strength, 1.0 *M* with KCl; nitrogen atmosphere), except that in Fig. 1 Cu^{2+} ions were present in 10-fold excess over 5-deoxypyridoxal, in Fig. 2 Cu^{2+} ions were present at a concentration equal to one-half that of the 5-deoxypyridoxal, and in Fig. 3, Zn^{2+} ions were present in 10-fold excess over 5-deoxypyridoxal.



(II)



(III)

(IV)

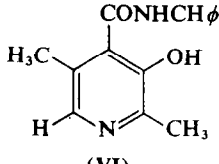
The products in the peaks in Fig. 1 were identified as Cu^{2+} (peak 1A), phenylglyoxylic acid (**IV**) (peak 1B), α-phenylglycine (**V**) (peak 1C), and *N*-5-deoxypyridoxoyl-α-phenylglycine (**VI**) (peak 1D). The only 5-deoxypyridoxal-derived product obtained from this reaction is **VI**.

When the Cu^{2+} concentration is decreased by a factor of 20, so that the ratio of Cu^{2+}/5-deoxypyridoxal is 0.5, the products in peaks 2A, 2B, and 2C are again Cu^{2+}, phenylglyoxylic acid (**IV**), and α-phenylglycine (**V**), respectively. However, the product isolated from peak 2D in a preparative scale reaction was determined to be 5-deoxypyridoxamine (**VII**).

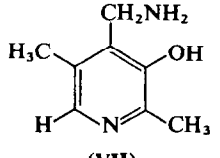
In Fig. 3, where Zn^{2+} ions are present at a concentration of 0.01 *M* (10-fold excess over 5-deoxypyridoxal), the products in peaks 3B–3D are identical to the products in peaks 2B–2D, respectively. Therefore, the only 5-deoxypyridoxal-derived product in the presence of excess Zn^{2+} ions is 5-deoxypyridoxamine (**VII**).

Hence, in this system, excess Zn^{2+} ions and limiting Cu^{2+} ions cause the exclusive formation of the same 5-deoxypyridoxal-derived product, **VII**. On the other hand, excess Cu^{2+} ions cause the exclusive formation of an entirely different product, **VI**, which arises by an oxidative reaction (*19*).

(V)



(VI)



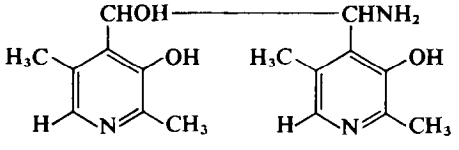
(VII)

DISCUSSION

In contrast to pyridoxal phosphate dependent enzymatic reactions, reactions catalyzed by pyridoxal phosphate and its analogs in chemical systems generally proceed by multipath reaction routes leading to mixtures of products. There have been relatively few examples of the imposition of reaction selectivity in pyridoxal catalyzed reactions in chemical systems. Demonstration of such reaction selectivity in model systems may provide insight into the factors that operate at the active sites of pyridoxal phosphate

dependent enzymes. Among those studies that have demonstrated reaction specificity are the experiments of Tenenbaum et al. (*20*), who showed that there is a pH dependency of the Al^{3+} and pyridoxal catalyzed deuteration at the α and β positions of amino acids. Experiments from Bruice's laboratory (*21*) have shown that the addition of imidazole can impart reaction specificity in the direction of transamination. Also, experiments from this laboratory have established that the substitution at the α-carbon of α-aminomalonates (**II**) can affect reaction pathways in the 5-deoxypyridoxal catalyzed reactions of α-aminomalonates (*1*, *13*, *14*).

The experiments reported herein provide examples of reaction selectivity resulting from the addition of metal ions, specifically, Cu^{2+} and Zn^{2+}. Previous experiments (*1*) have shown that, at pH 5.2, the 5-deoxypyridoxal-derived products isolated from a reaction mixture containing 5-deoxypyridoxal and α-phenyl-α-aminomalonic acid are 5-deoxypyridoxamine (**VII**) and a dimer-like product (**VIII**) that is formed from the condensation of the 4′-carbon atoms of 5-deoxypyridoxamine (**VII**) and 5-deoxypyridoxal (**III**). At pH 6.2, in the absence of metal ions, **VII** and **VIII** are again the only 5-deoxypyridoxal-derived products; these are formed in a ratio of 4:1 at this pH.



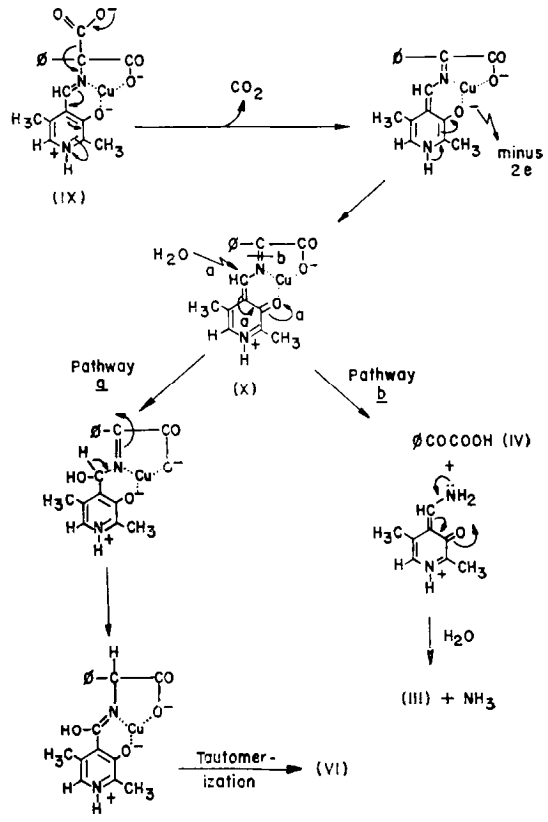
(VIII)

However, if one adds Cu^{2+} ions to this system at a concentration 10 times that of the 5-deoxypyridoxal, then the sole 5-deoxypyridoxal-derived product is *N*-5-deoxypyridoxoyl-α-phenylglycine (**VI**). This product can arise only by an oxidative mechanism; it results from the formation of a peptide bond between C4′ of the vitamin analog **III** and the amino nitrogen of the decarboxylation product of α-phenyl-α-aminomalonic acid, α-phenylglycine (**V**). This is an unusual type of reaction for vitamin B_6 and its analogs. The proof of structure of **VI** and a mechanistic proposal for its formation have been discussed (*19*). The other products obtained from this particular reaction mixture are α-phenylglycine (**V**) and phenylglyoxylic acid (**IV**). α-Phenylglycine can arise from catalytic decarboxylation of the substrate, α-phenyl-α-aminomalonic acid, by 5-deoxypyridoxal, and will also be formed by spontaneous decarboxylation of the acid-sensitive parent compound during the acidification and workup of the reaction mixture (*1*). Phenylglyoxylic acid (**IV**) can arise from this reaction mixture only by oxidative mechanism; if it were to arise by a mechanism involving decarboxylative transamination (see below), then 5-deoxypyridoxamine would have to be formed as well; this is not the case. Scheme I provides pathways for the formation of the products obtained in this experimental system.

The key intermediate in Scheme I is the *o*-quinone methide, X. This intermediate can undergo an addition reaction at C4′ (pathway *a*) leading to **VI**.[4] Alternatively, the

[4] A quinone methide derived from pyridoxine recently has been proposed as an intermediate in the nucleophilic addition of a number of reagents at the C4′ position of the vitamin (*30*).

intermediate X can undergo hydrolysis (pathway *b*) and yield phenylglyoxylic acid (**IV**).[5]

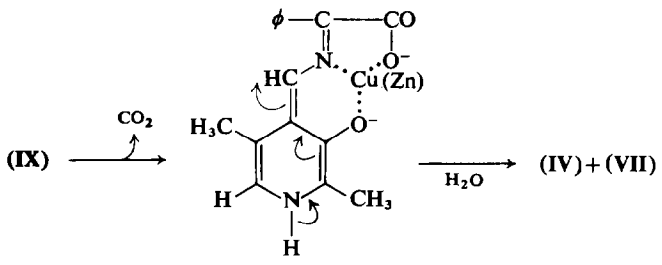


SCHEME I.

In the presence of a Cu^{2+} concentration equivalent to one-half that of the 5-deoxypyridoxal, the only 5-deoxypyridoxal-derived product formed is 5-deoxypyridoxamine (**VII**). As expected, phenylglyoxylic acid (**IV**) and α-phenylglycine (**V**) are also isolated (Fig. 2). In view of the fact that 5-deoxypyridoxamine (**VII**) is formed exclusively under these conditions, then phenylglyoxylic acid (**IV**) most likely arises by way of a decarboxylative transamination (*22*) as shown in Scheme II, rather than by the oxidative mechanism shown in Scheme I.

Having obtained the results discussed above, the effects of added Zn^{2+} ions were examined. This metal does not participate in oxidation–reduction reactions, and it seemed likely that the reaction of 5-deoxypyridoxal with α-phenyl-α-aminomalonic acid in the presence of excess Zn^{2+} ions would resemble the reaction in the case of limiting Cu^{2+} rather than the case of excess Cu^{2+} ions. In fact, this proved to be the case as shown in Fig. 3 where the products in peaks 3B–3D are identical to the products in

[5] If phenylglyoxylic acid (**IV**) is formed by the oxidative deamination mechanism proposed in Scheme I, this system may prove to be interesting with respect to the mechanism of pyridoxal phosphate and Cu^{2+}-dependent amine oxidases (*8–10*) which remove amino groups by an oxidative mechanism. Model systems and mechanisms for such reactions have been discussed by Hamilton (*31*, *32*).



SCHEME II.

peaks 2B–2D, rather than the products in peaks 1B–1D. The mechanisms for the formation of 5-deoxypyridoxamine (VII) and phenylglyoxylic acid (IV) are proposed to be the same as the mechanisms described above for the system with limiting Cu^{2+} ions, i.e., Scheme II.

The results are summarized in Table I and demonstrate that these metal ions can impart selectivity to potential multipath reaction schemes, that the selectivity can be

TABLE 1

EFFECTS OF Zn^{2+} AND Cu^{2+} ON PRODUCT DISTRIBUTION

Metal (m*M*)	Percentage of 5-deoxypyridoxal-derived product identified as:		
	5-Deoxypyridoxamine (VII)	Condensation product (VIII)	*N*-5-deoxypyridoxoyl-α-phenylglycine (VI)
None	81	19	0
Cu^{2+} (0.5)	97	0	3
Cu^{2+} (10.0)	0	0	100
Zn^{2+} (10.0)	100	0	0

dependent on the concentration of the metal ion, and that the selectivity is dependent on the chemical reactivity of the metal ion.

In addition to experiments of the type described in Table 1, differential selectivity by these two metals also is seen in experiments concerned with the absolute amount of CO_2 evolution resulting from the decarboxylation of α-phenyl-α-aminomalonic acid. These data are provided in Fig. 4 where it can be seen that effects of Zn^{2+} and Cu^{2+} on total

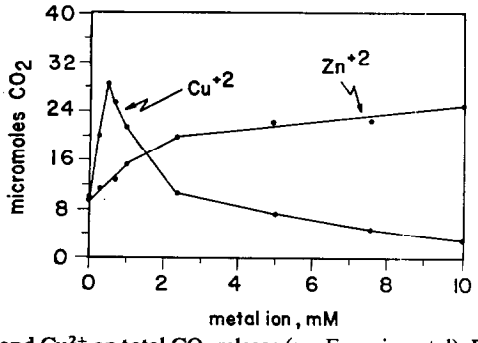


FIG. 4. Effects of Zn^{2+} and Cu^{2+} on total CO_2 release (see Experimental). Reaction mixtures (2.5 ml) were 0.001 *M* in 5-deoxypyridoxal and 0.025 *M* in α-phenyl-α-aminomalonic acid.

CO_2 production are completely different. In the case of Zn^{2+}, increasing concentrations cause a steady increase in total CO_2 production. In the case of Cu^{2+}, there is a maximum stimulation that occurs at a molar ratio of Cu^{2+}/5-deoxypyridoxal of 0.5. As the molar ratio of Cu^{2+}/5-deoxypyridoxal increases to 10, CO_2 production decreases to an amount approaching the amount of 5-deoxypyridoxal present (2.5 μmol); i.e., 1 mol of CO_2 is produced per mol of 5-deoxypyridoxal.

Total CO_2 production is an indicator of "turnover" in this system. That is, any CO_2 production greater than a molar equivalency of one can only arise if the vitamin analog is acting in a catalytic fashion. Therefore, the total amount of CO_2 liberated is an index of the number of decarboxylation reactions that take place before the 5-deoxypyridoxal is inactivated as a catalyst by competing reactions that lead to compounds such as **VI**, **VII**, or **VIII**. Catalytic decarboxylation means that CO_2 is formed by decarboxylation of the amino acid, reprotonation of the resulting carbanion occurs at C_α of the amino acid, and the Schiff base-bound α-phenylglycine that is formed exchanges with a molecule of α-phenyl-α-aminomalonic acid to complete the cycle. Cu^{2+}, at a molar concentration equal to one-half that of 5-deoxypyridoxal, enhances the catalytic decarboxylation by a factor greater than three when compared to a control containing no Cu^{2+}. Excess Cu^{2+}, on the other hand, causes CO_2 evolution to approach a 1:1 molar ratio with 5-deoxypyridoxal. Under these conditions, the oxidative reaction leading to **VI** becomes the pathway-determining reaction and effectively prevents the catalytic decarboxylation of α-phenyl-α-aminomalonic acid. In contrast to Cu^{2+}, Zn^{2+} continually increases the catalytic decarboxylation of α-phenyl-α-aminomalonic acid leading to an approximate 3-fold increase in total CO_2 production at a ratio of Zn^{+2}/5-deoxypyridoxal of 10. In addition to the different electrochemical properties of these two metals, it seems likely that different geometries of the metal chelates and different rates of ligand exchange are factors in determining the reaction pathways found in these experiments.

The details of the nature of the reactive metal complexes in these experiments are difficult to establish. Definitive studies of the equilibrium constants for Schiff base–metal complex formation, and for the acid–base hydrogen ion equilibria of these complexes, require systems that are either nonreactive or slow to react, as in the experiments of Felty et al. (*23*).[6] Model systems containing 5-deoxypyridoxal and α-aminomalonates are extremely reactive (*1*, *13–15*) and are not amenable to equilibrium measurements that will define the Schiff base–metal structures that are in solution.

The simplest explanation for the results summarized in Table I is that when Cu^{2+} is limiting, it is all complexed in the form of a Schiff base and there are no free Cu^{2+} ions available to act in oxidation–reduction reactions. It seems likely that in the case of excess Zn^{2+}, the predominant species in solution is a 1:1:1 complex of 5-deoxypyridoxal: α-phenyl-α-aminomalonate: Zn^{2+}. Leussing and his coworkers have shown (*23*) that at pH values around 6, a 1:1:1 species predominates in a system containing pyridoxal phosphate:alanine: Zn^{2+} when the molar ratios of these components are 5:10:2. Since the corresponding molar ratios in our experiments are 1:25:10, with α-phenyl-α-aminomalonate and Zn^{2+} in significant excess over the vitamin analog, a predominantly 1:1:1 species appears very likely. Similar arguments can be made for the system containing excess Cu^{2+} ions. In the case of limiting Cu^{2+} ions, there is probably a mixture of both 1:1:1 and 2:2:1 complexes.

[6] Holm has reviewed the chemistry of metal complexes of vitamin B_6 (*33*).

Both of the metal ions employed in these experiments can coordinate tridentate ligands. Gansow and Holm (*24*) discuss tridentate ligand binding by Cu^{2+} and Zn^{2+} in Schiff base complexes derived from pyridoxal and amino acids. Murakami et al. (*25*) suggest that the particular effectiveness of Cu^{2+} ions in catalyzing a β-elimination reaction of *o*-phosphothreonine lies in the fact there can be tridentate coordination of the phenolic oxygen, imine nitrogen, and carboxylate group to the Cu^{2+} atom in the Schiff base complex, and that these ligands can all lie in the same plane. The importance of such coplanarity in pyridoxal catalysis was initially recognized by Snell and his colleagues (*5*).

It seems likely, therefore, that in our experiments, Zn^{2+} and Cu^{2+} (limiting concentrations) impart selectivity by coordinating to the Schiff base of α-phenyl-α-aminomalonic acid and 5-deoxypyridoxal. A carboxyl group of the α-dicarboxylic acid that is not coordinated to the metal can occupy the reactive position *a* in **I** and undergo decarboxylation by delocalizing the bonding electron pair into the extended, conjugated Schiff base system; the metal ion would provide additional electrophilic catalysis for this step (*5*). In this sterically constrained system, catalytic decarboxylation is enhanced but there is also selection of a reaction pathway that ultimately leads to an overall decarboxylative transamination as outlined in Scheme II. Condensations of the type leading to **VIII**, observed in the absence of added metal ions, are selected against in this system. In the presence of excess Cu^{2+} ions, a different and unusual oxidative pathway is selected, leading to the exclusive formation of *N*-5-deoxypyridoxoyl-α-phenylglycine (**VI**) (Scheme I); under these conditions catalytic decarboxylation becomes suppressed.

These results illustrate, in chemical systems, that metal ions introduce into the Schiff base of 5-deoxypyridoxal and α-phenyl-α-aminomalonate, template factors that can be pathway-determining. Template effects of this kind are no doubt important in establishing the mechanistic discrimination essential to the reaction specificity in vitamin B_6-dependent enzymes.

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