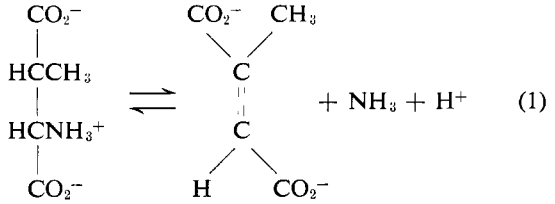
Divalent Metal Activation of β-Methylaspartase. The Importance of Ionic Radius*

Harold J. Bright

ABSTRACT: (1) As a prerequisite for a systematic study of the interactions of divalent metal ions with β-methylaspartase it was established that K^+, like substrate and divalent metal ion, added to the enzyme in random-order, rapid equilibrium fashion. (2) The interactions of seven divalent metal activators (M^{2+}) and two divalent metal inhibitors (I^{2+}) with the enzyme were analyzed by conventional kinetic methods. Divalent metal ions smaller than Ca^{2+} (ionic radius 1.0 A) were found to be activators, while Ca^{2+} and larger ions were inhibitors. The most stable complexes of M^{2+} and I^{2+} with E (or EK^+) and ES (or ESK^+) occurred with those ions having crystal ionic radii between 0.82 (Co^{2+}) and 0.97 A (Cd^{2+}). Divalent metal ions smaller than Co^{2+} and larger than Cd^{2+} formed progressively less stable complexes. The binding specificity shown by the enzyme for divalent metal ions was discussed in terms of decreasing entropies of complex formation with increasing ionic radii and the mutual repulsion of bulky (probably carboxylate) ligands surrounding the smaller divalent metal ions. (3) The rate of conversion of K^+ESM^{2+} to mesaconate and ammonium, k_{cat}, increased regularly with decreasing ionic radius for those divalent metal ions with closed electronic structures. This kinetic specificity was shown to be consistent with the formation of K^+ESM^{2+} in which M^{2+} interacts with the β-carboxylate group of the substrate, thus aiding in the activation (acidification) of the substrate β proton for β-carbanion formation. Small divalent ions, of high charge density, are the most effective in promoting electron withdrawal from the β carbon.

Although large divalent cations such as Ca^{2+} and Sr^{2+} are bound effectively by the enzyme, they are incapable of activating the β hydrogen and thus act as inhibitors.

The enzyme β-methylaspartase (*threo*-3-methyl-L-aspartate ammonia-lyase, EC 4.3.1.2) from *Clostridium tetanomorphum* catalyzes the reversible conversion of *threo*-β-methyl-L-aspartate to mesaconate and ammonia (eq 1).



(1)

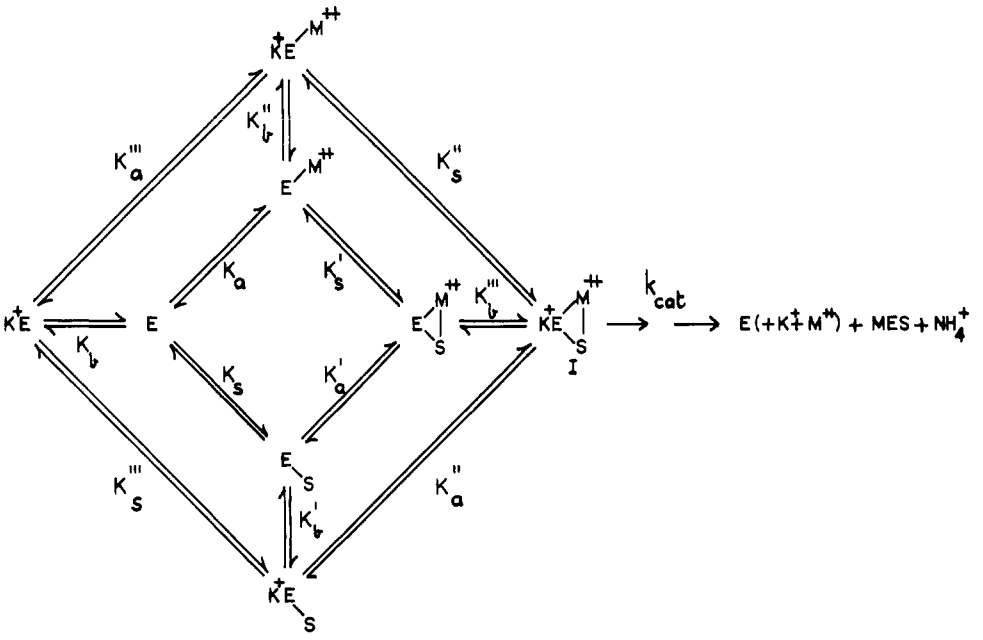
The divalent metal activation of β-methylaspartase has been the subject of several investigations. In their original paper, Barker *et al.* (1959) found only Mg^{2+} to be an activator. Bright and Ingraham (1960) later showed that the enzyme can in fact utilize divalent activators other than Mg^{2+}. Recently, Williams and Selbin (1964) reported K_m and V_{max} values measured at pH 8.2 in the presence of 0.02 M β-methyl-DL-aspartate, for seven divalent metal activators of β-methylaspartase. However, in none of these investigations was attention focused on the possibility that the divalent metal–amino acid complexes might not be substrates for the enzyme.

Bright and Silverman (1964) argued from kinetic data that the true substrate for the enzyme is probably the uncomplexed amino acid. In addition, it was pointed out (Bright, 1965) that several other pieces of evidence suggest that the divalent metal–amino acid complex is not a substrate for β-methylaspartase. It would appear that the divalent metal requirement of this enzyme is most easily demonstrated and studied with Mg^{2+} simply because the stability constant for the Mg^{2+}–amino acid complex is smaller than that for any other divalent activator. The formation of the amino acid–divalent metal complex occurs *via* the unprotonated α-amino (pK_a = 9.9) and α-carboxylate (pK_a < 3.5) groups and the fraction of metal or amino acid which is complexed will depend on the concentrations of amino acid and metal, the pH, and the stability constant of the particular amino acid–metal complex. The experimental conditions, therefore, if not properly chosen, can introduce great complexity in the interpretation of the kinetics of divalent metal activation of β-methylaspartase.

A detailed kinetic study of divalent metal activation of β-methylaspartase has been described (Bright, 1965)

* From the Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received October 27, 1966.* Presented in part at the 1966 meeting of the American Society of Biological Chemists, Atlantic City, N. J. Financial assistance for this project by the National Institutes of Health, U. S. Public Health Service (Grant GM 11040-03S1), is gratefully acknowledged.

SCHEME I



using conditions which resulted in negligible complexing of the divalent metal activator by either the substrate or buffer components. The kinetic data for the forward reaction were uniquely satisfied by the random-order, rapid equilibrium mechanism of addition of β-methylaspartate and M^{2+} when the results from both Mg^{2+} and Co^{2+} activation were taken into consideration. It was found that the substrate was bound more firmly by both E–Mg^{2+} and E–Co^{2+} than by E (metal-less enzyme). Studies of the reverse reaction showed that mesaconate and ammonia also add to the metal–enzyme in a random-order, rapid equilibrium fashion, and that the values of the dissociation constants governing the interactions of ammonium with the Co^{2+}–enzyme and Mg^{2+}–enzyme are identical, whereas mesaconate, like β-methylaspartate, is more firmly held by the Co^{2+}–enzyme than it is by the Mg^{2+}–enzyme. It was pointed out that these results are consistent with the β-carbanion mechanism proposed for the β-methylaspartase reaction if the function of the enzyme-bound divalent metal activator in the ternary complex is to bond to the β-carboxylate group of the substrate and activate (acidify) the β proton.

Considerable differences were noted previously (Bright, 1965) between the binding of Mg^{2+} and Co^{2+} by β-methylaspartase. The ligands on the enzyme behave like those of many small organic molecules, to the extent that enzyme–Co^{2+} is thermodynamically more stable than enzyme–Mg^{2+}. It was, therefore, of interest to determine whether the pattern of metal binding by the enzyme completely parallels that of small organic ligands. In this communication we show that the interaction of divalent metals with β-methylaspartase, as determined by conventional steady-state kinetic analysis, in fact bears some resemblance to model organic ligand–metal systems. The basis for the specificity of β-methylaspartase in its interactions with divalent metal activators appears to be the ionic radius. We also demonstrate that ionic radius plays an important part in determining the catalytic efficiency of divalent metal activators having closed electronic shell structures. The relative catalytic efficiencies of these metal ions can be explained in terms of the carbanion mechanism which we have favored for some time (Bright *et al.*, 1964; Bright, 1965).

Results

Kinetics of Monovalent Ion Activation. Since the enzyme also requires a monovalent ion for catalytic activity, it was essential to examine the kinetic behavior of the monovalent ion (K^+ in these studies) before the divalent metal kinetic data could be properly analyzed. The interactions of Mg^{2+} and β-methylaspartate with the enzyme were, therefore, studied at two concentrations of K^+. Figures 1 and 2 show the resulting kinetic data plotted in double-reciprocal form. It will be noted that the K_m values of Mg^{2+} and β-methylaspartate are not functions of the concentration of K^+. These results, together with those described previously (Bright, 1965), indicate that Mg^{2+}, K^+, and β-methylaspartate all add to the enzyme in a random-order, rapid equilibrium fashion. This conclusion is strengthened by the finding that the K_m values of Mg^{2+} and β-methylaspartate are independent of the nature, as well as the concentration, of monovalent cation activator.[1]

[1] Unpublished experiments with Na^+, K^+, Cs^+, Li^+, Rb^+, and NH_4^+.

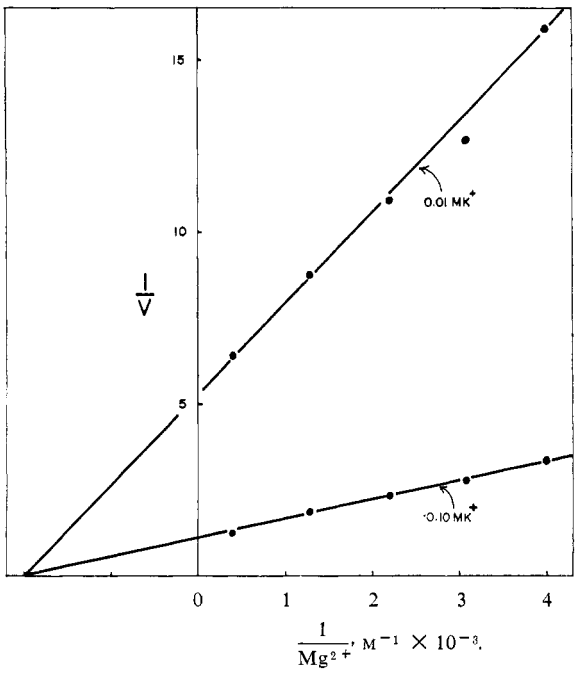


FIGURE 1: Effect of K^+ on Mg^{2+} kinetics. Measurements carried out at 25° and pH 5.9 with 10^{-3} M β-methylaspartate and ionic strength 0.3. For other details, see Experimental Section.

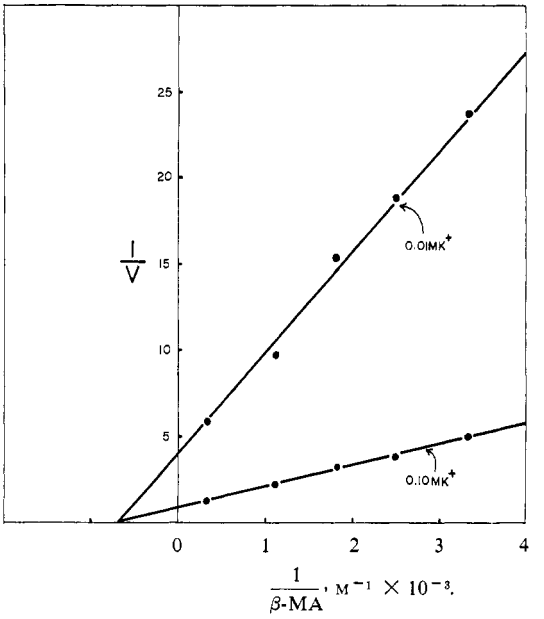


FIGURE 2: Effect of K^+ on β-methylaspartate kinetics. Measurements carried out at 25° and pH 5.9 with 5×10^{-4} M Mg^{2+} and ionic strength 0.3. For other details, see Experimental Section.

We may, therefore, depict the additions of Mg^{2+} M^+,[2] and β-methylaspartate to the enzyme as shown in Scheme I. The data of Figures 1 and 2 indicate that (i) $K_b = K_b' = K_b'' = K_b'''$, (ii) $K_a = K_a'''$, (iii) $K_s = K_s'''$, (iv) $K_a' = K_a''$, and (v) $K_s' = K_s''$. If it is assumed that these relationships hold with all other divalent metal ion activators, then the velocities ($V_{\infty S,\infty M^{2+}}$) measured at infinite concentration of M^{2+} and β-methylaspartate, and at a constant K^+ concentration, can be directly compared, since

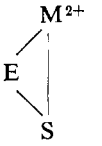
$$k_{cat} = \frac{V_{\infty S,\infty M^{2+}}}{[E_0]}\left[1 + \frac{K_b}{[K^+]}\right] \qquad (2)$$

The value of K_b can be obtained if necessary by replotting the data of Figures 1 and 2. Potassium was used as the monovalent ion activator in the studies to be described, at a concentration of 0.135 M, because k_{cat} is larger with K^+ than with any other monovalent metal cation. It also served to maintain constant ionic strength.

Kinetics of Divalent Metal Ion Activation. The dissociation constants K_a, K_s, K_a', and K_s' (see Scheme I) were determined for each of the divalent metal activators[3] (Mg^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, and Ni^{2+}) from kinetic data according to the procedures described previously (Bright, 1965). Those procedures were based on the conclusion that M^{2+} and substrate interact with the enzyme in a random-order, rapid equilibrium fashion (Scheme I) for which the rate equation is

$$v = \frac{V_{\infty S,\infty M^{2+}}}{1 + \frac{K_a'}{[M^{2+}]} + \frac{K_s'}{[S]} + \frac{K_a'K_s}{[M^{2+}][S]}} \qquad (3)$$

The tacet assumption was made that the third known effector (besides H^+), namely the monovalent ion required for catalysis, did not affect the affinity of M^{2+} or S for the enzyme. This would be a critical assumption if experiments were carried out at nonsaturating concentrations of the monovalent ion, since different M^{2+} might give rise to differing affinities of EM^{2+} and



[2] Abbreviations used: M^{2+}, divalent metal activator; M^+, monovalent metal activator; S and β-MA, *threo*-β-methyl-L-aspartate; E, active site of β-methylaspartase; I^{2+}, divalent metal inhibitor; L, ligand; ATP, adenosine triphosphate.

[3] Cu^{2+} was also found to be an activator of β-methylaspartase under the experimental conditions described here (*cf.* Williams and Selbin, 1964). However, concomitant inhibition by Cu^{2+} caused difficulties in the interpretation of the activation kinetics. The inhibition is almost certainly due to the formation of catalytically inert $E-(Cu^{2+})_n$, possibly through binding to peptide nitrogens.

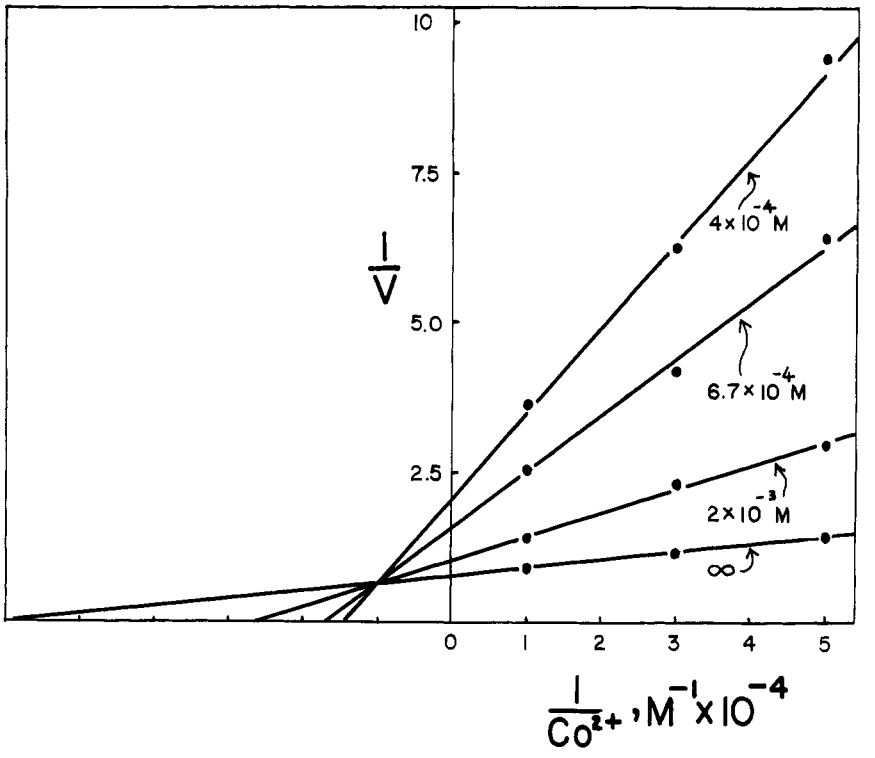


FIGURE 3: Effect of β-methylaspartate on Co^{2+} kinetics. The concentration of β-methylaspartate is indicated by each line. The points representing infinite substrate concentration are the ordinate intercepts of Figure 4. Measurements carried out at 25° and pH 5.1 and ionic strength 0.135. For other details, see Experimental Section.

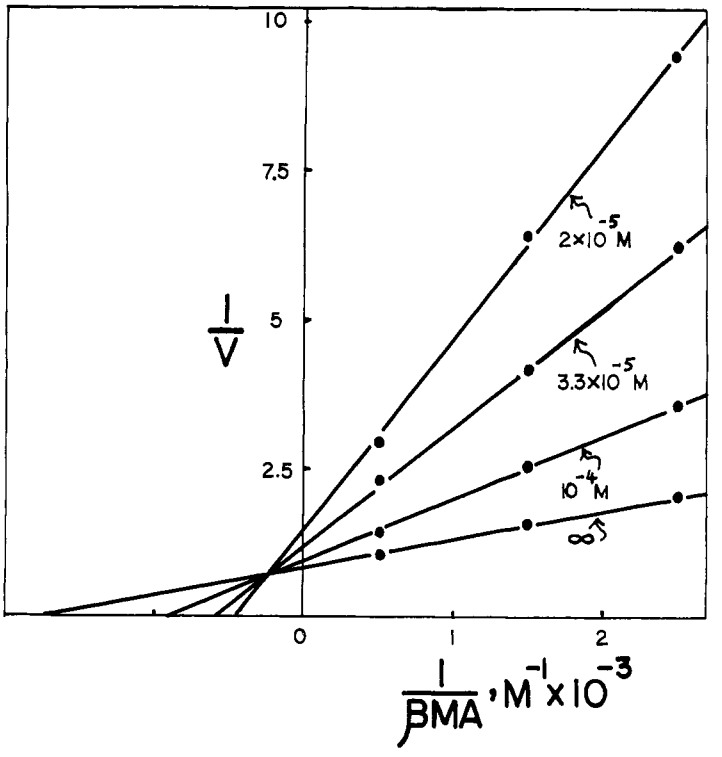


FIGURE 4: Effect of CO^{2+} on β-methylaspartate kinetics. These are the data of Figure 3 replotted, with the concentration of Co^{2+} indicated by each line. Points representing infinite Co^{2+} concentration are the ordinate intercepts of Figure 3.

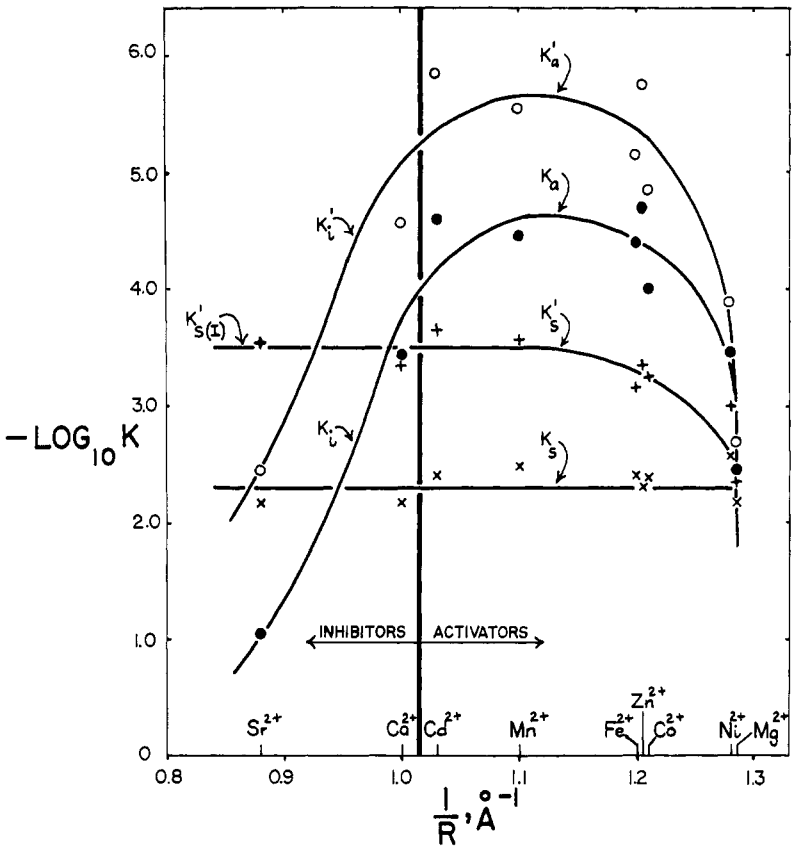


FIGURE 5: The dependence of the dissociation constants measured for divalent activators and inhibitors of β-methylaspartase on the crystal ionic radii of the divalent ions. All measurements made at pH 5.1, 25°, and ionic strength 0.135.

for M^+ and hence lead to complications in the analysis of the rate data. However, we used saturating concentrations of K^+ in the previous studies (Bright, 1965) and, moreover, in the previous section it was shown that the affinities of S and M^{2+} are not functions of the concentration of K^+. The graphical analysis of the rate data which was proposed by Bright (1965) is, therefore, justified. Under our carefully chosen conditions, linear double-reciprocal plots are readily obtained and are highly reproducible. As before, it was necessary to include EDTA in the reaction mixtures in order to abolish both the activation and inhibition of the enzyme by traces of metal ions in the system. The concentration of EDTA was never more than 10% of the concentration of the added divalent metal ion. In our discussion of the effects of EDTA on this reaction (Bright, 1965) it was pointed out that the concentration of EDTA necessary to eliminate the activity due to contaminating metal ions, in the presence of low concentrations of substrate, decreased markedly as the pH is lowered. It was found convenient in that study to use 5×10^{-6} M EDTA for all rate measurements performed at pH 5.9 with Co^{2+} and Mg^{2+} as activators. In the present study the K_m values of several of the metal activators are sufficiently smaller than those of Co^{2+} that the concentration of EDTA had to be decreased to 2×10^{-6} M, in order that a significant dependence of initial velocity on metal ion concentration could be observed with an excess of metal over EDTA of at least tenfold. Under these conditions pH 5.1 is the highest pH value at which

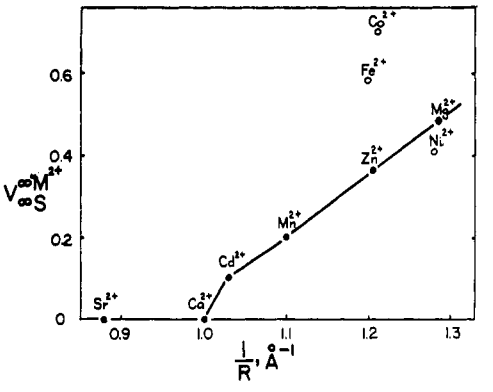


FIGURE 6: The dependence of $V^{6}_{\infty S,\infty M^{2+}}$ (and hence k_{cat}, see text) on the crystal ionic radii of divalent metal activator of β-methylaspartase. All measurements made at pH 5.1, 25°, and ionic strength 0.135.

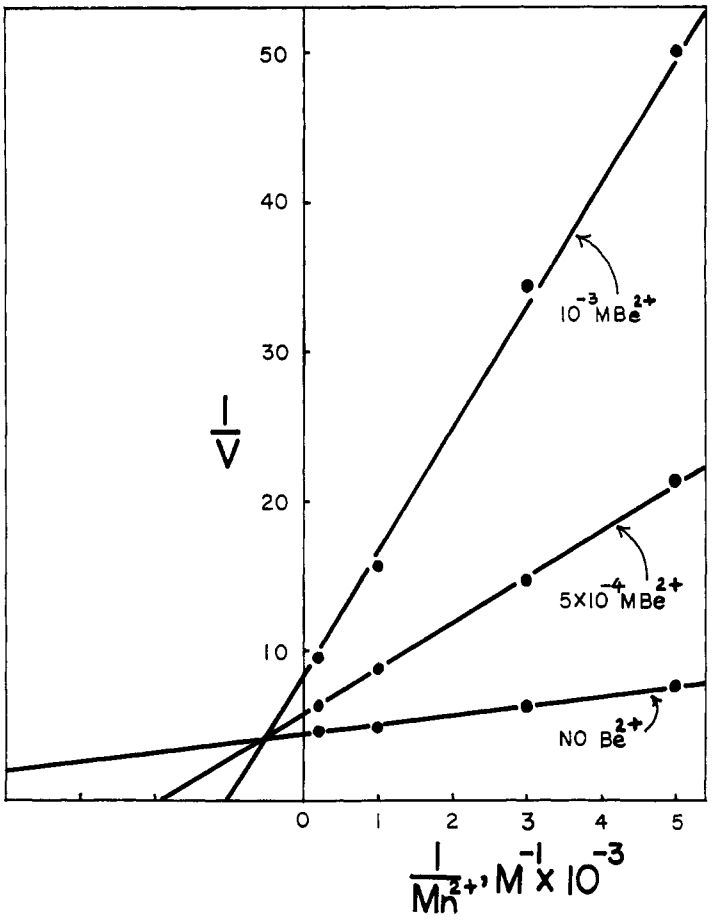


FIGURE 7: Effect of Be^{2+} on activation of β-methylaspartase by Mn^{2+}. Measurements carried out at 25° and pH 5.1 with 4×10^{-4} M β-methylaspartate and ionic strength 0.135. For other details, see Experimental Section.

2×10^{-6} M EDTA is sufficient to completely eliminate the activity due to contaminant metal ions. We have, therefore, carried out all rate measurements (except those concerned with the effect of K^+ in Figures 1 and 2) at pH 5.1 rather than pH 5.9.

Shown in Figures 3 and 4 are representative double-reciprocal plots, for the case of Co^{2+}, demonstrating the dependence of the initial velocity of mesaconate formation on the concentrations of substrate and Co^{2+}. Included in each plot are the ordinate intercepts of the other plot representing the extrapolated initial velocities corresponding to infinite concentrations of Co^{2+} and substrate. The graphically determined value of the initial velocity at infinite concentrations of substrate and metal is $V_{\infty S,\infty M^{2+}}$. Corresponding double-reciprocal plots were obtained for each of the seven divalent metal activators studied. These plots were analyzed as described previously (Bright, 1965). The values of K_s and K_a are determined from the intersections of lines in the substrate–velocity and M^{2+}–velocity plots, respectively. The values of K_s' and K_a' are determined, respectively, from the abscissa intercepts of the lines corresponding to infinite M^{2+} and infinite substrate concentrations. Because K^+ adds in a random-order, rapid equilibrium manner to the enzyme, the constants $V_{\infty S,\infty M^{2+}}$, K_s, K_a', K_s', and K_b are sufficient to describe the kinetic behavior of Scheme I.

In Figure 5 we demonstrate the dependence of K_s, K_a, K_s', and K_a', obtained with each of the seven divalent metal activators, on the crystal ionic radii of these ions. The dissociation constants K_a and K_a' vary quite similarly with ionic radius although it will be noted that K_a is consistently larger than K_a'. The values of K_s' increase as the ionic radii decrease. Since K_s governs a process not involving the divalent ion, its value should be constant for all metals. The data of Figure 5 show this to be the case.

We also found that K_a and K_a' measured in the presence of Mn^{2+}, are invariant with temperature between 1 and 40°. The implications of this finding will be discussed.

Figure 6 shows that there is also a marked dependence of $V_{\infty S,\infty M^{2+}}$ (and hence k_{cat}) on the crystal ionic radii of the divalent metal ions, especially those having closed electronic shell structures (or half-

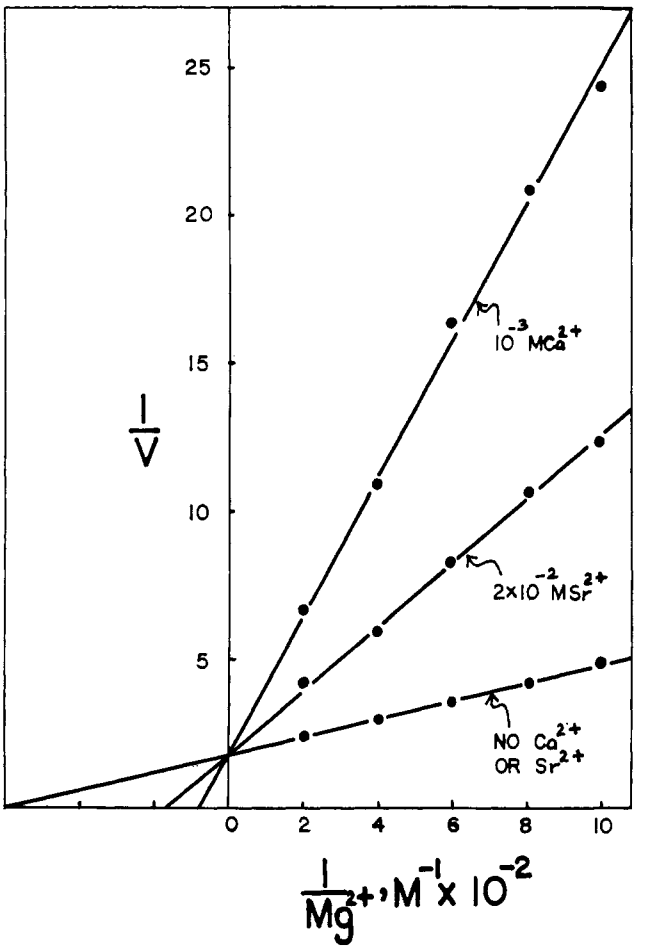


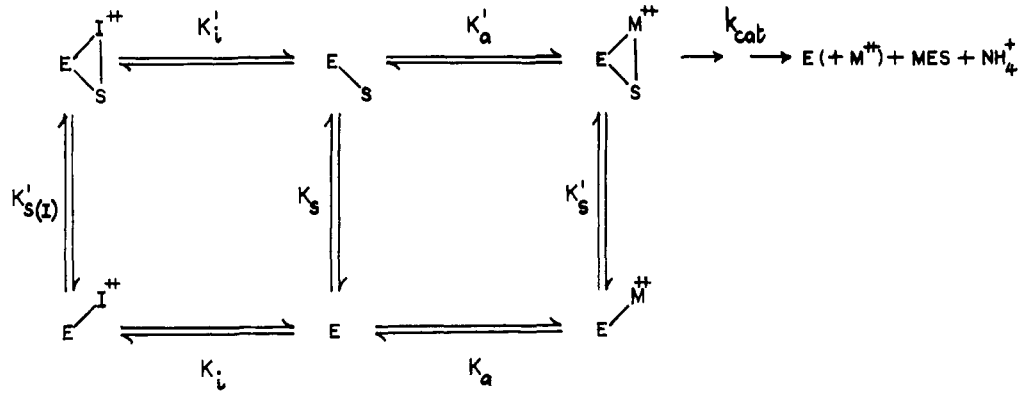
FIGURE 8: Competitive inhibition by Sr^{2+} and Ca^{2+} of Mg^{2+} activation of β-methylaspartase. Measurements carried out at 25° and pH 5.1 with 5×10^{-3} M substrate and ionic strength 0.135. For other details, see Experimental Section.

closed in the case of Mn^{2+}). As will be discussed, Fe^{2+}, Co^{2+}, and Ni^{2+} would not be expected to conform to an ionic radius relationship.

It was of interest to determine the behavior in this system of ions, such as Be^{2+}, which are smaller than Mg^{2+} and of those ions, such as Ca^{2+} and Sr^{2+}, which are larger than Cd^{2+}. Although it is difficult to establish unequivocally that a given divalent metal ion cannot act as an enzyme activator, we can state that if Be^{2+}, Ca^{2+}, and Sr^{2+} do in fact activate β-methylaspartase, their $V_{\infty S,\infty M^{2+}}$ values are less than one-tenth that of Cd^{2+}. All three metal ions, however, combine readily with the enzyme, as evidenced by their ability to inhibit the β-methylaspartase reaction. However, the inhibition by Be^{2+} of the Mn^{2+}-activated reaction, as shown in Figure 7, is not of the competitive type in that the graphically determined velocity at infinite Mn^{2+} concentration decreased as the Be^{2+} concentration is raised. On the other hand, the relatively large Ca^{2+} and Sr^{2+} ions are clearly competitive inhibitors of Mn^{2+}, as shown in Figure 8. Although we have not presented the data, Ca^{2+} also acts as a purely competitive inhibitor of Mg^{2+}. The competitive inhibition by Ca^{2+} of Mg^{2+} activation of β-methylaspartase was noted by Barker *et al.* (1959) in experiments performed at pH 9.7 and with relatively high substrate concentrations. The nonlinearity of their double-reciprocal plot, which can be seen at the higher Mg^{2+} concentrations, is probably due to the considerable formation under their experimental conditions of the Mg^{2+}-amino acid complex which is now believed to be kinetically inert.

From the fact that the inhibition observed for both Ca^{2+} and Sr^{2+} is purely competitive it is likely that these ions are bound at the divalent metal activator site on the enzyme. It is therefore reasonable to assume that Ca^{2+} and Sr^{2+} are catalytically inert because the quaternary complex formed from either of these ions cannot undergo chemical transformation to mesaconate and ammonia. We may, therefore, depict the reactions occurring in the presence of a divalent metal activator and either Ca^{2+} or Sr^{2+} (indicated as I^{2+}) in Scheme II. Although we assumed that K^+ can interact with all forms of the enzyme in this scheme,

SCHEME II



these interactions are omitted here and in the following discussion for the sake of clarity. The rapid equilibrium for this scheme leads to the rate equation

$$v = \frac{V_{\infty S,\infty M^{2+}}}{1 + \frac{K_a'}{[M^{2+}]} + \frac{K_s'}{[S]} + \frac{K_a'K_s}{[M^{2+}][S]} + \frac{[I^{2+}]K_a'}{[M^{2+}]K_i'} + \frac{[I^{2+}]K_sK_a}{[M^{2+}][S]K_i}} \quad (4)$$

It is also known that

$$K_iK_{s(I)}' = K_i'K_s \quad (5)$$

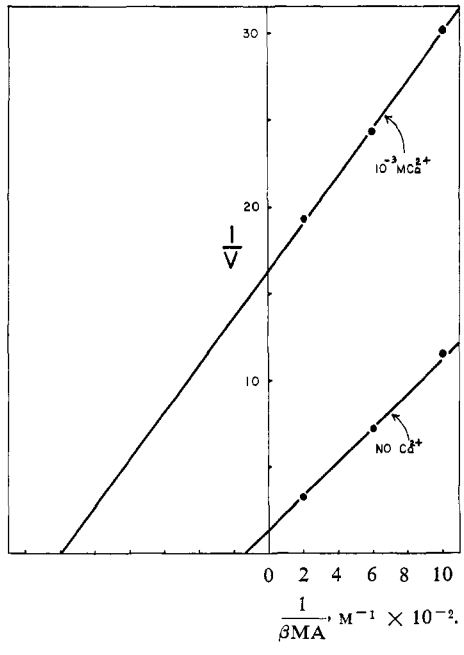


FIGURE 9: Effect of Ca^{2+} on β-methylaspartate kinetics. Measurements carried out at 25° and pH 5.1 with 5×10^{-3} M Mg^{2+} and ionic strength 0.135. For other details, see Experimental Section.

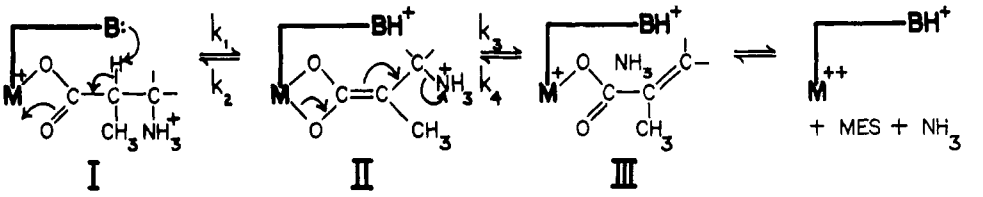
The dissociation constants K_i', $K_{s(I)}'$, and K_i are readily obtained from double-reciprocal plots of initial velocity *vs.* substrate concentration, at constant inhibitor and activator concentrations. The ordinate intercept for such a plot will be

$$\frac{1}{v} = \frac{1}{V_{\infty S,\infty M^{2+}}}\left[1 + \frac{K_a'}{[M^{2+}]} + \frac{[I^{2+}]K_a'}{[M^{2+}]K_i'}\right] \quad (6)$$

while the abscissa intercept is

$$\frac{1}{[S]} = \frac{1 + \frac{K_a'}{[M^{2+}]} + \frac{[I^{2+}]K_a'}{[M^{2+}]K_i'}}{K_s' + \frac{K_a'K_s}{[M^{2+}]} + \frac{[I^{2+}]K_sK_a'}{[M^{2+}]K_i}} \quad (7)$$

In practice (see Figure 9 for the case of Ca^{2+}), we measured the dependence of initial velocity on substrate concentration, in the presence of constant concentrations of M^{2+} and I^{2+} and then repeated these measurements in the absence of I^{2+}. When plotted in double-reciprocal form, the ordinate intercepts of the plot obtained in the absence of I^{2+} could be inserted into eq 3, thus obtaining $V_{\infty S,\infty M^{2+}}$ for the particular sample of enzyme being used. The values of K_i, K_i', and $K_{s(I)}'$ could then be determined from eq 5–7, since K_a, K_s, K_a', and K_s' for the activator (Mg^{2+}) had been previously determined. According to our model (Scheme II) the dissociation constants K_i, K_i', and $K_{s(I)}'$ are strictly analogous to the constants K_a, K_a', and K_s', respectively. The constants K_i, K_i', and $K_{s(I)}'$ obtained for Ca^{2+} and Sr^{2+} have, therefore, been included in Figure 5. There is no obvious discontinuity between the binding behavior of divalent activators and divalent inhibitors of the enzyme, but merely an increase in K_i and K_i' as the ionic radius increases. Similarly, the $K_{s(I)}'$ values for Ca^{2+} and Sr^{2+} seem to follow the pattern of K_s' values determined for the activators. It is of interest that the substrate is held as strongly in the ternary and quaternary complexes by the two inhibitors as it is by any of the activators.



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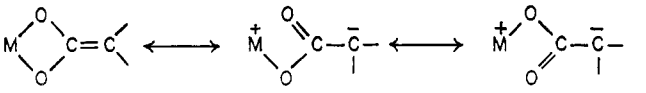

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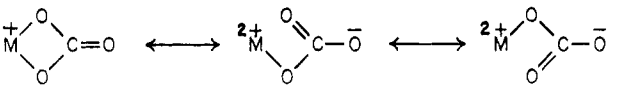

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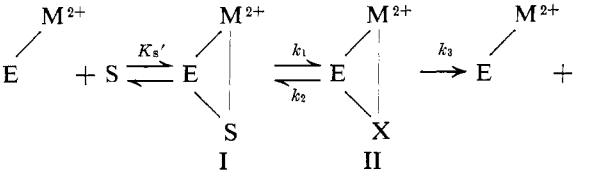
Discussion

The β-methylaspartase reaction is unusually favorable for studies of divalent metal activation because it is possible to choose experimental conditions (*i.e.*, low pH, low substrate, and metal concentrations) under which even the most electronegative metals, such as Ni^{2+}, remain uncomplexed by substrate (or buffer). Even with Ni^{2+}, it is estimated, using the stability constant for aspartate (Sillen and Martell, 1964) that not more than 10% of the added substrate or metal ion is ever present as the metal–substrate complex. In this respect, the reaction might be contrasted to phosphotransferase reactions, for example, in which the stability constants and kinetic behavior of the numerous metal–substrate complexes are often not known with great precision. We believe, therefore, that our experimental data are relatively free from many of the difficulties which often beset investigations of this kind. The discussion will be initially concerned with the interpretation of the kinetic specificity exhibited by β-methylaspartase for the divalent metal ions and will then consider the thermodynamic specificity shown by the enzyme in binding these ions in so far as such specificity is reflected by the dissociation constants which have been assigned in these studies.

The Kinetic Efficiencies of the Divalent Metal Ions. In order to explain the dependence of catalytic efficiency (k_{cat}) on the radius of the metal ion it is necessary to consider the chemical mechanism which we have proposed for the β-methylaspartase reaction. The demonstration of a substrate β-proton exchange reaction, together with studies of the properties of this exchange reaction, led to the postulate that an enzyme-bound β-carbanion intermediate is formed, from which ammonia either ionizes or is displaced in the rate-determining step of the reaction (Bright *et al.*, 1964; Bright, 1964). We have also suggested that a reasonable function for M^{2+} would be to activate (acidify) the β proton of the substrate and facilitate its extraction by a base at the active site of the enzyme (Bright, 1965). The chemical transformations of the quaternary complex I of Scheme I may, therefore, be depicted as eq 8.

The type of coordinated bidentate structure which is proposed for the enzyme-bound carbanion–metal complex II (eq 8) has been demonstrated by X-ray diffraction methods for the nitrate (Cotton and Soderberg, 1963) and carbonate (Barclay and Hoskins, 1962) ligands bonded to Co^{2+} and Co^{3+}, respectively. The similarity between the postulated intermediate (eq 9) and the carbonate complex (eq 10) is particularly close.

For the case of rate-determining dissociation of ammonia from the enzyme–carbanion complex II (eq 8) and considering only initial velocities the reaction in the presence of saturating M^{2+} ($M^{2+} \gg K_a$) and K^+ ($K^+ \gg K_b$) may be written as



mesaconate + NH_4^+ (11)

where X denotes the β-carbanion intermediate and where the substrate β-carboxylate group displaces two water molecules from M^{2+} in EM^{2+} on forming complex I. Allowing the first quaternary complex (I) to be in thermodynamic equilibrium with EM^{2+} and S, and making the steady-state approximation for the second quaternary complex (II), leads to

$$V_{\infty K^+,\infty M^{2+}} = \frac{k_3[E_0]}{1 + \dfrac{K_s'(k_2 + k_3)}{k_1[S]} + \dfrac{k_2 + k_3}{k_1}} \quad (12)$$

When $[S] \gg K_s'$

$$V_{\infty M^{2+},\infty S,\infty K^+} = \frac{k_3[E_0]}{1 + \dfrac{k_2 + k_3}{k_1}} \quad (13)$$

It, therefore, follows that the constant, k_{cat}, of Scheme I, which controls the beakdown of the first quaternary

complex I, would have the following structure

$$k_{cat} = \frac{k_1 k_3}{k_1 + k_2 + k_3} \quad (14)$$

if the mechanism depicted in eq 8 is correct. In order to see how k_{cat} might increase with decreasing ionic radius of M^{2+}, the condition $k_2 > k_3, k_1$ must hold for the divalent metals examined. That k_2 is actually considerably larger than k_3 follows from the fact that the rate of the β-proton exchange reaction can be many times larger than the rate of the over-all reaction (Bright, 1964). No experimental evidence exists to support the assumption that $k_2 > k_1$. However, it would seem perfectly reasonable to suppose that the hypothetical carbanion–enzyme complex (II), existing to some extent with a negative charge on carbon, would have a considerably larger free-energy content than the amino acid–enzyme complex (I). If both of these assumptions are correct

$$k_{cat} \approx \frac{k_1 k_3}{k_2} \quad (15)$$

Since k_1 governs a process involving bond formation between the β-carboxylate oxygen anion and the metal ion, it would be expected that this rate constant would increase as the radius of the activator metal is decreased, provided that the bonding is purely electrostatic in nature. The rate constants k_2 and k_3, on the other hand, both control steps in which an electron pair is instead being withdrawn from the metal ion. Consequently, the magnitudes of k_2 and k_3 would be expected to decrease as the radius of the metal ion decreases. If there is indeed a parallel change in k_3 and k_2 with variation of the metal ion radius, it will be noted from eq 15 that k_{cat} will indeed increase with decreasing ionic radius since k_1 will exert a dominating effect in the expression for k_{cat}.

If, instead of being eliminated from the carbanion, ammonia is in fact displaced from the carbanion by an enzyme nucleophile in the rate-determining step of the reaction (a possibility which has not been excluded experimentally), then the interpretation of the effect of ionic radius on k_{cat} would be more straightforward. Again, k_2 would have to be larger than k_1. However, the rate of nucleophilic displacement of ammonia would probably not be sensitive to the electron-withdrawing power of the divalent metal ion. The increase in k_{cat} with decreasing ionic radius would then simply be due to the increase in the ratio $k_1:k_2$ in eq 15. In other words, the rate of the over-all reaction would be increased owing to an increase in the steady-state concentration of the carbanion which is decomposing in the rate-determining step of the reaction.

It would appear, therefore, that the kinetic specificity which β-methylaspartase displays for divalent metal activators can be interpreted in terms of the carbanion mechanism proposed for this reaction. The inability of large ions such as Ca^{2+} or Sr^{2+}, which are readily bound by the enzyme, to serve as activators is related in this interpretation to the relatively small electrostatic forces these ions can exert on the electrons in the β-carboxylate group. It should be noted in this regard that the β-proton-exchange reaction is inhibited in the presence of Ca^{2+} to approximately the same extent as the over-all reaction (Bright, 1964). Thus neither the over-all reaction nor β-proton exchange can occur in the presence of Ca^{2+}. This is consistent with our electrostatic interpretation of the function of the divalent metal ion.

We have thus far omitted discussion of the kinetic behavior of the transition metal ions, with the exception of Mn^{2+}. The inclusion of Mn^{2+} is justified on the basis that with its five half-filled 3d orbitals it behaves in many respects very much like ions having "closed" electronic structures such as Mg^{2+}. The k_{cat} values determined for Fe^{2+}, Co^{2+}, and Ni^{2+} do not fit the sample pattern shown by the other divalent metal ions (see Figure 6). This is not considered surprising, in view of the importance of covalent bonding and crystal field effects in the chemistry of these ions. The rate of water dissociation from hydrated metal ions, for example, which in general controls the rate of metal–ligand interaction, shows a simple dependence on ionic radius for most of the nontransition metal ions, but shows no such simple dependence in the cases of Fe^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} (Eigen and Kustin, 1963). In this regard it should be noted that there is an approximate and positive correlation between the reciprocal of the rates of water substitution on the divalent ions used in this study and their catalytic efficiencies in the β-methylaspartase reaction. This probably reflects only the fact that ionic radius and crystal field effects (when they occur) play dominant roles in both processes. The rate of water substitution on divalent metal ions is in general far too large to be directly responsible for the kinetic specificities found in divalent metal activation of enzymes. Thus the maximum turnover rate of β-methylaspartase is, like that of many nonoxidative enzymes, around 10^3 sec^{-1}, whereas the rates of ligand substitution on the metals studied here (Eigen and Kustin, 1963) vary from $\sim 10^9$ (Sr^{2+}) to $\sim 10^4$ sec^{-1} (Ni^{2+}).

It was of interest to attempt to demonstrate activation of β-methylaspartase by a trivalent metal ion having an ionic radius within the range spanned by Sr^{2+} and Mg^{2+}, these being the largest and smallest ions, respectively, which have so far been demonstrated to bind to the enzyme. Yttrium (ionic radius 0.93 A) did not activate the enzyme. It was bound by the enzyme, however, as evidenced by the fact that it inhibited the reaction when Mg^{2+} was present. We suggest that the inability of metal ions having the appropriate radius but a very high charge density, such as yttrium, to activate the enzyme, even though they may be bound very securely at the activator site, may be caused by the fact that they stabilize the β-carbanion intermediate to such an extent that k_3 becomes too small for the over-all reaction to be de-

tected under the condition used. Large divalent cations and monovalent cations with small charge density, on the other hand, as discussed above for Ca^{2+}, are probably incapable of activating the β hydrogen of the substrate. The monotonic relationship between k_{cat} ionic radius would be expected, therefore, to hold for only a small range of ionic radius. Whether Be^{2+}, which is by far the smallest of the alkaline earth ions (0.34 A) forms an inert enzyme-bound carbanion intermediate, as suggested for Y^{3+}, is not known. According to Figure 5, the enzyme is unable to bond a metal ion as small as Be^{2+} at the active site. At concentrations up to 5×10^{-3} M, Be^{2+} did not cause an observable production of mesaconate. The anomalous inhibitory properties of Be^{2+} (Figure 7) are perhaps due to strong binding of this ion to the enzyme at sites other than the metal activator site. We wish to emphasize that direct experimental evidence, kinetic or otherwise, is not as yet available for the functions of the divalent metal ions which we have postulated.

The Binding of Divalent Metal Ions by β-Methylaspartase. According to the model that was deduced for divalent metal activation of β-methylaspartase (Scheme I) the metal ion is able to combine both with E and ES. The dissociation constants governing these interactions are K_a and K_a', respectively. We shall briefly discuss possible reasons for the variations in K_a and K_a' which are observed in the presence of different divalent metal activators.

There are two reasons for believing that some, if not all, of the ligands for the metal which are contributed by the amino acid residues of the enzyme at the active site are carboxylate groups. First, since Mg^{2+} in general exhibits a strong preference for oxygenous ligands (Williams, 1959) it is likely that the carboxylate group would be the only ligand which is sufficiently ionized at pH 5.1 to account for the values of K_a and K_a' found with Mg^{2+}. Second, a range of dissociation constants as narrow as we have found with the nine divalent metal ions is only commonly found in small metal ion complexes when the ligands are oxyanions. The metal ion complexes of oxalate and citrate are good examples. In the case of citrate there is only a 100-fold difference between the dissociation constants (for the reaction $L^{3-} + M^{2+} \rightleftharpoons ML^{-}$) of the most stable ($Ni^{2+}$) and the least stable ($Sr^{2+}$) complexes (Sillen and Martell, 1964). The difference in the stability of Ni^{2+} and Sr^{2+} complexes is much greater when nitrogenous ligands are present. The stability constants for the Sr^{2+} and Ni^{2+} complexes of glycine differ for example by a factor of 10^5 (Sillen and Martell, 1964). Arguments of this kind were used by Vallee *et al.* (1961) for the identification of ligands at the metal binding site of carboxypeptidase. Their plots of the observed binary complex equilibrium constants *vs.* the stability constants of possible model complexes afforded evidence for the existence of nitrogenous and sulfurous ligands at the metal binding site. We have made several such plots for the dissociation constants K_a and K_a' and find that least scatter is obtained when no ligands other than oxygen are present in the model complex (*i.e.*, oxalate and citrate complexes). However, it should be noted that the K_a and K_a' values obtained for β-methylaspartase are apparent equilibrium constants only, since their values are much smaller at pH 8.2, for example. Nor do they necessarily describe the equilibria of elementary steps in the reaction.

The finding that the values of K_a and K_a' are invariant with temperature (at least in the case of Mn^{2+}) suggests that the driving force for complex formation is the release of water from the aquated metal cations. It should be emphasized, however, that this interpretation is not unequivocal owing again to the fact that the measured K_a and K_a' values are apparent equilibrium constants. Our K_a, for example, presumably had the form (Sillen and Martell, 1964)

$$K_a = \frac{[M^{2+}][H_xL^{z-}]}{[MH_yL^{2-z-x+y}][H^+]^{x-y}} \qquad (16)$$

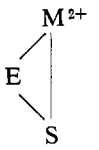
It is, therefore, possible that a temperature-dependent ionization of the ligand could compensate for a temperature-dependent complexing step and thus give rise to the observed lack of over-all temperature dependence of K_a and K_a'. However, such an exact compensation seems unlikely and furthermore, the heat of ionization of carboxylate groups is typically very small (Sillen and Martell, 1964).

If entropy is the main driving force for metal complex formation on the enzyme then the variation of K_a and K_a' with the different types of divalent metal ions might be very simply accounted for. The increase in K_a and K_a' values observed with ions having radii greater than 0.97 A (Cd^{2+}) would be due to the fact that water of hydration becomes less ordered as the ionic radius increases and consequently gives rise to a smaller entropy change upon displacement by enzyme and substrate ligands. The complexes of oxalate owe their stability in large part to the release of bound water upon complex formation. The Co^{2+}–, Mn^{2+}–, and Ni^{2+}–oxalate complexes for example form with very small values of ΔH° but with ΔS° values of about +24 cal deg^{-1} (Sillen and Martell, 1964). In this respect, therefore, oxalate is a model for β-methylaspartase. We explain the increase in K_a and K_a' as the ions become smaller than 0.82 A (Co^{2+}) by invoking the mutual repulsion of oxyanion (carboxylate) ligands around the smaller ions. Thus Ca^{2+}, although larger than Mg^{2+}, often forms the more stable complexes with polydentate ligands (Williams, 1959). It should also be pointed out that the parallelism which is observed between K_a and K_a' suggests that the ligands contributed by the enzyme in the ternary complex are chemically similar to the ligand contributed by the substrate in the ternary complex. Since indirect evidence suggests that it is the β-carboxylate group of the substrate which bonds to the metal in the ternary complex, we can infer that the enzyme also contributes carboxylate groups for metal bonding.

Although the foregoing explanation can account for the observed trends in the stabilities of the com-

plexes formed by β-methylaspartase with many of the divalent metal ions studied, the relative stabilities of the complexes involving Mn^{2+}, Co^{2+}, and Ni^{2+}, for example, are somewhat unusual and require comment. The usual order of stability (Williams, 1959) is $Mn^{2+} < Co^{2+} < Ni^{2+}$, but exceptions are known. Co^{2+}–pyrophosphate and Co^{2+}–tripolyphosphate, for example, are more stable then the corresponding Ni^{2+} complexes (Hammes and Morrell, 1964). In addition, Mn^{2+}–ATP is more stable than Co^{2+}–ATP (Khan and Martell, 1962) and these investigators reported that the Ni^{2+}–ATP and Co^{2+}–ATP complexes are of equal stability. Hammes and Morrell (1964) have suggested that highly charged ligands can form more stable complexes with Co^{2+} than with Ni^{2+} because they can accelerate the rate of dissociation of water molecules from the inner hydration shell of Co^{2+}. Moreover, when a metal ion interacts with a site on a protein molecule, it is possible that the steric relationships of the ligands comprising the site play a role in determining the stereochemistry and stability of the complex. Thus Ni^{2+}, for example, which rarely forms tetrahedral complexes (Orgel, 1960) may be forced to form a relatively weak tetrahedral complex with β-methylaspartase because the energy necessary to distort tetrahedrally arranged ligands into a configuration more suitable for bonding with Ni^{2+} is greater than the energy to be gained from the formation of such a complex.

Experimental evidence for the nature of the ligands bonded to the metal in EM^{2+} and



could be obtained from studies of the pH dependence of the apparent dissociation constants. Although we have made several attempts to carry out such studies our efforts have been thwarted by the problem of divalent metal contamination. Experiments with Mg^{2+} at pH 8.2 illustrate the difficulties involved. In the absence of EDTA and with 4×10^{-4} M substrate a K_m value for Mg^{2+} activation of 10^{-4} M was obtained. In the presence of 2×10^{-5} M EDTA, the smallest concentration which is sufficient to abolish all activity in the absence of added Mg^{2+} the K_m of Mg^{2+} could not be accurately determined but was of the order of 10^{-5} M. We have not yet been able to identify the source of the metal contamination. The substantial affinity of the enzyme for Mg^{2+}, which is the least well bound of all the activators, suggests that the actual amount of contaminating metal could be very small.

Experimental Section

Materials. β-Methylaspartase was prepared from *C. tetanomorphum* as described previously (Bright, 1965). The specific activity of the enzyme (Barker *et al.*, 1959) was 150 units/mg initially, but had diminished to about 100 units/mg toward the end of these studies. Protein was determined by the method of Lowry *et al.* (1951). *threo*-β-Methyl-L-aspartate was prepared enzymically by the method of Barker and Smyth (1960) and titrated with KOH or Tris base to the appropriate pH before use. Except for Be^{2+}, where the nitrate was used, all divalent metal ions were used as their chlorides. The concentrations of stock solutions of the metal ion salts were determined by atomic absorption measurements, using standard solutions obtained from Hartman–Leddon Co., Philadelphia, Pa., and by chemical analysis performed by Crobaugh Laboratories, Cleveland, Ohio.

Rate Measurements. Rates of mesaconate formation were measured at 230 mμ in a Beckman DB recording spectrophotometer, the cell compartment of which was maintained at the desired temperature by circulating water from a constant-temperature bath. Quartz cells of 4-cm path length were used, in which the total liquid volume was 4 ml. Beer's law was obeyed up to a total absorbance of 2, with a slit width of 1.0 mm. A typical rate measurement was carried out as follows. Enzyme (0.1 ml of a stock solution in 1.0 M KCl, containing 2×10^{-6} M EDTA and about 1.5 mg of protein/ml), potassium *threo*-β-methyl-L-aspartate (pH 5.1), potassium acetate (pH 5.1), KCl, and EDTA were incubated in a total volume of between 3.8 and 3.95 ml in the spectrophotometer for 3 min. The reaction was then begun by adding the desired volume of the divalent metal ion salt solution and mixing immediately. The total volume of the mixture was 4.0 ml and the final concentration of potassium acetate and EDTA were 0.01 and 2×10^{-6} M, respectively. The final concentration of KCl was 0.125 M. Total ionic strength, including the potassium acetate buffer, was therefore 0.135. Under these conditions, no mesaconate was formed in the absence of added divalent metal ion. Furthermore, the initial rates of mesaconate formation were constant for several minutes after the addition of the divalent metal. In order to adjust for the fact that the specific activity of the enzyme declined during the course of these studies, a standard assay was run with each set of rate measurements. All velocities reported here are initial velocities and are expressed as the change in absorbance per minute.

In the studies of the effect of K^+ on the reaction kinetics (Figures 1 and 2) several modifications of the typical rate measurement procedure described above were required as follows. The stock enzyme solution was in 1.0 M tetramethylammonium chloride and the Tris salt of β-methylaspartate was used. In addition, owing to the large K_m values of K^+, total ionic strength was maintained at 0.3, rather than 0.135, by the addition of tetramethylammonium chloride as required. The measurements were carried out at pH 5.9 with 0.01 M Tris-acetate buffer. Otherwise, the data of Figures 1 and 2 were obtained in the manner described in the previous paragraph.

Acknowledgments

The author wishes to thank Dr. Berton C. Pressman for the atomic absorption measurements and Miss Roberta Catlett for excellent technical assistance.

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Cobamides and Ribonucleotide Reduction. IV. Stereochemistry of Hydrogen Transfer to the Deoxyribonucleotide*

T. J. Batterham, R. K. Ghambeer,† R. L. Blakley, and Carol Brownson

ABSTRACT: Determination of coupling constants from the proton magnetic resonance (pmr) spectrum of deoxyadenosine in deuterated dimethyl sulfoxide has permitted calculation of the approximate dihedral angles for vicinal protons in the deoxyribose moiety. The agreement between these and the angles calculated for the C-2′ *endo* conformation of the deoxyribose moiety indicate that deoxyadenosine in dimethyl sulfoxide solution has considerable conformational purity and that the preferred conformation is the C-2′ *endo* form. The chemical shifts of the $2'_\alpha$ and $2'_\beta$ protons are sufficiently different for these protons to give separate octets, the downfield octet owing to the $2'_\beta$ proton. Deuterated deoxyadenosine was derived from the deoxyadenosine triphosphate formed by the action of ribonucleotide reductase of *Lactobacillus leichmannii* on adenosine triphosphate (ATP) in deuterium oxide. In the pmr spectrum of the deuterated deoxyadenosine the H-$2'_\alpha$ octet was absent and the H-1′, H-$2'_\beta$, and H-3′ peaks showed simplification consistent with deuteration predominantly, if not exclusively, in the $2'_\alpha$ position. It is concluded that in the reductase reaction the 2′-hydroxyl group of ATP is replaced by a hydrogen with retention of the configuration at C-2′.

In studies on the deoxyadenosylcobalamin-dependent reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleoside triphosphates by ribonucleotide reductase of *Lactobacillus leichmannii*, proton magnetic resonance (pmr) has been used to show that when ATP[1] is enzymically reduced in deuterium oxide, a deuterium atom is incorporated into the deoxyribose moiety of the dATP product at the 2′ position (Blakley *et al.*, 1966). We have now obtained an unequivocal analysis of peak patterns from the deoxyribose protons of unlabeled deoxyadenosine

* From the Departments of Medical Chemistry and Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia. *Received November 21, 1966.*

† Australian National University Research Scholar.

[1] Abbreviations used: ATP, adenosine triphosphate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; CoA, coenzyme A.