consistent with the simulated changes of other climatic variables. These results are generally supported by independent investigations with simpler models.

In spite of this qualified success, further analysis of both simulated and verification data is needed to establish the details of ice-age climate, especially the precipitation regimes, and to document the role of eddy fluxes in maintaining the heat, momentum, and moisture balances of the ice-age general circulation. New paleoclimatic data bases for both July and January of 18,000 B.P. are being assembled by CLIMAP and will be used in new simulations of the seasonal ice-age climate.

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Metal Ions in Enzymes Using Ammonia or Amides

A simple generalization provides fresh insights and makes specific predictions about some enzymes.

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The discovery that highly purified urease (E.C. 3.5.1.5) from jack beans (*Canavalia ensiformis*) contained stoichiometric amounts of nickel (*1*) developed out of two observations: (i) inhibition by two inhibitors of markedly disparate structure (acetohydroxamic acid and phosphoramidate) was slowly achieved and reversed (*2, 3*); and (ii) the enzyme showed a weak tail absorption extending from ~ 320 nm to a shoulder at ~ 425 nm, together with broad, weak maxima at 725 and 1060 nm (*1*).

In attempting to understand the role of nickel in the mechanism of action of urease, we have turned to other enzymes whose biological reactions involve ammonia as substrate, product, or as part of a transfer reaction. Many of these enzymes display apparently non-simple inhibition and activation effects, and, where the near-ultraviolet spectra are available, they are reminiscent of that of jack bean urease. In this article, we summarize evidence which suggests that various enzymes involved in reactions of ammonia may be partially understood in terms of two speculative postulates. (i) In every case there is a transition metal ion (or possibly an alkaline earth metal ion) more or less tightly bound to the enzyme; and (ii) the frequently postulated "enzyme-ammonia" intermediate in reactions catalyzed by these enzymes involves ammonia complexed to the bound metal ion.

An initial stumbling block in our thinking was the classical notion that the lack of an effect of ethylenediaminetetraacetate (EDTA) on an enzyme is substantial evidence for the absence of an essential metal ion. However, jack bean urease, with tightly bound active-site nickel ion (*1*), is at least the third example [together with yeast alcohol dehydrogenase (E.C. 1.1.1.1) and zinc ion (*4*), and chicken liver pyruvate carboxylase (E.C. 6.4.1.1) and manganous ion (*5*)] of an enzyme from which an active-site metal ion has thus far resisted all attempts at reversible removal (for example, by exhaustive dialysis in the presence of chelating agents or by exchange with a radioactive metal ion). Each of these enzymes is stable in the presence of EDTA at neutral *p*H, and in the case of urease, the enzyme is fully active in the presence of 0.5 m*M* EDTA at neutral *p*H (*6*). These three examples establish firmly the proposition (*4*) that the absence of an effect of EDTA on an enzyme is not a sufficient basis on which to exclude the possibility of a tightly bound active-site transition metal ion. Fur-

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ther, there is at least one example of an alkaline earth ion [Mg^{2+}, in calf liver pyruvate carboxylase (*7*)] which survives the presence of EDTA throughout the purification procedure.

We now turn to a consideration of individual enzymes and the available evidence supporting our two postulates.

Amino Acid Ammonia-Lyases

The conversion of L-histidine into urocanate and ammonia, catalyzed by bacterial histidine ammonia-lyase (E.C. 4.3.1.3), was postulated by Peterkofsky (*8*) to involve an "amino-enzyme" intermediate which could be formed reversibly in the deamination of L-histidine but not readily from ammonia. The essential observation was that [^{14}C]urocanate and a proton from [^{3}H]H_2O, but not nitrogen from [^{15}N]NH_3, became incorporated into residual L-histidine after partial enzymatic deamination (*8*). A similar observation has been made with L-phenylalanine and [^{14}C]cinnamate in the case of phenylalanine ammonia-lyase (E.C. 4.3.1.5) from potato tubers (*9, 10*). Both of these enzymes are irreversibly inhibited by borohydride ion and by nitromethane, and analysis of the inhibited enzymes (using radioactive inhibitors) has led to the suggestion of a dehydroalanine moiety at the active site (*9, 11, 12*). Hanson and Havir suggested a mechanism applicable to both these enzymes, in which the α-amino group of the substrate forms a covalent adduct with the putative dehydroalanine moiety (*9, 11*). However, there appears to be absolutely no direct evidence (such as trapping) demanding such an adduct (*9, 11*), and an alternative proposition is given below.

We have noted (*1*) that the published ultraviolet and visible absorption spectra of phenylalanine ammonia-lyase from potato tubers and maize (*13*) and *Rhodotorula glutinis* (*14*), that of histidine ammonia-lyase from *Pseudomonas* (*15, 16*), and that of vitamin B_6–independent threonine dehydratase from *Pseudomonas putida* (*17*) are all consistent with as yet unreported tightly bound transition metal ions in the highly purified enzymes. The key feature in each spectrum (with the enzyme at a concentration of $\sim$ 1 mg/ml) is an evident tail or shoulder (or both) in the region 310 to 340 nm. This tail is reminiscent of that associated with a tightly bound metal ion in jack bean urease (*1*). A tightly bound transition metal ion in the amino acid ammonia-lyases could serve as a binding site for the α-amino group of the substrate amino acid. Further, the coordinated metal ion, acting as a Lewis acid, could provide impetus for the elimination reaction, producing thereby a relatively stable complex between ammonia and the metal ion—the "amino-enzyme"—together with a readily dissociated *trans* α,β-unsaturated carboxylate product.

Certain properties of this postulated transition metal ion in phenylalanine ammonia-lyase are defined by observations made on the highly purified enzymes from potato tubers and from a fungus (*Ustilago hordei*): (i) EDTA in the assay system is not inhibitory (*10, 18*); (ii) added Mn^{2+} and Mg^{2+} ions do not stimulate activity in the assay system (*10, 18*); and (iii) maximal activity (*10, 18, 19*) is obtained without exposure to sulfhydryl compounds.

Many heretofore confusing observations on histidine ammonia-lyase may be understood in terms of a role for two different metal ions.

1) A tightly bound transition metal ion, which is inaccessible to EDTA (analogous to the one described above) provides a catalytic activity (that is, turnover number, k_{cat}) with histidine comparable to that of phenylalanine ammonia-lyase acting on phenylalanine.

2) A loosely bound divalent metal ion which coordinates with the enzyme and the imidazole ring of the substrate histidine provides an enzymatic activity which is five- to tenfold greater than in its absence. The binding site for this second metal ion (and hence activation by metal ions) is dependent on suitable treatment of the enzyme with sulfhydryl compounds.

Granted the presence of a tightly bound metal ion in histidine ammonia-lyase, evidence in support of a role for a second metal ion in the action of this enzyme is as follows:

1) Histidine ammonia-lyase is stable to exhaustive dialysis against EDTA (*12*). The enzyme prepared in the absence of added Mn^{2+} ion is maximally active (in nonchelating buffers and after suitable treatment with sulfhydryl compounds) when Mn^{2+} or Cd^{2+} is present, although the presence of one of a number of other divalent metal ions (Mg^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) provides substantial activation (*12, 20*).

2) The enzyme which has not been treated with a sulfhydryl compound has a specific activity about 20 percent of that of the maximally active, sulfhydryl-treated enzyme (*20*). This activity is essentially independent of added Mn^{2+} or Cd^{2+} (*20*).

3) In the absence of added Mn^{2+} or in the presence of EDTA, the sulfhydryl-treated enzyme has essentially the same activity as the untreated enzyme (*15, 20*).

4) After treatment with dithiothreitol and subsequent dialysis, one sulfhydryl group per subunit becomes reactive to 5,5′-dithiobis(2-nitrobenzoic acid), apparently with the formation of a stable mixed disulfide. After reaction with this compound, the enzyme retains in the presence of Mn^{2+} ion about 20 percent of its maximal activity (*20*). Maximal activity is restored by reduction with sulfhydryl compounds (*20, 21*).

5) Michaelis-Menten kinetics are obeyed by the sulfhydryl-treated enzyme in the presence of EDTA and by the untreated enzyme in the absence or presence of EDTA. The maximum velocity is identical in all three cases, as is the Michaelis constant (K_m) for L-histidine (*20*).

6) The sulfhydryl-treated enzyme obeys Michaelis-Menten kinetics in the presence of a sufficiently high concentration of metal ion (apparently $\sim$ 0.1 m*M* Mn^{2+}). The Michaelis constant for L-histidine is one half that, and the maximum velocity is ten times that of the sulfhydryl-treated enzyme in the presence of EDTA.

7) The product urocanate binds to the enzyme (in the presence of histidine and 0.1 m*M* Mn^{2+} ion) with an apparent dissociation constant of 10 m*M* (*12*). Proton magnetic resonance studies (details of which are as yet unpublished) of this interaction are interpreted in terms of coordination of the imidazole ring of urocanate with enzyme-bound Mn^{2+} ion (*22*).

8) The maximal turnover number (k_{cat}) per active site of histidine ammonia-lyase is about 60 times greater than that of phenylalanine ammonia-lyase (*9*). Because of the tenfold activation by the second metal ion in the case of the histidine enzyme, the turnover numbers associated with the postulated tightly bound transition metal ions are within an order of magnitude of each other for the two enzymes.

All these facts are consistent with the proposition that the activity of histidine ammonia-lyase is dependent on a tightly bound metal ion, in addition to the more loosely bound one which produces further activation of the enzyme. An essential metal ion was proposed by Peterkofsky and Mehler to account for reversible inhibition by cysteine and EDTA (*23*). More recently, Klee considered this possibility in order to account for the ultraviolet absorption shoulder displayed by histidine ammonia-lyase at $\sim$ 315 nm in the presence of β-mercaptoethanol (*16, 24*), but later reported (without giving detailed results) that, after treatment with dithiothreitol followed by dialysis, the fully active enzyme "did not contain significant amounts of copper or any other metal ions in amounts greater than those found in the dialysate" (*20*). Nonetheless, the unexplained absorption of ultraviolet light by

this enzyme near 315 nm (*15, 16*) could be associated with a tightly bound transition metal such as iron, which is stated (*25*) to be "tightly bound" in "metal-depleted enzyme preparations" of histidine ammonia-lyase.

This proposed catalytic role for a tightly bound transition metal ion in these amino acid ammonia-lyases is not inconsistent with the presence of the postulated dehydroalanine residue (*9, 11, 12*). Although the incorporation into these enzymes of radioactivity from various labeled inhibitors has been interpreted in terms of propositions related to dehydroalanine (*9, 11, 12, 26*), the incorporation data are not entirely reproducible, nor is the stoichiometry of incorporation simple. The detailed understanding of all these studies therefore may lie in a hitherto unappreciated role of a tightly bound transition metal ion (*27*). Furthermore, this argument, developed in detail above for the specific cases of the histidine and phenylalanine ammonia-lyases, may be of general significance in enzymatic eliminations of ammonia.

Glutamine and Ammonia Utilization

In Table 1 are listed 13 enzymes, most of which have been highly purified and all of which are concerned with the utilization of ammonia and the amide nitrogen of glutamine in anabolic reactions (*28, 29*). Number 1 has been independently suggested to transfer the amide nitrogen directly from glutamine to the carbon to which it is attached in the final product or, in common with suggestions concerning numbers 2, 3, 7, 8, and 10, to transfer the amide nitrogen

Table 1. Some enzymes which utilize either ammonia or L-glutamine in anabolic reactions, and which are covalently inhibited by certain analogs of L-glutamine. The molecular weights given are approximate. Abbreviations: CTP, cytidine triphosphate; NAD^+, nicotinamide adenine dinucleotide.

Enzyme	Source	Isolated enzymatic species			Properties after covalent reaction with analog of L-glutamine*‡				Binding of L-glutamine to native enzyme (mole/mole)	References
					Substrate					
		Molecular weight*	Subunit structure*†	Glutaminase activity*	Ammonia	L-Glutamine	Cysteine blocked	Bound analog (mole/mole)		
1. Amidophosphoribosyl transferase (E.C. 2.4.2.14)	Chicken liver	210,000			(+)	–	(+)	1		(*31*)
2. Amidophosphoribosyl transferase (E.C. 2.4.2.14)	Pigeon liver	200,000	α_4							(*32*)
3. CTP synthetase (E.C. 6.3.4.2)	*Escherichia coli*	105,000	α_2	(+)	+	–	+	1	1.8	(*89*)
4. Asparagine synthetase (E.C. 6.3.1.1)	Mouse leukemia cells	105,000		+	+	–				(*83*)
5. Xanthosine-5′-phosphate aminase (E.C. 6.3.4.1)	*E. coli* (B-96)	126,000	α_2		+	(–)				(*90*)
6. NAD^+ synthetase (E.C. 6.3.5.1)	*Saccharomyces cerevisiae*	630,000			+	–				(*91*)
7. Phosphoribosylformylglycineamidine synthetase (E.C. 6.3.5.3)	Chicken liver	133,000	α	+	+	–	+	1	1.0	(*34, 92*)
8. Carbamoyl phosphate synthase (E.C. 2.7.2.9)	*E. coli*	170,000 130,000	H_1L_1§ H	+ –	+ +‖	– –‖	+	~1 ~0¶	1	(*93*)
9. Anthranilate synthase (E.C. 4.1.3.27)	*E. coli***	(260,000) 60,000 (70,000)	(I_2II_2)†† I II	(+)	(+)†† +‖	(–)†† –‖				(*35–37, 94*)
10. Anthranilate synthase (E.C. 4.1.3.27)	*Salmonella*** *typhimurium*	280,000 64,000 63,000	I_2II_2§ I II	+ – –	(+) +‖	– –‖	+	~2		(*95*)
11. Anthranilate synthase (E.C. 4.1.3.27)	*Bacillus subtilis*	(96,000) 67,000 19,000	E_1X_1 E X		+ +‖	– (–)‖	+			(*96*)
12. Anthranilate synthase (E.C. 4.1.3.27)	*Pseudomonas putida*	≠75,000 63,000 18,000	I_1II_1§ I II	(+)	+ +‖	– –‖	(+)			(*97*)
13. Anthranilate synthase (E.C. 4.1.3.27)	*Serratia marcescens*	141,000 60,000 21,000	I_2II_2§ I II	(+)	+	–	+	2.2 ~1		(*98*)

*Parentheses are used where doubt exists or proof is not rigorous. The symbols + and – indicate that the enzyme species does or does not (respectively) possess this property. †The notation used for individual subunits is that used in the original articles, except that, where no such notation has been previously ventured, α denotes the subunit. ‡Covalent inhibitors that have been used in these studies are 6-diazo-5-oxo-L-norleucine (enzyme numbers 1, 3, 5, 9, 10, 11, 12, and 13), L-2-amino-4-oxo-5-chloropentanoic acid (numbers 4 and 8), *O*-diazoacetyl-L-serine (L-azaserine, numbers 6 and 7), and L-2-amino-3-ureidopropionic acid (L-albizziin, number 7). §This subunit contains the site of alkylation or carbamoylation by the analog of L-glutamine. ‖This activity pattern refers only to subunits as isolated from uninhibited enzyme. ¶This entry refers to the subunit isolated *after* the ability of the aggregate to utilize L-glutamine was blocked by reaction with the alkylating agent. **The anthranilate synthases of *E. coli* and *S. typhimurium* are aggregates of two enzymes anthranilate synthase (E.C. 4.1.3.27, subunit I) and anthranilate phosphoribosyltransferase (E.C. 2.4.2.18, subunit II). On treatment with trypsin, subunit II of the *S. typhimurium* aggregate is cleaved to a species (molecular weight of 15,000 to 18,000) that does not have phosphoribosyltransferase activity, but still contains a functional glutamine site, and that interacts with subunit I to reconstitute glutamine-dependent anthranilate synthase activity. This reconstituted aggregate is thus similar to the native anthranilate synthases of *B. subtilis, P. putida*, and *Serratia marcescens*. ††The *E. coli* enzyme is generally assumed to have a subunit structure similar to that of *S. typhimurium* (enzyme number 10). However, the results of studies of its reaction with 6-diazo-5-oxo-L-norleucine are inconclusive (*35, 37*).

first to an ammonia site on the enzyme, and thence to form the product. Despite the different molecular weights, metabolic functions, and sources of these 13 enzymes, there are several major similarities among most of them.

1) Either L-glutamine or ammonia may be the source of nitrogen for the anabolic reaction.

2) In the absence of the complete substrate system, at least six (numbers 3, 4, 7, 8, 10, and 12) of these enzymes act as L-glutamine hydrolases. Many (numbers 1, 6, 7, 8, 9, 10, and 13) have γ-glutamyl transferase activity under appropriate conditions (*30*).

3) For every enzyme in Table 1, the transfer of nitrogen from glutamine (and in each tested case, the glutamine hydrolase or γ-glutamyl transferase activity as well), but not the related ammonia reaction, is prevented by covalent reaction of the enzyme with a structural analog of L-glutamine. In each case, the analog has a potential alkylating or carbamoylating group corresponding roughly in position to the γ-carbonyl of L-glutamine. In several cases, the site of reaction on the enzyme has been established or inferred to be a cysteine residue.

4) Carbamoyl phosphate synthase (number 8) and the anthranilate synthases (numbers 9 through 13) contain subunits of two types, of which one contains the site of alkylation by the L-glutamine analog and the other is capable (in isolation or in the covalently inhibited "native" aggregate) of utilizing ammonia in the anabolic reaction.

5) In the absence of other substrates, several of the enzymes (numbers 3, 7, and 8) form a stoichiometric complex with L-glutamine which is sufficiently stable to be isolated by rapid gel filtration.

It is generally supposed that transfer of the nitrogen from glutamine is associated with formation of a transient γ-glutamyl enzyme on that cysteine residue which is susceptible to the various analogs of L-glutamine. For nearly every enzyme in Table 1, the catalytic efficiency (that is, turnover number, k_{cat}) with ammonia as substrate is within an order of magnitude of that for L-glutamine as substrate. This fact would be explained if each of the enzymes had an ammonia site that could either bind ammonia from solution or receive the amide nitrogen from glutamine as the γ-glutamyl enzyme was formed. Because the postulated ammonia sites have proven refractory of definition, and because of the long-overlooked presence of nickel at the active site of jack bean urease (*1*), it occurred to us that a further, previously unsuspected, common factor among the enzymes in Table 1 might be a transition metal ion acting as a binding site for ammonia. Evidence in support of the common occurrence of such a transition metal ion is as follows.

1) Highly purified amidophosphoribosyl transferase from chicken liver (*31*) and pigeon liver (*32*) is a brown metalloenzyme, and the enzyme from the latter source contains 2.2 to 2.4 gram-atoms of iron per mole of subunit (*32, 33*).

2) Highly purified liver phosphoribosylformylglycineamidine synthetase has an ultraviolet spectrum (*34*) whose absorbance in the region 320 to 400 nm, by comparison with that of jack bean urease (*1*), is suggestive of an as yet unreported transition metal ion.

3) Anthranilate synthase (*Escherichia coli*) has two types of subunit (*35*). Purified subunit I by itself catalyzes the reaction of chorismate with ammonia to produce anthranilate and pyruvate (*36*). However, subunit I does not use glutamine for this purpose except in the presence of subunit II (*35*). After treatment with EDTA, the enzymatic activity of subunit I is as fully restored by Co^{2+} at $10^{-4}M$ as it is by Mg^{2+} at $10^{-2}M$ (*35*). Further, the optimal effect of Fe^{2+} ion (at $10^{-4}M$) is more than half as great as that of Co^{2+} ion (*35*). The ultraviolet spectrum of the highly purified subunit I (apparently never exposed to EDTA) from *E. coli* (*36*) is not inconsistent with the presence of a transition metal ion (*37*).

4) The presence of a metal ion in *E. coli* xanthosine-5′-phosphate aminase was suggested in order to account for the apparently reversible inhibition of the enzyme by *o*-phenanthroline (*38*).

The possibility that the role of iron in amidophosphoribosyltransferase includes the complexing of ammonia appears to have been discarded on the grounds that "related metal ions have not been detected in other enzymes" involved in the utilization of the amide nitrogen of glutamine (*39*). The similarities summarized in Table 1, together with evidence for transition metal ions in four of the eight different types of enzymes in Table 1, suggests that more or less tightly bound metal ions will be a common factor in the active-site chemistry of all of these enzymes.

A logical role for such a transition metal ion in these enzymes would be as an ammonia binding site. Buckingham, Sargeson, and co-workers have established that ammonia (*40*) and hydroxide ion (*41*), complexed with Co(III), are efficient nucleophiles. Their work provides a reasonable model on which to propose that all the enzymes in Table 1 might use analogous chemistry in the transfer of ammonia from the metal ion binding site to the carbon to which it is attached in the final product. Further, the simplest way for the amide nitrogen of glutamine to be transferred to the binding site for ammonia would be for the metal ion to act as a Lewis acid in complexing the amide nitrogen, so as to promote nucleophilic attack by the active-site cysteine residue on the γ-carbonyl of glutamine (*42, 43*).

Carboxypeptidase A

The role of enzyme-bound zinc ion in the hydrolysis of carboxamides catalyzed by bovine carboxypeptidase A (E.C. 3.4.12.2) has been discussed in terms of two general possibilities, each of which has found support in unambiguous mechanisms established for Co(III)-promoted hydrolysis of carboxamides in model studies (*41*).

1) The carbonyl oxygen of the carboxamide moiety to be cleaved could coordinate with the active-site zinc ion, thereby rendering the carbonyl carbon more susceptible to nucleophilic attack (*44*).

2) A hydroxide ion coordinated with the enzyme-bound zinc ion could act as a nucleophile toward the carbonyl carbon of the carboxamide moiety to be cleaved (*44*).

A third possibility arises from consideration of the (H_3O^+)-catalyzed hydrolysis of carboxamides, the mechanism of which is unknown (*45*). While it has been firmly established that the carbonyl oxygen is the predominant site of protonation of carboxamides in strongly acidic solutions (*45, 46*), there has been no proof that protonation on oxygen is mechanistically significant in the hydrolysis reaction (*45*). The lifetime of the *N*-protonated form of a carboxamide $[RC(O)NH_3^+]$ has been compared with the rate of rotation about the C-N bond (*47*), and the logical possibility has been raised that the acid-catalyzed hydrolysis of amides may occur "*via* the minor *N*-protonated form" (*45*). The zinc ion (a Lewis acid) in carboxypeptidase A could play a similar role in the enzymatic hydrolysis of carboxamides (*48, 49*).

A detailed set of proposals for the mechanism of action of carboxypeptidase A, based on the first general possibility above, has been made by Ludwig and Lipscomb (*50*). Partial support for the proposals was drawn from x-ray crystallographic studies which demonstrated that the carbonyl oxygen of the poor substrates, Gly-L-Tyr and Phe-Gly-Phe-Gly, was coordinated with the active-site zinc ion (*50*). The question of the mechanistic significance of such a complex is not different in principle from the as yet unresolved question of the mechanistic significance of *N*- or *O*-protonation in the (H_3O^+)-catalyzed hydrolysis of carboxamides (*45*). Many oligopeptides form chelates with transition metal ions through a carbonyl oxygen (*49*), and it is entirely possible that Gly-L-Tyr and Phe-Gly-Phe-

Gly are poor substrates for carboxypeptidase A because coordination with the active site zinc ion is through a carbonyl oxygen (*51*).

The chemical principles and questions discussed above regarding the role of the active-site zinc ion in the mechanism of action of carboxypeptidase A are, in general, applicable to all metallopeptidases.

"Superacid" Catalysis by Metal Ions

It was suggested in 1968 by Vallee and Williams that metalloenzymes were efficient catalysts by virtue of strain associated with an unusual metal ion coordination geometry in the enzyme (*52*). They based this proposition in part on spectral differences observed in a comparison of copper and nonheme-iron enzymes with simpler complexes of the same metals (*52*). We believe that it is unnecessary to assign special strain to active-site metal ions in general, and that a better working hypothesis is the earlier proposition (*53*) that "metal-ion catalysis can perhaps be described as superacid catalysis in neutral solution." Some of our reasons for taking the latter view are as follows.

1) The rates of enzyme-catalyzed reactions are many orders of magnitude greater than those of corresponding nonenzymatic reactions. However, there are now many examples of synthetic metalloenzymes in which the "natural" metal ion has been replaced by other metal ions with reconstitution of essentially full enzymatic activity (*54–58*). In most, but not all of these examples, the ions which produce similar activity in a given enzyme have similar ionic radii (*59*). This would be explained if the Lewis acid properties of the metal ion were the essential aspect of the enzymatic catalysis, moderated somewhat by the particular circumstance of ion, enzyme, and substrate (*60*).

2) Chicken liver pyruvate carboxylase (E.C. 6.4.1.1.) has tightly bound active-site Mn^{2+} ion nearly stoichiometric with the biotin content (*5*). In purified enzyme from chickens grown on low and differing levels of dietary manganese, the sum of Mg^{2+} and Mn^{2+} was always roughly equivalent to the biotin content even though the ratio of Mg^{2+} to Mn^{2+} ranged from 0.1 to 33 (*7*). Moreover, in the enzyme with high Mg^{2+} content, at least half the Mg^{2+} could be replaced by any one of Mn^{2+}, Ni^{2+}, or Co^{2+} (*7*). The striking feature of this work is that the specific enzymatic activity was the same within a factor of less than 2, and was unrelated to the Mg^{2+} content. This lack of dependence of specific activity on the particular divalent metal ion is most simply accounted for in terms of the Lewis acid proposition (*61*).

The first example of a metalloenzyme precursor that contained stoichiometric amounts of metal ions only if two or more metal ions were summed was bovine procarboxypeptidase A (*62*). In this case, the zinc was partially replaced by iron (~ 14 percent) and nickel (~ 5 percent). Given that Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} are all remarkably efficient in several different metalloenzyme systems, the actual metal found in an enzyme from a particular organism may reflect in part the status of that organism with respect to trace element nutrition, as has been demonstrated with chicken liver pyruvate carboxylase (*7*).

3) A flexible active site is indicated by the high enzymatic activity (toward carboxamide substrates) found for Co^{2+}–, Ni^{2+}–, and Zn^{2+}–carboxypeptidase A (*63*), in which the metal ion has 5-coordinate (*63*), octahedral (*63*), and distorted tetrahedral (*50*) coordination, respectively. A flexible active site is unlikely to be strained. Indeed, even in cases where the simple crystal field effects of transition metal ions are outweighed by geometrical constraints of a chelating ligand, the resulting "unusual" coordination geometry is not necessarily associated with "unusual" chemistry (*64*).

4) Fainberg and Winstein showed that solvolysis reactions occurring in solvents of low dielectric constant were subject to very large rate enhancements by added salts (*65*). This effect has been rationalized in terms of dipole-dipole interactions between ion pairs of the salt and the developing charge separation as solvolysis occurs (*66*). A Lewis acid (metal ion) more or less buried in a region of low dielectric constant at the active site of an enzyme (*50*) may well produce similar rate effects with a suitably disposed substrate.

5) The nickel metalloenzymes, Ni^{2+}–carboxypeptidase A (*63*) and Ni^{2+}-phosphoglucomutase (*67*), which are comparable in catalytic efficiency to the natural metalloenzymes (*54*), as well as jack bean urease (*1*), have electronic absorption spectra which are very ordinary in comparison with a large number of suitable simple octahedral Ni^{2+} complexes (*63*). In these cases, at least, it seems likely that it is the chemistry of the metal ion in its unstrained association with the enzyme which produces the observed catalysis and not any additional geometrical constraints imposed on the metal ion by this association.

Active-Site Metal Mercaptides

The relatively low reactivity observed by Gorin and Chin (*68*) and subsequently by ourselves for the active-site sulfhydryl group of jack bean urease could be explained by coordination of the active-site nickel (*1*) to the unreactive cysteine. Indeed, coordination with a metal ion may be a general explanation for the phenomenon of "masked" (*4*) thiol groups. Surprisingly, apart from the various iron-containing oxidation-reduction metalloproteins, there is apparently only one extant example of an enzyme in which a cysteine side chain forms a well-characterized complex with an active-site transition metal ion (*4, 69*). Further, characterized complexes of simple alkanethiols with Ni^{2+} involve coordination of two (or more) sulfhydryl groups per nickel ion (*70*), and the spectral properties of these complexes are different from those (*1*) of jack bean urease. Characterization of transition metalloenzymes in general would thus be greatly aided by characterization of simple model chelates involving only one thiol ligand, models which as yet are not commonly available (*71*).

Jack Bean Urease

Speculation on the biological significance of jack bean urease began when it was established that it constitutes as much as 0.15 percent of the dry weight of the jack bean seed (*72*). Yet ureases apparently similar to the jack bean enzyme are widely distributed, the enzyme being found in the seeds of many higher plants (*72*), in the pulmonate snails *Helix aspersa* and *Otala lactea* (*73*), and when urea is the sole source of nitrogen, in certain bacteria [*Bacillus pasteurii* (*74*) and *Proteus mirabilis* (*75*), for example].

While an involvement in nitrogen assimilation is a satisfactory justification for the presence of urease in bacteria, the function of the enzyme in higher plants is less clear. In these plants, urease is localized in the seeds, in particular the cotyledons. In the jack bean (*Canavalia ensiformis*), essentially all of the enzymatic activity remains in the cotyledons throughout the development of the embryo and seedling (*76, 77*). With development of the fertilized seed, urease activity increases over the first 5 to 7 weeks, followed by a cessation of net synthesis at maturation (*76*). After germination, the total activity in the cotyledons remains fairly constant for 7 to 10 days, then falls to a low level (*76*). Arginase and arginine are present in many seeds, and in soybeans arginase levels parallel urease levels during development (*78*). In the *Canavalia* spp., the arginine analog, canavanine is present in large amounts and serves as a principal nitrogen storage metabolite in the developing plant (*79*). In addition, the studies of Rosenthal indicate: (i) a pathway of canavanine synthesis and utilization in jack beans similar to the urea

cycle for arginine, involving the same enzymes, with the use of carbamoyl phosphate for the synthesis of *O*-ureidohomoserine from canaline, and producing urea in the breakdown of canavanine to canaline (*80*); (ii) a close correlation between the rise and fall of canavanine levels in the developing embryo and germinating seed (*81*) and the corresponding rise and fall in urease activity of jack beans; and (iii) a correlation between the presence of urease and canavanine in a wide range of canavanine-synthesizing legumes (*79*). It is likely, therefore, that urease produces ammonia from the urea produced by arginase and thus plays an important role in the mobilization of the seed nitrogen stored as canavanine (or arginine) during the early stages of growth of the seedling.

We noted previously that the restrictive specificity of urease is unusual for an enzyme involved in the hydrolysis of carboxamide derivatives, and that a biological role for the enzyme may be something other than simply the hydrolysis of urea (*82*). In this article, we have postulated that a metal ion may serve as a binding site for ammonia in enzymes involved in the utilization of ammonia or amides. Thus, while urea hydrolysis is presumably one function of urease, the enzyme may also serve in a synthetic capacity, such as in the utilization of the carbamoyl, ureido, or amidino moiety of urea in reactions or pathways as yet unrecognized. A precedent for such dual reactivity exists in mouse leukemia asparagine synthetase (*83*), for which the transfer of the amide nitrogen of glutamine to water is catalytically more efficient than to aspartate.

Evidence adduced by Slocum, Kouba, and Varner which suggested the formation of enzyme-bound carbamoyl phosphate in the urease-catalyzed hydrolysis of urea in the presence of phosphate at *p*H 6 (*84*) is consistent with a carbamoyl-transfer role for the enzyme. Certainly the initial product of urea hydrolysis by urease is ammonium carbamate which is subsequently hydrolyzed to ammonia and carbon dioxide (*3*). In addition, a phosphate binding site—possibly a nickel ion—is indicated both by the rapidly reversible inhibition produced by phosphate at *p*H 6 (*85*) and by the slower inhibition produced by phosphoramidate (*2*).

This question of the biological role of urease is intimately related to the mechanism of action of the enzyme. A demonstration of the production of an "amino-enzyme" or of a "carbamoyl-enzyme" intermediate in the urease-catalyzed hydrolysis of urea (or other specific substrate) would certainly further limit the number of possible alternative functions. The converse is also true.

An attractive proposition for the mechanism of action of urease involves coordination of urea with the active-site nickel ion (*1*). Octahedral complexes of the form $[NiL_6]X_2$ (L being urea or mono-*N*-alkylurea) have nickel coordinated with the carbonyl oxygen as donor atom (*86*). In a similar urease-urea complex, nickel could act as a Lewis acid to facilitate the attack of a suitable active-site nucleophile on the carbonyl carbon of urea to form an intermediate carbamoyl-enzyme (*87*). An alternative proposition involves coordination of the amide nitrogen of urea with the enzyme-bound nickel. Nucleophilic attack or general base catalysis by a suitable active-site group would then lead to an active-site nickel-ammonia complex similar to that proposed above for the glutamine amidotransferases.

Characterization of Highly Purified Enzymes

To the now classical criteria of homogeneity of an enzyme preparation (maximal and reproducible specific activity, minimal and reproducible equivalent weight, simple and "clean" polyacrylamide gel electrophoretograms under normal as well as fully reducing or denaturing conditions), should be added ultraviolet, visible, and near-infrared absorption spectra at high concentration together with thorough and exhaustive metal ion analyses. In our own experience with jack bean urease, the most important clue to the existence of nickel at the active site (*1*) was the observation of a characteristic metal ion spectrum which is resolved only at high concentrations, and which appears merely as a small, but significant tail absorption in the region 320 to 360 nm in routine ultraviolet spectra (*88*). In recommending such spectral and metal ion analyses, we hope that our long-standing error in ignoring the "abnormalities" in the urease spectrum will facilitate the recognition of the role of metal ions in other enzyme systems.

Summary

In an attempt to understand the role of nickel in jack bean urease (*1*), we turned to a variety of other enzymes important in the utilization, production, or transfer of ammonia. We found several, including the L-histidine and L-phenylalanine ammonia-lyases and some enzymes that utilize glutamine or ammonia in amidotransferase reactions, all of which show evidence for the involvement of as yet unreported transition metal ions in their mechanism of action.

We support the view that catalysis by metalloenzymes may be a reflection of the chemistry of the metal ion itself as a Lewis acid, and that perhaps too much emphasis has been placed on supposed special characteristics (such as strain, "entasis") of the enzyme–metal ion association. In this context, we have discussed the mechanism of catalysis of hydrolysis of specific substrates by carboxypeptidase A, and have returned to urease to examine the role of nickel in its mechanism of action.

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