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Magnetic Resonance Studies of Metal Activation of Enzymic Reactions of Nucleotides and Other Phosphate Substrates*

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The requirement of a divalent metal ion is common to all reactions of nucleoside triphosphates, and advantage has been taken of the paramagnetic property of the manganous ion to study interactions of metal, substrate, and enzyme. The effect of the paramagnetic ion Mn^{++} on the relaxation rate of the nuclear magnetic resonance of water protons in the reaction mixture is very sensitive to the type of metal complex formed. Upon formation of binary or ternary complexes with proteins, enhancement of the effect relative to the manganese aquocation (easily observable at 1×10^{-4} M) has been measured. The interactions of four crystalline kinases, creatine kinase, 3-phosphoglycerate kinase, hexokinase, and pyruvate kinase, with their respective substrates and Mn^{2+} have been investigated with this technique as well as another metal-requiring enzyme, yeast enolase. These enzymes fall into two groups: (1) enhancement of the binary complex (Mn-enzyme) $\gg$ enhancement of ternary complex (Mn-enzyme-substrate), and (2) enhancement of ternary complex $\gg$ binary complex. The first group includes enolase and pyruvate kinase, and the second group includes creatine kinase and 3-phosphoglycerate kinase. With yeast hexokinase a quaternary complex of enzyme-glucose-Mn-ADP was required to cause enhancement. For creatine kinase the enhancement data suggested that manganese does not act as a bridge between enzyme and substrate, but is complexed only to the nucleotide, and the metal-nucleotide is then bound to enzyme. This configuration of the ternary complex was verified by electron-spin-resonance spectra of the binary and ternary complexes. The measurement of enhancement has been used for quantitative determination of association constants for metal enzyme, metal substrate, and substrate-metal enzyme. For those enzymes such as enolase and pyruvate kinase, where the evidence indicates that the metal ion is bound at the active site, the enhancement of the proton relaxation rate of water due to the Mn-enzyme is characteristic of a particular site and not only can be used for titration of the site but is also a very sensitive indicator of changes in configuration occurring solely at the active site.

The nucleoside triphosphates are the almost universal repository of chemical energy in living systems and mediate the transfer of energy from one metabolic process to another. Adenosine triphosphate, hereafter referred to as ATP, is the most commonly encountered nucleoside triphosphate. The concept of "energy-rich phosphate compounds" formulated by Lipmann in 1941 (Lipmann, 1941) led to a rationale from the thermodynamic point of view for the central role of ATP in a multitude of metabolic and biosynthetic pathways. In recent years, attention has been focused on the kinetic aspects of the participation of nucleoside triphosphates in phosphoryl transfer reactions catalyzed by enzymes. Although a great deal of progress has been achieved in unraveling the events in enzymic catalysis, not a single enzymic mechanism is yet understood in the detail possible for nonenzymic reactions.

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The minimum information required to elucidate the mechanisms of the enzymic reactions of ATP and other nucleoside triphosphates includes, in the order of ease of attainment, the establishment of (1) the bond which is cleaved, (2) the active species of the substrate, (3) the groups of the substrate and enzyme molecule which interact in binding and catalysis, and (4) the relation of structure to function of the enzyme. The first objective has been achieved by studies from several laboratories using O^{18} as a tracer. The mechanism problem has been simplified by the universality of the findings illustrated in Figure 1: first, that the γ-P—O bond of the nucleoside triphosphate is always cleaved in the kinase reactions (phosphotransferases), which have been investigated (Cohn, 1959), including hexokinase, 3-phosphoglycerate kinase, adenylate kinase, creatine kinase, and a number of ATPases, and; second, that the α-P—O bond is always cleaved in pyrophosphorylase reactions, i.e., nucleotidyltrans-

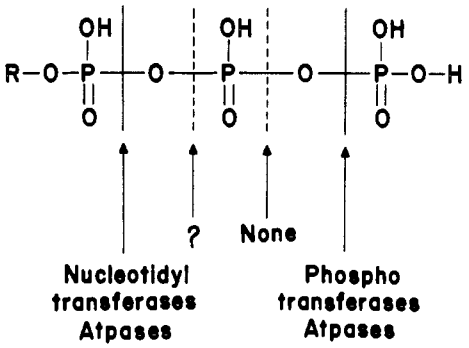

FIG. 1.—Bond cleavage in ATP reactions.

ferases, which have been investigated (Boyer, 1960), including acetate and amino acid activation, adenylic and pantothenic acid formation, and some hydrolytic reactions (Cohn, 1960).

In casting about for a method which would reveal the nature of the enzyme-substrate interactions, it occurred to us that advantage could be taken of a property common to all reactions of nucleoside di- and triphosphates—the requirement of a divalent metal ion for activation. Although the normal activator is probably Mg^{2+}, Mn^{2+} invariably activates also. Since Mn^{2+} is paramagnetic, it has become feasible with the rapid development of magnetic resonance techniques and theory to use various manifestations of its paramagnetic properties to study interactions of the metal ion with its immediate environment.

It has been possible not only to gain some insight into the role of the metal ion in these reactions but also to use the metal ion as a probe to investigate substrate-enzyme interactions. Some problems include the specification of which binary complex is the active species, metal-substrate or metal-enzyme; and the determination of the site of the metal ion in the ternary complex, i.e., whether the metal atom acts as a bridge between enzyme and substrate. In addition, information can be adduced concerning the qualitative and quantitative aspects of metal ion specificity; why, for example, Mn^{2+} is more effective than Mg^{2+} at low concentrations and why an ion like Ca^{2+} sometimes inhibits and sometimes activates. Furthermore, since a property of the binary and, more important, ternary complexes can be measured, binding constants can be determined quantitatively.

We have investigated four reactions involving transfer of the phosphoryl group from ATP to different types of acceptor molecules indicated in Table I.

TABLE I
TYPES OF BONDS FORMED IN FOUR ENZYME SYSTEMS UNDER INVESTIGATION

Enzyme	Acceptor	Bond Formed
ATP: creatine phosphotransferase	R—C(=NH)—NH_2	Phosphoamidate
ATP: D-hexose 6-phosphotransferase	R—OH	Ester
ATP: D-3-phosphoglycerate 1-phosphotransferase	R—C(=O)—OH	Carboxylic anhydride
ATP: pyruvate phosphotransferase	R—C(CH_2)—OH	Enol phosphate

In the reverse direction all these reactions lead to ATP as product with the formation of a phosphoanhydride bond; the equilibria differ considerably for these reactions, lying far in the direction of glucose-6-phosphate in the hexokinase reaction and in the opposite direction, i.e., formation of ATP in the pyruvate kinase reaction. In addition we have investigated in some detail the metal-activated enzymic reaction catalyzed by enolase, which involves the dehydration of 2-phosphoglycerate to phosphoenolpyruvate.

We have employed two types of measurement: first, the electron-spin-resonance absorption of Mn^{2+} observed with a Varian EPR spectrometer at 9000 megacycles/sec; and second, the proton relaxation time of the nuclear magnetic resonance of water observed at 25 megacycles/sec with a pulsed apparatus (Carr and Purcell, 1954) constructed by Mr. John Leigh of our laboratory. Once the equipment is available, such measurements are not difficult nor time consuming; an electron-spin-resonance spectrum may be recorded in about 10 minutes and a relaxation time may be determined in considerably less time. The electron-spin-resonance absorption spectra of Mn and its complexes have been of limited use since the spectral lines of most complexes, and in particular those with proteins, are so broad that they have thus far eluded detection. However the technique has proved to be a valuable adjunct to the proton relaxation rate measurements which we shall describe. The paramagnetic effect which has proved most useful as a probe for metal-substrate-enzyme interactions is the effect of Mn^{2+} and its various binary and ternary complexes on the longitudinal proton relaxation rates ($1/T_1$), i.e., on the rate of attainment of equilibrium over nuclear spin states of the protons of water. The principal mechanism of relaxation in pure water resides in the magnetic dipolar interaction between protons, but in the presence of a paramagnetic ion the much larger proton-electron dipolar magnetic interactions dominate the relaxation rate. Because Mn^{2+} has a large magnetic moment due to its five unpaired electrons, it has a large effect on the proton relaxation rate of the water protons; in fact at a concentration of 1 M Mn^{2+} the relaxation rate of pure water, $\sim$0.33 sec^{-1}, is increased by a factor of approximately 10,000. But more important is the fact first recognized by Bloembergen *et al.* (1948) that the dominant contribution to the effect is due to the interaction of Mn^{2+} with the water in its hydration sphere. Consequently the magnitude of the effect is very sensitive to the immediate environment of the Mn^{2+}. The displacement of H_2O by other ligands in the Mn^{2+} aquocation would be expected to decrease the effectiveness of Mn^{2+} in increasing the relaxation rate of the water protons ($1/T_1$), and indeed such an effect has been observed upon chelation of Mn^{2+} with ethylene diamine tetraacetate (King and Davidson, 1958). On the other hand, an unexpected enhancement of the effectiveness of Mn^{2+} in increasing $1/T_1$ was observed upon complexing Mn^{2+} with DNA and with proteins (Cohn and Leigh, 1962). Eisinger *et al.* (1962) defined an enhancement factor ϵ equal to the ratio of relaxation rates of the water protons in the presence and absence of complexing agent, and with a few approximations of the fundamental equations pointed out the following relationships:

$$\epsilon = \frac{R_1^*}{R_1} = \frac{\frac{1}{T_1^*} - \frac{1}{T_1^*(0)}}{\frac{1}{T_1} - \frac{1}{T_1(0)}} \approx \frac{p^* \tau_c^*}{p \tau_c}$$

where ϵ = enhancement factor, R_1 = relaxation rate due to Mn^{2+}, T_1 = observed relaxation time, $T_1(0)$

TABLE II

COMPARISON OF CREATINE KINASE AND YEAST ENOLASE

Total $MnCl_2$ concentration 1×10^{-4} M, Tris-HCl buffer 0.05 M
Enolase reaction mixture contained 0.5 M KCl in addition. $T = 20°$.

Creatine Kinase			Enolase		
Additions	ϵ	% Mn^{2+} Bound	Additions[a]	ϵ	% Mn^{2+} Bound
CrK (6.35 mg/ml)	1.15	25	En (4.9 mg/ml)	8.40	67
ADP, 2×10^{-4} M	1.32	72	PEP, 2×10^{-4} M	1.0	0
CrK + ADP	8.85	72	En + PEP	3.93	80

[a] PEP, phosphoenolpyruvate.

= relaxation time in absence of Mn^{2+}, p = number of protons in hydration shell, τ_c = correlation time, and * indicates presence of complexing agent.

Since p can only decrease when H_2O is replaced in the Mn aquocation with ligand groups from the complexing agent, one would expect the enhancement to be less than 1 as found in MnEDTA; when the enhancement is greater than 1, it becomes necessary to invoke an increase in τ_c in the Mn complex. If it is assumed that τ_c in the complex is determined primarily by the rotational correlation time τ_r, as in the Mn aquocation (Bloembergen and Morgan, 1961), then it may be concluded that the relative rotational motion of the Mn and water is hindered by the proximity of the macromolecule and the relaxation rate thereby increased. It must be borne in mind that the observed relaxation rate is the weighted average of the H_2O in the hydration sphere of Mn^{2+} and of H_2O in the bulk of the solution which is normally exchanging rapidly with H_2O in the hydration sphere; an increase in relaxation rate upon complexing Mn^{2+} ($\epsilon > 1$) implies that the Mn^{2+} is still accessible to the water in the bulk of the solution and is therefore located at an external site of the macromolecule rather than buried within it.

With this physical picture in mind, let us now examine what occurs when Mn^{2+} interacts with substrate and enzyme. The two enzyme reactions which have been examined in greatest detail are those involving creatine kinase and enolase, because the kinetics and equilibria of these reactions have previously been carefully and extensively investigated by several groups. The generosity of Dr. Noda in supplying the crystalline muscle creatine kinase and of Drs. Malmstrom and Westhead in supplying the crystalline yeast enolase made the current investigations possible. As shown in Table II, stoichiometric concentrations of enzyme are used; however since the total volume required is 0.1 ml, the amounts needed are not prohibitive. As suggested by kinetic and equilibrium data of previous investigators, the two enzymes fall into different categories: with creatine kinase the metal substrate is the active species, with enolase the metal enzyme is the active species. From the data in Table II, it is apparent that binding of Mn to ADP alone or creatine kinase alone results in a small enhancement of the relaxation rate but the addition of all three components together results in an enhancement factor of about 9.0. It may be concluded that a ternary complex of MnADP and creatine kinase has been formed. Qualitatively similar results are obtained with ATP, but no evidence of ternary complexes of enzyme and metal with creatine or creatine phosphate are revealed by this method. However, addition of creatine to the MnATP·E ternary complex under conditions where the equilibrium greatly favors ATP and creatine leads to a de-enhancement characteristic of the quaternary complex.

The interactions of enolase with the substrate and Mn contrast sharply with those of creatine kinase. The substrates phosphoenolpyruvate or 2-phosphoglycerate alone yield no enhancement as expected from their low association constants, but addition of enzyme alone to Mn causes a large enhancement; upon addition of substrate to the Mn-enolase, the enhancement is reversed. Again a ternary complex must have been formed since the enhancement of the three components together cannot be accounted for by any type of summation of the pairs, that is, manganese substrate and manganese enzyme. The same value is obtained with 2-phosphoglycerate added as with phosphoenolpyruvate since an equilibrium mixture is established from either direction. Qualitative evidence that we are dealing with enzymically active complexes is adduced from the fact that the addition of a number of phosphate compounds which complex with Mn but which are not substrates for these enzymes had an insignificant effect on the proton relaxation rate of Mn and enzyme. Conversely a protein which is not an enzyme, bovine serum albumin, exhibits an enhancement of approximately 11 with one Mn^{2+} bound per molecule but shows no evidence of formation of ternary complexes with ADP, ATP, or phosphoenolpyruvate. Furthermore, the addition of 4 M urea, which inactivates creatine kinase, considerably lowered the enhancement of the ternary complex, and 6 M urea completely abolished the enhancement of the binary and ternary complexes of enolase.

The values of the observed enhancement shown in Table II are not the values of the binary and ternary complexes since they represent a sum of the contributions of each Mn species existing in the solution; the concentration of each species depends on the various association constants. To obtain the enhancement of a binary complex is fairly simple. For example, in a solution of Mn^{2+} and enolase, it is necessary only to measure the free Mn^{2+} concentration by electron spin resonance and the enhancement of the same solution. Since the observed enhancement is the sum of the contribution of the free and complexed Mn and since the enhancement of free Mn^{2+} is 1, the enhancement of the complex can be directly calculated. The enhancement of the binary complex Mn-enolase has also been determined solely from enhancement data by extrapolation of titration curves in which enhancement was measured either as a function of Mn^{2+} concentration or as a function of protein concentration. The results of eight experiments with Mn^{2+} and enolase using the various methods outlined gave an enhancement of 13.8 ± 1.1 in 0.5 M KCl. From the electron-spin-resonance data, measuring free Mn^{2+}, obtained in a titration of enzyme with Mn, a dissociation constant of the order of 1×10^{-5} M was calculated with one metal ion bound per protein molecule. The dissociation constant determined from enhancement data,

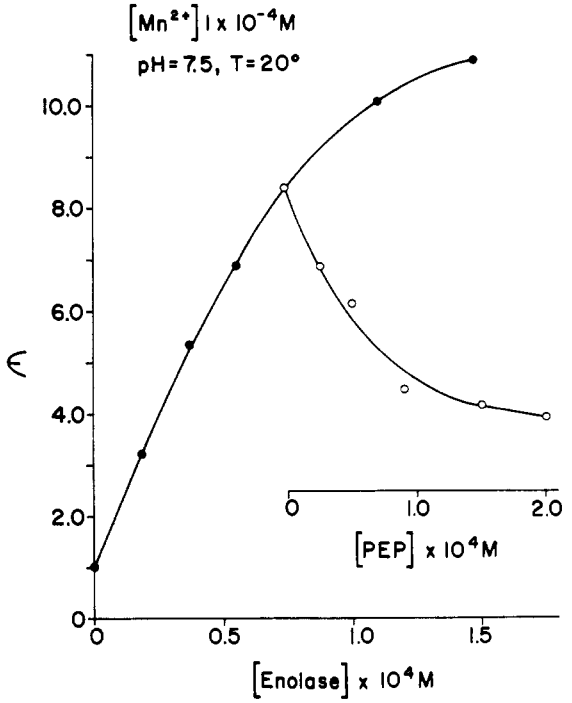

FIG. 2.—Enhancement of the binary complex, Mn-enolase, ascending curve, and of the ternary complex, Mn-enolase-substrate, descending curve. Each solution contained Tris-HCl buffer, 0.05 M, KCl 0.5 M, $MnCl_2$ 1 × 10^{-4} M, total volume 0.1 ml; in the ascending curve, the crystalline yeast enolase concentration varied as indicated; in the descending curve, enolase concentration was constant at 0.74 × 10^{4} M and phosphoenolpyruvate (PEP) was varied as indicated.

measuring bound Mn^{2+}, gave a value of 0.85 × 10^{-5} M. At very high Mn concentrations, other binding sites become significant and both the enhancement factor and binding constant values differ from those for the first binding site. A titration of Mn^{2+} with enolase is shown in Figure 2. In order to obtain the enhancement of the ternary complexes, Mn enolase was titrated with phosphoenolpyruvate, shown in the descending curve, and at each point free Mn^{2+} was also determined. The ϵ of the ternary complex is approximately 5.0. It must be remembered, of course, that one is unavoidably titrating with an equilibrium mixture of phosphoenolpyruvate and 2-phosphoglycerate. The dissociation constant for metal–enzyme–mixed substrate calculated from these data is 1.4 × 10^{-5} M, while K_m measured kinetically for phosphoenolpyruvate and 2-phosphoglycerate is 7.7 × 10^{-5} M and 10 × 10^{-5} M, respectively—approximately six times greater. The dissociation constant for metal-enzyme-substrate would be more profitably compared to K_m determined at equilibrium with isotopic methods, but such data are not yet available for enolase.

Another aspect of the interaction of metals with enolase was investigated by adding other metal ions to the Mn-enolase system. In Figure 3 the effect of Zn^{2+}, Mg^{2+}, and Ca^{2+} on the release of free Mn^{2+} and on the observed enhancement is illustrated. It will be noted that, as might be anticipated from the kinetics, Zn^{2+} is most effective, Ca^{2+} next, and Mg^{2+} least effective in replacing Mn^{2+} in the complex and lowering the enhancement. It is clear that these ions compete for the first binding site. However very high concentrations of Mg^{2+}, for example, are required to de-enhance completely, which would imply that Mg^{2+} does not compete effectively for the secondary sites occupied

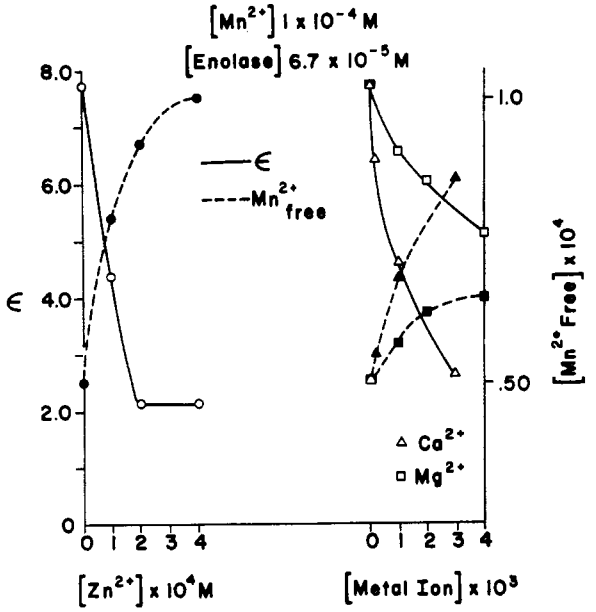

FIG. 3.—Variation of enhancement of the Mn-enolase complex and of free [Mn^{2+}] as a function of Zn(○), Ca(△), and Mg(□) concentration. All concentrations are molar. The solutions contained 0.05 M Tris-HCl buffer, *p*H 7.55, 0.5 M KCl, and the metals were added in the form of chloride salts. T = 19.2°.

by Mn^{2+}. This difference in the behavior of the two ions may well explain the inhibitory effect exhibited by Mn^{2+} but not Mg^{2+} at high concentrations.

If we now turn to the interactions of creatine kinase with it substrates, we find a more complex situation (cf. Kuby and Noltmann, 1962) because of the many species which exist in this system. To obtain the enhancement of each species and the related dissociation constants, it is necessary to titrate with each of the components. Such a titration of Mn^{2+} with ADP in the absence and presence of creatine kinase is shown in Figure 4. It should be pointed out that ADP as well as ATP causes a small enhancement, which reaches 1.5 for both binary complexes at saturation. From

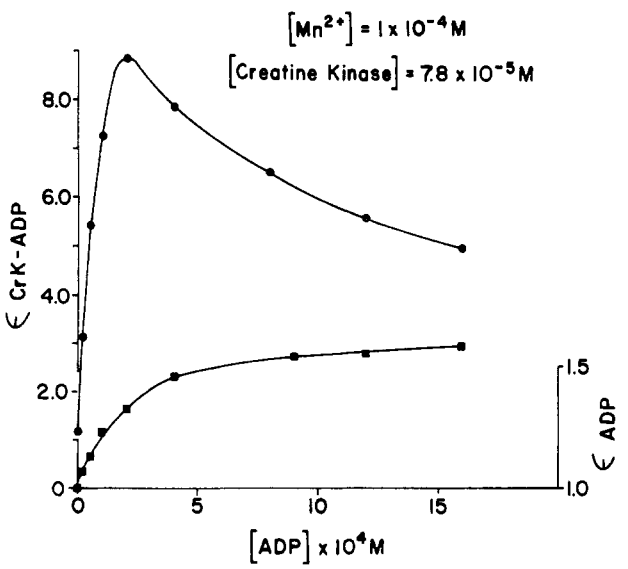

FIG. 4.—Enhancement as a function of ADP concentration in the absence and presence of crystalline creatine kinase from rabbit muscle. The solutions contained Tris-HCl buffer, 0.05 M, *p*H 7.50, T = 20°.

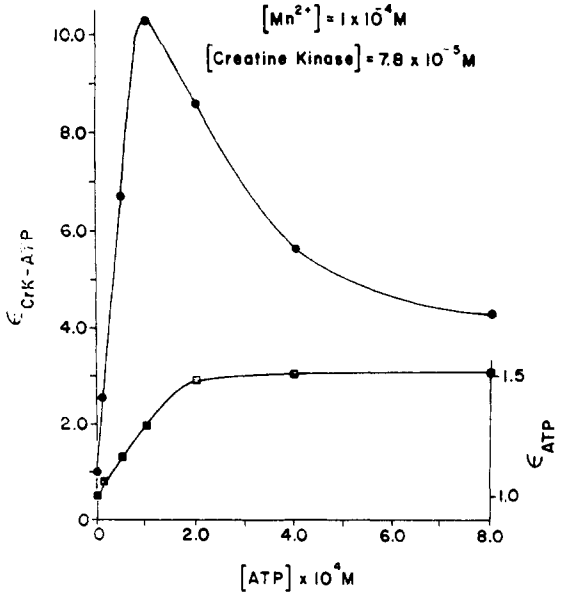

FIG. 5.—Enhancement as a function of ATP concentration. Conditions identical with those in Fig. 4.

the lower titration curve, one can obtain an association constant for MnADP of $\sim 1.0 \times 10^4$ mole^{-1}. In the presence of the enzyme, upon successive additions of ADP, the enhancement first rises and then begins to fall when the concentration of ADP is twice the concentration of Mn^{2+}. This phenomenon reflects the competition between $MnADP^-$ and free ADP^{3-} for the site on the enzyme. A simultaneous measurement of the free Mn^{2+} in each solution permits an extrapolation for a value of ϵ of the ternary complex, E · MnADP, equal to 15. Further titrations with enzyme as variable are required to evaluate the association constants of the two ternary complexes E·MnADP and E·ADP. A similar titration with ATP is shown in Figure 5. The data in the lower curve for MnATP yield an association constant of 3×10^4 mole^{-1}. In the upper curve the maximum falls at a Mn–ATP ratio of 1:1 consistent with kinetic data. The extrapolated value of ϵ is greater than 20. The de-enhancement with excess ATP falls more sharply than the analogous curve for ADP, indicating that free ATP competes more effectively with its metal complex for the binding site on the enzyme than ADP does with its metal complex. These relationships are in qualitative agreement with the analogous binding constants for the Mg complexes determined by ultracentrifugal and equilibrium dialysis techniques (Kuby *et al.*, 1962).

By a combination of electron-spin-resonance and enhancement measurements it has been possible to establish that in the creatine kinase–ADP–Mn complex the Mn does not serve as a bridge atom and is bonded only to the nucleotide and not to the protein. The results of the relevant experiment are listed in Table III. Since the lines of electron-spin-resonance spectrum of MnADP are much broader than those of free Mn^{2+}, it was necessary to use higher concentrations of Mn and ADP than usual to be able to observe the derivative curve of the absorption lines, and also to use a high ratio of ADP to Mn in order to suppress the free Mn^{2+} ion concentration. As a result the enhancement upon addition of the enzyme is not so striking as that attained in other experiments since the conditions correspond to the descending limb of the upper curve in Figure 4. Nevertheless, the essential result is clear-cut: the line widths of the electron-spin-resonance spectrum of Mn^{2+}, which are a measure of the relaxation time of the electron spin, are considerably altered upon addition of ADP, but no further alteration occurs upon addition of enzyme. On the other hand, the proton relaxation rate of H_2O changes somewhat on addition of ADP to Mn^{2+} but there is a further considerable change upon addition of enzyme. We may therefore conclude that addition of enzyme to MnADP causes a change in the relative rotational motion of water and Mn in the complex, which is reflected in the change in the proton relaxation rate, but causes no change in the type of motion which determines the electron-spin-relaxation rate, namely, a deformation of the complex arising from impact of molecules outside the complex (Bloembergen and Morgan, 1961). We may therefore conclude that the Mn bears the same relationship to ADP in the binary and ternary complex, but the binding of MnADP to the enzyme has affected the rotational motion in the hydration sphere of Mn.

TABLE III

RATIOS OF LONGITUDINAL NUCLEAR-SPIN-RELAXATION RATES OF PROTONS, R_P^*/R_P^0, (ϵ), AND ELECTRON-SPIN-RELAXATION RATES OF MANGANESE, R_e^*/R_e^0 IN THE PRESENCE (*) AND ABSENCE (0) OF COMPONENTS OF THE CREATINE KINASE REACTION

Additions to $MnCl_2$ 1×10^{-3} M in Tris-HCl Buffer (0.05 M), pH 7.55	R_P^*/R_P^0, (ϵ)	R_e^*/R_e^0
ADP (5×10^{-3} M)	1.92	~4
CrK (22.5 mg/ml)	2.16	—
ADP + CrK (22.5 mg/ml)	5.08	~4

The other two substrates for the creatine kinase reaction, creatine and phosphocreatine, have no effect on the enhancement of the relaxation rate when added to Mn^{2+} or to Mn^{2+} and enzyme. This is consistent with the finding that the metal ion is bound to the active site only through the nucleotide. Once the metal-nucleotide, MnATP, is bound to the enzyme, the addition of creatine causes a de-enhancement, again indicating a change of conformation in the region of binding of MnATP with the formation of a quaternary complex.

Three other kinase reactions have been surveyed: those involving 3-phosphoglycerate kinase, hexokinase, and pyruvate kinase. Of these, 3-phosphoglycerate kinase is qualitatively similar to creatine kinase in all respects. Hexokinase is of the same type, i.e., no marked enhancement of the relaxation rate is observed with Mn^{2+} and enzyme or Mn with nucleotide alone. Hexokinase differs from creatine kinase in that no enhancement is observed with enzyme, Mn, and ADP or ATP unless glucose is present. In other words, there is a compulsory order of addition and the metal nucleotide can be bound only to the glucose-enzyme complex. Unlike these three kinases, pyruvate kinase resembles enolase in that a large enhancement is observed with Mn and enzyme and each substrate added to the metal enzyme results in de-enhancement. This enzyme, which is now being investigated in detail by Dr. Mildvan in our laboratory, has given rise to the largest enhancement yet observed. Dr. Mildvan has found that the binary complex consisting of 2 moles of Mn per mole of protein, in agreement with the earlier finding (Reynard *et al.*, 1961) of two active sites per enzyme molecule, has an enhancement of 30. Upon titration with phosphoenolpyruvate nearly all the Mn^{2+} becomes bound in the ternary complex and the enhancement falls to 2; the effect is far greater than the comparable one for enolase where $\epsilon = 13.8$ for Mn-enolase and falls to 5.0 upon titration with phosphoenolpyruvate. Time does not permit discussion of this

TABLE IV
CLASSIFICATION OF ENZYME-METAL-SUBSTRATE INTERACTIONS INTO TWO GROUPS

Type I	Type II
Enhancement of relaxation rate in ternary complex ≫ binary complex	Enhancement of relaxation rate in binary complex ≫ ternary complex
Muscle creatine kinase	Yeast enolase
Muscle 3-phosphoglycerate kinase	Yeast inorganic pyrophosphatase
Yeast hexokinase[a]	Muscle pyruvate kinase

[a] Quaternary complex required for enhancement.

TABLE V
COMPARISON OF PARAMETERS CHARACTERIZING METAL-PROTEIN INTERACTION FOR DIFFERENT PROTEINS

Protein	No. of Binding Sites	K_{ass} mole^{-1}	ϵ
Bovine serum albumin	1.3	$2.8 \pm 0.6 \times 10^4$	11.6 ± 0.7
Yeast enolase	1.1	$1.0 \pm 0.2 \times 10^5$	13.8 ± 1.1
Muscle pyruvate kinase	2.1	$1.5 \pm 0.4 \times 10^4$	30.2 ± 1.0

interesting reaction, but suffice it to say that the K_{ass} for Mn-enzyme-phosphoenolpyruvate, unlike that for the enolase, agrees very well with the kinetically determined K_m, confirming the finding (Reynard *et al.*, 1961) that this reaction follows equilibrium kinetics and indicating that we are indeed looking at the kinetically active complex. An effect of K ion has been observed and is particularly striking in the ternary complex of Mn-enzyme-phosphoenolpyruvate, suggesting that the conformation at the active site is changed by K^+ in such a way as to influence most strongly the binding of this substrate.

Table IV presents a summary of the enzymes that we have examined and it will be noted that they fall into two groups. The first group, represented by creatine kinase, shows very little enhancement of binary complexes, i.e., metal-enzyme or metal-substrate, but a large enhancement results from the formation of the ternary complexes. The second group,[1] which includes enolase and pyruvate kinase, shows a large enhancement in the binary metal enzyme complex and a de-enhancement upon binding of substrate. It may be tentatively concluded that for members of the first group the metal atom does not function as a bridge atom between the substrate and the enzyme, but remains bound to the nucleotide only, even in the ternary complex. In the second group, the metal is bound to the enzyme and may or may not be acting as a bridge atom in the ternary complex. The decrease in enhancement upon binding of the substrate is consistent with a postulated bridge structure which would result in the replacement of water by other ligands, but an alternate explanation invoking only a change in conformation in the region of the metal binding site resulting from binding of substrate directly to the enzyme cannot be ruled out at the present time. In fact the observed de-enhancement of Mn–pyruvate kinase from 30 to 2 by binding of phosphoenolpyruvate is too large to be attributed solely to a change in the number of water protons in the hydration sphere of Mn^{2+}, and must be due in part to a change in conformation which causes a change in rotational correlation time. It is perhaps not accidental that the three enzymes of Type I are all activated by Ca^{2+} but the enzymes of Type II are all inhibited by Ca^{2+}. Far less metal ion specificity and steric restriction would be anticipated in complexes of the creatine kinase–nucleotide–metal type in which the metal is bound only to the nucleotide.

It should be pointed out that the enhancement factor in Mn protein complexes is a characteristic of the site of binding, and, for those enzymes which bind the metal at the active site, it is characteristic of the structure of the ligands and the conformation of the protein in that region. The number of binding sites, association constants, and enhancement factors for three proteins, determined by a combination of pulsed nuclear-magnetic-resonance and electron-spin-resonance measurements, are shown in Table V. It will be noted that we find one binding site on enolase, in agreement with earlier work (Malmstrom *et al.*, 1958), and two sites on pyruvate kinase, in agreement with ultracentrifugal measurements (Reynard *et al.*, 1961). Bovine serum albumin has one tight binding site, as found for human serum albumin by equilibrium dialysis (Holeysovska, 1961), and five weaker ones which are not indicated. Although enolase has a higher association constant with Mn^{2+} than pyruvate kinase, the latter has more than twice as large an enhancement factor. As already mentioned, the ternary complex of Mn enolase with the equilibrium mixture phosphoenolpyruvate and 2-phosphoglycerate is reduced from 13.8 to 5.0, and for Mn pyruvate kinase with phosphoenolpyruvate is reduced from 30 to 2.

I have outlined the type of data which can be obtained with these methods to yield metal-protein association constants and metal-protein-substrate association constants, and have indicated what one can learn about the structure and conformation of these complexes. I should like to emphasize that for those enzymes which bind the metal at the active site we now have a parameter, the enhancement factor, in addition to the binding constant which characterizes the site and is sensitive to the conformation at the site. This is best illustrated by studies carried out by Dr. Mildvan in our laboratory with Mn–serum albumin. Various denaturing agents which have the same quantitative effect on over-all parameters of the conformation of the protein molecule, such as optical rotation, exhibit quite different quantitative effects on the local region of the binding site as reflected in the enhancement values. The enhancement factor of the metal-protein complex reflects different features of protein structure than the strength of binding, as shown in studies with denaturing agents and in Figure 6, where the enhancement and association constants of Mn-albumin are compared as a function of *p*H. Although the K_{ass} is chiefly affected by a group with a $pK > 8.2$, the enhancement of the binary complex is not affected by this group but is strongly affected by a group with a pK in the region of 7. This type of study of *p*H dependence will be extended to binary, ternary, and quaternary complexes of the enzymes.

Thus far we have limited our investigations to reactions in which a phosphoryl group is transferred from ATP to an acceptor molecule. This group was chosen for initial study because it represents the simplest of the ATP reactions involving only two substrates and a metal ion, and because of the availability of these

[1] Inorganic pyrophosphatase is included in this group because of the large enhancement observed upon complexing the enzyme with Mn^{2+}. However, it is uncertain whether the Mn^{2+} is bound at the active site, since three metal ions are bound per molecule of protein. Furthermore it is difficult to study the ternary complex with substrate since pyrophosphatase is a hydrolytic enzyme, but preliminary data suggest a de-enhancement.

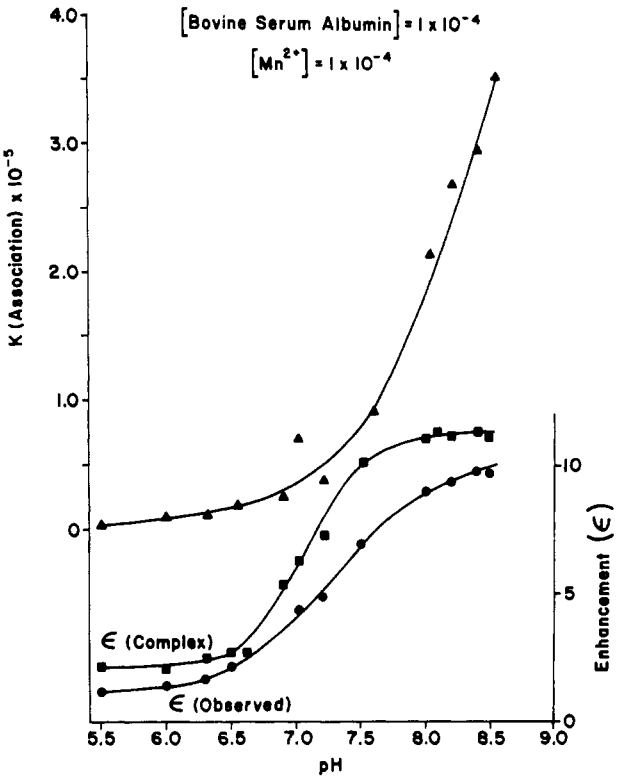

FIG. 6.—Comparison of association constants and enhancement values of Mn-bovine serum albumin complexes as a function of pH; K_{ass}▲, ϵ_{obs}●, $\epsilon_{complex}$ ■. All concentrations are molar. The pH was adjusted with tetramethyl ammonium hydroxide, and all solutions contained 0.17 M tetramethyl ammonium chloride. $T = 24 \pm 1°$.

enzymes in pure form. Even this small sample has revealed the variations of detailed mechanisms possible with different enzymes which catalyze the same group transfer from the same substrate, ATP. This approach can be extended to differentiate mechanistic aspects of more complex reactions involving phosphoryl transfer of the glutamine synthetase type, in which three substrates and a metal ion are interacting, and also to substituted phosphoryl transfer reactions of the type of amino acid activation, in which the adenyl moiety of ATP is transferred.

I should like to close on a note of optimism concerning the extension of magnetic resonance techniques to the study of enzymic mechanisms. With the improvement of techniques and with increased sensitivity, it is well within the realm of probability that one will be able to abandon the underhanded methods of the kind I have been describing this morning, which make it necessary to infer what the substrates and enzymes are doing by measuring parameters of water. Instead, one will hopefully be able to use continuous-wave nuclear magnetic resonance to look at individual protons and phosphorus atoms in the substrate when it is interacting with the enzyme. It is even within the realm of possibility that one will be able to distinguish directly which groups on the enzyme are involved, particularly if the enzyme obliges us by having a paramagnetic ion bound at the active site.

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