Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.
Piérard, A., and Wiame, J. M. (1964), *Biochem. Biophys. Res. Commun. 15*, 76.
Raijman, L., and Grisolia, S. (1964), *J. Biol. Chem. 239*, 1272.
Rogers, P., and Novelli, G. D. (1962), *Arch. Biochem. Biophys. 96*, 398.
Singer, T. P., and Kearney, E. B. (1950), *Arch. Biochem. 29*, 190.

A Kinetic Study of Nucleotide Interactions with Pyruvate Kinase*

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ABSTRACT: The apparent broad specificity of the enzyme pyruvate kinase for the nucleoside diphosphate substrate has been re-examined. Nucleoside diphosphokinase activity was found to be present but was active only when exogenous adenosine triphosphate was present.

The apparent K_m and V_{max} values were determined at several pH values between 6.0 and 9.0 for a number of nucleoside diphosphates. A doubly ionized phosphoryl group on the nucleotides and/or an imidazole group on the enzyme appear to be essential for nucleotide binding. Both the apparent K_m and V_{max} values decrease between pH 7.5 and 9.0, which suggests the involvement of an ionized α-amino group in the reaction. The results of the determinations of kinetic parameters in the pH range of 7–8 are discussed in relation to a proposed mechanism in which the nucleoside diphosphate is bound to the enzyme on two regions in an obligatory order, with the β-phosphoryl group binding first and the nucleoside portion binding second.

A broad specificity for the nucleoside diphosphate substrate in the pyruvate kinase catalyzed reaction has been reported previously (Strominger, 1955; Tietz and Ochoa, 1958; Adam, 1961). However, doubt has been cast on this specificity by Davidson (1959), who claimed that the apparent rate obtained, using pyrimidine nucleoside diphosphates, resulted from contamination of the reaction mixture with nucleoside diphosphokinase and adenosine phosphates. An upper limit for nucleoside diphosphokinase activity under varying conditions will be presented. Evidence will be given that two regions of the nucleotide are bound, that the phosphate must be doubly ionized for binding, and that an imidazole and an α-amino group on the enzyme are involved in binding.

Methods

Materials. The sodium salts of PEP,[1] AMP, ADP, ATP, GDP, IDP, UDP, CDP, dADP, dCDP, NADH, and $NADP^+$; crystalline lactic dehydrogenase, type II; and crystalline yeast hexokinase were purchased from the Sigma Chemical Co. The crystalline glucose 6-phosphate dehydrogenase was prepared by C. F. Boehringer und Soehn. The [^{32}P]ATP was prepared by mitochondrial phosphorylation of ADP by [^{32}P]inorganic phosphate. The [^{32}P]PEP was labeled by an exchange reaction using [^{32}P]ATP and pyruvate kinase by a method to be published elsewhere. Product separation was effected in both instances by chromatography on Dowex-1 chloride using the method of Khym and Cohn (1953).

Enzyme Preparation. Pyruvate kinase was prepared from rabbit muscle by the "fluorokinase" method of Tietz and Ochoa (1958). Two different preparations were used in this work. One batch had a specific activity of 60 μmoles of pyruvate formed/min/mg of protein at pH 7.0 and 25.0° and is referred to later

* From the Department of Biochemistry, University of Miami School of Medicine, Miami, Florida. *Received August 2, 1965.* This investigation was supported in part by the Julia Meador Palmer Memorial Institutional Grant of the American Cancer Society.

† Work done in partial fulfillment of the requirements for the Ph.D. degree and supported by a predoctoral fellowship (GM-24602) from the U. S. Public Health Service. Present address: Department of Biochemistry, University of Wisconsin, Madison 6, Wis.

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[1] Abbreviations used in this work: PEP, phosphoenolypyruvate; AMP, ADP, ATP, monophosphate, diphosphate, and triphosphate of adenosine; GDP, IDP, UDP, CDP, dADP, dCDP, guanosine, inosine, uridine, cytidine, deoxyadenosine, and deoxycytidine diphosphates; NADH, reduced nicotinamide–adenine dinucleotide; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate.

as "low specific activity pyruvate kinase." The specific activity of the second batch under the same conditions was 380. This preparation was used throughout this work unless otherwise indicated. The specific activity of this latter preparation is about 40% higher than the values of 260–280 under similar conditions listed by Boyer (1962). Two attempts to repeat the isolation of a high specific activity preparation yielded a product with a specific activity of 280. The two batches of enzyme used in this work were assayed for adenosine triphosphatase and adenyl kinase activities by the procedure of Tietz and Ochoa (1958). Both activities were less than 0.05% of pyruvate kinase activity at pH 7.0.

Nucleoside Diphosphokinase Assay. The maximum amount of nucleoside diphosphokinase activity in the presence of ATP was measured by chromatography of the products on Dowex-1 formate with gradient elution by ammonium formate using the method of Hurlbert *et al.* (1954). The following reaction mixture was employed at 25.0°: 0.04 M imidazole buffer at pH 7.0, 0.075 M KCl, 0.004 M $MgCl_2$, 1.0×10^{-3} M CDP, 1.0×10^{-3} M PEP, $0.1\text{–}1.1 \times 10^{-3}$ M ATP, and 1.5–3.7 μg/ml pyruvate kinase for a total volume of 6.0–11.2 ml. Either the terminal phosphate on ATP or the PEP was labeled with ^{32}P in each case. When [^{32}P]PEP was used, $HgCl_2$ was added to hydrolyze the remaining PEP, which would otherwise obscure the radioactive peak of CTP. The concentrations of the nucleotides in the column effluent were determined spectrophotometrically and the radioactivity measurements were taken by plating 1 ml on a planchet, drying, and counting with a thin-window gas flow scaler.

Initial Velocity Measurements. The 2,4-dinitrophenylhydrazine method modified by Reynard *et al.* (1961) was used in the preincubation experiment for measuring pyruvate formation. The measurements of initial rates of pyruvate formation used in the determination of kinetic parameters for the various nucleoside diphosphates at various pH values were made by the coupling of lactic dehydrogenase and NADH to the system as described by Tietz and Ochoa (1958). The rate of oxidation of NADH was measured at 25.0° at 339.0 mμ in a Cary Model 14 recording spectrophotometer. The reaction mixture at pH 6.5, 7.0, and 7.5 consisted of the following: 0.02 M imidazole, 0.075 M KCl, 0.004 M $MgCl_2$, 1×10^{-4} M NADH, 1.0×10^{-3} M PEP, $0.467\text{–}2.0 \times 10^{-3}$ M nucleoside diphosphate, 10 μg of lactic dehydrogenase, and 0.224–2.24 μg of pyruvate kinase. For determinations at other pH values, the following changes were made: pH 8.0, 0.04 M imidazole; pH 9.0, 0.04 M glycine, 25 μg of lactic dehydrogenase; pH 6.0, 0.04 M maleic acid. The total volume was always 3.0 ml.

A few experiments using hexokinase and glucose 6-phosphate dehydrogenase were performed to measure the rate of ATP formation. The procedure was the same as with the lactic dehydrogenase coupling method except that the lactic dehydrogenase and the NADH were replaced with the following: 1×10^{-3} M glucose, 1×10^{-4} M $NADP^+$, 16 μg of glucose 6-phosphate dehydrogenase, and 32 μg of hexokinase.

Results

Nucleoside Diphosphokinase

A series of experiments was performed in which a varying pool of terminally labeled [^{32}P]ATP was added to a pyruvate kinase reaction mixture with CDP as the nucleotide substrate to determine how much label from the ATP became incorporated into CTP. Under these conditions, label is incorporated into CTP if the CTP is formed by nucleoside diphosphokinase activity, and the label is not incorporated into CTP if the CTP is synthesized by direct phosphorylation of CDP with PEP by pyruvate kinase activity. The choice of CDP in this system was based on its low relative reactivity in the pyruvate kinase system.

Table I presents the ratio of nucleoside diphospho-

TABLE I: Determination of the Ratio of Nucleoside Diphosphokinase Activity to Total CTP-Forming Activity Using [^{32}P]ATP.[a]

Experiment	1	2	3	4	5
Initial concentration ATP (mM)	0.079	0.79	1.10	0.79	1.10
[^{32}P]CTP formed (μmole)	0.068	0.230	0.506	0.097	0.315
(NUDIKI activity[b])/(Total CTP formed)	0.161	0.546	0.786	...	...

[a] Experiments 1–3 contained 1 mM PEP which was omitted from experiments 4 and 5. All experiments were performed at 25.0° in a 0.04 M imidazole buffer adjusted to pH 7.0 and containing 0.075 M KCl, 0.004 M $MgCl_2$, and 3.7 μg/ml "low specific activity pyruvate kinase" and were incubated for 10 min. For other details, consult text. [b] Nucleoside diphosphokinase (NUDIKI) activity is defined here as the amount of CTP necessary to account for the radioactivity in that fraction with a specific radioactivity equal to the ATP at the termination of each experiment.

kinase activity to total CTP-forming activity at different concentrations of ATP. The nucleoside diphosphokinase activity was taken as the number of μmoles of CTP necessary to account for the amount of radioactivity in the CTP fraction assuming a specific radioactivity equal to that of the ATP at the termination of the experiment. The total μmoles of CTP were corrected for the 1–4% CTP in the CDP reagent, as indicated by a zero enzyme control. The actual figure of 2.32% CTP in the CDP was based on zero PEP controls. The use of this procedure tends to maximize the apparent amount of nucleoside diphosphokinase activity. Thus it is apparent that a considerable amount of nucleoside di-

phosphokinase activity can occur if ATP is present. The rate of [^{32}P]CTP formation in experiment 3 was 1.36 μmoles/min/mg of pyruvate kinase. Another feature of these experiments was observed in the zero PEP controls. The absolute number of counts incorporated into the CTP was only 40–60% of the amount in the corresponding experiments with PEP present, suggesting that a considerable amount of the nucleoside diphosphokinase activity was PEP dependent.

To confirm that the unlabeled CTP in the previous experiments came directly from PEP rather than through cytidine triphosphate–cytidine monophosphokinase activity, experiments using [^{32}P]PEP and an unlabeled ATP pool were performed. Table II presents the

TABLE II: Determination of the Ratio of Nucleoside Diphosphokinase Activity to Total CTP-Forming Activity Using [^{32}P]PEP.[a]

Experiment	1	2
Initial concentration ATP (mM)	0.10	0.10
(NUDIKI activity[b])/ (Total CTP formed)	0.239	0.260

[a] Conditions were identical with those in experiments 1–3 of Table I except that the PEP rather than the ATP was labeled with ^{32}P and 3.0 and 1.5 μg/ml of "high specific activity pyruvate kinase" were used in experiments 1 and 2, respectively. [b] Nucleoside diphosphokinase (NUDIKI) activity is defined here as the amount of CTP formed which is in excess of the amount necessary to account for the radioactivity of the fraction with a specific radioactivity equal to that of the [^{32}P]PEP.

results. In these experiments the μmoles of CTP necessary to account for the total radioactivity of the CTP with a specific radioactivity equal to that of the PEP was used as a measure of pyruvate kinase activity. The μmoles of CTP in excess of this figure after correction for the CTP in the CDP reagent was assigned to nucleoside diphosphokinase activity. The correction for CTP initially present was determined by replacing the ATP with ADP so that all CTP formed during the course of the reaction would have the same specific radioactivity as the PEP. Since a different batch of CDP was being used at this time, the correction of 4.16% is somewhat larger than before. The ratio of nucleoside diphosphokinase activity to total CTP formed is in rather good agreement with the previous ratio at an equivalent ATP concentration.

Since the results of the experiments in Tables I and II indicated that nucleoside diphosphokinase is active at ATP concentrations as low as 1×10^{-4} M, it became necessary to determine the extent of this activity in the absence of exogenous ATP. Table III presents the results of preincubation with the enzyme and initiation of the CDP–pyruvate kinase reaction with either PEP

TABLE III: Assay of Nucleoside Diphosphokinase Activity by Preincubation.[a]

Initiating Component (Concn)	Pyruvate Formation (μmoles/min/mg of protein)	
PEP (1.00 mM)	1.67	1.67
CDP (0.96 mM)	1.55	1.67
PK[b] (1.86 μg/ml)	1.61	1.67

[a] A 10-min preincubation containing the complete system minus the initiating component was followed by a 10-min reaction period at 25.0° and pH 7.0. Pyruvate was assayed for by the 2,4-dinitrophenylhydrazine method. For other details, consult the text. [b] "Low specific activity pyruvate kinase" (PK) was used.

or CDP. If adenosine phosphates were present at sufficient concentrations and ATP were the predominant form, the tubes initiated with PEP would have the greatest rate.

The second method for estimating the maximal amount of nucleoside diphosphokinase activity possible with no exogenous ATP present is based on a determination of the amount of adenosine phosphates present. By comparing the rate of apparent pyruvate kinase activity by the lactic dehydrogenase method with the hexokinase–glucose 6-phosphate dehydrogenase method, one can determine the ratio of pyruvate formed to ATP formed. If CDP is the nucleotide substrate, the yeast hexokinase with an apparent Michaelis constant (K_m) for ATP of 1×10^{-4} M (Sols *et al.*, 1958) and present in great excess can mimic nucleoside diphosphokinase by converting any ATP to ADP for recycling by pyruvate kinase. The amount of ATP formed at any time is measured by reduction of $NADP^+$. At 2×10^{-3} M CDP and pH 7.0, the lactic dehydrogenase rate was 18–20 μmoles/min/mg of protein and the hexokinase rate was about 0.2 μmole/min/mg of protein or about 1% of the rate of pyruvate formation. The hexokinase system was completely functional since the apparent Michaelis constant and maximal velocities (V_{max}) determined for ADP with pyruvate kinase by this method were in good agreement with the values obtained by the lactic dehydrogenase method.

Kinetic Parameters of Nucleoside Diphosphates. The apparent K_m and V_{max} values for six different nucleoside diphosphates at six different pH values were determined. The best fit straight line was fitted to a $1/V$ *vs.* $1/S$ plot of triplicate velocity measurements at five concentrations using the weighting procedure and Fortran program described and provided by Cleland (1963). Computations were performed using the IBM 7040 digital computer at the University of Miami. Table IV contains the values of apparent K_m values with the standard errors, and Figure 1 is a plot of K_m against pH. Table V and Figure 2 are similar presentations for the V_{max} values. The pH 6.0 data are included for the sake

TABLE IV: The Apparent Michaelis Constants $\times 10^3$ M with Standard Errors for Several Nucleotides with Pyruvate Kinase.[a]

pH	ADP	GDP	IDP	UDP	CDP	dADP
6.0	36.8 ± 65.8	2.88 ± 0.48	−3.85 ± 0.36	5.61 ± 1.63	256 ± 3718	6.42 ± 2.25
6.5	0.417 ± 0.041	0.772 ± 0.38	2.71 ± 0.47	2.22 ± 0.28	8.11 ± 0.73	2.95 ± 0.28
7.0	0.227 ± 0.041	0.830 ± 0.219	1.85 ± 0.23	2.68 ± 0.22	4.39 ± 1.07	2.52 ± 0.22
7.5	0.197 ± 0.012	0.841 ± 0.038	1.81 ± 0.14	2.66 ± 0.20	6.87 ± 0.39	3.01 ± 0.53
8.0	0.202 ± 0.021	0.758 ± 0.059	1.80 ± 0.11	2.45 ± 0.13	2.80 ± 0.20	1.67 ± 0.13
9.0	0.122 ± 0.009	0.572 ± 0.062	1.78 ± 0.31	1.53 ± 0.25	1.49 ± 0.16	1.09 ± 0.08
	dCDP					
7.0	3.26 ± 0.36					

[a] "High specific activity pyruvate kinase" was used for this work. Pyruvate formation was measured by the lactic dehydrogenase method at 25.0°. For further details, consult the text.

TABLE V: The Apparent Maximal Velocities in μmoles/min/mg with Standard Errors for Several Nucleotides with Pyruvate Kinase.[a]

pH	ADP	GDP	IDP	UDP	CDP	dADP
6.0	2123 ± 4518	80 ± 10	−48 ± 6	15 ± 3	179 ± 4469	67 ± 21
6.5	483 ± 7	223 ± 6	400 ± 53	119 ± 9	63 ± 5	106 ± 23
7.0	380 ± 22	420 ± 62	219 ± 20	150 ± 8	67 ± 12	145 ± 10
7.5	279 ± 6	289 ± 7	234 ± 13	162 ± 8	80 ± 4	173 ± 24
8.0	300 ± 11	203 ± 9	249 ± 11	150 ± 5	44 ± 2	111 ± 6
9.0	96 ± 2	127 ± 7	112 ± 14	115 ± 10	19 ± 1	76 ± 3
	dCDP					
7.0	0.660 ± 0.050					

[a] "High specific activity pyruvate kinase" was used for this work. Pyruvate formation was measured by the lactic dehydrogenase method at 25.0°. For further details, consult the text.

of completeness, although the extrapolations for the K_m' and V_{max}' values of some of the nucleotides, in particular IDP, CDP, and ADP, are not valid owing to the distance of the concentration range studied from the K_m. The primary conclusion on the pH 6.0 data is that the apparent K_m values for all of the nucleotides tested are increasing in value from pH 6.5.

Discussion

Nucleoside Diphosphokinase. Although nucleoside diphosphokinase activity is apparently present, sufficient ADP and ATP are not present to affect the measurements of initial velocities of pyruvate formation by the other nucleoside diphosphates. However, one interesting observation is the partial dependence of apparent nucleoside diphosphokinase activity on the presence of PEP. This phenomenon suggests that at least one-half of the measured nucleoside diphosphokinase activity may be a function of pyruvate kinase. It may be hypothesized that the apparent K_m values for substrates of pyruvate kinase are inversely related to the "dwell time" distributions, *i.e.*, as the K_d increases the specific rate constant controlling the dissociation reaction increases relatively more rapidly than the association rate constant decreases. Reynard *et al.* (1961) have shown that the apparent K_m for PEP is nearly an order of magnitude lower than that of ADP. This study has demonstrated that the apparent K_m of CDP is more than an order of magnitude larger than that of ADP. Thus one might imagine that [^{32}P]ATP and pyruvate would react to form [^{32}P]PEP and ADP. If PEP has a greater "dwell time" distribution than ADP, the probability is increased that the same [^{32}P]-PEP molecule formed would remain on the enzyme while the ADP diffused off and a CDP diffused on. Thus, [^{32}P]CTP and pyruvate could be formed. Pyruvic kinase would then behave as a PEP-dependent nucleoside diphosphokinase by this somewhat specialized exchange reaction which would also label the PEP pool but at a lower rate than it labels CTP. The specific radioactivity of the PEP was always far lower than that of either the ATP or the CTP.

Apparent Michaelis Constants. The mechanism pro-

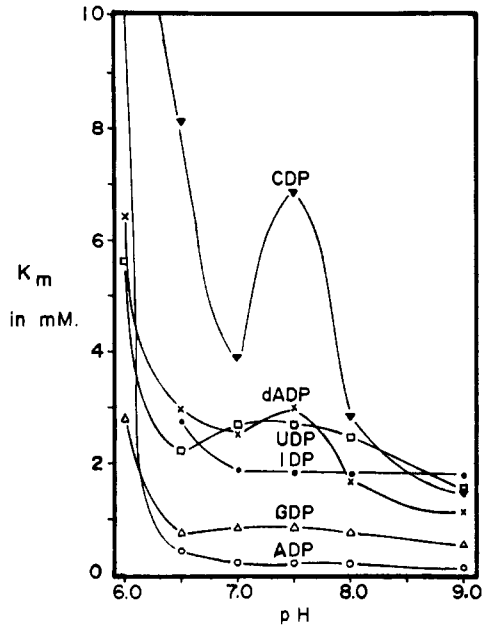


FIGURE 1: A plot of the data from Table IV.

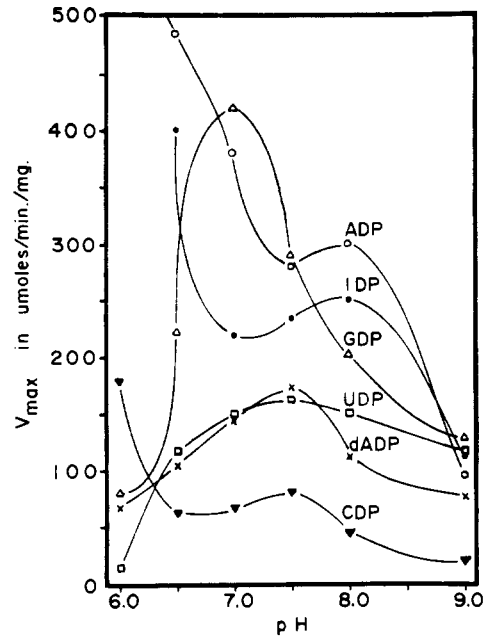
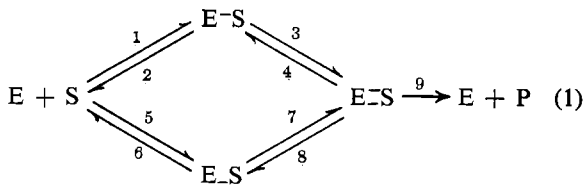


FIGURE 2: A plot of the data from Table V.

posed for pyruvate kinase by Reynard *et al.* (1961) is rapid equilibrium–random order for the binding and release of reactants and products. This model was formulated using ADP as the nucleotide substrate and requires that the apparent K_m and the dissociation constant (K_d) of the enzyme–substrate complex be equivalent. The plot of apparent K_m against pH for ADP is very similar in shape to the corresponding plot of the K_d for ADP published by Mildvan and Cohn (1965). The steeply descending portion of the ADP curve in Figure 1 between pH 6.0 and pH 7.0 could represent either a requirement for doubly ionized phosphate groups or a requirement for a metal–enzyme complex in which the metal is bound to an imidazole group on the enzyme. Rose (1959) has demonstrated that doubly ionized phosphate is necessary for one activity of the enzyme. Mildvan and Cohn (1965) have shown that the metal–enzyme is the active form of this enzyme and that the metal binding has a pK_a of 6.8 corresponding to an imidazole group. The curves in Figure 1 may be compound, representing a requirement for both of these conditions which have similar pK_a values.

The inactivity of AMP has been well documented by Reynard *et al.* (1961) and others. However, the considerable differences in apparent K_m values indicate that at least one other portion of the nucleoside diphosphate, besides the β-phosphoryl group, is involved in binding. If only two binding regions exist at the nucleoside diphosphate site, there are three possible orders to the binding, two ordered sequences and the random sequence. Although the absolute value for the apparent K_m of ADP at pH 6.0 is not very reliable, it does indicate that the phosphoryl group binding is important since the adenosine portion undergoes no ionization in this pH range. The apparent lack of binding by AMP strengthens this view. If no ionization of the enzyme occurs between pH 6.0 and 7.0 in the region involved with binding of the adenosine portion of ADP, it is apparent that at pH 7.0 the β-phosphoryl portion of ADP is the dominant term of K_m and, hence, K_d. If one considers the random order of binding indicated in eq 1 as the general case, one may place certain limitations on this scheme, using the information that the phosphoryl binding term is dominant.



(1)

Under this limitation the random order model degenerates into a functionally obligatory order with the phosphoryl portion normally binding first and the nucleoside portion normally binding second. By arbitrarily assigning the upper bond between E and S to the phosphoryl–enzyme ligand and the lower bond to the nucleoside–enzyme ligand, one may express the K_m and the V_{max} derived from the steady-state rate equations in eq 2 and 3.

$$K_m = \frac{k_2(k_4 + k_9) + k_3k_9}{k_1(k_3 + k_4 + k_9)} \quad (2)$$

$$\frac{V_{max}}{(E_0)} = \frac{k_3k_9}{k_3 + k_4 + k_9} \quad (3)$$

If the rapid equilibrium mechanism postulated by

Reynard *et al.* (1961) for ADP is true, then k_9 is much smaller than k_1, k_2, k_3, and k_4, and the K_m and V_{max} may be simplified as in eq 4 and 5.

$$K_m = \frac{k_2}{k_1}\left[\frac{k_4}{k_3 + k_4}\right] \quad (4)$$

$$\frac{V_{max}}{E_0} = k_9\frac{k_3}{k_3 + k_4} \quad (5)$$

By considering the data for pH 7.0 and 7.5, one notes that the apparent V_{max} values for ADP and GDP are essentially the same although the apparent K_m values are different by about threefold. This condition would be expected from the above model if the best substrates have a favorable equilibrium constant for the binding of the nucleoside portion, *i.e.*, $k_3 > k_4$. The apparent V_{max} becomes equal to k_9 and the apparent K_m becomes equal to the K_d, as would be expected from the model by Reynard *et al.* (1961). Assuming that the phosphoryl binding remains approximately constant for all nucleoside diphosphates, the apparent K_m would eventually approach the phosphoryl binding constant of k_2/k_1 with poor substrates. It should be noted that the apparent K_m values for CDP and dCDP are nearly equal, although the V_{max} values are greatly different. Another relationship that must hold is given in eq 6 which is derived from eq 2 and 3 with the deletion of the k_3k_9 term from the numerator of eq 2.

$$\frac{V_{max}}{[E_0]K_m}\left[\frac{E_0}{V_{max}} - \frac{1}{k_9}\right] = \frac{k_1}{k_2} = \text{constant} \quad (6)$$

By using the V_{max} for ADP as k_9, the values for k_1/k_2 are calculated in Table VI. These values are nearly constant for each given pH.

TABLE VI: The Values of k_1/k_2 (M^{-1}) Calculated by Eq 6 from the Data of Tables IV and V.

pH	GDP	IDP	UDP	CDP	dADP	dCDP
7.0	—	243	233	190	265	306
7.5	—	97	161	105	118	—
8.0	428	95	204	304	377	—

If one uses the average value of k_1/k_2 for any given pH in eq 4 and the V_{max} for ADP as k_9, one may calculate the ratios of k_4/k_3 for each nucleotide at each pH in this region. These values should be a first approximation to the binding constant and/or the inhibition constant (K_I) for the various nucleoside monophosphates or nucleosides. For AMP the K_I should be approximately 0.055 M at pH 7.0. Previous studies indicating a lack of inhibition by AMP utilized only 1.1 mM AMP (Reynard *et al.*, 1961).

In the region above pH 8, the interpretation of the data is more obscure. If the two-step obligatory order mechanism applies fully to this region, it is not possible to say definitively which region of the nucleotide binding is being affected. The pK for the decrease in K_m and V_{max} values is in the pH region 8.2–8.5, which corresponds to the ionization point of an α-amino group or a sulfhydryl group. Since the V_{max} for ADP, which is being considered here as equivalent to k_9 in eq 1, is decreasing, it appears more probable that the β-phosphoryl group of the nucleotide is being affected. Mildvan and Cohn (1965) have proposed that an α-amino group is involved in the binding of metal to the enzyme, an interpretation which supports our conclusion in this respect.

References

Adam, H. (1961), *Biochem. Z. 335*, 25.

Boyer, P. D. (1962), *Enzymes 6*, 95.

Cleland, W. W. (1963), *Nature 198*, 463.

Davidson, E. Z. (1959), *Biochim. Biophys. Acta 33*, 238.

Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209*, 23.

Khym, J. X., and Cohn, W. E. (1953), *J. Am. Chem. Soc. 75*, 1153.

Mildvan, A. S., and Cohn, M. (1965), *J. Biol. Chem. 240*, 238.

Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961), *J. Biol. Chem. 236*, 2277.

Rose, I. A. (1960), *J. Biol. Chem. 235*, 1170.

Sols, A., de la Fuente Sanchez, G., Villar-Palasi, C., and Asensio, C. (1958), *Biochem. Biophys. Acta 30*, 92.

Strominger, J. L. (1955), *Biochim. Biophys. Acta 16*, 616.

Tietz, A., and Ochoa, S. (1958), *Arch. Biochem. Biophys. 78*, 477.