ATP are well represented by the rate laws for these two mechanisms. The most reasonable explanation of the K_i(ATP) term is that MgATP is the true substrate, while ATP inhibits the reaction. In fact, this inhibition permits a distinction to be made between mechanisms 7 and 8.

The competitive inhibition in these two mechanisms can be written as

7. $E + ATP \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} EATP$ (inactive)

8. $EG + ATP \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} EGATP$ (inactive)

The steady state initial velocities for mechanisms 7 and 8, respectively, are now

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)}[1 + K_i(ATP)] + \frac{\phi_2\phi_4}{(MgATP)(G)}[1 + K_i(ATP)] \quad (9)$$

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)} + \frac{\phi_3}{(MgATP)}[1 + K_i(ATP)] + \frac{\phi_3\phi_4}{(MgATP)(G)} \quad (10)$$

where $K_i = k_7/k_{-7}$. Equation 9 fits the data very poorly, while equation 10 is identical with the experimentally determined equation given in the results section. Inhibition by combination of of ATP with X_1, EMgATP (in 7) and E (in 8) were also considered, but the resulting rate laws were inconsistent with the experimental results. Thus the conclusion of this study is that only mechanism 8 (of those considered) explains all of the data in a satisfactory manner. The formation of an enzyme–glucose complex is postulated, but it is *not* "undissociable" as proposed in the mechanism of Najjar and McKay[4] on the basis of their exchange data. In fact, the mechanism proposed here is consistent with their results, but their mechanism is *not* consistent with the steady state kinetic data. An enzyme–phosphate intermediate cannot be ruled out *if* the phosphate transfer occurs only when both substrates are bound to the enzyme. Although this mechanism may not sound attractive, it certainly is possible that both substrates are necessary in order for the enzyme to have the correct conformation for phosphate transfer. In this same vein, a very attractive hypothesis is that the binding of glucose by the enzyme changes the enzyme conformation sufficiently to enhance greatly the binding of MgATP occuring in the next step. This matter is being investigated further.

Only two of the rate constants can be calculated exactly from the steady state data presented; however, lower bounds can be found for many of the rate constants using a procedure similar to that employed by Peller and Alberty.[17] The results obtained are

$k_2 = 1/\phi_2 = 3.7 \times 10^6\ M^{-1}$ sec.$^{-1}$ $\quad k_4 > 1/\phi_1 = 750$ sec.$^{-1}$

$k_{-2} = \phi_4/\phi_2 = 1.5 \times 10^3$ sec.$^{-1}$ $\quad k_5 > 1/\phi_1 = 750$ sec.$^{-1}$

$k_3 > 1/\phi_3 = 3.3 \times 10^6\ M^{-1}$ sec.$^{-1}$

Due to the difficulty of obtaining the absolute value of (E_0) accurately, the rate constants are probably certain to only ± 40%. From previous work,[13,14] it is also known that at 25° and in 0.1 M KNO_3, $k_1 = 1.2 \times 10^7\ M^{-1}$ sec.$^{-1}$, $k_{-1} = 1.2 \times 10^3$ sec.$^{-1}$, $k_6 = 3 \times 10^6\ M^{-1}$ sec.$^{-1}$ and $k_{-6} = 2.5 \times 10^3$ sec^{-1}. The association constant for the enzyme-glucose complex is $2.5 \times 10^3\ M^{-1}$ (± 25%). This is about 60% smaller than the value found by Trayser and Colowick[18] by an indirect method. The rate constants will be discussed more fully in the next paper of this series.[19]

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(17) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).

(18) K. Trayser and S. P. Colowick, *Arch. Biochem. Biophys.*, **94**, 169 (1961).

(19) G. G. Hammes and D. Kochavi, *J. Am. Chem. Soc.*, **84**, 2073 (1962).

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Studies of the Enzyme Hexokinase. II. Kinetic Inhibition by Products

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The rate of transfer of a phosphate group from ATP to D-glucose is retarded by the presence of initial concentrations of the products, ADP and glucose-6-phosphate. This inhibition is in quantitative agreement with the previously proposed mechanism of action for the enzyme hexokinase. From these studies, the binding constants for glucose-6-phosphate and free enzyme and for MgATP and an enzyme–glucose complex were found to be $1.1 \times 10^2\ M^{-1}$ and $6.2 \times 10^3\ M^{-1}$, respectively. The latter constant is only about six times larger than the binding constant for the enzyme–glucose complex and free ATP. ($1.1 \times 10^3\ M^{-1}$). This suggests that Mg is not a very important factor for binding of the substrate to the enzyme; instead the primary role of Mg is probably to aid in the bond breaking step in the reaction. Combining all of the kinetic data available with the equilibrium constant for the over-all reaction permits all twelve of the rate constants in the mechanism to be determined.

Introduction

The previous paper in this series[1] proposed the following mechanism for the hexokinase catalyzed transfer of a phosphate group from adenosine triphosphate (ATP) to D-glucose

(1) G. G. Hammes and D. Kochavi, *J. Am. Chem. Soc.*, **84**, 2069 (1962).

$$Mg^{++} + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MgATP \qquad EG6P \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + G6P$$

$$E + G \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EG \qquad MgADP \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} Mg^{++} + ADP$$

$$EG + MgATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X_1 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EG6P + MgADP$$

$$EG + ATP \rightleftharpoons EGATP(inactive)$$

Here E represents hexokinase, G is glucose, ADP is adenosine diphosphate, G6P is glucose-6-phosphate, X_1 is an intermediate complex and other symbols have their obvious meanings. Kinetic studies of the reaction can be made only in the forward direction since the over-all equilibrium lies quite far towards the products. Therefore, the assumption was made that the mechanism is symmetrical with respect to MgATP and MgADP and consequently with respect to D-glucose and G6P.

A considerable amount of additional information can be found by studying the inhibitory effect of ADP and G6P on the initial reaction velocity. In addition to confirming the above mechanism, such studies, when coupled with the usual steady state experiments, and independent determinations of the equilibrium constant and the rate constants k_1, k_{-1}, k_6 and k_{-6}, allow calculation of all twelve of the rate constants in the mechanism. The results for the hexokinase system provide some insight into the molecular details of the reaction.

Theory.—The rate equation for the initial velocity of the forward reaction with the concentrations of ADP and G6P equal to zero has been given previously.[1] If the concentrations of both products are simultaneously non-zero, the equation for the initial velocity becomes quite complex. However, if only one of the product concentrations is unequal to zero, the equations are relatively simple.

If the initial concentration of ADP is equal to zero, the ratio of the total enzyme concentration (E_0) to the initial velocity for the above mechanism is

$$\frac{(E_0)}{v} = \phi_1 + \frac{\phi_2}{(G)}[1 + \phi_5(G6P)] + \frac{\phi_3}{(MgATP)}[1 + K_i(ATP)] + \frac{\phi_3\phi_4}{(G)(MgATP)}[1 + \phi_5(C_6P)] \quad (1)$$

where (E_0) is the total enzyme concentration, K_i is the equilibrium binding constant for ATP and EG and the ϕ's are independent functions of the rate constants defined in Table I of the results section. All of the ϕ's other than ϕ_5 can be obtained by experiments where the concentration of G6P is initially zero (*cf.* ref. 1). Further experiments with several different G6P concentrations will allow the determination of ϕ_5.

The situation where the concentration of G6P is equal to zero, while that of ADP is varied, is slightly more complex. In the first place, only the metal complexed species MgADP occurs in the enzyme reaction proper, but since free ATP inhibits the reaction, uncomplexed ADP would be an inhibitor also. The easiest way to avoid this complication is to work at high enough metal concentrations so that both ATP and ADP are essentially completely complexed. The second point which must be taken into account is that since ATP can inhibit the reaction by combining with EG, MgADP could cause inhibition in a similar manner. Therefore, the following additional step, with an equilibrium quotient K_i', must be included in the mechanism

$$MgADP + EG \rightleftharpoons EGMgADP(inactive)$$

The initial velocity function under the conditions discussed above is

$$\frac{(E_0)}{v} = \phi_1 + \phi_6(MgADP) + \left[\frac{\phi_3}{(MgATP)} + \frac{\phi_6\phi_7(MgADP)}{(MgATP)}\right][1 + K_i'(MgADP)] + \frac{\phi_2}{(G)} + \frac{\phi_3\phi_4}{(G)(MgATP)} + \frac{\phi_4\phi_6\phi_7(MgADP)}{(G)(MgATP)} \quad (3)$$

where the ϕ's are defined in Table I. Thus two new independent functions of the rate constants can be obtained, one of them (ϕ_7) being the equilibrium binding constant for MgATP and EG.

Neglecting the two steps in the mechanism involving the reaction of Mg^{++} with ATP and ADP (which can be studied independently[2,3]), seven independent functions of the eight rate constants can be obtained from steady state kinetic experiments. An eighth independent function of the rate constants is the equilibrium quotient for the over-all reaction

$$MgATP + G \rightleftharpoons MgADP + G6P$$

$$K_{eq} = \frac{k_2k_3k_4k_5}{k_{-2}k_{-3}k_{-4}k_{-5}} \quad (4)$$

(or ATP + G $\rightleftharpoons$ ADP + G6P since the binding constants for Mg^{++} to ATP and ADP are known[1]). The hexokinase equilibrium has been studied in great detail with tracer techniques by Robbins and Boyer.[4]

Thus in principle all eight of the rate constants can be calculated. However, in practice this may not always be true: if $k_{-3} >> k_4$, ϕ_3 is no longer an independent function of the rate constants, or if $k_5 << k_4$, ϕ_1 is not an independent function. Even in these cases, at least a lower bound can be obtained for all of the rate constants.[1]

Experimental

The procedure for preparing the solutions, determining the binding constants and carrying out the kinetic runs was exactly as previously described.[1] The only new chemicals used were the disodium salts of ADP(Pabst) and G6P (Calbiochem, Grade A). These were converted to the hydrogen form with Dowex 50 resin and their concentrations were determined spectrophotometrically and by titration with standard base respectively.

For the binding constant determinations, the concentrations of ADP and G6P were 10^{-3} *M* while Mg^{++} was 10^{-2} *M* and 5×10^{-2} *M*, respectively. In the kinetic experiments, the total Mg concentration was 10^{-2} *M* in all cases when ADP was used in order to insure that ATP and ADP were

(2) H. Diebler, M. Eigen and G. G. Hammes, *Z. Naturforsch.*, **15b**, 554 (1960).

(3) M. Eigen and G. G. Hammes, *J. Am. Chem. Soc.*, **82**, 5951 (1960); **83**, 2786 (1961).

(4) E. A. Robbins and P. D. Boyer, *J. Biol. Chem.*, **224**, 121 (1957).

over 90% in the form of the Mg complex. For G6P the total Mg^{++} concentration was 5×10^{-3} M. The total ATP concentration was either 1×10^{-3} M or 5×10^{-4} M, while those of ADP and G6P were varied from 0 to 2×10^{-3} M.

Results

The equilibrium quotients of interest for ADP and G6P in the kinetic experiments are

$$K_{ADPH} = \frac{(ADPH)}{(ADP)a_H} = 4.5 \times 10^6\ M^{-1}$$

$$K_{ADPM} = \frac{(MgADP)}{(Mg^{++})(ADP)} = 1.2 \times 10^3\ M^{-1}$$

$$K_{G6PH} = \frac{(G6PH)}{(G6P)a_H} = 2.0 \times 10^6\ M^{-1}$$

$$K_{G6PM} = \frac{(MgG6P)}{(Mg^{++})(G6P)} = 34\ M^{-1}$$

The results are for 25.0° and 0.3 M $(CH_3)_4NCl$. Similar quotients have already been reported for ATP.[1]

For all kinetic measurements, $(E_0)/v$ at constant total concentrations of ATP and ADP or G6P was plotted against 1/(G) and the slopes and intercepts were determined. When the concentration of ADP is initially equal to zero and the total concentration of ATP is constant, equation 1 predicts that the slope should be a linear function of G6P, while the intercept should be independent of G6P. The intercept for (MgATP) = 0.98×10^{-3} M was 1.7 ($\pm$0.02) 10^{-3} sec. while a plot of the slope *versus* the total concentration of G6P was linear within experimental error. In these experiments the amount of G6P complexed was always less than 15% of the total concentration. Since MgG6P undoubtedly inhibits the reaction to some extent also, the use of the total G6P concentration cannot be seriously in error. The ratio of the slope to intercept of such a plot is equal to ϕ_5. The value found, 1.1×10^2 M^{-1}, is in reasonable accord with that obtained by Trayser and Colowick[5] (2.5×10^2 M^{-1}) by an indirect method.

When the initial concentration of G6P is equal to zero, the treatment of the data is somewhat more complex. The slopes of the $(E_0)/v$ — 1/(G) plots are linear functions of MgADP at constant MgATP. As predicted by equation 3, the slope of the plots of $(E_0)/v$ — 1/(G) slopes *versus* MgADP is, within experimental error, inversely proportional to MgATP: 60 ($\pm$5) $\times 10^{-6}$ sec. for (MgATP) = 5×10^{-4} and 35 ($\pm$5) $\times 10^{-6}$ sec. for (MgATP) = 1×10^{-3}. Using the known values of ϕ_4[1] and the MgATP concentrations, the average value of $\phi_6\phi_7$ is found to be 8.1 ($\pm$ 0.7) $\times 10^{-5}$ sec. The intercepts of the $(E_0)/v$ — 1/(G) plots are not simple linear functions of MgADP. A term proportional to $(MgADP)^2$ is also present. A method of successive approximations was used to determine ϕ_6 and K_i'. The square term was first considered negligible and ϕ_6 and K_i' were calculated. Since the values of all the kinetic constants were then known, the numerical value of the square term could be calculated and subtracted from the experimental values of the intercepts. Using these new values of the intercepts ϕ_6 and K_i' were again determined neglecting the square term. This procedure was repeated until constant values of ϕ_6 and K_i' were obtained. Fig. 1 shows the intercepts plotted *versus* the concentration of MgADP at two different concentrations of MgATP. The solid lines in Fig. 1 are calculated from equation 3 with the ϕ's and K_i' in Table I. The dashed lines are the calculated curves with the square term subtracted off.

(5) K. Trayser and S. P. Colowick, *Arch. Biochem. Biophys.*, **94**, 169 (1961).

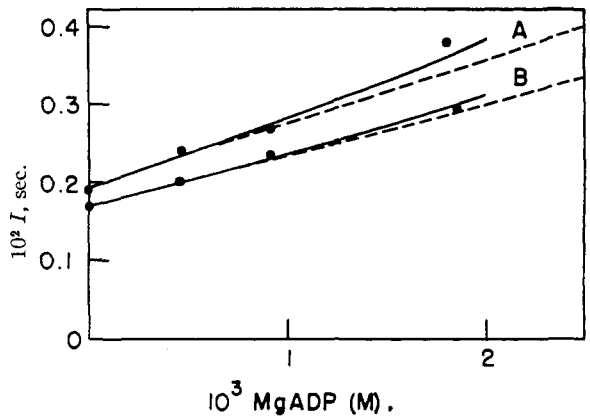

Fig. 1.—The intercepts of $(E_0)/v$ — 1/(G) plots *versus* the MgADP concentration: A, (MgATP) = 4.95×10^{-4} M; B, (MgATP) = 1.00×10^{-3} M. The solid lines are calculated from equation 3 and Table I while the dashed lines are the same with the term containing $(ADP)^2$ subtracted off.

The experimentally determined kinetic constants from this and previous work[1] are summarized in Table I.

TABLE I

Constant	Definition	Experimental value[a]
ϕ_1	$1/k_3 + 1/k_5$	1.34×10^{-3} sec. ($\pm$15%)
ϕ_2	$1/k_2$	2.7×10^{-7} M sec. ($\pm$15%)
ϕ_3	$(k_{-3} + k_4)/k_3k_4$	3.0×10^{-7} M sec. ($\pm$15%)
ϕ_4	k_{-2}/k_2	4.0×10^{-4} M ($\pm$25%)
ϕ_5	k_{-5}/k_5	1.1×10^2 M^{-1} ($\pm$25%)
ϕ_6	k_{-4}/k_4k_5	0.49 M^{-1} sec. ($\pm$25%)
ϕ_7	k_{-3}/k_3	1.6×10^{-4} M ($\pm$30%)
K_i	See text	1.1×10^3 M^{-1} ($\pm$20%)
K_i'	See text	3.3×10^2 M^{-1} ($\pm$25%)

[a] All results are for *p*H 8.0 ($\pm$ 0.05), 25.0 $\pm$ 0.1° and 0.3 M $(CH_3)_4NCl$.

Discussion

The results of the product inhibition studies all support the proposed mechanism for hexokinase. In addition, some inferences concerning the molecular details of the reaction can be made. The fact that the binding constant of glucose and enzyme (2.5×10^3 M^{-1}) is much larger than that for G6P and enzyme (1.1×10^2 M^{-1}) suggests either that the OH group of the 6 carbon on glucose is important in the binding process or that the electrostatic effect of the charged phosphate group inhibits the binding. The binding constant of

MgATP and EG ($6.2 \times 10^3\ M^{-1}$) is only about six times that for ATP and EG which indicates that although MgATP is the actual substrate rather than ATP, the role of the metal ion cannot be only to enhance the binding of ATP to the enzyme. This is contrary to the idea frequently brought forth that the metal ion acts as a strong structural cement between substrate and enzyme. Instead, a good deal of the binding seems to occur through the adenine and/or ribose portions of ATP which is consistent with the extended conformation of MgATP in solution proposed on the basis of nuclear magnetic resonance experiments.[6] The primary role of Mg^{++} is most likely to polarize the oxygen–phosphate bond being broken while anchoring the phosphate group of ATP to the enzyme. A more detailed discussion of the function of the metal ion will be presented elsewhere.[7]

If the equilibrium constant were known, enough data is available to calculate all of the rate constants which are still unknown. Robbins and Boyer[4] have determined the equilibrium constant under different conditions (pH 6, 30° and variable ionic strength) than those employed in our experiments, but at least an approximate value of the desired constant can be obtained from their data. A reasonable estimate is that the desired equilibrium constant (as defined by equation 4) is about 300. Using this value and the ϕ values in Table I, the rate constants can be calculated to within a factor of 2 or 3. Of course, k_1, k_{-1}, k_2, k_{-2}, k_6 and k_{-6} have all been determined independently and are known to about ± 30%.[1-3] A tabulation of all of the rate constants is

$^*k_1 = 1.2 \times 10^7\ M^{-1}$ sec.$^{-1}$	$^*k_{-1} = 1.2 \times 10^3$ sec.$^{-1}$
$k_2 = 3.7 \times 10^6\ M^{-1}$ sec.$^{-1}$	$k_{-2} = 1.5 \times 10^3$ sec.$^{-1}$
$k_3 = 4 \times 10^6\ M^{-1}$ sec.$^{-1}$	$k_{-3} = 6.5 \times 10^2$ sec.$^{-1}$
$k_4 = 3 \times 10^3$ sec.$^{-1}$	$k_{-4} = 2 \times 10^6\ M^{-1}$ sec.$^{-1}$
$k_5 = 1 \times 10^3$ sec.$^{-1}$	$k_{-5} = 1 \times 10^5\ M^{-1}$ sec.$^{-1}$
$^*k_6 = 2.5 \times 10^3$ sec.$^{-1}$	$^*k_{-6} = 3 \times 10^6\ M^{-1}$ sec.$^{-1}$

The constants marked with * were determined at 25° and an ionic strength of 0.1 M KNO_3.

The magnitudes of k_2 and k_3 are fairly large but not nearly as large as many other enzyme–substrate reactions which approach the theoretical upper limit for a bimolecular rate constant ($\sim 10^9\ M^{-1}$ sec.$^{-1}$).[8,9] An interesting point is that MgADP binds to both EG and EG6P ($K = 3.3 \times 10^2\ M^{-1}$ and $2 \times 10^3\ M^{-1}$, respectively); on the other hand no experimental evidence exists for the binding of MgATP and EG6P. This binding should be detectable if the binding constant is greater than about 50. This indicates that the phosphate group on G6P blocks the binding of MgATP very effectively.

Finally it should be pointed out that the procedure used for obtaining this rather detailed mechanism for the hexokinase system is applicable to phosphate transferring enzymes in general.

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Addendum.—Since this work was completed a kinetic study of the enzyme pyruvate kinase was carried out by Reynard, Hass, Jacobsen and Boyer.[10] The mechanism involved is apparently somewhat different than that proposed for hexokinase.

(6) G. G. Hammes, G. E. Maciel and J. S. Waugh, *J. Am. Chem. Soc.*, **83**, 2394 (1961).

(7) G. G. Hammes and D. Kochavi, *ibid.*, **84**, 2076 (1962).

(8) L. Peller and R. A. Alberty, *ibid.*, **81**, 5907 (1959).

(9) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).

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

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Studies of the Enzyme Hexokinase. III. The Role of the Metal Ion

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Steady state kinetic experiments were carried out with hexokinase using Ca^{++} as the metal ion activator. Although Ca^{++} is a much poorer activator than Mg^{++}, both metals utilize the same mechanism. Comparison of the two metal ions as activators indicates that the rates of complex formation are not rate determining. In fact, Ca^{++} in general builds complexes about 100 times faster than Mg^{++}. Furthermore, the equilibrium quotients involving the metal ions are approximately the same. However, the breakdown of the quaternary intermediate complex occurs over one hundred times faster for the Mg^{++} system indicating that the rate controlling step involves polarization of the media (probably breaking of a chemical bond) since Mg^{++} should be much more effective than Ca^{++} for this purpose. Comparison of the rate constants for the two systems suggests that at least two quaternary intermediates are necessary to explain the results in a satisfactory manner.

Introduction

Recent studies[1,2] of the enzyme hexokinase have indicated that the most probable mechanism for the transfer of a phosphoryl group from ATP to glucose is

$$\mathrm{Me} + \mathrm{ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MeATP} \qquad \mathrm{EG6P} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{G6P}$$

$$\mathrm{E} + \mathrm{G} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EG} \qquad \mathrm{MeADP} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{Me} + \mathrm{ADP}$$

$$\mathrm{EG} + \mathrm{MeATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{X_1} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{EG6P} + \mathrm{MeADP}$$

$$\mathrm{EG} + \mathrm{ATP} \rightleftharpoons \mathrm{EGATP(inactive)}$$

Here E respresents hexokinase, ATP is adenosine triphosphate, Me is the metal ion activator (Mg^{++} in previous studies), G is glucose, X_1 is a quaternary

(1) G. G. Hammes and D. Kochavi, *J. Am. Chem. Soc.*, **84**, 2069 (1962).

(2) *Ibid.*, 2073 (1962).