UNIVERSITY OF OTTAWA

DEPARTMENT OF CHEMISTRY

KINETICS OF MUSCLE ADENOSINE TRIPHOSPHATASE

by

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INTRODUCTION

In the presence of myosin, a muscle protein, adenosine triphosphate (ATP) is hydrolyzed to adenosine diphosphate (ADP) and inorganic orthophosphate (P_i).

$$ATP \xrightarrow{\text{Myosin}} ADP + P_i \qquad (1)$$

This reaction discovered by Engelhardt and Ljubimova (8) was shown by Szent-Gyorgyi (28) to be associated with the phenomenon of muscular contraction.

On the basis of ultracentrifugal and viscometric data, Mommaerts (22), Weber (31), Johnson and Landolt (13,14), assumed that myosin dissociates into smaller particles upon addition of ATP. On the other hand Blum and Morales (3) have shown by light - scattering experiments that ATP causes a structural change in the myosin without depolymerization. This last fact is consistent with the muscular contraction theory of Morales and Botts (24,25) who assume that a balanced between electrostatic and entropic forces determines the length of the myosin particles.

Under certain conditions (to be discussed later) a kinetic study of this enzymatic reaction provides information on the thermodynamics of the formation of the myosin-ATP complexes and as a result on the nature of the forces involved. The purpose of this investigation is to obtain more information on the myosin-ATP system as a particular case of an enzymatic reaction.

Various aspects of the kinetics of this reaction have been studied in the past. Mommaerts (23) has found that the rate of the reaction is activated by calcium ions and inhibited by magnesium ions. He also observed that by increasing the pH from 7 to 9 the rate was enhanced in the presence of calcium ions.

More recently Mommaerts (10,11,21,27) has made a further study on pH, calcium, magnesium and ADP effects at 27°C.

Ouellet, Laidler and Morales (26) have shown that the reaction follows a law of the form

$$\frac{d\,[ATP]}{dt} = \frac{k_2\ K\ [Myosin]\ [ATP]}{1\ +\ K\ [ATP]} \qquad (2)$$

At pH 7.0, in the presence of 0.001M Ca^{++} and 0.6M KCl, K and k_2 were determined at different temperatures. There was some evidence to suggest that K is an equilibrium constant for the formation of the enzyme-substrate complex. This provided the basis for the calculation of thermodynamic functions (ΔH, ΔS°, ΔF°) for the formation of the myosin - ATP complex. Solvent and structural effects were investigated by Laidler and Ethier (18) by carrying out the reaction in solvents of different dielectric constants, (water and ethanol). Laidler and Beardell (17) studied the reaction over a range of hydrostatic pressures and at different ionic strengths.

The above work indicates a conformational change in the protein during the reaction, and direct evidence for such a change was obtained by the light-scattering experiments of Blum and Morales (3).

Further kinetic studies were conducted by Watanabe (29,30) who investigated the effect of Ca^{++} and Mg^{++} ions on muscle ATPase activity.

THEORETICAL PART

Rate Laws

A general reaction mechanism for an enzyme catalyzed reaction involving two substrates or a substrate and a modifier can be represented by the following equations.

$$E + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM \tag{3}$$

$$E + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES \xrightarrow{k_o} E + \text{Products} \tag{4}$$

$$EM + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EMS \tag{5}$$

$$ES + M \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EMS \xrightarrow{k_r} E + \text{Products} \tag{6}$$

In this scheme E is the enzyme molecule, S and M are molecules that form with E the binary complexes ES and EM and the ternary complex EMS. In this discussion only the complexes ES and EMS will be considered as dissociating into products. The rate constants k_o and k_r are first-order rate constants for the breakdown of ES and EMS into enzyme and the products of the reaction. All other rate constants are for simple adsorption and desorption processes.

The following general steady state solution for this system was obtained recently by Laidler (15).

To facilitate solution of the steady-state equations the following definitions are made:

$$K_1 = \frac{k_1}{k_{-1}} \qquad (7) \qquad K_2 = \frac{k_2}{k_{-2}} \qquad (8)$$

$$K_3 = \frac{k_3}{k_{-3}} \qquad (9) \qquad K_4 = \frac{k_4}{k_{-4}} \qquad (10)$$

$$\overline{K_2} = \frac{k_2}{k_{-2} + k_o} \qquad (11)$$

Thermodynamics requires that

$$K_2 K_4 = K_1 K_3 \qquad (12)$$

Dimensionless variable U_{ES}, U_{EM}, U_{EMS}, are introduced and are defined by the following equations.

$$\frac{[ES]}{[E]} = \overline{K_2}\,[S] + U_{ES} \qquad (13)$$

$$\frac{[EM]}{[E]} = K_1\,[M] + U_{EM} \qquad (14)$$

$$\frac{[EMS]}{[E]} = \overline{K_2}\,K_4\,[S]\,[M] + U_{EMS} \qquad (15)$$

Dimensionless variables of this type were first introduced by Botts and Morales (4) in their treatment of modifier effects, and were also used (16) in a theory of pH effects on enzyme systems.

Four relationships are required in order to solve for the four concentrations [E], [ES], [EM], and [EMS]. One of these is given by the fact that the total concentration of enzyme $[E]_o$, is the sum of the concentration of the four forms in which it can exist; thus

$$[E]_o = [E] + [ES] + [EM] + [EMS] \tag{16}$$

$$= [E] (1 + \overline{K_2}\,[S] + K_1\,[M] + \overline{K_2}\;K_4\,[S]\,[M] + U_{ES} + U_{EM} + U_{EMS}) \tag{17}$$

The three remaining equations are steady-state equations; of these, although four can be written down, only three are independent. The steady-state equation for ES is

$$\frac{d\,[ES]}{dt} = k_2\,[E]\,[S] + k_{-4}\,[EMS] - (k_{-2} + k_4\,[M] + k_o)\,[ES] = 0 \tag{18}$$

Using Eqs. (13) and (15), this becomes

$$k_{-4}\;U_{EMS} - (k_{-2} + k_4\,[M] + k_o)\,U_{ES} + k_2\,[S] + k_{-4}\;\overline{K_2}\;K_4\,[M]\,[S] - (k_{-2} + k_4\,[M] + k_o)\,\overline{K_2}\,[S] = 0$$

With the use of Eqs. (8) and (10) this reduces to

$$k_{-4}\;U_{EMS} - (k_{-2} + k_4\,[M] + k_o)\,U_{ES} = 0 \tag{19}$$

Similarly, the steady-state equations for E and EM give rise to

$$(k_{-2} + k_o)\,U_{ES} + k_{-1}\;U_{EM} + k_r\;U_{EMS} + k_r\;\overline{K_2}\;K_4\,[M]\,[S] = 0 \tag{20}$$

$$k_{-3}\;U_{EMS} - (k_{-1} + k_3\,[S])\,U_{EM} + k_3\;K_1\,(\frac{\overline{K_2}}{K_2} - 1)\,[M]\,[S] = 0 \tag{21}$$

These three equations may be written in the form of the matrix

U_{ES}	U_{EM}	U_{EMS}	Constant
$-(k_{-2} + k_4\,[M] + k_o)$	0	k_{-4}	0
$(k_{-2} + k_o)$	k_{-1}	k_r	$k_r\;\overline{K_2}\;K_4\,[M]\,[S]$
0	$-(k_{-1} + k_3\,[S])$	k_{-3}	$k_3\;K_1\,(\frac{\overline{K_2}}{K_2} - 1)\,[M]\,[S]$

The solutions for U_{ES}, U_{EM}, U_{EMS} are given by the ratios of Δ^{ES}, Δ^{EM}, Δ EMS to Δ where the Δ's are the appropriate determinants. With the aid of these solutions the steady-state rate expressions can now be readily obtained for a number of cases.

The simplest system is the one involving the formation of only one complex between the enzyme and the substrate (ES).

In this case, only the constants k_2, k_{-2}, and k_o need be considered, all others being equal to zero. The general matrix now reduces to

U_{ES}	Constant
$-(k_{-2} + k_o)$	0
$(k_{-2} + k_o)$	0

The solution which is $U_{ES} = U_{EM} = U_{EMS} = 0$

Equation (17) therefore reduces to

$$[E]_o = [E] (1 + \overline{K_2} [S]) \tag{22}$$

and equation (13) to

$$[ES] = [E] \overline{K_2} [S] \tag{23}$$

The rate is therefore

$$v = k_o [ES]$$

$$v = \frac{k_o \overline{K_2} [E]_o [S]}{1 + \overline{K_2} [S]} \tag{24}$$

Briggs and Haldane (6) obtained this equation from a simple steady-state treatment. In the case where k_o is much smaller than k_{-2}, $\overline{K_2}$ reduces to K_2 and equation (24) then becomes identical with the equilibrium equation derived by Michaelis and Menten (20).

If the three complexes, ES, EM, and EMS are formed, steady-state equations become much more complex, since the solutions for U_{ES}, U_{EM}, and U_{EMS} do not in general vanish.

From equations (13), (15) and (17) it may be seen that the rates of breakdown of ES and EMS to give products are respectively

$$v' = k_o [ES] = \frac{k_o [E]_o (\overline{K_2} [S] + U_{ES})}{1 + \overline{K_2}[S] + K_1[M] + \overline{K_2} K_4[M][S] + \Sigma U} \qquad (25)$$

Where ΣU represents $U_{ES} + U_{EM} + U_{EMS}$.

$$v'' = k_r [EMS] = \frac{k_r[E]_o (\overline{K_2} K_4 [M][S] + U_{EMS})}{1 + \overline{K_2}[S] + K_1 [M] + \overline{K_2} K_4 [M][S] + \Sigma U.} \qquad (26)$$

However the problem is greatly simplified if equilibrium rather than steady-state conditions apply to the complexes ES, EM, and EMS.

If this is the case the constants k_o and k_r can be neglected in comparison with the remaining constants. The term $k_r \overline{K_2} K_4 [M][S]$ in the right-hand column of the matrix vanishes, as also does $k_3 K_1 \left(\frac{\overline{K_2}}{K_2} - 1 \right) [M][S]$ since $\overline{K_2} = K_2$. There is thus a column of zeros in each of the determinants ES, EM, EMS, and the "perturbation" terms U_{ES}, U_{EM}, and U_{EMS} therefore vanish.

The rate is now given simply by

$$v = v'' + v'$$

$$= \frac{k_r K_2 K_4 [E]_o [M][S]}{1 + K_2[S] + K_1[M] + K_2 K_4[M][S]} + \frac{k_o K_2 [E]_o [S]}{1 + K_2[S] + K_1[M] + K_2 K_4[M][S]}$$

Rearranging,

$$v = \frac{k_r[E]_o}{1 + \frac{1}{K_3[S]} + \frac{1}{K_4[M]}\left[1 + \frac{1}{K_2[S]}\right]} + \frac{k_o[E]_o}{1 + \frac{1}{K_2[S]} + K_4[M]\left[1 + \frac{1}{K_3[S]}\right]} \quad (27)$$

The first term in this expression is identical to one obtained by Alberty (1) for mechanisms of enzymatic reactions involving two reactants and two products, assuming equilibrium conditions.

Evaluation of Constants

If the conditions under which the reaction is carried out are varied systematically, it is possible to evaluate all the constants in this expression.

It can easily be seen by inspection of the expression that if the concentrations of M and S are kept constant, the rate will be proportional to the enzyme concentration. This has been observed in a number of cases.

Evaluation of K_3

If the conditions are such that the product $K_4[M]$ is much larger than one, the second part of the expression (27) becomes negligible with respect to the first part, since M appears in the denominator. For the same reason, the terms $\frac{1}{k_4[M]}$ and $\frac{1}{K_2 K_4[M][S]}$ in the first part of the expression can be neglected.

The expression (27) for the rate of the reaction then reduces to

$$v = \frac{k_r[E]_o}{1 + \frac{1}{K_3[S]}} \qquad (28)$$

From this expression K_3 can be obtained by the Lineweaver and Burk (19) method. Taking the reciprocal of equation (28) and rearranging, we obtain

$$\frac{1}{v} = \frac{1}{k_r[E]_o} + \frac{1}{k_r K_3[E]_o [S]} \qquad (29)$$

If 1/v is plotted against 1/[S] a straight line should result if the law is obeyed with a slope equal to $\frac{1}{k_r K_3[E]_o}$ and an intercept equal to $\frac{1}{k_r[E]_o}$.

From these results K_3 is easily calculated.

It can be shown that $K_3[S]$ is equal to 1 or that $K_3 = 1/[S]$ when the rate v is equal to 1/2 the maximum rate v_m. This provides a simple and rapid method of evaluating K_3 graphically. If the intercept on the 1/v axis is multiplied by 2, then the corresponding 1[S] value gives the constant K_3 directly.

G.S. Eadie (7) proposed a further method of evaluating constants such as K_3. If the expression is expanded to

$$v + K_3[S]\, v = k_r K_3[S][E]_o$$

and divided through by [S], we obtain

$$\frac{v}{[S]} = k_r K_3[E]_o - K_3 v$$

When v/[S] is plotted against v, the slope of the resulting straight line gives $-K_3$ directly.

Evaluation of K_4

If the concentration of S is made large equation (27) reduces to

$$v = \frac{k_r[E]_o}{1 + \frac{1}{k_4[M]}} + \frac{k_o[E]_o}{1 + K_4[M]} \qquad (30)$$

If v_o, the maximum rate of the reaction in absence of M, is substracted from both sides of this equation, we obtain

$$v - v_o = \frac{k_r[E]_o}{1 + \frac{1}{K_4[M]}} + \frac{k_o[E]_o}{1 + K_4[M]} - k_o[E]_o$$

$$v - v_o = \frac{(k_r - k_o)\,[E]_o}{1 + \frac{1}{K_4[M]}} = \frac{(k_r - k_o)\,K_4[E]_o\,[M]}{1 + K_4[M]} \qquad (31)$$

Taking the reciprocal equation (31) becomes

$$\frac{1}{v - v_o} = \frac{1}{(k_r - k_o)\,[E]_o} + \frac{1}{(k_r - k_o)\,K_4[E]_o\,[M]} \qquad (32)$$

This equation is of the same form as equation (29) and K_4 can therefore be obtained graphically by plotting $\frac{1}{(v-v_o)}$ against 1/[M].

Evaluation of K_2

Two methods are available for the determination of K_2, the equilibrium constant for the formation of the ES complex. If the reaction is carried out in absence of M, only the complex ES will be formed. K_3 and K_4, the equilibrium constants for the formation of the EMS complexes and k_r its dissociation constant become zero and equation (27) reduces to

$$v = \frac{k_o [E]_o}{1 + \frac{1}{K_2[S]}}, \tag{33}$$

from which K_2 can be obtained.

A second method of evaluating K_2 consists in carrying out the reaction in such conditions that the product $K_4[M]$ will be equal or very close to one.

Equation (27) now becomes

$$v = \frac{k_r [E]_o}{2 + \frac{1}{K_3[S]} + \frac{1}{K_2[S]}} + \frac{k_o [E]_o}{2 + \frac{1}{K_2[S]} + \frac{1}{K_3[S]}}$$

$$= \frac{(k_r + k_o) [E]_o}{2 + \frac{K_2 + K_3}{K_2 K_3[S]}} \tag{34}$$

Taking the reciprocal

$$\frac{1}{v} = \frac{2}{(k_r + k_o) [E]_o} + \frac{K_2 + K_3}{K_2 + K_3[S]} \quad \frac{1}{(k_r + k_o) [E]_o} \tag{35}$$

When 1/v is plotted against 1/[S], a straight line should result with a slope equal to $\frac{K_2 + K_3}{K_2 K_3 (k_r + k_o) [E]_o}$

and an intercept equal to $\frac{2}{(k_r + k_o) [E]_o}$

If K_3 has been previously determined K_2 can be calculated by the values of the slope and intercept.

Evaluation of K_1

The constant K_1 is obtained from the thermodynamic relation $K_1 K_3 = K_2 K_4$.

EXPERIMENTAL PART -

Reagents:

The di-potassium salt of adenosine triphosphate (Pabst Laboratories, Milwaukee, Wis.) was used throughout these experiments.

Tris (Hydroxymethyl) aminomethane (THAM) (Fisher purified grade) and potassium chloride (Fisher certified reagents) were recrystallized from solutions in glass-distilled water.

Trichloroacetic acid, calcium chloride, potassium carbonate (anhydrous) and potassium bicarbonate, were certified reagents (Fisher). The acetic acid was of the Merck reagent grade.

Myosin -

Rabbit muscle myosin of the Weber-Edsall type (5) was used throughout these experiments. Glass-distilled water was used in the preparation of solutions, and for the washing of glassware, since metallic ions are easily adborded on myosin and change its properties. The extraction mixture was 0.6M in potassium chloride, 0.04M in potassium bicarbonate, 0.01M in potassium carbonate. All operations were carried out in a cold room at approximately $4^{\circ}C$.

A rabbit was stunned and bled. The back muscles were removed, cut into small pieces, and weighed. For each gram of muscle, 3 ml. of extraction mixture were added. The whole was then poured into a "Waring Blendor" and agitated for one minute. The resulting mixture was stirred slowly for five hours and centrifuged. The extract was poured rapidly into ten times its volume of cold glass-distilled water which had been previously acidified by adding 20 ml. of 0.1 N acetic acid for every 100 ml. of extract. The pH was always kept over 6.5. The mixture was then left at approximately $4^{\circ}C$, at a pH of 6.55 measuredwith a pH meter, for 2 hours or more (overnight).

The supernatant liquid was decanted when a good enough separation had been obtained. The rest was centrifuged and the viscous part redissolved in 0.6M KCl. The resulting solution was stirred for 20 minutes and centrifuged. It was then decanted, measured, and poured into 10 volumes of cold glass-distilled water. The pH was adjusted at 6.55 with 0.1N acetic acid. The mixture was then allowed to rest for 2 hours and theabove procedure was repeated.

The final solution of myosin extract in KCl was centrifuged at high speed for 1 hour. (18000 rpm for 1 hour).

Myosin concentrations were determined by a micro-Kjehldahl nitrogen determination method (12). The protein sample was digested by means of sulfuric acid and hydrogen peroxide and determined colorimetrically after nessalorization. The myosin had a specific activity of 5.2×10^{-6} mole/sec/g. at 25°C. This value is consistent with values previously reported (17,26).

Procedure -

All reactions were carried out in buffered solutions containg 0.6 mole of KCl and 0.1 mole of THAM per liter adjusted at different pH's with acetic acid.

These buffers were so adjusted at a series of temperatures since the pH of THAM varies considerably with temperature. Three other solutions, one of ATP, one of myosin, and one of calcium chloride were prepared in 0.6 molar KCl. 5 ml of myosin solution and 5 ml of calcium chloride solution were added to a flask containing 15 ml of buffered solution. 5 ml of ATP solution were placed in another flask. With this system, ATP and CaCl2 concentrations could easily be varied by diluting the original solutions with 0.6 molar KCl.

The solutions were kept in a constant temperature bath for 15 minutes, then mixed. At measured intervals of time, 5 ml samples were pipetted into 3 ml of 20% trichloroacetic acid. The precipitated myosin was removed by filtration through a dry filter paper. 5 ml of the filtrate were set aside for a phosphate determination by the Fiske-SubbaRow method (33)

Phosphate Analysis -

In these experiments, 1 ml of ammonium molybdate reagent (25 grams of hydrated ammonium molybdate in one liter of 3N sulfuric acid) and 0.4 ml of aminonaphthol sulfonic acid (ANSA) solution (2.5 grams of purified ANSA in 975 ml of 15% sodium bisulfite and 25 ml of 20% sodium sulfite) were added to a 5 ml sample of the phosphate solution. The sample was left standing at room temperature for ten minutes, and the absorbance was read at 750 mμ on a Beckmann DU spectrophotometer. Values obtained were compared with a dibasic potassium phosphate calibration curve.

RESULTS

Determination of Constants

The enzymatic myosin - ATP system was studied in some detail in the presence and in the absence of an activator, Ca^{++}. Equilibrium conditions were assumed to exist and the "apparent" equilibrium constants for the formation of the different complexes during the reaction were calculated. The basis for this assumption will be presented later in the discussion.

The equilibrium constant K_2 for the formation of the enzyme-substrate complex from the enzyme and substrate was determined by carrying out the reaction at different substrate concentrations in absence of Ca^{++}. The rate of the reaction v was calculated from the slope of the curve obtained by plotting the phosphate concentrations as a function of time during the course of the reaction.

Figure (1) is a typical curve showing the variation of the rate of the reaction with the concentration of substrate. From plots of 1/v against 1/[ATP] (fig. 2) K_2 was determined at four temperatures. Log K_2 was plotted against the reciprocal of the absolute temperature (fig. 3) and the slope of the line drawn through the points gave a value of +12 kcal/mole for the enthalpy change (ΔH). The corresponding ΔF° and ΔS° were -6.7 kcal/mole and 62.5 e.u. respectively.

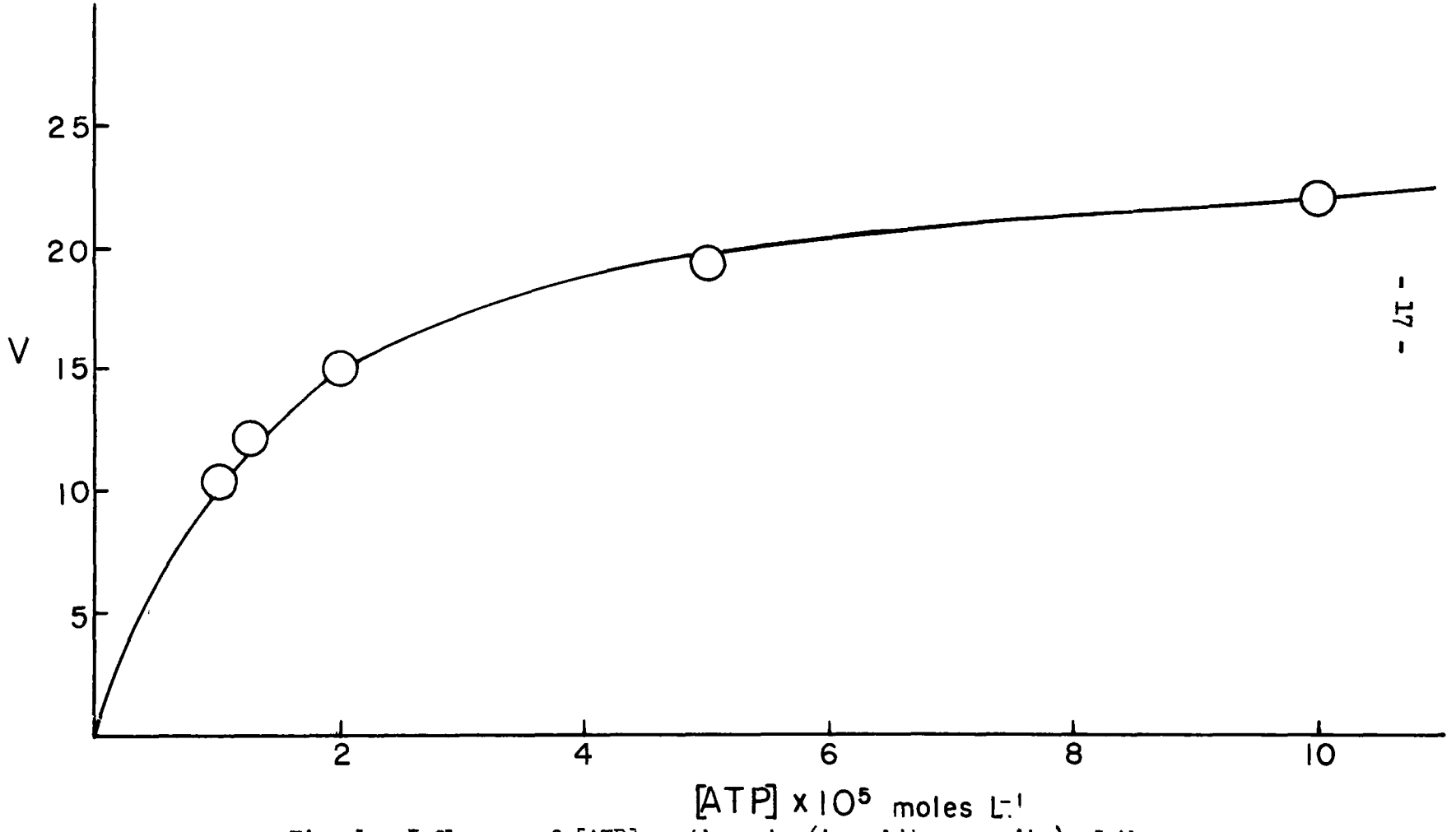

Fig. 1. Influence of [ATP] on the rate (in arbitrary units) of the myosin catalyzed dephosphorylation of ATP at 25°C at a pH of 7.5.

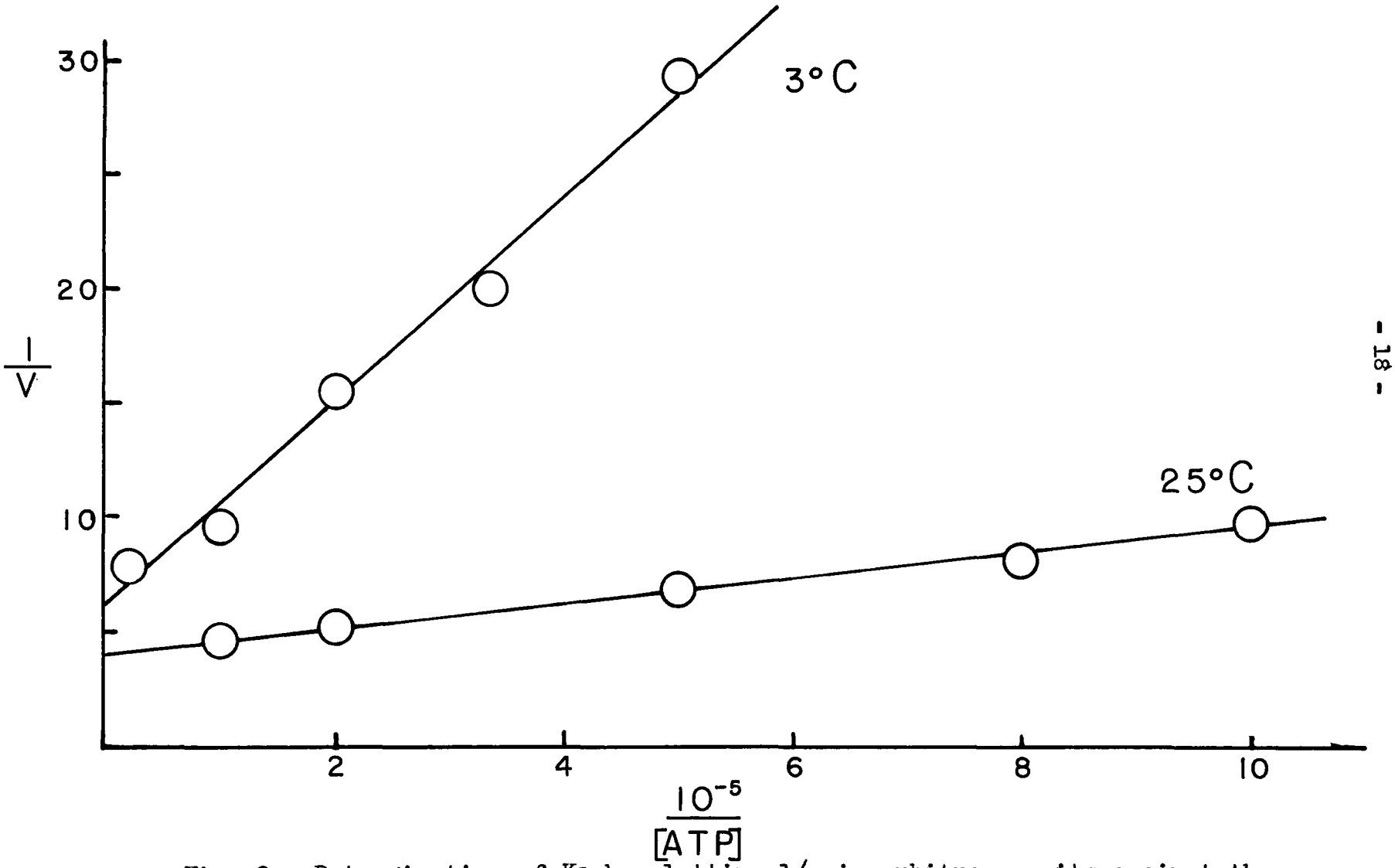

Fig. 2. Determination of K_2 by plotting 1/v in arbitrary units against the reciprocal of the ATP concentration in moles per liter at a pH of 7.5 in absence of Ca^{++}.

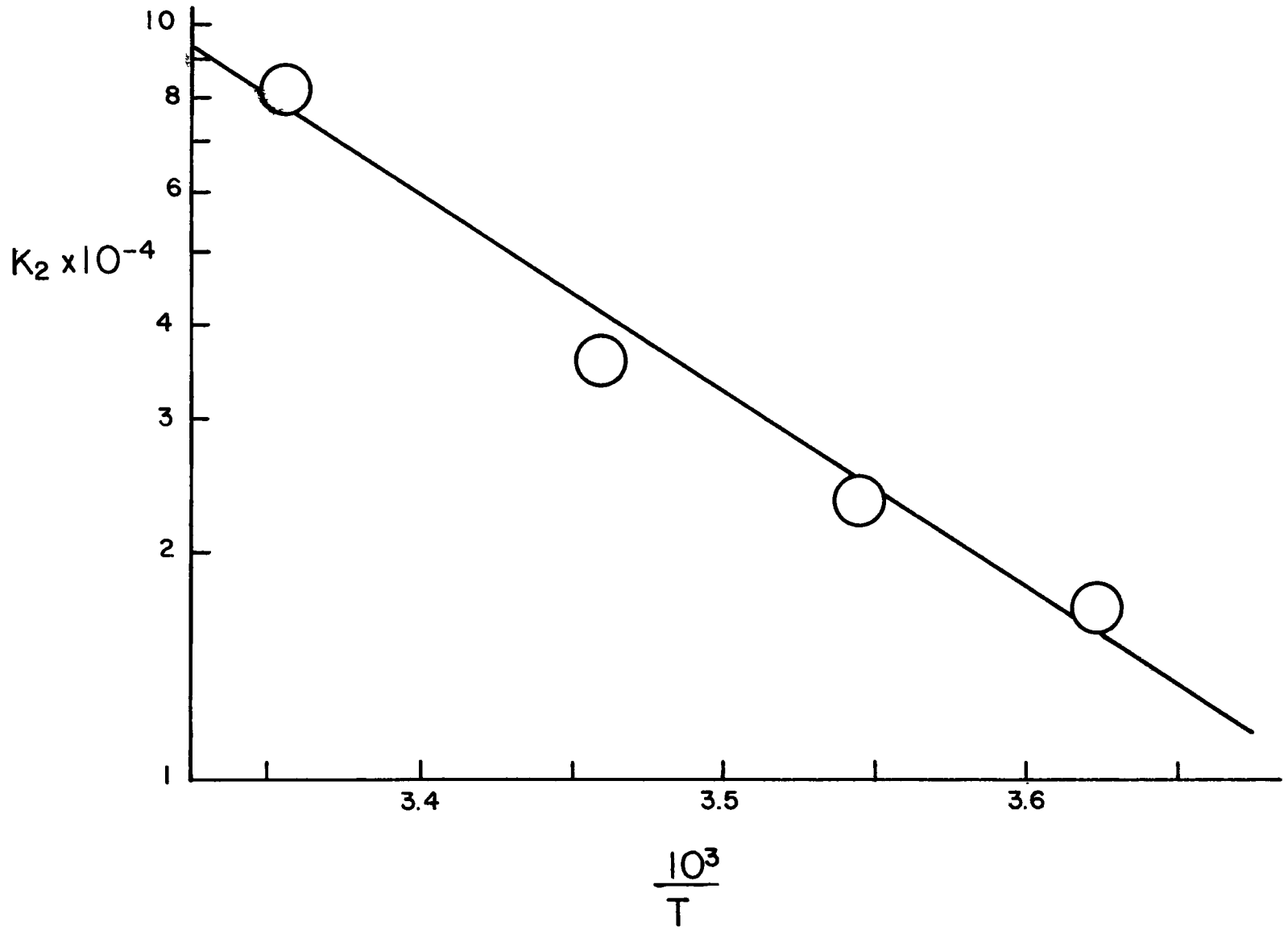

Fig. 3. Dependence of log K_2 (l. mole^{-1}) on 1/T at pH 7.5

The equilibrium constant K_3 for the formation of the enzyme-activator-substrate complex from the enzyme-activator complex and the substrate was determined by carrying out the reaction at different substrate concentrations in the presence of 0.01 molar Ca^{++}, rates being determined as in the previous case. Figure (4) shows the results obtained when 1/v was plotted against 1/[ATP] at 3°C and at 25°C. From Figure (5) a value of 9.2 kcal/mole was calculated for the enthalpy change. ΔF° and ΔS° values were -6.6. kcal/mole and 53 e.u. respectively.

However, these thermodynamic values include contributions from acid dissociation constants. This was established from an investigation of pH effect on K_3. Values for K_3 at different pH's are summarized in Table I.

TABLE I

Influence of pH on K_3 at 25°C

pH	K_3 liters/mole × 10^{-3}
7	92
7.5	66
8.5	29

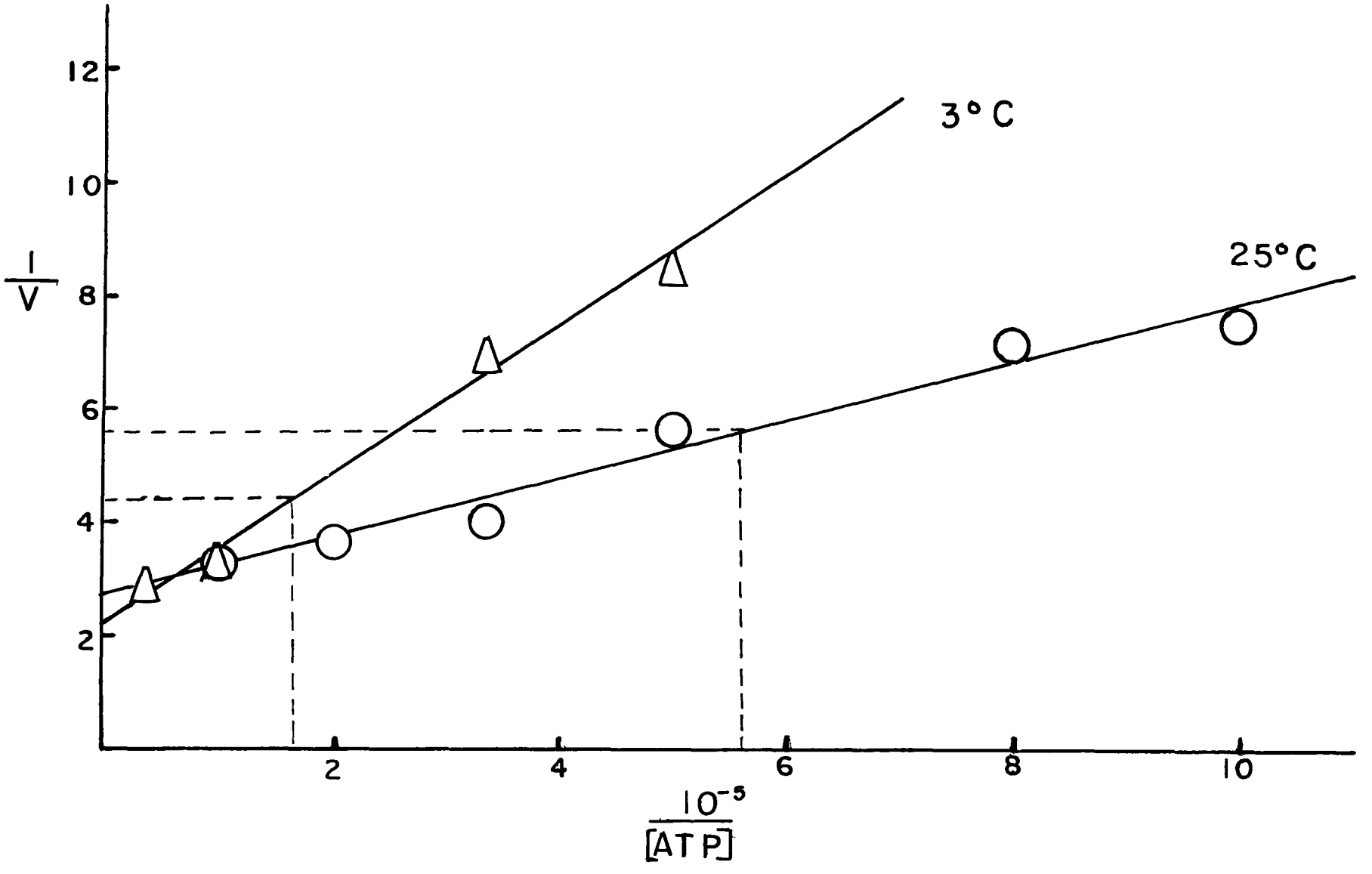

Fig. 4. Determination of K_3 by plotting 1/v in arbitrary units against the reciprocal of the ATP concentration in moles per liter in the presence of 0.01M Ca^{++} at pH 7.5.

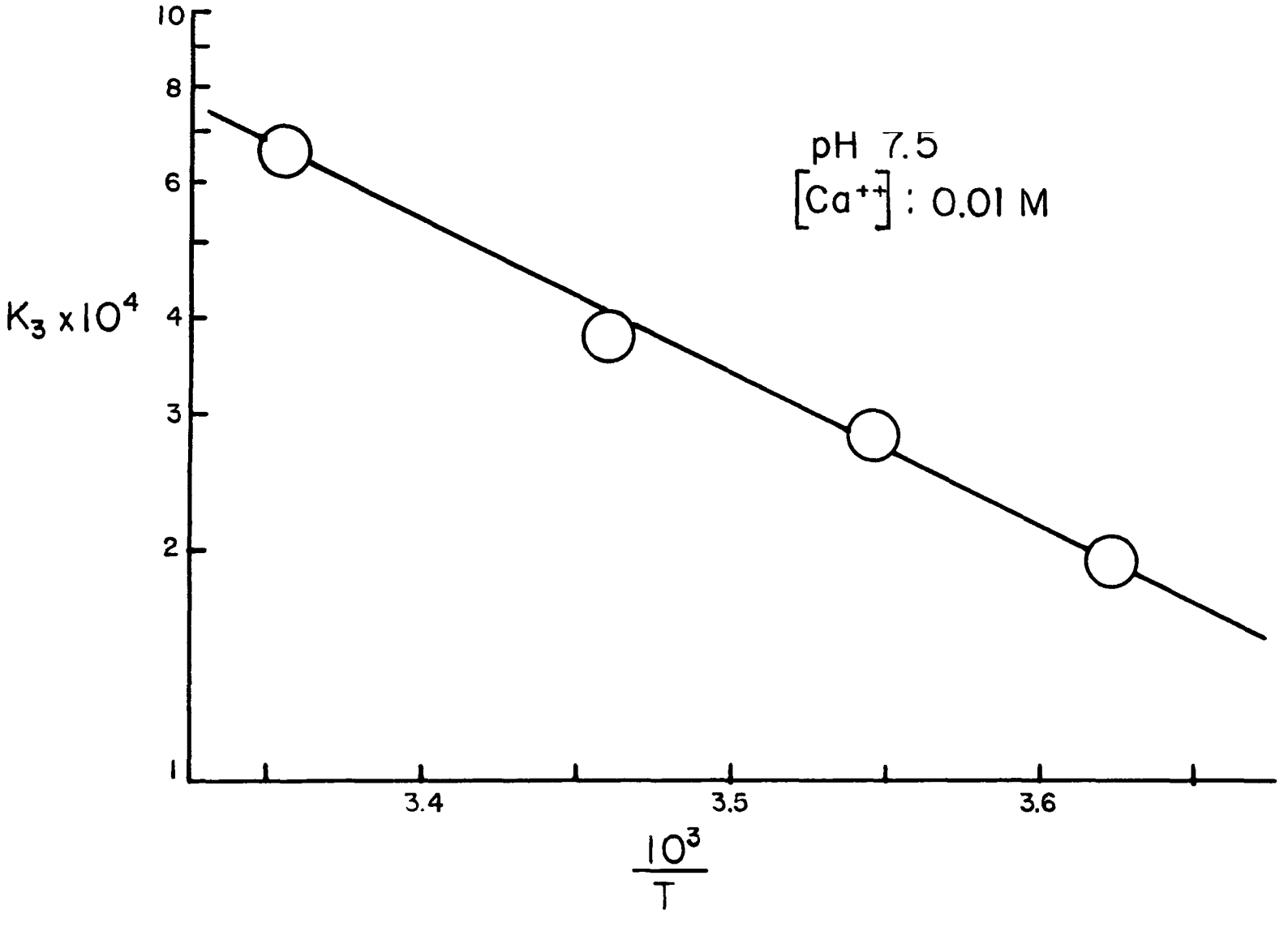

Fig. 5. Dependence of log K_3 (in liters per mole) on 1/T at pH 7.5.

The equilibrium constant K_4 for the formation of the enzyme-activator-substrate complex from the enzyme-substrate complex and the activator was determined by studying at a high concentration of substrate ($K_3[S]$ and $K_2[S] \gg 1$) the effect of the concentration of Ca^{++} on the rate of the reaction. As seen previously the reaction proceeds in the absence as well as in the presence of calcium ions. This fact is well illustrated in figure (6) where the curve obtained by plotting the rate of the reaction against the Ca^{++} concentration does not pass through the origin. For the determination of the constant, the rates at zero concentration of Ca^{++} were subtracted from the rates obtained with Ca^{++} and these values were plotted against the reciprocal of the Ca^{++} concentrations. (fig. 7). From these plots K_4 was determined at three temperatures. The enthalpy change calculated from figure (8) was -3.5 kcal/mole. The corresponding ΔF^o and ΔS^o were -4.3 kcal/mole and 2.7 e.u. respectively.

In a further study of the calcium ion effect, it was found that a decrease in the enzyme concentration produced an appreciable increase of the constant K_4 (fig. 9). At 25°C and pH 7.5 a linear relationship was found to exist between K_4 and the enzyme concentration and a value of 1480 liters per mole was obtained for the extrapolated value of K_4 at zero concentration of enzyme.

This dependence of the constant K_4 on the total concentration of enzyme permits a rough calculation of the weight of myosin that combines with one mole of Ca^{++}. From values in figure (9) it appears that this weight is of the order of 10^3 grams.

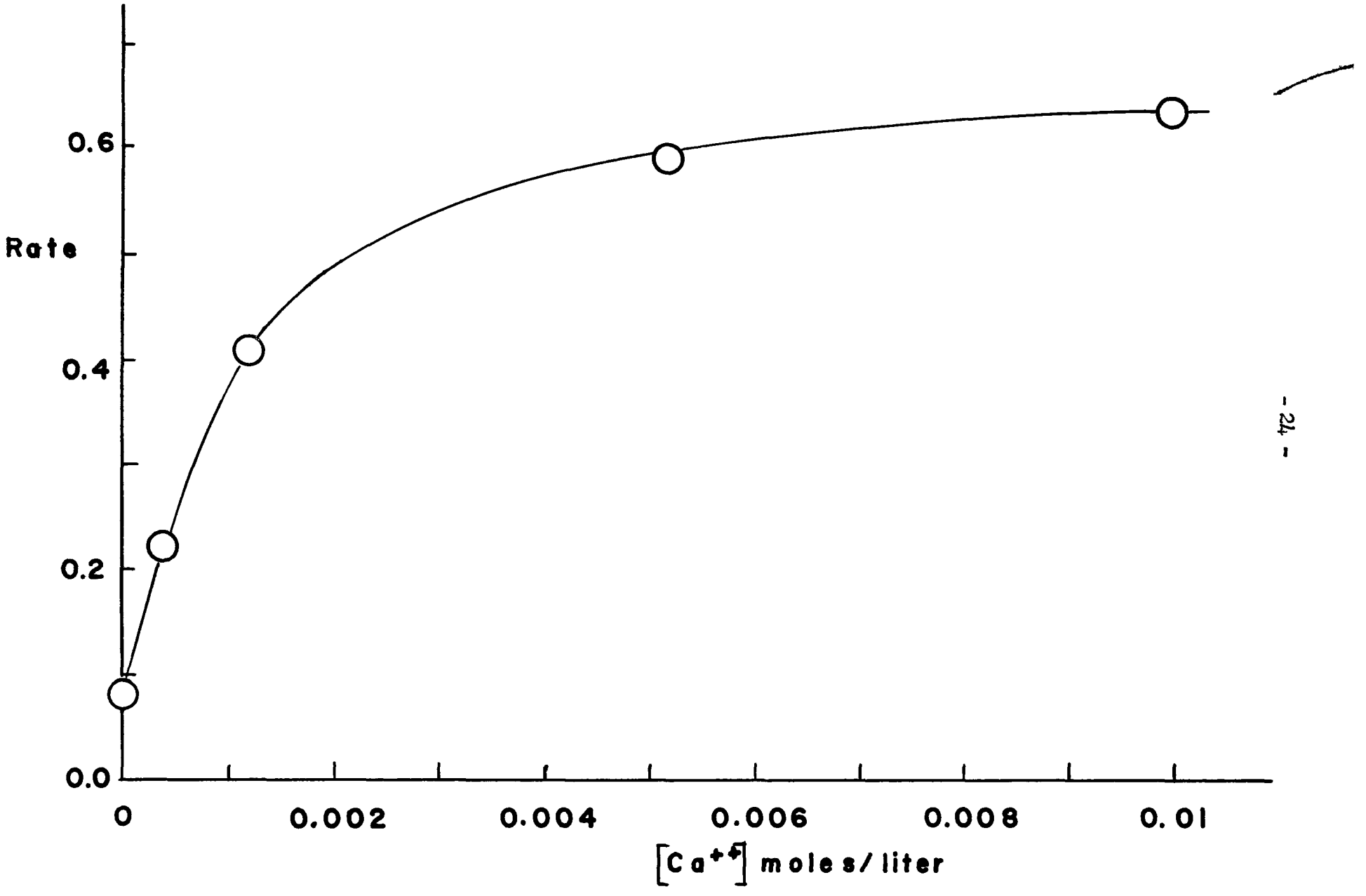

Fig. 6. Influence of [Ca^{++}] on the rate (in arbitrary units) of the myosin catalyzed dephosphorylation of ATP at 25°C and pH 7.5.

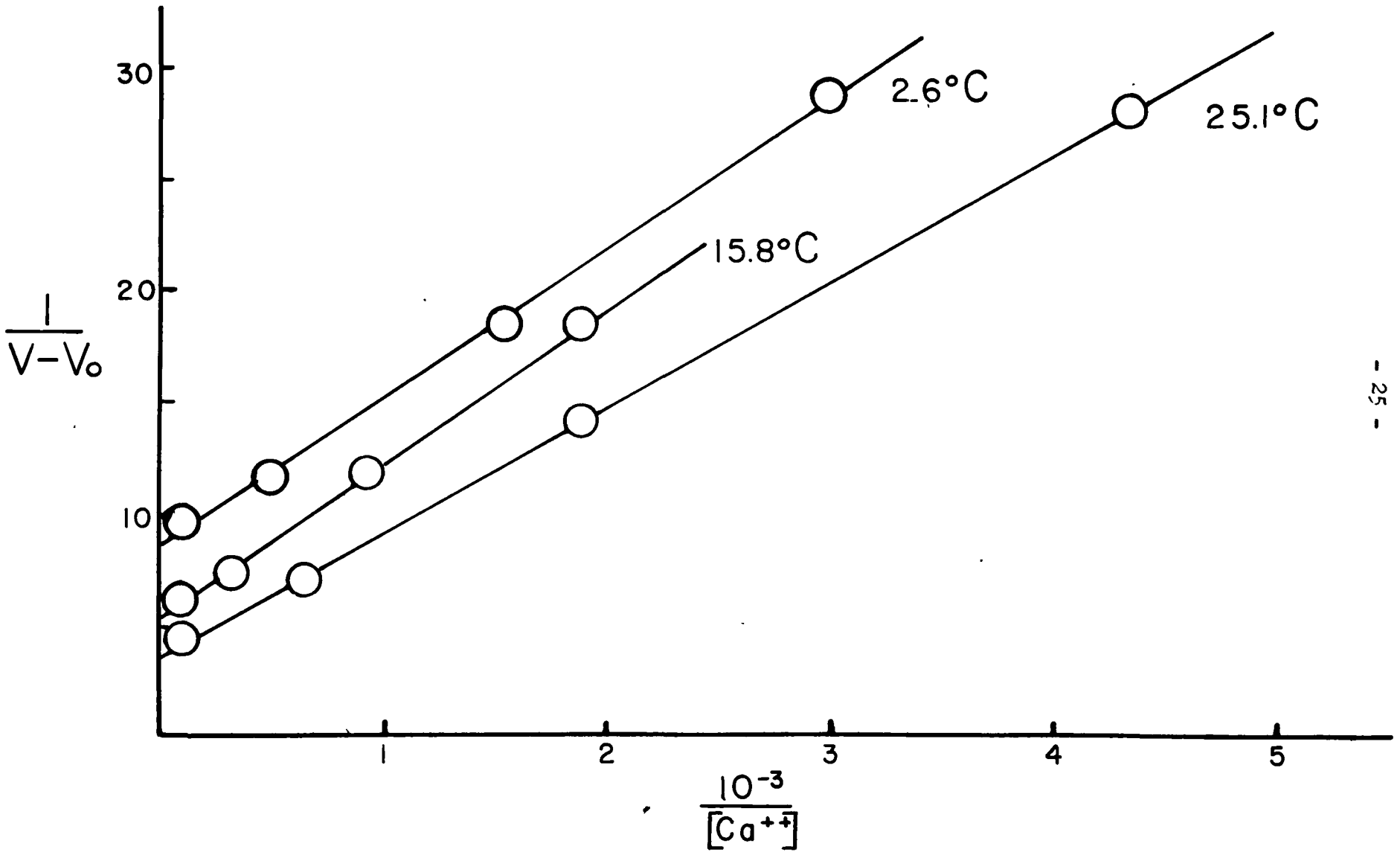

Fig. 7. Determination of K_4 in liters per mole by plotting $\frac{1}{v - v_o}$ against the reciprocal of the Ca++ concentration in moles per liter at pH 7.5.

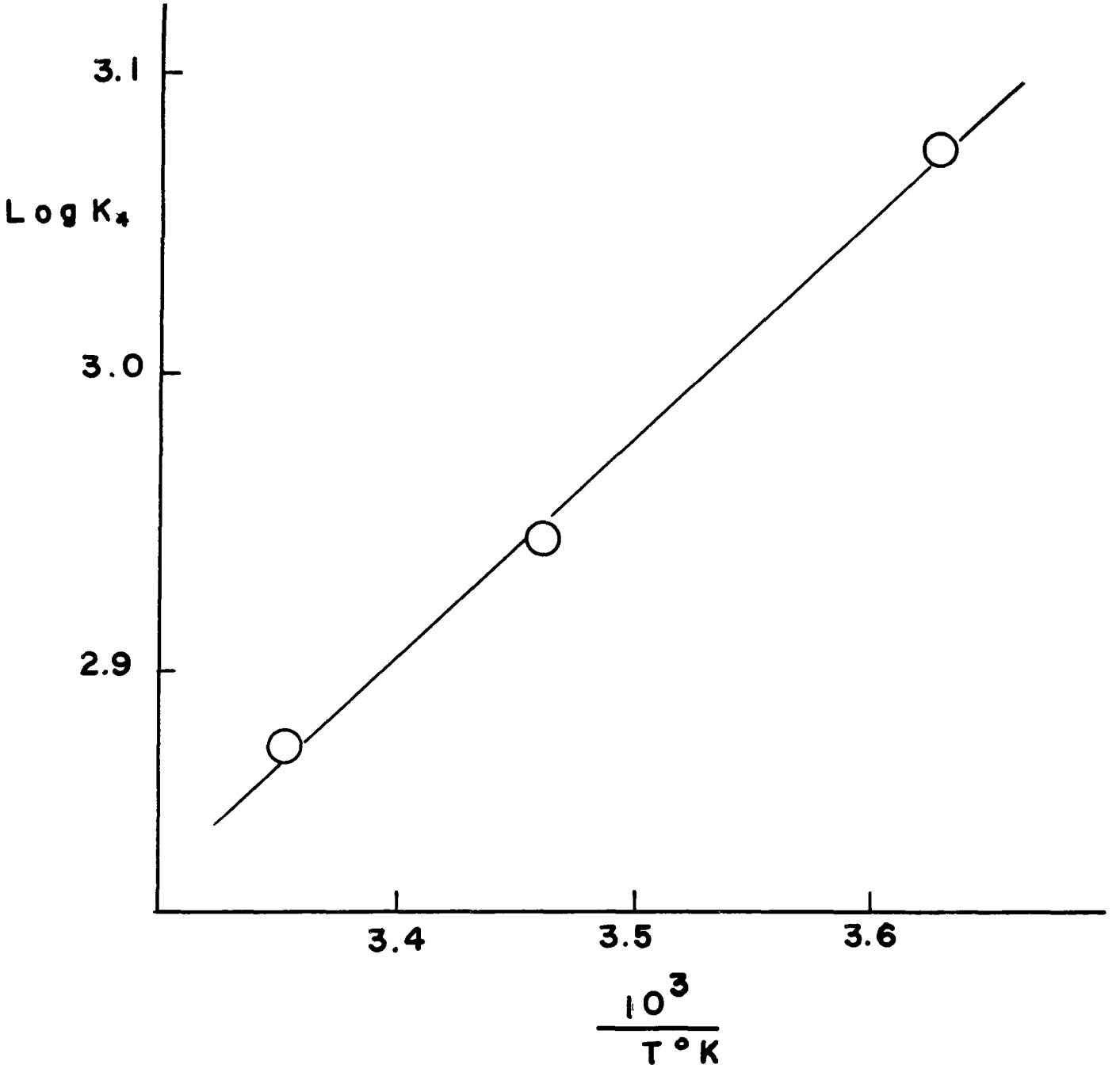

Fig. 8. Dependence of log K_4 in liters per mole on 1/T at pH 7.5.

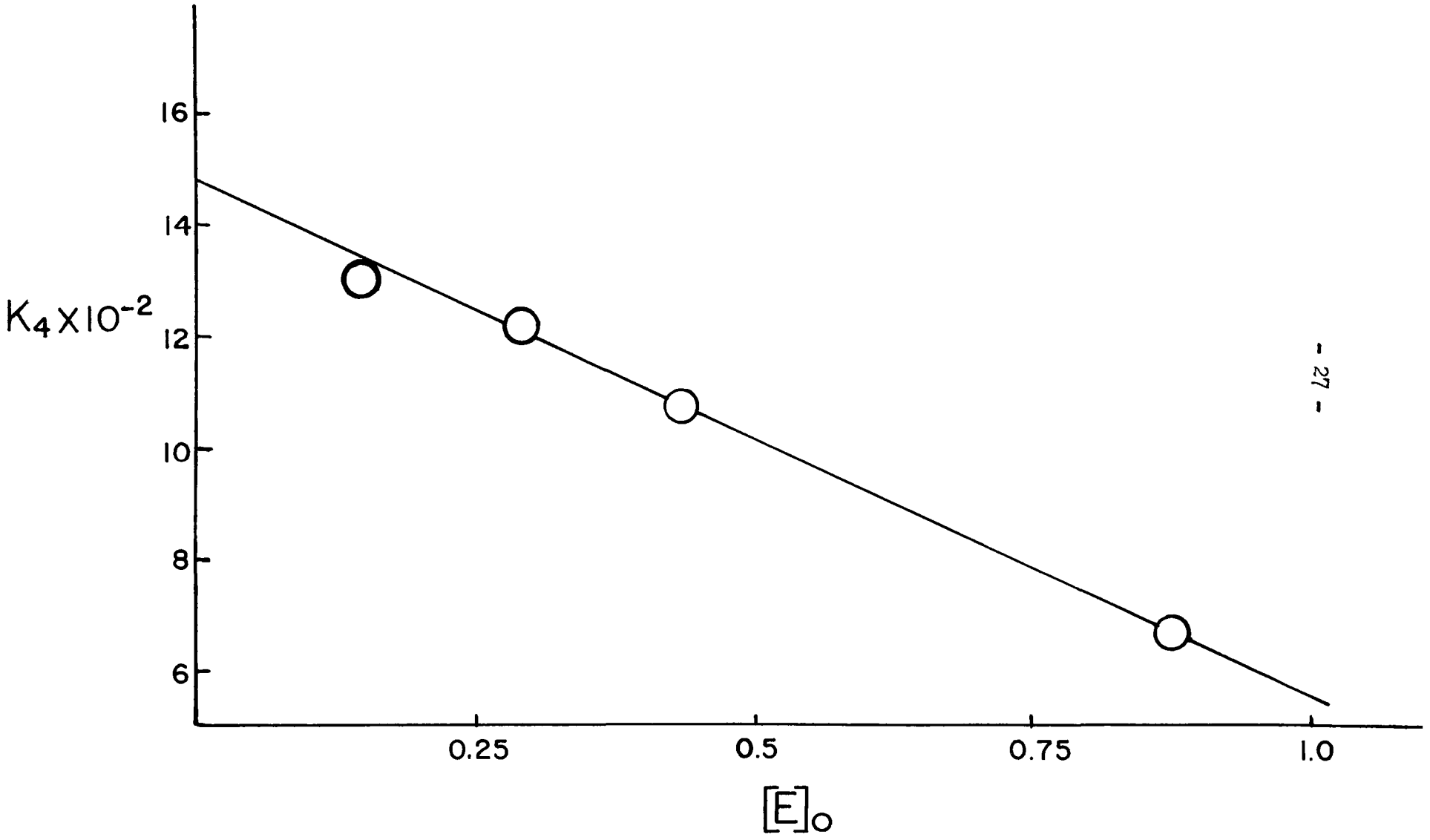

Fig. 9. Influence of the total concentration of enzyme in grams/liter on the constant K_4, expressed in liters/mole, at 25°C and pH 7.5.

Seemingly, the pH has little influence on the constant K_4. At pH 7.5 and 8.5, values obtained at comparable concentrations of enzyme differed by only about $10^0/_0$. For example, values at 23°C were 700 and 800 respectively.

Values for the equilibrium constant K_1 for the formation of the enzyme-activator complex from the enzyme and the activator were obtained from the relation $K_1 K_3 = K_2 K_4$ on the assumption that these are all equilibrium constants.

In Table II are listed values for the above determined equilibrium constants with their corresponding ΔH°, ΔF° and ΔS° values.

Energy of Activation

From Arrhenius plots of log v against the reciprocal of the absolute temperature in the presence of 0.01M Ca^{++} at pH 7.5 a value of 4.1 kcal/mole was obtained for the energy of activation (fig. 10) at a high concentration of ATP (1×10^{-3}M). The corresponding entropy of activation calculated from Eyring's relation (9).

$$k = \frac{RT}{Nh} \exp(\Delta S/R) \exp -(\Delta H/RT)$$

where N is Avogadro's number, and using a molecular weight of 5×10^6 for the enzyme, was -40 entropy units.

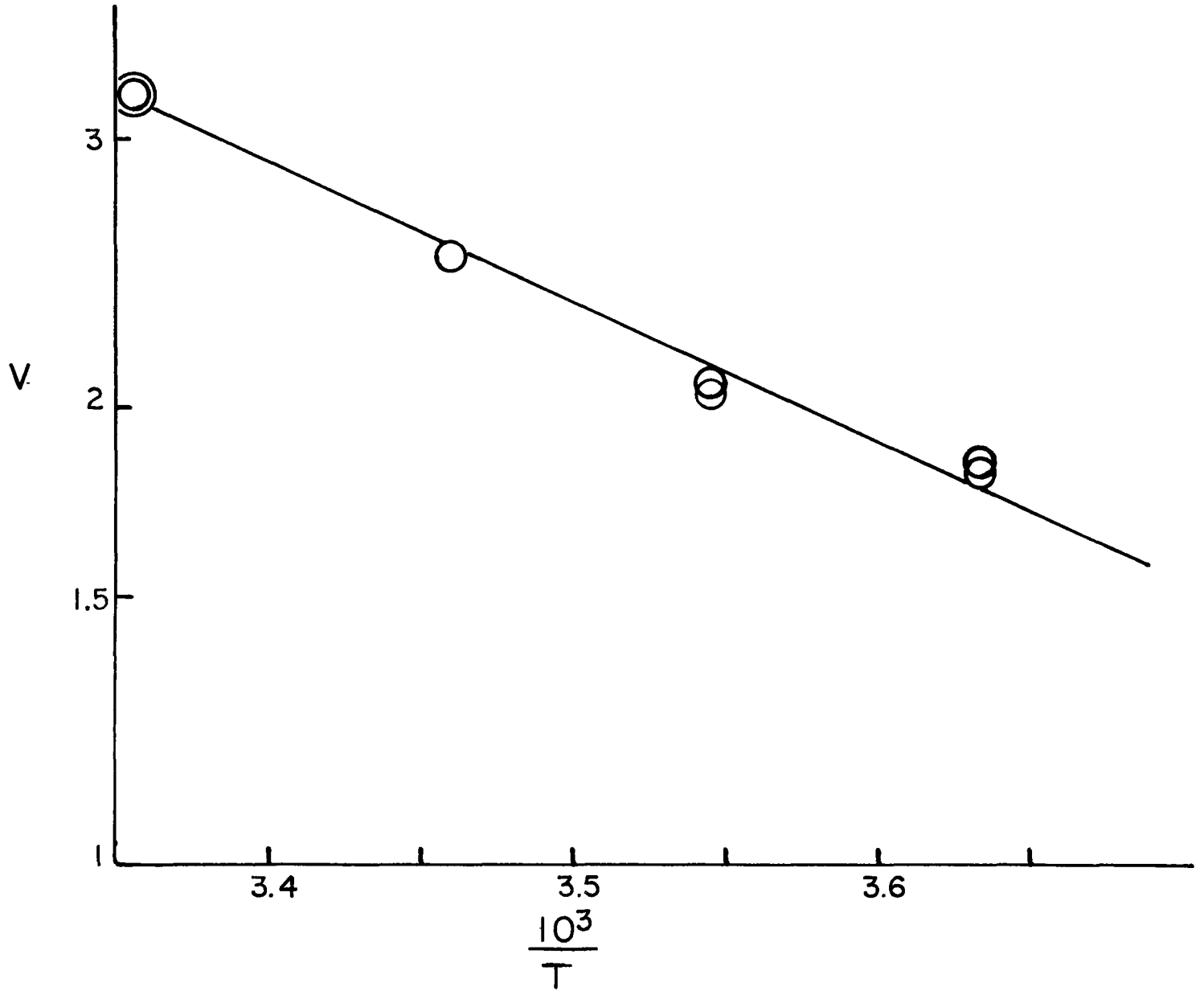

Fig. 10 . Arrhenius plot of log V in arbitrary units against 1/T at pH 7.5 in presence of 0.01M Ca^{++}.

When Ca^{++} was not added to the reaction mixture, values obtained were found to be very susceptible to variations of pH. Addition of ATP to make a 0.001M solution was found to lower the pH of the prepared THAM buffer from 7.5 to about 6.7 at 3°C. When the pH of the solution was readjusted to 7.5 at each temperature, essentially the same value (4.3 kcal/mole) was obtained for the energy of activation, with a corresponding entropy of activation of -42.5 e.u. A similar observation for energies of activation was made by Yon (32) for the system trypsin-calcium-lactoglobulin.

Effect of pH on the Reaction Rate

The pH of the solution has a marked effect upon enzyme catalyzed reactions. In the case of the myosin-catalyzed hydrolysis of ATP it was found that at 15°C at a high concentration of ATP the rate of the reaction increased slowly from 6.5 to 7.5, then more rapidly to reach a maximum at approximately pH 9.75 after which it dropped considerably. The drop in the rate after pH 9.75 is possibly due to an irreversible alkaline inactivation of the enzyme (2). Figure (11) shows the type of curve obtained when the rate of the reaction was plotted against the concentration of OH^- ions between pH's 8.4 and 9.46 at 15°C.

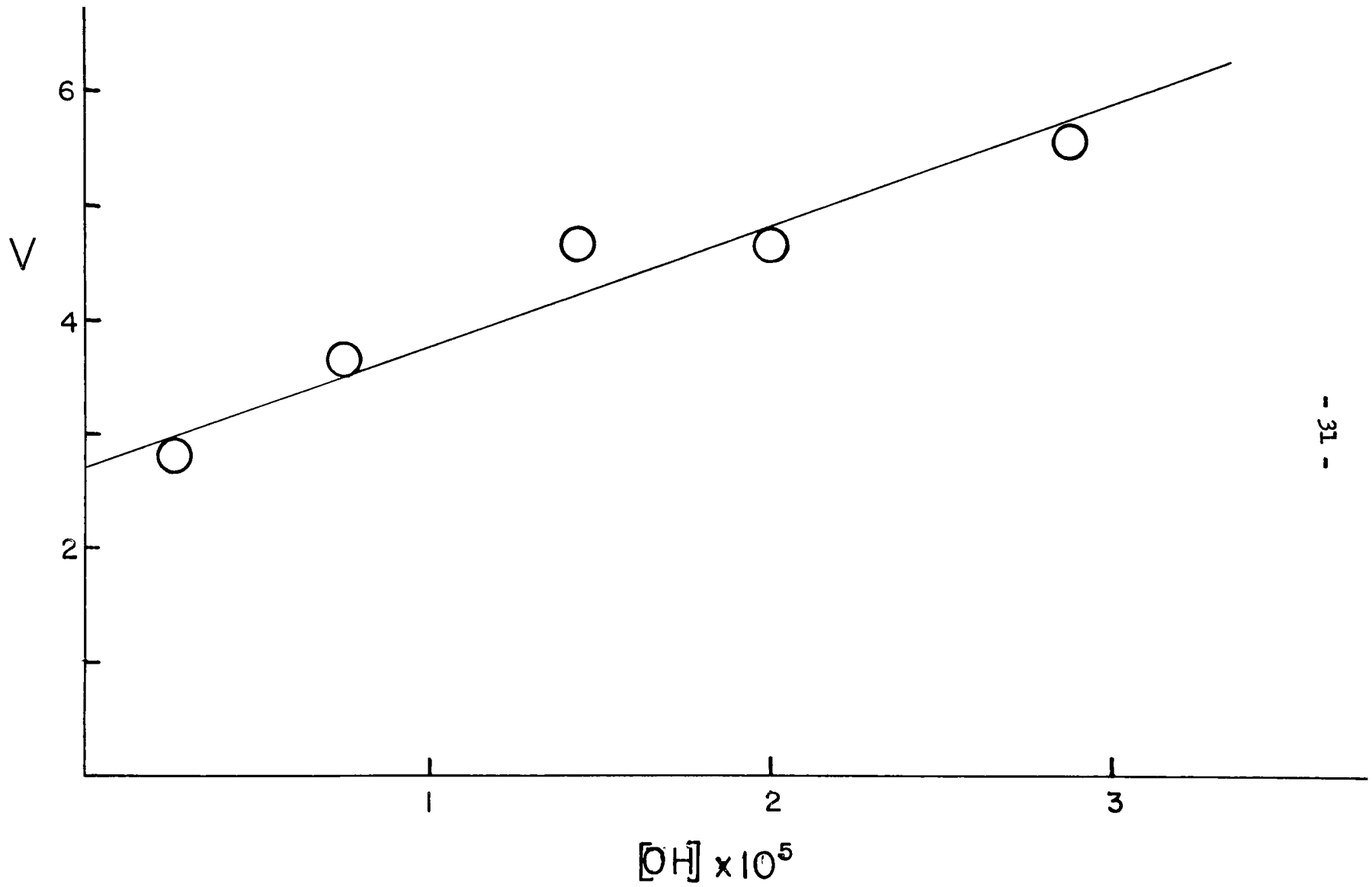

Fig. 11. Influence of [OH] in moles per liter on the rate (in arbitrary units) of the myosin catalyzed dephosphorylation of ATP at 15°C.

TABLE II

Summary of data obtained on the hydrolysis of ATP by myosin at pH 7.5

	Temp. °C	K liters/mole	ΔH° kcal/mole	ΔF° kcal/mole	ΔS° e.u.
K_1	25°	9.3×10^2	- 0.9	- 4.4	11.8
	3°	10.5×10^2			
K_2	25	82×10^3	12	-6.7	62.5
	16	36×10^3			
	9	24×10^3			
	3	17×10^3			
K_3	25	66×10^3	9.2	- 6.6	53
	16	38×10^3			
	9	28×10^3			
	3	19×10^3			
K_4	25	7.5×10^2	- 3.5	- 4.3*	2.7*
	16	9.0×10^2			
	3	12×10^2			
		sec^{-1}			
k_o	25	3.5	4.3	17	- 42.5
k_r	25	15	4.1	16	- 40

* From extrapolated value of K_4 (fig. 9)

DISCUSSION

In order to obtain the maximum information from the experimental data, it is necessary to consider the nature of the determined constants. It has always been a problem in enzyme kinetics to determine whether the constants are steady-state or equilibrium constants. Laidler (15) has treated in some detail the problem for a generalized system.

Within experimental error, it has been found in the present investigation that the myosin-ATP system follows a rate law of the form

$$v = \frac{k_r [E]_o}{1 + \frac{1}{K_3[S]} + \frac{1}{K_4[M]} + \frac{1}{K_2 K_4 [M][S]}} \quad \text{(see eq. 27)}$$

where M represents the Ca^{++}.

To obtain an expression of this form from the proposed mechanism on page 3, the u's in the general solution (eq. 26) for the mechanism must disappear, otherwise the expression will involve terms in $[S]^2$ and $[M]^2$. This would imply that the constants determined by plotting 1/v against 1/[S] and 1/[M] would be dependent upon [S] and [M] respectively.

In figures (2,4,7), plots used for the determination of the constants give rather good straight lines, indicating no dependence on the concentrations of S or M. Referring to the matrix on page 5, the only way the u's in the general solution can be made to vanish is to have a column of zeros for the constant terms. The term $k_r \overline{K}_2 K_4 [M][S]$ will reduce to zero only if k_r is very small or zero. The term

$k_3 K_1 \left(\frac{\overline{K_2}}{K_2} - 1 \right) [M] [S]$ will reduce to zero only if $\overline{K_2} = K_2$, which

implies that k_o is very small as compared to k_{-2} and can be neglected. It can then be concluded that if an expression of the form of eq. (27) is followed experimentally, the rate constants k_7 and k_o must be very small compared to the other rate constants, and that therefore equilibrium conditions exist.

The coincidence case discussed by Laidler (15) cannot, we feel, be applied here since it would involve formation of products from the enzyme-calcium complex.

An inspection of table II shows that the constant k_1 is approximately equal to the constant K_4. Under these conditions the denominator of equation (27) can be factorized to assume the form $(1 + K_1 [M]) (1 + K_2 [S])$. This form is typical of systems displaying simple non-competitive interaction. Assuming no blockage of reaction paths, Morales (34) has shown that when such a behaviour is shown the Michaelis constants are equilibrium constants.

Laidler (15) has extended the treatment to include the possibility of blockage. In the myosin-Ca^{++}-ATP system blockage between the myosin-Ca^{++} complex and the myosin-Ca^{++}-ATP complex can be considered as improbable since at high concentration of Ca^{++}, the myosin exist almost exclusively under the form of myosin-Ca^{++}. Blockage between the myosin-ATP complex and the myosin-Ca^{++}-ATP complex would require equilibrium with respect to these two complexes. Since the myosin-Ca^{++} complex is necessarily at equilibrium, then even in the event of blockage, the whole system would still be at equilibrium.

The above discussion is in agreement with conclusions reached by Ouellet, Laidler, and Morales (26) in an investigation of Ca^{++} and Mg^{++} effects on the same myosin-ATP system. These ions are in fact "non-competitive" modifiers of ATPase.

In the myosin-calcium-ATP system, there are two distinct paths the reaction can follow to convert the substrate into products. The enzyme E can react with the substrate S to form a binary enzyme-substrate complex ES which dissociates into the enzyme and the products of the reaction. The second path involves formation of the ternary complex enzyme-activator-substrate which dissociates into the enzyme and products. This last ternary complex can be formed in two equivalent ways. The enzyme E can form with the activator M the complex EM which can combine with the substrate S to form the EMS complex. On the other hand the enzyme can form with the substrate the ES complex which can add M to form the EMS complex.

The two paths can therefore be represented by the following equations:

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

$$\begin{matrix} E + M \rightleftharpoons EM \rightleftharpoons EMS \\ E + S \rightleftharpoons ES \rightleftharpoons EMS \end{matrix} \longrightarrow E + P$$

Figure (12) shows the variations of the thermodynamic functions ΔS°, ΔH° and ΔF° for each step in the different paths of the reaction. The formation of the ES and EMS complexes appear to be endothermic processes and to involve large increases in entropy. These increases in entropy can be attributed partly to structural changes (24), contraction of the myosin molecule, and partly to the liberation of water molecules through charge neutralisation (18) resulting when the enzyme and ATP molecules come together. If the increase in entropy were entirely due to the last factor, then compared to the process of fusion an increase of 62.5 entropy units for the formation of the ES complex would correspond to the "unfreezing" of about 12 molecules of water. When the charge and size of the molecules concerned are taken into consideration, the liberation of 12 molecules of water seems at least reasonable. It does not however eliminate the possibility of structural changes which in fact have been observed for this system (3).

The process of activation of the complexes is accompanied by a decrease in entropy, meaning that expansion occurs in the myosin molecule if structural effects are considered and that water molecules, 8 for the activation of the ES complex, are gained if solvent effects are considered.

However, since the constant K_3 depends on the pH, the corresponding values for ΔS° and ΔH° represent overall effects which could be broken down further into H^{+} and substrate contributions.

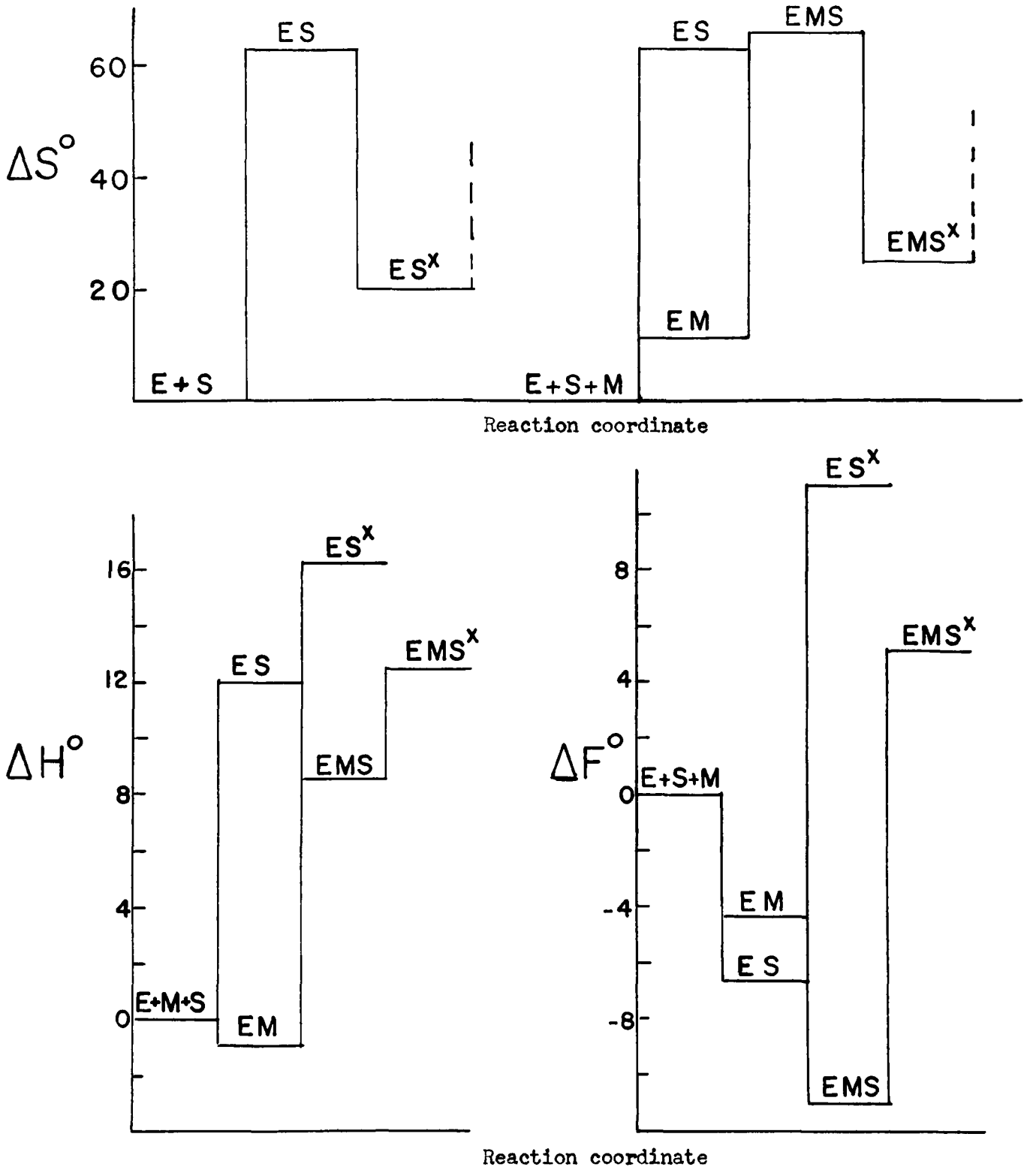

Fig. 12. Graphical representation of the variations of the thermodynamic functions ΔS° (e.u.), ΔH° and ΔF° in kcal/mole, for each step of the reaction.

When M is present in the reaction, the two complexes ES and EMS will be formed. Since the rate of breakdown of the EMS complex is approximately five times greater than that of the ES complex, any factor favouring the formation of the EMS complex will increase the rate of the overall reaction. It is readily seen in figure (12) that the entropy change is greater for the formation of the EMS complex than for that of the ES complex. The same is true for the activation of these complexes. Also the enthalpy changes for the formation and activation of the ES complex are greater than that for the EMS complex. Taken together these factors will favour the formation of the EMS^x complex and increase the rate of the reaction. This is well summarized in figure (12) where the ΔF^o values for the formation and activation of the different complexes are plotted against the reaction coordinates. The EMS and EMS^x complexes are seen to lie on much lower energy levels than the corresponding ES and ES^x complexes, and will therefore be formed preferentially.

Since within experimental error the energies of activation for the reaction in the presence and in the "apparent" absence of Ca^{++} are the same, (refer p. 30) the rate of the reaction will depend on the entropy of activation. From entropy values given in table II, the rate of breakdown of the EMS complex will be greater than that of the ES complex. It is interesting to note that the difference between the entropy of activation of the myosin-calcium-ATP complex and the myosin-ATP is approximately equal to the entropy change (ΔS^o) for the binding of the Ca^{++} to the ES complex.

It has been shown in a study of pH effect (p. 20) that the constant K_3 is a composite equilibrium constant since it includes a term $[H^+]$. The hydrogen ion can be treated as a simple modifier along the same lines as the Ca^{++}. If the reaction is carried out at a high concentration of Ca^{++}, the myosin will exist almost exclusively in the form of the myosin-Ca^{++} complex, and M in the rate expression (27) can be replaced by H. This treatment permits the calculation of the association constants for the myosin-calcium-hydrogen complex from the myosin-Ca^{++} complex and the H^+, and the myosin-calcium-ATP-hydrogen complex from the myosin-calcium-ATP and the H^+. From values in table (I) these association constants have been found to be 3.5×10^7, and 1.7×10^8 liters/mole respectively.

CONCLUSION

The enzymatic myosin-calcium-ATP system has been shown to follow a rate law of the form

$$v = \frac{k_r[\text{Myosin}]_o}{1 + \frac{1}{K_3[\text{ATP}]} + \frac{1}{K_4[Ca^{++}]}\left[1 + \frac{1}{K_2[\text{ATP}]}\right]} + \frac{k_o[\text{Myosin}]_o}{1 + \frac{1}{K_2[\text{ATP}]} + K_4[Ca^{++}]\left[1 + \frac{1}{K_3[\text{ATP}]}\right]}$$

The determined constants for the formation of the different complexes are believed to be equilibrium constants. On this basis, the thermodynamic functions (ΔH^o, ΔS^o, ΔF^o) involved in the formation of the different complexes have been calculated. It appears that the formation of the myosin-calcium-ATP complex from the myosin-ATP complex and the calcium ion is an exothermic reaction and that it involved a small increase in entropy. The increase in the rate of the reaction in the presence of Ca^{++} appears to be due to an increase in the entropy of activation as the energies of activation in the presence and in the "apparent" absence of Ca^{++} have been found to be the same.

The equilibrium constant K_3 for the formation of the myosin-calcium-ATP from the myosin-calcium complex and the ATP varied with pH and this permitted the calculation of acid association constants for the two complexes, myosin-calcium-hydrogen and myosin-calcium-ATP-hydrogen.

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