# KINETIC STUDIES OF PHOSPHOTRANSFERASES:

1. FUNCTION OF METAL IONS IN THE CREATINE KINASE REACTION;

2. MECHANISM OF THE ARGININE KINASE REACTION.

A THESIS

submitted for the Degree

of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

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November, 1966.

This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from September, 1963, to November, 1966, during the tenure of an Australian National University Research Scholarship.

## STATEMENT

The regulations of the Australian National University require that a statement be made describing which parts of the work in this thesis have been carried out by myself:

The method for the preparation of arginine kinase, as described in Chapter II, was devised in collaboration with Mr. F. Marcus, a Fellow of the Rockefeller Foundation working in this Department. The results reported in all other Chapters have been obtained independently.

Candidate's Signature:

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## ACKNOWLEDGEMENTS

It is a great pleasure to express my thanks to my supervisor, Dr. J.F. Morrison, for his guidance and encouragement during this work, and for his interest and readiness for discussion at any time.

I wish to thank both Professor Sir Hugh Ennor and Dr. R.L. Blakley for the privilege of working in the Department; to express my appreciation to the members of the Department for the help they have given me; and to thank Dr. A.B. Roy of the Department of Physical Biochemistry, for carrying out the ultracentrifuge analysis for me.

I am very grateful to Mrs. M. Labutis for skilful and most patient technical assistance. I also wish to thank Mr. I. Reid for his very ready co-operation in many matters.

The photographic prints for this thesis were prepared by Mr. R. Westen and his staff, whose help is greatly appreciated, and I wish to thank Mrs. A. Aldrich for preparation of lettering for the illustrations. I am deeply indebted to Miss P. Smith, whose skill and care in typing the thesis have been of great assistance.

I am grateful for the award of an Australian National University Scholarship during the tenure of which the work described has been carried out.

# PREFACE

The enzyme nomenclature in this thesis is in accordance with the recommendations of the Report of the Commission of Enzymes of the International Union of Biochemistry in that the recommended trivial names have been used throughout, with the number given at the first mention of enzymes discussed.

Temperatures are expressed in °C.

Figures and tables are presented on separate pages, a particular figure or table following in most instances the page on which first reference to it has been made. The identification numbers consist of two parts: Roman numerals indicating the chapter, and Arabic numerals the number of the figure or table within the chapter.

The following abbreviations are used:

| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| PA | phosphoarginine |
| PC | phosphocreatine |
| $NADH_2$ | reduced nicotinamide-adenine dinucleotide |
| NADP | nicotinamide-adenine dinucleotide phosphate |
| DEAE- | diethylaminoethyl- |
| EDTA | ethylenediaminetetraacetic acid |
| DCTA | Trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid |

In addition, abbreviations have been used to describe the computer programmes of Cleland (1963b) which have been used to analyse the kinetic data: below are listed the equations to which the individual programmes fit the data along with the abbreviated names which have been used.

| No. | Equation | Name | Title |
|---|---|---|---|
| 1. | $v = \frac{VA}{K + A}$ | hyperbola | HYPER |
| 2. | $y = ax + b$ | line | LINE |
| 3. | $y = a + bx + cx^2$ | parabola | PARA |
| 4. | $v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB}$ | sequential mechanism | SEQUEN |
| 5. | $v = \frac{VAB}{K_aB + K_bA + AB}$ | Ping-Pong mechanism | PING-PONG |
| 6. | $v = \frac{VA}{K(1 + \frac{I}{K_i}) + A}$ | competitive inhibition | COMP |
| 7. | $v = \frac{VA}{K(1+\frac{I}{K_{is}}) + A(1+\frac{I}{K_{ii}})}$ | non-competitive inhibition | NONCOMP |
| 8. | $v = \frac{VA}{K + A(1 + \frac{I}{K_i})}$ | uncompetitive inhibition | UNCOMP |
| 9. | $v = \frac{VA}{K + A + \frac{A^2}{K_i}}$ | substrate inhibition | SUBINH |

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# INTRODUCTION

The work reported in this thesis is concerned with the elucidation of the function of bivalent metal ions in the reaction catalysed by creatine kinase, and with the determination of the mechanism of the arginine kinase reaction. For both projects a kinetic approach has been used. Therefore, the first part of this Introduction will deal briefly with the development and uses of steady-state kinetics as applied to reactions with one substrate and to reactions with two substrates and two products. Then, since both kinases belong to the class of enzyme known as phosphotransferases, and have an essential requirement for a bivalent metal ion, the present state of knowledge with respect to this type of reaction will be discussed, as also will the current ideas concerning metal-activated enzymes. Finally, some attention will be paid to the biological role of guanidino phosphotransferases.

## General Introduction

In 1926, Sumner demonstrated the catalytic properties of the crystalline protein, urease. Since that discovery, extensive studies have been directed towards the elucidation of the mechanism by which certain proteins are able to accelerate markedly the rate of specific reactions. Accumulated evidence indicates that both substrate specificity and catalytic activity result from the unique three-dimensional structure of the protein that allows formation of an active site

(Boyer, 1963). It is this site that has the potential not only to bind specific molecules, but also, if certain strict structural requirements are met, to catalyse reaction between them. Koshland (1962) has calculated that the proximity and orientation effects brought about by this binding are not in themselves sufficient to account for the large rate increases achieved with enzymic catalysis; the enzyme must, therefore, play an integral part in the reaction sequence.

For an understanding of an enzyme-catalysed reaction therefore, it is necessary to know more than just the overall chemical reaction and the groups on the enzyme and substrate responsible for binding at the active site. It becomes important to study the structural properties of the enzyme in relation to the active site; the manner of binding of substrates, products and inhibitors; the requirement for specific activators or cofactors; the transition states through which the enzyme-substrate complexes pass during the reaction, and the rate constants for these individual steps (Boyer, 1960). While no single technique can provide all this information, kinetic studies, correlating the rate of the reaction with varying concentrations of substrates and inhibitors, can yield information about the order in which substrates are bound to, and products released from the enzyme, the minimum number of transient complexes and the rate limiting step in the reaction.

## The Development of Enzyme Kinetics

The present use of such measurements to gain information

about a reaction mechanism has its origin in the recognition that chemical reactions occur by means of a series of transient complexes formed between the reactants, and that the formation and breakdown of these complexes are time-dependent processes. Since early chemical studies were concerned with the discovery of new compounds and new reactions, it was not until the middle of the nineteenth century that attention was turned to the mechanism of these reactions. It was then that Laurent (1854) and Kekulé (1858) suggested the idea of the transient or intermediate state. The authors proposed that molecules came together during reaction to produce an unstable complex which broke down to form products. At this time, too, it was recognised (e.g. Williamson, 1851-1854) that measurement of the time taken for a reaction to occur was important for an understanding of the phenomena of reaction. Thus realistic rate equations for chemical reactions were formulated. Then when it was realised that the same two basic principles applied also to biochemical reactions, enzyme kinetics were able to develop.

As pointed out by Segal (1959) in a discussion of the historical development of enzyme kinetic theory, it was Brown (1902) who realised that the formation of an intermediate enzyme-substrate complex, requiring a finite time to break down, could account for the limiting velocity attained with invertase at high concentrations of sucrose. These ideas were developed further by Henri (1903) who, assuming

that this complex was in rapid equilibrium with free enzyme and substrate, and that the concentration of the substrate was much greater than that of the enzyme, derived an equation which can be expressed in the familiar form:

$$v = \frac{k(E)(S)}{K_s + S}$$

The importance of Henri's work lies not only in the derivation of this equation but also in the formulation of the method for the derivation of any enzyme rate equation.

Michaelis and Menten (1913) used the same assumptions as had Henri to derive a similar rate equation now associated with their names. In the formulation,

$$v = \frac{V_m S}{K_m + S}$$

$V_m$ was taken to be the maximum velocity that could be attained with a fixed concentration of enzyme at infinite substrate concentration; $K_m$ (Michaelis constant) was both the dissociation constant of the enzyme-substrate complex and the concentration of substrate required to give half maximum velocity. These authors also showed how values for the above kinetic constants could be obtained from initial velocity data.

It was later demonstrated by Briggs and Haldane (1923) that the restrictive assumption of rapid equilibrium between

free enzyme, substrate and enzyme-substrate complex was unnecessary: it could be replaced by a more general steady-state assumption now in general use. This assumption implies that the concentration of intermediate complexes is essentially constant after a brief initial period and remains so over the period during which initial velocity measurements are made. While this does not result in any change in the form of the initial velocity equation, the meaning of the Michaelis constant is altered so that it is no longer a dissociation constant.

The Michaelis-Menten equation adequately expresses the relationship between substrate concentration and initial velocity of a reaction involving only one substrate. But with hydrolytic reactions in which two products are formed, it does not take into account the order in which they are released from the enzyme. Further, it is not applicable to reactions which have two or more substrates, although this point has not always been considered.

Kinetics of Two-substrate, Two-product Reactions

Analysis of two-substrate reactions was concerned first with the derivation of equations expressing the initial velocity of the reaction. It had been considered (Woolf, 1931), by analogy with the binary complex of one-substrate reactions, that such reactions proceeded by way of a ternary complex. Furthermore, when it was realized that two substrates could react in either an ordered or random manner to form this complex,

corresponding rate equations were derived (Segal et al., 1952; Alberty, 1953). These showed that random reactions give rise to non-linear kinetics unless the breakdown of the ternary complex is the rate-limiting step of the reaction. In the latter case, the initial velocity equation for such a rapid equilibrium, random reaction becomes the same as that for all other mechanisms which require the addition of both substrates before any product is formed (sequential mechanisms), including the Theorell-Chance mechanism in which the steady-state concentration of the ternary complex is very low. In addition to these mechanisms, it appeared from studies of transaminase reactions that two-substrate reactions need not proceed via a ternary complex, but that the product of the first substrate to react may be released from the enzyme before the second substrate is added. For this type of reaction (named Ping-Pong by Cleland, 1963a) Alberty (1953) derived a different initial velocity equation.

Therefore, if the kinetics are linear, initial velocity data obtained in the absence of products suffice to categorize a reaction mechanism as either sequential or Ping-Pong; they also yield values for maximum velocities and Michaelis constants of all reactants, as well as inhibition constants of the reactants of a sequential mechanism that form non-central transitory complexes with the enzyme. In fact, the distinction between sequential and Ping-Pong mechanisms can be made most simply by varying the concentrations of the two substrates

at a constant ratio, and plotting the data as a double reciprocal plot. If the mechanism is sequential, the resulting plot will be that of a parabola, while if the mechanism is Ping-Pong, the plot will be linear (Morrison and Cleland, 1966).

For a reaction with two substrates and two products, the elucidation of the mechanism requires an investigation of all the steps occurring from the addition of the first substrate through to the release of the second product and the formation again of the enzyme form with which the substrates react. This knowledge cannot be obtained from analysis of the initial velocity equation. The value of product inhibition studies in this regard has been pointed out by a number of authors including Alberty (1958), Fromm and Nelson (1962) and Cleland (1963a). As indicated by Cleland (1963a) the product inhibition pattern pertaining to a particular mechanism is most easily found by deriving the complete rate equation for that mechanism, and then altering the equation by setting the concentration of each reactant, in turn, to zero. To formulate a full rate equation, it is necessary to propose a series of reactions leading from substrates to products, write individual rate equations for each step consistent with the law of mass action, and solve the series of equations for the velocity of the reaction by assuming, where possible, that steady-state conditions exist. The derivation is simplified by the use of the methods of King and Altman (1956)

and Volkenstein and Goldstein (1966). The full rate equation for two-substrate, two-product reactions expressed in individual rate constants is unwieldy, and not easily amenable to laboratory analysis. Therefore various systems have been devised for collecting rate constants into groups, and thus expressing the equation in terms of discrete, measurable kinetic constants. Examples of these methods are found in Dalziel (1957), Hearon et al. (1959), Alberty (1958), Cleland (1963a) and Dixon and Webb (1964, p.70).

Methods for the Analysis of Kinetic Data

To ascertain the possible mechanism of a particular reaction, experimental data are analysed for consistency with the rate equation of the proposed mechanism and for inconsistency with those of other mechanisms. Evaluation of the kinetic parameters of an equation is achieved by means of the simple linear method popularized by Lineweaver and Burk (1934), and extended to two-substrate reactions by Florini and Vestling (1957). Variations of the simple reciprocal velocity against reciprocal substrate concentration have been introduced. Recently an analysis of the comparable accuracy of the different methods of plotting has been given by Dowd and Riggs (1965) who, while demonstrating the relative inaccuracy of the Lineweaver-Burk plot, note that if proper statistical methods are utilized to fit the data, the method of presentation of plots becomes unimportant. Computer programmes have been written to make such a fit (Cleland, 1963b).

Haldane Relationships

It was first shown by Haldane (1930) that the kinetic parameters thus evaluated are not independent, but are related to one another and to the equilibrium constant of the overall reaction. This relationship may be expressed in general form (Cleland, 1963a) as:

$$K_{eq} = \frac{V_1^n K_{(p)} K_{(q)}}{V_2^n K_{(a)} K_{(b)}}$$

Evaluation of the Haldane relationships predicted by different rate equations using experimentally determined kinetic constants may help to decide which particular reaction mechanism is applicable (Alberty, 1953; Dalziel, 1957). A large number of constants is involved, however, and any one of them may be slightly in error, so it is difficult to obtain a definitive answer. As pointed out by Cleland (1963a), the greatest value of such calculations is in discovering isomerizations of transitory complexes from discrepancies between the calculated and observed values of the equilibrium constant.

Isotope Exchange Studies of Reaction Mechanisms

Isotope exchange studies have been used since the pioneer experiment of Duodoroff, Barker and Hassid (1947) to show that a reaction occurs by a Ping-Pong mechanism and to indicate the possible existence of a stable enzyme-bound intermediate. Boyer (1960) has warned that the demonstration of partial exchange reactions is not proof for the presence of

a reaction intermediate that is covalently bound to the enzyme; but a reaction occurring by means of such an intermediate would necessarily consist of two separate equilibria demonstrable by means of isotope exchange. Boyer (1959) has also introduced the technique of measuring the rate of isotope exchange of a reaction at or near equilibrium. The rate of exchange of isotope between reactants is measured as the concentrations of a like pair of reactants are raised simultaneously, and since different mechanisms yield different patterns, a distinction can be made between them.

As a result of the application of these kinetic procedures, the reaction mechanism of a number of two-substrate, two-product reactions has been established, and representative examples are listed in the accompanying Table.

## Metal Ions and Enzymic Catalysis

### Binding of the Metal to the Protein

Many enzymes depend for activity on the presence of a metal ion. Such enzymes have been divided into two broad categories, metalloenzymes and metal-enzyme complexes, according to the strength of binding of the metal to the enzyme (Vallee, 1955). But Vallee has stressed the operational character of the definition, which is based solely on the results of enzyme isolation and is not intended to imply functional differences.

Metalloenzymes refer to those proteins containing functional metal atoms which are bound firmly enough to remain

TABULATION OF SOME ENZYMES AND THEIR PROPOSED REACTION MECHANISMS AS WELL AS THE KINETIC METHODS USED FOR THE DETERMINATION OF THESE MECHANISMS

| Enzyme | Reaction Mechanism | Method | Reference |
|---|---|---|---|
| Alcohol Dehydrogenase (EC. 1.1.1.1) | Ordered Bi Bi * | Product Inhibition | Wratten & Cleland, 1963. |
| | | Isotope Exchange Rates | Silverstein & Boyer, 1964a. |
| Lactate Dehydrogenase (EC. 1.1.1.27) | Iso Theorell Chance | Product Inhibition | Zewe & Fromm, 1962; 1965; |
| | | Isotope Exchange Rates | Silverstein & Boyer, 1964b. |
| Hexokinase (EC. 2.7.1.1) | Rapid Equilibrium Random | Product Inhibition | Fromm & Zewe, 1962. |
| | | Isotope Exchange Rates | Fromm et al., 1964. |
| Creatine kinase (EC. 2.7.3.2) | Rapid Equilibrium Random | Product Inhibition | Morrison & James, 1965. |
| | | Isotope Exchange Rates | Morrison & Cleland, 1966. |
| Aspartic transaminase (EC. 2.6.1.1) | Ping-Pong | Initial Velocity & Product Inhibition | Henson & Cleland, 1964. |
| Nucleoside Diphospho-kinase (EC. 2.7.4.6) | Ping-Pong | Initial Velocity & Alter-native substrates | Mourad & Parks, 1966. |

* The results are consistent also with a random mechanism in which dissociation of coenzyme from the binary complex is favoured over dissociation from the ternary complex.

associated with the protein throughout isolation procedures, and to be present in the final purified material in stoichiometric amounts relative to the protein. The ratio of the number of atoms of metal to the number of molecules of protein is a small integer. The metal is usually bivalent and is chelated by ligands containing sulphur, nitrogen and oxygen (Vallee and Coleman, 1964). And so it is in keeping with the known ligand-binding properties of the metal to find that the essential zinc of carboxypeptidase A (EC. 3.4.2.1) appears to be bound to the thiol group of the only cysteine residue and to the terminal amino group of the N-terminal asparagine (Coombs et al., 1964), while one of the ligands of the zinc of carboxypeptidase B (EC. 3.4.2.2) would also seem to be a cysteine residue (Wintersberger et al., 1965).

As a result of either thermodynamic or kinetic factors, the dissociation constant of the metalloenzyme complex is essentially zero. Hence the metal can be considered as a stable part of the protein structure and need not be considered during kinetic studies to elucidate the order of substrate binding and product release.

The general name metal-enzyme complex has been given to all enzymes which, after isolation, require the addition of a metal ion for maximum activity. Metal ions that may be associated with the protein in vivo tend to dissociate during purification procedures, so that, since the final product does not contain stoichiometric amounts of functional

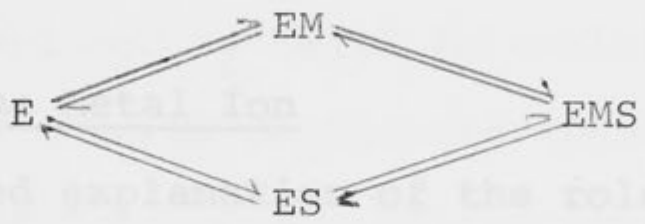
metal, it is not apparent which is the metal ion activating *in vivo*. Because of the high dissociation constants it becomes much more difficult, too, to ascertain the characteristics of enzyme-metal interaction, or indeed to determine whether or not direct enzyme-metal interaction is necessary for catalytic activity. From investigations of this point utilizing the paramagnetic property of $Mn^{2+}$ to measure metal-protein interactions, Cohn (1963) has proposed that enzymes Vallee classified generally as metal-enzyme complexes can themselves be subdivided into two groups:

(1) enzymes for which it is possible to demonstrate a strong enzyme-metal binding;

(2) enzymes with low affinity for free metal ions.

It has therefore been concluded tentatively that enzymes of group (1) function as enzyme-metal complexes, while those of group (2) function as free enzymes catalysing reactions of metal-substrate complexes. An interesting corollary is the ability of $Ca^{2+}$ to activate enzymes belonging to group (2) but not those belonging to group (1). While only a limited number of enzymes has been examined, no exception has yet been found to the generalization that enzymes which react strongly with activating metal ions are not activated by $Ca^{2+}$. It does not follow, however, that the group (1) enzymes must react first with the metal ion before they are able to bind substrates at the active site. Substrates for these enzymes, as for those of group (2), react non-enzymically with metal

ions forming metal-substrate complexes, so that the reaction mixture contains free metal ion, free substrates and metal-substrate complexes, with the concentration of the various ionic species depending not only on total concentrations of reactants but also on the pH, ionic strength, and stability constants of the complexes. It is possible for a ternary complex of enzyme (E), metal (M) and substrate (S) to be formed in any of the three ways illustrated:




and there is no evidence yet that strong metal-enzyme binding is indicative of an ordered pathway with the metal as the first reactant. Indeed, kinetic measurements of the reaction catalysed by inorganic pyrophosphatase (EC. 3.6.1.1) from E. coli are consistent with a mechanism in which a 1:1 complex of $Mg^{2+}$ and pyrophosphate, rather than the pyrophosphate ion, is the active form of the substrate (Josse, 1966), although pyrophosphatases are not activated by $Ca^{2+}$ and appear to bind $Mn^{2+}$ strongly (Cohn, 1964[3]). An additional activating effect by direct Mg-enzyme interaction is not indicated but cannot be ruled out.

While various kinetic equations have been written to distinguish between metal-enzyme and metal-substrate reactions (e.g. Dixon & Webb, 1964, p.429), it seems difficult in practice to obtain unequivocal results by these means. If, in fact, the formation of the ternary EMS complex is an entirely

random reaction (as has been suggested for pyruvate kinase (EC. 2.7.1.40) by Mildvan and Cohn, 1965) and none of these steps is rate limiting, then the distinction becomes fairly arbitrary. Perhaps any subdivision of metal-enzyme complexes based on the strength of metal-enzyme binding should be considered - like the original division into metalloenzymes and metal-enzyme complexes - of purely operational value giving little indication of the real function of the metal ion.

Function of the Metal Ion

A detailed explanation of the role of a metal ion in any particular enzymic reaction cannot yet be given. Nevertheless, evidence accumulated from studies of various enzymes points to the possible importance of functions such as:

(1) stabilization of the tertiary structure of the protein in an active conformation;

(2) participation in the binding of substrates at the active site;

(3) activation of the enzyme-substrate(s) complex by means of the electrophilic property of cations;

(4) participation in oxidation - reduction reactions by undergoing a valency change during the reaction (Vallee & Coleman, 1964).

These are not mutually exclusive, so that a metal may serve more than one function in any reaction. For instance, carnosinase (EC. 3.4.3.3) would seem to require metal ions both to stabilize and to activate the enzyme, and from kinetic

studies and from the different metal ion specificities for the two functions it appears that two sites are present (Rosenberg, 1960). The $Zn^{2+}$ of carboxypeptidase B may also stabilize the protein structure since the apoenzyme is stable under only very limited conditions (Wintersberger et al., 1965).

The idea that the metal was important for the binding of the substrate was postulated by Hellerman and Perkins (1935) and developed by Smith to explain the metal ion requirement of several peptidases (Smith & Spackman, 1955). Smith suggested that enzyme action took place through the formation of a ternary enzyme-metal-substrate complex, with the metal chelated to both enzyme and substrate, and that lysis resulted from the weakening of the peptide bond by the electrophilic property of the metal ion. This idea has received considerable popularity because it is consistent with the known properties of the activating ions (Williams, 1959), but there is little direct evidence to support it. It need not be necessary for the metal to form a bridge between enzyme and substrate. Instead, it is possible that it is bound either to one or the other in a position suitable to influence the electronic environment of the active complex (cf. Mahler, 1961, p.839; Vallee & Coleman, 1964, p.229).

The discovery that metals can be removed from some metalloenzymes to form stable apoenzymes, which can be re-activated, is helping to delineate the role of the metal ion

in these enzymes. The preparation of stable but inactive apoenzymes, as has been achieved with carboxypeptidase A (Coleman & Vallee, 1961) and carbonic anhydrase (EC. 4.2.1.1) (Lindskog & Malmstrom, 1962), indicates the absolute requirement for the metal for catalytic activity. With these preparations it is possible, also, to incorporate different metals into the apoenzyme producing in some cases active, and in other cases inactive enzyme. Thus the $Zn^{2+}$ of both carbonic anhydrase (Lindskog & Malmstrom, 1962) and alkaline phosphatase (EC. 3.1.3.1) (Plocke & Vallee, 1962) can be replaced by $Co^{2+}$ with the retention of considerable activity, while $Cu^{2+}$ produces inactive complexes. The Cu-complex of carboxypeptidase A is also found to be inactive having neither the peptidase nor the esterase activity of the native Zn-enzyme. The importance of the properties of the metal ion for both these activities is borne out by the effects that replacement of $Zn^{2+}$ by other metals has on the two activities. For instance, $Mn^{2+}$, $Co^{2+}$ and $Ni^{2+}$ restore both peptidase and esterase activity, with the Co- and Zn-enzymes being more active towards carbobenzoylglycyl-L-phenylalanine (but not towards other substrates) than the native enzyme. $Hg^{2+}$, $Cd^{2+}$ and $Pb^{2+}$ restore only esterase activity, but in no case does substrate binding seem to be affected seriously (Vallee, 1961).

In contrast to these examples in which the apoenzyme can be reactivated by a variety of metal ions, the apoenzyme of

a $Cu^{2+}$-containing enzyme such as ceruloplasmin can only be restored with $Cu^{2+}$ and not with other bivalent metal ions (Morell & Scheinberg, 1958). This result is compatible with the evidence put forward by Broman et al. (1963) that the catalytic activity of ceruloplasmin involves reduction of the $Cu^{2+}$ by the substrate and reoxidation by $O_2$. If the biological role of $Cu^{2+}$ in metalloenzymes involves a direct participation in oxidation - reduction reactions, it is not surprising that apoenzymes are not reactivated by metal ions that do not undergo a reversible valency change.

Mechanism and Metal Activation of Phosphotransferase Reactions

Phosphotransferases can be classified as those enzymes which catalyse the reversible transfer of the terminal phosphoryl group of a nucleoside triphosphate to an acceptor molecule (Bock, 1960) since, in all the phosphotransferase reactions that have been investigated, bond cleavage occurs between the γ-P and bridge O of the nucleotide (Cohn, 1959). This enzyme-catalysed transfer requires the addition of a bivalent metal ion, so that the enzymes belong, by Vallee's criterion, to the class of metal-enzyme complexes. This requirement is met in all cases by either $Mg^{2+}$ or $Mn^{2+}$ and in some cases by other bivalent metal ions such as $Co^{2+}$ or $Ca^{2+}$. In addition, the requirement for a monovalent cation for pyruvate kinase has been recognised by Boyer et al. (1942), while certain ATPases (EC. 3.6.1.3) have been shown to require both $Na^{+}$ and $K^{+}$ in addition to $Mg^{2+}$ (Skou, 1957).

Because of the ability of nucleotides to chelate strongly with bivalent cations that activate phosphotransferases (Burton, 1959; O'Sullivan & Perrin, 1964), it has been suggested that these metal-nucleotide complexes might be the true substrates of the reactions (e.g. Hers, 1952). This is difficult to ascertain since the metal ion may form a complex with the enzyme as well as one with the nucleotide, and the rates of ligand exchange reactions on bivalent cations such as $Mn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ are very much more rapid than the maximum velocity of these enzymic reactions (Connick & Poulson, 1959; Hammes & Levison, 1964). It can be said, however, that kinetic studies performed to date are consistent with the proposal that metal-nucleotide complexes are substrates for phosphotransferase reactions.

While the order (if any) of metal ion and nucleotide binding has not yet been established, detailed kinetic studies have been carried out with several enzymes to determine the order in which organic substrates combine with and the products leave the enzyme. These indicate that reaction can occur by one of two mechanisms: (a) Sequential, in which the phosphoryl group is transferred directly from donor to acceptor while both are on the enzyme; and (b) Ping-Pong, in which the phosphoryl group is transferred first to the enzyme to form a phosphoenzyme intermediate and then to the acceptor.

These general findings can best be illustrated by reference to some of the specific kinetic data that have been

obtained with phosphotransferases. For example, the reaction catalysed by yeast hexokinase has been shown by initial velocity measurements carried out in the presence of products, analogues or alternative substrates to have a rapid equilibrium, random reaction mechanism when $Mg^{2+}$ is used as the activating ion. Qualitatively similar results were obtained when $Mn^{2+}$ was used instead of $Mg^{2+}$, emphasizing that a change in the activating ion did not alter the reaction mechanism (Fromm & Zewe, 1962; Zewe, Fromm & Fabiano, 1964). This mechanism has been confirmed by Fromm, Silverstein and Boyer (1964) who measured isotope exchange rates at equilibrium and found that high concentrations of one substrate - product pair did not inhibit the rate of isotope exchange between the other pair. The initial velocity data were mainly obtained with the nucleotide, ATP, present as the metal-ATP complex. The consideration of $MgATP^{2-}$ and $MnATP^{2-}$ as substrates is in accord with the observation from nuclear magnetic resonance studies that $Mn^{2+}$ appears to have a low affinity for yeast hexokinase (Cohn, 1963).

In contrast to the results with hexokinase, nuclear magnetic resonance measurements have indicated that pyruvate kinase binds the activating ion $Mn^{2+}$ strongly (Cohn, 1963; Mildvan & Cohn, 1965), but the difference in metal binding between hexokinase and pyruvate kinase may not be paralleled by a difference in reaction mechanism. Binding studies (Reynard et al. 1961) demonstrated separate sites for nucleotide and non-nucleotide substrates, and from this finding

and from initial velocity measurements in the absence and presence of products it was concluded that the reaction mechanism is also a rapid equilibrium, random one. It should be pointed out, however, that the product inhibition experiments with ATP as an inhibitor were carried out without regard to $MgATP^{2-}$ formation. Thus the results are complicated by the reduction in $MgADP^{-}$ concentration and the concomitant increase in $ADP^{3-}$; both effects could contribute to the inhibition. The same problem arises with the work of Mildvan and Cohn (1965) in which concentrations of free $Mn^{2+}$ and of total ADP were treated as substrates with the result that replots of slopes and intercepts are non-linear. The conclusion of Reynard et al. (1961) that the reaction is rapid equilibrium, random, has been questioned by Melchior (1966) since a double reciprocal plot of the initial velocity against the concentration of $MgADP^{-}$ was found to be non-linear. The curve appears to be a hyperbola of a 2/1 nature (Cleland, 1963c) suggesting alternative reaction pathways, that is, that the reaction when studied in the direction of ATP formation may be random, but not rapid equilibrium. The overall conclusion would seem to be that the problem of the mechanism of the reaction catalysed by pyruvate kinase has not yet been resolved.

In spite of the uncertainty about the mechanism of the pyruvate kinase reaction, it is clear that this enzyme, as well as yeast hexokinase and creatine kinase (Morrison & James, 1965; Morrison & Cleland, 1966; see also below p.27) cata-

lyse sequential reactions. On the other hand, initial velocity measurements of the reaction between ATP and glucose catalysed by hexokinase from calf brain (Fromm & Zewe, 1962) and rat skeletal muscle (Hanson & Fromm, 1965) would appear to indicate that both reaction occur by a Ping-Pong mechanism. But clearly the reaction mechanism is more complex than a basic Ping-Pong, to judge from the type of inhibitions caused by products and substrate analogues. Though it would seem very likely that a phosphotransferase reaction with a Ping-Pong mechanism would have a phosphoenzyme as an intermediate, the authors were unable to demonstrate a partial exchange reaction between $^{14}$C-glucose and glucose-6-phosphate and thus proposed instead a glucose-enzyme as an intermediate. Subsequently, they have been unable to demonstrate any exchange reactions with the complete reaction mixture at equilibrium (Fromm, H.J., personal communication). It follows then that the failure to demonstrate the exchange of glucose is not an argument against the existence of a phosphoenzyme as a reaction intermediate in the reactions catalysed by these mammalian hexokinases.

Good evidence for a phosphoenzyme intermediate in a Ping-Pong reaction mechanism has been presented by Mourad and Parks (1965) in a study of the reaction catalysed by nucleoside diphosphokinase from erythrocytes. The mechanism of the reaction was investigated by initial velocity studies in the absence and presence of an alternative substrate and

of 5'-monophosphate nucleotide inhibitors (Mourad & Parks, 1966); the phosphoenzyme was then isolated. While the amino acid residue to which the phosphoryl group is attached has not yet been identified, it may be relevant that phosphohistidine has been detected in the nucleoside diphosphokinase purified from Jerusalem Artichoke mitochondria (Norman et al., 1965). In the course of their initial velocity studies Mourad and Parks found that high concentrations of the nucleoside diphosphate substrates caused inhibition of the reaction. Substrate inhibition is a feature common to several enzymes with Ping-Pong mechanisms. It has, for instance, been noted in studies of pig heart aspartic transaminase (Velick & Vavra, 1962; Henson & Cleland, 1964) and of pig heart alanine transaminase (EC. 2.6.1.2) (Bulos & Handler, 1965).

It is becoming apparent from studies of phosphotransferases that a similarity in chemical reaction is no guarantee of a similarity in enzymic reaction mechanism. The mechanism is a property of the enzyme, and therefore cannot be deduced by analogy with the mechanism of another reaction between chemically similar compounds. It is necessary to carry out detailed kinetic studies on individual enzymes to elucidate the way in which substrates and inhibitors react at the active site. When this understanding can be coupled with a knowledge of the chemistry of the active sites, it should reveal whether or not any basic principle of phosphotransferase reaction mechanism exists.

## Phosphorylated Guanidines and Guanidino Phosphotransferases

### Distribution of Phosphorylated Guanidines

Distributed throughout the animal kingdom is a group of N-phosphorylated guanidine derivatives that have been given the trivial name of phosphagens. The first of these was discovered in skeletal muscle by Eggleton and Eggleton (1927), and isolated and identified as phosphocreatine by Fiske and SubbaRow (1929). Subsequently other phosphagens have been discovered: phosphoarginine in crayfish muscle (Meyerhof & Lohmann, 1928); phosphotaurocyamine and phosphoglycocyamine in the marine worms, Arenicola marina and Nereis diversicolor, respectively (Thoai et al., 1953); phospholombricine in the earthworm Lumbricus terrestris (Thoai & Robin, 1954); phosphohypotaurocyamine in the Gephyrian, Phascolosoma vulgara (Robin & Thoai, 1962); and phosphoopheline in the polychete, Ophelia neglecta (Thoai et al., 1963). The general chemistry and distribution of the phosphagens have been summarized by Morrison and Ennor (1960). Although the original finding of phosphocreatine in vertebrate muscle and phosphoarginine in invertebrate muscle led to the idea that the distribution of the phosphagens might be of use as a phylogenetic criterion, neither the survey by Ennor and Morrison (1958), nor the more recent analyses by Virden and Watts (1964) and by Thoai and Robin (1965) indicate any simple relationship between the phosphagen(s) and kinase(s) present and evolutionary

development.

Function of Phosphagens

While highly purified preparations of both alkaline and acid phosphatases (EC. 3.1.3.2) have been shown to hydrolyse phosphagens (Morrison et al., 1958; Morton, 1955), the main biological function of phosphagens would seem to involve the phosphotransferase reactions catalysed by specific guanidino kinases (Morrison & Ennor, 1960). Moreover, since the original isolations of phosphocreatine and phosphoarginine and of creatine kinase and arginine kinase (EC. 2.7.3.3) (Lohmann, 1934; 1935) were made from muscle tissue, the biological roles of both phosphagens and of their kinases have been considered in terms of muscular contraction. Early experiments with iodoacetate-poisoned frog muscle showed that the energy for contraction was supplied by phosphocreatine breakdown; phosphoarginine was shown to play a similar role in crustacean muscle (Lundsgaard, 1930; 1931). Recent work, however, in which the creatine kinase of frog muscle was poisoned with 1-fluoro-2,4-dinitrobenzene has provided good evidence that the direct energy source for muscle contraction is ATP which is hydrolysed by an ATPase to ADP and Pi and then reconstituted by the action of either adenylate kinase (EC. 2.7.4.3) or creatine kinase (Davies, 1965). Thus phosphocreatine, and presumably other phosphagens, can be considered as a store of energy inside a muscle cell. It is known, though, that phosphocreatine and creatine kinase are widely distributed throughout the animal body where their function is

unknown (Morrison & Ennor, 1960; Kuby & Noltman, 1962). The enzyme has been purified from ox brain (Wood, 1963) and enzyme activity has also been found associated with mitochondria (Jacobs et al., 1964). The finding that creatine kinase bound to pigeon breast mitochondria requires extramitochondrial nucleotides suggested to Bessman and Fonyo (1966) that the enzyme could be important for the regulation of respiration especially in response to muscular activity. Similar studies of the distribution of other phosphagens and their kinases have not been carried out, but it would seem reasonable, by comparison, to consider them to have a more general function than just an energy store for muscular contraction.

General Properties of Guanidino Phosphotransferases

Reference has been made to the discovery of creatine kinase and arginine kinase which were found in vertebrate and invertebrate muscle respectively. Kinases specific for the other known phosphagens have been discovered, purified and partially characterized by van Thoai and his collaborators in a series of papers as tabulated below:

| Enzyme | Source | Reference |
|---|---|---|
| glycocyamine kinase (EC. 2.7.3.1) | Nephthys coeca | Thoai, 1957; Pradel, Kassab & Thoai, 1964. |
| taurocyamine kinase (EC. 2.7.3.4) | Arenicola marina | Thoai, 1957; Thoai & Pradel, 1962a, b; Kassab, Pradel & Thoai, 1965. |

| Enzyme | Source | Reference |
|---|---|---|
| lombricine kinase (EC. 2.7.3.5) | Lumbricus terrestris | Kassab, Pradel & Thoai, 1965. |
| hypotaurocyamine kinase (EC. 2.7.3.6) | Phascolosoma vulgara | Thoai, Robin & Pradel, 1963; Thoai, Kassab & Pradel, 1965. |
| opheline kinase | Ophelia neglecta | Thoai, di Jeso, Robin & der Terrossian, 1966. |

From the experimentally determined molecular weights of 81,000 for creatine kinase (Noda et al., 1954) and of 43,000 for arginine kinase from crab muscle (Elodi & Szorenyi, 1956), the molecular weights of taurocyamine kinase, hypotaurocyamine kinase, glycocyamine kinase and lombricine kinase have been estimated by gel filtration to be in the range 70,000 - 90,000 (Thoai, Kassab & Pradel, 1965). All guanidino kinases are sensitive to sulphydryl reagents, and, with the exception of arginine kinase from the Australian sea-crayfish, Jasus verreauxi (Morrison et al., 1957), are activated by $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$. The pH optima in the direction of ATP formation are in the vicinity of pH 7.0 and in the direction of ATP breakdown in the range pH 8.4 - 9.0.

## Mechanism of Creatine and Arginine Kinase Reactions

Since creatine kinase and arginine kinase are the two enzymes with which this Thesis is concerned, and since the work with creatine kinase is dependent on a knowledge of the reaction mechanism while that with arginine kinase is concerned with the elucidation of the mechanism, a brief

summary of the pertinent findings will be given.

(a) Creatine Kinase

Creatine kinase was isolated and crystallized from rabbit muscle by Kuby, Noda and Lardy (1954), and it is with enzyme from this source that studies of mechanism have largely been carried out. The existence of separate binding sites for nucleotide and guanidino substrates was demonstrated by thermodynamic studies (Kuby, Mahowald & Noltmann, 1962). This result suggested that the enzyme might have a random mechanism. From kinetic studies it was shown that the magnesium complexes of the nucleotides were able to act as substrates, while the guanidino substrates react as uncomplexed species (Kuby & Noltmann, 1962, p.576, p.553), and so experiments designed to elucidate the mechanism were performed with the Mg-nucleotide complexes and the free guanidino compounds as substrates. Product inhibition experiments (Morrison & James, 1965) and measurements of isotope exchange rates at equilibrium (Morrison & Cleland, 1966) verified the conclusion from binding studies and early kinetic studies (Kuby & Noltmann, 1962), indicating that the reaction mechanism is indeed a rapid equilibrium, random one.

(b) Arginine Kinase

Insufficient data are available to indicate the mechanism of the reaction catalysed by arginine kinase. Moreover, while studies of creatine kinase have nearly all been done with enzyme from rabbit muscle, studies of arginine kinase

have been done with enzymes prepared from several different members of the crustacean class and there is no indication that the results are relevant one to another. For instance, in addition to the variation in cation activation mentioned above (p.26), the two preparations whose molecular weights have been measured have been found to differ: the molecular weight of the enzyme from crab appears to be 43,000 (Elodi & Szorenyi, 1956) and that of enzyme from the lobster, 37,000 (Virden et al., 1966).

Hence for a study of the mechanism of the reaction catalysed by arginine kinase from the sea-crayfish it is interesting, but may not be relevant, that initial velocity measurements of phosphoarginine formation at varying concentrations of arginine and $MgATP^{2-}$ indicate that the lobster enzyme catalyses the reaction by means of a sequential mechanism (Virden, Watts & Baldwin, 1965). Some kinetic studies have already been performed with a partially purified preparation from the sea-crayfish (Griffiths et al., 1957), but since only one substrate was varied in any one experiment, and since allowance was not always made for the non-enzymic formation of Mg-substrate complexes, they are insufficient to give any indication of the reaction mechanism.

# CHAPTER I

## THE FUNCTION OF BIVALENT METAL IONS IN THE REACTION CATALYSED BY ATP:CREATINE PHOSPHOTRANSFERASE

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## INTRODUCTION

The reaction catalysed by creatine kinase has an essential requirement for a bivalent metal ion and this may be met by $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$ or $Co^{2+}$ (Kuby & Noltmann, 1962). The function of the metal ion in the catalytic process has not been established, and any kinetic studies designed to investigate this question are complicated by the ability of the metal ions which activate the enzyme to undergo non-enzymic reaction with the fully ionized forms of the nucleotide substrates to yield complexes of the type $MATP^{2-}$ and $MADP^{-}$. This difficulty has been overcome in the study of the $Mg^{2+}$-activated enzyme with the knowledge that these complexes can function as substrates for the reaction as also can the free forms of phosphocreatine and creatine (Kuby & Noltmann, 1962, pp.576, 553), so that the reaction may be considered to be bireactant and written as:

$$MgATP^{2-} + \text{creatine} \rightleftharpoons MgADP^{-} + \text{phosphocreatine}^{2-}$$

Moreover, the apparent stability constants for these complexes have been measured under conditions suitable for kinetic studies (O'Sullivan & Perrin, 1964). Therefore, it has been possible to investigate the mechanism of this reaction by measurements of product inhibition (Morrison & James, 1965)

and isotope exchange rates (Morrison & Cleland, 1966), and these experiments have shown that the reaction has a rapid equilibrium, random mechanism. This means that the enzyme possesses two distinct sites, one for the nucleotide and the other for the guanidino substrate and the slowest step in the reaction sequence is the interconversion of the central complexes. Since O'Sullivan and Perrin (1964) have obtained values also for the $Ca^{2+}$ and $Mn^{2+}$ complexes of ATP and ADP under the same conditions, it should be possible to analyse the kinetics of the reaction when $Mg^{2+}$, $Ca^{2+}$ or $Mn^{2+}$ is used as the activating ion.

Comparative studies have been made of the effect of various bivalent metal ions on the kinetics of the reaction by Rosenberg and Ennor (1955) and by Cho, Haslett and Jenden (1960), and it has been shown by Cho et al. and by Watts (1963) that higher concentrations of the activating ions can give rise to inhibition. It is difficult, however, to reach any firm conclusions from these results even in the light of the known reaction mechanism, since no allowance was made for the complexing of the metal ions by the reaction components.

While the elucidation of the mechanism has shown that the formation of an enzyme-Mg-nucleotide complex can be part of the reaction sequence, it does not indicate whether this complex involves a direct interaction of metal ion and enzyme. The equilibrium dialysis studies of Kuby et al. (1962) with $Mg^{2+}$

and the nuclear magnetic resonance studies of Cohn (1963) with $Mn^{2+}$ indicated only weak binding between these ions and creatine kinase. Therefore, it has been suggested (Cohn, 1963) that the metal ion does not function by forming a bridge between enzyme and nucleotide. Mg-enzyme interactions have also been examined kinetically by measuring the inhibition caused by high concentrations of $Mg^{2+}$: inhibition is non-competitive with respect both to Mg-nucleotide and to guanidino substrates, indicating that, while $Mg^{2+}$ can combine with the enzyme so as to reduce the initial velocity of the reaction, this combination is not at the Mg-nucleotide binding site (O'Sullivan & Morrison, 1965; James, 1965). Though these experiments show that formation of a Mg-enzyme complex would seem to be an unlikely first step in the reaction sequence, other experiments, measuring the initial velocity with $Mg^{2+}$ as a variable substrate, indicate that, at least under certain conditions, the complex can be of kinetic importance (James, 1965). These kinetic and thermodynamic findings, together with the fact that the isolated enzyme does not contain any essential metal ion in bound form (Kuby & Noltmann, 1962), have prompted further investigations of the reaction with the object of elucidating the role played by metal ions.

The conditions chosen were similar to those described previously for the product inhibition studies (Morrison & James, 1965), and on the basis that the reaction mechanism

is a property of the enzyme, initial velocity studies of the reaction with $MgADP^-$ and phosphocreatine as substrates have been used to obtain values for the various kinetic constants associated with the Mg, Ca and Mn complexes of ADP. The results indicate that, while the nature of the metal ion has little influence on the binding of the metal-nucleotide complex to the free form of the enzyme, it does affect the maximum velocity of the reaction. Further, it has been found that the combination of metal-nucleotide complexes with the enzyme-phosphocreatine complex is a function of the metal ion present in the system.

## THEORY

At pH 8.0, ADP can be considered to exist in its fully ionized form as $ADP^{3-}$ so that in the presence of a bivalent metal ion (M), the non-enzymic reaction

$$M^{2+} + ADP^{3-} \rightleftharpoons MADP^-$$

occurs (O'Sullivan & Perrin, 1964). Thus when $MADP^-$ is used as the nucleotide substrate for the creatine kinase reaction, it is necessary to take into account the concentrations of $ADP^{3-}$ and $M^{2+}$ which will vary according to the apparent stability constant of the $MADP^-$ complex. $ADP^{3-}$ has been shown to be a strong competitive inhibitor of the reaction with respect to the metal-nucleotide complex (Morrison & O'Sullivan, 1965) while $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ are

relatively weak inhibitors (see also Results). Therefore, in all experiments the concentration of $ADP^{3-}$ has not been allowed to exceed 0.4 times its $K_i$ value of 0.25 mM and its effect on the value of the kinetic constants has been assumed to be negligible. On the other hand, the inhibitory effect of the bivalent metal ions has been taken into account since experiments have been carried out at relatively high concentrations of these ions.

Phosphocreatine can also complex with $Mg^{2+}$ and $Mn^{2+}$ (O'Sullivan & Perrin, 1964) and while allowance has been made for the resulting reduction in the concentrations of free metal ion and free phosphocreatine, it has been considered that the metal-phosphocreatine complexes are inert (Kuby & Noltmann, 1962, p.553).

Under conditions where the concentrations of both $ADP^{3-}$ and $M^{2+}$ are low compared with their respective $K_i$ values, the initial velocity of the creatine kinase reaction in the reverse direction may be expressed as

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \qquad (1)$$

where A and B represent the metal-nucleotide complex and free phosphocreatine, respectively, $K_{ia}$ represents the dissociation constant for the reaction of A with free enzyme and V represents the maximum velocity of the reaction (Morrison & James, 1965). Because the reaction has a rapid equilibrium, random

mechanism, the Michaelis constants, $K_a$ and $K_b$, are the dissociation constants for the reaction of A and B with the enzyme-B and enzyme-A complexes, respectively. It also follows that $K_{ia}K_b = K_aK_{ib}$ where $K_{ib}$ represents the dissociation constant for the reaction of the free enzyme with B.

If the reaction is studied in the presence of relatively high concentrations of metal ions which are added as their chloride salts, then allowance should be made for

(a) the possible inhibitory reaction of the metal ion (M) with each of the four enzyme species: free enzyme ($K_1$), enzyme-MADP ($K_2$), enzyme-phosphocreatine ($K_3$) and enzyme-MADP-phosphocreatine ($K_4$); and

(b) the inhibition that is known to occur as a result of the reaction of $Cl^-$ with the binary enzyme forms: enzyme-MADP ($K_5$) and enzyme-phosphocreatine ($K_6$).

(The unpublished observations of Morrison and James indicate that $Cl^-$ acts as an inhibitor of the creatine kinase reaction by combining with the enzyme-MgADP and enzyme-phosphocreatine complexes). (The constants given in brackets represent dissociation constants for the reaction of either M or Cl with the indicated forms of enzyme: $K_5$ and $K_6$ would be apparent constants because of the presence in the buffer of 40 mM $Cl^-$). When this is done, the initial velocity equation becomes:

$$v = \frac{VAB}{K_{ia}K_b\left(1 + \frac{M}{K_1}\right) + K_aB\left(1 + \frac{M}{K_3}\right)\left(1 + \frac{Cl}{K_6}\right) + K_bA\left(1 + \frac{M}{K_2}\right)\left(1 + \frac{Cl}{K_5}\right) + AB\left(1 + \frac{M}{K_4}\right)} \quad (2)$$

As the concentration of Cl is equivalent to twice the concentration of the metal ion, the Cl in equation (2) can be replaced by 2M. It would, therefore, be expected that both the slopes and vertical intercepts of primary plots of 1/v against 1/A and 1/v against 1/B, at different concentrations of $MCl_2$, would be parabolic functions of the concentrations of $MCl_2$. No evidence was obtained for non-linear secondary plots using $MgCl_2$, $CaCl_2$ or $MnCl_2$ (Morrison & O'Sullivan, 1965; and see Results) and hence it has been concluded that under the chosen experimental conditions, the effect of the additional $Cl^-$ is negligible. This conclusion is in accord with the unpublished results of James and Morrison who showed that little difference could be expected between the inhibition of creatine kinase by $MgCl_2$ and $Mg(acetate)_2$, even though the acetate ion is much less inhibitory than $Cl^-$. If the inhibition by $Cl^-$ is neglected, equation (2) may be simplified to

$$v = \frac{VAB}{K_{ia}K_b\left(1 + \frac{M}{K_1}\right) + K_aB\left(1 + \frac{M}{K_3}\right) + K_bA\left(1 + \frac{M}{K_2}\right) + AB\left(1 + \frac{M}{K_4}\right)} \quad (3)$$

In this equation, M can be considered to represent the concentration of $MCl_2$ (which will be referred to hereafter as $M^{2+}$) and the values for the inhibition constants, $K_1$ - $K_4$, as

obtained from apparently linear secondary plots, might be regarded as close to, although not necessarily identical with the true values. However, it is true that the concentration of $Cl^-$ over and above that which is considered in association with the metal ion and which is released as a result of the reaction of $MCl_2$ with ADP, is low compared with the $K_5$ and $K_6$ values for $Cl^-$ of 50 and 70 mM, respectively. The highest concentration of $MADP^-$ used in the present work was 0.4 mM so that the maximum concentration of $Cl^-$ released would be 0.8 mM.

Initial velocity equations with $MADP^-$ (A) and free phosphocreatine (B) as the variable substrates

With A and B as the variable substrates, equation (3) may be rearranged in reciprocal form as

$$\frac{1}{v} = \frac{K_a}{V}\left\{\frac{K_{ib}}{B}\left(1 + \frac{M}{K_1}\right) + 1 + \frac{M}{K_3}\right\}\frac{1}{A} + \frac{1}{V}\left\{\frac{K_b}{B}\left(1 + \frac{M}{K_2}\right) + 1 + \frac{M}{K_4}\right\} \qquad (4)$$

and

$$\frac{1}{v} = \frac{K_b}{V}\left\{\frac{K_{ia}}{A}\left(1 + \frac{M}{K_1}\right) + 1 + \frac{M}{K_2}\right\}\frac{1}{B} + \frac{1}{V}\left\{\frac{K_a}{A}\left(1 + \frac{M}{K_3}\right) + 1 + \frac{M}{K_4}\right\} \qquad (5)$$

so that at a fixed concentration of M, plots of 1/v against 1/A or 1/v against 1/B will be linear with both the slopes of the lines and the vertical intercepts varying with the concentration of B or A, respectively. Secondary plots of the slopes and vertical intercepts against 1/B or 1/A will be linear and give apparent values for the kinetic constants associated with B and A. The true values may be calculated from the relation-

ships :

$$K_{ib} = \text{app. } K_{ib}\left\{\frac{1 + \frac{M}{K_3}}{1 + \frac{M}{K_1}}\right\} \quad : \quad K_b = \text{app. } K_b\left\{\frac{1 + \frac{M}{K_4}}{1 + \frac{M}{K_2}}\right\}$$

$$K_{ia} = \text{app. } K_{ia}\left\{\frac{1 + \frac{M}{K_2}}{1 + \frac{M}{K_1}}\right\} \quad : \quad K_a = \text{app. } K_a\left\{\frac{1 + \frac{M}{K_4}}{1 + \frac{M}{K_3}}\right\}$$

Equations for the calculation of the inhibition constants associated with metal ions

From equations (4) and (5), it is apparent that if each of the metal ions is capable of inhibiting the reaction as a result of reacting with each of the four enzyme species so as to form dead-end complexes, then the inhibition will be non-competitive with respect to both A and B. However, secondary plots of the slopes and vertical intercepts of the primary plots would not yield values for any of the inhibition constants. These values must be obtained by solution of the four simultaneous equations:

equation (4):

$$K_i \text{ slope} = \frac{1 + \frac{K_{ib}}{B}}{\frac{1}{K_3} + \frac{K_{ib}}{K_1 B}} \quad : \quad K_i \text{ vertical intercept} = \frac{1 + \frac{K_b}{B}}{\frac{1}{K_4} + \frac{K_b}{K_2 B}}$$

equation (5):

$$K_i \text{ slope} = \frac{1 + \frac{K_{ia}}{A}}{\frac{1}{K_2} + \frac{K_{ia}}{K_1 A}} \quad : \quad K_i \text{ vertical intercept} = \frac{1 + \frac{K_a}{A}}{\frac{1}{K_4} + \frac{K_a}{K_3 A}}$$

using appropriate values for $K_{ib}$, $K_b$, $K_{ia}$ and $K_a$.

If the metal ion does not react with the free form of enzyme or the enzyme-phosphocreatine complex so as to cause inhibition, as appears to be the case with $CaCl_2$ and $MnCl_2$ (see Results), then $K_1$ and $K_3$ become infinity and equations (4) and (5) reduce to

$$\frac{1}{v} = \frac{K_a}{V}\left\{\frac{K_{ib}}{B}\right\}\frac{1}{A} + \frac{1}{V}\left\{\frac{K_b}{B}\left(1 + \frac{M}{K_2}\right) + 1 + \frac{M}{K_4}\right\} \qquad (6)$$

$$\frac{1}{v} = \frac{K_b}{B}\left\{\frac{K_{ia}}{A} + 1 + \frac{M}{K_2}\right\}\frac{1}{B} + \frac{1}{V}\left\{\frac{K_a}{A} + 1 + \frac{M}{K_4}\right\} \qquad (7)$$

The inhibition by $Ca^{2+}$ and $Mn^{2+}$ would still be non-competitive with respect to B (equation 7), but become uncompetitive with respect to A (equation 6). Apparent values for $K_2$ and $K_4$ can be obtained from secondary plots of the slopes and vertical intercepts of the primary plot (equation 7) against the concentration of M. The true values can be calculated from the relationships

$$K_2 = \frac{\text{app. } K_2}{1 + \frac{K_{ia}}{A}} \quad \text{and} \quad K_4 = \frac{\text{app. } K_4}{1 + \frac{K_a}{A}}$$

If metal ions react with the ternary complex, enzyme-MADP-phosphocreatine, to form a dead-end complex, then the true maximum velocity of the reaction can be determined from the relationship $V = \text{app. } V\left(1 + \frac{M}{K_4}\right)$ (equations 4 - 7). Alternatively, it can be obtained by extrapolation to zero of a plot of apparent $\frac{1}{V}$ against the concentration of M. Such a plot would be linear.

It will be noted that calculation of the true values for

the kinetic constants of the substrates from the apparent values determined from secondary plots, requires a knowledge of the values for the inhibition constants of the metal ion. The reverse is also true. Theoretically, it is possible to obtain these values from tertiary plots of the apparent constant values at different concentrations of the metal ion against the concentration of the metal ion by fitting either a hyperbola (equations 4 and 5) or a straight line (equations 6 and 7). However, as it was not practical to use a wide range of metal ion concentrations, the values so obtained would not be accurate. Therefore, it has been assumed that the kinetic constants for the substrates as obtained from initial velocity studies with the free metal ion at a concentration of 1-2 mM, are close to the correct values and these have been used to determine the inhibition constants for the metal ions. This assumption would appear to be reasonable because of the relatively high values obtained for $K_1$ to $K_4$. Corrections to the directly determined values for the kinetic constants of the substrates have been applied only when the free metal ion concentration was in excess of 2 mM.

## EXPERIMENTAL

### Materials

Phosphocreatine was prepared by the method of Ennor and Stocken (1948) as modified by Peanasky, Kuby and Lardy (1957);

solutions were treated with Chelex 100 ($Na^+$ form; 200-400 mesh) obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. as described by Morrison and O'Sullivan (1965). The free creatine content was about 0.03 mole/100 moles of phosphocreatine.

The sodium salt of ADP was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and purified by the method of Morrison, O'Sullivan and Ogston (1961). After purification it showed only one spot which absorbed ultraviolet light after chromatography in isobutyric acid - $NH_3$ (sp. gr. 0.88) - water (66:1:33 by vol.). Stock solutions (10 mM) were adjusted to pH 7.6 with 0.5 N NaOH and stored at -10°. The concentration was determined in 0.01 N HCl at 259 mμ (Bock, Ling, Morell & Lipton, 1956).

N-ethylmorpholine (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was purified by distillation (Morrison et al., 1961). Stock aqueous solutions were prepared by weight and adjusted to pH 8.0 with 5 N HCl (microanalytical reagent; British Drug Houses Ltd.).

Laboratory grade EDTA and Analar grade $CaCl_2.6H_2O$, $MnCl_2.4H_2O$ and diphenylthiocarbazone (dithizone) were products of British Drug Houses Ltd. $MgCl_2.6H_2O$ (Guaranteed reagent) and carbon tetrachloride were supplied by E. Merck AG. Darmstadt. Aqueous solutions of $MgCl_2$, $MnCl_2$ and $CaCl_2$ were standardized as described by Morrison et al. (1961).

Trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid

(DCTA) was obtained from Fluka AG, Buchs SG, Switzerland. Creatine kinase was isolated in crystalline form by the procedure of Kuby et al. (1954). Stock solutions of the enzyme were prepared, stored, and diluted as described by Morrison et al. (1961).

## Methods

### Measurement of creatine kinase activity:

The methods used were essentially those elaborated by Morrison, O'Sullivan and Ogston (1961). Reaction mixtures contained in a total volume of 1.0 ml: N-ethylmorpholine-HCl buffer (pH 8.0) 0.1 M; EDTA, 0.01 mM; substrates and the required bivalent salt at concentrations as indicated. After the addition of the components the tubes were kept in ice, and before the addition of the enzyme were incubated for 3 min. at 30°. The amount of enzyme added corresponded to 0.58 µg of protein for the experiments with $Mg^{2+}$ and $Ca^{2+}$ and to 0.29 µg for those in which $Mn^{2+}$ was used. All experiments were run for at least two time periods (between 0.5 and 2 min.) to ensure that initial velocities were being measured. Reactions involving $Mg^{2+}$ were stopped by the addition of 0.6 ml of 3 N NaOH containing 0.14 M EDTA; those involving $Mn^{2+}$ were stopped by the addition of 0.5 ml of 0.08 M DCTA (adjusted to pH 12 with NaOH) and 0.3 ml of 3 N NaOH was added just prior to the estimation of creatine. Usually creatine was estimated in a final volume of 3.0 ml as described by Morrison et al. (1961),

but when $Ca^{2+}$ was used as the activating ion, the volume was reduced to 1.5 ml. The reaction was stopped by the addition of 0.2 ml of 5.3 N NaOH containing 0.42 M EDTA. All extinction measurements were made with a Shimadzu Spectrophotometer.

Purification of metal chloride salts:

Aqueous solutions (50%, w/v) of the metal chloride salts were extracted repeatedly with equal volumes of a 0.001% (w/v) solution of dithizone in carbon tetrachloride until no colour change could be detected in the carbon tetrachloride layer. The aqueous solution was then shaken twice with the same volume of carbon tetrachloride so as to remove any dithizone, after which the carbon tetrachloride was removed by blowing air through the solution. A neutralized solution of DCTA was then added to a final concentration of 0.005 M and the salt crystallized by evaporation. The salt was recrystallized from glass distilled water. Solutions of the metal salts were made up and standardized as described by Morrison et al. (1961).

Calculation of substrate concentrations:

The concentrations of total metal ion, total ADP and total phosphocreatine that were necessary to give the required concentrations of $M^{2+}$, $MADP^{-}$ and free phosphocreatine were calculated by the procedure outlined by Morrison et al. (1961). For this purpose the values used for the apparent stability constants of $MgADP^{-}$, $CaADP^{-}$ and $MnADP^{-}$ were 4,000 $M^{-1}$, 2,200 $M^{-1}$, 25,000 $M^{-1}$, respectively. The apparent stability con-

stants for the Mg, Ca and Mn complexes of phosphocreatine were taken to be 40 $M^{-1}$, zero and 50 $M^{-1}$ (O'Sullivan & Perrin, 1964).

Determination of the maximum velocity of the reaction:

The maximum velocity of the reaction with different metal-nucleotide substrates at a fixed, low concentration of the free metal ion was determined under conditions where the concentrations of $MADP^-$ and phosphocreatine were varied simultaneously while their ratio was maintained constant. As pointed out by Morrison and Cleland (1966), when reciprocals of the initial velocities are plotted against the reciprocals of the concentrations of one of the substrates, a parabola is obtained for a reaction possessing a sequential mechanism. The vertical intercept of the parabola gives a measure of the maximum velocity.

Analysis of results:

All initial velocity data were plotted graphically in double reciprocal form, and occasional points that departed markedly from the general pattern were discarded. Precise analysis of the remaining data was carried out using the appropriate computer programme of Cleland (1963b) with the aid of an IBM 1620 computer and programmes written in Fortran II(D). Primary initial velocity data that gave a linear double reciprocal plot were analysed by the HYPER programme (preface, equation (1) ) to give the slope (K/V) and vertical

intercept (1/V) and their standard errors. These constants were used to draw the lines of the primary plots. Analyses of the slopes and intercepts of any one experiment by the LINE programme (preface, equation (2) ) were used to draw the secondary plots. Primary initial velocity data that gave a parabolic double reciprocal plot were analysed by the PARA programme (preface, equation (3) ). The results of these analyses indicated the particular rate equation which fitted all the primary data of any one experiment. The values for and the standard errors of the apparent kinetic constants were obtained by making this fit with the aid of the SEQUEN, NONCOMP and UNCOMP programmes (preface, equations (4), (7) and (8), respectively). The weighted mean values of the apparent constants, together with their standard errors, were calculated according to the formulae:

$$\text{Weighted mean of x values} = \frac{\sum W_i X_i}{\sum W_i} \quad \text{and}$$

$$\text{S.E. of weighted mean value} = \frac{1}{\sqrt{\sum W_i}} \quad \text{where } W_i = \frac{1}{[\text{S.E.}(X_i)]^2}$$

The true values for the kinetic constants were calculated from the relationships given in the Theory section and the standard error ($\sigma$) of these values was determined by means of the relationships:

$$\sigma\left(\frac{x}{y}\right) = \frac{x}{y}\sqrt{\left(\frac{\text{S.E.}(x)}{x}\right)^2 + \left(\frac{\text{S.E.}(y)}{y}\right)^2} \quad \text{and}$$

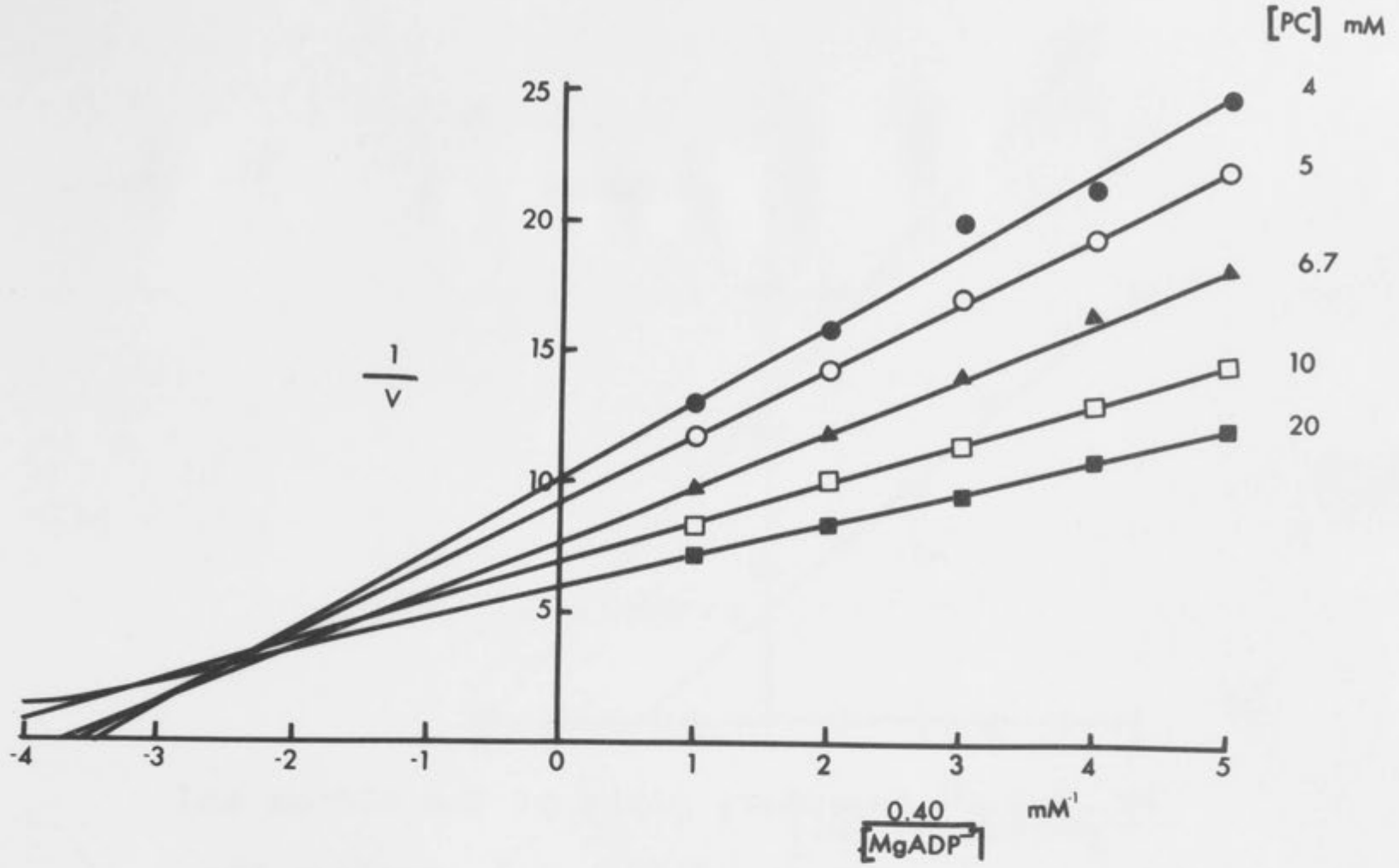
$$\sigma(xy) = xy\sqrt{\left(\frac{S.E.(x)}{x}\right)^2 + \left(\frac{S.E.(y)}{y}\right)^2}$$

## RESULTS

### Initial velocity studies with $Mg^{2+}$ as the activating ion:

A series of investigations was made in which free phosphocreatine was held constant at various concentrations and the initial velocities of the reaction were determined as a function of the concentration of $MgADP^-$. At the same time, the concentration of free $Mg^{2+}$ was kept at a fixed value. The results obtained with free $Mg^{2+}$ at a concentration of 10.0 mM are illustrated in Fig. I.1(a) and similar results were obtained with free $Mg^{2+}$ at concentrations of 1.0 and 5.0 mM. Thus all primary plots gave families of straight lines which intersected at a point to the left of the ordinate and above the abscissa. When the same data were plotted with phosphocreatine as the variable substrate at different fixed concentrations of $MgADP^-$, similar linear plots were obtained (Fig. I.1(b) ). Secondary plots of the slopes and vertical intercepts of these primary plots against the reciprocals of the concentrations of the fixed variable substrates were linear (cf. Fig. I.2) and the results are, therefore, in agreement with those predicted by equations (4) and (5). Analysis of the data by means of the SEQUEN computer programme gave the values for the apparent maximum velocities and apparent kinetic constants which are listed in Table I.1. Although equations (4) and (5) predict that the values for

FIG. I.1. Effect of phosphocreatine on the initial velocity of the reaction catalysed by creatine kinase with $MgADP^-$ as the variable substrate (a), and the effect of $MgADP^-$ on the initial velocity of the reaction with phosphocreatine as the variable substrate (b). The concentration of free $Mg^{2+}$ held constant at 10 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.


[PC] mM
4
5
6.7
10
20
25
20
15
10
5
1/V
-4
-3
-2
-1
0
1
2
3
4
5
0.40 / [MgADP] mM⁻¹

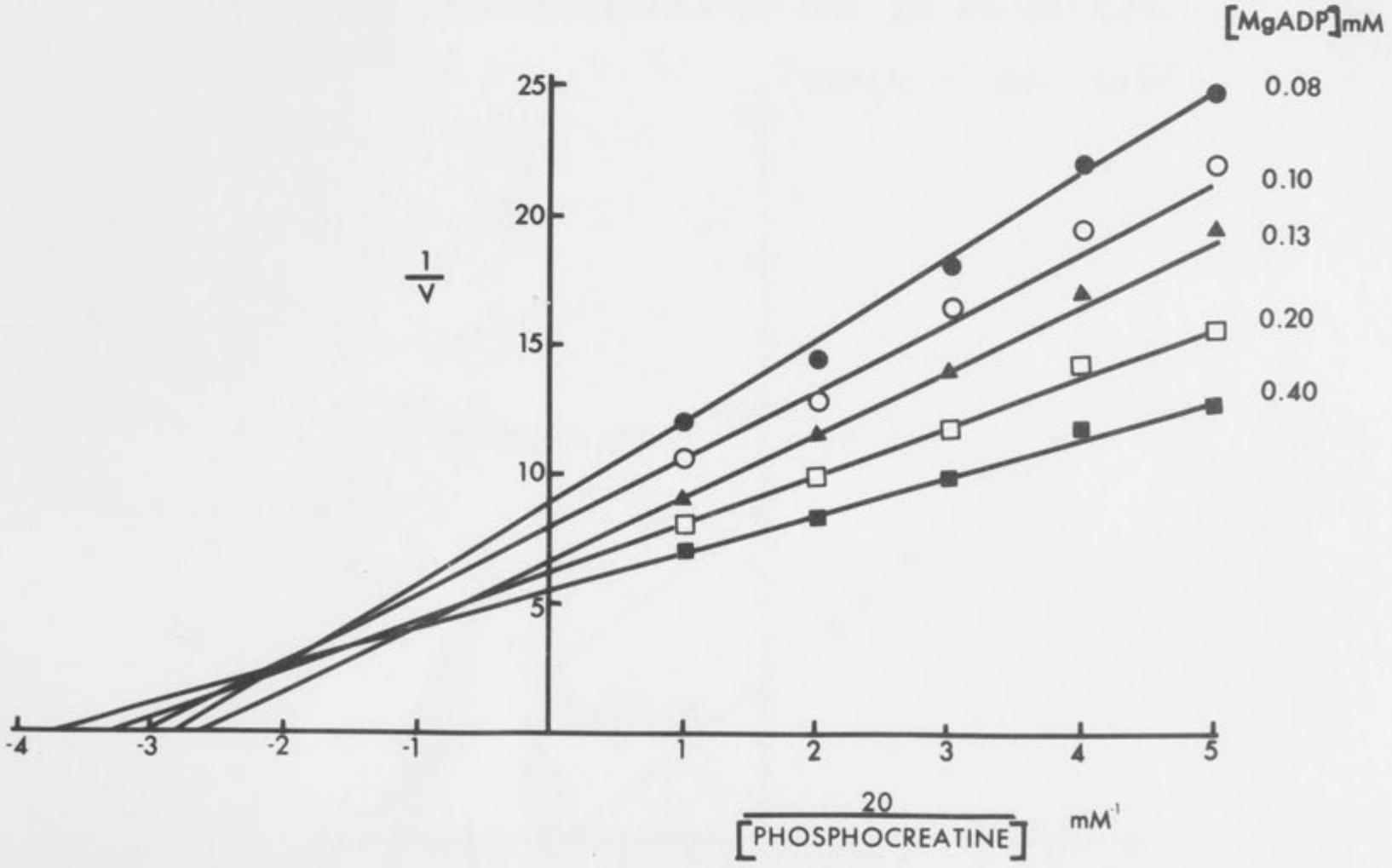
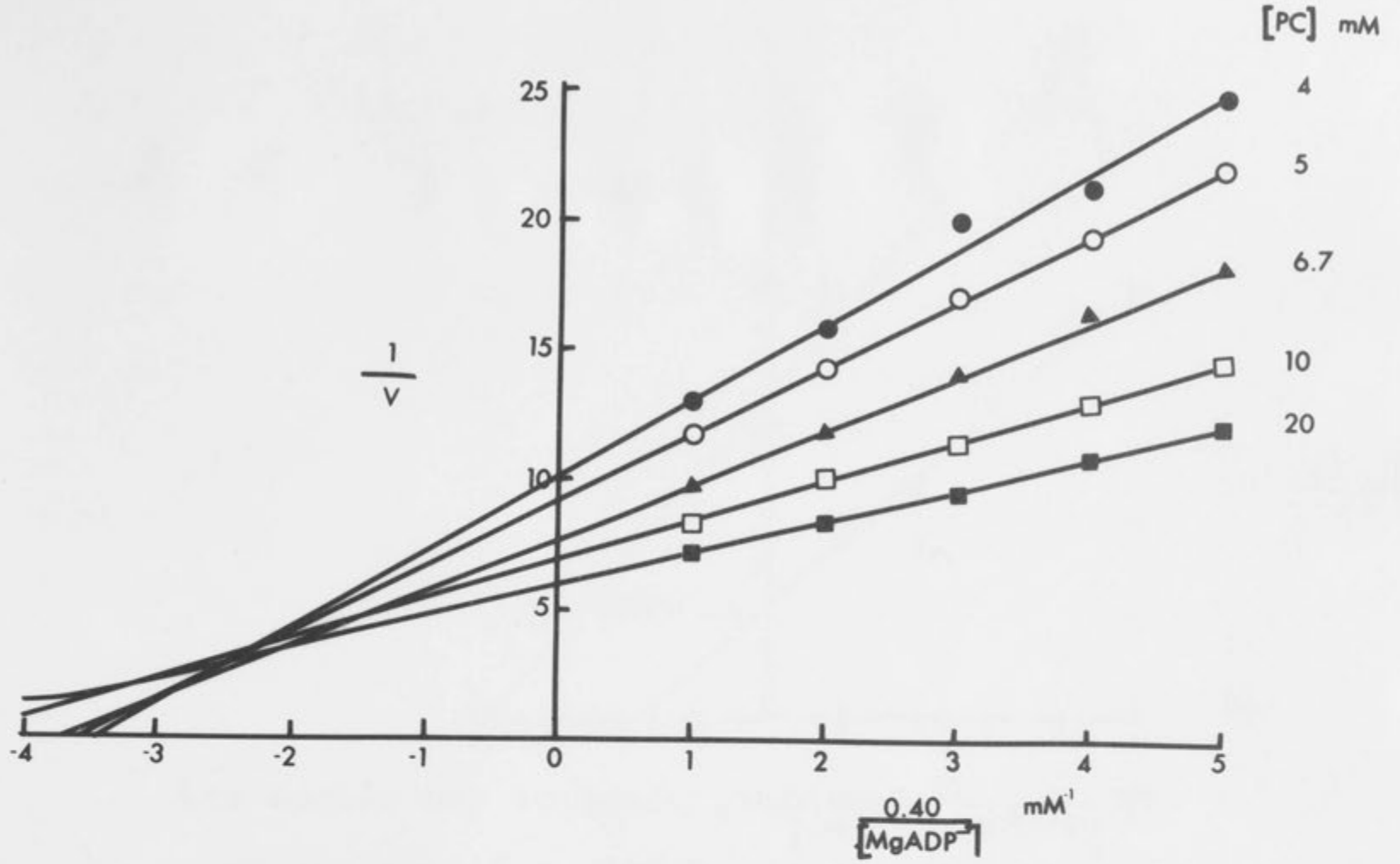

[MgADP]mM
0.08
0.10
0.13
0.20
0.40
25
20
15
10
5
1/V
-4
-3
-2
-1
1
2
3
4
5
20 / [PHOSPHOCREATINE] mM⁻¹

FIG. I.1. Effect of phosphocreatine on the initial velocity of the reaction catalysed by creatine kinase with $MgADP^-$ as the variable substrate (a), and the effect of $MgADP^-$ on the initial velocity of the reaction with phosphocreatine as the variable substrate (b). The concentration of free $Mg^{2+}$ held constant at 10 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.


[PC] mM
4
5
6.7
10
20
25
20
15
10
5
1/V
-4
-3
-2
-1
0
1
2
3
4
5
0.40
[MgADP⁻]
mM⁻¹

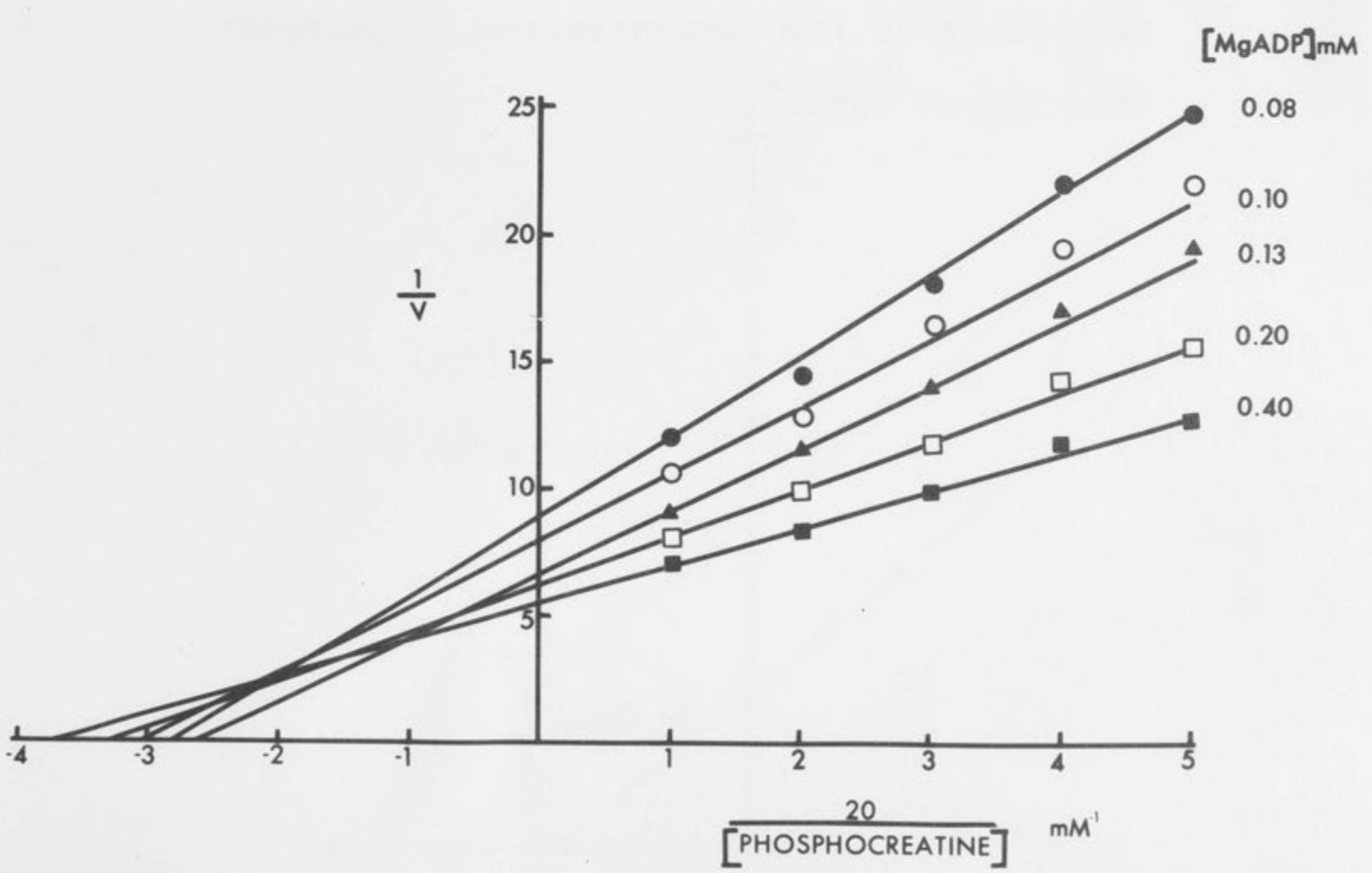
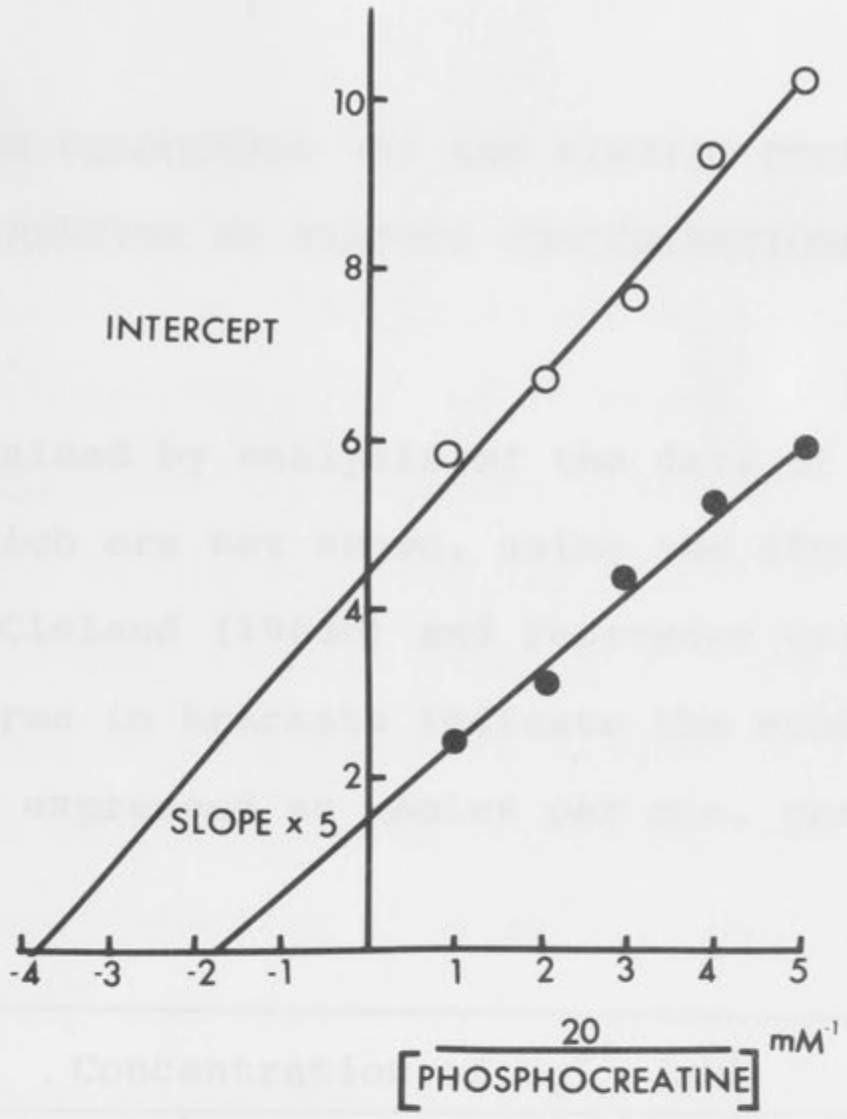

[MgADP]mM
0.08
0.10
0.13
0.20
0.40
25
20
15
10
5
1/V
-4
-3
-2
-1
1
2
3
4
5
20
[PHOSPHOCREATINE]
mM⁻¹

FIG. I.2. Secondary plots of the slopes and vertical intercepts of Fig. I.1. against the reciprocal of the concentration of phosphocreatine or $MgADP^-$.


10
8
INTERCEPT
6
4
2
SLOPE x 5
-4
-3
-2
-1
1
2
3
4
5
20
[PHOSPHOCREATINE]
mM⁻¹

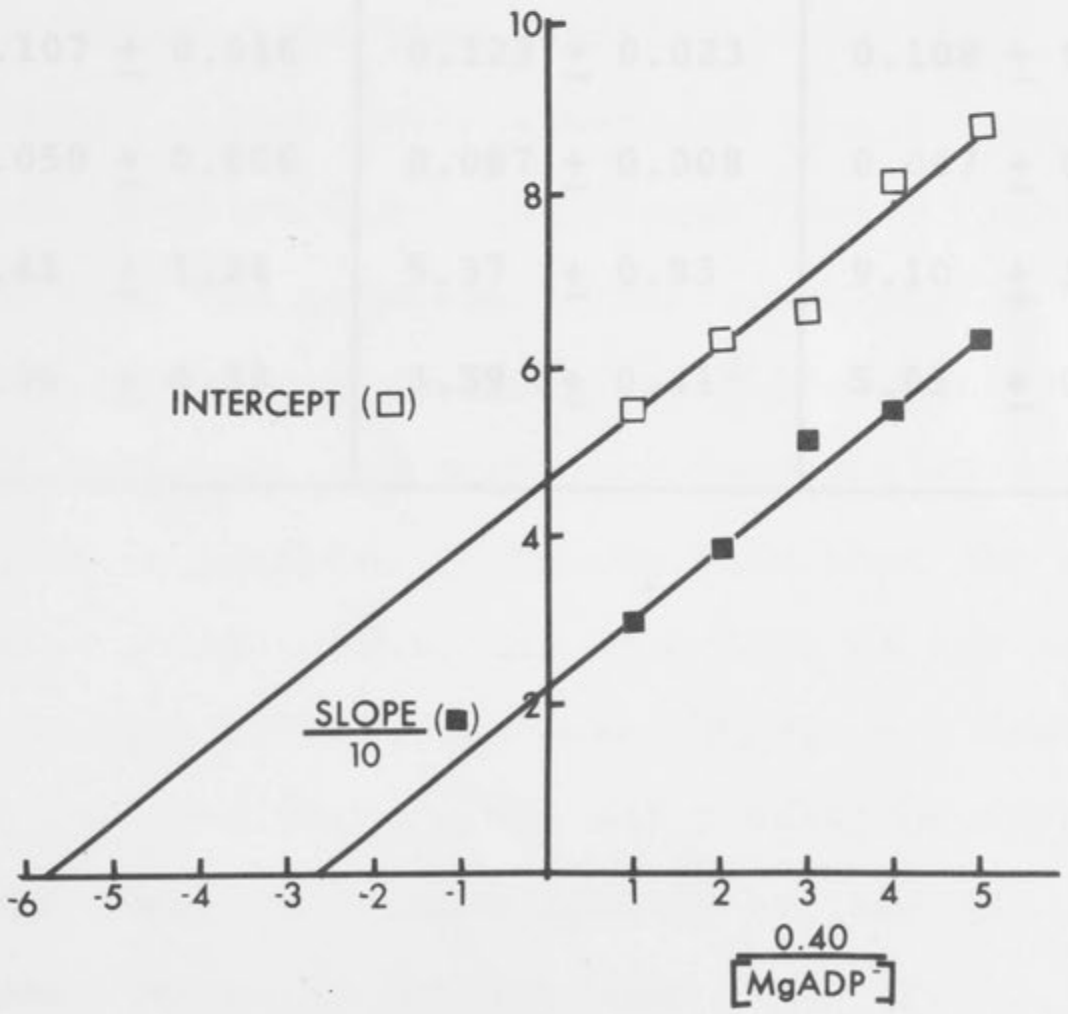

10
8
6
INTERCEPT (□)
4
SLOPE (■)
10
2
-6
-5
-4
-3
-2
-1
1
2
3
4
5
0.40
[MgADP⁻]

TABLE I.1 : MAXIMUM VELOCITIES (V) AND KINETIC CONSTANTS FOR $MgADP^-$ AND PHOSPHOCREATINE AT VARIOUS CONCENTRATIONS OF FREE $Mg^{2+}$.

The values were obtained by analysis of the data of Fig. I.1 and other plots, which are not shown, using the SEQUEN computer programme of Cleland (1963b) and represent weighted mean values. Figures in brackets indicate the number of experiments. V is expressed as μmoles per min. per μg. of creatine kinase.

| Kinetic Constant | Concentration of $Mg^{2+}$ (mM) | | |
|---|---|---|---|
| | 1.0 (3) | 5.0 (2) | 10.0 (2) |
| V | 0.220 ± 0.006 | 0.217 ± 0.008 | 0.207 ± 0.008 |
| $K_{ia}$ (mM) | 0.107 ± 0.016 | 0.123 ± 0.023 | 0.108 ± 0.018 |
| $K_a$ (mM) | 0.059 ± 0.006 | 0.087 ± 0.008 | 0.067 ± 0.008 |
| $K_{ib}$ (mM) | 7.41 ± 1.24 | 5.37 ± 0.93 | 9.10 ± 1.63 |
| $K_b$ (mM) | 4.36 ± 0.38 | 3.59 ± 0.41 | 5.05 ± 0.50 |

the apparent kinetic constants will vary hyperbolically with the concentration of free $Mg^{2+}$, it is found that there is only a small random variation and this is in accord with $Mg^{2+}$ having a weak inhibitory effect on the reaction. Nevertheless, allowance has been made for the inhibitory effect of $Mg^{2+}$ at concentrations of 5.0 and 10.0 mM and the corrected values for the kinetic constants are given in Table I.2, together with the uncorrected values obtained with $Mg^{2+}$ equal to 1.0 mM. The maximum velocity of the reaction varies to only a small extent with the concentration of free $Mg^{2+}$ so that the values at zero and 1.0 mM $Mg^{2+}$ are virtually the same.

Calculation of the inhibition constants for $Mg^{2+}$

The inhibition constants for the reaction of $Mg^{2+}$ with free enzyme, enzyme-MgADP, enzyme-phosphocreatine and enzyme-MgADP-phosphocreatine have been reported by Morrison and O'Sullivan (1965). They were determined by assuming that the creatine kinase reaction has a rapid equilibrium, random mechanism, and that the presence of one substrate on the enzyme does not affect the combination of the other. While it has subsequently been shown that the assumption with regard to the mechanism is tenable, it is not true that the presence of one substrate on the enzyme has no effect on the degree to which the other substrate combines (Morrison & James, 1965). Reanalysis of the data of Morrison and O'Sullivan (1965) using the relationships derived from equations (4) and (5) and the kinetic constants given by Morrison and James (1965), has

TABLE I.2 : SUMMARY OF THE KINETIC CONSTANTS FOR $MgADP^-$ AND PHOSPHOCREATINE

The values obtained for kinetic constants with free $Mg^{2+}$ at 5.0 and 10.0 mM (Table I.1) were corrected using the relationships derived from equations (4) and (5) and values for $K_1$, $K_2$, $K_3$ and $K_4$ of 10.6 mM, 8.8 mM, 25.7 mM and 21.8 mM, respectively. The directly determined values for the constants with free $Mg^{2+}$ at 1.0 mM are included for comparison. The constants are expressed as mM.

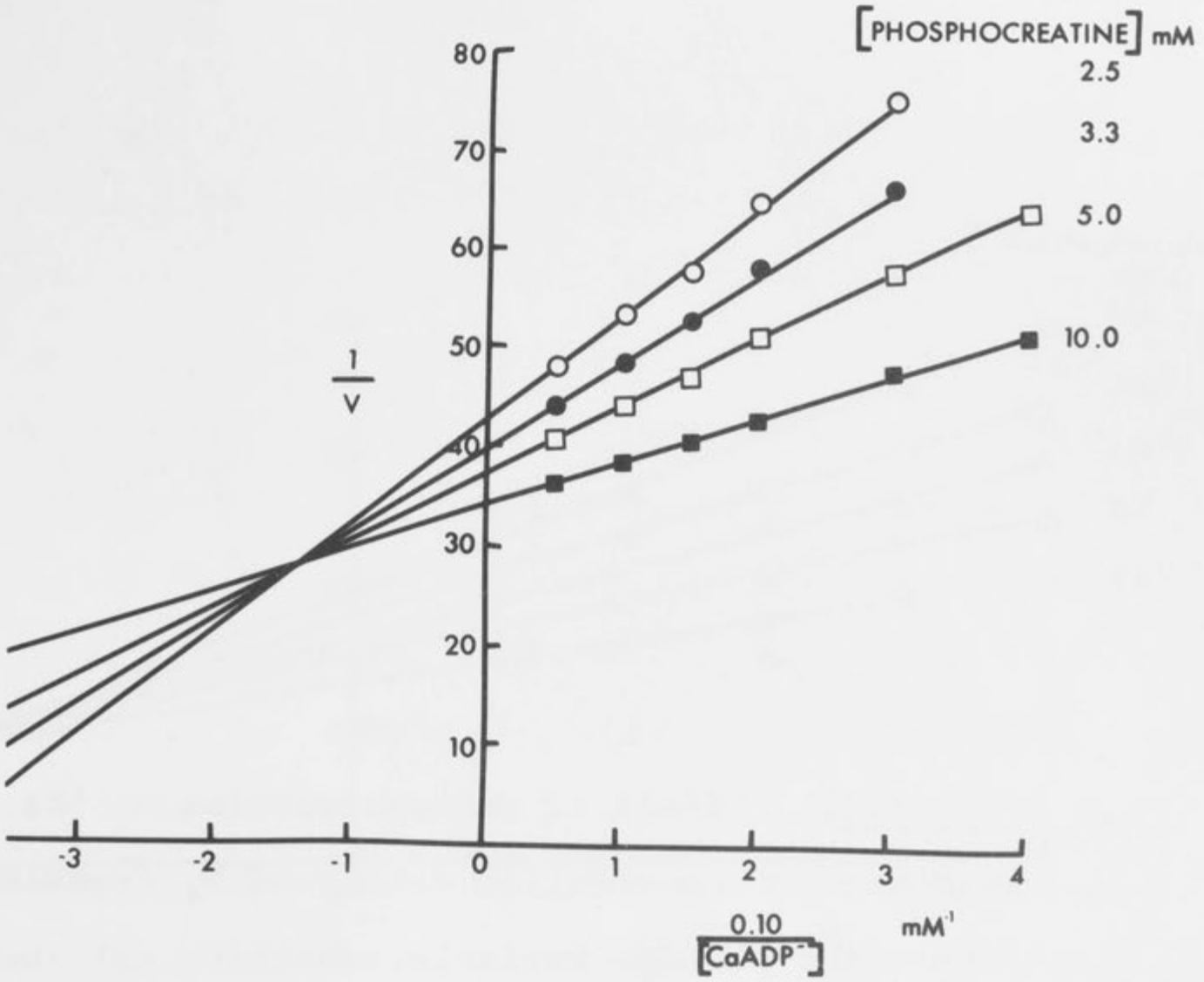
| Kinetic Constant | Concentration of Free $Mg^{2+}$ | | |
|---|---|---|---|
| | 1.0 mM | 5.0 mM | 10.0 mM |
| $K_{ia}$ | 0.11 | 0.13 | 0.12 |
| $K_a$ | 0.06 | 0.09 | 0.07 |
| $K_{ib}$ | 7.4 | 4.4 | 6.5 |
| $K_b$ | 4.4 | 2.8 | 3.4 |

yielded values for $K_1$, $K_2$, $K_3$ and $K_4$ of 10.6 mM, 8.8 mM, 25.7 mM and 21.8 mM, respectively. These may be compared with the previously reported values of $K_1 = K_2 = 10.5$ mM and $K_3 = K_4 = 21$ mM. The former values were used to obtain the results recorded in Table I.2.

Initial velocity studies with $Ca^{2+}$ and $Mn^{2+}$ as the activating ions

Experiments similar to those described above for $Mg^{2+}$ have been carried out using $Ca^{2+}$ and $Mn^{2+}$ as the activating metal ions. With $CaADP^-$ and phosphocreatine as variable substrates (Fig. I.3) and with $MnADP^-$ and phosphocreatine as variable substrates (Fig. I.4) at fixed concentrations of free metal ion, the results were similar to those obtained using $MgADP^-$. Thus primary plots consisted of families of straight lines which intersected at a point which lay to the left of the ordinate and above the abscissa. Within experimental error, the vertical co-ordinates of the intersection points of the two graphs of Fig. I.3 are equal, as are those of Fig. I.4. It may, therefore, be concluded that both reactions are sequential, which result is in accord with the assumption (equations 4 and 5) that a change in the metal ion does not alter the reaction mechanism. The results obtained with free $Ca^{2+}$ at a concentration of 2.0 mM and free $Mn^{2+}$ at a concentration of 5.0 mM were qualitatively similar to those of Fig. I.3 and Fig. I.4, respectively. The secondary plots of the slopes and vertical intercepts of the primary plots

FIG. I.3. Effect of phosphocreatine on the initial velocity of the reaction catalysed by creatine kinase with $CaADP^-$ as the variable substrate (a) and the effect of $CaADP^-$ on the initial velocity of the reaction with phosphocreatine as the variable substrate (b). The concentration of free $Ca^{2+}$ held constant at 10 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.


[PHOSPHOCREATINE] mM
2.5
3.3
5.0
10.0
80
70
60
50
40
30
20
10
1/V
-3
-2
-1
0
1
2
3
4
0.10 / [CaADP⁻] mM⁻¹

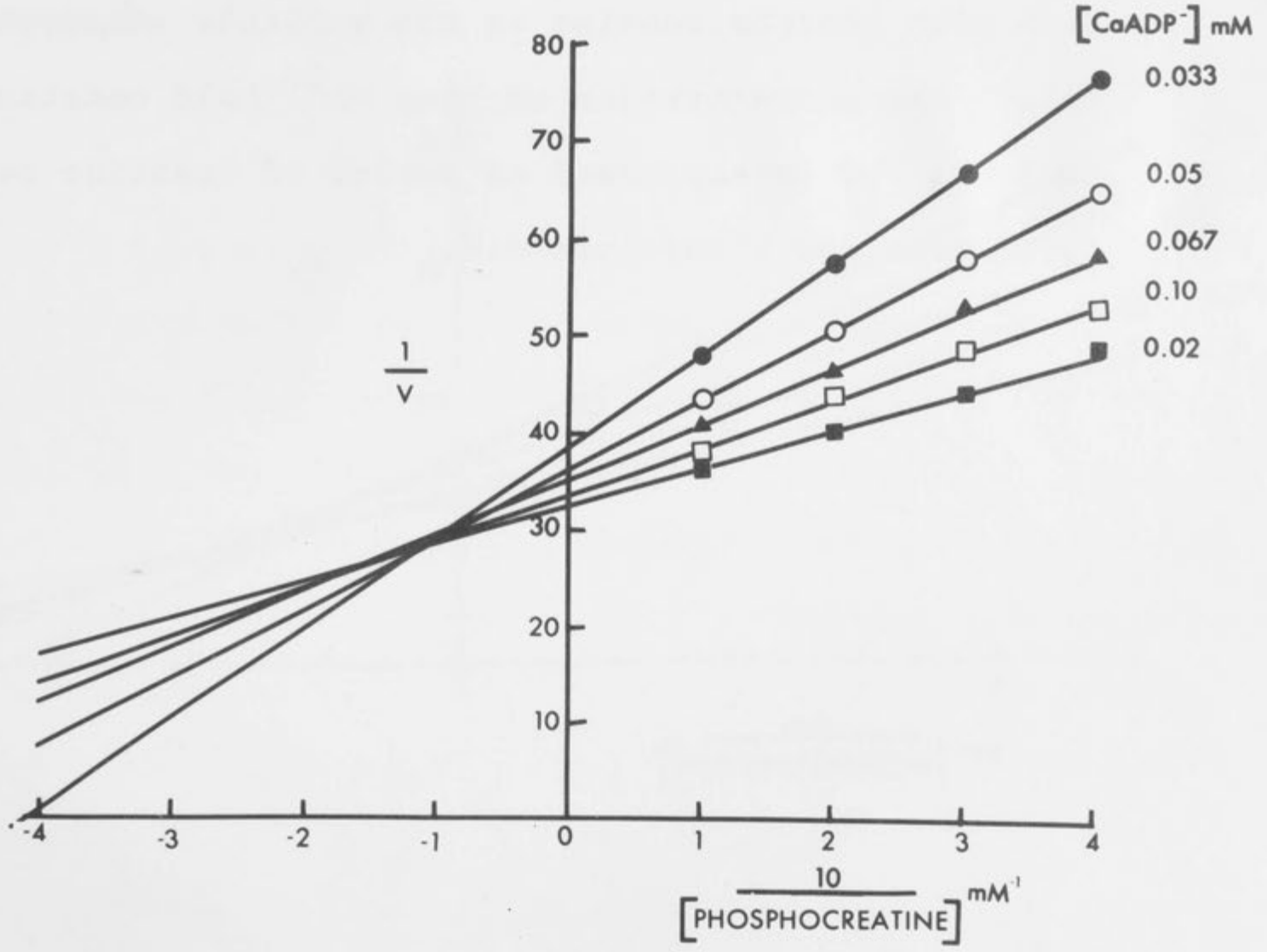
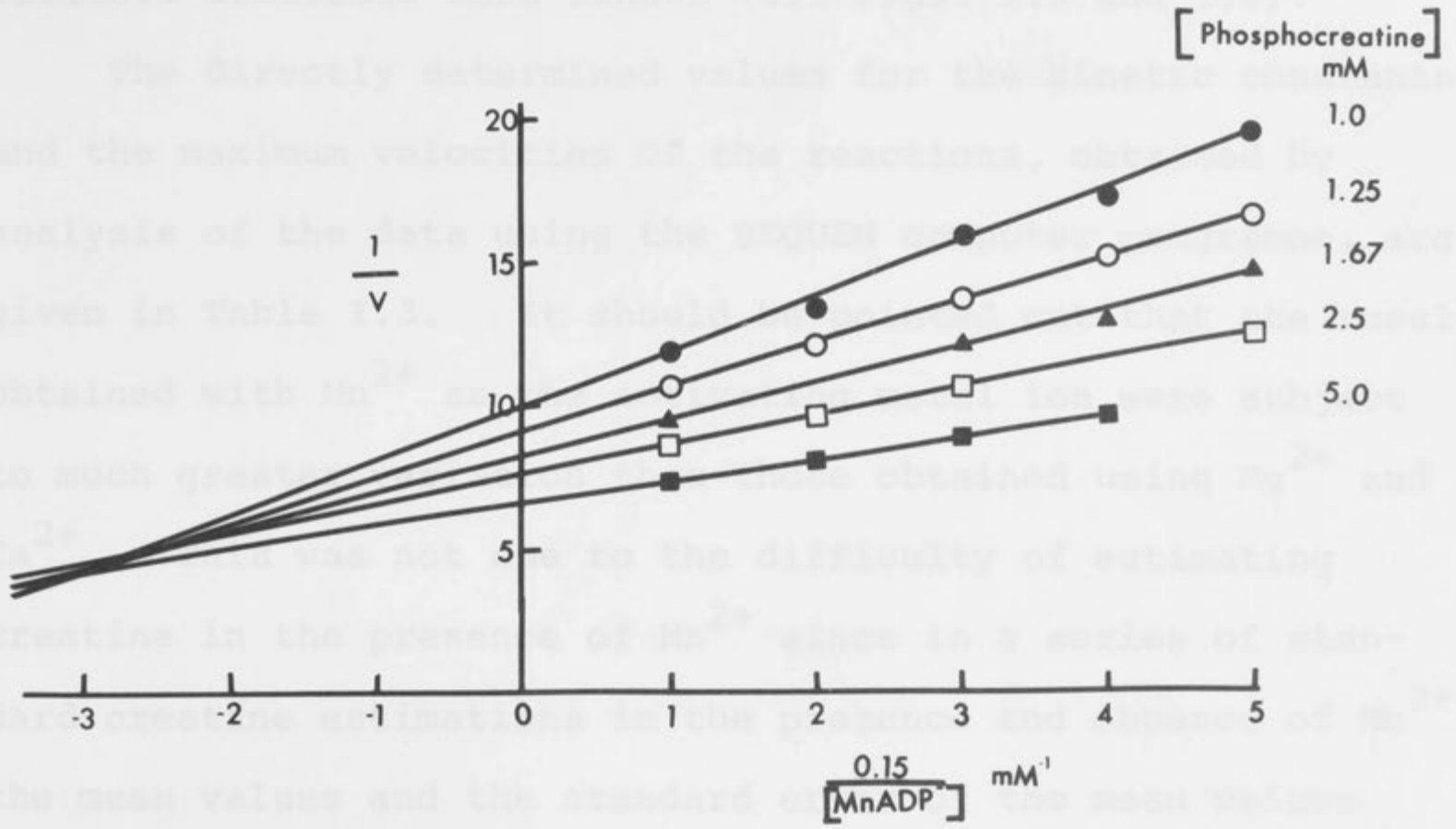
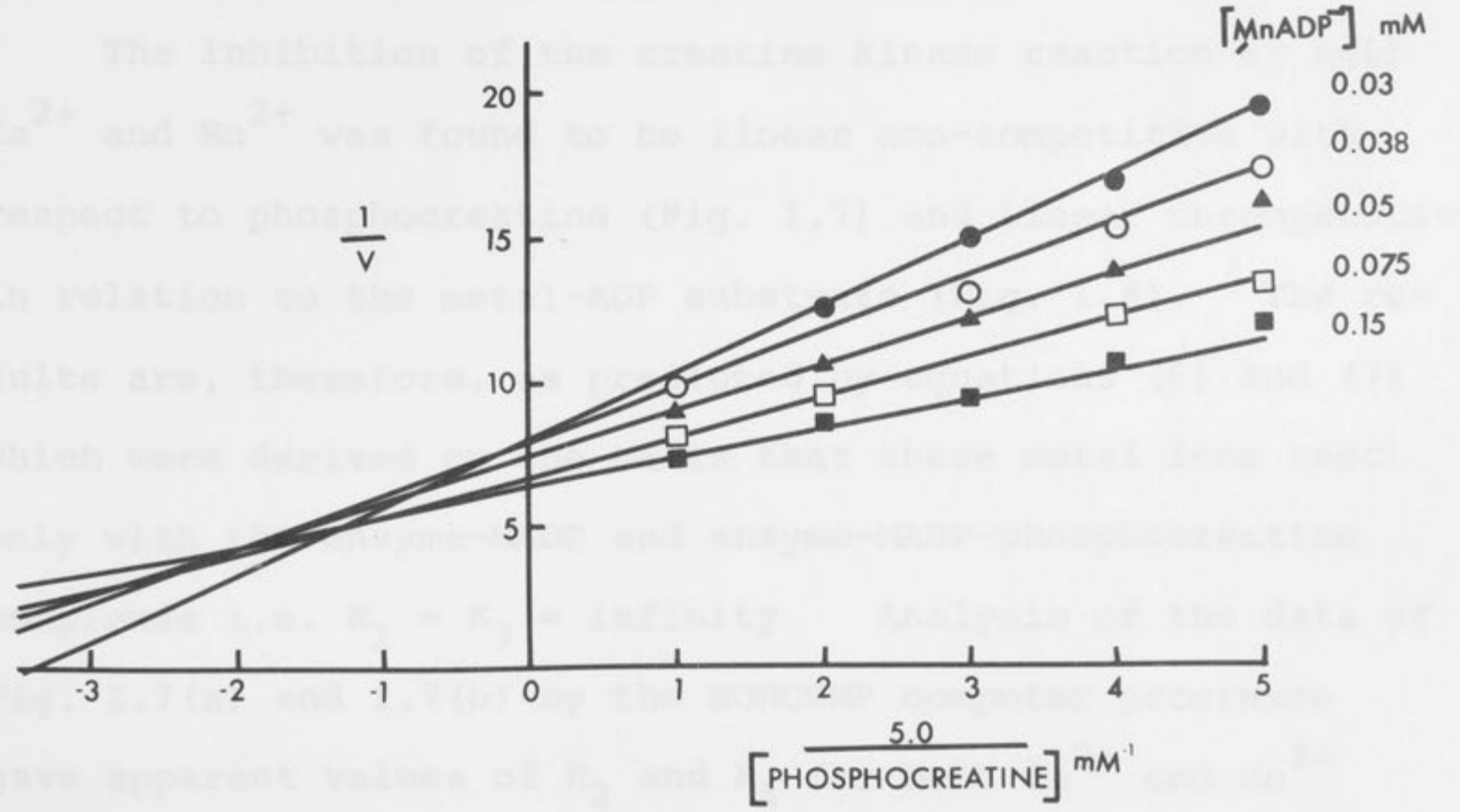

[CaADP⁻] mM
0.033
0.05
0.067
0.10
0.02
80
70
60
50
40
30
20
10
1/V
-4
-3
-2
-1
0
1
2
3
4
10 / [PHOSPHOCREATINE] mM⁻¹

FIG. I.4. Effect of phosphocreatine on the initial velocity of the reaction catalysed by creatine kinase with $MnADP^-$ as the variable substrate (a) and the effect of $MnADP^-$ on the initial velocity of the reaction with phosphocreatine as the variable substrate (b). The concentration of free $Mn^{2+}$ held constant at 1 mM. v is expressed as μmoles of creatine per μg of creatine kinase per min.


[Phosphocreatine]
mM
1.0
1.25
1.67
2.5
5.0
1/V
20
15
10
5
-3
-2
-1
0
1
2
3
4
5
0.15 / [MnADP⁻] mM⁻¹


[MnADP⁻] mM
0.03
0.038
0.05
0.075
0.15
1/V
20
15
10
5
-3
-2
-1
0
1
2
3
4
5
5.0 / [PHOSPHOCREATINE] mM⁻¹

against the reciprocal of the concentration of the fixed variable substrate were linear (cf. Figs. I.5 and I.6).

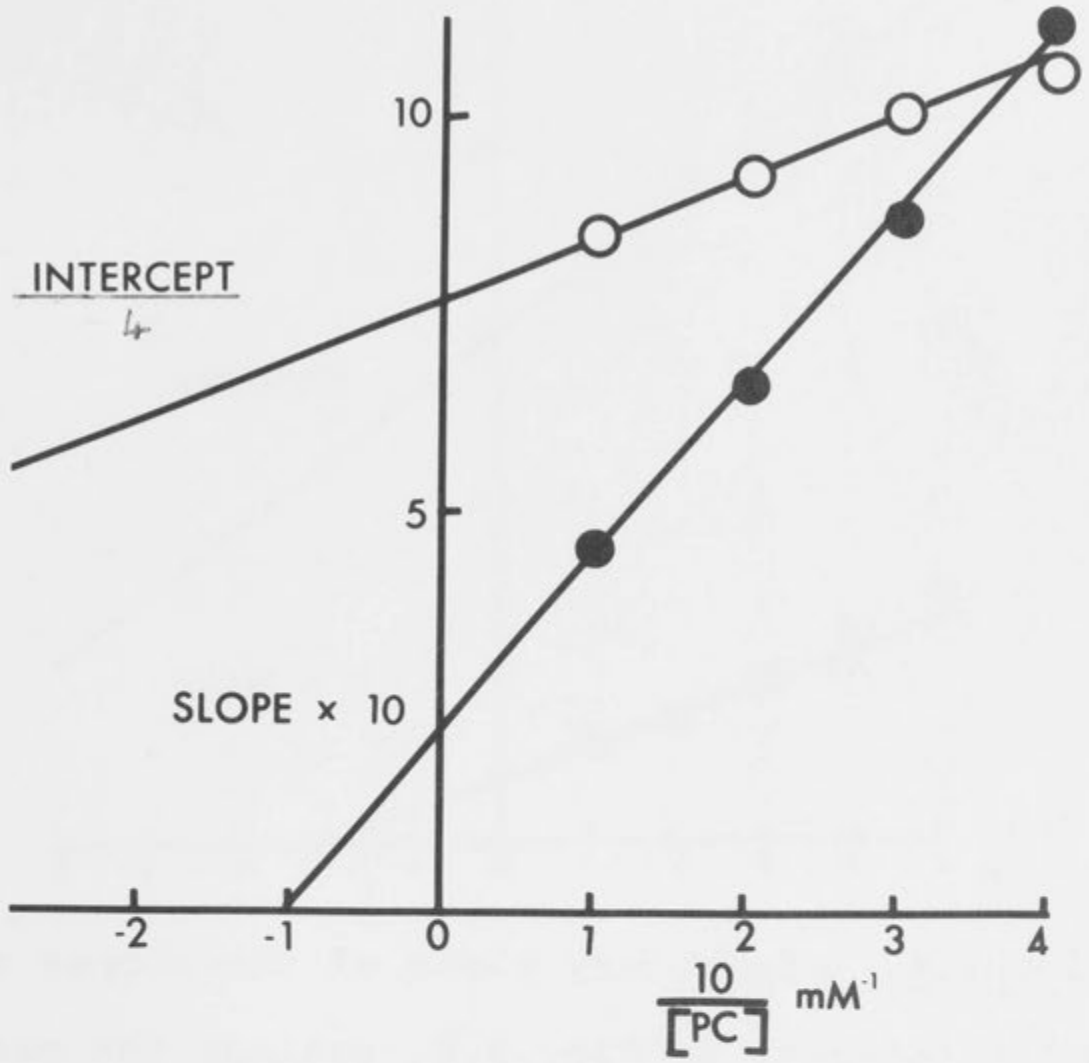
The directly determined values for the kinetic constants and the maximum velocities of the reactions, obtained by analysis of the data using the SEQUEN computer programme, are given in Table I.3. It should be pointed out that the results obtained with $Mn^{2+}$ as the activating metal ion were subject to much greater variation than those obtained using $Mg^{2+}$ and $Ca^{2+}$. This was not due to the difficulty of estimating creatine in the presence of $Mn^{2+}$ since in a series of standard creatine estimations in the presence and absence of $Mn^{2+}$, the mean values and the standard error of the mean values were virtually identical.

Inhibition of the reaction by $Ca^{2+}$ and $Mn^{2+}$

The inhibition of the creatine kinase reaction by both $Ca^{2+}$ and $Mn^{2+}$ was found to be linear non-competitive with respect to phosphocreatine (Fig. I.7) and linear uncompetitive in relation to the metal-ADP substrate (Fig. I.8). The results are, therefore, as predicted by equations (6) and (7) which were derived on the basis that these metal ions react only with the enzyme-MADP and enzyme-MADP-phosphocreatine complexes i.e. $K_1 = K_3 =$ infinity. Analysis of the data of Fig. I.7(a) and I.7(b) by the NONCOMP computer programme gave apparent values of $K_2$ and $K_4$ for both $Ca^{2+}$ and $Mn^{2+}$ (Table I.4).

Because tests with dithizone showed that solutions of

FIG. I.5. Secondary plots of the slopes and vertical intercepts of Fig. I.3 against the reciprocal of the concentration of phosphocreatine or $CaADP^-$.


10
INTERCEPT
4
5
SLOPE × 10
-2
-1
0
1
2
3
4
10
[PC]
mM⁻¹

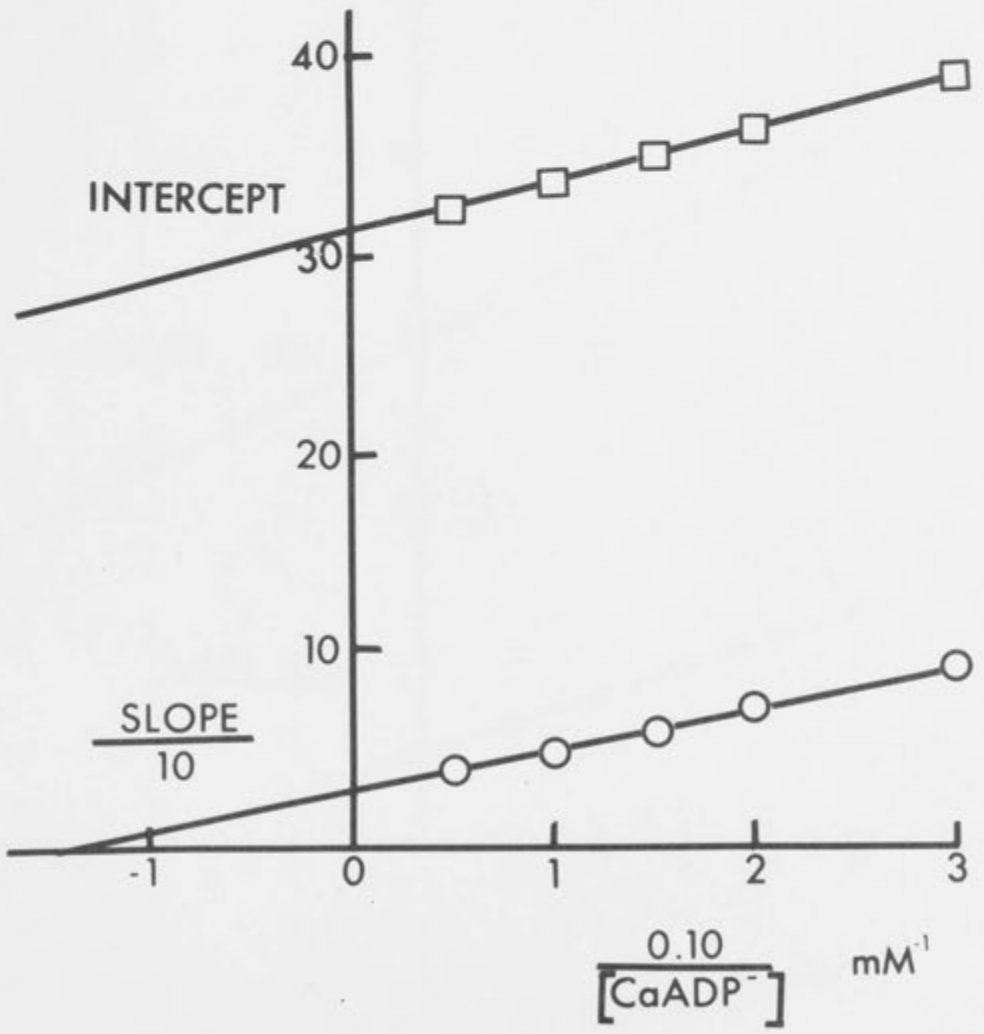
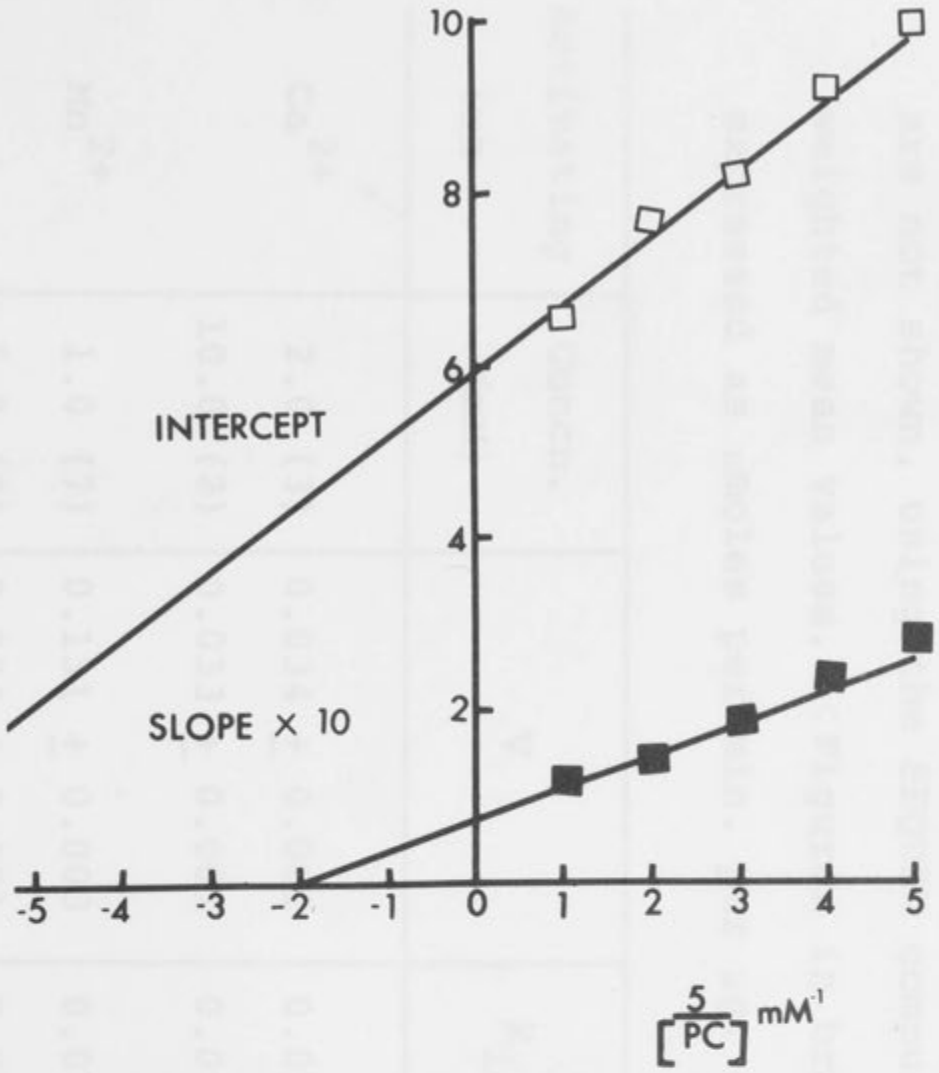

40
INTERCEPT
30
20
10
SLOPE
10
-1
0
1
2
3
0.10
[CaADP⁻]
mM⁻¹

FIG. I.6. Secondary plots of the slopes and vertical intercepts of Fig. I.4. against the reciprocal of the concentration of phosphocreatine or $MnADP^-$.


10
8
6
4
2
INTERCEPT
SLOPE × 10
-5
-4
-3
-2
-1
0
1
2
3
4
5
$\left[\frac{5}{PC}\right]$ mM$^{-1}$

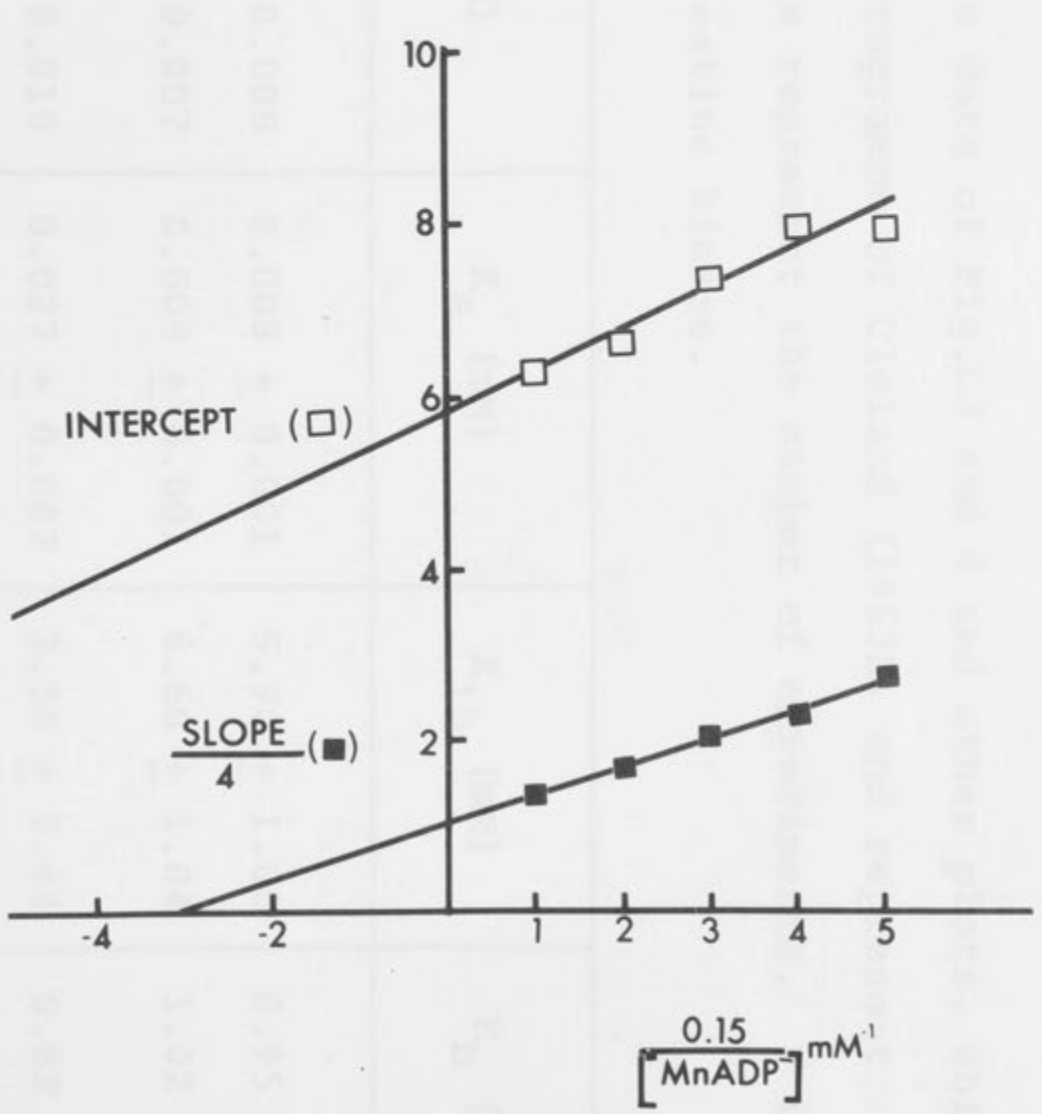
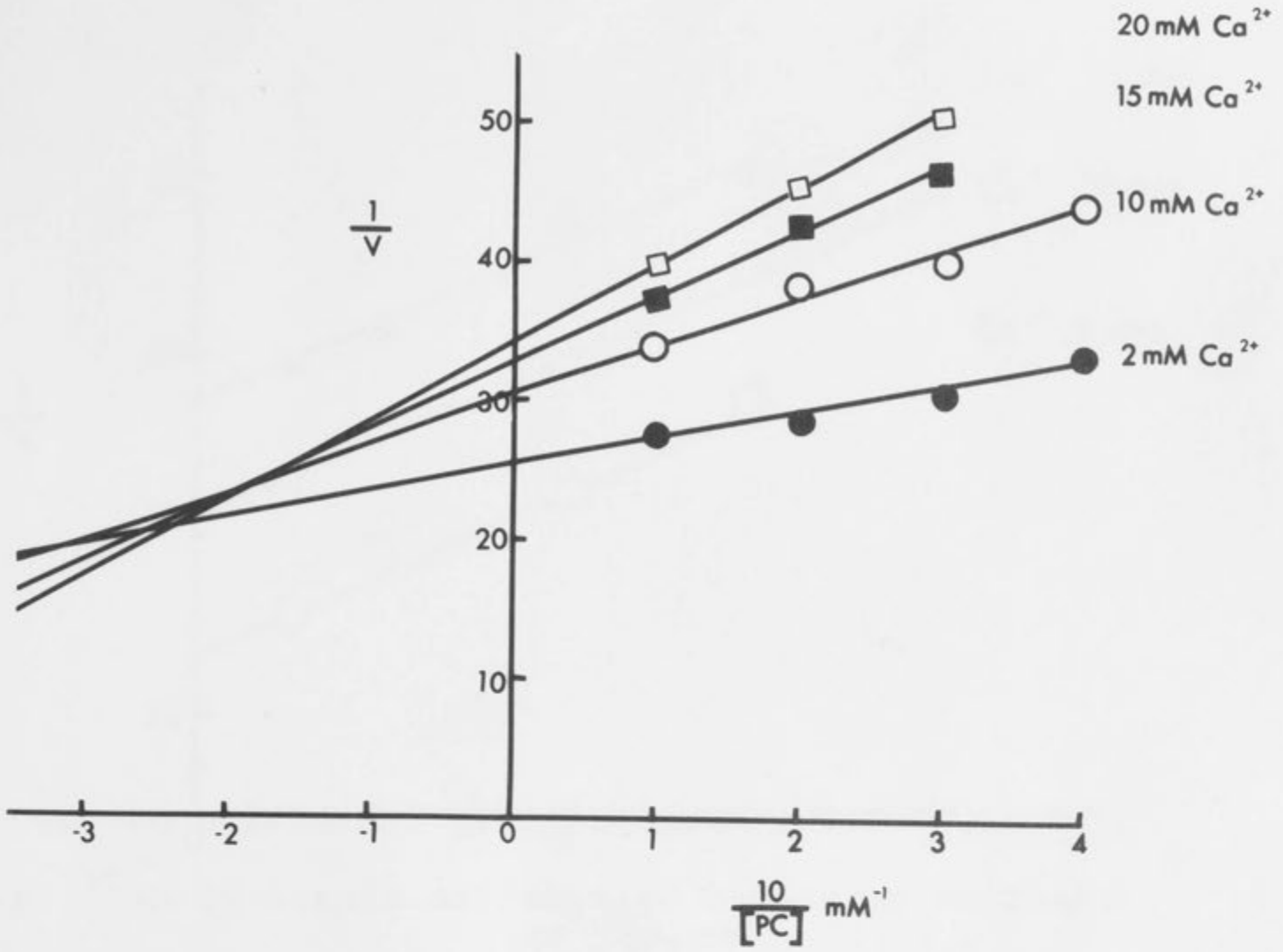

10
8
6
4
2
INTERCEPT (□)
SLOPE / 4 (■)
-4
-2
1
2
3
4
5
$\left[\frac{0.15}{MnADP^-}\right]$ mM$^{-1}$

TABLE I.3 : MAXIMUM VELOCITIES (V) AND KINETIC CONSTANTS FOR PHOSPHOCREATINE AND $CaADP^-$ OR $MnADP^-$ AT FIXED CONCENTRATIONS OF FREE $Ca^{2+}$ OR FREE $Mn^{2+}$.

The values were obtained by analysis of the data of Fig.I.3 and 4 and other plots, which are not shown, using the SEQUEN computer programme of Cleland (1963b) and represent weighted mean values. Figures in brackets represent the number of experiments. V is expressed as μmoles per min. per μg. of creatine kinase.

| Activating ion | Concn. (mM) | V | $K_{ia}$ (mM) | $K_a$ (mM) | $K_{ib}$ (mM) | $K_b$ (mM) |
|---|---|---|---|---|---|---|
| $Ca^{2+}$ | 2.0 (3) | 0.034 ± 0.001 | 0.055 ± 0.009 | 0.008 ± 0.001 | 5.90 ± 1.06 | 0.95 ± 0.15 |
| | 10.0 (2) | 0.033 ± 0.001 | 0.055 ± 0.007 | 0.009 ± 0.001 | 6.60 ± 1.04 | 1.02 ± 0.08 |
| $Mn^{2+}$ | 1.0 (7) | 0.131 ± 0.003 | 0.074 ± 0.010 | 0.027 ± 0.002 | 2.56 ± 0.44 | 0.82 ± 0.05 |
| | 5.0 (2) | 0.094 ± 0.001 | 0.067 ± 0.016 | 0.016 ± 0.002 | 2.52 ± 0.77 | 0.65 ± 0.08 |

FIG. I.7. The non-competitive inhibition of the reaction catalysed by creatine kinase by $Ca^{2+}$ (a) and $Mn^{2+}$ (b) with respect to phosphocreatine as the variable substrate, and with the concentrations of $CaADP^-$ (a) and $MnADP^-$ (b) held constant at 0.1 mM and 0.3 mM, respectively. Purified metal salts were used. v is expressed as μmoles of creatine per μg of creatine kinase per min.


20 mM Ca 2+
15 mM Ca 2+
10 mM Ca 2+
2 mM Ca 2+
1/V
50
40
30
20
10
-3
-2
-1
0
1
2
3
4
10/[PC] mM-1

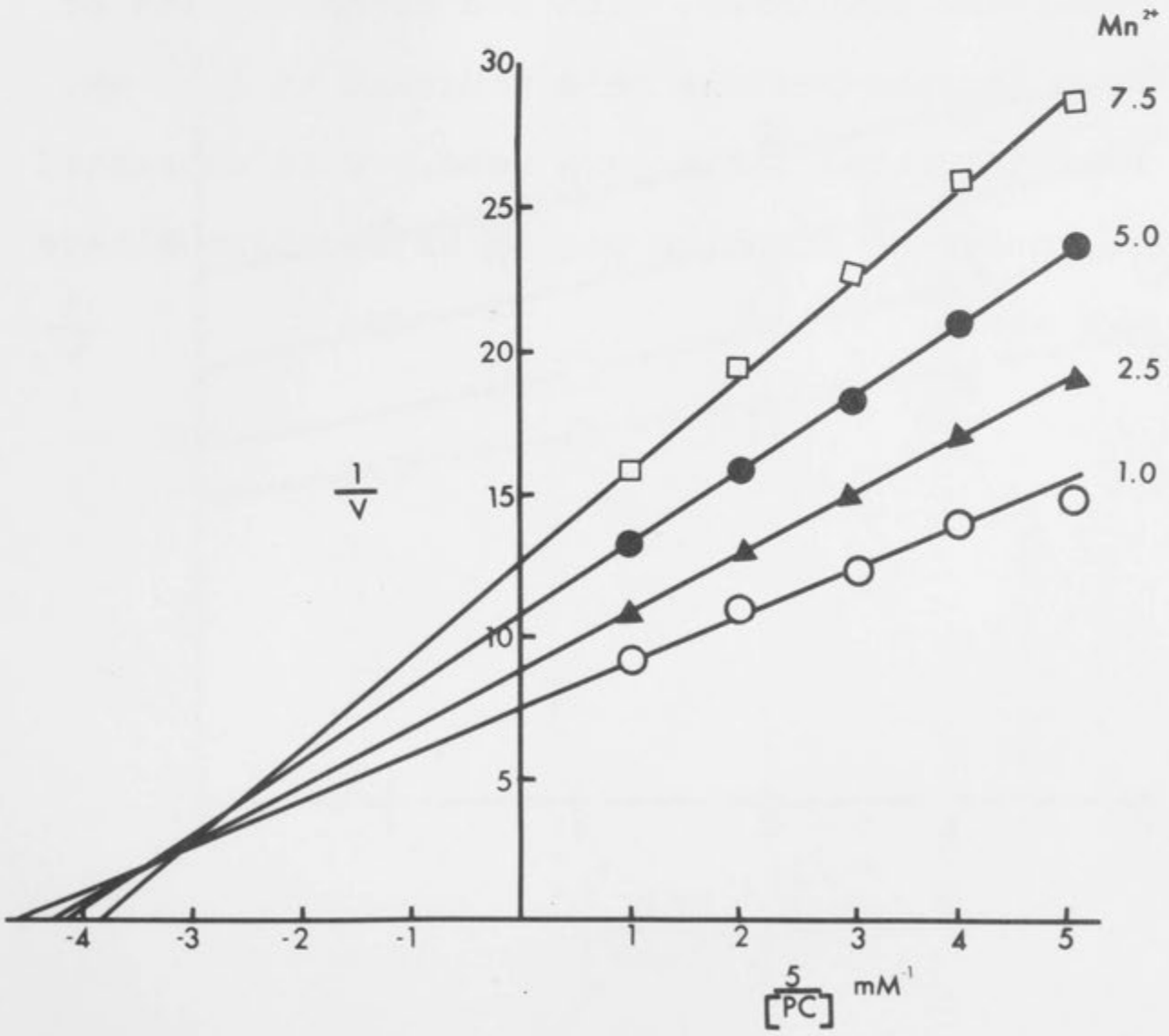
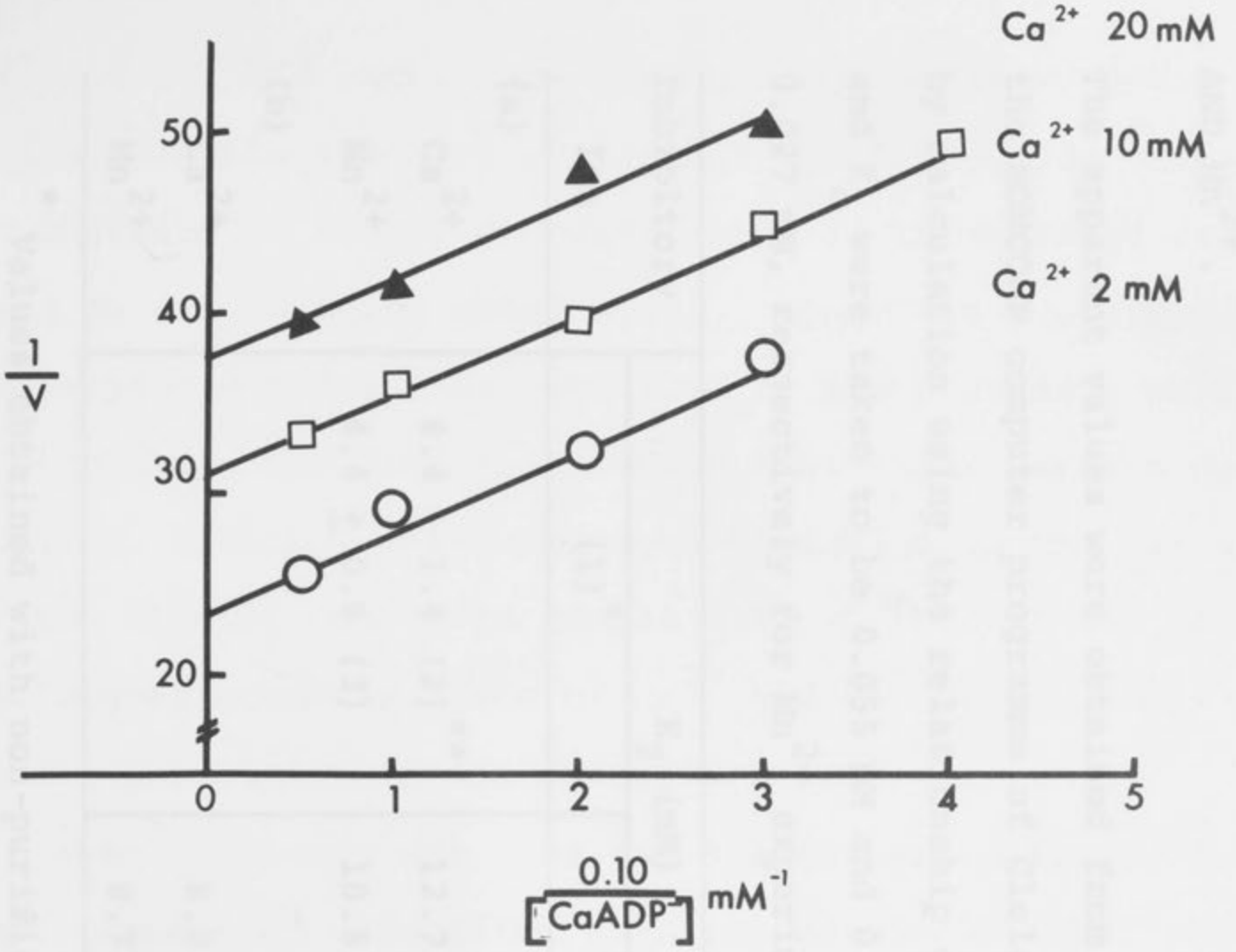

Mn 2+
7.5
5.0
2.5
1.0
1/V
30
25
20
15
10
5
-4
-3
-2
-1
1
2
3
4
5
5/[PC] mM-1

FIG. I.8. The uncompetitive inhibition of the reaction catalysed by creatine kinase by $Ca^{2+}$ (a) and $Mn^{2+}$ (b) with respect to their corresponding metal-ADP complexes, with the concentration of free phosphocreatine held constant at 10.0 mM. Purified metal salts were used. v is expressed as μmoles of creatine per μg of creatine kinase per min.


Ca 2+ 20 mM
Ca 2+ 10 mM
Ca 2+ 2 mM
1/V
50
40
30
20
0
1
2
3
4
5
0.10 / [CaADP-] mM-1

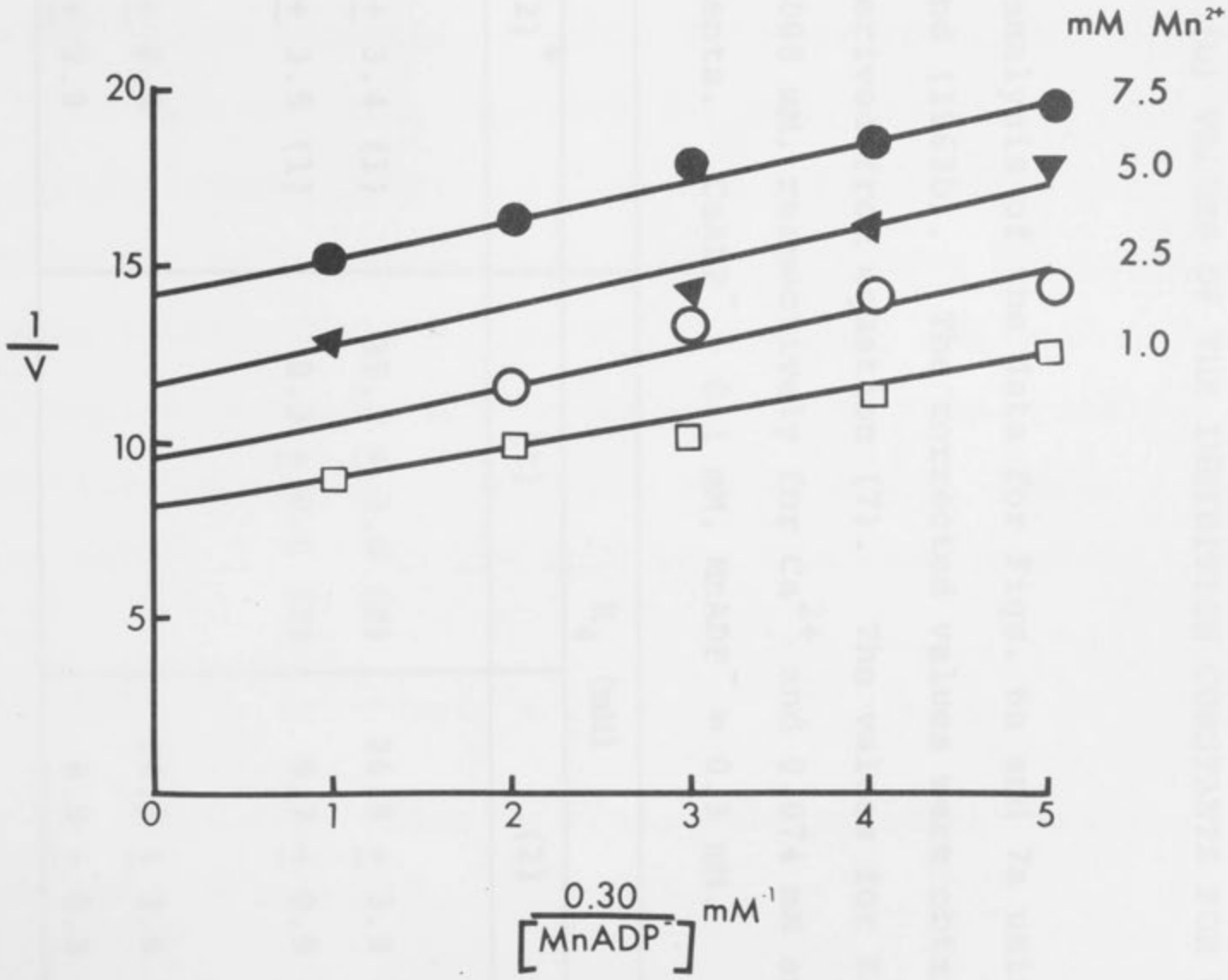

mM Mn2+
7.5
5.0
2.5
1.0
1/V
20
15
10
5
0
1
2
3
4
5
0.30 / [MnADP-] mM-1

TABLE I.4 : APPARENT (a) AND CORRECTED (b) VALUES OF THE INHIBITION CONSTANTS FOR $Ca^{2+}$ AND $Mn^{2+}$.

The apparent values were obtained from analysis of the data for Figs. 6a and 7a using the NONCOMP computer programme of Cleland (1963b). The corrected values were obtained by calculation using the relationship derived from equation (7). The values for $K_{ia}$ and $K_a$ were taken to be 0.055 mM and 0.008 mM, respectively for $Ca^{2+}$ and 0.074 mM and 0.027 mM, respectively for $Mn^{2+}$ experiments. $CaADP^-$ = 0.1 mM, $MnADP^-$ = 0.3 mM.

| Inhibitory Ion | $K_2$ (mM) | | $K_4$ (mM) | |
|---|---|---|---|---|
| | (1)* | (2)* | (1) | (2) |
| (a) | | | | |
| $Ca^{2+}$ | 8.4 ± 1.4 (2)** | 12.7 ± 3.4 (1) | 37.3 ± 3.0 (2) | 26.8 ± 3.9 (1) |
| $Mn^{2+}$ | 4.4 ± 0.6 (3) | 10.8 ± 3.5 (1) | 8.3 ± 0.6 (3) | 9.7 ± 0.9 (1) |
| (b) | | | | |
| $Ca^{2+}$ | | 8.2 ± 2.3 | | 24.8 ± 3.6 |
| $Mn^{2+}$ | | 8.7 ± 2.9 | | 8.9 ± 0.8 |

* Values obtained with non-purified (1) and purified (2) metal salts.

** Figures in brackets represent the number of experiments used for the calculation of weighted mean values.

Analar $CaCl_2$ and $MnCl_2$ were contaminated by heavy metal ions, it was possible that all or part of the inhibition caused by the addition of higher concentrations of these metal salts could be due to heavy metal ions. Hence the experiments were repeated using salts purified by the procedure outlined in Methods. The same qualitative inhibition pattern was obtained, although there was some variation in the values for the kinetic constants (Table I.4). Thus it was concluded that higher concentrations of $Ca^{2+}$ and $Mn^{2+}$ are capable of causing inhibition of the creatine kinase reaction.

When allowance was made for the concentration of and the kinetic constant associated with the metal-ADP substrate, the corrected values reported in Table I.4 were obtained.

It is apparent from equation (6) that replots of the vertical intercepts of Fig. I.8(a) and I.8(b) against the concentration of the metal ions will give values for complex constants involving $K_2$, $K_4$ and $K_b$. However, the values for these complex constants may be compared with those obtained by substitution of the true values for $K_2$ and $K_4$ (Table I.4) and the values for $K_b$ (Table I.3) into the relationship:

$$\text{Apparent } K_i \text{ intercept} = 1 + \frac{K_b}{B} \Big/ \left(\frac{K_b}{K_2 B} + \frac{1}{K_4}\right)$$

Table I.5 shows that there is reasonable agreement between the two sets of values.

TABLE I.5 : COMPARISON OF THE DETERMINED AND CALCULATED VALUES FOR APPARENT $K_i$ INTERCEPT OBTAINED FROM THE UNCOMPETITIVE INHIBITION BY $Ca^{2+}$ AND $Mn^{2+}$.

The calculated values were obtained from the relationship

$$\text{Apparent } K_i \text{ intercept} = \frac{1 + \frac{K_b}{B}}{\frac{1}{K_4} + \frac{K_b}{K_2 B}}$$

using values for $K_2$, $K_4$ and $K_b$ of 8.2 mM, 24.8 mM (Table I.4) and 0.95 mM (Table I.3) for the $Ca^{2+}$ experiments and 8.7 mM, 8.9 mM (Table I.4) and 0.82 mM (Table I.3) respectively for the $Mn^{2+}$ experiments. Concentration of phosphocreatine (B) was 10.0 mM.

| Inhibitory Ion | Variable Substrate | Apparent $K_i$ intercept (mM) | |
|---|---|---|---|
| | | Observed | Calculated |
| $Ca^{2+}$ | $CaADP^-$ | 22.0 ± 1.4 | 21.1 ± 2.8 |
| $Mn^{2+}$ | $MnADP^-$ | 7.6 ± 0.3 | 8.9 ± 0.8 |

Correction of the kinetic constants for the substrates given in Table I.3

The values for the kinetic constants reported in Table I.3 with $Ca^{2+}$ and $Mn^{2+}$ at concentrations of 10.0 and 5.0 mM, respectively have been corrected by using the relationships derived from equations (4) and (5) and setting $K_1 = K_3 =$ infinity. The values so obtained are given in Table I.6 as also are the directly determined values with $Ca^{2+}$ and $Mn^{2+}$ at concentrations of 2.0 and 1.0 mM, respectively. The corrected values for the maximum velocities are also included.

Maximum velocities with different metal-nucleotide substrates

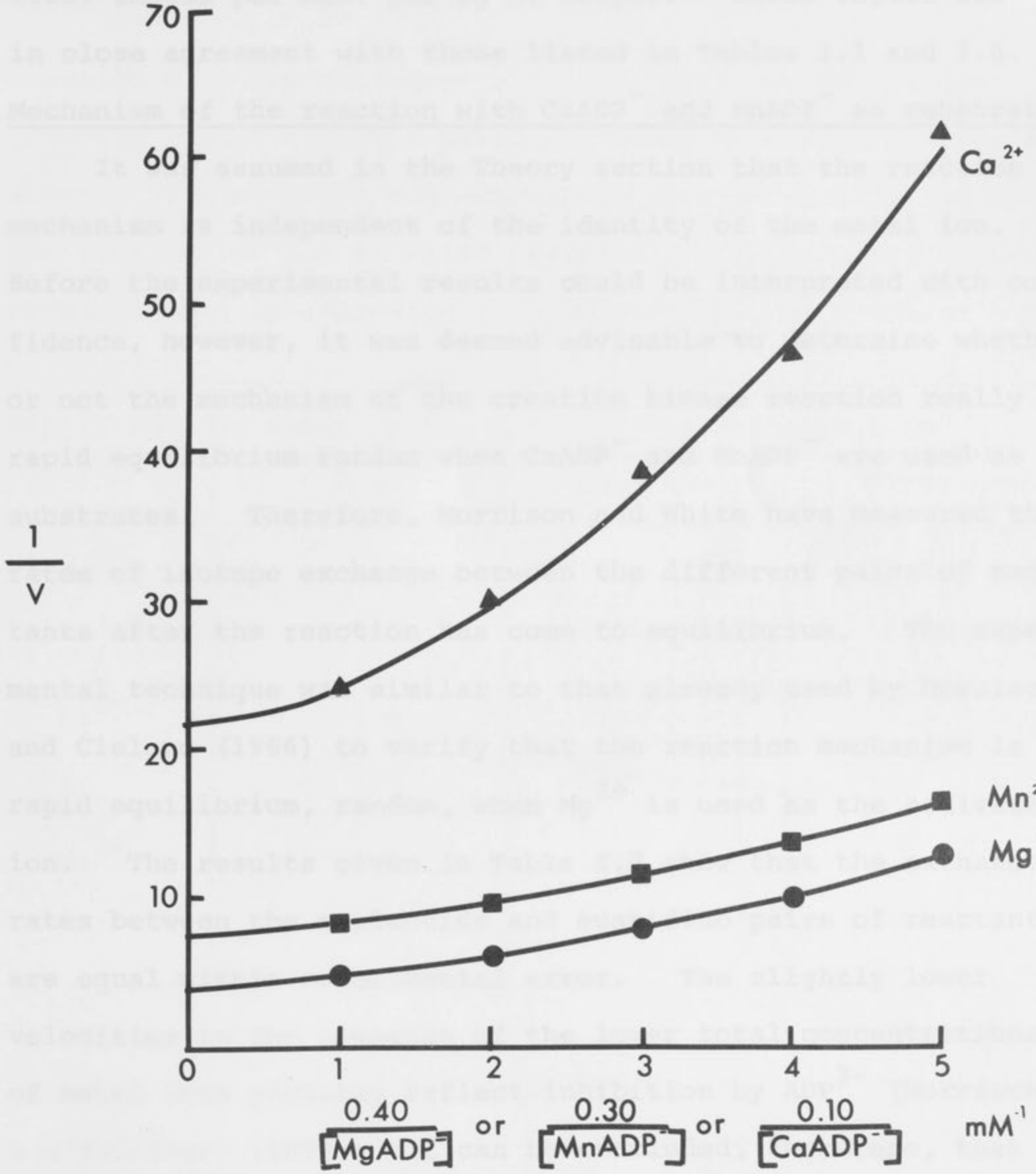
Since the maximum velocity is dependent on enzyme concentration, it is desirable when comparisons are to be made of the values obtained with each metal-ADP complex to carry out the determinations with the same enzyme solution and within a reasonably short period of time. The procedure outlined in Methods, whereby the ratio of the reactants is maintained constant while the absolute concentration of each is varied, permits of this as only five concentrations of each of the variable substrates are required to determine the maximum velocity. The results of such experiments (Fig. I.9), which were obtained under conditions of low free metal ion concentration, show that the maximum velocity with $MgADP^-$ is greater than that with $MnADP^-$ which is in turn greater than that with $CaADP^-$. By computer analysis of the data of Fig. I.9 using the PARA programme, it was found that the respective maximum

TABLE I.6 : SUMMARY OF THE KINETIC CONSTANTS USING $Ca^{2+}$ AND $Mn^{2+}$ AS THE ACTIVATING IONS.

The values for the kinetic constants with free $Ca^{2+}$ and free $Mn^{2+}$ at concentrations of 10.0 and 5.0 mM, respectively (Table I.3), were corrected using the relationships derived from equations (4) and (5). $K_1$ and $K_3$ were set equal to infinity; $K_2$ and $K_4$ were taken to be 8.2 $\pm$ 2.3 mM and 24.8 $\pm$ 3.6 mM, respectively for $Ca^{2+}$ and 8.7 $\pm$ 2.9 mM and 8.9 $\pm$ 0.8 mM for $Mn^{2+}$ (Table I.6). Maximum velocities were calculated from the relationship $V = \text{app. } V \left(1 + \frac{M}{K_4}\right)$. The directly determined values for the constants with $Ca^{2+}$ = 2.0 mM and $Mn^{2+}$ = 1.0 mM are included for comparison. V is expressed as µmoles per min. per µg. of creatine kinase.

| Activating Ion | Concn. (mM) | $K_{ia}$ (mM) | $K_a$ (mM) | $K_{ib}$ (mM) | $K_b$ (mM) | V |
|---|---|---|---|---|---|---|
| $Ca^{2+}$ | 2.0 | 0.06 $\pm$ 0.01 | 0.008 $\pm$ 0.001 | 5.9 $\pm$ 1.1 | 0.95 $\pm$ 0.15 | 0.034 |
| | 10.0 | 0.12 $\pm$ 0.03 | 0.013 $\pm$ 0.002 | 6.6 $\pm$ 1.5 | 0.65 $\pm$ 0.11 | 0.046 |
| $Mn^{2+}$ | 1.0 | 0.07 $\pm$ 0.01 | 0.027 $\pm$ 0.002 | 2.6 $\pm$ 0.4 | 0.82 $\pm$ 0.05 | 0.131 |
| | 5.0 | 0.11 $\pm$ 0.03 | 0.025 $\pm$ 0.003 | 2.5 $\pm$ 0.8 | 0.64 $\pm$ 0.11 | 0.147 |

FIG. I.9. Determination of the maximum velocity of the reaction catalysed by creatine kinase by simultaneously varying the concentrations of free phosphocreatine and metal-ADP complexes while maintaining their ratios and the concentration of free metal ion constant. Phosphocreatine : $MgADP^-$ :: 50 : 1, with free $Mg^{2+}$ at 1.0 mM; phosphocreatine : $MnADP^-$ :: 33.3 : 1, with free $Mn^{2+}$ at 1.0 mM; phosphocreatine : $CaADP^-$ :: 100 : 1, with free $Ca^{2+}$ at 2.0 mM. The lines were drawn using the constants obtained from analysis of the data by means of the PARA computer programme of Cleland (1963b). Purified metal salts were used. v is expressed as μmoles of creatine per μg of creatine kinase per min.


70
60
50
40
30
20
10
1/V
Ca 2+
Mn2+
Mg2+
0
1
2
3
4
5
0.40
[MgADP-]
or
0.30
[MnADP-]
or
0.10
[CaADP-]
mM-1

velocities were 0.239 ± 0.019, 0.134 ± 0.011 and 0.046 ± 0.007 μmoles per min. per μg of enzyme. These values are in close agreement with those listed in Tables I.1 and I.6.

Mechanism of the reaction with $CaADP^-$ and $MnADP^-$ as substrates

It was assumed in the Theory section that the reaction mechanism is independent of the identity of the metal ion. Before the experimental results could be interpreted with confidence, however, it was deemed advisable to determine whether or not the mechanism of the creatine kinase reaction really is rapid equilibrium random when $CaADP^-$ and $MnADP^-$ are used as substrates. Therefore, Morrison and White have measured the rates of isotope exchange between the different pairs of reactants after the reaction has come to equilibrium. The experimental technique was similar to that already used by Morrison and Cleland (1966) to verify that the reaction mechanism is rapid equilibrium, random, when $Mg^{2+}$ is used as the activating ion. The results given in Table I.7 show that the exchange rates between the nucleotide and guanidino pairs of reactants are equal within experimental error. The slightly lower velocities in the presence of the lower total concentrations of metal ions probably reflect inhibition by $ADP^{3-}$ (Morrison & O'Sullivan, 1965). It can be concluded, therefore, that the mechanism of the reaction catalysed by creatine kinase is a rapid equilibrium random one, regardless of whether $Mg^{2+}$, $Ca^{2+}$ or $Mn^{2+}$ is used as the activating ion.

TABLE I.7 : ISOTOPE EXCHANGE RATES AT EQUILIBRIUM WITH $Mg^{2+}$, $Ca^{2+}$ AND $Mn^{2+}$ AS THE ACTIVATING METAL IONS.*

Reaction mixtures contained ATP, ADP, phosphocreatine and creatine kinase with the metal ion added at concentrations as shown. The reaction was allowed to come to equilibrium before the addition of the labelled reactant. The velocity of the exchange rate is expressed as mμmoles/min/μg of creatine kinase.

| Metal Ion | Total Concn. of Metal Ion (mM) | Exchange Rate | |
|---|---|---|---|
| | | Creatine-PC | ADP-ATP |
| $Mg^{2+}$ | 2.0 | 10.2 ± 1.7 | 8.8 ± 0.4 |
| | 8.0 | 12.6 ± 0.7 | 11.7 ± 0.4 |
| $Ca^{2+}$ | 2.0 | 8.2 ± 1.4 | 6.1 ± 0.5 |
| | 8.0 | 10.6 ± 1.1 | 8.7 ± 0.3 |
| $Mn^{2+}$ | 2.0 | 12.8 ± 1.2 | 11.3 ± 0.4 |
| | 8.0 | 13.2 ± 1.2 | 14.6 ± 0.7 |

* Data taken from a paper presented by J.F. Morrison and Anne White to a meeting of the Australian Biochemical Society, held in Brisbane, 23rd - 26th May, 1966.

## DISCUSSION

The function of metal ions in the reaction catalysed by creatine kinase has been investigated by means of initial velocity studies using the ADP complexes of three different metal ions as substrates under conditions where the inhibitory effect of $ADP^{3-}$ was negligible. In addition, allowance has been made for the inhibition of the reaction by higher concentrations of the free metal ions. It was assumed in the Theory section, and has since been verified by Morrison and White, that the reaction mechanism is a rapid equilibrium, random one when $MgADP^{-}$, $CaADP^{-}$ or $MnADP^{-}$ is used as substrate. A similar result has been obtained with yeast hexokinase: Zewe, Fromm and Fabiano (1964) found that the mechanism is not altered when $Mn^{2+}$ is substituted for $Mg^{2+}$ as the activating ion. These results emphasize that the reaction mechanism is a property of the enzyme and may not be affected by a change in the activating ion. Since the rapid equilibrium, random mechanism holds for all experimental conditions, the values obtained for the kinetic constants are all dissociation constants for the reaction of substrates either with the free form of enzyme or with the enzyme saturated with the second substrate (Michaelis constants).

From a comparison of the $K_{ia}$ values (Tables I.2 and I.6), which are dissociation constants giving a measure of the ability of the metal-nucleotide complexes to combine with the

free enzyme, it would appear that the binding of these substrates is not influenced to any extent by the identity of the metal ion. Furthermore, a value of 0.07 $\pm$ 0.01 mM for the dissociation constant of the enzyme-MnADP complex is in reasonable agreement with the value of 0.05 mM recently reported by O'Sullivan and Cohn (1966) and obtained both from kinetic data and from determinations of the dissociation constant by magnetic resonance techniques. This agreement would appear to substantiate the conclusion that the identity of the metal ion is unimportant in determining the dissociation constants. Since these values are lower than the dissociation constant for the reaction of $ADP^{3-}$ with free enzyme (0.2 - 0.3 mM) (Morrison & O'Sullivan, 1965), it might be argued that the metal ion does play some part in the binding. But this effect could be due to the reduction of the overall negative charge on the nucleotide.

In contrast to the above results, the values of the Michaelis constants ($K_a$) for the metal-ADP complexes (Tables I.2 and I.6), representing the degree to which they combine with the enzyme-phosphocreatine complex, are dependent on the identity of the metal ion. They are also lower than the corresponding values for $K_{ia}$ and this indicates that the presence of phosphocreatine on the enzyme facilitates the binding of the metal-ADP substrate. A similar finding has been reported by Morrison and James (1965) from their studies with $MgADP^-$. The binding of the metal-ADP complexes to the

enzyme-phosphocreatine complex decreases in the order: $CaADP^- > MnADP^- > MgADP^-$, which is the same order as the decrease in the non-hydrated ionic radii of the metal ions. This suggests that the metal ion may be involved in the formation of the ternary complex. But such a suggestion does not necessarily imply that the metal moiety of the metal-nucleotide complex is bound to the enzyme. Indeed, Cohn (1963) and Cohn and Leigh (1962) have concluded that the metal moiety of $MnADP^-$ is not bound to, but is orientated away from the enzyme and these conclusions are in agreement with the present findings that $Ca^{2+}$ and $Mn^{2+}$ do not inhibit by combining at the nucleotide binding site. Whether or not the metal moiety of the metal-nucleotide is bound to the enzyme, it might well be available for chelation with the phosphoryl groups of both ADP and phosphocreatine. Admittedly, in free solution phosphocreatine has at the best only a poor ability to chelate $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ (O'Sullivan & Perrin, 1964), but when combined with the enzyme it could bind metal ions more strongly. It may well follow from this idea that the larger is the bivalent metal ion, the less is the strain introduced into the ternary complex and the slower is the rate at which the phosphoryl group of phosphocreatine is polarized. Support for this hypothesis comes from the finding that the maximum velocity of the reaction with the metal-ADP complexes decreases in the order : $MgADP^- > MnADP^- > CaADP^-$. Thus the maximum velocity decreases with increasing size of the metal

ion. It may then be concluded that the metal ion is involved in the polarization of the N-P bond of phosphocreatine, rather than in the binding of the metal-nucleotide substrates to the enzyme. The conclusions are thus similar to those reached by Hammes and Kochavi (1962) with regard to the role played by metal ions in the hexokinase reaction. In connection with the above results, it would be of interest to determine if other metal ions with the same ionic radius as $Ca^{2+}$, but with higher charge density, would be equally as effective as $Ca^{2+}$ in forming the ternary complex and give rise to a higher maximum velocity.

While the metal ion may be involved directly in the formation of a central enzyme-MADP-phosphocreatine complex, it is also possible that the combination of phosphocreatine with the enzyme results in a conformational change at the metal-nucleotide binding site. Such a change could result in an enhanced combination of the metal-nucleotide complexes, the enhancement being dependent on the structure of the complex. It has been reported that in solution, there is no difference in the structures of $MgADP^-$ and $CaADP^-$, although they differ from that of $MnADP^-$ (Hammes, Maciel & Waugh, 1961; Cohn & Hughes, 1962). Nevertheless, it is conceivable that only small changes in the conformation of the ADP moiety by $Mg^{2+}$ or $Ca^{2+}$ would be required to affect its binding to the enzyme, presuming that the metal ion itself were not involved.

A comparison of Table I.2 with Table I.6 shows that the

values for $K_b$ also vary with the identity of the activating ion. Since the mechanism of the reaction is rapid equilibrium, random, the four Michaelis and dissociation constants have been calculated using the relationship: $K_{ia}K_b = K_aK_{ib}$. Therefore any change in the ratio $K_{ia}:K_a$ (which has been discussed) will be reflected in the ratio $K_{ib}:K_b$, so that there seems little point in discussing the variation in $K_b$ values in detail. It should be mentioned, however, that it was expected that the binding of free phosphocreatine to the free enzyme form ($K_{ib}$) would be independent of the metal ion present. While this was true using $Mg^{2+}$ and $Ca^{2+}$, the value obtained using $Mn^{2+}$ was significantly lower. It is difficult to account for this, especially if the Mn-phosphocreatine complex is inert as assumed. But as mentioned in Results, it was difficult to obtain reproducible results for initial velocities when $Mn^{2+}$ was used as the activating metal ion.

The uncompetitive inhibition of the reaction by $Ca^{2+}$ and $Mn^{2+}$ with respect to their corresponding metal-ADP complexes indicates that these ions differ from $Mg^{2+}$ in that they do not react with the free form of the enzyme or the enzyme-phosphocreatine complex (see Morrison & O'Sullivan, 1965). O'Sullivan and Cohn (1966) have also examined kinetically the inhibition by $MnCl_2$ with respect to $MnADP^-$ as the variable substrate. While the inhibition appears non-competitive, the increase in slope is so slight that no attempt was made to extract the (presumably large) apparent inhibition constant.

A value of 4 mM is given for the apparent $K_i$ intercept, as compared with 7.6 $\pm$ 0.3 mM found in this study under different conditions of temperature and ionic strength. In addition, the authors have examined the interaction of $Mn^{2+}$ and creatine kinase with the use of nuclear magnetic resonance techniques, measuring enzyme-Mn binding from changes in the proton relaxation rate of the water molecules in the hydration sphere of the $Mn^{2+}$, and the concentration of $Mn^{2+}$ from its electron paramagnetic resonance. The results obtained indicated only very weak binding which can be eliminated almost entirely by increasing the ionic strength, so it is concluded that any interaction of $Mn^{2+}$ and creatine kinase is largely or entirely non-specific. These results, with those reported here, are in accord with the idea mentioned above (p. 54) that the metal moiety of the metal-ADP complex is not bound to the enzyme.

The non-competitive inhibition of the reaction by $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ with respect to phosphocreatine indicated that all three metal ions are capable of reacting with the enzyme-MADP and enzyme-MADP-phosphocreatine complexes to cause inhibition. The difference in the inhibitions obtained with $Ca^{2+}$ and $Mn^{2+}$ on the one hand, and $Mg^{2+}$ on the other, might be related to the difference in the ionic radii of these ions. Perhaps the larger $Mn^{2+}$ and $Ca^{2+}$ cannot combine with the enzyme at an inhibitory site until the reaction of the metal-ADP complex causes some conformational change. Such a change

is apparently not a prerequisite for the combination of the smaller $Mg^{2+}$ ion.

Since the presence of a metal-nucleotide complex on the enzyme is essential for the inhibition by $Ca^{2+}$ and $Mn^{2+}$, it is possible that the inhibition is due to the reaction of these ions with the bound metal-nucleotide complex to form a dead-end enzyme-2:1 metal-nucleotide complex. The results for the inhibition of the reaction by $Mg^{2+}$, however, could not be explained by this scheme.

## SUMMARY

1. The reverse reaction catalysed by ATP:creatine phosphotransferase has been studied kinetically at pH 8.0 using different metal-ADP complexes and free phosphocreatine as substrates. The concentration of the free metal ion was held constant.

2. It has been shown that the reaction mechanism is rapid equilibrium, random irrespective of which metal ion is added; this allowed the kinetic parameters to be determined and the results to be interpreted.

3. The nature of the metal ion does not affect to any extent the combination of the metal-ADP complex with the free form of enzyme, although it does influence the binding of the metal-nucleotide to the enzyme-phosphocreatine complex. Thus the binding decreases in the order: $CaADP^- > MnADP^- > MgADP^-$, and this may be due either to con-

formational changes in the enzyme or to the involvement of the metal ion in the formation of the ternary complex.

4. It has been concluded that the metal ion is concerned with the polarization of the N-P bond of phosphocreatine since the maximum velocity of the reaction varies with the metal-ADP complex, in the order: $MgADP^- > MnADP^- > CaADP^-$.

5. Higher concentrations of free $Ca^{2+}$ and free $Mn^{2+}$ inhibit the reaction non-competitively with respect to phosphocreatine and uncompetitively in relation to the corresponding metal-ADP complex.

# CHAPTER II

# THE PURIFICATION AND SOME PROPERTIES OF ATP:ARGININE PHOSPHOTRANSFERASE (ARGININE KINASE)

# CHAPTER II

# THE PURIFICATION AND SOME PROPERTIES OF ARGININE KINASE

## INTRODUCTION

Arginine kinase, the ATP:guanidino phosphotransferase commonly found among invertebrates, was first discovered in crude extracts of crab and octopus muscle by Lohmann (1935, 1936) and Lehmann (1935, 1936). Although these extracts were used for studies of the reaction (e.g. Lehmann, 1936) no attempts was made to purify the enzyme. Considerable attention over the following years, however, has been directed towards determining the distribution of arginine kinase and its substrate, phosphoarginine (see Ennor & Morrison, 1958; Virden & Watts, 1964; Thoai & Robin, 1965; Jeso, Malcovati & Speranza, 1965). From such investigations, it has become apparent that both the enzyme and the substrate occur in many different classes of invertebrates, including the unicellular organism, Tetrahymena pyriformis (Robin & Viala, 1966). At the same time, it has been found that phosphoarginine is only one of at least seven phosphorylated guanidines to occur in the invertebrates.

Crystalline arginine kinase was obtained first by Szorenyi, Dvornikhova and Degtyar (1949) from fresh-water crab muscle extracts and subsequently by Elodi and Szorenyi (1956) from muscle extracts of different species of crab, using only ammonium sulphate fractionation. The latter

authors showed that their preparation was homogeneous as judged by ultracentrifugation and electrophoretic analysis. Since 1956, there has been a number of reports concerning the preparation of enzyme from various crustacean sources. Pradel, Kassab, Regnouf and Thoai (1964) obtained arginine kinase from lobster muscle extracts and their product appeared homogeneous on ultracentrifugation, electrophoresis and chromatography on DEAE-cellulose, but a more recent report from the same laboratory (Pradel et al., 1965) has shown that crystallization results in a two-fold increase in specific activity. Virden, Watts and Baldwin (1965) purified the enzyme from extracts of muscle of the lobster, Homarus vulgaris. Ultracentrifugation showed a single symmetrical peak, but electrophoresis in starch gel indicated a second minor component, and chromatography on G-100 Sephadex suggested that the preparation was contaminated with 5% of heavier material. The enzyme has also been obtained in crystalline form as a result of ammonium sulphate fractionation of extracts of muscle from the lobster, Homarus americanus (Sacktor & Hurlbut, 1966), but no details of the method or the product have been given.

From the measurements of the sedimentation and diffusion constants of the enzyme from crab muscle extracts, Elodi and Szorenyi (1956) have calculated its molecular weight to be 43,000; but they also reported that considerable denaturation of the protein occurred during the second half of the 6 to 8

hour ultracentrifuge run. Since Pradel et al. (1964) obtained a sedimentation constant of 2.69 which was similar to that for the crab enzyme (2.5), they assumed the molecular weights of the two enzymes to be identical. However, as pointed out above, crystallization of the lobster enzyme results in an almost two-fold increase in specific activity and hence the sedimentation constant obtained may not represent a true value for the lobster enzyme. The best estimate of the molecular weight of the lobster enzyme would appear to come from the work of Virden, Watts, Watts, Gammack and Raper (1966). From studies involving sedimentation and diffusion constants, sephadex filtration and sucrose density-gradient analysis, they have obtained a figure of 37,000.

The enzymic activity of all preparations of arginine kinase is sensitive to sulphydryl reagents, and both Pradel et al. (1964) and Virden and Watts (1966) have examined the relationship between alkylation of SH groups and loss of enzyme activity, in an endeavour to elucidate the role of thiol groups in the activity. It appears that there are five thiols per 37,000 molecular weight : one of these is essential for activity, while two others may be in the area of nucleotide binding (Virden & Watts, 1966).

Virden et al. (1965) have found also that increasing concentrations of salts such as NaCl and sodium acetate and in particular $NaNO_3$ and $KNO_3$ have inhibitory effects, with only 2% of the activity remaining in the presence of 0.5 M

nitrate. The enzyme is activated by a large number of bivalent cations including $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Sn^{2+}$ and $Fe^{2+}$ and hence differs from the Australian sea-crayfish enzyme (cf. Introduction, p.26 and also below).

No lobster or closely related species is present in Australian coastal waters. There abound instead three species known as sea-water crayfish, which belong to a small group of decapods not closely related either to the real lobster or to the fresh-water crayfish (Dakin, 1952, p.183). Of these, the species Jasus verreauxi, which inhabits the coastal waters of New South Wales, is the most accessible, so the tail muscle of this organism has been used as the source of arginine kinase. Enzyme was partially purified by means of ammonium sulphate fractionation (Morrison et al., 1957) and shown to be sensitive to sulphydryl reagents and to be activated by $Mg^{2+}$ and $Mn^{2+}$ but not by $Ca^{2+}$, $Co^{2+}$, $Fe^{2+}$ or five other bivalent cations tested. The preparation, however, was not homogeneous, was contaminated with adenylate kinase activity, and was unsuitable for kinetic studies at 30°. One reason for this appeared to be an inactivation caused by the dilution that was necessary to obtain accurate initial velocities at 30°.

The purpose of the work reported in this Chapter was the preparation of arginine kinase which would be suitable for investigation of the reaction mechanism by means of kinetic studies. This has been accomplished. In addition,

preliminary kinetic investigations have been carried out to determine the effects of various components of the reaction mixtures on the initial velocity of the reaction. These experiments show that the enzyme is inhibited by higher concentrations of free $Mg^{2+}$ as well as by relatively low concentrations of the free nucleotides $ADP^{3-}$ and $ATP^{4-}$.

## EXPERIMENTAL

### Materials

The sodium salts of ADP and ATP were obtained from P-L Biochemicals and the Sigma Chemical Co., respectively. ADP was used without further purification while ATP was recrystallized twice at 2° by the method of Berger (1956). Both nucleotides showed the presence of only single spots which absorbed ultraviolet light after chromatography in isobutyric acid - $NH_3$ (sp.gr. 0.88) - water (66:1:33, v/v). Stock solutions of the nucleotides were adjusted to pH 7.6 with 0.5 N NaOH and stored at -10°. Their concentrations (10 to 25 mM) were checked by measurement of the absorption at 259 mμ in 0.01 N HCl (Bock et al., 1956). L-arginine (free base) was purchased from Mann Research Laboratories and after adjustment to pH 8.0 with N HCl, was stored at -10°. Phosphoarginine was isolated from crayfish muscle by the procedure of Marcus and Morrison (1964) and stored at -10° as the barium salt. NaCl, $NaNO_3$, $NaClO_4$ and sodium acetate were Analar grade products from British Drug Houses Ltd. Triethanolamine

(puriss) from Fluka was used without further purification. N-ethylmorpholine, $MgCl_2$, EDTA and dithizone were purchased and treated as described in Chapter I (p.40). Solutions of EDTA were brought to pH 8.0 with N NaOH before use.

DEAE-cellulose (Cellex D) was purchased from Bio-Rad Laboratories, Sephadex G-100 from Pharmacia, Sweden, and bentonite from British Drug Houses Ltd. Dialysis tubing from Visking Co., Illinois, U.S.A. was soaked at least 2 hours in 20 mM EDTA before use.

$NADH_2$ was from the Sigma Chemical Co. while phosphoenolpyruvate was used as the monocyclohexylamine salt. Crystalline preparations of muscle pyruvate kinase and lactate dehydrogenase were from the California Corp. for Biochemical Research.

## Methods

### Preparations of Sodium Phosphoarginine

The barium salt of phosphoarginine (500 mg) was suspended in 10 ml of water and added to the top of a column (10 x 1 cm) of Zeo-Karb 225 ($Na^+$ form). The suspension was stirred into the upper part of the column which was then washed with water. The effluent (20 ml) was collected and the concentration of phosphoarginine (about 50 mM) was determined by estimating the arginine released after hydrolysis in N HCl for 7 min. at 100° (Rosenberg, Ennor and Morrison, 1956).

Determination of Protein

This was carried out by the Biuret method of Gornall, Bardawill and David (1949) and bovine serum albumin was used as a standard. Interference of the method by ammonium sulphate was prevented by varying the volume of the ammonium sulphate fractions so that the concentration was less than 3% (w/v).

Fractionation with Ammonium Sulphate

The weight (w) of ammonium sulphate required to give the various degrees of saturation was calculated from the formula

$$w = \frac{0.515\ V\ (S_2 - S_1)}{1.0 - 0.292\ S_2}$$

where V represents the volume in millilitres and w is expressed in g. $S_1$ and $S_2$ represent the initial and desired degree (0 to 1.0) of saturation at 0° (Kunitz, 1952; Noltmann, Gubler & Kuby, 1961).

Measurement of Initial Velocities

The arginine kinase activity of the various fractions obtained during the purification procedure was determined by measuring the rate at which arginine was released from phosphoarginine. To 3 ml graduated test tubes was added 0.8 ml of a stock solution containing 0.2 ml of 0.5 M N-ethylmorpholine-HCl buffer (pH 8.0), 0.1 ml of 25.0 mM phosphoarginine, 0.1 ml of 15.0 mM $MgCl_2$, 0.1 ml of 6.25 mM ADP, 0.1 ml of 0.1 mM EDTA and sufficient water to bring the final volume of the reaction mixture to 1.0 ml. The tubes were equili-

brated in a water bath for 3 min. at 30° and the reaction started by the addition of 0.01 - 0.02 ml of arginine kinase solution. After 3 min., the reaction was stopped by the addition of 0.6 ml of 1.5 N NaOH containing 0.14 M EDTA and the arginine release determined by the method of Rosenberg et al. (1956). One unit of activity was taken as being the amount of enzyme which releases one μmole of arginine from phosphoarginine per min. under the above conditions. Specific activity is defined as units per mg. of protein.

Kinetic investigations were made at 30° in 0.1 M triethanolamine-HCl buffer (pH 8.0) in the presence of 0.01 mM EDTA; $Mg^{2+}$ was used as the activating metal ion. It was assumed that the reaction, like that of creatine kinase (Morrison & James, 1965; Chapter I), could be considered as being bireactant, and so written as :

$$MgATP^{2-} + arginine^{+} \rightleftharpoons MgADP^{-} + phosphoarginine^{-}$$

Thus it was the concentrations of the Mg-nucleotide complexes and the free forms of the guanidino compounds that were considered as the variable substrates. The Mg-guanidino complexes, the Mg-acetate complex and the Na-nucleotide complexes were considered to be inert. The concentrations of total inorganic salts and total substrates required to give the desired concentrations of different ionic species were calculated as described by Morrison et al. (1961) and for this purpose the apparent stability constants for $MgATP^{2-}$, $MgADP^{-}$,

Mg-phosphoarginine, Mg-arginine, magnesium acetate and $NaADP^{2-}$ were taken to be 70,000 $M^{-1}$, 4,000 $M^{-1}$, 20 $M^{-1}$, 7 $M^{-1}$ and 6 $M^{-1}$ respectively (O'Sullivan & Perrin, 1964; Pelletier, 1960; Davies, R.H., personal communication to Noda et al., 1960).

Initial velocities in the direction of phosphoarginine and ADP formation were determined by estimating the amount of ADP from the equivalent amount of $NADH_2$ oxidized by means of the coupled reactions catalysed by pyruvate kinase and lactate dehydrogenase. Initial velocities in the direction of ATP and arginine formation were determined by measuring the amount of arginine formed by the method of Rosenberg (1956). All kinetic experiments were run for two time periods to ensure that initial velocities were being measured. The amount of enzyme added for these experiments corresponded to 0.15 µg of protein per ml of reaction mixture.

Analysis of data

Kinetic data were analysed as described in Chapter I (p.43), using the computer programmes HYPER, LINE, NONCOMP and also COMP (preface, equation (6) ). The Figures illustrating the inhibitions by $MgCl_2$, $ADP^{3-}$ and $ATP^{4-}$ have been drawn using the apparent kinetic constants obtained by analysis of the primary data by the COMP or NONCOMP programme. The weighted mean values and their standard errors were calculated using the formulae given in Chapter I, p.44.

# RESULTS

## Purification of Arginine Kinase

### Collection and storage of crayfish muscle

Live crayfish (Jasus verreauxi) were obtained from sea water storage tanks and the tail muscle was dissected rapidly. After freeing from chitinous material, it was dropped into liquid $N_2$. When the muscle reached the temperature of liquid $N_2$, it was removed, placed in polythene bags and stored in solid $CO_2$ for transport to the laboratory. Subsequent storage was at -15°. (The total arginine kinase activity of crayfish muscle remained constant for up to two years after which time there was an appreciable loss).

### Extraction of muscle

A sample of muscle (200 g) was allowed to thaw at room temperature and then transferred to the coldroom at 2° where all further operations were carried out. The muscle was then ground, stirred with 5 vol. of 1.3 mM EDTA (pH 7.0) and homogenised for 30 sec. in a Waring blendor. The mixture was centrifuged for 20 min. at 3000 x g and the supernatant retained. The residue was re-extracted in a Waring blendor for 30 sec. with 2 vol. of EDTA solution and after centrifuging as described above, the two opalescent supernatants were combined.

### Bentonite treatment

The pH of the combined extracts was adjusted from pH

6.2 - 6.8 to pH 8.0 by the addition of 5 N NaOH and bentonite (2 g per g of protein in the extract) was added with efficient stirring over a period of 5 min. The suspension was stirred for another 5 min. after which the bentonite was removed by centrifuging for 20 min. at 3000 x g.

Ammonium sulphate fractionation

To the clear supernatant from the previous step was added 0.125 vol. of 0.5 M N-ethylmorpholine-HCl buffer (pH 8.0) and the solution was brought to 0.72 saturation by the addition of solid ammonium sulphate over a period of not less than 2.5 hours. After the addition was complete, stirring was continued for a further 20 min. before the precipitate was removed by centrifuging for 20 min. at 4000 x g. The supernatant was then brought to 0.95 saturation by adding solid ammonium sulphate over a period of 1 hour. After stirring for an additional 30 min., the precipitate was collected by centrifuging for 30 min. at 4000 x g and dissolved in 15 ml of 0.01 M N-ethylmorpholine-HCl buffer containing 1.0 mM EDTA (pH 8.0). Any insoluble material was removed by centrifuging at 10000 x g for 15 min. and the clear supernatant dialysed for 17 hr. against two changes of 2.5 l of the same buffer.

Chromatography on DEAE-cellulose

The 100-300 mesh fraction of DEAE-cellulose, after equilibration for 48 hr. with the N-ethylmorpholine buffer mentioned above, was packed into a column (2 x 45 cm) and washed with

200-300 ml of the same buffer. The dialysed supernatant from the previous step was applied to the top of the column and after it had sunk in, the column was washed with 50 ml of buffer to remove protein which does not adsorb on the cellulose. The enzyme was eluted by using a linear gradient formed from 0.01 M N-ethylmorpholine-HCl buffer containing 1.0 mM EDTA (pH 8.0) and the same buffer at a concentration of 0.25 M. Elution of arginine kinase occurred soon after application of the gradient; the leading edge was sharp, but there was some trailing.

The fractions containing arginine kinase activity were pooled and the protein precipitated by the addition of solid ammonium sulphate to 0.95 saturation. After collection of the precipitate by centrifuging for 15 min. at 10000 x g, it was dissolved in 15 ml of 0.01 M N-ethylmorpholine-HCl buffer containing 1.0 mM EDTA (pH 8.0) and dialysed against the same buffer for 17 hr. The enzyme was then rechromatographed on a 2 x 30 cm column of DEAE-cellulose and precipitated from those fractions possessing activity by ammonium sulphate. The resulting precipitate was dissolved in 5 ml of 1.0 mM N-ethylmorpholine-HCl buffer containing 1.0 mM EDTA (pH 8.0) and after dialysis against 100 vol. of the same buffer and buffer from which the EDTA had been omitted, the enzyme solution was stored at 4° with the protein at a concentration of 7 - 10 mg per ml. Some protein precipitation

occurred during the first two weeks of storage, but there was no decrease in specific activity. After removal of this precipitate, the solution remained clear and there was no loss of enzyme activity over a period of six months, but after this time the activity began to fall.

A summary of the yield and the specific activity of the fractions obtained during the purification procedure is given in TableII.1. It should be noted that when muscle, which has been stored at -15° for many months, is used for the purification of the enzyme, the number of units in, and therefore the specific activity of the first three fractions is considerably less than that obtained with relatively fresh muscle. However, the specific activity of the final product is not a function of the time for which muscle is stored.

Properties of purified arginine kinase

The ultracentrifuge pattern of an arginine kinase preparation obtained by the above procedure showed the presence of a single, symmetrical peak (see Fig. II.1) with an $s_{4.3}$ of 1.9953 ($s_{20} = 3.07$). This value was obtained at a protein concentration of 8 mg per ml in 0.03 M veronal buffer (pH 8.0) which was brought to an ionic strength of 0.1 with NaCl. A similar value was also obtained at a protein concentration of 0.8 mg per ml. Chromatography on Sephadex G-100 also gave rise to a single, symmetrical peak, but the specific activity of the more dilute fractions was less than that of the sample applied to the column. Presumably this is because of the

TABLE II.1 : SUMMARY OF YIELDS AND SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED DURING THE PURIFICATION OF ARGININE KINASE FROM STORED CRAYFISH MUSCLE.

Wt. of muscle, 200 g. Details are given in text. Figures in brackets represent the specific activities of fractions obtained from fresh crayfish muscle.

| Fraction | Volume (ml) | Protein (mg) | Total Units | Specific Activity |
|---|---|---|---|---|
| Extract | 680 | 8160 | 113,560 | 14 (47) |
| Supernatant after bentonite treatment | 605 | 3146 | 104,650 | 31 (86) |
| Ammonium sulphate precipitate (0.72 - 0.95 sat.) | 43 | 1247 | 89,000 | 72 (156) |
| Eluate from first DEAE-cellulose column | 58 | 232 | 51,620 | 225 (248) |
| Eluate from second DEAE-cellulose column precipitated with $(NH_4)_2SO_4$ and dialysed | 7.3 | 64.6 | 15,880 | 246 |

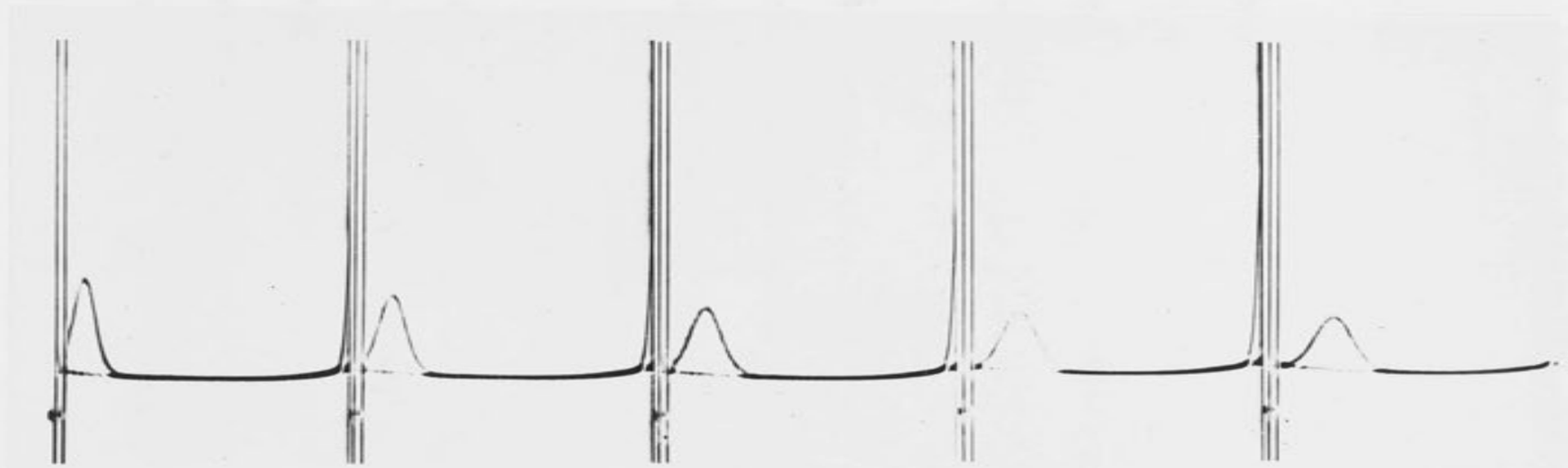


FIG. II.1. Ultracentrifuge pattern obtained with purified arginine kinase. The analysis was carried out in veronal buffer, 0.03 M (pH 8.0), made up to an ionic strength of 0.1 with NaCl, at a temperature of 4.3°, using a Model E Spinco Ultracentrifuge. The initial protein concentration was 8 mg./ml. The rotor speed was 50,740 rev./min., and the pictures were taken 27 min., 43 min., 59 min., 75 min. and 91 min. after reaching full speed. The phase plate angle was 70° and the exposure time 5 sec. The analysis was carried out by Dr. A. B. Roy.

instability of the enzyme in dilute solution as noted with earlier preparations by Morrison et al. (1957). No phosphoamidase, ATP-ase or adenylate kinase activities could be demonstrated when the procedures of Morrison et al. (1957) were used with the enzyme at a concentration ten times greater than those employed for the kinetic studies and with incubation times up to 60 min. It appeared, then, that this enzyme preparation was free from enzymic activities which would interfere with the reaction and that it was sufficiently stable for use in kinetic studies.

Investigation of suitable experimental conditions

Preliminary experiments were designed to determine if the Mg complexes of ADP and ATP could function as substrates at pH 8.0. These experiments were carried out by studying the initial velocity of the reaction as a function of the concentration of nucleotide (or $MgCl_2$) at a fixed concentration of $MgCl_2$ (or nucleotide) and the results indicated that maximum velocities were attained under conditions where all the nucleotide was present as its magnesium complex. Thus $MgADP^-$ and $MgATP^{2-}$ could be considered as the nucleotide substrates for arginine kinase and, in addition, it was assumed that this enzyme is similar to creatine kinase in utilizing the free forms of the guanidino substrates (cf. Morrison & James, 1965). Therefore, in the subsequent kinetic experiments it was the concentrations of free arginine or phosphoarginine and $MgATP^{2-}$ or $MgADP^-$ that were varied.

Since the Mg-nucleotide complexes will exist in non-enzymic equilibrium with $Mg^{2+}$ and free nucleotides, variation in the concentrations of the complexes may be accompanied by variations in the concentrations of both $Mg^{2+}$ and free nucleotide; but if one of these latter species is held constant then variation in the concentration of the complex will result only in a corresponding variation in the other species. It has been shown that both $Mg^{2+}$ and $ADP^{3-}$ inhibit the creatine kinase reaction (Morrison & O'Sullivan, 1965). Therefore, it seemed advisable, before beginning detailed kinetic studies, to examine the effects of $Mg^{2+}$, $ADP^{3-}$ and $ATP^{4-}$ on the initial velocity of the reaction with the Mg-nucleotide complexes as substrates. In this connection, consideration was given first to what magnesium salt should be used. The chloride ion has been found to inhibit the creatine kinase reaction (James, 1965; Morrison, unpublished data) and Virden et al. (1965) have demonstrated that arginine kinase from lobster muscle is inhibited by NaCl, sodium acetate and $NaNO_3^-$. While $MgCl_2$ was used for the preliminary experiments, it was necessary to determine the inhibitory effect of $Cl^-$ and several other anions before selecting the salt of magnesium for use in the detailed kinetic work.

Anion inhibition of arginine kinase

Four sodium salts of monovalent anions, NaCl, sodium acetate, $NaNO_3$ and $NaClO_4$, were tested at a concentration of 40 mM for their effects on the initial velocity of the reaction.

All salts were found to be weak non-competitive inhibitors with respect to $MgADP^-$ (Fig. II.2). Nevertheless, it is apparent that NaCl and sodium acetate are less inhibitory than either $NaNO_3$ or $NaClO_4$ and hence it seemed that either $MgCl_2$ or magnesium acetate would be suitable as a source of $Mg^{2+}$. But as there is some complexing in solution between $Mg^{2+}$ and the acetate ion, it was decided to use $MgCl_2$ in all future experiments.

Because $MgCl_2$ was to be used throughout this study, more extensive investigations were made of the effect of $Cl^-$ on the velocity of the reaction. These showed that inhibition by NaCl appears to be linear non-competitive with respect to both $MgADP^-$ and phosphoarginine as variable substrates (Fig. II.3). The apparent inhibition constants obtained from NONCOMP analysis of such experiments (Table II.2) are not necessarily true dissociation constants, but do indicate, as predicted by Fig. II.2, that inhibition is likely to be of significance only in the presence of higher concentrations of $Cl^-$. It should be mentioned that inhibition is considered in terms of $Cl^-$ rather than $Na^+$ and $Cl^-$ because it is the $Cl^-$ that has been found inhibitory to creatine kinase and because, in preliminary experiments, inhibition by triethanolamine-HCl appeared to be equal to that produced by NaCl.

Inhibition of arginine kinase by $MgCl_2$

Inhibition by $MgCl_2$ was examined with respect to both

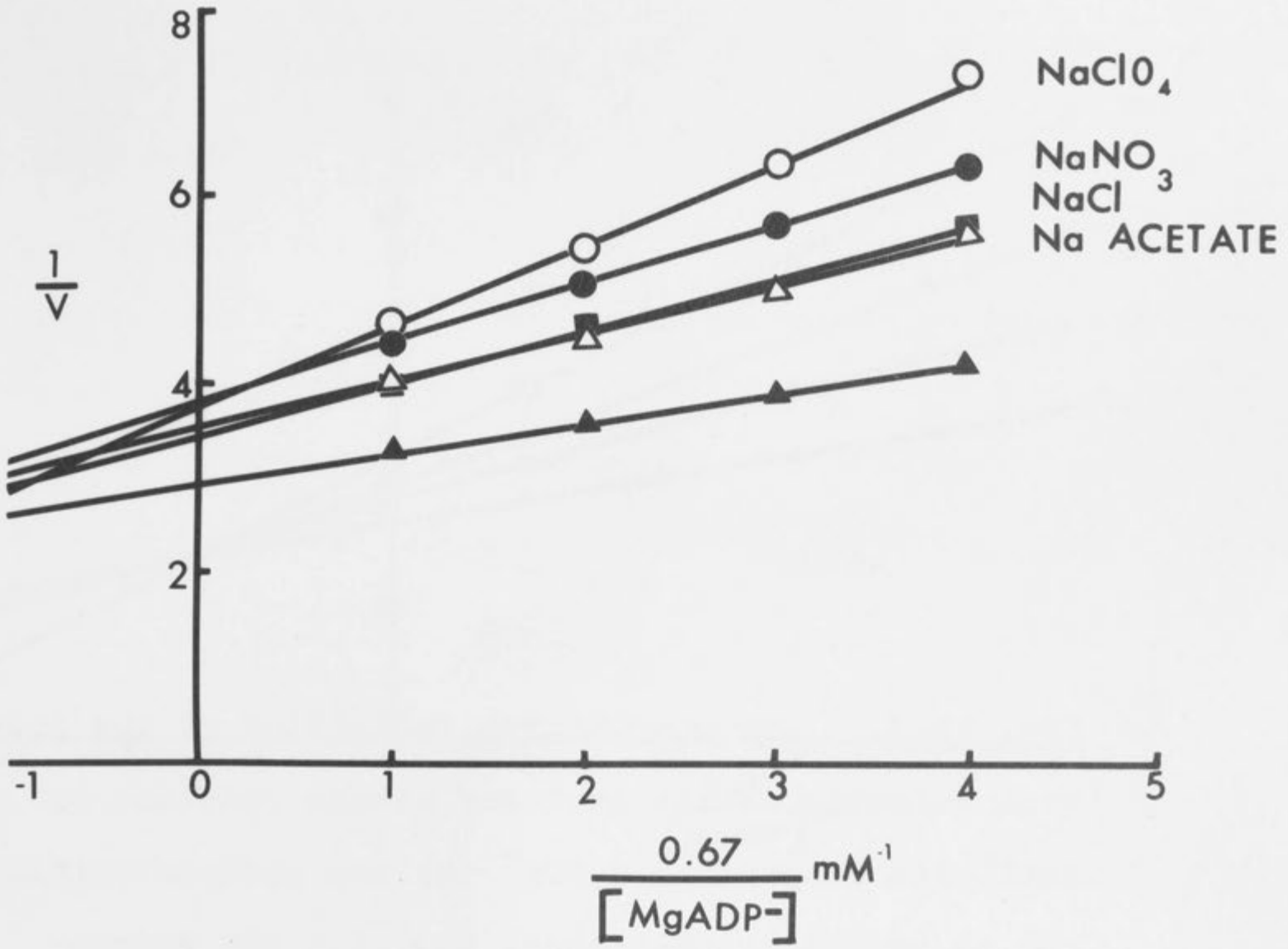
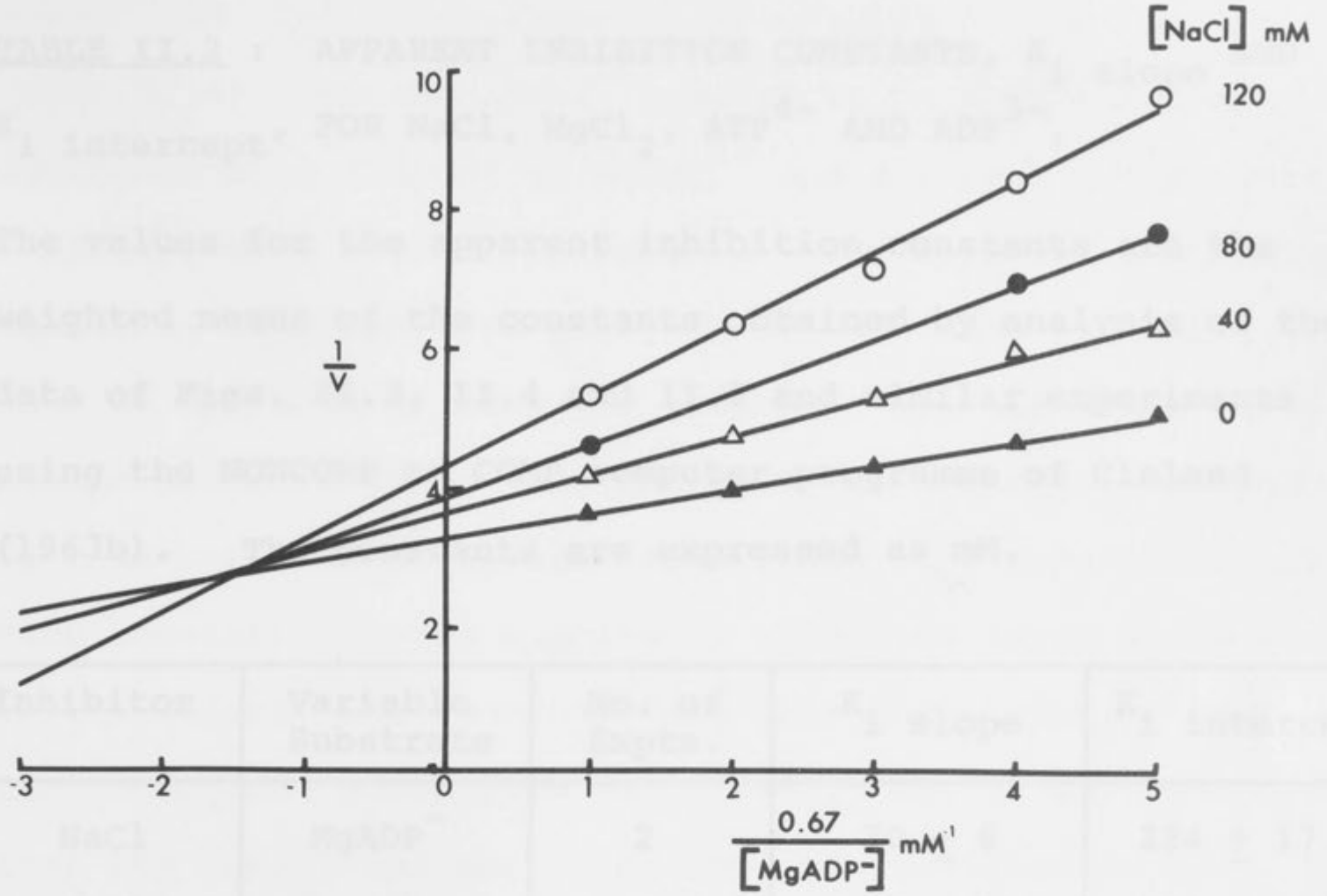



FIG. II.2. Inhibition of the initial velocity of the reaction catalysed by arginine kinase by 40 mM of NaCl, sodium acetate, $NaNO_3$ or $NaClO_4$ with respect to $MgADP^-$, the variable substrate, and with the concentrations of free phosphoarginine and $Mg^{2+}$ held constant at 5 mM and 1mM, respectively. The lines were drawn using kinetic constants obtained from analysis of the data by the HYPER computer programme (Cleland, 1963b). v is expressed as µmoles of arginine per µg of arginine kinase per min.

FIG. II.3. Non-competitive inhibition of the initial velocity of the arginine kinase reaction by NaCl with respect to $MgADP^-$ (a) and phosphoarginine (b) as variable substrates, and with the concentrations of free phosphoarginine (a) and $MgADP^-$ (b) held constant at 5 mM and 1.0 mM, respectively. The lines were drawn using the kinetic constants obtained from analysis of the data by the NONCOMP computer programme (Cleland, 1963b). v is expressed as μmoles of arginine per μg of arginine kinase per min.


[NaCl] mM
120
80
40
0
10
8
6
4
2
1/V
-3
-2
-1
0
1
2
3
4
5
0.67 / [MgADP⁻] mM⁻¹

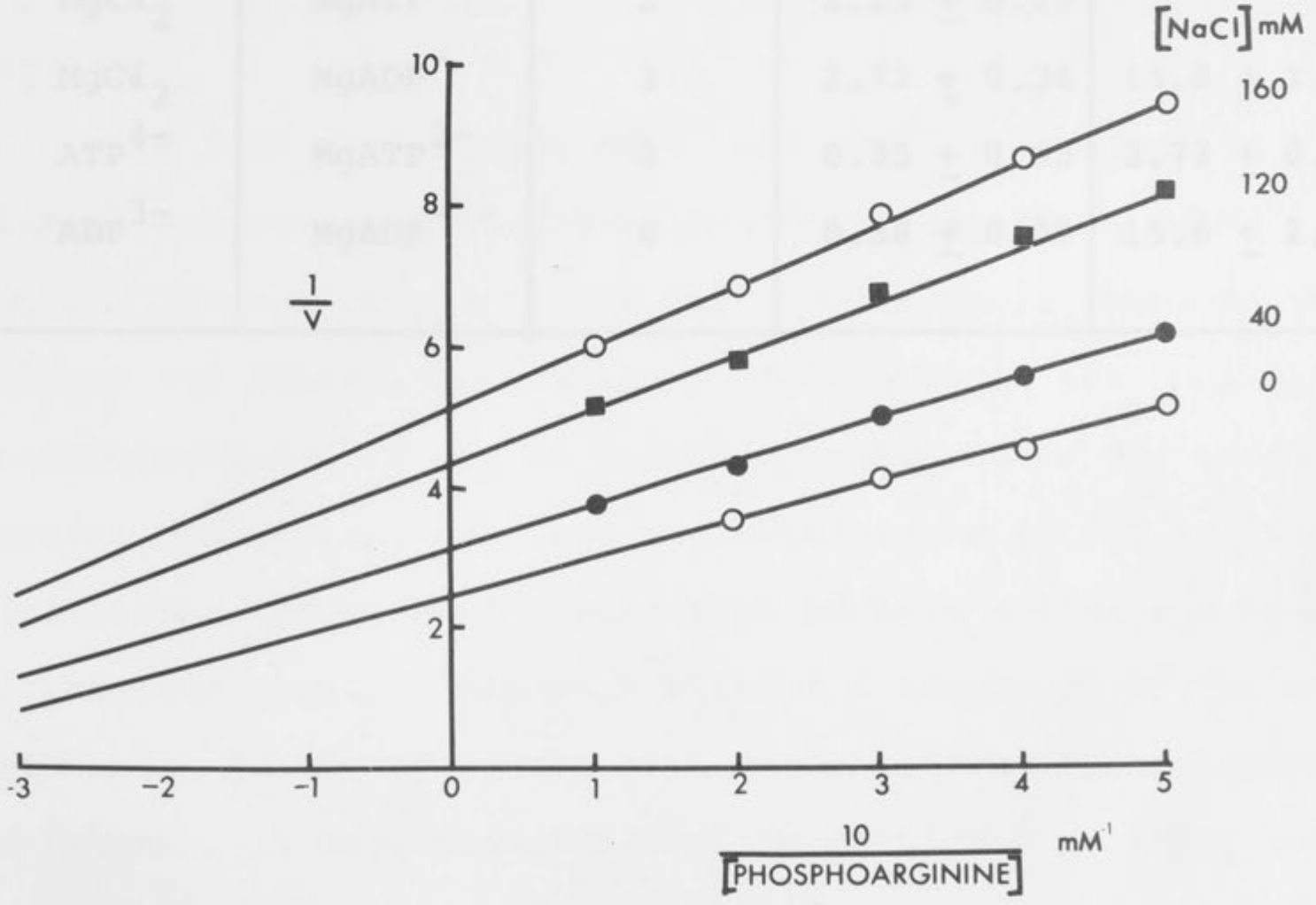

[NaCl] mM
160
120
40
0
10
8
6
4
2
1/V
-3
-2
-1
0
1
2
3
4
5
10 / [PHOSPHOARGININE] mM⁻¹

TABLE II.2 : APPARENT INHIBITION CONSTANTS, $K_{i\ slope}$ AND $K_{i\ intercept}$, FOR NaCl, $MgCl_2$, $ATP^{4-}$ AND $ADP^{3-}$.

The values for the apparent inhibition constants are the weighted means of the constants obtained by analysis of the data of Figs. II.3, II.4 and II.5 and similar experiments using the NONCOMP or COMP computer programme of Cleland (1963b). The constants are expressed as mM.

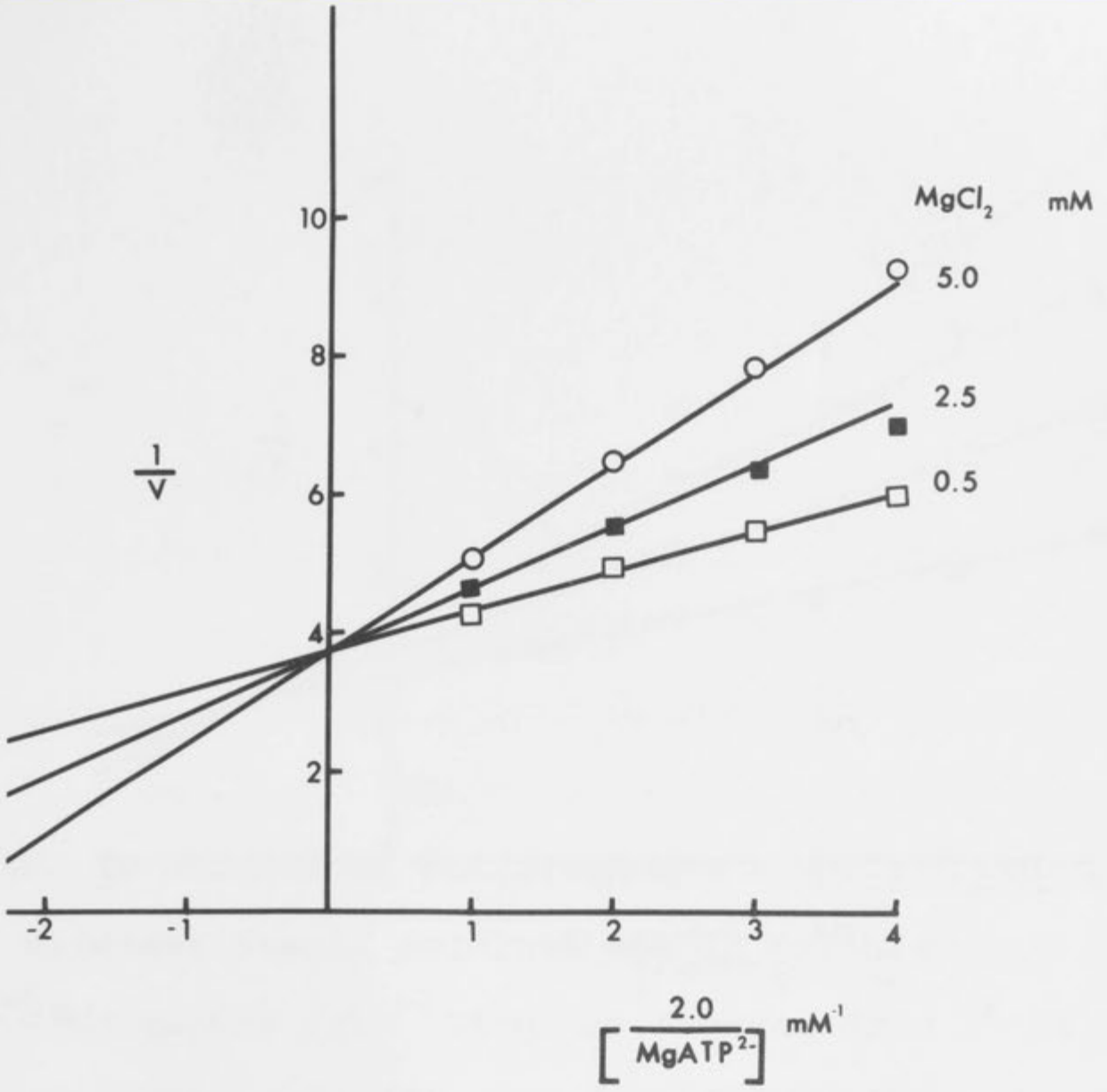
| Inhibitor | Variable Substrate | No. of Expts. | $K_{i\ slope}$ | $K_{i\ intercept}$ |
|---|---|---|---|---|
| NaCl | $MgADP^-$ | 2 | 70 ± 6 | 224 ± 17 |
| NaCl | Phospho-arginine | 2 | 200 ± 31 | 154 ± 10 |
| $MgCl_2$ | $MgATP^{2-}$ | 2 | 2.15 ± 0.28 | |
| $MgCl_2$ | $MgADP^-$ | 3 | 2.72 ± 0.34 | 15.8 ± 2.5 |
| $ATP^{4-}$ | $MgATP^{2-}$ | 3 | 0.35 ± 0.03 | 2.72 ± 0.21 |
| $ADP^{3-}$ | $MgADP^-$ | 4 | 0.58 ± 0.09 | 15.6 ± 2.3 |

$MgATP^{2-}$ and $MgADP^{-}$ as variable substrates. The results (Fig. II.4) show that $MgCl_2$ appears to be a linear competitive inhibitor with respect to $MgATP^{2-}$ and a linear non-competitive inhibitor with respect to $MgADP^{-}$. The apparent inhibition constants obtained from analysis of such data by either the COMP or the NONCOMP computer programme are listed in Table II.2. As the inhibitions by NaCl and $MgCl_2$ with respect to $MgADP^{-}$ were measured under similar conditions, the apparent inhibition constants may be compared : from the large difference in the values, and from the fact that the maximum concentration of $Cl^{-}$ added with $MgCl_2$ was 15 mM, it may be concluded that the inhibition resulting from the addition of $MgCl_2$ is caused largely if not entirely by $Mg^{2+}$.

Inhibition of arginine kinase by the free nucleotides, $ATP^{4-}$ and $ADP^{3-}$

The free nucleotides $ATP^{4-}$ and $ADP^{3-}$ were found to be linear non-competitive inhibitors with respect to $MgATP^{2-}$ and $MgADP^{-}$ respectively (Fig. II.5). Furthermore, because the slopes and intercepts are linear functions of the inhibitor concentrations, it may be concluded that, under the experimental conditions, the free nucleotides are acting as dead-end inhibitors rather than giving rise to less active enzyme-substrate complexes. Although without a knowledge of the reaction mechanism it is not possible to estimate true dissociation constants, it may, nevertheless, be concluded from the marked reduction in velocity caused by the presence of excess $ATP^{4-}$

FIG. II.4. Inhibition of the initial velocity of the arginine kinase reaction by $MgCl_2$ with $MgATP^{2-}$ (a) and $MgADP^-$ (b) as the variable substrates and the concentrations of free arginine (a) and free phosphoarginine (b) held constant at 10 mM and 5 mM, respectively. The lines were drawn using kinetic constants obtained by analysis of the data of (a) by the COMP and of (b) by the NONCOMP computer programmes (Cleland, 1963b). v is expressed as μmoles of ADP (a) and μmoles of arginine (b) per μg of arginine kinase per min.


MgCl2 mM
5.0
2.5
0.5
1/V
10
8
6
4
2
-2
-1
1
2
3
4
2.0 / [MgATP2-] mM-1

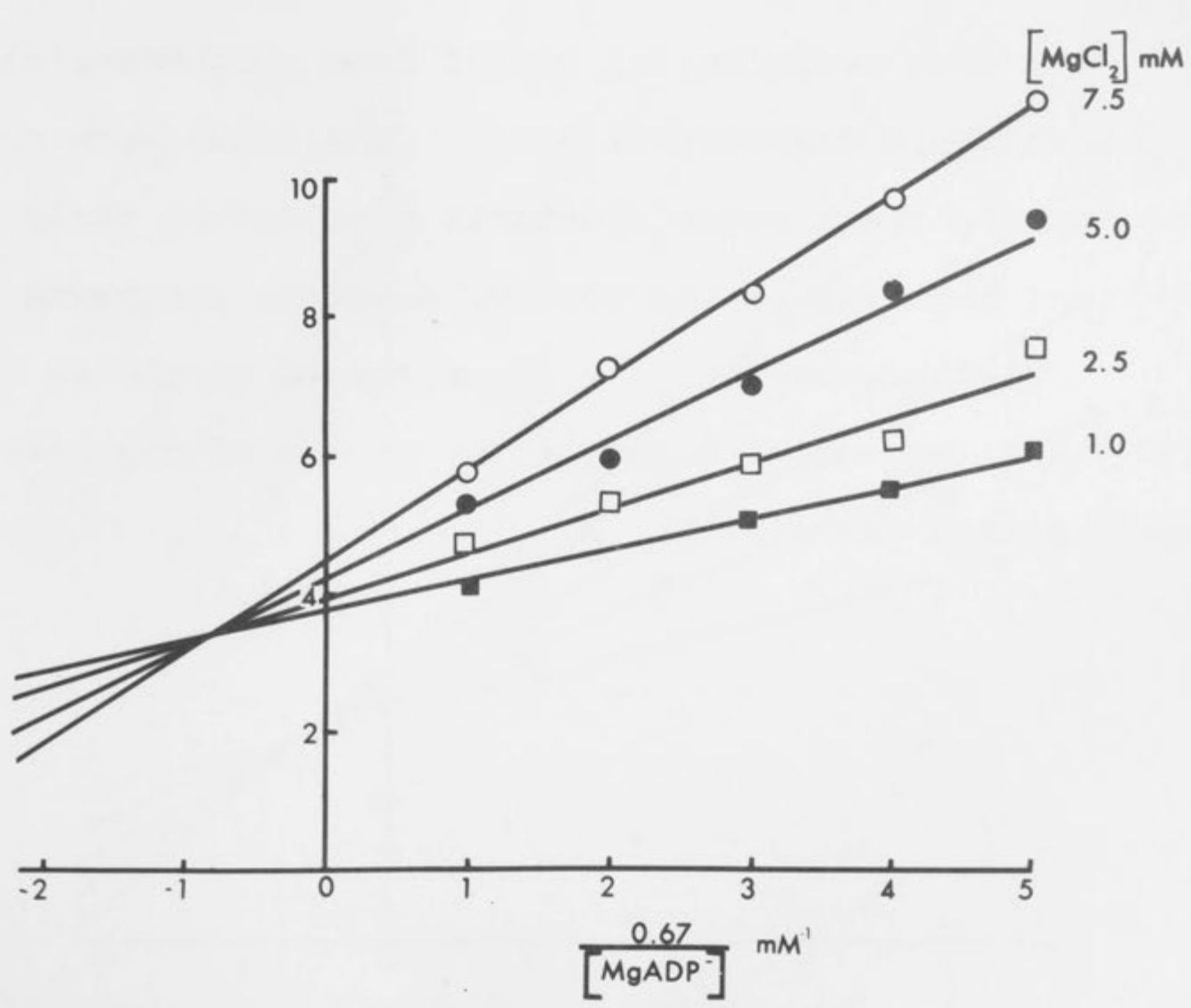
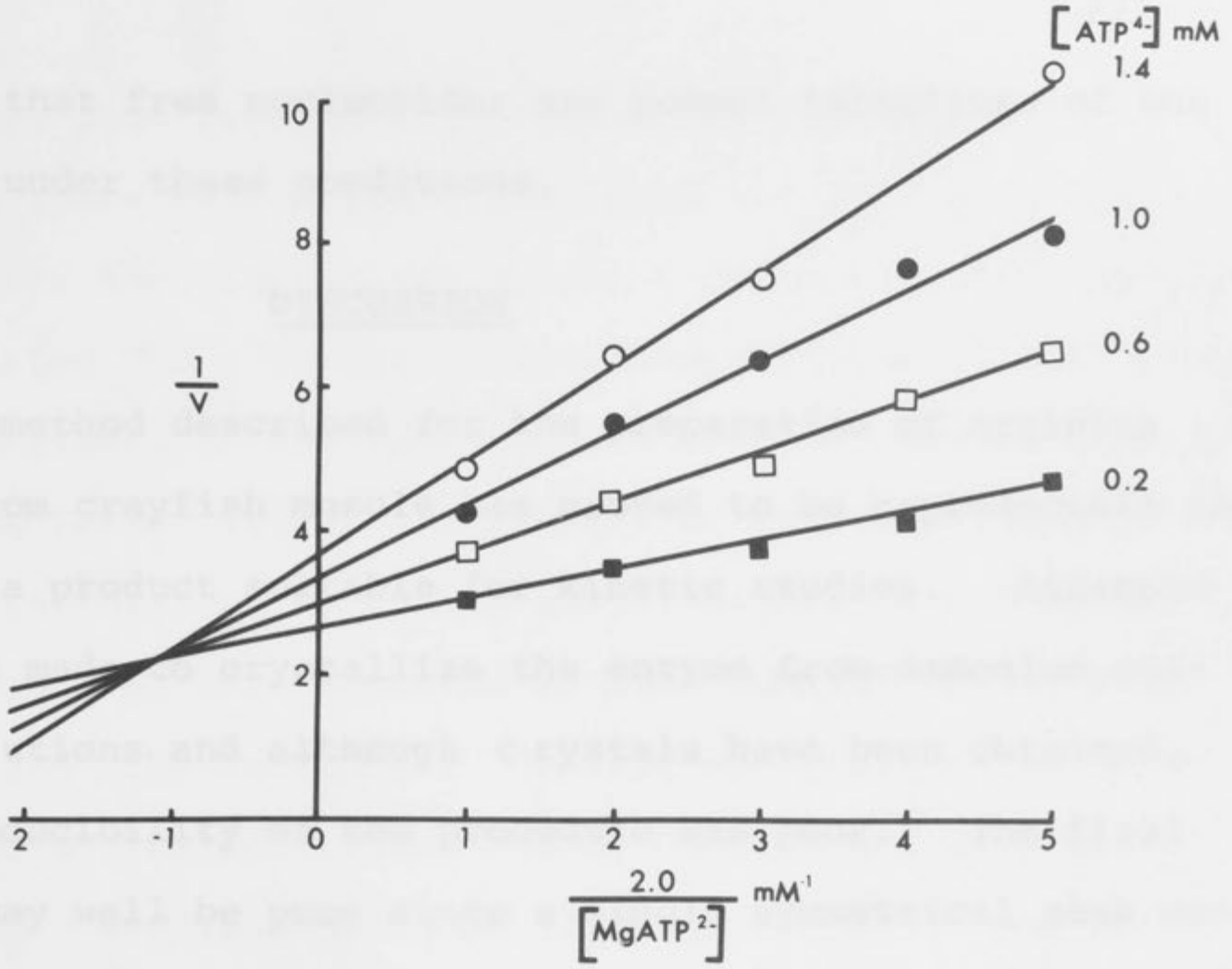
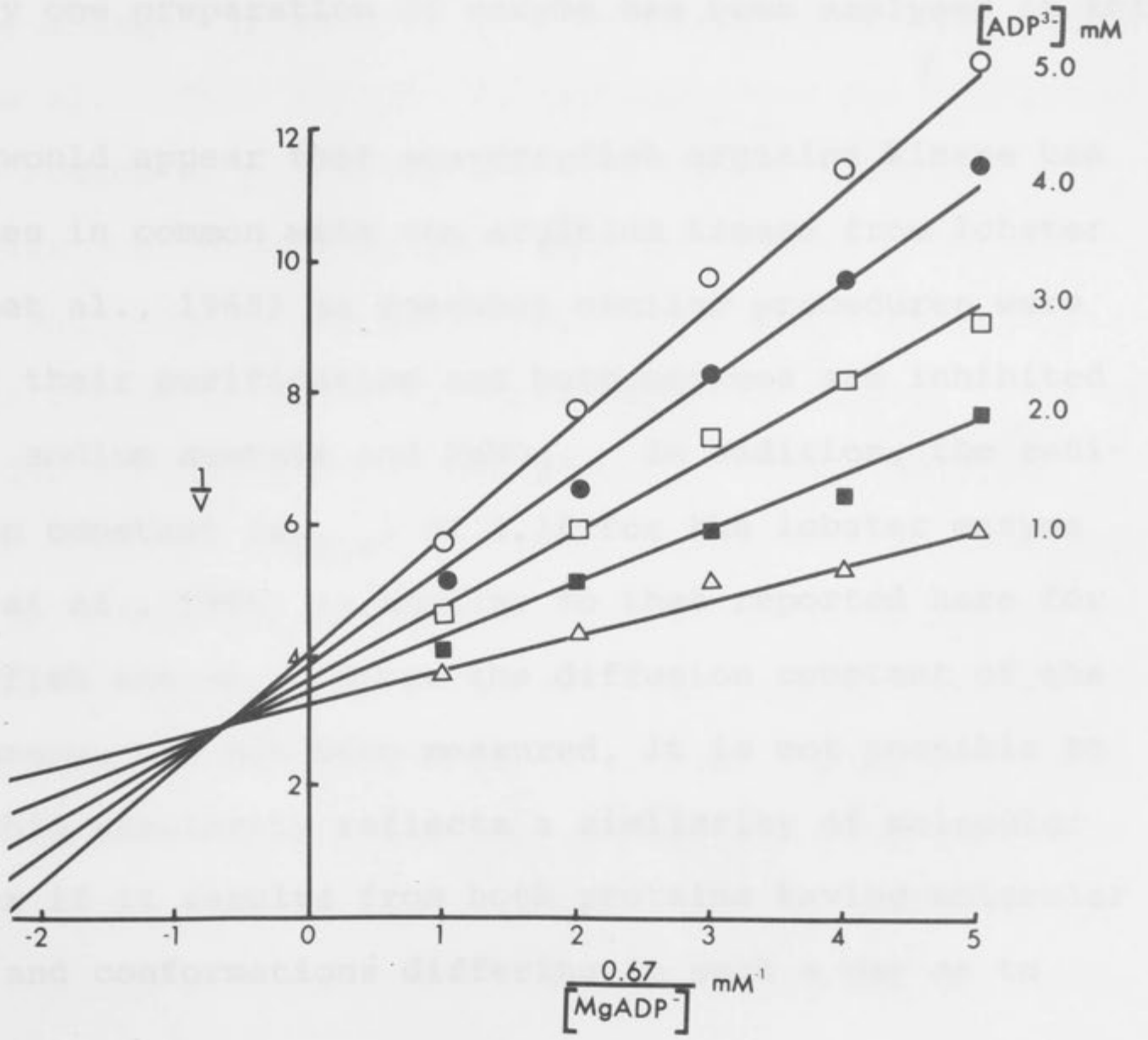

[MgCl2] mM
7.5
5.0
2.5
1.0
10
8
6
4
2
-2
-1
0
1
2
3
4
5
0.67 / [MgADP-] mM-1

FIG. II.5. Non-competitive inhibition of the initial velocity of the arginine kinase reaction by $ATP^{4-}$ with respect to $MgATP^{2-}$ (a) and by $ADP^{3-}$ with respect to $MgADP^{-}$ (b) and with the concentrations of free arginine (a) and of free phosphoarginine (b) held constant at 5 mM. The lines were drawn using the kinetic constants obtained by analysis of the data by the NONCOMP computer programme (Cleland, 1963b). v is expressed as μmoles of ADP (a) and of arginine (b) per μg of arginine kinase per min.


[ATP4-] mM
1.4
1.0
0.6
0.2
10
8
6
4
2
1/V
2
-1
0
1
2
3
4
5
2.0 / [MgATP2-] mM-1


[ADP3-] mM
5.0
4.0
3.0
2.0
1.0
12
10
8
6
4
2
1/V
-2
-1
0
1
2
3
4
5
0.67 / [MgADP-] mM-1

or $ADP^{3-}$ that free nucleotides are potent inhibitors of the reaction under these conditions.

## DISCUSSION

The method described for the preparation of arginine kinase from crayfish muscle has proved to be reproducible and to yield a product suitable for kinetic studies. Attempts have been made to crystallize the enzyme from ammonium sulphate solutions and although crystals have been obtained, the reproducibility of the procedure was poor. The final product may well be pure since a single symmetrical peak was obtained on ultracentrifugation, but it should be emphasised that only one preparation of enzyme has been analysed in this manner.

It would appear that sea-crayfish arginine kinase has properties in common with the arginine kinase from lobster (Virden et al., 1965) as somewhat similar procedures were used for their purification and both enzymes are inhibited by NaCl, sodium acetate and $NaNO_3$. In addition, the sedimentation constant ($s_{20,w}$) of 3.18 for the lobster enzyme (Virden et al., 1966) is similar to that reported here for the crayfish enzyme. Since the diffusion constant of the latter enzyme has not been measured, it is not possible to say if this similarity reflects a similarity of molecular weight or if it results from both proteins having molecular weights and conformations differing in such a way as to

produce the same sedimentation constant. It may also be noted that both these values are higher than the $s_{20}$ values of 2.5 reported for the crab enzyme (Elodi & Szorenyi, 1956) and of 2.69 reported for a lobster preparation (Pradel et al., 1964). But as mentioned in the Introduction to this Chapter (p.61), both these latter values are subject to some uncertainty.

The inhibitions by sodium salts, by $MgCl_2$, and by the free nucleotides were measured to find conditions suitable for detailed kinetic studies. While the addition of 40 mM of any one of the four salts - NaCl, sodium acetate, $NaNo_3$ and $NaClO_4$ - sufficed to cause noticeable inhibition, this inhibition was more marked with $NaNO_3$ or $NaClO_4$ than with NaCl or sodium acetate (Fig. II.2). The result resembles that of Virden et al. (1965) who found that $NaNO_3$ was more inhibitory than the sodium or potassium salts of $Cl^-$ or acetate.

It was mentioned when reporting the results of the inhibitions by NaCl, $MgCl_2$ and free nucleotides that the inhibition constants listed in Table II.2 are apparent values. Without a knowledge of the reaction mechanism it is not possible to ascertain their relationships to real dissociation constants. Nevertheless, the results are sufficient to allow suitable conditions to be chosen for the detailed kinetic studies. It could be concluded from a study of NaCl inhibition that the $Cl^-$ added as $MgCl_2$ would not be sufficient to influence the initial velocity of the reaction. While the presence in all reaction mixtures of 0.1 M triethanolamine-HCl buffer,

and hence of 40 mM $Cl^-$, would reduce the maximum velocities that could be achieved, it is important to use a cationic, non-chelating buffer and this strength of buffer was considered necessary to achieve constant pH and almost constant ionic strength in the presence of varying concentrations of substrates and products. Similar experimental conditions were chosen to determine the kinetic constants, which would be true values for this particular set of experimental conditions.

The marked inhibitions resulting from increasing concentrations of $Mg^{2+}$, $ADP^{3-}$ and $ATP^{4-}$ make it clear that in order to achieve experimental conditions of the minimum complexity, it is necessary to maintain low concentrations of these ionic species. Moreover, when the Mg-nucleotide complexes are used as the variable substrates, the concentration of either $Mg^{2+}$ or the free nucleotide must vary also. And since kinetic constants for both directions of the reaction may be compared most simply when the same experimental conditions are used, it seemed advisable to keep the concentration of $Mg^{2+}$ constant for all experiments. This means that the concentration of free nucleotide will vary in proportion to the concentration of the Mg-nucleotide complex, the actual concentrations depending on the concentration and apparent stability constant of the Mg-nucleotide complex as well as on the concentration of $Mg^{2+}$. The effect that this free nucleotide has on the reaction will depend on the relationship between the concentrations present and the true inhibition constants. While

these true constants are not known, it is apparent that both free nucleotides are strong inhibitors under the experimental conditions used for Fig. II.5. With $Mg^{2+}$ at 1 mM, the concentration of $ATP^{4-}$ will always be small because of the large apparent stability constant for $MgATP^{2-}$, and it may be calculated that if the highest concentration of $MgATP^{2-}$ is 3.0 mM, then the highest concentration of $ATP^{4-}$ will be 0.043 mM, a figure well below the values of the apparent inhibition constants. Because of the much lower value of the apparent stability constant for $MgADP^{-}$, at 1 mM $Mg^{2+}$ the concentration of $ADP^{3-}$ will always be one-quarter of the concentration of $MgADP^{-}$, so that if the highest concentration of $MgADP^{-}$ to be used in the initial velocity experiments is 0.5 mM, then the highest concentration of $ADP^{3-}$ will be 0.125 mM. Though this is considerably more than the concentration of $ATP^{4-}$, it is still sufficiently below the apparent inhibition constants to have little influence on the kinetic values for $MgADP^{-}$ that will be obtained from initial velocity experiments.

## SUMMARY

1. A simple and reproducible method is described for the preparation of arginine kinase from the tail muscle of the sea-crayfish, Jasus verreauxi.
2. The preparation appears homogeneous in the ultracentrifuge and is similar to the enzyme from the lobster Homarus vulgaris as regards sedimentation constant and inhibition

by NaCl, sodium acetate and $NaNO_3$.

3. While NaCl inhibits the reaction non-competitively with respect to $MgADP^-$ and phosphoarginine, the apparent inhibition constants are large enough to indicate that $MgCl_2$ is a suitable salt to use for detailed kinetic studies.

4. $Mg^{2+}$, $ATP^{4-}$ and $ADP^{3-}$ are potent inhibitors of the arginine kinase reaction when the Mg-nucleotide complexes are used as substrates. Therefore detailed kinetic studies are to be carried out with the concentration of $Mg^{2+}$ constant at 1 mM and with low concentrations of the free nucleotides.

# CHAPTER III

## INITIAL VELOCITY STUDIES OF THE REACTION CATALYSED BY ARGININE KINASE

# CHAPTER III

# INITIAL VELOCITY STUDIES OF THE REACTION CATALYSED BY ARGININE KINASE

## INTRODUCTION

Arginine kinase, prepared as described in Chapter II, has been used for a kinetic investigation of the reaction mechanism. In the first instance, initial velocity measurements in the absence of added product (referred to hereafter simply as initial velocity studies) were made to determine if the reaction mechanism were sequential or Ping-Pong. Such a differentiation is possible for a reaction with two substrates and two products because of the differing forms of the initial velocity equations (Cleland, 1963a). Before presenting the results of these studies, brief mention will be made of other kinetic work carried out with arginine kinase.

The only kinetic study that has been made of the reaction catalysed by arginine kinase is that by Virden, Watts and Baldwin (1965). Using enzyme prepared from extracts of muscle of the lobster, Homarus vulgaris, they measured the initial velocity of phosphoarginine formation as a function of the concentrations of $MgATP^{2-}$ and arginine, and the results, when plotted in double reciprocal form, consisted of families of straight lines intersecting to the left of the ordinate. Thus it may be concluded that the reaction has a sequential mechanism. But in the absence of product inhibition data, no further

conclusions can be reached as to the precise type of sequential mechanism, although the authors analysed their data on the basis that the reaction mechanism is rapid equilibrium, random.

The conditions chosen for the kinetic studies with arginine kinase were based on the experimental data reported in Chapter II, and velocities were determined as functions of the concentrations of the Mg-nucleotide complexes and of the free forms of the guanidino compounds. The results were consistent with a Ping-Pong mechanism, which means that the first substrate adds to, and the first product dissociates from the enzyme before the second substrate reacts. It follows that the reaction should be divisible into two partial reactions and this has been verified by demonstration of partial exchange reactions between ADP and ATP in the absence of the guanidino substrates and between arginine and phosphoarginine in the absence of the nucleotides. As these results suggest that a phosphorylated enzyme may form as an intermediate in the reaction sequence, a preliminary investigation of this possibility was undertaken, but the results are not conclusive. In addition, it has been found that increasing concentrations of the substrate phosphoarginine give rise to inhibition.

## EXPERIMENTAL

### Materials

ATP, ADP arginine, phosphoarginine and $MgCl_2$ were purchased and/or prepared as described in Chapter II p.64.

EDTA and DCTA were obtained as stated in Chapter I, p.40; solutions of both compounds were brought to pH 8.0 with N NaOH before use. N-ethylmorpholine and triethanolamine buffers were obtained and treated as described in Chapters I and II respectively (pp.40 & 64). Phosphocreatine and creatine kinase were prepared and stored as described in Chapter I, pp. 39 & 41, while arginine kinase was prepared as described in Chapter II, p.68.

[8-$^{14}$C]ATP-$Li_4$ (28 mC/mmole), [8-$^{14}$C]ADP-$Li_3$, both in 50% ethanol, and [$^{14}$C]L-arginine (240 mC/mmole) were obtained from Schwarz Bioresearch Inc. Before using the labelled nucleotides, the solutions were evaporated to dryness in a stream of dry air and reconstituted by the addition of water. This procedure was necessary to prevent the inactivation of the enzyme by ethanol (Morrison, Griffiths & Ennor, 1957). [$\gamma$-$^{32}$P]ATP (496 mC/mmole) was purchased as the tetraammonium salt from The Radiochemical Centre, Amersham, England.

Naphthelene was purchased from British Drug Houses, Ltd., 2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene, both Scintillation Grade were from the Packard Instrument Co., Illinois, U.S.A. and dioxane from Fluka. Sephadex G-25 (fine) was obtained from Pharmacia, Sweden; DEAE-cellulose paper (Whatman paper, DE-20) was made by W & R Balston Ltd., England.

## Methods

### Measurement of Initial Velocities

Detailed kinetic studies were made at 30° in 0.1 M

triethanolamine-HCl buffer (pH 8.0) in the presence of 0.01 mM EDTA. The Mg-nucleotide complexes and the free forms of the guanidino compounds were assumed to be the substrates, so that the reaction could be considered as being bireactant and written as :

$$MgATP^{2-} + arginine^{+} \rightleftharpoons MgADP^{-} + phosphoarginine^{-}$$

Thus it was the concentrations of the Mg-nucleotide complexes and the free forms of the guanidino substrates which were varied while the concentration of free $Mg^{2+}$ was maintained constant at 1.0 mM. The amounts of total $MgCl_2$ and total substrates required to give the desired concentrationsof the various ionic species in a total volume of 1.0 ml were calculated as described by Morrison et al. (1961), using the stability constants as listed in Chapter II, p.67. After all components except enzyme were added, the tubes were kept in ice until ready for use. Care was taken, however, to ensure that tubes containing ATP were not kept longer in ice than a few hours because some hydrolysis occurs. The tubes were incubated at 30° for 3 min. before reaction was initiated by addition of enzyme. The amount of enzyme added corresponded to 0.18 µg of protein per ml of reaction mixture. Initial velocities of the reaction in the forward and the reverse direction of the reaction as written above were determined by estimating the rate of ADP formation from ATP and the rate of arginine release from phosphoarginine, respectively, as described in Chapter II, p.68.

All experiments were run for two time periods to ensure that initial velocities were being measured.

Determination of the Equilibrium Constant for the Reaction

Reaction mixtures contained 0.1 M triethanolamine-HCl buffer (pH 8.0), 1.0 mM ATP, 5.0 mM ADP, 5.0 mM phosphoarginine and 21 µg of arginine kinase per ml, as well as various concentrations of $MgCl_2$. Equilibrium was established within 10 min. of the addition of enzyme, but samples (0.3 ml) were taken at 5 min. intervals between 10 and 30 min. to ensure that the amount of arginine released did not vary. To each sample was added 5.0 ml of 0.08 M DCTA (adjusted to pH 12 with NaOH) and 4.7 ml of water. After mixing, 0.7 ml samples were taken for the estimation of arginine (Rosenberg et al., 1956). The total concentrations of ATP, ADP and phosphoarginine present at equilibrium were calculated from the average value for the equilibrium concentration of arginine, as the concentration of arginine in the samples taken after 10 min. incubation did not vary by more than 2%. The equilibrium concentrations of $MgATP^{2-}$, $MgADP^{-}$ and free phosphoarginine were calculated by means of a computer programme which was used in conjunction with an IBM 1620 computer. For this purpose, the complexing between $Mg^{2+}$ and arginine was considered to be negligible.

Determination of Isotope Exchange Rates

The rates of the ADP-ATP and arginine-phosphoarginine

exchange were determined under the conditions described in the legend to Fig. III.4. The reaction was stopped by application of 50 µl samples to DEAE-cellulose paper which was used for the separation of the pairs of reactants. Details of the experimental technique and method of counting have been given by Morrison and Cleland (1966).

Investigation of the Formation of a Covalently-bound, phosphorylated intermediate

The method used was basically that described by Mourad and Parks (1965). 16.3 mg of arginine kinase were incubated in a volume of 2.0 ml with N-ethylmorpholine-HCl buffer (pH 8.0), 0.1 M; EDTA, 0.01 mM; [$\gamma$-$^{32}$P]ATP (40 mC/mmole), 1 mM; $MgCl_2$, 2 mM; and creatine kinase, 2.5 µM and phosphocreatine, 7 mM, to displace the equilibrium in the direction of phosphorylated-enzyme formation by converting the ADP formed to ATP. After 3 min. at 30°, reaction was initiated by the addition of the ATP, and after 4 min. at 30° was stopped by the addition of 0.2 ml of 0.2 M EDTA (pH 8.0). The protein was precipitated by the addition of solid ammonium sulphate to 95% saturation and redissolved in 1.0 ml of 0.1 M N-ethylmorpholine-HCl buffer (pH 8.0) containing 5 mM EDTA and, after repetition of the procedure, the solution was dialysed in two portions against the same buffer. One of these portions was chromatographed on G-25 (fine) Sephadex. The other was incubated with ADP and $MgCl_2$ to transfer any $^{32}$P attached to

the active site of the enzyme to ADP to reform [$\gamma$-$^{32}$P]ATP. After precipitation of the protein with ethanol, the nucleotides were separated by chromatography on DEAE-cellulose paper (Morrison & Cleland, 1966) and the radioactivity of the ATP compared with that of an enzyme sample incubated without ATP. The experimental details of the chromatography and the incubation with ADP are given in the legends to Fig. III.5 and Table III.3. Replacement of [$\gamma$-$^{32}$P]ATP in the reaction mixture by [8-$^{14}$C]ATP was used to determine the amount of non-specific binding of ATP to the enzyme.

Estimation of Radioactivity

Radioactivity was measured with a Packard Tri-Carb Scintillation Counter. Samples on DEAE-cellulose paper were immersed in a scintillation fluid with toluene as the solvent as described by Morrison and Cleland (1966). The efficiency of counting was 40% for $^{14}$C and 23% for $^{32}$P. Aqueous samples were mixed with a scintillation fluid composed of 100 g naphthelene, 10 g of 2,5-diphenyloxazole and 0.25 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 litre of dioxane. The overall efficiency of counting was 45% for $^{14}$C and 28% for $^{32}$P.

Analysis of Data

Kinetic data were analysed as described in Chapter I, p.43 using one or more of the programmes - HYPER, LINE, PARA, PING-PONG and SUBINH (preface, equations 1, 2, 3, 5, & 9).

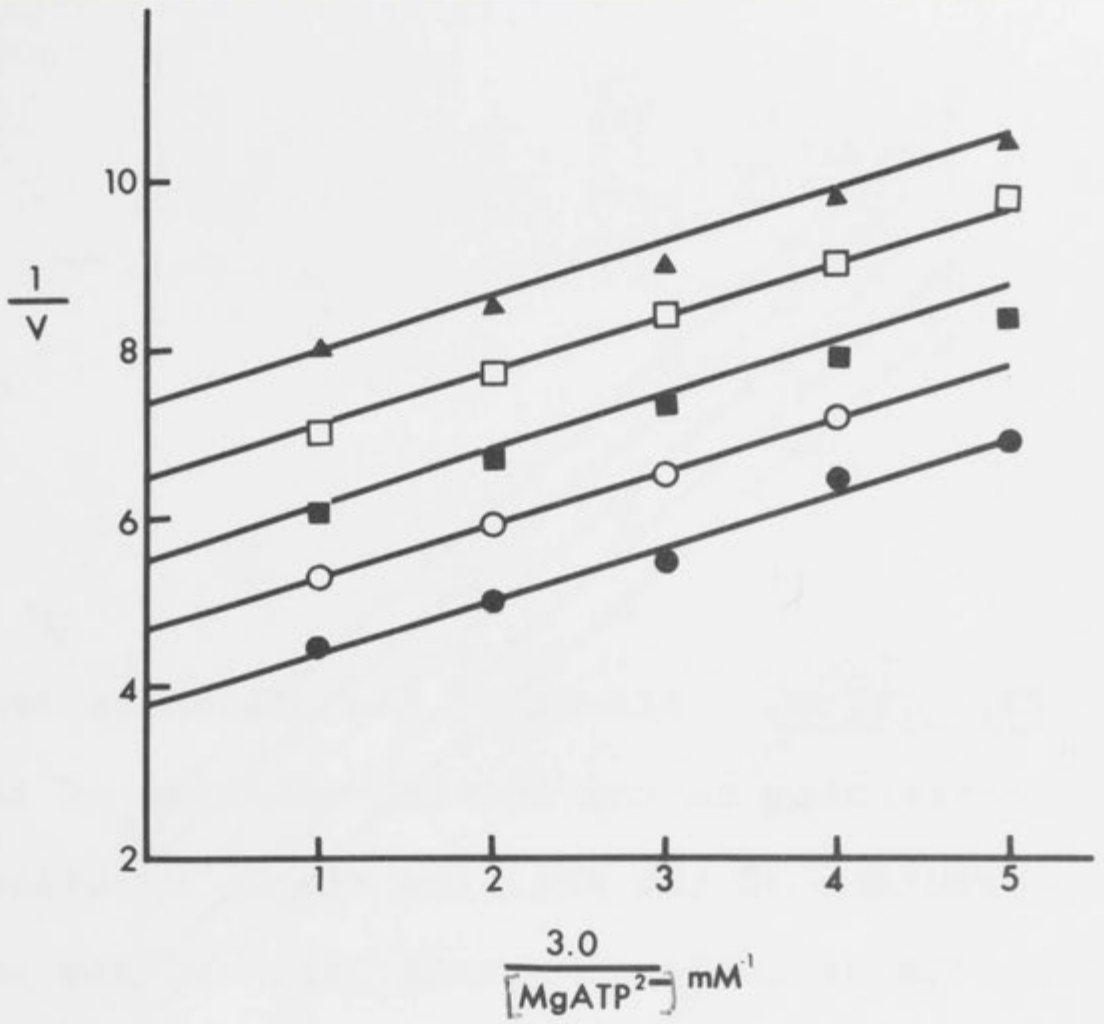
Illustrations have been prepared with the aid of constants obtained from the particular programmes as noted. Weighted mean values and their standard errors were calculated using the formulae given in Chapter I, p.44. The standard error of $K_{eq}$, calculated from the Haldane relationship, was determined by modifying the PING-PONG programme to obtain the standard errors of the products $K_a K_b$ and $K_p K_q$ by making allowance for their covariances and using the formulae for the standard errors of products and quotients given in Chapter I, pp.44 & 45. I am grateful to Dr. W. W. Cleland for making this modification to the PING-PONG computer programme.

## RESULTS

### Initial Velocity Studies

The effect of the concentration of $MgATP^{2-}$ on the initial velocity of the forward reaction at different fixed concentrations of the arginine is illustrated in the form of a double reciprocal plot. The same data are shown also plotted with arginine as the variable substrate at several fixed concentrations of $MgATP^{2-}$ (Fig. III.1). The results of similar investigations of the reverse reaction with $MgADP^{-}$ and phosphoarginine as variable substrates are shown in Fig. III.2. It is apparent that the slopes of the lines are independent of the concentration of the fixed substrate. Thus it may be concluded that the arginine kinase reaction

FIG. III.1. Effect of the concentration of arginine on the initial velocity of the forward direction of the arginine kinase reaction with $MgATP^{2-}$ as the variable substrate (a), and the effect of the concentration of $MgATP^{2-}$ on the initial velocity of the reaction with arginine as the variable substrate. The concentration of free $Mg^{2+}$ held constant at 1 mM. The lines are drawn using the kinetic constants obtained by analysis of the data by the PING-PONG computer programme (Cleland, 1963b). v is expressed as μmoles of ADP per μg of arginine kinase per min.


1/V
10
8
6
4
2
1
2
3
4
5
3.0 / [MgATP2−] mM-1

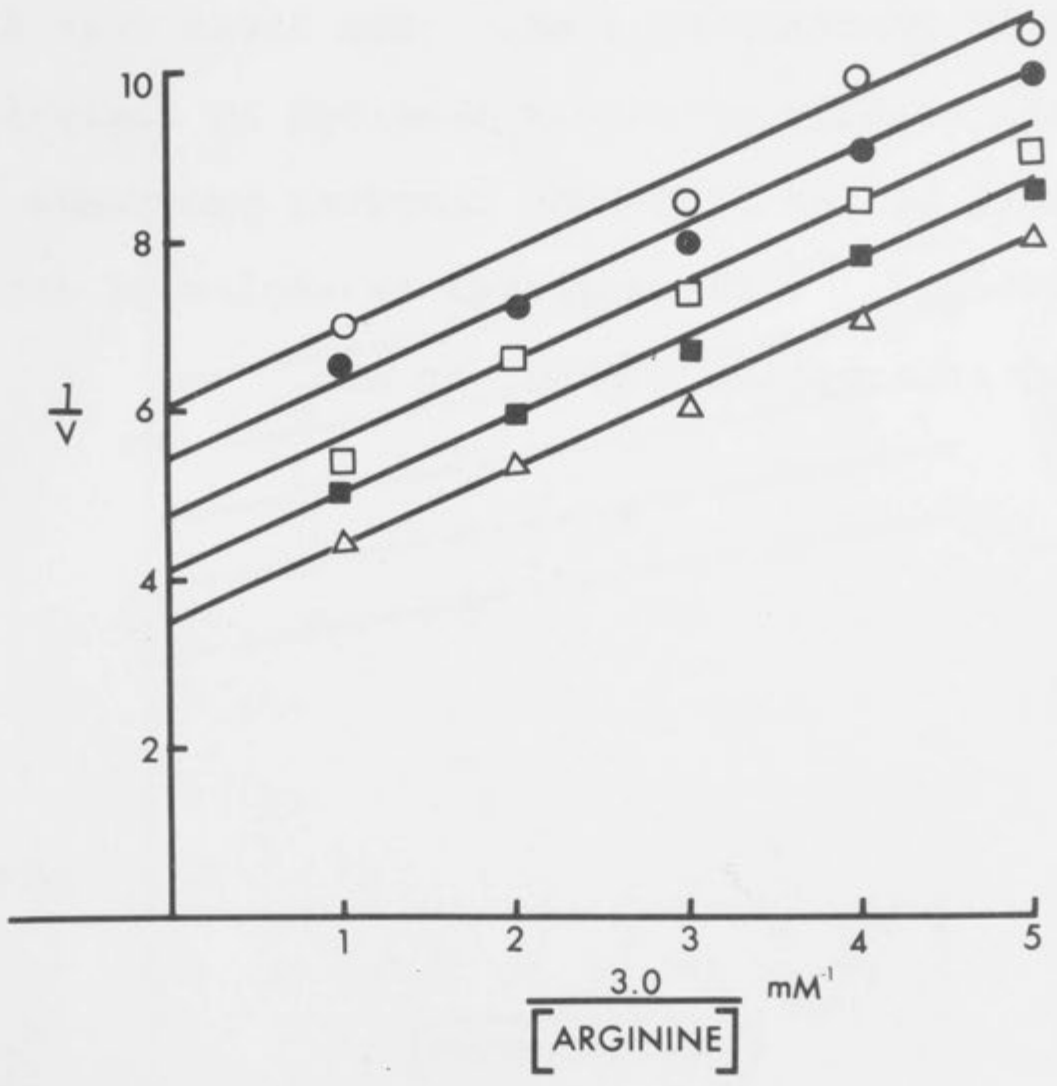
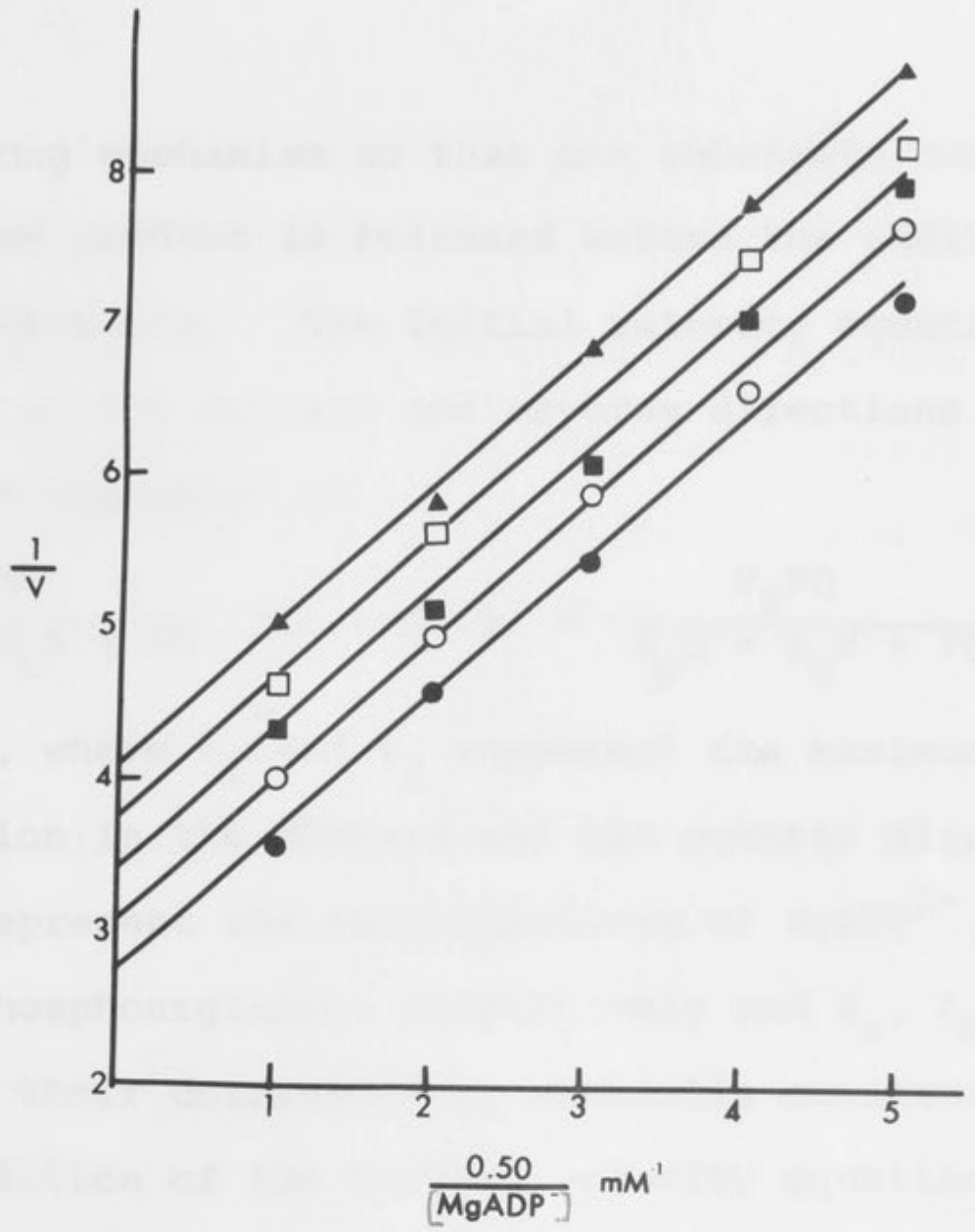
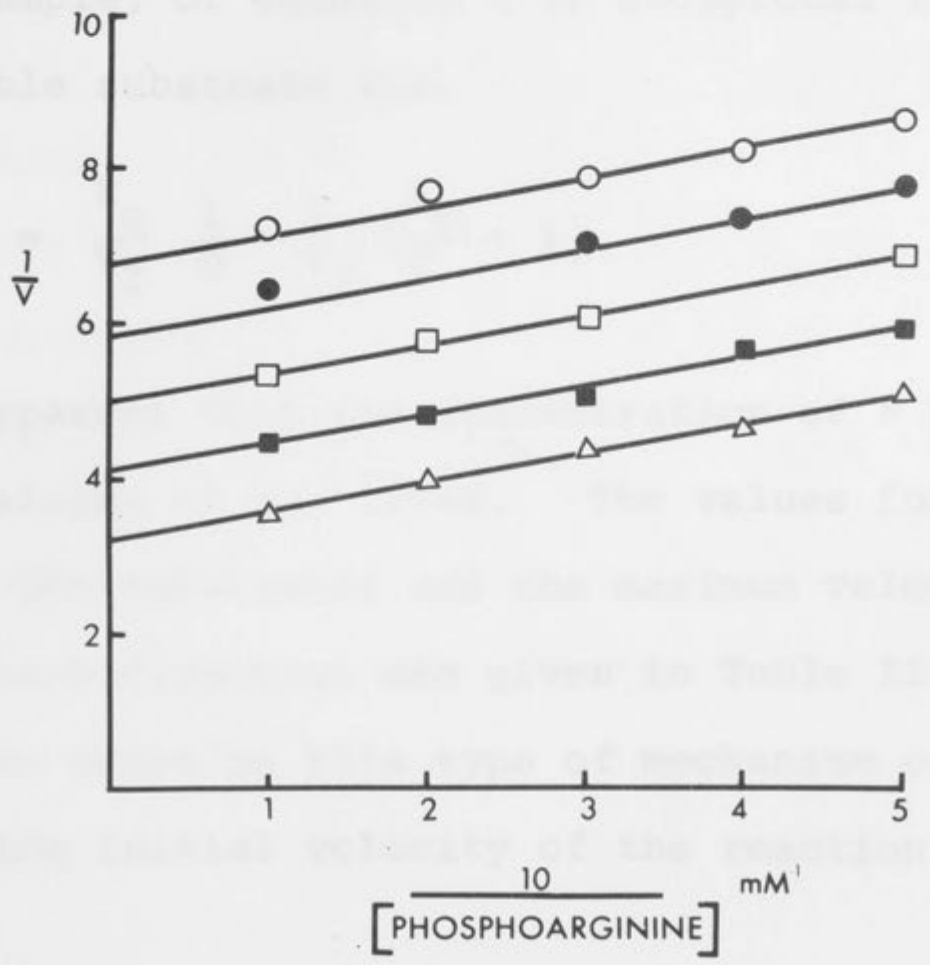

1/V
10
8
6
4
2
1
2
3
4
5
3.0 / [ARGININE] mM-1

FIG. III.2. Effect of the concentration of phosphoarginine on the initial velocity of the reverse direction of the arginine kinase reaction with $MgADP^-$ as the variable substrate (a), and the effect of the concentration of $MgADP^-$ on the initial velocity of the reaction with phosphoarginine as the variable substrate (b). The concentration of free $Mg^{2+}$ held constant at 1 mM. The lines were drawn using the kinetic constants obtained by analysis of the data by the PING-PONG computer programme (Cleland, 1963b). v is expressed as μmoles of arginine per μg of arginine kinase per min.


8
7
6
5
4
3
2
1
2
3
4
5
1/V
0.50
[MgADP⁻]
mM⁻¹


10
8
6
4
2
1
2
3
4
5
1/V
10
[PHOSPHOARGININE]
mM⁻¹

has a Ping-Pong mechanism so that one substrate adds to the enzyme and one product is released before the addition of the second substrate. The initial velocity equations for the reaction in the forward and reverse directions may, therefore, be expressed as

$$v = \frac{V_1 AB}{K_a B + K_b A + AB} \quad (1) \text{ and } \quad v = \frac{V_2 PQ}{K_p Q + K_q P + PQ} \quad (2)$$

respectively, where $V_1$ and $V_2$ represent the maximum velocities of the reaction in the forward and the reverse direction; A, B, P and Q represent the concentrations of $MgATP^{2-}$, arginine, $MgADP^{-}$ and phosphoarginine, respectively and $K_a$, $K_b$, $K_p$ and $K_q$ represent their corresponding Michaelis constants. That such a formulation of the initial velocity equations is in accord with the experimental results may be seen by re-arrangement, for example, of equation 2 in reciprocal form with Q as the variable substrate viz.

$$\frac{1}{v} = \frac{K_q}{V_2} \frac{1}{Q} + \frac{1}{V_2} \left(\frac{K_p}{P} + 1\right) \quad (3)$$

when it is apparent that the concentration of P has no influence on the slopes of the lines. The values for the Michaelis constants of the substrates and the maximum velocities of the reaction in each direction are given in Table III.1.

A further check on this type of mechanism can be made by studying the initial velocity of the reaction as a function

TABLE III.1. KINETIC PARAMETERS OF THE ARGININE KINASE REACTION AS DETERMINED FROM INITIAL VELOCITY STUDIES

The values for the Michaelis constants and maximum velocities are weighted means of the values obtained by analysis of the results of three experiments, including those of Fig. III.1 and III.2 using the modified Ping-Pong computer programme of Cleland (1963b).

| Substrate | Michaelis Constant (mM) | Maximum Velocity (μmoles/min/μg of enzyme) |
|---|---|---|
| **Forward Reaction** | | |
| $MgATP^{2-}$ | $0.64 \pm 0.03$ ($K_a$) | $0.341 \pm 0.006$ ($V_1$)* |
| Arginine | $0.87 \pm 0.04$ ($K_b$) | |
| **Reverse Reaction** | | |
| $MgADP^{-}$ | $0.17 \pm 0.005$ ($K_p$) | $0.359 \pm 0.002$ ($V_2$)* |
| Phosphoarginine | $1.60 \pm 0.06$ ($K_q$) | |

* From the same experimental data, values for the relationships $\frac{K_a K_b}{(V_1)^2}$ and $\frac{K_p K_q}{(V_2)^2}$ were determined to be $4.52 \pm 0.35$ and $1.86 \pm 0.15$.

of the concentration of one substrate while maintaining constant the ratio of the two substrates. If the ratio of P:Q is held equal to x, then equation 3 becomes

$$\frac{1}{v} = \left\{ \frac{K_q + \frac{K_p}{x}}{V_2} \right\} \frac{1}{Q} + \frac{1}{V_2} \qquad (4)$$

Under these conditions, a plot of 1/v against 1/Q would yield a straight line with a vertical intercept corresponding to the reciprocal of the maximum velocity. Such a plot is obtained when $MgADP^-$ and phosphoarginine are varied in constant ratio as shown in Fig. III.3. The maximum velocity was determined to be 0.439 $\pm$ 0.006 µmoles per min. per µg of enzyme.

Calculation and Determination of the Equilibrium Constant of the Reaction

The equilibrium constant ($K_{eq}$) of the reaction was calculated from the Haldane relationship,

$$K_{eq} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_p K_q}{K_a K_b}$$

which is one of four applicable to a Ping-Pong reaction (Cleland, 1963a). Substitution of the values for

$$\frac{K_a K_b}{(V_1)^2} \quad \text{and} \quad \frac{K_p K_q}{(V_2)^2}$$

(Table III.1) into this relationship gave a value of 0.41 $\pm$ 0.05. For comparative purposes, the experimental value for

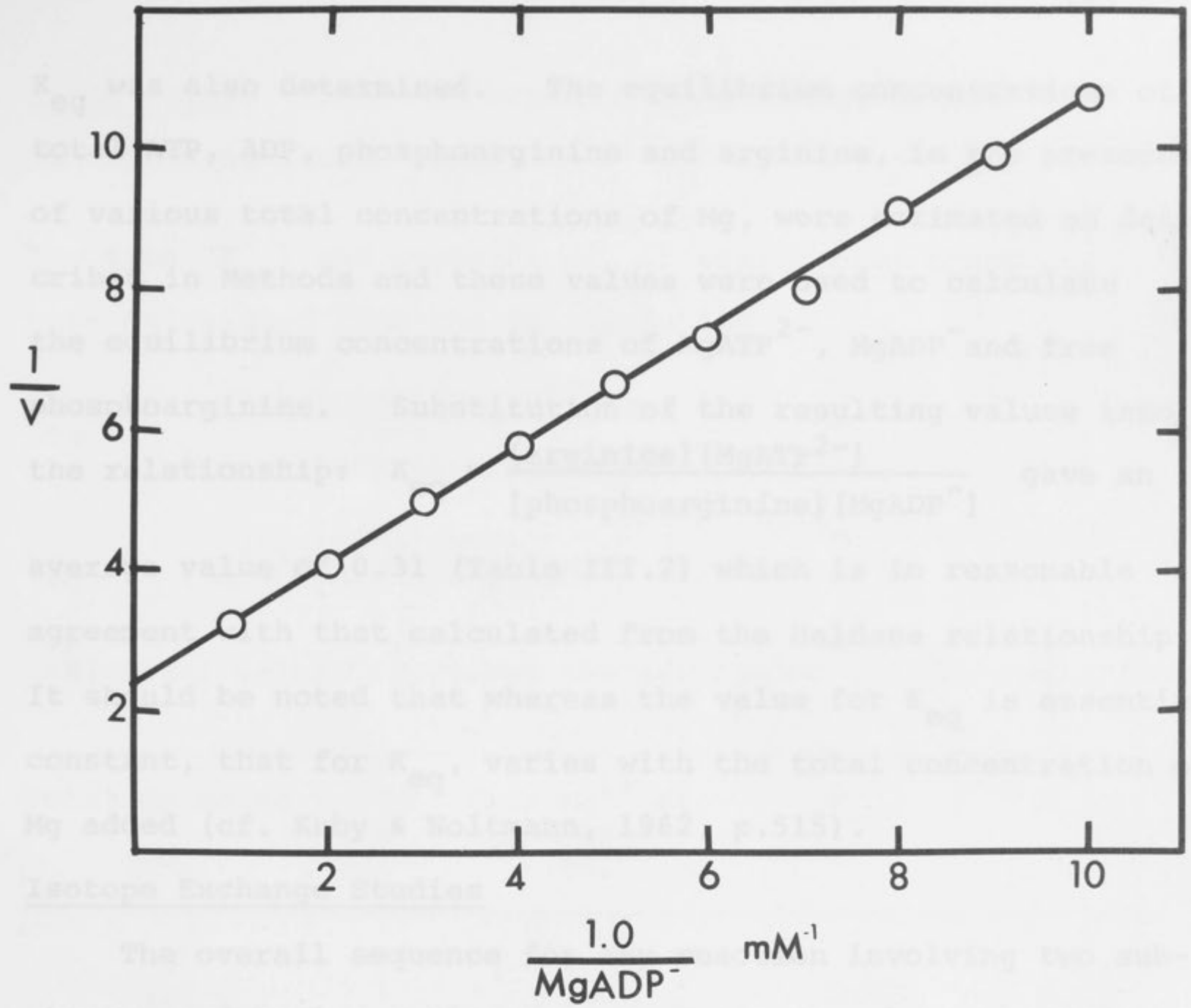



FIG. III.3. A plot of the reciprocals of the initial velocities of the reverse direction of the arginine kinase reaction against the reciprocals of the concentrations of $MgADP^-$ with the ratio of the concentration of phosphoarginine to the concentration of $MgADP^-$ held constant at 10 : 1, and the concentration of free $Mg^{2+}$ held constant at 1 mM. The data were analysed with the HYPER computer programme (Cleland, 1963b). v is expressed as μmoles of arginine per μg of arginine kinase per min.

$K_{eq}$ was also determined. The equilibrium concentrations of total ATP, ADP, phosphoarginine and arginine, in the presence of various total concentrations of Mg, were estimated as described in Methods and these values were used to calculate the equilibrium concentrations of $MgATP^{2-}$, $MgADP^{-}$ and free phosphoarginine. Substitution of the resulting values into the relationship: $K_{eq} = \frac{[\text{arginine}][MgATP^{2-}]}{[\text{phosphoarginine}][MgADP^{-}]}$ gave an average value of 0.31 (Table III.2) which is in reasonable agreement with that calculated from the Haldane relationship. It should be noted that whereas the value for $K_{eq}$ is essentially constant, that for $K_{eq}$, varies with the total concentration of Mg added (cf. Kuby & Noltmann, 1962, p.515).

Isotope Exchange Studies

The overall sequence for any reaction involving two substrates and having a Ping-Pong mechanism may be expressed as the sum of two partial reactions. A set of possible partial reactions for the arginine kinase reaction is

$$E + MgATP^{2-} \rightleftharpoons E\text{-}P + MgADP^{-} \qquad (a)$$

and

$$E\text{-}P + \text{arginine} \rightleftharpoons E + \text{phosphoarginine} \qquad (b)$$

where E represents the free form of enzyme and E-P a second stable form of enzyme to which the phosphoryl group (P) is not necessarily bound covalently (Boyer, 1960). If such reactions do occur, then it should be possible to demonstrate nucleotide exchange in the absence of guanidino substrates

TABLE III.2. TRUE AND APPARENT EQUILIBRIUM CONSTANTS FOR THE ARGININE KINASE REACTION

The true ($K_{eq}$) and apparent ($K_{eq}'$) equilibrium constants were calculated from the relationships :

$$K_{eq} = \frac{[\text{arginine}]_{\text{free}}[MgATP^{2-}]}{[\text{phosphoarginine}][MgADP^{-}]} \quad \text{and}$$

$$K_{eq}' = \frac{[\text{arginine}][ATP]}{[\text{phosphoarginine}][ADP]}$$

Individual values represent the average of two determinations.

| Total Mg added (mM) | Free $Mg^{2+}$ at equilibrium (mM) | $K_{eq}'$ | $K_{eq}$ |
|---|---|---|---|
| 4.0 | 0.12 | 0.82 | 0.29 |
| 6.0 | 0.61 | 0.47 | 0.32 |
| 8.0 | 1.93 | 0.41 | 0.31 |
| | | | Average value for $K_{eq} = 0.31$ |

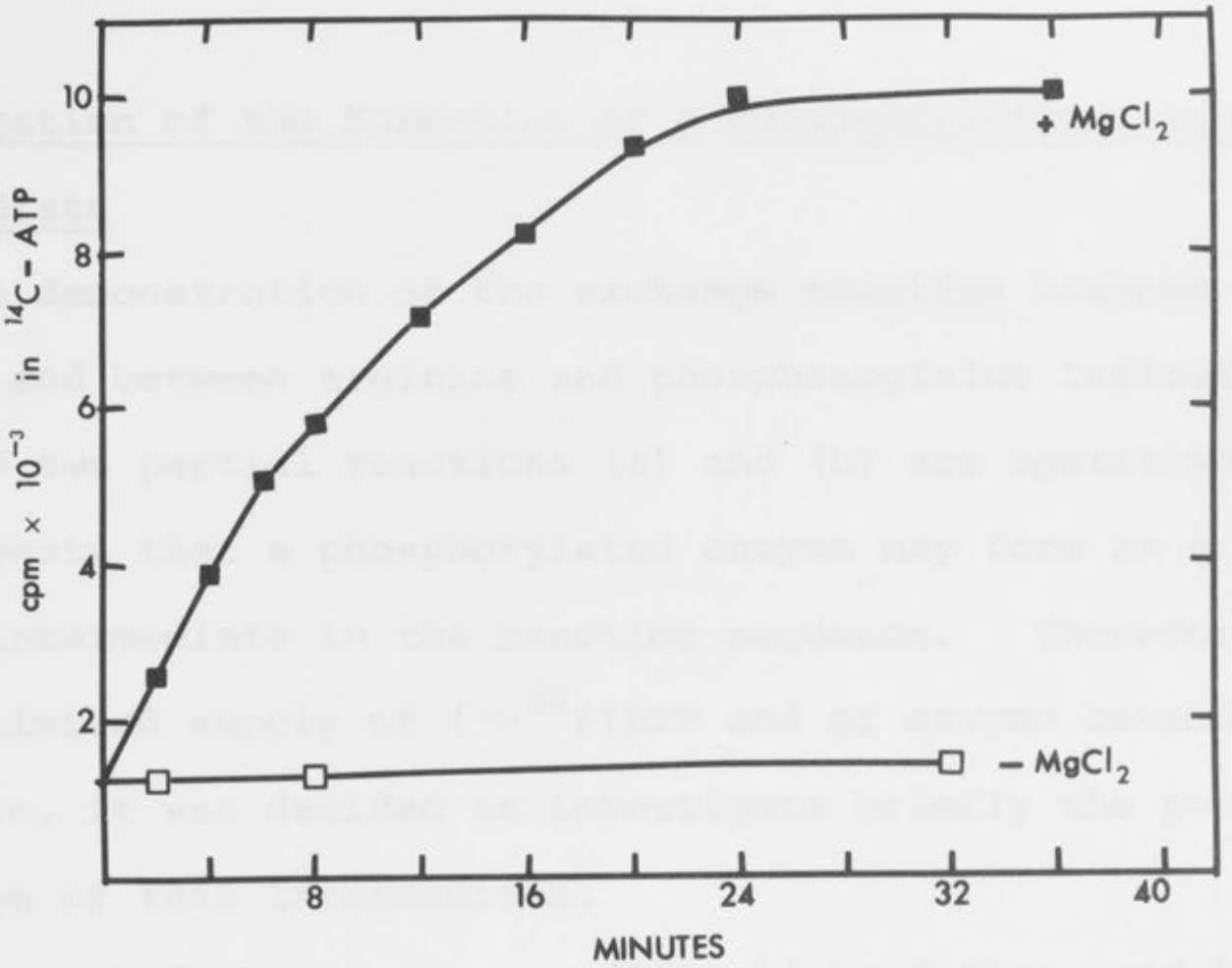
and guanidino exchange in the absence of nucleotides. Incubation of ATP and $^{14}$C-ADP with the enzyme gave rise to the incorporation of label into ATP while incubation of phosphoarginine and $^{14}$C-arginine with the enzyme resulted in the formation of labelled phosphoarginine. The approach to equilibrium of these reactions is illustrated in Fig. III.4. It might be noted that the curves do not pass through the origin. This is because of the presence of $^{14}$C-ATP in the $^{14}$C-ADP sample and of a radioactive contaminant in the $^{14}$C-arginine which could not be removed from DEAE-cellulose paper by washing. Qualitative evidence was obtained for the ATP-ADP exchange also.

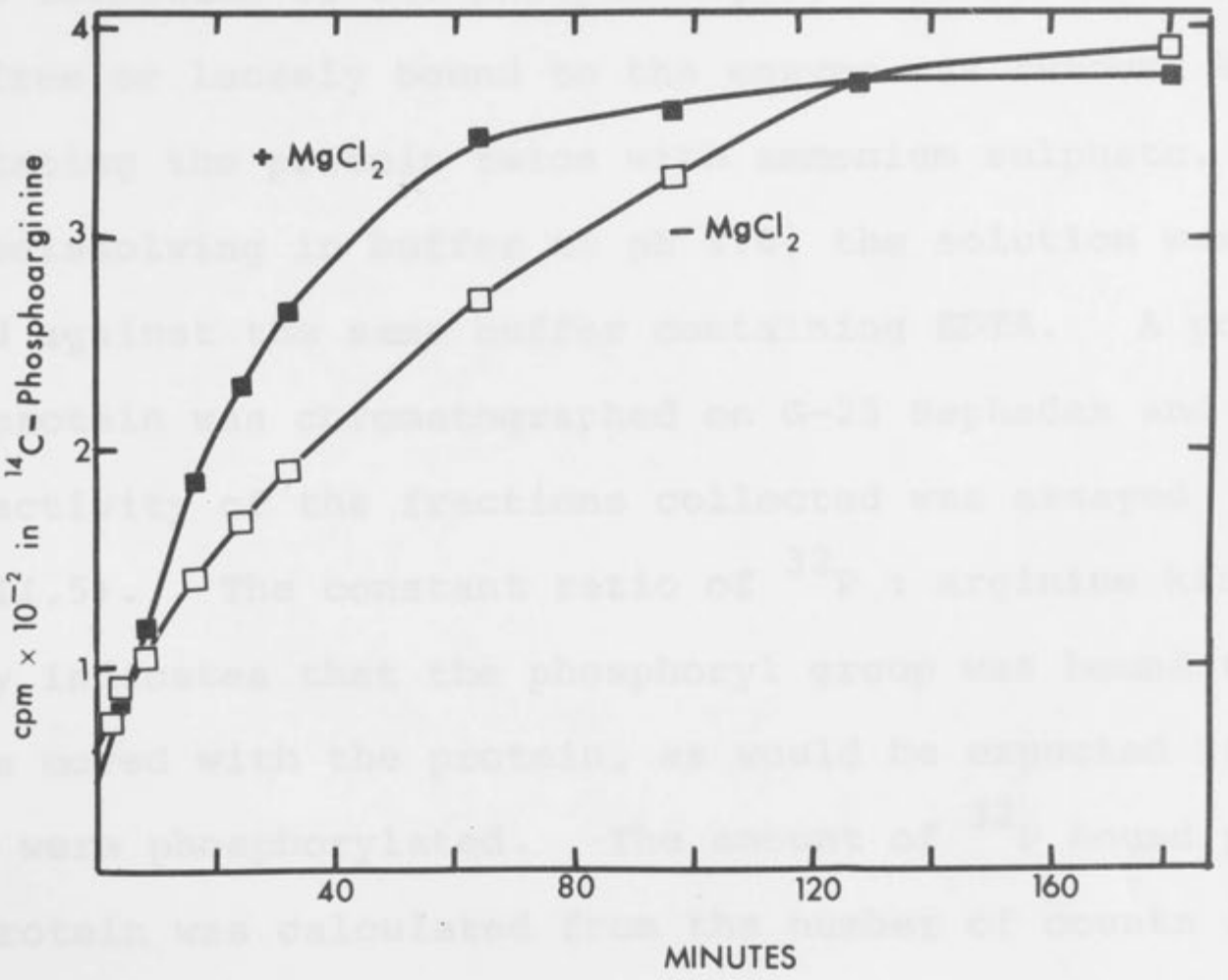
When $Mg^{2+}$ was omitted from the reaction medium, there was a marked reduction in the initial rate of the ADP-ATP exchange (Fig. III.4a) while the arginine-phosphoarginine exchange rate was affected only to a small extent (Fig. III.4b). The latter exchange was not inhibited by concentrations of EDTA or DCTA up to 0.03 mM, but both compounds at a concentrations of 0.1 mM produced a time-dependent inhibition of the exchange. Treatment of phosphoarginine with Chelex-100 ($Na^+$ form), together with replacement of triethanolamine with N-ethylmorpholine, which had been purified by distillation, did not result in a reduction of the arginine-phosphoarginine exchange rate in the absence of added metal ion.

FIG. III.4. Incorporation of label from [$^{14}$C]ADP into ATP in the absence of guanidino substrates (a) and the incorporation of label from [$^{14}$C]arginine into phosphoarginine in the absence of nucleotide substrates (b).

(a) The reaction mixture contained in a final volume of 0.5 ml : triethanolamine-HCl buffer (pH 8.0), 50 µmoles; EDTA, 5 mµmoles; ATP, 0.25 µmole; [8-$^{14}$C]ADP, 0.2 µC (6.7 mµmoles); arginine kinase, 3.6 µg and either with or without $MgCl_2$, 0.5 µmoles. Incorporation is expressed as counts per min. in [$^{14}$C]ATP per µg of arginine kinase.

(b) The reaction mixture contained in a final volume of 0.5 ml : triethanolamine-HCl buffer (pH 8.0), 50 µmoles; EDTA, 5 mµmoles; phospho-arginine, 0.5 µmole; [$^{14}$C]L-arginine, 0.2 µC (0.83 mµmole); arginine kinase, 36 µg and either with or without $MgCl_2$, 0.5 µmole. Incorporation is expressed as counts per min. in [$^{14}$C]phospho-arginine per µg of arginine kinase.


10
8
6
4
2
cpm × 10⁻³ in ¹⁴C – ATP
+ MgCl₂
– MgCl₂
8
16
24
32
40
MINUTES

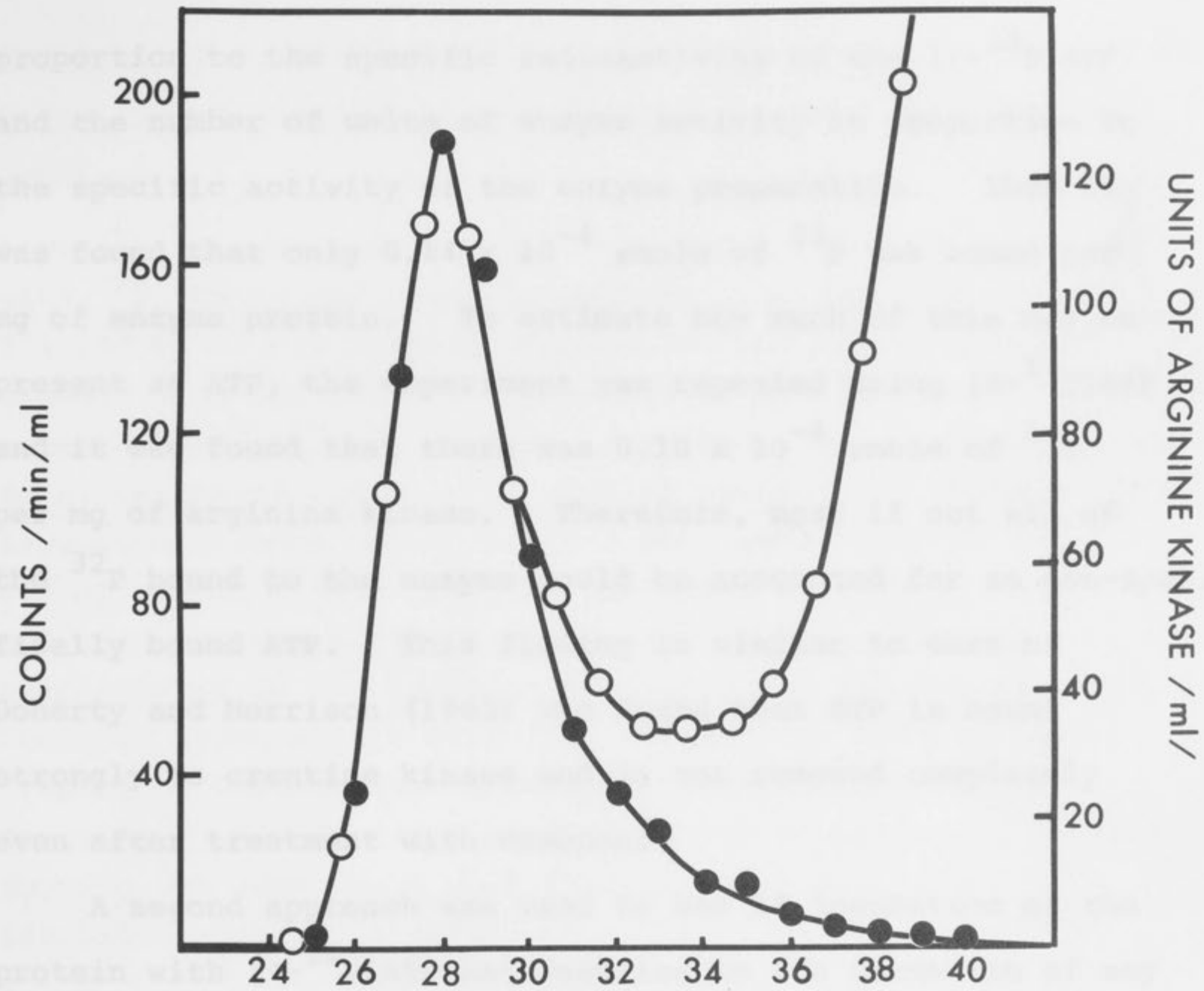

4
3
2
1
cpm × 10⁻² in ¹⁴C – Phosphoarginine
+ MgCl₂
– MgCl₂
40
80
120
160
MINUTES

Investigation of the Formation of a Phosphorylated-enzyme intermediate

The demonstration of the exchange reaction between ADP and ATP and between arginine and phosphoarginine indicates that the two partial reactions (a) and (b) are operative, and suggests that a phosphorylated enzyme may form as a stable intermediate in the reaction sequence. Therefore, when a limited supply of [$\gamma$-$^{32}$P]ATP and of enzyme became available, it was decided to investigate briefly the possible formation of this intermediate.

The method chosen was a modification of that used by Mourad and Parks (1965) to isolate the phosphoryl-enzyme intermediate in the reaction catalysed by nucleoside diphospho-kinase. Enzyme was incubated with [$\gamma$-$^{32}$P]ATP at pH 8.0 to allow formation of the phosphoenzyme, and then the ATP either free or loosely bound to the enzyme was removed by precipitating the protein twice with ammonium sulphate. After redissolving in buffer at pH 8.0, the solution was dialysed against the same buffer containing EDTA. A portion of the protein was chromatographed on G-25 Sephadex and the enzyme activity of the fractions collected was assayed (Fig. III.5). The constant ratio of $^{32}$P : arginine kinase activity indicates that the phosphoryl group was bound to, and thus moved with the protein, as would be expected if the protein were phosphorylated. The amount of $^{32}$P bound per mg of protein was calculated from the number of counts in

FIG. III.5. Chromatography of arginine kinase on G-25 Sephadex, after reaction of the enzyme with radioactive ATP. 0.8 ml of the arginine kinase that had been incubated with [$^{32}$P]ATP as described in METHODS, was dialysed against 2 x 250 ml of 0.1 M N-ethylmorpholine buffer (pH 8.0) containing 5 mM EDTA (5) for 2½ hours. The protein was then applied to the top of a G-25 (fine) Sephadex column (53 x 2.2 cm), which had been equilibrated previously with 0.1 M N-ethylmorpholine buffer (pH 8.0) containing 5 mM EDTA, and this same buffer was used to elute. The flow rate was 60 ml per hour. Fractions of 1.9 ml (60 drops) were collected using a Gilson Medical Electronics Linear Fractionator equipped with a Drop Counter-Timer combination unit connected to an ultraviolet absorption meter (Model UV-265IF) attached to a Model RR Texas Instruments Recorder. Samples of 0.22 ml were taken from each fraction for radioactivity assay. The enzyme activity of the fractions were tested as described for fractions collected during the enzyme purification (Chapter II, p. 66).


COUNTS /min/ml
200
160
120
80
40
UNITS OF ARGININE KINASE /ml/
120
100
80
60
40
20
24
26
28
30
32
34
36
38
40
TUBE NO

proportion to the specific radioactivity of the [$\gamma$-$^{32}$P]ATP and the number of units of enzyme activity in proportion to the specific activity of the enzyme preparation. Thus it was found that only $0.14 \times 10^{-4}$ µmole of $^{32}$P was bound per mg of enzyme protein. To estimate how much of this may be present as ATP, the experiment was repeated using [8-$^{14}$C]ATP and it was found that there was $0.10 \times 10^{-4}$ µmole of $^{14}$C per mg of arginine kinase. Therefore, most if not all of the $^{32}$P bound to the enzyme could be accounted for as non-specifically bound ATP. This finding is similar to that of Doherty and Morrison (1963) who found that ATP is bound strongly to creatine kinase and is not removed completely even after treatment with charcoal.

A second approach was used to see if incubation of the protein with [$\gamma$-$^{32}$P]ATP had resulted in the formation of any phosphorylated enzyme. To this end, another portion of the incubated and precipitated protein was dialysed against buffer free from EDTA and then incubated in the presence and absence of ADP, to allow any $^{32}$P-phosphoenzyme to form free enzyme by transferring the phosphoryl group back to ADP, with the formation of [$\gamma$-$^{32}$P]ATP. Similar experiments were carried out with enzyme that had been incubated with [8-$^{14}$C]ATP rather than [$\gamma$-$^{32}$P]ATP. After precipitation of the protein with ethanol, the ATP in the supernatant solutions was chromatographed and the radioactivity of the ATP spot determined (Table III.3). The presence of radioactive ATP in

TABLE III.3. INVESTIGATION OF THE FORMATION OF A PHOSPHORYLATED ENZYME INTERMEDIATE BY FORMING ATP FROM PHOSPHOENZYME AND ADP.

Arginine kinase (6 mg. in 0.45 ml) which had been incubated with $^{32}$P ATP as described in Methods, was dialysed against 2 x 250 ml of 0.1 M N-ethylmorpholine-HCl buffer (pH 8.0) without EDTA for 2.5 hr. Then 0.2 ml was incubated in the same buffer with 0.01 mM EDTA, 2 mM $MgCl_2$ and 1 mM ADP in a total volume of 0.4 ml. A similar quantity of protein was incubated without ADP. After 4 min. incubation at 30°, 0.1 ml of 0.1 M EDTA (pH 8.0) was added to stop the reaction, and then ADP was added to the control tube. Protein was precipitated by the addition of 0.25 ml of 95% ethanol, and then the supernatants with cold carrier ATP were chromatographed on DEAE-cellulose paper to isolate the ATP for counting. Non-specific binding of ATP was measured by performing the experiment with arginine kinase that had been incubated with 8-$^{14}$C ATP.

| | Radioactive form of ATP with which the arginine kinase was incubated. | | | |
|---|---|---|---|---|
| | [$^{32}$P]ATP | | [8-$^{14}$C]ATP | |
| phosphorylated enzyme incubated with ADP | + | - | + | - |
| mg. protein per assay | 2.8 | 3.1 | 1.1 | 1.1 |
| µmoles x $10^3$ radioactive ATP in supernatant | 1.05 | 0.74 | 0.15 | 0.13 |
| µmoles x $10^3$ radioactive ATP per mg. protein | 0.38 | 0.24 | 0.13 | 0.12 |

samples that had not been incubated first with [$\gamma$-$^{32}$P]ATP and then with ADP indicates that ATP must have remained with the protein throughout the ammonium sulphate precipitations and dialysis and then been released when the protein was denatured with ethanol, As this amount of ATP is more than 10 times greater than the ATP present after passage down the Sephadex column (Fig. III.5), presumably it represents ATP that is non-specifically adsorbed and dissociates slowly from the protein. In addition to this ATP, there is a further amount present after incubation with ADP, so that it would seem reasonable to conclude that this is formed by the reaction of the ADP with a phosphorylated form of the enzyme; this suggests that a phosphorylated enzyme is an intermediate in the reaction catalysed by arginine kinase. It will be noticed, however, that the amount of ATP formed, and therefore the amount of phosphorylated enzyme, is small when compared with the total amount of arginine kinase present, indicating that such a phosphorylated enzyme must be unstable with very little surviving the 4 hour time lapse that occurs during the experimental period.

Substrate Inhibition by Phosphoarginine

The early experiments designed to demonstrate the arginine-phosphoarginine exchange were carried out with phosphoarginine at a concentration of 10 mM. This concentration was used with the object of forcing the enzyme into the E-P

form for reaction with labelled arginine and to increase the number of counts in phosphoarginine at equilibrium when the specific activity of phosphoarginine and arginine would be equal. It was difficult, however, to demonstrate any exchange. Because it was possible to show the exchange with a lower concentration of phosphoarginine (1.0 mM, Fig. III.4), it appeared likely that higher concentrations of phosphoarginine may give rise to substrate inhibition; that is, the phosphoarginine may bind not only to the E form of the enzyme but also to the E-P form to yield a dead-end complex.

The above considerations prompted an investigation of the effect of a range of phosphoarginine concentrations on the initial velocity of the reaction at a fixed concentration of $MgADP^-$. Fig. III.6 shows that at relatively high concentrations of phosphoarginine there is a marked inhibition of the reaction. Analysis of these data by means of the SUBINH computer programme (Cleland, 1963b) gave an apparent inhibition constant of 14.7 $\pm$ 2.9 mM, but it should be noted that the overall fit is not good and that this is especially true in the region of highest velocities. It appeared that this discrepancy might be due to the formation of more than one phosphoarginine dead-end complex. In order to investigate the nature of this substrate inhibition and to obtain true inhibition constants, the initial velocity of the reaction was measured with $MgADP^-$ as the variable substrate at increasing concentrations of phosphoarginine. The fixed

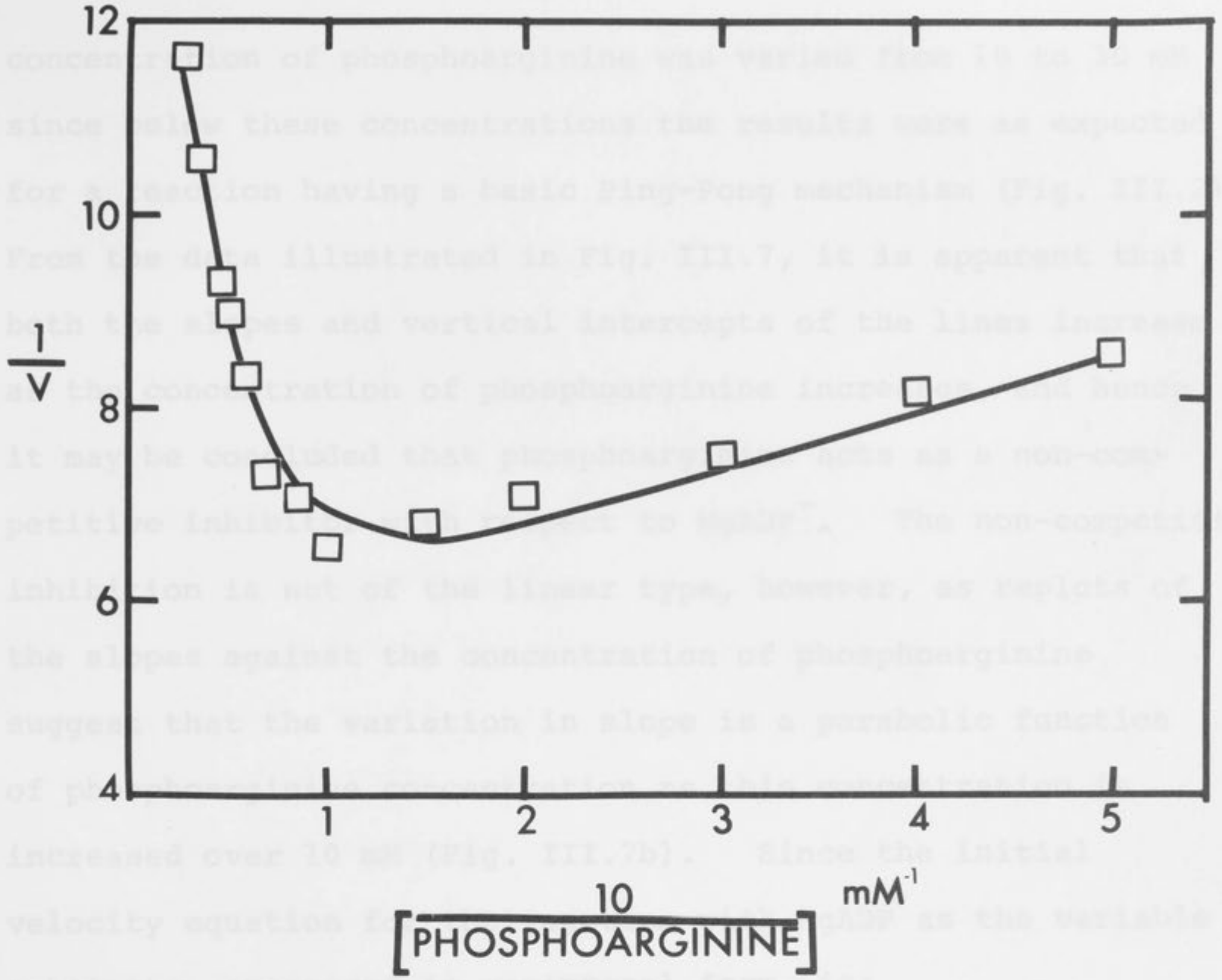


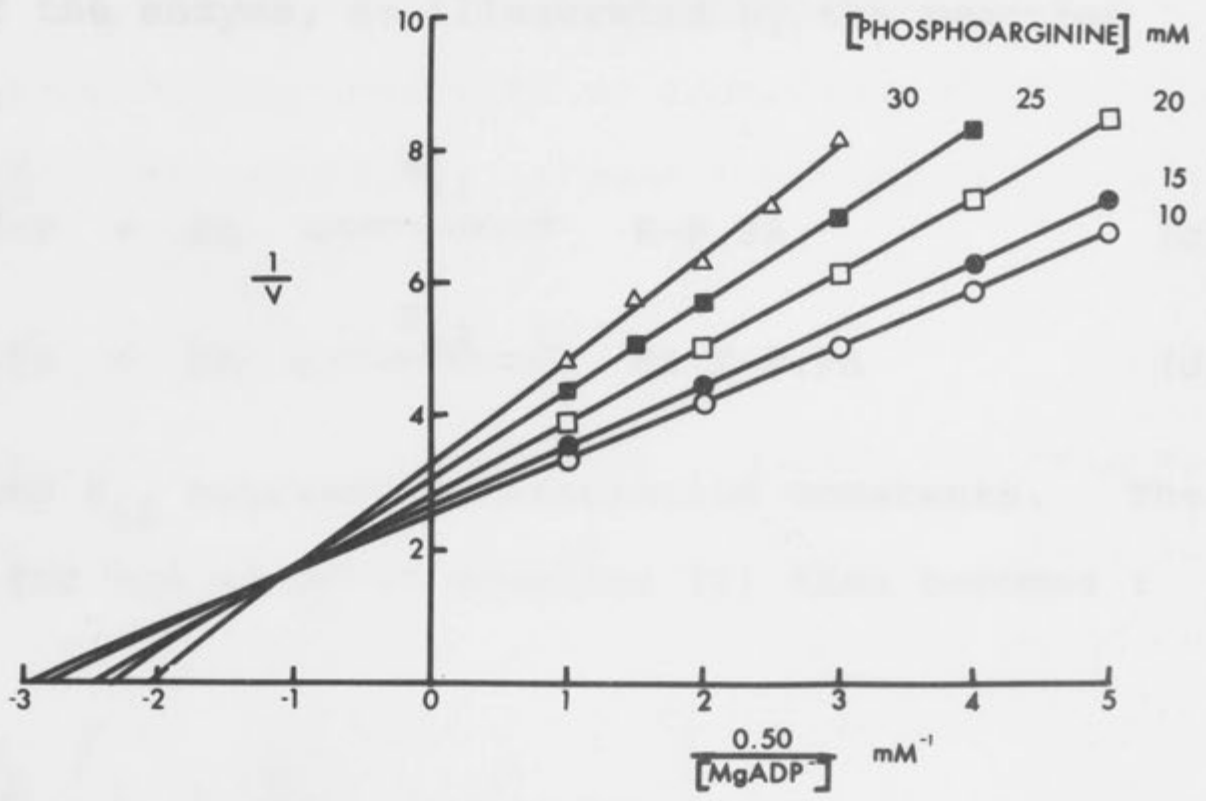

FIG. III.6. A plot of the reciprocals of the initial velocities of the reverse direction of the arginine kinase reaction against the reciprocals of the concentrations of free phosphoarginine, with the concentrations of $MgADP^-$ and free $Mg^{2+}$ held constant at 0.1 mM and 1.0 mM, respectively. The curve is drawn using kinetic constants obtained from analysis of the data by the SUBINH computer programme (Cleland, 1963b). v is expressed as μmoles of arginine per μg of arginine kinase per min.

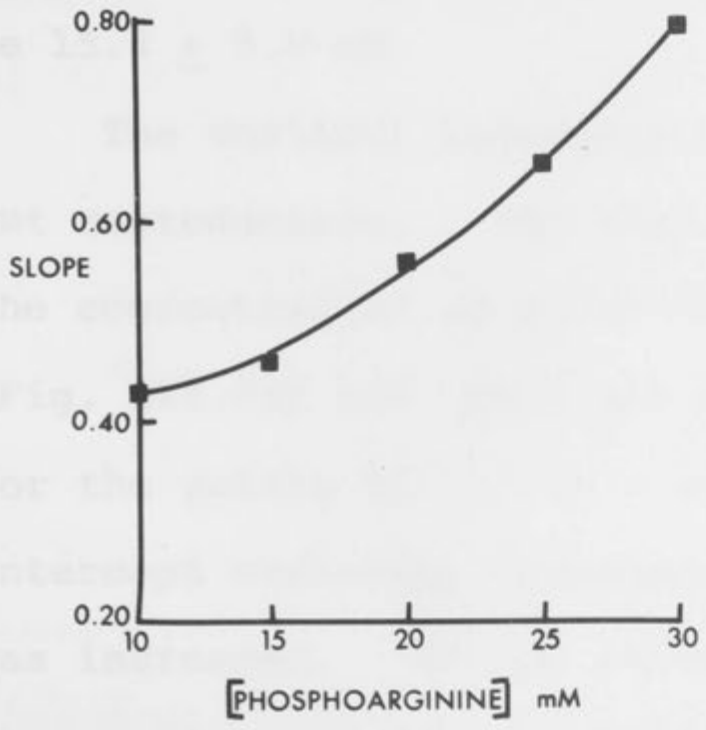
concentration of phosphoarginine was varied from 10 to 30 mM since below these concentrations the results were as expected for a reaction having a basic Ping-Pong mechanism (Fig. III.2). From the data illustrated in Fig. III.7, it is apparent that both the slopes and vertical intercepts of the lines increase as the concentration of phosphoarginine increases, and hence it may be concluded that phosphoarginine acts as a non-competitive inhibitor with respect to $MgADP^-$. The non-competitive inhibition is not of the linear type, however, as replots of the slopes against the concentration of phosphoarginine suggest that the variation in slope is a parabolic function of phosphoarginine concentration as this concentration is increased over 10 mM (Fig. III.7b). Since the initial velocity equation for the reaction with $MgADP^-$ as the variable substrate, expressed in reciprocal form, is:

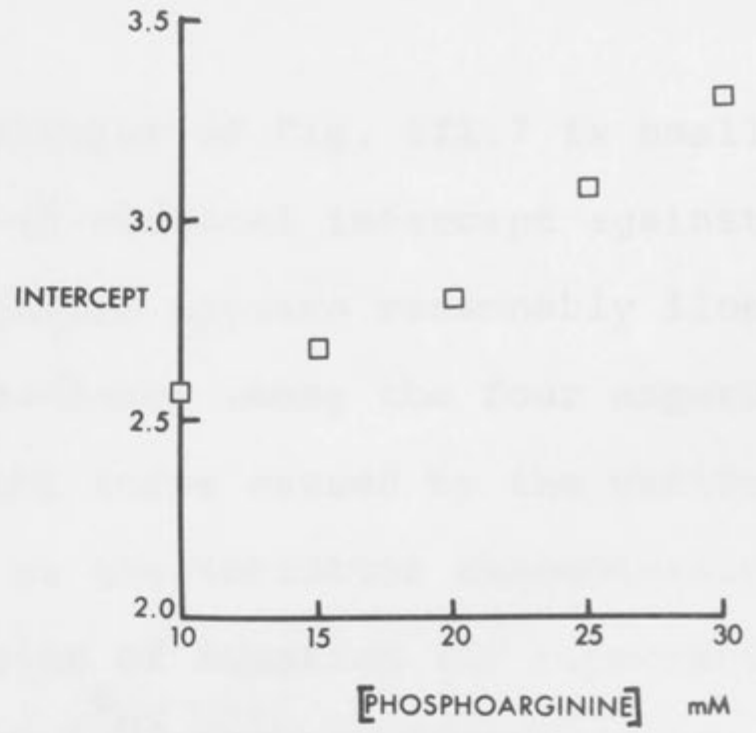
$$\frac{1}{v} = \frac{K_p}{V_2}\frac{1}{P} + \frac{1}{V_2}\left(\frac{K_q}{Q} + 1\right) \qquad (5)$$

(cf. equation 3, p.90), the slope can be a parabolic function of the inhibitor concentration only if the inhibitor is combining twice with the form of the enzyme with which the variable substrate combines. If it may be assumed for the purpose of this analysis that the mechanism of the reaction catalysed by arginine kinase is a basic Ping-Pong one in which the stable enzyme forms do not isomerize (but see Chapter IV), then it can be concluded from the slope data

FIG. III.7. Effect of the concentration of free phosphoarginine on the initial velocity of the reverse reaction catalysed by arginine kinase with $MgADP^-$ as the variable substrate and with the free phosphoarginine concentration increased from 10 mM to 30 mM. (a) The non-competitive inhibition by phosphoarginine with respect to $MgADP^-$; (b) a replot of the slopes of the lines of (a) as a function of the concentration of phosphoarginine; and (c) a replot of the vertical intercepts of (a) as a function of the concentration of phosphoarginine. The lines of (a) were drawn using kinetic constants obtained from analysis of the data by the HYPER computer programme, the points of (b) and (c) were obtained fromthis HYPER analysis while the parabola of (b) was drawn from kinetic constants obtained from analysis of the points by the PARA computer programme (Cleland, 1963b). v is expressed as μmoles of arginine per μg of arginine kinase per min.


[PHOSPHOARGININE] mM
30
25
20
15
10
1/v
10
8
6
4
2
-3
-2
-1
0
1
2
3
4
5
0.50 / [MgADP⁻] mM⁻¹


0.80
0.60
SLOPE
0.40
0.20
10
15
20
25
30
[PHOSPHOARGININE] mM


3.5
3.0
INTERCEPT
2.5
2.0
10
15
20
25
30
[PHOSPHOARGININE] mM

that two molecules of phosphoarginine are combining with the E-P form of the enzyme, as illustrated by the reaction sequences :

$$E\text{-}P + PA \underset{}{\overset{K_{i1}}{\rightleftharpoons}} E\text{-}P.PA \qquad (c)$$

$$E\text{-}P.PA + PA \underset{}{\overset{K_{i2}}{\rightleftharpoons}} PA.E\text{-}P.PA \qquad (d)$$

where $K_{i1}$ and $K_{i2}$ represent dissociation constants. The expression for the slope of equation (5) then becomes :

$$\frac{K_p}{V_2}\left\{1 + \frac{Q}{K_{i1}} + \frac{Q^2}{K_{i1}\ K_{i2}}\right\}$$

From the fit of the slope data of four such experiments to the equation for a parabola, the weighted mean value for $K_{i1}$ has been estimated to be 78.7 $\pm$ 37.8 mM and that for $K_{i2}$ to be 15.4 $\pm$ 5.0 mM.

The vertical intercept variation of Fig. III.7 is small but reproducible. The replot of vertical intercept against the concentration of phosphoarginine appears reasonably linear (Fig. III.7c) but there was a tendency among the four experiments for the points to lie on a slight curve caused by the vertical intercept variation increasing as the inhibitor concentration was increased. In the expression of equation (5) representing the vertical intercept, the term $\left(\frac{K_q}{Q}\right)$ represents the proportion of total enzyme present in form E while the term (1) represents

the proportion of enzyme distributed among the binary forms of enzyme. If one molecule of phosphoarginine were combining with the E form of the enzyme as an inhibitor, then the expression for the vertical intercept would become

$$\frac{1}{V_2}\left\{\frac{K_q}{Q} + \frac{K_q}{K_I} + 1\right\}$$

where $K_I$ represents the dissociation constant for phosphoarginine combining with form E as an inhibitor. This term shows that the vertical intercept would continue to decrease to a limiting value not equal to $\frac{1}{V_2}$ as the concentration of Q is raised. Thus the variation in the vertical intercepts recorded in Figs. III.7a & c cannot result from the reaction of one molecule of phosphoarginine with E, but could be due to two molecules of phosphoarginine combining with E as an inhibitor to give an expression of the form

$$\frac{1}{V_2}\left(\frac{K_q}{Q} + \frac{K_q}{K_{I1}} + \frac{K_q Q}{K_{I1}\ K_{I2}}\right)$$

But as phosphoarginine combines with E as substrate, it is difficult to envisage the presence of three molecules on the enzyme in positions capable of affecting the initial velocity, so this possibility will not be considered further. On the other hand, the vertical intercept variation could come from phosphoarginine combining with one or both of the binary enzyme forms. Since the distribution equation for these

forms cannot be expressed in kinetic terms, it is not possible to evaluate a real dissociation constant for combination with either of these forms. However, if Q is considered to combine equally with both binary forms of the enzyme, then the vertical intercept term would be of the form

$$\frac{1}{V_2}\left(\frac{K_q}{Q} + 1 + \frac{Q}{K_I}\right)$$

This expression is consistent with the vertical intercept variation shown in Fig. III.7 but in view of the paucity of the data and the impossibility of obtaining true dissociation constants from them, no further analysis has been attempted.

After the demonstration of substrate inhibition by phosphoarginine, the effect of higher concentrations of the other three substrates were examined after the manner of Fig. III.6. No inhibition was observed by increasing the concentration of $MgADP^-$ to 2 mM in the presence of 2 mM phosphoarginine, by increasing the concentration of $MgATP^{2-}$ to 8 mM in the presence of 0.6 mM arginine, or by increasing the concentration of arginine to 12 mM in the presence of 0.6 mM $MgATP^{2-}$.

## DISCUSSION

While the kinetic data of Virden, Watts and Baldwin (1965) indicate that the reaction mechanism of arginine kinase from lobster muscle is sequential, the results of

this study clearly shows that the mechanism of the reaction catalysed by the enzyme from the crayfish is of the Ping-Pong type. Because the mechanism can be described as Ping-Pong, the enzyme must exist in two stable forms under steady state conditions and oscillate back and forth between these forms during the course of reaction. This situation is achieved because the product of the first substrate to react is released before the addition of the second substrate to the enzyme. If the reaction mechanism were sequential so that both substrates added to the enzyme before reaction occurred, then the concentration of the second substrate would affect the slope of the lines of double reciprocal plots. Furthermore, when the concentrations of both substrates were varied at a constant ratio, a plot of 1/v against the reciprocal of the concentration of either substrate would be parabolic (cf. Chapter I, Fig. I.9) and partial reactions would not occur.

From the initial velocity data, it seems logical to consider MgATP and phosphoarginine, which possess the transferable phosphoryl group, as the substrates that react with the free enzyme in the forward and the reverse reaction, respectively. It follows, then, that the enzyme would exist in both free and phosphorylated forms and this is consistent with the results of the isotope exchange experiments. In this connection, the equilibrium constants, $K_1$ and $K_2$ for the partial reactions (a) and (b) are of interest. These

were calculated from the relationships

$$K_1 = \frac{V_1 K_p}{V_2 K_a} \quad \text{and} \quad K_2 = \frac{V_1 K_q}{V_2 K_b}$$

to be 0.25 and 1.75, respectively. Thus both equilibria favour the free form of the enzyme and this suggests that, if a covalent bond is formed between the enzyme and its transferable group, its free energy of hydrolysis is greater than that of the same group in these substrates.

In an attempt to demonstrate the phosphorylated form, the enzyme has been incubated with [$\gamma$-$^{32}$P]ATP, but the results (Fig. III.5 and Table III.3) are inconclusive. The data of Table III.3 suggest that some phosphorylated enzyme may have been present 4 hrs. after the reaction took place, but the quantity is so small that it could possibly be explained by experimental variation. Even if it can be inferred from the results that a quantity of enzyme - of the order of 0.5% - is phosphorylated, it is apparent that this phosphoenzyme is unstable, presumably hydrolysing to free enzyme and inorganic phosphate. In spite of this, the enzyme showed no ATPase activity in 5 min. at 30° at a concentration of 0.16 mg per ml. Other enzyme-bound intermediates of reactions with Ping-Pong type mechanism have proved to be unstable. For instance, the $CO_2$-enzyme complex that forms in the methylmalonyl-CoA carboxytransferase reaction (EC. 2.1.2.1) has a half life of about 3 min. at 30° (Kaziro & Ochoa, 1962).

Furthermore, direct demonstration of a glucose-enzyme intermediate in the reaction catalysed by sucrose glucosyltransferase (EC. 2.4.1.7) has been achieved only recently by precipitating inactive enzyme after a very brief incubation with radioactive substrate (Voet & Abeles, 1966), although the existence of such an intermediate was first postulated as a result of isotope exchange experiments in 1947 (Duodoroff et al., 1947).

An experiment involving inactivation of the enzyme would be an important first step in any additional experiments designed to study the association between the enzyme and the transferable phosphoryl group in the reaction catalysed by arginine kinase. Moreover, it can be seen from the equilibrium constants for the partial reactions that the partial reaction involving arginine and phosphoarginine is more favourable for the formation of E-P than is the partial reaction involving ATP and ADP. Therefore a better yield of phosphoenzyme might be achieved if the enzyme were incubated with [$^{32}$P]phosphoarginine rather than [$\gamma$-$^{32}$P]ATP, with arginase included in the reaction mixture to hydrolyse the arginine released and so displace the equilibrium in favour of phosphoenzyme formation :

$$E + [^{32}P]\text{phosphoarginine} \rightleftharpoons E\text{-}^{32}P + \text{arginine}$$

$$\text{arginine} + H_2O \xrightleftharpoons{\text{arginase}} \text{ornithine} + \text{urea}$$

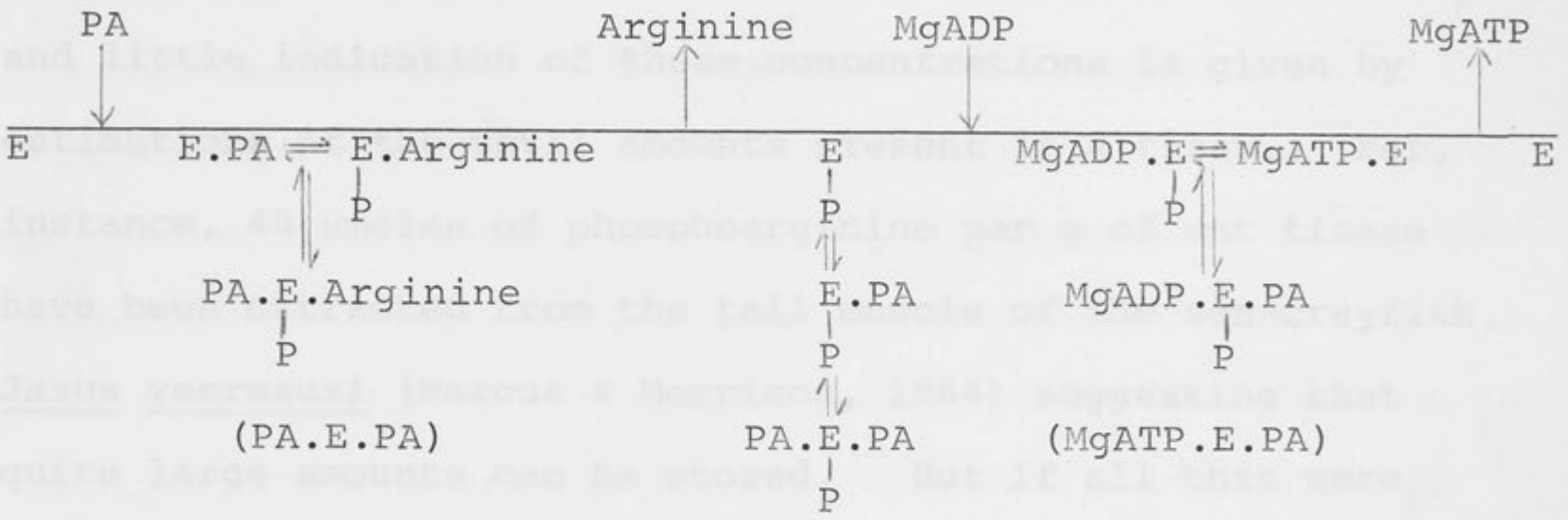
When enzyme was reacted with [$\gamma$-$^{32}$P]ATP and then chromato-

graphed directly on Sephadex, the emerging protein peak was not separated from a continually increasing quantity of radioactive ATP, although the bulk of the ATP, as recognized by ultraviolet absorption, did not commence to emerge from the column until 30 ml after the end of the protein peak. Under these conditions it was impossible to recognise any phosphorylated enzyme. It was not until most of the ATP had been removed by protein precipitation and dialysis that it was possible to separate protein-bound radioactivity from free radioactivity, and even under these conditions (Fig. III.5) radioactivity began to rise sharply towards the end of the protein peak. This $^{32}P$ may represent ATP that was bound to the enzyme but dissociates from it during chromatography, as it can be seen that the ATP bound to the enzyme after chromatography is only 1/20th of that associated with the protein before chromatography (Table III.3). It would be preferable to be able to separate [$^{32}P$]phosphoenzyme from $^{32}P$ by direct chromatography on Sephadex without preliminary precipitation with ammonium sulphate. Indeed Mourad and Parks (1965) have found that immediate transfer to a column is necessary to obtain the phosphorylated form of nucleoside diphosphokinase.

The inhibition caused by higher concentrations of phosphoarginine would appear to be of a complex nature. The non-competitive inhibition is consistent with phosphoarginine

reacting as an inhibitor with the E-P form and also with one or both of the binary forms of the enzyme. The more complex nature of this inhibition, as compared with the substrate inhibition found with aspartic and alanine transaminases (Henson & Cleland, 1964; Bulos & Handler, 1965) may indicate that, while the reaction mechanism is a Ping-Pong one, the enzyme has separate binding sites for the nucleotide and guanidino substrates, whereas the transaminases appear to have only one site which binds both the amino and keto acids. This would seem likely in view of the dissimilarity of the two substrates for the arginine kinase reaction. If this is so, combination of one molecule of phosphoarginine with the E-P form may be at the arginine binding site, while combination of the second molecule may occur through binding to the phosphoryl group to the ADP site. Alternatively, it is possible that this second binding may be non-specific. Although it is not possible to identify a compulsory order in which these bindings may occur, the fact that $K_{i2}$ is considerably less than $K_{i1}$ indicates that binding of the first molecule facilitates the attachment of the second molecule, if it can be assumed that the mechanism of the reaction is a basic Ping-Pong one so that $K_{i1}$ and $K_{i2}$ are dissociations constants, but the possible isomerizations of the stable enzyme forms (Chapter IV) would considerably complicate this. If combination of phosphoarginine as an inhibitor is occurring at both the nucleotide and guanidino

sites, then binding to binary enzyme forms could occur in a similar manner, with binding to an E-P arginine form occurring at the nucleotide binding site, and binding to an E-P.MgADP binary form at the guanidino site. The formation of the dead-end complexes can be depicted by the diagram :




It must be considered possible that some or all of the inhibition caused by higher concentrations of phosphoarginine could be due not to the phosphoarginine itself, but to a contaminant present in the phosphoarginine preparation. To be present, such a compound must have properties and an elementary composition very similar to phosphoarginine, in view of the number of steps necessary for the isolation of sodium phosphoarginine from crayfish muscle and the apparent homogeneity of this phosphoarginine as judged by elementary analysis (Marcus & Morrison, 1964). While these criteria render it unlikely that such a contaminant is present in appreciable quantities, they do not eliminate the possibility of a compound present in very small amounts with a very high affinity for the enzyme.

It is difficult to ascertain the biological importance of this inhibition by increased concentrations of phosphoarginine. The velocity of the reaction *in vivo* will be dependent on the steady-state concentrations of $Mg^{2+}$, nucleotides and guanidino compounds that are available to the enzyme, and little indication of these concentrations is given by estimations of the total amounts present in a tissue. For, instance, 40 μmoles of phosphoarginine per g of wet tissue have been extracted from the tail muscle of the sea-crayfish, *Jasus verreauxi* (Marcus & Morrison, 1964) suggesting that quite large amounts can be stored. But if all this were available to the enzyme under conditions similar to those used in the laboratory to examine substrate inhibition, the velocity of reaction would be very slow indeed, so that, if any storage occurs, it is likely to be in an area of the cell separated from the arginine kinase.

Like other phosphotransferases, arginine kinase has an essential requirement for a bivalent metal ion, but the function of the metal ion has not been established. Thus it is not known if reaction of the metal ion with the enzyme is a compulsory first step in the overall reaction sequence, if the metal ion function is related to the formation of metal-nucleotide complexes or if both reactions are essential (Cohn, 1963). While the complete reaction sequence is yet to be elucidated, any scheme must take into account the fact that

while an added metal ion is essential for the ADP-ATP exchange, it is not essential for the arginine-phosphoarginine exchange. In this context it is of interest that a recent spectrographic analysis of an enzyme preparation indicates the presence of calcium and of magnesium but not of manganese. If either of the metals present should prove to be an integral part of the enzyme, then it is possible it may participate in this arginine-phosphoarginine phosphoryl exchange reaction, just as it would seem that the manganese bound to pyruvate carboxylase (EC. 6.4. 1.1) participates in the pyruvate-oxalacetate exchange reaction catalysed by this enzyme (Mildvan, Scrutton & Utter, 1966).

## SUMMARY

1. The initial velocity of the reaction catalysed by arginine kinase has been measured with $MgATP^{2-}$ and arginine as variable substrates (forward direction) and with $MgADP^{-}$ and phosphoarginine as variable substrates (reverse direction). The results indicate that the enzyme catalyses the reaction by means of a Ping-Pong mechanism.

2. Further evidence for a Ping-Pong reaction mechanism has come from the demonstration of the partial exchange reactions (a) between ADP and ATP in the absence of guanidino substrate and (b) between arginine and phosphoarginine in the absence of nucleotide substrates. The nucleotide

but not the guanidino exchange is dependent on the addition of a bivalent metal ion.

3. It seems likely that the enzyme exists in both free and phosphorylated forms, although definitive evidence for the phosphorylated form has not yet been obtained.

4. Higher concentrations of phosphoarginine give rise to substrate inhibition and this inhibition is non-linear non-competitive with respect to $MgADP^-$ as the variable substrate.

# CHAPTER IV

# PRODUCT INHIBITION STUDIES OF THE REACTION CATALYSED BY ARGININE KINASE

# CHAPTER IV

# PRODUCT INHIBITION STUDIES OF THE REACTION CATALYSED BY ARGININE KINASE

## INTRODUCTION

The results of initial velocity studies in the absence of added product and of isotope exchange measurements are consistent with the proposal that arginine kinase purified from the tail muscle of the sea crayfish, Jasus verreauxi, has a Ping-Pong reaction mechanism (Chapter III). From the initial velocity data it has been possible to calculate the maximum velocities for the forward and reverse reactions as well as the Michaelis constants for the four substrates. The Michaelis constants for an enzyme with a Ping-Pong mechanism are complex constants, rather than dissociation constants and thus give no indication of the affinity of the substrate for the enzyme. While in the absence of complicating effects values for the dissociation constants could be determined from thermodynamic experiments involving the two stable forms of the enzyme, it is also possible to obtain them by means of product inhibition studies. These studies, moreover, give additional information about the reaction mechanism.

The phenomenom of inhibition of a reaction by the products of that reaction was recognised early in the development of enzyme kinetics - both Henri (1903) and Michaelis and Menten (1913) considered the effects of product inhibition

in the derivation of their rate equations - but it is only recently that such inhibition has been utilized to gain insight into reaction mechanisms. Alberty (1958) showed how product inhibition studies could be used to differentiate between sequential mechanisms of reactions involving two substrates and two products. This work was extended by Fromm and Nelson (1962) to allow for the effect of products in giving rise to dead-end complexes, that is, complexes which are not an integral part of the reaction sequence and which can dissociate only to yield the reactants from which they were formed. The formation of such complexes can be inferred from the finding that double reciprocal plots become non-linear in the presence of product, or from the non-linearity of secondary plots of the slopes and/or vertical intercepts of a primary plot against the product concentration.

Developments in the analysis of product inhibition have been summarized by Walter and Frieden (1963) who emphasize that products need not be competitive inhibitors with respect to the substrate even though both reactants may show a marked chemical similarity and bind to the same site on the enzyme (cf. Crane & Sols, 1954). As pointed out by Cleland (1963a), the actual product inhibition pattern can be found most easily by writing the complete rate equation for the proposed mechanism, altering it by setting one or more of the product concentrations to zero, and then rearranging this equation in reciprocal form with each substrate, in turn, as

the variable one. A list of product inhibition patterns for various two-substrate, two-product reactions is given by Cleland (1963a).

Because of the additional information that can be obtained from product inhibition studies, it was decided to use each reactant as a product inhibitor of the arginine kinase reaction with respect to each of the two substrates and to measure the initial velocity as a function of varying concentrations of both substrates. The conditions chosen were similar to those used for the initial velocity studies in the absence of added product. The results indicate that arginine kinase does not appear to have the product inhibition pattern expected for a basic Ping-Pong mechanism. It is possible that this could be due to the effects of dead-end complexes but it seems more likely, though it is by no means certain, that it is due to isomerizations of both stable forms of the enzyme.

## EXPERIMENTAL

### Materials

#### Chemicals

Triethanolamine buffer, arginine, phosphoarginine, ADP, ATP, DCTA, $MgCl_2$, phosphoenolpyruvate, $NADH_2$ and EDTA were purchased and/or prepared as described in Chapter II, p.64. $^{14}C$-arginine was obtained from Schwarz Bioresearch, Inc., the sodium salt of NADP from California Corporation for Biochemical

Research and D-glucose from the British Drug Houses Ltd. Scintillation materials were purchased as described in Chapter III, p.84 and the fluid prepared as described by Morrison and Cleland (1966).

Enzymes

Hexokinase, pyruvate kinase and lactate dehydrogenase were purchased from the California Corporation for Biochemical Research, glucose 6-phosphate dehydrogenase (EC. 1.1.1.49) from Nutritional Biochemicals Corp., and arginase (EC. 3.5.3.1) from Worthington Biochemical Corporation, New Jersey. Arginine kinase was prepared as described in Chapter II, and was the same preparation as was used for the initial velocity and isotope exchange studies (Chapter III).

## Methods

### Initial velocity measurements of the arginine kinase reaction

Reaction mixtures contained in a total volume of 1.0 ml: triethanolamine-HCl buffer (pH 8.0), 0.1 M; EDTA, 0.01 mM; substrates and a product at the concentrations indicated; in addition to sufficient $MgCl_2$ to maintain the concentration of $Mg^{2+}$ at 1 mM. In experiments in which [$^{14}$C]phosphoarginine was being assayed, the reaction volume was reduced to 0.5 ml and the amount of protein to 0.045 µg from the usual amount of 0.18 µg. All experiments were run for at least two time periods to ensure that initial velocities were being

measured.

Estimation of reaction products

Arginine and ADP were measured by the methods described in Chapter II, p.68.

ATP was measured by means of the coupled reactions catalysed by hexokinase and glucose 6-phosphate dehydrogenase, as described by Morrison and James (1965).

Two different methods were used for the estimation of phosphoarginine :

1. Measurement of arginine liberated from phosphoarginine by acid hydrolysis after enzymic hydrolysis of the substrate arginine by means of arginase.

The enzymic reaction was stopped by the addition of 0.1 ml of 2 N NaOH containing 0.1 M DCTA and after 30 min. the pH was readjusted to pH 8.3 to 8.4 with 2 N HCl. To each tube was added arginase (0.2 mg) to remove free arginine, and, after 2 hr. at room temperature, 0.3 ml of 5 N HCl was added. The tubes were placed in a boiling water bath for 10 min. and, after cooling to room temperature, the reaction mixture was neutralized with NaOH. The arginine released from phosphoarginine was estimated by the method of Rosenberg et al. (1956).

2. Because traces of arginine remained after the 2 hr. of arginase treatment, a second method was used in which the velocity was measured in terms of the production of [$^{14}$C]phosphoarginine.

[$^{14}$C]arginine (0.2 to 0.4 μC) was included in the reaction mixture. Samples (100 μl) were taken at intervals using Hamilton microliter syringes and applied to DEAE-cellulose paper to stop the reaction. The paper was washed with water to remove [$^{14}$C]arginine and to leave the [$^{14}$C]phosphoarginine bound to the DEAE groups of the paper, and the radioactivity was counted in the manner used for the isotope exchange experiments (Chapter III, p. 86) and as described by Morrison and Cleland (1966). At least four samples were taken from each reaction mixture. The results were checked graphically to ensure linearity, and although no evidence for non-linearity was obtained, the points did not fit exactly on a straight line. An IBM computer was then used to fit a least-squares plot to the points and the slope of the line taken to be a measure of the amount of phosphoarginine formed in 1 min.

Analysis of results

The kinetic data were analysed in the manner described in Chapter I (p.43) using the programmes HYPER, LINE, NONCOMP, PARA (preface, equations 1, 2, 7, 3) and either an IBM 1620 computer with the programmes written in Fortran II(D) or an IBM 360 computer for which the programmes were written in Fortran IV(E). All illustrations of product inhibitions have been drawn with the aid of the constants obtained from analysis of the data with the NONCOMP computer programme. Weighted means and their standard errors have been estimated

using the formulae given in Chapter I (p.44). Values for the standard errors of products and quotients have been estimated using the formulae listed in Chapter I (pp. 44 & 45).

## RESULTS

### Measurement of initial velocities by estimation of phosphoarginine formation

Before presenting the results of the product inhibition studies, mention must be made of the problem that arose in estimating phosphoarginine.

When one product of the reaction was added as a product inhibitor, the initial velocity of the reaction was measured by estimating the formation of the other product. As the four substrates of the arginine kinase reaction were used as product inhibitors, methods were required for the estimation of all four substrates. Standard assays were available for the estimation of arginine, ADP and ATP (see Methods) but not for the estimation of phosphoarginine. Morrison and James (1965) were able to estimate phosphocreatine by estimation of the inorganic phosphate released after hydrolysis in acid molybdate at room temperature. The complete hydrolysis of phosphoarginine does not occur under these mild conditions, however, and it is necessary to heat solutions with 1 N HCl for 7 min. at 100° in order to hydrolyse quantitatively the N-P bond of this compound. This treatment results in considerable hydrolysis of ATP and so the

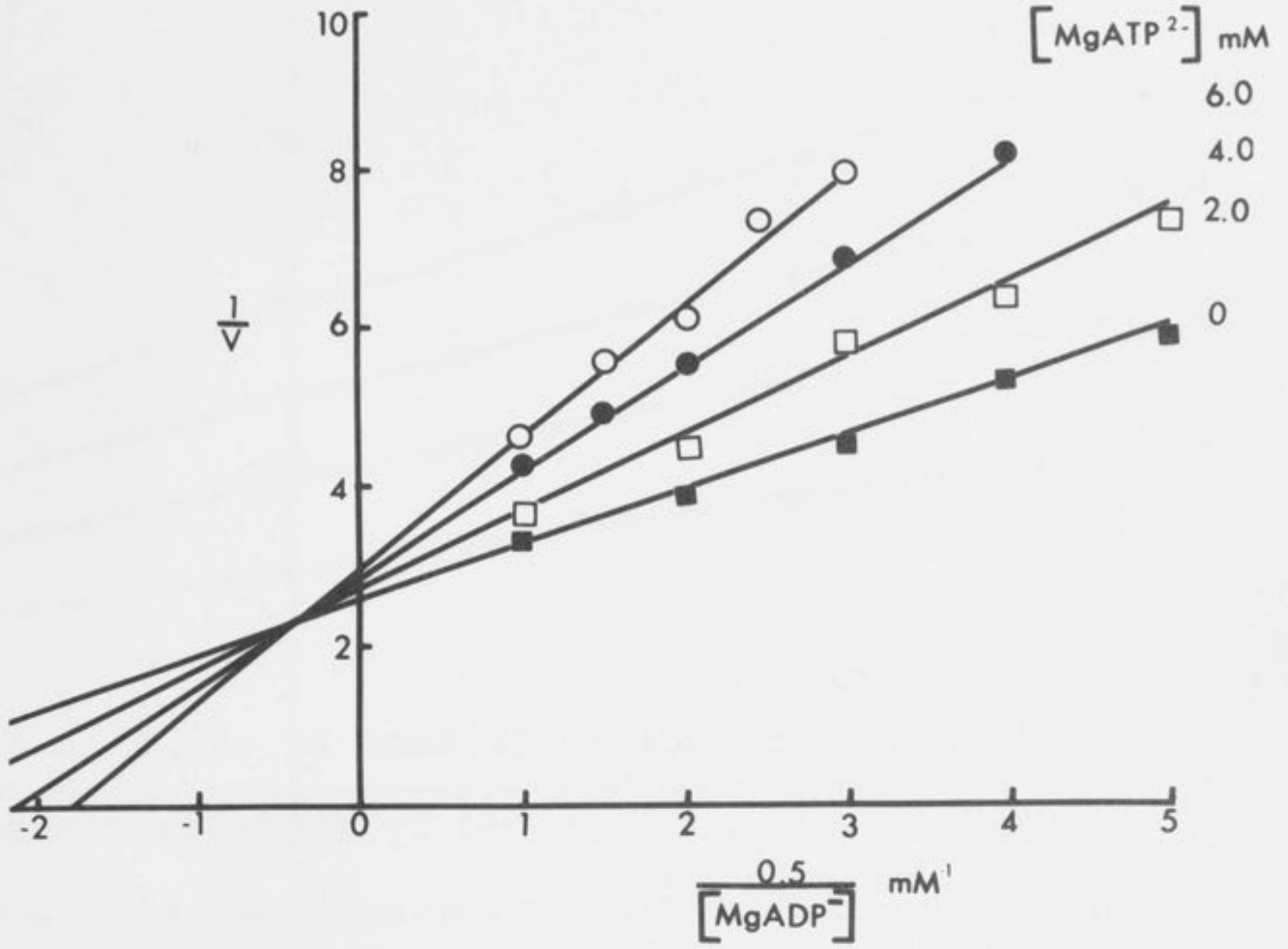
estimation of phosphoarginine as inorganic phosphate is precluded. Instead, phosphoarginine has been estimated by measuring the arginine released from phosphoarginine by acid hydrolysis after the arginine present as substrate has been hydrolysed by the action of added arginase. In addition, there has been used a second method which involves the conversion of [$^{14}$C]arginine to [$^{14}$C]phosphoarginine which is estimated by counting, after the guanidino compounds have been separated by the use of DEAE-cellulose paper. The two methods gave equivalent results when tested in an experiment in which MgATP was the variable substrate, but since the [$^{14}$C]arginine contains a radioactive contaminant not removed by washing the DEAE-cellulose paper and since a small amount (usually 0.005 μmole) of arginine remained after the arginase treatment, both methods gave high blank values. Thus it was necessary to estimate the velocity from the slope of the line drawn by plotting phosphoarginine produced against time. Because the amount of arginine remaining after the arginase was dependent on but not linearily proportional to the amount of arginine added as substrate, this method was not used when arginine was the variable substrate.

Inhibition by products of the arginine kinase reaction

The four substrates of the arginine kinase reaction have been used, in turn, as product inhibitors of the reaction with respect to each of the appropriate substrates. The concentration of free $Mg^{2+}$ was kept constant at 1 mM. In

every case the inhibition appears to be non-competitive (Figs. IV.1, IV.2, IV.3, IV.4). When the primary data were analysed by the HYPER programme and the slopes and vertical intercepts of these lines were fitted to a straight line, the goodness of fit of these replots (Figs. IV.5 & IV.6) indicates that, in addition, all inhibitions can be described as being linear non-competitive. For this reason, the illustrations of Figs. IV.1 to IV.4 have been drawn using the constants obtained from analysis of the data by the NONCOMP computer programme. From these analyses, values were obtained for the apparent inhibition constants, $K_{i\ slope}$ and $K_{i\ intercept}$, and also for the intersection point, that is the reciprocal of the horizontal coordinate of the point where the lines of the double reciprocal plot of a non-competitive inhibition intersect (Table IV.1). True dissociation constants cannot be calculated from the apparent inhibition constants (Table IV.1) without a knowledge of the reaction mechanism and the formulation of the complete rate equation. Nevertheless, the apparent inhibition constants, $K_{i\ slope}$ and $K_{i\ intercept}$, represent the concentrations of product inhibitors required to double the values for the slope of the line and the vertical intercept in the absence or product, but do not represent the concentrations that halve the initial velocity. The intersection point is equal to $K(\frac{K_{i\ intercept}}{K_{i\ slope}})$ where K is the apparent Michaelis constant for the variable substrate

FIG. IV.1. Product inhibition by $MgATP^{2-}$ of the reverse reaction catalysed by arginine kinase with $MgADP^{-}$ as the variable substrate and with phosphoarginine held constant at 10 mM (a), and with phosphoarginine as the variable substrate and with $MgADP^{-}$ held constant at 0.5 mM (b). The lines were drawn using kinetic constants obtained from analysis of the data by the NONCOMP computer programme (Cleland, 1963b). v is expressed as µmoles of arginine per µg of arginine kinase per min.


[MgATP 2-] mM
6.0
4.0
2.0
0
1/V
10
8
6
4
2
-2
-1
0
1
2
3
4
5
0.5
[MgADP-]
mM-1

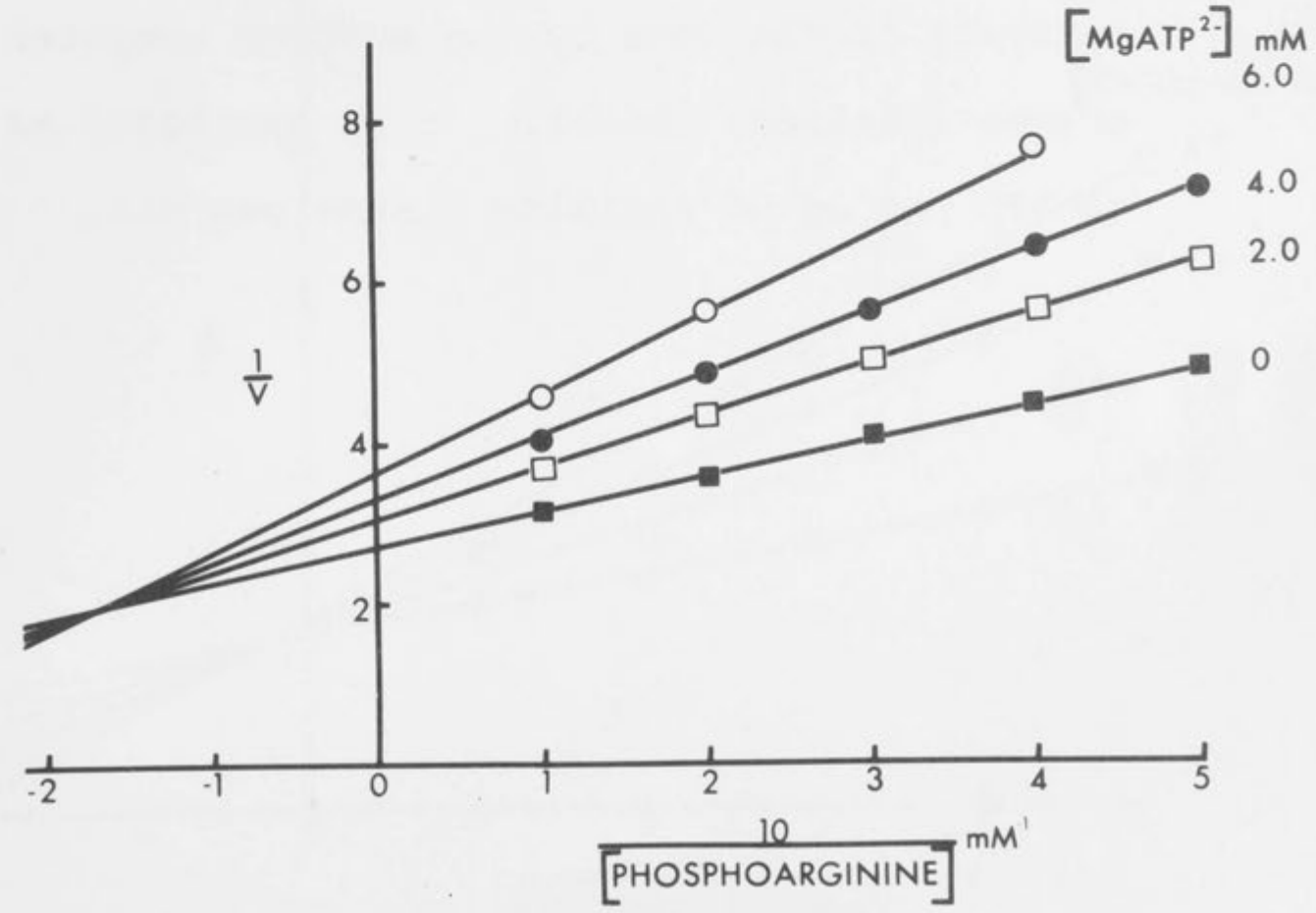
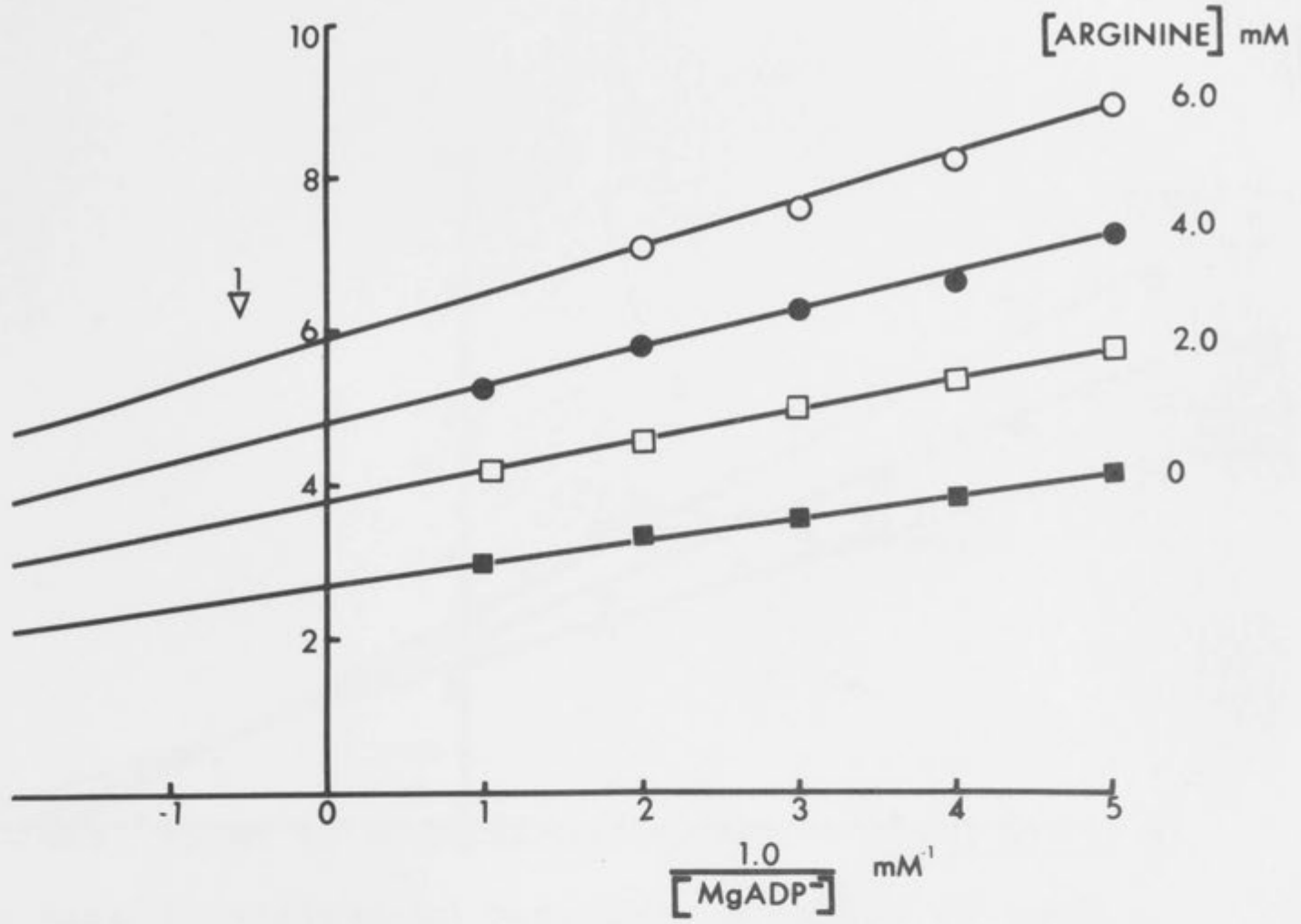

[MgATP 2-] mM
6.0
4.0
2.0
0
1/V
8
6
4
2
-2
-1
0
1
2
3
4
5
10
[PHOSPHOARGININE]
mM-1

FIG.IV.2. Product inhibition by arginine of the reverse reaction catalysed by arginine kinase with $MgADP^-$ as the variable substrate and with phosphoarginine held constant at 5 mM (a), and with phosphoarginine as the variable substrate and with $MgADP^-$ held constant at 0.5 mM (b). The lines were drawn using kinetic constants obtained from analysis of the data by the NONCOMP computer programme (Cleland, 1963b). v is expressed as μmoles of ATP per μg of arginine kinase per min.


[ARGININE] mM
6.0
4.0
2.0
0
10
8
6
4
2
1/V
-1
0
1
2
3
4
5
1.0 / [MgADP⁻] mM⁻¹

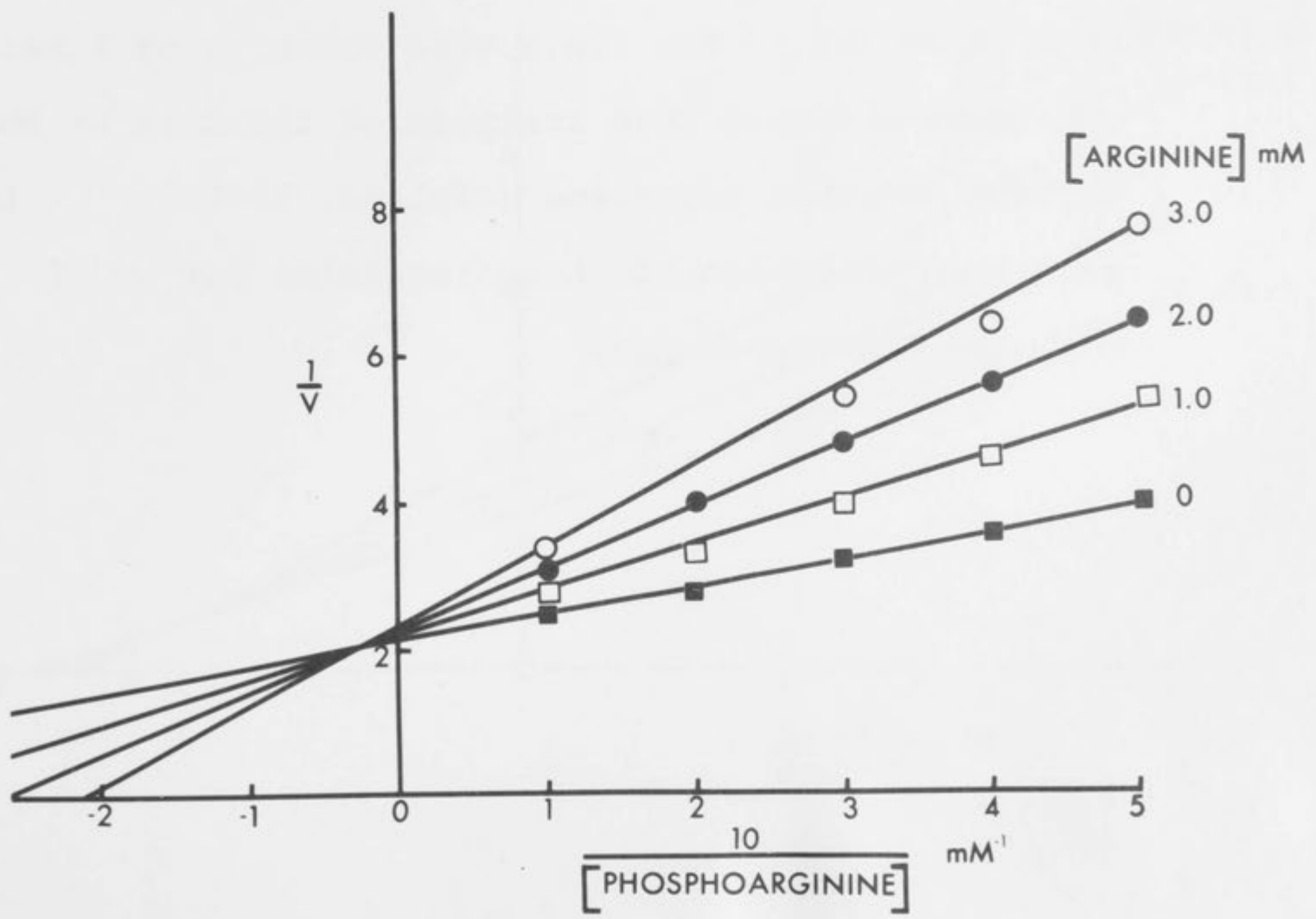
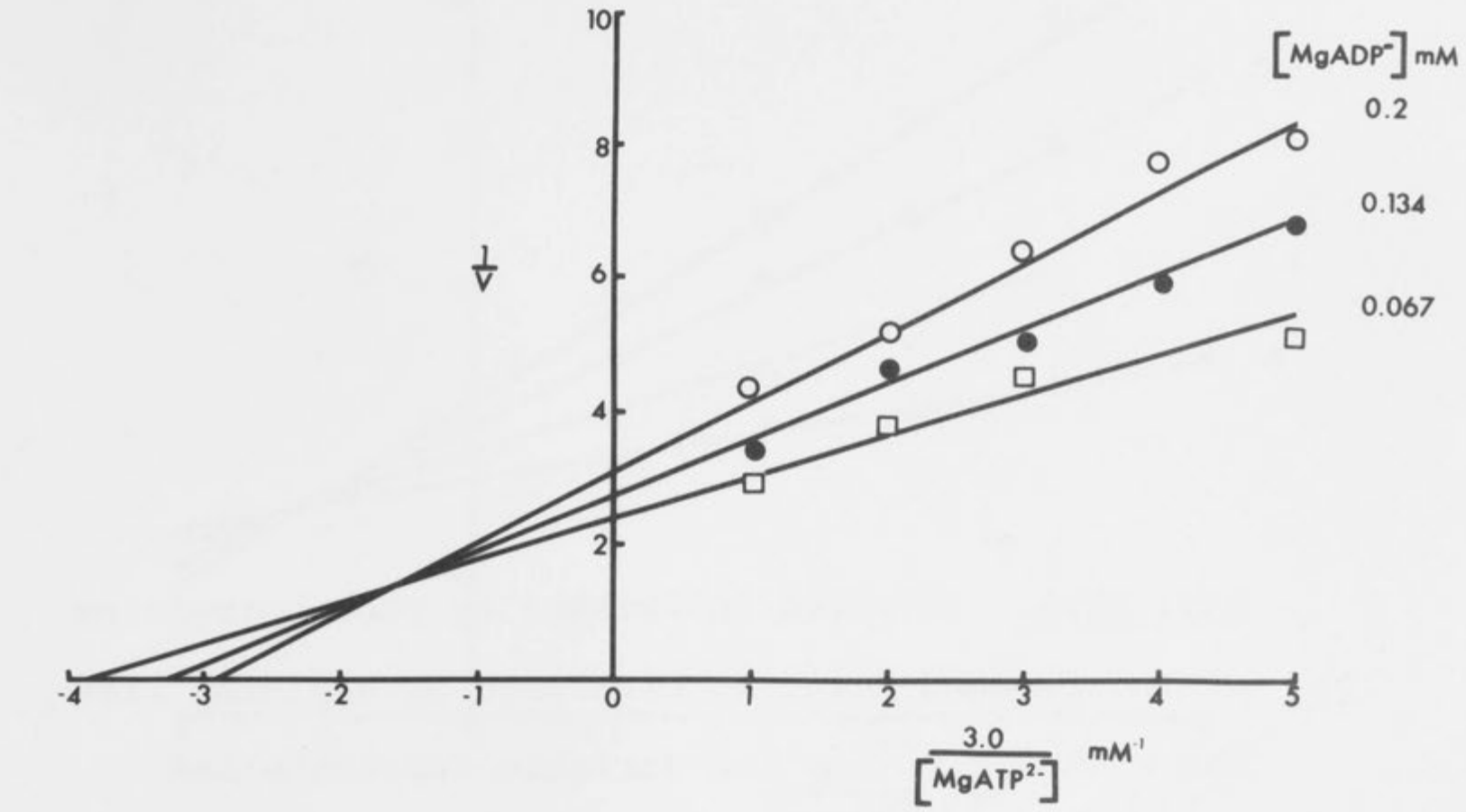

[ARGININE] mM
3.0
2.0
1.0
0
8
6
4
2
1/V
-2
-1
0
1
2
3
4
5
10 / [PHOSPHOARGININE] mM⁻¹

FIG. IV.3. Product inhibition by $MgADP^-$ of the forward reaction catalysed by arginine kinase with $MgATP^{2-}$ as the variable substrate and with arginine held constant at 3.0 mM (a) and with arginine as the variable substrate and $MgATP^{2-}$ held constant at 3. 0 mM (b). The lines were drawn using kinetic constants obtained from analysis of the data by the NONCOMP computer programme (Cleland, 1963b). v is expressed as μmoles of phosphoarginine per μg of arginine kinase per min.


10
8
6
4
2
1/V
-4
-3
-2
-1
0
1
2
3
4
5
3.0 / [MgATP2-] mM-1
[MgADP-] mM
0.2
0.134
0.067

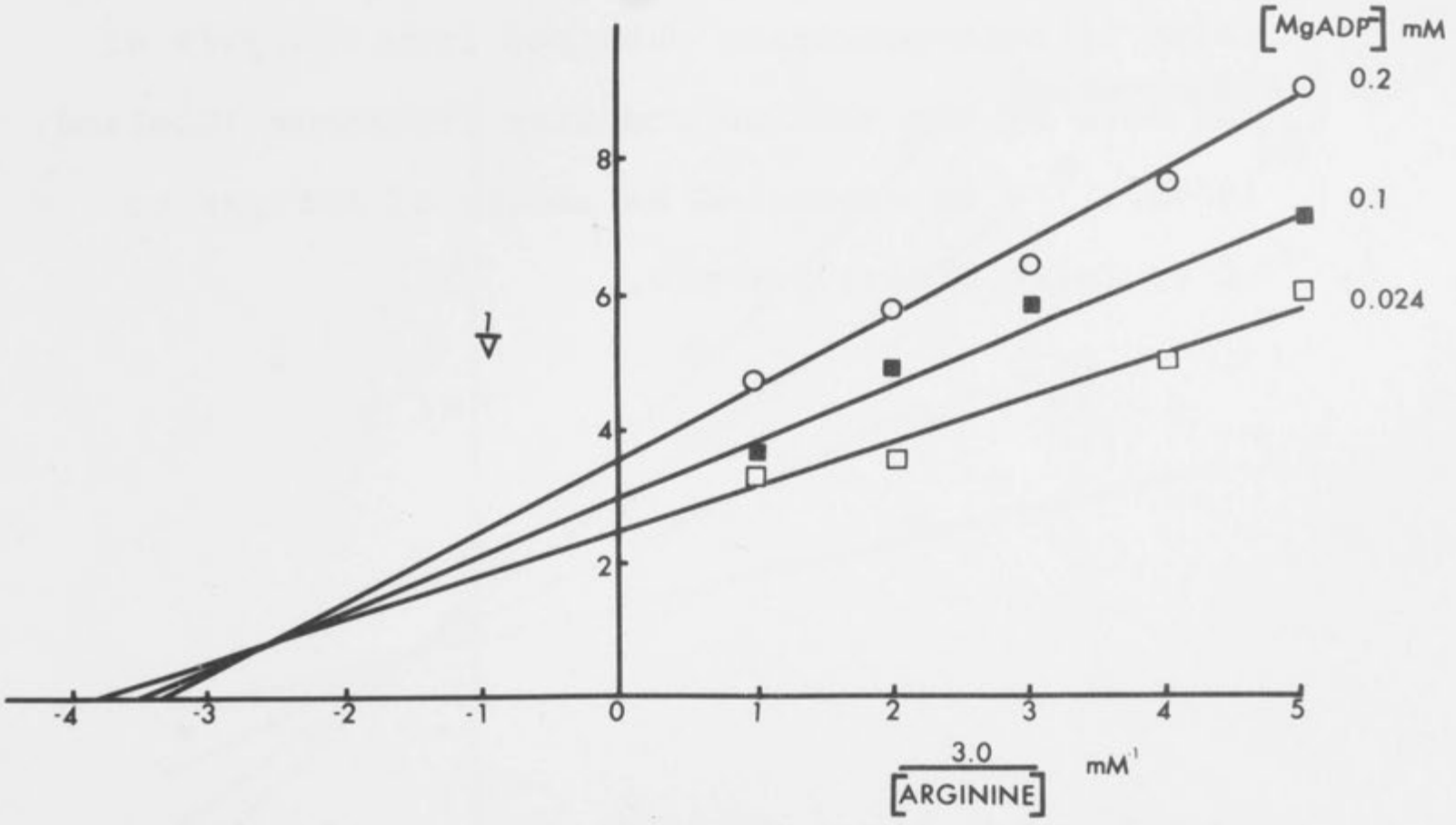
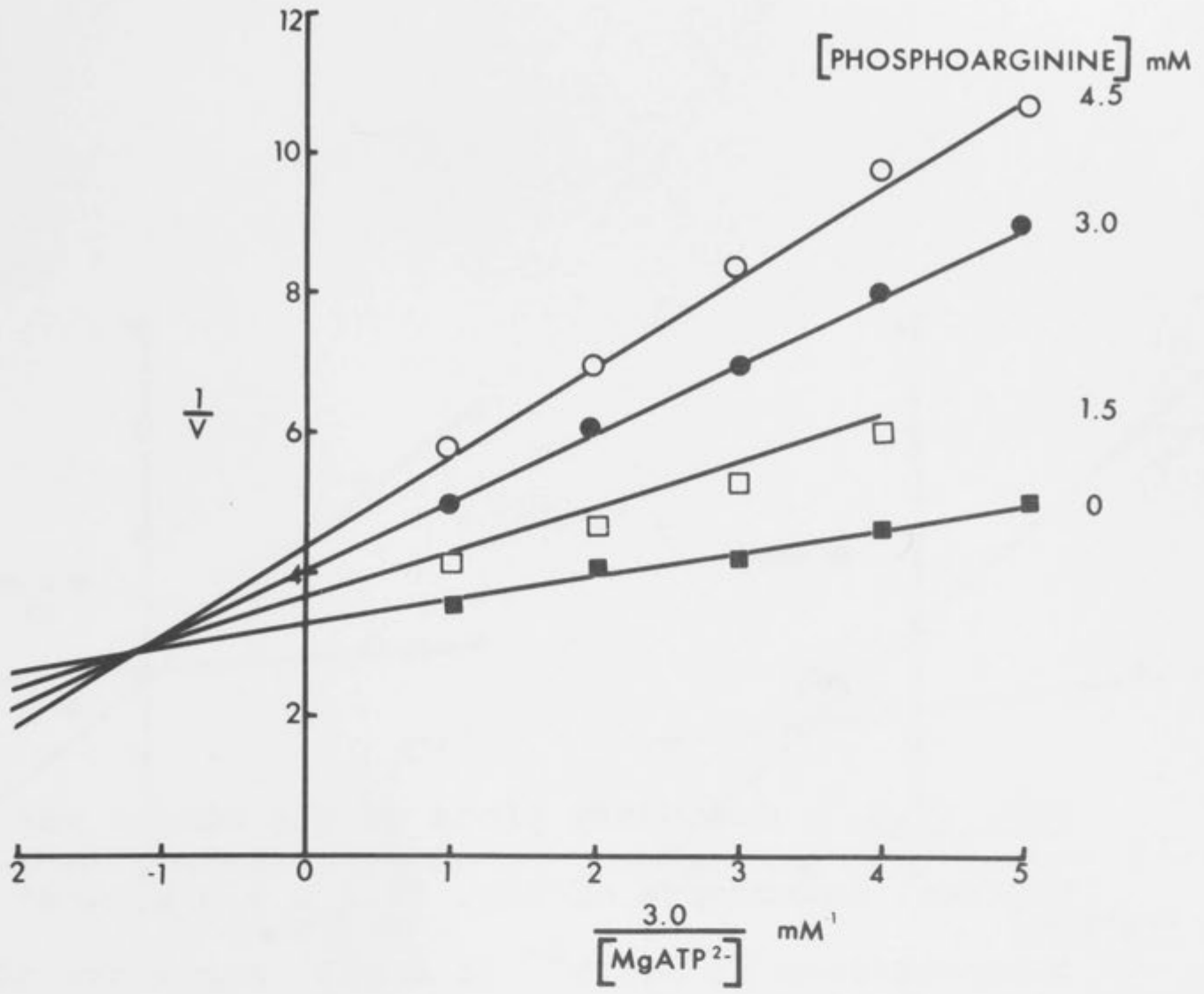

8
6
4
2
1/V
-4
-3
-2
-1
0
1
2
3
4
5
3.0 / [ARGININE] mM-1
[MgADP-] mM
0.2
0.1
0.024

FIG. IV.4. Product inhibition by phosphoarginine of the forward reaction catalysed by arginine kinase with $MgATP^{2-}$ as the variable substrate and arginine held constant at 3.0 mM (a), and with arginine as the variable substrate and $MgATP^{2-}$ held constant at 3.0 mM (b). The lines were drawn using kinetic constants obtained from analysis of the data by the NONCOMP computer programme (Cleland, 1963b). v is expressed as µmoles of ADP per µg of arginine kinase per min.


[PHOSPHOARGININE] mM
4.5
3.0
1.5
0
1/V
12
10
8
6
4
2
2
-1
0
1
2
3
4
5
3.0 / [MgATP 2-] mM-1

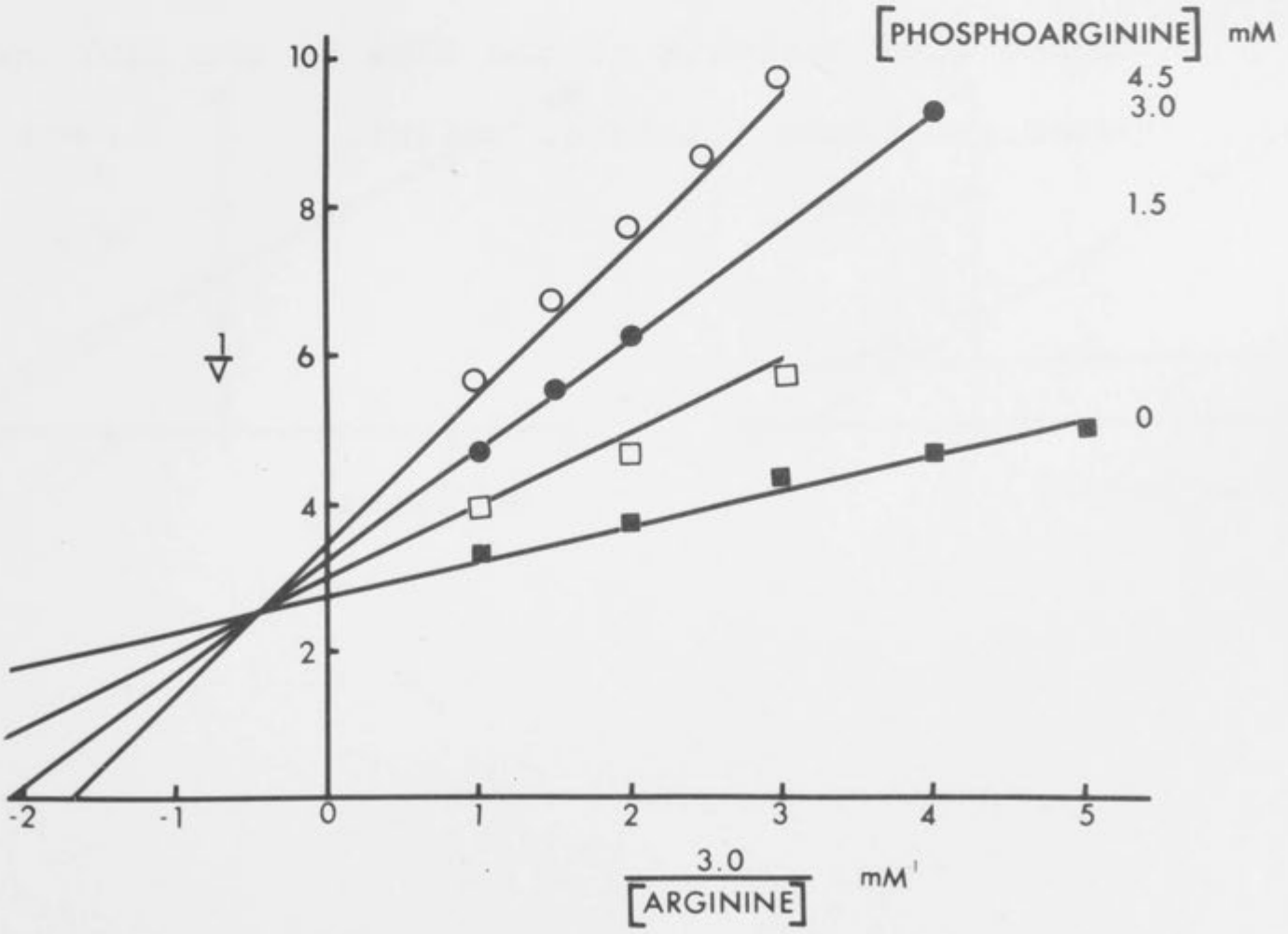
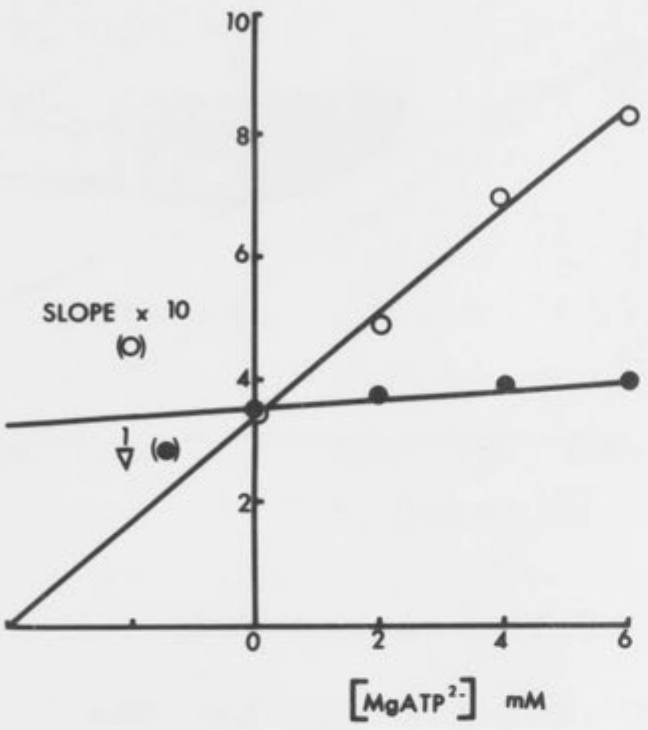
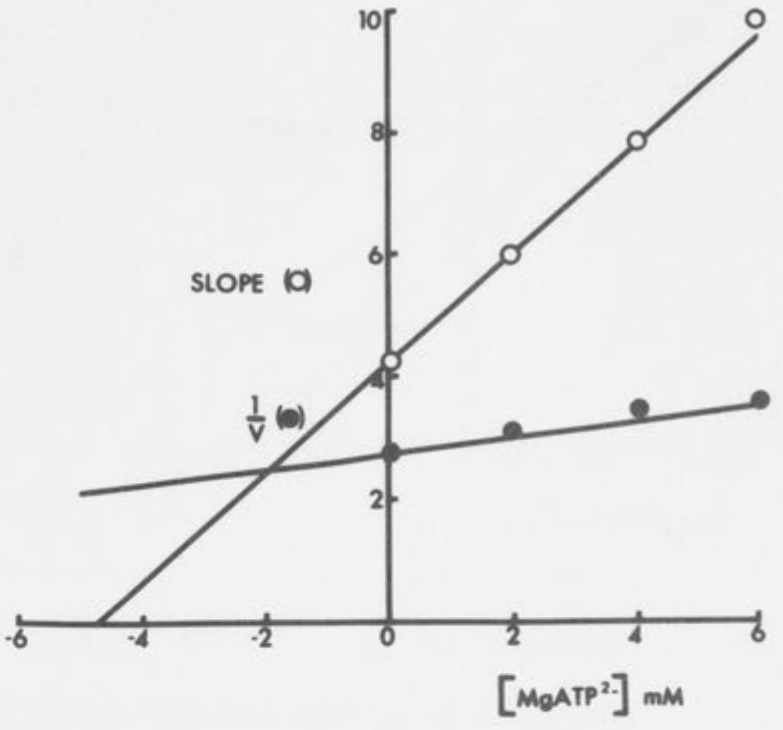

[PHOSPHOARGININE] mM
4.5
3.0
1.5
0
1/V
10
8
6
4
2
-2
-1
0
1
2
3
4
5
3.0 / [ARGININE] mM-1

FIG. IV.5. Secondary plots of the slopes and vertical intercepts of Fig. IV.1 a & b against the concentration of $MgATP^{2-}$ (a & b); secondary plots of the slopes and vertical intercepts of Fig. IV.2 a & b against the concentration of arginine (c & d). The lines were drawn using kinetic constants obtained from analysis of the data by the LINE computer programme (Cleland, 1963b).

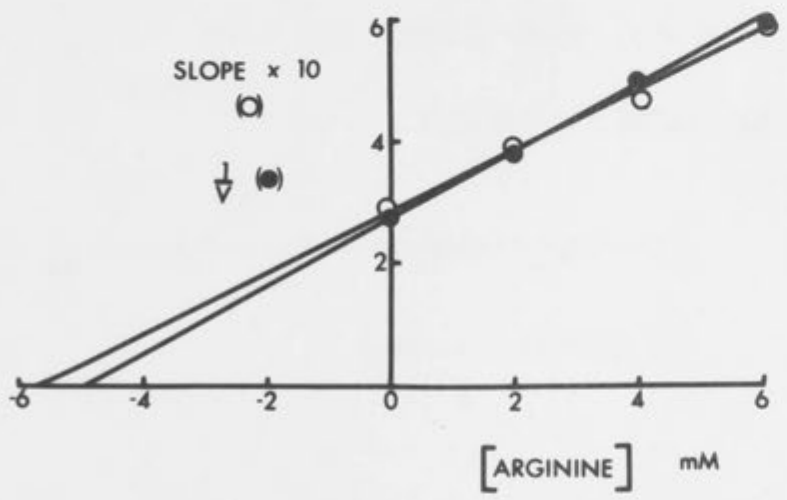
(a)


SLOPE x 10
(○)
1/V (●)
10
8
6
4
2
0
2
4
6
[MgATP2-] mM

(b)


SLOPE (○)
1/V (●)
10
8
6
4
2
-6
-4
-2
0
2
4
6
[MgATP2-] mM

(c)


SLOPE x 10
(○)
1/V (●)
6
4
2
-6
-4
-2
0
2
4
6
[ARGININE] mM

(d)

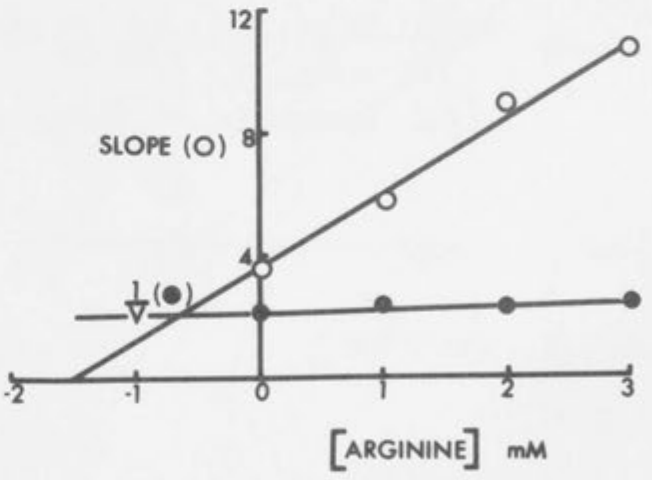
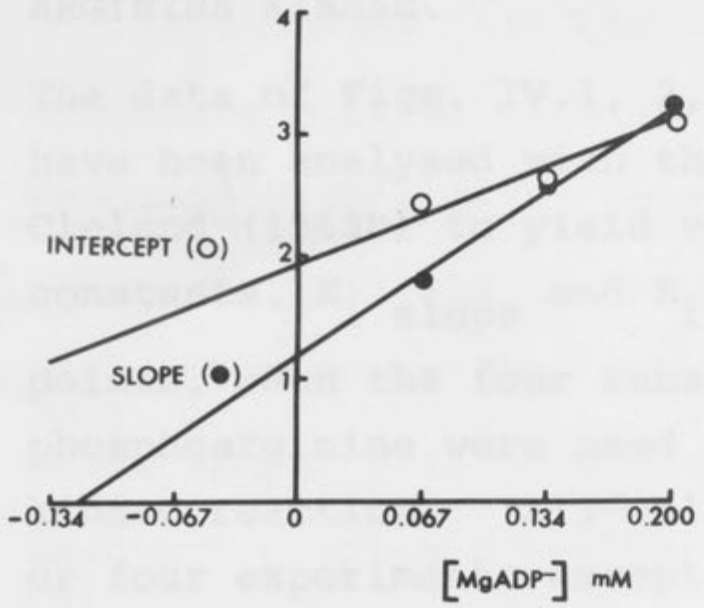
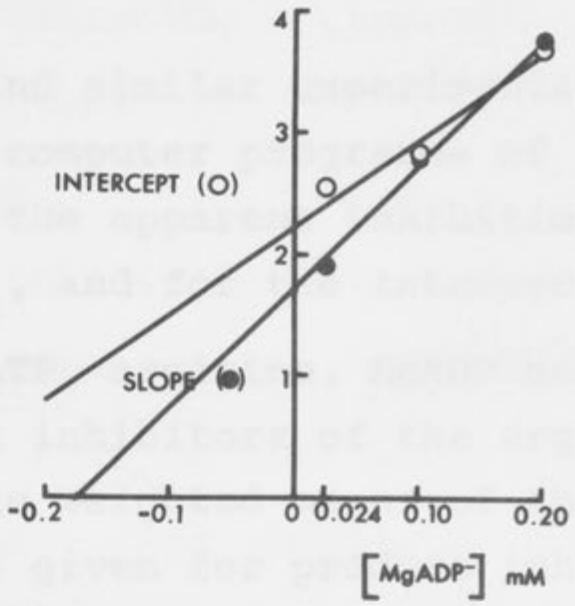

SLOPE (○)
1/V (●)
12
8
4
-2
-1
0
1
2
3
[ARGININE] mM

FIG. IV.6. Secondary plots of the slopes and vertical intercepts of Fig. IV.3 a & b against the concentration of $MgADP^-$ (a & b); secondary plots of the slopes and vertical intercepts of Fig. IV.4 a & b against the concentration of phosphoarginine (c & d). The lines were drawn using kinetic constants obtained from analysis of the data by the LINE computer programme (Cleland, 1963b).

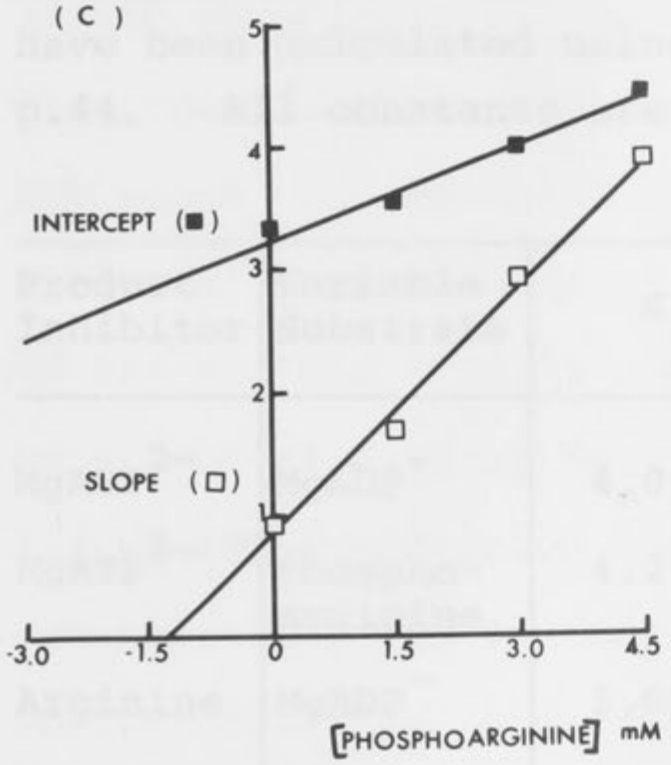
( A )


4
3
2
1
INTERCEPT (O)
SLOPE (●)
-0.134
-0.067
0
0.067
0.134
0.200
[MgADP⁻] mM

( B )


4
3
2
1
INTERCEPT (O)
SLOPE (●)
-0.2
-0.1
0
0.024
0.10
0.20
[MgADP⁻] mM

( C )


5
4
3
2
1
INTERCEPT (■)
SLOPE (□)
-3.0
-1.5
0
1.5
3.0
4.5
[PHOSPHOARGININE] mM

( D )

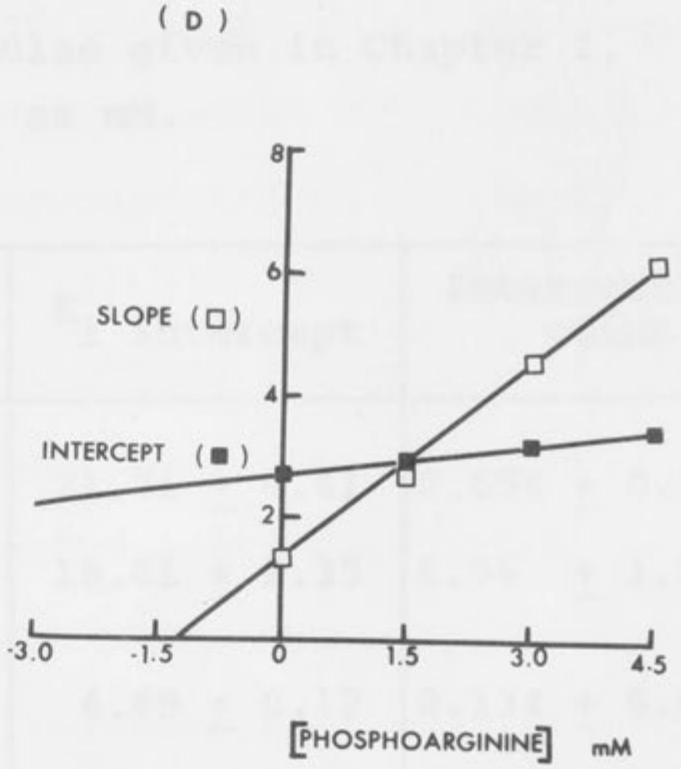

8
6
4
2
SLOPE (□)
INTERCEPT (■)
-3.0
-1.5
0
1.5
3.0
4.5
[PHOSPHOARGININE] mM

TABLE IV.1. APPARENT INHIBITION CONSTANTS AND INTERSECTION POINTS FOR LINEAR NON-COMPETITIVE PRODUCT INHIBITION OF ARGININE KINASE.

The data of Figs. IV.1, 2, 3 and 4 and similar experiments have been analysed with the NONCOMP computer programme of Cleland (1963b) to yield values for the apparent inhibition constants, $K_{i\ slope}$ and $K_{i\ intercept}$, and for the intersection points, when the four substrates MgATP, arginine, MgADP and phosphoarginine were used as product inhibitors of the arginine kinase reaction. All values are the weighted means of three or four experiments except for those given for product inhibition by MgADP and phosphoarginine with respect to MgATP (2 experiments) and for MgADP inhibition with respect to arginine (1 experiment). The weighted means and their standard errors have been calculated using the formulae given in Chapter I, p.44. All constants are expressed as mM.

| Product Inhibitor | Variable Substrate | $K_{i\ slope}$ | $K_{i\ intercept}$ | Intersection point |
|---|---|---|---|---|
| $MgATP^{2-}$ | $MgADP^{-}$ | 4.09 ± 0.32 | 21.71 ± 4.41 | 0.654 ± 0.177 |
| $MgATP^{2-}$ | Phospho-arginine | 4.28 ± 0.35 | 19.01 ± 2.35 | 6.56 ± 1.21 |
| Arginine | $MgADP^{-}$ | 5.65 ± 0.51 | 4.69 ± 0.12 | 0.134 ± 0.011 |
| Arginine | Phospho-arginine | 1.66 ± 0.08 | 18.16 ± 3.79 | 18.15 ± 5.32 |
| $NgADP^{-}$ | $MgATP^{2-}$ | 0.115 ± 0.036 | 0.264 ± 0.080 | 1.72 ± 0.67 |
| $MgADP^{-}$ | Arginine | 0.273 ± 0.097 | 0.334 ± 0.093 | 1.10 ± 0.26 |
| Phospho-arginine | $MgATP^{2-}$ | 1.85 ± 0.21 | 11.26 ± 1.43 | 1.75 ± 0.33 |
| Phospho-arginine | Arginine | 1.40 ± 0.09 | 17.09 ± 3.17 | 4.45 ± 1.18 |

under the conditions of the particular experiment (Cleland, 1963c). In view of the complexity of the results, reference to calculations that have been made using these apparent constants will be made in the Discussion.

Product inhibition by phosphoarginine

From analysis of the substrate inhibition caused by higher concentrations of phosphoarginine (Chapter III) it would appear that phosphoarginine is able to form several dead-end complexes with arginine kinase, and therefore it might be expected that, if these complexes are important, product inhibition by phosphoarginine would be non-linear rather than linear. To eliminate or minimize this non-linearity the concentration of phosphoarginine used as an inhibitor did not exceed 4.5 mM which value is less than 1/17th of the $K_{i1}$ value and less than 1/3rd of the $K_{i2}$ value. The linearity of the replots (Fig. IV.6c & d) and the good fit of the experimental data of Fig. IV.4 to the lines drawn assuming linear non-competitive inhibition both suggest that this non-linearity is, in fact, unimportant under the experimental conditions. It is possible to fit the slopes of Fig. IV.6c & d to the equation for a parabola (Fig. IV.7). The resulting parabolas, however, deviate little from straight lines. Moreover, the first inhibition constants obtained from the PARA analysis (Table IV.2) do not differ markedly from the $K_{i\ slope}$ values obtained on the assumption that the slopes are linear functions of the phosphoarginine concentration

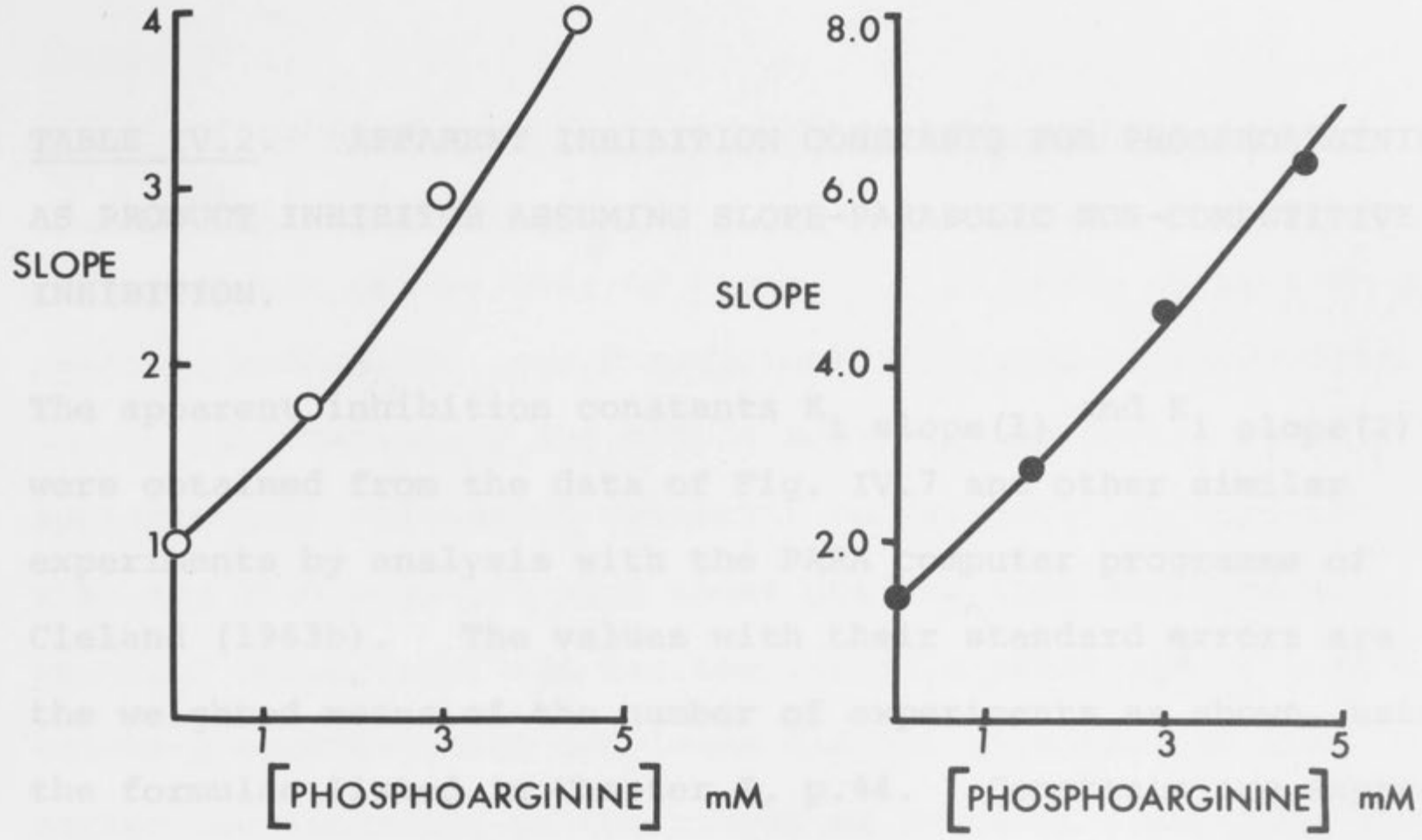



FIG. IV.7. Replots of the slopes of the lines of double reciprocal plots obtained using phosphoarginine as product inhibitor with respect to $MgATP^{2-}$ (a) and arginine (b) (Figs. IV.4a & b) against the concentration of phosphoarginine. The curves have been drawn using kinetic constants from analysis of the data by the PARA computer programme of Cleland (1963b).

TABLE IV.2. APPARENT INHIBITION CONSTANTS FOR PHOSPHOARGININE AS PRODUCT INHIBITOR ASSUMING SLOPE-PARABOLIC NON-COMPETITIVE INHIBITION.

The apparent inhibition constants $K_{i\ slope(1)}$ and $K_{i\ slope(2)}$ were obtained from the data of Fig. IV.7 and other similar experiments by analysis with the PARA computer programme of Cleland (1963b). The values with their standard errors are the weighted means of the number of experiments as shown, using the formulae listed in Chapter I, p.44. Constants are expressed as mM.

| Variable substrate | No. of experiments | $K_{i\ slope(1)}$ | $K_{i\ slope(2)}$ |
|---|---|---|---|
| $MgATP^{2-}$ | 2 | 2.64 ± 0.65 | 6.08 ± 2.94 |
| Arginine | 4 | 1.66 ± 0.05 | 8.88 ± 4.18 |

(Table IV.1).

Effect of substrate inhibition when phosphoarginine is used as the variable substrate

Analysis of the data of Table IV.1 in terms of Ping-Pong reaction mechanisms (see Discussion) indicated that the data do not fit completely any simple scheme. Moreover, it appeared that calculated constants consistently at variance with any rate equation were those derived from experiments in which phosphoarginine was the variable substrate. Dead-end enzyme-phosphoarginine complexes did not give rise to any marked non-linearity of the slopes or vertical intercepts of double reciprocal plots when phosphoarginine was the product inhibitor, but it appeared that they may be of importance when phosphoarginine was used at a higher range of concentrations as the variable substrate. From a study of substrate inhibition (Chapter III, Fig. III.6) it is known that reciprocal plots of velocity against substrate concentration become non-linear as the substrate concentration is increased. Therefore, true values for the apparent inhibition constants will be obtained only when the substrate concentrations are sufficiently low for the slopes of the individual lines of the experiment to be unaffected by the dead-end complex formation. This is true only if the slopes are asymptote values for the curve that would be obtained if phosphoarginine were varied over a wide range. If phosphoarginine formed only one dead-end complex, it would be possible to

evaluate these asymptotes by using a wide range of phosphoarginine concentrations and analysing individual lines with the SUBINH computer programme (preface, equation 9), but in view of the complexity of the inhibition, as illustrated in Fig. III.7, this cannot be done. Instead, the experiment using MgATP as product inhibitor was repeated with phosphoarginine varied over the range 5 mM to 1 mM as compared with the range 10 mM to 2 mM which was used for Fig. IV.1b and for the initial velocity experiments of Chapter III, Fig. III.2 (Fig. IV.8). The apparent inhibition constants (Table IV.3) and the intersection points of the lines are different from those listed in Table IV.1. In addition, reanalysis of the experimental data obtained with arginine as product inhibitor and phosphoarginine as variable substrate (Fig. IV.2b) when the velocities obtained with 10 mM phosphoarginine are omitted, alters the apparent inhibition constants (cf. Table IV.1 with Table IV.3). These results indicate that the apparent inhibition constants obtained with phosphoarginine as variable substrate are subject to error arising from substrate inhibition, so it is necessary to analyse the product inhibition data without reference to these experiments.

## DISCUSSION

As a result of the experiments described in Chapter III, it was concluded that the reaction catalysed by the sea-crayfish

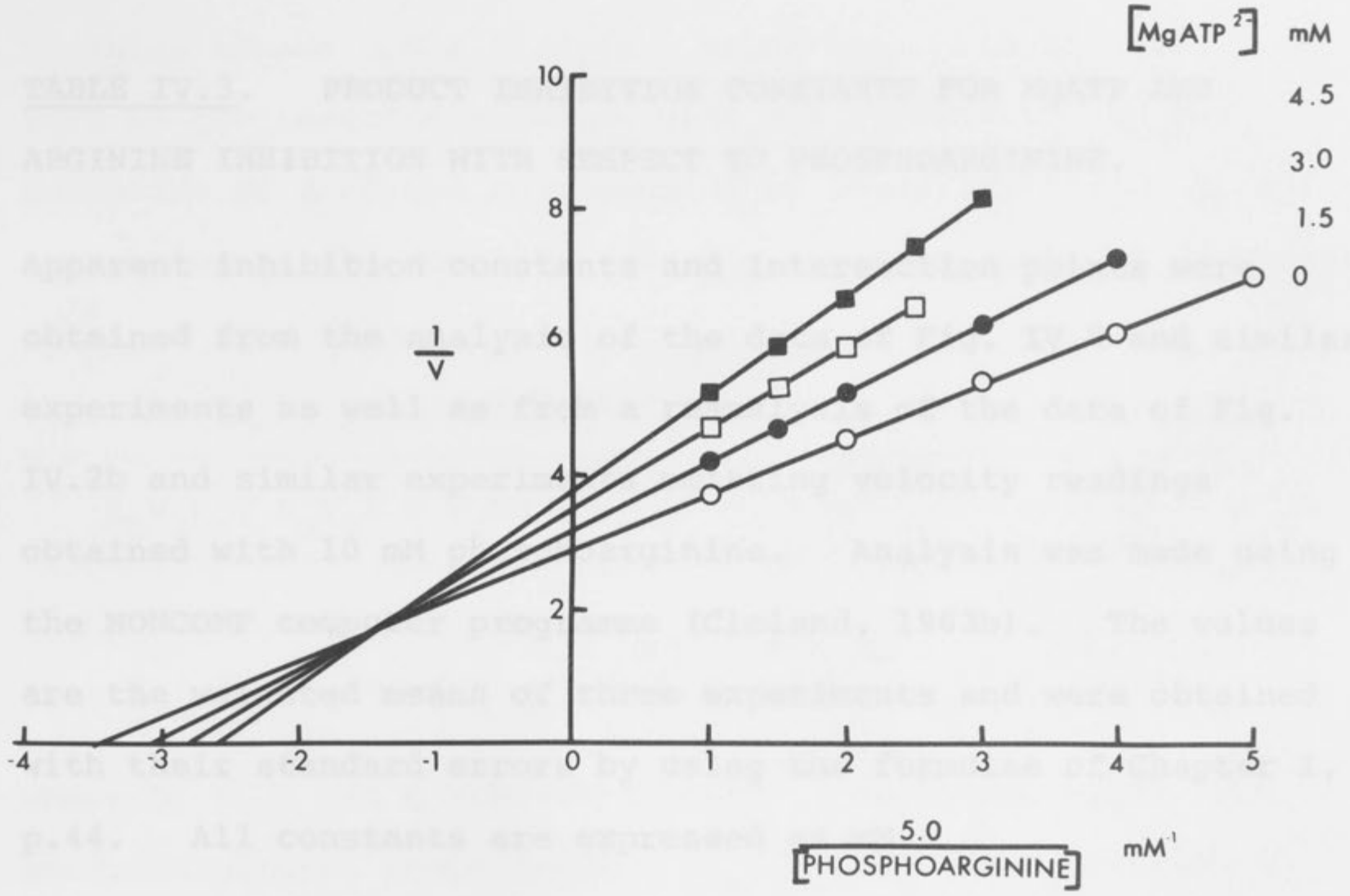



FIG. IV.8. Product inhibition by $MgATP^{2-}$ of the reverse reaction catalysed by arginine kinase with phosphoarginine as the variable substrate at concentrations ranging from 5 mM to 1 mM. The concentration of $MgADP^{-}$ held constant at 0.5 mM. The lines were drawn using kinetic constants obtained from analysis of the data by the NONCOMP computer programme (Cleland, 1963b).

TABLE IV.3. PRODUCT INHIBITION CONSTANTS FOR MgATP AND ARGININE INHIBITION WITH RESPECT TO PHOSPHOARGININE.

Apparent inhibition constants and intersection points were obtained from the analysis of the data of Fig. IV.8 and similar experiments as well as from a reanalysis of the data of Fig. IV.2b and similar experiments omitting velocity readings obtained with 10 mM phosphoarginine. Analysis was made using the NONCOMP computer programme (Cleland, 1963b). The values are the weighted means of three experiments and were obtained with their standard errors by using the formulae of Chapter I, p.44. All constants are expressed as mM.

| Product Inhibitor | $K_i$ slope | $K_i$ intercept | Intersection point |
|---|---|---|---|
| $MgATP^{2-}$ | 6.77 ± 0.54 | 10.59 ± 0.72 | 2.30 ± 0.30 |
| arginine | 1.77 ± 0.23 | 9.58 ± 2.81 | 8.87 ± 3.40 |

arginine kinase has a Ping-Pong mechanism. It follows, then, that if the reaction sequence were not complicated by the formation of dead-end complexes or by isomerization of stable enzyme forms, the complete velocity equation could be expressed as :

$$v = \frac{V_1V_2AB - \frac{V_1V_2PQ}{K_{eq}}}{K_bV_2A + K_aV_2B + V_2AB + \frac{V_2K_{ia}K_bP}{K_{ip}} + \frac{V_2K_aK_{ib}Q}{K_{iq}} + \frac{V_2K_aK_{ib}PQ}{K_pK_{iq}} + \frac{V_2K_bAP}{K_{ip}} + \frac{V_2K_aBQ}{K_{iq}}} \qquad (1)$$

where A, B, P and Q represent $MgATP^{2-}$, arginine, $MgADP^{-}$ and phosphoarginine, respectively; $K_a$, $K_b$, $K_p$ and $K_q$ represent Michaelis constants and $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ represent dissociation constants for A, B, P and Q (Cleland, 1963a). The product inhibition pattern expected for this velocity equation is set out below and may be compared with the pattern actually obtained with arginine kinase for which the results are given in brackets :

| | Variable substrate | | | |
|---|---|---|---|---|
| Product inhibitor | MgATP | arginine | MgADP | phospho-arginine |
| MgATP | | | NC (NC) | C (NC) |
| arginine | | | C (NC) | NC (NC) |
| MgADP | NC (NC) | C (NC) | | |
| phosphoarginine | C (NC) | NC (NC) | | |

where NC and C represent non-competitive and competitive inhibition respectively. It is apparent that the results obtained are not consistent with the pattern for a basic Ping-Pong mechanism, as all product inhibitions are non-competitive. Thus it appears likely that either dead-end complex formation or isomerization of stable enzyme forms must occur, since either of these effects will yield a product inhibition pattern consisting only of non-competitive inhibitions.

In order to distinguish between dead-end complex formation and isomerization of stable enzyme forms, it is necessary to consider the quantitative aspects of the results, and, in the first instance, the experimental data will be discussed in terms of dead-end complex formation. A dead-end complex may be formed by combination of a reactant with either :

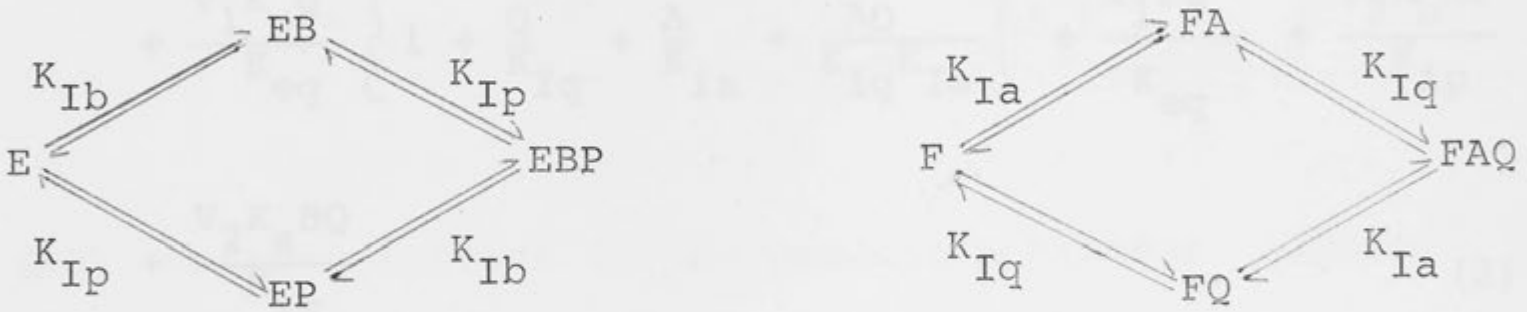
1. the stable enzyme form with which it does not combine as substrate; or
2. one of the binary enzyme forms.

1. Inhibition brought about by combination of a reactant with one of the stable forms of the enzyme.

Inhibition resulting from reaction with a stable form of enzyme can be analysed kinetically since the distribution equations for these forms are given by :

$$\frac{E}{E_t} = \frac{K_a V_2 B + \frac{K_q V_1 P}{K_{eq}}}{\text{denom. of rate equation}} \quad ; \quad \frac{F}{E_t} = \frac{K_b V_2 B + \frac{K_p V_1 Q}{K_{eq}}}{\text{denom. of rate equation}}$$

where E and F represent the free form and the E-P form of the enzyme, respectively. It is necessary for the most general case to consider the reactions of A (MgATP) and Q (phosphoarginine) with F, and of B (arginine) and P (MgADP) with E. Since the nucleotide and guanidino substrate are chemically dissimilar, it would seem reasonable to assume that there are separate binding sites on the enzyme for each type of reactant and that the formation of the ternary complexes FAQ and EBP could occur possibly by reactions of the type :




Scheme 1.

For the purposes of analysis, it is being assumed that the binding of one substrate does not affect the binding of a second substrate to the same enzyme form.

The dead-end complexes involving phosphoarginine (Q) have been considered in detail already (Chapter III, p.98) and it would appear that, in the presence of relatively high concentrations of this substrate, a second molecule is able to combine with F and that at least one molecule is able to combine with one of the other forms of the enzyme. The combination of only one molecule is considered here because

the concentration of phosphoarginine used for product inhibition did not exceed 4.5 mM.

When allowance is made for the reactions illustrated in scheme (1), the complete rate equation may be written:

$$v = \frac{V_1V_2AB - \frac{V_1V_2PQ}{K_{eq}}}{V_2K_bA\left\{1 + \frac{Q}{K_{Iq}} + \frac{A}{K_{Ia}} + \frac{AQ}{K_{Ia}K_{Iq}}\right\} + V_2K_aB\left\{1 + \frac{P}{K_{Ip}} + \frac{B}{K_{Ib}} + \frac{PB}{K_{Ip}K_{Ib}}\right\} + V_2AB + \frac{V_1K_qP}{K_{eq}}\left\{1 + \frac{P}{K_{Ip}} + \frac{B}{K_{Ib}} + \frac{PB}{K_{Ip}K_{Ib}}\right\} + \frac{V_1K_pQ}{K_{eq}}\left\{1 + \frac{Q}{K_{Iq}} + \frac{A}{K_{Ia}} + \frac{AQ}{K_{Iq}K_{Ia}}\right\} + \frac{V_1PQ}{K_{eq}} + \frac{V_2K_bAP}{K_{ip}} + \frac{V_2K_aBQ}{K_{iq}}} \qquad (2)$$

where the additional kinetic constants, $K_{Ia}$, $K_{Ib}$, $K_{Ip}$ and $K_{Iq}$, represent the dissociation constants for the reactions shown in scheme (1). To predict the type of product inhibition to be expected from a reaction conforming to this rate equation, the complete equation is modified by setting to zero the concentration of the product that is not present. The resulting equations may be rearranged in reciprocal form according to the substrate that is being varied to give the expressions as shown below:

(i) $MgADP^-$ (P) as product inhibitor. $MgATP^{2-}$ (A) and arginine (B) as variable substrates :

$$\frac{1}{v} = \frac{K_a}{V_1}\left[1 + \frac{B}{K_{Ib}} + \frac{P}{K_{Ip}} + \frac{BP}{K_{Ib}K_{Ip}} + \frac{K_{ia}K_bP}{K_aK_{ip}}\left\{\frac{1}{B} + \frac{1}{K_{Ib}} + \frac{P}{K_{Ip}B} + \frac{P}{K_{Ib}K_{Ip}}\right\}\right]\frac{1}{A} + \frac{K_bA}{V_1K_{Ia}B} + \frac{1}{V_1}\left[\frac{K_b}{B} + 1 + \frac{K_bP}{K_{ip}B}\right] \qquad [2(i)]$$

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{A}{K_{Ia}} + \frac{P}{K_{ip}}\left\{1 + \frac{K_{ia}}{A} + \frac{K_{ia}P}{K_{Ip}A}\right\}\right]\frac{1}{B} + \frac{K_aBP}{V_1K_{Ib}K_{Ip}A} + \frac{1}{V_1}\left[\frac{K_a}{A} + 1 + \frac{K_aP}{K_{Ip}A} + \frac{K_{ia}K_bP}{K_{ip}K_{Ib}A} + \frac{K_{ia}K_bP^2}{K_{ip}K_{Ip}K_{Ib}A}\right] \qquad [2(ii)]$$

(ii) Phosphoarginine (Q) as product inhibitor. $MgATP^{2-}$ (A) and arginine (B) as variable substrates :

$$\frac{1}{v} = \frac{K_a}{V_1}\left[1 + \frac{B}{K_{Ib}} + \frac{Q}{K_{iq}}\left\{1 + \frac{K_{ib}}{B} + \frac{K_{ib}Q}{K_{Iq}B}\right\}\right]\frac{1}{A} + \frac{K_bAQ}{V_1K_{Ia}K_{Iq}B} + \frac{1}{V_1}\left[\frac{K_b}{B} + 1 + \frac{K_bQ}{K_{Iq}B} + \frac{K_aK_{ib}Q}{K_{iq}K_{Ia}B} + \frac{K_aK_{ib}Q^2}{K_{iq}K_{Iq}K_{Ia}B}\right] \qquad [2(III)]$$

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{A}{K_{Ia}} + \frac{Q}{K_{Iq}} + \frac{AQ}{K_{Ia}K_{Iq}} + \frac{K_aK_{ib}Q}{K_bK_{iq}}\left\{\frac{1}{A} + \frac{1}{K_{Ia}} + \frac{Q}{K_{Iq}A} + \frac{Q}{K_{Iq}K_{Ia}}\right\}\right]\frac{1}{B} + \frac{K_aB}{V_1K_{Ib}A} + \frac{1}{V_1}\left[\frac{K_a}{A} + 1 + \frac{K_aQ}{K_{iq}A}\right] \qquad [2(iv)]$$

Because the complete velocity equation is symmetrical, similar equations may be written for the reaction with MgADP and phosphoarginine as substrates and MgATP and arginine as product inhibitors.

All equations predict that substrate inhibition will be observed as the concentration of the variable substrate is raised. While there was no clear-cut evidence for substrate inhibition (Figs. IV.1, IV.2, IV.3, IV.4), indications of substrate inhibition with phosphoarginine as the variable substrate were found by repeating the experiment of Fig. IV.1b over a lower range of phosphoarginine concentrations (Fig. IV.8). This resulted in different values for the apparent inhibition constants (cf. Table IV.1 & Table IV.3). In addition, it is known (Chapter III) that phosphoarginine forms several dead-end complexes with arginine kinase, so it would seem that the concentrations used are sufficient for these complexes to affect the initial velocity. On the other hand, no sign of substrate inhibition has been found by increasing the concentration of MgADP to 2.0 mM in the presence of 2.0 mM phosphoarginine, by increasing the concentration of arginine to 12 mM in the presence of 0.6 mM MgATP, or by increasing the concentration of MgATP to 8 mM in the presence of 0.6 mM arginine. (It was difficult to use higher concentrations of MgATP as the velocity was measured by assaying the ADP released and it was not possible to obtain ATP that contained less than 1% ADP). Therefore,

it would seem likely that, with the highest concentrations of the variable substrates MgATP (3.0 mM), arginine (3.0 mM) and MgADP (1.0 mM) well below these levels, the experiments follow linear kinetics. It is appropriate, then, to consider as equal to zero those terms of equation (2) which express substrate inhibition solely as a function of the concentration of one of these three substrates.

From the rearrangements of equation (2), it can be seen that substrate inhibition should arise also when the variable substrate and product inhibitor form a ternary dead-end complex, and that the inhibition observed will be a function of the products of the concentrations of the variable substrate and the product inhibitor. Therefore, inhibition should be observed in the presence of lower concentrations of substrate as the product concentration is increased. Since this effect is not observed in any of the experiments (Figs. IV.1 to IV.4), it may be concluded that, at least under the experimental conditions, such ternary complexes are unimportant.

The equations predict also that, even without ternary dead-end complex formation and substrate inhibition, the slopes of all and the vertical intercepts of four of the double reciprocal plots will be parabolic functions of the inhibitor concentration. Experimentally, there was no evidence for non-linearity of the vertical intercept replots. The only possible evidence for non-linearity of slope replots comes from the experiments in which phosphoarginine was used

as product inhibitor, and here, as shown in Fig. IV.7, the parabolas deviate so little from straight lines that this deviation could be within the range of experimental variation. Therefore, if the equation derived from a consideration of dead-end complexes is to fit the experimental data, terms expressing the parabolic nature of slope or vertical intercept must be set equal to zero. When this is done, the equations as rearranged in the manner of equations 2(i - iv) will predict linear non-competitive inhibition.

Regardless of the number and nature of the dead-end complexes, the vertical intercept terms of four of the eight different rearrangements of equation (2) are linear and from the apparent $K_{i\ intercept}$ values it is possible to calculate values for the dissociation constants $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ (Table IV.4). These constants, along with the values for the Michaelis constants and maximum velocities (Chapter III, Table III.1), can be used to calculate the Haldane relationships applicable to a Ping-Pong reaction mechanism (Table IV.5). Such calculations show that there is a marked variation in the values for $K_{eq}$ and that only one is in agreement with the experimental value of 0.3. In this connection, it should be pointed out that the results obtained using $K_{ib}$ could be in error as this value was obtained from an experiment in which phosphoarginine was the variable substrate. Nevertheless, it is clear that the Haldane relationship not involving $K_{ib}$ does not agree with the

TABLE IV.4. DISSOCIATION CONSTANTS FOR A PING-PONG MECHANISM

The dissociation constants have been calculated on the basis that arginine kinase has a basic Ping-Pong mechanism by equating the relationships for $K_{i\ intercept}$ obtained from the four experiments in which non-competitive inhibition is expected for a Ping-Pong mechanism with the experimental values for these $K_{i\ intercept}$ constants as listed in Table IV.1 and Table IV.3. The values for the Michaelis constants are listed in Table III.1. The standard errors of the dissociation constants have been calculated with the formulae for the standard errors of products and quotients as given in Chapter I, pp. 44 & 45. All constants are expressed as mM.

| $K_{i\ intercept}$ | Experimental value | Dissociation constant | Calculated value |
|---|---|---|---|
| $\frac{K_{ia}(K_q + Q)}{K_q}$ | 21.71 ± 4.41 | $K_{ia}$ | 2.99 ± 0.13 |
| $\frac{K_{ib}(K_p + P)}{K_p}$ | 18.16 ± 3.79 | $K_{ib}$ | 4.63 ± 0.98 |
| | 9.58 ± 2.81 | | 2.44 ± 0.21 |
| $\frac{K_{ip}(K_b + B)}{K_b}$ | 0.264 ± 0.080 | $K_{ip}$ | 0.059 ± 0.018 |
| $\frac{K_{iq}(K_a + A)}{K_a}$ | 17.09 ± 3.17 | $K_{iq}$ | 2.99 ± 0.57 |

TABLE IV.5. THE HALDANE RELATIONSHIPS OF A PING-PONG MECHANISM

The Haldane relationships applicable to a Ping-Pong mechanism have been calculated using the values for the dissociation constants given in Table IV.4 together with the values for the maximum velocities and Michaelis constants as listed in Table III.1. The standard errors for the Haldane relationships have been calculated using the formulae for the standard errors of products and quotients as given in Chapter I, pp. 44 & 45.

| $K_{eq}$ = | $\frac{K_{ip}\,K_{iq}}{K_{ia}\,K_{ib}}$ = | $\frac{V_1 K_{ip}\,K_q}{V_2 K_{ia}\,K_b}$ = | $\frac{V_1 K_p K_{iq}}{V_2 K_a K_{ib}}$ |
|---|---|---|---|
| | $0.024 \pm 0.003$ | $0.034 \pm 0.011$ | $0.313 \pm 0.068$ |

experimental value and therefore does not support the idea that equation (2) represents the mechanism of the arginine kinase reaction.

While the same values for $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ will be obtained regardless of the number and nature of the dead-end complexes, it is not necessary to consider the formation of all four dead-end complexes in order to obtain an equation for which all product inhibitions are non-competitive. Non-competitive inhibitions would be obtained also if there were only two dead-end complexes as long as one of these was formed by a reactant combining with form E of the enzyme while the other was formed by a reactant combining with form F. If either $K_{Ia}$ or $K_{Iq}$ and either $K_{Ib}$ or $K_{Ip}$ are set equal to infinity, rearrangement of equation (2) will still predict non-competitive inhibition. Because phosphoarginine has been shown to form dead-end complexes, it would seem reasonable to retain $K_{Iq}$ and set $K_{Ia}$ equal to infinity, but as neither MgADP nor arginine has been shown to exhibit substrate inhibition it is not clear which of these should be retained to explain the non-competitive inhibition observed when arginine is used as product inhibitor with respect to MgADP and visa versa. Because of the resemblance between phosphoarginine and arginine it has been decided to set $K_{Ip}$ equal to infinity and examine the experimental data in terms of phosphoarginine and arginine dead-end complexes.

When $K_{Ia}$ and $K_{Ip}$ of equation (2) are set equal to infinity, the equation simplifies to :

$$v = \frac{V_1V_2AB - \frac{V_1V_2PQ}{K_{eq}}}{V_2K_bA\left(1 + \frac{Q}{K_{Iq}}\right) + K_aV_2B\left(1 + \frac{B}{K_{Ib}}\right) + V_2AB + \frac{V_2K_{ia}K_bP}{K_{ip}}\left(1 + \frac{B}{K_{Ib}}\right)}$$

$$+ \frac{V_2K_aK_{ib}Q}{K_{iq}}\left(1 + \frac{Q}{K_{Iq}}\right) + \frac{V_2K_aK_{ib}PQ}{K_pK_{iq}} + \frac{V_2K_bAP}{K_{ip}} + \frac{V_2K_aBQ}{K_{iq}} \qquad (3)$$

This equation predicts that :

1. all product inhibition experiments will show non-competitive inhibition;

2. product inhibition by either B (arginine) or Q (phospho-arginine) will be of a slope-parabolic, intercept-linear nature;

3. when either B or Q is the variable substrate, higher concentrations will give rise to substrate inhibition.

As has been discussed, all inhibitions fit the equation for slope-linear, intercept-linear non-competitive inhibition and there is no sign of substrate inhibition with B as variable substrate, but some effect of substrate inhibition when phosphoarginine is the variable substrate in Figs. IV.1b and IV.2b, though this is not apparent on inspection of the figures. If equation (3) is rearranged in the manner shown for equation (2) and terms containing the second power of the product inhibitor set equal to zero, then the relationships expressing the

values for the apparent inhibition constants and the intersection points of the lines are as listed in Table IV.6. Because the same relationships hold for the apparent $K_{i\ intercept}$ constants, the values for the dissociation constants and for the Haldane relationships will be the same (Tables IV.4 and IV.5). Therefore the same criticism holds (p.131).

With the use of the experimental $K_{i\ intercept}$ data and the relationships of Table IV.6, the inhibition constants $K_{Ib}$ and $K_{Iq}$ were calculated to be 0.65 $\pm$ 0.03 mM and 2.53 $\pm$ 0.34 mM, respectively. The value for $K_{Ib}$ would not seem compatible with the linear kinetics observed with arginine as the variable substrate over the range 3.0 to 0.6 mM used in all these experiments and is difficult to reconcile with the complete lack of substrate inhibition by arginine at a concentration of 12 mM. The value of 2.53 mM for $K_{Iq}$ is very much lower than those calculated from the experiments studying substrate inhibition (Chapter III). If MgADP, rather than arginine, is considered to form the dead-end complex with E, $K_{Ip}$ is calculated to be 0.058 mM, a value that would seem to be incompatible with the linear kinetics observed when MgADP is used as the variable substrate at concentrations up to 1.0 mM.

Since the same apparent $K_{i\ intercept}$ values and relationships are used to calculate the dissociation constants for any mechanism involving dead-end constants, then if it is assumed that dead-end complexes are responsible for the

TABLE IV.6. APPARENT INHIBITION CONSTANTS FOR A PING-PONG REACTION MECHANISM WITH TWO DEAD-END COMPLEXES, ASSUMING LINEAR NON-COMPETITIVE INHIBITION.

The relationships between the kinetic constants of equation (3) and the apparent inhibition constants have been derived on the basis that all reactants give rise to linear or non-competitive inhibition when used as product inhibitors and that substrate inhibition is not effective when the two reactants forming dead-end complexes (B and Q) are used as variable substrates.

| Product Inhibitor | Variable Substrate | $K_i$ slope | $K_i$ intercept | Intersection point |
|---|---|---|---|---|
| A | P | $\frac{K_{ia}K_pQ}{K_qK_{ip}}$ | $\frac{K_{ia}(K_q+Q)}{K_q}$ | $\frac{K_{iq}}{K_{ip}(K_{iq}+Q)}$ |
| | Q | $\frac{K_{ia}P}{(K_{ip}+P)}$ | $\frac{K_{ia}K_{Iq}(K_p+P)}{K_qK_{ip}}$ | $\frac{K_{ip}}{K_{Iq}(K_{ip}+P)}$ |
| B | P | $\frac{K_{ib}Q(K_{Iq}+Q)}{K_{Iq}(K_{iq}+Q)}$ | $\frac{K_{Ib}(K_q+Q)}{K_q}$ | $\frac{K_qK_{ib}}{K_{Ib}(K_pK_{iq}+K_pQ)}$ |
| | Q | $\frac{K_{Ib}K_qK_{ib}P}{K_qK_{ib}P+K_{Ib}K_{iq}K_p}$ | $\frac{K_{ib}(K_p+P)}{K_p}$ | $\frac{K_pK_{Ib}}{K_qK_{ib}P+K_{Ib}K_{iq}K_p}$ |
| P | A | $\frac{K_{ip}K_aK_{Ib}}{K_{ia}K_b}$ | $\frac{K_{ip}(K_b+B)}{K_b}$ | $\frac{K_{Ib}}{K_{ia}(K_{Ib}+B)}$ |
| | B | $\frac{K_{ip}A}{(K_{ia}+A)}$ | $\frac{K_{ip}K_{Ib}(K_a+A)}{K_{ia}K_b}$ | $\frac{K_{ia}}{K_{Ib}(K_{ia}+A)}$ |
| Q | A | $\frac{K_{iq}B(K_{Ib}+B)}{(K_{ib}+B)K_{Ib}}$ | $\frac{K_{Iq}(K_b+B)}{K_b}$ | $\frac{K_bK_{iq}}{K_{Iq}(K_aK_{ib}+K_aB)}$ |
| | B | $\frac{K_{Iq}K_bK_{iq}A}{K_aK_{ib}K_{Ib}+K_bK_{iq}A}$ | $\frac{K_{iq}(K_a+A)}{K_a}$ | $\frac{K_aK_{Iq}}{K_bK_{iq}A+K_aK_{ib}K_{Iq}}$ |

non-competitive inhibition observed between MgADP and arginine and between MgATP and phosphoarginine the decision as to which complexes form must be made from the values for the inhibition constants. The feasibility of the values calculated from the experimental data must be judged from their relationships with the range of concentrations used as variable substrates. In addition, from the values for the dissociation and inhibition constants, it is possible to calculate theoretical values for the apparent $K_{i\ slope}$ constants and the intersection points of the lines of the double reciprocal plots, and these may be compared with the experimental values. When this is done for equation (3) and the resulting values are compared with those in Table IV.1 - using a value of 2.44 mM for $K_{ib}$ - the calculated values do not agree with the experimental for any one experiment (Table IV.7). Therefore, it would seem reasonable to conclude that, in this case at least, the experimental data do not fit an equation for a Ping-Pong mechanism with dead-end complex formation.

2. Ternary dead-end complexes formed by combination of reactants with binary enzyme forms.

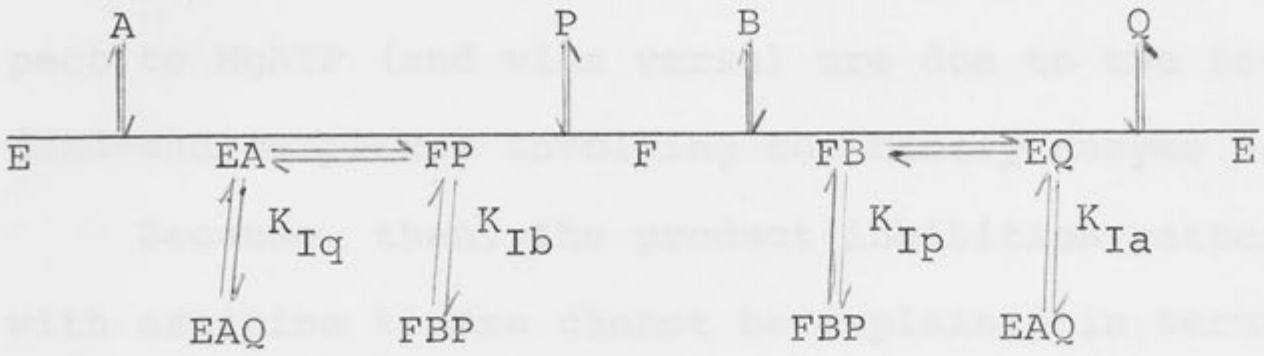
The formation of ternary dead-end complexes was considered as a likely mechanism to explain the non-competitive nature of the inhibition when phosphoarginine was used as an inhibitor with respect to MgADP as the variable substrate (Chapter III, p. 98). If the four substrates of the reaction were able to

TABLE IV.7. CALCULATED VALUES FOR THE APPARENT INHIBITION CONSTANTS AND INTERSECTION POINTS DERIVED FROM EQUATION 3.

Values for $K_{i\ slope}$, $K_{i\ intercept}$ and the intersection points have been calculated from the relationships given in Table IV.6 using values of 2.99, 2.44, 0.059 and 2.99 mM for $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ respectively, and 0.65 and 2.53 for $K_{Ib}$ and $K_{Iq}$ respectively. Theoretical values have been calculated for those apparent inhibition constants and intersection points that were not used to derive the dissociation and inhibition constants listed above. All values are expressed as mM.

| Product Inhibitor | Variable Substrate | $K_{i\ slope}$ | $K_{i\ intercept}$ | Intersection point |
|---|---|---|---|---|
| MgATP | MgADP | 54.2 | | 3.42 |
| MgATP | phospho-arginine | 2.7 | 53.8 | 0.042 |
| arginine | MgADP | 4.54 | | 4.40 |
| arginine | phospho-arginine | 0.56 | | 0.049 |
| MgADP | MgATP | 0.01 | | 0.06 |
| MgADP | arginine | 2.31 | 0.054 | 0.77 |
| phospho-arginine | MgATP | 9.26 | | 0.298 |
| phospho-arginine | arginine | 2.24 | | 0.137 |

form such dead-end complexes, the reaction mechanism could be represented diagrammatically as :



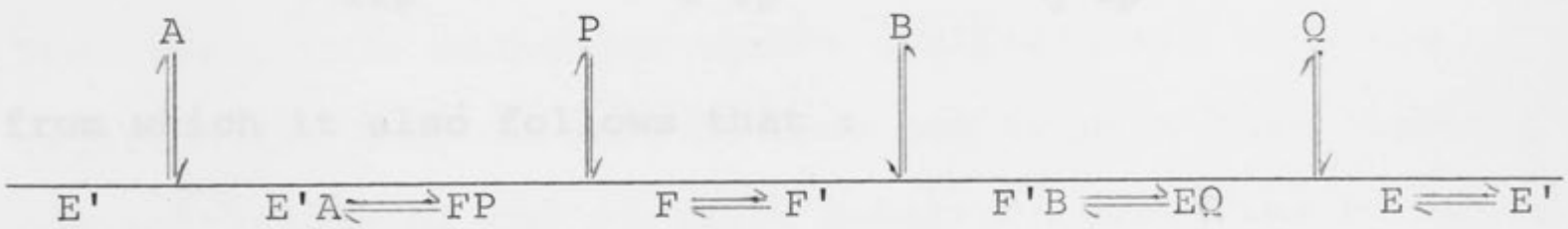

It can be seen from inspection, and verified by derivation of the rate equation, that such dead-end complexes would result in a product inhibition pattern in which all product inhibitions were non-competitive. However, as the distribution of enzyme among the binary forms cannot be expressed in kinetic terms, the velocity equation cannot be written in kinetic terms, and the quantitative effects of the dead-end complexes cannot be compared with the experimental results. But it must be pointed out that such a mechanism predicts that all substrates will give rise to substrate inhibition. In addition, if we consider, for example, the experiment in which arginine (B) is product inhibitor with respect to MgADP (P) as variable substrate, (Fig. IV.2a) then the inhibitor (B) combines with F as part of the normal reaction sequence and with FP as a dead-end inhibitor; at non-saturating concentrations of MgADP (P) these two points of addition are connected along the reaction sequence, so that the inhibition should be of a slope-parabolic, intercept-linear nature, whereas the replots of both slope and intercept (Fig. IV.5c) are linear.

Thus there is no quantitative evidence to suggest the idea that the non-competitive inhibitions by arginine with respect to MgADP (and vice versa), and by phosphoarginine with respect to MgATP (and vice versa) are due to the formation of dead-end complexes involving the binary enzyme forms.

Because, then, the product inhibition pattern obtained with arginine kinase cannot be explained in terms of dead-end complex formation, it becomes necessary to examine quantitatively a second Ping-Pong reaction mechanism which will give rise to a product inhibition pattern in which all product inhibitionsare non-competitive. This is a Ping-Pong mechanism in which both stable forms of the enzyme isomerize.

Product inhibition for a Di Iso Ping-Pong reaction mechanism

This mechanism assumes that each stable form of the enzyme exists in two different conformational states which are in equilibrium with each other, and that the change from one conformation to the other occurs as part of the reaction sequence. The reaction mechanism can be represented diagrammatically as :




It will be noticed that the substrates A and Q, which with a basic Ping-Pong mechanism combine with the same form of enzyme, now combine with different conformations of the same form,

viz. E' and E. Thus if Q is used as a product inhibitor with respect to A, saturation with A, which combines with E', will not prevent Q from combining with E so that the inhibition will be non-competitive rather than competitive. Similarly, B and P become non-competitive inhibitors.

In order to translate the equation for a Ping-Pong mechanism with two isomerizations of stable enzyme forms from rate constants into kinetic constants, additional kinetic constants must be defined. For conversion of the rate equation into kinetic terms only two constants need be defined, provided that one of these is associated with a reactant that combines with one of the E forms and that the other is associated with a reactant combining with one of the F forms. It is possible, however, to define four constants, one associated with each reactant, that satisfy the relationships :

$$(1) \qquad \frac{K_{iia}}{K_{iiq}} = \frac{V_1 K_{ia}}{V_2 K_{iq}} = \frac{K_a K_{ip}}{K_p K_{iq}}$$

$$(2) \qquad \frac{K_{iib}}{K_{iip}} = \frac{V_1 K_{ib}}{V_2 K_{ip}} = \frac{K_b K_{iq}}{K_q K_{ip}}$$

from which it also follows that :

$$\frac{K_{iia} K_{iip}}{K_{iib} K_{iiq}} = \frac{K_{ia} K_{ip}}{K_{ib} K_{iq}}$$

and

$$\frac{K_{iia} K_{iib}}{K_{iip} K_{iiq}} = \frac{K_a K_b}{K_p K_q}$$

When all four of the additional kinetic constants are used, in addition to those previously defined (Cleland, 1963a), the complete rate equation becomes :

$$v = \frac{V_1V_2AB - \frac{V_1V_2PQ}{K_{eq}}}{V_2K_bA + V_2K_aB + V_2AB + \frac{V_1K_qP}{K_{eq}} + \frac{V_1K_pQ}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{V_2K_bAP}{K_{ip}} + \frac{V_2K_{ia}BP}{K_{iip}} + \frac{V_2K_{ib}AQ}{K_{iiq}} + \frac{V_2K_aBQ}{K_{iq}} + \frac{V_2ABP}{K_{iip}} + \frac{V_1APQ}{K_{iia}K_{eq}} + \frac{V_2ABQ}{K_{iiq}} + \frac{V_1BPQ}{K_{iib}K_{eq}}} \quad (4)$$

It should be noted that the constants $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ are no longer simple dissociation constants but represent the dissociation of substrate-enzyme complex into free substrate and two isomerizing forms of enzyme.

Rearrangement of equation (4) in the manner of equation (2) shows that, for a Ping-Pong mechanism in which the two stable forms isomerize, all product inhibitions are non-competitive. Moreover, they are all linear non-competitive. Therefore, this mechanism agrees qualitatively with the experimental data in that it does not require that higher concentrations of the variable substrate give rise to inhibition, nor that slopes or vertical intercepts are parabolic functions of the inhibitor concentration. The relationships expressing the values for the apparent inhibition constants

and the intersection points of the lines of the double reciprocal plots are given in Table IV.8 for product inhibition by MgATP and arginine (A & B) and in Table IV.9 for product inhibition by MgADP (P) and phosphoarginine (Q).

From the relationships of Table IV.8 and IV.9 with the data of Table IV.1, values have been calculated for the constants $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ as well as for $K_{iia}$, $K_{iib}$, $K_{iip}$ and $K_{iiq}$ (Table IV.10). In this connection, some further mention must be made of the determination of the constants. The experiments in which phosphoarginine was used as the variable substrate have not been used in these calculations for reasons previously outlined (p.122). The value for $K_{iq}$ should be given by the intersection point of the experiment in which arginine is used as product inhibitor with respect to phosphoarginine (Table IV.8). When phosphoarginine is used over the range from 10 mM to 2 mM (Fig. IV.2b) the value for $K_{iq}$ is found to be 18.15 $\pm$ 5.32 mM (Table IV.1). But when the velocities at 10 mM substrate are omitted and the experiments reanalysed, then the value for $K_{iq}$ reduces to 8.87 $\pm$ 3.40 mM; and it is possible that lower ranges of phosphoarginine concentrations would give even lower values for $K_{iq}$. From these experiments it can only be said that the value for $K_{iq}$ is equal to or less than 8.87 mM. However, $K_{iq}$ can be obtained from the $K_{i\ slope}$ when phosphoarginine is used as product inhibitor with respect to MgATP and it is this value that is recorded in Table IV.10.

TABLE IV.8. APPARENT INHIBITION CONSTANTS FOR A PING-PONG REACTION MECHANISM IN WHICH BOTH STABLE FORMS OF ENZYME ISOMERIZE, WITH A AND B AS PRODUCT INHIBITORS

The relationships between the kinetic constants of equation (4) and the apparent inhibition constants are given for the experiments in which A and B are used as product inhibitors while P and Q are variable substrates. The apparent inhibition constants are expressed as $K_{i\ slope}$, $K_{i\ intercept}$ and the intersection point of the lines of the double reciprocal plot $K(\frac{K_{i\ intercept}}{K_{i\ slope}})$.

| | Variable substrate | | | |
|---|---|---|---|---|
| | P | | Q | |
| Product inhibitor | A | B | A | B |
| $K_{i\ slope}$ | $\frac{K_{ia}K_{iia}K_pQ}{K_{iia}K_{ip}K_q+K_{ia}K_{ip}Q}$ | $\frac{K_{ib}Q}{K_{iq}+Q}$ | $\frac{K_{ia}P}{K_{ip}+P}$ | $\frac{K_{ib}K_{iib}K_qP}{K_{iq}(K_pK_{iib}+K_{ib}P)}$ |
| $K_{i\ intercept}$ | $\frac{K_{ia}K_{iia}(K_q+Q)}{K_{ia}Q+K_{iia}K_q}$ | $\frac{K_{iib}(K_q+Q)}{(K_{iq}+Q)}$ | $\frac{K_{iia}(K_p+P)}{(K_{ip}+P)}$ | $\frac{K_{ib}K_{iib}(K_p+P)}{K_{iib}K_p+K_{ib}P}$ |
| Intersection point | $K_{ip}$ | $\frac{K_pK_{iib}}{K_{ib}}$ | $\frac{K_qK_{iia}}{K_{ia}}$ | $K_{iq}$ |

TABLE IV.9. APPARENT INHIBITION CONSTANTS FOR A PING-PONG REACTION MECHANISM IN WHICH BOTH STABLE FORMS OF ENZYME ISOMERIZE, WITH P AND Q AS PRODUCT INHIBITORS.

The relationships between the kinetic constants of equation (4) and the apparent inhibition constants are given for the experiments in which P and Q are used as product inhibitors while A and B are variable substrates. The apparent inhibition constants are expressed as $K_{i\ slope}$, $K_{i\ intercept}$ and the intersection point of the lines of the double reciprocal plot $K\ (\frac{K_{i\ intercept}}{K_{i\ slope}})$.

| | Variable substrate | | | |
|---|---|---|---|---|
| | A | | B | |
| Product Inhibitor | P | Q | P | Q |
| $K_{i\ slope}$ | $\frac{-K_a B K_{ip} K_{iip}}{K_{ia}(K_b K_{iip} + K_{ip} B)}$ | $\frac{K_{iq} B}{(K_{ib} + B)}$ | $\frac{K_{ip} A}{(K_{ia} + A)}$ | $\frac{K_b A K_{iq} K_{iiq}}{K_{ib}(K_{iip} K_a + K_{iq} A)}$ |
| $K_{i\ intercept}$ | $\frac{(K_b + B) K_{ip} K_{iip}}{K_b K_{iip} + K_{ip} B}$ | $\frac{K_{iiq}(K_b + B)}{(K_{ib} + B)}$ | $\frac{K_{iiq}(K_b + B)}{(K_{ia} + A)}$ | $\frac{(K_a + A) K_{iq} K_{iiq}}{(K_{iiq} K_a + K_{iq} A)}$ |
| Intersection point | $K_{ia}$ | $\frac{K_{iiq} K_b}{K_{iq}}$ | $\frac{K_{iip} K_b}{K_{ip}}$ | $K_{ib}$ |

TABLE IV.10. INHIBITION CONSTANTS CALCULATED FOR A PING-PONG MECHANISM WITH ISOMERIZATION OF STABLE ENZYME FORMS.

The inhibition constants associated with equation (4) have been calculated from the data of Table IV.1 and the relationships tabulated in Tables IV.8 and IV.9 using the values for the Michaelis constants listed in Table III.1. The standard errors of the constants were calculated with the aid of the formulae for the standard errors of products and quotients given in Chapter I, pp.44 & 45. All constants are expressed as mM.

| $K_{ia}$ | $K_{ib}$ | $K_{ip}$ | $K_{iq}$ |
|---|---|---|---|
| 1.72 ± 0.67 | 4.45 ± 1.18 | 0.65 ± 0.18 | 4.59 ± 0.90 |
| | 10.80 ± 1.40 | 0.43 ± 0.14 | |
| $K_{iia}$ | $K_{iib}$ | $K_{iip}$ | $K_{iiq}$ |
| 7.05 ± 3.04 | 3.49 ± 0.98 | 0.43 ± 0.11 | 21.67 ± 4.41 |
| | 7.51 ± 4.15 | 0.39 ± 0.29 | 12.66 ± 3.49 |

As this value has been calculated using the experimental value for $K_{ib}$ it is dependent on the value for $K_{ib}$, so that use of the ratio $K_{ib}/K_{iq}$ in a Haldane relationship tests the correctness of the ratio rather than that of the individual values.

The Haldane relationships are not altered as a result of the isomerization of stable enzyme forme. Therefore the values for the various kinetic constants (Table IV.10) may be substituted into the relationships given in Table IV.5. The calculated values for the equilibrium constant so obtained agree reasonably well with the value calculated from the maximum velocities and Michaelis constants and with the experimentally determined value (Table IV.11). Other equalities defined by equation (4) are also listed in Table IV.11 and, considering the number of calculations involved to derive first the constants and then their relationships, the agreement would appear reasonable. Thus it may be concluded that the six experiments used to calculate the constants do indeed indicate a mechanism that can be represented by equation (4). Certainly, the values for the Haldanes are in better agreement with one another and with the experimentally determined value when the data are analysed on the basis of a Ping-Pong mechanism with isomerizations of both stable enzyme forms than when they are analysed on the basis of a Ping-Pong mechanism with dead-end complex formation. The difference in the two series of calculations

TABLE IV.11. HALDANE RELATIONSHIPS AND OTHER EQUALITIES FOR A PING-PONG MECHANISM WITH ISOMERIZATIONS OF STABLE FORMS

The Haldane relationships for a Ping-Pong mechanism have been calculated with the constants derived from the experimental data analysed in accordance with equation (4), i.e. that the mechanism of the reaction is Ping-Pong with isomerizations of both stable enzyme forms. In addition, the data of Table IV.10 have been used to estimate the numerical values of the equalities connecting the additional constants defined for equation (4) with the constants $K_{ia}$, $K_{ib}$, $K_{ip}$, $K_{iq}$, the Michaelis constants and the maximum velocities.

| $K_{eq} = \frac{V_1^2 K_p K_q}{V_2 K_a K_b}$ | $= \frac{V_1 K_p K_{iq}}{V_2 K_a K_{ib}}$ | $= \frac{V_1 K_{ip} K_q}{V_2 K_{ia} K_b}$ | $= \frac{K_{ip} K_{iq}}{K_{ia} K_{ib}}$ |
|---|---|---|---|
| 0.30 | 0.41 ± 0.05 | 0.26 ± 0.09 | 0.66 ± 0.32 | 0.39 ± 0.23 |

| $\frac{K_{iia}}{K_{iiq}} = \frac{V_1 K_{ia}}{V_2 K_{iq}} = \frac{K_a K_{ip}}{K_p K_{iq}}$ | | |
|---|---|---|
| 0.33 ± 0.16 | 0.36 ± 0.16 | 0.53 ± 0.18 |
| $\frac{K_{iib}}{K_{iip}} = \frac{V_1 K_{ib}}{V_2 K_{ip}} = \frac{K_b K_{iq}}{K_q K_{ip}}$ | | |
| 8.12 ± 3.08 | 6.51 ± 2.50 | 3.84 ± 1.32 |

| $\frac{K_{iia} K_{iip}}{K_{iib} K_{iiq}} = \frac{K_{ia} K_{ip}}{K_{ib} K_{iq}}$ | | $\frac{K_{iia} K_{iib}}{K_{iip} K_{iiq}} = \frac{K_a K_b}{K_p K_q}$ | |
|---|---|---|---|
| 0.040 ± 0.024 | 0.055 ± 0.032 | 2.64 ± 1.61 | 2.03 ± 0.16 |

for $K_{eq}$ lies mainly in the value determined for $K_{ip}$, as the values for $K_{ia}$, $K_{ib}$ and $K_{iq}$ do not differ by more than two-fold and such variation could arise from experimental error.

The above analyses draw attention to the difficulties associated with quantitative differentiation between mechanisms, especially when it is not possible to use sensitive, continuous methods for the determination of initial velocities. Hence they emphasize the merits of using qualitative distinctions to determine reaction mechanisms. For this reason, it should be noted that equation (4) predicts that all product inhibitions will be linear non-competitive inhibition, whereas equations (2) and (3) predict that the reactants forming dead-end complexes will give rise to substrate inhibition and slope-parabolic, intercept-parabolic non-competitive inhibitions.

While the experimental data suggest that the arginine kinase reaction involves isomerization of stable enzyme forms, it is apparent that additional experiments and alternative approaches will be necessary to reach a definitive conclusion. It would be possible, for instance, to utilise the difference in the $K_{i\ intercept}$ relationships for a Ping-Pong mechanism with dead-end complexes as opposed to one with isomerization of stable enzyme forms. In the former, the $K_{i\ intercept}$ relationship is a linear function of the concentration of the second, non-varied substrate, while in the latter it is a

hyperbolic function. Therefore, if an experiment is carried out at a number of concentrations of the non-varied substrate and the values for the $K_{i\ intercept}$ are plotted as a function of this substrate concentration, the shape of the plot should indicate whether the mechanism involves dead-end complexes or isomerizations.

An alternative approach to the problem would be the direct determination of the values for $K_{ia}$, $K_{ib}$, $K_{ip}$ and $K_{iq}$ for comparison with those obtained from product inhibition studies. This can be done by measuring the initial velocity of the partial exchange reactions as a function of the concentrations of the two reactants since the equation expressing the initial velocity is :

$$v = \frac{V_1 \frac{K_{ia}}{K_a}}{\frac{K_{ia}}{A} + \frac{K_{ip}}{P} + 1} \qquad (5)$$

when A (MgATP) and P (MgADP) are the two reactants. This equation is similar in form to the initial velocity equation for the overall Ping-Pong reaction. If, then, the initial velocity of the exchange is measured at varying concentrations of A at several fixed concentrations of P and the results are plotted in reciprocal form, a series of parallel lines should result. Hence, $K_{ia}$ and $K_{ip}$ can be evaluated in a manner similar to that used to evaluate $K_a$ and $K_b$ from the data of Fig. III.1. This procedure may be used to determine the

values regardless of whether the mechanism is a basic Ping-Pong or one with isomerizations of the stable enzyme forms.

Finally, it is of some interest to compare the results of the product inhibition studies with arginine kinase with those obtained with other Ping-Pong mechanisms. Product inhibition studies on aspartic transaminase (Henson & Cleland, 1964) clearly indicate that the enzyme has a basic Ping-Pong mechanism and it is possible that alanine transaminase also has a basic mechanism (Bulos & Handler, 1965), although product inhibition by one keto acid with respect to the other keto acid has not been measured. Nevertheless, it is apparent that in neither case do the reactants causing substrate inhibition at higher concentrations lead to an alteration of the product inhibition pattern to be expected for a basic mechanism. More complex results have been found with enzyme whose pairs of reactants are chemically dissimilar. For instance, studies with hexokinase from calf brain (Fromm & Zewe, 1962) show that ADP is an uncompetitive (rather than a competitive) inhibitor with respect to glucose. By assuming certain relationships between the rate constants, ADP inhibition was explained by the formation of a glucose-enzyme dead-end complex, although substrate inhibition by glucose was not demonstrated and other possibilities, such as a ternary ADP-glucose-enzyme complex or isomerization of the E-P form, have not been considered. Similarly, product inhibition by pyrophosphate of the reaction catalysed by adenine phospho-

ribosyltransferase (EC. 2.4.2.7) appears uncompetitive with respect to 5-phosphoribosylpyrophosphate when sufficient $Mg^{2+}$ is present to complex with both inhibitor and substrate (Hori & Henderson, 1966). Because a large variation in vertical intercept was found even in the presence of high concentrations of the second, non-varied substrate, it has been suggested that pyrophosphate forms a dead-end complex with one of the binary enzyme forms, rather than with one of the stable forms. As mentioned in discussing the results with arginine kinase, this inhibition cannot be quantitated, and isomerization of the stable enzyme form with which product and substrate react has not been considered.

Thus there has not been any previous consideration of isomerization of stable enzyme forms as part of the reaction sequence of an enzyme with a Ping-Pong mechanism (except in the theoretical analysis of kinetic theory by Cleland (1963a)), so it is impossible to surmise the frequency of this phenomenon. Good evidence has been presented by Hsu, Cleland and Anderson (1966) for the isomerization of the E-P form of acid phosphatase (EC. 3.1.3.2) from potatoes; in addition these authors have stated that analysis of the kinetic data of Hass and Byrne (1960) for glucose-6-phosphatase (EC. 3.1.3.9) leads to the necessity to postulate an isomerization of the enzyme-phosphate complex. But while the mechanism of these enzymes indicates that the first product leaves the enzyme before the second substrate, $H_2O$, reacts so that such hydrolytic

reactions could be considered of a Ping-Pong type, thermodynamic considerations of the isomerization suggests that it involves the hydrolysis of a covalent bond and a large free energy change. Hence it would be quite different from the isomerization of the E-P form of arginine kinase which would appear to be a change of a conformational nature since in one form it is able to phosphorylate MgADP and in the other it is able to phosphorylate arginine. The results with arginine kinase also suggest that the free enzyme exists in two different forms, one of which reacts with MgATP and the other with phosphoarginine. Although Monod, Wyman and Changeux (1965) postulated that allosteric enzymes exist in two different conformations, no kinetic evidence has been presented up till now to indicate that free enzymes may undergo a conformational change as part of the normal reaction sequence.

## SUMMARY

1. The four reactants, $MgATP^{2-}$, arginine, $MgADP^{-}$ and phosphoarginine have been used, in turn, as product inhibitors of the arginine kinase reaction with respect to each of the appropriate substrates.
2. All product inhibition appear to be linear non-competitive.
3. Experiments in which phosphoarginine is used as the variable substrate over the range of 10 mM to 2 mM are affected by substrate inhibition, and therefore cannot be used in

quantitative analysis of the data.

4. The product inhibition pattern of all non-competitive inhibitions could be due either to dead-end complex formation or to isomerization of both stable enzyme forms. The qualitative evidence that all product inhibition replots are linear and that there is no sign of substrate inhibition with substrates other than phosphoarginine points to isomerization rather than dead-end complex formation. This tentative conclusion is reinforced by detailed quantitative analysis of the data.

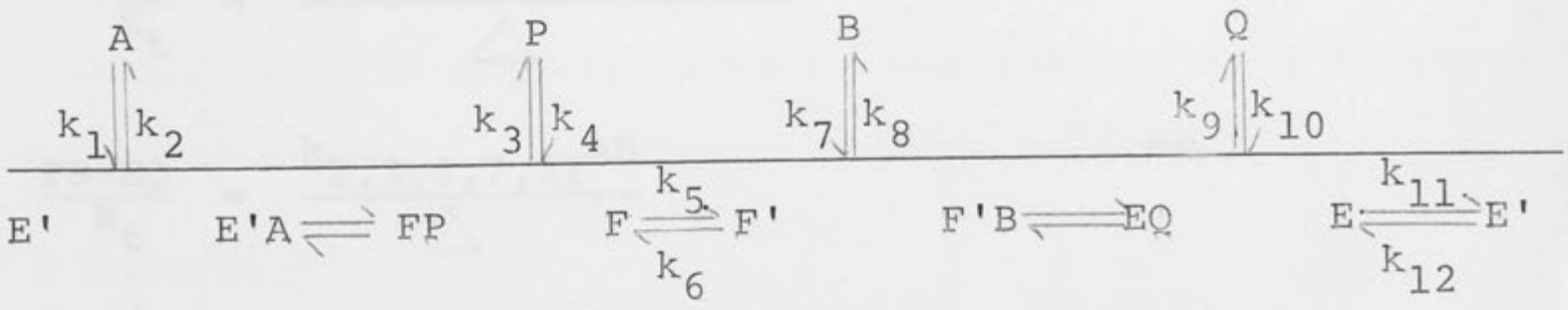
# ADDENDUM

## ADDENDUM

### Consideration of the Inhibitions Recorded in Chapter II in the Light of the Reaction Mechanism.

To find conditions suitable for detailed kinetic studies, the inhibition of the initial velocity of the arginine kinase reaction by NaCl, $MgCl_2$, $ATP^{4-}$ and $ADP^{3-}$ was measured and the results recorded in terms of apparent inhibition constants (Chapter II, Table II.2). It was mentioned then that true dissociation constants could not be calculated from the apparent constants because nothing was known of the reaction mechanism. The studies reported in Chapters III and IV have been directed towards the elucidation of that mechanism and it has been concluded that arginine kinase has a Ping-Pong mechanism. Furthermore, it appears likely that both stable forms of the enzyme isomerize. In the light of this knowledge, it is of interest to re-examine the inhibitions recorded in Chapter II to see what additional information can be gained about the reaction of the inhibitors with the enzyme.

The reaction sequence for the arginine kinase reaction with isomerization of both stable enzyme forms can be represented diagrammatically as :



where A, B, P and Q represent $MgATP^{2-}$, arginine, $MgADP^{-}$ and phosphoarginine, respectively. With A and B as substrates, in the absence of P and Q, the initial velocity equation is the same as that of a basic Ping-Pong reaction :

$$v = \frac{V_1 AB}{K_a B + K_b A + AB} = \frac{V_1}{\frac{K_a}{A} + \frac{K_b}{B} + 1}$$

However, as there are now two additional forms of enzyme, E' and F', the equations expressing the distribution of the total enzyme, $E_t$, among the various forms are different from those of the basic Ping-Pong and may be written as :

$$\frac{E}{E_t} = \frac{k_{1,3,5,7,9}AB + k_{5,7,9,12}(k_2 + k_3)B}{\triangle}$$

$$\frac{E'}{E_t} = \frac{k_{5,7,9,11}(k_2 + k_3)B}{\triangle}$$

$$\frac{EA-FB}{E_t} = \frac{k_{1,7,9,11}(k_4 + k_5)AB}{\triangle}$$

$$\frac{F}{E_t} = \frac{k_{1,3,7,9,11}AB + k_{1,3,6,11}(k_8 + k_9)A}{\triangle}$$

$$\frac{F'}{E_t} = \frac{k_{1,3,5,11}(k_8 + k_9)A}{\triangle}$$

$$\frac{FB-EQ}{E_t} = \frac{k_{1,3,5,7,11}AB}{\triangle}$$

where $\triangle$ represents the denominator of the rate equation and is equal to the sum of all the numerator terms. Therefore, any one term in the denominator of the rate equation represents enzyme in at least two forms. For instance, the $K_aB$ term, which becomes the $\frac{K_a}{A}$ term when the equations is put into reciprocal form, measures the proportion of the total enzyme which is in the form E' together with part of the proportion which is in the form E. Therefore, if A is the variable substrate, the slope of the lines of a double reciprocal plot will be affected by an inhibitor combining with either E or E', while the vertical intercept will be affected by an inhibitor combining with E or with any of the other enzyme forms. Hence, in general, it can be said that the slope of the lines will be affected if an inhibitor is combining with either or both of the two conformations of the stable form (E or F) with which the variable substrate combines; the vertical intercepts will be a function of the concentration of an inhibitor if the inhibitor is combining with any form of the enzyme except that particular conformation with which the variable substrate combines. Because the distribution equations cannot be expressed in kinetic constants and because the inhibitory effects cannot be attributed to combination of an inhibitor with a specific form of the enzyme, true dissociation constants cannot be calculated from values for $K_{i\ slope}$ and $K_{i\ intercept}$.

Values for $K_{i\ slope}$ and $K_{i\ intercept}$ for inhibition of arginine kinase by NaCl, $MgCl_2$, $ATP^{4-}$ and $ADP^{3-}$ were recorded in Table II.2. It was stated in Chapter II (p. 78) that dissociation constants could not be calculated because the reaction mechanism was not known, implying that such constants could be found when the mechanism was elucidated. However, this is not possible for a mechanism as complex as a Di Iso Ping-Pong one. All that can be said is that the non-competitive inhibition of NaCl with respect to $MgADP^-$, which combines with F, and with respect to phosphoarginine, which combines with E, indicates that $Cl^-$ is able to combine with at least one of the E forms and one of the F forms. Similarly, the same conclusion can be drawn with respect to $Mg^{2+}$ from the results of the inhibitions with respect to $MgATP^{2-}$ and $MgADP^-$. At this point, it may be noted that $Mg^{2+}$ inhibition with respect to $MgATP^{2-}$ should be non-competitive, rather than competitive as it appears to be in Fig. II.4a. The variation in vertical intercept, however, is a function of $\frac{K_b}{B}$ and, because these experiments were carried out before the Michaelis constants were known, the concentration of arginine used was 10 mM as compared with the Michaelis constant of 0.87 mM. Therefore, the expected variation in vertical intercept would be small. The free nucleotides, $ATP^{4-}$ and $ADP^{3-}$, are non-competitive inhibitors with their respective Mg-nucleotide complexes as variable substrates, indicating combination of the inhibitors with at least two of the four stable enzyme con-

formations (ignoring combination with binary enzyme forms). But apart from the fact that one of these forms must be a conformation of the stable form with which the Mg-nucleotide substrate combines, these forms cannot be delineated.

If it were assumed that an inhibitor, such as $ATP^{4-}$, combines equally with both conformations of that stable form (E or F) with which the variable substrate combined, then it would be possible to equate the values for $K_{i\ slope}$ (Table II.2) with inhibition constants for combination of the inhibitor with those forms. For example, 0.35 mM would represent the inhibition constant for combination of $ATP^{4-}$ with the E forms of arginine kinase. Experimental evidence, however, points to the fact that $MgATP^{2-}$ reacts only with E' and not at all with E, so that there would not appear to be any good reason for making this assumption.

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