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THE KINETIC MECHANISM OF RABBIT MUSCLE GLYCOGEN PHOSPHORYLASE

by

Ⓒ HOWARD DONALD ENGERS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE KINETIC MECHANISM OF RABBIT MUSCLE GLYCOGEN PHOSPHORYLASE" submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Rabbit muscle phosphorylase _b_ has always been considered to possess an absolute dependence upon 5'-AMP for activity. However, Buc recently presented results suggesting that at high concentrations of P_i (120 mM) the enzyme loses its requirement for AMP. Since several other anions had previously been shown to affect the activity of various forms of phosphorylase, the effect of P_i and other anions on the activity of phosphorylase _b_ was reinvestigated in the presence and absence of AMP. The effect of P_i on the activity of this enzyme compares with those results obtained for other anions high in the Hofmeister series and is most likely non-specific in nature.

In order to investigate the kinetic mechanism of phosphorylase _b_ and _a_ from rabbit muscle, initial rate studies were conducted, in the presence of AMP for phosphorylase _b_, and in the presence and absence of AMP for phosphorylase _a_. Initial velocity rates were measured with varied concentrations of both substrates in each reaction direction and data analyzed with double reciprocal plots and secondary replots of intercepts and slopes. The resulting kinetically derived dissociation constants agreed reasonably well with those determined by independent means. These results indicate that the kinetic mechanism of phosphorylase _a_ and _b_ is rapid equilibrium random bi bi.

To confirm the kinetic mechanism suggested by initial velocity experiments, isotope exchange studies at equilibrium were performed with both enzyme forms, in the presence of AMP for phosphorylase _b_, and in the presence and absence of AMP for phosphorylase _a_.

Both the initial velocity and the equilibrium isotope exchange studies support a reaction mechanism in which substrates bind in a noncompulsory order to the enzyme and in which the interconversions of ternary complexes are the rate limiting steps.

The method of isotope exchange at equilibrium provides a means of ascertaining whether or not rapid equilibrium conditions do in fact prevail for any given enzyme mechanism. Utilizing this method, it has been shown that for rabbit muscle glycogen phosphorylase, in the presence of effectors which cause the velocity versus substrate concentration curves to become sigmoid, the kinetic mechanism remains rapid equilibrium random. Hence, the sigmoidicity observed is in fact due to cooperativity among ligand binding sites. The application of equilibrium isotope exchange methods to enzymes exhibiting sigmoidal kinetics should be a useful test to distinguish between the possible conditions which could result in sigmoidicity; i.e., cooperative effects ("allosteric" effects), or a breakdown in the kinetic mechanism.

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TABLE OF CONTENTS

Page

Abstract iii

Acknowledgements v

List of Tables viii

List of Illustrations ix

List of Abbreviations xii

Chapter

I. INTRODUCTION 1

II. EXPERIMENTAL METHODS 10

- A. Preparation of Materials 10
- B. Initial Velocity Assays 11
- C. Isotope Exchange Measurements 13

III. THE EFFECT OF ANIONS ON THE ACTIVITY OF PHOSPHORYLASE b . . 16

- A. Results and Discussion 16
- B. Summary 21

IV. THE KINETIC MECHANISM OF PHOSPHORYLASE b. INITIAL VELOCITY STUDIES AND ISOTOPE EXCHANGE AT EQUILIBRIUM 22

- A. Results and Discussion 22
- B. Summary 49

V. THE KINETIC MECHANISM OF PHOSPHORYLASE a. INITIAL VELOCITY STUDIES 51

- A. Results and Discussion 51
- B. Summary 81

Chapter Page

VI. THE KINETIC MECHANISM OF PHOSPHORYLASE a. ISOTOPE EXCHANGE RATES AT EQUILIBRIUM 82

A. Results and Discussion 82

B. Summary 89

VII. ISOTOPE EXCHANGE STUDIES AT EQUILIBRIUM AS APPLIED TO ALLOSTERIC ENZYMES 91

A. Results and Discussion 91

B. Summary 98

BIBLIOGRAPHY 100

APPENDIX

Published Papers Arising From This Work 104

LIST OF TABLES

Table Page

I. Summary of Data from Figures 4 through 12 35

II. Relationships Between Coefficients and Constants for Phosphorylase b 36

III. Dissociation Constants for Substrates and Phosphorylase b 38

IV. Kinetic Constants for Glycogen Phosphorylases 72

V. Dissociation Constants for AMP and Phosphorylase a . . 75

LIST OF ILLUSTRATIONS

Figure		Page
1.	The effect of high concentrations of P_i and glucose-1-P on the activity of phosphorylase b	17
2.	The effect of anions high in the Hofmeister series on the activity of phosphorylase b in the absence of AMP	18
3.	The effect of anions low in the Hofmeister series on the activity of phosphorylase b in the absence of AMP	19
4.	Proposed rapid equilibrium random bi bi kinetic mechanism for muscle phosphorylase b	24
5.	Velocity of muscle phosphorylase b activity as a function of glycogen concentration at six levels of P_i concentrations	26
6.	Velocity of muscle phosphorylase b activity as a function of P_i concentration at five levels of glycogen	27
7.	Velocity of muscle phosphorylase b activity as a function of glycogen concentration at six levels of glucose-1-P	28
8.	Velocity of muscle phosphorylase b activity as a function of glucose-1-P concentration at five levels of glycogen	29
9.	Secondary plots of the intercepts from Figures 5 and 6 .	30
10.	Secondary plots of the intercepts from Figures 7 and 8 .	31
11.	Secondary plots of the slopes from Figures 5 and 6 . . .	32
12.	Secondary plots of the slopes from Figures 7 and 8 . . .	33
13.	Isotope exchange rates measured from a single equilibrium mix	40
14.	First order plot showing the effect of prior incubation of equilibrium mixes before isotope exchange	42
15.	The effect of glucose-1-P and P_i concentrations on equilibrium exchange rates with muscle phosphorylase b .	44

Figure Page

16. The effect of glycogen concentrations on equilibrium exchange rates with muscle phosphorylase b 45

17. The effect of glucose-1-P and P_i concentrations on equilibrium exchange rates at very high glycogen concentration 47

18. Proposed rapid equilibrium random kinetic mechanism for rabbit muscle phosphorylase a 52

19. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at six levels of P_i, with AMP present at 1 mM 54

20. Velocity of muscle phosphorylase a activity as a function of P_i concentration at five levels of glycogen, in the presence of 1 mM AMP 55

21. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at six levels of P_i concentrations, in the absence of AMP 56

22. Velocity of muscle phosphorylase a activity as a function of P_i concentration at five levels of glycogen concentrations, in the absence of AMP 57

23. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at six levels of glucose-1-P, in the presence of 1 mM AMP 59

24. Velocity of muscle phosphorylase a activity as a function of glucose-1-P concentration at five levels of glycogen in the presence of 1 mM AMP 60

25. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at six levels of glucose-1-P in the absence of AMP 61

26. Velocity of muscle phosphorylase a activity as a function of glucose-1-P concentration at five levels of glycogen in the absence of AMP 62

27. Secondary plots of the intercepts from Figures 19 and 20. 64

28. Secondary plots of the intercepts from Figures 23 and 24. 65

29. Secondary plots of the intercepts from Figures 21 and 22. 66

30. Secondary plots of the intercepts from Figures 25 and 26. 67

Figure Page

31. Secondary plots of the slopes from Figures 19 and 20 . . 68

32. Secondary plots of the slopes from Figures 23 and 24 . . 69

33. Secondary plots of the slopes from Figures 21 and 22 . . 70

34. Secondary plots of the slopes from Figures 25 and 26 . . 71

35. Velocity of muscle phosphorylase a activity as a function of P_i concentration with varied concentrations of UDP-glucose 77

36. Velocity of muscle phosphorylase a activity as a function of glycogen concentration with varied concentrations of UDP-glucose 79

37. Secondary plot of slopes and intercepts from Figure 36 . 80

38. The effect of glucose-1-P (G-1-P) and P_i concentrations on equilibrium isotope exchange rates with muscle phosphorylase a in the presence of AMP 83

39. The effect of glycogen concentration on equilibrium isotope exchange rates with muscle phosphorylase a in the presence of AMP 84

40. The effect of glucose-1-P and P_i concentrations on equilibrium isotope exchange rates with muscle phosphorylase a in the absence of AMP 86

41. The effect of glycogen concentration on equilibrium isotope exchange rates with muscle phosphorylase a in the absence of AMP 87

42. The effect of AMP on equilibrium isotope exchange rates with muscle phosphorylase b in the absence and presence of ATP 92

43. The effect of ATP on equilibrium isotope exchange rates of muscle phosphorylase b with varied glucose-1-P and P_i 94

44. The effect of glucose on equilibrium isotope exchange rates of muscle phosphorylase a in the absence of AMP, with varied glucose-1-P and P_i 96

LIST OF ABBREVIATIONS

AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
Glucose-1-P	α-D-glucose-1-phosphate
K_{diss}	dissociation constant
K_{eq}	equilibrium constant
K_m	apparent Michaelis constant
NADP	nicotine-adenine dinucleotide phosphate
P_i	inorganic phosphate
R_{max}	equilibrium reaction rate at infinite substrate concentrations
UDP-glucose	uridine 5'-diphosphate-glucose
V_{max}	initial velocity at infinite substrate concentrations

CHAPTER I

INTRODUCTION

Muscle glycogen phosphorylases a and b (α-1,4 glucan:orthophosphate glucosyl transferase, E.C. 2.4.1.1.) catalyse the reaction:

$$\text{glycogen}_{(n)} + P_i \rightleftharpoons \text{glycogen}_{(n-1)} + \text{glucose-1-P}$$

where n refers to the number of glucose residues on the glycogen macromolecule. The role of this enzyme in glycogen metabolism was first elucidated by the Coris around 1936 (1). Since then, glycogen phosphorylase from many varied species has been the subject of considerable investigation, as is evidenced in recent review articles (Brown and Cori (2); Helmreich (3); Fischer and Krebs (4,5). However, the actual kinetic mechanism of either phosphorylase a or b has yet to be delineated in a complete unequivocal manner, and hence the subject of this dissertation.

Rabbit muscle phosphorylase b has always been considered to possess an absolute dependence upon 5'-AMP for activity. In this laboratory, control assays run in the absence of AMP, at "kinetic" levels of P_i (0 - 25 mM), routinely gave 0 to 2% the activity observed in the presence of saturating levels of AMP (1 mM). However, Buc (6) has presented results suggesting that in the presence of high concentrations of P_i (120 mM), the enzyme loses its requirement for AMP. While attempting to repeat these results, it was found that P_i at concentrations over 50 mM inhibited severely the conventional coupled auxiliary enzyme assay system of Helmreich and Cori (7), used by Buc.

This inhibition is in accord with that reported by Glaser and Brown (8) for glucose-6-phosphate dehydrogenase, one of the enzyme components of the phosphorylase assay system.

Several anions other than P_i have previously been shown to affect the activity of various phosphorylase preparations. Sulfate was found to activate the phosphorylase from lobster muscle (Cowgill (9)), beef adrenal (Riley and Haynes (10)), and liver (Appleman _et al_. (11)). Sealock and Graves (12) have reported that phosphorylases _a_ and _b_ from rabbit muscle were activated by fluoride and inhibited by perchlorate. In view of these observed ion effects on phosphorylase, it was decided to reinvestigate the effect of P_i and other anions on the activity of phosphorylase _b_ in the presence and absence of AMP, using a modified phosphorolysis assay system which is independent of the possible effect of any added substance on the rate of action of the auxiliary enzymes.

In contrast to phosphorylase _b_, which lacks the one covalently bound phosphate group per monomer (13), phosphorylase _a_ retains substantial activity in the absence of the modifier AMP. The 15 - 20% activation of phosphorylase _a_ that is observed in the presence of AMP appears to be mediated by an effect on the affinity constants for the substrates rather than on the maximal velocity of the reaction (7, 14). In the presence of AMP, phosphorylase _a_ does not exhibit pronounced allosteric phenomena as compared to phosphorylase _b_ (15, 16); however, in the absence of the activator AMP, the enzyme does exhibit some homotropic cooperativity (16). Also, Helmreich _et al_. (17) have recently shown that glucose behaves as an allosteric inhibitor in the phosphorylase _a_ system, whereas in contrast to phosphorylase _b_ (18), ATP does not. It was therefore of interest to determine whether or not the presence of the modifier AMP alters the kinetic mechanism of the phosphorylase _a_ reaction, as has been shown for the case of the allosteric isocitrate dehydrogenase from _Neurospora_, by Sanwal _et al_. (19, 20).

The allosteric and structural properties of phosphorylase _b_ have been the subject of much extensive investigation. Analyses of the kinetic behaviour of phosphorylase _b_ have shown this enzyme to exhibit allosteric properties interpretable in terms of the concerted transition model proposed by Monod _et al_. (21), although the data indicate that certain modifications have to be made (22, 23, 24, 25, 26). In addition, physical studies have verified kinetic predictions of homotropic cooperativity for AMP binding, and heterotropic interactions among substrates, activator and inhibitors (24, 25, 26). Despite this progress, the actual kinetic mechanism of the catalytic reaction has not yet been presented.

Work on other glycogen phosphorylases has suggested that the kinetic mechanism is rapid equilibrium random bi bi, in Cleland's terminology (27). For example, Maddaiah and Madsen (28) proposed this mechanism for liver phosphorylase on the basis of a kinetic analysis by the methods of Dalziel (29). Their data for the inhibition by UDP-glucose provided strong evidence for this conclusion. This suggestion was later verified by a comparison of kinetically and physically derived dissociation constants for glucose-1-phosphate (26).

Chao _et al_. (30), by the use of initial velocity experiments, studies with inhibitors and isotope exchange measurements at equilibrium, have deduced the kinetic mechanism for the maltodextrin phosphorylase from Escherichia coli. Initial rate studies were conducted in the direction of phosphorolysis utilizing maltoheptaose as a substrate, with maltotetraose serving as a competitive inhibitor for their inhibition studies. They concluded that this form of phosphorylase also exhibited a random rapid equilibrium kinetic mechanism; i.e., the addition of substrates to the enzyme can occur in a random order, with the interconversion of ternary complexes as the rate limiting step in the reaction sequence.

The possibility that glycogen phosphorylase a may have an ordered bi bi mechanism has been suggested by Helmreich and his co-workers (15, 31), based on studies with muscle phosphorylase a from both rabbit (31) and frog (15). Lowry et al. (14, 32) have fitted their available kinetic data for rabbit muscle and brain phosphorylase a to a rapid equilibrium random bi bi mechanism. They did not, however, carry out a detailed analysis under standard conditions (i.e., pH 6.8, 30°), and also did not include additional proof for their suggestions.

It would seem necessary, therefore, to verify the actual mechanism for phosphorylase a and b since this has profound significance for the interpretation of much kinetic data used for testing theoretical allosteric models. Implicit in the treatment of allosteric kinetics for this enzyme has been the assumption that the mechanism is rapid equilibrium random bi bi.

An assessment of the kinetic mechanism for an allosteric enzyme presents theoretical and practical difficulties not encountered in a more conventional enzymatic system. For example, reciprocal plots are curved at low substrate concentrations, so it is necessary to work at substrate concentrations above the nominal "K_m" such that the velocity vs. substrate relationship is rectangular hyperbolic. One might therefore object that only a partial picture is being presented. In addition, product inhibition studies are not possible with glycogen phosphorylase because one of the substrates (glycogen) is changed to an essentially indistinguishable product, while the equilibrium between the other two substrates (P_i and glucose-1-P) is close to unity.

Fortunately, the development and verification of the enzymic equilibrium reaction rate theory by Boyer and his colleagues (33, 34, 35, 36) provides a useful means of confirming possible kinetic mechanisms suggested by initial rate studies. We therefore decided to investigate the rates of isotope exchange at equilibrium for both the phosphorylase _b_ and _a_ systems, using ^{14}C-glucose-1-P and $^{32}P_i$ as the isotopically labeled substrates, in order to complement the data obtained from conventional initial rate studies. It was felt that a detailed kinetic analysis in conjunction with subsequent isotope exchange studies at equilibrium would provide conclusive proof as to the kinetic mechanism of rabbit muscle glycogen phosphorylases _b_ and _a_.

The concept of "allosterism" as developed by Monod _et al_. (21, 37) (i.e., the control of an enzyme's catalytic efficiency by the binding of small molecular weight effectors, not necessarily structurally related to the substrate) has been a favorite explanation for sigmoid rate versus ligand concentration curves. The usual postulate involves the cooperative interaction of several catalytically active substrate binding sites, in addition to various combinations of positive and/or negative effector-binding sites. Implicit in such models is the proposal that the enzyme consists of two or more subunits or protomers capable of interaction with each other. However, several investigators have been able to provide reasonable alternative explanations for apparent sigmoidal rate data without resorting to the requirement for more than one catalytic binding site, or for subunit interactions, to support their models. For example, Atkinson *et al*. (38, 39, 40) have implicated certain concentration ratios as a control parameter, initially using data obtained for rat liver citrate cleavage enzyme to show that characteristic steep response curves may be obtained without special regulatory sites, cooperative binding, or even multiple binding sites. In addition, he points out that the "energy charge" of the adenylate system could function as a fundamental control parameter, with *Escherichia coli* phosphofructokinase as well as the citrate cleavage enzyme as examples.

Frieden (41) has considered the kinetic behaviour of enzyme systems using the single substrate-single modifier case. In this simple situation, his treatment shows that the dependence of velocity upon the substrate concentration is hyperbolic only if it is assumed that the attachment of the substrate and modifier is in rapid equilibrium compared to the step(s) involving product formation. If this condition does not apply, the steady state treatment yields rate equations with squared substrate terms and hence the corresponding reciprocal plots become non-linear. Henderson (42) has extended the steady state theory to enzymes with two substrates, and has tabulated a variety of kinetic mechanisms which can lead to apparent sigmoidal velocity versus substrate dependence in the presence of a single modifier.

Ferdinand (43) presented a model which could explain the kinetic data observed for phosphofructokinase, based on the proposed existence of a kinetically preferred pathway. He recalled the fact that when an alternative pathway exists for a two-substrate enzyme, this kinetic mechanism can result in apparent substrate inhibition or substrate activation (44, 45, 46). If these phenomena occur with appropriate rate constants, apparent sigmoid curves could result. In theory, one should be able to distinguish between true sigmoidal curves and apparent sigmoidicity, but in practice, the experimental data usually obtained does not permit such a verification. Katsumata (47), in an elegant theoretical paper, has expanded upon the ideas of Ferdinand and others, and has provided a convincing argument for the possibility that certain interrelationships of the rate constants for a two-substrate random mechanism can lead to apparent sigmoidicity (non-linear Lineweaver-Burk plots). Sweeny and Fisher (48) have also considered these possibilities, and have presented a group of fifteen steady state models which can yield sigmoid rate data, on the basis of single independent active sites with various alternative pathways of substrate attachment. There also exists the possibility that for a two substrate random rapid equilibrium mechanism at low substrate concentration (less than 10^{-4} M) the rapid equilibrium condition may be lost and as a result apparent sigmoidicity could appear.

Those considerations which have been discussed above raise the possibility that modifiers in certain enzyme systems might induce a change in a given kinetic mechanism. For example, an enzyme which exhibits a rapid equilibrium random mechanism in the absence of modifier may lose the rapid equilibrium condition in the presence of modifier, due to an effect on certain rate constants. Thus the appearance of sigmoid curves in the presence of a modifier could be a result of a change in the mechanism and not due to subunit interactions or cooperativity among binding sites. In fact, Sanwal *et al.* (19, 20) have indeed shown a modifier-dependent change in kinetic mechanism for the NAD-dependent isocitrate-dehydrogenase of *Neurospora crassa*. In this system the mechanism was found to be ordered in the presence of the activator AMP, but in the absence of AMP it became random, with the addition steps partially rate limiting. As a result the reciprocal plots for NAD^+ in the absence of AMP became non-linear. The authors found no evidence for the binding of more than one NAD^+ molecule to the enzyme surface.

Madsen (18), and also Helmreich and Cori (7), showed in 1964 that the activator AMP and the substrate glucose-1-P appeared to exhibit homotropic cooperativity between their binding sites on rabbit muscle phosphorylase *b*. This enzyme was subsequently cited by Monod and his group (21, 49) as a model enzyme for their allosteric theories. In Chapters IV - VI, it has been shown that the kinetic mechanism of both phosphorylase *b* and *a* from rabbit muscle was rapid equilibrium random bi bi. However, the possibility exists that in the presence of "allosteric" effectors such as ATP, the rapid equilibrium conditions break down, resulting in apparent sigmoidal curves. Isotope exchange at equilibrium provides a unique kinetic tool with which to investigate a possible breakdown in the kinetic mechanism. Since it was implicity assumed in Monod's model that rapid equilibrium conditions prevail throughout, it was decided to examine the isotope exchange at equilibrium patterns obtained for these enzyme forms in the presence of their allosteric effectors. These experiments should provide an indication as to whether or not in the case of phosphorylase the sigmoid data is in actual fact due to cooperativity between substrate binding sites or due to a breakdown in the rapid equilibrium conditions of the mechanism.

CHAPTER II

EXPERIMENTAL PROCEDURE

A. Preparation of Materials

Phosphorylase b was prepared from rabbit muscle by the method of Fischer and Krebs (50) and recrystallized a minimum of three times. Before use, phosphorylase b crystals were centrifuged out of suspension, dissolved in 0.04 M sodium β-glycerophosphate, 0.001 M EDTA, 0.005 M mercaptoethanol, pH 6.8, and purified on a column of Sephadex G-25. For experiments in Chapter III, phosphorylase b was further fractionated on a 2.5 x 77 cm Sephadex G-200 column to remove any possible contamination by phosphorylase a, recrystallized, and passed through a Sephadex G-25 column to remove Mg^{++} and AMP.

Crystalline rabbit muscle phosphorylase a was prepared from crystallized phosphorylase b using purified phosphorylase b kinase according to the method of Krebs and Fischer (51). The resulting phosphorylase a was recrystallized a minimum of four times and treated with Norit A to remove any remaining nucleotides.

Before use, phosphorylase a crystals were centrifuged out of suspension and dissolved in 0.02 M sodium β-glycerophosphate, 0.0015 M EDTA, 0.005 M mercaptoethanol, pH 6.8. All experiments reported in Chapter V were carried out with the same enzyme preparation, within two weeks of isolation, with the exception of the data presented in Table IV for aged enzyme, which was obtained from experiments repeated with phosphorylase a aged for three months at 4° in the crystalline form.

Chemicals and auxiliary enzymes were purchased from Sigma when possible and were the highest grade available. The salts tested in Chapter III were utilized without further purification. Also, for experiments reported in Chapter III, possible contamination by AMP was removed from glucose-1-P by a charcoal-celite column, and from the bovine serum albumin Fraction V (Mann) by a Sephadex G-25 column. The rabbit liver glycogen was routinely passed through a Dowex-1-chloride column in order to remove any possible AMP contamination. It was assayed against a glucose standard by the method of Dische, as described by Ashwell (52). Its concentration is expressed as the molar equivalent of its glucose residues, except that for experiments in Chapter VII, its concentration is expressed in terms of glucose end groups, with 2% glycogen equivalent to 10.1 mM glucose end groups.

B. Initial Velocity Assays

Phosphorylase b activities are expressed as micromoles of P_i esterified (or released from glucose-1-P) per minute per mg of protein in the presence of saturating levels of AMP, at 30°. Phosphorylase was incubated with AMP and glycogen prior to activity measurements in either reaction direction. Activities in the direction of glycogen synthesis were assayed by following the release of inorganic phosphate using the Fiske-SubbaRow test (53). In a typical experiment the 0.5 ml reaction mixture contained 4 mM sodium β-glycerophosphate, pH 6.8, 0.55 mM mercaptoethanol, 0.1 mM EDTA, 1 mM AMP and 5 - 10 μg protein. Initial reaction rates were calculated from the pseudo-first order reaction constants by multiplying the latter by the concentration of P_i calculated to be present when equilibrium is attained. Glycogen and glucose-1-P concentrations are given for each experiment. Appropriate precautions were taken to ensure that the average increase of chain length did not exceed 2 glucose units (28).

The phosphorolysis of glycogen was coupled to a large excess of phosphoglucomutase and glucose-6-P dehydrogenase so that the rate limiting step in the reduction of NADP was the phosphorylase activity. Assay conditions were essentially those described by Maddaiah and Madsen for liver phosphorylase (28), with the glycogen and P_i concentrations given for each experiment.

Phosphorylase a activities were determined in a similar manner, but with 2.5 mM instead of 4 mM glycerophosphate buffer. For the phosphorylase a experiments in Chapter V, two sets of experiments were carried out, one in the presence of saturating AMP (1mM) and the other in the absence of the activator.

For the ion effect experiments described in Chapter III, phosphorylase b activity was assayed in the direction of glycogen phosphorolysis in a 0.2 ml reaction mixture containing the desired concentration of ion(s) and P_i, 10 mM sodium glycerophosphate, 1.5 mM mercaptoethanol, 0.25 mM EDTA, 1% glycogen, 1 mM AMP (if present), 0.05 mg of bovine serum albumin and 8-10 μg of enzyme, all at pH 6.8 and 30°. Reaction times were adjusted to ensure that the release of product with time was linear. The reaction was terminated by immersion in boiling water for 1 minute and the glucose-1-P formed was determined in a 0.1 ml aliquot by measuring the total reduction of NADP in an auxiliary enzyme reaction mixture similar to that of Helmreich and Cori (7). This assay system is independent of the possible effect of any added substance on the rate of action of the auxiliary enzymes.

Also for experiments in Chapter III, activity in the direction of glycogen synthesis was determined by measuring the P_i released from glucose-1-P (Fiske and SubbaRow, 53) or by measuring the radioactivity incorporated into glycogen from ^{14}C-glucose-1-P. The radioactive glycogen was isolated on millipore filter discs by precipitation with cold ethanol, and the filter discs counted in Bray's scintillation solution (54).

C. Isotope Exchange Measurements

$^{32}P_i$ (Atomic Energy of Canada Limited) and ^{14}C-glucose-1-P (uniformly labelled) (Calbiochem or Amersham Searle) were used without added carrier, and the respective radioactive products were counted in a Beckman CPM-100 liquid scintillation counter, using Bray's solution (54) as a counting medium. $^{32}P_i$ and ^{32}P-glucose-1-P were separated by means of the iso-butanol-benzene extraction procedure for phosphomolybdate (55). When the glucose-1-P concentration in the reaction mixtures was less than 2 mM, carrier glucose-1-P and P_i (0.05 ml of a 50 mM P_i, 32 mM glucose-1-P stock solution) was added to the incubation mixture which had been treated with 2 ml of 2.5% ammonium molybdate in 0.5 N H_2SO_4 in order to stop the reaction. This mixture was extracted three times with 3 ml of a water saturated iso-butanol-benzene solution (50:50), and the lower aqueous layer containing the glucose-1-P was added to a scintillation vial and counted. ^{14}C-glucose-1-P and ^{14}C-glycogen were separated and the ^{14}C-glycogen isolated by a modification of the method of Gold and Segal (56). First the incubation mixture, which had been treated with 1 ml of 45% KOH to stop the reaction, was boiled for 10 minutes. At this point, if the glycogen concentration in the incubation mixes was less than 5 mM (total residues), 0.05 ml of a 10% glycogen solution was added as carrier. Then 0.5 ml saturated (42 gm per 100 ml) sodium sulfate was added, followed by 2.5 ml 95% ethanol. The tubes were heated to incipient boiling, cooled in ice for at least one-half hour, and then centrifuged at 12,000 x g for 10 min at 2°. The pelleted glycogen was dissolved with 2 ml hot distilled water, and reprecipitated with ethanol. After chilling and recentrifugation, the purified glycogen was again dissolved in 2 ml water and counted in Bray's solution (54).

For the phosphorylase b isotope exchange experiments in Chapter IV, equilibrium mixes were set up for each concentration of substrates desired. P_i:glucose-1-P ratios were consistent with the anticipated equilibrium constant at pH 6.8 (K = .21, Trevelyan et al., 57), and AMP was present in all experiments at saturating levels (1 mM). Initially, in the experiments described in Chapter IV, complete equilibrium mixes were incubated at 30° for at least 2-1/2 hours prior to the addition of isotopes, in order to ensure the attainment of equilibrium. However, for the experiments described in Chapters VI and VII, equilibrium mixes of 0.4 ml containing all components except glycogen were incubated at 30° for at least one hour, then the desired concentration of glycogen in 0.1 ml was added and the complete equilibrium mix incubated for a further five minutes prior to the addition of isotope. This order of addition of substrates prevented extensive disproportionation of the glucose end groups on the glycogen molecules. For the phosphorylase a experiments in Chapter VI, two sets of experiments were carried out, one in the presence of saturating AMP (1 mM) and the other in the absence of the activator.

Reactions were started by the addition of either 10 or 20 μl of radioactive substrate ($^{32}P_i$ or ^{14}C-glucose-1-P), to 0.5 ml aliquots of the final equilibrium mix. In this way, very small volume changes occurred and the amount of labeled substrate added was insignificant when compared to the total substrate concentration, thus avoiding net reaction. Reactions were stopped, after appropriate time intervals, with 1 ml 45% KOH for the ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange and 2 ml acid molybdate for the $^{32}P_i \rightleftharpoons$ glucose-1-P exchange. The radioactive products, either ^{14}C-glycogen or ^{32}P-glucose-1-P, were then isolated and counted as described earlier in this section.

Equilibrium reaction rates were calculated from the exchanging substrate concentrations, time of reaction, and the amount of radioactivity incorporated into product, using reactions in which not more than 80% of the radioactive label appeared in the product (33, 34). Equilibrium reaction rates are expressed as μmoles product produced per minute per mg protein. For the experiments reported in Chapter VII, where the glycogen concentration was high (>2%) and the effective concentration of glycogen was calculated in terms of end groups, experiments were set up so as to ensure that first-order conditions prevailed. Reaction times were adjusted such that less than 15% of the radioactive label was incorporated into the isolated product. Under these conditions, the possibility of disproportionation effects was reduced to a minimum.

CHAPTER III

THE EFFECT OF ANIONS ON THE ACTIVITY OF PHOSPHORYLASE b

A. Results and Discussion

The effect of increasing concentrations of P_i on the activity of phosphorylase b is shown in Figure 1. The maximum activity obtained in the absence of AMP was approximately 30% of the V_{max} with AMP present, and was observed at a concentration of 575 mM P_i, in contrast to the 80% V_{max} figure reported by Buc (6), at only 120 mM P_i. The results reported here are analogous to those obtained with Na_2SO_4 by Appleman *et al.* (11) for the activation of inactive liver phosphorylase and the inhibition of active liver phosphorylase, although in their system AMP was present in both cases. Results obtained for high concentrations of glucose-1-P, minus AMP, are also shown in Figure 1. It is evident that there is very little activity with this substrate in the absence of AMP, and that which is observed (approximately 10% of V_{max} at 540 mM glucose-1-P) is most likely due to an anionic effect of the very high organic phosphate concentration.

The effect of anions high on the Hofmeister or lyotropic series (58) on the activity of phosphorylase b in the absence of AMP is shown in Figure 2. The control value was that activity obtained with 24 mM P_i alone and amounted to 1.8% of that for 24 mM P_i plus AMP. A marked similarity exists between the results for these activating anions and the activation observed for P_i.

Figure 3 shows that those anions low on the Hofmeister series produce the expected inhibitions. It is evident from the results with

Figure 1. The effect of high concentrations of P_i and glucose-1-P, in the presence and absence of AMP, on the activity of phosphorylase _b_. i. Left hand scale: phosphorolysis with P_i as substrate; ●, P_i plus AMP; ∇, P_i minus AMP. ii. Right hand scale: glycogen synthesis with glucose-1-P as substrate; X, glucose-1-P in the absence of AMP. V_{max} for glucose-1-P in the presence of AMP was 80 μmoles/min/mg.

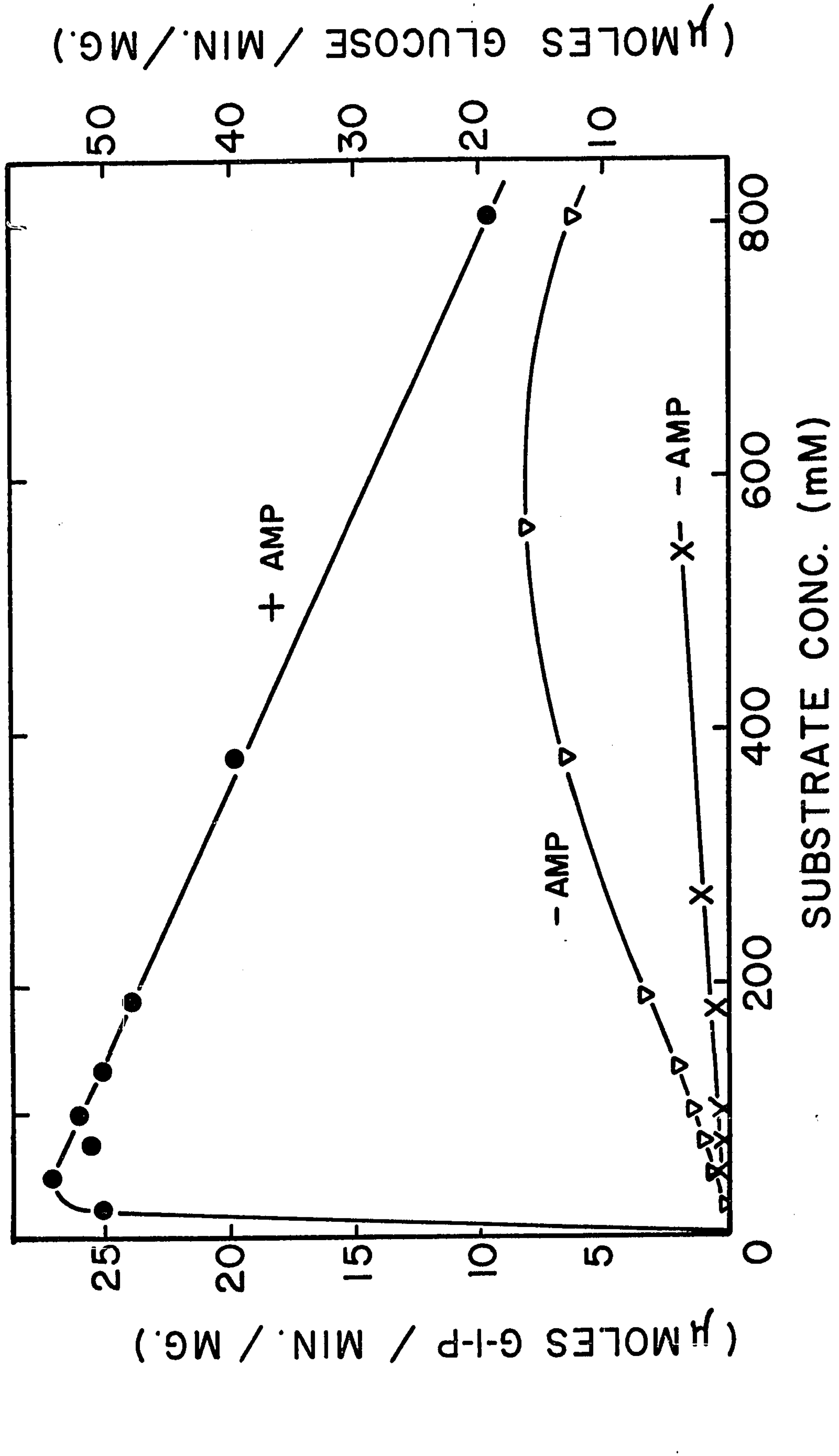

(μMOLES GLUCOSE / MIN./MG.)
50
40
30
20
10
+ AMP
– AMP
–AMP
25
20
15
10
5
0
200
400
600
800
SUBSTRATE CONC. (mM)
(μMOLES G-I-P / MIN. / MG.)

Figure 2. The effect of anions high in the Hofmeister series on the activity of phosphorylase b in the absence of AMP. X, Na_2SO_4; O, $(CH_3O)_2PO_2Na$; ∇, Na Tartrate; ●, Na citrate; ▲, KF.

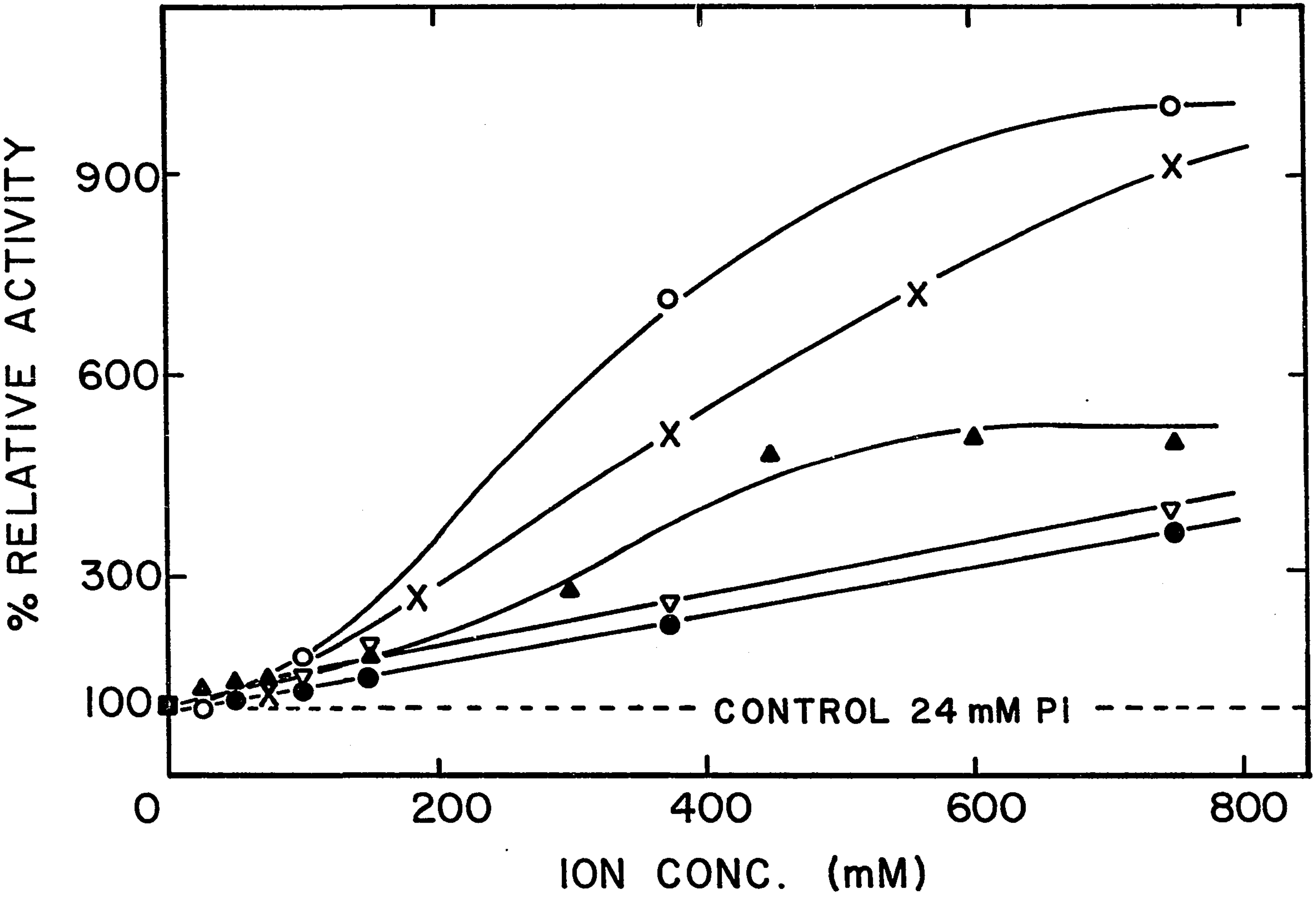
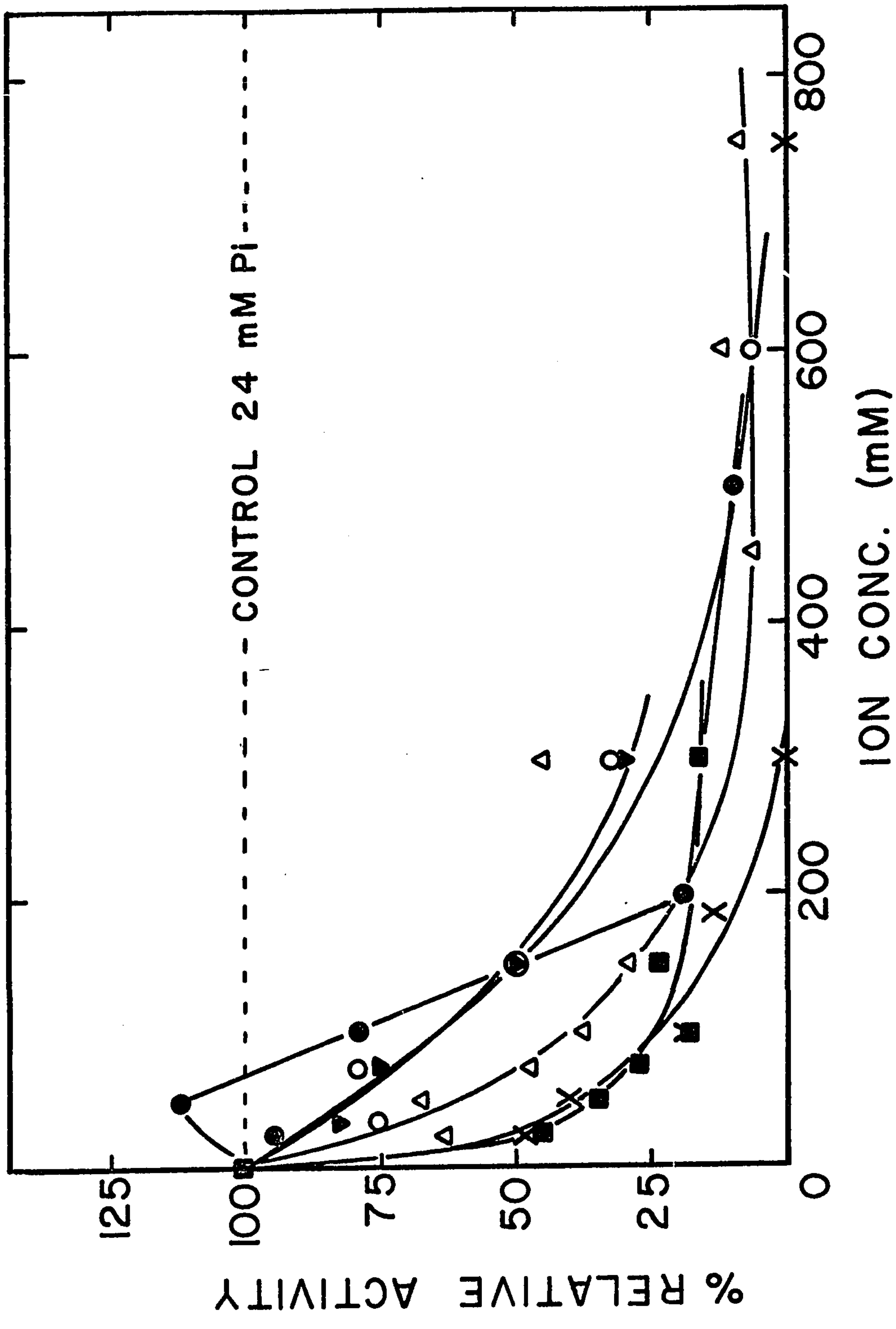

% RELATIVE ACTIVITY
900
600
300
100
0
200
400
600
800
ION CONC. (mM)
CONTROL 24 mM PI

Figure 3. The effect of anions low in the Hofmeister series on the activity of phosphorylase b in the absence of AMP. ◘, KI; Δ, KBr; ●, Na Tosylate; X, $NaNO_3$; O, NH_4Cl; ▼, NaCl.


CONTROL 24 mM Pi
% RELATIVE ACTIVITY
125
100
75
50
25
ION CONC. (mM)
0
200
400
600
800

the halides that the nature of the effect is due primarily to the anion.

These effects of anions in the Hofmeister series on the activity of phosphorylase b, as studied in the absence of AMP, are in direct qualitative agreement with those effects observed by Robinson and Jencks (59) on the solubility of acetyltetraglycine ethyl ester, a synthetic peptide. As shown in the above data for phosphorylase b, those anions that activate are high on the series, while those that inhibit are low on the series, i.e., those ions which tend to salt out protein activate, while those tending to salt in proteins inhibit.

Studies published by several laboratories suggest that the model for allosteric enzymes proposed by Monod et al. (21) may be applied to phosphorylase b. Of a few discrepancies so far noted, one of the most serious is the negligible activity in the absence of AMP (Madsen and Shechosky, 22). The interesting observation by Buc (6) that very high concentrations of P_i will activate the enzyme in the absence of AMP now appears to be related to a non-specific phenomenon. That glucose-1-P, the substrate in the reverse direction, provides only a limited activation also supports our contention. As suggested by Avramovic and Madsen (26) and others (23, 24, 25), conformational states in addition to the R and T states originally proposed may have to be invoked. It is possible that the activating effect of the anions is mediated by causing a conformational change in the enzyme similar to that induced by AMP and that a detailed study of these interactions may aid in explaining the role of conformational changes as related to the allosteric control of enzymic activity.

B. Summary

Rabbit muscle phosphorylase b has always been considered to possess an absolute dependence upon 5'-AMP for activity. However, Buc recently presented results suggesting that at high concentrations of P_i (120 mM) the enzyme loses its requirement for AMP. Since several other anions had previously been shown to affect the activity of various forms of phosphorylase, the effect of P_i and other anions on the activity of phosphorylase b was reinvestigated in the presence and absence of AMP. The effect of P_i on the activity of this enzyme compares with those results obtained for other anions high in the Hofmeister series and is most likely non-specific in nature. It is suggested that the activating effect of the anions is mediated by causing a conformational change in the enzyme similar to that induced by the activator AMP, and that a detailed study of these interactions may aid in explaining the role of conformational changes as related to the allosteric control of enzymic activity.

CHAPTER IV

THE KINETIC MECHANISM OF PHOSPHORYLASE b. INITIAL VELOCITY STUDIES AND ISOTOPE EXCHANGE AT EQUILIBRIUM

A. Results and Discussion

Initial Velocity Studies

Phosphorylase carries out a rather unique reaction in which, in a single catalytic event, one glucose moiety is added onto, or removed from, one of the end-groups of an enormous branched polymer of glucose. 6.8% of the glucose units of rabbit liver glycogen are at these end groups and the outer branches have an average length of 9 residues (60). Thus the product of the phosphorylase reaction is essentially equivalent to the substrate and, in fact, as shown by Hestrin (61), glycogen does not affect the chemical equilibrium (ratio of P_i:glucose-1-P) until it is either degraded to a limit dextrin or the chains are elongated considerably. In either case, the reaction rate slows down (61, 62, 63). In our experiments, care has been taken to ensure that an average of 2 or less glucose units per end group are added to or subtracted from the glycogen.

Therefore, under the conditions of the initial velocity studies reported in this study, one of the products of the reaction is present at all times, at a concentration equivalent to one of the substrates. Furthermore, as shown below and as demonstrated earlier by Maddaiah and Madsen (28), the dissociation constant is the same when measured from either direction. In Cleland's nomenclature (27), P = A and $K_{ip} = K_{ia}$. Making these substitutions in Cleland's general rate equation for a rapid equilibrium random bi bi mechanism, and substituting the terminology used in Figure 4, one obtains equation [1], shown in the legend for Figure 4. The most important practical effect of the number 2 in the numerator of the third expression on the right is that the kinetically derived values for K_1 and K_7 are actually one half the true dissociation constant for glycogen and the free enzyme, were one to use the usual rate equation. As will be seen below, the corrected values for K_1 and K_7 do agree reasonably well with the dissociation constant for glycogen which was determined by physical methods.

The other effect of the modified rate equation for glycogen phosphorylase is that the Michaelis constants for phosphate and glucose-1-P, (K_3 and K_5, respectively), determined with saturating levels of glycogen, are twice the theoretical values. This result is that expected from having equal concentrations of a product and substrate, with equal dissociation constants, present at all times when the activity of the enzyme is being measured. The phenomenon is probably of only academic interest in the case of phosphorylase. However, for those enzymes which carry out analogous reactions on both a homopolymer or a heteropolymer, the Michaelis constants for given monomer substrates may well vary depending on the type of polymer which is being synthesized or degraded. Naturally, the discussion about the dissociation constants for the polymer itself would also apply. An example of an enzyme for which these considerations should be borne in mind when evaluating the kinetics is polynucleotide phosphorylase (E.C. 2.7.7.8.).

Initial rate data for the phosphorolysis of glycogen at varying concentrations of both glycogen and P_i are shown in the primary

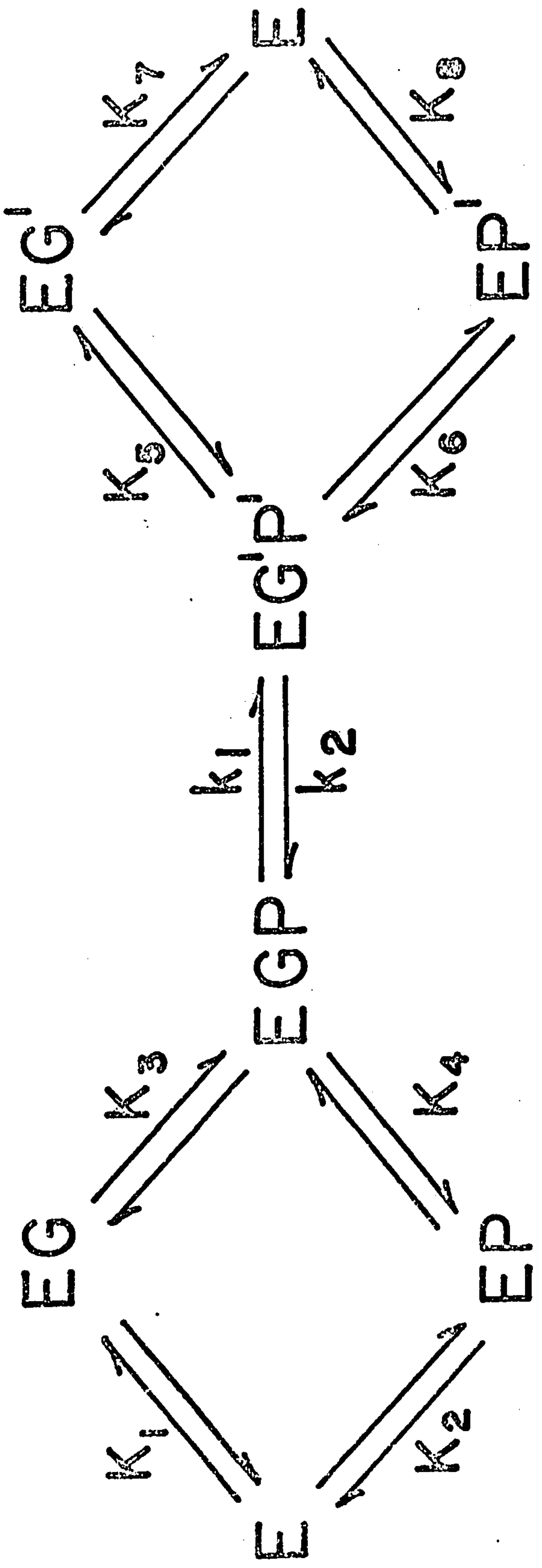
Figure 4. Proposed rapid equilibrium random bi bi kinetic mechanism for muscle phosphorylase b. E, G and P are, respectively, enzyme-AMP complex, glycogen and P_i, G' is glycogen with one less terminal glucose residue while P' is glucose-1-P. The eight dissociation constants and two rate constants are indicated. The overall rate equation relating the initial velocity (V_o), total enzyme concentration (E) and substrate concentrations for the forward direction is, as explained in the text,

$$\frac{E}{V_o} = \frac{1}{k_1} + \frac{K_4}{k_1(G)} + \frac{2K_3}{k_1(P)} + \frac{K_1K_3}{k_1(G)(P)} \qquad [1]$$

which can be written in the general form of Dalziel (29),

$$\frac{E}{V'_o} = \phi_o + \frac{\phi_1}{(G)} + \frac{\phi_2}{(P)} + \frac{\phi_{12}}{(G)(P)} \qquad [2]$$

Analogous equations may be written for the reverse reaction, with the appropriate constants for Equation 1 and adding primes in Equation 2.

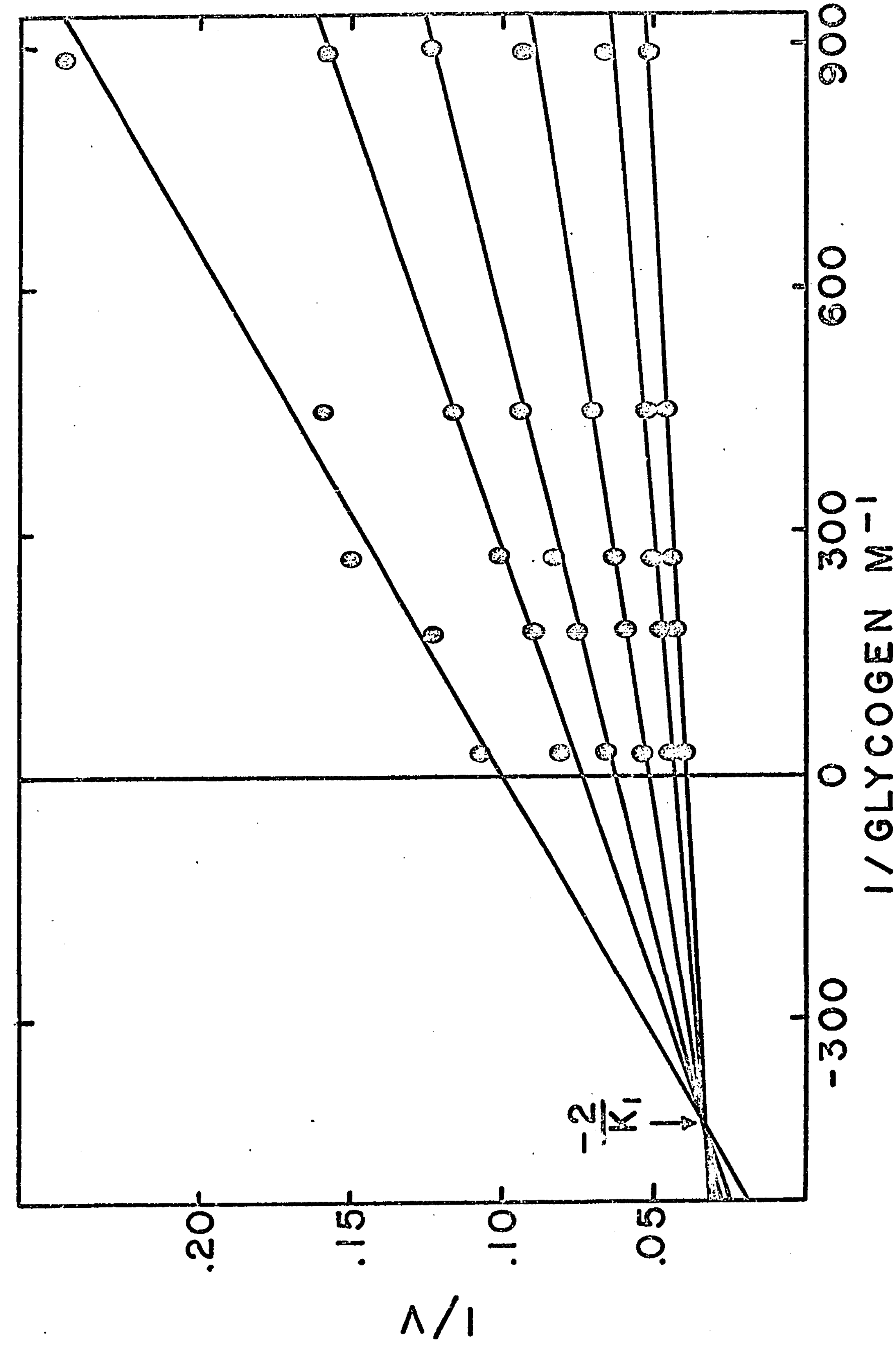

E
EG'
EP'
EG'P'
EGP
EG
EP
E
K7
K8
K5
K6
k1
k2
K3
K4
K1
K2

plots of Figures 5 and 6. In Figure 5, the intersection point in the upper left hand quadrant yields the dissociation constant for glycogen and enzyme·AMP, and corresponds to the appropriate constant in Figure 4. Similarly, in Figure 6, the point of intersection yields the dissociation constant for P_i and enzyme·AMP. The upward curvature of these lines at the lower substrate concentrations is typical for phosphorylase b and is most likely due to an expression of homotropic cooperativity between the P_i binding sites (22). However, it could also be due to a breakdown of the rapid equilibrium condition for a random bi bi mechanism, resulting in the appearance of squared substrate concentration terms in the rate equation (43, Chapter VII). Experiments to distinguish between these two possibilities are reported below and also in Chapter VII, but for the time being the first explanation will be assumed. Where the lines on reciprocal plots are curved, the extrapolations have been drawn from the data for the higher range of substrate concentrations. Initial rate data have been interpreted on the basis of a rapid equilibrium random bi bi mechanism, as illustrated in Figure 4.

The initial rate data for the reaction in the direction of glycogen synthesis at varying concentrations of glycogen and glucose-1-P are given in Figures 7 and 8. The dissociation constants for glycogen derived from Figures 5 and 7 are very similar, i.e., $K_1 = K_7$, as would be expected for this enzyme.

Figures 9-12 show the intercepts and slopes from the double reciprocal plots replotted according to the method of Dalziel (29). Internal consistencies of the data are indicated by common intercepts on the intercept plots, and parallel lines on the slope plots. The

Figure 5. Velocity of muscle phosphorylase b activity as a function of glycogen concentration at six levels of P_i concentrations. (1/v in this and all other reciprocal plots is in units of mg of protein x min per μmole product, either glucose-1-P or P_i). Concentrations of P_i, reading from the upper line downward, and corresponding apparent Michaelis constants for glycogen, are as follows: (a) 0.97 mM P_i, 1.55 mM glycogen; (b) 1.5 mM P_i, 1.28 mM glycogen; (c) 2.0 mM P_i, 1.1 mM glycogen; (d) 3.0 mM P_i, 0.86 mM glycogen; (e) 5.2 mM P_i, 0.54 mM glycogen; (f) 11.2 mM P_i, 0.38 mM glycogen.



.20
.15
.10
0.5
1/V
-300
0
300
600
900
1/GLYCOGEN M^{-1}
$-\frac{2}{K_I}$

Figure 6. Velocity of muscle phosphorylase b activity as a function of P_i concentration at five levels of glycogen. Concentrations of glycogen and corresponding apparent Michaelis constants for P_i are as follows: ○, 1.12 mM glycogen, 4.45 mM P_i; X, 2.25 mM glycogen, 3.33 mM P_i; Δ, 3.75 mM glycogen, 3.0 mM P_i; ●, 5.6 mM glycogen, 2.8 mM P_i; □, 28 mM glycogen, 2.56 mM P_i.

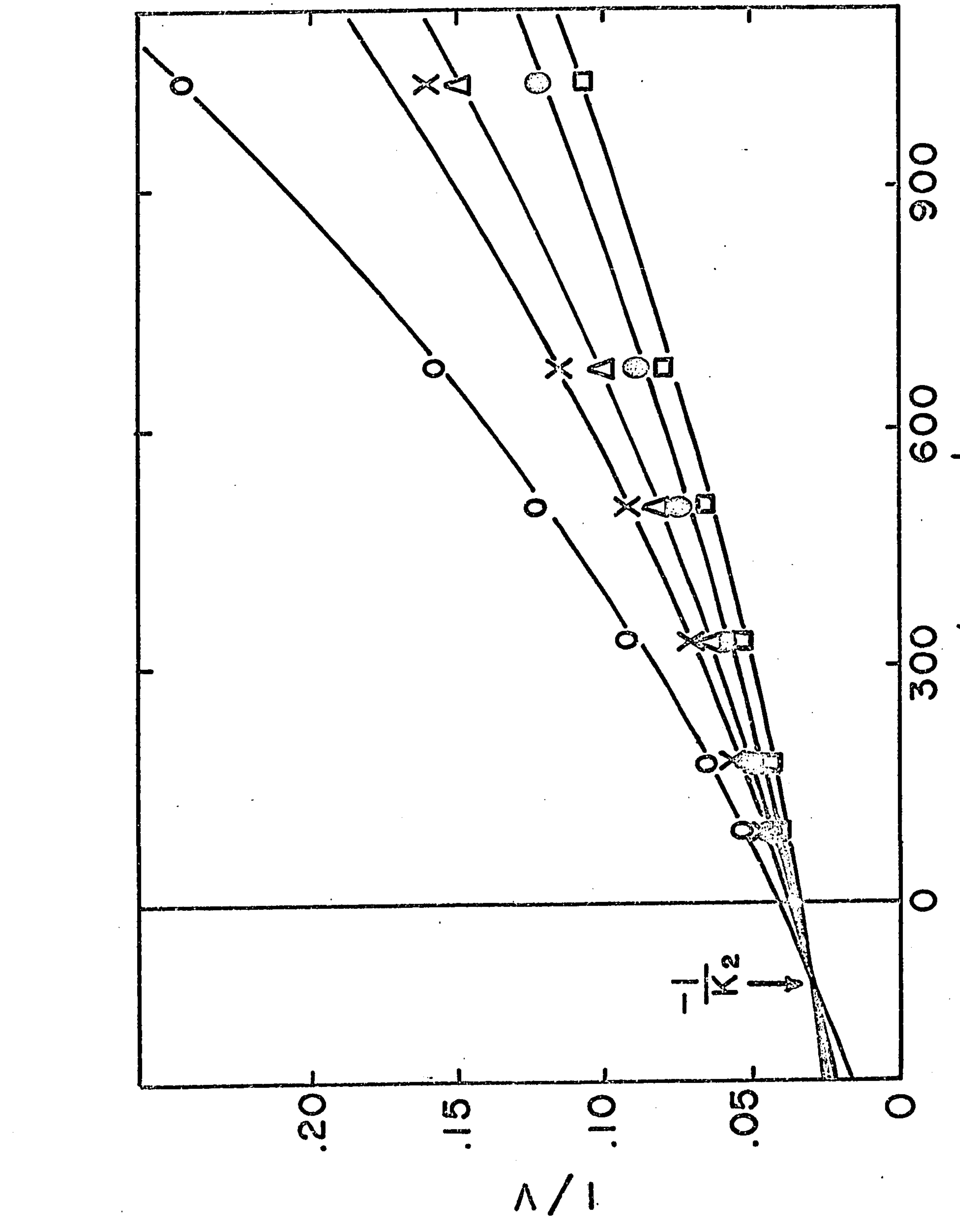

1 / V
.20
.15
.10
.05
0
0
300
600
900
1 / Pi M⁻¹
$-\frac{1}{K_2}$

Figure 7. Velocity of muscle phosphorylase _b_ activity as a function of glycogen concentration at six levels of glucose-1-P. Concentrations of glucose-1-P and corresponding apparent Michaelis constants for glycogen are as follows: ○, 2.16 mM glucose-1-P, 1.57 mM glycogen; Δ, 2.98 mM glucose-1-P, 1.40 mM glycogen; X, 4.26 mM glucose-1-P, 1.22 mM glycogen; ●, 8.52 mM glucose-1-P, 1.11 mM glycogen; □, 12.8 mM glucose-1-P, 0.975 mM glycogen, ▼, 25.6 mM glucose-1-P, 0.79 mM glycogen.

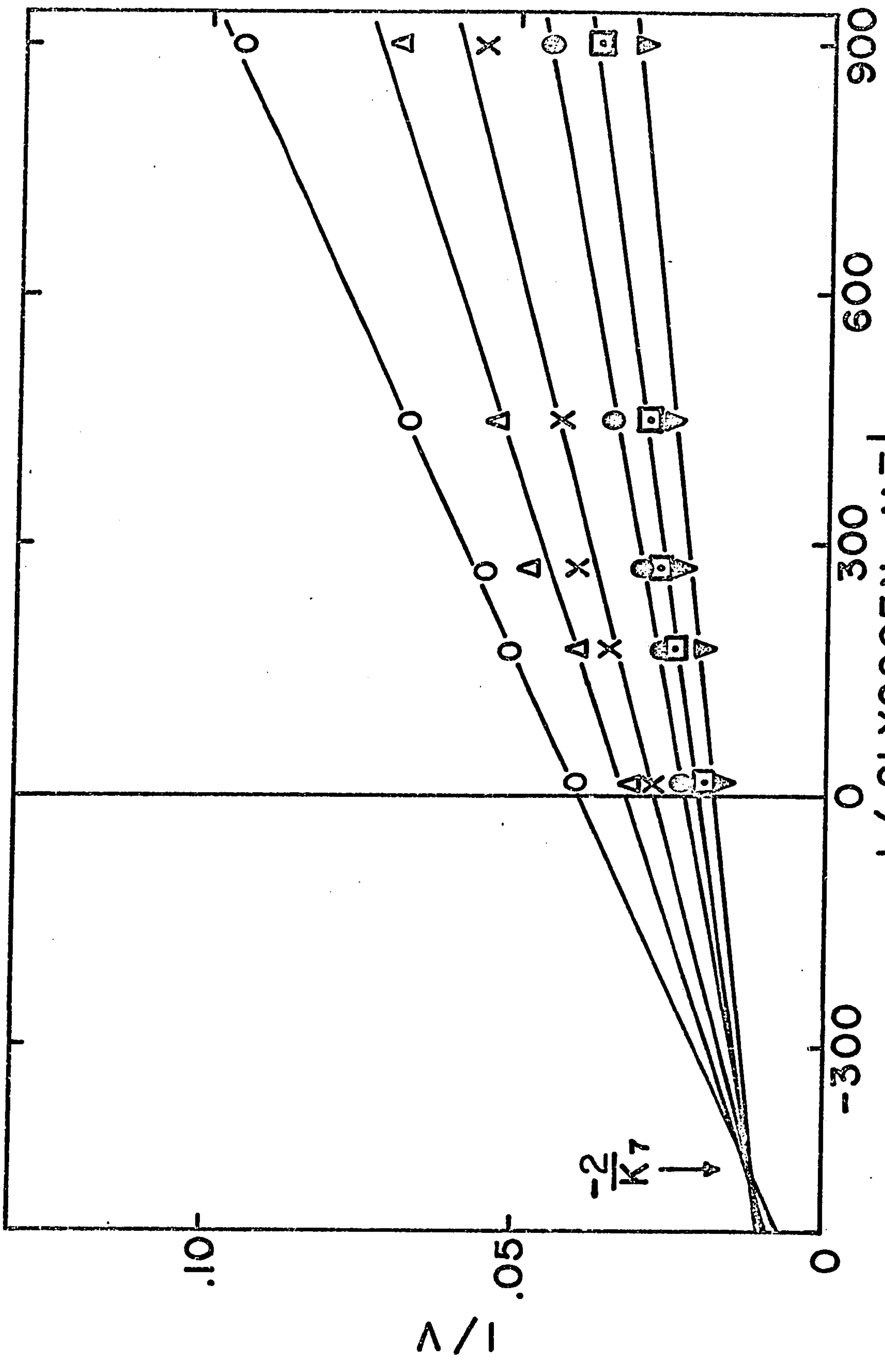
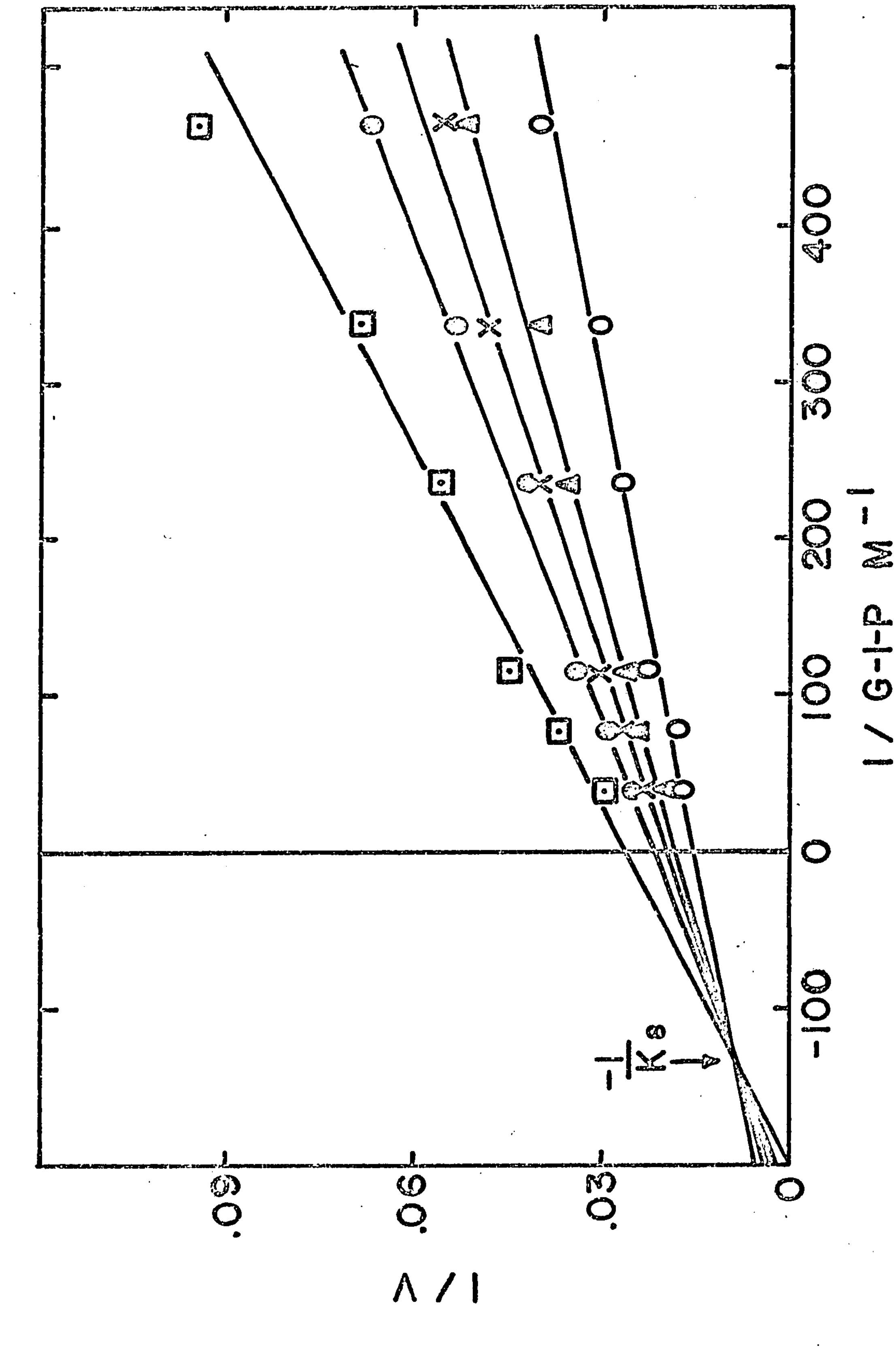

1 / V
0
.05
.10
1 / GLYCOGEN M⁻¹
-300
0
300
600
900
-2/K7

Figure 8. Velocity of muscle phosphorylase b activity as a function of glucose-1-P concentration at five levels of glycogen. Concentrations of glycogen and corresponding apparent Michaelis constants for glucose-1-P are as follows: □, 1.11 mM glycogen, 5.15 mM glucose-1-P; ●, 2.22 mM glycogen, 4.56 mM glucose-1-P, X, 3.67 mM glycogen, 4.27 mM glucose-1-P; ▲, 5.56 mM glycogen, 3.8 mM glucose-1-P; O, 55.6 mM glycogen, 3.28 mM glucose-1-P.



1 / V
.09
.06
.03
0
-100
0
100
200
300
400
1 / G-1-P M⁻¹
-1/Ks

Figure 9. Secondary plots of the intercepts (mg of protein x min per μmole) from the two primary Lineweaver-Burk plots in the direction of glycogen degradation. O, Figure 5; ▲, Figure 6.

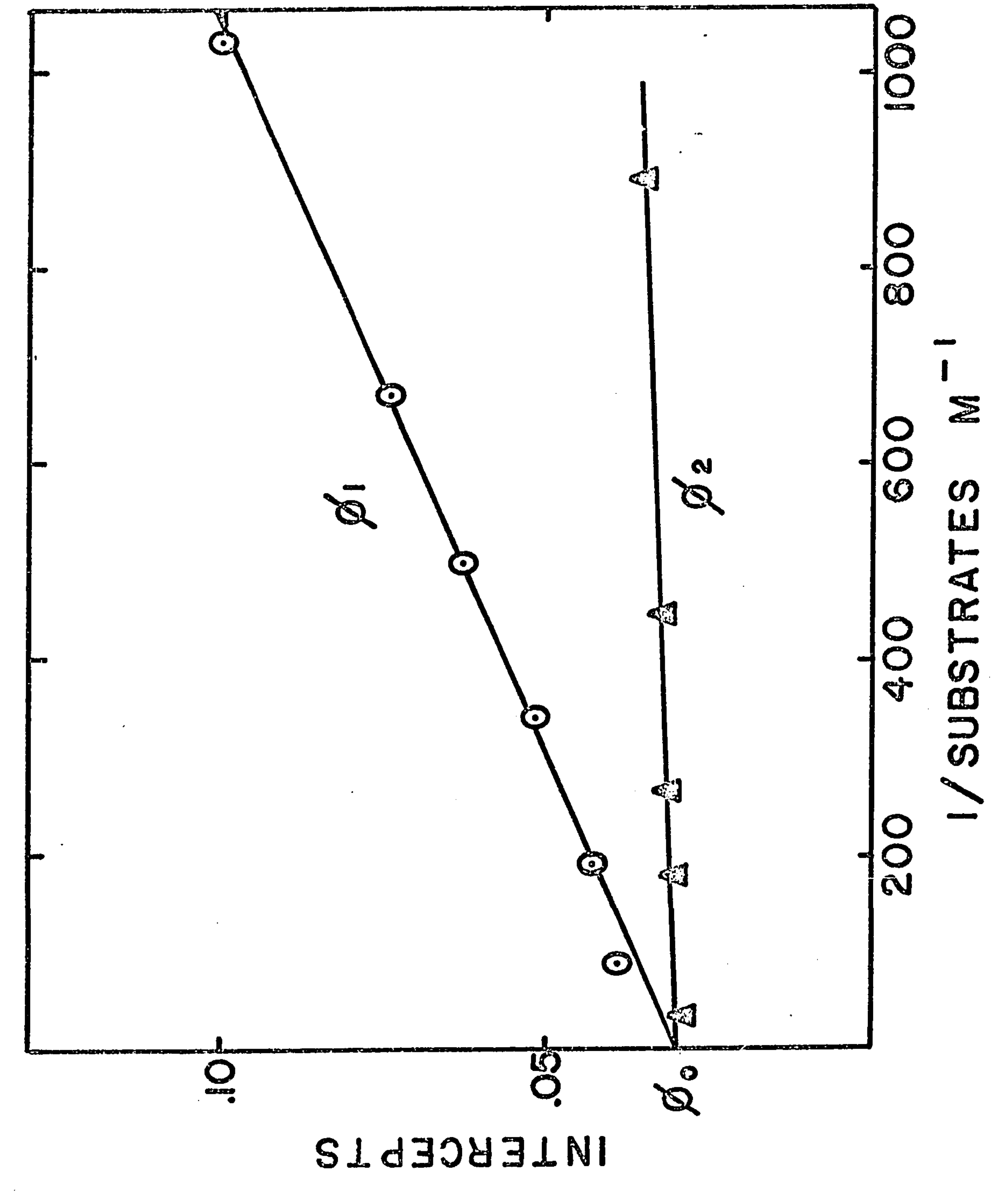
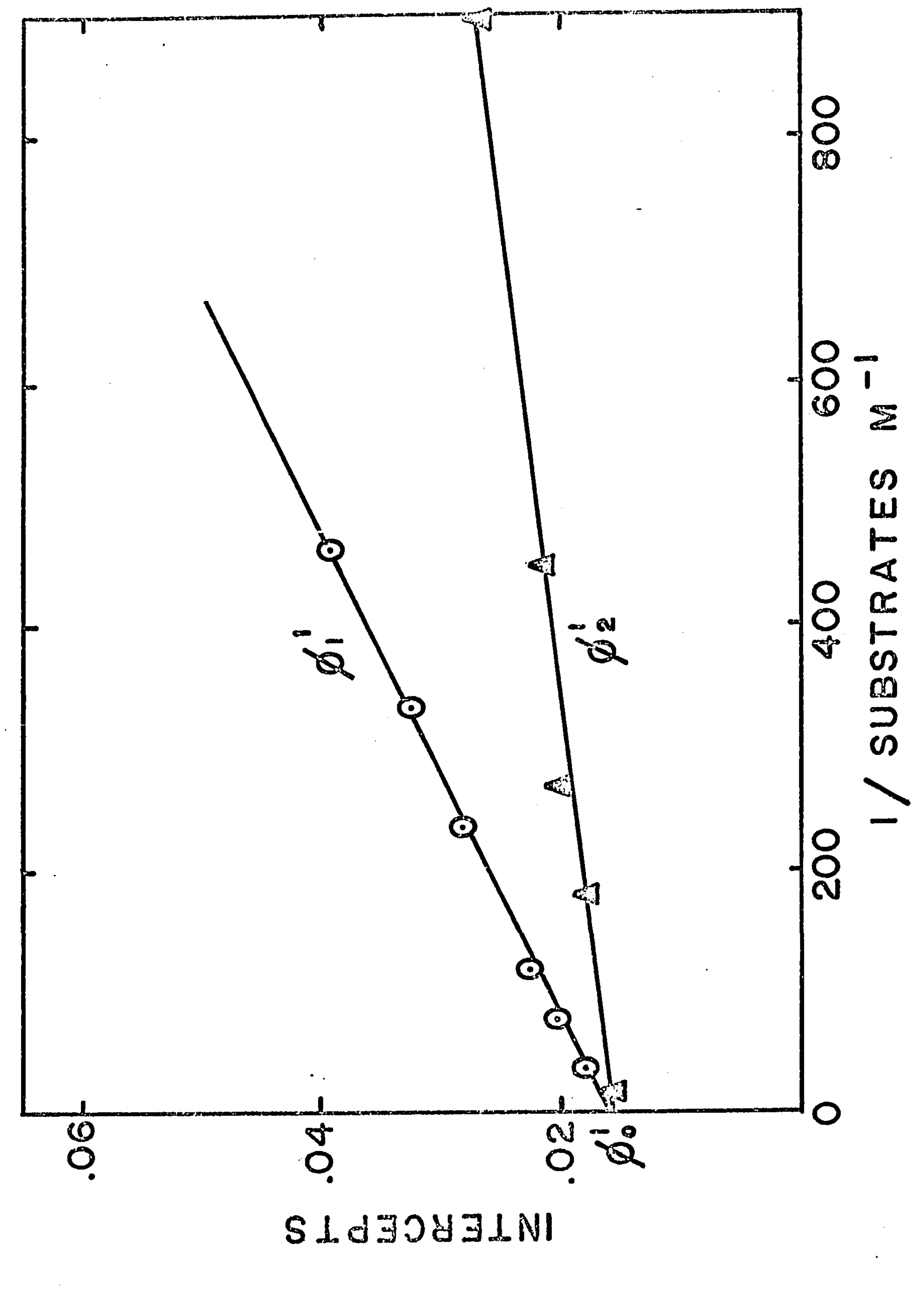

INTERCEPTS
.10
.05
ϕ₀
ϕ₁
ϕ₂
200
400
600
800
1000
1/SUBSTRATES M⁻¹

Figure 10. Secondary plots of the intercepts (mg of protein x min per μmole) from the two primary Lineweaver-Burk plots in the direction of glycogen synthesis. ○, Figure 7; ▲, Figure 8.



INTERCEPTS
.06
.04
.02
ø'₀
0
200
400
600
800
1/ SUBSTRATES M⁻¹
ø'₁
ø'₂

Figure 11. Secondary plots of the slopes (mg of protein x min) from the two primary Lineweaver-Burk plots in the direction of glycogen degradation. X, Figure 5; ●, Figure 6.

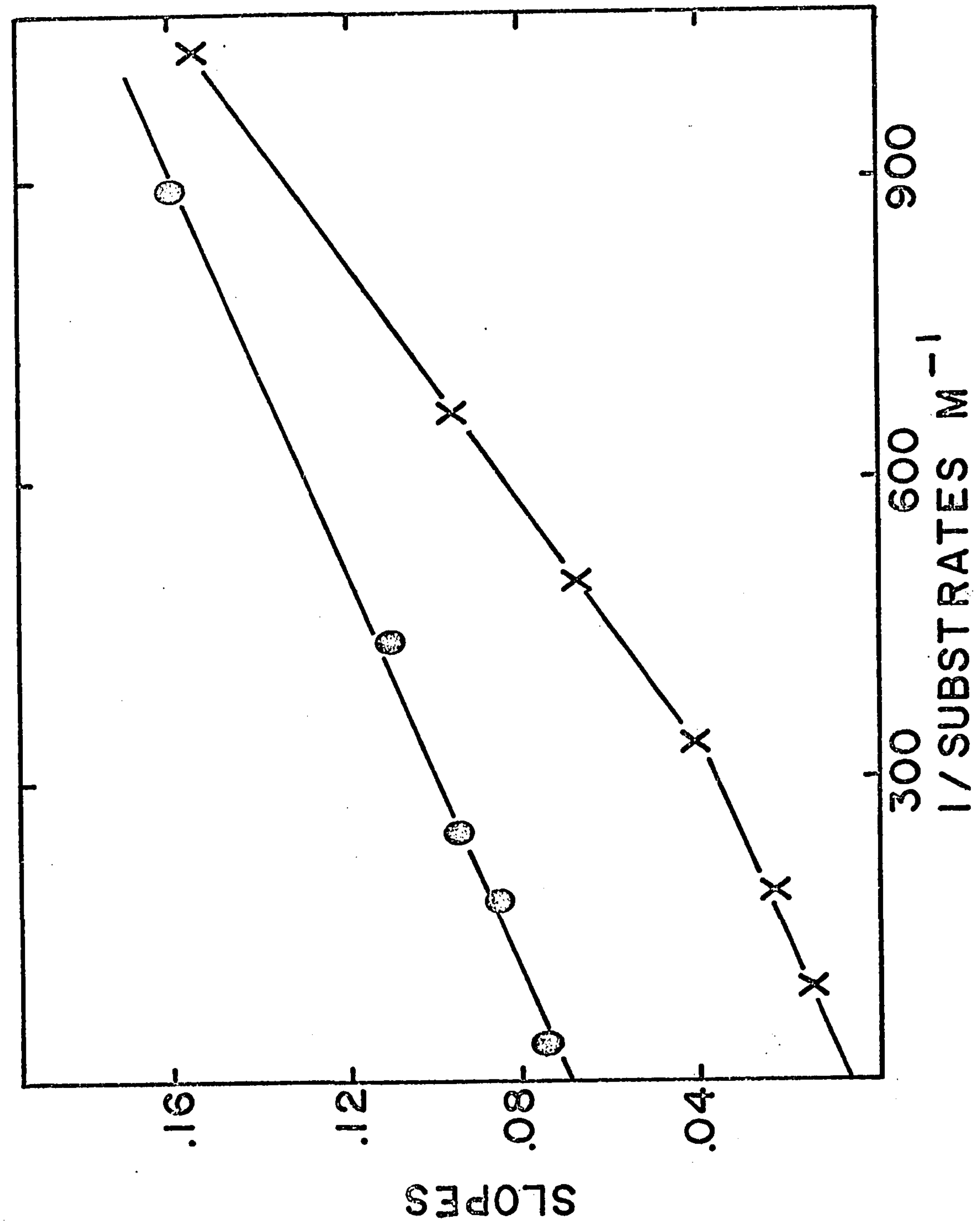
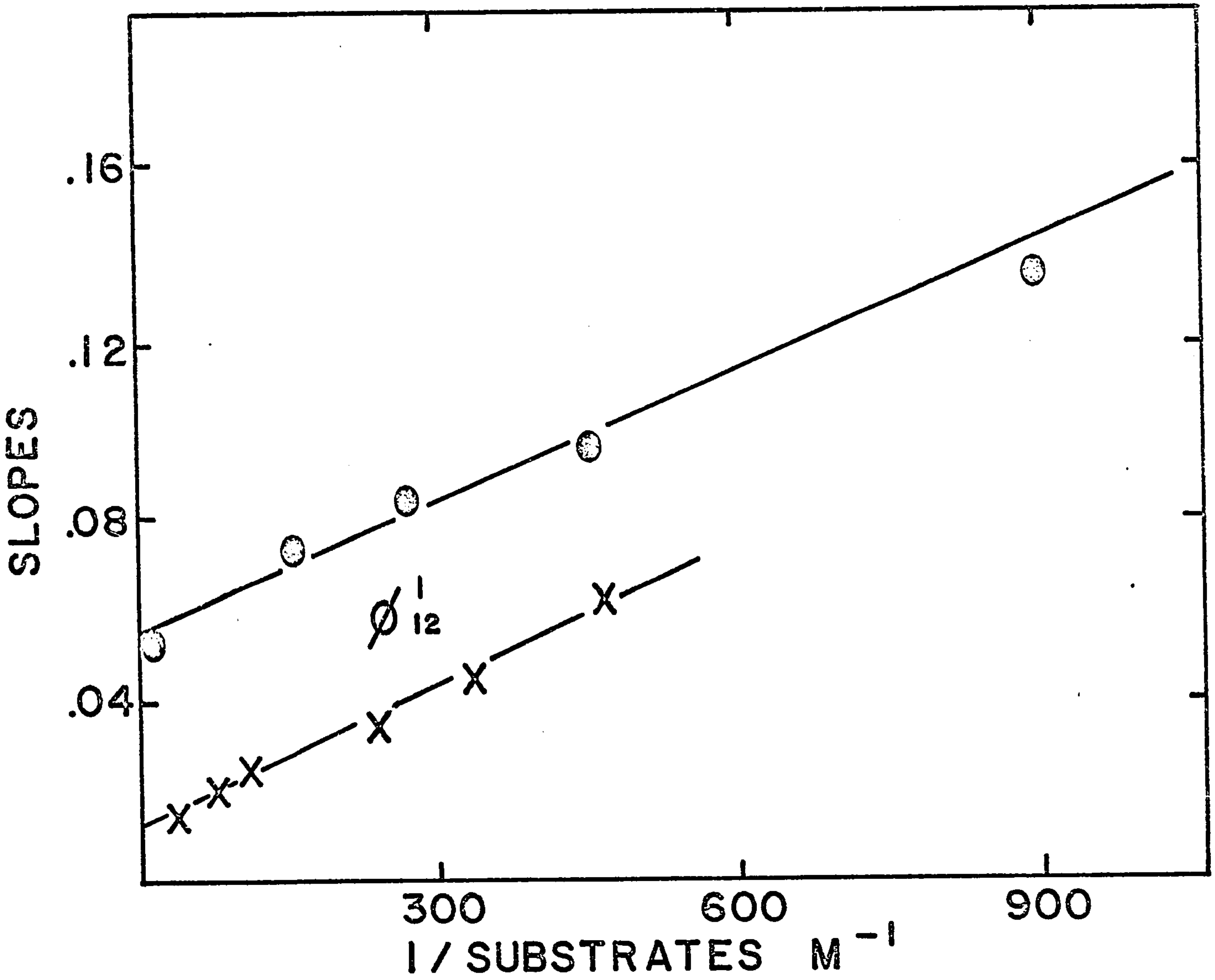

SLOPES
.16
.12
.08
.04
300
600
900
1/SUBSTRATES M^{-1}

Figure 12. Secondary plots of the slopes (mg of protein x min) from the two primary Lineweaver-Burk plots in the direction of glycogen synthesis. X, Figure 7; ●, Figure 8.


SLOPES
.16
.12
.08
.04
ϕ^{1}_{12}
300
600
900
1 / SUBSTRATES M^{-1}

replot of slopes from Figure 5, as shown on Figure 12, has an upward curvature at the low substrate concentrations, reflecting the same phenomenon (presumably homotropic cooperativity) seen in Figure 6. Table I summarizes the various kinetic coefficients derived from the replots, which are generalized entities applicable to any kinetic mechanism. The specific kinetic constants, applied to the kinetic mechanism in Figure 4, are also given in Table I.

Table II summarizes certain relationships between the kinetic coefficients and kinetic constants. The equilibrium constant calculated from initial rate data using the Haldane relationship (27, 29) does not agree with that obtained by direct measurement (57) as it did for other phosphorylase systems. The latter include liver phosphorylase (28), rabbit muscle phosphorylase a (calculated from the data of Lowry et al. (14)) and frog muscle phosphorylase a (calculated from the data of Metzger et al. (15)). Thus the only exception noted so far is the allosteric phosphorylase b, since those phosphorylases which have escaped allosteric control by having phosphorylated serine residues exhibit the correct Haldane relationship for a rapid equilibrium random bi bi mechanism. One would thus tend to associate the incorrect Haldane relationship of phosphorylase b with its allosteric properties. For example, it was suggested by Avramovic and Madsen (26), on the basis of chemical inactivation studies, that the binding of P_i or glucose-1-P to phosphorylase b already saturated with AMP is correlated with a conformational change. This suggestion received support from the studies of Kastenschmidt et al. (25), who proposed two R states which differ in their affinity for AMP and glucose-1-P. Black and Wang also suggested that a two-stage allosteric transition

TABLE I

Summary of data from Figures 4 through 12.

Kinetic coefficients	Corresponding kinetic constants
ϕ_o = .030 mg of protein x min per μmole	k_1 = 33 μmoles/min/mg
ϕ_1 = 67 min x mg	K_4 = 0.20 mM glycogen
ϕ_2 = 6 min x mg	2 K_3 = 2.2 mM P_i
ϕ_{12} = 0.09 mole x min x mg	1/2 K_1[a] = 2.3 mM glycogen
	K_2[a] = 15 mM P_i
ϕ'_o = 0.0155 mg of protein x min per μmole	k_2 = 64.5 μmoles/min/mg
ϕ'_1 = 51 min x mg	K_6 = 0.90 mM glycogen
ϕ'_2 = 13 min x mg	2 K_5 = 3.0 mM glucose-1-P
ϕ'_{12} = 0.104 mole x min x mg	1/2 K_7[a] = 2.2 mM glycogen
	K_8[a] = 7.4 mM glucose-1-P

[a] From the intersections in the upper left-hand quadrants of Figures 5 through 8.

TABLE II

Relationships between coefficients and constants

$K_1K_3 = 5 \times 10^{-6}$ $K_2K_4 = 3 \times 10^{-6}$

$$\phi_{12} = \frac{K_1K_3}{k_1} = \frac{K_2K_4}{k_1} = 0.120 \quad \text{(average)}$$

ϕ_{12} from Figure 6 = 0.090

$K_5K_7 = 7 \times 10^{-6}$ $K_6K_8 = 6 \times 10^{-6}$

$$\phi'_{12} = \frac{K_5K_7}{k_2} = \frac{K_6K_8}{k_2} = 0.101 \quad \text{(average)}$$

ϕ'_{12} from Figure 7 = 0.104

$$K_{eq} = \frac{\phi'_{12}}{\phi_{12}} = 0.84$$

K_{eq} by direct measurement = 0.21[a] is involved in the activation of the enzyme (23). Thus, extra enzyme isomerization steps might have to be added to the scheme in Figure 4. Significantly, the major difference in the kinetic constants of phosphorylase b which leads to the incorrect Haldane relationship concerns the relative binding affinities of the anionic substrates; glucose-1-P not showing the markedly greater binding affinity and lower Michaelis constant compared to P_i that is seen in the other phosphorylases. However, it is admittedly hard to see how the insertion of these extra isomerization steps into a supposedly symmetrical kinetic mechanism could lead to an alteration in the calculated equilibrium constant.

---

[a] From Trevelyan _et al_. (57).

Table III illustrates the kinetically determined dissociation constants for phosphorylase b as compared to those obtained by independent physical methods. The K_{diss} of 7.4 mM for glucose-1-P observed kinetically agrees well with the value of 6 mM determined by Avramovic and Madsen (26). The kinetic K_{diss} for P_i, 10 to 15 mM, also agrees with the 11 mM value obtained from the isocyanate inactivation studies (26). It should be noted that the values published by other investigators for the P_i dissociation constant also fall in this range (64, 65).

Rabbit liver glycogen (Sigma) was fractionated in the preparative ultracentrifuge to remove light fractions which did not sediment at 96,000 x g for 60'. This material was then used to determine the dissociation constant for glycogen and the enzyme saturated with AMP by the ultracentrifugal separation method of Madsen and Cori (63). The results, shown in Table III, are similar to those obtained by Kastenschmidt et al. (24), for corn phytoglycogen and the

TABLE III

Dissociation constants for substrates and phosphorylase b

Source of dissociation constant	Glucose-1-P	P_i	Glycogen
Initial velocity kinetics:			
(1) From Table I	7.4	15	4.6
(2) From two other similar experiments	(7.5, 8.0)	(10, 10)	(3.9, 4.4)
Physical Studies	6.3[a]	11[a] 10[b] 20[c]	5 to 10[d] 4[e]

Units are millimolar, for enzyme saturated with AMP, except as noted.

[a] Avramovic and Madsen (26)

[b] Buc and Buc (64)

[c] Bresler and Firsov (65)

[d] This Chapter

[e] Kastenschmidt, Kastenschmidt and Helmreich, for the apoenzyme in the absence of AMP. The holoenzyme gave similar results (24).

enzyme in the absence of AMP, and also for enzyme from which the pyridoxal phosphate had been removed. There is reasonable agreement between the results from these physical measurements and those derived from the kinetic studies.

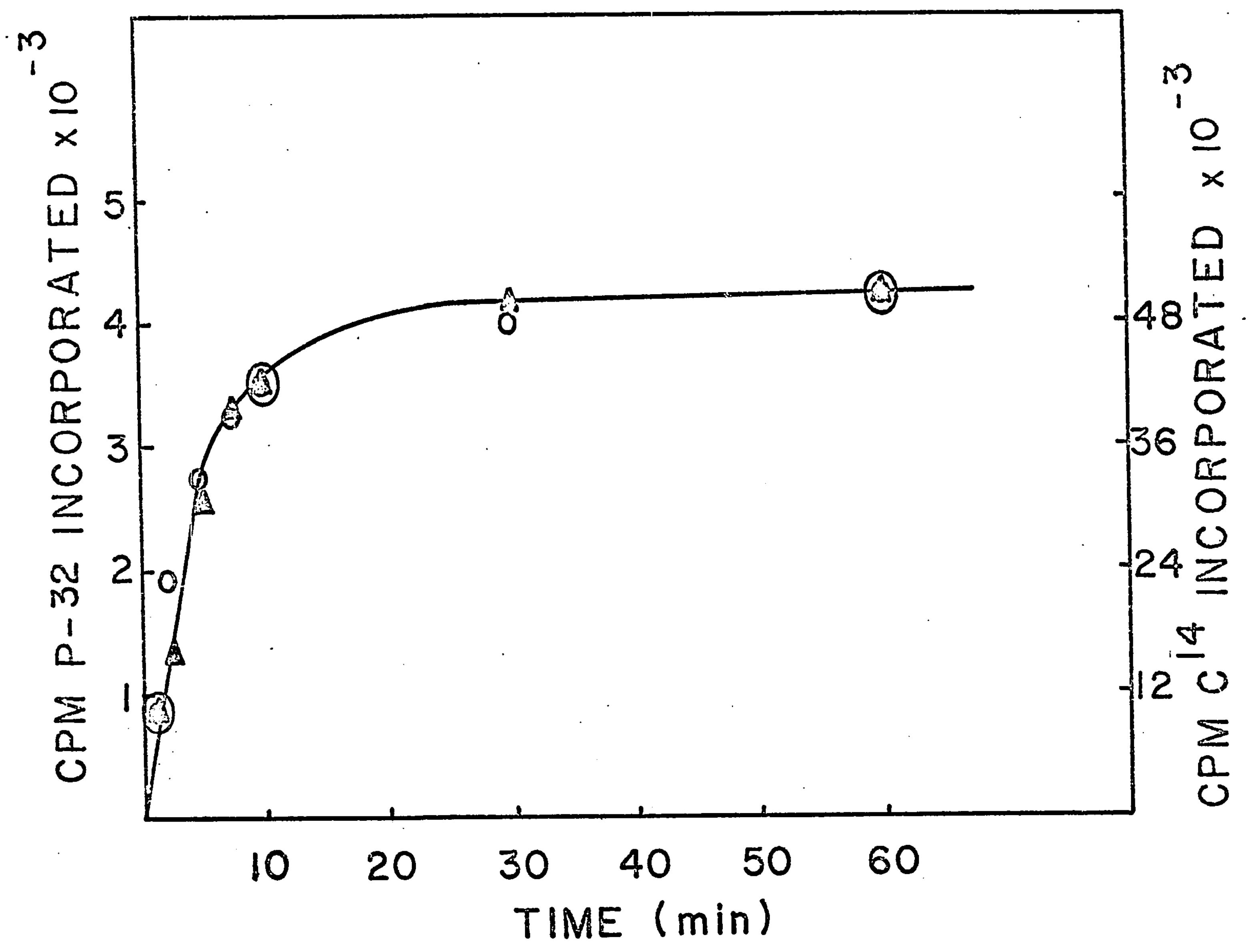
The favorable comparison of kinetic dissociation constants with those determined by independent physical methods supports the assignment of the rapid equilibrium random bi bi mechanism (27) to phosphorylase _b_.

Isotope Exchange Studies

Preliminary experiments showed that the rate of isotope exchange was directly proportional to the concentration of enzyme present. In order to confirm the fact that the reaction is rapid equilibrium, one equilibrium mix was divided into several 0.5 ml aliquots and the $P_i \rightleftharpoons$ glucose-1-P rate followed in one half of them, the glucose-1-P $\rightleftharpoons$ glycogen rate in the remainder. It can be seen in Figure 13 that the two rates of incorporation are very similar. This experiment demonstrates that the interconversion of ternary complexes is the rate limiting step in the conversion of substrate to products. Since in this experiment, the concentrations of P_i and glucose-1-P are in the vicinity of K_m, these data do not completely rule out an ordered mechanism with glycogen adding first in a rapid equilibrium step, regardless of the relative rate of ternary complex interconversion (34).

The approach to equilibrium of the $^{32}P_i \rightleftharpoons$ glucose-1-P exchange followed first order kinetics as would be expected. However, the rate of the ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange slowed down with

Figure 13. Isotope exchange rates measured from a single equilibrium mix containing 20 μg phosphorylase b per ml, 1.17 mM glucose-1-P, 3.94 mM P_i, 55.5 mM glycogen, 1 mM AMP, in 3 mM Na-glycerophosphate, 1.5 mM EDTA, 2.5 mM mercaptoethanol, pH 6.8. O, incorporation of ^{14}C-glucose-1-P into glycogen; ▲, incorporation of $^{32}P_i$ into glucose-1-P.

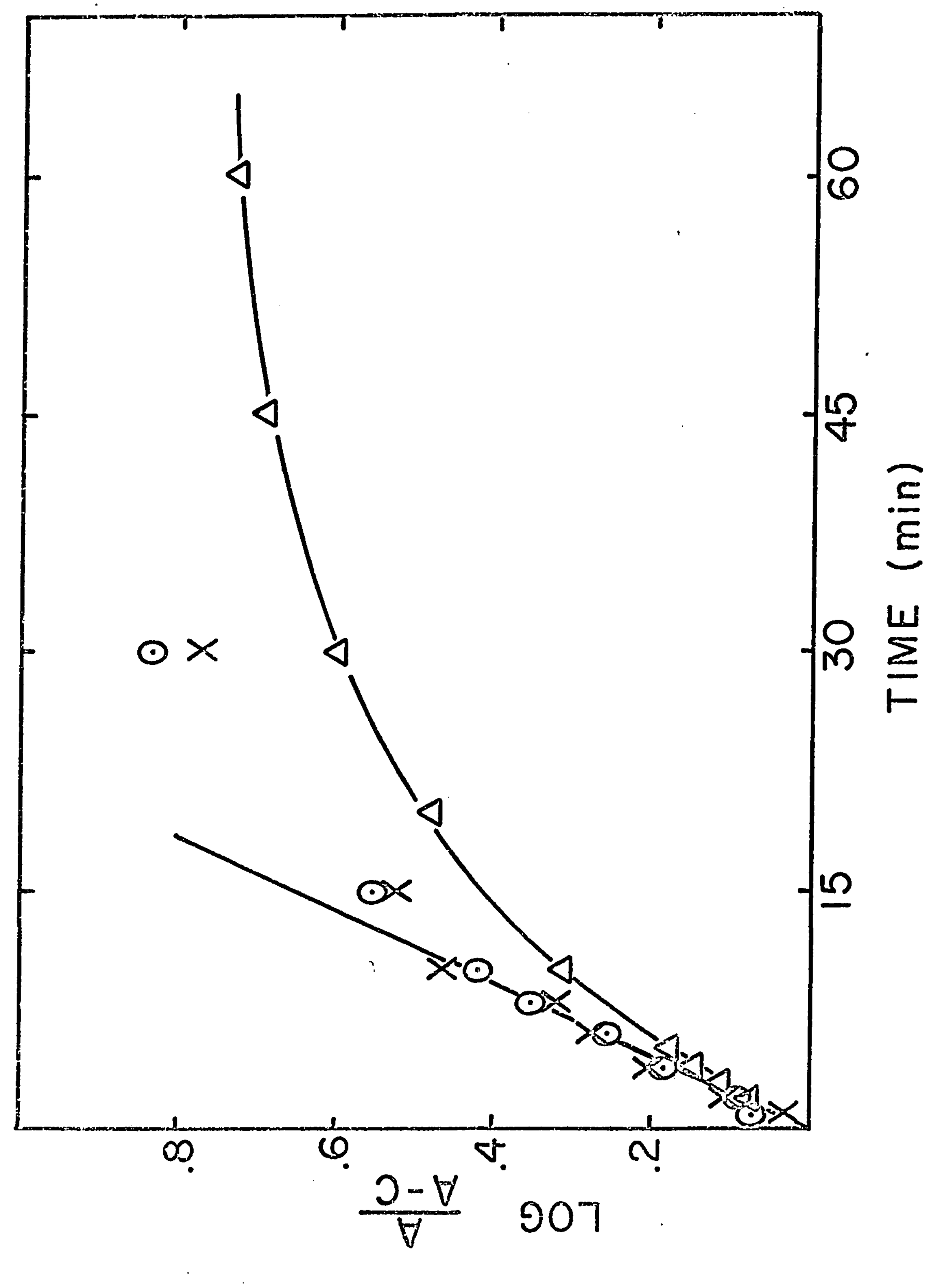

CPM P-32 INCORPORATED x 10^{-3}
1
2
3
4
5
CPM C^{14} INCORPORATED x 10^{-3}
12
24
36
48
10
20
30
40
50
60
TIME (min)

time, so that successive rate constants decreased as shown in Figure 14. For the experiments reported in this Chapter, this deviation from first order kinetics was not serious enough to prevent using the rates calculated from the early periods. The ratio of isotopes found in the two exchanging substrates after equilibrium has been established is that expected from the chemically derived equilibrium constant for the phosphorylase reaction (57). For example, approximately 20% of the total $^{32}P_i$ added is found in the form of glucose-1-P, while approximately 80% of the total ^{14}C is found in the form of glycogen.

The deviation of the glucose-1-P $\rightleftharpoons$ glycogen exchange may be due to the disproportionate attack of phosphorylase on the outer chains of glycogen. As shown by Illingworth _et al_. (66), the pre-incubation period would have allowed the enzyme to lengthen some of the outer chains of glycogen and to shorten others. Examination of the iodine color of our incubation mixes showed a shift in the spectra similar to that reported by these authors (66) and suggested that, by the time isotope was added, the outer chain glucose units have become asymmetrically distributed. During the isotope incorporation, the loss of ^{14}C-glucose moieties most recently incorporated might then be greater than expected from statistical considerations.

If this hypothesis is correct, then elimination of the pre-incubation period should allow the glucose-1-P $\rightleftharpoons$ glycogen exchange to achieve a first order character. An experiment to test this is shown in Figure 14. Without preincubation, both the exchanges are similar and follow first order kinetics for 75% of the path toward isotope equilibrium. With pre-incubation, the ^{14}C exchange began to deviate from first order as early as 11% of the attainment of equilibrium,

Figure 14. First order plot showing the effect of pre-incubation of equilibrium mixes before isotope exchange. A = cpm incorporated at infinite time, c = cpm incorporated at time t. Conditions as in Figure 13, with glucose-1-P = 5 mM, P_i = 20 mM. ⊖, $^{32}P_i \rightleftharpoons$ glucose-1-P exchange, no pre-incubation; X, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange, no pre-incubation; Δ, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange with the usual 2-1/2 hours pre-incubation.


LOG $\frac{A}{A-C}$
.8
.6
.4
.2
15
30
45
60
TIME (min)

whereas the $^{32}P_i$ exchange rate (not shown) remained constant.

Figures 15 and 16 present the results of the isotope exchange at equilibrium experiments for the phosphorylase b system. In Figure 15, the glycogen concentration was held constant while glucose-1-P and P_i concentrations were varied at a constant ratio up to values approaching 10 times K_m. The $P_i \rightleftharpoons$ glucose-1-P exchange rate rose and then leveled off as saturation of the enzyme with these two substrates was reached. The glucose-1-P $\rightleftharpoons$ glycogen rate paralleled the $P_i \rightleftharpoons$ glucose-1-P rate. The results of Figure 15 negate the possibility that glycogen is the initial substrate to be bound in a compulsory mechanism. If this had been so, one would have expected a depression of the glucose-1-P $\rightleftharpoons$ glycogen exchange rate at the elevated P_i and glucose-1-P concentrations (34).

The unlikely possibility that glucose-1-P is the leading substrate in an ordered sequence of addition was explored as shown in Figure 16. The results indicate that both exchange rates investigated reach a maximum rate of exchange at saturating glycogen concentrations. Since no lowering in the $P_i \rightleftharpoons$ glucose-1-P exchange rate was observed, these results support the view that glucose-1-P does not bind first in an ordered pathway of substrate interaction with the enzyme·AMP complex.

It may be appropriate to comment on the rates calculated for the glucose-1-P $\rightleftharpoons$ glycogen exchange. The equation used is (33)

$$R = \frac{-(P')\ (G)}{(P')+(G)}\ \frac{\ln(1-F)}{t} \qquad [3]$$

where P' and G are glucose-1-P and glycogen, and F is the fraction of isotope equilibrium attained at time t. Because glycogen is involved,

Figure 15. The effect of glucose-1-P and P_i concentrations on equilibrium exchange rates with muscle phosphorylase *b*. Equilibrium reaction mixes as in Figure 13, with glucose-1-P and P_i as shown: ▲, ^{14}C glucose-1-P $\rightleftharpoons$ glycogen, 14 mM glycogen, 11.1 μg enzyme per ml; O, ^{14}C glucose-1-P $\rightleftharpoons$ glycogen, 55.5 mM glycogen, 20.1 μg enzyme per ml; X, $^{32}P_i$ $\rightleftharpoons$ glucose-1-P, 55.5 mM glycogen, 20.1 μg enzyme per ml.

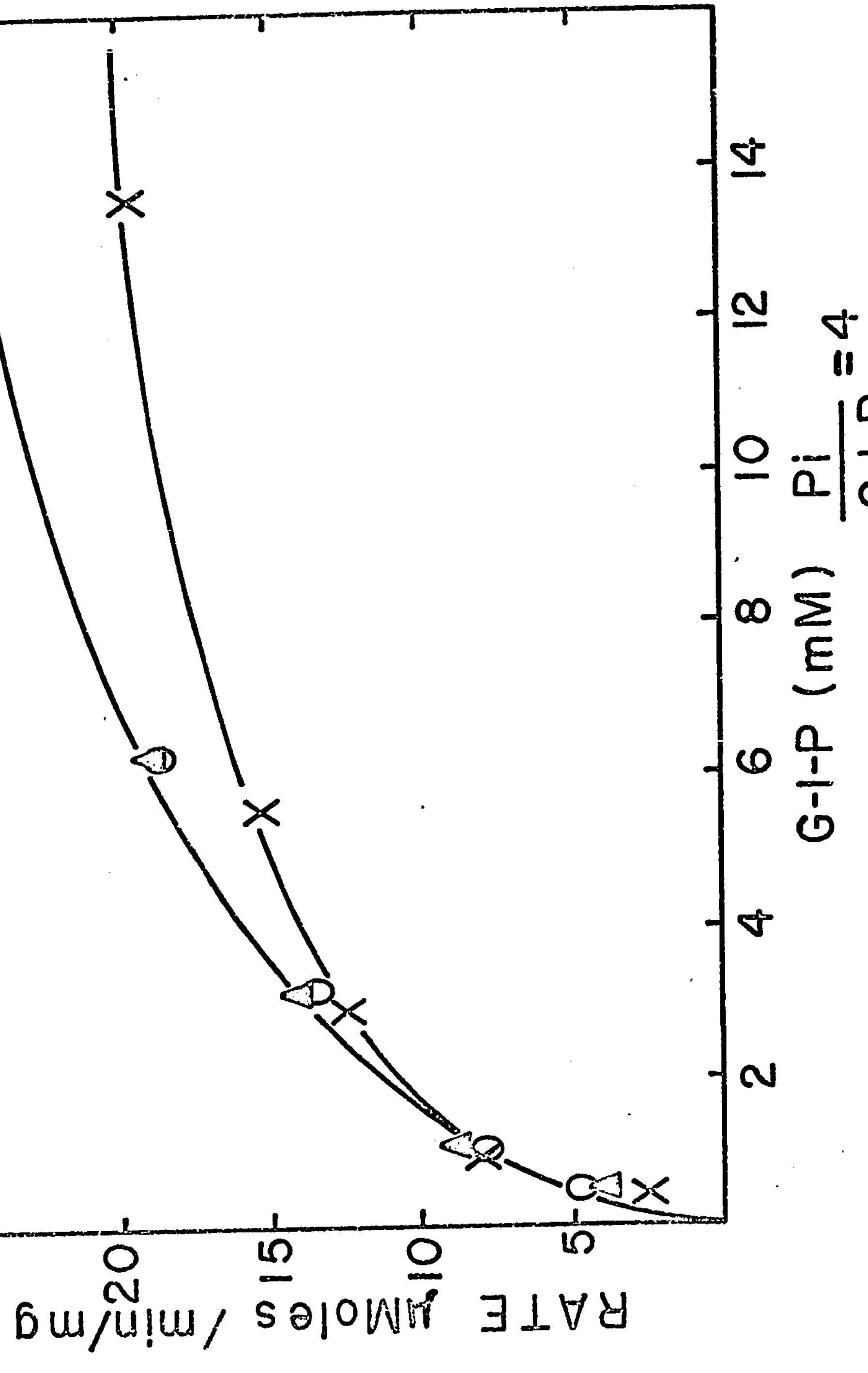
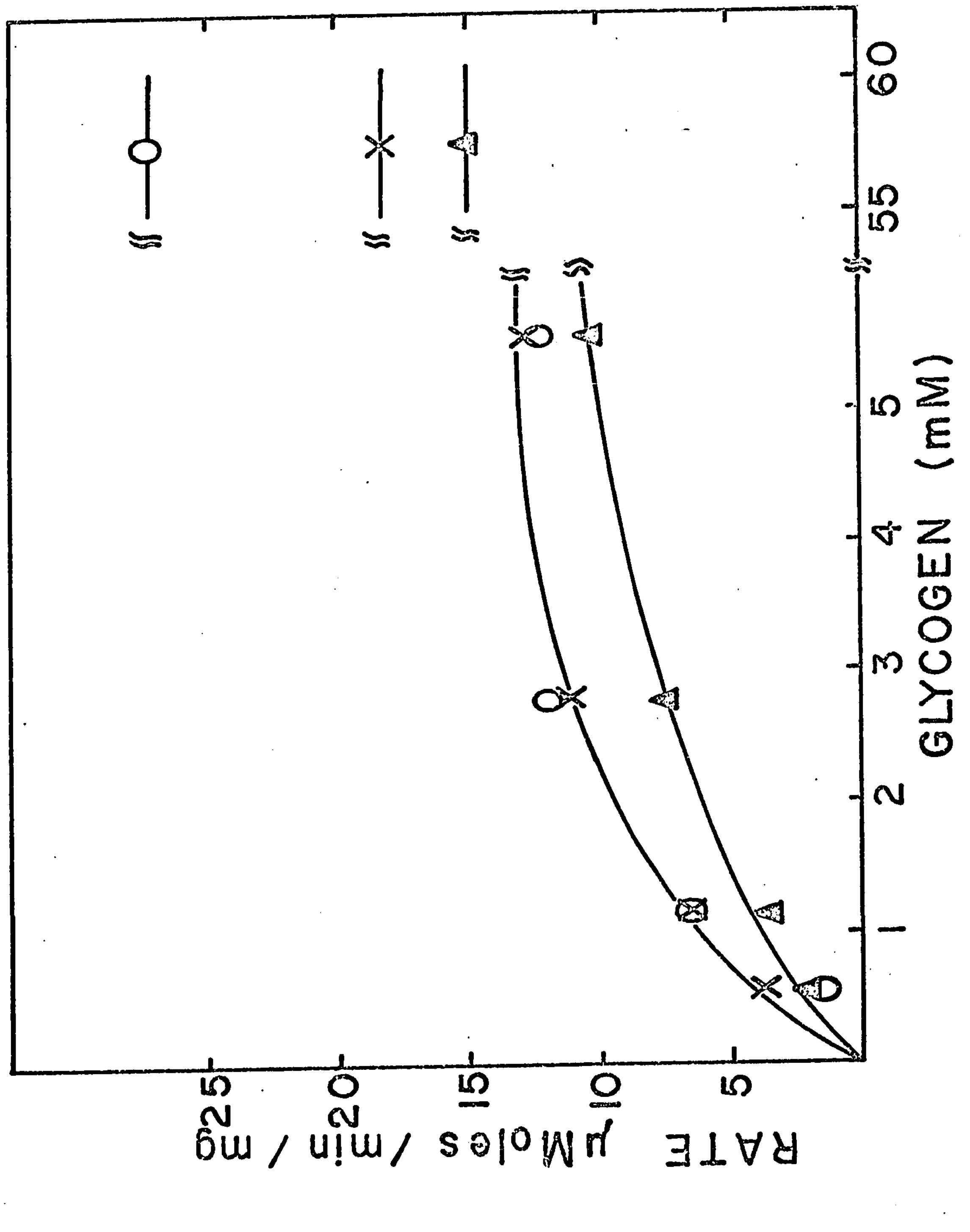

RATE μMoles / min/mg
5
10
15
20
25
2
4
6
8
10
12
14
G-1-P (mM) $\frac{Pi}{G\text{-}1\text{-}P} = 4$

Figure 16. The effect of glycogen concentrations on equilibrium exchange rates with muscle phosphorylase b. Equilibrium reaction mixes as in Figure 13, with glucose-1-P and P_i 2.9 mM and 11.8 mM respectively and glycogen as shown. X, O, $^{32}P_i \rightleftharpoons$ glucose-1-P; ▲, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen.


RATE μMoles /min / mg
5
10
15
20
25
GLYCOGEN (mM)
1
2
3
4
5
55
60

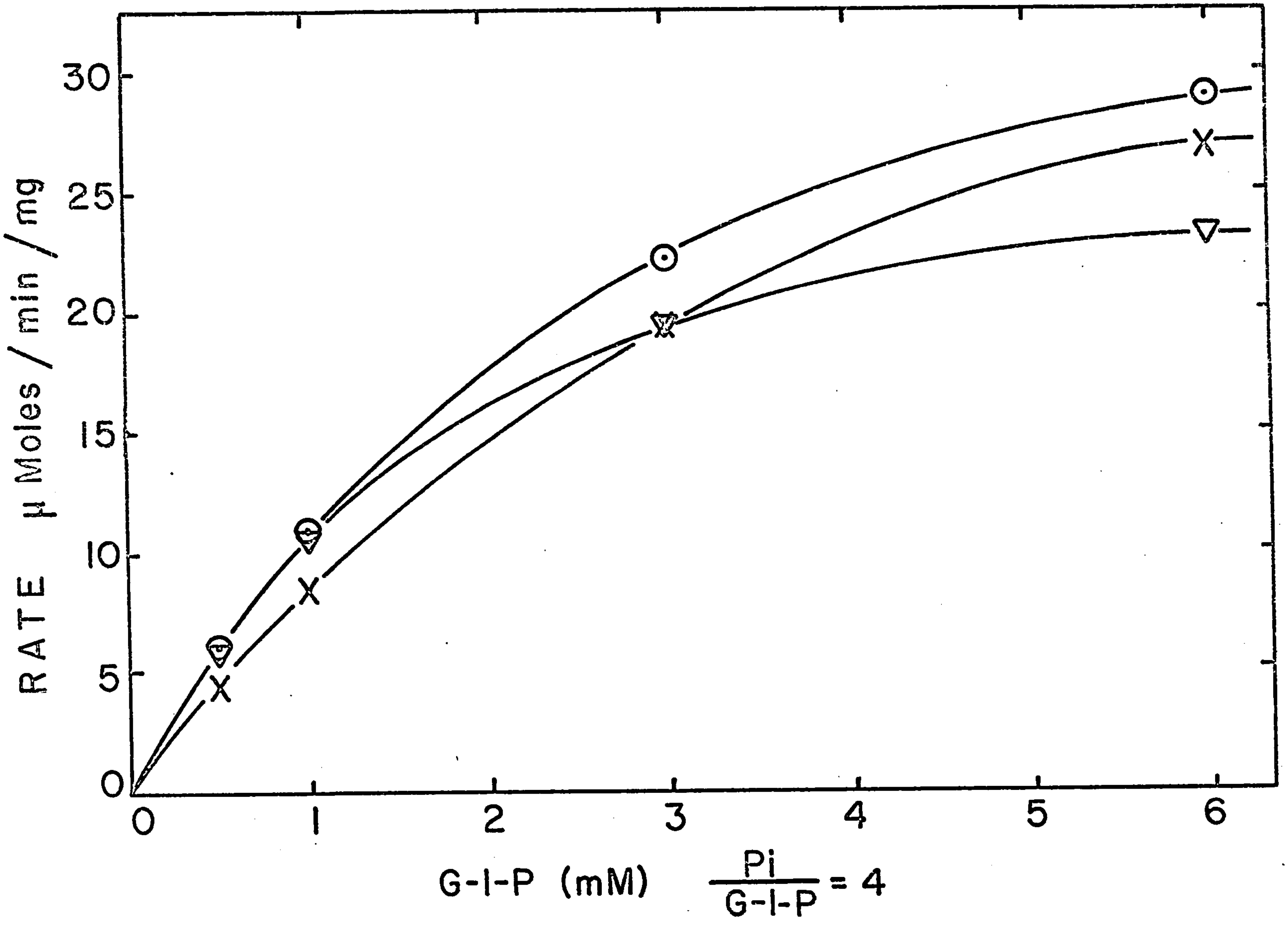
we are unable to assign a concentration which is a realistic measure of the substrate concentration available to the enzyme. We have, for convenience, used the total molar concentration of glucose residues throughout this Chapter. This probably gives unrealistically high rates of isotope exchange in Figures 15 and 16. It could be argued that the concentration of glucose end groups should be used but this figure is probably low because of the disproportionate attack of the enzyme on the outer chains, as discussed above. The total exchanging pool of glucose units would thus be greater than the end groups alone.

If the concentration of glucose end groups is used in equation [3], the calculated rates of the ^{14}C-isotope exchange are decreased below the values for the $^{32}P_i$ exchange, but the two exchange rates remain parallel. At very high concentrations of glycogen, the values for G in equation [3] would tend to cancel out and make the choice of a value less critical. The experiment reported in Figure 15 was repeated at 320 mM glycogen (22 mM end groups). Figure 17 shows that, within experimental error, the two exchange rates are equivalent, no matter which concentration of glycogen was used to calculate the glucose-1-P $\rightleftharpoons$ glycogen exchange rate. These results are confirmed by experiments reported in Chapter VII.

As discussed by Fromm _et al._ (35), R_{max}, the equilibrium reaction rate at infinite concentrations of substrates, may be obtained for an enzyme exhibiting rapid equilibrium random bi bi kinetics by the relationship

$$R_{max} = \frac{Vf \times Vr}{Vf + Vr}$$

Figure 17. The effect of glucose-1-P and P_i concentrations on equilibrium exchange rates at very high glycogen concentration. Equilibrium reaction mixes as in Figure 13 with glucose-1-P and P_i as shown, but with 320 mM glycogen. X, $^{32}P_i \rightleftharpoons$ glucose-1-P exchange; O, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange, using 320 mM as the glycogen concentration in equation 3; ▽, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange, using 22 mM (the end group concentration) as the glycogen concentration in equation 3.


30
25
20
15
10
5
0
RATE μ Moles / min / mg
0
1
2
3
4
5
6
G-I-P (mM)
$\frac{Pi}{G\text{-}I\text{-}P} = 4$

where Vf and Vr are the maximal velocities obtained from initial rate kinetics for the forward and reverse directions, respectively. For phosphorylase b, under the conditions of our experiments, the observed Vf and Vr values, from Table I, yield an R_{max} of 21.8 μmoles/min/mg. This compares favourably with the values for the $P_i \rightleftharpoons$ glucose-1-P exchange rates at high substrate concentrations.

The application of the isotope exchange studies to phosphorylase a yields results which are similar to those reported in this Chapter and suggest that the two forms of the enzyme have the same kinetic mechanism (Chapters V and VI). While this work was in progress, an abstract appeared by Johnson and Gold (67) which confirms our results for phosphorylase a.

The isotope exchange studies reported in this Chapter verify the kinetic mechanism for phosphorylase b as being rapid equilibrium random bi bi. The rapid equilibrium conditions were shown to apply at low concentrations of substrate where homotropic cooperativity between substrate binding sites had been suggested on the basis of non-Michaelian kinetics (22). Further studies, reported in Chapter VII, confirm that the rates of the $P_i \rightleftharpoons$ glucose-1-P and glucose-1-P $\rightleftharpoons$ glycogen exchanges remain equivalent in the presence of such allosteric inhibitors as ATP. Therefore the various homotropic cooperativities seen with this enzyme are not due to the loss of the rapid equilibrium state for a random bi bi mechanism. The general application of isotope exchange studies to the kinetics of allosteric enzymes would appear quite promising and will be discussed in Chapter VII.

B. Summary

In order to assign a kinetic mechanism to phosphorylase b, initial rate studies were conducted in conjunction with isotope exchange studies at equilibrium. Initial velocity rates were measured with varied concentrations of both substrates in each direction, in the presence of saturating levels of AMP. Data were analyzed with double reciprocal plots and secondary replots of intercepts and slopes. The resulting kinetically derived dissociation constants agreed reasonably well with those determined by independent means. These results indicate that the kinetic mechanism of phosphorylase b is rapid equilibrium random bi bi in nature.

The rate equation for this mechanism has been modified to take into account the fact that one of the substrates, glycogen, gives rise to a product which is chemically and kinetically indistinguishable under the conditions used to determine initial rates. This phenomenon, equivalent to having one of the two products present at all times, results in the number two being introduced into the rate equation, so the kinetically derived dissociation constant for glycogen is half the true value while the observed K_m's for phosphate and glucose-1-phosphate are twice the theoretical values. This rate equation should apply wherever an enzyme synthesizes or degrades a homopolymer by one unit at a time.

To confirm the mechanism suggested by initial velocity experiments, isotope exchange studies at equilibrium were performed for the phosphorylase b system, again in the presence of saturating levels of AMP. The ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen equilibrium reaction rate increased as the concentrations of either glucose-1-P and P_i or glycogen were increased, and reached a plateau as the concentration of varied substrates became saturating. The same results were obtained for $^{32}P_i \rightleftharpoons$ glucose-1-P exchange. There was thus no evidence of an inhibition of the exchange of one pair of substrates when the concentration of the other substrate pair was raised. Similar exchange rates were observed in either direction, indicating that rapid equilibrium conditions apply. A reasonable agreement existed between the maximal velocities calculated from the initial rate data and those determined from the isotope exchange rates, assuming a rapid equilibrium random bi bi mechanism.

Both the initial velocity and the equilibrium isotope exchange studies support a reaction mechanism in which substrates bind in a non-compulsory order to the enzyme and in which the interconversions of ternary complexes are the rate limiting steps.

CHAPTER V

THE KINETIC MECHANISM OF PHOSPHORYLASE a. INITIAL VELOCITY STUDIES

A. Results and Discussion

The proposed rapid equilibrium random bi bi kinetic mechanism for rabbit muscle phosphorylase a is shown in Figure 18. This scheme allows for the participation of the modifier AMP in the mechanism, provided that the extrapolated maximal velocities both in the presence and the absence of AMP are the same, as has been suggested in the literature (7, 14, 15), and described by Monod *et al*. as a "K" system (21). In the data presented in this Chapter, the V_{max} in the absence of AMP approaches ninety percent of that obtained in the presence of AMP.

The overall rate equation relating the initial velocity (V_o), total enzyme concentration (E) and substrate concentrations for the forward reaction is: [1]

$$\frac{k_1 E}{V_o} = 1 + \frac{1}{1 + A/K_{14}}\left(\frac{K_4}{G} + \frac{2K_3}{P} + \frac{K_1K_3}{GP}\right) + \frac{1}{1 + K_{14}/A}\left(\frac{K'_4}{G} + \frac{2K'_3}{P} + \frac{K'_1K'_3}{GP}\right)$$

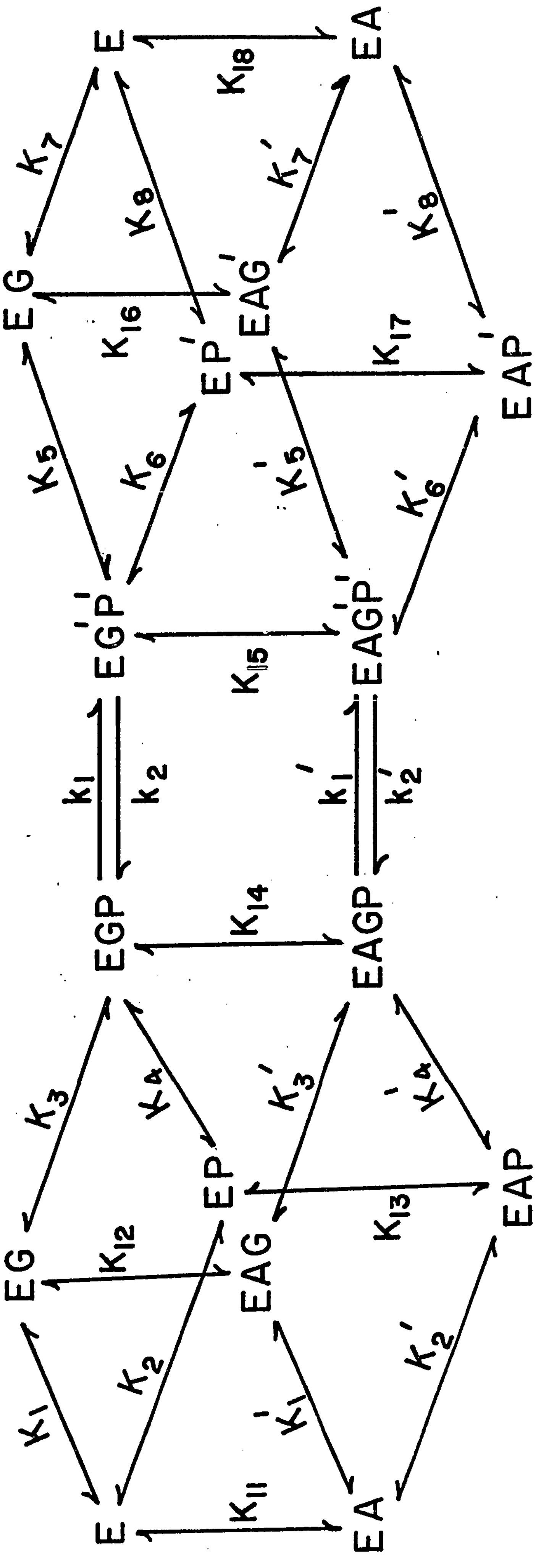
One should note that when A = 0, then equation [1] reduces to:

$$\frac{k_1 E}{V_o} = 1 + \frac{K_4}{G} + \frac{2K_3}{P} + \frac{K_1K_3}{GP} \qquad [2]$$

and also when $A \gg K_{14}$, then:

$$\frac{k_1 E}{V_o} = 1 + \frac{K'_4}{G} + \frac{2K'_3}{P} + \frac{K'_1K'_3}{GP} \qquad [3]$$

Figure 18. Proposed rapid equilibrium random kinetic mechanism for rabbit muscle phosphorylase a. A, E, G and P are, respectively, 5'-AMP, enzyme, glycogen and P_i. G' is glycogen with one less terminal glucose, while P' is glucose-1-P. The twenty-four dissociation constants and four rate constants for enzyme in the presence or absence of AMP are indicated.


E
EA
EG
EAG
EP
EAP
EGP
EAGP
EG'P'
EAG'P'
EG'
EAG'
EP'
EAP'
K_1
K_2
K_1'
K_2'
K_3
K_4
K_3'
K_4'
K_5
K_6
K_5'
K_6'
K_7
K_8
K_7'
K_8'
k_1
k_2
k_1'
k_2'
K_{11}
K_{12}
K_{13}
K_{14}
K_{15}
K_{16}
K_{17}
K_{18}

By replacing the appropriate constants in the above equations, analogous equations may be written for the reverse reaction.

It should be noted that since glycogen is a substrate on both sides of the reaction scheme, one of the products is essentially identical with one of the substrates. Therefore, the kinetically derived dissociation constants for glycogen are actually one-half the true dissociation constant for glycogen and the free enzyme, if one were to use the usual rate equation (Chapter IV). The rate equation given in equation [1], with removal of the number 2 from the numerator terms, should apply to any enzyme-modifier system in which the enzyme exhibits a random rapid equilibrium mechanism and the modifier affects the Michaelis constants and not maximal velocities; i.e., a "K" system in Monod's terminology (21). Thus, this rate equation provides a further method of kinetic analysis for the action of a modifier on two-substrate reactions (41, 42).

Initial rate data for the reaction in the direction of glycogen degradation at varying concentrations of both glycogen and P_i are shown in the primary plots of Figures 19-22 with AMP present for Figures 19 and 20 at a concentration of 1 mM. In Figure 19 the intersection point in the upper left hand quadrant yields the dissociation constant for glycogen from enzyme·AMP, while that in Figure 21 yields the K_{diss} for glycogen and free enzyme. Similarly, the intersection points in Figures 20 and 22 determine the K_{diss} for P_i from enzyme·AMP and P_i from free enzyme, respectively.

Recent investigations have shown that phosphorylase a, in the presence of AMP, does not exhibit the pronounced curvature in Lineweaver-Burk plots (15, 16), as compared to phosphorylase b (Chapter IV).

Figure 19. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at six levels of P_i, with AMP present at 1 mM. (1/v in this and all other reciprocal plots is in units of milligram of protein x min per μmole of product, either glucose-1-P or P_i.) Concentrations of P_i and corresponding apparent Michaelis constants for glycogen are as follows: □, 0.54 mM P_i, 0.81 mM glycogen; ▼, 0.91 mM P_i, 0.70 mM glycogen; O, 1.4 mM P_i, 0.64 mM glycogen; ▣, 2.7 mM P_i, 0.48 mM glycogen; Δ, 5.4 mM P_i, 0.33 mM glycogen; ◕, 10 mM P_i, 0.30 mM glycogen.

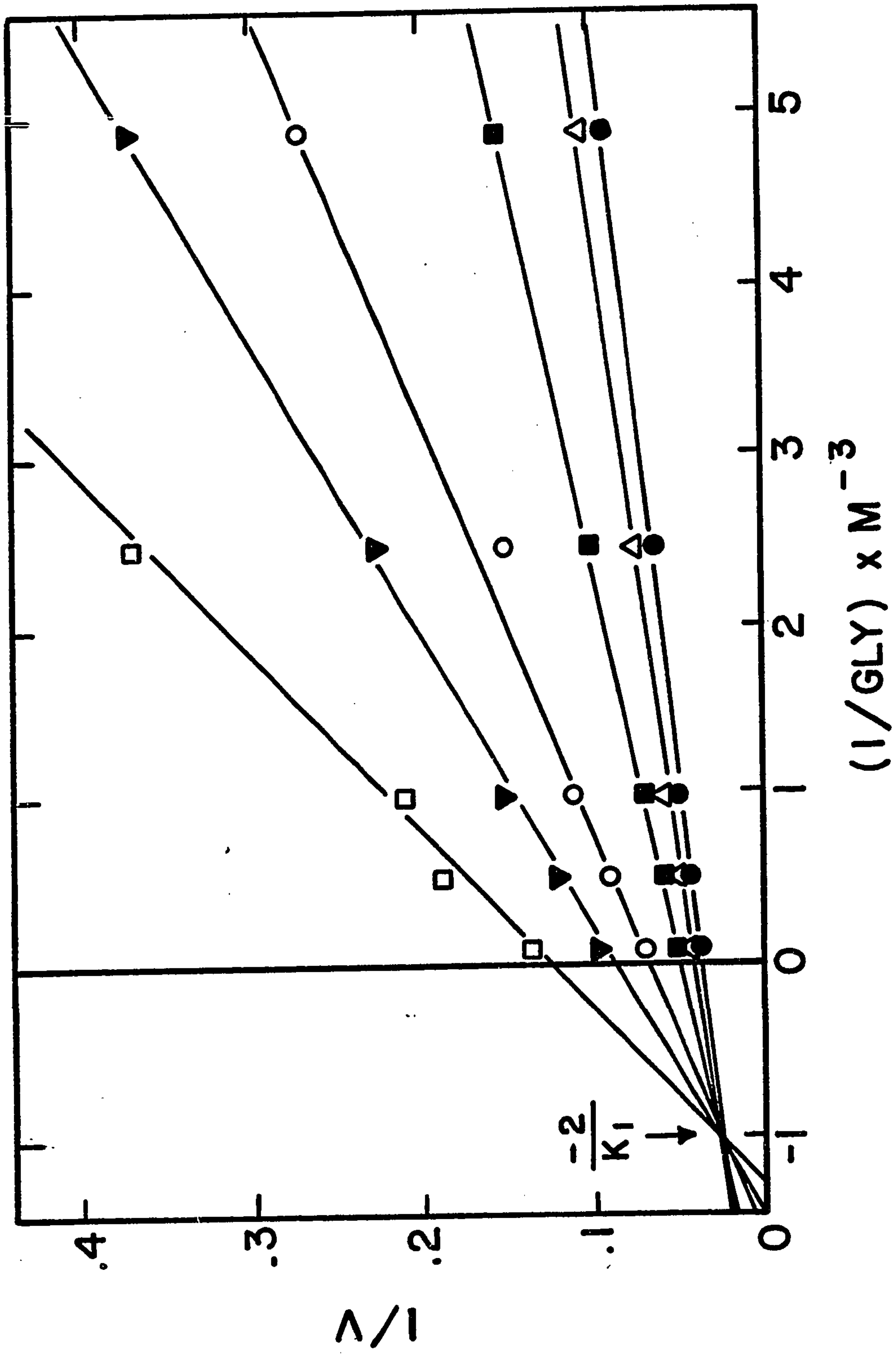

1/V
0
.1
.2
.3
.4
(1/GLY) x M^{-3}
-1
0
1
2
3
4
5
$\frac{-2}{K_1}$

Figure 20. Velocity of muscle phosphorylase *a* activity as a function of P_i concentration at five levels of glycogen, in the presence of 1 mM AMP. Concentrations of glycogen and corresponding apparent Michaelis constants for P_i are as follows: ■, 0.20 mM glycogen, 4.6 mM P_i; ○, 0.41 mM glycogen, 3.1 mM P_i; Δ, 1.0 mM glycogen, 2.8 mM P_i; ●, 2.0 mM glycogen, 2.3 mM P_i; ▼, 10.2 mM glycogen, 1.9 mM P_i.

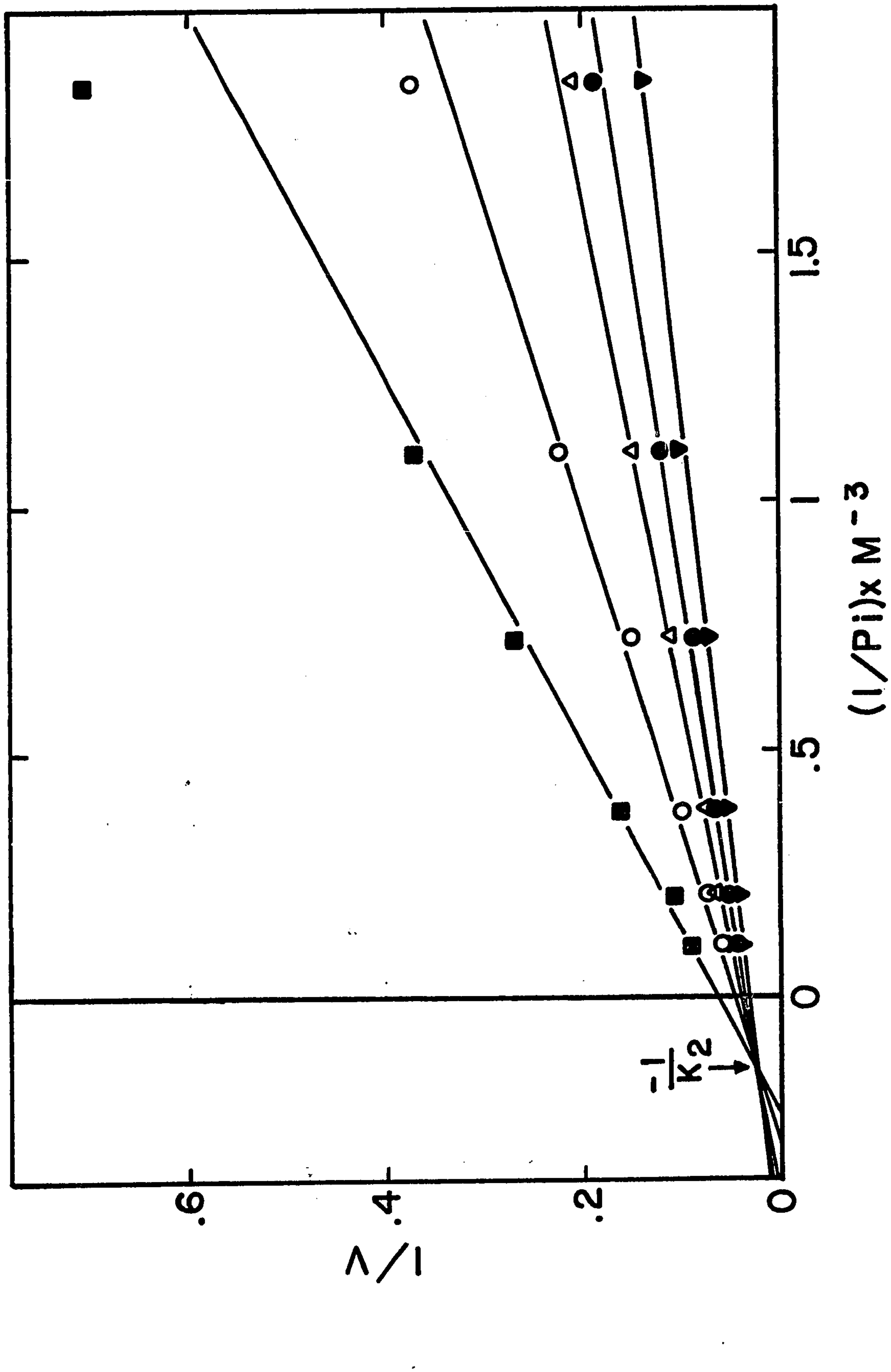

1/V
.6
.4
.2
0
0
.5
1
1.5
(1/Pi)x M^{-3}
$-\frac{1}{K_2}$

Figure 21. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at six levels of P_i concentrations, in the absence of AMP. Concentrations of P_i and corresponding apparent Michaelis constants for glycogen are as follows: □, 0.54 mM P_i, 2.6 mM glycogen; ▽, 0.91 mM P_i, 2.3 mM glycogen; ○, 1.4 mM P_i, 2.0 mM glycogen; ▲, 2.7 mM P_i, 1.6 mM glycogen; ◐, 5.4 mM P_i, 1.2 mM glycogen; X, 10 mM P_i, 0.91 mM glycogen.

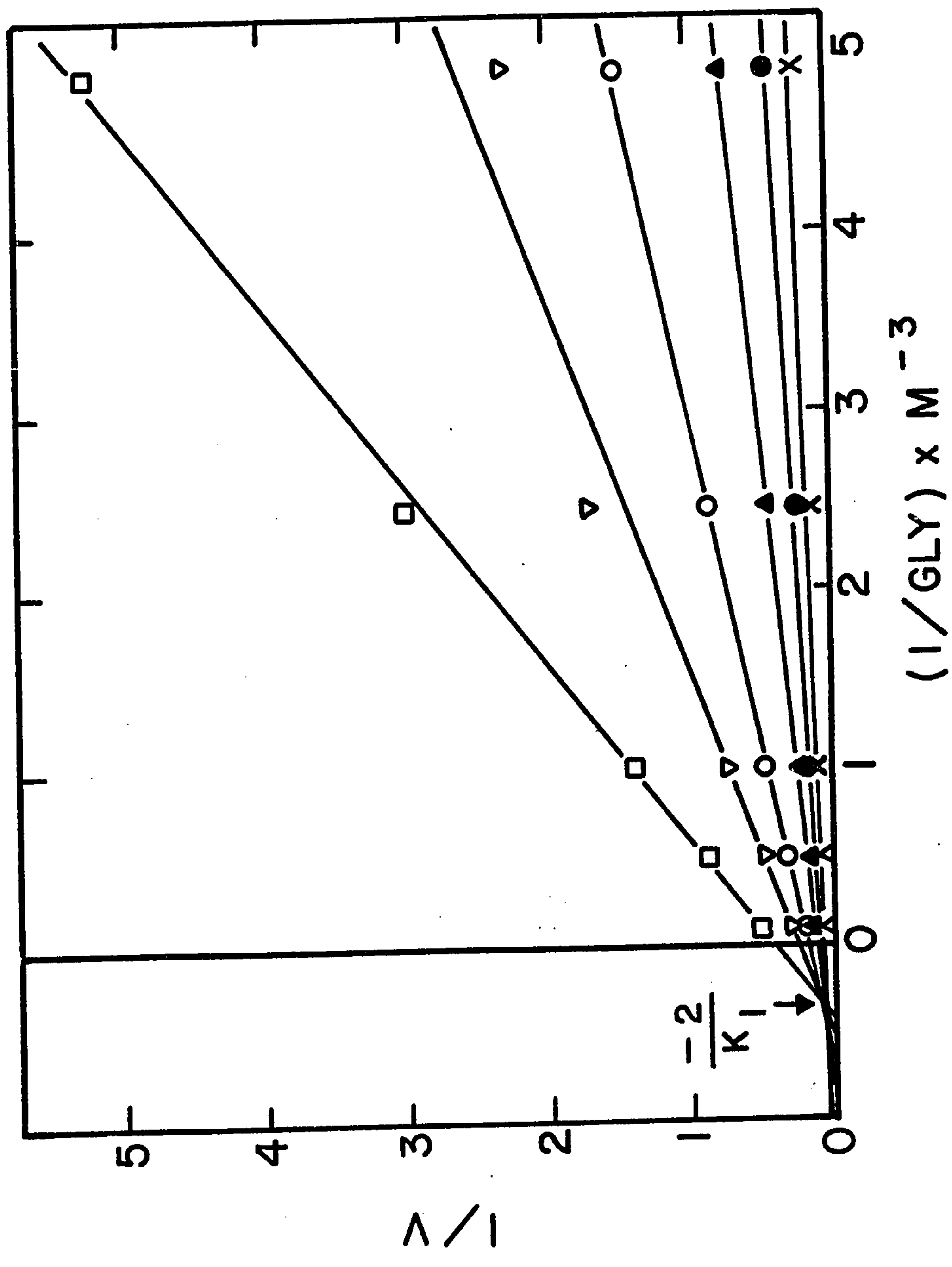
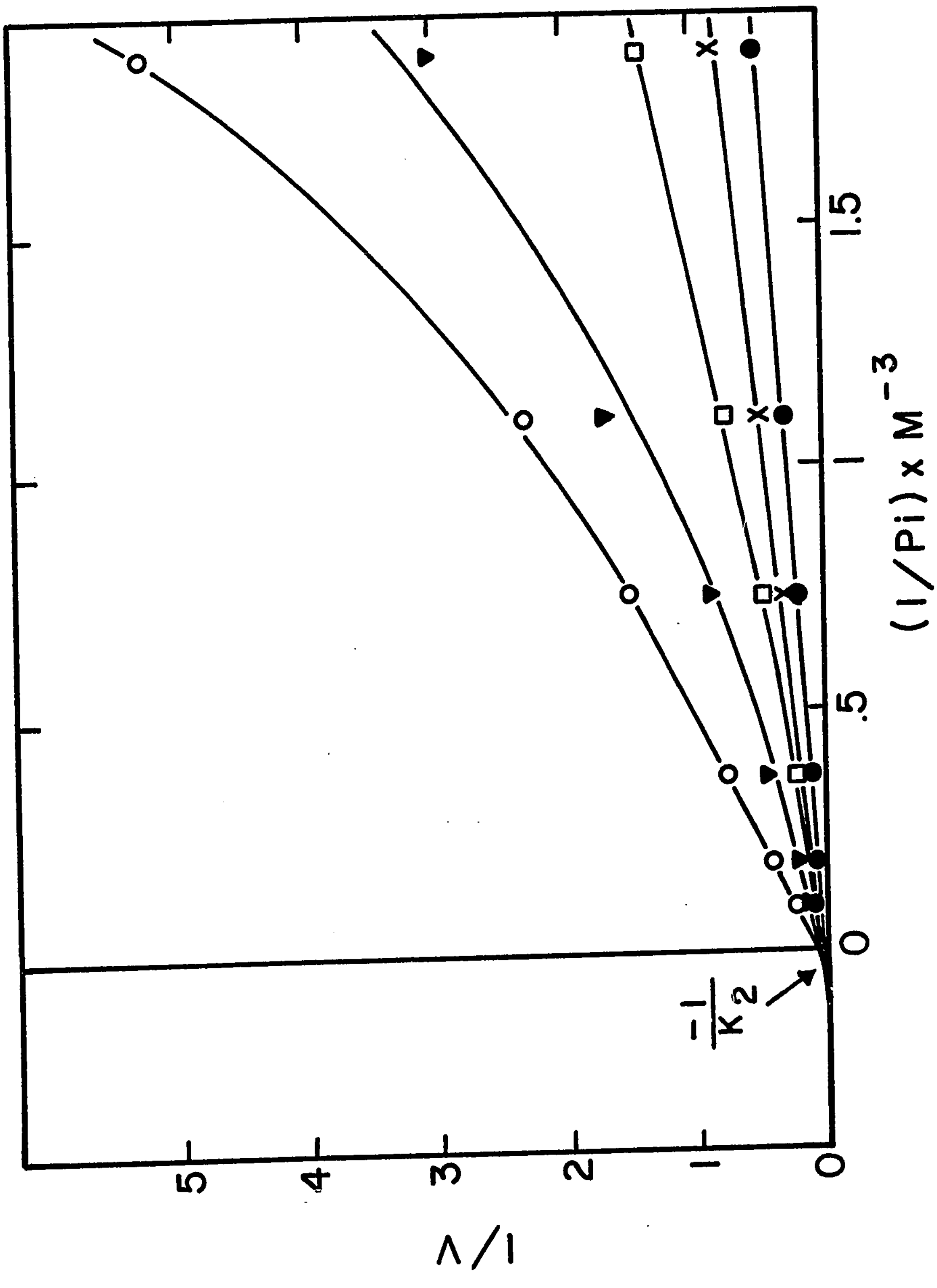

1/V
0
1
2
3
4
5
$-\frac{2}{K_I}$
0
1
2
3
4
5
(1/GLY) x M^{-3}

Figure 22. Velocity of muscle phosphorylase a activity as a function of P_i concentration at five levels of glycogen concentrations, in the absence of AMP. Concentrations of glycogen and corresponding apparent Michaelis constants are as follows: O, 0.20 mM glycogen, 18.2 mM P_i; ▼, 0.41 mM glycogen, 11.7 mM P_i; □, 1 mM glycogen, 8.3 mM P_i; X, 2 mM glycogen, 7.2 mM P_i; ●, 10.2 mM glycogen, 5.0 mM P_i.


1/V
0
1
2
3
4
5
(1/Pi) x M^{-3}
0
.5
1
1.5
$-\frac{1}{K_2}$

However, in the absence of the activator AMP, the enzyme exhibits considerable curvature (Figures 22 and 26); this curvature is usually taken as an expression of allosteric behaviour, i.e., homotropic cooperativity between substrate binding sites. Recently, several other investigators have also shown similar curvature for phosphorylase a in the absence of AMP (15, 16). This curvature could also have resulted from a breakdown of the rapid equilibrium conditions for a random bi bi mechanism, as is discussed by Ferdinand (43). The isotope exchange results reported in the following chapters rule out the latter possibility.

The initial rate data at varying concentrations of glycogen and glucose-1-P in the direction of glycogen synthesis are given in Figures 23 - 26. The dissociation constants for glycogen from the enzyme, in the absence of other substrates, are similar when determined from either reaction direction, as was the case for both liver phosphorylase (28) and phosphorylase b (Chapter IV).

It is of interest that the average value determined for the true dissociation constant for glycogen and enzyme in the absence of AMP is 7.5 mM, which agrees reasonably well with the value of 12 mM determined for corn phytoglycogen and phosphorylase a by Madsen and Cori (63), using an ultracentrifugal binding method. Since the K_m values for liver glycogen are approximately 80% of those values for corn phytoglycogen, the comparison of the dissociation constants by the kinetic and physical methods is not unreasonable. Helmreich et al. (17) estimate a K_{diss} of about 5 mM for glycogen from their studies on the interaction of 5'-AMP with phosphorylase a. Another aspect to be taken into consideration when evaluating the binding studies is

Figure 23. Velocity of muscle phosphorylase *a* activity as a function of glycogen concentration at six levels of glucose-1-P, in the presence of 1 mM AMP. Concentrations of glucose-1-P and corresponding apparent Michaelis constants for glycogen are as follows: □, 0.49 mM glucose-1-P, 0.73 mM glycogen; △, 0.80 mM glucose-1-P, 0.62 mM glycogen; ●, 1.2 mM glucose-1-P, 0.61 mM glycogen; ■, 2.4 glucose-1-P, 0.61 mM glycogen; ○, 4.9 mM glucose-1-P, 0.58 mM glycogen; ▼, 18 mM glucose-1-P, 0.49 mM glycogen.

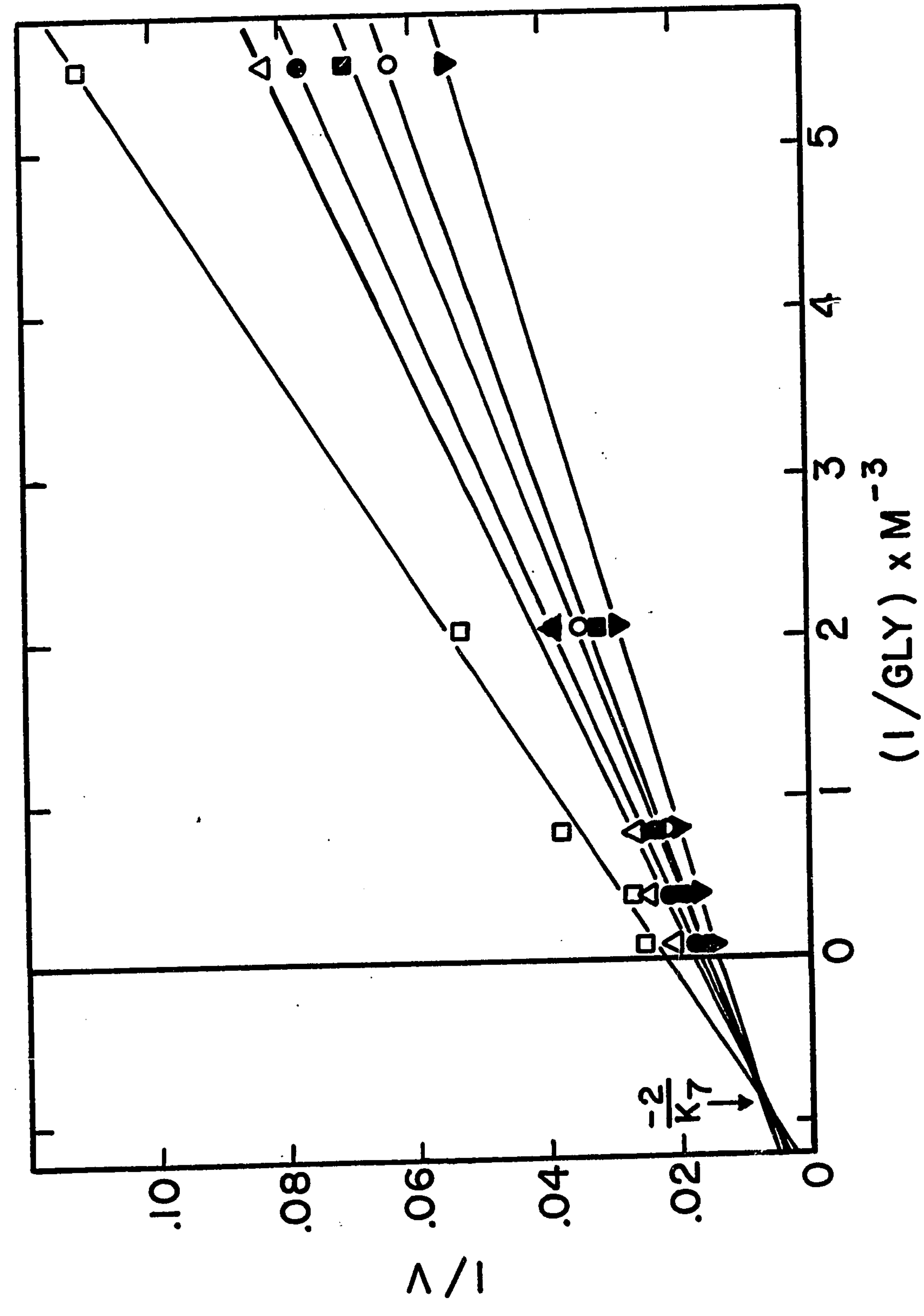

1/V
.10
.08
.06
.04
.02
0
0
1
2
3
4
5
(1/GLY) x M^{-3}
$\frac{-2}{K_7}$

Figure 24. Velocity of muscle phosphorylase a activity as a function of glucose-1-P concentration at five levels of glycogen in the presence of 1 mM AMP. Concentrations of glycogen and corresponding apparent Michaelis constants for glucose-1-P are as follows: X, 0.18 mM glycogen, 0.55 mM glucose-1-P; O, 0.49 mM glycogen, 0.42 mM glucose-1-P; ▼, 1.2 mM glycogen, 0.38 mM glucose-1-P; ●, 2.4 mM glycogen, 0.23 mM glucose-1-P; □, 12.2 mM glycogen, 0.13 mM glucose-1-P.

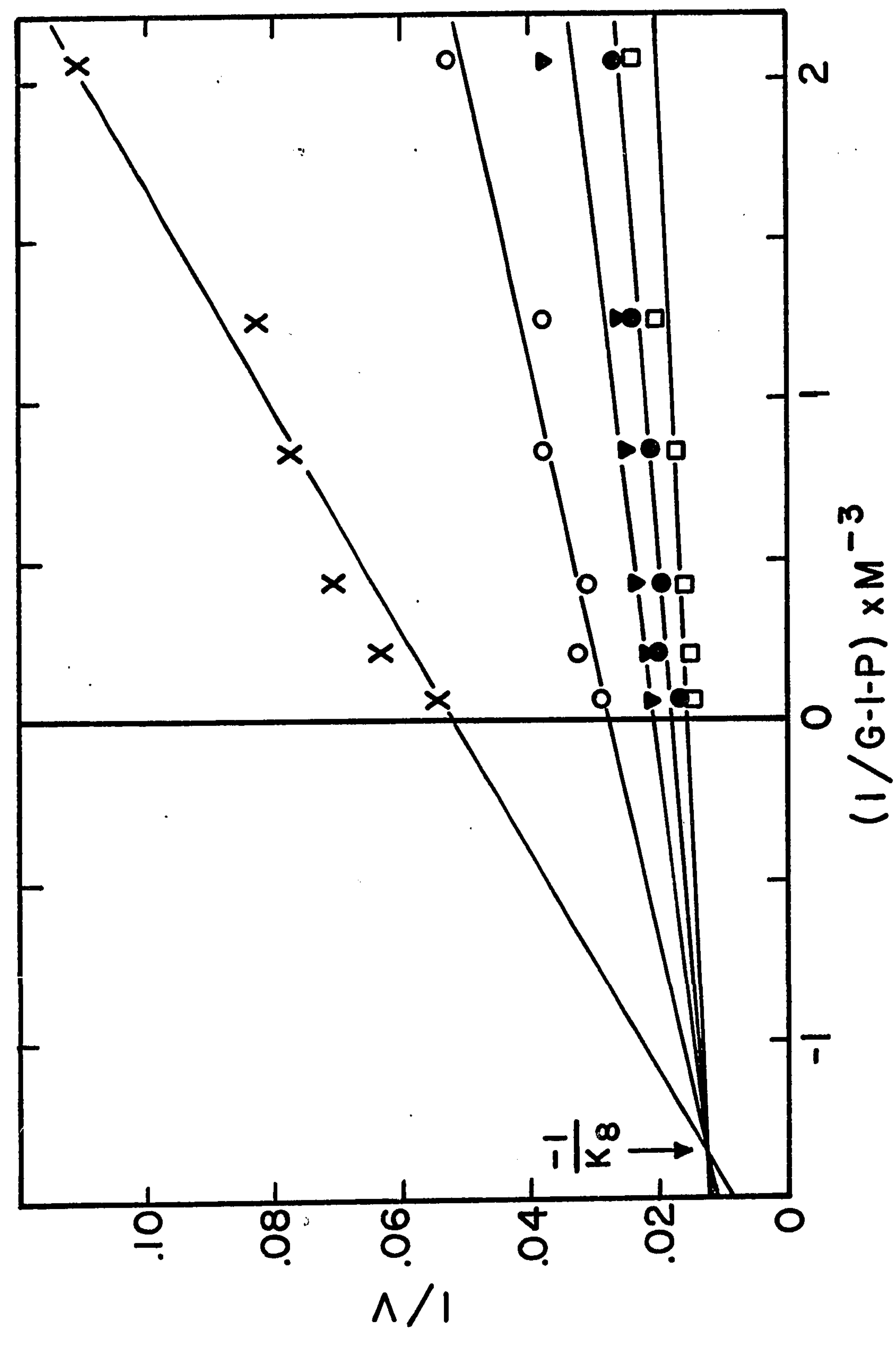

1/V
.10
.08
.06
.04
.02
0
-1
0
1
2
(1/G-1-P) x M^-3
-1/K8

Figure 25. Velocity of muscle phosphorylase *a* activity as a function of glycogen concentration at six levels of glucose-1-P in the absence of AMP. Concentrations of glucose-1-P and corresponding apparent Michaelis constants for glycogen are as follows: O, 0.49 mM glucose-1-P, 3.8 mM glycogen; □, 0.80 mM glucose-1-P, 3.2 mM glycogen; ▲, 1.2 mM glucose-1-P, 2.3 mM glycogen; ●, 2.4 mM glucose-1-P, 1.4 mM glycogen; X, 4.9 mM glucose-1-P, 1.4 mM glycogen; ■, 18 mM glucose-1-P, 1.3 mM glycogen.

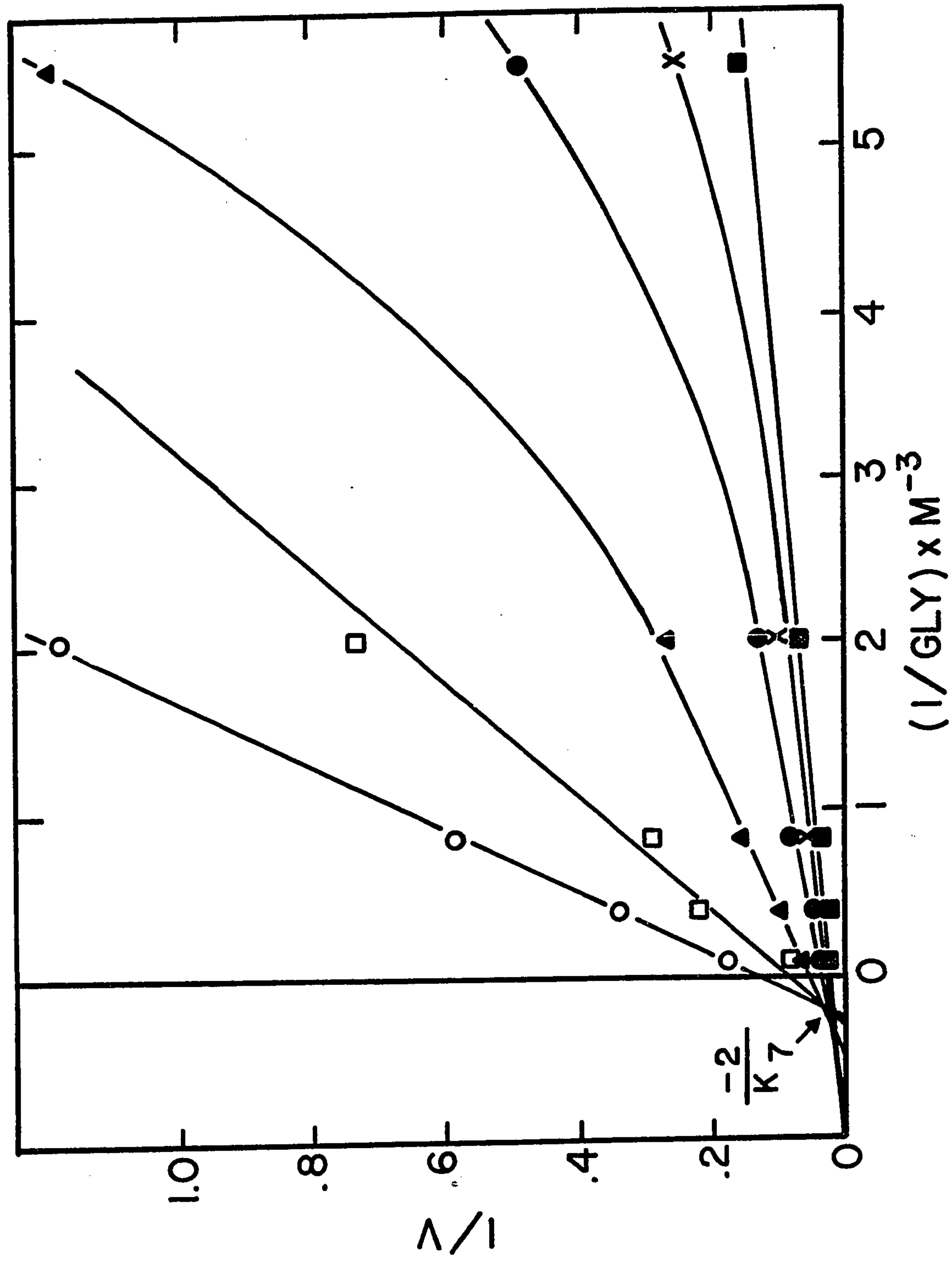
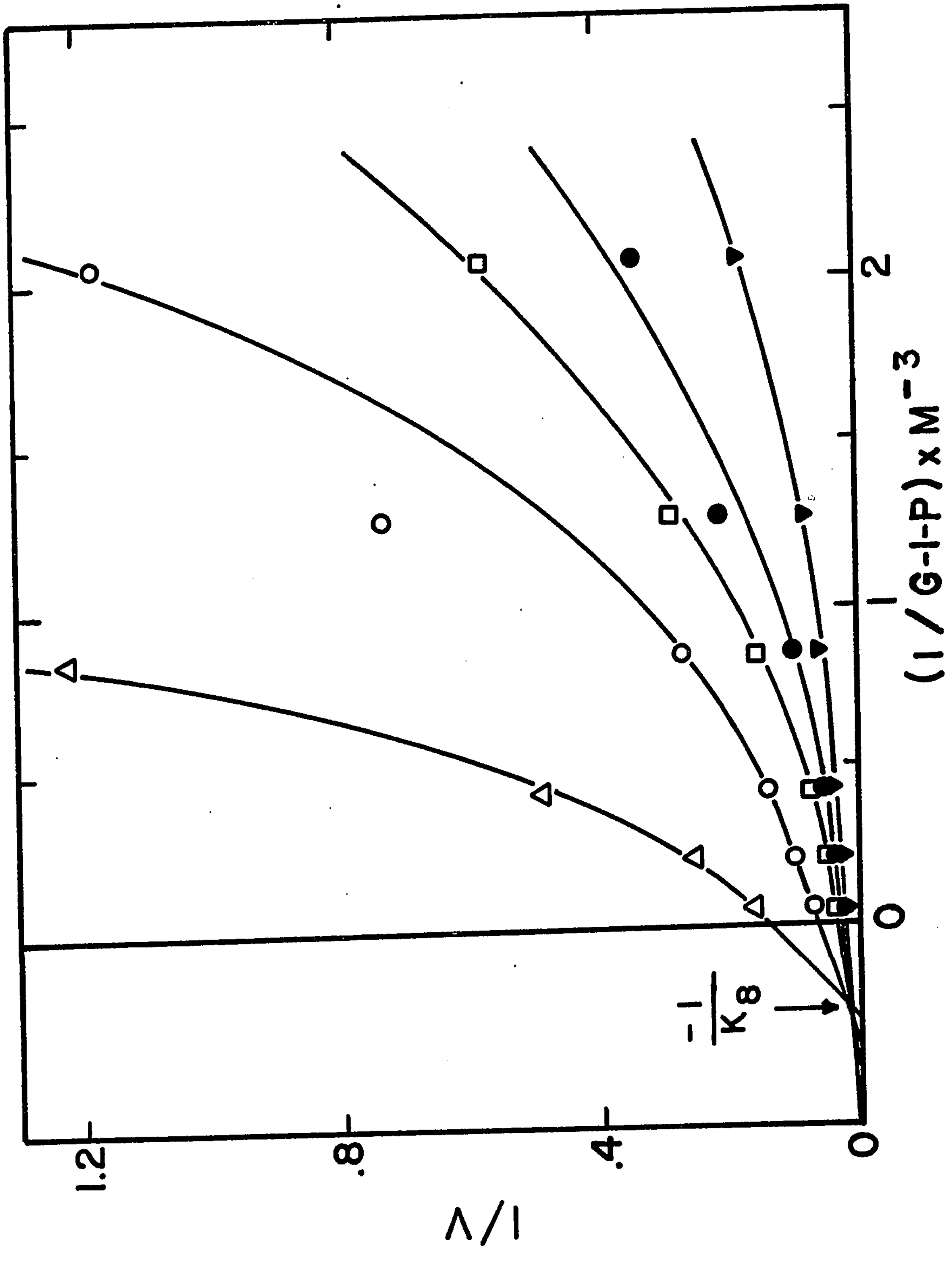

1/V
0
.2
.4
.6
.8
1.0
(1/GLY) x M^{-3}
0
1
2
3
4
5
$\frac{-2}{K_7}$

Figure 26. Velocity of muscle phosphorylase *a* activity as a function of glucose-1-P concentration at five levels of glycogen in the absence of AMP. Concentrations of glycogen and corresponding apparent Michaelis constants for glucose-1-P are as follows: Δ, 0.18 mM glycogen, 3.5 mM glucose-1-P, O, 0.49 mM glycogen, 3.1 mM glucose-1-P; □, 1.2 mM glycogen, 3.0 mM glucose-1-P; ●, 2.4 mM glycogen, 2.8 mM glucose-1-P; ▼, 12.2 mM glycogen, 2.4 mM glucose-1-P.


1/V
1.2
.8
.4
0
0
1
2
(1/G-1-P) x M⁻³
-1/K₈

that of the dimer $\rightleftharpoons$ tetramer equilibrium set up in solutions of phosphorylase *a* at a concentration of greater than 1 mg per ml, since the tetramer is believed to bind glycogen less tightly (68, 69).

Secondary replots of intercepts and slopes from the double reciprocal plots are given in Figures 27 - 34. Again one notices the curvature in the data for glucose-1-P and P_i, in the absence of AMP. The values obtained from these replots for the appropriate dissociation constants agreed reasonably well with those corresponding constants obtained from the intersection points in the primary plots, illustrating the consistency of the data. Where the secondary plots are linear, the minimal deviations from the lines indicate low internal errors in the primary data. The Dalziel kinetic coefficients obtained from the secondary plots, as shown by ϕ_0, ϕ_1, ϕ_2, etc., can be used to confirm the calculated Haldane relationships (29, Chapter IV).

Table IV summarizes the various kinetic constants derived from the primary plots and replots, as applied to the kinetic mechanism in Figure 18. Also shown in this table are various kinetic constants that have been reported in the literature for several forms of glycogen phosphorylase. The values in brackets are kinetic constants obtained from experiments repeated with aged phosphorylase *a* that had been stored in the crystalline state for three months at 4°. These "aged" values indicate that the kinetically derived dissociation constants are essentially the same, although Hill plots of this data confirm the suggestions of several authors (16, 17) that aged phosphorylase *a* exhibits increased allosteric interactions over those observed for fresh enzyme. Since the maximal velocities of the aged enzyme in the

Figure 27. Secondary plots of the intercepts (milligrams of protein x min per μmole) from Figures 19 and 20. ●, Figure 19; □, Figure 20.

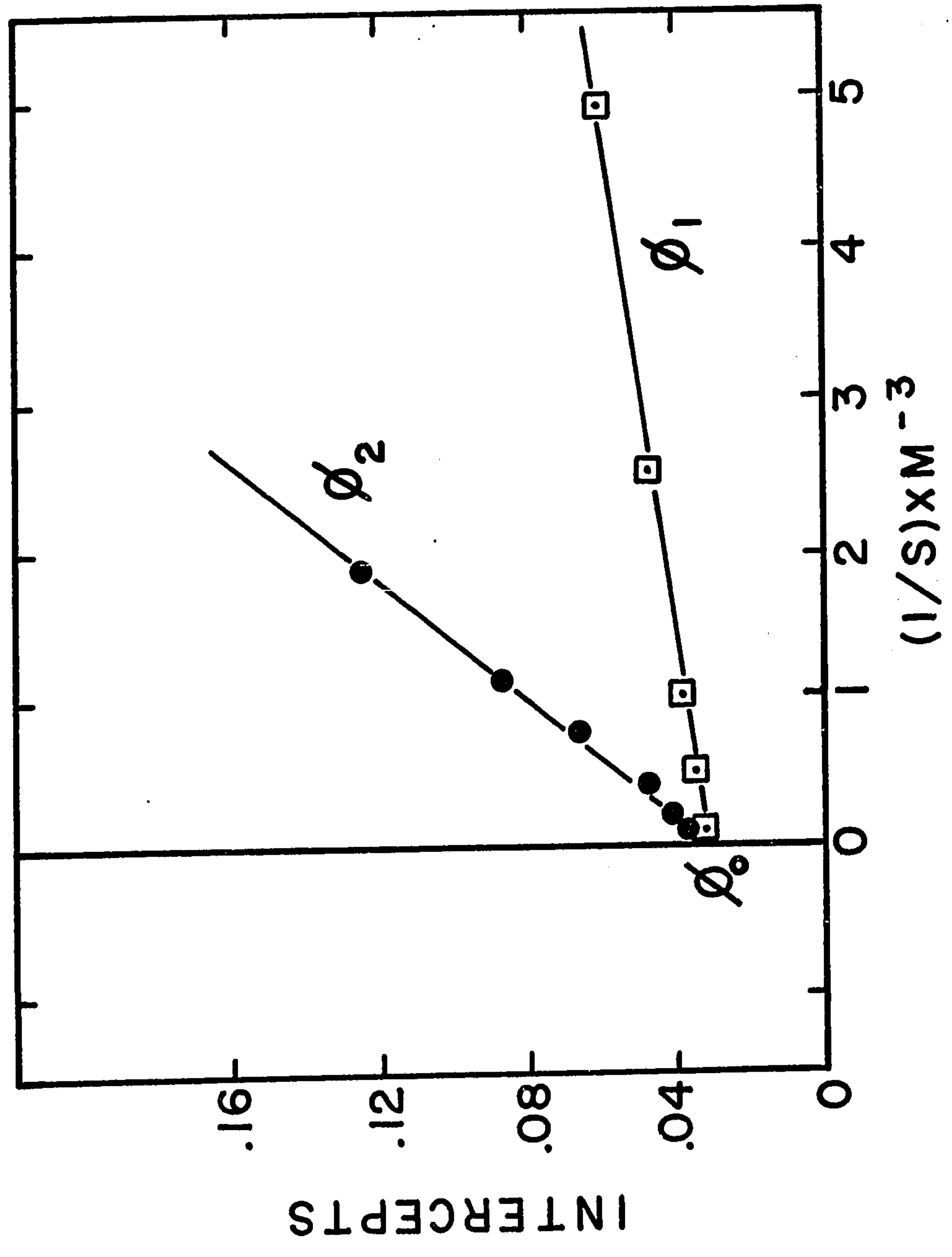

INTERCEPTS
0
.04
.08
.12
.16
$(1/S) \times M^{-3}$
0
1
2
3
4
5
ϕ_0
ϕ_1
ϕ_2

Figure 28. Secondary plots of the intercepts (milligrams of protein x min per μmole) from Figures 23 and 24. ●, Figure 23, Δ, Figure 24.

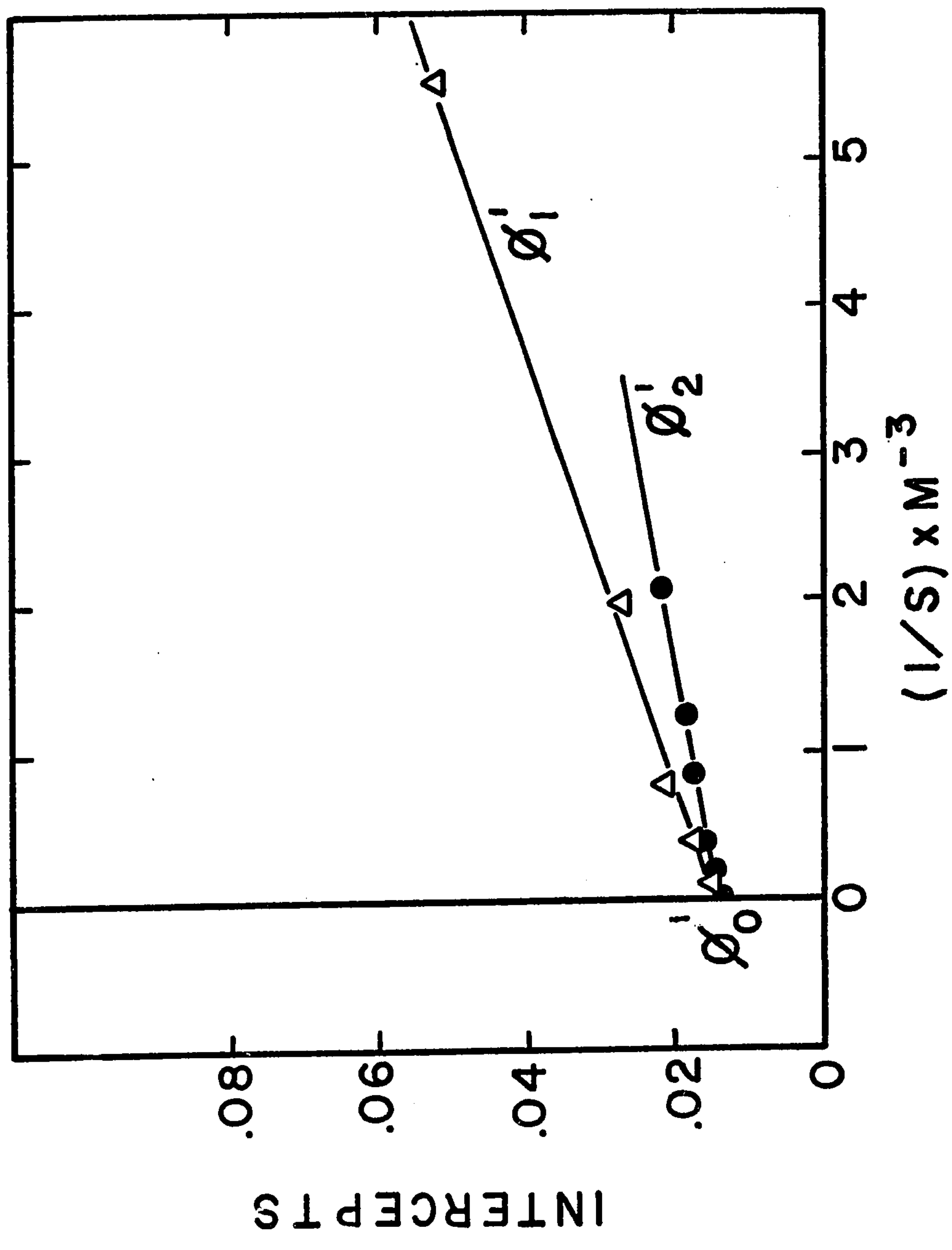

INTERCEPTS
.08
.06
.04
.02
0
0
1
2
3
4
5
$(1/S) \times M^{-3}$
$\emptyset'_1$
$\emptyset'_2$
$\emptyset'_0$

Figure 29. Secondary plots of the intercepts (milligrams of protein x min per μmole) from Figures 21 and 22. ●, Figure 21; □, Figure 22.

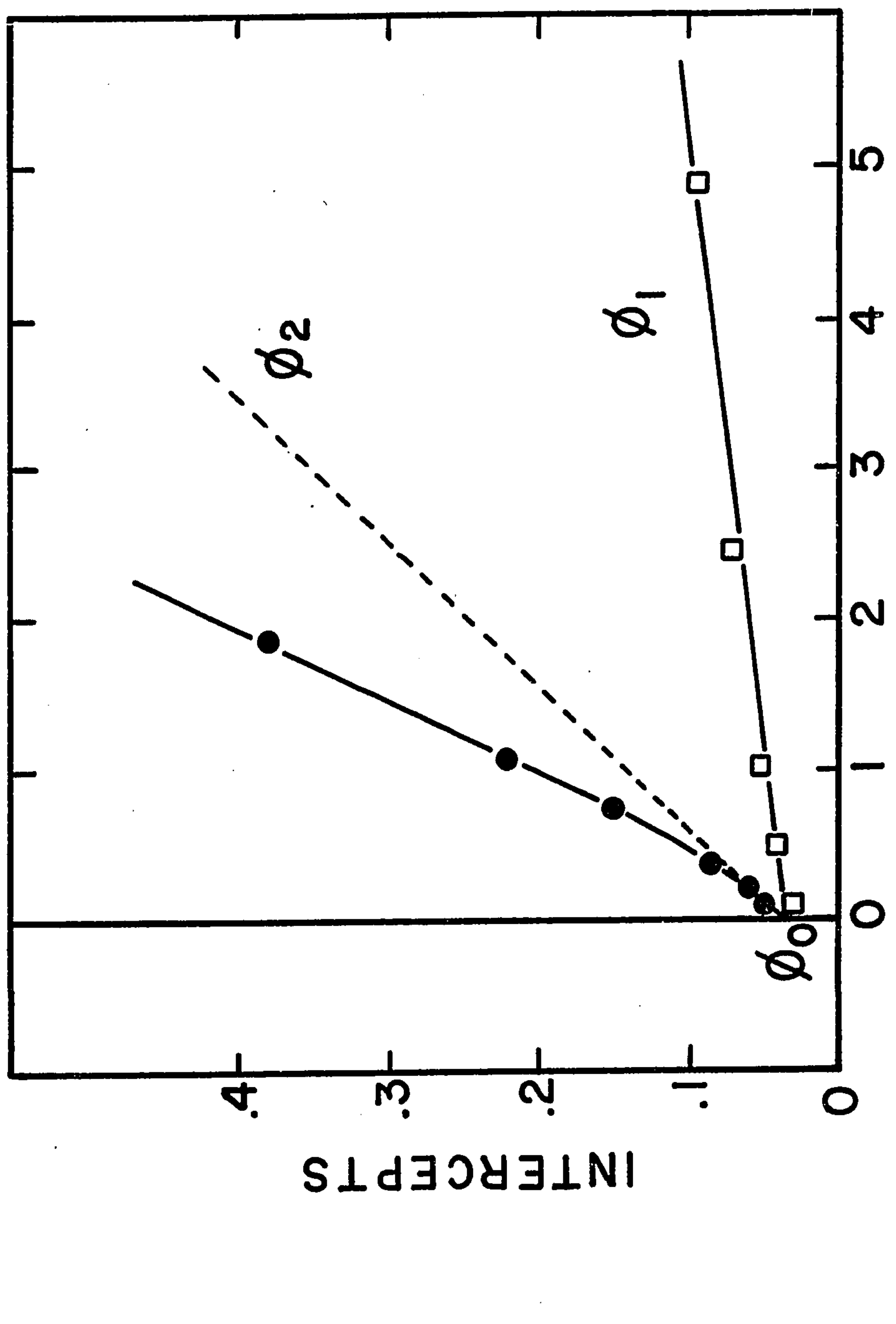

INTERCEPTS
0
.1
.2
.3
.4
(1/S) x M^{-3}
0
1
2
3
4
5
ϕ_0
ϕ_1
ϕ_2

Figure 30. Secondary plots of the intercepts (milligrams of protein x min per μmole) from Figures 25 and 26. ●, Figure 25; Δ, Figure 26.

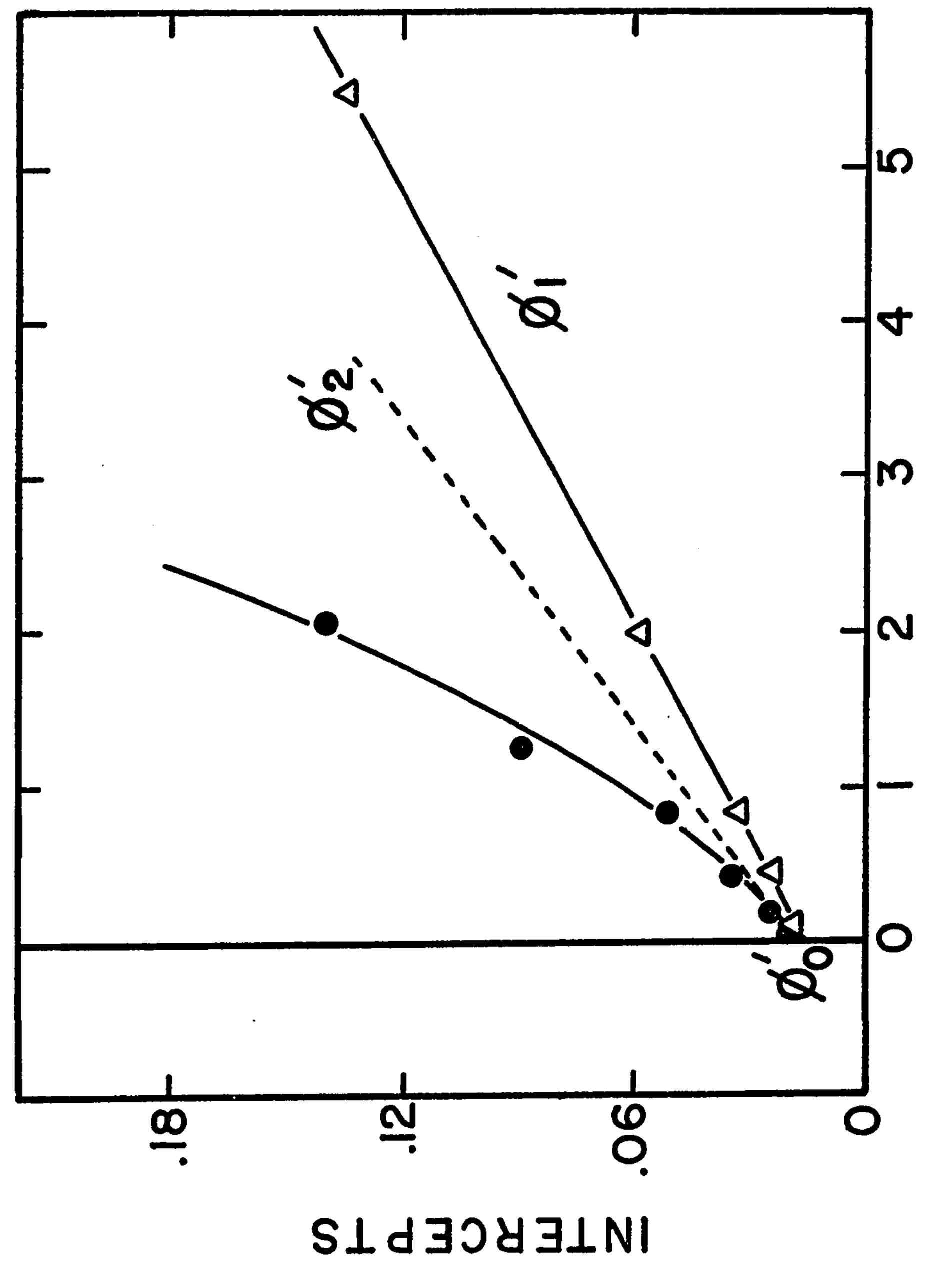

INTERCEPTS
.18
.12
.06
0
(1/S) x M^{-3}
0
1
2
3
4
5
ϕ'_0
ϕ'_1
ϕ'_2

Figure 31. Secondary plots of the slopes (milligrams of protein x min) from Figures 19 and 20. ●, Figure 19; V, Figure 20.

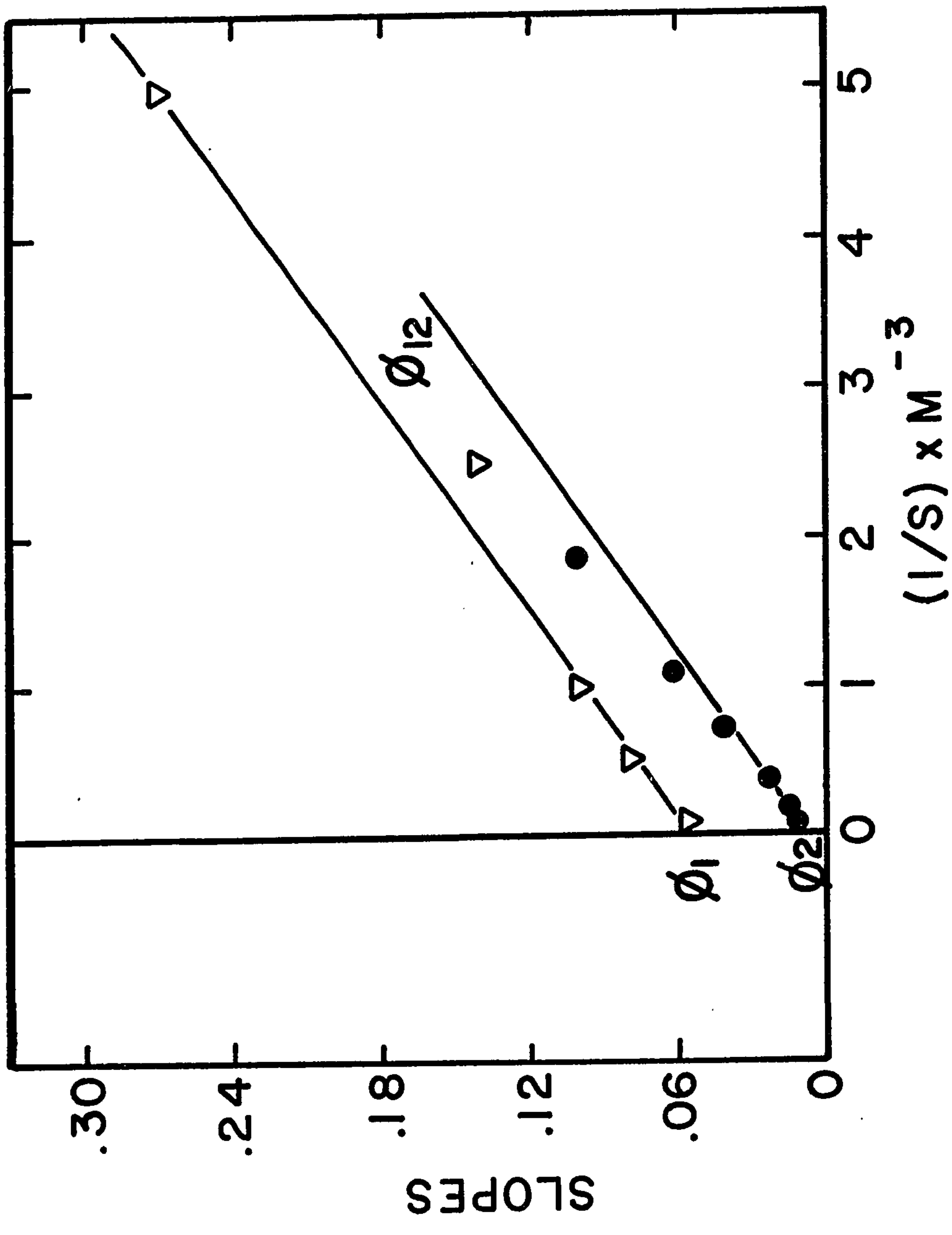

SLOPES
.30
.24
.18
.12
.06
0
0
1
2
3
4
5
(1/S) x M^{-3}
ϕ_1
ϕ_2
ϕ_{12}

Figure 32. Secondary plots of the slopes (milligrams of protein x min) from Figures 23 and 24. ●, Figure 23, □, Figure 24.

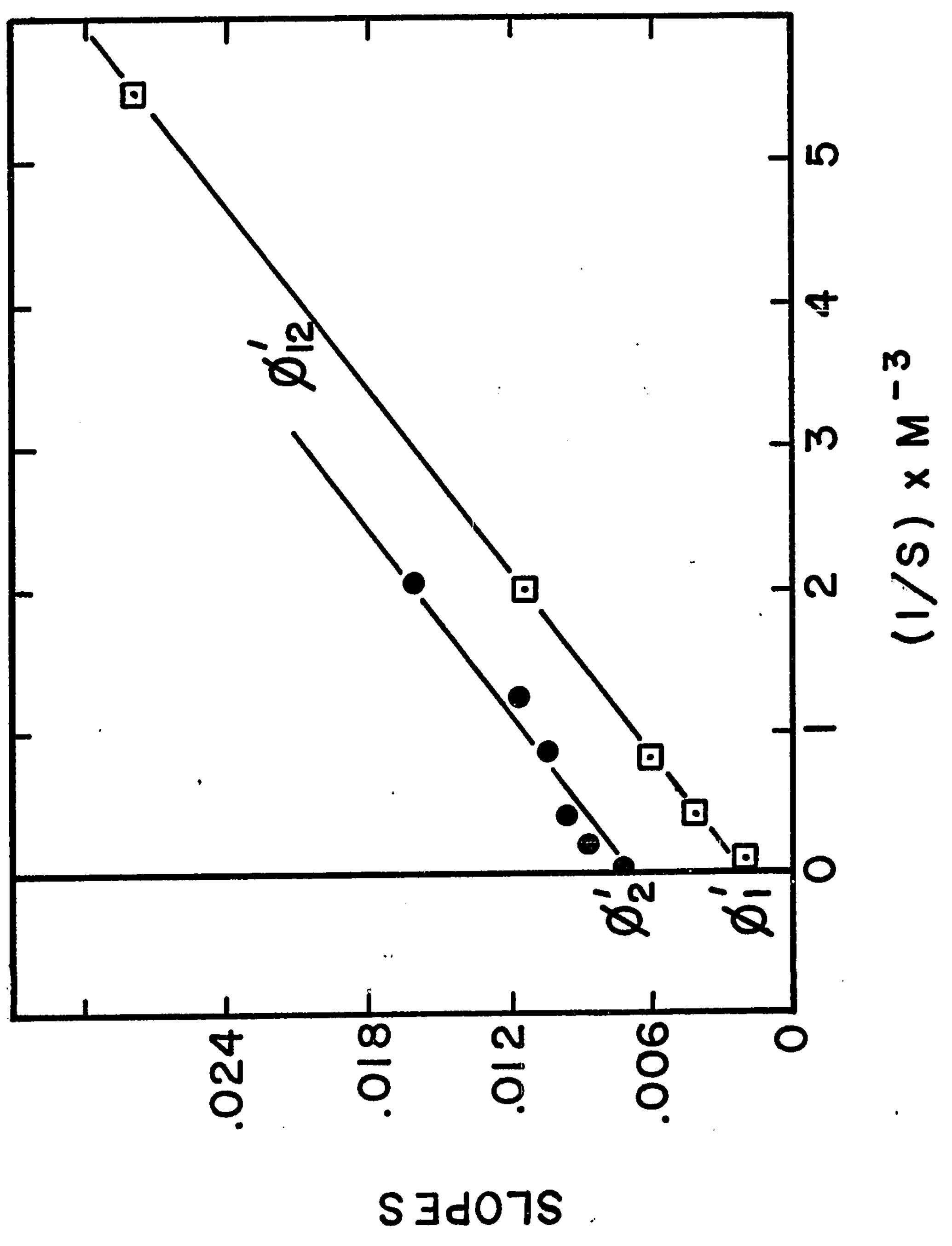

SLOPES
0
.006
.012
.018
.024
0
1
2
3
4
5
(1/S) x M^{-3}
ϕ'_{12}
ϕ'_{2}
ϕ'_{1}

Figure 33. Secondary plots of the slopes (milligrams of protein x min) from Figures 21 and 22. ●, Figure 21; □, Figure 22.

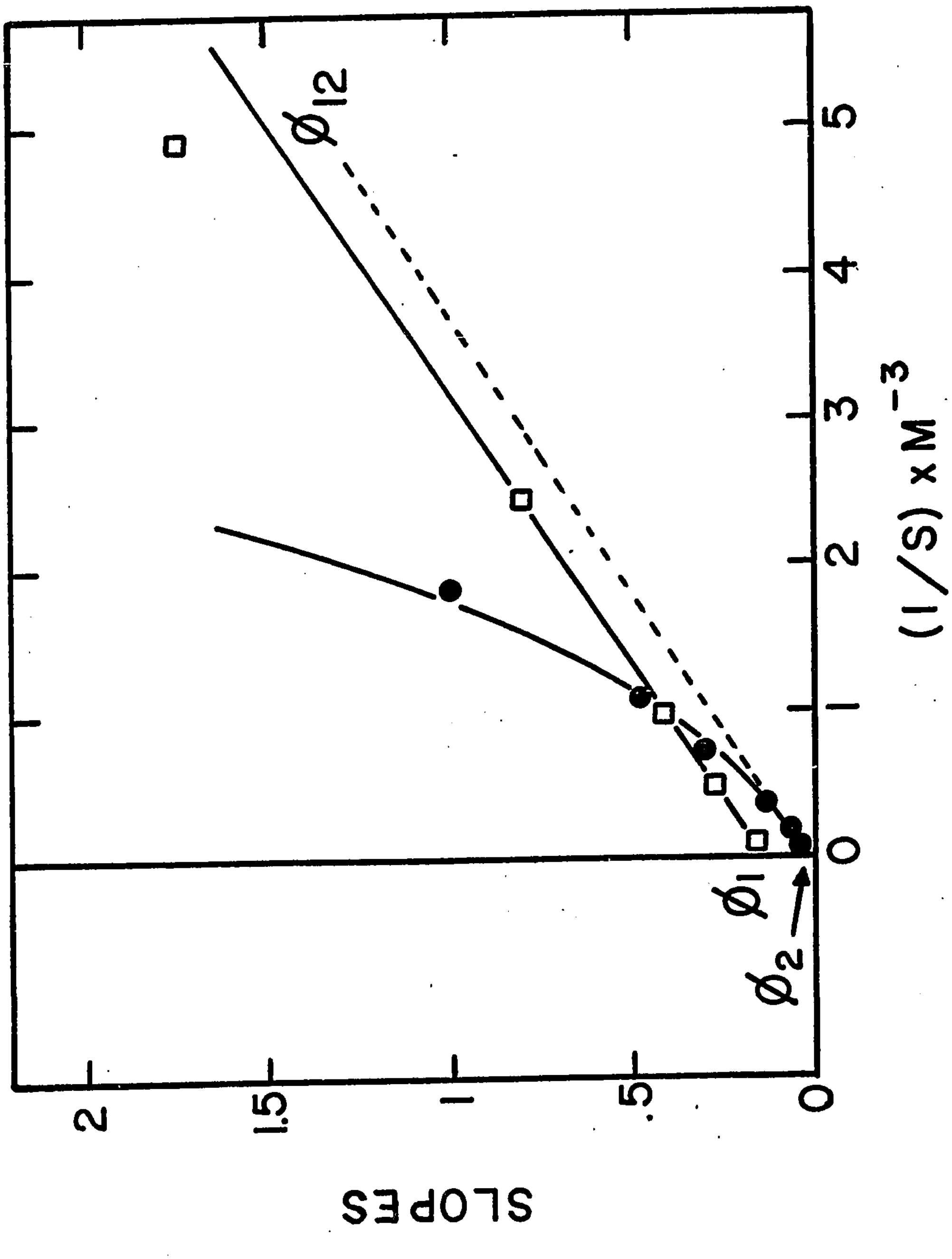
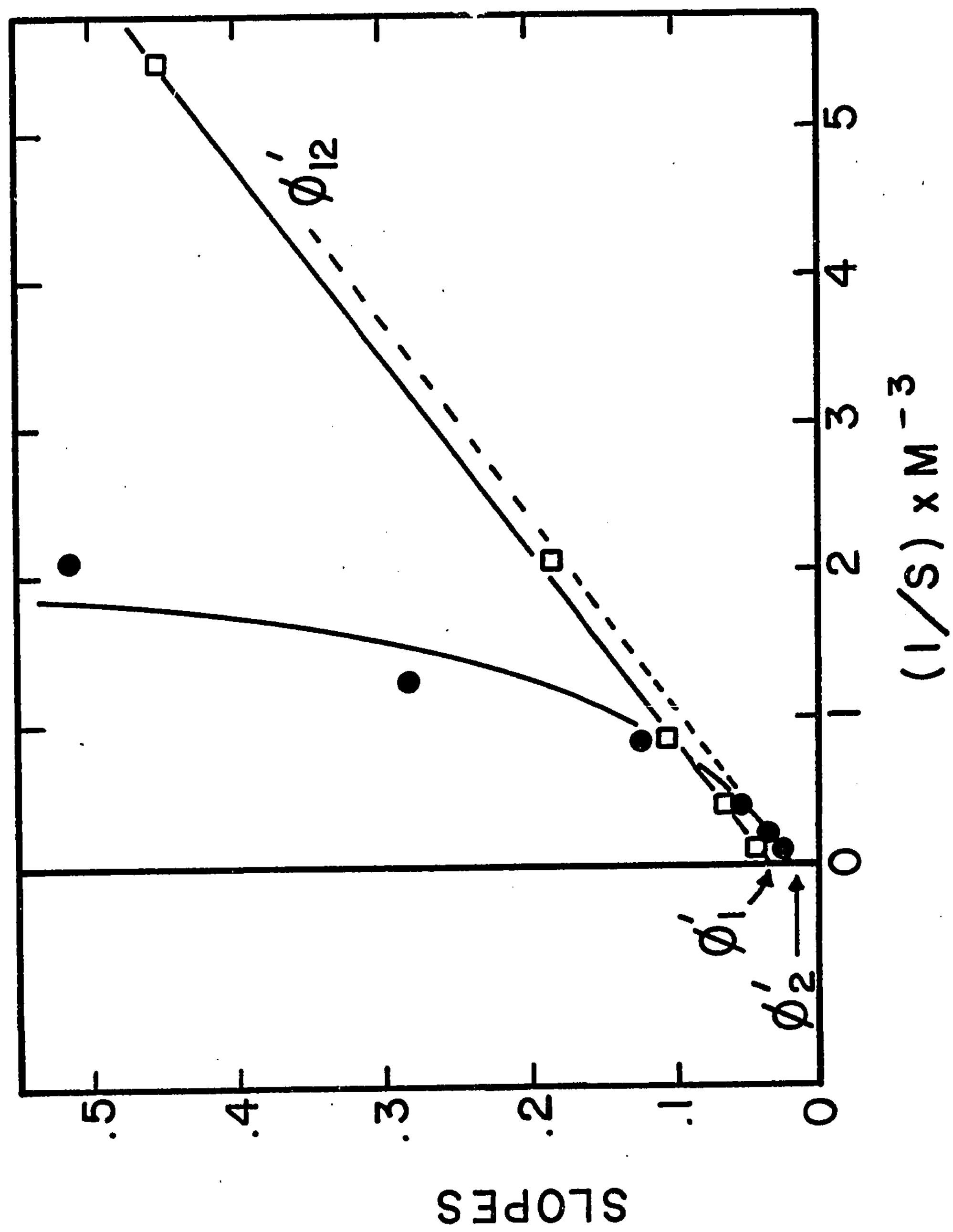

SLOPES
0
.5
1
1.5
2
(1/S) x M^{-3}
0
1
2
3
4
5
ϕ_{12}
ϕ_1
ϕ_2

Figure 34. Secondary plots of the slopes (milligrams of protein x min) from Figures 25 and 26. ●, Figure 25; □, Figure 26.


SLOPES
0
.1
.2
.3
.4
.5
(1/S) x M^{-3}
0
1
2
3
4
5
ϕ'_{12}
ϕ'_1
ϕ'_2

A. This chapter, data from Figures 18 to 34. Values in brackets were obtained from a preparation aged for three months at 4°.

B. Rabbit muscle phosphorylase _a_, Lowry, Schulz and Passonneau (32).

C. Rabbit brain phosphorylase _a_, Lowry, Schulz and Passonneau (32).

D. Frog muscle phosphorylase _a_, calculated from data of Metzger, Glaser and Helmreich (15).

E. Rabbit liver phosphorylase, plus AMP, Maddaiah and Madsen (28); minus AMP, unpublished results.

F. Rabbit muscle phosphorylase _b_, Chapter IV.

*Units for these rate constants are μMoles/min/mg.

TABLE IV

Kinetic constants for glycogen phosphorylases

Kinetic constant	Source of phosphorylase						
	A		B	C	D	E	F
$1/2\ K_1$	3.0	(5)	1.0	44	14	21	
K_2	25	(40)	35	6.2	10	22	
$2\ K_3$	2.9	(3)	7.0	0.6	4.0	3.5	
K_4	0.3	(0.1)	0.2	4.3	1.7	2	
$2\ K_5$	1.7	(1.2)	1.9		2.4	0.5	
K_6	1.2	(0.6)			7.1	4.1	
$1/2\ K_7$	4.0	(4.5)	1.0		11	20	
K_8	3.6	(3.9)	1.9		20	1.8	
$*k_1$	27	(18)	14		10	17	
$*k_2$	61	(42)	22		40	33	
$1/2\ K'_1$	1	(1)	0.3	0.9	3.4	14	2.3
K'_2	5.5	(3.6)	20	6.0		11	15
$2\ K'_3$	1.5	(1.7)	1.1	10	1.1	0.8	2.2
K'_4	0.2	(0.3)	0.02	0.3	1.2	0.9	0.2
$2\ K'_5$	0.2	(0.3)	0.2			0.3	3
K'_6	0.5	(0.5)				2.9	0.9
$1/2\ K'_7$	1.1	(0.9)	0.3			14	2.2
K'_8	0.8	(1.1)	0.2			1.3	7.4
$*k'_1$	32	(26)	14		11	14	33
$*k'_2$	69	(67)	22		40	37	65

absence of the modifier AMP are considerably decreased, one would conclude that under these conditions, phosphorylase a begins to depart from a "K" system, tending towards a mixed "K and V" system (21).

Upon comparison with the corresponding kinetic constants for rabbit liver phosphorylase (28) and rabbit muscle phosphorylase b (Chapter IV), those values obtained for phosphorylase a exhibit several similarities. For instance, in all cases glycogen is bound more tightly to the enzyme saturated with P_i than to the enzyme saturated with glucose-1-P. Possible explanations for this have been presented in a previous paper (28). Also, in general, the free enzyme binds glucose-1-P better than P_i, which could reflect the added availability of the glucose moiety of glucose-1-P to an additional binding site.

All of the phosphorylase a enzymes from various tissues would appear to be "K" systems, similar to that of the prototype phosphorylase a from rabbit skeletal muscle. Thus, the modifier AMP has a minimal effect on the V_{max} but causes a consistent decrease in the dissociation constants for the substrates. Aging the enzyme shifts it to a mixed "K and V" system, while removal of the phosphate from its serine moiety yields the extreme case of phosphorylase b which is almost a true "V" system in that it exhibits only about 1% of V_{max} in the absence of AMP (22). However, certain anions can replace AMP to some extent (6, Chapter III) while the binding of substrates is progressively improved by AMP, and vice versa (7, 18).

Comparison of Columns A and B of Table IV show a reasonable degree of correspondence between our data and those of Lowry, Schulz and Passonneau (32) for most of the kinetic constants. Some of the more striking differences (e.g., in K'_4, k_1 and k_2) may be due to the different assay methods and physical conditions, as well as the much lower enzyme concentrations used in the earlier work.

In comparing phosphorylase a with phosphorylase b, it may be seen that phosphorylase a is a much more "efficient" enzyme, not only because it is active in the absence of AMP but also because it binds substrates much more tightly when AMP is present, and it also binds AMP more tightly. Other differences in allosteric properties have been discussed elsewhere (17, 22, 32). The phosphorylase a system of muscle shows remarkable kinetic similarities to that of liver, except that the muscle enzyme binds glycogen much more tightly, perhaps reflecting the lower concentration of glycogen in muscle (32).

Similar comparisons could be made with the enzymes from brain and frog muscle, but these have already been discussed by the authors who supplied the data (15, 32).

In Table V the values for K_{11} through K_{18} are taken from a paper by Helmreich et al. (17), which deals with the binding of AMP to phosphorylase a. These results, obtained by equilibrium dialysis methods, represent the best estimate that one may make for the various AMP constants, excluding K_{14} and K_{15} which may be determined kinetically. The kinetically derived value for K_{15} is approximately 1.5 μMolar (62), which is reasonably close to that determined by the binding studies. It should be mentioned that the K_{diss} for AMP and phosphorylase a is very sensitive to changes in ionic strength; for instance, there is roughly a six-fold increase in the K_{diss} for AMP and enzyme$\cdot P_i$, from 1.1 to approximately 6μM, when one raises the ionic

TABLE V

Dissociation constants for AMP and phosphorylase a*

Kinetic constant	Value of K_{diss}
K_{11}	1.8
K_{12}	1.2
K_{13}	1.8
K_{14}	1.0
K_{15}	1.0
K_{16}	1.2
K_{17}	<1.8
K_{18}	1.8

Units are μmolar, and the symbols are defined in Figure 18.

* Ionic strength $\tau/2 = .10$, pH 6.8, Helmreich, Michaelides and Cori (17).

strength from $\tau/2 = 0$ to $\tau/2 = 0.28$ (17).

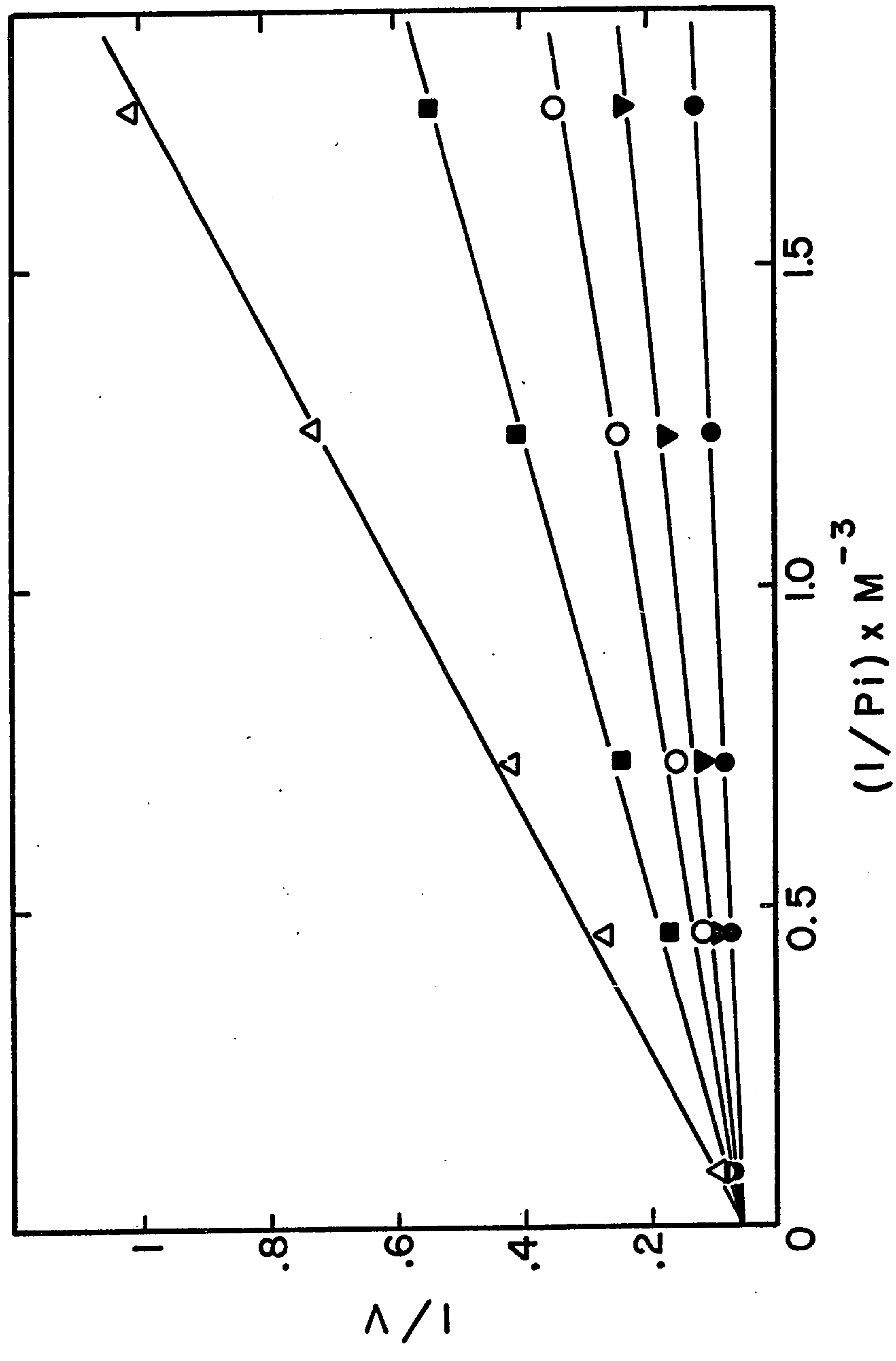
The fact that the substrate glucose-1-P binds to the free enzyme, or enzyme·AMP complex, and hence affords protection against inactivation by isocyanate (70) would tend to rule out the likelihood of an ordered sequence, with glycogen as the initial substrate bound. The protection against inactivation which is afforded by glucose-1-P can be used to obtain an estimate of the K_{diss} for glucose-1-P and the enzyme (26). Preliminary experiments with phosphorylase a yielded rough estimates of 7 mM for the dissociation constant in the absence of AMP and 0.5 mM in the presence of 1 mM AMP. These estimates agree reasonably well with the corresponding values for K_8 and K'_8 in Table IV, considering the complications that can arise when working with the high ionic strength buffer system, which is necessary to ensure adequate control of pH (70).

Using the Haldane relationship (27, 29), the equilibrium constants calculated from the initial rate data are in fair agreement with the value of 0.21 obtained by direct measurement (57); the value in the presence of AMP being 0.11 and, in the absence of AMP, 0.29. As mentioned in the previous chapter, several other phosphorylase a systems yielded a Haldane value in reasonable accord with the chemical $K_{equilibrium}$.

Inhibition by UDP-glucose

Figure 35 illustrates the inhibition pattern of phosphorylase a by UDP-glucose as a function of P_i concentration. The inhibition by UDP-glucose is competitive with respect to P_i at all concentrations tested. The effect of various concentrations of UDP-glucose at various

Figure 35. Velocity of muscle phosphorylase a activity as a function of P_i concentration at 5 mM glycogen, 1 mM AMP, and the following levels of UDP-glucose: ●, 0 (apparent K_m for P_i = 0.87 mM); ▼, 0.52 mM; ○, 1.0 mM; ▣, 2.1 mM; △, 4.1 mM.


1/V
1
.8
.6
.4
.2
0
0.5
1.0
1.5
(1/Pi) x M^{-3}

glycogen levels and a constant level of P_i is shown in Figure 36. The resulting inhibition is non-competitive with respect to glycogen, as would be expected for our suggested mechanism when a competitive inhibitor for a second substrate is added to various concentrations of the first substrate (30, 71).

Figure 37 illustrates the results obtained when one plots the slopes and intercepts from Figure 36 against the UDP-glucose concentration present. Using the $K_{i\ slope}$ value of 2.3 mM UDP-glucose, one can calculate a value of 0.54 mM for the dissociation of UDP-glucose from the enzyme·AMP·glycogen complex. Similarly, using the $K_{i\ intercept}$ value of 0.51 mM from Figure 37, one calculates a K_{diss} of 0.27 mM for UDP-glucose and enzyme·AMP. These two values are in the same range as those obtained for liver phosphorylase, i.e., 0.27 and 0.36 mM UDP-glucose in the presence and absence of glycogen respectively. As discussed previously (28), these results reported for the inhibition of the phosphorylase a reaction by UDP-glucose lend additional support to the assignment of a rapid equilibrium random bi bi kinetic mechanism to rabbit muscle phosphorylase a.

It is of interest to note that Karpatkin and Langer (72) have recently shown that ATP and ADP exhibit competitive inhibition of phosphorylase a from human platelets when P_i was the varied substrate, and simple linear non-competitive inhibition, in Cleland's nomenclature (27), when glycogen was varied. This pattern of inhibition would tend to suggest that human platelet phosphorylase also operates via a random reaction sequence.

Figure 36. Velocity of muscle phosphorylase a activity as a function of glycogen concentration at 5 mM P_i, 1 mM AMP, and the following levels of UDP-glucose: ●, 0 (apparent K_m for glycogen = 0.39 mM); ∇, 0.52 mM; ■, 1.0 mM; ○, 2.1 mM; ▲, 4.1 mM.

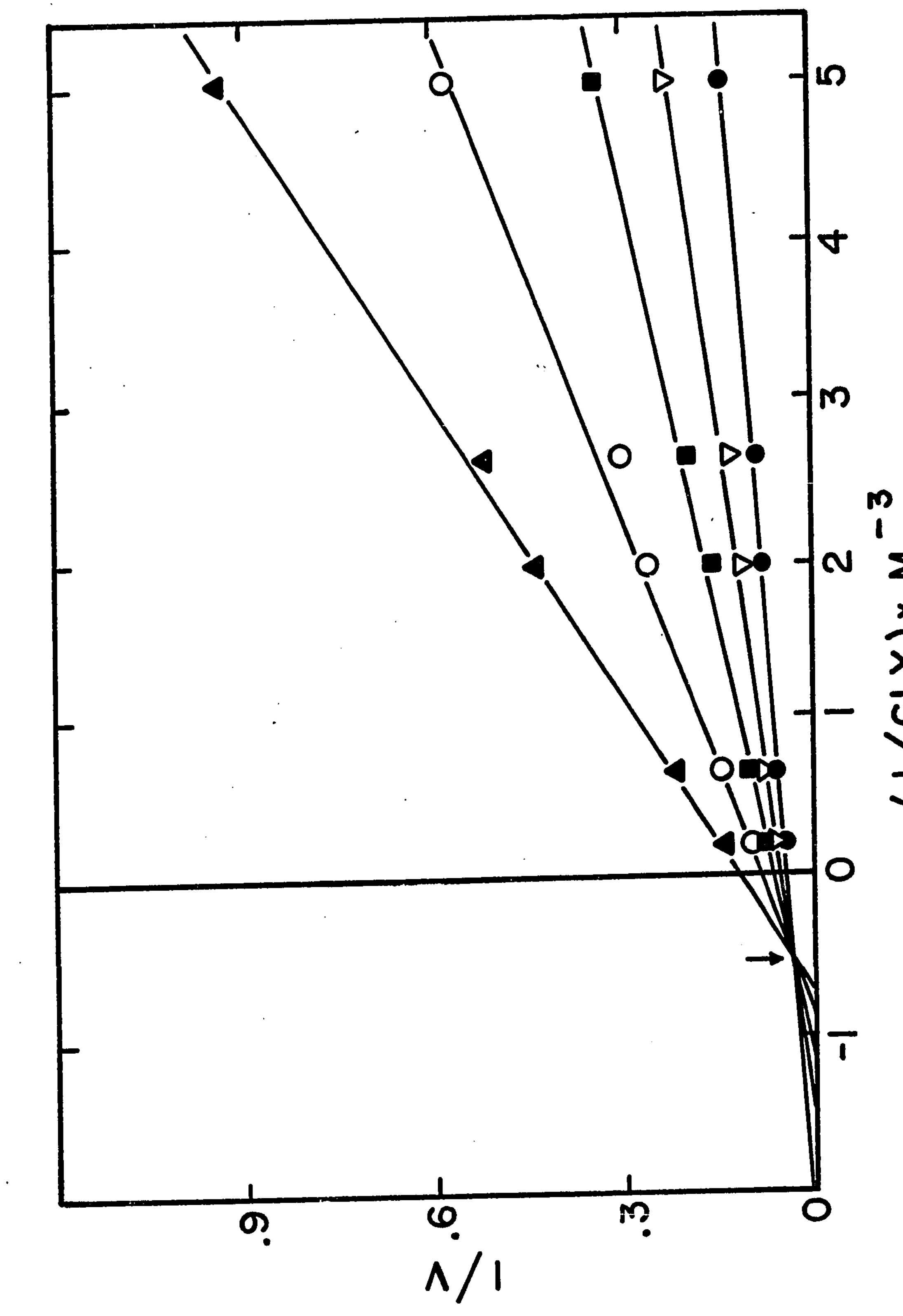
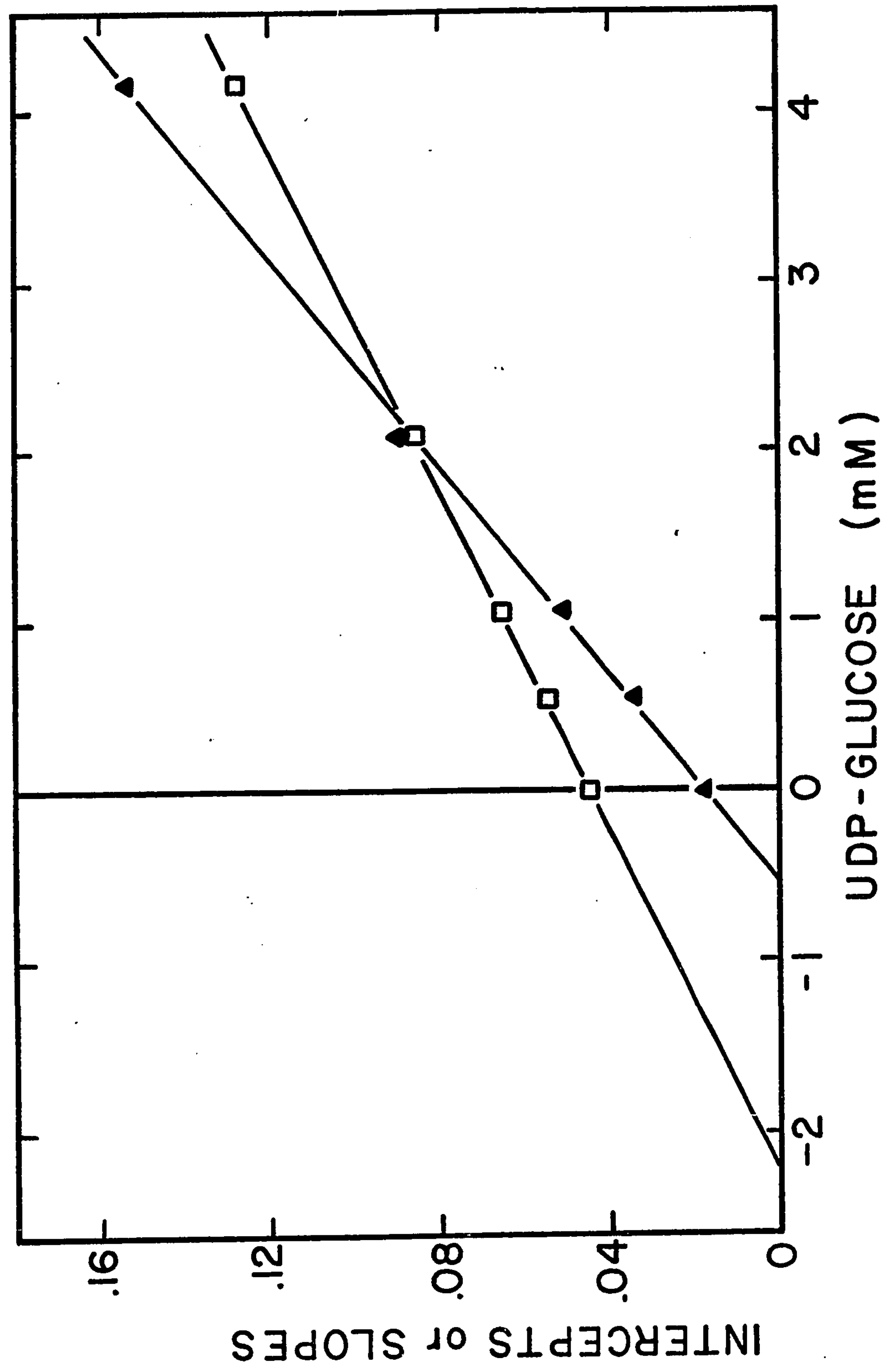

1/V
9.
6.
3.
0
(1/GLY) x M^{-3}
-1
0
1
2
3
4
5

Figure 37. Secondary plot of slopes and intercepts from Figure 36. ▲, slopes, 5 mM P_i, K_i slope for UDP-glucose = 2.3 mM. □, intercepts, 5 mM P_i, K_i intercept for UDP-glucose = 0.51 mM.


INTERCEPTS or SLOPES
0
.04
.08
.12
.16
UDP-GLUCOSE (mM)
-2
-1
0
1
2
3
4

Results reported in the following chapter on the equilibrium isotope exchange patterns obtained with phosphorylase a provide independent confirmation of the proposed rapid equilibrium random kinetic mechanism.

B. Summary

Initial rate studies on rabbit muscle phosphorylase a were carried out in order to assign a kinetic mechanism to this enzyme, which plays an important role in the control of glycogen metabolism. Initial velocities were measured with varied concentrations of both substrates in each reaction direction, both in the presence and the absence of the modifier AMP. Data were analysed with double reciprocal plots and secondary replots of slopes and intercepts to yield kinetically derived dissociation constants which may be compared with dissociation constants determined by independent methods. Inhibition studies using UDP-glucose as a substrate analogue are also reported. Inhibition is competitive with glucose-1-P and P_i and non-competitive with glycogen.

A suitable rate equation for this system has been derived, and it should apply to other two-substrate enzyme-modifier systems exhibiting similar kinetics. The results of this detailed kinetic analysis, in conjunction with the isotope exchange studies at equilibrium which are reported in the following chapter, suggest the kinetic mechanism of phosphorylase a to be rapid equilibrium random bi bi; i.e., random addition of substrates to the enzyme, with ternary complex interconversion as the rate limiting step in the reaction sequence.

CHAPTER VI

THE KINETIC MECHANISM OF PHOSPHORYLASE a.

ISOTOPE EXCHANGE RATES AT EQUILIBRIUM

A. Results and Discussion

Preliminary experiments showed that the rate of isotope exchange was directly proportional to the concentration of enzyme present. As discussed in Chapter IV, prior incubation of the complete assay system for any appreciable length of time will allow disproportionation of glycogen end groups to take place. In order to avoid this problem, the desired concentration of glycogen was added only five minutes prior to starting the reaction by the addition of isotope.

Figures 38 and 39 illustrate the results of the isotope exchange at equilibrium experiments for phosphorylase a in the presence of 1 mM AMP, a concentration which is saturating for this activator. In Figure 38, the glycogen concentration was held constant at 5.6 mM total residues, while glucose-1-P and P_i concentrations were varied at a constant ratio up to saturating levels. Both the $^{32}P_i \rightleftharpoons$ glucose-1-P and ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange rates rose and leveled off as the enzyme became saturated with the substrates P_i and glucose-1-P. These results rule out the possibility that glycogen is the initial substrate to be bound in a compulsory mechanism. One would have expected a decrease in the glucose-1-P $\rightleftharpoons$ glycogen exchange rate at the saturating levels of P_i and glucose-1-P, had the mechanism been ordered in nature (35). Similarly, the hyperbolic curves obtained in Figure 39 negate the possibility that glucose-1-P is the initial

Figure 38. The effect of glucose-1-P (G-1-P) and P_i concentrations on equilibrium isotope exchange rates with muscle phosphorylase *a* in the presence of AMP. Equilibrium mixes contained 9.5 μg phosphorylase *a* per ml, 5.6 mM glycogen, 1 mM AMP, 3 mM sodium-β-glycerophosphate, 1.5 mM EDTA, 0.8 mM mercaptoethanol, pH 6.8, with glucose-1-P and P_i as shown: ▨, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen; O, $^{32}P_i \rightleftharpoons$ glucose-1-P.

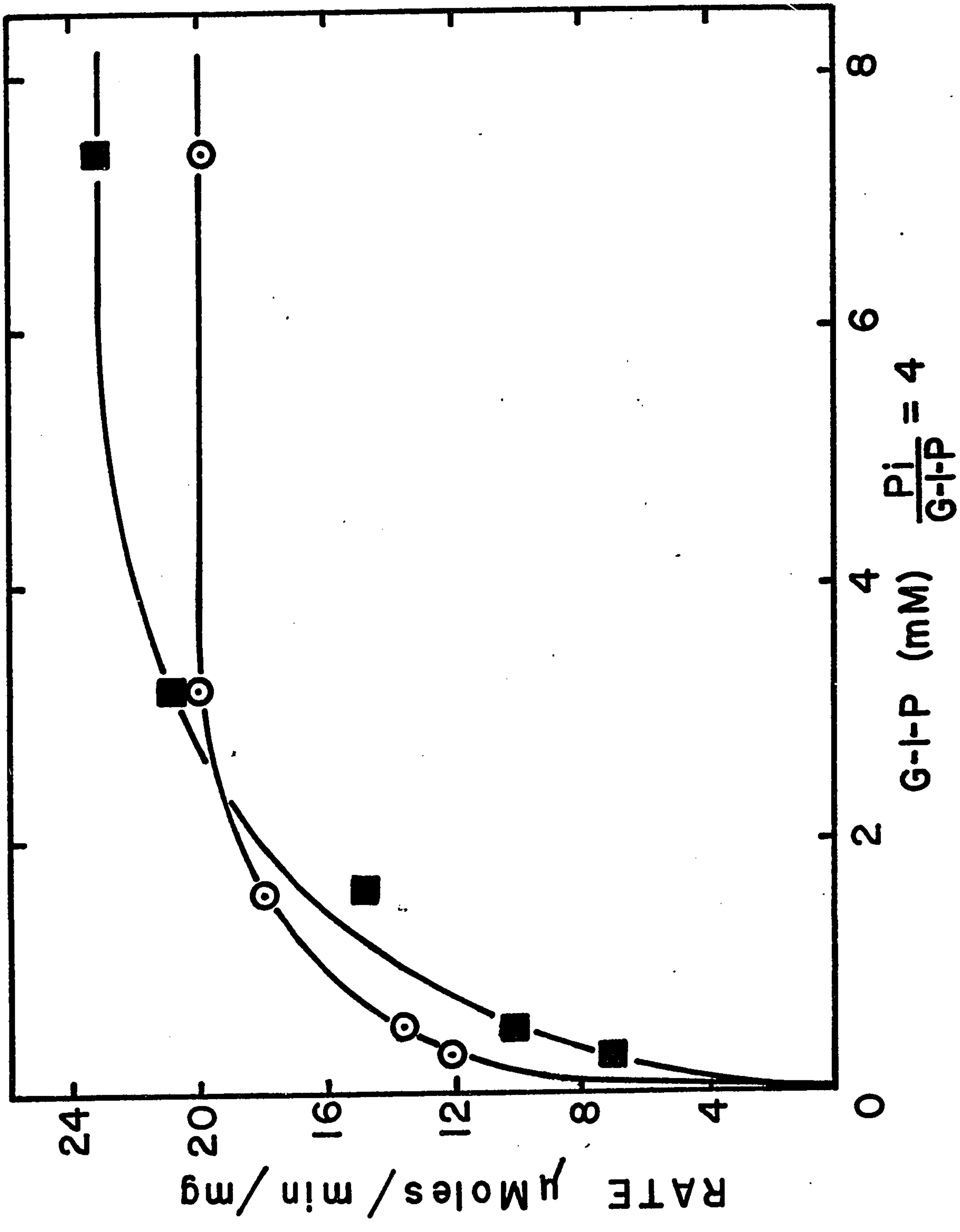
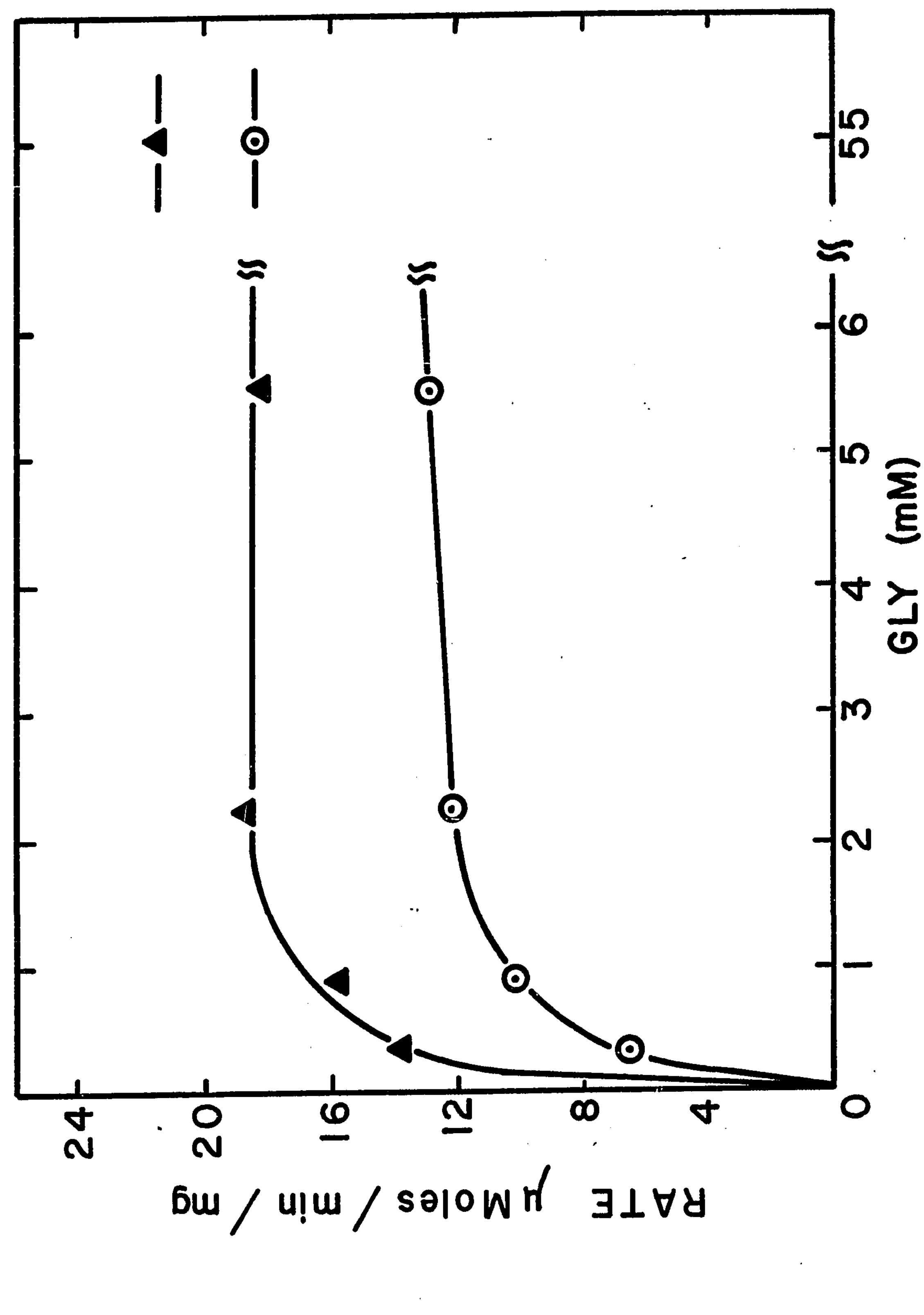

RATE μMoles/min/mg
24
20
16
12
8
4
0
2
4
6
8
G-I-P (mM)
$\frac{Pi}{G\text{-}I\text{-}P} = 4$

Figure 39. The effect of glycogen concentration on equilibrium isotope exchange rates with muscle phosphorylase a in the presence of AMP. Equilibrium reaction mixes as in Figure 38, with 1.03 mM glucose-1-P, 4.12 mM P_i, and glycogen as shown: ▲, $^{32}P_i \rightleftharpoons$ glucose-1-P; O, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen.


RATE μMoles / min / mg
24
20
16
12
8
4
0
1
2
3
4
5
6
55
GLY (mM)

substrate bound in an ordered sequence of substrate addition.

The $^{32}P_i \rightleftharpoons$ glucose-1-P and ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchange rates are well within a factor of two of each other (35), confirming that rapid equilibrium conditions apply to this mechanism. As was discussed in an earlier chapter on phosphorylase b kinetics, the actual concentration of glycogen residues participating in the exchange reaction is difficult to assess; hence we have calculated the glycogen concentration in terms of total residues, and therefore the exchange rates observed for ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen represent an upper limit for isotope exchange in that direction.

Figures 40 and 41 illustrate the results obtained for the isotope exchange experiments in the absence of AMP. It can be seen that when either glucose-1-P:P_i or glycogen were the varied substrates, the curves rose and then leveled off as the varied substrate levels became saturating, indicating a random mechanism. Again, the rates of exchange for $^{32}P_i \rightleftharpoons$ glucose-1-P and ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen are comparable, suggesting that rapid equilibrium conditions hold in the absence of the modifier AMP as well as in its presence. This knowledge is important, since it confirms the fact that AMP does not alter the kinetic mechanism of muscle phosphorylase a, as it appears to do in the case of isocitrate dehydrogenase from Neurospora (19, 20).

Using the maximal velocities obtained in initial rate studies, one can calculate R_{max}, the equilibrium reaction rate at infinite substrate concentration, for a rapid equilibrium random bi bi kinetic mechanism (35). For phosphorylase a plus AMP the calculated R_{max} is 21.7 μmoles per min per mg; this value agrees well with that observed for the isotope exchange studies, in Figures 38 and 39. It is of

Figure 40. The effect of glucose-1-P and P_i concentrations on equilibrium isotope exchange rates with muscle phosphorylase a in the absence of AMP. Equilibrium reaction mixes as in Figure 38, with 11 μg enzyme per ml and glucose-1-P and P_i as shown: ▲, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen; O, $^{32}P_i \rightleftharpoons$ glucose-1-P.

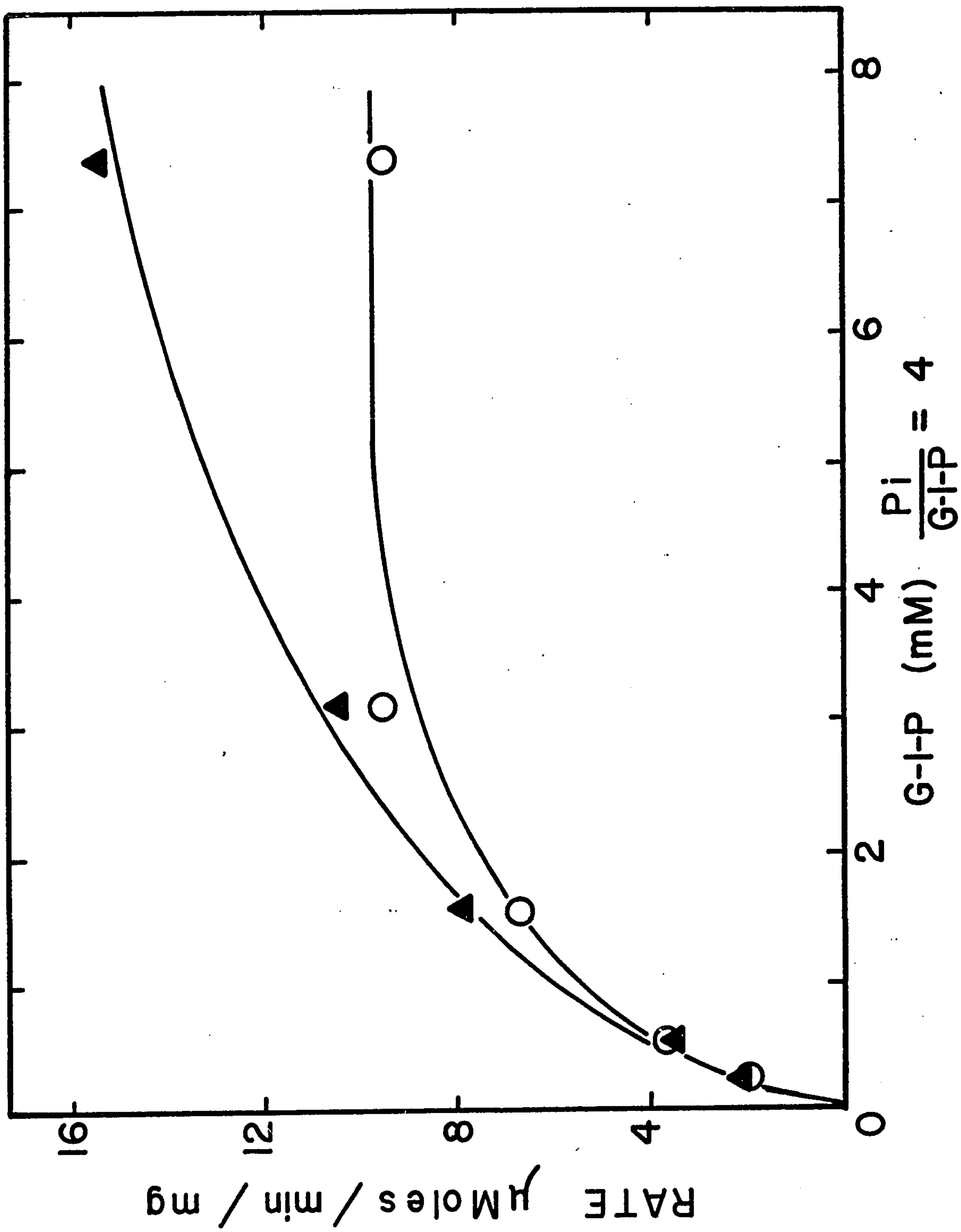
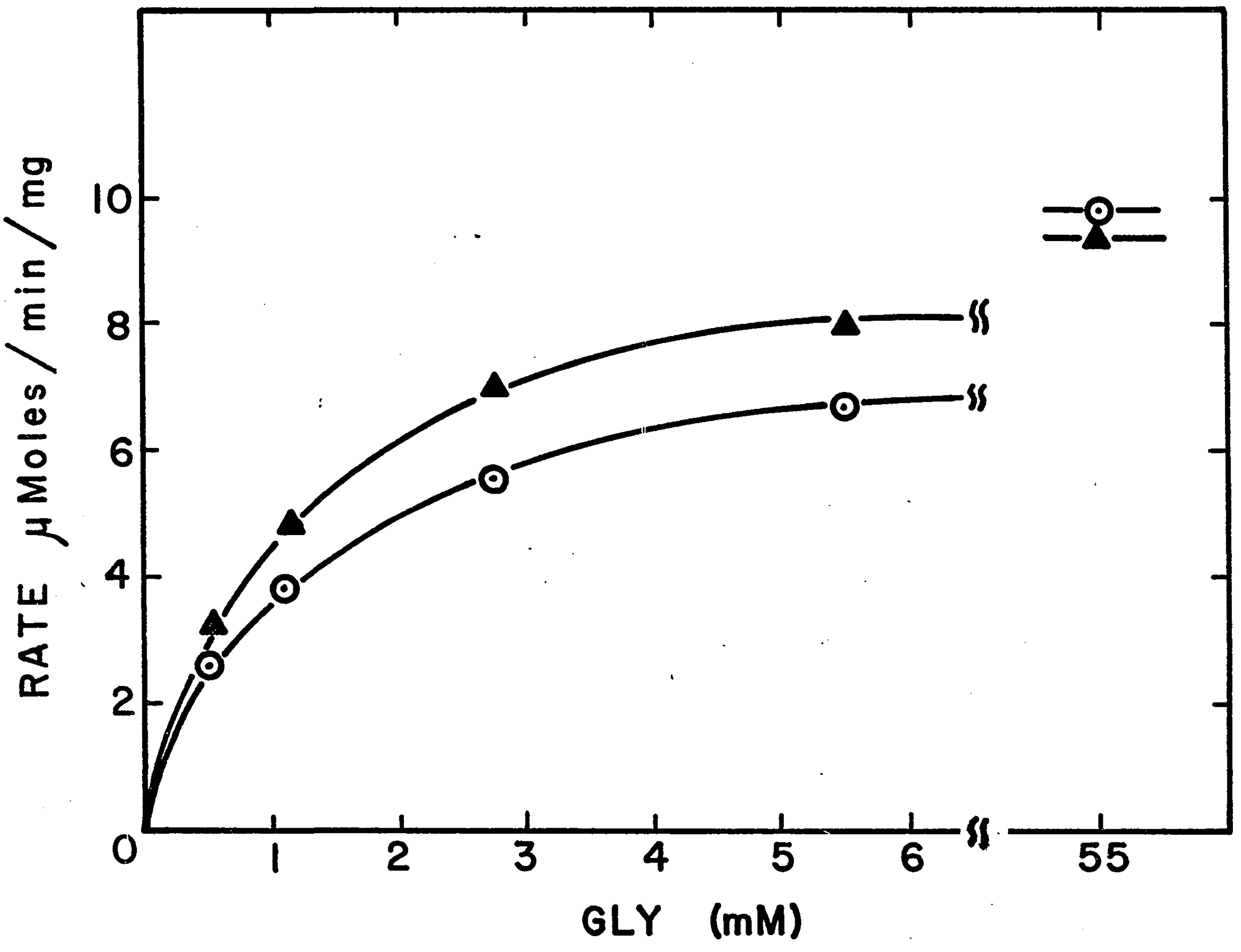

RATE µMoles / min / mg
0
4
8
12
16
0
2
4
6
8
G-I-P (mM)
$\frac{Pi}{G\text{-}I\text{-}P} = 4$

Figure 41. The effect of glycogen concentration on equilibrium isotope exchange rates with muscle phosphorylase a in the absence of AMP. Equilibrium reaction mixes as in Figure 38, with 1.03 mM glucose-1-P, 4.12 mM P_i and 11.2 μg enzyme per ml: O, $^{32}P_i \rightleftharpoons$ glucose-1-P; ▲, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen.


RATE µMoles / min / mg
0
2
4
6
8
10
1
2
3
4
5
6
55
GLY (mM)

interest to note that the R_{max} calculated for phosphorylase b in the presence of AMP is 21.8 μmoles per min per mg (Chapter IV).

In the absence of AMP, the phosphorylase a system yields a calculated R_{max} of 18.5 μmoles per min per mg protein, which is somewhat higher than those values observed in Figures 40 and 41. This may be explained by the fact that the concentration of the constant substrates (glycogen in the case of Figure 40 and glucose-1-P and P_i in the case of Figure 41) are in the neighborhood of the K_m values for these substrates (Chapter V) and hence one would not expect to observe a maximal rate under these conditions.

One other important conclusion can be drawn from these isotope exchange experiments; that concerning the non-linear reciprocal plots and replots observed for initial velocity kinetic experiments performed in the absence of AMP (Chapter V, 16). As discussed previously (Chapter IV, 16), these curved plots can originate as a consequence of two possible alternatives: (i) due to allosteric interactions between substrate binding sites; (ii) due to a breakdown in the rapid equilibrium conditions, thus introducing a squared term in the rate equation (43). Since the isotope exchange rates for both the $^{32}P_i \rightleftharpoons$ glucose-1-P and ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen exchanges are virtually identical at the lower levels of substrates; i.e., the substrate levels at which one would expect to observe allosteric interactions, the kinetic mechanism must still be rapid equilibrium in nature and no breakdown of such a mechanism has occurred. This type of experiment points out the usefulness of the isotope exchange method as applied to enzymes exhibiting "apparent" allosteric kinetics.

The isotope exchange results reported herein confirm the mechanism suggested by the initial rate studies described in the preceding chapter; i.e., the kinetic mechanism of rabbit muscle phosphorylase a is rapid equilibrium random bi bi, with random addition of substrates, and the interconversion of the ternary complexes is the rate limiting step in the reaction sequence. While this work was in progress, an abstract appeared by Johnson and Gold (67) which confirms the results presented in this chapter.

B. Summary

In order to confirm the kinetic mechanism which was proposed for rabbit muscle phosphorylase a on the basis of initial rate studies and UDP-glucose inhibition experiments, isotope exchange studies at equilibrium were performed, both in the presence and absence of the modifier AMP.

Both the ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen and the $^{32}P_i \rightleftharpoons$ glucose-1-P equilibrium exchange rates increased to a maximum as the concentrations of the varied substrates became saturating, either in the presence or absence of AMP. The plateaus observed in these experiments indicate the lack of inhibition of the exchange of one pair of substrates when the concentration of the other substrate pair was raised, and confirms the proposed random addition of substrates to the enzyme.

The fact that similar exchange rates were observed for either reaction direction reinforced the concept that rapid equilibrium conditions apply to the phosphorylase a mechanism; i.e., the interconversion of the ternary complexes tends to be the rate limiting step in the reaction sequence.

Maximal velocities determined from initial rate data reported in the previous chapter agreed with those calculated from isotope exchange rates.

CHAPTER VII

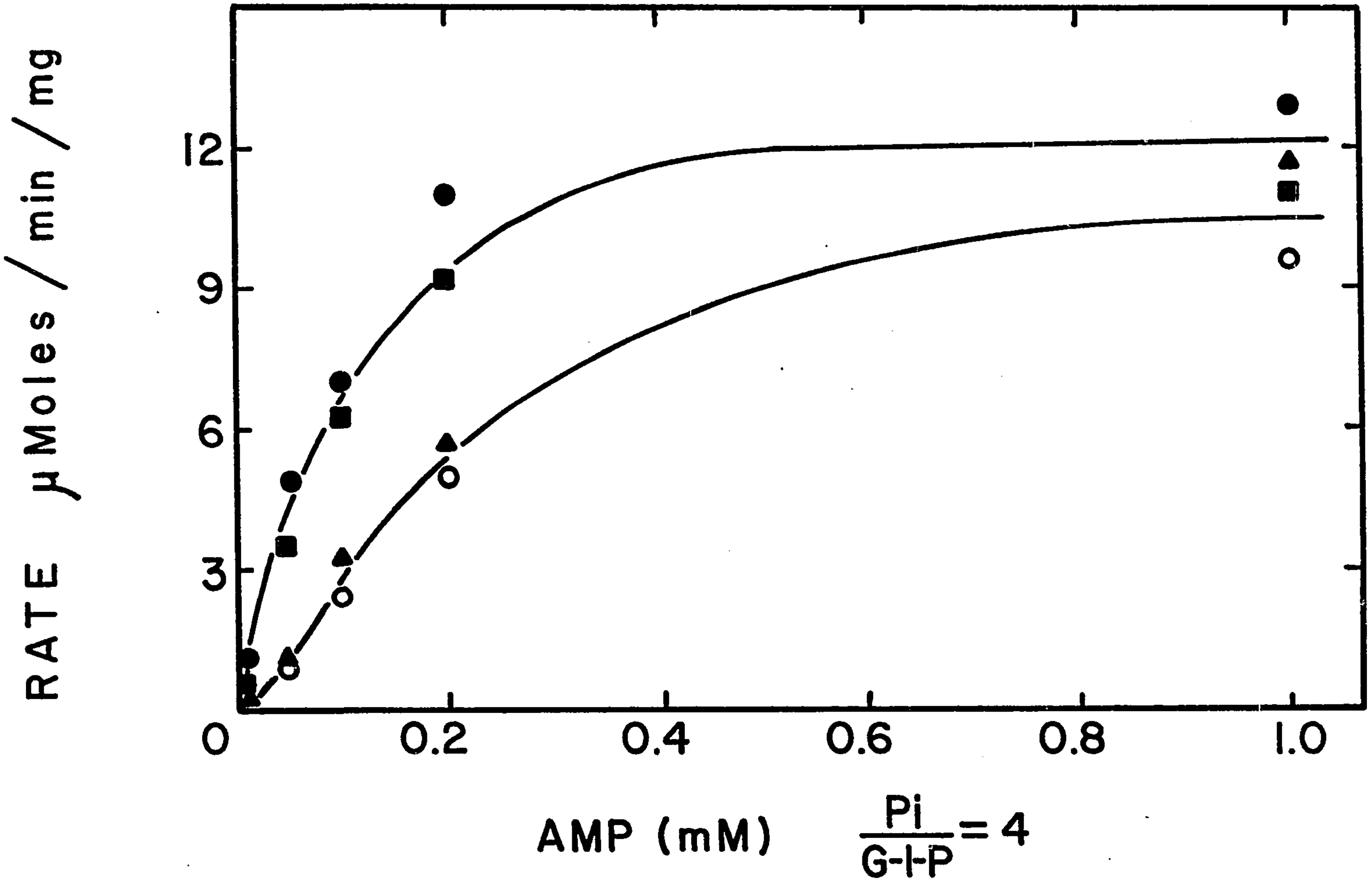
ISOTOPE EXCHANGE STUDIES AT EQUILIBRIUM AS APPLIED TO ALLOSTERIC ENZYMES

A. Results and Discussion

Figure 42 illustrates the results obtained for an isotope exchange experiment using phosphorylase b in which glucose-1-P, P_i and glycogen were held constant at 1 mM, 4 mM and 11.4 mM (end groups) respectively. The activator AMP was varied from 0.01 to 1 mM in the presence and absence of the inhibitor ATP. The control curve in the absence of ATP shows that both exchange rates; i.e., $^{32}P_i \rightleftharpoons$ glucose-1-P and ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen are identical, and exhibit a normal hyperbolic saturation curve, with one-half maximal rate of exchange occurring at a value of approximately 0.13 mM AMP, as determined from a plot of 1/rate versus 1/AMP. This value is in good agreement with the "K_m" values obtained in initial velocity experiments, using either glucose-1-P or P_i as substrate (Madsen and Shechosky, Figures 10 and 12, ref.22). In addition, this value of 0.13 mM is in the same range as the values determined by Avramovic and Madsen (26) for the dissociation constant of AMP binding to enzyme, as measured by equilibrium dialysis and protection against inactivation by isocyanate.

The inclusion of 1 mM ATP results in a sigmoidal inhibition pattern, as is shown in the lower curve in Figure 42. The $^{32}P_i \rightleftharpoons$ glucose-1-P exchange rate is equivalent to the ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen rate in the presence of the "allosteric" inhibitor ATP; this observation confirms the suggestion that the sigmoidicity is due to a

Figure 42. The effect of ATP on equilibrium isotope exchange rates with muscle phosphorylase *b*. The 0.5 ml equilibrium reaction mixes contained 11.4 mM glycogen (end groups), 1 mM glucose-1-P, 4 mM P_i, with AMP as shown, in 5 mM sodium-glycerophosphate, 1.5 mM EDTA, 2 mM mercaptoethanol, pH 6.8. The *upper curve* represents results obtained in the absence of ATP, with 7 μg per ml phosphorylase *b*. ●, $^{32}P_i \rightleftharpoons$ glucose-1-P; ■, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen. The *lower curve* represents results obtained in the presence of 1 mM ATP, with 21 μg per ml phosphorylase *b*. ▲, $^{32}P_i \rightleftharpoons$ glucose-1-P; O, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen.

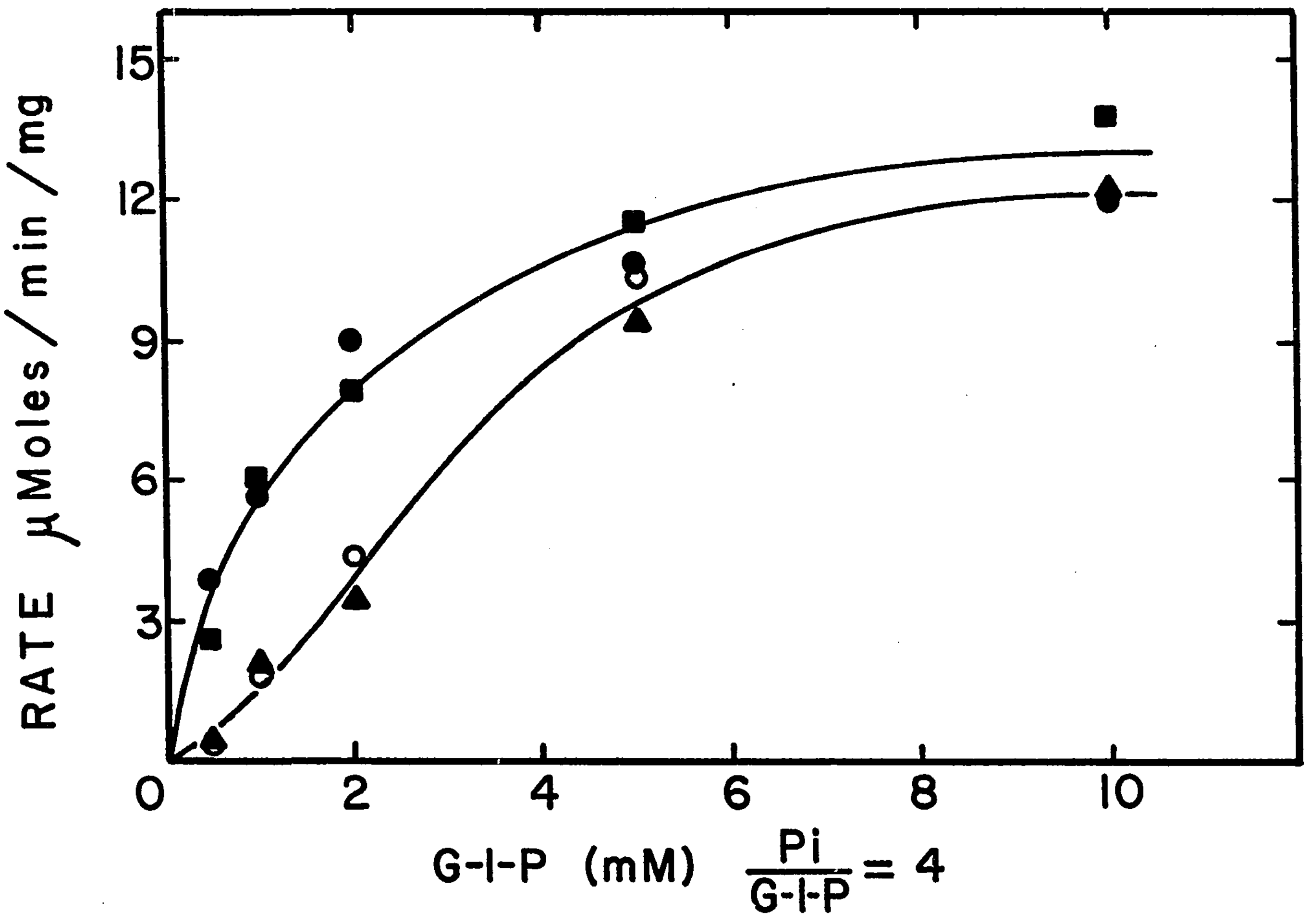

RATE µMoles / min / mg
12
9
6
3
0
0.2
0.4
0.6
0.8
1.0
AMP (mM)
$\frac{Pi}{G\text{-}1\text{-}P} = 4$

cooperativity among binding sites (18, 22), and not due to a breakdown of the random rapid equilibrium conditions.

Avramovic and Madsen (26), and also Kastenschmidt et al. (24, 25), have confirmed, by the use of binding studies, that there are two AMP binding sites per mole of phosphorylase b (73), an obligate requirement for the expression of homotropic allosteric cooperativity by AMP. In fact, the binding studies demonstrated homotropic cooperativity among the AMP binding sites (24, 25, 26). Of added interest is the fact that the concentrations used in Figure 42, as well as those used in the binding studies mentioned above, approach those expected for in vivo conditions, and reinforce the belief that these cooperative effects may be involved as a control mechanism in living muscle.

The results shown in Figure 43 are from an experiment conducted with phosphorylase b in the presence and absence of 10 mM ATP. The glucose-1-P and P_i concentrations were varied at a constant ratio, with glycogen and AMP held constant. The control curve in the absence of ATP is hyperbolic, with both exchange rates identical. In the presence of ATP, the curve becomes sigmoidal in shape, analogous to those results obtained for initial velocity studies (18, 22). Here again, the two exchange rates were equivalent, confirming that the mechanism is still rapid equilibrium. A Hill plot of the data in Figure 43 gives a slope of 1.1 for exchange rates obtained minus ATP and 1.75 for the data obtained in the presence of ATP. These "n" values for the Hill plots are virtually identical to those obtained for initial velocity studies, varying either glucose-1-P or P_i under similar conditions (22). These data also support the conclusion that

Figure 43. The effect of ATP on equilibrium isotope exchange rates with muscle phosphorylase *b*. The 0.5 ml equilibrium reaction mixes contained 10.6 mM glycogen (end groups), 1 mM AMP, with glucose-1-P and P_i as shown, in 5.7 mM sodium-glycerophosphate, 1.5 mM EDTA, 2 mM mercaptoethanol, pH 6.8. The *upper curve* represents results obtained in the absence of ATP, with 4 μg per ml phosphorylase *b*. ■, $^{32}P_i \rightleftharpoons$ glucose-1-P; ●, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen. The *lower curve* represents results obtained in the presence of 9.6 mM ATP, with 12 μg per ml phosphorylase *b*. ▲, $^{32}P_i \rightleftharpoons$ glucose-1-P; O, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen.

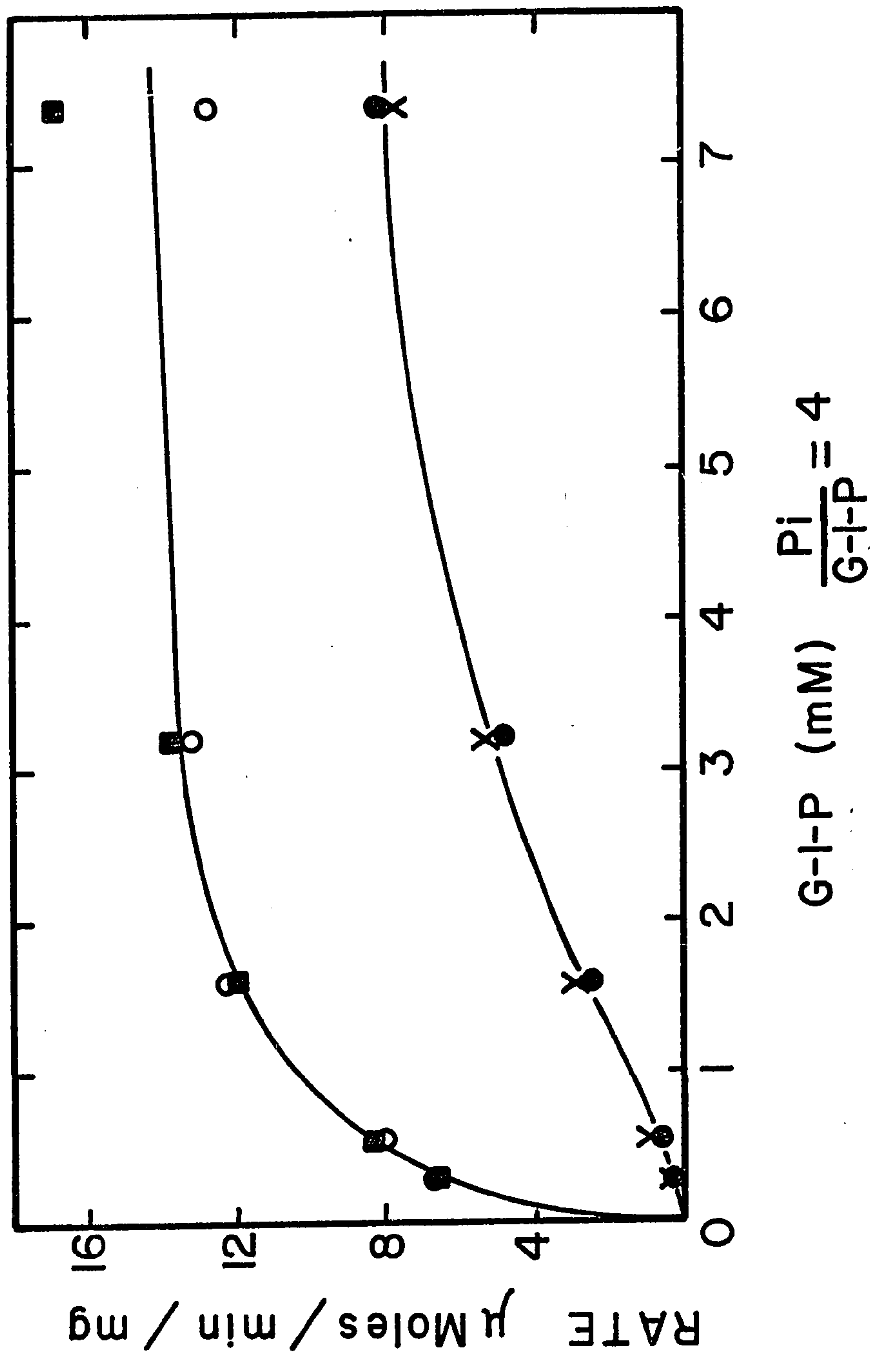

RATE μMoles / min / mg
15
12
9
6
3
0
2
4
6
8
10
G-1-P (mM) $\frac{Pi}{G\text{-}1\text{-}P} = 4$

there exists more than one binding site per mole of enzyme for the substrates glucose-1-P and P_i.

Figure 44 illustrates the results obtained when equilibrium isotope exchange rates were measured for phosphorylase a in the presence and absence of glucose, which has been shown to be an allosteric effector for phosphorylase a (17). The upper curve represents the control experiment, conducted in the absence of glucose. As can be seen, the $^{32}P_i \rightleftharpoons$ glucose-1-P and ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen isotope exchange rates follow a hyperbolic curve, and are the same. The lower curve shows the results obtained in the presence of 50 mM glucose. Again, in the presence of the "allosteric" effector, the rate curve is sigmoidal, and the isotope exchange rates are the same. These results agree with the observed effect of glucose on the initial velocity of phosphorylase a, as reported by Helmreich et al. (17).

It had been shown previously for glycogen phosphorylase, by initial velocity studies in conjunction with isotope exchange studies at equilibrium (Chapters IV, V, VI), that the kinetic mechanism of both phosphorylase b and a was random rapid equilibrium bi bi. In this chapter, we have shown that in the presence of the allosteric effectors ATP (18, 22), and glucose (17), the kinetic mechanisms remain random rapid equilibrium; i.e., the rate limiting step in the reaction sequence is still the interconversion of ternary complexes. Hence, the sigmoidicity which results is in fact most likely due to some form of cooperativity between binding sites or possibly subunits, presumably resulting from a conformational change induced by the binding of the ligands in question.

Figure 44. The effect of glucose on equilibrium isotope exchange with muscle phosphorylase *a* in the absence of AMP. The 0.5 ml equilibrium reaction mixes contained 10.6 mM glycogen (end groups), with glucose-1-P and P_i as shown, in 3 mM sodium-glycerophosphate, 1.5 mM EDTA, 0.8 mM mercaptoethanol, pH 6.8. The *upper curve* represents results obtained in the absence of glucose, with 4 μg per ml phosphorylase *a*. ■, $^{32}P_i \rightleftharpoons$ glucose-1-P; O, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen. The *lower curve* represents results obtained in the presence of 50 mM glucose, with 11.1 μg per ml phosphorylase *a*. ●, $^{32}P_i \rightleftharpoons$ glucose-1-P; X, ^{14}C-glucose-1-P $\rightleftharpoons$ glycogen.


RATE µMoles / min / mg
0
4
8
12
16
G-I-P (mM)
0
1
2
3
4
5
6
7
$\frac{Pi}{G\text{-}I\text{-}P} = 4$

Isotope exchange studies at equilibrium are particularly suited to the phosphorylase system, since they allow the circumvention of certain technical difficulties encountered when one attempts to measure initial velocities at low substrate concentrations. These technical difficulties (3) are well known to those working in the phosphorylase field, and in this laboratory, initial velocity experiments at low substrate concentrations are designed in such a manner as to minimize these difficulties (28). However, the isotope exchange at equilibrium methods developed by Boyer (33) allow one to work at relatively low substrate concentrations (less than 1 mM), without the inherent measurement problems, since the system is at equilibrium and the rate of incorporation of radioactive label into product can be measured quite accurately. One apparent drawback to the method is that all substrates must be present at once; therefore, any effects observed, such as results obtained in the presence of a modifier, can only be interpreted in a qualitative manner, as compared to initial velocity measurements. However, this apparent drawback may also be an advantage, since the presence of all substrates and modifiers in the system at once most likely approaches _in vivo_ conditions, and hence equilibrium isotope exchange studies may provide one with a starting point in the necessary task of applying our vast stores of enzymic knowledge obtained _in vitro_ to actual enzyme function and regulation _in vivo_.

The application of equilibrium isotope exchange methods to other enzymes exhibiting sigmoidal kinetics should be a useful test for discriminating between conditions which can result in the expression of apparent sigmoidicity; i.e., cooperative "allosteric" effects, or a breakdown in the kinetic mechanism.

B. Summary

In recent years, the apparent sigmoid relationships observed between velocities and substrate concentrations for certain key regulatory enzymes have been explained by several types of cooperative models. These models have involved both allosteric interactions between subunits and multiple binding sites for effectors. However, cooperativity among binding sites is not essential, and models utilizing single, independent active sites with multiple reaction pathways by which these binding sites are filled can also explain sigmoid data.

Certain conditions, nevertheless, would rule out the possibility of a squared term being introduced into the rate equation for a mechanism with multiple reaction pathways, and one of these conditions is that case where the interconversion of ternary complexes is rate limiting (i.e., rapid equilibrium). The method of isotope exchange at equilibrium provides a means of ascertaining whether or not rapid equilibrium conditions do in fact prevail for any given enzyme mechanism. Utilizing this method, it has been shown that for both rabbit muscle glycogen phosphorylases b and a, in the presence of effectors which cause initial velocity versus substrate concentration curves to become sigmoid, the equilibrium isotope exchange rate versus substrate concentration curves are also sigmoid. The fact that both exchange rates were identical would indicate that the kinetic mechanism remains rapid equilibrium random, and hence the sigmoidicity observed is in fact most likely due to cooperativity among effector binding sites.

The application of equilibrium isotope exchange methods to other enzymes exhibiting sigmoidal kinetics should be a useful test for discriminating between conditions which can result in the expression of apparent sigmoidicity; i.e., cooperative "allosteric" effects, or a breakdown in the kinetic mechanism.

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APPENDIX

Published Papers Arising From This Work

1. Engers, H.D. and N.B. Madsen. The Effect of Anions on the Activity of Phosphorylase b. Biochem.Biophys.Res.Comm., 33, 49 (1968).

2. Engers, H.D., W.A. Bridger and N.B. Madsen. The Kinetic Mechanism of Phosphorylase b. J.Biol.Chem., 244, 5936 (1969).

3. Engers, H.D., S. Shechosky and N.B. Madsen. The Kinetic Mechanism of Phosphorylase a. Initial Velocity Studies. Can.J.Biochem. (in press).

4. Engers, H.D., W.A. Bridger and N.B. Madsen. The Kinetic Mechanism of Phosphorylase a. Isotope Exchange Rates at Equilibrium. Can. J.Biochem. (in press).

5. Engers, H.D., W.A. Bridger and N.B. Madsen. Isotope Exchange Studies at Equilibrium as Applied to Allosteric Enzymes. (To be submitted to Biochemistry).