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# A STUDY OF THE ENZYME PYRUVATE CARBOXYLASE FROM *ASPERGILLUS NIGER*

by

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For My Parents

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# ABSTRACT

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The enzyme, pyruvate carboxylase (pyruvate:$CO_2$ ligase (ADP), EC 6.4.1.1), was extracted and purified 18-fold from a citrate accumulating fungus, *Aspergillus niger* A-1-233. Enzyme activity in crude extracts was found to depend on the age of the culture and, if the sucrose concentration in the medium was high (10%), on the species of Aspergillus used. No conclusions as to the possible role of pyruvate carboxylase in citrate accumulation were reached.

A 9-fold purified enzyme from *A. niger* A-1-233 was used for both the physical and kinetic studies. The activity of pyruvate carboxylase from this source was found to be stimulated by $K^+$ ions and inhibited by $Na^+$ ions. It was proportional with time and protein concentration. The enzyme was found to be subject to cold inactivation and subsequent thermal reactivation. Glycerol, in a concentration of 25%, was used for complete protection against cold inactivation. The enzyme was markedly labile at 60° C and 55° C and lost all activity within 18 minutes at 48° C. The pH optimum was determined to be 7.9-8.0. Pyruvate carboxylase from *A. niger* was shown to be a biotin-

containing enzyme by its inactivation by avidin and protection against such inactivation by excess biotin. Acetyl CoA was found to have no effect on enzyme activity.

Apparent and true Michaelis constants were determined for the substrates involved in this reaction; pyruvate, bicarbonate, ATP and $Mg^{++}$. The reaction was inhibited by both mesoxalate and L-aspartate. Product inhibition studies were carried out with each product (oxalacetate, ADP and $P_i$) in combination with every substrate (pyruvate, bicarbonate and ATP). From these kinetic results, a mechanism was proposed for the action of pyruvate carboxylase which involves 3 independently active sites on the enzyme, one for each substrate. The bicarbonate on site II was visualized as swinging from the ATP site (site I) where it was activated to the pyruvate site (site III) where it combined with pyruvate to form oxalacetate. This proposed mechanism explains satisfactorily all the kinetic results obtained in this study.

## ABBREVIATIONS

The following abbreviations have been used:

Acetyl CoA - acetyl coenzyme A

ADP - adenosine-5'-diphosphate

ATP - adenosine-5'-triphosphate

Bicine - N,N-bis(2-hydroxyethyl) glycine

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

LDH - lactate dehydrogenase; L-lactate:NAD oxidoreductase, EC 1.1.1.27

MDH - malate dehydrogenase; L-malate:NAD oxidoreductase, EC 1.1.1.37

MES - 2-(N-morpholino) ethanesulfonic acid

NADH - β-dihydronicotinamide adenine dinucleotide

Tris - Tris(hydroxymethyl)aminomethane

TABLE OF CONTENTS


PAGE

INTRODUCTION .................................... 1

HISTORICAL ...................................... 3

MATERIALS AND METHODS ........................... 19

Cultures ........................................ 19
Maintenance of Cultures ......................... 19
Growth Medium ................................... 20
Preparation of Cell-free Extracts ............... 21
Chemicals ....................................... 22
Enzyme Assays ................................... 23
Purification of Pyruvate Carboxylase ............ 26

RESULTS ......................................... 28

Dependence of Enzyme Level on Culture Age ....... 28
Comparisons of Various Aspergillus Strains ...... 28
Purification of Pyruvate Carboxylase ............ 31
Stability of Pyruvate Carboxylase ............... 31
1. Cold lability and reactivation ........... 31
2. Stabilization against cold inactivation .. 34
3. Effect of freezing ....................... 36
4. Storage of pyruvate carboxylase .......... 38
5. Thermal inactivation ..................... 38
pH Optimum ...................................... 38
Effect of $K^+$ and $Na^+$ Ions on Pyruvate
Carboxylase Activity ........................ 41
Lack of Effect of Acetyl CoA .................... 41
Effect of Avidin ................................ 42
Kinetics of Pyruvate Carboxylase from
*Aspergillus niger* .......................... 44
Determination of Apparent $K_m$ Values for
Reaction Components ......................... 44

TABLE OF CONTENTS (CONT'D)


PAGE

RESULTS (CONT'D)

1. Apparent $K_m$ for pyruvate ................. 45
2. Apparent $K_m$ for bicarbonate ............. 45
3. Apparent $K_m$ for $Mg^{++}$ ..................... 45
4. Apparent $K_m$ for ATP ...................... 45

Determination of True $K_m$ Values for Reaction Components .................................. 50
1. Pyruvate : ATP .......................... 50
2. Pyruvate : bicarbonate .................. 53
3. ATP : bicarbonate ....................... 57

Product Inhibition Studies ...................... 62
1. $P_i$ : ATP ................................ 62
2. $P_i$ : bicarbonate ........................ 63
3. $P_i$ : pyruvate ........................... 63
4. ADP : ATP ............................... 69
5. ADP : bicarbonate ....................... 69
6. ADP : pyruvate .......................... 69
7. Oxalacetate : ATP ....................... 73
8. Oxalacetate : bicarbonate ............... 73
9. Oxalacetate : pyruvate .................. 73

Inhibitor Studies ............................... 73

DISCUSSION ........................................ 80

Role of Pyruvate Carboxylase in Citrate Accumulation ................................ 80
Cold Inactivation and the Structure of Pyruvate Carboxylase ............................... 82
Lack of Effect of Acetyl CoA .................... 83
Inhibition by Avidin and Protection by Biotin ... 84
Kinetics and Mechanism of Pyruvate Carboxylase .. 86

REFERENCES ........................................ 98

INDEX OF TABLES


| TABLE | | PAGE |
|---|---|---|
| I | Dependence of enzyme level in *A. niger* A-1-233 on culture age .................. | 30 |
| II | Comparisons of strains of Aspergillus ... | 30 |
| III | Purification of pyruvate carboxylase from *Aspergillus niger* .................. | 32 |
| IV | Effect of $K^+$ and $Na^+$ concentrations on pyruvate carboxylase activity ........... | 43 |
| V | Effect of avidin and biotin on pyruvate carboxylase activity .................... | 43 |
| VI | Summary of kinetic data for pyruvate carboxylase from *A. niger* ............... | 87 |
| VII | Comparison of Michaelis and inhibitor constants for pyruvate carboxylase from several sources .................... | 88 |

LIST OF FIGURES


| FIGURE | | PAGE |
|---|---|---|
| 1. | Proportionality of pyruvate carboxylase activity with time ..................... | 33 |
| 2. | Proportionality of pyruvate carboxylase activity with protein concentration ..... | 33 |
| 3. | Cold inactivation and reactivation of pyruvate carboxylase and stabilization of enzyme activity by addition of glycerol ............................... | 35 |
| 4. | Effect of freezing and thawing on pyruvate carboxylase activity ........... | 37 |
| 5. | Thermal inactivation of pyruvate carboxylase of _A. niger_ ................ | 39 |
| 6. | The effect of hydrogen ion concentration (pH) on pyruvate carboxylase activity ... | 40 |
| 7. | Apparent $K_m$ for pyruvate ................ | 46 |
| 8. | Apparent $K_m$ for bicarbonate ............ | 47 |
| 9. | Apparent $K_m$ for $Mg^{++}$ .................... | 48 |
| 10. | Apparent $K_m$ for ATP ..................... | 49 |
| 11a. | Double reciprocal plots of velocity versus varying pyruvate concentrations with ATP as fixed variable .............. | 51 |
| 11b. | Double reciprocal plots of velocity versus varying ATP concentrations with pyruvate as fixed variable .............. | 51 |

LIST OF FIGURES (CONT'D)


| FIGURE | | PAGE |
|---|---|---|
| 12a. | Replot of intercepts versus reciprocal of ATP from Fig. 11a in which pyruvate was varying and ATP the fixed variable .. | 52 |
| 12b. | Replot of intercepts versus reciprocal of pyruvate from Fig. 11b in which ATP was varying and pyruvate the fixed variable ................................ | 52 |
| 13. | Double reciprocal plots of velocity versus varying pyruvate concentrations with ATP as fixed variable .............. | 54 |
| 14. | Replot of intercepts versus reciprocal of ATP from Fig. 13 in which pyruvate was varying, ATP the fixed variable and bicarbonate held at 0.9 mM .............. | 54 |
| 15a. | Double reciprocal plots of velocity versus varying pyruvate concentrations with bicarbonate as fixed variable ...... | 55 |
| 15b. | Double reciprocal plots of velocity versus varying bicarbonate concentrations with pyruvate as fixed variable ......... | 55 |
| 16a. | Replot of intercepts versus reciprocal of bicarbonate from Fig. 15a in which pyruvate was varying and bicarbonate the fixed variable ...................... | 56 |
| 16b. | Replot of slopes versus reciprocal of bicarbonate from Fig. 15a in which pyruvate was varying and bicarbonate the fixed variable ...................... | 56 |

LIST OF FIGURES (CONT'D)

PAGE

FIGURE

17a. Replot of intercepts versus reciprocal of pyruvate from Fig. 15b in which bicarbonate was varying and pyruvate the fixed variable ..................... 58

17b. Replot of slopes versus reciprocal of pyruvate from Fig. 15b in which bicarbonate was varying and pyruvate the fixed variable ..................... 58

18a. Double reciprocal plots of velocity versus varying ATP concentrations with bicarbonate as fixed variable ........... 59

18b. Double reciprocal plots of velocity versus varying bicarbonate concentrations with ATP as fixed variable ............. 59

19a. Replot of intercepts versus reciprocal of bicarbonate from Fig. 18a in which ATP was varying and bicarbonate the fixed variable ......................... 60

19b. Replot of slopes versus reciprocal of bicarbonate from Fig. 18a in which ATP was varying and bicarbonate the fixed variable ................................ 60

20a. Replot of intercepts versus reciprocal of ATP from Fig. 18b in which bicarbonate was varying and ATP the fixed variable ......................... 61

20b. Replot of slopes versus reciprocal of ATP from Fig. 18b in which bicarbonate was varying and ATP the fixed variable .. 61

LIST OF FIGURES (CONT'D)


PAGE

FIGURE

21. Double reciprocal plots of velocity versus varying ATP concentrations with $P_i$ as the fixed variable product ........ 64

22a. Replot of intercepts versus $P_i$ concentration from Fig. 21 in which ATP was varying and $P_i$ the fixed variable product ................................ 65

22b. Replot of slopes versus $P_i$ concentration from Fig. 21 in which ATP was varying and $P_i$ the fixed variable product ....... 65

23. Double reciprocal plots of velocity versus varying bicarbonate concentrations with $P_i$ as the fixed variable product ... 66

24a. Replot of intercepts versus $P_i$ concentration from Fig. 23 in which bicarbonate was varying and $P_i$ the fixed variable product .................. 67

24b. Replot of slopes versus $P_i$ concentration from Fig. 23 in which bicarbonate was varying and $P_i$ the fixed variable product ................................ 67

25. Double reciprocal plots of velocity versus varying pyruvate concentrations with $P_i$ as the fixed variable product ... 68

26. Replot of intercepts versus $P_i$ concentration from Fig. 25 in which pyruvate was varying and $P_i$ the fixed variable product ....................... 68

27. Double reciprocal plots of velocity

LIST OF FIGURES (CONT'D)


| FIGURE | | PAGE |
|---|---|---|
| | versus varying ATP concentrations with ADP as the fixed variable product ....... | 70 |
| 28. | Replot of slopes versus ADP concentration from Fig. 27 in which ATP was varying and ADP the fixed variable product ...... | 70 |
| 29. | Double reciprocal plots of velocity versus varying bicarbonate concentrations with ADP as the fixed variable product .. | 71 |
| 30. | Replot of intercepts versus ADP concentration from Fig. 29 in which bicarbonate was varying and ADP the fixed variable product .................. | 71 |
| 31. | Double reciprocal plots of velocity versus varying pyruvate concentrations with ADP as the fixed variable product .. | 72 |
| 32. | Replot of intercepts versus ADP concentration from Fig. 31 in which pyruvate was varying and ADP the fixed variable product ........................ | 72 |
| 33. | Double reciprocal plots of velocity versus varying ATP concentrations with oxalacetate as the fixed variable product ................................ | 74 |
| 34. | Replot of intercepts versus oxalacetate concentration from Fig. 33 in which ATP was varying and oxalacetate the fixed variable product ........................ | 74 |
| 35. | Double reciprocal plots of velocity versus varying bicarbonate concentrations | |

LIST OF FIGURES (CONT'D)


| FIGURE | | PAGE |
|---|---|---|
| | with oxalacetate as the fixed variable product ........ | 75 |
| 36. | Replot of slopes versus oxalacetate concentration from Fig. 35 in which bicarbonate was varying and oxalacetate the fixed variable product ........ | 75 |
| 37. | Double reciprocal plots of velocity versus varying pyruvate concentrations with oxalacetate as the fixed variable product ........ | 76 |
| 38. | Replot of slopes versus oxalacetate concentration from Fig. 37 in which pyruvate was varying and oxalacetate the fixed variable product ........ | 76 |
| 39. | Double reciprocal plots of velocity versus varying pyruvate concentrations with mesoxalate as the fixed variable inhibitor ........ | 78 |
| 40. | Double reciprocal plots of velocity versus varying pyruvate concentrations with L-aspartate as the fixed variable inhibitor ........ | 79 |
| 41. | Diagram proposed to illustrate the mechanism of action of pyruvate carboxylase from *A. niger* ........ | 93 |

# INTRODUCTION

# INTRODUCTION

The ability of certain strains of Aspergillus to produce citric acid is a well-known fact (Karow and Waksman, 1947; Shu and Johnson, 1948). The importance of the composition of the culture medium has also been reported. The additions of certain metal ions in strictly controlled concentrations at various times (Shu and Johnson, 1947) and of high concentrations of sucrose (Shu and Johnson, 1948) have been found to stimulate citric acid accumulation. Attempts have been made to determine the mechanism of citrate formation and accumulation in this organism by studying the levels of the various Krebs cycle enzymes (Ramakrishnan et al., 1955; la Nauze, 1966).

The enzyme, pyruvate carboxylase, catalyzes the formation of oxalacetate by the direct carboxylation of pyruvate with energy supplied by the breakdown of ATP to ADP and $P_i$. The possibility that it might play a role in citrate accumulation resulted in the initiation of this study. It was thought that perhaps the accumulation of citrate by certain strains of Aspergillus niger might be due, at least in part, to an increase in the activity of

this enzyme.

It was thought to be of interest to try to determine the mechanism of action of pyruvate carboxylase. To this end, a complete kinetic study was done. An advantage of working with the enzyme from _A. niger_ was that acetyl CoA has no effect on its activity. With some of the other reported sources, acetyl CoA has been found to be either absolutely required or stimulatory. By using this source, however, one variable has been eliminated. The reaction was found not to be a simple one involving only one active site. Rather, it was postulated from the results that 3 active sites, one for each substrate, were probably present on the enzyme surface. There have been no reports of kinetic studies on enzymes which have more than one active centre. Attempts made in this study to determine rate equations for such a complex system proved to be unsuccessful. However, it was possible to apply the analytical principles of Cleland (1963c) in order to elucidate the reaction mechanism.

# HISTORICAL

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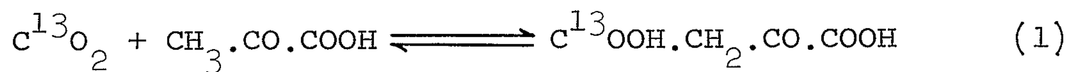
The direct carboxylation of a 3-carbon compound, pyruvate, to yield a 4-carbon compound, oxalacetate, was first postulated by Wood and Werkman in 1938. Earlier experiments (Wood and Werkman, 1936) had shown for the first time that $CO_2$ was fixed by heterotrophic non-photosynthetic bacteria in the case of the fermentation of glycerol by the propionic acid bacteria. The mechanism of its utilization was not clear at that time however. The results obtained in 1938 led these workers to suggest that succinic acid was formed from a 3-carbon compound through the addition of $CO_2$. They had found that succinic acid formed and $CO_2$ utilized were approximately equimolar and that in the absence of $CO_2$, little or no succinic acid was formed. They felt that $CO_2$ utilization might in some way be involved in the synthesis of citrate by its condensation with a 3-carbon compound to form oxalacetate.

In 1940, in a continuation of their studies into bacterial $CO_2$ fixation, Wood and Werkman postulated that $CO_2$ utilization involved phosphopyruvic acid. The ability of NaF to inhibit both $CO_2$ utilization and phosphoglycerate

fermentation indicated that $CO_2$ fixation had a close connection with phosphorylation. This assumption was logical since the high energy content of phosphorylated compounds could have been the source of energy for $CO_2$ uptake.

Krampitz *et al*. (1943), working with *Micrococcus lysodeikticus*, reported that the reaction catalyzed by the enzyme, oxalacetate β-carboxylase, was reversible and that the enzyme could induce the incorporation of $CO_2$ into oxalacetate according to reaction (1):



$$C^{13}O_2 + CH_3.CO.COOH \rightleftharpoons C^{13}OOH.CH_2.CO.COOH \qquad (1)$$

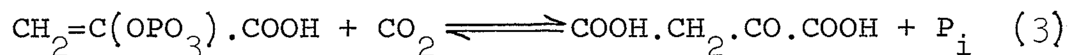
Despite evidence that $CO_2$ fixation occurred in animal tissues, direct demonstration of the reaction in these preparations was not accomplished. It was just assumed that such a mechanism was involved. However, in 1946, Utter and Wood demonstrated that the decarboxylation of oxalacetate by a pigeon liver extract was reversible when ATP was added to the enzyme. The function of ATP was thought either to be stimulation through phosphorylation of a cofactor of the reaction or stimulation by direct participation of ATP in the reaction. Their results again suggested that a high-

energy derivative might be involved in the reaction. This contention was supported by Wood in his review of $CO_2$ fixation (1946).

The hypothesis of the direct carboxylation of pyruvate to oxalacetate, as postulated by Wood and Werkman, assumed less significance at this time since there was no conclusive enzymatic evidence and also since alternate pathways for the formation of dicarboxylic acids were found by various enzyme studies. One of these enzymes was the NADP-specific malic enzyme which catalyzed reaction (2):

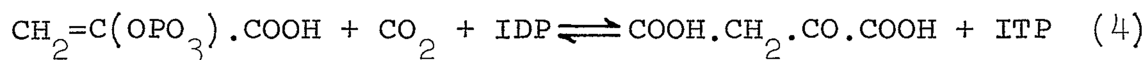
$$COOH.CH_2.CHOH.COOH \underset{}{\overset{NADP \quad NADPH + H^+}{\rightleftharpoons}} CH_3.CO.COOH + CO_2 \quad (2)$$

This enzyme was discovered by Ochoa *et al*. (1948). Bandurski and Greiner (1953) first reported the existence of another such enzyme, phosphoenolpyruvate carboxylase, which catalyzed reaction (3):



$$CH_2{=}C(OPO_3).COOH + CO_2 \rightleftharpoons COOH.CH_2.CO.COOH + P_i \quad (3)$$

A third enzyme which was found to catalyze the formation of a dicarboxylic acid was oxalacetate carboxylase (phosphoenol-pyruvate carboxykinase) (reaction (4)):



$$CH_2{=}C(OPO_3).COOH + CO_2 + IDP \rightleftharpoons COOH.CH_2.CO.COOH + ITP \quad (4)$$

This enzyme was first isolated by Utter and Kurahashi (1954).

A direct carboxylation of pyruvate was postulated for a reaction catalyzed by a crude enzyme system isolated from Escherichia coli and Proteus morganii (Kaltenbach and Kalnitsky, 1951a). The reaction required high concentrations of bicarbonate. A requirement for ATP could not be demonstrated (Kaltenbach and Kalnitsky, 1951b) as had been found by Utter and Wood (1946). However, a marked stimulation was observed with $P_i$ in a concentration of 90 mM.

Then it was reported that extracts prepared from Aspergillus niger could form aspartate and malate from pyruvate, $CO_2$ and ATP (Woronick and Johnson, 1960). These workers felt that this was a new system and that it probably yielded oxalacetate as an initial product. At the same time, Utter and Keech (1960) published evidence for the occurrence of a similar enzyme system in avian and beef liver. It was postulated to catalyze the following reaction (5):

$$CH_3.CO.COOH + CO_2 + ATP \rightleftharpoons COOH.CH_2.CO.COOH + ADP + P_i \quad (5)$$

The nature of the reaction was first described in 1963 by Utter and Keech. The enzyme was tentatively given the trivial name "pyruvate carboxylase" and has since been classified systematically as pyruvate:$CO_2$ ligase (ADP), EC 6.4.1.1. Although most of the work done on the enzyme has made use of chicken liver as the source, pyruvate carboxylase has also been isolated from *Pseudomonas citronellolis* (Seubert and Remberger, 1961), *Aspergillus niger* (Bloom and Johnson, 1962), rat liver (Henning and Seubert, 1964), baker's yeast (Ruiz-Amil *et al.*, 1965), sheep kidney (Ling and Keech, 1966) and recently from mammalian brain cortex (Felicioli *et al.*, 1967).

No assumptions have been made as yet as to the active species of $CO_2$ involved in the reaction since there is no evidence available at present on this question (Keech and Utter, 1963).

In 1960, when Woronick and Johnson first observed a reaction catalyzed by cell-free extracts of *A. niger* which resulted in the formation of oxalacetate from pyruvate and ATP, they found it was stimulated 1.5-fold by addition of 100 μmoles $K^+$. Bloom and Johnson (1962) reported a 4-fold stimulation of *A. niger* pyruvate carboxylase activity by

100 μmoles KCl. The yeast enzyme was also found to be stimulated by $K^+$ ions whereas it was inhibited by $Na^+$ ions (Ruiz-Amil *et al.*, 1965).

Magnesium has been found to be necessary for the reaction independent of the source of the enzyme (Utter and Keech, 1960; Seubert and Remberger, 1961; Bloom and Johnson, 1962; Henning and Seubert, 1964; Ruiz-Amil *et al.*, 1965; Ling and Keech, 1966). It has been found, in the case of the chicken liver enzyme (Scrutton *et al.*, 1966), that manganese can replace magnesium in the reaction. Recently, using kinetic methods, Keech and Barritt (1967) have investigated the role of $Mg^{++}$ in the pyruvate carboxylase reaction of sheep kidney. Their results have led them to suggest that $Mg^{++}$ might play at least 2 roles in the reaction mechanism; (a) to form a complex with ATP yielding $MgATP^{=}$ which is the true substrate and (b) to form a complex with the enzyme resulting in activation of the reaction. The kinetics for the $MgATP^{=}$ complex indicated a homotropic cooperative effect involving the binding of at least 2 molecules of ligand. Two possible binding mechanisms for $Mg^{++}$ and $MgATP^{=}$ have been postulated. The $Mg^{++}$ ion could bind to a specific effector site. In this case, there

would be 2 interdependent $MgATP^{=}$-binding sites. Alternatively, $Mg^{++}$ could bind at one of the $MgATP^{=}$-binding sites which is capable of functioning as an effector site. As yet, no choice has been made between these 2 postulates.

The cold lability of pyruvate carboxylase was first reported by Utter et al. (1964), working with the chicken liver enzyme. In the absence of any protective agents, partial reactivation was obtained by simple rewarming to 23° C. This reactivation was rapid and its extent varied somewhat with the experiment. Higher temperatures did not increase the degree of reactivation. Later research with pyruvate carboxylase from yeast (Ruiz-Amil et al., 1965) and from sheep kidney (Ling and Keech, 1966) demonstrated the cold lability of the enzyme from these sources as well.

Research was carried out to find means of stabilizing the enzyme against cold inactivation. Utter et al. (1964) found that pyruvate carboxylase was protected by high concentrations of sucrose (1.5 M) and to a lesser degree by combinations of some of the reaction components. The yeast enzyme has been stabilized by addition of 1.0 M sucrose or 45% saturated $(NH_4)_2SO_4$ (Ruiz-Amil et al., 1965) and the sheep kidney enzyme by 1.5 M sucrose (Ling and Keech, 1966).

Studies with pyruvate carboxylase from chicken liver (Scrutton and Utter, 1965a) showed that the enzyme had a molecular weight of approximately 660,000. Activity was found to be associated with the 14.8S band which was the major component. There was also a slower-sedimenting 6.75S band of a minor component. Cold inactivation was accompanied by a transfer of protein from the 15S to the 7S peak. Thus, it appeared that the loss of activity was due to the dissociation of subunits. In 1966, Valentine *et al*. presented very convincing electron micrographs of molecules of pyruvate carboxylase which showed 4 subunits at the corners of a square. The result of cold inactivation was the dissociation of these subunits, each of which had an assumed molecular weight of 165,000. The result of subsequent thermal reactivation was the reformation of the intact molecule.

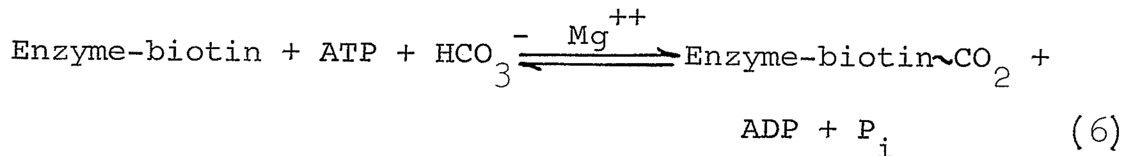
Avidin inhibition has been verified for the pyruvate carboxylase from all sources thus far studied. This inhibition is almost complete in all cases and can be prevented by preincubation of the avidin with biotin. These results demonstrated that pyruvate carboxylase is a biotin-containing enzyme. The fact that this vitamin had a

function in $CO_2$ fixation had long been postulated since it had been observed in many systems that $CO_2$ fixation was decreased in biotin deficiencies (Ochoa and Kaziro, 1961).

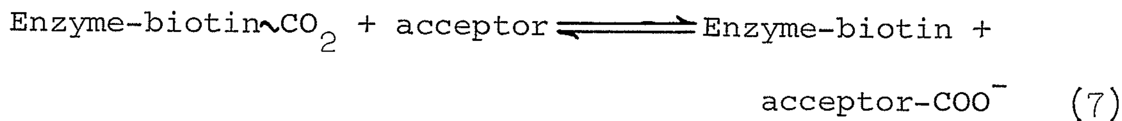
Lardy *et al*. (1947), working with *Lactobacillus arabinosus*, having suggested again that the synthesis of oxalacetate might occur by a condensation of pyruvate and $CO_2$, reported that bicarbonate elicited no appreciable growth response in a biotin-low medium. However, in the presence of biotin it greatly stimulated growth. The results of Shive and Rogers (1947) indicated that biotin functioned in yeast in the carboxylation of pyruvate to form oxalacetate.

More recent enzyme studies have showed that many carboxylases are biotin-containing enzymes. In this respect, pyruvate carboxylase is similar to propionyl carboxylase (Kaziro and Ochoa, 1961), acetyl CoA carboxylase (Waite and Wakil, 1962) and to the transcarboxylase, methylmalonyl-oxalacetic transcarboxylase (Wood *et al*., 1963). Also, they all appear to be composed of 4 subunits.

The general mechanism for all biotin-containing carboxylases as proposed by Ochoa and Kaziro (1961) has been stated as follows (reactions (6) and (7)):



$$\text{Enzyme-biotin} + \text{ATP} + HCO_3^- \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \text{Enzyme-biotin}{\sim}CO_2 + \text{ADP} + P_i \qquad (6)$$



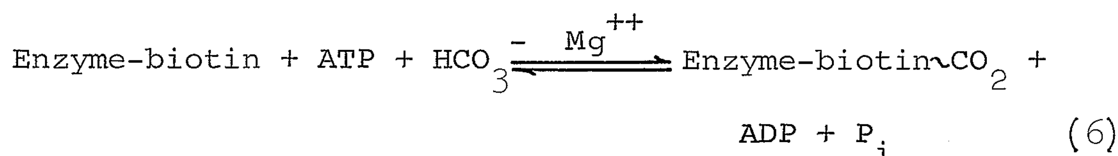
$$\text{Enzyme-biotin}{\sim}CO_2 + \text{acceptor} \rightleftharpoons \text{Enzyme-biotin} + \text{acceptor-}COO^- \qquad (7)$$

From microbiological assay studies with _Lactobacillus arabinosus_, Scrutton and Utter (1965a) have determined that there are approximately 4 moles of biotin bound per mole of pyruvate carboxylase. Thus, each subunit of the enzyme could be assumed to contain one biotin site. This biotin is not free, but rather is bound to the enzyme from which it can be released by hydrolysis. It has been postulated that the linkage is covalent in nature through the carboxyl group of its valeric acid side chain to the $\epsilon$-amino group of lysine (Ochoa and Kaziro, 1961). Green (1963) postulated that the biotin must be mobile enough to permit its movement between the 2 active centres; the ATP-$Mg^{++}$-$HCO_3^-$ centre and the substrate-carboxylating centre. Mildvan _et al_. (1966) have made use of this postulate and have suggested that the biotin ring is on a long, flexible arm.

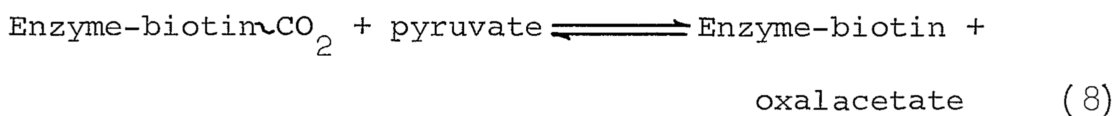
In 1966, Scrutton _et al_. reported the presence of tightly bound manganese in the pyruvate carboxylase of

chicken liver. No definite evidence was presented as to the valence state of manganese when bound to the enzyme. They did, however, determine the metal content to be 2.5-4.3 moles of Mn per mole of enzyme or 0.6-1.1 moles of Mn per mole of biotin. This is not the same form of the metal that can replace $Mg^{++}$ and that is necessary for enzyme activity. In this latter case, it is readily dissociable.

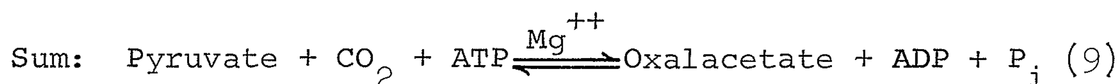
According to the general mechanism proposed for carboxylases by Ochoa and Kaziro (1961), the reaction mechanism for pyruvate carboxylase may be written in 2 steps (reactions (6) (as described previously) and (8)):



$$\text{Enzyme-biotin} + \text{ATP} + \text{HCO}_3^- \xrightleftharpoons{Mg^{++}} \text{Enzyme-biotin}{\sim}\text{CO}_2 + \text{ADP} + \text{P}_i \quad (6)$$



$$\text{Enzyme-biotin}{\sim}\text{CO}_2 + \text{pyruvate} \rightleftharpoons \text{Enzyme-biotin} + \text{oxalacetate} \quad (8)$$



$$\text{Sum:} \quad \text{Pyruvate} + \text{CO}_2 + \text{ATP} \xrightleftharpoons{Mg^{++}} \text{Oxalacetate} + \text{ADP} + \text{P}_i \quad (9)$$

The overall reaction is given in (9). The results obtained by Mildvan *et al*. (1966) support the hypothesis that the bound Mn plays a functional role in the transcarboxylation part of the reaction; ie. the transfer of a carboxyl group

from the enzyme-biotin$\sim CO_2$ complex to pyruvate (reaction (8)). If this is the case, then the locus of action of the bound metal ion differs from that of the added divalent metal ion which participates in the formation of the enzyme-biotin$\sim CO_2$ intermediate from bicarbonate and ATP (reaction (6)). This group has proposed that the pyruvate is held at the enzyme surface through 2 bonds with the bound manganese.

A striking feature of pyruvate carboxylase is the variability of effect of acetyl CoA. In 1960, research showed that the pyruvate carboxylase of chicken liver had an absolute requirement for acetyl CoA that could not be replaced by any other compound or combination of compounds. The indirect role of acetyl CoA was confirmed. Its function was not that of an initial $CO_2$ acceptor, as shown by the fact that labelled acetyl CoA was not incorporated into oxalacetate (Utter and Keech, 1960). Later work with a more purified form of the enzyme verified these results (Scrutton *et al*., 1965).

Work has since been done on the effect of acetyl CoA on pyruvate carboxylase from a variety of different sources. The reaction in sheep kidney has been shown to share the absolute requirement for acetyl CoA demonstrated by the

chicken liver enzyme (Ling and Keech, 1966). The reaction in rat liver (Henning and Seubert, 1964) and baker's yeast (Ruiz-Amil et al., 1965; Cooper and Benedict, 1966) is stimulated by acetyl CoA. Pyruvate carboxylase from Ps. citronellolis (Seubert and Remberger, 1961) and from A. niger (Bloom and Johnson, 1962) is not affected by addition of acetyl CoA.

Ultracentrifugal studies have showed that the presence of acetyl CoA has no effect on the sedimentation rates of the 2 peaks (14.8S and 6.75S) or on the distribution of protein between the 2 fractions (Utter et al., 1964). Thus, the action of acetyl CoA is not to cause aggregation of subunits into an active molecule. Its effect has to be more subtle; perhaps involving changes in the conformation of the protein molecule.

More recently, Scrutton et al. (1965) have showed that acetyl CoA is required for the carboxylation step (reaction (6)) but not the transcarboxylation step (reaction (8)) (Scrutton and Utter, 1964; Gailiusis et al., 1964). The activation by acetyl CoA was thus localized in the formation of the enzyme-biotin$\sim CO_2$ intermediate from ATP and bicarbonate.

Cooper and Benedict (1966), working with the yeast enzyme, have found that the addition of acetyl CoA results in a change in the tertiary structure of the protein and causes a lower $K_m$ value for bicarbonate. Recently, results for the sheep kidney enzyme have indicated that the kinetics observed with acetyl CoA are more complex than previously assumed (Barritt et al., 1966). These authors suggested that acetyl CoA activation of the enzyme from this source is an allosteric effect involving homotropic cooperative interaction between at least 2 activating sites.

Some work has been done on the effect of sulfhydryl inhibitors on pyruvate carboxylase. Keech and Utter (1963) found that N-ethylmaleimide, iodoacetate and iodoacetamide were all virtually without effect. Mercuric salts, such as $HgCl_2$, and $\rho$-chloromercuribenzoate were found to be powerful inhibitors. Ling and Keech (1966) reported 45% inhibition by $\rho$-chloromercuribenzoate and 47% inhibition by iodoacetate. In the latter case, the difference with the results of Keech and Utter (1963) were perhaps due to the fact that they used a more concentrated solution and incubated it with the enzyme for a longer period of time. Keech and Utter (1963) have suggested that free sulfhydryl

groups are essential for activity although they have never been able to demonstrate increased stability or activity of the enzyme in the presence of reduced glutathione. On the other hand, Ling and Keech (1966) have suggested that since there is a slow rate of inactivation, free sulfhydryl groups are not necessary for activity but possibly the inhibitor is reacting at a distance from the active centre or that it is acting on another type of group, such as a free amino or hydroxyl group.

The work with the chicken liver enzyme was begun in order to investigate a mechanism for the formation of phosphoenolpyruvate (Utter and Keech, 1963). Liver extracts do not contain enzymes for the direct conversion of pyruvate to phosphoenolpyruvate. Since the latter can be synthesized from oxalacetate, the possibility that there was an enzyme to carboxylate pyruvate directly to oxalacetate was studied.

The enzyme, phosphoenolpyruvate carboxykinase (Utter and Kurahashi, 1954), has been found to be closely associated with pyruvate carboxylase in certain tissues. This association has led to the suggestion that a combination of the 2 reactions catalyzed by these enzymes

represented a major pathway for the synthesis of phosphoenolpyruvate from pyruvate during gluconeogenesis (Keech and Utter, 1963).

In 1964, it was found that there was a simultaneous rise in glycogen and pyruvate carboxylase activity within 6 hours in livers of cortisol-treated rats (Henning _et al_., 1964). In the same year, a similar rise in levels of pyruvate carboxylase was reported in the livers of alloxan diabetic rats (Wagle, 1964). Further studies by Henning _et al_. (1966) showed again that diabetes, cortisol and starvation all increase pyruvate carboxylase activity. The evidence presented suggests that the physiological significance of pyruvate carboxylase, in conjunction with phosphoenolpyruvate carboxykinase, is to act as pacemaker of gluconeogenesis.

# MATERIALS AND METHODS

## Cultures

A citrate accumulating strain of Aspergillus, *Aspergillus niger* NRC A-1-233, was used throughout this study. *A. niger* NRC A-1-215, another citrate accumulating strain, two non-accumulating strains of *Aspergillus flavus*, NRC A-2-74 and NRC A-2-88, and *Aspergillus ustis* NRC A-7-7 were used for comparison studies. All these organisms were obtained from the National Research Council, Division of Biosciences, Ottawa, Ontario.

## Maintenance of Cultures

All stock cultures were routinely maintained on slants of Brain Heart Infusion Agar (Baltimore Biological Laboratory). Subculturing was carried out approximately every month. Luxuriant growth was obtained after 48 hours incubation at $28^{\circ}$ C in the case of *A. niger* and after 72 hours in the cases of *A. flavus* and *A. ustis*. The slants were then stored at $4^{\circ}$ C.

Growth Medium

The liquid medium was a modification of the medium described by Marshall and Alexander (1962). It was prepared in four parts. Part A contained: sucrose, 8.8 g; $(NH_4)_2PO_4$, 1.65 g; $FeSO_4.7H_2O$, 0.001 g; $MnSO_4.4H_2O$, 0.001 g; and distilled water, 300 ml. Part B contained: $MgSO_4.7H_2O$, 0.2 g; and distilled water, 100 ml. Part C contained: $CaCl_2.2H_2O$, 0.1 g; and distilled water, 100 ml. Part D contained: $K_2HPO_4$, 0.1 g; $Na_2HPO_4.7H_2O$, 28.8 g; $NaH_2PO_4$, 13.9 g; and distilled water, 500 ml. The pH of Part D was adjusted to 6.6-6.8.

The four solutions were autoclaved separately at $121^{\circ}$ C for 20 minutes, then mixed aseptically after having been cooled to room temperature.

A "high sucrose" medium was prepared in a similar manner using 10% sucrose (100g/l).

For inoculation into the liquid medium, the conidia were washed from the slants with sterile distilled water. Five millilitres of a heavy suspension were transferred aseptically into 500 ml of liquid medium in 2-litre Erlenmeyer flasks.

The fungi were grown on a New Brunswick rotary shaker at 180 revolutions per minute at $28^{\circ}$ C. The *A. niger* cultures were incubated for 48 hours and the *A. flavus* and *A. ustis* cultures for 72 hours.

Preparation of Cell-free Extracts

All cultures were harvested on four layers of cheesecloth. The mycelia were washed with sufficient dilute KOH to bring the pH to neutrality and then with approximately one litre of ice-cold glass-distilled water. The mycelial mat was pressed dry and frozen until required.

All subsequent procedures were carried out at $0\text{-}3^{\circ}$ C. The mycelia were thawed and the nearly dry mass was extracted in the ratio of 1 g to 5 ml of cold 0.1 M Tris-HCl buffer (pH 7.9) to which had been added 25% glycerol. Disruption was achieved by grinding at approximately 240 revolutions per minute with a tissue homogenizer (Bellco), the pestle of which had been attached to an Eberbach Zero-Max motor. Satisfactory breakage was obtained after 10 minutes. Debris was removed by centrifugation in an RC-2 Sorvall centrifuge at 27,000 x g for 15 minutes. The creamy opaque supernatant solution was used for assays and further

purification.

Chemicals

The following chemicals were obtained from the Sigma Chemical Company:

Pyruvic acid, sodium salt, Type III

β-Diphosphopyridine Nucleotide, reduced form (β-Dihydronicotinamide Adenine Dinucleotide; β-DPNH; β-NADH) from yeast, disodium salt, Grade III

S-acetyl Coenzyme A, sodium salt, Grade II

Adenosine-5'-Triphosphate (ATP) from equine muscle, disodium salt

Adenosine-5'-Diphosphate (ADP) from equine muscle, sodium salt, Grade I

Oxalacetic acid

L-aspartic acid

D-aspartic acid, crystalline

The following chemicals were obtained from Nutritional Biochemicals Corporation:

Avidin, 2273 units/g

D-biotin (Vitamin H), crystalline

meso Oxalic acid

The following chemicals were obtained from Calbiochem:

Protamine sulphate (salmine)

2-(N-morpholino) ethanesulfonic acid (MES), A grade

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), A grade

N,N-bis(2-hydroxyethyl) glycine (bicine), A grade

The following chemical was obtained from Boehringer Mannheim Corporation:

Malate dehydrogenase (MDH) from pig heart, analytical reagent grade, specific activity approximately 720 units/mg

## Enzyme Assays

The conversion of pyruvate, $CO_2$ and ATP to oxalacetate, ADP and $P_i$ as catalyzed by pyruvate carboxylase (pyruvate:$CO_2$ ligase (ADP), EC 6.4.1.1) was followed spectrophotometrically by a modification of the method of Utter and Keech (1963). The rate of decrease in optical density was measured at 340 mμ with a Model 2000 Gilford recording spectrophotometer. Successive readings on any single cuvette were made at 13-second intervals.

Reaction mixtures routinely contained: Tris-HCl buffer (pH 7.9), 50 mM; sodium pyruvate, 5 mM; $NaHCO_3$, 5 mM; $MgCl_2.6H_2O$, 3.3 mM; ATP, 1.25 mM; KCl, 33.3 mM; NADH, 0.12 mM; MDH, 1 unit; diluted enzyme, 0.1 ml; and distilled water to a final volume of 3.0 ml. Cuvettes with a 10-mm light path were used. The reaction was initiated by addition of the enzyme and proceeded at room temperature. Under these conditions, the reaction was linear for approximately 3 minutes.

For the determination of NADH oxidase, the usual reaction mixture from which ATP, pyruvate and bicarbonate were omitted was used. The usual reaction mixture from which ATP and bicarbonate had been omitted was used for the determination of LDH activity.

For the product inhibition studies with oxalacetic acid, a somewhat different procedure was used, the difficulty being that any oxalacetate added would immediately be trapped by the NADH-MDH coupling system.

Reaction mixtures which contained all the components with the exception of NADH, and included varied concentrations of oxalacetate were prepared. The reaction was initiated by the addition of enzyme and was allowed to

incubate at room temperature for 5 minutes. Pyruvate carboxylase activity was then stopped by addition of oxalic acid in a final concentration of 10 mM (Ruiz-Amil et al., 1965). Oxalate had no effect on MDH activity but inhibited pyruvate carboxylase completely. Then sufficient NADH was added to reduce all the oxalacetate present. This reaction ran to completion in 5-8 minutes.

Residual NADH was calculated from optical density readings made at 340 mμ on a Unicam SP. 700 spectrophotometer using cuvettes of 10-mm light path. From this, knowing the amount of oxalacetate added, the quantity of oxalacetate formed in the reaction could be determined.

It was of interest to calculate the endogenous bicarbonate concentration of the reaction mixture since, at the pH used, it was not possible to eliminate it by simple gassing and evacuating procedures. To accomplish this, the components, with the exception of added bicarbonate, were mixed in a sealed cuvette. A final concentration of 1 mM NADH was used. Initial absorbance was measured at 375 mμ on the Unicam SP. 700 spectrophotometer. The reaction was initiated by the addition of enzyme and allowed to proceed to completion. During the incubation period of approximately

2 1/2 hours at room temperature the cuvette was shaken intermittently. From initial and final optical density readings, the NADH consumed, and from this, endogenous bicarbonate present, was calculated.

One unit of pyruvate carboxylase activity was defined as the amount of enzyme which caused a decrease in optical density of 0.001 per minute at 340 mμ. One unit of activity of NADH oxidase or LDH was also defined as that amount of enzyme which resulted in a decrease in optical density of 0.001 per minute at 340 mμ. Specific activity was expressed per mg protein. Protein was determined by the spectrophotometric method of Warburg and Christian as described by Layne (1957).

## Purification of Pyruvate Carboxylase

All subsequent steps were carried out at 0-4$^{\circ}$ C. To the crude extract which contained approximately 20 mg protein per ml was added a 2% solution of protamine sulphate in the proportion of 0.1 mg protamine sulphate per mg protein. The mixture was stirred for 10 minutes and centrifuged for 20 minutes at 27,000 x g. The precipitate was discarded. Solid ammonium sulphate was added to the

supernatant solution to bring it to 65% saturation. This mixture was stirred for 15 minutes, centrifuged at 27,000 x g for 20 minutes and the supernatant fraction discarded. The precipitate was suspended in a solution of 50% saturated $(NH_4)_2SO_4$, stirred for 10 minutes and centrifuged for 20 minutes at 27,000 x g. The resulting precipitate was suspended in a 40% saturated $(NH_4)_2SO_4$ solution, allowed to stir for 10 minutes and centrifuged at 27,000 x g for 20 minutes. When this precipitate was suspended in a 30% saturated $(NH_4)_2SO_4$ solution, pyruvate carboxylase remained soluble. An outline of the purification procedure is given in Table III.

After the 65% $(NH_4)_2SO_4$ step, the enzyme preparation was virtually free of NADH oxidase and LDH. The level of MDH, however, throughout all purification steps remained very high, making a study of the reverse reaction impossible.

The 65% ammonium sulphate precipitate was the enzyme form used for all kinetic and physical studies.

# RESULTS

# RESULTS

## Dependence of Enzyme Level on Culture Age

*Aspergillus niger* A-1-233 was grown under the standard conditions for either 2 or 4 days. The mycelia were then harvested and crude extracts prepared. The protein concentration was determined for each extract and assays were made for pyruvate carboxylase and NADH oxidase. The results are summarized in Table I. Although the protein concentration was higher in the 4-day culture, the pyruvate carboxylase level was less than 17% of that in the 2-day culture. The level of NADH oxidase dropped by 50% in the 4-day culture.

## Comparisons of Various Aspergillus Strains

The five strains of Aspergillus; *A. niger* A-1-215 and A-1-233; *A. flavus* A-2-74 and A-2-88; and *A. ustis* A-7-7; were grown in each of the normal sucrose and "high sucrose" media which had been dispensed in 200-ml amounts into 500-ml Erlenmeyer flasks. The *A. niger* cultures were incubated with shaking at 28$^{o}$ C for 48 hours and the *A. flavus* and *A. ustis* cultures for 72 hours. The pH of the medium after

growth was measured. Crude extracts of all samples were prepared. Measurements were made of protein concentration and activities of pyruvate carboxylase, NADH oxidase and LDH. The results of this study are presented in Table II.

It was noted that the pH of the "high sucrose" medium after growth, in the case of the 2 citrate accumulators (the A. niger strains), was lower than in any of the other samples. In his review of citric acid production, von Loesecke (1945) reported that a high sucrose concentration resulted in greater yields of citric acid. Shu and Johnson (1948) found that sucrose concentrations of 14.5 - 26.4% resulted in high citrate accumulation. Lower yields were obtained with a concentration of 6.8% sucrose. This lower pH then, was probably the result of increased citric acid accumulation. The non-accumulating strains of A. flavus and A. ustis showed no marked variation in pH with sucrose concentration.

Among the 3 citrate non-accumulating strains, there was very little difference in the level of pyruvate carboxylase whether the organism had been grown in low or high sucrose concentrations. With the 2 citrate accumulating strains of A. niger, however, the level of pyruvate

Table I

Dependence of enzyme level in *A. niger* A-1-233 on culture age.

| Culture Age | Protein Concentration (mg/ml) | Pyruvate Carboxylase Activity (units/mg) | NADH Oxidase Activity (units/mg) |
|---|---|---|---|
| 2 days | 22.7 | 840 | 60 |
| 4 days | 36.9 | 140 | 30 |

Table II

Comparisons of strains of Aspergillus.

| Strain | Sucrose (%) | pH of Medium | Protein (mg/ml) | Pyruvate Carboxylase Activity | NADH Oxidase Activity | LDH Activity |
|---|---|---|---|---|---|---|
| | | | | (activity in units/mg) | | |
| *A. niger* | | | | | | |
| A-1-215 | 0.88 | 6.3 | 28.7 | 303 | 24 | 14 |
| | 10.0 | 5.7 | 21.2 | 28 | 24 | - |
| A-1-233 | 0.88 | 6.2 | 24.7 | 231 | 32 | 28 |
| | 10.0 | 5.8 | 17.7 | 17 | 22 | 73 |
| *A. flavus* | | | | | | |
| A-2-74 | 0.88 | 6.5 | 8.9 | 216 | 352 | - |
| | 10.0 | 6.2 | 12.1 | 289 | 107 | 58 |
| A-2-88 | 0.88 | 6.6 | 12.7 | 121 | 102 | 64 |
| | 10.0 | 6.4 | 23.1 | 163 | 45 | 13 |
| *A. ustis* | | | | | | |
| A-7-7 | 0.88 | 6.6 | 12.7 | 197 | 110 | 197 |
| | 10.0 | 6.5 | 14.6 | 185 | 73 | 73 |

carboxylase in the presence of 10% sucrose was only 10% of the level when the organism had been grown in 0.88% sucrose.

Pyruvate carboxylase activity was generally lower in the *A. flavus* strains and in *A. ustis* than in the *A. niger* strains. The activity of NADH oxidase appeared to be lower in the case of the two strains of *A. niger*. LDH activity remained fairly constant in all strains.

## Purification of Pyruvate Carboxylase

An 18-fold purification of pyruvate carboxylase was obtained with a combination of procedures involving protamine sulphate and ammonium sulphate. An outline of the purification scheme is presented in Table III.

As shown in Fig. 1, pyruvate carboxylase activity was linear with time for at least 3 minutes with the four protein concentrations used. Activity was also proportional to the amount of protein added (Fig. 2).

## Stability of Pyruvate Carboxylase

### 1. Cold lability and reactivation

The cold lability of pyruvate carboxylase from

Table III

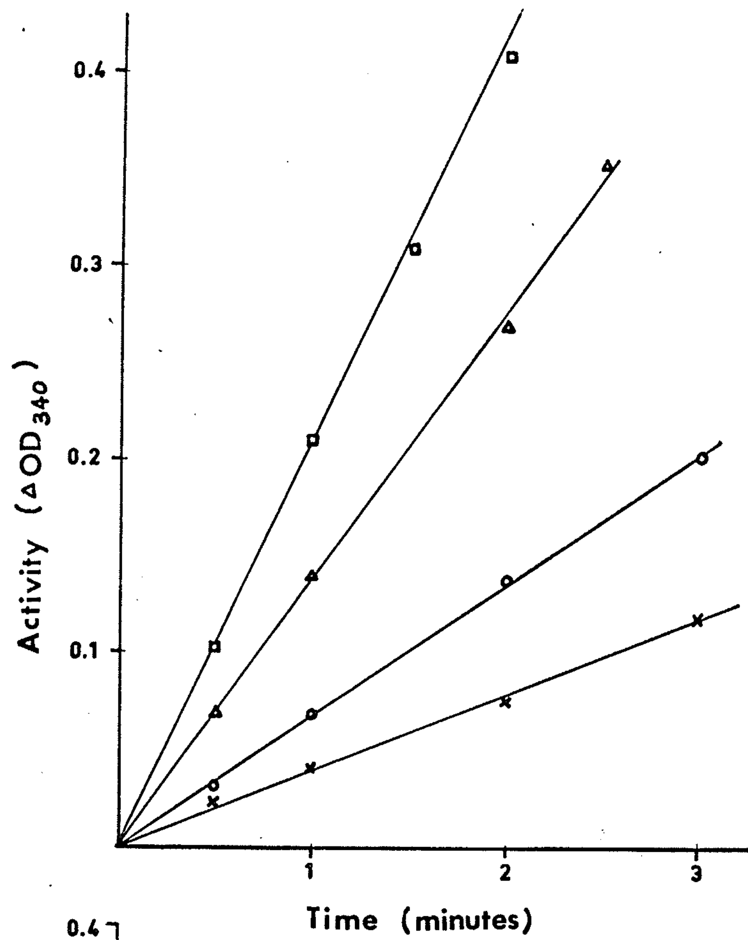
Purification of pyruvate carboxylase from *Aspergillus niger*.

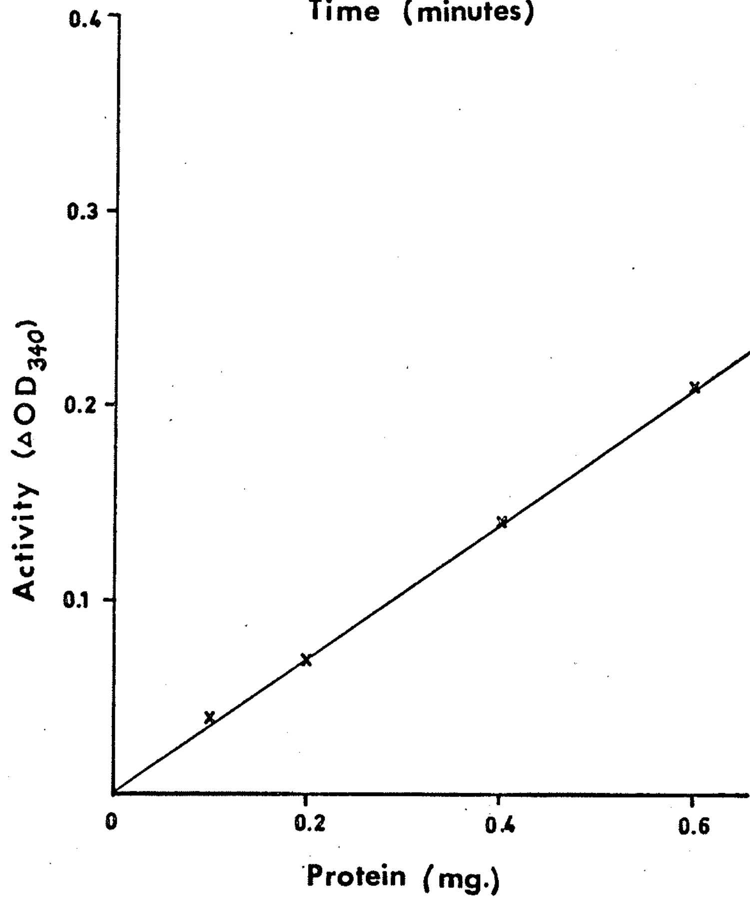
| Stage | Total Protein (mg) | Total Activity (units) | Recovery (%) | Specific Activity (units/mg) |
|---|---|---|---|---|
| I. Crude extract | 343.8 | 30,000 | 100 | 87.3 |
| II. Protamine sulphate supernatant | 102.3 | 26,550 | 88.5 | 259.5 |
| III. $(NH_4)_2SO_4$ precipitate, 0-65% | 35.8 | 27,300 | 91 | 762.6 |
| IV. $(NH_4)_2SO_4$ precipitate, 50-65% | 22.1 | 21,200 | 71 | 959.3 |
| V. $(NH_4)_2SO_4$ precipitate, 40-50% | 12.2 | 15,000 | 50 | 1229.5 |
| VI. $(NH_4)_2SO_4$ supernatant, 30-40% | 8.2 | 12,710 | 42 | 1550.0 |

FIGURE 1. Proportionality of pyruvate carboxylase activity with time. Assay mixtures were prepared as described in Materials and Methods.

× - 0.1 mg protein
○ - 0.2 mg protein
△ - 0.4 mg protein
□ - 0.6 mg protein

FIGURE 2. Proportionality of pyruvate carboxylase activity with protein concentration. Assay mixtures were prepared as described in Materials and Methods.


0.4
0.3
0.2
0.1
Activity ($\Delta OD_{340}$)
1
2
3
Time (minutes)


0.4
0.3
0.2
0.1
Activity ($\Delta OD_{340}$)
0
0.2
0.4
0.6
Protein (mg.)

A. niger was tested after solubilizing the 65% $(NH_4)_2SO_4$ precipitate in 0.1 M Tris-HCl buffer (pH 7.9). The enzyme preparation was held either at $0^\circ$ C in an ice bath or at $22^\circ$ C. Aliquots were removed at various time intervals and activity assayed. The results are shown in Fig. 3. It was seen that the enzyme, when held at $0^\circ$ C, underwent a fairly rapid initial loss in activity which later became more gradual. After 105 minutes, the enzyme activity was 39% that of the original. At this point it was warmed to $22^\circ$ C with an immediate rapid increase in activity which reached a maximum of 61% of the original activity and later decreased gradually again. In the absence of protective agents, the enzyme preparation held at $22^\circ$ C lost 16% of its activity in the same length of time.

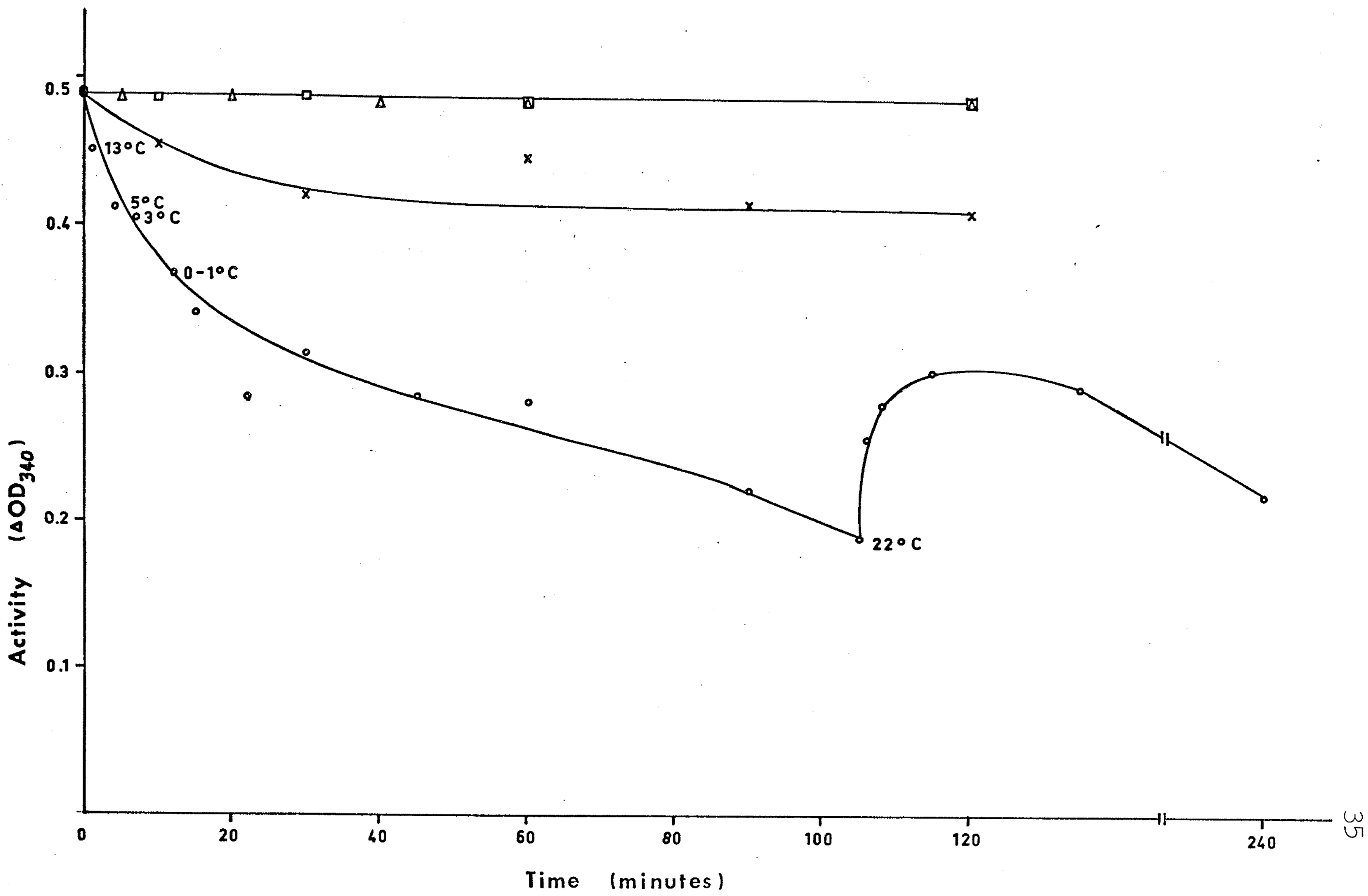
2. Stabilization against cold inactivation

The effectiveness of glycerol in protecting pyruvate carboxylase from A. niger was tested by solubilizing the enzyme in Tris-HCl buffer to which had been added 25% glycerol. The results of this study are shown in Fig. 3. At either $0^\circ$ C or $22^\circ$ C, the 25% glycerol solution completely protected pyruvate carboxylase activity for at

FIGURE 3. Cold inactivation and reactivation of pyruvate carboxylase and stabilization of enzyme activity by addition of glycerol. Assay mixtures were prepared as described in Materials and Methods.

× - $22^{o}$ C
○ - $0^{o}$ C
0% glycerol

△ - $22^{o}$ C
□ - $0^{o}$ C
25% glycerol



Activity ($\Delta OD_{340}$)
0.5
0.4
0.3
0.2
0.1
13°C
5°C
3°C
0-1°C
22°C
0
20
40
60
80
100
120
240
Time (minutes)

least 2 hours. Thus, buffered glycerol was used routinely to solubilize all enzyme preparations.

3. Effect of freezing

A comparison study was set up to see what effect multiple freezings, as compared with one, had on enzyme activity.

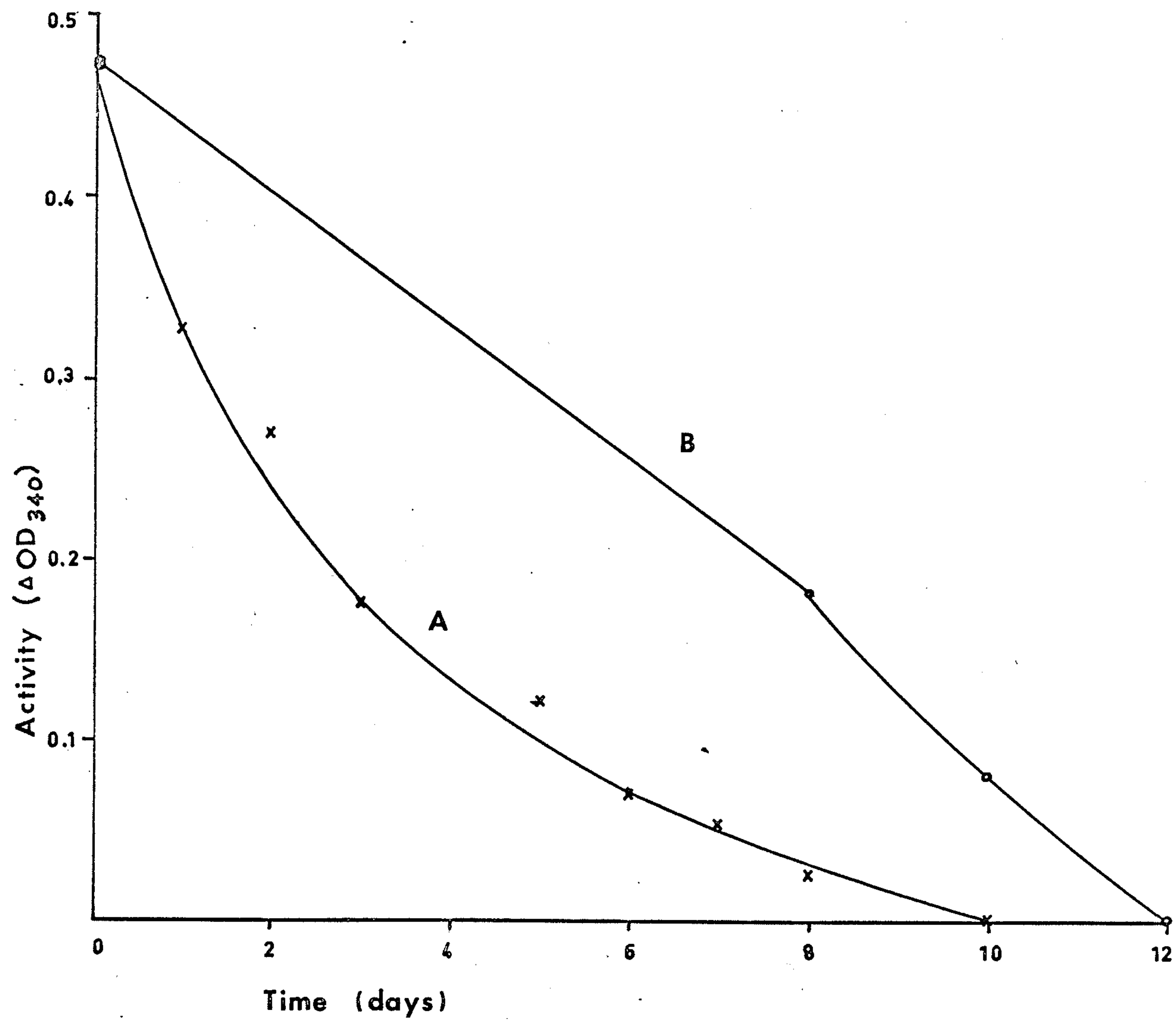
A 65% $(NH_4)_2SO_4$ precipitate was solubilized in 25% buffered glycerol (0.1 M Tris-HCl (pH 7.9)). Pyruvate carboxylase activity was assayed, the preparation divided into 2 parts and frozen. One part (A) was thawed approximately daily for 8 days and assayed for enzyme activity. On the eighth day, the other part (B) was thawed for the first time and assayed also.

It was found that the activity of part (A) had dropped to 50% after the second freezing. All activity was lost after 10 days. On the eighth day when part (B) had been frozen and thawed once, its activity was 38% of the original. After this, the activity dropped off more sharply and was negligible after 12 days.

These results are represented graphically in Fig. 4 and demonstrate the inactivation of the enzyme by repeated

FIGURE 4. Effect of freezing and thawing on pyruvate carboxylase activity. At the indicated times the enzyme was thawed and assayed for activity. Assay mixtures were prepared as described in Materials and Methods. For further descriptions, see the text.

A - several freezings and thawings
B - one freezing and thawing


0.5
0.4
0.3
0.2
0.1
Activity ($\Delta OD_{340}$)
B
A
0
2
4
6
8
10
12
Time (days)

freezing and thawing.

4. Storage of pyruvate carboxylase

The 65% $(NH_4)_2SO_4$ precipitate, which represented a 9-fold purification, was used as the source of enzyme throughout this work. The enzyme could be stored in the precipitated pellet form at $-20^{o}$ C for at least 2 weeks without any loss in activity.

5. Thermal inactivation

This study was carried out with an enzyme preparation stabilized in 25% buffered glycerol. The enzyme was rapidly heated to the desired temperature and then held in a water bath at that temperature. At various time intervals samples were removed and assayed for pyruvate carboxylase activity.

The results are shown in Fig. 5. It was seen that the enzyme was extremely labile at both $55^{o}$ C and $60^{o}$ C. At $48^{o}$ C, all activity was lost after 17 minutes.

pH Optimum

As shown in Fig. 6, the pH optimum for pyruvate

FIGURE 5. Thermal inactivation of pyruvate carboxylase of _A_. _niger_. Assay mixtures were prepared as described in Materials and Methods.

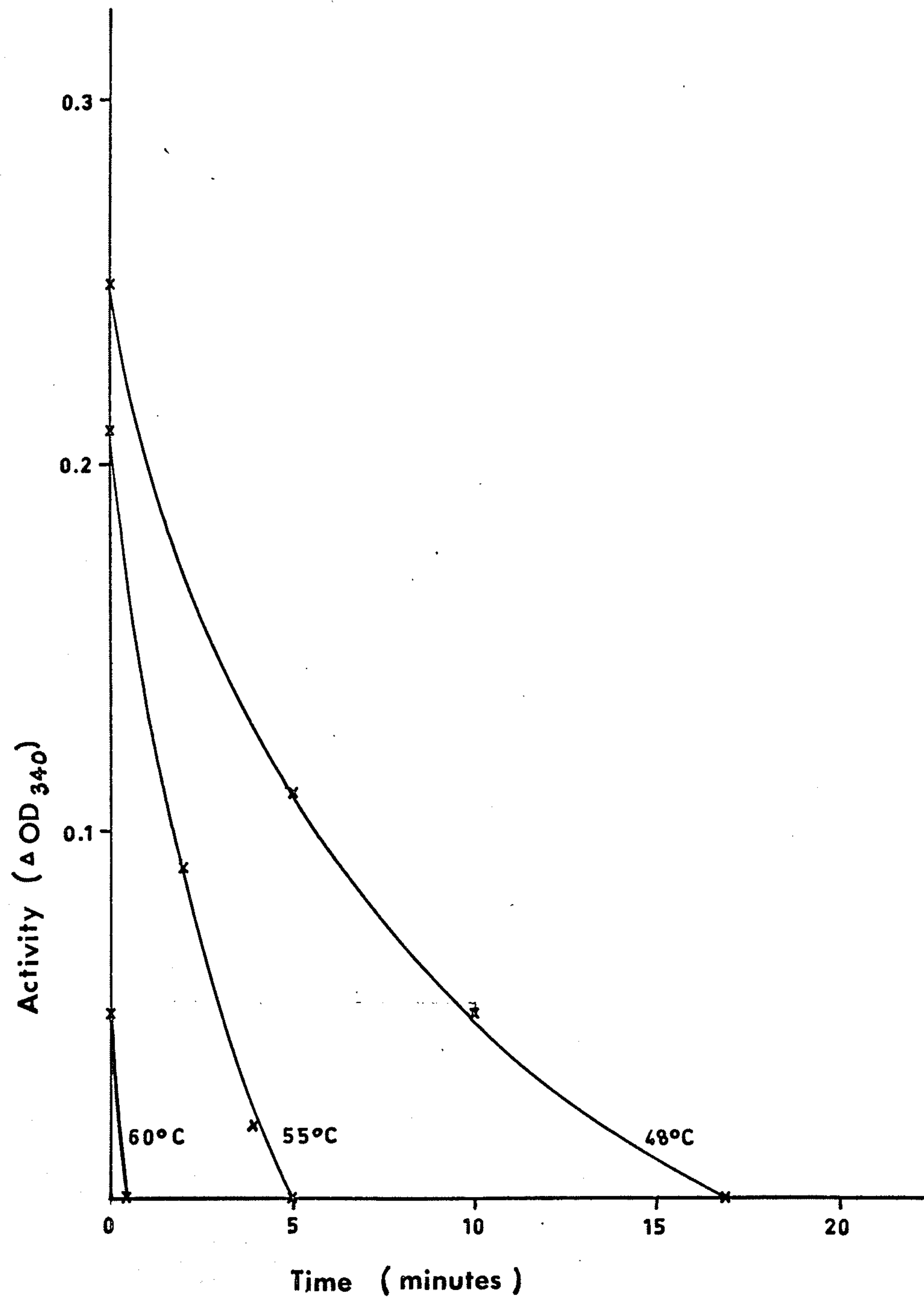
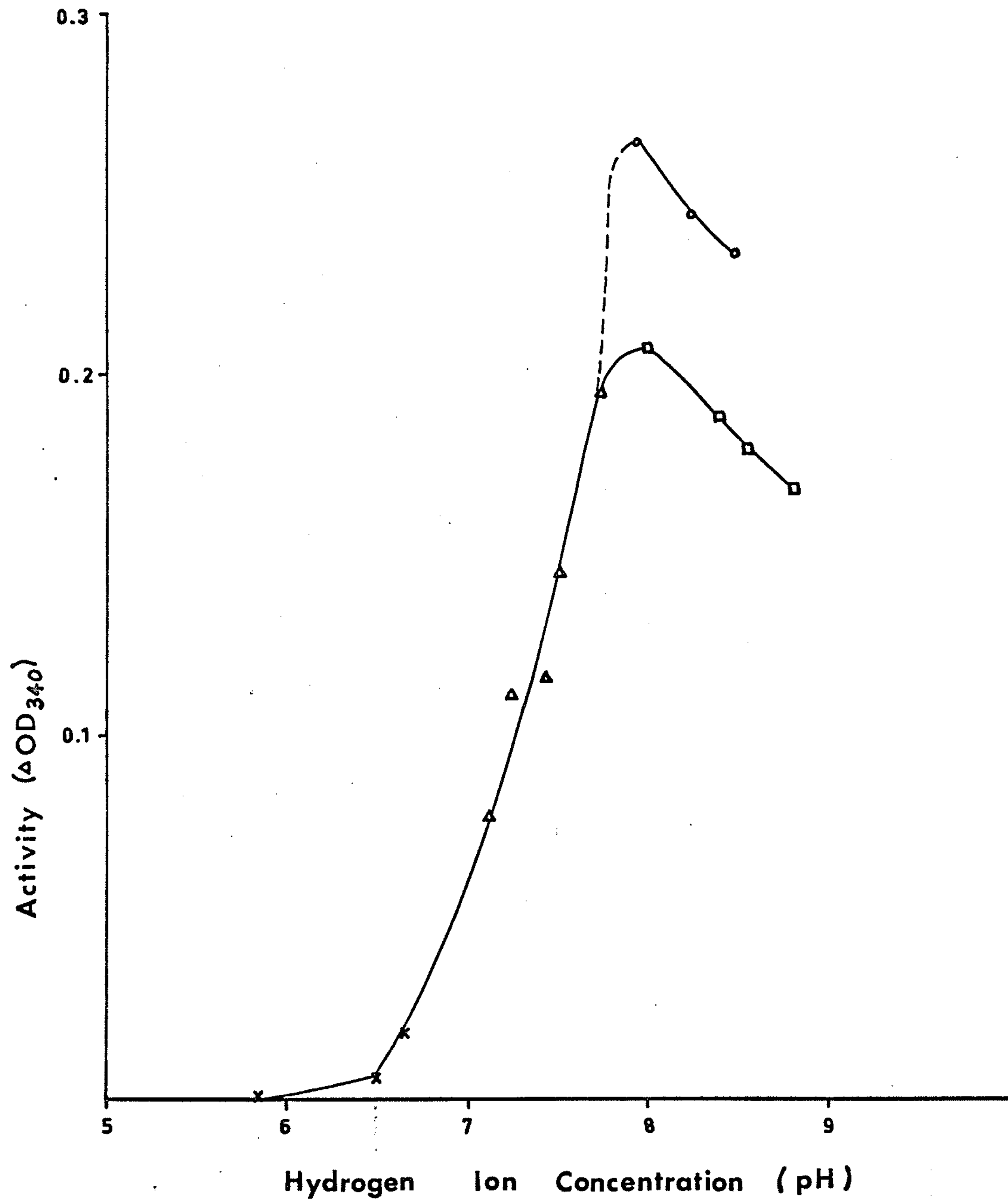

0.3
0.2
0.1
Activity ($\Delta OD_{340}$)
60°C
55°C
48°C
0
5
10
15
20
Time ( minutes )

FIGURE 6. The effect of hydrogen ion concentration (pH) on pyruvate carboxylase activity. Assay mixtures were prepared as described in Materials and Methods with different buffers as noted.

× - MES buffer
△ - HEPES buffer
□ - Tris-HCl buffer
○ - bicine buffer


0.3
0.2
0.1
Activity ($\Delta OD_{340}$)
5
6
7
8
9
Hydrogen Ion Concentration ( pH )

carboxylase was pH 7.9-8.0. This curve was determined using a series of different buffers. As well as Tris-HCl buffer which was used for all subsequent studies, three of the new zwitterionic buffers described by Good *et al*. (1966), MES, HEPES and bicine, were also used. The bicine buffer was found to stimulate enzyme activity at high pH values.

All buffers were used at a concentration of 0.1 M and were brought to the desired pH with HCl or KOH.

## Effect of $K^+$ and $Na^+$ Ions on Pyruvate Carboxylase Activity

The effect of both $K^+$ and $Na^+$ ions on *A. niger* pyruvate carboxylase activity was studied. The results are shown in Table IV. The usual assay mixtures were prepared and KCl or NaCl added as indicated. It was found that 100 mM KCl resulted in 21% stimulation while 100 mM NaCl resulted in 24% inhibition. Potassium chloride in a final concentration of 33.3 mM was routinely added to all assay mixtures.

## Lack of Effect of Acetyl CoA

Acetyl CoA was added to the reaction mixture for the

assay of *A. niger* pyruvate carboxylase in a final concentration of 0.12 mM and 0.24 mM. No effect on activity was observed with either of these additions. These results confirmed the findings of Bloom and Johnson (1962).

## Effect of Avidin

Pyruvate carboxylase from all sources thus far studied has been found to be inhibited by avidin. This inhibition was eliminated if the enzyme was protected by biotin.

The effects of these compounds on *A. niger* pyruvate carboxylase were tested. The assays were carried out spectrophotometrically with the standard reaction mixture except for the avidin and biotin additions. Avidin was incubated for 2 minutes with the enzyme at room temperature before addition of the latter to the reaction cuvette. When biotin was used, it was added to the avidin 2 minutes before the latter was mixed with the enzyme.

The results of this study are shown in Table V. The almost complete inhibition of activity by avidin and protection against this effect by preincubation with biotin, demonstrated that the Aspergillus enzyme, like that of

Table IV

Effect of $K^+$ and $Na^+$ concentrations on pyruvate carboxylase activity.

| Experiment | $K^+$ (mM) | $Na^+$ (mM) | Activity ($\Delta$OD/min) |
|---|---|---|---|
| 1 | 0 | 0 | 0.214 |
| | 10 | 0 | 0.232 |
| | 100 | 0 | 0.260 |
| 2 | 0 | 50 | 0.172 |
| | 0 | 100 | 0.162 |

Table V

Effect of avidin and biotin on pyruvate carboxylase activity.

| Experiment | Avidin (units) | Biotin ($\mu$g) | Activity ($\Delta$OD/min) |
|---|---|---|---|
| 1 | 0 | 0 | 1.380 |
| | 0.10 | 0 | 0.310 |
| | 0.21 | 0 | 0.190 |
| | 0.31 | 0 | 0.100 |
| 2 | 0 | 25 | 1.360 |
| | 0.31 | 0 | 0.090 |
| | 0.31 | 25 | 1.290 |

chicken liver (Keech and Utter, 1963), yeast (Gailiusis *et al*., 1964), Pseudomonas (Seubert and Remberger, 1961) and sheep kidney (Ling and Keech, 1966) is a biotin enzyme.

## Kinetics of Pyruvate Carboxylase from *Aspergillus niger*

All initial velocity data, unless otherwise stated, were plotted in the double reciprocal form (1/v versus 1/S) according to Lineweaver and Burk (1934), where v is the initial velocity and S is the concentration of the variable substrate. The nomenclature of reaction mechanisms and definitions of kinetic constants were those proposed by Cleland (1963a).

## Determination of Apparent $K_m$ Values for Reaction Components

Apparent $K_m$ values for various substrates were calculated from the intercepts at the horizontal axis of double reciprocal plots according to the equation (1):

$$\frac{1}{v} = \frac{1}{V'} + \frac{K_m'}{V'}\left(\frac{1}{S}\right) \qquad (1)$$

where $K_m'$ is the apparent $K_m$ value for the variable substrate, S, and V' is the apparent maximum velocity.

1. Apparent $K_m$ for pyruvate

The plots for v versus S and 1/v versus 1/S are shown in Fig. 7. The apparent $K_m$ for pyruvate was calculated to be 0.28 mM.

2. Apparent $K_m$ for bicarbonate

The plots for v versus S and 1/v versus 1/S for bicarbonate are shown in Fig. 8. The endogenous bicarbonate was calculated to be 0.8 mM by the method described in Materials and Methods. The apparent $K_m$ for bicarbonate was calculated to be 1.33 mM.

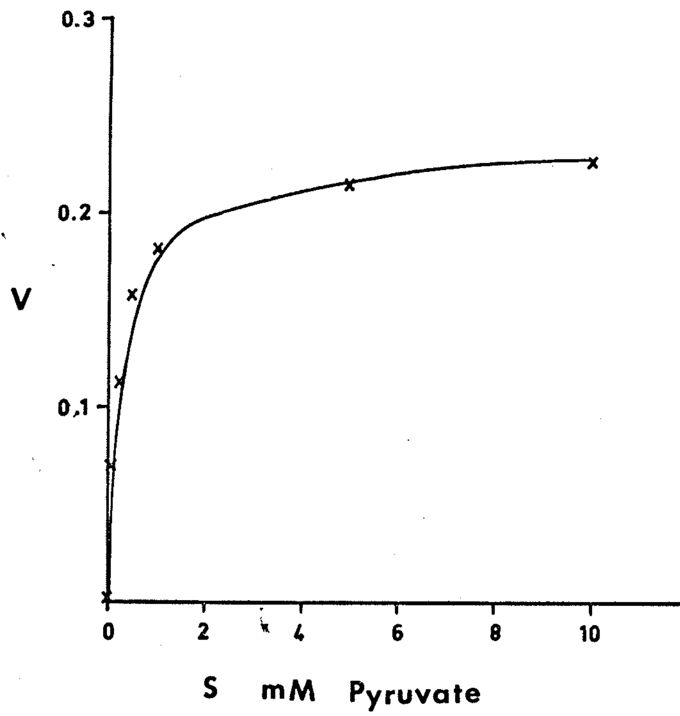
3. Apparent $K_m$ for $Mg^{++}$

The plots for v versus S and 1/v versus 1/S for $Mg^{++}$ are shown in Fig. 9. To avoid complications which might have arisen due to chelation of the divalent cation by ATP, the concentration of the latter was decreased to 0.25 mM for this study. The apparent $K_m$ for $Mg^{++}$ was calculated to be 0.69 mM.

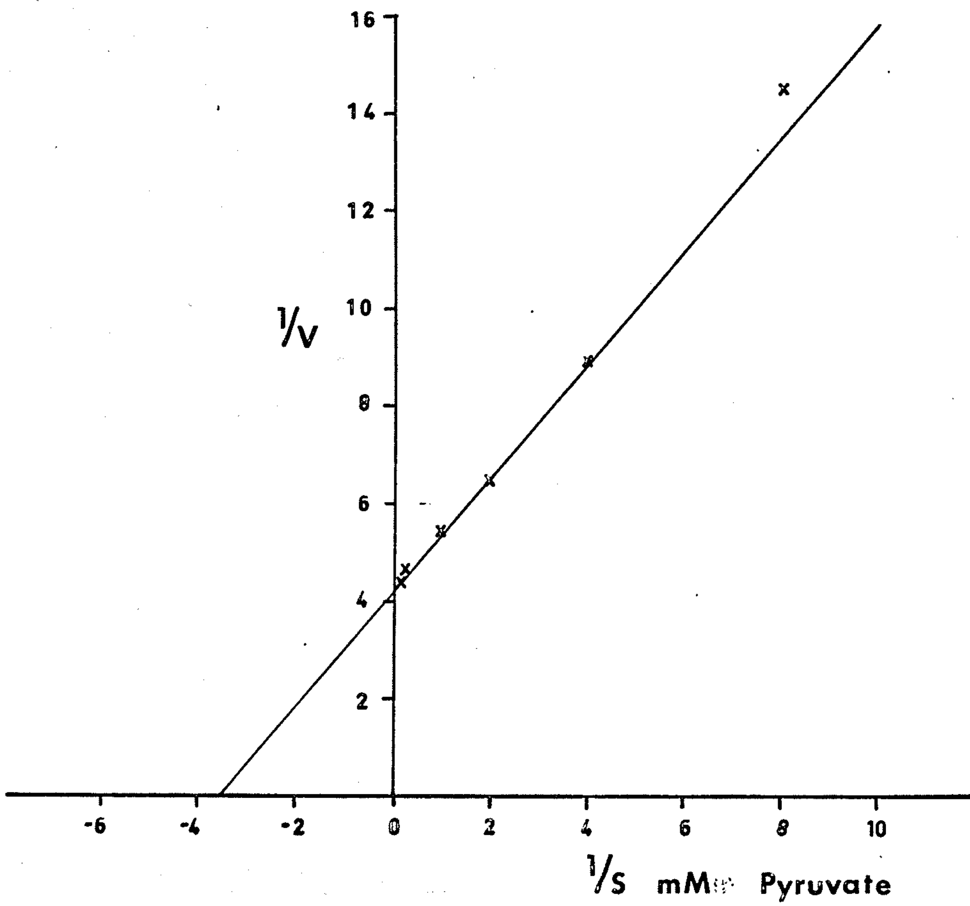
4. Apparent $K_m$ for ATP

The plots for v versus S and 1/v versus 1/S are

FIGURE 7. Apparent $K_m$ for pyruvate. Assay mixtures were prepared as described in Materials and Methods except that pyruvate concentration was varied as indicated.

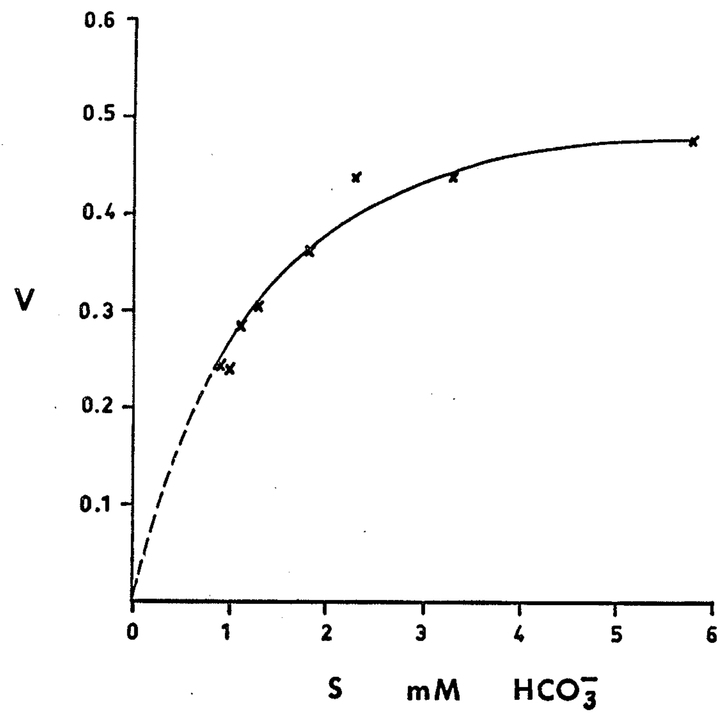


0.3
0.2
0,1
V
0
2
4
6
8
10
S mM Pyruvate

16
14
12
10
8
6
4
2
1/V
-6
-4
-2
0
2
4
6
8
10
1/S mM Pyruvate

FIGURE 8. Apparent $K_m$ for bicarbonate. Assay mixtures were prepared as described in Materials and Methods except that bicarbonate concentration was varied as indicated and endogenous bicarbonate was determined to be 0.8 mM as described in detail in Materials and Methods.


0.6
0.5
0.4
V
0.3
0.2
0.1
0
1
2
3
4
5
6
S mM $HCO_3^-$

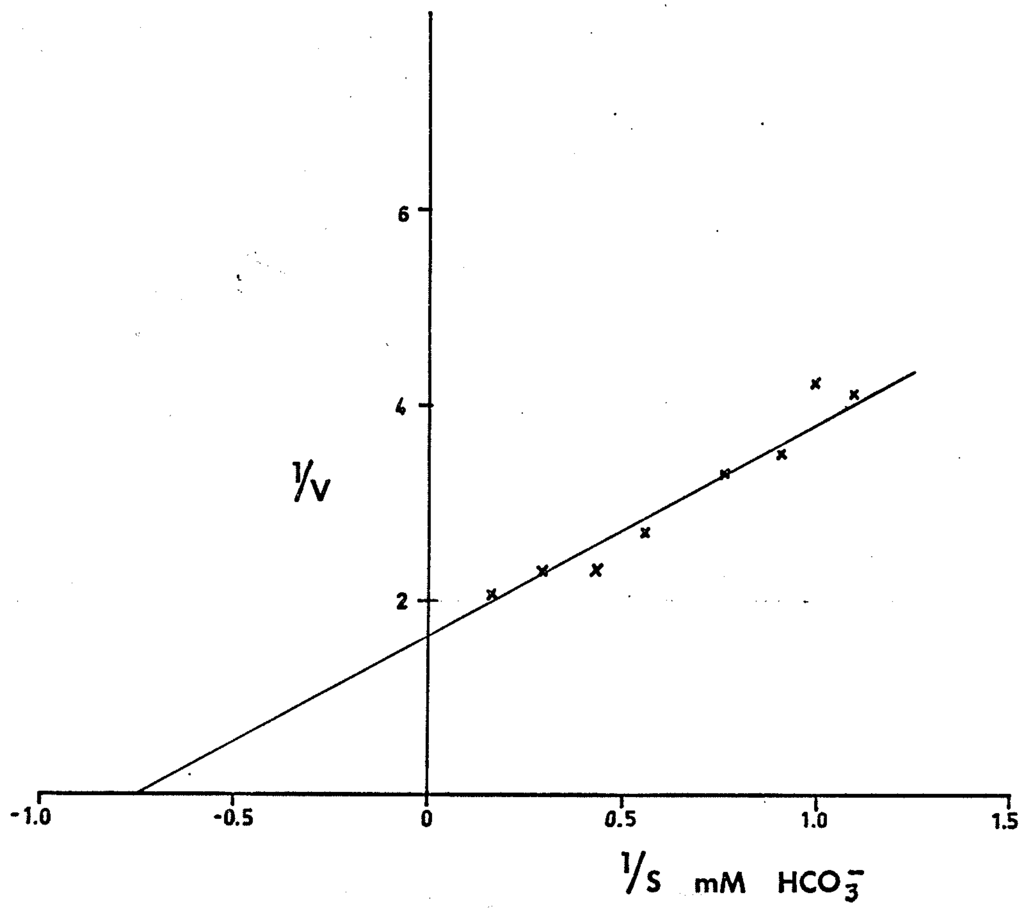
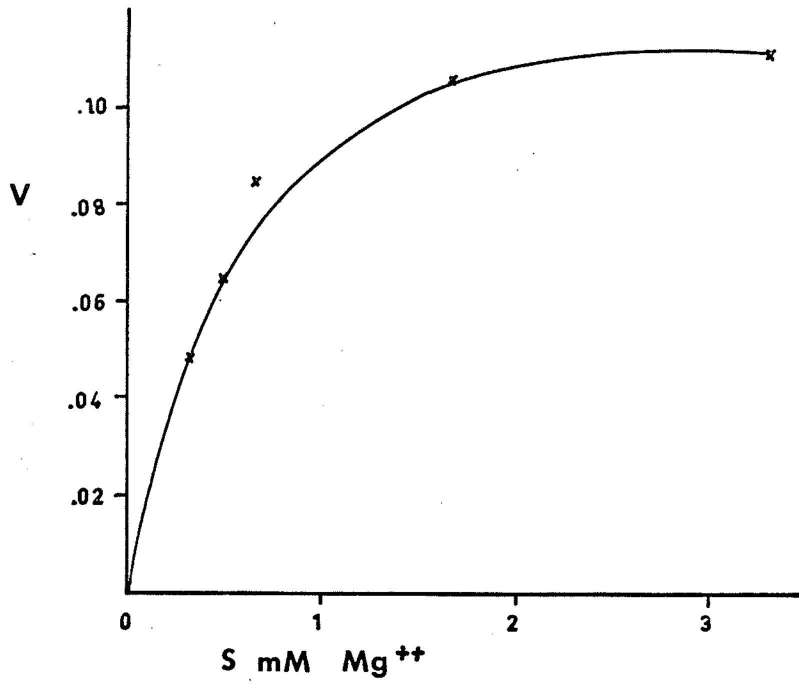

6
4
1/V
2
-1.0
-0.5
0
0.5
1.0
1.5
1/S mM $HCO_3^-$

FIGURE 9. Apparent $K_m$ for $Mg^{++}$. Assay mixtures were prepared as described in Materials and Methods except that ATP concentration was reduced to 0.25 mM and $Mg^{++}$ concentration was varied as indicated.


V
.10
.08
.06
.04
.02
0
1
2
3
S mM Mg ++

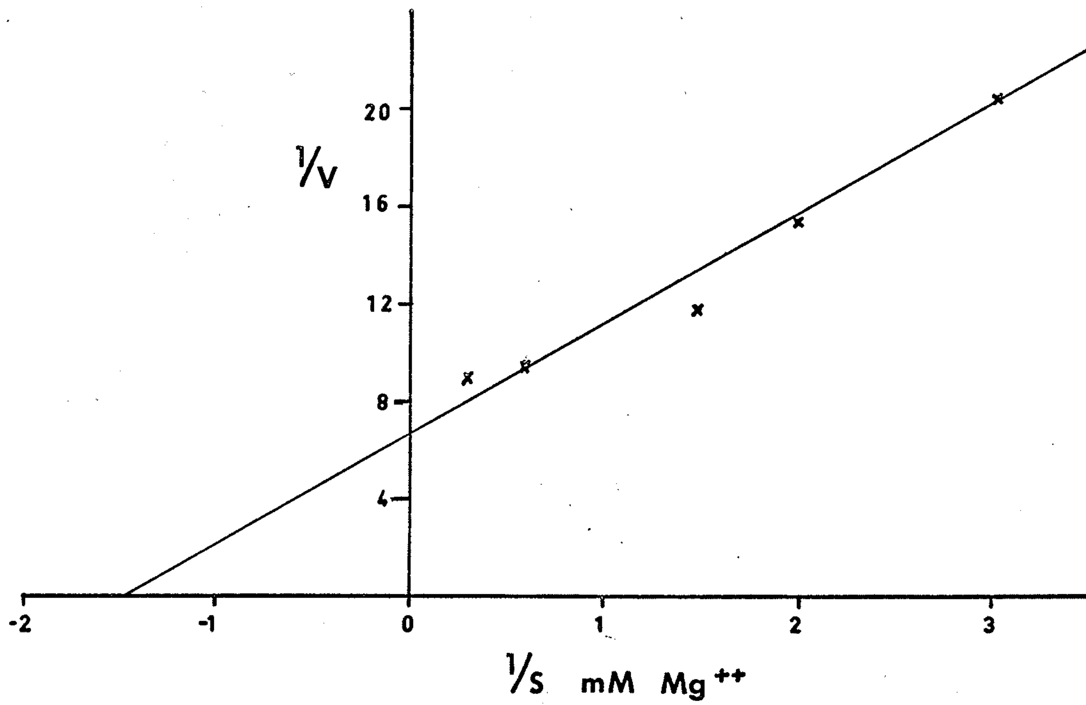
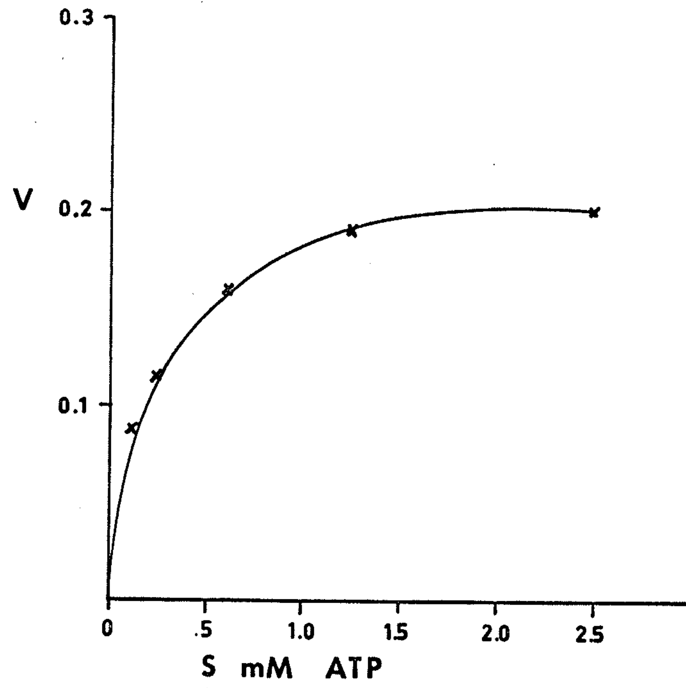
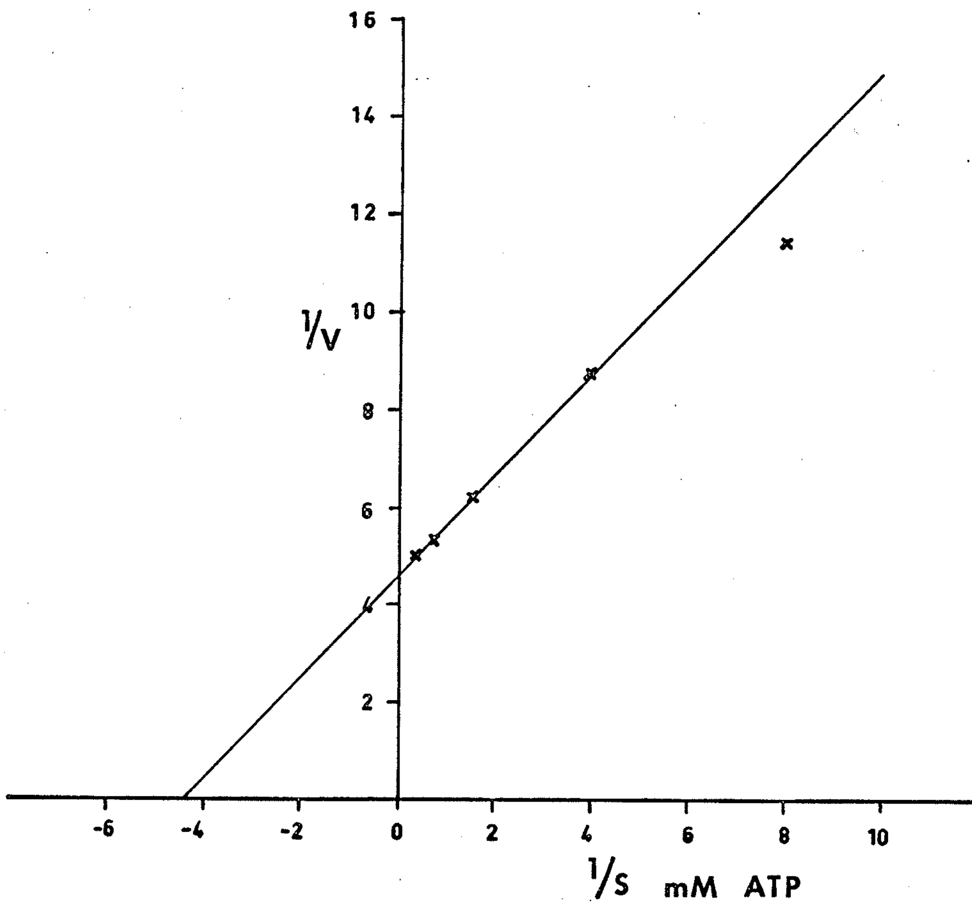

1/V
20
16
12
8
4
-2
-1
0
1
2
3
1/S mM Mg ++

FIGURE 10. Apparent $K_m$ for ATP. Assay mixtures were prepared as described in Materials and Methods except that ATP concentration was varied as indicated.



0.3
V
0.2
0.1
0
.5
1.0
1.5
2.0
2.5
S mM ATP

16
14
12
1/V
10
8
6
4
2
-6
-4
-2
0
2
4
6
8
10
1/S mM ATP

shown in Fig. 10. The apparent $K_m$ for ATP was calculated to be 0.23 mM.

## Determination of True $K_m$ Values for Reaction Components

True Michaelis constants ($K_m$) were determined by measuring initial velocities at varied concentrations of one substrate and several fixed concentrations of the second substrate. The third substrate was kept at the concentration used for the standard assays. From the double reciprocal plots, the intercepts ($1/V'$) and slopes ($K_m'/V'$) were replotted against the reciprocal of the fixed substrate concentration (Florini and Vestling, 1957). $K_m$ values were obtained from the intercepts at the horizontal axis.

### 1. Pyruvate : ATP

When pyruvate was used as the variable substrate at several fixed concentrations of ATP, the double reciprocal plots (Fig. 11a) were linear. Replots of values for intercepts against reciprocals of ATP concentration were also linear (Fig. 12a) and yielded a true $K_m$ for ATP of 0.21 mM.

With ATP as the variable and pyruvate the changing

FIGURE 11a. Double reciprocal plots of velocity versus varying pyruvate concentrations with ATP as fixed variable.

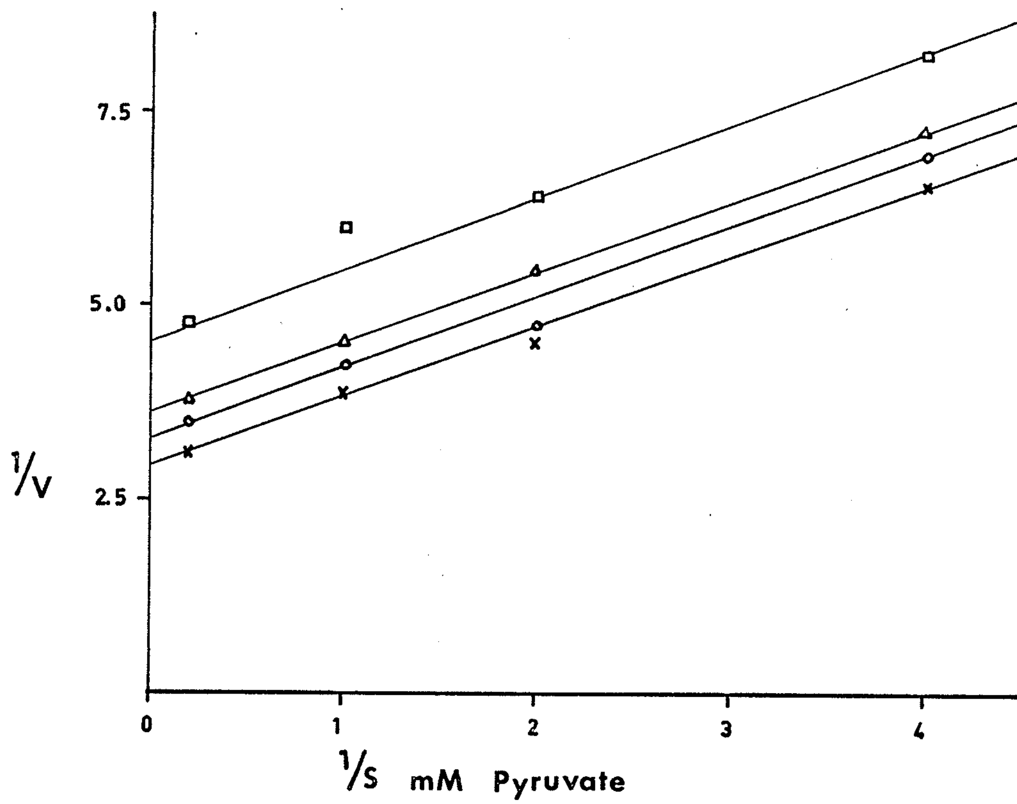
ATP concentrations:

× - 1.25 mM
o - 0.75 mM
Δ - 0.50 mM
□ - 0.25 mM

FIGURE 11b. Double reciprocal plots of velocity versus varying ATP concentrations with pyruvate as fixed variable.

Pyruvate concentrations:

× - 5.0 mM
o - 1.0 mM
Δ - 0.5 mM
□ - 0.25 mM


7.5
5.0
2.5
1/V
0
1
2
3
4
1/S mM Pyruvate

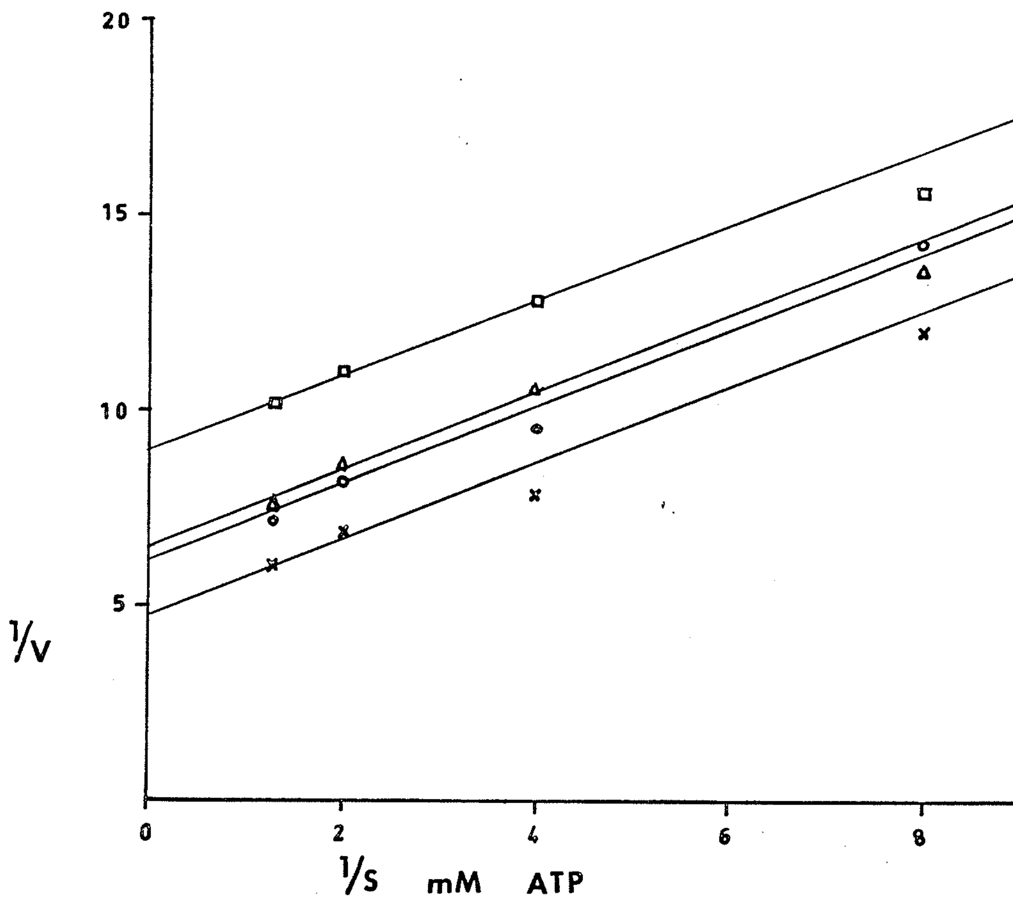
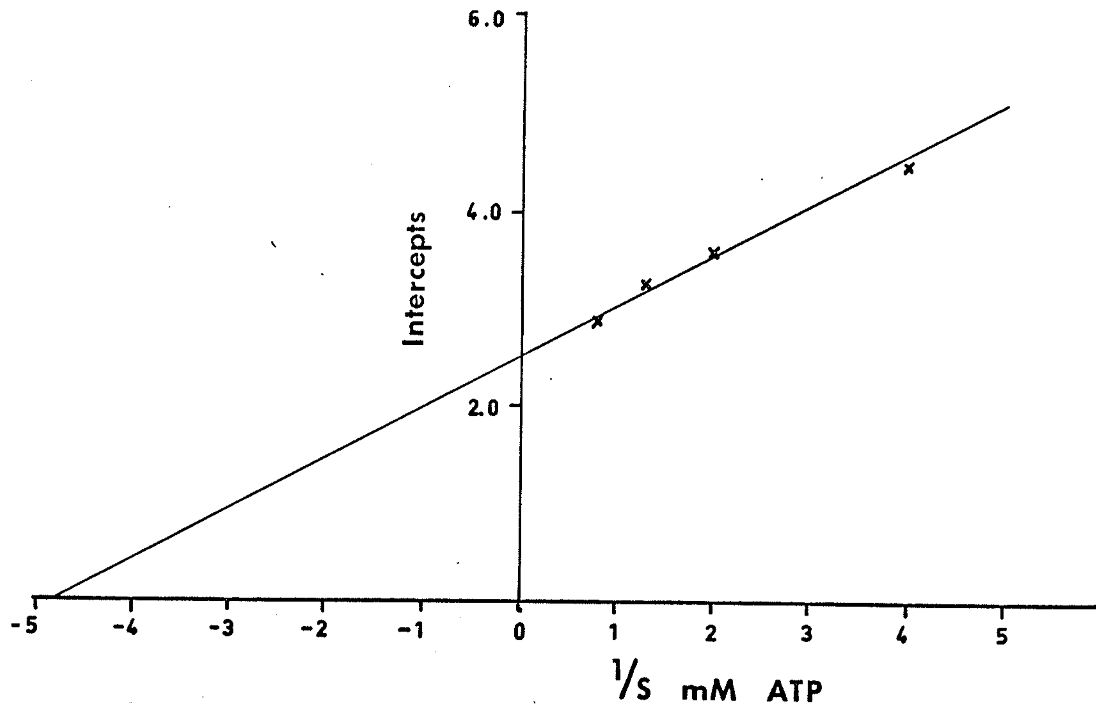
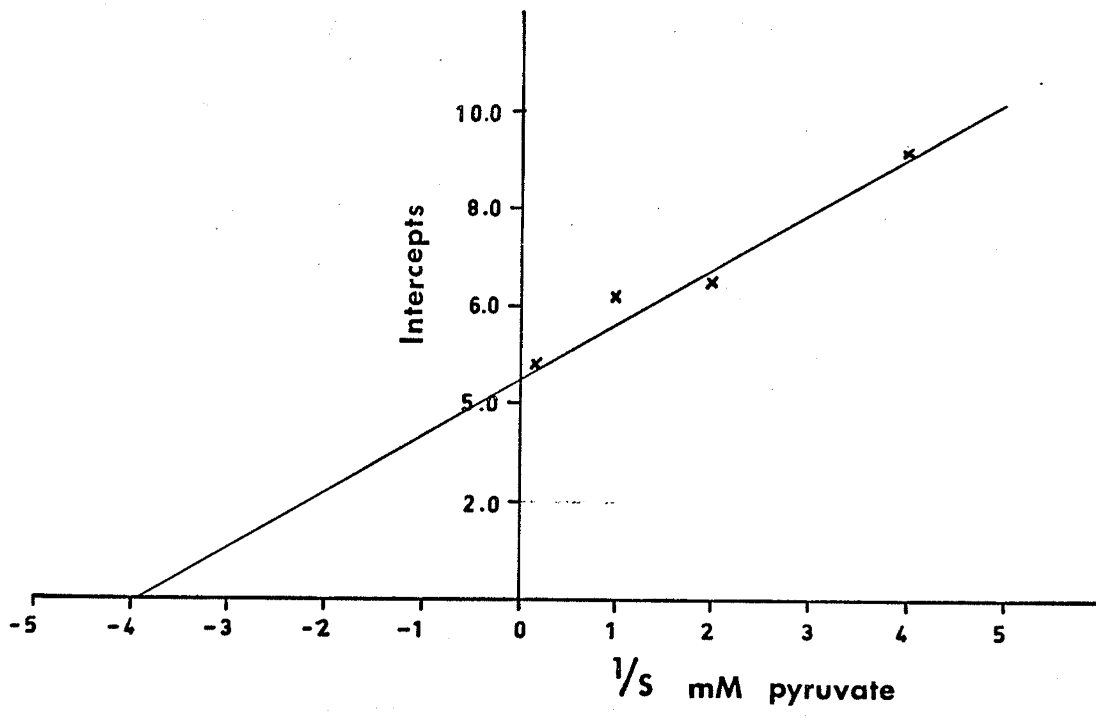

20
15
10
5
1/V
0
2
4
6
8
1/S mM ATP

FIGURE 12a. Replot of intercepts versus reciprocal of ATP from Fig. 11a in which pyruvate was varying and ATP the fixed variable.

FIGURE 12b. Replot of intercepts versus reciprocal of pyruvate from Fig. 11b in which ATP was varying and pyruvate the fixed variable.


6.0
4.0
2.0
Intercepts
-5
-4
-3
-2
-1
0
1
2
3
4
5
1/S mM ATP


10.0
8.0
6.0
5.0
2.0
Intercepts
-5
-4
-3
-2
-1
0
1
2
3
4
5
1/S mM pyruvate

fixed substrate, the double reciprocal plots (Fig. 11b) and replot of (1/V') against reciprocals of pyruvate concentrations were linear (Fig. 12b). The intercept replot yielded a true $K_m$ for pyruvate of 0.26 mM.

The double reciprocal plots in both cases resulted in families of lines which were parallel to one another.

If pyruvate was the variable substrate at several fixed concentrations of ATP and bicarbonate was held at an unsaturating concentration (0.9 mM), double reciprocal plots resulted in linear parallel lines (Fig. 13). A replot of values for intercepts against reciprocals of ATP concentrations was also linear (Fig. 14).

2. Pyruvate : bicarbonate

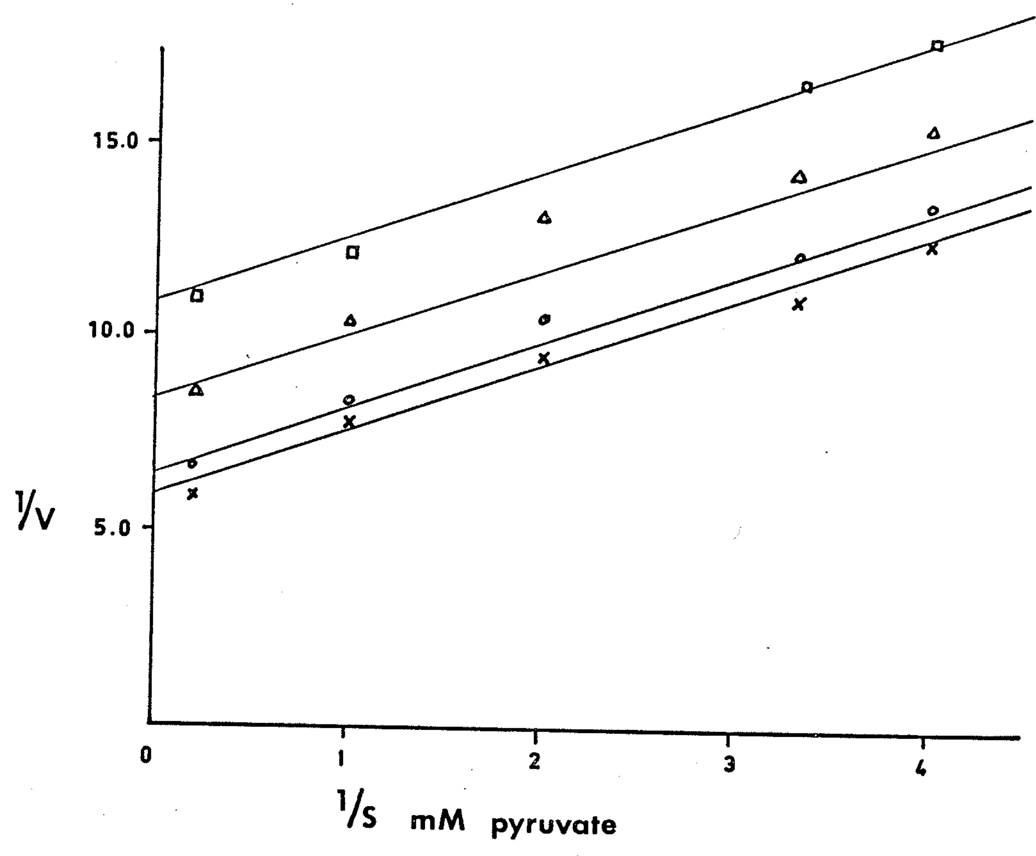
Double reciprocal plots obtained when pyruvate was varied at several fixed concentrations of bicarbonate yielded a set of lines which were linear and intersecting (Fig. 15a). Replots of values for (1/V') and ($K_m$'/V') against reciprocals of bicarbonate concentrations were also linear (Figs. 16a and 16b). A replot of intercepts yielded a true $K_m$ for bicarbonate of 1.02 mM.

When bicarbonate was varied at several fixed

FIGURE 13. Double reciprocal plots of velocity versus varying pyruvate concentrations with ATP as fixed variable. Assay mixtures were prepared as described in Materials and Methods except that bicarbonate was held at 0.9 mM.

ATP concentrations:
- × - 0.75 mM
- ○ - 0.50 mM
- △ - 0.25 mM
- □ - 0.125 mM

FIGURE 14. Replot of intercepts versus reciprocal of ATP from Fig. 13 in which pyruvate was varying, ATP the fixed variable and bicarbonate held at 0.9 mM.


15.0
10.0
5.0
1/v
0
1
2
3
4
1/s mM pyruvate

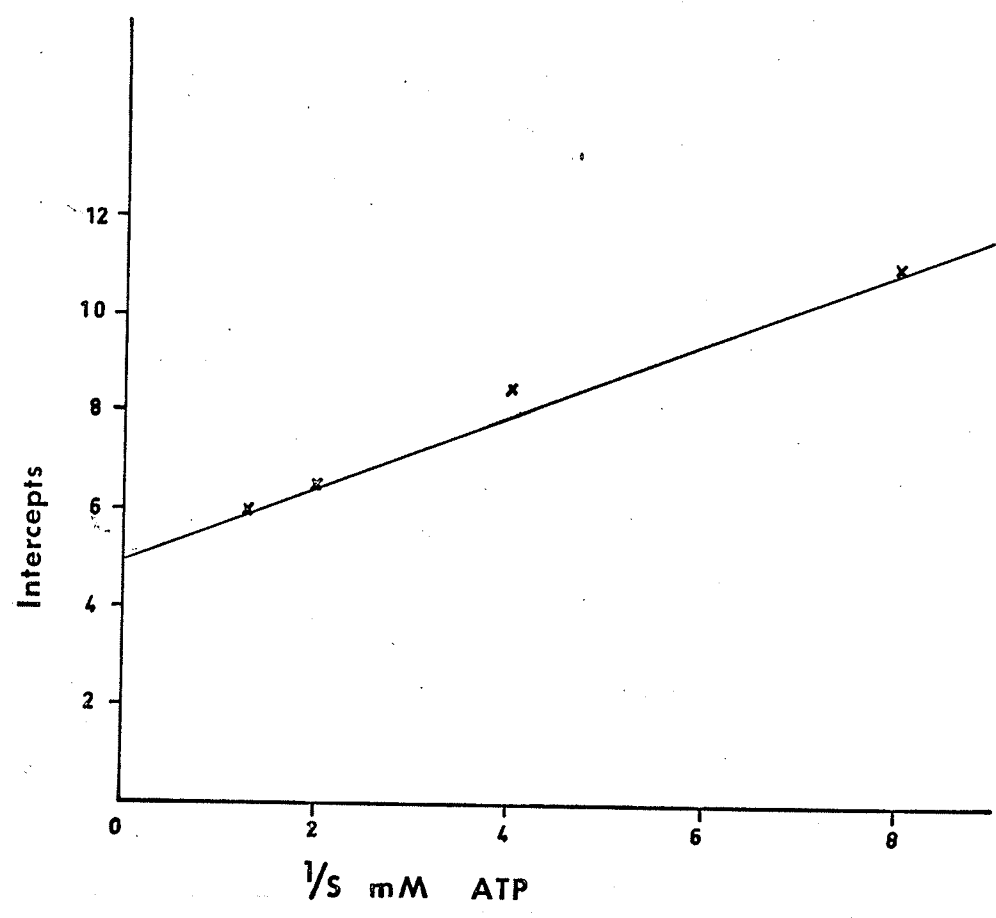

12
10
8
6
4
2
Intercepts
0
2
4
6
8
1/s mM ATP

FIGURE 15a. Double reciprocal plots of velocity versus varying pyruvate concentrations with bicarbonate as fixed variable. Endogenous bicarbonate was estimated at 0.8 mM.

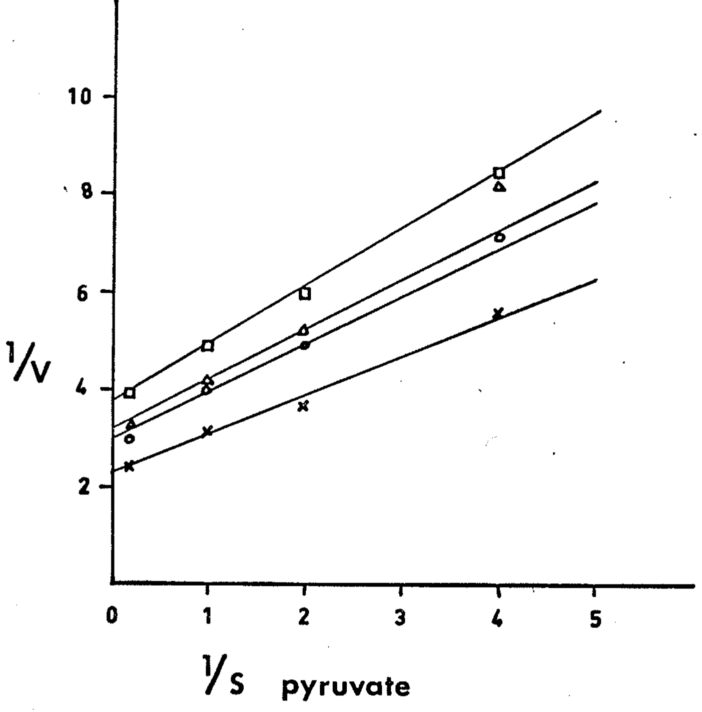
Bicarbonate concentrations:

× - 5.8 mM
○ - 1.8 mM
△ - 1.3 mM
□ - 1.0 mM

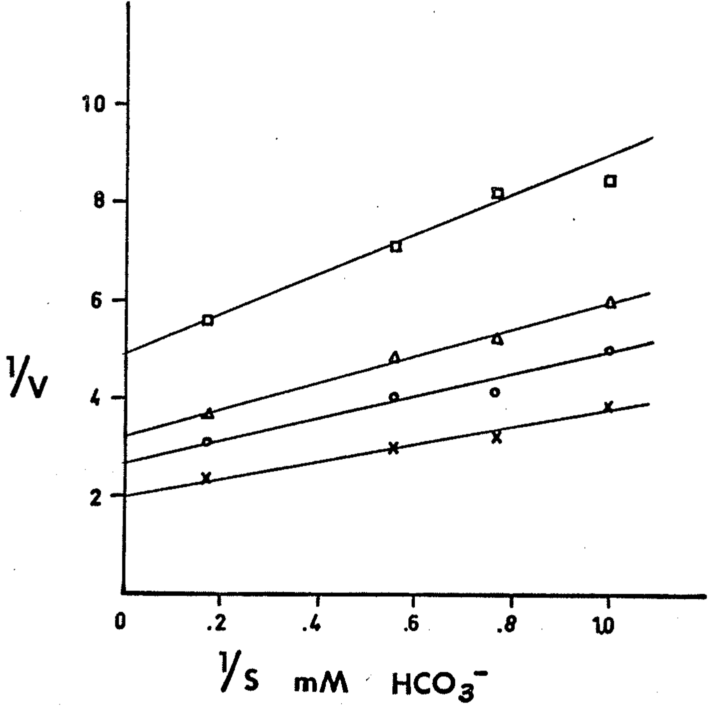
FIGURE 15b. Double reciprocal plots of velocity versus varying bicarbonate concentrations with pyruvate as fixed variable. Endogenous bicarbonate was estimated at 0.8 mM.

Pyruvate concentrations:

× - 5.0 mM
○ - 1.0 mM
△ - 0.5 mM
□ - 0.25 mM



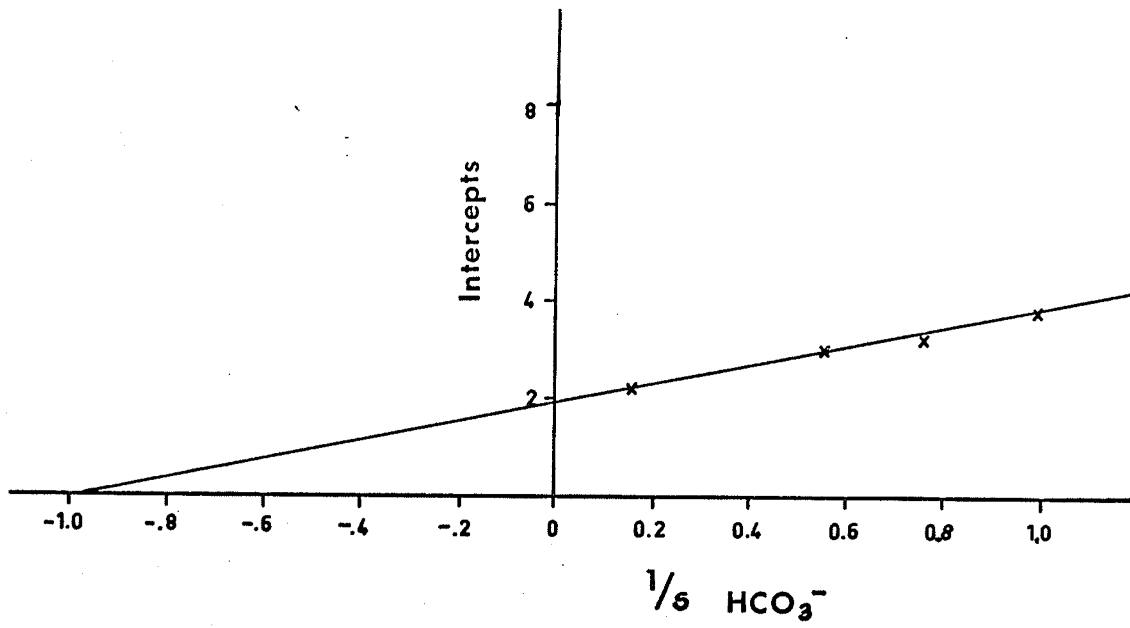
10
8
6
4
2
1/v
0
1
2
3
4
5
1/s pyruvate

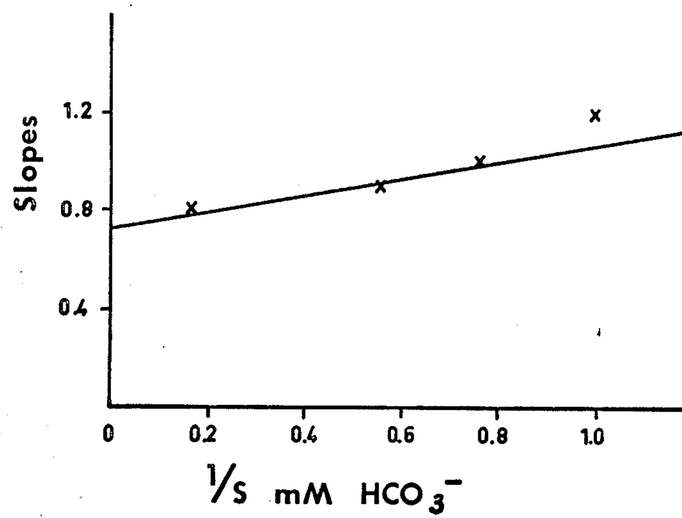



10
8
6
4
2
1/v
0
.2
.4
.6
.8
1.0
1/s mM HCO3-

FIGURE 16a. Replot of intercepts versus reciprocal of bicarbonate from Fig. 15a in which pyruvate was varying and bicarbonate the fixed variable.

FIGURE 16b. Replot of slopes versus reciprocal of bicarbonate from Fig. 15a in which pyruvate was varying and bicarbonate the fixed variable.


Intercepts
8
6
4
2
-1.0
-.8
-.6
-.4
-.2
0
0.2
0.4
0.6
0.8
1.0
1/s HCO3⁻


Slopes
1.2
0.8
0.4
0
0.2
0.4
0.6
0.8
1.0
1/s mM HCO3⁻

concentrations of pyruvate, the double reciprocal plots were both linear and intersecting (Fig. 15b). Replots of (1/V') and ($K_m$'/V') against reciprocals of pyruvate concentrations were linear as well (Figs. 17a and 17b). The former yielded a true $K_m$ value of 0.28 mM for pyruvate.

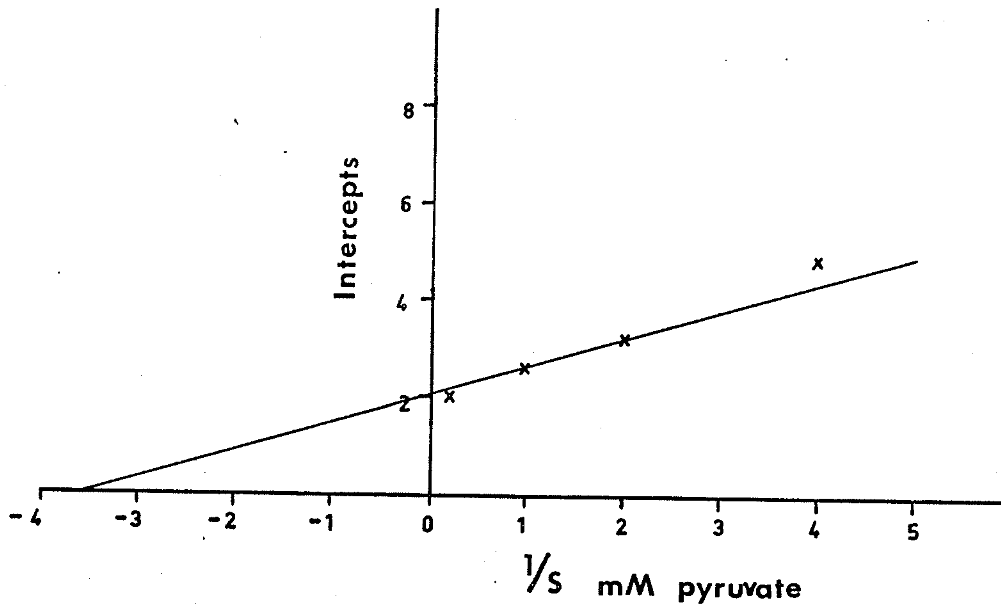
3. ATP : bicarbonate

With ATP as the variable substrate and bicarbonate at several fixed concentrations, double reciprocal plots resulted in linear intersecting lines (Fig. 18a). Replots of (1/V') and ($K_m$'/V') against reciprocals of bicarbonate concentrations were linear (Figs. 19a and 19b). The true $K_m$ for bicarbonate was calculated from the intercept replot to be 0.58 mM.

When bicarbonate was varied against several fixed concentrations of ATP, the double reciprocal plots were linear and intersecting (Fig. 18b). Replots of values for intercepts and slopes against reciprocals of ATP concentrations were also linear (Figs. 20a and 20b). The replot of intercepts yielded a true $K_m$ value for ATP of 0.22 mM.

FIGURE 17a. Replot of intercepts versus reciprocal of pyruvate from Fig. 15b in which bicarbonate was varying and pyruvate the fixed variable.

FIGURE 17b. Replot of slopes versus reciprocal of pyruvate from Fig. 15b in which bicarbonate was varying and pyruvate the fixed variable.


Intercepts
8
6
4
2
-4
-3
-2
-1
0
1
2
3
4
5
1/S mM pyruvate

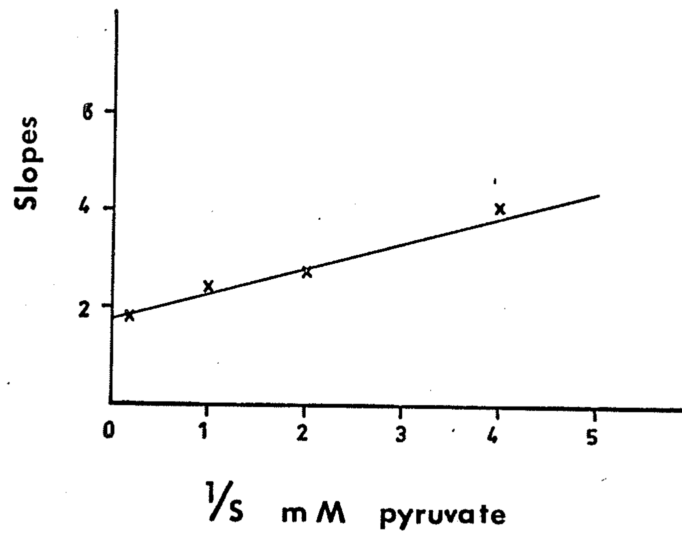

Slopes
6
4
2
0
1
2
3
4
5
1/S mM pyruvate

FIGURE 18a. Double reciprocal plots of velocity versus varying ATP concentrations with bicarbonate as fixed variable. Endogenous bicarbonate was estimated at 0.8 mM.

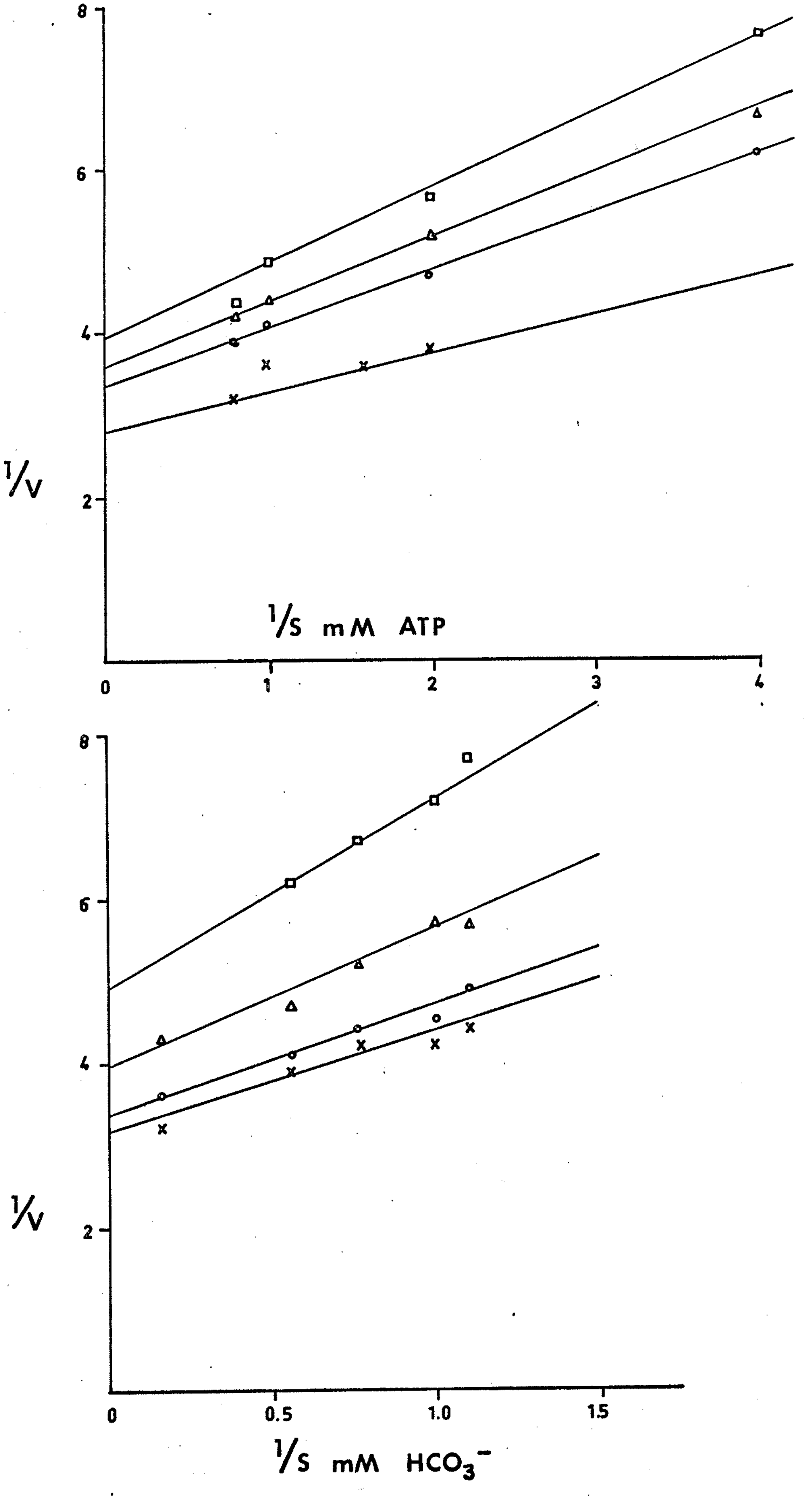
Bicarbonate concentrations:

× - 5.8 mM
○ - 1.8 mM
△ - 1.3 mM
□ - 0.9 mM

FIGURE 18b. Double reciprocal plots of velocity versus varying bicarbonate concentrations with ATP as fixed variable. Endogenous bicarbonate was estimated at 0.8 mM.

ATP concentrations:

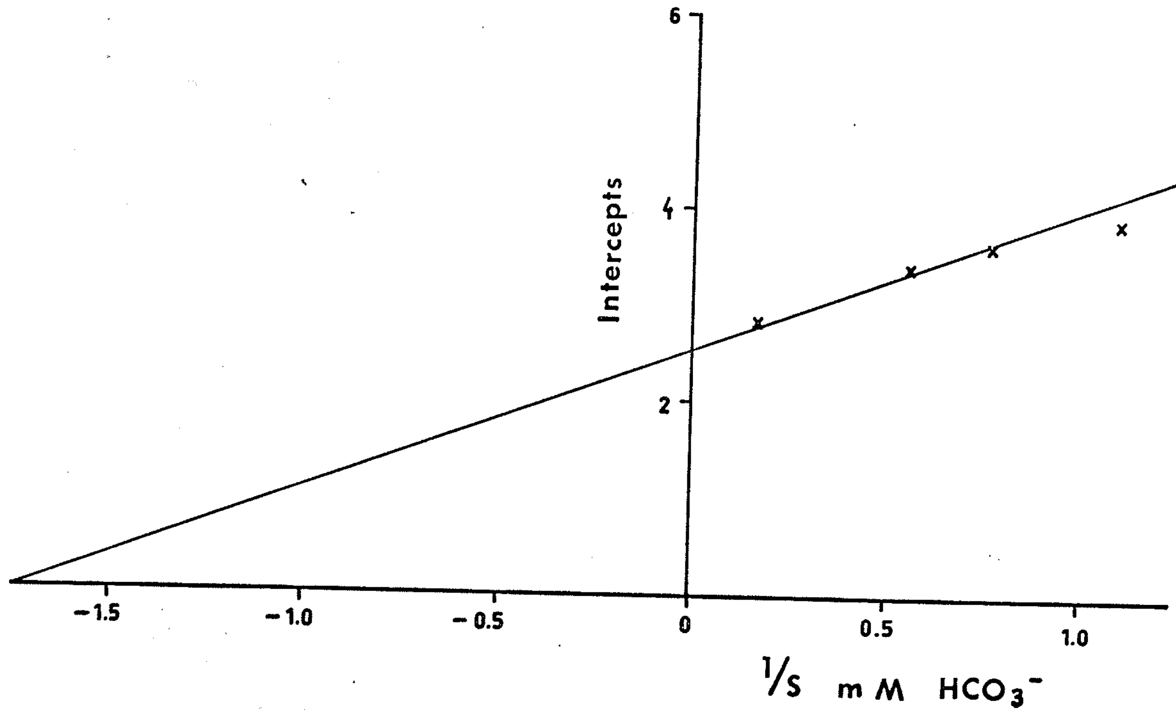
× - 1.25 mM
○ - 1.0 mM
△ - 0.5 mM
□ - 0.25 mM



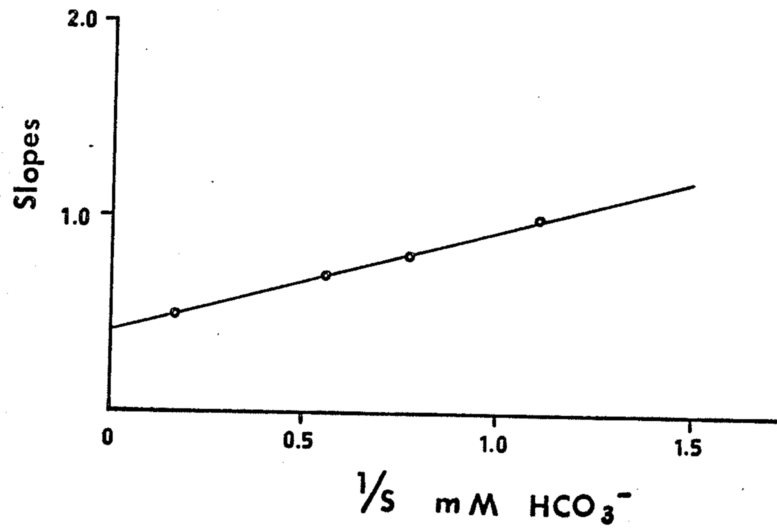
8
6
4
2
1/V
1/S mM ATP
0
1
2
3
4
8
6
4
2
1/V
0
0.5
1.0
1.5
1/S mM HCO3-

FIGURE 19a. Replot of intercepts versus reciprocal of bicarbonate from Fig. 18a in which ATP was varying and bicarbonate the fixed variable.

FIGURE 19b. Replot of slopes versus reciprocal of bicarbonate from Fig. 18a in which ATP was varying and bicarbonate the fixed variable.



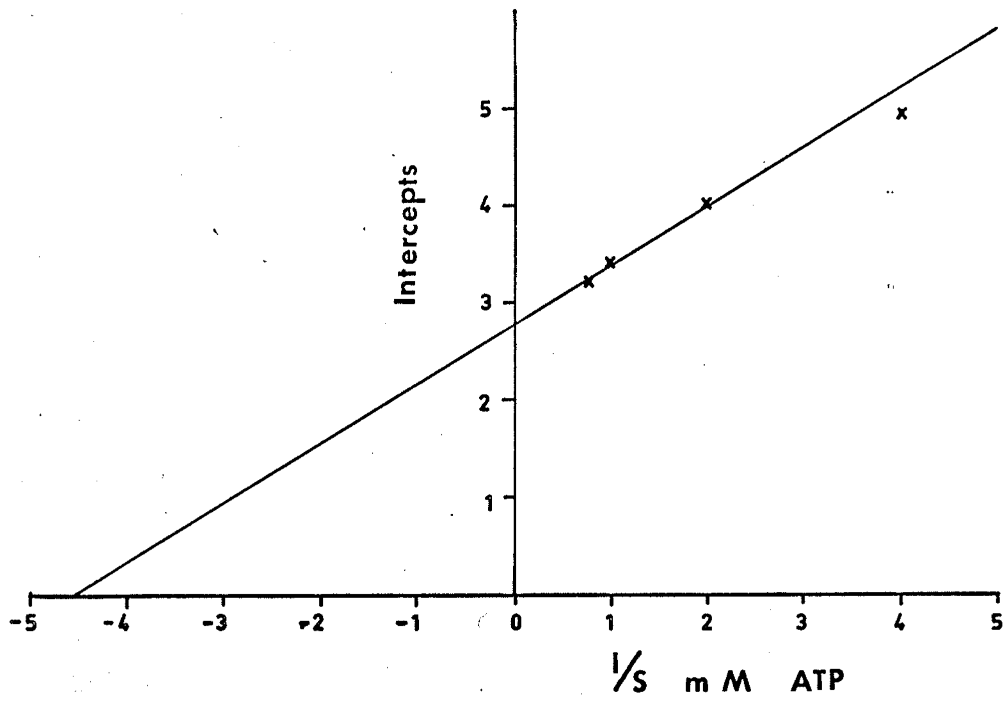
Intercepts
6
4
2
−1.5
−1.0
−0.5
0
0.5
1.0
1/S mM $HCO_3^-$

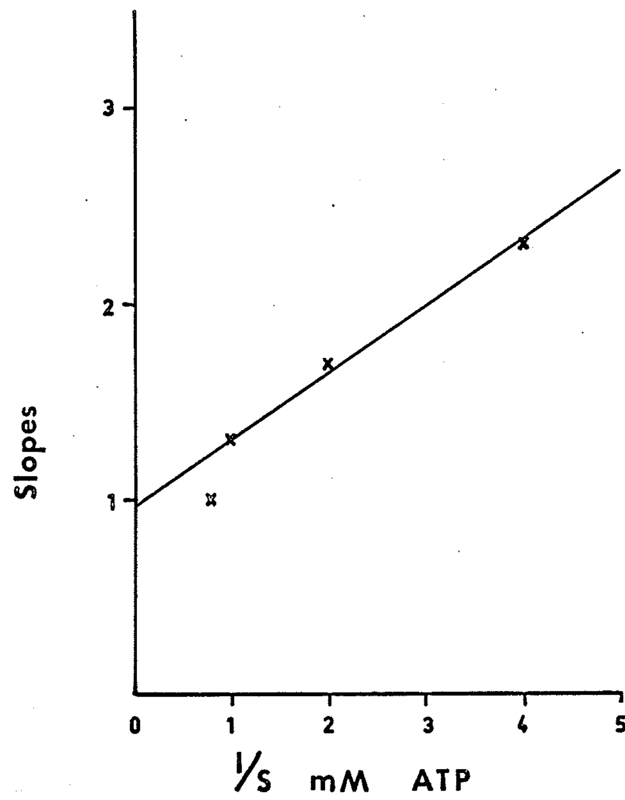



Slopes
2.0
1.0
0
0.5
1.0
1.5
1/S mM $HCO_3^-$

FIGURE 20a. Replot of intercepts versus reciprocal of ATP from Fig. 18b in which bicarbonate was varying and ATP the fixed variable.

FIGURE 20b. Replot of slopes versus reciprocal of ATP from Fig. 18b in which bicarbonate was varying and ATP the fixed variable.


Intercepts
5
4
3
2
1
-5
-4
-3
-2
-1
0
1
2
3
4
5
1/S mM ATP


Slopes
3
2
1
0
1
2
3
4
5
1/S mM ATP

## Product Inhibition Studies

Product inhibition studies were carried out with each product in combination with every substrate. Inhibition constants ($K_i$'s) were determined by measuring initial velocities at varied concentrations of a substrate and several fixed concentrations of the product. From the double reciprocal plots, the intercepts and slopes were replotted versus inhibitor concentration. $K_i$ values, $K_{i\ intercept}$ ($K_{i_I}$) and $K_{i\ slope}$ ($K_{i_S}$), were obtained from the intercepts at the horizontal axis.

The terms used to describe the various types of inhibition were those of Cleland (1963b). Thus, the inhibition is called competitive if the inhibitor increases the slope but does not affect the vertical intercepts. The inhibition is noncompetitive if the inhibitor increases both the slope and the vertical intercepts and uncompetitive if its effect is only on the vertical intercepts.

1. $P_i$ : ATP

When ATP was the variable substrate at several fixed concentrations of $P_i$, the double reciprocal plots were

linear (Fig. 21) and indicated noncompetitive inhibition; ie. both the slopes and vertical intercepts were increased by addition of inhibitor. Replots for both intercepts and slopes (Figs. 22a and 22b respectively) were also linear. The values for $K_{i_I}$ and $K_{i_S}$ were calculated to be 92 mM and 40 mM respectively.

2. $P_i$ : bicarbonate

When bicarbonate was the variable substrate at several fixed concentrations of $P_i$, double reciprocal plots were linear (Fig. 23) and indicated noncompetitive inhibition. Replots of intercepts and slopes (Figs. 24a and 24b) were linear. The values for $K_{i_I}$ and $K_{i_S}$ were calculated to be 140 mM and 37 mM respectively.

3. $P_i$ : pyruvate

When pyruvate was varied at several fixed concentrations of $P_i$, the double reciprocal plots were linear (Fig. 25) and indicated uncompetitive inhibition; ie. only the intercepts were affected by addition of inhibitor. The replot for intercepts (Fig. 26) was linear and yielded a $K_{i_I}$ value of 190 mM.

FIGURE 21. Double reciprocal plots of velocity versus varying ATP concentrations with $P_i$ as the fixed variable product.

$P_i$ concentrations:

× - 0 mM

o - 10 mM

△ - 50 mM

□ - 100 mM

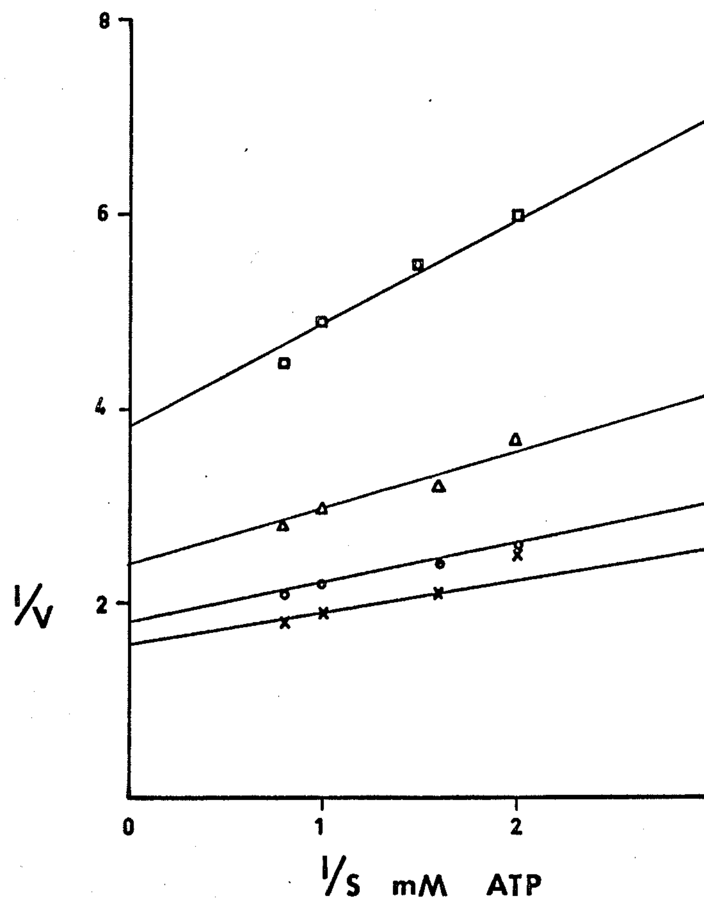
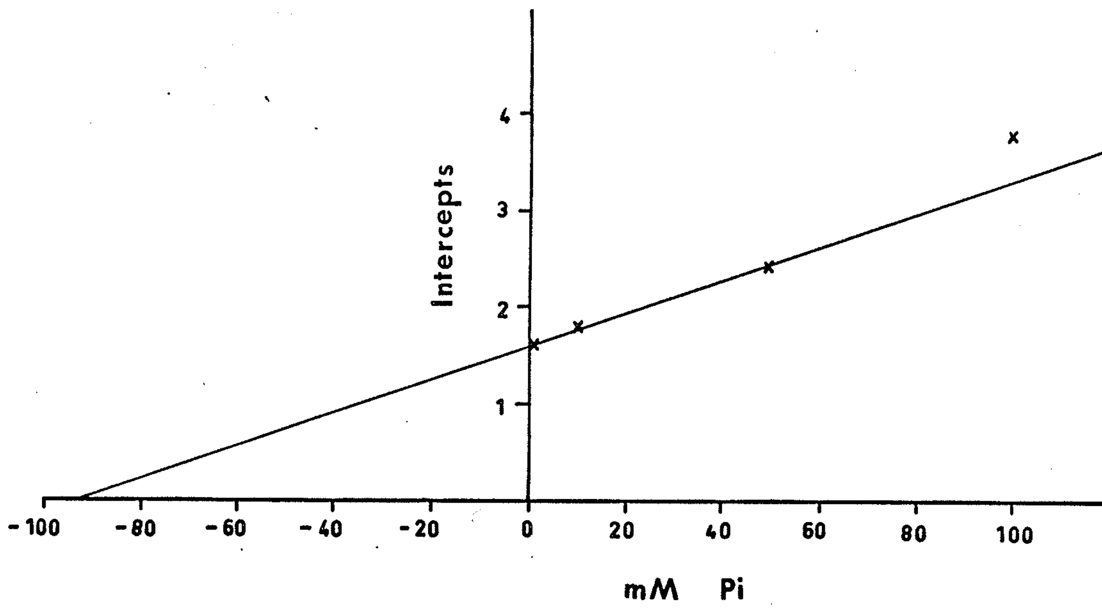

8
6
4
2
1/V
0
1
2
1/S mM ATP

FIGURE 22a. Replot of intercepts versus $P_i$ concentration from Fig. 21 in which ATP was varying and $P_i$ the fixed variable product.

FIGURE 22b. Replot of slopes versus $P_i$ concentration from Fig. 21 in which ATP was varying and $P_i$ the fixed variable product.


Intercepts
4
3
2
1
- 100
- 80
- 60
- 40
- 20
0
20
40
60
80
100
mM Pi

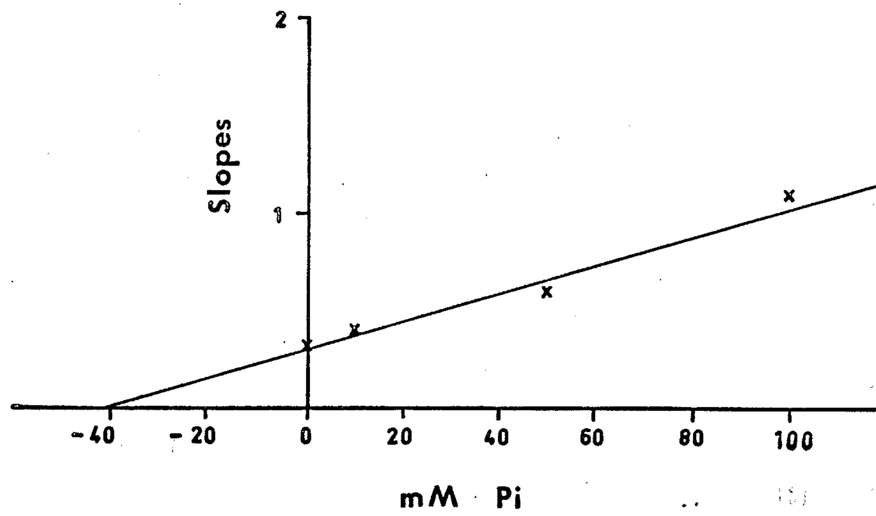

Slopes
2
1
- 40
- 20
0
20
40
60
80
100
mM Pi

FIGURE 23. Double reciprocal plots of velocity versus varying bicarbonate concentrations with $P_i$ as the fixed variable product. Endogenous bicarbonate was estimated at 0.8 mM.

$P_i$ concentrations:

× - 0 mM

○ - 10 mM

△ - 50 mM

□ - 100 mM

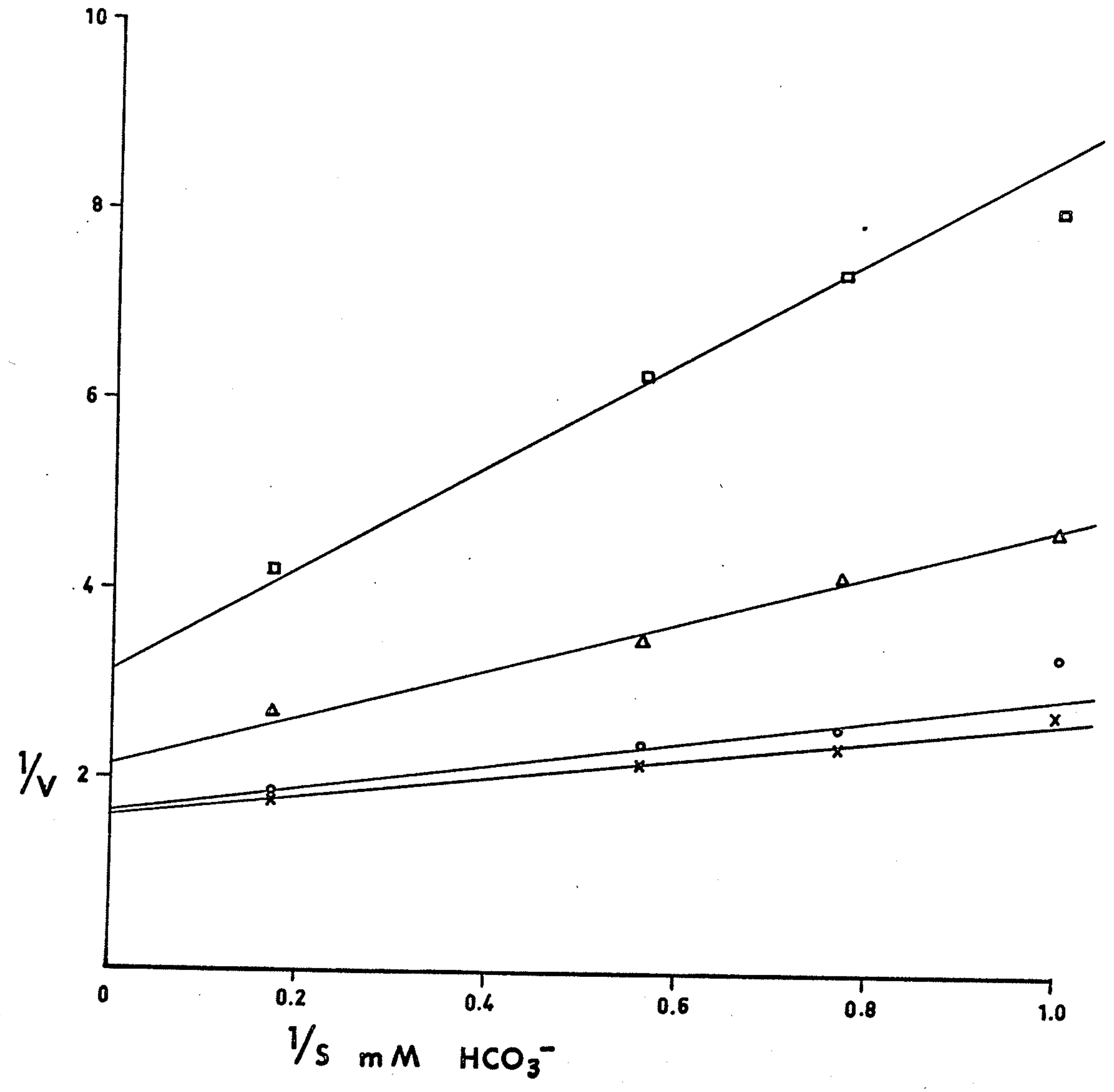
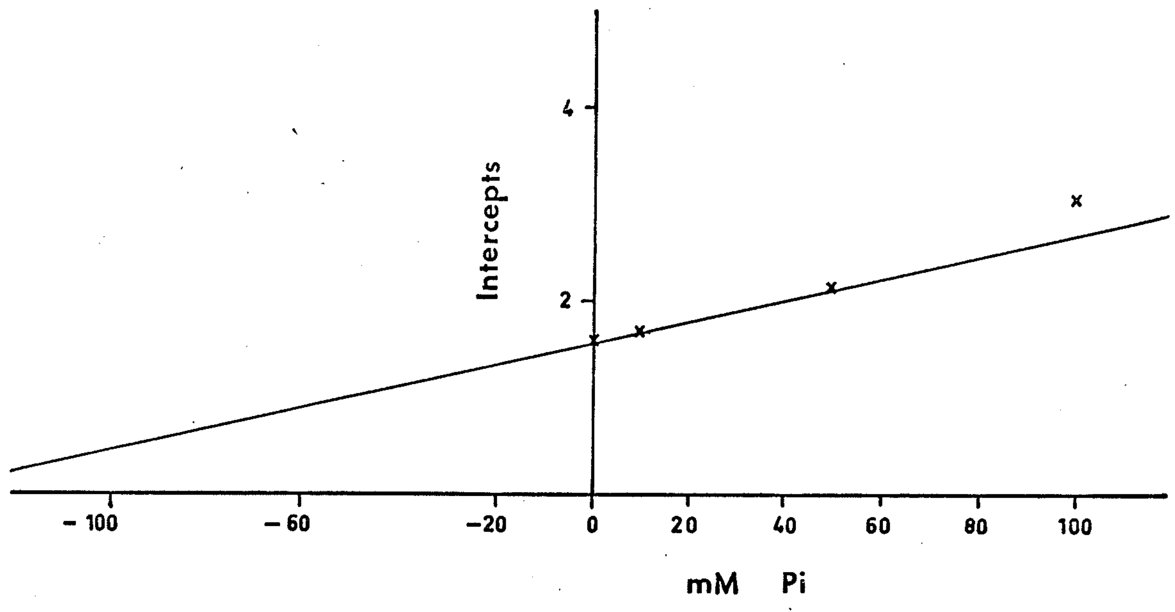
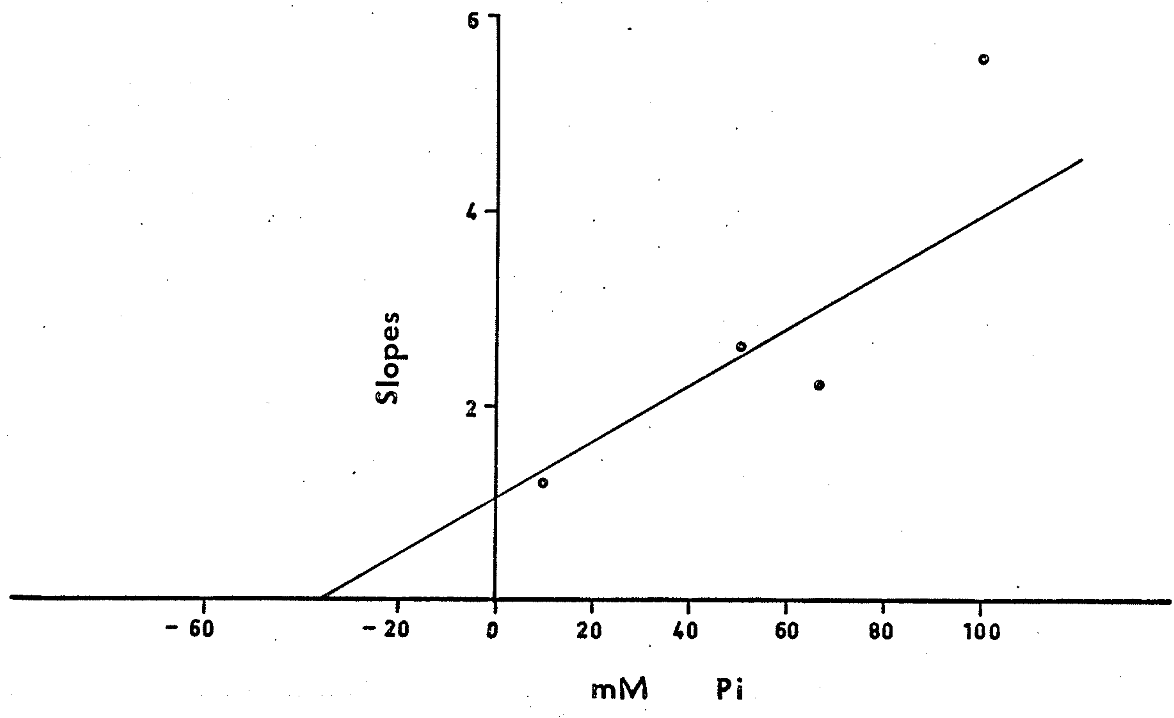

10
8
6
4
2
1/V
0
0.2
0.4
0.6
0.8
1.0
1/S mM HCO3-

FIGURE 24a. Replot of intercepts versus $P_i$ concentration from Fig. 23 in which bicarbonate was varying and $P_i$ the fixed variable product.

FIGURE 24b. Replot of slopes versus $P_i$ concentration from Fig. 23 in which bicarbonate was varying and $P_i$ the fixed variable product.



Intercepts
4
2
- 100
- 60
-20
0
20
40
60
80
100
mM Pi

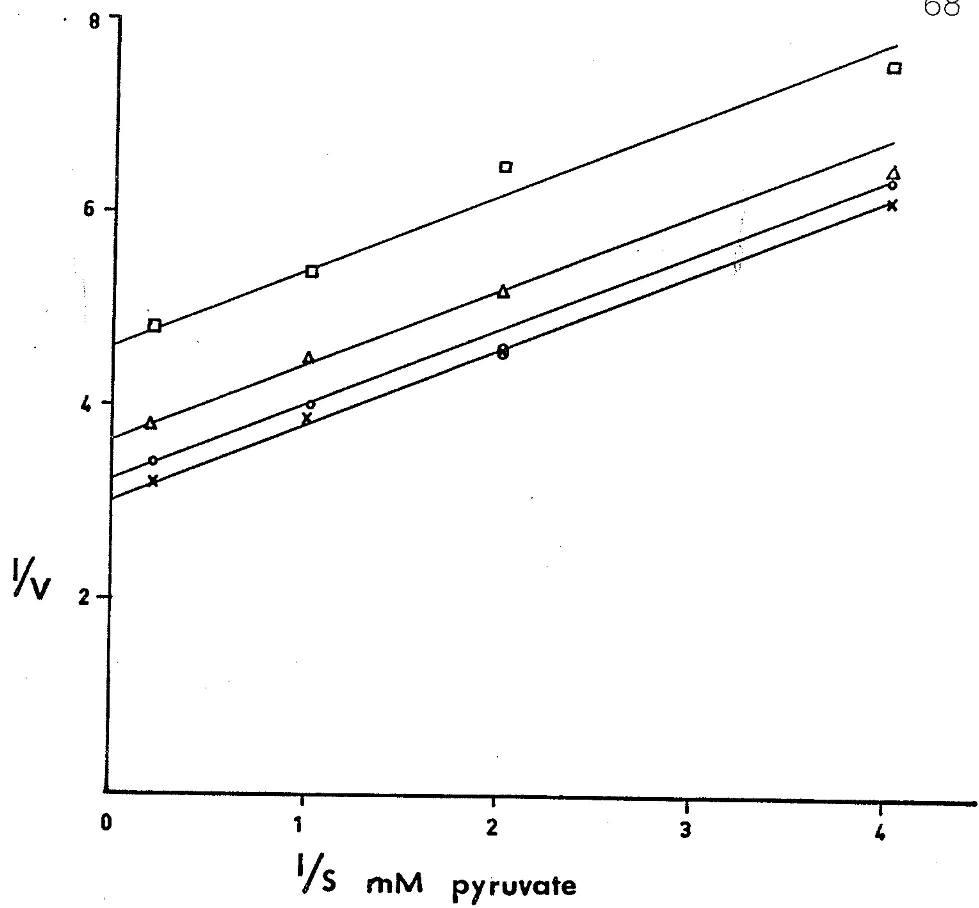
Slopes
6
4
2
- 60
- 20
0
20
40
60
80
100
mM Pi

FIGURE 25. Double reciprocal plots of velocity versus varying pyruvate concentrations with $P_i$ as the fixed variable product.

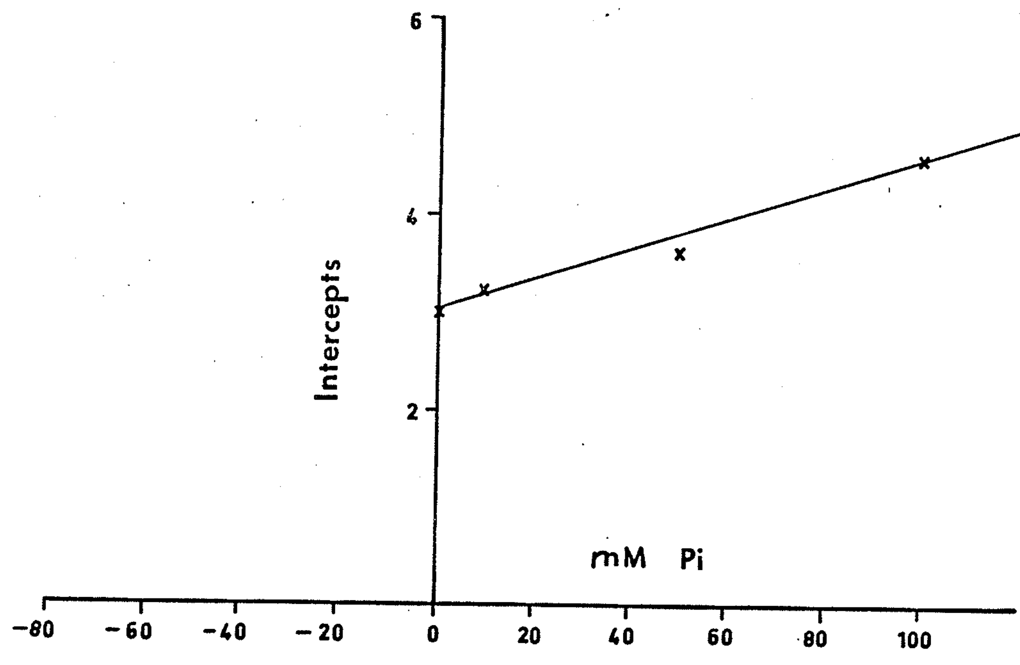
$P_i$ concentrations:

× - 0 mM
o - 10 mM
Δ - 50 mM
□ - 100 mM

FIGURE 26. Replot of intercepts versus $P_i$ concentration from Fig. 25 in which pyruvate was varying and $P_i$ the fixed variable product.



8
6
4
2
1/V
0
1
2
3
4
1/S mM pyruvate

6
4
2
Intercepts
mM Pi
-80
-60
-40
-20
0
20
40
60
80
100

4. ADP : ATP

When ATP was varied at several fixed concentrations of ADP, the double reciprocal plots were linear (Fig. 27) and indicated competitive inhibition; ie. only the slopes were affected by addition of inhibitor. The replot of slopes (Fig. 28) was linear and resulted in a $K_{i_S}$ value of 0.15 mM.

5. ADP : bicarbonate

When bicarbonate was the variable substrate at several fixed concentrations of ADP, the double reciprocal plots were linear (Fig. 29) and indicated uncompetitive inhibition. The replot of intercepts (Fig. 30) was linear and resulted in a $K_{i_I}$ value of 0.7 mM.

6. ADP : pyruvate

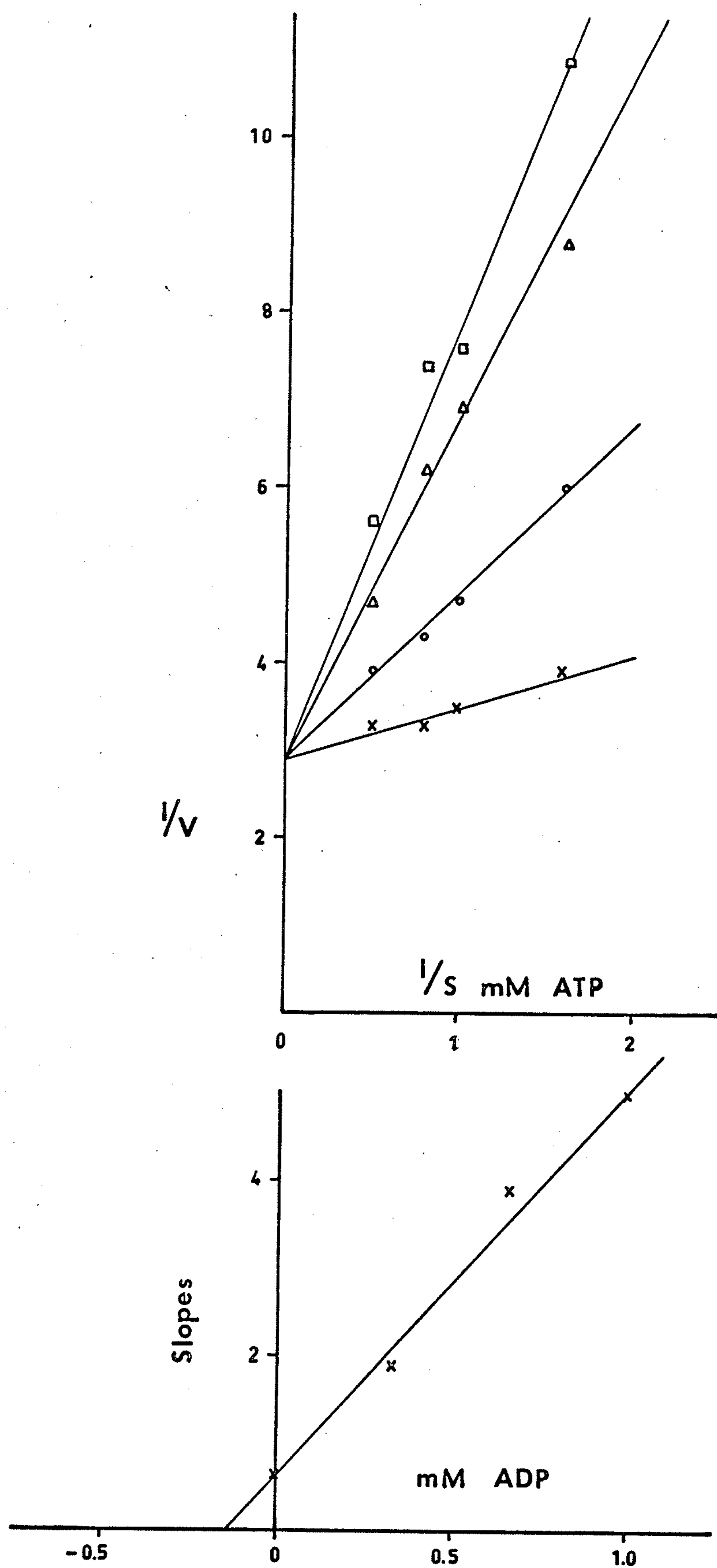
When pyruvate was varied at several fixed concentrations of ADP, the double reciprocal plots were linear (Fig. 31) and indicated uncompetitive inhibition. The replot of intercepts versus ADP concentration was linear (Fig. 32) and resulted in a $K_{i_I}$ of 1.1 mM.

FIGURE 27. Double reciprocal plots of velocity versus varying ATP concentrations with ADP as the fixed variable product.

ADP concentrations:

- x - 0 mM
- o - 0.33 mM
- Δ - 0.67 mM
- □ - 1.0 mM

FIGURE 28. Replot of slopes versus ADP concentration from Fig. 27 in which ATP was varying and ADP the fixed variable product.



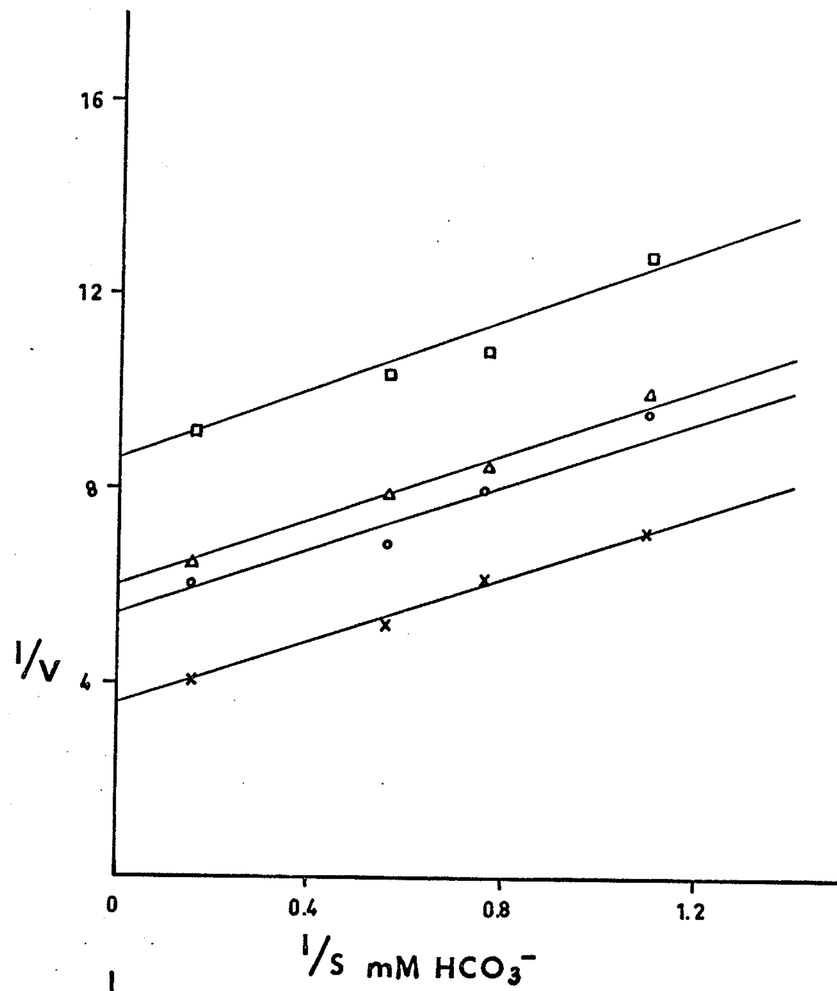
10
8
6
4
2
1/v
1/s mM ATP
0
1
2
4
2
Slopes
mM ADP
-0.5
0
0.5
1.0

FIGURE 29. Double reciprocal plots of velocity versus varying bicarbonate concentrations with ADP as the fixed variable product. Endogenous bicarbonate was estimated at 0.8 mM.

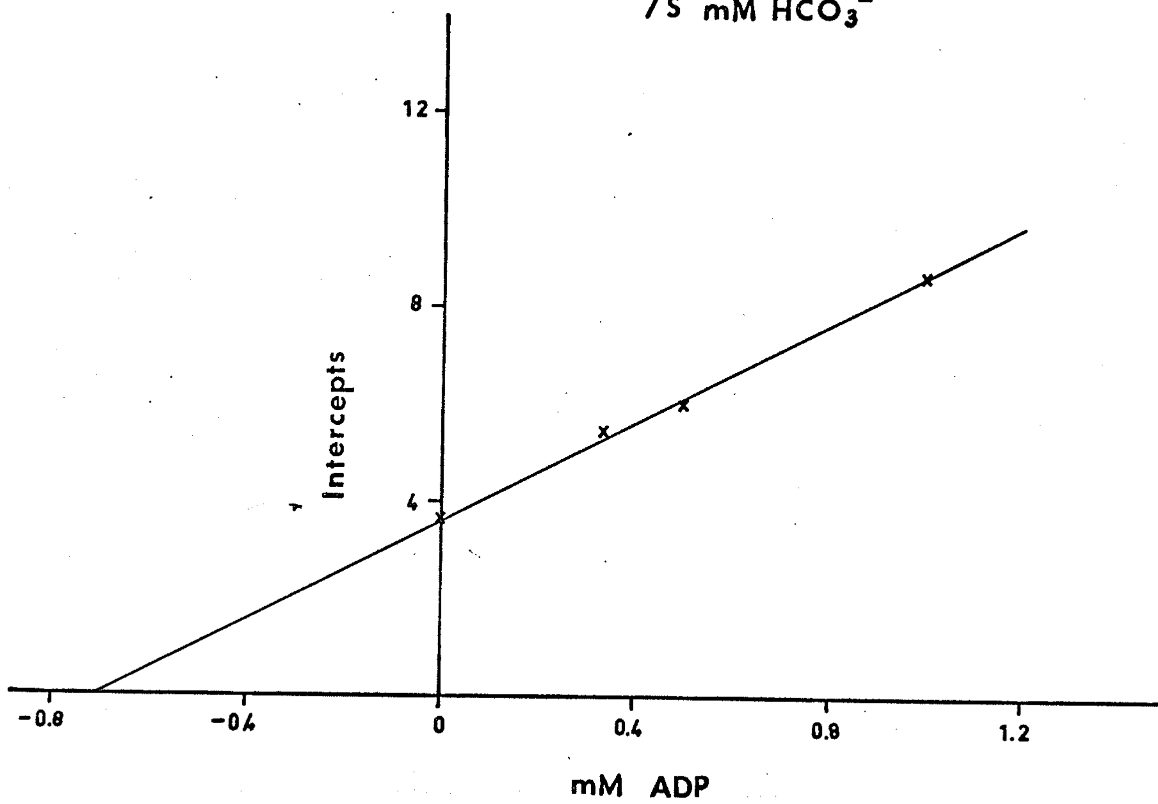
ADP concentrations:

× - 0 mM
○ - 0.33 mM
Δ - 0.50 mM
□ - 1.0 mM

FIGURE 30. Replot of intercepts versus ADP concentration from Fig. 29 in which bicarbonate was varying and ADP the fixed variable product.



16
12
8
4
1/V
0
0.4
0.8
1.2
1/S mM $HCO_3^-$

12
8
4
Intercepts
-0.8
-0.4
0
0.4
0.8
1.2
mM ADP

FIGURE 31. Double reciprocal plots of velocity versus varying pyruvate concentrations with ADP as the fixed variable product.

ADP concentrations:

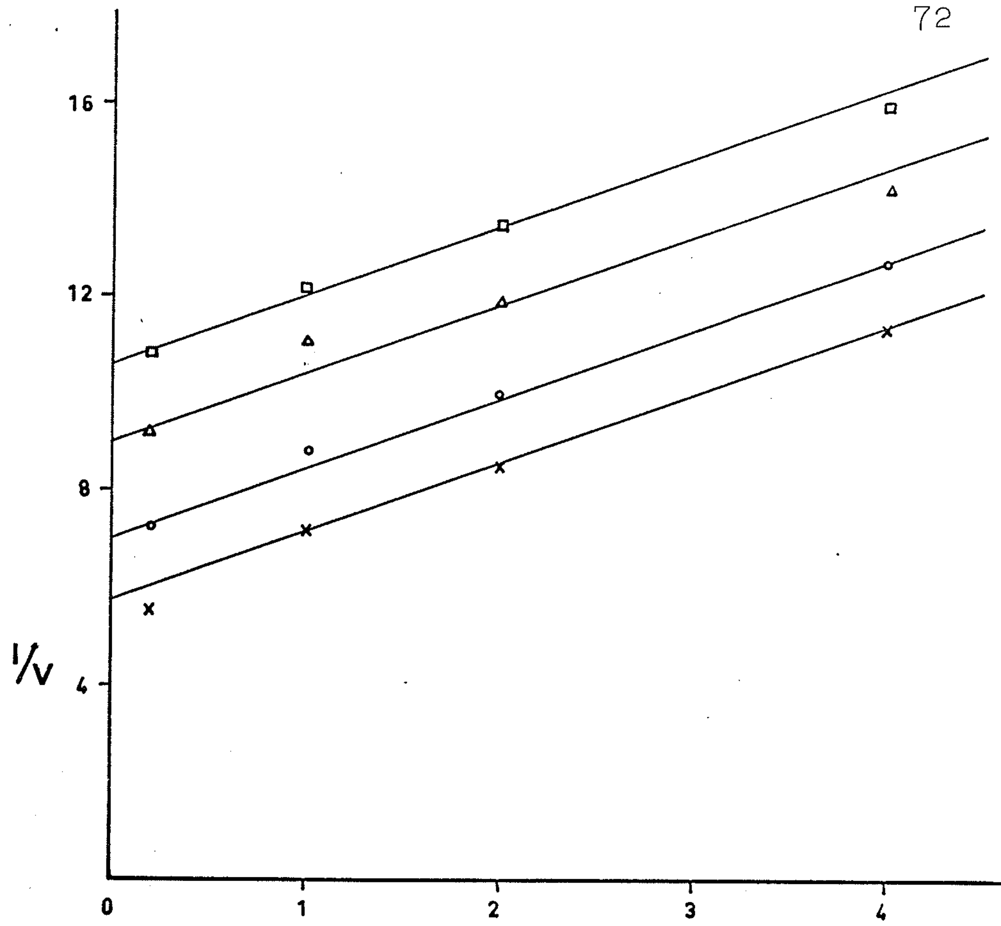
× - 0 mM

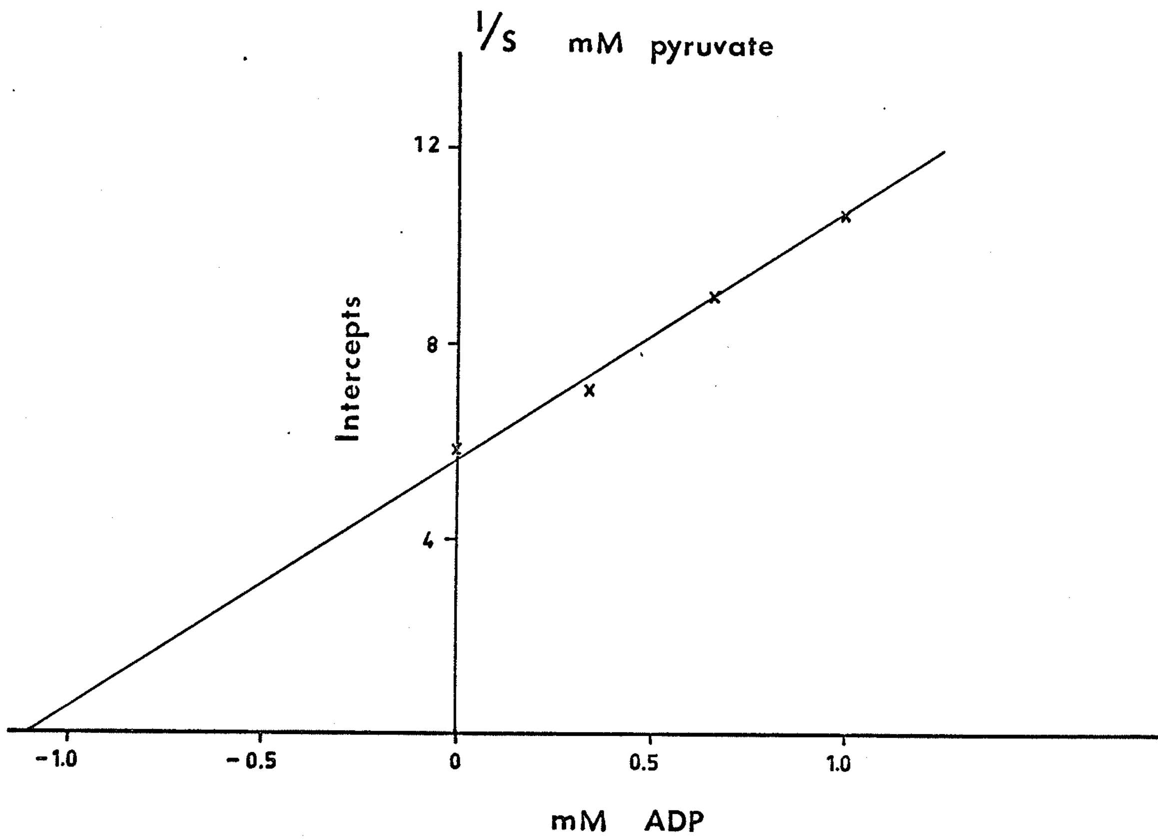
○ - 0.33 mM

△ - 0.67 mM

□ - 1.0 mM

FIGURE 32. Replot of intercepts versus ADP concentration from Fig. 31 in which pyruvate was varying and ADP the fixed variable product.


16
12
8
4
1/V
0
1
2
3
4
1/S mM pyruvate


12
8
4
Intercepts
-1.0
-0.5
0
0.5
1.0
mM ADP

7. Oxalacetate : ATP

When ATP was varied at several fixed concentrations of oxalacetate, the double reciprocal plots were linear (Fig. 33) and indicated uncompetitive inhibition. The replot of intercepts was linear also (Fig. 34) and resulted in a $K_{i_I}$ of 0.48 mM.

8. Oxalacetate : bicarbonate

When bicarbonate was varied at several fixed concentrations of oxalacetate, the double reciprocal plots were linear (Fig. 35) and indicated competitive inhibition. The replot of slopes versus oxalacetate concentration was linear (Fig. 36) and a $K_{i_S}$ of 0.21 mM was calculated from it.

9. Oxalacetate : pyruvate

When pyruvate was varied at several fixed concentrations of oxalacetate, the double reciprocal plots were linear (Fig. 37) and indicated competitive inhibition. A replot of slopes yielded a $K_{i_S}$ value of 0.12 mM (Fig. 38).

Inhibitor Studies

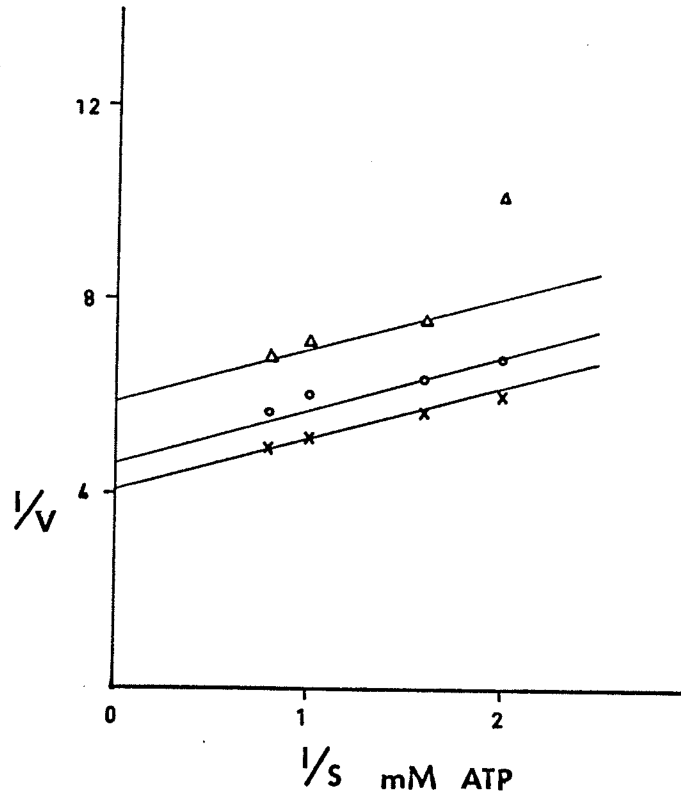
Studies were carried out to characterize the type of

FIGURE 33. Double reciprocal plots of velocity versus varying ATP concentrations with oxalacetate as the fixed variable product.

Oxalacetate concentrations:

× - 0 mM
o - 0.1 mM
Δ - 0.2 mM

FIGURE 34. Replot of intercepts versus oxalacetate concentration from Fig. 33 in which ATP was varying and oxalacetate the fixed variable product.


12
8
4
1/V
0
1
2
1/S mM ATP

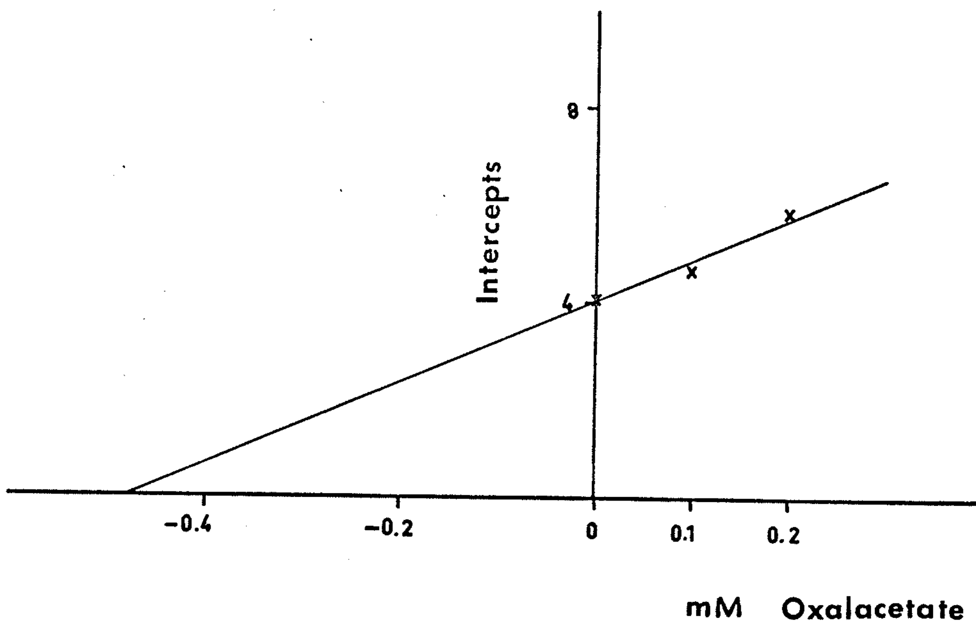

8
Intercepts
4
-0.4
-0.2
0
0.1
0.2
mM Oxalacetate

FIGURE 35. Double reciprocal plots of velocity versus varying bicarbonate concentrations with oxalacetate as the fixed variable product. Endogenous bicarbonate was estimated at 0.5 mM.

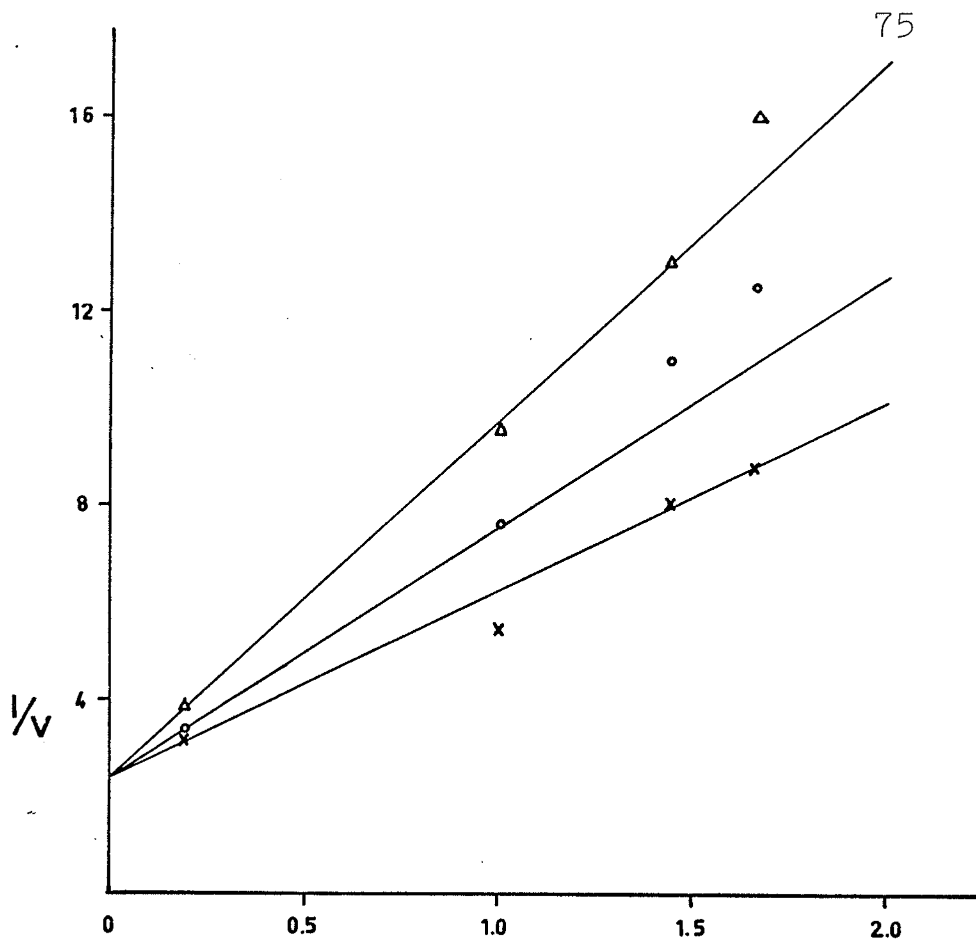
Oxalacetate concentrations:

× - 0 mM

o - 0.1 mM

Δ - 0.2 mM

FIGURE 36. Replot of slopes versus oxalacetate concentration from Fig. 35 in which bicarbonate was varying and oxalacetate the fixed variable product.


16
12
8
4
1/V
0
0.5
1.0
1.5
2.0
1/S mM HCO3⁻

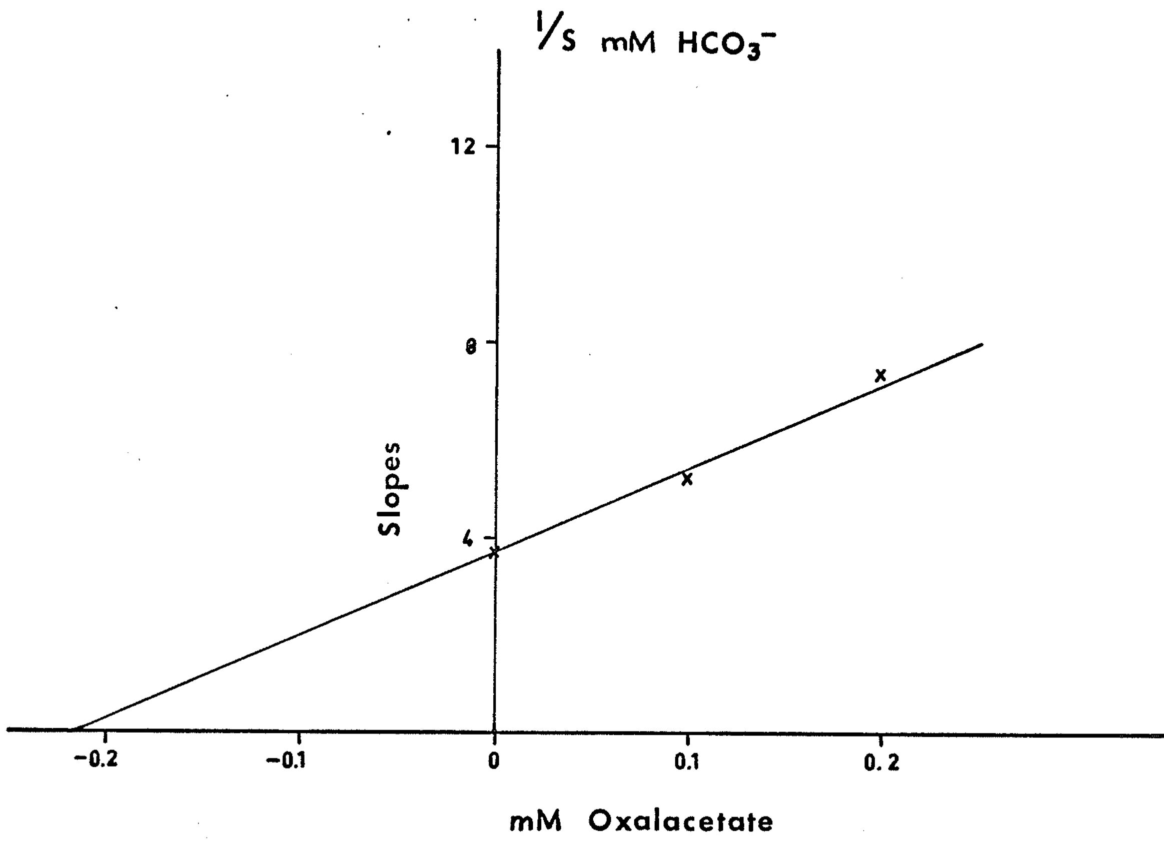

12
8
4
Slopes
-0.2
-0.1
0
0.1
0.2
mM Oxalacetate

FIGURE 37. Double reciprocal plots of velocity versus varying pyruvate concentrations with oxalacetate as the fixed variable product.

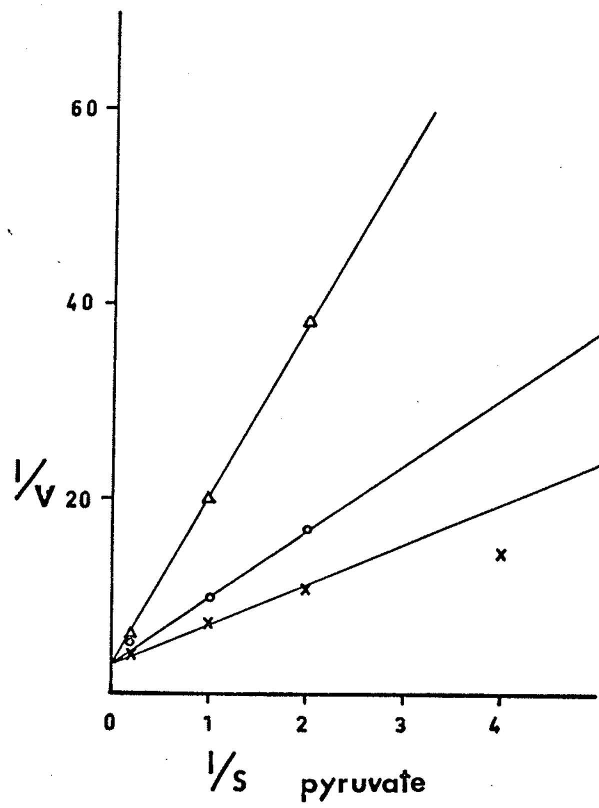
Oxalacetate concentrations:

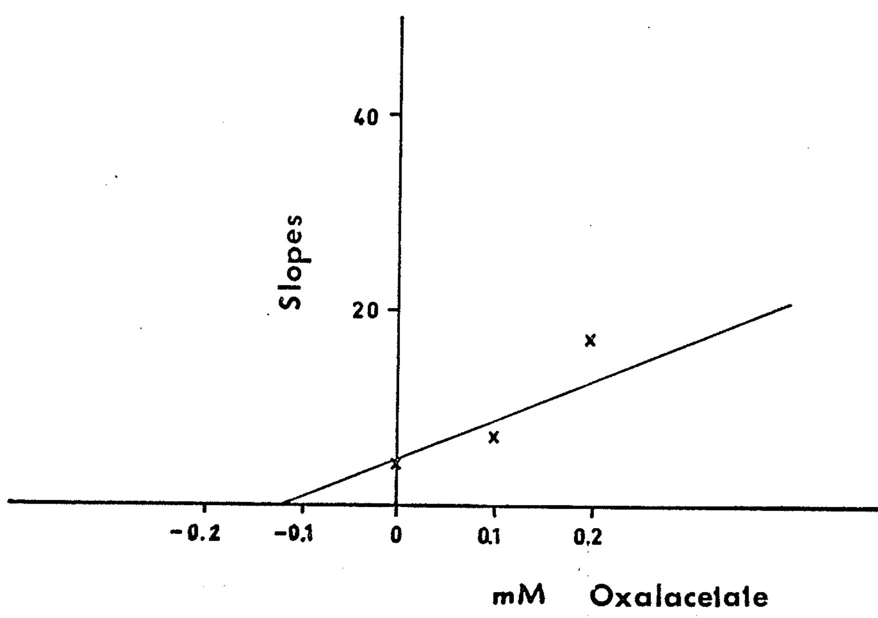
× - 0 mM

o - 0.1 mM

Δ - 0.2 mM

FIGURE 38. Replot of slopes versus oxalacetate concentration from Fig. 37 in which pyruvate was varying and oxalacetate the fixed variable product.


60
40
1/V 20
0
1
2
3
4
1/S pyruvate


40
Slopes
20
-0.2
-0.1
0
0.1
0.2
mM Oxalacetate

inhibition brought about by oxalic acid. When pyruvate was the varied substrate at several fixed concentrations of mesoxalate, the double reciprocal plots were linear (Fig. 39) and indicated noncompetitive inhibition. The $K_{i_I}$ value was determined to be 0.24 mM.

The inhibition of pyruvate carboxylase by L-aspartic acid was also studied. The double reciprocal plots of velocity versus pyruvate concentration at several fixed concentrations of L-aspartate were linear (Fig. 40) and indicated noncompetitive inhibition. The $K_{i_I}$ value was calculated to be 5.0 mM. D-aspartic acid, at a concentration of 2.5 mM, had no effect on the activity of pyruvate carboxylase.

FIGURE 39. Double reciprocal plots of velocity versus varying pyruvate concentrations with mesoxalate as the fixed variable inhibitor.

Mesoxalate concentrations:

× – 0 mM

o – 0.1 mM

Δ – 0.15 mM

□ – 0.2 mM

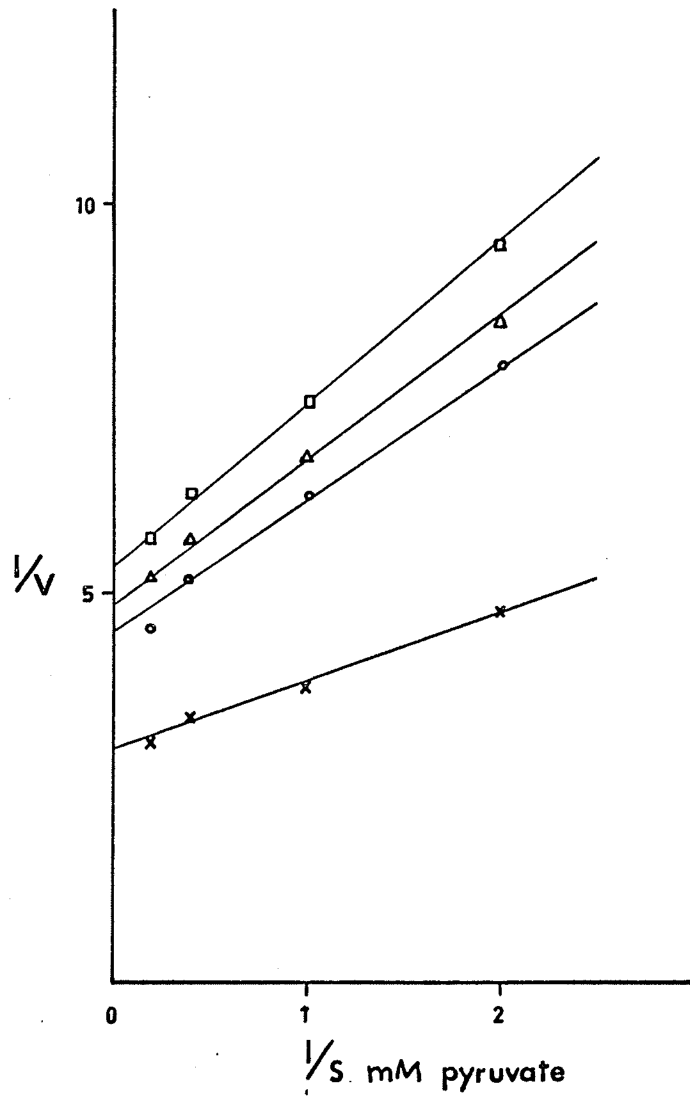

10
5
1/V
0
1
2
1/S. mM pyruvate

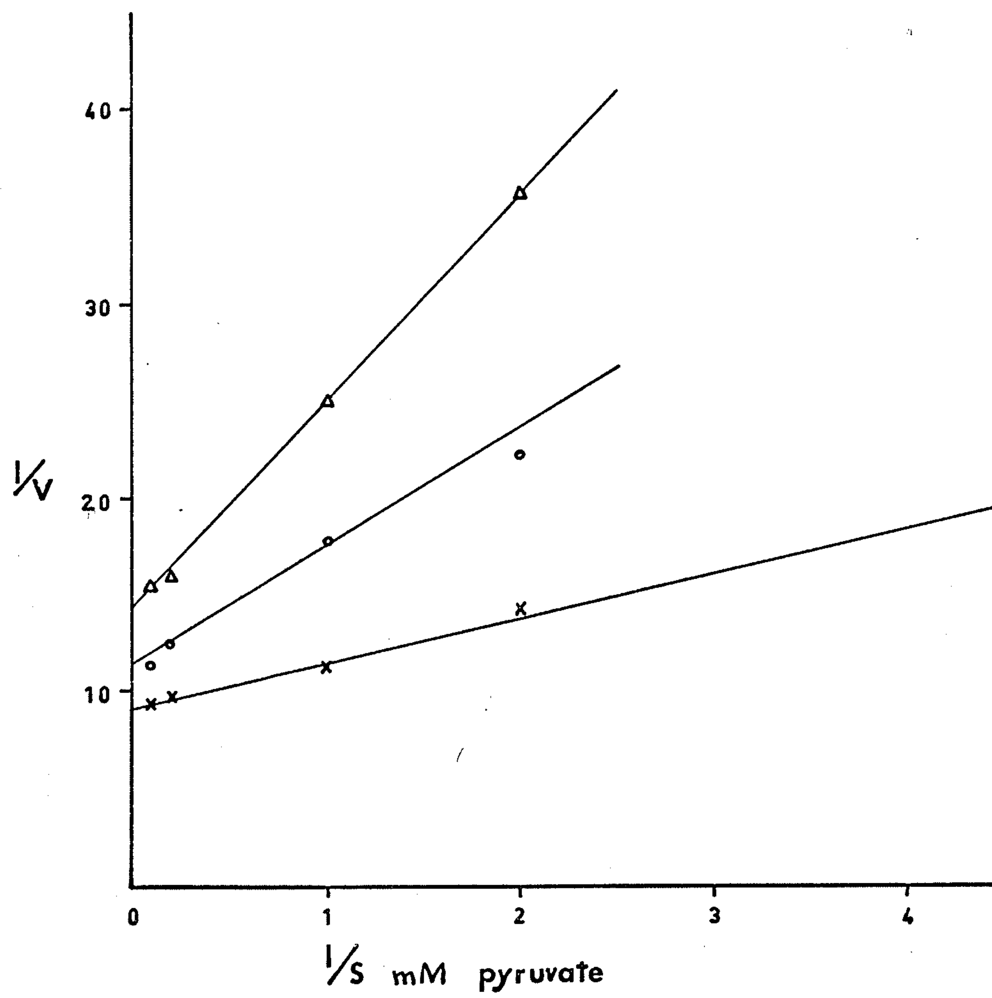
FIGURE 40. Double reciprocal plots of velocity versus varying pyruvate concentrations with L-aspartate as the fixed variable inhibitor.

L-aspartate concentrations:

× - 0 mM

○ - 1.5 mM

Δ - 3.0 mM



1/V
40
30
20
10
0
1
2
3
4
1/S mM pyruvate

# DISCUSSION

# DISCUSSION

## Role of Pyruvate Carboxylase in Citrate Accumulation

This study was begun to investigate the possibility that the enzyme, pyruvate carboxylase, might have some role in the accumulation of citrate by certain strains of *Aspergillus niger*. The results of an experiment which had been designed to test this possibility were shown in Table II. They were, for the most part, inconclusive. A more detailed study would have been necessary to draw any definite conclusions. When grown in a 0.88% sucrose medium, however, the level of pyruvate carboxylase did not seem to be a function of strain, whether a citrate accumulating or non-accumulating strain. In the "high sucrose" medium, the pyruvate carboxylase level in the 3 non-accumulators, the strains of *A. flavus* and *A. ustis*, remained almost constant. This was not the case in the 2 citrate accumulating strains of *A. niger* where the enzyme level in the presence of 10% sucrose was lower by a factor of ten. There was a possibility that the lower specific activity could have been due to the destruction of the enzyme during extraction by the more acidic conditions in this high sucrose medium where,

presumably, citrate accumulation was occurring. However, when the pH of each extract was determined, it was found that it remained constant at approximately 7.5.

If it was assumed that pyruvate carboxylase played a role in citrate accumulation, then it would be expected that under growth conditions which included a high concentration of sucrose which was known to be favourable to citrate accumulation (Shu and Johnson, 1948), the level of pyruvate carboxylase would be greatly increased. These results indicated, however, that the opposite was true; that a high level of sucrose in fact decreased drastically the pyruvate carboxylase level. To test the possibility that excess citrate might be repressing pyruvate carboxylase activity, citrate in a final concentration of 1.0 mM was added to the usual reaction mixture. It was found to have no effect on pyruvate carboxylase activity.

The effect of $D_2O$ on both citrate accumulation and pyruvate carboxylase activity reported by Henderson and Lamonds (1966) may have been coincidental. The lack of citrate formation in the presence of $D_2O$ might have been due to the repression of another enzyme which was not tested for in this study.

Further experiments are indicated in order to verify the exact role of pyruvate carboxylase.

## Cold Inactivation and the Structure of Pyruvate Carboxylase

The enzyme isolated from *A. niger* displayed some similar physical characteristics to the enzyme from chicken liver (Scrutton and Utter, 1965a). Both were inactivated by the cold and showed subsequent thermal reactivation. Thus, the *A. niger* pyruvate carboxylase might also be expected to be composed of 4 subunits although sedimentation studies were not carried out.

This study showed that pyruvate carboxylase from *A. niger* was completely protected against the cold by 1.5 M sucrose and by high concentrations of glycerol. Thus, 25% buffered glycerol was used routinely for all extraction and purification procedures. The effect of glycerol and other such stabilizing agents is still obscure although Jarabak *et al*. (1966) have proposed that these compounds share the property of networks of "structured" water molecules which are necessary to maintain the correct spatial configuration of the protein in its native state.

Lack of Effect of Acetyl CoA

A striking feature of pyruvate carboxylase as isolated from different sources is the variability of the effect of acetyl CoA. Utter and Keech (1960) found that it was absolutely required by the enzyme from chicken liver. This absolute requirement was found to be shared by the sheep kidney enzyme (Ling and Keech, 1966). Acetyl CoA stimulation of activity has been observed in rat liver (Henning and Seubert, 1964) and baker's yeast (Ruiz-Amil *et al*., 1965; Cooper and Benedict, 1966). Pyruvate carboxylase from *Pseudomonas citronellolis* (Seubert and Remberger, 1961) was not affected by acetyl CoA. These studies showed a lack of effect of acetyl CoA on the enzyme from *A. niger* as well and thus confirmed the results of Bloom and Johnson (1962).

In the various sources of pyruvate carboxylase where acetyl CoA exerts some influence, either absolute or stimulatory, it offers an excellent means of control for the formation of oxalacetate and thus influences the rates of the initial steps in the Krebs cycle. An accumulation of acetyl CoA from pyruvate or fatty acids would activate or stimulate the formation of oxalacetate and the further

oxidation of acetyl CoA. The variability of effect would be a simple reflection of the evolution of a more complex system for controlling the activity of pyruvate carboxylase.

Inhibition by Avidin and Protection by Biotin

Pyruvate carboxylase isolated from *A. niger* is inhibited by avidin. This fact indicates that it is one of the biotin-containing enzymes as is the pyruvate carboxylase from any source thus far tested.

In 1963, Keech and Utter noted that the $CO_2$-fixing enzymes could be divided into 2 groups on the basis of whether or not they contained biotin and were inhibited by avidin. They suggested that those reactions which were inhibited by avidin were those in which acyl CoA compounds were involved. This latter statement is not true, however, since pyruvate carboxylase from *Ps. citronellolis* (Seubert and Remberger, 1961) and from *A. niger*, as shown by this study, does not involve any acyl CoA compounds.

The work done by Bloom and Johnson (1962) indicated almost complete inhibition by avidin and complete protection by preincubation with biotin. The results of this study confirmed these findings. Utter and Keech's work with the

enzyme from chicken liver (1963) also showed the complete inhibition or protection by avidin or biotin respectively. Avidin inhibition of pyruvate carboxylase from sheep kidney, however, has been found to differ quantitatively from that of the enzyme from these sources (Ling and Keech, 1966). They found 16% residual activity even with amounts of avidin greater than 1 unit per 180 μg of enzyme protein. Also, even when avidin was preincubated with concentrations of biotin 90 times greater than the theoretical amount required for the saturation of all biotin-binding sites on the avidin, there was still a 60% loss in enzyme activity. To determine this theoretical value, these workers have used the assumption that 4 moles of biotin are bound per mole of avidin (Green, 1964).

These results have not as yet been explained. Ling and Keech (1966) suggest that the difference may be in the pH of the mixture used for preincubation of biotin with avidin. It is possible that a different pH could affect the conformation of the avidin molecule and thus the biotin-binding sites that are exposed. They also suggest that their enzyme may bind non-specifically with commercial avidin; ie. inhibition not prevented by excess biotin.

Kinetics and Mechanism of Pyruvate Carboxylase

In the discussion of kinetic results, the terminology and analytical methods used were those of Cleland (1963a, b, c). A summary of these results has been presented in Table VI. A comparison of Michaelis and inhibitor constants for pyruvate carboxylase from *A. niger*, as determined in this study, with those from other sources of the enzyme has been presented in Table VII. The results for the enzyme from baker's yeast were those of Ruiz-Amil *et al*. (1965) and of Palacián *et al*. (1966). The work done on pyruvate carboxylase from chicken liver was that of Scrutton and Utter (1965a, b) and Mildvan *et al*. (1966), and that on the enzyme from sheep kidney by Ling and Keech (1966). It can be seen that the values are generally of the same order of magnitude. The $K_m$ for $Mg^{++}$ from the *A. niger* enzyme is smaller than that from the other sources. The $K_i$ for mesoxalate is much smaller in the case of *A. niger* when compared with that from chicken liver.

The kinetic results obtained could not be explained by a mechanism in which there is only one active site on the enzyme that can be operative at one time as is assumed

Table VI

Summary of kinetic data for pyruvate carboxylase from *A. niger*.

| | Intercept | Slope | Inhibition |
|---|---|---|---|
| Substrates | | | |
| ATP - $HCO_3^-$ | change | change | |
| ATP - pyr | change | no change | |
| pyr - $HCO_3^-$ | change | change | |
| Product Inhibition | | | |
| ATP - ADP | no change | change | C |
| ATP - $P_i$ | change | change | NC |
| ATP - OAA | change | no change | UC |
| $HCO_3^-$ - ADP | change | no change | UC |
| $HCO_3^-$ - $P_i$ | change | change | NC |
| $HCO_3^-$ - OAA | no change | change | C |
| pyr - ADP | change | no change | UC |
| pyr - $P_i$ | change | no change | UC |
| pyr - OAA | no change | change | C |

pyr = pyruvate, OAA = oxalacetate, C = competitive, NC = noncompetitive, UC = uncompetitive

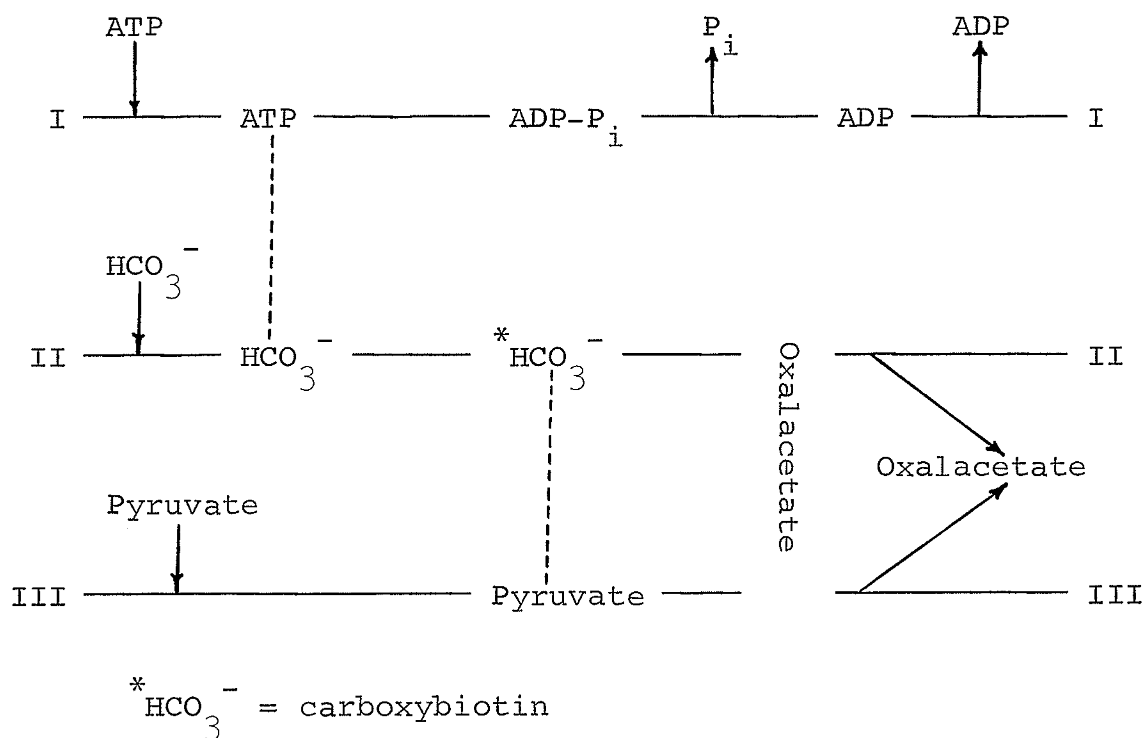
Table VII

Comparison of Michaelis and inhibitor constants for pyruvate carboxylase from several sources.

| | *A. niger* | Baker's yeast | Chicken liver | Sheep kidney |
|---|---|---|---|---|
| Michaelis constants (mM) | | | | |
| pyruvate | 0.28 | 0.80 | 0.44 | 0.43 |
| bicarbonate | 1.33 | 2.7 | 1.0 | 0.62 |
| ATP | 0.23 | 0.24 | 0.058 | 0.17 |
| $Mg^{++}$ | 0.69 | 4.2 | 2.8 | 2.5 |
| acetyl CoA | no effect | stimulation | absolute requirement | absolute requirement |
| Inhibitor constants (mM) | | | | |
| oxalacetate | 0.12 | 0.22 | | |
| ADP | 0.15 | | 0.075 | |
| mesoxalate | 0.24 | | 2.1 | |
| L-aspartate | 5.0 | 1.9 | | |

for all the kinetic discussions by Cleland. The results can be interpreted satisfactorily, however, by assuming three separate sites for the three substrates; ATP, bicarbonate and pyruvate. Each site is specific for its particular substrate and can be occupied by that substrate independent of the addition of other substrates to their respective sites. This interpretation does not conflict with a mechanism for biotin enzymes proposed by Ochoa and Kaziro (1961) and by Green (1963). It is suggested that the enzyme-bound biotin mediates the transfer of bicarbonate between the ATP and pyruvate sites with the intermediate formation of carboxybiotin. The mechanism is also consistent with a conclusion that all of the reaction components of pyruvate carboxylase can form binary complexes with the enzyme (Scrutton and Utter, 1965b; Mildvan _et al_., 1966).

If it is assumed that there are three separate sites, I, II and III, then the reaction catalyzed by pyruvate carboxylase may be visualized as follows (Scheme 1):




$^{*}HCO_3^-$ = carboxybiotin

Scheme 1

The reaction at site I is essentially a UniBi reaction with ATP as substrate and $P_i$ and ADP as products. The noncompetitive inhibition by $P_i$ and competitive inhibition by ADP obtained when ATP was the variable substrate fit this mechanism.

The reaction at site II is a UniUni reaction with bicarbonate as substrate and oxalacetate as product. This site interacts with site I through enzyme-bound ATP and enzyme-bound bicarbonate. Since this interaction is

essential for the hydrolysis of ATP to ADP and $P_i$ and also for the formation of carboxybiotin-enzyme from bicarbonate, the ATP concentration should affect both the slope and intercept of 1/v versus $1/HCO_3^-$ plots and vice versa. The results of the present study indicate that these conclusions are correct. Oxalacetate should give a competitive type of inhibition when bicarbonate is used as variable substrate. Noncompetitive and uncompetitive inhibitions are expected when $P_i$ and ADP respectively are used as inhibitors. In the former case, there is a reversible sequence between the points of combination of bicarbonate and $P_i$, but in the latter, the reversibility is broken by the release of $P_i$ between bicarbonate and ADP. Again, these expected results have been verified by this study.

The reaction at site III is again a UniUni reaction with pyruvate as substrate and oxalacetate as product. Oxalacetate inhibits the reaction competitively when pyruvate is the variable substrate since they both bind the same site and form of the enzyme. Site III interacts with site II through enzyme-bound pyruvate and enzyme-bound carboxy-biotin. The interaction leads to the formation of oxalacetate which occupies the two sites. Since this

interaction is brought about by the movement of carboxybiotin from site I to site III; a transfer which is mediated by a long flexible arm of a lysine group; the connection between site I and site II is broken before a connection between site II and site III is established. This leads to the obvious conclusion that there is no connection between site I and site III at any time during the pyruvate carboxylase reaction. Thus there is a reversible connection between the points of combination of bicarbonate and pyruvate but not between those of ATP and pyruvate. Pyruvate concentration therefore affects both the slope and intercept of 1/v versus $1/HCO_3^-$ plots but only the intercept of 1/v versus 1/ATP plots. The uncompetitive inhibition by $P_i$ and ADP which is observed when pyruvate is the variable substrate may also be explained by the non-interaction of site I and site III. The same reasoning applies to the uncompetitive inhibition by oxalacetate when ATP is the variable substrate.

The noncompetitive inhibition by mesoxalate or L-aspartate with pyruvate as the variable substrate suggests that these compounds may possibly bind to the bicarbonate site (site II).

Thus, the scheme presented satisfactorily explains all the kinetic results obtained in the study of A. niger pyruvate carboxylase. The mechanism of action may be represented pictorially as in Fig. 41.

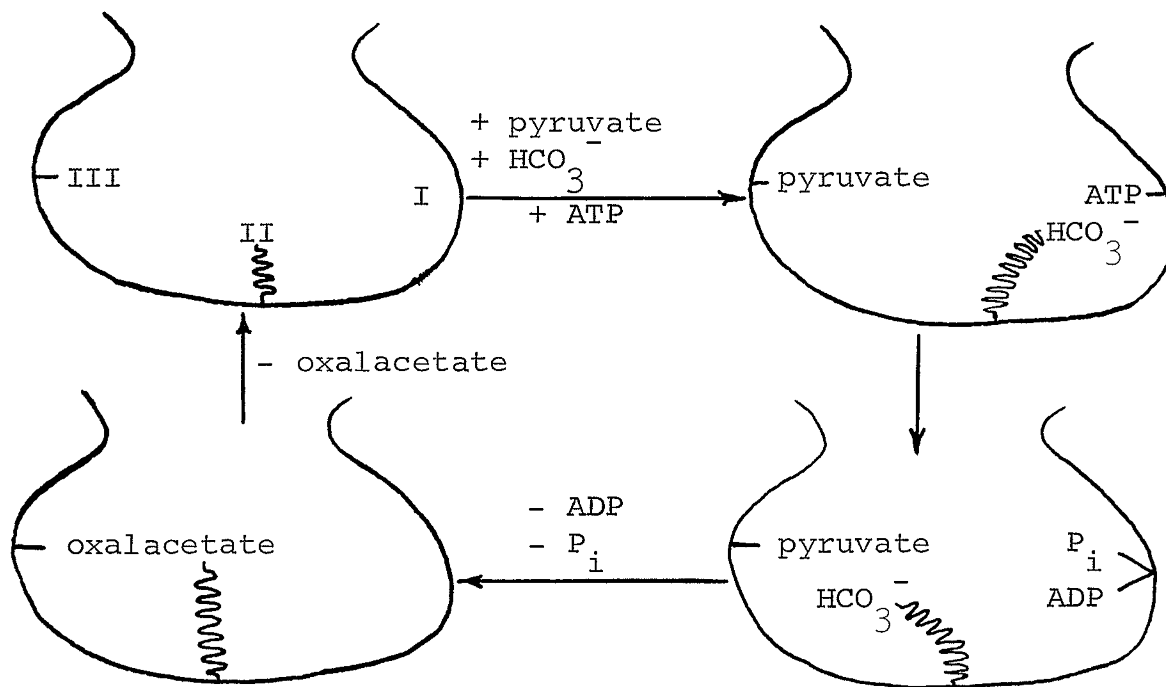



FIGURE 41. Diagram proposed to illustrate the mechanism of action of pyruvate carboxylase from A. niger

Some work has been done on product inhibition studies with this enzyme. In 1962, Bloom and Johnson, working with

the enzyme from _A. niger_, reported that ADP, when used in the same concentration as ATP, had no effect on pyruvate carboxylase activity. The results of this study, however, indicated that when the concentrations of both ATP and ADP were 1.0 mM, the inhibition obtained was greater than 50%. There was no satisfactory explanation for this discrepancy. The work of Keech and Utter (1963) with pyruvate carboxylase from chicken liver indicated that ADP gave a competitive type of inhibition with respect to ATP, although only at low concentrations of ADP. They felt that the kinetics at higher concentrations were involved and not subject to simple interpretation. In this study, the inhibition by ADP when ATP was the variable substrate was also found to be competitive in nature.

Inorganic phosphate in a concentration of 50 mM has been found to give approximately 50% inhibition with the enzyme from _A. niger_ (Bloom and Johnson, 1962). The results of this study were comparable. An inhibition of approximately 35% was found with 50 mM $P_i$.

Previous work with the inhibition by oxalacetate when pyruvate was the variable substrate (Palacián _et al_., 1966) showed that it was competitive in nature. A $K_i$

value of 0.22 mM was determined. From this present study, the inhibition by oxalacetate with pyruvate as the variable substrate was also found to be competitive. A $K_i$ value of 0.12 mM was determined.

Oxalate inhibition of pyruvate carboxylase from baker's yeast was first reported by Losada et al. (1964) with concentrations of oxalate as low as 0.05 mM. A more detailed study indicated noncompetitive inhibition by oxalate when pyruvate was the variable substrate (Ruiz-Amil et al., 1965). The $K_i$ was determined to be 0.07 mM. Mildvan et al. (1966), working with pyruvate carboxylase from chicken liver found that the type of inhibition depended on whether oxalate or mesoxalate was used. They classified oxalate as a Type I inhibitor. As such, it produced uncompetitive inhibition with respect to pyruvate and competitive inhibition with respect to oxalacetate. A Type II inhibitor such as mesoxalate resulted in noncompetitive inhibition with respect to both pyruvate and oxalacetate. The report of Ruiz-Amil et al. (1965) did not state if they used mesoxalate. If oxalate itself was used, their finding of noncompetitive inhibition with respect to pyruvate does not agree with the uncompetitive inhibition

reported by Mildvan *et al*. (1966). The results of the present study indicated that mesoxalate yielded noncompetitive inhibition when pyruvate was the variable substrate and were thus in agreement with those of Mildvan's group.

In 1966, working with the enzyme from baker's yeast, Palacián *et al*. reported noncompetitive inhibition by L-aspartate with respect to pyruvate. The value for $K_i$ was calculated to be 1.9 mM. The same type of inhibition was found in this study although the $K_i$ was 5.0 mM. These workers also found that D-aspartate in a concentration of 2.5 mM did not inhibit pyruvate carboxylase. Similar results were obtained in this study. Since oxalacetate can be used to synthesize aspartate, Palacián's group considered pyruvate carboxylase to be the first enzyme in the pathway from pyruvate to aspartate. Thus, inhibition by aspartate was thought to be allosteric inhibition by an end-product.

Cleland has suggested the possibility of enzymes' having more than one active site operating at one time for complex reactions. He has, however, limited his discussion to reaction mechanisms where there is only one active site. There has been no report of kinetic studies conducted on an

enzyme with 2 or more active centres. Our effort to derive a rate equation that describes the pyruvate carboxylase reaction has been unsuccessful. However, the application of analytical principles laid down by Cleland (1963c) on kinetic studies of initial velocity and product inhibition proved successful in elucidating the reaction mechanism of a complex system in the present study.

# REFERENCES

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Bandurski, R.S. and Greiner, C.M. (1953). The enzymatic synthesis of oxalacetate from phosphoryl-enolpyruvate and carbon dioxide. J. Biol. Chem. 204: 781-786.

Barritt, G.J., Keech, D.B. and Ling, A.-M. (1966). Apparent co-operative effect of acetyl-CoA on sheep kidney pyruvate carboxylase. Biochem. Biophys. Res. Commun. 24: 476-481.

Bloom, S.J. and Johnson, M.J. (1962). The pyruvate carboxylase of _Aspergillus niger_. J. Biol. Chem. 237: 2718-2720.

Cleland, W.W. (1963a). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim. Biophys. Acta. 67: 104-137.

Cleland, W.W. (1963b). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. Biochim. Biophys. Acta. 67: 173-187.

Cleland, W.W. (1963c). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. Biochim. Biophys. Acta. 67: 188-196.

Cooper, T.G. and Benedict, C.R. (1966). The participation of acetyl-CoA in pyruvate carboxylase. Biochem. Biophys. Res. Commun. 22: 285-290.

Felicioli, R.A., Gabrielli, F. and Rossi, C.A. (1967). Pyruvate carboxylase activity of mammalian brain cortex. Life Sci. 6: 133-144.

Florini, J.R. and Vestling, C.S. (1957). Graphical determination of the dissociation constants for two-substrate enzyme systems. Biochim. Biophys. Acta.

25: 575-578.

Gailiusis, J., Rinne, R.W. and Benedict, C.R. (1964). Pyruvate-oxaloacetate exchange reaction in baker's yeast. Biochim. Biophys. Acta. 92: 595-601.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S. and Singh, R.M.M. (1966). Hydrogen ion buffers for biological research. Biochemistry, 5: 467-477.

Green, N.M. (1963). Avidin. I. The use of [$^{14}$C] biotin for kinetic studies and for assay. Biochem. J. 89: 585-591.

Green, N.M. (1964). The molecular weight of avidin. Biochem. J. 92: 16C-17C.

Henderson, T.R. and Lamonds, M.R. (1966). Effects of $D_2O$ on citric acid and pyruvate carboxylase formation by *Aspergillus niger*. Arch. Biochem. Biophys. 115: 187-191.

Henning, H.V., Huth, W. and Seubert, W. (1964). An *in vitro* effect of cortisol on pyruvate carboxylase and gluconeogenesis. Biochem. Biophys. Res. Commun. 17: 496-501.

Henning, H. and Seubert, W. (1964). Zum Mechanismus der Gluconeogenese und ihrer Steuerung. I. Quantitative Bestimmung der Pyruvatcarboxylase in Rohextrakten der Rattenleber. Biochem. Z. 340: 160-170.

Henning, H.V., Stumpf, B., Ohly, B. and Seubert, W. (1966). On the mechanism of gluconeogenesis and its regulation. III. The glucogenic capacity and the activities of pyruvate carboxylase and PEP-carboxylase of rat kidney and rat liver after cortisol treatment and starvation. Biochem. Z. 344: 274-288.

Jarabak, J., Seeds, Jr., A.E. and Talalay, P. (1966). Reversible cold inactivation of a 17β-hydroxysteroid dehydrogenase of human placenta: protective effect of

glycerol. Biochemistry, 5: 1269-1279.

Kaltenbach, J.P. and Kalnitsky, G. (1951a). The enzymatic formation of oxalacetate from pyruvate and carbon dioxide. I. J. Biol. Chem. 192: 629-639.

Kaltenbach, J.P. and Kalnitsky, G. (1951b). The enzymatic formation of oxalacetate from pyruvate and carbon dioxide. II. J. Biol. Chem. 192: 641-649.

Karow, E.O. and Waksman, S.A. (1947). Production of citric acid in submerged culture. Ind. Eng. Chem. 39: 821-825.

Kaziro, Y. and Ochoa, S. (1961). Mechanism of the propionyl carboxylase reaction. I. Carboxylation and decarboxylation of the enzyme. J. Biol. Chem. 236: 3131-3136.

Keech, B. and Barritt, G.J. (1967). Allosteric activation of sheep kidney pyruvate carboxylase by the magnesium ion ($Mg^{2+}$) and the magnesium adenosine triphosphate ion ($MgATP^{2-}$). J. Biol. Chem. 242: 1983-1987.

Keech, D.B. and Utter, M.F. (1963). Pyruvate carboxylase. II. Properties. J. Biol. Chem. 238: 2609-2614.

Krampitz, L.O., Wood, H.G. and Werkman, C.H. (1943). Enzymatic fixation of carbon dioxide in oxalacetate. J. Biol. Chem. 147: 243-253.

la Nauze, J.M. (1966). Aconitase and isocitric dehydrogenases of *Aspergillus niger* in relation to citric acid production. J. Gen. Microbiol. 44: 73-81.

Lardy, H.A., Potter, R.L. and Elvehjem, C.A. (1947). The role of biotin in bicarbonate utilization by bacteria. J. Biol. Chem. 169: 451-452.

Layne, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. In Methods in Enzymology, Vol. III, S.P. Colowick and N.O. Kaplan (eds.), Academic Press, Inc., New York, pp. 447-454.

Lineweaver, H. and Burk, O. (1934). The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658-666.

Ling, A.-M. and Keech, D.B. (1966). Pyruvate carboxylase from sheep kidney. I. Purification and some properties of the enzyme. Enzymologia, XXX: 367-380.

Losada, M., Cánovas, J.L. and Ruiz-Amil, M. (1964). Oxalacetate, citramalate and glutamate formation from pyruvate in baker's yeast. Biochem. Z. 340: 60-74.

Marshall, K.C. and Alexander, M. (1962). Nitrification by *Aspergillus flavus*. J. Bacteriol. 83: 572-578.

Mildvan, A.S., Scrutton, M.C. and Utter, M.F. (1966). Pyruvate carboxylase. VII. A possible role for tightly bound manganese. J. Biol. Chem. 241: 3488-3498.

Ochoa, S. and Kaziro, Y. (1961). Biotin enzymes. Federation Proc. 20: 982-988.

Ochoa, S., Mehler, A.H. and Kornberg, A. (1948). Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidative decarboxylation of l-malic acid. J. Biol. Chem. 174: 979-1000.

Palacián, E., de Torrontegui, G. and Losada, M. (1966). Inhibition of yeast pyruvate carboxylase by L-aspartate and oxaloacetate. Biochem. Biophys. Res. Commun. 24: 644-649.

Ramakrishnan, C.V., Steel, R. and Lentz, C.P. (1955). Mechanism of citric acid formation and accumulation in *Aspergillus niger*. Arch. Biochem. Biophys. 55: 270-273.

Ruiz-Amil, M., de Torrontegui, G., Palacián, E., Catalina, L. and Losada, M. (1965). Properties and function of

yeast pyruvate carboxylase. J. Biol. Chem. 240: 3485-3492.

Scrutton, M.C., Keech, D.B. and Utter, M.F. (1965). Pyruvate carboxylase. IV. Partial reactions and the locus of activation by acetyl coenzyme A. J. Biol. Chem. 240: 574-581.

Scrutton, M.C. and Utter, M.F. (1964). Studies on the mechanism of action and temperature sensitivity of pyruvate carboxylase. Federation Proc. 23: 162.

Scrutton, M.C. and Utter, M.F. (1965a). Pyruvate carboxylase. III. Some physical and chemical properties of the highly purified enzyme. J. Biol. Chem. 240: 1-9.

Scrutton, M.C. and Utter, M.F. (1965b). Pyruvate carboxylase. V. Interaction of the enzyme with adenosine triphosphate. J. Biol. Chem. 240: 3714-3723.

Scrutton, M.C., Utter, M.F. and Mildvan, A.S. (1966). Pyruvate carboxylase. VI. The presence of tightly bound manganese. J. Biol. Chem. 241: 3480-3487.

Seubert, W. and Remberger, U. (1961). Reinigung und Wirkungweise der Pyruvatcarboxylase aus *Pseudomonas citronellolis*. Biochem. Z. 334: 401-414.

Shive, W. and Rogers, L.L. (1947). Involvement of biotin in the biosynthesis of oxalacetic and α-ketoglutaric acids. J. Biol. Chem. 169: 453-454.

Shu, P. and Johnson, M.J. (1947). Effect of the composition of sporulation medium on citrate production by *Aspergillus niger* in submerged culture. J. Bacteriol. 54: 161-167.

Shu, P. and Johnson, M.J. (1948). Citric acid: production by submerged fermentation with *Aspergillus niger*. Ind. Eng. Chem. 40: 1202-1205.

Utter, M.F. and Keech, D.B. (1960). Formation of oxalacetate from pyruvate and $CO_2$. J. Biol. Chem. 235: PC17.

Utter, M.F. and Keech, D.B. (1963). Pyruvate carboxylase. I. Nature of the reaction. J. Biol. Chem. 238: 2603-2608.

Utter, M.F., Keech, D.B. and Scrutton, M.C. (1964). A possible role for acetyl CoA in the control of gluconeogenesis. In Advan. Enz. Regul., Vol. 2, G. Weber (ed.), Pergamon Press, Oxford, pp. 49-68.

Utter, M.F. and Kurahashi, K. (1954). Purification of oxalacetic carboxylase from chicken liver. J. Biol. Chem. 207: 787-802.

Utter, M.F. and Wood, H.G. (1946). The fixation of $CO_2$ in oxalacetate by pigeon liver. J. Biol. Chem. 164: 455-476.

Valentine, R.C., Wrigley, N.G., Scrutton, M.C., Irias, J.J. and Utter, M.F. (1966). Pyruvate carboxylase. VIII. The subunit structure as examined by electron microscopy. Biochemistry, 5: 3111-3116.

von Loesecke, H.W. (1945). A review of information on mycological citric acid production. Chem. Eng. News, 23: 1952-1959.

Wagle, S.R. (1964). Studies on pyruvate carboxylase activity in alloxan diabetic and normal animals. Biochem. Biophys. Res. Commun. 14: 533-536.

Waite, M. and Wakil, S.J. (1962). Studies on the mechanism of fatty acid synthesis. XII. Acetyl coenzyme A carboxylase. J. Biol. Chem. 237: 2750-2757.

Wood, H.G. (1946). The fixation of carbon dioxide and the interrelationships of the tricarboxylic acid cycle. Physiol. Rev. 26: 198-246.

Wood, H.G., Allen, S.H.G., Stjernholm, R. and Jacobson, B.

(1963). Transcarboxylase. III. Purification and properties of methylmalonyl-oxaloacetic transcarboxylase containing tritiated biotin. J. Biol. Chem. 238: 547-556.

Wood, H.G. and Werkman, C.H. (1936). The utilisation of $CO_2$ in the dissimilation of glycerol by the propionic acid bacteria. Biochem. J. 30: 48-53.

Wood, H.G. and Werkman, C.H. (1938). The utilization of $CO_2$ by the propionic acid bacteria. Biochem. J. 32: 1262-1271.

Wood, H.G. and Werkman, C.H. (1940). The fixation of $CO_2$ by cell suspensions of *Propionibacterium pentosaceum*. Biochem. J. 34: 7-14.

Woronick, C.L. and Johnson, (1960). Carbon dioxide fixation by cell-free extracts of *Aspergillus niger*. J. Biol. Chem. 235: 9-15.