# THE ATP-DEPENDENT REDUCTIVE CARBOXYLATION OF 2-OXOGLUTARATE

A thesis submitted by

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To the University of Adelaide

South Australia,

for the Degree of

Doctor of Philosophy

DEPARTMENT OF BIOCHEMISTRY

UNIVERSITY OF ADELAIDE,

SOUTH AUSTRALIA

May, 1978

CONTENTS

## SUMMARY

The work presented in this thesis was carried out to determine whether or not the reductive carboxylation of 2-oxoglutarate to form isocitrate in the "2-oxoglutarate reductive carboxylation pathway" was a function of the reversal of isocitrate dehydrogenase (NADP). This function was found to be attributable to an enzyme in cytosol from rat hepatocytes which has not been previously described and has been given the trivial name "isocitrate synthase". Partial characterisation of the synthase has included studies on the requirement and specificity of the reaction, product identification, dietary influence on the level of enzyme, physical, chemical and kinetic investigations.

Using a radiochemical assay it has been shown that this enzyme is dependent upon the presence of $HCO_3^-$, $MgATP^{2-}$ and 2-oxoglutarate whilst paper chromatography has revealed that the product is OAS if NADPH is omitted from the assay mixture and isocitrate if NADPH is included. The carboxylating species was $HCO_3^-$ and not $CO_2$ and NADPH could not be substituted by NADH.

Studies using alternate substrates revealed that pyruvate is also a keto acid substrate for the synthase and has a Vmax 2 to 2.5-fold greater than 2-oxoglutarate although the Km values for both of these substrates are identical. The carboxylation of pyruvate in this system was not catalysed by the well known pyruvate carboxylase since isocitrate synthase was not inhibited by avidin, did not require acetyl-CoA for activation and has significantly different Km values for MgATP and $HCO_3^-$. Furthermore

pyruvate carboxylase cannot utilize 2-oxoglutarate nor CTP as substrates both of which are properties of isocitrate synthase.

The role of ATP is not entirely clear although the reaction under all conditions required the presence of ATP for maximum enzymic activity. The ratio of $H^{14}CO_3^-$ fixed to [$\gamma$-$^{32}$P]ATP hydrolysed was not 1:1 with $H^{14}CO_3^-$ fixation being far in excess of the [$\gamma$-$^{32}$P]ATP hydrolysed. This led to the search for a phosphorylated intermediate which was essential for activity and thus a possible control mechanism for the enzymic activity.

Gel chromatography of enzyme previously incubated with [$\gamma$-$^{32}$P]ATP resulted in the formation of a radio-active labelled protein which had a higher ATP-dependent activity than in a similar experiment without prior incubation with ATP. Attempts to remove the bound $^{32}P_i$ with either acid or alkaline phosphatase failed but acid precipitation of the enzyme completely removed all radio-activity. The bound radio-activity was stable to alkaline treatment and chloroform/methanol extraction indicating that the $^{32}P_i$ was covalently bound to the enzyme and not attached to a phospholipid.

The specificity of ATP for enzymic activity was tested by replacing ATP with the other nucleotide triphosphates. Only CTP could replace ATP in the reaction mixture. The addition of CTP resulted in a higher Vmax (2-3 fold) than that obtained using ATP. This activation was exhibited when either 2-oxoglutarate or pyruvate was the keto acid substrate.

Studies on the dietary influence on the level of

isocitrate synthase revealed that the level of enzyme fluctuated in a manner parallel to the utilization of the 2-oxoglutarate reductive carboxylation pathway. The contribution to fatty acid synthesis by this pathway has been shown to depend upon the nutritional state of the animal. Labelling studies have shown that starvation decreased the utilization of this pathway whilst refeeding increased the level above that of ad libitum fed animals. Similarly the level of isocitrate synthase in rat hepatocyte cytosol decreased upon starvation and increased above the level of ad libitum fed rats upon refeeding.

Only partial purification of the enzyme system has been achieved in this study. Some of the problems encountered during attempts to work out a purification procedure have been (a) dissociation (b) $(NH_4)_2SO_4$ fractionation and (c) ion exchange chromatography. Dissociation during gel filtration suggested that isocitrate synthase was a multi-component complex and some enzymic activity was recovered upon recombining three fractions of different elution positions.

Glucose was the most successful stabilising agent. It prevented much of the dissociation that occurred during gel filtration and resulted in a higher recovery of enzymic activity. This allowed a tentative estimate of molecular weight of the complex. Using a calibrated Sepharose 6B column, the molecular weight of the complex in the presence of glucose was $0.9x10^5$-$1x10^5$ daltons.

Kinetic studies involving alternate substrates indicated that the reaction mechanism was sequential but further work will be required before the order of addition

of substrates can be established. Non-classical kinetics were exhibited when $MgATP^{2-}$ was the variable substrate in multi-substrate kinetic studies and this combined with product inhibition studies led to the conclusion that two ATP binding sites exist on the enzyme. The presence of glucose in the assay mixture increased the Vmax of the reaction (2.5-fold) without altering the appKm value of all the substrates.

Isocitrate synthase appears to have two possible control mechanisms (a) a glucose mediated effect and (b) an ATP effect. The increase in Vmax obtained by the addition of glucose means that the activity of the enzyme may be controlled by the availability of glucose. ATP can control the enzymic activity by covalent modification, i.e. phosphorylation to increase activity and dephosphorylation to decrease activity.

There are quite distinct differences between the isocitrate synthase reaction, which is,

$$\text{2-oxoglutarate} + HCO_3^- + MgATP^{2-} \rightleftharpoons \text{oxalosuccinate} + MgADP^- + P_i$$

$$\text{oxalosuccinate} + NADPH^+ + H^+ \rightleftharpoons \text{isocitrate} + NADP^+ + H^+$$

The net result is the overall reaction

$$\text{2-oxoglutarate} + HCO_3^- + MgATP^{2-} + NADPH^+ + H^+ \rightleftharpoons \text{isocitrate} + NADP^+ + H^+ + MgADP^- + P_i$$

and the reaction catalysed by isocitrate dehydrogenase (NADP). (a) The synthase reaction is ATP-dependent, (b) $HCO_3^-$ and not $CO_2$ is the carboxylating species, and (c) OAS is a product of the reaction and has been isolated from the reaction mixture. In the isocitrate dehydrogenase

reaction, OAS is the proposed intermediate but remains bound to the enzyme. Furthermore the level of isocitrate synthase responds to dietary manipulation in a manner identical to the 2-oxoglutarate reductive carboxylation pathway whilst the level of isocitrate dehydrogenase (NADP) remains constant. These results suggest that isocitrate synthase and not isocitrate dehydrogenase (NADP) could possibly fit into and explain the original data of D'Adamo and Haft (1965) and the many studies done on different tissues by many other authors after the original postulate of this pathway.

## STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material that has been previously published, or written by another person, except where due reference is made in the text.

Mary J. J. Carabott

## ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

I am grateful to my supervisors, Dr. D.B. Keech and Dr. J.C. Wallace, for their advice, criticisms and encouragement throughout the course of this work, and in the preparation of this thesis. In addition I wish to thank Dr. A.K. Mattoo for his collaboration and encouragement during the initial stages of this work.

Many thanks go to Ms. J. Anderson and Mrs. N. Willoughby for friendly and reliable technical assistance, to the typist Ms. C. Carabott and to Mr. P. Cohen for help with the computing and his tolerance during the course of this work.

I wish to acknowledge the financial support of an Adelaide University Research Grant for the duration of the project.

## ABBREVIATIONS

In addition to those accepted for use in the Journal of Biological Chemistry, the following abbreviations are used in this thesis.

| | |
|---|---|
| AMP-PCP | 5'-adenosyl-methylenediphosphonate |
| AMP-PNP | 5'-adenylylimidodiphosphate |
| appKm | apparent Km |
| appVmax | apparent Vmax |
| BSA | bovine serum albumin |
| C.A. | carbonic anhydrase |
| DNP- | dinitrophenyl- |
| DTE | dithioerythritol |
| EDTA | ethylenediamine tetraacetic acid |
| GF/A | glass microfibre papers |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| Km | Michaelis-Menten constant |
| NEM | N-ethylmorphiline |
| OAA | oxaloacetate |
| OAS | oxalosuccinate |
| 2-OG | 2-oxoglutarate |
| PEG | polyethylene glycol |
| Pi | orthophosphate |
| Tris | tris-(hydroxymethyl)-aminomethane |
| Ve | elution volume |
| Vmax | maximum velocity |
| Vo | void volume |
| Cp 2m. | Counts per 2 minutes. |
| Cp 5m | Counts per 5 minutes |

# CHAPTER I

# INTRODUCTION

## 1. The Sources of Acetyl-CoA

Acetyl-CoA is formed in liver cells from the pyruvate produced in glycolysis and also as a product of the oxidation of fatty acids. A small additional amount of acetyl-CoA is formed from certain amino acids (Kornacker and Lowenstein, 1965). These amino acids are transaminated to α-keto acids and then further metabolized to acetyl-CoA or other products. Since the acetyl-CoA is formed in the mitochondria and is itself very slowly transported from mitochondria to the cytoplasm, where de novo synthesis of fatty acids occurs, another method must exist for the transport of acetyl-CoA across the mitochondrial membrane (Srere, 1965).

### 1.1 Transfer of Acetyl-CoA: Possible Mechanisms

Several mechanisms for the transfer of acetyl-CoA from mitochondria to the cytoplasm have been proposed. These are; transfer as acetate (Spencer and Lowenstein,1963) acetyl-carnitine (Fritz and Yue, 1964) and citrate (Srere and Bhaduri, 1962). Present evidence indicates that the diffusion of acetate through the mitochondrial membrane plays only a minor role in the synthesis of fatty acids (Spencer et al., 1964) whilst attempts to establish acetyl-carnitine as a carrier in the transfer of acetate out of mitochondria have been inconclusive (Srere, 1965). Thus the mechanism of choice is the transport of acetate from mitochondria as citrate (Olson, 1966).

Citrate is formed in mitochondria by the transfer of acetate from acetyl-CoA to oxaloacetate. Although data directly concerning the permeability of the mitochondria to citrate are not available, there is evidence showing that cytoplasmic citrate is poorly oxidized by mitochondria (Plaut and Plaut, 1952). This might suggest that a barrier exists to the movement of citrate across the mitochondrial membrane. However, 2-oxoglutarate, or glutamate which is readily transaminated to 2-oxoglutarate, is transported from mitochondria. The 2-oxoglutarate would then be converted in the cytoplasm to citrate by a reversal of the reactions in which citrate is converted to 2-oxoglutarate. Evidence for the latter possibility and the elucidation of the pathway involved was first reported by D'Adamo and Haft (1962) in the isolated, perfused rat liver.

### 1.1.1 Proposed transfer of Acetyl-CoA via 2-oxoglutarate

The pathway reported by D'Adamo and Haft is termed the 2-oxoglutarate reductive carboxylation pathway. It has also appeared in the literature as the 2-oxoglutarate shunt and the "backward pathway" since it represents, in part, a reversal of the carbon flow through the citric acid cycle.

As depicted in Fig.1.1 the pathway involves the following steps: (i) the condensing enzyme, present in mitochondria, converts acetyl-CoA and oxaloacetate to

citrate (ii) the action of aconitase upon citrate produces isocitrate (iii) mitochondrial isocitrate dehydrogenase converts isocitrate to 2-oxoglutarate, $CO_2$ and a reduced pyridine nucleotide. The 2-oxoglutarate may leave the mitochondria, or it may be converted to glutamic acid which is transported to the cytoplasm; here 2-oxoglutarate is formed again by transamination (iv) once in the cytoplasm 2-oxoglutarate is converted to isocitric acid (v) aconitase converts isocitric acid to citric acid (vi) citrate cleavage enzyme, ATP-citrate lyase, which is cytoplasmic converts citrate irreversibly to oxaloacetate and acetyl-CoA. Thus the consequence of these conversions is that acetyl groups and equivalent amounts of oxaloacetate are transported from the mitochondria to the cytoplasm without the migration of CoA.

### 1.1.2 The existence of the 2-oxoglutarate reductive carboxylation pathway

D'Adamo and Haft (1965) used labelling studies to establish the existence of the 2-oxoglutarate reductive carboxylation pathway. Using the isolated, perfused rat liver and [2-$^{14}$C] and [5-$^{14}$C] DL glutamate they proposed that if the 2-oxoglutarate derived from the labelled glutamate is oxidized solely via the Krebs cycle, the labelling pattern of products from the [2-$^{14}$C] and [5-$^{14}$C] labelled substrate would be as in Fig.1.2. Thus, it can be seen that unlabelled fatty acids and [3-$^{14}$C] and [4-$^{14}$C] glucose would result from both radiochemical isomers.

However, if the 2-oxoglutarate reductive carboxylation pathway is operative, then [2-$^{14}$C] glutamate would produce the labelling pattern shown in Fig.1.3(a) and Fig.1.3(b).

The pathway outlined in Fig.1.2 and Fig.1.3 includes randomization of isotope into both central carbon atoms of oxaloacetate, since such randomization is practically complete in the liver oxaloacetate derived from [3-$^{14}$C] aspartate (Bloom and Foster, 1962) or [3-$^{14}$C] malate (Hoberman and D'Adamo, 1960). With the [5-$^{14}$C] labelled precursor, the pathway would produce unlabelled glucose, and fatty acids labelled in carbon atoms 1,3,5,7,etc.

Existence of the pathway will be confirmed if (a) there is labelling of fatty acids from either [2-$^{14}$C] or [5-$^{14}$C] glutamate; (b) carbon atom 6 of glucose is labelled in experiments with the [2-$^{14}$C] but not with the [5-$^{14}$C] glutamate and (c) fatty acids synthesized from the [2-$^{14}$C] labelled compound have a ratio of carboxyl to average carbon activity of 1 while the ratio for the [5-$^{14}$C] labelled compound is 2.

The labelling patterns predicted in the products, as illustrated in Fig.1.3(a),1.3(b) and detailed above, have been obtained experimentally by several authors (Madsen *et al.*, 1964a; D'Adamo and Haft, 1965; Leveille and Hanson, 1966a). The existence of the 2-oxoglutarate reductive carboxylation pathway is thus confirmed.

### 1.1.3 The discovery of the pathway from different sources

Since the discovery of the pathway in rat liver many tissues have been shown to utilise the 2-oxoglutarate

reductive carboxylation pathway. These include adipose tissue (Madsen et al., 1964b; Leveille and Hanson, 1966a), lactating and prelactating mammary gland (Madsen et al., 1964a; Kopelovich and McGrath, 1970), brain (D'Adamo and D'Adamo, 1968), hibernators liver (Klain, 1976) and ruminants liver and udder (Hardwick, 1965; Hanson and Ballard, 1967).

Perfusion studies with the isolated ruminant udder show that the reductive carboxylation of 2-oxoglutarate accounts for 13-55% of the entry of $CO_2$ into citrate (Hardwick, 1965). However, the citrate does not contribute significantly to fatty acid production. The ruminant liver shows a similar situation with the 2-oxoglutarate pathway providing only one-tenth of the acetyl-CoA production for lipogenesis as compared to liver slices of the adult rat (Hanson and Ballard, 1967 and 1968). In both cases the lack of label into the acetyl-CoA is attributable to the low levels of ATP-citrate lyase activity.

In contrast to this, in the fetal liver of ruminants the 2-oxoglutarate pathway as measured by [5-$^{14}$C] glutamate conversion to fatty acids is almost 200 times more active than in the adult liver. There is approximately 20 fold more ATP-citrate lyase activity in the fetal liver than in the adult (Hanson and Ballard, 1968). This profound difference between fetal and adult ruminant utilization of the 2-oxoglutarate pathway may be due to the difference in the nutritional state. Fetal ruminants are supplied

with glucose, which illustrates that the pathway requires carbohydrates for its function. In the adult bacterial production of acetic acid in the rumen provides large amounts of this precursor for acetyl-CoA production (Ballard et al., 1969), whereas all glucose requirements must be synthesised from propionate, lactate or glucogenic amino acids.

In hibernating animals, the rate of hepatic fatty acid synthesis follows a yearly cycle. Lipogenesis is maximal during the summer and progressively decreases to minimal levels during hibernation and arousal. Klain (1976) has found that the 2-oxoglutarate pathway functions in hibernators and that glutamate utilization is subject to this annual rhythm. The pathway uses 71-74% of the glutamate metabolized in summer then drops sharply in autumn to about 25% of the value during the summer months. During the period of hibernation virtually no glutamate was converted to fatty acids. This yearly rhythm is also observed with enzymes which produce cytoplasmic NADPH, namely; glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and NADP malate dehydrogenase (Whitten and Klain, 1969), which would be required for the reductive carboxylation step.

### 1.1.4 Methods of estimation of glutamate utilization by this pathway

Several methods have been used to estimate the relative utilization of glutamate via the reductive

carboxylation pathway and the tricarboxylic acid cycle. D'Adamo and Haft (1965) have utilized glucose labelling data and thus this method can only be used with gluconeogenic tissue. The method of Madsen et al.,(1964a) assumes that acetyl-CoA from acetate activation, citrate cleavage and pyruvate decarboxylation mixes in a common pool and that its subsequent fate is independent of its origin. This is not true for all tissues and all precursors e.g. in the brain of the new born rat (D'Adamo et al., 1975).

The method of Leveille and Hanson (1966a) does not have either of these disadvantages. This method entails separate experiments using [2-$^{14}$C], [5-$^{14}$C] and [3,4-$^{14}$C] glutamic acid and accounts for the contribution of the recycling of the labelled oxaloacetate through either pathway.

Naruse et al., (1966) in their study of the role of the reductive carboxylation of 2-oxoglutarate in citrate production, have developed a method for the degradation of citrate. After incubation of tissue with $H^{14}CO_3$ the portion of radioactivity in C-6 and C-1 of the citrate is determined and compared with the distribution of radioactivity in the carboxyl groups of the tissue aspartic acid. This can be used to estimate the relative contribution to $CO_2$ fixation by $C_3$ acids (pyruvate or phosphoenol pyruvate) and by reductive carboxylation of 2-oxoglutarate.

1.1.5 Relative contribution of this pathway in metabolism

The relative contributions of the two pathways have

been evaluated in many studies. D'Adamo and Haft (1965) in their studies with perfused liver estimated that the backward pathway contributed 40-60% of the 2-oxoglutarate carbon to glucose. Studies by Hardwick (1965) with perfused goat udder estimated a 13-55% conversion of $NaH^{14}CO_3$ into citrate via the carboxylation of 2-oxoglutarate. Using rat adipose tissue and liver Leveille and Hanson (1966a) estimated that 50% or more of the glutamate or 2-oxoglutarate converted to lipid involves flow via the 2-oxoglutarate reductive carboxylation pathway. Kopelovich and McGrath (1970) studying prelactating and hyperplastic alveolar nodule outgrowth tissue calculated that between 37-54% of glutamate is metabolized via this pathway. Hence, relative to its total metabolism glutamate contributes appreciably to fatty acid synthesis.

These data merely permit a relative evaluation of the flow of 2-oxoglutarate (or glutamate) via the two pathways. However, the data obtained with cell-free preparation do permit an estimation of the possible significance to lipogenesis of the 2-oxoglutarate reductive carboxylation. The amount of citrate formed from 2-oxoglutarate (glutamate) exceeds the total amount of citrate cleaved and incorporated into fatty acids. This suggests that 2-oxoglutarate may be an important precursor of cytoplasmic citrate and, hence, of acetyl-CoA via citrate cleavage (Leveille and Hanson, 1966a).

### 1.1.6 Nutritional dependence of the pathway

The choice of pathways is dependent on the nutritional

state. In the perfused rat liver the synthesis of radioactive fatty acids from glutamate was depressed 98-99%, in experiments with fasted animals as compared to glucose fed animals (D'Adamo and Haft, 1965). Similarly Leveille and Hanson (1966b) using meal-fed (animals fed a single daily 2 hr meal) and nibbling (ad libitum-fed) rats showed that the 2-oxoglutarate pathway was significantly more active in adipose tissue from meal-eating than from nibbling animals. The effect on the 2-oxoglutarate reductive carboxylation of the annual rhythm in hibernators liver has already been discussed. Using rat epididymal fat pad Madsen et al., (1964b) attained the highest fatty acid-$C^{14}$ yields using [5-$^{14}$C] glutamate from rats fasted and then refed a 60% glucose diet. Hill et al., (1957) have shown in rat liver that the substitution of a 60% glucose diet for a stock diet containing no free hexoses results in an 8- to 10-fold increase in the liver's capacity to convert glucose carbon to fatty acids. This diet does not increase significantly the liver's capacity for oxidizing acetate to $CO_2$ whilst there is a 2-fold increase in its capacity to incorporate acetate carbon into fatty acids.

It is clear that carbon flow through the 2-oxoglutarate pathway is dependent upon the presence of carbohydrate. With normally fed animals, the presence of glucose in the incubation medium is essential for the functioning of the 2-oxoglutarate reductive carboxylation pathway. Studies of the glucose effects show that the increase of glutamate conversion to fatty acid is accompanied by a decrease in

oxidation of glutamate. Kopelovich and McGrath (1970) using prelactating tissues and hyperplastic alveolar nodule outgrowths showed that the incorporation of $^{14}C$-labeled carbon from [2-$^{14}C$] and [5-$^{14}C$] glutamate was significantly increased (up to 8-fold) by the addition of glucose. Insulin stimulated the formation of $^{14}C$-labelled fatty acids about 2-fold above levels observed when glucose alone was present. Neither glucose, nor insulin in the presence of glucose, had any marked effect on the $^{14}CO_2$ evolution from these substrates in either tissue. From $^{14}C$-fatty acid recoveries using [2-$^{14}C$] and [5-$^{14}C$] glutamate, in experiments with epididymal fat pads from rats fed a stock diet, Madsen et al., (1964b) calculated that of the total amount of glutamate metabolized 6% proceeded via the 2-oxoglutarate reductive carboxylation in the absence of glucose, 17% in the presence of glucose and 35% when both glucose and insulin were added to the incubation mixture. Lactating rat mammary gland slices also show an increase in the percentage of the glutamate metabolized via this pathway from 7-3% to 20-30% upon the addition of glucose (Madsen et al., 1964a).

Hardwick (1965) has suggested that the role of carbohydrate is to provide NADPH for the reduction step. In addition, it is also possible that the operation of the pentose pathway provides a high local concentration of the required bicarbonate by oxidation of 6-phosphogluconic acid. The enzymes involved exhibit activities which parallels the requirement for fatty acid synthesis (Whitten and Klain, 1969).

### 1.1.7 Importance of carboxylation in the pathway

The availability of $CO_2$ for the carboxylation step also plays an important role in the conversion of 2-oxoglutarate to lipids. Leveille and Hanson (1966a) have shown that in adipose tissue bicarbonate buffer increases the incorporation of labelled carbon into fatty acids as compared to phosphate buffer. Similar results have been obtained using brain slices from neonatal rats and it has been suggested that the carboxylation step is the rate limiting process for the 2-oxoglutarate reductive carboxylation pathway (D'Adamo et al., 1975).

## 1.2 The assumed mechanism

### 1.2.1 Reversibility of Isocitric Dehydrogenase (NADP)

The reductive carboxylation of 2-oxoglutarate is thought to occur by the action of the cytoplasmic NADPH requiring isocitrate dehydrogenase (EC1.1.1.42). D'Adamo and Haft (1965) who first investigated this pathway, suggested that this was a plausible sequence of events. Ochoa (1945), and Grisolia and Vennesland (1947) had demonstrated the reversibility of this enzyme and thus it was reasonable to suggest that the conversion of 2-oxoglutarate to isocitric acid was achieved in this manner. Other authors who subsequently discovered evidence for the existence of the reductive carboxylation of 2-oxoglutarate in other tissues similarly assumed that this reaction was carried out by the reversal of isocitrate dehydrogenase (NADP).

Ochoa (1948) has shown that the conversion of

isocitric acid to 2-oxoglutarate and $CO_2$ is the result of two distinct, reversible enzyme-catalyzed reactions.

(1) d-isocitric acid + NADP $\xleftarrow[\text{dehydrogenase}]{\text{isocitrate}}$ oxalosuccinic acid + NADPH

(2) oxalosuccinic acid $\xrightarrow[Mn^{++}]{\text{oxalosuccinate carboxylase}}$ 2-oxoglutarate + $CO_2$

The net result of reactions 1 and 2 is the over-all reaction 3.

(3) d-isocitric acid + NADP $\underset{Mn^{++}}{\rightleftharpoons}$ 2-oxoglutarate + $CO_2$ + NADPH

Reaction 1 occurs in the absence of $Mn^{++}$ whilst reaction 2 and the reversal of reaction 3 occur in the presence of $Mn^{++}$. The equilibrium of reaction 3 is so far to the right that if NADP is present in excess practically all the d-isocitric acid is converted to 2-oxoglutarate and $CO_2$ (Adler et al., 1939). The equilibrium can be shifted to the left by linking it to another dehydrogenase system capable of reducing NADP. This has been accomplished by using glucose-6-phosphate dehydrogenase (Ochoa,1948). Further shifting of the equilibrium towards $CO_2$ fixation occurs in the presence of aconitase, since over 90% of the isocitric acid is removed to form cis-aconitic and citric acid.

The work of Ochoa suggested that reactions 1 and 2 are catalyzed by two distinct enzymes, isocitrate dehydrogenase and oxalosuccinate carboxylase. Reinvestigation of this problem with purified preparations has shown that the reactions 1,2 and 3 are inherent properties of isocitrate dehydrogenase (Plaut, 1963). The presence of oxalosuccinate

decarboxylase and reductase activities in the same protein suggests that the oxidative decarboxylation of isocitrate [Equation 3] involves, sequentially, the oxidation of isocitrate to oxalosuccinate [Equation 1] followed by its decarboxylation to 2-oxoglutarate. However, several observations suggest that free oxalosuccinate does not participate in the overall reaction. Firstly the formation of oxalosuccinate from isocitrate or from 2-oxoglutarate and $CO_2$ could not be demonstrated under a variety of conditions (Moyle, 1956; Siebert et al., 1957a) and secondly only minor incorporation of radioactivity into an oxalosuccinate pool could be observed either from labelled isocitrate or from $^{14}CO_2$ (Plaut, 1963). The work of Dalziel and Londesborough (1968) is also consistent with this view. It is possible that the true intermediate of the reaction is an enzyme-bound form of oxalosuccinate in equilibrium with free oxalosuccinate (Moyle, 1956; Siebert et al., 1957a).

The reversibility of the reaction

oxalosuccinate $\rightleftharpoons$ 2-oxoglutarate + $CO_2$

shown by Ochoa (1948), indicated the possibility of biosynthesis of tricarboxylic acids through fixation of $CO_2$ by 2-oxoglutarate. Whilst the equilibrium is far to the right, it can be shifted to the left through reduction of oxalosuccinate to d-isocitrate by NADPH in the presence of isocitrate dehydrogenase.

### 1.2.2 Another type of carboxylation

The type of carboxylation reaction considered in the work presented by Ochoa is β-carboxylation and is distinguished from "reductive" carboxylation. Lipmann and Tuttle (1945) discovered that "reductive" carboxylation occurs by reversal

of the oxidative decarboxylation of α-keto acids. "Reductive" carboxylation involves a much greater change of free energy which is supplied in part by energy-rich phosphate through the generation of an acyl phosphate bond by reaction with ATP. Ochoa (1947) emphasized that the isocitrate dehydrogenase reactions he dealt with (see Section 1.2.1) whether partial or over-all, proceed in either direction in the absence of inorganic phosphate and ATP. Further, no generation of phosphate bonds is connected with the oxidation of isocitric acid to 2-oxoglutarate and $CO_2$.

Chen and Plaut (1963) in their studies of the possible role of nucleotides in cellular isocitrate oxidation, presented evidence that the activity of the NADP-linked isocitrate dehydrogenase from bovine heart is not influenced by ADP, ATP, NAD or NADH. These results further reinforces the work of Ochoa that ATP is not needed for the NADP-isocitrate dehydrogenase reaction.

### 1.2.3 The substrate for the carboxylation step

In considering the mechanisms of enzymes involved in carbon dioxide metabolism, the question arises as to which of the three forms existing in equilibrium $CO_2$, $H_2CO_3$ and $HCO_3^-$ is the substrate or product. Dalziel and Londesborough (1968) showed unequivocally that dissolved $CO_2$ is the real substrate of NADP-linked isocitrate dehydrogenase. The $HCO_3^-$ (or $H_2CO_3$) was found to be either a very poor substrate or not a substrate at all. They also concluded that it is unlikely that the same mechanisms of carboxylation would serve for these different molecular

species, or that an enzyme would use both species in different mechanisms.

## 1.3 Adaptation of enzymes

Enzyme concentrations and activities are influenced by many dietary and metabolic conditions. Fitch and Chaikoff (1960) in their studies of adaptation of enzyme activities in rats subjected to various dietary conditions came to the following conclusions:

(a) The level of an enzyme's activity is related to the metabolic activity of the pathway in which the enzyme participates; (b) a change in this level reflects, qualitatively, alterations in usage (throughput) of that metabolic pathway; and (c) alterations in enzymatic activity may be broad in extent, rather than involving only one key, rate-limiting enzyme in each pathway.

### 1.3.1 Adaptation of lipogenesis

Lipogenesis is decreased during fasting (Lyon et al., 1952) and increases rapidly to levels above normal upon refeeding (Medes et al., 1952). It follows an annual cycle in hibernators as discussed before (Klain, 1976) and is affected by meal-feeding (animals fed a single daily 2 hr meal) (Leveille and Hanson, 1966b) and dietary effects such as the substitution of a 60% glucose diet for a stock diet containing no free hexoses (Hill et al., 1957). These fluctations in fatty acid synthesis prompted the study of the activities of various enzymes involved in this pathway by several authors.

Lardy et al., (1964) suggested that malic enzyme is

an important source of NADPH for fat synthesis. Shrago et al., (1963) showed the malic enzyme activity was decreased by fasting and increased above normal upon refeeding. That is, it adapts in a manner identical to overall lipogenesis. It was also observed that a longer period of fasting led to a greater refeeding response of malic enzyme in both liver and adipose tissue. Similar results were obtained for glucose-6-phosphate dehydrogenase, an enzyme known to contribute to lipogenesis by the production of NADPH, needed for reductive synthesis of fatty acid, via the hexose monophosphate oxidative pathway (Cohn and Joseph, 1959). These enzymes exhibited an identical pattern when there was an increase of fatty acids synthesis following the feeding of the 60% hexose diet (Fitch and Chaikoff, 1960). α-glycerophosphate dehydrogenase, which is responsible for the glycerol moiety of neutral fats, was also greatly elevated by the feeding of the hexose-containing diet. Studies with hibernators showed that malic enzyme and hexose monophosphate shunt dehydrogenases were lowest during hibernation when fatty acid synthesis is blocked and attained maximum level in the summer when there is maximum lipogenesis (Whitten and Klain, 1969).

1.3.2 Adaptation of the oxoglutarate reductive carboxylation pathway

The contribution to fatty acid synthesis by the 2-oxoglutarate reductive carboxylation pathway has also been shown to increase significantly as lipogenesis increases (see Section 1.1.3). The enzyme claimed responsible for the reductive carboxylation of 2-oxoglutarate

is NADP-dependent isocitrate dehydrogenase for reasons already discussed. Thus it is of interest to measure the level of this enzyme under conditions of varying lipogenic activity since D'Adamo et al., (1975) suggested that the reductive carboxylation of 2-oxoglutarate may be the rate-limiting step for the pathway.

### 1.3.3 Isocitrate dehydrogenase (NADP): A non-adaptive enzyme

Young et al., (1964) using liver and adipose tissue, showed that isocitrate dehydrogenase does not adapt to a starvation and refeeding regime. This regime has been shown to increase both lipogenesis and the contribution of the 2-oxoglutarate reductive carboxylation pathway to lipogenesis. The activity of isocitrate dehydrogenase was not affected by meal-feeding (Leveille and Hanson, 1966b) nor by 60% glucose diets (Fitch and Chaikoff, 1960) conditions favouring lipogenesis.

In the rat, the level of isocitrate dehydrogenase is relatively lower in the fat pad than in the liver and not adaptive to diets which increased lipogenesis (Young et al., 1964). This low level in adipose tissue is unexpected in view of the fact that there is a rapid rate of lipid synthesis in this tissue and an increased activity of enzymes in pathways supporting lipogenesis was therefore expected (Leveille and Hanson, 1966b).

These properties of NADP-dependent isocitrate dehydrogenase suggest that perhaps this enzyme is not responsible for the reductive carboxylation of 2-oxoglutarate. There had been no studies done to verify

or disprove the role of NADP-dependent isocitrate dehydrogenase in the 2-oxoglutarate reductive carboxylation pathway. D'Adamo and Haft (1965) suggested the utilization of this enzyme only as a plausible mechanism. In view of these more recent results an appraisal of this pathway was considered necessary.

## 1.4 A proposed alternative to isocitrate dehydrogenase (NADP)

### 1.4.1 Limitations of isocitrate dehydrogenase (NADP)

Prior to this work the role of isocitrate dehydrogenase as the 'carboxylase' in the reductive carboxylation of 2-oxoglutarate had not been questioned. Recent studies on isocitrate dehydrogenase (NADP) have revealed properties which cast doubts on the involvement of this enzyme as the 'carboxylase' of 2-oxoglutarate.

Uhr et al., (1974) using pig heart NADP-isocitrate dehydrogenase have shown that in the reverse reaction the enzyme is inhibited at high $CO_2$ levels presumably caused by bicarbonate. Further more 2-oxoglutarate gives strong linear substrate inhibition which is thought to occur by 2-oxoglutarate binding in dead-end fashion with enzyme-NADP and preventing release of the nucleotide (Northrop and Cleland, 1974; Uhr et al., 1974). From these studies the authors also calculated that the ratio of the maximum velocities in forward (decarboxylation) and reverse (carboxylation) directions was 4.4. Ingebretsen et al., (1975) calculated that the ratio of forward and reverse direction was 6 again showing that the carboxylation

reaction is the unfavourable direction of the reaction. Thus it can be argued that the reversal of isocitrate dehydrogenase (NADP) does not fit the role for the reductive carboxylation of 2-oxoglutarate especially when the animal requires an increased throughput in this pathway. Such a situation arises in several instances e.g. hibernating animals and starving and refeeding (see Section 1.1.3) and results in a greater utilization 2-oxoglutarate by this pathway and an increase in the local concentration of the required bicarbonate due to an increase in the pentose pathway. These increases would lead to inhibition of the already unfavourable reversal of isocitrate dehydrogenase (NADP) at a time when there is a many fold increase in the utilization of the reductive carboxylation of 2-oxoglutarate pathway.

### 1.4.2 An analogous situation

Due to increasing evidence that isocitrate dehydrogenase (NADP) may not be suitable for the carboxylation reaction and by analogy with pyruvate carboxylation Dr. D.B. Keech postulated that this reaction is carried out by a carboxylase.

Pyruvate is a substrate for two enzymes, pyruvate carboxylase and pyruvate dehydrogenase. The pyruvate carboxylase reaction carboxylates pyruvate thus producing oxaloacetate whilst the pyruvate dehydrogenase reaction produces (the acetate moiety of) acetyl CoA. Analogous to this, oxoglutarate dehydrogenase produces (the succinate moiety of) succinyl CoA whilst the 2-oxoglutarate reductive

carboxylation pathway requires the carboxylation of 2-oxoglutarate. The postulate is that a carboxylase, analogous to pyruvate carboxylase, can fit the role of the carboxylase of 2-oxoglutarate.

### 1.4.3 The postulated carboxylase

The postulated carboxylase would have many advantages over the reversal of isocitrate dehydrogenase (NADP). Firstly a carboxylase would require $HCO_3^-$ (or $CO_2$) as its substrate and not as one of the products of the forward (decarboxylating) reaction as is the case with isocitrate dehydrogenase (NADP). This could possibly result in a lower Km for the carboxylating species which would be advantageous since the Km of $CO_2$ for isocitrate dehydrogenase (NADP) is 2.2mM (Uhr *et al.*, 1974) which is limiting under physiological conditions (Cleland, 1967a). Secondly, as 2-oxoglutarate is also the substrate in the forward direction it would be less likely to get substrate inhibition at low levels as occurs with the reversal of isocitrate dehydrogenase (NADP).

The proposed reaction is as follows:

$$\text{2-oxoglutarate} + HCO_3^- + MgATP^{2-} \longrightarrow \text{oxalosuccinate} + MgADP^- + P_i^{2-}$$

$$\text{oxalosuccinate} + NADPH^+ + H^+ \longrightarrow \text{isocitrate} + NADP^+ + H^+$$

The net result is the overall reaction:

$$\text{2-oxoglutarate} + HCO_3^- + MgATP^{2-} + NADPH^+ + H^+ \longrightarrow \text{isocitrate} + NADP^+ + MgADP^- + P_i^{2-} + H^+.$$

With this postulate at hand the work of this thesis is the search for a carboxylating enzyme which carboxylates

2-oxoglutarate to oxalosuccinate. It should be stressed here that oxalosuccinate has never been isolated in the reversal of isocitrate dehydrogenase (NADP) though several methods were attempted (Moyle, 1956; Siebert et al., 1957a) and it is thought that oxalosuccinate is an enzyme bound intermediate in the isocitrate dehydrogenase (NADP) reaction (Plaut, 1963). The oxalosuccinate would then be reduced to isocitrate by a reducing enzyme. The reductase could be part of a multi-component system which includes the carboxylase or it could be an additional reaction performed by the same carboxylase or by a separate reductase.

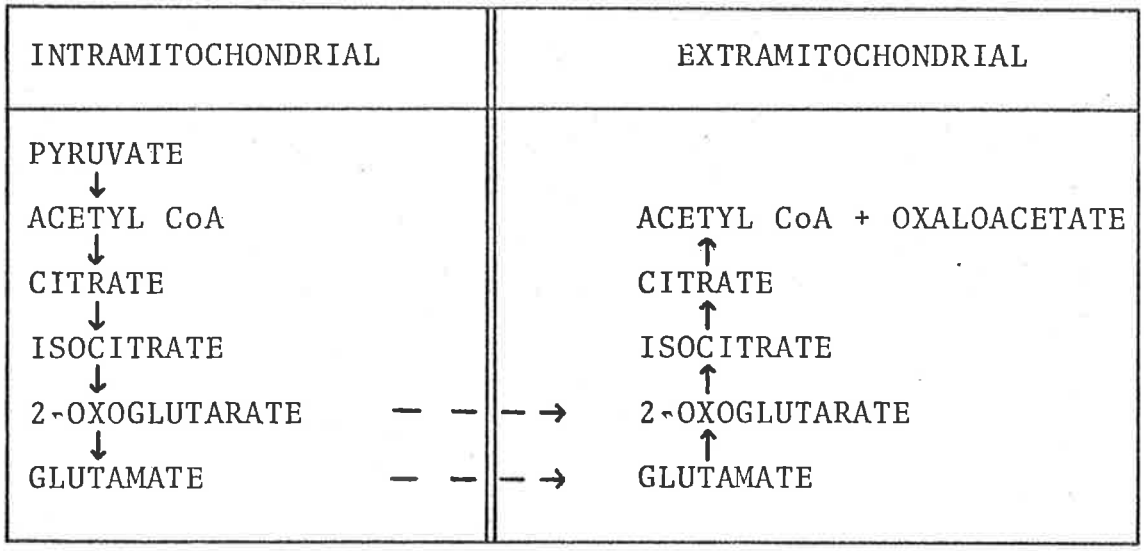
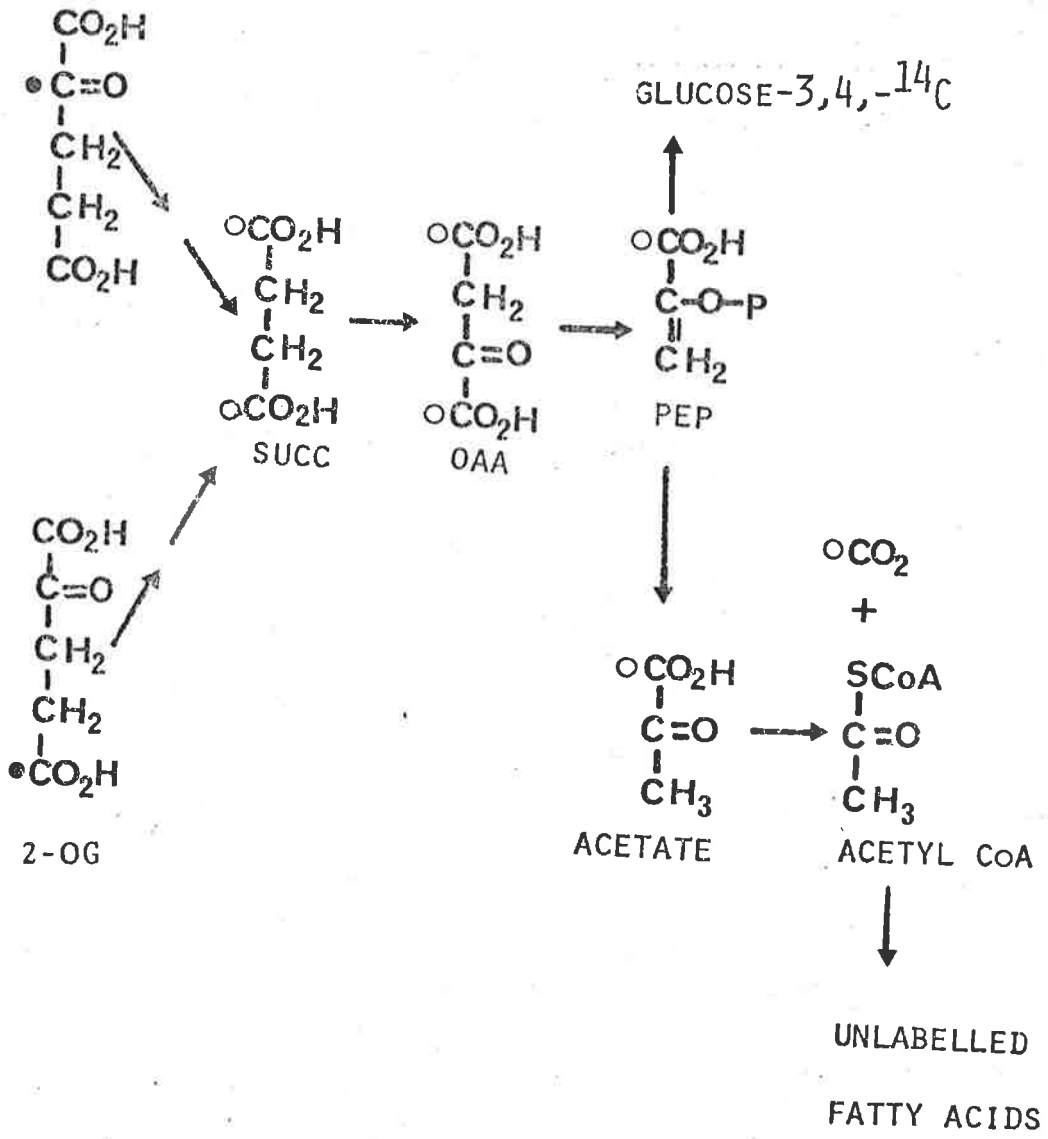



Fig.1.1 Proposed shuttle for generation of extramitochondrial acetyl-CoA (D'Adamo and Haft,1965).

Fig.1.2 Labelling of products from [2-$^{14}$C] and [5-$^{14}$C] 2-oxoglutarate by the Krebs cycle




● , initial $^{14}$C specific activity
○ , dilution of $^{14}$C by $^{12}$C in the randomizing step

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Enzymes and proteins

Glyceraldehyde 3- phosphate dehydrogenase (D-glyceraldehyde-3-phospate: NADoxidoreductase (phosphorylating), EC1.2.1.12), from rabbit muscle, 3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate phosphotransferase, EC2.7.23), hexokinase (ATP:D-hexose 6-phosphotransferase, EC2.7.1.1) type IV from yeast, isocitrate dehydrogenase (Ls-isocitrate; NADP oxidoreductase (decarboxylating) EC1.1.1.42) type IV from pig heart and pyruvate kinase (ATP:pyruvate phosphotransferase EC2.7.1.40) type III from rabbit muscle were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Avidin was supplied by Worthington Biochemical Corporation, N.J., U.S.A. Carbonic anhydrase (carbonate hydro-lyase; EC4.2.1.1) B grade was supplied by Calbiochem.

### 2.1.2 Animals and diet ingredients

Wistar hooded female rats approximately three months old were used throughout this study. They were maintained in a temperature and light controlled animal house which was set for 12 hr light and 12 hr dark.

Casein was supplied by Colac Dairying Co. Ltd., Vic., Aust. Non-fat skim-milk powder was supplied by Dairy Vale Metro Co-op., Adel. Aust. Sucrose was supplied by C.S.R. Aust., and flour and M&V mouse cubes were supplied by Charlick Ltd., Adel., Aust. Vitamin and minerals were premixed and supplied by a pharmaceutical firm, Adel. Aust.

### 2.1.3 Radioactive chemicals

Sodium [$^{14}$C] bicarbonate, [8-$^{14}$C] ATP, were obtained

from The Radiochemical Centre, Amersham, England. [$^{32}$P] orthophosphate was supplied by The Australian Atomic Energy Commission, Lucas Heights, Australia.

### 2.1.4 General chemicals

ATP (disodium salt, Grade I), NAD, NADH, NADP, NADPH, 2-oxoglutarate, sodium pyruvate (type II, dimer free), DTE, OAA, OAS, isocitric acid, ADP, CTP, TTP, GTP, ITP, 3PGA streptomycin sulphate, and α-keto adipic acid were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. N-ethylmorpholine was obtained from Eastman Organic Chemicals, PEG (molecular weight 6,000) from Union Carbide Corporation. 1,4-bis-2(4-methyl-5-phenoxazolyl)-benzine and 2,5-diphenyloxazole were supplied by Koch-Light Laboratories Ltd., Bucks., England. Polyethyleneimine thin layers were obtained from Machery-Nagel and Co., Duren, Germany. Cellulose and Silica gel thin layers were obtained from Eastman Kodak Co., N.Y., U.S.A. $MgCl_2$ was prepared from Spec-pure magnesium (Hilger-Watts Ltd., London) and redistilled HCl, and was standardised by titration against EDTA, using Eriochrome Black as an indicator (Vogel, 1961) Triton X-100 was supplied by ICI (Australia) Ltd., Melbourne.

## 2.2 Methods

### 2.2.1 Preparation and purification of nucleotides

[$\gamma$-$^{32}$P] ATP was prepared by the method of Glynn and Chappell (1964), and purified by ion exchange chromatography using a Dowex-1 (formate form) column (1 x 5cm.) eluted with 25ml 0.2M ammonium formate, (pH4.0), followed by 25ml 0.2M ammonium formate (pH3.45), 25ml 0.4M ammonium formate

(pH3.45), and finally 15ml IMHCl. The eluate from the last wash was neutralised with NaOH, concentrated by freeze-drying, and remaining contaminating [$^{32}$P] orthophosphate removed by gel filtration using Sephadex G-10.

2.2.2 Determination of radioactivity

Samples dried on to solid supports (2.5cm x 2.5cm squares of Whatmans 3MM paper) were placed in vials containing 2ml scintillation fluid (0.3% ($^{w}$/v) 2,5-diphenyloxazole, 0.03% ($^{w}$/v) 1,4-bis-2 (4-methyl-5-phenoxazolyl)-benzene, in sulphur-free toleune; Bosquet and Christian, 1960) and counted in a Packard Scintillation Spectrometer. When the samples contained coloured material, (as when reaction was stopped with 2,4-Dinitrophenylhydrazine in 6NHCl) correction was made for colour quenching using the channels ratio method (Baille, 1960). Liquid samples were placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toleune scintillation fluid, as above, containing Triton X-100, 7:3 $^{v}$/v), and counted in a Packard Scintillation Spectrometer.

2.2.3 The high protein diet

The "normal" rat food is M&V Mouse cubes which consisted of 21.4% protein, 3.9% fat, 57% carbohydrate and is supplemented by vitamins. To obtain a high protein diet consisting of 40% protein, 51% carbohydrate and 1.5% fat the following were mixed together, per 300gm ; 100gm flour, 71 gm caesin, 29gm sucrose and 100 gm skim-milk powder. This mixture was made into a dough with

water and baked in an oven until it was a cutting consistency. The "cake" was diced into 2cm squares and fed to the rats in place of mouse cubes for the duration of the high protein diet. A vitamin supplement was also given for the duration of the diet. This method of feeding was used in preference to giving the rats the mixed diet in powder form as it was easier for the rats to eat the cubes.

The diet regime consisted of starving the rats for 72 hr during which time they had free access to water only. After this time they were fed the high protein diet for an additional 72 hr .

2.2.4 Liver extraction

Rats were stunned by a sharp blow to the head, decapitated and the livers were quickly removed and placed in ice. All stages of the extraction were carried out at 4°C. The livers were diced and homogenized in three volumes of 0.25M sucrose containing 0.02M NEM, pH7.5 and 0.001M EDTA using a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 48,000g for 60 min and the supernatant was freeze-dried and stored dessicated at -15°C. When mitochondria were prepared the precipitate of the 48,000g centrifugation step was suspended in 0.1mM EDTA, freeze dried and stored dessicated at -15°C.

2.2.5 Measurement of enzymic activity

2.2.5.1a Radiochemical assay system

In this procedure $H^{14}CO_3^-$ fixed in an acid-stable form is measured, while unreacted $H^{14}CO_3^-$ is driven off on acidification and subsequent drying on paper squares

(cf., Gailiusis et al., 1964). Assay solutions contained, in μmoles, in a final volume of 0.25ml: NEM ($Cl^-$, pH7.5), 25; ATP, 0.5; $MgCl_2$, 1.0; $NaH^{14}CO_3$ (0.25μ Ci per μmole), 2.5; NADPH, 0.025; and 2-oxoglutarate, 1.0. Unless otherwise stated, assays were initiated by addition of enzyme and allowed to proceed for 5 min at 30°C in a fume hood before being quenched with 0.05ml of a saturated solution of 2, 4 dinitrophenylhydrazene in 6NHCl. After standing for 30 min in a fume hood denatured protein was removed by centrifuging and 0.05ml samples of the supernatant solution were spotted in triplicate onto 2.5cm squares of Whatman 3MM paper, dried for 5 min at 90°C, and counted as described in Section 2.2.2.

2.2.5.1b A modified radiochemical assay system

The procedure for this assay is identical to that of Section 2.2.5.1a except that the assay solutions contained, in μmoles, in a final volume of 0.25ml: NEM ($Cl^-$, pH7.5), 25; ATP, 1.25; $MgCl_2$, 2.5; $NaH^{14}CO_3$ (0.76 μ Ci per μmole), 25, NADPH, 0.025; and 2-oxoglutarate, 1.0.

2.2.5.1c Radiochemical assay system for estimation of $P_i$ release

When orthophosphate release from [$\gamma$-$^{32}$P]ATP was measured, the assay system described in Section 2.2.5.1b was used except [$\gamma^{32}$-P]ATP and $NaH^{12}CO_3$ were used. After five minutes reaction at 30°C, the reaction was quenched by addition of 0.05ml 6M HCl. Carrier orthophosphate (20 μmoles) was added, and the orthophosphate in a 0.25ml

aliquot was extracted into the organic phase of a water-saturated iso-butanol (4ml), ammonium molybdate (1ml), (40 mM in 1.25M $H_2SO_4$) separation system. Duplicate samples were taken and their radioactivity determined.

2.2.5.2 Spectrophotometric assay for isocitrate dehydrogenase (NADP) activity

The enzyme activity in the forward direction was measured in tris buffer, pH7.4 by the method of Ruffo et al., (1975). The reverse reaction was measured by the method of Siebert et al., (1957a). The reactions were followed at 340nm, using a Varian-Techtron 635-0 spectrophotometer. The rate of the reactions were calculated assuming an extinction coefficient at 340nm for NADP(H) of $6.22 mM^{-1} cm^{-1}$ (Dawson et al., 1969).

2.2.5.3 Spectrophotometric assay for lactate dehydrogenase

The reaction rate was followed at 340nm and the rate calculated assuming an extinction coefficient at 340nm for NADH of $6.22 mM^{-1} cm^{-1}$. Assay solutions contained, in µmoles, in a final volume of 1.0ml: NEM ($Cl^-$, pH7.5), 200; NADH, 0.12; $MgCl_2$, 7; and pyruvate, 0.75.

2.2.5.4 Spectrophotometric assay for malate dehydrogenase (NADH)

The procedure for this assay is identical to that in Section 2.2.5.3 except oxaloacetate replaces pyruvate as the substrate.

2.2.5.5 Spectrophotometric assay for malate dehydrogenase (Decarboxylating) (NADP)

This enzyme is also known as the 'Malic Enzyme' and was assayed by following the reaction rate at 340nm and the rate calculated assuming an extinction coefficient at 340nm for NADPH of $6.22 mM^{-1} cm^{-1}$. Assay solutions contained, in µmoles, in a final volume of 1.0ml:NEM($Cl^-$, pH7.5), 100; NADPH, 0.12; $MgCl_2$, 7; pyruvate, 0.75; and $NaHCO_3$, 100.

2.2.5.6 Spectrophotometric determination of ATP

Assay solutions contained, in µmoles, in a final volume of 1.0ml; Triethanolamine hydrochloride ($Na^+$,pH7.6), 42; glucose, 222; $MgCl_2$, 6.7; NADP, 0.73; and 0.5 units glucose-6-phosphate dehydrogenase and 0.1µg hexokinase. The assay was initiated with the solution contained ATP and the reaction rate at 340nm was calculated assuming an extinction coefficient at 340nm for NADP of $6.22 mM^{-1} cm^{-1}$.

2.2.6 Protein determination

Protein concentrations were routinely determined from absorption at 260 and 280nm using a Varian-Techtron 635-0 spectrophotometer and applying the data to the formula, for a 1cm light path.

$mg/ml = 1.55A_{280nm} - 0.76A_{260nm}$ (Layne, 1957)

When the protein solutions contained interfering substances such as ATP protein was determined by the method of Lowry et al., (1951). The method of Blumel and Uecker (1976) was used to determine protein concentrations in solutions containing glycerol.

# CHAPTER 3

# ATP-DEPENDENT REDUCTIVE CARBOXYLATION OF 2-OXOGLUTARATE

## 3.1 Introduction

### 3.1.1 Choice of starting material

Studies on the reductive carboxylation in intact tissues have shown that both fatty acid synthesis and the relative contribution of the 2-oxoglutarate reductive carboxylation pathway to lipogenesis increase rapidly to levels above normal upon refeeding starved rats (Madsen et al., 1964a). The lipogenic response is more marked the longer the animal is starved, up to a 72 hr limit (Lyon et al., 1952). Thus the choice of tissue for the work reported in this investigation is rat liver, obtained by sacrificing rats which had undergone a diet regime of 72 hr starvation followed by 72 hr on a high protein diet. Furthermore since the pathway is located in the cytosol (Leveille et al., 1966a) and there is a need to study this pathway with cell-free preparations, the rat liver cytosol was extracted and used throughout.

### 3.1.2 Studies involving a variety of nutritional states

Several laboratories have reported that the activity of isocitrate dehydrogenase (NADP), the postulated reductive carboxylase, remains unchanged under conditions favouring lipogenesis and an increased usage of the 2-oxoglutarate reductive pathway (Fitch and Chaikoff, 1960; Leveille et al., 1966b). Other enzymes proven to be involved in lipogenesis exhibit activities parallel to lipogenesis. It is thus desirable in investigating the activity of this pathway to look at the level of the reductive carboxylase in liver extracts from rats fed different diets which thus exhibit

different levels of lipogenesis. This aspect of the work is important in testing the hypothesis that an enzyme different from isocitrate dehydrogenase (NADP) catalyses the carboxylation of 2-oxoglutarate as it is predicted that isocitrate synthase would show fluctuations in activity comparable with the changes in lipogenesis.

### 3.1.3 The reaction

The reaction from the 2-oxoglutarate reductive pathway that is under scrutiny is the carboxylation of 2-oxoglutarate to oxalosuccinate and further the reduction of oxalosuccinate to isocitrate. These reactions can be written as follows if the reversal of isocitrate dehydrogenase (NADP) is responsible for the end products;

$$\text{2-oxoglutarate} + CO_2 \rightleftharpoons \text{oxalosuccinate} \quad ..(1)$$

$$\text{oxalosuccinate} + \text{NADPH} + H^+ \rightleftharpoons \text{isocitrate} + NADP^+ \quad ..(2)$$

The net results of (1) and (2) is the overall reaction (3)

$$\text{2-oxoglutarate} + CO_2 + \text{NADPH} + H^+ \rightleftharpoons \text{isocitrate} + NADP^+ \quad ..(3)$$

Oxalosuccinate has never been shown to be a free product and has been assumed to be an enzyme-bound intermediate. Thus in the analysis of the overall reaction isocitrate would be the only identifiable product. If the reaction is carried out by the postulated method viz a carboxylase then the reactions may possibly be the following;

$$\text{2-oxoglutarate} + HCO_3^- + MgATP^{2-} \rightleftharpoons \text{oxalosuccinate} + MgADP^- + P_i$$

$$\text{oxalosuccinate} + NADPH^+ + H^+ \rightleftharpoons \text{isocitrate} + NADP^+ + H^+$$

The net result is the overall reaction

$$\text{2-oxoglutarate} + HCO_3^- + MgATP^{2-} + NADPH^+ + H^+ \rightleftharpoons \text{isocitrate} + NADP^+ + MgADP^- + P_i + H^+$$

The second part of these reactions is identical whilst the first part reflect the differences between the present postulate and the presumed reaction sequence of previous literature.

### 3.1.4 An energy requirement

The reaction of isocitrate dehygrogenase (NADP) whether partial or overall proceeds in either direction in the absence of inorganic phosphate and ATP (Ochoa, 1945). This is in contrast to carboxylases which have an energy requirement, since the formation of a C-C bond is endergonic, which is supplied in part by energy-rich phosphates such as inorganic phosphate, nucleotide diphosphate and nucleotide triphosphate. This is the critical difference between the assumed and the proposed mechanism.

It should also be stressed at this point that whilst isocitrate dehydrogenase (NAD) is allosterically affected by ADP and ATP isocitrate dehydrogenase (NADP) has been shown to be unaffected by ATP, ADP as well as NAD and NADH. (Chen et al., 1963).

## 3.2 Material and methods

### 3.2.1 Materials

The lyophilized rat liver cytosol prepared as in Section 2.2.4 was the starting material. Bromophenol blue, bromocresol purple and methyl red were supplied by British Drug Houses, Poole, England.

### 3.2.2 Methods

3.2.2.1 Extraction of the lyophilized rat liver cytosol

Isocitrate synthase activity was extracted from the lyophilized material using 2.5 volumes of a solution containing 20mM NEM, pH7.5 containing 2.4mM EDTA. The solution was centrifuged at 5 x $10^4$g for 60 min to remove any precipitated protein and the supernatant was used for enzyme assays. All procedures were carried out at 4°C as the enzyme is heat labile.

3.2.2.2 The radiochemical assay

The radiochemical assay for isocitrate synthase described in Section 2.2.5.1a was the method of assay used and the spectrophotometric assay described in Section 2.2.5.2 was used for isocitrate dehydrogenase (NADP) assays.

3.2.2.3 Assay for the carboxylating species

The radiochemical method of Cooper et al., (1968) was used for recording the rate of $^{14}C$-product formation in a reaction mixture containing in a total volume of 0.5ml; 2.5µmoles ATP, 3.5µmoles $MgCl_2$, 2µmoles 2-oxoglutarate, 0.005µmoles NADPH, 50µmoles NEM-ClpH7.5 and 1.76µmoles $HCO_3^-$ plus 1.76µmoles HCl (for the $CO_2$ species) or 1.76µmoles $HCO_3$ (the indicated radioactive species containing 0.66µC/µmole). Temp., 11°C and 50µg carbonic anhydrase per 0.5ml assay was used where indicated.

3.2.2.4 Identification of products

Chromatography was effected on both cellulose thin layer plates and Whatman 3MM paper with reference compounds as markers. $^{14}C$-isocitrate was prepared by reversal of

isocitrate dehydrogenase (NADP) using $H^{14}CO_3$ and the method of Siebert et al., (1957a). The DNP derivatives of OAS and 2-oxoglutarate were freshly prepared by reacting 2mg of these compounds with 1ml DNP saturated 6MHCl for 1 hr and extracting the DNP derivatives with ethyl acetate.

The solvents used for running the chromatograms are as indicated in the respective diagrams (Figs3.3 to 3.6). The detection of the products was achieved by drying the chromatograms to remove the solvents and spraying with a mixture of 0.3% bromophenol blue and 0.1% methyl red in 95% ethanol. The acidic products were revealed as yellow spots on a violet background (Ting and Dugger, 1965). Alternatively the detection spray consisted of 0.04% aqueous solution of bromocresol purple pH7 followed by a brief exposure of the plates to ammonia vapour to accentuate the colour. Again yellow spots on a violet background (Myers and Huang, 1966) revealed the position of the acidic compounds.

Electrophoresis on cellulose thin layers was conducted using 0.02M ammonium bicarbonate pH8.5 at 20V/cm.

## 3.3 Results

### 3.3.1 The isocitrate synthase reaction

The enzyme system being investigated has been described as "Isocitrate Synthase" (Keech et al., 1976) and to avoid confusion with isocitrate dehydrogenase (NADP) will be referred to as such henceforth. Fig.3.1 shows the reaction sequence of the ATP-dependent reductive carboxylation of 2-oxoglutarate as postulated. The 2-oxoglutarate is carboxylated to oxalosuccinate. There is

an essential requirement for ATP which is hydrolysed. The oxalosuccinate produced is unstable in neutral or slightly alkaline solutions and must be stabilized by reduction to isocitrate. Thus to establish the existence of this enzyme the assay must show that there is complete dependence of product formation upon $MgATP^{2-}$, $HCO_3^-$ and 2-oxoglutarate and dependence on NADPH to the extent of the instability of oxalosuccinate.

Using the radiochemical assay and supernatant of the extraction of lyopholized rat liver cytosol it can be seen in Table 3.1 that the complete assay mix resulted in the fixation of radioactive carbon into an acid stable product. However, when either ATP, $Mg^{2+}$ or 2-oxoglutarate was omitted the $^{14}CO_2$ fixed fell dramatically to very low levels whilst with the omission of NADPH the activity was approximately 10% of the complete system. Thus, to obtain acid stable fixation of radioactive carbon from $H^{14}CO_3$ there is an absolute need for the substrates, 2-oxoglutarate, ATP, $Mg^{2+}$ and NADPH which satisfied the requirements of reactions (i) and (ii).

### 3.3.2 The carboxylating species

The carboxylating species may be either $CO_2$ or $HCO_3^-$ (as carboxylases have been known to utilize either species). The results of Kaziro *et al.*, (1962) indicate that $HCO_3^-$ (or $H_2CO_3$) is the reactant in $CO_2$ fixation by biotin carboxylases whilst Cooper *et al.*, (1968) showed that the carboxylating enzymes P-enolpyruvate carboxykinase (EC4.1.1.32) and P-enolpyruvate carboxytransphosphorylase (EC4.1.1.38) use $CO_2$ as the active species. The reverse

reaction of isocitrate dehydrogenase utilizes $CO_2$ as the carboxylating species (Uhr et al., 1974). Thus this could be a point of difference between the two enzymes that are being considered.

Fig.3.2(i) shows the theoretical incorporation of $^{14}C$ into isocitrate for this assay: A, if the active species used in the fixation is $CO_2$ and B, if it is $HCO_3^-$. It is seen in Fig.3.2(i)A that when $CO_2$ is the active species and $CO_2$ is the source of $^{14}C$, that the incorporation of $^{14}C$ into product is rapid and then levels off to a rate similar to that when carbonic anhydrase is present. When $H^{14}CO_3^-$ is added, the rate of $^{14}C$ incorporation is slower than that with carbonic anhydrase present. Fig.3.2(i)B shows the results when $HCO_3^-$ is the active species. The rate of $^{14}C$ incorporation is faster if $H^{14}CO_3^-$ is the initial labelled species than it is in the presence of carbonic anhydrase, and when the initial labelled species is $CO_2$ it is slower.

Partially purified enzyme was used for this assay and the results obtained are shown in Fig. 3.2(ii). A high initial rate of formation of $^{14}C$-isocitrate is observed when $H^{14}CO_3^-$ and $^{12}CO_2$ are added whilst a low initial rate is obtained when $H^{12}CO_3^-$ and $^{14}CO_2$ are added. These results indicate that $HCO_3$ and not $CO_2$ is the carboxylating species for this reaction. This is in contrast to the reverse reaction of isocitrate dehydrogenase (NADP) which has been shown to carboxylate 2-oxoglutarate using $CO_2$ as the reactant.

### 3.3.3 Divalent metal ion requirement

The enzyme reaction required a divalent metal ion for expression of full activity. To check whether the choice of $Mg^{2+}$ ions was the best for the reaction a series of divalent metal ions was tested. The $Cl^-$ ion was chosen as the constant anion as it did not have any effect on the enzyme. $Mg^{2+}$ ion was found to be essential for this reaction with $Mn^{2+}$ and $Co^{2+}$ exhibiting 35% and 24% respectively of the $Mg^{2+}$ value at the concentration used (4mM).

### 3.3.4 Product identification

The product of the carboxylation of 2-oxoglutarate is OAS which is unstable in solution making it difficult to isolate. In attempting to isolate OAS the enzyme assay contains only the reactants for the first part of the reaction so that OAS is not reduced to isocitrate. To stabilise OAS the reaction is terminated by a HCl solution saturated with DNP. The DNP-OAS is extracted in chloroform-ethyl alcohol and chromatographed as shown using freshly prepared 2,4-dinitrophenylhydrazone of authentic OAS as the standard. Derivatives of DNP are easily detected on paper due to their bright yellow colour.

Fig.3.3 depicts the counts incorporated into acid stable DNP-product. The DNP-OAS authentic marker with an $R_f$=0.77 co-chromatographed with the radioactive spot of the applied DNP-acid stable product. This result was further confirmed by the use of thin layer electrophoresis (Walker and Coop, 1974). The labelled product co-migrated with

the yellow spot of the standard DNP-OAS as shown in Fig.3.4. These results show that OAS is the product of the ATP-dependent carboxylation of 2-oxoglutarate and that it can be stabilized to some extent and observed on chromatograms.

To demonstrate that isocitrate is the product of the overall reaction the complete assay mixture is used (i.e. with NADPH added) so that the OAS is reduced to the more stable isocitrate. Fig.3.5 shows that the acid stable isocitrate synthase product co-chromatographed with the $^{14}$C-isocitrate standard in ether:formic acid:water (5:2:1) with an $R_f$=0.64. This result was verified using the solvent system ethanol:ammonia:water (80:5:15) as presented in Fig.3.6 where the $R_f$=0.71.

The results positively identify the acid stable product of the ATP-dependent isocitrate synthase reaction which carboxylates 2-oxoglutarate to produce OAS which is reduced further to the more stable isocitrate.

### 3.3.5 pH optimum for the reaction

The activity of the enzyme is dependent upon the assay pH used. As shown in Table 3.2 the pH optimum is between pH7 and pH7.5 with large losses of activity resulting when the reaction is allowed to proceed in either very alkaline or acid conditions. The buffer used for all subsequent assays is NEM pH7.5 as this was the optimum pH for the extraction of the lyophilized rat cytosol powder and for the retention of enzymic activity on standing at 4$^{o}$C after extraction.

### 3.3.6 Subcellular fractionation

Leveille et al., (1966a) found that the activity of the 2-oxoglutarate reductive carboxylation pathway was located in the cytosol of the cell by fractionation using high speed centrifugation. The results of similar studies done on the localization of the isocitrate synthase reaction within the cell are shown in Table 3.3. Section A of the table gives three examples of fractionation at $5x10^4$g for 1 hr. Between 70-90% of the isocitrate synthase activity was in the supernatant indicating a cytosolic site for the enzyme. This was further examined by centrifugation at $1x10^5$g (Section B) and $1.5x10^5$g (Section C) for 1 hr. In both instances the enzyme activity resided in the supernatant. It is concluded from these results that isocitrate synthase and the 2-oxoglutarate reductive carboxylation pathway are both located in the cell cytosol.

### 3.3.7 A brief survey of the distribution of isocitrate synthase activity in other species and tissues

The 2-oxoglutarate reductive carboxylation pathway has been demonstrated in a variety of animals and tissue under different nutritional conditions. To determine whether isocitrate synthase could be extracted from tissue other than the rat liver several other animals were studied. All tissues were extracted and assayed in an identical manner to the rat liver and all animals were fed ad libitum.

The data presented in Table 3.4 are the extraction of isocitrate synthase of rabbit heart, kidney, liver and brain. Fractionation of the crude homogenate into mitochondrial and cytosolic components reveals that the

enzyme is in the cell cytoplasm in these four tissues. However the rabbit tissues were not used for further studies as the level of enzyme present did not warrant the change from the rat liver as the starting material.

Results of the extraction of avian livers are shown in Table 3.5. Both turkey and chicken livers exhibited very little or no isocitrate synthase activity using the assay system developed for the rat liver enzyme. It is possible that the enzyme from different animals has different optimal conditions but this was not investigated.

The extraction of enzyme was also attempted from the livers of normal mice and from mice with induced hepatomas (Mouse hepatomas were kindly supplied by Dr. J. Sabine, Waite Institute, Adel.). All hepatomas examined thus far have been shown to utilize the 2-oxoglutarate reductive carboxylation pathway (Kopelovich, 1970; Kopelovich *et al.*, 1970; Sabine *et al.*, 1973). From Table 3.6 it can also be concluded that hepatomas also have isocitrate synthase activity. The activity is almost two-fold greater compared to the normal liver cells although it should be pointed out that there were not enough hepatomas for statistical analysis. However this is similar to results for the hyperplastic alveolar nodule outgrowths (Kopelovich *et al.*, 1970) where there is a two-fold greater incorporation of radioactivity from [2-$^{14}$C] and [5-$^{14}$C] glutamate relative to prelactating tissue with an accompanying five-fold greater conversion of [5-$^{14}$C] glutamate into labelled fatty acids. In addition Sabine *et al.*, (1973) using transplantable hepatomas found that glutamate can serve as an efficient precursor of citrate,

even more effectively, under their experimental conditions, than in the normal or host liver. Previous work by Sabine et al., (1967) had shown that the overall rate of lipogenesis by the same mouse tumor is greater than by its host liver. However Sabine et al., (1973) stressed that the increase in the utilization of glutamate as a citrate precursor in tumors does not account for the lack of control of hepatomal lipogenesis.

### 3.3.8 The effect of diet on isocitrate synthase activity in rat liver

A number of studies using intact tissues have demonstrated the dependence of the 2-oxoglutarate reductive carboxylase pathway on the nutritional state of the animal. A decrease in the activity of the pathway is seen when the animal is starved followed by increases above normal upon refeeding. It is thus of great interest to discover whether isocitrate synthase undergoes a trend parallel to this change in the activity of the pathway or whether its activity remains unchanged irrespective of the level of utilization the pathway as is the situation with isocitrate dehygronenase (NADP).

Wistar hooded female rats were divided into four groups, each group given a different diet for the duration of the experiment. The first group was fed the normal rat pellets for 6 days, the second a diet of normal pellets for 3 days then 3 days of a high protein diet, the third were fed normal pellets for 3 days then starved for 3 days and the fourth were starved for 3 days and refed a high

protein diet for 3 days. After the various dietary treatments, the animals were sacrificed and their livers extracted, and the activity of isocitrate synthase was determined. The results presented in Table 3.7 show that the ATP-dependent carboxylation of 2-oxoglutarate is dependent upon the nutritional state of the rat in a fashion parallel to the activity of the 2-oxoglutarate reductive carboxylation pathway and lipogenesis. In contrast to this and in agreement with published results Table 3.8 shows that the level of isocitrate dehydrogenase (NADP) remains constant irrespective of the nutritional state and whether the forward or backward reaction is measured.

## 3.4 Discussion

### 3.4.1 The existence of isocitrate synthase

Since the demonstration by D'Adamo and Haft (1965) of the existence of the 2-oxoglutarate reductive carboxylation pathway there has been a need to investigate the presumed participation of the reversal of isocitrate dehydrogenase (NADP) in the carboxylation of 2-oxoglutarate to isocitrate. The reversal of isocitrate dehydrogenase (NADP) has been shown to be 4.4 (Uhr *et al.*, 1974) to 10 times (Howard and Becker, 1970) less than the rate of the forward reaction under optimal conditions. These values are most probably an overestimate of the extent of the reversal of isocitrate dehydrogenase *in vivo* as it would be difficult to get the high concentration of $CO_2$ required for optimal activity at physiological pH. Also the total activity of the enzyme remains unchanged under nutritional

states that increase the usage of the pathway. This however does not eliminate the possibility that *in vivo* isocitrate dehydrogenase may be regulated by a mechanism other than enzyme concentration.

It was thus postulated that the carboxylation of 2-oxoglutarate in the reductive carboxylation pathway is performed by the forward reaction of a carboxylase instead of the reverse reaction of isocitrate dehydrogenase (NADP). To study this proposal it is necessary to fractionate the liver and show that the pathway and the proposed enzyme co-exist in the same cell compartment. Having achieved this it was then shown that the enzyme reaction of the proposed enzyme (isocitrate synthase) and the reverse reaction differed. The isocitrate synthase was shown here to differ in its substrate requirement in that ATP was essential for isocitrate synthase activity whilst having no effect on the isocitrate dehydrogenase (NADP) activity. Furthermore, it has been shown (Fig.3.2) that $HCO_3^-$ is the substrate of isocitrate synthase, whereas it is known that $CO_2$ is the substrate of isocitrate dehydrogenase (NADP). The use of $HCO_3^-$ as the carboxylating species has physiological advantage as it would be difficult to attain saturation with respect to $CO_2$ under physiological pH due to the high Km (appKm=2.2mM) for $CO_2$ (Uhr *et al.*, 1974). These authors further observed competitive substrate inhibition when high levels of $CO_2$ were used, as they suggested, is presumably caused by bicarbonate, rather than by $CO_2$. However studies with intact tissue have shown that the 2-oxoglutarate reductive pathway is stimulated by the use of bicarbonate buffers (Leveille *et al.*, 1966a).

Another difference which has been reported here is the isolation of free OAS which has not yet been achieved by studies on isocitrate dehydrogenase (NADP). The probable explanation for this is thought to be that OAS is an enzyme-bound intermediate and not a released product (Dalziel et al., 1968). The product of isocitrate dehydrogenase is isocitrate if the reducing agent NADPH is added to the reaction mix. In this respect both enzymes produce an identical product but the above evidence indicates that the two mechanisms are entirely different.

### 3.4.2 Effect of nutrition on enzyme activity

The utilization of the 2-oxoglutarate reductive carboxylation pathway has been shown to decrease during starvation and increase after refeeding to levels above normal. Measurements of isocitrate synthase activity in the cell cytosol also shows this to be the situation for this enzyme in contrast to isocitrate dehydrogenase (NADP) which does not change significantly under these drastically different nutritional states.

Thus isocitrate synthase is a carboxylating enzyme with the level of its activity related to the metabolic activity of the pathway in which it participates. The change in the enzymatic activity is reflected, qualitatively, in the usage of the 2-oxoglutarate carboxylase pathway. This does not mean however that the altered levels of isocitrate synthase activity alone accounts for the fluctuations in the pathway or that this is the only method of control of isocitrate synthase in vivo. Alterations in enzymatic activity may be broad in extent, rather than

involving only one key, rate-limiting enzyme in the pathway.

In this pathway the citrate cleavage enzyme has also been shown to fluctuate in different nutritional and developmental stages in a pattern parallel to changes in lipogenesis (Kornacker et al., 1965; Leveille et al., 1966b; and Whitten et al., 1969). However the results of Foster and Srere (1968) indicate that the citrate cleavage enzyme does not regulate the rate of fatty acid synthesis in rat liver. They found that fatty acid synthesis increased markedly without any change in citrate cleavage enzyme actively in animals fasted and then refed. Further work on the levels of citrate cleavage enzyme will be needed to clarify this controversy.

Isocitrate synthase strengthens the evidence for the existence of the 2-oxoglutarate reductive carboxylation pathway. Although the change in isocitrate synthase in a fashion parallel to lipogenesis may not explain the control of lipogenesis in its entirety it is significant that it does change with the lipogenic response. This property of isocitrate synthase as well as the influence of ATP and the carboxylating species being $HCO_3^-$ and not $CO_2$ makes this enzyme a more likely candidate than the reversal of isocitrate dehydrogenase (NADP) for the reductive carboxylation of 2-oxoglutarate.

Fig. 3.1 The Isocitrate Synthase Reaction

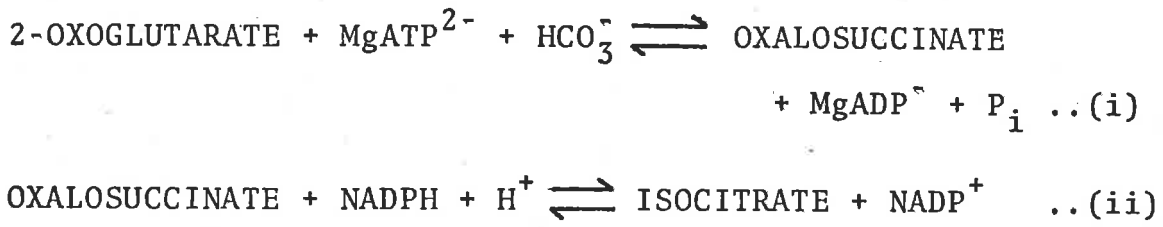


TABLE 3.1 Requirements for the isocitrate synthase reaction

| | Enzymic activity ($nmoles\ H^{14}CO_3$ fixed/min/mg of protein |
|---|---|
| Complete system | 27.03 |
| minus ATP | 0.07 |
| minus $Mg^{2+}$ | 0.02 |
| minus NADPH | 3.12 |
| minus 2-oxoglutarate | 0.85 |

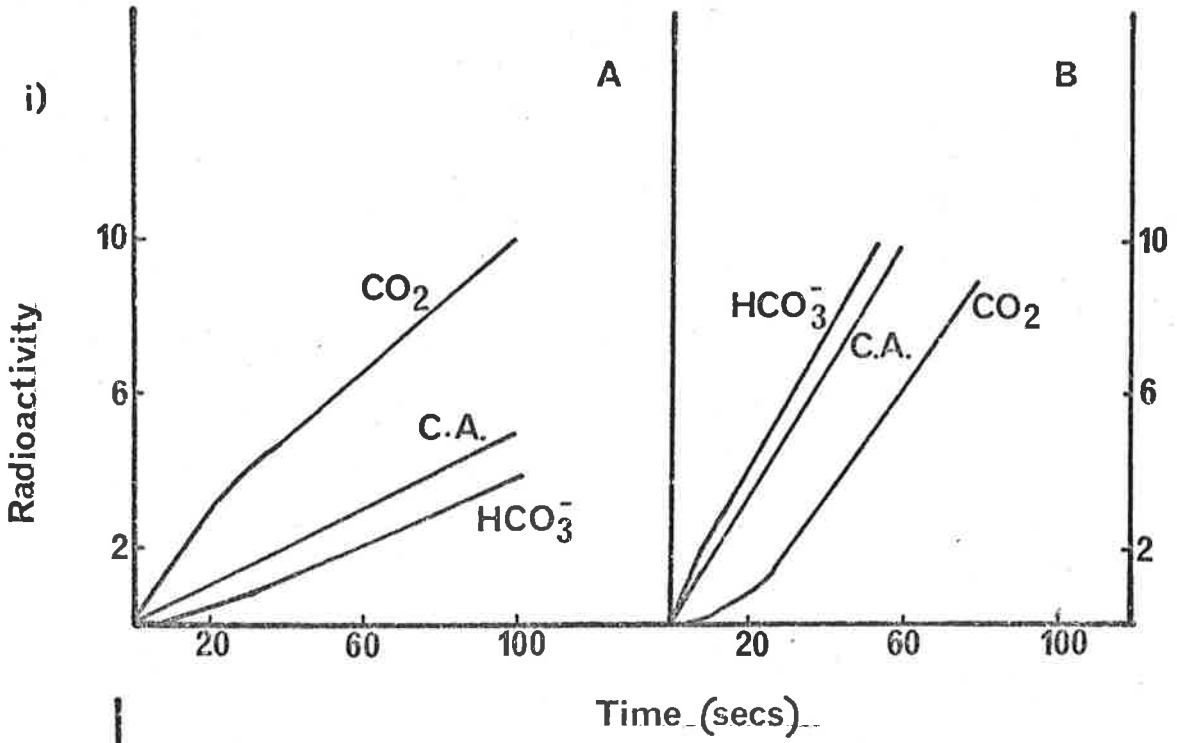
The assays were carried out as described in Section 2.2.5.1a. The complete assay system contained in µmoles, in a final volume of 0.25ml:NEM($Cl^-$,pH7.5), 25; ATP, 0.5; $MgCl_2$, 1.0; $NaH^{14}CO_3$ (0.25µCi per µmole), 2.5; NADPH, 0.025; and 2-oxoglutarate, 1.0. The enzyme used was partially purified up to Stage 4 (Table 4.10).

Fig.3.2 The active species for the carboxylation of isocitrate synthase

(i) Theoretical curves:A, if the active species used in the fixation is $CO_2$ and B, if it is $HCO_3^-$. The lines designated $CO_2$ is radioactive and the $HCO_3^-$ is unlabelled, those designated $HCO_3^-$ is radioactive and $CO_2$ is unlabelled, and those designated C.A. pertain to the instance when either member is radioactive and the other unlabelled and carbonic anhydrase is present.

(ii) Assay of isocitrate synthase activity when either $H^{12}CO_3^-$ plus $^{14}CO_2$ (—■—), $H^{12}CO_3^-$ plus $^{14}CO_2$ plus C.A. (—●—), $H^{14}CO_3^-$ plus $^{12}CO_2$ (—□—) and $H^{14}CO_3^-$ plus $^{12}CO_3$ plus C.A. (—▲—). The procedure is as set out in Section 3.2.2.3 and the enzyme used was purified to Stage 5 (Table 4.10).

i)


A
B
Radioactivity
10
6
2
$CO_2$
C.A.
$HCO_3^-$
$HCO_3^-$
C.A.
$CO_2$
20
60
100
Time (secs)

ii)

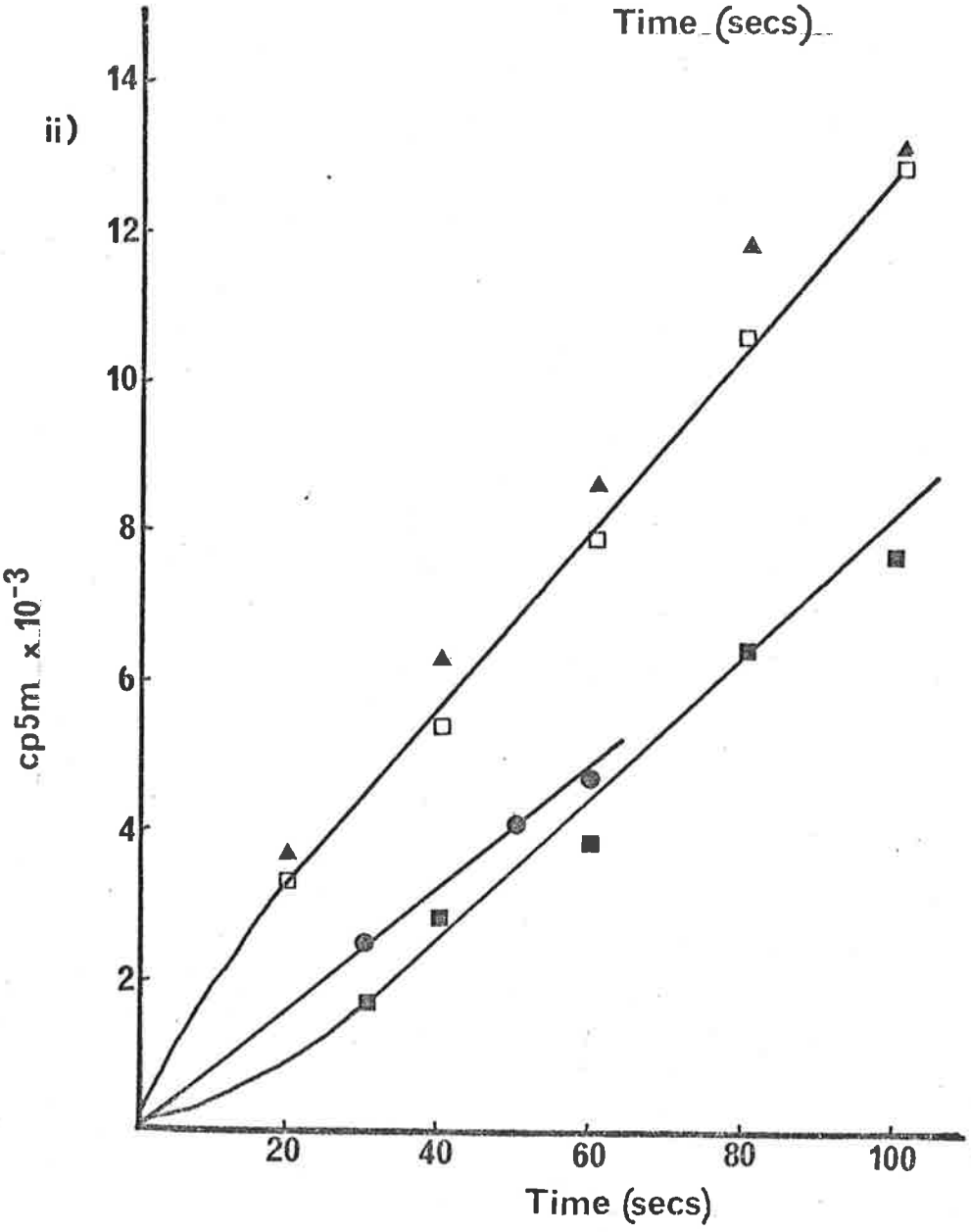

cp5m x $10^{-3}$
14
12
10
8
6
4
2
20
40
60
80
100
Time (secs)

Fig.3.3 Chromatography of the reaction product versus OAS standard

The assay mixture in Section 2.2.5.1a was used except NADPH was omitted. The reaction was terminated by a 6NHCl-DNP solution and the DNP-OAS extracted in chloroform (twice) and ethyl alcohol (once). These extracts were combined, evaporated and spotted onto the paper. Descending chromatography on Whatman 3MM was performed using 5%$NaHCO_3$ as the solvent and authentic DNP-OAS as a reference. The DNP-OAS was detected by its bright yellow colour and the radioactivity was determined as in Section 2.2.2.  represents authentic DNP-OAS.

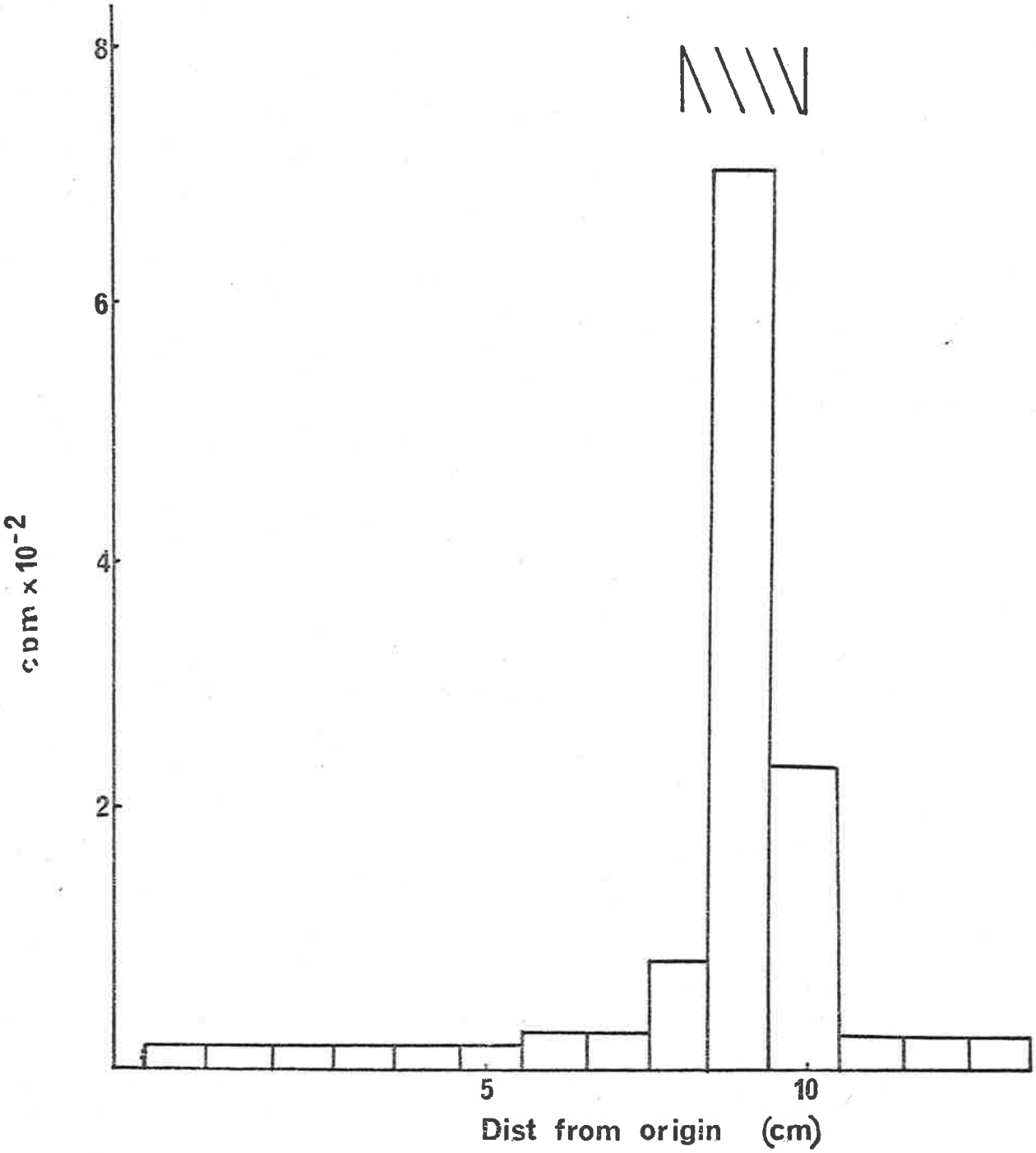

8
6
4
2
cpm x 10⁻²
5
10
Dist from origin (cm)

Fig.3.4 Thin layer electrophoresis of reaction product and authentic DNP-OAS

Reaction product as in Fig.3.3 was spotted on cellulose thin layer plate and electrophoresed with authentic DNP-OAS using 0.02M $NH_4HCO_3$,pH8.5 for 1 hr at 20 V/cm. DNP-OAS was detected by its bright yellow colour and this co-electrophoresed with the radioactivity of the assay samples applied.

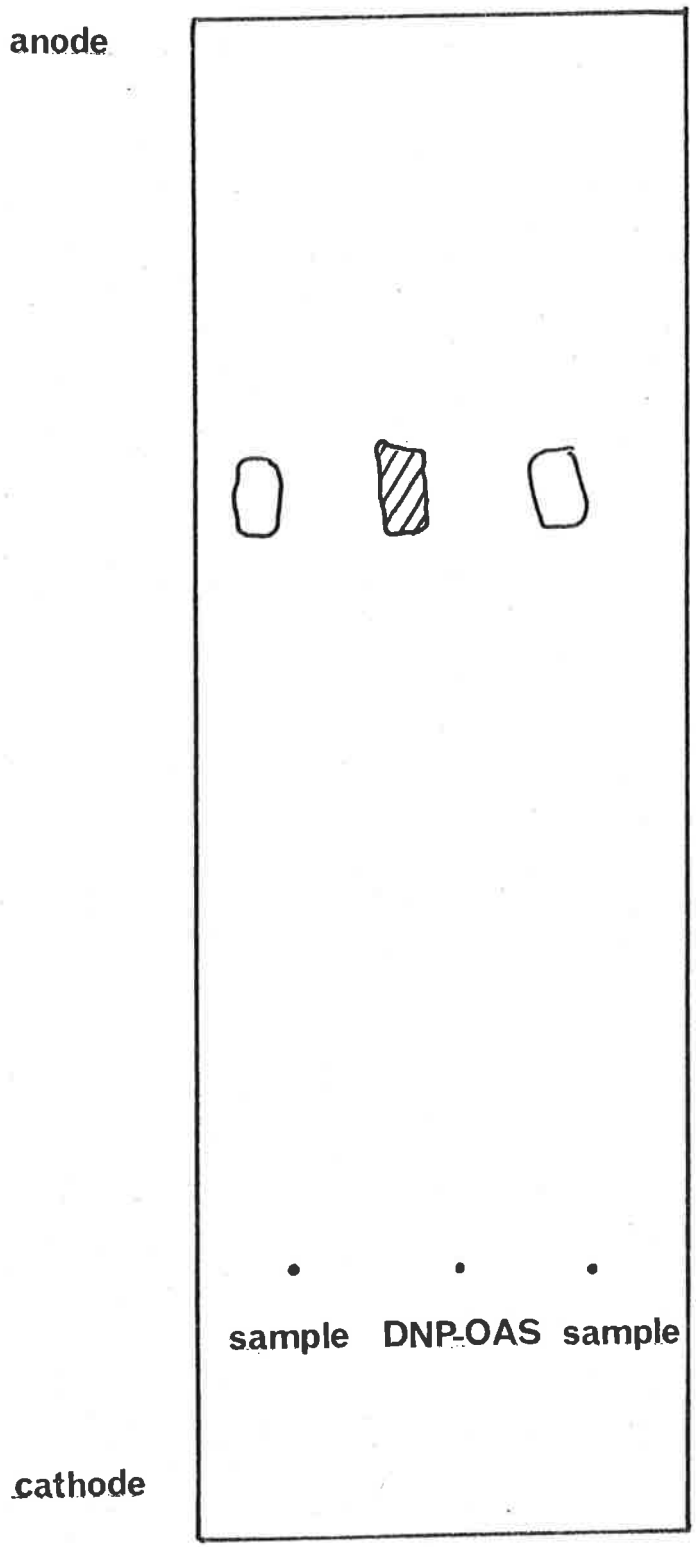

anode
sample
DNP-OAS
sample
cathode

Fig.3.5 Thin layer chromatograph of the isocitrate synthase reaction product and $^{14}$C-isocitrate

The assay mixture in Section 2.2.5.1a was used and extraction of the product was as in Fig.3.3 $^{14}$C-isocitrate used as the standard was obtained as described in Section 3.2.2.4. Ascending chromatography was carried out on cellulose thin layer plates using the solvent ether:formic acid:water (5:2:1)

(a) represents the $^{14}$C-isocitrate standard

(b) represents the reaction product in the presence 
and absence 
of ATP in the reaction mixture.

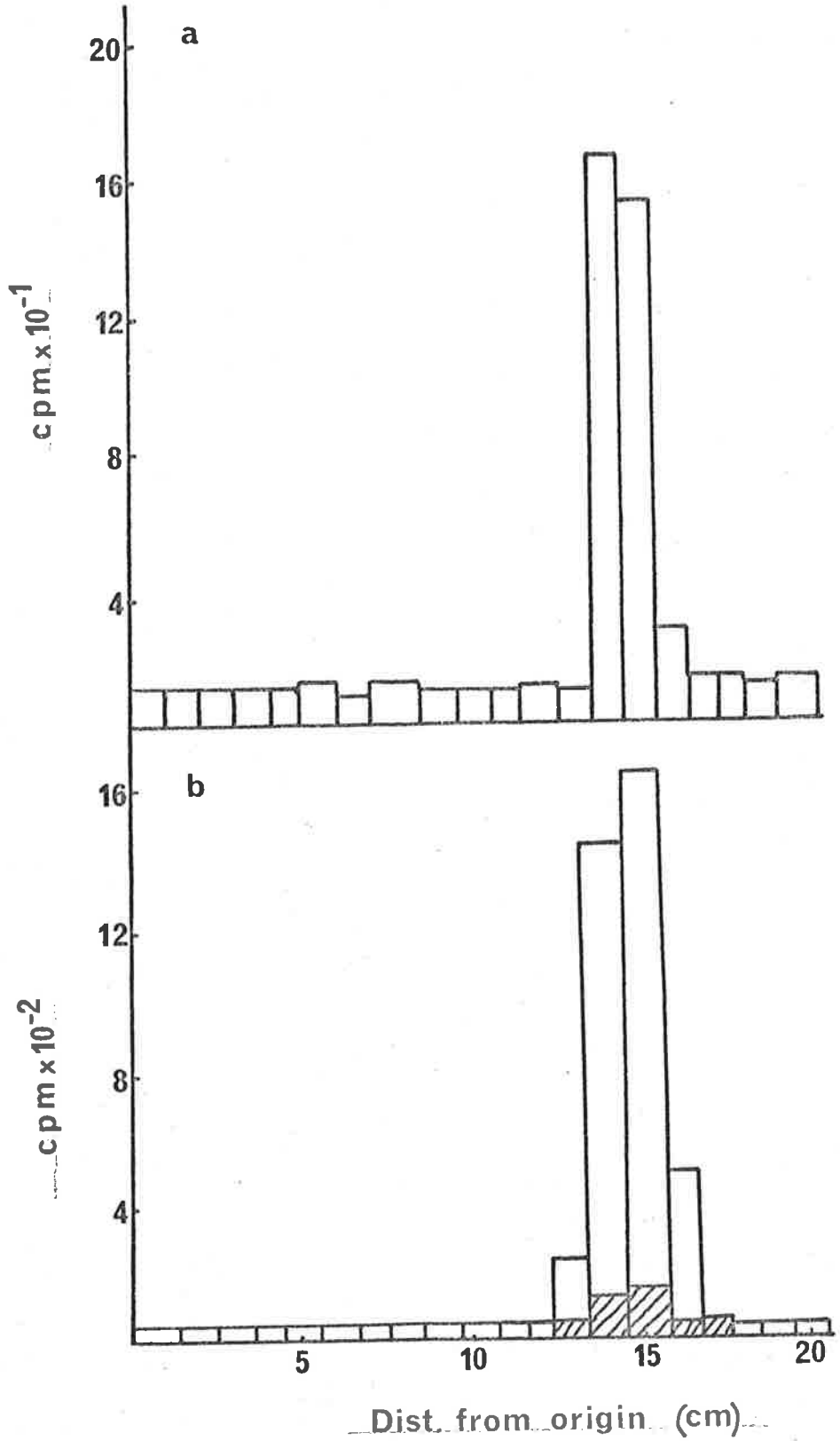

a
20
16
12
8
4
cpm x 10-1
b
16
12
8
4
cpm x 10-2
5
10
15
20
Dist. from origin (cm)

Fig.3.6 This is identical to Fig.3,5 except that the solvent used was ethanol:ammonia:water(80:5:15) pH6.5 with acetic acid.

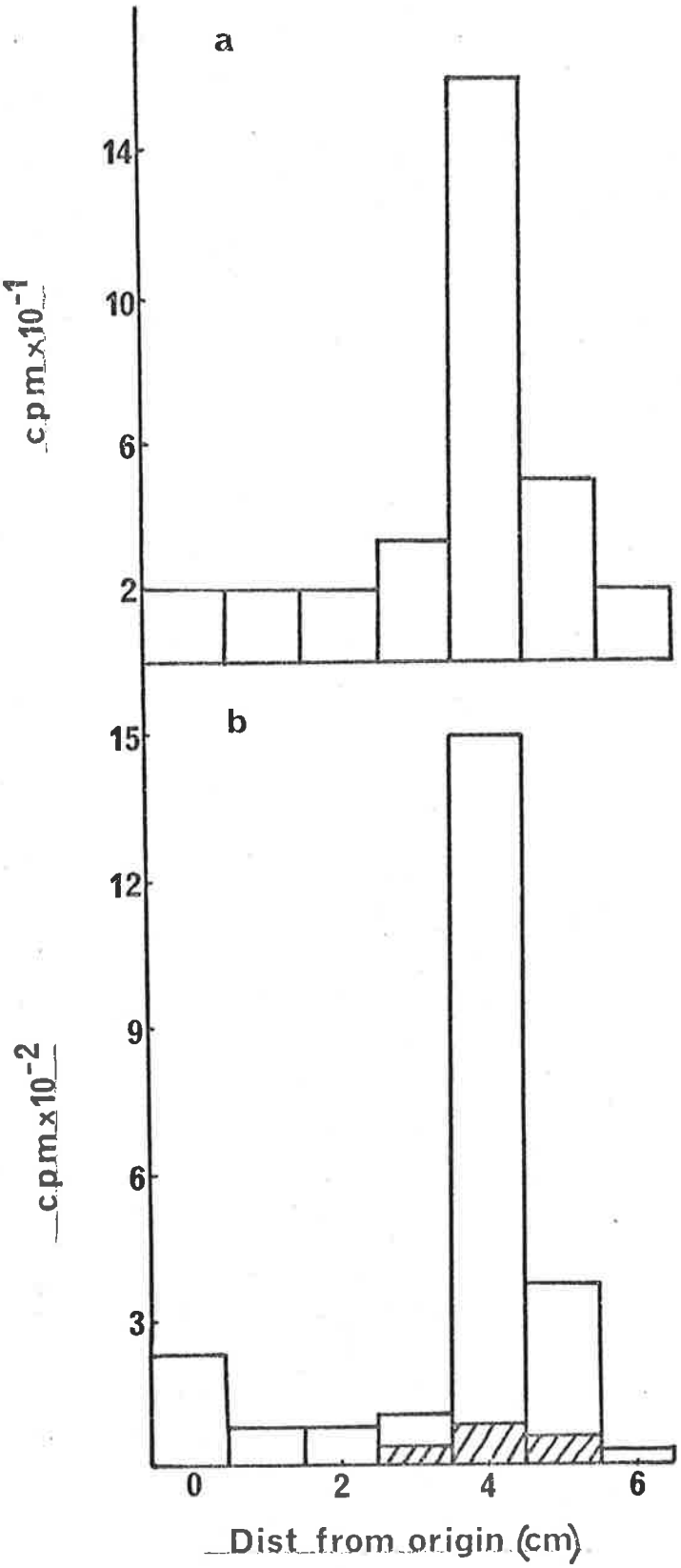

a
14
10
6
2
cpm x10⁻¹
b
15
12
9
6
3
cpm x10⁻²
0
2
4
6
Dist from origin (cm)

TABLE 3.2 pH optimum for the isocitrate synthase reaction

| Assay pH | Enzymic Activity nmoles $H^{14}CO_3$ fixed/min/mg of protein |
|---|---|
| 6.0 | 1.05 |
| 6.5 | 0.7 |
| 7.0 | 3.6 |
| 7.5 | 2.27 |
| 8.0 | 1.76 |
| 8.5 | 0.65 |
| 9.0 | 0.66 |

The assay mixture in Section 2.2.5.1a was used at each pH except that imidazole-HCl buffer was used at pH6.0 and pH6.5 NEM-Cl buffer was used at pH7-pH9.

TABLE 3.3 Fractionation by high speed centrifugation

| | | cpm/mg protein | % of total cell activity |
|---|---|---|---|
| A. | | | |
| Supernatant | (i) | 5198 | 92 |
| | (ii) | 5093 | 71 |
| | (iii) | 2473 | 80 |
| Precipitate | (i) | 432 | 8 |
| | (ii) | 2060 | 29 |
| | (iii) | 633 | 20 |
| B. | | | |
| Supernatant | | 311 | 92 |
| Precipitate | | 29 | 8 |
| C. | | | |
| Supernatant | | 338 | 79 |
| Precipitate | | 92 | 21 |

A: Liver extract was centrifuged at $5x10^4$g for 1 hr at $4^oC$

B: Liver extract was centrifuged at $1x10^5$g for 1 hr at $4^oC$

C: Liver extract was centrifuged at $1.5x10^5$g for 1 hr at $4^oC$

All precipitates were dissolved in 50mM NEM-Cl, pH7.5 and all fractions assayed as in Section 2.2.5.1a.

TABLE 3.4 Extraction of various rabbit tissues

| | ATP-dependent $H^{14}CO_3$ fixation nmoles/mg protein/min |
|---|---|
| Heart extract | 0.23 |
| (a) Supernatant | 3.20 |
| (b) Mitochondria | 0.36 |
| Kidney Cortex extract | 0.64 |
| (a) Supernatant | 3.06 |
| (b) Mitochondria | 0.44 |
| Liver extract | 0.74 |
| (a) Supernatant | 1.75 |
| (b) Mitochondria | 0.16 |
| Brain extract | 0.04 |
| (a) Supernatant | 0.79 |
| (b) Mitochondria | 0.17 |

All tissues were extracted as described in Section 2.2.4. The mitochondria were suspended in 0.25M sucrose containing 0.02MNEM, ph7.5 and 0.001M EDTA and all extracts were assayed as in Section 2.2.5.1a.

TABLE 3.5 Extraction of Avian Livers Post Mitochondrial Supernatant

| | ATP-dependent $H^{14}CO_3$ fixation nmoles/mg protein |
|---|---|
| Chicken | 0.076 |
| | 0.0 |
| | 0.92 |
| Turkey | 0.125 |
| | 0.0 |

Extraction of avian livers is as described in Section 2.2.4 and assayed as in Section 2.2.5.1a.

TABLE 3.6 Extraction of Mouse Liver and Hepatomas

| | $H^{14}CO_3^-$ fixation<br>µmoles/g wet weight |
|---|---|
| Mouse liver cytosol | 4.54 |
| Mouse hepatoma | 8.08 |

The hepatomas were removed from the liver and homogenized as was the liver in 0.25M sucrose containing 0.02MNEM, pH7.5 and 0.001MEDTA. Both extracts were assayed as in Section 2.2.5.1a.

TABLE 3.7 Effect of different diet regime on isocitrate synthase activity

| Diet | $H^{14}CO_3$ fixation *nmoles/min/g wet weight liver |
|---|---|
| Starved (S) | 61 ± 12 |
| Fed (F) | 119 ± 22 |
| Protein Fed (PF) | 97 ± 18 |
| Starved Refed (SF) | 191 ± 23 |

* Average values of seven rats

S vs F $p < .05$

S vs SF $p < .001$

F vs SF $p < .05$

PF vs SF $p < .01$

Rat livers were extracted as described in Section 2.2.4 and assayed using the assay mixture in Section 2.2.5.1a. Data analysis by the t-test show that the level of $^{14}CO_2$ fixation was statistically significant for S vs F; S vs SF and F vs SF as shown by the p value.

TABLE 3.8 The activity of isocitrate dehydrogenase under different diet regimes

| Diet | Isocitrate dehydrogenase activity | |
|---|---|---|
| | forward direction *µmoles/min/mg | backward direction +nmoles/min/mg |
| Starved (S) | 0.13 ± 0.03 | 0.17 ± 0.12 |
| Fed (F) | 0.20 ± 0.08 | 0.63 ± 0.32 |
| Protein Fed (PF) | 0.15 ± 0.01 | 1.37 ± 0.37 |
| Starved Refed (SF) | 0.17 ± 0.04 | 1.01 ± 0.42 |

* Average of 3 rats

+ Average of 2 rats

| | | |
|---|---|---|
| S vs F | $p < 0.5$ | $p < 0.4$ |
| S vs SF | $p < 0.5$ | $p < 0.2$ |
| F vs SF | $p < 0,8$ | $p < 0.6$ |
| PF vs SF | $p < 0,7$ | $p < 0.6$ |

Liver extraction was performed as described in Section 2.2.4. The forward reaction of isocitrate dehydrogenase activity is as described in Section 2.2.5.2 whilst the reaction in the backward direction was measured by the method of Siebert et al., (1957a). Data analysis using the t-test shows that the differences between the diets is not statistically significant as shown by the p values.

# CHAPTER 4

# PURIFICATION

## 4.1 Introduction

In order to further characterise isocitrate synthase it is desirable to purify it from the many other enzymes in the cell cytosol. Since the enzyme's physical properties are unknown a variety of purification techniques had to be attempted to discover the best methods applicable to this enzyme.

## 4.2 Materials and methods

### 4.2.1 Materials

All the G-series Sephadex, Sepharose 4B,6B, DEAE-Sephadex A-25, Sephacryl S-200 superfine, Octyl-Sepharose Cl-4B, Phenyl-Sepharose Cl-4B were purchased from Pharmacia (South Seas) Pty., Ltd. N.S.W. Australia and Bio-Gel P-300 was obtained from Bio-Rad Laboratories, Calif. U.S.A. Agarose-NAD type I and agarose-NADP type IV were from PL Biochemicals Inc., Milwaukee, Wis. U.S.A. P-cellulose P11 from Whatman. Special enzyme grade $(NH_4)_2SO_4$ was purchased from Schwarz/Mann, Orangeburg, N.Y. U.S.A. The enzyme source is rat liver cytosol of rats which had undergone 3 days starvation followed by 3 days of high protein diet.

### 4.2.2 Methods

The rat cytosol was obtained as in Section 2.2.4 and the radiochemical assay as in Section 2.2.5.1a is used throughout. Radioactivity was determined as described in Section 2.2.2. Isocitrate dehydrogenase (NADP), lactate dehydrogenase, malate dehydrogenase and

malic enzyme were measured spectrophotometrically as described in Sections 2.2.5(2), (.3), (.4) and (.5) respectively.

PEG-6000 was further purified by dissolving in acetone and then precipitating with ether (Albertsson,1967). Acetone was purified by refluxing over $KMnO_4$ for 1 hr and collecting the distillant at the constant boiling point. NEM was redistilled after refluxing with ninhydrin for 60 min.

Acetone fractionation was performed at $-10^{o}C$ following closely the method of Kaufman, (1971). Blue Dextran Sepharose was prepared by the method of Ryan and Vesting, (1974).

## 4.3 Result

### 4.3.1 Extraction of lyophilized cytosol

The best conditions for the extraction of the lyophilized starved-refed rat cytosol were investigated. Since NEM-Cl buffer was found to be the most suitable in the extraction of rat livers this buffer was chosen for the extraction of the cytosol. Table 4.1 reveals that 10-100mM NEM-Cl is preferable to $KP_i$ buffer. This buffer showed no variation in activity from 10-100mM and the enzyme remained active after 210 hr with only a loss of 33% of the original activity. In contrast to this, the $KP_i$ buffer resulted in a lower yield with increasing concentration from 5-300mM. Though the enzyme is more stable with time in 10-200 mM $KP_i$ compared to the activity at the time of extraction the lower initial yield still

indicates that NEM is the preferred buffer. Other buffers attempted included Tris-acetate, Tris-Cl and HEPES but these buffers did not better the results with NEM-Cl when activity and stability were taken into consideration.

Since isocitrate synthase is an ATP-dependent enzyme the presence of MgATP in the extraction buffer was tested. Table 4.2 shows that the use of $MgCl_2$ and ATP in NEM buffer increase the yield of enzyme activity when compared to NEM buffer alone. Thus the lyophilized cytosol is routinely extracted with the NEM-MgATP buffer used here.

### 4.3.2 Purification by "salting out" of proteins

The selective precipitation of proteins by "salting out" is a widely used technique in the laboratory. Commonly used precipitating agents are ammonium sulphate sodium sulphate, and streptomycin sulphate. Ammonium sulphate is the most commonly used agent because of its high solubility, its low cost and its protective effect on many enzymes (Charm and Matteo, 1971).

#### 4.3.2.1 Ammonium Sulphate

All attempts to fractionate isocitrate synthase by using ammonium sulphate resulted in almost complete loss of activity. Some activity was recovered after dialysis against 50mM NEM, pH7.5 but this did not warrant the inclusion of this step in the purification procedure. Table 4.3 shows that it is the ammonium ion and not the sulphate ion which is inactivating the enzyme. Concentrations of $(NH_4)_2\ SO_4$ or $NH_4Cl$ as low as 40mM completely inhibited

the enzyme whilst the same concentration of NaCl caused only slight inhibition of the enzymic activity. These results also show that the enzyme cannot tolerate high salt concentrations as 200mM NaCl inhibits the enzyme although not to the same extent as $(NH_4)_2SO_4$ and $NH_4Cl$.

4.3.2.2 Sodium Sulphate

Since the sulphate ion was not inhibitory attempts were made to precipitate the enzyme using sodium sulphate. In Table 4.4 it can be seen that up to 35% sodium sulphate did not precipitate isocitrate synthase. Further increases in salt concentration in order to precipitate the protein could not be achieved due to the low solubility of $Na_2SO_4$ compared to $(NH_4)_2SO_4$.

4.3.2.3 Streptomycin sulphate

Streptomycin sulphate was also attempted. Table 4.5 shows that low levels of this salt are not detrimental to this enzyme and up to 1% salt may be used without losses in activity. Higher concentrations cannot be used due to loss of enzyme activity, for example 50% loss of activity at 5% streptomycin sulphate concentration. Again with this step the enzyme is not precipitated but other proteins and nucleic acids are removed. It should be noted that the precipitate from 0.5-5% streptomycin sulphate did not exhibit any isocitrate synthase activity.

4.3.2.4 Polyethylene glycol

Another separation based on solubility is the use of

water-soluble nonionic polymers such as PEG. Precipitation of proteins from solution by the polymer is due to the ability of the polymer to exclude the protein, sterically, from part of the solvent. This brings the protein solution to its solubility limit. In general the larger the protein the less soluble it is in high polymer solutions (Fried and Chun, 1971).

This polymer is used in an attempt to precipitate the protein since the organic salts could not fulfil this function. Several series of different PEG concentration ranges were used and Table 4.6 shows the result of two such experiments. The enzyme activity residues in the PEG precipitates of 4-8% and 8-16% as shown in Table 4.6(a). In an attempt to pinpoint the exact concentration range Table 4.6(b) shows that the enzyme activity has mainly between 3-10% PEG precipitation. However there is still some activity in the 10% supernatant. The majority of the enzyme activity is in the 4-12% fraction shown in Table 4.6(c) and this concentration range was used for the purification of the enzyme. Due to viscosity problems with PEG the upper concentration range must be maintained as low as possible to obtain the desired separation.

4.3.2.5 Acetone fractionation

Organic solvents such as ethanol or acetone may be used for protein fractionation provided a rigid technique is used, as organic solvents can potentially denature protein. The most important factor in this fractionation is to work at temperatures below $0^{o}$ and thus it cannot be

used for cold labile enzymes. The advantages, disadvantage and the technique in detail is well set out by Kaufman, (1971).

Acetone fractionation at -10°C was attempted. Stepwise addition of acetone as shown in Table 4.7 reveals that 30% ($^{v}/_{v}$) acetone destroys enzymic activity in the supernatant. Further work has shown that the enzyme can tolerate up to 20% ($^{v}/_{v}$) acetone with the activity remaining in the supernatant. Thus this method cannot be used to precipitate isocitrate synthase but can remove other proteins from solution.

4.3.2.6 pH fractionation

Precipitation is an important tool for the concentration of proteins from solution, allowing further fractionation to be carried out with smaller volumes. Jagannathan and Schweet, (1952) employed the technique of variable solubility of enzymes at differing pH to attain highly purified pyruvic oxidase. In this technique the desired protein may be brought into minimum solubility at a precise pH resulting in a precipitate which may then be solubilized for further purification. This method may also be used to denature unwanted proteins which may be discarded after precipitation. The limit of this technique lies in the range of pH that the enzyme under investigation can tolerate without significant loss of activity.

Attempts to precipitate isocitrate synthase by stepwise variation of pH from pH7.5 to 4.5 is shown in Table 4.8. The original pH7.5 enzyme solution is adjusted to pH6.5, centrifuged to remove precipitate and the

supernatant readjusted to pH6. This step is repeated until pH4.5 is attained. The results of Table 4.8(a) show that the enzyme is either not precipitated at these pH values or on precipitation it is denatured. Table 4.8(b) shows that up to pH6 the enzyme is still active and in the supernatant whilst Table 4.9 shows that the enzyme is still active and in the supernatant at pH5.5. Since the enzyme activity does not residue in the pH4.5 supernatant it can be concluded that at levels below pH5.5 the enzyme is inactivated. This step is useful in the purification of isocitrate synthase as unwanted proteins can be precipitated between pH7.5 and pH5.5 without affecting the enzyme. It is stressed at this point that isocitrate synthase is not stable for long periods of time between pH6.8-pH5.5 and care must be taken to adjust the enzyme solution to pH7-pH7.5 immediately after the removal of the precipitated proteins.

### 4.3.3 Partial purification of isocitrate synthase

Partial purification of isocitrate synthase was attained by combining the foregoing procedures in the sequence shown in Table 4.10. Both the activity of isocitrate synthase and isocitrate dehydrogenase (NADP) were measured in order to discover whether the two enzyme co-purified. There is approximately a nine-fold purification of isocitrate synthase activity whilst concurrently the ratio of synthase to dehydrogenase activity increases. Thus these two enzymes are not being co-purified which is indicative that the two enzymes are distinct.

4.3.4 Storage conditions and stability of enzyme

Enzyme purified up to Stage 4 (see Table 4.10) was used to test a large series of conditions of storage to discover the preferred environment for the largest time interval. Table 4.11(a) shows the fifteen conditions used were the enzyme solution was freeze-dried before stored at -80°C whilst Table 4.11(b) is the same experiment but the solutions were snap frozen only before stored at -80°C. The zero time values are those of enzyme with the respective additions prior to snap freezing or freeze-drying. Both groups were assayed one, three and 21 months after storage. The activity after one month is identical to the first day of storage. After 3 months in the group that were freeze-dried the sucrose containing tubes lost some of their activity whilst the rest remained almost constant given that the assays were done two months apart. In the experiment which included snap freezing the loss of activity after three months was in the mannitol samples. The activity after 21 months in both experiments is identical to the values shown after 3 months.

It can be concluded from these results that enzyme after Stage 4 can be stored for a long time under a variety of conditions without loss of activity. Enzyme which has had no additional protective agent, that is, it is in 50mM NEM, pH7.5 maintains its activity over this time period. This allows the enzyme to be partially purified in large batches ready for further purification without having to remove any interfering protective agents. Thus this was the method of choice used throughout the following work.

4.3.5 Ion exchange chromatography

An ion exchanger is an insoluble material containing chemically bound charged groups and mobile counter ions. Adsorption of proteins to ion-exchange celluloses involves the formation of multiple ionic bonds between charged groups on the protein and groups of opposite charge on the absorbent. Chromatographic separation depends on the differential elution of the absorbed proteins. There are several techniques for elution which are based either upon alteration of the charge state of the protein (pH), or upon the use of substances which "compete" with the absorbed protein for the charged sites on the absorbent.

When a positively charged group is incorporated on the gel, the counter ions will be negative. Such an ion exchanger will exchange negative ions and is therefore termed an anion exchanger. Similarly a gel with negative groups incorporated has positive counter ions which are exchangeable, hence termed a cation exchanger. In general proteins will be absorbed to anion exchangers at pH's above their isoelectric point and to cation exchangers at pH's below their isoelectric points.(Himmelhoch,1971).

4.3.5.1 Cation exchange chromatography

P-cellulose P11 was used as the cation exchanger in attempts to further purify isocitrate synthase. The enzyme used for this purpose was the 4-12% PEG precipitate as this could be easily dissolved in the buffer with which the column was equilibrated. The most successful separation using this technique yielded 45% recovery and the elution profile is shown in Fig.4.1. The gradient

used is 50-200mM$KP_i$ pH7.0 and resulted in one major peak coming through unbound at 50mM$KP_i$ and three minor broad peaks coming off as the gradient is applied.

A gradient of 5-250mM$KP_i$ pH7 was tried to further fractionate the unbound peak that came off at 50mM. The result of this is that only 24% enzyme activity was recovered and as shown in Fig.4.2 the enzyme activity was smeared throughout the broad protein peak.

The lack of binding of the enzyme to P-cellulose even at very low buffer concentration and the loss of enzymic activity precluded the further use of this method for purification.

4.3.5.2 Anion exchange chromatography

The anion exchange resin used for this study is DEAE-Sephadex which is a weakly basic anion exchanger. Many varied conditions were attempted including pH elution, Tris-maleate, with and without NaCl, NEM with and without NaCl and NEM with sucrose. All attempts resulted in loss of enzymic activity. A typical profile obtained is shown in Fig.4.3. The protein profile shows four peaks before the gradient is applied and then one continuous peak at the end of the gradient. There is no enzyme activity anywhere along the peaks and attempts to recombine them failed to improve the situation. It can be concluded that either the enzyme binds to DEAE-Sephadex so strongly that it cannot be eluted from the column even with high buffer concentrations or once the enzyme binds it can only be removed in an inactive form.

4.3.6 Gel filtration

There are several different gel matrices available for this technique. Sephadex, polyacrylamide gels and agarose gels are the gels commonly used. The starting material used for the production of Sephadex is a linear dextran with glyceryl side chains and cross-linkages within and between the dextran molecules. This anhydro-glucose polymer has a high content of hydroxyl groups in the polysaccharide chains making it strongly hydrophilic. Sorption of aromatic and heterocyclic solutes to the gel is a commonly encountered phenomenon which results in retarded solute migration.

Polyacrylamide gels (Bio-GelP) are gels produced by copolymerizing acrylamide with a cross-linking agent. Sorption of very acidic, very basic, and aromatic compounds may occur, but this effect is less pronounced than with dextran gels.

Agarose gels (Sepharose, Bio-GelA) are linear polysaccharides obtained from agar and composed of alternating residues of D-galactose and 3,6-anhydro-L-galactose. These gels are hydrophilic in nature and show nearly complete absence of charged groups. These gels fractionate in a range above Sephadex and therefore form a complement to the Sephadex series.

Several gel matrices were used to purify isocitrate synthase on the basis of its size, as its molecular weight was unknown. Sephadex G100 with a fractionation range of $4x10^3$ - $1.5x10^5$ daltons for globular proteins resulted in enzyme activity associated with protein very

close to the Vo of the column as shown in Fig.4.4. All the protein applied to the column eluted at or near Vo with only a little peak towards Ve. Isocitrate synthase activity was only 8% of that applied onto the column and this, compounded with the poor resolution, made Sephadex-G100 unsuitable for this enzyme. However from the elution position of the enzyme activity along this column the molecular weight of isocitrate synthase is approximately in the 100,000 daltons range.

Sepharose 4B was next used as its Ve is $20x10^6$ daltons and thus the proteins that cluster together at the Vo when Sephadex-G100 is used should be separated. An agarose gel was chosen at this stage as these gels have superior flow properties than the very fragile Sephadex G200. Fig.4.5 is the resulting profile of applying isocitrate synthase after the PEG precipitation step to a Sepharose 4B column and chromatographing in 20mM NEM, pH7.5. The result once more is not a series of distinct peaks but a continuous broad profile with little or no separation of proteins. The recovery of isocitrate synthase activity is a very low 5% of the activity applied to the column.

Another gel attempted is the new product Sephacryl S-200 Superfine which has a fractionation range 5,000-250,000 daltons. This gel is prepared by covalently cross-linking allyl dextran with N,N' -methylene bisacrylamide resulting in a rigid but highly porous gel. However even using this gel the proteins applied did not separate and a profile similar to Fig.4.5 is obtained with the bulk of protein at Vo and almost complete loss of

isocitrate synthase activity.

Bio-Gel P-300 is porous polyacrylamide beads similar in composition to polyacrylamide gels with an exclusion limit of 400,000 daltons. Several attempts were made to utilise this gel for there is less sorption than with dextran gels. However columns of this gel poured and run as prescribed by the manufacturer shrink considerably upon being loaded with partially purified protein solution to be purified. The gel structure is not rigid enough and this physical limitation prevented the utilization of this gel.

4.3.7 Affinity chromatography

Affinity chromatography is absorption chromatography in which the bed material has biological affinity for the protein to be isolated. The specific absorptive properties of the bed material are obtained by covalently coupling an appropriate binding ligand to an insoluble matrix. This technique provides opportunities for the isolation of substances according to their biological function, and thus differs radically from conventional chromatographic techniques in which separation depends on gross physical and chemical differences between the proteins. Examples of such gel matrices are ATP-Sepharose, agarose-NAD, agarose-NADP and Blue Dextran Sepharose.

4.3.7.1 Blue Dextran-Sepharose affinity chromatography

Through the use of X-ray crystallography it has been observed that many $NAD^+$ utilizing enzymes have a "dinucleotide fold" (Ohlsson et al., 1974). This is a

particular arrangement of polypeptide chains which appears to be heavily conserved and binds $NAD^+$. Furthermore Thompson et al., (1975) have shown that blue dextran, a sulphonated polyaromatic blue dye covalently attached to dextran, behaves as an analogue of $NAD^+$ and so, when coupled to Sepharose 4B can be used for affinity chromatography of those enzymes possessing the dinucleotide fold. These authors further showed that some enzymes utilising NADPH, ATP and other nuceotide phosphates also bind to this matrix. Since isocitrate synthase is dependent upon both ATP and NADPH it should possess affinity for this matrix and be retarded thus purifying the enzyme from proteins which do not have any affinity for the ligand. Binding should occur at low ionic strength and the protein can then be eluted by the use of a gradient of increasing ionic strength.

Isocitrate synthase activity did not bind to this matrix even at 5mMNEM pH7.5 (Fig.4.6) and eluted unretarded with only 5% of enzyme activity recovered. However on applying a gradient up to 1.9MNEM pH7.5 three more peaks appeared. By combining the unretarded peak with peak one after the gradient, which itself had no isocitrate synthase activity, there resulted a four fold increase in activity. This was not attained using the other peaks eluted after the gradient. It therefore appears that blue-dextran sepharose at this low buffer concentration is binding part of the protein or proteins responsible for the isocitrate synthase activity but not the whole entity.

In an attempt to retain all the activity in one peak a series of higher concentrations of buffer were used. Glycerol was also added to the buffer in the hope of preventing any inactivation by dissociation of the proteins. Buffer concentrations 20mM NEM to 500mM NEM with 10% Glycerol resulted in recovery of the enzyme in the unbound state with the best recovery range being 65-95%. A representation of these results is shown in Fig.4.7. The peaks appearing after the application of the gradient were assayed for a variety of dehydrogenase activities as the matrix has been shown to bind many of these enzymes (Thompson et al., 1975).

The enzyme fraction applied to the column was contaminated by small amounts of isocitrate dehydrogenase (NADP), and malic enzyme and large amounts of lactate dehydrogenase and malate dehydrogenase. The first peak which exhibits isocitrate synthase activity had only 0.4% of the applied lactate dehydrogenase, 1.3% of the malate dehydrogenase, 64% of isocitrate dehydrogenase (NADP) and 68% of malic enzyme. The peaks resulting after the gradient contained the remaining lactate dehydrogenase and malate dehydrogenase. Table 4.12 shows a summary of results using different buffer concentrations to elute the enzyme of the column. Thus this step in the purification is most useful in removing or reducing the contaminating dehydrogenases.

4.3.7.2 Agarose-NAD(P)$^+$ and ATP-Agarose affinity chromatography

The lack of binding of total isocitrate synthase

activity to Blue Dextran-Sepharose led to an investigation of the more specific affinity ligand agarose-$NAD^+$ and-$NADP^+$. However as with the Blue Dextran-Sepharose, agarose-$NAD^+$ did not bind isocitrate synthase activity using a variety of buffer concentrations and conditions. The best result attained with this gel is 78% recovery and approximately 2.5 fold purification which is shown in Fig.4.8.

Since the enzyme is highly specific for $NADP^+$ and due to the lack of binding to agarose-$NAD^+$ the more specific agarose-$NADP^+$ was used. The results are identical to those using agarose-$NAD^+$ which tends to indicate that the NADP binding site is not available for interaction with the ligand or the conditions chosen did not favour such an interaction.

Another substrate used as an affinity column was ATP-Sepharose. Again the enzyme did not bind to the column, and the protein and activity profiles are identical to those obtained with agarose-$NAD^+$. The lack of consistent success with these affinity gels and their expense did not warrant their use in the purification procedure.

### 4.3.8 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography is a new technique in which substances are separated on the basis of the differing strengths of their hydrophobic interactions with an unchanged bed material which contains hydrophobic groups (Hofstee,1976). Many proteins have hydrophobic sites exposed on their surfaces.

Raising the ionic strength of a solution increases the strength of hydrophobic interaction. Absorbed proteins are fractionated by altering the elution conditions to give selection desorption based on the differing strengths of their hydrophobic interactions with the matrix, for example, by lowering the ionic strength or raising the pH of the eluent. The gel matrix used for this purpose is Phenyl-Sepharose Cl-4B.

Fig.4.9 shows that there is no binding or hydrophobic interaction of isocitrate synthase with this matrix as the activity is eluted before gradient application. It should be stressed here that this enzyme has very low tolerance of high ionic strength and this was a limiting factor. However this matrix does purify the enzyme as it binds contaminating hydrophobic proteins. Table 4.13 is a summary of a variety of conditions used and the yield and purification achieved. As is shown here enzyme applied in 200mMNEM is purified four fold but with 70% loss of activity. Applying a gradient down to 1mMNEM did not release any of the bound proteins and thus lower buffer concentrations were used. The enzyme is seen to be more stable at 100mMNEM as this increases the amount of enzyme recovered with the purification being from 1.7-5.0 fold. Therefore although the enzyme does not exhibit any hydrophobic interaction with this matrix, the method can still be used to purify isocitrate synthase.

## 4.4 Discussion

The aim of any purification procedure is to develop reliable and reproducible methods for isolation of a homogeneous protein in reasonable yield. Many and various techniques were used in this attempt as isocitrate synthase is a newly discovered enzyme with unknown properties.

Of the buffer systems used in this study NEM-Cl is the most suitable with constant enzyme activity in the concentration range 20mM-100mM. The enzyme is very susceptible to denaturation by ammonium salts and ionic strengths greater than 200mM. This prohibited the use of ammonium sulphate precipitation which is a useful and widely used step in protein purification. It also limits attempts at affinity and hydrophobic interaction chromatography which use salt gradients for elution. Thus PEG a nonionic polymer was the only useful reagent for precipitating the enzyme.

The pH optimum for enzymic activity is pH7.5 and the enzyme is inactivated at alkaline pH. However during purification isocitrate synthase can tolerate acid pH to pH5.5 for short periods of time and this is used to advantage by removing precipitating enzymes in the range pH7.5-pH5.5.

Ion exchange chromatography reveals that the enzyme is denatured by ion exchange procedures and the lack of binding to P-cellulose at 50mM$KP_i$ or higher indicates that the enzyme is negatively charged at pH7 and is acidic in nature. Such a protein will bind to DEAE. However the eluted protein that bound to this matrix did

not exhibit any isocitrate synthase activity. The enzyme could be denatured, as a high ionic strength is required to elute the bound protein, or it could be inactivated by the dilution that it had undergone, as the protein was smeared over a large volume. If the enzyme was in the unbound peaks than it must have been denatured by the passage down the matrix. The lack of activity can also be due to tight binding to the matrix which can be difficult to reverse without denaturing conditions as could occur if the protein was very high negatively charged, that is, very acidic.

Recent studies using the periodate-resorcinol assay of Jourdian et al., (1971) revealed the presence of protein-bound sialic acids in the enzyme fraction applied to these gels. These acidic sugars would attribute to the very acidic nature of the enzyme as is evident from these ion exchange chromatography results.

The work with several gel chromatography matrices shows that the enzyme spreads over a broad range instead of being a distinct peak. There is also large losses in enzymic activity. This can be rationalized by the sorption of very acidic proteins to the gel matrices based on agarose and sephadex which retards the protein and causes the broadening of the protein peak. Another probable explanation is that the enzyme is not one protein but a complex of several proteins or subunits and the gel matrix is dissociating the complex resulting in loss of activity and a wide spreading of the associating-dissociating complex. This aspect is fully discussed in

chapter six.

Affinity chromatography of isocitrate synthase indicates that the enzyme's active sites for both NADP and ATP are not readily available on the surface of the molecule. The work with Blue Dextran Sepharose also reflects the possible multicomponent nature of the enzyme, that is, that the enzyme is a complex and not a single protein. The components have different affinities to Blue Dextran Sepharose when the enzyme is applied at low ionic strength. One component or components having no affinity for the column whilst another or other components bind to the column and elute off after a gradient of higher ionic strength is applied. The apparent multicomponent nature of the enzyme will be discussed further in chapter six but with respect to attempts to purify the enzyme this feature of the enzyme could well be responsible for the lack of success when column chromatography is used. If the different components have different ionic properties or molecular weight, then the active complex may be dissociated resulting in the large losses of activity as reported in these studies.

Studies using hydrophobic chromatography show that isocitrate synthase does not possess many or any hydrophobic sites exposed to the surface. However the hydrophobic interactions between protein and the gel is maximum at high ionic strength and since the enzyme is not stable under such conditions this technique could not be used to full advantage.

Isocitrate synthase remains only partially purified at the conclusion of this work. The problems encountered here could be due to the multicomponent nature of the enzyme as indicated here and shown in more detail in chapter six.

TABLE 4.1 Extraction of lyophilized cytosol into various buffers

| Buffer | ATP-dependent $CO_2$ fixation at time of extraction | Stability of enzyme after 210 hr % of zero time |
|---|---|---|
| 10-100mMNEM | 100% | 67% |
| 5mM$KP_i$ | 96 | 62 |
| 10mM | 89 | 73 |
| 20mM | 74 | 72 |
| 50mM | 68 | 85 |
| 100mM | 67 | 77 |
| 200mM | 53 | 79 |
| 300mM | 60 | 54 |

The lyophilized rat liver cytosol was extracted as described in Section 3.2.2.1 except the buffer was as shown above. In addition all buffers contained 1.6mM$Mg^{2+}$, 0.8mM$ATP^{2-}$, and 2.4mMEDTA and all extractions were at pH7.2. All extracts were stored at 4°C for the duration of the experiment and assayed as described in Section 2.2.5.1a.

TABLE 4.2 Enzyme extraction in the presence and absence of MgATP

| Extraction Buffer | ATP-dependent cpm/ml |
|---|---|
| MgATP, NEM, EDTA | 654 |
| | 522 |
| NEM, EDTA | 273 |
| | 445 |

The lyophilized rat liver cytosol was extracted as described in Section 3.2.2.1. The buffer contained 20mMNEM, pH7.5, 2.4mMEDTA and when used 0.8mMATP and 1.6mM$Mg^{2+}$. Results shown are for duplicate extractions in each buffer system. ATP was removed from the extracts prior to assaying by passage down a Sephadex G15 column.

TABLE 4.3 The effect of ammonium ions on isocitrate synthase activity

| Salt added to enzyme | ATP-dependent $H^{14}CO_3$ fixation cpm/mg protein |
|---|---|
| 0 (Starting Material) | 958 |
| 40mM $(NH_4)_2SO_4$ | 0 |
| 200mM $(NH_4)_2SO_4$ | 0 |
| 40mM $NH_4Cl$ | 19 |
| 200mM $NH_4Cl$ | 0 |
| 40mM NaCl | 765 |
| 200mM NaCl | 171 |

The starting material was the extracted liver cytosol obtained as described in Section 3.2.2.1. The enzyme was preincubated in the added salt for 30 min at 20°C prior to assaying as described in Section 2.2.5.1a.

TABLE 4.4 Precipitation with sodium sulphate ($Na_2SO_4$)

| % $Na_2SO_4$ ($^w/_v$) | $H^{14}CO_3$ fixation nmoles/mg protein |
|---|---|
| 0 (Starting Material) | 15.7 |
| 25 supernatant | 17.1 |
| precipitate | 1.14 |
| 25-35 precipitate | 2.9 |
| 35 supernatant | 14.0 |

The starting material is enzyme partially purified to Stage 4 (see Table 4.10). All precipitates were suspended in 20mMNEM, pH7.5 containing 2.4mMEDTA and the precipitates and supernatants were dialysed for 2 hr against this buffer to remove the salt prior to assaying as described in Section 2.2.5.1a.

TABLE 4.5 Precipitation by streptomycin sulphate

| % Streptomycin Sulphate ($^{w}/_{v}$) | ATP-dependent $H^{14}CO_3$ fixation cpm/mg protein |
|---|---|
| 0 (Starting Material) | 2061 |
| 0.5 | 1712 |
| 1.0 | 1978 |
| 2.5 | 1682 |
| 5.0 | 1039 |

The starting material was the supernatant obtained after extraction of the liver cytosol as described in Section 3.2.2.1. The precipitate obtained at each step was inactive and the results shown are for the activity in the supernatant as measured by the assay described in Section 2.2.5.1a.

TABLE 4.6 PEG fractionation

The starting material in each case is the supernatant as extracted from liver cytosol and described in Section 3.2.2.1. All precipitates were dissolved in 20mMNEM, pH7.5 containing 2.5mMEDTA and assayed as described in Section 2.2.5.1a.

TABLE 4.6 PEG fractionation

| % PEG ($^{w}/_{v}$) | $H^{14}CO_3$ fixation cpm/mg protein |
|---|---|
| (a) | |
| 0 (Starting Material) | 2961 |
| 0-4 precipitate | 110 |
| 4-8 " | 3166 |
| 8-16 " | 1736 |
| 16-20 " | 103 |
| (b) | |
| 0 (Starting Material) | 7083 |
| 3 precipitate | 325 |
| 3-10 " | 16729 |
| 10 supernatant | 1130 |
| (c) | |
| 0 (Starting Material) | 2079 |
| 4 supernatant | 785 |
| 4-12 precipitate | 1251 |
| 12 supernatant | 534 |

TABLE 4.7 Acetone Fractionation

| Acetone conc. (v/v %) | | ATP-dependent $H^{14}CO_3$ fixation cpm/mg protein | |
|---|---|---|---|
| | | Expt 1 | Expt 2 |
| 0 | (Starting Material) | 4835 | 6853 |
| 14 | supernatant | 6238 | 6062 |
| | precipitate | 467 | 1080 |
| 30 | supernatant | 339 | 419 |
| | precipitate | 599 | 1308 |
| 50 | supernatant | 36 | 72 |
| | precipitate | 158 | 246 |

The starting material is the supernatant resulting from the extraction of liver cytosol as described in Section 3.2.2.1. The precipitates were dissolved in 20mMNEM, pH7.5 containing 2.4mMEDTA and the supernatant from each step was dialysed against this buffer prior to assaying as described in Section 2.2.5.1a.

TABLE 4.8 pH fractionation from pH 7.5-pH4.5

| | pH | % activity |
|---|---|---|
| (a) | 7.5 | 100 |
| | 6.5 | 92 |
| | 6.0 | 80 |
| | 5.5 | 80 |
| | 4.5 | 0 |
| (b) | 7.5 | 100 |
| | 7.25 | 86 |
| | 6.8 | 83 |
| | 6.3 | 85 |
| | 6.0 | 86 |

(a) The enzyme was the supernatant obtained after the extraction of liver cytosol as described in Section 3.2.2.1. The pH was adjusted by the addition of 0.5M acetic acid and all activities shown are for the enzymic activity remaining in the supernatant after precipitated material was removed by centrifugation.

(b) The procedure above was used except that the enzyme used was partially purified up to Stage 3 (see Table 4.10) and the buffer used was 50mMNEM, pH7.5.

TABLE 4.9 pH fractionation from pH6.4-pH5.5

| pH | $H^{14}CO_3$ fixation nmoles/min/mg protein |
|---|---|
| 6.4 supernatant | 397 |
| 6.1 supernatant | 806 |
| precipitate | 0 |
| 5.9 supernatant | 513 |
| precipitate | 74 |
| 5.7 supernatant | 770 |
| precipitate | 49 |
| 5.5 supernatant | 756 |
| precipitate | 69 |

The supernatant obtained from the extraction of liver cytosol as described in Section 3.2.2.1 was made to pH6.4 with 0.5M acetic acid and the supernatant was divided into 4 equal aliquots which were then each adjusted to one of the pH shown using 0.5M acetic acid. All precipitates were suspended in 20mMNEM, pH7.5 prior to assaying in the system described in Section 2.2.5.1a.

TABLE 4.10 Partial Purification of the Isocitrate Synthase System

| Fraction | Specific Activity (nmoles $^{14}CO_2$-fixed/min/mg of protein) | % Yield | Ratio of synthase to dehydrogenase activity |
|---|---|---|---|
| 1. Crude supernatant | 4.9 | 100 | 0.009 |
| 2. Streptomycin sulphate supernatant | 6.5 | 90 | 0.013 |
| 3. 4-12% polyethylene glycol precipitate | 13.8* | 72 | 0.028 |
| 4. pH5.5 supernatant | 25.8* | 63 | |
| 5. 20% acetone supernatant | 39.4* | 49 | 0.066 |

* Values obtained when the enzyme solution had been incubated with ATP and $Mg^{2+}$ for 60 min at 25°C prior to assay (5mMATP, 10mM$Mg^{2+}$)

\+ Isocitrate dehydrogenase activity measured in the forward direction as described in Section 2.2.5.2.

TABLE 4.11 Storage of isocitrate synthase

Enzyme purified up to Stage 3 (see Table 4.10) and dissolved in 0.025mNEM-Cl, pH7.5 was used. The freeze dried samples (a) were reconstituted in 0.025MNEM-Cl, pH7.5. The concentrations of the added reagents were; BSA, 1mg/ml; KCl, 0.9% ($^{w}/_{v}$); Mg, 2mM; ATP, 1mM, sucrose, 0.88M; DTE, 1mM; EDTA, 1mM; and mannitol, 0.44M.

TABLE 4.11 Storage of isocitrate synthase

| Addition for Storage | Time after Storage (months) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 3 | 1 | 3 |
| | nmoles of $H^{14}CO_3$ fixed | | | | |
| | | (a) | | (b) | |
| None | 124 | 124 | 127 | 132 | 103 |
| BSA | 122 | 122 | 134 | 112 | 141 |
| KCl | 100 | 99 | 150 | 134 | 113 |
| Mg | 120 | 122 | 122 | 154 | N.D. |
| ATP | 115 | 111 | 143 | 101 | N.D. |
| MgATP | 120 | 120 | 94 | 139 | 115 |
| Sucrose | 115 | 115 | 78 | 110 | 98 |
| + DTE | 100 | 96 | 69 | 115 | 91 |
| + DTE+EDTA | 120 | 122 | 74 | 108 | 98 |
| +MgATP | 125 | 128 | 81 | 105 | 134 |
| +MgATP+DTE | 130 | 132 | 76 | 115 | N.D. |
| Mannitol | 120 | 123 | 116 | 143 | 84 |
| +DTE | 125 | 129 | 110 | 172 | 87 |
| +DTE+EDTA | 125 | 127 | N.D. | 140 | 108 |
| +MgATP | 130 | 144 | N.D. | 153 | 95 |

Fig.4.1 P-cellulose chromatography using 50mM-200mM$KP_i$

Rat liver cytosol purified up to Stage 3 (see Table 4.10) was dissolved in 3ml of 50mM$KP_i$, pH7.5 and loaded in a P-cellulose P11 column.

Column size : 12cmx3cm

Buffer gradient : 50mM-200mM$KP_i$, pH7 (250mlx250ml)

Flow rate : 20ml/hr

Fraction size : 3ml

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

- - - - $KP_i$ (mM)

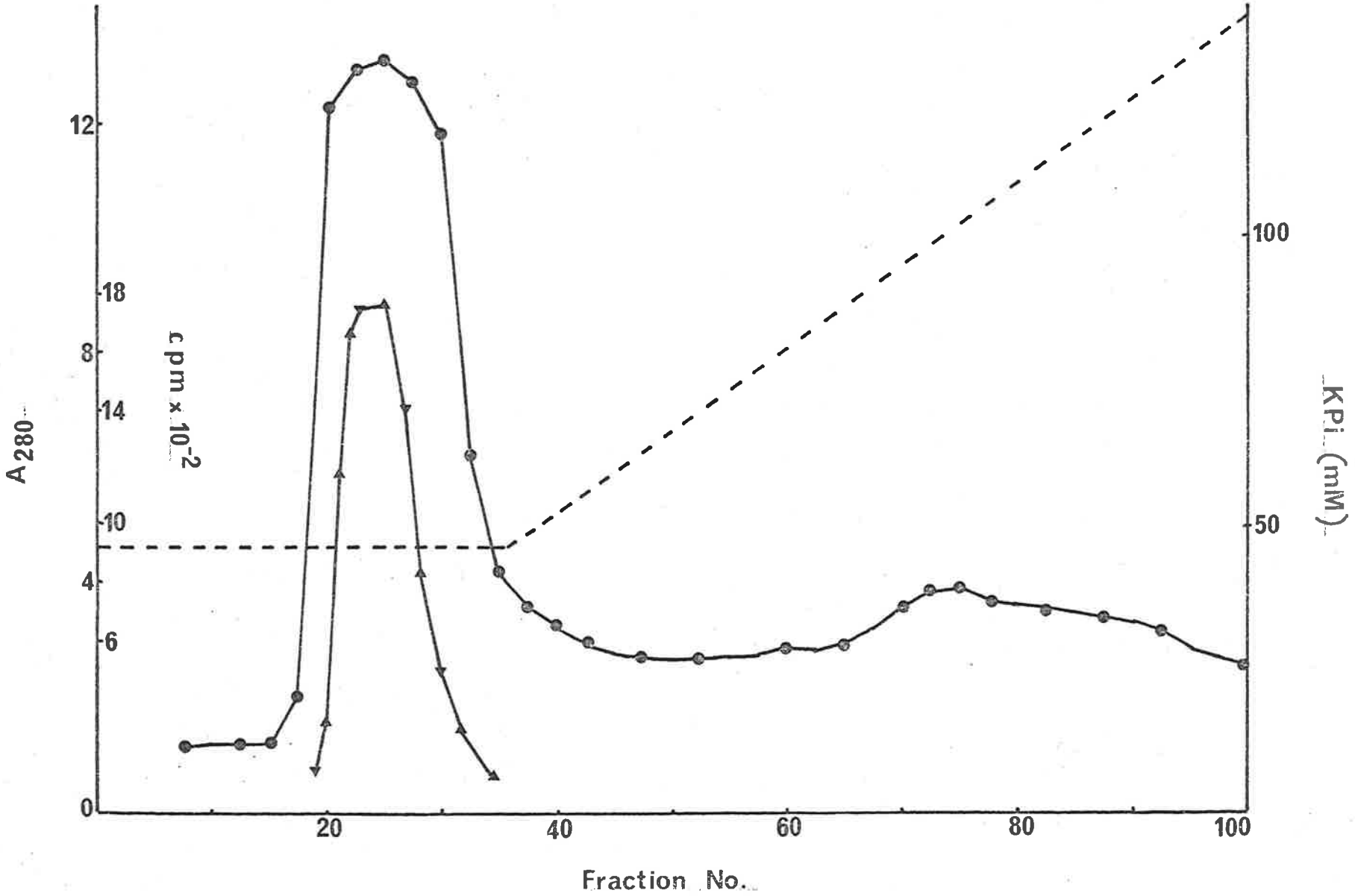

12
8
4
0
$A_{280}$
18
14
10
6
cpm x $10^{-2}$
20
40
60
80
100
Fraction No.
100
50
KPi (mM)

Fig.4.2 P-cellulose chromatography using 5-250mM$KP_i$

The unbound enzyme (fraction 20-35) from Fig.4.1 was concentrated and re-chromatographed on an identical P-cellulose column in identical conditions except the buffer gradient was 5-250mM$KP_i$, pH7.

—●— $A_{280}$

—■— $^{14}CO_2$-fixation

- - - - $KP_i$

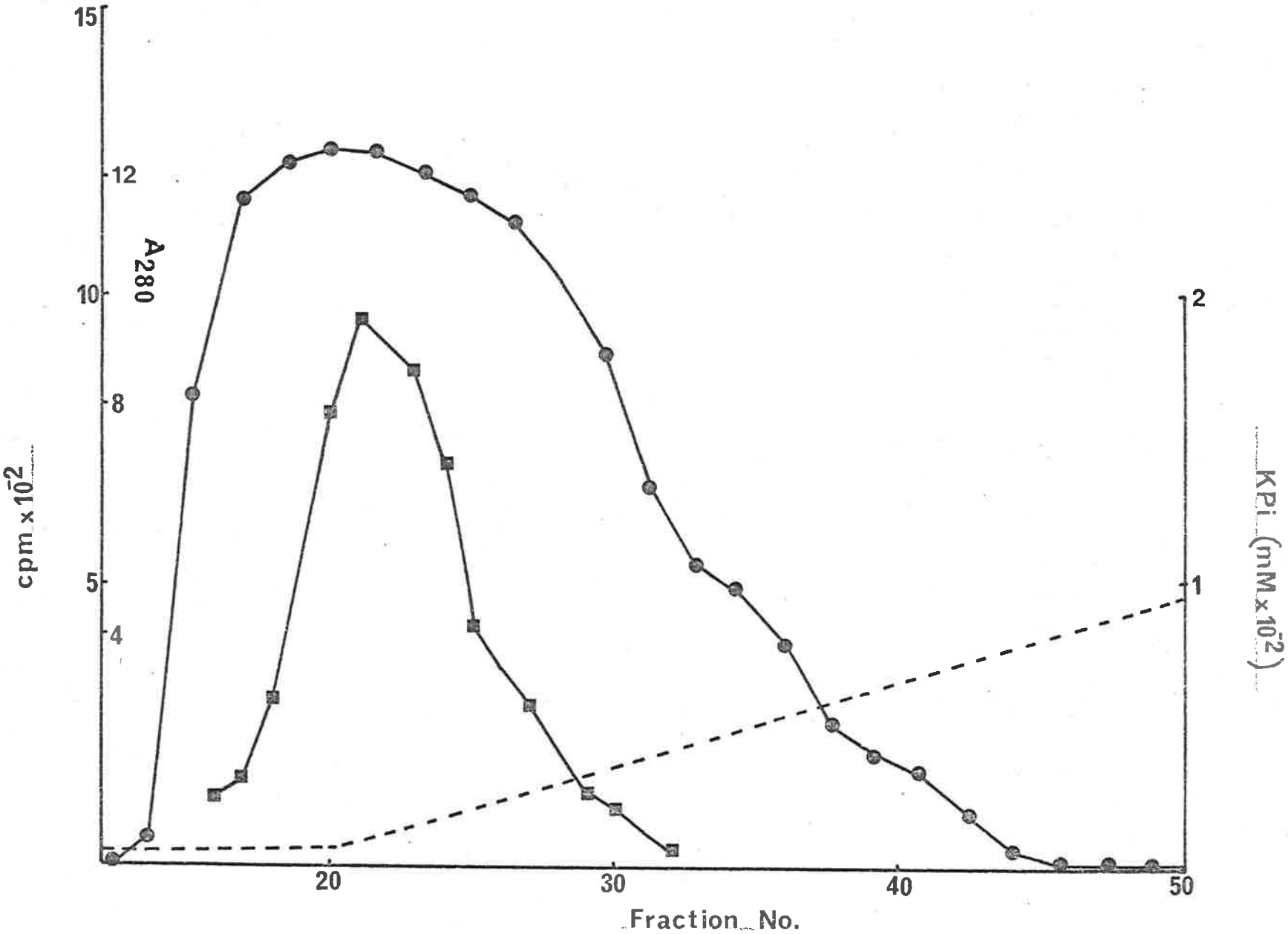

15
12
10
8
5
4
$A_{280}$
cpm x $10^{-2}$
2
1
KPi (mM x $10^{-2}$)
20
30
40
50
Fraction No.

Fig.4.3 DEAE-Sephadex anion exchange chromatography

Enzyme partially purified up to Stage 3 (see Table 4.10) was dissolved in 3mls of 10mMNEM-Cl, pH7.5 and loaded onto a DEAE-Sephadex column and the enzyme eluted by application of a gradient.

Column size : 12cmx3cm

Buffer gradient : 10-700mMNEM-Cl, pH7.5 (400mlx400ml)

Flow rate : 60mls/hr

Fraction size : 10ml

—●— $A_{280}$

- - - NEM(M)

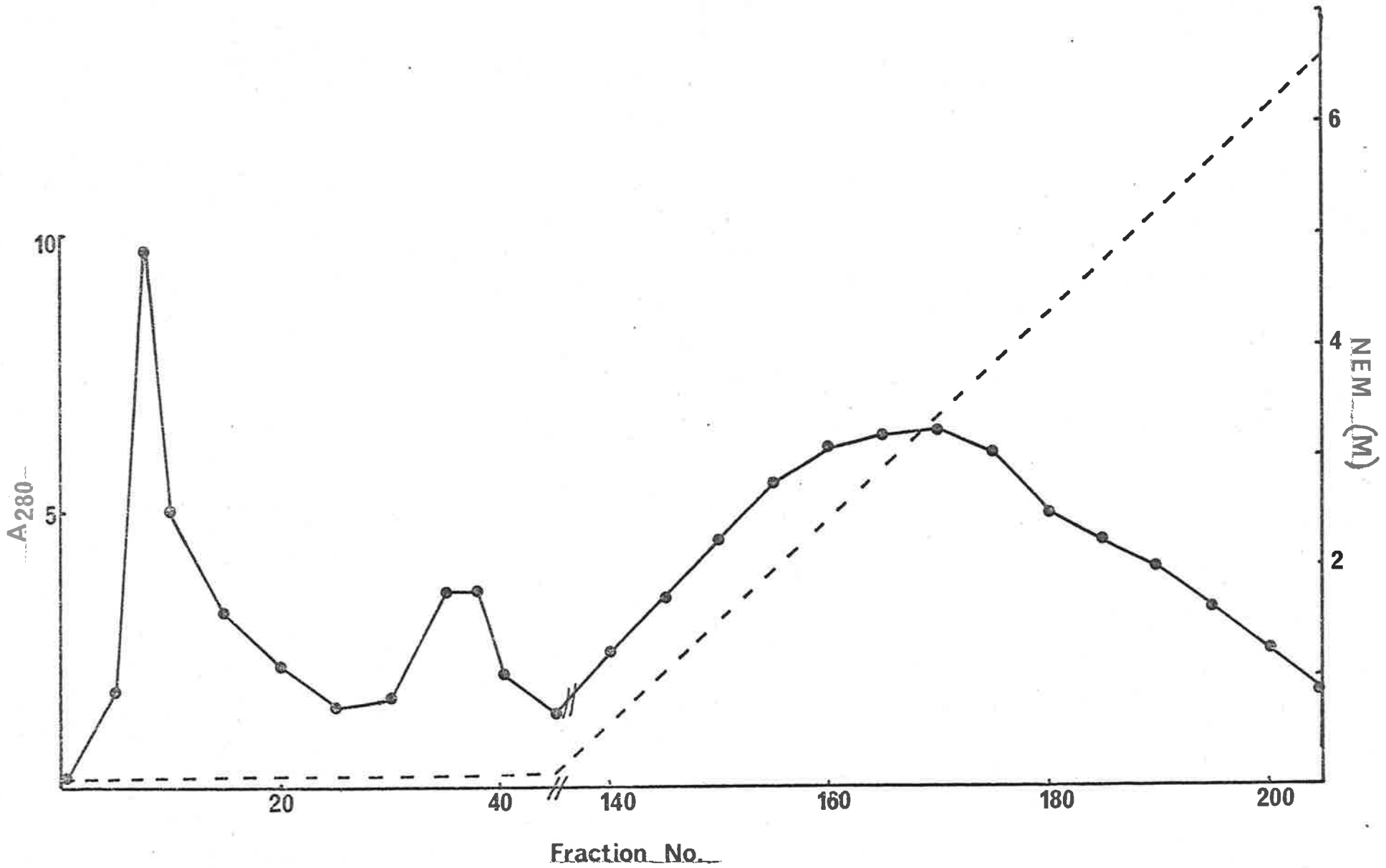

10
5
A280
20
40
140
160
180
200
Fraction No.
6
4
2
NEM (M)

Fig.4.4 Sephadex-G100 chromatography

Partially purified enzyme, up to Stage 4 (see Table 4.10), was applied to a Sephadex-G100.

Column size : 60cmx2cm

Buffer : 100mMNEM-Cl, pH7.5

Flow rate : 18ml/hr

Fraction size : 3ml

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

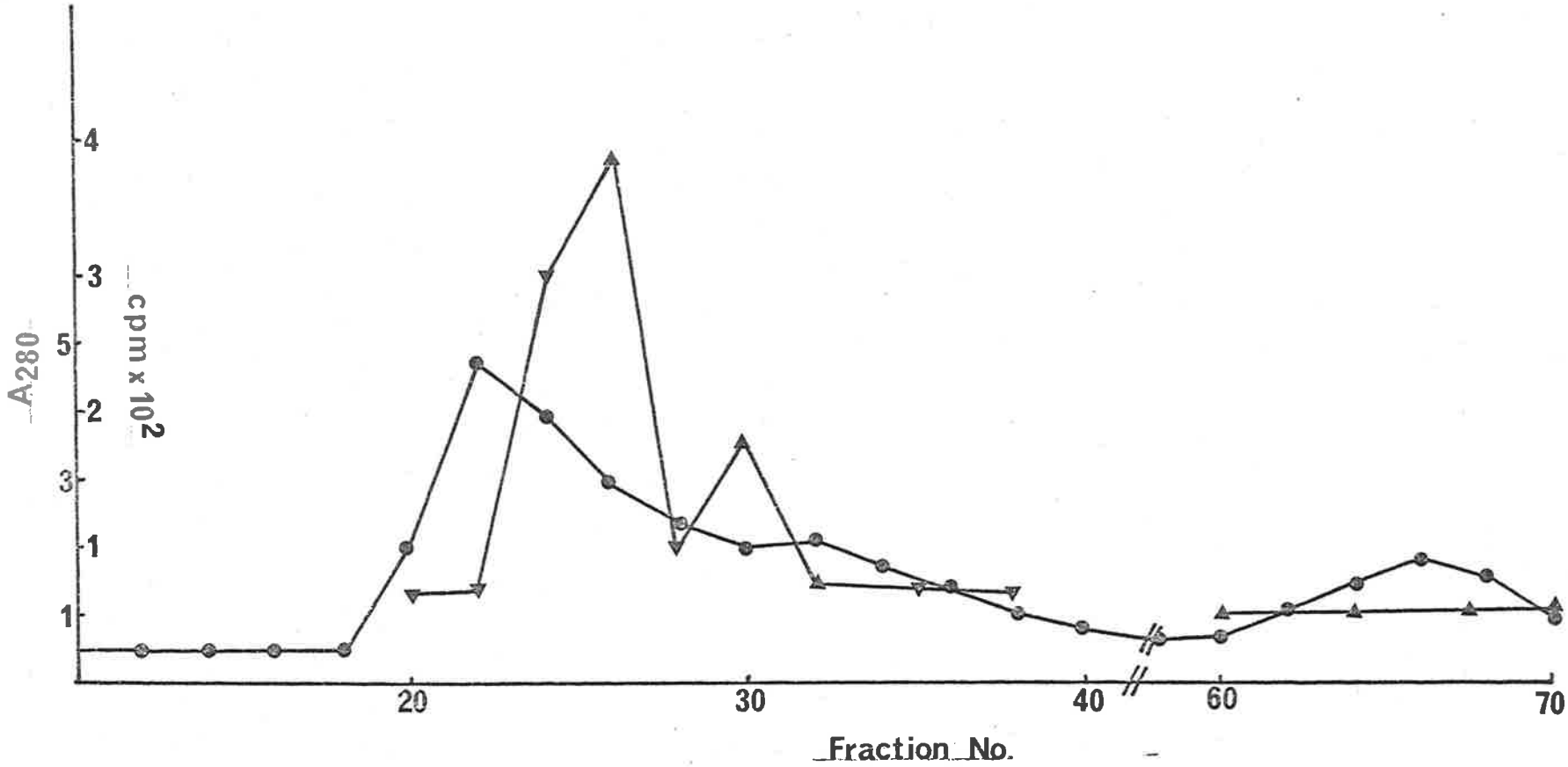

A280
cpm x 10²
4
3
5
2
3
1
1
20
30
40
60
70
Fraction No.

Fig.4.5 Sepharose 4B chromatography

Enzyme purified up to Stage 3 (see table 4.10) was dissolved in 4ml of 20mMNEM, pH7.5 and loaded onto a Sepharose 4B column.

Column size : 70cmx2.5cm

Buffer : 20mMNEM, pH7.5

Flow rate : 15ml/hr

Fraction size : 4ml

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

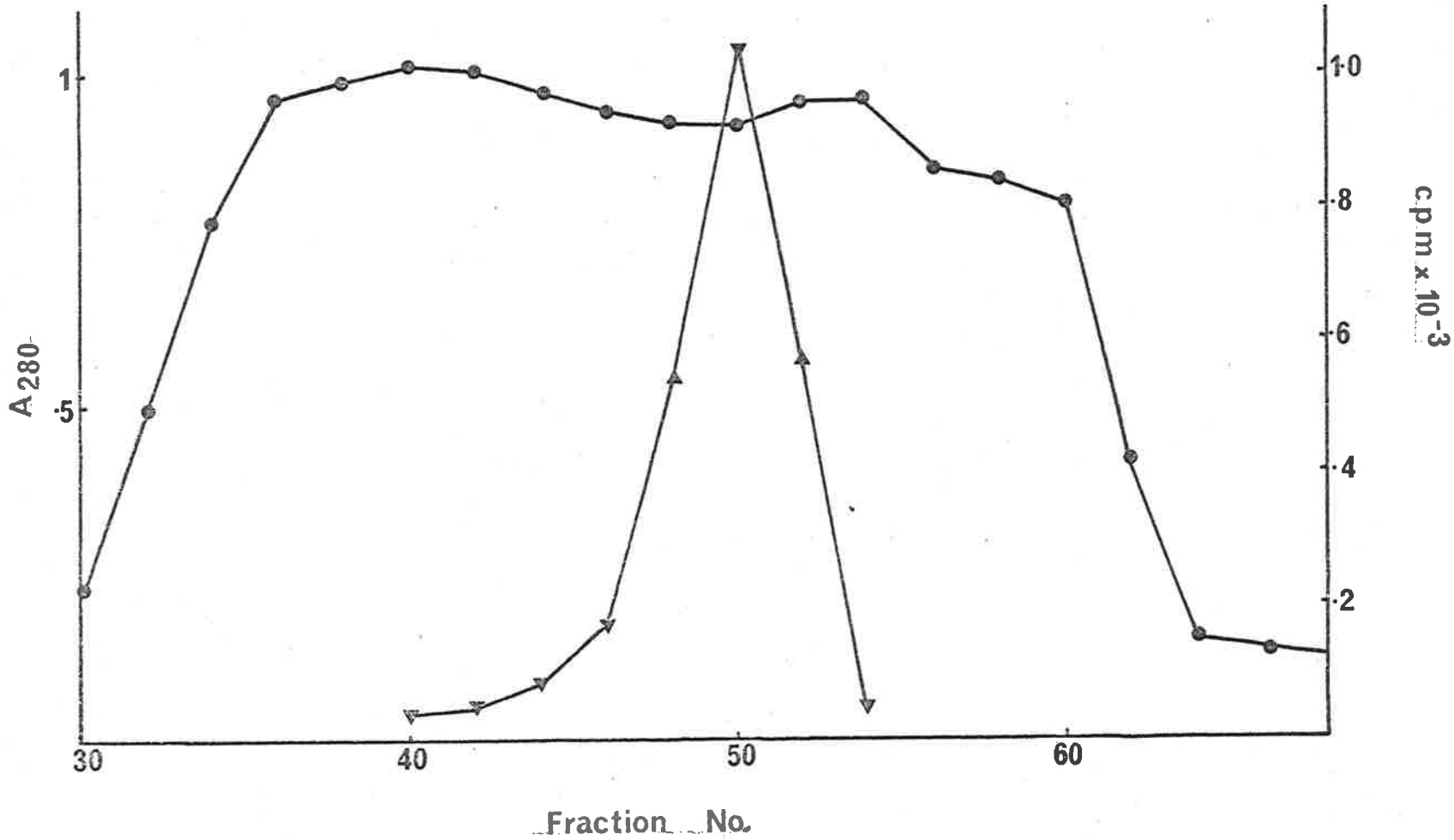

A280
1
·5
1·0
·8
·6
·4
·2
c.p.m x 10⁻³
30
40
50
60
Fraction No.

Fig.4.6 Sepharose 4B-Blue Dextran affinity chromatography in NEM buffer

The enzyme loaded on this column was partially purified up to Stage 3 (see Table 4.10) and dissolved in 1ml of 5mMNEM, pH7.5 containing 230mg of protein.

Column size : 20cmx1.5cm

Buffer gradient : 5mM-1.9MNEM, pH7.5

Flow rate : 9ml/hr

Fraction size : 2.5ml

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

– – – NEM(M)

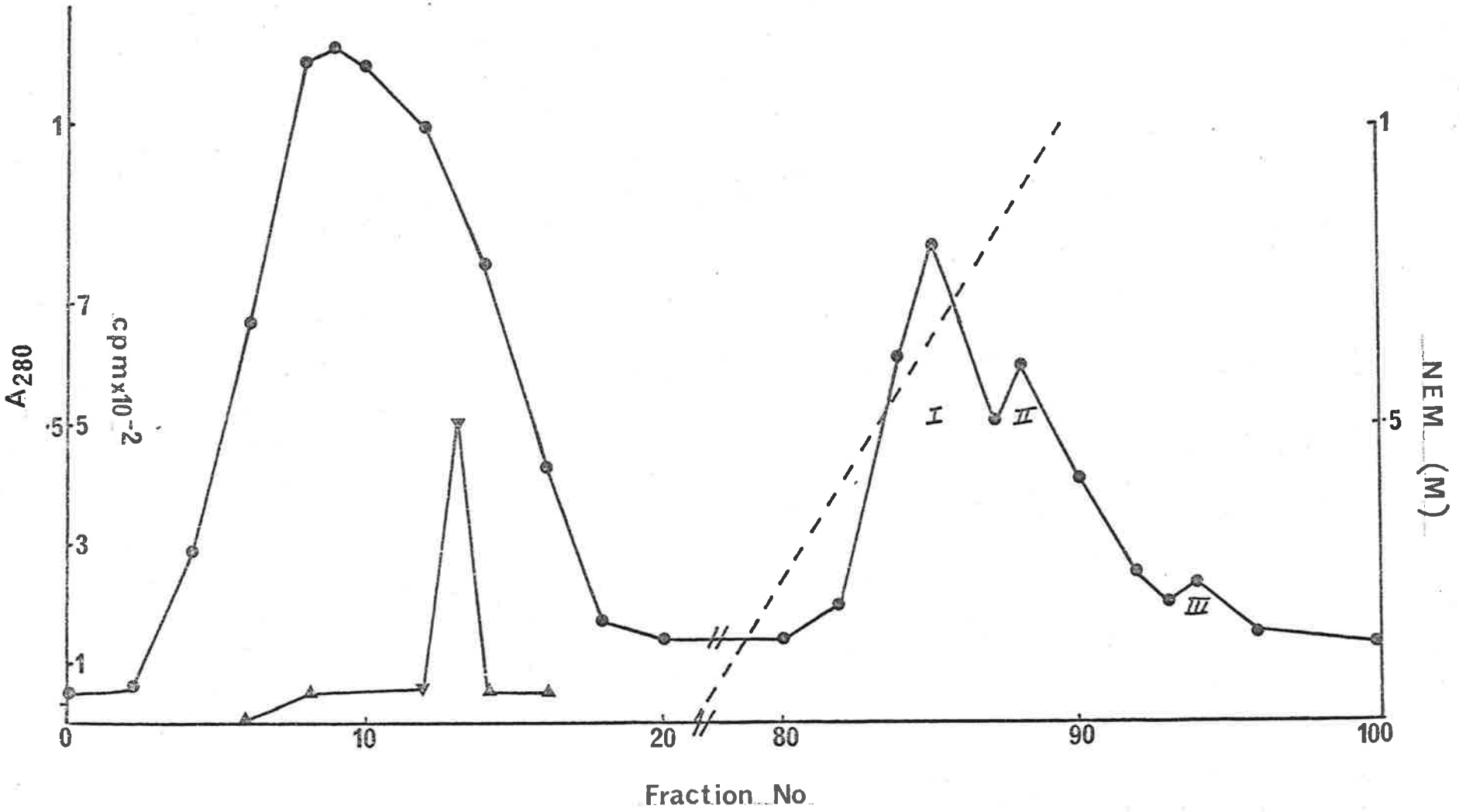

A280
cpm×10⁻²
NEM (M)
1
·7
·5
·3
·1
0
10
20
80
90
100
I
II
III
Fraction No.

Fig.4.7 Sepharose 4B-Blue Dextran chromatography in NEM-glycerol buffer

This column is identical to that of Fig.4.6 except the equilibrating buffer was 20mMNEM, pH7.5 containing 10% ($^{v}/_{v}$) glycerol.

Buffer gradient : 20mM-500mMNEM, pH7.5 containing 10% ($^{v}/_{v}$) glycerol

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

– – – NEM(M)

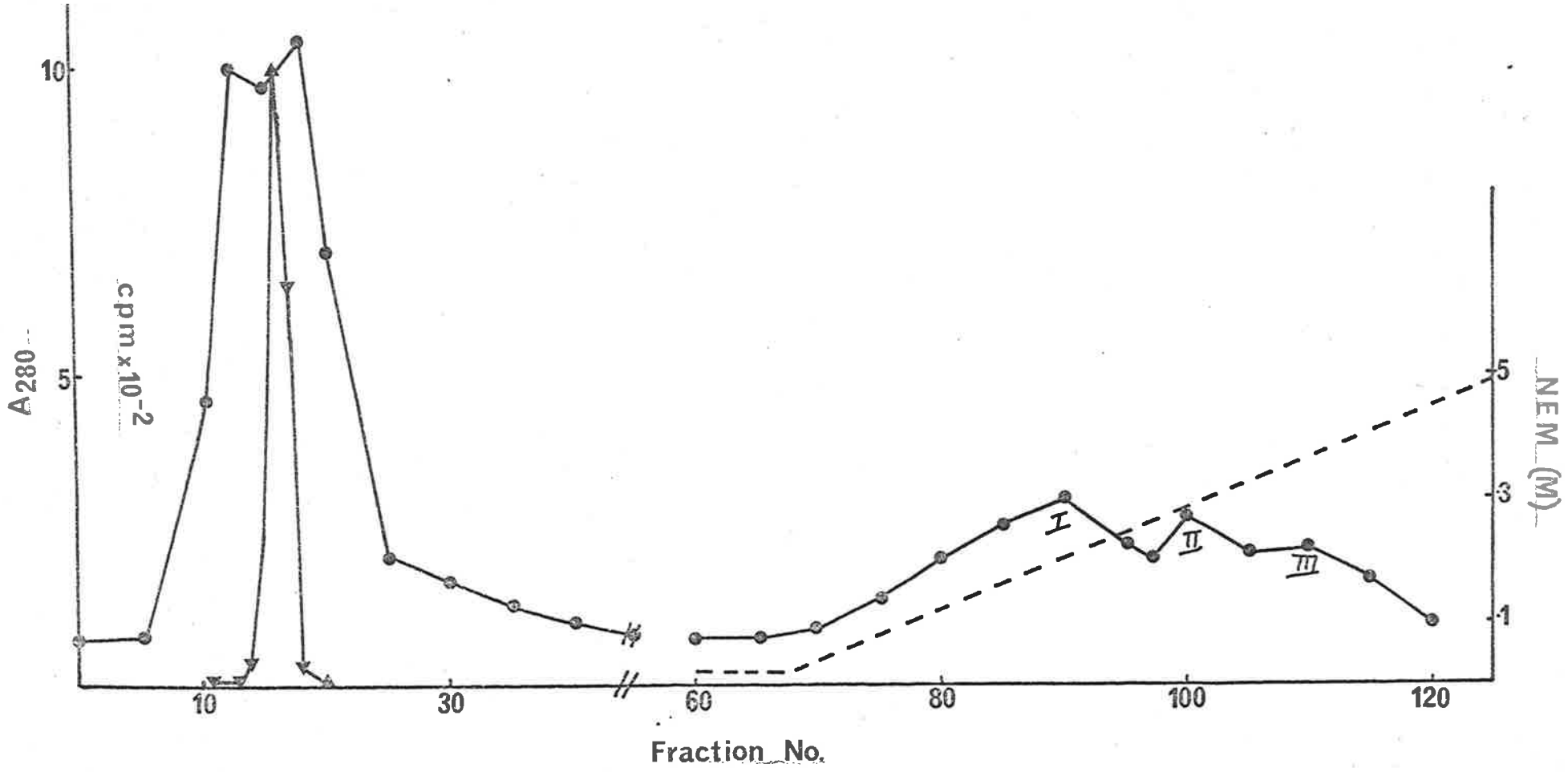

10
5
A280
cpm x 10-2
10
30
60
80
100
120
Fraction No.
I
II
III
5
3
1
NEM (M)

TABLE 4.12 Separation of isocitrate synthase from several dehydrogenases

A. Measurement of the dehydrogenase activities in the unbound peak containing isocitrate synthase activity using 100mMNEM, pH7.5 containing 10% ($^{v}/_{v}$) glycerol.

B. As for A except the buffer used was 50mMNEM, pH7.5 containing 20% ($^{v}/_{v}$) glycerol and 10mM$MgCl_2$.

TABLE 4.12 Separation of isocitrate synthase from several dehydrogenases

| ENZYME | level of enzyme (total units) | | |
|---|---|---|---|
| | before elution | *after elution | % after elution |
| A. | | | |
| Isocitrate dehydrogenase | 4 | 2.5 | 63 |
| Malic enzyme | 1.5 | 1.1 | 74 |
| Lactate dehydrogenase | 1350 | 9.0 | 0.67 |
| Malate dehydrogenase | 428 | 112 | 26 |
| B. | | | |
| Isocitrate dehydrogenase | 25 | 2.9 | 11 |
| Malic enzyme | 3.2 | 0.0 | 0 |
| Lactate dehydrogenase | 2940 | 10 | 0.3 |
| Malate dehydrogenase | 3215 | 19 | 0.06 |

* level after elution in the peak corresponding to the isocitrate synthase activity

Fig.4.8 Agarose-NAD affinity chromatography

Enzyme purified up to stage 3 (see Table 4.10) was dissolved in 1ml 0.02MNEM, pH7.5 containing 1mMEDTA. This was then loaded on the agarose-NAD column.

Column size : 12cmx1cm

Buffer : 0.02MNEM, pH7.5 containing 1mMEDTA

Flow rate : 10ml/hr

Fraction size : 2ml

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

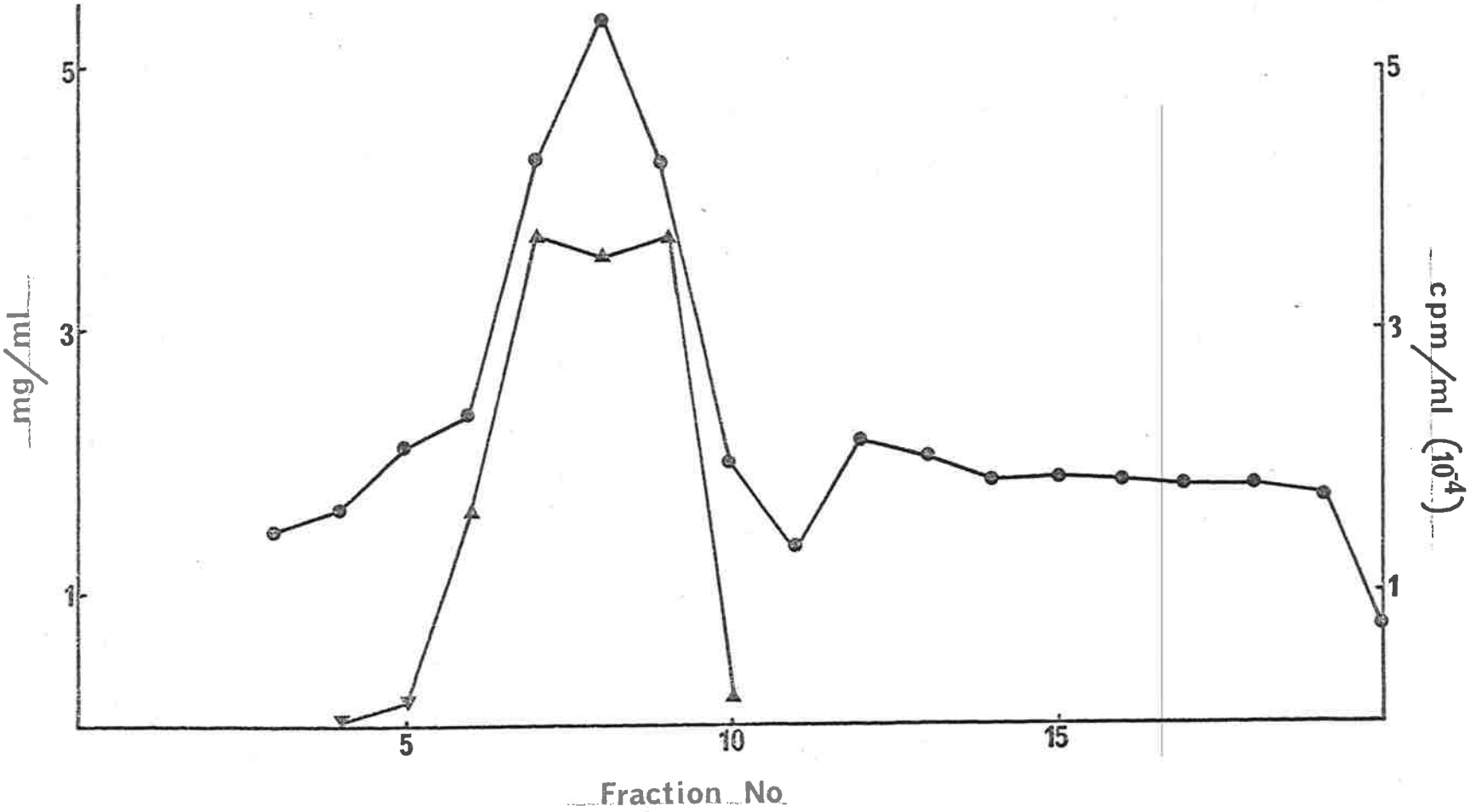

5
3
1
mg/ml
5
3
1
cpm/ml ($10^{-4}$)
5
10
15
Fraction No

Fig.4.9 Phenyl Sepharose Cl-4B chromatography elution profile

Enzyme was partially purified to Stage 4 (see Table 4.10) and dissolved in 1ml of 0.2M NEM-Cl, pH7.5 containing 1mMEDTA, 2mMMgCl$_2$ and 1mMATP. The gel matrix was also equilibrated in this buffer. The enzyme solution contained 55mg/ml protein and the column was run as follows

| | | |
|---|---|---|
| Column size | : | 10cmx2cm |
| Buffer | : | As above |
| Flow rate | : | 10ml/hr |
| Fraction size | : | 3.5ml |

—●— $A_{280}$

—▲— $^{14}CO_2$-fixation

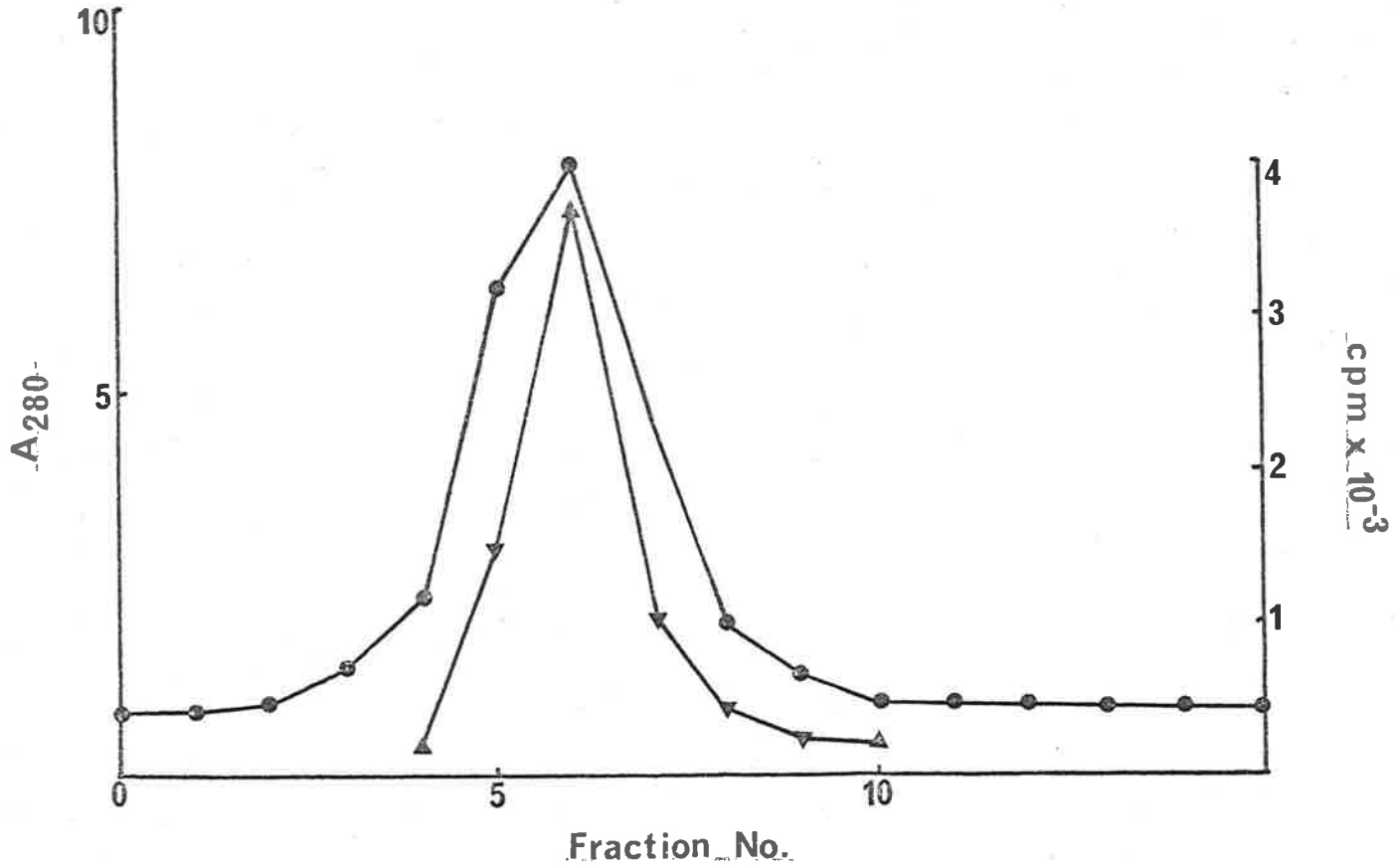

10
5
$A_{280}$
4
3
2
1
cpm x $10^{-3}$
0
5
10
Fraction No.

TABLE 4.13 Phenyl Sepharose Cl-4B Chromatography

| Buffer | Recovery (%) | Extent of purification (X) |
|---|---|---|
| 100mMNEM MgATP EDTA | 73 | 2.1 |
| | 68 | 3.0 |
| | 43 | 3.0 |
| | 90 | 5.0 |
| 200mMNEM MgATP EDTA | 33 | 4.2 |

The buffers were pH7.5 and 1mMEDTA, 1mMATP and 2mM$MgCl_2$ were the concentrations used in the buffer. All other conditions are as for Fig.4.9.

# CHAPTER 5

# KINETIC STUDIES

## 5.1 Introduction

It is desirable in describing the characteristics of an enzyme not previously described, to (a) identify the reaction products and (b) obtain as many kinetic constants as possible. Kinetic analyses are also required for a complete understanding of enzyme function and to unravel the events occurring in complex multi-substrate enzyme system and thus to establish the reaction sequence.

### 5.1.1 Initial velocity studies

#### 5.1.1(a) Single substrate kinetics

With multi-substrate enzymes the Michaelis constant for the variable substrate is almost always a function of the concentration of the fixed variable substrate and therefore the value obtained at one concentration of the fixed variable is referred to as the apparent Michaelis constant (appKm). Single substrate kinetic studies are used to obtain the appKm value for all the substrates. To achieve this, one substrate is varied keeping all other components constant. The results are expressed diagramatically in the double reciprocal form, i.e. by the use of the Lineweaver-Burk plot. The appKm value obtained from such a plot is then used as a guide to the concentration range to be tested when the multi-substrate kinetic approach is attempted.

#### 5.1.1.(b) Multi-substrate kinetics

There are two different basic sequences by which a reaction involving two substrates and two products may proceed, e.g.

$$A + B \rightleftharpoons P + Q$$

can occur:

(a) By a sequential pathway which is the case where all substrates must be present on the enzyme before any products leave.

(b) By a non-sequential or "ping-pong" pathway, which means that one or more products are released before all the substrates have added to the enzyme.

Sequential mechanisms can be (i) random reactions or (ii) ordered reactions. An enzyme catalysing a random mechanism would possess two distinct sites, one for each substrate (or product), so that the reaction of one substrate with the enzyme may occur before or after the other. Ordered reactions are those in which the two substrates (or products) have a compulsory order of addition to the enzyme. The Theorell-Chance mechanism is a special case of the ordered mechanism in which the steady state concentrations of the central complexes are sufficiently low as to be kinetically insignificant and the first product P appears to be formed directly from the substrate B by interaction of the EA-complex. These mechanisms are outlined in Fig.5.1.

To determine which of the mechanisms holds for a particular enzyme, the concentration of one substrate (variable substrate) is varied while the other substrate is held constant at several non-saturating levels (fixed variable substrate), and then making double reciprocal plots for each level of the non-varied substrate. The rate equation for the sequential reaction mechanisms is;

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB}$$

where $K_{ia}$ represents the dissociation constant for EA, $K_a$ and $K_b$ are the Michaelis constants for A and B, respectively, and V is the maximum velocity.

By taking reciprocals and rearranging, this equation can be written in the form of a straight line,

$$\frac{1}{v} = \frac{K_a}{V}\left[\frac{K_{ia}K_b}{K_aB} + 1\right]\frac{1}{A} + \frac{1}{V}\left[\frac{K_b}{B} + 1\right]$$

so that if $\frac{1}{v}$ is plotted as a function of $\frac{1}{A}$, the slope of the line would be,

$$\frac{K_a}{V}\left[\frac{K_{ia}K_b}{K_aB} + 1\right] \text{ and the intercept is } \frac{1}{V}\left[\frac{K_b}{B} + 1\right]$$

Since both the slope term and the intercept term are functions of the fixed variable, B, double reciprocal plots ($\frac{1}{v}$ as function of $\frac{1}{A}$) at different fixed non-saturating concentrations of B will be a family of straight lines which cross at a point to the left of the ordinate and this point may be above, below or on the abscissa.

The rate equation for the non-sequential reaction mechanism is;

$$v = \frac{VAB}{K_aB + K_bA + AB}$$

when this equation is rearranged into the form of the equation of a straight line it becomes:

$$\frac{1}{v} = \frac{K_a}{V}\cdot\frac{1}{A} + \frac{1}{V}\left[\frac{K_b}{B} + 1\right]$$

where the slope term is $\frac{K_a}{V}$ and the intercept term is the same as for the sequential mechanism.

Since the slope term is independent of the fixed variable, B, double reciprocal plots of $\frac{1}{v}$ as a function of $\frac{1}{A}$ at different fixed non-saturating concentrations of B will be a family of straight parallel lines, i.e. slope will not vary with different concentrations of B. On the other hand, the intercept term is a function of B and at each different concentration of B there is a different intercept point on the ordinate. These plots are presented in Fig.5.2.

While the Michaelis constant (Km) for single-substrate enzymes may be defined as the substrate concentration at half the maximal velocity, this definition no longer holds for multi-substrate enzymes as the Km value for a particular substrate is a function of the other substrate(s). The Michaelis constant can be obtained from secondary plots of slope and intercept. For a sequential reaction mechanism,

$$\text{slope} = \frac{K_a}{V}\left[\frac{K_{ia}K_b}{K_a B} + 1\right]$$

$$= \frac{K_{ia}K_b}{V}\cdot\frac{1}{B} + \frac{K_a}{V}$$

Again this equation is of the form of the equation of a straight line and if the value of the slope is plotted as a function of $\frac{1}{B}$ the new slope is $\frac{K_{ia}K_b}{V}$ and the new intercept in the secondary plot is $\frac{K_a}{V}$. Similarly

$$\text{intercept} = \frac{1}{V}\left[\frac{K_b}{B} + 1\right]$$

$$= \frac{K_b}{V}\cdot\frac{1}{B} + \frac{1}{V}$$

When the values of the intercepts obtained in the primary plots are replotted as a function of $\frac{1}{B}$ then in this secondary plot, the slope is $\frac{K_b}{V}$ and the intercept is $\frac{1}{V}$.

For the non-sequential mechanism a secondary plot for slope cannot be made as the slope remains constant. However, a replot of the intercepts of the primary plots as a function of $\frac{1}{B}$ yields a secondary plot with a new slope, $\frac{K_b}{V}$ and an intercept, $\frac{1}{V}$. Thus all kinetic constants can be obtained from secondary plots irrespective of the reaction mechanism.

5.1.2 Product inhibition studies

The product of an enzymically catalysed reaction inhibits the rate of an enzymic reaction by combining with the enzyme form that results when that particular product dissociates from the complex e.g.

$$EPQ \rightleftharpoons EQ + P$$

therefore P will combine with EQ. In studying this type of inhibition, the reaction velocity is measured in the presence of a fixed, non-saturating level of the product while the concentration of one of the substrates is varied. This procedure is repeated several times at different fixed levels of the product. The data are plotted in double reciprocal form and the following may be deduced. If the slope of the primary plot is a function of the product concentrations, the family of lines will intersect on the ordinate and the inhibition will be "competitive" i.e. the product inhibitor will be competitive with respect to the varied substrate. When the intercept of the primary plot is a function of the product concentration a family of parallel lines results and the inhibition will be "uncompetitive" i.e. the product inhibitor can combine only with an enzyme-substrate complex. A third type of

inhibition results when both slope and intercept are a function of product concentrations. This is "non-competitive" inhibition i.e. the product inhibitor combines equally well with the free enzyme or the enzyme-substrate complex. These types of inhibition are shown in Fig.5.3.

Initial velocity studies cannot distinguish between the three possible sequential mechanisms. However, this can be achieved by determining the type of inhibition pattern obtained for each product against each substrate.

### 5.1.3 Alternative substrate kinetics

The use of alternative substrates has proved a powerful tool in investigations of the reaction pathways of enzyme catalysed reactions (Webb et al., 1976; Easterbrook-Smith et al., 1978). For a two substrate reaction e.g.

$$A + B \rightleftharpoons P + Q$$

if $B_1$ is an alternate substrate for B then

$$A + B_1 \rightleftharpoons P_1 + Q$$

The reaction velocity is measured when varying A at fixed levels of B and $B_1$ and the data are plotted in double reciprocal form. For a ping-pong mechanism, the double reciprocal plots will have identical slopes whilst for a sequential mechanism the plots will have different slopes.

### 5.1.4 Aim of the kinetic studies of isocitrate synthase

Initial velocity studies are required as a guideline for optimal assay conditions for this previously undescribed enzyme, isocitrate synthase. Single-substrate kinetics yield

only appKm values but serve as an approximation of Km values which can then be obtained using multi-substrate kinetic approach. Multi-substrate initial velocity studies are one line of evidence required for the sequence of the reaction mechanism to be established but other evidence e.g., product inhibition studies and alternative substrate kinetics are required to confirm or clarify the sequence of the reaction mechanism. Therefore these studies were undertaken to determine the optimal assay conditions for this enzyme and to elucidate its reaction mechanism.

## 5.2 Methods

### 5.2.1 Assay methods

The isotopic assay procedure for the overall reaction as described in Section 2.2.5.1a was used in all initial velocity studies for single-substrate kinetics. To reduce the concentration of endogenous $HCO_3^-$ in the assay solution, all reagents were prepared from freshly boiled, double distilled water and kept stoppered in a vacuum dessicator. For multi-substrate kinetic experiments and subsequent studies, the assay procedure described in Section 2.2.5.1b was used.

### 5.2.2 Data analysis

Unless otherwise stated, initial velocity data were analysed by computing the hyperbolae of best fit to the primary data using the FORTRAN programme HYPER (Cleland, 1963a). In initial velocity experiments where two substrates were varied together, the goodness of fit of the

sequential and non-sequential mechanisms was examined using programmes SEQUEN and PNPONG (Cleland, 1963a) respectively. Lines shown in the double-reciprocal plots were drawn from the computed kinetic constants.

To determine the inhibition patterns kinetic data were plotted graphically and then fitted to the appropriate rate equations by using the method of least squares, with the Fortran computer programmes of Cleland (1967). Where appropriate other data were fitted to a straight line using a Fortran least mean squares programme.

## 5.3 Results

### 5.3.1 Single-substrate kinetics

The results of varying one substrate whilst keeping the other substrates at near-saturation level are shown in Table 5.1. The data, which yield the appKm values, were processed by the HYPER programme and the resulting kinetic constants indicated the need of alteration of the concentrations of substrates previously used in the isocitrate synthase assay system. Changes in the assay system were necessary as the concentrations of 2-oxoglutarate and $HCO_3^-$ that were used here are equal to or only a several fold greater than their appKm value. Since a concentration nine times greater than the Km value will produce only 90% saturation of the enzyme, a concentration ten times greater than the appKm value should be used for a substrate which is required at a saturation or near-saturation level.

These values were also required to give a range of concentration values to be used in multi-substrate initial

velocity studies from which the true Km values for each substrate can be determined.

5.3.2 Multi-substrate kinetics

The double reciprocal patterns obtained when the substrates, $MgATP^{2-}$, $HCO_3^-$ and 2-oxoglutarate were varied in pairs with the third substrate held at near-saturating concentration are shown in Figs.5.4;5.5;5.6. The Michaelis constants obtained from secondary plots of the data presented in Figs.5.4;5.5;5.6 are presented in Table 5.2. When $HCO_3^-$ and 2-oxoglutarate were the variable and the fixed variable substrates respectively, a family of intersecting lines was obtained indicating sequential addition of these two substrates to the enzyme. The $K_a$ value was less than the value of $K_{ia}$ computed according to the SEQUEN programme irrespective of whether $HCO_3^-$ or 2-oxoglutarate was taken to be substrate A. This indicates that the binding of each of these substrates facilitates the binding of the other, and implies a random binding of these substrates to the enzyme. The positive interaction between the two substrates is seen in Fig.5.4 where the appKm value for $HCO_3^-$ decreased as the concentration of 2-oxoglutarate was increased. The Km value for 2-oxoglutarate is one-third the value of the appKm thus showing that there is a greater affinity for this substrate when the $HCO_3^-$ concentration is raised above the level used in the initial experiments. By contrast to this the appKm and Km value for $HCO_3^-$ are identical.

When either 2-oxoglutarate or $MgATP^{2-}$ were the variable substrate, the data in double reciprocal form

yielded parallel lines. In Fig.5.5, the results of varying 2-oxoglutarate at several different constant concentrations of $MgATP^{2-}$ are shown. The Km value for $MgATP^{2-}$ is of the same order as its appKm and the Km for 2-oxoglutarate is similar to that in Fig.5.4.

The data obtained from varying $HCO_3^-$ and $MgATP^{2-}$ are presented in Fig.5.6. Individual double-reciprocal lines were obtained by fitting hyperbolae (Programme HYPER) to the primary data for fixed $HCO_3^-$ concentrations. The data obtained from this series of experiments could not be analysed with any degree of precision since double-reciprocal plots yield two series of parallel lines which could not be fitted to the non-sequential (PNPONG) programme. The slope and intercept replots show this "transition" from one set of parallel lines to the second set to occur between 1mM and 1.5mM $MgATP^{2-}$.

### 5.3.3 Product inhibition studies

Each of the three products of the reaction catalyzed by this enzyme, $MgADP^-$, $P_i$ and OAS were tested with respect to each substrate. Furthermore the stabilized derivative of OAS, that is, isocitrate was tested to the substrate 2-oxoglutarate. The results are presented in full in Figs.5.7, 5.8 and 5.9 and summarised in Table 5.3. When $HCO_3^-$ was the varied substrate, secondary plots, where the slopes (and intercepts) of the primary plots were plotted as functions of product concentration, were linear indicating that a single molecule of inhibitor was acting to cause the inhibition. When 2-oxoglutarate was the varied substrate, all secondary plots, except the parabolic

slope replot in the case where isocitrate was used as the product inhibitor, were also linear. However when the varied substrate was $MgATP^{2-}$, all secondary plots of the slope were non-linear. OAS is a parabolic non-competitive inhibitor whilst both $P_i$ and $MgADP^-$ are hyperbolic competitive inhibitors of $MgATP^{2-}$. Hyperbolic competitive inhibition occurs when both the inhibitor and the substrate are present on the enzyme at the same time thus indicating that both products of $MgATP^{2-}$ can combine with the enzyme in the presence of $MgATP^{2-}$. Competitive interactions were found only for $MgATP^{2-}$ and $MgADP^-$, $MgATP^{2-}$ and $P_i$, and 2-oxoglutarate and OAS.

### 5.3.4 Alternate substrate kinetics

The alternate substrates to the keto acid substrate 2-oxoglutarate chosen for this study were the keto acids, pyruvate and α-ketoadipic acid. The results of experiments carried out using these two compounds to replace 2-oxoglutarate are presented in double reciprocal form in Fig.5.10. Table 5.4 presents an analysis of the slopes of the plots of the primary data shown in Fig.5.10. Both of these compounds can be used by isocitrate synthase and pyruvate was unexpectedly a better substrate, that is, it has a higher Vmax than the "true" substrate 2-oxoglutarate. The analyses of the slope values show that the slopes for the alternate substrates are statistically significantly different from that for 2-oxoglutarate.

## 5.4 Discussion

Initial velocity studies in which one substrate was varied at a time, revealed that the level of $HCO_3^-$ initially used in the search for isocitrate synthase was not near-saturating as judged by the appKm value. The concentration of 2-oxoglutarate used was also non-saturating when compared to its appKm value, however the true Km value was lower than the appKm value and the concentration used was indeed near-saturating when the level of $HCO_3^-$ was elevated. Both $MgATP^{2-}$ and NADPH were used at near-saturating concentrations and thus the only alteration to the original assay mixture was the increase of the $HCO_3^-$ level. The appKm value for NADPH of $8.6 \times 10^{-6}$M is of the same order as the Km value of $9.2 \times 10^{-6}$M given by Rose (1960) for the detritiation reaction by hog heart isocitrate dehydrogenase (NADP) and also of the Km value given for other NADPH dehydrogenases (Rutter et al., (1958) and Balinsky et al., (1961)). No kinetic studies were carried out using NADPH as a variable ligand since this study was concentrated upon the ATP-dependent carboxylation of 2-oxoglutarate.

The true Km value for the substrates (Table 5.2) reveal that the Km value for $HCO_3^-$ is identical to the value reported by Cleland (1967) for the reverse reaction for isocitrate dehydrogenase (NADP). However it is emphasised that the substrate for isocitrate dehydrogenase (NADP) is $CO_2$ whilst the substrate for isocitrate synthase is $HCO_3^-$ (see Chapter 3). A Km of $3.16 \times 10^{-4}$M for 2-oxoglutarate is higher than the values of $1.3 \times 10^{-4}$M (Rose, 1960) and $0.24 \times 10^{-4}$M (Cleland, 1967) calculated for isocitrate dehydrogenase (NADP). This

higher requirement for 2-oxoglutarate could be supplied under physiological condition as this enzyme is "switched on" when there would be a high level of this substrate from glutamate arising from protein digestion after feeding. Similarly the high $HCO_3^-$ levels required could be supplied as was suggested by D'Adamo (1978) by the increased rate of oxidation of 6-phosphogluconic acid in the pentose pathway which provides a high local concentration of $HCO_3^-$.

Product inhibition studies resulted in competitive inhibition patterns only between 2-oxoglutarate and OAS and $MgATP^{2-}$ and its two products. These results suggest that $MgATP^{2-}$ and 2-oxoglutarate bind at separate sub-sites on the enzyme as if a single site was involved, competitive interactions between one substrate and the product of the other substrate would be expected. All other sets of substrate-product inhibition combinations were non-competitive. When $HCO_3^-$ was the substrate, all slope and intercept replots were linear indicating a single mode of action of the product. Similarly with 2-oxoglutarate as the variable substrate, the slope and intercept replots when $MgADP^-$, $P_i$ or OAS were the product inhibitors were linear. However, when isocitrate was the product inhibitor, the replot of the slopes was parabolic. Thus the inhibition by isocitrate appeared to be via a mixed mode of action. The difference in product inhibition patterns seen here with isocitrate and OAS can be contrasted with the expected result of the reverse reaction of isocitrate dehydrogenase (NADP).

The situation with isocitrate dehydrogenase (NADP) is

that OAS is not released but bound to the enzyme where it is then reduced to the stable isocitrate. Thus it would be expected that competitive product inhibition would be obtained between 2-oxoglutarate and both OAS and isocitrate. However, for isocitrate synthase this was not the case. Linear competitive inhibition resulted when OAS was the product inhibitor, whilst non-linear non-competitive inhibition was seen when isocitrate was the product inhibitor. Thus OAS appeared to be binding to the 2-oxoglutarate binding site and excluding 2-oxoglutarate whilst isocitrate can bind to the enzyme when 2-oxoglutarate is bound at a position other than the active site. It is concluded, therefore, that the reduction of OAS to isocitrate occurs at a site different from the carboxylation site. This conclusion is supported by the ability to isolate OAS as a product which has not been achieved for the reversal of isocitrate dehydrogenase (NADP).

Inhibition studies with $MgATP^{2-}$ resulted in non-linear, hyperbolic, slope replots with both $MgADP^{-}$ and $P_i$ as product inhibitors. This implies that $MgADP^{-}$ can bind to the enzyme in the presence of $MgATP^{2-}$ and similarly $P_i$ can bind at the same time as $MgATP^{2-}$. Therefore it appears that the enzyme has two phosphate binding sites; perhaps one site for catalytic activity and one for an allosteric control effect. Further experiments such as direct binding studies would have to be carried out before the two sub-site proposal can be verified and purification of the enzyme to homogeneity is essential prior to such studies.

When 2-oxoglutarate was the variable substrate and $MgATP^{2-}$ the fixed variable substrate, the double reciprocal plots yielded a family of straight, parallel lines. This result indicates a non-sequential or ping-pong mechanism for these two substrates and thus for the overall reaction although it should be pointed out that in some instances, parallel initial velocity patterns alone are not sufficient to identify a ping-pong mechanism. The reason for this situation is that in the equation for a sequential mechanism (see Section 5.1.1(b)), the term $K_{ia}K_b$ is constant and if this is so small as to be negligible, then the rate equation for the sequential mechanism reduces to the rate equation for a non-sequential mechanism. Again this data does not allow the order of addition of the substrates to be established but if this part of the reaction is truly ping-pong, then there is a product released from one of the substrates prior to the binding of the second substrate.

A non-classical kinetic situation is seen when $HCO_3^-$ is the varied substrate whilst $MgATP^{2-}$ is the fixed variable substrate. There are two different series of parallel lines which again suggests non-sequential addition of these two substrates. However this data cannot be fitted to the non-sequential rate equation as there is a change of slope from one series of parallel lines at low levels of $MgATP^{2-}$ to an increase in slope for the series of lines at higher $MgATP^{2-}$ concentrations. No explanation can be advanced for this phenomenon but points to the non-classical kinetic nature of isocitrate

synthase. Furthermore, the complex nature of the interaction between this enzyme with $MgATP^{2-}$ will be discussed in detail in Chapter 6.

The double reciprocal plots of reaction velocity plotted as a function of $HCO_3^-$ concentration at several fixed non-saturating levels of 2-oxoglutarate intersect at a point to the left of the ordinate above the abscissa. This indicates that there is a sequential addition of these two substrates to the enzyme but from these initial velocity studies no further conclusion can be made as to whether the addition of these two reactants is ordered or random sequential.

Alternative substrate kinetics results showing a change in slope of the double reciprocal plots when various keto acid substrates were used implies a sequential mechanism for $MgATP^{2-}$ and 2-oxoglutarate. This is in contrast to the non-sequential plots obtained when $MgATP^{2-}$ was the varied substrate in the two substrate initial velocity studies. However, as has been previously stated, parallel lines attained with multi-substrate kinetics may not necessarily reflect a non-sequential mechanism and this result with alternative substrate kinetics verifies this. Recently such alternate substrate studies were used by Easterbrook-Smith *et al.*, (1978) to refute the previously accepted ping-pong mechanism for pyruvate carboxylase. This enzyme exhibited a change in the slope when alternate substrates were used indicating a sequential mechanism for the enzyme.

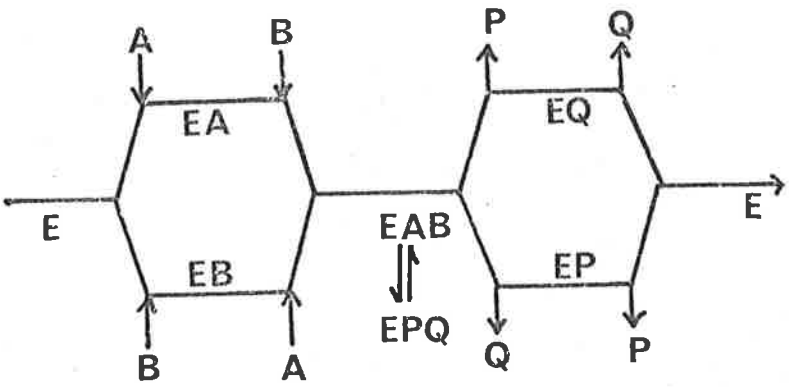
The product inhibition results showing competitive inhibition pattern only between substrates and their

corresponding products combined with the alternate substrate kinetics lead to the conclusion that the enzyme mechanism is sequential and has a Theorell-Chance mechanism. The order of addition of substrates cannot be elucidated from this product inhibition data as all inhibitions are non-competitive, other than 2-oxoglutarate versus OAS and $MgATP^{2-}$ versus $MgADP^{-}$ or $P_i$. Where the slopes and intercept replots versus inhibitor concentration are linear, these product inhibitors add only to one enzyme form but since the inhibition is non-competitive it is not known whether the inhibitor is binding to the free enzyme or to the enzyme substrate complex.

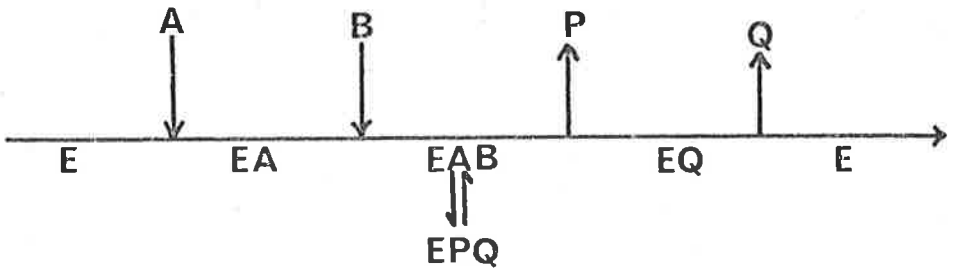
Further experimentation, such as isotope exchange studies at equilibrium is required before much credence can be placed on the proposed mechanism. Such studies will also provide evidence on the order of substrate addition in the ordered sequential mechanism.

Fig.5.1 Enzyme Mechanisms

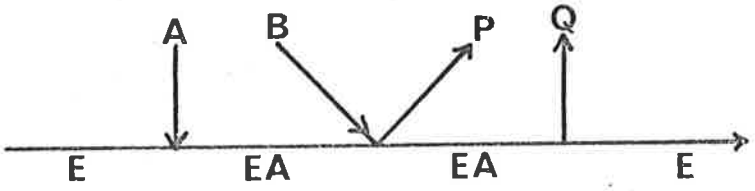
(a) Random Sequential




($b_1$) Ordered Sequential




($b_2$) Theorell-Chance (ordered sequential)




(c) Non-sequential (Ping-Pong)

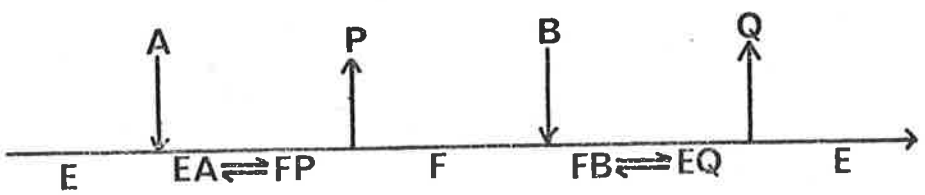
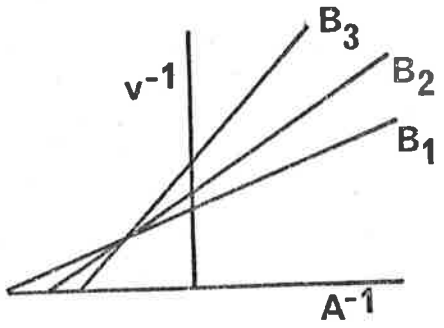
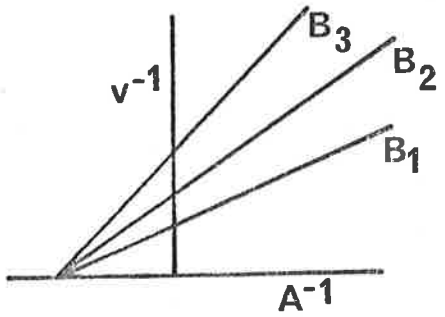
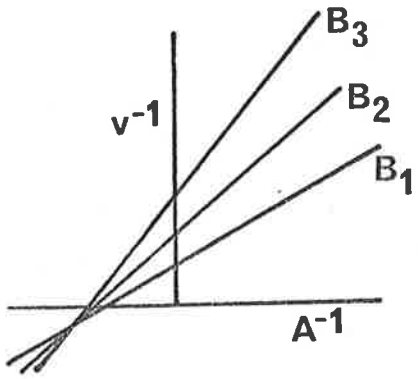


Fig.5.2. Double Reciprocal Plots of Sequential and Non-sequential Mechanism

(a) Sequential




(b) Non-sequential

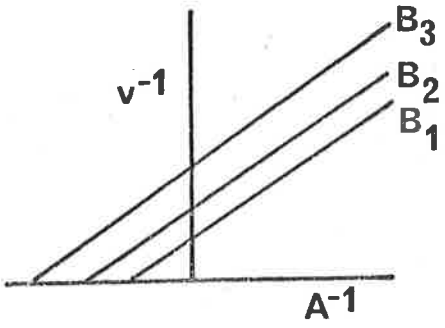
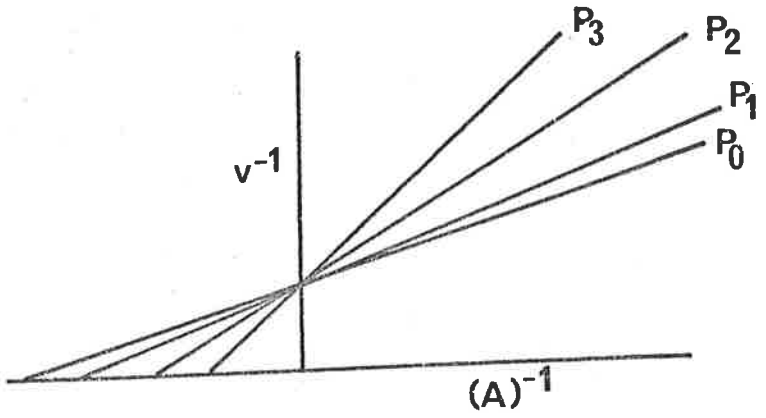
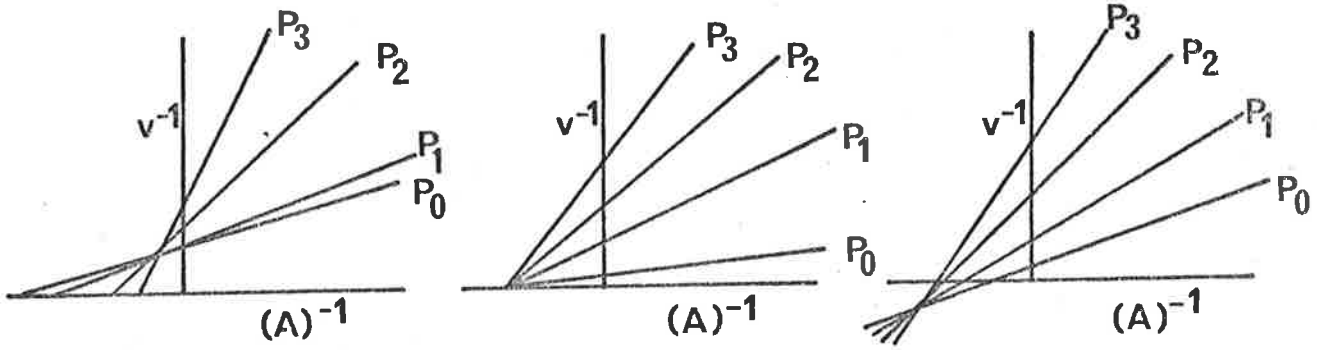


Fig.5.3 Double Reciprocal Plots of Product Inhibition Studies

(a) Competitive Inhibition




(b) Non-competitive Inhibition




(c) Un-competitive Inhibition

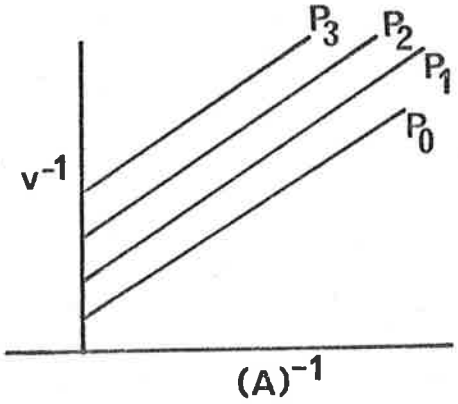


TABLE 5.1 The appKm values for Isocitrate Synthase

| Substrate Varied | appKm value mM |
|---|---|
| 2-oxoglutarate | 1.47 ± 0.09 |
| $MgATP^{2-}$ | 0.54 ± 0.08 |
| $HCO_3^-$ | 10.89 ± 1.13 |
| NADPH | 0.0086 ± .0025 |

All reactions were carried out in 0.1MNEM-Cl, pH7.5 as described in Section 2.2.5.1a using the following concentrations for the constant substrates: ATP,2mM; $MgCl_2$,4mM; $NaH^{14}CO_3$,10mM; NADPH,0.1mM and 2-oxoglutarate,4mM. The enzyme used was partially purified to Stage 5 (see Table 4.10). The kinetic constants were obtained by computer analysis using the HYPER programme.

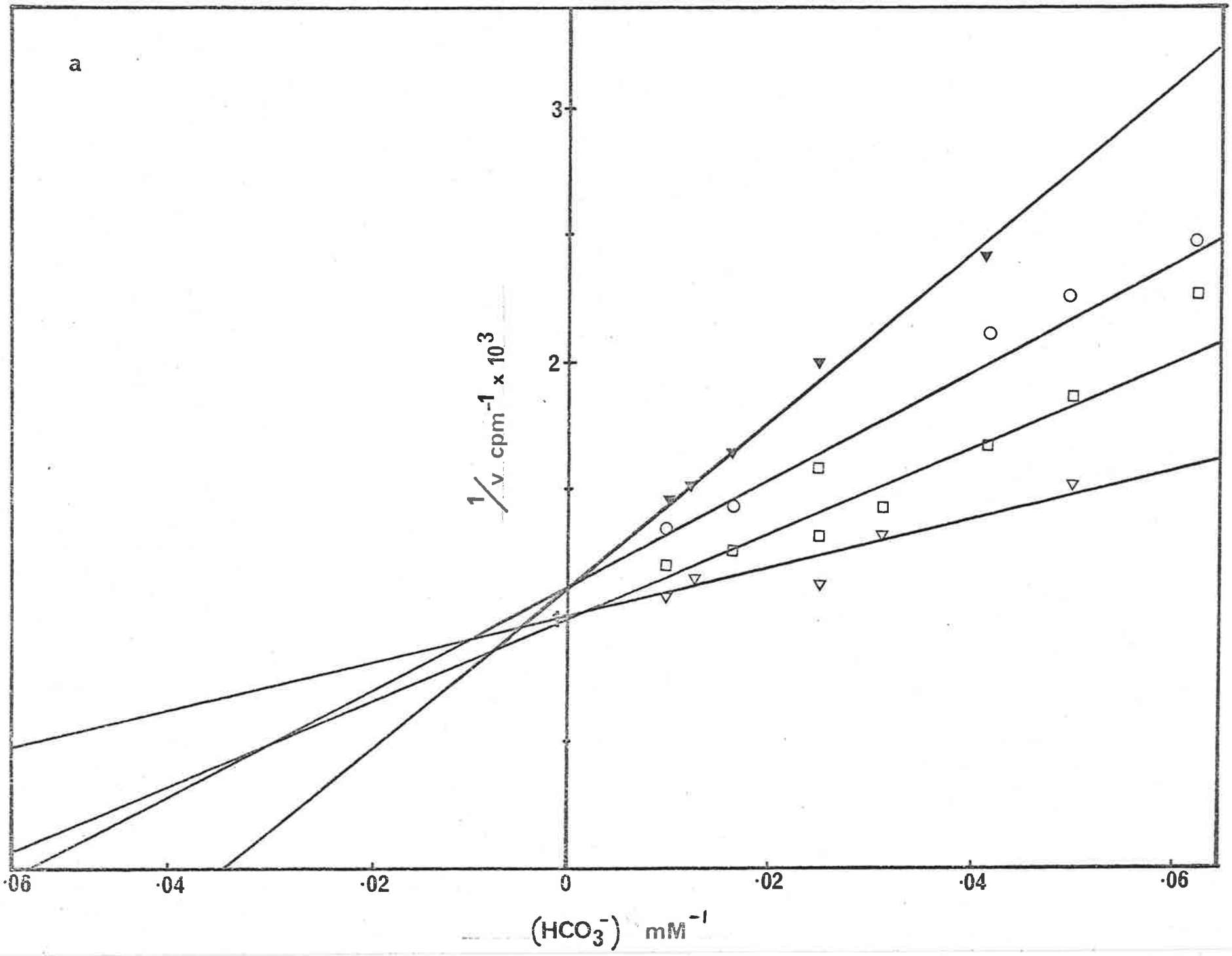
Fig.5.4a Primary plot of varying $HCO_3^-$ concentration at fixed levels of 2-oxoglutarate

Radiochemical assays were initiated by the addition of 0.05ml of enzyme purified up to Stage 4 (see Table 4.10). The $MgATP^{2-}$ concentration was 5mM and 2-oxoglutarate were —△—, 14mM; —□—, 4mM; —○—, 3mM; —▲—, 1.5mM and 12mM; 6mM; and 1.5mM which are not shown for clarity.

The lines were computed according to the SEQUEN programme which gave the following values for the kinetic constants: $Km(HCO_3^-)$, 9.47 ± 1.89mM; $K_i(HCO_3^-)$, 105.1 ± 61.5mM; Km(2-oxoglutarate), 0.367 ± 0.162mM; $K_i$ (2-oxoglutarate), 5.11 ± 2.64mM.

Fig.5.4b Secondary plots of the data presented in Fig.5.4a

The data shown were analysed using the Fortran programme SEQUEN.


a
3
2
$\frac{1}{v}$ cpm$^{-1}$ x 10$^3$
·06
·04
·02
0
·02
·04
·06
$(HCO_3^-)$ mM$^{-1}$

b

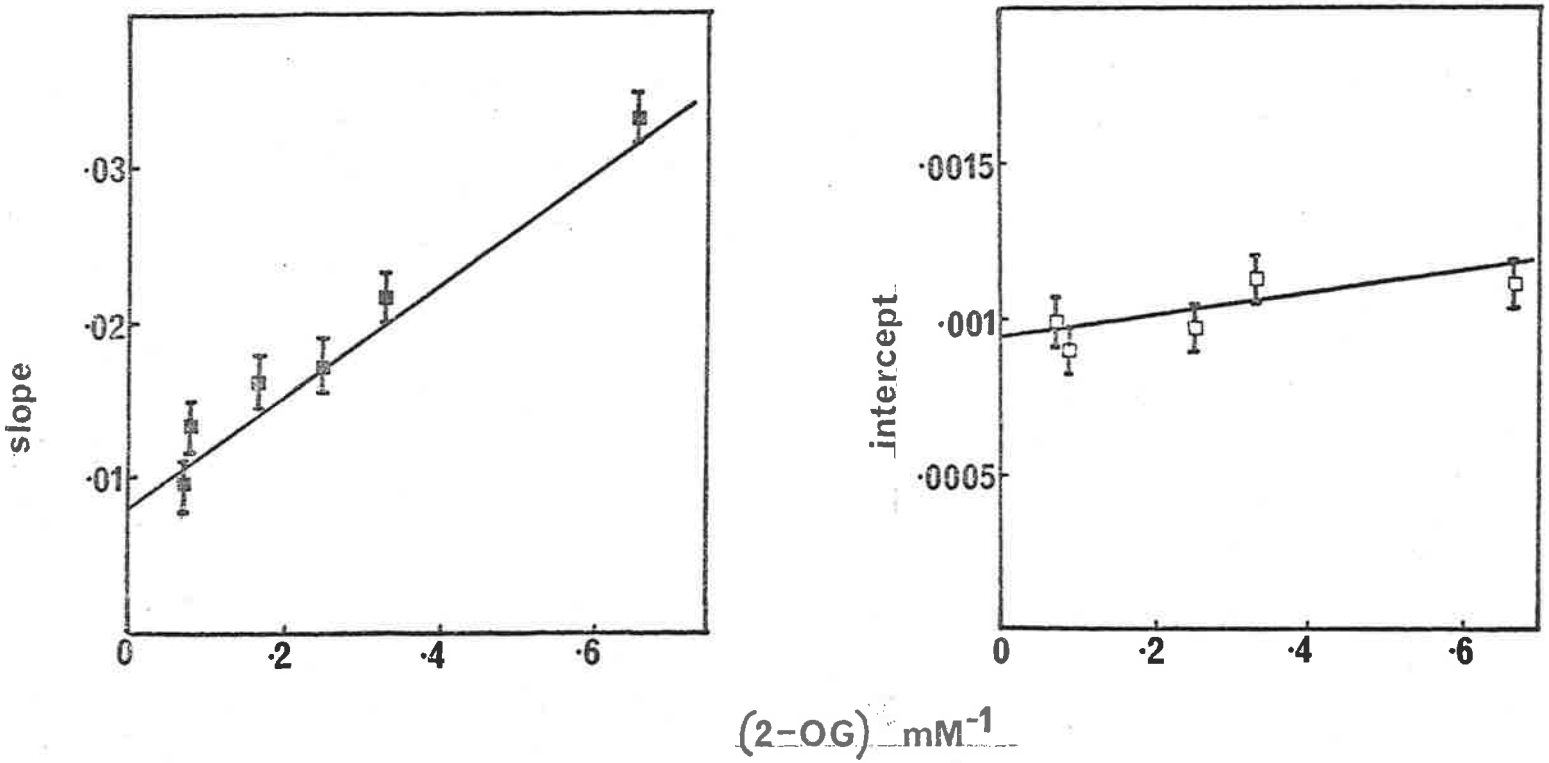


Fig.5.5a Primary plot of varying 2-oxoglutarate at fixed levels of $MgATP^{2-}$

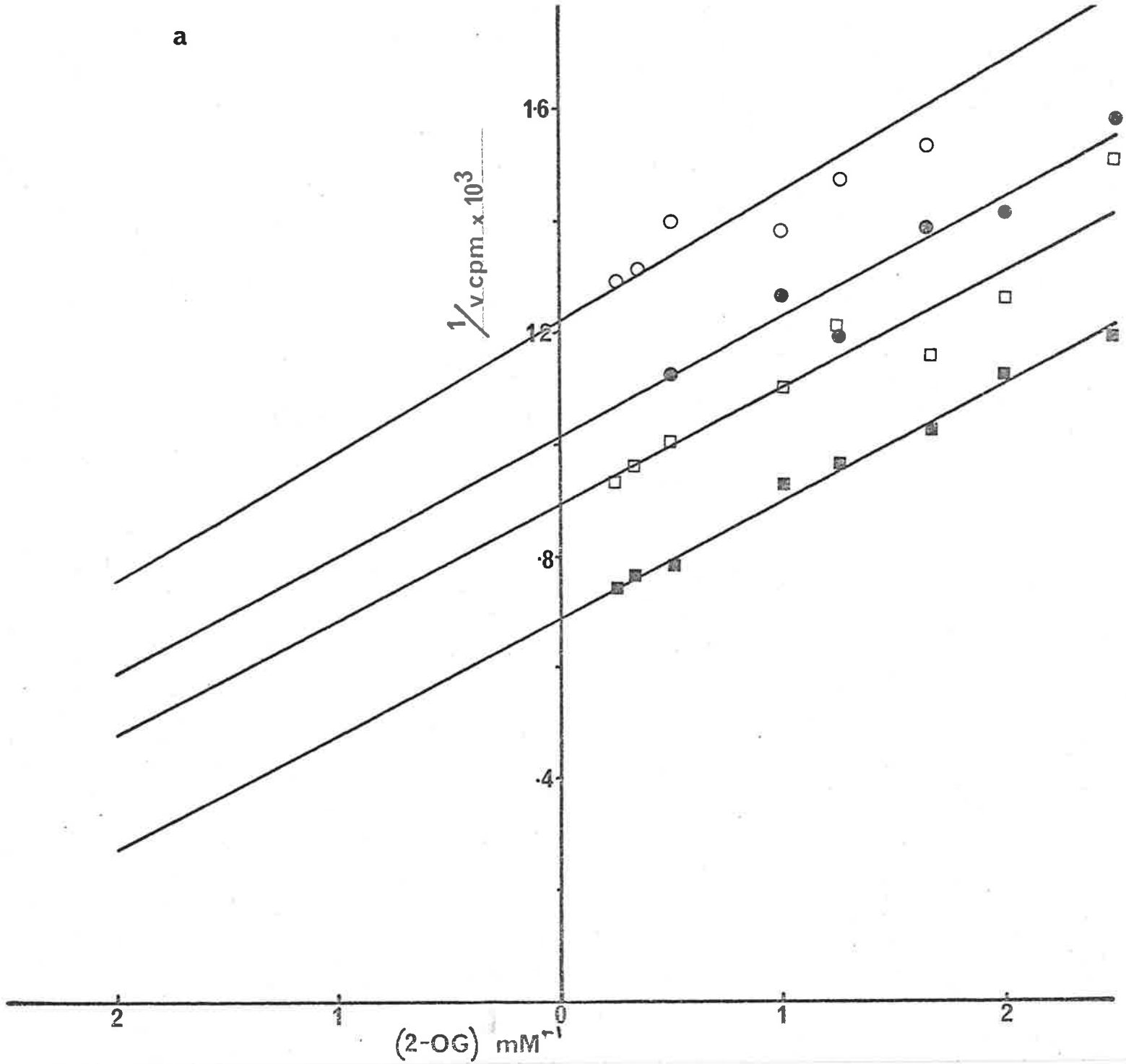
Radiochemical assays were initiated by the addition of 0.05ml of enzyme as in Fig.5.4a.

The $HCO_3^-$ concentration was 100mM and $MgATP^{2-}$ concentrations were —■—, 5mM; —□—, 0.8mM; —●—, 0.6mM; —○—, 0.5mM and 3mM; and 1mM which are not shown for clarity.

The lines were computed according to the PNPONG programme which gave the following values for the kinetic constants: Km(2-oxoglutarate), 0.316 ±0.028mM; Km($MgATP^{2-}$), 0.433 ±0.343mM.

Fig.5.5b Secondary plots of the data presented in Fig.5.5a

The data shown for the intercept plot were analysed using the Fortran programme PNPONG.


a
1·6
1·2
·8
·4
1/v cpm x 10^3
2
1
0
1
2
(2-OG) mM^-1

b

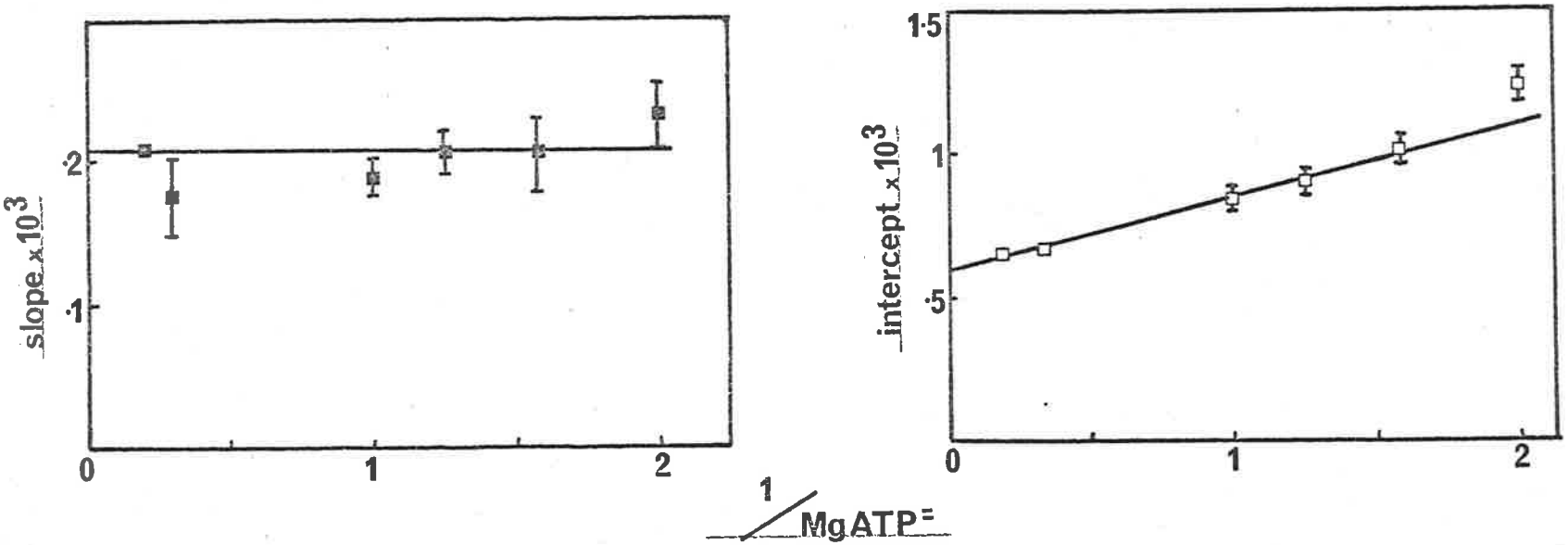


Fig.5.6a Primary plot of varying $HCO_3^-$ at fixed levels of $MgATP^{2-}$

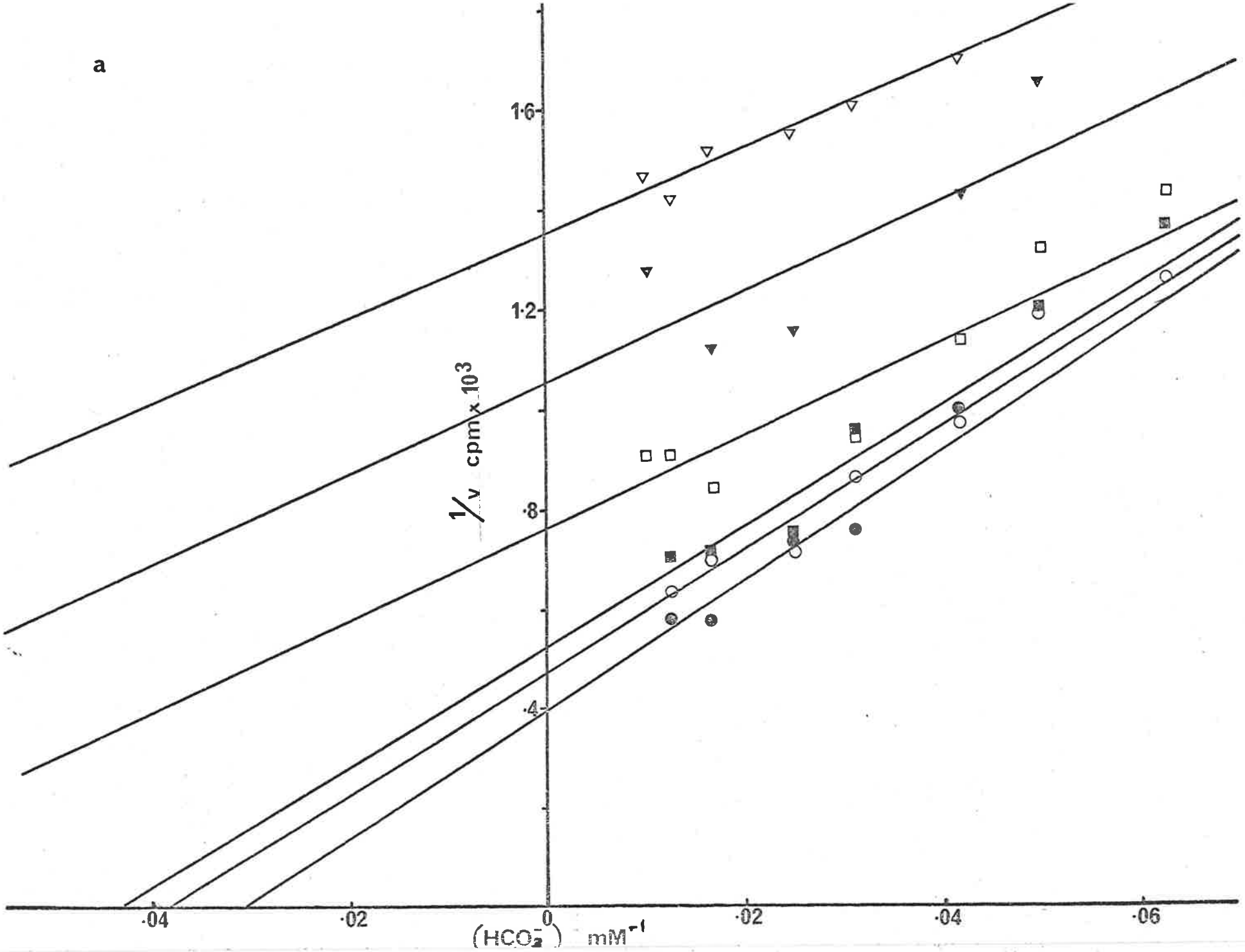
Radiochemical assays were initiated by the addition of 0.05ml of enzyme as in Fig.5.4a.

The 2-oxoglutarate concentration was 4mM and $MgATP^{2-}$ concentrations were —●—,5mM; —○—,2mM; —■—,1.5mM; —□—,1mM; —▲—,0.7mM; and —△—,0.6mM and 3mM which is not shown for clarity.

The lines were drawn according to the kinetic constants computed using HYPER.

Fig.5.6b Secondary plots of the data presented in Fig.5.6a

The data could not be fitted to either the SEQUEN nor PNPONG programme.


a
1·6
1·2
·8
·4
1/v cpm x 10³
·04
·02
0
·02
·04
·06
(HCO₃⁻) mM⁻¹

b

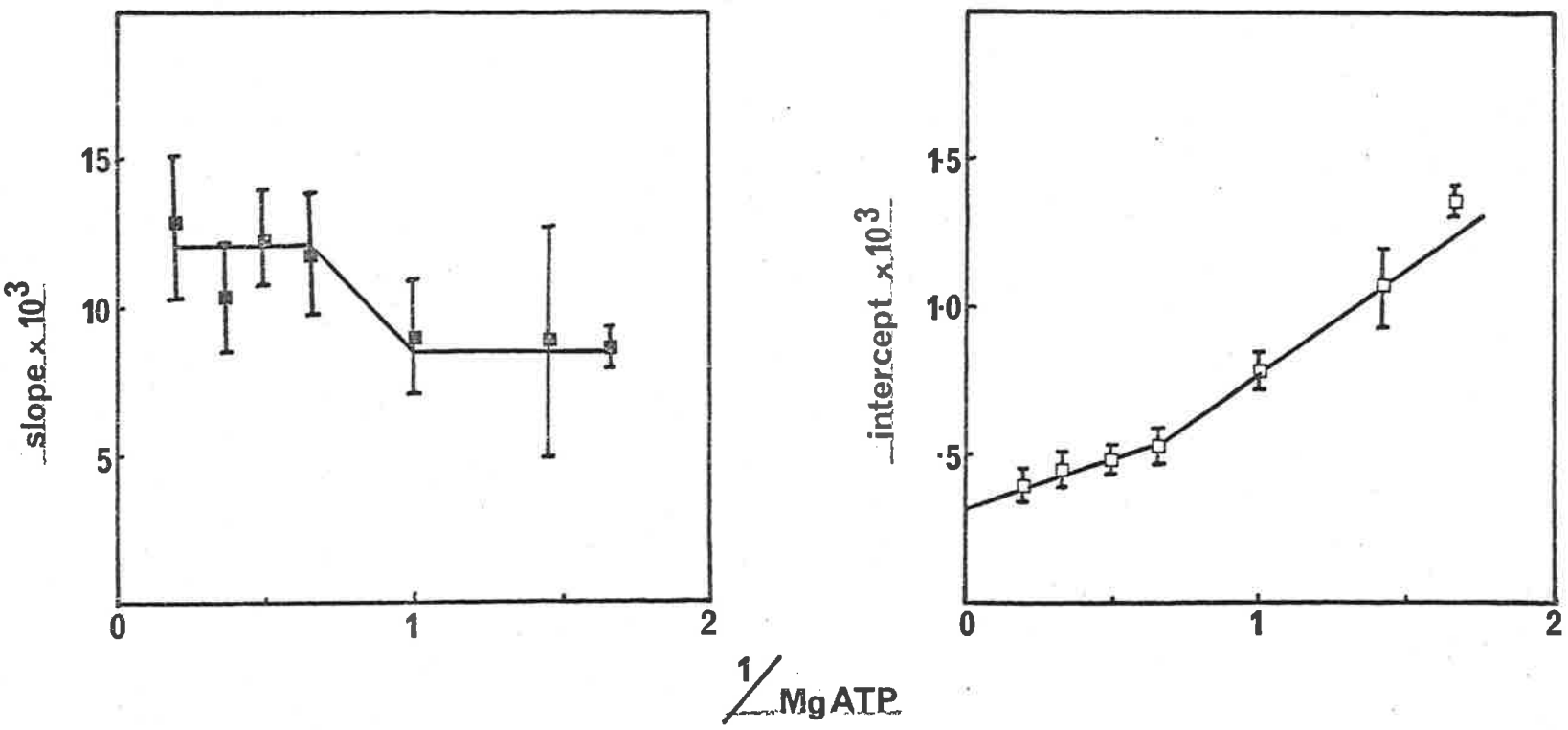


TABLE 5.2 The Km values summarised from Fig.5.4;5.5;5.6

| Variable Substrate | Fixed Variable | Km Value (mM) |
|---|---|---|
| 2-oxoglutarate | $HCO_3^-$ | 0.367 ± 0.162 |
| | $MgATP^{2-}$ | 0.316 ± 0.028 |
| $MgATP^{2-}$ | 2-oxoglutarate | 0.433 ± 0.343 |
| | $HCO_3^-$ | * |
| $HCO_3^-$ | 2-oxoglutarate | 9.47 ± 1.89 |
| | MgATP | * |

* These values cannot be obtained as the slope and intercept replots are not linear.

Fig.5.7 Product inhibition with $HCO_3^-$ as varied substrate

The enzyme was purified to Stage 4 (see Table 4.10) and assayed as in Section 2.2.5.1b.

$A_1$. MgADP as product inhibitor at the following concentrations: (—△—),0mM; (—●—),1mM; (—□—),2mM; (—▲—),3mM.

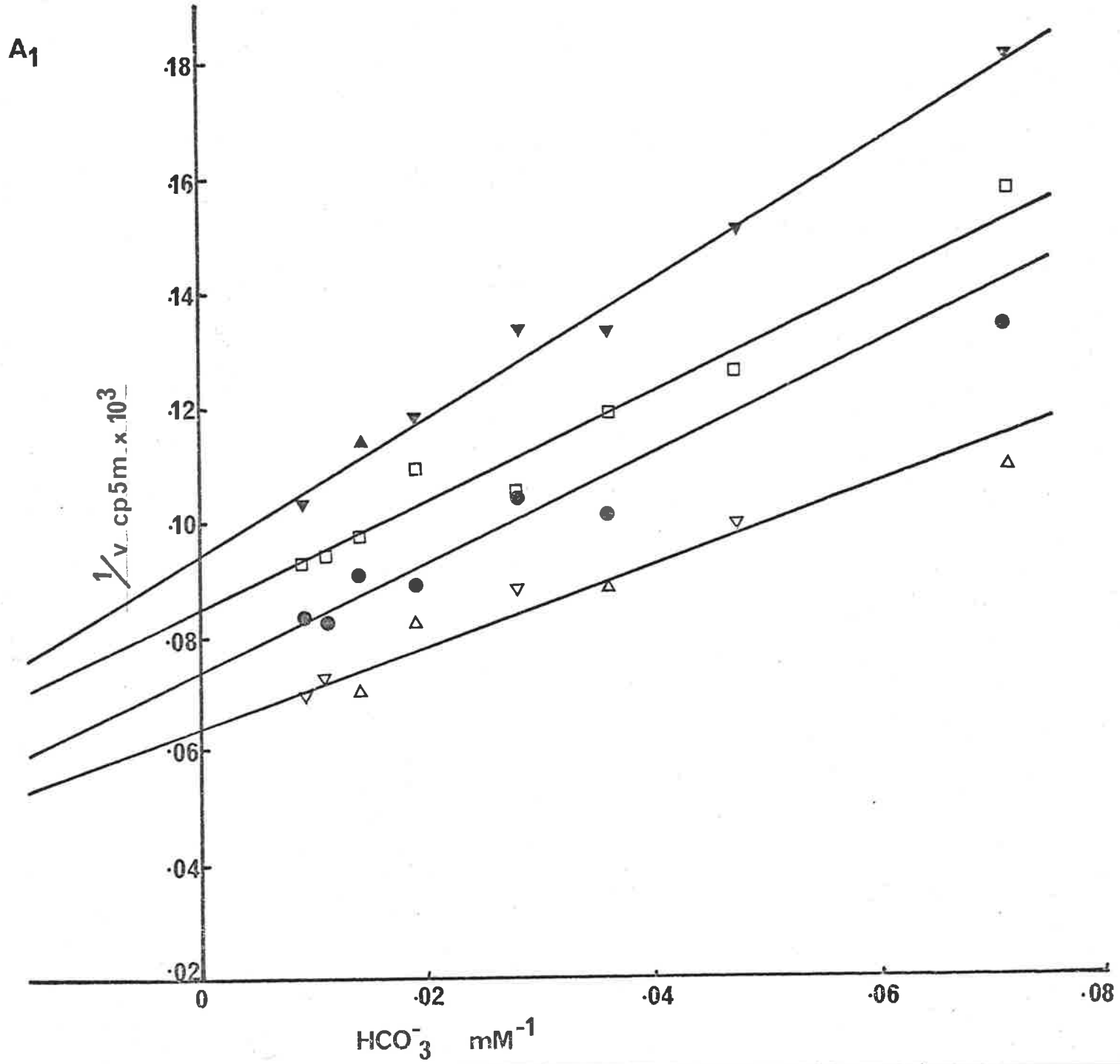
$A_2$ is the slope and intercept secondary plots.

$B_1$. $P_i$ as product inhibitor; $P_i(Na^+)$ and NaCl were added so that the concentration of $Na^+$ was constant. Final $P_i$ concentrations were: (—△—),0mM; (—●—),30mM; (—□—), 40mM; (—▲—)60mM.

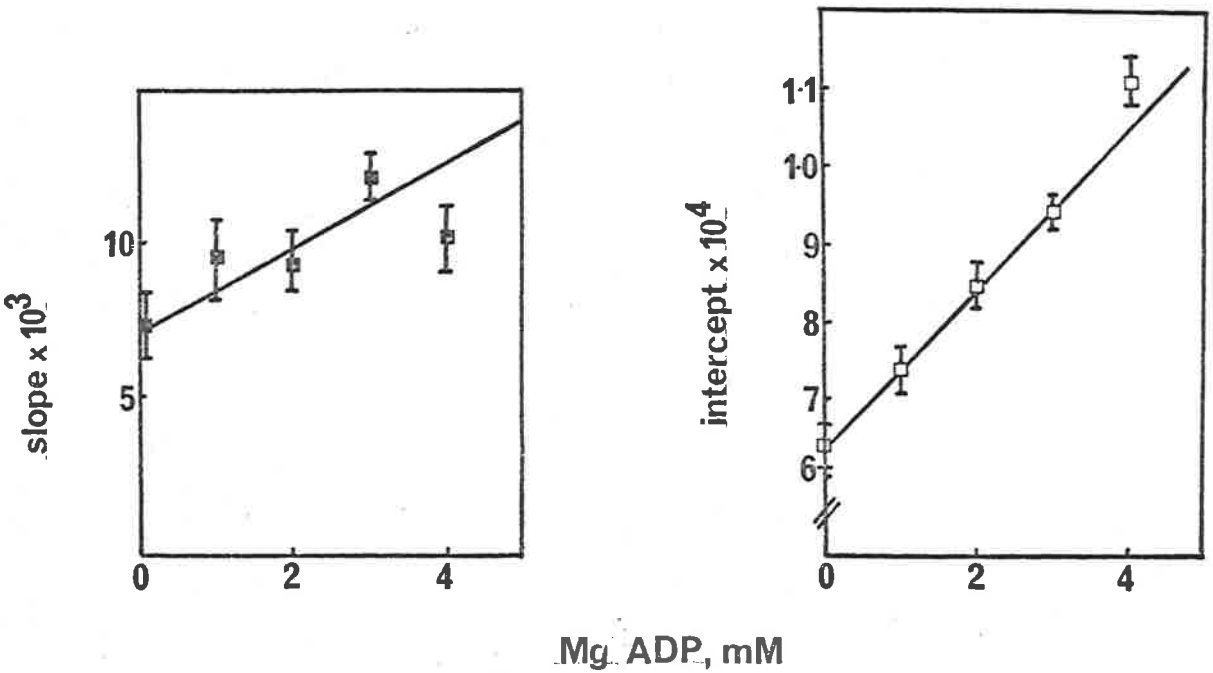
$B_2$ is the slope and intercept secondary plots.

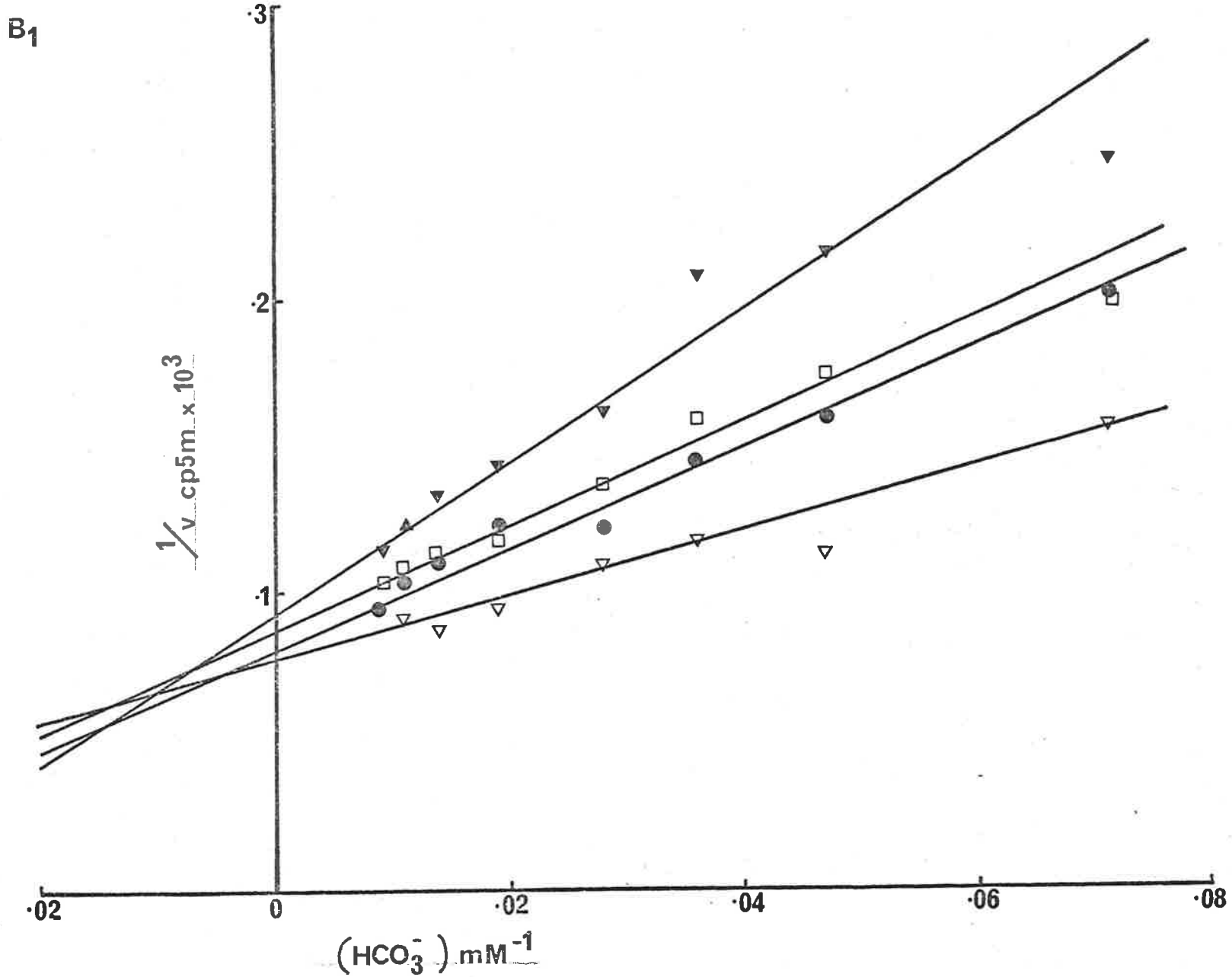
$C_1$. OAS as product inhibitor: the final concentrations were: (—■—),0mM; (—□—),12mM; (—▲—),24mM; (—△—),36mM; (—○—),48mM.

$C_2$ is the slope and intercept secondary plots.

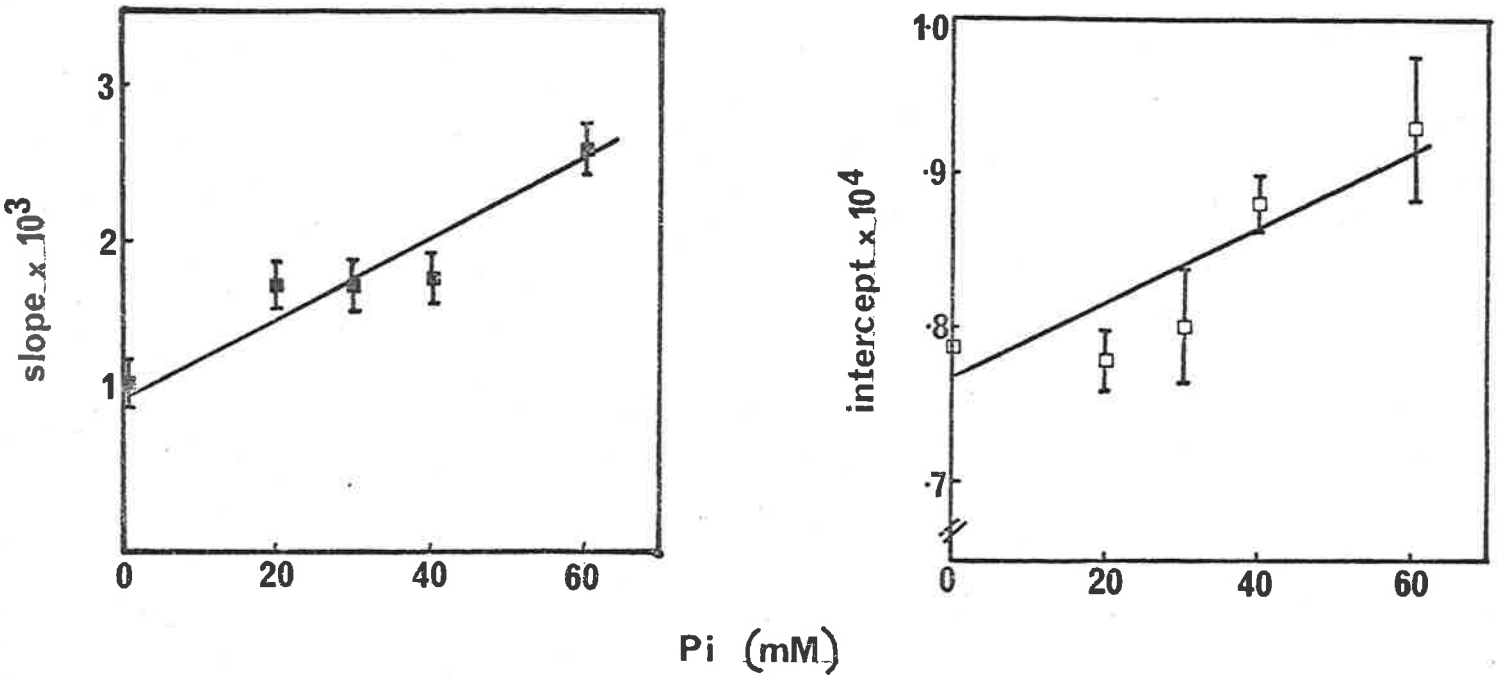

A1
·18
·16
·14
·12
·10
·08
·06
·04
·02
1/v cp5m x 10^3
0
·02
·04
·06
·08
HCO3- mM-1

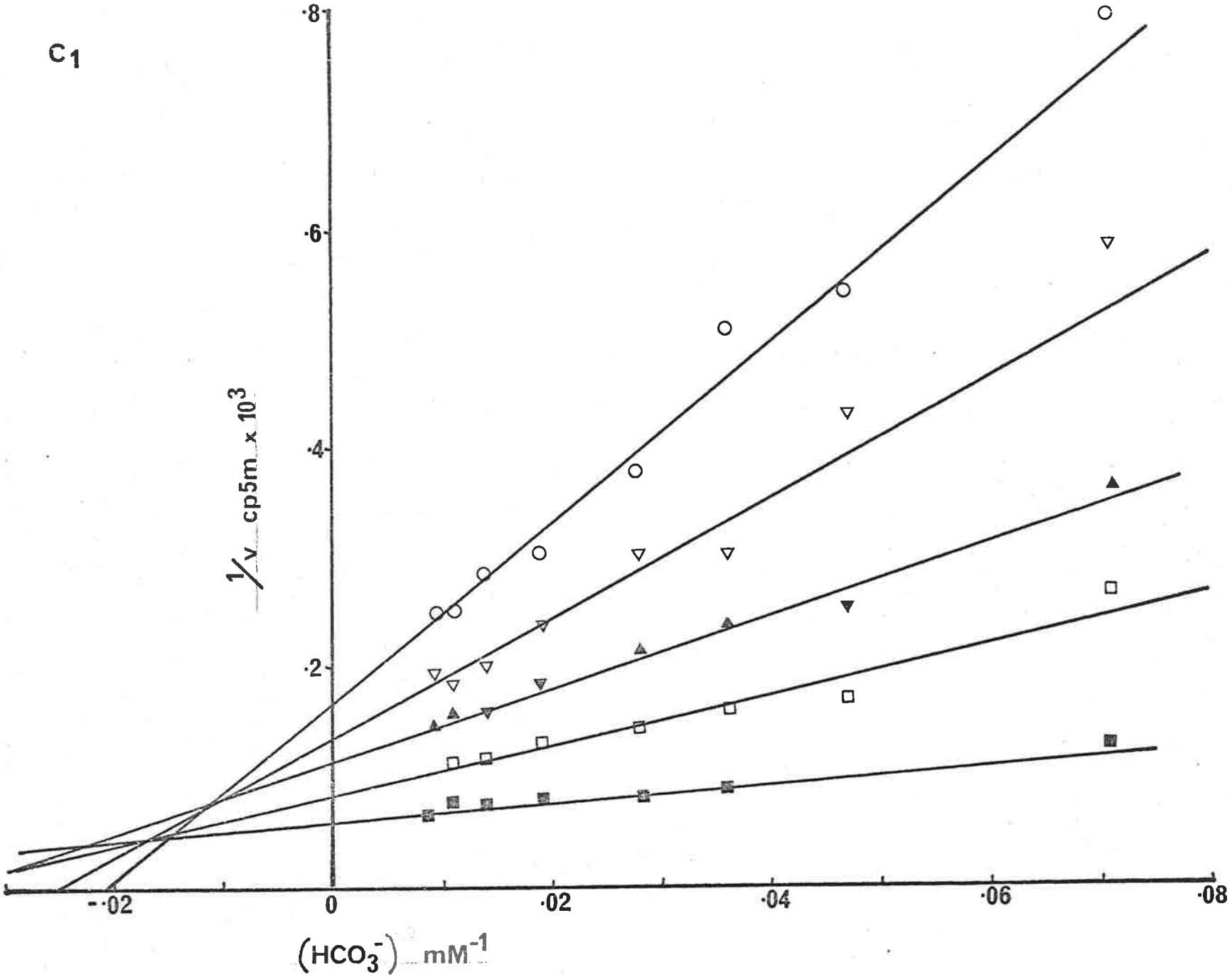
$A_2$




B1
·3
·2
·1
1/v cp5m x 10^3
·02
0
·02
·04
·06
·08
(HCO3-) mM-1

$B_2$




C1
·8
·6
·4
·2
1/v cp5m x 10^3
-·02
0
·02
·04
·06
·08
(HCO3-) mM-1

$C_2$

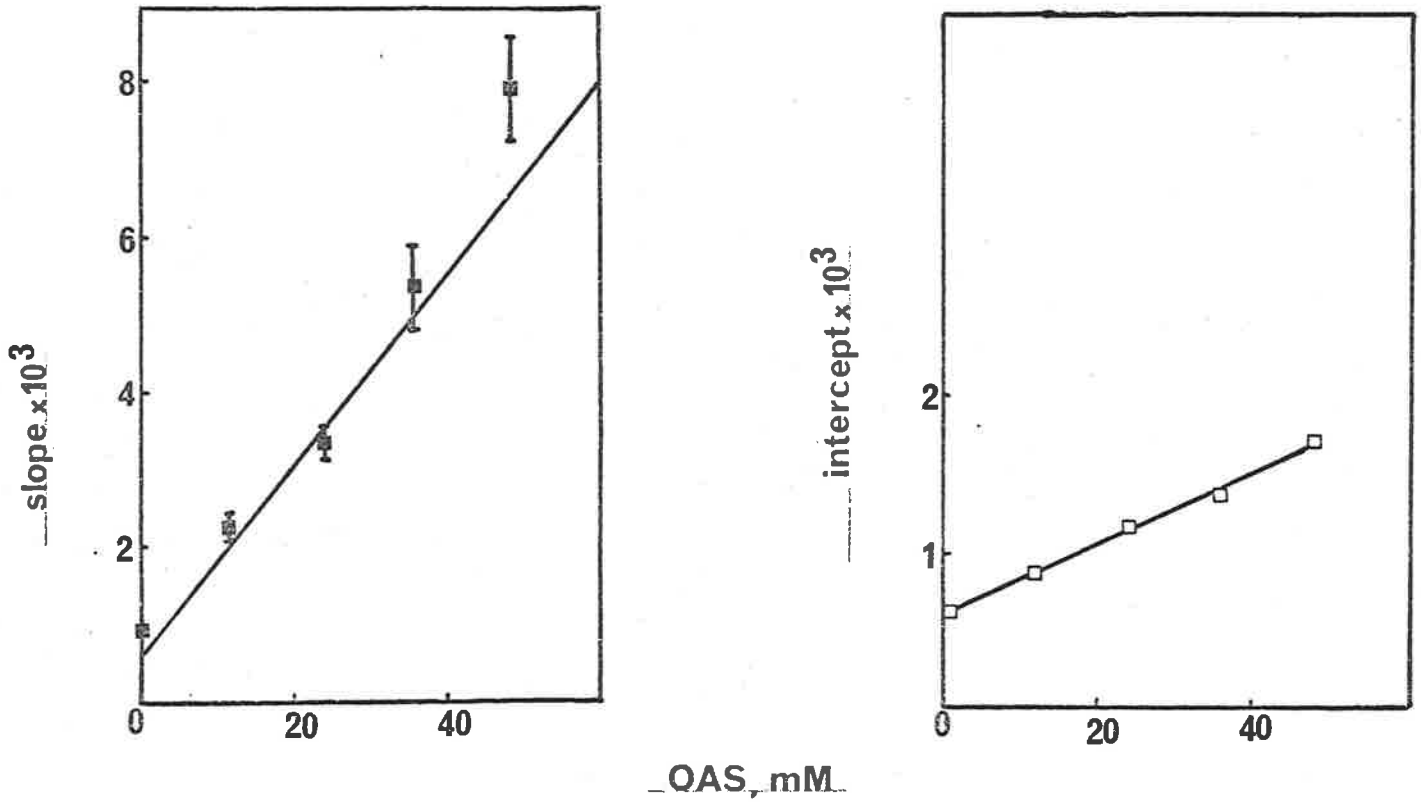


Fig.5.8 Product inhibition with 2-oxoglutarate as varied substrate

The enzyme was purified to Stage 4 (see Table 4.10) and assayed as in Section 2.2.5.1b.

$A_1$. MgADP as product inhibitor at the following concentrations:

(—▲—),0mM; (—○—),2mM; (—■—),3mM; (—△—),5mM

$A_2$ is the slope and intercept secondary plots.

$B_1$. $P_i$ as product inhibitor; $P_i(Na^+)$ and NaCl were added so that the concentration of $Na^+$ was constant. Final $P_i$ concentrations were: (—▲—),0mM; (—□—),25mM; (—○—), 75mM; (—●—)150mM.

$B_2$ is the slope and intercept secondary plots.

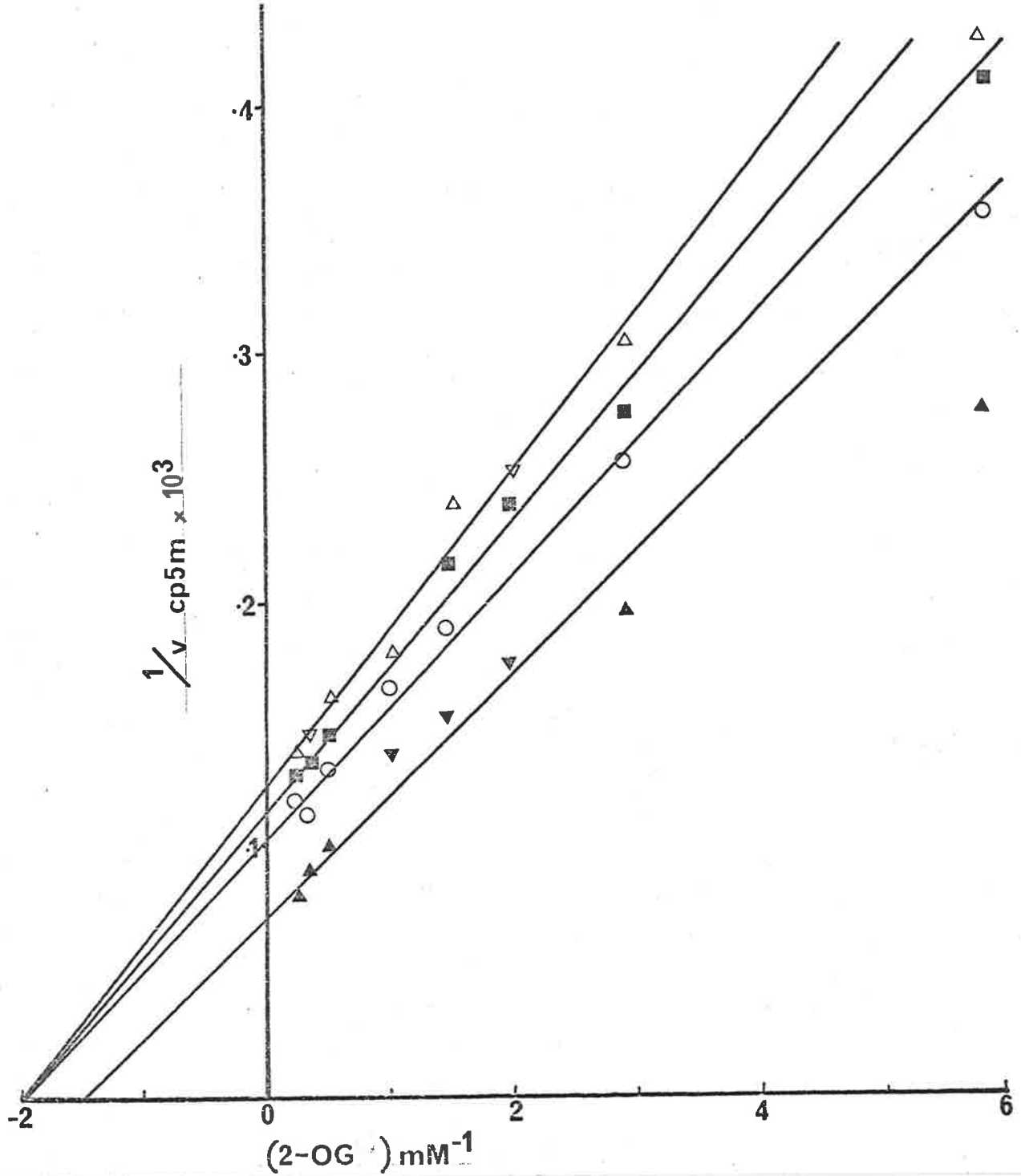
$C_1$. OAS as product inhibitor: the final concentrations were: (—▲—),0mM; (—●—),0.19mM; (—□—),0.388mM.

$C_2$ is the slope and intercept secondary plots.

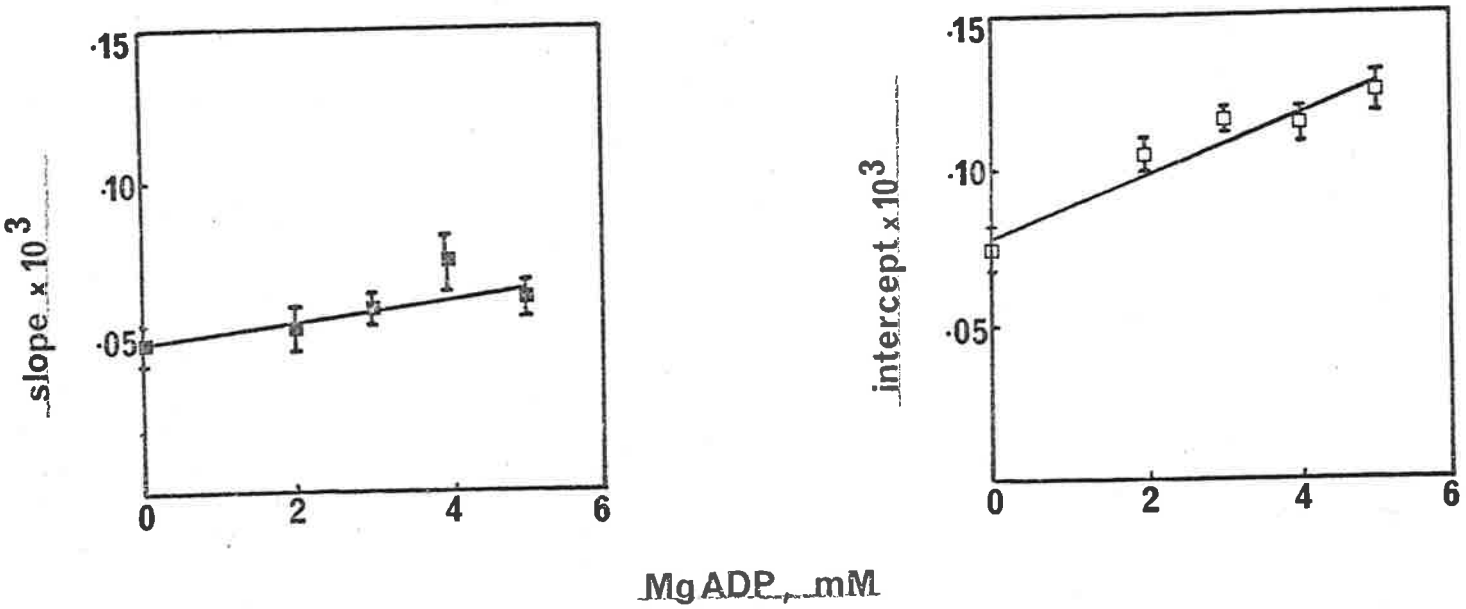
$D_1$. Isocitrate as product inhibitor: the final concentrations were: (—▲—),0mM; (—△—),1mM; (—□—),2mM; (—■—),3mM.

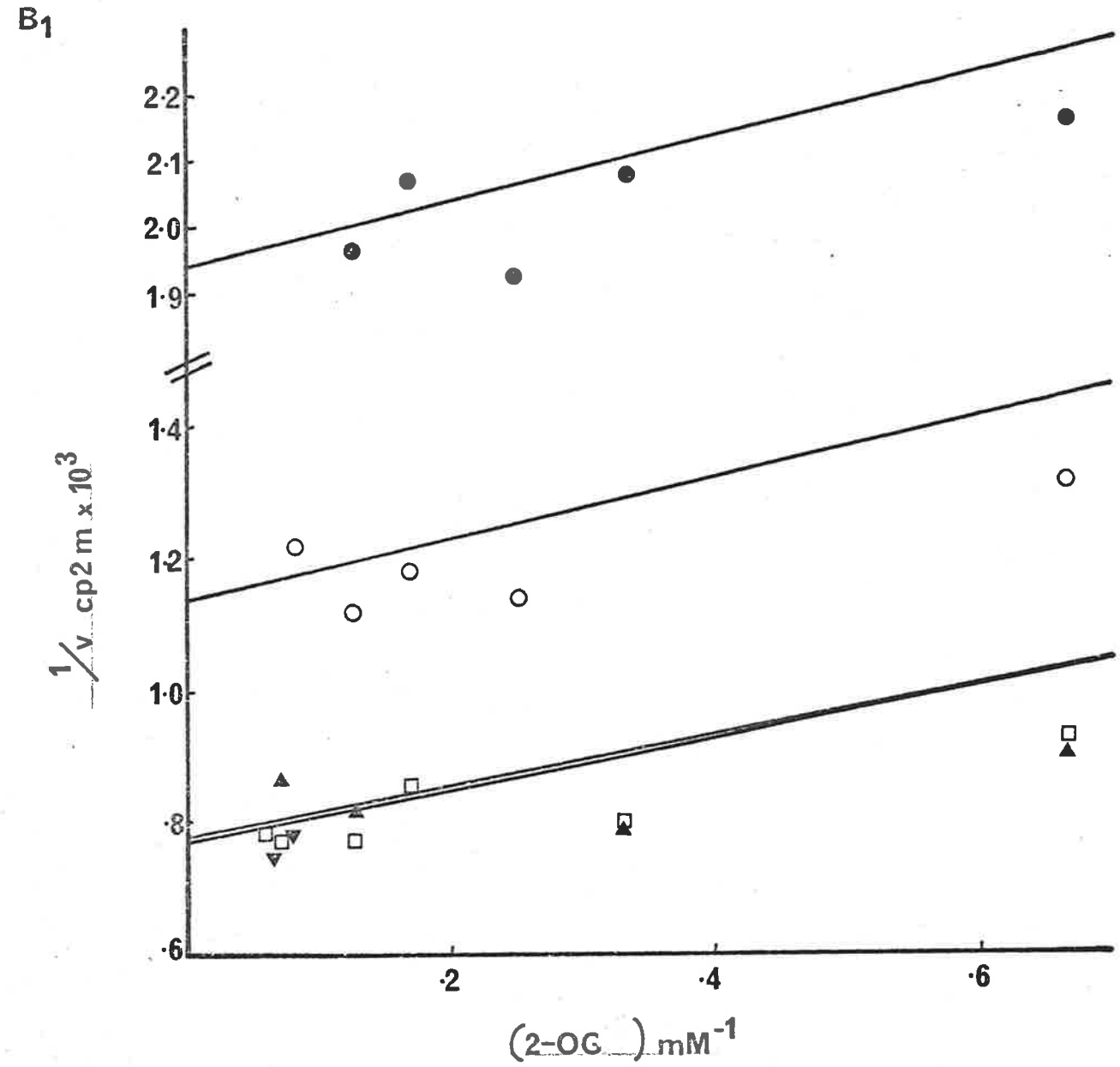
$D_2$ is the slope and intercept secondary plots.

$A_1$

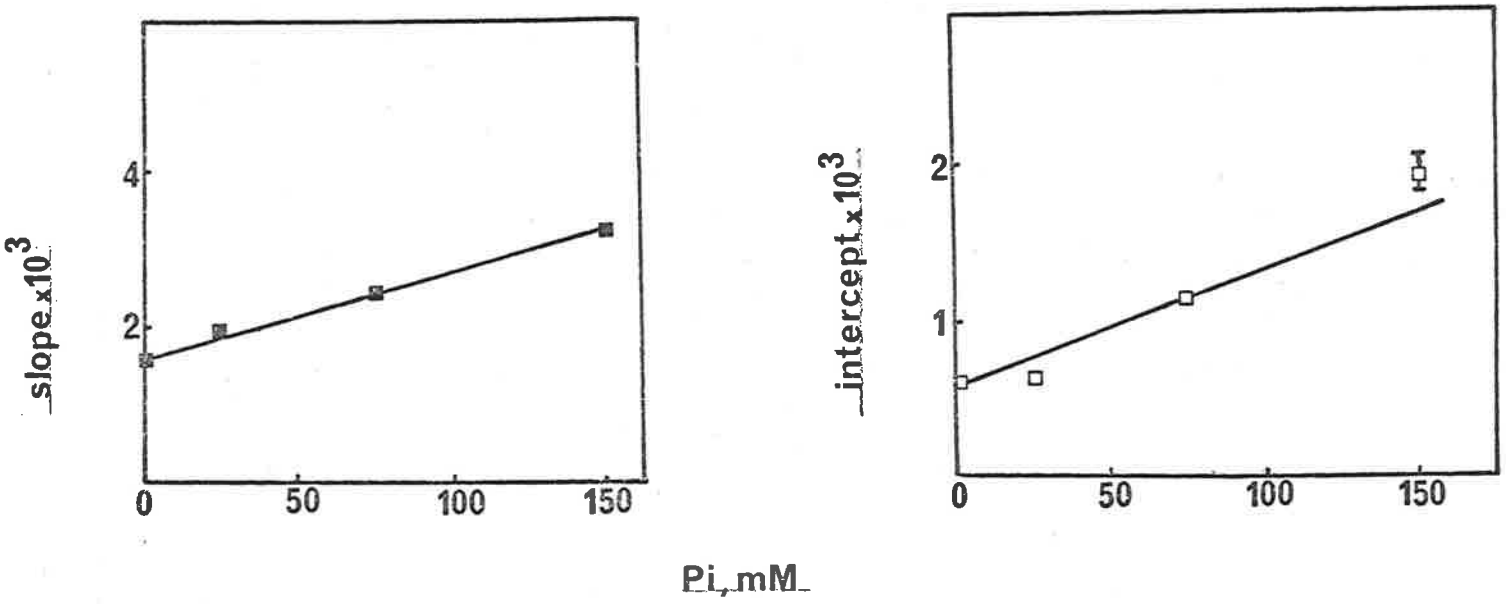


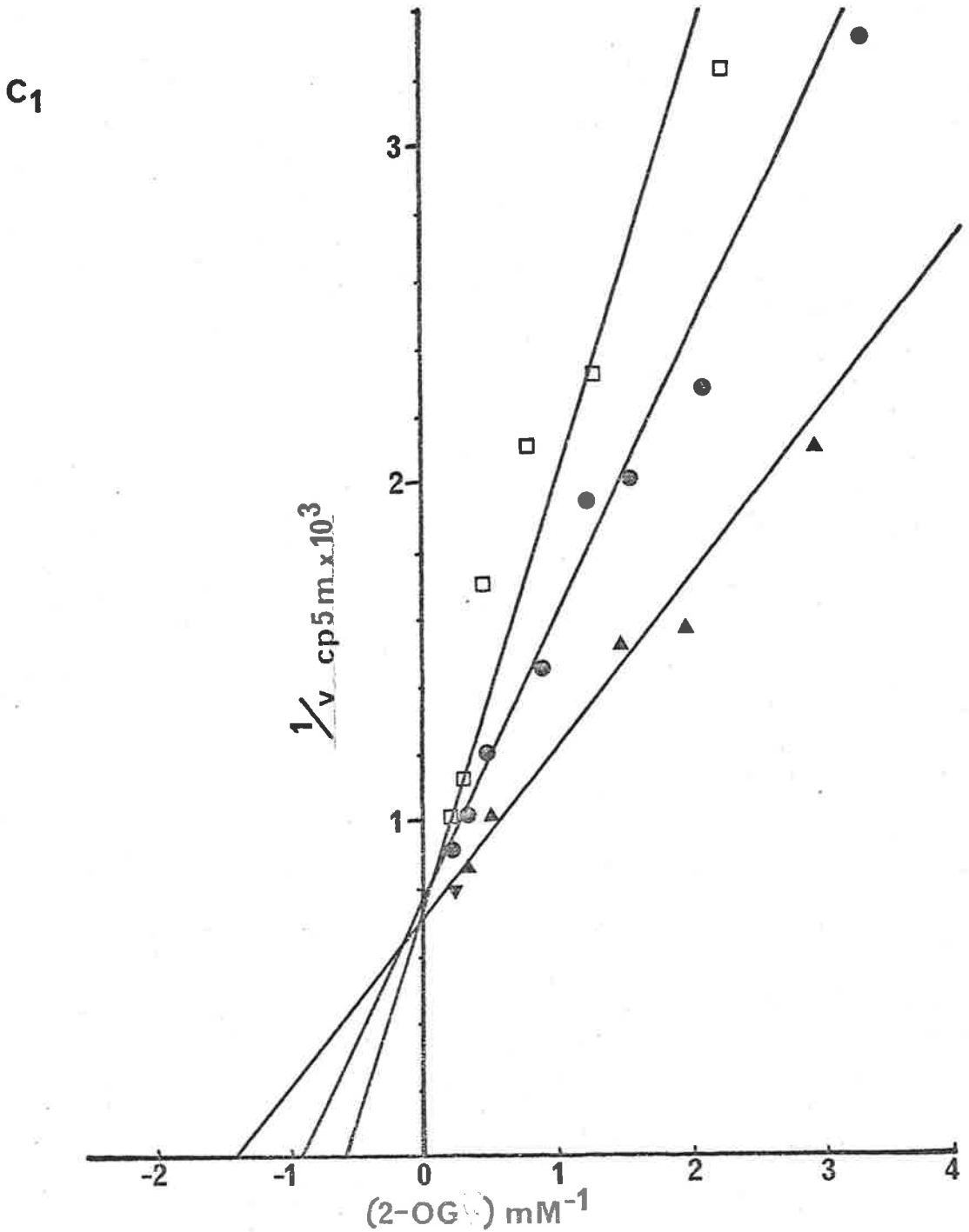
$A_2$



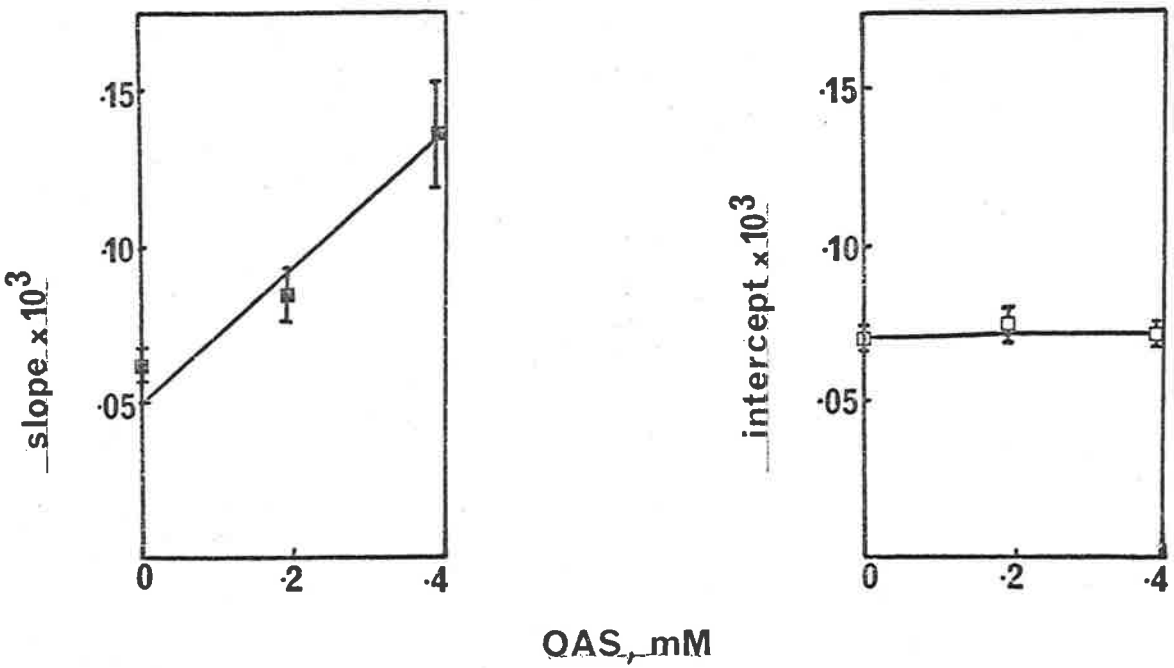

B1
2·2
2·1
2·0
1·9
1·4
1·2
1·0
·8
·6
·2
·4
·6
1/v cp2 m x 10^3
(2-OG) mM^-1

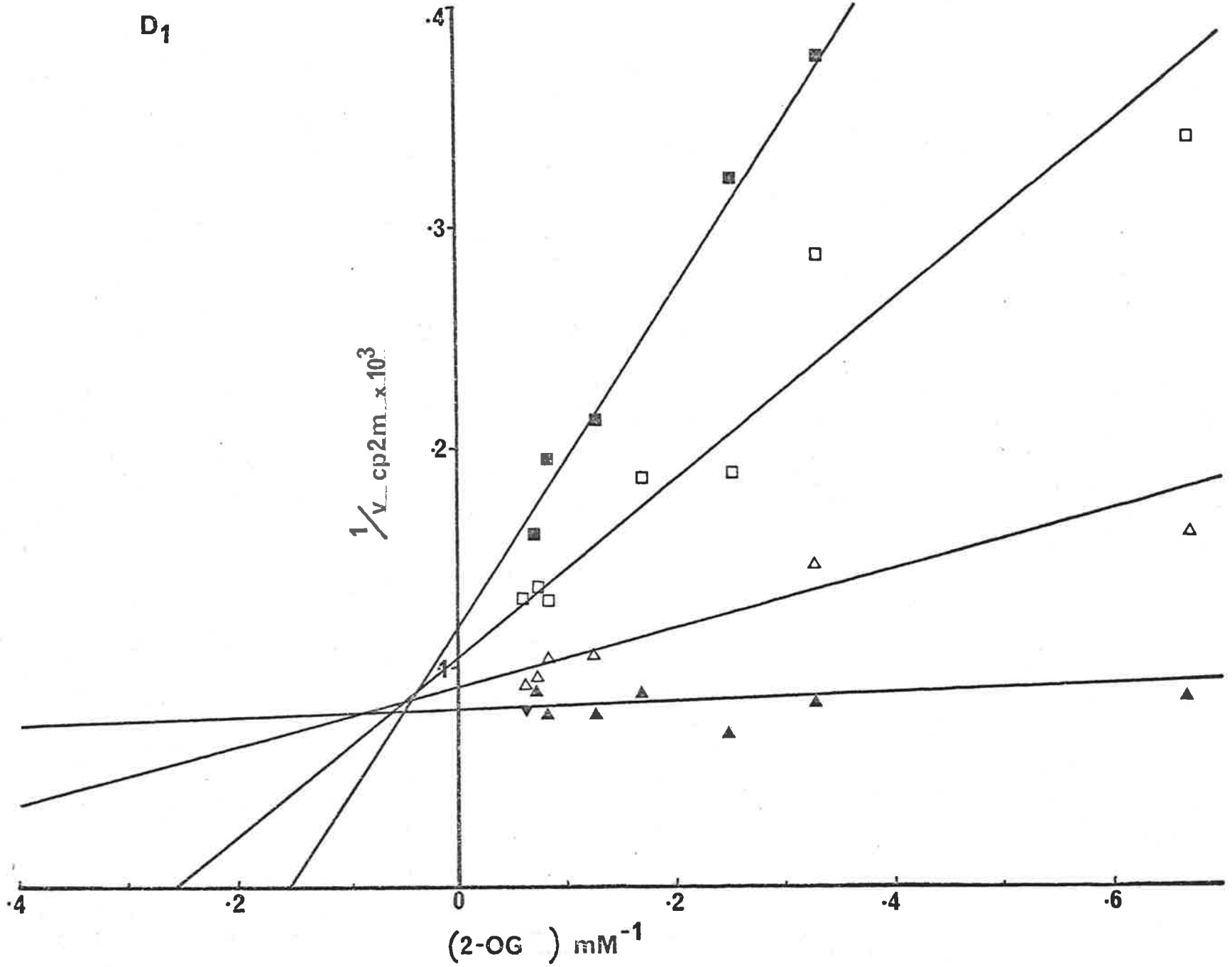
$B_2$




C1
3
2
1
1/v cp5m.x10^3
-2
-1
0
1
2
3
4
(2-OG) mM^-1

$C_2$




D1
·4
·3
·2
·1
0
1/v cp2m x 10^3
·4
·2
0
·2
·4
·6
(2-OG ) mM^-1

$D_2$

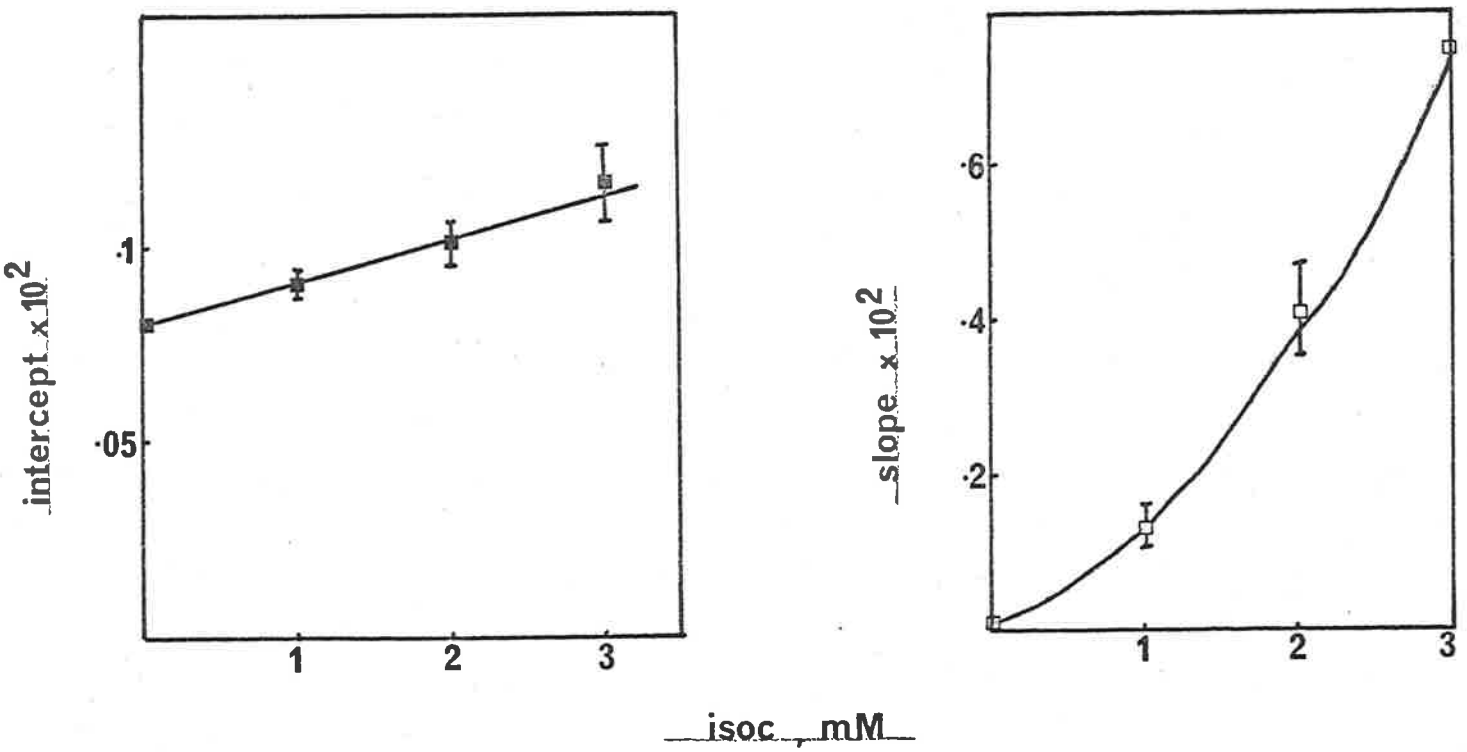


Fig.5.9 Product inhibition with $MgATP^{2-}$ as varied substrate

The enzyme was purified to Stage 4 (see Table 4.10) and assayed as in Section 2.2.5.1b.

$A_1$ MgADP as product inhibitor at the following concentrations: (—○—),0mM; (—●—),1mM; (—□—),2mM; (—△—),5mM.

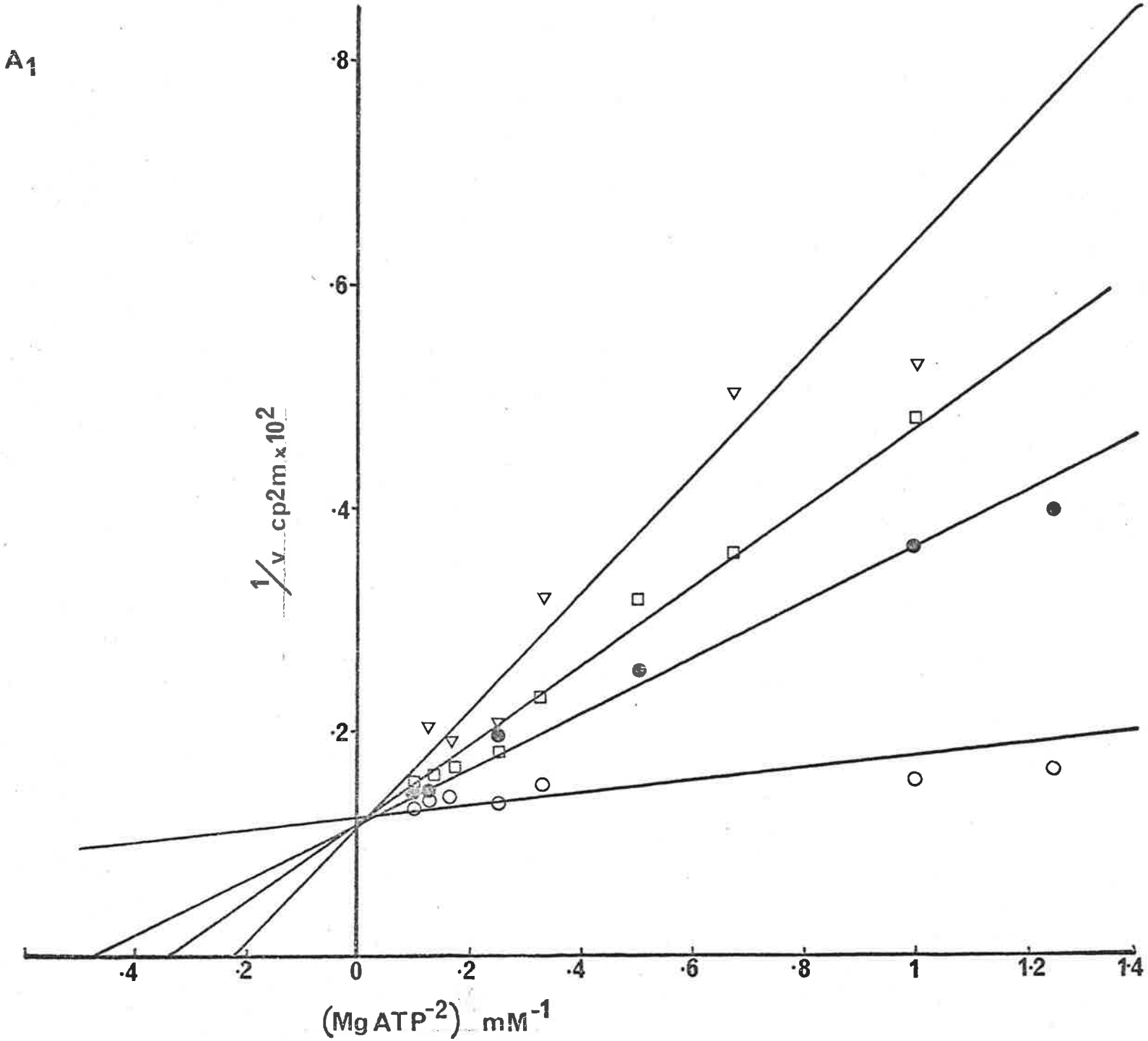
$A_2$ is the slope and intercept secondary plots.

$B_1$ $P_i$ as product inhibitor; $P_i$ ($Na^+$) and NaCl were added so that the concentration of $Na^+$ was constant. Final $P_i$ concentrations were: (—▲—),0mM; (—●—),40mM; (—□—),80mM; (—△—),120mM.

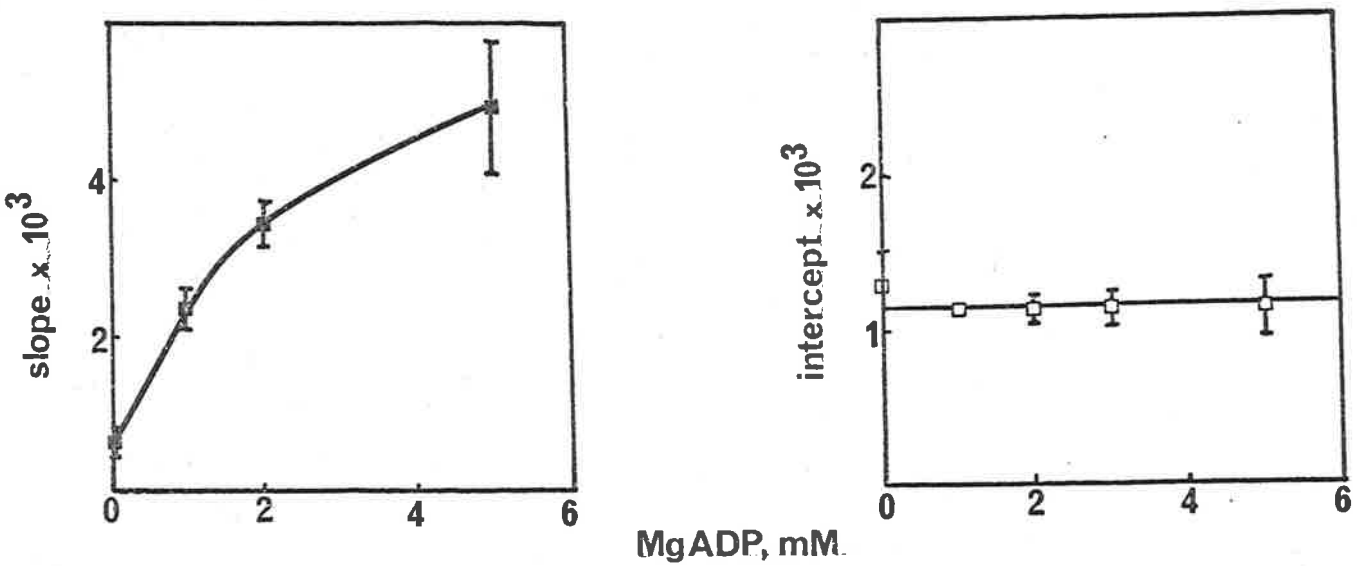
$B_2$ is the slope and intercept secondary plots.

$C_1$ OAS as product inhibitor at the following concentrations: (—□—),0mM; (—▲—),24mM; (—△—),48mM.

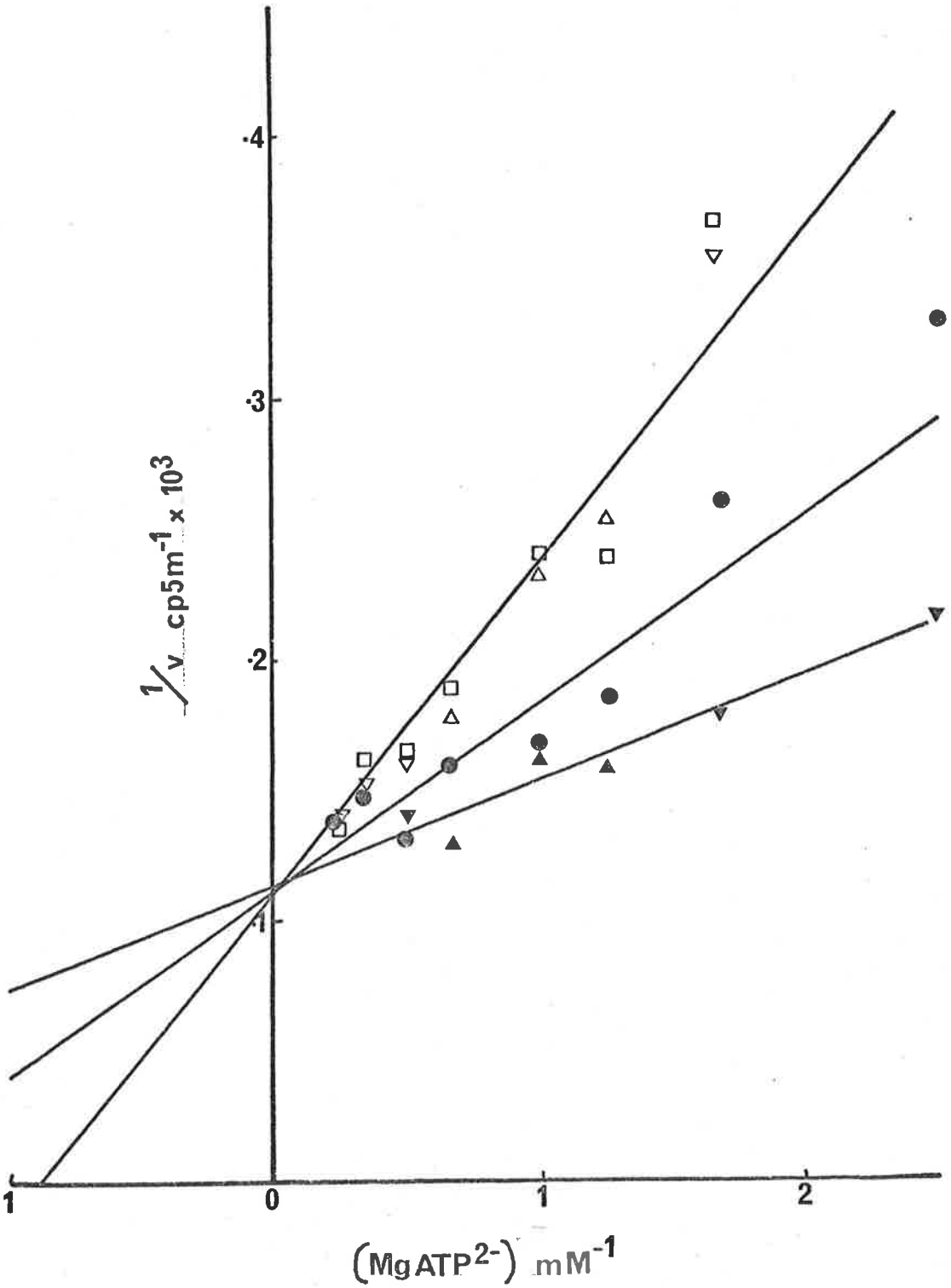
$C_2$ is the slope and intercept secondary plots.


A1
·8
·6
·4
·2
1/v cp2m x 10^2
·4
·2
0
·2
·4
·6
·8
1
1·2
1·4
(Mg ATP^-2) mM^-1

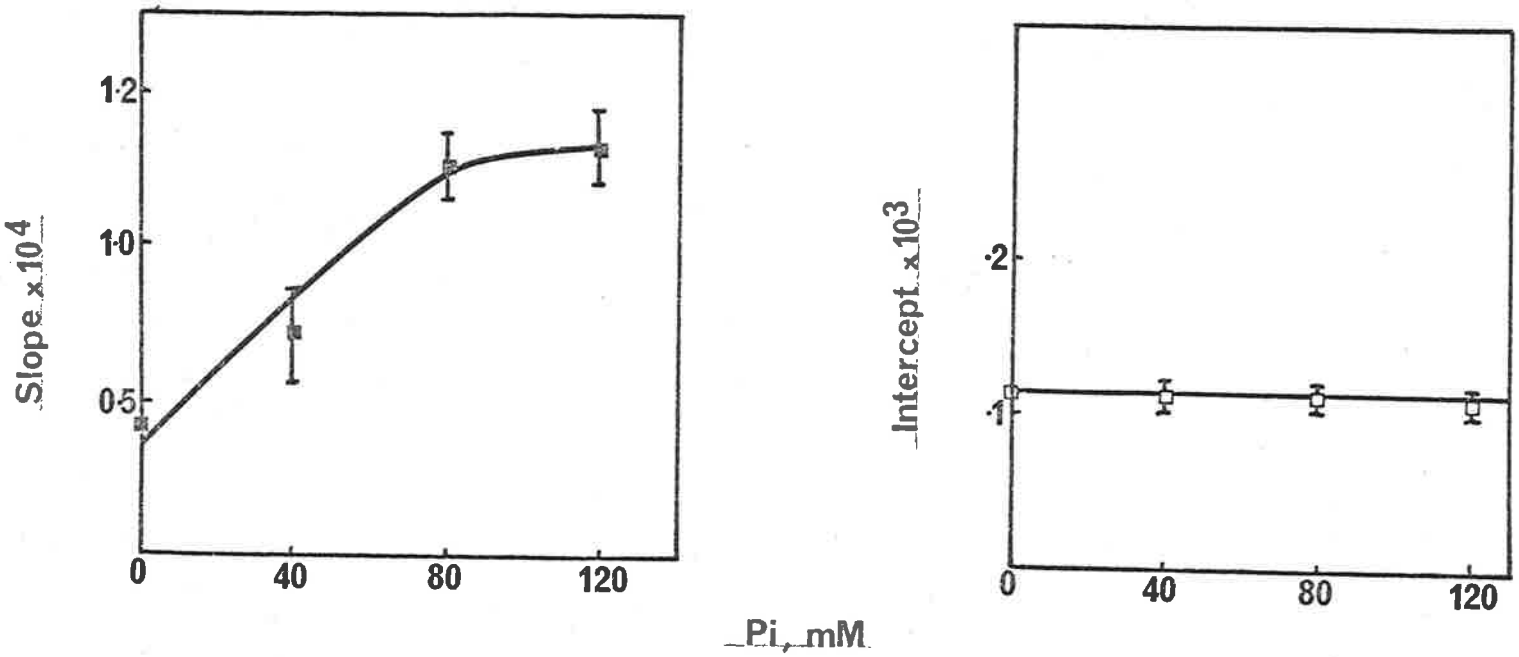
$A_2$



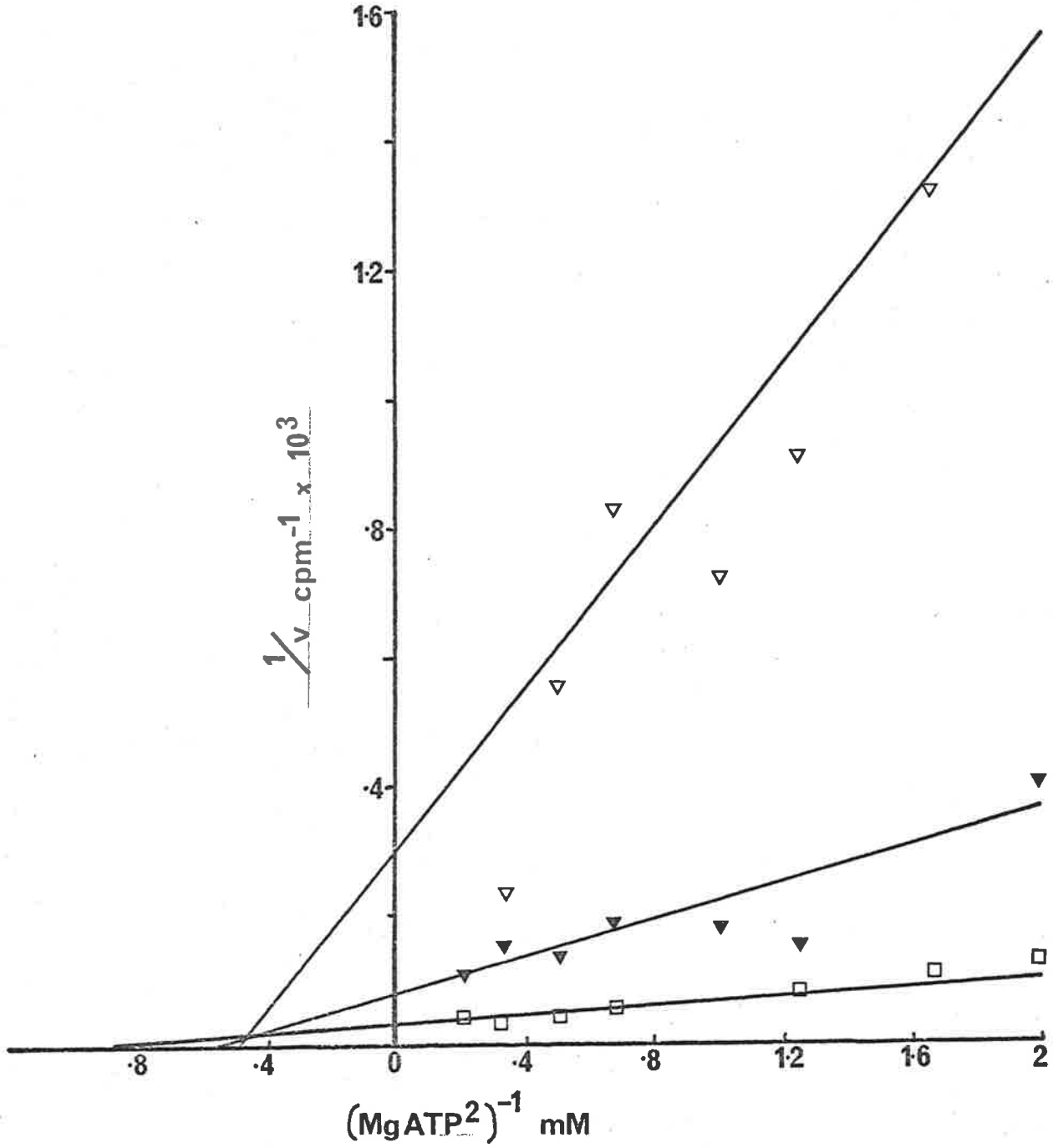
$B_1$



$B_2$


Slope x10⁴
1·2
1·0
0·5
0
40
80
120
Pi, mM
Intercept x10³
·2
·1
0
40
80
120

$C_1$



$C_2$

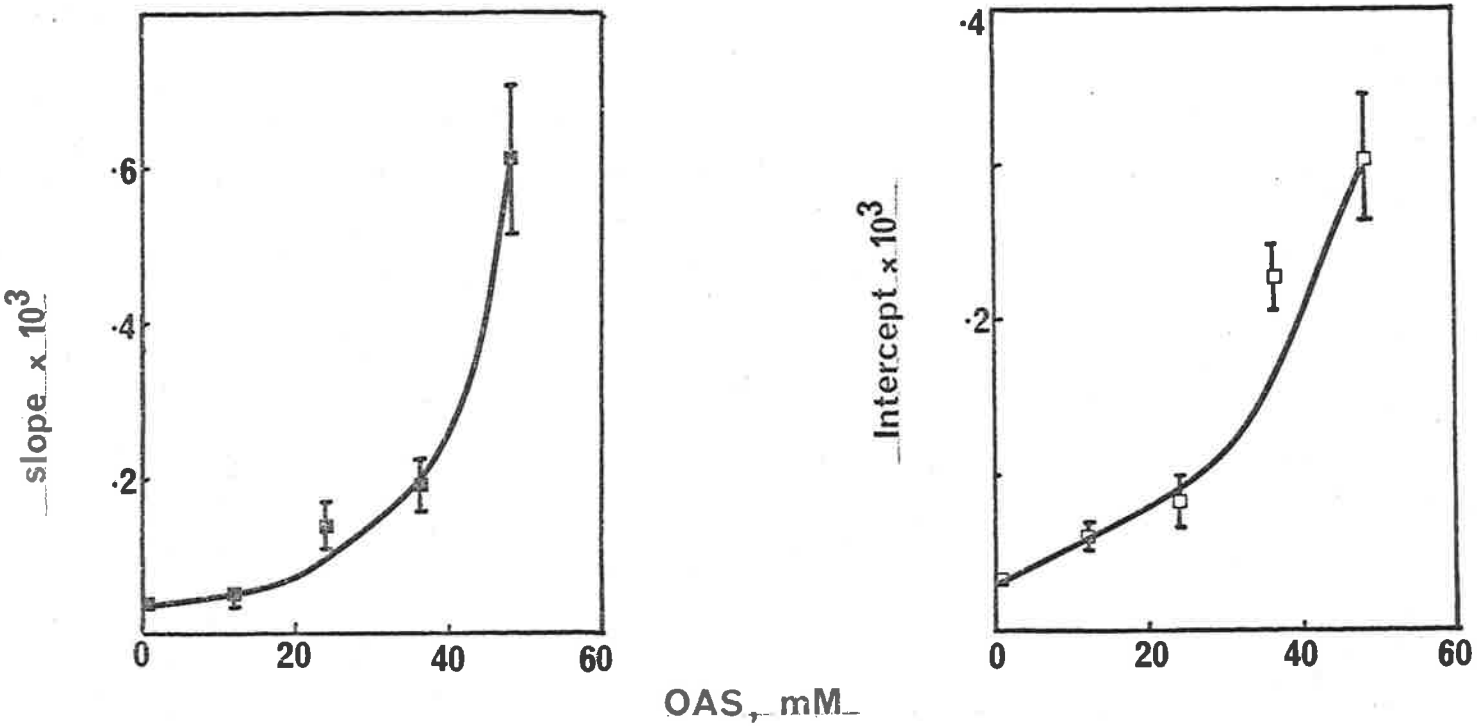


TABLE 5.3 Product Inhibition Studies

The results summarised below are shown in detail in Figs.5.7;5.8;5.9. The levels of the fixed substrates were: MgATP,5mM;$HCO_3^-$,100mM and 2-oxoglutarate, 4mM. The types of inhibition are C, competitive and NC, non-competitive.

| Varied Substrate | Product Inhibitor | Type of Inhibition | Slope-linear $K_i$ (mM) | Intercept-linear $K_I$ (mM) |
|---|---|---|---|---|
| $MgATP^{2-}$ | $MgADP^-$ | C | non-linear | |
| | $P_i$ | C | non-linear | |
| | OAS | NC | non-linear | non-linear |
| $HCO_3^-$ | $MgADP^-$ | NC | 5.0 ± 1.6 | 6.3 ± 0.2 |
| | $P_i$ | NC | 41.8 ± 13.9 | 321 ± 123 |
| | OAS | NC | 3.22± 3.3 | 2.7 ± 2 |
| 2-oxoglutarate | $MgADP^-$ | NC | 17 ± 2 | 7.9 ± 2 |
| | $P_i$ | NC | 149 ± 14 | 81 ± 25 |
| | isocitrate | NC | non-linear | 6.5 ± 0.6 |
| | OAS | C | 0.22± 0.05 | |

Fig.5.10 Alternate keto acid substrate kinetics

The assay system as described in Section 2.2.5.1b using enzyme after Stage 4 of purification (see Table 4.10) was used to determine the level of carboxylation for each of the keto acid substrates. The $MgATP^{2-}$ concentration range was 0.3mM to 5mM, the $HCO_3^-$ concentration was 100mM and the keto acids were 4mM. The data was plotted in double reciprocal form, and the slopes and intercepts of the lines obtained by a least mean squares analysis.

(—■—), pyruvate;

(—△—), 2-oxoglutarate;

(—○—), α-ketoadipic acid.

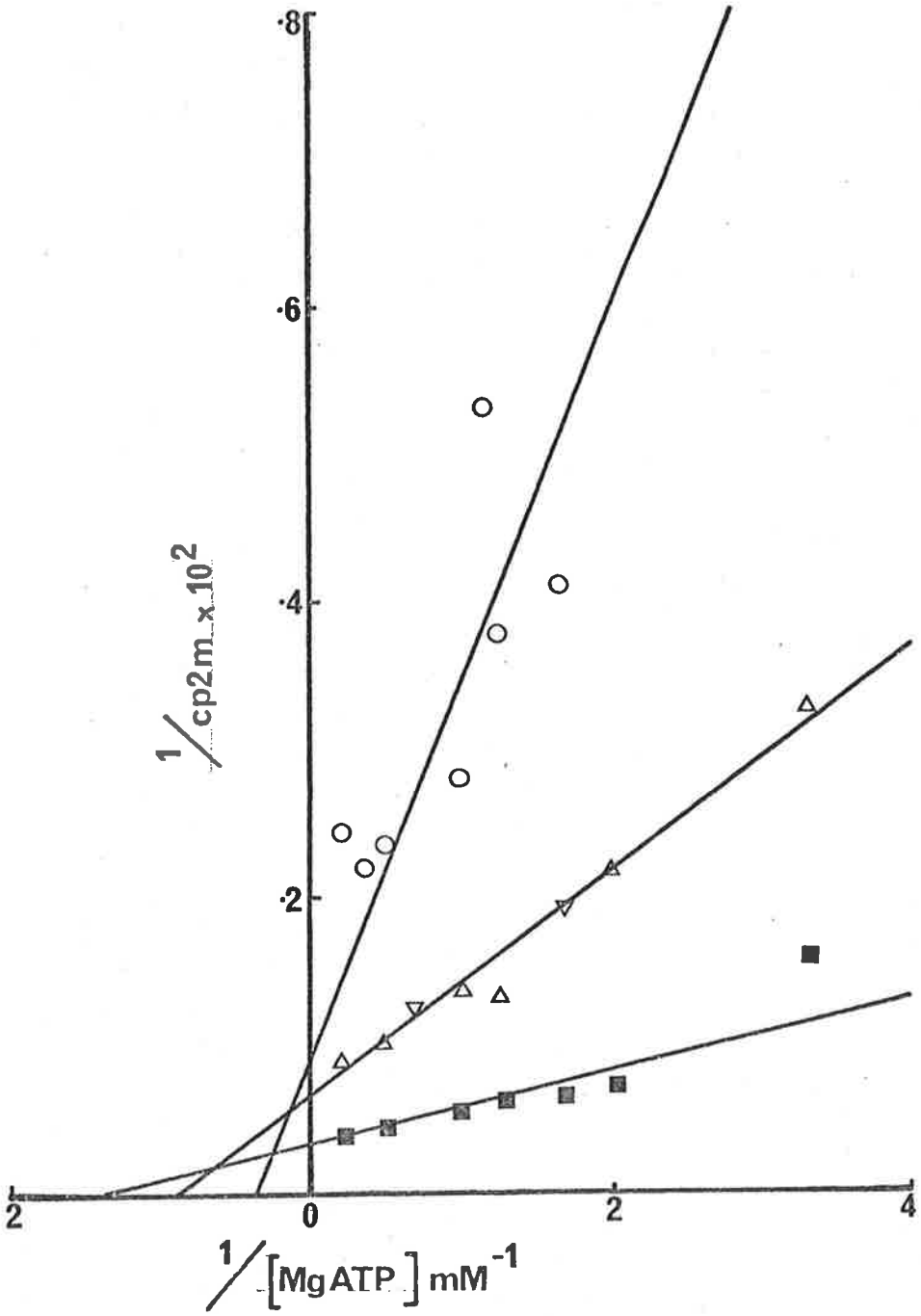

·8
·6
·4
·2
0
2
4
1/cp2m x 10^2
1/[MgATP] mM^-1

TABLE 5.4 Analysis of the slopes of the data presented in Fig.5.10

| Keto acid substrate | Slope | Slope ratio | V(%) | appKm mM |
|---|---|---|---|---|
| 2-oxoglutarate | .000770 ± .000048 | 1 | 100 | 1.1 ± 0.19 |
| pyruvate | .000248 ± .000013 | .322 ± .017 | 250 | 0.82 ± 0.09 |
| α-ketoadipic acid | .00264 ± .00031 | 3.43 ± .04 | 41 | 2.8 ± 1.6 |

Statistical analysis using the t-test shows that the slopes for the alternate substrates are significantly different from that for 2-oxoglutarate.

2-oxoglutarate vs pyruvate $p < .01$

2-oxoglutarate vs α-ketoadipic acid $p < .05$

The slope ratio represents the slope of the line relative to that using 2-oxoglutarate as the keto acid substrate.

# CHAPTER 6

# PHYSICAL AND CHEMICAL PROPERTIES

## 6.1 Introduction

### 6.1.1 Multi-component enzyme systems

The complete loss of enzymic activity upon elution of an enzyme from chromatographic gels illustrated in Chapter 4 suggests that the enzyme is part of a multi-component complex. The recovery of enzymic activity when two or more fractions from such an elution are combined lends further support to the multi-component nature of the system. For a number of reasons it is considered to be advantageous to the cell for the enzymes catalysing sequential reactions in a metabolic pathway to exist in a multi-component complex.

Pyruvate dehydrogenase is one example of such a complex system. This complex consists of three enzymes which catalyse successive reactions in the oxidative decarboxylation of pyruvate and two enzymes which possess regulatory functions (a kinase and a phosphatase). All of these enzymes have been separated from the purified complex (Severin and Feigina, 1976).

### 6.1.2 Phosphorylation and dephosphorylation

Another interesting aspect of the pyruvate dehydrogenase complex is that it also belongs to the group of proteins which depend on either phosphorylation or dephosphorylation before exhibiting biological activity. (Reed et al., 1973). Phosphorylation is catalyzed by a protein kinase which transfers the terminal phosphoryl moiety of ATP to the enzyme. For pyruvate dehydrogenase, phosphorylation results in a decrease or complete suppression of the decarboxylase activity. Activity is

restored by hydrolysing off of the phosphoryl group by the corresponding phosphatase. The phosphorylation and dephosphorylation processes are a means of an *in vivo* regulation of pyruvate dehydrogenase activity.

The topic of phosphoproteins has been comprehensively reviewed by Taborsky (1974) with sections II and VI being of relevance to this work.

### 6.1.3 The glucose effect

In Section 1.1.6 a number of examples were quoted where an increase in fatty acid synthesis occurred from glutamate when animal diets were altered to include glucose. The stimulation of fatty acid synthesis by glucose was also observed when glucose was added *in vitro* to tissues of animals fed a glucose-free diet. Using epididymal fat pads from rats maintained on a glucose-free diet, Madsen *et al.*, (1964b) observed an increase of from 6% to 17% of the incorporation of radioactive label from [2-$^{14}$C] and [5-$^{14}$C] glutamate into labelled fatty acid in the presence of glucose. Similarly, when lactating rat mammary gland slices were studied, the level of glutamate metabolized via the 2-oxoglutarate reductive carboxylation pathway increased from 3-7% to 20-30% upon the *in vitro* addition of glucose (Madsen *et al.*, 1964a). The total amount of glutamate metabolized was of the same order of magnitude in the presence or absence of glucose. Therefore, the addition of glucose *in vitro* does not increase the amount of glutamate metabolized but does appreciably increase the contribution of the 2-oxoglutarate reductive carboxylation pathway to fatty

acid synthesis. The explanation and locus of action for this glucose effect remains an enigma. However, with the isolation of the enzyme, isocitrate synthase, as a result of this investigation, it may be possible to elucidate the mechanism of this effect.

6.1.4 The carboxylation of pyruvate

The results presented in Section 5.3.4 show that pyruvate is an alternate substrate to 2-oxoglutarate of the enzyme containing isocitrate synthase activity. Therefore it is essential to establish that pyruvate is being utilised by isocitrate synthase and not another contaminating enzyme in the partially purified enzyme preparation. The major source for the carboxylation of pyruvate in the cell is catalysed by pyruvate carboxylase (EC6.4.1.1), a mitochondrial biotin-containing carboxylase which is inactivated by avidin. This reaction is ATP-dependent and requires activation by acetyl-CoA for full expression of activity, yielding OAA as the product.

Another enzyme which utilises pyruvate and $CO_2$ is the cytoplasmic enzyme malate dehydrogenase (decarboxylating) (NADPH)EC1.1.1.40, which is referred to as the malic enzyme. The carboxylation of pyruvate requires NADP but no ATP and results in malate formation.

It was also necessary to show that pyruvate dependence of the isocitrate synthase co-chromatographs on gel chromatograph with 2-oxoglutarate dependence by assaying each fraction eluted from the gel with both keto acid substrates.

### 6.1.5 ATP-dependence

Although the isocitrate synthase activity has shown complete dependence on the presence of ATP, some questions have remained unanswered. (a) Whether or not there is a one-to-one stoichiometric relationship between the utilisation of ATP and 2-oxoglutarate, (b) Whether ATP is utilised to phosphorylate one of the components of the complex to an activated form. Alternatively, (c) ATP could be acting as an allosteric activator.

Non-hydrolysable analogues of ATP are most useful in elucidating the action of ATP in enzymic reactions. AMP-PCP and AMP-PNP are two such analogues which cannot be hydrolysed. The O between the $\beta$ and $\gamma$ phosphorus atoms is replaced by a carbon (C) and a nitrogen (N) respectively. The substitution of these derivatives instead of ATP would indicate whether there is a requirement for ATP hydrolysis in the reaction mechanism.

## 6.2 Materials and methods

### 6.2.1 Materials

AMP-PNP was supplied by P.L. Biochemicals and AMP-PCP was a gift from Dr. R. Symons of this department. Human prostatic acid phosphatase was a gift from Dr. R. Bais of the Institute of Medical and Veterinary Science, Adelaide and alkaline phosphatase type III was purchased from Sigma and GF/A filters from Whatman Biochemicals Ltd., Kent, England. All other reagents were of analytical grade and bi-distilled water was used to prepare all solutions.

### 6.2.2 Methods

Preparation and purification of [$\gamma$-$^{32}$P] ATP was described in Section 2.2.1. The spectrophotometric assays for ATP estimation, level of malic dehydrogenase, malic enzyme and lactate dehydrogenase are described in Sections 2.2.5.3.-2.2.5.5 respectively. Measurement of $^{32}P_i$ release from [$\gamma$-$^{32}$P] ATP is described in Section 2.2.5.1c.

Determination of alkaline-stable phosphoryl groups incorporated into proteins was achieved by addition to the enzyme of 5 volumes of a solution containing 7M urea and 10mMNaOH and 5 volumes cold acetone (-15$^{o}$C). This solution was allowed to stand on ice for 10 min then placed onto GF/A filter. The filters were washed extensively with cold acetone, dried and their radioactive content determined. When chloroform and methanol were substituted for the acetone, the procedure was as above except that a mixture of chloroform:methanol (2:1$^{v}/_{v}$) was used to precipitate the enzyme and for washing the filter paper.

Acid-stable phosphoryl groups incorporated into protein were determined by precipitation of the protein using 10% TCA final concentration. Upon centrifuging, the supernatant was removed and the precipitate suspended in 0.1M NaOH and neutralized with HCl. This procedure was repeated three times and the final suspension is placed onto a filter, dried and counted.

## 6.3 Results

### 6.3.1 Fractionation of isocitrate synthase on Sephadex G-150

Gel chromatography on Sephadex G-150 as shown in Fig.6.1(a) resulted in complete loss of enzymic activity.

Preliminary experiments showed that some recovery of isocitrate synthase activity was possible by combining certain fractions. Most enzyme activity was obtained by combining the proteins that were eluted in fractions 94-102, 104-113 and 158-180 and designated A,B and C in order of decreasing molecular weight. It appeared that this procedure resolved the isocitrate synthase system into at least three fractions on the basis of molecular weights. Experiments in which the fractions were incubated for different time intervals at 25°C with different reaction components before determining the level of enzymic activity showed that the presence of $Mg^{2+}$ and ATP together enhanced enzymic activity 5-fold (Table 6.1). Of the other reaction components tested, either alone or in combination, none was effective in restoring the enzymic activity except $Mg^{2+}$, which promoted reconstitution 2.5-fold above the control value. The combinations in which the various fractions were incubated before assaying for enzymic activity are shown in Table 6.2. It is clear that all three fractions were required for maximum reconstitution in the presence of ATP and $Mg^{2+}$. However, an appreciable amount of enzymic activity was regained when fractions A and C were added together.

To investigate whether ATP and $Mg^{2+}$ were, in fact, facilitating the reconstitution of the isocitrate synthase system by promoting the association of the different constituent proteins of the 'native complex', gel filtration was carried out using a Sephadex G-150 column equilibrated with the reconstitution medium i.e. 50mMNEM-Cl, pH7.5

containing 2mMATP and 4mM$Mg^{2+}$. As is shown in Fig.6.1(b) some isocitrate synthase activity was eluted in a single peak from the column. This single peak was still quite broad and spanned a wide molecular weight range. Furthermore, this peak represented only 15% of the activity originally put onto the column. However, these data suggest that ATP and $Mg^{2+}$ may to some extent lead to an associated form of the isocitrate synthase system, which is active.

To ascertain whether this apparent association, measured in terms of specific activity of the enzyme system was dependent on protein concentration, the eluted material was examined for enzymic activity over a wide concentration range of protein with and without prior incubation with ATP and $Mg^{2+}$. As shown in Fig.6.2 the specific activity of the enzyme incubated with $MgATP^{2-}$ remained constant over the range 0.5-2.0mg of protein per assay. However, the specific activity of the enzyme incubated in the absence of $MgATP^{2-}$ increased 3-fold over this same concentration range and only approached 35% of the specific activity of the $MgATP^{2-}$-treated enzyme. It thus appears that concentration-dependent association of the isocitrate synthase system may occur and that ATP and $Mg^{2+}$ promotes this association.

### 6.3.2 Fractionation of isocitrate synthase on Sepharose-6B gel filtration

Since the peak of enzyme activity eluted close to the Vo of the Sephadex G-150 column elution profile, for further work the gel matrix chosen for the estimation of

the molecular weight of isocitrate synthase was Sepharose-6B. The results of the fractionation of isocitrate synthase on a Sepharose-6B column were similar to those seen using Sephadex G-150 in that the complex dissociated into at least three components.

In an attempt to prevent this dissociation glycerol and glucose were added to the equilibrating and elution buffer as both of these reagents have been used to stabilise enzyme complexes. In Fig.6.3 is shown the elution profile of protein and enzymic activity using as the eluting buffer 50mMNEM, pH7.5 containing 20% ($^{v}/_{v}$) glycerol and 5mM$MgCl_2$. Substitution of 0.125M glucose for the 20% ($^{v}/_{v}$) glycerol resulted in the shifting of the enzymic activity to a larger molecular weight protein and two peaks of enzymic activity appeared instead of three. (Fig.6.4). It appears from comparison between these two fractionations that glucose is preventing some of the dissociation that had been occurring upon gel fractionation.

### 6.3.3 Molecular weight estimation

Since glucose reduced the extent of dissociation of the complex, the molecular weight estimation of the complex was calculated from the elution volume off a Sepharose-6B column using 50mMNEM, pH7.5 containing 0.125M glucose. Fig.6.5 shows the result of calibrating the Sepharose-6B using five markers of known molecular weight. From the Ve of the isocitrate synthase activity peak shown in Fig.6.4, the molecular weight can be estimated to be of the order of 100,000 daltons for the higher molecular weight species and 80,000 daltons for the

second peak. This is in contrast to the molecular weights calculated when glycerol was used in the buffer instead of glucose. The molecular weights when glycerol was used in the buffer were 100,000; 74,000; and 64,000 daltons. It thus appears that glucose prevents the dissociation of the "complex" to the lower molecular weight components.

### 6.3.4 The glucose effect

#### 6.3.4(a) Specificity

The effect of glucose on isocitrate synthase was not restricted to its effect on the physical integrity of the complex. As is shown in Fig.6.6, glucose has a large stimulatory effect upon the enzymic reaction increasing the activity by 2.5 fold between 0 and 20mM glucose. There was a rapid increase in enzymic activity which then plateaus with no further increase up to 100mM glucose.

A series of hexoses was tested in an attempt to ascertain how specific this effect is. The hexoses used in this study and their effects on the enzymic activity is seen in Table 6.3. Glucose has the greatest stimulatory effect whilst fructose also shows some activation. The two diastereomers of glucose used, D-galactose and D-mannose, had no effect on the reaction and inhibited the reaction by 60-70% respectively. Thus the activation is stereo specific for glucose but activation is also observed to a smaller extent with fructose.

#### 6.3.4(b) Kinetic aspect

The effect of glucose upon the appKm value for the substrates of the reaction was studied by varying each substrate in turn while holding the others at saturating levels in presence and absence of 50mM glucose. It can be concluded from the results shown in Table 6.4 that the presence of glucose did not alter the appKm value for any of the substrates. It follows therefore that glucose must be exerting a V effect without a concomitant K effect.

### 6.3.5 Keto acid specificity of the isocitrate reaction

Specificity studies using a partially purified preparation of isocitrate synthase showed that pyruvate was a more effective substrate than 2-oxoglutarate. A detailed study of this phenomenon was essential to elucidate whether this reaction was due to the contamination of the enzyme preparation by pyruvate carboxylase, malic enzyme or some other enzyme or whether the isocitrate synthase complex has the ability to utilize more than one keto acid substrate.

#### 6.3.5.1 ATP-dependence and product identification

ATP-dependent carboxylation of pyruvate has been shown to yield oxaloacetate (OAA) by the enzyme pyruvate carboxylase (EC6.4.1.1.) isolated from the mitochondria of a number of animal species. The partially purified cell cytosol fraction which contains the isocitrate synthase activity also exhibits ATP-dependent pyruvate carboxylation as shown in Table 6.5. The ATP-dependence suggests that the incorporation of $H^{14}CO_3$ is not due to malic enzyme (EC1.1.1.40). A 5-fold increase in $^{14}CO_2$

fixation results in the presence of NADPH. This large increase may be a consequence of the presence of large amounts of malic dehydrogenase (EC1.1.1.37) in these enzyme preparations (see Table 6.6). When NADPH is omitted from the reaction mixture, the OAA produced spontaneously decarboxylates and only a fraction of the true enzymic activity is observed. However when NADPH is added malic dehydrogenase, which can oxidize NADPH at a rate approximately 30 times slower than the rate of oxidation of NADH, reduces the OAA to the stable malate.

That this was the situation was supported by product identification as shown in Fig.6.7. The products of the above mentioned reactions were chromatographed. When NADPH was omitted from the reaction mixture, the radioactive product co-chromatographed with marker OAA, however, when NADPH was added, two radioactive products were obtained. One of these co-chromatographed with OAA while the other co-chromatographed with the malate marker.

6.3.5.2. Effect of glucose

The appKm value for pyruvate was attained in a manner similar to that used for obtaining appKm constants for 2-oxoglutarate. The concentration of pyruvate was varied whilst the concentration of all other substrates were held at near-saturating levels. Similarly the appKm value for all other substrates was measured using pyruvate as the keto acid substrate held constant at near-saturating concentration. All reactions were performed in the presence and absence of 50mM glucose. The results summarised in Table 6.7(a) show that the appKm value of

pyruvate was the same as the appKm value for 2-oxoglutarate and that pyruvate did not alter the appKm values of the other substrates. The glucose effect was also identical for the two keto acids, that is, glucose did not alter significantly the appKm values of the substrates but increased the Vmax of the reaction (Table 6.7(b)).

6.3.5.3 Gel filtration of the pyruvate activity using Sepharose 6B chromatography

The enzymic activity eluted from the Sepharose 6B column equilibrated in 50mMNEM, pH7.5, containing 5mM$MgCl_2$ and 20% ($^{v}/_{v}$) glycerol was determined in parallel assays using 2-oxoglutarate and pyruvate as the keto acid substrate (cf.,Fig.6.3 with Fig.6.8). Similarly when the Sepharose 6B column was equilibrated, and the enzyme eluted with, 50mMNEM, pH7.5 containing 5mM$MgCl_2$ and 0.125M glucose parallel assays with 2-oxoglutarate gave results similar to those presented in Fig.6.4, whilst the results when pyruvate was the substrate are shown in Fig.6.9. Qualitatively, the elution profile shown in Fig.6.8 is identical to that shown in Fig.6.3. There appears to be a dissociation of the complex resulting in a low recovery and smearing of the activity over a large portion of the elution profile. When glucose was added to the buffer the elution profile was again qualitatively the same irrespective of whether 2-oxoglutarate or pyruvate was the substrate (cf., Fig.6.9 with Fig.6.4). Thus both the pyruvate- and the 2-oxoglutarate-dependent

carboxylation reactions co-chromatographed under these conditions.

6.3.5.4 The effect of avidin upon the pyruvate carboxylation by isocitrate synthase

Since pyruvate carboxylase, a mitochondrial enzyme, catalyses an ATP-dependent carboxylation of pyruvate, it was essential to establish that the activity observed in the cytosol enzyme fraction under study was not contaminated by this enzyme. Pyruvate carboxylase a biotin-containing enzyme has been shown to be sensitive to avidin inhibition (Keech and Utter, 1963).

Table 6.8 shows that for both pyruvate and 2-oxoglutarate there was no avidin inhibition of the reaction even though the avidin concentration used was many times greater than the amount required to inhibit pyruvate carboxylase. This result was contrary, to our previous report using 2-oxoglutarate as substrate (Mattoo et al., 1976). Our result which showed some avidin inhibition could not be repeated despite many attempts using enzyme which had higher specific activity and assayed under the new assay system derived after the initial velocity studies. The low activity of the enzyme at this earlier stage of the project and the sub-optimal condition of the assay system at that time could explain this fortuitous result. The lack of avidin inhibition of the isocitrate synthase complex when either keto acid substrate was utilized provided strong evidence that the pyruvate carboxylation in the cytosol fraction was not

due to pyruvate carboxylase activity. Furthermore, an aliquot of the cytosolic fraction assayed for pyruvate carboxylase activity using the standard assay for this enzyme gave no $H^{14}CO_3$-fixation, again confirming the absence of this enzyme in the cytosolic fraction.

### 6.3.6 The interaction of ATP with isocitrate synthase

Although the fixation of $HCO_3$ into 2-oxoglutarate was shown to be ATP-dependent, experiments designed to demonstrate the stoichiometry of the reaction have failed to show the release of ADP and/or $P_i$ in a 1:1 relationship with the amount of $HCO_3$ fixed (Table 6.9). Thus, the role of ATP in the reaction was investigated further.

#### 6.3.6.1 Enzyme bound ATP

The omission of 2-oxoglutarate in the assay solution results in $H^{14}CO_3$ fixation to a level identical to that when enzyme is omitted. However, it has been repeatedly observed that some $H^{14}CO_3$ is fixed above this level in the absence of added ATP. There are at least three possible explanations for this result. (a) ATP is in the preparation, (b) the enzyme can catalyse, slowly, an ATP-independent reaction, (c) the activated form of the enzyme is phosphorylated and the apparent ATP-dependence is simply to provide ATP to phosphorylate the rest of the enzyme.

In order to ascertain which of these possibilities applies, the enzyme was assayed using the complete assay mixture, in the absence of 2-oxoglutarate and in the absence of ATP over

several time intervals up to 5 min. The rationale being that if the enzyme preparation contained ATP, this will result in $H^{14}CO_3$-fixation in the absence of added ATP which will attain a level dictated by the level of ATP and then remain constant. If the enzyme catalyses an ATP-independent reaction then the level of $H^{14}CO_3$-fixation should increase with time over the 5 min reaction. Similarly if ATP is required to phosphorylate the rest of the enzyme then there should be a linear increase with time of $H^{14}CO_3$-fixation by enzyme which is already phosphorylated.

Results depicted in Fig.6.10 show that in the complete assay mixture there was linear $H^{14}CO_3$-fixation over the 5 min interval, similarly in the absence of 2-oxoglutarate. However in the absence of ATP the assay was not linear and the amount of counts fixed remained constant with time after the first 1-2 min. This indicated that there was ATP present in the enzyme preparation for $H^{14}CO_3$-fixation over the 1-2 min period and the $H^{14}CO_3$-fixation was not due to a slow ATP-dependent reaction. However, the ATP requirement could still be necessary to phosphorylate the enzyme. If this was the case, then the above result could be explained by the dephosphorylation of enzyme during the first 1-2 min resulting in loss of $H^{14}CO_3$-fixation.

Further evidence shown in Fig.6.11 supports the above conclusions. When the enzyme was incubated in the assay mixture in the absence of ATP and initiated after five minutes with $H^{14}CO_3$ and the reaction followed for a further ten minutes the $H^{14}CO_3$ fixed in the absence of

ATP and in the absence of 2-oxoglutarate were identical. Thus the endogenous ATP was utilized so that upon initiation with $H^{14}CO_3$, there was no further $HCO_3$-fixation without additional ATP. Alternatively, any phosphorylated enzyme became dephosphorylated and additional ATP was required to phosphorylate the enzyme and result in $H^{14}CO_3$-fixation. That the reaction was linear for 10 min even after 5 min preincubation shows that the substrate levels were high enough for a 15 min reaction time. Therefore, a 5 min preincubation and 5 min reaction after initiation with $H^{14}CO_3$ was a suitable assay procedure for this enzyme system and both controls are valid for isocitrate synthase.

The amount of ATP in the enzyme fraction was estimated using the spectrophotometric assay of hexokinase coupled to glucose-6-phosphate dehydrogenase. It can be seen in Table 6.10 that the enzyme preparation contained 39nmoles of ATP per 0.05ml and this was equal to the estimate of the level of ATP as calculated from the $H^{14}CO_3$ fixed into product in the absence of ATP as shown in Fig.6.10. Thus the high level of $H^{14}CO_3$-fixation in the absence of ATP compared to that in the absence of 2-oxoglutarate can be attributed to the presence of ATP in the enzyme preparation.

### 6.3.6.2 Requirement of ATP hydrolysis for enzyme activity

To determine whether ATP was acting as an allosteric activator or was hydrolysed as part of the reaction mechanism could be tested by using two ATP non-hydrolysable analogues AMP-PNP and AMP-PCP the structures of which are shown in Fig.6.12(a).' Although AMP-PCP was not

contaminated with ATP, as shown by cellulose thin layer chromatography against authentic ATP, the AMP-PNP had traces of ATP as shown by this method. These findings were confirmed using the hexokinase/glucose-6-phosphate dehydrogenase assay, although this latter method may not be valid as these ATP analogues may inhibit the hexokinase reaction. Taking this into account, it is shown in Table 6.11 that both AMP-PNP and AMP-PCP result in complete loss of enzymic activity. It can be concluded that either there is dependence on ATP hydrolysis for enzymic activity or that if the ATP is solely an allosteric effector it might be structually very specific for the phosphate part of the molecule and any change would result in loss of activity.

6.3.6.3 Specificity of the nucleotide triphosphate

To test the specificity of the ATP binding site of this enzyme the series of nucleotide triphosphates shown in Fig.6.12(b) were tested at the same concentration as ATP. Results shown in Table 6.12 clearly show that the only nucleotide triphosphates utilized by isocitrate synthase were ATP and CTP. The most effective of the polyphosphates tested was CTP which increased enzymic activity 2 to 3-fold over the activity obtained using ATP. On comparing the structures of the nucleotide triphosphates it is tempting to suggest that the 6-amino group on the purine/pyrimidine ring may be important in the binding of the triphosphate to the nucleotide site.

No matter which of the keto acid substrates,

2-oxoglutarate or pyruvate, were used the appKm value for CTP was not significantly different (Table 6.13a). However although the appKm value for CTP (2.5mM) was approximately 4 times greater than the appKm value for ATP(0.54mM), the Vmax obtained using CTP is greater. Similarly even though the appKm value for CTP is identical with either 2-oxoglutarate or pyruvate as substrate, the Vmax when pyruvate was used was approximately twice the Vmax when 2-oxoglutarate was the substrate. This situation also arose when ATP was the nucleotide triphosphate. Table 6.13b shows the appKm value of both pyruvate and 2-oxoglutarate are approximately the same value whether CTP or ATP are used as the substrate. There is however a five-fold increase in the Vmax value when pyruvate is the keto acid substrate and when CTP is the nucleotide triphosphate utilized. Thus it appears that isocitrate synthase lacks specificity for both the keto acid substrate and for the nucleotide triphosphate requirement with the greatest level of $H^{14}CO_3$ fixation occurring when pyruvate and CTP are substrates. However ATP binds to the enzyme better than CTP (as measured by their appKm value) and this will influence the utilization of these substrates by the enzyme.

### 6.3.6.4 Labelling of the isocitrate synthase fraction with [$\gamma$-$^{32}$P]ATP

The ATP-dependence of the isocitrate synthase reaction and the lack of correlation between the amount of $H^{14}CO_3$ fixed and the amount of ATP hydrolysed led to the hypothesis that ATP was required to phosphorylate

the enzyme to convert it to the active form. To test this theory, the enzyme was incubated with [$\gamma$-$^{32}$P] ATP in the presence of $MgCl_2$ for 2 hr and then chromatographed on a Sepharose-6B column in 50mMNEM, pH7.5, containing 5mM$Mg^{2+}$ and 0.25M glucose. The elution profile (Fig.6.13) shows that $^{32}$P counts were associated with the enzymic activity and these counts were distinct from any unbound [$\gamma$-$^{32}$P]ATP which eluted at the Ve of the column. Furthermore, the increased level of ATP-independent activity indicated that there was less requirement of ATP for enzymic activity after the incubation with ATP prior to column elution although further addition of cold ATP to the assay mixture is necessary to attain maximum activity.

6.3.6.5 Attempts to remove $^{32}$P-label from protein

If the $^{32}P_i$ can be removed from the protein it should be reflected in the assay of isocitrate synthase as an increase in the ATP-dependence of the enzymic activity. Attempts were made to remove the $^{32}P_i$ from the enzyme using both acid and alkaline phosphatase but these attempts were unsuccessful as there was no decrease in the ATP-independent activity. This result could reflect the need of a specific phosphatase for the isocitrate synthase complex.

Further attempts were made to remove the $^{32}P_i$, this time by chemical procedures. The $^{32}P_i$-enzyme was precipitated several times using 10% TCA resulting in loss of all radioactivity from the protein. This is shown in Table 6.14(a). In contrast to this, an enzyme aliquot treated with 7M urea containing 10mMNaOH and

cold acetone, retained 95% of the count upon filtration and extensive washing as is shown in Table 6.14(b).

To ensure that the $^{32}P$ is not present as phospholipid, a chloroform:methanol (2:1 $^{v}/_{v}$) extraction of the enzyme was carried out. Table 6.14(c) shows that about 90% of the radioactivity remained bound to the enzyme after this treatment.

## 6.4 Discussion

### 6.4.1 Stability of the isocitrate synthase complex

Fractionation of the partially purified isocitrate synthase enzyme fraction revealed that the overall enzymic activity can be separated into three inactive components which upon recombination regain enzymic activity. To regain maximal enzymic activity of the recombined fractions, it was necessary to incubate the fractions in the presence of $MgATP^{2-}$ prior to assaying. It thus appears that $MgATP^{2-}$ is a factor in maintaining the complex as a biologically active unit *in vitro*. This view was confirmed by the retention of some overall activity in a single peak upon elution of the enzyme from a gel filtration column using $MgATP^{2-}$ in the equilibrating and eluting buffer.

The molecular weight of the complex responsible for overall activity could not be estimated until a way of stabilising the complex during gel filtration chromatography was discovered. To achieve this several additives known to stabilise other enzyme and enzyme complexes were investigated and although 20% glycerol afforded the same degree of protection as $MgATP^{2-}$, glucose was by far the

most effective. The molecular weight of $9x10^4$-$1x10^5$ daltons is larger than the $6.1x10^4$ daltons of hog heart isocitrate dehydrogenase (NADP) (Siebert et al., 1957a) Further purification is needed before much can be said about the molecular weight of the different components and before a more accurate molecular weight can be established.

### 6.4.2 Glucose activation

The effect of glucose upon the reaction of the partially purified isocitrate synthase is in accord with the effect of added glucose to the 2-oxoglutarate reductive carboxylation pathway (Madsen et al., 1964b). An increase in the extent of labelling fatty acids from [2-$^{14}$C] and [5-$^{14}$C] glutamate upon addition of glucose to tissue from animals fed on a stock diet when the total amount of glutamate metabolized was of the same order of magnitude was observed by Madsen et al., (1964a) but so far no satisfactory explanation has been proposed to account for this behaviour.

Results presented here suggest that the glucose is activating the isocitrate synthase complex and the resulting increase in isocitrate produced leads to an increase in citrate which is subsequently cleaved to yield OAA and acetyl CoA. Thus, the end result of an activated isocitrate synthase is the increase in the utilization of the 2-oxoglutarate reductive carboxylation pathway for the synthesis of fatty acid without having an effect on the amount of glutamate metabolished by the cell.

6.4.3 Pyruvate as the keto acid substrate

That pyruvate is indeed an alternate keto acid substrate of isocitrate synthase was established by the ATP-dependence of the reaction and the lack of avidin inhibition of this reaction. Furthermore, the characteristics of the pyruvate carboxylation such as the activation by glucose and the ability of CTP to replace ATP as the nucleotide triphosphate are similar to those of the 2-oxoglutarate carboxylation. This is in contrast to isocitrate dehydrogenase (NADP) which cannot utilize pyruvate as a substrate (Rose, 1960) is not stimulated by glucose, ATP or CTP.

Although the affinity of the enzyme for pyruvate (on the basis of the appKm values) is the same as for 2-oxoglutarate the Vmax of the reaction is higher when pyruvate is the substrate. This difference in magnitude of the reaction velocity may be due to the difference in stability of the products OAA and OAS. When pyruvate was the keto acid substrate and the reaction was assayed in the presence and absence of NADPH there was more acid stable radioactivity fixed into product when NADPH was present. This indicated that OAA was not very stable at 30°C and when NADPH was present, the malate dehydrogenase that contaminates the enzyme fraction converted the OAA into the more stable malate. These two products were confirmed by chromatography.

The difference in magnitude in $H^{14}CO_3$-fixation between pyruvate and 2-oxoglutarate as keto acid substrates may be attributable to the greater liability of OAS compared to OAA at 30°C. Furthermore, the level of isocitrate

dehydrogenase which reduces the OAS to isocitrate is very small in comparison to the level of malate dehydrogenase (see Table 4.12). These two properties in combination can produce far less radioactivity in acid-stable compounds than obtained when pyruvate was the substrate.

The role of the 2-oxoglutarate reductive carboxylation pathway in gluconeogenesis in providing oxaloacetate obtained from citrate cleavage has been examined by D'Adamo and Haft (1965). These authors show that the operation of this pathway is important in supplying the $C_4$ dicarboxylic acids required for gluconeogenesis. Since isocitrate synthase produces OAA directly from pyruvate and also produces isocitrate which will be converted to citrate and cleaved to yield OAA the $C_4$ dicarboxylic acids required are supplied by the 2-oxoglutarate reductive carboxylase pathway via two mechanisms. Furthermore glucose formation can occur prior to the formation of OAA from citrate cleavage (OAA being supplied by pyruvate carboxylation) and this can supply the glucose which has been shown to increase the contribution to fatty acid synthesis via the 2-oxoglutarate reductive carboxylation pathway.

### 6.4.4 The ATP interaction

As has been already indicated by kinetic studies the interaction of ATP with isocitrate synthase is complex. In the enzyme preparation there appears to be ATP which may be removed by incubation of the enzyme with the assay mixture. This ATP also becomes available to hexokinase when this enzyme was used to estimate the ATP levels. The

purification procedure (see Table 4.10) by which this enzyme has been partially purified apparently has not removed all of the ATP associated with the enzyme. The partial prevention of the dissociation of the complex by $MgATP^{2-}$ can explain the need for ATP associated with isocitrate synthase.

In contrast to the above, there is also a requirement for ATP hydrolysis for enzymic activity which can also be fulfilled by CTP but no other nucleotide triphosphate. Replacement of ATP with either AMP-PNP or AMP-PCP resulted in zero activity indicating that there is a need for the hydrolysis of the γ-phosphate of ATP (or CTP) but is not available from these analogues. The requirement for ATP hydrolysis may be explained by the labelling of isocitrate synthase with [$\gamma$-$^{32}$P]ATP.

Labelling of the complex with [$\gamma$-$^{32}$P]ATP resulted in a higher level of ATP-independent activity. That the label is indeed covalently bound and not behaving in an associating-disassociating manner was established by acid and alkaline dispersion and precipitation of the enzyme onto GF/A filters. When the labelled enzyme was acid precipitated the label was removed from the protein however when alkali was used to denature the enzyme, the label was still attached to the protein. This situation was also the case when chloroform and methanol were used to disperse the enzyme thus indicating that the $^{32}P_i$ was not labelling a phospholipid bound to the enzyme as the chloroform, methanol extraction would have removed such a label from the phospholipid.

The complete ATP-dependence of the reaction whilst at the same time the greater $H^{14}CO_3$-fixation compared to $[^{32}P_i]$ release from $[\gamma\text{-}^{32}P_i]$ATP suggests that at least part of the ATP requirement was for the phosphorylation of the enzyme to exhibit full activity. This conclusion would mean that ATP is not a substrate of the reaction but an essential enzyme modifier.

Reports in the literature (Taborsky, 1974; Smith et al., 1976) of phosphoenzymes which are acid-labile and alkali-stable have ruled out phosphorylation of a serine side chain and isolation of the phosphorylated amino acid revealed a phosphohistidine or a phospho-arginine. The results presented in this thesis would also indicate that serine was not phosphorylated and histidine or arginine are candidates for the phosphorylation site. Positive identification of the phosphorylation site can only be made by protein digestion and isolation of the phosphorylated amino acid. This still remains to be done.

Attempts to remove the bound $[^{32}P_i]$ so as to increase the extent of ATP-dependence of the enzymic activity, failed when either acid phosphatase or alkaline phosphatase was used. This could indicate that the phosphatase required to remove the bound phosphate is very substrate specific or that the conditions used were not optimal for this particular enzyme system. Further work is necessary to clarify the situation.

That CTP can replace ATP as the nucleotide phosphate points to the specificity of the binding to the enzyme as

both of these compounds have a 6-amino group on the purine/pyrimidine ring which the other nucleotide triphosphates lacked. This group could be instrumental in the binding of the ATP/CTP to the enzyme for correct positioning of the $\gamma$-$P_i$ to be hydrolyzed.

CTP has a higher appKm, that is, has less affinity for the enzyme, than does ATP but produces a higher Vmax for the enzymic reaction. This effect was seen when both pyruvate and 2-oxoglutarate were used as keto acid substrates with a greater increase in Vmax when pyruvate was used. The appKm for pyruvate and 2-oxoglutarate are identical whether ATP or CTP are used and thus the increase in reaction velocity was not due to an increase in affinity for the keto acid substrate. This effect was a very recent finding and further work will be required prior to making any conclusion concerning the interaction of CTP with the enzyme.

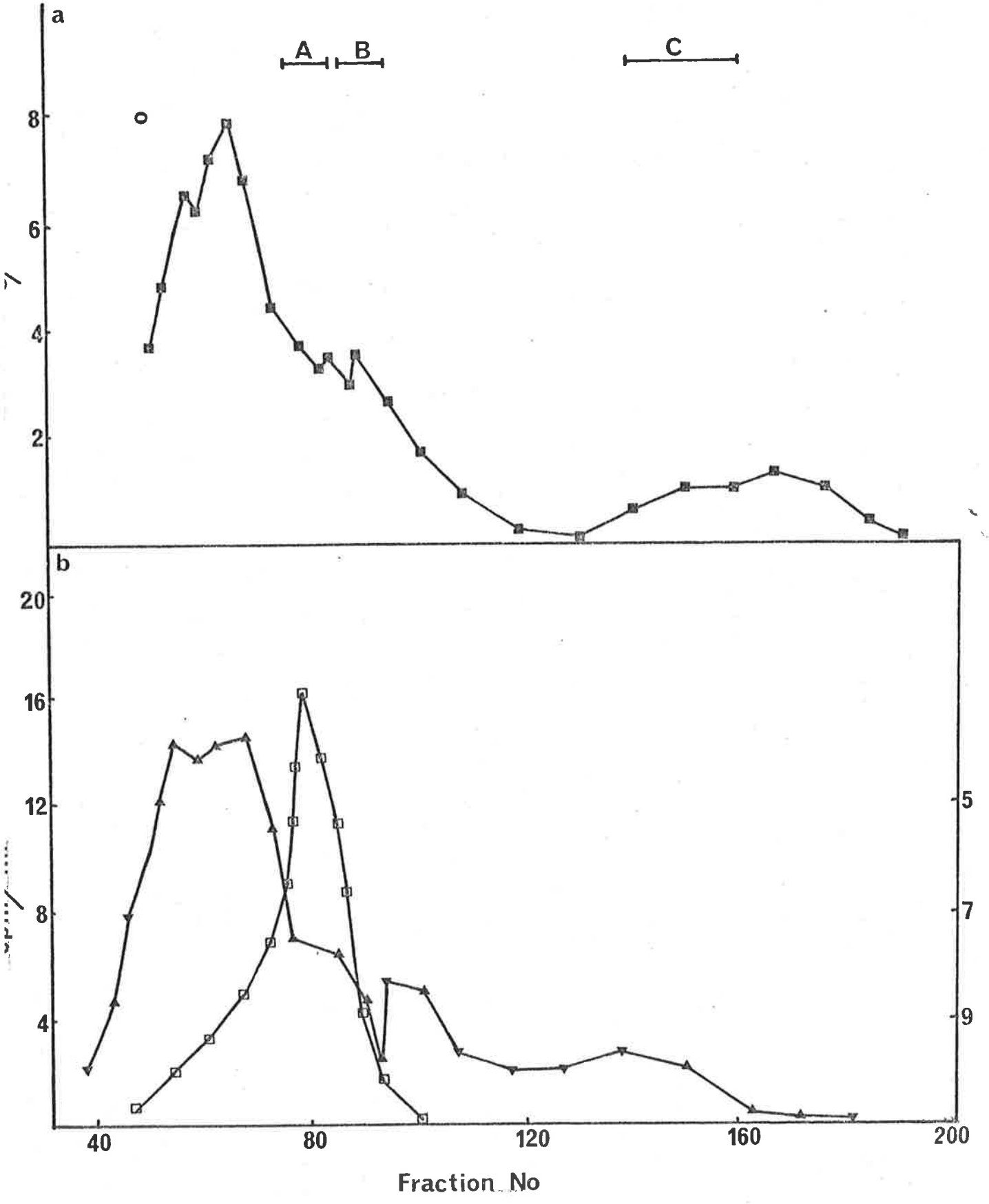

a
A
B
C
8
6
4
2
b
20
16
12
8
4
5
7
9
40
80
120
160
200
Fraction No

Fig.6.1 Sephadex G-150 chromatography

Chromatography of the partially purified isocitrate synthase up to Stage 4 of purification (see Table 4.10).

(a) The column was equilibrated and eluted with 0.02MNEM, pH7.5 containing 1mMEDTA and for

(b) the column was equilibrated and eluted with 0.05MNEM, pH7.5 containing 2mMATP, 4mMMgCl$_2$ and 1mMEDTA.

The enzyme was dissolved in the respective buffer (3.5ml, 180mg/ml).

Column size : 60cmx2.5cm

Buffer : as above

Flow rate : 13ml/hr

Fraction size : 2.5ml

—■— mg/ml

—□— $^{14}CO_2$-fixation

—▲— relative fluorescence

TABLE 6.1 Effect of assay components on the reconstitution of the isocitrate synthase system from fractions A, B and C

Fractions (50µl) of A,B and C were mixed together before the addition of the indicated components followed by an incubation period of 1 h at 25°C and then assayed.

| Components tested | Isocitrate synthase activity (nmol of $CO_2$ fixed/min per mg) |
|---|---|
| Buffer alone | 0.99 |
| Oxoglutarate (3.5mM) | 1.06 |
| ATP (2mM) | 0.53 |
| $Mg^{2+}$ (4mM) | 2.65 |
| $NADP^+$ (H) (0.1mM) | 0.79 |
| $NaH^{14}CO_3$ (10mM) | 0.79 |
| ATP (2mM) + $Mg^{2+}$ (4mM) | 4.77 |
| ATP (2mM) + $Mg^{2+}$ (4mM) + oxoglutarate (3.5mM) | 4.28 |
| ATP (2mM) + $Mg^{2+}$ (4mM) + $NADP^+$ (H) (0.1mM) | 4.67 |
| ATP (2mM) + $Mg^{2+}$ (4mM) + $NaH^4CO_3$ (10mM) | 3.31 |
| ATP (2mM) + $Mg^{2+}$ (4mM) + oxoglutarate (3.5mM) + $NADP^+$ (H) (0.1mM) | 5.02 |
| ATP (2mM) + $Mg^{2+}$ (4mM) + $NADP^+$H (0.1mM) + $NaH^{14}CO_3$ (10mM) | 3.79 |

TABLE 6.2 Reconstitution of the isocitrate synthase system on recombining fractions A,B and C in the presence of ATP and $MgCl_2$

Each fraction as indicated was incubated for 75 min at 25°C with 0.05M-N-ethyl-morpholine,pH7.5,2mM-ATP and 4mM-$MgCl_2$ in a total volume of 0.2ml. The enzyme reaction was initiated by the addition of the rest of the assay components in a final volume of 0.25ml.

| Fraction | Isocitrate synthase activity (nmol/min per mg) |
|---|---|
| A | 0.01 |
| B | 0.02 |
| C | 0.44 |
| A+B | 0.19 |
| A+C | 5.04 |
| B+C | 0.00 |
| A+B+C | 6.47 |

Fig.6.2 Protein concentration dependent association of isocitrate synthase

The activity peak eluted off G150 (Fig.6.1b) was dialysed to remove MgATP, concentrated and tested at the protein concentrations shown with (—■—) and without (—●—) prior incubation with 2mMATP and 4mM$Mg^{2+}$. The assay procedure is as described in Section 2.2.5.1a. Similar results were obtained if the enzyme used was partially purified to Stage 4 of purification (Table 4.10)

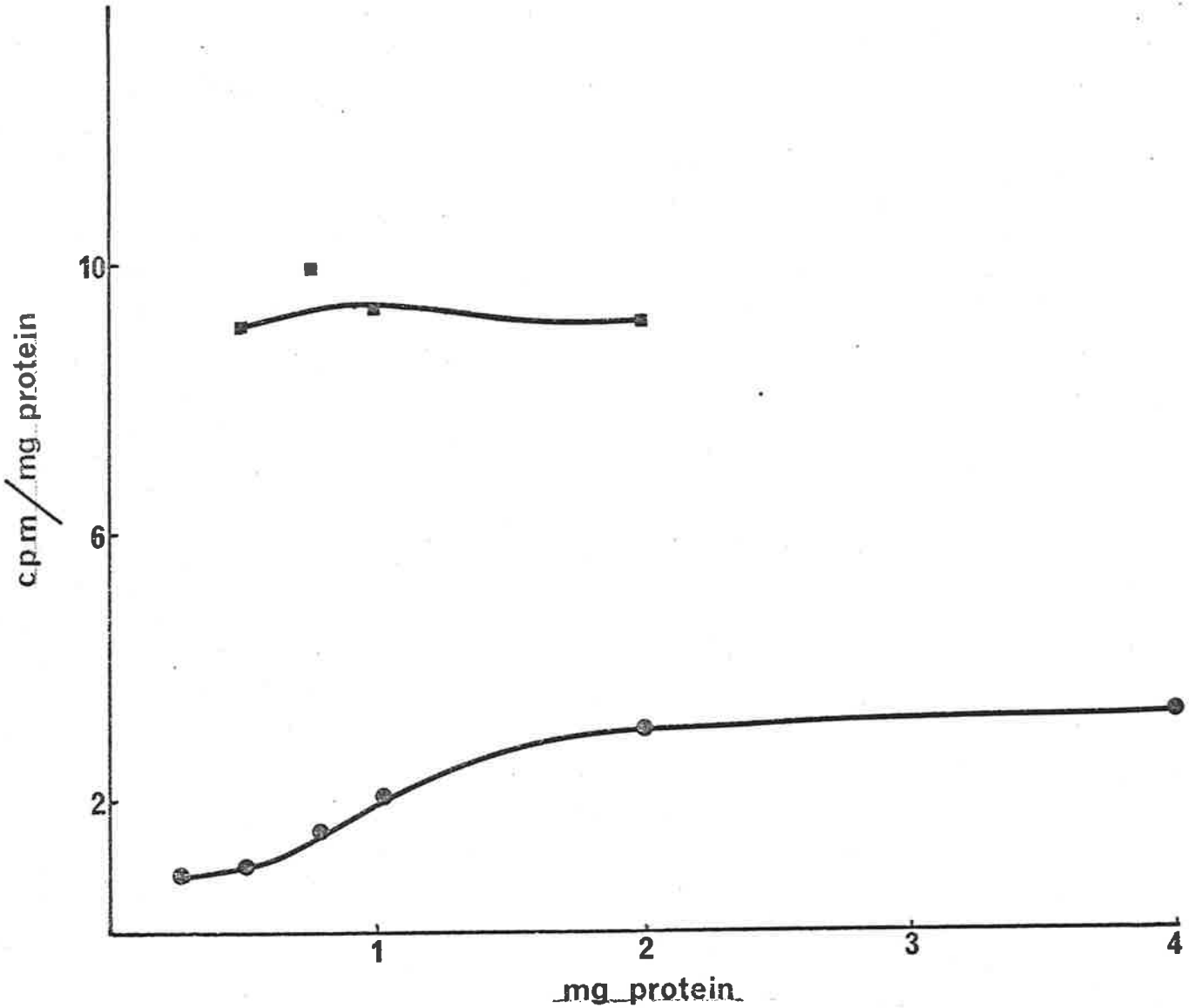

10
6
2
c.p.m / mg protein
1
2
3
4
mg protein

Fig.6.3 Sepharose 6B chromatography in the presence of glycerol

Enzyme partially purified to Stage 5 (see Table 4.10) was extracted into the equilibration and elution buffer shown below and 3ml(225mg/ml) was loaded onto the column. The enzymic activity was assayed as described in Section 2.2.5.1b.

| | | |
|---|---|---|
| Column size | : | 70cmx2.5cm |
| Buffer | : | 50mMNEM, pH7.5 containing 20% ($^{v}/_{v}$) glycerol |
| Flow rate | : | 18ml/hr |
| Fraction size | : | 3mls |

—■— $A_{280}$

—▲— $^{14}CO_2$-fixation

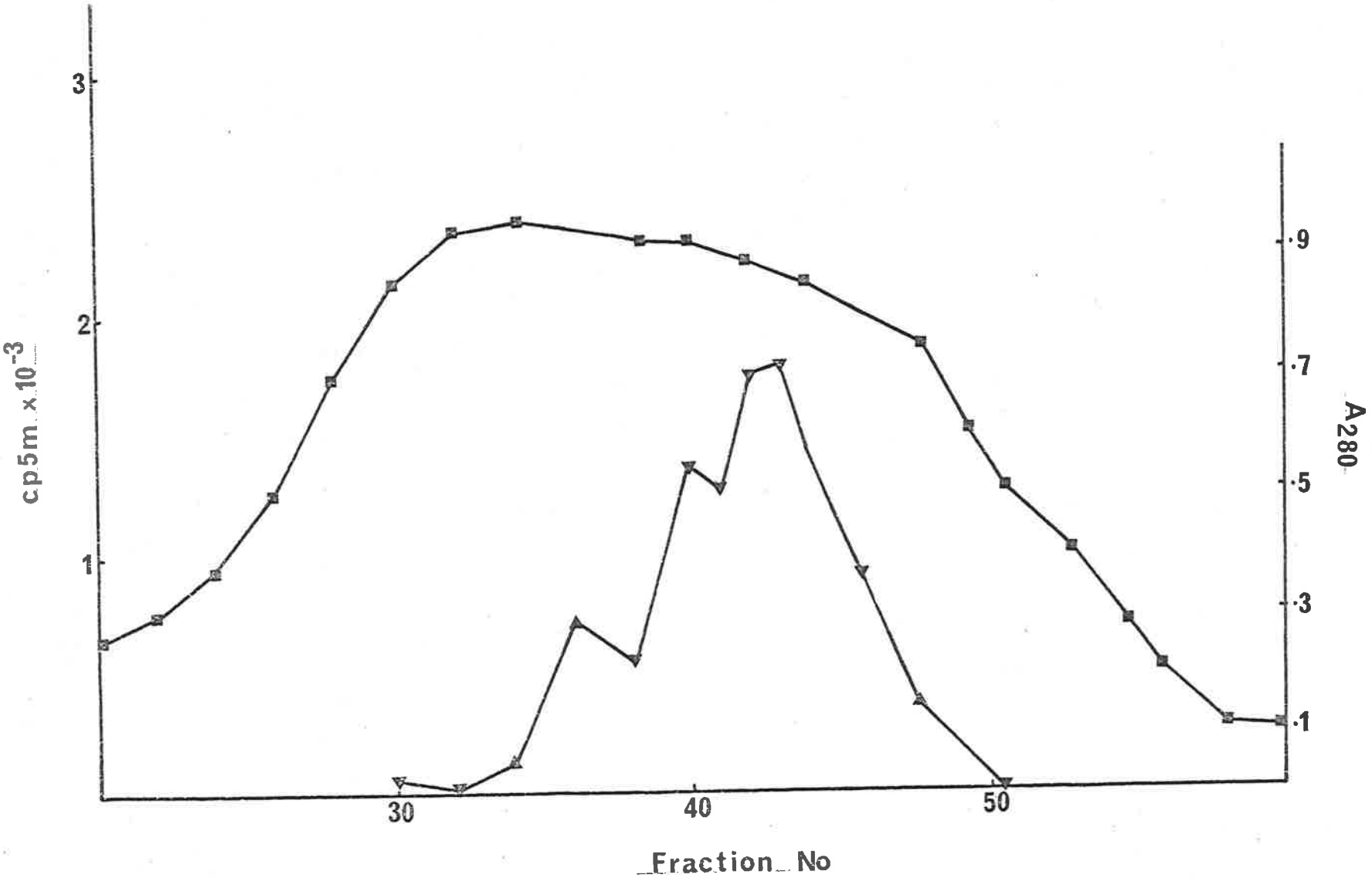

3
2
1
cp5m x10⁻³
·9
·7
·5
·3
·1
A280
30
40
50
Fraction No

Fig.6.4 Sepharose 6B chromatography in the presence of glucose

This column is identical to that of Fig.6.3 except that the equilibration and eluting buffer was 50mMNEM, pH7.5 containing 0.125M glucose and 5mMMgCl$_2$. The enzyme loaded on the column was dissolved in 3ml of the above buffer and contained 180mg/ml protein. Enzymic activity was assayed as described in Section 2.2.5.1b.

—■— $A_{280}$

—▲— $^{14}CO_2$-fixation

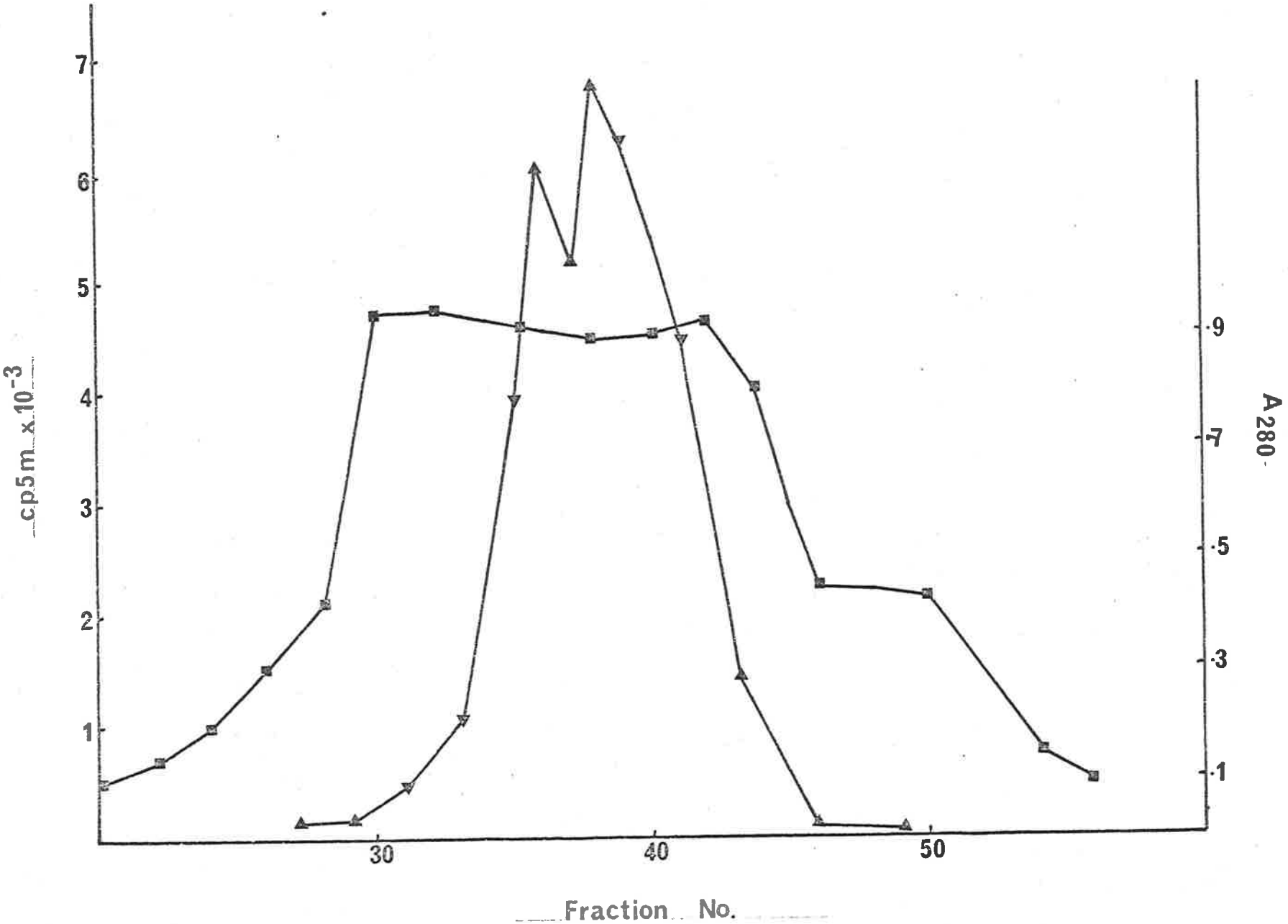

7
6
5
4
3
2
1
cpsm x10⁻³
·9
·7
·5
·3
·1
A280
30
40
50
Fraction No.

Fig.6.5 Molecular weight estimation of isocitrate synthase on Sepharose 6B gel

Column size : 70cmx2.5cm

Buffer : 50mMNEM, pH7.5 containing 0.125M glucose

Flow rate : 10ml/hr

The proteins used were:

(a) Ovalbumin (43,000 daltons)

(b) Malate dehydrogenase (70,000 daltons)

(e) Hexokinase (102,000 daltons)

(f) Lactate dehydrogenase (140,000 daltons)

(g) Pyruvate carboxylase (480,000 daltons)

The two peaks of activity shown in Fig.6.4 were

(c) lower molecular weight peak (80,000 daltons)

(d) higher molecular weight peak (100,000 daltons)

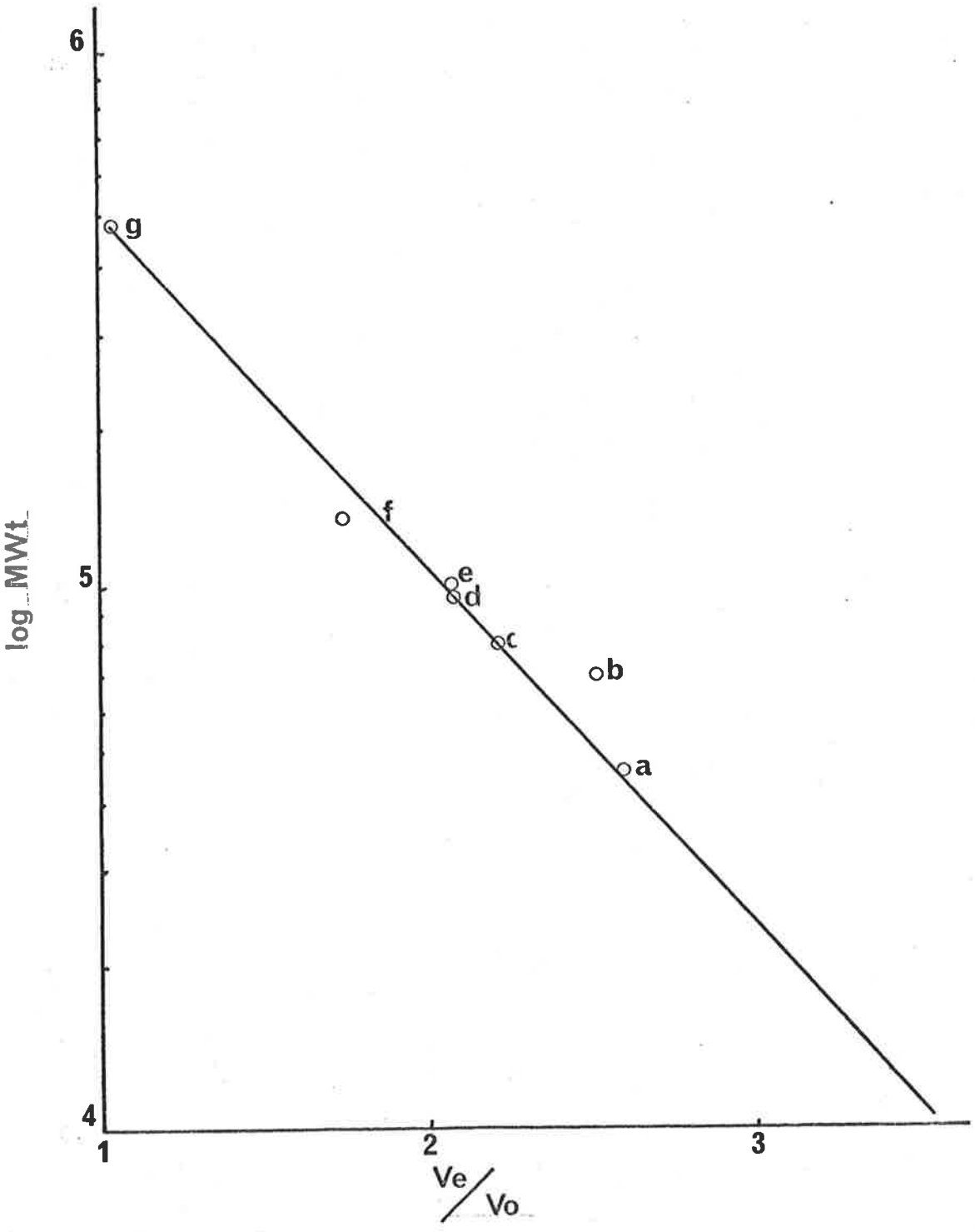

6
5
4
log MWt
g
f
e
d
c
b
a
1
2
3
Ve/Vo

Fig.6.6 The effect of glucose on the level of $^{14}CO_2$ fixation

The enzyme purified to Stage 5 (see Table 4.10) was assayed as described in Section 2.2.5.1b with the addition of increasing concentrations of glucose (0mM-100mM final concentration in the assay mixture), using 0.02ml enzyme (90mg/ml).

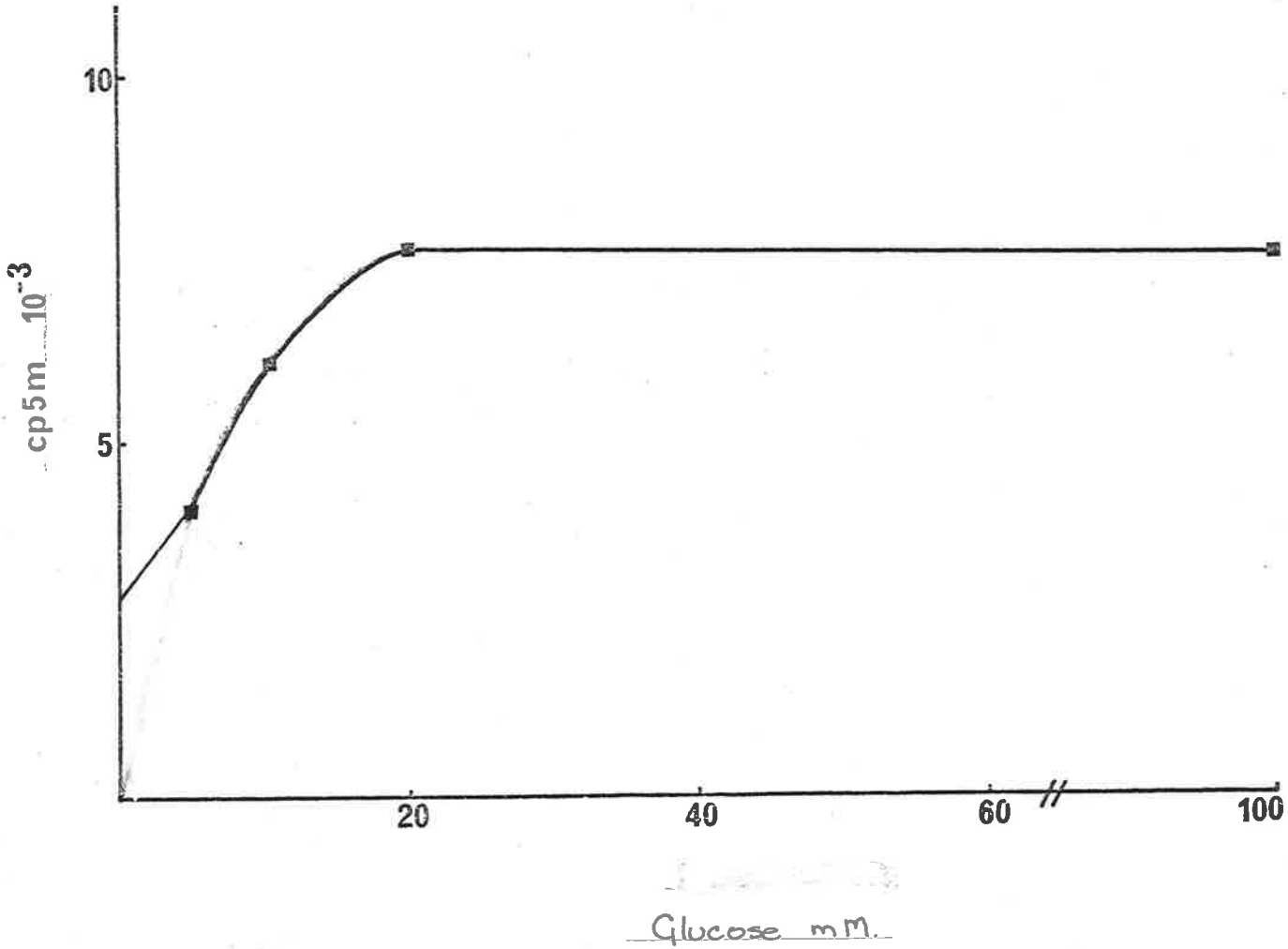

10
5
cp5m 10⁻³
20
40
60
100
Glucose mM.

TABLE 6.3 Effect of hexoses on Isocitrate Synthase

| | % ATP-dependent Activity |
|---|---|
| Complete assay mixture | 100 |
| + Glucose | 194 |
| + Galoctose | 116 |
| + Fructose | 148 |
| + Mannose | 37 |

The complete assay mixture was as described in Section 2.2.5.1b. Final concentration of all the hexoses was 50mM.

TABLE 6.4 Glucose effect upon appKm for the substrates of isocitrate synthase

| Substrate | appKm | |
|---|---|---|
| | - Glucose | + 50mM Glucose |
| $HCO_3^-$ | 9.01 ± 0.96 | 12.5 ± 2.0 |
| 2-oxoglutarate | 0.538 ± 0.078 | 0.509 ± 0.027 |
| $MgATP^{2-}$ | 0.54 ± 0.08 | 0.40 ± 0.05 |
| NADPH | 0.0155± 0.005 | 0.0146 ± 0.003 |

The assays were carried out as described in Section 2.2.5.1b and the data analysed by the computer programme HYPER.

TABLE 6.5 ATP-dependent pyruvate carboxylation of isocitrate synthase protein fraction

| | -ATP | +ATP | ATP-dependent cpm |
|---|---|---|---|
| pyruvate + complete mixture | 289 | 5005 | 4716 |
| pyruvate + complete mixture - NADPH | 117 | 1042 | 925 |

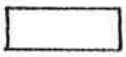
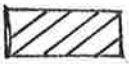
The complete assay mixture is as described in Section 2.2.5.1b except 2-oxoglutarate was omitted. The final concentration of pyruvate was 4mM and the enzyme used was partially purified to Stage 5 (see Table 4.10).

TABLE 6.6 Levels of contamination by several dehydrogenases

| Enzyme | μ/ml |
|---|---|
| lactate dehydrogenase | 394 |
| malate dehydrogenase | 95 |
| malic enzyme | 0.41 |
| isocitrate dehydrogenase | 3.11 |

The dehydrogenases were assayed spectrophotometrically as described in Section 2.2.5.3., 2.2.5.4, 2.2.5.5. and 2.2.5.2 respectively. The enzyme preparation tested was partially purified to Stage 5 (see Table 4.10).

Fig. 6.7 Product identification when pyruvate was the keto acid substrate

Enzyme partially purified up to Stage 5 (see Table 4.10) was assayed as described in Section 2.2.5.1b with pyruvate instead of 2-oxoglutarate as the keto acid in the presence  and absence  of NADPH. The reaction was terminated after 10 min and 0.025ml spotted onto a cellulose thin layer with authentic DNP-OAA and malate. Ascending chromatography using diethyl ether:formic acid: $H_2O$ 7:2:1 was used.

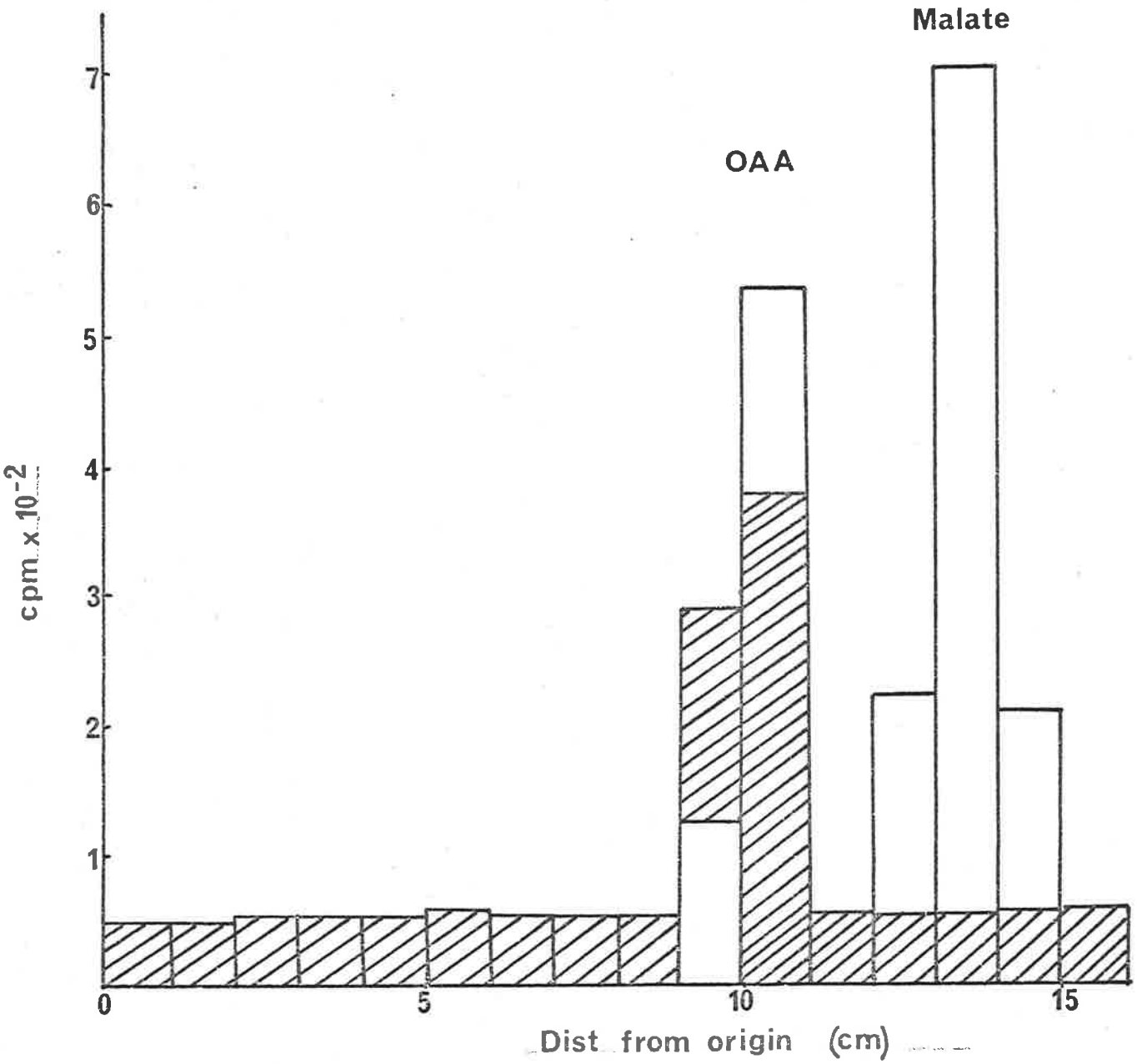

Malate
OAA
7
6
5
4
3
2
1
cpm x 10⁻²
0
5
10
15
Dist from origin (cm)

TABLE 6.7 Kinetic Constants when pyruvate is the keto-acid substrate

| (a) Substrate varied | appKm | appKm (+50mMGlu) |
|---|---|---|
| pyruvate | 0.532 ± 0.133 | 0.488 ± 0.118 |
| ATP | 1.03 ± 0.130 | 0.862 ± 0.175 |
| $HCO_3^-$ | 8.57 ± 2.82 | 10.23 ± 2.29 |
| NADPH | 0.159 ± 0.0386 | 0.050 ± 0.0179 |
| (b) pyruvate | Vmax cpm | |
| | - Glucose | + Glucose |
| | 9591 ± 493 | 11192 ± 535 |

The assay system described in Section 2.2.5.1b was used except that pyruvate and not 2-oxoglutarate was the keto acid substrate. The data was analysed using the computer programme HYPER.

Fig.6.8 Sepharose 6B chromatography of the pyruvate activity in the presence of glycerol

This is the same column shown in Fig.6.3 except the assay mixture contained pyruvate as the keto acid substrate.

—□— $A_{280}$

—■— $^{14}CO_2$ fixation

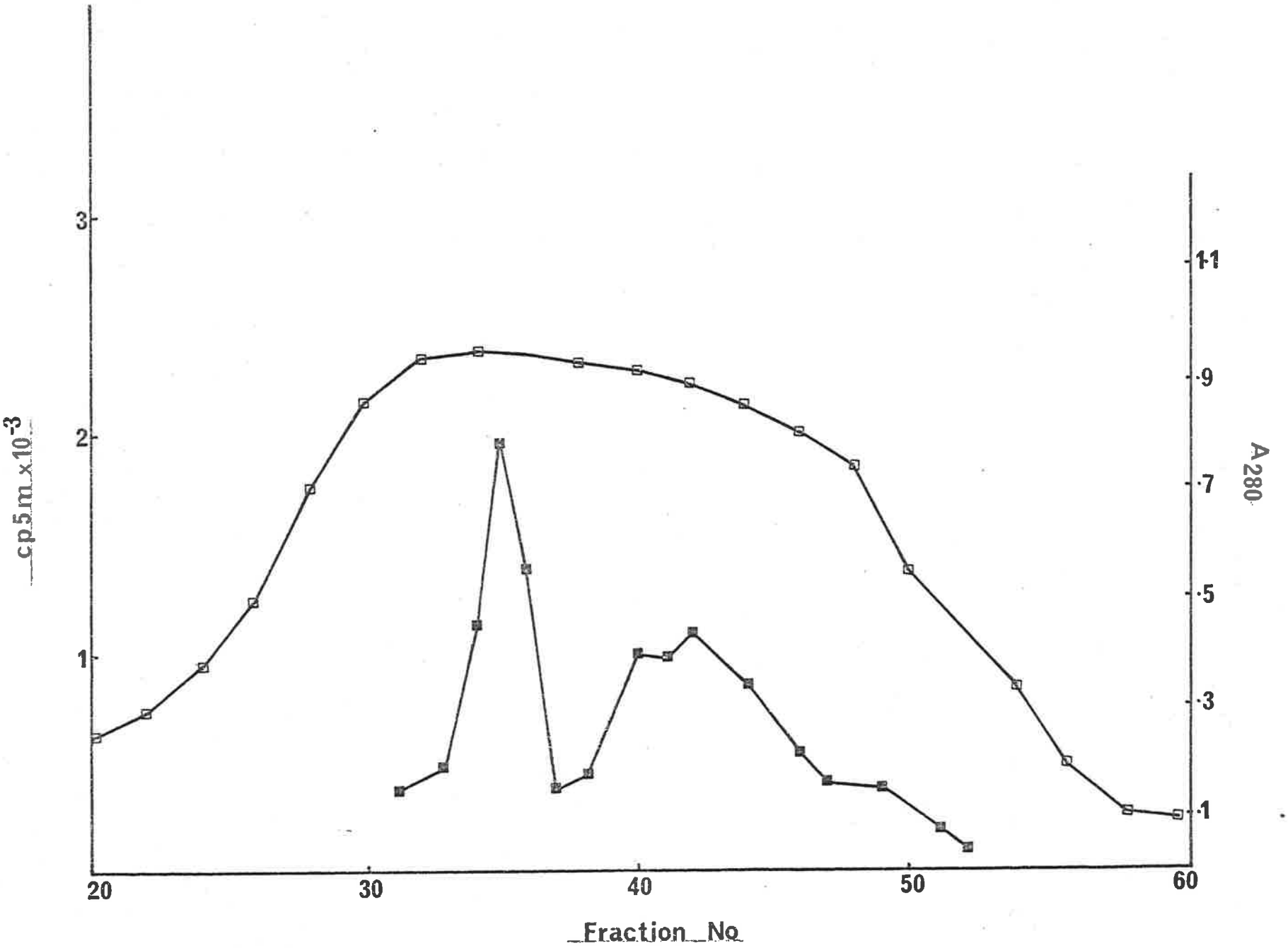

cp5m x10-3
3
2
1
A280
1·1
·9
·7
·5
·3
·1
20
30
40
50
60
Fraction No

Fig.6.9 Sepharose 6B chromatography of the pyruvate activity in the presence of glucose

This is the same column shown in Fig.6.4 except the assay mixture contained pyruvate as the keto acid substrate.

—□— $A_{280}$

—■— $^{14}CO_2$ fixation

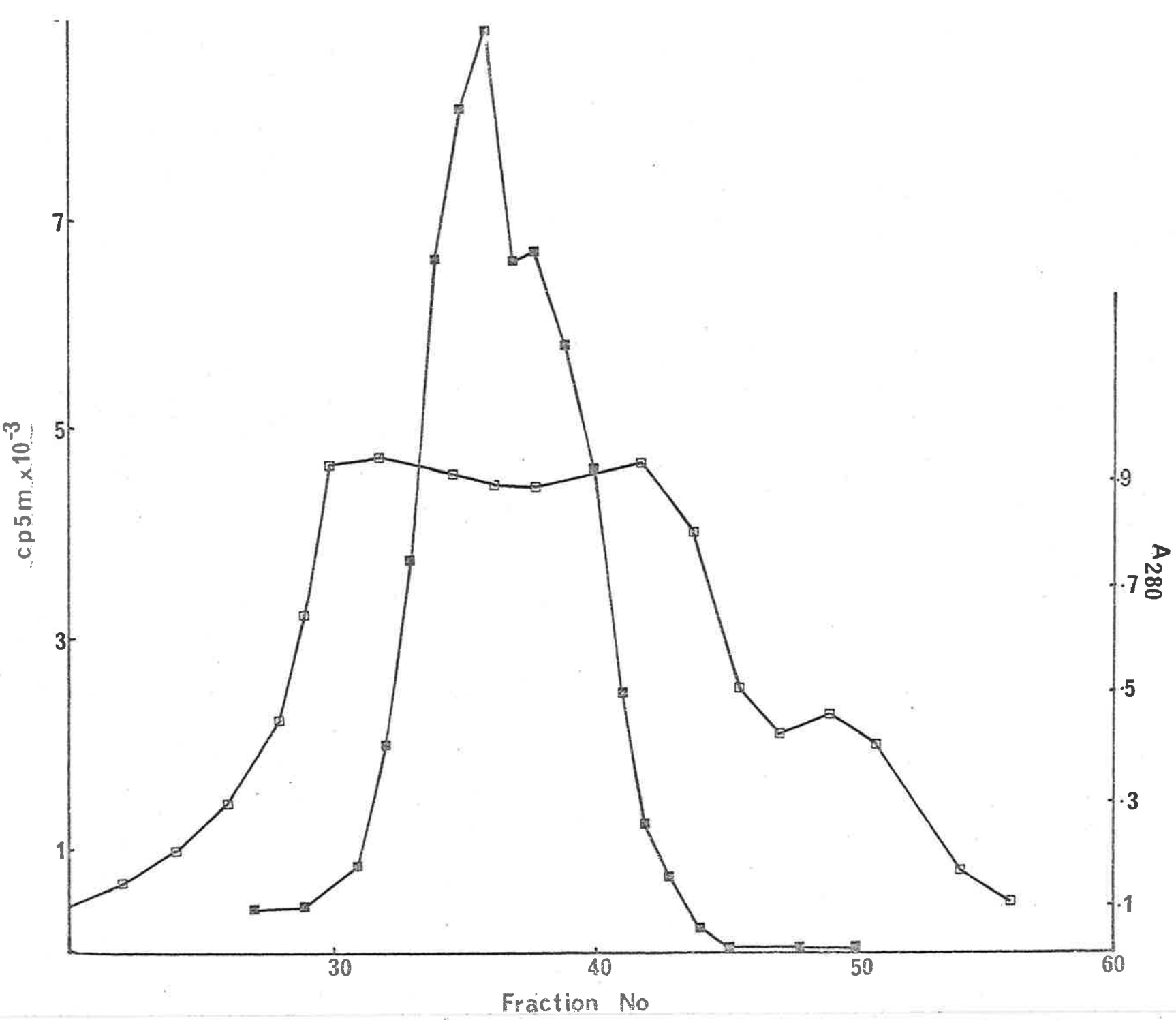

cp5 m x10$^{-3}$
7
5
3
1
·9
·7
·5
·3
·1
A$_{280}$
30
40
50
60
Fraction No

TABLE 6.8 Effect of avidin upon keto-acid carboxylation

| Substrate | $H^{14}CO_3$ fixation, cp5m |
|---|---|
| pyruvate | |
| no addition | 3952 |
| + Avidin | 4155 |
| + Avidin + Biotin | 4222 |
| + Biotin | 4106 |
| 2-oxoglutarate | |
| no additions | 1668 |
| + Avidin | 1584 |
| + Avidin + Biotin | 1581 |
| + Biotin | 1588 |

Partially purified enzyme (Stage 5, Table 4.10) in 50mMNEM-Cl, pH7.5 was assayed as described in Section 2.2.5.1b using pyruvate or 2-oxoglutarate as shown. When avidin and biotin were added, they were incubated together prior to addition to the enzyme. The concentrations used were; Avidin, 2U/0.05ml enzyme; biotin 10µg/0.05ml enzyme. Incubation with the added reagent was for 2 hrs at 25°C.

TABLE 6.9 Comparison of $H^{14}CO_3^-$ fixation and $[^{32}P_i]$ release from $[\gamma\text{-}^{32}P]ATP$

| Substrate | $H\ ^{14}CO_3^-$ fixation nmoles/5 min | $[^{32}P_i]$ release nmoles/5 min |
|---|---|---|
| pyruvate | 159.3 | 3.31 |
| 2-oxoglutarate | 40.3 | 4.02 |

The assay system described in Section 2.2.5.1b was used to measure $^{14}CO_2$-fixation and the assay for $^{32}P_i$ is as described in Section 2.2.5.1c. All assay mixtures contained 50mM Glucose and 0.01ml enzyme (70mg/ml) purified to Stage 5 (see Table 4.10).

Fig.6.10 Time study of the ATP-dependent isocitrate synthase reaction

The enzyme used was purified up to Stage 5 (see Table 4.10) and 0.2ml enzyme/1ml assay (70mg/ml) was assayed as described in Section 2.2.5.1b.

—●— Complete mixture

—■— " " minus ATP

—▲— " " " 2-oxoglutarate

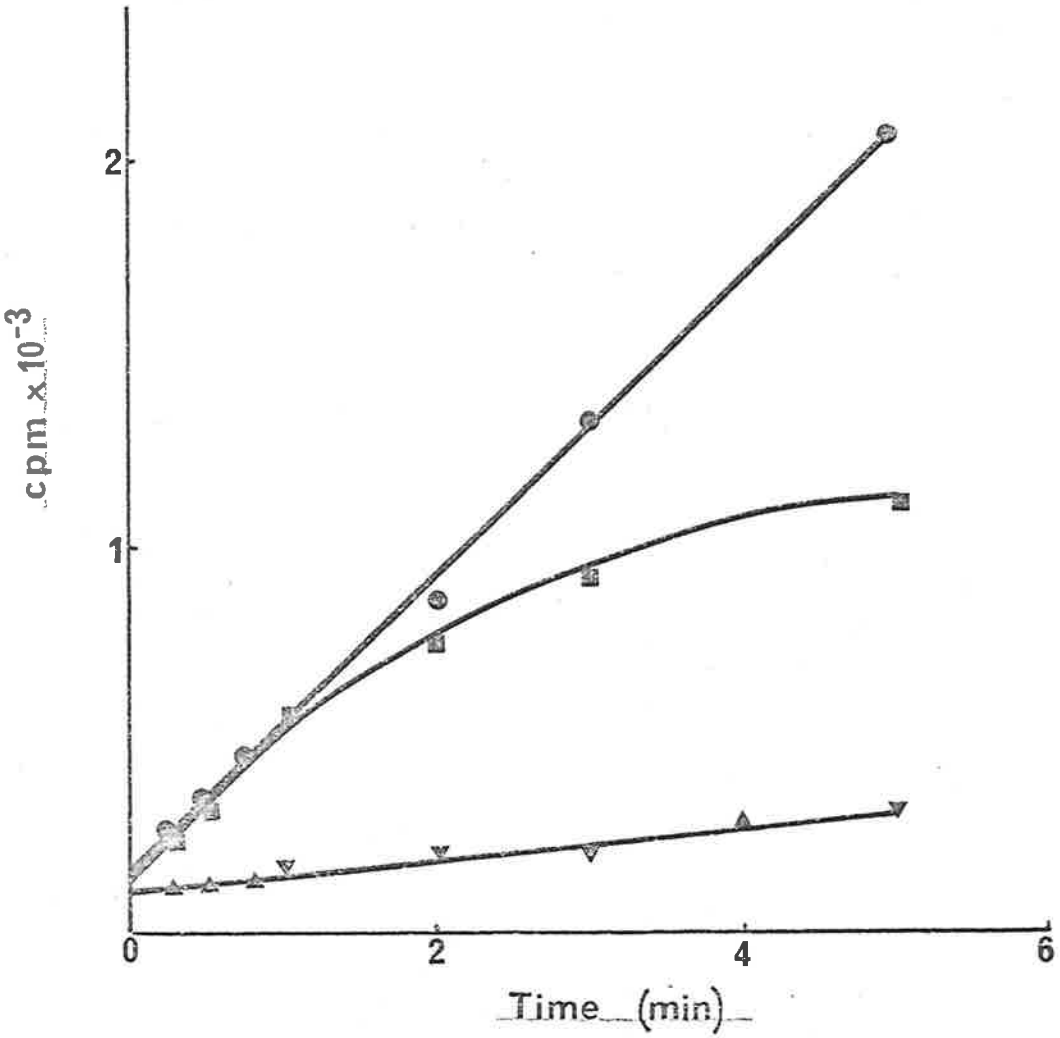

2
1
0
2
4
6
cpm x10⁻³
Time (min)

Fig.6.11 Time study of the ATP-dependent isocitrate synthase following the preincubation of the enzyme

This is identical to the experiment shown in Fig.3.10 except that the enzyme was incubated with the assay mixture (minus $HCO_3$) for 5 min prior to initiation with $H^{14}CO_3^-$.

—□— Complete mixture

—△— " " minus ATP

—■— " " " 2-oxoglutarate

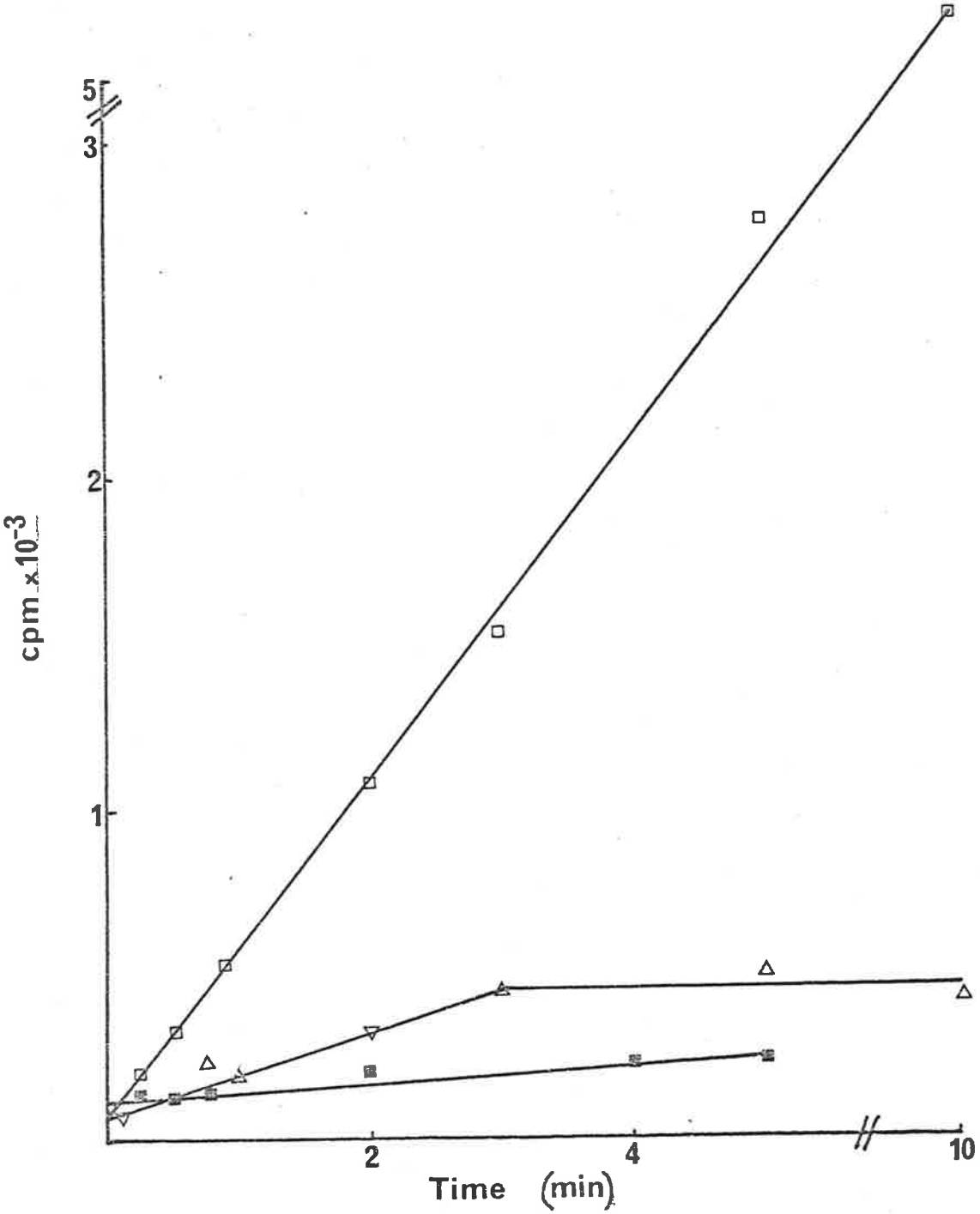

5
3
2
1
cpm x10⁻³
2
4
10
Time (min)

TABLE 6.10 Level of ATP in the isocitrate synthase fraction

| Method of measurement | Amount of ATP/50λ enzyme nmoles |
|---|---|
| Spectrophotometric | 39 |
| Radioisotope | 39 |

The enzyme was as for Fig.6.10. The spectrophotometric measurement of ATP was as described in Section 2.2.5.6 and the value for the radioisotope assay was calculated from data in Fig.6.10.

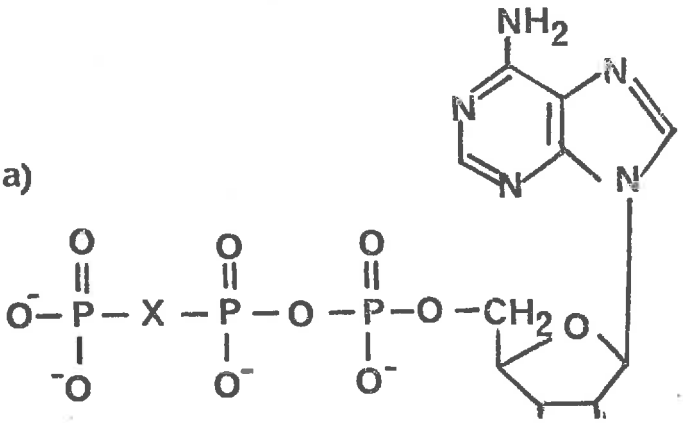
TABLE 6.11 Effect of NON-HYDROLYSABLE ATP Analogues

| Substrate | Counts fixed into products cpm | % of ATP counts |
|---|---|---|
| ATP | 3545 | 100 |
| AMP-PNP | 758* | 21 |
| AMP-PCP | 0 | 0 |

* The level of ATP contamination as measured spectrophotometrically using hexokinase would produce 793cpm, therefore the counts fixed by AMP-PNP is zero.

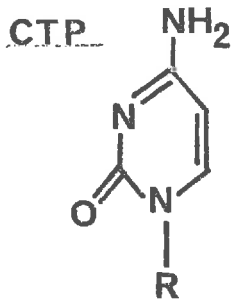
The assay mixture used was as described in Section 2.2.5.1b. The final concentrations of ATP and the analogues was 5mM and 0.05ml of enzyme (70mg/ml) was used per assay.

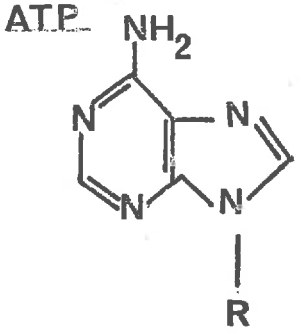
Fig.6.12 Structure of nucleotide triphosphates and derivatives of ATP

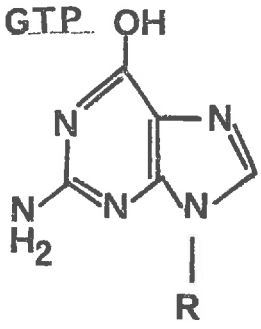


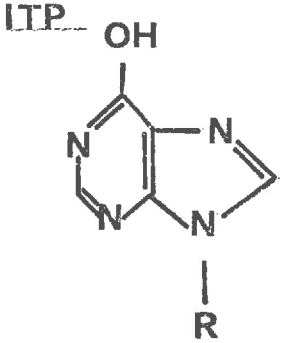
ATP : X = O
AMP-PNP: X = NH
AMP-PCP: X = $CH_2$

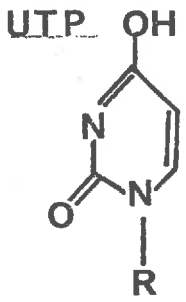
b)

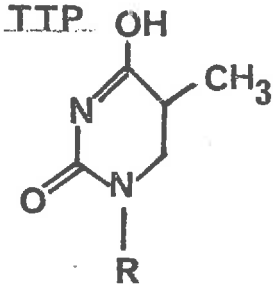


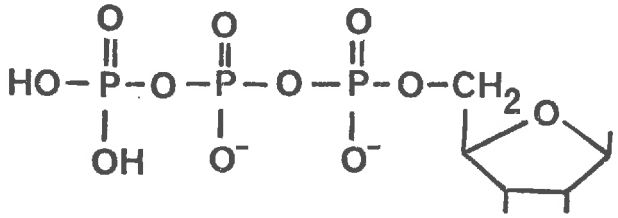

R = 

TABLE 6.12 Specificity of Nucleotide Triphosphate

| Nucleotide Triphosphate | % activity of ATP |
|---|---|
| ATP | 100 |
| CTP | 274 |
| GTP | 0 |
| ITP | 0 |
| UTP | 0 |
| TTP | 0 |

The assay system was as described in Section 2.2.5.1b using 0.05ml enzyme (70mg/ml) and 5mM final concentrations of the nucleotide triphosphates.

TABLE 6.13a appKm CTP

| Nucleotide Triphosphate | appKm mM | Vmax cp5m |
|---|---|---|
| $^{1}$CTP | 2.85 ± 0.93 | 11500 ± 2700 |
| $^{2}$CTP | 2.36 ± 1.25 | 22500 ± 8200 |

1-keto acid substrate is 2-oxoglutarate

2- " " " " pyruvate

TABLE 6.13b appKm for the keto acids in presence of CTP

| Substrate | appKm mM | Vmax cp5m |
|---|---|---|
| 2-oxoglutarate | 0.623 ± 0.106 | 5500 ± 120 |
| | *0.538 ± 0.078 | |
| pyruvate | 0.93 ± 0.269 | 29500 ± 5200 |
| | *0.532 ± 0.133 | |

* ATP as substrate

All assays were as described in Section 2.2.5.1b using 5mM CTP and 4mM pyruvate (final concentrations) were applicable. The data were analysed by the computer programme HYPER.

Fig.6.13 Sepharose 6B chromatography of $^{32}P$ labelled isocitrate synthase

This column is identical to that shown in Fig.6.9 except that the buffer used was 50mMMNEM, pH7.5 containing 5mM$Mg^{2+}$ and 0.25M glucose. Partially purified enzyme (Stage 5, see Table 4.10) was dissolved in 3mls of the above buffer and 0.5ml [$\gamma$-$^{32}P$]ATP (100$\mu$C total) was added. The final concentration of MgATP was 5mM. This was then incubated at 30°C for 2 hr prior to column chromatography.

Column size : 70cmx2.5cm

Buffer : as above

Flow rate : 18ml/hr

Fraction size : 3ml

—□— $^{32}P$ counts

—○— ATP-dependent $^{14}CO_2$ fixation

—▲— ATP-independent $^{14}CO_2$ fixation

—■— $^{14}CO_2$ fixation in presence of ATP

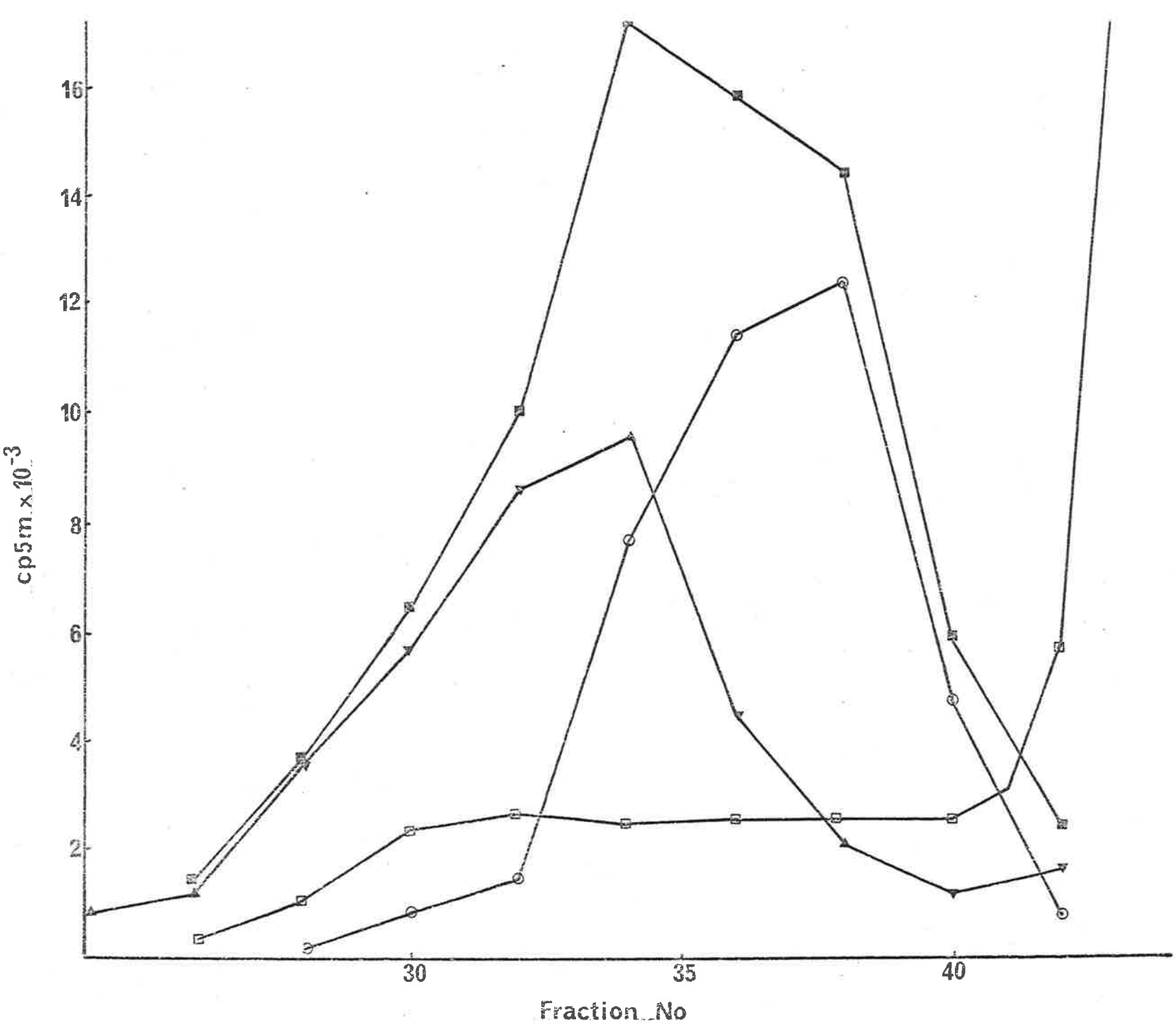

16
14
12
10
8
6
4
2
cp5m x 10^-3
30
35
40
Fraction No

TABLE 6.14 Precipitation of Labelled Protein

| $^{32}$Pcpm before precipitation | $^{32}$Pcpm after precipitation |
|---|---|
| (a) Acid Precipitation | |
| 19,005 | 122 |
| (b) Alkaline Precipitation | |
| 16,688 | 15,854 |
| (c) $CHCl_3$/MeOH Extraction | |
| 12,075 | 10,647 |

The $^{32}$P-enzyme eluted as in Fig.6.14 was used in attempts to remove the $^{32}$P-label. Enzyme aliquots were treated as described below and the precipitates washed onto GF/A papers, dried and counted.

(a) 10% TCA

(b) 7M Urea containing 10mMNaOH followed by washing in cold acetone

(c) chloroform:methanol ($2:1^{v}/_{v}$)

# CHAPTER 7

# GENERAL DISCUSSION

## 7.1 General Discussion

An enzyme, not previously described, and given the trivial name "isocitrate synthase" was investigated in some detail in this work. Evidence presented in Chapter 3 has shown that the synthase is different from isocitrate dehydrogenase (NADP). It is proposed that the properties of isocitrate synthase make this enzyme a far more feasible candidate than isocitrate dehydrogenase (NADP) for the reductive carboxylation of 2-oxoglutarate to isocitrate in the 2-oxoglutarate reductive carboxylation pathway.

There are several controls of the isocitrate synthase not exhibited by isocitrate dehydrogenase (NADP). Previous work appearing in the literature as well as results presented in this thesis have shown that the level of isocitrate dehydrogenase (NADP) activity remains constant, irrespective of the dietary regime of the animal. However the 2-oxoglutarate reductive carboxylation pathway has been shown to fluctuate in response to the cell's fatty acid requirement as discussed elsewhere in this work. Results presented here show that isocitrate synthase activity fluctuates in a manner parallel to the activity of the pathway and this combined with the other two controlling factors, glucose and ATP, indicates a tight multi-facet control usually bestowed upon regulatory enzyme in a metabolic pathway.

The 2-oxoglutarate pathway is not only involved in lipogenesis but also in gluconeogenesis as citrate cleavage produces oxaloacetate as well as the acetyl moiety of acetyl CoA. The discovery presented in this work that pyruvate is also a substrate for isocitrate synthase leads

to the conclusion that this enzyme contributes to gluconeogenesis via two different oxaloacetate sources. Thus if there is a low level of citrate cleavage enzyme, as has been shown under some dietary conditions, isocitrate synthase can still participate in gluconeogenesis. The enzyme can thus participate in gluconeogenesis without concomitant participation in fatty acid synthesis. Furthermore since the enzyme is activated by glucose it will increase its involvement in lipogenesis provided no other step in the lipogenic cycle is limiting.

The absolute requirement for ATP in the isocitrate synthase reaction is clearly established here whilst the mechanism of its involvement in the reaction is not as yet clearly defined. There is not a 1:1 stoichiometry between $^{14}C$-fixation and $^{32}P$-hydrolysis while product inhibition studies indicate that there are two distinct phosphate binding sites on the enzyme. Furthermore it appears highly likely that the enzyme can be labelled with protein-bound acid labile, alkaline stable $^{32}P$ when incubated with [$\gamma$-$^{32}P$]ATP after which the enzyme loses some of its ATP-dependence. These data imply that one of the interactions of ATP with the enzyme is for the purpose of protein phosphorylation. The ATP dependence would thus be partly due to a requirement for a phosphorylated enzyme for the expression of enzymic activity. Protein phosphorylation has been shown to control the activity of a number of enzymes (Taborsky, 1974), and isocitrate synthase could possibly be an example of such a group of enzymes.

Further work is necessary before this conclusion is verified. It is necessary to purify the enzyme to homogeneity before attempts are made to identify the phosphorylated protein. The phosphorylated residue is possibly histidine, however this has yet to be established and highly purified enzyme is required for an unambiguous result.

Another possible interaction of ATP with the enzyme is that of allosteric activation. The reversable binding of ATP to the enzyme could enhance the rate of the reaction by facilitating the correct positioning of the other substrates with ATP remaining unchanged after the reaction. Since AMP-PNP and AMP-PCP did not result in enzymic activity then, if allosteric activation is an obligatory step in the enzyme mechanism, then alteration of the phosphate moiety of the ATP cannot be tolerated and only ATP and CTP could activate the enzyme. For allosteric activation to be established direct binding studies using highly purified enzyme are necessary.

The failure to purify intact isocitrate synthase by column chromatography is not surprising considering the enzyme appears to consist of several components differing in molecular weight. Another factor which could possibly hinder the purification is the interaction of glucose with the enzyme. Since Sephadex and Sepharose are anhydroglucose polymers (see Section 4.3.6) isocitrate synthase could interact with the gel, be absorbed, retarded and perhaps denatured. If the complex consists of components which differ in their affinity for glucose then the complex will be disassociated upon filtration on

these gels since retardation will only affect components involved in the glucose interaction. The introduction of glucose in the buffer system prevented much of the breakdown occurring on these gels, thus supporting this idea. Glycerol which has been used to stabilise other enzymes during purification procedures had little effect upon the elution profile, showing that the effect is specific for glucose.

This work is the discovery and not the total story of isocitrate synthase. Much work is needed before the complexity of its structure, the mechanism of action of the reaction and its complex control factors are elucidated.

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PUBLICATIONS

1. Papers published

   a) The ATP-dependent reductive carboxylation of 2-oxoglutarate using cytosol from rat liver.
   (with D.B. Keech, A.K. Mattoo and J.C. Wallace)
   Biochem. Biophys. Res. Commun. 71, 712 (1976)

2. Papers presented at meetings

   a) The ATP-dependent synthesis of isocitrate from 2-oxoglutarate.
   (with D.B. Keech, A.K. Mattoo and J.C. Wallace)
   Proc. Aust. Biochem. Soc., 9, 23, (1976)

   b) Properties of the isocitrate synthase system from rat liver.
   (with A.K. Mattoo, D.B. Keech and J.C. Wallace)
   Biochem. Soc. Trans., 4, 1058, (1976)

   c) The ATP-dependent synthesis of isocitrate from 2-oxoglutarate.
   (with D.B. Keech, A.K. Mattoo and J.C. Wallace)
   Abstr. 10th I.U.B. Congr. p399 (1976).