THE CONTROL OF CITRIC ACID CYCLE ENZYMES IN ACINETOBACTER

a thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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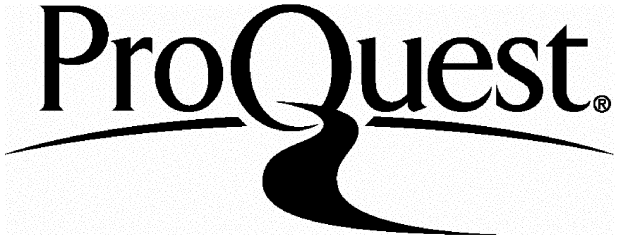
May 1975



to my husband Roy and my parents

Acknowledgements

I would like to express my sincere thanks to all those without whose help and encouragement this thesis would not have been possible. In particular I would like to thank my husband Roy for living through it with me, my supervisor Dr David Weitzman for his helpful advice and enthusiasm, Professor Kornberg for use of departmental facilities and the Medical Research Council for financial support.

ABSTRACT

The activities of Acinetobacter lwoffi pyruvate dehydrogenase and succinate thiokinase have been shown to be under adenylate control. Pyruvate dehydrogenase was stimulated by AMP and ADP whereas ATP was inhibitory. The products NADH and acetyl-CoA inhibited the enzyme and AMP decreased the latter inhibition. On the other hand, A. lwoffi succinate thiokinase utilised ADP (Km = 1.18mM), or GDP (Km = 0.026mM), or IDP (Km = 0.023mM) as nucleotide substrate, and the GDP- or IDP- dependent activities were inhibited by ATP in the presence of nucleoside diphosphate kinase. Thus adenylate control of this enzyme may either be a consequence of the sensitive operation of the enzyme over the range in which the intracellular ADP concentrations may vary, or it may involve a nucleoside diphosphate kinase mediated interaction of ATP with the GDP or IDP pools.

No adenylate control was observed with A. lwoffi aconitase, succinate dehydrogenase, fumarase and malate dehydrogenase. Therefore, these results together with those previously observed with citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase suggest that adenylate control of the citric acid cycle enzymes in A. lwoffi is exerted only on those enzymes which occur at the metabolic branch-points. This novel form of regulation in which several enzymes of a metabolic sequence are controlled by the same effector has been called "multipoint" control and presumably ensures that cycle activity is not limited by the withdrawal of intermediates. An examination of a large number of diverse bacterial species showed that the elements of the control are generally found together and were detected in all species of Acinetobacter examined, as well as in a few other Gram-negative strictly aerobic bacteria.

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I INTRODUCTION

Metabolism consists of a vast number of interrelated pathways, the sheer complexity of which one might expect to result in chemical chaos. However, it is a characteristic of the living cell that metabolism occurs in an ordered and co-ordinated fashion. If this is the case, then clearly control processes must be present. Metabolic regulations are exerted at two principal levels and control enzyme activity and the amount of an enzyme. The control of enzyme activity is essentially immediate, but the control of enzyme amount is a coarse regulatory device and relatively slow.

Enzyme concentration is mainly governed by the rate of protein synthesis and is controlled genetically by the mechanisms of induction and repression (Jacob and Monod, 1961). Inducible enzymes are often additionally subject to catabolite repression (Magasanik, 1961). Evidence of these mechanisms controlling the synthesis of the citric acid cycle enzymes was obtained by growing organisms under a variety of conditions. Three factors were shown to bring about these responses - anaerobiosis, the presence of glucose and the nutritional state of the culture (Englesberg et al., 1954 a & b; Umbarger, 1954; Collins and Lascelles, 1962; Strasers and Winkler, 1963; Amarasingham and Davis, 1965; Gray et al., 1966; Hanson and Cox, 1967). These parameters were found to be interactive and depending on the demands on the cycle, a large number of responses was observed. Not all the cycle reactions were affected equally and responses varied in different organisms. The extensive studies by Gray et al. (1966) illustrate how these factors influence the biosynthesis of Escherichia coli citric acid cycle enzymes. During anaerobic growth, a general decrease in the concentration of these enzymes was obtained; however, each enzyme was affected differently and further variations were observed

depending on the growth medium. Aerobic growth with glucose repressed the formation of these enzymes, especially if complex rather than synthetic mineral salts medium was used. Those enzymes leading to the biosynthesis of glutamate were derepressed, although other cycle enzymes were not derepressed proportionally. This reduction in the repressive effect of glucose was due to a biosynthetic requirement for cycle activity during growth on the latter medium, whereas preformed intermediates were supplied in the complex medium. These mechanisms are therefore extremely important for cellular economy ; enzymes are produced only when required, thereby avoiding a wasteful consumption of energy and intermediates.

The investigations reported in this thesis are not concerned with the control of enzyme amount, however, but with a complementary type of regulation - the control of enzyme activity. This is responsible for the compensation of short term changes in the cell and is therefore finely poised. The first examples of this type of control were reported by Umbarger (1956), and by Yates and Pardee (1956), when they described the regulation of isoleucine and pyrimidine biosynthesis, respectively. These pathways were found to be regulated by end-product inhibition of the first committed enzyme in each pathway. The controlled enzyme was recognised as the pacemaker because the rate of the whole sequence was dependent on it. Since these original observations there have been numerous others in which regulation is achieved by modulation of the activity of key pacemaker enzymes.

The control of the citric acid cycle poses a special problem, however, because unlike the linear pathways studied previously there is no readily identifiable first enzyme or end-product.

Notwithstanding, citrate synthase has been suggested as the first enzyme in the citric acid cycle because it effects the entry of carbon in the form of acetyl-CoA units by catalysing their condensation with oxaloacetate. Thus Krebs and Lowenstein (1960) have demonstrated that the addition of cycle intermediates to respiring cells increases the rate of oxygen consumption and the rates of individual cycle reactions. They therefore concluded that at normal endogenous substrate concentrations the activity of those enzymes responsible for the individual steps is submaximal and that citrate is oxidised through the cycle as rapidly as it is formed from oxaloacetate and acetyl-CoA, i.e. citrate synthase is the rate determining step of the cycle. Cycle activity results in the formation of NADH, the subsequent oxidation of which is coupled to the formation of ATP. NADH can therefore be regarded as the direct end-product of the citric acid cycle, and ATP the indirect end-product. If feedback control mechanisms do operate one might expect either or both substances to act as effectors. Because ATP is formed at the expense of AMP, stimulation by AMP will be functionally equivalent to inhibition by ATP. It is thus consistent with this hypothesis that the citrate synthases from eukaryotic organisms and Gram-positive bacteria are inhibited by ATP (Hathaway and Atkinson, 1965; Bogin and Wallace, 1966; Shepherd and Garland, 1966; Jangaard et al., 1968; Flechtner and Hanson, 1969), whereas those from Gram-negative bacteria are inhibited by NADH; this inhibition can be relieved with AMP in Gram-negative strictly aerobic bacteria (Weitzman, 1966; Weitzman and Jones, 1968).

Citrate synthase is not the only cycle enzyme with regulatory properties, which is hardly surprising because the citric acid cycle is a highly complex pathway with many reactions leading

in and out (Fig. 1). The activities of the following cycle enzymes from diverse bacterial and eukaryotic sources are modified by NADH and the adenylates and may contribute to the overall control of cycle activity: isocitrate dehydrogenase, α-oxoglutarate dehydrogenase, fumarase and malate dehydrogenase. The diversity of the regulatory mechanisms imposed on metabolically identical pathways is illustrated by the following results.

The regulatory properties of the first of these enzymes, isocitrate dehydrogenase, has been examined in great detail. In eukaryotic systems there are two isocitrate dehydrogenases, one specific for NAD^+, and the other for $NADP^+$. It is the NAD-linked enzyme which exhibits regulatory properties. The enzyme from the fungi, yeast, Aspergillus niger and Neurospora crassa, is stimulated by AMP (Ramakrishnan and Martin, 1955; Hathaway and Atkinson, 1963; Sanwal et al., 1964). The latter enzyme is also inhibited by NADH (Sanwal et al., 1965). The mammalian enzyme, on the other hand, is activated by ADP, rather than AMP, and the yeast enzyme is also inhibited by NADH and ATP (Chen and Plaut, 1963; Klingenberg et al., 1965). In contrast to the fungal and mammalian enzymes, however, the plant enzyme is insensitive to the adenine nucleotides but is powerfully inhibited by the direct end-product of the cycle, NADH (Cox and Davies, 1967; Coultate and Dennis, 1969). The regulatory properties of bacterial isocitrate dehydrogenases on the other hand, are very different. Bacteria generally possess only one isocitrate dehydrogenase, and this is usually specific for $NADP^+$. Some of these NADP-linked enzymes are also regulated. The enzymes from Pseudomonas fluorescens and Salmonella typhimurium are inhibited by ATP (Marr and Weber, 1968; Hampton and Hansen, 1969), whereas the Acinetobacter lwoffi enzyme, like the NAD-linked enzyme from higher organisms, is activated by AMP and ADP (Parker and Weitzman, 1970).

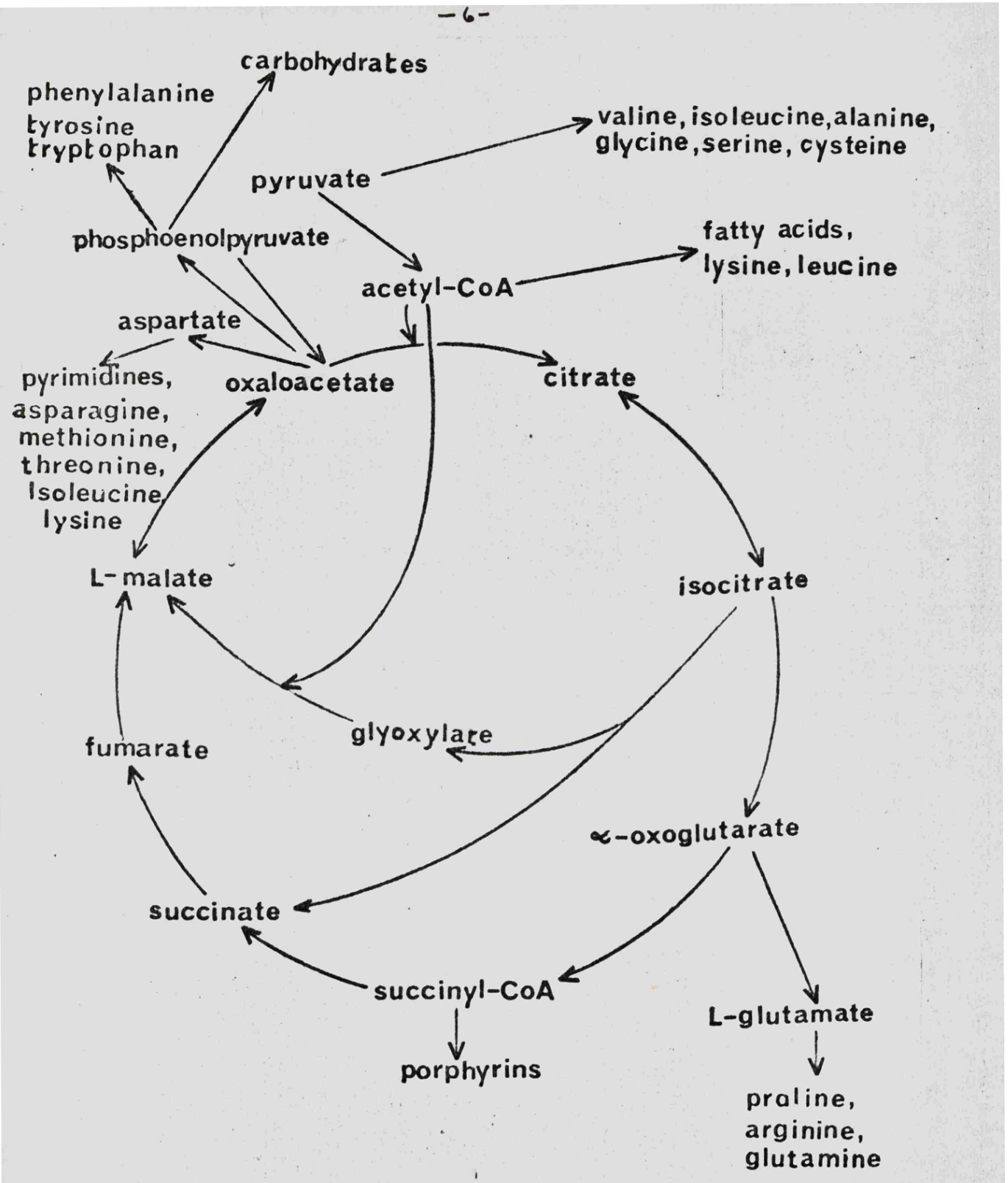

Figure 1. The citric acid cycle and related pathways of metabolism.

In contrast to the many reports illustrating the control of isocitrate dehydrogenase, the instances describing the regulation of α-oxoglutarate dehydrogenase are much less common. NADH, the direct product of the citric acid cycle, is an inhibitor of the enzyme from pig heart, E. coli, cauliflower florets and A. lwoffi, (Garland, 1966; Hansen and Henning, 1966; Wedding and Black, 1971; Weitzman, 1972), but in the case of the latter enzymes, this inhibition can be overcome with AMP. Interestingly, the citrate synthase from this organism is controlled in a similar manner. Here again, enzyme activity is inhibited by NADH and this inhibition can be relieved with AMP (Weitzman and Jones, 1968). However, NADH is not the only modulator of α-oxoglutarate dehydrogenase activity and further regulatory responses are brought about by the adenylates. The enzyme from cauliflower florets and A. lwoffi is stimulated by AMP, whereas ATP is an inhibitor of the enzyme from blowfly flight muscle, and AMP reverses this inhibition (Hansford, 1972).

The regulatory properties of yeast and pig heart fumarase activities are unusual; both enzymes are inhibited by free ATP but not by $MgATP^{2-}$. This results in a sigmoid dependence of inhibition on ATP concentration, in the presence of Mg^{2+}, because ATP inhibition is not expressed until all the Mg^{2+} has been titrated with ATP (Penner and Cohen, 1969). It has been suggested that this mechanism may operate in vivo to increase the sensitivity of fumarase to small changes in ATP concentration.

Finally, E. coli malate dehydrogenase is inhibited by NADH, and although AMP, ADP or ATP can also bring about this response, the concentrations of these compounds required for half-maximal inhibition are high (~4mM), and therefore unlikely

to be physiologically significant (Sanwal, 1969). In addition, pig heart malate dehydrogenase is stimulated by AMP, ADP and ATP, when assayed in the direction associated with citric acid cycle activity (Kuramitsu, 1966). However, ATP is more effective than AMP, a response at variance with the postulated role of the adenylates as feedback modifiers of cycle activity.

The response of enzymes to the adenine nucleotides is called adenylate control and because the intracellular concentration of ATP increases under high energy conditions, whereas that of AMP increases under low energy conditions, these responses have a particular significance for energy metabolism. The citric acid cycle results in the production of energy and this energy is subsequently used for the diverse energy-consuming reactions of the cell. These responses may therefore suggest a mechanism for balancing the intracellular supply and demand for energy. Because the adenine nucleotides are inessential components of the reactions which they were found to modify, they can be considered to function as metabolic signals. Thus, when the intracellular energy levels are high, as signalled by ATP, cycle activity may be decreased and conversely when they are low, as signalled by AMP, cycle activity may be increased.

Although the concentration of the adenine nucleotides is a useful parameter against which to examine the control of citric acid cycle enzymes, nevertheless it is a poor reflection of the *in vivo* situation. In the cell, the range from complete absence of adenylates through increasing values of any one of them does not exist, rather changes in their concentration within a fixed adenylate pool. A physiologically more realistic parameter is perhaps energy charge (Atkinson and Walton, 1967). Energy charge

is defined as half the average number of anhydride-bound phosphate groups per adenosine moiety or,

$$\frac{(ATP) + \frac{1}{2}(ADP)}{(ATP) + (ADP) + (AMP)}$$

and it has numerical values from 0 (all AMP) to 1.0 (all ATP). This single parameter describes the energy state of the cell in terms of the degree of phosphorylation of the adenylate pool, and therefore represents the adenylate mixture. General response curves for enzymes sensitive to energy charge control have been described by Atkinson (1968a) and are illustrated in Fig. 2. Enzymes involved in ATP-regenerating systems exhibit an R-type of response, and are fully active at low energy charge but show a sharp decrease in activity at high values; in contrast, enzymes involved in ATP-utilising systems exhibit a U-type of response and are almost inactive at low energy charge but show a rapid increase in activity at high values. Both curves are steepest at charge values above about 0.8, thus permitting a very sensitive adjustment of enzyme activity in this region. The two curves cross near the mid-point of their activity range and this has two important implications. First, catabolism and anabolism are balanced around this cross-over point and secondly, because they cross in a region where both are steep, a very strong response is possible in either direction. Thus, yeast citrate synthase and isocitrate dehydrogenase, *E. coli* phosphofructokinase and pyruvate dehydrogenase and *A. Iwoffi* isocitrate dehydrogenase and α-oxoglutarate dehydrogenase exhibit R-type responses (Atkinson, 1968 b; Shen *et al.*, 1968; Parker and Weitzman, 1970, 1973), whereas *E. coli* aspartokinase, phosphoribosyladenosine triphosphate synthetase and phosphoribosyl pyrophosphate synthetase exhibited U-type responses (Klungsøyr *et al.*, 1968). This consistency of response observed *in vitro* strongly suggests that these enzymes

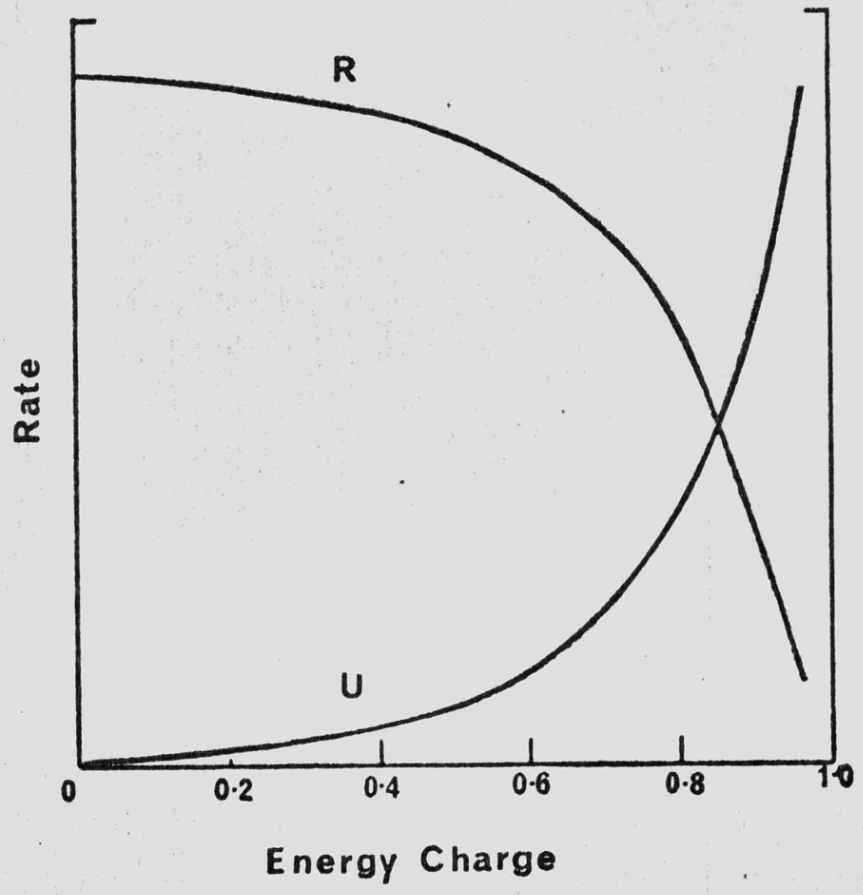

Figure 2. General response curve for enzymes sensitive to energy charge control.

R: enzymes involved in ATP-regenerating systems.
U: enzymes involved in ATP-utilising systems.

have been designed to maintain the energy charge *in vivo* at values between 0.8 and 0.9.

The usefulness of this concept in assessing the physiological significance of observed responses is illustrated by its application to the regulatory properties of yeast citrate synthase and *E. coli* pyruvate dehydrogenase. AMP, ADP and ATP were all found to inhibit the former enzyme (Hathaway and Atkinson, 1965) and activate the latter (Shen *et al.*, 1968), and although there was a gradation of responses in the physiologically meaningful direction, the regulatory significance of these responses was unclear. However, when enzyme activity was examined as a function of energy charge, the responses followed the pattern predicted for enzymes involved in ATP-regenerating sequences.

In spite of the several reports described above, information concerning the control of the citric acid cycle is still inadequate, and an investigation of this pathway as a functioning unit in metabolism by examining all the citric acid cycle enzymes from one organism has not been undertaken. The studies described in this thesis are therefore concerned with such an examination, because it was felt that an investigation of this kind would not only show the extent to which the citric acid cycle may be regulated, but it may also give an indication of the particular significance of cycle activity for that organism. For this investigation, the citric acid cycle enzymes from *A. lwoffi*, a Gram-negative strictly aerobic bacterium were selected, because this organism had already exhibited interesting regulatory properties of its citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase. Thus NADH inhibition of citrate synthase, a feature of all Gram-negative bacteria is overcome by AMP in this organism (Weitzman and Jones, 1968). The larger molecular weight isocitrate dehydrogenase isoenzyme

is stimulated by AMP and ADP, although the latter nucleotide is less effective (Parker and Weitzman, 1970; Self et al., 1973) and the α-oxoglutarate dehydrogenase in the organism is also stimulated by AMP, less so by ADP, whereas ATP is slightly inhibitory (Weitzman, 1972, Parker and Weitzman, 1973). In view of the marked sensitivity of these enzymes to the adenine nucleotides, the other A. lwoffi citric acid cycle enzymes, aconitase, succinate thiokinase, succinate dehydrogenase, fumarase, malate dehydrogenase and pyruvate dehydrogenase, were examined for their response to the adenylates. The latter enzyme, although not strictly part of the citric acid cycle, fulfils a crucial role in providing the acetyl -CoA required for condensation with oxaloacetate. Enzymes sensitive to the adenine nucleotides were examined in greater detail to investigate the mechanisms of these responses. An investigation of this kind, in which all the enzymes of a pathway are examined for a uniformity of response to a particular effector has not been reported previously, and was therefore of special interest.

To gain a better insight into the role of regulated enzymes in controlling the overall activity of the citric acid cycle, those enzymes which exhibited adenylate control were examined for their response to other metabolites. The citric acid cycle exhibits a duality of function, providing the cell with primary biosynthetic intermediates as well as energy. Davis (1961) has coined the term 'amphibolic' to describe sequences such as the citric acid cycle which fulfil both a catabolic (or degradative) and anabolic (or biosynthetic) role. These two functions are intimately related because biosynthesis requires a source of energy. However energy-utilisation does not always consume intermediates, e.g. mobility, transport, thus to some extent these

roles are independent. For efficient control of both functions one might expect constituent amphibolic enzymes to respond to energy signals and to be subject to product inhibition. Therefore, to investigate the interaction of these two functions and to examine the particular significance of cycle activity for this organism, the joint effects of adenylates and appropriate metabolites were also studied.

Having ascertained the extent to which the citric acid cycle of this organism was regulated by the adenylates, the significance of these responses for its overall metabolism was further investigated by examining other enzymes from sequences closely related to the cycle for a complementary form of control by the adenine nucleotides. Two groups of enzymes were studied: those which catalyse the withdrawal of intermediates from the cycle into biosynthetic pathways, namely isocitrate lyase, glutamate dehydrogenase and malic enzyme, and those which catalyse a C_3 to C_4 carboxylation reaction to provide cycle intermediates.

Although the citric acid cycle consists of the same reactions irrespective of the source organism, the control mechanisms imposed on it have already been shown to be very different. The novel regulatory properties of A. lwoffi citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase are not observed with these enzymes from E. coli (Weitzman and Jones, 1968; Weitzman, 1973). These differences presumably confer some advantage for the survival of the organism as a whole, and reflect the different metabolic demands on cycle activity in these organisms. Metabolic capabilities like other phenotypic characteristics are shaped by evolutionary processes. Therefore, one might expect the regulatory properties of

the cycle to be similar in closely related organisms. This possible correlation was investigated by examining a large number of diverse bacterial species for adenylate sensitivity of their citric acid cycle enzymes. Large scale comparative surveys are a novel approach in the study of regulation and provide a new tool for the study of taxonomic classification. Weitzman and Jones (1968) conducted the first survey of this kind when they examined the regulatory properties of a large number of bacterial citrate synthases. They observed three types of effect, no inhibition by NADH, inhibition by NADH but no relief of inhibition with AMP, inhibition by NADH and relief of this inhibition with AMP. These responses could be correlated with the source of enzyme as follows; Gram-positive species, Gram-negative facultatively anaerobic species and Gram-negative strictly aerobic species. The application of this approach was particularly useful in the reclassification of Brevibacterium leucinophagum, a supposedly Gram-positive organism, as a Gram-negative strictly aerobic bacterium (Jones and Weitzman, 1974).

Although the regulatory properties of an enzyme may be correlated with the taxonomic classification of the source organism this relationship may be limited and members of related species need not have the same metabolic capabilities, and conversely the metabolic properties of taxonomically unrelated bacteria may be similar. Thus, although Weitzman and Dunmore (1969) have shown that inhibition of citrate synthase by α-oxoglutarate is a feature of Gram-negative facultatively anaerobic species, several other bacteria also exhibit this response. Thus the citrate synthases from Gram-negative strictly autotrophic Thiobacilli are inhibited by α-oxoglutarate, even though these organisms are

strictly aerobic (Taylor, 1970); this effect has also been observed with the citrate synthases from the Gram-positive facultative anaerobes Bacillus polymyxa and B. macerans, the Gram-positive strict anaerobe Clostridium acidi-urici and the blue-green bacteria (Weitzman and Danson, to be published). Weitzman and Dunmore have explained their results in terms of a feedback mechanism. Under anaerobic conditions the citric acid cycles of these organisms serves a purely biosynthetic role and is modified to a branched non-cyclic pathway in which α-oxoglutarate dehydrogenase is missing (Amarasingham and Davis, 1965). Therefore citrate synthase is the first enzyme leading to the biosynthesis of α-oxoglutarate and inhibition of this enzyme by αoxoglutarate is a typical example of feedback control. Interest-ingly, α-oxoglutarate dehydrogenase is also missing from other organisms whose citrate synthases exhibit this response. Therefore, the original restriction of this regulatory property to Gram-negative facultatively anaerobic bacteria may need modification because the significant factor may be the absence of α-oxoglutarate dehydrogenase; this makes α-oxoglutarate an end-product of citrate synthase and it is expressed in regulatory terms as a feedback inhibition of the enzyme. Thus regulatory mechanisms may be better correlated with life-style, rather than the taxonomic classification of the source organism, although taxonomically related bacteria may be expected to share similar modes of life.

Because the studies described in this thesis involve the examination of many enzymes, one of the main considerations in undertaking these investigations was the ease with which these enzymes could be assayed. A prerequisite of these examinations was, therefore, the use of crude enzyme extracts without further

purification. However, many existing assay methods were unsuitable for use with crude enzyme preparations because either other enzymes in these crude extracts interfered with the procedures employed, or the assay methods did not permit an examination of the effects of the adenylates. New assay procedures were therefore developed as required. In addition, because a modifier need not affect the forward and backward directions of a reversible reaction equally (except at equilibrium (Atkinson, 1969)), special attention was paid to the examination of such reactions: these were always investigated in the direction normally associated with citric acid cycle activity.

As a result of these studies a novel type of regulation has been described. The properties of this form of control, its significance for metabolism, and it occurrence in bacterial species has been discussed in some detail.

II MATERIALS AND METHODS

1. Materials

Most chemicals were obtained from Sigma London Chemical Co. Ltd., BDH Chemicals Ltd., or The Boehringer Corporation (London) Ltd., and were of the finest grade available. Sephadex and Blue Dextran were obtained from Pharmacia Fine Chemicals Ltd., and Folin-Ciocalteu reagent from Fisons Chemical Company. Enzymes used in this work together with their sources were as follows: isocitrate dehydrogenase (pig heart; Sigma), hexokinase (yeast; Boehringer), glucose-6-phosphate dehydrogenase (yeast; Boehringer), lactate dehydrogenase (rabbit muscle; Boehringer), pyruvate kinase (rabbit muscle; Boehringer), citrate synthase (pig heart; Boehringer), malate dehydrogenase (pig heart, mitochondrial; Boehringer) and adenylate kinase (rabbit muscle; Sigma).

Preparation of acetyl-CoA and succinyl-CoA

Acetyl-CoA and succinyl-CoA were prepared by acylation of free CoA with the appropriate acid anhydride (Simon and Shemin, 1953). 10mg. of CoA were dissolved in 1ml. of water and cooled to 5°C. After adjusting the pH of the solution to 7.5 with 200μmoles of ice-cold $KHCO_3$, the freshly prepared ice-cold acid anhydride solutions were added: 14μmoles of acetic anhydride or 24μmoles of succinic anhydride were usually required. The solutions were mixed thoroughly and left for 10 min. at 5°C to ensure complete acylation. This was checked by testing for free sulphydryl groups with the chromogenic thiol reagent 5,5'-dithiobis-(2-nitrobenzoate). Any yellow colour produced indicated the presence of unacylated CoA; however, by adding a further 2 μmoles of the acid anhydrides, complete acylation was generally achieved.

The concentrations of acetyl-CoA and succinyl-CoA were determined by cleaving the esters to form a free sulphydryl group, which was then determined by measuring the yellow colour produced at 412nm. due to the formation of thionitrobenzoate from excess 5,5'-dithiobis-(2-nitrobenzoate). Cleavage of acetyl-CoA was achieved with citrate synthase and excess oxaloacetate, whereas cleavage of succinyl-CoA was achieved with hydroxylamine. The latter determination was carried out as follows: 10µl of succinyl-CoA solution were added to 0.3µmoles of 5,5'-dithiobis-(2-nitrobenzoate) in a total volume of 2.85ml. 150µl (300µmoles) of freshly prepared, neutralised hydroxylamine were then introduced and the mixture left at room temperature for 15 min. to ensure complete cleavage of the thioester bond. Assays were performed in 1cm. glass cells and the molar extinction coefficient of thionitrobenzoate at 412nm. was taken as 13.6×10^3 litre.mol.$^{-1}$cm.$^{-1}$ (Ellman, 1959).

2. Maintenance and Growth of Organisms

Acinetobacter lwoffi, strain 4B, was isolated from a laboratory reservoir of distilled water by Dr. P.D.J. Weitzman, Other organisms used in this work were either stock cultures maintained in this laboratory or were obtained from Dr. Dorothy Jones of the M.R.C. Microbial Systematics Research Unit, University of Leicester. All organisms were maintained on nutrient agar slopes and periodically subcultured. A list of these organisms, together with their culture collection numbers is presented in Table 1.

Unless otherwise stated organisms were grown in 1.3% ($^W/V$) nutrient broth; where defined medium was used, this contained 50mM Na^+/K^+ phosphate buffer, pH 7.2, 50mM NH_4Cl, 0.18mM $CaCl_2$, 0.33mM $MgSO_4$, 0.18mM $MnSO_4$, 0.14mM $FeSO_4$, and either 50mM pyruvate or 25mM acetate as a carbon source.

TABLE 1

Organisms used in the present work, showing their culture collection numbers

BACTERIUM	CULTURE COLLECTION NUMBER
Acinetobacter anitratus (1)	NCTC 8102
Acinetobacter anitratus (2)	ATCC 14290
Acinetobacter lwoffi (1)	4B*
Acinetobacter lwoffi (2)	NCTC 5867
Alcaligenes faecalis	NCTC 8769
Arizona arizonae	NCTC 8297
Arthrobacter globiformis	NCIB 8602
Arthrobacter simplex	NCIB 8929
Azotobacter vinelandii	NCIB 8660
Bacillus megaterium*	
Bacillus stearothermophilis (M.R.E., Porton)	
Bacillus subtilis*	
Bordetella bronchiseptica (1)	ATCC 10580
Bordetella bronchiseptica (2)	ATCC 4617
Brevibacterium leucinophagum	ATCC 13809
Cellulomonas cellasea	NCIB 8078
Chromobacterium violaceum	D263*
Corynebacterium fascians	NCIB 9433
Corynebacterium rubrum	ATCC 12974
Escherichia coli	K12*
Flavobacterium devorans	NCIB 8195
Klebsiella (Aerobacter) aerogenes	NCTC 10006
Klebsiella pneumoniae	NCTC 9633
Kurthia zopfii	C5*
Microbacterium thermosphactum	C4*
Mima polymorpha*	
Moraxella calcoacetica (1)	73*
Moraxella calcoacetica (2)	94*
Mycobacterium rhodocrous	C144*
Proteus vulgaris	NCTC 4175
Pseudomonas aeruginosa (1)	NCIB 8295
Pseudomonas aeruginosa (2)	D17*
Pseudomonas fluorescens	D1086*
Pseudomonas ovalis (Chester)*	
Pseudomonas stutzeri (1)	ATCC 11607
Pseudomonas stutzeri (2)	ATCC 17592
Rhodopseudomonas spheroides (M.R.E., Porton)	
Salmonella anatum	Uetake 293*
Serratia marcescens	NCTC 10211
Xanthomonas campestris	NCPPB 528
Xanthomonas hyacynthi	NCPPB 599
Xanthomonas uredovorans	NCPPB 800

ATCC: American Type Culture Collection
NCIB: National Collection of Industrial Bacteria
NCPPB: National Collection of Plant Pathogenic Bacteria
NCTC: National Collection of Type Cultures
* Cultures from culture collections of the MRC Microbial Systematics Research Unit and the Department of Biochemistry, University of Leicester
M.R.E., Porton: Microbial Research Establishment, Porton, Wilts. (These organisms were grown by M.R.E. and supplied as a frozen cell

Cells were grown in either 500ml. or 2l flasks, containing 200ml. and 1 litre of medium, respectively. These were incubated for 12h on a Brunswick shaker operating at 130 rev/min. and 37°C, until the cells had reached late stationary phase. They were then collected by centrifugation for 10 min. with an MSE "High Speed 18" centrifuge operating at 12,000g and 5°C, and washed in a buffer containing 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA.

3. Techniques used during Enzyme Purification

(a) Preparation of cell-free extracts

Cell-free extracts were prepared by resuspending the washed cells in 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA (1ml. for every 100ml. of initial culture) and sonicating the cooled suspension with an MSE 100 watt disintegrator at full power. Sonication was usually performed for 2 min, except when pyruvate dehydrogenase activity was required when sonication was carried out for only 30s. Cell debris and unbroken cells were removed by centrifugation for 30 min. at 20,000g. and 5°C.

(b) High-speed centrifugation

Cell-free extracts were spun for 1h in an MSE 'Superspeed 50' untracentrifuge with a 10 x 10 ml. angle rotor operating at 160,000 g. and 5°C.

(c) Gel filtration

Gel filtration was performed on a column of Sephadex G-200. The gel was first equilibrated with buffer containing 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA and then packed into columns (45cm. x 2.5cm.). These were equilibrated with three column volumes of starting buffer, after which their packing was checked by passing a solution of Blue Dextran through the gel bed; if the Blue Dextran travelled unevenly the columns were repacked.

After making the protein solutions 10% ($^{W}/V$) in sucrose, they were carefully layered on top of the gel bed under a head of buffer, run into the gel, and eluted from the column by passage of the buffer solution. A flow rate of 2 drops/min. was used and fractions of 30 drops were collected with an LKB Ultrorac fraction collector. After use, the columns were washed with at least five column volumes of buffer.

(d) Estimation of protein

(i) During the initial stages of enzyme purification protein concentrations were determined by the method of Lowry et al. (1951), for which a standard curve was obtained with bovine serum albumin.

(ii) The protein concentration in column fractions was estimated from the ratio E^{280}/E^{260} by comparing these values with those presented on a nomograph by E. Adams, based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (1941). Absorbanc-es were measured with a Unicam SP 1800 Spectrophotometer, using 1mm quartz semi-micro cu-vettes.

4. Assay of Enzyme Activities

The systematic names and enzyme commission numbers of the enzymes examined in this thesis are found in Table 2. Unless otherwise stated the following conditions apply to the assays described below. Experiments were performed at 25°C, with saturating concentrations of the fixed substrates, and activities have been expressed as numbers of units/mg protein. 1 unit of enzyme activity represents the conversion of 1nmole of substrate to product per min. Where the effects of various compounds on enzyme activity have been examined, they were included in assay mixtures prior to the addition of enzyme, which was used to initiate the reactions. Any stimulatory effects observed have been calculated

TABLE 2

Enzymes examined in the present studies, together with their systematic names and enzyme commission numbers

TRIVIAL NAME	SYSTEMATIC NAME	ENZYME COMMISSION NUMBER
Malate dehydrogenase	L-Malate: NAD oxidoreductase	1.1.1.37
Malic enzyme	L-Malate: NAD oxidoreductase (decarboxylating)	1.1.1.40
Isocitrate dehydrogenase	threo-Ds-Isocitrate: NADP oxidoreductase (decarboxylating)	1.1.1.42
Pyruvate dehydrogenase	Pyruvate: lipoate oxideroductase (acceptor-acetylating)	1.2.4.1
α-Oxoglutarate dehydrogenase	α-Oxoglutarate: lipoate oxidoreductase (acceptor succinylating)	1.2.4.2
Succinate dehydrogenase	Succinate (acceptor) oxidoreductase	1.3.99.1
Glutamate dehydrogenase	L-Glutamate: NADP oxidoreductase (deaminating)	1.4.1.4
Phosphoenolpyruvate carboxylase	Orthophosphate: oxaloacetate carboxy-lyase (phosphorylating)	4.1.1.31
Isocitrate lyase	threo-Ds-Isocitrate glyoxylate-lyase	4.1.3.1
Fumarase	L-Malate hydro-lyase	4.2.1.2
Aconitase	Citrate (isocitrate) hydrolyase	4.2.1.3
Succinate thiokinase	Succinate: CoA ligase (GDP) or (ADP)	6.2.1.4 or 6.2.1.5

in terms of activation, which is defined as the activity measured in the presence of the activator divided by the activity in its absence. Where kinetic parameters have been reported these are apparent and not true values.

Spectrophotometric assays

These were carried out with a Unicam SP 1800 recording spectrophotometer, fitted with a thermostatted cuvette holder and connected to a Unicam AR 25 external recorder. Assays were performed in 1.5ml. quartz semi-micro cuvettes and reaction mixtures were made up to 1.0 ml.

(a) Pyruvate dehydrogenase

Pyruvate dehydrogenase activity was determined by measuring the increase in absorbance at 340nm. due to the formation of NADH. Assay mixtures contained 0.15M Tris-HCl, pH 8.0, 10mM $MgCl_2$, 0.1mM thiamin pyrophosphate, 2.5mM cysteine hydrochloride, 0.1mM CoA, 0.5mM NAD^+ and concentrations of pyruvate as indicated. The molar extinction coefficient of NADH at 340nm was taken as 6.2×10^3 litre.mol.$^{-1}$cm^{-1}.

(b) α-Oxoglutarate dehydrogenase

α-Oxoglutarate dehydrogenase activity was determined in a similar manner to pyruvate dehydrogenase. Assay mixtures contained 0.1M Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 0.1mM thiamin pyrophosphate, 2.5mM cysteine hydrochloride, 0.1mM CoA, 0.5mM NAD^+ and a subsaturating concentration of α-oxoglutarate.

(c) Lactate dehydrogenase

Lactate dehydrogenase activity was determined by measuring the decrease in absorbance at 340nm. due to the disappearance of NADH. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 0.2mM NADH and 0.2mM pyruvate.

(d) NADH oxidase

NADH oxidase activity was determined by measuring the decrease in absorbance at 340nm. due to the disappearance of NADH. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA and 0.2mM NADH.

(e) Isocitrate dehydrogenase

NADP-linked isocitrate dehydrogenase activity was determined by measuring the increase in absorbance at 340mn. due to the formation of NADPH. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 1mM EDTA, 10mM $MgCl_2$, 0.2mM $NADP^+$, and 1mM threo-Ds-isocitrate (added as 2mM DL-isocitrate).

(f) Malic enzyme

NADP-linked malic enzyme activity was determined by measuring the increase in absorbance at 340nm. due to the formation of NADPH. Assay mixtures contained 20mM Tris-HCl, pH 7.25, 1mM $MnCl_2$ and concentrations of L-malate and $NADP^+$ as indicated.

(g) Glutamate dehydrogenase

Glutamate dehydrogenase activity was determined by measuring the decrease in absorbance at 340nm. due to the disappearance of NADPH. Reaction mixtures contained 0.1M Tris-HCl, pH 7.6, 10mM $MgCl_2$, 1mM EDTA, 50mM NH_4Cl, 0.2mM NADPH and concentrations of α-oxoglutarate as indicated.

(h) Aconitase

The conversion of citrate to isocitrate, catalysed by aconitase, was assayed by coupling the reaction to isocitrate dehydrogenase and estimating the NADPH produced by measuring the increase in absorbance at 340nm. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA with 0.5mM $NADP^+$, 0.2 Units of isocitrate dehydrogenase and citrate at the concentrations indicated.

(i) Nucleoside diphosphate kinase

Nucleoside diphosphate kinase activity was determined by estimating the ATP produced from GTP and ADP by coupling the reaction to hexokinase and glucose-6-phosphate dehydrogenase in the presence of glucose and $NADP^+$. The NADPH produced was estimated by measuring the increase in absorbance at 340nm. Assay mixtures contained 20mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 28mM glucose, 0.2mM GTP, 0.2mM ADP, 0.2mM $NADP^+$, 0.56 Units of hexokinase and 0.14 Units glucose-6-phosphate dehydrogenase.

(j) Adenylate kinase

Adenylate kinase activity was assayed by two methods. The first procedure involved estimating the ADP produced from AMP and ATP by coupling the reaction to pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and NADH. The NADH removed was determined by measuring the decrease in absorbance at 340nm. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 50mM KCl, 2mM AMP, 2mM ATP, 2mM phosphoenolpyruvate, 0.12mM NADH, 1.38 Units of lactate dehydrogenase and 1 Unit of pyruvate kinase.

The second method was a modification of the procedure used to assay nucleoside diphosphate kinase and was used when extracts were assayed for both nucleoside diphosphate kinase and adenylate kinase activities. Assay mixtures were identical to those used for nucleoside diphosphate kinase, with the omission of GTP.

(k) Isocitrate lyase

Isocitrate lyase activity was assayed by measuring the increase in absorbance at 324nm. when glyoxylate was converted to its phenylhydrazone (Dixon and Kornberg, 1959). Stock solution

containing 125mM imidazole-HCl, pH 6.8, 25mM $MgCl_2$, 5mM EDTA and 20mM phenylhydrazine hydrochloride was kept in ice and freshly prepared every four hours. Assay mixtures contained 0.2ml. stock buffer, enzyme, the appropriate concentration of threo-Ds-isocitrate (added as DL-isocitrate) and distilled water in a total volume of 1.0ml. The molar extinction coefficient of the phenylhydrazone at 324nm. was taken as 16.8×10^3 litre.$mol^{-1}$$cm.^{-1}$.

Polarographic assays

Dropping mercury electrode

When a constantly increasing voltage is applied at an electrode to an electro-active solution a current flows, the intensity of which is dependent on the magnitude of the applied voltage. The automatic recording of such current-voltage dependences forms the basis of polarographic assays. In these studies, measurements were made with a Radiometer PO4 recording polarograph, using a reaction vessel and procedure as described by Weitzman (1969). The apparatus is illustrated in Fig. 3 and shows a dropping mercury cathode and a saturated calomel reference anode. When an outside voltage is applied only the dropping mercury becomes polarised, permitting electrolytic processes to occur at the surface of the drops. As long as the potential of the dropping mercury electrode is insufficient to enact a specific electrolytic process, the outside voltage is compensated by an equal but oppositely directed voltage, generated by the polarisation of the dropping electrode. A very small 'capacity current' flows and is recorded by a sensitive galvanometer. As the potential is gradually increased until it is sufficient to cause the transfer of electrons, either from the electrode to a specific electro-active species in solution (electro-reduction), or

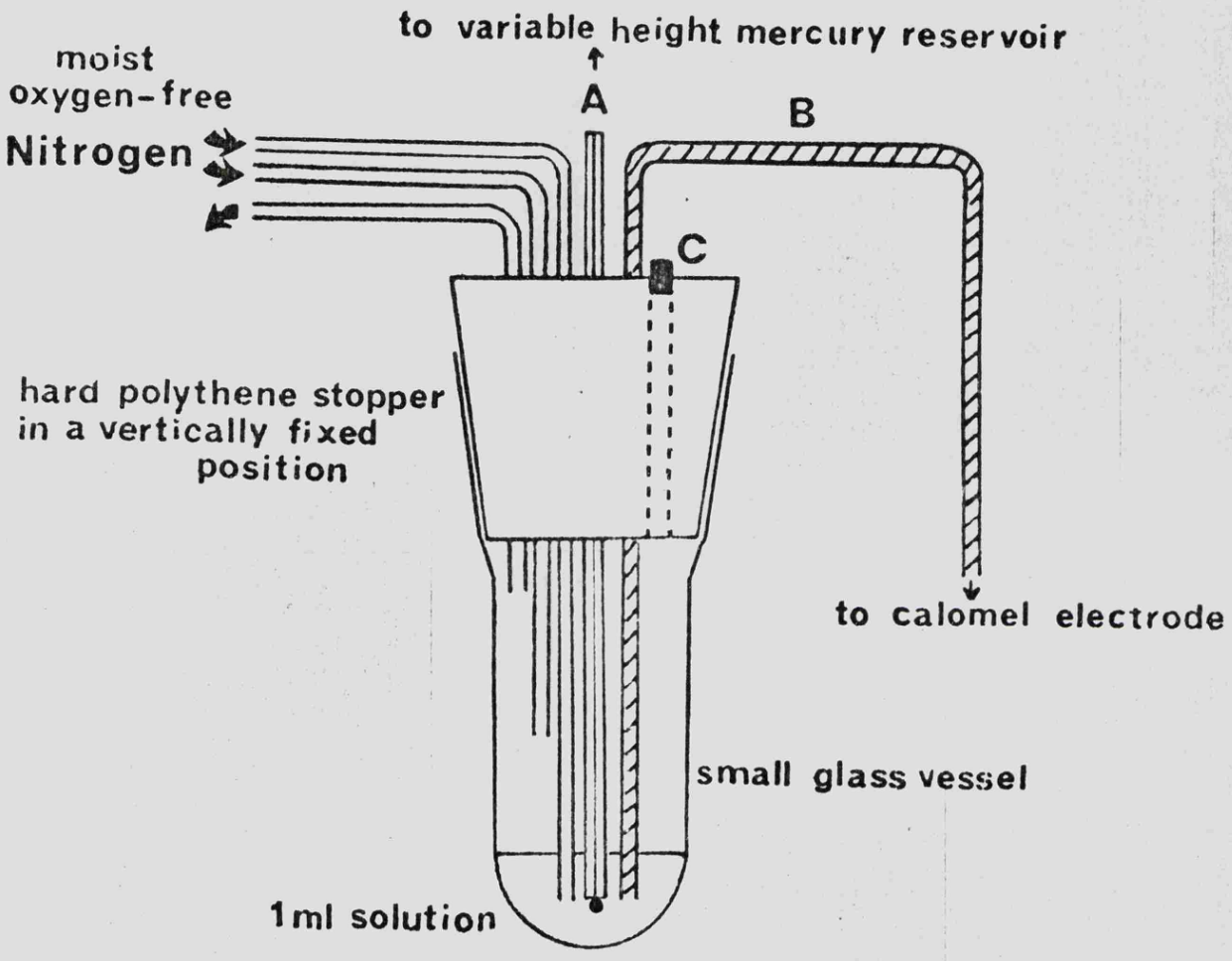

Figure 3. Diagram of the reaction vessel used for polarographic assays.

A: dropping electrode mercury capillary.
B: salt bridge (2% agar in saturated KCl).
C: small capped hole (to enable material to be added to vessel).

in the opposite direction (electro-oxidation), a current will start to flow. Because these processes decrease the enforced polarity of the dropping mercury electrode, they result in a rapid increase in current. As the potential is increased further, a point is reached at which the rate of discharge of the species at the electrode is equal to the rate at which material reaches it by diffusion from the body of the solution; a 'limiting current' is produced. Such a current-voltage curve is known as a polarographic wave and is illustrated in Fig. 4. In cases of electro-reduction, the waves and currents are termed cathodic and in cases of electro-oxidation, anodic. The borderline dividing them is the zero line of the galvanometer, corresponding to the currentless state. Cathodic waves are then represented above, and anodic waves below this zero line.

The half-wave potential, which is the potential of the dropping mercury electrode at which the current intensity is half the intensity corresponding to the limiting current, is characteristic of the material under examination. Since the magnitude of the limiting current (the wave height) is controlled by the rate of diffusion of the electro-active material, it is directly proportional to the concentration of the latter. Thus changes in concentration of such species can be determined by measuring differences in the limiting current, at a particular voltage. However, it is first necessary to calibrate the responses of the electrode by constructing a linear calibration plot relating current intensity to concentration at this voltage.

(1) Succinate thiokinase

Succinate thiokinase activity was determined in the directions of both succinyl-CoA de-acylation and CoA acylation by measuring the respective increase or decrease in current at -0.2V

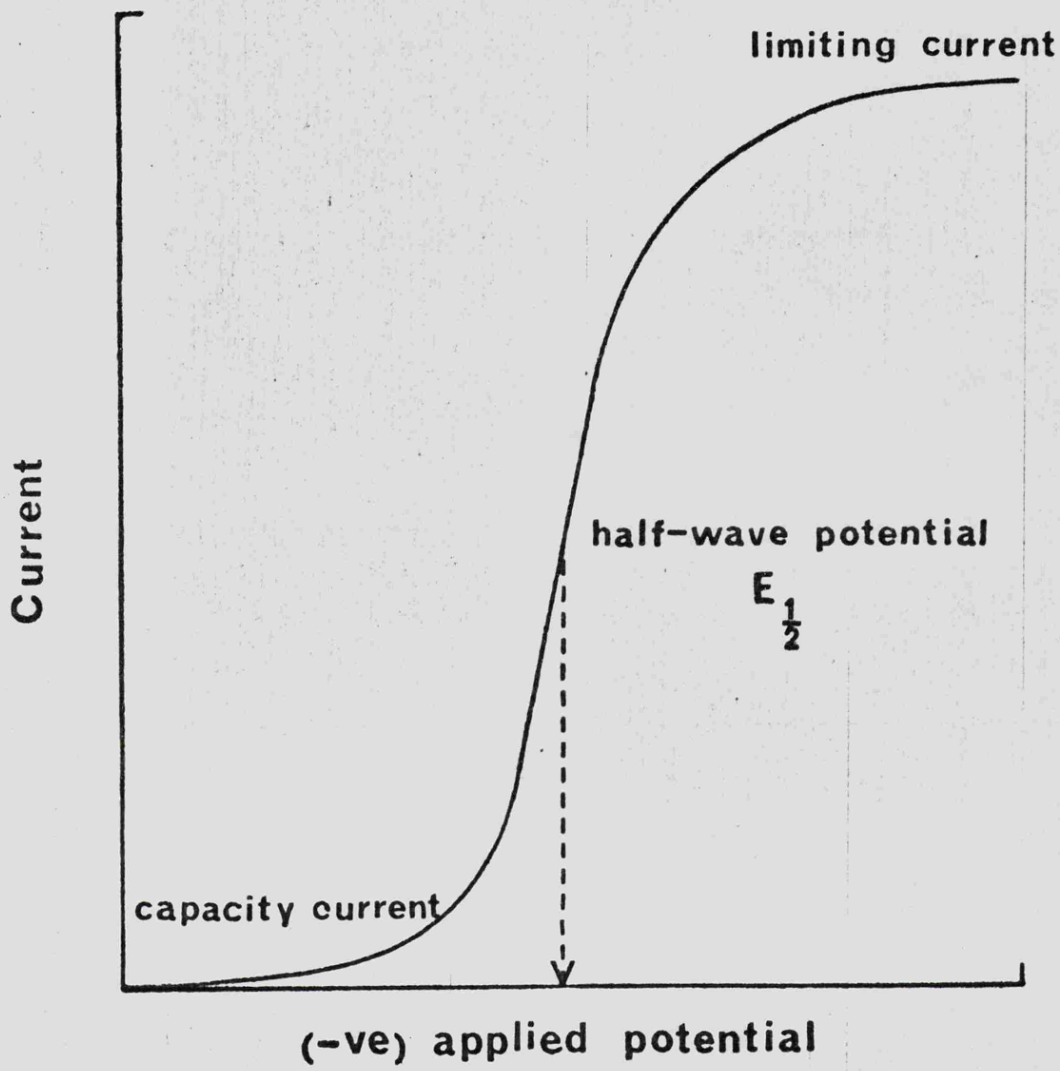

Figure 4. Diagram of a polarographic wave.

due to the formation or removal of CoA, which exhibits an anodic wave (Weitzman and Hewson, 1973). The rates of succinyl-CoA de-acylation was determined in assay mixtures containing 0.1M Na^+/K^+ phosphate buffer, pH 7.8, 10mM $MgCl_2$, 0.14mM succinyl-CoA and concentrations of ADP or GDP as indicated. The succinylation of CoA was determined in assay mixtures containing 0.1M Tris-HCl, pH 8.0, 10mM $MgCl_2$, 2mM succinate, 0.1mM CoA and concentrations of ATP or GTP as indicated.

The solutions were deoxygenated by bubbling oxygen-free nitrogen for 2-3 min. and the reaction initiated by the addition of enzyme to bring the total volume to 1.0ml. The conversion factor employed was based on a current of 1.4μA for 1mM CoA.

(m) Malate dehydrogenase

Malate dehydrogenase activity was determined by estimating the rate of oxaloacetate production with citrate synthase and acetyl-CoA. The CoA produced was determined by measuring the increase in current at -0.2V. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 0.14mM acetyl-CoA, 0.5mM NAD^+, 1.1 Units of citrate synthase and concentrations of L-malate as indicated.

(n) Fumarase

Fumarase activity was assayed by estimating the rate of L-malate production by coupling the reaction to malate dehydrogenase and citrate synthase in the presence of NAD^+ and acetyl-CoA. The CoA produced was determined by measuring the increase in current at -0.2V. Assay mixtures contained 20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 0.5mM NAD^+, 0.14mM acetyl-CoA, 11 Units of malate dehydrogenase, 1.1 Units of citrate synthase and concentrations of fumarate as indicated.

(o) Phosphoenolpyruvate carboxylase

A polarographic assay for phosphoenolpyruvate carboxylase has been developed in which the rate of oxaloacetate production is estimated with malate dehydrogenase and NADH. The NAD^+ produced was determined by measuring the increase in current at -1.3V, due to its formation. Assay mixtures contained 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 5mM $KHCO_3$, 0.2mM NADH, 11 Units of malate dehydrogenase and concentrations of phosphoenolpyruvate as indicated. The conversion factor was based on a current of 2.4μA for 1mM NAD^+.

This method is also applicable to pyruvate carboxylase by modifying the assay mixture to include ATP and substituting pyruvate for phosphoenolpyruvate.

Oxygen Electrode

(p) Succinate dehydrogenase

Succinate dehydrogenase activity was determined polarographically but with an oxygen electrode. A Clark YSI 4004 oxygen electrode with a reaction chamber of 3.0ml capacity was used. Assay mixtures contained 0.1M Na^+/K^+ phosphate buffer, pH 7.4 and enzyme. This was allowed to equilibrate for 30s and any oxidase activity due to the presence of endogenous substrates was noted. Succinate was then added with a drawn out pipette and the rate of oxygen consumption measured. All assays were performed at 30°C and enzyme activities are expressed in ng atom O/min/mg protein. The recorder was calibrated by equilibrating the electrode with air-saturated buffer for 30s and calculating the oxygen concentration from the data of Chappell (1964).

5. Energy Charge

Mixtures of varying energy charge were prepared by dissolving the appropriate quantities of AMP and ATP in buffer containing 0.15M Tris-HCl, pH 8.0, 10mM $MgCl_2$ and incubating them to equilibrium with 0.32 Units of adenylate kinase at 37°C. Aliquots of these mixtures were then incorporated into enzyme assay mixtures at the required adenine nucleotide concentration.

When A. lwoffi pyruvate dehydrogenase was being examined the energy charge mixtures were prepared by a slightly modified method and commercial adenylate kinase was first freed from the 3.2M ammonium sulphate it contained by passage through a column of Sephadex G-25 with 20mM Tris-HCl, pH 8.0, 1mM EDTA.

III RESULTS

1. Pyruvate Dehydrogenase

The extent to which A. lwoffi citric acid cycle activity is controlled by the adenylates was first investigated with pyruvate dehydrogenase, the enzyme which precedes citrate synthase and provides the acetyl-CoA "fuel" for citric acid cycle activity. However, crude extracts of A. lwoffi, obtained after 2 min sonication of the cells, showed no enzyme activity when assayed with 2mM pyruvate. An examination of enzyme activity in extracts obtained from cells which had been sonicated for selected times between 0 and 1 min showed maximum activity (12 units/mg protein) in cell-free extracts prepared after 30 s sonication of the cells; the enzyme was virtually inactive after sonication for 1 min. Therefore when pyruvate dehydrogenase activity was subsequently required, cells were sonicated for only 30 s. The possibility that adenine nucleotides regulate the activity of the enzyme was investigated by including 1mM AMP, 1mM ADP or 1mM ATP in assays performed with 2mM pyruvate. AMP and ADP markedly stimulated enzyme activity, but ATP was inhibitory. Prior to examining these apparent regulatory properties, a partial purification of the enzyme was attempted.

(a) Purification of pyruvate dehydrogenase

Because NADH oxidase may interfere with pyruvate dehydrogenase assays by removing NADH in some crude preparations where the concentration of this enzyme was particularly high, pyruvate dehydrogenase activity was undetectable. Various attempts were therefore made to remove NADH oxidase and to purify the enzyme. However, these attempts were unsuccessful. The problems were two-fold: first, experience in this laboratory with A. lwoffi has shown that cell-free extracts contain material which interferes with

various fractionation procedures; secondly, the enzyme was very unstable during purification.

The following conventional techniques of enzyme purification have been examined: removal of nucleic acids either by precipitation with protamine or digestion with ribonuclease or a mixture of ribonuclease and deoxyribonuclease, salt fractionation with ammonium sulphate, freezing and thawing, isoelectric precipitation, enzyme precipitation with protamine, high-speed centrifugation, ion-exchange chromatography on DEAE-cellulose and gel filtration on Sepharose 4B and Sepharose 6B. None of these methods resulted in significant increases in specific activity, nor did they separate NADH oxidase and pyruvate dehydrogenase activities from each other.

Use of "aged" cells

A useful procedure was found which minimised the interference by NADH oxidase with pyruvate dehydrogenase assays. Stationary phase cells were left shaking for a further 12 h and extracts prepared from them; these showed little or no NADH oxidase activity and pyruvate dehydrogenase was readily assayed. A closer examination of this procedure showed that the NADH oxidase activity decreased from 105 units/mg. protein at the beginning of stationary phase, to 60.0 units/mg. protein by late stationary phase, and then dramatically to 10.8 units/mg protein on "ageing". Pyruvate dehydrogenase activity, on the other hand, remained constant throughout stationary phase at approximately 11 units/mg protein and remained virtually unchanged on "ageing".

Extracts prepared from these "aged" cells were particularly useful in the examination of pyruvate dehydrogenase activity in the presence of added NADH.

(b) Adenylate control of pyruvate dehydrogenase

The initial observation that AMP, ADP and ATP produced a graded response, in the physiologically meaningful direction, prompted a more thorough examination of this apparent regulatory phenomenon. First, the dependence of enzyme activation on adenine nucleotide concentration was examined with 2mM pyruvate (Fig. 5). AMP and ADP were stimulatory, producing maximum activations of 6.0-fold and 3.0-fold, respectively; ATP was inhibitory. These responses were brought about by 0.5mM nucleotides. Next, the effects of these nucleotides on the rate dependence on pyruvate concentration was examined (Fig. 6). The Km values for pyruvate, determined from double reciprocal plots, were

3.0mM without adenine nucleotides and 0.46mM, 1.8mM and 5.6mM, with AMP, ADP and ATP, respectively. AMP and ADP also increased V_{max} from 44.4 units/mg protein without adenine nucleotides to 89.4 units/mg protein and 71.5 units/mg protein, respectively; ATP, on the other hand, decreases V_{max} to 41.7 units/mg protein.

Effect of AMP on Escherichia coli pyruvate dehydrogenase activity

For comparative purposes, the effect of AMP on E. coli pyruvate dehydrogenase activity was investigated by examining the effect of 0.5mM AMP on the dependence of enzyme activity on pyruvate concentration (Fig. 7). AMP was stimulatory and double reciprocal plots showed that with AMP, V_{max} increased from 88.0 units/mg protein to 135 units/mg protein; the pyruvate Km was 0.67mM both with and without AMP.

Dependence of enzyme activation on energy charge

The differential response of A. lwoffi pyruvate dehydrogenase to the three adenine nucleotides prompted an investigation of the dependence of enzyme activity on energy charge

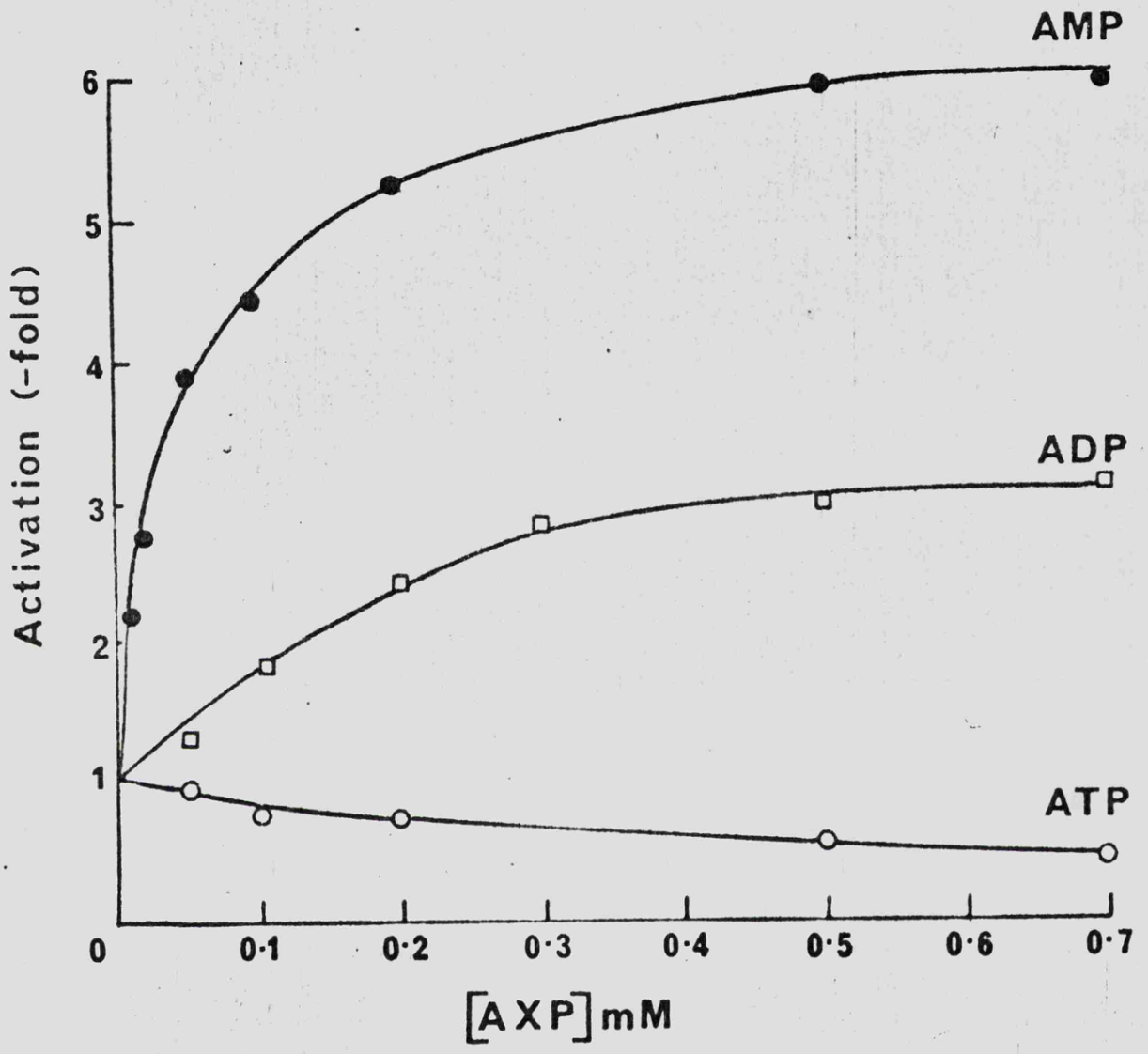

Figure 5. Dependence of the activation of A. lwoffi pyruvate dehydrogenase on adenine nucleotide concentration.

Assays were performed under standard conditions with 2mM pyruvate and AMP (●), ADP (□), or ATP (○).

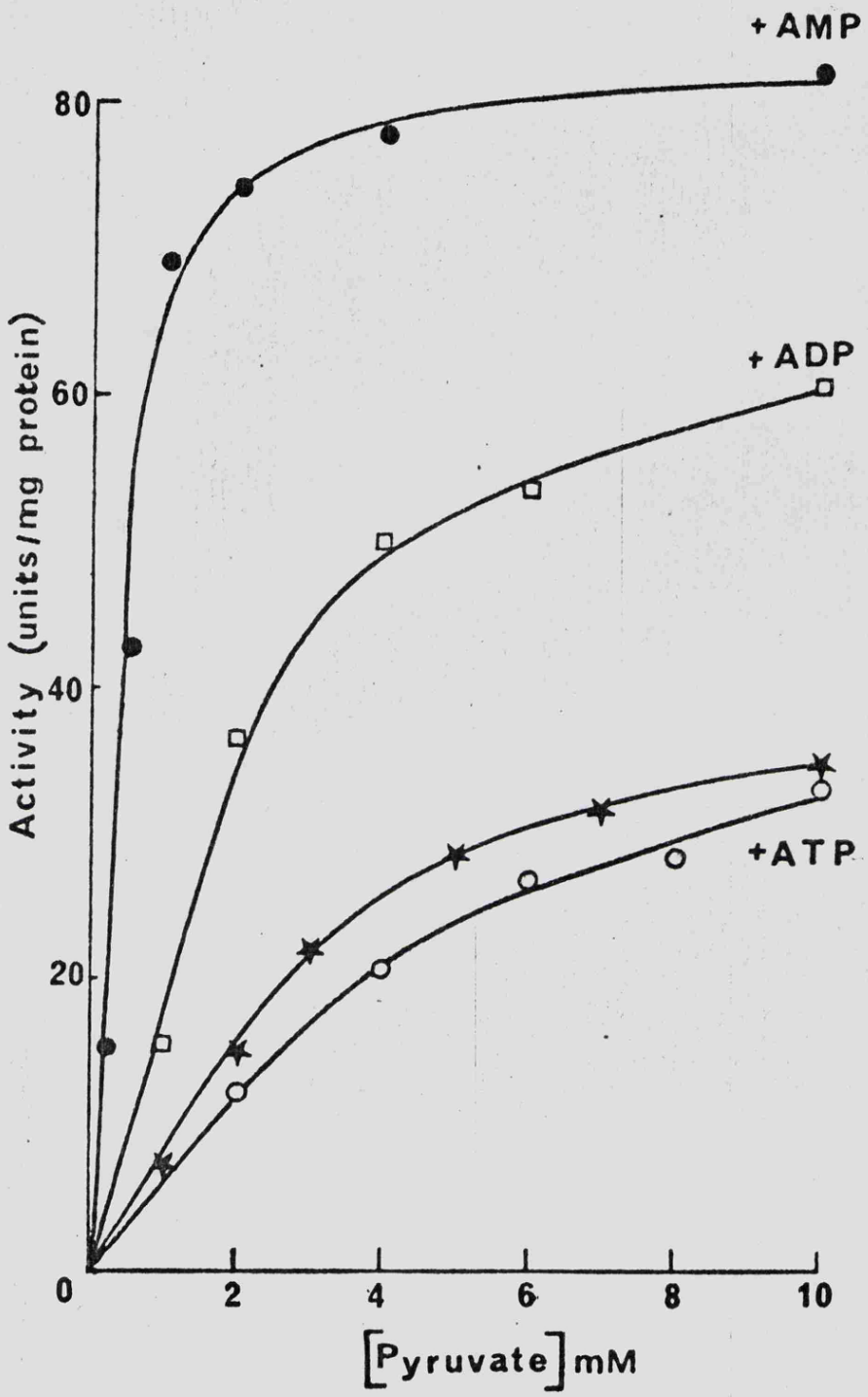

Figure 6. Effects of adenine nucleotides on the dependence of A. lwoffi pyruvate dehydrogenase activity on pyruvate concentration.

Assays were performed under standard conditions, without adenylates (✱), or with 0.5mM AMP (●), 0.5mM ADP (□), or 0.5mM ATP (○).

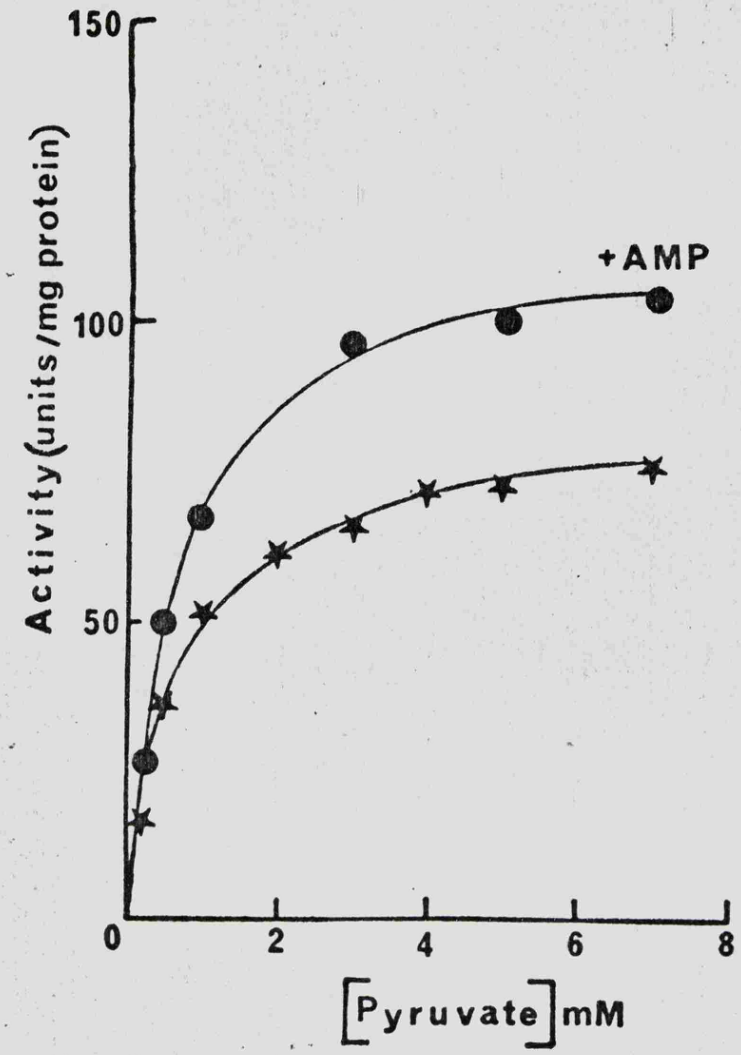

Figure 7. Effect of AMP on the dependence of E. coli pyruvate dehydrogenase activity on pyruvate concentration

Assays were performed under standard conditions, in the absence (✱), or presence (●) of 0.5mM AMP.

(Atkinson, 1968 a). Fig. 8 shows this dependence examined with 5mM total nucleotide concentration and 2mM pyruvate. Mixtures of varying energy charge were produced as described. Maximum activity was achieved at energy charge values between 0 and 0.7 and a marked reduction in activity and ultimately inhibition was found in the region 0.8 to 1.0.

Influence of pH on the dependence of pyruvate dehydrogenase activity on pyruvate concentration. Effect of AMP

The effect of pH on the pyruvate K_m was investigated by examining the rate-dependence on pyruvate concentration in Tris-HCl buffers varying from pH 7.0 to pH 8.5 (Fig. 9). The K_m values for pyruvate determined from double reciprocal plots were 0.58mM at pH 7.0, 0.74mM at pH 7.5, 3.0mM at pH 8.0 and 5.5mM at pH 8.5. When 0.5mM AMP was included in these assays (Fig 10), the corresponding values for the pyruvate K_m were 0.15mM, 0.20mM, 0.46mM and 0.77mM.

pH Optimum

The pH dependence of enzyme activity was examined with 20mM pyruvate (Fig. 11) and irrespective of the presence of AMP, a pH optimum of 8.0 was obtained.

Dependence of enzyme activity on the nature of the buffer

In these studies, pyruvate dehydrogenase activity was assayed in 0.15M Tris-HCl buffer. However, when the Tris-HCl concentration was decreased, a marked stimulation of enzyme activity was observed. Fig. 12 shows the dependence of enzyme activity on buffer concentration for a range of buffers at pH 8.0 with and without 0.5mM AMP. In general, enzyme activity decreased as the buffer concentration was increased, although the degree of inhibition varied with the buffer used. For each buffer, at a particular

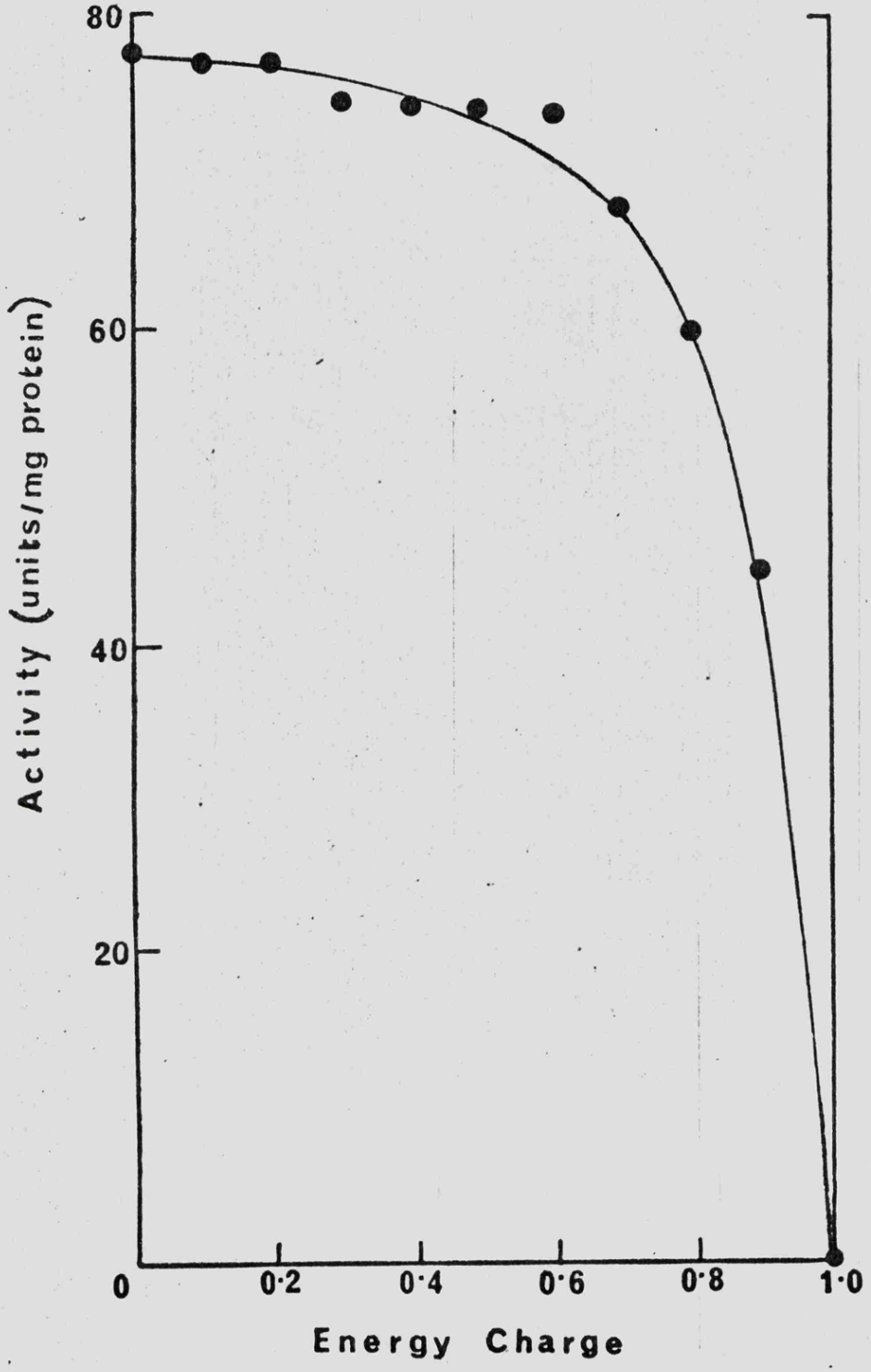

Figure 8. Dependence of A. lwoffi pyruvate dehydrogenase activity on energy charge

Assays were performed under standard conditions with 5mM total adenine nucleotide concentration and 2mM pyruvate.

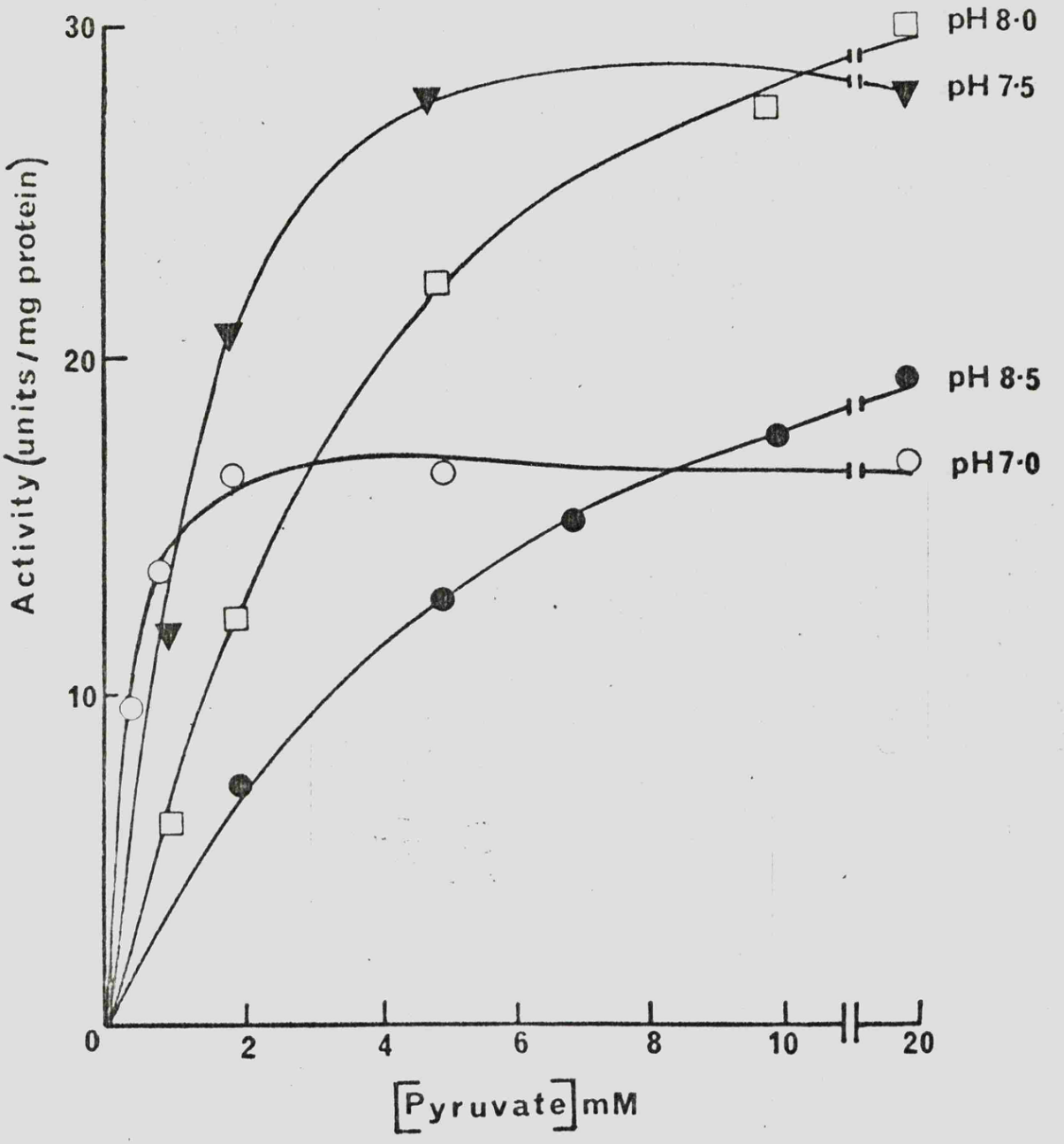

Figure 9 Effect of pH on the dependence of A. lwoffi pyruvate dehydrogenase activity on pyruvate concentration

Assays were performed in 0.15mM Tris-HCl buffers at pH 7.0 (O), pH 7.5 (▼), pH 8.0 (□), or pH 8.5 (●), under otherwise standard conditions.

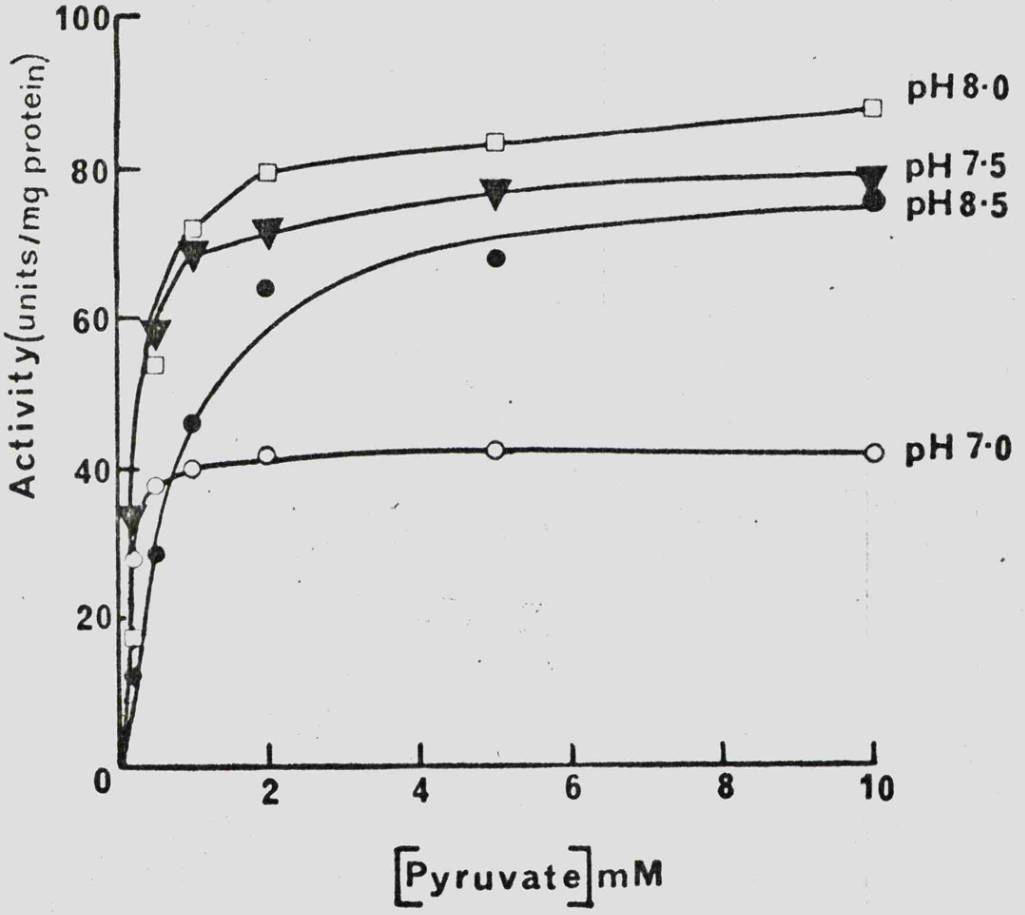

Figure 10 Effect of pH on the dependence of A. lwoffi pyruvate dehydrogenase activity on pyruvate concentration in the presence of AMP

Assays were performed as described for Figure 9, but in the additional presence of 0.5mM AMP.

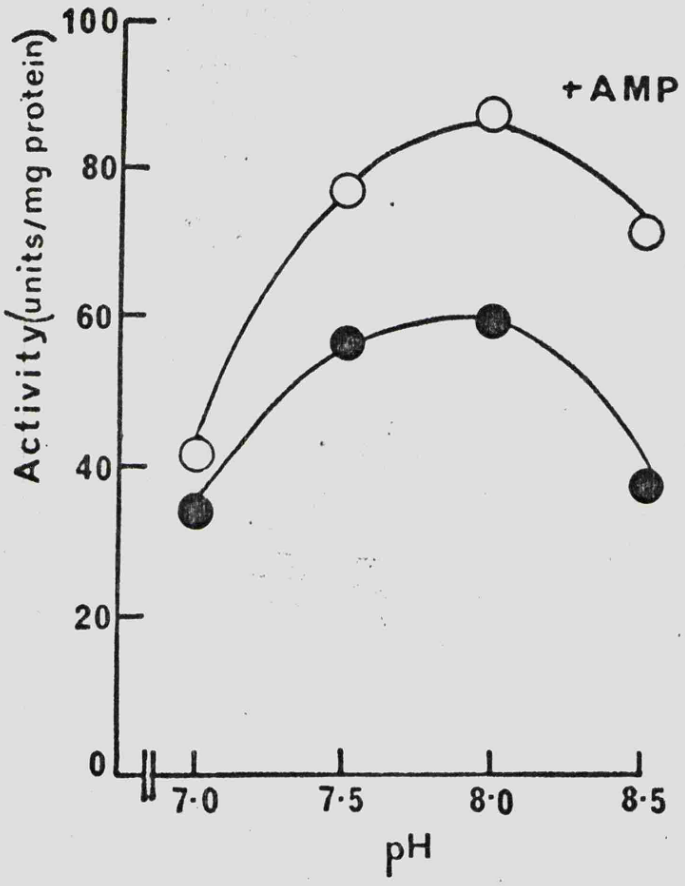

Figure 11 Dependence of A. lwoffi pyruvate dehydrogenase activity on pH

Assays were performed under standard conditions in 0.15mM Tris-HCl buffers with 20mM pyruvate in the absence (●) or presence (O) of 0.5mM AMP.

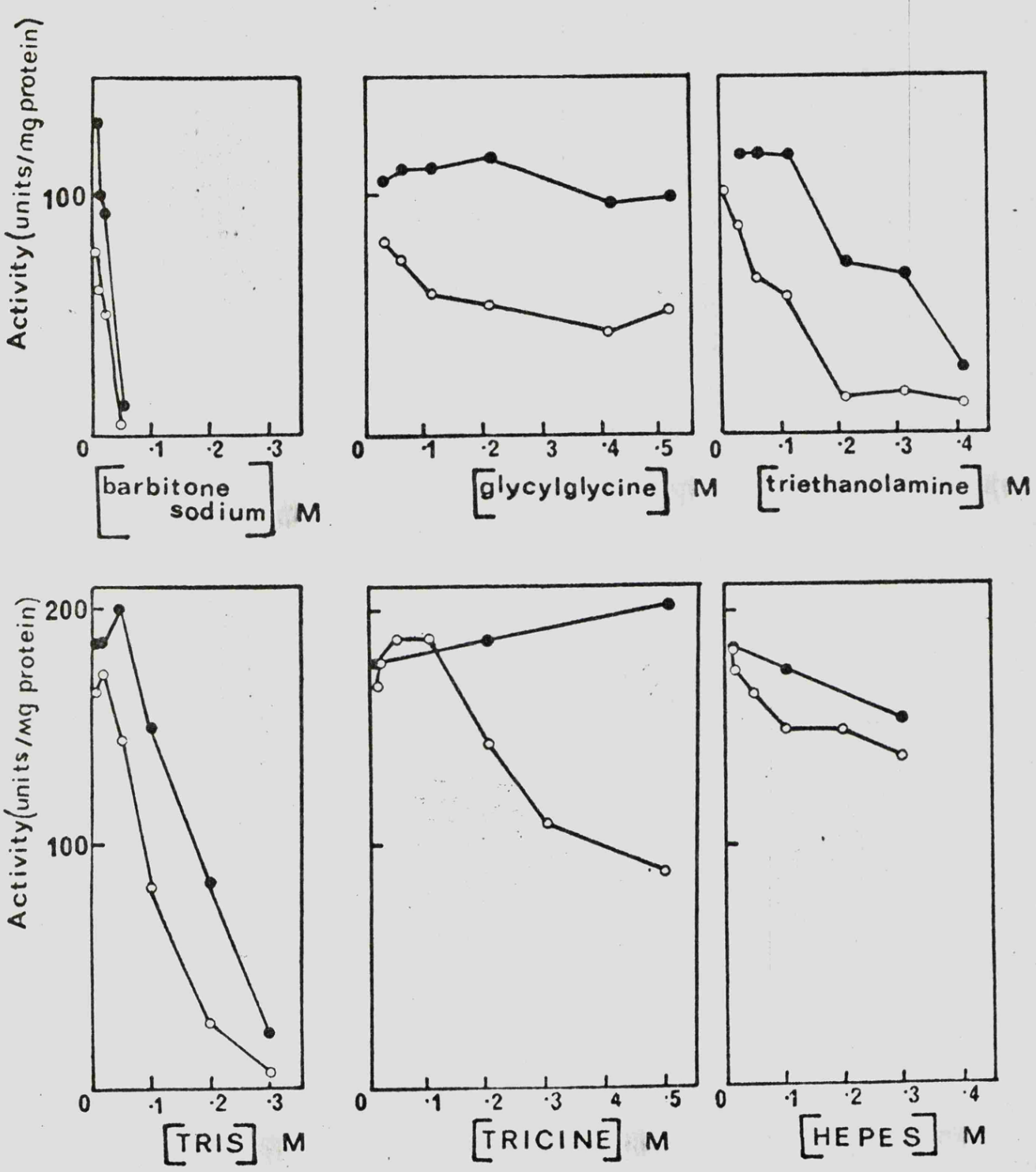

Figure 12 Dependence of A. lwoffi pyruvate dehydrogenase activity on buffer concentration for a range of buffers

Assays were performed in the buffers above with 10mM pyruvate, in the absence (O), or presence (●) of 0.5mM AMP, under otherwise standard conditions.

Tricine: N-Tris(hydroxymethyl)methylglycine.
Hepes: N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid.

concentration, the activity with AMP was usually greater than in its absence.

(c) Product inhibition of pyruvate dehydrogenase

Acetyl-CoA and NADH, products of the pyruvate dehydrogenase reaction, are potent inhibitors of the enzyme from various sources (See DISCUSSION). Preliminary experiments indicated that such inhibitions also occurred with A. lwoffi pyruvate dehydrogenase and were thus investigated in more detail.

Inhibition by acetyl-CoA

The dependence of inhibition on acetyl-CoA concentration was examined with 10mM pyruvate (Fig. 13). Without AMP, the inhibition increased with increasing acetyl-CoA concentration in a hyperbolic manner; however with AMP, the extent of inhibition decreased over the whole range of acetyl-CoA concentration and the dependence was sigmoid. Virtually no inhibition was observed below 0.1mM acetyl-CoA, but between 0.1mM and 0.2mM acetyl-CoA, the inhibition increased sharply. Thus, whereas without AMP, 50% inhibition was achieved with 0.075mM acetyl-CoA, in its presence, 0.24mM acetyl-CoA was required. An examination of the dependence of enzyme inhibition on acetyl-CoA concentration with increasing concentrations of AMP (Fig. 14) showed a reduced maximum inhibition and an enhanced sigmoidicity of the dependence.

The kinetics of acetyl-CoA inhibition were next investigated. The dependence of enzyme activity on CoA and pyruvate concentrations was examined, with and without 0.058mM acetyl-CoA. This concentration of effector produced 45% inhibition of enzyme activity assayed under standard conditions with 10mM pyruvate. Figs. 15 and 16 illustrate the respective rate

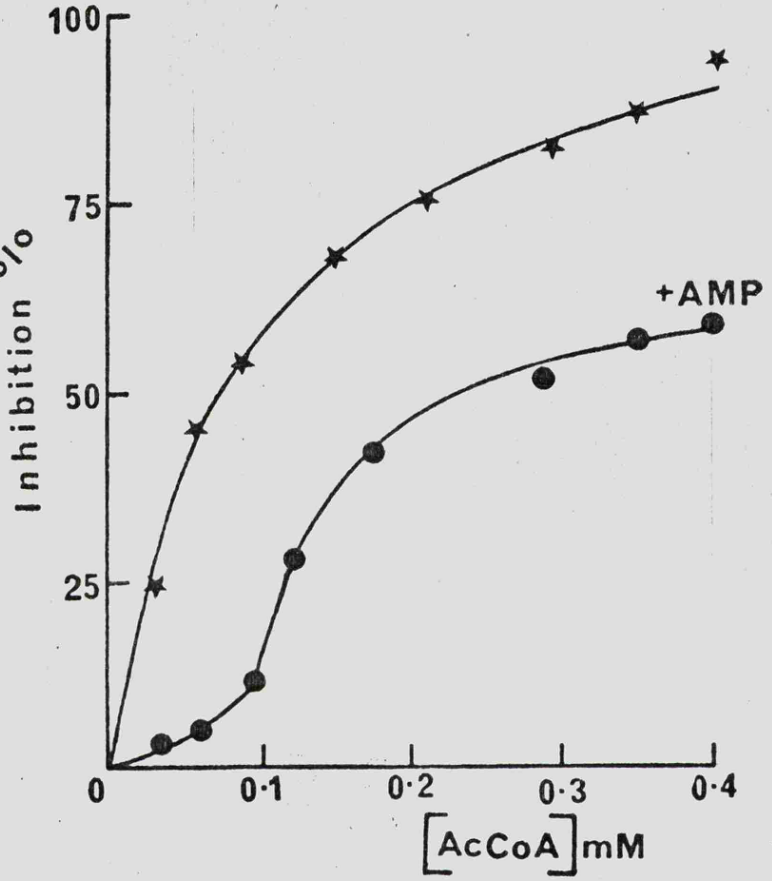

Figure 13 Dependence of inhibition of A. lwoffi pyruvate dehydrogenase on acetyl-CoA concentration

Assays were performed under standard conditions with 10mM pyruvate, in the absence (✦) or presence (●) of 0.5mM AMP.

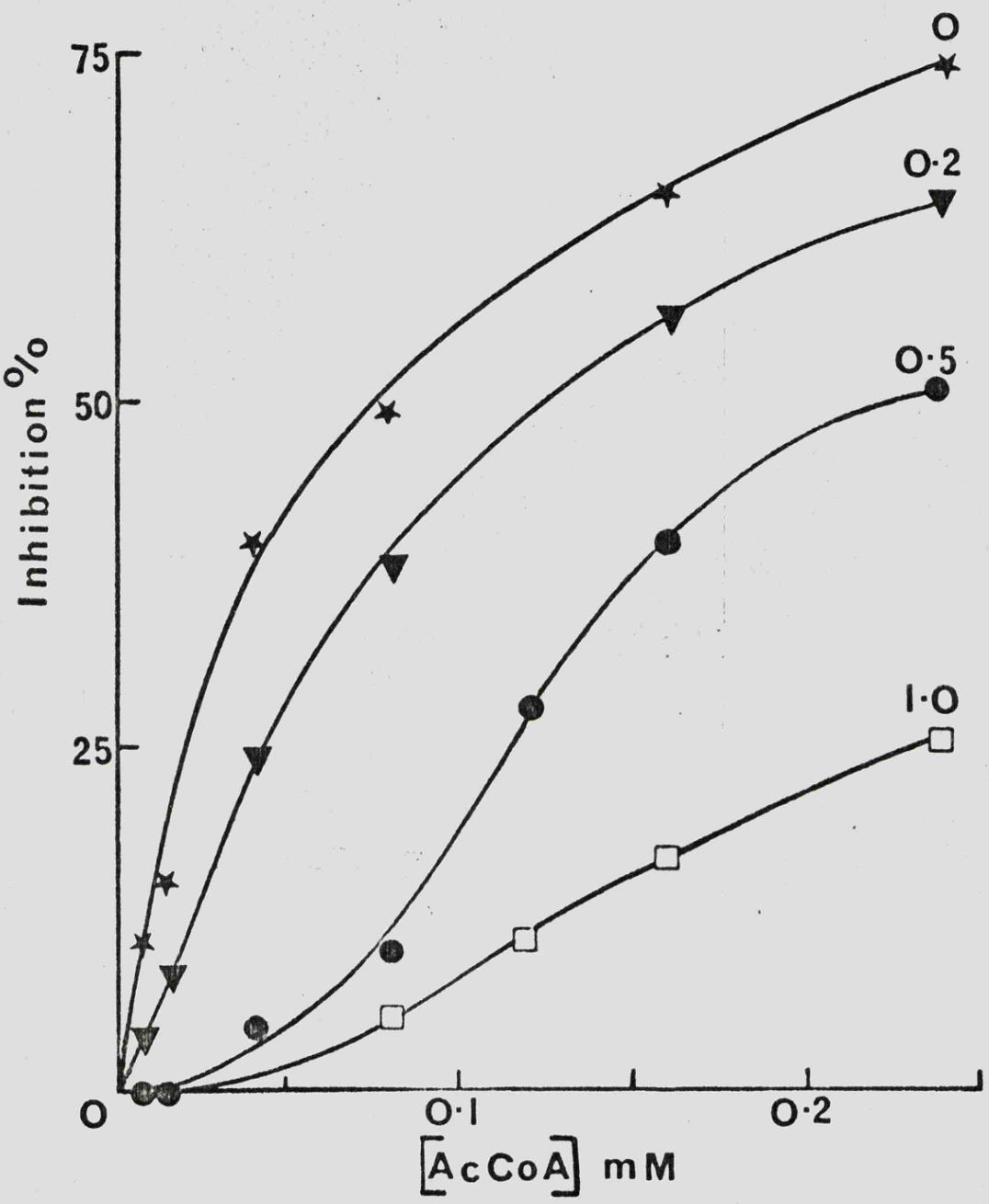

Figure 14 Effect of increasing AMP concentration on the dependence of inhibition of A. lwoffi pyruvate dehydrogenase on acetyl-CoA concentration

Assays were performed under standard conditions with 10mM pyruvate, without AMP (✱), or with 0.2mM AMP (▼), 0.5mM AMP (●) or 1.0mM AMP (□).

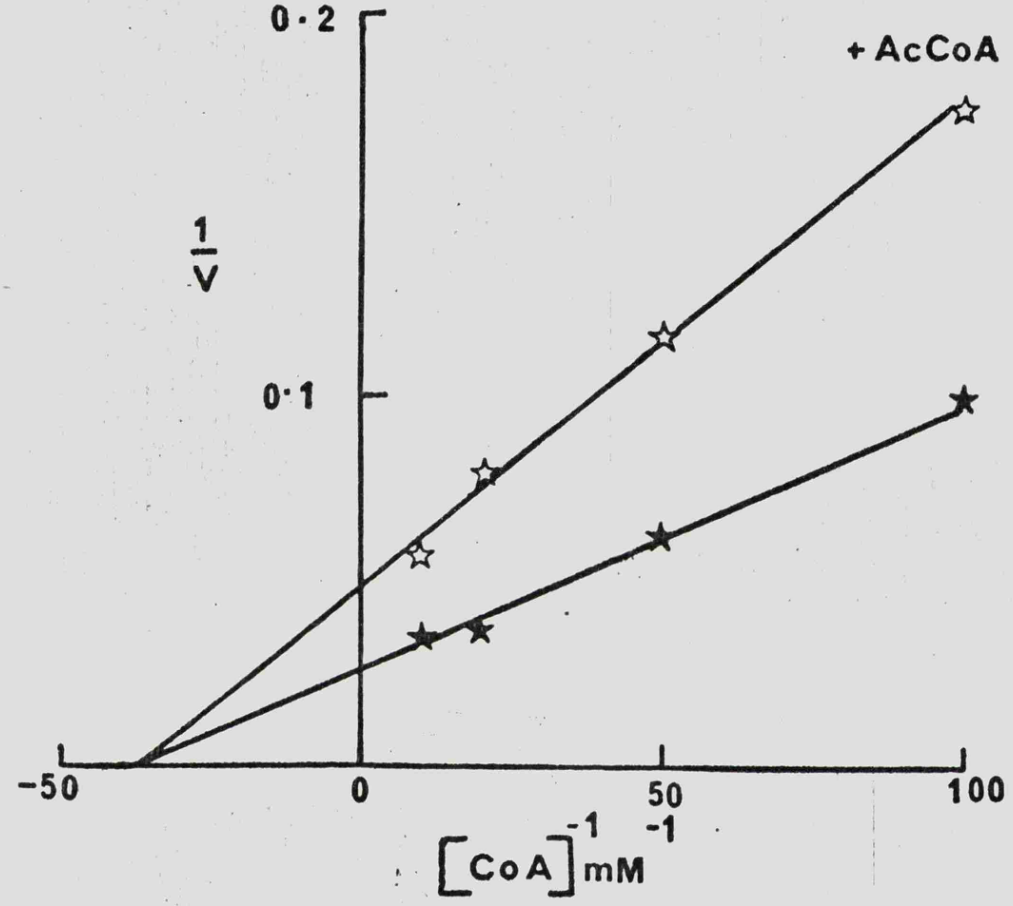

Figure 15 Effect of acetyl-CoA on the dependence of A. lwoffi pyruvate dehydrogenase activity on CoA concentration

Assays were performed under standard conditions with 10mM pyruvate, in the absence (★) or presence (☆) of 0.058mM acetyl-CoA. The results are presented in the form of double reciprocal plots.

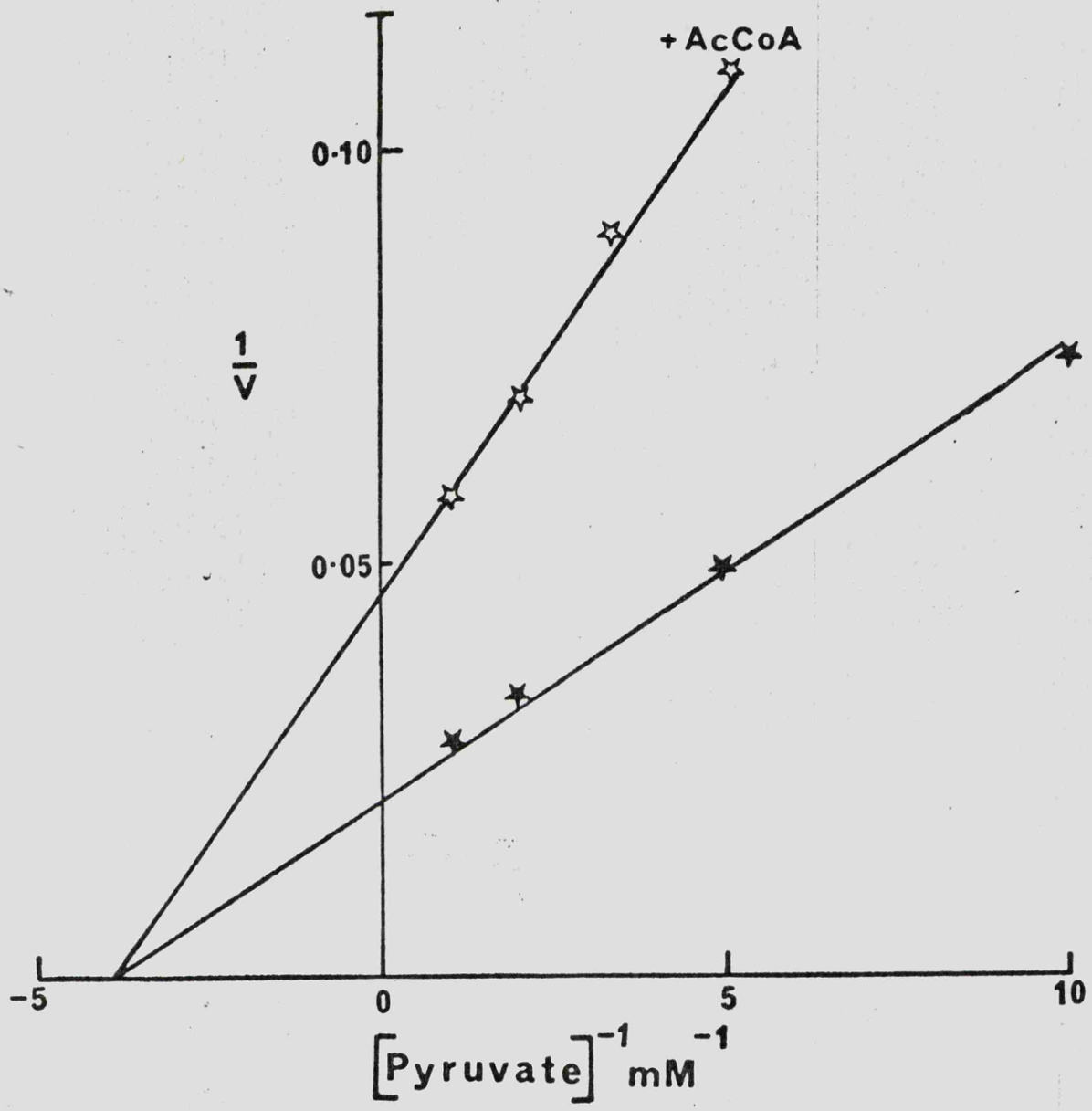

Figure 16 Effect of acetyl-CoA on the dependence of A. lwoffi pyruvate dehydrogenase activity on pyruvate concentration

Assays were performed under standard conditions, in the absence (★), or presence (☆) of 0.058mM acetyl-CoA. The results are presented in the form of double reciprocal plots.

dependences on CoA and pyruvate concentration, the results being presented in the form of double reciprocal plots.

Inhibition by NADH

The dependence of inhibition on NADH concentration was examined at 0.1mM NAD^+ concentration, with and without 0.5mM AMP: the results are presented in Fig.17. The dependence was hyperbolic and 50% inhibition was achieved with 0.2mM NADH. In order to investigate whether AMP had any effect on this dependence, the percentage inhibition computed by comparing the activity in the presence of NADH + AMP, with the activity in the presence of AMP, was contrasted with the percentage inhibition calculated by comparing the activity in the presence of NADH, with the activity in the absence of effectors. There was no difference in these two percentage inhibitions.

The effect of 0.1mM NADH on the dependence of enzyme activity on NAD^+ and pyruvate concentrations was next investigated. The results are presented in the form of double reciprocal plots in Figs.18 and 19 respectively.

(d) Activation of pyruvate dehydrogenase by divalent anions

Initial experiments investigating the dependence of enzyme activation on energy charge produced an unexpected result. At an energy charge value of 1.0, at which ATP is the only nucleotide present, activation rather than inhibition was observed. Commercial adenylate kinase was supplied as a suspension in 3.2M ammonium sulphate and further experiments showed that the stimulation at energy charge 1.0 was due to the presence of ammonium sulphate. When 2mM ammonium sulphate, ammonium chloride,

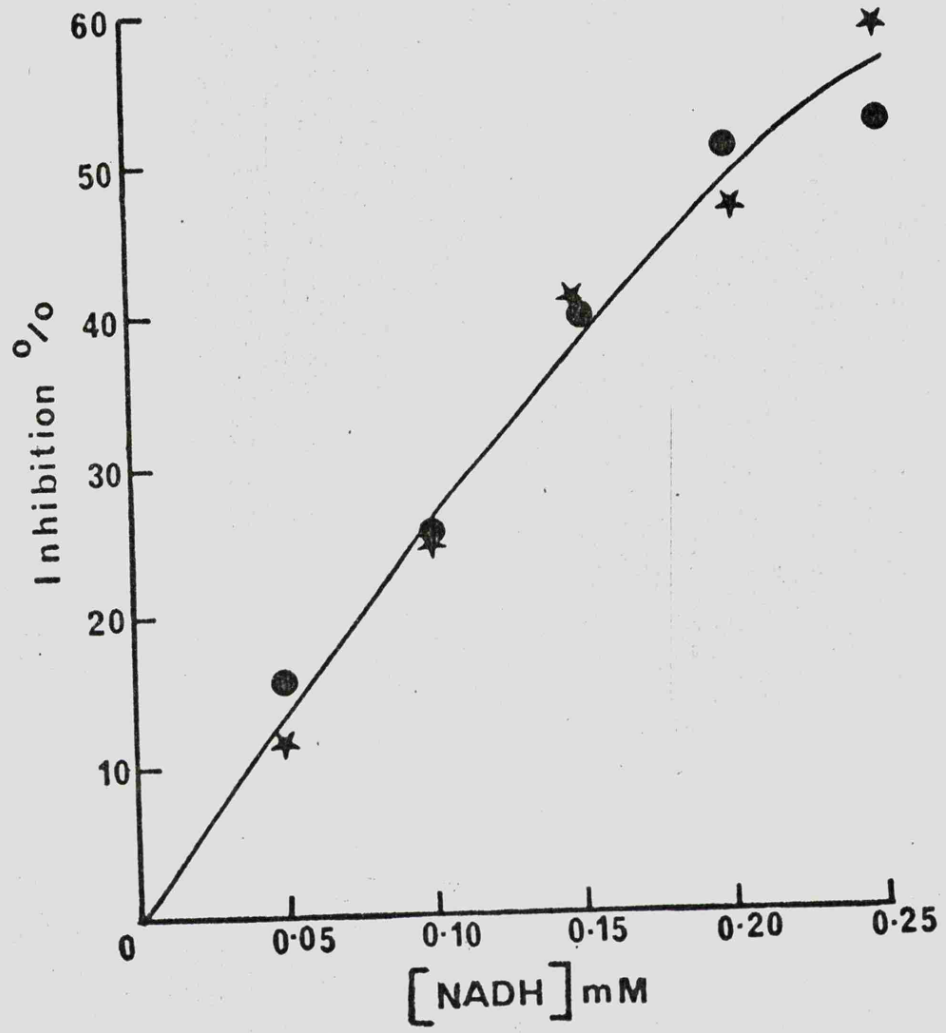

Figure 17 Dependence of inhibition of A. lwoffi pyruvate dehydrogenase on NADH concentration

Assays were performed with 0.1mM NAD^+, in the absence (✱), or presence (●) of 0.5mM AMP, under otherwise standard conditions.

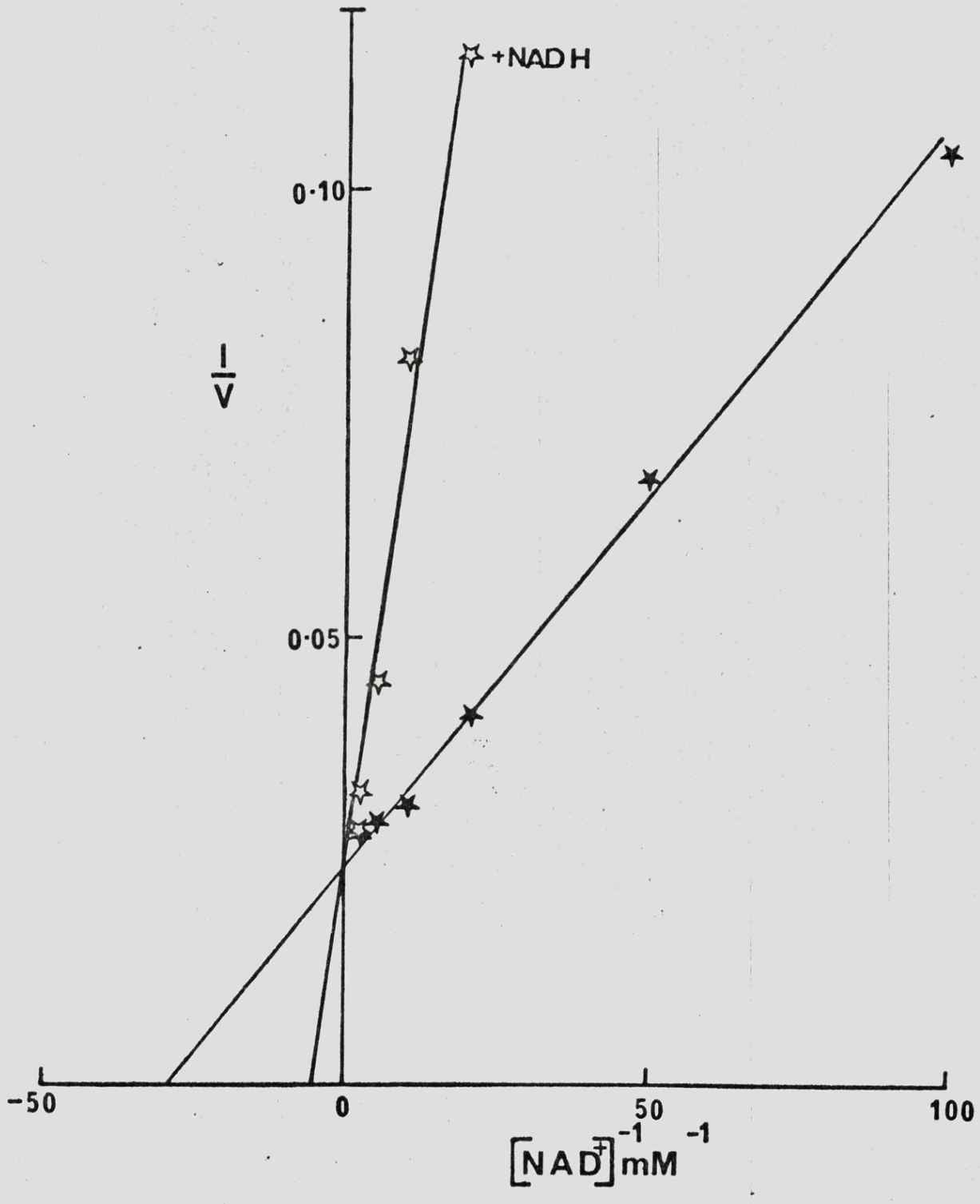

Figure 18 Effect of NADH on the dependence of A. lwoffi pyruvate dehydrogenase activity on NAD^+ concentration

Assays were performed under standard conditions with 10mM pyruvate, in the absence (★), or presence (☆) of 0.1mM NADH. The results are presented in the form of double reciprocal plots.

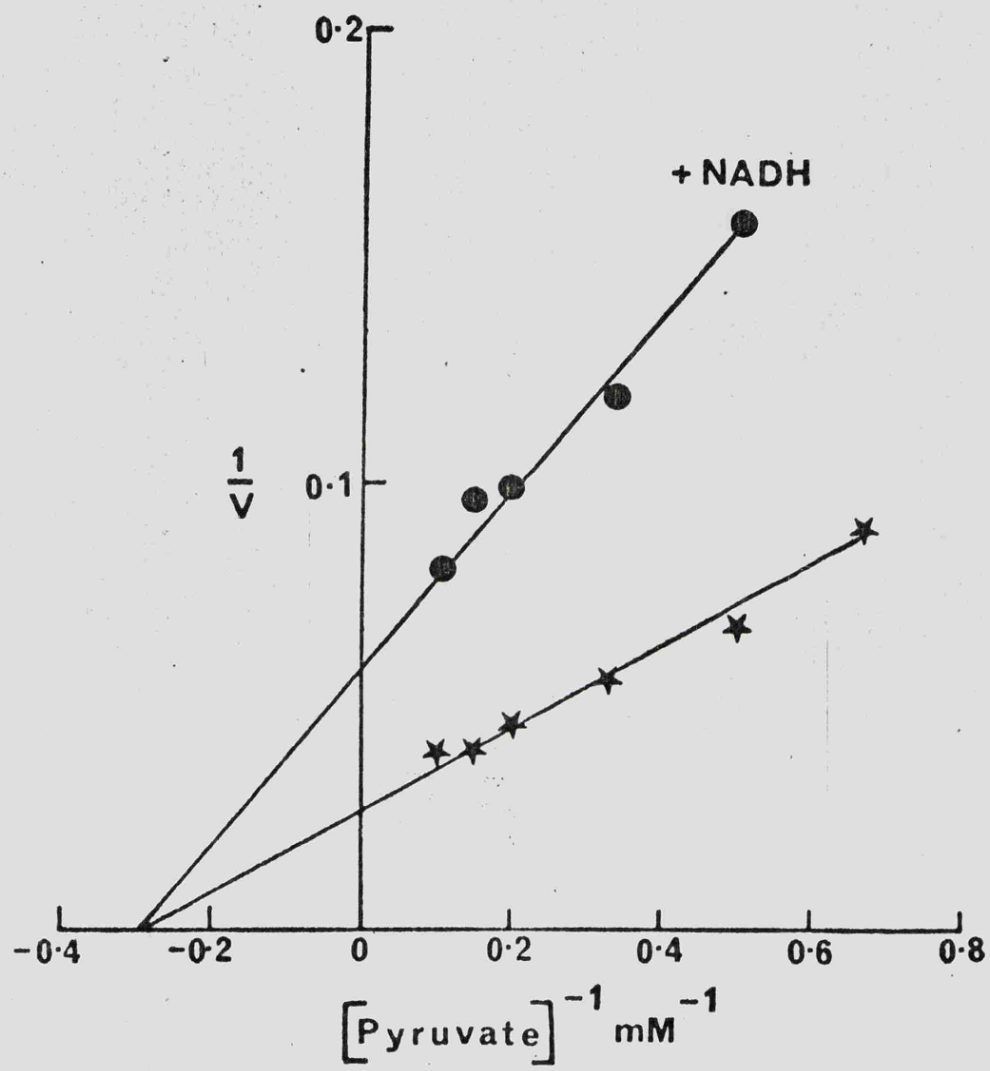

Figure 19 Effect of NADH on the dependence of A. lwoffi pyruvate dehydrogenase activity on pyruvate concentration

Assays were performed with 0.1mM NAD^+, in the absence (✶), or presence (●) of 0.1mM NADH, under otherwise standard conditions. The results are presented in the form of double reciprocal plots.

potassium sulphate or potassium chloride were included in assays performed with 2mM pyruvate, both potassium sulphate and ammonium sulphate stimulated the enzyme activity, whereas ammonium chloride and potassium chloride had little or no effect. The activations produced by potassium sulphate and ammonium sulphate were comparable, 4.2-fold and 4.1-fold respectively, suggesting that the activations observed above were due to sulphate anions. Other anions were therefore examined for their ability to stimulate A. lwoffi pyruvate dehydrogenase activity. Table 3 illustrates the results obtained with inorganic anions and shows that only divalent anions were stimulatory. The inhibition observed in the presence of pyrophosphate was probably due to the chelation and removal of Mg^{2+} ions which are essential for enzyme activity. When organic anions were examined (Table 4), activation was only observed with divalent anions, but not all divalent organic anions were stimulatory; only oxalate, malate, fumarate and phthalate were activators.

This unusual anion activation was therefore investigated in greater detail using sulphate, oxalate and phosphate as representative anions. Fig. 20 shows the dependence of enzyme activation on anion concentration assayed with 2mM pyruvate. The maximum activations achieved by sulphate and oxalate were similar (5.5-fold and 5.2-fold respectively), whereas the maximum activation achieved by phosphate was 3.6-fold. These activations were brought about by 5mM sulphate, 7mM oxalate and 10mM phosphate respectively.

The effects of these anions on the rate-dependence on pyruvate concentration was next investigated (Fig. 21). The Km values for pyruvate obtained from double reciprocal plots were 3.0mM, without effectors, and 0.56mM, 0.56mM, and 1.7mM, with

TABLE 3

The effect of inorganic anions on

A. lwoffi pyruvate dehydrogenase activity

SALT	PRODOMINANT ANIONIC SPECIES AT pH 8.0	ACTIVATION (-FOLD)
None	-	1.0
Ammonium chloride	Cl^-	1.1
Potassium chloride	Cl^-	1.1
Sodium nitrate	NO_3^-	1.0
Sodium bicarbonate	HCO_3^-	1.1
Ammonium sulphate	SO_4^{2-}	4.1
Potassium sulphate	SO_4^{2-}	4.2
Sodium sulphate	SO_4^{2-}	4.1
Disodium hydrogen phosphate	HPO_4^{2-}	1.8
Sodium sulphite	SO_3^{2-}	1.6
Sodium arsenate	$HAsO_4^{2-}$	1.5
Potassium metabisulphite	$S_2O_5^{2-}$	2.3
Sodium thiosulphate	$S_2O_3^{2-}$	4.3
Sodium molybdate	MoO_4^{2-}	7.4
Sodium pyrophosphate	$HP_4O_7^{3-}$	0.7

Assays were performed under standard conditions with 2mM pyruvate and 2mM salt concentration.

Each value represents the average of three measurements.

TABLE 4

The effect of organic anions on

A. lwoffi pyruvate dehydrogenase activity

SALT	ACTIVATION (-FOLD)
None	1.0
Sodium acetate	1.0
Sodium propionate	1.0
Sodium n-butyrate	1.0
Sodium iso-valerate	1.0
Sodium benzoate	1.1
Sodium oxalate	3.5
Sodium malonate	1.1
Sodium succinate	1.0
Sodium malate	1.8
Sodium maleate	1.0
Sodium fumarate	1.7
Sodium phthalate	4.5
Sodium citrate	1.0

Assays were performed under standard conditions with 2mM pyruvate and 2mM salt concentration.
Each value represents the average of three measurements.

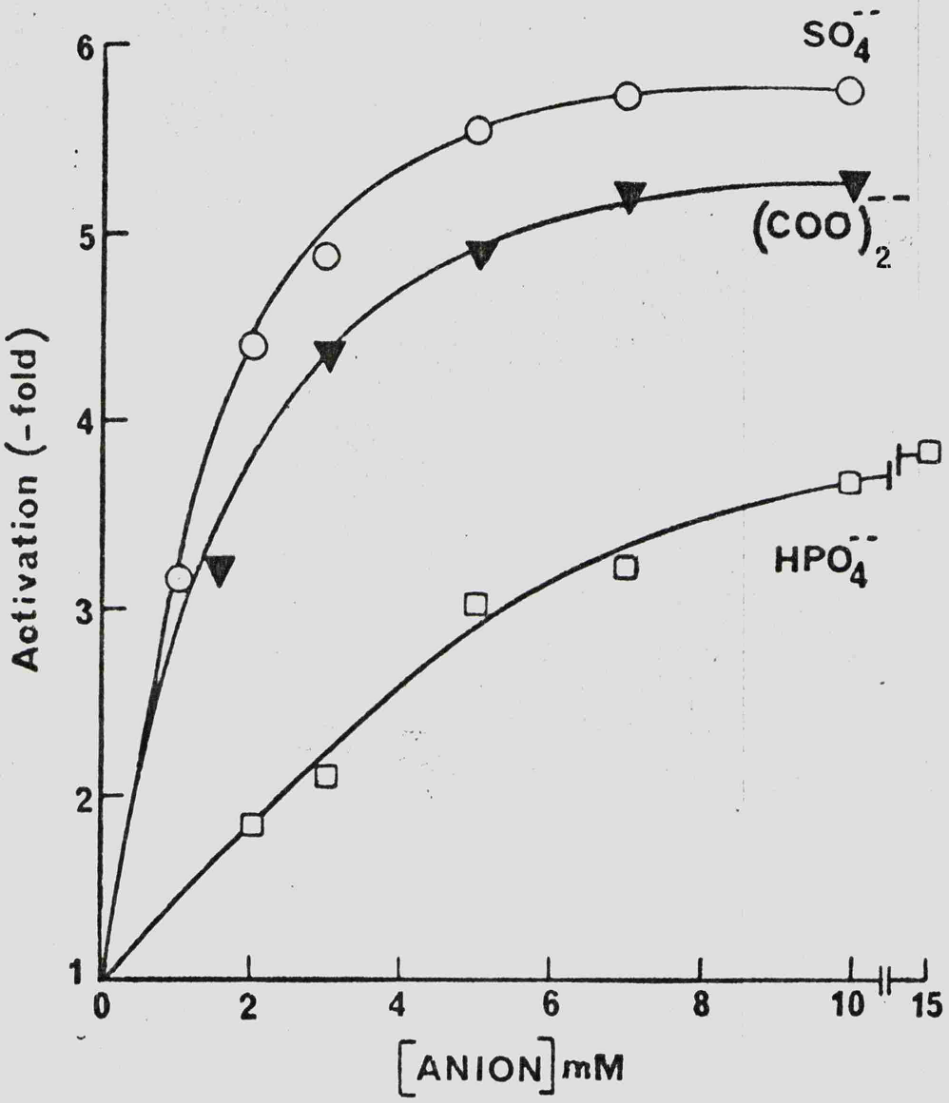

Figure 20 Dependence of activation of A. lwoffi pyruvate dehydrogenase on anion concentration

Assays were performed under standard conditions with 2mM pyruvate and SO_4^{2-} (○), $(COO)_2^{2-}$ (▼), or HPO_4^{2-} (□).

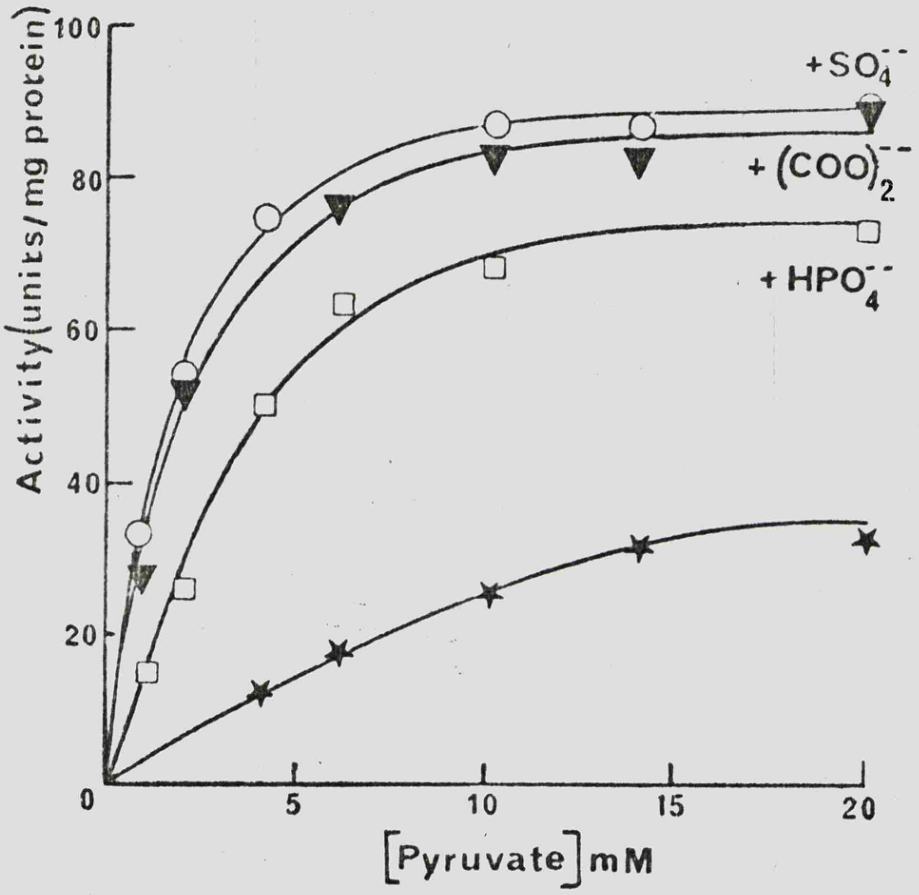

Figure 21 Effect of divalent anions on the dependence of A. lwoffi pyruvate dehydrogenase activity on pyruvate concentration

Assays were performed under standard conditions without anions (✱), or with 5mM SO_4^{2-} (O), 7mM $(COO)_2^{2-}$ (▼), or 10mM HPO_4^{2-} (□).

sulphate, oxalate and phosphate, respectively. These anions also increased V_{max}, phosphate being less effective than sulphate or oxalate, which were comparable.

The effect of sulphate on the acetyl-CoA inhibition of the enzyme

AMP affected A. lwoffi pyruvate dehydrogenase in two ways: the nucleotide activated the enzyme, primarily by decreasing the K_m for pyruvate and also partially relieved the inhibition produced by acetyl-CoA. In view of the similar effects produced by AMP and divalent anions in activating the enzyme, the effect of sulphate (as representative of these anions) on acetyl-CoA inhibition was investigated (Fig. 22). For comparison, the results with AMP have been included. With sulphate the inhibition was decreased over the whole range of acetyl-CoA concentration and dependence became sigmoid. At the concentrations of AMP and sulphate chosen (0.5mM and 5mM respectively), these effectors were almost equally active in relieving the acetyl-CoA inhibition and in converting the dependence from a hyperbolic to a sigmoid form; however, AMP was present at one-tenth the concentration of sulphate.

Non-additivity of the stimulations produced by AMP and sulphate

Because AMP and divalent anions affected A. lwoffi pyruvate dehydrogenase in a similar manner, a common binding site on the enzymes molecule was suggested for these effectors. If this was the case, one would not expect the stimulations produced by AMP and sulphate to be additive. The non-additivity of these two effects was demonstrated in the following way. The dependence of enzyme activation on either AMP or sulphate concentration was examined and the results are shown in Fig. 23(a). Although the results have been displayed on the same axes to illustrate the

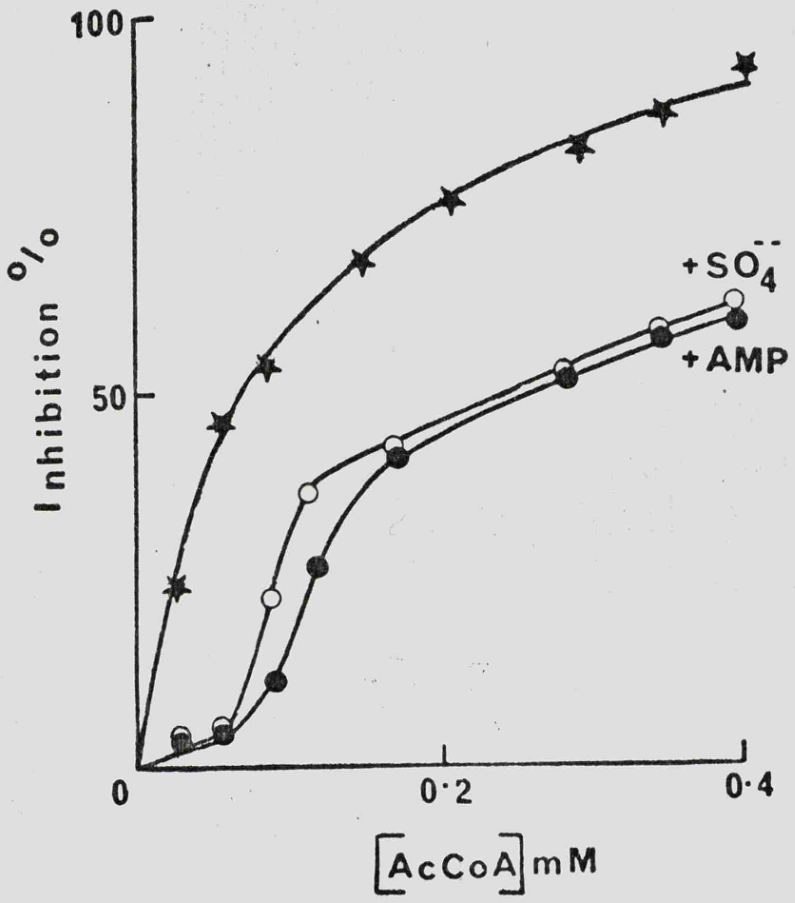

Figure 22 Effect of sulphate on the dependence of inhibition of A. lwoffi pyruvate dehydrogenase on acetyl-CoA concentration

Assays were performed under standard conditions with 10mM pyruvate, in the absence (✱), or presence (O) of 5mM SO_4^{2-}. The results with 0.5mM AMP are included for comparison(●).

similarity of the dependences on effector concentration, nevertheless AMP was considered the more potent activator because ten-times more sulphate was required to achieve comparable activation. Fig. 23(b) shows the effect of increasing the sulphate concentration in assay mixtures until half the maximum activation was achieved. The sulphate concentration was then kept constant at this value (1mM) and increasing quantities of AMP were added until the enzyme was fully activated. The maximum activation thereby achieved was similar to that produced by either AMP or sulphate alone.

(e) Taxonomic division of bacterial species and the regulation of their pyruvate dehydrogenases

The difference in response of E. coli and A. lwoffi pyruvate dehydrogenases to AMP prompted an examination of the effect of AMP on the pyruvate dehydrogenase activity of a large number of bacterial species. This investigation was undertaken to examine whether differences in regulatory behaviour could be correlated with the taxonomic classification of organisms. Crude enzyme extracts were prepared from nutrient-grown cells (Methods 2 and 3(a)) and the sensitivity of pyruvate dehydrogenases to AMP was examined with 2mM pyruvate and 0.5mM AMP. Enzymes were considered sensitive to AMP if a minimum activation of 1.5-fold was achieved. Table 5 illustrates the results of these experiments and shows that none of the pyruvate dehydrogenases of Gram-positive bacteria, or Gram-negative facultatively anaerobic bacteria exhibited AMP sensitivity. Gram-negative strictly aerobic species, on the other hand, could be sub-divided into two groups - those showing pyruvate dehydrogenase sensitive to AMP, and those whose enzymes were unaffected.

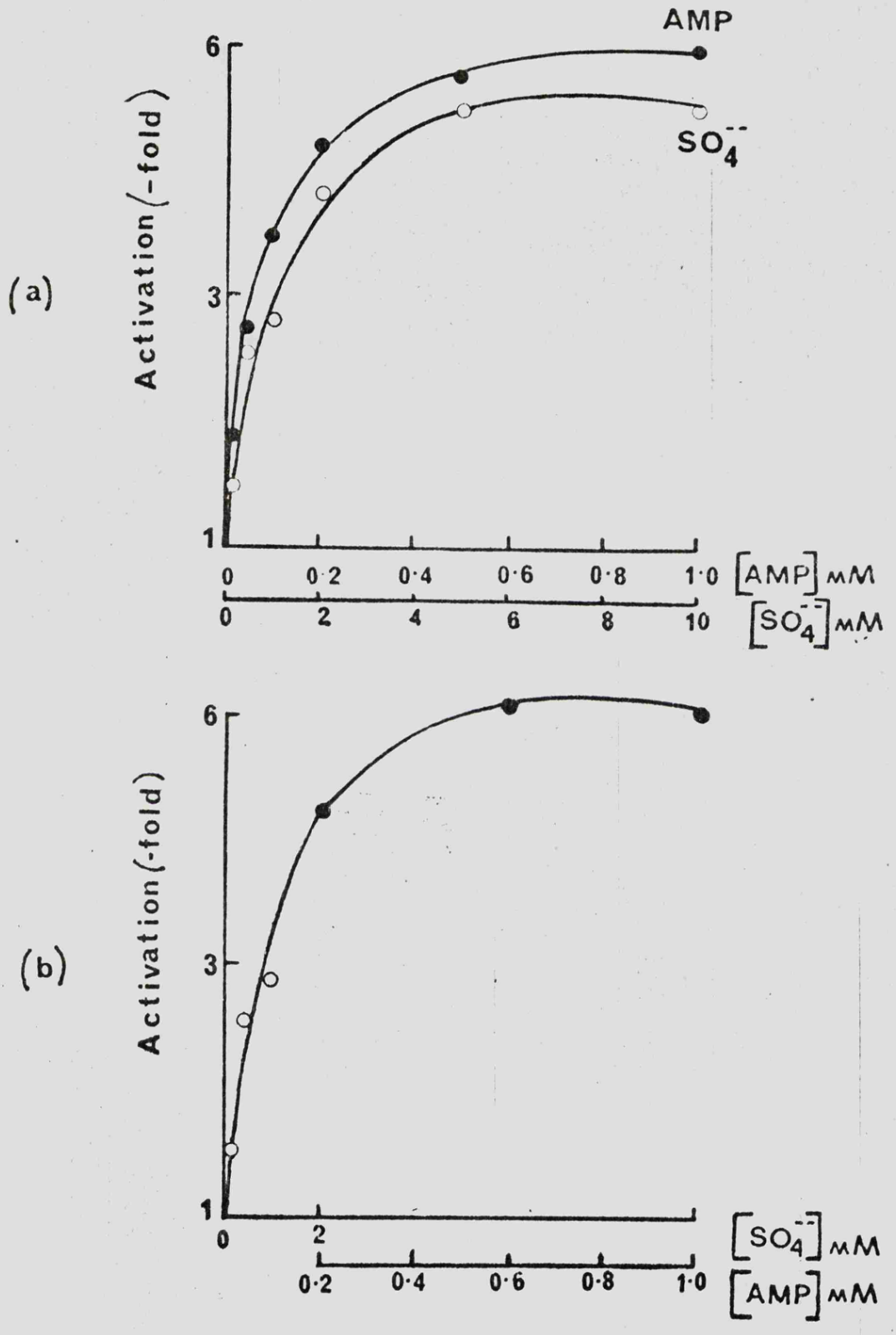

Figure 23 Demonstration of the non-additivity of the stimulations produced by AMP and sulphate

(a) the dependence of enzyme activation on AMP or sulphate concentration.

(b) the dependence of enzyme activation on AMP and sulphate concentration when used together.

Assays were performed under standard conditions with 2mM pyruvate and with AMP (●) or SO_4^{2-} (○).

TABLE 5

Division of bacteria on the basis of the

sensitivity of their pyruvate dehydrogenases to AMP

	GRAM-POSITIVE	GRAM-NEGATIVE	
		FACULTATIVE ANAEROBES	STRICT AEROBES
AMP-sensitive			*Acinetobacter lwoffi* (1) and (2) *Acinetobacter anitratus* (1) and (2) *Alcaligenes faecalis* *Bordetella bronchiseptica* (1) and (2) *Brevibacterium leucinophagum* *Chromobacterium violaceum* *Mima polymorpha* *Moraxella calcoacetica* (1) and (2) *Pseudomonas stutzeri* (1) and (2) *Xanthomonas hyacinthi*
AMP-insensitive	*Arthrobacter globiformis* *Bacillus subtilis* *Kurthia zopfii* *Microbacterium thermosphactum* *Mycobacterium rhodocrous*	*Escherichia coli* *Klebsiella (Aerobacter) aerogenes* *Proteus vulgaris* *Salmonella anatum* *Serratia marcescens*	*Azotobacter vinelandii* *Flavobacterium devorans* *Pseudomonas aeruginosa* (1) and (2) *Pseudomonas ovalis* *Xanthomonas campestris* *Xanthomonas uredovorans*

Assays were performed under standard conditions with 2mM pyruvate and 0.5mM AMP.
(1) and (2) denote different strains and their culture
collection numbers are presented in Table 1.

Further studies on AMP activation and divalent anion stimulation

Because divalent anions mimic AMP in their effects on A. lwoffi pyruvate dehydrogenase, other AMP sensitive pyruvate dehydrogenases were examined for divalent anion activation. A selection of AMP-sensitive and AMP-insensitive pyruvate dehydrogenases was chosen and the effect of 10mM sulphate (as representative of these anions) on the enzyme activity assayed with 2mM pyruvate was examined. Enzymes were considered sensitive to sulphate if they were activated at least 1.5-fold by 10mM sulphate. Of the enzymes examined, anion activation was observed only where AMP was also stimulatory. In all cases activation was predominantly due to a marked decrease in the K_m for pyruvate.

2. Succinate Thiokinase

Four enzymes involved in A. lwoffi citric acid cycle activity appear to be under adenylate control. Therefore another enzyme in this sequence, succinate thiokinase, was examined for sensitivity to this type of regulation. Because E. coli succinate thiokinase utilises ADP for its reaction (Smith et al., 1957), initial examinations of A. lwoffi succinate thiokinase were performed with this nucleotide.

(a) Properties of succinate thiokinase in crude enzyme extracts

ADP-dependent enzyme activity

Succinate thiokinase catalyses the only citric acid cycle reaction for which an adenine nucleotide is a substrate. Therefore the dependence of A. lwoffi succinate thiokinase activity on ADP concentration was examined. Experiments were performed with crude sonic extracts of nutrient-grown cells and the results are presented in Fig. 24. Double reciprocal plots of these data showed the ADP Km to be 1.18mM and V_{max} 0.32 units x 10^{3}/mg protein. For comparative purposes, the dependence of E. coli succinate thiokinase activity on ADP concentration was examined under identical conditions. The ADP Km was 0.017mM and V_{max} 0.61 units x 10^{3}/mg protein (Fig. 25).

The pH profile of the A. lwoffi enzyme was next determined. Enzyme activity was examined in 0.1M phosphate

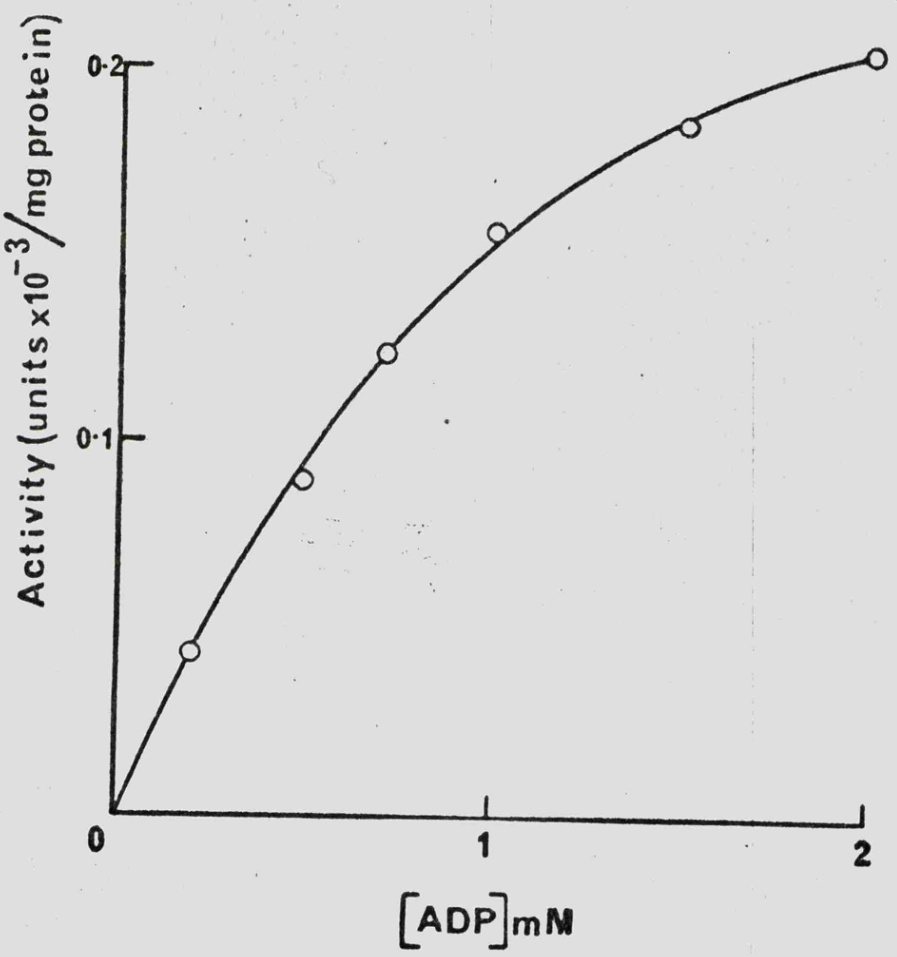

Figure 24 Dependence of A. lwoffi succinate thiokinase activity on ADP concentration

Assays were performed under standard conditions with crude enzyme preparations.

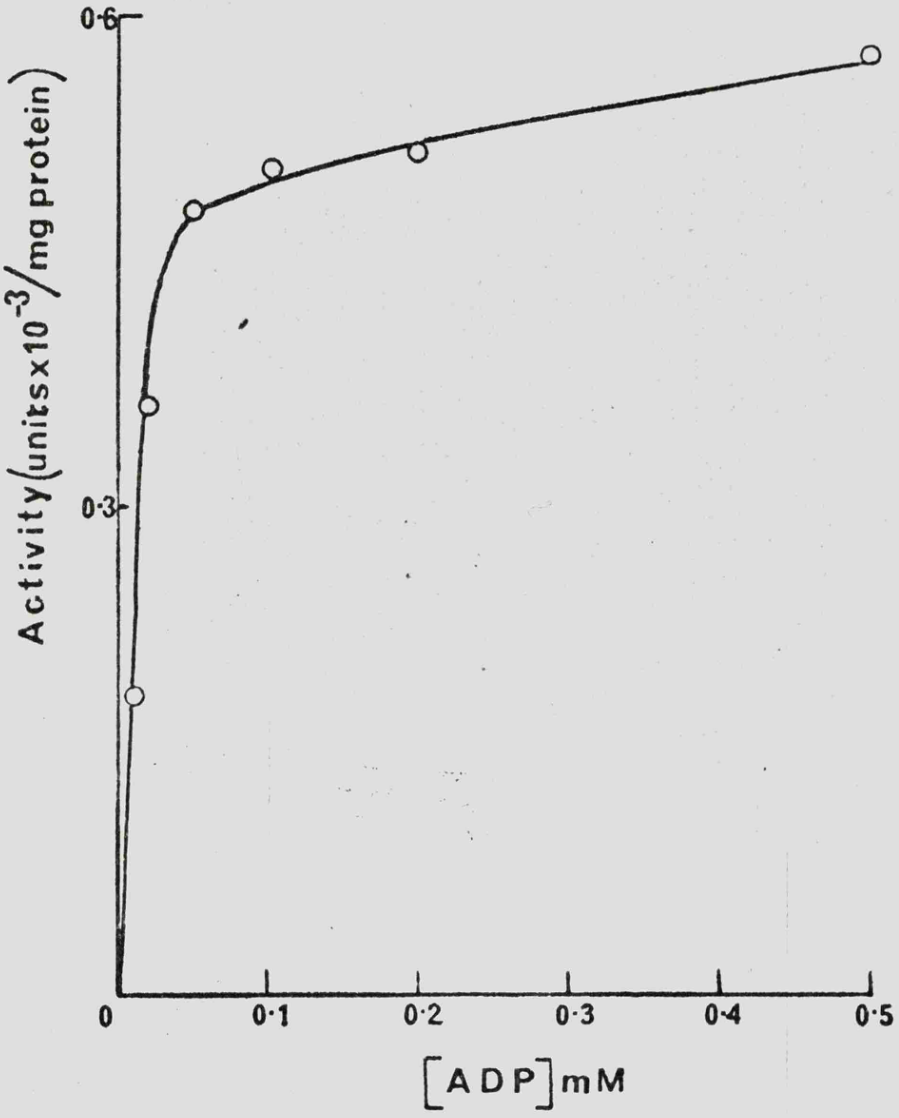

Figure 25 Dependence of E. coli succinate thiokinase activity on ADP concentration

Assays were performed under standard conditions with crude enzyme preparations.

buffers, varying between pH 6.0 and pH 7.8, and in 0.1M Tris-HCl, 20mM Na_2HPO_4 buffers varying between pH 7.5 and pH 9.0 (Fig. 26). Although the activities measured in Tris-HCl were lower than those measured in phosphate buffers, at the same pH values, nevertheless the enzyme activity showed an optimum between pH 7.4 and pH 7.8.

Adenylate control of the ADP-dependent enzyme activity

Adenylate control of this enzyme activity was investigated by examining the dependence of A. lwoffi succinate thiokinase activity on ADP concentration, with and without 1mM AMP or 1mM ATP. The results, which are illustrated in Fig. 27, show that AMP and ATP have no effect on enzymic activity.

In view of the high Km for ADP exhibited by this enzyme compared with E. coli succinate thiokinase, it was considered possible that this high Km may enable the enzyme to be sensitive to changes in ADP concentration over the physiological range and thereby render it sensitive to energy charge control. The dependence of ADP-dependent enzyme activity on energy charge was examined in the following manner. Mixtures possessing energy charge values between 0.5 and 1.0 were prepared as described in Methods and these mixtures were used as a source of ADP (at 1mM total adenine nucleotide concentration) for succinate thiokinase assays performed under otherwise standard conditions. The results which are presented in Fig. 28, show that the enzyme was fully active between energy charge

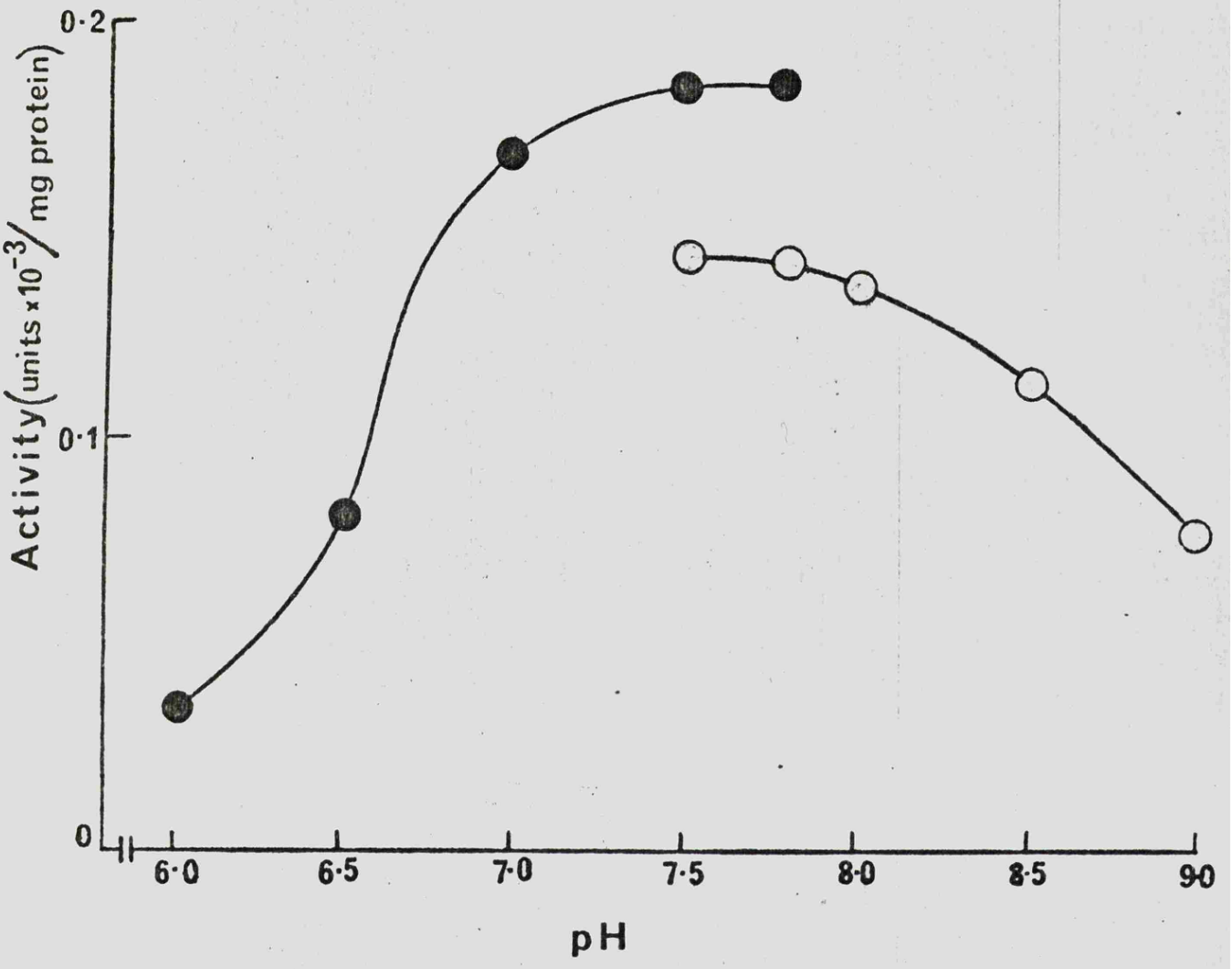

Figure 26 Dependence of A. lwoffi succinate thiokinase activity on pH

Assays were performed with 2mM ADP in 0.1M phosphate buffers (●) or in 0.1M Tris-HCl, 20mM Na_2HPO_4 buffers (○), under otherwise standard conditions.

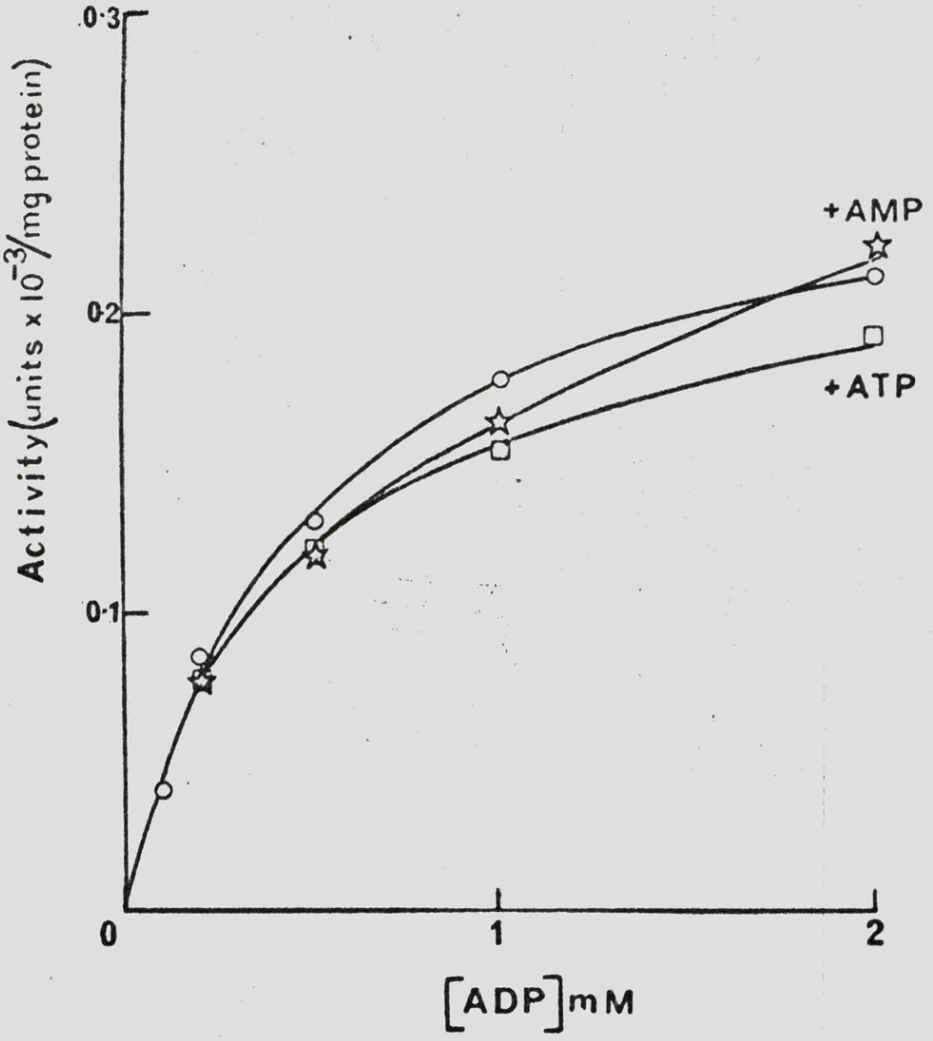

Figure 27 Effect of AMP and ATP on the dependence of A. lwoffi succinate thiokinase activity on ADP concentration

Assays were performed under standard conditions with crude enzyme preparations, without effectors (○), or with 1mM AMP (☆), or 1mM ATP (□).

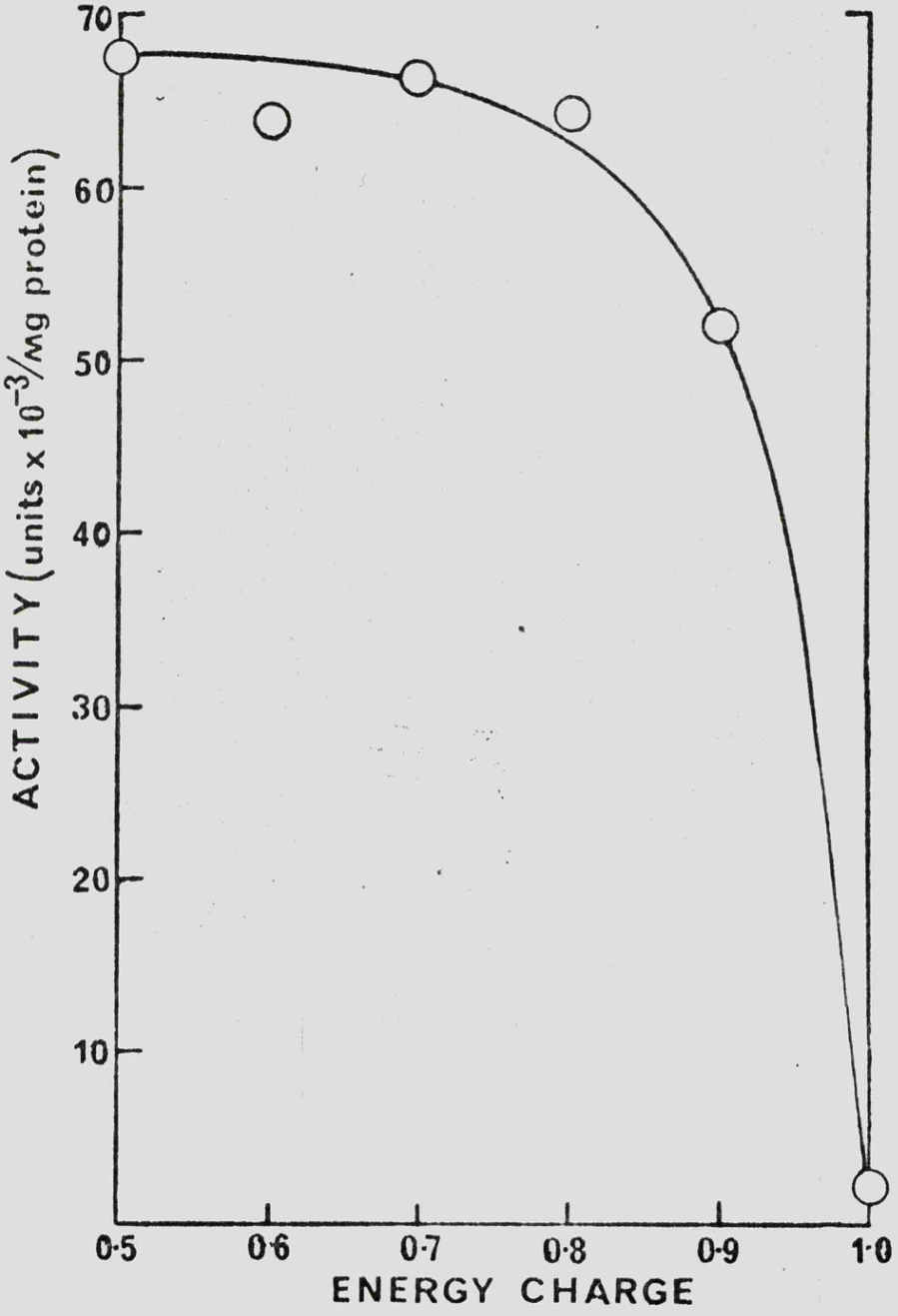

Figure 28 Dependence of A. lwoffi ADP-linked succinate thiokinase activity on energy charge

Assays were performed with crude enzyme preparations as described in the text.

As the Km for ADP is 1.18mM the ADP concentrations were not saturating.

values 0.5 and 0.8, and slightly less so at 0.9. At an energy charge value of 1.0, the enzyme was virtually inactive.

GDP- or IDP-dependent enzyme activity

Because of the high Km for ADP exhibited by the A. lwoffi enzyme, other nucleoside diphosphates were examined to see if they were better substrates. Preliminary experiments performed with 2mM GDP or 2mM ADP showed that A. lwoffi succinate thiokinase could utilise GDP and ADP equally well, moreover, decreasing the GDP concentration as far as 0.2mM did not reduce the enzyme activity, whereas a similar decrease in ADP concentration resulted in an almost complete loss of enzyme activity. IDP behaved similarly to GDP.

The dependence of A. lwoffi succinate thiokinase activity on GDP and IDP concentrations was therefore examined, and the results are presented in Figs. 29(a) and (b). Kinetic parameters were obtained from reciprocal plots of these data and were as follows: the Km for GDP and the value of V_{max} with this nucleotide were 0.026mM and 0.36 x 10^{3} units/mg protein, whereas the Km for IDP and the corresponding V_{max} value was 0.023mM and 0.37 x 10^{3} units/mg protein.

Adenylate control of the GDP- or IDP-dependent enzyme activities

Adenylate control of the GDP- or IDP-dependent succinate thiokinase activities was investigated by examining the dependences of enzyme activity on GDP and IDP concentrations, with and without 1mM AMP or 1mM ATP. Figs. 30(a) and (b) show that when ATP was included in assay mixtures, there was a marked inhibition of both GDP-dependent and IDP-dependent succinate thiokinase activities. AMP on the other hand, had little effect on enzymic activitity, producing slight stimulation at higher GDP and IDP concentrations, which could be attributed to a reduction in substrate inhibition observed at these high GDP and IDP concentrations.

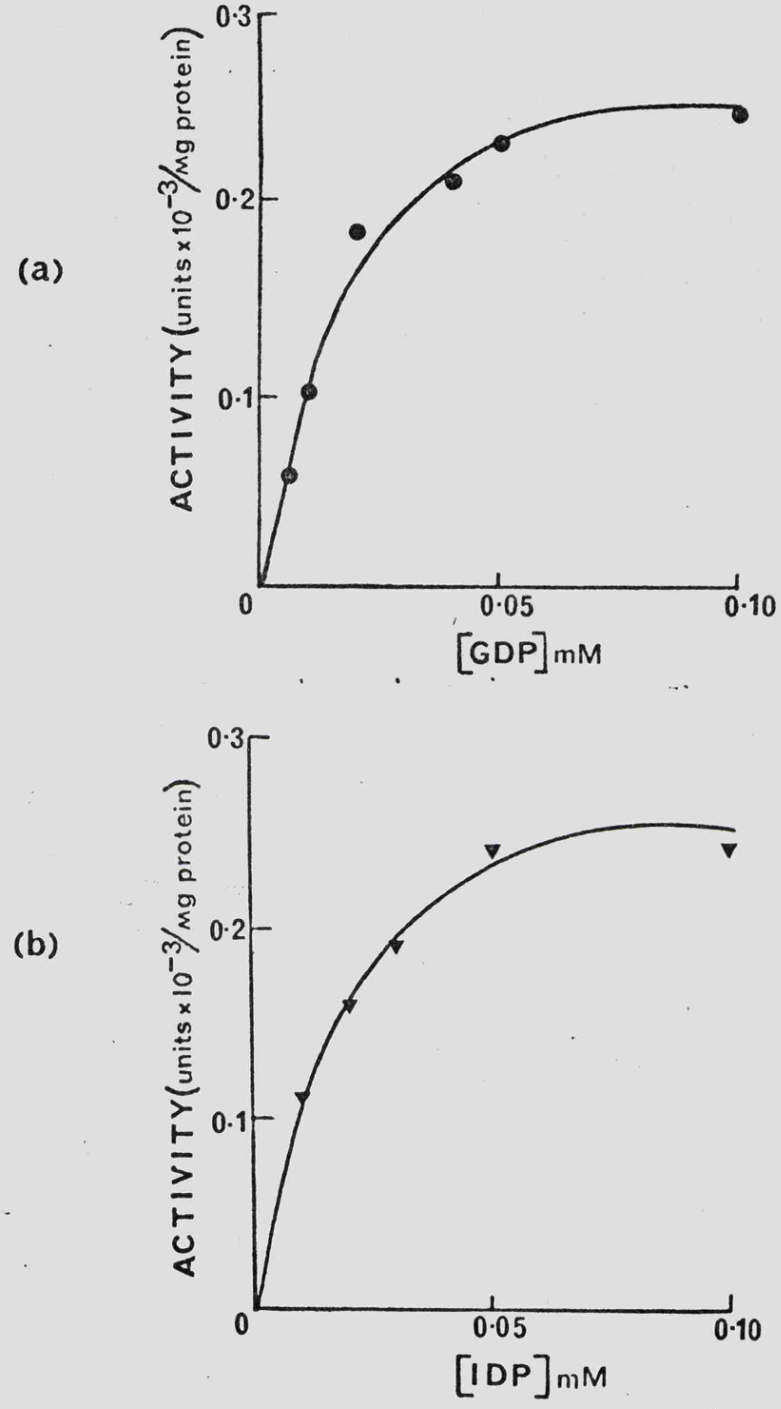

Figure 29 Dependence of A. lwoffi succinate thiokinase activity on (a) GDP and (b) IDP concentration

Assays were performed under standard conditions with crude enzyme preparations.

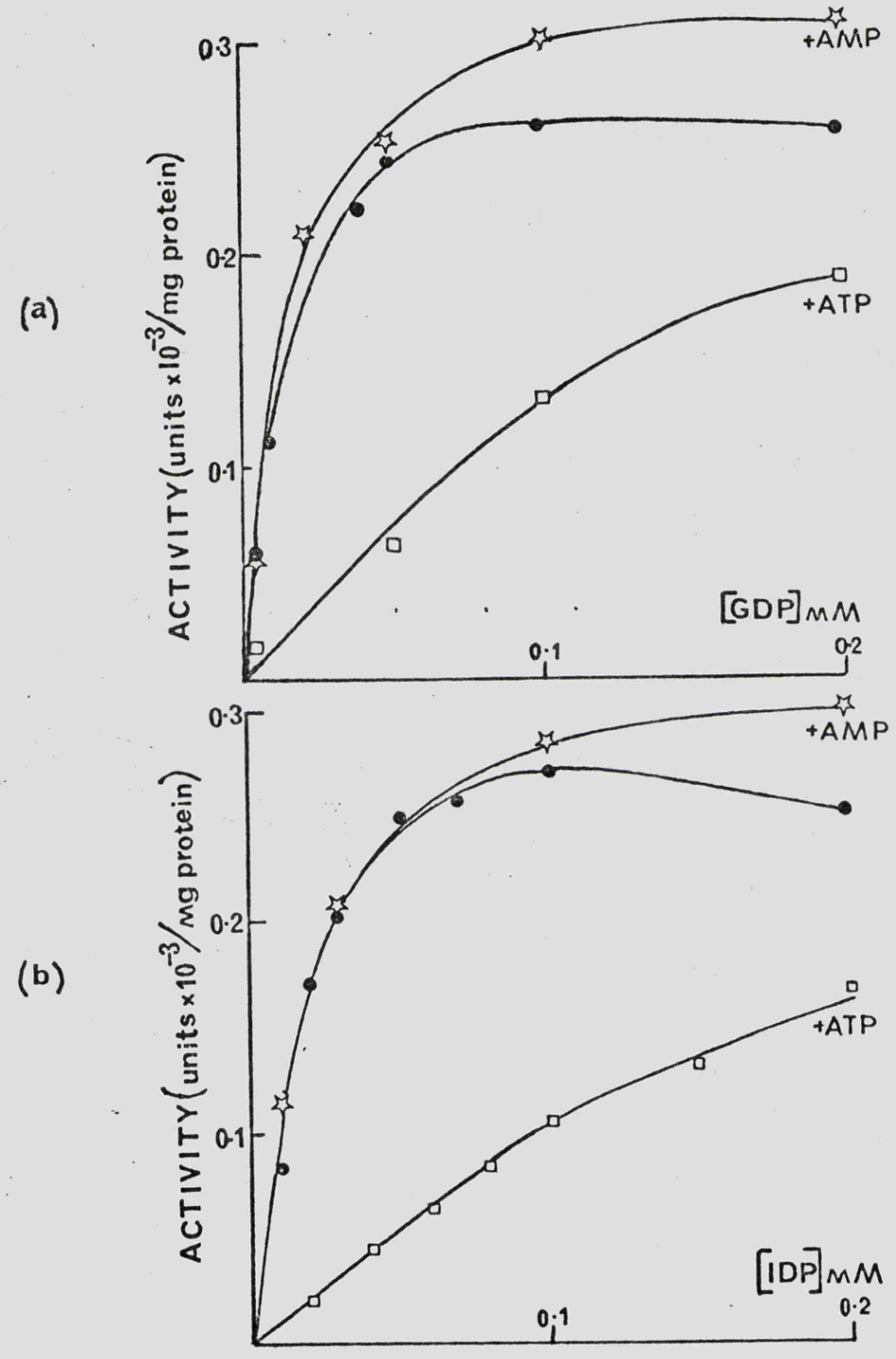

Figure 30 Effect of AMP and ATP on the dependence of A. lwoffi succinate thiokinase activity on (a) GDP and (b) IDP concentration

Assays were performed under standard conditions with crude enzyme preparations, without effectors (●), or with 1mM ATP (□), or 1mM AMP (☆).

Because ATP was inhibitory when GDP was the nucleotide substrate, the dependence of GDP-dependent succinate thiokinase activity on energy charge was examined. Experiments were performed with 0.05mM GDP and 2mM total adenine nucleotide concentration. The results, which are presented in Fig. 31, show that the enzyme was active at all values of energy charge, but maximum activity was exhibited between energy charge values 0 and 0.7. Between energy charge values 0.7 and 1.0 there was a rapid decrease in enzyme activity, falling to a minimum at an energy charge value 1.0. A similar dependence was observed when 0.05mM IDP was used as substrate.

The energy charge diagrams obtained above, using GDP or IDP as nucleotide substrates for the succinate thiokinase reaction, and the energy charge diagram obtained previously using ADP, are typical of an enzyme which serves a catabolic role and is sensitive to regulation by the adenine nucleotides.

(b) Partial purification of succinate thiokinase and properties of the partially purified enzyme

Partial purification of the enzyme

The high Km for ADP observed for the A. lwoffi enzyme might have been an artefact due to a particularly active adenylate kinase, which removes the substrate for succinate thiokinase activity, ADP. In addition, it was possible that the apparent inhibition of the GDP- or IDP-dependent succinate thiokinase activities by ATP was the result of GDP or IDP removal through the action of ATP and nucleoside diphosphate kinase. Although ADP would be produced, this has already been shown to be a poorer substrate.

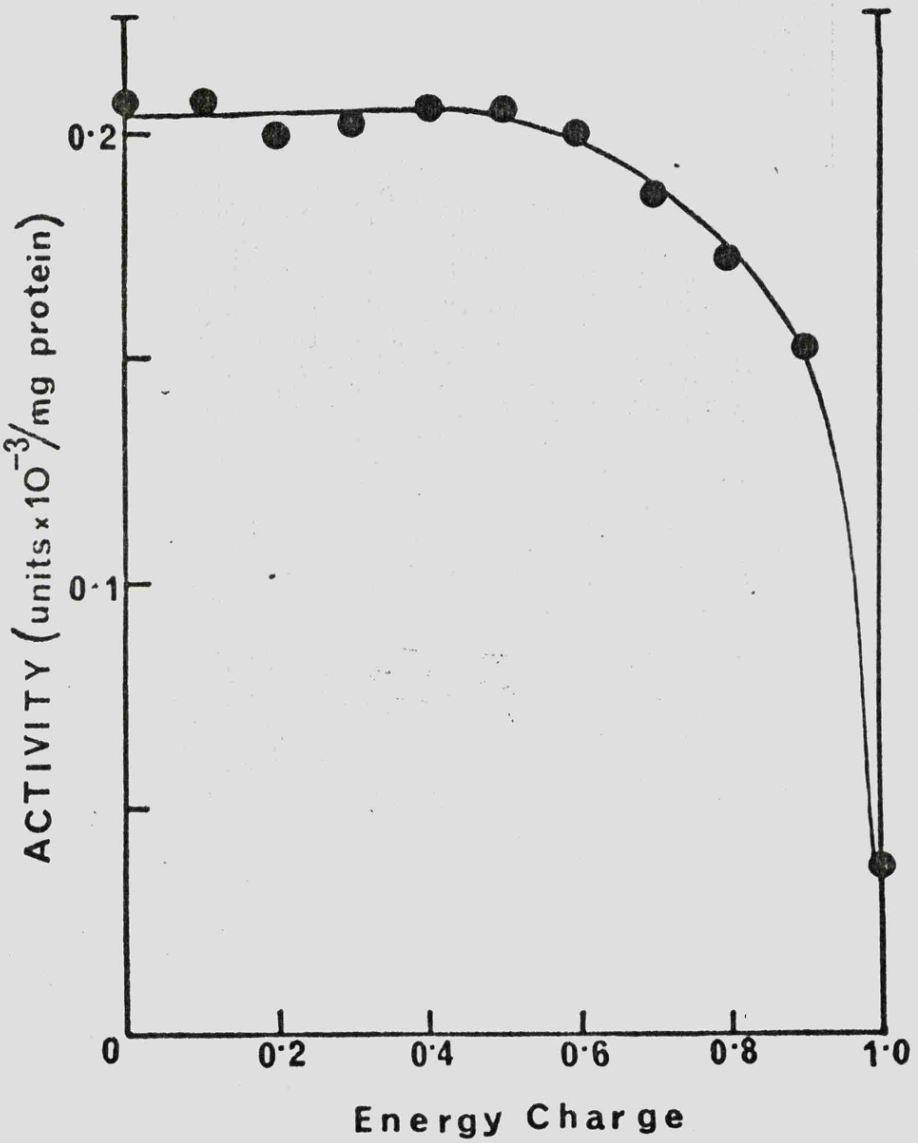

Figure 31 Dependence of A. lwoffi GDP-dependent succinate thiokinase activity on energy charge

Assays were performed under standard conditions with crude enzyme preparations and 0.05mM GDP. The total adenine concentration was 2mM.

The crude extracts contained nucleoside diphosphate kinase which may have removed GDP to produce ADP.
See p.73 Fig.28.

Separation of succinate thiokinase from both adenylate kinase and nucleoside diphosphate kinase was achieved by the following procedure. Cell-free extracts, prepared from 200ml of initial nutrient-grown A. lwoffi culture, were centrifuged at high speed and the supernatant solution so obtained was then applied to a Sephadex G-200 column, eluted and fractions collected as described in Methods 3(c). These were assayed for succinate thiokinase, adenylate kinase and nucleoside diphosphate kinase and their protein contents also determined. The elution profiles obtained are illustrated in Fig. 32 and show that there was a good separation of succinate thiokinase from both adenylate kinase and nucleoside diphosphate kinase, although there was no resolution of the latter two enzyme activites from each other. A summary of the results obtained when this procedure was applied to crude enzyme extracts of A. lwoffi is shown in Table 6. The succinate thiokinase obtained after this treatment exhibited an 8.2-fold increase in specific activity over that measured in crude enzyme preparations.

Properties of the partially purified enzyme

ADP-dependent enzyme activity

The dependence of enzyme activity on ADP concentrations was re-examined, using the partially purified enzyme, with and without 1mM AMP or ATP (Fig. 33). The Km for ADP was 1.11mM, and AMP and ADP had no effect on enzyme activity.

The effect of 1mM AMP or 1mM ATP on the dependence of enzyme activity on succinyl-CoA concentration was also examined, to investigate the possibility of adenylate control with respect to the other substrate for the succinate thiokinase reaction, succinyl-CoA. Fig. 34 illustrates the results of these experiments and shows no

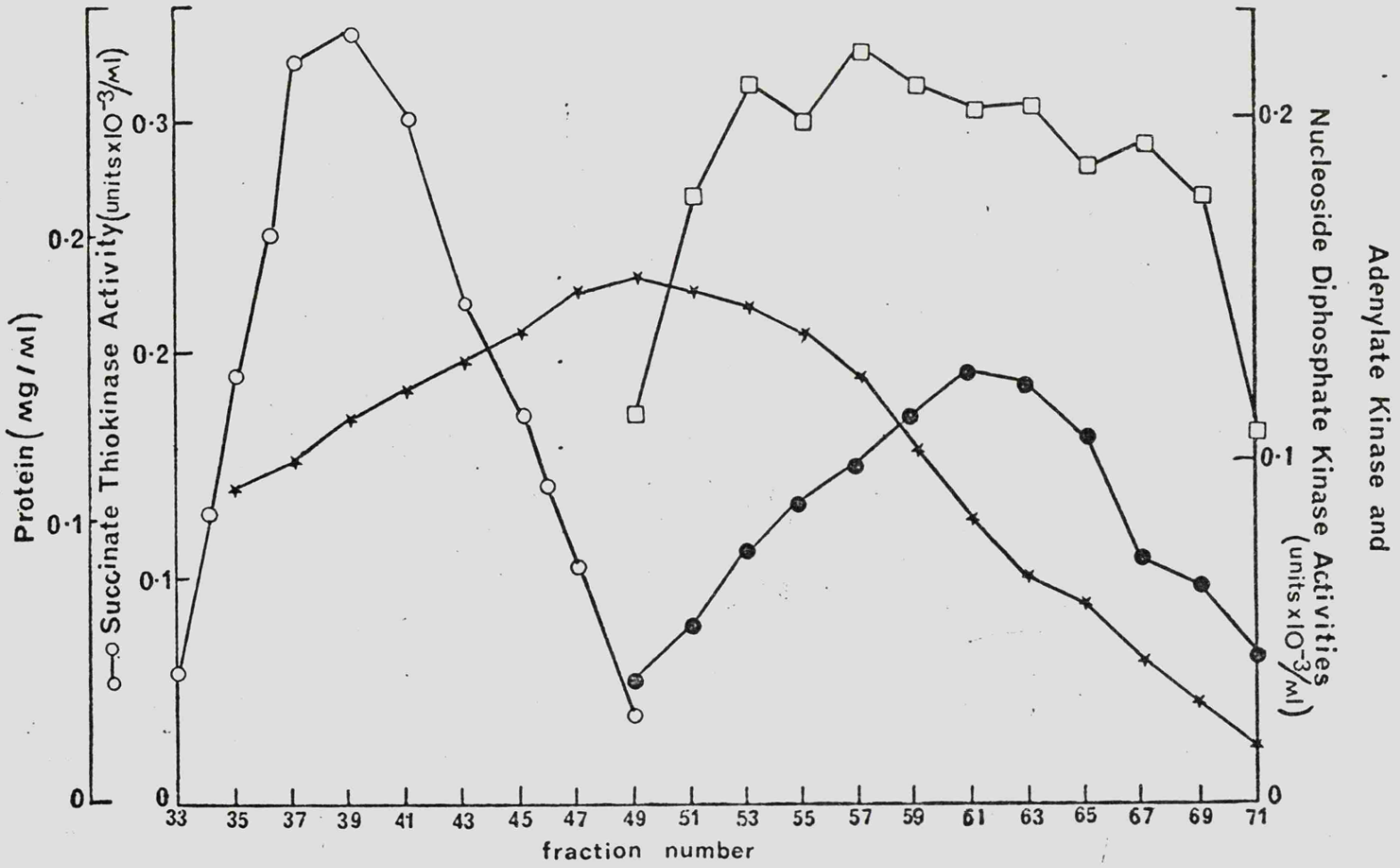

Figure 32 Separation of A. lwoffi succinate thiokinase, adenylate kinase and nucleoside diphosphate kinase by gel filtration on Sephadex G-200

(○) Succinate thiokinase (assayed under standard conditions with 2mM ADP)
(□) Nucleoside diphosphate kinase (assayed under standard conditions)
(●) Adenylate kinase (assayed under standard conditions)
(✶) Protein

ENZYME	FRACTION	TOTAL ACTIVITY (units x 10^{-3})	SPECIFIC ACTIVITY (units x 10^{-3}/mg)	PURIFICATION (-fold)	YIELD (%)
Succinate thiokinase	crude	10.25	0.21	1.0	100
	high-speed supernatant	8.2	0.27	1.3	80
	G-200, ǂ34 - 46 inc	5.65	1.71	8.2	51
Adenylate kinase	crude	7.25	0.15	1.0	100
	high-speed supernatant	5.8	0.19	1.3	80
	G-200, ǂ 51 - 69 inc	3.15	0.84	5.6	43
Nucleoside diphosphate kinase	crude	10.0	0.21	1.0	100
	high-speed supernatant	8.0	0.26	1.3	80
	G-200, ǂ51 - 69 inc	6.6	1.72	8.2	66

TABLE 6 Separation of succinate thiokinase from adenylate kinase and nucleoside diphosphate kinase activities of crude A. lwoffi extracts.

Succinate thiokinase activity was assayed under standard conditions with 2mM ADP.

Adenylate kinase and nucleoside diphosphate kinase were both assayed under standard conditions.

G-200, ǂ 34 - 46 inc: pooled Sephadex G-200 fractions numbered 34 - 46 inclusive.
G-200, ǂ 51 - 69 inc: pooled Sephadex G-200 fractions numbered 51 - 69 inclusive.

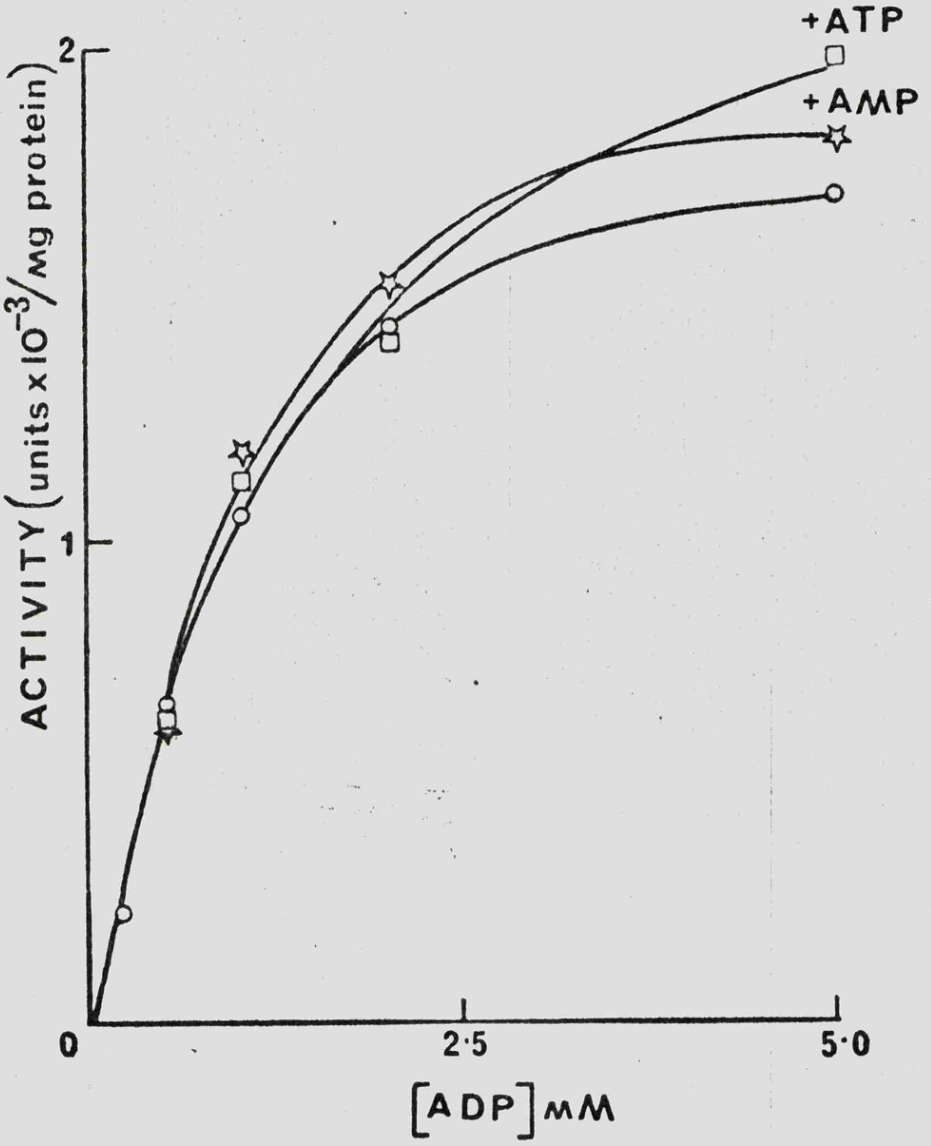

Figure 33 Effect of AMP and ATP on the dependence of A. lwoffi succinate thiokinase activity on ADP concentration

Assays were performed under standard conditions with partially purified enzyme preparations, without effectors (○), or with 1mM AMP (☆), or 1mM ATP (□).

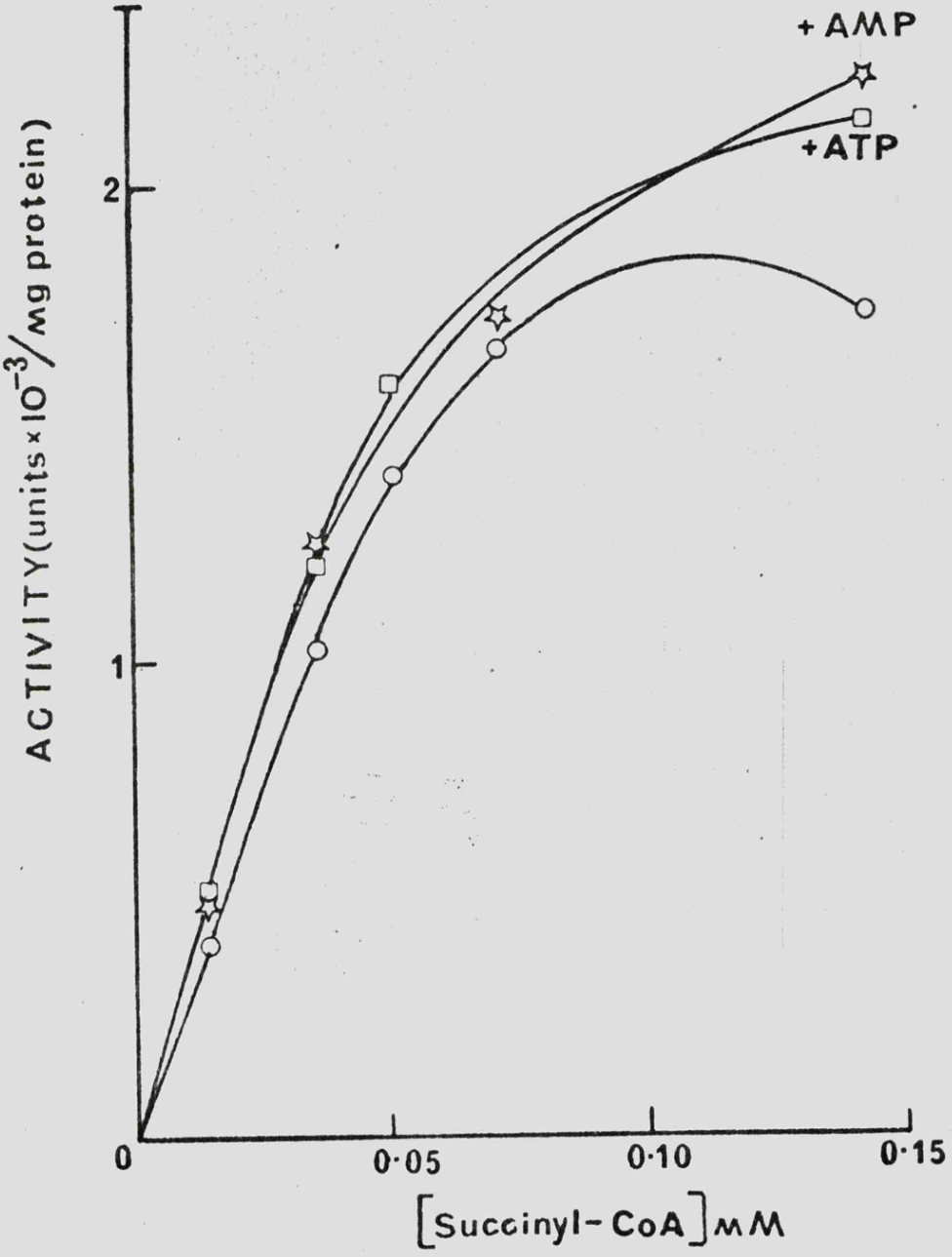

Figure 34 Effect of AMP and ATP on the dependence of A. lwoffi succinate thiokinase activity on succinyl-CoA concentration

Assays were performed under standard conditions with partially purified enzyme preparations and 2mM ADP: no effectors present (O); with 1mM AMP (☆); with 1mM ATP (□).

such effect. However, without AMP or ATP, the activity of the enzyme was inhibited at high succinyl-CoA concentrations, but in the presence of these nucleotides substrate inhibition was relieved.

GDP- or IDP-dependent enzyme activity

When the dependence of enzyme activity on GDP concentration was re-examined using partially purified enzyme preparations, with and without 1mM AMP or 1mM ATP (Fig.35), ATP was not inhibitory; neither did ATP inhibit the IDP-dependent enzyme activity. This observation was therefore investigated further.

If ATP inhibition of GDP- or IDP-dependent enzyme activity was indeed a consequence of nucleoside diphosphate kinase activity, this inhibition should also be observed in assays performed with partially purified succinate thiokinase preparations if aliquots of the previously separated nucleoside diphosphate kinase are included in the assay mixtures. Succinate thiokinase activity was measured at 0.02mM GDP, both in the absence and presence of 1mM ATP, using a partially purified enzyme preparation. No ATP inhibition was observed. However, when a similar assay mixture was prepared containing 1mM ATP but in the further presence of an aliquot (0.1ml) of partially purified nucleoside diphosphate kinase, and the mixture left to stand at room temperature for 5 min, prior to the addition of succinyl-CoA, a marked reduction in enzyme activity was observed. Under the assay conditions described, 1mM ATP produced 69% inhibition. of the enzyme activity measured with 0.02mM GDP. In order to check that both ATP and nucleoside diphosphate kinase were required for this inhibition, control mixtures were prepared, one without 1mM ATP but containing 0.1ml nucleoside diphosphate kinase, the other containing 1mM ATP but no nucleoside diphosphate kinase. These controls were allowed to stand for 5 min at room temperature

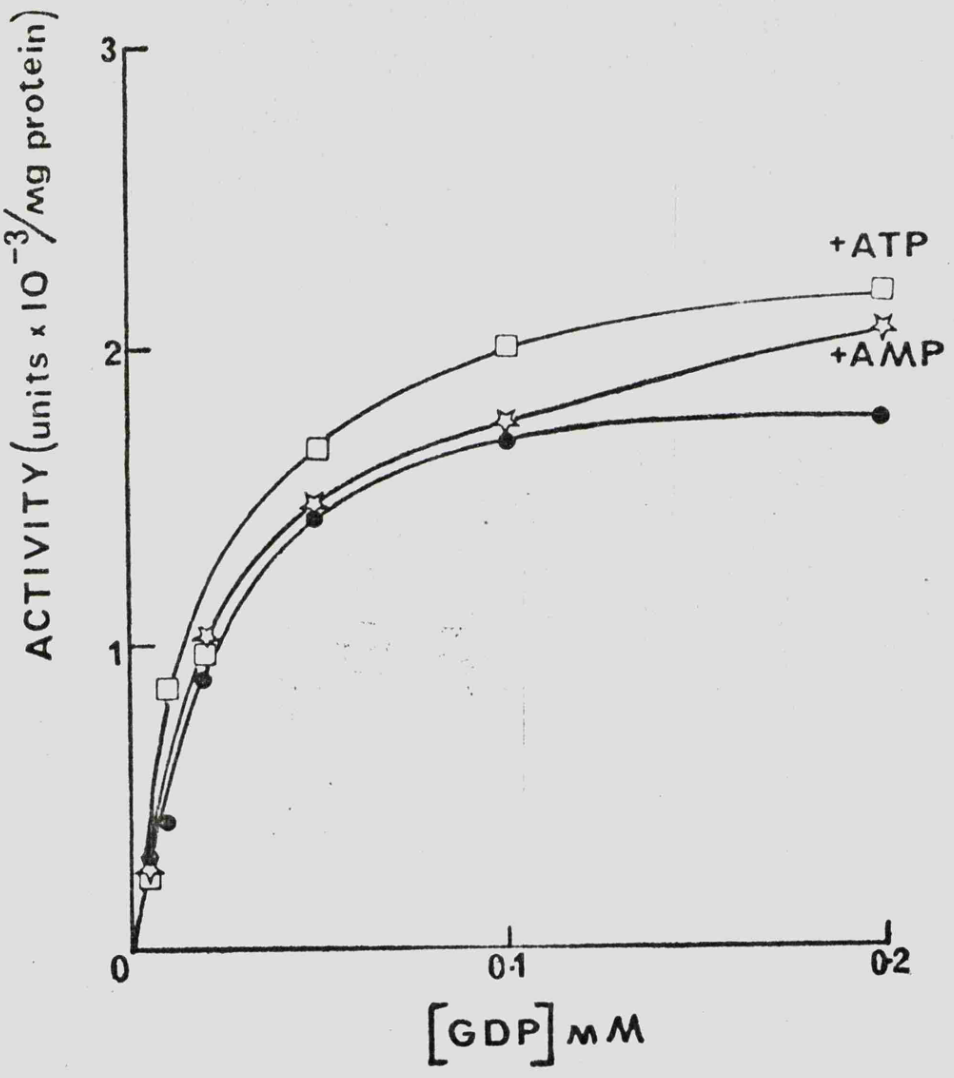

Figure 35 Effect of AMP and ATP on the dependence of A. lwoffi succinate thiokinase activity on GDP concentration

Assays were performed under standard conditions with partially purified enzyme preparations, without effectors (●), or with 1mM AMP (☆), or 1mM ATP (□).

prior to the addition of succinyl-CoA. In neither case was there any inhibition of A. lwoffi GDP-dependent succinate thiokinase activity. A summary of these results is presented in Table 7.

Estimation of molecular size

The molecular size of A. lwoffi succinate thiokinase was examined. 50µl of lactate dehydrogenase (140 units) were added to the supernatant solution obtained after high-speed centrifugation of the crude extract. The mixture was applied to a Sephadex G-200 column, eluted and fractions collected as described previously for the partial purification of the enzyme. These were assayed for succinate thiokinase and lactate dehydrogenase, and the results are presented in Fig.36. The two enzyme activities were eluted virtually coincidentaly. When a similar preparation of E. coli succinate thiokinase was mixed with lactate dehydrogenase and examined by gel filtration on Sephadex G-200, the two activities again ran coincidentally.

(c) Taxonomic significance of succinate thiokinase nucleotide specificity

It has been proposed that plant and bacterial succinate thiokinases are specific for ADP whereas mammalian succinate thiokinases utilise GDP and IDP (Bridger et al.,1969). However, A. lwoffi succinate thiokinase was active with ADP, and GDP or IDP, therefore the nucleotide specificity of the enzyme from a wide range of bacterial species was investigated. Crude enzyme extracts were prepared from nutrient-grown cells (Methods 2 and 3(a)) and by varying the concentrations of ADP and GDP, approximate Km values for these substrates were determined. The results are presented in Table 8.

ADDITIONS	PRE-INCUBATION TIME (MIN)	ACTIVITY (UNITS/MG)	INHIBITION (%)
None	0	90	0
1mM ATP	0	91.5	0
1mM ATP, 0.1ml NDPK	5	28	69
0.1ml NDPK	5	91.5	0
1mM ATP	5	92	0

TABLE 7 The effect of ATP and nucleoside diphosphate kinase on A. lwoffi GDP-dependent succinate thiokinase activity.

Succinate thiokinase activity was assayed under standard conditions with 0.02mM GDP.

NDPK: nucleoside diphosphate kinase (G-200, ≡ 51 - 69 inc.)

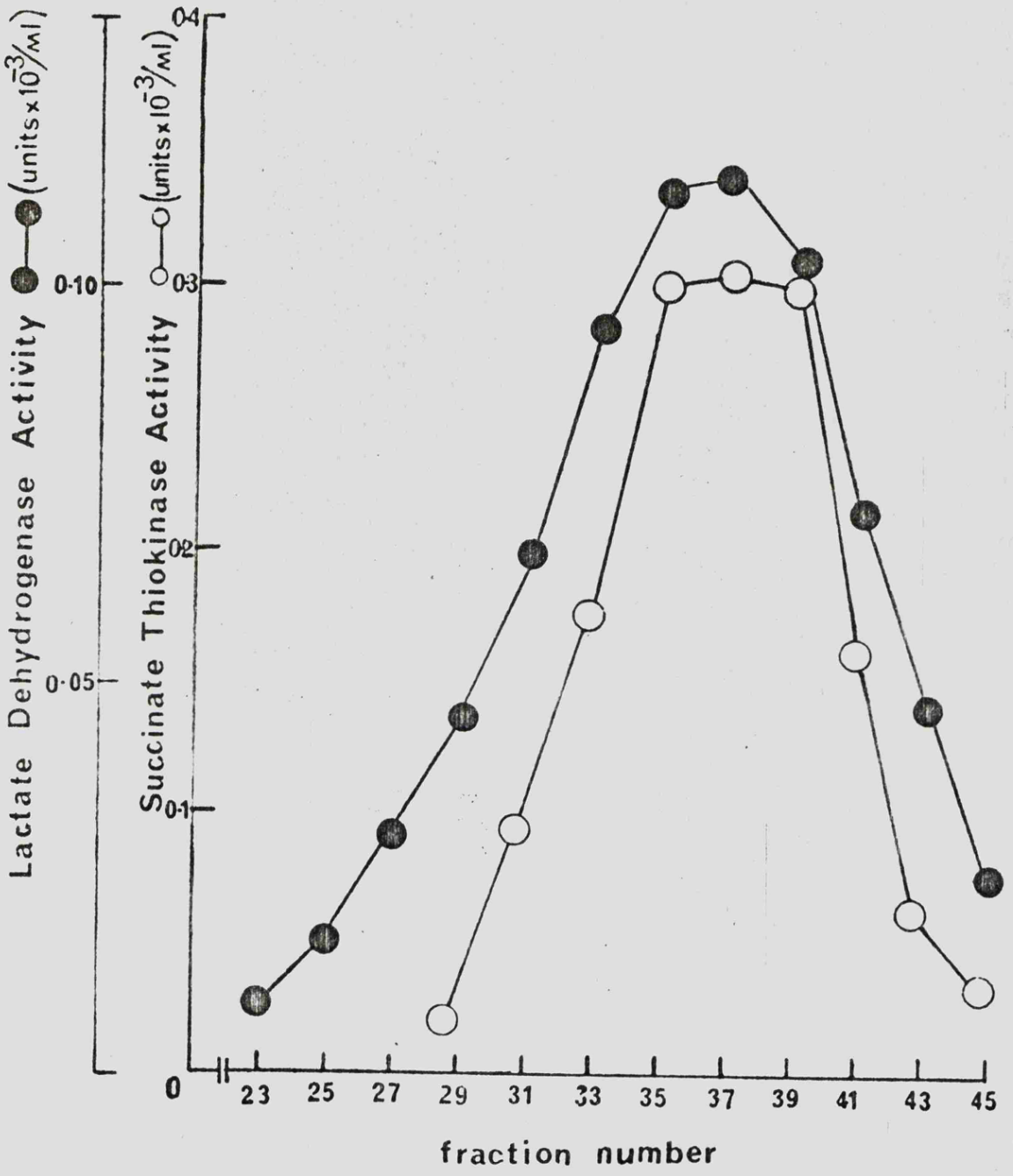

Figure 36 Estimation of the molecular size of A. lwoffi succinate thiokinase by gel filtration on Sephadex G-200

(○) succinate thiokinase (assayed under standard conditions with 2mM ADP)

(●) lactate dehydrogenase (assayed under standard conditions)

ORGANISM	GRAM STAIN	Km ADP	Km GDP
Acinetobacter lwoffi (1) and (2)	-ve	high	+++
Acinetobacter anitratus (1) and (2)	-ve	high	+++
Bordetella bronchiseptica (1) and (2)	-ve	high	+++
Chromobacterium violaceum	-ve	high	+++
Moraxella calcoacetica (1) and (2)	-ve	high	+++
Mima polymorpha	-ve	high	+++
Xanthomonas hyacinthi	-ve	high	+++
Corynebacterium fascians	+ve	low	++
Corynebacterium rubrum	+ve	low	++
Pseudomonas aeruginosa (1) and (2)	-ve	low	++
Pseudomonas fluorescens	-ve	low	++
Pseudomonas stutzeri (1) and (2)	-ve	low	++
Rhodopseudomonas spheroides	-ve	low	++
Alcaligenes faecalis	-ve	low	+
Arizona arizonae	-ve	low	+
Cellulomonas cellasea	-ve	low	+
Escherichia coli	-ve	low	+
Klebsiella (Aerobacter) aerogenes	-ve	low	+
Serratia marcescens	-ve	low	+
Bacillus megaterium	+ve	low	O
Bacillus stearothermophilus	+ve	low	O
Kurthia zopfii	+ve	low	O
Arthrobacter simplex	+ve	low	O

TABLE 8 Nucleotide specificity of succinate thiokinase from a range of bacterial species.

Assays were performed under standard conditions with varying concentrations of ADP and GDP.

(1) and (2) denote different strains and their culture collection numbers are presented in Table 1.

high: approximately 1mM
low: less than 0.1mM
+: Km^{GDP} greater than Km^{ADP}
++: Km^{GDP} comparable with Km^{ADP}
+++: Km^{GDP} less than Km^{ADP}
O: no activity detectable at any GDP concentration tested.

Four types of succinate thiokinase were identifiable on the basis of their nucleotide specificity. The first type showed a high Km for ADP, i.e. approximately 1mM and a Km for GDP which was at least an order of magnitude lower. The second class of enzyme exhibited similar Km values for ADP and GDP and both were less than 0.1mM. Succinate thiokinases belonging to the third category again showed low (less than 0.1mM) Km values for ADP, but the GDP Km was appreciably higher. The last type of enzyme was only active with ADP, and the Km was less than 0.1mM

Organisms which possessed succinate thiokinases of type 1 were those Gram-negative strictly aerobic bacterial species which also showed adenylate control of their pyruvate dehydrogenase citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase. Pseudomonads and Corynebacteria possessed succinate thiokinase of type 2, whereas all the other Gram-negative species tested showed succinate thiokinases of type 3 and the rest of the Gram-positive bacterial species examined were specific for ADP (type 4). With the exception of A. lwoffi, the ability of IDP to substitute for GDP as nucleotide substrate was not examined.

The nucleotide specificity of mammalian succinate thiokinase

The nucleotide specificity of mammalian succinate thiokinase was investigated by examining the dependence of enzyme activity on both GDP and ADP concentrations. The source of the enzyme was sheep liver which had been homogenised in 0.1M Tris-HCl, pH 8.0 and centrifuged at 12,000g for 30 min to obtain a clear supernatant solution. Fig. 37. illustrates the dependence of this activity on GDP concentration. The enzyme was extremely active with GDP as substrate, exhibiting a Km of 0.024mM and a V_{max} value of 0.7×10^{3} units/mg protein, but no activity was detectable

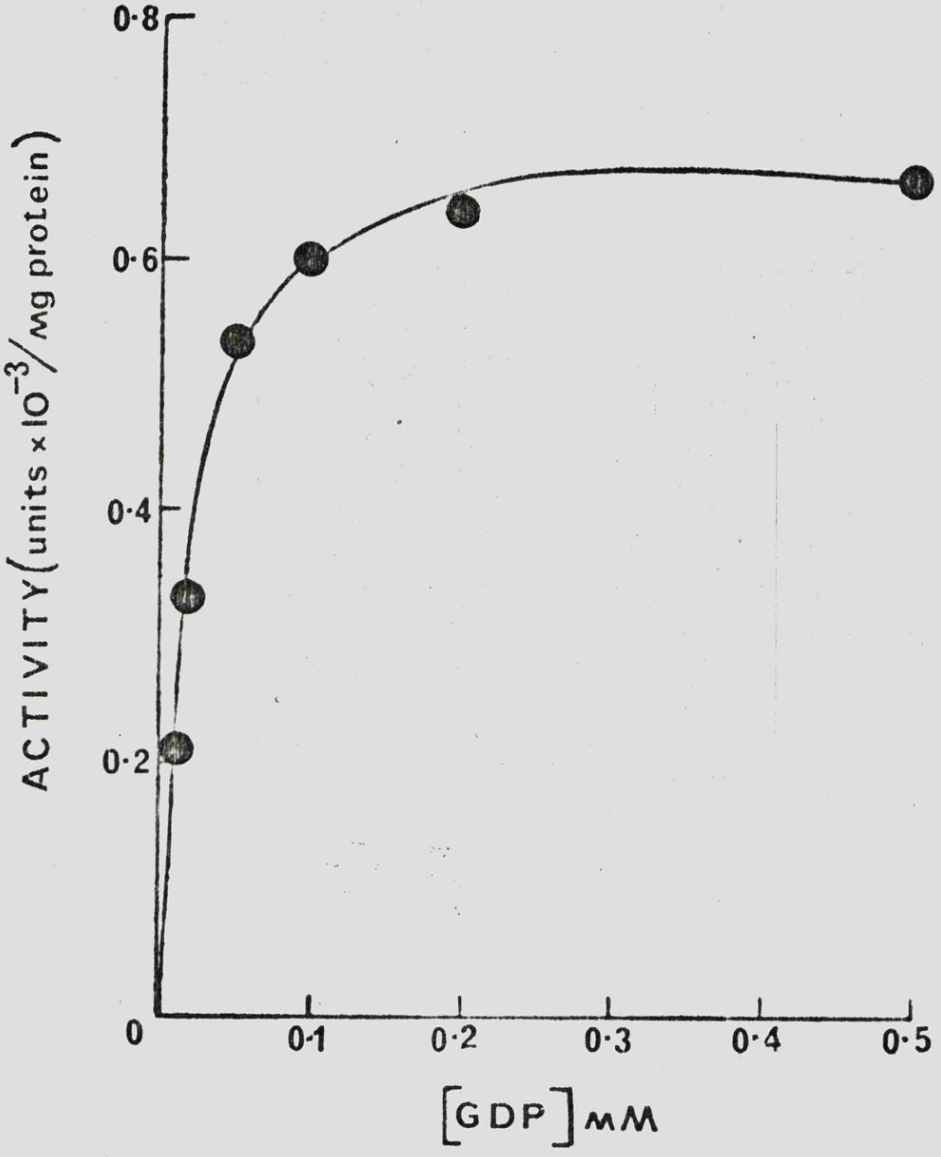

Figure 37 . Dependence of sheep liver succinate thiokinase activity on GDP concentration

Assays were performed under standard conditions.

with ADP, even when 5mM concentrations were used. The effect of IDP was not tested.

(d) Comparison of the succinate thiokinases from A. lwoffi, Pseudomonas aeruginosa and E. coli

Those succinate thiokinases which were active with both ADP and GDP were examined in greater detail. A. lwoffi, Ps. aeruginosa and E. coli were selected as representative of types 1, 2 and 3 respectively, and experiments were performed with crude extracts of nutrient-grown cells (Methods 2 and 3(a)). The dependences of enzyme activity on ADP and GDP concentrations were first examined for each organism. An extensive examination of the kinetic properties of A. lwoffi succinate thiokinase has already been described and the Km values for ADP and GDP shown to be 1.18mM and 0.023mM respectively; the corresponding V_{max} values were 0.32×10^{3} units/mg protein and 0.37×10^{3} units/mg protein. Because the data were obtained with different enzyme preparations, these examinations were repeated with the same enzyme extracts and the kinetic parameters thus obtained were shown to be similar to previous values. Fig. 38 illustrates the results obtained with Ps. aeruginosa succinate thiokinase. The Km values for ADP and GDP determined from double reciprocal plots were 0.043mM and 0.023mM respectively; the corresponding V_{max} values were 0.33×10^{3} units/mg protein and 0.35×10^{3} units/mg protein. A similar examination of these dependences with E. coli succinate thiokinase (Fig. 39) gave Km values for ADP and GDP of 0.014mM and 0.11mM respectively, and V_{max} values for both substrates of 0.61×10^{3} units/mg protein.

The nucleotide specificity of A. lwoffi, Ps. aeroginosa and E. coli succinate thiokinases was also investigated by examining the dependences of enzyme activity on ATP and GTP

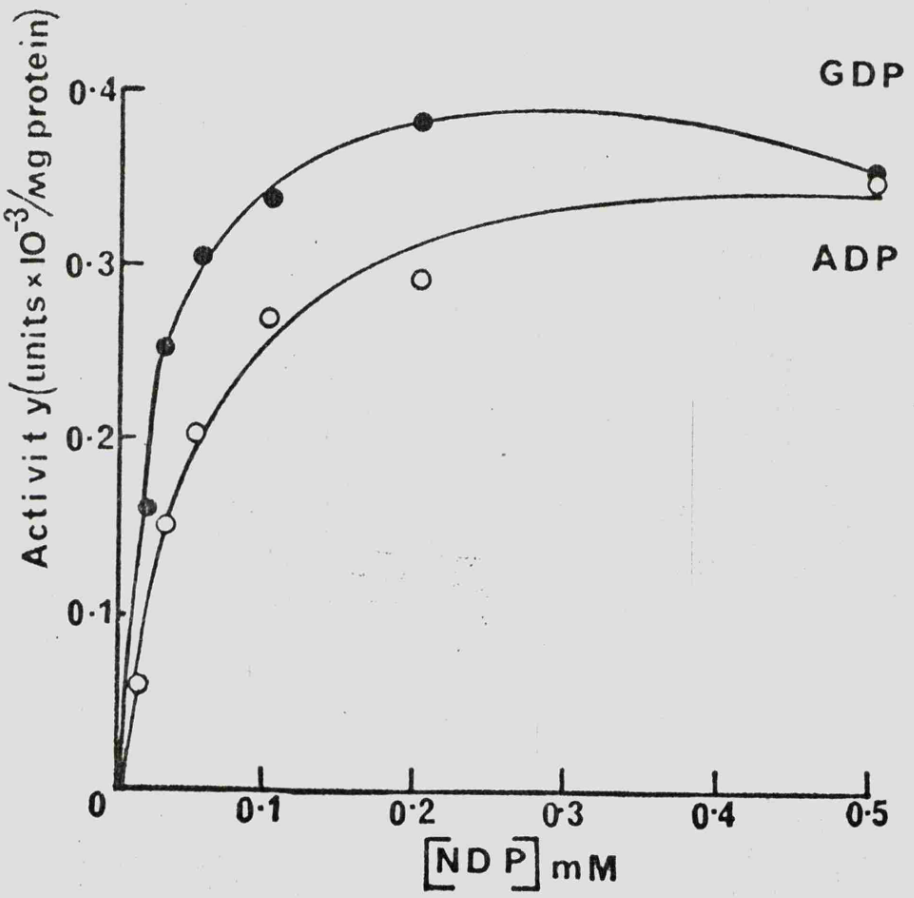

Figure 38 Dependence of Ps. aeruginosa succinate thiokinase activity on ADP and GDP concentration.

Assays were performed under standard conditions with crude enzyme preparations and ADP (○), or GDP (●) as nucleotide substrate.

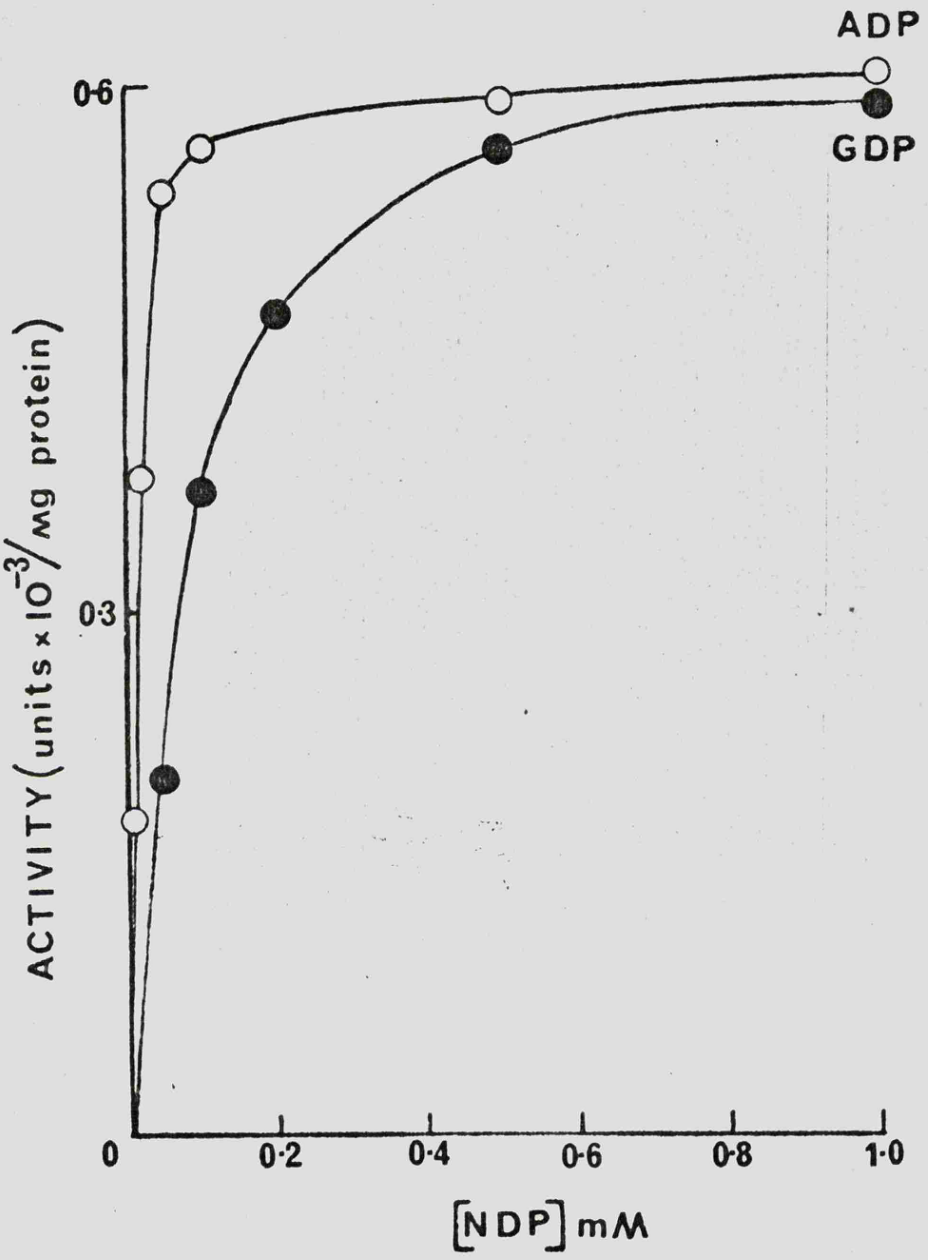

Figure 39 Dependence of E. coli succinate thiokinase activity on ADP and GDP concentration

Assays were performed under standard conditions with crude enzyme preparations and ADP (○), or GDP (●) as nucleotide substrate. .

concentrations by determining the rate of succinyl-CoA formation with these nucleotides. Fig. 40 illustrates the results of these experiments and shows that A. lwoffi succinate thiokinase exhibited a lower Km for GTP (0.13mM) than ATP (7.0mM), whereas the Ps. aeruginosa enzyme possessed comparable values for these parameters (0.20mM) and E. coli succinate thiokinase exhibited appreciably lower Km values for ATP (0.15mM) than GTP (0.63mM). (These parameters were determined from double reciprocal plots.)

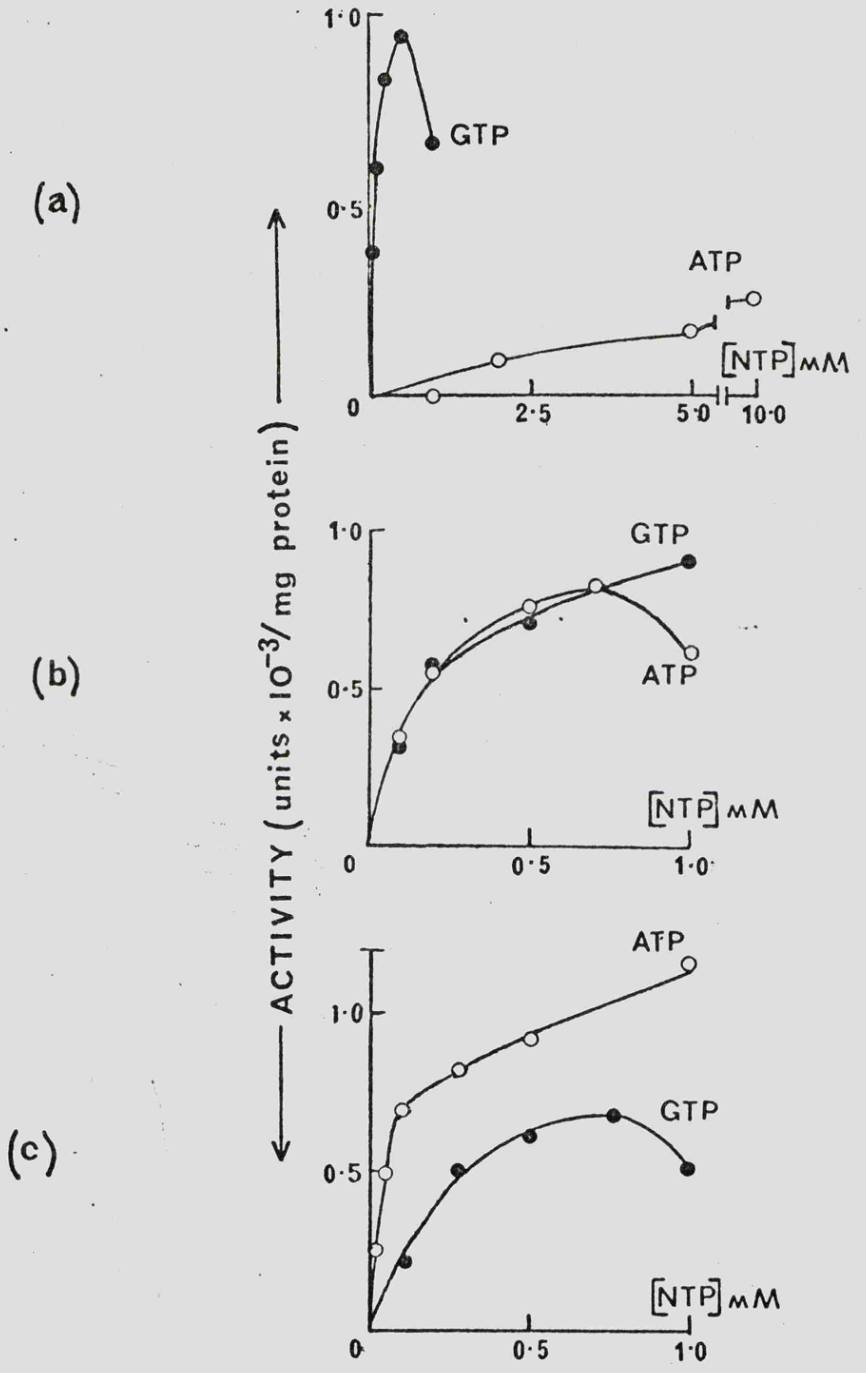

Figure 40 Dependence of (a) A. lwoffi (b) Ps. aeruginosa and (c) E. coli succinate thiokinase activity on ATP and GTP concentration

Assays were performed under standard conditions with crude enzyme preparations and ATP (○) or GTP (●) as nucleotide substrate.

3. Other Enzymes

The remaining A. Iwoffi citric acid cycle enzymes were examined for adenylate sensitivity and the extent to which other enzymes were subject to this type of regulation was also investigated. For this purpose those enzymes closely related to the cycle were selected. The effects of 1mM AMP or 1mM ATP on the rate-dependences on substrate concentration were examined with crude enzyme extracts which, unless otherwise stated, were prepared from nutrient-grown cells. Experiments were repeated with at least three different enzyme preparations and the date presented here illustrates one such experiment. Where appropriate, new assay methods have been developed.

(a) Citric acid cycle enzymes

Aconitase

Aconitase activity is generally determined by measuring the decrease in absorbance at 240nm due to disappearance of cis-aconitate. However, this method was unsuitable for these investigations because of the high initial absorbances associated with high protein concentrations or the presence of adenine nucleotides. By determining the conversion of citrate to isocitrate by coupling the reaction to NADP-linked isocitrate dehydrogenase these problems were avoided. The dependence of the reaction velocity on aconitase concentration was first examined by varying the amount of crude extract present in assay

mixtures. The observed rate was proportional to the concentration of enzyme protein (Fig. 41). Aconitase activity was then determined, with and without 1mM AMP or 1mM ATP (Fig. 42). Enzyme activity increased with increasing concentrations of citrate, but this dependence was unaffected by either AMP or ATP.

Succinate dehydrogenase

When estimating succinate dehydrogenase activity by determining succinoxidase activity, it is first essential to check that cytochrome oxidase is not the rate-determining step. This can be demonstrated by showing an increase in the rate of oxygen consumption when NADH is included in assays performed with a low concentration of succinate. Thus in one typical experiment the rate of oxygen consumption increased from 5.8ng atom O/min/mg protein, with 0.67mM succinate, to 16.3ng atom O/min/mg protein with 0.67mM succinate + 0.067mM NADH. Succinate dehydrogenase activity was then determined, with and without AMP and ATP (Fig. 43); again adenylates had no significant effect.

Fumarase

Fumarase activity was determined by a new polarographic method which was particularly suitable for use with crude enzyme extracts. The spectrophotometric method usually used involves measuring the decrease in absorbance due to the disappearance of fumarate. However, the wavelength selected for these measurements is between 250nm and 300nm, therefore this method is unsuitable for these investigations because of the high initial absorbances associated with high protein concentrations or the presence of the adenine nucleotides. The dependence of the reaction velocity on fumarase concentration was first examined by varying the amount of crude extract present in assay mixtures. The observed rate was

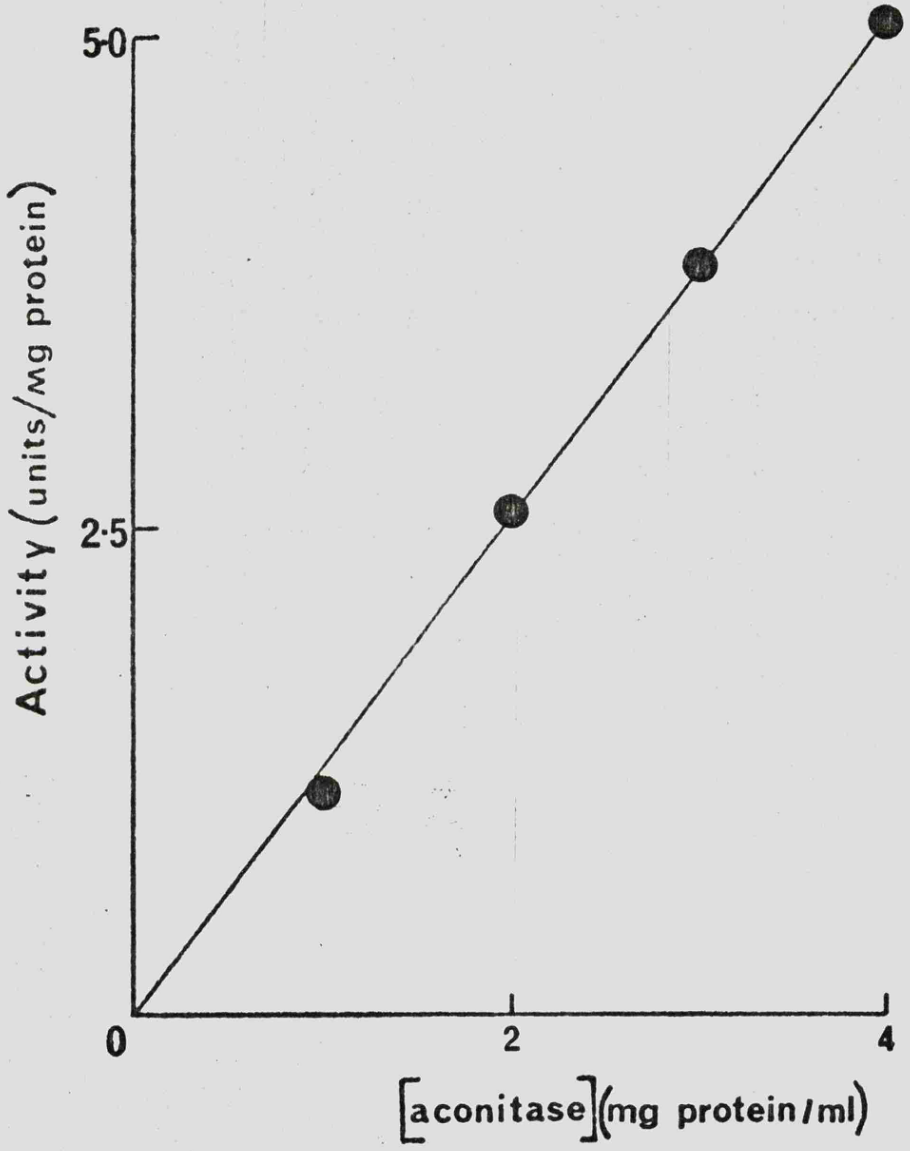

Figure 41 Dependence of reaction velocity on aconitase concentration

Assays were performed under standard conditions with crude A. lwoffi extracts and 5mM citrate.

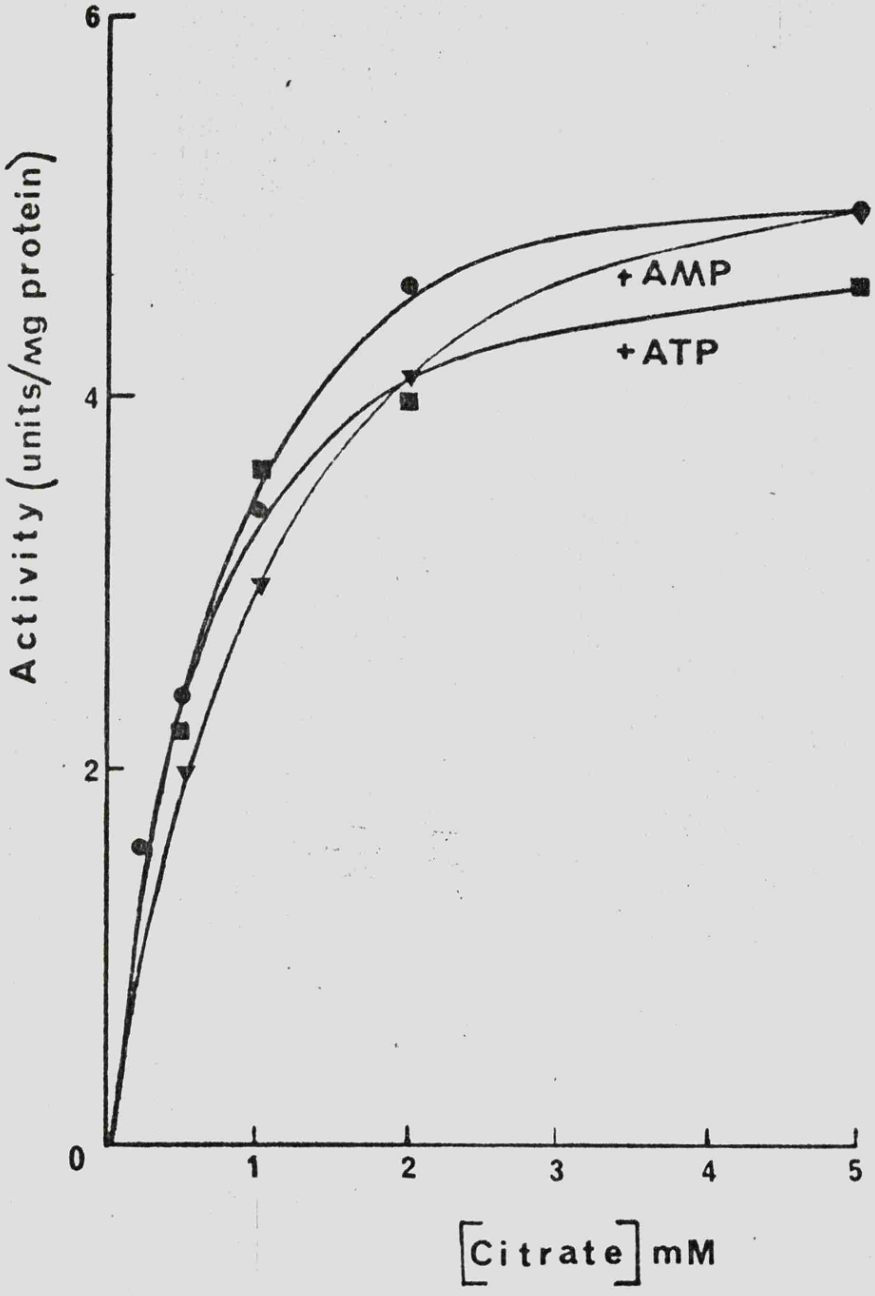

Figure 42 Effects of AMP or ATP on the dependence of A. lwoffi aconitase activity on citrate concentration

Assays were performed under standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

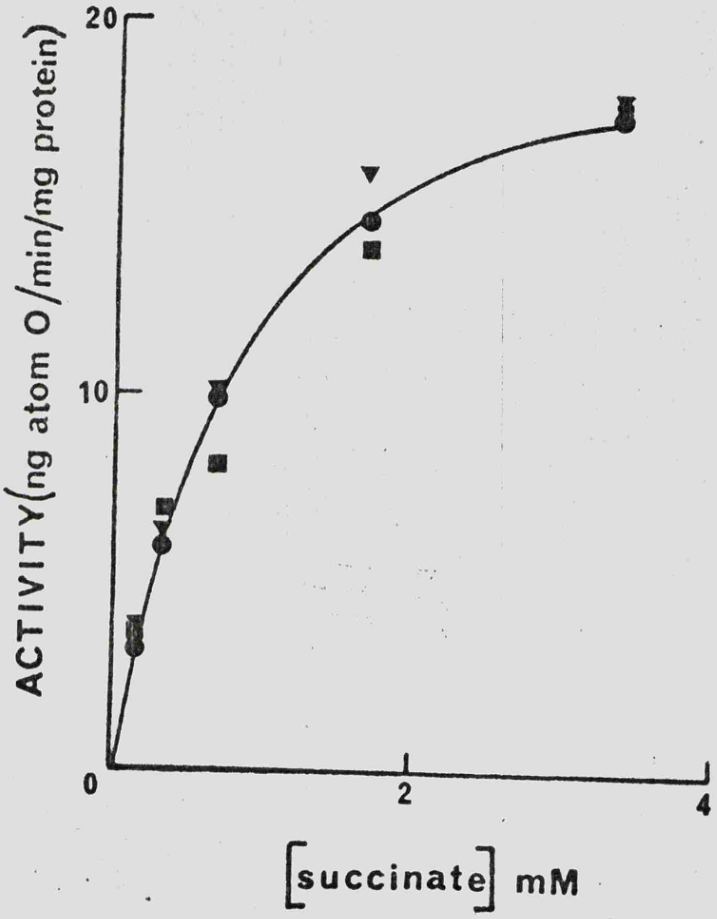

Figure 43 Effects of AMP or ATP on the dependence of A. lwoffi succinate dehydrogenase activity on succinate concentration

Assays were performed under standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

proportional to the concentration of enzyme protein (Fig. 44). Fumarase activity was then determined, with and without 1mM AMP or 1mM ATP (Fig. 45). Marked substrate inhibition was observed with high fumarate concentrations, but this dependence was unaffected by either AMP or ATP. The small effects of these nucleotides at high concentrations of fumarase were small and not reproducible with different enzyme preparations.

Malate dehydrogenase

Malate dehydrogenase activity can be assayed by measuring the increase in absorbance at 340nm due to NADH formation. If the product, oxaloacetate, is rapidly removed with citrate synthase and acetyl-CoA, this reaction proceeds in the thermodynamically unfavoured direction. However, NADH oxidase may interfere with these assays by removing NADH so that these methods may be unsuitable for use with crude enzyme preparations. However, malate dehydrogenase activity was determined polarographically in these studies and measurements were made in anaerobic conditions so that NADH oxidase did not interfere with these assays. The reaction rate was proportional to the concentration of enzyme protein (Fig. 46) and enzyme activity increased with increasing L-malate concentration (Fig. 47). Although AMP and ATP inhibited this enzyme in some experiments, this inhibition was small and not reproducible with different enzyme preparations.

(b) Reactions which deplete citric acid cycle intermediates

Those enzymes which catalyse the withdrawal of intermediates from the citric acid cycle, into biosynthetic pathways, have also been examined for a response to adenine nucleotides. It was possible that the sensitivity of the cycle enzymes may be complemented by a reverse form of control over these branch-point enzymes.

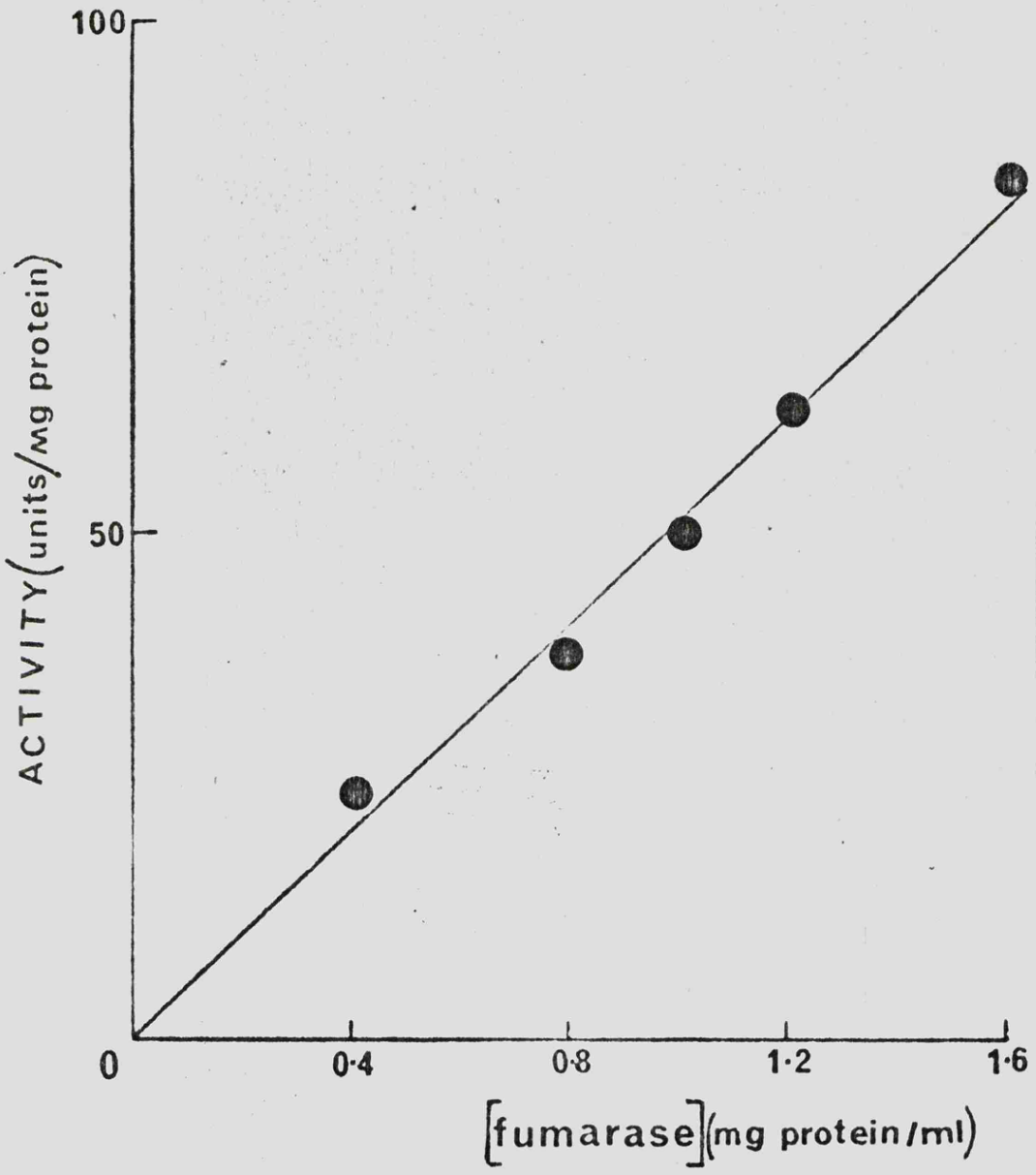

Figure 44 Dependence of reaction velocity on fumarase concentration

Assays were performed under standard conditions with crude A. lwoffi extracts and 5mM fumarate.

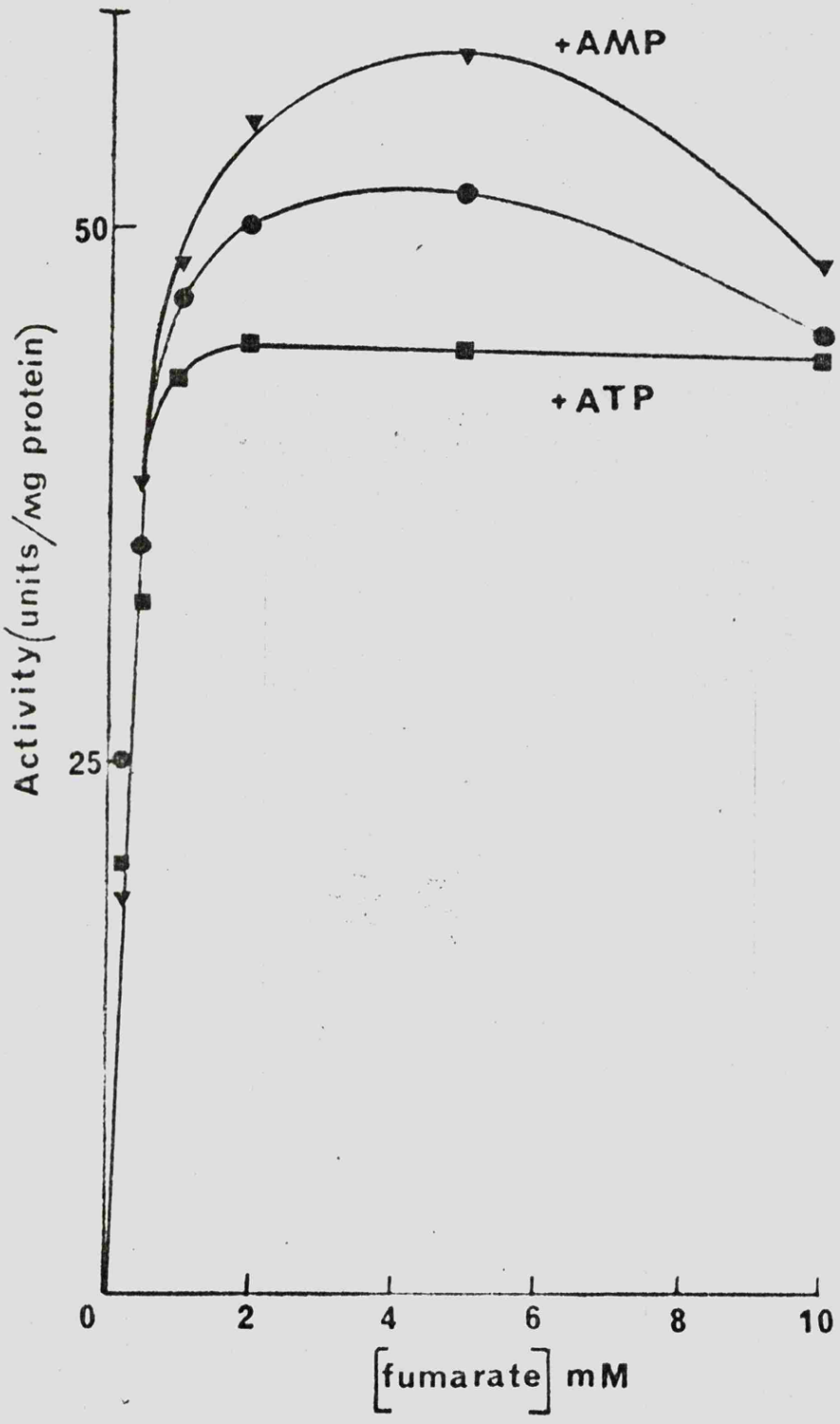

Figure 45 Effects of AMP or ATP on the dependence of A. lwoffi fumarase activity on fumarate concentration

Assays were performed under standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

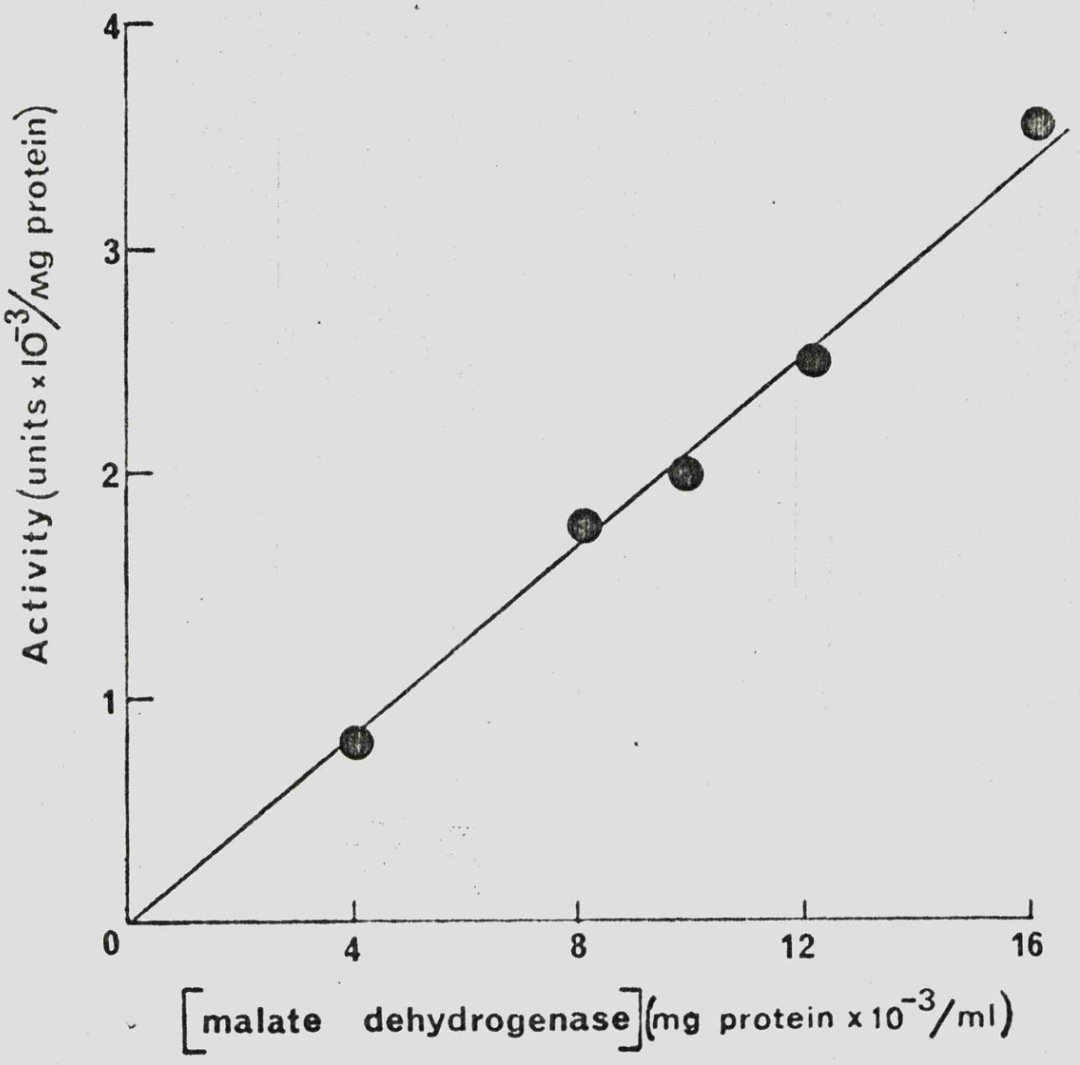

Figure 46 Dependence of reaction velocity on malate dehydrogenase concentration

Assays were performed under standard conditions with crude A. lwoffi extracts and 5mM L-malate.

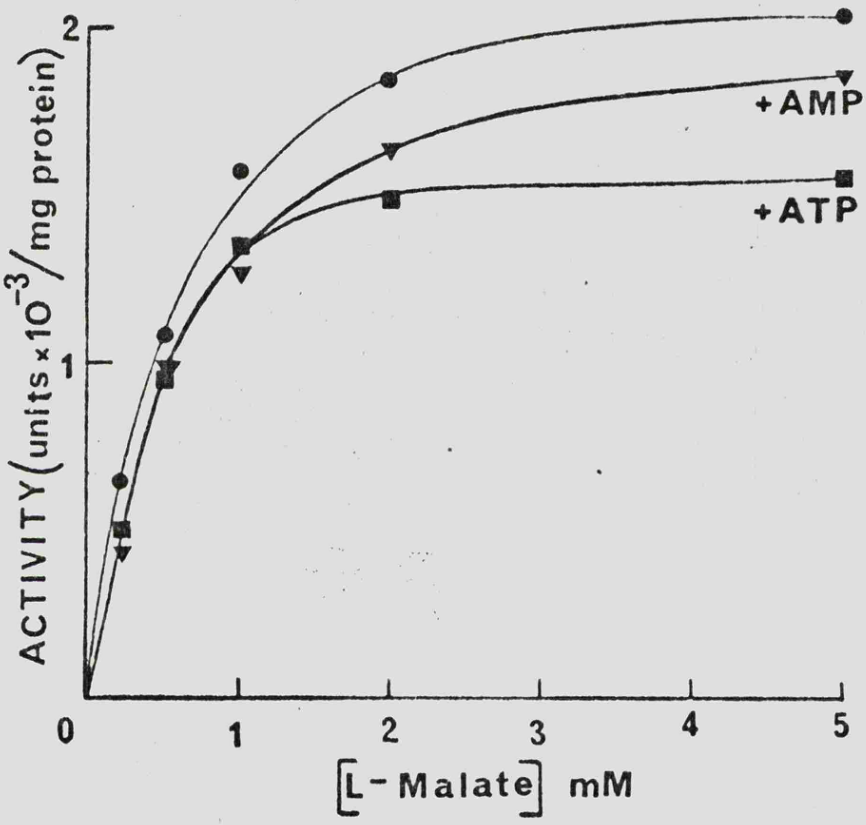

Figure 47 Effects of AMP or ATP on the dependence of A. lwoffi malate dehydrogenase activity on L-malate concentration

Assays were performed under standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

Isocitrate lyase

Isocitrate lyase activity was determined in cell-free extracts of A. lwoffi which had been grown with acetate as the carbon source. An examination of isocitrate lyase activity, with and without AMP or ATP (Fig. 48) showed marked substrate inhibition but this dependence was unaffected by these nucleotides. Phosphoenolpyruvate inhibited the enzyme but AMP and ATP had no significant effect on this inhibition.

Glutamate dehydrogenase

Glutamate dehydrogenase activity was determined in cell-free extracts of A. lwoffi which had been grown with acetate as the carbon source. The dependence of enzyme activity on α-oxoglutarate concentration was investigated, with and without 1mM AMP or 1mM ATP (Fig. 49). Substrate inhibition was observed at concentrations of α-oxoglutarate above 1mM, but the response to varying concentrations of substrate was unaltered by AMP or ATP.

Malic enzyme

Malic enzyme activity was first determined with various concentrations of $NADP^+$, and a constant concentration of L-malate. Fig. 50 illustrates the sigmoid dependence observed. The sigmoidicity was not enhanced by AMP or ATP and although both nucleotides stimulated this enzyme in some experiments, the activations were small and irreproducible. The apparent sigmoidicity of this dependence was not enhanced by compounds which are effectors of this enzyme in other systems, e.g. oxaloacetate, acetyl-CoA, NADPH or NADH (Sanwal and Smando, 1969). When the concentration of L-malate was varied at a constant concentration of $NADP^+$, the activity was slightly higher with AMP or ATP, at high concentrations of L-malate (Fig. 51). Once again, these stimulations were small and not always observed.

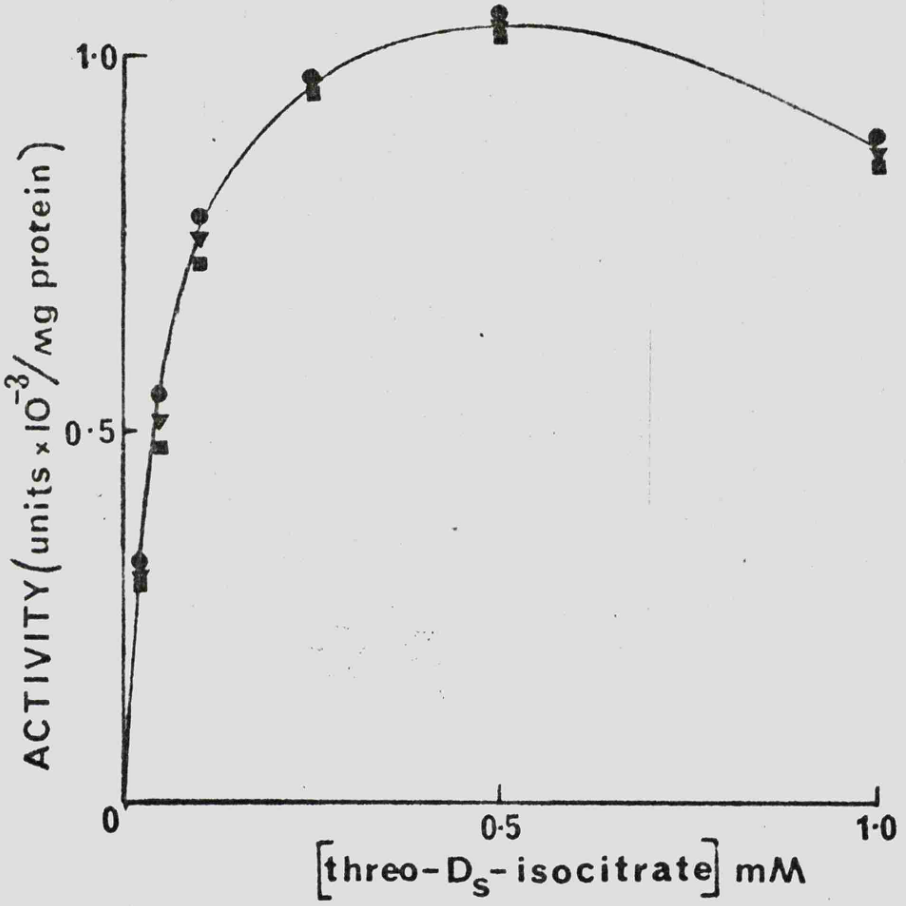

Figure 48 Effects of AMP or ATP on the dependence of A. lwoffi isocitrate lyase activity on threo-Ds-isocitrate concentration

Assays were performed under standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

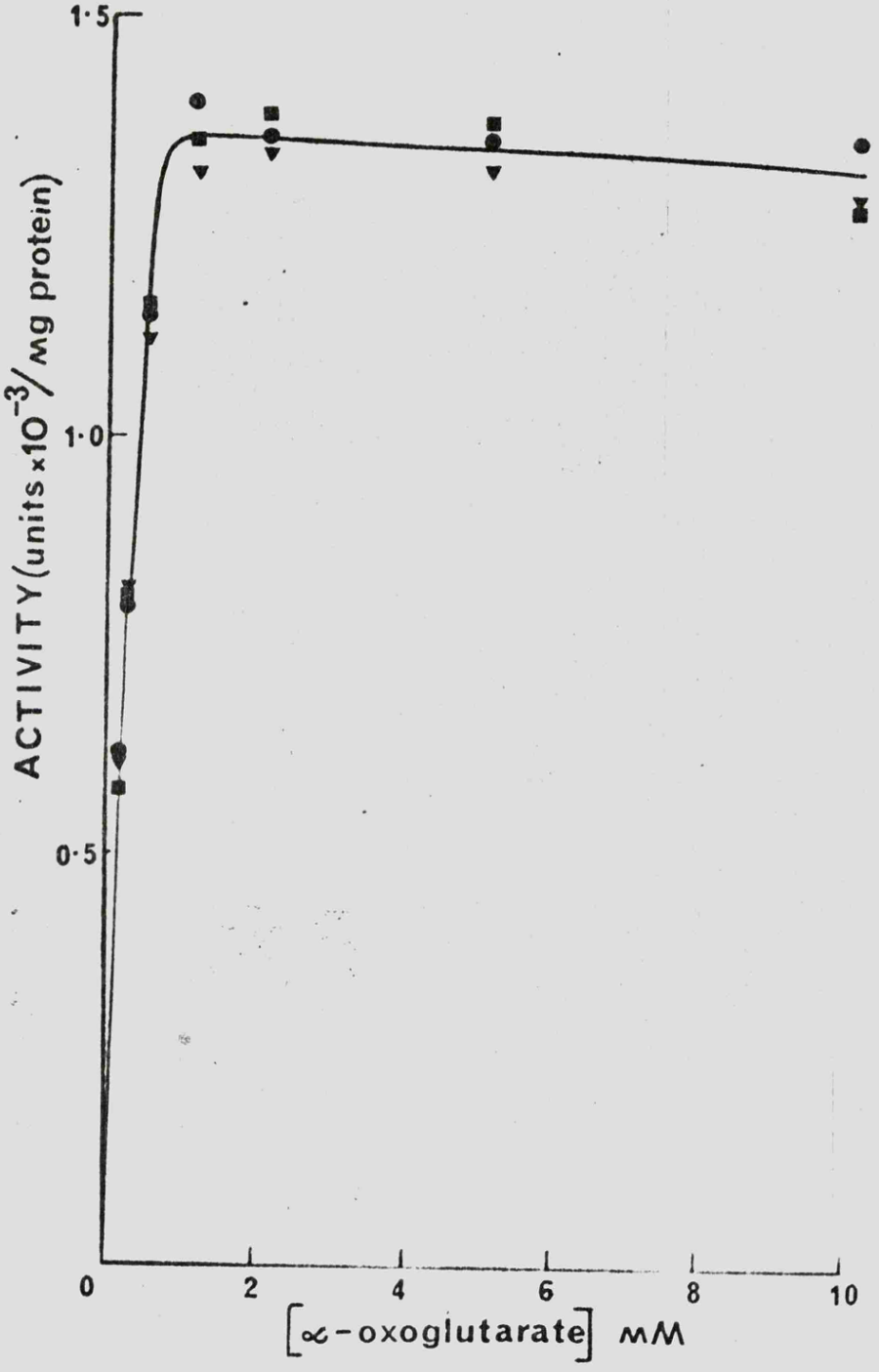

Figure 49 Effects of AMP or ATP on the dependence of A. lwoffi glutamate dehydrogenase activity on ∝-oxoglutarate concentration

Assays were performed under standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

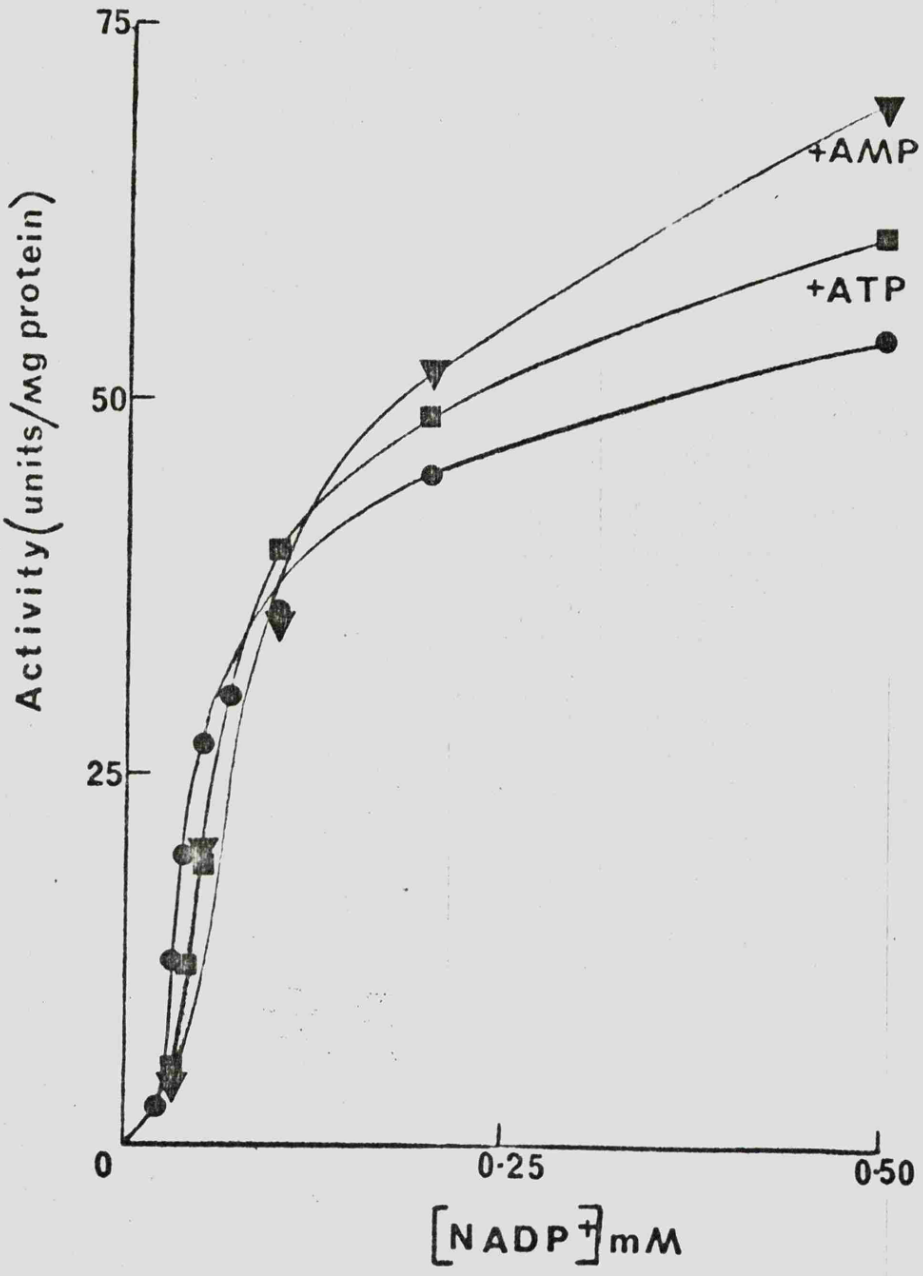

Figure 50 Effects of AMP or ATP on the dependence of A. lwoffi malic enzyme on $NADP^+$ concentration

Assays were performed under standard conditions with 5mM L-malate, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

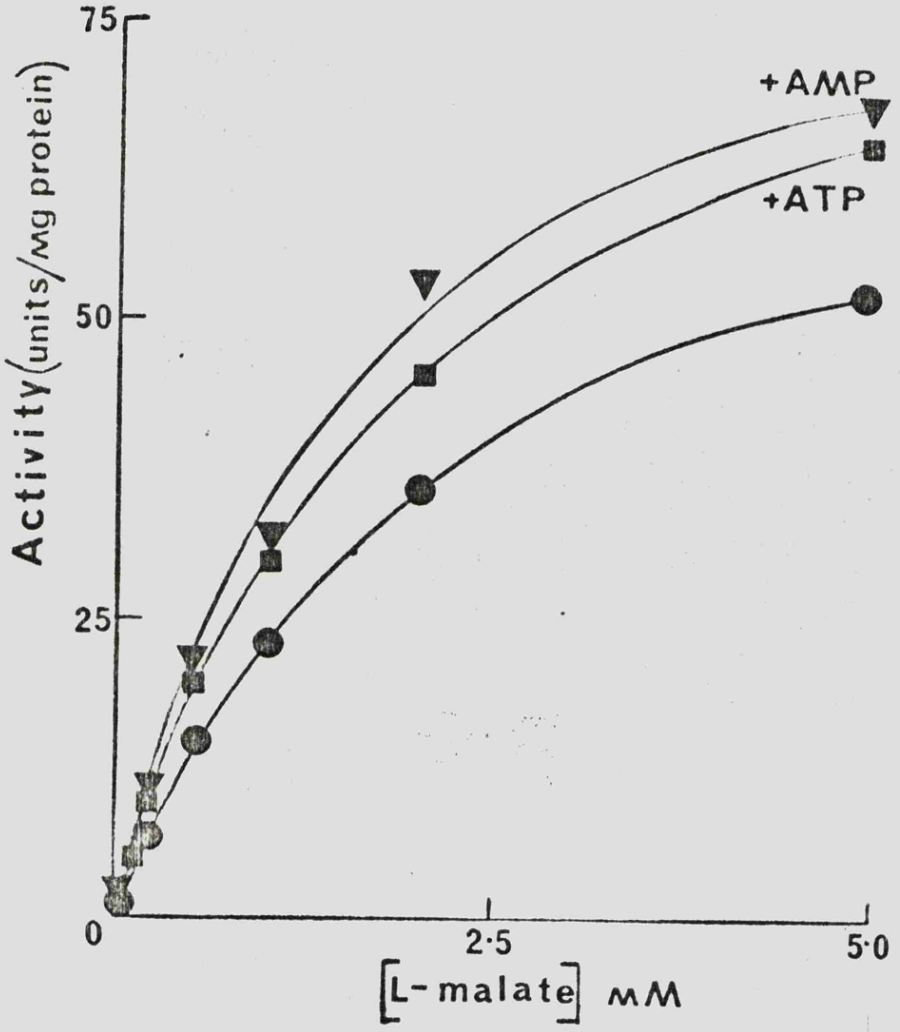

Figure 51 Effects of AMP or ATP on the dependence of A. lwoffi malic enzyme on L-malate concentration

Assays were performed under standard conditions with 0.5mM $NADP^+$, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

(c) C_3 to C_4 carboxylation reactions

Pyruvate occurs at a metabolic branch-point for several reactions, one of which is its carboxylation, in the presence of ATP, to form oxaloacetate. This reaction, catalysed by pyruvate carboxylase, was of special interest because the product formed is a citric acid cycle intermediate. A polarographic assay for pyruvate carboxylase (and phosphoenolpyruvate carboxylase) has been developed. These carboxylases are usually determined by estimating oxaloacetate production with malate dehydrogenase and measuring the rate of NADH oxidation. However, this method may be unsuitable for use with crude enzyme preparations which contain high concentrations of NADH oxidase. Polarographic assays are performed under anaerobic conditions and therefore have the advantage that they are unaffected by this contaminating enzyme activity. The assay procedure used retained the coupling system of the spectrophotometric method, but the reaction was followed by measuring the increase in current at -1.3V, due to NAD^+ formation.

NAD^+ is polarographically active, producing a cathodic wave at the dropping mercury electrode (Fig. 52). The trace obtained shows the familiar oscillation pattern and a line is drawn through their mid-points. Because the wave height is proportional to the concentration of NAD^+, by keeping the applied potential constant at -1.3V (at which a limiting current is reached), changes in NAD^+ concentration can be determined. First, the dependence of the height of the NAD^+ wave on NAD^+ concentration was examined. Increasing concentrations of NAD^+ were prepared in buffer solutions containing 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA and current-voltage polarograms recorded for each mixture (Fig. 53). A linear dependence was observed, relating the response

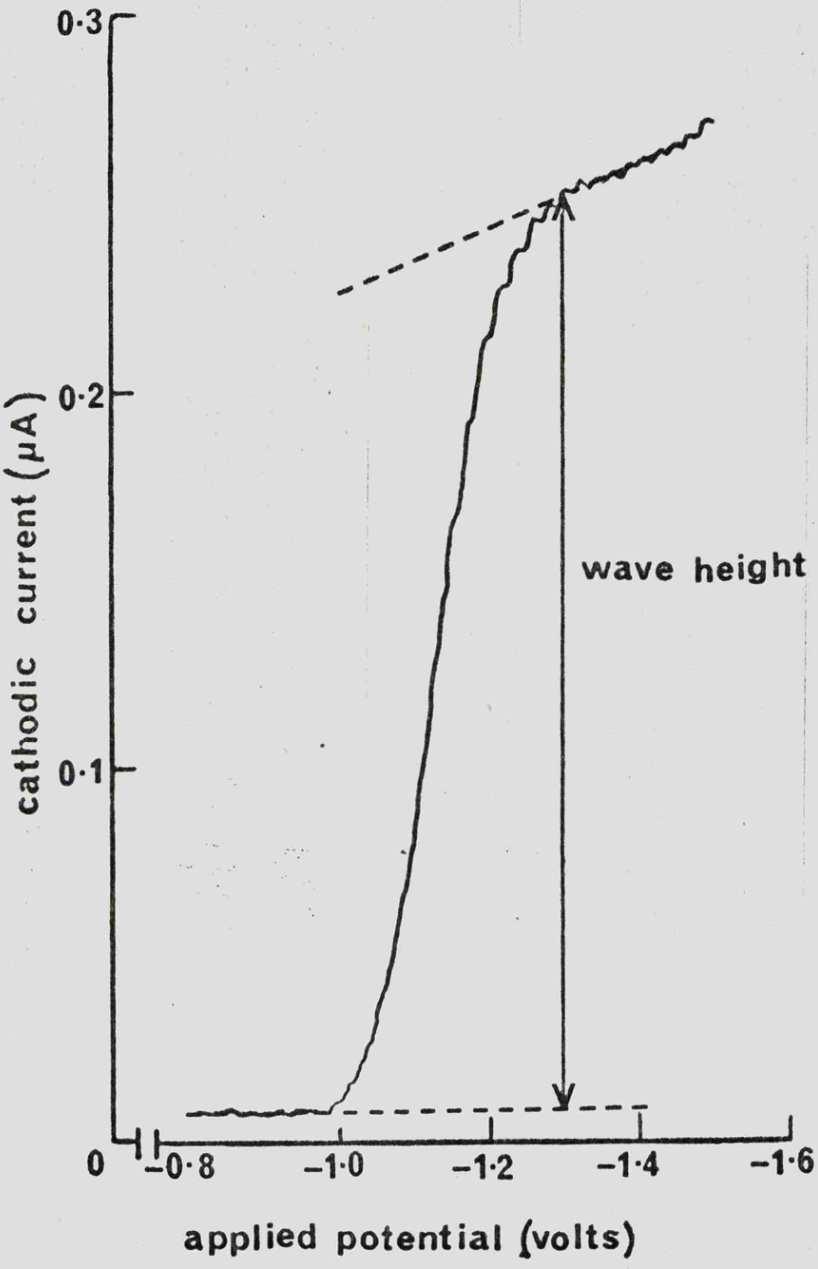

Figure 52 Polargraphic wave of NAD^+

Current-voltage polarograms were recorded in 20mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA.

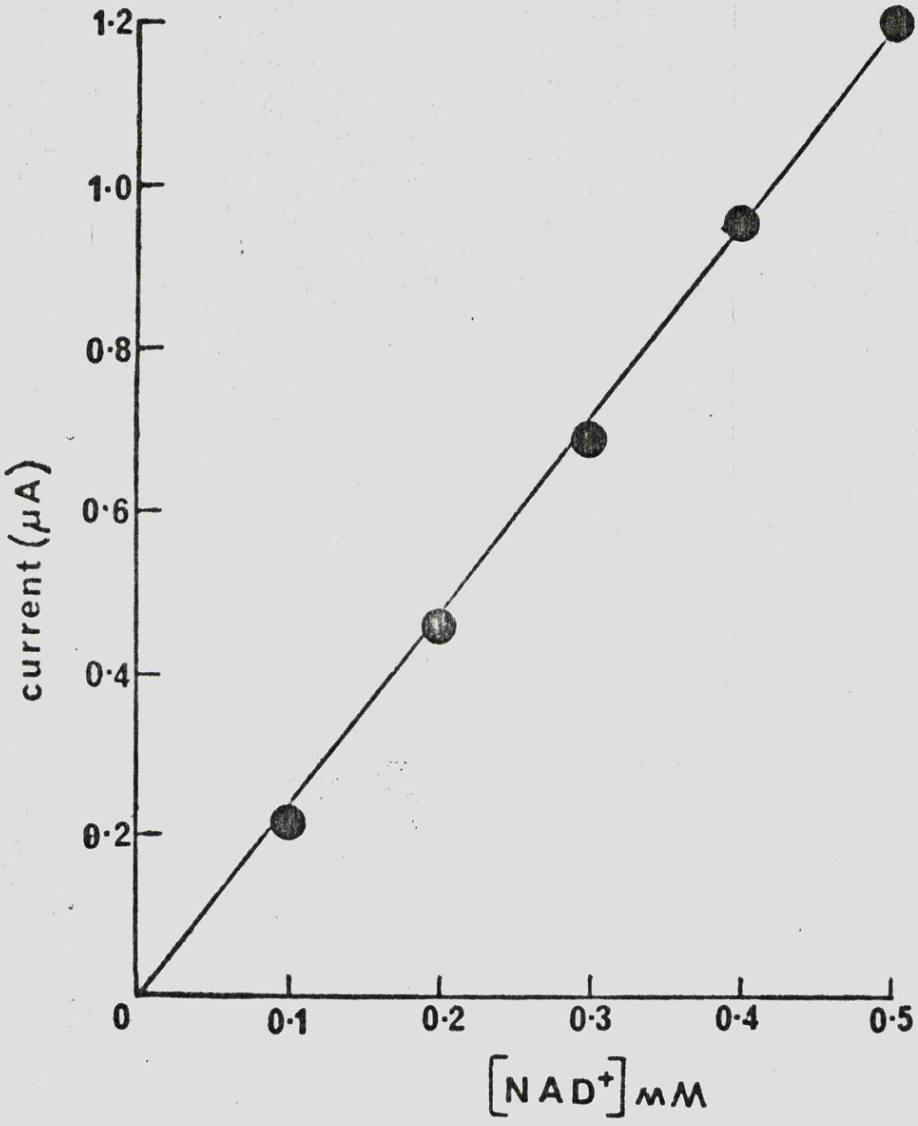

Figure 53 Dependence of polarographic wave height on NAD^+ concentration

Current-voltage polarograms were recorded at -1.3V as in Fig.52.

of the electrode in μA to NAD^+ concentration. Cell-free A. lwoffi extracts, which had been grown with pyruvate as the carbon source, were then examined for pyruvate carboxylase activity, but none was detectable. The assay mixtures were therefore modified to assay phosphoenolpyruvate carboxylase and appreciable levels of this enzyme activity were measured. Because the carboxylation reaction in A. lwoffi involves phosphoenolpyruvate carboxylase, rather than pyruvate carboxylase, this was examined for sensitivity to AMP or ATP.

The dependence of the measured reaction rate on phosphoenolpyruvate carboxylase concentration was investigated by varying the amount of sonic extract in the assay mixture. A linear dependence was observed (Fig. 54). Next, the dependence of enzyme activity on phosphoenolpyruvate concentration was examined, with and without 1mM AMP or 1mM ATP. The reaction velocity was slightly higher with AMP and ATP, but these stimulations were small and not always observed (Fig. 55). Enzyme activity was much higher with acetyl-CoA (Fig. 56), and a maximum activation of 6-fold was achieved with 0.034mM acetyl-CoA and 0.2mM phosphoenolpyruvate. Enzyme activity was therefore re-examined with 0.034mM acetyl-CoA and either without adenylates or with 1mM AMP or 1mM ATP (Fig. 57). Again adenylates had no significant effect. From double reciprocal plots the Km values for phosphoenolpyruvate were 0.19mM and 3.5mM, with and without 0.034mM acetyl-CoA, respectively.

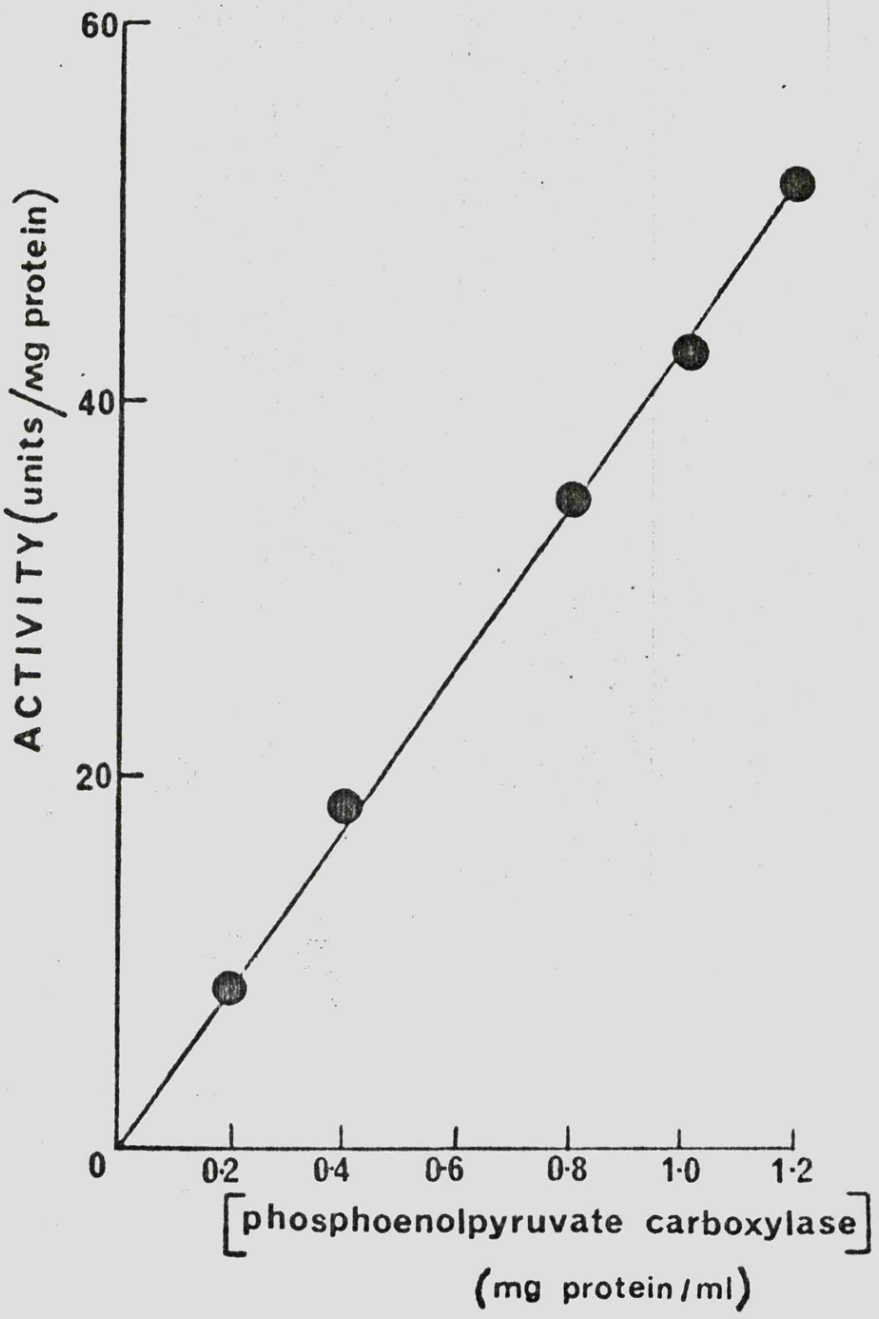

Figure 54 Dependence of reaction velocity on phosphoenolpyruvate carboxylase concentration

Assays were performed under standard conditions with crude A. lwoffi extracts and 2mM phosphoenolpyruvate.

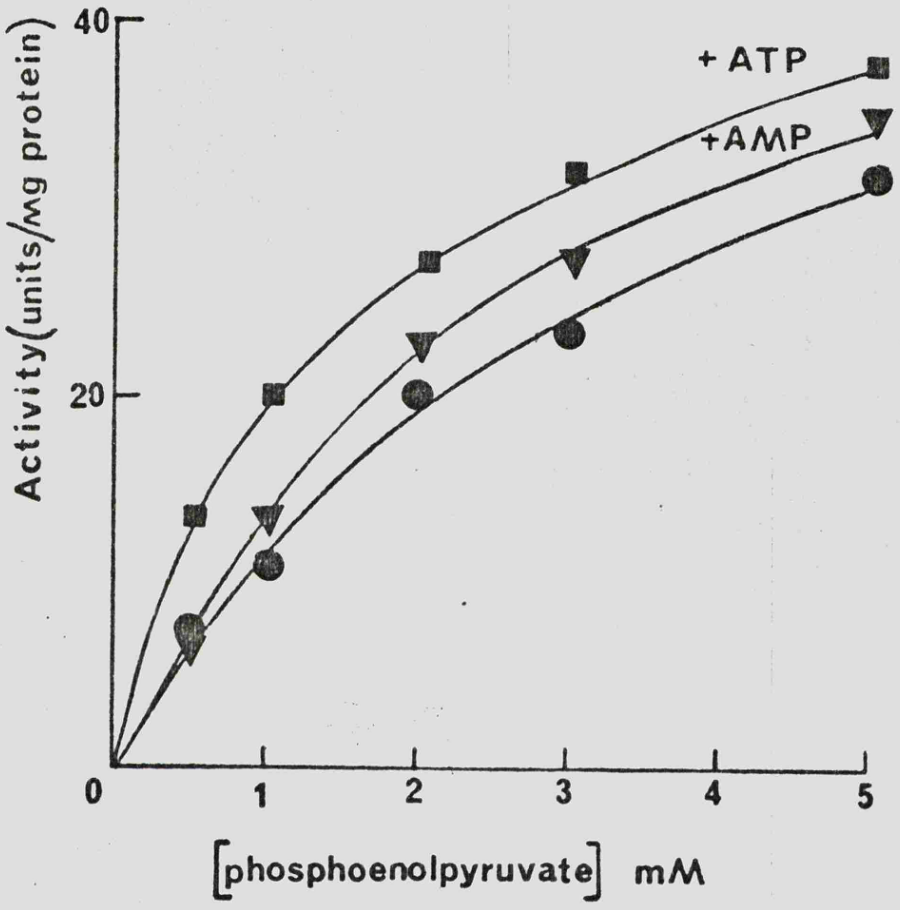

Figure 55 Effects of AMP and ATP on the dependence of A. lwoffi phosphoenolpyruvate carboxylase activity on phosphoenolpyruvate concentration

Assays were performed under standard conditions, without adenylates (●), or with lmM AMP (▼), or lmM ATP (■).

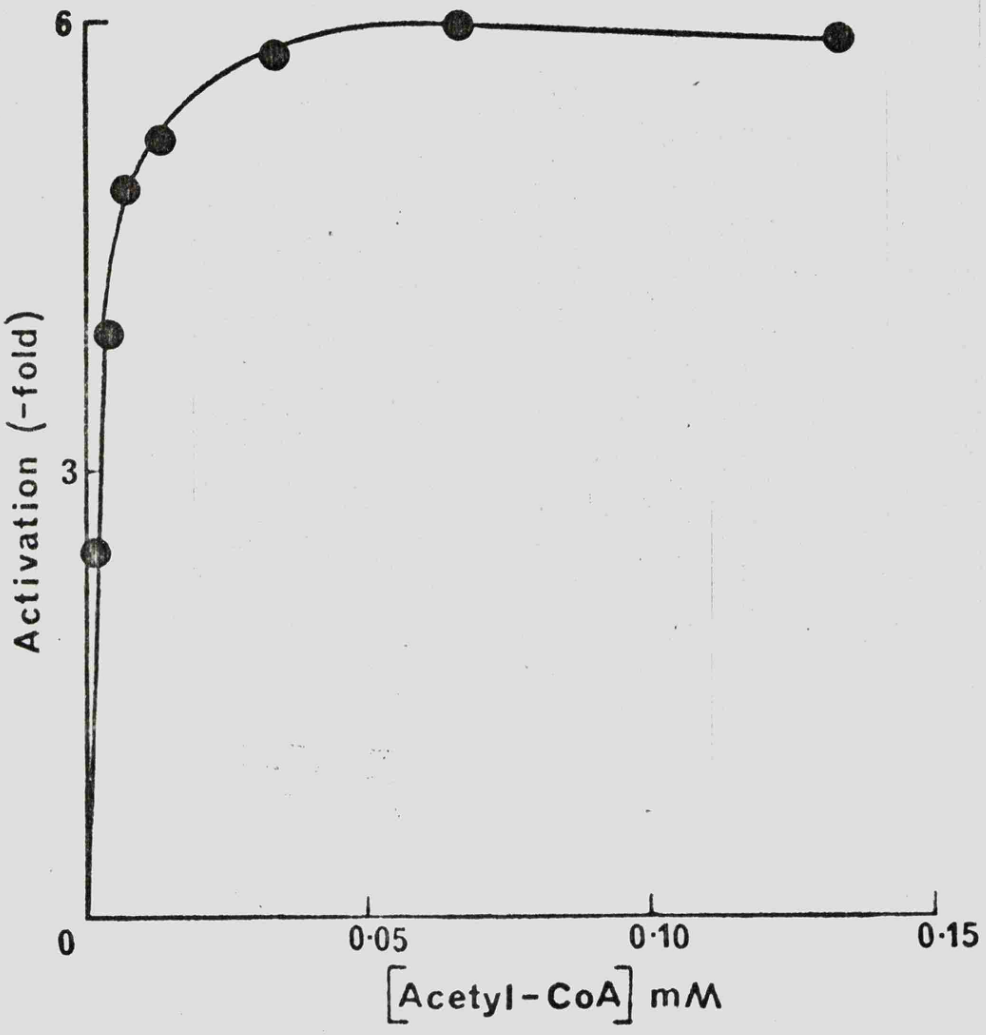

Figure 56 Dependence of the activation of A. lwoffi phosphoenolpyruvate carboxylase on acetyl-CoA concentration

Assays were performed under standard conditions with 0.2mM phospheonolpyruvate.

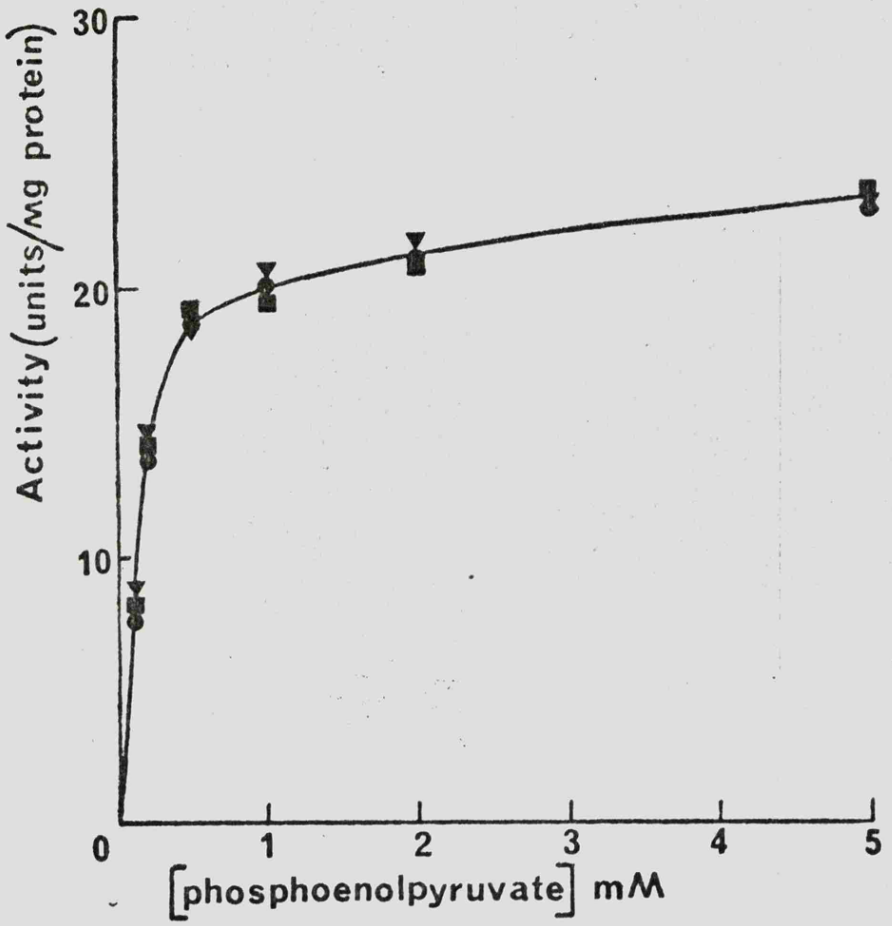

Figure 57 Effect of AMP or ATP on the dependence of acetyl-CoA stimulated-A. iwoffi phosphoenolpyruvate carboxylase activity on phosphoenolpyruvate concentration

Assays were performed with 0.034mM acetyl-CoA, under otherwise standard conditions, without adenylates (●), or with 1mM AMP (▼), or 1mM ATP (■).

4. Regulation of the citric acid cycle and taxonomic classification of bacterial species

Adenylate control of citrate synthase by relief of NADH inhibition with AMP is a feature of all Gram-negative strictly aerobic bacteria (Weitzman, 1966; Weitzman and Jones, 1968). However, A. lwoffi and a number of other Gram-negative strictly aerobic species also exhibit adenylate control of their pyruvate dehydrogenase and succinate thiokinase. The former enzyme is stimulated by AMP and by ADP, whereas ATP is inhibitory. Adenylate control of the latter enzyme is either a direct consequence of an unusually high Km for ADP, or an indirect result of this arising from ATP inhibition of the GDP-dependent enzyme activity, in the presence of nucleoside diphosphate kinase. Because A. lwoffi also exhibits adenylate control of its NADP-linked isocitrate dehydrogenase (Parker and Weitzman, 1970) and its α-oxoglutarate dehydrogenase (Weitzman, 1972; Parker and Weitzman, 1973), the extent to which other organisms showed adenylate control of their isocitrate dehydrogenase and α-oxoglutarate dehydrogenase was investigated. A. lwoffi isocitrate dehydrogenase is stimulated by AMP and ADP, whereas its α-oxoglutarate dehydrogenase is activated by AMP and ADP, and ATP is inhibitory. The adenylate sensitivity of other isocitrate dehydrogenases and α-oxoglutarate dehydrogenases was therefore investigated by examining the extent of activation produced by 0.5mM AMP. Enzymes were considered sensitive to AMP stimulation if a minimum activation of 1.5-fold was achieved. Because A. lwoffi isocitrate dehydrogenase is also stimulated by pyruvate and glyoxylate (Self et al., 1973), sensitivity of isocitrate dehydrogenases to activation by pyruvate and glyoxylate was additionally examined. 0.5mM glyoxylate was used and isocitrate

dehydrogenases were considered sensitive to glyoxylate stimulation if a minimum activation of 1.5-fold was achieved. Experiments were performed with crude extracts of cells which had been grown aerobically on nutrient broth and unless otherwise stated, assay conditions were as described in Methods. These examinations were performed in conjunction with Mr M.G.Parker of this Department and have also been presented elsewhere (Parker, 1972).

Table 9 shows the results of these experiments, together with the data already presented for citrate synthase (Weitzman and Jones, 1968), pyruvate dehydrogenase and succinate thiokinase. None of these five enzymes from Gram-negative facultative anaerobes or Gram-positive species was affected by the presence of adenine nucleotides. In contrast, the Gram-negative strictly aerobic species could be divided into three categories. The first category comprised organisms in which all five enzymes were sensitive to adenylates, the second group consisted of those organisms which, with the exception of the adenylate control at citrate synthase, a feature of all Gram-negative strictly aerobic species, showed no adenylate control of these citric acid cycle enzymes, and a third group showed adenylate control only at pyruvate dehydrogenase (in addition to that of citrate synthase). Glyoxylate activation was usually only observed in those isocitrate dehydrogenases which were also sensitive to AMP stimulation. There were two exceptions to this; the isocitrate dehydrogenases from Alcaligenes faecalis and Pseudomonas stutzeri were sensitive to glyoxylate activation but insensitive to AMP activation. These two organisms also constituted the third category of Gram-negative strict aerobes described above and showed adenylate control of their citric acid cycle activity only at citrate synthase and pyruvate dehydrogenase.

			PYRUVATE DEHYDROGENASE	CITRATE SYNTHASE	ISOCITRATE DEHYDROGENASE	α-OXOGLUTARATE DEHYDROGENASE	SUCCINATE THIOKINASE
Gram-negative	Strict aerobes	Acinetobacter lwoffi (1) and (2)	+	+	+‡	+	high
		Acinetobacter anitratus (1) and (2)	+	+	+‡	+	high
		Bordetella bronchiseptica (1) and (2)	+	+	+‡	+*	high
		Chromobacterium violaceum	+	+	+‡	+*	high
		Mima polymorpha	+	+	+‡	+*	high
		Moraxella calcoacetica (1) and (2)	+	+	+‡	+	high
		Xanthomonas hyacinthi (1)	+	+	+‡	+	high
		Azotobacter vinelandii	O	+	O	O	low
		Flavobacterium devorans	O	+	O	O	low
		Pseudomonas aeruginosa (1) and (2)	O	+	O	O	low
		Pseudomonas fluorescens	O	+	O	O	low
		Pseudomonas ovalis	O	+	O	O	low
		Xanthomonas campestris	O	+	O	O	low
		Xanthomonas uredovorans	O	+	O	O	low
		Alcaligenes faecalis	+	+	O‡	O	low
		Pseudomonas stutzeri (1) and (2)	+	+	O‡	O	low
	facultative anaerobes	Escherichia coli	O	O	O	O	low
		Klebsiella (Aerobacter) aerogenes	O	O	O	O	low
		Klebsiella pneumoniae	O	O	O	O	low
		Proteus vulgaris	O	O	O	O	low
		Salmonella anatum	O	O	O	O	low
		Serratia marcescens	O	O	O	O	low
Gram-positive		Arthrobacter globiformis	O	O	O	O	low
		Bacillus subtilis	O	O	O	O	low
		Kurthia zopfii	O	O	O	O	low
		Microbacterium thermosphactum	O	O	O	O	low
		Mycobacterium rhodocrous	O	O	O	O	low

TABLE 9 Patterns of adenylate regulation of bacterial citric acid cycle enzymes.

For legend see overleaf

TABLE 9 Patterns of adenylate regulation of bacterial citric acid cycle enzymes.

+: denotes a minimum AMP activation of 1.5-fold, except in the case of citrate synthase where it denotes that AMP overcomes the NADH inhibition.
0: denotes that the enzyme activity was unaffected by adenylates
low: denotes a Km for ADP less than 0.1mM
high: denotes a Km for ADP of approximately 1mM
(1) and (2): denote different strains and their culture collection numbers are presented in Table 1.
*: assays were performed at 0.1mM α-oxoglutarate concentration.
‡: denotes a minimum glyoxylate activation of 1.5-fold.

IV DISCUSSION

1. General Considerations

The studies described in this thesis have been performed in vitro and require translation into metabolic terms. Such extrapolations may be hazardous, however, because the very nature of these experimental methods disturbs the conditions of the phenomenon under examination, and may give rise to spurious effects. Perhaps the first consideration must be the suitability of the parameter used to examine these responses. In the study of regulation a physiologically applicable variable must be used and for these investigations the concentration of the adenine nucleotides has been selected. Intracellular cycle activity results in the formation of reducing equivalents which, when fed into the electron-transport chain, result in the regeneration of ATP. It would seem reasonable, by analogy with the end-product feedback control of biosynthetic pathways, that citric acid cycle activity should be inhibited by ATP. Because the intracellular concentrations of AMP and ATP vary in an opposite manner, stimulation by AMP will be functionally equivalent to inhibition by ATP. However, the intracellular concentration of AMP may be a more sensitive indicator of the supply of ATP than the concentrations of either ATP or ADP, because the concentration of AMP varies much more than the concentrations of ATP or ADP in the cell (Krebs, 1964; Sanwal, 1970). A simple calculation illustrates this (Krebs, 1964). In the cell the adenine nucleotides are maintained in an equilibrium mixture by adenylate kinase, which has an equilibrium constant $\left((AMP) \times (ATP)/(ADP)^2\right)$ of 0.44 (Eggleston and Hems, 1952). If a total adenine nucleotide concentration of 5mM is assumed, then by increasing the ADP concentration from 1mM to 2mM (100%), the ATP concentration will decrease 43% (from 3.87mM to 2.21mM) but the AMP concentration will increase 720% (from 0.11mM to 0.79mM). Therefore

the absolute concentration of AMP may be a more sensitive control signal than the concentrations of ADP or ATP. Control by the adenylates will not only apply to the citric acid cycle but also to other pathways which furnish electrons for electron transport phosphorylation, and includes all pathways with a degradative function (Sanwal, 1970). These latter pathways convert nonessential carbon sources into the intermediates of amphibolic pathways, such as the citric acid cycle, and are therefore regulated by the end-products of energy metabolism.

In assessing the physiological significance of observed responses, the concentration of metabolite required to bring about these effects must be considered. Ideally, the amount of a metabolite included in *in vitro* assays should reflect the intracellular concentration of the compound in that particular organism. However, this value is often unknown and the effector concentration used is based on measurements made in other systems. Thus the regulatory properties of *A. lwoffi* enzymes have been investigated with concentrations of AMP, ADP or ATP which have been shown to exist in *E. coli* (Frazer and Binkley, 1961; Lowry *et al.*, 1971) and in other systems (Sols and Marco, 1970). Here the total adenine nucleotide concentration has been estimated to be between 1mM and 5mM, and a value as high as 10mM has been reported in rabbit skeletal muscle (Hasselbach, 1957). These concentrations have been calculated with the assumptions that there are 2.5g H_2O/g dry weight (Schultz and Solomon, 1961; Winkler and Wilson, 1966; Zwaig *et al.*, 1970) and that the nucleotides are uniformly distributed in the cell water, with no concentration gradients or cellular compartmentation. Withinthis nucleotide pool, the ratio ATP:ADP:AMP is 50:6:1 so that in a 1-5mM adenylate pool, the intracellular concentrations of ATP, ADP and AMP will be 0.88mM - 4.4mM, 0.11 - 0.55mM and 0.018mM - 0.09mM

respectively. It is very likely that similar adenylate concentrations are found in A. lwoffi.

Although the concentration of the adenine nucleotides is a useful parameter with which to examine the control of the citric acid cycle, nevertheless it is a physiologically unrealistic variable. The range from complete absence of adenylates, through increasing values of any one of them does not occur in the cell. Therefore, the activity of cycle enzymes has been examined as a function of energy charge. This parameter reflects the changes in concentration of the adenine nucleotides within a fixed adenylate pool, and it is particularly useful when enzyme activity responds to more than one adenine nucleotide. Because the adenylates are involved in many reactions, either as substrates or effectors, energy charge is a widely applicable parameter, and can be used to interpret the metabolic interactions between them. Thus, A. lwoffi pyruvate dehydrogenase (Fig. 8) and succinate thiokinase (Figs. 28 and 31) exhibit energy charge dependenc-es which are typical of enzymes involved in ATP-regenerating sequences. Similar dependenc-es have also been observed with A. lwoffi citrate synthase (Weitzman, personal communication), isocitrate dehydrogenase (Parker and Weitzman, 1970) and α-oxoglutarate dehydrogenase (Parker and Weitzman, 1973).

For energy charge to be a meaningful concept it must apply to the in vivo situation. Thus normally growing E. coli cells have been shown to possess energy charge values of 0.8 or above (Chapman et. al., 1971). Between energy charge values of 0.8 and 0.5 the cells did not grow but remained viable, forming colonies, when removed from the limiting cultures in which their energy charge was between 0.5 and 0.8. Below energy charge values of 0.5 the cells died. These results indicate that an energy charge of at least 0.8

is required for normal growth, whereas charge values between 0.5 and 0.8 are only compatible with the maintenance of viability, but not growth. Below an energy charge value of 0.5 even this minimal level of homeostasis cannot be maintained. This is hardly surprising because an examination of the general energy charge response curves illustrated in Fig. 2 shows that enzyme activity is virtually independent of energy charge below a value of 0.5. This represents an absence of strict control and because metabolic regulation is a prerequisite of life, it seems inevitable that a decrease in energy charge to below 0.5 must lead to a fatal disruption of cellular economy, i.e. death. These workers also computed energy charge values for many tissues and organisms from published data in the intracellular concentrations of the adenine nucleotides. They showed this parameter to be stabilised near 0.85, and although the value of energy charge has not been estimated in A. lwoffi, a similar value may well be observed in this organism.

It may be argued that energy charge is not a useful control parameter because it varies so little. However, the very constancy of energy charge might be more properly taken as evidence of an efficient control system (Shen et al., 1968; Atkinson, 1970): the more sensitive a regulatory system, the less the control parameter will vary. Indeed a constant concentration of intermediates cannot be achieved in vivo without regulation, and it may be that the stabilisation of energy charge places regulatory enzymes in a position where they are sensitive to changes in concentration of other metabolites (Atkinson, 1968b; Lowry et al., 1971). This will be particularly important for amphibolic enzymes which supply the cell with energy as well as providing the primary biosynthetic intermediates. Thus, E. coli pyruvate dehydrogenase and phosphofructokinase are

controlled by both energy charge and the concentrations of acetyl-CoA and citrate, respectively (Shen et al., 1968)

When monitoring the response of initial reaction velocities to energy charge, several factors must be taken into consideration. First, the intracellular concentration of free uncomplexed magnesium may be important, because magnesium forms complexes with the adenine nucleotides. This will alter the mass action ratio of adenylate kinase, which is based on the concentrations of the free nucleotides (Rose, 1968; Blair, 1970; Purich and Fromm, 1972). Atkinson (1968a) originally assumed an equilibrium constant of 0.8; however this value only applies to certain conditions. He also only considered the total concentration of the adenylates and overlooked those parts linked to magnesium. These will depend on the magnesium concentration. Thus, the distributions of the mole fractions of AMP, ADP and ATP may be different to those originally proposed by Atkinson, depending on the concentration of magnesium. Other factors may also alter the response of enzymes to energy charge. These are the total adenine nucleotide pool size, the concentration of substrates and products present, and the mechanism under study (Purich and Fromm, 1972).

Although the concentration of the adenylates is a useful parameter with which to investigate the regulatory properties of the citric acid cycle, the contribution made by responses to NADH must also be considered. It has been suggested that this compound acts as a feedback modifier of citric acid cycle activity, because it is generally produced at the dehydrogenation steps. Thus, NADH has been shown to inhibit a variety of pyruvate dehydrogenases, citrate synthases, isocitrate dehydrogenases, α-oxoglutarate dehydrogenases and malate dehydrogenases (see INTRODUCTION). The responses to NADH and the adenylates may be complementary, because NADH oxidation is linked to electron transport phosphorylation . Under high energy conditions

the rate of NADH oxidation may well be reduced so that the intracellular concentration of NADH may be temporarily increased. This situation, together with the increased concentration of ATP, or the decrease in AMP concentration, may reduce cycle activity by the appropriate controls exerted over regulated enzymes.

The importance of using physiological concentrations of metabolites to study in vitro regulation has already been pointed out. However, values obtained for the intracellular concentrations of metabolites are estimates of the total concentration of these compounds, and not their free concentration. (Free concentration is an operational concept used to describe the amount of a metabolite actually available for interacting with proteins (Sols and Marco, 1970). There may be a considerable difference between free concentration and total concentration, especially for those metabolites, such as the adenylates and the pyridine nucleotides, which are involved in a number of intracellular reactions. An appreciable concentration of these compounds may be enzyme bound, particularly if the metabolite is present at a low concentration in the cell. Thus, in the adenylate pool, AMP is present at the lowest concentration. A large proportion of it may therefore be enzyme bound, and its free concentration may be considerably lower than its total concentration. ATP, on the other hand, is at the highest concentration in the adenylate pool. It is therefore unlikely that its free concentration will be affected by binding to proteins, although its free concentration may be reduced by forming complexes with divalent metals, such as magnesium (Rose, 1968; Blair, 1970; Purich and Fromm, 1972).

Similarly, the intracellular concentration of NADH is also low. Therefore an appreciable proportion of this metabolite

may also be enzyme bound. Indeed, Chance and Baltscheffsky, (1958) have suggested that there is no unbound NADH in the cell; a result consistent with the observation that dehydrogenases have a high affinity for NADH (Velick, 1958). However, calculations of the $NAD^+_{free}/NADH_{free}$ ratio by Williamson et al. (1967) indicate that free NADH does exist in the cell, although Sols and Marco suggest that its concentration must be 1% or less of that measured, and therefore unlikely to be of value as a regulatory signal. The concentration of NAD^+ may therefore be the determining regulatory factor, particularly as dehydrogenases appear to exhibit a variable affinity for this cofactor (Velick, 1958). Thus, where the intracellular concentration of a metabolite is low, the assumption that the free concentration is the same as the total concentration may be incorrect. Under such circumstances the concentrations of the enzymes using these metabolites and their relative affinities, must be taken into account.

The high concentration of enzymes found in cells may well pose another problem when translating in vitro responses into metabolic terms. The apparent concentration of enzymes in cells and mitochondria has been calculated to be between 10^{-6} and 10^{-5} moles/kg tissue (Srere, 1967). However, concentrations of only 10^{-7} - 10^{-10} moles/l are used for in vitro studies because of the high sensitivity of conventional techniques. This deviation from in vivo conditions (which may be several orders of magnitude) may give rise to apparently different regulatory responses (Srere, 1965; Frieden and Colman, 1967; Sanwal and Suzuki, 1970; Weitzman and Hewson, 1973). Perhaps the most popular examples of such a variation is the effect of high enzyme concentration on the regulatory properties of bovine liver glutamate dehydrogenase (Frieden and Colman, 1967). A co-operative interaction exists between the GTP and GDP inhibitor

sites at high but not at low protein concentrations. It has been suggested that this is due to a preferential binding of the inhibitors to one form of the enzyme. Bovine liver glutamate dehydrogenase is known to undergo a protein-dependent association, existing as a monomer at low protein concentrations and a mixture of monomer and polymer at high protein concentration (Freiden, 1958). GTP and GDP bind more strongly to the monomer than to polymer. Although enzyme dissociation at low protein concentrations may account for these particular apparent regulatory phenomena, gross physical changes need not always occur, and subtle conformational changes at high protein concentration may be sufficient to bring about apparently different regulatory properties.

High enzyme concentrations can be used *in vitro*, using highly complex stopped flow techniques. However, these enzymic studies are still performed under conditions far removed from those of the cell. In preparing cell-free extracts not only are enzymes diluted, but also all the macromolecular constituents. This destroys the particular environment the enzyme experienced in the cell, and may give rise to apparently different regulatory behaviour in extracts compared to *in vivo*. To examine the regulatory properties of enzymes in their intracellular environment and to overcome the problems of high enzyme concentration, enzymes have been studied *in situ* (Reeves and Sols, 1973; Weitzman, 1973). These methods involve permeabilising cells with toluene or other reagents to render them freely permeable to low molecular weight metabolites, whilst retaining the protein and other macromolecular constituents intracellularly (Monod *et al.*, 1951; Torriani, 1960; Bridgeland and Jones, 1965; Kornberg and Reeves, 1972; Weitzman and Hewson, 1973). Enzymes will therefore be present at similar concentrations to those of living cells, and because the other

macromolecules are also present, some of the molecular interactions characteristic of the living cell may also be preserved. Toluenised cells are therefore a closer approximation to the living cell than cell-free extracts so that the properties of enzymes observed *in situ* may be a closer approximation to *in vivo* behaviour. Thus more physiological significance can be attributed to the phosphoenolpyruvate inhibition of *E. coli* isocitrate lyase because it is observed in toluenised cells, as well as in cell-free extracts (Ashworth, 1965). In contrast, *Arthrobacter globiformis* serine dehydratase only exhibited a lag in pyruvate formation, a cation requirement, and a sigmoid serine saturation curve in cell-free extracts (Bridgeland and Jones, 1965), and this was subsequently shown to be due to a slow serine-dependent dimerisation of the enzyme in crude extracts (Gannon, 1973). On the other hand, Sawula and Suzuki (1970) have used purified *Klebsiella (Aerobacter) aerogenes* lactate dehydrogenase to examine the effects of high protein concentration on the regulatory properties of the enzyme. They demonstrated a sigmoid pyruvate-dependence at low but not at high protein concentrations, and concluded that at normal intracellular enzyme concentrations this dependence would be hyperbolic. However, Weitzman (1973) has demonstrated a sigmoid pyruvate dependence with this enzyme in toluenised cells. He therefore suggested that such a dependence may operate *in vivo*, because permeabilised cells not only contain high protein concentrations, but they also approximate the intracellular environment of the living cell. These results emphasise the danger of considering enzyme concentration in isolation, without taking into account the modifying effects of the intracellular environment.

Weitzman (personal communication) has also examined the regulatory properties of *A. lwoffi* pyruvate dehydrogenase, citrate

synthase, isocitrate dehydrogenase, α-oxoglutarate dehydrogenase and succinate thiokinase in toluenised cells. The adenylate control of these enzymes observed in cell-free extracts was also found in permeabilised cells. This emphasises the metabolic significance of these responses for *in vivo* regulation.

The use of crude enzyme extracts rather than purified preparations may be of a particular advantage in the study of regulation because in these extracts the enzyme has been subject to the least modification. This may be important in preserving their regulatory properties. Thus Johnson and Hanson (1974) could not demonstrate NADH inhibition of purified *A. anitratus* citrate synthase although Jones and Weitzman (1974) have observed NADH inhibition and AMP reactivation with this enzyme in crude extracts. Another advantage which crude preparations may have over isolated pure enzymes is that the former may give an indication of the possible *in vivo* interactions between enzymes. Thus ATP inhibition of *A. lwoffi* GDP- or IDP- dependent succinate thiokinase activities was not observed in partially purified preparation unless nucleoside diphosphate kinase was also added.

The determination of enzyme activity in crude extracts may pose several problems, especially if spectrophotometric methods are used. First, the high initial absorbances of crude extracts may render these assays unsuitable, particularly for those enzymes whose activities are determined at ultraviolet wavelengths. In this thesis, steps have been taken to overcome this problem and several new polarographic assays have been developed. These were of particular advantage because polarographic measurements are unaffected by turbidity. This permitted the examination of enzymes at high protein concentrations, without recourse to more complex stopped flow techniques, because enzymes could be assayed *in situ* with

suspensions of cells normally too dense for spectrophotometric assays.

Many of the enzymes which have been investigated involve measuring the initial rate of NADH oxidation or formation. However, this method may be unsuitable for use with crude enzyme preparations, which generally contain high concentrations of NADH oxidase. Polarographic assays are performed under anaerobic conditions and are therefore unaffected by this contaminating enzyme activity. This was a particular advantage with malate dehydrogenase and phosphoenolpyruvate carboxylase.

Before assessing the physiological significance of observed responses, the data on which they are based must be examined. Experiments are subject to experimental error, and different values may be obtained when experiments are repeated. To minimise these variations, the experiments described in this thesis have been repeated at least three times, each time with a different enzyme preparation. A 'typical' set of results obtained from one experiment has been presented. Where the data have been tabulated each value represents the average of three measurements, but where the data have been presented graphically, each point is a single estimate. However, by drawing the best curve through all the points, variations due to experimental error have been minimised. The effects of various compounds on enzyme activity have been investigated by comparing the reaction velocities measured with and without these substances. If these values differed reproducibly by more than 10%, the responses were considered significant.

2. Pyruvate Dehydrogenase

The sensitivity to adenylate control previously observed with A. lwoffi citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase (see INTRODUCTION) was also observed with pyruvate dehydrogenase, the enzyme which precedes this group of citric acid cycle enzymes and provides the acetyl-CoA 'fuel' for cycle activity. AMP and ADP considerably stimulated the activity of this enzyme, AMP being more effective than ADP, whereas ATP was inhibitory. An examination of the effects of AMP on the kinetics of the enzyme with respect to the substrates pyruvate NAD^+ and CoA, showed that the prime effect was exerted on the pyruvate-dependence. With AMP, there was a marked reduction in the pyruvate Km, but there was no comparable decrease in the Km values for the other substrates. This permits a flexible expression of enzyme activity, because in the area bounded by the two extreme pyruvate-dependences activity depends on both pyruvate and AMP concentration. Thus, at a particular AMP concentration, enzyme activity depends on the concentration of pyruvate and conversely, at a fixed pyruvate concentration, enzyme activity is proportional to the concentration of AMP. Thé area between the curves therefore represents the operational range of the enzyme. Although the response of A. lwoffi pyruvate dehydrogenase to ADP was smaller than its response to AMP, nevertheless, the property may contribute to the control of the enzyme in a similar manner to AMP. In contrast, the effect of ATP was small, and may therefore be insignificant for in vivo regulation. Notwithstanding, both ADP and ATP are formed at the expense of AMP, and an increase in their concentration will automatically result in an increase in the pyruvate Km because the concentration of AMP will be decreased. It

has been suggested that AMP is a better indicator of the ATP supply than ADP or ATP (Krebs, 1964; Sanwal, 1970), therefore it is interesting that the most dramatic response was to changes in the concentration of AMP.

Because AMP is a reflection of the energy state of the cell, A. lwoffi pyruvate dehydrogenase may be controlled by the intracellular energy level. This has been examined in vitro by investigating the response of the enzyme to energy charge. Because pyruvate dehydrogenase is an ATP-regenerating system, an R-type of response was expected. This was indeed the case, and enzyme activity was maximal over the charge range 0 - 0.7, but decreased sharply above this value (Fig. 8). Thus, above an energy charge of 0.7, very sensitive adjustments of enzyme activity can be made to compensate for fluctuations of energy charge, and this may operate in vivo to stabilise the intracellular energy charge at about 0.8. Thus, although the responses of enzyme activity to ADP, and particularly ATP were small (compared with the response to AMP), nevertheless, the particularly steep response to energy charge in vitro suggests that these nucleotides may sharpen the response of this enzyme to energy charge in the cell.

For comparative purposes, the effect of AMP on E. coli pyruvate dehydrogenase was examined. In contrast to the marked response of A. lwoffi pyruvate dehydrogenase, the E. coli enzyme was relatively insensitive to this effector. The small degree of activation which was observed was not accompanied by a reduction in the pyruvate Km. Thus, in A. lwoffi, pyruvate dehydrogenase, like citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase, is sensitive to adenylate control, whereas in E. coli none of these enzymes is so regulated. Therefore adenylate sensitivity of A. lwoffi pyruvate dehydrogenase may be a method of ensuring that

the supply of acetyl-CoA is kept in step with the demands of the subsequent enzymes. It was also interesting to compare the Km values for pyruvate in these two organisms. In E. coli, the values of this parameter (0.67mM) was very much lower than that of the A. lwoffi enzyme (3.0mM), but similar to the value of this parameter in the presence of AMP (0.46mM). AMP activation of E. coli pyruvate dehydrogenase has been reported previously, but there is discrepancy in the literature concerning the reduction of the pyruvate Km. In agreement with the results reported in this thesis, Schwartz and Reed (1968) observed no reduction in the pyruvate Km (with 0.5mM AMP). In contrast, Shen and Atkinson (1970) demonstrated a 6-fold increase in this parameter, but the concentration of AMP was high (5mM) and therefore unlikely to be physiological.

This response of A. lwoffi pyruvate dehydrogenase to the adenine nucleotides is very similar to the adenylate control of A. lwoffi α-oxoglutarate dehydrogenase (Weitzman, 1972; Parker and Weitzman, 1973). With 0.5mM α-oxoglutarate, and either 0.2mM AMP or 0.2mM ADP, α-oxoglutarate dehydrogenase activity is stimulated 5.5-fold and 4.3-fold, respectively. ATP, on the other hand, is inhibitory. Activation is accompanied by marked reduction of the Km for α-oxoglutarate, and the value of this parameter is decreased from 2.5mM, without adenylates, to 0.2mM and 0.43mM, with 0.2mM AMP and 0.2mM ADP, respectively. The response of enzyme activity to energy charge is also similar. Enzyme activity is maximal over an energy charge range of 0 - 0.7, but exhibits a sharp decrease at 0.7 to 0.9.

Interestingly, pyruvate dehydrogenase and α-oxoglutarate dehydrogenase catalyse similar reactions and are multienzyme complexes of high molecular weight. These complexes consist of three component

enzymes: pyruvate decarboxylase or α-oxoglutarate-decarboxylase, dihydrolipoyl transacetylase or dihydrolipoyl transuccinylase and dihydrolipoyl dehydrogenase. In the case of pyruvate dehydrogenase, the first component enzyme (E_1) catalyses two reactions. First, the decarboxylation of pyruvate to an α-hydroxy-ethyl-thiamine pyrophosphate complex, and secondly the transfer of the α-hydroxy-ethyl-thiamine pyrophosphate group to the lipoyl moiety of the second component enzyme (E_2). The lipoyl moiety is present as lipoamide which is joined to E_2 through a lysine ε-amino group and the transfer results in the reductive fission of the lipoyl disulphide bond. E_2 then catalyses the transfer of the acetyl group from lipoamide to the thiol group of CoA (to form acetyl-CoA) leaving the lipoamide thiol groups in a fully reduced form. Dihydrolipoamide is subsequently reoxidised a the expense of NAD^+ by the third component enzyme (E_3), the reaction being mediated through FAD bound to E_3. These reactions are summarised in Fig. 58.

In E. coli and mammalian systems, the component enzymes of pyruvate dehydrogenase and α-oxoglutarate dehydrogenase appear to be assembled into multienzyme complexes. Electron micrographs of these complexes and their component enzymes show that the transacetylase or transuccinylase have a structural as well as a catalytic role, and form different basic frameworks around which the molecules of the carboxylases and flavoproteins appear to be regularly distributed (Ishikawa et al., 1966; Reed and Oliver, 1968; Harding et al., 1970). Such an arrangement presumably increases the efficiency of these multistep sequences. An intermediate formed by one enzyme will probably encounter the next enzyme in the sequence more rapidly if the enzymes are physically associated, than if they are structurally independent.

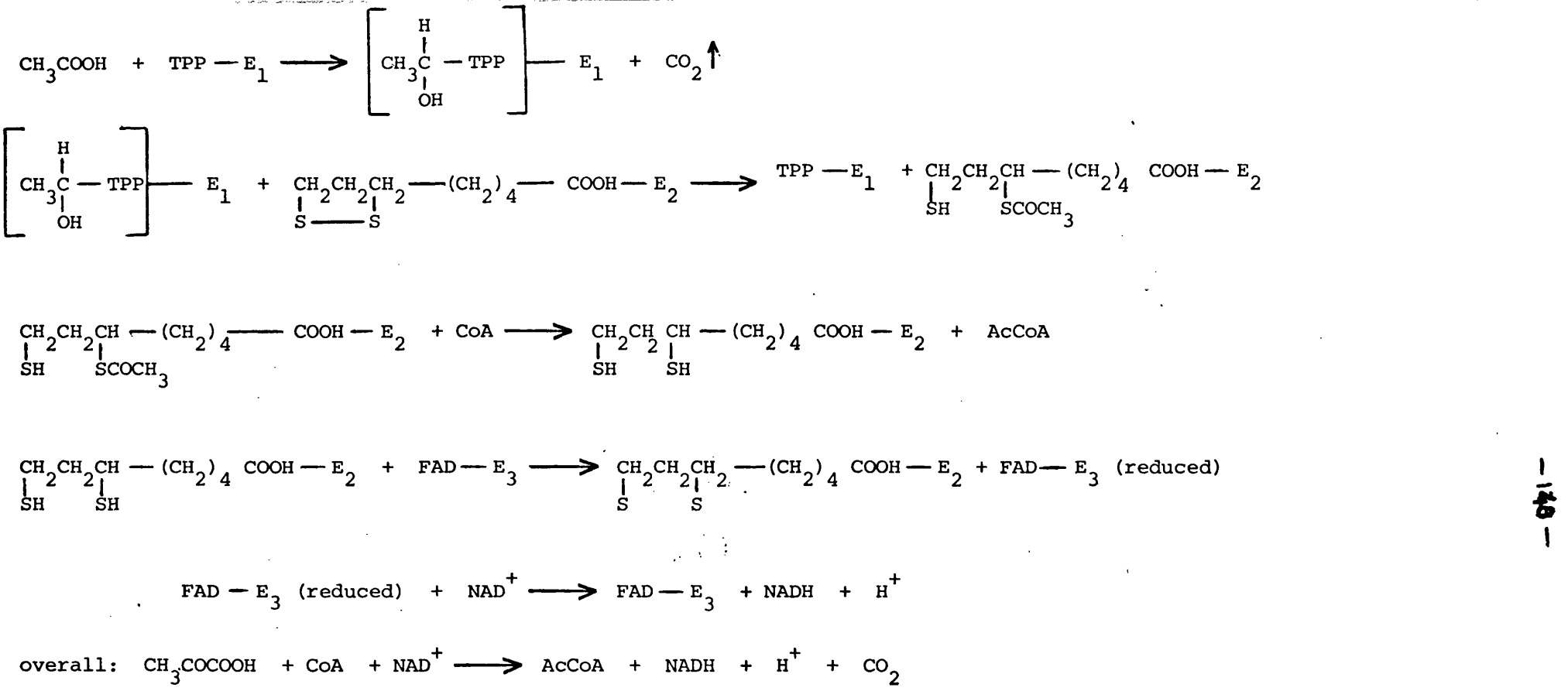

Figure 58 Summary of the reactions catalysed by the component enzymes of the pyruvate dehydrogenase complex

See text for details of the abbreviations used.

The structural and functional similarities between the various α-oxo acid dehydrogenase complexes are particularly useful for comparative purposes. Thus, although it has not been possible to demonstrate a site of action for the adenylates on A. lwoffi pyruvate dehydrogenase complex (due to lack of sufficiently concentrated and purified enzyme), nevertheless, a site of action on E_1 is suggested because AMP stimulation of the whole complex activity with A. lwoffi α-oxoglutarate dehydrogenase (Parker and Weitzman, 1973) and E. coli pyruvate dehydrogenase (Schwartz et al., 1968; Shen and Atkinson, 1970) has been attributed to activation of the first component enzyme. It is very likely that a similar mechanism may operate in the A. lwoffi pyruvate dehydrogenase complex, because not only does E_1 catalyse the first and, apparently, irreversible step of the reaction sequence, but also AMP exerts its prime effect on the pyruvate dependence.

A. lwoffi pyruvate dehydrogenase was also very sensitive to a wide variety of agents. pH and the nature of the assay buffer have been studied in greater detail. An examination of the rate-dependence of enzyme activity on pyruvate concentration as the pH of the assay mixture was increased showed a corresponding increase in the pyruvate Km. This was not the case in the additional presence of AMP. It was therefore interesting to compare the pyruvate Km values under these conditions. At pH 7.0 and without AMP, the Km value (0.58mM) was very similar to the values of this parameter with AMP irrespective of the pH, but an order of magnitude lower than the Km value at pH 8.5 without AMP (5.5mM). This suggests that at high pH the conformation of the enzyme is similar to that of the enzyme bound to AMP. The activity of A. lwoffi pyruvate dehydrogenase is also influenced by the nature of the buffer. As the concentration in assay mixtures of Tris-HCl, barbitone sodium,

triethanolamine, Tricine (N-Tris(hydroxymethyl) methylglycine), glycylglycine and Hepes (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid) was increased, enzymic activity was generally decreased (although the latter two buffers were somewhat less effective) but AMP activation was observed at all buffer concentrations. The rate dependence on Tricine concentration in the presence of AMP did not show inhibition, but on the contrary slight activation. A. lwoffi pyruvate dehydrogenase is also easily inactivated by sonication with maximum activity exhibited in cell-free extracts obtained from cells sonicated for 30s. Presumably this represents the time at which the rate of cell disruption is equal to the rate of enzyme inactivation.

Similar sensitivities have been reported for other pyruvate dehydrogenases. Thus the enzyme from E. coli suffers a gradual and irreversible inactivation in Tris-HCl but not in Tricine buffer (Schwartz and Reed, 1970). The activity of potato pyruvate dehydrogenase is influenced by the nature of the buffer in which extracts are prepared: those prepared in Tris-HCl or Hepes buffers contained 20% of the activity of comparable phosphate extracts (Crompton and Laties, 1971).

In common with a wide variety of mammalian, plant and bacterial pyruvate dehydrogenases (Garland and Randle , 1964; Hayakawa et al., 1964; Hansen and Henning, 1966; Bremer, 1969; Harding et al., 1970; Schwartz and Reed, 1970; Crompton and Laties, 1971; Bresters et al., 1972; Wieland et al., 1972) A. lwoffi pyruvate dehydrogenase is subject to end-product inhibition by NADH and acetyl-CoA. NADH inhibits all these enzymes in competition with NAD^+, but acetyl-CoA inhibits them in competition with CoA or pyruvate. Thus, acetyl-CoA inhibition of mammalian, potato and Neurospora crassa pyruvate dehydrogenase is competitive with respect

to CoA (Garland and Randle, 1964; Hayakawa et al., 1964; Bremer, 1969; Harding et al., 1970; Crompton and Laties, 1971; Weiland et al., 1972) whereas that of Azotobacter vinelandi pyruvate dehydrogenase is competitive with respect to pyruvate (Bresters et al., 1972). Acetyl-CoA inhibition of E. coli pyruvate dehydrogenase, on the other hand, can be reversed with CoA (Hansen and Henning, 1966; Schwartz et al., 1968) or with pyruvate (Schwartz et al., 1968; Schwartz and Reed, 1970; Shen and Atkinson, 1970). When similar experiments were performed with A. lwoffi pyruvate dehydrogenase, acetyl-CoA inhibition appeared to be non-competitive with respect to the pyruvate and CoA. Interestingly, acetyl-CoA inhibition of A. lwoffi pyruvate dehydrogenase could be partially relieved with AMP and sulphate, and in the presence of these effectors the hyperbolic dependence of inhibition on acetyl-CoA concentration became sigmoid. Thus, at low acetyl-CoA concentrations there was virtually no inhibition with low concentrations of acetyl-CoA, but as this was increased above a threshold value of 0.1mM, there was a sharp increase of inhibition; the curve then followed the form of the dependence observed without AMP and sulphate, although the extent of inhibition was lower.

Pyruvate dehydrogenase is an amphibolic enzyme, producing acetyl-CoA which may be used either for energy production, when channelled into the citric acid cycle, or for biosynthetic purposes such as the manufacture of fatty acids. These two functions require two different methods of control if acetyl-CoA is to be partitioned efficiently according to the particular demands of the cell. Under conditions of high energy, acetyl-CoA may not be required for citric acid cycle activity, although there may be a biosynthetic requirement for this compound. This is reflected in the regulation of pyruvate dehydrogenase which is controlled by biosynthetic and

energetic signals. Thus, the mammalian enzyme is inhibited by acetyl-CoA and by ATP, the latter effector acting in competition with ADP (Linn et al., 1969a and b; Wieland and Jagow-Westermann, 1969). The effects of the adenylates are mediated through a phosphorylation-dephosphorylation mechanism, phosphorylation and concomitant inactivation being brought about by an ATP-specific kinase, dephosphorylation and concomitant re-activation by a magnesium-dependent phosphatase. These reactions modify the pyruvate carboxylase component (E_1) of the complex (Linn et al., 1969a). On the other hand, E. coli pyruvate dehydrogenase is controlled in a somewhat different manner. The enzyme has been shown to be inhibited by acetyl-CoA (Hansen and Henning, 1966) and to be stimulated by the nucleoside monophosphates, AMP, GMP and GDP (Schwartz and Reed, 1968). It was therefore interesting that these nucleotides also reversed the inhibition by acetyl-CoA (Schwartz and Reed, 1970). This response is similar to that observed with A. lwoffi pyruvate dehydrogenase. In both cases AMP overcomes the acetyl-CoA inhibition, but with the latter enzyme this effect is more marked and the form of the dependence of inhibition on acetyl-CoA concentration becomes sigmoid. Acetyl-CoA therefore acts as a feedback modifier to pyruvate dehydrogenase and prevents its own wastful overproduction. However, in E. coli and A. lwoffi, energetic considerations appear to overcome these biosynthetic ones, presumably to ensure that cycle activity is not limited, even when the supply of acetyl-CoA is adequate.

It has been suggested that adenylate control of A. lwoffi pyruvate dehydrogenase is more stringent than that of E. coli pyruvate dehydrogenase; this is reflected in the above response. Differences in regulatory behaviour presumably reflect the different demands on cycle activity in these organism. E. coli is a facultative anaerobe and even under most aerobic conditions obtains its energy by fermentation. It is interesting, therefore, that the pyruvate dehydrogenase of this organism is stimulated by glycolytic intermediates such as phosphoenolpyruvate (Schwartz et al., 1968), fructose diphosphate and glyceraldehyde-3-phosphate (Shen and Atkinson, 1970; Sanwal, 1970). These metabolites may feed-forward and stimulate pyruvate dehydrogenase activity in anticipation of an increased flow through the citric acid cycle. Acetyl-CoA, the nucleoside monophosphates and glycolytic intermediates have been shown to modify the activity of the pyruvate decarboxylase component of the E. coli complex (Schwartz and Reed, 1970; Shen and Atkinson, 1970).

Inhibition of A. lwoffi pyruvate dehydrogenase by NADH, the other end-product of the reaction, shows different regulatory behaviour compared with the response of A. lwoffi α-oxoglutarate dehydrogenase to the effector. NADH inhibition of the latter enzyme can be relieved with AMP and ADP (Weitzman, 1972) but no similar response is observed with the former enzyme. In addition, NADH inhibits pyruvate dehydrogenase in strict competition with NAD^+ but the inhibition of α-oxoglutarate dehydrogenase is not strictly competitive with respect to NAD^+, and shows an element of competition with α-oxoglutarate. This implies that there are two binding sites for NADH on the multienzyme complex: one which can interact with α-oxoglutarate, the other with NAD^+. If this is the case, the following anomalous observations can be explained. Using

assays specific for the carboxylase (E_1) and flavoprotein (E_3) components of the α-oxoglutarate dehydrogenase multenzyme complex, Parker and Weitzman (1973) have shown that AMP or ADP activation of the whole complex is due to activation of the carboxylase component (E_1); the flavoprotein component (E_3) is not involved. They have also demonstrated that NADH inhibits the flavoprotein component but in contrast to the relief of inhibition by the AMP and ADP observed with the whole complex, there was no effect of the adenylates in this inhibition. A second binding site for NADH was therefore suggested, at which the adenylates could also interact. Because component E_1 has already been implicated in the binding of adenylates, this was the obvious location of the second NADH binding site. However, a direct examination of the effects of NADH on component E_1 could not be undertaken, because NADH non-enzymically reduces the dichlorophenol-indophenol used to follow the oxidative decarboxylation of α-oxoglutarate. Indirect kinetic evidence has therefore been used. An examination of NADH inhibition as a function of α-oxoglutarate concentration only exhibited strict competition with respect to this substrate with near-saturating concentrations of NAD^+. Conversely, strict competition with respect to NAD^+ was only observed with near-saturating concentrations of α-oxoglutarate. This suggests that NADH inhibits component E_1 in competition with α-oxoglutarate and component E_3 in competition with NAD^+. It is the interaction between the NADH and the adenylate binding site on the first component enzyme that may account for the relief of NADH inhibition by AMP and ADP in the whole complex. Further evidence for an allosteric site for NADH has been obtained by Hall and Weitzman (to be published) by examining the multiple inhibition kinetics obtained in experiments performed according to the method of Yonetani and

Theorell (1964). By this method two competitive inhibitors can be shown to bind at different sites on the enzyme by examining the dependence of the reciprocal of the reaction velocity on the concentration of one inhibitor, in the absence and presence of the other; intersecting plots indicate separate inhibitor sites, parallel plots indicate identical sites. Thus NADH and α-oxoadipate inhibit A. lwoffi α-oxoglutarate dehydrogenase activity in competition with α-oxoglutarate (at high NAD^+ concentrations) and multiple inhibition kinetics with these inhibitors yield intersecting plots. This illustrates the lack of identity between the NADH and α-oxoadipate sites. Because α-oxoadipate is a substrate analogue of α-oxoglutarate it probably binds at the same site; thus, there is likely to be a separate site for NADH. The pyruvate dehydrogenase of this organism, on the other hand, has probably only one site of action for NADH, and that on the flavoprotein component of the enzyme. This is in keeping with the observations that the inhibition is strictly competitive with respect to NAD^+ and that inhibition is unaffected by the adenylates. NADH has also been shown to have its sole site of action on E_3 in E. coli (Hansen and Henning, 1966) and mammalian (Tsai et al., 1973) pyruvate dehydrogenases.

Initial examinations of the response of A. lwoffi pyruvate dehydrogenase activity to energy charge did not show the expected sharp negative slope at higher energy charge values. With a charge value of 1.0, stimulation was observed, whereas inhibition was predicted. This effect was attributed to the activation of the enzyme by ammonium sulphate which was present in commercial preparations of adenylate kinase. Stimulation was due to the sulphate anion and other anions were also found to produce this effect. Of the inorganic anions tested, only those divalent at pH 8.0 exhibited this property (Table 3),

whereas this was not the case with organic anions. Again monovalent and trivalent anions were without effect, however divalent anions exhibited a range of responses from stimulation to no effect (Table 4) suggesting that the arrangement and proximity of the carboxyl groups may be important for activation.

Anion activation was accompanied by a marked reduction in the pyruvate Km and sulphate, as representative of these anions, was also found to partially relieve the inhibition by acetyl-CoA, converting the form of the dependence on acetyl-CoA from a hyperbolic to a sigmoid form. These responses were similar to those observed with AMP. However, the concentrations of sulphate required to bring about these responses was an order of magnitude higher than those of AMP, indicating the greater sensitivity of the enzyme to AMP. The similarity of these responses suggests that divalent anions may bind to the same site on the enzyme molecule as AMP and thereby mimic its effects. The activations produced by AMP and sulphate were not additive and by saturating the enzyme to maximum stimulation with one effector, no further activation could be achieved by adding the other. However, at a sub-saturating concentration of either substrate, the resulting stimulation could be increased by adding the other compound (Fig. 23). The maximum activation produced in their joint presence was similar to that observed when either effector was used alone to saturate the enzyme. This type of non-additivity can be contrasted with the additivity of the pyruvate and AMP stimulations of A. lwoffi NADP-linked isocitrate dehydrogenase. The larger molecular weight isoenzyme, isoenzyme II, is stimulated by pyruvate or glyoxylate (Self and Weitzman, 1970) and by AMP or ADP (Parker and Weitzman, 1970). The enzyme can be saturated with either pyruvate or glyoxylate to maximum activation and this

activation can be increased further by adding AMP or ADP. The maximum activation produced in their joint presence was such that the stimulatory ability of each effector was expressed; the activations were therefore strictly additive (Fig. 59). These results suggest different binding sites for pyruvate or glyoxylate and for AMP or ADP, both of which are distinct from the catalytic site. Some selective desensitisation has indicated that this may indeed be the case (Self et al., 1973).

The regulation of catalytic activity by anions has been reported by other workers. Thus Warren and Cheatum (1966) have shown that the activities of trypsin, α-chymotrypsin, renal acylase, wheat germ lipase, estradiol-17β dehydrogenase, β-amylase and β-galactosidase are inhibited by neutral salts and similar responses have been observed with glucose dehydrogenase, (Anderson and Nordlie, 1968) and chicken liver pyruvate carboxylase (Scrutten and Fung, 1972). However, phosphorylase b appears to be particularly sensitive to this type of control. Thus the enzyme from lobster muscle (Cowgill, 1959), beef adrenal cortex (Riley and Haynes, 1963; Yunis and Assaf, 1970), liver (Appleman et al., 1966) and rabbit muscle (Sealock and Graves, 1967; Engers and Madsen, 1968) are stimulated by anions such as sulphate, phosphate, fluoride, tartrate and citrate. These anions salt out proteins and interestingly, those anions which salt in protein (chloride, bromide, iodide and nitrate) have no effect on the enzyme, or inhibit it (Cowgill, 1959; Engers and Madsen, 1968). Phosphorylase exists in two forms, an inactive one, phosphorylase b and an active one, phosphorylase a. The former enzyme requires AMP for activity, whereas the latter one is active without it. In the presence of salts which salt out proteins, rabbit muscle phosphorylase b loses its absolute requirement for AMP

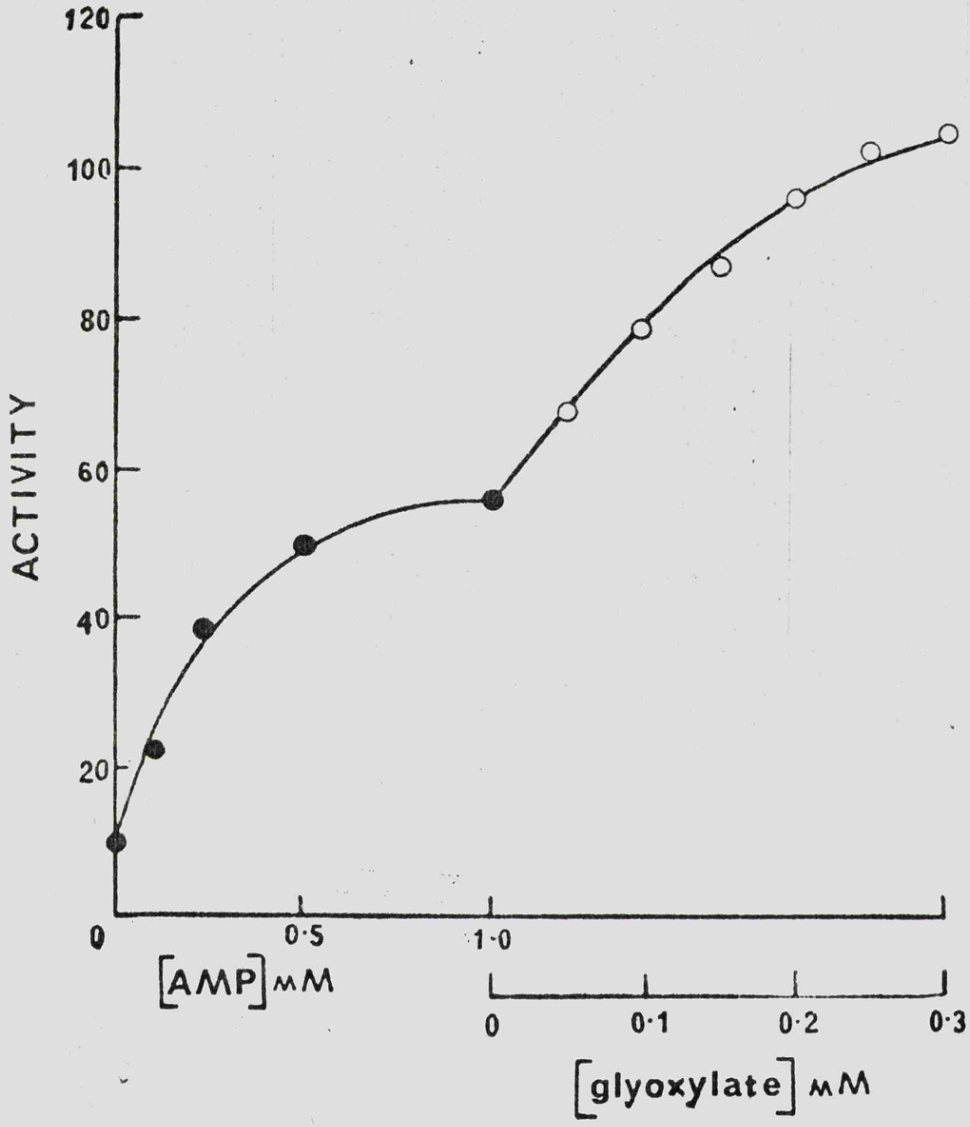

Figure 59 Additive nature of the A. lwoffi isocitrate dehydrogenase glyoxylate and AMP activations

Enzyme activities (arbitrary units) were measured under standard conditions first with increasing AMP concentration from 0 to 1.0mM (●), and then at fixed 1mM AMP, with increasing glyoxylate concentrations from 0 - 0.3mM (○).

From Self et al., 1973

(Engers and Madsen, 1968). Therefore it was suggested that the binding of these anions to the enzyme brings about a conformational change similar to that induced by AMP. Activation of lobster muscle phosphorylase b by sodium or potassium sulphate is accompanied by a marked reduction of the glucose-1-phosphate Km, the value of this parameter in the presence of sulphate closely resembling that obtained with phosphorylase a. Thus the binding of sulphate may modify the enzyme so that its capacity for binding glucose-1-phosphate is equivalent to that of phosphoylase a. Anion activation of A. lwoffi pyruvate dehydrogenase is therefore similar, because in the presence of sulphate the pyruvate Km is decreased. Thus certain anions can mimic the effect of AMP on several phorphorylases and A. lwoffi pyruvate dehydrogenase. However, the latter enzyme is more sensitive to these anions. Only 7mM sodium sulphate was required for maximum activation of A. lwoffi pyruvate dehydrogenase, whereas concentrations in the region of 800mM were required with the various phosphorylases.

Activation of pyruvate dehydrogenase by AMP was not restricted to A. lwoffi but was observed with a number of Gram-negative strictly aerobic species; none of the pyruvate dehydrogenases from Gram-negative facultatively anaerobic bacteria or Gram-positive bacteria were activated by AMP. Stimulation by AMP always appeared to be accompanied by sensitivity to divalent anion activation. It was interesting that those organisms which exhibited adenylate control of pyruvate dehydrogenase were also those Gram-negative strictly aerobic bacteria which in addition to adenylate control of citrate synthase (a feature of all Gram-negative species), exhibited adenylate control of their isocitrate dehydrogenase, α-oxoglutarate dehydrogenase and succinate thiokinase. However, there were two exceptions to this,

Alkaligenes faecalis and Pseudomonas stutzeri. In these two organisms only pyruvate dehydrogenase and citrate synthase appear to be under adenylate control. However, the isocitrate dehydrogenase, although not under adenylate control, is stimulated by pyruvate and glyoxy-late like those organisms which exhibit adenylate control of all five enzymes. These responses may be a method of ensuring an adequate flow of metabolites through the citric acid cycle. Thus, under low energy conditions, not only are pyruvate dehydrogenase and citrate synthase activities increased by appropriate adenylate controls exerted over them, but pyruvate may feed-forward (Shen and Atkinson, 1970; Sanwal, 1970) and activate isocitrate dehydrogenase. Isocitrate stands at a metabolic branch-point and can be metabolised further to α-oxoglutarate by isocitrate dehydrogenase or to glyoxylate and succinate by isocitrate lyase. Thus the activation of isocitrate dehydrogenase by glyoxylate, the product of this alternative branch, may ensure that cycle activity is not limited by the withdrawal of isocitrate.

An examination of the regulatory properties of A. lwoffi, E coli and mammalian pyruvate dehydrogenases has revealed that this enzyme exhibits different regulatory behaviour depending on the source organism, although the chemistry of the reaction is the same in all cases. In addition to the properties already described with E. coli pyruvate dehydrogenase, the enzyme is also inhibited by GTP. This inhibition is non-competitive with respect to pyruvate and it can be reversed by GDP (Schwartz and Reed, 1970). The control of mammalian pyruvate dehydrogenases is very different to the control of bacterial pyruvate dehydrogenases in one particular respect; the overall activity of mammalian pyruvate dehydrogenases is regulated by a phosphorylation-dephosphorylation mechanism, (Linn et al., 1969a and b; Wieland

and Jagow-Westerman, 1969). Phosphorylation is accompanied by inactivation, and is catalysed by an ATP specific kinase, whereas dephosphorylation and re-activation is catalysed by magnesium-dependent phosphatase. These different regulatory phenomena highlight the diversity of the control processes imposed on metabolically identical pathways.

3. Succinate Thiokinase

These studies of succinate thiokinase have been facilitated by the development of a polarographic assay procedure which involves measuring the current change at -0.2V, due to the appearance or disappearance of CoA (Weitzman and Hewson, 1973). Other methods of assaying succinate thiokinase often monitor succinyl-CoA formation, but these are less suitable for the present studies because this direction is not the one associated with citric acid cycle activity. One such method can only be used in the direction of succinyl-CoA formation, because it involves measuring the amount of succinyl hydroxamate produced when the reaction is carried out in the presence of hydroxylamine (Kaufman et al., 1953). Another method can be used to monitor the reaction in either direction, because it involves measuring the change in absorbance at 235nm due to the formation or degradation of the succinyl-CoA thioester bond (Cha and Parks, 1964). However, to measure succinyl-CoA degradation high initial absorbances must be used. This limits the amount of the nucleoside diphosphates which can be added, and makes an examination of the effects of adenine nucleotides difficult. The method is also inappropriate for use with crude enzyme preparations. The polarographic procedure, on the other hand, does not suffer from these disadvantages and is particularly suitable for the present studies. The reaction can be followed in either direction with crude enzyme preparations, and the method permits an examination of those succinate thiokinases with high Km values for their nucleotide substrates, as well as allowing an examination of the effects of non-substrate nucleotides on catalytic activity.

A comparison of succinate thiokinase activity in crude extracts of E. coli and A. lwoffi revealed that the latter enzyme had a 70-fold higher Km for ADP. The ADP-dependence of the A. lwoffi

enzyme was unaffected by AMP or ATP, both in crude and partially purified extracts, but the enzyme did respond to energy charge in a manner consistent with its role in ATP-regeneration. It is therefore suggested that the high ADP Km exhibited by the A. lwoffi enzyme may be of some regulatory significance in so far as the intracellular ADP concentration may vary over a range within which enzymatic activity changes. Alterations in ADP concentration, which reflect changes in the energy state or energy charge of the cell, would therefore exert a compensating regulatory effect on succinate thiokinase. On the other hand, E. coli succinate thiokinase, which has a low Km for ADP, may always be operating under conditions approximating V_{max}, so that fluctuations of the intracellular ADP concentration may have little effect.

The intracellular concentration of ADP has been measured in E. coli and similar concentrations may well be present in A. lwoffi. During logarithmic growth with various carbon sources the intracellular concentration of ADP was shown to average just over 1mM (Lowry et al., 1971), a similar value to that of rat liver cells (1.18mM) (Burch et al., 1970). These concentrations have been calculated with the same assumptions as those used in DISCUSSION 1). If the intracellular concentration of ADP in A. lwoffi is indeed approximately 1mM, then an ADP Km of 1.18mM may well serve to control succinate thiokinase activity in vivo. However, no such regulatory behaviour would be expected with the E. coli enzyme, because the ADP Km (0.017mM) is 60-times smaller than the intracellular concentration of this nucleotide.

In view of the unusually high ADP Km of A. lwoffi succinate thiokinase, crude enzyme extracts were examined with other nucleoside diphosphates to see if these were better substrates. Surprisingly, the enzyme was not only active with GDP and IDP, but

the GDP or IDP Km was 50-fold lower than the ADP Km; V_{max} values were comparable however. ATP inhibited the GDP- or IDP-dependent enzyme activity in crude but not in partially purified preparations, unless nucleoside diphosphate kinase was also present. Inhibition was attributed to the conversion of GDP or IDP, good substrates for A. lwoffi succinate thiokinase activity, to ADP a poorer one. Thus adenylate control of A. lwoffi succinate thiokinase may not simply be due to the sensitive operation of the enzyme within the intracellular range of ADP concentrations, but it may be brought about by the nucleoside diphosphate kinase mediated interaction of ATP with the GDP or IDP pools. This situation may be physiologically more realistic because it additionally takes into account the presence of guanosine and inosine nucleotides in the nucleotide pool. Thus A. lwoffi succinate thiokinase can exhibit two types of nucleoside diphosphate-dependence: an ADP-dependence with a high Km for ADP and GDP- or IDP-dependence with a low Km for the nucleotide substrates. At a particular nucleoside diphosphate concentration, the activity with ADP will be lower than the activity with GDP or IDP, so that in the presence of ATP and nucleoside diphosphate kinase the enzyme will be apparently inhibited as GDP (or IDP) is converted to ADP. Thus, with a fixed nucleoside diphosphate pool, the actual activity expressed will depend on the ATP concentration because this will define the relative amounts of GDP or IDP, and ADP. However, the concentration of ATP is an indicator of the energy state of the cell, therefore A. lwoffi succinate thiokinase may be controlled by the energy charge of the cell. For these responses to be metabolically significant, the intracellular concentration of GDP or IDP must be lower than that of the intracellular ADP concentration. This is indeed believed to be the case.

The physiological significance of these responses for *in vivo* regulation was investigated by examining the dependence of *A. lwoffi* GDP-linked succinate thiokinase activity on energy charge with crude extracts. The expected R-type response curve was obtained, typical of an enzyme involved in ATP-regeneration. The particularly marked response to energy charge indicates the significance of this response for energy metabolism, and strongly suggests that succinate thiokinase may contribute to the stabilisation of energy charge *in vivo*.

It has been suggested that mammalian succinate thiokinases are specific for GDP and GTP, or IDP and ITP, whereas the enzyme from plant and bacterial sources utilise ADP and ATP (Bridger *et al.*, 1969). Thus an examination of the succinate thiokinases from kidney (Sanadi *et al.*, 1956), porcine heart (Cha and Parks, 1964) and pigeon pectoral muscle (Meshkova and Matveeva, 1970) has shown the mammalian enzyme to be specific for guanosine and inosine nucleotides, whereas similar examinations of the spinach (Kaufman and Alivisatos, 1955) wheat (Nandi and Waygood, 1965) and Jerusalem artichoke (Palmer and Wedding, 1966) succinate thiokinases have shown the plant enzyme to be specific for adenosine nucleotides. Bacterial succinate thiokinases, on the other hand, appear to be less specific in their nucleotide requirement. Thus, the *E. coli* enzyme appeared to be specific for the adenine nucleotides (Smith *et al.*, 1957; Gibson *et al.*, 1967), whereas the enzyme from *Rhodopseudomonas spheroides* was active with both ATP and GTP or ITP (Burnham, 1963). In the present studies, *A. lwoffi* succinate thiokinase was also found to be active with both ADP and GDP or IDP; this prompted an examination of the nucleotide specificity of a large number of bacterial species.

Interestingly, the lack of nucleotide specificity observed with the Rhodopseudomonas spheroides and A. lwoffi enzymes was seen with most bacterial succinate thiokinases. There were four types of bacterial succinate thiokinase (defined on the basis of the Km values for GDP and ADP) and in general there was an apparent correlation with the taxonomic classification of the source organisms. Most Gram-positive species, with the exception of organisms belonging to the genus Corynebacter, possessed succinate thiokinases which were specific for ADP (Km $<$ O.lmM), whereas succinate thiokinases from Gram-negative species were active with both ADP and GDP. They were of four types. One type of succinate thiokinase exhibited a low Km value for ADP (i.e. less than O.lmM) and a GDP Km approximately 10x greater. This succinate thiokinase was present in most Gram-negative species. Another type of succinate thiokinase exhibited low and similar values for the Km of both ADP and GDP. This type of succinate thiokinase was observed in organisms from the Gram-negative genus Pseudomonas and the Gram-positive genus Corynebacter. The final type of bacterial succinate thiokinase showed an unusually high ADP Km (approximately lmM), but the GDP Km was at least an order of magnitude lower. The latter type of succinate thiokinase was observed in those Gram-negative strictly aerobic species which additionally showed adenylate control of their pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase. In agreement with published data, mammalian succinate thiokinase was specific for GDP.

It was at first thought possible that the activity of A. lwoffi succinate thiokinase with GDP might be due to contamination of commercial GDP with ADP. However, several Gram-negative strictly

aerobic species showed greater activity with GDP than with ADP. In addition bacterial succinate thiokinases exhibit nucleotide specificites which appear to be correlated with their taxonomic classification. With these considerations in mind, it was thought unlikely that GDP-dependent succinate thiokinase activity was due to contamination.

The succinate thiokinases from E. coli, Ps. aeruginosa and A. lwoffi were studied in greater detail as representatives of the three types of succinate thiokinase active with both ADP and GDP. An examination of the rate-dependences on ADP and GDP concentration gave Km values for these substrates which bore the expected relative magnitudes to each, although with each enzyme the V_{max} values were comparable. This suggests that it is the affinity of these enzymes for their substrates which is different, rather than the catalytic ability in their presence. The nucleotide specificity of E. coli, Ps. aeruginosa and A. lwoffi succinate thiokinase was also examined by determining the rate of succinyl-CoA formation with ATP and GTP. The pattern of nucleotide specificities was the same as that observed when succinyl-CoA cleavage was examined. The Michaelis constants for ATP and GTP were generally an order of magnitude lower, however. This observation is similar to that made by Mazumander et al., (1960) with hog kidney succinate thiokinase. These workers reported Km values for the nucleoside triphosphates which were a 100-fold higher than those of the nucleoside diphosphates. Thus the GTP and ITP Km values were both 3.3mM, whereas the GDP and IDP Km values were 0.017mM and 0.038mM, respectively.

Although it is hazardous to predict the metabolic significance of in vitro responses without detailed knowledge of

intracellular conditions in each particular organism, nevertheless it is tentatively suggested that there should be no adenylate control of the GDP- or IDP- dependent succinate thiokinase activity in those organisms where the ADP and the GDP or IDP Km values are similar, or where the GDP or IDP Km values are greater than the ADP Km value. This is readily understood in the former case because the action of ATP and nucleoside diphosphate produces a comparable substrate. However, in the latter case, one might expect the conversion of a poorer substrate to a better one to result in activation. This will probably not be the case because the intracellular concentration has been suggested to be approximately 1mM, whereas the ADP Km of succinate thiokinase in these organisms is less than 0.1mM. Under these circumstances the enzyme is likely to be operating at V_{max} and will probably be unaffected by increasing the ADP concentration.

To test these hypotheses, the GDP-dependent succinate thiokinase activies in E. coli and Ps. aeruginosa were examined as a function of energy charge; similar conditions to those previously employed with the A. lwoffi enzymes were used. However, in contrast to the marked dependence on energy charge observed with the latter enzyme, there was no comparable dependence on energy charge exhibited by the E. coli or Ps. aeruginosa succinate thiokinases. These results may well reflect the significance of observed responses for in vivo regulation. The different regulatory behaviour exhibited by these enzymes is probably associated with their different molecular structures. A comparison of the molecular sizes of A. lwoffi and E. coli succinate thiokinases by gel filtration on Sephadex G-200 suggested that these were not gross differences.

4. Multipoint Control

The principles of controlling enzyme activity by small molecular weight metabolites have been derived from work on simple, linear, purely biosynthetic pathways, where regulation is achieved by modulating the activity of the first enzyme committed to that sequence by the end-product of the pathway. Control of the first enzyme is an efficient method of regulation because it prevents wasting energy and primary intermediates in synthesising the various compounds leading to the biosynthesis of the ultimate end product. Thus histidine inhibits phosphoribosyl-ATP-pyrophosphorylase (Moyed and Magasanik, 1960; Ames et al., 1961; Martin, 1962), isoleucine inhibits threonine deaminase (Umbarger, 1956; Changeux, 1961, 1963), tryptophan inhibits anthranilate synthetase (Edwards et al., 1974) and CTP inhibits aspartate transcarbamylase (Yates and Pardee, 1956; Gerhart and Pardee, 1962). In a highly branched pathway however, the 'first' enzyme is not always readily identifiable. Thus, in the highly branched pathway leading to lysine biosynthesis in E. coli lysine inhibits the conversion of aspartate to aspartyl phosphate and the conversion of aspartate semi-aldehyde to dihydropicolinic acid, although neither step is unique to lysine biosynthesis (Stadtman et al., 1964; Yugari and Gilvarg, 1962). The former reaction is also involved in threonine, isoleucine methionine and diaminopimelate biosynthesis, the latter in both lysine and diaminopimelate biosynthesis. Control is therefore not restricted to the 'first' step of a pathway rather to key reactions which affect the overall rate of the sequences in which they participate.

Branched pathways pose special problems of regulation because part of the sequence is involved in the biosynthesis of two

or more end-products. Thus, independent inhibition of the first common step by any one of the ultimate end-products may lead to a reduction in the others where any of the former are in excess. Such a situation can be avoided in a number of ways. The first of these is enzyme multiplicity, where the first enzyme exists in a number of forms each susceptible to inhibition by a different end-product (Umbarger and Brown, 1957 and 1958; Stadtman et al., 1961; Smith et al., 1962). Another method is concerted inhibition, where all the ultimate end-products must be in excess before the first common step is inhibited (Datta and Gest, 1964; Paulus and Gray, 1964). Alternatively, regulation may be achieved by co-operative inhibition, here any of the end-products causes a partial inhibition of the first common step, and the simultaneous excess of two or more products results in greater inhibition than the sum of the individual independent inhibitions (Caskey et al., 1964; Nierlick and Magasanik, 1965). Finally, cumulative feedback inhibition (Woolfolk and Stadtman, 1964) is the mechanism by which each of the several end-products acts independently of the others in causing a partial inhibition of the first common enzyme at saturating concentrations, however, their joint presence, each at saturating concentrations results in a residual enzyme activity (relative to the uninhibited enzyme) equal to the products of the residual activities obtained with saturating concentrations of each individual metabolite. Examples of these forms of regulation have been elegantly reviewed by Stadtman (1966, 1970).

The problem of identifying the 'first' citric acid cycle enzyme is particularly difficult; not only is the pathway highly branched, but also it is non-linear. Nevertheless the injection of acetyl-CoA has been suggested as the first cycle reaction, and indeed

many citrate synthases exhibit regulatory properties in keeping with this postulated role (see INTRODUCTION). However, citrate synthase is not the only cycle enzyme with regulatory properties, suggesting that several pacemaker reactions exist in the citric acid cycle. This is particularly apparent in A. lwoffi where pyruvate dehydrogenase, citrate synthase (Weitzman and Jones, 1968) isocitrate dehydrogenase (Parker and Weitzman, 1972), α-oxoglutarate dehydrogenase (Weitzman, 1972; Parker and Weitzman, 1973) and succinate thiokinase are all under adenylate control.

Encompassing its branched nature and posing another problem of regulation is the duality of function of the citric acid cycle. Here, common intermediates are used to provide the cell with energy and to supply the primary biosynthetic intermediates. Thus at several points in the citric acid cycle, metabolites have a choice of continuing their progress round the cycle or diverging into various biosynthetic routes. Thus pyruvate may be made available for cycle activity by its conversion to acetyl-CoA with pyruvate dehydrogenase or it may be used for valine isoleucine, alanine, glycine, serine and cysteine biosynthesis. Acetyl-CoA may be condensed with oxaloacetate to form citrate or it may be channelled into lysine, leucine or fatty acid biosynthesis. Oxaloacetate can also have alternative fates and may be transaminated to aspartate, and thus give rise to pyrimidines and the amino acids lysine, threonine, methionine, isoleucine, asparagine, or it may be decarboxylated to phosphoenolpyruvate and thereby be available for gluconeogenesis and the biosynthesis of phenylalanine, tyrosine and tryptophan. Similarly, isocitrate may be oxidised to α-oxoglutarate with isocitrate dehydrogenase, or it may be converted to glyoxylate and and succinate with isocitrate lyase.(Isocitrate lyase appears to be

constitutive in this organism). α-Oxoglutarate may be metabolised via α-oxoglutarate dehydrogenase to succinyl-CoA or it may be converted to glutamate, either by transamination or by reduction with ammonia and glutamate dehydrogenase; glutamate is then available for proline, arginine and glutamine biosynthesis. Finally, succinyl-CoA may be metabolised to succinate with succinate thiokinase, or it may be used for porphyrin biosynthesis (Fig. 60). However, biosynthesis and degradation are oppositely directed sequences. Therefore to achieve a balance between them, the fate of the common intermediates must be controlled.

It is thus interesting, that of the enzymes involved in citric acid cycle activity in A. lwoffi it is those which occur at the metabolic branch-points which are regulated. Thus at every point in the cycle where intermediates may be metabolised further by continuing round the cycle or by diverging into biosynthetic or anaplerotic pathways, it is the energy-yielding sequences that are regulated. This rigorous control of the citric acid cycle presumably ensures that cycle activity is not limited by the withdrawal of intermediates. Aconitase, succinate dehydrogenase, fumarase and malate dehydrogenase do not appear to be under adenylate control. The response of these enzymes to AMP and ATP were considered insignificant for metabolic regulation, because the enzyme activity measured with these nucleotides was within 10% of the activity in their absence. The first three of these enzymes do not occur at metabolic branch points. Therefore their substrates do not have alternative fates. However, malate was originally considered to be a branch-point metabolite on which malate dehydrogenase and malic enzyme may act. Notwithstanding, malate dehydrogenase does not appear to be under adenylate control. It may well be that adenylate control of citrate synthase is indirectly exerted over malate dehydrogenase,

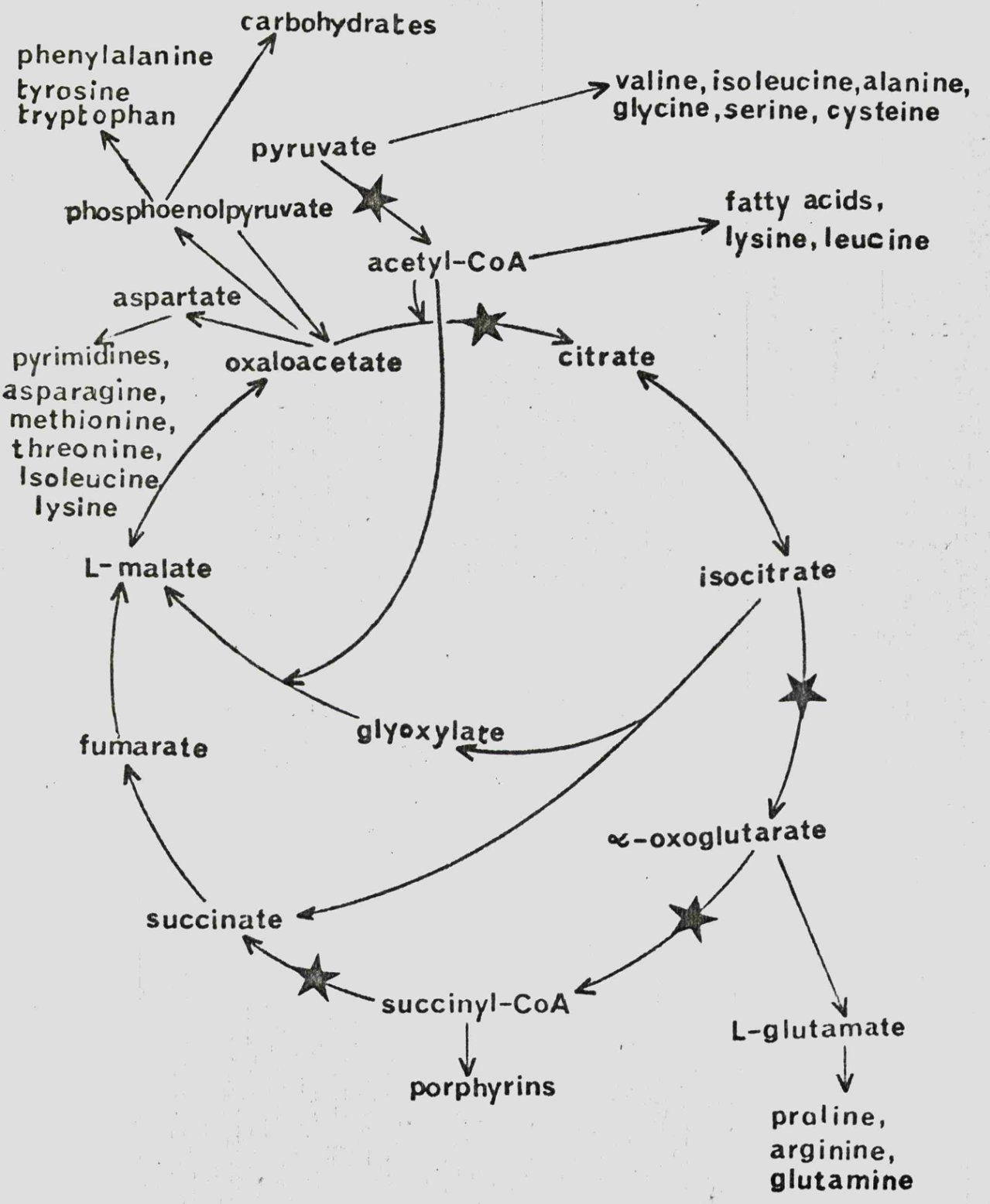

Figure 60 Citric acid cycle with its biosynthetic and anaplerotic branches

★ Enzymes under adenylate control (for definition see text)

because the malate dehydrogenase equilibrium lies in the direction of malate formation and only produces oxaloacetate when the latter is removed. It is also possible that *in vivo* malic enzyme functions in the reverse direction, so that malate does not occur at a metabolic branch-point. This is highly likely because, together with malate dehydrogenase, malic enzyme may perform an anaplerotic function converting pyruvate to oxaloacetate, especially as no pyruvate carboxylase has been detected in this organism.

In many branched biosynthetic pathways, common metabolites are directed towards the biosynthesis of particular end-products rather than others by regulating the activity of the first unique steps in the biosynthesis of those end-products. Thus in the biosynthesis of aromatic amino acids in *B. subtilis*, each of the first divergent steps in the synthesis of tryp-tophan, phenylalanine and tyrosine are inhibited by their respective end-products (Nester and Jensen, 1966). By comparison with this method of regulation, one might expect the anabolic branches of the cycle to exhibit regulatory properties; this would ensure a more efficient partitioning of metabolites between biosynthesis and degradation. Because of the marked sensitivity of the cycle enzymes to the adenylates, these effectors were used to examine the biosynthetic pathways which branch from the cycle. It was considered possible that these anabolic sequences may be modulated by a reverse form of adenylate control in which ATP would be stimulatory or AMP inhibitory. Thus under high energy conditions, there would be an active channelling of metabolites into these pathways. However ATP and AMP had no significant effect on the substrate dependences of isocitrate lyase, glutamate dehydrogenase or malic enzyme (using the same criteria of significance as described previously). In addition the $C_3 \longrightarrow C_4$ carboxylation reaction was examined, because one of the branch reactions from

pyruvate may have been its carboxylation with ATP to form oxoloacetate. However, extracts of A. lwoffi contained phosphoenolpyruvate carboxylase not pyruvate carboxylase. This phosphoenolpyruvate carboxylase was stimulated by acetyl-CoA, as are other phosphoenolpyruvate carboxylases, e.g. E. coli (Canovas and Kornberg, 1965 and 1966), Salmonella typhimurium (Maeba and Sanwal, 1965) and Azotobacter vinelandii Liao and Atkinson, 1969), but AMP and ATP exerted no effect on enzymic activity either with or without acetyl-CoA.

It would thus appear that energetic signals do not control the anabolic branch reactions from the cycle. However, this may not be entirely surprising. Sanwal (1970) has pointed out that the regulation of biosynthetic and catabolic pathways differs in the nature of the regulatory signal employed. In the former case, the signal is an ultimate end-product of the pathway, whereas in the latter it is an ultimate end-product of energy metabolism. This is because the products of catabolic sequences usually feed into amphibolic pathways, and these are energy producing. However, it must be borne in mind that energy production and biosynthesis are intimately related processes; energy production results in the formation of biosynthetic intermediates and biosynthesis is energy-consuming. Thus when the intracellular energy level falls and cycle activity is increased as a result of the appropriate controls exerted over regulated enzymes, not only will energy-production be increased but so will the supply of biosynthetic intermediates.

This situation will favour biosynthesis, and obviously under high energy conditions biosynthesis will be reduced. Thus the divergent biosynthetic sequences of the cycle are controlled by the energy state of the cell, albeit indirectly; direct control over these pathways is presumably brought about by their ultimate end-products. The use of the adenylates as modulators of citric acid cycle activity is ideal. These effectors not only encompass the biosynthetic demands on the cycle but also those energy-consuming demands which do not deplete its intermediates, such as motility

Working in conjunction with the adenylates and contributing to the overall control of the cycle is the NADH inhibition of pyruvate dehydrogenase, citrate synthase and α-oxoglutarate dehydrogenase. This effector has been described as the direct end-product of the cycle because it is generally produced at the dehydrogenation steps. Thus Weitzman and Jones (1968) have considered the NADH inhibition of a number of bacterial citrate synthases as a form of feedback control. However, NADH is an actual end-product of pyruvate dehydrogenase and α-oxoglutarate dehydrogenase. Therefore inhibition of these enzymes may simply be product inhibition due to the binding of substrate and product to the catalytic site. In the cell, the effects of NADH and the adenylates may interact and under high energy conditions (when the ATP concentration is high and that of AMP low) not only will the activity of those cycle enzymes under adenylate control be decreased, but this condition will presumably reduce the rate of NADH oxidation (which is coupled to oxidative phosphorylation) so that the concentration of NADH will temporarily be increased. This will further reduce the activities of pyruvate dehydrogenase, citrate synthase and α-oxoglutarate dehydrogenase.

In a similar manner, the concentration of acetyl-CoA may also contribute to the overall regulation of the citric acid cycle. Plant, animal and bacterial pyruvate dehydrogenases are inhibited by acetyl-CoA (see DISCUSSION 2); this presumably prevents the wasteful overproduction of acetyl-CoA when it is not being removed with citrate synthase or the other enzymes operating at this branch-point. However, in E. coli and in A. lwoffi inhibition can be overcome with AMP. Thus cycle activity can be increased, either to increase the energy state of the cell or to replenish those biosynthetic intermediates which occur later in the cycle, even when the intracellular concentration of acetyl-CoA is adequate.

The adenylate sensitivity of pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, α-oxoglutarate dehydrogenase and succinate thiokinase was examined in a large number of diverse bacterial species and adenylate control over all five enzymes was observed, not only in A. lwoffi, but in all species of Acinetobacter examined, and in a number of Gram-negative strictly aerobic bacteria. Srere (1969) suggested that regulation be viewed as a pervasive factor in metabolism, operating at every enzyme step; however, certain enzymes more than others will contribute to the overall regulation of the various pathways. He speculated that the citric acid cycle may show multiple control points because several cycle reactions exhibit regulatory properties. This situation has indeed been shown to exist in A. lwoffi and in a number of Gram-negative strictly aerobic bacteria. Here, several enzymes of a metabolic sequence are regulated by the same effector, and the term 'multipoint' control is suggested for this novel form of regulation.

In almost all the bacterial species examined there was an involvement of all five enzymes. Two exceptions were

found, Alkaligenes faecalis and Pseudomonas stutzeri, where only pyruvate dehydrogenase and citrate synthase appear to be under adenylate control, although the isocitrate dehydrogenase activity of these organisms is stimulated by pyruvate and glyoxylate. That some Gram-negative strictly aerobic species only exhibit adenylate control at citrate synthase, others at citrate synthase and pyruvate dehydrogenase, and yet others at all five enzymes, may indicate the evolutionary development of multipoint control.

Studies of this kind, in which the regulatory properties of a number of enzymes are examined in a wide range of bacterial species, have been reported previously (Weitzman and Jones, 1968; Sanwal, 1970; Self and Weitzman, 1972), and serve to highlight the diversity of the regulatory processes imposed on metabolically identical pathways. Because these control mechanisms are probably shaped by evolutionary pressures (Atkinson, 1968a; Stadtman, 1970), one might expect related organisms to control identical pathways in a similar manner; this type of investigation may therefore prove a useful tool for taxonomic classification. Thus Brevibacterium leucinophagum was originally classified as Gram-positive but its citrate synthase exhibited regulatory properties consistent with those of Gram-negative strictly aerobic bacteria. Further, morphological, physiological and electron microscope data showed that the latter was indeed the case (Jones and Weitzman, 1974). Using the regulatory properties of the other citric acid cycle enzymes as a diagnostic test, these workers showed that the organism belonged to the genus Acinetobacter (or a closely related species), because it exhibited multipoint control over its cycle enzymes. Similarly Mima polymorpha and Moraxella calcoacetica have been classified as belonging to the genus Acinetobacter (Baumann et al., 1968), whereas Bordetella

species are closely related to Acinetobacter (Johnson, 1971). That control processes can be related to taxonomy poses a fascinating phenomenon of evolutionary diversion. However, regulatory behaviour may also be correlated with the nutritional background of these organisms. In the laboratory these organisms generally do not grow on glucose and are therefore totally dependent on cycle activity for energy. Acinetobacter spp. tend to inhabit soil and water, as do Xanthomonas hyacinthi, Chromobacterium violaceum and Bordetella bronchiseptica (although these latter organisms also grow with other food sources). It would thus appear that multipoint control of the citric acid cycle is found in those organisms which are either closely related to Acinetobacter or share their nutritionally poor habitat. Stringent control of the citric acid cycle may therefore be particularly important for the economical growth of these organisms, and may be of a particular advantage when there is competition between cells.

Thus, the simplistic view of metabolic regulation in which a pathway is controlled at a single rate-limiting step at the beginning of a sequence is particularly inadequate when considering the citric acid cycle. Not only is the pathway highly branched and non-linear, but it also serves a dual metabolic role. Regulation at the beginning of the sequence may not be enough for efficient control and this is exemplified by the regulation of A. lwoffi citric acid cycle enzymes. However, there is a great diversity in the regulatory processes imposed on this pathway, and this may reflect the different demands on cycle activity in the various organisms.

In the course of this work a number of new assay procedures have been developed, several of them based on polarographic techniques. These methods have offered some advantage over existing methods, particularly in the examination of crude enzyme extracts, and may prove of general value in other studies.

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ABSTRACT

The activities of Acinetobacter lwoffi pyruvate dehydrogenase and succinate thiokinase have been shown to be under adenylate control. Pyruvate dehydrogenase was stimulated by AMP and ADP whereas ATP was inhibitory. The products NADH and acetyl-CoA inhibited the enzyme and AMP decreased the latter inhibition. On the other hand, A. lwoffi succinate thiokinase utilised ADP (Km = 1.18mM), or GDP (Km = 0.026mM), or IDP (Km = 0.023mM) as nucleotide substrate, and the GDP- or IDP- dependent activities were inhibited by ATP in the presence of nucleoside diphosphate kinase. Thus adenylate control of this enzyme may either be a consequence of the sensitive operation of the enzyme over the range in which the intracellular ADP concentrations may vary, or it may involve a nucleoside diphosphate kinase mediated interaction of ATP with the GDP or IDP pools.

No adenylate control was observed with A. lwoffi aconitase, succinate dehydrogenase, fumarase and malate dehydrogenase. Therefore, these results together with those previously observed with citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase suggest that adenylate control of the citric acid cycle enzymes in A. lwoffi is exerted only on those enzymes which occur at the metabolic branch-points. This novel form of regulation in which several enzymes of a metabolic sequence are controlled by the same effector has been called "multipoint" control and presumably ensures that cycle activity is not limited by the withdrawal of intermediates. An examination of a large number of diverse bacterial species showed that the elements of the control are generally found together and were detected in all species of Acinetobacter examined, as well as in a few other Gram-negative strictly aerobic bacteria.